Note

A rapid method for detection of mutations induced by CRISPR/Cas9-based genome editing in common wheat

Yoko Kamiya¹, Fumitaka Abe², Masafumi Mikami³, Masaki Endo³,
Kanako Kawaura^{1,*}

¹Kihara Institute for Biological Research, Yokohama City University, Yokohama 244-0813, Japan; ²Division of Basic Research, Institute of Crop Science, National Agriculture and Food Research Organization, Ibaraki 305-8518, Japan; ³Division of Applied Genetics, Institute of Agrobiological Sciences, National Agriculture and Food Research Organization, Ibaraki 305-8604, Japan

*E-mail: kawaura@yokohama-cu.ac.jp Tel: +81-45-820-2401 Fax: +81-45-820-2466

Received October 30, 2019; accepted April 4, 2020 (Edited by T. Komari)

Abstract Genome editing using CRISPR/Cas9 is useful for common wheat because common wheat has allohexaploid nature and it can induce mutations simultaneously in three homoeologous genes. Although *Agrobacterium*-mediated transformation has advantages in genome editing, it still has low efficiency and requires relatively long time in wheat. Therefore, the use of guide RNAs (gRNAs) with efficient mutagenesis in vivo is one of the critical factors for producing genome-edited mutant lines in a short time. In this study, we targeted three genes in common wheat and established a rapid method for detection of mutations induced by the biolistic transient expression system. Biolistic transient expression of the gRNAs and Cas9 was achieved in immature wheat embryos. Mutations were detected a week later using PCR-RFLP and verified by the sequencing of genomic clones. We confirmed several types of mutations that occurred at different rates depending on the target sequences. Furthermore, frequencies of mutations tended to be higher at the targets that were edited at higher rates in the plants transformed by *Agrobacterium*. These results show that this method of rapid detection of edited mutations could be used for variety of applications, such as screening of target sequences or modified vectors for efficient CRISPR/Cas9 genome editing in wheat.

Key words: biolistic transient expression, common wheat, CRISPR/Cas9, rapid editing detection.

The CRISPR/Cas9 system is a very powerful tool for the genome editing of various crops. Genome editing via CRISPR/Cas9 is especially valuable for use in the common wheat (*Triticum aestivum* L.) because it is a hexaploid organism. In the common wheat, CRISPR/Cas9 can simultaneously generate mutations in three sets of homoeologous genes derived from three sub-genomes, named A, B, and D. Since the CRISPR/Cas9 system needs only a 20-bp genome sequence next to protospacer adjacent motif (PAM) for the target, we can select targets conserved across the three homoeologous genes more easily than is possible with other targeted genome editing methods, such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). Recently, we successfully produced a triple-recessive mutant common wheat line using *Agrobacterium*-mediated CRISPR/Cas9 genome editing (Abe et al. 2019). In the previous study, we designed two guide RNAs (gRNAs) for the *TaQsd1* gene. However, we succeeded in obtaining mutant lines from only one target and were unable to obtain any mutant lines from the other. Similarly, mutation efficiency differing for different

target sequences has been reported in various plants, such as rice (Mikami et al. 2015) and grape (Nakajima et al. 2017). These results demonstrate that the selection of a target sequence with highly efficient mutagenesis in vivo is one of the most important factors for obtaining mutant lines in a short amount of time, because the efficiency of *Agrobacterium*-mediated transformation is still so low in wheat that it takes relatively long time to produce mutant lines. Therefore, to screen for efficient target sequences in common wheat for *Agrobacterium*-mediated genome editing by CRISPR/Cas9, we established a rapid method for detection of mutations using the biolistic transient expression of Cas9 and gRNA in the immature embryos of common wheat.

Three genes, *TaQsd1* (Abe et al. 2019), *TraesCS4A02G110300* (International Wheat Genome Consortium (IWGSC) 2018), and *TaLOX2* (Zhang et al. 2016), were targeted in this study. Two of them were previously studied; two efficient targets, namely *TaQsd1_t1* (Abe et al. 2019) and *TaLOX2* (Zhang et al. 2016) and one inefficient target, *TaQsd1_t2*. *TraesCS4A02G110300* was chosen as the third target

Figure 1. Secondary structure of gRNAs with a scaffold sequence, estimated by Quickfold software. Asterisks in blue represent the position of 20-bp target sequences. (A) *TaQsd1*_t1 (5'-ACGGATCCACCTCCCTGCAG-3'), (B) *TaQsd1*_t2 (5'-GCTGCAGGGAGGTGGATCCG-3'), (C) *TraesCS4A02G110300*_t1 (5'-ACATGGAGCTCGTCTCGGGC-3') (D) *TraesCS4A02G110300*_t2 (5'-CGCCGCGCGCTCGCCAAGGC-3'), (E) *TraesCS4A02G110300*_t3 (5'-GGCCGGCCTTGCGAGCGCG-3'), (F) *TaLOX2* (5'-GTGCCGCGCGACGAGCTCTT-3').

Figure 2. In vitro cleavage assay of target sequences. Fragments of genomic DNA were synthesized and tested against the target in *TraesCS4A02G110300* and *TaQsd1*. - indicates uncleaved DNA fragment. M indicates 100 bp DNA ladder marker.

gene, and three candidate target sites were considered (t1, t2, and t3). These six targets were examined for GC contents, secondary structure of gRNA, and in vitro cleavage activity. The GC contents of these 20-bp target sequences were above 60%. Since previous studies suggested that the GC contents of target sequences above 60% showed a high editing efficiency (Wang et al. 2014; Zhang et al. 2016), we selected these six targets for subsequent assays. We then estimated the secondary structure of each gRNA containing a 20-bp target sequence, including the scaffold sequence necessary for Cas binding, using Quickfold software (<http://unafold.rna.albany.edu/?q=DINAMelt/Quickfold>) (parameters: RNA, 3.0; sequence type, circular) (Figure 1). One target sequence for *TaQsd1* (t1), three for *TraesCS4A02G110300* (t1, t2, and t3), and *TaLOX2* had similar conserved stem-loop structures and did not form long linear chains by hydrogen bonding. One gRNA for *TaQsd1* (t2), which was chosen as an inefficient target, had long linear chains and did not have clear loop structures. All 20bp target sequences were conserved among the three sub-genomes in common wheat. These target sequences were then tested for in vitro cleavage assay using the Guide-it sgRNA In Vitro Transcription and Screening Systems (TaKaRa Bio) according to the manufacturer's instructions. Modifications were made to the amount of

gRNA (5 ng) and Cas9 protein (100 ng) used. In the assay, *TaQsd1*_t2 and *TraesCS4A02G110300*_t2 showed lower cleavage activity and *TaQsd1*_t1, *TraesCS4A02G110300*_t1, and *TraesCS4A02G110300*_t3 showed higher cleavage activity (Figure 2). In rice, gRNAs showing low cleavage efficiency in vitro also had low mutagenesis efficiency in vivo (Mikami et al. unpublished). For this reason, we selected the other two targets described (t1 and t3) for *TraesCS4A02G110300* to perform the subsequent experiments.

To construct vectors for the transient in vivo assay, oligo DNAs of the target sequences were synthesized and ligated into the pOsU6gRNA (pU6gRNA-oligo) and pTaU6gRNA vectors, as previously described (Abe et al. 2019). The pTaU6gRNA vector was constructed by replacing the OsU6 promoter region with the TaU6 promoter of common wheat (Shan et al. 2014). The vector pE(R4-R3)ZmUbi_OsCas9_ver.3 (Hamada et al. 2018) was used for Cas9 expression.

A scheme of the transient in vivo assay is shown in Figure 3. Common wheat cultivar Fielder (Accession number KT020-061) was used here because it was the same one used in our previous study that applied *Agrobacterium*-mediated CRISPR/Cas9 genome editing (Abe et al. 2019). The plants were grown under the same conditions as previously described (Abe et al. 2019). Spikes were harvested at 17 days after flowering and incubated at 4°C overnight. Immature embryos were collected from sterilized grains and centrifuged in a liquid medium (0.1×MS salt with vitamins (Sigma), 0.2 mg l⁻¹ ZnSO₄·7H₂O, 10 g l⁻¹ glucose, and 0.5 g l⁻¹ 2-(N-morpholino)-ethanesulfonic acid, pH 5.8) (Ishida et al. 2015) at 20,000×g and 4°C for 10 min. Next, the immature embryos were transferred to a high osmotic medium (1×MS salt with vitamins (Sigma), 0.1 g l⁻¹ myo-inositol, 0.15 g l⁻¹ L-asparagine, 150 g l⁻¹ maltose, 2.5 mg l⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D), and 0.8% agar, pH 5.8) (Ogawa et al. 2008) and cultured in the dark at 25°C for at least 1 h. Approximately 50 embryos were placed in one petri dish.

Biolistic transient expression was achieved using a PDS-1000/He particle delivery device (Bio-Rad). Five micrograms of each plasmid (i.e., one containing the

Figure 3. Scheme of the transient in vivo assay for rapid detection of gene editing in common wheat. Arrowhead in red indicates mutation(s) detected in the lane by PCR-RFLP. When the mutation is induced in the targeted site, uncleaved band is detected. More gel images of mutation detection by PCR-RFLP are shown in Supplementary Figure 1.

Table 1. Mutation frequencies by biolistic transient expression and *Agrobacterium*-mediated transformation.

Gene	Target	GC content (%)	gRNA expression vector	No. of mutants/ bombarded embryos (%)	No. of mutants ^a / transformants ^b (%)
<i>TaQsd1</i>	t1	65	OsU6gRNA	15/492 (3.0)	7/57 (12.3)
<i>TaQsd1</i>	t1	65	TaU6gRNA	3/93 (3.2)	20/52 (38.5)
<i>TaQsd1</i>	t2	70	OsU6gRNA	0/195 (0)	0/10 (0)
<i>TraesCS4A02G110300</i>	t1	65	TaU6gRNA	11/287 (3.8)	5/75 (6.7)
<i>TraesCS4A02G110300</i>	t3	85	TaU6gRNA	0/192 (0)	N/A
<i>TaLOX2</i>		70	TaU6gRNA	51/734 (6.9)	N/A

N/A: not available ^a Number of mutant lines (T_0) detected mutations at the target site. ^b Number of transformants (T_0) generated by *Agrobacterium*-mediated transformation method (Abe et al. 2019; Abe et al. unpublished).

gRNA and one with Cas9) was mixed and then used to coat 1 mg of 0.6 μ m gold particles (Bio-Rad) according to the manufacturer's instructions. These gold particles were suspended in 50 μ l of ethanol, and 15 μ l of this mixture was then applied to one particle bombardment shot into the embryos. The bombardment was conducted at 900 psi helium pressure with a target distance of 8 cm from the stopping plate. After bombardment, the immature embryos were incubated in the dark at 25°C for 16 h. The embryos were then transferred to a callus induction medium containing 1 $\times$ MS salt with vitamins (Sigma), 0.1 g l⁻¹ myo-inositol, 0.15 g l⁻¹ L-asparagine, 30 g l⁻¹ sucrose, 2.5 mg l⁻¹ 2,4-D, and 0.8% agar (pH 5.8) and cultured for 1 week. To evaluate the mutations caused by the transient expression of Cas9 and gRNA, genomic DNA was extracted from a whole tissue containing callus derived from one immature embryo. Each embryo was mixed with 300 μ l of DNA extraction buffer (0.2 M Tris-HCl, 250 mM NaCl, 25 mM EDTA, pH 8.0) using TissueLyser II (QIAGEN) and zirconia beads at 25 oscillations s⁻¹ for 20 s twice. The sample was mixed with 75 μ l of 20% SDS and incubated for 15 min at room temperature. The sample was then centrifuged at 2,700 $\times$ *g* for 10 min. The 100 μ l of supernatant was used for ethanol precipitation, and purified DNAs were eluted in 100 μ l of 0.1 $\times$ TE (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). These DNA samples were used in PCR-restriction fragment length polymorphism (RFLP) analysis. Each target region was amplified by PCR using Tks Gflex DNA polymerase (Takara Bio) containing 0.8 μ l of DNA sample in 15 μ l

reaction volume and the following primer sets: *TaQsd1*-ABD (Abe et al. 2019), *TraesCS4A02G110300*-F3 (5'-GGG AAA TGC ATC GGG AAG GG-3'), *TraesCS4A02G110300*-R3 (5'-GAG GCA CTT GTC GAG GAA GTC-3'), *LOX2*-F, and *LOX2*-R (Zhang et al. 2016). The PCR products were digested with the restriction enzymes *Pst*I, *Bam*HI, *Ava*I, *Bss*HII, and *Sac*I for the targets *TaQsd1*-t1, *TaQsd1*-t2, *TraesCS4A02G110300*-t1, *TraesCS4A02G110300*-t3, and *TaLOX2*, respectively. These samples were then subjected to agarose gel electrophoresis (Supplementary Figure S1). The PCR products containing mutations were extracted from the agarose gel (MinElute Gel extraction kit, QIAGEN) and cloned. The sequences were determined using the ABI 3500 Genetic Analyzer (Applied Biosystems).

We identified various rates of mutation among these target sequences (Table 1). In the embryos with gRNAs targeting *TaLOX2*, 51 out of 734 embryos exhibited *TaLOX2* mutations, as revealed by the PCR-RFLP assay (Supplementary Figure S1). We found 15 out of 492 embryos (3.0%) and 3 out of 93 embryos (3.2%) with mutations in the target *TaQsd1*-t1, using the OsU6gRNA and TaU6gRNA expression vectors, respectively (Table 1). In the case of *TraesCS4A02G110300*-t1, the mutation frequency in the bombarded embryos was 3.8% (Table 1). The mutation frequency in the target *TaLOX2* (6.9%) was the highest among the targets. This result agrees with the previous report that the target *TaLOX2* had high mutagenesis efficiency when Cas9 was transiently expressed in wheat (Zhang et al. 2016). These mutant variants were verified by sequencing (Figure 4). The

TaLOX2	
GTGCCGCGCAGAGCTCTTCGG	WT
GTGCCGCGCAGAGC-CTTCGG	-1 (x4)
GTGCCGCGCAGAG-TCTTCGG	-1 (x4)
GTGCCGCGCAGAG--CTTCGG	-2 (x19)
GTGCCGCGCAGCA--TCTTCGG	-2 (x1)
GTGCCGCGCAGCA---CTTCGG	-3 (x1)
GTGCCGCGCAGC---TCTTCGG	-3 (x2)
GTGCCGCGCAGAGCT---CGG	-3 (x1)
GTGCCGCGCAGC---CTTCGG	-4 (x2)
GTGCCGCGCAGCA---TTCGG	-4 (x1)
GTGCCGCGCAGC---CTTCGG	-5 (x1)
GTGCCGCGCGA---TCTTCGG	-5 (x2)
GTGCCGCGCGA---CTTCGG	-6 (x2)
GTGCCGCGCAGCA---GG	-7 (x1)
GTGCCGCG---TTCGG	-11 (x1)
G---CTTCGG	-16 (x1)
-----GG	-21 (x1)
GTGCCGCGCAGAGCT/CTTCGG	+94 (x1)
GTGCCGCGCAGAGCT/AGATGG	+145 s4 (x1)
TaQsd1_t1	
ACGGATCCACCTCCCTGCAGCGG	WT
ACGGATCCACCTCCCTGTACGCGG	+1 (x6)
ACGGATCCACCTCCCTGACGCGG	+1 (x2)
ACGGATCCACCTCCCTGCAGCGG	+1 (x1)
TraesCS4A02G110300_t1	
GGCCGGCCTTGGCAGCGCGG	WT
GGCCGGCCTTGG-----GG	-9 (x2)
GGCCGGCCTTGGCAGC-CGCGG	-1 (x3)
GGCCGGCCTTGGCAGGGCGCGG	s1 (x1)
GGCCGGCCTTGGCTGCGCGCGG	s1 (x6)
GGCCGGCCTTGGCAGCGAGCGG	s1 (x1)
GGCCGGCCTTGGCAGAGCGCGG	s1 (x1)
GGCCGGCCTTGGCAGTCGCGCGG	+1 (x2)
GGCCGGCCTTGGCAGGCGCGCGG	+1 (x2)
GGCCGGCCTTGGCAGCTGCGCGG	+1 (x6)
GGCCGGCCTTGGCAGCAGCGCGG	+1 (x2)

Figure 4. Mutations detected in target sequences. The wild-type sequence is shown (top) and the protospacer adjacent motif (PAM) sequence is underlined. Dashes indicate deleted bases. Changes in length are shown to the right of each sequence (+, insertion; -, deletion; s, base substitution). The number of clones is shown in parentheses.

mutation patterns were variable in these three targets. Mutations were drastic especially in *TaLOX2*, with 16 kinds of deletions and 2 kinds of large insertions (Figure 4). Only one nucleotide insertions in the same position were found in *TaQsd1_t1* (Figure 4). Various mutation patterns were observed in *TraesCS4A02G110300_t1*, with 2 kinds of small deletions, 4 kinds of one nucleotide substitutions, and 4 kinds of one nucleotide insertions (Figure 4). In contrast, no mutation was detected in the targets *TaQsd1_t2* and *TraesCS4A02G110300_t3* by the transient expression method (Table 1). Although the secondary structure of gRNA (Figure 1) and the cleavage assay in vitro (Figure 2) showed reasonable high mutagenesis efficiency in the case of *TraesCS4A02G110300_t3*, no mutant was obtained by the transient expression method. Furthermore, we could efficiently establish mutant lines for the target *TraesCS4A02G110300_t1* by *Agrobacterium*-mediated CRISPR/Cas9 transformation method, by which we successfully produced mutant lines for *TaQsd1_t1* (Abe et al. 2019) (Table 1). Although the number of experiments is limited, these results suggest that the mutagenesis efficiency of gRNAs screened by the biolistic transient expression method is reflected by the

Agrobacterium-mediated methods.

Agrobacterium-mediated transformation in wheat is an ideal method for CRISPR/Cas9 genome editing because CRISPR/Cas9 activity is stable through generations (Zhang et al. 2019). Additionally, because transformants derived from the *Agrobacterium*-mediated method have only one or at most a few copies of the transgene, transgene-free mutant lines are relatively easy to obtain (Abe et al. 2019). The rapid method for mutation detection established in this study does not require the preparation of cultured cell lines such as protoplasts, and only takes 1 week before results can be obtained. This method can also be used in other applications, such as for the screening of modified enzymes and the availability of constructed vectors for CRISPR/Cas9 genome editing. Given that the transformation efficiency in wheat is low, this method is ideal for selecting efficient gRNAs in vivo for use in accelerating *Agrobacterium*-mediated CRISPR/Cas9 genome editing in wheat as well as in other crops.

Acknowledgements

We thank Dr. F. Kobayashi (Institute of Crop Science, NARO) for providing sequence information, and Drs. K. Sato and H. Hisano (Institute of Plant Science and Resources, Okayama University) for helpful discussion. This research was supported by the Cabinet Office, Government of Japan, Cross-ministerial Strategic Innovation Promotion Program (SIP). The seed sample was provided by the National BioResource Project-Wheat, Japan.

References

- Abe F, Haque E, Hisano H, Tanaka T, Kamiya Y, Mikami M, Kawaura K, Endo M, Onishi K, Hayashi T, et al. (2019) Genome-edited triple-recessive mutation alters seed dormancy in wheat. *Cell Rep* 28: 1362–1369.e4
- Hamada H, Liu Y, Nagira Y, Miki R, Taoka N, Imai R (2018) Biolistic-delivery-based transient CRISPR/Cas9 expression enables in planta genome editing in wheat. *Sci Rep* 8: 14422
- International Wheat Genome Consortium (IWGSC) (2018) Shifting the limits in wheat research and breeding using a fully annotated reference genome. *Science* 361: 6403
- Ishida Y, Tsunashima M, Hiei Y, Komari T (2015) Wheat (*Triticum aestivum* L.) transformation using immature embryo. *Methods Mol Biol* 1223: 189–198
- Mikami M, Toki S, Endo M (2015) Parameters affecting frequency of CRISPR/Cas9 mediated targeted mutagenesis in rice. *Plant Cell Rep* 34: 1807–1815
- Nakajima I, Ban Y, Azuma A, Onoue N, Moriguchi T, Yamamoto T, Toki S, Endo M (2017) CRISPR/Cas9-mediated targeted mutagenesis in grape. *PLoS One* 12: e0177966
- Ogawa T, Kawahigashi H, Toki S, Handa H (2008) Efficient transformation of wheat by using a mutated rice acetolactate synthase gene as a selectable marker. *Plant Cell Rep* 27: 1325–1331
- Shan Q, Wang Y, Li J, Gao C (2014) Genome editing in rice and wheat using the CRISPR/Cas system. *Nat Protoc* 9: 2395–2410
- Wang Y, Cheng X, Shan Q, Zhang Y, Liu J, Gao C, Qiu J-L (2014)

- Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. *Nat Biotechnol* 32: 947–951
- Zhang Y, Liang Z, Zong Y, Wang Y, Liu J, Chen K, Qiu JL, Gao C (2016) Efficient and transgene-free genome editing in wheat through transient expression of CRISPR/Cas9 DNA or RNA. *Nat Commun* 7: 12617
- Zhang Z, Hua L, Gupta A, Tricoli D, Edwards KJ, Yang B, Li W (2019) Development of an *Agrobacterium*-delivered CRISPR/Cas9 system for wheat genome editing. *Plant Biotechnol J* 17: 1623–1635