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SHATTERING ABORTION3 controls rice seed shattering by promoting abscission zone separation

Dear Editor,

The selection of non-shattering seeds has been a primary target during crop domestication, making wild rice suitable for human cultivation. Seed shattering involves development and separation of the abscission zone (AZ). The AZ consists of several layers of thin-walled, iso-diametrically flattened cells, marking the point at which the cells of the falling organ separate from the parent plant (Sexton and Roberts, 1982). Many previously identified factors that regulate seed shattering, such as *SH4*, *qSH1*, *qSH3*, *SHAT1*, *SSH1*, and *SH5*, all participate in development of the AZ in rice (Wu et al., 2023a; 2023b). However, what occurs at the cellular level to enable abscission and what induces the separation of the AZ in rice remain unclear. Here, we report that the major facilitator superfamily (MFS) gene *SHATTERING ABORTION3* (*SHAT3*) functions as a novel regulator of seed shattering in rice (*Oryza sativa)* by facilitating jasmonic acid (JA) accumulation in the AZ and pedicel (AZ-P) region to promote AZ separation. However, the non-shattering phenotype of *shat3* cannot be complemented by exogenous JA, indicating that *SHAT3* has unique functions that have yet to be explored.

To examine the regulatory network underlying seed shattering in rice, we mutagenized SL4 (a wild rice chromosomal segment substitution line with a natural seed-shattering phenotype) using ^{60Co} ϒ-rays and screened the resulting M_2 mutant generation, thus identifying the loss-of-shattering mutant *shat3* (Figure 1A and Supplemental Figure 1A) (Zhou et al., 2012). Phenotypic observations showed that the pedicel of *shat3* had a higher breaking tensile strength (BTS) at the harvest stage than that of the wild type (WT). The WT showed a dramatic decrease in BTS 11 days after pollination (DAP) and displayed a completely shattering phenotype by 15 DAP, whereas the BTS of *shat3* did not decrease significantly (Figure 1B). Longitudinal sections of spikelets at the anthesis stage showed that both the WT and *shat3* had a complete AZ (Figure 1C), and the pedicel fracture surfaces of both remained relatively intact, even when the *shat3* seeds were removed with external force (Figure 1D). Close-up observations of the pedicel fracture surface at 0 DAP revealed no critical differences in morphology between the WT and *shat3*. However, at 15 DAP, the WT exhibited a smoother surface than *shat3* (Figure 1D). Notably, the WT had a naturally dissociated smooth surface with no obvious covering over the pedicel and glume, whereas the parenchyma cells of *shat3* were tougher and exhibited a jagged plane of broken cell walls in longitudinal section, indicating that forcible separation involved breakage of the primary walls (Figure 1E and 1F). These results suggest that *shat3* affects seed shattering by disrupting breakdown of the AZ cells.

To identify *SHAT3*, we developed several mapping populations (Supplemental Figure 1B and 1C) and performed map-based cloning and whole-genome sequencing. We detected a 1-bp deletion in the first exon of Os05g0475700 in *shat3*, which caused a frameshift and premature termination (Figure 1G and Supplemental Figure 2A–2C). qPCR analysis showed that *SHAT3* transcripts accumulated in the AZ, with higher levels at 13 DAP (Supplemental Figure 2D). We introduced a 4744-bp wild rice genomic fragment, which included the gene region and 2911-bp flanking regions, into *shat3*. The positive transgenic complementation (CP) lines showed a natural shattering phenotype (Supplemental Figure 2E–2G). *SHAT3* expression was significantly reduced in *shat3* but significantly increased in the CP lines (Supplemental Figure 2H). We also used CRISPR–Cas9 to generate loss-of-function (CR) lines in the background of GLA4 (a cultivated rice with a partially developed AZ) (Supplemental Figure 3Q). Compared with GLA4, the CR lines showed no significant differences in AZ and panicle type (Supplemental Figure 3A–3I and 3P), but the pedicel fracture surface was relatively rough, especially near the vascular bundle (Supplemental Figure 3J–3O). The BTS of the CR lines was significantly increased, with a corresponding decrease in seed shattering rate (Supplemental Figure 3R and 3S), suggesting that *SHAT3* plays a role after the AZ develops.

SHAT3 encodes a protein from the MFS family with 12 transmembrane α helices, whereas the *shat3* variant has only 5 such helices, as predicted by TMHMM-2.0 (Supplemental Figure 4A–4C). MFS proteins are ubiquitously distributed across organisms, playing roles in the transport of a broad spectrum of ions and solutes across membranes (Yan, 2015). However, the biological functions of MFS proteins containing nodulin-like motifs are still unknown. The SHAT3 protein is localized primarily to the plasma membrane, with cytosolic puncta (Figure 1H).

An *in situ* hybridization assay revealed no obvious signals in young, 6–8-cm panicles; signals became visible in the AZ-P region when panicles reached about 10 cm and persisted until the panicle length reached 20 cm (Figure 1I). To determine the regulatory relationship between *SHAT3* and other dominant genes that regulate shattering, we investigated *SHAT3* expression patterns in GLA4 (an *sh4* mutant line), *shat1*, *shat2*, and Nipponbare (a *qSH1* mutant line) (Zhou et al., 2012). *SHAT3* expression commenced at the bottom of the seed and pedicel at the same time in the different genetic backgrounds (Figure 1I). These data indicate that *SHAT3* transcripts are initially expressed from the In8 stage (inflorescence length, 4–22cm; inflorescence developmental stage characterized by rapid elongation of the rachis and branches) (Itoh et al., 2005) independently of *SH4*, *qSH1*, and *SHAT1*. Interestingly, the

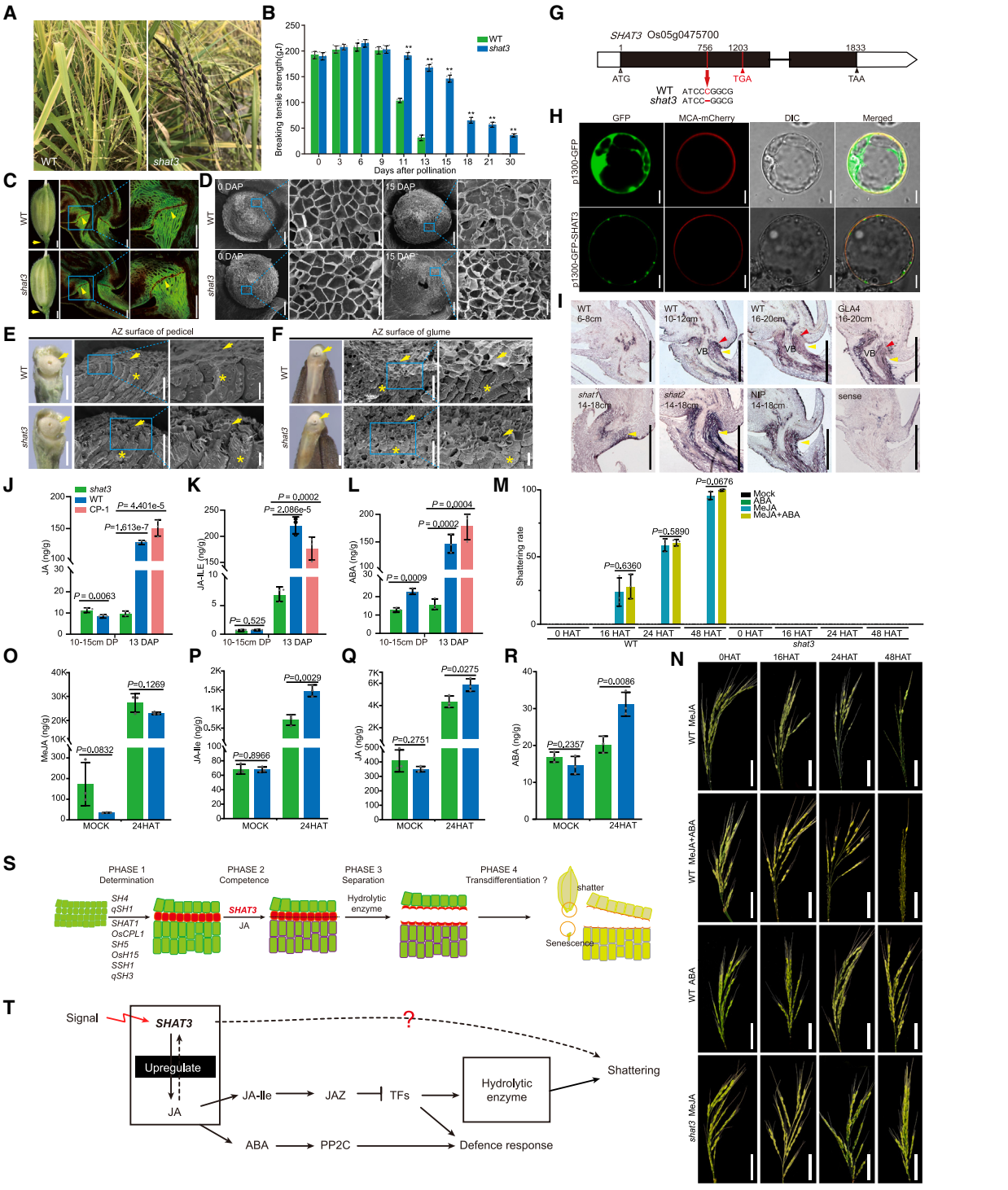

Figure 1. Cloning and functional validation of *SHAT3*.

(A) Phenotypes of WT and *shat3*.

(B) Comparison of breaking tensile strength (BTS) between the WT and *shat3*. The g.f. is the gravitational unit of force. Values are means±SD (n = 6 panicles, ≥25 seeds per panicle). $^{**}P<0.01$, unpaired Student's t test.

(C) Characterization of AZ morphology. The yellow arrows indicate the position of the AZ. The blue boxed regions are enlarged on the right. Bars = 1 mm (spikelets) and 100 μm (fluorescence images).

(D) SEM images of pedicel fracture surfaces of the WT and *shat3* at 0 DAP and 15 DAP. The blue boxed regions are enlarged on the right. Bars = 100 μm and 10 μm (close-up views).

(legend continued on next page)

signal was seemingly absent in AZ parenchyma cells and the vascular bundle (Figure 1I).

Plant hormones affect tissue and organ shedding (Patharkar and Walker, 2019), and gibberellin modulates rice seed shattering by regulating lignin biosynthesis (Wu et al., 2023a; 2023b). We therefore monitored the dynamic changes in phytohormone levels in the AZ-P region of developing and 13-DAP panicles of the WT, *shat3*, and CP lines. Compared with *shat3*, the WT and CP lines accumulated more JA, JA-Ile (jasmonoyl-isoleucine), and ABA (abscisic acid) at the shattering stage (Figure 1J–1L). We then treated the WT and *shat3* plants with MeJA (7.5 mg/mL in lanolin) and ABA (1 mM in water) to observe the effects of these hormones on seed shattering. To avoid the influence of the natural shedding process and clarify the relationship between shedding and maturation, we selected inflorescences less than 7 DAP for treatment. We found that the MeJA and MeJA+ABA treatments led to rapid seed shedding in the WT, and the entire shedding process was complete within 48 h. The MeJA+ABA treatment did not further accelerate the detachment process compared with MeJA alone, and the spikelets displayed a non-shattering phenotype when treated with ABA alone (Figure 1M and 1N). We therefore speculated that ABA does not affect the detachment process. Surprisingly, *shat3* did not complement the shattering phenotype after hormone treatment (Figure 1N and Supplemental Figure 5). After treatment with MeJA, the levels of MeJA, JA, and JA-Ile increased by thousands of times in both the WT and *shat3* (Figure 1O–1Q). The ABA content increased in the WT but was much lower than that observed during the natural shedding process; it showed little change in *shat3* before and after treatment (Figure 1R). These results indicate that JAs, not ABA, promote seed shattering in rice and that *SHAT3* enhances JA accumulation. Although exogenous MeJA can be metabolized to JA and JA-Ile in the mutant, it does not induce shedding. This suggests that both *SHAT3* and JA accumulation may be vital for AZ cell separation.

To gain insights into the likely regulatory network of *SHAT3*, we performed transcriptome analysis, identifying 1620 upregulated and 965 downregulated genes in the AZ-P region of the WT compared with *shat3* at 13 DAP. Gene Ontology analysis showed that the upregulated genes were involved in multiple biological processes, including the JA-mediated signaling pathway and the regulation of wounding and defense responses (Supplemental Figure 6; Supplemental Tables 1 and 2). Consistent with the previous results, genes related to JA synthesis and signal transduction (ZIM domain; JAZ protein) were upregulated during seed shattering (Chini et al., 2007). Exogenous application of MeJA induced the upregulation of cell-wall-related genes and genes encoding glycoside hydrolase enzymes, primarily in the WT (Supplemental Figure 7A and 7B). In addition, some transcription factor genes were upregulated, and *SHAT3* itself appeared to be upregulated by JA (Supplemental Figure 8; Supplemental Table 3). Despite the restoration of JAZ signaling in *shat3*, the phenotype could not be complemented, possibly owing to a missing SHAT3 function such as transport ability. However, microscale thermophoresis experiments showed no binding between the SHAT3 protein and JA, JA-Ile, or ABA, suggesting that the underlying mechanism still needs to be studied.

In summary, the primary function of *SHAT3* is to regulate the dissociation of the rice AZ and control the process of seed abscission by regulating the accumulation of JAs in the AZ-P region. *SHAT3 is* the first gene identified in rice that does not participate in the formation of AZ morphology but does participate in the process of abscission (Figure 1S). *SHAT3* is also the first member of the MFS gene family shown to participate in the regulation of seed shattering. As summarized in Figure 1T, the membrane localized SHAT3 protein may receive a specific signal and induce JA signaling to initiate the hydrolysis process of AZ cells and complete the process of seed shedding. Moreover, initiation of the detachment process requires both *SHAT3* and JA accumulation, although the mechanism remains unknown. In addition, JAs and ABA activate a series of defense responses, which may serve to protect the plants during seed shedding.

ACCESSION NUMBERS

Sequence data for the major genes in this article can be found at GenBank under the following accession numbers: *SHAT3*/*OsMFS3*, Os05g0475700; *SH4*, Os04g0670900; *qSH1*, Os01g0848400; *qSH3*/*OsSh1*/*ObSH3*, Os03g0650000; *SHAT1*, Os04g0649100; *SSH1*/*OsSNB*, Os07g0235800; *OsCPL1*, Os07g0207700; *SH5*, Os05g0455200; *OsMCA1*, Os03g0157300.

FUNDING

This work was funded by the National Natural Science Foundation of China (31788103, 31601281, and 31401367).

ACKNOWLEDGMENTS

We thank Jiqin Li for the resin sections, Zhiping Zhang for scanning electron microscopy observations, and Wenjuan Cai and Shuining Yin (CAS Center

(E and F) Photographs and SEM images showing AZ surfaces of the pedicel and glume in longitudinal sections of WT and *shat3*. The blue boxed regions are enlarged on the right. Yellow arrows indicate the AZ surface; yellow asterisks indicate adjacent sclerenchyma cells. The WT shows a smooth surface, whereas *shat3* shows a jagged and tough fracture surface. Bars = 50 μm and 10 μm (close-up views).
(G) Gene structure of *SHAT3*. Red letter C, mutation site; TGA, premature termination codon.
(H) Subcellular localization of the GFP-SHAT3 fusion protein in rice protoplasts. An OsMCA-mCherry fusion protein was used as a plasma membrane marker (Kurusu et al., 2012), and GFP alone was used as the control. Bars = 20 μm.
(I) *In situ* hybridization of *SHAT3* in different genetic backgrounds. Yellow arrows indicate *SHAT3* expression; red arrows indicate the AZ parenchyma cells; VB, vascular bundles. Bars = 0.5 mm.
(J–L) Contents of JA, JA-ILE, and ABA in the AZ-P region.
(M) Shattering rate of WT spikelets after exogenous application of hormones. HAT, hours after treatment.
(N) Shattering phenotypes of panicles after exogenous application of hormones.
(O–R) Contents of JA, JA-ILE, and ABA in the AZ-P region after exogenous application of Me-JA.
(S) Schematic diagram of the proposed model for AZ separation in rice.
(T) Proposed model of the regulatory network by which *SHAT3* influences abscission in rice.

for Excellence in Molecular Plant Sciences) for support with confocal microscopy observations. We also thank Yao-Guang Liu (South China Agricultural University) for providing the CRISPR–Cas9 vector. Additionally, we thank Jia-Wei Wang, Da-Peng Li, Mingrui Fan (all from CAS Center for Excellence in Molecular Plant Sciences), and Xuehui Huang (Shanghai Normal University) for discussions. No conflict of interest is declared.

AUTHOR CONTRIBUTIONS

B.H., D.L., and B.D. initiated the project and designed all experiments. D.L., B.D., Y.Zhou, J.L., R.S., Q.H., Y.W., Q.F., D.F., C.Z., Y.L., Q.T., and Y.Y. performed the experiments and analyzed the data. Z.G., Z.Z., Y.Zhao, and J.M. performed bioinformatics analyses. B.H., D.L., B.D., Z.W., and H.-X.L. participated in discussions and in revision of the manuscript. D.L. and B.D. wrote the manuscript. B.H. and H.L. revised the manuscript.

SUPPLEMENTAL INFORMATION

Supplemental information is available at *Plant Communications Online*.

Received: October 26, 2024
Revised: December 20, 2024
Accepted: February 10, 2025
Published: February 11, 2025

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https://doi.org/10.1016/j.xplc.2025.101282

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