Modulation of defence and iron homeostasis genes in rice roots by the diazotrophic endophyte *Herbaspirillum seropedicae*

Brusamarello-Santos,[1] L.C.C, Alberton,[2] D., Valdameri,[2] G., Camilios-Neto,[1,6] D., Covre,[3] R., Lopes,[3] K., Tadra-Sfeir,[1] M.Z., Faoro,[1,7] H., Monteiro, R. A.[1], Silva,[3,8] A.B., Broughton[4], W. J., Pedrosa,[1] F.O., Wassem,[5] R., Souza*,[1] E.M.

[1]Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná, Curitiba, PR, Brasil

[2]Departamento de Patologia Médica, Setor Ciências da Saúde, Curitiba, PR, Brasil

[3]Setor de Educação Profissional e Tecnológica, Universidade Federal do Paraná, Curitiba, PR, Brasil

[4]Federal Institute of Materials Research and Testing, Division 4 Environment, Berlin, Germany

[5]Departamento de Genética, Universidade Federal do Paraná, Curitiba, PR, Brasil

[6] Present address: Departamento de Bioquímica e Biotecnologia, Universidade Estadual de Londrina, Londrina, PR, Brasil

[7] Present address: Instituto Carlos Chagas – Fiocruz, Curitiba, PR, Brasil

[9] Present address: Luxembourg Centre for Systems Biomedicine (LCSB), University of Luxembourg, Esch-sur-Alzette, Luxembourg.

*** Corresponding author:**

Address: Universidade Federal do Paraná/UFPR, Rua Coronel Francisco Heráclito dos Santos, s/n Caixa Postal 19046, 81531-980 – Curitiba,

E-mail: souzaem@ufpr.br

Telephone and fax : +55413361-1667

E-mail of authors:

Brusamarello-Santos, L.C.C: lizianebrusa@gmail.com

Alberton, D.: dayalberton@yahoo.com.br

Valdameri, G.: glaucio_valdameri@hotmail.com

Camilios-Neto, D.: camiliosneto@yahoo.com.br

Covre, R.: Covrebio06@gmail.com

Lopes, K.: katiaplopes@gmail.com

Tadra-Sfeir,1 M.Z.: mzitadrasfeir@gmail.com

Faoro, H.: hfaoro@gmail.com

Monteiro, R. A.: roseadele@gmail.com

Silva, A.B.: adriano.bioinfo@gmail.com

Broughton, William J.: williamebroughton@gmail.com

Pedrosa, F.O.: fpedrosa@ufpr.br

Wassem, R.: wassem@ufpr.br

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Running title: Modulation of defence-related rice genes by *H. seropedicae*

Highlights

RNASeq of *H. seropedicae* colonised rice roots showed remarkable regulation of defence, metal transport, stress and signalling genes. Fe-uptake genes were highly induced with implications in plant nutrition and immunity.

Abstract

Rice is staple food of nearly half the world's population. Rice yields must therefore increase to feed ever larger populations. By colonising rice and other plants, *Herbaspirillum* spp. stimulate plant growth and productivity. However the molecular factors involved are largely unknown. To further explore this interaction, the transcription profiles of Nipponbare rice roots inoculated with *Herbaspirillum seropedicae* were determined by RNA-seq. Mapping the 104 million reads against the

Oryza sativa cv. Nipponbare genome produced 65 million unique mapped reads that represented 13,840 transcripts each with at least two-times coverage. About 7.4 % (1,019) genes were differentially regulated and of these 256 changed expression levels more than two times. Several of the modulated genes encoded proteins related to plant defence (e.g. a putative probenazole inducible protein), plant disease resistance as well as enzymes involved in flavonoid and isoprenoid synthesis. Genes related to the synthesis and efflux of phytosiderophores (PS) and transport of PS-iron complexes were also induced by the bacteria. These data suggest that the bacterium represses the rice defence system while concomitantly activating iron uptake. Transcripts of *H. seropedicae* were also detected amongst which genes involved in nitrogen fixation, cell motility and cell wall synthesis were the most expressed.

Keywords: *Herbaspirillum seropedicae*, rice, biotic stresses, flavonoids, nitrogen fixation, pathogenic responses, pathogen-associated molecular patterns (PAMPs)

Introduction

To answer the ever increasing demand for cereals, genetic improvement of rice and the concomitant development of bio-fertilisers are promising, low environmental-impact solutions. After water, the most limiting nutrient in plant development is nitrogen and nitrogenous fertilisers have been heavily used in rice cultivation (Ladha and Reddy, 2003). Heavy use of nitrogen fertilisers causes environmental damage including contamination of ground-water and the release of nitrogen oxides.

An alternative to the use of nitrogenous fertilisers is to employ plant-associated micro-organisms that fix nitrogen. *Herbaspirillum seropedicae* is an endophytic diazotrophic that can colonise many plants and improve their productivity (reviewed by Monteiro *et al.*, 2012; Chubatsu *et al.*, 2012). Inoculation of rice with *H. seropedicae* increased root and shoot biomass by 38 to 54 % and 22 to 50 % respectively (Gyaneshwar *et al.*, 2002) part of which was attributable to biological nitrogen fixation (James, 2000; Gyaneshwar *et al.*, 2002; James *et al.*, 2002; Roncato-Maccari *et al.*, 2003). Pankievicz *et al.* (2015) showed that the nitrogen fixed by *H. seropedicae* and *Azospirillum brasilense* was rapidly incorporated into *Setaria viridis*. Other factors, including production of phyto-hormones by the bacteria stimulate plant growth and several authors have observed that the increase in biomass of inoculated plants is dependent on the plant genotype (Gyaneshwar *et al.*, 2002; Sasaki *et al.*, 2010).

Transcriptome based studies are powerful tools to detect differentially expressed genes and discover novel molecular processes (Nobuta *et al.*, 2007; Mizuno *et al.*, 2010; Xu *et al.*, 2012, Xu-H *et al.* 2015; Wakasa *et al.*, 2014; Magbanua *et al.*, 2014; Shankar *et al.*, 2016). Expression analyses (using EST sequencing and RT-qPCR) of rice roots inoculated with *H. seropedicae* suggested that genes related to auxin and ethylene syntheses as well as defence are modulated by the microorganism in a cultivar dependent manner (Brusamarello-Santos *et al.*, 2011). Here we used RNA-seq to profile the transcriptome of rice inoculated with *H. seropedicae.*

Materials and Methods

Plant material and growth conditions

Testas were removed from seeds of rice (*Oryza sativa* ssp *japonica* cv. Nipponbare, kindly provided by the Instituto Riograndense do arroz, IRGA – Avenida Missões 342, Porto Alegre, RS, Brazil), then disinfected with 70 % (v/v) ethanol for 5 min followed by 30 min soaking in 8 % sodium hypochlorite (1 mL per seed) containing 0.1 % v/v Triton-X100. After rinsing 20 times with sterile water, the seeds were treated with 0.025 % (v/v) Vitavax-Thiram (Chentura, Avenida Nações Unidas 4777, Alto de Pinheiros, São Paulo, SP, Brazil) fungicide solution and stirred (120 rpm) for 24 h in the dark at 30 ºC. The seeds were then transferred to 0.7 % water-agar and left for two days to germinate after which the seedlings were inoculated with 1 mL of *Herbaspirillum seropedicae* strain SmR1 (10^8 cells/seedling) for 30 minutes while control seedlings were treated with 1 mL of N-free NFbHP-malate medium (Klassen *et al.*, 1997) (controls). Seedlings were washed with sterile water and transferred to glass tubes (25 cm long, 2.5 cm diameter) containing propylene beads and 25 mL of modified Hoagland's solution (Hoagland and Arnon, 1950) without nitrogen (1mM KH_2PO_4, 1mM K_2HPO_4, 2mM $MgSO_4.7H_2O$, 2mM $CaCl_2.2H_2O$, 1mL/L micronutrient solution (H_3BO_3 2.86 $g.L^{-1}$, $MnCl_2$.4H2O 1.81 $g.L^{-1}$, $ZnSO_4.7H_2O$ 0.22 $g.L^{-1}$, $CuSO_4.5H_2O$ 0.08 $g.L^{-1}$, $Na_2MoO_4.2H_2O$ 0.02 $g.L^{-1}$) and $1mL.L^{-1}$ Fe-EDTA solution ($Na_2H_2EDTA._2H_2O$ 13.4 $g.L^{-1}$ and $FeCl_3.6H_2O$ 6 $g.L^{-1}$)), pH 6.5-7.0. Plants were cultivated at 24 ºC under 14 h light and 10 h dark for 3 days. *H. seropedicae* was cultivated in NFbHP malate medium containing 5 mM glutamate as the nitrogen source. Cells were shaken (120 rpm) overnight at 30 °C, then centrifuged, washed once with N-free NFbHP-malate and suspended in the same medium to OD_{600} = 1 (corresponding to 10^8 $cells.mL^{-1}$). Strain SmR1 (Souza *et al.*, 2000) is a spontaneous streptomycin resistant mutant of strain Z78 (Baldani *et al.*, 1986). Root colonisation was monitored by counting the number of colony forming units (CFU) per gram of fresh root.

Approximately 100 mg of roots were washed in 70 % v/v ethanol for 1 min, 1% chloramine T for 1 min followed by three washes with sterile water (Dobereiner *et al.*, 1995). The roots were then crushed with a mortar and pestle in 1 mL of NFbHP-malate and serial dilutions (10^{-1} to 10^{-5}) were plated onto solid NFbHP-malate containing 20 mM NH_4Cl. Two days later the cells were counted.

RNA isolation and construction of libraries

The roots were separated from the aerial part and immediately stored in RNA later™ (Life Technologies, Foster City, CA, USA) Total RNA was extracted from roots of five rice plants using a RNAqueos kit (Ambion, Austin, TX, USA). Contaminating genomic DNA was eliminated with RNase-free DNase I (Ambion) for 30 min at 37 °C. Total RNA (7 to 10 µg) was depleted of ribosomal RNA by treatment with a RiboMinus™ Plant Kit for RNA-Seq (Invitrogen, Carlsbad, CA, USA). The integrity and quality of the total RNA was checked spectrophotometrically and by agarose gel electrophoresis. Whole Transcriptome Analysis RNA Kits™ (Life Technologies) were used on 500 ng purified RNA to construct the libraries and sequencing was performed in a SOLiD4 (Life Technologies) sequencer.

Sequencing and analysis of short reads

SOLiD sequencing produced 161 million 50 bp reads that were analysed by SAET software (Applied Biosystems – Foster City, CA, EUA) to improve base calling, followed by quality trimming using the CLC Genomics Workbench (CLC bio, a QIAGEN Company, Silkeborgvej 2, DK-8000 Aarhus C, Denmark) (quality scores higher than 0.05 and reads with less than 20 bp were discarded). Then the reads were mapped to the rice genome database from the MSU Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/) using the following parameters: a minimum length fraction of 95 %, minimum similarity of 90 % and only one hit. Differential expression was analysed using DESeq (Anders and Huber, 2010) from RobiNA software (Lohse *et al.*, 2012). Genes covered at least twice were considered expressed and regulated when expression changed two times and the P-value was lower than 0.05.

Quantification of mRNA levels using RT-qPCR

Reverse transcription quantitative PCR (RT-qPCR) analyses were used to evaluate gene expression under the conditions described above. Total RNA was isolated from rice roots using the

TRI Reagent (Sigma, St. Louis, MO, USA) and contamination with genomic DNA was removed with DNase I (Life Technologies). The integrity and quality of the total RNA was confirmed by spectrophotometric analyses and electrophoresis. cDNA was produced from 1 µg DNase-treated total RNA using high-capacity cDNA reverse transcription kits (Life Technologies). The cDNA reaction was diluted 60 times before quantitative PCR using Power SYBR-Green PCR Master Mix on a Step One Plus Real Time-PCR System (both from Life Technologies). Primer sequences are listed in Table S1 and were designed with the Primer express 3.0 software (Applied Biosystems) and the NCBI primer designing tool using the genome sequence of *O. sativa* ssp. japonica cv. Nipponbare. Calibration curves for all primer sets were linear over four orders of magnitude (R^2 = 0.98 to 0.99) and efficiencies were 90 % or higher. mRNA expression levels were normalised using the expression levels of actin 1, tubulin beta-2 chain (beta-2 tubulin) (Jain *et al.*, 2006) and a hypothetical protein (protein kinase) (Narsai *et al.*, 2010) using geNorm 3.4 software (Vandesompele *et al.*, 2002). The relative expression level was calculated according to Pfaffl (2001). Three independent samples were analysed for each condition and each sample was assayed in triplicate.

Results and Discussion

Transcriptional analyses

H. seropedicae enters rice roots via cracks at the points of lateral root emergence and later (three to 15 days) colonises the intercellular spaces, aerenchyma, cortical cells and vascular tissue (Elbeltagy *et al.*, 2001; Gyaneshwar *et al.*, 2002; James *et al.*, 2002; Roncato-Maccari *et al.*, 2003). 14 days after inoculation we observed an increase of weight of roots and leaf but this increase was not statistically significant. The number of endophytic *H. seropedicae* reached approximately 10^5 to 10^6 CFU per gram of fresh root weight one to two days after inoculation (DAI), with a peak at three DAI (data not shown). For this reason roots were collected for RNA-seq analyses three DAI when the population of *H. seropedicae* had stabilised in the intercellular spaces and xylem (Roncato-Maccari *et al.*, 2003). Rice plants inoculated in parallel with the samples used for RNA extraction contained 4.2 x 10^5 endophytic CFU.g^{-1} of fresh roots and 4.4 x 10^8 epiphytic CFU.g^{-1} of root at three DAI.

Sixty-four percent of the reads (103,563,118) were finally used for mapping to the reference rice (www.rice.plantbiology) and *H. seropedicae* genomes. Illustration of RNA-seq analyses is shown in Figure 1 and the numbers of reads mapped to each reference genome are listed in Table 1. Mapping

on the rice genome database (http://rice.plantbiology.msu.edu/) produced 22 million unique mapped reads representing 13,837 expressed transcripts.

Differentially expressed genes

Statistical analyses were performed using DESeq software (Anders and Huber, 2010) and comparison of non-inoculated with inoculated samples revealed 1,015 differentially expressed genes ($P < 0.05$). Amongst these, 255 had fold-changes higher than two and they were functionally categorised using MapMan (http://mapman.gabipd.org/) (Figure 2). Considering the number of regulated genes in relation to the number of expressed genes in each category, the main categories down-regulated by *H. seropedicae* were: metal handling (9.0%; 4/41), polyamine metabolism (9.1%; 1/11), secondary metabolism (9%; 15/167), hormone metabolism (4.7%; 11/233); stress (4.8%; 24/503) and nucleotide metabolism (4.9%; 6/123). Among genes up-regulated by bacteria were those involved in gluconeogenesis/glyoxylate cycles (28.6%; 01/07), polyamine metabolism (27.3%; 03/11), metal handling (24.4%; 10/41), fermentation (23.1%; 03/13) and nitrogen metabolism (18.8%; 03/16) (Figure 2). Some of these categories of regulated genes were analysed in more detail below.

Biotic and abiotic stresses

Among the 255 differentially expressed genes (> 2fold), 59 were stress-related (30 repressed and 29 induced) in the following categories: secondary metabolites, hormone signalling, cell-wall, proteolysis, PR-proteins, signalling, transcription factors, redox-state, abiotic stress and peroxidases (Figure 3 and Table S2).

Secondary metabolism

Amongst the secondary metabolic pathways with higher numbers of regulated genes were those involved in phenylpropanoid and isoprenoid synthesis (Table S2). Phenylpropanoids synthesised by deamination of L-phenylalanine are eventually converted to *p*-coumaric acid, a precursor of flavonoids and lignin (Ferrer *et al.*, 2008; Hassan and Mathesius, 2012). *H. seropedicae* modulated expression of four genes involved in flavonoid synthesis. Amongst them, the gene encoding chalcone isomerase (CHI), which catalyses the synthesis of naringenin from tetrahydroxy-chalcone (Naoumkina *et al.*, 2010), was repressed 2.1-fold. Naringenin is a key intermediate in the synthesis of other compounds including flavonol [flavonol synthase (FS), repressed 8.3-fold by *H. seropedicae*], and

anthocyanins [dihydroxiflavonol 4-reductase (DRF), repressed 2.5-fold]. In addition, isoflavone reductase (IFR) gene, involved in isoflavone synthesis, was repressed 1.3-fold (P = 0.01). RT-qPCR of FS confirmed repression by *H. seropedicae* (Figure 4) but to a much lower extent (1.8-fold). Flavonols such as quercetin (Hassan and Mathesius, 2012) exhibit antimicrobial activity possibly by binding and inhibiting DNA gyrase (Plaper *et al.*, 2003). Previously, Balsanelli *et al.* (2010) showed that narigenin has antimicrobial activity against *H. seropedicae*.

Naoumkina *et al.* (2010) reported that flavonone-3-β-hydroxylase was induced in rice following infection with *Xanthomonas oryzae*. Nematode cysts stimulate isoflavone synthesis (including chalcone reductases, chalcone isomerase, isoflavon 2'-hydroxylase, isoflavones and isoflavone reductase synthase) in soybeans. Our data thus indicate that down-regulation of flavonoid/isoflavone synthesis by *H. seropedicae* is part of the attenuation of the defence system in rice roots that is necessary to host an endophyte.

As mentioned above *p*-coumaric acid is also a precursor of lignin which is assembled from monolignols (Vanholme *et al.*, 2010). In this pathway cinnamoyl-CoA esters are converted into monolignols by two enzymes, cinnamoyl-CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) (Boerjan *et al.*, 2003). RNA-seq data showed that a gene (LOC_Os02g56700.1) encoding a cinnamoyl CoA reductase was induced 2.8-fold in rice inoculated with *H. seropedicae* (Table S2) while Kawasaki *et al.* (2006) reported that cinnamoyl-CoA reductase 1 (OsCCR1 - LOC_Os02g56460.1) is an effector of the small GTPase Rac that is involved in defence responses in rice (Kawasaki *et al.*, 1999; Ono *et al.*, 2001; Suharsono *et al.*, 2002; Akamatsu *et al.*, 2013). Transcriptome analyses of rice inoculated with the endophyte *Harpophora oryzae* or the pathogen *Magnaporthe oryzae* showed that a cinnamoyl CoA reductase (LOC_Os08g34280.1) like other enzymes in lignin synthesis was repressed in beneficial interactions and induced in pathogenic ones (Xu *et al.*, 2015). LOC_Os08g34280.1 was also slightly repressed in our experiments (≈1.3 fold). It therefore seems as if a balance between induction and repression of defence-related genes allows *H. seropedicae* to survive inside rice tissues while concomittantly activating some defence responses to protect the plant from pathogens.

Isoprenoid (or terpenoid) synthesis was also modulated by *H. seropedicae* (Table S2 and Figure 5). Isoprenoids are derived from isomeric compounds including isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) (Chen *et al.*, 2011) via two pathways: the mevalonate pathway (MEV) and the methyl-erythritol phosphate pathway (MEP) (Figure 5). The MEV pathway

247 occurs in the cytoplasm and the MEP pathway in plastids (Vranová *et al.*, 2013) producing an
248 estimated that 55,000 isoprenoid compounds. As a consequence, the biological function of these
249 molecules is diverse and includes precursors of hormones, components of membranes, defence agents
250 and carotenoids.

251 In rice, *H. seropedicae* repressed genes of the MEP pathway (Table S2 and Figure 5). The
252 most repressed gene (5.9 times) encodes 1-deoxy-D-xylulose 5-phosphate synthase (DXS) the first
253 enzyme of the pathway that synthesises 1-deoxy-D-xylulose 5-phosphate (DXP) from pyruvate and D-
254 glyceraldehyde 3-phosphate. DXP is a precursor of antimicrobial compounds including phytoalexins.
255 Chitin induces the MEP pathway in cultured rice cells and as a result phytoalexins accumulate (Okada
256 *et al.*, 2007). Furthermore treatment of the cells with inhibitors of the enzymes DXS and 1-deoxy-D-
257 xylulose 5-phosphate reductoisomerase (DXR) impeded chitin-dependent accumulation of
258 phytoalexin. The authors suggest that activation of the MEP is required to meet the demand for
259 isoprenoid and phytoalexin synthesis in infected cells. In our experiments and those of Drogue *et al.*
260 (2014), the gene encoding stemar-13-ene synthase (OsDTC2), an enzyme involved in synthesis of
261 phytoalexins in rice, was repressed 1.9-fold in *H. seropedicae* treated rice roots (Figure 5) and two-
262 fold when infected with *Azospirillum* spp. (Drogue *et al.*, 2014). It thus seems as if *H. seropedicae*
263 represses production of defence-related isoprenoids in rice roots perhaps to allow the bacteria to enter
264 and rapidly colonise the intercellular spaces and xylem.
265
266 *Oxidative stress responses*
267 In this study *H. seropedicae* modulated expression of five redox state genes including those
268 for non-symbiotic hemoglobin 2 and peroxiredoxin. One peroxiredoxin gene (LOC_Os07g44440.1)
269 induced 3.7 and the other LOC_Os01g16152.1 was repressed 2.1 fold. Peroxiredoxins are a group of
270 H_2O_2-decomposing antioxidant enzymes related to the redox state. In addition to the reduction of
271 H_2O_2, peroxiredoxin proteins also detoxify alkyl hydroperoxides and peroxinitrite (Rhee *et al.*, 2005;
272 Dietz *et al.*, 2006).
273 Plants respond to attacks by pathogens with rapid increases in reactive oxygen species (ROS)
274 such as superoxide and H_2O_2 (Apostol *et al.*, 1989). Peroxidases produce ROS that could cause
275 oxidative damage to proteins, DNA, and lipids. Many defects in the immune system of mature
276 *Arabidopsis thaliana* plants with reduced expression of two key peroxidase genes, PRX33 or PRX34,
277 were observed (Daudi *et al.*, 2012). Silencing the French-bean class III peroxidase (FBP1) in *A.*

thaliana impaired the oxidative burst and rendered plants more susceptible to bacterial and fungal pathogens (Bindschedler *et al.*, 2006). Proteomic studies of rice roots seven days after inoculation with *H. seropedicae* showed induction of ascorbate peroxidases (Alberton *et al.*, 2013), though the genes encoding these enzymes were not affected in our RNA-seq analyses. Moreover, seven days after inoculation, ROS levels in *Herbaspirillum rubrisubalbicans* attached to rice roots had increased suggesting that the bacteria were subject to oxidative stresses (Valdameri *et al.*, 2017). In this work, two peroxidase genes were induced by *H. seropedicae* in inoculated roots.

Cell wall

A variety of diazotrophic microorganisms such as *H. seropedicae* Z67, *H. rubrisubalbicans* and *A. brasilense* produce cell-wall degrading enzymes (Elbeltagy *et al.*, 2001; James *et al.*, 2002). Interestingly, the *H. seropedicae* SmR1 genome did not reveal genes coding for known cellulases, pectinases or any other cell-wall degrading enzymes (Pedrosa *et al.*, 2011). Nevertheless, rice roots inoculated with *H. seropedicae* induced a gene (2.0-fold) encoding a β-D-xylosidase and repressed 2.4-fold a gene coding for a polygalacturonase suggesting re-modeling of plant cell wall. In *A. thaliana*, β-D-xylosidase has been shown to be involved in secondary cell-wall hemi-cellulose metabolism and plant development (Goujon *et al.*, 2003), but little is known about the function of this enzyme.

Other differentially expressed genes involved in cell-wall metabolism code for proteins similar to an expansin11 (induced 2.6-fold). Expansins have a loosening effect on plant cell-walls and function in cell enlargement as well as in diverse developmental processes in which cell-wall modification occurs including elongation. In addition, they promote elongation of root-hairs (Lin *et al.*, 2011; ZhiMing *et al.*, 2011) and root-hair initiation (Kwasniewski and Szarejko, 2006).

Expansins have been correlated with plant-bacteria interactions. In tobacco, *Bacillus subtilis* G1, a plant growth promoting bacterium, induced the expression of two expansins NtEXP2 and NtEXP6 (Wang *et al.*, 2009). Also, inoculation of *Melilotus alba* with *Sinorhizobium meliloti* lead to enhanced MaEXP1 mRNA levels in roots and nodules (Giordano and Hirsch, 2004). Together, these results suggest that inoculation with *H. seropedicae* also led to modification of plant cell wall which may facilitate bacterial colonization of inner tissue by loosening cell wall.

Plant immune responses

The plant immune system can sense and respond to pathogen attacks in two manners: the first involves recognition of pathogen/microbe associated molecular patterns (PAMPs) by surface pattern-recognition receptors (PRRs) resulting in pattern-triggered immunity (PTI). Second, resistance proteins (R) that recognise pathogen effectors are expressed leading to effector-triggered immunity (ETI) (Abramovitch *et al.*, 2006; Jones and Dangl, 2006; McDowell and Simon, 2008). Among the regulated genes that have functions involved in defence (in dark gray squares in Figure 3), as determined by MapMan, three genes encoding PR-proteins were repressed (LOC_Os10g25870.1, LOC_Os11g07680.1, LOC_Os02g38392.1, 2.5, 3.4 and 2.7-fold respectively) while one transcript that encoded a lipase called EDS1 (enhanced disease susceptibility 1) was induced 2-fold.

The role of EDS1 in defence is well described in *Arabidopsis* and is required for resistance conditioned by R genes which encode proteins that contain nucleotide-binding sites and leucine-rich repeats (NBS-LLR) (Aarts *et al.*, 1998; McHale *et al.*, 2006; Bartsch *et al.*, 2006; Jones and Dangl, 2006; Venugopal *et al.*, 2009). Mutant *eds1* seedlings exhibited enhanced susceptibility to the biotrophic oomycete *Peronospora parasifica* (Parker *et al.*, 1996). Analysis of *EDS1* and *PR*-gene expression showed induction of both genes after inoculation with *Pseudomonas syringae* or treatment with salicylic acid (SA) (Falk *et al.*, 1999). In addition, in the *Arabidopsis eds1* mutant, the expression of PR-proteins was undetectable. When the *eds1* mutant was treated with SA however, expression of PR was detected. The authors suggest that EDS1 functions upstream of *PR1*-mRNA accumulation. Among the 3 genes for PR-proteins repressed, one (LOC_Os02g38392.1) is a NBS-LRR disease resistance protein. Therefore in *H. seropedicae*- rice interaction an inverse correlation between PR-protein and EDS1 expression was observed, perhaps suggesting a fine regulation of these defence systems by *H. seropedicae.*

In previous work down-regulation of genes associated with defence was observed in rice plants inoculated with *H. seropedicae* such as a putative probenazole inducible protein (PBZ1, LOC_Os12g36840.1) that was repressed 3.6-fold as shown by RT-qPCR analysis (Brusamarello-Santos *et al.*, 2011). RNA-seq data showed that this gene (LOC_Os12g36840.1) was repressed 2.9-fold. Another two genes coding proteins similar to PBZ1 (LOC_Os12g36830.1 and LOC_Os12g36850.1) were also repressed (7.2 and 4.1, respectively). Kawahara *et al.* (2012) using an RNA-seq approach to study the transcriptome of rice inoculated with the blast fungus *M. oryzae* observed that the same PBZ1 genes detected in our study were induced 273 and 233-fold upon

inoculation with the pathogen. The induction of PBZ1 gene by pathogens has been considered as a molecular marker for rice defence response (Kim *et al*., 2008).

Another gene regulated by *H. seropedicae* that is related to defence codes for a thionin, a small cysteine-rich protein that occurs in a broad range of plant species (Florack and Stiekema W.J., 1994). Thionins are known for their toxicity to plant pathogens and several studies showed that their over-expression is related to increased resistance to diseases (Iwai *et al.*, 2002; Choi *et al.*, 2008; Muramoto *et al.*, 2012; Ji *et al.*, 2015).

Brusamarello-Santos *et al.* (2011) showed 5-fold repression of thionin genes from chromosome 6 seven days after inoculation with *H. seropedicae* but these thionins from chromosome 6 were not regulated in roots three days after inoculation with *H. seropedicae*. Since there are 15 thionin genes (according to the Rice Genome Annotation Project RGAP7) sharing high identity in chromosome 6, we mapped the unmapped reads to the rice genome as well as to chromosome 6 separately and observed an average of 480 mapped reads for control libraries and 136 for inoculated libraries mapping on thionin genes, a result that is in accordance with repression patterns observed in rice roots seven days after inoculation with *H. seropedicae* (Brusamarello-Santos *et al.*, 2011). Interestingly, we observed a thionin transcript from chromosome 7 (LOC_Os07g24830.1) that was induced 7.2-fold in the presence of *H. seropedicae* three DAI. Time-dependent regulation of thionin was also observed by Ji *et al.* (2015) in rice roots infected with *Meloidogyne graminicola*. In a by Straub and co-workers study only a few defence-related genes were induced in the transcriptome of *Miscanthus sinensis* inoculated with *Herbaspirillum frisingense* helping to explain why this bacterium can effectively invade and colonise plants (Straub *et al.*, 2013).

Signalling

Plant Receptor

The plant immune system employ, at the cell surface, receptors to perceive pathogen associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs). Theses receptors have an ectodomain potentially involved in ligand binding, a single transmembrane domain and, most of times, a intracellular kinase domain (Couto and Zipfel, 2016).

Amongst the genes with the highest expression differences seen in RNA-seq data was a wall-associated receptor kinase-like (WAK) 22 precursor gene (LOC_Os10g07556.1), repressed 93-fold (30-fold by RT-qPCR) in *H. seropedicae* inoculated roots. Cell wall-associated receptor kinases

(WAKs) contain an extracellular domain composed of one or more epidermal growth factor repeats (EGF). Animal proteins containing these repeats are known to bind small peptides (Hynes and MacDonald, 2009). In *A. thaliana* WAKs bind to cross-linked pectin cell wall, pathogen- or damage-induced pectin fragments and oligogalacturonides, thus regulating cell expansion or stress response depending on the state of the pectin (Kohorn and Kohorn, 2012; Kohorn, 2015, 2016). Several studies have described the role of WAK genes in rice resistance to pathogens (Li *et al.*, 2009; Cayrol *et al.*, 2016; Harkenrider *et al.*, 2016; Delteil *et al.*, 2016). Plant proteins show a similar domain architecture consisting of cell-wall pectin binding extracellular region, the EGF-like domain, and a kinase domain. Analyses of rice loss-of-function mutants of WAK genes showed that individual genes are important for resistance against *M. oryzae*. OsWAK14, OsWAK91 and OsWAK92 positively regulate resistance while OsWAK112d is a negative regulator of blast resistance (Delteil *et al.*, 2016). Cayrol et al. (2016) demonstrated that OsWAK14, OsWAK91 and OsWAK92 can form homo- and hetero-complexes and hypothesized that the loss of function of any of these proteins may destabilized the complex and affect their functioning. In *A. thaliana* the WAK22 orthologous gene (AtWAKL10) encodes a functional guanylyl cyclase which is co-expressed with pathogen defence related genes (Meier *et al.*, 2010). Thus the wall-associated receptor kinase-like 22 gene of rice could be a candidate for surface pattern recognition receptor and its repression may allow *H. seropedicae* to evade activation of the plant-defence system.

A cysteine-rich receptor-like protein kinase (CRK) gene (LOC_Os04g56430.1) and a serine/threonine-protein kinase At1g18390 precursor (LOC_Os05g47770.1) were induced 3.2-fold and 2-fold, respectively, while a lectin-like receptor kinase 7 (LOC_Os07g03790.1) and SHR5-receptor-like kinase (LOC_Os08g10320.1) were repressed 2.7 and 2.5-fold respectively in the presence of *H. seropedicae* (Table S2). The latter protein has 75% identity with sugarcane SHR5 receptor kinase repressed by colonisation with diazotrophic endophytes (Vinagre *et al.*, 2006). The authors suggested that the expression levels of this gene were inversely related to the efficiency of beneficial plant-bacterial interactions (Vinagre *et al.*, 2006). Besides in *Arabidopsis* cysteine rich receptor-like kinase 5 protein is involved in regulation of growth, development, and acclimatory responses (Burdiak *et al.*, 2015).

Auxin signalling

Auxins regulate diverse physiological processes such as vascular tissue differentiation, lateral root initiation and have also been linked to defence in plant-pathogen interactions (Rogg and Bartel, 2001; Kazan and Manners, 2009; McSteen, 2010; Adamowski and Friml, 2015). Auxin response elements (AuxREs), when bound to auxin response factors (ARFs), control auxin-dependent gene expression. The Aux/IAA protein family members that inhibit ARFs mediate this regulation (Guilfoyle, 2015).

Four differentially expressed genes related to auxin signalling were identified in rice roots inoculated with *H. seropedicae*. The gene LOC_Os08g24790.1 encoding an auxin-responsive protein was repressed 2.1-fold (Table 2) whereas the genes coding for auxin-induced proteins, LOC_Os09g25770.1 and LOC_Os05g01570.1, were repressed 1.8 and 2.5-fold, respectively. Repression of genes related to auxin signalling was reported in rice roots inoculated with *H. seropedicae* (Brusamarello-Santos et al. 2011). Brusamarello-Santos et al. (2011) found that ARF2-like, IAA 11 and IAA18 were repressed, 1.4, 1.5 and 2.8-fold respectively, in the presence of *H. seropedicae* 7 days after inoculation. These genes were not regulated in our data probably due to the time difference of the cDNA library construction and too low expression levels.

H. seropedicae can produce IAA in the presence of tryptophan, thus suggesting the plant growth promotion effect may be due to bacterial derived auxin (Bastian *et al.*, 1998). In addition exogenous application of auxin lead to increase in lateral root numbers (Inukai *et al.*, 2005). However the number of lateral roots in rice plants inoculated and non-inoculated with *H. seropedicae*, was determined (data not shown) but no significant difference was observed.

In the absence of clear regulation of auxin-dependent genes and phenotype, the results suggest that auxin does not play an important role in Nipponbare rice roots colonization by *H. seropedicae*. On the other hand the repression of auxin signalling has been correlated to defence responses. Auxin levels have been correlated with susceptibility to pathogens (Kazan and Manners, 2009) that are able to produce high levels of auxin (Jameson, 2000). It has also been shown that pathogen-associated molecular patterns (PAMPs) induce the expression of a miRNA that negatively regulates mRNAs for F-box auxin receptors leading to resistance to *P. syringae* in *Arabidopsis* (Navarro *et al.*, 2006). The observed repression in several genes involved with defence in the rice-*H. seropedicae* interaction opens the question whether auxin could be important for bacteria survival inside the plant by down-regulation defence system. Further studies are needed to elucidate if and how auxin signalling participates in plant-bacterial interactions.

Ethylene signalling

Ethylene is also involved in several biological processes that activate defence responses and adventitious root-growth in rice and other plants (Lorbiecke and Sauter, 1999; Glazebrook, 2005; Robert-Seilaniantz *et al.*, 2011). Ethylene can be synthesised by oxidation of 1-aminocyclopropane-1-carboxylate (ACC) by ACC oxidase. ACC is synthesised from adenosylmethionine (AdoMet) by ACC synthase (ACS). ACS is up regulated 6-fold and ACC oxidase is repressed 2-fold by inoculation with *H. seropedicae*. An ethylene response factor (ERF) (LOC_Os01g04800.1) gene was also induced 2.2-fold in inoculated roots. These data indicate that ethylene synthesis is attenuated in the presence of bacteria. Moreover Alberton et al. (2013) measured the level of ethylene in inoculated rice roots (seven days after inoculation) and found a decrease of ethylene levels. Furthermore, Valdameri *et al.* (2017) detected induction of ACC oxidase in rice plants inoculated with the pathogen *H. rubrisubalbicans*. These results suggest that the ethylene pathway is differentially modulated in the presence of pathogens and beneficial endophytic bacteria that promote plant growth.

SA signalling

Salicylic acid is derived from phenolic compounds and is involved in response to attack by pathogens (Vlot *et al.*, 2009; An and Mou, 2011). In rice roots inoculated with *H. seropedicae*, a SA-dependent carboxyl methyltransferase family protein (LOC_Os11g15340.2) was repressed 40-fold. A member of this family is salicylic acid carboxyl methyltransferase (SAMT) that catalyses the formation of methyl salicylate (MeSA) from SA (Ross *et al.*, 1999). MeSA is an essential signal for systemic acquired resistance (SAR) in tobacco plants. In addition mutations in SAMT showed that this gene is required for SAR (Park *et al.*, 2007).

SA signalling is differentially regulated by members of the WRKY transcription factor family (Eulgem and Somssich, 2007). In inoculated rice roots, two WRKY transcription factors were regulated, one repressed (2.3-fold) and one induced (2.0-fold). The induced gene encodes a WRKY51 similar to WRKY11 of *Arabidopsis*, whereas the repressed gene encodes WRKY46, which was shown to be induced by SA in *Arabidopsis*. WRKY11 is a negative regulator of resistance (Journot-Catalino *et al.*, 2006) and *Arabidopsis* plants in which WRKY46 was over-expressed were more resistant to *P. syringae* (Hu *et al.*, 2012). These results are in agreement with the hypothesis that the SA signalling and defence system are attenuated in the presence of the *H. seropedicae*.

462

463 **Metal ion metabolism**

464 Several genes related to metal transport were differentially expressed, most of them up
465 regulated in the presence of *H. seropedicae*. Amongst the 20 most highly regulated rice genes, eight
466 were related to metal transport (Table 3; Table 4). Phyto-siderophore synthesis starts with production
467 of nicotianamine (NA) from S-adenosylmethionine, which in turn is derived from 5′-
468 methylthioadenosine of the methionine salvage pathway (MTA cycle) (Figure 6). Transcripts of
469 enzymes of the MTA cycle encoding SAM, MTA nucleosidase, MTR kinase, E1 enolase/phosphatase
470 and acireductone dioxygenase (ARD) were induced 1.9, 2.6, 6.0, 1.9 and 7.5-times, respectively, in
471 roots colonised by *H. seropedicae* (Figure 6). Using proteomic and RT-qPCR analyses, Alberton
472 (2013) observed similar expression patterns in rice roots inoculated with *H. seropedicae*.

473 The synthesis of NA and MAs involves a set of enzymes including S-adenosylmethionine
474 syntase (SAM) that catalyses the adenylation of L-methionine to S-adenosylmethionine, nicotianamine
475 synthase (NAS) that converts S-adenosylmethionine to nicotianamine, and nicotianamine
476 aminotransferase (NAAT) that catalyses the amino transfer of NA to produce the 3’’-keto intermediate
477 that is reduced by deoxymugineic acid synthase (DMAS) to produce 2´- deoxymugineic acid (DMA)
478 (Shojima *et al.*, 1990; Higuchi *et al.*, 1999; Bashir *et al.*, 2006; Inoue *et al.*, 2008).

479 Two nicotianamine synthase (NAS) genes of rice - LOC_Os03g19420.2 and
480 LOC_Os03g19427.1 were induced (52- and 32-fold, respectively) in inoculated rice roots.
481 LOC_Os03g19427.1 was also induced in rice roots inoculated with *Azospirillum spp.* (Drogue *et al.*,
482 2014). Increased levels of nicotianamine have been shown to increase Fe uptake in rice plants (Lee *et*
483 *al.*, 2009). Furthermore, in *Lotus japonicus* inoculated with *Mesorhizobium loti*, nicotianamine
484 synthase 2 was expressed only in nodules pointing to a role in symbiotic nitrogen fixation (Hakoyama
485 *et al.*, 2009). A tomato mutant defective in the synthesis of nicotianamine was affected in iron
486 metabolism (Ling *et al.*, 1996). In addition NAAT (LOC_Os02g20360.1) and DMAS
487 (LOC_Os03g13390.2) were also induced 10-fold and 19-times respectively in colonised rice roots.
488 Iron deficiency provoked induction of rice gene OsNAAT1 (Inoue *et al.*, 2008).

489 Recently, members of a major facilitator super-family have been described as essential to the
490 efflux of MA and NA in rice. TOM1 (transporter of mugineic acid 1) is involved in the efflux of MA,
491 while ENA1 (efflux transporters of nicotianamine 1) and ENA2 in the efflux of NA (Nozoye *et al.*,

2011). TOM1 (LOC_Os11g04020.1) and ENA1 (LOC_Os11g05390.1) were induced 40 and 3-fold in colonised roots. Induction of TOM1 was confirmed by RT-qPCR (Figure 4).

Fe^{+++} ions chelated by PS need to be transported inside the cell. In gramineous plants, two groups of Fe-MA transporters are present: ZmYS1 (Curie *et al.*, 2001) and the YSL (yellow strip-like) transporter family (Inoue *et al.*, 2009). Inoue et al. (2009) analysed the expression of 18 YSL genes in rice and observed induction of OsYSL15 and OSYSL16 genes under iron-deficiency. Other studies have shown that OsYSL2 (LOC_Os02g43370) takes up Fe^{2+}-NA (Ishimaru *et al.*, 2010) and OsYSL16 Fe^{3+}-DMA (LOC_Os04g45900.1) (Kakei *et al.*, 2012). Here we showed induction (3.2-fold) of OsYSL16 (LOC_Os04g45900.1) and 21-fold increase of transcripts encoding a gene similar to OSYSL15 (LOC_Os02g43410.1). Induction of OSYSL15 was confirmed by RT-qPCR (Figure 4). Furthermore, OsIRT1 (iron-regulated transporter 1) and OsIRT2 were also induced under low Fe conditions (Ishimaru *et al.*, 2006). We detected induction of OsIRT2 (LOC_Os03g46454.1) (34-fold in RNA-seq and 93-fold in RT-qPCR) (Figure4).

Other metal transporters such as Nramp6 (LOC_Os07g15460.1) were also up regulated (27-fold) in colonised rice roots, a result confirmed by RT-qPCR (Figure 4). Members of the natural resistance-associated macrophage protein (NRAMP) family are transition metal cation/proton co-transporters or anti-porters of broad specificity. AtNRAMP6 of *Arabidopsis* is up-regulated in response to iron deficiency and is involved with metal mobilization from vacuoles to cytosol (Thomine *et al.*, 2003). Induction of Nramp6 in rice roots colonised by *H. seropedicae*. In a previous study in rice inoculated with *Azospirillum* spp the expression of this gene was also induced (Drogue et al. 2014). In addition a gene for an integral membrane protein (LOC_Os09g23300.1) named OsVIT2 was 17.7-fold repressed by *H. seropedicae*. This gene is involved in transport of Fe/Zn into vacuoles and is up-regulated in rice roots with excess Fe. Knockout/ knockdown of this gene led to Fe accumulation in seeds (Zhang *et al.*, 2012; Bashir *et al.*, 2013). Together, these results suggest that colonised roots respond in such a manner as to accumulate Fe.

Interestingly the transcript with the highest fold change [104-times – a result confirmed using RT-qPCR (Figure 4)] in colonised roots codes for the non-symbiotic hemoglobin 2 (LOC_Os03g12510.1). High levels of non-symbiotic hemoglobin 2 could help buffer free oxygen and protect bacterial nitrogenase. Arredondo-Peter *et al.* (1997) described two hemoglobins, Hb1 and Hb2, in rice. The Hb2 induced by *H. seropedicae* is very similar to the one described by Arredondo-Peter et al. (1997) (coverage 82 % with an identity of 97 %). In addition, Lira-Ruan *et al.* (2001) studied the

synthesis of hemoglobins in rice under normal and stress conditions, coming to the conclusion that Hbs are not part of a generalised stress response. They demonstrated that Hb1 is expressed in different rice organs (root and leaves) during plant development. In etiolated rice plants under 0_2 limiting conditions the Hb levels increase (Lira-Ruan et al. 2001). This increase suggest that Hb expression maybe due to reduced O_2 levels in the presence of the bacteria which make the root environment microaerophilic. The higher requirement for Fe needed for incorporation into Hb may partially explain activation of siderophore synthesis and Fe accumulation. We also found a *H. seropedicae* bacterioferritin (Hsero_1195) gene induced (2.2-fold), but symptoms of Fe deficiency in colonised rice plants were not observed.

Iron homeostasis has been related to plant defence, ROS accumulation and immunity. Also, Fe deficiency triggers accumulation of antimicrobial phenolics compounds. It has been suggested recently that Fe sequestration by bacterial siderophore could be a signal for pathogen infection (Aznar *et al.*, 2015). However, bacterial genes involved in siderophore biosynthesis were not observed among the *H. seropedicae* genes expressed in rice roots. Also, transcriptomic analysis of *H. seropedicae* attached to wheat and mayze roots did not show iron metabolism genes up-regulated (Pankievicz *et al.*, 2016; Balsanelli *et al.*, 2016). These results suggest that the effect of bacteria on plant iron mebabolism is more complex than those caused by iron sequestration.

Transcripts expressed in interactions with bacteria

The libraries from inoculated roots were mapped against the *H. seropedicae* genome (24,278 reads). Amongst the 4,085 annotated genes, 287 had at least one-fold coverage (this set of genes was called *H. seropedicae* expressed genes). Functional classification of expressed genes was performed using the COG system. After ribosomal genes, the most abundant functional classes found were “unknown”, “energy production and conversion”, “amino acid transport and metabolism”, “cell motility and cell wall” (Table 5). Comparison of expressed genes of *H. seropedicae* detected in plants with bacterial genes expressed in culture revealed only 16 differences [p-value < 0.05 using the DESeq statistical package (Table 5)].

Genes involved in nitrogen fixation (*nif*) were found amongst genes classified as “energy production and conversion” and “amino acid transport and metabolism”. *Nif*-genes encode proteins involved in the synthesis, maturation and assembly of the nitrogenase complex (Chubatsu *et al.*, 2012). *nifD* and *nifH* (coverage 1.7 and 1.2 respectively) were highly expressed in *H. seropedicae* colonising

554 wheat and maize roots (Pankievicz *et al.*, 2016; Balsanelli *et al.*, 2016). We also found two ferredoxin
555 genes important for nitrogenase activity: *fdxA* and *fdxN* (coverage 1.9 and 1.6) (Souza *et al.*, 2010).
556 The promoters of *nif* genes are activated by NifA that is regulated by the Ntr system. When
557 comparisons were made between free-living *H. seropedicae* and those grown in association with rice,
558 *glnK* and *amtB* of the Ntr system were induced 43 and 20-times respectively in the plant-bacterial
559 interaction. Pankievicz *et al*. (2016) also found that *amtB* was induced in *H. seropedicae* attached to
560 maize roots. Furthermore, *fixN* and *fixP* (both 1.6X coverage) were also detected *in planta* along with
561 an *urtA* ABC-type urea transport system (Hsero_4713) (27-fold of induction *in planta*).
562 Twenty-three genes related to cell motility were found, four with > 2X coverage. Amongst
563 these were *cheW* (a positive regulator of CheA), *flhD, fliC* and *pilZ* (Hsero_2062) that encodes a type
564 IV pilus assembly protein. A methyl-accepting chemotaxis trans-membrane protein (Hsero_2723) was
565 induced 90-fold when compared with expression in culture (Tadra-Sfeir *et al.*, 2015). This gene was
566 also found to be up-regulated in epiphytic *H. seropedicae* colonising wheat and maize (Pankievicz *et*
567 *al*., 2016; Balsanelli *et al.*, 2016). Methyl-accepting chemotaxis proteins interact with Che proteins to
568 detect signals from the environment. Although other T4SS genes were not found perhaps these data
569 suggest that type IV secretion plays a role in *H-seropedicae*-rice interactions. In the nitrogen-fixing,
570 endophytic bacterium *Azoarcus* spp., mutation of type IV pilus *pilAB* genes negatively affects
571 colonisation of rice roots (Dörr *et al.*, 1998).
572 Among the 19 cell-wall related genes, seven were covered at least 2X and an outer-membrane
573 porin (Hsero_4295) was induced 28X. Another membrane-protein (Hsero_0083) of unknown function
574 was induced 14-fold. These could be proteins that *H. seropedicae* uses to recognise rice.
575
576 **Supplementary Data**
577 Table S1 – Oligonucleotides used in this research
578 Table S2 – Genes involved in secondary metabolism and abiotic and biotic stresses
579 modulated in rice roots colonised with *H. seropedicae.*
580 Table S3 - Differentially expressed genes in rice roots colonised by *H. seropedicae*
581
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References

Aarts N, Metz M, Holub E, Staskawicz BJ, Daniels MJ, Parker JE. 1998. Different requirements for EDS1 and NDR1 by disease resistance genes define at least two R gene-mediated signaling pathways in Arabidopsis. Proceedings of the National Academy of Sciences of the United States of America **95**, 10306–10311.

Abramovitch RB, Anderson JC, Martin GB. 2006. Bacterial elicitation and evasion of plant innate immunity. Nature reviews. Molecular cell biology **7**, 601–611.

Adamowski M, Friml J. 2015. PIN-dependent auxin transport: action, regulation, and evolution. The Plant Cell **27**, 20–32.

Akamatsu A, Wong HL, Fujiwara M, *et al.* 2013. An OsCEBiP/OsCERK1-OsRacGEF1-OsRac1 module is an essential early component of chitin-induced rice immunity. Cell Host & Microbe **13**, 465–476.

Alberton D, Müller-Santos M, Brusamarello-Santos LCC, Valdameri G, Cordeiro FA, Yates MG, de Oliveira Pedrosa F, de Souza EM. 2013. Comparative proteomics analysis of the rice roots colonized by Herbaspirillum seropedicae strain SmR1 reveals induction of the methionine recycling in the plant host. Journal of Proteome Research **12**, 4757–4768.

An C, Mou Z. 2011. Salicylic acid and its function in plant immunity. Journal of integrative plant biology **53**, 412–428.

Anders S, Huber W. 2010. Differential expression analysis for sequence count data. Genome biology **11**, R106.

Apostol I, Heinstein PF, Low PS. 1989. Rapid Stimulation of an Oxidative Burst during Elicitation of Cultured Plant Cells□: Role in Defense and Signal Transduction. Plant physiology **90**, 109–116.

Arredondo-Peter R, Hargrove MS, Sarath G, Moran JF, Lohrman J, Olson JS, Klucas RV. 1997. Rice hemoglobins. Gene cloning, analysis, and O2-binding kinetics of a recombinant protein synthesized in Escherichia coli. Plant physiology **115**, 1259–1266.

Aznar A, Chen NWG, Thomine S, Dellagi A. 2015. Immunity to plant pathogens and iron homeostasis. Plant Science: An International Journal of Experimental Plant Biology **240**, 90–97.

Baldani JI, Baldani VLD, Seldin L, Döbereiner J. 1986. Characterization of Herbaspirillum seropedicae gen. nov., sp. nov., a Root-Associated Nitrogen-Fixing Bacterium. International Journal of Systematic Bacteriology **36**, 86–93.

Balsanelli E, Serrato RV, de Baura VA, Sassaki G, Yates MG, Rigo LU, Pedrosa FO, de Souza EM, Monteiro RA. 2010. Herbaspirillum seropedicae rfbB and rfbC genes are required for maize colonization. Environmental Microbiology **12**, 2233–2244.

Balsanelli E, Tadra-Sfeir MZ, Faoro H, Pankievicz VC, de Baura VA, Pedrosa FO, de Souza EM, Dixon R, Monteiro RA. 2016. Molecular adaptations of Herbaspirillum seropedicae during colonization of the maize rhizosphere. Environmental Microbiology **18**, 2343–2356.

Bartsch M, Gobbato E, Bednarek P, Debey S, Schultze JL, Bautor J, Parker JE. 2006. Salicylic acid-independent ENHANCED DISEASE SUSCEPTIBILITY1 signaling in Arabidopsis immunity and cell death is regulated by the monooxygenase FMO1 and the Nudix hydrolase NUDT7. The Plant cell **18**, 1038–1051.

Bashir K, Inoue H, Nagasaka S, Takahashi M, Nakanishi H, Mori S, Nishizawa NK. 2006. Cloning and characterization of deoxymugineic acid synthase genes from graminaceous plants. The Journal of biological chemistry **281**, 32395–32402.

Bashir K, Takahashi R, Akhtar S, Ishimaru Y, Nakanishi H, Nishizawa NK. 2013. The knockdown of OsVIT2 and MIT affects iron localization in rice seed. Rice (New York, N.Y.) **6**, 31.

Bastian F, Cohen A, Piccoli P, Luna V, Baraldi R, Bottini R. 1998. Production of indole-3-acetic acid and gibberellins A1 and A3 by Acetobacter diazotrophicus and Herbaspirillum seropedicae in chemically-defined culture media. Plant Growth Regulation **24**, 7–11.

Bindschedler LV, Dewdney J, Blee KA, *et al.* 2006. Peroxidase-dependent apoplastic oxidative burst in Arabidopsis required for pathogen resistance. The Plant journal: for cell and molecular biology **47**, 851–863.

Boerjan W, Ralph J, Baucher M. 2003. Lignin Biosynthesis. Annual Review of Plant Biology **54**, 519–546.

Brusamarello-Santos LCC, Pacheco F, Aljanabi SMM, Monteiro RA, Cruz LM, Baura VA, Pedrosa FO, Souza EM, Wassem R. 2011. Differential gene expression of rice roots inoculated with the diazotroph Herbaspirillum seropedicae. Plant Soil.

Burdiak P, Rusaczonek A, Witoń D, Głów D, Karpiński S. 2015. Cysteine-rich receptor-like kinase CRK5 as a regulator of growth, development, and ultraviolet radiation responses in Arabidopsis thaliana. Journal of Experimental Botany **66**, 3325–3337.

Cayrol B, Delteil A, Gobbato E, Kroj T, Morel J-B. 2016. Three wall-associated kinases required for rice basal immunity form protein complexes in the plasma membrane. Plant Signaling & Behavior **11**, e1149676.

Chen F, Tholl D, Bohlmann J, Pichersky E. 2011. The family of terpene synthases in plants: a mid-size family of genes for specialized metabolism that is highly diversified throughout the kingdom. The Plant journal: for cell and molecular biology **66**, 212–229.

Choi Y, Choi YD, Lee JS. 2008. Antimicrobial activity of gamma-thionin-like soybean SE60 in E. coli and tobacco plants. Biochemical and biophysical research communications **375**, 230–234.

Chubatsu LS, Monteiro RA, Souza EM de, *et al.* 2012. Nitrogen fixation control in Herbaspirillum seropedicae. Plant and Soil **356**, 197–207.

Couto D, Zipfel C. 2016. Regulation of pattern recognition receptor signalling in plants. Nature Reviews. Immunology **16**, 537–552.

Curie C, Panaviene Z, Loulergue C, Dellaporta SL, Briat JF, Walker EL. 2001. Maize yellow stripe1 encodes a membrane protein directly involved in Fe(III) uptake. Nature **409**, 346–349.

Daudi A, Cheng Z, O'Brien JA, Mammarella N, Khan S, Ausubel FM, Bolwell GP. 2012. The apoplastic oxidative burst peroxidase in Arabidopsis is a major component of pattern-triggered immunity. The Plant cell **24**, 275–287.

Delteil A, Gobbato E, Cayrol B, Estevan J, Michel-Romiti C, Dievart A, Kroj T, Morel J-B. 2016. Several wall-associated kinases participate positively and negatively in basal defense against rice blast fungus. BMC plant biology **16**, 17.

Dietz K-J, Jacob S, Oelze M-L, Laxa M, Tognetti V, de Miranda SMN, Baier M, Finkemeier I. 2006. The function of peroxiredoxins in plant organelle redox metabolism. Journal of experimental botany **57**, 1697–1709.

Dobereiner J, Baldani VLD, Baldani JI. 1995. *Como isolar e identificar bactérias diazotróficas de plantas não-leguminosas.* Brasília: EMBRAPA.

Dörr J, Hurek T, Reinhold-Hurek B. 1998. Type IV pili are involved in plant-microbe and fungus-microbe interactions. Molecular microbiology **30**, 7–17.

Drogue B, Sanguin H, Chamam A, Mozar M, Llauro C, Panaud O, Prigent-Combaret C, Picault N, Wisniewski-Dyé F. 2014. Plant root transcriptome profiling reveals a strain-dependent response during Azospirillum-rice cooperation. Frontiers in Plant Science **5**, 607.

Elbeltagy A, Nishioka K, Sato T, Suzuki H, Ye B, Hamada T, Isawa T, Mitsui H, Minamisawa K. 2001. Endophytic colonization and in planta nitrogen fixation by a Herbaspirillum sp. isolated from wild rice species. Applied and environmental microbiology **67**, 5285–5293.

Eulgem T, Somssich IE. 2007. Networks of WRKY transcription factors in defense signaling. Current opinion in plant biology **10**, 366–371.

Falk A, Feys BJ, Frost LN, Jones JDG, Daniels MJ, Parker JE. 1999. EDS1, an essential component of R gene-mediated disease resistance in Arabidopsis has homology to eukaryotic lipases. Proceedings of the National Academy of Sciences of the United States of America **96**, 3292–3297.

Ferrer J-L, Austin MB, Stewart C Jr, Noel JP. 2008. Structure and function of enzymes involved in the biosynthesis of phenylpropanoids. Plant physiology and biochemistry: PPB / Société française de physiologie végétale **46**, 356–370.

Florack DEA, Stiekema W.J. 1994. Thionins: properties, possible biological roles and mechanisms of action. Plant Molecular Biology **26**, 25–37.

Giordano W, Hirsch AM. 2004. The expression of MaEXP1, a Melilotus alba expansin gene, is upregulated during the sweetclover-Sinorhizobium meliloti interaction. Molecular plant-microbe interactions: MPMI **17**, 613–622.

Glazebrook J. 2005. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annual review of phytopathology **43**, 205–227.

Goujon T, Minic Z, El Amrani A, Lerouxel O, Aletti E, Lapierre C, Joseleau J-P, Jouanin L. 2003. AtBXL1, a novel higher plant (Arabidopsis thaliana) putative beta-xylosidase gene, is involved in secondary cell wall metabolism and plant development. The Plant Journal: For Cell and Molecular Biology **33**, 677–690.

Guilfoyle TJ. 2015. The PB1 domain in auxin response factor and Aux/IAA proteins: a versatile protein interaction module in the auxin response. The Plant Cell **27**, 33–43.

Gyaneshwar P, James EK, Reddy PM, Ladha JK. 2002. Blackwell Science Ltd Herbaspirillum colonization increases growth and nitrogen accumulation in aluminium-tolerant rice varieties. New Phytologist **154**, 131–145.

Hakoyama T, Watanabe H, Tomita J, Yamamoto A, Sato S, Mori Y, Kouchi H, Suganuma N. 2009. Nicotianamine synthase specifically expressed in root nodules of Lotus japonicus. Planta **230**, 309–317.

Harkenrider M, Sharma R, De Vleesschauwer D, Tsao L, Zhang X, Chern M, Canlas P, Zuo S, Ronald PC. 2016. Overexpression of Rice Wall-Associated Kinase 25 (OsWAK25) Alters Resistance to Bacterial and Fungal Pathogens. PloS One **11**, e0147310.

Hassan S, Mathesius U. 2012. The role of flavonoids in root–rhizosphere signalling: opportunities and challenges for improving plant–microbe interactions. Journal of Experimental Botany.

Higuchi K, Suzuki K, Nakanishi H, Yamaguchi H, Nishizawa NK, Mori S. 1999. Cloning of nicotianamine synthase genes, novel genes involved in the biosynthesis of phytosiderophores. Plant physiology **119**, 471–480.

Hoagland D, Arnon D. 1950. *The water-culture method for growing plants without soil*. California.

Hu Y, Dong Q, Yu D. 2012. Arabidopsis WRKY46 coordinates with WRKY70 and WRKY53 in basal resistance against pathogen Pseudomonas syringae. Plant science: an international journal of experimental plant biology **185–186**, 288–297.

Hynes NE, MacDonald G. 2009. ErbB receptors and signaling pathways in cancer. Current Opinion in Cell Biology **21**, 177–184.

Inoue H, Kobayashi T, Nozoye T, Takahashi M, Kakei Y, Suzuki K, Nakazono M, Nakanishi H, Mori S, Nishizawa NK. 2009. Rice OsYSL15 is an iron-regulated iron(III)-deoxymugineic acid transporter expressed in the roots and is essential for iron uptake in early growth of the seedlings. The Journal of biological chemistry **284**, 3470–3479.

Inoue H, Takahashi M, Kobayashi T, Suzuki M, Nakanishi H, Mori S, Nishizawa NK. 2008. Identification and localisation of the rice nicotianamine aminotransferase gene OsNAAT1 expression suggests the site of phytosiderophore synthesis in rice. Plant molecular biology **66**, 193–203.

Inukai Y, Sakamoto T, Ueguchi-Tanaka M, Shibata Y, Gomi K, Umemura I, Hasegawa Y, Ashikari M, Kitano H, Matsuoka M. 2005. Crown rootless1, which is essential for crown root formation in rice, is a target of an AUXIN RESPONSE FACTOR in auxin signaling. The Plant cell **17**, 1387–1396.

Ishimaru Y, Masuda H, Bashir K, *et al.* 2010. Rice metal-nicotianamine transporter, OsYSL2, is required for the long-distance transport of iron and manganese. The Plant journal: for cell and molecular biology **62**, 379–390.

Ishimaru Y, Suzuki M, Tsukamoto T, *et al.* 2006. Rice plants take up iron as an Fe3+-phytosiderophore and as Fe2+. The Plant journal: for cell and molecular biology **45**, 335–346.

Iwai T, Kaku H, Honkura R, Nakamura S, Ochiai H, Sasaki T, Ohashi Y. 2002. Enhanced resistance to seed-transmitted bacterial diseases in transgenic rice plants overproducing an oat cell-wall-bound thionin. Molecular plant-microbe interactions: MPMI **15**, 515–521.

Jain M, Nijhawan A, Tyagi AK, Khurana JP. 2006. Validation of housekeeping genes as internal control for studying gene expression in rice by quantitative real-time PCR. Biochemical and Biophysical Research Communications **345**, 646–651.

James EK. 2000. Nitrogen fixation in endophytic and associative symbiosis. Field Crops Research **65**, 197–209.

James EK, Gyaneshwar P, Mathan N, Barraquio WL, Reddy PM, Iannetta PPM, Olivares FL, Ladha JK. 2002. Infection and colonization of rice seedlings by the plant growth-promoting bacterium Herbaspirillum seropedicae Z67. Molecular plant-microbe interactions: MPMI **15**, 894–906.

Jameson P. 2000. Cytokinins and auxins in plant-pathogen interactions – An overview. Plant Growth Regulation **32**, 369–380.

Ji H, Gheysen G, Ullah C, Verbeek R, Shang C, De Vleesschauwer D, Höfte M, Kyndt T. 2015. The role of thionins in rice defence against root pathogens. Molecular Plant Pathology **16**, 870–881.

Jones JDG, Dangl JL. 2006. The plant immune system. Nature **444**, 323–329.

Journot-Catalino N, Somssich IE, Roby D, Kroj T. 2006. The transcription factors WRKY11 and WRKY17 act as negative regulators of basal resistance in Arabidopsis thaliana. The Plant cell **18**, 3289–3302.

Kakei Y, Ishimaru Y, Kobayashi T, Yamakawa T, Nakanishi H, Nishizawa NK. 2012. OsYSL16 plays a role in the allocation of iron. Plant molecular biology **79**, 583–594.

Kawahara Y, Oono Y, Kanamori H, Matsumoto T, Itoh T, Minami E. 2012. Simultaneous RNA-Seq Analysis of a Mixed Transcriptome of Rice and Blast Fungus Interaction. PloS one **7**, e49423.

Kawasaki T, Henmi K, Ono E, Hatakeyama S, Iwano M, Satoh H, Shimamoto K. 1999. The small GTP-binding protein Rac is a regulator of cell death in plants. Proceedings of the National Academy of Sciences of the United States of America **96**, 10922–10926.

Kawasaki T, Koita H, Nakatsubo T, Hasegawa K, Wakabayashi K, Takahashi H, Umemura K, Umezawa T, Shimamoto K. 2006. Cinnamoyl-CoA reductase, a key enzyme in lignin biosynthesis, is an effector of small GTPase Rac in defense signaling in rice. Proceedings of the National Academy of Sciences of the United States of America **103**, 230–235.

Kazan K, Manners JM. 2009. Linking development to defense: auxin in plant-pathogen interactions. Trends in plant science **14**, 373–382.

Kim ST, Kim SG, Kang YH, Wang Y, Kim J-Y, Yi N, Kim J-K, Rakwal R, Koh H-J, Kang KY. 2008. Proteomics analysis of rice lesion mimic mutant (spl1) reveals tightly localized probenazole-induced protein (PBZ1) in cells undergoing programmed cell death. Journal of proteome research **7**, 1750–1760.

Klassen G, Pedrosa FO, Souza EM, Funayama S, Rigo L. 1997. Effect of nitrogen compounds on nitrogenase activity in Herbaspirillum seropedicae. Canadian Journal of Microbiology **43**, 887–891.

Kohorn BD. 2015. The state of cell wall pectin monitored by wall associated kinases: A model. Plant Signaling & Behavior **10**, e1035854.

Kohorn BD. 2016. Cell wall-associated kinases and pectin perception. Journal of Experimental Botany **67**, 489–494.

Kohorn BD, Kohorn SL. 2012. The cell wall-associated kinases, WAKs, as pectin receptors. Frontiers in Plant Science **3**.

Kwasniewski M, Szarejko I. 2006. Molecular cloning and characterization of beta-expansin gene related to root hair formation in barley. Plant Physiology **141**, 1149–1158.

Ladha JK, Reddy PM. 2003. Nitrogen fixation in rice systems: state of knowledge and future prospects. Plant and Soil **252**, 151–167.

Lee S, Jeon US, Lee SJ, *et al.* 2009. Iron fortification of rice seeds through activation of the nicotianamine synthase gene. Proceedings of the National Academy of Sciences **106**, 22014–22019.

Li H, Zhou S-Y, Zhao W-S, Su S-C, Peng Y-L. 2009. A novel wall-associated receptor-like protein kinase gene, OsWAK1, plays important roles in rice blast disease resistance. Plant Molecular Biology **69**, 337–346.

Lin C, Choi H-S, Cho H-T. 2011. Root hair-specific EXPANSIN A7 is required for root hair elongation in Arabidopsis. Molecules and cells **31**, 393–397.

Ling HQ, Pich A, Scholz G, Ganal MW. 1996. Genetic analysis of two tomato mutants affected in the regulation of iron metabolism. Molecular & general genetics: MGG **252**, 87–92.

Lira-Ruan V, Sarath G, Klucas RV, Arredondo-Peter R. 2001. Synthesis of hemoglobins in rice (Oryza sativa var. Jackson) plants growing in normal and stress conditions. Plant Science **161**, 279–287.

Lohse M, Bolger AM, Nagel A, Fernie AR, Lunn JE, Stitt M, Usadel B. 2012. RobiNA: a user-friendly, integrated software solution for RNA-Seq-based transcriptomics. Nucleic Acids Research **40**, W622-627.

Lorbiecke R, Sauter M. 1999. Adventitious Root Growth and Cell-Cycle Induction in Deepwater Rice. Plant Physiology **119**, 21–30.

Magbanua ZV, Arick M, Buza T, Hsu C-Y, Showmaker KC, Chouvarine P, Deng P, Peterson DG, Lu S. 2014. Transcriptomic dissection of the rice – Burkholderia glumae interaction. BMC Genomics **15**.

McDowell JM, Simon SA. 2008. Molecular diversity at the plant-pathogen interface. Developmental and comparative immunology **32**, 736–744.

McHale L, Tan X, Koehl P, Michelmore RW. 2006. Plant NBS-LRR proteins: adaptable guards. Genome biology **7**, 212.

McSteen P. 2010. Auxin and Monocot Development. Cold Spring Harbor Perspectives in Biology **2**.

Meier S, Ruzvidzo O, Morse M, Donaldson L, Kwezi L, Gehring C. 2010. The Arabidopsis wall associated kinase-like 10 gene encodes a functional guanylyl cyclase and is co-expressed with pathogen defense related genes. PloS one **5**, e8904.

Mizuno H, Kawahara Y, Sakai H, *et al.* 2010. Massive parallel sequencing of mRNA in identification of unannotated salinity stress-inducible transcripts in rice (Oryza sativa L.). BMC genomics **11**, 683.

Monteiro RA, Balsanelli E, Wassem R, *et al.* 2012. Herbaspirillum-plant interactions: microscopical, histological and molecular aspects. Plant and Soil **356**, 175–196.

Muramoto N, Tanaka T, Shimamura T, Mitsukawa N, Hori E, Koda K, Otani M, Hirai M, Nakamura K, Imaeda T. 2012. Transgenic sweet potato expressing thionin from barley

gives resistance to black rot disease caused by Ceratocystis fimbriata in leaves and storage roots. Plant cell reports **31**, 987–997.

Naoumkina MA, Zhao Q, Gallego-Giraldo L, Dai X, Zhao PX, Dixon RA. 2010. Genome-wide analysis of phenylpropanoid defence pathways. Molecular plant pathology **11**, 829–846.

Narsai R, Ivanova A, Ng S, Whelan J. 2010. Defining reference genes in Oryza sativa using organ, development, biotic and abiotic transcriptome datasets. BMC Plant Biology **10**, 56.

Navarro L, Dunoyer P, Jay F, Arnold B, Dharmasiri N, Estelle M, Voinnet O, Jones JDG. 2006. A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. Science (New York, N.Y.) **312**, 436–439.

Nobuta K, Venu RC, Lu C, *et al.* 2007. An expression atlas of rice mRNAs and small RNAs. Nature biotechnology **25**, 473–477.

Nozoye T, Nagasaka S, Kobayashi T, Takahashi M, Sato Y, Sato Y, Uozumi N, Nakanishi H, Nishizawa NK. 2011. Phytosiderophore efflux transporters are crucial for iron acquisition in graminaceous plants. The Journal of biological chemistry **286**, 5446–5454.

Okada A, Shimizu T, Okada K, Kuzuyama T, Koga J, Shibuya N, Nojiri H, Yamane H. 2007. Elicitor induced activation of the methylerythritol phosphate pathway toward phytoalexins biosynthesis in rice. Plant molecular biology **65**, 177–187.

Ono E, Wong HL, Kawasaki T, Hasegawa M, Kodama O, Shimamoto K. 2001. Essential role of the small GTPase Rac in disease resistance of rice. Proceedings of the National Academy of Sciences **98**, 759–764.

Pankievicz VCS, do Amaral FP, Santos KFDN, *et al.* 2015. Robust biological nitrogen fixation in a model grass-bacterial association. The Plant Journal: For Cell and Molecular Biology **81**, 907–919.

Pankievicz VCS, Camilios-Neto D, Bonato P, *et al.* 2016. RNA-seq transcriptional profiling of Herbaspirillum seropedicae colonizing wheat (Triticum aestivum) roots. Plant Molecular Biology **90**, 589–603.

Park S-W, Kaimoyo E, Kumar D, Mosher S, Klessig DF. 2007. Methyl salicylate is a critical mobile signal for plant systemic acquired resistance. Science (New York, N.Y.) **318**, 113–116.

Parker JE, Holub EB, Frost LN, Falk A, Gunn ND, Daniels MJ. 1996. Characterization of eds1, a mutation in Arabidopsis suppressing resistance to Peronospora parasitica specified by several different RPP genes. The Plant Cell Online **8**, 2033–2046.

Pedrosa FO, Monteiro RA, Wassem R, *et al.* 2011. Genome of Herbaspirillum seropedicae strain SmR1, a specialized diazotrophic endophyte of tropical grasses. PLoS genetics **7**, e1002064.

Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research **29**, e45.

Plaper A, Golob M, Hafner I, Oblak M, Solmajer T, Jerala R. 2003. Characterization of quercetin binding site on DNA gyrase. Biochemical and biophysical research communications **306**, 530–536.

Rhee SG, Chae HZ, Kim K. 2005. Peroxiredoxins: a historical overview and speculative preview of novel mechanisms and emerging concepts in cell signaling. Free radical biology & medicine **38**, 1543–1552.

Robert-Seilaniantz A, Grant M, Jones JDG. 2011. Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism. Annual review of phytopathology **49**, 317–343.

Rogg LE, Bartel B. 2001. Auxin signaling: derepression through regulated proteolysis. Developmental cell **1**, 595–604.

Roncato-Maccari LDB, Ramos HJO, Pedrosa FO, Alquini Y, Chubatsu LS, Yates MG, Rigo LU, Steffens MBR, Souza EM. 2003. Endophytic Herbaspirillum seropedicae expresses nif genes in gramineous plants. FEMS microbiology ecology **45**, 39–47.

Ross JR, Nam KH, D'Auria JC, Pichersky E. 1999. S-Adenosyl-L-methionine:salicylic acid carboxyl methyltransferase, an enzyme involved in floral scent production and plant defense, represents a new class of plant methyltransferases. Archives of biochemistry and biophysics **367**, 9–16.

Sasaki K, Ikeda S, Eda S, *et al.* 2010. Impact of plant genotype and nitrogen level on rice growth response to inoculation with Azospirillum sp. strain B510 under paddy field conditions. Soil Science and Plant Nutrition **56**, 636–644.

Shankar R, Bhattacharjee A, Jain M. 2016. Transcriptome analysis in different rice cultivars provides novel insights into desiccation and salinity stress responses. Scientific Reports **6**, 23719.

Shojima S, Nishizawa NK, Fushiya S, Nozoe S, Irifune T, Mori S. 1990. Biosynthesis of Phytosiderophores□: In Vitro Biosynthesis of 2'-Deoxymugineic Acid from l-Methionine and Nicotianamine. Plant physiology **93**, 1497–1503.

Souza ALF, Invitti AL, Rego FGM, Monteiro RA, Klassen G, Souza EM, Chubatsu LS, Pedrosa FO, Rigo LU. 2010. The involvement of the nif-associated ferredoxin-like genes fdxA and fdxN of Herbaspirillum seropedicae in nitrogen fixation. Journal of microbiology (Seoul, Korea) **48**, 77–83.

Souza EM, Pedrosa FO, Rigo LU, Machado HB, Yates MG. 2000. Expression of the nifA gene of Herbaspirillum seropedicae: role of the NtrC and NifA binding sites and of the -24/-12 promoter element. Microbiology (Reading, England) **146 (Pt 6)**, 1407–1418.

Straub D, Yang H, Liu Y, Tsap T, Ludewig U. 2013. Root ethylene signalling is involved in Miscanthus sinensis growth promotion by the bacterial endophyte Herbaspirillum frisingense GSF30(T). Journal of Experimental Botany **64**, 4603–4615.

Suharsono U, Fujisawa Y, Kawasaki T, Iwasaki Y, Satoh H, Shimamoto K. 2002. The heterotrimeric G protein alpha subunit acts upstream of the small GTPase Rac in disease resistance of rice. Proceedings of the National Academy of Sciences of the United States of America **99**, 13307–13312.

Tadra-Sfeir MZ, Faoro H, Camilios-Neto D, *et al.* 2015. Genome wide transcriptional profiling of Herbaspirillum seropedicae SmR1 grown in the presence of naringenin. Frontiers in Microbiology **6**, 491.

Thomine S, Lelièvre F, Debarbieux E, Schroeder JI, Barbier-Brygoo H. 2003. AtNRAMP3, a multispecific vacuolar metal transporter involved in plant responses to iron deficiency. The Plant Journal **34**, 685–695.

Valdameri G, Alberton D, Moure VR, Kokot TB, Kukolj C, Brusamarello-Santos LCC, Monteiro RA, Pedrosa F de O, de Souza EM. 2017. Herbaspirillum rubrisubalbicans, a mild pathogen impairs growth of rice by augmenting ethylene levels. Plant Molecular Biology **94**, 625–640.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology **3**, research0034.1-research0034.11.

Vanholme R, Demedts B, Morreel K, Ralph J, Boerjan W. 2010. Lignin Biosynthesis and Structure. Plant Physiology **153**, 895–905.

Venugopal SC, Jeong R-D, Mandal MK, *et al.* 2009. Enhanced disease susceptibility 1 and salicylic acid act redundantly to regulate resistance gene-mediated signaling. PLoS genetics **5**, e1000545.

Vinagre F, Vargas C, Schwarcz K, Cavalcante J, Nogueira EM, Baldani JI, Ferreira PCG, Hemerly AS. 2006. SHR5: a novel plant receptor kinase involved in plant-N2-fixing endophytic bacteria association. Journal of experimental botany **57**, 559–569.

Vlot AC, Dempsey DA, Klessig DF. 2009. Salicylic Acid, a Multifaceted Hormone to Combat Disease. Annual Review of Phytopathology **47**, 177–206.

Vranová E, Coman D, Gruissem W. 2013. Network Analysis of the MVA and MEP Pathways for Isoprenoid Synthesis. Annual review of plant biology.

Wakasa Y, Oono Y, Yazawa T, Hayashi S, Ozawa K, Handa H, Matsumoto T, Takaiwa F. 2014. RNA sequencing-mediated transcriptome analysis of rice plants in endoplasmic reticulum stress conditions. BMC Plant Biology **14**, 101.

Wang S, Wu H, Qiao J, Ma L, Liu J, Xia Y, Gao X. 2009. Molecular mechanism of plant growth promotion and induced systemic resistance to tobacco mosaic virus by Bacillus spp. Journal of microbiology and biotechnology **19**, 1250–1258.

Xu H, Gao Y, Wang J. 2012. Transcriptomic analysis of rice (Oryza sativa) developing embryos using the RNA-Seq technique. PloS one **7**, e30646.

Xu X-H, Wang C, Li S-X, *et al.* 2015. Friend or foe: differential responses of rice to invasion by mutualistic or pathogenic fungi revealed by RNAseq and metabolite profiling. Scientific Reports **5**.

Zhang Y, Xu Y-H, Yi H-Y, Gong J-M. 2012. Vacuolar membrane transporters OsVIT1 and OsVIT2 modulate iron translocation between flag leaves and seeds in rice. The Plant Journal: For Cell and Molecular Biology **72**, 400–410.

ZhiMing Y, Bo K, XiaoWei H, ShaoLei L, YouHuang B, WoNa D, Ming C, Hyung-Taeg C, Ping W. 2011. Root hair-specific expansins modulate root hair elongation in rice. The Plant journal: for cell and molecular biology **66**, 725–734.

Table 1 – Statistics of RNA-seq reads mapping

	Control	Inoculated
Total number of reads	101,587,721	59,591,404
Total number of reads after trimming	65,099,320	38,463,798
Reads uniquely mapped to RGAP7 (%)	12,255,809 (18.8)	9,919,863 (25.8)
Reads mapped uniquely to the rice mitochondrial genome (%)	626,587 (0.96)	253,771 (0.66)
Reads mapped uniquely to the rice chloroplast genome (%)	102,730 (0.16)	36,915 (0.09)
Total number of reads mapped to rice rRNA (%)	41,744,369 (64.1)	26,875,340 (69.9)
Reads mapped to SmR1 genome (%)		16,878 (0.04)
Total number of reads mapped (%)	54,803,125 (84.2)	32,813,183 (85.3)
Unmapped reads (%)	10,296,195 (15.8)	5,650,615 (14.7)

Table 2 – Rice genes modulated by colonisation with *H. seropedicae* that are involved in phyto-hormone signalling and the MTA cycle

Gene ID	Gene product/description*	Fold-change	Pvalue
LOC_Os01g67030.1	Auxin-responsive protein, putative, expressed	2.5	4.99E-06
LOC_Os08g24790.1	AIR12, putative, expressed	0.49	0.04
LOC_Os09g25770.1	Auxin-induced protein 5NG4, putative, expressed	0.55	0.01
LOC_Os05g01570.1	Auxin-induced protein 5NG4, putative, expressed	0.40	0.05
LOC_Os09g28050.1	1-aminocyclopropane-1-carboxylate synthase family protein (ACC synthase) (RAPDB) Asparate aminotransferase (MSU)	6.1	2.11E-54
LOC_Os03g63900.1	1-aminocyclopropane-1-carboxylate oxidase 2 (ACC oxidase)	0.50	7.88E-04
LOC_Os01g04800.1	B3 DNA binding domain containing protein (MSU) APETALA2/ethylene-responsive element binding protein 129 (RAPDB)	2.2	3.67E-03
LOC_Os06g02220.1	MTA/SAH nucleosidase	2.6	1.83E-11

LOC_Os04g57400.1	Methylthioribose kinase	6.1	3.20E-18
LOC_Os04g57410.1	Methylthioribose kinase	10.2	1.16E-12
LOC_Os06g04510.1	Similar to enolase 1	1.9	0.03
LOC_Os03g06620.1	1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase	7.5	5.94E-65
LOC_Os10g28360.1	1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase	0.47	6.14E-03
LOC_Os01g22010.3	S-adenosylmethionine synthetase (SAM)	1.9	1.72E-05

* Gene product name/description of the both rice database were used: MSU (Rice Genome Annotation Project) and RAPDB (The Rice Annotation Project).

Table 3 – Rice genes highly regulated (fold change > 10) by inoculation with *H. seropedicae*

Gene ID	Gene product/description*	Fold change	P-value
	Up-regulated rice genes		
LOC_Os02g20360.1	Ttyrosine aminotransferase, putative, expressed, Similar to Nicotianamine aminotransferase A (RAPDB)	10	5.24E-30
LOC_Os04g57410.1	Methylthioribose kinase. putative. Expressed	10.2	1.16E-12
LOC_Os03g26210.1	Helix-loop-helix DNA-binding domain containing protein. Expressed	12.6	4.10E-09
LOC_Os01g72370.1	Helix-loop-helix DNA-binding domain containing protein. Expressed	16.4	3.36E-74
LOC_Os11g15624.1	Expressed protein	17.7	1.22E-25
LOC_Os03g13390.2	Oxidoreductase. aldo/keto reductase family protein. putative. Expressed	19.4	1.61E-10
LOC_Os02g43410.1	Transposon protein. putative. unclassified. Expressed	21	3.97E-81
LOC_Os10g11889.2	Expressed protein	23	3.29E-113
LOC_Os01g65110.1	POT family protein. Expressed	26	6.63E-14
LOC_Os07g15460.1	Metal transporter Nramp6. putative. Expressed	27	3.56E-61
LOC_Os03g03724.1	Expressed protein	28	0.02
LOC_Os03g19427.1	Nicotianamine synthase. putative. expressed	32	1.02E-22
LOC_Os03g46454.1	Metal cation transporter. putative.	34	4.02E-08

	expressed		
LOC_Os06g19095.1	Expressed protein	40	1.51E-14
LOC_Os11g04020.1	Major facilitator superfamily antiporter. putative. expressed	40	8.84E-51
LOC_Os03g19420.2	Nicotianamine synthase. putative. expressed	52	5.57E-80
LOC_Os12g18410.1	Expressed protein	70	2.07E-05
LOC_Os03g12510.1	Non-symbiotic hemoglobin 2. putative. expressed	104	5.58E-24
LOC_Os04g51660.1	Transferase family protein, putative, expressed	0.08	2.79E-03
LOC_Os04g59020.1	Integral membrane protein, putative, expressed	0.08	3.41E-03
LOC_Os09g23300.1	Integral membrane protein, putative, expressed	0.06	3.63E-05
LOC_Os11g15340.2	SAM dependent carboxyl methyltransferase family protein, putative, expressed	0.03	1.54E-03
LOC_Os01g55690.1	Glutelin, putative, expressed	0.02	0.02
LOC_Os12g38040.1	Metallothionein family protein, expressed	0.02	3.72E-22
LOC_Os10g07556.1	Wall-associated receptor kinase-like 22 precursor, putative, expressed	0.01	5.22E-08

* Gene product name/description of the both rice database were used: MSU (Rice Genome Annotation Project) and RAPDB (The Rice Annotation Project).

Table 4: Differentially expressed genes in rice roots colonised by *H. seropedicae* involved in uptake and transport of metals

ID	Gene product/description*	Fold change	p-value
LOC_Os01g22010.3	S-adenosylmethionine synthetase, putative, expressed	1.9	1.72E-05
LOC_Os03g19427.1	Nicotianamine synthase. putative. expressed	32	1.02E-22
LOC_Os03g19420.2	Nicotianamine synthase. putative. expressed	52	5.57E-80
LOC_Os02g20360.1	tyrosine aminotransferase, putative, expressed (MSU), Similar to Nicotianamine aminotransferase (RAPDB)	10	5.24E-30
LOC_Os03g13390.2	Oxidoreductase. aldo/keto reductase family protein. putative. Expressed (MSU) Similar to NADPH-dependent codeinone reductase, gene name: deoxymugineic acid synthase1 (RAPDB)	19.4	1.61E-10
LOC_Os11g04020.1	Major facilitator superfamily antiporter, putative, expressed (TOM1)	40	8.84E-51
LOC_Os11g05390.1	Transporter, major facilitator family, putative, expressed (ENA1)	3.1	5.80E-03
LOC_Os03g46470.1	Metal cation transporter, putative, expressed (OsIRT1)	4.3	4.09E-14
LOC_Os03g46454.1	Metal cation transporter, putative,	34	4,02E-

	expressed (OsIRT2)		08
LOC_Os04g45900.1	Transposon protein, putative, unclassified, expressed (MSU) Similar to Metal-nicotianamine transporter YSL2, Gene symbol synonym: OsYSL16 (RAPDB)	3.2	1.23E-07
LOC_Os02g43410.1	Transposon protein. putative. unclassified. Expressed (MSU) Iron-phytosiderophore transporter, Iron homeostasis (Os02t0650300-01); Similar to Iron-phytosiderophore transporter YSL15. (Os02t0650300-02) (RAP-DB)	21	3.97E-81
LOC_Os07g15460.1	Metal transporter Nramp6. putative. Expressed	27	3.56E-61

* Gene product name/description of the both rice database were used: MSU (Rice Genome Annotation Project) and RAPDB (The Rice Annotation Project).

Table 5 – ORFs of *H. seropedicae* expressed following contact with rice roots

ID	Fold Change	p-value	ID Feature	Description	COG
Hsero_3499	0.4	7.0E-04	qor	qor NADPH:quinone oxidoreductase protein 4007887:4008915 forward	C;R - Energy production and conversion;General function prediction only
Hsero_1gy130	30	6.20E-03	Hsero_1130	Hsero_1130 ABC-type dipeptide transporter, periplasmic peptide-binding protein 1287409:1289031 forward	E - Amino acid transport and metabolism
Hsero_4713	27	7.70E-03	urtA	urtA ABC-type urea transport system, periplasmic component protein 5407999:5409252 forward	E - Amino acid transport and metabolism
Hsero_0084	43	0.02	glnK	glnK nitrogen regulatory PII-like protein 99052:99390 forward	E - Amino acid transport and metabolism

Hsero_1469	0.2	2.0E-04	ttuC	ttuC tartrate dehydrogenase protein 1690575:1691654 reverse	G - Carbohydrate transport and metabolism
Hsero_1123	84	0.02	Hsero_1123	Hsero_1123 family II aminotransferase protein 1278563:1279939 reverse	H - Coenzyme transport and metabolism
Hsero_1509	0.1	2.0E-03	rplS	rplS 50S ribosomal subunit protein L19 1731532:1731915 forward	J - Translation, ribosomal structure and biogenesis
Hsero_0677	0.3	0.02	ompW2	ompW2 outer membrane W protein 741190:741927 forward	M - Cell wall
Hsero_2580	0.4	5.0E-03	lon	lon ATP-dependent protease LA protein 2940893:2943301 reverse	O - Posttranslational modification, protein turnover, chaperones
Hsero_0085	20	0.04	amtB	amtB ammonium transporter transmembrane protein 99406:100938 forward	P - Inorganic ion transport and metabolism

Hsero_2905	0.1	1.10E-03	Hsero_2905	Hsero_2905 conserved hypothetical protein 3301039:3301272 reverse	S - Function unknown
Hsero_0083	15	0.03	Hsero_0083	Hsero_0083 membrane protein 98251:99039 forward	S - Function unknown
Hsero_2723	90	1.90E-03	Hsero_2723	Hsero_2723 methyl-accepting chemotaxis transmembrane protein 3101951:3103597 reverse	T;N - Signal transduction mechanisms;Cell motility
Hsero_1215	0.3	0.04	trnK	trnK tRNA-Lys 1374570:1374645 reverse	trnK
Hsero_1466	0.2	1.7E-03	trnL	trnL tRNA-Leu 1689660:1689744 reverse	trnL
Hsero_1546	0.1	0.03	trnS	trnS tRNA-Ser 1781783:1781873 forward	trnS

Figure 1 – Diagram showing how rice roots were grown, inoculated with *H. seropedicae*, prepared for extraction of RNA and the RNA-seq analyses performed.

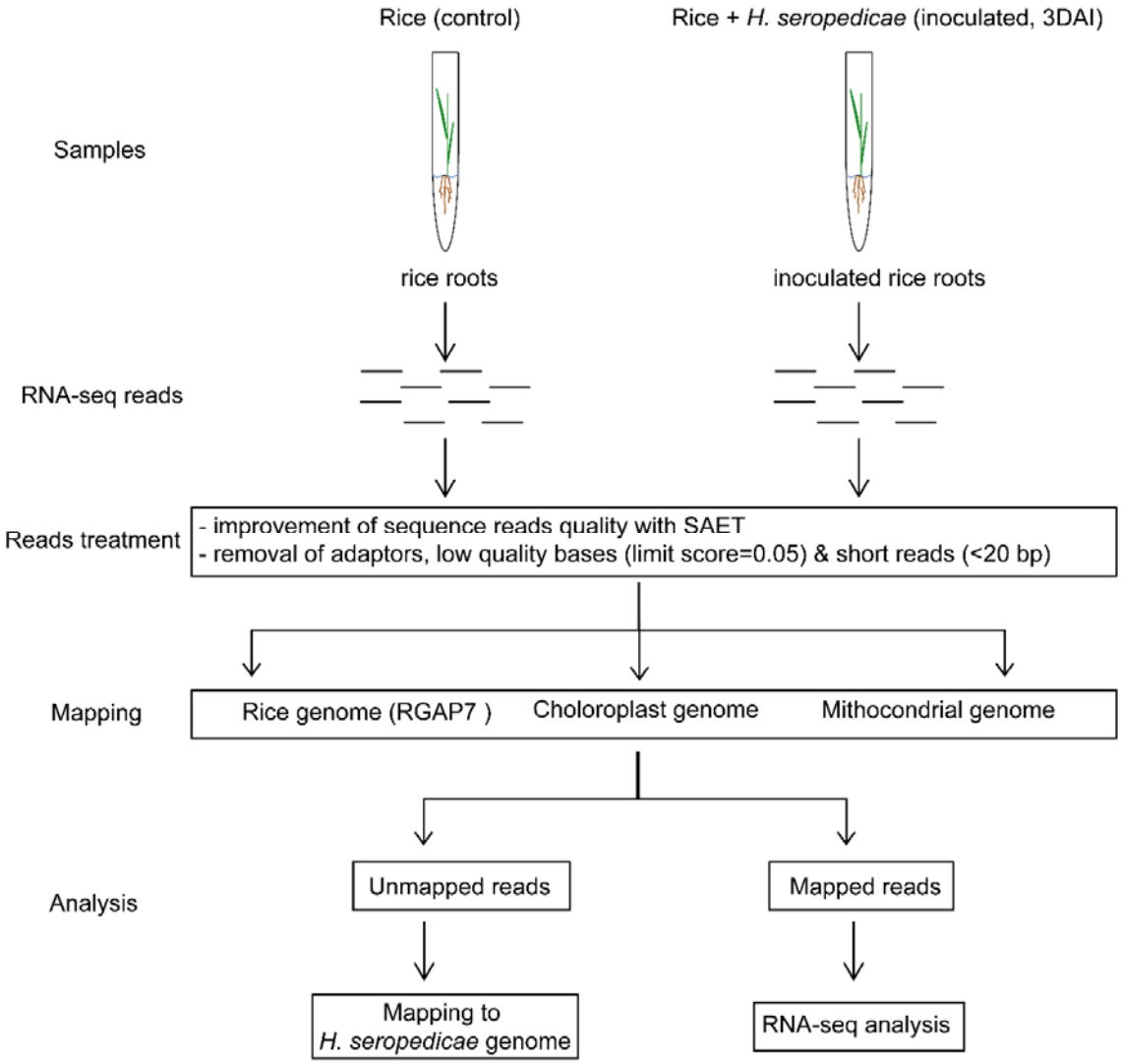
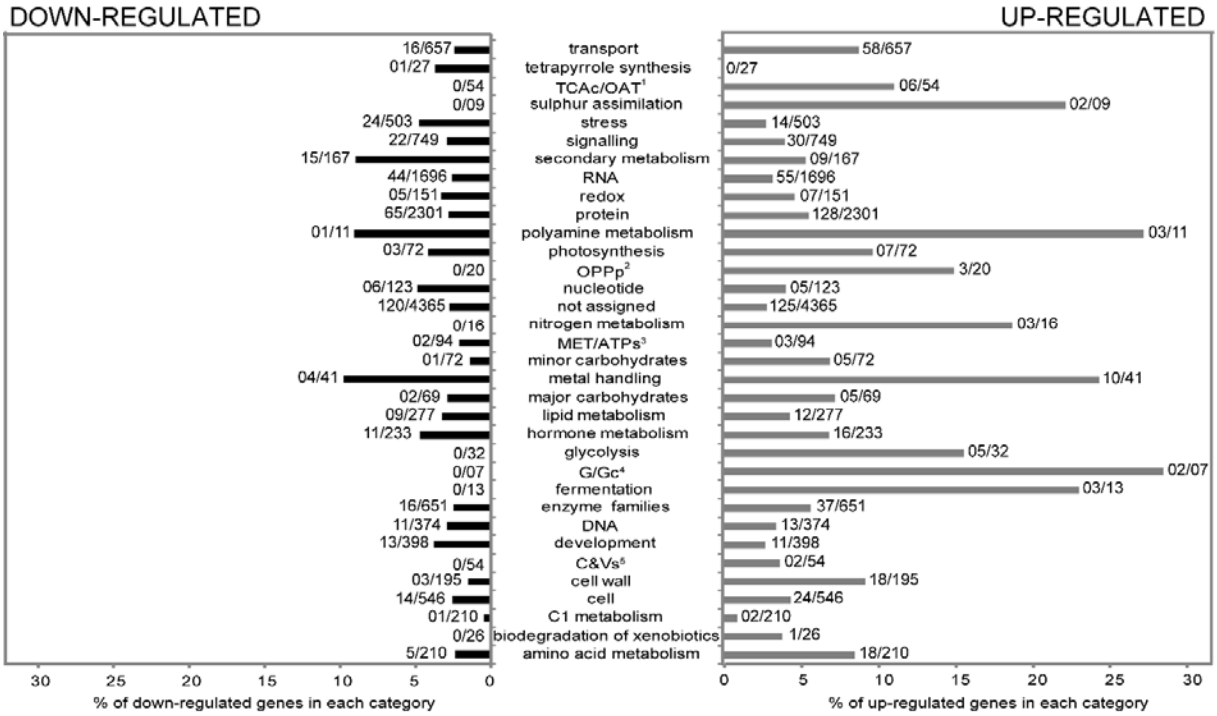


Figure 2 – Transcripts differentially expressed in rice roots colonised by *H. seropedicae*. Up-regulated genes are shown in the right-hand column (in gray) with down-regulated genes to the left (in black). Numbers of regulated genes and total numbers of expressed genes are shown beside each column. Transcripts are grouped according to metabolic categories determined using MapMan.



[1]TCA cycle/organic acids; [2]oxidative pentose phosphate pathway; [3]mitochondrial electron transport/ATP synthesis; [4]gluconeogenesis/glyoxylate cycle; [5]cofactor and vitamin synthesis. To be considered differentially transcripts must have had at least two-fold coverage and P-values < 0.05.

Figure 3 –Biotic stress pathways regulated by *H. seropedicae* in rice roots. The scheme was constructed using MapMan (only genes with fold change ≥2, ≤2). Dark gray squares represent genes shown in these experiments to be involved in the biotic stress. On the left- and right-hand sides (shaded in light grey) are genes possibly involvement in biotic stress. Smaller squares represent up-regulated (blue) or down-regulated (red) genes.

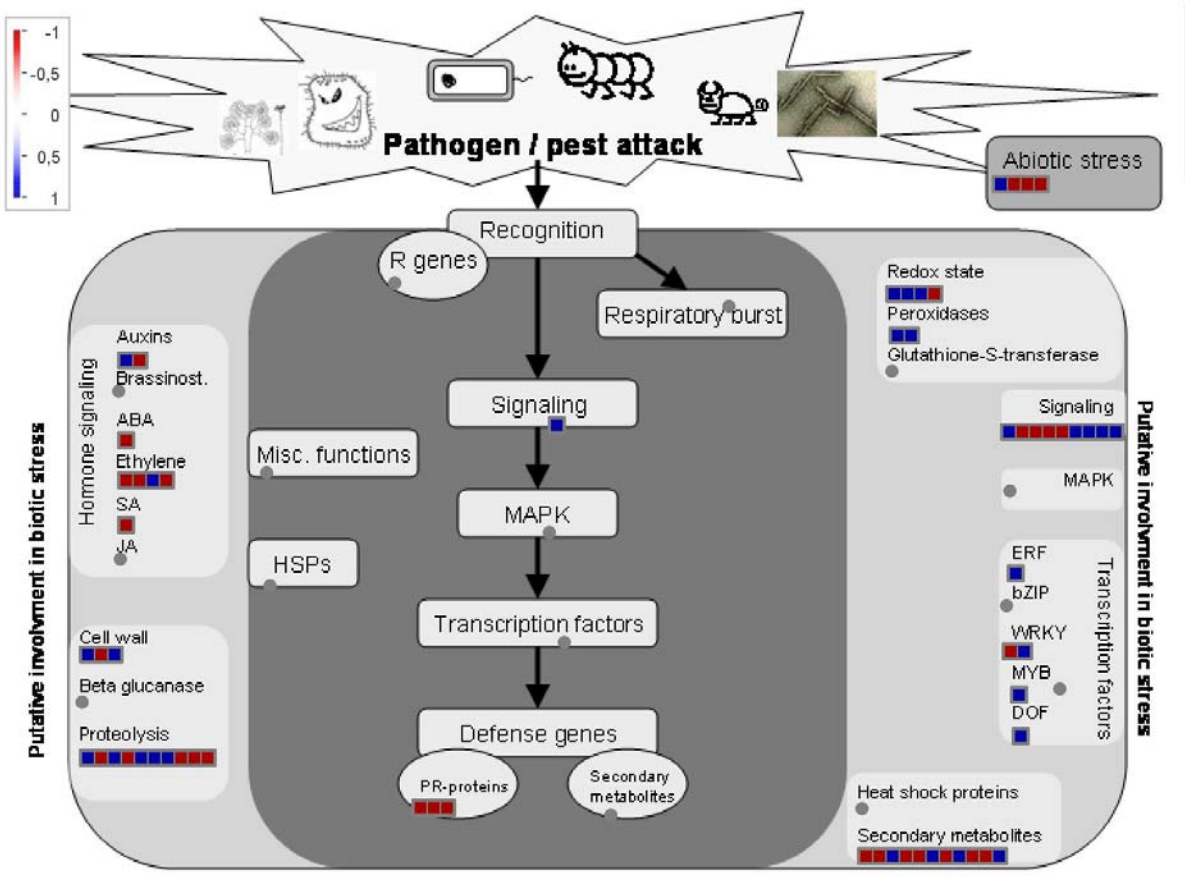


Figure 4: Confirmation of differential expression of rice genes by qRT-PCR and RNA-Seq. The results are average of three independent samples and error bars represent, the standard deviation. The reference genes used for the analysis were actin 1, tubulin beta-2 chain and conserved hypothetical protein.

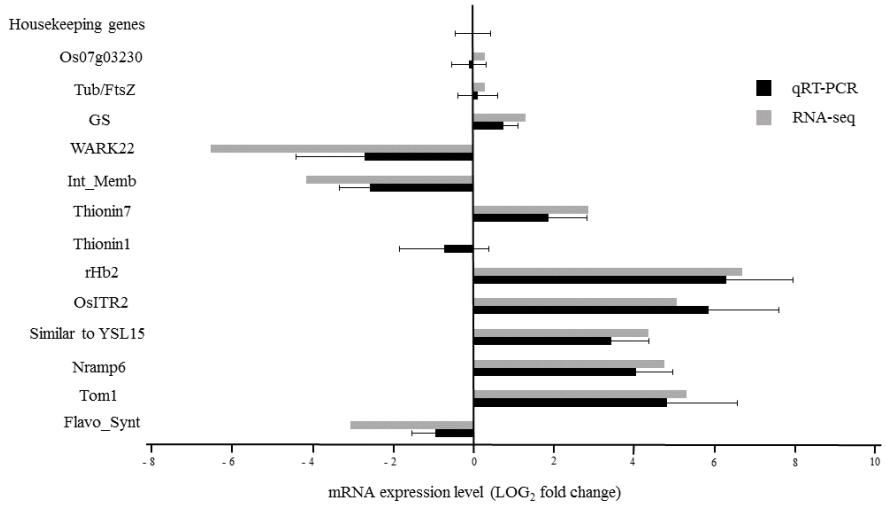


Figure 5: Isoprenoid synthesis genes down-regulated in rice roots colonised by *H. seropedicae*. The names of the genes differentially expressed are shown and the numbers in parentheses represent the fold change. The components of the MEP pathway leading to geranylgeranyl diphosphate synthesis and the diterpenoid- phytoalexin pathway are: G3P, glyceraldehyde-3-phosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; MEP, 2-C-methyl-D-erythritol 4-phosphate; CDP-ME, 4-(cytidine 5-diphospho)-2-C-methyl-D-erythritol; CDP-ME2P, 2-phospho-4-(cytidine 5´-diphospho)-2-C-methyl-D-erythritol; MEC-DP, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate; HMBDP, 1-hydroxy-2-methyl-2-butenyl 4-diphosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GGDP, geranylgeranyl diphosphate; and CDP, copalyl diphosphate. Enzymes are indicated in rose coloured circles: DXS, 1-deoxy-D-xylulose 5-phosphate synthase; DXR, DXP reductoisomerase; CMS, CDP-ME synthase; CMK, CDP-ME kinase; MCS, MECDP synthase; HDS, HMBDP synthase; HDR, HMBDP reductase; IPI, IPP isomerase; GGPS, GGDP synthase; OsCyc1, syn-CDP synthase; OsCyc2, ent-CDP synthase; OsDTC2, stemar-13-ene synthase.

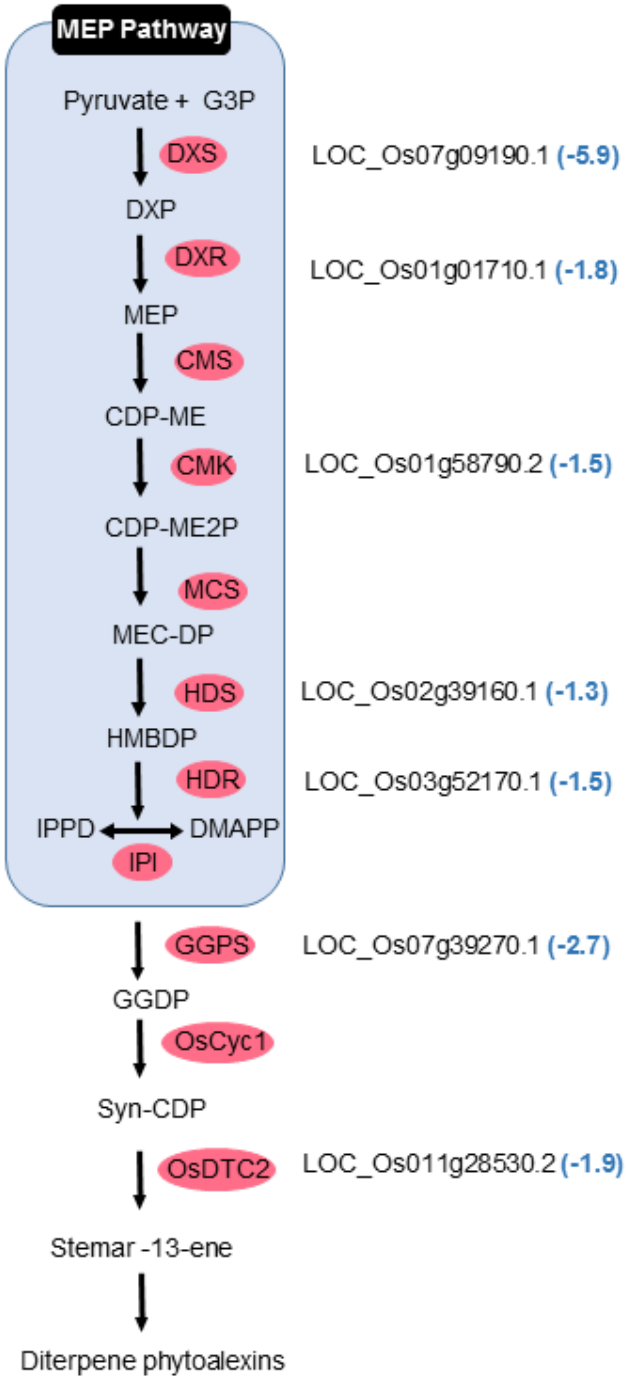
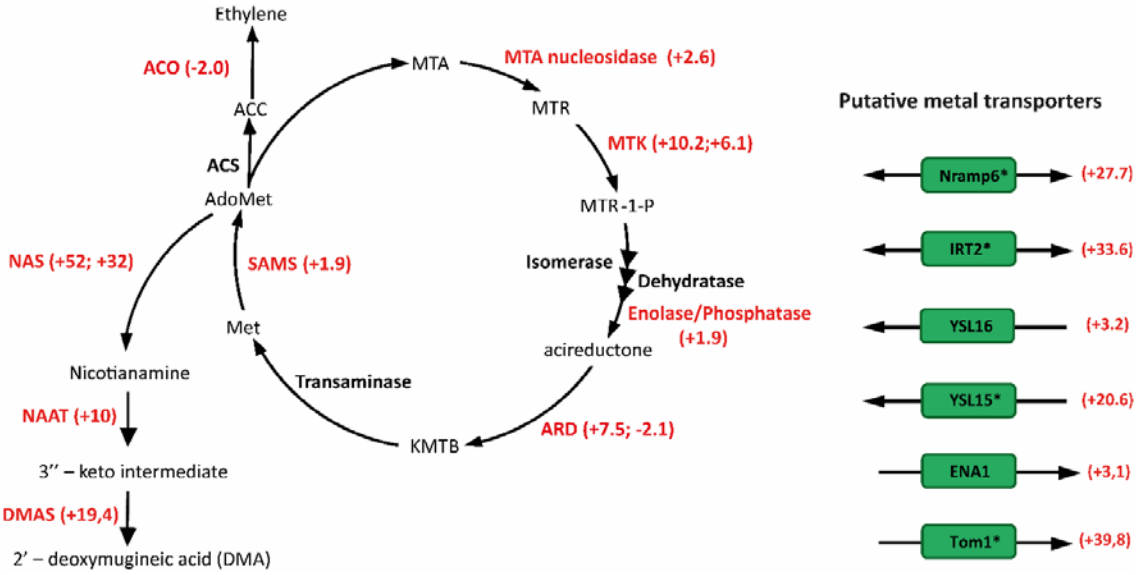


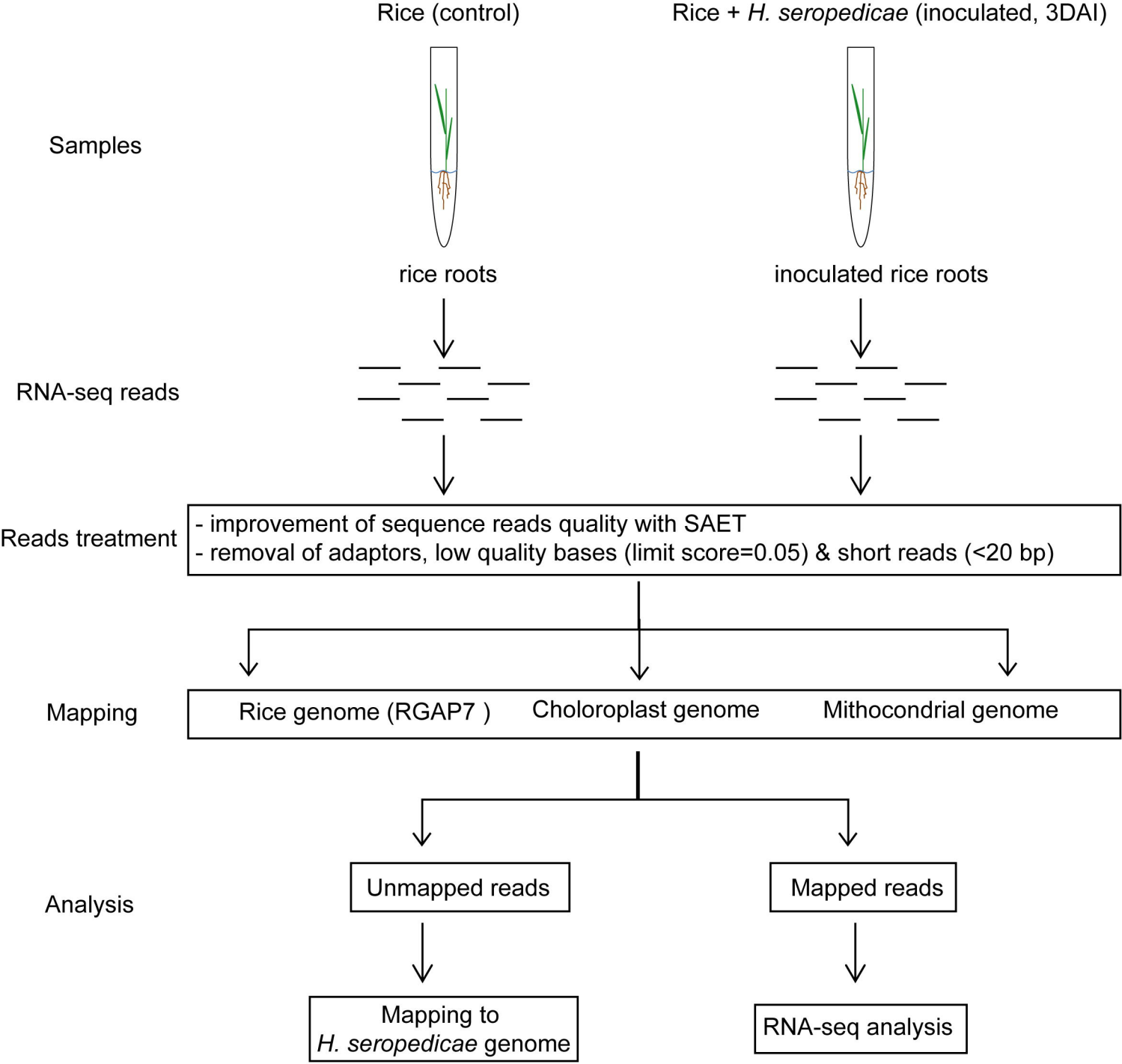
Figure 6: Differentially expressed genes in rice roots following colonisation by *H. seropedicae*. Genes involved in siderophore synthesis and transport, the methionine salvage pathway and ethylene synthesis are shown. Numbers in parentheses represent the fold change.

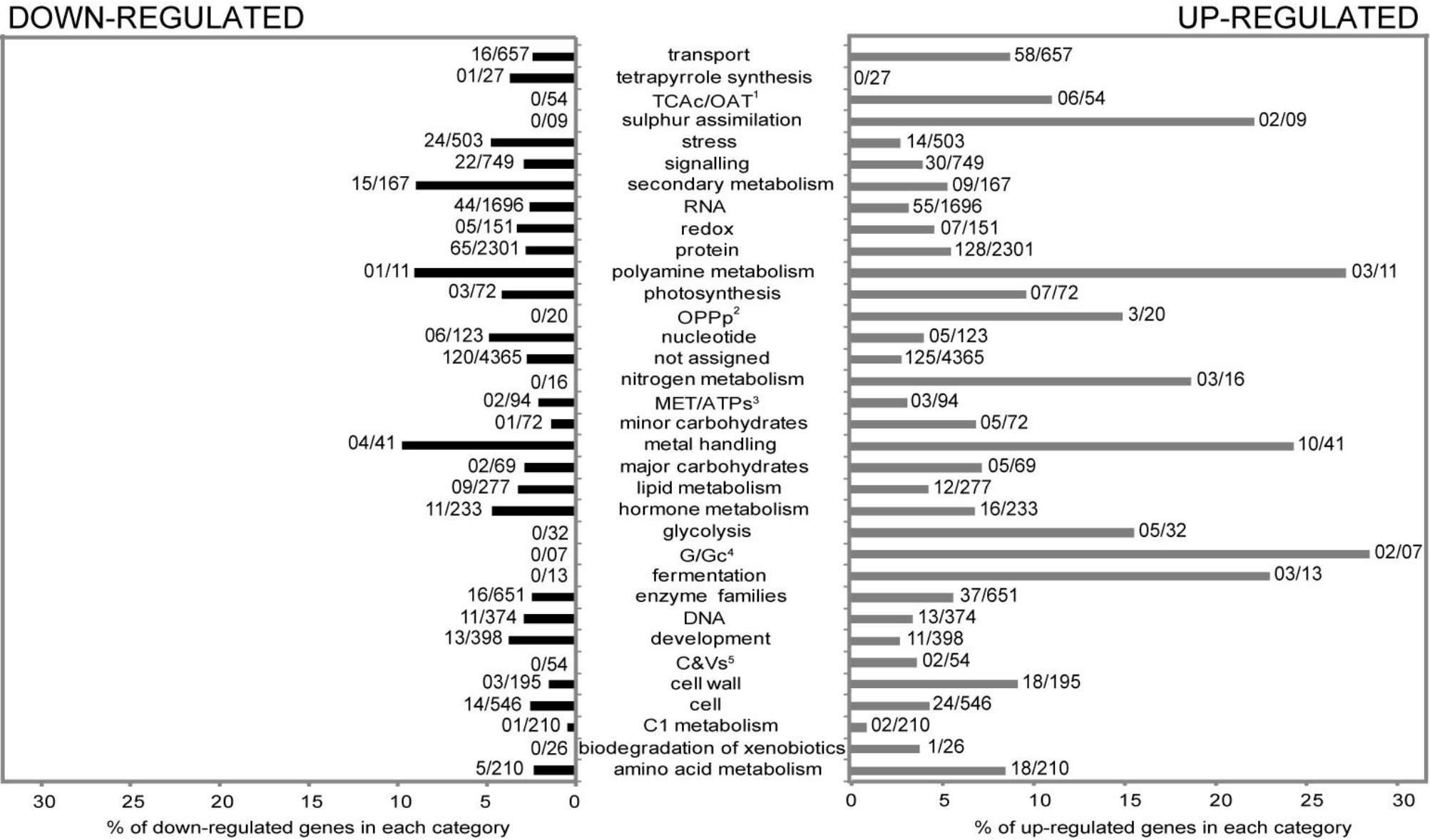


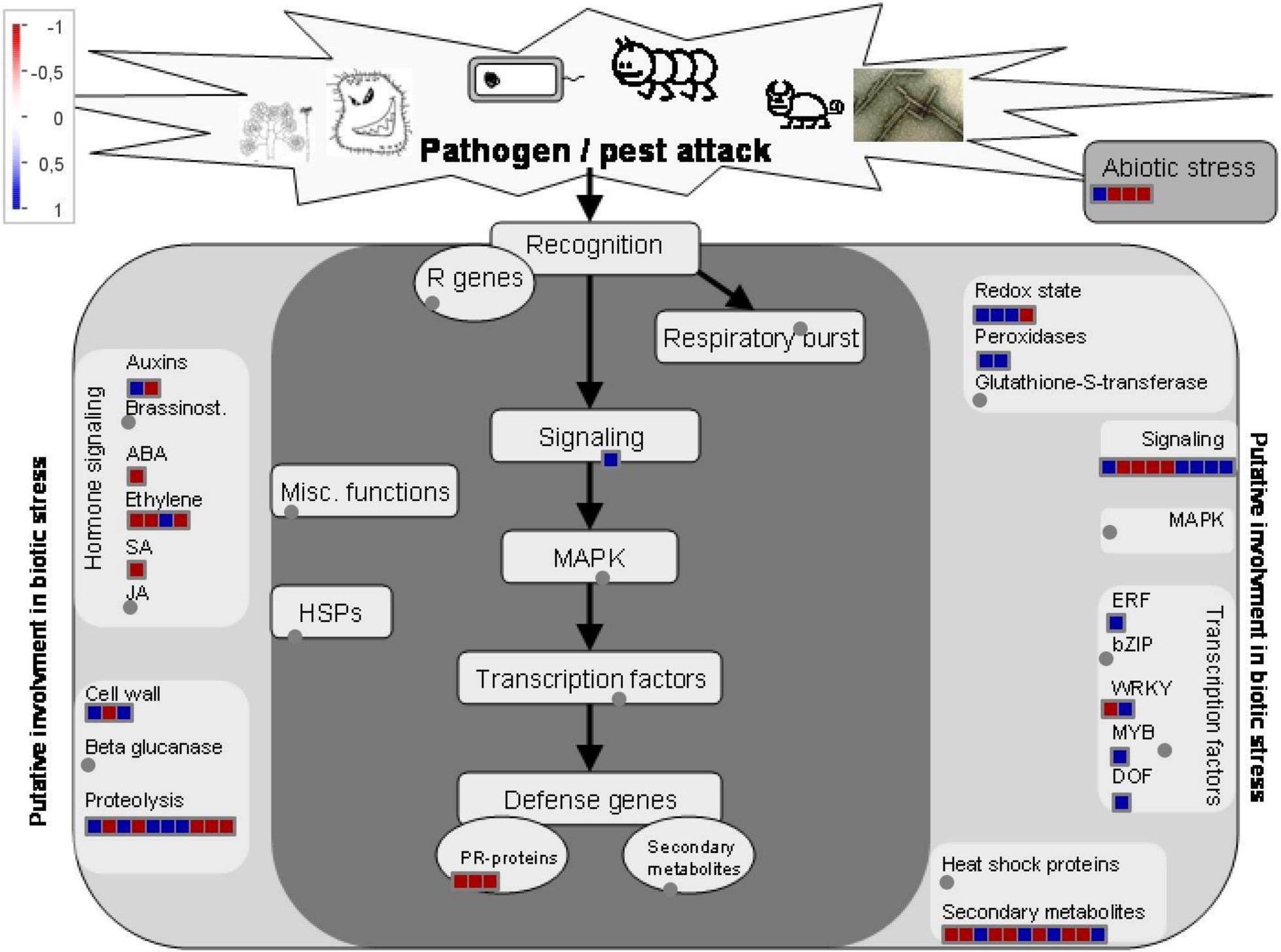
H. seropedicae SmR1 induces methionine recycling and mugineic acid (MA) synthesis as well as the expression of transporters involved in iron metabolism. The expression of those genes marked with an asterisk was confirmed by RT-qPCR

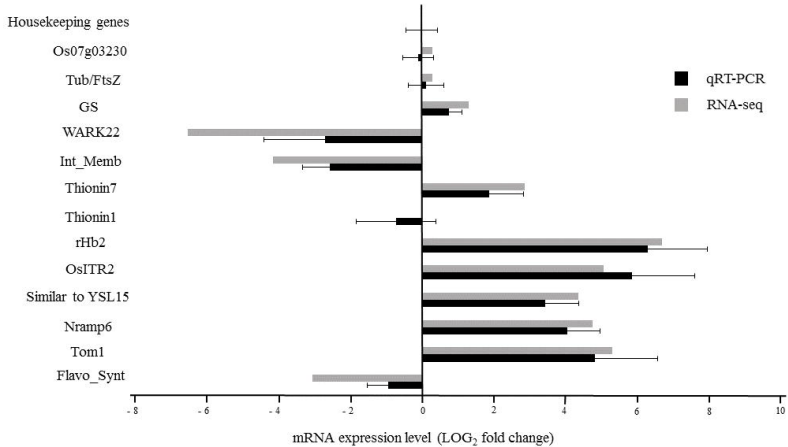
Abbreviations: AdoMet, S-adenosylmethionine; ACC, 1-aminocyclopropane-1-carboxylate; ACS, 1-aminocyclopropane-1-carboxylate synthase; ACO, 1-aminocyclopropane-1-carboxylate oxidase; MTA, 5′-methylthioadenosine; MTR, 5′-methylthioribose; MTK, methylthioribose kinase; MTR-1-P, 5′-methylthioribose-1-phosphate; KMTB, 2-keto-4-methylthiobutyrate; ARD, acireductone dioxygenase; SAMS, S-adenosylmethionine synthetase; NAS, nicotianamine synthase ; NAAT nicotianamine aminotransferase ; DMAS, deoxymugineic acid synthase ; Tom1, transporter of mugineic acid 1 ; ENA1 (efflux transporters of nicotianamine 1); Nramp6, Natural Resistance-Associated Macrophage

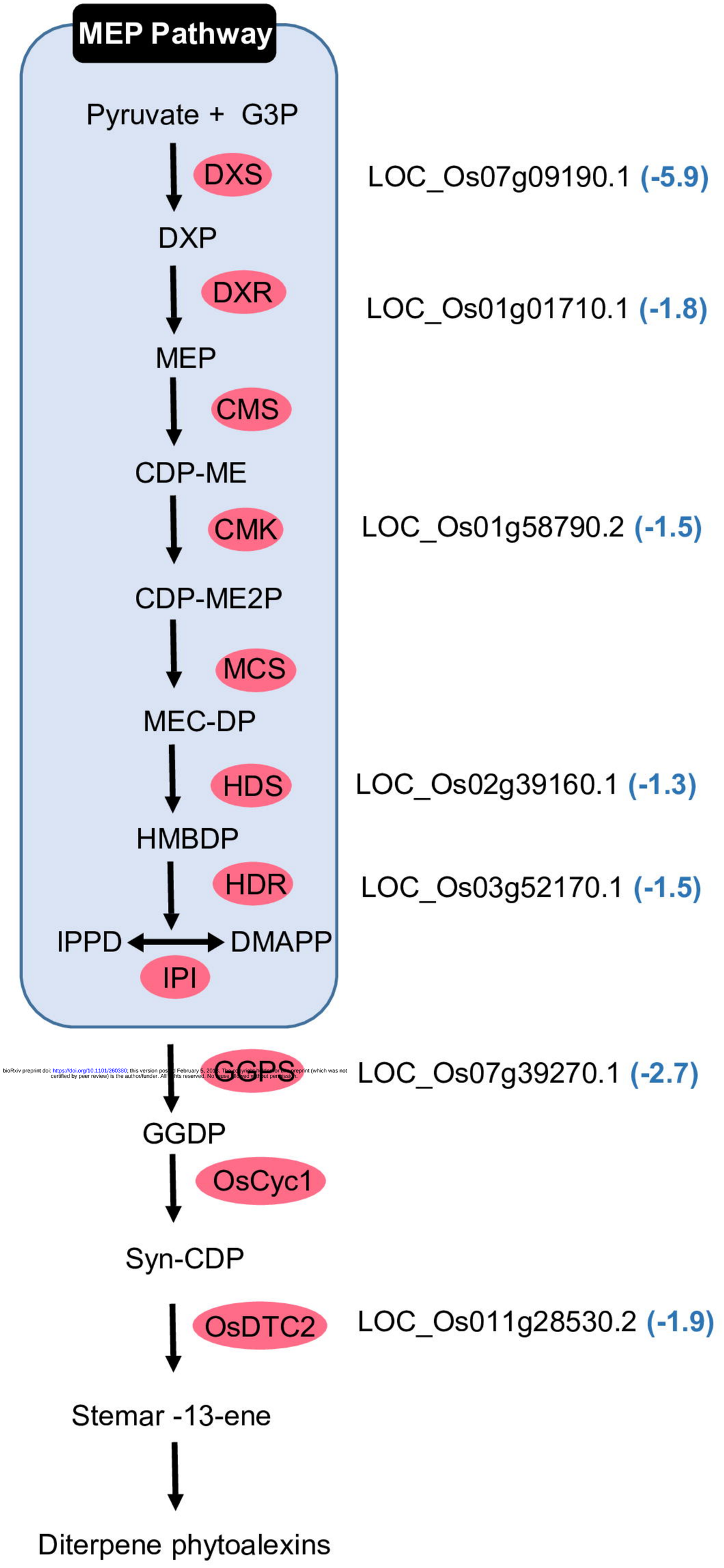
Protein; IRT2(iron-regulated transporter 2); YSL16 (yellow strip-like gene 16); YSL15 (yellow strip-like gene 15)

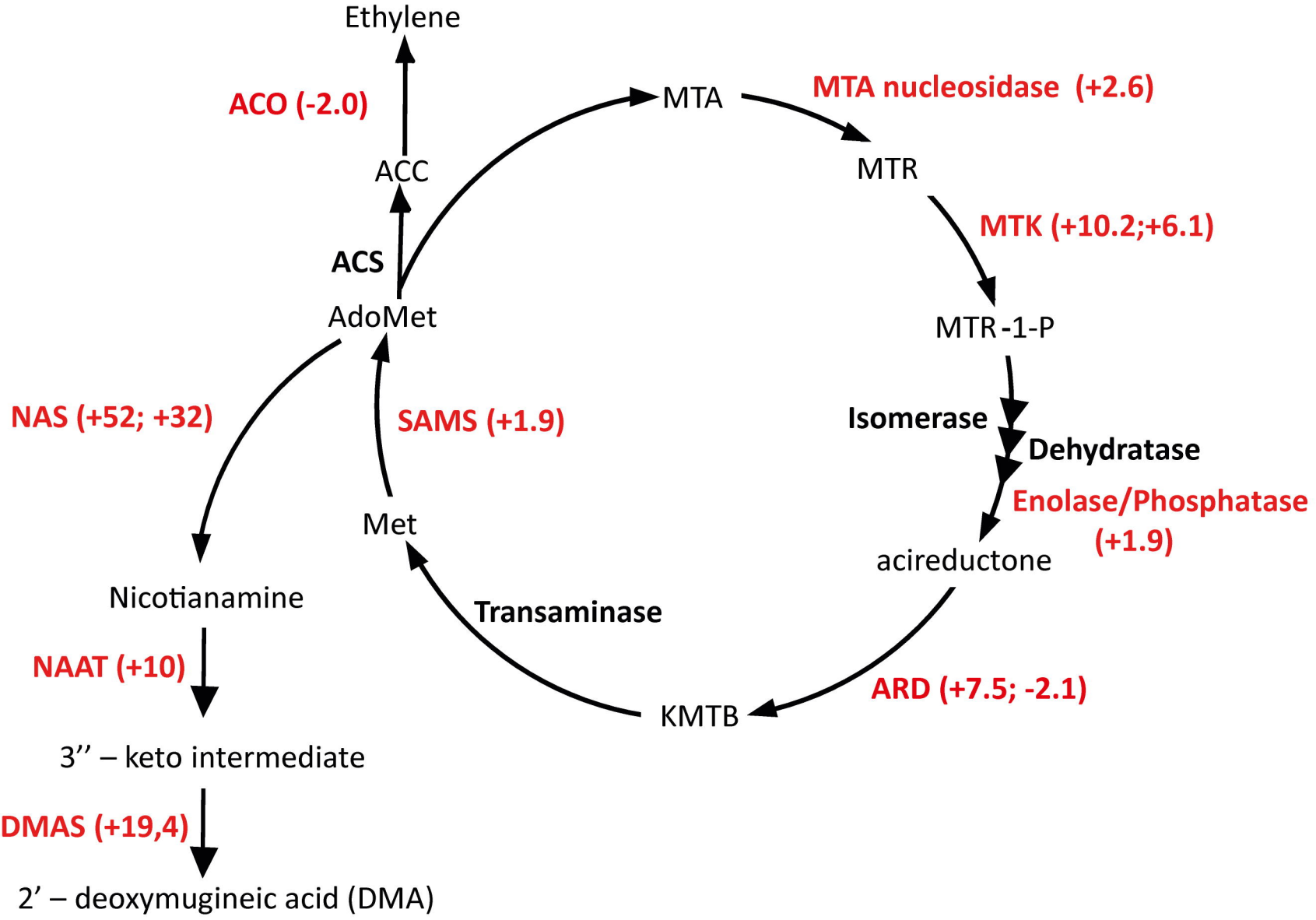

Rice (control)
Rice + H. seropedicae (inoculated, 3DAI)
Samples
rice roots
inoculated rice roots
RNA-seq reads
Reads treatment
- improvement of sequence reads quality with SAET
- removal of adaptors, low quality bases (limit score=0.05) & short reads (<20 bp)
Mapping
Rice genome (RGAP7)
Choloroplast genome
Mithocondrial genome
Analysis
Unmapped reads
Mapped reads
Mapping to H. seropedicae genome
RNA-seq analysis


DOWN-REGULATED
UP-REGULATED
16/657
01/27
0/54
0/09
24/503
22/749
15/167
44/1696
05/151
65/2301
01/11
03/72
0/20
06/123
120/4365
0/16
02/94
01/72
04/41
02/69
09/277
11/233
0/32
0/07
0/13
16/651
11/374
13/398
0/54
03/195
14/546
01/210
0/26
5/210
transport
tetrapyrrole synthesis
TCAc/OAT[1]
sulphur assimilation
stress
signalling
secondary metabolism
RNA
redox
protein
polyamine metabolism
photosynthesis
OPPp[2]
nucleotide
not assigned
nitrogen metabolism
MET/ATPs[3]
minor carbohydrates
metal handling
major carbohydrates
lipid metabolism
hormone metabolism
glycolysis
G/Gc[4]
fermentation
enzyme families
DNA
development
C&Vs[5]
cell wall
cell
C1 metabolism
biodegradation of xenobiotics
amino acid metabolism
58/657
0/27
06/54
02/09
14/503
30/749
09/167
55/1696
07/151
128/2301
03/11
07/72
3/20
05/123
125/4365
03/16
03/94
05/72
10/41
05/69
12/277
16/233
05/32
02/07
03/13
37/651
13/374
11/398
02/54
18/195
24/546
02/210
1/26
18/210
30 25 20 15 10 5 0
% of down-regulated genes in each category
0 5 10 15 20 25 30
% of up-regulated genes in each category


-1
-0,5
0
0,5
1
Pathogen / pest attack
Abiotic stress
Recognition
R genes
Respiratory burst
Signaling
Misc. functions
MAPK
HSPs
Transcription factors
Defense genes
PR-proteins
Secondary metabolites
Hormone signaling
Auxins
Brassinost.
ABA
Ethylene
SA
JA
Cell wall
Beta glucanase
Proteolysis
Redox state
Peroxidases
Glutathione-S-transferase
Signaling
MAPK
Transcription factors
ERF
bZIP
WRKY
MYB
DOF
Heat shock proteins
Secondary metabolites
Putative involvment in biotic stress
Putative involvment in biotic stress


Housekeeping genes
Os07g03230
Tub/FtsZ
GS
WARK22
Int_Memb
Thionin7
Thionin1
rHb2
OsITR2
Similar to YSL15
Nramp6
Tom1
Flavo_Synt
qRT-PCR
RNA-seq
- 8
- 6
- 4
- 2
0
2
4
6
8
10
mRNA expression level (LOG2 fold change)





Putative metal transporters