CRISPR plasmid design: identifying the right elements for the job

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Monsanto
Cas9 and Cpf1 are power players to edit your gene of interest

Cas9 binding motif:
TTTVTGGAGGAGGAGGTGAAAGCTGNGG
AAABACCTCCTCTCCACTTTCGACNCC

PAM: UGGAGGAGAGGUGAAAGCUG

S. pyogenes
Cas9

Cpf1 binding motif:
TTTVTGGAGGAGGAGGTGAAAGCTGAGG
AAABACCTCCTCTCCACTTTCGACTCC

PAM: UGGAGGAGAGGUGAAAGCUGAGG

Lachnospiraceae bacterium ND2006
Cpf1

Fired up? Time to build that construct!
When it comes down to making plasmids, Cas9 and Cpf1 share a common set of requirements.

- **Where and how much**
- **Coding optimization matters**
- **Supports promoter decision**
- **Pol II or Pol III**
- **TTTTTTT**
Outline of the talk

• Nuclease selection and cassette

• gRNA cassette creation

• Overall configuration of the final plasmid

• Multiplexing
Nuclease cassette
Nuclease choice is a matter of PAM and reagent availability

- Cas9:
  - SpCas9 PAM variants
    - Hu et al. 2018 in human
    - Kleinstiver et al. 2015
    - Anders et al. 2016

- Cpf1:
  - FnCpf1 Zhong et al. 2018 in rice
  - AsCpf1 Gao et al. 2017
  - Fn and LbCpf1 expanded PAMs Li et al. 2018

Commercially available reagents
A variety of promoters have been validated in plants to drive nucleases

Constitutive:
- 35S (many such as Yan et al. 2015)
- Ubq (Mao et al. 2015 Char et al. 2016)
- RPS5A (Tsutsui and Higashiyama 2013)

Egg-specific
- EC1.2 (Wang et al. 2015)
- ZmDcm1 (Feng et al. 2018)

Meristematic/actively dividing cells
- Yao (Yan et al. 2015)

Pollen-specific
- AtSPL (Mao et al. 2015)
Promoter choice impacts in which generation the mutations can be detected

Constitutive expression

- See edits in first generation
- Need to produce more transgenic plants
- More chimerism
- Plant seed from only edit positive plants

Germline specific

- See edits in second generation
- Need fewer transgenic plants
- Need to plant our more seed to see edits
Coding optimization of Cpf1 impacts activity

FAD2-1A target site from Kim et al. 2017
Even when coding optimization has no impact on activity, it can impact ease of use.

Unstable in *E. coli*

gRNA targeting the Psy1 gene from Zhu et al. 2016
Wang et al. showed terminator choice impacts nuclease activity

ETC2, TRY, and CPC triple mutant-tricomb clusters

<table>
<thead>
<tr>
<th></th>
<th>rbcS-E9t</th>
<th>Nost</th>
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<tbody>
<tr>
<td><strong>EC1.2p</strong></td>
<td>triple knockout 9/108 (8.3%)</td>
<td>triple knockout 0/54 (0)</td>
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<tr>
<td><strong>EC1.1p</strong></td>
<td>triple knockout 4/224 (1.8%)</td>
<td>triple knockout 0/102 (0)</td>
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<tr>
<td><strong>2X35Sp</strong></td>
<td>Double knockout 33/109 (30.3)</td>
<td><strong>Double knockout 26/33(78.8)</strong></td>
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Wang et al. 2015
gRNA cassette
For the gRNA cassettes, Pol III or Pol II promoters can be used

**Pol III**
- RNA is nuclear localized
- Constitutive
- Easy to identify in new species
- Small <1 kb

**Pol II**
- RNA is exported to cytoplasm
- Can make tissue/timing specific
- Can be hard to identify required elements
- Usually large >1 lb

But what if you are working in a new species and want to test the promoter first?
TaqMan® assays can be used to test Pol III promoter strength for your favorite crop

TTTTTTT (U3, U6, 7SL)
or
ACAATTCAAAAACAAGTTTTTAT (U2 and U5)

~200-400nt
The Cas9-Transcription factor (Cas9-TF) fusion can also be used to identify functional promoters.

GUS expression as measured by MUG assay is a combination of:
- Whether gRNA is transcribed
- Whether the gRNA is stable
- Whether the gRNA can bind the nuclease
- Whether the nuclease/gRNA complex can bind DNA
Cas9 TF system can screen new promoters in a high-throughput fashion.

Promotor | gRNA
--- | ---
~200 nt | TTTTTTTT (U3, U6, 7SL)
or
| ACAATTCAAACAAGTTTTAT (U2 and U5)

![Graph showing normalized GUS activity for different samples: Control, 7SLCR07, U6Chr08, U3_8B, U2_1, U5_e. The graph indicates varying levels of activity with U3_8B showing the highest activity compared to the others.](image-url)
Overall configuration
The location of the gRNA relative to the nuclease did not impact activity in maize

<table>
<thead>
<tr>
<th>Marker</th>
<th>Cas9</th>
<th>gRNA</th>
<th>vs</th>
<th>Marker</th>
<th>gRNA</th>
<th>Cas9</th>
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<tr>
<td>14/80 plants 17.5%</td>
<td>vs</td>
<td>15/71 plants 21%</td>
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The nuclease-gRNA configuration drove 73% of Cas9 guides and 56% of Cpf1 rates to greater than 20%
gRNAs can be expressed from the same cassette as the nuclease

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<tr>
<th>Promoter</th>
<th>Cas9</th>
<th>Poly(A)</th>
<th>R</th>
<th>B</th>
<th>gRNA1</th>
<th>R</th>
<th>B</th>
<th>gRNA2</th>
<th>Full Ribozyme</th>
<th>Term</th>
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<td>Ding et al. 2018 (Cas9 and Cpf1)</td>
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Multiplexing
Cpf1 is highly amenable to multiplexing as an array of mature gRNAs

Xudong Ye; Monsanto 2018

FAD2-1A target site from Kim et al. 2017
Cas9 and Cpf1 gRNAs can also be multiplexed with tRNAs

Cas9
Xie et al 2015 in Rice
Qi et al 2016 in maize
Wang et al. 2018 wheat

Cpf1
Shown to reduce cutting relative to array
Ding et al 2018 in Rice protoplasts

Can be used with both Pol III and Pol II promoter
Cas9 and Cpf1 gRNAs can also be multiplexed with ribozymes or Csy4

Cermak et al. 2017
Gao and Zhao 2014 in yeast
Cermak et al. 2017

Can be used with both Pol III and Pol II promoter
Putting the pieces all together

• Choose a promoter and terminator that will give you mutations in the desired time frame

• Coding optimization of nuclease can impact both activity and ease of use

• gRNAs can be driven off of both Pol III and Pol II promoters by themselves or in conjunction with the nuclease

• For new species, Pol III promoters can be identified using TaqMan® for RNA levels or a nuclease-TF fusions system in protoplast

• gRNA multiplexing can be achieved through a variety of methods such as tRNAs, Csy4, ribozymes or using gRNA arrays (Cpf1)
Acknowledgements

AGeM (Applied Genome Modification)

Applied Molecular Biology

Applied Cellular Biology

And many other great support teams that help take the idea from plasmid to plant!