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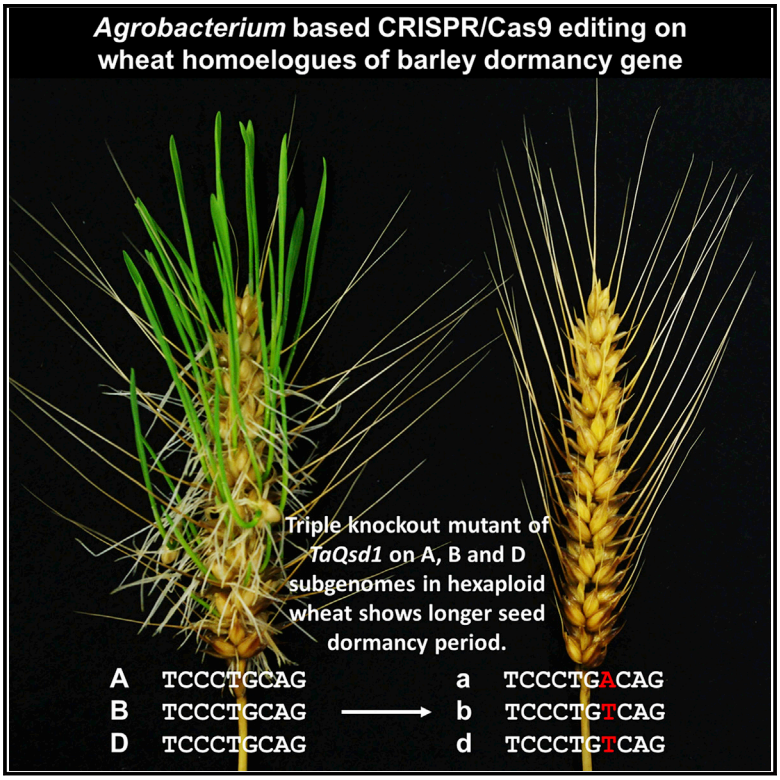
Genome-Edited Triple-Recessive Mutation Alters Seed Dormancy in Wheat

Graphical Abstract



Highlights

- Genome editing via *Agrobacterium*-delivered CRISPR/Cas9 was performed in wheat.
- A triple-knockout mutation of *TaQsd1* was successfully generated.
- Orthologous genes of barley *Qsd1* regulate seed dormancy levels in wheat.
- A transgene-free null-segregant was identified by PCR and whole-genome sequencing.

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In Brief

Using *Agrobacterium*-delivered CRISPR/Cas9, Abe et al. developed a loss-of-function triple mutation of *Qsd1*, which controls seed dormancy in barley, resulting in longer seed dormancy in wheat. This serves as a model technique for the improvement of wheat traits, particularly genetically recessive traits, based on locus information for diploid barley.

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Genome-Edited Triple-Recessive Mutation Alters Seed Dormancy in Wheat

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SUMMARY

Common wheat has three sets of sub-genomes, making mutations difficult to observe, especially for traits controlled by recessive genes. Here, we produced hexaploid wheat lines with loss of function of homeoalleles of *Qsd1*, which controls seed dormancy in barley, by *Agrobacterium*-mediated CRISPR/Cas9. Of the eight transformed wheat events produced, three independent events carrying multiple mutations in wheat *Qsd1* homeoalleles were obtained. Notably, one line had mutations in every homeoallele. We crossed this plant with wild-type cultivar Fielder to generate a transgene-free triple-recessive mutant, as revealed by Mendelian segregation. The mutant showed a significantly longer seed dormancy period than wild-type, which may result in reduced pre-harvest sprouting of grains on spikes. PCR, southern blotting, and whole-genome shotgun sequencing revealed that this segregant lacked transgenes in its genomic sequence. This technique serves as a model for trait improvement in wheat, particularly for genetically recessive traits, based on locus information from diploid barley.

INTRODUCTION

Common wheat (*Triticum aestivum* L., 2*n* = 6*x* = 42, AABBDD) harbors three closely related sub-genomes that were originally derived from three homeologous species. This complex allohexaploid genetic system and the large genome size of common wheat (16 Gbp) (IWGSC, 2018) represent obstacles for targeted gene analysis of this crop. Traits controlled by recessive genes are particularly difficult to observe due to their multiple homeologs.

Genome editing with sequence-specific nucleases is a promising approach for obtaining knockout mutations in a target gene sequence in various crop species (Voytas and Gao, 2014; Zhang et al., 2018b). The CRISPR/CRISPR-associated protein 9 (Cas9) system is the most commonly employed genome-editing system due to the ease of vector construction and its applicability to any animal or plant species. During CRISPR/Cas9 gene editing in plants, exogenous DNA including expression cassettes is delivered into the plant chromosomes by biolistics or *Agrobacterium tumefaciens*-mediated transformation. The Cas9 nuclease then cleaves the target sites and induces mutations (Bortesi and Fischer, 2015). The biolistic-based CRISPR/Cas9 system is frequently employed in wheat using plasmid DNA, RNA, or RNA-Cas9 protein complexes (Wang et al., 2014; Zhang et al., 2016; Liang et al., 2017). *Agrobacterium*-meditated transformation is a straightforward approach to plant transformation resulting in the insertion of only one or a few copies of the transgene (Kohli et al., 2003; Travella et al., 2005). Several reports describe the use of *Agrobacterium*-delivered CRISPR/Cas9 for wheat genome editing, despite the low transformation efficiency of this crop (Zhang et al., 2018a, 2019; Howells et al., 2018; Okada et al., 2019).

Seed dormancy is one of the most important agronomic/commercial traits of cereal crops. Grain germination on the spike before harvest (pre-harvest sprouting) causes a serious decrease in grain quality, especially in Asian monsoon areas. Longer seed dormancy prevents pre-harvest sprouting. Among the dormancy genes reported in small grain cereals, barley *Qsd1* (quantitative trait locus on seed dormancy 1) has the largest effect on dormancy and is frequently identified in mapping populations (Han et al., 1996; Takeda and Hori, 2007). Barley *Qsd1* encodes alanine amino transferase. The loss of function of the *Qsd1* allele in knockdown T2 barley plants exhibited longer seed dormancy periods than the negative control lines (Sato et al., 2016). Due to the closely related genome structures of barley and wheat, many orthologous genes share similar functions, as demonstrated by the seed dormancy gene *Qsd2* in barley (Nakamura et al., 2016) and its ortholog *Phs1* in hexaploid

wheat (Torada et al., 2016). However, orthologous loci of *Qsd1* have not yet been reported in hexaploid wheat, although several mapping populations have been used to detect seed dormancy loci (Flintham et al., 2002; Mori et al., 2005).

Barley *Qsd1* is controlled by a recessive genetic system (Sato et al., 2009). Knockout of homeologous loci in wheat may result in longer dormancy, which is desirable, as it helps to prevent pre-harvest sprouting. Here, we performed genome editing of the homeologous *Qsd1* loci by *Agrobacterium*-mediated CRISPR/Cas9 in wheat and obtained a triple-knockout mutant showing longer dormancy than the wild-type (WT). The aim of this study was to develop a model technique for wheat improvement based on locus information from diploid barley.

RESULTS

Targeted Knockout of *TaQsd1* Using CRISPR/Cas9 via *Agrobacterium*-Mediated Transformation

We previously performed genomic and transcriptomic sequencing of three homeologs, *TaQsd1-A*, *-B*, and *-D* (with 15 exons), in common wheat cultivar (cv.) Fielder, a haplotype amenable to *Agrobacterium*-mediated transformation with a certain level of dormancy under genetically modified (GM) growth chamber conditions (Ashikawa et al., 2014). To perform genome editing of the *TaQsd1* homeoalleles, we initially designed two 20-bp sequence of guide RNAs (gRNAs) at the conserved region among three sub-genomes in exon 14 for use as a target of CRISPR/Cas9 (*TaQsd1_t1* and *TaQsd1_t2* in Figures 1A and S1A). These sequences are common to the A, B, and D sub-genomes and contain restriction enzyme recognition sites located 3 bp upstream of the PAM (protospacer-adjacent motif sequence) and are closely located to the causal SNP in *Qsd1* controlling seed dormancy in barley (see also Method Details). We constructed all-in-one plasmid vectors carrying both gRNA and the *Cas9* gene (Mikami et al., 2015) and introduced them into Fielder via *Agrobacterium*-mediated transformation (Ishida et al., 2015). Since *TaQsd1_t2* showed low activity in an *in vitro* transient assay (Figure S1B) and failed to induce mutations in transgenic plants, we only used *TaQsd1_t1* for subsequent analysis.

Eight independent transgenic transformed generation 0 (T0) plants were successfully developed from 342 explants. Three of the eight carried mutations in the target site, detected in initial screening by PCR-restriction fragment-length polymorphism (RFLP) using the primer set TaQsd1-ABD (Table S1) and the restriction enzyme *Pst*I (T0 plants #1, #2, and #7 in Figure 1B). The sub-genome-specific PCR-RFLP revealed that mutations were present in all A, B, and D homeologs within the three plants (Figure 1B). Sequencing of these mutants confirmed that all mutations were single-nucleotide insertions (Figure 1C). The genotypes of the T0 plants at *TaQsd1* were *aaBbdd* (#1), *AABbDd* (#2), and *AABBDd* (#7) (Figure 1C). All mutations caused frameshifts at the C-terminal regions in the predicted amino acid sequences (Figures 1D, S1C, S1D, and S1E) and maintained the wild-type sequences of Fielder in other regions of the loci. The edited *TaQsd1-A*, *-B*, and *-D* sequences were confirmed to be localized to the respective positions on chromosomes 5A, 5B, and 5D of the wheat reference genome (IWGSC, 2018).

Acceleration of Generation Advancement for Homozygous Edited Lines of *TaQsd1_t1*

To obtain a triple-recessive homozygous mutant lacking transgenes (null-segregant) by genetic segregation, we crossed cv. Fielder (*AABBDD*) with *aaBbdd* (#1) (Figure 2A). Of the 158 F1 plants obtained, 11 were negative for *Cas9*, as revealed by PCR using the primer set 05 (Table S1), thus giving a segregation ratio of four loci (*Cas9* positive:negative = 15:1; Table S2). Of the 11 individuals, three were triple heterozygotes (*AaBbDd*). Subsequently, a total of 240 F2 individuals were obtained from the triple heterozygotes, which could produce all possible combinations of triple homozygotes: *AABBDD*, *aaBBDD*, *AAbbDD*, *AABBdd*, *aabbDD*, *aaBBdd*, *AAbbdd*, and *aabbdd*. All homozygotes appeared at a probability of 1/64 via Mendelian segregation of triple loci. Figure S2 shows an example of a gel image of marker segregation for homeoalleles in the A sub-genome. We accelerated the process of F1 and F2 generation advancement using embryo culture to shorten the grain-filling period (Figure 2B), thereby developing a triple-homozygous mutant in only 14 months (Figure 2A).

Triple-Recessive Homozygous Mutant Showed Clearly Longer Seed Dormancy

We evaluated the phenotypes of the eight triple homozygotes. No visible phenotypic differences, e.g., plant height, were detected in these eight homozygous lines (Figure 3A; Table S3). The time to 50% seed germination in *aabbdd* was delayed more than 5 days compared to *AABBDD* and other homozygous lines (Figures 3B, 3C, S3A, and S3B). The time required for grains to complete germination was more than 30 days for *aabbdd*, whereas this process was mostly completed within 7–8 days for the other homozygotes (Figure 3D). We compared the germination rates of the homozygotes on day 7 (Figure S3B). Plants with the genotype *aabbdd* showed significantly different germination rates from those of the other homozygotes. However, the germination rates of the other single- and double-mutant homozygotes did not significantly differ from that of *AABBDD*.

The Null-Segregant of Edited Triple-Recessive Homozygous Lacked Transgenes Including *Cas9* in Its Genomic Sequence

To determine whether the triple-recessive mutant satisfied the regulatory requirements for genetically modified organisms, we checked for the presence/absence of vector DNA sequence by PCR amplification with primer sets 01–14 (Table S1; Figure 4A). PCR products of transfer DNA (T-DNA) (primer sets 01–07) and other vector sequences (primer sets 08–14) were not obtained for the null-segregant or Fielder (negative control), while T-DNA was detected in T1 plants derived from T0 event #1 (positive control, Figure 4B). Next, we identified the flanking sequences of T-DNA insertions in the T1 plants to estimate their copy numbers and positions in the genome. By sequencing borders of T-DNA insertions, we mapped single or tandem repeat insertions of T-DNA at two locations on chromosome 2D and one location each on chromosomes 3B and 7A of the wheat reference genome (IWGSC, 2018) (Figures S4A–S4C). The number of T-DNA integrated loci supported a 15:1 segregation ratio for

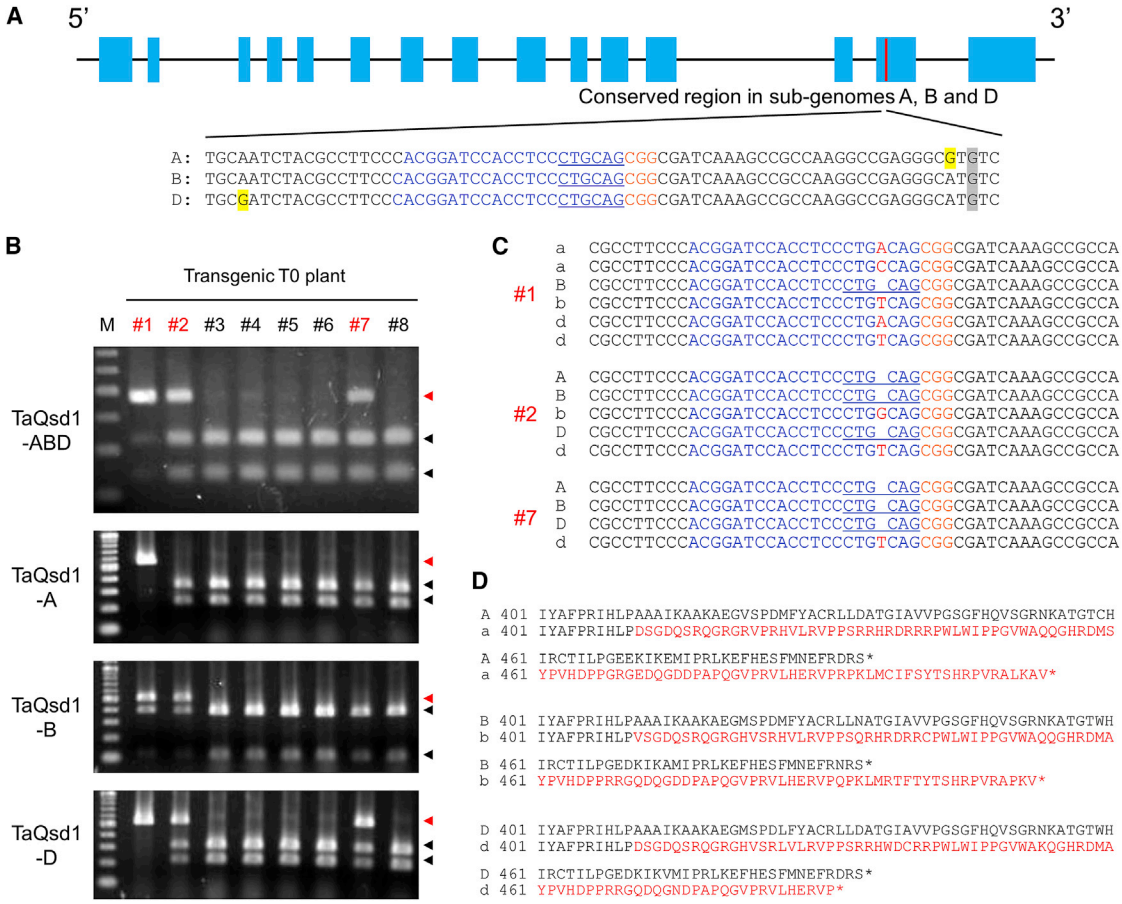

Figure 1. Targeted Knockout of *TaQsd1* Using CRISPR/Cas9 via *Agrobacterium*-Mediated Transformation

(A) Exons of wheat *Qsd1* (*TaQsd1*) homeolog are shown as blue squares, and introns are shown as black lines. A conserved region of exon 14 of the wheat *Qsd1* (*TaQsd1*) homeolog (shown as red line) targeted by the CRISPR/Cas9 system. CRISPR/Cas9-targeted sequence 1 in *TaQsd1* (*TaQsd1_t1*) is shown in blue letters, and the *Pst*I restriction site is underlined. The protospacer-adjacent motif sequence (PAM) is shown in orange, and the SNPs of *TaQsd1* homeoalleles are highlighted in yellow. The mutation position in the barley dormancy orthologous gene (Sato et al., 2016) is highlighted in gray (see also Figures S1A and S1B).

(B) PCR-RFLP analysis of the mutation site at *TaQsd1* using A, B, and D sub-genome-specific or non-specific primers. PCR was conducted with A, B, and D sub-genome-specific (TaQsd1-A, TaQsd1-B, and TaQsd1-D, respectively) or non-specific (TaQsd1-ABD) primer sets (see also Table S1) using the DNA samples derived from eight T0 transgenic plants as template. T0 plant including mutations are indicated in red. Fragment sizes of amplicons are 549, 503, 523, and 186 bp with the primer sets of TaQsd1-A, TaQsd1-B, TaQsd1-D, and TaQsd1-ABD, respectively. Amplicons were digested with restriction enzyme *Pst*I in the Cas9 cleavage site to detect wild-type (digested, black arrowheads) and mutant (undigested, red arrowhead) plants. M indicates 100-bp size marker in TaQsd1-A, TaQsd1-B, and TaQsd1-D and 50-bp size marker in TaQsd1-ABD.

(C) Nucleotide sequences showing genome editing in the *TaQsd1* site induced by *TaQsd1_t1*. Target sequence, *Pst*I restriction site, and PAM are shown as in Figure 1A. Nucleotides inserted are shown in red letters.

(D) Deduced amino acid sequences around the target sites. One base insertion causes a frameshift. The resulting amino acid sequences are shown below in red (see also Figures S1C–S1E).

the presence/absence of transgenes in T0 event #1 (Table S2). To confirm the segregation of these four T-DNA loci, we performed Southern blot analysis using the *hpt* (hygromycin phosphotransferase) gene as a hybridization probe in the null-segregant, WT, and six T0 #1 derived T1 plants positive for all four T-DNA copies. As shown in Figure S4D, no hybridization signals were detected for the null-segregant and WT, whereas positive signals were detected for the T1 plants. Finally, we used whole-genome shotgun sequencing to detect residual short fragments of the transgene in the null-segregant. We obtained shotgun reads at high redundancy (36×) from each of the null-segregant, WT, and T1 (#1–8) plants and mapped these reads on the T-DNA in the vector sequence (Figures 5 and S5). We did not detect any possible T-DNA sequence contamination in null-segregant (Figure S5; Table S4), which is assumed to be an acceptable level to declare the absence of transgenes in the genomic sequence of a null-segregant.

DISCUSSION

Of the vascular plant species, 24% were reported as polyploid (Barker et al., 2016). Genetic analysis of polyploids is a

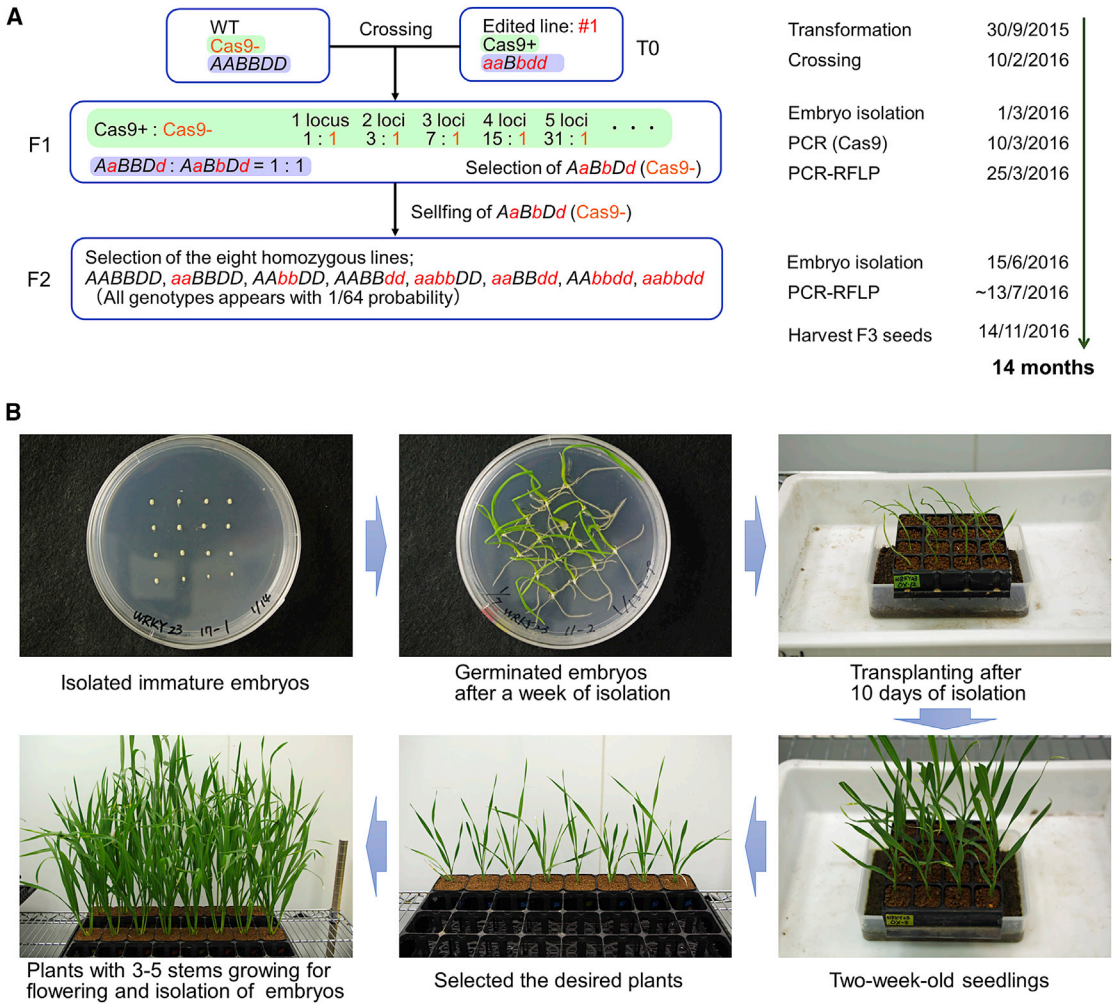

Figure 2. Acceleration of Generation Advancement for Homozygous Edited Lines of *TaQsd1_t1*

(A) Developmental scheme of eight homozygous lines from the cross between T0 #1 (*aaBbdd* for three homeoalleles) with cv. Fielder (*AABBDD*).

(B) Acceleration of generation advancement and genotypic selection (see also Figure S2) with the aid of embryo culture.

complicated process, including both the genotyping and phenotyping of sub-genomes. Thus, most recessively controlled traits in polyploid species have not been explored in detail. An example of a mutant with three mutated homeoalleles in wheat plants is the waxy-grain mutant harboring natural mutations of the *waxy* locus from three sub-genomes (Nakamura et al., 1995). Multiple recessive homeoalleles may also be acquired by inducing mutations and sequencing individuals in a population, a process known as TILLING (targeting induced local lesions in genomes) (McCallum et al., 2000). The development of mutant populations requires several years of generation advancement and the planting of thousands of individuals to harvest seed and DNA samples. After mutants in homeoalleles have been selected, multiple crosses must be performed to obtain a transgene-free multiple-recessive mutant. By contrast, we developed a triple-recessive mutant in hexaploid wheat by *Agrobacterium*-mediated CRISPR/Cas9 genome editing (Figure 2). The major advantage of this method is that multiple mutagenesis of homeoalleles on all three sub-genomes can be performed simultaneously. The mutation of five alleles in T0 event #1 (*aaBbdd*) was an especially efficient way to enable the development of all possible combinations of homeoalleles (Figure 1C). All three T0 mutation-positive plants contained high copy numbers (4–7) of transgenes, whereas the five other mutation-negative T0 plants contained 1–3 copies of the transgenes (Table S2). To obtain mutations in all three homeologs in common wheat, a reasonable number of T-DNAs are required despite the use of *Agrobacterium*-mediated transformation, which might reduce the T-DNA copy number.

Barley and wheat share closely related genomes due to their recent differentiation (8–9 mya) from a common ancestor (Middleton et al., 2014). The gene and genome structures of diploid barley and hexaploid wheat share high similarity (Mayer et al., 2011), which results in the common regulation of traits by orthologous genes, e.g., the vernalization requirement gene *Vrn3* (Yan et al., 2006). Despite the high genomic similarity between barley

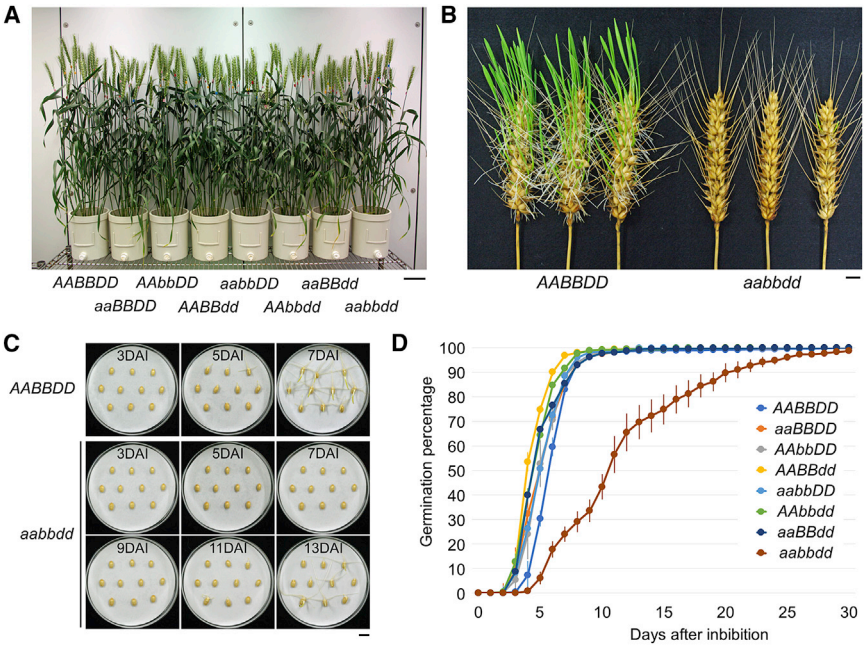

Figure 3. Triple-Recessive Homozygous Mutant Showed Clearly Longer Seed Dormancy

(A) Morphological observation of eight homozygous lines harboring recessive alleles of the homeologs. Plants were grown in a growth chamber and are shown at 21 days after flowering. Scale bar, 10 cm.

(B) Spike samples of triple-dominant (*AABBDD*) and -recessive (*aabbdd*) genotypes allowed to germinate in a dew chamber for 6 days in the dark followed by 7 days in the light at 20°C. Scale bar, 1 cm.

(C) Germination tests of triple-dominant and -recessive genotypes. Seeds were soaked in Petri dishes and allowed to germinate in a growth chamber in the dark at 20°C. Scale bar, 1 cm. (See also Figure S3A.)

(D) Germination behaviors of eight homozygous lines. Four pots (six plants/pot) were used for each genotype as replications (n = 4). Two spikes from each plant, and thus a total of 48 spikes from 24 plants, were subjected to germination tests. The germination percentages of two Petri dishes including 20 seeds (10 seeds from each spike) from each plant were averaged. Data are represented as mean ± SEM. (See also Figure S3B.)

and wheat, it was challenging to focus on the barley seed dormancy gene *Qsd1*, which might be controlled by a recessive genetic system but has never been reported in hexaploid wheat despite the extensive genetic studies of seed dormancy. Barley *Qsd1* is a quantitative trait locus encoding alanine amino transferase (Sato et al., 2016). Loss of function of the *Qsd1* allele in knockdown T2 barley plants led to a germination rate of <5% after 21 days, whereas that of the negative control lines was 80%–90% (Sato et al., 2016). We previously identified the orthologous sequence of *Qsd1* in hexaploid wheat cv. Chinese Spring (IWGSC, 2018; Onishi et al., 2017). However, *Qsd1* orthologs have not been functionally characterized in hexaploid wheat lines harboring spontaneous or induced mutations, likely due to its polyploid nature and the difficulty in collecting all three homeoalleles in a single plant. Here, we successfully performed targeted mutagenesis of all three *TaQsd1* homeologs at high efficiency. The triple-recessive mutant had a significantly longer germination period than other genotypes. These results indicate that a frameshift of the nucleic acid sequence of *TaQsd1* at the 14th exon caused a loss of function of TaQsd1, which only altered the phenotype of the triple mutant. The success of this method for inducing multiple mutations based on the common traits between barley and wheat demonstrates that loci of agronomic importance can be identified through mutation studies in the diploid barley and transferred to wheat. There are likely several other important genes for wheat improvement that could

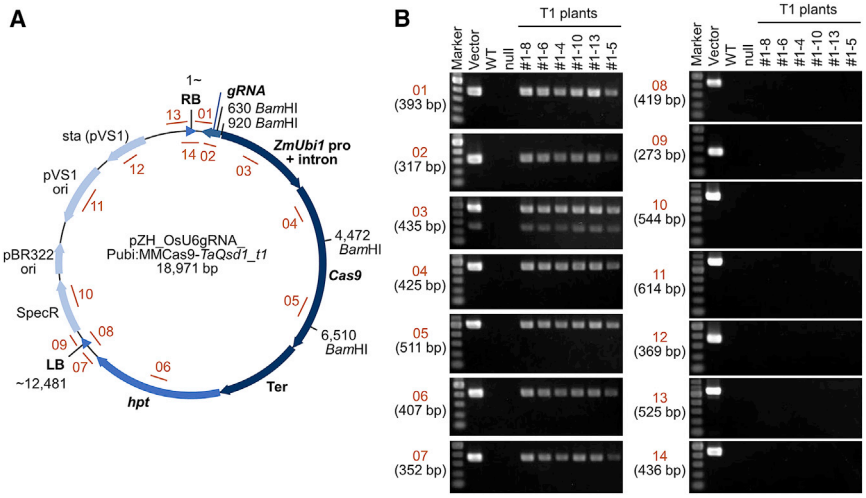

Figure 4. Detection of Vector Sequence in the Null-Segregant, Transgene-Positive T1, and Wild-Type Plants by PCR

(A) Schematic structure of the plasmid vector, pZH_OsU6gRNA_PubiMMCas9-*TaQsd1_t1*, and primer sets 01–14 used for transgene detection (see also Table S1).

(B) Gel images of PCR assays to detect transgene using primer sets 01–14. Wild-type (WT: cv. Fielder), null-segregant (null), and six representative transgene-positive T1 plants were tested (see also Figure S4). Product size is indicated in parentheses. Marker indicates 100-bp size marker.

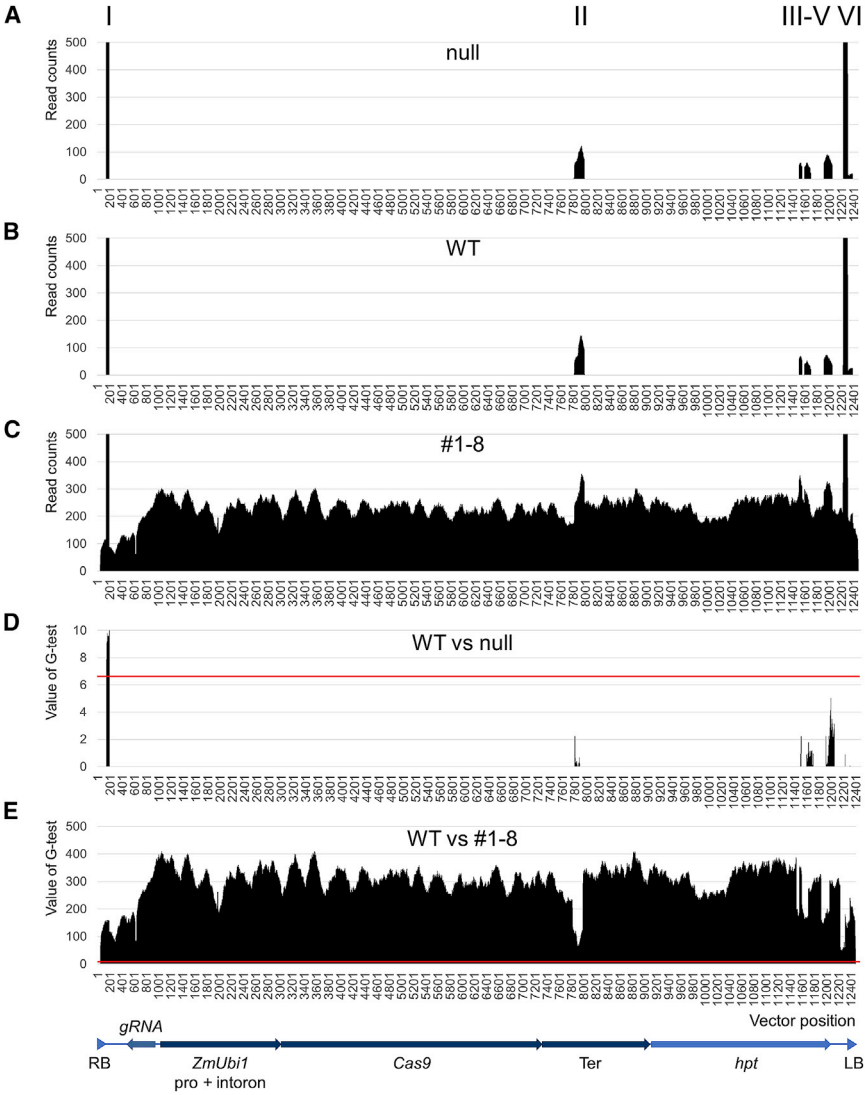

Figure 5. Detection of T-DNA Sequence in Plasmid Vector in the Null-Segregant, Transgene-Positive T1, and Wild-Type Plants by Whole-Genome Shotgun Sequencing Analyses

(A–C) The 36× (612 Gbp) shotgun reads from (A) null-segregant (null), (B) cv. Fielder (WT), and (C) transgene-positive T1 plants (#1–8) are mapped on the T-DNA sequence of the plasmid vector (12,481 bp) used in this study. The horizontal axis shows the sequence position on the vector. Schematic structure of the T-DNA region used in this study is shown in the bottom. The vertical axis shows read counts. (See also Figure S5.)

(D and E) Differences in read counts on the T-DNA sequence are shown between (D) WT and the null, and (E) WT and #1–8. The horizontal axis shows sequence position on the T-DNA. The vertical axis shows the value of the G-test. The 1% level of significance in the G-test is denoted by the red line (6.634). I–VI indicate positions showing peaks on read counts in the null and WT. Annotations of these peaks are shown in Table S4.

be identified using the method described in this report. Successful results have also been reported for the simultaneous editing of three homeoalleles for race non-specific resistance to powdery mildew, a trait that has been widely used in barley breeding, but this trait has not been identified in hexaploid wheat due to its recessive nature (Wang et al., 2014).

The current regulations for genome-edited plants might pose some limitations to trait evaluation in these plants. The delayed germination data obtained for edited wheat plants in the current study (Figure 3) were produced and evaluated in a GM growth chamber with relatively low temperature conditions (20/13°C). We previously obtained germination data at day 7 for Japanese wheat cv. Zenkoji Komugi (15%: tolerant to pre-harvest sprouting) and Fielder (90%) using the same GM growth chamber (Ashikawa et al., 2014). The germination of *aabbdd* (24%) in Figure 3D indicated that the triple mutant might have shorter germination period than Zenkoji Komugi. After breaking dormancy, we confirmed that grains of the triple mutant germinated within 5 days using seed samples that had been stored for over 21 months. Compared to these GM growth chamber conditions, germination is likely to be accelerated in samples grown at higher temperatures in the field. Since the level of dormancy is quite sensitive to the environmental conditions of grain filling period, we plan to carefully evaluate the level of dormancy in genome-edited plants under field conditions in the future.

The avoidance of residual transgenes, i.e., the production of non-genetically modified organisms, might be the most important goal for the practical use of genome editing in crop breeding. In response to the public acceptance of new cultivars generated by genome editing, substantial efforts have been made to optimize the CRISPR/Cas9 system, e.g., the peptide-mediated delivery of gRNA/Cas9 protein (Ramakrishna et al., 2014). In this study, we used a number of techniques to confirm the presence/absence of transgenes to produce transgene-free wheat mutants (Figures 4 and 5). Of these, whole-genome shotgun sequencing of mutant and control wheat plants requires much effort and substantial financial resources due to the large genome size of hexaploid wheat. The techniques that have been used to confirm that edited plants are free from transgenes (Huang et al., 2016) usually require genome sequencing to map the reads from mutant plants.

The read mapping technique used in this study based on the T-DNA sequences in the plasmid vector does not require complete genome sequence information or substantial computations for mapping. Read mapping onto vector sequences is especially useful for polyploid species, since reads are evenly derived from the sub-genomes, and it is not necessary to determine the

genomic positions of the reads, which is often a difficult process. The recent release of a wheat reference genome (IWGSC, 2018) also helped us to precisely identify T-DNA fragments on the genome (Figure S4). We mapped four T-DNA integrations on respective chromosomal positions of sub-genomes by sequencing border junctions of the T-DNA and wheat genome, which was quite difficult before the release of the wheat reference genome due to the sequence similarity among homeologs. Based on all of these results, we are confident that our null-segregant lacked transgenes including *Cas9* in its genomic sequence.

In conclusion, we designed a technique for producing genome-edited triple-recessive mutants in wheat with altered seed dormancy. Perhaps many similarly important genes have not been previously identified in wheat despite extensive genetic studies due to their recessive nature. This strategy could be utilized to study loci underlying traits of agronomic and industrial importance, particularly traits of a genetically recessive nature, in diploid barley and likely other crops for the improvement of new common wheat varieties.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2019.06.090.

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AUTHOR CONTRIBUTIONS

Conceptualization, F.A., H.H., T.T., K.K., and K.S.; Formal Analysis, T.T. and T.H.; Investigation, F.A., E.H., Y.K., and K.O.; Resources, H.H., M.M., and M.E.; Data Curation, T.T. and K.S.; Writing – Original Draft, F.A., E.H., and K.S.; Writing – Review & Editing, F.A., E.H., H.H., and K.S.; Funding Acquisition, F.A., H.H., T.T., K.K., and K.S.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and Virus Strains		
Agrobacterium tumefaciens EHA101	Hood et al. (1986)	N/A
Chemicals, Peptides and Recombinant Proteins		
Hygromycin B	Sigma-Aldrich	Cat# 10843555001
Critical Commercial Assays		
Guide-it Complete sgRNA Screening System	TaKaRa Bio	Cat# 632636
Straight Walk Kit	BEX	Cat# 02-0201
TruSeq DNA PCR-free Library Prep Kit	Illumina	Cat# 20015962
Deposited Data		
NGS Raw Data	This paper	BioProject accession PRJDB7455
Experimental Models: Organisms/Strains		
Triticum aestivum cv. Fielder wild type	National Bio Resource Project_Japan	Accession number KT020-061
T. aestivum cv. Fielder *qsd1-A* mutant (*aaBBDD*)	This paper	N/A
T. aestivum cv. Fielder *qsd1-B* mutant (*AAbbDD*)	This paper	N/A
T. aestivum cv. Fielder *qsd1-D* mutant (*AABBdd*)	This paper	N/A
T. aestivum cv. Fielder *qsd1-A,B* double mutant (*aabbDD*)	This paper	N/A
T. aestivum cv. Fielder *qsd1-B,D* double mutant (*AAbbdd*)	This paper	N/A
T. aestivum cv. Fielder *qsd1-A,D* double mutant (*aaBBdd*)	This paper	N/A
T. aestivum cv. Fielder *qsd1-A,B,D* triple mutant (*aabbdd*)	This paper	N/A
Oligonucleotides		
Primers used in this study, see Table S1	This paper	N/A
Recombinant DNA		
pCR2.1-TOPO vector	ThermoFisher Scientific	Cat# K450001
pGEM-T Easy vector	Promega	Cat# A1360
pZH_OsU6gRNA_MMCas9	Mikami et al. (2015)	N/A
pZH_OsU6gRNA_PubiMMCas9-*TaQsd1_t1*	This paper	N/A
pZH_OsU6gRNA_PubiMMCas9-*TaQsd1_t2*	This paper	N/A
Software and Algorithms		
R 3.5.1	The R project for Statistical Computing	https://www.R-project.org; RRID: SCR_001905
Trimmomatic 0.36	Bolger et al. (2014)	http://www.usadellab.org/cms/index.php?page=trimmomatic; RRID: SCR_011848
Bwa 0.7.15	Li and Durbin (2009)	http://bio-bwa.sourceforge.net/; RRID: CR_010910
Samtools 1.4	Li (2011)	http://samtools.sourceforge.net/; RRID: SCR_002105

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Kazuhiro Sato (kazsato@okayama-u.ac.jp). There are restrictions to the availability of genome edited plants and/or plasmids due to the regulations of National Agriculture and Food Research Organization (NARO) to disclose only after signing MTA of NARO.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Plant material

Seed samples of spring wheat (*Triticum aestivum* L.) cv. Fielder (Accession number. KT020-061) was provided by the National BioResource Project–Wheat, Japan. Wheat plants used in transformation experiments were grown in soil-filled plastic pot in a greenhouse at approximately 15/10°C (day/night) with an 8 h light/16 h dark photoperiod for 12 weeks, followed by their transfer to a controlled environmental chamber maintained at 20/13°C under a 14 h light/10 h dark photoperiod. All transgenic lines and edited homozygous mutant lines listed in KEY RESOURCES TABLE were grown in a controlled environmental chamber maintained at 20/13°C under a 14 h light/10 h dark photoperiod.

METHOD DETAILS

gRNAs and CRISPR/Cas9 vectors

Two gRNA targets (designated as *TaQsd1_t1*: 5′-ACGGATCCACCTCCCTGCAG-3′ and *TaQsd1_t2*: 5′- GCTGCAGGGAGGTGGATCCG-3′) for CRISPR/Cas9 were designed as follows: first, the targets were selected based on the protospacer-adjacent motif (PAM) sequence in the 14th exon of *TaQsd1*, the orthologous region corresponding to a non-synonymous spontaneous mutation in *Qsd1* controlling seed dormancy in barley; second, the targets in the conserved sequences of the wheat A, B and D sub-genomes (Figure 1A) were selected; third, two targets on the cleavage site of gRNA/Cas9, including the recognition sequences of common restriction enzymes (underlined in the gRNA targets in the figure), *Pst*I and *Bam*HI for *TaQsd1_t1* and *TaQsd1_t2*, respectively, were selected for PCR-RFLP analysis.

The CRISPR/Cas9 vectors pZH_OsU6gRNA_PubiMMCas9-*TaQsd1_t1/t2* used in this study were constructed as previously described (Mikami et al., 2015). In brief, the complementary DNA for each gRNA target was cloned into a *Bbs*I-digested site in pU6gRNA-oligo, and the expression cassettes of targets with gRNA scaffold sequences under the control of the *OsU6* promoter were excised with *Pac*I and *Asc*I and cloned into destination vector pZH_gYSA_PubiMMCas9 harboring the maize *ubiquitin* promoter instead of the 2 × 35S promoter in pZH_OsU6gRNA_MMCas9 (Mikami et al., 2015). The all-in-one vectors pZH_OsU6gRNA_PubiMMCas9-*TaQsd1_t1/t2* included expression cassettes for one gRNA (OsU6pro: *TaQsd1*_gRNA: polyT), one Cas9 (ZmUbipro: *Cas9*: T35STnos) and one hygromycin phosphotransferase (*hpt*) gene (CaMV35Spro: *hpt*: Thsp17.3). The *in vitro* cleavage assay was performed using a Guide-it Complete sgRNA Screening System (Takara Bio) according to the manufacturer's instructions with modifications of the volume of gRNA (5 ng) and Cas9 nuclease (100 ng).

Agrobacterium-mediated transformation

Agrobacterium-mediated transformation of wheat (*Triticum aestivum* L.) cv. Fielder was performed using immature embryos as described previously (Ishida et al., 2015). In brief, the plants were grown in a greenhouse at approximately 15/10°C (day/night) with an 8 h light/16 h dark photoperiod for 12 weeks, followed by their transfer to a controlled environmental chamber maintained at 20/13°C under a 14 h light/10 h dark photoperiod. Immature embryos were collected from ripening grains at 17 days after anthesis and co-cultivated with *Agrobacterium tumefaciens* EHA101 (Hood et al., 1986) harboring the CRISPR/Cas9 vectors (pZH_OsU6gRNA_PubiMMCas9-*TaQsd1_t1/t2*) at 23°C for 2 days in the dark. After co-cultivation, the entire axes were removed from the embryos using a fine blade, placed scutellum side up on medium containing carbenicillin at 25°C and incubated for 5 days. The embryos were then cultured on screening medium containing 15 mg/L hygromycin B (Sigma-Aldrich) at 25°C in the dark for 2 weeks, followed by 30 mg/L hygromycin B medium under the same conditions for 2 weeks. Proliferated calli were transferred to regeneration medium and cultured at 25°C under a 14 h light/10 h dark photoperiod for 2 weeks. Regenerated plants were transferred to rooting medium and cultured under the same conditions. Rooted plants were transplanted to soil and cultivated in a growth chamber 20/13°C (day/night) under a 14 h light/10 h dark photoperiod.

PCR analysis for transgenes

Genomic DNA samples were isolated from the leaf tips of regenerated plants as described previously (Taniguchi et al., 2010). The presence of *Cas9* and *hpt* in the regenerated plants was confirmed by PCR using primer sets 05 and 06, respectively (Table S1). The target loci were amplified from plants with transgenes using primer set TaQsd1-ABD, TaQsd1-A, TaQsd1-B and TaQsd1-D (Table S1) for PCR-RFLP analysis. The PCR products were digested with restriction enzymes (*Pst*I and *Bam*HI for *TaQsd1_t1* and *TaQsd1_t2*, respectively) and subjected to agarose gel electrophoresis. The PCR products from candidate genome-edited plants were cloned into the pCR2.1-TOPO vector (Thermo Fisher Scientific) and sequenced on an ABI3130 sequencer (Thermo Fisher Scientific).

Generation advancement for edited lines

The T0 plant of event #1 (genotype *aaBbdd* for sub-genomes) was crossed with cv. Fielder (*AABBDD*). F1 plants with the *AaBbDd* genotype were selfed to develop all possible haplotypes for three homeoalleles. Generation advancement for genotypic selection was accelerated using embryo culture (Figure 2B). In brief, immature embryos of about 15 days after anthesis were aseptically isolated and germinated on half-strength MS medium. After one week, the germinated embryos were transplanted to soil. After 2 weeks

of growth, DNA was extracted from the leaves and examined for the presence/absence of the transgene by PCR using primer sets 05 and 06 (Table S1). Heterozygous genotypes for three homeoalleles were selected by PCR-RFLP as described above. Only transgene-free plants heterozygous for three homeoalleles were retained for cultivation. The culms of these plants were trimmed, leaving 3–5 for subsequent harvesting of immature embryos in the F2 generation. These F2 plants were genotyped, and plants with every possible genotype of the three homeoalleles were selected. Homogeneous F3 lines produced from F2 plants were subjected to seed dormancy scoring.

Seed dormancy assay

The level of seed dormancy in the F3 lines of all genotypes for three homeoalleles were measured as previously reported (Ashikawa et al., 2014), with some modifications. Six healthy seeds per line were directly sown 3-cm deep in a soil-filled 1/5000a Wagner pot. A pot contained 2.58 kg soil comprising a 2:1 mixture of Sakata Soil Mix (Sakata Seed Co.) and Kureha fertilized granulated soil (Kureha Co.). Four pots (six plants/pot) were used for each line as replications (n = 4). The plants were grown in a controlled environmental chamber maintained at 20/13°C (day/night) under 14 h light/10 h dark photoperiod. The pots were watered daily to equal weight (measured on a balance), and their positions were rotated daily.

All tillers with inflorescences were retained for harvest, and seeds from only primary and secondary spikes (12 spikes/pot) were used for the seed dormancy assay. We harvested each plant and accordingly, a total of 48 spikes from 24 plants were tested for each eight line. The seeds were harvested at 60 days after flowering, and 10 seeds harvested from the center of each spike were used for dormancy scoring. Each set of 10 seeds was placed on two sheets of No. 2 filter paper (8.3-cm diameter, Advantec) moistened with 3 mL of water in a 9-cm Petri dish and incubated in the dark at 20°C. A total of 46–48 Petri dishes for each genotype were used for dormancy scoring. To evaluate the level of dormancy, the number of germinated seeds was counted daily and used to calculate the mean and standard error of the germination percentage.

Southern hybridization for transgenes

Genomic DNA was isolated from leaf tissues of edited and control plants using the CTAB protocol (Rogers and Bendich, 1994). Genomic DNA (30 μg) was digested with *Bam*HI, electrophoresed on a 0.9% w/v agarose gel and transferred to a positively charged nylon membrane (Roche Diagnostics). The blot was hybridized with PCR-generated digoxigenin (DIG)-labeled probes produced with primer set 06 (Table S1) and detected by chemiluminescence according to the DIG Application Manual for Filter Hybridization (Roche Diagnostics, Rotkreuz).

Sequences flanking T-DNA insertions

In order to identify the sequences flanking T-DNA insertions, ligation-mediated genome walking (Tsuchiya et al., 2009) was performed using the Straight Walk kit (BEX Co. Ltd.) according to the manufacturer's instructions. In brief, genomic DNA was extracted from T1 plants harboring the transgene as described above and digested with *Spe*I. Partially end-filled DNA fragments generated with Klenow enzyme were ligated to the adaptor. T-DNA specific primers 01R and 14R (Table S1) were used for first and second amplification, respectively. All clear, robust bands were cloned into pGEM-T Easy vector (Promega) and sequenced. To determine the position of the T-DNA insertion, flanking sequences were subjected to BLAST searches against IWGSC RefSeq v1.0 (IWGSC, 2018).

Whole-genome shotgun sequencing

DNA samples from the null-segregant, cv. Fielder (negative control) and the transgene-positive T1 plant #1-8 (positive control) were used to prepare a sequencing library with an insert size of 500 bp with a TruSeq DNA PCR-free Library Prep Kit (Illumina). Each library was sequenced using 151 bp paired-end reads on the HiSeq X platform (Illumina) to generate highly redundant sequences (36x, 612 Gbp).

Transgene sequences from the shotgun reads

Raw reads were processed using Trimmomatic 0.36 (Bolger et al., 2014) to remove low-quality bases with the following option: LEADING:15 TRAILING:15 SLIDINGWINDOW:4:15 MINLEN:32. The trimmed reads were mapped to the insert region of the vector sequence by bwa (0.7.15) (Li and Durbin, 2009) with the option (mem -M -T 30). Using the samtools (1.4) (Li, 2011), multiple hit reads, low mapping quality reads (< 30) and unproper paired reads were discarded.

QUANTITATIVE AND STATISTICAL ANALYSIS

For statistical analysis of seed dormancy, the germination rates of the homozygous lines on day 7 were compared (Figure S3B). Twenty-four plants for each of the eight genotypes were grown in four pots (replicates), each containing six plants, and 10 seeds were sampled from each of two spikes per plant to evaluate germination rates. Statistical analysis was conducted based on the arcsine-transformed score of the average germination rate for six plants in a pot with four replicates, where significance for pairwise differences among eight genotypes was determined by Tukey's HSD test.

In order to analyze the data derived from whole-genome shotgun sequencing, differences of read counts on the vector sequence between WT and the null, and between WT and T1plant #1-8 were evaluated by G-test.

DATA AND CODE AVAILABILITY

The whole-genome shotgun data for the null-segregant, cv. Fielder (negative control) and the transgene-positive T1 plant #1-8 (positive control) have been deposited in the DDBJ Sequence Read Archive under BioProject Accession PRJDB7455.