CRISPR/Cas9 sgRNA-Mediated Mutagenesis in Switchgrass (Panicum virgatum L.)

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1. Introduction
2. Transient assay of CRISPR/Cas9 system
3. Efficiency of CRISPR/Cas9 system in generating stable target gene mutations in switchgrass
4. Conclusion

Introduction:


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Rice CRISPR/Cas9 System

Cas9 expression vector
(Provided by Dr. Bing Yang)
Isolation switchgrass protoplast

We demonstrate transient expression of PEG-mediated DNA uptake in the isolated protoplasts by detecting the GFP (green fluorescent protein) gene driven by rice ubiquitin promoter. Transformation efficiency is around 25%.

Strategy to detect Cas9 and sgRNA activity in switchgrass

We co-transform pUC19:35sGFP, pENTER4: gRNA, pBY:OsCas9 vectors to switchgrass protoplasts. After 60 hours, we detected fluorescent signal with NIKON ECLIPSE E200 microscope.

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Target Sequence Design

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Target Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphoglycerate Mutase (PGM)</td>
<td>TACGCGGCTGGTGGTGGTG</td>
</tr>
</tbody>
</table>

Phosphoglycerate mutase

3-phosphoglycerate (3-PGA) → 2-phosphoglycerate (2-PGA)

Agrobacteria-mediated switchgrass transformation:

All of the calli in one callus line come from the same genetic background.
Selection of Hygromycin-resistant callus

After 6-8 weeks, with 100 mg L⁻¹ hygromycin selection, actively growing calli were picked out.

Agrobacteria-mediated switchgrass transformation:

Regeneration:
Calli that were actively growing were placed on regeneration medium with 50 mg L⁻¹ hygromycin.

Detection of mutations:
If the Cas9 functions as expected, it will cut the DNA at the site 3 bases upstream of the PAM sequence within the target gene and likely generating deletions or insertions.

For gene PGM, the mutation frequency is around 11.1% (1 out of 9 independent events contain mutants).

PGM target sequence: GCACGGAGCTGGTGGTGCGG

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Independent Events</th>
<th>Plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGM</td>
<td>9</td>
<td>25</td>
</tr>
</tbody>
</table>

| WT1 | CAGGAGCTGGTGGTGCGG |
| WT2 | CATGGACAGACGGAGTGGAACGTCTCCAG |
| mutant1 | ACAGGAGCTGGTGGTGCGG |
| mutant2 | CATGGACAGACGGAGTGGAACGTCTCCAG |
| mutant3 | ACAGGAGCTGGTGGTGCGG |
| mutant4 | CATGGACAGACGGAGTGGAACGTCTCCAG |
| mutant5 | ACAGGAGCTGGTGGTGCGG |
| mutant6 | CATGGACAGACGGAGTGGAACGTCTCCAG |
| mutant7 | ACAGGAGCTGGTGGTGCGG |
| mutant8 | CATGGACAGACGGAGTGGAACGTCTCCAG |

PGM 5-4-1 (Homozygous)

| WT1 | CAGGAGCTGGTGGTGCGG |
| WT2 | CATGGACAGACGGAGTGGAACGTCTCCAG |
| mutant1 | ACAGGAGCTGGTGGTGCGG |
| mutant2 | CATGGACAGACGGAGTGGAACGTCTCCAG |
| mutant3 | ACAGGAGCTGGTGGTGCGG |
| mutant4 | CATGGACAGACGGAGTGGAACGTCTCCAG |
| mutant5 | ACAGGAGCTGGTGGTGCGG |
| mutant6 | CATGGACAGACGGAGTGGAACGTCTCCAG |
| mutant7 | ACAGGAGCTGGTGGTGCGG |
| mutant8 | CATGGACAGACGGAGTGGAACGTCTCCAG |

PGM 5-4-2 (Heterozygous)
Teosinte and Maize

Teosinte branched 1 (tb1) is found to affect the differentiation in branch architecture from teosinte to maize (John Doebley 2001)

Detection of mutations:

DNAs from 21 independent events were digested by Ncol and Bbvcl enzyme respectively to enrich the mutant alleles.

TB1 second target region:

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<th>Gene Name</th>
<th>Target Sequences</th>
</tr>
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<tbody>
<tr>
<td>Teosinte Branched 1 (TB1)</td>
<td>GGTAAAGCGGTAAGTCCATG</td>
</tr>
</tbody>
</table>

**TB1 second target region:**

- 5'------GGTAAAGCGGTAAGTCCATG----------3'
- 3'------C CATTTCGCCATCGCC---------5'

**PAM:**

- **TB1A**
  - Primer
- **TB1B**
  - Primer

TB1 first target region:

- 5'------TACCGAGCTGGTAGCTGAGG----------3'
- 3'------A TGGCCTGACCATCGCCC---------5'

**PAM:**

Detection of mutations:

**TB1A**

1. From 21 independent events, 84 plants were digested by Ncol and Bbvcl enzyme to enrich the mutant alleles.

**TB1B**

**Conclusion:**

1. Rice CRISPR/Cas9 system can induce mutation in switchgrass. For gene PGM, the mutation frequency is around 11.1% (1 out of 9 independent events contain mutants).
2. For TB1, we could use one construct with two sgRNAs to mutant two different genes simultaneously.
3. Detection is critical.

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Thanks and Questions