Epistatic Interaction of QTLs Controlling Leaf Bronzing in Rice (*Oryza sativa* L.) Grown in a Saline Paddy Field

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We analyzed quantitative trait loci (QTLs) in rice (*Oryza sativa* L.) for leaf bronzing induced by growing plants in a saline paddy field. The mapping population comprised 98 backcross-inbred lines (BILs) derived from crosses between Nipponbare, the recurrent parent, and Kasalath varieties. Fifteen of the BILs showed obvious leaf bronzing, unlike the parent varieties. We identified two QTLs for leaf bronzing: one (*qLb-3*) was located close to the R1925 marker on the long-arm of chromosome 3, the other (*qLb-11*) lay in the interval between the C1350 and C477 markers on the short-arm of chromosome 11. The Kasalath allele of *qLb-3* and the Nipponbare allele of *qLb-11* were found to promote leaf bronzing. Moreover, it was observed that the two QTLs were epistatic, the Kasalath *qLb-3* allele causing obvious leaf bronzing in the presence of the Nipponbare *qLb-11* allele. To confirm this interaction, we investigated leaf bronzing in three chromosome segment substitution lines: two of these lines, SL2 and SL61, carried an introgressed Kasalath *qLb-3* allele in a Nipponbare background while the third line, SL47, carried introgressed Kasalath alleles of both *qLb-3* and *qLb-11*. Leaf bronzing occurred in the SL2 and SL61 plants, but not in the SL47 plants. We further investigated the interaction between the two QTLs using F₂ plants derived from crosses between the SL47 and SL61. In the F₂ plants, segregants harboring both the Kasalath *qLb-3* allele and the Nipponbare *qLb-11* allele showed leaf bronzing. These results indicate that leaf bronzing required the epistatic interaction of the Kasalath *qLb-3* allele on chromosome 3 and the Nipponbare *qLb-11* allele on chromosome 11.

Key Words: chromosome segment substitution lines, complementary gene, leaf bronzing, quantitative trait loci, rice (*Oryza sativa* L.), saline paddy field, salt stress.

Introduction

Salinity is by far the most important toxicity problem encountered by rice (*Oryza sativa* L.). Although rice is the staple cereal for more people than any other crop, it is one of the most salt-sensitive among crop species (Yeo *et al.* 1985). Consequently, increased salt tolerance in rice would enable to promote global rice production and contribute to solving future food shortage problems. The efficient development of salt-tolerant rice cultivars requires a better understanding of the genetic characteristics that increase the tolerance of rice plants to higher levels of salinity in paddy fields.

Much of the genetic variation associated with tolerance to environmental stresses is controlled by a large number of genes, scattered throughout the genome, each of which with

a small effect. Identification and mapping of these quantitative trait loci (QTLs) have been facilitated by the development of both DNA markers and detailed linkage maps (Tanksley 1993). In many QTL analyses using DNA markers and molecular genetic maps, loci controlling environmental stress tolerance have been identified in various crop plants, particularly in rice (Ma *et al.* 2002, Sato *et al.* 2003, Ueda *et al.* 2004, Wissuwa *et al.* 1998, Wu *et al.* 2000). Furthermore, molecular cloning of genes at QTLs for resistance to environmental stress has been achieved using a map-based strategy (Ren *et al.* 2005, Ueda *et al.* 2005).

Regarding QTL analyses of salt tolerance in rice, loci that influence many of the relevant traits (e.g., dry weight of shoots, Na⁺ uptake, K⁺ uptake, Na⁺/K⁺ ratio of shoots, rate of seed germination, seedling root length and seedling vigor) have been identified (Flowers *et al.* 2000, Koyama *et al.* 2001, Lin *et al.* 2004, Prasad *et al.* 2000). These QTL analyses for salt-tolerance were conducted using hydroponics or pot cultivation in a greenhouse. However, mineral deficiencies,

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submergence, water depth and drought, in addition to salt stress, compound problems of salinity in paddy fields (Gregorio *et al.* 2002). In an earlier study, we identified 3 QTLs that control vegetative growth in saline paddy fields in all four cropping seasons: 1 QTL influenced shoot length, while 2 QTLs controlled tiller number (Takehisa *et al.* 2004).

Salinity not only affects various aspects of growth in rice, but also the withering of leaf blades and the dying of older leaves (Neue *et al.* 1990). We observed a bronzing phenotype on leaf blades that was associated with the suppression of subsequent leaf growth in a saline paddy field. Leaf bronzing in rice occurs widely in the wetlands of Sri Lanka and in paddy fields of other Asian countries (Ota and Yamada 1962). It markedly reduces the growth and yield of rice in these areas (Ota 1968). Based on these observations, leaf bronzing might be linked to depressed rice yields. Therefore, it is important to gain an understanding of this phenomenon, as part of the process to achieve efficient rice production in saline paddy fields. In some studies, it was reported that physiological factors such as nutrient deficiency and increased toxicity in paddy fields determine leaf bronzing (Inada 1965, Yamauchi 1989). However, the number and chromosomal location of the loci associated with leaf bronzing in paddy fields have not yet been determined.

The objective of the present study was to elucidate the genetic mechanism of leaf bronzing in order to gain a better understanding of salt tolerance of rice in saline paddy fields. We firstly aimed at identifying QTLs associated with leaf bronzing in rice plants grown in a saline paddy field and secondly at characterizing the epistatic interactions of these QTLs. To achieve these objectives, we used backcross-inbred plant lines (BILs) derived from a Nipponbare $\times$ Kasalath cross, chromosome segment substitution lines (SLs), and F_2 plants of a cross between these SLs.

Materials and Methods

Plant materials

To identify QTLs, we used 98 backcross-inbred lines (BILs) that were developed from BC_1F_1 (Nipponbare/Kasalath//Nipponbare) by the single-seed descent method. Nipponbare is a Japanese lowland rice variety belonging to the *japonica* group; Kasalath is an *indica* landrace from Assam, India. Three chromosome segment substitution lines (SLs), SL2, SL47 and SL61, were used, which contained Kasalath chromosomal segments carrying the QTLs in a Nipponbare background (Fig. 4A). These SLs were selected from advanced backcross progenies using Nipponbare as the recurrent parent and Kasalath as the donor parent by marker-assisted selection. Genotype information for SL2 and SL47 can be found at <http://www.rgrc.dna.affrc.go.jp/data/NK-SL54-20030430.xls>, while the information for SL61 is unlisted. The marker information about BILs and SLs can be found at <http://rgp.dna.affrc.go.jp/publicdata/geneticmap2000/index.html>. The Rice Genome Project of

the National Institute of Agrobiological Sciences, Japan developed these lines and the Rice Genome Resource Center (<http://www.rgrc.dna.affrc.go.jp/index.html.en>) provided the seeds. Original F_2 plants were derived from a cross between SL47 and SL61.

Growth conditions

In April, seeds were placed in distilled water at 30°C for 2 days. Germinated seeds were then sown on seedling cell-trays (30 cm $\times$ 60 cm) and grown in a greenhouse for four weeks. Field experiments were conducted at the Experimental Farm Station, Graduate School of Life Sciences, Tohoku University, in Kashimadai, Miyagi, Japan. Basal fertilizer (N, P and K) was applied to the paddy fields at 7 days before transplanting: nitrogen, phosphoric acid and potassium were applied at rates of 30, 30 and 30 kg/ha, respectively. Four-week seedlings were arranged with single plantings per hill. The space between hills was 30 cm.

Flooding with salt water

Initially, we irrigated the paddy fields with fresh water immediately after rice transplanting to promote the growth of the plants. Two weeks after transplantation, one paddy field was flooded with salt water (Na^+ ; 120 mM) and a second one with fresh water (Na^+ ; less than 5 mM). The Na^+ concentration of the floodwater in the saline paddy field was maintained at 50–120 mM Na^+ by flooding with salt water or fresh water every week to compensate for the effects of evaporation and rainfall. The Na^+ concentration of floodwater in the control paddy field was maintained at below 10 mM by flooding only with fresh water. The floodwater depth was maintained at 5–10 cm in both paddy fields. The Na^+ concentration in floodwater was determined using a Na^+ -specific electrode (ION/pH Meter IM-22P; DKK-TOA Corp., Tokyo). Plants subjected to three replications of the BIL experiment were transplanted to the control and saline paddy fields in late May 2005. In late May 2004 and 2005, 15 plants of the parent varieties and each SL were transplanted to the control and saline paddy fields. F_2 plants were transplanted to the saline paddy field in late May 2005.

Evaluation of leaf bronzing

Leaf bronzing scores were determined in mid-July, during the phase of vegetative growth. Leaf bronzing was scored using the following grading system: score 0, no bronzing; score 1, bronzing present on tips of old leaves; score 2, bronzing present throughout old leaves; score 3, bronzing present on tips of new leaves and throughout old leaves; score 4, bronzing present on all the leaves. In the QTL analyses using BILs, leaf bronzing scores were calculated as the mean values of the three replicating in the saline paddy field. Leaf bronzing scores were evaluated using the “Standard evaluation system for rice” for leaf spot disease that had been modified by IRRI in 1996 (INGER 1996).

Genotype data of BILs and QTL analysis

To map the QTLs associated with leaf bronzing in the BILs, we used genotype data generated using 245 RFLP markers (<http://rgp.dna.affrc.go.jp/publicdata/genotypedataBILs/genotypedata.html>). QTL analysis was performed by composite interval mapping (CIM) using the QTL Cartographer ver. 2.0 computer program (Wang *et al.* 2004). CIM analysis was performed using the forward-backward stepwise regression, model 6 with a 10 cM window size. A log-likelihood (LOD) score threshold of 2.5 was adopted to identify the regions containing loci putatively associated with leaf bronzing. QTL positions were assigned to the point of maximum LOD score in the target regions. The percentage of the total phenotypic variance, accounted for by each QTL was estimated based on the R^2 value. Two-way interactions between pairs of QTLs were tested by ANOVA.

Genotype mapping of the F_2 plants and QTL analysis

DNA was extracted from each of the 94 F_2 plants derived from a cross between SL47 and SL 61. A piece of leaf, 2 cm in length, was cut from the tip of each leaf blade, placed in a microtube containing extraction buffer [200 mM Tris-HCl (pH 8.0), 250 mM NaCl, 25 mM EDTA, 0.5% (w/v) sodium dodecyl sulfate (SDS)], and homogenized with a pestle. DNA was precipitated with isopropanol and then re-suspended in 50 μ l of TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA].

We used 21 markers in total on chromosomes 3, 4, 6, 7, 8, 9, 10, 11 and 12 (at least one marker on each segment of Kasalath chromosome introgressed in SL47). Six sequence-tagged site (STS) and three cleaved amplified polymorphic sequence (CAPS) markers for genotyping mapping were obtained from a database constructed by the Rice Genome Research Program (<http://rgp.dna.affrc.go.jp/publicdata/caps/index.html>). Eleven simple sequence repeat (SSR) markers were selected from the information presented by McCouch *et al.* (2002). Primer sequences for the 10-01 marker, an STS marker on chromosome 10, were designed using the genome sequence database for Nipponbare constructed by the Rice Genome Research Program (<http://rgp.dna.affrc.go.jp/publicdata/geneticmap2000/index.html>): forward primer sequence, 5'-taacacctcccaactttcg-3'; reverse primer sequence: 5'-ttggcctagtgtgcagaaaa-3'. The PCR amplification fragment was separated using electrophoresis on a 2% agarose gel. A total of 21 markers were mapped using the program MAPMAKER/EXP v.3.0 (Lincoln *et al.* 1993). The QTL analyses were performed by single marker analysis using the computer program QTL Cartographer v.2.0. (Wang *et al.* 2004). The QTLs were considered to be significant at P -values above 0.01.

Results

Leaf bronzing in BILs

In the control paddy field, leaf bronzing was not observed in either the BILs or the parental varieties Nipponbare

and Kasalath. In the saline paddy field, 15 BILs showed bronzing (i.e. with scores above 1), while the remaining 83 lines were designated as normal (i.e. with scores less than 1) (Fig. 1). The observed ratio of bronzing to normal BILs did not differ significantly from the ratio of 3: 13 expected for a trait governed by two complementary genes originating from each parent ($\chi^2=0.38$, $P=53.6\%$). On the other hand, the parental varieties failed to display leaf bronzing. Our results suggested that two complementary genes originating from Nipponbare and Kasalath controlled leaf bronzing in the plants grown under saline conditions.

QTL analysis for leaf bronzing in the saline paddy field

We used the BILs to identify the QTLs associated with leaf bronzing in the rice plants grown in the saline paddy field. Two QTLs were identified, one was located near the R1925 marker on the long arm of chromosome 3 (*qLb-3*) and the second lay in the interval between the C1350 and C477 markers on the short arm of chromosome 11 (*qLb-11*) (Fig. 2). The *qLb-3* allele from Kasalath promoted leaf bronzing (Table 1). The LOD score of 31.7 for the QTL accounted for 83% of the phenotypic variance, and the additive effect was -3.12. The *qLb-11* allele from Nipponbare also promoted leaf bronzing (Table 1). The LOD score of 3.5 for this QTL accounted for 8% of the phenotypic variance, and the additive effect was 0.76. The analyses showed that the two QTLs, *qLb-3* and *qLb-11*, controlled leaf bronzing in the BILs grown in a saline paddy field.

Epistatic interaction between *qLb-3* and *qLb-11*

Two-way interactions were analyzed between the

Fig. 1. Frequency distribution of leaf bronzing scores of backcross-inbred lines (BILs) and of their parents, Nipponbare and Kasalath, grown in the saline paddy field. Leaf bronzing was scored using the following grading system: score 0, no bronzing; score 1, bronzing present on tips of old leaves; score 2, bronzing present throughout old leaves; score 3, bronzing present on tips of new leaves and throughout old leaves; score 4, bronzing present on all the leaves. Leaf bronzing scores in the BILs were calculated as the mean values of the three replications in the saline paddy field.

Fig. 2. Mapping of QTLs for leaf bronzing in the backcross-inbred lines (BILs) grown in the saline paddy field. Black vertical bars denote the genomic regions giving a LOD score of more than 2.5 for leaf bronzing. Arrowheads indicate the position of the highest LOD peak for each QTL. Open and closed arrowheads indicate, respectively, that the Nipponbare and Kasalath alleles enhanced the extent of leaf bronzing.

Table 1. Location and effect of QTLs on the leaf bronzing score of backcross-inbred lines grown in the saline paddy field

Chromosome	Marker ¹⁾	LOD ²⁾	R^2 ³⁾	Add ⁴⁾
3	R1925	31.7	0.83	-3.12
11	<u>C1350</u> /C477	3.5	0.08	0.76

¹⁾ Marker nearest to QTL is underlined.

²⁾ LOD score (threshold > 2.5)

³⁾ Proportion of the phenotypic variance explained

⁴⁾ Estimated effect of replacing Kasalath alleles by Nipponbare alleles

QTLs, *qLb-3* and *qLb-11* in the BILs (Fig. 3). This analysis revealed the presence of a significant interaction between *qLb-3* and *qLb-11* ($P < 0.01$). The Nipponbare *qLb-3* allele did not enhance the leaf bronzing score, regardless of the *qLb-11* genotype. However, the Kasalath *qLb-3* allele interacted with the Nipponbare *qLb-11* allele to enhance the average leaf bronzing score by 2.2, while interactions between the Kasalath *qLb-3* allele and Kasalath *qLb-11* allele led to the increase of bronzing by 0.8. These results suggested that the Kasalath *qLb-3* allele enhanced the leaf bronzing score depending on the genotype at *qLb-11*.

We used three chromosome segment substitution lines (SLs), SL2, SL47 and SL61, to confirm the interaction between *qLb-3* and *qLb-11*. These SLs were selected from advanced backcross progenies, based on marker information for the two putative QTLs. All the SLs harbored Kasalath chromosome segments in the *qLb-3* region of chromosome 3 (Fig. 4A). Two SLs, SL2 and SL61, carried Nipponbare seg-

Fig. 3. Two-way interactions between *qLb-3* and *qLb-11*. The graph points indicate the mean leaf bronzing scores of the four genotypic classes of BILs for *qLb-3* (R1925 marker) and *qLb-11* (C1350 marker). N and K denote the Nipponbare and Kasalath alleles. In the 98 BILs, 57 lines carried the Nipponbare *qLb-3* allele and the Nipponbare *qLb-11* allele, 6 lines bore the Kasalath *qLb-3* allele and the Kasalath *qLb-11* allele, 17 lines harbored the Nipponbare *qLb-3* allele and the Kasalath *qLb-11* allele, and, 18 lines bore the Kasalath *qLb-3* allele and the Nipponbare *qLb-11* allele.

ments in the *qLb-11* region of chromosome 11, while the third SL, SL47, bore a Kasalath segment in this region of chromosome 11. In the control paddy field, none of the SLs showed leaf bronzing. In the saline paddy field, two SLs, SL2 and SL61, displayed severe leaf bronzing (score 4). In contrast, SL47 did not exhibit leaf bronzing (score 0) (Fig. 4B). The donor and background varieties, Kasalath and Nipponbare, again did not display leaf bronzing, either in the control or saline paddy fields. These results suggested that the two QTLs, *qLb-3* and *qLb-11*, contributed to leaf bronzing in a complementary manner in the BILs grown in a saline paddy field.

We performed single marker analysis using 94 F_2 plants derived from a cross between SL47 and SL61 to examine epistatic interactions between *qLb-3* and *qLb-11*. In the F_2 plants, the *qLb-3* alleles were homozygous for Kasalath, while the *qLb-11* alleles were homozygous for Kasalath, for homozygous Nipponbare, or heterozygous for Kasalath and Nipponbare. Information about the 21 DNA markers used in this analysis is presented in the Materials and Methods. Single marker analysis revealed that the S21074 allele (located between the C477 and G320 markers, the DNA markers nearest to *qLb-11* on chromosome 11), was closely linked to leaf bronzing. For S21074, a LOD score of 20.5 was obtained. The allele from Nipponbare promoted leaf bronzing. The leaf bronzing scores of the F_2 plants in each of the three genotype classes for S21074 (homozygous for Nipponbare, homozygous for Kasalath, and heterozygous for Nipponbare and Kasalath) are shown in Fig. 5. The leaf-bronzing scores of the F_2 plants carrying the Nipponbare S21074 allele were above 1, whereas in the plants carrying the Kasalath allele, the leaf bronzing score was less than 1. The leaf bronzing score of the F_2 plants was

Fig. 4. (A) Graphical representation of the genotypes of the chromosomal segment substitution lines (SLs), SL2, SL47 and SL61. White and black bars on each chromosome denote, respectively, the Nipponbare and Kasalath segments. Ellipses indicate approximate positions of the QTLs identified in the backcross-inbred lines, that enhance leaf bronzing. (B) Leaves of Nipponbare (N), SL2, SL47, SL61 and Kasalath (K) grown in the saline paddy field. These leaves were photographed at 2 months after the plants were transplanted to the saline paddy field. The numbers indicate the assessed leaf bronzing score determines for each line.

enhanced by the Nipponbare *S21074* allele and was suppressed by the Kasalath allele. These results indicated that leaf bronzing in the BILs in the saline paddy field was induced by the complementary interaction of the Kasalath *qLb-3* allele on chromosome 3 and the Nipponbare *qLb-11* allele on chromosome 11.

Discussion

Yano and Sasaki (1997) showed that the use of a series of near-isogenic lines or substitution lines provided a valuable approach for clarifying the epistatic interaction between QTLs. Although in previous studies, epistatic interactions between QTLs that determine the resistance to environmental stress had been detected (Li *et al.* 2005, Nguyen *et al.* 2003, Sripongpankul *et al.* 2000, Wu *et al.* 2000, Zhang *et al.* 2005), in none of these analyses near-isogenic lines or substitution lines had been used. This is the first report in which that has used substitution lines were used to identify an epistatic interaction between QTLs for leaf bronzing in rice. Our analyses using the substitution lines, SL2, SL47 and SL61, and the F_2 plants of a SL47 $\times$ SL61 cross, clearly demonstrated that leaf bronzing was induced by the epistatic interaction between the Kasalath *qLb-3* allele on chromosome 3 and the Nipponbare *qLb-11* allele on chromosome 11. Therefore, the absence of leaf bronzing in the parental

Fig. 5. Frequency distribution of leaf bronzing scores of F_2 plants of the three genotype classes for the *S21074* allele on chromosome 11: homozygous for Nipponbare, homozygous for Kasalath, and heterozygous for Nipponbare and Kasalath. The F_2 plants were derived from crosses between SL47 and SL61 and grown in the saline paddy field. White, black and gray bars indicate the genotypes homozygous for Nipponbare, homozygous for Kasalath and heterozygous for Nipponbare and Kasalath for *S21074*, respectively.

varieties, Nipponbare and Kasalath, could be explained by the fact that they did not carry either the Kasalath *qLb-3* or Nipponbare *qLb-11* alleles (Fig. 4).

In the present study, some of the F_2 plants that were homozygous for the Kasalath *S21074* allele showed leaf bronzing (leaf bronzing score: 1) (Fig. 5), suggesting that either other minor genes control leaf bronzing with *qLb-3* and *qLb-11* or that *S21074* is incompletely linked to *qLb-11*. To test these assumptions, fine mapping and characterization of both *qLb-3* and *qLb-11* should be carried out in the future.

In the present study, leaf bronzing in rice was induced in a saline paddy field. In earlier studies, plants grown in culture solutions had been used and it was reported that leaf bronzing occurred where there was an excess of metal ions, e.g. Fe^{2+} , Mn^{2+} and Zn^{2+} (Dong *et al.* 2006, Inada 1965, Tanaka *et al.* 1966, Wang *et al.* 2002). In some studies, QTLs that control leaf bronzing induced by stress associated with an excess of metal ions had been screened. As for ferrous iron stress, for example, Wu *et al.* (1997) used a doubled haploid (DH) population consisting of 123 lines derived from the *japonica* variety Azucena and the *indica* variety IR64, to identify a QTL on chromosome 1 for leaf bronzing in rice. Wan *et al.* (2003) also reported that QTLs on chromosome 1 for leaf bronzing were induced by ferrous iron stress. In this latter study, a mapping population of 96 BILs derived from a backcross of Nipponbare/Kasalath/Nipponbare had been used. As for manganese stress, Wang *et al.* (2002) used a recombinant inbred line (RIL) population, derived from a cross between the *japonica* variety Azucena and the *indica* variety IR1552, to show that QTLs for brown spots on leaves were positioned near the centromere of chromosome 3 and on chromosomes 4, 5, 6 and 10. Dong *et al.* (2006) reported that, under conditions of zinc stress, QTLs for leaf

bronzing were present on chromosome 1, the short arm of chromosome 3 and on chromosome 10 in a RIL population from a cross between the *japonica* variety Asominori and the *indica* variety IR24. None of the QTLs described in these reports corresponded to those identified in the present study. Therefore, it is possible that *qLb-3* and *qLb-11* are new QTLs for leaf bronzing that are independent of those induced by metal stress. Further investigations of the molecular mechanisms where by *qLb-3* and *qLb-11* act, should enable to clarify not only the leaf bronzing development processes, but also environmental stress tolerance, including salt tolerance, of rice in paddy fields.

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