Identification and Characterization of Quantitative Trait Loci for Shattering in Japonica Rice Landrace Jiucaiqing from Taihu Lake Valley, China

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ABSTRACT

Easy shattering reduces yield from grain loss during rice (Oryza sativa L.) harvest. We characterized a nonshattering japonica rice landrace Jiucaiqing from Taihu Lake valley in China. The breaking tensile strength (BTS; grams force, gf) of the grain pedicel was measured using a digital force gauge to evaluate the degree of shattering at 0, 7, 14, 21, 28, and 35 d after heading (DAH). The BTS of Jiucaiqing did not significantly decrease with increasing DAH, maintaining a level of 152.2 to 195.9 gf, while that of indica IR26 decreased greatly during 0 to 14 DAH and finally stabilized at ~100 gf. Then the chromosome segment substitution lines (CSSLs) and near isogenic lines (NILs) of Jiucaiqing in IR26 background were developed for quantitative trait loci (QTL) mapping. Four putative QTL (qSH1JCQ, qSH3JCQ, qSH6JCQ, and qSH11JCQ) for shattering were detected, and qSH1JCQ was confirmed on chromosome 1. We further mapped qSH1JCQ to a 98.4-kb region, which contains 14 genes. Os01g62920 was considered to be a strong candidate for qSH1JCQ, which colocated with qSH1. Further quantitative real-time polymerase chain reaction (PCR) analyses confirmed that the QTL qSH1JCQ can significantly decrease the expression of shattering related genes (qSH1, OsCPL1, Sh4, SH5, and SHAT1) especially at the middle development stage at 10 and 15 cm panicle length, which causes rice shattering decrease. The elite allele and the NIL with desirable agronomic traits identified in this study could be useful for rice breeding.

Rice is the most important food crop in China. In rice breeding, seed shattering remains an important target trait for improvement (Konishi et al., 2006). Seed shattering habit causes yield loss during crops harvest (Zhou et al., 2012), while nonshattering also confounds those yields by hampering the harvest process (Ji et al., 2006). It is important to cultivate rice with an appropriate level of shattering in rice breeding programs (Subudhi et al., 2014). The identification of shattering genes and loci will positively impact rice breeding programs by transgenic and marker-assisted selection (MAS) approaches.

Several shattering genes have been cloned in rice such as sh4 (Li et al., 2006; Lin et al., 2007), qSH1 (Konishi et al., 2006), OsCPL1 (Ji et al., 2010), SHAT1 (Zhou et al., 2012), and SH5 (Yoon et al., 2014). The sh4 encodes a transcription factor with a Myb3 DNA binding domain and promotes hydrolyzing of abscission zone cells during the abscission process (Li et al., 2006; Lin et al., 2007). The OsCPL1 encodes a carboxy-terminal domain phosphatase-like protein, which represses development of the abscission zone (Ji et al., 2010). The SHAT1 gene encodes a transcription factor with an APETALA2 domain containing the gene and affects the abscission zone development (Zhou et al., 2012). These results indicated that seed shattering depends on development of an abscission zone.

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Abbreviations: BTS, breaking tensile strength; cDNA, complementary DNA; CSSL, chromosome segment substitution line; DAH, days after heading; gf, grams force; MAS, marker-assisted selection; NIL, near isogenic line; ORF, open reading frame; PCR, polymerase chain reaction; QTL, quantitative trait loci; SSR, simple-sequence repeat.
Recently, Yoon et al. (2014) identified SH5 that is highly homologous to qSH1; both function together in controlling development of the abscission zone. qSH1 was revealed through a cross between shattering-type indica Kasalath and the nonshattering type japonica Nipponbare (Konishi et al., 2006). qSH1 encodes a BELL-type homeobox protein that is highly homologous to Arabidopsis RPL, which interacts with BP involved in cell differentiation by modulating the lignin biosynthesis pathway (Mele et al., 2003; Smith and Hake, 2003; Kanrar et al., 2006). Yoon et al. (2014) also predicted that SH5 functions in suppressing the deposition of lignin during early anther development. qSH1 and SH5 have a role in maintaining the expression of Sh4 and SHAT1 (Zhou et al., 2012; Yoon et al., 2014). The role of qSH1 in rice evolution is now considered to be important during the improvement of japonica varieties (Konishi et al., 2006; Thurber et al., 2010).

Cultivated rice exhibits a wide range of seed shattering. Generally, japonica varieties shatter more difficulty than indica varieties (Subudhi et al., 2014). Indica is not only an important rice subspecies widely planted in Asia, but it is also the genetic background of the majority of hybrid varieties in China (Zhang et al., 2013). Indica hybrid varieties are normally easy shattering, causing yield loss during hybrid seeds production, thus, decreasing the shattering level of indica rice is an important goal in rice breeding. There are a large number of farmers using japonica cultivars or landraces in the Taihu Lake valley of Yangtze River region in China (Dang et al., 2015). Of them, the elite japonica cultivars or landraces can serve as a source for many agronomical important genes. For example, the japonica landrace Jiucaiqing is a good source of genes for salt tolerance, seed germination, and seed vigor in our previous studies (Wang et al., 2011, 2013). Indica varieties are normally easy shattering, causing yield loss during hybrid seeds production, thus, decreasing the shattering level of indica rice is a major goal in rice breeding. There are a large number of farmers using japonica cultivars or landraces as a source of many agronomical important genes. For example, the japonica cultivar Jiucaiqing is a good source of genes for salt tolerance, seed germination, and seed vigor in our previous studies (Wang et al., 2011, 2013). Indica varieties are normally easy shattering, causing yield loss during hybrid seeds production, thus, decreasing the shattering level of indica rice is an important goal in rice breeding. There are a large number of farmers using japonica cultivars or landraces for salt tolerance, seed germination, and seed vigor in our previous studies. Indica hybrid varieties are normally easy shattering, causing yield loss during hybrid seeds production, thus, decreasing the shattering level of indica rice is an important goal in rice breeding. There are a large number of farmers using japonica cultivars or landraces for salt tolerance, seed germination, and seed vigor in our previous studies (Wang et al., 2011, 2013).

In this study, we identified that the japonica landrace Jiucaiqing has a reduced shattering performance. To mine elite genes or alleles of shattering, the CSSL population was developed from BC5F4 in which the region around QTL on chromosome 1 was heterozygous and all other QTL regions were homozygous for IR26. All plants were grown in a paddy field at the Experimental Station of Nanjing Agricultural University (Jiangsu Province, China; 32.020° N, 118.500° E) in 2014 and 2015. Field management was performed in accordance with the local standard methods (Cheng et al., 2014).

Materials and Methods

Plant Materials

A japonica landrace Jiucaiqing, derived from Taihu Lake valley in Jiangsu Province of China, was crossed with an indica IR26, and the resultant F1 plants were backcrossed with IR26. The backcrossing was performed five times, followed by one or two generations of selfing to develop the BC1F2, BC2F3, and BC3F4 populations for the selection of homozygous CSSLs (Supplemental Fig. S1). The BC3F4 was followed by one or two generations of selfing to develop the BC1F4 and BC1F5 population for fine mapping. A NIL population was developed from BC1F4 in which the region around QTL on chromosome 1 was heterozygous and all other QTL regions were homozygous for IR26. All plants were grown in a paddy field at the Experimental Station of Nanjing Agricultural University (Jiangsu Province, China; 32.020° N, 118.500° E) in 2014 and 2015. Field management was performed in accordance with the local standard methods (Cheng et al., 2014).

Molecular Marker Analyses

According to the published rice molecular map (http://www.granene.org), 192 simple-sequence repeat (SSR) markers scattered on 12 chromosomes with an average interval of ~2 cM were used in this study (Supplemental Table S1). Total DNA of each individual in each backcross generation and parents was extracted from the fresh leaf tissue of the 4-wk-old seedlings using CTAB method. The PCR was performed using the procedure of Chen et al. (1997) and the PCR products were separated on an 8% nondenaturing polyacrylamide gel and visualized by the silver staining method of Sanguinetti et al. (1994).

Shattering Degree Test

The BTS was measured using a digital force gauge as described in Konishi et al. (2006). The BTS is a measure of the maximum amount of grams force (gf) in a single flower or grain holding before releasing. One gf is the force exerted by 1 g of mass under the influence of standard gravity on Earth. Twenty grains on the main stem were tested at 35 DAH in CSSLs. Panicles from each individual were collected at 0, 7, 14, 21, 28, and 35 DAH in one NIL and two parents for shattering analyses during seed development. The average value was taken as the degree of shattering for each sample. The BTS values over 150 gf represent nonshattering in this study.

Quantitative Trait Loci Analyses and Fine Mapping

The mean BTS values of the individual CSSL at 35 DAH were compared with the recurrent parent IR26 in 2014 and 2015, and QTL were assigned to the introgressed region if the differences of BTS were significant at p < 0.01 in both years. The panicles of BC1F4 and BC2F4 populations were harvested at 35 DAH and the markers located in the H6 to H24 interval (Supplemental Table S2) were used for fine mapping.

Sequencing Analyses

The partial region ~12 kb upstream of qSH1 with an SNP responsible for shattering (Konishi et al., 2006) and the promoter ~2.0 kb upstream of qSH1 were amplified from the genomic DNA from the young leaf tissues of Jiucaiqing and IR26 using Phusion High Fidelity DNA Polymerase (Takara). Three independent purified PCR products were directly sequenced in both directions. The cis-acting elements of the promoter were determined based on the PLACE (Plant cis-acting Regulatory DNA Elements) and PlantPAN databases (Plant Promoter
RNA Isolation, Complementary DNA Synthesis and Quantitative Real-Time Polymerase Chain Reaction

Genes involved in seed shattering—\(qSH1\), \(OsCPL1\), \(sh4\), \(SH5\), and \(SHAT1\) —were assessed. The young spikes were quickly cut when 5, 10, 15, and 20 cm panicle length and stored in liquid nitrogen. Total RNA was extracted from 80 to 100 mg of spikes using the RNeasy plant mini kit (Qiagen). Total RNA was used to synthesize complementary DNA (cDNA) with random oligonucleotides using a reverse transcription system (Takara). Quantitative real-time PCR was performed using an ABI 7500 Fast Real-Time PCR System with the SYBR Green Master Mix (Applied Biosystems). The rice 18S ribosomal RNA gene was used as an internal control. Relative quantification of transcript levels was performed using the comparative Ct method (Livak and Schmittgen, 2001). Three biological replications were conducted. The primers were designed (http://www.quantprime.de; Supplemental Table S3).

Data Analyses

The experimental data were analyzed using SAS software version 9.3 (SAS Institute, 2012), and significant differences between samples were compared using Student’s \(t\)-test at the 5 and 1% levels of probability (Wang et al., 2012a, 2012b).

Results

Phenotypic Variation

The grains of IR26 shattered easily with a touch of the hand compared with Jiucaiqing at 35 DAH (Fig. 1a). The BTS value of Jiucaiqing did not decrease significantly with increasing DAH but retained a BTS level of 152.2 to 195.9 gf. In contrast, the BTS value of IR26 decreased dramatically during 0 to 14 DAH and finally stabilized at ~100 gf after that (Fig. 1b). Phenotypic variation revealed that the final
average BTS values at 35 DAH ranged from 82.5 to 296.0 gf in CSSLs. The BTS of most CSSLs were closer to those of the recurrent parent IR26 in both years (Fig. 1c). By comparison, a slight difference of shattering was observed in CSSLs between 2 yr, which might be due to the variation of temperature during seed development (Fig. 1d).

Quantitative Trait Loci Mapping
Among the CSSLs, two CSSLs (CSSL2 and CSSL14) had the highest BTS simultaneously in 2014 and 2015, which had significantly nonshattering degree compared with IR26 (Fig. 2a). These two CSSLs with Jiucaiqing introgressions on chromosome 1, 3, 6, and 11 had reduced BTS (Fig. 2b and 2c), which suggested that four QTL (qSH1JCQ, qSH3JCQ, qSH6JCQ, and qSH11JCQ) might be involved in shattering. Of them, QTL on chromosome 1 and 6 were simultaneously detected in both CSSLs. A residual heterozygous line of qSH1JCQ on chromosome 1 was selected from BC$_2$F$_3$ for the loci confirmation. Chi-square test demonstrated that nonshattering in BC$_2$F$_3$ was likely controlled by a complete dominance major gene (Fig. 2d). A NIL was developed for qSH1JCQ confirmation (Fig. 2e); the NIL had significantly higher BTS than IR26 (Fig. 2f).

Fine Mapping of qSH1JCQ
Thirteen recombinant plants were identified in BC$_2$F$_4$ introgression lines by six SSR markers. Based on their segregation of shattering phenotype, qSH1JCQ was found to be located in the 98.4-kb interval between Jm36 and Jm23 (Fig. 3), in which 14 genes were predicted (Table 1). Of them, Os01g62920 was considered to be a strong candidate for the qSH1JCQ locus, which colocated with qSH1 (Konishi et al., 2006). Other genes are annotated as encoding oxidoreductase, aldo/keto reductase family protein (Os01g62860, Os01g62870 and Os01g62880), prenylated rab acceptor (Os01g62890), amino acid kinase (Os01g62900), ras-related protein (Os01g62950), seed storage/LTP family protein precursor (Os01g62980), stress protein domain containing protein (Os01g63010) and unknown proteins (Os01g62910, Os01g62945, Os01g62960, Os01g62970, and Os01g62990).

Sequence Variation in qSH1
The ORFs of qSH1 were identical between Jiucaiqing and Nipponbare, while IR26 differed from others, having nucleotide change, A to G, at position +1977 and +3512 but without amino acid substitution. Nucleotide substitution of G with T at the 12 kb ahead of qSH1 position was early reported to contribute to the nonshattering phenotype (Konishi et al., 2006). The nucleotide sequences at the 12 kb ahead of qSH1 were identical (G) between Jiucaiqing and IR26, while the nonshattering T allele was present in Nipponbare (Fig. 4). The promoter of qSH1 were identical between Jiucaiqing and Nipponbare, while IR26 differed from others, having a TAAG insertion at position −1845 and nucleotide changes, T to C, GC to TG and G to A, at position −1787, −1262, and −380, respectively. Then the different cis-acting elements were predicted between Jiucaiqing and IR26 at the mutation positions; Jiucaiqing contained DOFCOREZM, BSIEGCCR, and SORLIP1AT while IR26 contained CACTFTPPCA1, and CAAT box.

Expression of Shattering Related Genes Regulated by qSH1JCQ
The BTS of IR26 decreased dramatically during 0 to 14 DAH, while NIL (IR26 background with qSH1JCQ) retained a high BTS of 237.9 to 266.3 gf during whole-seed development (Fig. 5a). The expression of Sh4 and SHAT1 was relatively lower than that of qSH1, OsCPL1, and SH5 in IR26 and NIL during seed development (Fig. 5b–f). The gene expression (except Sh4) rapidly increased at the early stage in IR26 and reached the highest levels when at 10 cm panicle length, and then they progressively declined with development. However, the opposite expression pattern was observed in NIL. By contrast, the NIL showed a significantly higher expression (except Sh4) at the early and late development stages when at 5 and 20 cm panicle length, while it had a significantly lower level of gene expression at the middle development stage when at 10 and 15 cm panicle length.

Characterization of Agronomy in Near-Isogenic Line
In addition to loss of shattering, the NIL showed no significant agronomic alterations in contrast with IR26. There were similar agronomic traits between IR26 and NIL such as heading date, 1000-grain weight, plant height, panicle length, effective tillers, and filled grains per panicle (Fig. 6). The elite allele of qSH1JCQ from Jiucaiqing introgressed into indica rice could reduce the shattering level without affecting other agronomic traits.

Discussion
Generally, indica varieties shatter more easily than japonica varieties (Subudhi et al., 2014); similar results were observed in this study that the japonica landrace Jiucaiqing had a relatively nonshattering trait. The abscission layer of rice might be created at 10 d after pollination (Thurber et al., 2011). At this stage, the BTS was similar for all weedy rice accessions and rice cultivars (Nunes et al., 2014). Therefore, the significant differences of BTS between Jiucaiqing and IR26 were observed after 14 DAH in this study. The BTS gradually decreased with development and reached the lowest level at 20 to 25 DAH and did not change significantly thereafter, which is similar with a previous study (Ji et al., 2006). Usually, the days from heading to harvest are 30 to 40 d for rice in Nanjing (Jiangsu Province, China; 32°02’ N, 118°50’ E). Therefore, the BTS value of CSSLs measured at 35 DAH is believed to represent the shattering degree at the maturity stage in this study. The BTS of CSSLs varied between 82.5 to 296.0 gf, which were in a similar range to other studies (Konishi et al., 2006; Ji et al., 2010; Thurber et al., 2010; Zhou et al., 2012; Nunes et al., 2015).
The CSSLs have been successfully used to discover new alleles for agronomic traits in rice (Tian et al., 2006). In this study, four QTL for shattering were successfully identified from the *japonica* landrace Juicaiqing using a CSSL population. In previous studies, several QTL or genes for seed shattering were identified on 12
Two major seed shattering loci, \textit{qSH1} (Konishi et al., 2006) and \textit{sh4} (Li et al., 2006; Lin et al., 2007), were detected on chromosomes 1 and 4, respectively. The \textit{SHAT1} (Lin et al., 2007; Zhou et al., 2012), \textit{OsCPL1} (Ji et al., 2006, 2010) and \textit{OsSh1} (Lin et al., 2012) were mapped to chromosome 4, 7, and 3, respectively. Recently, two regions of the QTL for seed shattering on chromosome 1 and chromosome 6 were detected in rice (Lee et al., 2016). By comparison, the positions of two QTL in previous studies were similar to the QTL detected in this study. \textit{qSH1}\textsuperscript{JCQ} and \textit{qSH3}\textsuperscript{JCQ} were near the regions of \textit{qSH1} (Konishi et al., 2006) and \textit{OsSh1} (Lin et al., 2012), respectively. These results indicate that the QTL identified in Jiucaiqing might be reliable, and two QTL, \textit{qSH6}\textsuperscript{JCQ} and \textit{qSH11}\textsuperscript{JCQ}, might represent novel genes in this study.

In this study, we found that \textit{qSH1}\textsuperscript{JCQ} introgressed into IR26 can significantly reduce the shattering of IR26.
Further comparison revealed that the early reported \( qSH1 \) and its regulatory region (Konishi et al., 2006) lie within the region of \( qSH1JCQ \). Therefore, the \( qSH1 \) was considered to be a strong candidate for \( qSH1JCQ \), and then the sequence comparison of parental alleles was conducted only on gene \( qSH1 \) in this study. Our results showed that there is no polymorphism in coding part of \( qSH1 \) influencing shattering between Jiucaiqing and IR26. The nucleotide

Fig. 4. Comparison of the nucleotide sequence at the 12 kb head of \( qSH1 \) and the promoter and the open reading frames (ORFs) region of \( qSH1 \) among Jiucaiqing, IR26, and Nipponbare. Black boxes indicate coding regions, and white boxes show the 5’ and 3’ untranslated regions. Nucleotides with red color indicate the sequence variation in IR26. Red boxes indicate cis-acting elements, and blue boxes show amino acid. Single asterisk (*) indicates complementary sequences.

Fig. 5. (a) Shattering levels and (b–f) expression levels of shattering related genes regulated by the Jiucaiqing allele of \( qSH1JCQ \) in various development stages. The breaking tensile strength (BTS) of grain pedicels was tested at 0, 7, 14, 21, 28, and 35 d after heading. The unit of BTS, gf, is the abbreviation of grams force. The transcript levels were determined with quantitative real-time polymerase chain reaction using gene specific primers when 5, 10, 15, and 20 cm panicle length. Transcript levels were calculated relative to the \( qSH1 \) when 5 cm panicle length. Bars indicate the mean values ± standard deviation. Single and double asterisks (*) and **) represent significant differences between IR26 and NIL at \( p < 0.05 \) and \( p < 0.01 \) level, respectively.
substitution of G for T in the 5' regulatory region 12 kb ahead of the qSH1 gene was associated with rice shattering (Konishi et al., 2006). However, no nucleotide variability was found between Jiucaiqing and IR26 in this study, and similar results were observed in the weedy and cultivated rice genotypes with a G at this position of the qSH1 (Nunes et al., 2015). Our further study indicated that the variation of qSH1 promoter between Jiucaiqing and IR26 might be responsible for seed shattering. Compared with Jiucaiqing, the nucleotide mutation (GC to TG) of qSH1 promoter was observed in IR26, causing a CAAT box contained, that might change the expression pattern of shattering related genes with seed development. The CAAT box signals the binding site for the RNA transcription factor, which is known as a core promoter that initiates gene transcription (Mantovani, 1999; Laloum et al., 2013). More experiments should be performed to confirm this hypothesis.

The expression change of shattering-related genes regulated by qSH1JCQ was confirmed in our further study. The significant decrease of qSH1 expression was observed in IR26 when the qSH1JCQ introgressed (NIL) in this study, especially at the middle development stage when the abscission layer formation. Recently, a genetic model proposed that the both qSH1 and SH5 positively affect Sh4 and SHAT1 gene expression (Zhou et al., 2012; Yoon et al., 2014). The interactions between the qSH1JCQ with other shattering related genes (OsCPL1, Sh4, SH5, and SHAT1) were therefore also analyzed in this study. The qSH1JCQ can significantly decrease the expression of OsCPL1, Sh4, SH5, and SHAT1 especially at the middle development stage. However, we found that the expression of Sh4 and SHAT1 was relatively lower, indicating they might play minor roles on shattering in this study. Additionally, the low OsCPL1 expression observed in NIL at the middle development stage is a challenge that OsCPL1 represses the development of abscission zone (Ji et al., 2010). More experiments should be performed to examine the interactions between qSH1JCQ and other shattering related genes and to further decipher the genetic network underlying seed shattering in rice (Nunes et al., 2014).

It is important to balance nonshattering and threshing traits in rice production. The advantage is to allow the harvest of mature grains at once without considerable grain loss (Zhang et al., 2009). The ideal shattering levels of rice might depend on the harvest method being used. To our knowledge, the middle level of shattering, for example, 100 to 200 gf, might be the ideal ranges of shattering for rice production. To perform MAS efficiently within breeding programs, it is important to identify genes for shattering that can serve as markers (Kwon et al., 2015). In this study, the introgression of qSH1JCQ into indica IR26 would reduce the shattering degree with desirable agronomic traits. The Jiucaiqing allele of qSH1JCQ may provide opportunities for rice breeders to reduce the indica shattering by MAS.

Supplemental Information Available
Supplemental information is available with the online version of this manuscript.
Laloum, T., S. De Mita, P. Gamas, M. Baudin, and A. Niebel. 2013. CCAAT-
Public Interest (Grant No. 201203052), and the Special Fund for Agro-scientific Research in the Public Interest (Grant No. 201203052).

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Acknowledgments
This work was supported by the National Natural Science Foundation of
China (Grant No. 31271806), the Natural Science Foundation of Jiangsu
Province (Grant No. BK2015067; BK20161451), the Fundamental Research
Funds for the Central Universities (Grant No. KY201402; 
KY201505), and the Special Fund for Agro-scientific Research in
the Public Interest (Grant No. 201203052).