Comparative proteomic analysis of the effects of nitric oxide on alleviating Cd-induced toxicity in rice (*Oryza sativa* L.)

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Abstract

Nitric oxide (NO) is a signaling molecule that mediates many physiological processes. To help understand the effect of NO on cadmium (Cd)-induced toxicity in rice (*Oryza sativa* L.), a hydroponic experiment was conducted to investigate the effects of exogenous sodium nitroprusside (SNP), an NO donor, on the physiology and proteomics of rice seedlings. Rice seedlings were treated with 0.1 mM Cd and the same Cd concentration with a range of SNP concentrations (0.005, 0.05, 0.1 and 0.2 mM) for 8 days. A concentration of 0.005 mM SNP significantly ameliorated the Cd-induced decrease in dry weight and length of both the shoots and roots, whereas the hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) content in the rice seedlings decreased with the addition of NO. The differential expression of proteins in the rice leaves and roots was analyzed using a two-dimensional electrophoresis and MALDI-TOF/MS approach. Compared with the plants under Cd stress, 92 protein spots from the rice leaves and roots were differentially expressed after NO application, and 41 of those 92 proteins were successfully identified. A total of 16 proteins were expressed in the rice leaves, and 25 proteins were expressed in the rice roots. As expected, the identified proteins were involved in photosynthesis, carbohydrate metabolism, nitrogen metabolism, oxidative phosphorylation, oxidative stress responses, signal transductions and cell division.

Keywords: Nitric oxide; Cadmium toxicity; Proteomic; Rice (*Oryza sativa* L.).
Abbreviations*: Cd, cadmium; DW, dry weight; H_2O_2, hydrogen peroxide; MDA, malondialdehy-de; NO, nitric oxide; SNP, sodium nitroprusside; 2-DE, two-dimensional gel electrophoresis; IEF, isoelectric focusing; IPG, immobilized pH gradient; MALDI-TOF/TOF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectrometry; TCA, tricarboxylic acid.

Introduction

Cadmium (Cd) is one of the most serious health-threatening pollutants found in the environment, and it easily contaminates both soil and water. It is commonly released into the environment through human activities such as smelting, mining and petroleum refining. Additionally, it is released from sewage sludge and through the agricultural use of phosphate fertilizers (Nriagu and Pacyna, 1988; Adams et al., 2004). The predominant source of Cd exposure in humans is through food contamination. The subject of Cd pollution has received increasing international concern. Cd toxicity can cause negative symptoms in plants, such as growth inhibition, leaf chlorosis and cell death (Larbi et al., 2002; Clemens, 2006; Maksymiec, 2007). Cd toxicity also induces oxidative stress at the cellular level through hydrogen peroxide (H_2O_2) accumulation, lipid peroxidation and oxidative burst (Romero-Puertas et al., 2004; Tang et al., 2005; Xu et al., 2010). In addition, Cd also stimulates the activity of antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (APX), glutathione reductase (GR), and catalase (CAT) (Xu et al., 2010; Kuo and Kao, 2004; Liu et al., 2005), in addition to the antioxidants glutathione (GSH) and phytochelatins (PCs). Nitric oxide (NO) is a small gaseous signaling molecule in plants. There is mounting evidence showing that NO plays a crucial role in plant physiological processes throughout the entire plant life cycle, including plant growth, development and defense against biotic and abiotic

stresses (Beligni and Lamattina, 2000; Wilson et al., 2008). An increasing number of studies have revealed the importance of exogenous NO with regard to the protection against the deleterious effects of heavy metals. Exogenous NO prevents Cd-induced increases in the levels of H_2O_2 and MDA, decreases in GSH and ASC levels, and increases in the activities of antioxidant enzymes in rice leaves (Hsu and Kao, 2004). It has also been demonstrated that exogenous NO reverses Cd-induced increases in the activities of antioxidant enzymes (Singh et al., 2008). The identification of genes or proteins that are involved in heavy metal stress responses is a fundamental step in understanding the molecular mechanisms of NO signaling in plants. With advances in the genomics technologies, it will soon be possible to integrate analyses of the effects of NO on alleviating Cd stress. Proteomics is not only a powerful molecular tool for describing the complete proteome but also for comparative proteomes that are affected by different physiological conditions, such as exposure to a heavy metal or other stressful environmental factors. Recently, several studies have been conducted to investigate the effects of environmental stress on gene expression and to elucidate response mechanisms in model plants such as *Arabidopsis* and rice under heavy metal stress at the proteome level (Semane et al., 2010; Lee et al., 2010). However, until now, there has not been any comparative proteomic report of the effects of NO on alleviating Cd-induced toxicity. Rice is an important crop

worldwide and is considered a useful model plant for genetic and molecular studies (Khan and Komatsu, 2004; Agrawal and Rakwal, 2006). In the present study, we aimed to analyze the protein expression profiles for the leaves and roots of rice seedlings that were treated with Cd or Cd + SNP for 8 days using two-dimensional gel electrophoresis (2-DE) combined with matrix-assisted laser desorption /ionization time-of-flight mass spectrometry (MALDI-TOF/T-OF MS).

Results

Nitric oxide alleviates rice growth inhibition under cadmium stress

As shown in Fig. 1, the length and dry weight of the shoots and roots first increased and then decreased with increasing SNP concentrations in the group of plantlets under Cd stress-free conditions. Under Cd stress, 0.005 mM, 0.05 mM and 0.1 mM SNP produced slightly positive effects with regard to alleviating Cd toxicity. Greater concentrations (0.2 mM SNP) were ineffective at reducing the toxic impacts of Cd stress. The concentration of 0.005 mM SNP exhibited the most significant alleviation effect on growth performance in the Cd-treated rice plantlets. Therefore, we selected a concentration of 0.005 mM SNP for the following experiments. To prove that it was specifically SNP-released NO rather than other substances from SNP decomposition that was responsible for the SNP-induced alleviation effect on Cd toxicity, Cd-treated rice was also treated with 0.005 mM potassium ferrocyanide and 0.005 mM sodium nitrate and nitrite (SNP analogues that do not release NO) simultaneously. As shown in Fig. 2, the Cd treatment significantly decreased the shoot length (22.7%), root length (15.5%), and shoot and root dry weight (19% and 20.3%, respectively) compared to the control (CK). NO treatment (Cd+S1) markedly alleviated the decreases in the lengths and dry weights of the shoots and roots. There were no significant differences in shoot and root lengths between the control and the seedlings with SNP alone. However, the SNP application did result in significantly higher shoot and root dry weights. The alleviating phenomenon was restricted by hemoglobin (Cd+S1+Hb). Moreover, neither application of 0.005 mM potassium ferrocyanide (Cd+Fe) nor 0.005 mM sodium nitrate and nitrite (Cd+N) generated NO and therefore had no obvious effect on the alleviation of heavy metal stress in rice plants.

Effect of nitric oxide on hydrogen peroxide concentration and lipid peroxidation

Lipid peroxidation in terms of MDA (a major TBARS) content was measured. Compared to CK, Cd treatment increased the MDA content by 25.6% and 29.5% in the rice leaves (Fig. 3A) and roots (Fig. 3B), respectively. NO treatment (Cd+S1) repressed the Cd-induced increase in MDA content. There were no significant differences that were observed between the SNP-treated plants and untreated plants. When hemoglobin was added to the NO treatment (Cd+S1), the repression effect was blocked. Addition of potassium ferrocyanide (Cd+Fe) or sodium nitrate and nitrite (Cd+N) had no significant impact on the MDA content under Cd stress. These results showed that NO significantly reduced the formation of MDA in response to Cd stress. Compared to CK, the Cd treatment considerably increased the H_2O_2 content by 30.4% and 30.3% in the rice leaves and roots, respectively. In contrast, the application of NO (Cd+S1) reduced the H_2O_2 content (Fig. 3C and D). Pretreatment with SNP minimized this reduction effect on the H_2O_2 content. Similarly, application of potassium ferrocyanide (Cd+Fe) or sodium nitrate and nitrite (Cd+N) had no

significant influence on the H_2O_2 content, and the effect caused by exogenous NO was also diminished through the addition of hemoglobin (Cd+S1+Hb).

Protein expressions changed in leaves and roots in response to nitric oxide under Cd stress

Physiological analysis indicated that low concentrations of SNP exerted significant alleviation on the seedlings' growth. Therefore, 0.005 mM SNP-treated and 0.05 mM SNP-treated rice plants were selected for further proteomic analysis. The 2D gels of the extracted proteins from the rice leaves and roots are shown in Fig 4. The results were analyzed with PDQUEST 8.0 software. Triplicate gels were obtained from three independent experiments. In total, 41 differentially expressed protein spots were analyzed by MALDI-TOF MS (Tables 1 and 2). Peptide sequence was provided as supplementary material (Supplementary Table 1 and 2).

Functional identification of differentially expressed proteins

The list of differentially expressed proteins in leaves and roots is summarized in Tables 1 and 2, respectively. In total, 16 differentially expressed proteins were identified in rice leaves (Fig. 5), and 14 out of the 16 protein spots were upregulated following Cd treatment (Fig. 6). After pretreatment with different concentrations of SNP, most proteins were downregulated. The identified proteins were involved in photosynthesis, carbohydrate metabolism, oxidative phosphorylation, oxidative stress responses, and the Calvin cycle. In total, 25 differentially expressed proteins were identified in the rice roots (Fig. 7). Fourteen of the 25 protein spots were upregulated, while 11 out of the 25 protein spots were downregulated (Fig. 8). After pretreatment with different concentrations of SNP, most of the proteins approached their endogenous levels. The identified proteins were involved in carbohydrate metabolism, oxidative phosphorylation, secondary metabolism, carbohydrate metabolism, signal transduction and cell division.

Discussion

NO response of differentially expressed proteins in rice leaves Energy-related proteins

In plants, ATP synthase, which is expressed in chloroplasts and is integrated into the thylakoid membrane, is a key enzyme in energy metabolism. It participates in oxidative phosphorylation during respiration. The proton transport of the respiratory electron chain is used to form a transmembrance force, which provides energy for material transport. Five identified enzymes from this experiment participate in energy metabolism. These enzymes include ATP synthase gamma chain 1 (spot 2106), ATP synthase beta chain (spot 2211), ATP synthase CF1 beta subunit (spot 3513), and ATP synthase CF1 alpha subunit (spot 4724, spot 5720). ATP synthase, a multisubunit complex protein localized in the membrane, is also a key enzyme during the ATP biosynthesis process. ATP synthase is composed of an integral membrane CFo portion and an extrinsic CF1 portion. The CFo is a hydrophobic protein complex that forms a transmembrane ion channel for the translocation of protons. The ATP synthase CF1 subunit catalyzes the formation of ATP from ADP and inorganic phosphate (Senior et al., 2002). ATP synthase plays essential roles in regulating the availability of short-lived proteins and in removing abnormal and damaged proteins. It has been reported that ATP synthase plays a key role in the removal of damaged proteins and in the fine control of

Table 1. Differentially regulated protein identified by MALDI-TOF-TOF in rice leaves.

| Spots ID	Protein name	Accession No.	Theoretical Mass/pI	Experimental Mass/pI	NMP	SC(%)	score	Biological function
306	hypothetical protein OsI_20474	gi|125552851	30.5/6.86	38.1/4.86	11	5	57	Carbohydrate metabolism
1413	Glyceraldehyde-3-phosphate dehydrogenase B	gi|108705994	34.0/4.99	41.6/5.13	62	20	209	Carbohydrate metabolism
1801	C1-like domain containing protein	gi|53370754	85.5/5.37	76.1/4.96	11	1	43	Calvin cycle
2106	putative ATP synthase gamma chain 1	gi|115472339	40.0/8.60	27.5/5.25	31	8	71	Photosynthesis
2211	ATP synthase beta chain	gi|108711272	37.4/5.03	32.8/5.30	21	7	34	Photosynthesis
2407	unnamed protein product	gi|115450493	47.5/6.22	41.4/5.24	54	12	315	Carbohydrate metabolism
3308	OSJNBa0036B21.24	gi|115458768	43.0/7.62	37.7/5.45	77	29	539	Carbohydrate metabolism
3506	monodehydroascorbate reductase	gi|4760483	43.0/5.36	47.6/5.42	31	10	140	Oxidative stress responses
3513	ATP synthase CF1 beta subunit	gi|11466794	54.0/5.47	45.3/5.46	32	10	191	Oxidative phosphorylation
4507	unnamed protein product	gi|115450493	47.5/6.22	42.3/5.59	77	23	393	Carbohydrate metabolism
4724	ATP synthase CF1 alpha subunit	gi|11466784	55.6/5.95	65.7/5.69	52	11	141	Oxidative phosphorylation
5720	ATP synthase CF1 alpha subunit	gi|11466784	55.6/5.95	65.0/5.80	43	9	210	Oxidative phosphorylation
6808	NADP dependent malic enzyme	gi|54606800	65.8/5.79	73.9/6.04	21	4	46	Photosynthesis
6902	putative phosphoglycerate mutase	gi|115440691	60.9/5.42	109.2/5.97	11	2	38	Photosynthesis
7202	OSJNBa0053K19.11	gi|115460656	44.3/8.53	34.9/6.18	32	8	102	Photosynthesis
7717	putative ferredoxin--nitrite reductase	gi|53792338	70.2/6.88	69.6/6.32	20	3	61	Oxidative stress responses
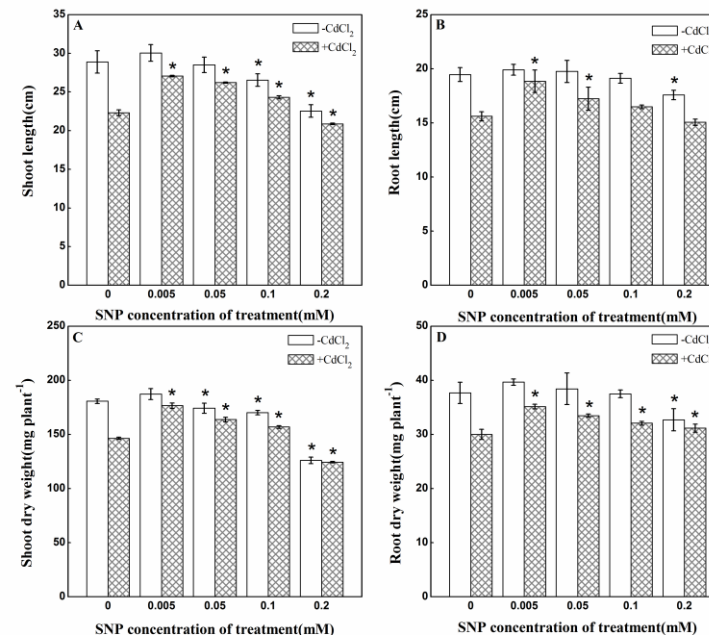
Fig 1. Effects of different concentrations of SNP on shoot length (A), root length (B), shoot dry weight (C) and root dry weight (D) of 8 days treatment rice plants grown in nutrient solutions without or with 0.1 mM CdCl$_2$. Asterisk represents significantly differences at P=0.05 level between rice plants treated without or with SNP.

certain key cellular components. Under Cd stress, the ATP synthase CF1 subunit was upregulated, which indicated that more energy was required to resist the Cd stress. ATP synthase gamma chain 1 and ATP synthase beta chain are involved in the synthesis of the ATPase. Compared with CK, the above five enzymes were upregulated by Cd stress, but they were downregulated with the application of NO. This indicates that plants must have enough energy to resist Cd stress.

Photosynthesis-related proteins

NADP-malic enzyme catalyzes the conversion of malate to pyruvate and CO_2, which is an important reaction during photosynthesis. Additionally, NADP-ME participates in many different metabolic pathways, and it has been confirmed that NADP-ME is involved in defense responses in plants (Drincovich et al., 2001). NADP-ME activity has been shown to increase under salt, osmotic and drought stress in rice, and overexpression of NADP-ME improved salt and osmotic tolerance in Arabidopsis (Liu et al., 2007; Ke et al., 2009). In this study, NADP-ME (spot 6808) was upregulated by Cd stress, which suggested that the enhanced expression of NADP-ME could also increase rice resistance to Cd. After application with NO, the NADP-ME levels approached endogenous levels. Spot 7202 was identified as aminomethyl transferase, which belongs to a family of glycine cleavage T-proteins that catalyze the catabolism of glycine (Okamura-Ikeda et al., 2005). Numerous studies have demonstrated that photorespiration could slow down the degradation of chlorophylls (Kozaki and Takeba, 1996). Photorespiration can increase Rubisco content (Muraoka et al., 2000), and it could prevent the reduction of electron carriers between PSI and PSII to ensure the normal transfer of electrons in the electron transport chain (Andrews and Baker, 1997). In this study, aminomethyl transferase, which eliminates the excess NADPH and ATP in the photosynthetic electron transport chain, was upregulated by Cd stress. Aminomethyl transferase was downregulated following the application of SNP. These results indicated that the expression of aminomethyl transferase might be related to the response of NO. Phosphoglycerate mutase catalyzes the conversion of glycerate-3-phosphate to glycerate -2-phosphate during glycolysis. This process provides protection for the generation of ATP. In the present study, phosphoglycerate mutase (spot 6902), which affects energy generation and photosynthesis, was downregulated by Cd stress. Increased expression of phosphoglycerate mutase with the application of SNP suggested that enough energy was supplied to the Cd-treated plants by the glycolytic pathway.

Carbohydrate metabolism

Spot 1413 was identified as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was up regulated by more than 2-fold following Cd treatment. Spots 2407, 3308 and 4507, similar to GAPDH, belonged to a tetrameric NAD-binding enzyme. The protein spots were also upregulated under Cd stress. GAPDH is a key enzyme during CO_2 fixation. During glycolysis, it reversibly catalyzes the conversion of fructose-1,6-bisphosphate to glyceraldehyde 3-phosphate and finally to 1,3-bisphosphoglycerate. GAPDH is involved in stress-responsive mitogen-activated protein kinase signaling and protects against the damage of reactive oxygen species to the plant (Danchenko et al., 2009). In this study, GAPDH was upregulated under Cd stress, which would change the metabolic flux from glycolysis to the pentose phosphate pathway. This process produces more NADPH for counteracting oxidative

stress (Ralser et al., 2007). With the application of SNP, the downregulation of GAPDH increased rice tolerance to Cd, which is positively correlated to the generation of spinosad. Phosphoglycerate kinase is a necessary enzyme for survival and is responsible for the phosphorylation of substrates that generate required energy. The first reaction of the Calvin cycle involves phosphoglycerate kinase utilizing ATP to phosphorylate 3-phosphoglycerate to form 1,3-bisphosphoglycerate. In this study, phosphoglycerate kinase (spot 306) was upregulated by Cd stress. The increased expression of the enzyme could indicate an increase in photosynthetic carbon assimilation under Cd stress. Following the application of SNP, phosphoglycerate kinase was downregulated.

Oxidative stress-related proteins

Ferredoxin-nitrite reductase (spot 7717) was downregulated by Cd stress in rice leaves. Nitrite reductase is a flavin protease that exists in the chloroplasts of higher plants to take advantage of the reduced iron oxygen protein transferring an electron to nitrite. Nitrite reductase is the enzyme of the iron porphyrin and requires Cu and Fe. Under Cd stress, the damage of the rice chloroplasts and the decrease of absorption of iron may damage and cause the degradation of the enzyme. With the application of SNP, the upregulation of nitrite reductase indicated that NO could alleviate the chloroplast damage and increase the absorption of the iron. Monodehydroascorbate reductase (MDHAR) plays an important role in maintaining the balance of reactive oxygen species (Leterrier et al., 2005). MDHAR is an enzyme in the ascorbate-glutathione cycle, the role of which is restoring monodehydroascorbate to ascorbate (ASA). ASA is a ROS scavenger in the intracellular glucose metabolism process. MDHAR (spot 3506) was upregulated by Cd stress, and it was also upregulated with the application of SNP, which indicated that the plant required more ASA to scavenge reactive oxygen species.

Signal transduction-associated protein

C1-like domain-containing protein is known to be involved in signaling pathways in a variety of organisms (Hurley et al., 1997). It is important as a signal transduction molecule in cells. In this study, C1-like domain-containing protein (spot 1801) was upregulated by Cd stress. The transmission of signals was impeded within the cell, and the cellular conditions became toxic. With the application of SNP, C1-like domain-containing protein was downregulated, which suggested that NO could regulate the expression levels of C1-like domain-containing protein to reduce the toxic effects of Cd in rice.

NO response of differentially expressed proteins in rice roots Energy-related proteins

ATP synthase, which is involved in oxidative phosphorylation and photophosphorylation, is widely present in the inner membrane of mitochondrial or chloroplast thylakoids. The ATP synthase β subunit catalyzes ATP synthesis and includes nucleotide binding sites. In the present study, the ATP synthase β subunit (spot 3404) was downregulated by Cd stress in the rice roots, which presumably affected the energy metabolism. With the application of SNP, the ATP synthase β subunit had no significant expression changes compared with the seedling treated with Cd. The ATP synthase Fo subunit 1 (spot 7703) is an energy-related protein that was upregulated in response to 0.005 mM SNP. The enhancement of the expression of this protein has been related to increased plant defenses against abiotic stress.

Table 2. Differentially regulated protein identified by MALDI-TOF-TOF in rice roots.

| Spot ID	Protein name	Accession No.	Theoretical Mass/pI	Experimental Mass/pI	NMP	SC(%)	score	Biological function
1320	Fructokinase II	gi|115474481	35.8/5.02	36.9/5.2	21	7	70	Carbohydrate metabolism
1327	5-methyltetrahydropteroyltriglutamate-hom ocysteine methyltransferase	gi|108862992	84.9/5.93	36.0/5.24	20	2	63	Nitrogen metabolism
1908	heat shock protein 90	gi|6863054	92.7/4.89	99.9/5.18	11	2	46	Heat shock proteins
1920	putative heat shock protein 82	gi|56202189	70.9/5.07	87.2/5.26	52	7	67	Heat shock proteins
2616	taxadienol acetyl transferase-like	gi|115438576	46.1/5.13	49.4/5.37	43	11	184	Secondary metabolism
2715	transferase family protein	gi|77551124	49.5/5.04	54.8/5.33	65	15	171	Other
2814	von Willebrand factor type A domain containing protein	gi|115488386	56.1/5.02	64.8/5.3	21	4	102	Other
3309	phosphoglycerate kinase	gi|114386664	42.2/5.64	37.7/5.46	32	10	157	Carbohydrate metabolism
3404	putative ATP synthase beta subunit	gi|56784991	45.9/5.33	38.7/5.44	66	20	586	Oxidative phosphorylation
3606	wheat adenosylhomocysteinase-like protein	gi|29367605	53.8/5.62	48.8/5.45	11	3	38	Other
3732	putative endo-1,3-beta-glucanase	gi|115442217	67.7/5.32	62.3/5.47	44	9	205	Carbohydrate Metabolism
3903	Cell division cycle protein 48	gi|115450773	90.4/5.12	100.9/5.44	41	5	57	Cell division
4502	hypothetical protein OsI_22156	gi|218197799	41.9/5.17	44.6/5.6	64	4	63	Stress responses
4914	putative oligopeptidase B	gi|115470116	84.7/5.77	87.4/5.76	30	19	322	Other
4916	orthophosphate dikinase	gi|2443402	104/5.98	99.3/5.76	20	2	43	Secondary metabolism
5318	putative PrMC3	gi|115434610	36.6/5.34	36.3/5.89	63	21	156	Other
5324	putative isoflavone reductase	gi|115434036	33.4/5.69	35.1/5.92	88	42	612	Secondary metabolism
5728	5-methyltetrahydropteroyltriglutamate-homocystei ne methyltransferase	gi|108862994	66.9/5.92	56.9/5.95	31	5	148	Nitrogen metabolism
5904	putative reductase	gi|115454943	82.1/5.86	84.1/5.9	53	11	237	Other
6515	5-methyltetrahydropteroyltriglutamate-homocystei ne methyltransferase	gi|108862994	66.9/5.92	45.3/6.07	20	3	51	Nitrogen metabolism
6522	putative cysteine conjugate beta-lyase	gi|115479507	50.9/6.69	46.3/6.1	22	6	58	Signal transduction
7703	ATP synthase F0 subunit 1	gi|89280711	55.5/5.85	56.4/6.15	66	17	238	Oxidative phosphorylation
7853	phenylalanine ammonia-lyase	gi|29367609	72.8/6.11	78.5/6.25	42	8	114	Secondary metabolism
7919	Sucrose-UDP glucosyltransferase 1	gi|115453437	93.3/5.94	88.5/6.25	72	9	218	Carbohydrate metabolism
8804	Phenylalanine ammonia-lyase	gi|115447403	76.0/6.07	75.7/6.31	43	7	169	Stress responses
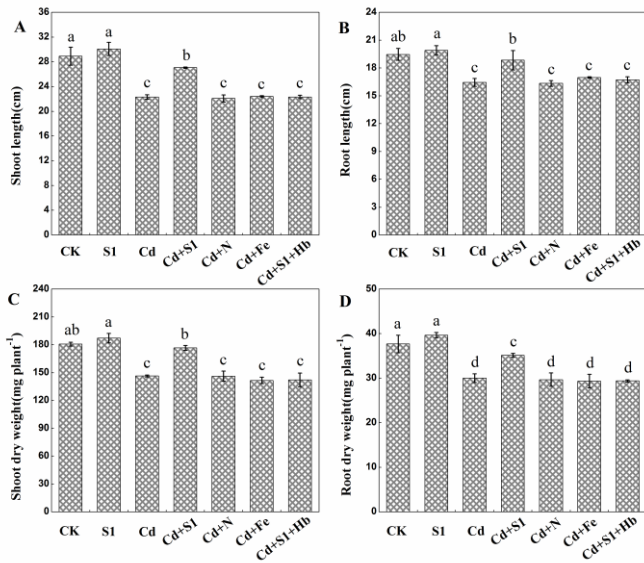
Fig 2. Effects of different treatments on shoot length (A), root length (B), shoot dry weight (C) and root dry weight (D) of 8 days treatment rice plants grown in nutrient solutions without or with 0.1 mM CdCl$_2$. Different letters are significantly different at the 0.05 level without or with 0.1 mM CdCl$_2$. Cd, 0.1 mM CdCl$_2$; S1, 0.005 mM SNP; Cd +S1, 0.1 mM CdCl$_2$+0.005 mM SNP; Cd +N, 0.1 mM CdCl$_2$+0.005 mM NaNO$_3$ + 0.005 mM NaNO$_2$; Cd +Fe, 0.1 mM CdCl$_2$+0.005 mM K$_4$Fe(CN)$_6$; Cd +S1 +Hb, 0.1mM CdCl$_2$ + 0.005 mM SNP + 0.1% Hb.
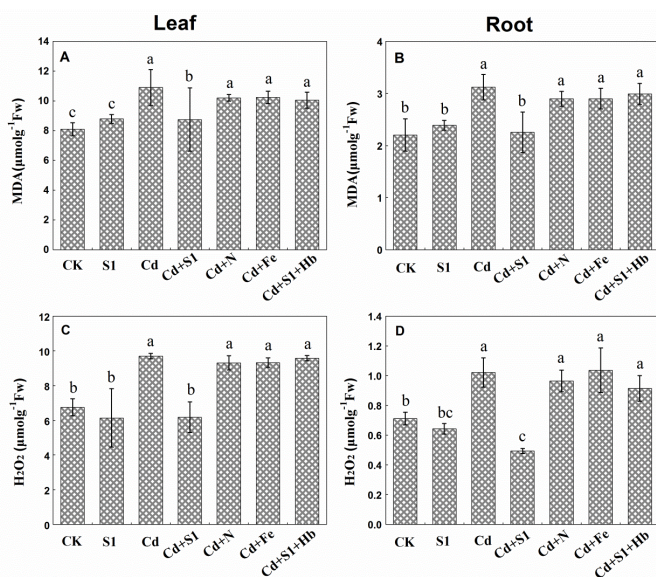
Fig 3. Effects of different treatments supply on MDA content (A), H$_2$O$_2$ content (C) in leaves and MDA content (B), H$_2$O$_2$ content (D) in roots of 8 days treatment rice plants grown in nutrient solutions without or with 0.1 mM CdCl$_2$. Different letters are significantly different at the 0.05 level without or with 0.1 mM CdCl$_2$. Cd, 0.1 mM CdCl$_2$; S1, 0.005mM SNP; Cd +S1, 0.1 mM CdCl$_2$ +0.005 mM SNP; Cd +N, 0.1mM CdCl$_2$ +0.005mM NaNO$_3$ + 0.005 mM NaNO$_2$; Cd +Fe, 0.1 mM CdCl$_2$ +0.005 mM K$_4$Fe(CN)$_6$; Cd +S1 +Hb, 0.1 mM CdCl$_2$ + 0.005 mM SNP + 0.1% Hb.

Carbohydrate metabolism

In plants, hexokinase plays a major role in regulating basal metabolism. Fructokinase catalyzes the phosphorlation of fructose and plays an essential role in hexose metabolism. It has been shown that the overexpression of hexokinase inhibits the growth and photosynthesis of tomato plants (Menu et al., 2004). Fructokinase II (spot 1320) was upregulated by Cd stress, which affected plant growth and development under abiotic stress by regulating carbohydrate metabolism. The expression of fructokinase II was decreased in the presence of SNP and Cd. The decrease may indicate a potentially decreased flux of carbohydrates to the citric acid cycle, which could contribute to WSC accumulation. The function of orthophosphate dikinase still remains enigmatic in C3 plants. Nevertheless, orth-ophosphate dikinase might be used for converting pyruvate phosphate into pyruvate, which promotes starch synthesis and aromatic amino acid biosynthesis (Chastain and Chollet, 2003). Orthophosphate dikinase was involved in the activation of nitrogen during leaf senescence in Arabidopsis (Taylor et al., 2010). Orthophosphate dikinase (spot 4916) was downregulated by Cd treatment. No significant change was observed following SNP treatment. Sucrose-UDP glucosyltransferase 1 is involved in catalyzing the decomposition of sucrose, which provides the substrate for the synthesis of the cell wall and regulates the synthesis of starch. In this study, sucrose-UDP glucosyltransferase 1 (spot 7919) was upregulated upon SNP treatment, which presumably provided UDPG for the formation of the root cell wall and allowed the normal growth and development of the rice roots.

Endo-1,3-beta-glucanase (spot 3732), a pathogenesis-related protein, was upregulated by Cd stress in rice roots. This enzyme has been implicated in not only a variety of physiological and developmental processes, but also in plant defenses against pathogens and stress (Akiyama et al., 2004). With the application of NO, endo-1,3-beta-glucanase experienced no significant changes under Cd stress in the rice roots. The increased expression of endo-1,3-beta-glucanase suggested that the defense pathway was activated by Cd treatment and offered more protection to the roots against Cd stress.

Nitrogen metabolism-related protein

The enzyme 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase (MHM) is critical during the biosynthetic pathway of methionine (Met). The deficiency of Met leads to impaired protein synthesis. Spots 1327, 5728 and 6515 were identified as MHM. With the application of SNP, MHM was upregulated, promoting the formation of Met.

Secondary metabolism-related proteins

Phenylalanine ammonia-lyase (PAL), which catalyzes the deamination of phenylalanine to trans-cinnamic acid and NH$_4$$^+$, is a key regulatory enzyme during phenylpropanoid metabolism (Wang et al., 2004). PAL is involved with both primary and phenylalanine metabolism. Compared with the Cd-treated seedlings, PAL (spots 7853 and 8804) was upregulated in the presence of SNP and Cd. This indicated that the synthesis of macromolecules was increased during the secondary metabolism for cell growth. In this study, isoflavone reductase
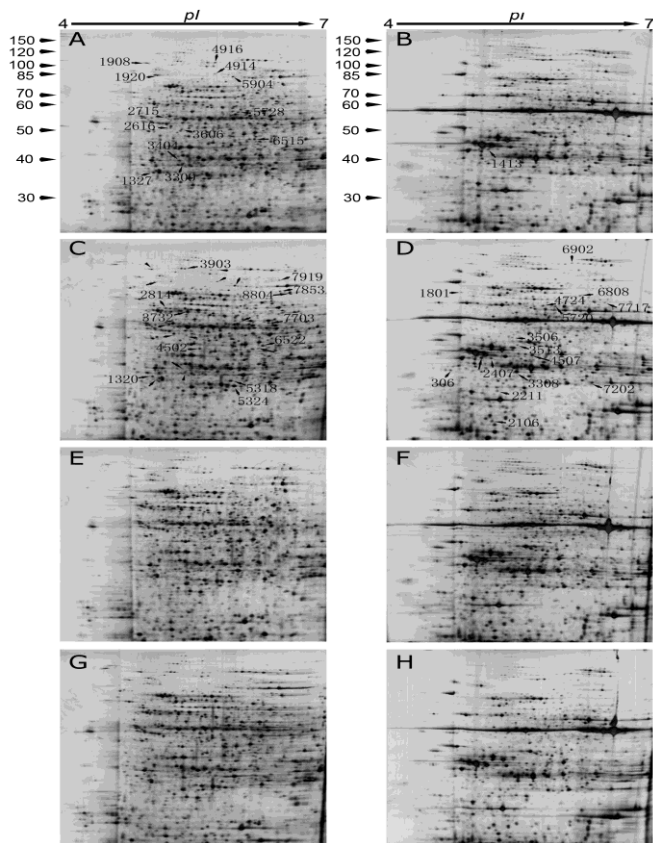
Fig 4. The 2-DE maps of proteins in rice leaves and roots under different treatment. Note: A-B, CK; C-D, 0.1 mM Cd; E-F, 0.1 mM Cd+ 0.005 mM SNP; G-H, 0.1 mM Cd+ 0.05 mM SNP A、C、E、G: 2-DE maps of rice roots (25 differentially expression proteins); B、D、F、H: 2-DE maps of rice leaves (16 differentially expression proteins).

Fig 5. 2-DE gels showing the changed expression of proteins affected by application of nitric oxide in rice leaves. Cd: 0.1 mM Cd; Cd+S1: 0.1 mM Cd+ 0.005 mM SNP; Cd+S2: 0.1 mM Cd+ 0.05 mM SNP.

(spot 5324) was upregulated by Cd stress. Isoflavone reductase has been shown to inhibit pathogen infection in rice (Kim et al., 2003). The cell walls in rice roots are damaged under Cd stress, which makes them susceptible to pathogen infection. Therefore, this damage induces the expression of certain proteins to increase the resistance of rice roots. Previous reports have demonstrated that isoflavone reductase was expressed in response to salt (Wen et al., 2010), drought (Salekdeh et al., 2002), and oxidative stress (Babiychuk et al., 1995). The expression of isoflavone reductase also changes in response to UV light irradiation, pathogen infection and wounding (Lers et al., 1998). Isoflavone reductase was slightly downregulated by SNP. Taxadienol acetyl transferase-like protein catalyzes the first acylation step in taxol biosynthesis (Kai et al., 2004). Paclitaxel induces cells to accumulae a large number of microtubules, which interferes with cell function, especially cell division. Taxadienol acetyl transferase-like protein (spot 2616) was downregulated by Cd stress. No significant change in the expression of this protein was observed following SNP treatment.

Oxidative stress-related proteins

Pathogenesis-related (PR) proteins are induced by biotic and abiotic stresses, and they are an important part of the plant defense system. Endo-1,3-beta-glucanase, a pathogenesis-related protein, is widely present in plants and hydrolyzes various types of β-glucan. Endo-1,3-beta-glucanase is the major cell wall component of fungal pathogens
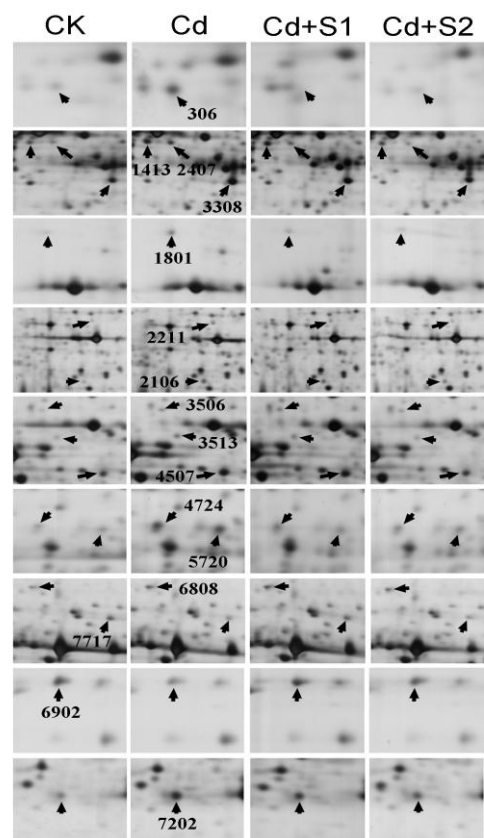
(Kauffmann et al., 1987). The β-1,3-glucanase protein is strongly regulated by environmental stresses such as UV, cold, and wounding. Endo-1,3-beta-glucanase (spot 3732) was up-regulated by Cd treatment, while it was slightly decreased following SNP treatment.
A hypothetical protein (spot 4502) belonging to the universal stress protein family was also identified.

Molecular chaperone

HSPs are expressed by stress stimulation in biological organisms. HSP synthesis is induced by conditions such as high temperature and heavy metal ions. HSP90 is an abundant, ubiquitous, and highly conserved molecular chaperone that is involved in modulating a multitude of cellular processes (Fan et al., 2011). In this study, HSPs were downregulated by Cd stress, while HSPs were upregulated by SNP treatment. HSP90 plays an important role in cell apoptosis inhibition and promoting cell survival. The upregulation of HSP90 helps assure the regulation of apoptotic signaling and mitochondrial stability.

Others

Wheat adenosylhomocysteinase-like protein is a key enzyme that regulates cellular methyl reactions. It catalyzes the conversion of S-adenosyl-L-homocysteine (SAH) to adenosine and homocysteine. S-adenosylmethionine is a methyl donor during gene methylation, which is catalyzed by SAH. S-adenosylmethionine methyltransferase activity is inhibited by
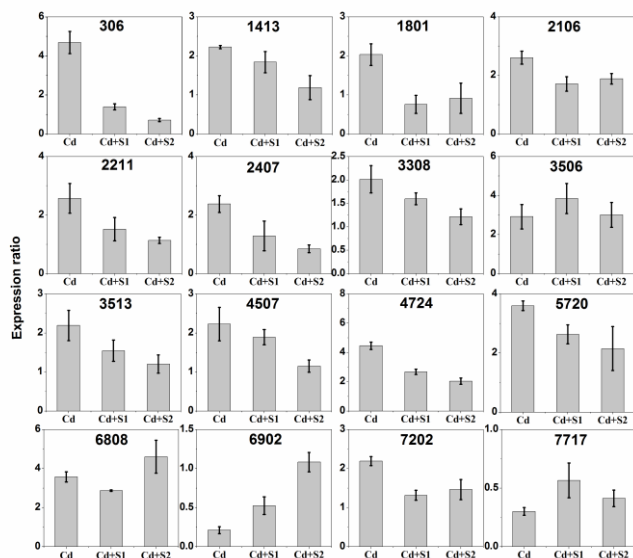
Fig 6. Relative expression ratio of altered proteins from rice leaves. Values are the means (±SD) of protein volumes from three independent experiments. The spot numbers are the same as those specified in Table 1.Cd: 0.1 mM Cd; Cd+S1: 0.1 mM Cd+ 0.005 mM SNP; Cd+S2: 0.1 mM Cd+ 0.05 mM SNP
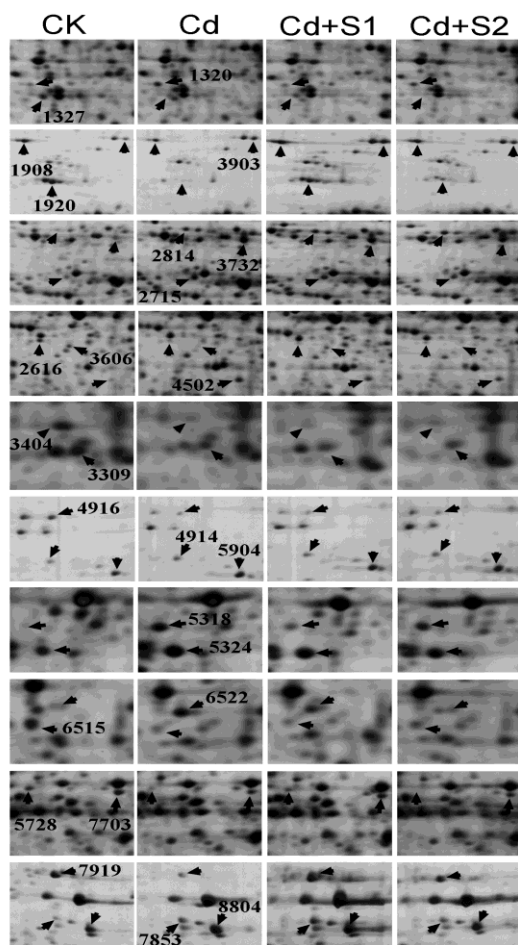

Fig 7. 2-DE gels showing the changed expression of proteins affected by application of nitric oxide in rice roots. Cd: 0.1 mM Cd; Cd+S1: 0.1 mM Cd+ 0.005 mM SNP; Cd+S2: 0.1 mM Cd+ 0.05 mM SNP

SAH, which serves to reduce gene methylation and regulate gene expression. In higher plants, the gene expression is related to DNA methylation. Wheat adenosylhomocysteinase-like protein catalyzes SAH hydrolysis, which is beneficial to SAH methyltransferase. Wheat adenosylhomocysteinase-like protein plays a similar role in the regulation of gene expression. Following the application of SNP, wheat adenosylhomocysteinase-like protein (spot 3606) was upregulated. PrMC3 hydrolyzes the esters of short-chain fatty acids (Marshall et al., 2003). However, its function is still unclear. It has been reported that esters produced by PrMC3 may negatively affect rice flavor and quality (Kim et al., 2009).

Materials and methods

Plant materials and treatment

Rice seeds (*Oryza sativa* L., cv. Xiushui63, from the Academy of Agricultural Sciences, Jiaxing, China) were surface sterilized with 2.5% sodium hypochlorite solution for 15 min and then rinsed thoroughly with sterilized water. These seeds were then germinated in Petri dishes with moist filter papers at 37 °C in the dark for 3 days. The uniformly germinated seeds were selected and transferred to 7-L plastic trays containing half-strength Yoshida's rice nutrient solution (Yoshida, 1975) during the first week and then the complete Yoshida's rice nutrient solution for the next 2 weeks. The nutrient solution consisted of 10 mg L^{-1} $NaH_2PO_4 \cdot 2H_2O$, 40 mg L^{-1} K_2SO_4, 40 mg L^{-1} $CaCl_2$, 40 mg L^{-1} $MgSO_4 \cdot 7H_2O$, 0.5 mg L^{-1} $MnCl_2 \cdot 4H_2O$, 0.05 mg L^{-1} $(NH_4)_6 \cdot Mo_7O_{24} \cdot 2H_2O$, 0.2 mg L^{-1} H_3BO_3, 0.01 mg L^{-1} $ZnSO_4 \cdot 7H_2O$, 0.01 mg L^{-1} $CuSO_4 \cdot 5H_2O$, 2.0 mg L^{-1} $FeCl_2 \cdot 6H_2O$ and 10 mg L^{-1} N (the concentration of N was regulated by NH_4NO_3) to sustain the growth of the rice plants. The pH of the nutrient solution was adjusted to 5.0 ± 0.2 with NaOH or HCl, and the nutrient solution was renewed every 2 days to maintain consistent concentrations. After 3 weeks, the rice seedlings were given 11 treatments as follows: (1) control, (2) 0.1 mM $CdCl_2$ (Cd), (3) 0.005 mM SNP (S1), (4) 0.05 mM SNP (S2), (5) 0.1 mM SNP (S3), (6) 0.2 mM SNP (S4), (7) 0.1 mM $CdCl_2$ + 0.005 mM SNP (Cd+S1), (8) 0.1 mM $CdCl_2$ + 0.05 mM SNP (Cd+S2), (9) 0.1 mM $CdCl_2$+ 0.005 mM $NaNO_2$ + 0.005 mM $NaNO_3$ (Cd+N), (10) 0.1 mM $CdCl_2$ + 0.005 mM $K_4Fe(CN)_6$ (Cd+Fe), or (11) 0.1 mM $CdCl_2$ + 0.005 mM SNP + 0.1% bovine hemoglobin (Cd+S1+Hb). In this experiment, the cadmium (Cd) toxic condition was applied as cadmium chloride ($CdCl_2 \cdot 2.5H_2O$), and its concentration (0.1 mM) was determined based on preliminary experiments. Hemoglobin was used as a specific NO scavenger, and sodium nitrite, sodium nitrate and potassium ferrocyanide were used as the controls of SNP decomposition. The experiment was performed at the Experimental Station of Nanjing Agricultural University (118.8ºE, 32.0ºN) with natural sunlight at 25-32 °C during the day and 19-26 °C during the night. The leaves and roots were harvested after 8 days of treatment, frozen in liquid nitrogen, and stored at -80 ºC. The experiment was conducted in triplicate for each treatment.

Growth analysis

After 8 days of treatment, the shoot and root lengths were measured. To measure the dry-weight (DW) biomass, the shoots and roots were dried at 105 °C for 30 min and then at 80 °C until a constant weight was reached.

Lipid peroxidation concentration

The tissue lipid peroxidation was determined by measuring

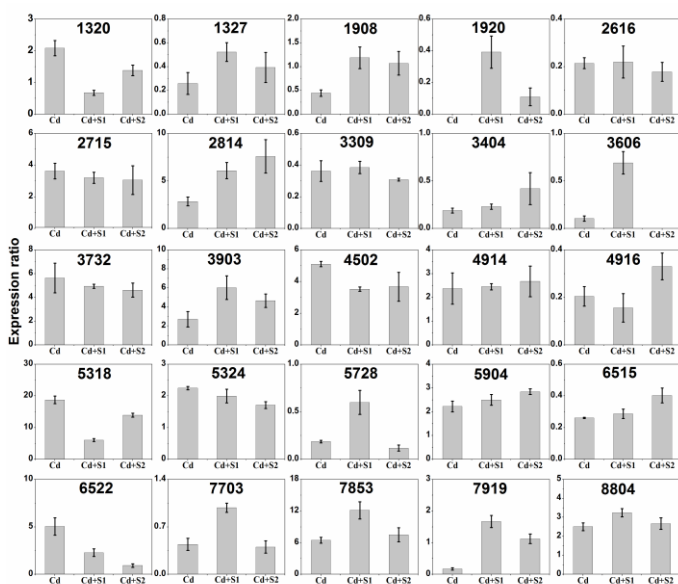**Fig 8.** Relative expression ratio of altered proteins from rice roots. Values are the means (±SD) of protein volumes from three independent experiments. The spot numbers are the same as those specified in Table 1. Cd: 0.1 mM Cd; Cd+S1: 0.1 mM Cd+ 0.005 mM SNP; Cd+S2: 0.1 mM Cd+ 0.05 mM SNP

malondialdehyde (MDA), a major thiobarbituric acid-reactive species (TBARS) and a product of lipid peroxidation (Heath and Packer, 1968). The tissue sample (0.1 g) was homogenized in 5 mL of TCA (0.1%, w/v), followed by centrifugation at 10,000 g for 10 min. To measure the MDA concentration, a 1-mL aliquot of the supernatant was mixed with 4 mL of 20% TCA that contained 0.5% TBA. The mixture was heated at 95 °C for 20 min, quickly cooled in an ice bath and then centrifuged at 10,000 g for 10 min at 4 °C. The absorbance of the supernatant was measured at 532 and 600 nm. The concentration of MDA was calculated using the MDA extinction coefficient of 155 $mM^{-1}cm^{-1}$. The MDA results are expressed as $\mu mol\ g^{-1}$ FW.

H_2O_2 determination

The concentration of H_2O_2 was determined using the following procedure with minor modifications (Patterson et al., 1984). First, fresh leaves and roots (0.5 ± 0.01 g) were homogenized in 3 mL of ice-cold acetone and centrifuged at 3,000 g for 10 min. Then, 5% Ti $(SO_4)_2$ was added to 1 mL of the extract and allowed to precipitate by adding 0.2 mL of 17 M ammonia solution per milliliter of extract. The precipitate was dissolved in 5 mL of 2 M H_2SO_4 after five consecutive washes with ice-cold acetone. The absorbance of the solution was measured at 415 nm, and the H_2O_2 concentration was calculated from a standard curve.

Protein extraction

Protein extraction was performed using the following method (Ding et al., 2011). Rice samples were finely ground in a mortar with liquid N_2 and then homogenized in an extraction buffer. The homogenate was suspended in ice-cold acetone that contained 10% trichloroacetic acid (TCA), 10 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride (PMSF) and incubated at -20 °C for 1 h, followed by centrifugation at 20,000 g for 30 min at 4 °C. The pellet was washed three times with ice-cold acetone that contained 10 mM DTT and 1 mM PMSF and incubated at -20 °C for 1 h before centrifuging at 20,000 g for 15 min at 4 °C. The pellet was dried under vacuum and resuspended in 200 µL lysis buffer (7 M urea, 2 M thiourea, 4% 3-[3-cholamidopropyl) dimethylammonio] propanesulfonic acid (CHAPS), 0.5% (v/v) immobilized pH gradient (IPG) buffer (pH 4-7 NL, Amersham Biosciences), and distilled water). The mixed sample was incubated overnight at 4 °C, centrifuged at 40,000 g for 15 min at 20 °C, and the resultant supernatant was used for 2-DE analysis. Protein concentrations were determined with the standard Bradford assay, using bovine serum albumin as the standard.

Two-dimensional gel electrophoresis analysis

Isoelectric focusing (IEF) was performed as described previously by Ding et al. (2011). The dried protein pellet was rehydrated in rehydration buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.5% (v/v) IPG buffer, 10 mM DTT and 0.1% bromophenol blue). A dry strip of PI 4-7 (17 cm) was loaded with 100 µg protein samples in a rehydration tray for 12 h at 20 °C. The IPG strips were run on a PROTEAN IEF apparatus (Bio-Rad). The IEF voltage was set at 500 V for 1 h, followed by 1,000 V for 1 h, a linear gradient increase to 8,000 V over 3 h and then 8,000 V for a total of 60,000 V h. After running the first dimension, the IEF strips were equilibrated for 15 min with an equilibrating buffer (6 M urea, 0.375 M Tris–HCl (pH 6.8), 20% (v/v) glycerol, 2% (w/v) SDS, and 1% (w/v) DTT) and then with another buffer (similar to the above buffer, with 2.5% iodoacetamide instead of DTT) for another 15 min. The second-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a Bio-Rad PROTEAN PLUS Dodeca cell at 200 V in the presence of 10% SDS. The protein spots in the analytical gels were stained with silver nitrate (Yan et al., 2000).

Protein visualization, image analysis and quantification

The stained gels were scanned using a VersaDoc4000 image system (Bio-Rad), followed by analysis of the protein spots with PDQUEST 8.0 software (Bio-Rad). For each sample, at least three 2-DE gels were performed. Only those with significant changes (P< 0.05) were considered for further analysis after a statistical analysis using Student's t-test. The target protein spots were automatically excised from the stained gels, dehydrated in acetonitrile, and dried at room temperature. The gel pieces were reduced with 10 mM DTT in 25 mM NH_4HCO_3 at 56 °C for 1 h. Then, 55 mM iodoacetamide/25 mM NH_4HCO_3 was added to the gel pieces, and the cysteine residues were alkylated in the dark for 45 min at room temperature. After washing with 25 mM NH_4HCO_3, 50% acetonitrile, and 100% acetonitrile, the dried gels were rehydrated with 2-3 µL of trypsin (Promega, Madison, WI, USA) solution. After the gel plugs were incubated at 37 °C for 10 h, trifluoroacetic acid (TFA) was added to quench the digestive reaction. Digestion was performed on 400 µm AnchorChips (Bruker Daltonics, Bremen, Germany) using a MALDI-TOF/TOF target plate together with a-cyano-4-hydroxycinnamic acid (HCCA). MALDI-TOF mass spectrometry and tandem TOF/TOF mass spectrometry were conducted on a time-of-flight AutoFlex III mass spectrometer (Bruker Daltonics, Bremen, Germany). A peptide tolerance of 150 ppm, fragment mass tolerance of ± 0.4 Da, and peptide charge of 1+ were selected when searching with MASCOT for protein hits. Only significant hits, as defined by the MASCOT probability analysis (p<0.05), were accepted. Peptides were

queried against the NCBInr protein database using the following search parameters: taxonomy of *Oryza sativa*, trypsin as the digestion enzyme, one allowed missed cleavage site, carbamidomethylation of cysteine and oxidation of methionine.

Statistical analysis

The experiments were performed using a randomized design. All results are presented as the mean ± standard deviation (S.D.) from three independent experiments. The results were subjected to ANOVA and the Tukey test using the statistical package SPSS 16.0. Differences between treatments were tested by the least significant difference (LSD) test at a 0.05 probability level.

Conclusion

In conclusion, NO plays an important protective role in alleviating Cd stress in rice plants. Few studies on the NO alleviation of Cd stress have been proposed. However, we present the following NO-responsive protein network in rice leaves and roots from this study (Supplementary Figure 1 and 2). In total, 41 differentially expressed proteins were successful identified, and these proteins were involved in carbon metabolism, oxidative stress, signal transduction, secondary metabolism, energy metabolism and other physiological processes. These results depict a panoramic view of the alleviating role of rice seedlings to Cd stress and deepen our understanding of the molecular basis of the NO response in plants.

Acknowledgements

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