Inhibition of abscission layer formation by an interaction of two seed-shattering loci, sh4 and qSH3, in rice

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Loss of seed shattering was one of the key phenotypic changes selected for in the domestication of many crop species. Asian cultivated rice, Oryza sativa L., was domesticated from its wild ancestor, O. rufipogon, and three seed-shattering loci, qSH1, sh4 and qSH3, have been reported to be involved in the loss of seed shattering in cultivated rice. Here, we analysed the seed-shattering behaviour of wild rice using introgression lines carrying the cultivated alleles from O. sativa Nipponbare in the genetic background of wild rice, O. rufipogon W630. We first carried out fine mapping of the qSH3 region and found that the qSH3 locus is localized in an 850-kb region on chromosome 3. We then analysed the effects of the Nipponbare alleles at sh4 and qSH3 on seed-shattering behaviour in wild rice, as a mutation at qSH1 was not commonly found in rice cultivars. Seed-shattering behaviour did not change in the two types of introgression line independently carrying the Nipponbare-homozygous alleles at sh4 or qSH3 in the genetic background of wild rice. However, the introgression lines having the Nipponbare-homozygous alleles at both sh4 and qSH3 showed a reduction in the degree of seed shattering. Histological and scanning electron microscopy analyses revealed that abscission layer formation was inhibited around the vascular bundles in these lines. Since the qSH3 region, as well as sh4, has been shown to be under artificial selection, the interaction of mutations at these two loci may have played a role in the initial loss of seed shattering during rice domestication.

Key words: abscission layer, domestication, Oryza rufipogon, Oryza sativa, seed shattering

INTRODUCTION

Rice is one of the most important crops to humans, and supports almost half of the world population (Khush, 2005). Asian cultivated rice, Oryza sativa L., was domesticated from its wild ancestor, O. rufipogon, about 10,000 years ago, and has mainly differentiated into two subspecies, Indica and Japonica (Oka, 1988; Fuller, 2007). During rice domestication, several key morphological changes were selected by early agriculturalists. Of these, the loss of seed shattering was one of the most obvious phenotypic changes to enable efficient harvesting (Harlan, 1975; Fuller and Allaby, 2009). Seed shattering is caused by the formation and degradation of the abscission layer at the basal part of the grain (Fuller and Allaby, 2009). In cultivated rice, mature seeds in most of the Japonica cultivars remain firmly attached on the panicle because the abscission layer does not form. Indica cultivars exhibit moderate seed-shattering behaviour with a partial defect in the abscission layer around the vascular bundle. Large variations in the degree of seed shattering have been observed in different cultivars (Konishi et al., 2006), suggesting that abscission layer formation is regulated by several alleles or loci that control seed-shattering behaviour.

In rice, several quantitative trait loci (QTLs) for seed shattering have been reported (Xiong et al., 1999; Cai and Morishima, 2000; Thomson et al., 2003; Lee et al., 2005; Li et al., 2006a; Onishi et al., 2007a; Ishikawa et al., 2010). Of these, three seed-shattering loci are of interest, as they were confirmed to be involved in the loss of
seed shattering in cultivated rice. First, \textit{sh4} has been reported as a locus involved in seed shattering based on comparisons between the Indica cultivar and an annual form of wild rice, \textit{O. nivara} (Li et al., 2006a, b). Second, \textit{qSH1} has been identified as a major locus, having a large effect on seed shattering in an \textit{F2} segregating population between the Japonica cultivar Nipponbare and the Indica cultivar Kasalath (Konishi et al., 2006). The third, \textit{qSH3}, was detected in crosses between \textit{O. sativa} Japonica and \textit{O. rufipogon} (Onishi et al., 2007a, b). Recently, we confirmed the allele effect of \textit{qSH3} using an \textit{F2} segregating population between \textit{O. sativa} cv. Nipponbare and an introgression line (IL) carrying the Nipponbare-homozygous alleles at \textit{qSH1} and \textit{sh4} in the genetic background of \textit{O. rufipogon} W630 (Htun et al., 2014). We further produced ILs carrying the Nipponbare-homozygous alleles at any one of these three loci (\textit{qSH1}, \textit{sh4} and \textit{qSH3}) in the genetic background of wild rice. Interestingly, their seed-shattering behaviour remained as strong as that of wild rice, suggesting that these loci redundantly regulate abscission layer formation (Htun et al., 2014). In contrast, ILs of wild rice carrying the Nipponbare-homozygous alleles at all three loci displayed significantly reduced seed shattering due to the partial loss of the abscission layer; abscission layer formation in the epidermal region was particularly inhibited. These studies suggest that genetic interactions at seed-shattering loci are important in understanding the initial loss-of-seed-shattering step during rice domestication.

A single nucleotide polymorphism in the regulatory region of \textit{qSH1} has been detected in Japonica rice (Konishi et al., 2006; Onishi et al., 2007b). This mutation at \textit{qSH1} led to a complete loss of shattering by eliminating abscission layer formation particularly in temperate Japonica rice. Therefore, a mutation at \textit{qSH1} may have been selected by farmers with threshing tools in the domestication of Japonica rice. Sequence analysis has revealed a mutation at \textit{sh4} in all cultivated rice tested, and population genetic studies have indicated that \textit{sh4} experienced artificial selection (Onishi et al., 2007b; Zhang et al., 2009). Based on these findings, it is widely accepted that \textit{sh4} was a major gene selected for the loss of seed shattering during rice domestication. However, we found that the ILs having the Nipponbare-homozygous allele at \textit{sh4} in the genetic background of wild rice showed strong seed-shattering behaviour (Ishikawa et al., 2010; Ishii et al., 2013; Htun et al., 2014). This finding strongly indicated that a single mutation at \textit{sh4} was not sufficient to confer the phenotypic change for seed shattering in wild rice. In addition, several weedy rice accessions also have the domesticated \textit{sh4} allele, but they exhibit strong seed shattering due to complete abscission layer formation (Zhu et al., 2012). Chromosomal regions harbouring \textit{qSH3} in cultivated rice underwent artificial selection (He et al., 2011), and the selective sweep was observed around the region containing \textit{qSH3} in both Indica and Japonica cultivated rice (Xu et al., 2012). However, we do not know how the two seed-shattering loci \textit{sh4} and \textit{qSH3} were involved in rice domestication. In this study, we investigated the genetic interaction of these two loci, which possibly contributed to the initial loss of seed shattering during rice domestication.

**MATERIALS AND METHODS**

Fine mapping of \textit{qSH3} and production of ILs for seed-shattering loci An annual wild rice, \textit{Oryza rufipogon} acc. W630, originating from Myanmar, and the Japonica cultivar \textit{O. sativa} cv. Nipponbare were used as parental plants. In the fine mapping experiment, an IL having the Nipponbare-homozygous alleles at \textit{qSH1} and \textit{sh4} and heterozygous alleles at \textit{qSH3} was self-pollinated to obtain the segregating population. A total of 1342 plants were screened to identify recombinants between the two simple sequence repeat (SSR) markers RM16 and RM426, covering the \textit{qSH3} region. Four additional SSR markers, RM15539, RM3513, RM3601 and RM15658, were employed for fine mapping. Two independent ILs of wild rice having the W630-homozygous allele at \textit{qSH1}, the Nipponbare-homozygous allele at \textit{sh4}, and heterozygous alleles at \textit{qSH3} were generated by backcrossing with W630. The genotypes at the three seed-shattering loci in the ILs and their progeny were confirmed by derived cleaved amplified polymorphic sequences (dCAPS) for \textit{sh4} and \textit{qSH1} (Htun et al., 2011, 2014), and two SSR markers, RM3513 and RM3601, for \textit{qSH3}.

Evaluation of the degree of seed shattering The degree of seed shattering was evaluated by measuring the breaking tensile strength (BTS, gf; gram-force) required to detach the seeds from the pedicels using a digital force gauge (FGP 0.5, Nidec-Shimpo, Japan). The BTS values of 10 spikelets were measured for each plant. Average BTS values of the lines were calculated with three independent plants. Since the flowering date of each spikelet within a panicle was different, each spikelet was coloured based on the flowering date, and their BTS values were measured on the indicated date after flowering. The difference in BTS values between the two genotypes of the progeny was calculated using the unpaired Student’s \textit{t}-test.

Histological analyses of abscission layer formation Samples of the abscission layer for histological analysis were collected from the pedicel tissue of grains at the flowering date. Samples were fixed based on a method previously described (Htun et al., 2014), and were embedded in Technovit 7100 resin (Heraeus Kulzer, Germany) according to the manufacturer’s instructions.
Sections that were 3-μm thick were produced using an RM2125RT rotary microtome (Leica Microsystems, Wetzlar, Germany) and stained with toluidine blue O solution. These sections were then photographed under a microscope.

**Scanning electron microscopy (SEM)** Sets of grain and pedicel were soaked with 2.5% glutaraldehyde (Nisshin EM, Tokyo, Japan) in 0.1 M phosphate-buffered saline, pH 7.4 (PBS) and kept in a vacuum desiccator to remove air from the tissues. They were then washed in PBS three times and postfixed with 1% osmium tetroxide (Nisshin EM) in PBS for 2 h at room temperature. After the samples were briefly rinsed in distilled water, they were continuously dehydrated using an ethanol series (50%, 70%, 90%, and 100% thrice, each for 10 min). After dehydration, they were transferred to t-butyl alcohol three times (10 min each). The samples were then placed in a butanol freeze-drier (VFD-21S; Vacuum Device, Mito, Japan) for a few minutes, after which they were evaporated for 35 min to remove the frozen t-butyl alcohol. The samples were set on foundations and osmium coating was carried out using an HPC-1SW Hollow Cathode Plasma CVD (Vacuum Device). Secondary electron images of the joint surfaces between grain and pedicel were observed using a JSM-6010LV scanning electron microscope (JEOL, Akishima, Japan) and then photographed.

**RESULTS**

**Fine mapping of qSH3** In our previous study, the seed-shattering locus qSH3 was identified by QTL analysis using an F2 segregating population between *O. sativa* Nipponbare and an IL having the Nipponbare-homozygous alleles at two major seed-shattering loci, qSH1 and sh4 (Htun et al., 2014). The QTL region of qSH3 was estimated to be between two marker loci at RM16 and RM3513. To locate the qSH3 candidate region more precisely, we selected one IL that had a heterozygous chromosomal segment between RM16 and RM426 and the Nipponbare-homozygous alleles at qSH1 and sh4 in the genetic background of wild rice (Fig. 1). The BTS value of the parental line (q3H-15) used for the fine mapping analysis was 50.0 ± 4.0 gf, which was much lower than that (116.0 ± 37.0 gf) of the IL having the Nipponbare-

![Fig. 1. Mapping of qSH3. Molecular markers between RM16 and RM426 on chromosome 3 were used to detect recombinants near the qSH3 locus. Chromosomal constitutions of six recombinants (plant numbers q3H-15-72, -112, -113, -114, -115, and -116) and heterozygote (q3H-15-122 and -125) between RM16 and RM426 are shown with their BTS values. The qSH3 locus was estimated to be within the 850-kb region between RM3513 and RM3601.](image-url)
homozygous alleles at the three seed-shattering loci (Htun et al., 2014). This result suggested that the W630 allele at qSH3 acts dominantly. Mapping analysis was conducted using 1342 segregating lines. Using four additional markers between RM16 and RM426, chromosomal constitutions of six recombinants and two control lines were analysed (Fig. 1). The BTS values of four of these six recombinants (q3H-15-14, -31, -112 and -113) were more than 100 gf, much higher than the other two (q3H-15-16 and -72). Based on the genotypes at these SSR markers, the candidate region of qSH3 was narrowed down to between RM3513 and RM3601, a length of approximately 850 kb according to the Nipponbare genome sequence (Fig. 1).

The degree of seed shattering in IL progeny with the Nipponbare alleles at sh4 and qSH3  We previously reported that ILs carrying the Nipponbare-homozygous alleles at any one of the three seed-shattering loci qSH1, sh4 and qSH3 in the genetic background of wild rice exhibited complete abscission layer formation, as does wild rice (Htun et al., 2014). These results clearly indicate that a single mutation at any one of the three loci was insufficient to confer non-shattering behaviour on wild rice. Of these three seed-shattering loci, a non-functional allele at qSH1 has been observed only in Japonica-type cultivated rice (Konishi et al., 2006; Onishi et al., 2007b; Zhang et al., 2009), suggesting that a mutation at qSH1 was selected for in the domestication of Japonica rice. Therefore, we focused on the genetic interaction of the cultivated alleles at sh4 and qSH3 to investigate the initial loss of seed shattering in rice domestication. Since it is widely accepted that a causal mutation at sh4 was fixed in all cultivated rice (Lin et al., 2007; Onishi et al., 2007b; Zhang et al., 2009), we selected...
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Two ILs carrying the W630-homozygous allele at qSH1, the Nipponbare-homozygous allele at sh4 (sh4-N), and heterozygous alleles at the qSH3 candidate region, between RM3513 and RM3601 (qSH3-H), in the genetic background of wild rice (Fig. 2). They were designated as sh4-N/qSH3-H lines to show the genotypes at sh4 and qSH3. These two independent ILs (IL1 and IL2) were self-pollinated to generate progeny having homozygous alleles of Nipponbare (sh4-N/qSH3-N) or W630 (sh4-N/qSH3-W) at qSH3. Genotypes at the three seed-shattering loci were confirmed by two dCAPS markers for qSH1 and sh4 and a pair of flanking SSR markers for qSH3 (Fig. 2).

Progeny derived from the ILs, as well as wild (O. rufipogon W630) and cultivated rice (O. sativa Nipponbare), were grown in the greenhouse and their seed-shattering behaviour was investigated at the flowering and two maturation stages (0, 20 and 35 days after flowering (DAF)). In wild rice, the BTS value at 0 DAF was similar to that in Nipponbare, indicating that the abscission layer had not yet been degraded. The progeny of the two ILs also exhibited similar BTS values to Nipponbare and W630 at flowering (Fig. 3A). At the seed maturation stages (20 and 35 DAF), significant differences in BTS values were observed between the two genotypes of the progeny (Fig. 3, B and C). At 35 DAF, the BTS values in the progeny carrying two Nipponbare alleles at sh4 and qSH3 (sh4-N/qSH3-N) were 30.5 ± 4.4 gf for IL1 and 19.5 ± 3.5 gf for IL2, while those in the progeny carrying the Nipponbare allele at only sh4 (sh4-N/qSH3-W) were 16.6 ± 6.9 gf for IL1 and 4.4 ± 3.3 gf for IL2. These results clearly indicate that the Nipponbare allele at qSH3 plays a role in the inhibition of seed shattering at the seed maturation stage.

Abscission layer formation in the IL progeny at the flowering stage

The abscission layer is composed of abscission cells, which are small and densely aligned transversely to the organ axis (Roberts et al., 2002). To investigate abscission layer formation in these IL progeny, we conducted histological analyses. Tissues surrounding the junction between pedicel and grain were harvested at the day of flowering and their transverse

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**Fig. 4.** Abscission layer formation at flowering stage (0 DAF) in the progeny of two ILs in the genetic background of wild rice. Wild rice, O. rufipogon W630, was used as a control. Magnified views of the abscission layer on the left are shown on the right. Black arrowheads indicate non-differentiated abscission cells. VB, vascular bundle. Bars = 50 μm.
sections were prepared. In wild rice, as well as in progeny having the Nipponbare-homozygous allele at sh4 only, complete abscission layer penetration from the vascular bundle to the outside epidermis was observed (Fig. 4). Interestingly, around the vascular bundles, progeny carrying the Nipponbare-homozygous alleles at both sh4 and qSH3 had partially connected tissues, which were not strongly stained (Fig. 4). We counted the number of these non-differentiated cells using five transverse sections for each progeny line. Approximately three cells (3.9 ± 0.7 and 3.3 ± 1.0 in progeny from IL1 and IL2, respectively) were not stained as abscission cells (Fig. 4, Supplementary Fig. S1). This observation suggests that the Nipponbare cultivated alleles at qSH3 and sh4 together caused a partial loss of abscission layer formation, ultimately resulting in an increase in the BTS value in the genetic background of wild rice.

**Structural analyses of abscission cells in IL progeny by SEM** To further investigate the structure of the abscission layer in the two progeny genotypes, we employed SEM. In the degree of seed shattering, the difference in BTS values between the two genotypes of progeny was significant at 20 DAF (Fig. 3B). Therefore, we harvested samples of the grain-pedicel junction for SEM analysis around 20 DAF. We also analysed wild rice at 12 DAF as their seeds had completely shattered by 15 DAF. Each sample for SEM analysis was carefully collected as a pair of grain and pedicel. The grain and pedicel became separated by spontaneous detachment during the fixation process and SEM photographs were taken of the interface surfaces of grain and pedicel. In this SEM analysis, strong signals are derived from the rough surface of the samples by secondary electrons. In wild rice, as well as in progeny having the Nipponbare-homozygous allele only at sh4, a smooth surface of abscission cells was observed over the vascular bundle to outside the epidermis, confirming that abscission cells had completely formed and had already separated from the pedicel (Fig. 5). We then analysed the abscission cells of progeny carrying the Nipponbare-homozygous alleles at both sh4 and qSH3. A rough surface was observed particularly around the vascular bundles, and tissue was broken due to the detachment of grains. The position of this broken tissue was similar to that observed by histological analysis, indicating that the cells around the vascular bundles were not differentiated into abscission cells at the seed maturation stage (Fig. 5). These results clearly demonstrate that both the Nipponbare cultivated alleles at sh4 and qSH3 were required to inhibit the formation of

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**Fig. 5.** Scanning electron micrographs of abscission layer formation in the progeny of two ILs in the genetic background of wild rice. *O. rufipogon* W630, was used as a control. White arrowheads indicate roughly separated cells. Bars = 50 μm.
abscession cells around the vascular bundles.

DISCUSSION

We previously estimated that qSH3 was located in the genomic region near RM16 on chromosome 3, and that the wild allele acted dominantly (Htun et al., 2014). In this study, we first narrowed down the candidate region of qSH3 to the approximately 850 kb between RM3513 and RM3601 (Fig. 1). This region harbours two seed-shattering genes, OsSh1 and OsWRKY, which are homologues of sorghum seed-shattering genes (Lin et al., 2012; Tang et al., 2013). We have previously reported that one and two amino acid changes between Nipponbare and W630 occur in the proteins of OsSh1 and OsWRKY, respectively (Htun et al., 2014).

Using ILs selected by two newly detected flanking markers at qSH3, we have shown that the Nipponbare-homozygous alleles at sh4 and qSH3 together conferred a partial inhibition of abscission layer formation in the genetic background of wild rice. As previously shown, independent mutations at sh4 or qSH3 were insufficient to change abscission layer formation in wild rice (Htun et al., 2014). These findings suggest that several mutations at seed-shattering loci were required to confer the phenotypic change in the abscission layer that resulted in the loss of seed shattering during rice domestication.

As shown by histological analyses, wild rice formed an abscission layer at the junction of the grain and the pedicel except for the vascular bundle at 0 DAF (Fig. 4). Upon fertilisation, abscission cells start to degrade and only the vascular bundle connects the grain and pedicel. Therefore, seeds could easily be shattered with spontaneous detachment (Fig. 3, B and C). The structure of the abscission layer in progeny having the Nipponbare-homozygous allele at sh4 only was similar to that in wild rice (Fig. 4), but these progeny kept seeds at 20 and 35 DAF, whereas wild rice shed seeds (Fig. 3, B and C); this could be due to other minor QTLs, or to the effect of sh4, which may control the degradation of abscission cells. An obvious change in BTS values and abscission cells was detected in progeny having the Nipponbare-homozygous alleles at both sh4 and qSH3 (Fig. 3, B and C, and Fig. 4). In these progeny, approximately three cells around the vascular bundle had not differentiated into abscission cells (Fig. 4, Supplementary Fig. S1). The broken and rough surface of these cells was observed by SEM at the maturation stage (Fig. 5), confirming that the grain and pedicel were connected through these cells. In most Indica cultivars, the development of abscission cells is also inhibited around the vascular bundle (Konishi et al., 2006), but the number of disrupted abscission cells in Indica cultivars is higher than that in the progeny having the Nipponbare-homozygous alleles at both sh4 and qSH3. Perhaps this can be explained by additional small QTLs, which may have been involved in the loss of seed shattering during rice domestication.

In our previous study, ILs of wild rice having any one of the three seed-shattering loci exhibited complete abscission layer formation, as in wild rice (Htun et al., 2014). These results clearly indicate that single mutations occurring at any of the three loci were not sufficient to result in phenotypic changes in the abscission layer. Several mutations may therefore have been required to trigger phenotypic change in the abscission layer, resulting in the loss of seed shattering. Genetic interaction at the three seed-shattering loci qSH1, qSH4 (synonymous with sh4) and qSH3 was previously studied using ILs having the wild alleles in the genetic background of Japonica cultivated rice (Onishi et al., 2007b). The results showed that wild alleles at qSH1 and sh4 had strong effects on seed shattering, but that at qSH3 was weak. On the other hand, our results showed that qSH3 contributed to seed-shattering behaviour together with other loci (Figs. 3, 4 and 5; Htun et al., 2014). This could be due to the genetic background or epistatic interaction among seed-shattering loci; in particular, cultivated rice may still harbour other unidentified mutation(s) at seed-shattering loci.

As demonstrated in the present study, the Nipponbare domesticated alleles at sh4 and qSH3 contributed to the partial inhibition of abscission cell differentiation, resulting in an increase in the BTS value (Figs. 3 and 4). To completely inhibit abscission layer formation (as observed in Nipponbare), mutations at four or more loci were required, as the IL carrying the Nipponbare-homozygous alleles at the three known loci (qSH1, sh4 and qSH3) still formed a partial abscission layer (Htun et al., 2014). We do not know the involvement of other unidentified loci that contributed to the loss of seed shattering in Japonica type rice, and the identification of all loci with causal mutations that were selected for the loss of seed shattering is therefore needed in future studies. These studies will clarify the loss-of-seed-shattering process that occurred during rice domestication. The investigation of seed-shattering behaviour in the genetic background of wild rice, as conducted in this study, is an important step in elucidating the genetic control of seed shattering in rice.

For ancient hunter-gatherers, weak shattering (but retention of the seeds in the panicle) would have been beneficial, as complete non-shattering requires mechanical power to thresh the seeds. Therefore, the degree of seed shattering could have been gradually modified in several steps, depending on the progress of harvesting styles during rice domestication. In particular, the degree of seed shattering as provided by the mutations at both sh4 and qSH3 would have been useful during the initial stages of domestication. If seeds were connected to the pedicel, they would have stayed longer in the
panicle. This would have been important for early agriculturists, as they could easily find wild rice plants whose seeds remained attached for a longer period than those in other plants. Recently, we reported the role of panicle spreading on rice domestication. Most cultivated rice has closed panicles, while wild rice has open panicles. This trait was shown to be regulated by a mutation in the regulatory region of OsLG1, an orthologue of maize LG1 (Ishii et al., 2013). As revealed by experiments using near-isogenic lines in the genetic background of wild rice, a closed panicle was beneficial for rice domestication as it increased the yield. Interestingly, a closed panicle was also shown to increase the self-fertilisation rate. These results suggest that a recessive mutation could have been fixed more easily in plants with a closed panicle than in those with an open panicle. Although we do not know the order of the selection of mutations, the selection of a closed panicle may have preceded that of the other traits, as this pheno- typic change would have contributed to the fixing of recessive mutations such as those involved in the loss of seed shattering. The use of genetic resources from wild and cultivated rice to genotype these domestication-related mutations at seed-shattering loci will continue to elucidate the process of rice domestication.

**CONCLUSION**

Our study demonstrates that naturally occurring alleles at sh4 and qSH3 played an important role in the initial loss of seed shattering during rice domestication. Ongoing studies to evaluate seed-shattering behaviour in the genetic background of wild rice will shed light on how the change in the abscission layer was selected for during rice domestication. Alternatively, marker-assisted breeding for a combination of naturally occurring alleles at seed-shattering loci, including those identified from wild and cultivated rice, should contribute to the future breeding of cultivated rice, and fine-tune the degree of seed shattering based on harvesting style.

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