RESEARCH PAPER

A major quantitative trait locus for increasing cadmium-specific concentration in rice grain is located on the short arm of chromosome 7

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Abstract

Large phenotypic variations in the cadmium (Cd) concentration of rice grains and shoots have been observed. However, the genetic control of Cd accumulation remains poorly understood. Quantitative trait loci (QTLs) determining the grain Cd concentration of rice grown in a Cd-polluted paddy field were identified. Using a mapping population consisting of 85 backcross inbred lines derived from a cross between the low-Cd-accumulating cultivar Sasanishiki (japonica) and high-Cd-accumulating cultivar Habataki (indica), two QTLs for increasing grain Cd concentration were found on chromosomes 2 and 7. A major-effect QTL, qGCd7 (QTL for grain Cd on chromosome 7), was detected on the short arm of chromosome 7. It accounted for 35.5% of all phenotypic variance in backcross inbred lines. qGCd7 was not genetically related to any QTLs for concentrations of essential trace metals (Cu, Fe, Mn, and Zn) or those for agronomic traits such as heading date, suggesting that this QTL is specific to Cd. Furthermore, the existence of qGCd7 was confirmed using chromosome segment substitution lines (CSSLs) and an F₂ population from a cross between the target CSSL and Sasanishiki grown in a Cd-polluted paddy soil. To our knowledge, qGCd7 is a novel QTL with major effects for increasing grain Cd concentrations.

Key words: Advanced mapping population, cadmium, essential trace metals, Oryza sativa L., quantitative trait loci.

Introduction

Since cadmium (Cd) was first officially recognized as a possible causative toxicant of Itai-itai disease in Japan in 1968, Cd contamination of rice—the major source of Cd dietary intake for Japanese people—has been monitored in accordance with the Agricultural Land–Soil Pollution Prevention Law established in 1970. Recently, the Codex Alimentarius Commission of the FAO/WHO determined the official maximum allowable limit of Cd concentration as 0.4 mg kg⁻¹ of polished rice (CODEX STAN 193-1995, 2008). On the basis of this international criterion for rice Cd, the Japanese government has discussed whether the domestic standard of Cd should be changed from 1 mg kg⁻¹ to 0.4 mg kg⁻¹ for unpolished rice. Thus, it is urgently necessary to develop agronomic management practices and low-Cd-accumulating cultivars to comply with the new standard for rice Cd concentration that will soon be established in Japan.

Large varietal differences in Cd concentrations of the aerial parts of rice, including grains, have been reported (Morishita et al., 1987; Arao and Ishikawa, 2006; Uraguchi et al., 2009). Generally, Cd concentrations are higher in indica-type rice varieties than in japonica-type ones. Using several rice varieties that accumulate high levels of Cd in shoots, a practical phytoextraction of Cd-polluted paddy...
fields has been launched (Ibaraki et al., 2009; Murakami et al., 2009). On the other hand, developing rice cultivars that accumulate only low levels of Cd in grains may help to minimize the dietary intake of Cd (Grant et al., 2008). Whether developing rice cultivars with high-Cd traits for phytoextraction or those with low-Cd traits for use in the diet, it is important to understand the genetic control mechanisms of Cd accumulation in rice.

Quantitative trait locus (QTL) mapping is a powerful tool for understanding the genetic controls underlying complex traits, many of which are important for agriculture (Yano and Sasaki, 1997; Yamamoto et al., 2009). QTL mapping has been used extensively to identify genetic loci determining mineral uptake and stress in rice (Wissuwa et al., 1998; Ishikawa et al., 2005; Cho et al., 2007; Lu et al., 2008; Ueno et al., 2009). Ishikawa et al. (2005) first reported that three putative QTLs controlling Cd concentration in brown rice were mapped on chromosomes 3, 6, and 8, and Kashiwagi et al. (2009) found that the interaction of two putative QTLs on chromosome 4 increased Cd concentration in brown rice. Xue et al. (2009) mapped six QTLs associated with Cd tolerance and three QTLs for root and shoot Cd concentrations during the seedling stage. A major QTL controlling the translocation of Cd from roots to shoots at the seedling stage was mapped on chromosome 11 (Ueno et al., 2009).

Although our knowledge of the genetic control of Cd uptake and accumulation in rice has gradually increased, these QTL effects have not yet been evaluated in Cd-polluted fields. Rice Cd uptake is greatly influenced by soil properties such as pH, clay content, organic carbon content, cation exchange capacity, and the coexistence of other heavy metals. Furthermore, soil redox potential is the most important factor controlling bioavailable Cd. Redox potential often varies in a paddy field depending on the extent of irrigation or rainfall (limura, 1981). Under such complex conditions, the detection of QTLs with stable effects is necessary for their practical use in agriculture.

In 69 rice varieties cultivated in Cd-polluted paddy soil, positive correlations were observed between the accumulation of Cd and that of essential trace metals (Cu, Zn, and Mn) by young shoots, except in the case of some varieties that accumulated extremely high levels of Cd (Uraguchi et al., 2009). Furthermore, studies have indicated that the rice Fe transporters OsIRT1 and OsIRT2 and Zn transporter OsZIP1 can mediate Cd uptake by roots (Ramesh et al., 2003; Nakanishi et al., 2006). In Arabidopsis thaliana, studies have shown that a P-type ATPase, AtHMA4, plays a role in the xylem loading of Cd as well as Zn (Verret et al., 2004). Thus, physiological and molecular experiments suggest that Cd accumulation is closely related to the transport of essential trace metals, but the genetic relationship between Cd and such metals, especially in rice grains, has not been investigated to date.

A previous study found a significant phenotypic correlation between grain Cd concentration and days to heading (DTH) in 39 chromosome segment substitution lines (CSSLs) in which a Koshihikari (japonica) allele was replaced by a Kasalath ( indica) allele (Ishikawa et al., 2005). Subsequent QTL analyses revealed that chromosomal regions with QTLs related to grain Cd concentration contained QTLs for DTH: Hdh on chromosome 3, Hd1 on chromosome 6, and Hd5 on chromosome 8 (Yano et al., 2001; Ishikawa et al., 2005). The phenotypic variation in DTH of the CSSLs was great, with a maximum difference of 40 d. A large difference in DTH might interfere with the detection of QTLs for grain Cd accumulation because of the different growth periods resulting within the rice population. Therefore, it becomes necessary to re-evaluate whether QTLs for grain Cd accumulation are associated with those for DTH by using a mapping population with little phenotypic variation in DTH.

The objectives of this study were to analyse QTLs and their effects on grain Cd concentration in rice grown in a Cd-polluted paddy field, to examine the genetic relationship between concentrations of Cd and essential trace metals (Cu, Fe, Mn, and Zn) in rice, and to investigate the effects of agronomic traits, especially DTH, on grain Cd concentration in rice. A mapping population of backcross inbred lines (BILs) derived from a cross between the japonica cultivar Sasanishiki and indica cultivar Habataki was used. This type of population is often used to analyse QTLs for grain ripening and panicle architecture and is characterized by minimal segregation in DTH (Nagata et al., 2002; Ando et al., 2008). In addition, previous work has shown that Habataki exhibits approximately five times higher grain Cd concentration than that of Sasanishiki when cultivated in pots filled with Cd-polluted soil (Arao and Ishikawa, 2006; Uraguchi et al., 2009). Our QTL analysis revealed that a major-effect QTL for increasing grain Cd concentration governed by the Habataki allele is localized on the short arm of chromosome 7 (named qGcd7). This QTL is not genetically related to any QTLs for trace metal concentrations of grains or those for DTH or grain yield traits.

Materials and methods

Plant materials and field and pot experiments

A population consisting of 85 BILs derived from the cross Sasanishiki/Habataki//Sasanishiki///Sasanishiki was obtained from the Rice Genome Resource Centre (RGRC; http://www.rgrc.dna.affrc.go.jp/index.html) in Japan. For phenotypic evaluation of Cd uptake, a field experiment was conducted in a paddy field that had been contaminated with Cd after being irrigated with river water originating from an abandoned mine area in the Tohoku District of eastern Japan. The paddy soil was an alluvial soil. The Cd concentration was 2.76 mg kg\(^{-1}\) dry weight of soil, as determined by 0.1 M HCl extraction as stipulated by the Agricultural Land–Soil Pollution Prevention Law in Japan. The concentrations of Cu, Fe, Mn, and Zn were 111, 73.9, 128, and 56.5 mg kg\(^{-1}\) dry weight, respectively, when extracted by 0.1 M HCl.

Seeds of 85 BILs and their parental cultivars were sown in nursery boxes filled with artificially prepared fertilized soil (Ponzol No. 1; Sumitomo Chemical Co., Ltd., Tokyo, Japan). One-month-old seedlings were transplanted in late May 2007 into the flooded paddy field that was contaminated with Cd. The planting rate was
30 seedlings per line in rows 30 cm apart, with the seedlings within each row spaced 30 cm apart. Because of limited field space and quantity of seeds, two replications of each line arranged in a randomized complete block design were laid out in the field. A commercial chemical fertilizer was applied at the rate of 70 kg N ha\(^{-1}\) as a base dressing. Flood-irrigation was conducted until mid-July, and then the irrigation water was withheld to increase the bioavailable Cd concentration in the soil and to enhance the subsequent Cd uptake by rice plants (Ishikawa et al., 2005). After drainage, the paddy field was managed with rain-fed cultivation until the grain matured in mid-October. DTH, defined as the number of days from transplanting to emergence of 40–50% of all panicles, was scored. Four out of 30 plants per line in each of two plots were collected randomly from the field for the analysis of Cd and trace metal concentrations in grains (brown rice) and straw (leaves and stems). Several agronomic traits that may influence the grain Cd concentration were also measured: culm length, grain weight per plant, 1000 g weight, straw dry weight per plant, and harvest index calculated as grain weight per plant / (grain weight per plant + straw weight per plant).

To verify a major QTL for grain Cd concentration detected in BILs, CSSLs that carried a chromosome segment of Habataki in a Sasanishiki genetic background were used. The CSSLs were developed by Ando et al. (2008) to facilitate genetic analyses of complex traits in rice, and they are available from the RGR. Of 39 CSSLs, three lines (SL422, SL423, and SL424) that carried one or two segments from Habataki on chromosome 7 were selected on the basis of the results of analyses of BILs. Furthermore, an F\(_2\) population was generated from SL422/Sasanishiki to delimit the chromosomal region containing the QTL with the major effect.

For phenotypic evaluation, 1-month-old seedlings of parental cultivars, the three CSSLs, and F\(_2\) progeny were transplanted into Wagner pots (one plant per pot) containing 2.5 kg of Cd-polluted soil under flooded conditions. The soil was collected from the upper layer (0–15 cm) of a different paddy field from that used for the BIL cultivation. The soil was classified as alluvial. Concentrations of Cd, Cu, Fe, Mn, and Zn were 1.8, 10.9, 248, 46.1, and 16.4 mg kg\(^{-1}\) dry weight, respectively, when extracted by 0.1 M HCl (Ishikawa et al., 2006). Fertilizer was applied at a rate of 0.25 g each of N, P\(_2\)O\(_5\), and K\(_2\)O as a base dressing. Flood-irrigation was conducted until mid-July, and then the irrigation water was withheld to increase the bioavailable Cd concentration in the soil and to enhance the subsequent Cd uptake by rice plants (Ishikawa et al., 2005). After drainage, the paddy field was managed with rain-fed cultivation until the grain matured in mid-October. DTH, defined as the number of days from transplanting to emergence of 40–50% of all panicles, was scored. Four out of 30 plants per line in each of two plots were collected randomly from the field for the analysis of Cd and trace metal concentrations in grains (brown rice) and straw (leaves and stems). Several agronomic traits that may influence the grain Cd concentration were also measured: culm length, grain weight per plant, 1000 g weight, straw dry weight per plant, and harvest index calculated as grain weight per plant / (grain weight per plant + straw weight per plant).

Analysis of Cd and related metals in plant tissues

After threshing, rice grains were husked to obtain brown rice, ground to a fine powder with a grinder, and filtered through a stainless-steel 0.5 mm mesh screen (P-14; Fritsch GmbH, Kastl, Germany). Rice straw samples were milled by the same method. Then 0.5 g of grain sample was placed in a 50 ml polyethylene tube and digested with 10 ml of 60% H\(_2\)PO\(_4\) solution using a heating block system (DigiPREP LS; SCP Science, Baie d’Urfe, Quebec, Canada) at 105 °C for 3 h. The dry straw sample was digested in 30 ml Teflon tubes with a solution of 10 ml H\(_2\)PO\(_4\), 3 ml HF, and 1 ml HClO\(_4\) on a heating block at 180 °C for 3 h. The digested grain and straw solutions were each filtered by using disposable 0.2 µm PTFE syringe filters after appropriate dilution with Milli-Q water.

The concentrations of Cd, Cu, Fe, Mn, and Zn in the plant tissues were determined by inductively coupled plasma–optical emission spectrometry, and then the irrigation water was withheld to increase the bioavailable Cd concentration in the soil and to enhance the subsequent Cd uptake by rice plants (Ishikawa et al., 2005). After drainage, the paddy field was managed with rain-fed cultivation until the grain matured in mid-October. DTH, defined as the number of days from transplanting to emergence of 40–50% of all panicles, was scored. Four out of 30 plants per line in each of two plots were collected randomly from the field for the analysis of Cd and trace metal concentrations in grains (brown rice) and straw (leaves and stems). Several agronomic traits that may influence the grain Cd concentration were also measured: culm length, grain weight per plant, 1000 g weight, straw dry weight per plant, and harvest index calculated as grain weight per plant / (grain weight per plant + straw weight per plant).

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QTL analyses

Paired-sample \(t\) tests were used to detect the differences in means of all traits between Habataki and Sasanishiki. Significant differences in all means of BILs were detected by one-way ANOVA, and then broad-sense heritability \((h^2_b)\) was estimated for each trait by the formula

\[
h^2_b = \frac{VAR(G)}{VAR(G) + VAR(E)}
\]

where \(VAR(G)\) is the genetic variance and \(VAR(E)\) is the environmental variance.

The linkage map and graphical genotypes of BILs were obtained from RGR (http://www.rgrc.dna.affrc.go.jp/jp/ineSHBIL85.html; see Supplementary Fig. S1 at JXB online). The linkage map was reconstructed from the genotype data with MAPMAKER/EXP ver. 3.0 (Lincoln et al., 1992) to obtain the datasets necessary for QTL analysis by QTL Cartographer ver. 2.5 (Wang et al., 2005). The population was designated as recombinant inbred lines, with the result that the linkage map calculated was entirely reduced in scale as compared with the linkage map distributed by RGR.

For QTL analysis, composite interval mapping was run with model 6, which is a standard model featuring five background cofactors determined by forward regression, 10 cM window size, and 2 cM walking speed along chromosomes. Permutation tests \((n=1000\) permutations) were performed to estimate threshold values \((\alpha=0.05)\) of the logarithm of odds (LOD) for declaring the presence of QTLs (Churchill and Doerge, 1994). The additive effect and percentage of phenotypic variance explained by each QTL were estimated at the peak LOD score. For F\(_2\) progeny, a small piece of the third leaf was collected from each of 100 seedlings before transplantation into the Cd-polluted soil. Then DNA was simply extracted using TRIS-phosphate buffer. Eight simple sequence repeat (SSR) markers were added in the interval between RM6728 and RM7273 located on the target QTL (McCouch et al., 2002; International Rice Genome Sequencing Project, 2005). The resultant markers were used for F\(_2\) plant genotyping. The linkage order and genetic distances of each marker were calculated by using MAPMAKER/EXP ver. 3.0. In addition, QTL analysis was performed by composite interval mapping with the software QTL Cartographer ver. 2.5, as described above.

Results

Phenotypic variation in Cd and related essential trace metals of BILs and their parental cultivars

The concentrations of Cd, Cu, and Zn in grains of Habataki were significantly higher than in grains of Sasanishiki. Although there was no significant difference in grain Mn concentration between the genotypes, the concentration tended to be higher in Habataki. For grain Fe concentration, a similar value was obtained in both parental cultivars (Fig. 1). Similar results were obtained for the metal concentrations in straw (Fig. 2). Among the trace metals compared, the largest difference between the parents was observed for the Cd concentration in grains, which was approximately four times higher in Habataki than in Sasanishiki when these plants were cultivated in the Cd-polluted paddy field. Grain metal concentrations in the BILs were 0.38–2.31 mg kg\(^{-1}\) for Cd, 3.11–6.04 mg kg\(^{-1}\) for Cu, 7.80–14.6 mg kg\(^{-1}\) for Fe, 13.0–35.0 mg kg\(^{-1}\) for Mn, and 19.6–47.5 mg kg\(^{-1}\) for Zn. Similarly, wide variations in straw metal concentrations were observed: 2.13–11.7 mg kg\(^{-1}\) for Cd, 4.03–11.1 mg kg\(^{-1}\) for Cu, 88–246 mg kg\(^{-1}\) for Fe, 419–1345 mg kg\(^{-1}\) for Mn, and 51–182 mg kg\(^{-1}\) for Zn. Broad-sense heritability values for metal concentrations in
grains and straw are shown in Figs 1 and 2. The high levels of heritability for all metal traits in grains were observed. The pattern of the frequency distribution in the BILs revealed a continuous variation in all metals analysed, suggesting that metal uptake and subsequent accumulation in rice are controlled by several QTLs. The Cd and Cu concentrations of grains and straw in the BILs were mostly segregated between Sasanishiki and Habataki, but the other metals exhibited a transgressive segregation.

**QTL mapping for metal traits in BILs**

A summary of the QTLs for metal concentrations in grains and straw of rice is given in Table 1, and the chromosomal locations of the QTLs are shown in Fig. 3. Two QTLs for grain Cd concentrations were detected on chromosomes 2 and 7 and designated tentatively as \( qGCd2 \) and \( qGCd7 \), respectively. The two QTLs increased the grain Cd concentration with additive effects of the Habataki allele. Especially noteworthy was a major-effect QTL mapped on chromosome 7 between restriction fragment length polymorphism (RFLP) markers C383 and G1068; this QTL had an LOD value of 13.6 and accounted for 35.5% of the total phenotypic variance. No QTL was detected for grain Cu concentration, although large phenotypic variation was observed among BILs and parental cultivars. One putative QTL for grain Fe concentration, \( qGFe4 \), was mapped on chromosome 4 between C107 and C445. It explained 14.4% of phenotypic variance, and the Sasanishiki allele increased the grain Fe concentration by 0.74 mg kg\(^{-1}\). Four QTLs for grain Mn concentration were detected on chromosomes 1, 2, 7, and 12. Individually, the QTLs accounted for 7.65–20.2% of the trait variance. The grain Mn concentrations were increased by the presence of Habataki alleles at \( qGMn1 \) and \( qGMn2 \) and by the presence of Sasanishiki alleles at \( qGMn7 \) and \( qGMn12 \). Although a large LOD peak for grain Zn was observed on the short arm of chromosome 3 (see Supplementary Fig. S2 at JXB online), it was omitted because the LOD value was not over the threshold (LOD <3.9) at the flanking marker points. Consequently, no QTL for grain Zn concentration was found.

For straw Cd concentration, when the presence of QTLs was declared at LOD \( >2.9 \) after 1000-permutation tests,
three QTLs were found, on chromosomes 2, 7, and 12, and were designated tentatively as \( q_{SCd2} \), \( q_{SCd7} \), and \( q_{SCd12} \), respectively. The presence of Habataki alleles at \( q_{SCd2} \) and \( q_{SCd7} \) increased the straw Cd concentration, and these QTLs were mapped within almost identical regions as those of the QTLs for grain Cd. Three QTLs for straw Cu concentration were located on chromosomes 1, 2, and 8. The Habataki alleles at all QTLs were responsible for increasing straw Cu concentration. There were two QTLs for straw Fe concentration, on chromosomes 1 and 12; neither of these is the chromosome on which the QTL for grain Fe concentration is localized. Two QTLs for straw Mn concentration were mapped, on chromosomes 5 and 12; the QTL on chromosome 12 (near marker C1069) colocalized with that for grain Mn. Two QTLs were found for straw Zn concentration, on chromosomes 2 and 12. The straw Zn concentration was increased by the presence of the Habataki allele at \( q_{SZn2} \) and the Sasanishiki allele at \( q_{SZn12} \).

Two clusters of QTLs for multiple metal concentrations in grains and straw were found on the long arms of chromosomes 2 and 12 (Fig. 3). One cluster comprised five QTLs (\( q_{GCd2} \), \( q_{GMn2} \), \( q_{SCd2} \), \( q_{SCu2} \), and \( q_{SZn2} \)) on chromosome 2 around markers C1221 and R2511. In all QTLs, the Habataki allele was responsible for increasing the metal concentrations. Another cluster included five QTLs (\( q_{GMn12} \), \( q_{SCd12} \), \( q_{SMn12} \), \( q_{SZn12} \), and \( q_{SFe12} \)) around markers R1709 and C1069 on chromosome 12. The Sasanishiki allele at all QTLs was related to the increased metal concentrations. The major QTL for grain Cd concentration (\( q_{GCd7} \)) that was mapped near marker C383 on chromosome 7 was not part of a QTL cluster.

**Phenotypic and genetic relationships between grain Cd concentration and heading date**

The difference in DTH between the parental cultivars was not significant (see Supplementary Fig. S3 at JXB online). The ears of all but nine BILs emerged at 70–80 d after transplanting (see Supplementary Fig. S3 at JXB online), indicating that this population is characterized by less DTH segregation than in CSSLs derived from Kasalath and Kashihikari, which have been used in previous studies (Ebitani et al., 2005; Ishikawa et al., 2005). The difference in DTH is unlikely to influence grain Cd concentration in rice, because no significant correlation was found between
some traits in this population ($r=0.114$; see Supplementary Fig. S3 at JXB online).

A putative QTL for DTH (a threshold LOD value of $>3.6$) was detected on chromosome 12 flanked by markers R3375 and R617 (Table 1). This QTL was designated $qDTH_{12}$. The Habataki allele at $qDTH_{12}$ was responsible for lengthened DTH. No QTL for DTH was declared around $qGC_{d7}$, although a small peak in the LOD value (LOD = 2.8) was observed near marker C383 on chromosome 7 (see Supplementary Fig. S2 at JXB online). The QTLs for other important agronomic traits (culm length, straw dry weight, grain yield per plant, 1000-grain weight, and harvest index) were also not detected on chromosome 7 (see Supplementary Table S1 at JXB online).

### Further analysis of a major QTL for grain Cd concentration ($qGC_{d7}$) using CSSLs

To confirm the existence of the major QTL for grain Cd concentration, $qGC_{d7}$, three CSSLs (SL422, SL423, and SL424) were used. These lines covered the whole genome on chromosome 7 with overlapping substituted segments of Habataki in a Sasanishiki genetic background. They carried a homozygous segment of Habataki for the defined region. On the basis of positional information on the genetic markers of RFLPs and SSRs, $qGC_{d7}$ was mapped in the chromosomal region flanked by SSR markers RM6728 and RM7273 (Fig. 4a). Only SL422 possessed this QTL. When the three CSSLs and their parental cultivars were cultivated in the Cd-polluted soil, the grain Cd concentration of SL422 was significantly higher than that of Sasanishiki, SL423, and SL424 and was comparable to that of Habataki (Fig. 4b). For other trace metal concentrations, SL422 was nearly equal to those of Sasanishiki (Fig. 4c–f). Consequently, $qGC_{d7}$ was confirmed as specific to increased Cd.

### Table 1. Quantitative trait loci (QTLs) for Cd, Cu, Fe, Mn, and Zn concentrations of grains (brown rice) and straw (leaves and stems) and for days to heading (DTH) in the BIL population

| Plant parts | Traits | QTL* | Chr. | Marker intervalb | LODc | % of variance explained | Additive effectd |
|-------------|--------|------|------|-----------------|-------|-------------------------|----------------|
| Grains      | Cd     | $qGCd_2$ | 2    | R2511–C1470     | 5.64  | 11.8                    | -0.17          |
|             | Fe     | $qGFe_4$ | 4    | C107–C445       | 4.27  | 14.2                    | 0.74           |
|             | Mn     | $qGMn_1$ | 1    | R1928–R3072     | 4.18  | 9.40                    | -2.06          |
|             |        | $qGMn_2$ | 2    | R2511–C1470     | 4.64  | 10.8                    | -1.96          |
|             |        | $qGMn_7$ | 7    | R1440–R2394     | 7.93  | 20.2                    | 2.74           |
|             |        | $qGMn_{12}$ | 12   | C1069–G1406     | 3.38  | 7.65                    | 1.69           |
| Straw       | Cd     | $qSCd_2$ | 2    | C1221–R2511     | 4.64  | 12.8                    | -1.03          |
|             | Fe     | $qSFe_1$ | 1    | C86–G2200       | 4.36  | 15.7                    | -1.18          |
|             | Mn     | $qSMn_5$ | 5    | R1553–C43       | 4.22  | 12.2                    | 117            |
|             |        | $qSMn_{12}$ | 12   | R1709–C1069     | 3.68  | 12.8                    | 88.7           |
|             | Cu     | $qSCu_1$ | 1    | C86–G2200       | 4.36  | 15.7                    | -1.18          |
|             | Fe     | $qSFe_1$ | 8    | C813–C86        | 5.68  | 19.4                    | -27.3          |
|             | Mn     | $qSMn_5$ | 12   | R1709–C1069     | 3.68  | 12.8                    | 88.7           |
|             | Zn     | $qSZn_2$ | 2    | R418–C1221      | 4.42  | 16.7                    | -16.0          |
|             |        | $qSZn_{12}$ | 12   | R1709–C1069     | 6.12  | 21.3                    | 15.9           |
|             | DTH    | $qDTH_{12}$ | 12   | R3375–R617      | 4.87  | 13.7                    | -1.99          |

| a | Individual QTLs are shown with the italic abbreviation of the trait and the chromosome number. |
| b | The marker nearest the putative QTL is underlined. |
| c | After 1000-permutation tests, threshold values of logarithm of odds (LOD) for metal concentrations of grains and straw were calculated as 4.4 and 2.9 for Cd, 3.3 and 4.2 for Cu, 3.0 and 3.2 for Fe, 2.9 and 2.9 for Mn, and 3.9 and 3.2 for Zn, respectively. For DTH, the LOD threshold value was 3.6. |
| d | A negative value indicates that the allele from Habataki increases the phenotypic value. |

To delimit the location of $qGC_{d7}$, a QTL analysis was conducted using F2 progeny ($n=100$) derived from a cross between SL422 and Sasanishiki. High LOD values were observed continuously between RM6728 and RM7273 (Fig. 5a). The peak LOD value for $qGC_{d7}$ (LOD = 19.0) was found adjacent to SSR marker RM5436, and the phenotypic variance was 57.7%, with the positive allele contributed by Habataki. On the basis of the genotype data of RM5436, the 100 F2 progeny lines were classified into three types: homoyzogous for the Sasanishiki or Habataki alleles or heterozygous. The plants homozygous for Habataki had higher grain Cd concentration than those homozygous for Sasanishiki (Fig. 5b). The average grain Cd concentration in the heterozygotes (1.75 mg kg$^{-1}$, $n=53$) was closer to that of the Habataki homozygotes (2.09 mg kg$^{-1}$, $n=25$) than to that of the Sasanishiki homozygotes (1.07 mg kg$^{-1}$, $n=22$).
However, the grain Cd concentration of the heterozygotes showed wide variation (0.99–2.75 mg kg\(^{-1}\); Fig. 5b).

**Discussion**

Contamination of rice with Cd is a serious threat to people, as Cd consumption may result in Itai-itai disease. Among crops, rice is the greatest source of the dietary intake of Cd. Therefore, it is important to understand the genetic aspects of Cd accumulation in order to devise a breeding plan for reducing Cd levels in rice grains.

**QTLs for Cd accumulation in rice grown in a Cd-polluted paddy field**

Parental cultivars that show wide phenotypic variation in the target traits are necessary for QTL analysis, because QTL detection is based on natural allelic differences between parental cultivars (Ashikari et al., 2005). The indica rice cultivar Habataki and the japonica rice cultivar Sasanishiki, were chosen not only because they exhibit a large difference in grain Cd concentration (Arao and Ae, 2003; Uraguchi et al., 2009) but also because mapping populations, such as BILs and CSSLs, derived from their cultivars are available at RGRC. Although several reports have described QTLs for Cd accumulation in rice, the studies were conducted in hydroponic systems that created toxic Cd levels in young seedlings (Xue et al., 2009; Ueno et al., 2009), or in pots filled with Cd-polluted soil (Ishikawa et al., 2005), or in a paddy field not polluted by Cd (Kashiwagi et al., 2009), rather than under conditions more realistically representing a Cd-polluted paddy field. The detection of QTLs for Cd accumulation under field conditions, however, may be affected by various environmental factors. Cd solubility in soil becomes especially unstable with the changes in redox potential induced by irrigation. Consequently, Cd uptake by rice varies greatly according to irrigation levels, and the detection of QTLs under normal field conditions is unreliable.

Here, to minimize changes in Cd solubility in the soil and to evaluate the intrinsic capacity of rice plants for Cd uptake at a relatively high level of bioavailable soil Cd, the paddy field was drained and kept under oxidative conditions from...
the early vegetative stage until grain maturing. Under such conditions, the grain Cd concentration of Sasanishiki (0.65 mg kg\(^{-1}\)) exceeded the CODEX standard threshold limit of 0.4 mg kg\(^{-1}\), and that of Habataki (2.82 mg kg\(^{-1}\)) was four times higher (Fig. 1). In addition, the high broad-sense heritability of grain Cd concentration in the BIL population indicated the strong genetic control of grain Cd concentration in rice, suggesting that the field conditions used in this
study were adequate for detecting the QTLs. Results of the QTL analyses indicated that QTLs related to grain Cd concentration were present on chromosomes 2 (qGCd2) and 7 (qGCd7) (Table 1; Fig. 3). A major-effect QTL was qGCd7, detected on the short arm of chromosome 7; a minor-effect QTL for straw Cd concentration (qSCd7) was also observed in the same chromosomal region. The existence of qGCd7 was also confirmed by using CSSLs (Fig. 4). Further analyses using advanced backcross F2 progeny verified that qGCd7 is responsible for increasing grain Cd concentration (Fig. 5a).

This QTL with major effect was not detected in CSSLs derived from Koshikihari and Kasalath, which were used our in previous study (Ishikawa et al., 2005). In addition, qGCd7 did not coincide with several QTLs found by Xue et al. (2009), Ueno et al. (2009), or Kashiwagi et al. (2009). QTL detection is affected by population size, threshold value, marker number, and the heritability of tagged traits, and it varies with the genetic background of the population, the plant tissues used for phenotypic evaluation, and the experimental conditions (Xue et al., 2009). Therefore, the discrepancies among reports in the detection of QTLs for Cd accumulation in rice may be explained by some of these factors. Nevertheless, this is the first report of a novel QTL with major effects for increasing grain Cd concentrations in rice grown in a Cd-polluted paddy field.

Genetic relationship between Cd and other essential trace metals in rice

The QTL qGCd7 for grain Cd concentration was independent of those for the essential trace metals Cu, Fe, Mn, or Zn in rice grains and straw (Table 1; Figs 3, 4). Similar observations were reported by Clarke et al. (1997, 2002), who found that a single dominant gene (Cdu1) for low grain Cd concentration in durum wheat appeared to be specific for Cd because there were no correlations between concentrations of Cd and other essential elements in the grains of near-isogenic durum wheat. Recently, the Cdu1 gene has been mapped on the long arm of Chromosome 5B (Knox et al., 2009). In addition, grain Zn concentration was not significantly different between low- and high-Cd-accumulating maize hybrids (Hinesly et al., 1982). These results suggest that a Cd-specific system of transport into grains functions in graminaceous crops. However, the clustering of QTLs for multiple metals on the long arm of chromosome 2 reflects either pleiotropic effects of a gene or close linkage of multiple genes.

Effect of agronomic traits on QTLs for rice Cd

Another reason why we selected the BIL population derived from a cross between Sasanishiki and Habataki is that the population shows little phenotypic variation in DTH. In our previous study, a mapping population was used with wide variation in DTH for QTL analysis, and the QTLs for grain Cd concentration were likely to be linked to those of DTH (Ishikawa et al., 2005). In the present study, however, the QTLs for grain Cd concentration were unrelated to those for DTH (Table 1; see Supplementary Fig. S3 at JXB online), suggesting that grain Cd concentration was not influenced by genotypic differences in DTH in this mapping population.

Liu et al. (2005) reported that Cd concentration in brown rice was correlated significantly with grain yield and agronomic traits governing the formation of grain yield, such as spikelets per panicle, spikelets per pot, and harvest index, when 52 rice cultivars of different subspecies were grown in a soil contaminated artificially with a high level of Cd. However, no QTL linkage were found between grain Cd concentration and grain yield (gram weight per plant) or harvest index in the BIL population derived from Sasanishiki and Habataki. Although the spikelets per panicle were not measured, the major QTLs for this trait were reported by Nagata et al. (2002) and Ando et al. (2008), who found the QTLs on chromosomes 1 and 6 when analysing BILs and CSSLs derived from the same parents as used in this study. Therefore, our findings indicate that it is possible to control grain Cd concentration genetically without imposing negative effects on the major agronomic traits in rice.

Physiological role of qGCd7 for grain Cd accumulation

The level of Cd in rice grains may be influenced by any of several physiological processes, such as root Cd uptake, xylem translocation from the root to the shoot, transfer from xylem to phloem, and phloem transport into grains during fruit development (Hart et al., 1998; Riesen and Feller, 2005). The major QTL qGCd7 could be responsible for any of these processes. Uraguchi et al. (2009) characterized the physiological properties involved in the differences in shoot and grain Cd accumulation between Sasanishiki and Habataki. The activity of root Cd uptake was significantly higher for Sasanishiki (low Cd in grains) than Habataki (high Cd in grains), suggesting that qGCd7 is not likely to be involved in Cd influx across the root plasma membrane. A large difference was observed between the cultivars in Cd concentration in the xylem sap; this concentration is determined by factors such as xylem loading of Cd and transpiration rate. The ratio of xylem Cd concentration between Habataki and Sasanishiki at the heading stage was approximately equal to the ratio of grain Cd concentration between the cultivars. This observation suggests that root-to-shoot Cd translocation via the xylem
is a key factor determining grain Cd concentration in Habataki and Sasanishiki, and it is speculated that the process is governed mainly by \( qGCd7 \). Although heavy-metal-association P-type ATPases have been well characterized as transporters in the xylem loading of Zn and Cd in *Arabidopsis thaliana* (Hussain et al., 2004; Verret et al., 2004; Wong and Cobbett, 2009), *Arabidopsis halleri* (Hanikenne et al., 2008), and *Thlaspi caerulescens* (Papoyan and Kochian, 2004), no gene encoding a transporter involved in Cd-specific xylem loading has yet been identified in rice.

Cd is probably not translocated directly via the xylem, because the xylem is not connected to the rice grains (Kawahara et al., 1977). Studies with graminaceous crops, such as wheat (Harris and Taylor, 2004), barley (Chen et al., 2007), and rice (Tanaka et al., 2003), have demonstrated that Cd moves into developing grains via the phloem. In rice, 91–100% of Cd in grains is transported via the phloem (Tanaka et al., 2007). QTLs that are likely to be associated with phloem transport of Cd into rice grains were identified by Kashiwagi et al. (2009), who used BILs and near-isogenic lines derived from Nipponbare and Kasalath. They found that two QTLs, \( qCd4-1 \) and \( qCd4-2 \), located on chromosome 4, synergistically increased the Cd translocation from leaves to grains after heading. \( qGCd7 \) may also be associated with phloem transport of Cd in rice grains, because this QTL showed a major effect for Cd concentration in the grains, but not in the straw (leaves and stems). \( qGCd7 \) could be responsible for either root-to-shoot Cd translocation via xylem or phloem transport into grains during fruit development, and several candidate genes that are likely to be associated with Cd transport are inferred around \( qGCd7 \) from analyses of the rice genome annotation database. However, further analyses using methods such as high-resolution mapping or gene cloning will be necessary to identify conclusively particular candidate genes for \( qGCd7 \) and to understand the physiological function of this QTL.

In summary, two QTLs for grain Cd concentration were found on chromosomes 2 and 7. The QTL with a major effect on grain Cd concentration, \( qGCd7 \), is located on chromosome 7. This QTL is specific to Cd, not other essential trace metals. Moreover, it is not influenced by the agronomic traits of plants, such as heading date. Thus, \( qGCd7 \) can be used to eliminate the high-Cd-accumulation allele through marker-assisted selection and to understand the genetic mechanism of grain Cd accumulation. At present, positional cloning is in progress by our group to identify the gene at this QTL.

**Supplementary data**

Supplementary data can be found at JXB online. **Figure S1.** Linkage map developed by using backcross inbred lines (BILs) derived from the cross Sasanishiki/Habataki//Sasanishiki//Sasanishiki.

**Figure S2.** Logarithm of odds (LOD) curves for metal concentrations of grains and straw and for days to heading (DTH).

**Figure S3.** Phenotypic correlation between grain Cd concentration and DTH.

**Table S1.** Quantitative trait loci (QTLs) for agronomic traits in the BIL population.

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