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Genetic analysis of seed-shattering genes in rice using an $F_{3:4}$ population derived from an *Oryza sativa* x *Oryza rufipogon* cross

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ABSTRACT. Seed shattering of wild plant species is thought to be an adaptive trait to facilitate seed dispersal. For rice breeding, seed shattering is an important trait for improving breeding strategies, particularly when developing lines use interspecific hybrids and introgression of genes from wild species. We developed $F_{3:4}$ recombinant inbred lines from an interspecific cross between *Oryza sativa* cv. *Ilpoombyeo* and *Oryza rufipogon*. In this study, we genetically analyzed known shattering-related loci using the $F_{3:4}$ population of *O. sativa/O. rufipogon*. CACTA-AG190 was significantly associated with the shattering trait

CACTA-TD according to bulked segregant analysis results, and was found in the *qSH-1* region of chromosome 1. Fine genetic mapping of the flanking regions around *qSH-1* based on CACTA-AG190 revealed multiple-sequence variations. The highest limit of detection based on quantitative trait locus analysis was observed between *shaap-7715* and a 518-bp insertion site. Two other quantitative trait locus analyses of seed-shattering-related loci, *qSH-4* and *sh-h*, were performed using simple sequence repeat and allele-pecific single nucleotide polymorphism markers. Our results can be applied for rice-breeding research, such as marker-assisted selection between cultivated and wild rice.

Key words: Quantitative trait loci; Rice; Seed-shattering genes; Sequence characterized amplified region marker; Simple sequence repeat

INTRODUCTION

Rice (*Oryza sativa*) is an important staple crop that supplies 20% of the global daily calories (World Rice Statistics, http://www.irri.org). According to archaeological records, rice is also an ancient crop species, and its cultivation dates to more than 10,000 years ago in the upstream region of the Yangtze River in southwest China (Khush, 1997; Xu et al., 2011). Ancient humans noted several agronomic characteristics in wild plant species and selected favorable plants (consciously or unconsciously) through domestication processes (Hillman and Davies, 1990). These characteristics included high seed numbers, changes in plant architecture, changes in seed size and shape, adaptation of flowering time to local areas, reduction in seed dormancy, and loss of seed shattering (Gepts, 2004; Doebley et al., 2006; Kovach et al., 2007; Sang and Ge, 2007; Izawa et al., 2009; Panaud, 2009).

Seed shattering is thought to have adapted for seed dispersal in wild plant species. Seeds that shatter easily are not ideal for use by farmers because these seeds reduce yield under all types of harvesting practice. Therefore, non-shattering or shattering-resistant seeds are important traits for transitioning from wild to modern crop species. Seed shattering is caused by either seed abscission and/or spikelet disarticulation (Li and Gill, 2006). In rice breeding, seed shattering remains an important target trait for improvement, particularly in indica-type rice breeding and when developing lines of interspecific hybrids for introgressing genes of wild species (Konishi et al., 2006).

Most rice traits are quantitative and governed by quantitative trait loci (QTLs). These traits show continuous genetic variation in natural populations and among inbred lines. Because most important agricultural traits are controlled by QTLs, characterizing these loci is important for advancing rice-breeding programs (Tao and Song, 2007). Advances in DNA technologies has led to DNA marker development, resulting in marker-assisted selection, and has been widely applied to rice genomics to facilitate characterization of the relationship between phenotype and genotype. Genetic studies examining seed shattering were performed using molecular markers in rice. The shattering degree of cereals is complex and influenced by many genetic and environmental factors (Tang and Morishima, 1989; Konishi et al., 2006). Seed shattering is controlled by various QTLs distributed throughout the genome (Xiong et

al., 1999; Cai and Morishima, 2000; Kennard et al., 2002; Thomson et al., 2003; Gu et al., 2005; Lee et al., 2005; Konishi et al., 2006; Onishi et al., 2007). QTL analysis is performed using a population of natural variations derived by crossing the shattering-type *indica* cultivar and the non-shattering-type *japonica* cultivar. Several QTLs have been reported, with 2 strongly associated with shattering. These QTLs were further fine-mapped and the genes were identified. The 1st QTL, *sh4*, was detected in the F_2 population between cultivated (*O. sativa*) and its wild ancestral species (*O. rufipogon*) (Li et al., 2006; Lin et al., 2007). This QTL was responsible for 69.0% of the total phenotypic variance in the population. The wild-type allele encodes a putative transcription factor containing the Myb3 DNA-binding domain, whereas the cultivated mutant allele contains a single-non-synonymous substitution in the domain. Lesions in this gene affect abscission layer formation between the pedicle and spikelet, which prevents seed dispersal (Li et al., 2006; Lin et al., 2007). The other QTL, QTL for shattering on chromosome 1 (*qSH-1*), accounted for 68.6% of the total variation in the shattering process, and was identified in the F_2 population between *O. sativa japonica* cv. Nipponbare and *O. sativa indica* cv. Kasalath. *qSH-1* encodes a BEL1-type homeobox gene; a single-nucleotide polymorphism (SNP) in the 5'-regulatory region of the *qSH-1* gene resulted in the loss of seed shattering because of the absence of abscission layer formation (Konishi et al., 2006).

Over the past few decades, 4 loci, including *sh1* on chromosome 11 (Nagao and Takahashi, 1963), *sh*2 on chromosome 1 (Oba et al., 1990), *Sh3* on chromosome 4 (Nagai et al., 2002), and *Sh4* on chromosome 4 (Fukuta and Yagi, 1998), were found to play a role in seed-shattering in rice. Four independent groups reported genes or gene blocks involved in seed-shattering in rice (Konishi et al., 2006; Li et al., 2006; Sweeney et al., 2006; Ji et al., 2006). Using a genomic approach and association analysis, Konishi et al. (2006) demonstrated a single nucleotide change T→G in a *qSH-1* locus at a QTL on chromosome 1 that caused seed-shattering and accounted for 68.8% of the total phenotypic variations in a segregating population between *japonica* cultivars Nipponbare/Kasalath. Using a similar approach, but with a segregating population from an interspecific hybrid of *Oryza sativa* spp *indica*/*O. nivara*, Li et al. (2006) showed that 7 single-nucleotide mutations at the LOC_Os04g57530 locus (TIGRE rice annotation number) were involved in seed shattering in wild *Oryza* species (*O. nivara*, *O. rufipogon*, *O. glumaepatulae*, *O. barthii*, *O. longistaminata*, and *O. meridionalis*). Using the easy-shattering mutant of *japonica*-type rice, Ji et al. (2006) localized the shattering gene, *sh-h*, to chromosome 7. The *sh-h* locus is closely associated with the anthocyanin synthase gene *Rc* (Sweeney et al., 2006).

We developed a recombinant inbred line (RIL) from an interspecific cross between *O. sativa* cv. *Ilpoombyeo* and *O. rufipogon*. *O. rufipogon*, a wild species related to *O. sativa*, has red seeds with very high shattering characteristics. The objective of this study was to identify QTLs associated with seed-shattering traits in the $F_{3:4}$ population.

MATERIAL AND METHODS

Hybrid development and DNA isolation

Twelve hybrid seeds were obtained from crosses between *O. sativa* var. *Ilpoombyeo*

(♀) and *O. rufipogon* W259 (♂). All 12 F_1 seeds were sown, and 185 F_2 seeds were randomly selected to proceed to the next generation. Four seeds from 185 F_2 plants were randomly selected to proceed to F_3, and seed-shattering characteristics were subsequently analyzed. A total of 743 $F_{3:4}$ plants were analyzed. DNA was extracted from 3-week-old leaves using the modified protocol of Delaporta et al. (1983).

Shattering analysis

Plants were grown in 5-inch pots in the greenhouse to reduce any environmental effects on seed shattering. Forty days after sowing, plants were grown for 10 h in light and 14 h in darkness for 4 weeks. After flowering, 2 heads with the greatest number of florets in each plant were bagged to isolate auto-shattered florets. Bagged heads were harvested 30 days after flowering. To measure shattering, heads were placed in the bottom of a 1-m wood panel slanted at 10 degrees. A concrete roller (1 kg) was then rolled twice from the top of the panel over the heads to shed the remaining florets.

Transposon display

Genomic DNA from 17 all-shattering (100%) and non-shattering (0%) plants were mixed at equal concentrations for bulk segregant analysis by transposon display using the *Rim2*/*Hipa* CACTA-TD protocol (Kwon et al., 2005). A total of 16 primer combinations were used; their nucleotide sequences are shown in Table 1. Specific markers for shattering or non-shattering were purified from the gel plate and cloned for nucleotide sequencing and subsequent sequence characterized amplified region (SCAR) analysis. Their genomic locations and other related genomic information were obtained from http://www.gramene.org/.

Fine genetic mapping of shattering loci

For SCAR markers, allele-specific polymerase chain reactions (PCR) were performed in a total volume of 20 μL, consisting of 10 ng genomic DNA, 10X PCR buffer, 0.5 μM primers, 0.2 mM dNTPs, and 1 U *Taq* DNA polymerase (Ex Taq, Takara, Shiga, Japan). PCR profiles included an initial denaturation at 94°C for 5 min, followed by 30 cycles of 10 s at 94°C, 20 s at 60°-68°C depending on the primer set, and 20 s at 72°C. A final extension step was conducted at 72°C for 3 min. Amplified products were resolved using 1.5% agarose gel electrophoresis and visualized using ethidium bromide staining.

Simple sequence repeat (SSR) analysis was performed at 13 SSR loci in the region of shattering locus *qSH-1* (Table 1). PCR amplifications were performed in a total volume of 25 μL, and contained 20 ng genomic DNA, 10X PCR buffer, 0.5 μM forward and reverse primers, 0.2 mM dNTPs, and 1 U Taq polymerase. The PCR profile consisted of an initial denaturation for 3 min at 95°C, followed by a 30-s denaturation at 95°C, a 30-s annealing at 55°C, and a 90-s extension at 72°C (repeated for 35 cycles). A final extension was conducted at 72°C for 5 min.

Table 1. Primer sequences of *Rim2/Hipa* CACTA-TD and fine mapping.

Primer name	Sequence	Primer name	Sequence	Primer name	Sequence
Rim2/Hipa CACTA-TD primers		RM5389	TCTTGCATGAGAGCCAACAC	Chromosome 4 fine mapping primers	
Adaptor			GCTATTGCGCGAGATTATCC	RM5771	GAAGAGGAGGAGCAGGAGG
KRMA-1	GACGATGAGTCCTGAG	RM3640	TACTGGTGCAAGGATACCCC		CATCACTCACGTTGTAGCGG
KRMA-2	TACTCAGGACTCAT		TGCTCCAAACCTCAGTCTCC	RM348	CCGCTACTAATAGCAGAGAG
*Mse*I anchors		RM7250	GTAAAGGCGAATCCGAACAC		GGAGCTTTGTTCTTGCGAAC
KRMP-0	GACGATGAGTCCTGAGTAA		CACGTGGTGGCTTGAGAAC	RM3648	TACCCTTTCTTCCCCAAACC
KRMP-AA	GACGATGAGTCCTGAGTAAAA	RM5407	CCGACTGAACAAGTACACAGC		ACCTCCTCCTCCACTTCTCC
KRMP-AC	GACGATGAGTCCTGAGTAAAC		GAGCAAGAACTGTCATCACC	RM1113	GGGCGCATGTGTATTTCTTC
KRMP-AG	GACGATGAGTCCTGAGTAAAG	shaap-2431(J)	CTCCGAACAAATTGATTCAG		TGGGGAAAAACCACAAGCC
KRMP-AT	GACGATGAGTCCTGAGTAAAT		TTGGTCTTCACCAGAACAAC	RM3333	GAAGCAAAAGCTATCGACAC
KRMP-CA	GACGATGAGTCCTGAGTAACA	shaap-2431(R)	CCTCCGAACAAATTGATAAAT		GAGCTTGTTGGCACCTTAC
KRMP-CC	GACGATGAGTCCTGAGTAACC		AGTCAGTGACGCCTATGTGAT	RM6473	GACGACAGGCTACAGGCATC
KRMP-CG	GACGATGAGTCCTGAGTAACG	shaap-3895	ATTATAGCCTAAGGTATGTACTTC		GTTAGCTAGCCATGGTTCGG
KRMP-CT	GACGATGAGTCCTGAGTAACT		ATTATAGCCTAAGGTATGTACTTT	RM5879	ACCAGAGATCGATTGGTAGC
KRMP-GA	GACGATGAGTCCTGAGTAAGA		ACTTGGTGATCTAAATAGTGG		GGCTGCCTATCGAGGCTAAC
KRMP-GC	GACGATGAGTCCTGAGTAAGC	shaap-5074	TACATTGTACTCCTACGTAGATT	RM1272	TCTATGGATCTGCATGCTGG
KRMP-GG	GACGATGAGTCCTGAGTAAGG		TACATTGTACTCCTACGTAGATG		CTGCCCTGTCCTTTTAATCG
KRMP-GT	GACGATGAGTCCTGAGTAAGT		AATTAGAACTAACCGTCCTTT	Chromosome 7 fine mapping primers	
KRMP-TA	GACGATGAGTCCTGAGTAATA	shaap-7715	GACGCACCACCGACAAGG	RM1243	TTTCGGGAGGGATTATGACC
KRMP-TC	GACGATGAGTCCTGAGTAATC		GACGCACCACCGACAAGC		GTGACCCCCGATACAAACAC
KRMP-TG	GACGATGAGTCCTGAGTAATG		CCGTAGCGTAGCTTTGCTAATCA	RM7121	GGAGATGGCACACGTCAAAC
KRMP-TT	GACGATGAGTCCTGAGTAATT	*rufipogon* 518	TTTGGGATGAAAAGAGTATGA		AGGATCCCGTTTTGTAGCAG
CACTA			CAAGCCAAAACTACTTGAATG	RM21194	TATGGCTACACGCCTACACG
CACTA-MAP	AGATGGTTTCTCCACCAGTG	RM8278	CTGCAACTGCGAACTACAC		GAAGCGTGGGATGTTTGTTT
			TGAAATCAATGACATGAACG	RID13	ACCTACGACACGATGTACAG
Chromosome 1 fine mapping primers		RM315	GAGGTACTTCCTCCGTTTCAC		ATGCCATGCGATCACAACTA
RM6547	TCCATCCTTCTCCTCTCGTG		AGTCAGCTCACTGTGCAGTG	RID12	TACAGGGGAGCAGAAACACC
	AGCCACCCCCATATATAGCC	RM5448	GGGGCCCTTAGAGTTTTTAC		AAAGGTACCAAAGATCGCAGAA
RM8085	TGCGTTTCGATTTCTTTTTA		CTGCTACAGGACAAAACCAG	RM652	GCCAAGGTCGTCGTTATGAT
	GGAAAGTTGTGTTCTTTGGC	RM8231	GCGTAAGATCTCCCTACCAC		CCAACCAATTCACCCACAC
RM8084	CATGACAACATTGTTCTGAA		CAACACATGATAGCACATGG	RM653	TACTCCCCCATTCCTCTCCT
	CCTGTGATCAAGTCCTGTAG	RM8232	AAACGGGTCAGCTACTAAGG		AGCCCTGCTCACTGTTCATT
RM3403	AACGACTGCTCCCTCTTCAG		ACCACGTGTGTACTCCTACC	RM1377	ATTAGATACATCAGCGGGGG
	AGCTTGCAAGGCATTAGCTC				GCTGCTGTACGATGTGATCC
				RM6767	ACAAGCAACTTGCACTGTGG
					CATCGCCTGGGAGATCATAC

Data analysis

Segregation of marker loci was analyzed for deviations from the expected 1:1 ratio based on χ^2 analysis at the 5% significance level. SSR markers were used as anchors and were selected from available mapping information in the rice database (http://www.gramene.org). Linkage analysis was conducted using the Mapmaker software, version 3.0 (Lincoln et al., 1992). A frame map of SSR markers was chosen for each mapping population to facilitate chromosome assignment of the markers and map comparisons. Linkage groups were created with limit of detection (LOD) scores of 5.0 and a 50-cM maximum distance as significant thresholds. All map distances were calculated according to the Kosambi mapping function (Kosambi, 1943). QTL analysis was performed to determine shattering of the segregating population using the MAPMAKER/QTL software (version 1.1b).

RESULTS

Shattering analysis of 743 $F_{3:4}$ plants

The wild species *O. rufipogon* showed almost complete seed shattering, while the cultigen *O. sativa* showed very high resistance to seed shattering. The 743 $F_{3:4}$ plants showed highly variable seed shattering, ranging from all-shattering (100%) to non-shattering (0%). The shattering mode of distribution revealed that most plants were shattering-resistant. Among the 743 plants, 35 were all-shattering, while 436 were 0-5% shattering plants. Plants classified into these categories accounted for 63.4% of the total number of plants. The remaining plants showed variable shattering, ranging from 5-95%, as shown in Table 2. While the female parent *O. sativa* cv. *Ilpoombyeo* showed a complete tolerance for shattering, the male parent *O. rufipogon* was all-shattering.

Table 2. Examination of shattering using F_3 recombinant inbred line 743 plants.

Shattering degree	No. of plants
0-5%	436
5-10%	68
10-15%	34
15-20%	16
20-25%	15
25-30%	9
30-35%	11
35-40%	13
40-45%	7
45-50%	8
50-55%	11
55-60%	10
60-65%	8
65-70%	10
70-75%	15
75-80%	7
80-85%	10
85-90%	13
90-95%	7
95-100%	35

Isolation of shattering-related *Rim2*/*Hipa* markers

The *Rim2/Hipa* CACTA-TD produced 2968 amplified fragments based on bulk segregant analysis. Five and 3 fragments were specific to the bulked DNA pools from non-shattering and all-shattering plants, respectively (Figure 1). After DNA sequences were determined for these polymorphic DNA fragments, their genomic locations were determined by BLAST analysis using NCBI and subsequently the Gramene website (http://www.gramene.org).

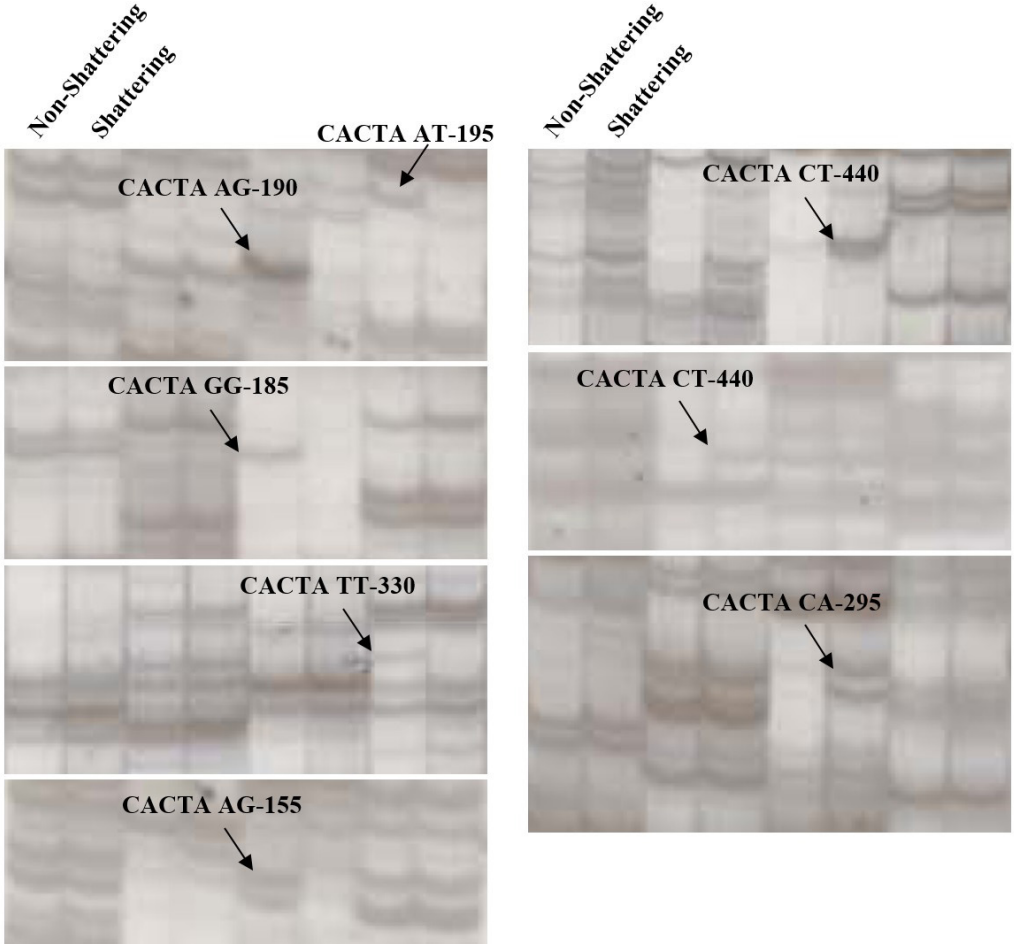

Figure 1. *Rim2/Hipa* CACTA transposon display. Bulk segregant analysis of *Rim2/Hipa* CACTA-TD produced 2968 amplified fragments. Five and 3 fragments were specific to the bulked DNA pools of non-shattering and all-shattering, respectively.

Among the 8 polymorphic fragments, CACTA-AG190 was located 250 kb (approximately 2.5 cM) from the *qSH-1* locus, a shattering-related locus on chromosome 1 (Konish et al., 2006). SCAR analysis of this fragment among the $F_{3:4}$ plants revealed that most easy-shattering plants contained the *O. rufipogon* allele, while non-shattering plants contained the

O. sativa allele (Figure 2). Among the 8 lines, 3 were non-shattering and 5 were all-shattering and contained alleles from both *O. sativa* and *O. rufipogon* as heterozygotes. One line showing all-shattering contained homozygous alleles from *O. sativa*.

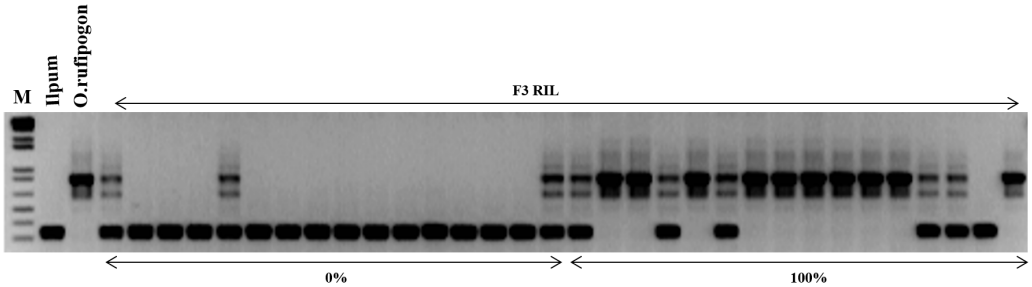

Figure 2. SCAR analysis of the CACTA-AG190 polymorphic fragment among the $F_{3:4}$ families. Among the 8 lines, 3 showed non-shattering and 5 showed all-shattering, which contained alleles from both *Oryza sativa* and *O. rufipogon* as heterozygotes. One line showing all-shattering contained homozygous alleles of the *O. sativa*. RIL = recombinant inbred line.

Fine mapping of the chromosomal region of *qSH-1*

Because CACTA-AG190 is located in the chromosomal region of *qSH-1*, we analyzed the *qSH-1* region in detail. Nucleotide sequences in the *qSH-1* region, from -2.5 to +30 kb of *qSH-1*, were compared between *O. sativa* cv. *Ilpoombyeo* and *O. rufipogon* and revealed several sequence variations (Figure 3). Numerous SNPs were detected, with an average of 1 SNP per 118 bp. A putative miniature inverted-repeat transposable element (MITE; MITE-adh, type D-like) sequence of 518 bp was present in *O. rufipogon*, but not in *O. sativa* (11.7 kb from the *qSH-1* locus). We also identified a $(GA)_7$ SSR in *O. sativa* +21.8 kb from *qSH-1*. Four SNP loci and 518-bp insertion polymorphisms were analyzed by single-nucleotide amplified polymorphism among the 743 $F_{3:4}$ inbred lines. In addition to the 32.5-kb regions that were sequenced, SSR analysis was performed in a wide region of *qSH-1* from -1750 to +1742 kb around the *qSH-1* locus. The single-nucleotide amplified polymorphism and SSR primers are shown in Table 1. Chromosomal distributions of these markers are shown in Table 3.

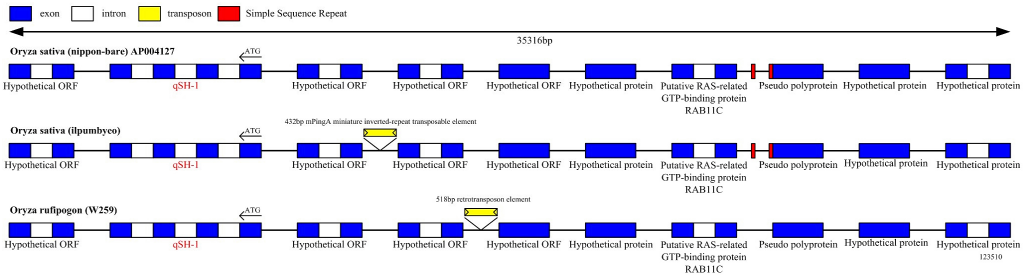

Figure 3. Nucleotide sequence comparison of *Oryza sativa* cv. *Ilpoombyeo* and *O. rufipogon* in the *qSH-1* region, from -2.5 to +30 kb.

Table 3. Genetic segregation of fine mapping primer loci nearby qSH-1.

Primer name	A	H	B
RM6547	58%	24%	18%
RM8085	58%	24%	18%
RM8084	59%	24%	17%
RM3403	59%	24%	17%
RM5389	60%	24%	16%
RM3640	61%	23%	16%
RM7250	61%	23%	16%
RM5407	61%	23%	16%
shaap-2431	61%	23%	16%
shaap-3895	61%	23%	16%
shaap-5074	61%	23%	16%
shaap-7715	61%	23%	16%
O. rufipogon 518 insertion	61%	23%	16%
RM8278	61%	23%	16%
RM315	61%	23%	16%
RM5448	62%	23%	15%
RM8231	64%	22%	14%
RM8232	64%	22%	14%
Average	61%	23%	16%

A = *Ilpoombyeo*; B = *O. rufipogon*; H = hetero.

Figure 4 shows the DNA fingerprinting results of these DNA polymorphisms in the *qSH-1* region associated with seed shattering among the 743 $F_{3:4}$ inbred lines.

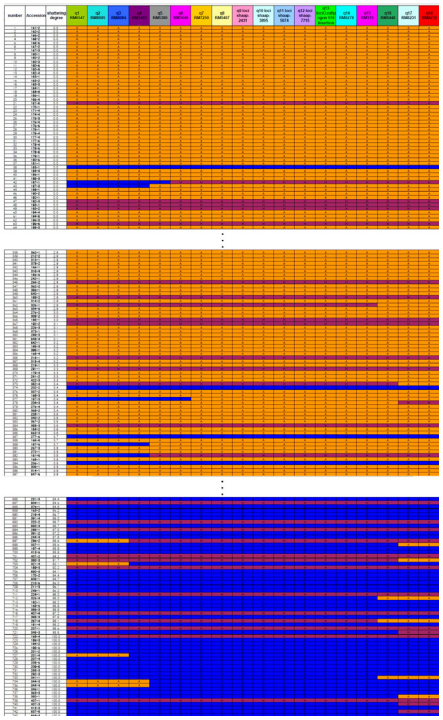

Figure 4. DNA polymorphisms based on DNA fingerprinting analysis in the *qSH-1* region association with seed shattering among the 743 $F_{3:4}$ families. Segregation ratio of the alleles was 2.4:1:0.6 for homozygotes of *Oryza. sativa* cv. *Ilpoombyeo*, homozygotes of *O. rufipogon*, and heterozygotes, respectively.

While the non-shattering lines contained alleles from *O. sativa* cv. *Ilpoombyeo*, all-shattering lines contained alleles from *O. rufipogon*. The segregation ratio of the alleles was 2.4:1:0.6 for homozygotes of *O. sativa* cv. *Ilpoombyeo*, homozygotes of *O. rufipogon*, and heterozygotes, respectively. Although generation advancement was observed in early stages ($F_{3:4}$), the results revealed that allele transmission from *O. sativa* was higher than that from *O. rufipogon*.

Table 4 shows the recombination frequency of 3474 kb around the *qSH-1* region. The region spanned 7.1 cM in our analysis and 7.5 cM in previous studies (McCouch et al., 2002). The average recombination frequency was 1% per 489 kb. No recombinant type was detected in the proximal region of the *qSH-1* (RM5407-RM8278), which spanned 350 kb among chromosome 1 of the 743 $F_{3:4}$ families.

Table 4. Frequency of crossing over nearby qSH-1.

Loci		Physical distance (bp)	Recombination ratio
RM6547	RM8085	168,653	0.010%
RM8085	RM8084	38,876	0.004%
RM8084	RM3403	94,497	0.013%
RM3403	RM5389	736,531	0.032%
RM5389	RM3640	104,947	0.004%
RM3640	RM7250	247,638	0.004%
RM7250	RM5407	296,453	0.001%
RM5407	shaap-2431	62,405	0%
shaap-2431	shaap-3895	1423	0%
shaap-3895	shaap-5074	1158	0%
shaap-5074	shaap-7715	2624	0%
shaap-7715	*O. rufipogon* 518 insertion	9718	0%
O. rufipogon 518 insertion	RM8278	162,479	0%
RM8278	RM315	112,053	0%
RM315	RM5448	662,996	0.008%
RM5448	RM8231	799,462	0.019%
RM8231	RM8232	5308	0.003%

QTL analysis of seed shattering was performed using segregation markers near the *qSH-1* locus. The overall genetic length of our markers covered 7.1 cM, and the highest QTL peak was observed between the *qSH-1-12* locus *shaap-7715*. The highest LOD value in our marker was observed at the polymorphic locus of the 518-bp insertion site with LOD 142 (Figure 5a). However, the biological or genetic significance of this MITE-related 514-bp insertion remains unknown.

QTL analysis of seed shattering in other loci

QTL analysis was also performed for other known shattering loci of *qSH4* (Li et al., 2006) and *sh-h* (Ji et al., 2006) to compare the effect of the QTL on *qSH-1* shattering in our population. Eight SSR markers around the *qSH-4* locus on chromosome 4 were analyzed (Table 1 and Figure 5b). The genetic length of the 8 SSR markers was 18.2 cM, and QTL genetic linkage with shattering in this region ranged from LOD 4-7. The physical location of qSH-4 was placed between RM3648 and RM1113, for which the LOD score was 4. The seed-shattering locus *sh-h* was present on chromosome 7 and linked very closely with the *Rc* locus, which determined the pericarp color in rice (Ji et al., 2006; Sweeney et al., 2006) (Figure 5c). We previously designed 5 sets of allele-specific primers using the in/del information (Table 1) (Sweeney et al., 2006). The 5 in/del sites spanned 0.7 cM and showed a QTL genetic linkage with shattering of 1.0 (Figure 5d).

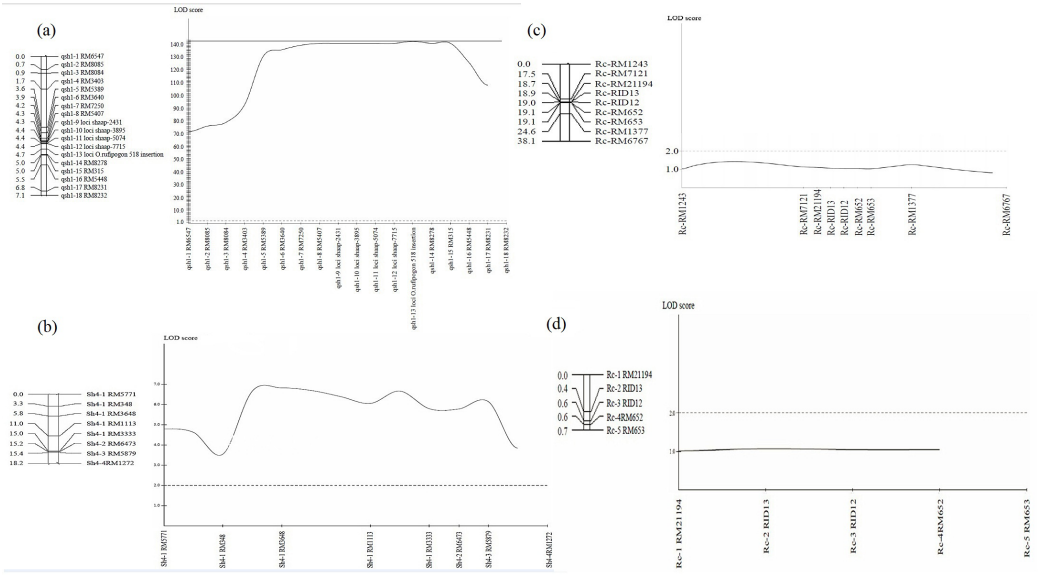

Figure 5. QTL analysis for shattering loci in 743 $F_{3:4}$ populations. (**a**) QTL analysis of the *qSH-1* locus. The overall genetic length of our markers covered 7.1 cM. The highest LOD value in our marker was observed at the polymorphic locus of the 518-bp insertion site with LOD 142. (**b**) QTL analysis of the *qSH-4* locus. Genetic length of the 8 SSR markers were 18.2 cM and QTL genetic linkage with shattering in this region ranged from LOD 4-7. (**c**) QTL analysis of *sh-h* locus. *sh-h* locus very closely linked with the *Rc* locus, which was associated with the pericarp color in rice. (**d**) QTL analysis of 5 in/del sites showed genetic linkage with shattering, and spanned 0.7 cM.

DISCUSSION

While deep seed dormancy and seed shattering are key traits for the survival of plants in the wild, these traits are not favorable for modern agriculture and are strongly selected against during crop domestication. Numerous studies have identified genetic loci or QTLs governing seed shattering in crop species, including rice (Ji et al., 2006). We analyzed the seed-shattering trait using a hybrid RIL from *O. sativa*/*O. rufipogon*.

Seed shattering in rice has been measured using several methods, including clasping the rice panicles with a clenched fist and hitting the panicles on a drum (Kwon and Shin, 1980). However, both methods are prone to error resulting from variations in clasping power and hitting force. Ji et al. (2006) developed a method for breaking tensile strength and recording shattering using a digital gauge. In this report, we efficiently measured seed shattering by minimizing variations in the physical force to the panicles. The skewed distribution of non-seed-shattering lines in the $F_{3:4}$ families may have resulted from preferential selection of the non-shattering lines during line development, since the high-shattering trait was dominant to the high seed shattering in F_1 of a cross between *O. sativa* and *O. rufipogon* (Xiong et al., 1999). However, the high-seed-shattering *O. sativa japonica* mutant was recessive to the wild-type in crosses with cultivars of *O. sativa japonica* (Ji et al., 2006). This inconsistency may have resulted from the involvement of multiple genes and QTLs in seed shattering. Seed non-shattering is a key trait for transitioning from wild to modern cultivars (Gepts, 2004). Without human interference, seeds that shatter easily may improve the survival of the species in nature.

Genetic markers using transposons have been reported in several plants, including

rice (Kwon et al., 2006), maize (Lee et al., 2006), and wheat (Queen et al., 2004), which are typically used for genetic mapping studies and subsequent chromosomal distributions. Transposons account for a large fraction of plant genomes, including 20% of the rice genome, and this value may be even higher in other cereal grasses such as corn and wheat (Turcotte et al., 2001). However, few studies have been conducted to tag agronomic traits using transposon markers. CACTA-AG190 was found to be located 250 kb from *qSH-1*. CACTA is a class 2 transposon and is abundant in cereal grass species (Wicker et al., 2003). At least 600-700 copies of *Rim2/Hipa* CACTA, an element of the CACTA superfamily transposons, are thought to be present in the rice genome (Wang et al., 2003).

In our analysis, CACTA-AG190 was highly associated with shattering traits through CACTA-TD according to bulked segregant analysis, which was observed in the *qSH-1* region of chromosome 1. However, the CACTA-AG190 marker may not be directly related to shattering. We also observed a 518-bp MITE element near *qSH-1* in *O. rufipogon*, as well as sequences with high-sequence homology on chromosomes 6, 9, and 11 according to BLAST analysis (data not shown).

Ishikawa et al. (2010) performed QTL analysis of rice shattering using the BC_2F_1 backcross population between *O. sativa* cv. *Nipponbare* (recurrent parent) and *O. rufipogon* acc. W630 (donor parent). As a result, 2 strong QTLs were detected in the 2 major seed-shattering loci, *qSH-1* and *sh4*. In the genetic background of cultivated rice, wild-type *qSH-1* alleles showed a stronger effect on seed shattering than did *sh4*. In addition, the wild-type alleles at both *qSH-1* and *sh4* loci showed semi-dominant effects. In this study, the backcrossed plants individually containing *Nipponbare* homozygous alleles at either shattering locus (*qSH-1* or *sh4*) shed all of their seeds. This indicated that non-shattering behavior was not due to a single mutation in the genetic background of wild rice. Zhou et al. (2012) identified a seed-shattering abortion1 (*shat1*) mutant in a wild rice introgression line. Genetic analyses revealed that *SHAT1* expression was positively regulated by the trihelix transcription factor *SH4*. They also identified a frame-shift mutant of *SH4* that showed non-shattering. *qSH-1* functioned downstream of *SHAT1* and *SH4* by maintaining *SHAT1* and *SH4* expression. This differed from the results of previous reports showing that *qSH-1* had a greater effect on seed shattering than *sh4-1* in the genetic background of *japonica* cv. *Nipponbare* (Onishi et al., 2007; Ishikawa et al., 2010).

Genetic analysis performed on an F_2 population derived from *O. sativa* ssp *indica* and the wild annual species *O. nivara* identified QTL, including *sh3*, *sh4*, and *sh8*, which are responsible for reducing grain shattering in cultivated rice (Li et al., 2006). This study found that the *sh4* allele of the wild species caused shattering and was dominant among the 3 QTLs. Additionally, QTL analysis identified *sh4* between SSR markers RC4-123 and RM280, which had a physical distance of ~1360 kb in the *O. sativa* genome. Thurber et al. (2010) assessed allelic identity and diversity at the major shattering locus, *sh4*, in weedy rice. They demonstrated that all cultivated and weedy rice, regardless of the population, shared similar haplotypes at *sh4* and all contained a single mutation associated with decreased seed shattering. The physical location of *qSH-4* was between RM3648 and RM1113 and the LOD score was 4 in our study.

Ji et al. (2006) identified and mapped the shattering gene *sh-h* on chromosome 7, but it appeared to be involved in the same process as previously mapped shattering genes in rice, including *sh2* on chromosome 1 (Oba et al., 1990, 1995) and *sh4* on chromosome 3 (Fukuta et al., 1994; Fukuta and Yagi, 1998), which play a role in abscission layer formation. However, the gene underlying the QTL remains unknown, but may correspond to a different allele at the *sh-h* locus.

Among the 3 QTL analyzed in our study, *qSH-1* is thought to be the major QTL related to shattering, which is consistent with the results of Konishi et al. (2006). They found that the *qSH-1* gene encodes a BEL1-type homeobox gene, and in the 5'-regulatory region of *qSH-1*, an SNP resulted in the loss of seed shattering due to the absence of abscission layer formation. This SNP has been highly associated with shattering among *japonica* subspecies. Our analysis revealed that this SNP site was polymorphic as thymine (T) in *O. sativa* cv. *Ilpoombyeo* and guanine (G) in *O. rufipogon*. However, high GC content in the genomic region of the SNP site prevented allele-specific PCR amplification in our analysis. This SNP site was found between the markers *shaap-3895* and *shaap-5074.* In contrast, the highest LOD was observed between *shaap-7715* and a 518-bp *O. rufipogon* insertion (LOD score 142) in this analysis. The high LOD values of shattering in this region agreed with the results of Konishi et al. (2006).

Our results differed somewhat from those of previous reports. For example, the *qSH-1* region spanned 7.1 cM in our analysis, but 7.5 cM in previous studies. This may be because our studied population differed from other studied populations. In our study, we applied $F_{3:4}$ progeny of *O. sativa*/*O. rufipogon*, which may have affected the results. Additionally, deviations between different analysis methods may have affected our results. Finally, QTL may need to be modified depending on the population. The putative feasibility and authenticity of QTL analysis should be validated in a future study.

Similarly, interactions among the identified rice shattering-related loci and genes should be further analyzed to understand the genetic network underlying abscission layer development and seed shattering in rice. In summary, these results indicate that the introgression of seed-shattering traits in *O. sativa*/*O. rufipogon* is facilitated using conventional and marker-assisted selection breeding efforts. To perform marker-assisted selection efficiently within breeding programs, it is important to identify genes or traits linked to the target gene that can serve as markers. Thus, detecting and confirming the *qSH-1* region associated with the seed-shattering traits presented here may provide opportunities for rice breeders to improve seed quality using marker-assisted selection.

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