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Detection of a novel locus involved in non-seed-shattering behaviour of Japonica rice cultivar, *Oryza sativa* ‘Nipponbare’

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Key message

A novel locus, *qCSS3*, involved in the non-seed-shattering behaviour of Japonica rice cultivar, ‘Nipponbare’, was detected by QTL-seq analysis using the segregating population with the fixed known seed-shattering loci.

Abstract

Asian cultivated rice, *Oryza sativa*, was domesticated from its wild ancestor, *O. rufipogon*. Loss of seed shattering is one of the most recognisable traits selected during rice domestication. Three quantitative trait loci (QTLs), *qSH1*, *qSH3*, and *sh4*, were previously reported to be involved in the loss of seed shattering of Japonica cultivated rice, *O. sativa* ‘Nipponbare’. However, the introgression line (IL) carrying ‘Nipponbare’ alleles at these three loci in the genetic background of wild rice, *O. rufipogon* W630, showed a lower value for detaching a grain from the pedicel than ‘Nipponbare’. Here, we investigated abscission layer formation

in the IL and found a partially formed abscission layer in the central region between the epidermis and vascular bundles. Based on QTL-seq analysis using the F₂ population obtained from a cross between ‘Nipponbare’ and the IL, we detected two novel loci *qCSS3* and *qCSS9* (*QTL for the Control of Seed Shattering in rice on chromosomes 3 and 9*), which were found to be involved in the difference in seed-shattering degree between ‘Nipponbare’ and W630. Then, we further focused on *qCSS3* in order to understand its potential role on the loss of seed shattering. The candidate region of *qCSS3* was found to be located within a 526 kb region using substitution mapping analysis. Interestingly, the *qCSS3* candidate region partially overlaps with the selective sweep detected for Japonica but not for Indica rice cultivars, suggesting that this region harbours the mutation at a novel seed-shattering locus specifically selected for non-seed-shattering behaviour in Japonica cultivars.

Introduction

Historically, the process of crop domestication involved the selection of several naturally occurring variations in wild plants that provided useful agronomic traits. These traits were related to seed size, plant architecture, seed shattering, seed dormancy, and photoperiod sensitivity (Doebley et al. 2006). Seed shattering is one of the most important characteristics for the propagation of wild plants (Dong and Wang 2015), and is caused by the degradation of the abscission layer formed between the grain and pedicel. Because seed shattering affects yield, hunter-gatherers and early farmers must have selected plants with non-seed-shattering behaviour to increase their yield (Fuller and Allaby 2009). Thus, loss of seed shattering is regarded as one of the most important domestication traits (Harlan 1975; Fuller 2007).

Oryza sativa L., Asian cultivated rice, was domesticated from its wild ancestor, *O. rufipogon* Griff. (Oka 1988; Fuller 2007). During the process of domestication, the seed-shattering ability of rice plants weakened or was lost as a result of the inhibition of abscission layer formation. Asian cultivated rice is generally classified into two groups; Indica and Japonica. The former normally exhibits a weak shattering habit with partial abscission layer formation, whereas the latter has a non-shattering habit with complete inhibition of abscission layer formation. Previous studies have shown that three quantitative trait loci (QTLs), *sh4*, *qSH1*, and *qSH3*, are involved in the loss of seed shattering (Li et al. 2006; Konishi et al. 2006; Htun et al. 2014). The major QTL is *sh4*, which was identified from a cross between *O. nivara* and *O. sativa* Indica explaining 69% of the phenotypic variation (Li et al. 2006). The cultivated allele of *sh4* is recessive and inhibits the formation of the abscission layer, resulting in the reduction of the degree of seed shattering (Li et al. 2006; Lin et al. 2007). *qSH1* was identified from a cross between *O. sativa* Indica ‘Kasalath’ and *O. sativa* Japonica ‘Nipponbare’, which explained 68.6% of the phenotypic variation (Konishi et al. 2006). A single nucleotide polymorphism (SNP) in the regulatory region of a downstream gene was shown to result in the absence of an abscission layer in ‘Nipponbare’ (Konishi et al. 2006). These two QTLs were found to be major loci involved in non-seed-shattering behaviour of most Japonica rice cultivars. However, we previously evaluated seed-shattering degree of introgression lines of wild rice carrying ‘Nipponbare’ alleles at *sh4* and *qSH1* (Ishikawa et al. 2010). Results show that a single introgression of ‘Nipponbare’ alleles at *sh4* or *qSH1*

display complete shattering behaviour as wild rice, and even a double introgression at the two loci display weak seed shattering inhibition. These findings suggest that additional loci may still be involved in a loss of seed shattering in Japonica rice cultivars. The third QTL is *qSH3*, which was originally detected from a cross between *O. rufipogon* and *O. sativa* Japonica (Onishi et al. 2007). We also detected *qSH3* by QTL analysis in a previous study, using an F₂ population generated by a cross between ‘Nipponbare’ and an introgression line (IL) carrying the ‘Nipponbare’ alleles at both *qSH1* and *sh4* in the genetic background of *O. rufipogon* W630 (Htun et al. 2014). Gene interaction of *qSH3* and *sh4* was also investigated in the genetic background of wild rice to understand the seed-shattering behaviour in early rice domestication (Inoue et al. 2015; Ishikawa et al. 2017), because the selection of *qSH1* is specific to Japonica rice cultivars. We also evaluated the seed-shattering degree of an F₂ segregating population between Indica rice cultivar *O. sativa* ‘IR36’ and an introgression line carrying ‘Nipponbare’ allele at *qSH3* and *sh4* in the genetic background of wild rice *O. rufipogon* W630 (Tsujimura et al. 2017). We observed the segregation of seed-shattering behaviour in the F₂ population, confirming that unknown mutation(s) other than *qSH3* and *sh4* may underlie the non-shattering behaviour of ‘IR36’. These studies suggest that several unidentified loci may still be involved in the non-shattering of seeds during rice domestication or breeding.

Here, we first investigated the abscission layer formation in an IL having the cultivated alleles of ‘Nipponbare’ at *qSH1*, *qSH3*, and *sh4* in the genetic background of wild rice, *O. rufipogon* W630. The IL was crossed with ‘Nipponbare’ and the seed-shattering degrees of their F₂ plants were measured to detect QTL(s) other than the three known seed-shattering loci. We performed QTL-seq analysis, and two novel loci on chromosomes 3 and 9 were detected. We further analysed the effect of the putative locus on chromosome 3, and the effect of the wild allele on seed-shattering degree was confirmed with two backcross recombinant inbred lines (BRILs) in the genetic background of ‘Nipponbare’ and by a progeny test of the F₂ plants. The candidate region of the locus on chromosome 3 was restricted to a 526 kb region by substitution mapping analysis.

Material and methods

Plant materials

A Japonica rice cultivar, *O. sativa* ‘Nipponbare’, and a wild accession of *O. rufipogon* W630 originated from Myanmar were used in this study. By backcrossing with W630, an IL carrying the ‘Nipponbare’ alleles at the *sh4*, *qSH1*, and *qSH3* loci in the genetic background of wild rice was produced. The IL was crossed with ‘Nipponbare’ and the resulting F₁ plant was self-pollinated to obtain the segregating F₂ population. A total of 174 individuals were grown in pots at Kobe University, Japan, and their seed-shattering behaviour was evaluated. To minimise the effect of the difference in heading time, short-day treatment was used for all the plants. The validation of the locus detected in this study was conducted using backcross recombinant inbred lines as previously reported by Thanh et al. (2011). A progeny test was also conducted using three lines having a heterozygous chromosomal constitution at the locus detected in this study. Further substitution mapping analysis was conducted by the progeny tests using one of the three lines.

Evaluation of seed-shattering degree

The seed-shattering degree was evaluated by measuring the breaking tensile strength (BTS, gf: gramme-force), which is the value required to detach a grain from the pedicel, measured with a digital force gauge (FGP 0.5, Nidec-Shimpo Co., Japan). The BTS values of 75 seeds (25 randomly selected seeds from three panicles) were measured, approximately a month after heading, and their average BTS values were calculated.

DNA extraction, bulking, and library construction for next-generation sequencing analysis

For QTL-seq analysis, bulked DNA samples were prepared as described in previous studies (Abe et al. 2012; Takagi et al. 2013). DNA was extracted from 100 mg of fresh rice leaves using DNeasy Plant Mini Kit (QIAGEN Sciences, Germany) and was quantified using Qubit® 3.0 Fluorometer and Qubit® dsDNA BR Assay Kit (Life Technologies, Japan). The extracted DNA samples were mixed in an equal ratio and were regarded as the bulked DNA. To survey the genotypes at the detected loci, simple sequence repeat (SSR) or InDel markers were used (Supplemental Table 1).

Detection of novel seed-shattering loci by QTL-seq analysis

All analyses were carried out using the previously developed QTL-seq Pipeline (Department of Genomics and Breeding, Iwate Biotechnology Research Center; <http://genome-e.ibrc.or.jp>). The short reads obtained from the IL were aligned to the ‘Nipponbare’ reference genome sequence obtained from the Rice Annotation Database Project (<http://rapdb.dna.affrc.go.jp>). Thereafter, the genome sequence of the IL was developed as a ‘reference sequence’. The short reads obtained from high (H-) and low (L-) bulks were aligned to the reference sequence using the Burrows-Wheeler Aligner software (Li and Durbin 2009). The aligned sequence files were converted to SAM/BAM files using SAM tools (Li et al. 2009) and were applied to Coval (Kosugi et al. 2013) to increase the SNP-calling accuracy. SNP-index was calculated for all the SNP positions. Then $\Delta(\text{SNP-index})$ was calculated by subtracting the SNP-index values of L-bulk from H-bulk. A sliding window analysis was applied by averaging the $\Delta(\text{SNP-index})$ values within a 4 Mb window size and a 50 kb increment.

Morphological and histological analysis of abscission layer formation

The abscission layer (axial images of detached spikelet) was examined using a LEICA S6D microscope and photographs were taken with the MC170HD and Leica Application Suite (Leica Biosystems, Germany). The samples for histological analysis were collected from the pedicel tissue of grains before heading, as previously reported (Htun et al. 2014; Inoue et al. 2015). The samples were fixed in FAA solution (formaldehyde: acetic acid: 70% ethanol = 1:1:18 (volume ratio)) with vacuum infiltration and were preserved at 4°C. They were dehydrated in an ethanol series (70%, 80%, and 90% ethanol) for 2 days at each stage and then embedded in Technovit 7100 resin (Heraeus Kulzer, Germany), according to the manufacturer’s instructions. The samples were cut into 3- μm sections with a rotary microtome, RM1215RT (Leica Biosystems, Germany), and stained with toluidine blue O solution. These sections were observed

under a microscope and photographed with a digital camera using the imaging software, ToupView ($\times 86$) (Amscope.com, US).

Results

Seed-shattering behaviour of the IL (*qSH1*, *qSH3*, and *sh4*)

We first surveyed backcross plants carrying *O. sativa* ‘Nipponbare’ alleles at the *qSH1*, *qSH3*, and *sh4* loci in the genetic background of wild rice, *O. rufipogon* W630. From these, the IL with the least other ‘Nipponbare’ chromosomal segments was selected. The graphical genotype of the IL with the ‘Nipponbare’ chromosomal segments covering the three seed-shattering loci and on chrs. 5, 7, 10, and 11 is shown in Fig. 1a. The appearance of the seed of the IL was similar to that of wild rice, *O. rufipogon* W630 (Fig. 1b). The IL showed an inhibition of seed-shattering behaviour, but it had a lower BTS value than ‘Nipponbare’ (Fig. 1c). We investigated the abscission layer formation in the IL after the detachment of seeds. In the basal part of the grain, no abscission layer was produced in ‘Nipponbare’ and the pedicel tissues were broken when a seed was strongly detached from the pedicel (Fig. 1d). In contrast, W630 formed a complete abscission layer from the epidermis to the region surrounding the vascular bundle (Fig. 1d). A partial abscission layer formation was observed in the inner region of the IL corresponding to the area where the complete abscission layer is formed in W630. The abscission layer formation in the IL was different from those of the parents. Furthermore, we also compared abscission layer formation using longitudinal sections. The abscission layer was inhibited outside the region of the pedicel and around the vascular bundle and partially formed only in the central region of the IL whereas a complete abscission layer is formed in W630 (Fig. 1e). Because the IL had significantly lower BTS values than ‘Nipponbare’, the partially formed abscission layer probably contributes to lowering the BTS value. These results indicated that mutations at *qSH1*, *qSH3*, and *sh4* are insufficient to explain the non-seed-shattering phenotype of ‘Nipponbare’, and other unknown loci are probably involved.

Seed-shattering degree in an F₂ population obtained by a cross between the IL and ‘Nipponbare’

We obtained a total of 174 F₂ plants from a cross between the IL and ‘Nipponbare’ and their BTS values were measured. In this segregating population, the three seed-shattering loci were all fixed with the ‘Nipponbare’ alleles. Differences in the BTS were approximately 70 to 210 gf (Fig. 2, Supplemental Table 2). The transgressive segregation of the BTS values in the population indicates that several genes might be involved in the difference in seed-shattering degree between the parent lines.

Detection of novel loci controlling the seed-shattering behaviour in rice

To detect the loci involved in the difference in seed-shattering degree between the IL and ‘Nipponbare’, we employed QTL-seq analysis (Takagi et al. 2013). The analysis requires bulked DNA of progeny showing extreme phenotypes (i.e., those exhibiting high and low BTS values in the population). We selected 12 and 14 F₂ plants with low (70–100 gf) and high (150–210 gf) BTS values, respectively (Fig. 2 and Supplemental

Table 2). The DNA of each bulk as well as that of the IL was subjected to whole-genome resequencing analysis. A total of 104.3, 107.1, and 48.2 million sequence reads (each 100 bp) were obtained from the DNA of H-bulk, L-bulk, and IL, respectively. By examining the $\Delta(\text{SNP-index})$ plot, we identified the two genomic regions exhibiting the $\Delta(\text{SNP-index})$ values exceeding the 95% confidence interval by a sliding window analysis (statistical significance under the null hypothesis: $P < 0.05$): the regions on chr. 3 from 8.30 to 13.65 Mb with maximum $\Delta(\text{SNP-index}) = 0.49$ (statistical significance under the null hypothesis: $P < 0.05$) and on chr. 9 from 21.1 Mb to the distal end with maximum $\Delta(\text{SNP-index}) = -0.46$ (Fig. 3, Supplemental Fig. 1). We named them as *QTL for the Control of Seed Shattering in rice on chrs. 3 and 9* (*qCSS3* and *qCSS9*). No other region over the confidence interval was detected in this analysis (Supplemental Fig. 1). According to the $\Delta(\text{SNP-index})$, ‘Nipponbare’ allele at *qCSS3* and W630 allele at *qCSS9* were found to inhibit seed shattering. To confirm the results of the QTL-seq analysis, each F_2 plant was genotyped using the five and four DNA markers covering the entire significant regions on chrs. 3 and 9, respectively (Supplemental Table 1). For *qCSS3* region, 32 (18%) and 20 (11%) plants were found to carry the ‘Nipponbare’ and W630 homozygous chromosomal segments, respectively. Moreover, 33 (19%) plants had heterozygous chromosomal segments and the rest (52%) showed recombination between any of the five SSR markers (Supplemental Table 3). We compared the BTS values of two homozygous groups based on the chromosomal constitutions (Supplemental Fig. 2), and found that the ‘Nipponbare’ and W630 chromosomal segments at the region were responsible for increasing and decreasing the BTS values, respectively. Similarly, the result of QTL-seq analysis at *qCSS9* region was also confirmed (Supplemental Table 2, Supplemental Fig. 2). These results confirm that both *qCSS3* and *qCSS9* detected in QTL-seq analysis are involved in the control of seed shattering in rice.

Validation of the effect of *qCSS3* on the seed-shattering degree

As we found that the ‘Nipponbare’ allele at *qCSS3* contributed to inhibition of seed shattering and possibly involved in non-shattering behaviour of *O. sativa* ‘Nipponbare’, we further studied *qCSS3*. To validate the effect of *qCSS3* on the seed-shattering degree, we first screened the individual lines of the previously produced backcross recombinant inbred lines (BRILs) (Thanh et al. 2011). These BRILs were generated by crossing W630, as the donor parent, and ‘Nipponbare’, as the recurrent parent. Using two SSR markers, flanking the *qCSS3* region (RM1002 and RM6080; Supplemental Table 1), we screened 159 BRILs. We found that lines 42 and 86 had homozygous chromosomal segments of W630, covering the candidate region of *qCSS3* (Supplemental Fig. 3a). Both lines showed significantly lower BTS values than ‘Nipponbare’, although the differences were very small (Supplemental Fig. 3b). Next, we carried out the progeny test of F_2 lines obtained by crossing the IL and ‘Nipponbare’. One of the F_2 plants (No. 57), with heterozygous chromosomal constitution between RM1002 and RM6080 covering the whole candidate region of *qCSS3* was selected (Fig. 4a). The self-pollinating seeds (F_3 generation) were germinated and their chromosomal constitutions were surveyed using the five SSR markers (Supplemental Table 1). The F_3 progeny of this plant showed similar days to heading, minimising the effect of differences in heading date on the seed-shattering degree (Supplemental Table 3). A significant difference in the BTS values was observed between the F_3 lines

with the ‘Nipponbare’ and W630 homozygous chromosomal segments (Fig. 4b, $P < 0.01$). In addition, partial abscission layer formation was found to be associated with the seed-shattering degree, depending on the genotypes at the region (Fig. 4b and c). To restrict the border of *qCSS3*, we selected two other F_2 plants having recombination within the *qCSS3* candidate region. nos. 54 and 129 F_2 plants were found to have recombination between RM5639 and RM232 and between RM5551 and RM3297, respectively (Fig. 4a). They produced F_3 progeny with similar heading dates (Supplemental Table 3). Their progeny tests showed a significant difference in the BTS values between F_3 lines having recombinant and W630 homozygous chromosomal segments (Fig. 4a, $P < 0.01$). These results indicated that the candidate region of *qCSS3* was within a 5.1 Mb region between RM5639 and RM3297.

We further carried out genetic dissection at *qCSS3* candidate region. A total of 78 recombinants out of 200 segregating plants were selected between RM5639 and RM3297 using F_3 generation of F_2 -129. We surveyed these recombinant plants with additional DNA markers and 25 of recombinant plants were self-pollinated to conduct substitution mapping analysis. The mean BTS values of recombinant lines were compared at $P < 0.05$ level. Comparison of the BTS values in the progeny test showed that the *qCSS3* candidate region was narrowed down to 1.3 Mb region between RM5639 and RM1284 by six critical recombinants (Nos. 129-43, -55, -88, -185, -195, and -282, Fig. 4d). We further investigated their genotypes at *qCSS3* using ten more additional DNA markers (Supplemental Table 1). No significant differences were observed for both progeny tests of 129-55 (recombination between RM14731 and OTS1) and 129-185 (recombination between RM232 and RM14764), respectively (Fig. 4d). These results indicated that the putative candidate position of *qCSS3* is mapped to a 526 kb interval defined by two SSR markers RM14731 and RM14764. In this 526 kb region, we found 96 genes based on the published sequence annotation for *O. sativa* Japonica ‘Nipponbare’ (Supplemental Table 4), although there might be additional genes in the corresponding region of *O. rufipogon* W630.

Discussion

Abscission layer formation in ILs with non-functional alleles at seed-shattering loci in the genetic background of wild rice

In the previous study, we evaluated the ‘Nipponbare’ allele at any of the *qSH1*, *qSH3*, or *sh4* loci in the W630 genetic background (Htun et al. 2014). All of them showed complete abscission layer formation as in W630, indicating that single mutation at any of the three seed-shattering loci was not sufficient to disrupt the abscission layer formation. In this study, we characterized the IL having the ‘Nipponbare’ alleles at the *qSH1*, *qSH3*, and *sh4* loci in the genetic background of wild rice, *O. rufipogon* W630. Although these three seed-shattering loci were fixed with the ‘Nipponbare’ alleles, the average BTS value was significantly lower than that of ‘Nipponbare’ (Fig. 1a, c). Microscopic and longitudinal section analyses showed the presence of a ring-shaped abscission layer in the IL (Fig. 1d, e). Although the mechanism underlying the partial formation of a ring-shaped abscission layer is not understood, the expression gradient of the genes involved in abscission layer formation might be responsible.

As the SNP at *qSH1* was specific to most Japonica rice cultivars and probably occurred late in the domestication process of rice (Konishi et al. 2006), we previously focused on the gene interaction at the other two loci (*sh4* and *qSH3*) in the genetic background of W630 to understand the process of loss of seed shattering in rice domestication. A couple of abscission cells around the vascular bundles were found to be disrupted in the IL having the ‘Nipponbare’ alleles at *qSH3* and *sh4*, suggesting that the interaction of the mutations at the two loci might have played an important role in the selection of the initial non-seed-shattering rice.

The ILs with the ‘Nipponbare’ alleles at the three (*qSH1*, *sh4*, and *qSH3*) and two (*sh4* and *qSH3*; Inoue et al. 2015) loci showed different abscission layer formation at the epidermis region (Fig. 1d, e). The disruption of abscission layer at the epidermis region increases the BTS values tremendously (Fig. 1c). The progeny test of No. 57 F₂ plant gave further interesting results. The F₃ lines with ‘Nipponbare’ homozygous allele at the *qCSS3* showed no abscission layer formation, similar to ‘Nipponbare’, whereas those with W630 homozygous allele had a partial abscission layer formation as the parental IL with the ‘Nipponbare’ alleles at the three seed-shattering loci (Fig. 4 b and c). A similar allele effect was also observed in the two BRILs with the W630 allele at *qCSS3* in the genetic background of ‘Nipponbare’. Both lines showed significantly lower BTS values than ‘Nipponbare’ (Supplemental Fig. 3). These results strongly indicate that the wild allele at *qCSS3* may act to promote the abscission layer formation. It would be of interest to investigate the tissue specific expression of the seed-shattering gene(s) in the developing stage of spikelets with or without the functional allele at *qCSS3*.

Allele effects of *qCSS3* and *qCSS9* on the seed-shattering degree

The QTL-seq analysis successfully detected *qCSS3* in a 5.1-Mb region on chr. 3, which is different from *qSH3*, the seed-shattering locus that was previously detected (Htun et al. 2014). We also detected *qCSS9* in a 1.9 -Mb region on chr. 9. As ‘Nipponbare’ allele at *qCSS9* was found to promote seed shattering, we speculated that the allele effect of *qCSS9* is not involved in a loss of seed shattering in ‘Nipponbare’. The allele effect at *qCSS3* on the seed-shattering degree was examined using two BRILs in the ‘Nipponbare’ genetic background (Supplemental Fig. 3) and in the progeny test of F₃ lines between the IL and ‘Nipponbare’ (Fig. 4). Although the BTS value of ‘Nipponbare’ was significantly higher than those of the two BRILs, their difference was about 15 gf. In the progeny test, large differences in the BTS values (30 gf) were observed between the F₃ lines from three independent F₂ plants. This may be owing to the different genetic background of the lines. Probably, ‘Nipponbare’ possesses cultivated alleles at some other minor QTLs for seed shattering..

Mapping of *qCSS3* and the selection signature at the candidate region

On the basis of progeny tests, the candidate region of *qCSS3* was estimated to be in a 526kb region between the two SSR markers, RM14731 and RM14764. In the region, we found a total of 96 annotated genes based on Rice Annotation Database for *O. sativa* ‘Nipponbare’ (Supplemental Table 4). Once the causal gene is identified by further genetic mapping experiments, expression analysis of the candidate genes should be

conducted in wild genetic background that has a functional pathway for promoting seed shattering.

In many domestication-related genes, reduction in nucleotide diversity is often observed (Doebley et al. 2006). Interestingly, a 526 kb candidate region partially overlaps with the selective sweep reported previously (Xu et al. 2012; Huang et al. 2013). Approximately, a 1.0-Mb region (9.0-10.0 Mb) on chr. 3 shows a reduction of diversity in Japonica rice cultivars (Xu et al. 2012), and the region carries the putative candidate region of *qCSS3*. This signature is specific to Japonica but not to Indica rice cultivars. Furthermore, a large scale genomic study using 446 wild and 1,083 cultivated rice shows that the putative region from 9.7 to 10.1 Mb on chr. 3 exhibits quite low diversity in Japonica rice population (Huang et al. 2012). An approximately 130 kb-region on RM14764 side of *qCSS3* were found to overlap partially with the sweep region. Interestingly, the selective sweep at the region was not detected in Indica nor full populations. These findings suggest that Japonica-specific domestication-related region may be located within a putative candidate region of the *qCSS3*. If a loss-of-function mutation at *qCSS3* is common only to Japonica cultivars, this mutation may be selected after the differentiation of Japonica rice cultivars diverged from the initial cultivated rice. In contrast, the *qSH3* region was found to be overlapping with the selective sweep region, both for the Japonica and Indica rice cultivars (Xu et al. 2012). These results suggest different evolutionary trajectories for the seed-shattering loci, which might be useful in understanding the process of rice domestication and distribution.

Loci involved in non-seed-shattering behaviour of cultivated rice

In previous studies, *sh4* was identified as a major locus selected for during rice domestication (Li et al. 2006; Lin et al. 2007). The mutation at *sh4* is conserved in all cultivated rice examined, indicating that *sh4* is a key locus playing an important role in the loss of seed shattering (Zhang et al. 2009). On the other hand, mutation at *qSH1* was found only in Japonica rice cultivars, suggesting that the mutation might have been selected after Japonica rice differentiated (Konishi et al. 2006; Zhang et al. 2009). In our recent study, we evaluated the non-seed-shattering behaviour of the Indica rice cultivar, ‘IR36’ (Ishikawa et al. 2017). The evaluation of the BRILs having ‘IR36’ derived chromosomal segments in the W630 genetic background identified a strong QTL on chr. 4, which overlaps with *sh4*. Additional genetic analysis suggested the involvement of *qSH3* and other minor loci in the non-seed-shattering behaviour of ‘IR36’. At present, it remains unclear whether *qCSS3* is involved in the loss of seed shattering in Indica rice. However, once the causal mutation at *qCSS3* is identified, genotyping survey at *qCSS3* in the Indica rice cultivars will clarify involvement of *qCSS3* in the non-shattering behaviour. The gene interaction among the four seed-shattering loci provides scope for future studies that will help in understanding the process of loss of seed shattering during rice domestication.

Conclusions

We successfully identified novel loci involved in the difference in seed shattering behaviour between ‘Nipponbare’, a typical Japonica rice cultivar, and wild rice *O. rufipogon*. Among two loci, ‘Nipponbare’ allele at *qCSS3* may have contributed to a loss of seed shattering in rice domestication. To our knowledge,

qCSS3 is the fourth detected locus involved in a loss of seed shattering in cultivated rice after *sh4*, *qSH1*, and *qSH3*. The genetic dissection of the non-seed-shattering behaviour of ‘Nipponbare’ reveals that at least four mutations (i.e. *qSH1*, *qSH3*, *sh4*, and *qCSS3*) are required to fully lose the abscission layer formation. Identification of the causal mutation at *qCSS3* will provide important information for rice breeding that will help in manipulating the degree of seed shattering. Moreover, combination of the alleles at the four seed-shattering loci will be useful in understanding the process and history of rice domestication.

Author contribution statement

RI conceived and designed the study. YT, SS, KO, TMT, CC and RI performed the experiments. YT, SS, KO, TI, and RI analysed the data. YT, KN and TA contributed to bioinformatic analysis. YT, TI and RI prepared the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Figure Legends

Fig. 1 Characterisation of the introgression line (IL) carrying *Oryza sativa* ‘Nipponbare’ (Npb) alleles at *qSH1*, *qSH3*, and *sh4* in the genetic background of wild rice, *O. rufipogon* W630.

(a) Graphical genotype of the IL. The positions of the three seed-shattering loci *qSH1*, *qSH3*, and *sh4* are shown. (b) Seeds of ‘Nipponbare’ (Npb), W630, and the IL. Scale bar = 5 mm. (c) The breaking tensile strength (BTS) values for ‘Nipponbare’ (Npb), W630, and the IL. Data are mean $\pm$ S.D. (n = 4). ** indicates $P < 0.01$ by unpaired Student’s *t*-test. N.D. indicates ‘Not Determined’ owing to complete seed shattering (d) An overview of spikelet base view (upper panels) and a close view of the spikelet base (lower panels) in ‘Nipponbare’ (Npb), W630, and the IL. The abscission scars were observed from the side of each dotted-line square (bottom). Scale bars = 1 mm. (e) Longitudinal sections of the spikelet base after seed detachment in ‘Nipponbare’ (Npb), W630, and the IL. vb = vascular bundle. ab = abscission layer. Black triangles indicate both edges of the abscission layer. Scale bars = 50 μ m

Fig. 2 Frequency distribution of breaking tensile strength (BTS) values for 174 F_2 individuals between *Oryza sativa* ‘Nipponbare’ and the introgression line (IL) carrying the ‘Nipponbare’ alleles at the *qSH1*, *qSH3*, and *sh4* loci. The BTS values for the parent lines and their F_1 plants are shown with black dots with S.D. ‘Nipponbare’: 157.7 ± 5.1 gf (n = 4), the IL: 111.4 ± 3.3 (n = 4), and F_1 : 129.6 ± 2.1 (n = 3) (mean $\pm$ S.D.). The DNA samples of F_2 plants with BTS values between 70 and 100 gf were selected as low (L) bulk and those with BTS values between 150 and 210 gf were selected as high (H) bulk

Fig. 3 Detection of novel loci for seed shattering on chromosomes 3 (*qCSS3*) and 9 (*qCSS9*) by QTL-seq analysis.

qCSS3 and *qCSS9* were detected in approximately the 5.4-Mb region on chromosome 3 and the 1.9-Mb region on chromosome 9. The Δ (SNP-index) plots with statistical intervals under the null hypothesis of no QTL (orange, $P < 0.01$; green; $P < 0.05$). The red line indicates the average Δ SNP-index calculated by a sliding window analysis.

Fig. 4 Genetic dissection of *qCSS3*.

(a, d) Graphical genotypes of three F_2 plants (Nos. 54, 57, and 129) and six critical recombinant F_3 plants (Nos. 129-43, -55, -88, -185, -195, and -282) in the candidate region of *qCSS3*. The BTS values of the F_3 lines from the three F_2 plants are shown on right. Data are mean $\pm$ S.D. (n = 6). Significant differences in the BTS values were observed for all pairs between the F_3 and F_4 lines having ‘Nipponbare’ and W630 homozygous chromosomal segments at the *qCSS3* candidate region. **, *, and n.s. indicates $P < 0.01$, $P <$

0.05, and not significant ($P \geq 0.05$) by unpaired Student's *t*-test. (b, c) Abscission layer formation for the F_3 progeny of No. 57 having 'Nipponbare' and W630 heterozygous chromosomal segments. vb = vascular bundle. ab = abscission layer. Black triangles indicate both edges of the partially formed abscission layer. Scale bars = 50 μ m

Supplemental Table 1 PCR-based molecular markers used in this study

Supplemental Table 2 Information of each 174 F_2 plant used for QTL-seq analysis

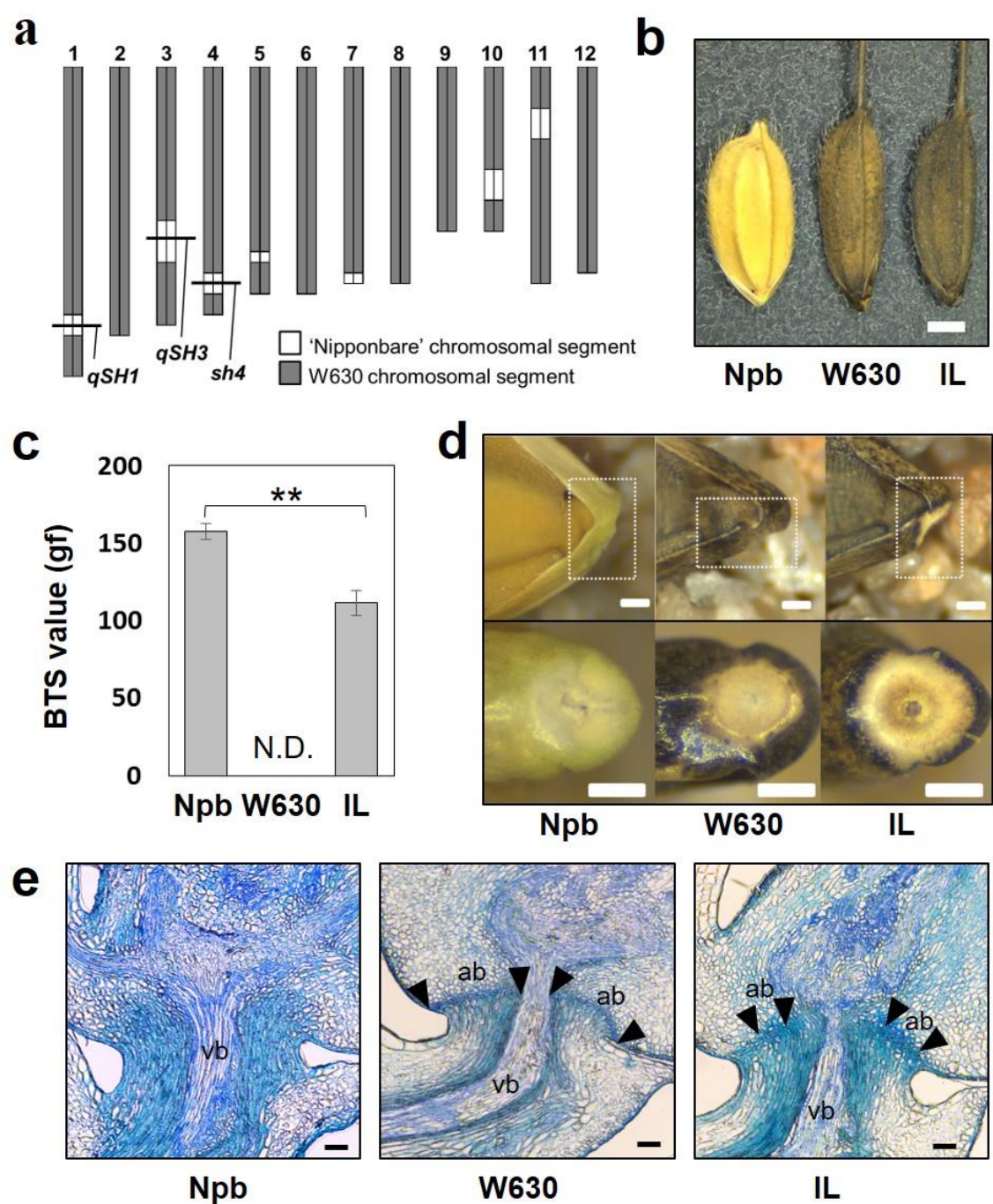
Supplemental Table 3 Days to heading observed for F_3 progeny from three F_2 plants (Nos. 57, 54, and 129). Data are mean $\pm$ S.D. (n = 6)

Supplemental Table 4 List of the 96 genes annotated in a 526 –kb candidate region of *qCSS3*.

Supplemental Fig. 1 Results of the QTL-seq analysis for all chromosomes. The Δ (SNP-index) plots with statistical intervals under the null hypothesis of no QTL (orange, $P < 0.01$; green; $P < 0.05$). The red line indicates the average Δ SNP-index calculated by a sliding window analysis.

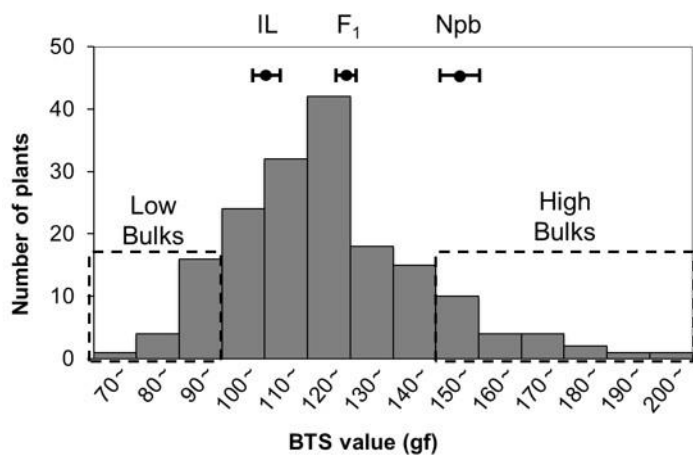
Supplemental Fig. 2 Box plots of the BTS values of two F_2 groups based on the genotypes at the candidate regions of *qCSS3* and *qCSS9*. All F_2 individuals were surveyed with five and four PCR-based molecular markers covering *qCSS3* and *qCSS9* regions, respectively (Supplemental Tables 1 and 2). Plants carrying the 'Nipponbare' (Npb) and W630 homozygous chromosomal segments for the entire candidate regions of *qCSS3* and *qCSS9* were selected.

Supplemental Fig. 3 Evaluation of seed-shattering degree of the two backcross recombinant inbred lines (BRILs) carrying the W630 chromosomal segment covering the *qCSS3* region in the 'Nipponbare' genetic background. (a) Graphical genotypes of two BRILs, BRIL42 and BRIL86. Red lines indicate the region covering *qCSS3*. (b) Seed-shattering degree of 'Nipponbare' (Npb) and the two BRILs. Data are mean $\pm$ S.D. (n = 6). ** indicates $P < 0.01$ by unpaired Student's *t*-test



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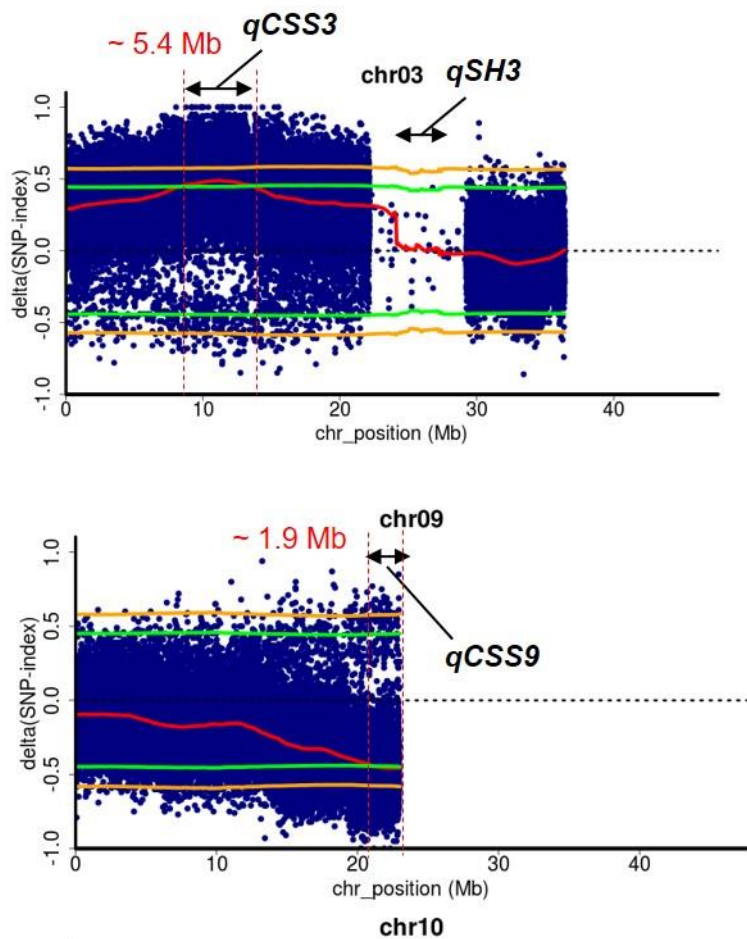
526 Fig. 2



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529 Fig. 3



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