Effects of Cadmium Stress on Alternative Oxidase and Photosystem II in Three Wheat Cultivars

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The effects of Cd stress (200 μmol/L, 8 days) on respiration and photosynthesis of three wheat cultivars were investigated: Chuanyu 12 (CY12), Chuanmai 42 (CM42), and Chuanmai 47 (CM47). Fifteen-day-old seedlings were exposed to 200 μmol/L $CdCl_2$ for 4 days and 8 days, respectively. The results indicated that Cd was accumulated largely in roots, but little in leaves of all three cultivars. CY12 accumulated the highest level of Cd in roots and showed the weakest resistance. On the contrary, the other two cultivars, CM42 and CM47, adapted better to Cd stress, and their thiobarbituric acid-reactive substances (TBARS) contents were lower than in CY12, but the chlorophyll contents and water contents were higher than in CY12. Additionally, Cd stress prompted the alternative oxidase (AOX) activity and upregulated the cyanide-resistant respiration in CM42 and CM47 after 8 days; no such induction was observed for CY12. The CO_2 assimilation rate, leaf stomatal conductance and chlorophyll fluorescence were inhibited by Cd stress in all cultivars, but more severe in the CY12 cultivar. Western blots indicated that the content of the photosystem II proteins LHCII and D1 decreased in CY12, but did not change in CM42 and CM47. While the content of the mitochondrial AOX protein increased markedly in CM42 and CM47, it did not in CY12. These results suggested that AOX and LHCII could be regarded as indicators of plant's resistance to heavy metals.

Key words: Alternative Oxidase, Cadmium Stress, Light-Harvesting Complex II, Wheat

Introduction

Cadmium (Cd) is one of the most toxic environmental pollutants in the atmosphere, soil and water, and in excessive amounts it can cause serious problems to all organisms (Ekmekci *et al.*, 2008). Plants can accumulate Cd during plant growth, and Cd has numerous negative effects on plant cells, such as membrane distortion, changes in nitrogen metabolism, disturbance of water status, production of toxic metabolites and reactive oxygen species (ROS) (Perfus-Barbeoch *et al.*, 2002; Krantev *et al.*, 2008; Meng *et al.*, 2009). Furthermore, it has been noticed that Cd accumulates mainly in roots but not in leaves (Tiryakioglu *et al.*, 2006). Cadmium tolerance in plants was thought to involve internal metal detoxification processes as well as physiological responses to this heavy metal, and can vary in the same species even among cultivars and populations, depending on genotypic and ecotypic differences (Lux *et al.*, 2004).

To date, most studies dealing with the effects of Cd contamination on plants examined the influence on the antioxidant capacity (Lin *et al.*, 2007). Whereas less information is available on respiration and photosynthesis, a variety of biotic and abiotic stress conditions have been shown to negatively impact the cytochrome pathway and induce alternative oxidase (AOX) (Mizuno *et al.*, 2008). Previous studies also showed that heavy-metal ions would reduce the efficiency of photosynthesis by inhibiting the key enzymes (ribulose-1,5-bisphosphate carboxylase, phosphoenol-pyruvate carboxylase) of the Calvin cycle. Photosystem II (PSII) is extremely sensitive to Cd, and its func-

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tion is inhibited to a much greater extent than that of PSI (Mallick and Mohn, 2003). In addition, damages to PSII are mostly localized at the D1 protein (Pagliano *et al.*, 2006; Komayama *et al.*, 2007; Murata *et al.*, 2007). However, little is known about the metabolic changes of the major functional proteins and the corresponding transcripts of PSII as well as the changes of the alternative respiratory pathway in mitochondria with cadmium stress, especially in different plant cultivars.

In our study, respiration and photosynthetic characteristics of three wheat cultivars with different cadmium stress resistance were studied at both RNA level and protein level. We proved the assumption that LHCII (light-harvesting complex II) a key family of PSII proteins, and AOX could be used as functional markers for stress tolerance in plant breeding to identify genotypes with high stabilities under various conditions.

Material and Methods

Plant growth and stress treatments

Three cultivars, Chuanyu 12 (CY12), Chuanmai 42 (CM42), and Chuanmai 47 (CM47), of wheat (*Triticum aestivum* L.) were used in the experiments. Seeds were germinated under dark conditions at 25 °C on humidified carbasus with distilled water for 4 d. The seedlings of cultivars were planted on vermiculite and watered with 1/2 Hoagland's solution with 16 h of light (approx. 100 μmol m^{-2} s^{-1}) at 25 °C, 8 h of dark at 20 °C, and 70% relative humidity. For Cd stress, fifteen-day-old seedlings were transferred to three 500-mL beakers containing 1/2 Hoagland's solution and 200 μmol/L $CdCl_2$ for 4–8 d. Control plants were grown in 1/2 Hoagland's solution and all samples were treated under the above conditions.

Estimation of Cd content in wheat roots and leaves

Wheat samples were dried in an oven at 80 °C until constant weight, then weighed and ground to fine powder with a waring blender. After digested with HNO_3, the Cd amount was determined using a flame atomic absorption spectrophotometer and expressed on the basis of the dry weight.

Measurement of absolute water contents

Approx. 0.5 g fresh leaves, determined as fresh weight (FW), were dried in an oven at 105 °C to constant weight (DW). The absolute water content in leaves was determined as the ratio of $[(FW - DW)/FW] \cdot 100\%$.

Measurement of thiobarbituric acid-reactive substances (TBARS)

The TBARS content was determined according to Sun *et al.* (2006) with some modifications. Approx. 0.5 g of fresh leaves were cut into small pieces and homogenized by addition of 5 mL 5% trichloroacetic acid (TCA) in an ice bath. The homogenate was transferred into a tube and centrifuged at 1,000 × *g* for 10 min at 4 °C. Aliquots of supernatant and 0.5% thiobarbituric acid (TBA) in 20% TCA were added into a new tube. This mixture was incubated at 98 °C for 40 min, then cooled to room temperature, and centrifuged at 8,000 × *g* for 10 min. The supernatant was examined spectrophotometrically at 532 nm, 600 nm and 450 nm.

Measurement of leaf total chlorophyll contents

Leaf total chlorophyll was extracted with 80% acetone from the fresh leaves and measured according to Lichtenthaler and Wellburn (1983). The absorbance of samples was read at the wavelength of 663 nm using a spectrophotometer (TU1800 spectrophotometer, P-general Limited Company, Beijing, China).

Measurement of leaf respiration

The respiratory oxygen consumption was measured using Clark-type electrodes (Hansatech, King's Lynn, UK) according to Lei *et al.* (2008) with some modifications. Measurements were done at 25 °C in a final volume of 2 mL containing 25 mg of leaves, and the cuvette was tightly closed to prevent diffusion of oxygen from the air. The alternative pathway was inhibited with 2 mM *n*-propyl gallate. To inhibit the cytochrome pathway, 1 mM KCN was added. The optimal inhibitor concentrations were determined from a titration curve as explained by Møller *et al.* (1988).

Analysis of gas exchange

Gas exchange analysis was made using an open system (TPS-1, PP systems, Hitchin, UK). The net

CO_2 assimilation rate (Pn) was determined at a CO_2 content of 360 cm^3 m^{-3}, 80% relative humidity and 180 μmol m^{-2} s^{-1} light intensity at 25 °C. The leaf stomatal conductance (Gs) was measured under the same conditions with a steady-state porometer (TPS-1, PP systems).

Measurement of chlorophyll fluorescence

According to Yuan *et al.* (2007), chlorophyll fluorescence quenching analysis was carried out at room temperature (25 °C) with a portable fluorometer (PAM-2100, Walz, Germany). Using both light and dark fluorescence parameters, we calculated: (1) the maximum efficiency of PSII photochemistry in the dark-adapted state, *F*v/*F*m; (2) the non-photochemical quenching, NPQ = *F*m/*F*m' – 1; (3) the quantum yield of PSII electron transport, Φ_{PSII} = (*F*m' – *F*s)/*F*m'; (4) the efficiency of excitation energy capture by open PSII reaction centres, *F*v'/*F*m' = (*F*m' – *F*o')/*F*m'. All samples were dark-adapted for 10 min before chlorophyll fluorescence was determined.

SDS-PAGE and Western blot analysis

According to the mothod of Liu *et al.* (2009), isolated thylakoids were solubilized in the presence of 6 M urea, and the polypeptides were separated by SDS-PAGE using 15% (w/v) acrylamide gels with 6 M urea. The quantities of proteins were measured according to the standard Bradford method (Liu *et al.*, 2009). For Western blotting, the proteins were electrotransferred onto a nitrocellulose film according to Yuan *et al.* (2005). Then antisera to D1 (provided by Dr. Eva-Mari Aro), LHCII b1 (purchased from AgriSera, Stockholm, Sweden), and AOX (provided by Dr. Anthony L. Moore) were applied. The signals were revealed by using secondary antibodies of alkaline phosphatase goat anti-rabbit IgG. The intensity of the signals of Western blotting was analyzed densitometrically by a thin-layer scanner.

Preparation of total RNA and Northern blot hybridization

Total RNA was extracted from leaves according to Zhang *et al.* (2004). For Northern blot analysis, equal amounts of total RNA (20 μg) were separated on formaldehyde agarose gels, then RNA was transferred to nitrocellulose filters (Boehringer Mannheim, Dassel, Germany) for subsequent probe hybridization. DNA fragments of the *Lhcb* gene, *PsbA* gene, and *Aox* gene were used as probes for RNA blot analyses. Semi-quantitative changes in the steady state of transcripts were obtained by scanning the signal intensity of individual RNA blots.

Statistical analysis

Means of 3 triplicates were measured. Student's *t* test was used for comparison between different treatments. A difference was considered to be statistically significant when $p < 0.05$.

Results

Cd accumulation in wheat plants

Exposure to 200 μmol/L $CdCl_2$ for 8 days resulted in dramatic accumulation of Cd in the roots, but small amounts were transferred to the leaves of all three wheat cultivars (Fig. 1). Cd concentrations in wheat roots were about more than 100 times those of the leaves. CY12 accumulated the most Cd in the roots, and almost the double amount of the other two cultivars (CM42 and CM47).

Effects of cadmium stress on leaf water status, lipid peroxidation, and chlorophyll changes

The water status was measured in leaves of the three wheat cultivars (Fig. 2). Eight days after Cd stress, the absolute water content decreased dramatically. However, the fastest decline was showed in CY12 compared to the other cultivars.

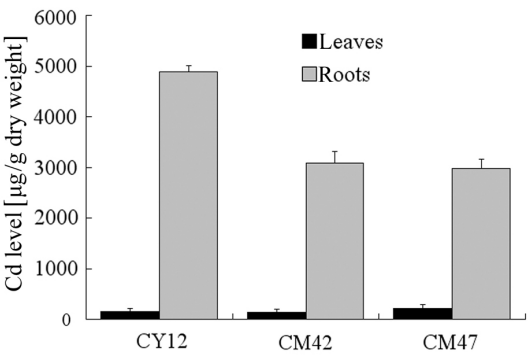

Fig. 1. Cadmium contents in CY12, CM42 and CM47 roots and leaves after 8 days of exposure to 200 μmol/L $CdCl_2$. Bars represent standard deviations of 3 independent replicates.

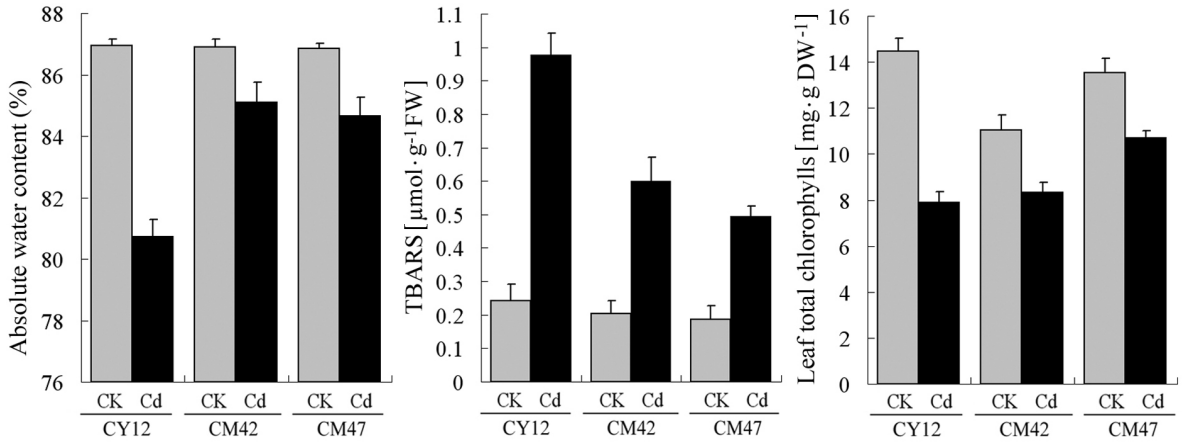

Fig. 2. Effects of cadmium stress on leaf absolute water, thiobarbituric acid-reactive substances (TBARS), and leaf total chlorophylls contents of the three wheat cultivars. Control plants (CK) are shown with grey pillars, and cadmium-stressed seedlings (Cd) are shown with black pillars. Bars represent standard deviations of 3 independent replicates.

To further confirm the effects of Cd stress on membrane integrity, TBARS were determined (Fig. 2). Cd stress resulted in an accumulation of lipid peroxidation products in leaves especially in CY12, whose TBARS values were about four times that of the control, while the oxidative damages in CM42 and CM47 were not as serious as in CY12.

Overall, chlorophylls *a* and *b* also decreased under Cd stress after 8 days in the three wheat cultivars (Fig. 2). Nonetheless, chlorophylls *a* and *b* were much lower in CY12 compared to CM42 and CM47.

Effects of cadmium stress on leaf respiration

To determine leaf respiration changes under Cd stress, the cytochrome *c* pathway respiration and the cyanide-resistant respiration were measured after 4 and 8 days, respectively. After 4 days of Cd stress, cyanide-resistant respiration was a little induced in all three wheat cultivars. However, only in CM42 and CM47 it continued to increase after 8 days. Contrastingly, cyanide-resistant respiration was inhibited obviously in CY12 at the 8th day; although its total respiration was not changed. These results suggest that CY12 adapts worse to long-time Cd stress (Fig. 3).

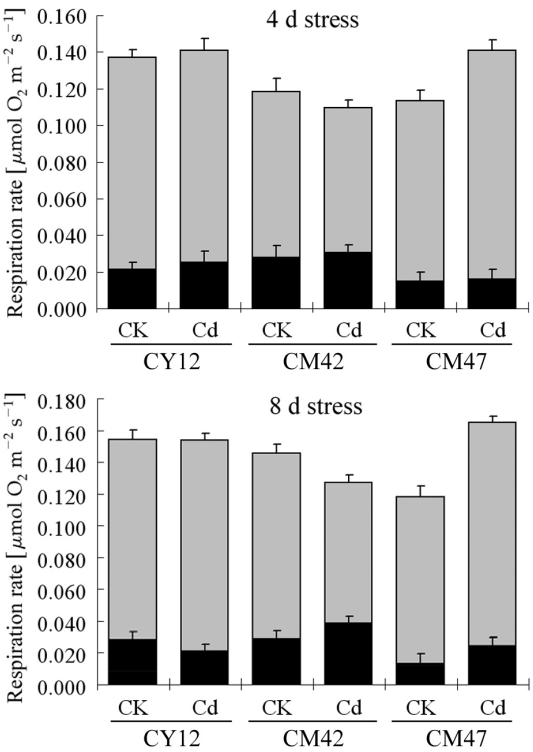

Fig. 3. Effects of cadmium stress on leaf respiration of the three wheat cultivars. Cytochrome *c* pathway respiration (grey pillar) and cyanide-resistant respiration (black pillar) were measured under cadmium stress for 4 and 8 days, respectively. CK, control plants; Cd, cadmium-stressed seedlings. Bars represent standard deviations of 3 independent replicates.

Gas exchange parameters

After 8 days of Cd stress, Pn and Gs were significantly reduced in the three wheat cultivars when compared to the control (Fig. 4). Fig. 4 shows that less decreases of stomatal conductance and CO_2 assimilation rate were observed in CM42 and CM47 than in CY12 after 8 days of Cd stress. The CO_2 assimilation rate of CY12 decreased to one quarter of the control, and was about one half that of CM42 or CM47. In addition, the transpiration rate changed similarly to the stomatal conductance (data not shown).

Changes of chlorophyll fluorescence parameters under cadmium stress

Fv/Fm, *Fv'/Fm'*, and Φ_{PSII} declined and NPQ increased when compared to the control in all three wheat cultivars after 8 days of Cd stress (Fig. 4). However, the decrease of CM42 and CM47 was not as high as in CY12. NPQ only increased significantly in CY12.

Effects of cadmium stress on alternative oxidase and mRNA

We indentified the expression of AOX by Western blot and Northern blot analysis. As shown in Fig. 5, the AOX protein was almost not changed in all three wheat cultivars after 4 days of Cd stress. Nevertheless, it increased significantly in both CM42 and CM47 after 8 days of Cd stress, although there was no such induction in CY12.

Northern blot analysis showed the same variations as the Western blots (Fig. 6). *Aox* mRNA even decreased in CY12 after 8 days of Cd stress.

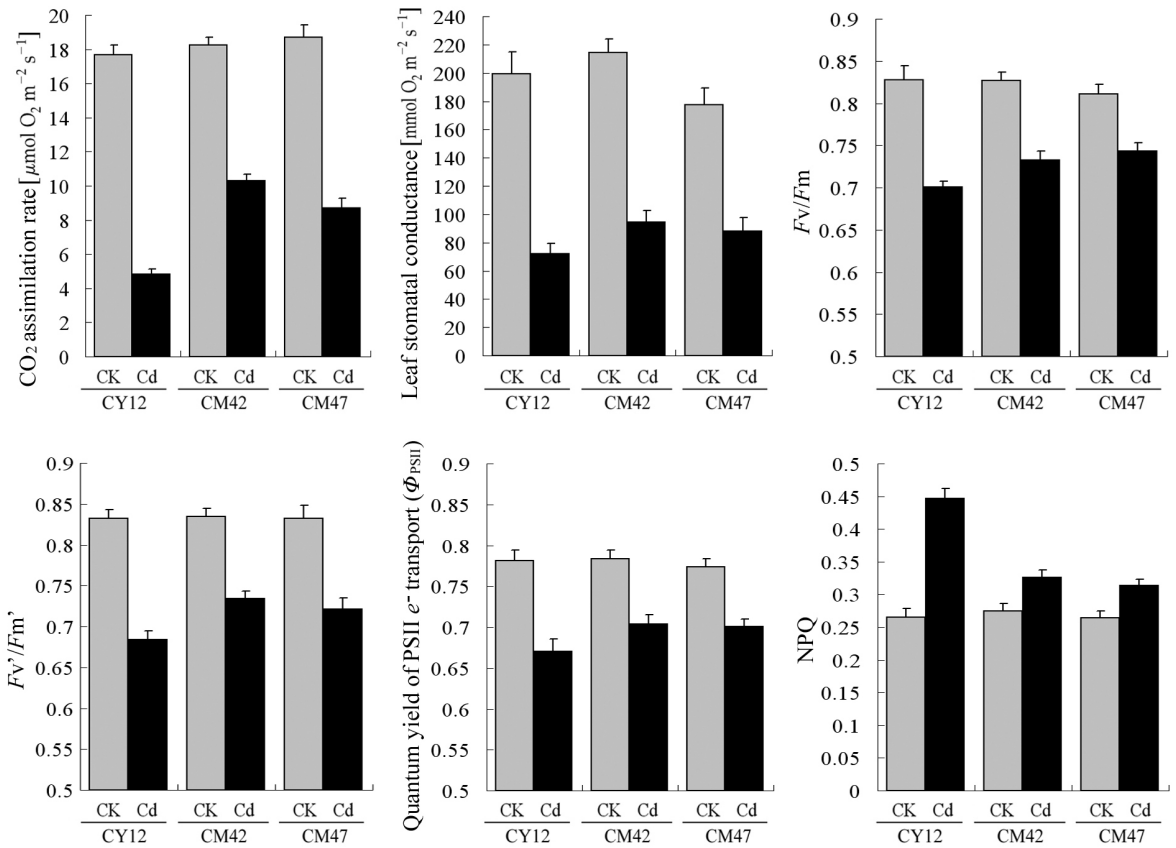

Fig. 4. Changes of gas exchange (indicated by CO_2 assimilation rate and leaf stomatal conductance) and PSII photochemical activities (indicated by *Fv/Fm*, *Fv'/Fm'*, Φ_{PSII}, and NPQ) of the three wheat cultivars under cadmium stress. CK, control plants; Cd, cadmium-stressed seedlings. Bars represent standard deviations of 3 independent replicates.

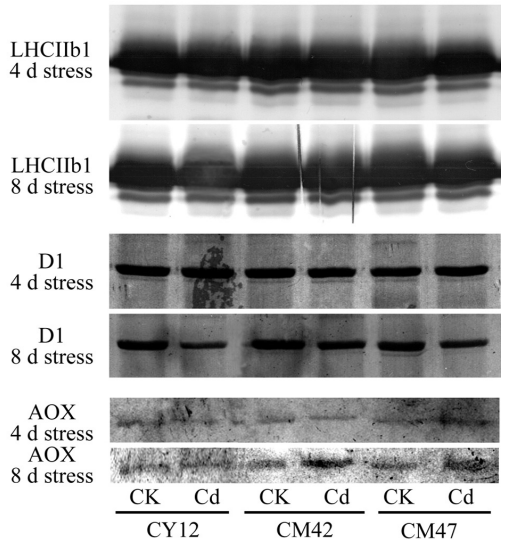

Fig. 5. Western blot analysis of steady-state levels of the LHCIIb1, D1, and AOX proteins of the three wheat cultivars during Cd stress. CK, control plants; Cd, cadmium-stressed seedlings.

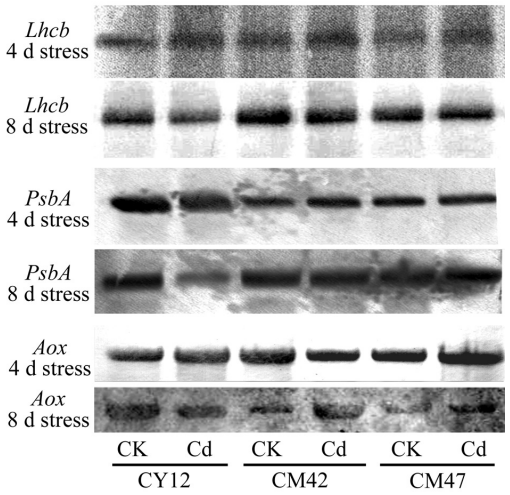

Fig. 6. Northern blot analysis of chloroplast genes *Lhcb* and *PsbA*, and mitochondria gene *Aox* transcripts of the three wheat cultivars. Total RNA was prepared from cadmium-stressed (4 and 8 days) and control wheat seedling leaves. *Lhcb*, *PsbA*, and *Aox* transcripts were detected by hybridization with specific DNA probes. CK, control plants; Cd, cadmium-stressed seedlings.

Again, the results suggest that CY12 suffered severe oxidative damages after 8 days of Cd stress, when the antioxidant genes were repressed.

Effects of cadmium stress on PSII proteins and their mRNAs

In order to detect the effects of Cd stress on the steady-state levels of some thylakoid polypeptides, Western blots for LHCIIb1 and D1 proteins were performed. As shown in Fig. 5, LHCIIb1 and D1 proteins did not significantly change after Cd stress for 4 days in all three wheat cultivars, whereas at the 8th day, D1 and LHCIIb1 proteins declined apparently in CY12, but not in the other cultivars.

Northern blots showed similar changes for their corresponding transcripts *Lhcb* and *PsbA* (Fig. 6). These data suggest that CM42 and CM47 were much more resistant to Cd stress than CY12, especially under the long-time stress condition.

Discussion

The experimental Cd concentration was rather high, contrasting to the most literatures. However, this concentration (200 μmol/L) is close to that in some waste water. This Cd stress damages wheat seedlings apparently within one week, and can be reversed after another week (data not shown). Higher concentrations of Cd (500 μmol/L) usually result in unrecoverable photobleaching and wilting (data not shown), while an apparent injury at low concentrations of Cd (50 μmol/L) occur only after one month, when the effect of seedling growth can not be neglected (data not shown). Therefore, we chose a concentration of 200 μmol/L to perform the experiments.

In our study, all three wheat cultivars accumulated more Cd in roots than in leaves, and the amount of Cd accumulation was the highest in CY12. Correspondingly, the CY12 cultivar suffered more severe damages than CM42 or CM47. This result conforms to previous studies that excess of Cd in nutrient solution can cause iron deficiency and decrease plant fresh weight (Perfus-Barbeoch *et al.*, 2002; Meng *et al.*, 2009). Likewise, water contents and chlorophyll levels of leaves were higher in CM42 and CM47 than in CY12, though decreased in all three cultivars after Cd stress, implying different cadmium absorption and translocation mechanisms in different cultivars.

Previous studies put biomass, leaf and root length as indicators of metal toxicity in plants

(Baker and Walker, 1989). However, little attention was payed on respiration and photosynthesis, *i. e.* on AOX and LHCII proteins. Previous studies demonstrated that Cd interferes with the enzyme activities of the Krebs cycle (Smiri *et al.*, 2009) and the mitochondria state 3 respiration and state 4 respiration (Sokolova *et al.*, 2005). Here we first report that cyanide-resistant respiration in wheat is also regulated under Cd stress. AOX (responsible for cyanide-resistant respiration) was reported to be induced in multiple environmental stresses, and could maintain stabilization of respiratory electron chain, scavenge ROS, and regulate plant growth rate homeostasis (Lei *et al.*, 2008). Siedow and his colleagues suggested that AOX affects the metabolism through more pervasive effects, including some that are extramitochondrial (Fiorani *et al.*, 2005; Umbach *et al.*, 2005). They further proposed an idea that the *Aox* gene functions, as a marker for genetic variation in cell reprogramming, yield stability and stress resistance (Arnholdt-Schmitt *et al.*, 2006). In our experiment, the AOX protein level, mRNA level, and cyanide-resistant respiration are tightly correlated with Cd stress resistance, varying among the different wheat cultivars. Therefore, we testified the hypothesis that AOX can be used as an indicator for plant's resistance to environmental stress.

Early studies indicated that Cd ions affect the oxidizing side of PSII and lead to the uncoupling of electron transport in the chloroplasts (Krantev *et al.*, 2008). Net photosynthesis is also sensitive to Cd because it directly affects chlorophyll biosynthesis (Krantev *et al.*, 2008). The same results were acquired in our experiments: the leaf total chlorophylls reduced significantly in all three wheat cultivars. It should be noticed that different reductions occurred corresponding to the different tolerance. In our previous work (Liu *et al.*, 2006), we found that different drought stress resistance of different wheat cultivars could be mainly attributed to the differences in steady levels of LHCII proteins and *Lhcb* mRNAs, and we brought out the idea that LHCII and *Lhcb* gene also function as genetic markers for stress resistance of plants. Here, in the present research about metal stress, this point of view has been proved again.

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