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# Integrated physiological and proteomic analysis reveals underlying response and defense mechanisms of *Brachypodium distachyon* seedling leaves under osmotic stress, cadmium and their combined stresses

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ABSTRACT

Drought stress, a major abiotic stress, commonly occurs in metal-contaminated environments and affects crop growth and yield. In this study, we performed the first integrated phenotypic, physiological, and proteomic analysis of *Brachypodium distachyon* L. seedling leaves under polyethylene glycol (PEG) mock osmotic stress, cadmium ($Cd^{2+}$), and their combined stresses. Combined osmotic and $Cd^{2+}$ stress had more significant effects than each individual stress on seedling growth, and the physiological traits and ultrastructures of leaves. Totally 117 differentially accumulated protein (DAP) spots detected by two-dimensional difference gel electrophoresis (2D-DIGE) were identified, and representing 89 unique proteins under individual and combined stresses. These DAPs were involved in photosynthesis/respiration (34%), energy and carbon metabolism (21%), stress/defense/detoxification (13%), protein folding and degradation (12%), and amino acid metabolism (7%). Principal component analysis (PCA) revealed that DAPs from the $Cd^{2+}$ and combined stresses grouped much closer than those from osmotic stress, indicating $Cd^{2+}$ and combined stresses resulted in more changes to the leaf proteome than osmotic stress alone. Protein-protein interaction analyses showed that a 14-3-3 centered sub-network could play important roles in responses to abiotic stresses. An overview pathway of proteome metabolic changes in Bd21 seedling leaves under combined stresses is proposed, representing a synergistic responsive network and underlying response and defense mechanisms.

*Significance:* Drought stress is one of the major abiotic stresses, which commonly occurs in metal-contaminated environments, and affects crop growth and yield performance. We performed the first integrated phenotypic, physiological and proteomic analysis of *Brachypodium distachyon* L. seedling leaves under drought (PEG), cadmium ($Cd^{2+}$) and their combined stresses.

## 1. Introduction

Drought, cadmium (Cd), and their combined stresses are common, particularly in metal-contaminated environments, and they always affect crop growth and yield [1–3]. The plant response to drought stress or osmotic stress is a complex process that involves morphological, physiological, and biochemical changes [4–7]. It normally involves a mixture of stress avoidance and tolerance responses that vary with plant species and genotype [4,8]. Meanwhile, the pollution of soils by heavy metals is an ever-growing problem throughout the world, and is the result of human activities as well as geochemical weathering of rocks and other environmental causes such as volcanic eruptions, acid rain, and continental dusts. Heavy metals discharged onto farmlands directly lead to excessively high levels in crops, a serious problem for agriculture [9]. The uptake of heavy metals not only constrains crop yields but can be a major hazard to human health and the entire ecosystem [10]. Metal-rich soils often have poor structure with low microbial activity and low organic matter, resulting in a low water-holding capacity.

Among these heavy metals, Cd is of great concern because of its severe phytotoxic effects on plants, as they can take up Cd ions easily, and accumulates in grains, thereby entering the food chain and contributing to bioconcentration [10–11]. Cd poisoning can lead to serious health problems in humans, such as itai-itai disease [12]. According to

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many previous studies, the contamination of Cd in rice is serious, particularly in China [9,13–14]. Cd induces several toxicity symptoms in plants, including the inhibition of whole plant and cell growth as well as photosynthesis, by interfering with different steps such as acting on photosystem II (PSII) or plastoquinone, or interfering with Rubisco activation in Calvin cycle [15].

When plants suffer from drought or osmotic stress, photosynthesis is reduced either through stomatal closure or metabolic impairment, and changes in mitochondrial respiration and photosynthetic electron transport lead to the generation of highly toxic reactive oxygen species (ROS) such as superoxide and peroxides, which cause chemical damage to DNA and proteins and produce serious effects on cellular metabolism [16–17]. To date, the molecular basis of heavy metal stress and tolerance in wheat has been investigated by numerous proteomic studies and supplemented by a rapidly advancing understanding of genomics [18–23]. In one study, treatment of wheat plants with heavy metals resulted in less dry matter, grain yield, nitrogen in tissues, and protein in grains [18]. In another study that exposed wheat seedlings to increasing Cd concentrations in a nutrient solution, the lengths of roots and shoot-leaves as well as shoot-leaf biomass progressively decreased [19]. Treatment of *Triticum durum* with municipal solid waste compost and sewage sludge at high doses ($\geq 300\ ha^{-1}$) leads to a substantial reduction in growth, concomitant with increasing heavy metal levels [20]. Then, permine in wheat leaves may play certain antioxidant functions by protecting the tissues from metal-induced oxidative damage [21], and $H_2O_2$, lipid peroxidation and malondialdehyde (MDA) levels significantly increased at higher Cd concentrations [22–23]. However, most of these studies involved only a single stress treatment, and little is known about the underlying molecular mechanisms of plant responses to a combination of abiotic stresses [24]. Therefore, it is highly important to understand plants' synergistic response mechanisms to multiple abiotic stresses, particularly under dual stresses such as osmotic stress and heavy metal stress.

*Brachypodium distachyon* L. has emerged as a valuable experimental model for studying small-grain cereals due to its small genome (272 Mb), short life cycle (8–12 weeks), small stature (15–20 cm), diploid accessions, self-fertility, and simple growth requirements [25–26]. Genome sequencing and analysis of *B. distachyon* accession Bd21 were completed in 2010. This revealed that *Brachypodium* is much more closely related to wheat and barley than to rice, sorghum, or maize [26–28], making it an attractive model for molecular, genetic, and proteomic studies of those species, which have much larger genomes are thus more complicated to study. In the present study, we performed the first integrated phenotypic, physiological, and proteomic analysis of Bd21 seedling leaves under osmotic stress, Cd, and their combined stresses.

## 2. Materials and methods

### 2.1. Plant materials and stress treatments

Seeds of *Brachypodium distachyon* L. (Bd21), kindly provided by Dr. John Vogel, USDA-ARS, Albany, CA, were surface-sterilized in 5% sodium hypochlorite for 5 min, and rinsed 4 times in sterile distilled water. Seeds were submerged in water for 12 h at room temperature, and then transferred to wet filter paper for 24 h to germinate at room temperature (22–25 °C). The uniformly germinated seeds were selected to grow in plastic pots containing Hoagland solution that was changed every two days. At the three leaf stage, the seedlings were treated by control (initial nutrient solution, and the osmotic potential (ψs) was − 0.044 MPa determined by a vapor pressure osmometer (Wescor Vapro 5520, USA)), osmotic stress (15% (w/v) polyethylene glycol PEG6000, (ψs) = − 0.562 MPa), $Cd^{2+}$ (500 μM $CdC1_2$) and their combination (15% PEG6000 + 500 μM $CdC1_2$) in three biological replicates. After 1 day to 7 days treatments, the physiological indicators from different stress treatment times were measured. Then, the remaining leaf samples were kept frozen in − 80 °C for later RNA and protein extraction and proteome analysis.

### 2.2. Phenotypic performance and physiological parameter measurements

The phenotypic traits of the Bd21 seedlings were profiled, including the length of the third leaf, plant height, the fresh weight and dry weight of whole seedling. Then, chlorophyll contents (chlorophyll *a* and *b*) were measured according to the previously described method [29] with minor modifications. Chlorophyll was extracted from fresh leaves (0.5 g) with 5 mL of 95% alcohol. The homogenate was filtered by filter paper and the filtrate was saved and pooled together to a final volume of 25 mL with 95% alcohol. The absorbance of the extract was taken at 663 nm and 645 nm for chlorophyll *a* and *b* measurement, respectively, using an Ultrospec 3100 Pro (GE Healthcare). The concentrations of chlorophyll *a*, chlorophyll *b*, and total chlorophyll content were calculated using Arnon's equations [29]. Malondialdehyde (MDA) and soluble sugar contents were determined according to Zhang et al. [30] with minor modifications. Leaf samples (0.5 g fresh weight) were homogenized in 1 mL 10% trichloroacetic acid (TCA) and then 4 mL 10% TCA was added for further grinding and centrifuged for 10 min at 4000*g*. A volume of 1 mL of supernatant sample was combined with 2 mL 0.6% thiobarbituric acid (TBA) and incubated in boiling water for 15 min, and then quickly cooled in an ice bath. The mixture was centrifuged at 10,000*g* for 5 min and absorbance of supernatant was measured at 532, 600 and 450 nm. The content of soluble sugars ($C_1$) (mmol $L^{-1}$) and MDA ($C_2$) (μmol $L^{-1}$) was calculated by the following formulae (D532, D600 and D450 represent the absorbance in the wavelengths of 532, 600 and 450 nm, respectively): $C_1 = 11.71 \times D450$, $C_2 = 6.45 \times (D532 - D600) - 0.56 \times D450$. Then, leaf free proline content was measured according to Bates et al. [31] with minor modifications. Approximately 0.5 g of leaf samples was homogenized with 5 mL of 3% (w/v) aqueous sulfosalicylic acid solution. The homogenate was centrifuged at 3000*g* for 20 min. The supernatant, acid–ninhydrin agent and glacial acetic acid (2 mL each) were mixed and boiled for 1 h. The reaction mixture was extracted with 4 mL toluene. Then the homogenate was centrifuged at 3000*g* for 10 min. Absorbance at 520 nm was determined using L-proline as a standard. Proline content was expressed in micrograms per gram per unit fresh weight. Betaine content was measured by using reagent kit from the Suzhou Comin Biotechnology Co., Ltd. of Jiangsu Province, China (Art. No. TCJ-1-G), and the experiments were conducted in strict accordance with the manufacturer's instructions. Three biological replicates were used to minimize experimental error.

### 2.3. Transmission electron microscope (TEM) observation

TEM observation of leaf ultrastructures was performed according to the method recently reported by Bian et al. [32]. The small pieces of sample from tips of top middle section of the fully expanded third leaf (1 mm × 2 mm) of each treatment after 4 days were used. After fixation, samples were dehydrated in a series of graded alcohol and propylene oxide and embedded in resin and polymerized in thermostatic chamber at 37 °C (12 h), 45 °C (12 h), 60 °C (48 h). Ultrathin sections (70–90 nm in thickness) were generated using a Lycra UC6 ultramicrotome and stained with 2% uranyl acetate for 30 min followed by lead citrate for 15 min. Then the sections were examined on a Hitachi H7500 TEM at 100 kV.

### 2.4. Protein extraction, 2D-DIGE, image acquisition and data analysis

Leaf total proteins from 2 days and 4 days treatments with three biological replicates of each treatment were extracted according to the method of Lv et al. [33]. Each sample was extracted three times, and protein concentrations were determined with a 2D-Quant-Kit (GE Healthcare, USA). The differentially accumulated protein (DAP) spots