Ecotoxicology and Environmental Safety 124 (2016) 255–266

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Proteomic analysis of chromium stress and sulfur deficiency responses in leaves of two canola (*Brassica napus* L.) cultivars differing in Cr(VI) tolerance

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ARTICLE INFO

Article history:
Received 8 June 2015
Received in revised form
17 September 2015
Accepted 20 October 2015

Keywords:
Brassica napus L.
Chromium
2-DE
MALDI-TOF/TOF MS
Proteomics
Sulfur

ABSTRACT

Sulfur (S) is an essential macronutrient for plant growth and development, and it plays an essential role in response to environmental stresses. Plants suffer with combined stress of S deficiency and hexavalent chromium [Cr(VI)] in the rhizosphere. Little is known about the impact of S deficiency on leaf metabolism of canola (*Brassica napus* L.) under Cr(VI) stress. Therefore, this study is the first to examine the effects of Cr(VI) stress and S deficiency in canola at a molecular level. A comparative proteomic approach was used to investigate the differences in protein abundance between Cr-tolerant NK Petrol and Cr-sensitive Sary cultivars. The germinated seeds were grown hydroponically in S-sufficient (+S) nutrient solution for 7 days and then subjected to S-deficiency (−S) for 7 days. S-deficient and +S seedlings were then exposed to 100 μM Cr(VI) for 3 days. Protein patterns analyzed by two-dimensional electrophoresis (2-DE) revealed that 58 protein spots were differentially regulated by Cr(VI) stress (+S/+Cr), S-deficiency (−S/−Cr) and combined stress (−S/+Cr). Of these, 39 protein spots were identified by MALDI-TOF/TOF mass spectrometry. Differentially regulated proteins predominantly had functions not only in photosynthesis, but also in energy metabolism, stress defense, protein folding and stabilization, signal transduction, redox regulation and sulfur metabolism. Six stress defense related proteins including 2-Cys peroxiredoxin BAS1, glutathione S-transferase, ferritin-1, L-ascorbate peroxidase, thiazole biosynthetic enzyme and myrosinase-binding protein-like At3g16470 exhibited a greater increase in NK Petrol. The stress-related proteins play an important role in the detoxification of Cr(VI) and maintaining cellular homeostasis under variable S nutrition.

1. Introduction

Chromium (Cr) is one of the most hazardous environmental pollutants, and increasing industrial pollution and urban activities result in the contamination of ecosystems (Shanker et al., 2005). The risk of Cr transfer into the food chain from the soil is significant owing to the process of uptake and accumulation by crop plants (Dube et al., 2003). Chromium exists in nature in both trivalent [Cr(III)] and hexavalent [Cr(VI)] forms (Panda and Patra, 1997). Cr(VI) is the most persistent form in the soil and is highly toxic to biota. Chromium toxicity leads to alterations in various enzymes related to nitrogen and carbohydrate metabolism, and photosynthesis (Prado et al., 2010; Gill et al., 2015). Plants have developed different strategies to cope with Cr(VI) toxicity by manipulating the expression level of stress-related genes (Xie et al., 2015). Therefore, identification of the proteins involved in plants' cell detoxification systems is a fundamental step for understanding responses to Cr(VI) stress. On the other hand, heavy metals cannot be degraded through any known biological process, thus there is a need for effective and environment-friendly remediation technology such as phytoremediation to restore the Cr contaminated areas (Pilon-Smits, 2005). For this reason, the molecular and physiological bases of Cr tolerance and accumulation in plant species tolerant to toxic levels of Cr(VI) have attracted considerable interest, recently.

Sulfur (S) is an essential element for plant growth because it is found in amino acids, glutathione, phytochelatins, vitamins, cofactors, and a variety of secondary products such as glucosinolates (Leustek, 2002). Since S is known to play an important role in heavy metal tolerance (Bashir et al., 2013), it is important to assess the severity of the impact of Cr(VI) under S-deficiency. It has been also suggested that Cr(VI) is actively transported through plasma membranes and seems to be mediated by transporters, which are primarily responsible for the uptake of sulfate (Skeffington et al., 1976; Schiavon et al., 2008). To date, transcriptomic studies focusing on the effects of Cr on sulfate transport in plants have

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http://dx.doi.org/10.1016/j.ecoenv.2015.10.023
0147-6513/

considered the effect of S nutrition on the variation of gene expression profiles. Additionally, Cr(VI) can influence not only sulfate transport, but also S assimilation (Schiavon et al., 2012). To further investigate the importance of the potential role of S metabolism in Cr tolerance, the interactions between S nutrition and Cr tolerance were considered in this study.

The ability of plants to respond to biotic and abiotic stresses is revealed by the interaction of various proteins. Identification of the functional genes or proteins involved in plant responses to heavy metal toxicity is important to understanding the molecular mechanisms of metal tolerance (Ahsan et al., 2009). Recently, transcript analysis has been revealed the expression patterns of genes under Cr stress and S deficiency conditions (Xie et al., 2015; Bielecka et al., 2015). Proteomics provides unique insights into biological systems which cannot be acquired from genomic or transcriptomic approaches. Proteomics has been used extensively to investigate protein expression patterns under heavy metal stress. Up to now, only few reports on the proteome alterations under Cr (VI) stress and S-deficiency have been published (Labra et al., 2006; Bah et al., 2010; Sharmin et al., 2012; D'Hooghe et al., 2013). In Cr(VI)-treated maize seedlings, differentially expressed 22 proteins included oxidative stress-related proteins and carbohydrate metabolism-related proteins (Labra et al., 2006). Analysis of root proteomes of *Miscanthus sinensis* showed that ion transporters, energy and nitrogen metabolism-related proteins, oxidative stress-related regulatory proteins might work together to establish a new homeostasis in response to chromium stress (Sharmin et al., 2012). D'Hooghe et al. (2013) reported that proteins involved in photosynthesis and carbon metabolism were modulated by S restriction. These authors also suggested that the maintenance of chlorophyll in S-limited conditions is related to an accumulation of water soluble chlorophyll binding proteins, involved in the protection of chlorophyll against reactive oxygen species. However, no proteomic study has been carried on plants in response to Cr(VI) stress under S-deficiency. Thus, this study would be new inputs to our understanding of the mechanism of Cr(VI) tolerance in relation to S metabolism.

Canola (*Brassica napus* L.) has greater S requirements than other crop species, making it particularly sensitive to S-deficiency (Blake-Kalff et al., 2000). Canola is also characterized by its fast growing, high biomass accumulation and remarkable resistance to heavy metal stress (Yu et al., 2012; Terzi and Yıldız, 2014). The priority of the current study is to gain basic information on the proteome changes in exposure to Cr(VI) and S-deficiency in Cr-tolerant NK Petrol and Cr-sensitive Sary canola cultivars to increase understanding of the molecular mechanisms involved in heavy metal tolerance in pursuit of advancements to phytoremediation. In this study, proteins were separated by two-dimensional electrophoresis (2-DE), quantified by digital analysis, and identified by MALDI-TOF/TOF (matrix assisted laser desorption ionization time-of-flight/time-of-flight) mass spectrometry, to gain new insights into the molecular mechanisms related to Cr(VI) tolerance and S availability. To our knowledge, this is the first comparative proteomic analysis of Cr(VI) and S-deficiency responsive proteins between canola cultivars differing Cr(VI) tolerance.

2. Materials and methods

2.1. Plant growth condition, sulfur and Cr(VI) stress treatments

In a previous study, canola (*B. napus* L.) cultivars NK Petrol and Sary were identified as Cr-tolerant and Cr-sensitive cultivars, respectively (Terzi and Yıldız, 2014). The seeds were allowed to germinate for 2 d in the dark at 23 ± 1 °C on filter papers saturated with distilled water. Ten uniformly germinated seeds were transplanted into 1 L plastic pots containing nutrient solution with the following composition (μM): 200 KNO_3, 200 $Ca(NO_3)_2$, 40 KH_2PO_4, 1000 $MgSO_4$, 10 $FeNa_2EDTA$, 4.6 H_3BO_3, 0.16 $CuCl_2$, 0.9 $MnCl_2 \cdot 2H_2O$, 0.09 $ZnCl_2$ and 0.01 $NaMoO_4 \cdot 2H_2O$ (Schiavon et al., 2008). The seedlings were then grown hydroponically in a growth chamber under controlled environmental conditions (constant temperature of 23 ± 1 °C, a 16 h photoperiod with photosynthetically active radiation at photon flux density of 260 μmol m^{-2} s^{-1}, and relative humidity of 60%) for 7 days. After this period, the seedlings were transferred to nutrient solutions supplied with a different level of sulfur supply: deficient (0 mMS; −S) and sufficient (1 mMS; +S). After keeping the seedlings in the two sets of S supply for 7 days, they were exposed to 100 μM Cr(VI) (supplied as potassium dichromate) under the above-mentioned conditions for 3 days. Preliminary experiments with Cr(VI) at 0–300 μM was carried out to determine the appropriate test concentration and duration. The exposure duration was limited to 3 days, because–S plants suffered too much toxicity and damage. The following four experimental treatments were formed: (1) control, 1 mM sulfate (+S/−Cr); (2) 1 mM sulfate and 100 μM Cr(VI) (+S/+Cr); (3) S-deficiency, 0 mM sulfate (−S/−Cr), and (4) combined stress, 0 mM sulfate and 100 μM Cr(VI) (−S/+Cr). In the S-deficient treatments, $MgCl_2$ was used instead of $MgSO_4$. The pH of the nutrient solution was adjusted to 6.0 ± 0.1 using 0.05 M KOH, and the nutrient solution was renewed on alternate days. The second and third leaves from the seedlings were harvested after 3 days of Cr(VI) stress application.

2.2. Protein extraction and 2-DE

Leaf proteins were extracted using a phenol extraction method (Hurkman and Tanaka, 1986) with modifications (Ahsan et al., 2008). Leaf tissues (1 g) were ground with a mortar in liquid nitrogen and homogenized in 5 mL of ice-cold Mg/NP-40 extraction buffer containing 0.5 M Tris–HCl (pH 8.3), 2% (v/v) NP-40, 20 mM $MgCl_2$, 2% (v/v) β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.7 M sucrose (Kim et al., 2001). The homogenates were then fractionated with an equal volume of Tris–HCl-saturated phenol (pH 8.0), followed by centrifugation at $3500 \times g$ for 15 min. After centrifugation, the top phenol phase was collected, and proteins were precipitated overnight by adding of four volumes of cold methanol containing 0.1 M ammonium acetate at −20 °C. The precipitated proteins were recovered by centrifugation at $3500 \times g$ for 10 min followed by three washes with cold methanol containing 0.1 M ammonium acetate. The protein pellet was air dried and dissolved in a lysis buffer containing 7 M urea, 2 M thiourea, 4% CHAPS (3-[(3-cholanidopropyl)dimethylammonio]-1-propane sulfonate), 0.2% biolytes (pH 3–10), and 40 mM DTT (DL-dithiothreitol). Protein concentration was determined using the Bradford assay (Bradford, 1976).

The protein extracts were subjected to two-dimensional polyacrylamide gel electrophoresis (2-DE). For analytical and preparative gels, 80 μg and 500 μg proteins were used, respectively. The protein samples were diluted with a 300 μL of rehydration buffer with 0.002% bromophenol blue, and were loaded into a focusing tray. Immobilized pH gradient (IPG) strips (17 cm with 4–7 linear pH gradient; Bio-Rad) were passively rehydrated for 16 h. Isoelectric focusing (IEF) was carried out using Protean® i12™ IEF System (Bio-Rad) at 20 °C with the following settings: 250 V for 30 min with a linear ramp, 10,000 V for 2 h with a gradual ramp, and finally 10,000 V for 60,000 V-h with a rapid ramp. After IEF, the strips were first incubated in equilibration buffer I (6 M urea, 2% SDS, 0.05 M Tris–HCl pH 8.8, 20% glycerol, and 1% dithiothreitol) for 15 min and then in equilibration buffer II (6 M urea, 2% SDS, 0.05 M Tris–HCl pH 8.8, 20% glycerol, and 2.5%