Optimizing multiplex CRISPR/Cas9-based genome editing for wheat

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Abstract

Background: CRISPR/Cas9-based genome editing holds a great promise to accelerate the development of improved crop varieties by providing a powerful tool to modify genomic regions controlling major agronomic traits. The effective deployment of this technology for crop improvement still requires further optimization of the CRISPR/Cas9 system for different applications. Here we have optimized multiplex gene editing strategy for hexaploid wheat.

Results: The functionality of the various components of the CRISPR/Cas9 gene editing system was validated using the wheat protoplast transformation assay followed next-generation sequencing (NGS) of the targeted gene regions. The wheat codon optimized Cas9 was shown to be able to effectively edit targets in the wheat genome. Multiple guide RNAs (gRNAs) were evaluated for the ability to target the homoeologous copies of four genes controlling important agronomic traits in wheat. Low correspondence was found between the gRNA efficiency predicted bioinformatically and that assessed in the protoplast transient expression assay. Multiplex gene editing was successfully accomplished using the construct with several gRNA-tRNA units under the control of a single RNA polymerase III promoter.

Conclusions: The assessment of gRNAs' targeting efficiency using the transient expression in the protoplasts and NGS suggest that further optimization of the gRNA design tools will be required to improve the selection of functional gRNAs for the allopolyploid genome. The empirically selected gRNAs provided an effective method for designing a successful multiplex gene editing experiment in wheat. Wheat codon optimized Cas9 as well as the Golden Gate-compatible tRNA processing-based gRNA cassette developed here further expand the CRISPR/Cas9 toolbox available for editing the hexaploid wheat genome.

Keywords

CRISPR/Cas9, genome editing, polyploid wheat, multiplex editing, tRNA

Background

Genome editing approaches have been revolutionized by the discovery of clustered regularly interspaced short palindromic repeats (CRISPR) that can guide nuclease Cas9 to specific targets in genome and create double strand breaks (DSB) [1]. The DSB can be repaired through either the error-prone non-homologous end joining (NHEJ) process generating loss-of-function deletions or, if the exogenous template complementary to the DSB flanking sequences is provided, the homology-directed repair (HDR) resulting in the insertion or replacement of a specific sequence. This system provides a very simple, highly specific and a versatile tool to introduce targeted changes to genomes of any species amenable to transformation.

Since the first introduction of the CRISPR/Cas9 system for editing the mammalian genomes[2, 3], it has been applied to many model or crop plants including tobacco [4, 5], tomato [6, 7], barley [8], Arabidopsis [4], wheat [9-11], rice [11, 12] and maize [13]. In these studies, the components of the CRISPR/Cas9 editing system have been optimized for plants to improve the specificity, throughput and efficiency. Significant improvements in the expression level of Cas9 and the efficiency of genome editing have been achieved by developing plant codon optimized Cas9 [4]. However, most studies relied on Cas9 genes that were not optimized for the species of interest. For example, rice codon-optimized Cas9 was used to target the *Mlo* gene in wheat [9], or the *inox* and *pds* genes in wheat were mutagenized using the human-optimized SpCas9 [10].

The synthesized single-guiding RNA (sgRNA), which includes two functional domains, was widely used in most CRISPR/Cas9-based genome editing studies [14]. The first domain is a 20 nt-long leading sequence (gRNA), which defines the target specificity and guides Cas9/sgRNA complex to the target site. The second domain is a scaffold sequence, which was developed from CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA). The mature crRNA paired with tracrRNA forms a complex that can direct Cas9 to a target site [14]. While the minimal requirement for a gRNA target site is the presence of protospacer adjacent motif (PAM) NGG [15], there are other sequence features that can affect the efficiency of genome editing. For example, it was shown that the GC content of gRNA can impact the Cas9 cleavage efficiency [16, 17], or base C at position 16 [17] and base G at position 20 were preferentially found in the functional gRNAs [16-19]. It was also reported that the GG motif at the 3'-end of gRNA can increase the frequency of targeted mutagenesis in *C. elegans* [20]. To improve gRNA design, the large scale analyses of the various sequence features of the functional gRNAs were performed and integrated into the bioinformatical gRNA designer tools [17, 19, 21-28]. By taking into account such parameters as DNA sequence composition, thermodynamic stability, the presence of duplicated targets and the accessibility of genomic regions, these tools were shown can significantly improve the gRNA's targeting capability and specificity [19, 21, 22]. Only few of these tools were optimized to design gRNAs for wheat [25, 29] or other plants [30].

To increase the number of targets that can be simultaneously edited, several gRNA multiplexing strategies have been developed. Multiple gRNAs, each under the control of its own promoter, were combined into a single construct to edit multiple targets in maize and *Arabidopsis* [13]. Effective editing of multiple genes in rice was achieved by putting multiple gRNAs separated by tRNA spacers under the control of a single promoter [12]. Processing of this polycistronic gene through the endogenous tRNA processing system resulted in functional gRNAs capable of guiding Cas9 to multiple targets. These multiplex gene editing strategies provide a powerful tool for the targeting of several genes in multigene families [31] or gene networks, or for deleting multiple genomic regions simultaneously [3].

Here we have optimized the multiplex gene editing procedure for hexaploid wheat. To expand the set of tools available for CRISPR/Cas9-based editing of the wheat genome, we have developed a wheat optimized Cas9 gene and demonstrated its functionality by editing the *Q* [32], *TaGW2* [33], *TaLpx-1* [34] and *TaMLO* [9] genes. The gRNA selection procedure for multiplex gene editing included the transient expression assay in the wheat protoplasts followed by the deep NGS of multiplexed barcoded PCR products generated for different target regions. We demonstrate that the highly effective multiplex gene editing in wheat can be achieved using a single polycistronic gene construct including the array of gRNA-tRNA units [12] under the control of a single promoter.

Methods

Plasmids and vector construction

A fragment of vector pAHC17 between the cut sites for SphI and XhoI restriction enzymes (NEB) containing the maize ubiquitin gene promoter (*ubi*) was used to replace the 35S promoter region in vector pA7-GFP (Katrin Czempinski, Potsdam University, Germany). This new plasmid construct is referred to as pA9-GFP (Figure 1A). Wheat codon optimized Cas9 coding sequence (CDS) fused from both sides with the nuclear localization signals (NLS) was synthesized by Integrated DNA Technology (IDT). The XhoI and SmaI cut sites added to the 5'- and 3'-ends of the fragment during the synthesis were used to subclone it into the pBluescript SK (PSK) vector. Then the Cas9-containing fragment between XhoI and XbaI cut sites was excised from the PSK vector, and used to replace the GFP gene between the XhoI and XbaI sites in the pA9-GFP construct. Henceforth this construct will be referred to as pA9-Cas9 (Figure 1A and Supplementary figure 1A).

Wheat U6 promoter and sgRNA sequence flanked by BsaI cut sites was synthesized by IDT with the BamHI and SacI cut sites added to the 5'- and 3'- ends, respectively. The NOS poly-A terminator fragment between the SacI - EcoRI fragment was cut from the pA7-GFP construct and sub-cloned into pHSG299. The synthesized U6 promoter - sgRNA fragment was cloned into vector pHSG299 between the BamHI and SacI cut sites (henceforth, pU6sg plasmid) (Figure 1A and Supplementary figure 1B).

The following plasmids were ordered from the Addgene plasmid repository: 1) pBUN421 [13], pRGE32 [12] and pJIT163-2NLSCas9 [11] with the maize, human and rice codon optimized Cas9 genes, respectively (Figure 1A), and 2) pGTR [12] containing a gRNA-tRNA fused units. The latter was modified to create a polycistronic tRNA-gRNA (*PTG*) gene construct for wheat.

Targeting portion of the single gRNAs was synthesized as a pair of reverse complementary oligonucleotides (Supplementary Table 1 and Supplementary Fig. 1B), which created the 5' overhang tails after annealing. The reaction mix contained 2 μL of 10× annealing buffer (200 mM Tris-HCl, 100 mM (NH4)2SO4, 100 mM KCl, 1% Triton X-100, 20Mm MgCl2, pH9.0), 2 μL of 100 μM forward and reverse oligos, and 14 μL ddH2O. The mix was denatured for 5 min at 94 ℃ followed by the gradual 1 ℃ per min temperature decrease until it reached 30 ℃. Annealed oligos were sub-cloned into pU6sg or pBUN421 using Golden Gate cloning method [35]. The Golden Gate reaction was assembled by mixing 2 μL of annealed target, 0.5 μL of pU6sg (0.2 μg/μL) or pBUN421 (0.6 μg/μL), 2 μL of 10× T4 DNA ligase buffer, 2 μL of BSA (1mg/mL), 1 μL of T4 ligase (400 U/μL), 0.5 μL of BsaI-HF (20 U/μL), and 12 μL ddH2O. The mix was incubated for 50 cycles at 37 ℃ for 5 min and 16 ℃ for 10 min, followed by final incubation at 50 ℃ for 30 min. Golden Gate reaction products were transformed into the chemically competent cells of *E. coli* strain DH5α by heat shock. Plasmid DNA was extracted using QIAprep Spin Miniprep Kit (Qiagen) and validated by Sanger sequencing.

We have designed gRNAs for editing the set of four wheat genes including *Q* [32], *TaGW2* [33], *TaLpx-1* [34] and *TaMLO* [9]. A total of seven gRNAs were designed for the *Q* gene [32], and two gRNAs were designed to target each the *TaGW2* [33, 36] and *TaLpx-1* [34] genes (Supplementary Figure 2, Table 1 and Supplementary Table 1). A gRNA target sequence for the *TaMLO* gene on the wheat A genome was selected as a control from the previously published study (Table 1 and Supplementary Table 1) [9]. All gRNAs for genes *Q* and *TaGW2* were sub-cloned into the pU6sg and co-transformed with pA9-Cas9 into the wheat protoplasts.

Whereas, the gRNAs designed for the *TaLPX1* and *TaMLO* genes were delivered into the wheat protoplasts as single-plasmid constructs sub-cloned into pBUN421.

We have modified previously reported method for assembling the *PTG* gene construct [12]. Our modification allows subcloning multiple PCR fragments, each including a gRNA-tRNA block, into the pU6sg or pBUN421 plasmids in a single-step Golden Gate reaction. As shown in Supplementary Table 1 and Supplementary Figure 3, all primers for the PCR amplification of gRNA-tRNA blocks had 5'-end tails with the BsaI cut sites for Golden Gate assembly. The forward primer of the first gRNA-tRNA block and the reverse primer of the last gRNA-tRNA block in the *PTG* gene construct included the complete 20-nt long target gRNA sequences. The target sequence of the middle gRNA was divided among the two PCR primers, reverse primer of the first gRNA-tRNA block and forward primer of the next gRNA-tRNA block. These primers had four complementary nucleotides, which created sticky ends after the digestion with the BsaI enzyme and used for PCR fragment ligation (Supplementary Figure 3). PCR mix included the 10 μL NEB Next High Fidelity 2× PCR Master Mix, 2 μL plasmid pGTR (5 ng/μL), 2 μL each primer (5 μM), and 4 μL ddH2O. PCR conditions were as follows: 98 ℃ for 30 sec followed by 35 cycles of 98 ℃ for 10 sec, 56 ℃ for 15 sec, 72 ℃ for 10 sec, and final extension step at 72 ℃ for 5 min. The resulting PCR products were sub-cloned into pU6sg or pBUN421 in a single-step Golden Gate reaction by mixing each PCR product in equal amounts (20 – 50 ng) with 0.5 μL of pU6sg (0.2 μg/μL) or pBUN421 (0.6 μg/μL), 2 μL of 10×T4 DNA ligase buffer, 2 uL of BSA (1mg/mL), 1 μL of T4 ligase (400 U/μL), 0.5 μL of BsaI-HF (20 U/μL), and (14 - x) μL of ddH2O. The products of Golden Gate reaction were transformed into the DH5α strain of *E. coli*, followed by testing the accuracy of construct assembly using Sanger sequencing. This procedure was used for assembling plasmid pBUN421-GLM with the gRNAs targets *TaGW2T2*, *TaLPX1T2* and *TaMLOT1*.

The pU6sg-tQT1sgt construct including a single gRNA targeting the *Q* gene was designed for testing the efficiency of tRNA-based gRNA processing system in wheat [12]. The pU6sg-tQT1sgt was constructed using the strategy similar to that described for pBUN421-GLM (primers are shown in Supplementary Table 1).

Protoplast transformation and DNA isolation

Protoplast transformation was performed using a modified protocol developed for *Arabidopsis* [37]. The seedlings of *Triticum aestivum* cultivar Bobwhite were grown in dark at 25 ℃ for 1-2 weeks. Shoot tissues from 50 seedlings were sliced into 1-mm strips with a razor blade and placed into a flask with the digestion solution including 0.15 M Sorbitol, 0.25 M sucrose, 35 mM CaCl2, 20 mM KCl, 1.5% Cellulase R10 (From Trichoderma Viride, 7.5 U/mg), 0.75 % Macerozyme (R10 Macerating enzyme from Rhizopus sp. RPI) and 10 mM MES-KOH (pH 5.7). All chemicals were ordered from Sigma Aldrich. To infiltrate digestion solution into leaf tissues, vacuum (-600 MPa) was applied to the flask for 30 min in dark followed by incubation at room temperature for 2 hours with gentle shaking at 20-30 rpm. Digested tissues were filtered through a 40 um nylon mesh into a centrifuge tube followed by rinsing the mesh with 20 mL of W5 solution (0.1 % glucose, 0.08 % KCl, 0.9 % NaCl, 1.84 % $CaCl_2$•$2H_2O$, 2 mM MES-KOH, pH 5.7). Due to the difference in the concentration of sucrose, in the tube, the digestion solution (0.25 M sucrose) and W5 are separated into two phases. The tube was centrifuged for 7 min at 100 g at the room temperature. The protoplast fraction accumulated at the interface of the digestion solution and W5 was collected, diluted 2 times in W5 and precipitated by centrifuging for 5 min at 60 g. Protoplasts were resuspended in 10 mL of W5 and precipitated again by centrifugation. Finally, protoplasts were resuspended in 3 ml of W5 and incubated on ice for 30 min.

Before transformation, W5 was removed and protoplasts were resuspended in the MMG solution (0.4 M mannitol, 15 mM $MgCl_2$, 4 mM MES-KOH, pH 5.7). The protoplast count was adjusted to 10^6 protoplasts /mL. Ten microgram of plasmid DNA in 10-20 μL volume was gently mixed with 100 μL of protoplasts followed by adding 130 μL of PEG-calcium transfection solution (40 % PEG4000, 0.2 M mannitol, 100 mM $CaCl_2$). The resulting solution was gently mixed by agitating a tube and incubated at room temperature for 30 min. Then the transfection mix was diluted with 500 μL of W5, protoplasts were collected by centrifuging at 100 g for 2 min and resuspended in 1 mL of W5.

After 18 hours of incubation at room temperature, GFP signal in the protoplasts transformed with pA9-GFP was estimated using the Zeiss LSM 780 confocal microscope with the excitation wave length of 488 nm. The efficiency of transformation calculated as the fraction of GFP expressing protoplasts was close to 60%.

Protoplasts were incubated for 48 hours before DNA isolation. For DNA extraction, protoplasts were collected by centrifugation, mixed with 300 μL of TPS (100 mM Tris-HCl, 10 mM EDTA, 1 M KCl, pH8.0) by vortexing followed by adding 300 μL of isopropanol. After 20 min incubation, DNA was precipitated by centrifugation for 10 min at 13,000 rpm, rinsed with 1 mL of 70% ethanol, dried at the room temperature and resuspended in 20 μL ddH2O.

Validation of genome editing events

To detect mutations induced by the CRISPR/Cas9 system, genomic regions harboring the gRNA targets were PCR amplified and sequenced (Figure 1C). PCR was performed in 20 μL reaction volume using 10 μL NEB Next High Fidelity 2× PCR Master Mix, 2 μL protoplast DNA and 2 μL of each primer (5 μM) and 4 μL ddH2O.

The efficiency of target editing using gRNA QT1 was assessed by digesting PCR products with the StyI (NEB) enzyme (Figure 1B). The digestion was performed in a 20 μL reaction volume by mixing 5 μL of PCR product, 2 μL of 10× Cut Smart Buffer, 0.5 μL of StyI-HF (20 U/μL), and 12.5 μL of ddH2O. The reaction mix was incubated at 37 ℃ overnight, and analyzed on 3% agarose gel. The band intensities on the agarose gel images were measured with background subtracted using GelQuant.NET (biochemlabsolutions.com). To calculate the frequency of genome editing, the non-digested band intensity was divided by the sum of all band intensities.

Estimating gene editing efficiency by NGS

To simultaneously analyze the large number of edited genomic regions using the NGS approach, amplicons from the targeted regions were barcoded during the two rounds of PCR with the Illumina's TruSeq adaptors. During the first round, we have used target-specific forward and reverse PCR primers modified by adding to the 5'-end a 30-nucleotide long sequence complimentary to a part of the Illumina's TruSeq adaptors (Supplementary Table 1). The second round of PCR was performed using the primers complimentary to the 5'-ends of the first set of primers. This second set of primers completes the reconstruction of TruSeq adaptor sequences and adds amplicon-specific barcodes (Supplementary Fig. 4). PCR products were purified with MinElute PCR Purification Kit (Qiagen), normalized to 10 nM concentration, pooled in equal volumes, and then sequenced at the K-State Integrated Genomics Facility on the MiSeq instrument using the 600 cycles MiSeq reagent v3 kit.

De-multiplexed and quality-trimmed reads were aligned to each of the three homoeologous copies genes using BWA [38] followed by SNP and indel calling with SAMtools [39, 40]. The efficiency of target editing was estimated as the proportion of reads with indels or insertions relative to the total number of aligned reads using the custom Perl script.

Results

Genome editing using the wheat codon optimized Cas9

The pU6sg-QT1 construct expressing gRNA targeting the *Q* gene (Simons et al., 2006) was co-transformed into wheat protoplasts with the constructs expressing wheat (pA9-Cas9), maize (pBUN421) [13], humans (pRGE32) [12] or rice (pJIT163-2NLSCas9) [11] codon-optimized Cas9. The ability of all tested Cas9 versions to perform gRNA-guided genome editing was confirmed by the StyI digestion of amplicons from the target site (Figure 1B). Based on the gel image band intensities, the efficiency of construct pA9-Cas9 (8%) with wheat-optimized Cas9 was lower than that of pBUN421 (11.7%), but was higher than the efficiency of constructs pRGE32 (3.6%) or pJIT163-2NLSCas9 (4.1%). The Sanger sequencing of subcloned non-digested PCR products from the pA9-Cas9 transformed protoplast DNA revealed a number of deletions resulting from non-homologous end joining-mediated reparation of DNA (Figure 1C).

Analyzing genome editing events by next-generation sequencing (NGS)

The transient expression in the transformed protoplasts provides a simple and rapid method for assessing the editing capability of the CAS9/sgRNA constructs [10, 11]. Here we have adopted this approach to screen sgRNAs for the ability to generate Cas9-mediated changes in the wheat genome. Further, the protoplast expression assay was combined with the NGS for the rapid and cost-effective analysis of multiple genomic regions (Figure 2A and Supplementary Figure 4). This strategy was used to evaluate the gRNAs designed to target four genes controlling domestication (*Q* gene) [32], seed development (*TaGW2*) [33] and disease resistance (*TaLpx-1* and *TaMLO*) phenotypes in wheat [9, 34]. It was shown that nine out of eleven gRNAs effectively targeted different homoeologous copies of genes consistent with the gRNA design specificity (Table 1). The A and D genome copies of the *Q* gene were successfully targeted by three gRNAs on the 5'-end (Figure 2B and Supplementary Figure 5 and 6); only the A genome copy of the *Q* gene was targeted by two gRNAs on the 3'-end (Supplementary Figure 7). Only one of the two target sites in *TaGW2* was edited in all three wheat genomes (Figure 2B and Supplementary Figure 8). Both gRNAs designed for the *TaLpx-1* gene produced deletions in the B and D genomes, whereas no editing events were detected for the target in the A genome due to its high divergence from the guide sequence (Figure 2B and Supplementary Figure 9). The gRNA targeting *TaMLO* induced mutations only in the wheat A genome, as previously reported (Figure 2B and Supplementary Figure 10) [9].

Table 1. Efficiency of sgRNA design for wheat genome editing.

gRNA Name	Gene	Editing event	Efficiency (NGS)	Wheat genome	WU score[a]	E score[b]	sg score[c]	Mul score[d]
Q3endT	Q (3' end)	Yes	11.3%	A	0	0	67.4	0.04
Q3endT3	Q (3' end)	Yes	6.5%	A	0	48.2	78.5	0.41
Q5endT	Q (5' end)	Yes	17.9%	AD	0	0	36.1	0.1
Q5endT4	Q (5' end)	Yes	0.32%	AD	0	0	30.7	0.17
Q5endT5	Q (5' end)	No	0[e]		88	0	0	0
Q5endT6	Q (5' end)	Yes	8.5%	AD	0	54	59.9	0.38
GW2T2	TaGW2	Yes	4.0%	ABD	89	86.8	96.8	0.83
GW2T3	TaGW2	No	0		0	0	33.7	0.61
LPX1T1	TaLPX1	Yes	8.0%	BD	0	0	50.5	0.29
LPX1T2	TaLPX1	Yes	12.7%	BD	0	52	59.3	0.18
MLOT1	TaMLO	Yes	64.1%	A	0	60.5	70.8	0.28

a – WU scores (0-100) are calculated using the WU-CRISPR sgRNA designer [22]; the scores are predicted by a bioinformatics algorithm trained by reanalyzing empirical data from Doench *et.al.* [17].

b – E scores (0-100) are obtained using the E-CRISPR program [29].

c – sg scores (0-100) are the gRNA's editing activity scores calculated using the sgRNAScore 1.0; the scores are predicted based on a bioinformatics algorithm trained on the analysis of empirical data [19].

d – Mul scores (0-1) are gRNA's editing activity scores calculated using the CRISPR MultiTargeter [41]; the scores are predicted by a bioinformatics algorithm developed by Doench, *et.al.* [17].

e – "0" means that a gRNA did not have genome editing events detected or that a gRNA was not selected by a gRNA designer.

Validating the gRNA design tools

To test whether the failure of some sgRNAs to produce deletions is caused by suboptimal sgRNA design, we have used algorithms implemented in WU-CRISPR [22], E-CRISPR [29], sgRNAScore 1.0 [19] and CRISPR MultiTargeter [41], to analyze the gRNAs used in our study. Among these programs only the E-CRISP tool can specifically select gRNA for targets in the wheat genome.

Out of eleven gRNAs analyzed in the protoplast transformation assay, only two had prediction scores assigned by WU-CRISPR, five gRNAs matched the selection criteria of E-CRISP, and ten gRNAs had prediction scores assigned by sgRNAScore 1.0 or CRISPR MultiTargeter respectively (Table 1). Out of two gRNAs selected by WU-CRISPR, only one produced mutations in the target gene, while all five gRNAs selected by E-CRISPR resulted in editing events at the target sites. Among those sgRNAs that failed to meet the WU-CRISPR and E-CRISP design criteria, eight and four, respectively, produced mutations in the protoplast assay. Out of ten sgRNAs that had design scores assigned by sgRNAScore 1.0 or CRISPR MultiTargeter, nine were validated in the protoplast assays. However, the gRNA efficiency estimated by NGS and the gRNA design scores showed low correlation for sgRNAScore 1.0 and CRISPR MultiTargeter (R^2 = 0.088 and 0.016, respectively).

Multiplex gene editing in hexaploid wheat

There are several approaches developed for multiplex gene editing in humans, maize, *Arabidopsis* and rice [3, 4, 12, 13]. The recently developed method using a single polycistronic gene comprised of multiple tandemly arrayed tRNA – sgRNA blocks compatible with the endogenous tRNA processing system provides one of the most efficient and robust strategies for multiplexed gene editing [12]. Here we report the development of a PTG/Cas9 system for multiplex genome editing in hexaploid wheat.

To test the ability of the sgRNA subjected to tRNA processing to serve as guides for Cas9 in hexaploid wheat, we used a construct (pU6sg-tQT1sgt) with the QT1 sgRNA (Supplementary Table 1) flanked by the tRNA units (Figure 3A). The results of NGS suggest successful editing of the targeted region (Figure 3B) and indicate that the endogeneous tRNA processing system of wheat can produce functional sgRNAs from the tRNA – sgRNA constructs.

A higher level of multiplexing was tested by combining the GW2T2-sgRNA, LPX1T2-sgRNA and MLOT1-sgRNA spaced by two tRNAs into a single polycistronic gene construct pBUN421-GLM (Figure 3C). As a control, a single-sgRNA construct pBUN421-LPX1T2 was transformed into protoplasts. The NGS results obtained for the pBUN421-GLM transformants (Figure 3D, Supplementary figure 11 and 12) indicated that the polycistronic transcript was correctly processed resulting in functional sgRNAs capable of directing Cas9 to the respective genomic targets. The editing efficiencies obtained by multiplex editing for *TaGW2*, *TaLpx-1* and *TaMLO* gene targets were 22%, 7.2% and 6.5% respectively. The efficiency of *TaLpx-1* target editing using the LPX1T2 gRNA as a part of the multi-gRNA editing construct (7.2%) or as a single-gRNA editing construct (5.4%) were comparable.

Discussion

The emergence of CRISPR/Cas9 genome editing technology raised hopes for accelerating crop improvement through the targeted modification of genomic regions controlling major agronomic traits. However, large-scale application of this technology for crop genome editing requires further optimization and testing of various components of the CRISPR/Cas9 system. Here we describe a strategy for designing a multiplex gene editing experiment in wheat that includes procedures for pre-screening multiple gRNAs for their efficiency and assembling selected gRNAs into a single policistronic construct. In effort to expand the wheat genome editing toolbox, we have developed wheat codon optimized Cas9 and demonstrated its editing capability.

Because the development of transgenic wheat lines is a time-consuming and labor-intensive process, the selection of functional gRNAs, especially for multiplex target editing, before transformation is a critical step. The protoplast transient expression assay in combination with the multiplexed NGS of pooled amplicons was shown to be an effective strategy for large-scale gRNA pre-screening. Several studies reported the usage of NGS for evaluating the efficiency of Cas9-based editing. The Nextera kit (Illumina) was used for the NGS of PCR-amplified regions targeted by the sgRNA/Cas9 complex [42]. A high-throughput strategy for detecting the CRISPR-Cas9 induced mutations in mouse embryonic stem cells used unique barcodes added to the PCR products of different target sites followed by ligating Illumina adaptors before the NGS [43]. In contrast, the method used in our study allows for adding barcodes and Illumina adaptors during the two rounds of PCR generating products that can be directly sequenced on Illumina sequencers avoiding the time-consuming NGS library preparation step. Another advantage of the NGS-based analysis of Cas9 editing in allopolyploid wheat is that it provides valuable information about the efficiency of homoeologous target editing. Using this strategy, the gRNA design can be easily optimized to target either a specific homoeologous copy of a gene or all homoeologs simultaneously. The throughput of gRNA screening procedure can be increased by increasing the number of barcoded primers in the second round of PCR to include larger number of targets as needed.

Our study found some discrepancy between the bioinformatically predicted gRNA activity scores and the editing efficiency in the protoplast transformation assays. The majority of gRNAs matching the E-CRISP, sgRNAScore 1.0 or CRISPR MultiTargeter tool design criteria were shown to be able to produce editing events in the wheat protoplast assays. However, the effective gRNA design using E-CRISP is achieved at the cost of high false negative rate with the significant fraction of gRNAs classified as non-functional by the E-CRISP tool still showing good editing capability. One possibility for such high false negative classification could be that during the calculation of gRNA activity score using the E-CRISP tool, a penalty is applied if duplicated targets are identical or nearly identical to the original target in the genome [29]. Due to the polyploid nature of the wheat genome this criterion may reduce the number of gRNAs designed in the duplicated genomic regions [29]. While the majority of gRNAs functional in the protoplast assays were assigned prediction scores by the sgRNAScore 1.0 or CRISPR MultiTargeter tool, low correlation was observed between these scores and the editing efficiency assessed by the NGS. This result could be explained by the usage of empirical data obtained for humans [17, 19] for optimizing these bioinformatics algorithms, and indicates that for designing gRNAs optimized for genome editing in polyploid wheat further improvements of bioinformatical tools will be required.

Here we demonstrated that the editing of multiple gene targets in the wheat genome could be achieved using a construct with multiple gRNA-tRNA units under the control of a single RNA polymerase III promoter. A transcript produced from this construct was shown can produce functional gRNAs after being processed through the endogenous tRNA-processing system, consistent with the results reported for rice [12]. The high multiplexing capacity of the PTG/Cas9 system makes it well suited for studying the polyploid wheat genome function. Due to the presence of duplicated gene copies editing in the wheat genome might require multiple gRNAs for knocking out or deleting a specific set of homoeologs. Moreover, by designing gRNAs to sequences carrying homoeolog-specific nucleotide changes or to sequences conserved across all wheat genomes it will be possible to selectively edit either multiple genes from a specific wheat genome or to introduce modifications to all gene copies. When the useful gene variants for multiple agronomic traits are identified, the multiplex gene editing capability of the PTG/Cas9 system, can be used to simultaneously introduce multiple changes to the wheat genome in a single transformation experiment. For known genes, this strategy will allow for skipping the time-consuming step of gene pyramiding and accelerating the development of improved varieties.

Conclusions

The development of sgRNA/Cas9 genome editing system has a great potential to accelerate the development of new crop varieties by enabling rapid functional validation of candidate genes through reverse genetics approaches and by introducing novel variation into genomic regions controlling important agronomic traits. We demonstrate here that by integrating fast transient expression assays with multiplexed NGS it is possible to perform functional screens for a large number of gRNAs and to optimize constructs for effective editing of multiple independent targets in the wheat genome. We further showed that multiplex gene editing in wheat can be effectively accomplished using a construct with multiple tandemly arrayed tRNA–sgRNA units under the control of wheat RNA polymerase III promoter. The polycistronic gene construct that can be quickly assembled using the Golden Gate reaction along with wheat codon optimized Cas9 further expand the set of tools available for engineering the wheat genome.

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Figures

Figure 1. Genome editing using the wheat codon optimized Cas9.

(A) Constructs used in the current study. All promoters are shown as white arrows. zUbip -maize ubiquitin gene promoter; OsUbip - rice ubiquitin gene promoter; TaU3p and TaU6p - wheat U3 and U6 promoters, respectively; OsU3p - rice U3 promoter, 2X35Sp - duplicated 35S promoter from the tobacco mosaic virus. Cas9 gene terminator sequences are shown as gray rectangles. Yellow rectangles correspond to nuclear location signal signals (NLS), purple rectangles correspond to the target sequence insertion sites in the synthetic single-guide RNA (sgRNA), and blue rectangles stand for 3x FLAG sequences.

(B) Restriction enzyme digest of a PCR product obtained using the primers flanking the *Q* gene region targeted by Cas9 and QT1-sgRNA (Supplementary Fig. 2). Lane 1 and 6 - PCR products from the protoplasts transformed with pA9-GFP, Lanes 2 - 5 - PCR products from the protoplasts co-transformed with the sgRNA and the different versions of Cas9 from rice (Lane 2), humans (Lane 3), maize (Lane 4), and wheat (Lane 5), respectively. PCR products on Lanes 1 - 5 are digested with the StyI-HF, Lane 6 is non-digested PCR product. Percent of mutations (%) was calculated from the band intensities (see Methods).

(C) Sanger sequencing of the *Q* gene region targeted by the wheat optimized Cas9 and QT1-sgRNA. Wild type sequence is shown on the top row. Target sequence is shown in red rectangle; PAM sequence is underlined.

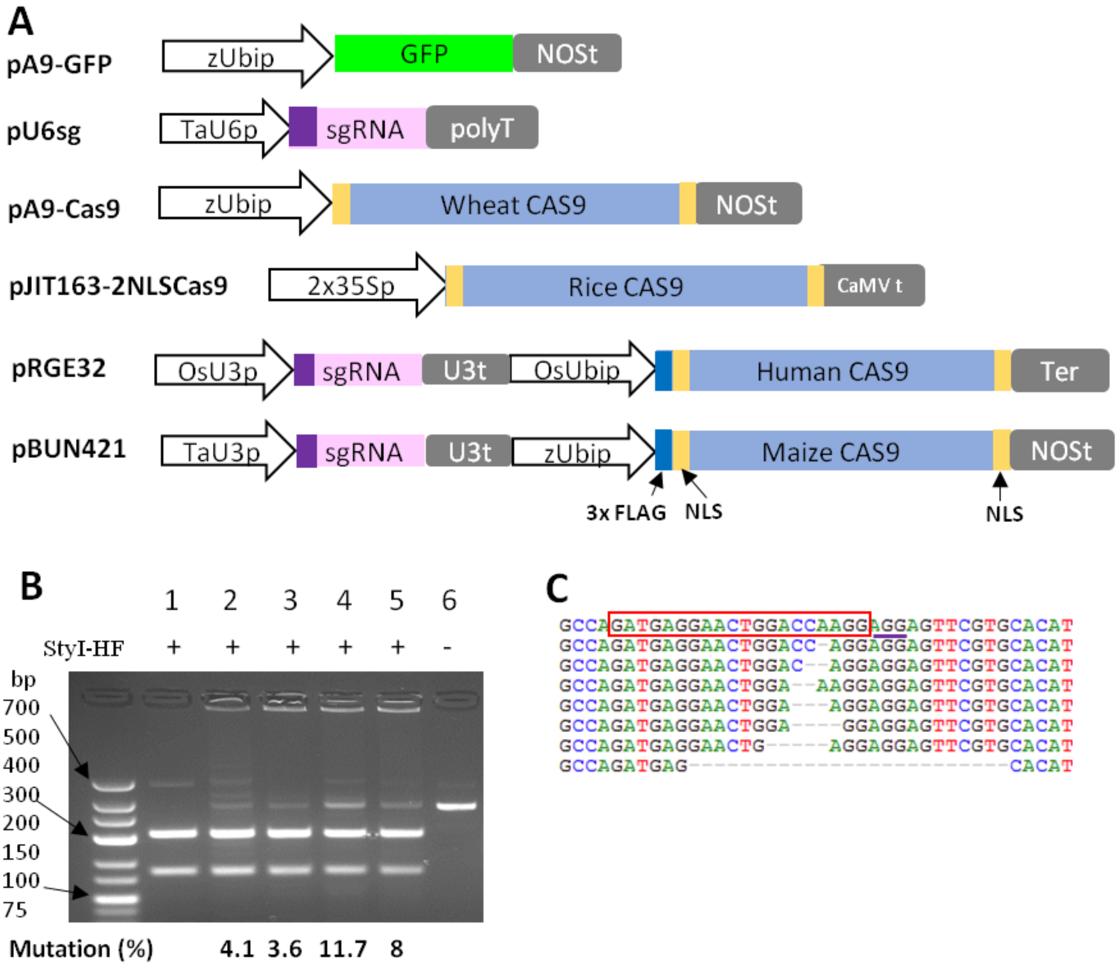

A
pA9-GFP
zUbip
GFP
NOSt
pU6sg
TaU6p
sgRNA
polyT
pA9-Cas9
zUbip
Wheat CAS9
NOSt
pJIT163-2NLSCas9
2x35Sp
Rice CAS9
CaMV t
pRGE32
OsU3p
sgRNA
U3t
OsUbip
Human CAS9
Ter
pBUN421
TaU3p
sgRNA
U3t
zUbip
Maize CAS9
NOSt
3x FLAG
NLS
NLS
B
1 2 3 4 5 6
StyI-HF + + + + + -
bp
700
500
400
300
200
150
100
75
Mutation (%) 4.1 3.6 11.7 8
C
GCCAGATGAGGAACTGGACCAAGGAGGAGTTCGTGCACAT
GCCAGATGAGGAACTGGACC-AGGAGGAGTTCGTGCACAT
GCCAGATGAGGAACTGGAC--AGGAGGAGTTCGTGCACAT
GCCAGATGAGGAACTGGA--AAGGAGGAGTTCGTGCACAT
GCCAGATGAGGAACTGGA---AGGAGGAGTTCGTGCACAT
GCCAGATGAGGAACTGGA----GGAGGAGTTCGTGCACAT
GCCAGATGAGGAACTG-----AGGAGGAGTTCGTGCACAT
GCCAGATGAG-------------------------CACAT

Figure 2. Next generation sequencing of barcoded PCR amplicons obtained for multiple Cas9-targeted genomic regions.

(A) Workflow of multiplexed PCR amplicon library preparation for NGS. Multiple targets are amplified and barcoded in two rounds of PCR (Supplementary figure 4), pooled and sequenced on the Illumina MiSeq instrument. (B) Illumina reads aligned to the wild type reference sequences. Target sequences are shown in the red rectangles; PAM sequence are underlined.

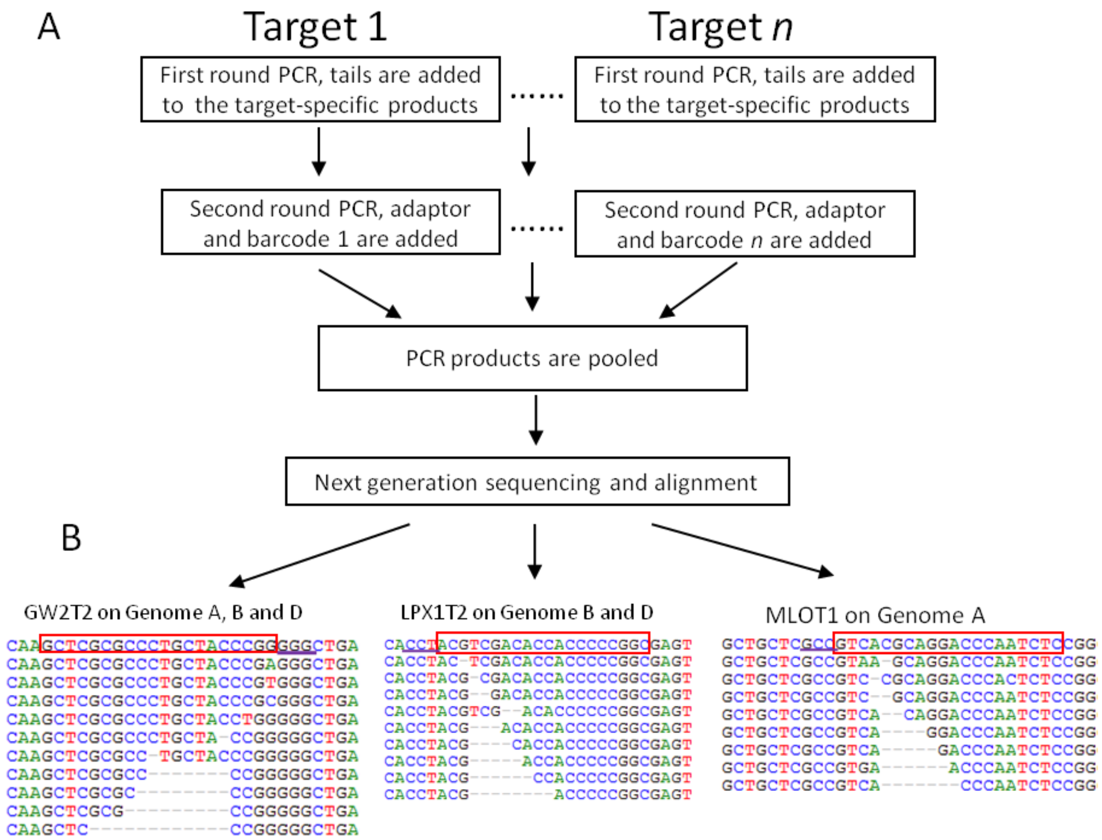


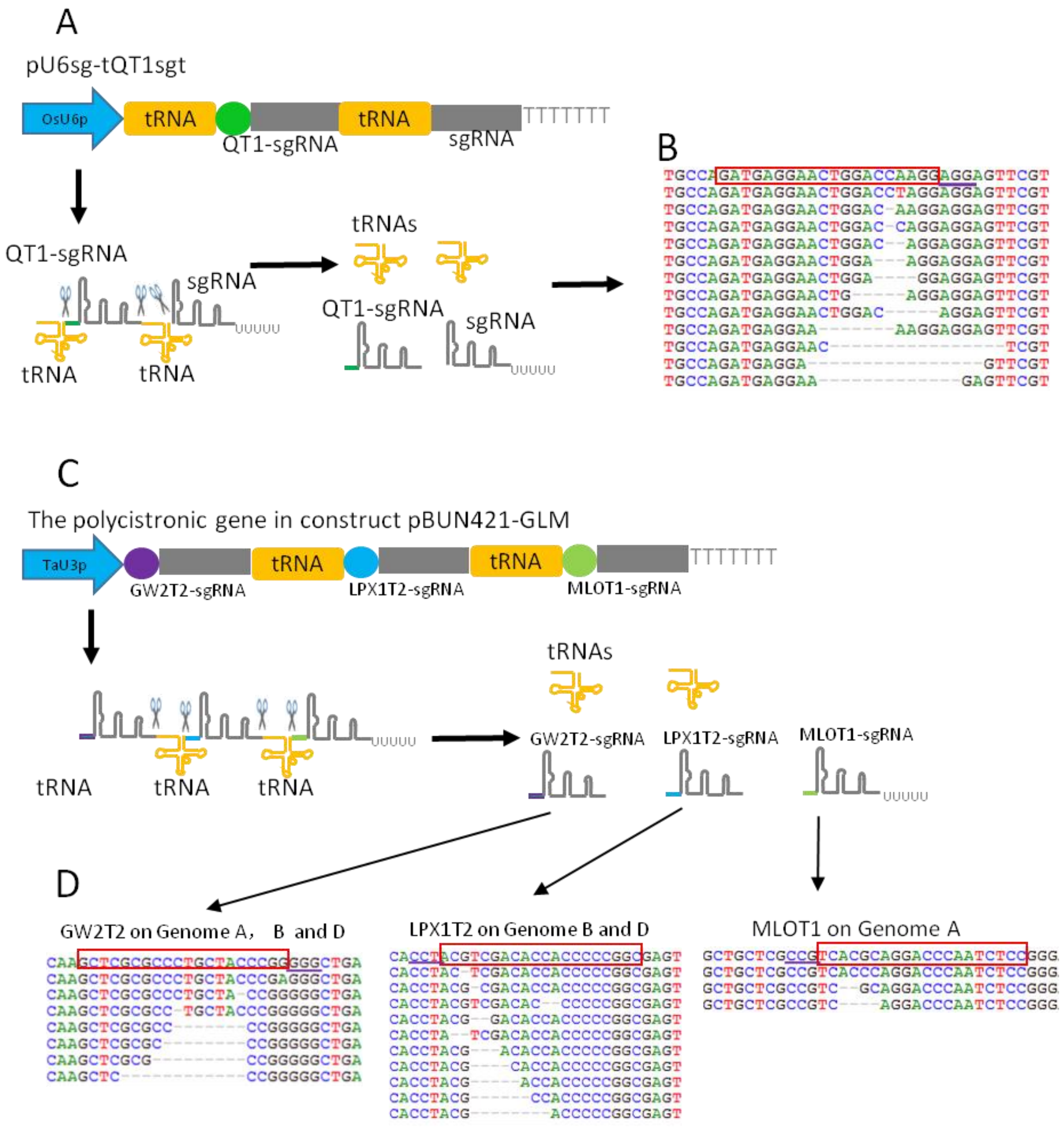
Figure 3. CRISPR/Cas9-mediated multiplex editing in hexaploid wheat using the endogenous tRNA-processing system.

(A) The structure of a test construct including QT1-sgRNA (targets the *Q* gene) flanked by two tRNAs is shown. TaU6p - wheat U6 promoter; QT1 is shown as green circle; sgRNA is shown as grey rectangle. sgRNAs are released after the tRNA processing of a transcript encoded by a polycistronic gene including QT1-sgRNA and one sgRNA without a guide sequence.

(B) Alignment of Illumina reads obtained for QT1-sgRNA-targeted regions of the *Q* gene. Wild type sequence is shown on the top. QT1 target sequence is shown in red rectangle; PAM sequence is underlined.

(C) Schematic of tRNA-based processing of a polycistronic gene construct (pBUN421-GLM) containing three tRNA-sgRNA blocks. Target sequences GW2T2, LPX1T2 and MLOT1 are shown in yellow, purple and blue color, respectively.

(D) The NGS of three genomic regions targeted by the pBUN421-GLM construct. Wild type sequence is shown on the top. Target sequences are shown in the red rectangles; PAM sequence is underlined.


A
pU6sg-tQT1sgt
OsU6p
tRNA
QT1-sgRNA
tRNA
sgRNA
TTTTTTT
QT1-sgRNA
sgRNA
tRNA
tRNA
tRNAs
QT1-sgRNA
sgRNA
B
C
The polycistronic gene in construct pBUN421-GLM
TaU3p
GW2T2-sgRNA
tRNA
LPX1T2-sgRNA
tRNA
MLOT1-sgRNA
TTTTTTT
tRNA
tRNA
tRNA
tRNAs
GW2T2-sgRNA
LPX1T2-sgRNA
MLOT1-sgRNA
D
GW2T2 on Genome A， B and D
LPX1T2 on Genome B and D
MLOT1 on Genome A