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Thiol-peptide level and proteomic changes in response to cadmium toxicity in *Oryza sativa* L. roots

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Abstract

In the present study, rice seedlings were exposed to a range of Cd concentrations (0.1 μM, 1 μM, 10 μM, 100 μM and 1 mM) for 15 days and a combination of different molecular approaches were used to evidence Cd effects and to assess the plants' ability to counteract metal toxicity. At a macroscopical level, only the highest Cd concentration (1 mM) caused a complete plant growth inhibition, whereas the lowest concentrations seemed to stimulate growth. At genome level, the amplified fragment length polymorphism (AFLP) technique was applied to detect DNA sequence changes in root cells, showing that all the Cd concentrations induced significant DNA polymorphisms in a dose-dependent manner. Data also evidenced the absence of preferential mutation sites.

Plant responses were analysed by measuring the levels of gluthatione (GSH) and phytochelatins (PCs), the thiol-peptides involved in heavy metal tolerance mechanisms. Results showed a progressive increase of GSH up to 10 μM of Cd treatment, whereas a significant induction only of PC_3 was detected in roots of plants exposed to 100 μM of Cd. As suggested by the proteome analysis of root tissues, this last concentration strongly induced the expression of regulatory proteins and some metabolic enzymes. Furthermore, the treatment with 10 μM of Cd induced changes in metabolic enzymes, but it mainly activated defence mechanisms by the induction of transporters and proteins involved in the degradation of oxidatively modified proteins.

Keywords: AFLP; Cadmium; Gluthatione; *Oryza sativa* L.; Phytochelatin; Two-dimensional electrophoresis

1. Introduction

Cadmium (Cd) is a toxic, widespread heavy metal, classified as a human carcinogen by the International Agency for Research on Cancer in 1993 (IARC, 1993; Beyersmann and Hechtenberg, 1997; Waalkes, 2000). Cd is easily absorbed by plants and it can be bioconcentrated through the food chain (Dudka and Miller, 1999). Since the diet is the main source of exposure to cadmium for the general population (Wagner, 1993; Tsukahara et al., 2003), intensive researches have been performed on the accumulation of cadmium in edible plant tissues (Stalikas et al., 1997; Kashem and Singh, 2001).

The effects of this toxic metal on plant and plant defence systems have also been investigated and reviewed by different authors (Prasad, 1995; Das et al., 1997; Sanità di Toppi and Gabbrielli, 1999). Cadmium is a non-essential element for plants and the most evident symptoms of its toxicity are chlorosis and stunting. Chlorosis seems to be the result of the effects of Cd on the uptake, transport and use of several elements (Ca, Mg, Fe, Mn, Cu, Zn, P and K), with the consequent reduction of Mn and Fe absorption and changes in Fe:Zn ratios (Das et al., 1997; Baryla et al., 2001). On the other hand, reduction of plant development seems to be the result of Cd interference with several important physiological processes: Cd alters the hormonal balance (Poschenrieder et al., 1989) and disturbs the plant water status through a decrease of water absorption, reduction of root hydraulic conductivity into xylem vessels, decrease of transpiration rate and increase of stomatal resistance (Barcelo and Poschenrieder, 1990; Vassilev and Yordanov,

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1997). At the cellular level, Cd causes turgor loss and cell expansion inhibition by decreasing the cell wall elasticity (Barcelo and Poschenrieder, 1990; Prasad, 1995), also causing damage to the photosynthetic apparatus (Siedlecka and Krupa, 1996; Vassilev et al., 1997). Several authors demonstrated that Cd binds the sulphydryl groups of proteins – leading to activity inhibition or to structure disruption – or displaces essential elements, resulting in deficiency effects (Van Assche and Clijsters, 1990; Hall, 2002).

A very important aspect of Cd toxicity is its induction of oxidative stress (Inzé and Van Montagu, 1995; Shah et al., 2001), displayed as an increase in lipid peroxidation, disruption of membrane integrity, oxidative modifications of proteins and DNA damage (Romero-Puertas et al., 2002). Chromosomal aberrations, micronucleus formation and chromatin fragmentation in Cd-treated plants have been reported by several authors (Koppen and Verschaeve, 1996; Panda et al., 1996; Fojtová and Kovarík, 2000). Because of oxidative stress, an increase in peroxidase and hydrolytic enzyme activity have often been observed in Cd-treated plants, representing signals of premature senescence (Prasad, 1995). To this regard, an increase in ethylene production and a reduction of soluble protein content in response to Cd have been shown in different plant species (Fuhrer, 1982; Kevresan et al., 1998; Hsu and Kao, 2003a).

Plants have evolved different mechanisms of defence against cadmium stress: they are able to avoid metal toxicity through metal binding to the cell wall, by reducing transport across the cell membrane and by active efflux (Cumming and Taylor, 1990; Li et al., 2002; Hall, 2002). Plants can also tolerate the presence of Cd by chelation of the metal ions and compartmentalisation into the vacuole (Salt et al., 1998). Metal-binding is a very important mechanism and is the most widely studied in the analysis of plant responses to Cd stress: heavy metals can be chelated by organic acids such as citric and malic acid (Rauser, 1999; Clemens, 2001), by metal binding proteins (MBP; Prasad, 1995) such as metallothioneins and also by metal binding complexes (MBC) such as phytochelatins (PCs) (Cobbet, 2000). In particular, PCs complex Cd ions through the thiolic group of Cys and the PC–Cd complexes are accumulated in the vacuole through the activity of ABC transporters, thus limiting the circulation of free Cd^{2+} inside the cytosol (Sanità di Toppi and Gabbrielli, 1999).

Furthermore, under Cd stress, several proteins, with a function in the protection and repair of proteins are expressed, such as the heat shock proteins (Hsp; Vierling, 1991). Pathogenesis-related (PR) proteins have also been reported to be induced by heavy metals (Hensel et al., 1999).

Rice (*Oryza sativa* L.), a plant of remarkable economic and alimentary importance (http://www.fao.org/rice2004), is often used as a model for monocotyledons in the laboratory. The advanced knowledge on its genome makes rice a good system for the study of Cd toxicity and plant responses (Izawa and Shimamoto, 1996; Karlowski et al., 2003).

The main aspects investigated have been the uptake of cadmium, its distribution in the plant's different organs and the possible consequences on human health (Wagner, 1993; Kashem and Singh, 2001; Liu et al., 2003).

In rice, Cd leads to the activation of antioxidant enzymes (SOD, CAT, POD and GR) and to the induction of GSH and PC (Klapheck et al., 1994; Yu et al., 2000; Shah et al., 2001). The effects of Cd on sugar metabolism and in protein and amino acid content of rice seedlings are also well documented (Shah and Dubey, 1997/1998; Verma and Dubey, 2001; Hsu and Kao, 2003a).

Moreover, many papers focus on important physiological aspects: for example, Llamas et al. (2000) investigated the effects of Cd^{2+} on transmembrane electrical potential difference, respiration and membrane permeability, whereas Hsu and Kao (2003b) recently proposed a role for ABA in the Cd tolerance of rice. A study on the changes in the rice leaf protein pattern in response to Cd exposure have shown a drastic effect on the photosynthetic apparatus (Hajduch et al., 2001).

Although there are a large number of reports, many aspects of plant's responses to Cd toxicity are still unknown. Moreover, according to Chen et al. (2003), the researches mainly focus on the influences of Cd on the above-ground parts of the plant and little is known about cadmium toxicity to the root system—the plant organ directly exposed to soil and water pollutants.

In the present work, the toxic and genotoxic effects of Cd on rice were first evaluated, by measuring dry weight, as an index of plant growth and detecting DNA sequence alteration by AFLP technique. The plant response was then analysed by measuring GSH and PC levels as these act as common mechanisms of plant tolerance to cadmium. Finally the specific changes in root protein pattern induced by cadmium were detected through bi-dimensional electrophoresis.

2. Materials and methods

2.1. Plant material and treatment conditions

O. sativa L. seeds cv. Baldo (Consorzio Agrario, Milan, Italy), after removal of the glumes, were surface sterilized in 70% ethanol for 2 min, washed in sterile water and placed in a water solution containing 5% chlorine and 0.01% Triton (Sigma–Aldrich, St. Louis, MO, USA) for 30 min on a shaking plate. Seeds were then rinsed in sterile water at least three times.

Seeds were germinated in Petri dishes supplemented with solid MS medium (2.15 g/l) including vitamins, 10% sucrose and 0.8% Plant Agar (Duchefa, Haarlem, The Netherlands), pH 5.8. After 3 days, seedlings were transferred to sterile glass pots containing solid MS medium supplemented with cadmium sulphate ($3CdSO_4{\cdot}8H_2O$, Sigma–Aldrich) at the final concentrations of: 0.1 μM, 1 μM, 10 μM, 100 μM and 1 mM. According to Sanità di Toppi and Gabbrielli (1999), these concentrations can be defined as very low, low, moderately high, high and very high, respectively. For each treatment, five different pots with 15 plantlets each were prepared and placed in a growth room (11 h dark at 19 °C/13 h light at 23 °C; 85% relative humidity; 150 $\mu mol\ m^{-2}\ s^{-1}$). After 2 weeks, the plant material was used for shoot and root growth determination, for the analysis of DNA sequence alteration by AFLP technique, for PC and GSH level determination and for root protein analysis.