Proteome of rice roots treated with exogenous proline

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Abstract

Proteomic analysis was conducted to identify the rice root proteins induced by exogenous proline and their involvement in root growth. Proteins were extracted from the root tissues grown under two conditions, T1 (control) and T2 (10 mM proline), and profiled by two-dimensional polyacrylamide gel electrophoresis. Seventeen of 30 differentially expressed proteins were identified by mass spectrometry. Proline-treated rice roots showed up-regulation and down-regulation of nine and eight proteins, respectively, when compared to those in the control. Among the differentially expressed proteins, the down-regulation of glutathione reductase and peroxidase could be involved in the regulation of cellular hydrogen peroxide and reactive oxygen species levels that modulate the root cell wall structure. Differentially expressed proteins identified as pathogenesis-related proteins might be related to stress adaptive mechanisms in response to exogenous proline treatment. In addition, differentially expressed protein identified as the fructose-bisphosphate aldolases and cytochrome c oxidase might be associated with energy metabolism, which is needed during root developmental process. This is the first attempt to study the changes in rice root proteome treated with proline. The acquired information could open new avenues for further functional studies on the involvement of proline in modulating root development and its relation to stress adaptation of plants.

Keyword Proline · Protein · Rice · Root

Introduction

Amino acids are not only the building blocks of protein; they are also involved in many cellular reactions that influence plant growth and development (Hildebrandt et al. 2015). Proline (Pro) is an amino acid which can be absorbed by plant roots as an organic form of nitrogen and its accumulation

in many plant species is generally viewed as a response to various stress conditions (Kavi Kishor et al. 2005; Kaur and Asthir 2015). Nevertheless, a significant accumulation of Pro was also reported in reproductive organs and tissues under non-stressed conditions during plant development (Matioli et al. 2009; Funck et al. 2012). For example, the Pro content in tomato flowers was 60-fold higher as compared to other organs analysed (Schwacke et al. 1999). Transgenic *Arabidopsis* with impaired Pro synthesis showed abnormal leaf morphology and defective inflorescences (Nanjo et al. 1999). In addition, *Arabidopsis* with a mutation in a Pro biosynthesis gene, *P5CS2*, was shown to have embryo abortion during seed development, indicating the involvement of Pro in embryo development (Székely et al. 2008). These studies showed that Pro accumulation does not only happen during stress but is also important for plant growth and development.

In addition, the involvement of Pro in regulating root growth has also been reported. The involvement of Pro in root growth was first reported in the adventitious roots induced by *Agrobacterium rhizogenes* (Costantino et al. 1994). The expression of *rolD* gene, a Pro-producing ornithine cyclodeaminase transferred by this bacterium, was found

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responsible for hairy root induction and elongation, suggesting a direct correlation between Pro availability and root growth (White et al. 1985; Trovato et al. 2001). In addition, Mattioli et al. (2009) reported that the exogenous application of Pro at micromolar concentrations promoted root elongation in *Arabidopsis*. Exogenous Pro can also modulate the size of root meristem in *Arabidopsis*, which subsequently controlled the cell division during postembryonic root development (Biancucci et al. 2015). Pro application also increased the number of roots in rice under salt-stressed and non-stressed conditions (Teh et al. 2016).

Roots perform a wide range of functions, including water and nutrient uptake, anchoring and mechanical support, synthesis of growth regulators, propagation and food storage (De Smet et al. 2012). In addition, roots can act as a sensory system which responds to various soil conditions, detecting changes in nutrient and water availability, maximising soil volume exploitation through root architecture alteration (Uga et al. 2013; Wu and Cheng 2014). Although evidence exists for the involvement of Pro in regulating root growth, the mechanisms involved remained largely obscure. To gain an enhanced understanding of the mechanism by which Pro regulates the root growth, it is necessary to identify the rice root proteins regulated by Pro since proteins are the key players in cellular events. The availability of protein databases and various mass spectrometry technologies has eased the protein identification process and allowed prediction of putative protein functions (Nozu et al. 2006). We hypothesised that the improved root growth in Pro-supplemented media reported by Teh et al. (2016) was associated with the changes in rice root proteome. Therefore, the objectives of this study are to identify the rice root proteins induced by exogenous Pro and to provide information on putative functions in root growth.

Materials and methods

Plant culture condition and treatments

The *in vitro* rice shoots of a Malaysian *indica* rice variety, MR253, were used as the experimental materials in this study. The explants and Murashige and Skoog (MSO) medium were prepared as described by Teh et al. (2015). To examine the root proteins regulated by Pro, rice shoot apices were cultured under two conditions: T1 (control, in MSO without Pro supply) and T2 (in MSO with 10 mM Pro). A single rice shoot apex was cultured into a test-tube (20 cm height $\times$ 2 cm diameter) containing 40 mL of the respective solidified medium for 30 days. A total of 10 replicates were included in each treatment and the treatments were repeated thrice over time. The Pro concentration and culture period

were chosen based on our previous findings (Teh et al. 2015, 2016).

Extraction of protein from rice roots

Proteins were extracted from fresh root samples using a protocol modified from Hurkman and Tanaka (1986). Briefly, the plants were removed from the test tubes and the whole root system was carefully harvested and washed thoroughly to remove excess media. Subsequently, 0.5 g (fresh weight) of sample was ground in liquid nitrogen into fine powder and suspended in 2 mL of extraction buffer containing 0.5 M Tris-HCl, pH 8.0, 50 mM EDTA, 0.9 M sucrose, 0.1 M KCl, 2 mM phenylmethylsulfonyl (PMSF), 1% (w/v) polyvinyl-pyrrolidone (PVPP) and 2% (v/v) β -mercaptoethanol (β -ME) and incubated on ice for 20 min. The mixture was mixed with an equal volume of phenol and incubated on ice for 30 min. After centrifugation for 15 min at 5000 $\times$ g, the supernatant was re-extracted with an equal volume of extraction buffer. The final phenol layer was mixed with five volumes of chilled methanol containing 0.1 M ammonium acetate and incubated overnight at -20°C . The precipitant was recovered by centrifugation at 5000 $\times$ g for 20 min and the pellet was washed three times with chilled methanol containing 0.1 M ammonium acetate and another three times with 100% (v/v) cold acetone. The protein pellet was air dried for 30 min and re-dissolved in 300 μL of lysis buffer containing 9 M urea, 4% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 40 mM Tris, 1% (w/v) dithiothreitol (DTT) and 2% (v/v) Bio-Lyte pH 3–10. The supernatant was either used for the determination of protein concentration or stored in aliquots at -80°C for further use. The protein concentrations were determined by Bradford assay using Bovine Serum Albumin (BSA) as a standard.

Two-dimensional polyacrylamide gel electrophoresis (2D PAGE)

The first dimension, isoelectric focusing (IEF) was performed using immobilised pH gradient (IPG) strips (13 cm, pH 3–10 non-linear gradient). The strips were rehydrated with rehydration buffer containing 8 M urea, 4% (w/v) CHAPS, 0.01% (w/v) bromophenol blue (BFB), 0.4% (w/v) DTT, 0.5% (w/v) and 700 μg of protein sample for 16 h at 20°C . Immediately after rehydration, IEF was performed using a PROTEAN i12 IEF system (Bio-Rad) with the following programme: a linear increase from 0 to 500 V for 1 h, gradient mode at 1000 V for 1 h and 8000 V for another 2.3 h and then maintained at 8000 V for 2 h. Subsequently, the strips were equilibrated with equilibration buffer containing 6 M urea, 0.375 M Tris-HCl, 2% (w/v) SDS, 30% (v/v) glycerol and 1% (w/v) DTT for 15 min. The second equilibration was carried out using the

same equilibration buffer by replacing DTT with 2.5% (w/v) iodoacetamide (IAA) for another 15 min. Equilibrated gel strips were rinsed with $1 \times$ Laemmli buffer (Laemmli 1970). For the second dimension protein separation, electrophoresis was performed in 12% (w/v) SDS-polyacrylamide gels using an SE 600 Hoefer Ruby electrophoresis system (GE Healthcare, UK). The equilibrated strips were placed on top of the gels and electrophoresis was performed at 10 mA per gel for 15 min followed by 20 mA per gel until the dye front reached the end of the gel. The gels obtained were stained with Coomassie Brilliant blue G-250 (CBB) solution.

Detection of proteins and image analysis

Gel images were documented using a calibrated GS800 densitometer (Bio-rad, USA) and analysed using Progenesis Samespots software (Nonlinear Dynamics, UK). The root proteome profiles of T2 were compared to T1 to identify the rice root proteins induced by Pro. Protein samples from three biological plant replicates and two technical laboratory replicates from each treatment were analysed. Protein spots that exhibited at least two-fold change in percent volume (% vol) in the T2 gel images compared to T1 gel images with $p < 0.05$ in analysis of variance (ANOVA) test were considered as differentially expressed proteins (DEPs) and were selected for further analysis.

In-gel digestion

Targeted protein spots were excised from 2D gels, washed three times with ultrapure water, destained and rehydrated with 50 mM ammonium bicarbonate in 50% (v/v) of acetonitrile (ACN). Subsequently, the proteins were reduced with 10 mM DTT in 100 mM ammonium bicarbonate for 30 min at 60 °C and alkylated with 55 mM IAA in 100 mM ammonium bicarbonate for 20 min in the dark. The gel plugs were then washed three times with a 50 mM ammonium bicarbonate in 50% (v/v) CAN solution, 20 min each time before completely dried with SpeedVac Concentrator (Thermo scientific). After that, the gel plugs were digested overnight at 37 °C with sequencing grade trypsin in 50 mM ammonium bicarbonate. The reaction was stopped by the addition of 50% ACN and incubated for 15 min. The digested samples were transferred to a new tube, and the second extraction was carried out with 100% ACN. The extracts were pooled and dried before storage at -80 °C for further analysis.

Matrix assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/TOF MS)

The digested peptides were reconstituted with 0.1% (v/v) formic acid and desalted using μ C18 ZipTips (Millipore,

Billerica, USA) according to the manufacturer's instructions. Mass spectrometry (MS) analysis was performed at the Medical Biotechnology Laboratory (University of Malaya, Malaysia) using a MALDI-TOF/TOF mass spectrometer (ABI 4800 plus, Applied Biosystems) with the following conditions: taxonomy restricted to *Oryza sativa* (146881 sequences), peptide mass tolerance of ± 100 ppm, fragment mass tolerance of ± 0.2 Da, peptide charge of $+1$ (monoisotopic), cysteine carbamidomethylation as fixed modification and methionine oxidation as a variable modification. All spectra obtained were searched using the online MASCOT program (Matrix Science, London, UK) against the National Center for Biotechnology Information (NCBI) non-redundant protein database for identification of proteins. Gene ontology analysis for individual protein was performed by searching the protein knowledgebase (UniProtKB) to reveal the biological functions of identified proteins.

Results and discussion

Identification of differentially expressed proteins in response to Pro

Previously, we reported that application of Pro significantly increased the number of roots and root fresh weight from 4 to 0.01 g in the non-supplemented media to 30 and 0.29 g in media supplemented with 10 mM of Pro, respectively (Teh et al. 2016). The morphological appearance of rice roots under T1 and T2 (Fig. 1a) showed that the supplementation of Pro (T2) altered the root structure and resulted in vigorous root growth as compared to T1. To compare the proteome profiles under normal and Pro-supplemented conditions, the proteins from these rice root were analysed by 2D-PAGE. As shown in Supplementary data Fig A, the protein spots were well resolved in both dimensions (pI and kDa) with little streaking. About 500–600 reproducible protein spots were detected on 2D gels for samples from T1 and T2. More than 90% of the proteins were found at pI range 4–9 with estimated molecular weights ranging from 11 to 75 kDa. Protein spots that exhibited at least twofold difference in Pro-supplemented condition (T2) compared to those in T1 in all gel replicates were considered as DEPs. A total of 30 DEPs (Fig. 1c) were detected using the above criteria and these proteins were excised for further analysis.

Protein identification through mass spectrometry successfully identified 17 out of 30 DEPs which corresponded to 14 unique proteins (Table 1). Among them, nine and eight proteins were up-regulated and down-regulated, respectively, as a result of Pro supplementation, and the positions of these proteins were indicated in 2-DE gels (Fig. 1d). The identified proteins can be further classified into five functional groups (Fig. 1b) based on their

Fig. 1 The root morphology and proteome profile as a result of proline treatment. **a** The MR253 root morphological appearance after 30 days of culture in T1 (control) and T2 (10 mM proline) respectively; **b** the distribution of identified DEPs based on their predicted biological functions; **c** positions of all DEPs on the reference gel; **d** positions of the identified up-regulated (indicated by triangle) and down-

predicted biological functions which were defence and stress response (47%), carbohydrate metabolism (20%), photosynthesis (13%), nucleic acid metabolism (13%) and redox regulation (7%). The relatively low number of DEPs could be due to the CBB staining method which is known to have less sensitivity as compared to silver staining and fluorescence dye (Rabilloud and Lelong 2011). Several reports on proteome analysis in rice also indicated that the number of DEPs detected using CBB staining ranged from 21 to 32 while the number of proteins identified by MS was from 12 to 26 (Yan et al. 2005; Xiang et al. 2013; Torabi et al. 2009).

regulated (indicated by circle) proteins as a result of proline treatment in the representative 2-DE gel. Samples (700 μ g proteins) were separated on pH 3–10 IPG strips (13 cm, non-linear) followed by SDS-PAGE with 12% (w/v) gels. The spots were visualised by Coomassie Brilliant Blue (CBB) staining

Proline-induced proteins and root growth

Among the 14 unique DEPs identified by MS, two proteins were identified as glutathione reductase (GR, spot 620) and peroxidase (Prx, spot 751) which are antioxidative enzymes responsible for the scavenging of hydrogen peroxide (H_2O_2) (Damanik et al. 2010). Since exogenously applied Pro can also act as a potent ROS scavenger, including H_2O_2 (Matsyik et al. 2002; Kaur and Asthir 2015), a high amount of GR and Prx in T2 might not be required and, thus, were down-regulated. A low level of H_2O_2 , as a result of Pro treatment, may promote cell elongation leading to enhanced root growth

Table 1 Differentially expressed proteins identified through mass spectroscopy in response to proline treatment

Spot ^a	Protein name	Mascot score ^b	Accession number	Mr ^c (kDa)/pI ^d experimental	Mr ^c (kDa)/pI ^d theoretical	Sequence coverage (%)	Number of matched peptide	Fold changes ^e (T2/T1)	p value
Up-regulated proteins by Pro									
830	Fructose-bisphosphate aldolase, cytoplasmic	222	Q5N725_ORYSJ	42.4/8.31	39.1/8.35	9	3	2.15	0.018
831	Fructose-bisphosphate aldolase, cytoplasmic	427	Q5N725_ORYSJ	45.2/8.88	39.1/8.35	18	6	2.31	0.007
993	Chitinase 2 (PR 3)	216	Q7DNA1 CHI2_ORYSJ	36.4/8.09	36.5/7.40	11	2	2.22	0.019
1383	Bowman-Birk trypsin inhibitor	56	E0×6V8_ORYSJ	18.5/4.91	29.2/5.70	7	2	2.22	0.014
1391	Pathogenesis related protein 1	59	A0A0D3QSW1_ORYSA	16.5/4.11	16.0/4.23	16	1	2.20	0.017
1404	Putative nucleic acid binding protein	438	A2YQW2_QRYSI	18.0/6.78	18.8/6.28	51	6	4.03	0.014
1455	Putative pathogenesis related protein Bet VI	77	A2XFP6_ORYSI	16.8/6.00	17.3/5.85	13	1	3.35	0.000053
1456	Pathogenesis-related protein (PR10)	650	B8BMF9_ORYSI	16.3/4.92	17.0/4.88	68	9	3.39	0.0000026
1458	Putative cytochrome c oxidase	139	A2Y952_ORYSI	16.3/4.72	17.2/5.40	24	3	2.49	0.00013
Down-regulated proteins by Pro									
620	Glutathione reductase (GR)	172	GSHRC_ORYSJ	69.0/7.67	53.8/6.24	12	4	0.48	0.001
638	Uncharacterised protein	338	B8BGM4_ORYSI	60.2/6.98	53.1/6.23	20	8	0.33	0.000027
751	Peroxidase (PR 9)	49	A2Y0P6_ORYSI	42.3/5.60	37.8/5.51	7	2	0.28	0.0000021
890	Fructose-bisphosphate adolase, chloroplastic	283	A2ZBX1_ORYSI	40.0/6.55	42.2/6.38	16	4	0.45	0.000027
997	Putative photo-system II PsbO	462	B8A8L8_ORYSI	34.5/5.81	35.1/6.01	33	8	0.44	0.002
999	Putative photo-system II PsbO	676	B8A8L8_ORYSI	34.0/5.43	35.1/6.01	40	9	0.47	0.000029
1017	Glycine-rich RNA-binding protein	43	Q7XI13_ORYSJ	33.2/9.00	33.4/9.31	7	1	0.38	0.024
1125	Putative uncharacterised protein	493	A2WLZ0_ORYSI	27.3/4.60	23.8/4.37	39	4	0.45	0.031

^aSpot, protein spot marked in proteome map^bMascot score, score using MOWSE algorithm;^cMr, molecular weight in kDa^dpI, isoelectric focusing point^eFold changes, when comparing the mean percentage volume of each proteins from three independent biological replicates in T2 to T1 (the accumulation graphs were provided in supplementary table 1); p value, significant level at ($p < 0.05$) using ANOVA test

since the accumulation of H_2O_2 inhibited elongation of root cell and root growth (Ivanchenko et al. 2013).

Root development and developmental processes can also be affected by other proteins identified in this study including fructose-bisphosphate aldolase (FBA) and cytochrome c oxidase (CCO) through their role in energy metabolism. FBA is a glycolytic enzyme which produces pyruvate and energy in the form of ATP (Konishi et al. 2004). Among the 3 FBAs identified, spots 830 and 831 were identified as cytoplasmic FBAs and found to be up-regulated by Pro treatment. Since both spots 830 and 831 shared similar peptide sequences, they are likely from the same protein. The difference in the *pI* value and molecular mass could be due to posttranslational modification or protein processing (Kim et al. 2010). Meanwhile, spot 890 could be a putative chloroplastic FBA and the protein abundance was reduced by Pro treatment. The accumulation of FBA in the root apical region as a result of gibberellin acid (GA) treatment was shown to be able to enhance root elongation in rice seedlings in a previous study (Tanaka et al. 2004). In addition, reduction of root growth was found in transgenic rice lines with low level of FBA in roots (Konishi et al. 2004). These findings suggested that root growth may be related to the levels of FBA in the root.

Pro biosynthesis pathway involves a high consumption of NADH and NADPH (Kaur and Asthir 2015). Oxidation of NADH is essential for energy production during cellular respiration. The addition of Pro could have reduced the endogenous Pro biosynthesis rate which increases the availability of NADH. An increase of CCO abundance could have oxidised the available NADH resulting in greater energy production since CCO is one of the protein complexes in plant electron transport chain (ETC) which is responsible for ATP production (Schertl et al. 2014).

Other up-regulated proteins identified include several putative pathogenesis-related (PR) protein such as PR-3 (spot 993), Bowman–Birk trypsin inhibitor (spot 1383), PR-1 (spot 1391), PR-BetVI (spot 1455) and PR-10 (spot 1456). PR proteins do not usually accumulate in healthy plants, but are rapidly induced during pathogen infection (Sinha et al. 2014). They can also be induced by application of plant growth regulators and chemicals (Jung et al. 1995; Seo et al. 2008). Despite its beneficial effects, when applied at excessive dosage, Pro can lead to toxicity in plants which disrupt the cellular metabolisms (Nanjo et al. 2003; Hayat et al. 2012). Excessive exogenous application of Pro may damage the ultra-structures of chloroplast and mitochondria (Hare et al. 2002). The exposure of rice root tissues to 10 mM Pro in this study was expected to induce certain stress response at the molecular level and led to the induction of PR proteins.

A nucleic acid-binding protein (NABP) was also found to be up-regulated in T2. NABP participates in many

fundamental cellular processes, including replication, transcriptional control, chromatin stability, gene silencing as well as epigenetic regulation (Hudson et al. 2014). However, the involvement of NABP in plant development and regulation remains elusive, which requires further investigation.

Putative photosystem II (PSII) PsbO and glycine-rich binding protein (GRBP) were among the other down-regulated proteins identified. In a plant system, PsbO plays a crucial role in maintaining optimal manganese, calcium and chloride concentrations in the active state of PSII (Bricker and Frankel 2011). A significant reduction in chlorophyll content, chlorophyll a/b ratio and PSI content was observed in low PsbO plants (Dwyer et al. 2012). However, the presence of this protein in the root remains unclear since this protein is responsible for photosynthetic activity. GRBP is one of the RNA-binding proteins that is involved in post-transcriptional gene regulation processes (Kwak et al. 2005). Functional analysis has demonstrated that the transcript level of GRBP increased with cold stress but decreased with salt or dehydration stress indicating that GRBP contributes differently in *Arabidopsis* under various stress conditions (Kwak et al. 2005). Nevertheless, the role of this protein in plant development is not yet fully understood.

Further studies can be carried out to establish a detailed regulatory network of Pro in plant growth and development through various omics approaches such as transcriptomics and metabolomics analyses (Ma et al. 2018). Besides that, the role of Pro in cell wall synthesis should be analysed to understand the involvement of Pro in regulating root development (Kavi Kishor et al. 2015).

Conclusions

In conclusion, this study provides evidence that supplementation of Pro significantly altered the abundance of some proteins of rice under non-stressed condition. Changes in the abundance of several proteins as a result of Pro supplementation could be important in enhancing root growth. Some of these proteins might be associated with the regulation of cellular H_2O_2 and ROS levels, stress and defence response and energy metabolism that could contribute to root development.

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Author contributions All the authors meet the essential criteria of the publication. CYT performed the experiments and wrote the manuscript. CLH, SNA and KSL designed the experiment and analysed the data. MM and CLH supervised the experiment and reviewed the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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