RESEARCH ARTICLE

Proteomic analysis of rice seedlings infected by *Sinorhizobium meliloti* 1021

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Rhizobial endophytes infect and colonize not only leguminous plants, but several non-leguminous species as well. Using green fluorescent protein tagging technique, it has been shown that Rhizobia infect different varieties of rice species and migrate from plant roots to aerial tissues such as leaf sheaths and leaves. The interaction between them was found to promote the growth of rice. The growth promotion is the cumulative result of enhanced photosynthesis and stress resistance. In addition, indole-3-acetic acid also contributes to the promotion. Gel-based comparative proteomic approaches were applied to analyze the protein profiles of three different tissues (root, leaf sheath and leaf) of *Sinorhizobium meliloti* 1021 inoculated rice in order to get an understanding about the molecular mechanism. Upon the inoculation of rhizobia, proteins involved in nine different functional categories were either up-regulated or down-regulated. Photosynthesis related proteins were up-regulated only in leaf sheath and leaf, while the up-regulated proteins in root were exclusively defense related. The results implied that there might have been an increase in the import and transport of proteins involved in light and dark reactions to the chloroplast as well as more efficient distribution of nutrients, hence enhanced photosynthesis. Although the initiation of defensive reactions mainly occurred in roots, some different defense mechanisms were also evoked in the aerial tissues.

Keywords:
2-DE / Infection / MS / Plant proteomics / Rice / Rhizobia

1 Introduction

Symbiosis is a widely existing phenomenon in nature, which attracts a lot of attention from scientists because of its potential application in agriculture. Several studies have reported that a variety of endophytic bacteria, including nitrogen fixers, can be isolated from surface sterilized healthy plant tissues [1–6]. Among these endophytic bacteria, rhizobia have been most extensively studied because of their interaction with legumes, which is believed to have a positive influence on plant growth. Rhizobia invade the root of legumes by producing Nod factors, which are recognized by plant LysM-like receptors. The interaction between Nod factors and Lysm-Like receptors initiates a signal transduction pathway that is critical for the invasion process [7–9]. Rhizobial infection of the host plant induces the formation of a special root structure (nodule) wherein ammonia is produced by reducing the atmosphere nitrogen [7–9]. The reduction of atmosphere nitrogen into ammonia

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Abbreviations: CRT, Calreticulin; **DAI,** days after inoculation; **GFP,** green fluorescent protein; **IAA,** indole-3-acetic acid; **POX,** peroxidase

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within root nodules is called biological nitrogen fixation reaction, and is of great importance in agriculture.

The interaction between rhizobia and legumes is highly specific and does not occur in the model plant Arabidopsis and rice. Most of the early studies were conducted in two legumes, *Medicago truncatula* and *Lotus japonicus* [10, 11]. In recent years, scientists have found that rhizobia were capable of endophytically infecting some other plant species, such as rice, maize, barley, wheat, canola and lettuce, and colonized both the intercellular and intracellular spaces of epidermis, cortex and vascular system [12–24].

The colonization of endophytic rhizobia in the host plants can promote their growth and also improve the grain yield of crop plants like rice, maize, wheat and barley. Besides biological nitrogen fixation, the presence of rhizobia in the roots also has many other beneficial effects such as production of plant hormones such as indole-3-acetic acid (IAA) and gibberellic acid (GA), which results in expansion of root surfaces and architecture, thus improving seedling vigor [12, 17, 18, 25]; more efficient nutrient uptake [13], solubilization of phosphate [18]; enhancement of photosynthesis in plant leaves [15, 25] and respiration in plant roots [26]; greater resistance to the stress and pathogens [27, 28].

It has been shown that many host genes are activated during nodule formation in legumes [9, 29–31]. However, previous studies have largely focused on the gene regulation that exists in roots where the infection occurs. Most of the studies were limited to a single gene or several genes. Recently, large-scale analysis using microarray technique has also been applied to study the plant–microbe interactions [32]. However, microarray analysis is constrained to the gene expression at transcriptional level. It is well known that the proteins are the final executor of most of biological processes. Proteome analysis, which focuses on investigating the accumulative changes and modifications of proteins, could help us to acquire a more comprehensive understanding of the responses that occur in host plants [33]. Since the advent of proteomics, it has been successfully applied to study many aspects of plant biology including plant–microbe interactions [34–36]. Compared with traditional molecular techniques, proteomics provides information on the consequences of large-scale changes of gene expression at the protein level. Hitherto, proteomics has been widely used in the study of the legumes-rhizobia symbiosis [37–43].

As a model monocotyledon and the most important cereal plant, rice has been sequenced and widely studied through proteomic techniques. Many studies focusing on rice response to different environmental factors, including various biotic factors, such as virus, bacterial and fungal pathogen, have been reported [34, 35, 44–46]. Endophytic rhizobia, as biotic environmental factors, have been shown to interact with rice [12–18]. Recently, our study has shown that rhizobia could not only colonize in rice roots, but also migrate upwards and colonize aerial tissues, such as leaf sheaths and leaves [25]. Does the colonization of rhizobia within leaf sheaths and leaves induce any changes of gene expression in these tissues? Are these changes beneficial to rice plant as a whole? Exploring these questions will certainly deepen our understanding of the interactions between rhizobia and their host plants.

In order to answer these questions, we inoculated rice roots with *Sinorhizobium meliloti* 1021, and systemically analyzed variations in protein patterns in roots, leaf sheaths and leaves using a 2-DE coupled to MS strategy. We hope that the identification of the differentially expressed proteins would contribute in explaining the molecular mechanisms of rice and rhizobia interaction, and further may provide clues as to how the growth promotion was accomplished.

2 Materials and methods

2.1 Bacteria, plasmids and plants

The *Rhizobium* strain used in this study is *Sinorhizobium meliloti* 1021, which contains plasmid pHC60. The resistance of this *Rhizobium* strain is Tc^r10, Sm^r50. The vector pHC60 (6) encodes for tetracycline resistance and contains the green fluorescent protein (*GFP)* gene that is constitutively expressed at fairly constant levels from a constitutive lacZ promoter sans *lacZ* expression. The detailed description of pHC60 is presented in our previous study [25]. *Japonica* rice (*Oryza sativa* L.) varieties Nipponbare was obtained from the National Institute of Agrobiological Sciences of Japan.

2.2 Growth conditions

For gnotobiotic culture of rice with rhizobia, seeds were dehulled, treated with 70% ethanol for 10 min, washed three times with sterile water, surface sterilized with 0.1% $HgCl_2$ for 10 min and then washed three times again with sterile water. Surface-sterilized seeds were germinated in sterile water in dark for 2 days at 28°C. Ten small axenic seedlings were transferred aseptically into sterilized glass tubes (40 cm in height, 5 cm in diameter) containing 250 cm^3 of sterile vermiculite and 125 mL of half-strength Hoagland's no. 2 plant growth media. Bacteria were cultured for 2 days at 28°C in TY liquid media, containing appropriate antibiotic, and then suspended in PBS (pH 7.4) to 10^8 cells/mL. Five microliters of bacterial inoculum were carefully introduced into the seedling rhizosphere using a pipette tip inserted 1 cm below the vermiculite surface in each tube in order to avoid contamination of the aboveground rice tissues. Each inoculated tube was covered with transparent tissue culture paper (12 cm by 12 cm) and incubated in a growth chamber programmed with 100 umol photons$^*m^{-2*}s^{-1}$ white light in a 14-h photoperiod and 28/25°C day/night cycle. The humidity was set as constant 90%.

For open culture of rice with rhizobia, the seeds were not sterilized and grown in boxes (20 cm in length, 12 cm in width and 8 cm in height) containing flooded culture soil

(mixture of 70% humus soil and 30% sands). Each box containing 18 seedlings was also incubated in the growth chamber in the same conditions as mentioned above. Each rice seedling was inoculated with 1 mL bacterial suspension using a pipette tip as mentioned above on the third day after germination with a density of 10^8 cells/mL in PBS. The controls were also inoculated with 1 mL PBS without rhizobia for each seedling.

2.3 Microscopy and image analysis

The GFP accumulated within the *GFP*-tagged bacteria produces a sufficiently bright fluorescent signal allowing for their single cell detection and quantification by computer-assisted fluorescence microscopy. Tissues of rice roots, leaf sheaths and leaves were excised from plants at 7, 14 and 21 days after inoculation (DAI), respectively. For each tissue and time point, five plants were used for the observation. Free-hand sections of the excised tissues were rinsed clean with sterile water, mounted on slides and observed using a Bio-Rad MRC 1024 laser confocal microscope with 488- and 568-nm band-pass filters to capture the green fluorescence from *GFP*-tagged bacteria and the red auto-fluorescence from host tissue, respectively. The images were acquired as confocal Z-section series using a Nikon E800 scanner and digital camera and then merged into loss-less montage images using Confocal Assistant Software (version 4.02; Todd Clerke Brelje (www.Imf.ludwig.ucl.ac.uk/CAScontent.htm)).

2.4 Population densities numeration

The rice roots from gnotobiotic seedlings were carefully removed from each tube, excised, washed with sterile water, blotted dry and weighed. Root samples were surface sterilized by vortexing for 1 min in a solution (1:20 w/v) of 1% bleach, 0.1% SDS and 0.2% Tween 20 in PBS. These samples were then rinsed four times with sterile water, placed on LB plate for 1 h and then removed. These plates developed no colonies after 2 days incubation at 28°C, verifying that the excised roots were surface sterilized. To enumerate the endophytic rhizobia, excised surface-sterilized samples of roots, leaf sheaths and leaves were macerated with a sterilized mortar and pestle, diluted in PBS solution containing 20% glycerol and spread on TY plates supplemented with tetracycline (10 μg/mL) and streptomycin (50 μg/mL). After 3 days incubation at 28°C, the numbers of colonies were counted to show the bacterial population densities in different rice tissues.

2.5 Protein extraction and 2-DE

The rice tissues cultured under gnotobiotic conditions were cut off, washed, dried and weighed. Denaturing protein extraction (Phenol extraction) procedure was applied here according to Saravanan and Rose [47] with minor modification. Two-gram roots, 0.5 g shoot tissues (leaf sheaths or leaves) harvested from ten different plants were ground, respectively, into fine powder in liquid nitrogen and homogenized on ice for 15 min in four volumes of pre-cooled extraction buffer (1:4 wt/vol, 250 mM sucrose, 20 mM Tris-Hcl, pH7.5, 10 mM EDTA, 1 mM PMSF, 1 mM DTT). After centrifugation (20 min, 15 000 × *g*, 4°C), the suspension was collected and an equal volume of ice-cold Tris-HCl, pH 7.5, saturated phenol was added. The mixture was vortexed on ice for 1 h. After centrifugation again (20 min, 15 000 × *g*, 4°C), the phenol phase was collected. Proteins were precipitated from the phenol phase with three volume of 100 mM ammonium acetate in methanol overnight at −20°C. The pellets were rinsed four times with ice-cold acetone containing 13 mM DTT and then lyophilized. The pellets were dissolved in sample buffer (7 M urea, 2 M thoiurea, 4% w/v CHAPS, 2% Ampholine, pH 3.5–10, 1% w/v DTT; 1 mg pellets for 0.1 mL buffer) through shaking at room temperature for 1 h.

2-DE was carried out according to Shen *et al.* [48]. The IEF rod gels were 13 cm long and 3 mm in diameter. The gel mixture was 8 M urea, 3.6% acrylamide, 2% NP-40 and 5% Ampholines (1 part pH 3.5–10, 1 part pH 5–8). IEF was performed at 200, 400 and 800 V for 30 min, 15 h and 1 h, respectively, with 20 mM NaOH and 20 mM H_3PO_4 as electrode solutions. After the first dimensional run, IEF gels were equilibrated in equilibration buffer (62.5 mM Tris-HCl, pH 6.8, 2.5% SDS, 10% v/v glycerol and 5% 2-mercapto-ethanol) for 15 min twice. The second dimension SDS-PAGE was performed with 15% resolving gels and 5% stacking gels (175 × 200 × 1 mm). The gels were stained with 0.1% CBB R-250.

2.6 Image and data analysis

The 2-DE gels were scanned at a 300 dpi resolution with UMAX Power Look 2100XL scannar (Maxium Tech, Taiwan, China), and gel images were analyzed with ImageMaster 2D Platinum version 5.0 (GE Healthcare BIO-Science). Spots were quantified on the basis of their relative volume, that is, the spot volume divided by the total volume over the whole set of gel spots. The identified protein spots were manually confirmed. A twofold change was set as criteria. Only those with an abundance change of more than twofold were taken into account. Statistical Package for the Social Sciences (SPSS) statistical software was used to analyze the data to ensure the significance of the changes.

2.7 In-gel digestion and MALDI-TOF MS analysis

Protein spots were manually excised from the gels and in-gel digested by trypsin according to Shen *et al.* [48] with some modification. Each small gel piece with protein was

washed with 25% v/v ethanol and 7% v/v acetic acid for about 12 h or overnight at room temperature, and destained with 50 mM NH_4HCO_3 in 50% v/v methanol for 1 h at 40°C. The protein in the gel piece was reduced with 10 mM DTT in 100 mM NH_4HCO_3 for 1 h at 60°C, and alkylated with 40 mM iodoacetamide in 100 mM NH_4HCO_3 for 30 min at room temperature in darkness. The gel pieces were minced and lyophilized, then rehydrated in 25 mM NH_4HCO_3 with 10 ng sequencing grade modified trypsin at 37°C overnight. The supernatant was harvested the following day and the fluid further extracted from gel pieces with 0.1% TFA in 50% ACN three times. All extracted fluid was pooled with the trypsin supernatant and vacuum-dried to approximately 2–3 μL final volumes. MS analysis was performed with an AXIMA Assurance MALDI-TOF mass spectrometer (Shimadzu Biotech, Kyoto, Japan). The spectra acquisition was performed in a reflector mode with a mass range of 600–3500 Da. Peak processing, including smoothing, baseline correction and mono-isotopic peak-picking, of which the default parameters were applied, was carried out to generate the peak list for each sample. The data were searched against the NCBI non-redundant database (NCBInr20080704 with 6 680 430 sequences entries) with the MASCOT Peptide Mass Fingerprint search engine which is available at http://www.matrixscience.com. The searching parameters were set as followed: peptide masses were assumed to be monoisotopic, 100 ppm was used as mass accuracy, a maximum of one missing cleavage site and modification of cysteines to carboxyamidomethyl cysteine (Cys_CAM) by iodoacetamide were considered. *Oryza sativa* was chosen for the taxonomic category. To determine the confidence of the identification results, the following criteria were used: in addition to a minimum of 60 in MOWSE score, sequence coverage of the protein by the matching peptides should be no less than 12%. Only the best matches with high confidence levels were selected.

3 Results

3.1 The inoculation of rhizobia can promote growth of rice seedlings

Previous studies have demonstrated that rhizobia could enhance the vigor of rice seedlings [17, 18]. In order to confirm this in our experimental system, promotion on the growth of rice seedlings by *Sinorhizobium meliloti* 1021 was examined. The roots of rice seedlings were inoculated with rhizobia and grown as described in Section 2. In parallel, un-inoculated seedlings were grown as control. Two weeks later, several growth parameters like root length, seedling height and fresh weight were measured on both the inoculated and un-inoculated seedlings. Comparison of these data showed that the averages of root length, height and fresh weight of shoots of rhizobia inoculated seedlings were 36.05, 7.43, and 44.24%, respectively, more than that of the control (Table 1). These data suggested that inoculation of *Sinorhizobium meliloti* 1021 could significantly promote the rice seedlings' growth, thus confirming the observations from previous studies and validating our experimental system.

Table 1. Growth responses of potted rice plants 14 DAI with *Sinorhizobium meliloti* 1021 cultured in growth chamber

Rhizobia	Root length (cm) ±SE	Shoot height (cm) ±SE	Shoot fresh weight (g) ±SE
Sm1021	11.17 ± 0.80^{A}	27.17 ± 2.54^{A}	0.1490 ± 0.0245^{A}
control	8.21 ± 0.76^{B}	25.29 ± 0.76^{B}	0.1033 ± 0.0180^{B}

Data are represented as means *per* pot from three pot replicates, each containing 18 rice seedlings *per* strain. Values followed by a different superscript capital letter are significantly different at the 95% confidence level according to Duncan's multiple-range test.

3.2 The colonization and migration of rhizobia within the rice seedlings

Our recent study reported the ascending migration of several species of rhizobia from roots to shoots in the rice species Zhuonghua 8 (*indica*) [25]. This phenomenon prompted us to examine whether *Sinorhizobium meliloti* 1021 could also migrate from roots to shoots in another sequenced rice species, Nipponbare (*japonica*). To confirm our hypothesis, confocal microscope was used to observe the infection, colonization and migration of *GFP*-tagged *Sinorhizobium meliloti* 1021 inside different tissues of rice seedlings cultured in gnotobiotic condition post-inoculation. The results indicated that *Sinorhizobium meliloti* 1021 could infect the roots and colonize in the root hairs, aerenchyma, intercellular and intracellular spaces of epidermis, cortex, *etc.* at 21 DAI (Fig. 1A). After 21 DAI, the fluorescence signal of rhizobia could also be detected in the intercellular and intracellular spaces of cortex and vascular system in leaf sheath and leaf (Figs. 1B and C). These results suggested *Sinorhizobium meliloti* 1021 behaves similarly in both rice species Nipponbare and Zhuonghua 8 during the process of colonization and migration inside the plants.

3.3 Population densities of rhizobia within the rice tissues

To determine the relative densities of rhizobia inside different rice tissues, plating experiment were conducted to count the bacteria numbers for each rice tissue at 21 DAI. The results showed the population densities of rhizobia inside roots, leaf sheaths and leaves were about $10^{6.8}$, $10^{5.5}$ and $10^{4.2}$, respectively. The density declines from the underground tissues to the above-ground tissues (Fig. 2). The

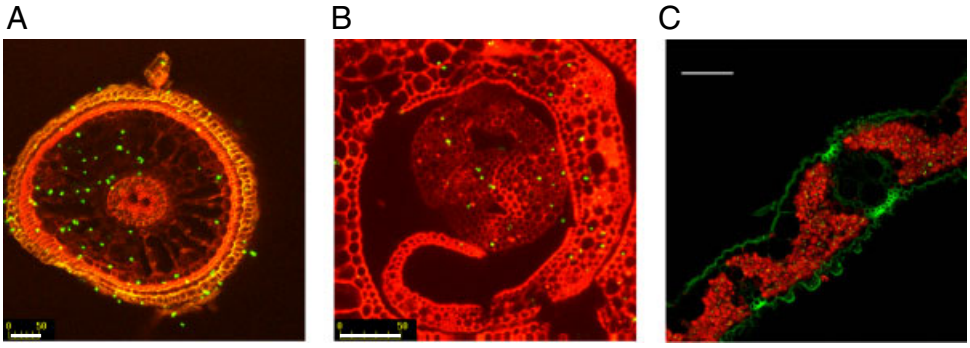

Figure 1. Confocal images of *GFP*-tagged cells of *Sinorhizobium meliloti* 1021 colonized within healthy rice tissues. (A) Cross-section image of roots at 21 DAI; (B) cross-section image of leaf sheath and (C) cross-section image of leaf at 21 DAI. Bar scales are 50 μm in (A–C).

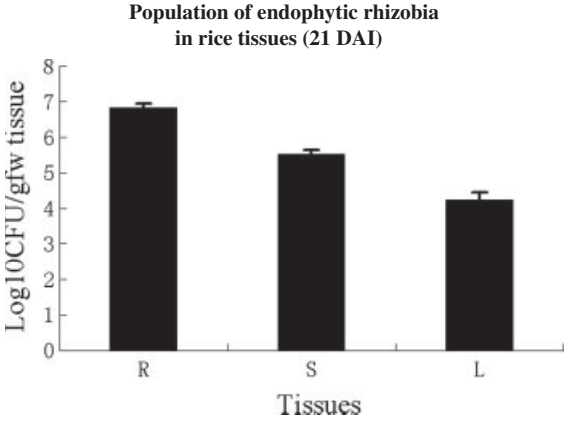

Figure 2. Population densities of *GFP*-tagged *Sinorhizobium meliloti* 1021 in different rice tissues after inoculation and growth in gnotobiotic culture for 21 days. Tissues were surface-sterilized. R: roots, S: leaf sheaths and L: leaves. Data are means and standard errors from three replicates.

high population densities of rhizobia inside the whole plants proved that *Sinorhizobium meliloti* 1021 could also colonize and migrate upwards within the Nipponbare rice seedlings with a high efficiency.

3.4 Protein profiles changes in different tissues

Because of the ascending migration of rhizobia inside its host plants, we suspected that rhizobia might cause changes of gene expression not only in roots, but also in leaf sheaths and leaves. To identify the changes in each tissue of rice seedlings during the process of this rice–rhizobia interaction, 2-DE was performed with proteins extracted from roots, leaf sheaths and leaves taken from rice seedlings with or without inoculations, respectively. Based on our previous results and experience, the rice seedlings cultured in gnotobiotic tubes at 21 DAI were used as materials. To obtain a reliable result, three biological repeat experiments were carried out. The gels were visualized by CBB R-250 staining. After scanning and digitizing with ImageMaster 2D Platinum software (GE Healthcare), general protein patterns in the range of pH 3.5–10 were built for each tissue (Fig. 3). The protein spots that could be reproducibly detected in the gels for each tissue were approximately 1000. The reproducibility between different repeats was analyzed as previous reported [49]. The overlay of the protein spots between different repeats was more than 95%.

Comparative analyses on the digitized 2-DE gels were conducted to highlight the changes in protein profiles of different tissues induced upon inoculation of rhizobia. Using the twofold abundance change as criteria, we found that 21, 19 and 12 protein spots displayed differentially in the gels of roots, leaf sheaths and leaves, respectively. Among these differentially expressed proteins, eight were down-regulated (R14–R21, Fig. 3A) and 13 were up-regulated (R1–R13, Fig. 3B) in roots, 12 were up-regulated (S9–S20, Fig. 3C) and seven were down-regulated (S1–S7, Fig. 3D) in leaf sheaths, and six were down-regulated (L1–L6, Fig. 3E) and six were up-regulated (L7–L12, Fig. 3F) in leaves. The changes of protein patterns occurred not only in roots, but also in shoot tissues, suggesting that the infection and colonization of *Sinorhizobium meliloti* 1021 does result in the changes of gene expression in different tissues. Functional annotation of these changed proteins might help us to get more detailed understanding about the plant–microbe interactions.

3.5 Protein identification and functional categorization

According to the stringent criteria described in Section 2, all the differentially displayed proteins were unambiguously identified by MALDI-TOF MS analysis and NCBI non-redundant database searching. In total, the 52 differentially displayed spots were identified as 44 individual proteins. The identification results were listed in Tables 2–4 for roots, leaf sheaths and leaves, respectively. Careful analysis of the results revealed that some of the proteins were represented by more than one spot. These spots included R2 and R3 (both identified as putative phosphoglycerate mutase) (Table 2), R8, R11

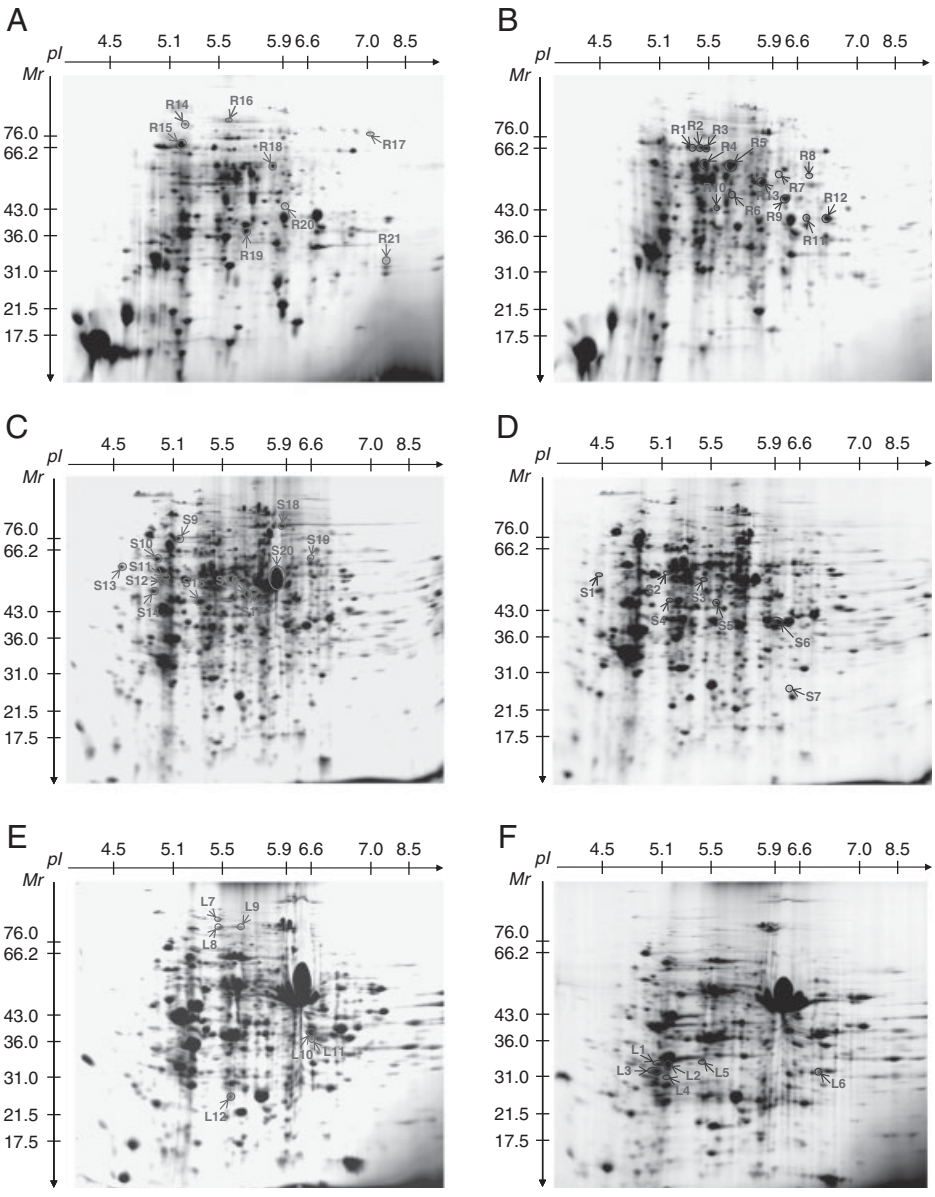

Figure 3. 2-DE maps showing the protein profiles of different tissues at 21 DAI. (A and B) Rice roots with inoculation (A) or without inoculation (B); (C and D) rice leaf sheaths with (C) or without inoculation (D); (E and F) leaves with (E) or without inoculation (F). The up-regulated proteins are marked in images (A, C and E); the down-regulated proteins are marked in images (B, D and F).

and R12 (all indentified as glyceraldehyde-3-phosphate dehydrogenase) (Table 2), S1 and S13 (both identified as putative Calreticulin (CRT) precursor) (Table 3), spots S2 and S12 (both identified as ATP synthase β subunit) (Table 3), and spot L7 and L8 (both identified as chloroplast inner envelope protein) (Table 4). These spots might be potential isoforms or different PTMs of one single protein.

According to gene annotation in database, the identified proteins in all three rice tissues could be classified into nine functional groups. They are primary metabolism proteins, energy-associated proteins, defense-related proteins, protein destination and storage, transporter, signal transduction protein, cell growth/division, cell structure and unknown function proteins. The distribution of different functional groups in the three tissues is shown in Fig. 4.

The energy-associated group was the major group that changed in all three tissues upon inoculation of rhizobia. Proteins in this group can be categorized into respiration and photosynthesis sub-groups. All the seven energy associated proteins identified in roots belong to the former sub-group, and are down-regulated by the inoculation (Table 2; Fig. 5A), while most of the energy-associated proteins identified in leaf sheaths and leaves were up-regulated and associated with photosynthesis sub-group (Tables 3 and 4; Fig. 5B).

In roots, all the up-regulated proteins were either signaling or defense related (Table 2; Fig. 5C). There were only two defense proteins identified in leaf sheaths (Table 3; Fig. 5D). They are catalase (S19) and *S*-adenosylmethionine (S17). Interestingly, the same signaling protein CRT was identified both as up-regulated (S13) and down-regulated

Table 2. Identification of differentially expressed proteins in roots

Spot no.	Mr[a] (kD)/ p*I*	MOWSE[b] score	NMP[c]	SC[d] (%)	Description	Function[e]	Changed folds[f]	Accession no.
Down-regulated proteins								
R1	73.08/5.49	67	9	21	Putative dnaK-type molecular chaperone precursor	4	2.5	XP_468043
R2	60.98/5.42	75	12	30	Putative phosphoglycerate mutase	2	3.5	BAD82294
R3	60.98/5.42	120	11	27	Putative phosphoglycerate mutase	2	3.5	BAD82294
R4	48.28/5.41	124	11	41	Enolase	2	2.1	AAP94211
R5	49.95/5.88	80	9	29	Adenosylhomocysteinase	8	3.6	ABA93501
R6	47.34/5.66	64	6	19	IAA-amino acid hydrolase	3	10	AAO25632
R7	53.13/6.23	63	7	20	Alanine aminotransferase	1	2.1	ABB47494
R8	53.96/6.57	65	6	13	Glyceraldehyde-3-phosphate dehydrogenase	2	2.8	XP_482618
R9	44.60/6.15	103	7	36	Glutamate dehydrogenase	1	12	XP_469970
R10	41.64/5.66	83	8	32	OSJNBa0044M19.9	9	3.1	XP_472275
R11	36.64/6.61	105	9	35	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	2	3.1	Q42977
R12	36.64/6.61	81	9	32	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	2	3.5	Q42977
R13	52.97/5.85	205	21	53	Putative phosphogluconate dehydrogenase	2	4.7	NP_910282
Up-regulated proteins								
R14	81.36/5.95	92	6	20	Putative subtilisin-like proteinase	8	2.2	XP_468091
R15	73.34/5.27	66	12	15	Arabinoxylan arabinofuranohydrolase isoenzyme AXAH-II	8	2.6	ABA96372
R16	98.60/5.42	96	12	23	Putative aminopeptidase N	8	2.4	XP_483801
R17	68.19/7.23	76	11	25	Putative exoglucanase precursor	8	2.9	XP_469751
R18	47.25/5.62	76	8	22	3′-*N*-debenzoyltaxol *N*-benzoyltransferase-like	8	3.8	BAD86875
R19	33.31/5.77	66	4	18	POX	8	2.3	CAA46916
R20	43.33/8.21	80	8	34	Peroxisomal targeting signal protein-like	7	1.9	BAD33323
R21	22.63/7.77	69	6	41	Probable germin protein 4	8	9.4	NP_912610

a) The MW and p*I* are theoretical.
b) MOWSE score represents the score acquired during the matching with NCBInr database with MASCOT software. When the score is more than 61, the identified result is creditable.
c) NMP is the abbreviation of number of matched peptides.
d) SC is the abbreviation for sequence coverage.
e) The functional categories were decided according to the system in Bevan *et al.*, 1998. The number represents the protein function category, respectively.: 1, Metabolism; 2, Energy associated; 3, Cell growth/division; 4, Protein destination and storage; 5, Transporters; 6, Cell structure; 7, Signal transduction; 8, Disease/defense; 9, Unknown function.
f) $p<0.05$

(S1) (Table 3; Fig. 5D) proteins in leaf sheaths. We suspect this is because of some PTM event, while the only defense proteins identified to be down-regulated in leaves was glyoxalase (L2; Table 4).

4 Discussion

4.1 Association of rhizobia with plant species other than legumes

The rhizobial species were first identified in 1889 and named thereafter. The interactions amongst rhizobia and legumes are highly specific [10, 11]. The beneficial nature of the interaction between rhizobia and plants and the potential for agricultural application has prompted scientists to explore this process in varied crop species. Such investigation were first carried out in rice and resulted in the discovery of rhizobial interactions therein [50]. It has also been confirmed that rhizobial species can successfully infect and colonize crops, such as wheat, barley, sorghum, canola and millet too [12–24]. Traditionally, surface-sterilized rice roots are used to detect and isolate rhizobia [51]. The development of molecular biology tools has resulted in the utilization of reporter genes (such as GFP) to observe the infection and colonization of rhizobia in the host plant. We used both of these approaches to validate the existence of rhizobial–rice interaction. Furthermore, in our study we found rhizobia not only in the root system, but also detected their presence in aerial plant tissues such as leaf sheaths and leaves [25]. Currently, there is no doubt that the beneficial interactions between rhizobia and crops exists widely in nature.

Table 3. Identification of differentially expressed proteins in leaf sheath

Spot no.	Mr[a] (kD)/p*I*	MOWSE[b] score	NMP[c]	SC[d] (%)	Description	Function[e]	Changed folds[f]	Accession no.
Down-regulated proteins								
S1	48.45/4.47	68	8	22	Putative CRT precursor	7	2.5	XP_477251
S2	54.02/5.38	127	12	37	ATP synthase β subunit	2	3	BAA90397
S3	48.29/5.41	71	5	21	Enolase	2	3.3	AAP94211
S4	39.41/5.51	64	5	27	Glutamine synthetase shoot isozyme	1	3.2	XP_467663
S5	46.02/5.90	142	12	42	Aspartate aminotransferase	1	2.7	AAO23563
S6	36.92/6.34	72	9	26	OJ000223_09.15	9	5.4	XP_472949
S7	30.09/5.73	76	7	33	β 5 subunit of 20S proteasome	4	2.1	BAA96838
Up-regulated proteins								
S9	64.37/5.15	68	8	18	Putative 70 kDa peptidyl prolyl isomerase	4	2	XP_483423
S10	61.15/5.12	170	14	35	Rubisco subunit binding-protein α subunit, chloroplast precursor	4	4.4	ABA97087
S11	64.33/5.60	74	7	15	Putative chaperonin 60 β precursor	4	3.7	NP_910308
S12	54.02/5.38	71		25	ATP synthase β subunit	2	2.7	BAA90397
S13	48.45/4.47	97	8	28	Putative CRT precursor	7	2	XP_477251
S14	52.39/5.59	113	11	36	RuBisCO activase small isoform precursor	2	6.09	AAX95414
S15	42.01/5.30	76	6	27	Actin	6	8	XP_469569
S16	43.15/5.75	126	12	39	Putative epimerase/dehydratase	1	8.6	NP_921492
S17	43.65/5.74	118	11	42	*S*-adenosylmethionine synthetase	8	2	AAT94053
S18	93.36/5.94	159	16	27	Sucrose-UDP glucosyltransferase 2	1	10.2	AAK52129
S19	57.07/6.75	99	11	27	Catalase (EC 1.11.1.6)	8	3.8	CSRZ
S20	52.86/6.45	74	7	20	RuBisCO large subunit	2	1.9	NP_920971

See Table 2 for footnotes and annotations.

Table 4. Identification of differentially expressed proteins in leaves

Spot no.	Mr[a] (kD)/p*I*	MOWSE[b] score	NMP[c]	SC[d] (%)	Description	Function[e]	Changed folds[f]	Accession no.
Down-regulated proteins								
L1	54.02/5.38	94	10	26	ATP synthase β subunit	2	2.9	BAA90397
L2	32.39/5.82	88	11	41	Putative glyoxalase I	8	2	BAD28547
L3	39.81/6.75	65	7	24	OSJNBa0084K20.14	9	4.3	XP_472987
L4	43.11/5.66	73	5	19	Putative ATP synthase β chain	2	2.9	XP_475256
L5	29.90/5.37	71	7	28	Proteasome subunit α type 1 (20S proteasome α subunit F)	4	3.3	P52428
L6	53.33/6.23	64	7	14	Ribulose bisphosphate carboxylase large chain	2	2.1	CAG34174
Up-regulated proteins								
L7	108.21/5.37	66	7	10	Chloroplast inner envelope protein	5	2.7	AAP54402
L8	108.21/5.37	116	16	19	Chloroplast inner envelope protein, putative	5	10	AAP54402
L9	103.59/5.98	94	14	18	Pyruvate orthophosphate dikinase	2	2	BAA22420
L10	36.92/6.34	68	5	20	OJ000223_09.15	9	2.2	XP_472949
L11	38.23/6.28	71	5	22	Putative auxin-induced protein	3	2.3	BAD61512
L12	27.09/8.66	94	8	38	Probable photosystem II oxygen-evolving complex protein 2 precursor	2	14	NP_911136

See Table 2 for footnotes and annotations.

4.2 Promotion of plant growth by rhizobial infection

It is well known that symbiosis of rhizobia to legumes can promote the growth of legumes. With the discovery of the interactions between rhizobia and other crops, more and more studies have shown that endophytic rhizobia could enhance the growth of these plants either through improving the photosynthesis ability of plant or increasing the levels of phytohormones [15, 17, 25].

As we described above, a series of proteins that are either directly or indirectly related to photosynthesis were up-regulated in both leaf sheaths and leaves after rhizobial infection (Fig. 5B), which suggested an enhancement of photosynthesis. Using a physiological approach the increase

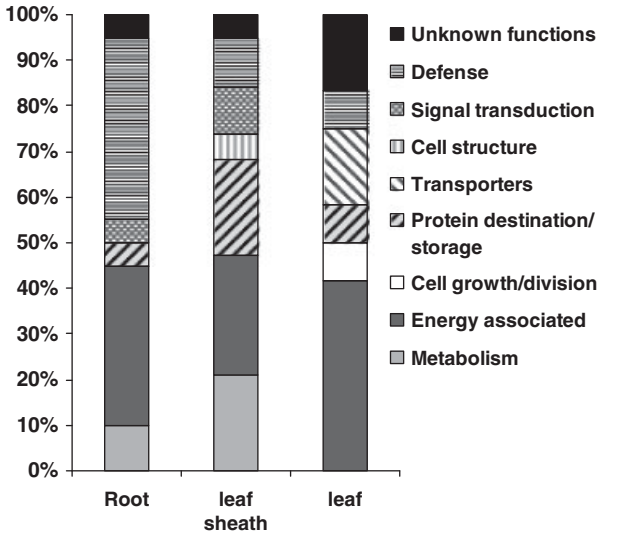

Figure 4. Functional categorization of the differentially expressed proteins in different tissues.

of photosynthesis by rhizobia infection has previously been shown [25]. Several changes to the chloroplast might contribute to the photosynthesis enhancement. First of all, the up-regulation of Rubisco activase (S 14) activates some of the passivated Rubisco, which will result in increase of CO_2 fixation efficiency [52]. Second, the up-regulation of pyruvate orthophosphate dikinase (L9) might increase CO_2 fixation efficiency. pyruvate orthophosphate dikinase is best recognized as a chloroplastic C4 cycle enzyme that catalyzes the production of PEP, the receptor of CO_2 in C4 plants. In C3 plants, its involvement in CO_2 fixation and amino acid synthesis has been implied [53]. Third, major proteins involved in both light and dark reactions of photosynthesis were up-regulated, which suggested that there was an overall increase of photosynthetic efficiency. In addition, the up-regulation of some chloroplast envelop proteins implies that the nutrients transportation might also be increased accordingly. Peptidyl-prolyl *cis-trans* isomerase (S9) is a chloroplast membrane protein involvement in protein folding, protein translocation through biological membranes and signal transduction. It plays an important role in the transport of many nuclear encoded proteins to chloroplast [54, 55]. The increasing of peptidyl-prolyl *cis-trans* isomerase suggested that the import of nuclear encoding chloroplast proteins was enhanced. The current evidence at the protein level along with physiological data suggests that the enhancement of photosynthesis is one of the major reasons for promotion of growth post rhizobial infection.

One auxin-induced protein (L11) was detected in the up-regulated group of proteins. This result is consistent with our recent work, which showed the increase of IAA level in both roots and shoots of rice seedlings [25]. Meanwhile, we also detected the down-regulation of IAA-amino acid hydrolase (R6), which hydrolyzes the IAA-l-amino acid conjugates into IAA [56]. So the increase of IAA biosynthesis induced by rhizobia might be through a tryptophan-independent route.

Recently, it was reported that *Sinorhizobium meliloti* bacteria could produce a rhizosphere signal molecule named lumichrome to promote the growth of different plant species, such as soybean, cowpea and maize by improving the root respiration, stomatal conductance, leaf transpiration and photosynthetic efficiency [26, 57, 58]. This signal molecule might also be involved in the growth promotion on rice seedlings. How this signal molecule helps in promoting plant growth is still unknown. Future proteomics study of lumichrome-treated rice seedlings might help in elucidating the mechanism underlying the growth promotion process.

4.3 Plant defense response induced by rhizobia

Defense initiation is a widely existing mechanism used by plants to respond to foreign microbes. Invasive microbes, irrespective of their species affiliations, invariably induce defensive reactions in host plants. Apart from the well-known plant–pathogen interaction, Schulz showed that even the infection of non-pathogenic endophytes could induce the defense response of host plant, which suggested that there is a balanceable antagonism between plant and endophytes [59, 60]. Freeman found some mutants of pathogens could live in plant like endophytes and inferred that endophytes probably evolved from pathogen [61]. Unlike non-compatible pathogen that can induce hypersensitive reactions of host plant, which prevent diffusion of pathogens, the endophytic rhizobium is far more inclusive, invasive and motive, involving the ascending migration of the endophyte from root to shoot. In this study, many differentially expressed proteins induced by rhizobia infection were identified to be defense-related proteins in both, roots as well as shoots.

4.3.1 Defensive reactions in roots

Roots are the primary tissue for rhizobia infection and colonization. In this study, seven of the ten defense-related proteins were found to have changed in roots. Furthermore, all these seven defensive proteins were up-regulated by the inoculation (Table 2; Fig. 4A). Among them, exoglucanase (R17) is an important PR protein that could antagonize exterior pathogen by lysing their cell walls [62–64]. Peroxidase (POX, R19) can detoxify the ROS. These two are common defense-related proteins that are usually involved in different plant–pathogen interactions. The increase in these two proteins indicates that some canonical defensive reactions were initiated upon the inoculation of rhizobia.

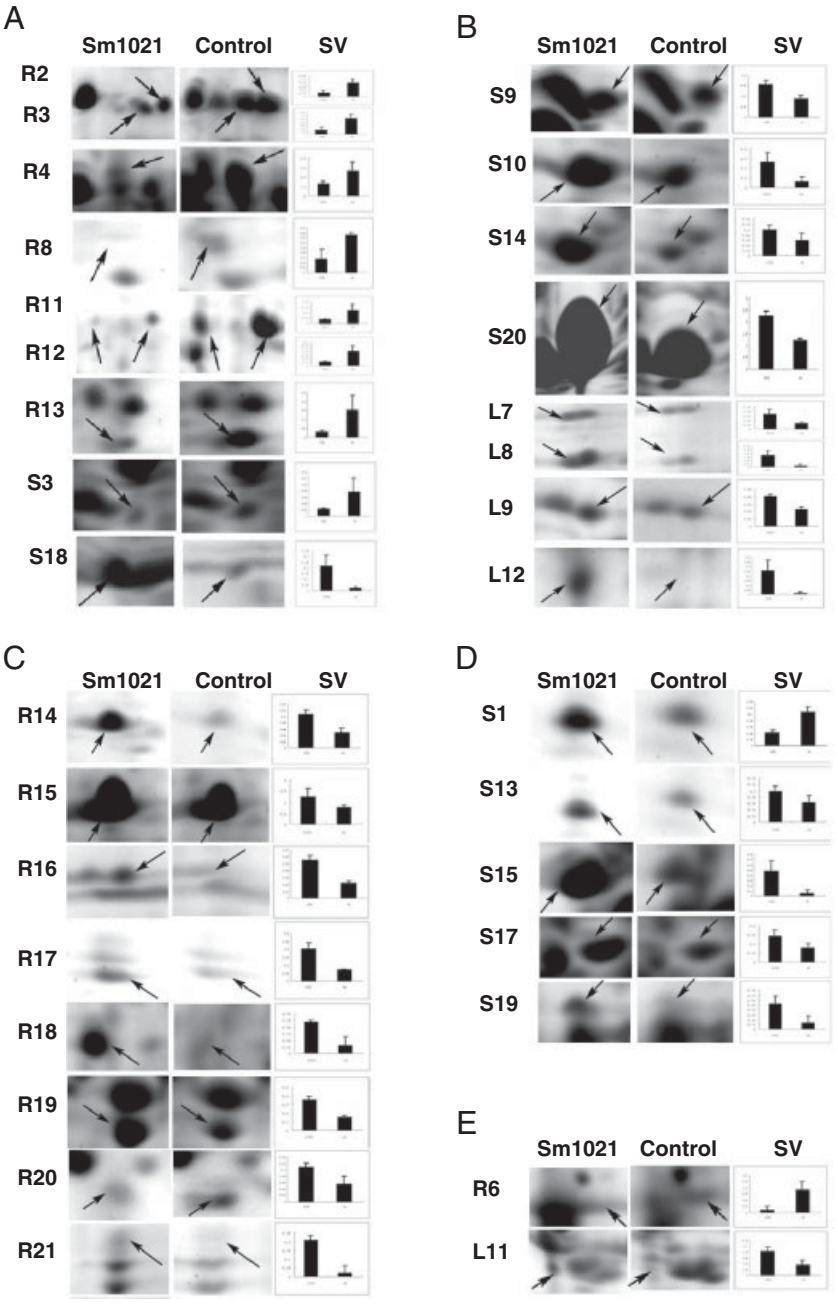

Figure 5. Enlarged area showing distribution of some important differentially expressed proteins. (A) Proteins involved respiration; (B) photosynthesis related proteins; (C) defense- and signaling-related proteins in roots; (D) defense- and signaling-related proteins in leaf sheathes; (E) proteins related with IAA. Sm1021 represents the samples inoculated with *Sinorhizobium meliloti* 1021; control represents the control samples without inoculation. In each panel, changed proteins were indicated by black arrows; spot numbers were shown on the first column, the quantitative changes of these proteins were shown in the last column (marked as SV: spot relative volume). Data are means and standard errors from three replicates.

Additionally, some other defensive mechanism might also be evoked. Arabinoxylan arabinofuranohydrolase isoenzyme (AXAH-II, R15) participates in the accumulation of arabinoxylan to the secondary cell wall, which causes thickening of the cell wall [65]. Germin protein 4 (R21) has also been reported to improve plant pathogen resistance by strengthening the cell wall structure, thus preventing pathogenic invasion [66, 67]. The increase of these two proteins suggests that the root cells thicken their walls to strengthen the defense barrier against attacks by other pathogens. Subtilisin-like proteinase (R14) is a calcium-activated endopeptidase acting in the posttranslational modification of proteins that participate in the defense response [68].

Aminopedeptidase *N* is an enzyme releasing the amino-terminal amino acid residues from proteins and peptides, and is involved in the turnover of proteins [69]. 3′-*N*-debenzoyltaxol *N*-benzoyltransferase–like (spot R18) is involved in the synthesis of methoxydianthramide B, a kind of phytoalexin that is an important anti-pathogen compound [70]. Increased abundance of these proteins implies that the invasion of rhizobia in rice root aids in enhancing the plants

defenses by strengthening the cell wall and promoting turnover of cellular proteins.

4.3.2 Defensive reactions in shoots

We also detected some defense-related proteins to be up-regulated in shoot tissues (Table 2). The results indicated that the initiation of defensive reactions occurs not only in roots but also in the aerial tissues. But it seems that different defensive mechanisms were activated in different tissues. *S*-adenosylmethionine synthetase (S17) catalyzes the formation of adenosylmethionine, which is the universal methyl donor in transmethylation reactions [71–73]. Under stress conditions, *S*-adenosylmethionine synthetase could possibly increase the lignifications by providing the methyl for the polymerization of lignin monomers and reinforce the cell walls to alleviate the damages caused by pathogen attacks [74]. Catalase (S19) was also up-regulated in leaf sheaths. It can remove excess ROS and protect the integrity of the cell membrane [74, 75]. Compared with the up-regulation of POX in roots, this result showed that different ROS detoxification mechanisms were activated in different tissues. CRT, a highly conserved protein mainly localized to the ER in plants, is implicated in a variety of cellular functions such as Ca^{2+} storage, signaling and chaperone activity [76]. In plant–pathogen interactions, the level of Ca^{2+} in cytoplasm is closely related to the hypersensitive reaction and the expression of PR genes [63]. CRT can regulate the Ca^{2+} level inside cytoplasm [77–79]. In our study, two different protein spots (S1 and S13) in leaf sheaths were identified as CRT precursor, which might represent different PTM of CRT. The spot S13 was up-regulated while spot S1 was down-regulated, which suggested the changes between two functional statuses of this protein. The change in CRT protein abundance was apparent post-inoculation indicating the regulation of Ca^{2+} level. Whether the defense mechanism in the aerial tissues was activated by rhizobia that migrated into these tissues or by the signal that originates in the roots, it is still unknown.

The results that defense-related proteins were increased are in conflict with previous microarray data from legumes [80, 81]. Those results showed that the infection of nitrogen fixation bacteria could repress the expression of defense-related genes, which enables the subsequent nodulation and symbiosis. Although the infection of rhizobia could promote rice growth similar to legume plants, the infection process and mechanism of infection in legumes and rice might be different.

4.4 Conclusions

Following up our previous study [25], we showed here that the nitrogen fixing bacteria *Sinorhizobium meliloti* 1021 could infect, colonize and migrate in different rice varieties. Although the process and mechanism of the infection are probably different with those that occur in legumes, the interactions between them are still mutually beneficial. The growth of rice was promoted by the inoculation. Furthermore, we analyzed the changes of proteome patterns that occurred in different rice tissues (including roots, leaf sheaths and leaves) after inoculation with *Sinorhizobium meliloti* 1021. To our knowledge, this is possibly the first study that explores the molecular mechanism of the beneficial plant–endophyte interaction using a proteomic approach. Our results showed that the promotion mechanism might follow these steps: (i) activation of the defense mechanisms in different tissues to minimize negative effects of environmental factors; (ii) enhancement of the anabolism (*e.g.* Photosynthesis) to increase biomass of the plant; (iii) regulation of the auxin level or status to promote growth. The results also raise new questions regarding the molecular mechanisms that govern rhizobia–rice interactions in the root and necessitate more comprehensive studies in the future to broader our understanding of this intriguing phenomena.

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