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Metabolic and transcriptional response of central metabolism affected by root endophytic fungus *Piriformospora indica* under salinity in barley

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Abstract The root endophytic fungus *Piriformospora indica* enhances plant adaptation to environmental stress based on general and non-specific plant species mechanisms. In the present study, we integrated the ionomics, metabolomics, and transcriptomics data to identify the genes and metabolic regulatory networks conferring salt tolerance in *P. indica*-colonized barley plants. To this end, leaf samples were harvested at control (0 mM NaCl) and severe salt stress (300 mM NaCl) in *P. indica*-colonized and non-inoculated barley plants 4 weeks after fungal inoculation. The metabolome analysis resulted in an identification of a signature containing 14 metabolites and ions conferring tolerance to salt stress. Gene expression analysis has led to the identification of 254 differentially expressed genes at 0 mM NaCl and 391 genes at 300 mM NaCl in *P. indica*-colonized compared to non-inoculated samples. The integration of metabolome and transcriptome analysis indicated that the major and minor carbohydrate metabolism, nitrogen metabolism, and ethylene biosynthesis pathway might play a role in systemic salt-tolerance in leaf tissue induced by the root-colonized fungus.

Keywords Endophyte · Salt stress tolerance · Metabolomics · Transcriptomics

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Introduction

Salinity is one of the major abiotic stresses that determine the success or failure of plant establishment and growth. The presence of excess ions in the rhizosphere injures plant roots and causes heavy damage to plant metabolism, which may lead to unfavorable growth and reduced yield (Mahajan and Tuteja 2005; Munns and Tester 2008; Barkla et al. 2013). Mutualistic symbiosis with mycorhizal and endophytic fungi can promote salt tolerance and decrease plant yield losses. The endophytic fungus *Piriformospora indica* (*P. indica*), a basidiomycete of the order *Sebacinales*, was isolated from the Indian Thar desert in 1997 (Varma et al. 1999). *P. indica* is similar to arbuscular mycorrhiza AM fungi (AMF) and has a diverse biological spectrum. The most important advantage of this fungus over AMF is that it is a facultative symbiont and can easily be grown on various synthetic media. Likewise, *P. indica* has a wide host range, colonizes the host roots, grows inter- and intracellularly, and forms pear-shaped chlamydospores within the cortex while improving the growth of many plant species. Furthermore, it enhances nutrient uptake, enables plants to cope with environmental conditions, and survives under abiotic stresses. It also confers resistance to toxins, pathogenic microorganisms, and increases seed biomass yield (Rai 2002; Rashmi et al. 2003; Sahay and

Varma 1999; Waller et al. 2008). As of recently, the gene and the metabolite regulatory networks that promote the growth of *P. indica*-colonized plants are still largely unknown. Nonetheless, some studies have shown that various factors induced by *P. indica* in host plants were responsible for its positive affects (Qiang et al. 2011).

In a previous study, we showed the proteome changes in *P. indica*-colonized barley plants under water deficiency (Ghabooli et al. 2013) and identified several proteins involved in photosynthesis, energy transport, and defense systems. Different functional categories consisting of photosynthesis, cell antioxidant defense, protein translation and degradation, energy production, signal transduction, and cell wall arrangement were detected in *P. indica*-colonized barley plants that were exposed to three levels of salinity (Alikhani et al. 2013). However, the advances in high-throughput genomics and metabolomics technologies have been shown to be valuable in providing better insight into pathways and mechanisms that are induced by beneficial microorganisms in plants. The use of transcriptomics have been shown to be very beneficial, considering most stress responsive pathways are transcriptionally initiated (Morán-Diez et al. 2012). For instance, global gene expression analysis revealed symbiotic association underpins of signaling components, such as ethylene biosynthesis in barley and *Arabidopsis thaliana* (Khatabi et al. 2012). There is also evidence that metabolic cues affect plant's ability to switch between biotrophy and saprotrophy during root colonization in different hosts (Lahrmann et al. 2013).

Integrating metabolomics and transcriptomics profiling were used to discover contrasting responses of two rice varieties to *Xanthomonas oryzae* pv. *oryzae* (Sana et al. 2010). These analyses yielded novel insights into the rice response to the bacterial blight pathogen, *X. oryzae* pv. *oryzae,* and revealed global metabolic and transcriptomic changes in the leaf tissue of two rice varieties. The identified primary and secondary metabolite signatures induced by roots colonized with *Rhizophagus irregularis* in *Medicago truncatula* grew under different phosphate conditions that were previously published (Schliemann et al. 2008). Recently, two active signal molecules comprising of propionyl- and butyryl-carnitines were discovered by the metabolite and transcript profiling of *M. truncatula* roots colonized with *R. irregularis* (Laparre et al. 2014). However, the connection between differentially expressed genes and their related metabolic pathways are still lacking.

In the current study, we analyzed the systemic response induced by a root endophyte, *P. indica*, at two levels of salt conditions in barley. Multi-omics profiling analyses of barley leaves were performed in *P. indica*-colonized and non-inoculated plants under 0 and 300 mM NaCl 4 weeks after inoculation. A comparative transcriptomics and metabolomics analysis suggested that major and minor carbohydrate metabolisms, nitrogen metabolism, and ethylene biosynthesis pathway play role in the induced systemic salt-tolerance supplemented by the root endophytic fungus *P. indica*.

Materials and methods

Fungal growth, plant inoculation, and co-culture of plant with *P. indica*

Piriformospora indica was grown on a complex medium (CM) at 24 °C (Huong et al. 2004). The spore suspension was collected after 4 weeks by gently scratching the fungus surface on the Petri dishes with a spatula until the spores were released. The spore concentration was adjusted to 5×10^5 spores per ml as described by Alikhani et al. (2013). Barley (*Hordeum vulgare* L.) cultivar Pallas was grown in a 2:1 mixture of sand and perlite in a growth chamber at 22/18 °C day/night cycle, 60 % relative humidity, and a photoperiod of 16 h (220 µmol m^{-2} s^{-1} photon flux density) and were irrigated by Wuxal Top N solution (Schering, N/P/K: 12/4/6). The experiment was conducted in a completely randomized design with two fungal treatments (*P. indica*-colonized and non-inoculated) and two salt levels (0 and 300 mM NaCl) in three independent biological replications. Barley kernels were sterilized with 1 % sodium hypochloride, rinsed in distilled water 3–4 times, and germinated. For plant inoculation, seedling roots were immersed in an aqueous solution of 0.02 % Tween-20 containing 5×10^5 spores ml^{-1}. Control seedling roots (non-inoculated) were immersed in the same solution without spores. *P. indica*-colonized and water-treated seedlings were transferred to pots and grown for 4 weeks. Before salinity treatment, root samples were tested for *P. indica*-colonization. After 14 days of fungal inoculation, plants were watered with double-distilled water containing either 0 or 300 mM NaCl in 2-day intervals. After 4 weeks of inoculation, leaf samples were collected from control and *P. indica*-colonized plants under different salt conditions. Three plants per pot (as one replication) were harvested from three pots (as biological replicates) for further analysis. The collected samples were immediately frozen in liquid N_2 and used for transcriptome metabolome and ion analysis.

Ionom analysis

The total dry weight, an indicator of plant productivity, was measured by drying the total number of fresh materials in an oven at 70 °C for 48 h and was thereafter used for the elements measurement. The elements were determined

using inductively coupled plasma mass spectrometry (ICP-OES, iCAP 6500 dual OES spectrometer; Thermo Fischer Scientific, Waltham, USA) after extraction with HNO_3 as previously described (Gruber et al. 2013). For the Nitrogen (N) and Carbon (C) analysis, the plant materials were freeze-dried and ground using a ball mill. The N and C concentrations of one mg of material were determined using an elemental analyzer (Euro-EA, HEKAtech, Germany).

Metabolome analysis

Targeted metabolite profiling was performed using ion chromatography, a mass spectrometry (IC-MS/MS) instrumentation consisting of a Dionex ICS 5000 (Dionex, Idstein, Germany) with a 6490 triple Quad LC/MSMS (Agilent, Germany). The anionic compounds were separated on a 250 × 2 mm AS11-HC column connected to a 10 × 2 mm AG 11-HC-guard column (Dionex, Idstein, Germany) and an ATC-1 anion trap column. The gradient was produced with H_2O (buffer A; MS grade water) and KOH, which was generated by an EGC III KOH eluent generator cartridge. The column was equilibrated with a mixture of buffer A (96 %) and 4 % KOH at a flow rate of 0.38 ml/min and heated to 37 °C during the measurement. The gradient was produced by changes of KOH concentration as follows: 0–4 min: 4 %; 4–15 min: 15 %; 15–25 min: 25 %; 25–28 min: 50 %; 28–31 min: 80 %; and 31–40 min; 4 %. Quantitative analysis of metabolites were performed using an Agilent 6490 triple quadrupole (QQQ) mass spectrometer (Agilent, Germany) by specific MS/MS transition methods described previously (Heinzel and Rolletschek 2011). The reliability and stability of the compounds were checked by the internal standard ^{14}C-pyruvate, which was added to each sample before analysis. The soluble amino acids were measured using reversed-phase HPLC system as described by Zurbriggen et al. (2009). Chromatograms were recorded using the software program Empower Pro (Waters, USA). The concentrations of sugar alcohols were determined using an ion chromatography system (Dionex, Idstein, Germany) consisting of a gradient pump (GS50), ED50 electrochemical detector, and an autosampler (AS50). The anionic compounds were separated on a CarboPac MA1 column (4 × 250 mm), connected to a guard column (4 × 10 mm), and an ATC-1 anion trap column was placed between the eluent and separation column. The eluent, 500 mM NaOH, was made from HPLC grade water (Millipore, Germany) and 50 % NaOH (Merck, Germany). The column was equilibrated for 1 h at a flow rate of 0.35 ml/min. The run time was 50 min and a linear gradient was accomplished with 52 % water and 48 % NaOH. The calibration and quantitative calculation of sugar alcohols were carried out using Chromeleon client software 6.6 SP, Build 1566 (Dionex, Idstein, Germany).

Transcriptome analysis

Total RNA was extracted from barley leaf samples of three independent biological replicates using Trizol reagent (Invitrogen, life technologies, Karlsruhe, Germany). RNA was treated with DNase (QIAGEN) and eluted with 10 µl of pre-warmed elution buffer. Then, quantity and quality of extracted RNA were determined using Nano-Drop (Ambion) and by gel electrophoresis, respectively. Probes were labeled with [^{33}P]-UTP during the second round of amplification to generate phosphorylated antisense RNA. Labeled probes were denatured (5 min, 70 °C) and cooled on ice (5 min) before hybridization. Reference probes for fidelity check of RNA amplification and tissue fixation were prepared according to Sreenivasulu et al. (2006). The purified RNA (35 µg) was used to synthesize [^{33}P]-dCTP-labeled cDNA probes using random primers. The barley PGRC2 13 K cDNA array was hybridized and processed, exposed to phosphor image screens for 24 h, and scanned using a Fuji BAS 3000 phosphor scanner (Fuji Photo Film, Tokyo, Japan) as previously described by Sreenivasulu et al. (2006). The images of hybridized nylon membranes were subjected to automatic spot detection using Automatic Image Data Analysis (AIDA) v 4.08 software (Raytest, Straubenhardt, Germany). A total number of 13,050 genes were covered per array by 13,824 double spots, enabling one technical replicate per gene for quality control. Additionally, we considered the gene expression levels from independent samples to check biological reproducibility. These combinations resulted in two technical and three biological data sets. Quantile normalization was carried out on the complete data set (Bolstad et al. 2003). Thereafter, the expression values were $\log^2$-transformed. Spots detected with *P* value higher than 0.05 were excluded. The characterizations of remained probes (genes) were identified using IPK Crop EST Database (http://pgrc.ipk-gatersleben.de/cr-est). The spots related to the same genes were eliminated. Then, the identified probes were BLAST searched against Arabidopsis transcriptome database TAIR 10 (ATtranscriptTAIR10). Furthermore, the Mapman bininng system was used to classified the regulated transcripts in 0 and 300 mM NaCl conditions.

Simple and multivariate statistical analysis

Metabolite and element concentrations expressed on FW and DW basis, respectively were used for statistical analysis. The ionomic and metabolomic data was $\log_2$-transformed prior to analysis of variation (ANOVA). Then, Benjamini-Hochberg's correction tests were computed to

produce adjusted *P* values. The metabolites and ions, which could pass pBH < 0.05, were considered as significant accumulated metabolites and ions. All metabolite and ion concentrations were then uploaded to Metaboanalyst v.3 web tool (Xia et al. 2015) for multivariate statistical analyses comprising of principal components analysis (PCA) and partial least squares discriminant analysis (PLS-DA). All data included were Pareto-scaled prior to PCA and PLS-DA. Discriminating metabolites and ions were identified using a statistically significant threshold of variable influence on projection (VIP > 1.0) values obtained from the PLS-DA model and were further validated by *t* test analysis and permutation test. Metabolites and ions with VIP values >1.0 were selected as discriminating traits among the classes of samples.

Visualization and classification of data

MapMan, which uses a hierarchical ontology system, was used to classify and visualize the transcriptome data (Usadel et al. 2009). The transcripts with the adjusted *P* values <0.05 used Benjamini-Hochberg's method and the transcripts with the fold changes $>+1.5$ or <-1.5 were considered differentially expressed to visualize in MapMan. The annotations on transcripts were carried out using a simple unidirectional BLAST search against already classified proteins from Arabidopsis to maximize their sensitivity. Based on the best BLAST search results and using a cutoff e-value of 10^{-30}, the barley genes were assigned to BINs/subBINS according to the most similar Arabidopsis genes. Heat maps were pictured using Metaboanalyst v.3. Functional categorization (Gene ontology, GO) using BINGO, a cytoscape plugin, to assess overrepresentation of GO categories (Maere et al. 2005).

Quantitative RT-PCR analysis

Total RNA was extracted using Trizol reagent (Invitrogen, life technologies) as described in the previous section. Two µg of each RNA sample used to construct cDNA by using a RevertAid First Strand cDNA Synthesis Kit (Thermo-scientific). Oligo-Analyzer 3.1 software was used to design primer pairs for the randomly selected candidate genes involved in carbon and nitrogen metabolism. Gene expression was assayed using a Mastercycler Realplex 2 cycler (Eppendorf, Hamburg, Germany) and the iQ SYBR Green Supermix Kit (Bio-Rad, Munich, Germany). All reactions were performed in six replications (three biological replications and two technical replications). The mRNA expression level of genes shown in Table 1 and Ubiquitin, chosen as a housekeeping gene, was determined in parallel for each sample. The comparative CT method (also known as the $2^{-\Delta\Delta CT}$ method) was used to present qRT-PCR data. In the way described, the data was interpreted as the expression of the gene of interest relative to the internal control (housekeeping) in the treated sample compared with the un-treated control (Schmittgen and Livak 2008). Mean and SEM was calculated after $2^{-\Delta\Delta CT}$ transformation had been performed. The fold induction resulted in three independent pools for each target gene was averaged and standard error of the mean was calculated.

Results

P. indica promoted barley growth under salinity

As it was shown in previous studies (Alikhani et al. 2013; Ghabooli et al. 2013), *P. indica* enhanced barley plants growth under two levels of salt conditions. Four weeks post plant inoculation, total shoot dry weight (DW) of *P. indica*-colonized plants increased up to 1.24-fold, compared to control plants at 0 mM NaCl condition. Furthermore, the results revealed an increase in DW 1.5-fold compared to non-inoculated barley plants on 300 mM NaCl (Supplementary Fig S1).

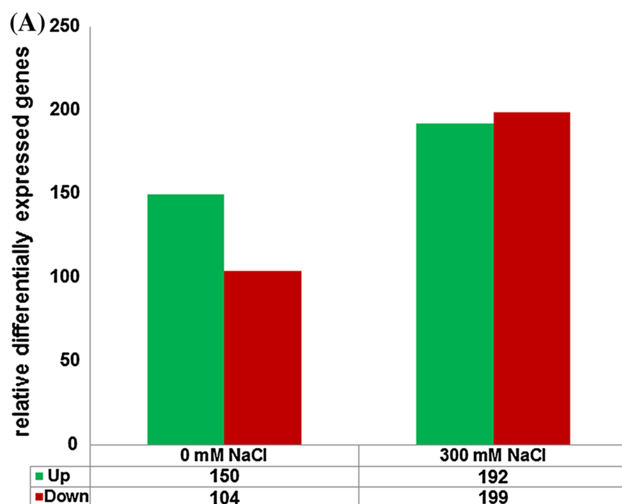
P. indica de-regulated genes involved in various metabolic pathways

A custom-made BarleyPGRC2 13 K cDNA array was used to identify differentially express transcripts in *P. indica*-colonized compared to non-inoculated (+*P. indica*/−*P. indica*) plants under two levels of the NaCl. The list of 4941 detected feature spot IDs is provided in Supplementary Table S1. Of these, 1048 plant-derived spotted features were categorized using MapMan binning system into the BINS 1-35 based on Arabidopsis gene IDs (Supplementary Table S2). Then, ANOVA analysis was used to compare the transcript levels in inoculated and non-inoculated plants at the 0 and 300 mM NaCl conditions. The results yielded unique 254 and 391 differentially regulated genes at 0 and 300 mM NaCl levels, respectively, of which 150 up- and 104 down-regulated genes belonged to 0 mM NaCl condition and 192 up- and 199 down-regulated genes at 300 mM NaCl compared to non-inoculated plants (Fig. 1a) and Supplementary Table S3. Likewise, there were 149 genes specifically regulated at the 0 mM NaCl and 242 differentially expressed genes (1.6 times more compared to 0 mM NaCl) were identified only at 300 mM NaCl condition (Fig. 1b). Functional analysis by gene ontology (GO) annotation was performed on up and down regulated genes and enriched biological processes with false discovery rates below 5 % were presented in Fig. 2. Interestingly, the results showed the enrichment of genes in

Table 1 List of genes validated by qRT-PCR for their expression in 0 and 300 mM NaCl conditions in inoculated versus non-inoculated barley plant

Gene ID	Gene	0 mM NaCl		300 mM NaCl	
		cDNA array	RT-PCR	cDNA array	RT-PCR
HF01G10	Hexose transporter	−2.03	−2.71	2.07	1.19
HA02O06	Putative glycosyltransferase	6.60	4.83	1.72	3.06
HZ01F01	Putative aminotransferase	4.04	4.06	−1.82	−3.96
HO13I18	UDP-D-glucuronate decarboxylase	2.02	3.37	−5.82	−4.15
HP01A07	Putative trehalose-phosphatase	2.19	5.11	3.24	4.24
HO14K14	Alanine aminotransferase	2.57	3.51	3.39	2.79

The values are the ratios of signal intensities between *P. indica*-colonized barley plants and control plants were quantified by array analysis and real-time PCR, respectively



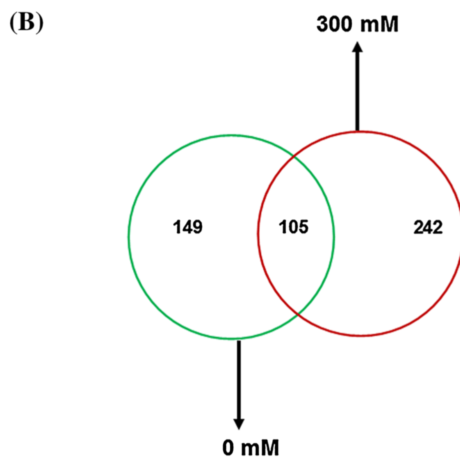


Fig. 1 Differentially expressed genes in *P. indica*-colonized versus non-inoculated barley plants under 0 mM NaCl and 300 mM NaCl. **a** Number of up- and down-regulated genes in non-inoculated (*green*) and *P. indica*-colonized plants (*red*). **b** Venn diagram indicates number of specifically and commonly up- and down-regulated genes in non-inoculated and *P. indica*-colonized plants under salt treatments

multi-organism process 54 (genes) of which 9 genes were down-regulated in 0 mM NaCl, whereas 19 and 26 genes were down- and up-regulated in 300 mM NaCl (Fig. 2). The enrichment of the multi-organism process category consists of the biological process subclasses, such as increase in size or mass of organism, when the organism is in symbiotic interaction lifestyle.

Additionally, MapMan software was used to integrate and visualize the differential expressed genes into their functions in the metabolic pathways (Figs. 3, 4). The two image annotator modules of overview and biological processes were used to map data in BINs/subBINS, according to the similarity of Arabidopsis genes. This allows exploration of the gene categories that are activated during *P. indica* colonization with higher emphasis on those related to energy metabolism, major and minor carbohydrate metabolism, hormone metabolism, transcription regulators, small RNA regulation, redox, and stress responses described in detail in the following sections.

Energy metabolism

The visualization of differentially expressed genes, by Mapman, showed that out of 149 and 391 transcripts 49 and 79 were differentially expressed at 0 and 300 mM NaCl, respectively. The differentially expressed genes with red and green colors for positive and negative regulations are shown in Fig. 3a, b, respectively. There was an increase in the number of down-regulated genes associated with light reaction of photosynthesis, Calvin cycle, and photorespiration at 300 mM NaCl (Fig. 3b). At 0 mM NaCl, seven genes including: phosphoglycerate kinase, triose phosphate isomerase, light harvesting complex 1–3, chlorophyll A/B binding protein, and peroxisome glycolate oxidate 3 were up-regulated. While three genes comprising of light harvesting complex 4, binding protein D1, and glycolate oxidate were down-regulated after 4 weeks of *P. indica* inoculation (Fig. 3a). Two isoforms of glycolate oxidate 1 and peroxisome glycolate oxidate 3, which were involved in hydrogen peroxidase biosynthesis and defense response signaling, were exclusively up- and down-regulated at 0 mM NaCl. The number of down-regulated genes

increased at 300 mM NaCl up to 22 of which chloroplastic bisphosphate aldolase (down-regulated to 2.54-fold) was the most prominent down-regulated gene amongst the transcripts (Fig. 3b).

Major and minor carbohydrate and nitrogen metabolism

Based on the array data, *P. indica* modified the expression pattern of major and minor CHO metabolism. Severe salt stress (300 mM NaCl) decreased the expression of three homologue genes of sucrose synthase (SUS3) and beta-amlyase in starch and sucrose degradation category, whereas ADP-glucose pyrophosphorylase, involved in starch biosynthesis, altered in *P. indica*-colonized plants without the addition of salt (Fig. 3a). Furthermore, we observed (Fig. 3a) the up-regulation of four genes involved in starch and sucrose breakdown is composed of cell wall invertase (CWI1), sucrose synthase (SUS1), vacuolar invertase (Vinv4), and cytosolic invertase. At 300 mM NaCl, the mapped data showed four up-regulated genes including invertase and sucrose synthase (SUS1) were involved in sucrose breakdown. Starch synthase, fructose

Fig. 3 Schematic overview of differentially expressed genes illustrated as metabolism overview in Mapman ontology. Up- and down-regulated genes in *P. indica*-colonized versus non-inoculated plants are shown as an increasingly *intense red* and decreasingly *intense green* at 0 mM NaCl (**a**) and 300 mM NaCl (**b**), respectively. The ratios of transcript abundance were calculated based on three biological replicates of BarleyPGRC2 13 K cDNA array. On the logarithmic scale ($\log^2$) ranging from 2 to −2, *green* shows down-regulated transcripts, and *red* shows up-regulated transcripts ▶

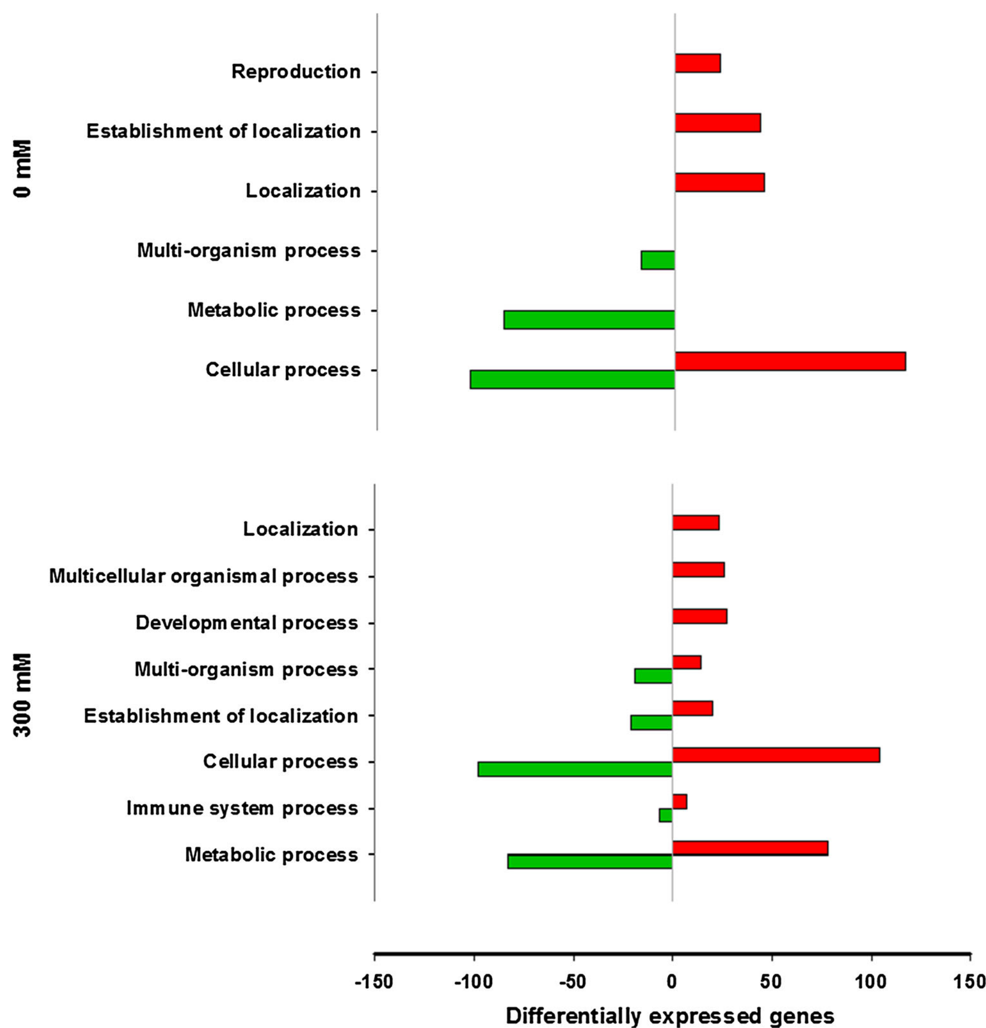
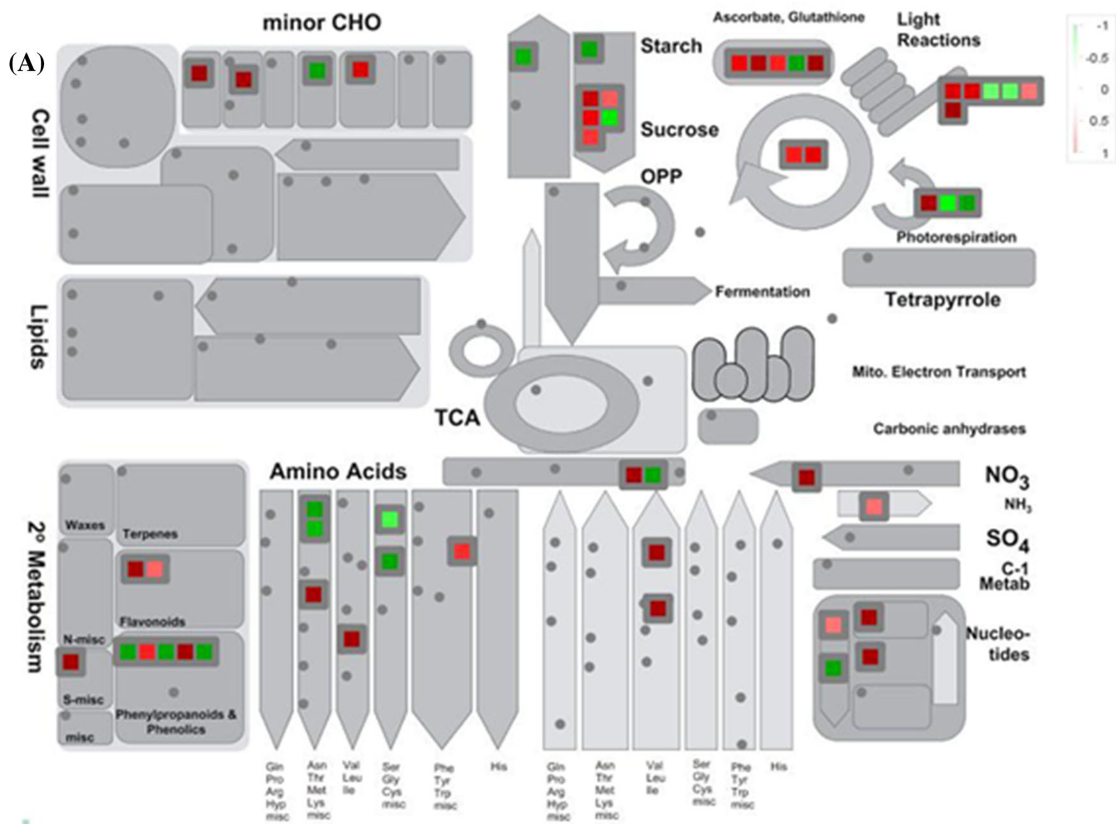
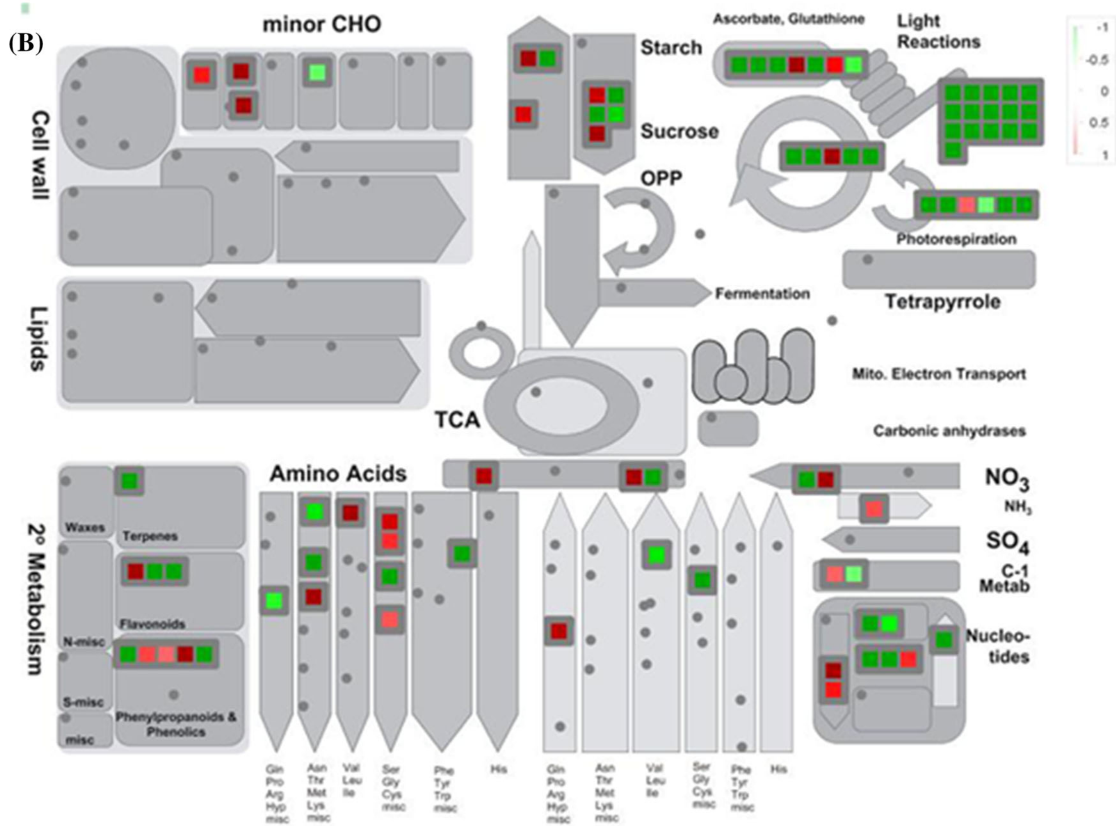


Fig. 2 Gene Ontology (GO) analysis of up- and down-regulated genes. The GO biological process associated with the best hit of the Arabidopsis genes were assigned to the corresponding barley transcript using BiNGO. The number of statistically significant enrichment of over- or under-represented genes in 0 and 300 mM NaCl conditions in a $P < 0.05$ after a multiple correction test are shown in *green* and *red colors*, respectively


(A)
minor CHO
Cell wall
Lipids
2° Metabolism
Starch
Sucrose
OPP
Ascorbate, Glutathione
Light Reactions
Photorespiration
Tetrapyrrole
Fermentation
Mito. Electron Transport
Carbonic anhydrases
TCA
Amino Acids
NO3
NH3
SO4
C-1 Metab
Nucleo-tides
Waxes
Terpenes
Flavonoids
N-misc
S-misc
misc
Phenylpropanoids & Phenolics
-1
-0.5
0
0.5
1


(B)
minor CHO
Cell wall
Lipids
2° Metabolism
Starch
Sucrose
OPP
Ascorbate, Glutathione
Light Reactions
Photorespiration
Tetrapyrrole
Fermentation
Mito. Electron Transport
Carbonic anhydrases
TCA
Amino Acids
NO3
NH3
SO4
C-1 Metab
Nucleo-tides
Waxes
Terpenes
Flavonoids
N-misc
S-misc
misc
Phenylpropanoids & Phenolics
-1
-0.5
0
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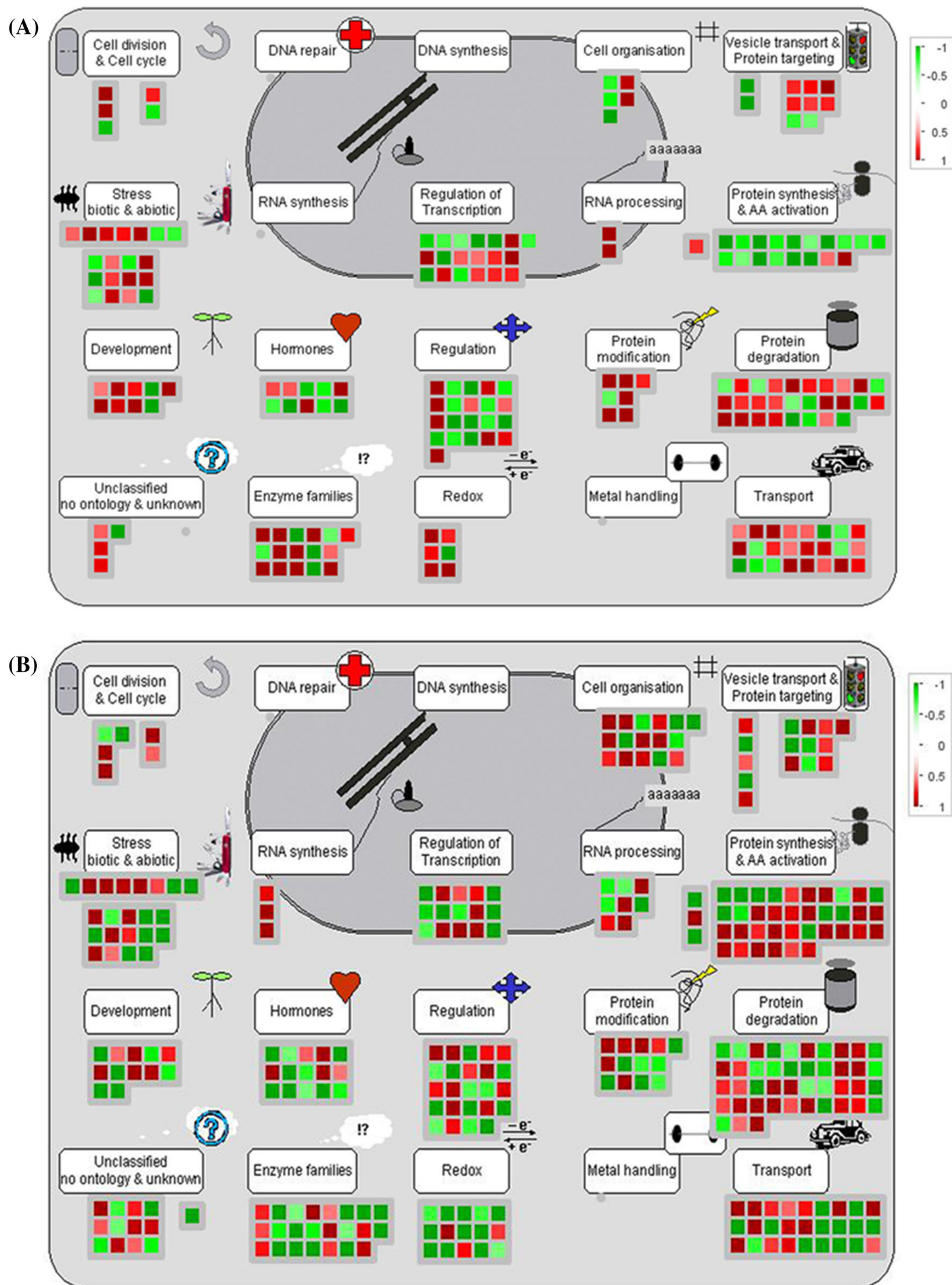

Fig. 4 MapMan cell function overview showing differences in transcript levels between 0 mM NaCl (**a**) and 300 mM NaCl (**b**) in inoculated versus non-inoculated barley. In the color logarithmic scale, *green* and *red* represent at least twofold higher or lower gene expression in *P. indica*-colonized versus non-inoculated barley in 0 mM NaCl and 300 mM NaCl conditions

1,6 bisphosphatase, and ADP-glucose pyrophosphorylase were involved in starch and sucrose biosynthesis (Fig. 3b). Likewise, the expression of two genes involved in trehalose metabolism, trehalse-6 phosphate synthase (TPS), and trehalose phosphate phosphatase (TPP) were up-regulated at 300 mM NaCl, whereas at the 0 mM NaCl only TPP showed up-regulation (Fig. 3a). Interestingly, glutamine synthetase 1 (GS1) gene was down-regulated, whereas glutamine synthetase (GS2) gene was up-regulated in *P. indica*-colonized plants at 300 mM NaCl (Fig. 3b).

Hormone regulations

The analysis of transcript data showed the specific expression patterns of genes involved in hormone metabolism at 0 and 300 mM NaCl included auxin, ethylene, jasmonic acid, abscisic acid (ABA), and brassinosteroid (BR) (Fig. 4). Overall, the 300 mM NaCl resulted in a much higher number of differentially expressed genes (5 up- and 9 down-regulated) compared to 0 mM NaCl in which 4 down- and 2 up-regulated unique genes were identified in the hormone metabolism during *P. indica*-colonization. This is described in detail in the following sections.

Auxin metabolism

Transcripts involved in auxin signal transduction comprising of amino acid transmembrane transporter (AUX1) Auxin resistant 1 (AXR1) were significantly up-regulated up to threefold at 300 mM NaCl. However, auxin inducible genes were only identified at 0 mM NaCl, of which ATB2 oxidoreductase showed negative and aldo/ketoreductase family member showed positive regulation (Fig. 4a, b).

Ethylene metabolism

In response to *P. indica*-root colonization, transcripts involved in the metabolism of ethylene, plant growth inhibitor as well as ethylene signaling molecule were significantly induced only at 300 mM NaCl. Specifically, the genes of universal stress protein (USP) and multi-protein bridging factor 1A (MBF1A) showed up- and down-regulation in the bins of induced-regulated-responsive-activated, respectively. The genes coding for ethylene response 1 in signal transduction bin, 1-aminocyclopropane-1-carboxylate oxidase (ACC oxidase) in ethylene biosynthesis, and degradation bin exhibited positive and negative regulation, respectively (Fig. 4a, b).

Bassinosteroids

Three genes involved in brassinosteroid pathway were identified, which were significantly up-regulated both 0 and 300 mM NaCl. The sterol delta 7 reductase gene of brassinosteroid synthesis-degradation bin showed negative regulation at 0 mM NaCl. However, brassinosteroid insensitive 1, a negative regulator of brassinosteroids, showed positive and squalene epoxidase gene showed negative regulation at 300 mM NaCl (Fig. 4a, b).

Abscisic acid (ABA) metabolism

Two de-regulated genes were identified in ABA pathway at 300 mM NaCl level. HV22D, a responsive gene to ABA showed down-regulation, while ABA deficient 1gene was down-regulated in 300 mM NaCl (Fig. 4a, b).

Transcription factors (TFs)

In total, 13 unique and differentially expressed Transcription Factors (TFs) were identified in *P. indica*-colonized barley at 0 mM NaCl. Five, of which, were down-regulated and eight were up-regulated in *P. indica*-colonized barley (Fig. 4a, b). In contrast, 17 TFs were identified in *P. indica*-colonized barley at 300 mM NaCl, of which 9 were down- and 8 were differentially up-regulated. The distinct pattern of TF expression was identified in response to *P. indica*- colonization between 0 and 300 mM NaCl conditions. The primary known family with significant response to *P. indica* colonization was bHLH, AP2/EREBP, SR, SNF7 and MYB. In the super family of bHLA there were three down-regulated genes comprising of BEE2 (BR enhanced expression 2) and PIL6 (phytochrome interacting factor 3-like 6) at 0 mM NaCl and bHLH protein at 300 mM NaCl. Furthermore, within the AP2/EREBP transcription factor family, there was a down-regulation for RAP2.7 genes at 300 mM NaCl. This contradicted *P. indica because it* did not change the expression of any of these genes at 0 mM NaCl. The SN7 gene family consisting of VPS32 and VPS2.1 were exclusively up-regulated only at 0 mM NaCl. In distinction, two genes of the SR family, including AKR2B and EICBP.B (ethylene induced calmodulin binding protein), were differentially up- and down-regulated exclusively at 300 mM NaCl, respectively (Fig. 4a, b).

Redox state metabolism

Reactive oxygen species (ROS) is one of the earliest plant responses to both a plant-pathogen and a plant-symbiont interaction. Our results showed an increase in the number of up-regulated redox-related genes at 0 mM NaCl, while at 300 mM NaCl only a few down-regulated genes could be detected (Fig. 4a, b). In particular, the gene encoding glutathione peroxidase was significantly up-regulated up to 2.5-fold at 0 mM NaCl. While 1.5-fold reduction was found in

redox.ascorbate and glutathione.glutathione bin at 300 mM NaCl. Moreover, in bins of redox.ascorbate and glutathione.glutathione, redox.ascorbate and glutathione. ascorbate, redox.ascorbate and glutathione.glutathione, two genes that belonged to peroxidases were identified, of which thylakoid ascrobate peroxidase (TAX) and ascorbate peroxidase 3 (APX3) were significantly up-regulated at 300 mM NaCl, while no changes were found for APX3 and TAPX at 0 mM NaCl. Likewise, the peroxiredox showed up-regulation only at 300 mM NaCl. Another differentially expressed gene identified within this study was the gene coding for vitamin c defective 2 (VTC2), which was involved in redox.ascorbate and glutathione.ascorbate. GDP-L-galactose-hexose-1-phosphate guanyltransferase were bins in both conditions. Furthermore, the down-regulation of the redox.glutaredoxins and redox.thioredoxin bins was found particularly at 300 mM NaCl (Fig. 4b).

P. indica modified the expression of abiotic stress-regulated genes

In the current study, we defined a distinct pattern of differentially expressed genes modified by abiotic stress in barley plants colonized by *P. indica* at both 0 and 300 mM NaCl (Fig. 4a, b). Overall, the higher number of genes (57 %) were down-regulated at 300 mM compared to 0 mM NaCl (40 %). The genes encoding HSC70-7, HSF3, heat shock protein-related, DNAJ heat shock N-terminal domain-containing protein, HSP81-3, HSP81-2, and heat shock cognate 70 kDa protein 3 showed an up-regulation at 300 mM NaCl. Furthermore, HSP81-3 and heat shock protein-related genes were significantly up-regulated at 0 mM NaCl (Fig. 4a, b).

P. indica affected ion hemostasis under salinity

Macro- and micro-nutrients were profiled in barley shoots using ICP-AES (Supplementary Table S4). Our results showed differential accumulation of some elements in different conditions (Supplementary Table S5). In a heat map, the color blue depicts down-regulated and the color red depicts up-regulation, respectively (Fig. 5). The macro- and micro-elements showed decreased at both 0 and 300 mM NaCl. The Mg^{2+} and Ca^{2+} levels were increased up to 1.4-fold at 300 mM NaCl compared to non-inoculated plants (Fig. 6). The Na^{+} concentration of the control and *P. indica*-colonized plants increased strongly up to 13 and sevenfold, separately (Fig. 6). A slight decrease in K^{+} concentration was detected (7 and 2 %) in control and *P. indica*-colonized plants, respectively. Moreover, the K^{+}/Na^{+} ratio was higher in *P. indica*-colonized plants compared to control plants under salt stress (2.13-fold induction in *P. indica*-colonized compared to 1.91-fold in control plants under severe salt stress). The similar results were obtained for Ca^{2+}/Na^{+} ratio (0.52 in *P. indica*-colonized plants compared to 0.37 in the control plants under severe salt stress) (data not shown).

P. indica changed the metabolic profile in response to salt stress

The metabolite profiling was performed using IC-MS/MS for all treatments in six replications to investigate metabolite changes in *P. indica*-colonized and non-inoculated plants under both normal and severe salt conditions (Supplementary Table 6). A total of forty-two compounds of known structures comprising of 20 amino acids, 18 phosphorylated metabolites, organic acids, nucleotides and sugar nucleotides and four sugars were quantified in barley shoot. The effect of different treatments, including the combination of fungus and salt, were analyzed by 2-way ANOVA with a *P* value of 0.05 using the Multi Experiment Viewer (MeV) in the TM4 software package (http://www.tm4.org). There were seven and eleven metabolites significantly affected by the fungus alone and the combination of fungus and salt treatment (Supplementary Table S7). Three metabolites were found commonly in both conditions; therefore, resulting in a signature of eleven compounds containing unique metabolites affected exclusively and significantly by fungus colonization. These metabolites are comprised of starch and glucose (Glc), two sugar phosphates, ribose-5 phosphate and trehalose-6 phosphate (Tre6P), five amino acids: Asp, Asn, Thr, Ala and GABA, two organic acids: citrate (Cit) and glucuronic acid, and ADP. The heat map of metabolites along with the other physiological parameters is shown in Fig. 6. In order to visualize and simplify graphical interpretation of the metabolic and physiological data, we further performed a multivariate principal component analysis (PCA) for the two levels of salt conditions. The leaf samples harvested at 0 (+*P. indica*/−*P. indica*) and 300 mM NaCl (+*P. indica*/−*P. indica*) were easily distinguishable along PCA1 (Fig. 7a). For the first and second principal components, PCA1 and PCA2 both covered 75.5 % of total variations using 53 features. Then, partial least squares discriminant analysis (PLSDA), a multivariate statistical test, was used to identify metabolic traits with stronger effects on the separation of samples. Two PCs of the PLSDA explained 74.3 % of the metabolic variation in the data set and clearly separated samples at 0 mM NaCl from the samples treated with 300 mM NaCl. The variable importance in the projection (VIP) value was validated by a permutation test to rank the influence of individual traits on the separation of the samples by the PLSDA model (Fig. 7c). VIP values being equal to or larger than 1.0 were considered as

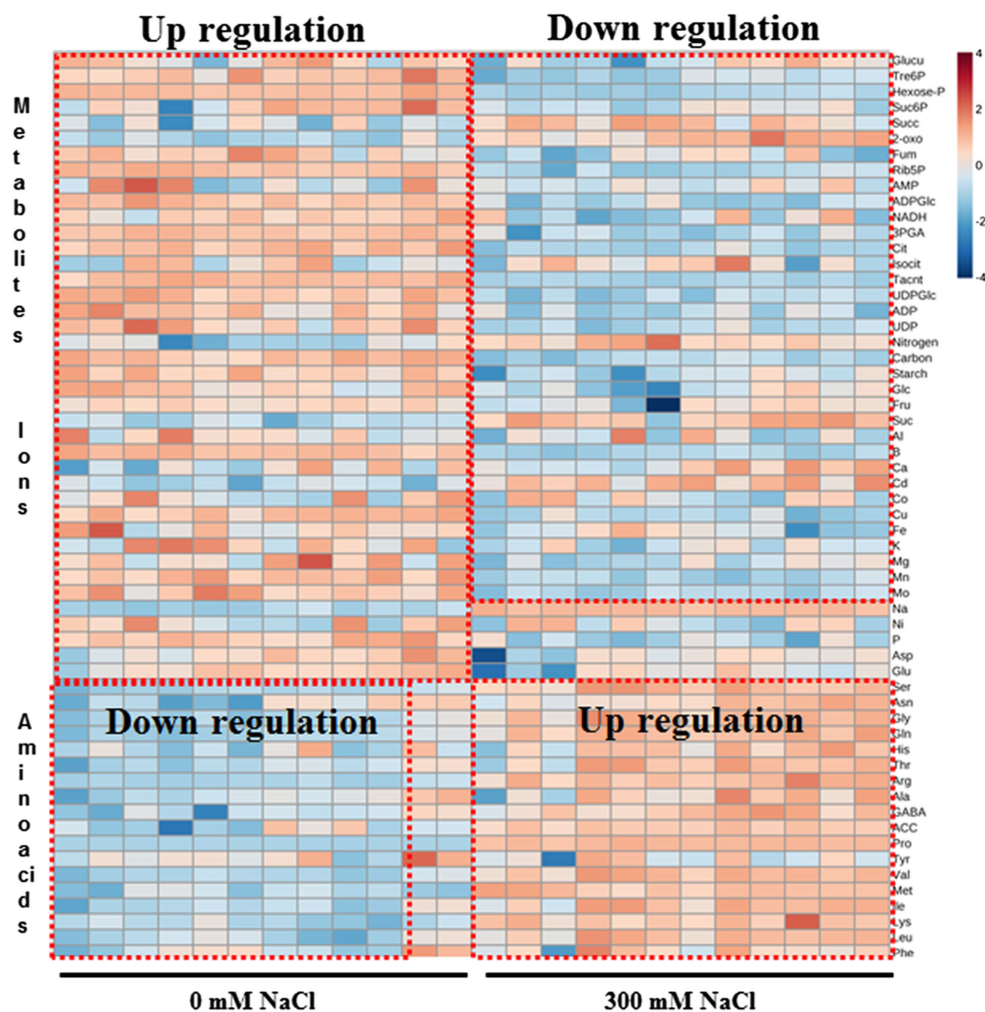

Fig. 5 Heat map of profiled metabolites, amino acids, and ions in *P. indica*- colonized versus non-inoculated plants at 0 and 300 mM NaCl. The significances were calculated by applying the analysis of variation, ANOVA that applied a correction test to produce an adjusted *P* values. The metabolites and ions, significant in a pBH < 0.05, considered as significant accumulated metabolites and ions. *Orange* and *blue colors* represent positive and negative regulations after $\log^2$ transformation respectively

significant (Fig. 7b). Proline (Pro), 3-phosphoglycerate (3PGA), methionine (Met), sodium (Na), 1-Aminocyclo-propane-1-carboxylic acid (ACC), ADP-glucose (ADPGlc), ribose-5 phosphate (Rib5P), glutamine (Gln), arginine (Arg), serine (Ser), asparagine (Asn), boron (B), glycine (Gly), and trans-aconitate (Tacnt) were among the metabolites with VIP values greater or equal to 1.0. This showed that they strongly contributed to separate samples observed in the PLSDA analysis (Fig. 7b).

Discussion

P. indica has been shown to promote the growth of many host plants. It increases the plant host adaptation to abiotic stresses, such as salt and drought stresses (Jouyban 2012; Läuchli and Grattan 2007; Mahajan and Tuteja 2005). Despite intensive studies on the molecular interaction between *P. indica* and salinity in plants, no reports have been presented to elucidate the beneficial effects of *P. indica* in crop plants like barley, especially how the metabolic activity and composition of central metabolism changes by *P. indica* (Alikhani et al. 2013; Ghabooli et al. 2013; Waller et al. 2005). For these reasons, we have grown barley cultivar Pallas either in the presence or in the absence of *P. indica* under two different salt levels, 0 and 300 mM NaCl, in the current study. Gene expression, metabolites, and ions levels were monitored in shoots 14 days post inoculation.

P. indica affected the composition of ions under salt condition

Salinity interferes with the capacity of plants to absorb water and to exchange available minerals. In the present

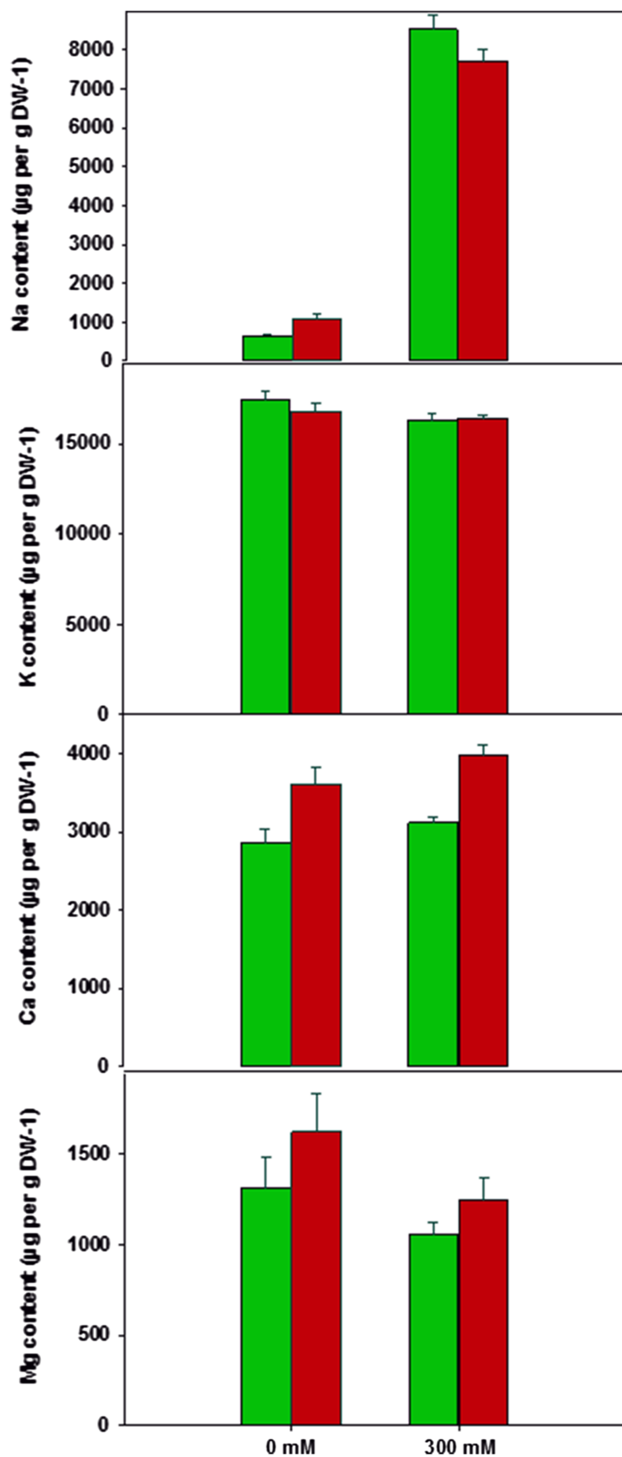

Fig. 6 Comparing of Na, Ca, Mg, and K contents in shoot dry weight of non-inoculated (*green columns*) and *P. indica*-colonized (*red columns*) barley plants. *Graphs* show significant difference in two levels of salt. *Vertical bars* depict standard error values for each treatment (n = 6)

study, the Na^+ and Ca^{2+} concentrations are the two major ions involved in salt stress and Mg^{2+}. These ions implicated in energy transfer and photosynthesis were profoundly changed in *P. indica*-colonized compared to non-inoculated plants in both conditions. Our results showed that salt stress increased Na^+ concentration. However, the K^+ concentration was decreased in the shoot of non-inoculated plant, while a slight increase in K^+ concentration and a decrease in Na^+ concentration was found in *P. indica*- colonized plants (Fig. 6). Similar results were obtained by Alikhani et al. (2013) displaying a strong antagonism between K^+ and Na^+ in terms of transport and partitioning (Rodrigues et al. 2013) and reducing the toxic effects of Na^+ in the cell. Likewise, our data also exhibited an increase in the Ca^{2+} concentration at 300 mM NaCl. The increase of the Ca^{2+} as a central regulator of growth and development in colonized plants by *P. indica* under ambient and severe salt stress is in the line with findings in *A. thaliana* (Vadassery et al. 2009). This supports the growing evidences that Ca^{2+} is likely an early signaling component initiating the stress signal transduction that leads to salt adaptation (Knight et al. 1997). As it is shown in Fig. 6, significant changes of Mg^{2+} concentration was observed in the shoot of *P. indica*-colonized plants compared to the non-inoculated under salt severe stress condition. Mg^{2+} is a major cofactor for tonoplast ATPase and its maintenance is necessary for protein translation in high presence of Na^+ (Läuchli and Grattan 2007). Furthermore, hindrance of Mg^{2+} in high salinity leads to chlorophyll degradation resulting in an increase of the activity of Rubisco. Thus, the significant change in the Mg^{2+} concentration hints on an increase in export of photosynthates. In turn, our results showed an increased biomass production that leads to plant host adaptation under high salt conditions.

P. indica down-regulated genes involved in energy metabolism as an adaptive mechanism to salt stress

The present transcriptome study showed many significant down-regulated genes of photosynthesis light reaction in severe salt conditions (Fig. 3a, b). Recently, it has been proposed that inactivation of photosystem light reaction by most environmental stresses caused inhibiting mechanisms for repairing photo damage rather than by directly attacking it (Murata et al. 2007). Likewise, the inhibition of photosystem light reaction generates reactive oxygen species that lead to a suppression of the transcription and translation of proteins of the PSII complex. The positive role of ROS in initiation of mutualistic interaction was previously shown in barley indicating that low photosynthesis activity is an adaptation for maintaining mutualistic interaction during prolonged severe stress (Alikhani et al. 2013). Furthermore, this supports the hypothesis proposed by Alikhani et al. (2013) indicating maintaining photosynthesis activity led to the higher fresh and dry weights of

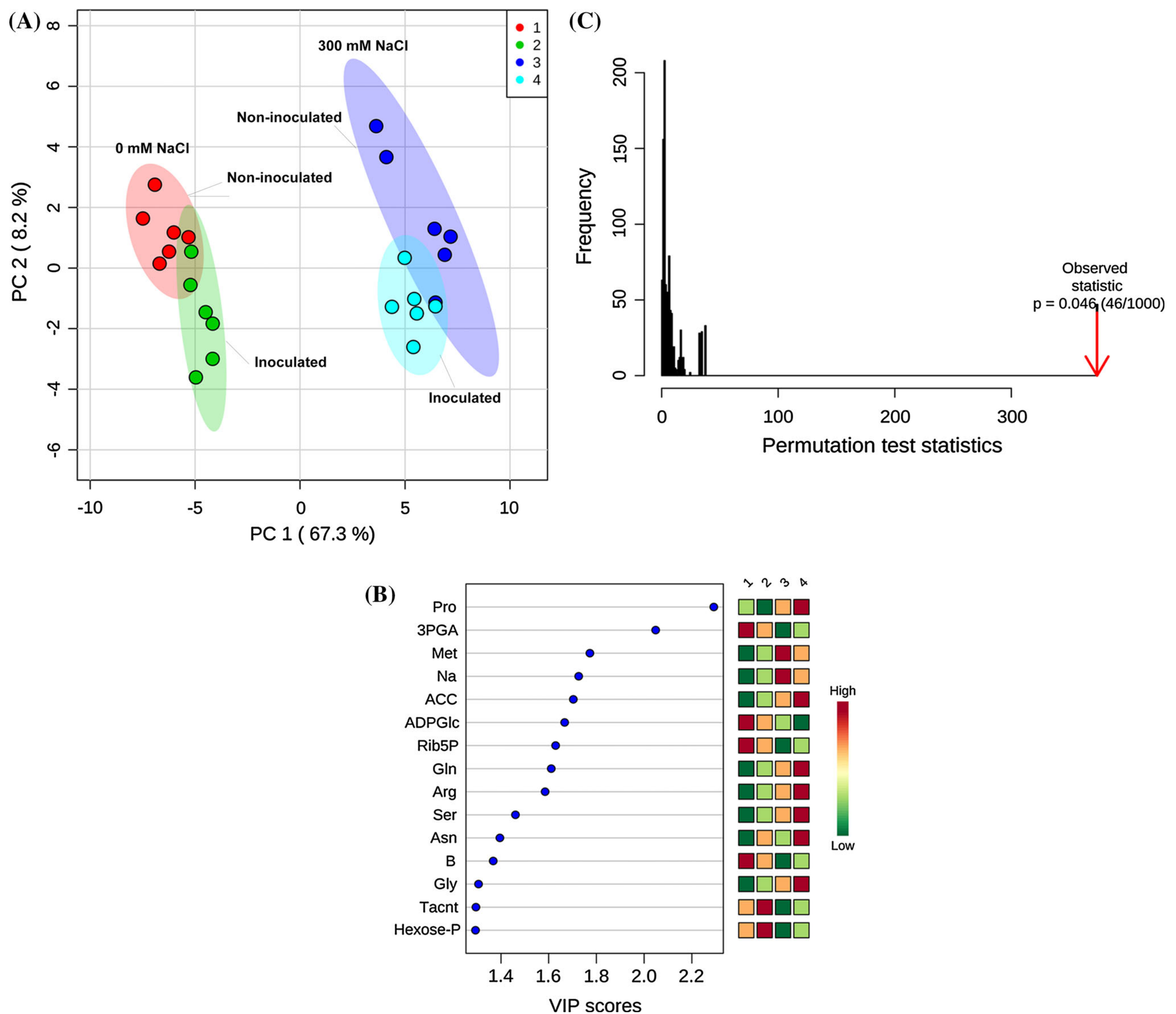

Fig. 7 **a** Principal component analysis (PCA) of leaf metabolites, amino acids, and ions profiled of *P. indica*-colonized and non-inoculated barley plants in 0 mM and 300 mM NaCl conditions. Principal components (PC1 and PC2) account for 75.5 % of the variance in the data. **b** Metabolites, amino acids, and ions with a higher rank of variance importance in the projection (VIP) are indicated. **c** The result of the permutation test was summarized by a histogram. Adjusted *P* values were calculated by using 1000 permutations for the partial least square

colonized barley compared to control condition (Supplementary Fig. S1).

P. indica altered carbon and nitrogen metabolisms in barley

The transcripts of sucrolytic enzymes, such as sucrose synthase (SUSY) and invertases, which play a major role in controlling plant metabolism and development, were deregulated in two salt levels suggesting a post transcriptional regulation in cell during salt-fungus interaction (Fig. 3a, b). Sucrose, as a major source of carbohydrate, is cleaved by two enzymes of invertase and sucrose synthase to make glucose, fructose, and UDP-glucose for a vast majority of biosynthesis. It has been documented the expression of the analogues of SUSY and invertase genes were regulated under abiotic and biotic stresses in response to high salinity, drought, low temperature, or hormone treatment in Arabidopsis and wheat (Baud et al. 2004; Maraña et al. 1990). In addition, it has been shown that SUSY increases during *Glomus intraradices* colonization of *Phaseolus vulgaris* (Blee and Anderson 2002) and promotes root nodule formation in *Pisum sativum* (Gordon et al. 1999). Considering that SUSY is often induced under stress

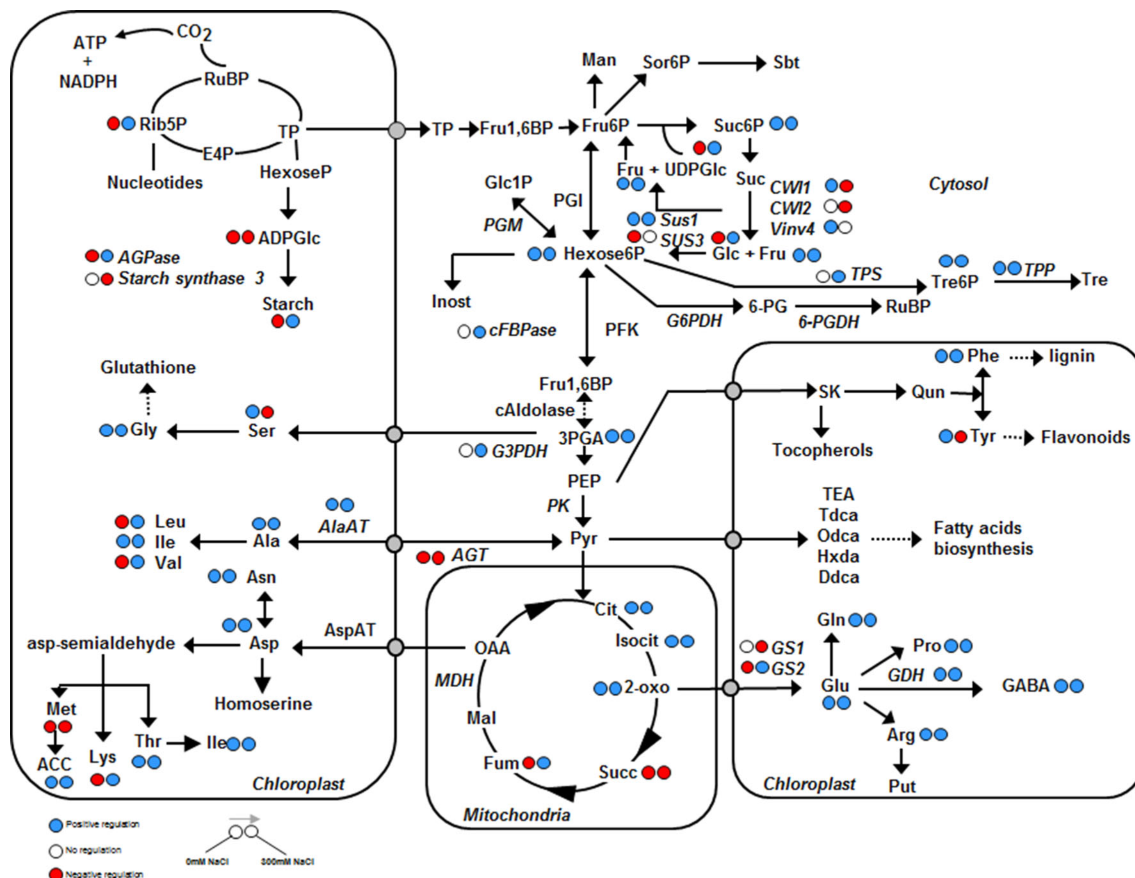

Fig. 8 Schematic representation of integrated metabolites and transcripts barley induced by *P. indica* under salt stress conditions. The full names and the abbreviation of transcripts and metabolites are presented in Supplemental Table S8. *Red* and *blue colors* indicate traits changes in relation to root fungal colonization

conditions rather than invertases suggests that *P. indica* may favor the buildup of UDP-glucose, which is serving as precursors for both starch or cell biosynthesis (Koch et al. 2004).

Our metabolomics results showed that *P. indica* increased the starch concentration in *P. indica*-colonized plants under the severe salt stress condition. The increase in the number of chloroplasts starch grains were previously shown in the halophyte and the llungiella plant leaf mesophyll cells under salinity stress (Wang et al. 2013). Partitioning of carbon in the form of starch may help plants to avoid metabolic alterations caused by accumulation of excess sucrose in the cytoplasm during salinity stress (Pattanagul and Thitisaksakul 2008). The increase in starch concentration is consistent with the significant transcript up-regulation of AGPase, which is a key enzyme in starch biosynthesis in *P. indica*-colonized plant. The relationship of presented changes in both transcripts and metabolites are illustrated in Fig. 8. Another interesting observation of our study was the change in the expression patterns of two major enzymes involved in trehalose metabolism, trehalose phosphate phosphatase (TPP), and trehalose phosphate synthase (TPS), were changed differentially under different salt conditions. Trehalose accumulated high amounts, conferring high tolerance levels to different abiotic stresses (Bianchi et al. 1993; Drennan et al. 1993). At sufficient levels, trehalose also functions as an osmolyte and stabilizes proteins and membranes. In plants, trehalose is synthesized in a two-step process (Paul et al. 2008). Trehalose-6-phosphate synthase (TPS) generates trehalose-6-phosphate (Tre6P) from UDPGlc and glucose-6 phosphate (Glc6P) followed by dephosphorylation to trehalose by trehalose-6-phosphate phosphatase (TPP). The transgenic plants overexpressed trehalose biosynthesis genes resulting in increased trehalose tolerance to abiotic stress (Krasensky and Jonak 2012). Furthermore, it has been proposed that the growth promoting effect initiated by *P. indica*-root colonization is accompanied by a co-regulated stimulation of enzymes involved in nitrogen metabolism (Sherameti et al. 2005). In our study, the expression of two analogs of glutamine synthetase, GS1, and GS2 were changed in *P. indica* root colonization barley (Fig. 4a, b). The transgenic

rice overexpressing chloroplastic GS showed enhanced tolerance to salt stress and indicated that the concentration of ammonium and Na^+ in the stressed plants correlate with the levels of GS2 isoform (Hoshida et al. 2000). Also, GS is involved in proline metabolism. Plants synthesize and accumulate compatible solute in forms of amino acids in which proline is an important osmolyte mitigating the membrane. The disruption of the membrane is effected by NaCl in playing non-exclusive roles to possibly limit or repair damage caused by water stress (Mansour 1998).

Although salt stress had a significant effect on proline concentration, no significant differences were found in relation to colonized and non-colonized plants. Interestingly, *P. indica* increased GABA content significantly under severe salt stress (Fig. 5). It is hypothesized that the GABA is required to limit the accumulation of reactive oxygen intermediates (Bouché et al. 2003) and it is required for maintaining the mutualistic interactions (Scott et al. 2007). Thus, GABA can likely be considered as a major metabolic biomarker either for mutualistic interaction or for environmental stresses.

P. indica induced transcripts involved in auxin, ethylene and brassinosteroid biosynthesis

Stress phytohormones are major endogenous effectors that modulate plant physiological responses leading to environmental adaptation. It has been previously reported that auxin plays a role in host mutualistic interaction with *P. indica* (Vadassery et al. 2008). Interestingly, *P. indica* has also shown to produce auxin, which increases root branching in *Arabidopsis* in response to IAA production (Sirrenberg et al. 2007). It has been shown that auxin increases the concentration of ROS and enhances antioxidant enzymes under severe stress condition (Baltruschat et al. 2008). Our comparative transcriptomics data revealed aldo/ketoreductase, an enzyme involved in antioxidant defense against the harmful effects of lipid peroxidation and two analogs of AXR1, involved in primary root growth and AUX1, a permease-like regulator of root gravitropism, were up-regulated in colonized plant (Fig. 4b). It has been shown that AUX1 mutant gene produces an auxin-resistant root growth phenotype in *A. thaliana* (Bennett et al. 1996). Our current findings show that the increase of auxin during the interaction with *P. indica* is likely a common strategy in *Arabidopsis* and barley in order to change the host root morphology and improve root accessibility by impairing plant defense. Likewise, one of the most important ethylene-producing enzymes, ACC oxidase was down-regulated in the colonized plant under the salt condition (Fig. 4a, b). ACC oxidase catalyzes the conversion of amino acid ACC and ascrobate to ethylene, which is a gaseous hormone and regulates plant growth and development (Fahad et al. 2014). The amino acid ACC, as nitrogen source, protects host plants from a number of environmental stresses (Fedorov et al. 2013). Though plant growth-promoting bacteria cleave the ethylene precursor, ACC, they thereby potentially reduce deleterious effects of ethylene accumulation in plant roots (Fedorov et al. 2013; Glick et al. 2007).

This is inconsistent with the significant accumulation of ACC and down-regulation of ACC oxidase in *P. indica*-colonized plants, which lead either to inhibition or reduction of ethylene biosynthesis under severe salt condition. It has been previously shown *Sebacina vermifera*, which is closely related endophyte to *P. indica*, profoundly down-regulates ethylene production in *Nicotiana attenuata* (Barazani et al. 2007). This finding is also supported by down-regulation of the ethylene-responsive factor (ERF) family and AP2/EREBP transcription factor in *P. indica*-colonized barley plants at severe salt condition. The biochemical analysis showed that LeERF2/TERF2 interacted with the dehydration-responsive element in the promoter of *Lycopersicon esculentum* ACC oxidase (LeACO3) resulting in the transcriptional activation of the genes involved in ethylene biosynthesis in tomato and tobacco (Zhang et al. 2009).

In addition to above described hormones, two major enzymes of squalene epoxidase 1 and brassinosteroid insensitive 1 involved in brassinosteroids biosynthesis, were differentially regulated in *P. indica*-colonized plants under severe salt condition (Fig. 4b). The brassinosteroids are a novel group of polyhydroxysteroids, their derivatives are composed of brassiniloide (BL) and castasetrone (CS) that counteract against abiotic and biotic stresses in plants. It is documented that brassinosteroids are known to ameliorate the effects of salinity on plant growth performance (Kartal et al. 2009; Krishna 2003; Wang et al. 2011; Zhu 2002).

Furthermore, it is shown that the down-regulation of brassinosteroid insensitive 1 gene produced a dwarf phenotype with reduced plant height, shortened internodes, narrowed and shortened the leaves, reduced expression of brassinosteroid signaling genes, and enhanced the expression of BR biosynthesis genes (Noguchi et al. 1999). However, the transgenic *Brachypodium distachyon* showed an enhanced drought tolerance, indicating brassinosteroids genes are significantly affected by brassinosteroids signaling pathway during the environmental conditions and regulated by other antagonist effects of other stress-related phytohormones (Feng et al. 2015; Friedrichsen et al. 2002).

Furthermore, plant hormone abscisic acid (ABA) was regulated during *P. indica*-barley mutualistic interactions. HV22D, an ABA-responsive gene and ABA deficient 1 which is involved in proline metabolism, were significantly down-regulated at 300 mM NaCl conditions (Koornneef

et al. 1982). Zhang et al. (2006) showed that the elevated ABA in leaves under salinity-protected plants by minimizes water loss through stomatal closure. This contradiction is consistent with the previous studies shown in *G. intraradices*, lettuce plants, endophytic fungi of *Penicillium funiculosum*, and *Aspergillus fumigatus* with soybean. It also indicated that ABA is more involved in the regulation of stress signaling against environmental stress rather than the plant growth promotion induced by endophytic fungus. The impact of ABA biosynthesis, induced by mutualistic fungus, is more dramatic when the plant is exposed to the abiotic stresses (Khan et al. 2012).

P. indica programmed the expression of stress-related genes

P. indica changed several genes involved in biotic stress under salt condition. This was obvious for changes in the expression pattern of a 70-kDa heat shock protein (Hsp70) (Fig. 4). Hsp70 and their co-chaperones prevented the aggregation of non-native proteins and folded them back to the native state through the association with hydrophobic patches of the substrate molecules. This then shields them from intermolecular interactions (Mayer and Bukau 2005). It has been reported that the molecular chaperone HvHsp70 is up-regulated in the leaves of *P. indica*-colonized barley plants and used as a practical biomarker to analyze systemic effects induced by *P. indica*-root colonization (Waller et al. 2008). In cooperation with other chaperones, Hsp70s stabilizes and maintains the pre-existing proteins against aggregation and degradation during mutualistic interaction when *P. indica* and barley plants are exposed to the severe stress.

P. indica altered transcript of genes coding for peroxidases under severe salt stress

The peroxidases, such as ascrobate peroxidases are detoxifying enzymes that catalyze the conversion of H_2O_2 into H_2O and peroxiredoxin, of which functions as peroxidase in the antioxidant defense network. In the current study, we observed the up-regulation of peroxidase genes in *P. indica*-colonized plants exposed to 300 mM NaCl (Fig. 4b). This is in agreement with the previous studies done by Alikhani et al. (2013) and Ghaboli et al. (2013) in barley under salt and drought stress, respectively. Furthermore, we showed that the reactive oxygen species (ROS) are one of the key elements for the initiation of mutualistic interaction in barley. *P. indica which* uses the plant redox system to establish and maintenance of *P. indica* root colonization during the symbiotic interactions (Alikhani et al. 2013).

P. indica induced small RNA metabolism

Most environmental stresses trigger epigenetic changes (Mirouze and Paszkowski 2011). For the first time, transcriptomics data has revealed a significant induction of three genes involved in RdDM pathway including NRPB11, RPB10, and NRPD9A in *P. indica*-colonized barley plants at 300 mM NaCl (Fig. 4b). The major siRNA-mediated epigenetic pathway in plants is RNA-directed DNA methylation (RdDM) that causes de novo DNA methylation (Matzke and Mosher 2014). Previous studies have shown that RdDM was important for the function of plant defense against pathogens and also stress responses (Chinnusamy and Zhu 2009; Matzke and Mosher 2014). Borsani et al. (2005) showed that the RdDM factor NRPD1 has an effect on the biogenesis of nat-siRNAs, which are required for salt stress tolerance in *A. thaliana.* Furthermore, it has been shown that salt stress is linked to the demethylation of coding regions of a glycerophosphodiesterase-like protein in tobacco (Choi and Sano 2007). This finding suggests that alteration in histone modification and DNA methylation are coordinated with changes in the expression of stress-responsive genes. Therefore, the epigenetic regulation of stress-responsive genes can act as an plant adaptive mechanism to the environmental stresses (Kim et al. 2015).

Conclusion

Plant symbionts can enhance crop yields and their ability to enhance tolerance to different abiotic stresses. A comprehensive understanding of mechanisms by which symbionts protect their plant hosts against the detrimental effects of abiotic stresses may provide new insights into the plant stress adaptation and pave the way to design a better strategy to cope with salt stress. Despite the fact that *P. indica* is not colonizing plant leaves, it induces systemic response to salt stress by changing physiology, metabolite, and transcriptome of *P. indica*-colonized barley plants. In addition to it ability to enhance the host tolerance to salt stress, *P. indica* may also serve as a model system to discover molecules and mechanisms affecting abiotic resistance in cereals. The application of *P. indica* for discovering stress-associated genes and mechanisms has a clear advantage over using contrasting genotypes by circumventing the complexity of genetic background in tolerant and susceptible genotypes. Functional analysis of key stress-associated genes in different genetic background and mutants will help to prove that the genes differentially regulated in this study will be critical for barley adaptation to salt stress.

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Author contributions MG prepared the plant samples; MRG, MRH and MG performed metabolome analysis; PS performed macroarray analysis; MRG and BK analyzed the data; GHS designed the experiment; MRG, BK and GHS wrote the manuscript.

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