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

2 'Nipponbare'

3

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24 **Key message**

25 A novel locus, *qCSS3*, involved in the non-seed-shattering behaviour of Japonica rice cultivar,

26 'Nipponbare', was detected by QTL-seq analysis using the segregating population with the fixed known

27 seed-shattering loci.

28

29 **Abstract**

30 Asian cultivated rice, *Oryza sativa*, was domesticated from its wild ancestor, *O. rufipogon*. Loss of seed

31 shattering is one of the most recognisable traits selected during rice domestication. Three quantitative trait

32 loci (QTLs), *qSH1*, *qSH3*, and *sh4*, were previously reported to be involved in the loss of seed shattering of

33 Japonica cultivated rice, *O. sativa* 'Nipponbare'. However, the introgression line (IL) carrying 'Nipponbare'

34 alleles at these three loci in the genetic background of wild rice, *O. rufipogon* W630, showed a lower value

35 for detaching a grain from the pedicel than 'Nipponbare', implying that additional loci might still be

36 involved in the complete loss of seed shattering in 'Nipponbare'. Here, we investigated abscission layer

37 formation in the IL and found a partially formed abscission layer in the central region between the
38 epidermis and vascular bundles. Based on QTL-seq analysis using the $F_2$ population obtained from a cross
39 between 'Nipponbare' and the IL, we detected two novel loci *qCSS3* and *qCSS9* (*QTL for the Control of*
40 *Seed Shattering in rice on chromosomes 3* and *9*), which were found to be involved in the difference in
41 seed-shattering degree between 'Nipponbare' and W630. Then, we further focused on *qCSS3* in order to
42 understand its potential role on the loss of seed shattering.. The candidate region of *qCSS3* was found to be
43 located within a 526kb region using substitution mapping analysis. Interestingly, the *qCSS3* candidate
44 region partially overlaps with the selective sweep detected for Japonica but not for Indica rice cultivars,
45 suggesting that this region harbours the mutation at a novel seed-shattering locus specifically selected for
46 non-seed-shattering behaviour in Japonica cultivars.

47

48 **Introduction**

49 Historically, the process of crop domestication involved the selection of several naturally occurring
50 variations in wild plants that provided useful agronomic traits. These traits were related to seed size, plant
51 architecture, seed shattering, seed dormancy, and photoperiod sensitivity (Doebley et al. 2006). Seed
52 shattering is one of the most important characteristics for the propagation of wild plants (Dong and Wang
53 2015), and is caused by the degradation of the abscission layer formed between the grain and pedicel.
54 Because seed shattering affects yield, hunter-gatherers and early farmers must have selected plants with

55 non-seed-shattering behaviour to increase their yield (Fuller and Allaby 2009). Thus, loss of seed shattering

56 is regarded as one of the most important domestication traits (Harlan 1975; Fuller 2007).

57 *Oryza sativa* L., Asian cultivated rice, was domesticated from its wild ancestor, *O. rufipogon*

58 Griff. (Oka 1998; Fuller 2007). During the process of domestication, the seed-shattering ability of rice

59 plants weakened or was lost as a result of the inhibition of abscission layer formation. Asian cultivated rice

60 is generally classified into two groups; Indica and Japonica. The former normally exhibits a weak

61 shattering habit with partial abscission layer formation, whereas the latter has a non-shattering habit with

62 complete inhibition of abscission layer formation. Previous studies have shown that three quantitative trait

63 loci (QTLs), *sh4*, *qSH1*, and *qSH3*, are involved in the loss of seed shattering (Li et al. 2006; Konishi et al.

64 2006; Htun et al. 2014). The major QTL is *sh4*, which was identified from a cross between *O. nivara* and *O.*

65 *sativa* Indica explaining 69% of the phenotypic variation (Li et al. 2006). The cultivated allele of *sh4* is

66 recessive and inhibits the formation of the abscission layer, resulting in the reduction of the degree of seed

67 shattering (Li et al. 2006; Lin et al. 2007). *qSH1* was identified from a cross between *O. sativa* Indica

68 'Kasalath' and *O. sativa* Japonica 'Nipponbare', which explained 68.6% of the phenotypic variation

69 (Konishi et al. 2006). A single nucleotide polymorphism (SNP) in the regulatory region of a downstream

70 gene was shown to result in the absence of an abscission layer in 'Nipponbare' (Konishi et al. 2006). These

71 two QTLs were found to be major loci involved in non-seed-shattering behaviour of most Japonica rice

72 cultivars. However, we previously evaluated seed-shattering degree of introgression lines of wild rice

73 carrying 'Nipponbare' alleles at *sh4* and *qSH1* (Ishikawa et al. 2010). Results show that a single
74 introgression of 'Nipponbare' alleles at *sh4* or *qSH1* display complete shattering behaviour as wild rice,
75 and even a double introgression at the two loci display weak seed shattering inhibition (Ishikawa et al.
76 2010). These findings suggest that additional loci may still be involved in a loss of seed shattering in
77 Japonica rice cultivars. The third QTL is *qSH3*, which was originally detected from a cross between *O.*
78 *rufipogon* and *O. sativa* Japonica (Onishi et al. 2007). We also detected *qSH3* by QTL analysis in a
79 previous study, using an $F_2$ population generated by a cross between 'Nipponbare' and an introgression line
80 (IL) carrying the 'Nipponbare' alleles at both *qSH1* and *sh4* in the genetic background of *O. rufipogon*
81 W630 (Htun et al. 2014). Gene interaction of *qSH3* and *sh4* was also investigated in the genetic background
82 of wild rice to understand the seed-shattering behaviour in early rice domestication (Inoue et al. 2015;
83 Ishikawa et al. 2017), because the selection of *qSH1* is specific to Japonica rice cultivars. We also
84 evaluated the seed-shattering degree of an $F_2$ segregating population between Indica rice cultivar *O. sativa*
85 'IR36' and an introgression line carrying 'Nipponbare' allele at *qSH3* and *sh4* in the genetic background of
86 wild rice *O. rufipogon* W630 (Tsujimura et al. 2017). We observed the segregation of seed-shattering
87 behaviour in the $F_2$ population, confirming that unknown mutation(s) other than *qSH3* and *sh4* may
88 underlie the non-shattering behaviour of 'IR36'. These studies suggest that several unidentified loci may
89 still be involved in the non-shattering of seeds during rice domestication or breeding.

90 Here, we first investigated the abscission layer formation in an IL having the cultivated alleles of

91 ‘Nipponbare’ at *qSH1*, *qSH3*, and *sh4* in the genetic background of wild rice, *O. rufipogon* W630. The IL

92 was crossed with ‘Nipponbare’ and the seed-shattering degrees of their $F_2$ plants were measured to detect

93 QTL(s) other than the three known seed-shattering loci. We performed QTL-seq analysis, and two novel

94 loci on chromosomes 3 and 9 were detected. We further analysed the effect of the putative locus on

95 chromosome 3, and the effect of the wild allele on seed-shattering degree was confirmed with two

96 backcross recombinant inbred lines (BRILs) in the genetic background of ‘Nipponbare’ and by a progeny

97 test of the $F_2$ plants. The candidate region of the locus on chromosome 3 was restricted to a 526 kb region

98 by substitution mapping analysis.

99

100 **Material and methods**

101 **Plant materials**

102 A Japonica rice cultivar, *O. sativa* ‘Nipponbare’, and a wild accession of *O. rufipogon* W630 originated

103 from Myanmar were used in this study. By backcrossing with W630, an introgression line (IL) carrying the

104 ‘Nipponbare’ alleles at the *sh4*, *qSH1*, and *qSH3* loci in the genetic background of wild rice was produced.

105 The IL was crossed with ‘Nipponbare’ and the resulting $F_1$ plant was self-pollinated to obtain the

106 segregating $F_2$ population. A total of 174 individuals were grown in pots at Kobe University, Japan, and

107 their seed-shattering behaviour was evaluated. To minimise the effect of the difference in heading time,

108 short-day treatment was used for all the plants. The validation of the locus detected in this study was

109 conducted using backcross recombinant inbred lines as previously reported by Thanh et al. (2011). A
110 progeny test was also conducted using three lines having a heterozygous chromosomal constitution at the
111 locus detected in this study. Further substitution mapping analysis was conducted by the progeny tests
112 using one of the three lines.
113
114 **Evaluation of seed-shattering degree**
115 The seed-shattering degree was evaluated by measuring the breaking tensile strength (BTS, gf:
116 gramme-force), which is the value required to detach a grain from the pedicel, measured with a digital force
117 gauge (FGP 0.5, Nidec-Shimpo Co., Japan). The BTS values of 75 seeds (25 randomly selected seeds from
118 three panicles) were measured, approximately a month after heading, and their average BTS values were
119 calculated.
120
121 **DNA extraction, bulking, and library construction for next-generation sequencing analysis**
122 For QTL-seq analysis, bulked DNA samples were prepared as described in previous studies (Abe et al.
123 2012; Takagi et al. 2013). DNA was extracted from 100 mg of fresh rice leaves using DNeasy Plant Mini
124 Kit (QIAGEN Sciences, Germany) and was quantified using Qubit® 3.0 Fluorometer and Qubit®
125 dsDNA BR Assay Kit (Life Technologies, Japan). The extracted DNA samples were mixed in an equal
126 ratio and were regarded as the bulked DNA. To survey the genotypes at the detected loci, simple sequence

127 repeat (SSR) or InDel markers were used (Supplemental Table 1).

128

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

130 All analyses were carried out using the previously developed QTL-seq Pipeline (Department of Genomics
131 and Breeding, Iwate Biotechnology Research Center; http://genome-e.ibrc.or.jp). The short reads obtained
132 from the IL were aligned to the 'Nipponbare' reference genome sequence obtained from the Rice
133 Annotation Database Project (http://rapdb.dna.affrc.go.jp). Thereafter, the genome sequence of the IL was
134 developed as a 'reference sequence'. The short reads obtained from high (H-) and low (L-) bulks were
135 aligned to the reference sequence using the Burrows-Wheeler Aligner (BWA) software (Li and Durbin
136 2009). The aligned sequence files were converted to SAM/BAM files using SAM tools (Li et al. 2009) and
137 were applied to Coval (Kosugi et al. 2013) to increase the SNP-calling accuracy. SNP-index was calculated
138 for all the SNP positions. Then $\Delta$(SNP-index) was calculated by subtracting the SNP-index values of
139 L-bulk from H-bulk. A sliding window analysis was applied by averaging the $\Delta$(SNP-index) values within
140 a 4 Mb window size and a 50 kb increment.

141 **Morphological and histological analysis of abscission layer formation**

142 The abscission layer (axial images of detached spikelet) was examined using a LEICA S6D microscope and
143 photographs were taken with the MC170HD and Leica Application Suite (Leica, Germany). The samples
144 for histological analysis were collected from the pedicel tissue of grains before heading, following Htun et

145 al. (2014) and Inoue et al. (2015). The samples were fixed in FAA solution (formaldehyde: acetic acid:
146 70% ethanol = 1:1 :18 (volume ratio)) with vacuum infiltration and were preserved at 4°C. They were
147 dehydrated in an ethanol series (70%, 80%, and 90% ethanol) for 2 days at each stage and then embedded
148 in Technovit 7100 resin (Heraeus Kulzer, Germany), according to the manufacturer's instructions. The
149 samples were cut into 3-μm sections with a rotary microtome, RM1215RT (Leica Biosystems, Germany),
150 and stained with toluidine blue O solution. These sections were observed under a microscope and
151 photographed with a digital camera using the imaging software, ToupView (×86) (Amscope.com, US).
152
153 **Results**
154 **Seed-shattering behaviour of the introgression line (*qSH1*, *qSH3*, and *sh4*)**
155 We first surveyed backcross plants carrying *O. sativa* 'Nipponbare' alleles at the *qSH1*, *qSH3*, and *sh4* loci
156 in the genetic background of wild rice, *O. rufipogon* W630. From these, the IL with the least other
157 'Nipponbare' chromosomal segments was selected. The graphical genotype of the IL with the 'Nipponbare'
158 chromosomal segments covering the three seed-shattering loci and on chrs. 5, 7, 10, and 11 is shown in Fig.
159 1a. The appearance of the seed of the IL was similar to that of wild rice, *O. rufipogon* W630 (Fig. 1b). The
160 IL showed an inhibition of seed-shattering behaviour, but it had a lower BTS value than 'Nipponbare' (Fig
161 1c). We investigated the abscission layer formation in the IL after the detachment of seeds. In the basal part
162 of the grain, no abscission layer was produced in 'Nipponbare' and the pedicel tissues were broken when a

163 seed was strongly detached from the pedicel (Fig. 1d). In contrast, W630 formed a complete abscission

164 layer from the epidermis to the region surrounding the vascular bundle (Fig. 1d). A partial abscission layer

165 formation was observed in the inner region of the IL corresponding to the area where the complete

166 abscission layer is formed in W630. The abscission layer formation in the IL was different from those of

167 the parents. Furthermore, we also compared abscission layer formation using longitudinal sections. The

168 abscission layer was inhibited outside the region of the pedicel and around the vascular bundle and partially

169 formed only in the central region of the IL whereas a complete abscission layer is formed in W630 (Fig. 1e).

170 Because the IL had significantly lower BTS values than 'Nipponbare', the partially formed abscission layer

171 probably contributes to lowering the BTS value. These results indicated that mutations at *qSH1*, *qSH3*, and

172 *sh4* are insufficient to explain the non-seed-shattering phenotype of 'Nipponbare', and other unknown loci

173 are probably involved.

174

175 **Seed-shattering degree in an $F_2$ population obtained by a cross between the IL and 'Nipponbare'**

176 We obtained a total of 174 $F_2$ plants from a cross between the IL and 'Nipponbare' and their BTS values

177 were measured. In this segregating population, the three seed-shattering loci were all fixed with the

178 'Nipponbare' alleles. Differences in the BTS were approximately 70 to 210 gf (Fig. 2, Supplemental Table

179 2). The transgressive segregation of the BTS values in the population indicates that several genes might be

180 involved in the difference in seed-shattering degree between the parent lines.

181

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

183 To detect the loci involved in the difference in seed-shattering degree between the IL and 'Nipponbare', we
184 employed QTL-seq analysis (Takagi et al. 2013). The analysis requires bulked DNA of progeny showing
185 extreme phenotypes (i.e., those exhibiting high and low BTS values in the population). We selected 12 and
186 14 $F_2$ plants with low (70–100 gf) and high (150–210 gf) BTS values, respectively (Fig. 2 and
187 Supplemental Table 2). The DNA of each bulk as well as that of the IL was subjected to whole-genome
188 resequencing analysis. A total of 104.3, 107.1, and 48.2 million sequence reads (each 100 bp) were
189 obtained from the DNA of H-bulk, L-bulk, and IL, respectively. By examining the Δ(SNP-index) plot, we
190 identified the two genomic regions exhibiting the Δ(SNP-index) values exceeding the 95% confidence
191 interval by a sliding window analysis (statistical significance under the null hypothesis: $P < 0.05$): the
192 regions on chr. 3 from 8.30 to 13.65 Mb with maximum Δ(SNP-index) = 0.49 (statistical significance under
193 the null hypothesis: $P < 0.05$) and on chr. 9 from 21.1 Mb to the distal end with maximum Δ(SNP-index) =
194 -0.46 (Table 1, Fig. 3, Supplemental Fig. 2). We named them as *QTL for the Control of Seed Shattering in*
195 *rice on chrs. 3* and *9* (*qCSS3* and *qCSS9*). No other region over the confidence interval was detected in this
196 analysis (Supplemental Fig. 1). According to the Δ(SNP-index), 'Nipponbare' allele at *qCSS3* and W630
197 allele at *qCSS9* were found to inhibit seed shattering. To confirm the results of the QTL-seq analysis, each
198 $F_2$ plant was genotyped using the seven and four DNA markers covering the entire significant regions on

199 chrs. 3 and 9, respectively (Supplemental Table 1). For *qCSS3* region, 31 (18%) and 20 (11%) plants were
200 found to carry the 'Nipponbare' and W630 homozygous chromosomal segments, respectively. Moreover,
201 33 (19%) plants had heterozygous chromosomal segments and the rest (52%) showed recombination
202 between any of the seven SSR markers (Supplemental Table 3). We compared the BTS values of two
203 homozygous groups based on the chromosomal constitutions (Supplemental Fig. 2), and found that the
204 'Nipponbare' and W630 chromosomal segments at the region were responsible for increasing and
205 decreasing the BTS values, respectively. Similarly, the result of QTL-seq analysis at *qCSS9* region was also
206 confirmed (Supplemental Table 3, Supplemental Fig. 2). These results confirm that both *qCSS3* and *qCSS9*
207 detected in QTL-seq analysis are involved in the control of seed shattering in rice.
208
209 **Validation of the effect of *qCSS3* on the seed-shattering degree**
210 As we found that the 'Nipponbare' allele at *qCSS3* contributed to inhibition of seed shattering and possibly
211 involved in non-shattering behaviour of *O. sativa* 'Nipponbare', we further studied *qCSS3*. To validate the
212 effect of *qCSS3* on the seed-shattering degree, we first screened the individual lines of the previously
213 produced backcross recombinant inbred lines (BRILs) (Thanh et al. 2011). These BRILs were generated by
214 crossing W630, as the donor parent, and 'Nipponbare', as the recurrent parent. Using two SSR markers,
215 flanking the *qCSS3* region (RM1002 and RM6080; Supplemental Table 1), we screened 159 BRILs. We
216 found that lines 42 and 86 had homozygous chromosomal segments of W630, covering the candidate

217 region of *qCSS3* (Supplemental Fig. 3a). Both lines showed significantly lower BTS values than
218 'Nipponbare', although the differences were very small (Supplemental Fig. 3b). Next, we carried out the
219 progeny test of $F_2$ lines obtained by crossing the IL and 'Nipponbare'. One of the $F_2$ plants (no. 57), with
220 heterozygous chromosomal constitution between RM1002 and RM6080 covering the whole candidate
221 region of *qCSS3* was selected (Fig. 4a). The self-pollinating seeds ($F_3$ generation) were germinated and
222 their chromosomal constitutions were surveyed using the seven SSR markers (Supplemental Table 1). The
223 $F_3$ progeny of this plant showed similar days to heading, minimising the effect of differences in heading
224 date on the seed-shattering degree (Supplemental Table 3). A significant difference in the BTS values was
225 observed between the $F_3$ lines with the 'Nipponbare' and W630 homozygous chromosomal segments (Fig.
226 4b, $P < 0.01$). In addition, partial abscission layer formation was found to be associated with the
227 seed-shattering degree, depending on the genotypes at the region (Fig. 4b and c). To restrict the border of
228 *qCSS3*, we selected two other $F_2$ plants having recombination within the *qCSS3* candidate region. Nos. 54
229 and 129 $F_2$ plants were found to have recombination between RM5639 and RM232 and between RM282
230 and RM5551, respectively (Fig. 4a). They produced $F_3$ progeny with similar heading dates (Supplemental
231 Table 3). Their progeny tests showed a significant difference in the BTS values between $F_3$ lines having
232 recombinant and W630 homozygous chromosomal segments (Fig. 4d, $P < 0.01$). These results indicated
233 that the candidate region of *qCSS3* was within a 5.1 Mb region between RM5639 and RM3297.

234 We further carried out genetic dissection at *qCSS3* candidate region. A total of 78 recombinants

235 out of 200 segregating plants were selected between RM5639 and RM3297 using $F_4$ generation of $F_2$-129.

236 We surveyed these recombinant plants with additional DNA markers and 25 of recombinant plants were

237 self-pollinated to conduct substitution mapping analysis. The mean BTS values of recombinant lines were

238 compared at $P < 0.05$ level. Comparison of the BTS values in the progeny test showed that the *qCSS3*

239 candidate region was narrowed down to 1.3 Mb region between RM5639 and RM1284 by six critical

240 recombinants (nos. 129-43, -55, -88, -185, -195, and -282, Fig. 4d). We further investigated their genotypes

241 at *qCSS3* using ten more additional DNA markers (Supplemental Table 1). No significant differences were

242 observed for both progeny tests of 129-55 (recombination between RM14731 and OTS1) and 129-185

243 (recombination between RM232 and RM14764), respectively (Fig. 4d). These results indicated that the

244 putative candidate poison of *qCSS3* is mapped to a 526 kb interval defined by two SSR markers RM14731

245 and RM14764. In this 526 kb region, we found 96 genes based on the published sequence annotation for *O.*

246 *sativa* Japonica 'Nipponbare' (Supplemental Table 4), although there might be additional genes in the

247 corresponding region of *O. rufipogon* W630.

248 **Discussion**

249 **Abscission layer formation in ILs with non-functional alleles at seed-shattering loci in the genetic**

250 **background of wild rice**

251 In the previous study, we evaluated the 'Nipponbare' allele at any of the *qSH1*, *qSH3*, or *sh4* loci in the

252 W630 genetic background (Htun et al. 2014). All of them showed complete abscission layer formation as in

253 W630, indicating that single mutation at any of the three seed-shattering loci was not sufficient to disrupt
254 the abscission layer formation. In this study, we characterized the IL having the 'Nipponbare' alleles at the
255 *qSH1*, *qSH3*, and *sh4* loci in the genetic background of wild rice, *O. rufipogon* W630. Although these three
256 seed-shattering loci were fixed with the 'Nipponbare' alleles, the average BTS value was significantly
257 lower than that of 'Nipponbare' (Fig. 1a, c). Microscopic and longitudinal section analyses showed the
258 presence of a ring-shaped abscission layer in the IL (Fig. 1d, e). Although the mechanism underlying the
259 partial formation of a ring-shaped abscission layer is not understood, the expression gradient of the genes
260 involved in abscission layer formation might be responsible.

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

267 The ILs with the 'Nipponbare' alleles at the three (*qSH1*, *sh4*, and *qSH3*) and two (*sh4* and *qSH3*;
268 Inoue et al. 2015) loci showed different abscission layer formation at the epidermis region (Fig. 1d, e). The
269 disruption of abscission layer at the epidermis region increases the BTS values tremendously (Fig. 1c). The
270 progeny test of no. 57 $F_2$ plant gave further interesting results. The $F_3$ lines with 'Nipponbare' homozygous

271 allele at the *qCSS3* showed no abscission layer formation, similar to ‘Nipponbare’, whereas those with

272 W630 homozygous allele had a partial abscission layer formation as the parental IL with the ‘Nipponbare’

273 alleles at the three seed-shattering loci (Fig. 4 b and c). A similar allele effect was also observed in the two

274 BRILs with the W630 allele at *qCSS3* in the genetic background of ‘Nipponbare’. Both lines showed

275 significantly lower BTS values than ‘Nipponbare’ (Supplemental Fig. 3). These results strongly indicate

276 that the wild allele at *qCSS3* may act to promote the abscission layer formation. It would be of interest to

277 investigate the tissue specific expression of the seed-shattering gene(s) in the developing stage of spikelets

278 with or without the functional allele at *qCSS3*.

279

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

281 The QTL-seq analysis successfully detected *qCSS3* in a 5.1-Mb region on chr. 3, which is different form

282 *qSH3*, the seed-shattering locus that was previously detected (Htun et al. 2014; Table 1). We also detected

283 *qCSS9* in a 1.9 -Mb region on chr. 9 (Table 1). As ‘Nipponbare’ allele at *qCSS9* was found to promote seed

284 shattering, we speculated that the allele effect of *qCSS9* is not involved in a loss of seed shattering in

285 ‘Nipponbare’. The allele effect at *qCSS3* on the seed-shattering degree was examined using two BRILs in

286 the ‘Nipponbare’ genetic background (Supplemental Fig. 3) and in the progeny test of $F_3$ lines between the

287 IL and ‘Nipponbare’ (Fig. 4). Although the BTS value of ‘Nipponbare’ was significantly higher than those

288 of the two BRILs, their difference was about 15 gf. In the progeny test, large differences in the BTS values

289 (30 gf) were observed between the $F_3$ lines from three independent $F_2$ plants. This may be owing to the
290 different genetic background of the lines. Probably, 'Nipponbare' possesses cultivated alleles at some other
291 minor QTLs for seed shattering..
292
293 **Mapping of *qCSS3* and the selection signature at the candidate region**
294 On the basis of progeny tests, the candidate region of *qCSS3* was estimated to be in a 526kb region between
295 the two SSR markers, RM14731 and RM14764. In the region, we found a total of 96 annotated genes based
296 on the Rice Annotation Database for *O. sativa* 'Nipponbare' (Supplemental Table 4). We investigated any
297 polymorphisms related to the gene function for these 96 genes between 'Nipponbare' and W630. We found
298 a gene (*Os03g0283000*) encoding *OsGSTL3*, *LAMBDA GLUTATHIONE S-TRANSFERASE 3*, that carries
299 missense mutation (*position*). Although the gene expression of *Os03g0283000* is detected in a leaf blade
300 according to gene expression database (RiceXPro, http://ricexpro.dna.affrc.go.jp), the involvement of the
301 gene in the control of seed shattering is unknown. Once the causal gene is identified by further genetic
302 mapping experiments, expression analysis of the candidate genes should be conducted in wild genetic
303 background that has a functional pathway for promoting seed shattering.
304 In many domestication-related genes, reduction in nucleotide diversity is often observed
305 (Doebley et al. 2006). Interestingly, a 526 kb candidate region partially overlaps with the selective sweep
306 reported previously (Xu et al. 2012; Huang et al. 2013). Approximately, a 1.0-Mb region (9.0-10.0 Mb) on

307 chr. 3 shows a reduction of diversity in Japonica rice cultivars (Xu et al. 2012), and the region carries the

308 putative candidate region of *qCSS3*. This signature is specific to Japonica but not to Indica rice cultivars.

309 Furthermore, a large scale genomic study using 446 wild and 1,083 cultivated rices shows that the putative

310 region from 9.7 to 10.1 Mb on chr. 3 exhibits quite low diversity in Japonica rice population (Huang et al.

311 2012). An approximately 130 kb-region on RM14764 side of *qCSS3* were found to overlap partially with

312 the sweep region. Interestingly, the selective sweep at the region was not detected in Indica nor full

313 populations. These findings suggest that Japonica-specific domestication-related region may be located

314 within a putative candidate region of the *qCSS3*. If a loss-of-function mutation at *qCSS3* is common only to

315 Japonica cultivars, this mutation may be selected after the differentiation of Japonica rice cultivars diverged

316 from the initial cultivated rice. In contrast, the *qSH3* region was found to be overlapping with the selective

317 sweep region, both for the Japonica and Indica rice cultivars (Xu et al. 2012). These results suggest

318 different evolutionary trajectories for the seed-shattering loci, which might be useful in understanding the

319 process of rice domestication and distribution.

320

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

322 In previous studies, *sh4* was identified as a major locus selected for during rice domestication (Li et al.

323 2006; Lin et al. 2007). The mutation at *sh4* is conserved in all cultivated rice examined, indicating that *sh4*

324 is a key locus playing an important role in the loss of seed shattering (Zhang et al. 2009). On the other hand,

325 mutation at *qSH1* was found only in Japonica rice cultivars, suggesting that the mutation might have been

326 selected after Japonica rice differentiated (Konishi et al. 2006; Zhang et al. 2009). In our recent study, we

327 evaluated the non-seed-shattering behaviour of the Indica rice cultivar, 'IR36' (Ishikawa et al. 2017). The

328 evaluation of the BRILs having 'IR36' derived chromosomal segments in the W630 genetic background

329 identified a strong QTL on chr. 4, which overlaps with *sh4*. Additional genetic analysis suggested the

330 involvement of *qSH3* and other minor loci in the non-seed-shattering behaviour of 'IR36'. At present, it

331 remains unclear whether *qCSS3* is involved in the loss of seed shattering in Indica rice. However, once the

332 causal mutation at *qCSS3* is identified, genotyping survey at *qCSS3* in the Indica rice cultivars will clarify

333 involvement of *qCSS3* in the non-shattering behaviour. The gene interaction among the four seed-shattering

334 loci provides scope for future studies that will help in understanding the process of loss of seed shattering

335 during rice domestication.

336

337 **Conclusions**

338 We successfully identified novel loci involved in the difference in seed shattering behaviour between

339 'Nipponbare', a typical Japonica rice cultivar, and wild rice *O. rufipogon*. Among two loci, 'Nipponbare'

340 allele at *qCSS3* may have contributed to a loss of seed shattering in rice domestication. To our knowledge,

341 *qCSS3* is the fourth detected locus involved in a loss of seed shattering in cultivated rice after *sh4*, *qSH1*,

342 and *qSH3*. The genetic dissection of the non-seed-shattering behaviour of 'Nipponbare' reveals that at least

343 four mutations (i.e. *qSH1*, *qSH3*, *sh4*, and *qCSS3*) are required to fully lose the abscission layer formation.

344 Identification of the causal mutation at *qCSS3* will provide important information for rice breeding that will

345 help in manipulating the degree of seed shattering. Moreover, combination of the alleles at the four

346 seed-shattering loci will be useful in understanding the process and history of rice domestication.

347

348 **Author contribution statement**

349 RI conceived and designed the study. YT, SS, KO, TMT, CC and RI performed the experiments. YT, SS,

350 KO, TI, and RI analysed the data. YT, KN and TA contributed to bioinformatic analysis. YT, TI and RI

351 prepared the manuscript. All authors read and approved the final manuscript.

352

353 **Compliance with ethical standards**

354

355 **Conflict of interest**

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

357

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365

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462

463 **Figure Legends**

464 **Fig. 1** Characterisation of the introgression line (IL) carrying *Oryza sativa* ‘Nipponbare’ (Npb) alleles at

465 *qSH1*, *qSH3*, and *sh4* in the genetic background of wild rice, *O. rufipogon* W630.

466 (a) Graphical genotype of the IL. The positions of the three seed-shattering loci *qSH1*, *qSH3*, and *sh4* are

467 shown. (b) Seeds of ‘Nipponbare’ (Npb, left), W630 (centre), and the IL (right). Scale bar = 5 mm. (c) The

468 breaking tensile strength (BTS) values for ‘Nipponbare’ (Npb), W630, and the IL. Data are mean ± S.D. (n

469 = 4). ** indicates P < 0.01 by unpaired Student's *t*-test. N.D., Not determined owing to complete seed

470 shattering (d) An overview of spikelet base view (upper panels) and a close view of the spikelet base (lower

471 panels) in 'Nipponbare' (left), W630 (centre), and the IL (right). The abscission scars were observed from

472 the side of each dotted-line square (bottom). Scale bars = 1 mm. (e) Longitudinal sections of the spikelet

473 base after seed detachment in 'Nipponbare' (Npb) (left), W630 (centre), and the IL (right). vb = vascular

474 bundle. ab = abscission layer. Black triangles indicate both edges of the abscission layer. Scale bars = 50

475 μm

476

477 **Fig. 2** Frequency distribution of breaking tensile strength (BTS) values for 174 $F_2$ individuals between

478 *Oryza sativa* 'Nipponbare' and the introgression line (IL) carrying the 'Nipponbare' alleles at the *qSH1*,

479 *qSH3*, and *sh4* loci. The BTS values for the parent lines and their $F_1$ plants are shown with black dots with

480 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 ±

481 S.D.). The DNA samples of $F_2$ plants with BTS values between 70 and 100 gf were selected as low (L)

482 bulk and those with BTS values between 150 and 210 gf were selected as high (H) bulk

483

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

485 analysis.

486 *qCSS3* and *qCSS9* were detected in approximately the 5.4-Mb region on chromosome 3 and the 1.9-Mb on

487 chromosome 9. The Δ(SNP-index) plots with statistical intervals under the null hypothesis of no QTL

488 (orange, $P < 0.01$; green; $P < 0.05$). The red line indicates the average ΔSNP-index calculated by a sliding

489 window analysis.

490

491 **Fig. 4** Genetic dissection of *qCSS3*.

492 (a, d) Graphical genotypes of three $F_2$ plants (nos. 57, 54, and 129) and six critical recombinant $F_3$ plants

493 (nos. 129-43, -55, -88, -185, -195, and -282) in the candidate region of *qCSS3*. The BTS values of the $F_3$

494 lines from the three $F_2$ plants are shown on right. Data are mean ± S.D. (n = 6). Significant differences in

495 the BTS values were observed for all pairs between the $F_3$ and $F_4$ lines having 'Nipponbare' and W630

496 homozygous chromosomal segments at the *qCSS3* candidate region. **, * and n.s. indicates $P < 0.01$,

497 $P<0.05$, and not significant ($P>0.05$) by unpaired Student's *t*-test. (b, c) Abscission layer formation for the

498 $F_3$ progeny of no. 57 having 'Nipponbare' and W630 heterozygous chromosomal segments. vb = vascular

499 bundle. ab = abscission layer. Black triangles indicate both edges of the partially formed abscission layer.

500 Scale bars = 50 μm

501

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

503

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

505

506 **Supplemental Table 3** Days to heading observed for $F_3$ progeny from three $F_2$ plants (nos. 57, 54, and

507 129). Data are mean ± S.D. (n = 6)

508

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

510 information based on Rice annotation data

511

512 **Supplemental Fig. 1** Results of the QTL-seq analysis for all chromosomes. The Δ(SNP-index) plots with

513 statistical intervals under the null hypothesis of no QTL (orange, $P < 0.01$; green; $P < 0.05$). The red line

514 indicates the average ΔSNP-index calculated by a sliding window analysis.

515

516 **Supplemental Fig. 2** Box plots of the BTS values of two $F_2$ groups based on the genotypes at the candidate

517 regions of *qCSS3* and *qCSS9*. All $F_2$ individuals were surveyed with seven and four PCR-based molecular

518 markers covering *qCSS3* and *qCSS9* regions, respectively (Supplemental Tables 1 and 2). Plants carrying

519 the 'Nipponbare' (Npb) and W630 homozygous chromosomal segments for the entire candidate regions of

520 *qCSS3* and *qCSS9* were selected.

521

522 **Supplemental Fig. 3** Evaluation of seed-shattering degree of the two backcross recombinant inbred lines

523 (BRILs) carrying the W630 chromosomal segment covering the *qCSS3* region in the 'Nipponbare' genetic

524 background. (a) Graphical genotypes of two BRILs, BRIL42 and BRIL86. Red lines indicate the region

525 covering *qCSS3*. (b) Seed-shattering degree of 'Nipponbare' (Npb) and the two BRILs. Data are mean ±

526 S.D. (n = 6). ** indicates $P < 0.01$ by unpaired Student's *t*-test