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Research article

Proteomic analysis of pakchoi leaves and roots under glycine–nitrogen conditions

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ABSTRACT

The physiological and differential proteomic responses of pakchoi leaves and roots to glycine–nitrogen (Gly–N) treatments were determined. Two pakchoi (*Brassica campestris ssp. chinensis* L. Makino. *var. communis Tsen et Lee*) cultivars, '*Huawang*' and '*Wuyueman*', were grown under sterile hydroponic conditions with different N forms (Gly–N and nitrate–N). Gly–N-treated pakchoi exhibited decreased fresh weights, total N uptake, leaf areas, and net photosynthetic rates than those treated with nitrate–N. Differentially regulated proteins were selected after image analysis and identified using MALDI-TOF MS. A total of 23 proteins was up- or down-regulated following Gly–N treatment. These spots are involved in several processes, such as energy synthesis, N metabolism, photosynthesis, and active antioxidant defense mechanisms, that could enhance plant adaptation to Gly–N. The superior Gly tolerance of '*Huawang*' was predominantly associated with a less severe down-regulation of proteins that are involved in the electron transport chain and N metabolism. Other factors could include less ribulose-1,5-bisphosphate carboxylase/oxygenase turnover or a higher up-regulation of stress defense proteins. These characteristics demonstrated that maintaining ATP synthesis, N metabolism, photosynthesis, and active defense mechanisms play a critical role in pakchoi adaptation to Gly–N.

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1. Introduction

Nitrogen (N) is one of the most important nutrients required for plant growth. An increasing number of studies has shown that many terrestrial plants can absorb intact low-molecular weight organic N, such as that found in amino acids (Jones et al., 2005; Näsholm et al., 2009). In addition, the soluble organic forms of N are generally regarded as significant pools of N in the soil (Murphy et al., 2000), facilitating organic N to function as a N source for plants. Although the exact contribution of amino acid uptake to plant N nutrition remains ambiguous, studies on plant organic nutrition can enrich plant nutrition theory, promote the reasonable utilization of organic waste, and drive the sustainable development of agricultural production.

Despite the ability of plants to use a wide range of N forms, research on plant N nutrition has predominantly focused on inorganic N forms. Additionally, while most of the studies on plant organic N have mainly compared inorganic and organic N acquisition by plants in a wide variety of terrestrial ecosystems, very few researchers have actually investigated the post-uptake conversion of absorbed amino acids (Näsholm et al., 2009; Persson et al., 2006). Therefore, a deeper understanding of the fate of organic N compounds following uptake is necessary.

It has been reported that the molecular pathways for the uptake and assimilation of nitrate, ammonium, and amino acids in plant roots are distinct (Miller and Cramer, 2005). The different forms of N are expected to alter the abundance of enzymes and transporters required to utilize these forms of N compared with inorganic N. These changes also affect the uptake and final biochemical fate of inorganic N. Proteomic analysis is an effective method for studying protein responses to different N conditions. For example, the responses of the whole plant proteome to different nitrate levels have

Abbreviations: 2-DE, two-dimensional gel electrophoresis; CA, carbonic anhydrase; CAN, acetonitrile; CBB, Coomassie brilliant blue; CCS, copper chaperone; CHAPS, 3-[(3-cholamido-propyl)-dimethylammonio]-1-propanesulfonate; DTT, dithiothreitol; FNR, ferredoxin-NADP$^+$ reductase; GR-RBP, Gly-rich RNA-binding protein; GS, glutamine synthetase; IPG, immobilized pH gradient; MALDI-TOF, matrix-assisted laser desorption and ionization time-of-flight; MS, mass spectrometry; Pn, net photosynthesis rate; PR, putative pathogenesis-related protein; ROS, Reactive oxygen species; RSS, reactive sulfur species; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; SAMS, *S*-adenosylmethionine synthase isozyme; TCTP, translationally-controlled tumor protein homolog.

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been studied in maize (*Zea mays*) (Prinsi et al., 2009), barley (*Hordeum vulgar*) (Møller et al., 2011), and wheat (*Triticum aestivum*) (Bahrman et al., 2004). Proteomics has also been demonstrated to be a powerful approach for the identification of the metabolic pathways related to the glycine–N supply (Thornton et al., 2007). Glycine (Gly) is the most common amino acid used in plant uptake studies and is thought to be particularly important as a N source for plants because of its low-molecular weight, low carbon-to-N ratio, relatively rapid diffusion rates in soil, and the fact that it is not well utilized by soil microorganisms (Näsholm et al., 2009). The separation of proteins using two-dimensional electrophoresis (2-DE) allows for the analysis of the expression of Gly-induced proteins in plants, and may help to identify the roles of these proteins under a variety of physiological and environmental conditions. However, up to now, little information is available concerning the proteomic responses to amino acid–nitrogen in plants.

The aim of this study was to identify Gly-responsive proteins in the leaves and roots of pakchoi using 2-DE and mass spectrometry (MS). The differentially expressed proteins in Gly-tolerant and Gly-sensitive cultivars exposed to long-term Gly–N treatment were also compared. Two pakchoi (*Brassica campestris ssp. chinensis* L. Makino. *var. communis Tsen et Lee*) cultivars, cv. '*Huawang*' and cv. '*Wuyueman*', were selected as the subject material. Pakchoi is one of the most important vegetable crops in China. '*Huawang*' is considered an elite pakchoi cultivar because of its high yield, adaptability, and resistance, whereas '*Wuyueman*' is known for its late bolting capability. Our pre-experiment showed that pakchoi cv. '*Huawang*' was more Gly tolerant than '*Wuyueman*' (Wang et al., 2014). The Gly-tolerant cultivar '*Huawang*' may possess unique or highly up-regulated proteins that allow better adaptation to the Gly–N supply, and/or these plants may contain more proteins that can maintain their expression levels despite Gly–N treatment. The mineral NO_3^--N was chosen as a control, as it can be easily taken up and utilized by pakchoi (Luo et al., 2006).

2. Materials and methods

2.1. Plant materials and treatments

Seeds of pakchoi cv. '*Huawang*' and cv. '*Wuyueman*' were surface sterilized with 70% ethanol for 1 min, rinsed five times in sterile deionized water, and infiltrated with 10% H_2O_2 for 5 min. The seeds were then rinsed extensively with sterile deionized water and planted in plastic pots containing autoclaved quartz sand. Plants were grown in a controlled greenhouse under a 14 h/10 h light/dark cycle with natural sunlight of photosynthetically active radiation in the range of 300–800 μmol m^{-2} s^{-1}. The substrate was kept continuously moist by the addition of sterile water.

After germination for 7 d, the emerging seedlings were transferred to plastic containers containing nitrate–N or Gly–N nutrient solution. The Gly–N nutrient solution contained 5 mM Gly, 1 mM $MgSO_4$, 1 mM KH_2PO_4, 0.1 mM $CaCl_2$, 0.1 mM $NaFeEDTA \cdot 3H_2O$, 46.3 μM H_3BO_3, 1.37 μM $ZnSO_4 \cdot 7H_2O$, 0.32 μM $CuSO_4 \cdot 5H_2O$, and 0.05 μM $NaMoO_3 \cdot 2H_2O$. The NO_3^--N nutrient solution possessed the same contents as the Gly–N nutrient solution but replaced the 5 mM Gly with 5 mM KNO_3. Our pre-experiment indicated that pakchoi could grow well under 5 mmol L^{-1} N in each N form than at 2.5, 10.0, and 20.0 mmol L^{-1} (data not shown). This result was also consistent with the results obtained by Wang (2006). The K concentration was maintained at the same level in all treatments using K_2SO_4. The pH of the solution was adjusted to 5.8, and the nutrient solution was continuously renewed every 3 days. Plants were grown hydroponically at 25 °C in the Gly–N or nitrate–N nutrient solutions prior to the experiments.

2.2. Measurements of physiological parameters

Fresh weights and total N content were recorded every 5 d. The total N content was determined using a Vario ELIII elemental analyzer (Elementar, Germany). The leaf area, net photosynthesis rate (Pn), and total protein concentrations of the leaves and roots were also recorded at the 10 d treatment. The leaf areas were analyzed using Epson Scan and WinRhizo software (Regent Instruments Inc., Québec, Canada). The Pn was measured using the third true leaves of the plantlets after transplanting. Measurements were conducted during mid-morning (9:30 AM–11:30 AM) on clear days using a Ciras-2 portable photosynthesis system (PP-Systems, Amesbury, MS, USA) with an LED light source at 600 μmol CO_2 m^{-2} s^{-2} photosynthetically active radiation. The protein concentrations were determined using a 2-D Quant Kit (GE Healthcare, USA).

2.3. Protein extraction

After 10 d treatment, the leaves and roots were harvested, washed, immediately frozen in liquid N, and then kept at −80 °C. To investigate the variations in protein levels associated with the biological material and the experimental techniques, three independent N treatment experiments (biological replicates) and three protein extracts (technical replicates) from three independent aliquots of the pulverized plant material of each N treatment experiment were performed. One biological sample was obtained by pooling the fully expanded leaves of different ages or whole roots from three plants. Leaf tissue was sampled using a leaf punch along the veins on both sides of the leaf. Approximately 0.5 g of plant material was homogenized and ground in liquid N, then incubated with 10 ml of precipitation solution [10% *w*/*v* trichloroacetic acid and 0.2% *w*/*v* dithiothreitol (DTT) in acetone] for 2 h at −20 °C. The precipitated proteins were pelleted and washed with ice-cold acetone containing 0.2% DTT until the supernatant was colorless. The protein pellets were vacuum dried and solubilized in lysis buffer [7 M urea, 2 M thiourea, 4% (*w*/*v*) CHAPS, 40 mM DTT, and 2% (*v*/*v*) pharmalyte (pH 4–7)] and centrifuged (15,000 rpm, 4 °C, 15 min). The clear supernatants were stored in aliquots at −80 °C until analysis. The protein concentration was determined using a 2-D Quant Kit (GE Healthcare, USA).

2.4. 2-DE and gel staining

A total of 350 μg of protein was loaded onto 13 cm immobilized pH gradient (IPG) strips (pH 4–7) that were equilibrated with rehydration buffer [7 M urea, 2 M thiourea, 2% (*w*/*v*) CHAPS, 0.002% (*w*/*v*) Bromophenol G, 20 mM DTT, and 0.5% (*v*/*v*) pharmalyte (pH 4–7)] for 12 h and focused at 500, 1000, 8000, 8000, and 200 V for 4, 1, 3, 1, and 2 h, respectively, using an Ettan IPGphor || platform (GE Healthcare, USA). Two-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (2-DE) was conducted as described by Laemmli (1970) using 12.5% polyacrylamide gels. The gels were stained with colloidal Coomassie brilliant blue (CBB) G-250 as described previously in reference (Kim et al., 2008).

2.5. Image acquisition and data analysis

The gels were scanned using an image scanner equipped with the LABSCAN version 5.0 (GE Healthcare, USA). The scanned gels were saved as TIFF images for subsequent analysis. The gel images were analyzed using the Imagemaster 2D platinum 6.0 software (GE Healthcare, USA). To correct for the variability caused by staining, the abundance of each protein spot was estimated by the percentage volume (%vol). Only spots that expressed up-

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