Cadmium tolerance is associated with the root-driven coordination of cadmium sequestration, iron regulation, and ROS scavenging in rice

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ABSTRACT

Excess cadmium (Cd) is a serious threat to agriculture and the environment. High Cd availability showed no significant decline in growth, chlorophyll synthesis, soluble protein, cell and membrane stability in Sonarbangla (Cd-tolerant), while these were severely affected in BRRI 72 (Cd-sensitive). Atomic absorption spectroscopy analysis demonstrated a huge increment of Cd and Fe in root and shoot of BRRI 72; however, Sonarbangla only exhibited a significant increase of Cd in roots. It suggests that excess Cd in Sonarbangla possibly retained in roots through vacuolar sequestration without interfering cell functions. This was further confirmed by the increased accumulation of cysteine, glutathione, and phytochelatin along with OsPCS1 and OsHMA3 upregulation, possibly facilitated by nitric oxide in roots of Sonarbangla. Further, Fe chelate reductase activity in conjunction with the genes (OsFRO1, OsNRAMP1, OsIRT1, and OsYSL15) associated with Fe availability significantly upregulated in BRRI 72 but not in Sonarbangla in response to Cd. It advises that Fe acquisition and transport were tightly regulated in Cd-tolerant Sonarbangla. Furthermore, elevated CAT, APX, GR, NO in root along with shoot sugar helps rice plants to withstand Cd-induced oxidative damage. Finally, reciprocal grafting combining Sonarbangla rootstock with either BRRI 72 or Sonarbangla scion showed Sonarbangla type tolerance along with no changes of H2O2 and Fe reductase activity in roots under high Cd. It indicates that the signal inducing the responses to adjust Cd stress is originated in the root system. These messages deliver essential background for further breeding program to produce Cd-free rice.

1. Introduction

Cadmium (Cd) is a harmful metal that causes plant damage and human diseases. Cd interacts with the essential metals or enzymatic cofactors and causes a disturbance in cell redox system (Hattab et al., 2014). Further, it hinders protein structure or membrane integrity (Rascio and Navarri-Izzo, 2011). Cd is present in most of the soils as a trace component. However, rapid industrialization and anthropogenic release cause high Cd availability in agricultural lands (Zhang et al., 2009). This elevated Cd is consumed by plants, which can turn into a potential hazard to human wellbeing. Rice (Oryza sativa L.) is an important staple crop. Cd-contaminated soil and poor water management in agricultural land may facilitate Cd buildup in rice and food chain (Cattani et al., 2008).

Cd toxicity often causes as chlorosis, wilting, leaf roll, and growth retardation in plants (Kabir et al., 2016; Prasad, 2004). Further, Cd stress reduces photosynthesis, gas exchange characteristics, and transpiration rate (Wang et al., 2014; Rascio et al., 2008). However, differential tolerance of Cd is seen in several plant genetic lines (Greger et al., 2016; Zhang et al., 2009). It is, therefore, decisive to explicate the mechanistic basis underlying Cd tolerance with a view to minimizing the toxicity of Cd in plant and human.

To withstand Cd toxicity, plants possess some coordinated mechanisms (Choppala et al., 2014; Shah and Nahakpam, 2012). Cd tolerance is often lined with vacuolar retention and reduced Cd in the aerial parts (Zhang et al., 2009). Also, high Cd can bind with cell walls and vacuoles in root (Zhang et al., 2009). Beside this, excess Cd may hold in roots through the chelation of non-protein thiols in root (Zhang et al., 2013). This evidence only provides preliminary indications on the physiological basis of Cd tolerance in rice. Therefore, molecular supports of these mechanisms still need attention.

Phytochelatins (PCs) is cysteine-rich molecules, which generally bind to heavy metals in plants (Jasinski et al., 2003) resulting in the inhibition of excess heavy metals in shoots (Emamverdian et al., 2015;
It is known that specific transporters for Cd uptake are not present in plants. The Cd is absorbed by plants through low-affinity cation and Fe transporters (Kabir, 2016; Takahashi et al., 2011). IRT1 (Fe-regulated transporter) is critical for Fe acquisition in plants although it carries Cd ion as a substrate (Rogers et al., 2000). Also, OsNRAMP1 (natural resistance-associated macrophage proteins) is associated with cellular Cd acquisition in rice (Takahashi et al., 2011). In addition, FRO gene responsible for ferric chelate reductase is crucial for Fe availability in roots (Ling et al., 2002). As rice possesses both Strategy-I and II Fe uptake mechanisms, YSL-like (YSL) transporters categorized as magnesium acid family phytosiderophores are essential for Fe transport in this species. Reports revealed that rice OsYSL15 is necessary for Fe uptake during the early stage of rice seedlings (Kabir et al., 2016).

Abiotic stresses cause excessive reactive oxygen species (ROS) and oxidative injury (Kabir et al., 2016; Dat et al., 2000). Upregulation of antioxidant system to overcome oxidative damage is common in plant although the magnitude varied (Kabir, 2016; Kabir et al., 2016). Enzymes that are mainly involved in neutralizing ROS damage include catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD), glutathione reductase (GR), etc. (Kabir, 2016; Cuypers et al., 2011). The behavior of antioxidant enzymes decreased due to a high level of Cd in rice (Hassan et al., 2005). However, APX, CAT and GR enzymes significantly increased in response to Cd in rice var. Dongjin (Ali et al., 2002). Further, guaiacol peroxidase and ascorbate peroxidase increased in IR-29 (salt-sensitive) and Nonabokra rice lines (Roychoudhury et al., 2012). This indicates that tolerance to Cd and antioxidant response may vary among the varieties or species. In addition, Cd stress caused different changes in glutathione in rice. Glutathione was increased in roots under excess Cd (Zhang and Ying, 2008) while another report demonstrated that glutathione notably accumulated in Cd-induced rice (Aina et al., 2007). Results also showed that soluble sugars pose a critical role in the cellular redox balance as they do have a close relationship with photosynthesis and respiration (Couée et al., 2006). Sugars function as ROS eliminator or cell signal in response to stresses in plants (Van den Ende and Valluru, 2009).

Cd toxicity is a critical agronomic and health problem in rice. However, our perception of the mechanistic explanation of Cd tolerance in a wide variety of rice is still ambiguous. In this study, we performed a progression of morphological and physiological examinations to confirm the differential variations subjected to Cd stress in contrasting rice genotypes. To characterize the molecular mechanisms, several physiochemical traits and their related genes were analyzed. We also sought to determine if Cd tolerance in rice is associated with Fe regulation or Cd sequestration in root/shoot. Moreover, analysis of antioxidant performance was analyzed to confirm if scavenging of ROS may trigger Cd tolerance in rice. Finally, reciprocal grafting of contrasting genotypes revealed the source of the signal triggering Cd tolerance in rice.

2. Materials and methods

2.1. Plant cultivation

Sonarbangla (Cd-tolerant) and BRRI 72 (Cd-sensitive) rice lines with differential Cd tolerance as ranked based on morphological markers in a preliminary screening study were used in this study (Supplementary Table S1). Sonarbangla (yield: 6.28 t/ha) is a Chinese imported cultivar while BRRI 72 (yield: 5.7 t/ha) is released by Bangladesh Rice Research Institute (BRRI). Firstly, the seeds were cleaned with 75% ethanol and rinsed 2–3 times with deionized water. The seeds were positioned in germination tray at room temperature in the dark. After germination, the uniform and healthy young seedlings were separated and transferred to the 2L pot which filled with hydroponic solution (Hoagland and Arnon, 1950). The basal nutrient solutions (pH 6.0) were containing the following nutrient components: KNO3 (16000 μM), NH4H2PO4 (1000 μM), MgSO4·7H2O (2000 μM), H3BO3 (25 μM), KCl (50 μM), Fe-EDTA (25 μM), Ca(NO3)2·4H2O (6000 μM), MnSO4·4H2O (2 μM), Na2MoO4·2H2O (0.5 μM) ZnSO4 (2 μM), and CuSO4·5H2O (0.5 μM). The containers with the seedlings were then kept into the growth chamber under 10 h light and 14 h dark. The Cd treatment was optimized by adding 10 μM CdSO4 to the hydroponic culture based on a prior pilot study (Supplementary Fig. S1). In this hydroponic system plants were cultivated for 7 days.

2.2. Morphological features and chlorophyll analysis

The length of root and shoot were measured (measuring range: 0–200 mm) using AOS Digital Caliper (Mitutoyo, United States). In addition, roots were cleaned with water and blotted softly in soft papers. These root and shoot samples were then put in 1.5 mL Eppendorf tube and dried in an oven for 2 days at 80˚C preceding dry weight estimation. Also, the total chlorophyll (a and b) was measured in leaves from the absorbance read at 662 nm and 646 nm in a spectrophotometer (Lichtenthaler and Wellburn, 1985).

2.3. Analysis of Cd and Fe

The shoots were directly stored on Eppendorf tube. In case of roots, samples were incubated in 1 mM CaSO4 for 5 min before washing with deionized water (Kabir et al., 2017). The samples were then dried at 80˚C for 2 days in an oven. These samples were then boiled in a glass beaker inside a microwave oven with 2 mL HClO4 and 5 mL HNO3. Cd and Fe concentrations were then tested separately using an air-acetylene atomization gas mixture of ASC-6100 auto-sampler connected with Flame Atomic Absorption Spectroscopy (Shimadzu).

2.4. Determination of total soluble protein and sugar in tissues

The total soluble protein in root and shoot was analyzed by the optical density taken at 595 nm in a spectrophotometer (GENESYS 10S UV–Vis). The calibration curve of bovine serum albumin (BSA) was used to measure the concentration of unknown sample (Guy et al., 1992). In addition, the total soluble sugar was measured in root and shoot using anthrone by a spectrophotometer as previously described (Dubois et al., 1956).

2.5. Determination of electrolyte leakage

The electrolyte leakage, a marker of membrane stability was performed both in root and shoot. Briefly, root surface was cleaned with deionized water. A while later, the roots and shoots incubated in 20 mL vial containing water with occasional shaking for 2 h. Lastly, the electrical conductivity of the solution containing sample was recorded (Lutts et al., 1996).

2.6. The activity of Fe chelate reductase assay

The activity of Fe (III) chelate reductase (FCR) in the excised roots and shoot, were analyzed as previously described (Kabir et al., 2015) through ferrozine (3-(2-pyridyl)-5,2,4-triazine, disodium salt) assay. At first, roots were rinsed in CaSO4 and deionized water and placed in a beaker filled with ice water. Then 100 mg of root and shoot tissue was...
transferred to 1.5 mL microcentrifuge tubes containing the assay solution (0.1 mM Fe-EDTA and 0.3 mM Ferrozine). Subsequently, the samples were then washed with water and were cut into pieces before transferring to 2 mL assay solution: 100 mM Fe(III) EDTA, 0.10 mM MES-NaOH (pH 5.5), 300 mM ferrozine. The samples and the corresponding blank solution were incubated in shaking water bath for 20 min at 23°C in a dark room and then centrifuged at 14,000 rpm at room temperature for 10 min. Afterward, the optical density of aliquot was monitored at 562 nm. The activity of ferric reductase was analyzed using the extinction coefficient of ferrozine (M−1 cm−1).

2.7. Analysis of \( H_2O_2 \) and lipid peroxidase

Excised plant samples were homogenized with 0.1% trichloroacetic acid (Alexieva et al., 2001) at 4°C and centrifuged at 10,000 rpm for 14 min. The exudates were separated in a falcon tube following the addition of 10 mM phosphate buffer and 1 M KI (pH 7.0). Afterward, the sample mixture was kept in dark condition for an hour and lastly, the OD was measured at 390 nm. In addition, we tested the activity of lipid peroxidase by the MDA (malondialdehyde) concentration in root and shoot as previously described by spectrophotometer (Kosugi and Kikugawa, 1985).

2.8. Analysis of cell death

The cell death was analyzed by Evans blue method with some modifications (Zhao et al., 2005). Briefly, 2 cm long ascertained roots and shoot were kept in 1 ml of 0.25% Evans blue solution at room temperature for 20 min. By treating 80% ethanol the trapped Evans blue was released from the plant sample and then centrifuged it with 12,000 rpm for 8 min. After centrifugation, the optical density of the solution was monitored at 600 nm. Finally, the cell death analysis in plant tissue was calculated based on fresh weight.

2.9. Isolation of RNA and relative gene expression

Expression of OsPCS1, OsHMA3, OsNRAMP1, OsIRT1, OsFRO1, OsYSI15 transcripts was analyzed by quantitative reverse transcription PCR (qRT-PCR). Briefly, cleaned root and shoot tissues (50–60 mg) were mashed with liquid nitrogen to a fine powder using homogenizer. The total RNA extraction procedure was followed based on the SV Total RNA Isolation System (Cat no. A5001, Promega Corporation, USA). The quality of RNA samples was verified using 0.8% agarose gel electrophoresis and further quantified by NanoDrop 2000 (UV–Vis Spectrophotometer). Then the synthesized RNA was converted to the first-strand complementary DNA (cDNA) using MultiGene™Optimax Thermal Cycler (TC9610-230 V). After synthesizing the cDNA, it was treated with RNase enzyme for avoiding RNA contamination. Finally, we performed the real-time PCR analysis by Eco™ Real-Time PCR system provided by Illumina, USA. Supplementary Table S2 presented the sequences of primers used in the Real-Time PCR system. Real-time PCR data was standardized with Actin as an internal control using the following program: 3 min at 95°C, 40 cycles of 30 s at 94°C, 15 s at 53°C and 30 s at 720°C (Eco Software v4.0.7.0).

2.10. Determination of metabolites

Cysteine (Cys), glutathione (GSH) and phytochelatin (PC) were determined in roots and shoots by HPLC (high-performance liquid chromatography) technique using Empower3™ software (Kabir et al., 2015). A Waters 515 HPLC pump and Waters In-line degasser AF was connected with the HPLC system (HPLC of Binary Gradient System, Waters Corporation, Massachusetts, Milford, USA). The system was also attached to a C18 reverse phase-HPLC column (pore size: 300 A, particle size: 5 μm, pH Range: 1.5–10, Dimension: 250 mm × 10 mm) for compound separation. The mobile phase consisted of buffer A (0.1% TFA and water) and buffer B (0.1% TFA and 80% acetonitrile) at the gradient of 1–24 min 100% A, 25–34 min 100% B and 35–40 min 100% A. Before injection, samples and standards were diluted (100 ×) and filtered using 0.22 μm Minisart Syringe Filters. Cys, GSH and PC were tracked out with a Waters 2489 dual absorbance detector at 280 and 360 nm wavelength (Lindberg et al., 2007).

2.11. Analysis nitric oxide (NO) in plant tissues

Nitric oxide was measured in the rice tissue based on the ability of HbO2 (oxyhemoglobin) to become methHb (methemoglobin) in the presence of NO (Orozco-Cardenas and Ryan, 2002). Briefly, harvested plant samples homogenized in cooled nitric oxide buffer containing 1 M NaCl, 0.1 M C5H5NaO2, and 1% (w/v) ascorbic acid (pH 6.0). The samples were centrifuged at 12,000 rpm at 4°C for 5 min before pouring the clear supernatant in a centrifuge tube. Afterward, 5 mM stock solution of HbO2 was mixed with the samples and incubated for 5 min. The rate of HbO2 to metHb conversion was calculated by means of OD at 401 nm.

2.12. Analysis of antioxidant enzymes (SOD, CAT, APX, GR)

Enzymes were extracted according to Goud and Kachole (2012) with few adjustments. Briefly, tissues were ground with mortar and pestle using 100 mM phosphate buffer (pH 7.0) and centrifuged for 12 min at 6000 rpm to separate the supernatant. The analysis of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) was performed by spectrophotometer as previously described by Sun and Zigman (1978), Almeselmani et al. (2006), Goud and Kachole (2012) and Halliwell and Foyer (1978), respectively.

2.13. Reciprocal grafting of tolerant and sensitive genotypes

Reciprocal grafting between Sonarbangla and BRRI 72 was performed on very young plants (Kabir et al., 2017; Supplementary Fig. S2). Micro-grafting experiment was performed not only with Sonarbangla and BRRI-72 but also with their self-grafting to ensure that grafting methods or any other process were affecting the results or not. Initially, after two days emergence of the germinated seeds, the small stems of Sonarbangla and BRRI 72 were cut in an oblique direction (45° from the horizontal) above 0.4 cm. Scion and rootstock of each genotype (Sonarbangla and BRRI-72) were grafted in four combinations (BRRI 72 rootstock + BRRI 72 scion, Sonarbangla rootstock + Sonarbangla scion, BRRI 72 rootstock + Sonarbangla scion and Sonarbangla rootstock + BRRI 72 scion). Each graft was joined together using a 0.05 mm diameter capillary tube positioned over the graft. All the Grafted plants were then cultivated in hydroponic culture with or without Cd.

2.14. Statistical analysis

Experiments were designed in randomly blocked design having at least three independent biological samples. Student t-test was employed to reveal statistical significance (5% level) using SPSS (20th edition). Further, GraphPad Prism 6 was used to prepare graphical figures.

3. Results

3.1. Root, shoot and chlorophyll features

Morphological features and total chlorophyll (a and b) concentrations showed considerable variations in Cd-tolerant (Sonarbangla) and Cd-sensitive (BRRI 72) genotypes subjected to Cd (Table 1; Fig. 1). Sonarbangla demonstrated no significant changes because of Cd stress in root length, shoot height, root dry weight, shoot dry weight and total
3.5. H$_2$O$_2$ and MDA concentrations

The total soluble sugar showed no significant variations in the shoot of Sonarbangla compared with controls (Fig. 3). However, conversely, Cd stress caused a significant increase of total soluble sugar in the shoot of BRRI 72 compared with controls (Fig. 3). Further, total soluble sugar demonstrated no remarkable variations due to Cd supplementation compared to the plants cultivated without Cd (Table 1).

3.6. Relative expression of key genes

Sonarbangla showed significant upregulation in OsPCS1 and OsHMA3 transcripts in roots, while these two transcripts showed no changes in shoot subjected to Cd stress compared with control plants (Fig. 4). In addition, the expression of OsNRAMP1, OsIRT1, OsFRO1, and OsYSL15 did not show any changes in either root or shoot of Sonarbangla following Cd supply compared with controls (Fig. 4). In BRRI 72, the expression of OsPCS1 and OsHMA3 did not differ in root and shoot. However, OsNRAMP1, OsIRT1, OsFRO1, and OsYSL15 expression significantly induced in both root and shoot of BRRI 72 subjected to Cd supply in comparison with the plants cultivated without Cd (Fig. 4).

3.7. Analysis of Cys, GSH, PC, and NO

The concentration of Cys, GSH, PC, and NO significantly enhanced in roots of Sonarbangla, while these compounds were not significantly changed in roots of BRRI 72 subjected to Cd supply compared to controls (Fig. 5). In the shoot, Sonarbangla and BRRI 72 showed no meaningful changes in Cys, PC and NO concentration in shoot following Cd supply compared with controls. Further, Sonarbangla showed a significant increase in GSH concentration in shoot under Cd supplementation compared with controls. However, GSH concentration did not demonstrate any changes in the shoot of BRRI 72 subjected to Cd treatment compared with non-treated controls (Fig. 5).

3.8. Changes in antioxidant enzymes (CAT, APX, SOD, GR) and antioxidant capacity

Antioxidant enzymes showed distinct variations depends on genotypes and tissues under Cd stress. The CAT activity significantly raised in roots of both Sonarbangla and BRRI72 in reply to Cd supply compared with non-treated plants (Table 2). However, none of the genotypes showed significant changes in CAT activity in shoot due to Cd treatment compared with controls (Table 2). Further, the activity of APX significantly increased in both root and shoot of Sonarbangla following Cd supply compared with non-treated plants. However, BRRI 72 showed no significant alternations in either root or shoot due to Cd supply compared with controls (Table 2).

In addition, SOD activities did not show any distinction in neither root nor shoot of Sonarbangla and BRRI 72 due to Cd supplementation. Further, Sonarbangla demonstrated a significant induction in GR activity in root while this enzyme showed no significant changes in shoot following Cd supply compared with non-treated plants (Table 2). In contrast, the activity of GR did not differ in roots of BRRI 72 under Cd stress compared with controls. However, BRRI 72 demonstrated a significant decrease in GR activity in shoot in the presence of Cd compared with controls (Table 2). Also, non-enzymatic antioxidant capacity showed no significant changes in roots of any of the cultivars due to Cd.
Fig. 1. The phenotype, Cd and Fe concentrations in roots and shoot in Sonarbangla and BRRI 72 grown in the absence and presence (10 μM CdSO₄) of Cd. Different letters indicate significant differences between means ± SD of treatments (n = 3).
stress. However, this phenomenon showed a substantial induction in Sonarbangla shoot under Cd supply compared with controls. In addition, BRRI 72 showed no significant changes in non-enzymatic antioxidant capacity following Cd treatment compared with non-treated controls (Table 2).

3.9. Analysis of reciprocally grafted plants

Shoot height, root length, shoot dry weight and root dry weight in type 2 (self-grafted BRRI-72) and type 4 (grafting between BRRI 72 rootstock and Sonarbangla scion) showed noteworthy retardation under Cd supply to that of controls (Table 3, Supplemented Fig. S3). However, these were unaffected following Cd supply in either self-grafted Sonarbangla (Type 1) or in plants (Type 3) joined with Sonarbangla rootstock and BRRI-72 scion (Table 3, Supplemented Fig. S3). Further, type 1 and type 3 plants showed a decline in Cd translocation (16.04–16.53%), while this was considerably higher in type 2 and type 4 following Cd supply.

Further, reciprocally grafted plants between Cd-tolerant Sonarbangla and Cd-sensitive BRRI 72 showed differential H2O2 concentration and activity of FCR in roots due to Cd. H2O2 concentration and FCR activity demonstrated no noteworthy changes in roots of type 1 and 3; in contrast, these were significantly augmented in type 2 and 4 following Cd supply compared with the plants cultivated without Cd (Table 4).

4. Discussion

Cd tolerance is the coordination of several biochemical and molecular processes in plants (Lamhamdi et al., 2010; Zhang et al., 2009). However, substantial evidence associated with Cd tolerance in rice was lacking. In the present study, Cd stress caused severe morphological decrease and chlorophyll reduction in BRRI 72 but not in Sonarbangla. The Cd causes an adverse effect on chloroplast ultrastructure and
chlorophyll synthesis (Rahman et al., 2016; Pietrini et al., 2010). Further, electrolyte leakage (an indicator of plasma membrane stability) and total soluble protein showed distinct variations among the contrasting rice genotypes under Cd stress. The loss of chlorophyll and protein in rice could have been the adverse effect of free radicals under Cd stress (Chien et al., 2001). Further, Cd stress causes cell death in plants (De Michele et al., 2009). However, Cd stress was unable to hamper total soluble protein, cell and plasma membrane stability in root and shoot of Sonarbangla, while BRRI 72 plants reverse characteristics. Several studies reveal that Cd does have the inhibitory effect on protein synthesis related to energy and carbohydrate metabolism in plants (Roy et al., 2016; Muneer et al., 2014). Interestingly, maintenance of protein level, cell and membrane stability in Sonarbangla further suggests that this genotype has the efficiency to Cd-induced cellular damage and to maintain healthy morphological development. It is possible that metabolites and antioxidant enzymes may induce stress proteins associated with Cd stress (Lamhamdi et al., 2010).
We analyzed the Cd and Fe level in root and shoot to elucidate the uptake and transport mechanisms in rice over Cd stress. Although Cd concentration increased in roots of both genotypes, Sonarbangla demonstrated no noteworthy changes in shoot Cd level under Cd stress. It suggests that translocation of Cd in Sonarbangla is tightly regulated, but it further pinpoints that Sonarbangla also retains excess Cd in root system without causing damage. Cd retention in Sonarbangla was further explored by our biochemical and molecular analysis of PC. PC synthesis is responsible for the vacuolar sequestration in roots is one of the essential mechanisms mediating Cd detoxification in plants (Verbruggen et al., 2009; Rauser, 2003). In this present study, GSH and its derivatives PC significantly increased in Cd-tolerant Sonarbangla, while Cd-sensitive BRRI 72 did not show any variations in any of these elements due to Cd stress. GSH and PC mediated metal sequestration are subsequently deported to the vacuole, reducing the arbitrary metal ions in the cell cytosol (Noctor et al., 2012; Najmanova et al., 2012). Our biochemical evidence is consistent with our real-time PCR analysis of OsPCS1 transcript. In this study, the upregulation of OsHMA3 transporter in Sonarbangla roots pinpoints that this gene might possess differential Cd accumulation in two contrasting rice genotypes. The OsHMA3 is primarily expressed in rice roots (Ueno et al., 2010) and poses more Cd transportation to the vacuoles (Miyadate et al., 2011). These findings are in compliance with Zhang et al. (2009) demonstrating that Cd detoxification in rice is related to Cd retention and compartmentation in roots. Our further study disclosed the significant enhancement of NO in roots of Cd-tolerant Sonarbangla following Cd supply, suggesting that NO possibly enhances PC synthesis in rice plants to counteract excessive Cd in the root system. This is in conformity with the previous study on arsenic tolerance in rice (Singh et al., 2016). Collectively, these findings confirm that elevated PC possibly binds to excess Cd and accumulated safely in root vacuole away from the cell cytosol facilitated by NO in Sonarbangla.

Several studies demonstrate that Fe transporters are also responsible for Cd acquisition (Bashir et al., 2014; Takahashi et al., 2011; Nakamichi et al., 2006). Taking these into account, we performed extensive studies on Fe uptake mechanisms that may have possible roles on Cd-tolerance in rice. AAS study showed distinct variations in Fe uptake and translocation between Sonarbangla and BRRI 72 in response to Cd stress. No significant change was observed in either root or shoot in Sonarbangla in Fe level; while Fe concentration dramatically increased in BRRI 72, suggesting that Fe uptake and translocation may tightly regulate Cd tolerance in Sonarbangla. Chien et al. (2001) provide a preliminary indication that Cd-induced toxicity in rice plants may require the sharing of Fe, but mechanisms were not proposed. Also, ferric chelate reductase activity, an important strategy found in rice plants to convert Fe$^{3+}$ to more available Fe$^{2+}$ form was also studied. Our assay revealed that regulation of Fe reductase activity in rice tissue is simultaneously involved in Fe regulation along with the transporters. The increase of Fe reduction capacity is coherence with the previous studies Parsley exposed to Cd stress (Ulusu et al., 2017). At the molecular level, genes related to Fe availability (OsFRO1) and transport (OsNRAMP1, OsIRT1, and OsYSL15) showed no significant changes under Cd stress in Cd-tolerant Sonarbangla genetic line. Rice shows a direct system of Fe$^{2+}$ uptake mediated by Fe$^{2+}$ transporter OsIRT1 (Bughio et al., 2002). Studies also demonstrated that IRT1 could transport various metals (Fe, Zn, Mn, and Cd) in plants (Kim and Guerinot, 2007). Uraguchi et al. (2014) reported that OsLCT1, a low-affinity cation transporter functions in the rice node to accumulate Cd in grains. Since this gene is devoted explicitly to grain Cd accumulation, we did not take consider it in our investigations. Our findings suggest that regulation of Fe acquisition and translocation coordinated by Fe chelate reductase activity and Fe-carriers confer a fundamental part of Cd-tolerance in Sonarbangla.

During metal stress, O$_2$ can be converted to superoxide anion (O$_2^{-}$) and H$_2$O which are often harmful to plant cells. Further, excessive accumulation of ROS causes a dramatic increase in lipid peroxide, resulting in cell membrane destruction (Cuypers et al., 2016; Sharma and Dietz, 2009). Here, H$_2$O$_2$ and MDA (an indicator of lipid peroxidase) significantly increased in Cd-sensitive BRRI 72 genotypes, while...
Fig. 5. Analysis of Cys, GSH, PC and NO in root and shoot of Sonarbangla and BRRI 72 cultivated in the absence and presence (10 μM CdSO₄) of Cd. Different letters indicate significant differences between means ± SD of treatments (n = 3).
Since SOD is considered to be the leading edge inhibitor for superoxide toxicity and MDA content at the same time. To withstand cellular damage, plants perhaps transmit signal in diverse ways depending on the participation of NO in ROS scavenging under Cd stress, which is associated with the oxidative pentose phosphate pathway (Bolouri-Moghaddam et al., 2010; Van den Ende and Valluru, 2009). Conversely, both high and low sugar accumulation may also provoke ROS accumulation by disturbing re- spiratory metabolism in plants (Xian et al., 2011; Couée et al., 2006). Apart from this, excess sugar accumulation in the shoot of Sonarbangla may also participate in reducing H2O2 and oxidative stress to withstand Cd toxicity. Sugar availability is involved in H2O2 scavenging linked to the oxidative pentose phosphate pathway (Bolouri-Moghaddam et al., 2010; Van den Ende and Valluru, 2009). This is associated with the indirect or direct signaling linked with ROS scavenging enzymes (Van den Ende and Valluru, 2009). Conversely, both high and low sugar accumulation may also provoke ROS accumulation by disturbing respiratory metabolism in plants (Xian et al., 2011; Couée et al., 2006). The participation of NO in ROS scavenging under Cd stress is previously reported (Hsu and Kao, 2004), which is in agreement with our findings. In this study, it is evident that scavenging of ROS through the induction of CAT, APX, GR, sugar, NO and non-enzymatic scavengers plays critical roles, at least in part to withstand Cdtoxicity in Cd-tolerant Sonarbangla. Increased DPPH is an indicator of tolerance in response to stress in plants (Doganlar et al., 2012; Kang and Saltveit, 2002). Apart from this, excess sugar accumulation in the shoot of Sonarbangla may also participate in reducing H2O2 and oxidative stress to withstand Cd toxicity. Sugar availability is involved in H2O2 scavenging linked to the oxidative pentose phosphate pathway (Bolouri-Moghaddam et al., 2010; Van den Ende and Valluru, 2009). Conversely, both high and low sugar accumulation may also provoke ROS accumulation by disturbing respiratory metabolism in plants (Xian et al., 2011; Couée et al., 2006). The participation of NO in ROS scavenging under Cd stress is previously reported (Hsu and Kao, 2004), which is in agreement with our findings. In this study, it is evident that scavenging of ROS through the induction of CAT, APX, GR, sugar, NO and non-enzymatic scavengers plays critical roles, at least in part to withstand Cd toxicity in Cd-tolerant Sonarbangla.

Although root is the principal location through which heavy metals enter, plants perhaps transmit signal in diverse ways depending on heavy metals. In this study, Sonarbangla type grafts tolerant to Cd stress, as evident by root H2O2 and Fe reductase activity, was observed in grafted plants comprising Sonarbangla rootstock (type 1 and 3) regardless of the part used for Scion. In contrast, grafts exhibited BRRI 72 rootstock (Type 2 and 4) showed elevated H2O2 and Fe reductase...
activity in roots which were related to Cd sensitivity. Sonarbangla roots may carry signal to govern the responses associated with Cd tolerance. Previously, Guimarães et al. (2009) demonstrated that Zn hyper-accumulation in the shoot of Thlaspi caerulescens is principally governed by its root system. It is also evident that BRRI 33 roots are inefficient to sense/transmit signal triggering Cd tolerance. This novel grafting experiment will be valuable for research involving metal-induced signaling in rice.

5. Conclusion

The disparity of tolerance in rice in response to Cd stress is associated with complex mechanisms. Morphological features and stress indicators reveal that Sonarbangla possesses adaptive mechanisms to manage with high Cd, while the Cd-sensitive BRRI 72 shows severe growth and cell retardation. Analysis of Cd and Fe indicates the involvement of Cd sequestration and Fe regulation in the presence of Cd in Sonarbangla, which was then supported by the molecular evidence of vacuolar sequestration (OsPCS1, OsHMA3) and Fe-related genes. Also, the enzymatic analysis suggests Cd tolerance in Sonarbangla is also linked to the induction of CAT, APX, GR and sugar leading H$_2$O$_2$ scavenging. Interestingly, reciprocally grafting of contrasting genotypes revealed the fact that it is the root that regulates Cd toxicity tolerance in Sonarbangla. Taken together, this study reveals the following complex mechanisms to alleviate Cd stress in Sonarbangla (Fig. 6): (i) PM-mediated vacuolar sequestration of Cd in roots, (ii) limiting Cd uptake through the regulation of Fe transporters and Fe chelate reductase activity, (iii) scavenging of ROS through the increased antioxidant defense (iv) root-originated signal driving tolerance in response to Cd. This paper presents advance knowledge on the mechanistic basis of Cd uptake and tolerance in rice. Molecular findings of candidate genes will be useful to produce Cd-tolerant transgenic rice for health safety and phytoremediation.

Contributions

MAB performed all the experiments and prepared the draft manuscript. MSA helped in a few experiments. MAR provided advice and few research facilities. AHK supervised the whole work and revised the manuscript.

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Appendix A. Supplementary data

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