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Responses of wheat seedlings to cadmium, mercury and trichlorobenzene stresses

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Abstract

The molecular response of wheat (*Triticum aestivum* L., cv. Yangmai 13) seedlings to heavy metal (Cd, Hg) and 1,2,4-trichlorobenzene (TCB) stresses were examined by two-dimensional gel electrophoresis, image analysis, and peptide mass fingerprinting. The results showed inhibitions of root and shoot growth by Cd, Hg, and TCB. These stresses led to water deficit and lipid phosphorylation in the seedling which also promoted protein phophorylation in the leaves. Hg stress inhibited protein synthesis while Cd and TCB stresses induced or up-regulated more proteins in the leaves. Most of these induced proteins played important roles in the biochemical reactions involved in tolerance of wheat to Cd and TCB stresses. The primary functions of Cd- and TCB-induced proteins included methionine metabolism, Rubisco modification, protein phosphorylation regulation, protein configuration protection, H^+ transmembrane transportation and also the synthesis of ethylene, defense substances and cell wall compounds.

Key words: wheat; proteomics; chemical pollutant; stress response

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Introduction

Chemical pollution has been a worldwide problem as a result of rapid increase in chemical inputs in agricultural production in the past decades. Most of chemical pollutants in soil and water cause disturbances in crop growth and development, and subsequently decreased crop productivity. Recent reports on the toxic effects of heavy metals (especially Cd) of wheat indicate that heavy metals inhibit root and shoot growth (Liu and Zhang, 2007; Cao *et al*., 2007), and also induce oxidative stress and lipid peroxidation (Singh *et al*., 2008). Cavallini *et al*. (1999) also reported that certain heavy metal ions (such as Hg) tend to form covalent bonds with DNA. Various defense mechanisms adopted by wheat to avoid heavy metal toxicity have been reported by several researchers, these include alteration of antioxidant enzyme level (Liu *et al*., 2007; Lin *et al*., 2007; Yannarelli *et al*., 2007), increase in the content of phytochelatin (Sun *et al*., 2005; Lindberg *et al*., 2007), and also increased generation of polyamine and ethylene (Groppa *et al*., 2003).

As compared to heavy metals, reports on the toxic effects of 1,2,4-trichlorobenzene (TCB) on crops are limited (Wang *et al*., 2006; Ge *et al*., 2007, 2008), and with no report on wheat. The present study reports the molecular responses of wheat to Cd, Hg, and TCB stresses, the physiological toxicity of Cd, Hg, and TCB in wheat, and the Cd-, TCB-induced proteins in wheat leaves.

1 Materials and methods

1.1 Seedlings cultivation, treatment and toxicity assay

Seeds of wheat (*Triticum aestivum* L.) variety Yangmai 13 were surface-sterilized in 3% (*V/V*) H_2O_2 for 5 min and then rinsed with deionized water before germination on moist filter paper at 30°C for 3 d. Seedlings were transferred into colored vitreous pots containing 100-mL Hoagland nutrient solution, and grown in a growth chambers with regulated day/night temperatures 25/18°C and the light intensity range 250–300 μmol/(m^2·s). Roots at three-leave stage seedlings were submerged in the test solutions containing 0.25, 0.5, 0.75, 1.0 mmol/L $CdCl_2$, 0.0125, 0.025, 0.0375, 0.05 mmol/L $HgCl_2$, and 0.014, 0.028, 0.042, 0.056 mmol/L TCB. Treatment combinations of the three chemicals were chosen based on preliminary studies in our laboratory (unpublished). Solutions were renewed daily within the 5 d treatment period to maintain their concentrations.

The inhibitory effects of Cd, Hg, and TCB on wheat seedlings were determined by root and shoot dry weight after 5 d treatment period. The levels of lipid peroxidation in wheat leaves were measured in terms of malondialdehyde

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(MDA) content as reported by Dhindsa *et al*. (1980), and MDA content expressed in μmol/g fw (fresh weight). All measurements were in three replicates with data presented in means ± standard deviations (SD). Statistical analyses were performed using SPSS statistical package software (version 10.0). Comparisons between control and other treatments were evaluated by one-way ANOVA and the least-significant-differences (LSD) test.

1.2 *In vitro* protein phosphorylation analysis

Fresh leaf tissues weighing 0.5 g each were sampled from seedlings treated with 0.5 mmol/L Cd and 0.056 mmol/L TCB for 5 d. These samples were homogenized in three volumes of extraction buffer (containing 50 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L EDTA, 5 μmol/L Na_3VO_4, 2 μmol/L Okadaic acid, 1 mmol/L PMSF). The homogenates were centrifuged at 12000 ×*g*, 4°C for 10 min. The soluble protein contents were determined by the Coomassie Brilliant Blue Method.

About 20 μL soluble protein was added to the reaction mixture containing 20 mmol/L Tris-HCl pH 7.5, 20 mmol/L β-glycerol phosphate, 10 mmol/L $MgCl_2$, 10 μmol/L Na_3VO_4, 0.5 μCi [γ-^{32}P] ATP. The reaction mixture was incubated at 30°C for 30 min, and phosphorylation reactions were stopped by adding protein loading buffer with 3 min boiling. Proteins in the reaction mixture were separated by SDS-PAGE (12.5% polyacrylamide). The gels were stained in Bio-safe colloidal Coomassie Blue G-250 (Bio-Rad Company, USA), and dried over a filter paper. Phosphorylated protein bands were detected by autoradiography using Kodak medical X-ray film (Machado *et al*., 2002).

1.3 Proteomic analysis

1.3.1 Protein extraction

Proteins from the leaves treated with 0.5 mmol/L Cd, 0.05 mmol/L Hg, and 0.056 mmol/L TCB for 5-d were extracted using polyethylene glycol (PEG) fractionation (Xi *et al*., 2006). All fractions of extracted proteins were rinsed with ice-cold acetone containing 0.07% (*V/V*) β-mercaptoethanol, then air-dried and stored at –20°C.

1.3.2 Two-dimensional gel electrophoresis

Above extracted proteins of F3 and F4 fractions were resuspended in rehydration buffer (Bio-Rad Company, USA) and incubated for 1 h at room temperature. After centrifugation at 12000 ×*g* at 4°C for 10 min, protein concentration in the supernatant was measured by Coomassie Brilliant Blue Method. IPG strip (Bio-Rad) of 11 cm (pH 4–7) was rehydrated in rehydration buffer (containing 400 μg protein sample) for 16 h to allow proteins to be uptaken. Iso-electric focusing (IEF) was performed using the PROTEAN IEF system (Bio-Rad Company, USA) at 20°C. Prior to second dimension analysis, the strips were equilibrated for 15 min in the equilibration buffer 1 (Bio-Rad), and equilibrated for 15 min in the equilibration buffer 2 (Bio-Rad). Protein separation of the second dimension was carried out on a 12.5% SDS-PAGE. After 2-DE separation, the gels were washed twice (5 min each) with double distilled water, and transferred into Bio-safe colloidal Coomassie Blue G-250 staining for 8 h. After which the gels were washed three times (1 h each).

1.3.3 Image analysis

Gels were scanned by Gel Doc XR system (Bio-Rad, USA). The protein spots were analyzed by PDQuest 2-D analysis software (Bio-Rad). The molecular weight and isoelectric point of differential proteins were calculated. After analysis, the selected protein spots were manually excised from the gel and stored at –20°C.

1.4 Identification of Cd- and TCB-induced proteins

The peptide mass fingerprints of Cd- and TCB-induced proteins were analyzed by mass spectrometer (MALDI-TOF MS, Applied Biosystem, USA) according to "Proteins and Proteomics: A Laboratory Manual" (Simpson, 2003). The peptide mass fingerprints were used for searching in MSDB, NCBInr or Swiss-Prot database using MASCOT software search engine (http://www.matrixscience.com) to identify Cd- and TCB-induced proteins. In order to evaluate protein identification, we considered that the protein scores must be significant ($P < 0.05$).

2 Results

2.1 Toxicity of Cd, Hg, and TCB to wheat seedlings

Figure 1 shows the effects of Cd, Hg, and TCB on the dry weight of roots and leaves of wheat seedlings compared to the control. The dry weights of both root and shoot were generally lower under stress conditions than in the control. There were highly significant reduction ($P < 0.01$) of both root and shoot dry weight at 1.0, 0.05, and 0.056 mmol/L for Cd, Hg, and TCB, respectively. The result showed 5.1% and 13.7% reduction at 0.5 and 1.0 mmol/L Cd for shoot dry weight and by 6.1% and 18.4% for root dry weight at 0.5 and 1.0 mmol/L Cd after 5 d treatment. TCB decreased shoot (or root) dry weights from 0.014 (or 0.028) to 0.056 mmol/L, while a concentration as low as 0.0125 of Hg decreased the shoot and root dry weights significantly ($P < 0.05$). These results confirm the inhibitory effects of Cd, Hg, and TCB on the growth of wheat.

The MDA contents were consistently higher in the treated leaves compared to treated roots. MDA from 0.196 μmol/L in the control roots increased to 0.338, 0.256 or 0.262 μmol/L in 1.0 mmol/L Cd, 0.05 mmol/L Hg or 0.056 mmol/L TCB treated roots (Fig. 2). Furthermore, MDA accumulation in the leaves was also significant ($P < 0.05$) at 0.5, 0.75 mmol/L Cd, 0.025, 0.0375, 0.05 mmol/L Hg and 0.028, 0.042, 0.056 mmol/L TCB. The results confirm that Cd, Hg, and TCB stresses caused lipid peroxidation in wheat seedlings.

2.2 Effect of Cd and TCB on protein phosphorylation in wheat leaves

Five phosphorylated proteins from the leaf extracts of