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Development of an Agrobacterium-delivered CRISPR/Cas9 system for wheat genome editing

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Summary
CRISPR/Cas9 has been widely used for genome editing in many organisms, including important crops like wheat. Despite the tractability in designing CRISPR/Cas9, efficacy in the application of this powerful genome editing tool also depends on DNA delivery methods. In wheat, the biolistics based transformation is the most used method for delivery of the CRISPR/Cas9 complex. Due to the high frequency of gene silencing associated with co-transferred plasmid backbone and low edit rate in wheat, a large T0 transgenic plant population are required for recovery of desired mutations, which poses a bottleneck for many genome editing projects. Here, we report an Agrobacterium-delivered CRISPR/Cas9 system in wheat, which includes a wheat codon optimized Cas9 driven by a maize ubiquitin gene promoter and a guide RNA cassette driven by wheat U6 promoters in a single binary vector. Using this CRISPR/Cas9 system, we have developed 68 edit mutants for four grain-regulatory genes, TaCKX2-1, TaGLW7, TaGW2, and TaGW8, in T0, T1, and T2 generation plants at an average edit rate of 10% without detecting off-target mutations in the most Cas9-active plants. Homozygous mutations can be recovered from a large population in a single generation. Different from most plant species, deletions over 10 bp are the dominant mutation types in wheat. Plants homozygous of 1160-bp deletion in TaCKX2-D1 significantly increased grain number per spikelet. In conclusion, our Agrobacterium-delivered CRISPR/Cas9 system provides an alternative option for wheat genome editing, which requires a small number of transformation events because CRISPR/Cas9 remains active for novel mutations through generations.

Keywords: Agrobacterium mediation, CRISPR/Cas9, genome editing, grain regulation, mutations, wheat.

Introduction
Targeted mutagenesis or precise genomic alteration has long been a dream of biologists and biotechnologists. This dream has come true thanks to the rapid development of genome editing technologies in the last decade using engineered nucleases such as zinc finger nucleases (ZFN) (Carroll, 2011), TAL effector nuclease (TALEN) (Cermak et al., 2011; Li et al., 2011) and Cluster Regularly Interspaced Short Palindromic Repeats (CRISPR)-associated (Cas) systems (Jinek et al., 2012). These systems operate in a similar fashion to create a double strand break (DSB) at a preselected and defined genomic site. The DSB is subsequently repaired by the error-prone non-homologous end joining (NHEJ) or by the error-free homologous recombination (HR) in the presence of a homologous DNA template (Symington and Gautier, 2011). While HR leads to gene correction or replacement, NHEJ causes insertion, deletion and other mutations at the cleavage locus. Engineered ZFN and TALEN nucleases involve selection and assembly of modular DNA binding units and have been successfully applied to plants (Cai et al., 2009; Christian et al., 2013; Clasen et al., 2015; Haun et al., 2014; Li et al., 2011, 2012; Maeder et al., 2008; Mahfouz et al., 2011; Shan et al., 2013a; Wang et al., 2014; Wright et al., 2005; Zhang et al., 2015). In contrast, the CRISPR/Cas9 system consists of a short guide RNA (sgRNA) and a conditional DNA nuclease Cas9 (Jinek et al., 2012). The 20 nucleotides (nt) at the 5’ end of the sgRNA guide the Cas9/sgRNA complex to search for the target sequence along the chromosomal DNA until an exact match is found. If an NGG trinucleotide, the protospacer adjacent motif (PAM), is located immediately downstream of the target site, Cas9 undergoes a conformational change, which activates two separate nuclease domains in Cas9 and leads to cleavage of the target (Jinek et al., 2014). Since the first success in editing genes in cultured human cell lines (Cong et al., 2013; Mali et al., 2013), application of the CRISPR/Cas9 system has been rapidly expanded into plants and ushered a flood of publication in last several years (reviewed in references: Soyars et al., 2018; Weeks et al., 2016). The predominant use of CRISPR/Cas9 technology in plants is to target genes of interest for knockout. More recently, the CRISPR system was engineered by Cas9 nickase fused with cytidine deaminase or adenosine deaminase for highly precise base editing (Gaudelli et al., 2017; Komor et al., 2016; Nishida et al., 2016), which has also been adopted in plants. With these features, the engineered CRISPR systems have been playing an important role...
in the basic understanding of plant biology and crop improvement. Common wheat, bread wheat, or hexaploid wheat (Triticum aestivum L., genomes AABBDD) is the most widely cultivated crop. It provides ~20% of our daily diets and thus plays an important role in global food security and rural economy. To meet the demand for the increasing population, we must increase wheat yield by 50% by the year 2054 (http://iwyp.org/). In this respect, the CRISPR technology is expected to contribute greatly to creating novel variations in wheat. Wheat was one of the plant species first modified by CRISPR/Cas9 (Shan et al., 2013b), and several traits have been targeted for mutations, including powdery mildew resistance (Wang et al., 2014), grain size (Wang et al., 2018; Zhang et al., 2018b), and grain quality (Liang et al., 2017; Sánchez-León et al., 2018; Zhang et al., 2018b). In all these researches, the Cas9 and sgRNA transgenes were delivered by the biolistic bombardment. In recent years, the efficiency of the Agrobacterium-mediated transformation has been improved significantly in wheat (Ishida et al., 2015; Richardson et al., 2014; Wang et al., 2017a). Compared to the biolistics approach, the improved Agrobacterium-mediating systems increased transformation efficiency and expanded the transformability of wheat genotypes. These improvements have not been integrated into CRISPR/Cas9-based genome editing or other genome editing platforms in wheat. During the preparation of this manuscript, a report was published on Agrobacterium-delivered CRISPR/Cas9 for wheat gene editing in which one gene (DA1) was tested in T0 transgenic plants with a high frequency of not yet proven inheritable mutations (Zhang et al., 2018a). In the present research, we developed an Agrobacterium-delivered CRISPR/Cas9 system for genome editing in wheat and applied it to target four grain-size regulatory candidate genes. Our result showed that a small number of transformation events are enough for recovering desired mutations in T0, T1, and T2 plants attributed to the continuing activity of CRISPR/Cas9 transgene through generations.

Results

Development of a CRISPR/Cas9 system for wheat genome editing

We first sought for strong U6 promoters from wheat genome to express guide RNA genes. Promoters from four wheat U6 RNA coding genes, i.e., TaU6.1, TaU6.2, TaU6.3, and TaU6.5 (Appendix S1), were tested individually for their abilities to express the guide RNA gene gGFP, which targets the 1-bp insertion site in the mutated GFP gene in the pUC-35S:GFP+1 for restoration of GFP, together with wheat codon-optimized Cas9 in wheat protoplasts (Figure 1a). To do so, we first evaluated the transfection rate of wheat protoplasts using a pOsUbi-GFP construct as a control. On average, 52.8% of protoplasts were successfully transfected by pOsUbi-GFP (Figure 1b). When transfected with the pUC-35S:GFP+1 construct, in contrast, no GFP signal was observed in the protoplasts (Figure 1c), indicating that the 1-bp frameshift insertion completely abolished the GFP-coding capacity. When transfected together with the U6 promoter-driven guide RNA gene gGFP and the ZmUbi-Cas9, the GFP signal was detected in the protoplasts (Figure 1d-g). After normalization with the transfection rate from pOsUbi-GFP, the desired editing occurred at 47.4%–68.5% in four U6 promoters tested (Figure S1). Among them, TaU6.3 showed significantly higher editing rate (68.5% ± 7.9%) than the rest three U6 promoters (P < 0.01). TaU6.1 ranked second in editing efficiency, but it was not significantly different from TaU6.2 (P > 0.08) and TaU6.5 (P = 0.28). In term of average GFP fluorescence intensity per cell, TaU6.3 also showed the best efficiency among the promoters tested (P < 0.05). This result indicated that the Cas9 functions properly in wheat cells, and TaU6.1 and TaU6.3 are best candidates for expressing guide RNAs in wheat. Thus, TaU6.1 was used to drive the sgRNA1 inserted into the BtgZI sites and TaU6.3 to drive the sgRNA2 inserted into the 8sil sites in pTaRNA4 (Figure S2).

To establish an Agrobacterium-based wheat CRISPR/Cas9 system, we chose a binary vector pLC41 (Japan Tobacco, Tokyo, Japan) that was modified as a Gateway recipient vector. A pENTR derived intermediate vector was constructed to express Cas9 under the strong maize ubiquitin gene promoter and for the construction of guide RNA genes under U6 promoters (Figure S3).

Identification of grain regulatory genes and design of guide RNAs

In a previous study, we identified 45 orthologous loci as candidate genes for wheat grain regulatory genes, 23 of which are negative regulators or expression is negatively regulated by microRNAs (Li and Yang, 2017). From these 23 genes, we selected two negative regulators TaCKX2-1 and TaGW2, and two microRNA-regulated positive regulators TaGLW7 and TaGW8 for the present research, two guide RNAs were designed for each gene to target the sequences conserved among the A, B and D genomes of the hexaploid wheat Fielder (Table S1). For TaCKX2-1 and TaGW2, guide RNA sequences are located in a conserved region of their first exon, and perfectly matched their targets. An SNP was found in the first position of PAM motif (5′-NGG-3′) in TaCKX2-D1 (Figures 2a and 3b), it should not affect the editing efficiency because the nucleotide at that position is degenerate. For TaGLW7 and TaGW8, the guide RNAs target the microRNA recognition sites (Figure 2c,d), the two guide RNA target sequences are overlapped on two strands. For TaGLW7 gene, a single nucleotide mismatch is found in the guide RNA target sequences among the homoeologous genes. Accordingly, two guide RNA genes were designed with sgRNA1 targeting the-TaGLW7-A and sgRNA2 targeting TaGLW7-B and TaGLW7-D (Figure 2c).

Agrobacterium-delivery of CRISPR/Cas9 system into wheat cells

Four CRISPR constructs were individually introduced into Fielder immature embryos through Agrobacterium-mediated gene transfer. After selection and regeneration, eight plants for TaCKX2-1, four for TaGW2, two for TaGLW7, and eight plants for TaGW8 were obtained. Plants grow normally in growth chamber or greenhouse without obvious morphological alteration. PCR screening with Cas9 and sgRNA specific primers identified that all plants except one from TaCKX2-1, contain both Cas9 and sgRNA (Table S2). Gene-specific primers flanking the target region were used successfully to PCR-amplify the expected fragments from all three loci of each gene. However, a T7 endonuclease 1 (T7E1) assay with PCR products did not reveal the expected digested pattern for the InDel (insertion/deletion) mutation. Sanger sequencing of the PCR products confirmed that all examined plants carry only the wild type allele for TaCKX2-1, TaGW2, TaGLW7, and TaGW8.
Detection of mutations in T0 plants by deep sequencing

We were not able to detect a mutation in T0 plants by T7E1 assay and Sanger sequencing directly on PCR products. This result could be due to low edit frequency in wheat as shown in prior studies (Wang et al., 2014, 2018), but somatic mutations may have occurred in the T0 plants. To test this hypothesis, we sequenced the PCR products derived from the TaCKX2-1 target sites by the HiSeq 2000 platform. A total of 1,101,752 raw reads were obtained from the deep sequencing, of which 486,890 reads meet the quality standards. Approximately 48% and 47% of the quality reads were mapped to the TaCKX2-B1 and TaCKX2-D1, respectively, but only 0.2% of them were mapped to the TaCKX2-A1. Both TaCKX2-A1 and TaCKX2-B1 showed 5.2% of mutation rate, while TaCKX2-D1 had a lower mutation frequency of 3.3% (Figure 3). Deletions occurred at much higher frequencies than insertions in all three homoeologous genes. Deletion frequency is 4.0%, 4.6%, and 3.1% for TaCKX2-A1, TaCKX2-B1 and TaCKX2-D1, respectively. By contrast, insertion frequency at these loci are 1.2%, 0.5%, and 0.2%, respectively. There are also rare cases of complex editing (simultaneous deletion and insertion) in TaCKX2-B1 and TaCKX2-D1, but they all happened at <0.05% (Figure 3).

The lengths of the deletion range from 1 to 37 bp in TaCKX2-A1, 1 to 47 bp in TaCKX2-B1, and 1 to 72 bp in TaCKX2-D1. Interestingly, a 37-bp deletion from position 136 to position 172 downstream of the translation start site (ATG) occurs at a high frequency. It is the most abundant mutant allele in TaCKX2-A1 and TaCKX2-B1, with 22.5% and 19.3% of the total mutations, respectively. This deletion, occurred at 10% in TaCKX2-D1, is the third most abundant mutation next to the 1-bp deletion at position 165 (16.7%) and 2-bp deletion at positions 172 and 173 (12.6%). In TaCKX2-A1 and TaCKX2-B1, the 1-bp and 2-bp deletions at 3 bp upstream of the PAM sites of two sgRNAs rank...
Figure 2  Schematics of gene structures with exons (black bars), introns (black lines) and sequences of target sites and guide RNAs. (a and b) Structures of TaCKX2-1 and TaGW2 with target sites in exon 1. Nucleotides in red and green indicate target sites and PAM sequences, respectively. Underlined nucleotides represent the single nucleotide polymorphism (SNP) in three homeologues. Boxed letters indicate the restriction enzyme site. The solid red bars are the conserved region of the target genes. (c) Structures of TaGLW7 with target sites located in 3' UTR and (d) structures of TaGW8 with the target sites in exon 3. Highlighted yellow regions indicate microRNA recognition sites.
in abundance just next to the 37-bp deletion (Figure 4). Overall, the deletion frequency at the sgRNA2 target site is higher than that at the sgRNA1 target site (Figure S4), suggesting that TaU6.3 may be more active in transcription of sgRNA1 or sgRNA2 for TaCKX2-J had the better capability in directing the Cas9-sgRNA binding to the target region. These results showed that our CRISPR/Cas9 system did work in the T0 transgenic plants.

**Screening mutations in T1 generation**

Considering that the CRISPR/Cas9 system functions in the somatic cells of the T2 transgenic plants, we expected mutations would also occur in gametes and embryonic cells, and screened T1 progenies carrying the Cas9 transgene for targeted mutations. To investigate CRISPR/Cas9 induced mutation at the sgRNA target sites, we used three gene-specific primer pairs to amplify each homoeologue, which contained the genomic region of two sgRNA target sites, and Sanger sequencing of PCR amplicons for all T1 plants was performed. The PCR restriction enzyme digestion assay (PCR-RE) and T7E1 cleavage assay were not conducted for two target sites, and gene-specific PCR primer binding sites were within the region of the 1160-bp deletion (complex edit with 880- and 280-bp deletions and a 1-bp substitution) (Table S4) in TaCKX2-D1, it is possible that the mutated allele was initially missed with the primers in the T0 plant #4.

We also screen T1 populations for transgene segregation. Transgenes segregated into 15 (present) to 1 (absent) in TaGLW7_T1-32 and _T1-38 families (P = 0.55698) and into 63 (present) to 1 (absent) in TaGW2_T1-12 family (P = 0.824132), indicating two and three copies of transgenes in the T2 transgenic plants, respectively. From these populations, we identified two transgene-free mutants, one for TaGLW7-A and one for TaGW2-D.

**Inheritance of induced mutations in T2 progenies**

To confirm inheritance of TaCKX2-1 mutations in T2 generation, two T1 lines, #4-1 that contained a 24-bp deletion and a 1-bp substitution mutation in TaCKX2-B1, were allowed to self-pollinate for generation of T2 populations. Totally, 24 T2 individuals were Cas9-positive and carried mutations at the TaCKX2-A1 and TaCKX2-B1. Of 17 T2 plants from #4-1, two were homozygous mutants, eight heterozygous mutants and seven wild type plants (Figure S8a), while two homozygous mutants, four heterozygous mutants, and one wild type plant were identified from TaGW2-A and TaGW2-B. Their segregation also followed a 1:2:1 ratio (P = 0.22313). Sanger sequencing results showed that all the T2 mutation types in the TaCKX2-A1 and TaCKX2-B1 were consistent with their T1 mutations. We screened 48 T2 progenies from the T1 mutants for TaGW2-A and TaGW2-D. Their segregation also followed a 1:2:1 ratio (P > 0.14370). These results indicate that the CRISPR/Cas9 induced mutations are heritable in wheat.

**Edit mutations in the T2 and T3 generations**

We found one new mutation in the T2 progenies of #4-1 (#4-1-9), which was due to a complex deletion of 218, 18 and 13 bp at the
**TaCKX2-A1**

| Sequence                        | WT     |
|---------------------------------|--------|
| GCGACCACCCACCGCACCCCTGGGCCTCCCAACGACTTCGGCAACATCACGCGCGCG | +1 bp (20%) |
| GCGACCACCCACCGCACCCCTGGGCCTCCCAACGACTTCGGCAACATCACGCGCGCG | +1 bp (4.4%) |
| GCGACCACCCACCGCACCCCTGGGCCTCCCAACGACTTCGGCAACATCACGCGCGCG | -1 bp (17%) |
| GCGACCACCCACCGCACCCCTGGGCCTCCCAACGACTTCGGCAACATCACGCGCGCG | -1 bp (11.3%) |
| GCGACCACCCACCGCACCCCTGGGCCTCCCAACGACTTCGGCAACATCACGCGCGCG | -2 bp (8.9%) |
| GCGACCACCCACCGCACCCCTGGGCCTCCCAACGACTTCGGCAACATCACGCGCGCG | -3 bp (4.0%) |
| GCGACCACCCACCGCACCCCTGGGCCTCCCAACGACTTCGGCAACATCACGCGCGCG | -5 bp (4.7%) |
| GCGACCACCCACCGCACCCCTGGGCCTCCCAACGACTTCGGCAACATCACGCGCGCG | -36 bp (4.3%) |
| GCGACCACCCACCGCACCCCTGGGCCTCCCAACGACTTCGGCAACATCACGCGCGCG | -37 bp (19.3%) |
| GCGACCACCCACCGCACCCCTGGGCCTCCCAACGACTTCGGCAACATCACGCGCGCG | -38 bp (1.0%) |

**TaCKX2-B1**

| Sequence                        | WT     |
|---------------------------------|--------|
| GCGACCACCGACGACCGACTTCGGCAACATCACGCGCGCG | +1 bp (20%) |
| GCGACCACCGACGACCGACTTCGGCAACATCACGCGCGCG | +1 bp (4.4%) |
| GCGACCACCGACGACCGACTTCGGCAACATCACGCGCGCG | -1 bp (11.3%) |
| GCGACCACCGACGACCGACTTCGGCAACATCACGCGCGCG | -1 bp (16.7%) |
| GCGACCACCGACGACCGACTTCGGCAACATCACGCGCGCG | -2 bp (12.6%) |
| GCGACCACCGACGACCGACTTCGGCAACATCACGCGCGCG | -5 bp (6.2%) |
| GCGACCACCGACGACCGACTTCGGCAACATCACGCGCGCG | -30 bp (2.8%) |
| GCGACCACCGACGACCGACTTCGGCAACATCACGCGCGCG | -37 bp (10.0%) |

**TaCKX2-D1**

| Sequence                        | WT     |
|---------------------------------|--------|
| GCGACCACCGACGACCGACTTCGGCAACATCACGCGCGCG | +1 bp (20%) |
| GCGACCACCGACGACCGACTTCGGCAACATCACGCGCGCG | +1 bp (4.4%) |
| GCGACCACCGACGACCGACTTCGGCAACATCACGCGCGCG | -1 bp (17%) |
| GCGACCACCGACGACCGACTTCGGCAACATCACGCGCGCG | -1 bp (17%) |
| GCGACCACCGACGACCGACTTCGGCAACATCACGCGCGCG | -8 bp (2.8%) |
| GCGACCACCGACGACCGACTTCGGCAACATCACGCGCGCG | -36 bp (8.5%) |
| GCGACCACCGACGACCGACTTCGGCAACATCACGCGCGCG | -37 bp (22.5%) |

Figure 4 Representative InDel mutations in TaCKX2-1 from T2 transgenic plants. Green letters indicate the PAM sequences. The dashed lines represent nucleotide deletions. Insertion is shown by lower case letters in black. The number in the parenthesis represents the frequency of the mutation type in the total mutations detected.

TaCKX2-D1 locus. No additional mutation was detected in the T2 progenies of line #4-35 due to small population size. To further investigate whether new mutations were induced by CRISPR/Cas9 system in the T2 generation, we screened progenies of three Cas9-positive T1 plants, #4-6, #4-10, and #4-18 for analysis of TaCKX2-1 targets because they produced more seeds than other plants. The T1 plant #4-6 is heterozygous for the 1160-bp deletion in TaCKX2-D1 and is wild type in TaCKX2-A1 and TaCKX-B1 (Figure S7). A total of 180 T2 plants from line #4-6 were screened using PCR-RE (Figure S9). Fifty-one new mutations were identified including 27 in TaCKX2-A1, 18 in TaCKX2-B1, and 6 in TaCKX2-D1 homoeologous loci in addition to the 1160-bp deletion in TaCKX2-D1 (Figure 6; Table S4). While most of the newly identified mutations were heterozygous, two mutations, 1-bp deletion in #4-6-226 and 2-bp deletion in #4-6-47 in TaCKX2-A1 (Figure S9a; Table S6), are homozygous. Because no heterozygotes were found in the T2 population, the homozygous mutations were most likely derived from fertilization of the two independent deletions at the same positions from the egg and sperm cells. From T2 and T3 populations, we recovered 35 homozygous double mutants (mutations in two of the three homoeologous genes) and 33 triple mutants (mutations in all three homoeologous genes), ten of which are homozygous in two homoeologous loci of TaCKX2-1 (Table S7).

We also screened 46 T2 plants from #1-32 line for TaGLW7 mutations using PCR-RNP assay (Figure S10) and found novel mutations in the TaGLW7-A and TaGLW7-D gene. Two independent mutant plants carried the same mutation type of 1-bp insertion in TaGLW7-A, and a novel 5-bp deletion was found in TaGLW7-D. The results suggest that the occurrence of novel mutations may be associated with the continuing activity of the Cas9/gRNA complex.

No off-target mutation detected via Sanger sequencing of PCR-amplicons in a CRISPR active population

We next assessed the potential off-target effect by the wheat CRISPR/Cas9 system. Considering that Cas9 and the guide RNA for TaCKX2-1 were most active to induce on-target mutations in
the #4-6 T2 family (i.e., 51 new mutations in 180 plants), we
CRISPR/Cas9 induced mutations in target genes. Sequencing chromatograms of PCR amplicons from the selected T1 mutants and corresponding
Figure 5
gRNA. The
of this family to assess the off-target effect by this most active
selected progeny, 24
complex and larger polyploid genomes like cotton (Li
PAMs and increase the potential off-target effect in more
also possible that genetic variations from parental plants can alter
methods such as deep sequencing of relevant PCR amplicons. It is
rare mutations that can be detected only by using more sensitive
possible that unintended mutations occur in some somatic cells,
showed that no mutation occurred at these four off-target sites
any sign of double tracing peaks at the target regions. The results
PCR amplicons (Table S8). Individual
the top four of eight loci to design gene and allele specific primers
in the wheat genome, while the remaining six contain two sites,
each of which are triplicated in the wheat genome. We selected
in the wheat genome, while the remaining six contain two sites,
each of which are triplicated in the wheat genome. We selected
the top four of eight loci to design gene and allele specific primers
for PCR amplification of relevant regions (Table S8). Individual
PCR amplicons (n = 192) were subjected to Sanger sequencing,
and the sequencing chromatograms were manually analysed for
PCR amplicons (#4-1)
#4-2
#4-31
#4-35
#4-4

Figure 5 CRISPR/Cas9 induced mutations in target genes. Sequencing chromatograms of PCR amplicons from the selected T1 mutants and corresponding regions are shown in (a–d) TaCKX2-1, TaGW2, TaGLW7, and TaGW8. The PAM sequences and target sequences are highlighted in green and red, respectively. The dashes lines indicate deletion, and the blue letters represent substitution. Black dots indicate nucleotides not shown.

Target sequences plus the PAM motifs were
TaCKX2-1
TaGW2
TaGLW7
TaGW8

Grain numbers increase in TaCKX2-1 mutants
While combining the mutations in the homoeologous copies of an orthologous gene or different orthologous loci, we evaluated the effect of mutation of the 1160-bp deletion in TaCKX2-D1 on grain number per spikelet and spikelet density per spike. Results showed that there was no significant difference in spike length, spikelet number/spike, and spikelet density between the mutant and the wild type segregants from the same T2 population (P > 0.76805). Compared to the wild type, the mutation increased grain number/spike from 42 to 55 (P = 0.03374; Figure 7c,e), grain number/spikelet from 2.45 to 3.25 (P = 0.00198; Figures 7b and 9d) and grain weight per spike from 1.12 to 1.69 g (P = 0.01717; Figure 7f). These results indicate that TaCKX2-1 negatively regulates the trait of grain number in wheat.

Discussion

Agrobacterium delivery of CRISPR/Cas9 system
CRISPR/Cas9 has been the primary choice for plant genome editing, and Agrobacterium-mediated transformation is the most common method for delivery of the CRISPR/Cas9 DNA components to plants (Soyars et al., 2018). Mainly due to historical reason, all genome editing systems in wheat were implemented through the biolistics-mediated transformation (Liang et al.,
Co-transfer of large DNA fragments from the vector backbone by biolistic transformation leads to the high frequency of transgene silencing (Anand et al., 2003; Tassy et al., 2014). A large number of transgenic plants are required for recovery of desired edit mutations in T0 generation. Thus, transformation poses a bottleneck for many wheat genome editing projects and application in wheat breeding. By contrast, Agrobacterium mediation usually generates simple insertion events of the transfer DNA (T-DNA), a DNA fragment defined by the border (left and right) sequences in a binary plasmid, with reduced frequency of transgene silencing (Dai et al., 2001).

Recent progress in improving Agrobacterium-mediated transformation efficiency (Ishida et al., 2015; Richardson et al., 2014) and expanding the spectrum of transformable genotypes (Wang et al., 2017a) makes it a more promising delivery method for wheat genome editing. In the present research, we developed an Agrobacterium-delivered CRISPR/Cas9 system and successfully generated targeted mutations in four grain-regulatory genes. The transgenes remain active and continuously induce site-specific mutations in T1 and T2 generations. Compared to a limited number of T0 transgenic plants, it is much easier to generate large pools of T1 and T2 plants and screen for independent and novel mutations using the efficient and cheap Cas9/gRNA RNP assay. Thus, the Agrobacterium-mediated system provides an alternative option for wheat genome editing, which requires a small number of transformation events but additional generations.

### TaCKX2-3.1-A

| Sequence                                                                 | Type       | Position |
|-------------------------------------------------------------------------|------------|----------|
| GCGACCCCAACGCCAACCTGGCGGCTCTCAACGGACTTGGGCAACATCACGGCGCGCTCC            | WT/WT      |          |
| GCGACACCCCAAC-CCGCCCTGGCGGCTCTCAACGGACTTGGGCAACATCACGGCGCGCTCC          | -1/WT      |          |
| GCGACCCCCACAC-ACCGCTGGGCTCTCAACGGACTTGGGCAACATCACGGCGCGCTCC             | -2/WT      |          |
| GCGACCCCCAA-----------------------GCGTTCACGGAGCTTGGGCAACATCACGGCGCGCTCC | -14/WT     |          |
| GGACC-----------------------------GCTGGCTTGGAACATCACGGCGCGCTCC            | -20/WT     |          |
| GCGACCCCCAACACACCTGGCGGCTCTCAACGGACTTGGGCAACATCACGGCGCGCTCC             | -21/WT     |          |
| GCGACCCCCAACAC-ACCGCTGGGCTCTCAACGGACTTGGGCAACATCACGGCGCGCTCC            | -33/WT     |          |
| GGGCAAA-ACCGGCCACCCCTGGCGGCTCTCAACGGACTTGGGCAACATCACGGCGCGCTCC          | -63 bp     |          |
| GTTTAC-164 bp-ACCGCTGGGCTCTCAACGGACTTGGGCAACATCACGGCGCGCTCC             | -164 bp    |          |
| GCGACCCCCAACACACCTGGCGGCTCTCAACGGACTTGGGCAACATCACGGCGCGCTCC             | +1/WT      |          |
| CCGCGT--122 bp--GCTGGCGGCAACAAT--507 bp--GTA--52 bp--CAACTCGC Mix        |            |          |

### TaCKX2-3.1-B

| Sequence                                                                 | Type       | Position |
|-------------------------------------------------------------------------|------------|----------|
| GCGACCCCAAACGCCAACCTGGCGGCTCTCAACGGACTTGGGCAACATCACGGCGCGCTCC          | WT/WT      |          |
| GCGACC-----------------------GCGGCGCTCcACGGACTTGGGCAACATCACGGCGCGCTCC | -11/WT     |          |
| GCGACC-----------------------TGGCGGCTCTCAACGGACTTGGGCAACATCACGGCGCGCTCC | -11/WT     |          |
| GCGACCCCCAACACACCTGGCGGCTCTCAACGGACTTGGGCAACATCACGGCGCGCTCC             | -11/WT     |          |
| GCGACCCCAA-----------------------GCGGCGCTCcACGGACTTGGGCAACATCACGGCGCGCTCC | -20/WT     |          |
| GCGACCCCCAA-----------------------GCGGCGCTCcACGGACTTGGGCAACATCACGGCGCGCTCC | -21/WT     |          |
| GCGACCCCCAA-----------------------GCGGCGCTCcACGGACTTGGGCAACATCACGGCGCGCTCC | -21/WT     |          |
| GCGACCCCCACAC-ACCGCTGGGCTCTCAACGGACTTGGGCAACATCACGGCGCGCTCC             | -21/WT     |          |
| CCGCGA---104 bp--GCTGGCGGCAACAAT--267 bp--GCATCA--97 bp--GTGGAGCGGA Mix  |            |          |

### TaCKX2-3.1-D

| Sequence                                                                 | Type       | Position |
|-------------------------------------------------------------------------|------------|----------|
| GCGACCCAACGCCAACCCCTGGCGGCTCTCAACGGACTTGGGCAACATCACGGCGCGCTCC          | WT/WT      |          |
| GCGACC-----------------------TGGCGGCGGCGCCTCAACGGACTTGGGCAACATCACGGCGCGCTCC | -11/WT     |          |
| ------17 bp------ACCGCGCGGCGGCGCCTCAACGGACTTGGGCAACATCACGGCGCGCTCC      | -17/WT     |          |
| G---------------GCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCTCC | -21/WT     |          |
| GCGACCCAAC-----------------------GCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCTCC | -42        |          |

Figure 6 Sequence information of new TaCKX2-A1, TaCKX2-B1 and TaCKX2-D1 mutations in the T2 progeny plants from event #4-6. The mutation types include deletions (dashed line), insertions (lower-case letters in black) and substitutions (blue letters). PAM sequences are in green.
Types and spectrum of edit mutations in wheat

Deep sequencing of the target region from the T0 plants and screening a large population of T1 and T2 plants give us the opportunity to investigate the spectrum and types of the edit mutations in wheat. Our results show that deletions are the dominant type of edit mutations in wheat. In T0 plants, deep sequencing indicates that over 99% of the mutations are due to deletions, and our genotyping data from T1 and T2 plants show that only five insertions were found of the 68 mutations detected, all of which are insertions of 1-bp A or T. Remaining 63 mutations are deletions (Figure S11). Similar scenarios were also found in other wheat studies (Liang et al., 2017; Sánchez-León et al., 2018; Wang et al., 2014, 2018). Compared to TaCKX2-1 and TaGW2, deletion in TaGLW7 and TaGW8 is much lower. This is because we intended to target the microRNA recognition sites of these two genes, which are highly conserved among the homologous gene recognition sites (Xie et al., 2006). Thus, there is no other option for designing gene-specific guide RNA genes. Despite the low edit efficiency compared to the model plants, we detected two homozygous mutants in TaCKX2-A1 and two triple mutants in a population of 231 T2 plants (Table S4 and S6).

Use of edit mutations in wheat breeding

In addition to studies of gene function, CRISPR technology can have a great impact on plant breeding by manipulating the agriculturally important traits such as grain quality and grain yield. Our result showed that 1160-bp deletion in TaCKX2-D1 as compared to TaCKX-A1 and TaCKX-B1 because the T0 plant #4 carried a 1160-bp deletion allele and the T1 plant #4-6 is heterozygous for this deletion. Therefore, the potential edit rate in TaCKX2-1 could be even higher. This is approximately threefold higher than the previously reports in wheat by Biolistic delivery (Liang et al., 2017; Sánchez-León et al., 2018; Wang et al., 2014, 2018). Compared to TaCKX2-1 and TaGW2, the mutation rate in TaGLW7 and TaGW8 is much lower. This is because we intended to target the microRNA recognition sites of these two genes, which are highly conserved among the squarosa promoter binding-like transcription factors (Xie et al., 2006). Thus, there is no other option for designing gene-specific guide RNA genes. Despite the low edit efficiency compared to the model plants, we detected two homozygous mutants in TaCKX2-A1 and two triple mutants in a population of 231 T2 plants (Table S4 and S6).
number needs to be further validated by using other independent homozygous mutants of single double or triple mutations of the same orthologous gene. In addition to TaCKX2-1, we also obtained mutation in three other grain regulatory genes, TaGLW7, TaGW2, and TaGW8. Two recent studies on TaGW2 showed that knockout mutations of this gene could significantly increase wheat grain size and grain weight (Wang et al., 2018; Zhang et al., 2018a,b). Combining and transferring the mutations of TaCKX2-1 and TaGW2 into the elite wheat cultivars are expected to have an additive effect on increasing the sink strength.

Wheat cultivars Bobwhite and Fielder are the most transformable genotypes, but they are outdated and not currently used in wheat production and by wheat breeding. For the use of the edit mutations in wheat improvement, two approaches may be considered. A straightforward approach would be transferring the desired mutation alleles into elite genotypes and purging the CRISPR/Cas9 transgene by backcrossing. Another approach would be transferring the CRISPR/Cas9 transgene into the elite cultivars and screen new mutations in the improved background. Compared to the former, the latter approach would reduce the risk of genetic vulnerability due to the reduction in diversity in the region surrounding the mutant alleles. One prerequisite for this approach is the inheritance of active CRISPR/Cas9 transgene through the backcrossing generations. In this respect, the Agrobacterium-mediated delivery system has an advantage over the biolistic delivery as demonstrated by the present research that the CRISPR/Cas9 transgene remains high activity in the T1 and T2 generation. Both approaches can be coupled with genomic selection for acceleration of the transgene-free mutants as novel germplasm or cultivars.

**Experimental procedures**

**Plants and growing conditions**

Wheat cultivar Fielder and its transgenic plants were grown in 6" pots containing Sunshine potting mix (Green State Gardener, Burlington, VT) mixed with Perlite and Vermiculite (Hummert, St Louis, MO) in equal ratio and supplied with Multicote controlled release fertilizer (Haifa Group, Haifa, Israel), one plant per pot. Plants were grown in greenhouse in the fall, winter and spring seasons and in growth chambers in summer to increase tillers. Plants were grown in greenhouse in the fall, winter and spring seasons and in growth chambers in summer to increase tillers. C with a 16 h of photoperiod, and the plants were watered every other day.

**Construction of plasmids**

Wheat U6 snRNA (Marshallsay et al., 1992) was used as a query to BLAST search against wheat reference genome IWGSC (v1.0) (International Wheat Genome Sequencing Consortium, 2018) to retrieve their promoter sequences. gBlocks for promoters of TaU6.1 and TaU6.2 were synthesized (Integrated DNA Technology, Iowa City, IA) and then cloned into pENTR4. Double-stranded oligonucleotides were sequentially cloned into TaU6.1 and TaU6.2. TaGW2 restriction sites were introduced during the gBlock synthesis for cloning of the sgRNA spacer sequences. After testing the efficiency of U6 promoters (Appendix S1) in wheat protoplasts, two most active U6 promoters, i.e., U6.1 and U6.3 promoters, were assembled into the same construct to facilitate the construction of two sgRNA genes in an intermediate vector, pTagRNA4.

Two versions of Cas9 were used. The first Cas9 was codon-optimized for expression in wheat and made from six gblocks through the Gibson assembly using Gibson Assembly™ Master Mix (New England Biolabs, Ipswich, MA). The assembled Cas9 was cloned into pMCaG1005 in which the selection marker Bar gene was replaced with hygromycin phosphotransferase (hpt) gene. After confirmation of the hpt activity in rice callus through Agrobacterium-mediated transformation, the construct was inserted with the AttR1-ccdB-AttR2 Gateway cassette to produce plasmid pTaCas9-Hyg-GW. The selection efficiency of the ccdB gene was examined by the introduction of the plasmid into Escherichia coli XL1-blue (Agilent Genomics, Santa Clara, CA) for minimal colony formation. pTaCas9-Hyg-GW was used for testing the activity of U6 promoters in protoplasts. The second Cas9 gene, Cas9.P2A:HA::GFP and referred to as Cas9-GFP hereinafter, was also wheat-codon optimized and derived from the fusion of Cas9 with the histone 2A (H2A) and GFP coding sequences. The Cas9 coding sequence was separated by a sequence encoding P2A, a translational slipping signal. This Cas9-GFP under the maize ubiquitin 1 gene promoter was used for wheat transgenics.

To transiently and quickly identify active U6 promoters for gRNA, a mutated green fluorescent protein (GFP), gene was constructed. To insert a 1-bp downstream of the start codon of GFP, a forward primer GFP-1-F, with the frameshift insertion, was paired with the reverse primer GFP-R (Table S9) to amplify the open reading frame (ORF) of the GFP gene. The PCR product was digested with BamHI and Spel and cloned into pUC19 under the 35S promoter, resulting in pUC-35SS-GFP-1. A sgRNA targeting the 1-bp insertion site in pUC-35SS-GFP-1 synthesized and cloned into the gRNA vector to produce the construct pTaRNA4-gGFP.

To make the constructs targeting the grain regulatory genes in wheat, two complementary oligonucleotides corresponding to the protospacer sequences of each of two sgRNAs, sgRNA1 and sgRNA2 for the same gene, were designed and synthesized. Double-stranded oligonucleotides were sequentially cloned into Btg2I sites and Bsal sites in pTagRNA4, in which sgRNA1 is driven by TaU6.1 and sgRNA2 by TaU6.3. gRNA cassette was then PCR-amplified and integrated into pCaS9-GFP (Cas9-GFP in pENTR4) at the 8ssHII site by Gibson assembly. Through Gateway recombination reaction, the Cas9-TagRNA4 cassette was finally incorporated into pLC41-GW (a Gateway binary vector) that is derived from pLC41_Exp1 Icat-GUS.

To make an in vitro transcription system for guide RNA that can be used along with Cas9 protein to in vitro screen for mutated plants, a gBlock containing the T7 promoter, guide RNA scaffold, and two Bsal cloning sites in between was synthesized (Integrated DNA Technology, Iowa City, IA). The gBlock was inserted into pENTR4 between NcoI and XbaI through Gibson assembly replacing the ccdB cassette, resulting in pT7-sgRNA. To make in vitro transcribed guide RNA molecule, a guide RNA gene was assembled in pT7-sgRNA vector. Specifically, two complementary oligonucleotides for the spacer sequence of sgRNAs were synthesized, annealed to form double stranded DNA fragment, and then cloned into Bsal sites in pT7-sgRNA.

**Isolation and transfection of wheat protoplasts**

Leaves from 1-week-old seedlings of wheat cultivar Fielder were chopped latitudinally into 0.5-mm strips using razor blades. The leaf strips were used for protoplast isolation.
following the procedure described by Zhou et al. (2014). The protoplasts were resuspended in a W5 solution (2 mM MES (pH 5.7), 154 mM NaCl, 125 mM CaCl$_2$, 5 mM KCl) at a concentration of 2 x 10$^6$ per mL. The pTaCas9-Hyg-GW, pUC-35S:GFP+1, and pTaU6-gGFP were mixed at a concentration of 400 ng/µL each plasmid. An aliquot of 200 µL protoplast solution was sequentially mixed with 30 µL of plasmid DNA and 230 µL of freshly prepared PEG solution (40% PEG-4000, 0.2 M Mannitol, 0.1 M CaCl$_2$) in a 2-mL microcentrifuge tube by inverting three times. After the mixture was incubated at room temperature in the dark for 20 min, 900 µL of W5 solution was added to the tube and mixed by inverting the tube three times. The transfected protoplasts were collected by centrifugation at 250 g for 5 min, washed once with W5 solution, resuspended in 800 µL of W5 solution, and incubated at room temperature. Constructs pOsuBi-GFP and pUC-35S:GFP+1 served as a positive and negative control, respectively, to transfect the protoplasts. Forty hours after transfection, protoplasts were examined under a fluorescence microscope for the proportion of protoplasts with GFP fluorescence and images were captured. Transfection was conducted with two replicates for each construct, and five random microscope views under 20X magnification were calculated. Images were analysed with Fiji-ImageJ (Schindelin et al., 2012), and integrated density was measured to quantify the fluorescence density in the selected view. Integrated density was then averaged by the total number of protoplasts in that view.

### Primers and guide RNA design

Total genomic DNA was isolated from the wheat leaf tissue following the protocol described by Li et al. (2008) except using the CTAB buffer (2% CTAB (hexadecyl trimethyl-ammonium bromide), 100 mM Tris, 20 mM EDTA, 1.4 M NaCl, 1% PVP (polyvinyl pyrrolidone (vinylpyrrolidine homopolymer) Mw 40 000), and pH5.0). Sequences of the homoeologous genes of *T. aestivum* cv Chinese Spring (CS) were retrieved from the Ensembl Plants database (https://plantsensembl.org/index.html), and gene-specific primers (Table S9) were designed for the homoeologous genes using online program GSP (https://probes.pw.usda.gov/GSP; Wang et al., 2016) and used to PCR-amplify the relevant genomic regions (~1 kb). Specificity of the primers was validated by use of DNA from the nulli-tetrasomic stocks (Sears, 1966), and the PCR products were separated through electrophoresis in 1% agarose gel. All the primers are listed in Table S9. The PCR reaction was performed for each homoeologous copy of an orthologous gene in the A, B, and D genome, which included 100 ng genomic DNA, 5 µl GoTaq® Green PCR buffer (Promega, Madison, WI), 125 µM dNTPs, 0.5 µM primers, and 0.2 µL DNA Taq polymerase. PCR cycling conditions were as follows: an initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1.5 min, and a final extension at 72°C for 7 min. The gene-specific PCR products were cleaned up with ExoSAP-IT (Thermo Fisher Scientific, Waltham, MA) following the manufacturer's instruction. After being denatured (95°C for 5 min) and re-annealed (ramp down to 25°C at 5°C/min), the PCR products were subjected to T7E1 (New England Biolabs, Ipswich, MA) digestion (1 unit, 0.2 µL at 37°C for 1.5 h and separated by agarose gel electrophoresis).

### Wheat transformation

Fielder was cultivated in a growth chamber. Immature seeds were harvested from panicles approximately 2 weeks after anthesis, surface-sterilized for 1 min in 70% ethanol followed by 10 min in 1.2% sodium hypochlorite and were washed three times with sterilized water. Immature embryos were isolated from the sterilized seeds under a stereoscopic microscope. The CRISPR/Cas9 constructs were transferred into *Agrobacterium tumefaciens* strain EHA105 by electroporation. The *Agrobacterium*-mediated transformation on the isolated immature embryos and plant regeneration were conducted following the protocol described by Ishida et al. (2015). The transgenic plants were further confirmed by PCR assays of the Cas9 and sgRNA transgenes.

### Purification of Cas9 protein, in vitro transcription of guide RNA and preparation of Cas9/sgRNA ribonucleoprotein

*Escherichia coli* strain Rosetta 2 DE3 carrying plasmid pMJ806 was obtained from Addgene. The Cas9 protein was prepared following the protocol as described (Anders and Jinek, 2014). For *in vitro* transcription of guide RNA, pt77-sgRNA derived gRNA-specific construct was linearized at a unique restriction enzyme site NcoI, followed by dephosphorylation with Shrimp Alkaline Phosphatase (rSAP) (New England Biolabs, Ipswich, MA), and then purified with DNA Clean and Concentrator Kit (Zymo Research, Irvine, CA). sgRNA was synthesized by using the HiScribe T7 Quick High Yield RNA Synthesis Kit (New England Biolabs) following the manufacturer's instructions and purified with RNA Clean and Concentrator (Zymo Research, Irvine, CA). For each DNA/ribonucleoprotein reaction, 1 µg of sgRNA and 1 µg of Cas9 protein were mixed in Cas9 reaction buffer and incubated at room temperature for 15 min to form a ribonucleoprotein (RNP) complex. The resulting RNP was used for 2–3 reactions each with about 1 µg of PCR product in a total volume of 10 µL at 37°C for 3 h. After inactivated at 65°C for 15 min, the reactions were analysed with electrophoresis in 2% agarose gel.

### Mutant screening

The PCR amplicons were digested with restriction enzymes (XcmI and BglII), T7E1 or Cas9/sgRNA ribonucleoprotein and separated by agarose electrophoresis. For digestion with restriction enzymes or T7E1, 1 unit of enzyme was used in 15 µL reaction including PCR product, following the manufacturer’s instruction. After being denatured (95°C for 5 min) and re-annealed (ramp down to 25°C at 5°C/min), the PCR products were subjected to T7E1 (New England Biolabs, Ipswich, MA) digestion (1 unit, 0.2 µL) at 37°C for 1.5 h and separated by agarose gel electrophoresis.

### DNA sequencing and sequence analyses

The PCR products were deep-sequenced by the high-throughput sequencing platform HiSeq 2000 (Illumina, San Diego, CA) or individually sequenced by Sanger sequencing in the ABI DNA analysers (Thermo Fisher Scientific, Waltham, MA). Genomic DNA samples from T0 plants transformed with Cas9 and TaCXX2-1 sgRNA was used in amplicon sequencing. First round PCR was conducted using gene-specific primers with universal tags for 35 cycles. PCR products were separated in 2.0% agarose gel, and the target bands were excised and column-purified. Approximately 10 ng of PCR product was used for a second round of PCR.
with primers matching the universal tags and containing a sample-specific barcode within the forward primer. PCR was conducted for 12 cycles. PCR products were separated on 2.0% agarose gel, and target bands were cut and column-purified. Purified amplicons were quantified with Qubit® 2.0 fluorometer (Life Technologies, Carlsbad, CA) and pooled equally to make a library at a concentration of 4 nmol/L. Paired-end (2 × 250) sequencing was conducted with a total of 500 cycles. Sequence analysis was conducted with CRISPR-DAV (Wang et al., 2017b) to discover the modification.

For Sanger sequencing, PCR amplicons were treated with ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher) following the manufacturer’s manual before subjected to sequencing. The sequencing chromatograms were fed to the computer program DSDecodeM (http://skl.scau.edu.cn/dsdecode/; Liu et al., 2015) to determine the allelic mutations.

Phenotyping

The spikes from the main stems were scored for spikelet and grain numbers; spikelet length, density and average grain number per spikelet were measured. The total grains from individual spike were weighed using a PB602-S digital precision scale (Mettler Toledo, Columbus, OH). Five biological replicates were used for each genotype.

Data analysis

The Student’s t-test was performed using pooled standard deviations to evaluate the statistical significance of the difference between different U6 promoters or between the mutant and wild type. ChiSq test was implemented for fitness of segregation of mutations in a T2 population to the 1 : 2 : 1 ratio. Both the Student’s and ChiSq tests were conducted using Microsoft Excel. The cut-off for statistical significance was set to a P-value of 0.05.

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Conflict of interest

The authors declare no conflicts of interest.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Comparison in editing efficiency of gfp driven by different wheat U6 promoters in terms of the proportion of fluorescence cell (a) and average fluorescence intensity per cell (b).

Figure S2 Structure of pTaGW4RNA in the pENTR4 backbone. Two sgRNAs are inserted into Btg2I-Btg2I and Bsal-Bsal sites sequentially.

Figure S3 Structure of pCas9-GFP used in the transgene. pTaU6-gRNA cassette is inserted into BssHI sites through Gibson assembly.

Figure S4 Deletion distribution along the ampiclon in TaCKX2-A1 (a), TaCKX2-B1 (b), and TaCKX2-D1 (c).

Figure S5 Identification of Cas9-positive transgenic plants in T1 progenies.

Figure S6 Targeted TaCKX2-T homoeologues using the CRISPR/Cas9 system in transgenic T1 plants.

Figure S7 Screen mutant plants in TaCKX2-D1 # 4 T1 plants using PCR assay.

Figure S8 Screen mutations in TaCKX2-1 of plants from the # 4-1 and # 4-35 T2 family by the PCR-RE assay.

Figure S9 Screen mutations in TaCKX2-1 of the T2 plants from the # 4-6 family by the PCR-RE assay.

Figure S10 Screening of mutations in TaGLW7 of the T2 plants from the # 1-32 family by the PCR-RNP assay.

Figure S11 Types and spectrum of 68 edit mutations.

Table S1 sgRNA target sites used in this study.

Table S2 Verification of transgenes in T0 plants.

Table S3 Frequencies of CRISPR/Cas9-induced novel mutations in T0, T1, and T2 populations.

Table S4 Types of CRISPR/Cas9-induced mutations for target genes in T0, T1, and T2 generations.

Table S5 Genotypes of mutant plants induced by CRISPR/Cas9 system in T1 population.

Table S6 Genotypes of mutant plants induced by CRISPR/Cas9 system in TaCKX2 T2 family #4-6.

Table S7 A summary of the double and triple mutants recovered in TaCKX2-1.

Table S8 Off-targeting of designed sgRNA for TaCKX2-1 gene.

Table S9 PCR primers used in this study.

Appendix S1 Sequences of U6 promoters.