CRISPR Plants
Development of a Course-based Undergraduate Research Experience

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Background

With the rapid advances in CRISPR-Cas9 gene editing technologies undergraduate students a re keen to learn about and experience these technologies[1]. One aim of this project was to give students the opportunity to explore the experimental workflow and trouble-shooting involved in a research project. Many biology programs require an "Experiential Learning" component, and a course-based approach enables this experience for a larger group of students.

The Plant Molecular Biology class of 2019 describe the preliminary steps in the development of a CURE (Course based Undergraduate Research Experience) to allow students to explore CRISPR Cas 9 gene editing in *Arabidopsis thaliana* using *Agrobacterium*-mediated transformation.

Approach

Project 1: Design a guide RNA sequence to knock out a chosen gene in *Arabidopsis thaliana*.

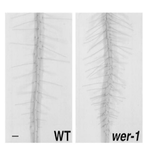
- Clone the guide sequence into pHAtC[2]:
  - a plant transformation plasmid encoding:
  - guide RNA sequence (U6 promoter)
  - Cas 9 gene (CaMV 35S promoter)
- Floral dip transformation of *Arabidopsis thaliana*

Project 2: Create a single guide RNA (sgRNA) by in vitro transcription, and test whether this is able to complex with Cas 9 nuclease and make a double strand break in DNA containing the target sequence.

Learning Gains:

1. Project planning: Although a broad outline of each project was presented, student teams designed their own workflow around class meeting times and classroom and personal availability outside of class.
2. Become proficient in standard molecular biology techniques. By the end of the course, student attitudes towards running gels and pouring agar plates had changed to " it won't take long to do this".
3. Become comfortable with bioinformatics tools such as Benchling, TAIR, CRISPRscan, Snapgene and NCBI.

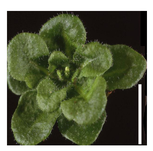
Selected *Arabidopsis* gene targets



Werewolf
At5G14750

Variegated 2
At2G30950

Agamous
At4G18960



Brassinosteroid insensitive 1
At4G39400

References

1. Wolyniak *et al* (2019) Integrating CRSPR-Cas 9 Technology into Undergraduate Courses: Perspectives from an NSF Workshop for Undergraduate faculty, June 2018 J. Microbiol. Biol. Educ. 20(1)

2. Kim *et al* (2016) A simple, flexible and high-throughput cloning system for plant genome editing via CRIPSR-Cas system. *Journal of Integrative Plant Biology 58*, 705-712

Project 1 Workflow and results

Chose the gene you want to edit

Use Benchling to check the exon/intron structure and design suitable guide sequences (Fig. 1)

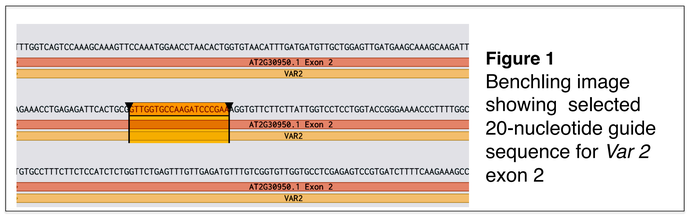


Figure 1
Benchling image showing selected 20-nucleotide guide sequence for *Var 2* exon 2

Order oligonucleotides with suitable overhangs to clone into the *Aar I* site of pHAtC[2] (Fig. 2)

5' GATTGGTTGCCAAGATCCCGAA 3'
3' CAACGGTTCTAGGGCTTCAAA 5'

Figure 2
Paired and annealed oligonucleotides corresponding to the guide sequence. These were ligated into *Aar* I digested pHAtC

Anneal oligonucleotides, digest pHAtC with *AarI* restriction endonuclease Perform ligation and transformation into competent *E.coli* cells. (Fig. 3)

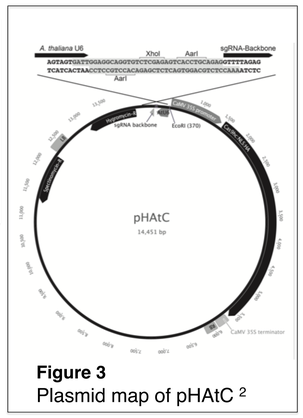


Figure 3
Plasmid map of pHAtC [2]

Verify successful plasmid construction by PCR and DNA sequencing and electroporate into *Agrobacterium tumefaciens* (Fig. 4 & 5).

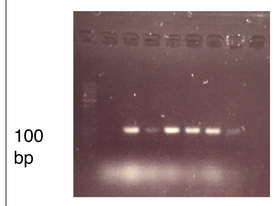


Figure 4
Gel electrophoresis of PCR using the primer set showing inserted guide sequence in pHAtC

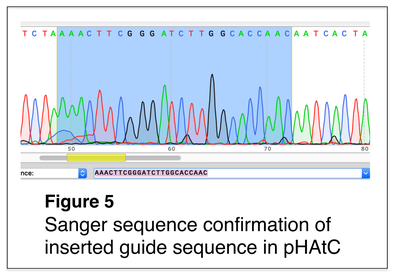


Figure 5
Sanger sequence confirmation of inserted guide sequence in pHAtC

Perform floral dip transformation of *Arabidopsis thaliana,* recover seeds and self pollinate. Verify CRISPR Cas 9 editing in T1 or T2 generations.

Project 2 Workflow and results

Design and order oligonucleotides to include the CRISPR guide sequence from the previous experiment (Fig. 6)

T7 Promoter **Guide sequence** **Tail annealing sequence**
5' TAATACGACTCACTATAGGGTTGCCAAGATCCCGAAGTTTTAGAGCTAGAA 3'

Tail oligo
5'AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTT ATTTTAACTTGCTAT TTCTAGCTCTAAAAC
Annealing sequence

Figure 6: Oligonucleotide sequences for generation of sgNA template

Anneal and extend the oligos using T4 polymerase or *Taq* polymerase (Fig. 7)

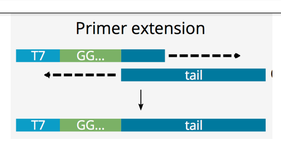


Figure 7
The next step is to anneal and extend the oligonucleotides to generate a DNA template for in vitro transcription
Adapted from CRISPRscan.org

In vitro transcription to make RNA from the DNA template (Fig. 8)

PCR-amplify the region containing the guide sequence from *Arabidopsis* genomic DNA

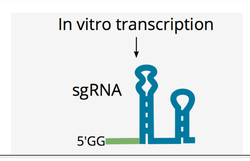


Figure 8
We used Lucigen T7 Flash In vitro Transcription kit to generate sgRNA from our DNA template
Adapted from CRISPRscan.org

Perform in vitro assay:
Allow Cas 9 nuclease to complex with sgRNA, then incubate with template DNA

Visualize results on an agarose or polyacrylamide gel (Fig. 9)

Figure 9
Lane 1: 800bp *Arabidopsis* gDNA PCR fragment containing target site for sgRNA

Lane 2: 800 bp *Arabidopsis* gDNA (as Lane 1) after exposure to sgRNA and Cas9. PCR fragment is cleaved at approximately 375 bp.

Assessments

Weekly quizzes tested student understanding of the various experimental procedures used during the semester.
Each student kept their own laboratory notebook, and complied a portfolio of relevant papers and files related to the project.
Two open-portfolio/notebook exams and two laboratory reports were completed during the semester.

Student comments

" "I really appreciate how the instructor pushed us to think independently and to troubleshoot our experiments. It was an opportunity to grow us scientists. I also really appreciate how she supported us to do experiments outside of class."

"The class focused on individual group projects but was still very organized. Students all had the freedom to design their experiments. The instructor and the TA provided help when students asked for it, so I wasn't lost. I also liked that this class was mostly labs. I think hands-on experiments are the most effective way to learn. In my experience, I can never focus on lecture for too long and I just don't learn anything from it. The exam questions focused on lab procedures and concepts instead of a bunch of random information that needs to be memorized".

Where did we get to?

- Four groups or 2-3 students designed guide RNA sequences for their chosen *Arabidopsis* gene and successfully cloned them into the pHAtC plasmid, and electroporated the plasmid DNA into *Agrobacterium tumefaciens*.
- Although we did a few *Arabidopsis thaliana* floral dip transformations, we did not recover any transformed seedlings. This was in large part a timing issue, as plants were not at the correct developmental stage.
- All student groups attempted the in vitro assay, and were partially successful in demonstrating that their CRISPR guide sequence was able to associate with Cas 9 nuclease and cleave the target DNA.

What's next?

- Optimize growth of *Arabidopsis* in our greenhouse, or under controlled conditions in a growth room.
- Optimize *Arabidopsis* floral dip transformation.
- Obtain several generations of seeds from transformed plants for analysis by Spring 2020 class.
- Optimize *in vitro* assay parameters to ensure student success.

Acknowledgments

Thanks are due to the 2019 Plant Molecular Biology class for their hard work and dedication, and for joining me on this journey. Spencer, Milky, Meghan, Maria, Ben, Trevor, Jake, Paul, Catherine, Cory and Joyce, you are amazing!

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