Direct identification of a mutation in OsSh1 causing non-shattering in a rice (Oryza sativa L.) mutant cultivar using whole-genome resequencing

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Loss of seed shattering has been regarded as a key step during crop domestication. Mutagenesis contributes to the development of novel crop cultivars with a desired seed-shattering habit in a relatively short period of time, but also to uncovering the genetic architecture of seed shattering. ‘Minamiyutaka’, a non-shattering indica rice cultivar, was developed from the easy-shattering japonica cultivar ‘Moretsu’ by mutation breeding via gamma-ray irradiation. In present study, we observed significant differences in shattering habit, breaking tensile strength, and abscission zone structure between ‘Moretsu’ and ‘Minamiyutaka’. Whole-genome mutation analysis of ‘Minamiyutaka’ newly identified a 13-bp deletion causing defective splicing in exon 3 of the OsSh1 gene which has previously been referred to as a candidate for controlling seed shattering. Using CRISPR/Cas9 gene editing, we demonstrated that loss-of-function mutation in OsSh1 causes non-shattering in rice. Furthermore, gene expression analysis suggests that OsSh1 may function downstream of qSH1, a known key gene involved in abscission zone differentiation. Nucleotide diversity analysis of OsSh1 in wild rice accessions and cultivars revealed that OsSh1 has been under strong selection during rice domestication, and a missense mutation might have contributed to the reduction of seed shattering from the wild progenitors to cultivated rice.

Loss or reduction of seed shattering is one of the key features in crop domestication, since it reduces yield losses from shattering and improves harvesting efficiency1,2. Asian cultivated rice (Oryza sativa L.), one of the most important cereal crops in the world, was domesticated from its Asian wild ancestor (O. rufipogon) more than ten thousand years ago3,4. In cultivated rice, the seed-shattering habit of wild rice was lost during domestication, and shattering degrees show a wide variation5. Generally, indica cultivars exhibit relatively easy shattering, whereas most japonica cultivars exhibit hard shattering6.

In rice, seeds shattering is implemented by an abscission zone in the junction of sterile lemma and pedicel6. The abscission zone, which is composed of one or two layers of small, isodiametrically shaped cells with thin cell walls, is formed at the young panicle development stage approximately 16–20 days before heading7, and gradually degrade after flowering8. The morphology and degradation behavior of the abscission zone differs in different rice varieties7–9. Seed shattering is controlled by a complex regulatory network8,9 and quantitative trait loci (QTLs) for seed shattering have been detected on almost all rice chromosomes10–15. Map-based cloning and genetic complementation experiments have revealed two domestication related mutations that facilitate the reduction of seed shattering. One mutation is a single amino acid substitution from Lysine residue to Asparagine at position 79 (K79N) in the gene SH4/SHA1, a transcription factor with an Myb3 DNA binding domain16,17. This

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mutation is responsible for the reduction of seed shattering from the wild progenitors to cultivated rice. Wild rice has a complete layer of abscission cells, while the SH4 domestication allele contributes to absent abscission cells near the vascular bundle, leading to incomplete development of the abscission zone in cultivated rice. Another mutation is a single nucleotide polymorphism (SNP) in the S′ regulatory region of the qSH1 gene, an ortholog of the Arabidopsis homeobox gene REPELUMLESS (RPL)\(^{16,17}\). This mutation results in the absence of abscission zone formation and thus loss of seed shattering in a subset of temperate japonica cultivar. Except for SH4 and qSH1, many minor QTLs involved in seed shattering have not been cloned\(^{10–15}\).

Using artificial mutagenesis, some mutants with changed seed-shattering habit were obtained and the causal genes were identified or speculated\(^{19–24}\). An insertion of a > 4-kb fragment in YABBY-like gene OsSh1, an ortholog of Sh1 involved in seed shattering in sorghum, was thought to be the cause of the non-shattering phenotype in a rice mutant\(^{19,25}\). More recently, a genomic segment deletion containing OhsH3, an ortholog of Sh1, was revealed to cause the loss of seed dispersal in populations of African cultivated rice (O. glaberrima Steud.)\(^{26}\). Loss-of-function mutations in the APETALA2 (AP2) gene, SHAT1\(^{20}\) or SHH1/SNB\(^{20}\), can inhibit the expression of qSH1, hence leading to loss of shattering. Furthermore, the natural variations in SHH1 were speculated to be associated with the domestication and improvement of seed shattering and yield-related traits in rice\(^ {20}\). In wheat, domestication-related Q gene, involved in controlling seed shattering, is also an AP2-like gene\(^ {27}\). These results suggest that the regulatory network controlling seed shattering is extensively conserved in grain crops, and that mutagenesis can facilitate the uncovering of the complex genetic architecture of seed shattering.

Minamiyutaka is a non-shattering indica rice cultivar for whole crop silage and is broadly cultivated in Japan. ‘Minamiyutaka’ was developed from a mutant obtained through gamma-ray irradiation of an easy-shattering cultivar ‘Moretsu’\(^ {28}\). The heading and maturing time, and the morphological characteristics of both cultivars ‘Minamiyutaka’ and ‘Moretsu’ were almost the same\(^ {28}\). In contrast, the reported mutants have pleiotropic phenotypes such as altered development of spikelet or inflorescence, besides the reduction of seed shattering. For example, SSH1 and inflorescence developmental defects, ssh2 shows smaller seeds\(^ {20}\), and while ssh1 shows larger seeds\(^ {20}\). We therefore hypothesized that a different causal gene was responsible for the non-shattering habit in ‘Minamiyutaka’. Identification of the causative mutation underlying the loss of shattering in ‘Minamiyutaka’ will be valuable for improving rice seed shattering without affecting other traits using molecular breeding strategies, and will also contribute to uncovering the complex regulatory pathways of seed shattering.

Recently, an approach combining bulked segregant analysis with whole-genome resequencing has dramatically accelerated the process of identifying candidate genes\(^ {20,21,29}\). Alternatively, since gamma-ray-irradiation induces less than one hundred mutations in the whole genome of rice, and since, furthermore, most of them are in the intergenic and intronic regions, with only several mutations highly or moderately impacting the gene function\(^ {40}\), we therefore supposed that the detection of the mutations in the whole genome and analysis of the effects of the mutations on gene function could directly identify the causal gene for an altered phenotype induced by gamma-ray-irradiation. In the present study, as expected, we successfully uncovered the putative causal gene for the loss of shattering in ‘Minamiyutaka’ with this approach and confirmed it by CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 gene editing.

**Results**

**Seed-shattering characteristics in the parental cultivars.** The cultivar ‘Moretsu’ has an easy-shattering habit, such that up to 40 of grains were shattered after grasping a panicle by hand at the maturity stage (Fig. 1a). In contrast, almost no grains were shattered in ‘Minamiyutaka’, confirming that it is of non-shattering. Subsequently, we evaluated the pulling strength (PS) and bending strength (BS), i.e., breaking tensile strength for detachment of a seed from the pedicel by pulling and bending, respectively. PS values of ‘Moretsu’ and ‘Minamiyutaka’ were 124.4 ± 29.6 and 211.2 ± 30.9 gf, respectively, showing a significant difference \( (P < 2.2 \times 10^{-18}) \) (Fig. 1b). BS values of ‘Minamiyutaka’ were 40.5 ± 18.9 gf, approximately three times that of ‘Moretsu’ (13.5 ± 6.2 gf) (Fig. 1c). These results revealed the strong resistance to shattering in ‘Minamiyutaka’. It should be noted that the PS and BS values may also be affected by the health status of a pedicel. If a pedicel was not well developed or was damaged, its PS or BS value would be small. While we endeavored to select spikelets that appeared healthy, standard deviation was still large for all evaluated spikelets of each cultivar.

To distinguish precisely the differences in anatomical aspects of abscission zone between the two cultivars, longitudinal sections of spikelets at heading stage were observed. In ‘Moretsu’, isodiametrically shaped and thin-walled abscission zone cells were aligned completely and transversely in the basal area near sterile lemmas except in the region near the vascular bundle. In contrast, the alignment of abscission cells was discontinuous and incomplete in ‘Minamiyutaka’ (Fig. 1d).

The interface where a mature grain separates from the pedicel was observed using a scanning electron microscope (SEM). The microstructure of the surface of the grain base of ‘Moretsu’ was clearly different from that of ‘Minamiyutaka’ (Fig. 1e,f). ‘Moretsu’ was relatively smooth, but ‘Minamiyutaka’ was broken and rough. It is obvious that a stronger force was needed to remove grain from the pedicels in ‘Minamiyutaka’ than in ‘Moretsu’ and this led to a rougher surface in ‘Minamiyutaka’.

**Segregation of non-shattering phenotype.** The shattering degrees of F\(_2\) plants \((n = 100)\) derived for the cross between ‘Moretsu’ and ‘Minamiyutaka’ were evaluated by grasping panicles by hand at the maturity stage. As a result, 27 and 73 plants showed a non-shattering and an easy-shattering phenotype, respectively (Fig. 2d). This segregation ratio fits a 1:3 ratio as determined by a chi-square test \((\chi^2 = 0.64, P = 0.42)\), indicating that a single recessive locus was the cause of the non-shattering phenotype in ‘Minamiyutaka’.
Identification of a candidate causal mutation using whole-genome resequencing. Using next-generation sequencing, a total of 78.3, and 79.2 million sequence reads (each 150 bp) were obtained for 'Moretsu' and 'Minamiyutaka', respectively (Supplementary Table S1 online). After removing low-quality, unpaired and duplicated reads, about 84.0% of the clean reads were mapped to the 'Nipponbare' reference genome. The average coverages were 20.2 times.

Homozygous mutations including 62 SNPs, 7 deletions (<=3 bp), and 5 insertions (<=5 bp) unique to 'Minamiyutaka' were identified (Supplementary Table S2 online). No structural variation (SV) was detected. Mutation annotation analysis revealed only one mutation (Mutation ID: MN19, 13-bp deletion) with high impact on gene function (gene ID: Os03t0650000-01) (Fig. 2a). This mutation was validated by Sanger sequencing (Fig. 2b). In a previous study, this gene has been identified as an ortholog of Sh1 controlling seed shattering in sorghum, and has been named OsSh125. OsSh1, also called as OsYABBY231, belongs to the YABBY gene family composed of an N-terminal zinc finger domain and a C-terminal YABBY domain (helix-loop-helix motif)32. Analysis of the protein coding sequence (CDS) region revealed that the 13-bp deletion caused exon 3 (127 bp) skipping and a reading frameshift leading to a putative translational termination codon (PTC) which can result in a loss of YABBY domain in 'Minamiyutaka' (Fig. 2c, Supplementary Fig. S1 online). We therefore inferred the 13-bp deletion in OsSh1 to be the candidate causal mutation resulting in loss of seed shattering in 'Minamiyutaka'.

A Cleaved Amplified Polymorphic Sequences (CAPS) marker designed based on the restriction enzyme site in the 13-bp deletion of OsSh1 was used to perform genotyping analysis of the F2 plants. As a result, 21, 52, and 27 plants were homozygous wild-type, heterozygous, and homozygous mutant-type, respectively (Fig. 2d). All the wild-type and heterozygous plants exhibited the easy-shattering phenotype, whereas all the mutant-type plants were of non-shattering phenotype (Fig. 2d). These results further suggested that OsSh1 is a strong candidate gene involved in seed shattering.

Genetic complementation using CRISPR/Cas9-based gene editing. 'Teqing', a Chinese high-yielding indica rice cultivar with an easy-shattering phenotype, was used for OsSh1 gene editing. In T0 plants, 8 genotypes with 11 different mutant alleles and 10 genotypes with 11 different mutant alleles were detected in the target regions of the exon 1 and exon 2 of OsSh1, respectively (Supplementary Table S3 online). T0 trans-
genic plants were self-pollinated and only partial T₁ lines were grown due to space limitation in the greenhouse. For seed-shattering evaluation, we selected three independent lines, each of which had more than four plants with homozygous Cas9-free mutation (Fig. 3a). All of them have one-base InDel (deletion/insertion) in exon1 or exon2, which may cause a reading frameshift and lead to loss of gene function. PS values of the three OsSh1-edited lines, i.e., T_Cas9-1, T_Cas9-2 and T_Cas9-3, were 180.0 ± 47.0, 195.9 ± 43.1, and 218.5 ± 46.5gf, respectively, significantly higher than that of ‘Teqing’, 110.1 ± 45.0 gf (P < 2.2 × 10⁻¹⁶) (Fig. 3b). The BS values of them were 26.6 ± 11.8, 38.4 ± 16.9, and 38.0 ± 15.4 gf, respectively, approximately 2 to 5 times higher than that of ‘Teqing’ (8.0 ± 5.9 gf) (P < 2.2 × 10⁻¹⁶) (Fig. 3c). The fracture surface of the grain base was investigated using an SEM. We found that ‘Teqing’ had a smooth fracture surface (Fig. 4a), whereas all the OsSh1-edited lines had...
a broken and rough fracture surface (Fig. 4b–d). These results indicated that OsSh1-edited lines had a non-shattering habit.

OsSh1 expression profile. The expression profile of OsSh1 in various rice tissues deposited in the rice gene expression database RiceXPro33 showed that OsSh1 was strongly expressed in leaf blade, leaf sheath and stem rather than in root during vegetative stage (Supplementary Fig. S2 online). During reproductive stage, the OsSh1 was mainly expressed in pistil, lemma, palea, ovary, embryo and young endosperm rather than in anther and old endosperm.

OsSh1 functions downstream of qSH1. To examine the gene expression of OsSh1 in developing panicles of 'Moretsu' and 'Minamiyutaka', we performed reverse transcription quantitative PCR (RT-qPCR) analysis using three primer sets P1, P2, and P3 (Fig. 5a). All the results showed a significantly lower level of OsSh1 expression in 'Minamiyutaka' than in 'Moretsu' (Fig. 5b–d) (P < 0.01). Since qSH1 is the key gene involved in abscission layer formation5,22, its transcript level was analyzed. However, no significant differences were observed between these two cultivars (Fig. 5e). These results led us to suppose that OsSh1 might function downstream of qSH1, or alternatively, that OsSh1 and qSH1 were in different pathways, respectively. To address this, firstly, we developed a non-shattering chromosome segment substitution line (CSSL) 'Takanari-qsh1' which harbors a dysfunctional allele of qSH1 derived from a japonica rice cultivar 'Koshihikari' in the 'Takanari' genetic background34. In contrast, 'Takanari' is an easy-shattering indica rice cultivar with a functional allele at qSH121,34. PS and BS values of the 'Takanari-qsh1' were 188.5 ± 56.2 and 22.6 ± 13.0, respectively, significantly higher than those of 'Takanari', 119.1 ± 38.2 and 12.3 ± 10.5 (Supplementary Fig. S3 online), confirming the dysfunctional allele of qSH1 causing reduction of seed shattering. Hence, we investigated the gene expressions of OsSh1 and qSH1 in the young pani-
cles of these two cultivars/lines. As a result, the transcript levels of qSH1 in ‘Takanari-qsh1’ were significantly lower than those in ‘Takanari’ (Fig. 6a), since an SNP in the 5′ regulatory region of the dysfunctional qSH1 can decrease its expression. Interestingly, OsSh1 in ‘Takanari-qsh1’ also notably decreased as compared with ‘Takanari’ (Fig. 6b). These data suggest that OsSh1 functions downstream of qSH1.

Nucleotide diversity in OsSh1. To investigate the nucleotide diversity of OsSh1 in wild and cultivated rice, we aligned the nucleotide sequences covering the entire OsSh1 gene (3,688 bp), a 1,653-bp 5′-flanking region, and a 1,354-bp 3′-flanking region, from 37 accessions of O. rufipogon, 84 indica varieties, 63 temperate japonica varieties, and 41 tropical japonica varieties (Supplementary Table S4 online). The nucleotide diversity (π) of OsSh1 was the highest in wild rice, moderate in indica rice, and lowest in japonica rice (Fig. 7a). The percentages of nucleotide diversity in indica/O. rufipogon and japonica/O. rufipogon were 23.2 and 1.5%, respectively, which are far below the percentages (53.3 and 20.0%, respectively) at the whole-genome level4,35. Furthermore, Tajima’s D based on the OsSh1 locus was negative and significantly (P < 0.01) different from neutral expectation in O. sativa (Supplementary Table S5 online). These results indicate that the OsSh1 was subjected to strong directional selection during rice domestication.

Variation annotation analysis revealed that a single nucleotide variant, c to t, at position + 70 relative to the translation start site (c70t), caused a single amino acid substitution from a Leucine residue (L) to Phenylalanine (F) at position 24 (L24F) (Fig. 7b). We named these two alleles OsSh1-c70 and OsSh1-t70, respectively. No other variant causing amino acid change was identified. The OsSh1-c70 was the major allele in O. rufipogon (frequency = 80.8%), in contrast, OsSh1-t70 was the major allele in indica rice (frequency = 91.7%), and no OsSh1-c70 was found in japonica cultivars (Fig. 7b). To confirm this result, we checked the allele frequencies of OsSh1 in 436 accessions of O. rufipogon (Supplementary Table S6 online), and found the allele frequencies of OsSh1-c70 in Or-I, Or-II and Or-III to be 71.0 to 88.0%, or 81.7% overall. OsSh1-c70 was found to be distributed extensively in the original producing area, while OsSh1-t70 was not found in Western India, Western New Guinea, Papua New Guinea or Australia (Supplementary Fig. S4 online). Subsequently, we analyzed 1,774 indica cultivars and 844 japonica cultivars (Supplementary Table S7 online). The frequencies of OsSh1-c70 in indica and japonica cultivars was 99.1% and 97.8%, respectively. Furthermore, OsSh1-c70 was only found in the tropical subpopulation with a frequency at 5.0%, and was not found in subtropical and temperate subpopulations. The reason that no OsSh1-c70 was found in the japonica cultivars collected in TASUKE + may be due to the limited number of tropical japonica varieties.

Amino acid alignment of YABBY2 proteins in different monocot and dicot plants indicated that the Zinc finger domain and YABBY domain were highly conserved (Supplementary Fig. S5 online). Leucine residue at position 24 (L24) was almost completely conserved in YABBY2 proteins of all seed plants (Supplementary Fig. S4 online), and the other YABBY genes as well31. These results suggested that amino acid substitution L24F in OsSh1 protein might partially affect its function in controlling seed shattering.
Figure 5. RT-qPCR analysis of OsSh1 and qSH1 in young panicles of 'Moretsu' and 'Minamiyutaka.' (a) The regions of OsSh1 were targeted using RT-qPCR with three primer sets, P1, P2, and P3, indicated by the black double-sided arrows. Note that the elements are not drawn to scale. Relative expression levels of OsSh1 and qSH1 are shown in (b)–(e). Data were normalized to 'Moretsu.' Bars indicate mean values ± standard deviation (n = 4). Double asterisks denote a significant difference at P < 0.01 using Student's t test.

Figure 6. RT-qPCR analysis of qSH1 (a) and OsSh1 (b) in young panicles of 'Takanari' and 'Takanari_qsh1'. Data were normalized to 'Takanari.' Bars indicate mean values ± standard deviation (n = 4). Double asterisks denote a significant difference at P < 0.01 using Student's t test.
Discussion

Non-shattering phenotype in ‘Minamiyutaka’ is defect in abscission zone formation. In the present study, we found remarkable improvement of resistance to shattering in ‘Minamiyutaka’ compared to its original cultivar ‘Moretsu’ (Fig. 1). This strong shattering resistance in ‘Minamiyutaka’ may be due to the defective abscission zone formation, as in the reported non-shattering mutants such as shat1 [22], shat2 [22], and ssh1 [20]. These results confirm that abscission zone formation is of key importance for controlling seed shattering.

Whole-genome resequencing may directly identify the causative mutation. Genetics analysis suggested that a single recessive locus was responsible for the non-shattering phenotype in ‘Minamiyutaka’ (Fig. 2), which may facilitate cloning of the candidate gene. Through whole-genome sequencing of ‘Moretsu’ and ‘Minamiyutaka’, we newly identified a 13-bp deletion in the OsSh1 gene of ‘Minamiyutaka’ (Fig. 2). The 13-bp deletion in OsSh1 caused exon 3 skipping and introduced a putative PTC in the coding regions of mRNA, which can lead to generation of a non-functional OsSh1 protein (Fig. 2). The PTC-containing mRNAs are known to be degraded via nonsense-mediated mRNA decay (NMD) [36,37]. We consistently observed a notable reduction in OsSh1 expression in ‘Minamiyutaka’ (Fig. 5), suggesting mRNAs with a PTC may be degraded. Furthermore, this 13-bp deletion was revealed to be completely associated with the non-shattering phenotype in an F2 population (Fig. 2). These results strongly indicate that OsSh1 was a candidate gene. These results also suggest that whole-genome sequencing of only the mutant generated by gamma-ray irradiation and its original wild type may directly identify the candidate causal gene. This strategy may be more cost-effective and time-saving than the bulked segregant analysis [21].

Loss-of-function mutation in OsSh1 can result in non-shattering phenotype. The YABBY gene family, specific to seed plants, is expressed in the abaxial region of leaf primordia and floral organs, and promotes abaxial cell fate and lateral organ development in Arabidopsis [38,39]. YABBY genes in poaceae such as rice, maize and wheat are not expressed in a polar manner [40–42], suggesting that the roles of YABBY genes have diversified.

Figure 7. Nucleotide diversity analysis of OsSh1 in wild and cultivated rice. The consensus sequences from 40 accessions of O. rufipogon, 96 indica varieties, 146 temperate japonica varieties, and 46 tropical japonica varieties were downloaded from TASUKE+. (a) Sliding-window analysis of nucleotide polymorphism (π) of OsSh1. The values were calculated for each sliding window of 100 bp with an increment of 10 bp. (b) shows a missense variant (c to t) at position +70 nearly fixed in rice varieties. Blue boxes represent exons, grey boxes represent UTRs, and thin black lines indicate flanking regions or introns. The transcription start site of exon 1 is taken as +1 position. Note that the elements are not drawn to scale. Pie graphs show the allele frequencies of OsSh1-c70 and OsSh1-t70 in O. rufipogon, indica varieties, and japonica varieties.
during the evolution of plants. There are 8 YABBY genes in rice \cite{31,41}, and they also show organ-specific expression patterns \cite{31,41}. In the present study, OsSh1/OsYABBY2 was revealed to be expressed in all organs except for the roots and anthers, which is similar to a previous study \cite{31,41}. In sorghum, Sh1, was identified as the gene controlling seed shattering using the mapping populations derived from the crosses between complete-shattering wild sorghum and non-shattering domesticated sorghum \cite{25}. Interestingly, Lin et al. \cite{25} found that syntenic blocks containing Sh1 correspond to the seed-shattering related QTLs in rice \cite{41,42,43} and YABBY3 (YAB3) play important regulatory roles in forming stripes of valve margin tissue that allow the fruit to shatter at maturity stage \cite{47}. Furthermore, the expression pattern of FIL is regulated by RPL (the ortholog of qSh1 in rice) \cite{47}. Our present study has revealed that the expression of qSh1 is not affected by loss-of-function of OsSh1 (Fig. 5), whereas the expression of OsSh1 decreases significantly when qSh1 is dysfunctional (Fig. 6). These results suggest that OsSh1 may function downstream of qSh1, similar to the pathway in Arabidopsis \cite{47}.

**OsSh1 functions downstream of qSH1.** Thus far, the pathway controlling the development of abscission zone located between the sterile lemma and the pedicel is still unclear. It has been revealed that qSH1 activity depends on SHAT1, SH4 and SHH1, indicating that qSH1 functions downstream of these genes \cite{30,22}. In Arabidopsis, the YABBY genes such as FILAMENTOUS FLOWER (FIL) and YABBY3 (YAB3) play important regulatory roles in forming stripes of valve margin tissue that allow the fruit to shatter at maturity stage \cite{47}. Furthermore, the expression pattern of FIL is regulated by RPL (the ortholog of qSh1 in rice) \cite{47}. Our present study has revealed that the expression of qSh1 is not affected by loss-of-function of OsSh1 (Fig. 5), whereas the expression of OsSh1 decreases significantly when qSh1 is dysfunctional (Fig. 6). These results suggest that OsSh1 may function downstream of qSh1, similar to the pathway in Arabidopsis \cite{47}.

**Artificial selection for OsSh1 in rice domestication.** Resequencing 50 accessions of cultivated rice and wild rice (O. rufipogon and O. nivara) suggested that OsSh1 is one of the putative artificially selected genes \cite{48}. We performed nucleotide diversity analysis and Tajima’s D test in many more wild rice accessions (O. rufipogon) and cultivars, and further confirmed very strong selection of OsSh1 during rice domestication (Fig. 7, Supplementary Table S5 online). Furthermore, only one missense variation c70t was identified in all accessions. Since the amino acid residue L24 harbored in the OsSh1-c70 allele was broadly conserved in seed plant species (Supplementary Fig. S5 online) as well as in the YABBY gene family \cite{31}, OsSh1-c70 might be an ancestral allele. The OsSh1-c70 is the major allele in all ecotypes of O. rufipogon, and is randomly distributed geographically, whereas OsSh1-c70 is mainly distributed in Southern China, Southeast Asia and Eastern South Asia (Supplementary Table S6 online, Supplementary Fig. S4 online), indicating that the c70t mutation might have occurred spontaneously in one of these regions and then gradually spread out from there. Interestingly, OsSh1-c70 has become a rare allele in indica and tropical japonica cultivars which are cultivated in geographically similar regions where O. rufipogon is distributed, but has disappeared in subtropical and temperate japonica cultivars (Supplementary Table S7 online). Based on these results, we speculate possible evolutionary scenarios for OsSh1 whereby a common ancient rice with the OsSh1-c70 allele was first domesticated before the indica-japonica differentiation, while a few indica and tropical japonica rice accessions were crossed to local wild rice with the OsSh1-c70 allele after differentiation. To clarify this, it might be necessary to further investigate the detailed genomic variations and DNA polymorphisms among cultivated rice containing the OsSh1-c70 allele and wild rice.

**Materials and methods**

**Materials and growth condition.** ‘Minamiyutaka’ and ‘Moretsu’ were crossed to generate an F1 population. A CSSL named ‘SL1303’, carrying a genomic region containing a dysfunctional allele of qSh1 and a functional allele of Semi dwarf1 (SD1) from a japonica rice cultivar ‘Koshihikari’ \cite{34} within the background of an indica rice cultivar ‘Takanari’, was kindly provided by Dr. Toshiro Yamamoto (NARO) \cite{34}. ‘Takanari’ is short-culm and easy-shattering, in contrast to which ‘Koshihikari’ and ‘SL1303’ show non-shattering and a long-culm phenotype. We backcrossed the ‘SL1303’ to ‘Takanari’ to develop a new non-shattering CSSL named ‘Takanari-qsh1’, whose morphological characteristics including the height of the culm is similar to that of ‘Takanari’.

**Evaluation of shattering degree.** To evaluate the seed shattering of ‘Moretsu’, ‘Minamiyutaka’, gene-edited plants (including wild types), ‘Takanari’, and ‘Takanari-qsh1’, panicles from the primary tillers were harvested at maturity stage and naturally air dried for more than two weeks in a room. PS and BS were measured according to Li et al. \cite{21}. A total of 100 to 120 grains from four panicles were measured. The seed shattering of the F1 plants derived from the cross between ‘Moretsu’ and ‘Minamiyutaka’ were evaluated using a method based on grasping panicles by hand at the maturity stage. In brief, three panicles from a plant were grasped by hand at one time, then the number of shattered grains then being counted. If the number of shattered grains was less than or equal to 3, this plant was regarded as non-shattering. If the number of shattered grains was more than or equal to 20, this plant was regarded as easy-shattering.

**Histological analysis.** The pedicles were collected at the anthesis stage, followed by fixation in FAA (Formalin-Acetic-Alcohol) solution. After dehydration and embedding, the tissues were longitudinally sectioned into 2-μm-thick sections according to the methods described by Li et al. \cite{31}. The sections were stained with 0.01% toluidine and were observed using an BX53 microscope (OLYMPUS, Tokyo, Japan).
Scanning electron microscopy. The bases of mature seeds were subjected to platinum sputter coating and observed using a SEM (JEOL JSM-5610 LV, Tokyo, Japan). High resolution images were obtained in high vacuum mode at 20 kV.

DNA extraction. The leaves were harvested from 30-day-old seedlings. For next generation sequencing, DNAs of 'Moretsu' and 'Minamiyutaka' were extracted using DNeasy Plant Maxi Kit (Qiagen Inc., Valencia, USA). For DNA marker analysis, DNAs of F1 plants and gene-edited plants were extracted using the simple DNA extraction method49.

Whole-genome resequencing. DNA samples of 'Moretsu' and 'Minamiyutaka' were subjected to paired-end (2 x 150 bp) sequencing using an Illumina HiSeq X Ten platform. The short reads were cleaned by removing low quality reads and unpaired reads using Trimmomatic (version 0.36)50 with the following parameters: LEADING:10, TRAILING:10, SLIDINGWINDOW:4:20, and MINLEN:36. Next, the clean reads were aligned to the Nipponbare reference sequence (IRGSP-1.0, http://rapdb.dna.affrc.go.jp) using the mapping tool Bow-

LEADING:10, TRAILING:10, SLIDINGWINDOW:4:20, and MINLEN:36. Next, the clean reads were aligned to the Nipponbare reference sequence (IRGSP-1.0, http://rapdb.dna.affrc.go.jp)51 using the mapping tool Bor-
rows Wheeler Aligner (version 0.7.17)52 and indexed as BAM files using SAMtools (version 1.3.1)53. Duplicate fragments were then marked and eliminated with MarkDuplicates tool in Picard-Tools (Version 2.7.1.0) (https://broadinstitute.github.io/picard/). SNP, insertion, and deletion calling was performed using the Haplotype-Callertool in GATK (Version 3.7-0)54. Detection of SV was performed using Pindel55 and Manta56 with default parameters.

Homozygous variations between 'Moretsu' and 'Minamiyutaka' were called with settings described below: (1) the read depth of the variant site was more than five and less than 100, (2) allele frequencies of both cultivars at a site were higher than 0.8, (3) genotypes of the two cultivars were different. To ensure the accuracy of variation detection, the candidate variations were visually confirmed using the Integrative Genomics Viewer57.

Variation annotation analysis was conducted based on the gene annotation of the 'Nipponbare' using SnpEff v4.258.

Validation of the candidate causal mutation. The causal mutation inferred by variation annotation analysis was first verified by Sanger sequencing. The methods described below have been reproduced in part from Li et al.21. Briefly, primers (Supplementary Table S8 online) were designed using the Primer3 program (https://bioinfo.ut.ee/primer3-0.4.0/). Purified PCR product was used for the sequencing reaction using the Big-

Dye Terminator V3.1 cycle sequencing kit (Thermo Fisher Scientific, MA, USA), followed by sequencing on a 3730xl Genetic Analyzer (Thermo Fisher Scientific).

For analysis of co-segregation of the causal mutation with non-shattering habit in the F2 population, a CAPS marker (Supplementary Table S8 online) was designed based on the restriction enzyme map analysis (https://www.restrictionmapper.org). PCR amplicons were digested by the restriction enzyme BspCNI (New England Biolabs, MA, USA) at 25 °C for 60 min, and then analyzed by electrophoresis on 2.0% PrimeGel Agarose PCR-Sieve HRS (Takara) gels for 40 min at 100 V.

Gene expression analyses. To determine the expression profile of OsSh1 in rice plants, we analyzed the microarray data from the rice gene expression database RiceXPro (https://ricexpro.dna.affrc.go.jp)59. RT-qPCR analysis was conducted to compare the gene expression levels between different cultivars or lines. Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), from the 70–90-mm long young panicles before heading, when the abscission stage is developing59. Each cultivar had four replications. The first-strand cDNA was synthesized from 1.0 μg of total RNA using a SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen by Life Technologies, Carlsbad, CA, USA). Ubiquitin gene was used as an endogenous control to normalize detected gene expression. RT-qPCR was performed using an SYBR Green SuperMix kit (Bio-Rad, Hercules, CA, USA) on a QuantStudio 1 System (Thermo Fisher Scientific). The PCR reaction mixture (15 μl) consisted of 0.2 μl forward and reverse primers, 1 x SYBR Advantage qPCR Premix and about 10 ng cDNA. The reactions were carried out using the following qPCR protocol: 2 min pre-incubation at 95 °C followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min, and finally a dissociation run from 60 to 95 °C. Primers used for RT-PCR experiments are listed in Supplementary Table S8 online.

cDNA sequence analysis. A cDNA clone containing the full open reading frame (ORF) of OsSh1 was generated by PCR using the primers listed in Supplementary Table S8 online. The PCR products were cloned into pCR-BluntII-TOPO (Invitrogen) and subjected to Sanger sequence analysis as mentioned above. The mRNA sequences of the OsSh1 gene in 'Moretsu' and 'Minamiyutaka' have been deposited in the DNA Data Bank of Japan (DDBJ; https://www.ddbj.nig.ac.jp) with accession numbers LC522940 and LC522941, respectively. The deduced amino acid sequences were aligned using Clustal Omega with default parameters (https://www.ebi.ac.uk/Tools/msa/clustalo/). To align amino acid sequences of YABBY2 proteins in different plants, we downloaded sequences from the GenBank (https://www.ncbi.nlm.nih.gov) as follows: O. sativa L. ssp. japonica (AAAX95527.1), O. sativa L. ssp. japonica (AXM44150.1), O. brachyantha (XP_006650352.1), Aegilops tauschii ssp. tauschii (XP_020155668.1), Triticum aestivum (ABW80974.1), Brachypodium distachyon (XP_003561769.1), Arabidopsis thaliana (NP_001077490.1), Camelina sativa (XP_010458084.1), Gossypium hirsutum (XP_01663147.1), Brassica oleracea (XP_013638657.1). In addition, YABBY2 protein sequences in O. sativa L. ssp. indica (AAX95527.1), O. rufipogon (A0A0E0NYX3), O. nivara (A0A0E0GR31), O. punctat (A0A0E0KGW2), O. glumipata (A0A0D9ZB45) were from UniProt (https://www.uniprot.org).
Vector construction and transformation. The sgRNA-Cas9 plant expression vector pZH_OsCas9 and the guide RNA expression vector pZK_sgrNA were kindly provided by Dr. Masaki Endo and Dr. Seiichi Toki (NARO). The oligos used in constructing the sgRNA vectors for OsSh1 are shown in Supplementary Fig. S6a online. The easy-shattering indica rice cultivar ’Teqing’ was subjected to Agrobacterium-mediated transformation using immature embryo-derived calli as described previously. In brief, immature embryos of ’Teqing’ from 10 to 14 days after flowering were infected by Agrobacterium carrying the pZM_OsU6gRNA_MMCas9 vector (Supplementary Fig. S6b online). After 5 days of co-cultivation, infected immature embryos were transferred to a fresh resting medium containing 400 mg/L carbenicillin disodium salt (Nakarai, Kyoto, Japan) to remove Agrobacterium. Following this, Hygromycin-resistant calli were selected over 4 weeks on a selection medium containing 400 mg/L carbenicillin disodium salt and hygromycin 30 mg/L (Wako Pure Chemicals, Osaka, Japan). Proliferating calli were then transferred to a fresh pre-regeneration medium containing 200 mg/L carbenicillin disodium salt and hygromycin 40 mg/L. After 8 days of culture, these calli were transferred to a fresh regeneration medium containing 30 mg/L hygromycin B and cultured for 2 weeks. The re-generated rice plants were grown in a closed greenhouse.

Analysis of CRISPR-induced mutations. To analyze the mutation induced in the regenerated plants, the first or second exon of OsSh1 were PCR amplified using the specified primers (Supplementary Table S8 online). PCR products were subjected to an Nsp I or MscI restriction enzyme reaction, followed by agarose gel electrophoresis. PCR products showing mutation by CAPS analysis were cloned into pCR-BluntII-TOPO (Invitrogen) and subjected to Sanger sequence analysis as mentioned above.

Statistical analysis. Statistical analyses were performed in R Software version 3.6.0. Violin plots were created in the R-package ggplot2. To determine the statistical significance, firstly, an F Test (two samples) or Bartlett's test (> =3 samples) was used to test whether variances were equal for all samples. Then, if the variances were equal, Student's t test (two samples) or pairwise comparisons using t tests with pooled SD (Standard Deviation) (> =3 samples) were performed, otherwise, Welch's t test (two samples) or Wilcoxon rank sum test (> =3 samples) was performed. Finally, a Bonferroni correction was used to control for the family-wise type I error rate across the comparisons.

DNA polymorphism analysis of candidate gene. We exported the consensus sequences covering the coding region and the flanking regions of OsSh1 in wild and cultivated rice from TASUKE +. The variant filter was set as follows: quality > =20 and depth > =4. The average number of reads that align the whole genome of an accession was set at more than eight. Nucleotide diversity analysis and test for neutral selection were performed using DnaSP (version 6.12.03) 65. The number of polymorphic (segregating) sites: π, the average number of pairwise nucleotide differences per site66; θ, Watterson's estimator of nucleotide polymorphism per site67, and Tajima's D test68 were calculated. The alleles of OsSh1 were also checked in the accessions of O. rufipogon in the database OryzaGenome (https://viewer.shigen.info/oryzagenome2detail/about/about.xhtml), and O. sativa in Rice SNP-Seek Database (https://snp-seek.irri.org/index.zul).

Data availability All NGS data files will be available in the DDBJ Sequenced Read Archive under the Accession Nos. DRA009647 and DRA009648, upon acceptance of this paper.

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**Author contributions**

H.K., A.S. and F.L. conceived and coordinated the research plan. F.L. analyzed the next-generation sequencing data and conducted gene expression analysis. A.K. and M.O. conducted CRISPR/Cas9 gene editing. H.E. observed the fracture surface of the grain base using scanning electron microscopy. F.L. and A.K. wrote the manuscript. All authors reviewed and approved the final manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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