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Self-cleaving ribozymes enable the production of guide RNAs from unlimited choices of promoters for CRISPR/Cas9 mediated genome editing

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Development of tools for targeted modifications of specific DNA sequences in plants is of great importance to basic plant biology research as well as crop improvement. The ability to cut DNA at specific locations in the genome to generate double-strand breaks (DSBs) *in vivo* is a prerequisite for any genome editing efforts. Several engineered nucleases including zinc finger nucleases (ZFNs) and transcriptional activator-like nucleases (TALENs) have

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been successfully used to edit genomes. However, development of sequence specific ZFNs and TALENs is laborious and technically challenging (Li et al., 2012; Gaj et al., 2013; Gupta and Musunuru, 2014; Kim and Kim, 2014). Recent discoveries of CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9) system have revolutionized the genome editing field because of its efficiency, simplicity, and versatility (Jinek et al., 2012; Cong et al., 2013; Mali et al., 2013).

Since the first reported successful genome editing cases by CRISPR/Cas9 in 2013 (Cong et al., 2013; Mali et al., 2013), this technology has succeeded in many organisms including various plant species. Major upgrades/modifications over the initial design of the CRISPR/Cas9 technology in plants have been introduced to improve editing efficiency, mutation heritability, and effectiveness to isolate transgene-free mutants. One of the major findings is that the choice of promoters for expressing Cas9 has a dramatic impact on the efficiency and heritability of gene editing in *Arabidopsis*. Another effort has been to isolate and characterize additional nucleases such as Cpf1 that have characteristically different PAM requirements and that generate DSBs with cohesive ends (Tang et al., 2017).

Proper production of guide RNAs (gRNAs) in cells determines whether a targeted gene modification is successful. The majority of reported gene editing cases relied on gRNA production from transcriptions driven by RNA Polymerase III (Pol III) promoters (Cong et al., 2013; Mali et al., 2013), whose physiological function is to transcribe ribosomal 5S rRNA genes, tRNA genes, and some small nuclear RNA genes such as *U6* and *U3 snRNA* genes. *U6* and *U3 snRNA* genes are constitutively and ubiquitously expressed. Thus, they cannot be used for conducting cell/tissue specific gene editing. It was reported that the endogenous tRNA-processing system can be modified to multiplex gRNA production (Xie et al., 2015). Precursors of tRNAs are precisely processed at both ends, a property that enables production of multiple gRNAs from one construct. The tRNA system, however, still uses Pol III promoters. There are usually multiple copies of *U6* and *U3* genes in a given genome and it is inherently difficult to determine which one would be the best for gRNA production. There is a strong need to develop a system for gRNA production from any promoters including both RNA Pol II and Pol III promoters. This is particularly important for systems in which limited genetics/genomic information is available.

We previously took advantages of the self-cleaving activities of ribozymes and developed an artificial gene named *RGR* (*Ribozyme-gRNA-Ribozyme*) that potentially allowed us to produce gRNAs from any promoters and in any organisms (Gao and Zhao, 2014; Gao et al., 2015). When transcribed, the primary transcripts from *RGR* contained the designed gRNA that was flanked with a ribozyme at each end (Fig. 1A). In addition to having more options in choosing a promoter, the ribozyme-based technology also made multiplexing gRNA production very easy because the primary sequences of the ribozymes can be altered as long as the required secondary structures are maintained (Fig. 1A). Moreover, multiple gRNAs can be produced from a single promoter so that each gRNA can have similar concentrations. Indeed, it has been reported that multiple gRNAs are successfully produced from RNA Pol II promoters using the ribozyme strategy in rice and in animal cells (Yoshioka et al., 2015; Tang et al., 2016). Furthermore, CRISPR-Cpf1 DNA-editing in rice has been successfully performed by utilizing gRNA produced from our RGR system, probably because our RGR

system produces gRNA molecules with precise ends (Tang et al., 2017). Despite the successful cases in several systems, it was still debatable whether our RGR system can use both Pol II and Pol III promoters for gRNA production.

To further test whether primary transcripts of a *RGR* unit generated from a RNA Pol III promoter can also undergo the expected self-cleavage to release the mature gRNA (Fig. 1A and B), we put two tandem *RGR* units (*RGR1-RGR2*) under the control of a rice *U6* (*OsU6*) promoter, a widely-used RNA Pol III promoter in the genome editing field (Fig. 1C). This construct not only can be used to determine the effectiveness of Pol III promoter for *RGR*-based gRNA production, but can also be used for evaluating the feasibility of multiplexing gRNA production by *RGR*. We used the maize *ubiquitin* (*ZmUBI*) promoter to drive the rice codon-optimized *Cas9* (Miao et al., 2013). The backbone of our CRISPR vector was based on the binary vector *pCXUN* (Chen et al., 2009) and the destination binary vector *pCXUN-Cas9* contains the *Cas9* expression cassette (Sun et al., 2016). We named the final plasmid ready for plant transformation *pU6:RGR1-RGR2*, which encodes two gRNAs targeting two distinct genes. We transformed the *pU6:RGR1-RGR2* construct into rice by *Agrobacterium*-mediated callus transformation (Supplementary data). We then amplified the target-containing regions by PCR using genomic DNA from the *Cas9*-positive T₀ plants as templates, and the PCR fragments were then directly sequenced by the Sanger sequencing method. As shown in Table S1, the two gRNAs produced from the tandem *RGR1-RGR2* driven by the single *OsU6* promoter very efficiently guided the editing of the target genes. Among the 15 T₀ plants analyzed, 11 plants (73%) contained various mutations at the target genes (Table S1). Consistent with previous reports, the majority of the mutations were small deletions or insertions (indels) (Table S1). Larger deletions or base-substitutions were also observed (Table S1). Interestingly, T₀ plants that were homozygous (43%) or bi-allelic (14%) at target 1 were also obtained (Table S1). We noticed that the mutation rate at target 2 was slightly lower than that of target 1. Only 53% (8/15) of the plants had mutations at target 2 and 50% (4/8) of the mutant plants were bi-allelic (Table S1). Surprisingly, no homozygous mutant plants were observed for target 2 (Table S1). Our strategy produced 27% plants with mutations homozygous or bi-allelic at both targets (Table S1), suggesting that our method can efficiently knockout two genes simultaneously. We observed that all of the plants that did not contain mutations at target 1 also did not have mutations at target 2 (Table S1), suggesting that perhaps the particular transgenic plants might have non-functional CRISPR construct or the CRISPR components were not expressed. Nevertheless, the two gRNAs produced here efficiently directed editing events at the intended target sites.

We then placed the tandem *RGR1-RGR2* under the control of the rice *Actin1* promoter, a RNA Pol II promoter, resulting in the final plasmid *pActin1:RGR1-RGR2* (Fig. 1C). Transformation of this construct into rice led to the isolation of 43 T₀ transgenic plants. Direct sequencing analysis clearly demonstrated that the gRNAs produced from *pActin1:RGR1-RGR2* were able to guide editing events at both targets (Table S2). The editing efficiencies for both targets were very similar with 36% for target 1 and 41% for target 2 (Table S2). Most of the mutations were small indels. Overall, we obtained about 14% plants with homozygous/bi-allelic mutations at both targets (Table S2), suggesting that multiplexing gRNA production from *RGR* driven by Pol II promoters is also effective.

To enable production of more gRNAs from a single plasmid and to provide a way to estimate and compare the efficiencies of the two gRNA production methods, we added an *OsU3*-driven gRNA unit (*pU3:gRNA3*) into *pU6:RGR1-RGR2* to generate a final plasmid *pU3:gRNA3-pU6:RGR1-RGR2*, which allows the production of three different gRNAs from the same plasmid (Fig. 1C). As shown in Table S3, all three targets had been successfully edited. The editing efficiencies for target 1 and target 2 were more than 70% and 45%, respectively, suggesting that adding the third gRNA production unit hardly affected the editing efficiencies of target 1 and target 2 (Tables S1 and S3). By contrast, the editing efficiency for target 3, whose editing was guided by the gRNA3 produced from the *pU3:gRNA3* unit, was relatively low (about 38%) (Table S3). Moreover, for target 3, the majority of the plants were heterozygous; no homozygous mutants were obtained, and the rate for bi-allelic mutations was also low (Table S3).

The low editing efficiency for target 3 could be due to intrinsic properties of target 3. To further test the system, we constructed another plasmid that could produce three gRNAs: one from the *OsU3*-driven gRNA unit (*pU3:gRNA6*), and two from the *OsU6*-driven tandem *RGR* units (*pU6:RGR4-RGR5*). We name this plasmid *pU3:gRNA6-pU6:RGR4-RGR5* (Fig. 1C). The overall editing efficiency for target 6, whose editing was guided by the gRNA6 produced from the *pU3:gRNA6* unit, was 75% (Table S4). For this particular construct, target 4 was very efficiently edited with an editing rate of 82% (Table S4). However, target 5 only had 35% editing efficiency (Table S4). These results suggested that the efficiency was mainly determined by target sequences and that it had less to do with how gRNA is produced. This construct produced about 10% plants that were homozygous or bi-allelic at all three target sites.

In summary, we have expanded the choices of promoters for gRNA transcription. With unlimited choices of both Pol II and Pol III promoters, sophisticated gene editing such as cell/tissue specific gene editing has become feasible. We envision that our RGR technology will be especially useful when modulation of gene expression in specific cells by a modified dCas9 system (Shalem et al., 2015). Because gRNAs produced through *RGR* have the precise ends, our method of gRNA production will be very important for using other nucleases such as Cpf1 that do not tolerate extra sequences at the ends of gRNA molecules (Tang et al., 2017). Further modifications of our RGR system will broaden the applications of the ribozyme-based method. For example, a single promoter may be used to produce gRNAs, *Cas9* mRNA, and even a fluorescence marker that can be used to visualize the cells in which *Cas9* mRNA and gRNAs are produced.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Production of guide RNAs (gRNAs) from either Pol II or Pol III promoters using the ribozyme-based strategy for CRISPR/Cas9 mediated genome editing. **A:** A schematic presentation of the production of multiple gRNAs using the ribozyme-based strategy. A gRNA production unit termed *RGR* (*Ribozyme-gRNA-Ribozyme*) is composed of three parts: the 5'-end encoding the Hammerhead (HH) ribozyme, the middle part encoding the gRNA, and the 3'-end encoding the hepatitis delta virus (HDV) ribozyme. A *RGR* unit can be transcribed from a promoter. The primary transcripts will undergo self-cleavage catalyzed

by the intrinsic nuclease activities of the ribozymes to release the mature and desired gRNA. Because the gRNA is flanked with ribozymes, modifications of the primary transcripts such as 50-end capping and 30-end polyadenylation will not affect the production of gRNA from *RGR*. *RGR* units can be placed in tandem and multiple gRNAs may be produced from a single promoter. The scissors in blue show the self-cleavage sites of the HH ribozyme. The scissors in green show the self-cleavage sites of the HDV ribozyme. Each target-specific spacer is labeled as different colors between HH and the gRNA scaffold. **B:** Each gRNA combining with the Cas9 protein will lead to the cleavage of different target sites. The black and white scissors show the double-strand breaks generated by two catalytic domains of *Streptococcus pyogenes* Cas9, respectively. **C:** The key structures of plasmids for genome editing in rice. In the schematic diagram of *pU6:RGR1-RGR2* plasmid, each *RGR* unit contains DNA elements encodes a HH ribozyme at the 5'-end, a gRNA molecule in the middle and an HDV ribozyme at the 3'-end. *RGR1* and *RGR2* were expressed from a single rice *U6* promoter. The two gRNAs produced by the two *RGR* units target Gene1 and Gene2, respectively. The schematic diagram of *pActin1:RGR1-RGR2* plasmid was similar to *pU6:RGR1-RGR2*, but the rice *U6* promoter in *pU6:RGR1-RGR2* was replaced by the rice *Actin1* promoter. In the schematic diagram of *pU3:gRNA3-pU6:RGR1-RGR2* plasmid, an extra gRNA transcription cassette driven by the rice *U3* promoter to target Gene3 was added to the plasmid *pU6:RGR1-RGR2*. The schematic diagram of *pU3:gRNA6-pU6:RGR4-RGR5* plasmid was similar to *pU3:gRNA3-pU6:RGR1-RGR2* plasmid, but the gRNAs produced from *RGR4*, *RGR5* and the gRNA6 transcription cassette target Gene4, Gene5, and Gene6, respectively. The tandem *RGR* units in every plasmid were separated by a 12-nt random sequence. The details of the construction of these plasmids can be found in the Materials and methods in the Supplementary data file. *OsU3P*, *OsU6P*, *Actin1P*, *UBIP*, and *35SP* refer to *OsU3*, *OsU6*, *Actin1*, maize *ubiquitin* and CaMV *35S* promoter, respectively. *ADH1T*, *U3T*, *NOST*, and *35ST* refer to *ADH1*, *OsU3*, *NOS* and CaMV *35S* terminator, respectively. L refers to a 12-nt DNA linker between two *RGR* units. *Cas9* refers to a rice codon-optimized *Cas9* from *Streptococcus pyogenes*. *HptII* refers to the hygromycin resistance gene.