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Proteomic changes and molecular effects associated with Cr(III) and Cr(VI) treatments on germinating kiwifruit pollen

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ABSTRACT

The present study is aimed at identifying molecular changes elicited by Cr(III) and Cr(VI) on germinating kiwifruit pollen. To address this question, comparative proteomic and DNA laddering analyses were performed. While no genotoxic effect was detected, a number of proteins whose accumulation levels were altered by treatments were identified. In particular, the upregulation of some proteins involved in the scavenging response, cell redox homeostasis and lipid synthesis could be interpreted as an oxidative stress response induced by Cr treatment. The strong reduction of two proteins involved in mitochondrial oxidative phosphorylation and a decline in ATP levels were also observed. The decrease of pollen energy availability could be one of the causes of the severe inhibition of the pollen germination observed upon exposure to both Cr(III) and Cr(VI). Finally, proteomic and biochemical data indicate proteasome impairment: the consequential accumulation of misfolded/damaged proteins could be an important molecular mechanism of Cr(III) toxicity in pollen.

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1. Introduction

Heavy metals are major industrial pollutants leading to phytotoxicity. Chromium is a heavy metal whose valency state ranges from −2 to +6. Trivalent [Cr(III)] and hexavalent [Cr(VI)] forms are of major environmental significance because of their stability in the natural environment where they are released in the form of liquid, solid and gaseous wastes (Kotas and Stasicka, 2000). Cr(III) prevails in effluents from tanneries and paint factories, whereas the sources of Cr(VI) include metallurgy, mining, wood preservation, cooling installation effluents and fossil fuel combustion. The metal is also present in the atmosphere in the form of particles and droplet aerosols which represent the major mechanism for long-range transfer of chromium (Nriagu et al., 1988). Chromium is taken up and accumulated by plant roots and aerial surfaces. As a consequence, it interferes with several metabolic and physiological processes. This causes alterations in seed germination as well as root, stem and leaf growth (Shanker et al., 2005; Scoccianti et al., 2006). Chromium was demonstrated to interfere with essential structures and functions of the male gametophyte (Speranza et al., 2007, 2009). In fact, both Cr(III) and Cr(VI) exerted a strong dose-dependent inhibitory effect on kiwifruit pollen germination and tube growth and produced profound alterations in pollen tube shape (Speranza et al., 2007). In particular, Cr(III) had dramatic effects on tube wall morphology and major polysaccharide assembly as indicated by the significant alteration of callose deposition and arabinogalactan protein distribution (Speranza et al., 2009). Contrary to many other systems (Cervantes et al., 2001; Han et al., 2004), Cr(III) was more toxic to kiwifruit pollen than Cr(VI), and complete germination inhibition was attained at much lower Cr(III) doses. Both the chromium species induced increases in lipid peroxide production, with a more pronounced effect upon Cr(VI) treatment (Scoccianti et al., 2008a). This result is consistent with the well-known Cr(VI) role as an oxidizing agent. Previously, it was reported that Cr(VI) reduction to Cr(III) generates the whole spectrum of ROS (Kotas and Stasicka, 2000). Both GSH and GSSG content increased in the presence of increasing chromium concentrations, but glutathione pool dynamics appeared to vary depending on chromium species (Scoccianti et al., 2008a). Finally, chromium exposure of kiwifruit pollen resulted in a marked increase of ubiquitin adducts and a

Abbreviations: 2-DE, two-dimensional electrophoresis; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; Hepes, [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]; IEF, isoelectrofocusing; ACN, acetonitril; DMF, dimethylformamide; CBB, Coomassie brilliant blue; LC–ESI-MS/MS, liquid chromatography–electro spray tandem mass spectrometry.

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parallel reduction of free ubiquitin content. This suggests a role for the ubiquitin–proteasome 26S proteolytic system in the chromium stress response (Scoccianti et al., 2008b). This proteolytic pathway plays an important role in eukaryotic cell growth and development, as well as plant stress response and environmental adaptation through the degradation of short-lived and abnormal proteins (Belknap and Garbarino, 1996; Callis and Vierstra, 2000; Vierstra, 2003).

Heavy metals such as Pb^{2+}, Ga^{3+}, and Cu^{2+} enhanced proteasomal activity in human SH-SY5Y cells (Grunberg-Etkovitz et al., 2009). Furthermore, a proteasome alpha (α)-subunit was found to be over-expressed in the alga *Scytosiphon gracilis* (Phaeophyceae). This may help to remove damaged proteins resulting from copper-mediated oxidative stress (Contreras et al., 2010). Cadmium (Cd) increased the 20S proteasome activity in maize (Pena et al., 2007). By contrast, Cd impact caused an upregulation in the gene expression and proteolytic activity of the proteasome and various proteases in *Arabidopsis* (Polge et al., 2009).

In addition to metabolic changes, ultrastructural alterations including swelling and loss of mitochondrial cristae, cytoplasmic vacuolization, perturbed endoplasmic reticulum cisternae arrangement and chromatin condensation were also observed as early as 30 min after incubation in the presence of 50 μM Cr(III) and 150 μM Cr(VI) (Speranza et al., 2007). These features have been observed in plant cells, including pollen, undergoing programmed cell death (PCD) (Wuang and Cui, 1998; Coimbra et al., 2004; Geitmann et al., 2004; Varnier et al., 2005).

The heavy metal genotoxicity has been reported for various crops including blackgram, soybean, paddy, tobacco, potato and kiwifruit (Gichner et al., 2006; Sankar Ganesh et al., 2006; Chidambaram et al., 2009; Sundaramoorthy et al., 2010). At higher concentrations, both Cr(VI) and Cr(III) lead to DNA damage by inducing DNA fragmentation, single- and double-strand breaks, DNA–protein crosslinks and oxidative DNA base modifications (Burkhardt et al., 2001; Rudolf and Červinka, 2003; Xie et al., 2005; Chidambaram et al., 2006; Sundaramoorthy and Sankar Ganesh, 2007). These previous studies led us to investigate the pollen response to metal by verifying DNA fragmentation (laddering). This is one of the characteristic features of PCD that occurs in cells during normal development and in response to biotic and abiotic stresses such as pathogen attacks or toxic compounds (Varnier et al., 2005; Chidambaram et al., 2009; Serrano et al., 2010).

Abiotic stresses, including heavy metals, induce changes in plant protein expression (Cuypers et al., 2005; Amme et al., 2006; Ndimba et al., 2005). Proteomic changes in response to Cr(VI) toxicity have been recently reported in bacteria, in the freshwater green alga *Pseudokirchneriella subcapitata* and in the leaves of *Typha angustifolia* (Chourey et al., 2006; Kılıç et al., 2010; Vannini et al., 2009; Bah et al., 2010). However, up to now there is no comparative proteomic report on protein pattern changes during Cr(III) and Cr(VI) stress in plants cells. Proteomics of pollen development and germination have been extensively investigated (see for example Fernando, 2005; Sheoran et al., 2006; Dai et al., 2006, 2007; Zou et al., 2009), whereas information on heavy metal (in particular chromium)-responsive proteins in the male gametophyte is still missing. Therefore, in the present study we use a proteomic approach to identify specific changes in the protein pattern of kiwifruit pollen during Cr(III) and Cr(VI) exposure. The purpose was to obtain insights into the molecular mechanism of chromium toxicity to pollen.

2. Results and discussion

2.1. 2-DE analysis of chromium treated pollen

High resolution 2-DE was used to separate soluble proteins from kiwifruit pollen treated for 1 h with 30 μM Cr(III) or 90 μM Cr(VI). These experimental conditions were based on our previous data in this area (Speranza et al., 2007, 2009; Scoccianti et al., 2008a).

About 600 spots were highly resolved and detected by colloidal CBB staining, over a pH range of 4–7 and a size range of 10–100 kDa. All spots were matched by gel to gel comparisons and differences in the relative abundance (vol%) of each spot were analyzed. We focused our attention on spots whose abundance varied at least ±1.5-fold between the Cr-treated and non-treated pollen. Using this criterion, we selected 28 statistically confirmed protein variations between control and treated samples.

The differentially expressed proteins are marked on the representative 2-DE gel shown in Fig. 1. Excised spots were in-gel digested and analyzed by LC–ESI-MS/MS. In total, 18 proteins were successfully identified: in 10 cases it was not possible to determine the protein spot identity. Some representative images of spot changes as induced by the chromium-treatments are shown in Fig. 1. The predicted molecular masses and pIs for the majority of the identified proteins were generally consistent with the experimental data, as judged from the location of spots on 2-D gels; however, there were some exceptions. For example, spot 883 had an apparent molecular mass lower than the corresponding identified protein, whereas spots 1756, 883, 1430, 2556 and 980 had a molecular pI very different from the predicted value. These deviations in molecular mass and pI, as well as multiple spots for the same protein (551/554 and 1658/749) could be due to various factors, including alternative RNA splicing, post-translational modifications, and protein degradation. Ten proteins were up-regulated and eight down-regulated in chromium treated versus non-treated pollen. Seven protein spots exhibited significant changes under both Cr-treatments, while five and six proteins changed specifically only in the presence of Cr(III) or Cr(VI), respectively. The identified proteins were categorized into three functional groups based on predicted protein function.

2.2. Differentially expressed proteins were involved in stress response

Cr(VI) exposure led to the accumulation of three spots corresponding to a dehydroascorbate reductase (DHAR, spot 475) and a thioredoxin-dependent peroxidase (PRX, spots 551 and 554) (Table 1). DHAR catalyzes the reduction of DHA into ascorbic acid (AsA) using glutathione as the reductant. DHAR allows plants to recycle oxidized AsA. Increased DHAR activity was earlier reported in pea plants and green gram leaves exposed to Cr(VI) stress (Pandey et al., 2009; Shanker et al., 2004). The authors attributed the increased DHAR activity to a possible signal transduction mechanism operational due to increased ROS generation by Cr(VI). PRXs are known to exert a protective antioxidant role through their peroxidase activity. Proteomic analysis of maize seeds and roots and germinating rice seeds showed that PRX-type proteins were up-regulated by potassium dichromate, arsenic and copper treatment respectively (Labra et al., 2006; Requejo and Tena, 2005; Ahsan et al., 2007).

In Cr(III)-treated pollen, we also found the up-regulation of an electron transporter protein (spot 599) containing a classic thioredoxin domain with a redox active CXXC motif. Its function could be to alter the redox state of target proteins via the reversible oxidation of its dithiol active site.

The overexpression of all these proteins following Cr exposure is in agreement with the ROS production generated by Cr, causing oxidative damage to plants (Panda and Patra, 2000; Panda et al., 2003; Choudhury and Panda, 2005). The differential defensive response caused by Cr(VI) and Cr(III) could putatively be due to a higher ROS production by Cr(VI) (Stohs and Bagchi, 1995).

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