A proteomics approach to study the molecular basis of enhanced salt tolerance in barley (*Hordeum vulgare* L.) conferred by the root mutualistic fungus *Piriformospora indica* †

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*Piriformospora indica* is a root-interacting mutualistic fungus capable of enhancing plant growth, increasing plant resistance to a wide variety of pathogens, and improving plant stress tolerance under extreme environmental conditions. Understanding the molecular mechanisms by which *P. indica* can improve plant tolerance to stresses will pave the way to identifying the major mechanisms underlying plant adaptability to environmental stresses. We conducted greenhouse experiments at three different salt levels (0, 100 and 300 mM NaCl) on barley (*Hordeum vulgare* L.) cultivar “Pallas” inoculated with *P. indica*. Based on the analysis of variance, *P. indica* had a significant impact on the barley growth and shoot biomass under normal and salt stress conditions. *P. indica* modulated ion accumulation in colonized plants by increasing the foliar potassium (K+)/sodium (Na+) ratio, as it is considered a reliable indicator of salt stress tolerance. *P. indica* induced calcium (Ca2+) accumulation and likely influenced the stress signal transduction. Subsequently, proteomic analysis of the barley leaf sheath using two-dimensional electrophoresis resulted in detection of 968 protein spots. Of these detected spots, the abundance of 72 protein spots changed significantly in response to salt treatment and *P. indica*-root colonization. Mass spectrometry analysis of responsive proteins led to the identification of 51 proteins. These proteins belonged to different functional categories including photosynthesis, cell antioxidant defense, protein translation and degradation, energy production, signal transduction and cell wall arrangement. Our results showed that *P. indica* induced a systemic response to salt stress by altering the physiological and proteome responses of the plant host.

Introduction

Abiotic stresses, including drought and salinity, are the major limiting factors of crop production worldwide. To improve growth performance and evade stresses, one strategy for a plant is to establish associations with the beneficial microbial microorganisms. 1 Mutualistic symbiosis with mycorrhizal and endophytic fungi can enhance plant stress tolerance by increasing the fitness of their host. 2 The most ancient mutualistic symbionts are arbuscular mycorrhizal fungi (AMF). 3 Endophytes are another group of symbionts that accomplish part of their life cycle within the living host tissues without causing apparent damage to hosts. 4 Endophytes may provide protection and improve survival of their hosts, thereby resulting in an enhanced resistance to stress, insects, and disease as well as improving the host yield. 4

*Piriformospora indica* is a root endophytic fungus, first isolated from the Indian Thar desert and recognized as a plant root symbiont. 5,6 *P. indica* is a member of *Sebacinales* and so far all members of this order are involved in mycorrhizal associations encompassing ecto-, orchid-, ericoid-, cavendishiodi- and

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Salt tolerance in barley has been explored in several studies. However, the molecular mechanism underlying the salt tolerance is not very clear. P. indica provides several more benefits to the host plants than merely plant growth promotion, such as a higher tolerance to various biotic and abiotic stresses. In addition, it improves plant fitness by increasing the plant growth performance both under normal and stressful conditions. It is likely that the mechanisms leading to abiotic stress (such as salt and drought) conferred by P. indica are based on the general, and not specific, plant-species mechanisms.

Salinity is one of the major abiotic stresses that restricts plant growth, development and productivity. Particular attention has been paid to the effects of salt stress on the morphological, physiological and cellular aspects of the host plants. However, the molecular mechanism underlying the salt tolerance has been less studied. The contribution of P. indica to improve salt tolerance in barley has been explored in several studies. P. indica has been found to elevate the plant antioxidant content to minimize the effect of salinity on barley. However, the detailed molecular mechanism associated with salt stress tolerance conferred by P. indica in barley is unclear.

Proteomics has proven to be a powerful approach in studying plant response to biotic and abiotic stresses. The application of proteomics can shed light on the mechanisms behind P. indica-mediated salt tolerance. Analyzing the changes in physiological traits and protein expression reveals some aspects of the adaptive mechanism occurring in P. indica-colonized plants. Presently, only a few proteomic studies are investigating the adaptive mechanisms of plant symbionts to the environmental stresses which are particularly important for agriculture. However, to the best of our knowledge, this is the first report showing the proteome analysis of symbionts under saline conditions.

We analyzed the proteome patterns of barley leaf sheaths in P. indica-colonized plants and then compared the colonized plants with control plants under different salt stress conditions using a two-dimensional gel electrophoresis (2-DE) based proteomics approach. We then coupled physiological data with data collected from mass spectrometry and identified differentially accumulated proteins associated with salinity and P. indica-root colonization. Our results showed that P. indica reprogrammed the host physiology by altering the ion content, and proteome patterns of barley leaf sheaths in order to cope with salt stress.

Materials and methods

Plant materials, inoculation, growth conditions, salt treatment and experimental conditions

Barley seeds of the cultivar “Pallas” were surface-sterilized with 70% ethanol (v/v) for 30 seconds followed by 6% sodium hypochlorite (NaOCl) for 15 minutes. They were then rinsed thoroughly with sterile water, and germinated for 2 days. P. indica was cultured on CM (complex medium) and the spore suspension collected after 28 days by gently scratching the fungus surface on the petri dishes with a spatula until the spores were released. The spore suspension was filtered through cheesecloth to remove the excess medium and washed three times with distilled water (dH2O) containing Tween-20. After each washing step, the spores were collected by centrifugation at 4000g for 7 minutes. The spore pellet was finally suspended again in dH2O and adjusted to ~5 × 10^5 spores per mL.

The two-day-old barley seedlings were inoculated by immersing in the spore suspension solution with gentle shaking for two hours. The mock-treated seedlings were dipped in sterile water only. Inoculated and mock-treated seedlings were later transferred into pots, filled with sand and perlite in the ratio of 2:1 and then placed in the greenhouse (16/8 h, day/night cycle, 22/18 °C regime and a 60% relative humidity). After fourteen days, the barley plants were exposed to 100 and 300 mM NaCl. To eliminate the effect of osmotic stress shock, plants were treated gradually by increasing the applied salt concentrations until the desired salt concentration was reached after seven days. The control group was grown under normal conditions (0 mM NaCl). The experiment was performed using a completely randomized design with four independent biological replicates.

Microscopic analysis

To check for the successful barley root colonization by P. indica under salt conditions, P. indica-inoculated barley roots were harvested 14 days after salt treatment. Three plants per pot (as one replication) were harvested for the physiological and proteomic analysis.

Physiological analysis

After harvesting plant materials, the shoot and root fresh weights were measured. The dry weight was then measured after drying fresh tissue in an oven at 70 °C for 48 hours. Physiological analyses were conducted by measuring the ion accumulation including sodium (Na^+), potassium (K^+) and calcium (Ca^{2+}) after being extracted in 500 mM HNO_3. The ion concentrations were measured in six replicates using inductively coupled plasma optical emission spectrometry (ICP-OES) equipment and reported as mg g^{-1} dry weight. In addition, the foliar K^+/Na^+ ratio of P. indica-colonized and non-inoculated barley plants was compared under both the control and the different salt treatments (100 and 300 mM NaCl).
Protein extraction
Proteomic analysis of barley leaf sheath from *P. indica*-colonized and non-inoculated plants was performed under both the control and the different salt stress conditions. Protein extraction and 2-DE analyses were performed as previously described. Leaf sheath samples (1 g) were pulverized to a fine powder with liquid nitrogen using a mortar and pestle. Protein extraction was performed via Trizol reagent (Invitrogen/Life technologies) according to manufacturer's instructions. The leaf sheath powder was suspended in 1 mL Trizol solution, homogenized and kept at room temperature (RT) for 10 minutes. Two-hundred μL chloroform was added to the mixture and after vortexing, the solution was held at RT for 5 minutes. The solution was then centrifuged at 12 000g for 15 minutes (all the centrifugation steps were conducted at 4 °C). Afterwards, the supernatant containing the protein was transferred to a 2 mL fresh tube and acetone was added in the ratio of 3:1. Samples were vortexed vigorously and held at RT for 10 minutes. The protein was precipitated by centrifugation at 12 000g for 10 minutes. Protein pellets were washed three times using 96% ethanol (containing 300 mM guanidine and 2.5% glycerol) followed by another wash with absolute ethanol containing 2.5% glycerol. The protein pellets were then solubilized in lysis buffer [7 M urea, 2 M thiourea, 2% (w/v) 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS), 0.8% (w/v) Pharmalyte pH 3–10, 1% (w/v), dithiothreitol (DTT)]. The protein concentration was determined by Bradford assay (Bio-Rad) and Bovine Serum Albumin (BSA).

Two-dimensional electrophoresis and image analysis
Two-dimensional electrophoresis was performed as previously described. Immobilized pH gradient gels (IPGs) (24 cm, pH 4–7 IPG strips) were rehydrated overnight with 450 μL of rehydration buffer [8 M urea, 0.5% CHAPS, 20 mM DTT, 0.5% (w/v) IPG buffers] in a re-swelling tray (GE Healthcare) at RT. For analytical and preparative gel electrophoresis studies, 300 μg and 3 mg of protein samples were loaded on IPG gels, respectively. Isoelectric focusing (IEF) was conducted using a Mutiphor II and a DryStrip kit (GE Healthcare) at 20 °C. The samples were run at 300 V for 30 minutes, 500 V for 1 hour, followed by 1 hour at 1000 V and finally 3500 V for 16 hours. The focused strips were equilibrated twice for 15 minutes in 10 mL equilibration solution. The first equilibration was performed in an equilibration solution [6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS, 1% (w/v) DTT and 50 mM Tris-HCl buffer, pH 8.8], followed by a second equilibration in a similar solution in which DTT was replaced with 2.5% (w/v) iodoaceticamide. Separation in the second dimension (SDS-PAGE) was performed in a vertical slab of acrylamide (12% total monomer with 2.6% crosslinker) using a PROTEAN II Multi Cell (Bio-Rad). Protein spots in analytical and preparative gels were stained with silver nitrate and coomassie brilliant blue colloidal (CBB) G-250, respectively. Gels were scanned using GS-800 densitometer (Bio-Rad) at a resolution of 600 dots per square inch. The scanned gels were analyzed using Melanie 4 software (GeneBio, Geneva, Switzerland). The molecular masses and pI (isoelectric point) of the protein spots on gels were determined by co-electrophoresis of standard protein markers (GE Healthcare) and migration of the protein spots on strips, respectively. Percent volume of each spot was estimated and analyzed to determine protein abundance. A single two-dimensional gel per plant was run for each of four biologically independent replicates and the percent volume of each spot was estimated and analyzed. The two treatments (*P. indica*-colonized and non-inoculated) and three salt levels (0, 100 and 300 mM NaCl) and four combinations were analyzed by two-way analyses of variance (ANOVA). ANOVA was performed by SPSS software and mean comparisons were performed using Duncan’s multiple range tests at a *p*-value ≤ 0.05, when appropriate. Spots were determined to be significantly up- or down-regulated when the *p*-value is ≤ 0.05. The induction factor was calculated by dividing the percent volume of spots in gels corresponding to 100 and 300 mM NaCl by the percent volume of spots corresponding to 0 mM NaCl.

Protein identification and database search
Protein spots were excised from CBB-stained gels and analyzed using a Bruker Ultraflex III MALDI TOF/TOF mass spectrometer as described previously. Bruker flex analysis software was used to perform the spectral processing and to generate the peak lists for the MS and MS/MS spectra. The combined MS and MS/MS data were subjected to database searching using a copy of Mascot version 2.1 (Matrix Science Ltd.) that was run locally through the Bruker BioTools interface (version 3.1). The search criteria included enzyme, trypsin; variable modifications, oxidation; peptide tolerance, 100 ppm; MS/MS tolerance, 0.5 Da; instrument, MALDITOF/TOF. The search included carbamidomethyl as a fixed modification for all alkylated samples. The database search was run against NCBI non-redundant protein database NCBI-nr 20090222 (26 August 2009: 7 894 593 sequences; 2 721 452 874 residues). The threshold for positive identification was a MOWSE score of >70 (*p*-value ≤ 0.05).

Results
Host beneficiaries from *P. indica* under salt stress conditions
We first characterized the interaction of *P. indica* with barley plants under different salt stress conditions. *P. indica* promoted the barley growth under normal and salt stress conditions (Fig. 1) and that effect could be observed during the whole growth and development stages of barley plants. Overall, *P. indica*-colonized barley plants appeared larger, stronger and contained more leaves compared with non-inoculated plants. Based on the microscopic analyses, *P. indica* colonized barley roots efficiently both under control and salt treatments as was seen by production of the number of chlamydomospores at 14 days after spore inoculation. No colonization was observed in non-inoculated plant roots. *P. indica*-root colonization was begun with the germination of chlamydomospores that penetrated the root cortex, ramifying intercellularly from the point of
penetration and subsequently, formed chlamydospores within cortical cells (Fig. 2). After 4 weeks of barley development, the total fresh and dry shoot weights of *P. indica*-colonized plants were increased up to 26% and 24%, respectively, as compared with non-inoculated plants under control conditions (0 mM NaCl) (Fig. 3). *P. indica* caused no visible changes in the root morphology. The effect of *P. indica* was expressed by the number of tillers and the leaf sheath expansion of colonized barley. Salinity negatively affected the barley growth in both non-inoculated and *P. indica*-colonized barley plants, as the shoot fresh and dry weights of colonized plants were significantly reduced by 43% and 26%, respectively, compared to 51% and 40% reduction in the shoot fresh and dry weights of non-inoculated plants, respectively (Fig. 3). The shoot fresh and dry weights of *P. indica*-colonized plants were 1.53 and 1.44 times higher, respectively, as compared to shoot fresh and dry weights of non-inoculated plants under severe salt conditions (300 mM NaCl) (Fig. 3).
Physiological response of \textit{P. indica} on barley plants under saline conditions

The Na\(^+\), K\(^+\) and Ca\(^{2+}\) concentrations were measured in barley leaf tissue of colonized and non-inoculated barley plants that were grown both under normal and salt conditions. Our results showed that the Na\(^+\) concentration in leaves of both \textit{P. indica}-colonized and non-inoculated barley plants were significantly affected by salinity (Table 1). The Na\(^+\) accumulation in the leaves of colonized and non-inoculated plants was increased up to 7 and 13 times, respectively, under severe salt conditions (300 mM NaCl). However, the K\(^+\) accumulation remained relatively constant between (300 mM NaCl). In colonized plants, the K\(^+\) accumulation was 7% to 7 and 13 times, respectively, under severe salt conditions. The Na\(^+\)/K\(^+\) ratio in barley roots were not significantly changed between colonized and non-inoculated plants (Table 1). The distribution patterns of Na\(^+\) and K\(^+\) in barley roots were not significantly changed between colonized and non-inoculated barley plants under normal and salt conditions. Although, the foliar K\(^+\)/Na\(^+\) ratio in \textit{P. indica}-colonized plants was higher compared with non-inoculated plants under salt stress conditions (2.13 in \textit{P. indica}-colonized plants compared with 1.91 in control plants under severe salt conditions) (Table 1).

Identification of proteins in response to salinity and \textit{P. indica}-root colonization

The protein patterns of barley leaf sheath of \textit{P. indica}-colonized barley plants were compared with mock-treated plants under different salt stress treatments (0, 100 and 300 mm NaCl). In total, 96 protein spots were detected in four independent biological replicates (Fig. 4). Of these detected spots, 72 protein spots showed significant differences in their abundance between \textit{P. indica}-colonized plants compared with non-inoculated plants in response to salinity. Protein identification via Matrix-assisted laser desorption/ionization-Time Of Flight (MALDI-TOF/TOF) resulted in identification of 51 proteins (Table 2). Of the 51 identified protein spots, 18 protein spots showed changes in abundance in response to 100 mM NaCl in \textit{P. indica}-colonized and/or non-inoculated plants under control conditions. Of these, the abundance of 17 protein spots was also changed in response to 300 mM NaCl in \textit{P. indica}-colonized and/or non-inoculated plants under control conditions. The plant response to salt stress was more pronounced under severe salt treatment (300 mM NaCl). We observed changes in the abundance of 50 protein spots under 300 mM NaCl in \textit{P. indica}-colonized and/or non-inoculated plants under control conditions.

Classification of altered proteins in response to 100 mM NaCl and \textit{P. indica}-root colonization

We found several proteins showing significant alteration in the expression levels under 100 mM NaCl and these were categorized into five groups (Fig. 5 and Table S1, ESI†). These groups included group (A) with a single member, 6-4 photolyase (protein spot 817) which decreased in \textit{P. indica}-inoculated and non-inoculated plants both under 100 and 300 mM NaCl conditions. The group (B) three proteins included nuclear transport factor 2 (protein spot 8), translation elongation factor EF-Tu (protein spot 606) and Ribulose bisphosphate carboxylase (Rubisco) small chain (protein spot 884) which were reduced in response to both 100 and 300 mM NaCl in the non-inoculated barley plants. \textit{P. indica}-root colonization resulted in abundant recovery of all three proteins under mild salt conditions (100 mM NaCl). However, the abundance of two proteins (nuclear transport factor 2 and Rubisco small chain) returned to control levels in the \textit{P. indica}-colonized plants under 300 mM NaCl. (C) ubiquinol-cytochrome c reductase (protein spot 928) was higher in all salt treated plants compared with the control condition except for inoculated plants under 100 mM NaCl. The group (D) seven proteins including cytochrome c oxidase polypeptide Vb (protein spot 140), 23 kDa jasmonate-induced protein (protein spot 301), tubulin-folding cofactor A (protein spot 95), ribosomal protein P1 (protein spot 117), cytochrome c oxidase subunit Vb (protein spot 122), peroxiredoxin-2E-2 (protein spot 119) and Rubisco small chain (protein spot 11) were up-regulated only in \textit{P. indica}-colonized plants. All these proteins were increased in \textit{P. indica}-colonized and non-inoculated plants at 300 mM NaCl. Finally, in-group (E) six proteins were found to increase at both levels of salinity. These proteins included vacuolar proton-translocating ATPase (V-ATPase) subunit E (YLPl) (protein spot 473), Mg-chelatase subunit (protein spot 621), Rubisco small chain (protein spot 66), endotransglucosylase/hydrolase XTH2 (protein spot 968), \(\alpha\)-expansin EXP2A (protein spot 205) and RNase S-like protein (protein spot 219). In this group, \textit{P. indica}-root colonization resulted in the recovery of only YLPl in response to 300 mM NaCl treatment.

Classification of altered proteins in response to 300 mM NaCl and \textit{P. indica}-root colonization

Fifty protein spots were found to change abundance under severe salt (300 mM NaCl) treatment. These proteins were divided into the seven major categories (Fig. 6 and Table S2, ESI†).

### Table 1 Mean and standard error of sodium (Na\(^+\)), potassium (K\(^+\)) and calcium (Ca\(^{2+}\)) concentration (mmol g\(^{-1}\) dry weight) and the K\(^+\)/Na\(^+\) ratio in barley leaves of \textit{P. indica}-colonized and non-inoculated plants under control (0 mM NaCl), moderate (100 mM NaCl) and severe salt stress (300 mM NaCl) conditions

<table>
<thead>
<tr>
<th>Salt treatment (NaCl)</th>
<th>Fungal treatment</th>
<th>Na(^+) (mmol g(^{-1}) DW)</th>
<th>K(^+) (mmol g(^{-1}) DW)</th>
<th>Ca(^{2+}) (mmol g(^{-1}) DW)</th>
<th>K(^+)/Na(^+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mM</td>
<td>Non-inoculated</td>
<td>1.30 ± 0.08</td>
<td>34.94 ± 0.98</td>
<td>5.73 ± 0.34</td>
<td>26.87 ± 0.53</td>
</tr>
<tr>
<td></td>
<td>Inoculated</td>
<td>2.16 ± 0.27</td>
<td>33.58 ± 0.85</td>
<td>7.23 ± 0.42</td>
<td>15.54 ± 0.56</td>
</tr>
<tr>
<td>100 mM</td>
<td>Non-inoculated</td>
<td>16.12 ± 0.81</td>
<td>26.43 ± 0.71</td>
<td>5.36 ± 0.45</td>
<td>1.639 ± 0.76</td>
</tr>
<tr>
<td></td>
<td>Inoculated</td>
<td>15.98 ± 0.36</td>
<td>26.75 ± 1.26</td>
<td>5.53 ± 0.31</td>
<td>1.673 ± 0.81</td>
</tr>
<tr>
<td>300 mM</td>
<td>Non-inoculated</td>
<td>17.05 ± 0.75</td>
<td>32.57 ± 0.80</td>
<td>6.24 ± 0.13</td>
<td>1.91 ± 0.77</td>
</tr>
<tr>
<td></td>
<td>Inoculated</td>
<td>15.41 ± 0.63</td>
<td>32.82 ± 0.41</td>
<td>7.95 ± 0.26</td>
<td>2.13 ± 0.52</td>
</tr>
</tbody>
</table>
In group A, eleven proteins were found which were reduced in both the *P. indica*-colonized and the non-inoculated plants. These proteins included putative eukaryotic translation initiation factor 6 (protein spot 253), importin subunit alpha (protein spot 827), Rubisco small chain (protein spots 41 and 884), Golgi associated protein se-wap41 (protein spot 765), S-adenosyl-methionine synthase 1 (protein spot 691), nuclear transport factor 2 (protein spot 8), fructokinase-2 (protein spot 527), legumin-like protein (protein spot 549), translation initiation factor (protein spot 166) and 6-4 photolyase (protein spot 817). In group (B) the abundance of 13 proteins which included Rubisco large subunit (protein spots 889, 946 and 947), Grx-S16-glutaredoxin subgroup II (protein spot 339), Myb family transcription factor (protein spot 338), profilin-1 (protein spot 1), biostress-resistance-related protein (protein spot 461), ankyrin repeat domain protein 2 (protein spot 814),

**Fig. 4** Two-dimensional gel electrophoresis of control protein samples. The first dimension was obtained using 300-microgram (μg) proteins on linear gradient IPG strips with pH 4–7. For the second dimension, 12% SDS-PAGE gels were used and the proteins were visualized using silver nitrate. The arrows represent protein spots that showed significant changes under salt stress as presented in Table 2.
translational elongation factor EF-Tu [protein spot 606], chitinase II (protein spot 681), chaperonin containing t-complex protein 1 (protein spot 903), germin-like protein [protein spot 221] and putative mRNA binding protein (protein spot 665) were decreased under 300 mM NaCl. Nonetheless, the abundance of these proteins returned to control levels after *P. indica* inoculation and (C) protein cp31BHv was down-regulated only in *P. indica*-colonized barley plants. (D) Chloroplast oxygen-evolving enhancer protein 1 (protein spot 521), YLP (protein spot 473) and fructan 6-fructosyltransferase (protein spot 791) were down-regulated only in non-inoculated barley plants. (E) The abundance of three proteins including xyloglucan endotransglycosylase (protein spot 412), tubulin folding cofactor A (protein spot 95) and endotransglucosylase/hydrolase XTH2 (protein spot 968) was increased only in *P. indica*-colonized barley plants. (F) The abundance of two proteins, Rubisco small chain (protein spot 11) and papain-like cysteine protease (protein spot 413) were expressed and changed in opposite directions in *P. indica*-colonized and non-inoculated plants.

(G) The abundance of seventeen proteins was increased in both *P. indica*-colonized and non-inoculated plants. (G) The abundance of seventeen proteins was increased in both *P. indica*-colonized and non-inoculated plants.
the non-inoculated and *P. indica*-treated barley plants under 300 mM NaCl. These proteins included oxygen-evolving enhancer protein 2 (chloroplast) (protein spot 325), polyamine oxidase (protein spot 377), triosephosphate isomerase (cytosolic) (protein spot 352), peroxiredoxin-2E-2, chloroplastic (protein spot 119), Rubisco small chain (protein spot 66), xyloglucan xyloglucosyl transferase (protein spot 636), cold-regulated protein (protein spot 125), cytochrome c oxidase subunit Vb (122), 23 kDa jasmonate-induced protein (protein spot 301), α-expansin (protein spot 205), ascorbate peroxidase (protein spot 342), thaumatin-like protein (protein spot 57), high light protein (protein spot 447), ubiquinol–cytochrome c reductase complex (protein spot 928), magnesium chelatase 40 kDa subunit (protein spot 621) and RNase S-like protein (protein spot 219). In this group, the abundance of the xyloglucan xyloglucosyl transferase and Rubisco small chain proteins were more reduced in the *P. indica*-colonized plants (Fig. 6).

**Discussion**

*P. indica* enhanced barley adaptation to salt stress by modulating the ion balance

In this study, the barley leaf sheath was used for proteome analysis, since the sheath is encased around the stem and expresses signs of stress sooner than leaf blades. By analyzing the physiological traits and molecular protein expression
changes indicated that the major aspects of the adaptive mechanism occurred in the *P. indica*-colonized barley plants. Salinity disturbs the plant nutrient relations by the availability, transport and partitioning of nutrients in plants. It also causes a deficiency, or imbalance of the ions Na⁺, K⁺ and Ca²⁺ due to the competition for minerals. In addition, many plant processes such as growth, photosynthesis, mineral nutrition uptakes, water and ion transport are affected by the ratios of these ions.²⁵

Moreover, the Na⁺ accumulation was remarkably lower in the leaf sheaths of *P. indica*-colonized plants compared to the non-inoculated barley plants (data not shown). In particular, the younger leaf sheaths accumulated less Na⁺ compared to the older leaf sheaths of *P. indica*-colonized barley. This difference was even more pronounced at a high salinity (data not shown). Although Na⁺ accumulation increased significantly in both *P. indica*-colonized plants and non-inoculated plants under salt conditions, the foliar ratio of K⁺/Na⁺ was higher in *P. indica*-colonized plants compared with non-inoculated barley plants. This ratio is a reliable indicator for plants with a high level of salt tolerance, which therefore indicates the adaptive mechanisms conferred by *P. indica* in colonized barley plants. K⁺ is a critical mineral nutrient that protects plants from salt-induced damages²⁶ and its impaired uptake by increased Na⁺ is well-documented in several plant species.²⁷ This reduction could be due to the negative effect of Na⁺ on K⁺ at root uptake sites, affecting transport into the xylem, or the inhibition of the uptake processes.²⁸ It has been reported that salt-tolerant barley plants maintain a lower amount of Na⁺ than non-tolerant ones.²⁹ Ca²⁺, which affects plant growth, photosynthesis, nutrition, water and ion transport, was greatly increased by the presence of *P. indica* in colonized barley plants under control and especially under salt stress conditions. Many of these responses can be linked to the direct function of Ca²⁺ in the plasma membrane and the subsequent Ca²⁺ signaling events. *P. indica* affects intracellular Ca²⁺ signaling and is likely to impact the stress signal transduction in *Arabidopsis*.³⁰ It is probable that this increase in Ca²⁺ concentration in *P. indica*-colonized

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**Fig. 6** Classification of 50 individual barley protein spots showed statistically significant changes in *Piriformospora indica*-inoculated (I) and non-inoculated (NI) barley plants only at 300 mM NaCl condition compared with control. The ratio is expressed as log² (abundance in salt-treated plants at 300 mM NaCl/abundance in control samples). Protein identification is obtained from Table 2. Proteins are grouped according to the expression pattern: (A) down-regulated in both NI and I plants, (B) down-regulated only in NI plants, (C) down-regulated only in I plants, (D) up-regulated only in NI plants, (E) up-regulated only in I plants, (F) modulated in both I and NI, but in opposite directions, (G) up-regulated in both I and NI plants. Solid markers represent a significant difference (*p*-value ≤ 0.05) and open markers represent a non-significant difference (*p*-value > 0.05) in response to salt stress in *P. indica*-inoculated and non-inoculated samples.
barley plants not only affects mutualistic interaction but also initiates the stress signal transduction leading to the increased plant host adaptation to salt stress.

**Salt-responsive proteins were altered in a similar direction in both** *P. indica*-colonized and non-inoculated barley plants

We found very few protein changes in abundance in response to *P. indica*-root colonization under control conditions (Table S3, ESI†). Some of them including quinone reductase 2, putative heat shock 70 KD protein (Hsp70), mitochondrial precursor, aspartate-semialdehyde dehydrogenase and Ribulose bisphosphate carboxylase small chain were increased in *P. indica*-colonized plants. In return, the abundance of actin and low temperature-responsive RNA-binding proteins were reduced in *P. indica*-colonized compared with non-inoculated barley plants under control conditions. The quinone reductase 2 protein was identified based on the proteomics analysis in wheat and introduced as the stress-responsive proteins. The aspartate-semialdehyde dehydrogenase is an important enzyme involved in the biosynthesis of amino acids, which forms an early branch point in the metabolic pathway forming lysine, methionine, leucine and isoleucine from aspartate. The Hsp70 was another identified protein increased in *P. indica*-colonized plant under control conditions. Waller et al. (2009) showed that Hsp70 was up-regulated in barley leaves of colonized plants, which can be a useful tool to analyze the systemic effects of fungal colonization. Hsp70 helps the proteins to maintain the tertiary structure and minimize the protein aggregation and degradation.

We observed that the abundance of several salt-responsive proteins was changed in a similar direction in *P. indica*-colonized and non-colonized plants (Fig. 5: groups A and E; Fig. 6: groups A and G). The production of reactive oxygen species (ROS) is increased under abiotic stresses, which leads to damage of cells.32 In return, plant antioxidant systems prevent damage induced by ROS and increased salt stress tolerance. A significant increase in the expression of ascorbate peroxidase (protein spot 342) was observed in both *P. indica*-colonized and non-inoculated plants under salt stress conditions. Ascorbate peroxidase is part of a plant’s enzymatic antioxidative defense systems and a higher level of ascorbate peroxidase was observed in the salt-tolerant plant varieties.33 We also identified polyamine oxidase (protein spot 377) as the salt-responsive protein, which was stimulated by salinity, and the expression of polyamine oxidase was significantly affected by *P. indica*. Polyamines play a role in reducing the negative effects of salinity. Polyamine oxidase (PAO) is a source of apoplastic H₂O₂, and the salinity induces changes in plant polyamine levels. ROS can modulate plant growth through a promotion of cell wall cleavage. The involvement of PAO in the leaf growth and elongation in maize under salt conditions was shown.34 In accordance with the previous proteomics analysis, PAO was highly expressed in the salt-tolerant cultivar compared to the expression in a salt-susceptible cultivar.35

Thaumatin-like protein (TLP) (protein spot 57) was another identified salt-responsive protein in which the expression level increased in both the *P. indica*-colonized and non-inoculated plants under severe salt stress conditions. TLP is associated with osmotic adaptation in plant cells, and some TLPs are known to protect plants against biotic and abiotic stresses such as the pathogen infections, osmotic stress and freezing.37

One of the identified salt-responsive proteins with a higher abundance in both colonized and non-inoculated barley plants was cytosolic triosephosphate isomerase (TPI) (protein spot 352). TPI is an important enzyme for carbon metabolism, especially in glycolysis and sugar metabolisms. The TPI activity increased the carbon metabolism through the pentose phosphate pathway, CO₂ uptake and the catabolism of sucrose to CO₂. Proteomic analysis revealed that TPI showed an increase in the expression under drought stress in maize.30 Over-expression of TPI showed a significant improvement of photosynthesis due to an increase in the rate of regeneration of Rubisco activity in transgenic cells.41

The most up-regulated identified protein in both *P. indica*-colonized and non-inoculated plants was an RNase S-like protein (protein spot 219). The amino acid sequence of an RNase S-like protein does not necessarily have S-RNase activity although it is closely related to RNase. It has been reported that the abundant S-like RNase protein was altered in response to the various environmental stresses.42 Up-regulation of this protein in response to salt stress exhibits the impact on senescence or nutrient deficiency processes that is induced by salt stress.33,43 However, exactly why the physiological function of noncatalytic S-like RNase was up-regulated under stress conditions is unclear. Further studies are needed to elucidate the exact function of the RNase S-like protein specifically under salt stress conditions.

An increase in the expression of ribosomal protein P1 was observed in both *P. indica*-colonized and non-inoculated plants under severe salt treatment. However, the expression increased only in *P. indica*-colonized plants under 100 mM NaCl treatment. Similar to the previous report, the ribosomal protein P1 was reported as a salt-responsive protein in which the expression level was increased in both the salt-tolerant and the salt-susceptible barley cultivars.35 Salinity also induced the expression of Magnesium chelatase (protein spot 621). Magnesium chelatase is an enzyme involved in the process, which regulates the chlorophyll biosynthesis. This protein is identified as an absicic acid (ABA) receptor in *Arabidopsis*.44 The ABA level was elevated in barley leaves under salinity, and therefore, we expected that salinity mediates the expression of a number of ABA-responsive proteins; however, expression of ABA related proteins was not altered in *P. indica*-colonized barley plants under salt stress conditions. The phytohormone ABA played a role in the *P. indica*-root colonization in barley.32 Nevertheless, a direct relationship between stress tolerance and increased levels of ABA has not always existed.47

**P. indica** modulated barley proteome in response to different levels of salinity

The proteome response of *P. indica*-colonized barley plants was less than the response in non-inoculated plants under severe salt stress (45 protein spots versus 33 protein spots in
non-inoculated and *P. indica*-colonized plants under 300 mM NaCl, respectively). Moreover, the trend of proteome change in the *P. indica*-colonized and non-inoculated plants was different. *P. indica*-colonized barley plants showed mostly increase in abundance (21 and 12 proteins were up- and down-regulated, respectively). However, the major response in non-inoculated plants was down-regulation under salt stress (19 and 26 proteins were up- and down-regulated, respectively). Aside from the physical and physiological impacts of *P. indica*, proteomic analysis revealed that *P. indica*-root colonization resulted in the alteration of some of the mechanisms involved in salt-tolerance such as photosynthesis, cell antioxidant defense, programmed cell death, protein synthesis and cell wall arrangement in barley leaves.

Under a low level of salinity, the three down-regulated proteins in non-inoculated plants showed no significant changes in the *P. indica*-colonized plants (Fig. 5; group B). However, these proteins showed a similar response to a high level of salt in *P. indica*-colonized and non-inoculated plants. These proteins, nuclear transport factor 2 (protein spot 8), translation elongation factor EF-Tu (protein spot 606) and Rubisco small chain (protein spot 884), are involved in major cell metabolisms such as translation and photosynthesis. Furthermore, seven proteins (Fig. 5, group D), which showed no changes in the abundance in response to a low level of salinity, were changed significantly in *P. indica*-colonized plants at a high level of salinity. A 23 kDa jasmonate-induced protein was identified in this group. This protein might be involved in systemic resistance against plant pathogens. The induction of systemic resistance in barley after *P. indica*-root colonization is documented and is similar to induced systemic resistance activated by a group of root-associated non-pathogenic bacteria which act through the jasmonic acid (JA) signaling pathway. It is shown that salinity induced the JA response in barley. In our study, the abundance of peroxiredoxins-2E-2 (protein spot 119) increased only in *P. indica*-colonized plants under 100 mM NaCl. However, the abundance of this protein increased in both *P. indica*-colonized and non-inoculated plants under 300 mM NaCl, but to a higher extent in *P. indica*-colonized plants. Peroxiredoxin is a universal family of antioxidant enzymes and a new component in the antioxidant defense network. It also controls the cytokine-induced peroxide levels and thereby mediates the signal transduction. The changes in the abundance of peroxiredoxins in response to various stresses have been previously reported.

By increasing the level of salinity to 300 mM NaCl, a number of salt-responsive proteins were up-regulated in both *P. indica*-colonized and non-inoculated proteins. *P. indica* specifically increased the expression of several proteins involved in photosynthesis under different salt stress conditions compared with non-inoculated plants. The down-regulation of photosynthetic machinery is a known phenomenon under salinity. Rubisco, which is involved in the first major step of carbon fixation, remained unchanged in *P. indica*-colonized barley plants, but was significantly down-regulated in non-inoculated plants under salinity stress conditions (protein spots 889, 946 and 947). The Rubisco small chain (RBCS) (protein spot 11) was highly expressed in *P. indica*-colonized barley plants, but significantly reduced in non-inoculated plants under saline stress conditions (Fig. 6). Two of the identified RBCS (protein spots 441 and 884) were reduced in both the *P. indica*-colonized and non-inoculated plants under salt stress conditions.

Salt stress affects the photosynthetic rate, due to this effect we hypothesized that *P. indica*’s strategy to improve stress tolerance can be partly explained by maintaining photosynthesis rates under salt conditions in order to prevent the salt-induced damage. This is in agreement with the higher fresh and dry shoot weights of colonized barley plants compared with non-inoculated plants under both the control and salt conditions.

Salinity hinders the plant growth by inhibition of shoot-growth, and therefore it is not surprising that salinity alters the expression of proteins involved in cell wall biosynthesis and cell elongation processes. Several proteins involved in cell wall synthesis or modifications were differentially accumulated in response to salt stress and *P. indica*-root colonization. The included proteins involved in cell wall biosynthesis and cell elongation processes such as xyloglucan endotransglycosylase (XET) (protein spot 636), tubulin-folding cofactor A (protein spot 95) and α-expansin (protein spot 205). The abundance of XET was increased in *P. indica*-colonized barley plants under different salt stress conditions, but the expression remained unchanged in non-inoculated plants under 300 mM NaCl treatment. XET has wall-loosening activity in plants and has been involved in many aspects of the cell wall biosynthesis.

In barley, the expression of XET was induced in response to the exposure of gibberellic acid (GA) and was stimulated during the cell elongation and leaf expansion. It was shown that GA biosynthesis and signaling were activated in barley during *P. indica*-root colonization. An additional cell wall-related identified protein, tubulin-folding cofactor A was required for plant cell division, but not cell growth. It was up-regulated upon exposure to different salt stress conditions (100 mM and 300 mM NaCl) in colonized barley plants, but the expression remained unchanged in non-inoculated plants.

The abundance of profilin (protein spot 1) decreased due to a high salinity in non-inoculated plants, but no significant protein expression differences were observed in *P. indica*-colonized plants. Profilins are low molecular weight ubiquitous proteins, which bind to the actin monomer and cause cytoskeleton remodeling, resulting in either the polymerization or depolymerization of actin filaments. The cytoskeleton remodeling induces a spatial and temporal response of the plant cells to internal and external signals and is important for the cell elongation and cell shape. Up-regulation of profilin may be associated with cytoskeleton remodeling, which may be required for the plant to adjust cellular behavior to substantial quantities of salt and, thereby, minimized the salt toxicity in plant cells.

Another member of group B (Fig. 6) was identified as an Myb family transcription factor. This protein is involved in the several aspects of the phloem development including the divisions of the phloem pole and sieve element differentiation. The loss-of
function of this transcription factor hindered the development of the phloem sieve tube elements or companion cells.60

The abundance of papain-like cysteine proteases (protein spot 413) decreased in non-inoculated plants under salt stress. However, P. indica-root colonization led to an increase of abundance of this protein. Papain-like cysteine protease is involved in the cell signaling pathways, which plays an essential role in the plant senescence and programmed cell death and in response to the biotic and abiotic stresses.35 P. indica-root colonization is associated with the biphasic trope and cell death-dependent phase.5 P. indica-induced cell death is molecularly and biochemically different to necrotrophic cell death, which has already been implicated by the absence of root-necrotization in P. indica-colonized roots.58 Providing more information about the function of papain-like cysteine proteases might provide a new insight into enhancing host tolerance to saline conditions.

Concluding remarks

The potential application of symbionts in agriculture is not only attributed to their ability to enhance crop yields but also is related to their ability to enhance tolerance to different abiotic stresses. Plant symbionts offer a long-term abiotic stress tolerance through host adaptation to environmental stress. In this respect, a deep understanding by which symbionts protect their plant hosts against the detrimental effects of soil salinity may provide new insights into the stress adaptation mechanisms in plants and assist in helping design a better strategy to cope with abiotic stresses. According to our results, it is likely that P. indica might induce systemic response to salt stress by changing physiology and proteome of P. indica-colonized barley plants, despite the fact that the fungus is not colonizing plant leaves. P. indica has a clear advantage with no obvious side effects for biotechnological applications. P. indica targets a few salinity host components over the higher manipulation of the host in control plant under salinity. P. indica may also serve as a model system to study molecular traits affecting abiotic resistance in cereals.61

References