Allelic interaction at seed-shattering loci in the genetic backgrounds of wild and cultivated rice species

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It is known that the common cultivated rice (Oryza sativa) was domesticated from Asian wild rice, O. rufipogon. Among the morphological differences between them, loss of seed shattering is one of the striking characters specific for the cultivated forms. In order to understand the genetic control on shattering habit, QTL analysis was carried out using BC2F1 backcross population between O. sativa cv. Nipponbare (a recurrent parent) and O. rufipogon acc. W630 (a donor parent). As a result, two strong QTLs were detected on chromosomes 1 and 4, and they were found to be identical to the two major seed-shattering loci, qSH1 and sh4, respectively. The allelic interaction at these loci was further examined using two sets of backcross populations having reciprocal genetic backgrounds, cultivated and wild. In the genetic background of cultivated rice, the wild qSH1 allele has stronger effect on seed shattering than that of sh4. In addition, the wild alleles at both qSH1 and sh4 loci showed semi-dominant effects. On the other hand, in the genetic background of wild rice, non-shattering effects of Nipponbare alleles at both loci were examined to inspect rice domestication from a viewpoint of seed shattering. It was serendipitous that the backcross plants individually having Nipponbare homozygous alleles at either shattering locus (qSH1 or sh4) shed all the seeds. This fact strongly indicates that the non-shattering behavior was not obtained by a single mutation in the genetic background of wild rice. Probably, some other minor genes are still associated with the formation or activation of abscission layer, which enhance the seed shattering.

Key words: domestication, Oryza rufipogon, O. sativa, rice, seed shattering

INTRODUCTION

Cereal crops providing the world’s primary food were domesticated from wild grass species by the ancient humans. Distinct morphological differences are observed between cultivated forms and their wild ancestors. The investigation of such domestication related traits shed light on the clarification how the ancient humans modified the wild plants for our life. Several studies have been reported to identify the genes or mutations underlying the domestication events using molecular techniques and genome information (reviewed in Glémin and Bataillon, 2009; Purugganan and Fuller, 2009; Sang, 2009). One of the striking characters specific for the cultivated forms is loss of seed shattering (Harlan, 1975). Originally, wild seed-shattering behavior at maturation stage guarantees the success of propagation through seed dispersal. This character, however, is undesirable for the ancient seed gatherers because of the difficulty of seed collection. Therefore, the reduction of seed-shattering degree was considered to be required in the early phase of domestication (Fuller, 2007).

It is known that cultivated rice (Oryza sativa L.) was domesticated from its wild ancestor, O. rufipogon, and several distinct morphological changes are observed between cultivated and wild forms including loss of seed shattering (Doebley et al., 2006; Kovach et al., 2007; Sang and Ge, 2007; Izawa et al., 2009; Panaud, 2009). The wide range of seed-shattering degree is observed among rice cultivars in the world (Konishi et al., 2006), suggesting that the shattering habit is a polygenic and complex trait. Many genetic approaches have been conducted to
identify the genes controlling shattering habit observed among cultivated and wild rice species (Xiong et al., 1999; Cai and Morishima, 2000; Thomson et al., 2003; Lee et al., 2005; Konishi et al., 2006; Li et al., 2006a; Onishi et al., 2007a). In these studies, several QTLs were reported, and two of them were found to be strongly responsible for the shattering habit. They were further fine-mapped and the genes were successfully isolated. The first QTL, \textit{sh4}, was detected in the F$_2$ population between cultivated (\textit{O. sativa}) and its wild ancestral species (\textit{O. rufipogon}) (Li et al., 2006b; Lin et al., 2007). This QTL explained 69.0% of the total phenotypic variance in the population. The wild allele encodes a putative transcription factor with Myb3 DNA binding domain, whereas the cultivated mutant allele has a single nonsynonymous substitution in the domain. Lesion in this gene affects the formation of abscission layer between pedicel and spikelet, resulting in the prevention from seed dispersal (Li et al., 2006b; Lin et al., 2007). The other QTL, \textit{qSH1}, showing 68.6% of the total variation of shattering habit was found in the F$_2$ population between \textit{O. sativa} Japonica cv. Nipponbare and \textit{O. sativa} Indica cv. Kasalath (Konishi et al., 2006). Interestingly, both cultivated alleles encode BEL1-type homeobox gene, however, the causative mutation was found only in Nipponbare as a single nucleotide polymorphism (SNP) in the 5' upstream region of the gene. This mutation causes the absence of abscission layer formation in Nipponbare and has strong association with non-shattering habit in Japonica population (Konishi et al., 2006).

The nucleotide divergence at \textit{sh4} and \textit{qSH1} was investigated to understand the process of rice domestication (Onishi et al., 2007b; Lin et al., 2007; Zhang et al., 2009). For example, the mutational and flanking sequences at \textit{sh4} locus were found to be conserved among the rice cultivars examined so far, while wild rice showed wide nucleotide divergence in these regions, suggesting that the non-shattering \textit{sh4} mutation could be monophyletic for rice cultivars and might cause the domestication event (Onishi et al., 2007b; Lin et al., 2007; Zhang et al., 2009). Regarding the allele effects, the wild shattering traits have always been evaluated in the genetic background of cultivars. However, in the early phase of rice domestication, the ancient humans might select non-shattering mutants in the wild natural populations. Therefore, in order to figure out the rice domestication, the effects of non-shattering alleles should be examined in the genetic background of wild rice. In this study, we conducted genetic analyses on seed shattering using the F$_2$ and BC$_2$F$_1$ populations between \textit{O. sativa} Nipponbare and \textit{O. rufipogon} W630. Subsequently, two backcross populations with reciprocal genetic backgrounds of Nipponbare and W630 were developed to examine the allelic effects and interaction at seed-shattering loci, \textit{sh4} and \textit{qSH1}.

**MATERIALS AND METHODS**

**Plant materials** A cultivar of \textit{O. sativa} Japonica Nipponbare and a wild accession of \textit{O. rufipogon} W630 were used in this study. The latter is a wild annual accession originated from Myanmar and was kindly provided from National Institute of Genetics, Japan. \textit{O. sativa} Nipponbare has non-seed-shattering behavior, whereas \textit{O. rufipogon} W630 completely disperses the seeds at maturation stage. They were crossed and the F$_2$ segregating population composed of 159 plants was produced (Fig. 1A). In addition, their F$_1$ hybrids were backcrossed with \textit{O. sativa} Nipponbare and \textit{O. rufipogon} W630, and two types of populations were generated, which consisted of the lines with small portions of wild and cultivated segments in the genetic backgrounds of cultivated and wild rice, respectively (Fig. 1). In the crossing, hot water treatment (44°C, 5 min) was successfully applied for the pollen emasculation of both wild and cultivated rice plants.

**QTL analysis for seed shattering** A total of 189 BC$_2$F$_1$ plants, which were generated by backcrossing twice with \textit{O. sativa} Nipponbare, were used for QTL analysis for seed shattering (Fig. 1A). They were planted in the paddy field of Kobe University, and the shattering phenotypes were examined to classify into three criteria, i.e., 0: non-shattering (same phenotype as Nipponbare), 1: weak shattering (matured seeds were detached by hand gripping), 2: strong shattering (no seeds remain on the panicle under natural condition). Their genomic DNA samples were prepared by potassium acetate method (Dellaporta et al., 1983). Eighty-three SSR markers covering all 12 chromosomes were used for PCR amplification, and the amplified products were electrophoresed in 4.0% polyacrylamide gels and the banding patterns were visualized by silver staining method (Panaud et al., 1996). Statistical analysis between marker and trait data was performed using qGene program (Nelson, 1997).

**Genotyping at seed-shattering loci** For the estimation of genotypes at seed-shattering loci in the backcross populations, two pairs of flanking SSR markers covering \textit{qSH1} on chromosome 1 and \textit{sh4} on chromosome 4 were used. The SSR markers were selected according to the map information on these loci, namely, RM315 and RM265 for \textit{qSH1}, and RM131 and RM349 for \textit{sh4}.

DNA sequencing around the causative SNPs at \textit{qSH1} and \textit{sh4} was also carried out with \textit{O. sativa} Nipponbare and \textit{O. rufipogon} W630. The amplified products after genomic PCR were sequenced with forward and reverse primers for target regions using BigDye terminator ver. 1.1 (Applied Biosystems).
Evaluation of seed-shattering degree in the backcross populations  In order to investigate allelic interaction at seed-shattering loci, seed-shattering degree was measured using two types of backcross populations with different genetic backgrounds. In the case of Nipponbare backcross population, a single plant having heterozygous alleles at both $qSH1$ and $sh4$ loci was selected at BC 2F1 generation based on the flanking marker genotypes. The selected plant was backcrossed twice with Nipponbare, and a BC 4F1 plant with wild heterozygous alleles was chosen. This plant was further self-pollinated and a backcross population (Population N-1) was produced at BC4F2 generation (Fig. 1A). Since the segregation (wild W630 homozygous, heterozygous and Nipponbare homozygous alleles) may independently occur at $qSH1$ and $sh4$ loci, a total of nine allelic combinations or genotypes for two loci were expected to be generated in this population. At the seedling stage, the plant genotypes in the population were examined using flanking marker pairs. Five plants for each genotype were selected and transferred to the pots for the evaluation of seed shattering. Panicles of these plants were collected on 35 days after heading. The other segregating population (Population N-2) was also produced from the BC4F1 plant having heterozygous alleles at both loci to repeat the evaluation (Fig. 1A). As for the wild backcross population, two plants were first selected at BC1F1 generation, and marker-assisted backcrossing was carried out to maintain Nipponbare non-shattering alleles at $qSH1$ and $sh4$ in the genetic background of wild rice. Finally, two segregating populations at BC2F2 generation (Populations W-1 and W-2) were produced (Fig. 1B), and four plants for each genotype were selected in both populations.

It is quite difficult to examine seed-shattering degree by hand gripping. Therefore, the precise value was measured in breaking tensile strength upon detachment of seeds from the pedicels using a shattering tester, TR-II (Fujiwara Scientific Co., Japan) (Ichikawa et al., 1990; Konishi et al., 2006). For each backcrossed plant, 50 seeds (10 seeds per panicle) were examined and the average strength was calculated.

RESULTS Segregation of seed-shattering behavior in F2 population between *O. sativa* Japonica Nipponbare and *O. rufipogon* W630  In order to understand the inheritance manner of shattering habit of *O. rufipogon* W630, F1 hybrids and F2 population between *O. sativa* Japonica Nipponbare and *O. rufipogon* W630 were produced. The F1 plants shed seeds at maturation stage as the wild parent, indicating that seed shattering was a dominant trait. A total of 159 F2 plants were grown in the university paddy field and their seed-shattering char-
acters were preliminary examined. However, the degree of seed shattering was continuous among the F$_2$ plants. They could be classified into only two groups, shattering (including weak shattering) and non-shattering. As a result, eight plants showed complete non-shattering habit as Nipponbare (Table 1). This segregation (151 shattering and 8 non-shattering) fitted to the 15:1 ratio, suggesting that wild seed shattering is mainly controlled by two dominant genes.

**Estimation of QTLs for seed shattering using BC$_2$F$_1$ population between O. sativa Nipponbare and O. rufipogon W630** In the previous experiment, F$_2$ population was used for seed-shattering evaluation, however, this population consisted of the plants with various kinds of plant morphologies and the degree of seed shattering was continuous. Therefore, further QTL analysis for seed shattering was carried out with the backcross population (BC$_2$F$_1$ population) in the genetic background of O. sativa Nipponbare. A total of 189 BC$_2$F$_1$ plants were planted in the university paddy field, and their shattering phenotypes and marker genotypes at 83 SSR loci covering 12 rice chromosomes were examined. As a result, two major QTLs on chromosomes 1 and 4 were detected (Table 2). They were mapped to the similar chromosomal regions of two loci, qSH1 and sh4, for seed shattering identified by Konishi et al. (2006) and Li et al. (2006a), respectively. In order to clarify the QTLs detected in this study are identical to those previously reported, the genetic regions of two shattering loci of O. rufipogon W630 were sequenced. As expected, O. rufipogon W630 was confirmed to have intact shattering genes at qSH1 and sh4 loci. Since O. sativa Nipponbare has non-functional alleles at both loci, we conclude that two QTLs detected here are identical to qSH1 and sh4.

<table>
<thead>
<tr>
<th>No. of plants</th>
<th>Shattering</th>
<th>Non-shattering</th>
<th>Total</th>
<th>$\chi^2$ (15:1)</th>
<th>P</th>
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<td>151</td>
<td>8</td>
<td>159</td>
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**Table 2.** Putative QTLs for seed shattering detected in BC$_2$F$_1$ population between O. sativa Nipponbare and O. rufipogon W630

- **Allelic interaction at seed-shattering loci in the genetic background of cultivated rice** Two major QTLs for seed shattering, i.e., qSH1 and sh4, were detected in the BC$_2$F$_1$ population in the genetic background of cultivated rice. The allelic interaction at these two loci was further examined using the backcross populations of N-1 and N-2 (Fig. 1A). In both populations, segregations were independently occurred at qSH1 and sh4 loci, and a total of nine genotypes at two loci were observed. The panicles of the plants for each genotype were subjected to the evaluation of shattering degree. We found that four genotypes (HH, WH, HW and WW) allelic combinations in genotypic order at qSH1 and sh4; where N, H and W indicate Nipponbare homozygous, heterozygous and wild W630 homozygous alleles, respectively) exhibited strong shattering whereas other five (NN, HW, WN, NH and NW) kept matured seeds on the panicles. Fig. 2 shows an example of the shattering behavior after hand gripping for the latter five genotypes in Population N-1. The precise degree of shattering was further measured by TR-II, the tester for breaking tensile strength. For each plant, 50 seeds (ten seeds per panicle) were examined and the average value with standard deviation was calculated (Fig. 3). In both populations, average strength values of NN genotypes (ca. 200 gf) were similar to that of Nipponbare. The plants with wild qSH1 and Nipponbare sh4 alleles (WN genotypes) showed significantly lower average values ($P < 0.01$) than those with Nipponbare qSH1 and wild sh4 alleles (NW genotypes).
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Genotypes), suggesting that wild qSH1 allele has stronger effect on seed shattering than that of sh4. In addition, wild homozygous genotypes at qSH1 (WN: 53.7 gf in N-1, 68.7 gf in N-2) and sh4 (NW: 125.2 gf in N-1, 131.9 gf in N-2) gave lower average values than heterozygous genotypes (HN: 117.2, 123.5 gf and NH: 174.3, 161.0 gf), respectively. Significant differences (P < 0.05) were detected between the values of NN, NH and WN, and between the values of NN, NH and NW in both populations. These results strongly indicate that the wild alleles at both qSH1 and sh4 loci showed semi-dominant effects in the genetic background of cultivated rice.

Allelic interaction at seed-shattering loci in the genetic background of wild rice

In the process of rice domestication, seed-gathering people might select non-shattering mutants in wild natural populations. In order to understand and discuss the early phase of rice domestication, the non-shattering alleles of cultivated rice should be evaluated in the genetic background of wild rice. Therefore, O. sativa Nipponbare was backcrossed with O. rufipogon W630, and allelic interaction at two shattering loci was examined with two segregating backcross populations of W-1 and W-2 (Fig. 1B). The plants of nine genotypes at two loci were prepared by the marker-assisted selection, and their shattering habit was examined on 35 days after heading. Surprisingly, all the plants showed strong seed shattering except for the plants having the homozygous alleles of cultivated rice at both qSH1 and sh4 (Fig. 4). Although they kept matured seeds on the panicles, the breaking tensile strength values were 22.3 ± 4.1 gf and 31.4 ± 15.0 gf in Populations W-1 and W-2, respectively. Their values were significantly lower (P < 0.01) than that examined for O. sativa Nipponbare, 213 ± 16.6 gf. These results suggest that the cultivated alleles at both qSH1 and sh4 can hardly contribute for non-seed shattering in the genetic background of wild rice.

DISCUSSION

Among several domestication related traits, non-seed shattering is often regarded as one of the most important key traits in many crops (Fuller, 2007). In rice, QTL analysis for seed shattering have been carried out by many researchers and the several QTLs were reported (Xiong et al., 1999; Cai and Morishima, 2000; Thomson et al., 2003; Lee et al., 2005; Li et al., 2006a; Konishi et al.,
2006; Onishi et al., 2007a). Of these, two loci with strong effects, sh4 and qSH1, were further investigated and mutations leading to non-shattering behavior were identified (Konishi et al., 2006; Li et al., 2006b; Lin et al., 2007). In this study, the inheritance of shattering habit was first shown to be controlled by two dominant genes using F_{2} population between Japonica cultivar Nipponbare and wild accession of O. rufipogon W630 (Table 1). The following QTL analysis using BC_{1}F_{1} population also confirmed that the two distinct loci had strong effect on seed shattering (Table 2). Moreover, their chromosomal positions were found to be identical to sh4 and qSH1 based on the sequencing analysis of wild alleles.

Previously, allelic interaction at the shattering loci, qSH1, qSH4 (same as sh4) and qSH3 (putative shattering locus) were investigated in the genetic background of O. sativa Japonica cv. A68 (Onishi et al., 2007b). The results showed that both qSH1 and qSH4 (sh4) loci played significant roles in seed shattering in comparison to qSH3. Furthermore, qSH1 was found to be genetically epistatic to qSH4 (sh4), supporting the notion that qSH4 (sh4) activates the abscission process after the seed shattering formation regulated by qSH1 (Konishi et al., 2006; Li et al., 2006b). In the present study, wild qSH1 allele exhibited stronger effect on seed shattering than that of sh4 in the genetic background of Japonica rice cultivar Nipponbare. According to Lin et al. (2007) and Zhang et al. (2009), the non-functional mutation at sh4 was common among all rice cultivars examined, whereas Nipponbare-type qSH1 allele was partly detected among Japonica cultivars. Although the qSH1 locus has stronger influence on the seed-shattering (or non-seed-shattering) behavior, we do not know why the mutation inactivating qSH1 allele was not widely observed among rice cultivars. In this study, the breaking tensile strength values of the plants carrying heterozygous alleles were also examined. As a result, the wild alleles at both qSH1 and sh4 loci showed semidominant effects on the seed shattering in the genetic background of cultivated rice (Fig. 3). It will be of interest to analyze whether the degree of shattering is associated with the formation of abscission layer in each genotype. The histological observation of the abscission layer will be informative to evaluate the shattering habit.

Using segregating populations in the genetic background of wild rice, we also evaluated the allelic interaction at qSH1 and sh4 loci. The results were unexpected because non-shattering effects of Nipponbare alleles were hardly observed at qSH1 and sh4 in the genetic background of wild rice. Most of the plants showed strong shattering behavior, and only the plants with Nipponbare homozygous alleles at both qSH1 and sh4 loci could keep the matured seeds on the panicles (Fig. 4). It was serendipitous that the plants having Nipponbare homozygous alleles at either shattering locus (qSH1 or sh4) shed all the seeds. This fact strongly indicates that the non-shattering behavior was not obtained by a single mutation in the genetic background of wild rice. Probably, some other wild minor genes are still associated with the formation or activation of abscission layer, which enhance the seed shattering.

The general question is how many genes and mutations were required for non-shattering behavior in the early phase of rice domestication. The genes involved in domestication can be evaluated by the analysis on selective sweep, which shows reduction of nucleotide diversity in surrounding sequences of domestication related genes (Wright et al., 2005; Yamazaki et al., 2005). Although the neighboring regions of the seed-shattering loci have not been well analyzed, the single nucleotide polymorphisms generating the alleles with non-shattering effects at qSH1 and sh4 loci were surveyed among wild and cultivated rice species (Onishi et al., 2007b; Lin et al., 2007; Zhang et al., 2009). According to the results, the non-shattering sh4 allele was fixed in all rice cultivars examined, whereas non-shattering qSH1 allele was detected partly in Japonica cultivars. This strongly indicates that the reduction of seed shattering in cultivated rice was first enhanced by the sh4 mutation in the early phase of domestication. Probably, the sh4 was the most important key locus for seed shattering, however, only the sh4 mutation can not cause a dramatic change on seed shattering. Therefore, some other mutations at shattering related loci would have also occurred during rice domestication, and the cultivar-like plants with non-shattering behavior were gradually selected by early agricultural people.

Recently, OsCPL1, a novel gene encoding a CDP phosphatase-like protein, was identified through the mutagenesis of cultivated rice and shown to inhibit the development of abscission layer in rice (Ji et al., 2010). Although it is not known that OsCPL1 is involved in rice domestication, the identification of such factor(s) underlying the control of seed shattering and analysis of nucleotide diversity around the genes will be an important subject in the future studies. In addition, the evaluation of shattering behavior in the genetic background of wild rice will give insights into the regulation of seed shattering and process of domestication in rice more clearly.

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