Detection of Chromosomal Regions Affecting Iron Concentration in Rice Shoots Subjected to Excess Ferrous Iron Using Chromosomal Segment Substitution Lines between Japonica and Indica

Akari Fukuda¹, Hiroyuki Shiratsuchi², Akira Fukushima², Hiromichi Yamaguchi², Hideyuki Mochida², Tomio Terao¹ and Hitoshi Ogiwara³

(¹Hokuriku Research Center, NARO Agricultural Research Center, NARO, 1-2-1 Inada, Joetsu, Niigata 943-0193, Japan; ²NARO Agricultural Research Center for Tohoku Region, NARO, 3 Shimo-furumichi, Yotsuya, Daisen, Akita 014-0102, Japan; ³NARO Agricultural Research Center, NARO, 3-1-1 Kannondai, Tsukuba, Ibaraki, 305-8666, Japan)

Abstract: Excess ferrous iron in lowland soil is known to inhibit the growth of rice. A quantitative trait locus (QTL) analysis for susceptibility to ferrous iron was performed using chromosomal segments substitution lines (CSSLs). Kasalath, an indica rice cultivar, is known to be susceptible to ferrous iron and accumulate excess iron in shoots. The shoot iron concentration was examined in 39 CSSLs carrying Kasalath chromosomal segments in a background of Koshihikari, a japonica cultivar. Kasalath grown in a hydroponic culture solution containing excess ferrous iron, had a higher shoot iron concentration than Koshihikari. Of the CSSLs, SL208, which carries the Kasalath chromosomal segment on chromosome 3, had a significantly higher shoot iron concentration than Koshihikari, and none of the CSSLs had a shoot iron concentration significantly lower than Koshihikari. This finding suggests that the putative QTL affecting the shoot iron concentration is between the markers R663 and S1571 on chromosome 3.

Key words: CSSLs, Ferrous iron, QTL, Rice (Oryza sativa L.).

Iron is an essential nutrient for plants, and is used in the redox center of enzymes for photosynthesis and respiration (Briat and Lobréaux, 1997). However, excess iron is toxic to plant tissues because it generates hydroxyl radicals that damage membrane lipids, proteins and DNA (Becana et al., 1998). For rice (Oryza sativa L.), excess ferrous iron in soil causes brown spots on leaves, known as ‘bronzing’, and retards the growth of shoots and roots, reducing grain yields (Tanaka et al., 1966b; Tadano, 1976; Genon et al., 1994). Decreased rice yields due to ferrous iron toxicity were reported to range from 15 to 100% (Becker and Asch, 2005). Excess ferrous iron in soil also inhibits seedling establishments, particularly in direct seeded rice on paddy soil (Hagiwara and Imura, 1993; Yamauchi, 2001).

Although iron is abundant in soils, most of it is in an insoluble ferric form (Fe³⁺) under aerobic and high pH conditions (Briat and Lobréaux, 1997; Mori, 1999). However, in waterlogged and acidic pH conditions, ferric iron is reduced to the soluble ferrous form (Fe²⁺) that is readily absorbed by plant roots (Sahrawat, 2004). Toxicity of excess ferrous iron is a major constraint for rice production, particularly on acidic lowland soils in Asia, South America and West Africa (Ponnamperuma et al., 1955; Genon et al., 1994; Audebert and Sahrawat, 2000). The optimum concentration of ferrous iron for rice growth was predicted to be between 0.0018 and 0.18 mM using hydroponic culture solutions (Ishizuka et al., 1961). On the other hand, in soil, ferrous iron concentrations reported to seriously inhibit rice growth range from 9.0 ~ 17.9 mM (Ponnamperuma et al., 1955; Tanaka and Navasero, 1966a), which are 50 to 100 times higher than the optimum concentrations in culture solution.

The improvement of rice cultivars to tolerate excess ferrous iron is more practical than the widespread reduction of ferrous iron levels in paddy soil. Rice varieties differ in their tolerance to ferrous iron (Gunawardena et al., 1982; Fageria and Rabelo, 1987), and breeding cultivars more tolerant to ferrous iron should, in principle, be possible. Marker-assisted selection is a powerful tool for breeding tolerant cultivars. To identify genetic markers, QTL analysis and the identification of the genes affecting...
the ferrous iron susceptibility are necessary.

Some QTL analyses of ferrous iron toxicity in rice have previously been reported (Wu et al., 1997, 1998; Wan et al., 2003; Shimizu et al., 2005b; Ouyang et al., 2007; Dufey et al., 2009). Wu et al. (1997, 1998) reported that the QTLs for the leaf bronzing index, enzyme activity of ascorbate peroxidase and glutathione reductase, and those affecting the concentration of the dehydroascorbate and ascorbate under ferrous iron stress were detected in the same region on chromosome 1 using Azucena /IR64 inbred lines. Wan et al. (2003) detected the QTLs on the same locus of chromosome 1 as those reported by Wu et al. (1997, 1998) for the leaf bronzing index, stem dry weight, tiller number and root dry weight using Nipponbare /Kasalath inbred lines. Other QTLs affecting stem dry weight and root dry weight under ferrous iron stress were detected on chromosome 3 (Wan et al., 2003). Shimizu et al. (2005b) reported QTLs affecting the iron concentration in rice seedlings on chromosome 3 and 4 under the excess ferrous iron using Gibouzu /Kasalath inbred lines. Ouyang et al. (2007) detected seven QTLs on chromosomes 1, 4, 5 and 7 that affect the coleoptile elongation rate under various ferrous iron concentrations using Zhenshan97B / Miyang46 inbred lines. Dufey et al. (2009) reported 24 putative QTLs on chromosomes 1, 2, 3, 4, 7 and 11 that affect the leaf bronzing index, shoot water content, shoot and root dry weight, shoot iron concentration, stomatal resistance and chlorophyll content under excess ferrous iron conditions using Azucena /IR64 inbred lines. However, no practical DNA markers or genes affecting susceptibility to ferrous iron have yet been identified.

Under the excess ferrous iron conditions, several mechanisms to prevent the influx of excess iron are suggested; oxidation of ferrous iron on root surface, retention of iron in root, stem and apoplast of the leaf tissues, storage of iron as ferritin in plastids (Tadano, 1976; Becker and Asch, 2005). However, molecular mechanisms under the excess ferrous iron conditions have not been studied in detail, except that the expression level of ferritin genes, OsFER1 and OsFER2, were increased in leaves under the excess ferrous iron conditions (Silveira et al., 2009; Stein et al., 2009).

For the iron uptake and transport in rice plants, several studies have been reported (Jeong and Guerinot 2009; Bashir et al., 2010; Kobayashi et al., 2010). Graminaceous plants are known to have Fe$^{3+}$-mugineic acids (MA) complexes transporters in roots, which contribute to iron uptake from soils (Bashir et al., 2010; Kobayashi et al., 2010). In addition to the Fe$^{3+}$-MA transporters, rice was reported to have the ferrous iron (Fe$^{2+}$) transporters, OsIRTI and OsIRT2, and their expression was induced in roots under iron deficient conditions (Ishimaru et al., 2006). In xylem tissue, iron is thought to be translocated as Fe$^{3+}$-citrate complex (Bashir et al., 2010; Kobayashi et al., 2010), and the citrate transporter, OsFRDL1, that localized on pericycle cells of the roots was suggested to contribute to the iron translocation to shoot (Yokosho et al., 2009).

Iron-chelate transporter genes; OsYSL2, OsYSL15 and OsYSL18, the ferrous iron transporter gene; OsIRT1, and the genes related to the biosynthesis of chelates, OsNAA1-3, OsNASI-3 and OsDMAS1, were expressed in the phloem companion cells, suggesting that they contribute to the iron translocation through phloem (Bashir et al., 2010; Kobayashi et al., 2010). However, most of the iron homeostasis genes have been studied under the iron deficient conditions, and it is unclear whether the known genes, which are related to the iron uptake and transport, affect the tolerance under the excess ferrous iron conditions.

Chromosomal segment substitution lines (CSSLs) are useful tools to simplify the genetic background (Ebitani et al., 2005). Because CSSLs usually carry one substituted region, the chromosomal regions with putative QTLs can be identified from known graphical genotypes. Moreover, backcrossing the CSSLs carrying the putative QTL could be useful to develop nearly isogenic lines, which can be used for identifying practical DNA markers for breeding and for narrowing the QTL region to identify the responsible gene. We used CSSLs in which the chromosome segment of an indica rice cultivar, Kasalath, were introgressed into a japonica cultivar, Koshihikari (Koshihikari / Kasalath CSSLs) (Ebitani et al., 2005). Kasalath is known to be susceptible to ferrous iron and accumulates high concentrations of iron in its shoots (Shimizu et al., 2005a). We screened for CSSLs that inherited the Kasalath susceptibility to ferrous iron during early seedling growth, which is an important trait for seedling establishment in direct seeding, by measuring shoot iron concentrations in the seedlings grown in a hydroponic culture solution containing excess ferrous iron.

**Materials and Methods**

1. **Seed materials**

We used CSSLs (SL201–239) from the japonica cultivar, Koshihikari, containing chromosome segments from the indica rice cultivar, Kasalath, that were developed with 130 DNA markers at Rice Genome Resource Center (http://www.rgrc.dna.affrc.go.jp/ineKKCSSL39.html; Ebitani et al., 2005). Seeds from the parental cultivars, Koshihikari and Kasalath, and the 39 CSSLs were harvested from the experimental paddy fields in Daisen campus of NARO Agricultural Research Center for Tohoku Region (Daisen, Akita, Japan).

2. **Hydroponic culture solution for Koshihikari and Kasalath**

Preliminary experiments were carried out to determine the ferrous iron concentration in the culture solution using Koshihikari and Kasalath. Floating plates for the
hydroponic culture solution were made from 96-well polypolypropylene PCR plates with the bottom of each well cut off. One germinated seed was sown in each well. Two lines per plate and forty-eight germinated seeds per line were grown in floating plates. The plates were floated on the hydroponic culture solution containing 0.5, 2.5 or 5 mM FeSO₄ with 0.35 mM (NH₄)₂SO₄, 0.17 mM Na₂HPO₄, 0.27 mM K₂SO₄, 0.47 mM MgSO₄, 0.37 mM CaCl₂, 0.16 μM CuSO₄, 0.15 μM ZnSO₄, 0.10 μM Na₂MoO₄, 15 μM H₃BO₃ and 3.6 μM MnSO₄ (Hayashi and Chino, 1986). Four floating plates were placed in a carton containing 2 L hydroponic culture solution, which was replaced weekly. The pH of the culture solution was maintained at pH 4.5 with 1N NaOH or HCl every two or three days. The seedlings were grown in a growth chamber for two weeks at 20ºC under a 12-hr artificial light and 12-hr dark photoperiod with 64–79 μmol m⁻² s⁻¹ of photosynthetically active radiation. The humidity in the growth chamber was not controlled. The experiments were repeated three times.

3. Hydroponic culture solution for CSSLs

Thirty nine CSSLs and their parental cultivars, Koshihikari and Kasalath, were used for the experiments. Two lines per plate and 48 germinated seeds per line were grown on floating plates as described above. Five plates were floated on the hydroponic culture solution containing 5 mM FeSO₄ and the same amounts of other nutrient factors as the preliminary experiment. Growth conditions were the same as those in the preliminary experiment.

4. Measurement of shoot iron concentration

The seedlings were harvested two weeks after sowing, and then oven dried at 80ºC for three days; the shoots and roots were weighed separately. The dried shoot samples were digested by wet combustion with 4mL of H₂SO₄ and 1 mL of 30% H₂O₂ at 310ºC for 1 hr. After cooling, 1 mL of 30% H₂O₂ was added and heated at 310ºC for 1 hr again. Addition of 1 mL of 30% H₂O₂ and heating was repeated three or four times until the samples were completely digested. The iron concentration was determined colorimetrically using the o-phenanthroline method, and the absorbance of the colored complex of iron and o-phenanthroline at pH 3.5 was measured at 508 nm.

Results

1. Shoot iron concentrations in Koshihikari and Kasalath rice varieties

The increase of the ferrous iron concentration in the hydroponic culture solutions increased the shoot iron concentrations in both Koshihikari and Kasalath (Fig. 1A). The shoot iron concentration in Kasalath was significantly higher than that in Koshihikari, particularly in the culture solution containing 0.5 and 5 mM ferrous iron (Fig. 1A). The shoot dry weights in Koshihikari and Kasalath were not significantly different (Fig. 1B). A slight suppression of shoot dry weight was observed in Koshihikari in the high ferrous iron culture solution (Fig. 1B). The effect of ferrous iron on the shoot dry weight of Kasalath was not clear (Fig. 1B).
Table 1. Shoot iron concentration, shoot dry weight and root dry weight in CSSLs cultured in a hydroponic solution containing 5 mM of ferrous iron for two weeks.

| Chr. | CSSL | Shoot iron concentration (mg g⁻¹ dry weight) | Shoot dry weight (mg) | Root dry weight (mg) |
|------|------|---------------------------------------------|----------------------|---------------------|
| Koshihikari | 7.98 ± 1.01 | 3.15 ± 0.17 | 1.25 ± 0.05 |
| Kasalath | 15.48 ± 2.74 ** | 3.05 ± 0.16 | 0.62 ± 0.11 ** |
| 1 | SL 201 | 8.14 ± 1.55 | 3.89 ± 0.27 ** | 1.22 ± 0.21 |
| 1 | SL 202 | 7.49 ± 1.72 | 3.98 ± 0.42 ** | 1.06 ± 0.19 |
| 1 | SL 203 | 7.10 ± 1.11 | 4.24 ± 0.50 ** | 1.13 ± 0.15 |
| 2 | SL 204 | 9.40 ± 1.14 | 3.55 ± 0.22 | 1.05 ± 0.03 |
| 2 | SL 205 | 9.21 ± 1.98 | 3.25 ± 0.25 | 1.03 ± 0.08 |
| 2 | SL 206 | 10.08 ± 2.49 | 3.19 ± 0.24 | 0.68 ± 0.03 ** |
| 3 | SL 207 | 8.68 ± 1.18 | 3.05 ± 0.23 | 0.64 ± 0.12 ** |
| 3 | SL 208 | 12.77 ± 2.97 * | 2.34 ± 0.15 ** | 0.55 ± 0.03 ** |
| 4 | SL 209 | 9.26 ± 1.84 | 3.34 ± 0.06 | 0.98 ± 0.08 |
| 4 | SL 210 | 8.60 ± 1.42 | 3.10 ± 0.29 | 1.06 ± 0.12 |
| 4 | SL 211 | 7.46 ± 2.49 | 3.29 ± 0.23 | 0.93 ± 0.11 |
| 5 | SL 212 | 8.25 ± 2.23 | 2.93 ± 0.21 | 0.91 ± 0.11 |
| 5 | SL 213 | 8.45 ± 2.88 | 3.36 ± 0.10 | 1.00 ± 0.07 |
| 5 | SL 214 | 6.63 ± 2.72 | 2.73 ± 0.19 | 0.88 ± 0.19 |
| 6 | SL 215 | 8.83 ± 2.63 | 2.95 ± 0.14 | 1.07 ± 0.14 |
| 6 | SL 216 | 7.58 ± 1.79 | 2.94 ± 0.12 | 0.91 ± 0.11 |
| 6 | SL 217 | 7.32 ± 0.87 | 3.25 ± 0.30 | 1.05 ± 0.16 |
| 6 | SL 218 | 7.75 ± 1.27 | 3.42 ± 0.08 | 0.99 ± 0.12 |
| 7 | SL 219 | 9.90 ± 0.73 | 3.23 ± 0.31 | 0.98 ± 0.15 |
| 7 | SL 220 | 9.40 ± 3.50 | 3.26 ± 0.14 | 0.73 ± 0.09 ** |
| 7 | SL 221 | 9.28 ± 4.71 | 3.42 ± 0.33 | 1.04 ± 0.12 |
| 7 | SL 222 | 7.16 ± 3.71 | 3.48 ± 0.40 | 1.07 ± 0.06 |
| 8 | SL 223 | 7.50 ± 4.47 | 3.03 ± 0.16 | 1.22 ± 0.21 |
| 8 | SL 224 | 5.72 ± 2.14 | 2.60 ± 0.30 | 0.97 ± 0.03 |
| 8 | SL 225 | 6.78 ± 1.75 | 2.90 ± 0.23 | 1.03 ± 0.14 |
| 9 | SL 226 | 8.11 ± 1.21 | 2.80 ± 0.18 | 1.29 ± 0.08 |
| 9 | SL 227 | 8.19 ± 0.21 | 2.73 ± 0.21 | 0.97 ± 0.05 |
| 9 | SL 228 | 7.01 ± 0.29 | 3.02 ± 0.17 | 1.19 ± 0.03 |
| 10 | SL 229 | 8.02 ± 0.82 | 3.36 ± 0.33 | 1.13 ± 0.13 |
| 10 | SL 230 | 6.61 ± 2.66 | 3.28 ± 0.28 | 1.21 ± 0.17 |
| 10 | SL 231 | 8.59 ± 2.61 | 3.11 ± 0.23 | 1.18 ± 0.30 |
| 10 | SL 232 | 7.28 ± 0.88 | 3.38 ± 0.11 | 1.35 ± 0.12 |
| 11 | SL 233 | 9.25 ± 0.34 | 3.38 ± 0.28 | 1.18 ± 0.16 |
| 11 | SL 234 | 7.42 ± 1.15 | 3.56 ± 0.25 | 1.28 ± 0.12 |
| 11 | SL 235 | 6.06 ± 0.85 | 4.04 ± 0.05 ** | 1.53 ± 0.15 |
| 4, 12 | SL 236 | 7.50 ± 0.44 | 3.74 ± 0.04 * | 1.19 ± 0.19 |
| 12 | SL 237 | 8.80 ± 1.75 | 3.30 ± 0.07 | 1.35 ± 0.06 |
| 12 | SL 238 | 7.76 ± 1.94 | 3.53 ± 0.31 | 1.30 ± 0.10 |
| 12 | SL 239 | 9.69 ± 0.90 | 3.33 ± 0.36 | 1.12 ± 0.08 |
| Average values of CSSLs | 8.18 ± 2.49 | 3.26 ± 0.46 | 1.06 ± 0.24 |

Chr. indicates the chromosome carrying the Kasalath chromosomal segment. Values are the averages of three independent replicates with standard deviations. * and ** indicate significant differences from Koshihikari at the 5% and 1% levels, respectively, detected by Dunnett's pairwise multiple t-test.
Root dry weight in Kasalath was approximately half of that in Koshihikari (Fig. 1C). The difference was significant, particularly at the 2.5 mM and 5 mM ferrous iron conditions (Fig. 1C). The suppressive effect of high ferrous iron on root dry weight was observed in both Koshihikari and Kasalath (Fig. 1C); a higher ferrous iron concentration in the hydroponic culture solutions led to lighter root dry weights in both Koshihikari and Kasalath (Fig. 1C).

2. The shoot iron concentration in CSSLs

Shoot iron concentrations as well as the shoot and root dry weights in the CSSLs and their parental cultivars, Koshihikari and Kasalath, grown in a hydroponic culture solution with 5 mM ferrous iron were measured (Table 1). The shoot iron concentration in Kasalath (15.48 mg g\(^{-1}\)) was significantly higher than that in Koshihikari (7.98 mg g\(^{-1}\); Table 1). The shoot iron concentrations in CSSLs ranged from 5.72 to 12.77 mg g\(^{-1}\) with an average of 8.18 mg g\(^{-1}\) (Table 1). The average value was close to the value in Koshihikari (Table 1). However, one of the CSSLs, SL208, which carries the Kasalath chromosome 3 segment, contained 12.77 mg g\(^{-1}\) iron in the shoot, which was a significantly higher value than that in Koshihikari (Table 1). No CSSLs had significantly lower shoot iron concentrations than Koshihikari (Table 1).

There was no significant difference in the shoot dry weight of the parental cultivars, Koshihikari and Kasalath (Table 1). The shoot dry weight in the CSSLs, which ranged from 2.34 to 4.04 mg, were similar to the parental values (Table 1). Among them, five CSSLs, SL201, SL202, SL203, SL205 and SL206, had a significantly heavier shoot dry weight than Koshihikari (Table 1), and SL208 had a significantly lighter shoot dry weight than Koshihikari (Table 1).

The roots of Kasalath had a significantly lighter dry weight (0.62 mg) than those of Koshihikari (1.25 mg; Table 1). The root dry weights in the CSSLs ranged from 0.55 to 1.53 mg, the average value, 1.06 mg, being slightly lighter than that of Koshihikari (Table 1). SL206, SL207, SL208 and SL220 had a significantly lighter root dry weight than Koshihikari, but no lines had a significantly heavier root dry weight than Koshihikari (Table 1).

The shoot iron concentration was not significantly correlated with the shoot dry weight in CSSLs (Fig. 2A). Only SL208 had a high iron concentration similar to Kasalath (Fig 2A). There was a significant negative correlation between shoot iron concentration and root dry weight in CSSLs (Fig. 2B).

3. The putative QTLs affecting shoot iron concentration

The putative QTLs for increased shoot iron concentration were suggested by the graphical genotype data of the CSSLs (Fig. 3). SL208 had a higher shoot iron concentration than Koshihikari and had one Kasalath chromosomal segment, on the longer arm side from a DNA marker named S1513 on chromosome 3 (Fig. 3). However, SL207 and SL214, which have Kasalath chromosomal segments on the shorter arm side from R663, and the longer arm side from S1571, respectively, did not show increased shoot iron concentrations (Fig. 3). Consequently, the putative QTL for the shoot iron concentration was suggested to be between R663 and S1571 on chromosome 3, which was replaced by a Kasalath allele only in SL208 (Fig. 3).

4. The putative QTLs affecting shoot dry weight

The CSSLs that had heavier shoot dry weights than
Koshihikari, SL201, SL202 and SL203, had Kasalath chromosomal segments on chromosome 1, suggesting that the putative QTL for increased shoot dry weight in Kasalath was located between R2635 and R2417 on chromosome 1 (Fig. 4). SL235 with a Kasalath chromosomal region on chromosome 11 also had a heavier shoot dry weight than Koshihikari, suggesting that the putative QTL for increased shoot dry weight in Kasalath was on the longer arm side from S10928 on chromosome 11 (Fig. 4). SL208, which had a lighter shoot dry weight than Koshihikari had a Kasalath chromosomal segment on chromosome 3, suggesting that the putative QTL for decreased shoot dry weight in Kasalath was between R663 and S1571 on chromosome 3 (Fig. 4), the same region as the putative QTL for increased shoot iron concentration (Fig. 3). Although SL236 with Kasalath segments on chromosomes 4 and 12 had a heavier shoot dry weight than Koshihikari at the 5% significance level, the putative QTLs were not discernible because other CSSLs with the same Kasalath region as SL236 on chromosomes 4 or 12 did not show any significant difference in shoot dry weight from Koshihikari (Fig. 4). The other possibility is that the Kasalath chromosomal segments on chromosomes 4 and 12 in SL236 are epistatic, and thus might complement one another to increase shoot dry weight.

5. The putative QTLs affecting root dry weight

SL206 with a Kasalath region on chromosome 2 had a lighter root dry weight than Koshihikari, suggesting that the putative QTL associated with the Kasalath alleles that decreased root dry weight was on the longer arm side from C747 on chromosome 2 (Fig. 5). SL207 and SL208 with lighter root dry weights than Koshihikari had Kasalath segments on chromosome 3, suggesting that the putative QTLs affecting root dry weight

[Diagram of chromosome 3 with markers and QTLs]

Fig. 3. The putative QTL for increased shoot iron concentrations on chromosome 3. The left side of the chromosome is the short arm. Dependent changes are shown as a percentage shoot iron concentration in the CSSL to that in Koshihikari. * indicates the lines with a significant difference from Koshihikari at the 5% level (Table 1).

[Diagram of chromosome 1 with markers and QTLs]

Fig. 4. The putative QTLs affecting the shoot dry weight. Dependent changes are shown as a percentage of the shoot dry weight in CSSL to that in Koshihikari. * and ** indicate the lines with a significant difference from Koshihikari at the 5% and 1% levels, respectively (Table 1).
QTL for decreased root dry weight due to Kasalath alleles was between C25 and G332 on chromosome 3 (Fig. 5). Although SL220, with a Kasalath segment on chromosome 7, also had a lighter root dry weight than Koshihikari, the other CSSLs with Kasalath chromosome 7 regions, SL219, SL221 and SL222, did not show any significant difference in root dry weight from Koshihikari; hence, the putative QTL was not detected (Fig. 5).

Discussion

Kasalath accumulated more shoot iron than Koshihikari in the hydroponic culture solution containing 5 mM ferrous iron for 2 wk after germination (Fig. 1 and Table 1). Since a high shoot iron concentration is an indicator of the inability to exclude excess ferrous iron, this suggests that Kasalath is an iron-susceptible cultivar as reported previously (Shimizu et al., 2005a). Using Koshihikari/Kasalath CSSLs, the putative QTL for increased iron concentration in Kasalath was found between markers R663 and S1571 on chromosome 3 (Fig. 3). Only this segment affected shoot iron concentration among all the other CSSLs covering whole chromosomes (Ebitani et al., 2005). This result strongly suggested that the region in chromosome 3 of Koshihikari contained the effective gene(s) for iron tolerance.

The QTL region for the increased shoot iron concentration detected in the present study contained the genes encoding ferrous iron transporters, OsIRT1 and OsIRT2 (Ishimaru et al., 2006). Both OsIRT1 and OsIRT2 were reported to express predominantly in roots (Ishimaru et al., 2006), and over-expression of OsIRT1 by the transgenic method led to an increased shoot iron concentration (Lee and An, 2009). However, whether the OsIRT genes are related to the increased shoot iron concentration under the excess ferrous iron conditions, and whether there are genotypic differences in the OsIRT genes with the cultivar remain unknown. If the genotypic differences of the iron-homeostasis genes affect the tolerance to excess ferrous iron stress, it may be possible to produce tolerant cultivars by breeding which does not depend on a transgenic method.

At the corresponding region on chromosome 3, the putative QTL for shoot iron concentration was detected using the lines derived from a cross between a japonica cultivar, Gimbozu, and an indica cultivar, Kasalath (Shimizu et al., 2005b). Kasalath alleles were associated with decreased shoot iron concentration in Gimbozu/Kasalath inbred lines (Shimizu et al., 2005b). However, in the present study, such alleles in Kasalath increased the shoot iron concentration. If the same locus is responsible for these effects, then one explanation is that they are multiple allelic; the Kasalath alleles have an iron accumulation effect weaker than that of Gimbozu alleles, but stronger than that of Koshihikari alleles. Another possibility is that the QTL found in the Gimbozu/Kasalath line is different from the QTL encountered in the present study. Further fine mapping is necessary to check whether these QTLs are located on the same chromosomal region.

SL208 had a higher shoot iron concentration and lighter shoot dry weight than Koshihikari (Table 1), and the QTLs affecting both shoot iron concentration and shoot dry weight were located in the same region on chromosome 3 (Fig. 3 and 4). However, it was not clear whether the decreased shoot dry weight in SL208 was due to its high iron concentration, because there was no significant difference in shoot dry weight between Koshihikari and Kasalath, although the shoot iron concentration in Kasalath was higher than that in Koshihikari under 5 mM ferrous iron conditions (Fig. 1).
and Table 1). Moreover, the shoot iron concentration was not correlated significantly with shoot dry weight in CSSLs. Only SL208 had a high shoot iron concentration, which was similar to Kasalath (Fig. 2A). This finding also suggested that the lighter shoot dry weight of SL208 might not be caused by its high shoot iron concentration. One possibility is that Kasalath has both the putative QTL for increased shoot dry weight on chromosome 1 and 11, and for decreased shoot dry weight on chromosome 3 in SL208 (Fig. 4). The effect of one QTL may cancel the effect of the other QTL leading to an unchanged shoot dry weight in Kasalath. In this case, the alleles for both light shoot dry weight and high shoot iron concentration in SL208 come from Kasalath. A precise analysis of the location of the QTLs for the decreased shoot dry weight and high shoot iron concentration and a linkage analysis between shoot dry weight and shoot iron concentration are necessary to clarify this issue. Nevertheless, Kasalath and SL208 might have a mechanism to accumulate a large amount of iron in the shoots.

The suppressive effect of ferrous iron on root dry weight was observed both in Koshihikari and Kasalath (Fig. 1C). Additionally, the correlation between shoot iron concentration and root dry weight in CSSLs was significant (Fig. 2B). Therefore, a high iron concentration is considered to be effective in reducing root dry weight. However, the positions of the QTLs for decreased root dry weight did not correspond to the QTL for increased shoot iron concentration (Fig. 3 and 5), suggesting that factors other than ferrous iron stress might affect root dry weight.

Kasalath, SL206, SL207, SL208 and SL220 had lighter root dry weights than Koshihikari, though they had higher or unchanged shoot iron concentrations as compared with Koshihikari (Table 1). It suggested that the root transported more iron per dry weight to the shoots in Kasalath, SL206, SL207, SL208 and SL220 than in Koshihikari. In rice, the roots are suggested to prevent the influx of excess ferrous iron by oxidation on the root surface (Tadano, 1976; Becker and Asch, 2005). The differences in the iron influx per root dry weight might be due to the power to eliminate excess ferrous iron on the root surface by oxidation. The lines which translocated more iron per root dry weight might have lower eliminating power and absorb more ferrous iron. Alternatively, the ability to store iron in root tissue might affect the translocation of iron to the shoot. A smaller amount of iron stored in root tissues might cause the increase in iron influx to shoots.

Rice plants have several resistance mechanisms against excess ferrous iron (Tadano, 1976; Becker and Asch, 2005). These mechanisms include (1) elimination of ferrous iron on the root surface by oxidation (Green and Etherington, 1977), (2) storage of excess iron in root tissue to block the translocation to the shoots (Tadano, 1976), (3) retention of iron in stem tissue and the apoplast of the leaf (Becker and Asch, 2005), (4) absorption of iron by ferritin in plastids (Briat and Lobréaux, 1997) and (5) detoxification of the active oxygen generated by iron in symplastic tissues (Becana et al., 1998). The QTL for increased shoot iron concentration detected in SL208 was considered to affect mechanism (1) or (2) because more iron was imported into shoots due to the lack of exclusion or compartmentation ability of iron in the roots. Determination of the effective gene on the QTL found in the present study might help elucidate the molecular mechanism of the system for preventing excess iron flow into shoots, helping contribute to the selection of genotypes that absorb less iron in shoots. Alternatively, the resistance mechanism after the iron is translocated to shoots might be regulated by different genetic factors. Yamauchi and Peng (1995) reported that the intensity of bronzing was not correlated with the increase in iron concentration in leaves among 16 rice genotypes. They suggested that cultivar differences in susceptibility to ferrous iron toxicity were caused not only by the concentration of iron in leaves, but also by the tolerance of leaf tissue to excess iron, which might involve the detoxification process. These QTLs for the leaf bronzing index were detected on chromosome 1 using Nipponbare / Kasalath inbred lines (Wan et al. 2003) and using Azucena / IR64 inbred lines (Wu et al., 1997, 1998; Dufey et al., 2009). The QTLs for the activity of ascorbate peroxidase and glutathione reductase, the enzymes involved in the scavenging system of active oxygen, and the increased concentration of dehydroascorbate and ascorbate were detected on the same locus of chromosome 1 under the ferrous iron stress conditions in Azucena / IR64 inbred lines (Wu et al., 1998). These QTLs did not correspond to the QTL for increased shoot iron concentration detected in the present study, suggesting that the location of the genetic factors that affect leaf tissue tolerance for ferrous iron toxicity might not be the same as that of the shoot iron concentration QTLs. Building up the genetic factors to eliminate ferrous iron uptake by roots and leaf tissue tolerance is expected to produce cultivars that are hyper-tolerant to iron toxicity. Among them, the putative QTL relating to the shoot iron concentration on chromosome 3 revealed in this study would be one of the factor for improving iron tolerance particularly in iron susceptible *indica* varieties of rice.

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