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Production of Guide RNAs *in vitro* and *in vivo* for CRISPR Using Ribozymes and RNA Polymerase II Promoters

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[Abstract] CRISPR/Cas9-mediated genome editing relies on a guide RNA (gRNA) molecule to generate sequence-specific DNA cleavage, which is a prerequisite for gene editing. Here we establish a method that enables production of gRNAs from any promoters, in any organisms, and *in vitro* (Gao and Zhao, 2014). This method also makes it feasible to conduct tissue/cell specific gene editing.

Keywords: Ribozyme, CRISPR, RNA polymerase II promoter, Genome editing, RNA transcription

[Background] Almost all of the reported cases of CRISPR-mediated gene editing used promoters of small nuclear RNAs such as the U6 and U3 snRNA promoters to drive the production of gRNAs *in vivo* (Cong *et al.*, 2013; Mali *et al.*, 2013). However, the U6 and U3 promoters have several major limitations: 1) They are constitutively active and not tunable; 2) They lack cell/tissue specificities; 3) They have not been well defined in many organisms; 4) U6 requires a G and U3 requires an A for transcription initiation, thus limiting target selections; 5) They are not suitable for *in vitro* transcriptions because of the lack of commercial RNA polymerase III. Unfortunately, RNA polymerase II promoters, which constitute the majority of the characterized promoters, cannot be directly used for gRNA production *in vivo* because of the following reasons: 1) The primary transcripts of RNA polymerase II promoters undergo extensive processing such as 5'-end capping, 3'-end polyadenylation, and splicing out of the introns. Some of the modifications may render the designed gRNA non-functional. 2) The mature RNA molecules are transported into cytosol; thus they are physically separated from the intended targets that are located in the nucleus. That is why production of gRNA *in vivo* using U6 and U3 snRNA promoters has been the dominant method (Gao and Zhao, 2014; Yoshioka *et al.*, 2015). In this protocol, we use a ribozyme-based strategy to overcome the aforementioned limitations of RNA polymerase III promoters, enabling gRNA production from any promoters and in any organisms. We design an artificial gene named *RGR* (Ribozyme-gRNA-Ribozyme) that, once transcribed, generates an RNA molecule with ribozyme sequences flanking both ends of the designed gRNA (Gao and Zhao, 2014). We show that the primary transcripts of *RGR* undergo self-catalyzed cleavage to precisely release the desired gRNA, which can efficiently guide sequence-specific cleavage of DNA targets *in vitro* and *in vivo* (Gao and Zhao, 2014).

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RGR can be transcribed from any promoters and thus allows for cell-and tissue-specific genome editing if appropriate promoters are chosen.

Materials and Reagents

1. *E. coli DH5a* and *Agrobacterium tumefaciens* strain GV3101
2. pRS316-RGR-GFP plasmid (Addgene, catalog number: plasmid 51056)
3. pHDE-35S-Cas9-mCherry-UBQ plasmid
4. Primers (Table S1)
5. Gibson assembly reagents
   You can either purchase commercial kits from (New England Biolabs, catalog number: E5510S), or prepare your own with the following individual reagents:
   a. 5x isothermal (ISO) reaction buffer (25% PEG-8000; 500 mM Tris-HCl, pH 7.5; 50 mM $MgCl_2$; 50 mM DTT; 1 mM each of the 4 dNTPs; and 5 mM NAD)
   b. T5 exonuclease (Epicentre, catalog number: T5E4111K)
   c. Phusion DNA polymerase (New England Biolabs, catalog number: M0530L)
   d. Taq DNA ligase (New England Biolabs, catalog number: M0208L)
6. Phusion High-Fidelity PCR Kit (New England Biolabs, catalog number: E0553L)
7. LB medium
8. Appropriate antibiotics
9. QIAGEN Plasmid Mini Kit
10. *Mfe*I (New England Biolabs, catalog number: R0589S)
11. 10X CutSmart® buffer
12. T7, SP6 or T3 RNA polymerase with transcription buffer
    For SP6/T7 (Thermo Fisher Scientific, Invitrogen™, catalog number: AM1320)
    For T3 (Thermo Fisher Scientific, Invitrogen™, catalog number: AM1316)
13. 5x transcription buffer
14. 1 M DTT (Thermo Fisher Scientific, catalog number: P2325)
15. 20 U/µl RNase inhibitor (Thermo Fisher Scientific, Applied Biosystems™, catalog number: N8080119)
16. 10 mM NTP mix (Thermo Fisher Scientific, Invitrogen™, catalog number: 18109017)
17. rNTPs
18. Inorganic pyrophosphatase
19. EDTA
20. 12% denaturing urea polyacrylamide gels
21. Ethidium bromide

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Equipment

1. 37 °C water bath (Temperature-controlled water bath) (Bio-Rad Laboratories, catalog number: 1660524)
2. Thermal cycler (Thermo Fisher Scientific, Applied Biosystems™, model: Applied Biosystems® 2720, catalog number: 4359659)
3. DNA electrophoresis apparatus (Bio-Rad Laboratories, model: PowerPac™ Basic Power Supply, catalog number: 1645050EDU)
4. Microcentrifuges (Eppendorf, model: 5424)
5. UV transilluminator (Bio-Rad Laboratories, model: UView™ Mini Transilluminator, catalog number: 1660531)

Procedure

A. Design, assemble, and clone an RGR (ribozyme-gRNA-ribozyme) unit

We design an artificial gene named *RGR*, whose primary transcripts would be flanked by ribozymes at both ends (Figure 1) (Gao and Zhao, 2014).

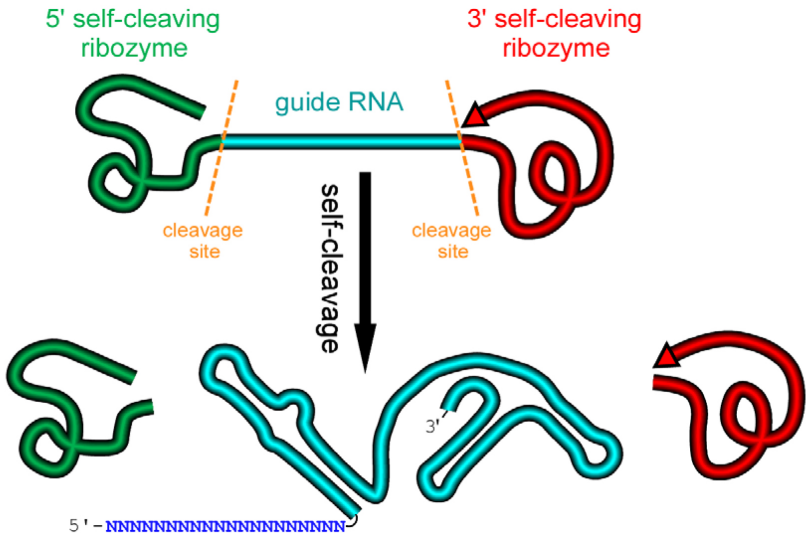

Figure 1. A schematic presentation of a ribozyme-flanked gRNA (RGR, ribozyme-gRNA-ribozyme) molecule. Once transcribed, the corresponding RGR DNA will produce an RNA molecule with a ribozyme at both the 5'- and 3'-end. The primary transcripts will undergo self-cleavage to release the mature gRNA. The RGR design allows the production of a functional gRNA molecule from any promoter, enabling production of gRNA molecules *in vitro*, *in vivo*, and in tissue/cell specific manner.

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1. Select a target sequence for editing the gene of interest
   The target should be 23 bp long and should contain the NGG Protospacer Adjacent Motif (PAM) site at the 3'-end. However, the NGG PAM site should not be included in the gRNA sequence itself. N refers to any nucleotide in the target $N^1N^2N^3N^4N^5N^6N^7N^8N^9N^{10}N^{11}N^{12}N^{13}N^{14}N^{15}N^{16}N^{17}N^{18}N^{19}N^{20}$NGG. Multiple web-based resources are publically available for selecting appropriate gRNA targets (http://cbi.hzau.edu.cn/crispr/; http://www.rgenome.net/cas-offinder/; http://www.genome.arizona.edu/crispr/CRISPRsearch.html).
2. Design an Ribozyme-gRNA-Ribozyme (RGR) unit
   a. We place a Hammerhead ribozyme at the 5'-end of the gRNA and a HDV ribozyme at the 3'-end of the gRNA (Figure 2). The complete sequences of the ribozymes were described in the plasmid pRS316-RGR-GFP (Gao and Zhao, 2014). Both the plasmid and the complete sequence of pRS316-RGR-GFP are available at Addgene.
   b. The RGR unit can then be placed under the control of a promoter for the production of the designed gRNA molecules. Any promoters including both RNA polymerase II and RNA polymerase III promoters can be used. For *in vitro* production of gRNAs, the RGR unit can be driven by an SP6 or T7 or T3 promoter, which can be transcribed using commercially available RNA polymerases (Gao and Zhao, 2014).
   c. Linearize the vector of your choice with a restriction enzyme and determine an 18 to 24 bp overlapping region (termed adaptor sequence) based on the Gibson assembly principle (Gibson *et al.*, 2009). Gibson assembly method seamlessly assembles overlapping DNA molecules into one molecule by the concerted action of a 5' exonuclease, a PHUSION DNA polymerase and a heat-stable Taq DNA ligase (Gibson *et al.*, 2009). Additional information about Gibson assembly can be found at Addgene (http://www.addgene.org/protocols/gibson-assembly/). Assembly kits are commercially available at New England Biolabs (https://www.neb.com/products/e5510-gibson-assembly-cloning-kit).
   d. If the adaptor sequences are **XXXXXXXXXXXXXXXXXXX** (upstream adaptor) and **YYYYYYYYYYYYYYYYYYY** (downstream adaptor), design two forward primers and one reverse primer as shown below. Adaptor sequences including X and Y provide the necessary overlapping sequences with your linearized vector so that the RGR unit can be assembled into the vector through Gibson assembly principle. The length of adaptor sequences varies depending on the actual sequences, but usually 17 bp to 24 bp is sufficient as long as the T_m value is above 50 °C.
      i. Forward primer 1: GENE-RGR-F1
         XXXXXXXXXXXXXXXXXXX*$M^6M^5M^4M^3M^2M^1$CTGATGAGTCCGTGAGGACGAAACG AGTAAGCTCGTC*
         The sequence X (bold) refers to the upstream adaptor sequence, which is complementary to the vector sequence. M sequence is reverse complementary to the

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first 6 bp of the target sequence. The italicized sequence is part of the Hammerhead ribozyme.

ii. Forward primer 2: GENE-RGR-F2
GACGAAACGAGTAAGCTCGTC$N^1N^2N^3N^4N^5N^6N^7N^8N^9N^{10}N^{11}N^{12}N^{13}N^{14}N^{15}N^{16}N^{17}N^{18}N^{19}N^{20}$*GTTTTAGAGCTAGAAATAGCAAG*. $N^1N^2N^3N^4N^5N^6$ and $M^6M^5M^4M^3M^2M^1$ are reverse complementary to ensure the correct secondary structure of the Hammerhead ribozyme. We designed the two overlapping PCR primers to avoid the synthesis of expensive long primers. The target sequence is $N^1N^2N^3N^4N^5N^6N^7N^8N^9N^{10}N^{11}N^{12}N^{13}N^{14}N^{15}N^{16}N^{17}N^{18}N^{19}N^{20}$NGG.
Note: The PAM site NGG is not included in the primer. The italicized sequence is part of the core gRNA sequence.

iii. Design a universal RGR-Reverse primer for the vector of your choice: UNIVERS-RGR-R: **ZZZZZZZZZZZZZZZZZZZZ***GTCCCATTCGCCATGCCGAAGC*. The UNIVERS-RGR-R primer should be reverse complementary to GCTTCGGCATGGCGAATGGGAC**YYYYYYYYYYYYYYYYYYYY,** where **YYYYYYYYYYYYYYYYYYYY** is the downstream adaptor sequence (Y and Z are reverse complementary). The italicized sequence is complementary to the 3' end of the HDV ribozyme.

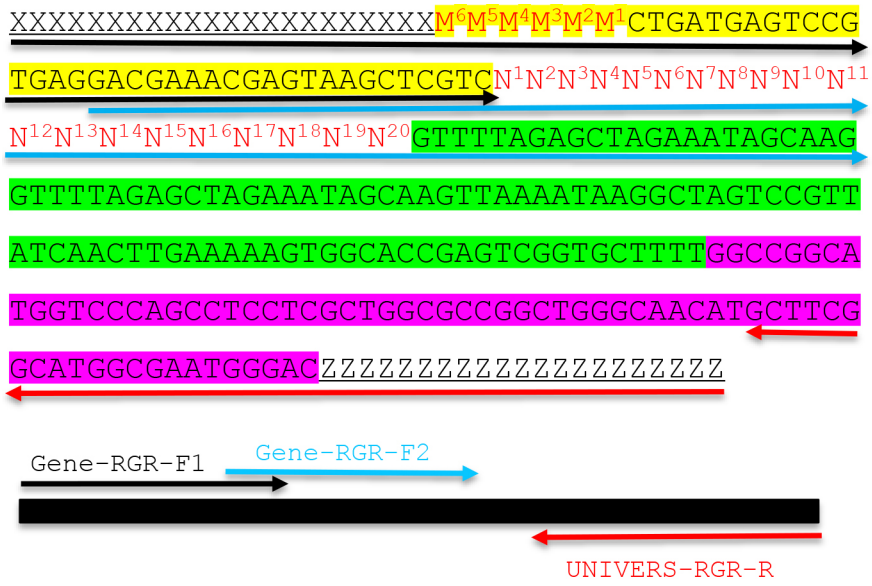

Figure 2. Molecular design of an RGR unit and the assembly of a complete RGR unit by PCR. The top panel shows the complete sequence of an RGR unit that produces a gRNA that targets $(N)^{20}$NGG sequence. The underlined sequences are adaptor sequences from the vector and are used for cloning the RGR unit into the final CRISPR vector. Note that the adaptor sequences vary accordingly if different vectors are chosen. N^1 to N^{20} (in red) are the target sequence prior to the NGG PAM site. It is essential that the $M^6M^5M^4M^3M^2M^1$ and $N^1N^2N^3N^4N^5N^6$ are reverse complementary so that the ribozyme can undergo self-cleavage as designed.

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Sequence highlighted in green is the core gRNA sequence. Sequence highlighted in yellow is the Hammerhead ribozyme. The HDV ribozyme is highlighted in purple. The primers are marked by different colors: black (Gene-RGR-F1), light blue (Gene-RGR-F2), and Red (UNIVERS-RGR-R). The Universal primer depends on the vector sequences and needs to be changed if different vectors are used. The complete RGR unit can be easily assembled by two over-lapping PCR reactions.

3. Assemble the Ribozyme-gRNA-Ribozyme (RGR) unit
   a. An RGR unit for targeting a new gene is assembled through two rounds of overlapping PCR reactions (Figure 2). There is a 21-bp overlap between the two forward primers (Figure 2).
   b. First PCR (PCR1): Use primer pair GENE-RGR-F2 + UNIVERS-RGR-R. Template: any existing RGR construct (*e.g.*, pRS316-RGR-GFP, available from Addgene. The expected PCR product is 233 bp).
   c. The second-round PCR (PCR2): Use primer pair GENE-RGR-F1 + UNIVERS-RGR-R. Gel-purified PCR product from PCR1 is used as the template. The expected PCR2 product is 277 bp, which is the complete RGR unit (Figure 2).

   A routine PCR reaction is set up according to the manufacturer's recommendation (https://www.neb.com/protocols/1/01/01/pcr-protocol-m0530). Briefly, the PCR components are mixed as described in Table 1.

Table 1. PCR setup for amplification of the RGR unit

DNA template	20 ng
dNTPs (2 mM)	10 µl
Forward primer (20 µM)	2.5 µl
Reverse primer (20 µM)	2.5 µl
5x PCR buffer	20 µl
Phusion DNA polymerase (NEB)	1 µl
Add nuclease free H_2O to 100 µl	

Table 2. PCR conditions for amplification of the RGR unit

98 °C	30 sec	1 cycle
98 °C	10 sec	30 cycles
58 °C	10 sec	
72 °C	15 sec	
72 °C	2 min	1 cycle

4. Ligate the final RGR product into a desired vector - clone the RGR (ribozyme-gRNA-ribozyme) unit

   The complete RGR unit assembled through two rounds of PCR is cloned into the final CRISPR vector using Gibson assembly protocol (Gibson *et al.*, 2009). Note that the PCR primers GENE-RGR-F1 and UNIVERS-RGR-R contain sequences that match part of the sequences in the vector to facilitate the *in vitro* assembly of the RGR unit into the vector (Figure 2).

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a. Incubate the DNA assembly mixture (Table 3) for 1 h at 50 °C. DNA assembly was conducted by following the manufacturer's recommended protocol (Table 3). (https://www.neb.com/products/e2611-gibson-assembly-master-mix#tabselect2)

Table 3. *In vitro* DNA assembly

Final PCR product and the vector	0.03-0.2 pmols, X µl
Recommended DNA ratio	Vector:Insert = 1:2
NEB HiFi DNA assembly master mix (Gibson Assembly® Cloning Kit)	10 µl
Water	10-X µl
Total Volume	20 µl

b. Then transform 3 µl ligated product to *E. coli* DH5α competent cells.
The ligated plasmid could be transformed into *E. coli* competent cells following routine cloning procedure (Sambrook and Russell, 2001).
   i. Transform *E. coli* DH5α competent cells (homemade or commercially available products) using 3 µl of ligation product.
   ii. Inoculate 2 to 4 colonies in LB medium with appropriate antibiotics.
   iii. Purify plasmids from the transformed DH5α cells using QIAGEN Plasmid Mini Kit.

c. Verify the positive clones by Sanger sequencing before introducing the final plasmid into your cell/organism of choice (Yoshioka *et al.*, 2015).

B. An example of constructing an RGR unit for CRISPR-mediated gene editing

1. Select a target gene and a vector
We show the construction of an RGR unit to target the *Arabidopsis* gene *Auxin Binding Protein 1* (*ABP1*) (Figure 3) as an example (Gao *et al.*, 2015). We use pHDE-35S-Cas9-mCherry-UBQ plasmid as our CRISPR vector (Gao *et al.*, 2016), which has been used for editing genes in *Arabidopsis* (the plasmid can be obtained through Addgene).
2. Linearize the plasmid
We digest pHDE-35S-Cas9-mCherry-UBQ plasmid with *Mfe*I (Figure 3). The *Mfe*I-linearized plasmid is used for assembling the RGR unit into the vector through Gibson assembly. The complete plasmid can express the *ABP1*-RGR unit from the *Arabidopsis UBQ10* promoter in plants (Figure 3).

Table 4. Linearize the vector by restriction digestion

pHDE-35S-Cas9-mCherry-UBQ	200 ng
*Mfe*I (10,000 U/ml)	1 µl
10x CutSmart® buffer	2 µl
Add sterile distilled H_2O	to 20 µl

Mix the components shown in Table 4 and digest the vector overnight at 37 °C. Then heat inactivation the enzyme at 80 °C for 15 min. The linearized plasmid was16,084 bp.

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3. Design the PCR primers

   The upstream adaptor sequence is GTTTTTCTGATTAACAGCTCGC. The downstream adaptor we used is GACCTAACTGAGTAAGCTAGC (Figure 3).

   a. Forward primer 1: ABP1-RGR-F1: GTTTTTCTGATTAACAGCTCGC**AGCTCC**CTGATGAGTCCGTGAGGACGAAACGAGTAAGCTCGTC. The 6 bp sequence in bold is reverse complementary to the first 6 bp of the gRNA target. The underlined sequence is part of the Hammerhead ribozyme.

   b. Forward primer 2: ABP1-RGR-F2: GACGAAACGAGTAAGCTCGTC**GGAGCTCCTTGTCCCATCAA***GTTTTAGAGCTAGAAATAGCAAG*. The underlined sequence is part of the Hammerhead ribozyme. Sequence in bold is the gRNA target sequence, and the italicized sequence is part of the core gRNA sequence.

   Note: The bold sequence in ABP1-RGR-F1 is reverse complementary to the first 6 bp of the bold sequence in ABP1-RGR-F2. ABP1-RGR-F1 and ABP1-RGR-F2 share the following overlapping sequences: GACGAAACGAGTAAGCTCGTC, which allows us to conduct two overlapping PCR reactions.

4. For cloning the ABP1-RGR unit into the *Mfe*I site in 35S-Cas9-mCherry-UBQ plasmid using Gibson assembly, the UNIVERS-RGR-R primer is:

   GCTAGCTTACTCAGTTAGGTC*GTCCCATTCGCCATGCCGAAGC*

   Note: The bold sequence is complementary to the downstream adaptor sequence. The italicized sequence matches the 3' end of the HDV ribozyme.

5. The complete RGR unit for producing a gRNA targeting the *ABP1* gene is shown in Figure 3. The RGR unit has led to successful isolation of several null alleles of *abp1* in *Arabidopsis* (Gao *et al.*, 2015 and 2016).

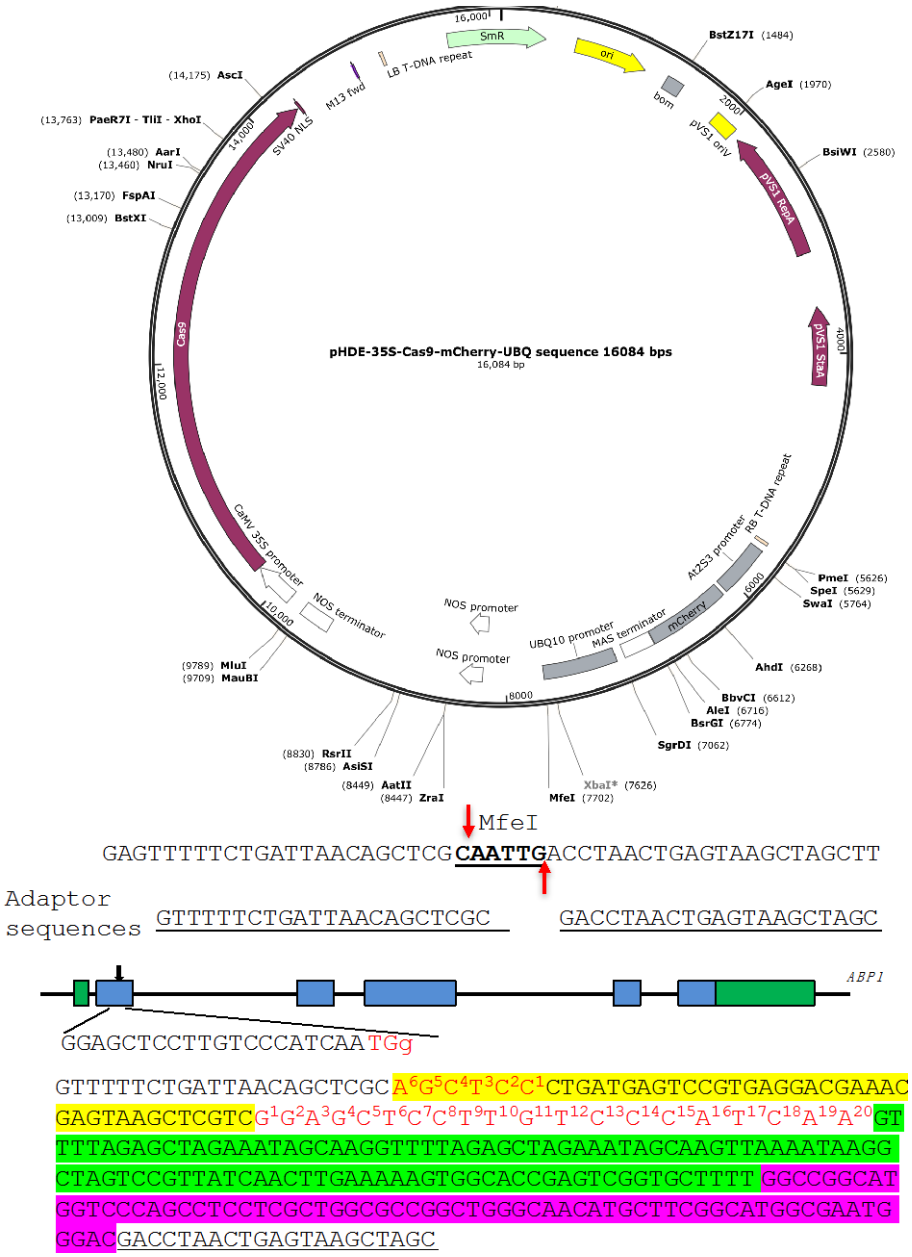

Figure 3. A Molecular design of an RGR unit for targeting the *ABP1* gene in *Arabidopsis*. The RGR unit is cloned into the pHDE-35S-Cas9-mCherry-UBQ plasmid at the *Mfe*I site using Gibson assembly. The adaptor sequences are underlined. Note that the downstream adaptor sequence has to be converted to reverse complementary sequence when designing the Universal primer. The target sequence is in red. The Hammerhead ribozyme and the HDV ribozyme are highlighted in yellow and purple, respectively.

C. Guide RNA production by *in vitro* transcription

Once an RGR unit is assembled and cloned into the final vector as described above, gRNAs can be easily produced by *in vitro* transcription using T7, SP6, or T3 RNA polymerases, which are commercially available.

1. Prepare a DNA template for *in vitro* transcription

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a. The templates for *in vitro* transcription are amplified by PCR from the assembled RGR constructs using two universal primers (any RGR units cloned into the same vector can be amplified using the same pair of primers). For example, RGR units cloned into the *Mfe*I site in pHDE-35S-Cas9-mCherry-UBQ can be amplified using the following pair of primers: SP6-P1: GTCACTATTTAGGTGACACTATA**G**AAGCGGTTTTTCTGATTAACAGCTCGC and Univers-P2: GCTAGCTTACTCAGTTAGGTC.

b. The Underlined sequence is the SP6 promoter and the bolded G in primer SP6-P1 is the transcription initiation site for SP6 RNA polymerase. If T7 or T3 RNA polymerases are used, the underlined 5'-end of the primer SP6-P1 needs to be changed to GTCACTAATACGACTCACTATA**G**GGAGA and GTCACAATTAACCCTCACTAAA**G**GGAGA respectively.

c. PCR conditions are described in Table 1 and Table 2.

2. *In vitro* transcription

a. Add the following reagents at room temperature in the order listed in Table 5:

Table 5. *In vitro* transcription of RGR to produce gRNA molecules

Total	20 µl
5x transcription buffer	4 µl
100 mM DTT	2 µl
~20 U/µl recombinant RNasin RNase inhibitor	0.5 µl
2.5 mM each rNTPs	4 µl
Template DNA (PCR fragment) (10-50 ng/µl [final])	x µl
T7, SP6 or T3 RNA polymerase	1 µl
H_2O	(8.5 - x) µl

Note: For higher yield, add 1 U inorganic pyrophosphatase into the reaction and incubate overnight.

b. Incubate the mixture for 2 to 8 h at 37 °C (T7, T3) or 42 °C (SP6).

c. Add 1 µl 0.5 M EDTA to terminate the reaction.

Note: There is no need to further purify the RNA transcripts for in vitro CRISPR cleavage assays (for in vitro cleavage assay example, follow the link: https://www.neb.com/products/m0386-cas9-nuclease-s-pyogenes. The quality of in vitro transcription and self-processing can be analyzed by electrophoresis in 12% denaturing urea polyacrylamide gels. The RNA bands are stained with ethidium bromide and visualized using a UV transilluminator. To analyze the DNA cleavage products generated by Cas9 and the gRNA from the in vitro transcription, regular 1% agarose gel is adequate.

D. Transformation of the RGR plasmid into *Arabidopsis*

The final plasmid with the RGR construct was transformed into *Arabidopsis* through *Agrobacterium*-mediated floral dipping method (Clough and Bent, 1998). The transgenic T1 plants were identified either as hygromycin-resistant or producing mCherry fluorescence (Gao *et al.*, 2015 and 2016). T1

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plants were screened for editing events using PCR and restriction digestion as described in Gao *et al.*, 2015 and 2016.

Data analysis

Our RGR-based production of functional gRNAs has been successful both *in vitro* and *in vivo* (Gao and Zhao, 2014; Gao *et al.*, 2015 and 2016). We initially produced a gRNA targeting GFP using the RGR design. The gRNA was produced *in vitro* from the SP6 promoter using the commercially available SP6 RNA polymerase (Gao and Zhao, 2014). *In vitro* digestion assays indicated that the gRNA was fully functional (Gao and Zhao, 2014). We used the same RGR design to produce gRNA in yeast using the ADH promoter, an RNA polymerase II promoter (Gao and Zhao, 2014). Such a construct led to successful editing of the *GFP* gene in yeast. We further introduced an RGR design in *Arabidopsis* to produce a gRNA targeting the *Arabidopsis ABP1* gene. Multiple stable heritable mutations in the *ABP1* gene have been obtained (Gao *et al.*, 2015 and 2016). For example, we made a construct that uses RGR for one gRNA production and the U6 promoter to produce another gRNA. The two gRNA molecules target two discrete sites of the *ABP1* gene in *Arabidopsis* and were designed to generate a 771 bp deletion (Gao *et al.*, 2016). We detected the intended deletions in 5 out of 61 T1 plants. Two of the T1 plants produced Cas9-free, stable homozygous mutants at the T2 generation (Gao *et al.*, 2016).

The RGR design has been adapted for editing genes in other organisms (Nissim *et al.*, 2014; Yoshioka *et al.*, 2015).

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