CRISPR-cas9 Vector System for *P. patens*

The CRISPR-cas9 vector system here is a modification of the vectors that were published by Miao J, Guo D, Zhang J, Huang Q, Qin G, et al. (2013) Targeted mutagenesis in rice using CRISPR-Cas system. Cell Res23: 1233–1236.

In this system, a protospacer is annealed and cloned into an entry vector. This entry vector contains the guide RNA expression cassette between attL sites. Using a simple LR clonase reaction the guide RNA expression cassette can be moved to a final vector that contains the maize ubiquitin promoter driving expression of Cas9.

Our modifications for moss include:

1. using a moss RNA polymerase III promoter in the entry clone
2. the destination vector is no longer an agro vector – it is a simple pGEM backbone that contains the attR sites, the UBI-Cas9 expression cassette, and an antibiotic selection cassette for transient expression in moss.

In our hands, we have had success using a single protospacer to target a gene. However, there are some caveats. First, editing efficiency can vary with the protospacer and with the locus that is being targeted. Second, if you are trying to target multiple genes with the same transformation, then the genotyping can be a bit of a nightmare. It is best to design your protospacer in such a way that downstream genotyping is not so labor intensive. While we have used T7 endonuclease as a diagnostic, it is not the most reliable method. We have recently started targeting genes with two protospacers and I think this increases the efficiency of editing and we have also seen larger deletions, about 10% of the time.

With the multi-site gateway vectors, it is possible to generate vectors with 4 different protospacers. It is also possible to transform three plasmids simultaneously (triple selection). This would give you the option of co-transforming 12 protospacers simultaneously.

Plasmids:

(1) Destination vector:
pMK-Cas9 (Ampicillin bacterial resistance, G418 plant resistance)
pMH-Cas9 (Ampicillin bacterial resistance, Hygromycin plant resistance)
pZeo-Cas9 (Ampicillin bacterial resistance, Zeocin plant resistance)
All destination vectors need to be propagated with Chloramphenicol and in ccdB (or DB3.1) cells, due to the presence of the Gateway cassette.

(2) Entry vector:
pENTR-PpU6p-sgRNA (Kan Resistance)
pENTR Multisite Plasmids enabling 2-,3-,and 4-way Gateway multi-site reactions (L1R5, L5L2, L1L4, etc…) (Kan bacterial resistance). For details on this system please refer to https://www.thermofisher.com/us/en/home/life-science/cloning/gateway-cloning/multisite-gateway-technology.html.

Protospacer Design

Use this website to help you choose the protospacer: http://crispor.tefor.net/
In step 1, put your genomic sequence into the blank box.
In step2, choose *Physcomitrella patens* phytozome V11.
In step3, choose NGG streptococcus pyogenes.

The website will generate a large table with candidate protospacers. Choose a high value for "Out of Frame", and keep in mind to minimize off-targets. It can be hard to find a protospacer that has no off-targets sometimes, so you need to balance between value of Out of Frame and off-targets. Also keep in mind possible loss of restriction sites in target sequence for downstream genotyping.

- Find the sequence with an 5'-NGG-3' (functions as the PAM motif). It is better to have restriction sites for further plasmid check, but it is okay if this 20 bp fragment does not have any restriction sites.
  E.g. FtsZ1: 5'-GGCGTTGTTTAGTGGGTGCTCGG-3' (green CGG is the PAM motif, yellow is the protospacer)
- Design primers for protospacer:
  5'-ccat-spacer_forward-3'
  5'-aaac-spacer_reverse-3'
  E.g. FtsZ1-cas9:
  Forward primer, 5'-ccatGGCGTTGTTTAGTGGGTGCT-3'
  Reverse primer, 5'-aaacAGCACCCACTAAACAACGCC-3'

  The overhangs are essential as complementary sequences will be present in the entry clone after digestion with BsaI, enabling efficient cloning.

NOTE: Do not include the PAM motif in your protospacer oligo design

Cloning Steps

Digest entry clone pENTR -sgRNA with BsaI

2μL miniprep plasmid
3μL cutsmart buffer
0.5μL BsaI
24.5μL ddH_2O

Incubate at 37°C at least one hour, but overnight is good to ensure complete digestion.

Run agarose gel and gel purify the linear plasmid. Measure the concentration for ligation

Anneal the two complementary protospacer oligos:

1. Suspend the primer powder with ddH_2O to 100 μM.
2. Mix the forward primer and the reverse primer with 1:1 ratio, which means each of your primer is 50μM.
3. PCR machine anneal

    1. 98°C 3min
    2. Slow ramp (set to 1%) to primer Tm, and then stay at Tm for 10 min. Slow ramp takes about one hour.
    3. Slow ramp (set to 1%) to 25°C and stay ever.

4. Dilute this annealed product 100-fold.

Ligate protospacer into BsaI digested entry clone

1. Transfer instant sticky-end ligation master mix (from NEB) to ice prior to reaction set up. Mix tube by finger flicking before use.
2. Combine 20-100 ng of linearized entry clone with a 3-fold molar excess of protospacer and adjust volume to 5μL with ddH_2O.
3. Add 5μL of instant sticky-end ligase master mix (NEB), mix thoroughly by pipetting up and down 7~10 times, and incubate room temperature for 5-10 minutes. The sample can now be transformed into *E. coli*.

Obtain the entry clone with your protospacer.

Transform to DH5α competent cell and plate LB-Kan plate. Pick colonies for overnight incubation and miniprep. If your protospacer has restriction sites, you can use these sites determine if the clone is good. And then you can choose positive samples to be sequenced. If not, you can digest with BsaI. Plasmids that have the inserted protospacer should no longer cut with BsaI (the BsaI site disappears after ligation) Those that no longer digest can be sequenced. Sequencing can be done with M13F sequencing primer.

Obtain the destination vector with your protospacer.

Conduct simple (one-way) or complex (multi-site) LR reaction to go into your choice of destination vector. Transform LR reaction products into DH5α competent cells and plate on LB-Amp plates. Pick colonies for overnight incubation and miniprep. To check whether the destination vector is correct, you can conduct a digest that releases a fragment. Suggested enzymes are: pZeo-Cas9: AscI; pMK-Cas9 and pMH-Cas9: AflII and SacI. We generally do not sequence this final vector unless there seems to be an issue with the digest, since LR reactions using sequenced entry vectors is unlikely to introduce mutations.

If your positive clones are right, they can be used for moss protoplast transformation. If you envision only doing a few transformations, then we usually just make a number of minipreps of the desired construct and then pool, instead of a maxiprep.

Plant selection:

- For single gene targeting, one transformation needs 15 μg of plasmid and should be plated onto three plates. No top agar is required. Protoplasts can be plated in plating media.
- After transformation, protoplasts are plated on PRMB plates (with cellophane) and stay on these plates for 4 days. PRMB is good for protoplast regeneration.
- Move the cellophane onto the antibiotic selection plates (PpNH4 + antibiotics). To fully kill the untransformed protoplasts, the regenerated plants need to be kept at least 7 days on antibiotic plates.
- Move the cellophane after 7 days on selection to a regular PpNH4 plate for maximal growth

Keep in mind that a CRISPR-induced mutation is a one-time thing. The plant will lose the destination plasmid along with the antibiotic resistance after moving to regular media without selection. A second selection step should not be performed.

CRISPR mutant genotyping

CRISPR/Cas9 has ~90% efficiency for single nonessential gene mutation. Mutations can be deletions, insertions or edited sequences. The scale of mutation tends to be less than 200bp. Genotyping primers should at least cover 400bp (upstream 200bp, downstream 200bp). We usually design primers that span 1kb, which also fits the requirement for T7 endonuclease.

For single gene mutation genotyping, amplify 1kb fragment in the locus (put the protospacer in the middle). Send the purified PCR products for sequencing.

For multiple gene mutations, we suggest to use T7 endonuclease (or other methods such as loss of a restriction site within the protospacer targeted region) to check whether targeted genes are mutated or not before sequencing. **Remember that T7 is unable to tell 1bp mutation.**

Using CRISPR/Cas9 for Homology-Directed Repair (HDR)

It is possible to repair a Cas9-induced double-strand break with co-transformation of a DNA donor homology plasmid that flanks the region of your protospacer. This allows precise genome editing (i.e. changing a single nucleotide, tagging a gene-of-interest with a probe, or knocking-in a "stop codon cassette" that has stop codons in all 3 frames to create a guaranteed truncated or null protein).

Plasmids for HDR:

- Destination vector:
    - pGEM-Gate
- Donor vectors for 5' and 3' arms, respectively, to create entry vectors:
    - pDONR-P1P4
    - pDONR-P3P2
- Entry vectors:
    - pENTR-R4R3-mEGFP (1X, 2X, or 3X), pENTR-R4R3-mRuby (1X, 2X, 3X) with or without stop codon for C- and N-terminal tagging, respectively.
    - pENTR-R4R3-stop (stop codon cassette with stops in all 3 frames)

*This system uses Gateway cloning technology. Please see Gateway if you are unfamiliar (https://www.thermofisher.com/us/en/home/life-science/cloning/gateway-cloning.html)

Designing the protospacer – same protocol as above

Use CRISPOR software to help choose a protospacer (http://crispor.tefor.net)

In step 1, put your genomic sequence into the blank box.
In step2, choose *Physcomitrella patens* phytozome V11.
In step3, choose NGG streptococcus pyogenes.

Things to think about when choosing a protospacer:

- If you are using the "stop codon cassette" to create a null, any protospacer within the beginning of the coding sequence of the gene will work—it is best to have the protein truncated towards the N-terminus than the C-terminus to ensure loss-of-function.
- If you are tagging a gene (i.e. with GFP, mRuby, etc.), the best choice of a protospacer is one that is very close to the N- or C-terminus (whichever end you choose to tag) and that can be destroyed by the insertion of the tag.
    - For N-terminal tagging, a protospacer + PAM that overlaps the 5' UTR and the coding region.
    - For C-terminal tagging, a protospacer + PAM that overlaps the coding region and the 3' UTR.
        - The insertion of the tag will effectively break the protospacer recognition site after repair, which will inhibit Cas9 from subsequently cutting this site.
    - If the protospacer does not overlap these regions, a protospacer close to the tag insertion site will require site-directed mutagenesis (see below).

- If you are editing the genome to your liking, choose a protospacer that is very close (or within) the region you are editing.
- **Don't forget about off-targets**. Try to minimize the amount of off-targets. You do not have to pay attention to the "out-of-frame score", since this has to do with Non-Homologous End Joining (NHEJ) repair.

Designing the DNA donor homology plasmid

Homology-directed repair has been shown to be effective using a DNA donor homology plasmid with regions of homology ("homology arms") of ~700-800 base pairs in length. We use Gateway cloning to make the DNA donor homology plasmid for insertions.

To generate entry clones for insertion (tagging, "stop codon cassette", etc.)

- 5' homology arm will be cloned into pDONR-P1P4 using BP clonase reaction
- The tag (or "stop codon cassette") into pDONR-P4rP3r using BP clonase reaction – we already have these plasmids made. If you are looking to insert a gene-of-interest, you will have to amplify using attB4r, attB3r primers followed by a BP reaction with pDONR-P4rP3r to make this entry plasmid.
- 3' homology arm will be cloned into pDONR-P3P2 using BP clonase reaction
    - **Note:** when designing the homology arms, include everything that you want to be included in the repair. Nucleotides may not be incorporated during repair if they are missing in the DNA donor homology plasmid, especially if they are missing in the arms very close to the site of the repair

To generate final clone

- 3-way LR reaction into pGEM-gate – a plasmid with a Gateway cassette and ampicillin bacterial resistance. There is no plant antibiotic resistance on this plasmid, and no plant-specific promoter. Keep in mind, if you use this for homology-directed repair, the attB sites between the homology regions and the insert will be integrated into the genome (you need to design the attB primers accordingly such that they are in frame, and act as a linker).

Note: To precisely edit a small region of the genome, the DNA donor homology plasmid will require a long stretch of homology flanking your specific gene changes (~800 base pairs total) into a plasmid of your choice. Site-directed mutagenesis of the plasmid will allow for specific changes, and HDR will use the homology region to repair the double-strand break, incorporating the desired changes. 3-way Gateway is not recommended for this, since undesirable attB sites will be incorporated into the repair.

Keeping the protospacer sequence in the DNA donor homology plasmid (IMPORTANT)

If the protospacer sequence will be incorporated into the DNA donor homology plasmid, it is possible Cas9 will cut your homology plasmid, rendering it useless. It is best to mutagenize the plasmid to create silent mutations so that (1) Cas9 will not recognize this plasmid and saturate its ability to cut genomic DNA, and (2) Cas9 will not recognize the genomic sequence after repair. The easiest silent mutation is one that disrupts the PAM (5'-NGG-3') to

inhibit Cas9 ability to cut this region. However, if it is not possible to change the PAM, it may be necessary to introduce a few silent mutations within the protospacer sequence of the homology plasmid itself to interrupt base pairing interactions with the gRNA (the more mismatches, the lower likelihood the gRNA will anneal – however, I have tried a single silent mutation and it seems to be sufficient to inhibit cleavage).

Transformation protocol

Co-transform 15 µg of Cas9/gRNA expression vector with 15 µg of the homology plasmid. Protoplasts will either take up both plasmids or neither plasmid – therefore a resistance cassette is only required on one plasmid (the Cas9/gRNA expression plasmid). Plant selection is the same as a regular CRISPR transformation, explained previously.

Genotyping

Choose genotyping primers upstream and downstream of the homology arms. It has been reported that HDR of Cas9-induced double-strand breaks can often result in concatenated copies of the homology region in the DNA donor plasmid (Collonnier *et al*., 2017), so it is best to genotype outside these arms to ensure single insertion. If using the "stop codon cassette," there are built-in multiple cloning restriction sites to help with genotyping. The PCR product will also be larger in size for easy genotyping.