RESEARCH PAPER

Physiological, genetic, and molecular characterization of a high-Cd-accumulating rice cultivar, Jarjan

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Abstract

Cadmium (Cd) in rice is a major source of Cd intake for people on a staple rice diet. The mechanisms underlying Cd accumulation in rice plant are still poorly understood. Here, we characterized the physiology and genetics of Cd transport in a high-Cd-accumulating cultivar (Jarjan) of rice (Oryza sativa). Jarjan showed 5- to 34-fold higher Cd accumulation in the shoots and grains than the cultivar Nipponbare, when it was grown in either a non-Cd-contaminated or a Cd-contaminated soil. A short-term uptake experiment showed no significant difference in Cd uptake by the roots between the two cultivars. However, Jarjan translocated 49% of the total Cd taken up to the shoots, whereas Nipponbare retained most of the Cd in the roots. In both concentration- and time-dependent experiments, Jarjan showed a superior capacity for root-to-shoot translocation of Cd. These results indicate that the high-Cd-accumulation phenotype in Jarjan results from efficient translocation of Cd from roots to shoots. Genetic analysis using an F2 population derived from Jarjan and Nipponbare revealed that plants showing high- and low-Cd-accumulation phenotypes segregated in a 1:3 ratio, indicating that high accumulation in Jarjan is controlled by a single recessive gene. Furthermore, we isolated OsHMA3, a gene encoding a tonoplast-localized Cd transporter from Jarjan. The OsHMA3 protein was localized in all roots cells, but the sequence has a mutation leading to loss of function. Therefore, failure to sequester Cd into the root vacuoles by OsHMA3 is probably responsible for high Cd accumulation in Jarjan.

Key words: Cadmium accumulation, OsHMA3, rice, sequestration, transport, vacuole.

Introduction

Cadmium (Cd) is one of the most toxic heavy metals for animals and plants. Many agricultural soils are contaminated with Cd due to anthropogenic inputs such as smelting, fossil fuel burning, and application of sewage sludge and phosphate fertilizers (Allaway and Steinnes, 1999; Kabata-Pendias, 2001). Crops produced on Cd-contaminated soils may contain elevated concentrations of Cd in the edible parts, leading to a risk of chronic toxicity with excessive intake (Grant et al., 2008). This is an especially serious case for rice because it is a staple food for nearly half the world’s population and is the largest source of dietary intake of Cd (Watanabe et al., 2004; Cheng et al., 2006). Therefore, minimizing Cd accumulation in rice from Cd-contaminated soil is a very important issue of food safety. However, the mechanisms underlying Cd accumulation in rice are still poorly understood.

There is a large genotypic variation in Cd accumulation in rice. Arao and Ae (2003) found a 23-fold difference in the grain Cd concentration among 49 rice cultivars grown on a Cd-contaminated soil. He et al. (2006) reported that the Cd concentration in brown rice ranged from 0.06 to 0.99 mg kg\(^{-1}\) in 38 cultivars. Liu et al. (2005) reported a variation in polished rice grain from 0.14 to 1.43 mg Cd kg\(^{-1}\). Recently, Ueno et al. (2009b) investigated genotypic variation in shoot Cd concentration using a rice core collection including 146 accessions, which represent minimal genetic
repetitiveness and cover the majority of the range of rice genetic diversity (Kojima et al., 2005; Ebana et al., 2008). They found a 13-fold difference in the shoot Cd concentration between the lowest and highest Cd-accumulating accessions (Ueno et al., 2009b).

Based on this genotypic variation, a number of quantitative trait loci (QTLs) for Cd accumulation have been identified. Three putative QTLs were detected on chromosomes 3, 6, and 8, for Cd concentration in brown rice by using chromosome segment substitution lines (CSSLs) derived from the cultivars Koshihikari and Kasalath (Ishikawa et al., 2005). By using a similar approach with the Kasalath/Nipponbare backcross inbred lines (BILs), Kashiwagi et al. (2009) identified three QTLs for Cd concentration in the upper plant parts of rice; two on chromosome 4 and one on chromosome 11. Xue et al. (2009) located a QTL for shoot Cd concentration on chromosome 7 using a double haploid population derived from a cross between the cultivars JX17 and ZYQ8. A major QTL controlling shoot Cd concentration was also detected on chromosome 11 in a population derived from two contrasting rice cultivars, Badari Dhan and Shwe War (Ueno et al., 2009b). Recently, a QTL for Cd accumulation showing a large effect was detected at a similar position on chromosome 7 in three different studies using different mapping populations (Ueno et al., 2009a; Ishikawa et al., 2010; Tezuka et al., 2010). Furthermore, the gene responsible for this QTL detected from the Anjana Dhan/Nipponbare population has been isolated and characterized (Ueno et al., 2010). This gene (OsHMA3) encodes a protein belonging to the family of P-type ATPases, which is localized at the tonoplast of all root cells. The gene from the low-Cd-accumulating cultivar is functional, whereas that from the high-Cd-accumulating cultivar is not (Ueno et al., 2010). Therefore, the loss of function to sequestrate Cd into the root vacuoles results in high root-to-shoot translocation in the high-Cd-accumulating cultivar Anjana Dhan.

Jarjan, an indica cultivar from Bhutan, also showed very high Cd accumulation in the shoots, being the highest among 146 accessions from a rice core collection (Ueno et al., 2009b). In this study, we characterized this cultivar in terms of physiological, genetic, and molecular attributes determining Cd accumulation. We found that a loss of function of OsHMA3, a tonoplast-localized Cd transporter, may be responsible for the high accumulation of Cd in this cultivar, similar to that in Anjana Dhan.

Materials and methods

Plant culture

Two rice (Oryza sativa L.) cultivars, Jarjan (indica, high Cd), and Nipponbare (japonica, low Cd), were used for all experiments in the present study. In hydroponic culture, seeds of both cultivars were soaked in tap water and germinated at 30 °C in the dark. The seedlings were transferred to nylon nets floating on 1 l of 0.5 mM CaCl₂ (pH 5.6) and grown for 5 d. After that, the seedlings were precultured in a 1/2 Kimura B nutrient solution (pH 5.4) containing the following macronutrients (mM): MgSO₄ (0.28), (NH₄)₂SO₄ (0.18), Ca(NO₃)₂ (0.18), KNO₃ (0.09), and KH₂PO₄ (0.09); and micronutrients (μM): Fe(II)SO₄ (10), H₂BO₃ (3), MnCl₂ (0.5), CuSO₄ (0.2), ZnSO₄ (0.4), and (NH₄)₆Mo₇O₂₄ (1). The solution was renewed every 2 d. All pot experiments were conducted in a glasshouse under natural sunlight at 20–30 °C.

Accumulation of Cd and other metals

The plants harvested in the paddy field (not contaminated with Cd) at the Institute of Plant Science and Resources, Okayama University, were used for analyses of the accumulation of Cd and other macro- or micro-elements. The seedlings were planted in mid-June 2008, and harvested in late September. Straw and brown rice were separated for the determination of elemental concentrations.

Cd accumulation by the two cultivars was also examined in a Cd-contaminated soil. Seeds were germinated in a pot (two plants per pot) containing 3.6 kg of soil enriched with Cd (as CdSO₄) on 17 May 2009. The 0.1 N HCl extractable Cd in the soil was 6.3 mg Cd kg⁻¹ soil. An N-P-K compound fertilizer (14-14-14) was applied to the soil at 2 g kg⁻¹ soil. The seedlings were cultured under continuously aerobic conditions. The shoots were harvested on 13 August 2009. The Cd concentration in the shoots was determined as described below.

Distribution of Cd in plants

Seedlings (18 d old) were exposed to 0.05 μM Cd in the 1/2 Kimura nutrient solution. After 10 d, the roots were washed with deionized water twice, and the shoots and roots were subjected to determination of Cd concentration as described below.

Cd uptake experiment

To investigate short-term Cd uptake by roots, five seedlings (21 d old) of each cultivar were transferred to a 3.5-l pot (10 plants per pot) and exposed to an uptake solution (pH 5.4) containing various concentrations of Cd (0, 0.2, 0.5, 1, 2, and 5 μM) at 25 °C and 2 °C (ice-cooled) for 20 min (Ueno et al., 2009b). The roots were then washed with 5 mM CaCl₂ buffered at pH 5.4 with 5 mM 2-(N-morpholino)-ethanesulfonic acid (MES) and distilled water, and then subjected to determination of Cd concentration as described below. All physiological experiments were repeated independently at least three times.

Concentration-dependent analysis of Cd translocation and accumulation

Seedlings (15 d old) were transferred to a 1.2-l pot (five plants per pot) and grown for another 18 d. After that, the seedlings were exposed to a nutrient solution (pH 5.4) containing different concentrations of Cd (0, 0.05, 0.1, 0.2, and 0.5 μM). The solution was renewed every 24 h. After 78 h, xylem sap was collected as described below, and then the roots were washed twice with deionized water. Cd concentration in the xylem sap, roots, and shoots was determined as described below.

Time-course experiment of Cd translocation and accumulation

Seedlings (13 d old) were transferred to a 3.5-l pot (15 plants per pot) and cultured for a further 15 d. The seedlings were then exposed to a nutrient solution containing 0.1 μM Cd. The roots and shoots were harvested at 0, 1, 2, 3, and 5 d after Cd treatment, washed with deionized water, and subjected to determination of Cd concentration.

Xylem sap collection

The shoots were excised 2 cm above the roots with a razor, and xylem sap was collected from the cut surface for 10 min using
micropipettes. To avoid contamination of symplastic Cd from the damaged cells, initial exudates (1–2 μl) were discarded.

Genetic analysis of shoot Cd concentration
For genetic analysis, Jarjan was crossed to Nipponbare and an F2 population was generated. Seedlings of 92 F2 plants were preincubated in a container (46 F2 with two parents per pot) filled with 10 l of the nutrient solution. After 14 d, the seedlings were treated with 0.05 μM Cd for 10 d. The shoots were then harvested and analysed for Cd concentration.

A cross between Jarjan and Anjana Dhan, another high-Cd-accumulating cultivar, was also performed. To compare Cd accumulation, seedling of F1 plants, Anjana Dhan, Jarjan, and Nipponbare (11 d old) were exposed to a nutrient solution containing 0.05 μM Cd for 10 d. The roots were washed with deionized water twice before harvest, and then separated from the shoots.

Cloning of OsHMA3 from Jarjan
To obtain the sequence of OsHMA3 from Jarjan, total RNA was extracted with an RNeasy Plant Mini Kit (Qagen). After the reaction of DNase I (Invitrogen, http://www.invitrogen.com/), the total RNA was converted to cDNA using the protocol attached to SuperScript II (Invitrogen). The full-length OsHMA3j (j denotes Jarjan) was amplified by RT-PCR using primers 5'-TTACCGGT-CATATTGCAACATCA-3' and 5'-GGGGTGGAACGAGCG-ACGGCGATG-3', designed according to the sequence of 5'- and 3'-untranslated region (UTR) of OsHMA3a, which were previously cloned from Anjana Dhan (Ueno et al., 2010). The sequence of OsHMA3j was determined using BigDye sequencing kit (Applied Biosystems, http://www.appliedbiosystems.com/) with gene-specific primers on an ABI 3130 Genetic Analyzer (Applied Biosystems).

Real time RT-PCR
To investigate the expression level of OsHMA3, total RNA was extracted from the roots and shoots of Nipponbare, Jarjan, and Anjana Dhan (12 d old) exposed to 0 or 1 μM CdSO4 for 24 h. The expression levels were analysed using Thunderbird™ pQPCR Mix (Toyobo, http://www.toyobo.co.jp/) with the following primers: 5'-TTACATCCAACACACCCGAAA-3' and 5'-GGGGTGGAACGAGCG-ACGGCGATG-3', designed according to the sequence of 5'- and 3'-untranslated region (UTR) of OsHMA3a. The expression levels of OsHMA3 were normalised to the expression levels of 35S actin, which were preamplified with primers 5'-TCCATCCAACACACCCGAAA-3' and 5'-GGGGTGGAACGAGCG-ACGGCGATG-3', designed according to the sequence of 5'- and 3'-untranslated region (UTR) of OsHMA3a, which were previously cloned from Anjana Dhan (Ueno et al., 2010).

Determination of metal concentration
Plant samples were dried at 70 °C and then digested with concentrated nitric acid (60%) at 140 °C. The concentrations of Cd and other metals in plant digests, xylem sap, and 0.1 N HCl extracted soil solution were determined by flame atomic absorption spectrometry (Hitachi Z-2000; Hitachi, Tokyo, Japan), after dilution with 0.1 N HNO3 to optimal concentration.

Cellular localization of OsHMA3
Cellular localization of OsHMA3 was investigated with immunostaining. The synthetic peptide C-CAKTMNGEIVK (positions 993–1004 of OsHMA3n was used to immunize rabbits to obtain antibodies to OsHMA3 as described previously (Ueno et al., 2010). Immunostaining was performed according to Yamaji and Ma (2007). Fluorescence of secondary antibody (Alexa Fluor 555 goat anti-rabbit IgG; Molecular Probes) was observed with a confocal laser scanning microscopy (LSM700; Carl Zeiss).

Modelling of metal speciation and statistical analysis
Free Cd2+ concentrations in the nutrient solutions were calculated using GEOCHEM-EZ (Shaif et al., 2010). Analysis of variance (ANOVA) was performed on all data sets. Tukey’s honest significant difference test was used to determine the significance of the difference between cultivars.

Results
Physiological characterization of a high-Cd-accumulating cultivar, Jarjan
Jarjan showed high Cd accumulation in the shoots in a hydroponic experiment (Ueno et al., 2009b). To confirm this trait, we compared Cd accumulation in Jarjan with that in Nipponbare grown in a Cd-uncontaminated field. Jarjan accumulated 34- and 18-times higher Cd concentrations in the shoots (stem, leaf sheath, and blade) and brown rice, respectively, than Nipponbare (Fig. 1A). However, both cultivars accumulated similar levels of micronutrients (Zn, Fe, Mn, and Cu) and macronutrients (K, Mg, and Ca) (Fig. 1B, C). When the two cultivars were grown in a Cd-contaminated soil in greenhouse, Jarjan also showed significantly higher Cd accumulation in the shoots (Fig. 1D).

To dissect the mechanism underlying the different Cd accumulation between the two cultivars, distribution of Cd in the roots and shoots was compared. Approximately 49% of the total Cd taken up by Jarjan was distributed to the shoots (Fig. 2). In contrast in Nipponbare, most Cd (99%) was retained in the roots.

A short-term (20 min) uptake experiment showed that an apparent uptake of Cd at both 25 °C and 2 °C was greater in Nipponbare than in Jarjan (Fig. 3A), but the net uptake, calculated by subtracting the uptake rate at 2 °C from that at 25 °C, was similar (Fig. 3B). The larger apparent uptake in Nipponbare is probably due to a larger capacity of Cd for cell wall binding. The values of Vmax and Km were computed to be 44.5±2.4 μg g−1 root dry weight (DW) and 1.1±0.1 μM in Jarjan, and 41.0±3.1 μg g−1 root DW and 0.6±0.1 μM in Nipponbare, respectively. These results indicate that there was no significant difference in Cd uptake by roots between Jarjan and Nipponbare.

In a further experiment with increasing concentrations of Cd in the uptake solution, the Cd concentration in the xylem sap was higher in Jarjan than in Nipponbare in all Cd concentrations tested (Fig. 4A). The shoot Cd concentrations were also higher in Jarjan than in Nipponbare (Fig. 4B). In contrast, the Cd concentrations in the roots were ~2-fold higher in Nipponbare than in Jarjan (Fig. 4C). The distribution of Cd to the shoots was 7.9–21.0% in Jarjan, compared with 1.3–1.7% in Nipponbare (Fig. 4D).

Further investigation with a time-course experiment showed that significant cultivar differences in Cd concentration in the xylem sap and roots were observed at all time points from 1 to 5 d after exposure to Cd (Fig. 5A, C), and in the shoots at 2–5 d (Fig. 5B). The Cd concentrations in the xylem sap and shoots of Jarjan increased with the length...
of time of Cd exposure and showed higher levels than those of Nipponbare (Fig. 5A, B). In contrast, the root Cd concentration was much higher in Nipponbare than in Jarjan; in both cultivars the concentration increased with exposure time (Fig. 5C). The distribution of total Cd to the shoots of Jarjan increased from 12% on day 1 to 27% on day 5; this compares with <4% in Nipponbare at all time points (Fig. 5D).

Genetic analysis of Cd accumulation in Jarjan

To examine the genetic basis of shoot Cd accumulation, segregation analysis was performed by using an F2 population derived from a cross between Jarjan and Nipponbare. Of 92 F2 seedlings, 74 plants showed low Cd concentrations (2.1–7.7 µg Cd g⁻¹ DW) and 18 plants displayed high Cd concentrations (8.2–18.9 µg Cd g⁻¹ DW) in the shoots (Fig. 6); this segregation pattern is consistent with a 1:3 ratio ($\chi^2=1.17$, 0.25<$P<$0.50), indicating that the high-Cd-accumulation phenotype in Jarjan is controlled by a single recessive gene.
Molecular characterization of Cd accumulation in Jarjan

Recently, it was reported that the loss of function of OsHMA3, a tonoplast-localized transporter for sequestration of Cd into vacuoles, is responsible for high Cd accumulation in the rice cultivar Anjana Dhan (Ueno et al., 2010). To examine whether this gene is also involved in high Cd accumulation in Jarjan, we isolated and sequenced this gene. The sequence of OsHMA3 from Jarjan was exactly the same as that from Anjana Dhan (see Supplementary Fig. S1) (Supplementary data are available at JXB online). This result suggests that loss of function of OsHMA3 is also responsible for high Cd accumulation in Jarjan.

To confirm this, Jarjan was crossed to Anjana Dhan. The concentrations of Cd in both the roots and shoots were similar between Anjana Dhan, Jarjan, and the generated F1 plants (Fig. 7). This pattern indicates that high Cd accumulation of both Jarjan and Anjana Dhan results from the loss of function of the same gene, OsHMA3.

The expression level of OsHMA3 was compared with that in Nipponbare and Anjana Dhan. OsHMA3 was dominantly expressed in the roots, with the level of expression in Jarjan being slightly higher ($P<0.01$) than that in Anjana Dhan and Nipponbare (Fig. 8). Furthermore, expression levels in both roots and shoots were not significantly affected by exposure to Cd (Fig. 8).
The localization of OsHMA3 in Jarjan roots was also investigated with immunostaining using an anti-OsHMA3 antibody. In both root tip (5 mm from the tip) and mature root zone (30 mm from the tip), OsHMA3 was localized in all root cells, especially the sclerenchyma cells, which showed the highest expression level (Fig. 9).

Discussion

Efficient root-to-shoot translocation contributes to high Cd accumulation in Jarjan

Jarjan showed high Cd accumulation in both straw and grains when grown in either Cd-contaminated or uncontaminated soils (Fig. 1), confirming previous screening results from hydroponic experiments (Ueno et al., 2009b). Furthermore, this trait is likely to be specific for Cd because there was no difference in the concentrations of other macro- and micronutrients (Fig. 1). Several processes may contribute to high accumulation of Cd in shoots, including efficient root uptake and/or efficient xylem loading. There was no difference in the short-term uptake of Cd between Jarjan and Nipponbare (Fig. 3), but the Cd concentration in the xylem sap was much higher in Jarjan than in Nipponbare (Figs 4, 5). As a result, a high proportion of the total Cd taken up by the roots was distributed to the shoots in Jarjan (Figs 2, 4, 5), whereas Nipponbare retained most of the Cd taken up in the roots (Figs 2, 4, 5). These results indicate that the difference in Cd accumulation between the high- and low-Cd-accumulating rice cultivars is not due to different capacities of the roots to take up Cd, but due to different efficiencies in Cd translocation from roots to shoots.

Efficient Cd translocation was also observed in other high-Cd-accumulating rice cultivars including Anjana Dhan, Badari Dhan, Cho-Ko-Koku, and Habataki (Ueno et al., 2009a, b; Uraguchi et al., 2009; Tezuka et al., 2010). Uraguchi et al. (2009) found good correlation between Cd concentrations in xylem sap and in shoots among 69 rice cultivars. Although Anjana Dhan showed a lower Cd uptake capacity by the roots than Nipponbare (Ueno et al., 2009b), Cd accumulation in the shoots was similar between Anjana Dhan and Jarjan, indicating that root-to-shoot translocation is a key factor determining Cd accumulation in the above-ground part. Similarly, Harris and Taylor (2004) reported that the difference in shoot Cd concentration between two near-isogenic durum wheat cultivars also results from different root-to-shoot Cd translocation rather than root uptake.

OsHMA3 is probably responsible for high Cd accumulation in Jarjan

Root-to-shoot translocation of Cd may be controlled by processes such as xylem loading and vacuolar sequestration. Several transporters involved in the xylem loading of Cd have been identified in Arabidopsis thaliana and some hyperaccumulators. For example, AtHMA2 and AtHMA4, which are members of the P-type ATPase family of proteins, were involved in xylem loading of Cd and Zn in A. thaliana (Hussain et al., 2004; Verret et al., 2004; Wong and Cobbett, 2009). In Cd/Zn hyperaccumulators Thlaspi caerulescens and Arabidopsis halleri, homologues of AtHMA4 are highly and constitutively expressed and contribute to Cd and Zn hyperaccumulation (Bernard et al., 2004; Papoyan and Kochian, 2004; Hanikenne et al., 2008). Rice has eight members of the HMA family of proteins (OsHMA1–8); OsHMA1–3 are classified into the Cd/Zn/Co/Pb transport subgroup, while OsHMA5–8 belong to the Cu/Ag transport subgroup (Williams and Mills,
Although it is still not known which HMA genes in rice are involved in the xylem loading of Cd, it is unlikely that transporters involved in xylem loading are responsible for high Cd accumulation in Jarjan as discussed below.

In the Zn hyperaccumulator T. caerulescens, efficient root-to-shoot translocation of Zn has been partly attributed to a smaller vacuolar sequestration of Zn in the root cells (Lasat et al., 1998). Recently, a rice OsHMA3 has been demonstrated to be involved in the vacuolar sequestration of Cd (Ueno et al., 2010). OsHMA3 is localized to the tonoplast of all root cells. There was no difference in expression level, cellular or subcellular localization of OsHMA3 between the low- and high-Cd-accumulating rice cultivars Nipponbare and Anjana Dhan, respectively (Ueno et al., 2010). However, OsHMA3 in Anjana Dhan is not functional due to an amino acid mutation at position 80 (Ueno et al., 2010); consequently, Cd becomes much more mobile from roots to shoots in this cultivar (Ueno et al., 2009b). Because the phenotypes of Cd accumulation are similar between Jarjan and Anjana Dhan (Figs 1, 3, 4; Ueno et al., 2009b) and, furthermore, high Cd accumulation in Jarjan was also controlled by a single recessive gene (Fig. 6), we examined whether loss of function of OsHMA3 is also the reason behind the phenotype observed in Jarjan, as in Anjana Dhan. The sequence of OsHMA3 isolated from Jarjan is an exact match of that from Anjana Dhan (see Supplementary Fig. S1). OsHMA3 from Jarjan also showed the same tissue and cellular localization, and expression pattern as OsHMA3 from Anjana Dhan (Figs 8, 9; Ueno et al., 2010). Therefore, OsHMA3 from Jarjan, like that from Anjana Dhan, is expected to be non-functional with regard to Cd transport across the tonoplast, and consequently, less Cd is sequestered in the root vacuoles and more is available for translocation from roots to shoots. This conclusion is also supported by a crossing analysis between Anjana Dhan and Jarjan: the F1 seedlings showed a Cd accumulation pattern similar to that of their parents Anjana Dhan and Jarjan (Fig. 7).

In summary, our results indicate that efficient root-to-shoot Cd translocation is responsible for high Cd accumulation in the rice cultivar Jarjan. This trait is most likely due to the loss of function of OsHMA3, which is a tonoplast-localized transporter of Cd in the roots.

Supplementary data
The following supplementary data are available at JXB online.

Supplementary Fig. S1. Alignment of amino acid sequences for OsHMA3 from the rice cultivars Jarjan (OsHMA3j), Anjana Dhan (OsHMA3a), and Nipponbare (OsHMA3n).

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