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CRISPR/Cas9 genome editing in wheat

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Abstract Genome editing has been a long-term challenge for molecular biology research, particularly for plants possess complex genome. The recently discovered Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system is a versatile tool for genome editing which enables editing of multiple genes based on the guidance of small RNAs. Even though the effi-ciency of CRISPR/Cas9 system has been shown with several studies from diploid plants, its application remains a challenge for plants with polyploid and complex genome. Here, we applied CRISPR/Cas9 genome editing system in wheat proto-plast to conduct the targeted editing of stress-responsive transcription factor genes, wheat dehydration responsive element binding protein 2 (*TaDREB2*) and wheat ethylene responsive factor 3 (*TaERF3*). Targeted genome editing of *TaDREB2* and *TaERF3* was achieved with transient expression of small guide RNA and Cas9 protein in wheat protoplast. The effec-tiveness of mutagenesis in wheat protoplast was confirmed with restriction enzyme digestion assay, T7 endonuclease as-say, and sequencing. Furthermore, several off-target regions for designed sgRNAs were analyzed, and the specificity of genome editing was confirmed with amplicon sequencing. Overall results suggested that CRISPR/Cas9 genome editing system can easily be established on wheat protoplast and it has a huge potentiality for targeted manipulation of wheat genome for crop improvement purposes.

Introduction

Plant genome editing aiming to generate more yielded and resilient varieties has always been a challenge. Thus far, several methods such as EMS mutagenesis and T-DNA insertions have been utilized to create random mutations; however, these methods do not provide a solution for targeted genome editing (Belhaj et al. 2015). Advances in technology promote the discovery and utilization of genome editing methods such as zinc finger nucleases (ZFNs) and TAL effector nucleases (TALENs) which enable the editing of a gene of interest in a precise manner. However, design and construction of gene editing via these technologies have been problematic and considerably expensive since protein engineering is required for the editing of the gene of interest (Gaj et al. 2013; Bortesi and Fischer 2014). Recently, an important tool for precise genome editing, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated protein 9 (Cas9), was discovered which is relatively easy to use and more cost-effective compared to other methods, and it has a potential to change plant improvement strategies in a revolutionary manner.

CRISPR/Cas9 system is a versatile tool for genome editing where multiple genes can be targeted based on the guidance of small RNAs (Doudna and Charpentier 2014). It is the key component of bacterial and archaeal adaptive immunity which was initially discovered in *Escherichia coli* in the 1980s (Ishino et al. 1987). However, the astonishing function of CRISPR/Cas9 system remained elusive until it was found that *Streptococcus thermophilus* can acquire resistance against a bacteriophage by integrating a

genome fragment of an infectious virus into its CRISPR locus (Barrangou et al. 2007). In this new system, destruction of target DNA relies on a double-stranded break which is guided by a crRNA transcript (Schiml and Puchta 2016). Following the targeted DNA breakage, there are two types of DNA repair mechanisms which can be activated: non-homologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ is an error-prone mechanism resulting in imperfect repair and causes the interruption of gene function. On the other hand, HDR uses a template for the repairing process and generates perfectly repaired new DNA (Schiml et al. 2014; Belhaj et al. 2015). NHEJ-based repairing mechanism may also cause nonspecific mutations which arises from possible off-targeting effect of designed sgRNAs on other regions in the genome. In order to increase the specificity of RNA-guided targeted mutagenesis and decrease the off-targeting, protein engineering methods were applied for editing Cas9 nuclease and Cas9 mutants. For example, Cas9D10A (Cas9 nickase variant) has been utilized for more precise editing of genomes (Ran et al. 2013).

Following the discovery of CRISPR/Cas9 system, several genome editing studies for plants have been performed both in monocots and dicots such as *Nicotiana benthamiana* (Li et al. 2015), *Nicotiana tobaccum* (Gao et al. 2014), *Arabidopsis thaliana* (Li et al. 2014), *Oryza sativa* (Shan et al. 2014), and *Sorghum bicolor* (Jiang et al. 2013). Even though genome editing via CRISPR/Cas9 system has certain advantages, there are several pitfalls which make its application troublesome for some plant species. For instance, the polyploid nature of several crop species increases the possibility of off-target mutations and decreases genome editing specificity (Peng et al. 2015). Additionally, the editing of each copy of a gene inside the genome is another controversial issue particularly for genes which possess a high copy number in several genomic locations. Bread wheat (*Triticum aestivum* L.) which is an important crop providing more than 20% of daily calorie intake for humans has a complex genome structure formed with the combination of three different genomes: A, B, and D (Ling et al. 2013; Choulet et al. 2014). The hexaploid nature of the wheat genome makes this plant an important model for studying and optimizing the genome editing system. In this study, we applied CRISPR/Cas9 genome editing system for two abiotic stress-responsive transcription factor genes, wheat ethylene responsive factor 3 (TaERF3) and wheat dehydration responsive element binding protein 2 (TaDREB2) in wheat protoplast. The targeted mutagenesis generated with CRISPR/Cas9 was confirmed with restriction enzyme digestion assay, T7 endonuclease I assay, and sequencing. The possible effect of off-targeting by designed sgRNAs was analyzed with in silico methods, and targeted editing of gene of interest was proven with amplicon sequencing. Current findings indicate that targeted editing of genes in polyploid plant genomes can be accomplished via CRISPR/Cas9 system where a combination of in silico off-target proofing and NGS can be used for improvement of genome editing specificity.

Materials and methods

In silico analysis of target genes and generation of sgRNA

For conducting CRISPR/Cas9-based genome editing in wheat protoplast, the full complementary DNA (cDNA) sequences of *TaDREB2* (GenBank ID DQ353852.1) and *TaERF3* (GenBank ID EF570122.1) were retrieved from NCBI nucleotide archive (https://www.ncbi.nlm.nih.gov/nucleotide/). The cDNA sequences were mapped to wheat genome assembly (v1) (provided by IWGSC) by utilizing Blast tool kit (Camacho et al. 2009), and the exon-intron boundaries were determined for each gene. In addition, each copy of the genes of interest located on different sub-genomes was analyzed in terms of their homology to each other at both sequence and protein levels. Small guide RNA (sgRNA) sequences were chosen, and associated oligos were manually designed based on GenBank sequences for each target gene (Table 1, Supplementary Figs. 1 and 2). Chosen sgRNAs were also analyzed with blasting against three different genomes of wheat: A, B, and D, to determine the editing ability of sgRNAs in different sub-genomes.

Cloning of target-specific sgRNA oligo into Cas9 vector

For cloning of target-specific sgRNAs, the protocol from Shan et al. (2014) was followed. The cloning was performed with utilizing pTaU6-gRNA plasmid which contains the wheat TaU6 promoter with a specific guide RNA cloning site and guide RNA scaffold (Shan et al. 2014). Followed by the design and synthesis of sgRNAs for targeted gene editing, the sgRNAs were sub-cloned into pU6-gRNA plasmid from BbsI enzyme site (NEB #R3539) after sgRNA annealing. For this ligation, synthesized oligos for target-specific sgRNAs were designed with overhangs, 5′-GTTGN(20)-3′ in the forward oligo and 5′-AAACN(20)-3′ in the reverse oligo, complimentary to BbsI enzyme site in pTaU6-sgRNA. The annealing of sgRNAs prior to the cloning was performed with 2 μl of 10× buffer (NEB CutSmart buffer 10×) and 9 μl of each 10 μM oligo pairs. The sgRNA annealing mixture was denatured at 95 °C for 5 min, and the annealing was realized with a gradual temperature decrease (1 °C per minute) to 25 °C. Subsequent to annealing, the sgRNA was ligated with BbsI-digested pTaU6 vector using T4 DNA ligase (NEB #M0202). Ligation reaction mix contained 1 μl of T4 ligation buffer, 50 ng of digested vector, 3 μl of annealed sgRNA oligos, 2.5 units of T4 DNA ligase, and ddH2O for a final reaction volume of 10 μl. The ligation mixture was incubated at room temperature for 1 h and transformed

Table 1 The sequences of oligos used in this study

Primer name	Sequence (5′-3′)	Purpose
TaERF3_oligo3	cttgGCGAGGGGCAAGCACTACCG	sgRNA
TaERF3_oligo4	aaacCGGTAGTGCTTGCCCCTCGC	sgRNA
TaDREB2_oligo1	cttgGCAGGACGTCGACGAGGACT	sgRNA
TaDREB2_oligo2	aaacAGTCCTCGTCGACGTCCTGC	sgRNA
TaU6_F	CCCAAGCTTGACCAAGCCCGTTATTCT	Sequencing
U6-SpeI-F	CGGACTAGTGACCAAGCCCGTTATTCTGAC	Cloning
sgRNA-SpeI-R	CGGACTAGTAAAAAAAGCACCGACTCGGTGCCA C	Cloning
ERF3_T7_F/R	ACTCCGACGACATGGTCGTCTA/GAATCCATTGCACTTGCGCA TT	Mutation validation
DREB2_T7_F/R	CCTCCCATTACCACGAGCGA/CCGTGAGCTGCTGCTCATTT	Mutation validation
TaActin-F/R	TTGCTGACCGTATGAGCAAG/ACCCTCCAATCCAGACACTG	qRT-PCR
qDREB2_F1/R1	CGACGACAAGAAGCGGAAC/TGATCTCCGACACCCACTT	qRT-PCR
qDREB2_F2/R2	GCAAGAAGTCCCGCATCT/CGAGGTCCGGGAAGTTAAG	qRT-PCR
qERF3_F1/R1	CGACATGGTCGTCTACGG/TCGAGGAAACCGAAGCAG	qRT-PCR
qERF3_F2/R2	CGACGACTCCGACGACAT/CTTGACGGCGGCAAAGG	qRT-PCR
DREB-OffT1-F/R	CCTTCAATGCTGACCCTAGC/AGAAACGAGTGGTAGTACTA TACC	Off-target
DREB-OffT2-F/R	CCAAATGAAACAACACAAGGCG/ACCATCATCCTACCGCGCAG	Off-target
DREB-OffT3-F/R	CAGCCTTCAATGCTGACCCT/GCTAGAATGTGCTTA ATTGGGGT GC	Off-target
ERF-OffT1-F/R	GGAGGGCAAGAGGATCATCTT/CCGTCAGCTCTGCATTTGTT	Off-target
ERF-OffT2-F/R	GTACTGTAATACATGAGTGAG/AAGCGGATCATCTTCACCAA	Off-target

into chemically competent cells of a DH5α strain of *E. coli*. Transformed cells were spread onto an LB plate containing ampicillin and incubated overnight at 37 °C. Plasmid DNA was isolated using Zyppy Plasmid DNA isolation kit (Zymo Research # ZD4019), and Sanger sequencing was performed with TaU6-F primer to confirm the successful cloning of sgRNA into the pTaU6 plasmid (Table 1).

For Cas9 construction, the procedure in Shan et al. (2014) was slightly modified from the previously used pJIT163-2NLSCas9 vector (Shan et al. 2014). In order to combine the previously cloned pTaU6-sgRNA and Cas9 vector (pJT163-2NLSCas9), sgRNA site from pTaU6-sgRNA scaffold vector was sub-cloned into Cas9 vector by PCR amplification (Fig. 1). SpeI-TaU6-F and SpeI-sgRNA-R primers (Table 1) were used for amplification of TaU6 promoter, sgRNA, and gRNA scaffold. The PCR fragment was ligated in the pJIT163-2NLSCas9 from SpeI enzyme site. This final constructed vector containing pTaU6 promotor site, single sgRNA site, and Cas9 sequence was utilized for protoplast transformation.

Protoplast isolation and transformation

Protoplast transformation was conducted by following the protocol from Shan et al. (2014)). The seedlings of *T. aestivum* cultivar Chinese spring were grown in 16-h light/8-h dark at 25 °C for 14 days. Fresh tissues were harvested from 40 to 60 seedlings and sliced into 0.5-mm strips with a sharp razor blade. The strips were transferred into a petri dish with 0.6 M of mannitol and incubated for 10 min in the dark for quick plasmolysis. After filtering through nylon meshes, the strips were transferred into a 150-ml conical flask containing 50 ml of filter-sterilized enzyme solution which contained 20 mM of MES (pH 5.7), 1.5% (*w*/*v*) of Cellulase R10, 0.75% (*w*/*v*) of Macerozyme R10, 0.6 M of mannitol, 10 mM of KCl, 10 mM of $CaCl_2$, and 0.1% (*w*/*v*) of BSA. In order to infiltrate the digestion solution into leaf tissues, a vacuum (380–508 mmHg) was applied for 30 min in the dark followed by incubation at room temperature for 5–6 h with gentle shaking (60–80 rpm). Subsequent to enzymatic digestion, 50 ml of the W5 solution containing 2 mM MES (pH 5.7), 154 mM of NaCl, 125 mM of $CaCl_2$ and 5 mM of KCl was added to the conical flask and shaken gently for 10 s to release the protoplasts. Protoplast cells were collected into three or four 50-ml falcon tubes by filtering the mixture through 40-μm nylon mesh and washing the tissue strips on the surface of the nylon mesh three to five times with W5 solution. The tube was centrifuged at room temperature for 3 min at 80×*g* in a swinging bucket rotor. The supernatant was then removed by pipetting, and the protoplasts were resuspended in 30 ml of W5 solution and placed on ice for 30 min. Without disturbing the protoplast pellet, the supernatant was again removed and the pellet resuspended in 4 ml of MMG solution (4 mM MES (pH 5.7), 0.4 M mannitol, and 15 mM $MgCl_2$) at a final concentration of 10^6 cells per milliliter.

Ten micrograms of plasmid DNA in 10–20 μg was gently mixed with 200 μl of protoplasts. Two hundred twenty microliters of freshly prepared PEG solution (40% (*w*/*v*) PEG 4000, 0.2 M mannitol, 100 mM $CaCl_2$) was added and mixed gently by tapping the tube. The mixture was then incubated for 20 min in the dark. After incubation, 880 μl of W5 solution was added to the tube and mixed by inverting the tube to stop the transformation process. The protoplasts were harvested by centrifuging at 80×*g* for 3 min at room temperature and then

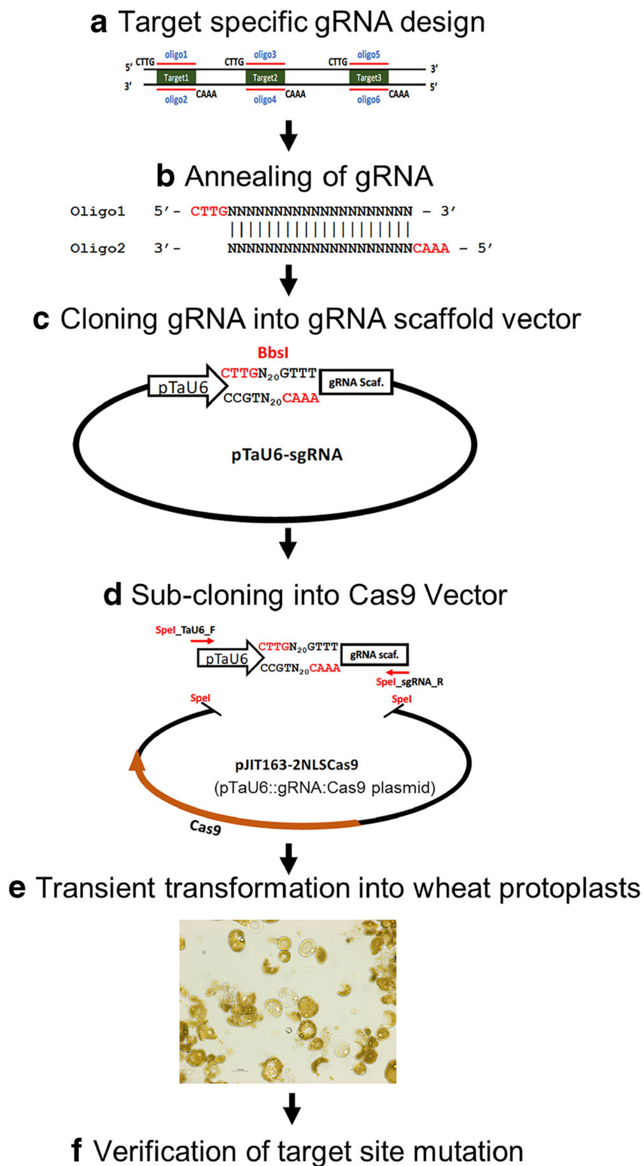


Fig. 1 Schematic description of CRISPR/Cas9-based genome editing application in wheat protoplasts. To begin with, forward and reverse oligos for selected target sites were designed and synthesized in order to insert into the sgRNA scaffold vector (pTaU6-sgRNA) (**a**). The oligos contained 20-nt target site together with 5′ and 3′ overhangs complementary BbsI digestion sites where the overhangs are 5′-CTTGN(20)-3′ for forward primer and 5′-AAACN(20)-3′ for reverse primer. Synthesized oligos were annealed with each other (**b**), and the paired sgRNAs were ligated into pTaU6-sgRNA plasmid which contained TaU6 promoter and sgRNA scaffold (**c**). After conformation of sgRNA insertion into pTaU6, sgRNA site from pTaU6-sgRNA scaffold vector was sub-cloned into Cas9 vector (pJIT163-2NLSCas9), and this combined plasmid was named as pTaU6::gRNA:Cas9 (**d**). For sub-cloning, TaU6 promoter, sgRNA, and gRNA scaffold were amplified from pTaU6-sgRNA scaffold vector with SpeI-Tau6-F and SpeI-sgRNA-R primers where SpeI enzyme site was used for two component ligations. Thus, the final construction of pTaU6::gRNA:Cas9 was completed, and constructed plasmid was transformed into protoplast (**e**). The mutation verification was performed with restriction enzyme digestion, T7EI assay, and sequencing (**f**)

resuspended in 2 ml of W5 solution. To detect the efficiency of wheat protoplast transfection, pGFPGUSplus plasmid (addgene #64401) which contains GFP expression construct was utilized. Ten micrograms of plasmid vector containing GFP was used for PEG (40%) mediated transfection which was infected for 20 min. After a 24, 48, and 72-h incubation of transfected protoplast, fluorescence microscopic analyses revealed that approximately 20, 40, and 70% of transfected protoplasts had GFP expression signal, respectively. The protoplast solution was transferred into 6-well plates, which were then wrapped in aluminum foil and incubated at 23 °C for 48–72 h.

Preparation of PCR amplicons for detection of the genome editing events in protoplasts

Genomic DNA was isolated from the protoplasts using Qiagen DNeasy Plant Mini Kit (Cat. # 69104) following the manufacturer's protocol. Genomic regions containing the gRNA targets were PCR amplified and subjected to digestion assay and T7 endonuclease I (T7E1) assay for validation of the mutation. PCR amplification was performed in a 25 μl reaction volume containing 5 μl of protoplast genomic DNA, 2.5 μl of primer mix, 0.5 μl of 10 mM dNTPs, 5 μl of 5× GC buffer (NEB# B0519), 3% DMSO, 0.25 μl of Phusion High Fidelity DNA Polymerase (NEB# 0530), and ddH2O up to a final volume of 25 μl with the following conditions: initial denaturation at 98 °C for 30 s, denaturation at 98 °C for 10 s, annealing at 60–72 °C for 30 s, extension at 72 °C for 15 s to 35 cycles, and final extension at 72 °C for 5 min. PCR products were run on a 1% agarose gel in TAE buffer, and purification of desired fragments was performed with Zymoclean Gel DNA Recovery Kit (Zymo Research # ZD4002) for restriction enzyme digestion PCR (RE-PCR) and T7E1 assay.

Restriction enzyme digestion and T7 endonuclease I assay for validation of genome editing

In order to detect the mutation at desired restriction enzyme sites, the PCR products were digested with *SalI* for wheat dehydration responsive element binding protein 2 (*TaDREB2*) at 37 °C for 2 h. The PCR fragments containing the gRNA-Cas9 target sites were then amplified by PCR (primer sequences in Table 1), and digested PCR product was analyzed by electrophoresis in 1.2% agarose gel. So as to identify targeted gene mutation, purified PCR products from the restriction enzyme-digested template were cloned to pMiniT vector (NEB# E1202). Resulting random colonies were used for plasmid extraction and Sanger sequencing.

For further confirmation of the presence of CRISPR/Cas9-based mutation at the target site, T7E1 assay was conducted. Firstly, DNA fragments containing the targeted sites were amplified from protoplast genomic DNA using a pair of primers (Table 1) with Phusion High fidelity DNA polymerase (NEB# 0530). The PCR products were purified

using Zyppy Plasmid DNA isolation kit (Zymo Research # ZD4036), and 100 ng of purified PCR product was denatured-annealed under 95 °C for 5 min and then ramped down to 15 °C (10 °C per minute). Annealed PCR products were then digested with 2.5 units of T7 endonuclease I (NEB# M0302) for 1 h at 37 °C. The T7 endonuclease I-digested product was separated by 1% agarose gel electrophoresis and used for conformation of mutation in the genes of interest. Digestion efficiency was calculated by measuring band intensities with ImageJ (NIH version 1.5). The gel was isolated, and its intensity measured, with background subtracted. Band intensities were summed to determine total intensities. To calculate the percent of digestion efficiency, the intensity of the non-cleaved band was divided by the total intensity (Shan et al. 2013).

Off-target analysis for CRISPR/Cas9 in wheat

In order to detect possible off-target potential of designed sgRNAs, specific locations were detected where two mismatched sgRNAs can bind via in silico blast analysis of designed sgRNAs to wheat genome assembly (IWGSC RefSeq v1.0). Based on the blast results, several candidate regions were chosen and oligo pairs were designed to amplify the 400–500 base pair off-target regions which contain the sgRNA target site in the middle (Table 1). The amplification of these regions was performed from both wild-type and mutated protoplast samples. The PCR amplification for all these regions was performed in a 25 μl reaction volume containing 5 μl of protoplast genomic DNA, 2.5 μl of primer mix, 0.5 μl of 10 mM dNTPs, 5 μl of 5× GC buffer (NEB# B0519), 3% of DMSO, 0.25 μl of Phusion High Fidelity DNA Polymerase (NEB# 0530), and ddH2O up to a final volume of 25 μl with the following conditions: initial denaturation at 98 °C for 30 s, denaturation at 98 °C for 10 s, annealing at 60 to 72 °C for 30 s, extension at 72 °C for 15 s to 35 cycles, and final extension at 72 °C for 5 min. PCR products were run on a 1% agarose gel in TAE buffer, and the desired fragment was purified with Zymoclean Gel DNA Recovery Kit (Zymo Research # ZD4002). The PCR fragments were then tagged with specific primers for amplicon sequencing, and deep sequencing of each amplicon was performed. Obtained deep amplicon sequencing data firstly analyzed with FastQC program to determine the qualified reads prior to analysis. The detected adaptor sequences were removed, and low-quality reads were discarded. The qualified reads were analyzed with two online tools: CRISPR-GA (Güell et al. 2014) and Cas analyzer (Park et al. 2016) (comparison range 70, minimum frequency 5, WT marker 5). The analysis was performed for wild type and mutated reads separately. The final mutation efficiency for the off-target region was calculated by subtraction of two mutation efficiencies.

Expression analysis of *TaDREB2* and *TaERF3* by quantitative real-time PCR

The TaDREB2 and ethylene responsive factor 3 (TaERF3) transcription levels were tested in 7-day-old seeding under short drought treatment (0, 1, and 4-h dehydration) by using qRT-PCR. RNA was extracted using Rapid Pure RNA Plant Kit (MP Biomedicals Cat# 112722000), and 2 μg of RNA was treated with DNase I (Sigma Cat# SLBR4100) and reverse-transcribed to cDNA using ProtoScript II first-strand cDNA synthesis kit (NEB Cat# E6560) following the manufacturer's suggestion. Quantitative real-time PCR was performed using a Bio-Rad CFX96 real-time system (C1000 Touch thermal cycler) with two primers per target gene (Table 1). For amplification, iTaq Universal SYBR Green supermix (Bio-Rad Cat# 172-5121) was used in a final volume of 10 μl. The cycler was programmed as follows: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min, and then 95 °C for 15 s. The constitutive gene of *Triticum aestivum actin* (*TaActin*) (Yue et al. 2015) was used as internal standard to normalize the transcripts using a gene-specific primer (Table 1). The $2^{-\Delta Ct}$ method was used to calculate the difference in expression of chosen genes (Livak and Schmittgen 2001).

Results

Target selection and vector construction for CRISPR/Cas9

Prior to sgRNA design and vector construction, the genomic region associated with DREB2 and ERF3 was analyzed, and each copy of genes located on A, B, and D genomes was defined. Based on in silico characterization of target genes, DREB2 was defined with three highly similar copies located on 3A, 3B, and 3D chromosomes where three homologs of ERF3 were detected on 2A, 2B, and 2D chromosomes. The sgRNA for DREB2 was designed at the beginning of the protein sequence which covers the 8th to 15th amino acids. For ERF3, the target site was located on 143th to 150th amino acid range which was near by the AP2 domain of the protein. Both DREB2 and ERF3 are characterized by the AP2 domain (Mizoi et al. 2012a), and the preference of a different target location on the protein used for testing and characterizing the editing specificity and its association with how far the sgRNA site was from protein domain. Homology analysis of sgRNA to A, B, and D genome showed that DREB2 sgRNA was not able to edit one copy of gene located in 3A chromosome because of two mismatches between designed sgRNA (Table 2). In 2A copy of ERF3, only one mismatch was

detected in the designed sgRNA which might result in non-editing or decreased editing results.

Transformation efficiency and transient expression of sgRNA and Cas9 protein in protoplast

The protoplast transient expression system is an effective and simple method to test the specific genome editing capacity of wheat genes via CRISPR/Cas9 system. In this experiment, the final isolated protoplast cells counted as approximately 1×10^6 cells per milliliter (Supplementary Fig. 3) which were sufficient for transfection experiments (5×10^5 cells for sample). After 24, 48, and 72-h incubation of transfected protoplast, fluorescence microscopic analyses revealed that approximately 20, 40, and 70% of transfected protoplasts had GFP expression signal, respectively (Fig. 2). The obtained rate for transformation efficiencies was lower compared to the results of a previous report in rice protoplast system (Xie and Yang 2013), in which 90% of GFP expression was reported after 72 h of incubation. However, the obtained efficiencies were sufficient to establish sgRNA and Cas9 expression in wheat protoplast system at the end of 72 h, and the transfection duration was detected as 72 h.

Validation of targeted gene mutation in wheat protoplast

To test the mutation efficiency of CRISPR/Cas9 system in wheat protoplast, three different methods were utilized. First, RE-PCR assay was used to detect mutations in the target site and to estimate the frequency of mutation as previously described (Gao et al. 2014). Second, the T7E1 assay was used to detect mismatched nucleotides in the target sites and confirm the mutagenesis (Kim et al. 2009). This was tested with mismatch-sensitive T7E1 after melting and annealing, and cleaved DNA fragments would be detected if amplified products contained both mutated and wild-type DNA (Xie and Yang 2013). Finally, Sanger sequencing of the cloned PCR products further confirmed that targeted mutations were introduced at the predicted Cas9 cleavage site, which is three bases upstream of PAM. After a 72-h incubation, the transfected wheat protoplasts were collected for genomic DNA isolation, with protoplasts transfected by pTaU6::sgRNA:Cas9 empty vector as negative control which did not contain sgRNA of target sites. PCR amplification was performed using the primer of target gene-specific oligos (Table 1) which resulted in products that were about 500–700 bases long. The PCR products of *TaDREB2* gene were digested with restriction digestion enzyme *Sal1* which recognizes and digests the target sequence near the PAM site. In *TaDREB2* transfection, undigested bands clearly appeared on the samples without *Sal1*. The PCR product of empty vector-transformed protoplasts were completely digested by the *Sal1* enzyme (Fig. 3a), whereas PCR product of *TaDREB2* sgRNA/Cas9 transfection digested into expected bands. According to band intensity, the targeting efficiency for *TaDREB2* was 50% (Fig. 3a). Additionally, mutation efficiency for *TaDREB2* was detected with T7E1 assay. In this assay, PCR products from genomic DNA from the *TaDREB2* sgRNA/Cas9 and empty vector-transfected protoplast were treated with mismatch-sensitive T7E1 after melting and annealing. Cleaved DNA fragments would be detected if amplified products contained both mutated and wild-type DNA. The T7E1-digested fragments were detected in the *TaDREB2* genomic DNA but not in the empty vector control (Fig. 3b). The percentage of digestion was about 6.7% in the *TaDREB2*. In addition, Sanger sequencing was performed with cloned PCR products to further confirm the presence of targeted mutations at the predicted Cas9 cleavage site. Sanger sequencing proved that various mutations, including deletion and nucleotide replacement, were detected at the TaDREB2 (Fig. 3c).

T7E1 assay was also performed on the *TaERF3* to detect mutation of the target site. The T7E1 digestion fragments were detected in the *TaERF3* genomic DNA but not in the empty vector control (Fig. 4a), and the percentage of digestion was about 10.2% in the *TaERF3*. In addition, Sanger sequencing results of *TaERF3* PCR products were analyzed to confirm the presence of the mutation in the target site. This result showed deletions and changing nucleotides at the targeted region with sgRNAs (Fig. 4b). Overall, these results suggested that the constructed CRISPR/Cas9 vector can be transiently expressed in wheat protoplast and targeted genome editing can be achieved by Cas9/sgRNA complex.

Specificity of CRISPR/Cas9

In order to detect the target specificity of CRISPR/Cas9-based mutations, three off-target regions for TaDREB2 and two off-target regions for TaERF3 were chosen with different mismatches compared to sgRNA (Table 3). Amplified PCR products for these regions were sequences with NGS. Amplicon sequencing was resulted with both intended and non-intended mutations which arise from the nature of PCR amplification process. To neglect the effect of non-intended mutations, the mutation rates of wild-type samples was substituted from mutated samples, and the difference was accepted as the off-target mutation efficiency. Off-target mutation rate was detected for DREB2-OffTarget1 and DREB2-OffTarget3 regions with an efficiency of 0.013 and 0.97% where the actual targeting efficiency for DREB2 target region was calculated as 6.7%. For DREB2-OffTarget2 region, no off-target effect was detected since the PAM sequence was placed four bases away from the mimicking sgRNA sequence. For those reasons, any off-target effect for ERF3 was not detected for the selected regions. The results confirmed the specificity of Crispr-Cas9-based editing for wheat genome.

Table 2 Sub-genomic specificity of designed sgRNAs

sgRNA name	sgRNA sequence	Target gene	Targeted amino acids	Targeted genome	Editing capability
sgRNA-DREB2	GCAGGACGTCGACG AGGACT	*TaDREB2*	ESSSTSC	3A	No
				3B	Yes
				3D	Yes
sgRNA-ERF3	GCGAGGGGCAAGCA CTACCG	*TaERF3*	V A R G K H Y	2A	No
				2B	Yes
				2D	Yes

The *TaDREB2* and *TaERF3* expression under dehydration treatment

To confirm the role of *TaDREB2* and *TaERF3* in the genotype used, their expression was investigated under shock drought stress by qRT-PCR. Quantitative gene expression results showed that shock drought treatment stimulated the upregulation of both genes (Fig. 5). The expression of *TaDREB2* and *TaERF3* was gradually increased in response to longer incubation of seedlings under dehydrated conditions. These results support the positive regulation of both *TaDREB2* and *TaERF3* under drought stress.

Discussion

CRISPR/Cas9 system is an effective method for targeted genome editing, and its efficiency has been shown in several plant species (Gao et al. 2014; Belhaj et al. 2015; Endo et al. 2015). Although this system is relatively easy to use and more precise compared to other genome editing technologies, there are still some issues, particularly for polyploid plants, such as editing efficiency and off-target mutation rate (Peng et al. 2015). Here, we conducted a series of experiments to show the efficient genome editing with CRISPR/Cas9 system in wheat protoplast. Our results confirmed that CRISPR/Cas9 system is a promising tool for further targeted editing of wheat genome.

Abiotic stress conditions such as drought stress, salt stress, and micronutrient deficiency are serious problems for wheat producers which cause tons of yield loss each year (Araus et al. 2008; Dolferus et al. 2011; Budak et al. 2015). Plants combat against the abiotic stress condition by utilizing a comprehensive signal mechanism where expression of many different genes is involved (Baldoni et al. 2015; Kuzuoglu-

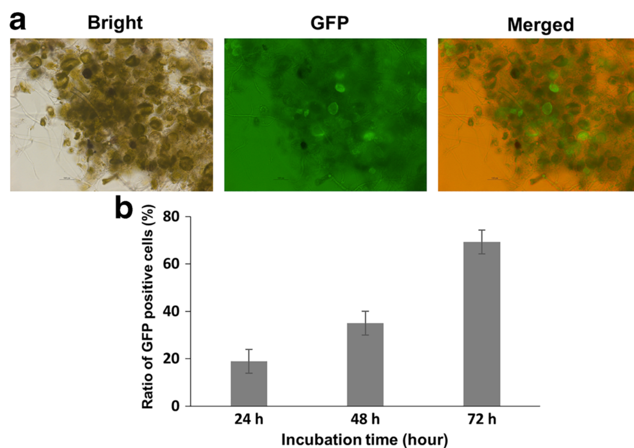


Fig. 2 Transient expression efficiencies for wheat protoplast. **a** pGFPGUSplus (35S::eGFP) plasmid was transiently expressed in protoplasts to observe the efficient transformation. Part a shows the same protoplast picture with different filters (bright and GFP) where the merged image was obtained from ImageJ (NIH version 1.5). **b** To detect the transfection efficiency, 10 μg of pGFPGUSplus plasmid DNA was used for 1 × 105 protoplast cells. The graph shows the ratio of GFP-positive cells to the total number of protoplasts (n ≥ 50). The represented mean values are generated based on at least three different transformation results

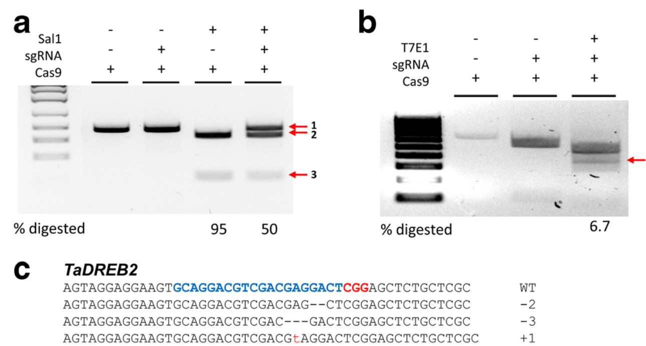
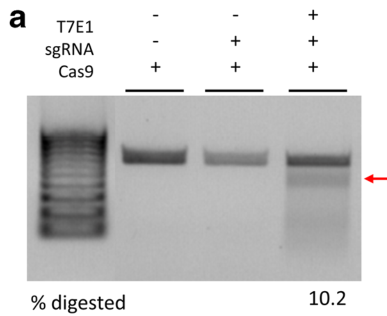


Fig. 3 Mutation detection for TaDREB2. **a** RE-PCR assay was performed to detect the mutations in TaDREB2 gene from gRNA-Cas9-induced protoplasts. Prior to RE assay, PCR amplification of 600-base region around target sites of TaDREB2 was performed. The negative control was an empty vector (without sgRNA but with Cas9) transformed protoplast genomic DNA. Arrows indicate the digested fragments (non-digested band (1), digested long fragments (2), digested short fragments (3)). **b** Targeted mutations revealed by the T7 endonuclease I (T7E1) assay. The DNA fragments were amplified around the CRISPR/Cas9 target sites by PCR using gene-specific primer from extracted genomic DNA from the protoplast. Mismatches resulting from deletion or insertion at the TaDREB2 PCR amplicon were detected by T7EI digestion. Arrows indicate the digested fragments by T7EI. The ratio of digested DNA band is shown at the bottom of the picture. **c** Detection of targeted mutation at the target sites in the TaDREB2 locus was performed with DNA sequencing. The target sequences are marked in blue, and PAM(NGG) motif is marked in red. The numbers on the sides indicate the type of mutations and involved nucleotides



b

TaERF3

```
GGCGGCCGCCGCGGTGGCGAGGGGCAAGCACTACCGCGGGGTGCGGCAGC    WT
GGCGGCCGCCGCGGTGGCGAGGGGCAAACACTACCGCGGGGTGCGGCAGC
GGCGGCCGCCGCGGTGGCGAGGGGCAAGCAC--CCGCGGGGTGCGGCAGC    -2
GGCGGCCGCCGCGGTGGCGAGGGGCAAGC----CCGCGGGGTGCGGCAGC    -4
```

Fig. 4 Detection of mutations in the TaERF3. **a** T7 endonuclease I (T7E1) assay result for TaERF3 is shown. The 600-bp region around target site was amplified by PCR from transformed protoplasts' genomic DNA. The negative control used PCR fragment from empty vector (without sgRNA but with Cas9) transformed protoplast genomic DNA and T7E1 non-treated PCR fragment. Arrows indicate the digested fragments by T7EI. The ratio of digested DNA band is shown at the bottom of picture. **b** Detection of targeted mutation at the target sites in the TaERF3 locus was performed with DNA sequencing. The target sequences are marked in blue, and PAM(NGG) motif is marked in red. The numbers at the sides indicate the type of mutations and involved nucleotides

Ozturk et al. 2012). Particularly, transcription factors are keystones for stress responses which affect the expression of many genes by resulting in the activation/deactivation of a comprehensive molecular pathways (Singh et al. 2002). In this study, we selected two important abiotic stress-responsive transcription factor genes, *TaDREB2* and *TaERF3* which are characterized by the AP2/ERF domain (Mizoi et al. 2012b; Lucas et al. 2011) for performing CRISPR/Cas9-based genome editing application. The effect of *DREB* genes on increased drought resistance has been shown in several studies (Agarwal et al. 2006; Lata and Prasad 2011; Lucas et al. 2011; Morran et al. 2011). Overexpression of *DREB* members in *Arabidopsis* and soybean increased the drought resistance without affecting the growth parameters (Nakashima et al. 2014). In wheat and barley, overexpressed *DREB2* and *DREB3* resulted with activated expression of many drought-responsive genes and provided more drought tolerance (Morran et al. 2011). It was also reported that DREB proteins are more abundant and strongly regulated in response to drought in root tissue than leaf tissue (Lucas et al. 2011). Additionally, in different maize varieties which show differential drought response, a natural variation was detected in *DREB2* promoters which were associated with drought tolerance. On the other hand, *ERF3* was defined as an important molecule for root development where its interaction with Wox11 protein causes activation of cytokine response (Zhao et al. 2015). This interaction is further linked with root elongation and root hair development which provide drought resistance (Cheng et al. 2016). In addition, expression of

Table 3 Off-targeting activity of designed sgRNA for *TaDREB2*

	Mutation occurrence	Genomic location	Off-target region		Total sequences	With both indicator sequences	More than minimum frequency	Insertions	Deletions	Indel frequency	Mutation frequency
DREB2-OffTarget1	Yes	chr1B:584365261–584365779	**C**CAGGACGT**G**GACG AGGAC**TCC**	WT	203,889	146,826	97,396	1453	2657	4.219885827	0.013241798
				Mutant	211,404	151,982	101,438	1430	2864	4.233127625	
DREB2-OffTarget2	No	chr4B:348692666–348693184	GCAGGACGTCGA**G**GAGG AC**A***GCAG***AGG**	WT	125,675	124,801	110,847	0	375	0.338304149	–
				Mutant	149,679	148,858	134,254	0	451	0.335930401	
DREB2-OffTarget3	Yes	chr6B:211588105–211588623	**C**CAGGACGT**G**GACG AGGAC**TCC**	WT	172,997	27,253	9544	650	488	11.92372171	0.970461892
				Mutant	101,807	21,558	4281	191	361	12.8941836	
ERF3-OffTarget1	No	chr2A:412471019–412471537	GGTAGTGCTTG**G**CCCT CGCGA**TGG**	WT	161,371	86,326	75,490	538	0	0.007126772	–
				Mutant	93,031	52,693	45,067	263	0	0.005835756	
ERF3-OffTarget2	No	chr2D:318566953–318567471	GGTAGTGCTTG**G**CCCT CGCGA**TGG**	WT	143,421	140,993	122,547	44	397	0.003598619	–

TaDREB2 and *TaERF3* showed upregulation in response to shock drought stress in variety Chinese spring (Fig. 5). Overall, these findings indicate that *DREB2* and *ERF3* are vital genes for abiotic stress tolerance, particularly for drought, and further characterization of functions of these genes is necessary for understanding their involvement in stress response. Regarding this purpose, these genes are thought as good candidates for targeted genome editing where their characterization with stable transformation will provide a deep insight about their functioning in abiotic stress response.

As the first step of CRISPR/Cas9, the target sites with PAM (NGG) sequence in the 3′-region were selected and associated oligos were synthesized. Chosen sgRNAs were inserted into the pJIT163-2NLSCas9 plasmid with a combined TaU6 promoter site. The efficient expression of sgRNA-Cas9 constructs was obtained for both *TaDREB2* and *TaERF3*. The results showed that rice codon-optimized Cas9 can be efficiently expressed in wheat and utilize for specific genome editing. The transient expression of the sgRNA-Cas9 construct was successfully achieved in wheat protoplast. Interestingly, the transformation efficiency was lower compared to other studies (Shan et al. 2014). This low transformation efficiency might arise from the fragile nature of wheat protoplast as mentioned in some previous works (Sun et al. 2013). In spite of this low efficiency, the obtained transformation rate was sufficient for effective editing of targeted genes in the wheat genome which was confirmed with three different mutation validation techniques. The restriction enzyme digestion assay was only conducted for *DREB2* since there was no defined restriction site in the *ERF3* target site. For both genes, the mutation efficiency was also confirmed with the T7EI assay and Sanger sequencing. Combination of three results suggested that mutation efficiency was lower in *DREB2* compared to *ERF3*. This situation might arise from the location of chosen sgRNA site and suggests that target sites which are chosen in a close proximity of protein domain work more efficiently compared to further target locations inside the protein.

There are a number of concerns about the specificity of genome editing with CRISPR/Cas9 because of the occurrence of random mutation during the genome editing process (Schaefer et al. 2017; Sharpe and Cooper 2017). In this study, the specificity of genome editing was further investigated with next-generation sequencing of off-target regions which were selected based on the homology of sgRNA to several regions in wheat. Three amplified off-target regions for *TaDREB2* were sequenced with amplicon sequencing, and results were analyzed with two different online tools CRISPR-GA (Güell et al. 2014) and Cas analyzer (Park et al. 2016). Results from both programs showed an accordance, however; Cas analyzer results were chosen as representative since the tool enables user to select the range of mutation analysis in given sequences. Furthermore, the minimum frequency parameter in this tool provides a control against randomly occurred mutations which is arisen from the nature of PCR amplification process. In fact, amplicon sequencing results showed the presence of random insertions/deletions in both wild-type and mutant samples which were occurred in the amplification process, probably because of high GC content of the amplicon of interest. Such mutations can easily affect the calculated mutation efficiency rate, particularly in pooled amplicon sequencing for mutation efficiency (Park et al. 2016). In order to avoid this effect of random mutations, the CRISPR mutation efficiency was calculated by subtraction of mutated and wild-type mutation rates. This analysis showed that genome editing efficiency for off-target regions with a real PAM sequence for *TaDREB2* was significantly lower compared to targeted sequence. This result highly supports the specificity of genome editing with CRISPR/Cas9 system in wheat even in the presence of some random indels in selected regions. However, the

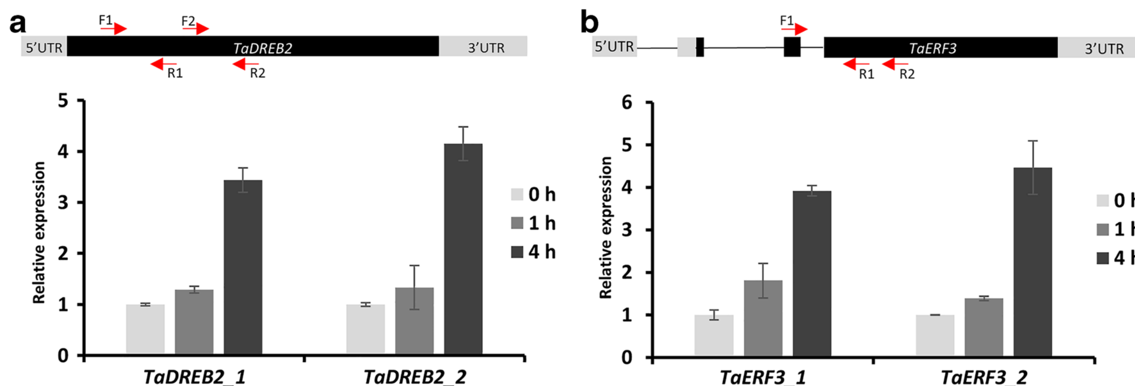


Fig. 5 *Expression patterns of TaDREB2 and TaERF3 upon drought treatment.* **a.** *Upper panel* shows gene model of *TaDREB2*. *Gray boxes* are 5′ and 3′ UTR, and *black boxes* are exon. *Red arrows* indicated two different qRT-PCR primers for gene expression analysis. The *bottom panel* shows the relative expression pattern of *TaDREB2* with short drought treatment (0, 1, and 5 h of drought incubation). *TaDREB2*_1 has used qRT-PCR primer F1 and R1, and *TaDREB2*_2 has used the primers F2 and R2. **b.** *Upper panel* shows the gene model of *TaERF3*. *Gray boxes* are 5′ and 3′ UTR; *black boxes* are exon, and *lines* are intron. *TaERF3_1* has used qRT-PCR primer F1 and R1, and *TaERF3_2* has used F1 and R2 primers. The short drought treatment was harvested after 0, 1, and 4-h treatments

length of such indels was relatively short (one to two bases) which provides support for their randomness.

In this study, the CRISPR/Cas9 genome editing system in wheat protoplast was effectively established. The transient expression of sgRNA and Cas9 protein was performed in wheat protoplast. The genome editing efficiency was shown with restriction enzyme assay, T7 endonuclease assay, and Sanger sequencing. Furthermore, the specificity of editing in wheat was confirmed with amplicon sequencing analysis. Overall, a successful application of CRISPR/Cas9 based genome editing in wheat protoplast was performed with two abiotic stress genes, *TaDREB2* and *TaERF3*. These current findings suggest that CRISPR/Cas9 system is an efficient tool for targeted genome editing for wheat and it has a potential application for manipulation of wheat genome aiming a better crop performance.

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