Transposon Insertion Drove the Loss of Natural Seed Shattering during Foxtail Millet Domestication

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Abstract

Loss of seed shattering was a key step during cereal domestication, and it greatly facilitated seed harvest of the staple cereal foxtail millet (*Setaria italica*) because the cereal has very small seeds. However, the genetic basis for this loss has been largely unknown. Here, we combined comparative and association mapping to identify an 855-bp Harbinger transposable element insertion in the second exon of the foxtail millet gene *shattering1* (*sh1*) that was responsible for the loss of seed shattering. The *sh1* gene encodes zinc finger and YABBY domains. The insert prevents transcription of the second exon, causing partial loss of the zinc finger domain and then loss of natural seed shattering. Specifically, *sh1* functions as a transcription repressor and represses the transcription of genes associated with lignin synthesis in the abscission zone, including *CAD2*. The diversity of *sh1* is highly reduced in foxtail millet, consistent with either a severe domestication bottleneck or a selective sweep. Phylogenetic analysis of *sh1* further revealed a single origin of foxtail millet in China. Our results support the theories that transposons were the most active factors in genome evolution driving loss of natural seed shattering during foxtail millet domestication and that *sh1* underwent parallel selection during domestication across different cereal species.

Key words: **seed shattering, comparative genomics, domestication, transposable element, parallel selection.**

Introduction

Cereals, including rice, maize, wheat, sorghum, and foxtail millet, were domesticated thousands of years ago from wild grass progenitors in different geographic regions (Doebley et al. 2006). A number of phenotypic and physiological traits have been reshaped to give rise to cereals distinct from their wild progenitors. Wild progenitors are characterized by self-propagation, loose plant architecture, and low seed production with generally poor edibility. In contrast, crop cereals are characterized by propagation that is completely dependent on humans, compact plant statures, and high production of grain with a pleasant taste. Among the transitions during cereal domestication, known collectively as the domestication syndrome (Harlan 1992), a crucial and common step is the change from natural seed shattering—the natural and timely shedding of ripe seeds to ensure their dispersal—in wild progenitors to a lack of shattering in domesticated cereals (Lin et al. 2012). Seed shattering allows self-planting and protects the seeds from small animals, especially birds, guaranteeing the self-propagation of wild progenitors. However, seed shattering in agricultural crops causes large production losses and greatly hinders harvest. Therefore, natural seed shattering was eliminated in cereals during domestication.

Foxtail millet is a model drought-tolerant C4 cereal that provides food and animal feed in semi-arid regions (Zhang et al. 2012). Compared with the progenitors of most other cereals, which have bigger seeds, the progenitor of foxtail millet, green millet, has small seeds that easily shatter and are hard to collect. This extremely small size and ease of shattering would have been major obstacles to the domestication of green millet; thus, the elimination of natural seed shattering must have been a key early step during foxtail millet domestication. However, the genetic basis for this change in foxtail millet remains largely unknown.

Seed shattering in cereals arises from the development of an abscission layer between the seed and pedicel (Hodge and Kellogg 2016). The development of the abscission layer is synchronized with the development of the panicle; the cell wall of the abscission layer then breaks down at maturity, and the seed is then shed from the mother plant (Li et al. 2006; Lin et al. 2007). Several genes that played key roles in the elimination of seed shattering during cereal domestication have been identified and cloned. These genes control the development of the abscission layer

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and the breakdown of the cell wall of the abscission layer during seed shattering. *Sha1* (*sh4*), which encodes an MYB domain, was first cloned in rice (Li et al. 2006; Lin et al. 2007). A single nucleotide change in the MYB domain of *Sha1* leads to failure in the breakdown of the abscission layer cell wall. A single nucleotide variant in the promoter region located 12 kilobase (kb) away from the coding region of *qSh1* with a BELL domain results in the loss of the abscission layer between the glume and pedicel in rice (Konishi et al. 2006). Variations in the wheat *Q* gene with an AP2 domain upregulate transcription to eliminate rachis fragility and give rise to free grain threshing (Simons et al. 2006). Sorghum *sh1*, which includes a YABBY domain, harbors multiple causal variants that decrease transcription and knock down gene function, thereby hindering abscission layer development (Lin et al. 2012). The homologs of *sh1* also underwent parallel domestication to decrease seed shattering in rice and maize (Lin et al. 2012). Whether *sh1* regulates seed shattering in other cereal species beyond these three species remains largely unknown. Additionally, the gene regulatory network of *sh1* for seed shattering remains little understood.

Here, we applied comparative and association mapping to identify a transposable element insertion in *sh1* that was responsible for the loss of natural seed shattering during foxtail millet domestication. The *sh1* gene acts as a transcription repressor and represses the expressions of several genes in the lignin synthesis pathway of the abscission layer. During domestication, the transposable element in *sh1* was fixed, potentially due to a selective sweep. Further phylogenetic analysis showed that foxtail millet might have a single origin in China. Our results suggest that transposons played an important role in foxtail millet domestication and that *sh1* was under parallel selection in several cereal species during domestication.

Results

Development of the Abscission Zone Was Repressed in Foxtail Millet during Domestication

A most conspicuous and key step in domestication syndrome in cereal is the loss of seed shattering. In the wild foxtail millet progenitor, green millet (*Setaria viridis*), seeds are naturally shed at maturity (fig. 1*a*), whereas seeds from foxtail millet are not shed naturally but stay firmly attached to the head of the plant (fig. 1*b*). Seed shattering is correlated with the development of the abscission zone, which is present between the seed and the pedicle (Doust, Mauro-Herrera, et al. 2014). To determine how the development of the abscission zone was changed during foxtail millet domestication, we conducted a multiplex histology analysis (fig. 1 and supplementary fig. S1, Supplementary Material online). We prepared sections from spikelets of both green millet and foxtail millet obtained before heading and stained them with toluidine blue and acridine orange. Cells with high lignification are stained green by toluidine blue and false-green by acridine orange. In rice and sorghum, the abscission zone is characterized by a band of cells with low lignification between the pedicle and glume. The abscission layer cells are distinct from the adjacent cells of the glume and pedicle, which have high lignification in these two species (Konishi et al. 2006; Lin et al. 2012). However, we found that the cells in the abscission zone were not distinct from the adjacent cells of the glume and pedicle in either the wild or the domesticated foxtail millets; all three types of cells had low lignification (fig. 1*c*, *d* and supplementary fig. S1, Supplementary Material online). In the seeds naturally shed from green millet, an abscission bowl was present and the abscission layer surface of the pedicle appeared smooth in scanning electron microscopy (SEM) images (fig. 1*e* and *f*). In contrast, in the seeds forcibly detached from the pedicle in domesticated foxtail millet, no abscission bowl was present and the surface of the pedicle cell was rough (fig. 1*g* and *h*). These findings indicated that the development of the abscission zone became inhibited in foxtail millet during domestication.

Loss of Natural Seed Shattering in Foxtail Millet Was Convergently Controlled by *sh1*

A major quantitative trait locus (QTL) for loss of seed shattering was repeatedly mapped to chromosome IX in foxtail millet (fig. 2*a*) (Doust, Lukens, et al. 2014; Odonkor et al. 2018). This major QTL for the loss of seed shattering was situated close to the previously identified *sh1* gene (Lin et al. 2012). To determine whether the *sh1* gene for the loss of seed shattering in domestication syndrome regulates seed shattering in other cereals beyond maize, rice, and sorghum, we performed a comparative genomic analysis across foxtail millet, sorghum, maize, rice, and wheat for this QTL (fig. 2*a*). Comparative genomic mapping identified a highly conserved syntenic chromosomal block, which contained several fragments on rice chromosome 3, sorghum chromosome 1, maize chromosomes 1 and 5, and wheat chromosome 5B. In this syntenic block, most genes and gene orders remained conserved despite two and one reversions on maize chromosome 1 and wheat chromosome 5b, respectively, and a 10-Mb deletion on wheat chromosome 5B (fig. 2*a*). This syntenic block was partially overlapped with the previously identified collinear genomic region based on molecular markers (Devos and Gale 2000) (fig. 2*a*).

To determine whether *sh1* also controlled seed shattering in foxtail millet during domestication, we then compared sequences of *sh1* orthologs in green millet A10 (Mamidi et al. 2020) and the domesticated foxtail millet Yugu1 (Bennetzen et al. 2012) with whole-genome sequences (https://phytozome.jgi.doe.gov/pz/portal.html). The two *sh1* orthologs correspond to Sevir.9G153200 in A10 and Seita.9G154300 in Yugu1. Sequence comparison revealed 24 single nucleotide polymorphisms (SNPs) and seven insertions/deletions (indels) present between Sevir.9G153200 and Seita.9G154300 (supplementary dataset 1, Supplementary Material online). All of these

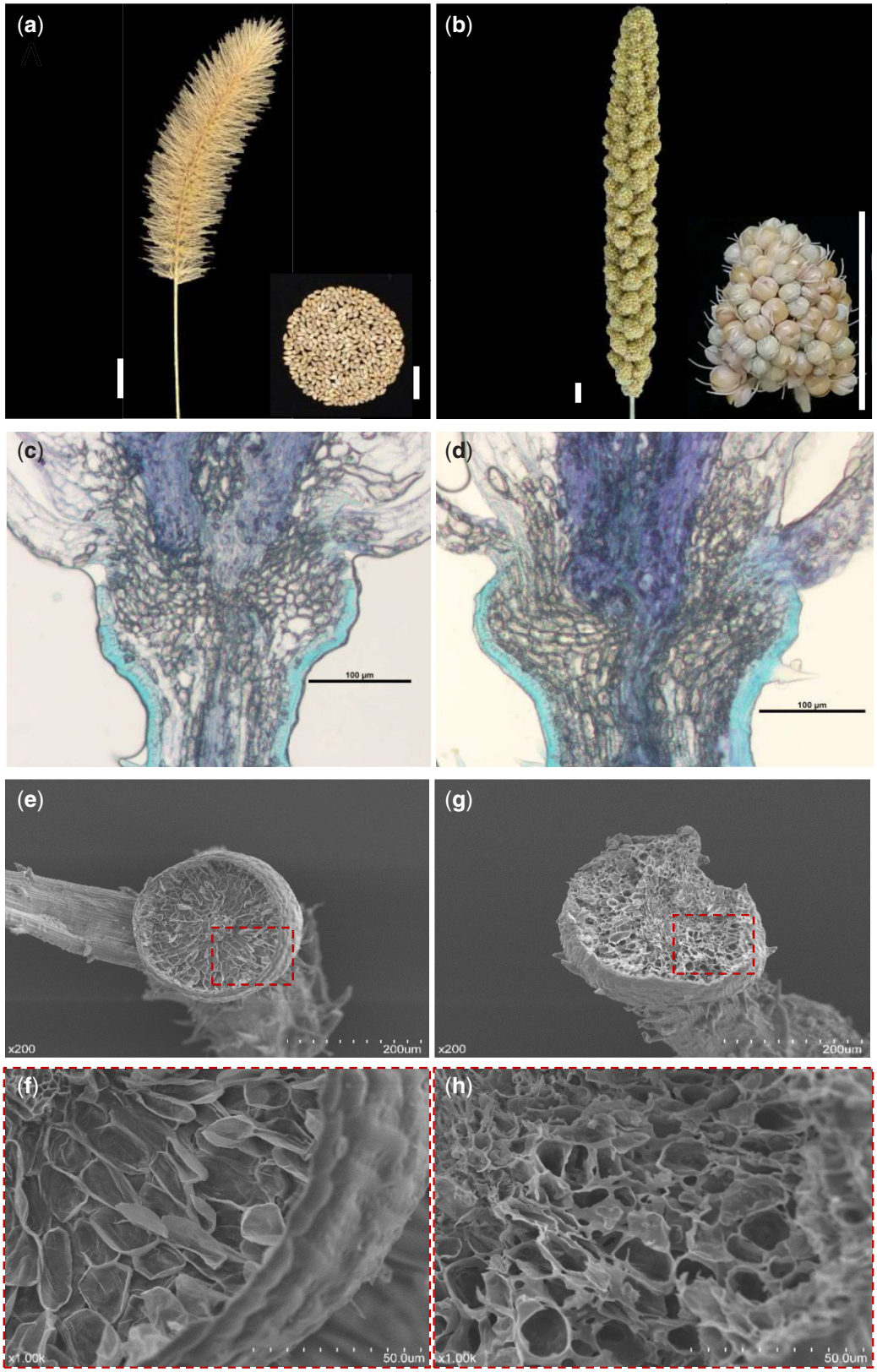

Fig. 1. Phenotype. (*a* and *b*) Seeds are shed freely at maturity from the wild progenitor green millet (*Setaria viridis*) (*a*) but remain firmly attached to the head of the foxtail millet (*Setaria italica*) plant (*b*). Scale bar, 1 cm. (*c* and *d*) Histology analysis revealed that no clear differences are present in longitudinal sections from pedicels of green millet and foxtail millet stained with toluidine blue. (*e*–*h*), Scanning electron microscopy of the surfaces of pedicels from green millet (*e* and *g*) and foxtail millet after forcibly detached from seed (*f* and *h*). (*g* and *h*) Close-up views of the pedicel surfaces, showing that the pedicel surface in green millet is smooth and contains an apparent abscission bowl (*g*), whereas the foxtail millet pedicel lacks the abscission bowl and has a rough surface (*h*). Red dashed lines represent the regions of close-up view.

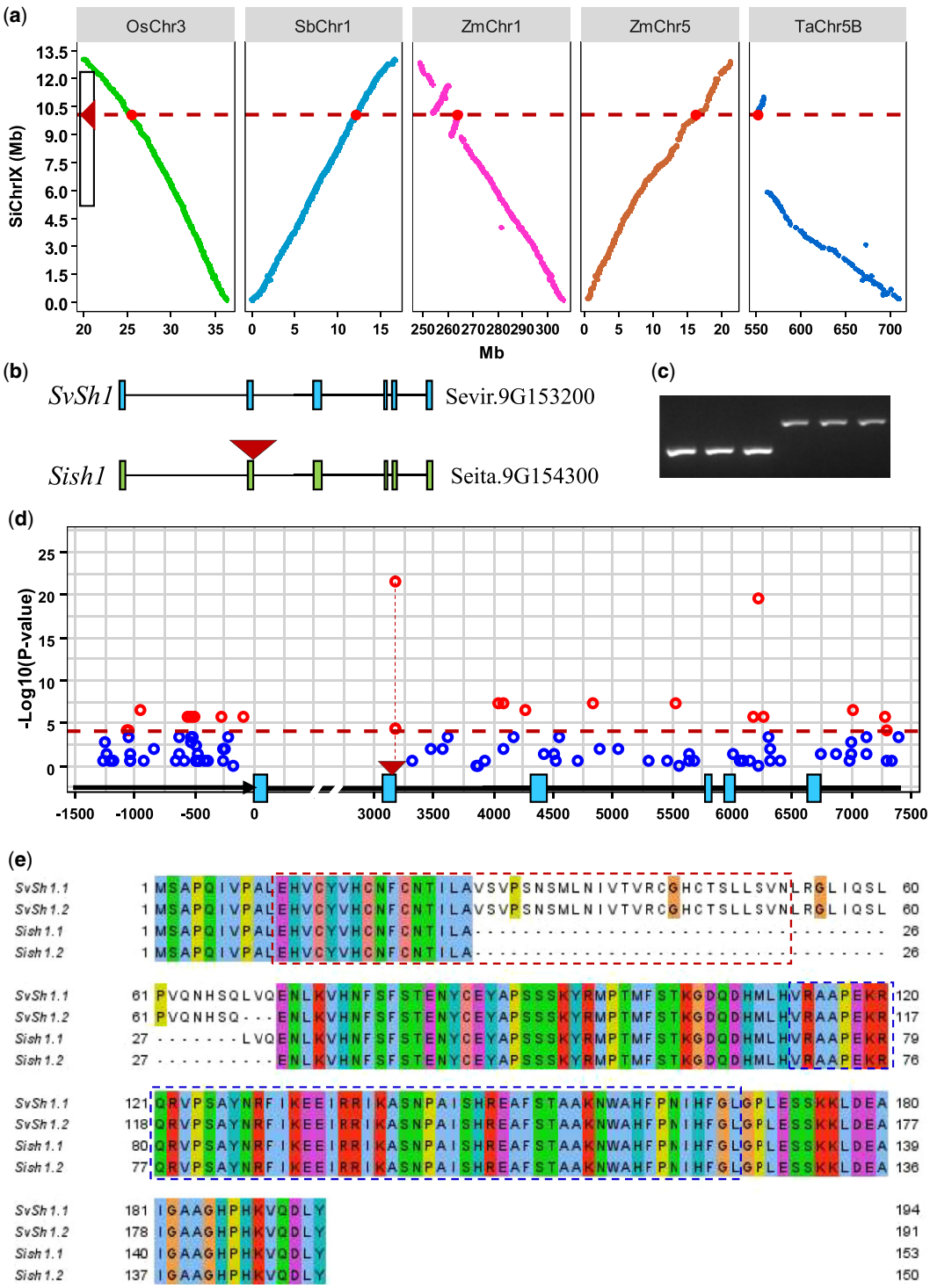

Fig. 2. The *sh1* gene is responsible for the loss of seed shattering in foxtail millet. (*a*) Comparative mapping showed high collinearity of genome sequences at the *sh1* loci among foxtail millet (*Setaria italica*), sorghum (*Sorghum bicolor*), maize (*Zea mays*), rice (*Oryza sativa*), and wheat (*Triticum aestivum*) (*OsChr3*, *SbChr2*, *ZmChr1*, *ZmChr5*, and *Ta Chr5B*, respectively). The position of the QTL for seed shattering in foxtail millet is marked on the *y*-axis. Blank box, QTL interval; triangle, QTL peak. (*b*) Sequence comparison of the *sh1* genes in green millet and foxtail millet. Green and blue boxes, exons; black bars, introns; red triangle, the transposable element insertion. (*c*) The transposable element insertion in *sh1* was absent in three random green millets and present in three random foxtail millets. (*d*) Association mapping showed that the transposable element insertion in the *sh1* gene caused the loss of seed shattering in foxtail millet. The red dashed line represents the threshold of significance at the $P = 0.01$ level with multiple testing correction for association mapping. The gene structure of *sh1* is shown on the *x*-axis, with the start codon set as position "0." Arrow, promoter; blue boxes, exons; black bars, intron and 3′ UTR. (*e*) The *sh1* gene encodes a protein with a zinc finger and a YABBY domain. The zinc finger and YABBY domain are highlighted in red and blue dashed line boxes, respectively.

SNPs and six of the indels were present in the introns (supplementary dataset 1, Supplementary Material online). The only indel located in the second exon was an insertion of the 855-base pair (bp) Harbinger transposable element (fig. 2*b* and *c*, supplementary fig. S2, Supplementary Material online) in the domesticated foxtail millet Yugu1. Sevir.9G153200 contains six exons and five introns and consists of 6,694 bp (fig. 2*b*). We then sequenced a 5,801-bp fragment of this gene from a global foxtail millet panel including 23 wild and 73 domesticated accessions (supplementary table S1, Supplementary Material online). The 5,801-bp fragment harbored a 1,269-bp promoter, an 824-bp 3′ untranslated region (3′ UTR), and a 3,708-bp gene region excluding the first intron with 2,986 bp, which was difficult to amplify due to a high AT content (61%). The large sequencing revealed 96 variants in the 5,801-bp fragment from these foxtail millet accessions (supplementary table S2, Supplementary Material online). We then performed association tests between the variants and phenotypes of seed shattering in the foxtail millet panel, finding that the gene was strongly ($P < 1.0 \times 10^{-4}$) associated with seed shattering in foxtail millet (fig. 2*d* and supplementary fig. S3*a*, Supplementary Material online). The strongest association signal ($P = 3.75 \times 10^{-22}$) was present on the transposable element inserted in the second exon (fig. 2*d*). Another strong signal ($P = 2.81 \times 10^{-20}$) occurred on the SNP at position 6,221, which was in high linkage disequilibrium ($r^2 = 0.89$) with the transposable element (fig. 2*d*, supplementary fig. S3*b* and table S2, Supplementary Material online). All the domesticated foxtail millet accessions examined harbored this transposable element in the second exon and exhibited nonshattering, and all the green millet without this insertion showed shattering (supplementary table S2, Supplementary Material online). These results indicated that *sh1* (Sevir.9G153200) controlled seed shattering and that the insertion of the 855-bp transposable element in the second exon resulted in the loss of natural seed shattering during foxtail millet domestication.

Transcription analysis revealed that the green millet *SvSh1* gene had two transcripts: *SvSh1.1* and *SvSh1.2* (fig. 2*e*). These transcripts differed by the presence or absence of a 9-bp sequence originating from alternative splicing of the 3′ end of the second intron (supplementary fig. S4 and dataset 1, Supplementary Material online). *SvSh1.1* and *SvSh1.2* encoded proteins with 194 and 191 amino acid residues (aa), respectively, with a zinc finger motif at the N terminus and a YABBY domain at the C terminus (fig. 2*e*). In contrast to green millet (A10) *SvSh1*, *Sish1* from the domesticated foxtail millet Yugu1 also had two transcripts with the 9-bp sequence difference (supplementary fig. S4, Supplementary Material online), but the transposable element insertion resulted in the complete loss of the second exon of 123 bp in both transcripts so that more than half of the zinc finger domain was lost in the corresponding *Sish1* proteins (fig. 2*e*). This result suggested that the transposable element insertion caused partial loss of the zinc finger domain of *sh1* and thus led to the loss of natural seed shattering during foxtail millet domestication.

Transgenic Analysis Revealed that *sh1* Controls Abscission Zone Development in Foxtail Millet

To validate whether *sh1* controls seed shattering in foxtail millet, we then conducted transformation through CRISPR/Cas9 and overexpression (fig. 3). Three homozygous editing events (T_1) with a 1-bp insertion and 16-bp and 19-bp deletions in the *sh1* coding sequence (CDS) were obtained from an easily shattering wild foxtail millet accession, ME034v, with a highly efficient transformation (Finley et al. 2021) (fig. 3*a*–*c* and supplementary fig. S5, Supplementary Material online). All three mutations resulted in a gene-frame shift and an early stop in the translation of the *sh1* gene (supplementary fig. S5*b*, Supplementary Material online). In comparison to wild-type ME034v, these three mutants exhibited nonshattering and the breaking tension strengths of seed detachment from the pedicle at maturity were greatly enhanced (fig. 3*b* and *c*). In parallel, we overexpressed the corresponding gene, *Sh1*, from a wild foxtail millet in a domesticated foxtail millet line, Ci846 (fig. 3*d*–*f*). The three overexpression transgenic lines showed extremely high expression of *Sh1*, more than 300-fold greater than in the nontransgenic Ci846 control plant (fig. 3*d*). These overexpression transgenic plants had more tillers, smaller panicles, and especially easy shattering at maturity (fig. 3*e*, *f* and supplementary fig. S6, Supplementary Material online). The breaking tension strengths of seed detachment from the pedicle were dramatically decreased in these three overexpression lines in comparison to the nontransgenic control plant (fig. 3*f*). The abscission bowl was generally absent from the pedicle after the detachment of the seed, and the abscission cell surface was often rougher in the three edited nonfunctional wild foxtail millet lines compared to the wild-type plants (fig. 3*g*–*n*). In contrast, the abscission bowl was restored and the abscission cell surface was smoother when *Sh1* was overexpressed in the domesticated foxtail millet (fig. 3*o*–*v*). These results suggested that *sh1* regulates seed shattering in foxtail millet during domestication via the control of abscission zone development.

sh1 Functions as a Transcription Repressor

The *sh1* gene had extremely low expression levels in the root, stem, and panicle bristle, low expression in the leaf and leaf sheath, and strong expression in the panicle (fig. 4*a*). *sh1* transcripts accumulated in the panicle before anthesis, reached a maximum at the anthesis stage, and then dropped during the grain-filling stage (fig. 4*a*).

Sh1 encodes a transcription factor with a zinc finger and a YABBY domain. To identify the subcellular localization of SH1, we transformed a construct with an SH1–GFP fusion protein into foxtail millet leaf protoplasts from Yugu1 (fig. 4*b*). We detected fluorescent signals from SH1–GFP in the nuclei of leaf protoplast cells (fig. 4*b*). To investigate whether SH1 acts as a transcription factor, we then

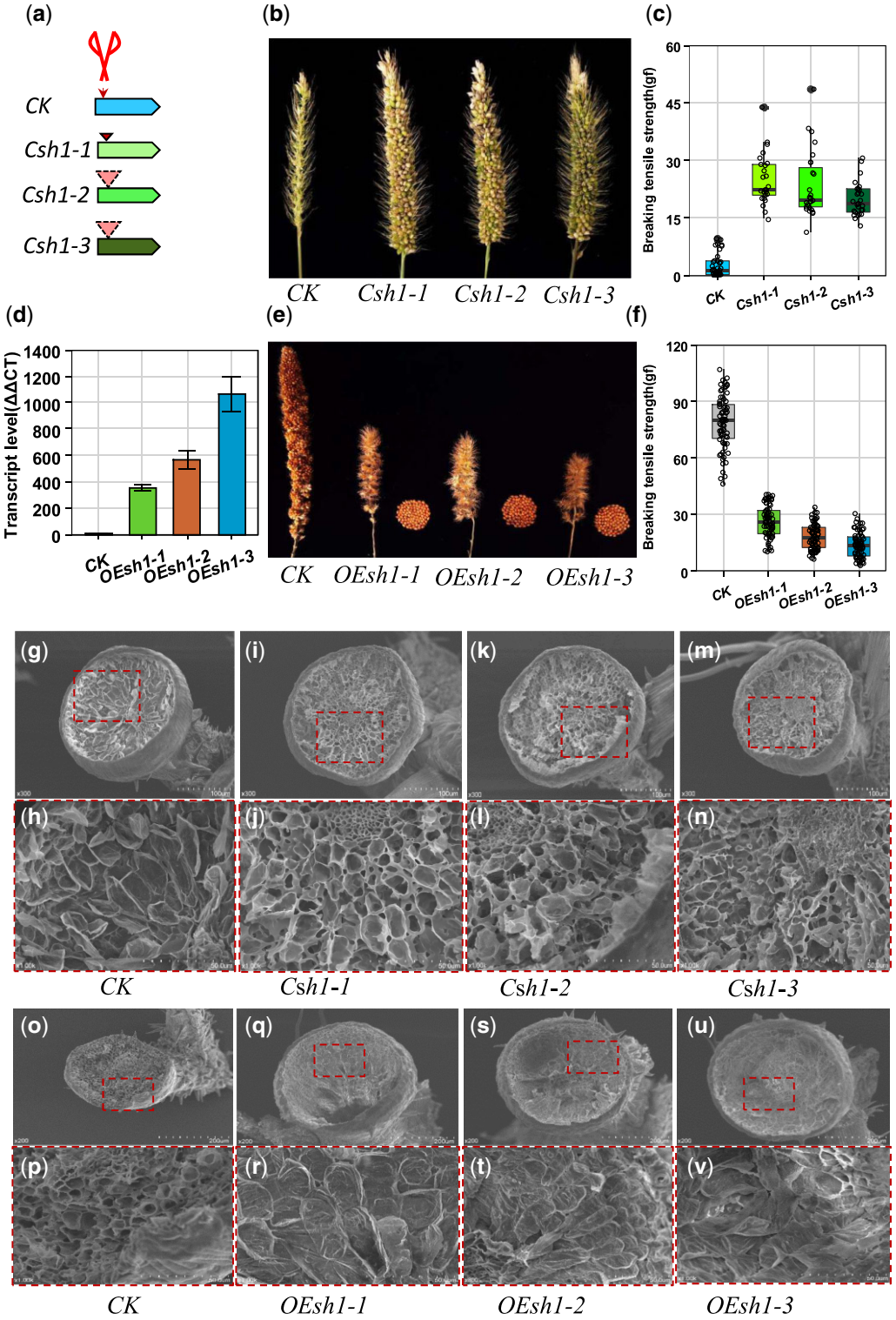

Fig. 3. Transformation through overexpression and CRISPR/Cas9 editing. (*a*) Gene editing of *Sh1* from green millet through CRISPR/Cas9 with two gRNA targets. Three editing events (T_1, *Csh1-1*, *Csh1-2*, and *Csh1-3*) contained a 1-bp insertion, a 16-bp deletion, and a 19-bp deletion in the CDS of *Sh1*, respectively, which resulted in gene-frame shifts and early stoppage of translation. (*b*) The seeds remained firmly on the panicles of the plants from the three editing events (T_1), while all seeds were shed freely from the head of the control green millet plants. (*c*) The breaking tensile strength of seed detachment from the pedicel remained low in the control green millet plant but was greatly enhanced ($P < 0.001$) in the plants descended from the three editing events. The breaking tensile strengths were recorded in gravitational units of force (gf). (*d*) Three overexpression transgenic events were obtained resulting in plants (T_1, *Osh1-1*, *Osh1-2*, and *Osh1-3*) with much higher expressions in comparison to the control foxtail millet plant. (*e*) Plants resulting from the three overexpression events exhibited easy shattering, while the control foxtail millet was nonshattering. (*f*) Breaking tensile strengths of seed detachment from the pedicel were greatly decreased ($P < 0.001$) in the transgenic overexpression plants as compared to the non-transgenic foxtail millet plants. (*g*–*n*) Scanning electron microscopy images of the surfaces of the pedicels from the edited and control green millet plants (*g*, *i*, *k*, and *m*) and close-up views of the surfaces (*h*, *j*, *l*, and n). (*o*–*v*) Scanning electron microscopy images of the surfaces of the pedicels from the overexpression transgenic and control foxtail millet plants (*o*, *q*, *s*, and *u*) and close-up views of the surfaces (*p*, *r*, *t*, and *v*).

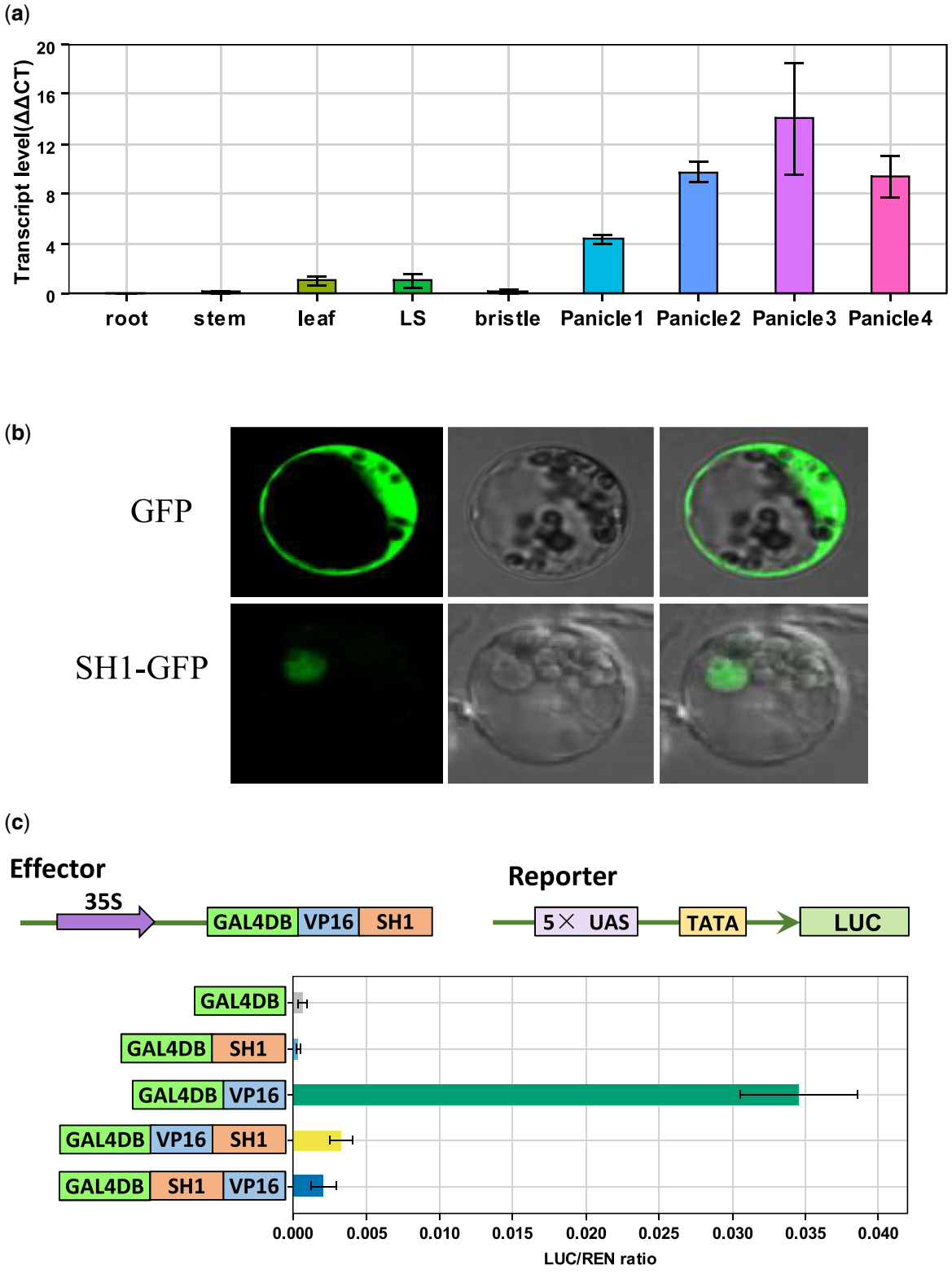

Fig. 4. Gene function analysis of *sh1* in foxtail millet. (*a*) Expression levels of *sh1* in multiple organs including the root, stem, leaf, leaf sheath (LS), bristle, and panicles at different stages (Panicle1, before heading; Panicle2, 3 d after heading; Panicle3, 8 d after heading; Panicle4, grain-filling stage). (*b*) Subcellular localization of the SH1–GFP fusion protein in foxtail millet leaf cells. (*c*) Dual-luciferase transient activity assays determined that SH1 functioned as a transcriptional repressor. The GAL4DB–VP16–SH1 fusion protein dramatically ($P = 6.9 \times 10^{-5}$) repressed luciferase activity in comparison to the control protein GAL4DB–VP16. **, strongly significant; error bars, SD ($n = 3$).

conducted transcriptional activity assays using the luciferase and yeast two-hybrid systems (fig. 4*c* and supplementary fig. S7, Supplementary Material online). The effector construct consisted of chimeric proteins to fuse SH1 to the DNA-binding domain from the yeast GAL4 gene (GAL4DB) and the activation domain from

herpes simplex virus protein 16 (VP16). The reporter constructs harbored the luciferase reporter gene controlled by a synthetic promoter with five copies of the upstream activating sequence (UAS) from GAL4 and a TATA box (fig. 4c). GAL4DB–VP16 greatly enhanced luciferase activities, whereas the fusion protein GAL4DB–VP16–SH1 strongly repressed the activities of the same reporter by over 7-fold (fig. 4c). The results from the yeast two-hybrid system were consistent with those from the luciferase system: The fusion protein of SH1 and the GAL4 DNA-binding domain did not activate the transcription of the reporter in yeast (supplementary fig. S7, Supplementary Material online). These results suggested that SH1 functions as a transcription repressor.

sh1 Represses Expression of Lignin Synthesis Genes in the Abscission Zone

To identify how *sh1* controls the downstream genes related to shattering, we then conducted RNA sequencing (RNA-seq) of samples from the panicles of a transgenic edited plant (*Csh1-1*) with a 1-bp-insertion mutation and the

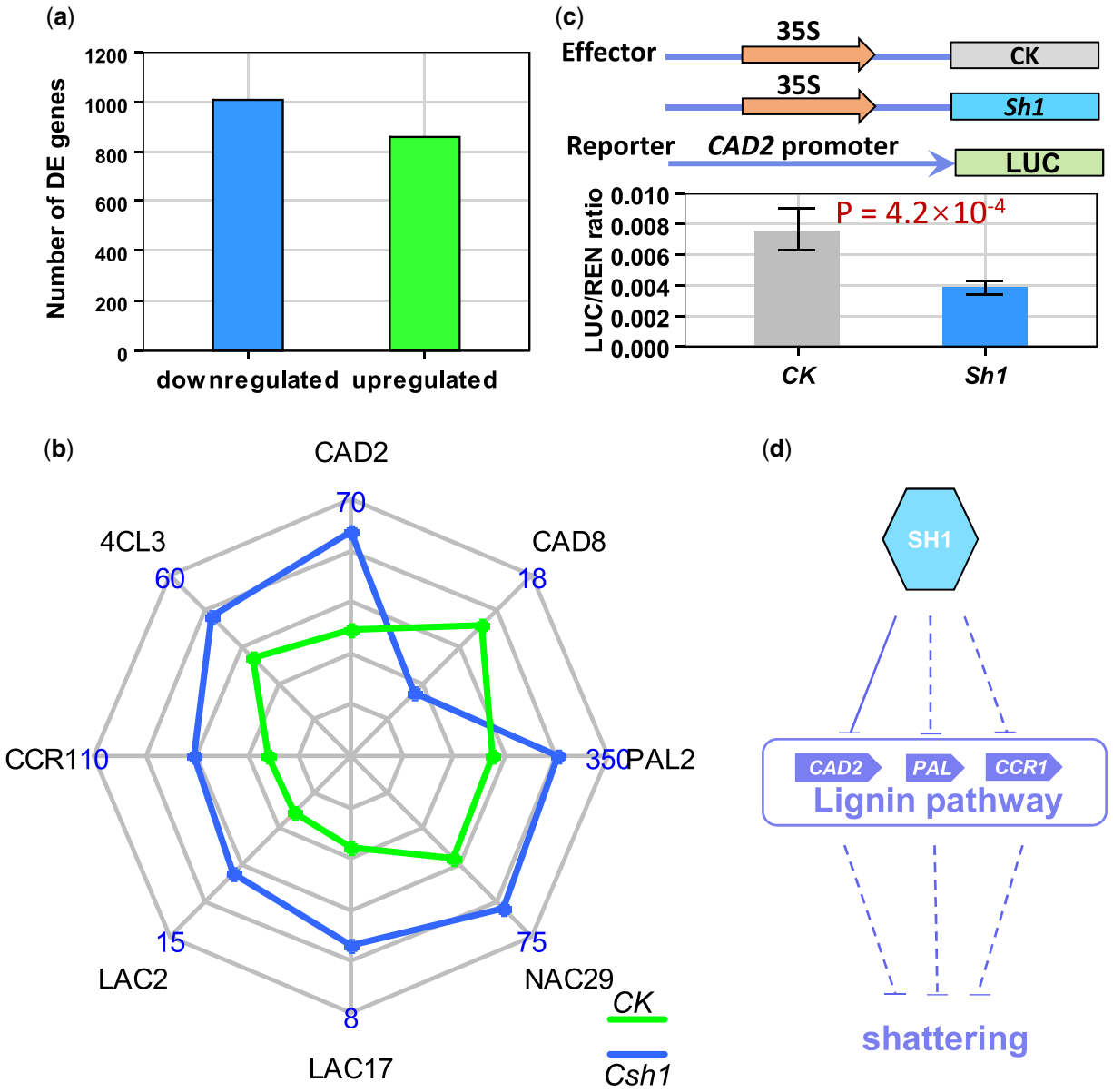

Fig. 5. Regulatory network of *sh1*. (*a*) 1,002 and 860 differentially expressed genes (DEGs) were downregulated and upregulated, respectively, in the panicles from the plants carrying a CRISPR/Cas9-edited *sh1* as compared to the unedited control plant based on RNA-seq. (*b*) Radar chart indicated that seven genes (*CAD2*, *4CL3*, *CCR1*, *LAC2*, *LAC17*, *PAL2*, and *NAC29*) and one gene (*CAD8*) related to lignin synthesis in the abscission zone whose transcription was upregulated and downregulated, respectively, in the edited plant (*Csh1*) compared with the control plant (CK). The numbers on the chart represent the transcription level (fragments per kilobase of exon model per million mapped fragments (FPKM)) based on RNA-seq. (*c*) Dual-luciferase transient activity assay revealed that the transcription of *CAD2* of lignin synthesis in the abscission zone was directly repressed ($P = 4.2 \times 10^{-4}$) by the *sh1* gene. Error bars, SD ($n = 5$). (*d*) Gene regulatory network of *Sh1*. *Sh1* directly and indirectly represses the transcriptions of the genes of lignin synthesis and then activates seed shattering in foxtail millet. Solid and dashed line "T" bars represent direct and indirect repression, respectively.

nontransgenic control plant ME034v. RNA-seq revealed that 1,002 and 860 differentially expressed genes (DEGs) were downregulated and upregulated, respectively, in the edited plant relative to the control (fig. 5*a*, supplementary table S3, Supplementary Material online). A Gene Ontology (GO) analysis (supplementary table S4, Supplementary Material online) through agriGO (Tian et al. 2017) then indicated that a top enriched GO term (FDR $= 8.7 \times 10^{-5}$) was the single-organism metabolic process. Lignin deposition in the abscission cell wall was strongly negatively correlated with shattering (Yoon et al. 2014, 2017). A careful search of the 1,862 DEGs revealed eight DEGs in the lignin synthesis pathway regulated by *sh1*: *CAD2*, *4CL3*, *CCR1*, *LAC2*, *LAC17*, *NAC29*, *PAL2*, and *CAD8* (fig. 5*b* and supplementary fig. S8, Supplementary Material online). Seven of these DEGs (the exception being *CAD8*) were upregulated in the *Csh1-1* plant compared with the control. We also performed dual-luciferase transient expression assays to detect the effect of *sh1* on these eight DEGs (fig. 5*c*). The *sh1* gene showed no direct effect on the transcriptions of any of these eight DEGs except *CAD2*. Compared with the control construct, the effector of *sh1* controlled by the 35S promoter significantly repressed luciferase activity under the control of the *CAD2* promoter (fig. 5*c*). This result indicated that *sh1* directly downregulated the expression of *CAD2* via banding to its promoter. Together, the above results indicated that *sh1* represses the expressions of lignin genes, resulting in seed shattering in foxtail millet (fig. 5*d*).

sh1 Shows a Severe Loss of Genetic Diversity and the Transposable Element Insertion Becomes Fixed during Foxtail Millet Domestication

In this study, we identified an 855-bp transposable element insertion in the shattering gene *sh1* in foxtail millet. To determine whether *sh1* was under selection during foxtail millet domestication, we performed a DNA diversity analysis of global foxtail millet accessions including 23 wild and 73 domesticated foxtail millets. Wild foxtail millets had abundant diversity in the promoter and coding regions and the 3′ UTR in the *sh1* gene (fig. 6*a*). However, no DNA diversity was present in any gene region except for a low diversity in the last intron and the 3′ UTR (fig. 6*a*). In total, only 2.3% of the DNA diversity of *sh1* remained in domesticated foxtail millets compared to wild accessions. Tajima's *D* test ($D = -2.1$, $P < 0.05$) suggested that selection might have shaped the *sh1* gene diversity of foxtail millet. Notably, further genome-wide diversity analysis will be needed to confirm this DNA diversity pattern because a severe domestication bottleneck originating from other regions would also induce such a diversity pattern in *sh1*. All 73 of the investigated domesticated foxtail millets harbored this transposable element insertion in *sh1*. These results indicated that *sh1* might be subject to human selection during the domestication of the small-seeded cereal foxtail millet.

Discussion

Transposon Drove the Domestication of Foxtail Millet

Foxtail millet was domesticated approximately 9,000 years ago (Diao and Jia 2017). As this is the cereal crop with small seeds, the first obstacle during its domestication would have been seed shattering, which would have made it extremely difficult to harvest the seeds. The loss of seed shattering must have greatly reduced seed loss and enhanced harvest efficiency and thus been a key early step toward domestication. Plant genomes generally contain numerous repeat sequences, composed mainly of different transposable elements. In maize, transposable elements comprise 85% of the genome (Schnable et al. 2009) and, as the most active factor in the maize genome, they played important roles in the domestication and improvement of this outcrossing species (Clark et al. 2006; Studer et al. 2011; Zhang et al. 2020). In contrast, in foxtail millet, only approximately 50% of the genome consists of transposable elements (Zhang et al. 2012; Mamidi et al. 2020), and it has been less clear whether transposable elements played a role during the domestication of this selfing species. In this study, we identified a transposon that was inserted into the second exon of *sh1* and was responsible for the loss of natural seed shattering during foxtail millet domestication. This indicates that, as in maize, transposons did play an important role during domestication in foxtail millet: a Harbinger transposon drove the loss of seed shattering and thus probably initiated the domestication of this species. When the transposon insertion occurred in the foxtail millet *sh1* gene, human selection might slowly sweep the diversity of *sh1* and the transposable element insertion finally became fixed in this cereal during domestication.

Even though foxtail millet is a selfing species, we detected a gene flow from domesticated foxtail millet to wild green millet in this study: one wild green millet, E28, was grouped with domesticated foxtail millets based on the *sh1* sequence (fig. 6*b* and supplementary table S3, Supplementary Material online). This green millet harbored the transposable element insertion in *sh1* and had nonshattering seeds (supplementary fig. S9, Supplementary Material online).

Presence of a Transposon in *sh1* Supports a Single Origin of Foxtail Millet in China

Foxtail millet was domesticated thousands of years ago. The origin(s) of foxtail millet and the location(s) where foxtail millet was domesticated were debated in the past, with origins in China, Europe, and West Africa variously proposed (Jones 2004; Nasu et al. 2007; Diao and Jia 2017). Recently, many studies consistently revealed a single origin in northern China for foxtail millet (d'Ennequin et al. 2000; Jia, Huang, et al. 2013; Jia, Shi, et al. 2013). Our identification of the role of *sh1* in the loss of shattering in foxtail millet provides a chance to test whether foxtail millet

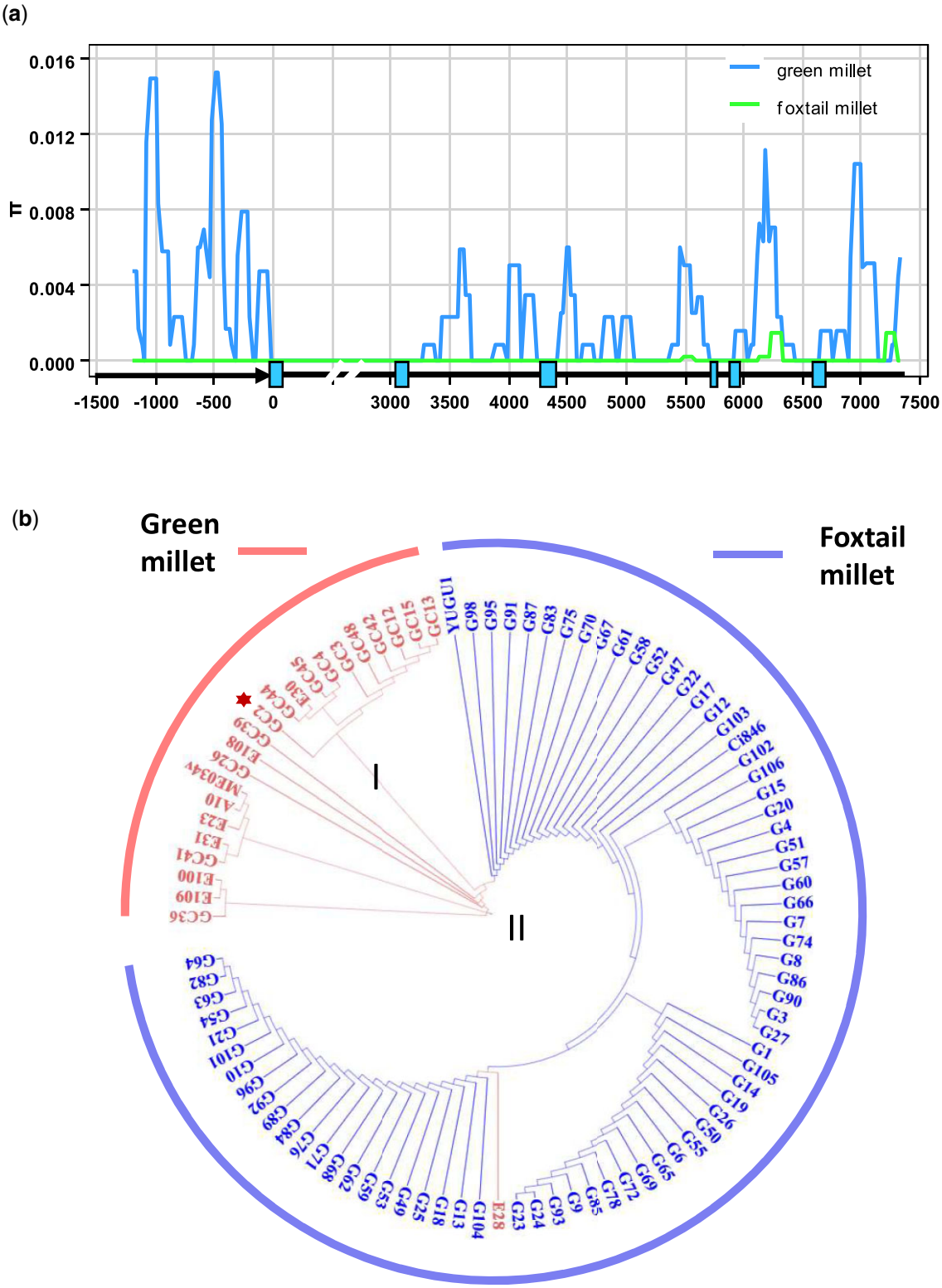

Fig. 6. DNA diversity analysis of *sh1*. (*a*) DNA diversity comparison in the *sh1* gene between green millet and foxtail millet. Gene structure is shown on the *x*-axis. Arrow, promoter; blue boxes, exons; black bars, intron, and 3′ UTR. (*b*) Phylogenetic tree based on the sequence of *sh1* revealed a single origin of foxtail millet. The foxtail millet cluster was derived from a Chinese green millet accession (GC2; red star). Green millet and foxtail millet accessions are marked in pink and blue, respectively. The cluster of green millet split into cluster I and II.

was singly originated in northern China. In this study, we investigated wild green millets collected from China, Europe (including Portugal and Russia), Central Asia (including Mongolia, Afghanistan, and Kazakhstan), West Asia (including Iran and Turkey), and the Americas (including the United States, Canada, and Chile), along with domesticated foxtail millets collected from more than 15 countries around the world (supplementary table S1,

Supplementary Material online). A phylogenetic tree based on the *sh1* gene revealed one main clade in wild green foxtail millets and a second main clade in domesticated foxtail millets. This supports a single origin for foxtail millet. The main wild green millet cluster was then further split into two subgroups, I and II. Subgroup I contained 11 green millets, all from northern China. The main clade of domesticated foxtail millet apparently descended from subgroup I of wild green millet, supporting that foxtail millet was first domesticated in northern China.

Whether Convergent Phenotypic Changes during Cereal Domestication, Diversification, and Improvement Have a Similar Genetic Basis

Staple cereals including rice, wheat, maize, sorghum, and foxtail millet belong to separate species. The genomes of all these species have been well sequenced, and comparative genomic analysis has revealed that many genomic regions are highly conserved across species (Schnable and Lyons 2011; Schnable et al. 2011; Jackson 2016), although these species vary widely in various phenotypes. However, over thousands of years of domestication, diversification, and improvement, human selections have reshaped these cereals into increasingly "similar" ideotypes. However, whether the parallel phenotypic changes during cereal domestication, diversification, and improvement share a similar genetic basis remains largely unknown. In this study, we investigated one common transition during cereal domestication, from seed shattering in wild grasses to nonshattering in domesticated cereals. In previous studies, *sh1* was shown to be responsible for the loss of seed shattering during sorghum, maize, and Asian and African rice domestication (Lin et al. 2012; Lv et al. 2018). Here, our comparative mapping showed that the *sh1* loci have high collinearity across foxtail millet, sorghum, maize, rice, and wheat. An 855-bp transposable element inserted in *sh1* caused nonshattering during foxtail millet domestication, indicating that *sh1* was under parallel selection during domestication across different cereal species. Cereals became better adapted to local environments and evolved into many landraces during diversification. These landraces are generally insensitive to the varying photoperiods in different regions around the world. The key genes *ZmCCT* in maize (Yang et al. 2013) (its ortholog *Ghd7* in rice; Xue et al. 2008); *Hd1*, encoding a CCT domain, in rice, sorghum, and foxtail millet (Yano et al. 2000; Liu, Liu, et al. 2015); and *Zcn8* in maize (Meng et al. 2011) (*hd3a* in rice; Kojima et al. 2002) were generally involved in reshaping flowering time during diversification across different species. Grain yield was the key target of human selection and has been greatly improved during domestication and improvement. Several key genes related to yield improvement, including *tb1* (Studer et al. 2011; Lyu et al. 2020), *tga1* (Wang et al. 2005) (*gw8* in rice; Wang et al. 2012), *tin1* (Zhang et al. 2019) (*prog1* in rice; Tan et al. 2008), *krn1* (Wang et al. 2019) (Q in wheat; Simons et al. 2006), and *krn4* (Liu, Du, et al. 2015) (*ipa1* in rice; Jiao et al. 2010), have undergone parallel selection across the staple cereals. All these above studies support the likelihood that the parallel phenotypic changes during cereal domestication, diversification, and improvement have a similar genetic basis.

Methods

Plant Materials

A foxtail millet panel consisting of 23 wild and 73 domesticated accessions (supplementary table S1, Supplementary Material online) from around the world was grown for phenotyping with three replicates at the China Agricultural University experimental station in Beijing in 2018. Natural shattering and nonshattering phenotypes were investigated 2 weeks after maturity. All green millet and foxtail millet accessions from different regions of the world were obtained from the Germplasm Resources Information Network (http://www.ars-grin.gov/) and the Chinese Crop Germplasm Resources Information System (http://www.cgris.net).

Comparative Mapping

The foxtail millet genomic sequence (of the line Yugu1, id28806) at the *sh1* locus on chromosome IX was compared with maize (B73, id333), rice (Nipponbare, id3), wheat (Chinese Spring, id54192), and sorghum (Tx623, id331) genomes in the comparative genomics database CoGe (http://genomevolution.org/CoGe/). A syntenic map was plotted using R based on the BLAST results for these comparative genomes at the *sh1* locus.

DNA Diversity Analysis

The whole *sh1* gene, including a 1,269-bp promoter region, a 3,708-bp gene body excluding the first intron, and an 824-bp 3′ UTR, was amplified from 23 wild and 73 domesticated accessions. The first intron was excluded for sequencing because of its high AT content (61%), which resulted in the failure of amplification. The resulting PCR products were cleaned with a QIAquick PCR Purification Kit (Qiagen) and then sequenced with Sanger sequencing. The obtained sequences were imported into ClustalW and DnaSPV5.1 to analyze DNA diversity (π) with a sliding window of 100 bp and a step size of 25 bp without a gap, and Tajima's *D* tests were conducted with DnaSPV5.1 (Librado and Rozas 2009) to determine whether the sequences were subjected to selection.

Association Mapping

To determine whether *sh1* was responsible for the loss of shattering in foxtail millet, associations between the variants from 23 wild and 73 domesticated foxtail millet accessions based on sequencing and phenotypes were tested with Fisher's exact test in R because the natural seed shattering trait exhibited a typical quality trait. The significance threshold was determined for multiple testing through Bonferroni correction based on the following

equation: $\alpha' \approx \alpha/n = 1.04 \times 10^{-4}$, where α is the nominal significance threshold ($\alpha = 0.01$) and n is the number of variants ($n = 96$).

Transformation

The wild-type *Sh1* gene from ME034v was edited with two gRNAs (TAGCACACATGCTCCAGCGCCGG and CTGCTGTCAGTGAACTTGAGAGG), targeting the first and second exons of *Sh1* through the pRLG103 vector with the CRISPR/Cas9 system (Čermák et al. 2017) in a wild foxtail millet accession (ME034v) suitable for transformation with natural seed shattering (Finley et al. 2021). The overexpression construct harboring the CDS of *Sh1* driven by the promoter of maize *Ubiquitin* was transformed into a domesticated foxtail millet cultivar (Ci846). The resulting three editing and three overexpressed T_0 transformation events were self-pollinated to generate homozygous T_1 plants. The *Sh1* sequences of all homozygous T_1 transformant plants were determined through Sanger sequencing.

The homozygous T_1 transformation plants were phenotyped 2 weeks after maturity. Each seed was pulled down by a clip linked to a force gauge. The breaking tensile strength was measured at the moment of seed detachment and recorded in gravitational units of force (gf).

Sectioning and Microscopy

Spikelets with pedicels from young panicles (3–4 cm) of wild green millet ME034v and Yugu1 before heading were fixed in 3.7% FAA and then dehydrated in an ethanol series for 1 h each, followed by a Histo-Clear series with ethanol as the solvent. Samples were further treated by replacing one-fourth of the volume of Histoclear/Paraplast mix with new molten Paraplast (Sigma, MO, USA) twice a day for 3 days in a 60 °C oven. Samples were then embedded, sectioned (8 µm thickness) with a Leica RM2265 automated microtome, spread in 37 °C water with a Leica HI1210 water bath, and mounted on microscope slides at 42 °C on a Leica HI1210 slide warmer. Sections were then deparaffinized by a Histo-Clear series, rehydrated in an ethanol series, and then stained with toluidine blue O and acridine orange. The sections were stained with 0.5% toluidine blue O for 30 min, rinsed with water, differentiated in 0.5% glacial acetic acid, rinsed with water again, dehydrated in ethanol, and cleared with Histo-Clear. Images were taken using a Leica biological microscope. In parallel, the sections were stained with 0.01% (w/v) acridine orange for 10 min in the dark, rinsed with water, and then observed using an Olympus FV1000 laser scanning microscope with 488- and 543-nm laser lines.

Scanning Electron Microscopy

Pedicels were collected from wild and domesticated foxtail millet as well as edited and overexpression transformation plants 2 weeks after maturity. The samples were then sputter-coated with gold and palladium for 60 s. After these treatments, the tissues were observed at 15 kV with a SEM (Hitachi S-3400N).

Phylogenetic Analysis

The phylogenetic tree was generated using MEGA7 (Kumar et al. 2016) with the maximum likelihood method based on the DNA alignment of the foxtail millet *sh1* gene.

RNA-seq Analysis

RNA was prepared from the panicles (~5 cm) of the edited transformed plants and the control plants (ME034v) with three biological replicates 2 days after heading. The RNA was then treated with RNase-Free DNase I (D2215, Takara) to remove DNA. The DNA-free RNA samples were subjected to library construction and sequencing on an Illumina HiSeq-2500 platform and resulted in 50 Gb of raw sequencing data. The raw RNA-seq data were treated with a common RNA-seq pipeline. Briefly, the raw RNA-seq reads were trimmed with Trimmomatic (Bolger et al. 2014), cleaned with fastq_clean (Zhang et al. 2014), and then aligned to the green millet reference genome (A10) with STAR (Dobin et al. 2013). Gene expression was further identified based on fragments per kilobase of exon per million fragments mapped (FPKM) with Cufflinks and cuffdiff2 (Trapnell et al. 2014). Finally, DEGs between the two edited plants and the control plant were determined based on their corrected *P*-values (q-value < 0.05).

RT-qPCR

Total RNA was extracted from roots, stems, leaves, leaf sheaths, bristle, and young panicles before heading (3–4 cm), 3 days after heading, 8 days after heading, and at the grain-filling stage using an RNA extraction kit (TianGen Biotech). After cDNA was synthesized with the TransScript-Uni cDNA Synthesis SuperMix (TransGen Biotech), quantitative PCR (qPCR) in three biological and three technical replications was conducted on a Bio-Rad CFX Maestro system using the foxtail millet *Actin* gene (Zhao et al. 2020) as an internal control. The relative expression levels were eventually determined through the ΔΔCT relative quantification method (Livak and Schmittgen 2001).

Luciferase Transient Expression Assay

To identify how the *Sh1* gene regulates the transcription of the genes related with lignin synthesis, promoters from *CAD2*, *CCR1*, *4CL3*, *PAL2*, *LAC2*, *LAC17*, *NAC29*, and *CAD8* (2.5–3-kb upstream fragments from the start codons of these genes from ME034v) were respectively fused with the firefly luciferase reporter gene (*LUC*) in the vector pGreenII 0800-LUC to generate reporter constructs, using the luciferase reporter gene from *Renilla reniformis* as an internal control driven by the 35S cauliflower mosaic virus (CaMV) promoter. The full-length CDS of *Sh1* was cloned into the pGreenII 62-SK vector to generate the effector construct, driven by the 35S CaMV promoter. These

effector and reporter constructs were cotransformed into foxtail millet Yugu1 etiolated leaf protoplasts at the two-leaf stage using the empty effector pGreenII 62-SK and respective reporter construct as a control.

Freshly isolated protoplasts were mixed with 20 μg DNA of the reporter construct in a PEG transfer solution for 18 min at room temperature. The transformed protoplasts were incubated for 16 h at 25 °C, then harvested by centrifugation, lysed in passive lysis buffer (PLB, Promega), and assayed according to the Dual-Luciferase Reporter Assay System (Promega). Three biological replicates of each construct were performed, and all assays were repeated three times.

Subcellular Localization

The CDS of *Sh1* was introduced into the pCAMBIA1300-GFP vector. The construct with the SH1–GFP fusion protein under the control of the 35S promoter was then introduced into Yugu1 leaf protoplasts by PEG. The signal of SH1–GFP was detected with a 488-nm laser line using an Olympus FV1000 laser scanning microscope.

Transcriptional Activity Assay

To identify the transcriptional activity of the SH1 protein, a dual-luciferase transient expression assay and a Matchmaker GAL4 Two-Hybrid assay (Clontech) were conducted. In the dual-luciferase transient assay, the full-length CDS of *Sh1* was fused with GAL4DB and VP16 to construct the effector *GAL4DB–VP16–SH1*, and a promoter with 5× GAL4 UAS sequence and a TATA box was cloned into pGreenII 0800-LUC to generate the reporter. Under the control of the empty effector construct, the reporter and effector constructs were cointroduced into foxtail millet (Yugu1) leaf protoplasts. In the two-hybrid assay, the full-length CDSs of *Sh1* and *sh1* were fused with the DNA-binding domain of GAL4 (GAL4BD) in the pGBKT7 vector using the construct containing a transcription factor *ZmCCT* fused with GAL4BD as a positive control and empty pGBKT7 as a negative control. These constructs were transformed into yeast strain AH109 based on the manufacturer's instructions, and the colonies were then diluted and grown onto yeast synthetic drop-out medium without Trp or without Trp, Ade, and His.

Primers

All primers used in this study are listed in supplementary table S5, Supplementary Material online, for details.

Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

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Author Contributions

Z.L.: designed the study. H.L., X.F., L.Z., Y.L., C.Z., J.L., Y.S., X.J., M.X., and L.D.: performed the research. H.L., X.F., and Z.L.: analyzed the data. H.L. and Z.L.: wrote the manuscript.

Data Availability

RNA-seq data were deposited under the accession number PRJNA780032 at the National Center for Biotechnology Information (NCBI).

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