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Cd^{2+} 诱导的镉敏感水稻突变体 *cadB-1* 叶片抗坏血酸循环的变化

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摘要:【目的】镉离子(Cd^{2+})为非必需的微量元素,植物易从土壤中吸收并积累 Cd^{2+},通过食物链进入人体内,对人类的健康造成重大威胁。为了阐明 Cd^{2+} 诱导氧化胁制和抑制生长的机制,对 Cd^{2+} 敏感水稻突变体(*cadB-1*)进行了水培试验。**【方法】**植物材料为水稻粳稻中花 11(*Oryza sativa* L. ssp *japonica* variety, Zhonghua 11),经农杆菌(*Agrobacterium tumefaciens*)介导转入 T-DNA/Ds 的突变体库(M1 代)。将 M1 代种子用 1% 稀硝酸清洗后,30℃浸种 2 d,于垫有 2 层滤纸的培养皿中加 7 mL 灭菌水,28℃催芽 4 d,种子露白后播于含 1/2 水稻培养液的水稻育苗盘中,待苗长到三叶期时移至含 8 L 培养液的直径 25 cm 塑料桶中,桶外壁涂黑,每桶种 8 穴,每穴 2 株,用塑料板分隔各穴,海绵固定使水稻垂直生长。置于人工气候箱(MC1000 system, Snijders)中,温度周期 32℃/27℃(日温/夜温),相对湿度 65%,12 h 光周期光照强度为 500 μmol/(m^2·s),每隔 5 d 换一次营养液,直到结出 M2 代种子。将中花 11 野生型与 M2 代突变体种子用以上同样方法培养,长到五叶期。以不加 Cd^{2+} 作为对照,分别加入 0.1、0.25、0.5 和 0.75 mmol/L Cd^{2+} 进行筛选,每种处理平行培养 3 桶,作为重复,共 6001 桶,每天定时观察。12 d 后,发现 0.5 mmol/L Cd^{2+} 中的中花 11 野生型没有死亡,而 M2 代突变体出现部分死亡。按所在位置,选取表型最明显的株系命名为 *cadB-1*。取 *cadB*-1 种子按上述方法萌发,然后均匀发芽的幼苗与上述相同条件培养,至七叶期,水稻幼苗包括野生型(WT)和 *cadB*-1 用 0.5 mmol/L $CdCl_2$ 处理 2、4、6、8 和 12 d。**【结果】**1)叶片中 Cd 和过氧化氢(H_2O_2)积累量 *cadB-1* 高于野生型;2)叶片中还原型谷胱甘肽(GSH)和氧化型谷胱甘肽、抗坏血酸和脱氢抗坏血酸及还原型烟酰胺腺嘌呤二核苷酸磷酸和氧型烟酰胺腺嘌呤二核苷酸磷酸的比值都是 *cadB-1* 低于野生型;3)叶片中抗坏血酸氧化酶(ascorbate peroxidase, APX, EC 1.11.1.11),还原型谷胱甘肽酶(glutathione reductase, GR, EC 1.6.4.2),脱氢抗坏血酸还原酶(dehydroascorbate reductase, DHAR, EC 1.8.5.1)和单脱氢抗坏血酸还原酶(monodehydroascorbate reductase, MDHAR, EC 1.6.5.4)活性都是 *cadB-1* 低于野生型。**【结论】***cadB-1* 具有低水平的抗氧化剂和抗氧化酶活性。此外,*cadB-1* 比 WT 积累更多的 Cd 从而产生更多的活性氧(reactive oxygen species, ROS)。也就是说,与野生型相比,*cadB-1* 更缺乏防御力来清除更多的活性氧,从而导致较低的生长势和对 Cd 的敏感。

关键词:抗坏血酸-谷胱甘肽循环;镉敏感突变体;生长抑制;过氧化氢;水稻
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Cd^{2+} induced changes of ascorbate-glutathione cycle in Cd sensitive rice mutant *cadB-1* leaves

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Abstract:【Objectives】 Cd^{2+} is easily absorbed from the soil by plants and accumulation in plants which health threat to humans through human food chain. To investigate the mechanism of cadmium (Cd^{2+}) induced oxidative stress and inhibit growth in a Cd sensitive rice mutant (*cadB-1*), a hydroponic experiment was conducted. **【Methods】** A *japonica* rice (*Oryza sativa*) variety Zhonghua 11 and the mutant rice seedlings obtained from the same rice variety as that formerly constructed with T-DNA/Ds insertion mediated by *Agrobacterium*. The

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transgenetic rice generations have stable hereditability and were used in this experiment. The seeds were surface sterilized in 0.5% sodium hypochlorite for 20 min, rinsed, and germinated in the dark on moistened filter paper at 30℃ for 2 d, and then moved to a plastic screen floating on distilled water at 28℃ for 4 d. Then uniformly germinated seedlings were transferred to black polyethylene barrels which contained 6 L of rice culture solution. Seedlings were grown in a growth chamber with a photo flux density of 500 μmol/(m^2 · s), relative humidity of approximately 65% and day/night temperatures of 32℃/27℃ (14 h/10 h). During the growth period, the solution was renewed every 5 d. At the seven leaf stage, rice seedlings include wild-type (WT) and *cadB-1* exposed to 0.5 mmol/L $CdCl_2$ for 0 (as control), 2, 4, 6, 8, or 12 d. **【Results】** 1) Cd and hydrogen peroxide (H_2O_2) accumulation were higher in *cadB-1* than in wild one; 2) The ratios of reduced glutathione (GSH) and oxidized glutathione (GSSG), ascorbate (ASC) and dehydroascorbate (DHA), or reduced nicotinamide adenine dinucleotide phosphate (NADPH) and oxidized nicotinamide adenine dinucleotide phosphate (NADP +) were lower in *cadB-1* than in WT; 3) Ascorbate peroxidase (APX, EC 1.11.1.11), glutathione reductase (GR, EC 1.6.4.2), dehydroascorbate reductase (DHAR, EC 1.8.5.1) and monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) activities were lower in *cadB-1* than in WT in leaves during $CdCl_2$ exposure periods. **【Conclusion】** *cadB-1* has lower level of antioxidants as well as lower activity of antioxidant enzymes. In addition, *cadB-1* accumulates more Cd means that it can produce more reactive oxygen species (ROS). Videlicet, *cadB-1* is deficient of the defense power against increased level of ROS which leads to a lower growth potential and sensitive to Cd.

Key words: ascorbate-glutathione cycle; cadmium sensitive mutant; growth inhibit; hydrogen peroxide; rice

Cadmium can be readily taken up by roots and often accumulates to a large number in plant system[1], the presence of Cd can induce the generation of reactive oxygen species (ROS). Plants have developed antioxidant mechanisms to alleviate hazardous effects imposed by oxide stress. These mechanisms include antioxidative enzymatic and antioxidative non-enzymatic systems. The ascorbate-glutathione (ASC-GSH) cycle is the keystone of the nonenzymatic antioxidative defense system and has been suggested as the source for H_2O_2 remova1 into organelles[2-3]. ASC and GSH, two low molecular weight antioxidants are of great importance in preserving a wide range of metabolic processes[4]. They can both react directly with ROS as well as participating in ROS detoxification through the ASC-GSH cycle[2, 5-7]. Moreover, ASC and GSH are also associated with the cellular redox balance and the ratios of ASC : DHA and GSH : GSSG may function as signals for the regulation of antioxidant mechanisms[8].

Previously, we screened a rice cadmium sensitive and hyper-accumulation mutant by *Agrobacterium tumefaciens* system, investigated the enzymatic defense system, root morphology and cadmium uptake kinetics[9-10]. In present research, we mainly compared the differences in the ASC-GSH metabolism between *cadB-1* and WT seedling leaves after increasing exposure periods of Cd. Although recently we reported some results of ASC-GSH metabolism[11-12], we also want more evidences to confirm that higher ASC, GSH, or NADPH are more able to resist Cd toxicity.

1 Materials and methods

1.1 Plant materials and culture conditions

Stable inheritance rice (*Oriza sativa* L.) cadmium sensitive mutant (*cadB-1*) and the same rice variety wild-type (WT) were used in this experiment. The seeds were surface sterilized in 0.5% sodium hypochlorite for 20 min, rinsed, and germinated in the dark on moistened filter paper at 30℃ for 2 d, and then moved to a plastic screen floating on distilled water at 28℃ for 4 d. Then uniformly germinated seedlings were transferred to black polyethylene barrels which contained 6 L of rice culture solution. Seedlings were grown in a growth chamber with a photo flux density of 500 μmol/(m^2 · s), relative humidity of approximately 65% and day/night temperatures of 32℃/27℃ (14 h/10 h). During the growth period, the solution was renewed every 5 d. At the seven leaf

stage, rice seedlings include wild-type (WT) and *cadB-1* exposed to 0.5 mmol/L $CdCl_2$ for 0 (as control), 2, 4, 6, 8, or 12 d.

1.2 Cd and H_2O_2 content analysis

The Cd contents in seedling leaves, stems and roots were determined according to the method of Shah and Dubey[13]. H_2O_2 content was determined according to the method described by Jana and Choudhuri[14].

1.3 Ratios of ASC/DHA, GSH/GSSG and NADPH/NADP$^+$ analysis

ASC and DHA content were determined according to the method of Law *et al.*[15]. GSH and GSSG content was determined according to the method of Anderson *et al.*[16]. NADPH and NADP+ content was determined according to the method of Nisselbaum and Gree[17].

1.4 Enzyme assays

Frozen materials (400 mg fresh weight) were homogenized in 4 mL of 50 mmol/L potassium phosphate buffer, pH 7.8, containing 0.1% Triton X-100. The homogenate was centrifuged at 15,000 × g for 20 min at 4℃ and the supernatant was used for enzyme assays. APX and GR activities were determined according to the method of Nakano *et al.*[18]. DHAR activity was determined according to the method of Dalton *et al.*[19]. NDHAR activity was determined according to the method of Arrigoni *et al.*[20].

1.5 Statistical Analysis

Data were analyzed with the statistical package SPSS15 for Windows on the website (www.nbs.ntu.edu.sg/userguide/SPSS/SPSS15/). Significance levels 0.05 and 0.01 were used in presenting the results. The experiments were repeated in triplicate, and the data presented are the mean values ± standard error (SE). The difference was considered significant at P levels lower than 0.05 ($P < 0.05$) and this significance is denoted in the figures by an asterisk (*) while significant at P levels lower than 0.01 ($P < 0.01$) denoted in the figures by double asterisk (**).

2 Results

2.1 Cadmium accumulation and effect on rice seedling growth

At the seven-leaf stage, $CdCl_2$ was added to the nutrient solution to achieve the final Cd^{2+} concentration is 0.5 mmol/L. After 12 d exposure to Cd^{2+}, the leaves of *cadB*-1 faded seriously and the roots were more exiguous than WT. The fresh weight of shoots, and roots of wild type seedlings declined by 45.97%, and 46.99%, respectively, while the percent decrease of *cadB-1* are 63.56% and 51.28%, compared to the control-cultivated seedlings (Table 1).

表1 侵染于0.5 mmol/L Cd^{2+} 12天后野生型(WT)和Cd-敏感型(*cadB-1*)水稻秧苗不同部位鲜重(g, FW)

Table 1 Fresh weights (FW) of different organs of wild type (WT) and Cd-sensitive (*cadB-1*) rice seedlings after 12 d of exposure to 0.5 mmol/L Cd^{2+}

处理 Treatment	地上部 Shoot		根 Root	
	WT	*cadB-1*	WT	*cadB-1*
对照 CK	3.72 ± 0.10	3.65 ± 0.09	0.83 ± 0.05	0.78 ± 0.03
镉处理 Cr^{2+} treatment	2.01 ± 0.14	1.33 ± 0.23	0.44 ± 0.05	0.38 ± 0.05

The Cd contents increased in all organs of both WT and *cadB-1* with 0.5 mmol/L Cd^{2+} exposure for 12 d (Table 2). A larger increase was seen in all organs of *cadB-1* compared to WT seedlings. However, the increase was not statistically significant.

2.2 H_2O_2 accumulation in rice seedlings

H_2O_2 contents increased in the leaves of WT and *cadB*-1 rice seedlings during Cd^{2+} exposure period (Fig. 1). In general, *cadB-1* rice seedlings accumulated more H_2O_2 than WT rice seedlings, except the 2nd day. Significant difference of H_2O_2 contents between WT and *cadB-1* rice seedling leaves was only detected the 12th exposure day.

表 2　侵染于 0.5 mmol/L Cd^{2+} 12 天后野生型(WT)和 Cd－敏感型(*cadB-1*)水稻秧苗根茎叶中 Cd 含量 (mg/kg, DW)

Table 2　Cadmium content in leaves, stems and roots in wild type (WT) and Cd-sensitive (*cadB-1*) rice seedlings after 12 d of exposure to 0.5 mmol/L Cd^{2+}

处理 Treatment	叶 Leaf		茎 Stem		根 Root	
	WT	*cadB-1*	WT	*cadB-1*	WT	*cadB-1*
对照 CK	6.19 ± 0.82	8.42 ± 0.28	16.96 ± 1.03	15.26 ± 1.21	21.95 ± 1.14	22.04 ± 0.98
镉处理 Cd^{2+} treatment	72.33 ± 1.15	88.21 ± 1.92	340.56 ± 3.32	381.98 ± 2.31	1685.42 ± 4.76	1898.18 ± 5.32

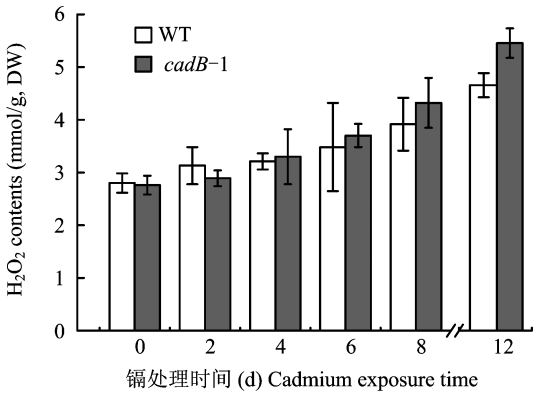

图 1　野生型(WT)和 Cd－敏感型(*cadB-1*)水稻秧苗叶片中 H_2O_2 含量

Fig. 1　H_2O_2 contents in leaves of WT and *cadB-1* rice seedlings

[注(Note): External Cd^{2+} concentration is 0.5 mmol/L 外源 Cd^{2+} 浓度为0.5 mmol/L. Error bars represent standard error (n = 3)误差线代表标准差(n = 3)].

2.3　Cd effect on ratios of ASC/DHA, GSH/GSSG and NADPH/$NADP^+$ in rice seedlings

During exposure period, leaf contents of ASC decreased both in WT and *cadB-1* (Fig. 2-a), however the opposite effect was observed with DHA contents in leaves (Fig. 2-b). Significant differences were seen in ASC content between WT and *cadB-1* rice seedling leaves at the 8th and 12th exposure day. Therefore, the ratio of ASC/DHA was reduced with prolonging exposure time (Fig. 2-c). Furthermore, in WT and *cadB-1* rice seedlings the ratio varied concomitantly with prolonging time at 0.5 mmol/L Cd^{2+}. After 12 d exposure to 0.5 mmol/L Cd^{2+}, the ratio of ASC/DHA at the 2nd, 4th, 6th, 8th and 12th exposure day compared to the control declined by 9.76%, 20.10%, 38.00%, 53.00% and 66.55%, respectively in the leaves of WT rice seedlings, while in *cadB-1* rice seedlings the percentage of decrease was 16.56%, 32.92%, 48.57%, 65.86% and 85.06%, respectively. Overall, the ratio of ASC: DHA declined more in leaves of *cadB-1* rice seedlings than in WT rice seedlings.

The GSH contents decrease and GSSG increase in leaves occurred not only in WT but also in *cadB-1* during the exposure (Fig. 2-d, e). Significant differences took place in GSH contents but not in GSSG between WT and *cadB-1* rice seedling leaves during exposure period. Changes in GSH and GSSG led to changes in GSH/GSSG ratio (Fig. 2-f), significant differences could be found in the GSH/GSSG ratio between WT and *cadB*-1 rice seedling leaves at the 4th, 8th and 12th exposure day. During exposure period, the GSH/GSSG ratio in leaves of *cadB-1* rice seedlings declined by 35.80%, 61.09%, 70.65%, 82.31% and 85.91%, respectively, while ratios for leaves of the WT rice seedlings were increased by 26.37%, 37.52%, 59.15%, 63.00% and 68.09%, respectively compared to the control. GSH/GSSG ratios decreased more in *cadB-1* rice seedlings than in WT rice seedlings.

Oxidized nicotinamide adenine dinucleotide phosphate ($NADP^+$) contents increased during exposure period (Fig. 2-h), while the NADPH contents meet the opposite to $NADP^+$ in leaves. NADPH: $NADP^+$ ratios were reduced (Fig. 2-i), similar to GSH/GSSG and ASC/DHA ratios. The leaves of *cadB-1* rice seedlings showed a decrease in the NADPH/$NADP^+$ ratios of 7.77%, 19.34%, 35.76%, 55.03%, and 67.94%, respectively. In the WT seedlings, the NADPH/$NADP^+$ ratios

compared to the control were decreased by 3.81%, 18.30%, 28.76%, 45.09%, and 58.37%. The decrease in the NADPH/NADP$^+$ ratios was more pronounced in *cadB-1* rice seedlings than in WT rice seedlings.

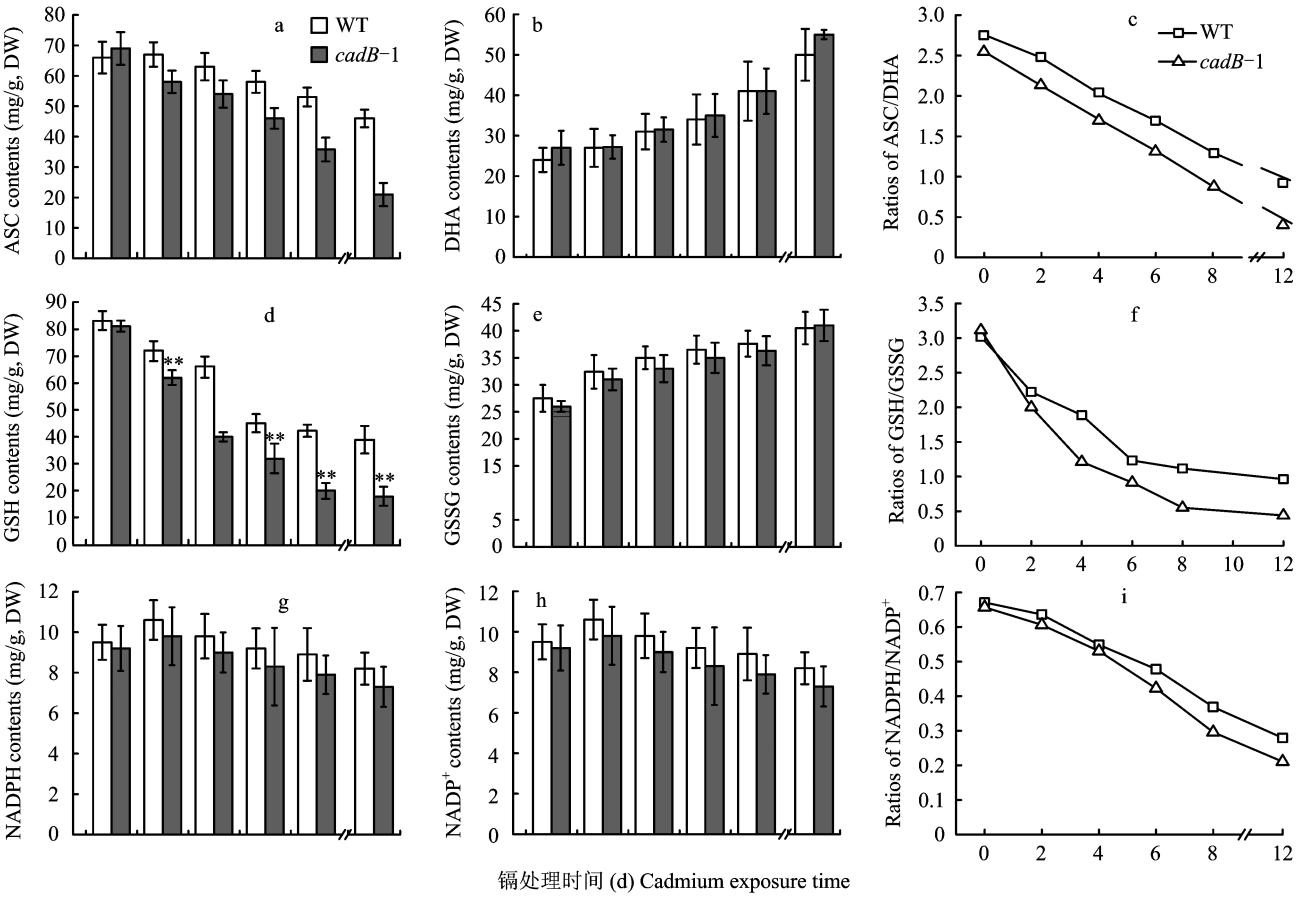

图2 野生型(WT)和Cd-敏感型(*cadB-1*)水稻秧苗叶片中ASC、DHA、GSH、GSSG、NADPH、NADP$^+$值和ASC/DHA, GSH/GSSG and NADPH/NADP$^+$比率

Fig. 2 The contesnt ASC, DHA, GSH, GSSG, NADPH, NADP$^+$ and the ratio of ASC/DHA, GSH/GSSG and NADPH/NADP$^+$ in leaves of WT and *cadB-1* rice seedlings

[注(Note): 外源Cd浓度为0.5 mmol/L External Cd concentration is 0.5 mmol/L; $*P<0.05$ and $**P<0.01$. 误差线代表标准差($n=3$) Error bars represent standard error ($n=3$).]

2.4 Cd effect on APX, GR, DHAR and MDHAR activities in rice seedlings

APX activities increased and then decreased both in WT and in *cadB*-1 rice seedlings during exposure (Fig. 3-a). APX activity reached its highest level in WT and in *cadB*-1 rice seedlings at the 6th exposure day, and the APX activities showed significant differences between WT and *cadB*-1 rice seedling. APX activities decreased more in the leaves of *cadB-1* seedlings than in WT seedlings during exposure.

GR activities increased first and then decreased in both WT and *cadB*-1 rice seedlings with the prolongation of exposure (Fig. 3-b). GR activities were highest in both WT and *cadB-1* rice seedlings at the 4th exposure day, GR activities were observed higher in WT than in *cadB-1* rice seedlings. to 0.5 mmol/L Cd^{2+}, on the 4th day of exprosure, comparing to the control, GR activities increased by 64.85% in *cadB-1* seedlings and 101% in the WT rice seedlings. At the 8th day, significant differences in GR activities were observed between *cadB-1* and the WT rice seedling.

In this experiment, DHAR activities were lower in *cadB-1* than in WT seedlings with Cd^{2+} exposure (Fig.

3-c). DHAR activities in the WT increased and then decreased, *cadB-1* varied concomitantly with WT. In the *cadB*-1 rice seedlings, DHAR activities reached maximum levels at the 4th exposure day, while those in WT seedlings reached maximum levels at the 8th day. Differences in DHAR activities between *cadB-1* and WT plants were significant at the 6th, 8th and 12th exposure day.

MDHAR activities increased and then decreased both in the *cadB-1* and WT rice seedlings (Fig. 3-d). MDHAR activities were maximum at the 4th day in *cadB-1*, and at the 6th day in WT seedlings. MDHAR activities decreased more in the *cadB-1* than the WT rice seedlings. At the 12th exposure day, MDHAR activities decreased by 56.82% in the MT, and 47.79% in the *cadB-1*. MDHAR activity differences were significant between *cadB-1* and WT rice seedlings at the 6th exposure day.

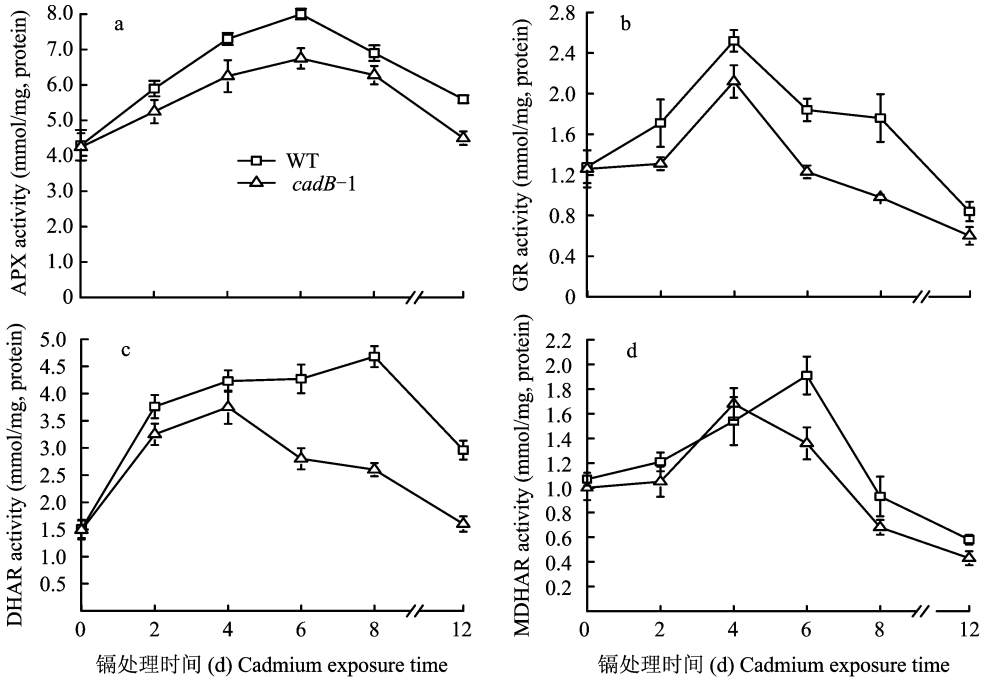

图 3　野生型(WT)和 Cd－敏感型(*cadB-1*)水稻秧苗根系中 APX, GR, DHAR and MDHAR 活性

Fig. 3　APX, GR, DHAR and MDHAR activities in roots of WT and *cadH*-5 rice seedlings

[注(Note): 外源 Cd 浓度为 0.5 mmol/L External Cd concentration is 0.5 mmol/L; *—$P<0.05$; **—$P<0.01$. 误差线代表标准差 ($n=3$) Error bars represent standard error ($n=3$).]

3 Discussion

H_2O_2 is induced in *Arabidopis* treated with Cd^{2+}[21], moreover, H_2O_2 considered, as a signaling molecule in stress, is well documented[22], it defense and provide acclimation during various abiotic and biotic stresses. In general, we found the H_2O_2 contents increased with prolongation of Cd^{2+} exposure in both *cadB*-1 and WT plants, and accumulated more in *cadB-1* than in WT (Fig. 1).

GSH has been to demonstrated play a key role in defense mechanisms[23]. It can alleviate H_2O_2 immediately or participate in the ASC-GSH cycle to scavenge H_2O_2[24]. GSH has been shown to combine with Cu^{2+} and alleviate Cu^{2+} toxicity, GSH is also the substrate for phytochelatin (PC) synthesis, which plays a central role in metal detoxification[23]. GSH is oxidized to GSSG and the balance of GSH and GSSG is crucial for the enzymatic systems that scavenge H_2O_2. A common response to heavy metal exposure is depletion of GSH and a subsequent rise in GSSG[25]. Here, the GSH content was slightly decreased both in *cadB-1* and WT in short time exposure. When the exposure prolonged, the GSH content went down, particularly in the mutant, and the GSSG content increased. Thus the GSH/GSSG ratio decreased with

increasing Cd^{2+} concentrations (Fig. 2-f). At short time Cd^{2+} exposure, GSH content decreased only slightly, suggesting one way plants might adapt to Cd^{2+} stress. Plants can use this ability to self-adjust and enhance resistance to Cd^{2+} stress in short time exposure. With the exposure time prolonged, GSH content decreased dramatically, perhaps because it attaches to heavy metals, or provides a substrate for PCs synthesis. Alternatively, it may behave as a antioxidant to scavenge ROS, or it may have lower NADPH, which may be ascribed to the corresponding decrease in GR activity, and the resulting decrease in GSH. During our study, the lower GSH contents in *cadB-1* than WT may be due to the lower GR activity or the lower activity of γ-glutamyl-cysteine synthetase (γ-ECS) and glutathione synthetase (GS). The lower GSH contents and GSH/GSSG ratio may be one reason why the mutant is more sensitive to Cd^{2+} toxicity.

ASC behaves as an electron donor for APX scavenging of H_2O_2, and would be oxidated to DHA. On the other hand, DHA could be regenerated to ASC by DHAR, using GSH as an electron donor. The balance of ASC and DHA is crucial for the enzymatic systems that scavenge H_2O_2. In the present study, we found that prolonged Cd^{2+} exposure time produced a decrease in the ASC content while increased in the DHA content both in *cadB*-1 and WT. These changes resulted in decreases in the ASC: HA ratio. The decrease in ASC content might be due to an inhibition of the DHAR activity, or more excessive use of ASC in metal detoxification, or because the activity of GSH, an electron donor, is lower. The ASC content and the ASC:DHA ratio were lower in the mutant than in the wild type. This could be the result of lower DHAR activities in *cadB-1* than in the wild type (Fig. 3). From this, we inferred that plants resistant to Cd^{2+} toxicity maintain a high ASC content and require a high ratio of ASC/DHA.

H_2O_2 scavenging by APX is the first step in the ASC-GSH cycle[26]. As demonstrated in the choroplasts of pea (*Pisum sativum*) and spinach (*Spinacea oleracea*), GR, DHAR, and MDHAR also participate in this cycle[26]. In this study, we found at the short time of Cd^{2+} exposure can induce APX activity, and may indicate the H_2O_2 content is increasing; after long time of Cd^{2+} exposure the APX activities decreased, which not suggesting the H_2O_2 content is decreasing but showing Cd^{2+} inhibition of APX activities. With the prolongation of exposure, APX activity reductions were more pronounced in the mutant than in the wild type, showing that Cd^{2+} strongly inhibits APX activity in the mutant. GR activities vary concomitantly with APX activities; short time Cd^{2+} exposure can induce GR activities, which catalyze GSSG to synthesize GSH by consuming NADPH as an electron donor. DHAR and MDHAR take part in the regeneration of ASC. At first can induce DHAR and MDHAR activity, possibly as the result of increased APX activities, hence DHAR and MDHAR caught regenerate enough ASC as an electron donor for APX scavenging for H_2O_2. With long time of Cd^{2+}, DHAR and MDHAR activities are inhibited, and DHAR and MDHAR activities decrease more in the mutant than in the wild type, showing that Cd^{2+} inhibited both DHAR and MDHAR activities in the mutant acutely. At the short time, ROS is effectively scavenged; at the long time the capability is overridden and they cannot scavenge ROS effectively. From this we inferred the plant is irreversibly damaged and inhibited by ROS at the long time to Cd^{2+} exposure.

4 Conclusion

The higher ASC, GSH and NADPH levels and the higher ratios of ASC/DHA, GSH/GSSG and NADPH/$NADP^+$, as well as the higher antioxidative enzymatic activities in plants will be more effective to resist Cd^{2+} toxicity. Compare to WT, the mutant *cadB-1* has lower level of antioxidants as well as lower activity of antioxidant enzymes; A little more Cd accumulated in *cadB-1* means a little more reactive oxygen species production (ROS). Videlicet, *cadB-1* is deficient of the defense power against increased level of ROS which leads to a lower growth potential and sensitive to Cd.

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References:

[1] Liu Y G, Wang X, Zeng G M *et al*. Cadmium-induced oxidative stress and response of the ascorbate-glutathione cycle in *Bechmeria nivea* (L.) Gaud[J]. Chemosphere, 2007, 69(1): 99-107.

[2] Anjum A N, Gill S S, Gill R *et al*. Metal/metalloid stress tolerance in plants: role of ascorbate, its redox couple, and associated enzymes [J]. Protoplasma, 2014, 251 (6): 1265-1283.

[3] Li Z, Su D, Lei B *et al*. Transcriptional profile of genes involved in ascorbate glutathione cycle in senescing leaves for an early senescence leaf (esl) rice mutant [J]. Journal of Plant Physiology, 2014, 176(25): 1-15.

[4] Chaparzadeh N D, Amico M L, Khavari-Nejad R A *et al*. Antioxidative responses of *Calendula officinalis* under salinity conditions[J]. Plant Physiology and Biochemistry, 2004, 42(9): 695-701.

[5] Kuzniak E. Maria S. Ascorbate, glutathione and related enzymes in chloroplasts of tomato leaves infected by *Botrytis cinerea*[J]. Plant Science, 2001,160(4): 723-731.

[6] Kuo M C, Kao C H. Antioxidant enzyme activities are upregulated in response to cadmium in sensitive, but not in tolerant rice (*Oryza sativa* L.) seedlings[J]. Botany Bulletin of Academy Sinica, 2004, 45: 91-299.

[7] Srivastava S, Tripathi R D, Dwivedi U N. Synthesis of phytochelatins and modulation of antioxidants in response to cadmium stress in *Cuscuta reflexa* - An angiospermic parasite[J]. Journal of Plant Physiology, 2004, 161(6): 665-674.

[8] Monrás J P, Collao B, Molina-Quiroz R C, Pradenas G A *et al*. Microarray analysis of the *Escherichia coli* response to CdTe-GSH Quantum Dots: understanding the bacterial toxicity of semiconductor nanoparticles [J]. BMC Genomics. 2014, 15 (1): 1099.

[9] 林冬, 朱诚, 孙宗修. 镉敏感水稻突变体在镉胁迫下活性氧代谢的变化[J]. 环境科学, 2006, 27(3): 561-566.
Lin D, Zhu C, Sun Z X. Alterations of oxidative metabolism respond to cadmium stress in Cd-sensitive mutant rice seedlings [J]. Environmental Sciences 2006, 27(3): 561-566.

[10] He J Y, Zhu C, Ren Y F *et al*. Root morphology and cadmium uptake kinetics of the cadmium-sensitive rice mutant [J]. Biologia Plantarum, 2007, 51(4): 791-794.

[11] Shen G M, Zhu C, Du Q Z, Shangguan L N. Ascorbate - Glutathione cycle alteration in a cadmium sensitive mutant from rice[J]. Rice Science, 2012, 19(3): 185-192

[12] Shen G M, Zhu C, Shangguan L N, Du Q Z. The Cd-tolerant rice mutant *cadH*-5 is a high Cd accumulator and shows enhanced antioxidant activity [J]. Journal of Plant Nutrition and Soil Science, 2012, 175(2): 309-318.

[13] Shah K, Dubey R S. A 18 kDa cadmium inducible protein Complex: its isolation and characterisation from rice (*Oryza sativa* L.) seedlings[J]. Journal of Plant Physiology, 1998, 152 (4): 448-454.

[14] Jana S, Choudhuri M A. Glycolate metabolism of three submersed aquatic angiosperms during aging [J]. Aquatic Botany, 1982, 12: 345-354.

[15] Law M Y, Charles S A, Halliwell B. Glutathione and ascorbic acid in spinach (*Spinacia oleracea*) chloroplasts. The effect of hydrogen peroxide and of Paraquat [J]. Biochemistry Journal, 1983, 210: 899-903.

[16] Anderson J V, Chevone B I, Hess J L. Seasonal variation in the antioxidant system of eastern white pine needles evidence for thermal dependence[J]. Plant Physiology, 1992, 98(2): 501-508.

[17] Nisselbaum J S, Green S. A simple ultramicro method for determination of pyridine nucleotides in tissues[J]. Analytical Biochemistry, 1969, 27(2): 212-217.

[18] Nakano Y, ASCda K. Purification of ascorbate peroxidase in spinach chloroplasts; its inactivation in ascorbate-depleted medium and reactivation by monodehydroascorbate radical [J]. Plant and Cell Physiology, 1987, 28(1): 131-140.

[19] Dalton D A, Russell S A, Hanus F J *et al*. Enzymatic reactions of ascorbate and glutathione that prevent peroxide damage in soybean root nodules[J]. Proceedings of the National Academy of Sciences, 1986, 83(11): 3811-3815.

[20] Arrigoni O, Dipierro S, Borraccino G. Ascorbate free radical recuctase: A key enzyme of ascorbic acid system [J]. FEBS Letters, 1981, 125: 242-244.

[21] Cho U, Seo N. Oxidative stress in *Arabidopsis thaliana* exposed to cadmium is due to hydrogen peroxide accumulation[J]. Plant Science, 2005, 168(1): 113-120.

[22] Chen C, Twito S, Miller G. New cross talk between ROS, ABA and auxin controlling seed maturation and germination unraveled in APX6 deficient Arabidopsis seeds [J]. Plant Signal & Behavior, 2014, 9(12): e976489.

[23] Cobbett C, Goldsbrough P. Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis[J]. Annual Review of Plant Biology, 2002, 53(1): 159-182.

[24] Dalton D A, Russell S A, Hanus F J *et al*. Enzymatic reactions of ascorbate and glutathione that prevent peroxide damage in soybean root nodules[J]. Proceedings of the National Academy of Sciences, 1986, 83(11): 3811-3815.

[25] Ortega-VillASCnte C, Rellán-álvarez R, Del Campo F F *et al*. Cellular damage induced by cadmium and mercury in *Medicago sativa* [J]. Journal of Experimental Botany, 2005, 56(418): 2239-2251.

[26] Jimenez A, Hernandez J A, Del Rio L A, Sevilla F. Evidence for the presence of the ascorbate-glutathione cycle in mitochondria and peroxisomes of pea leaves[J]. Plant Physiology, 1997, 114 (1): 275-284.