

An Undergraduate Research Project Utilizing CRISPR-Cas9 Gene Editing Technology to Study Gene Function in *Arabidopsis thaliana*[†]

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The CRISPR-Cas9 system functions in microbial viral pathogen recognition pathways by identifying and targeting foreign DNA for degradation. Recently, biotechnological advances have allowed scientists to use CRISPR-Cas9-based elements as a molecular tool to selectively modify DNA in a wide variety of other living systems. Given the emerging need to bring engaging CRISPR-Cas9 laboratory experiences to an undergraduate audience, we incorporated a CRISPR-based research project into our *Genetics* class laboratories, emphasizing its use in plants. Our genetic manipulations were designed for *Arabidopsis thaliana*, which despite serving as a plant research model, has traditionally been difficult to use in a classroom setting. For this project, students transformed plasmid DNA containing the essential CRISPR-Cas9 gene editing elements into *A. thaliana*. Expression of these elements in the plant genome was expected to create a deletion at one of six targeted genes. The genes we chose had a known seedling and/or juvenile loss-of-function phenotype, which made genetic analysis by students with a limited background possible. It also allowed the project to reach completion in a typical undergraduate semester timeframe. Assessment efforts demonstrated several learning gains, including students' understanding of CRISPR-Cas9 content, their ability to apply CRISPR-Cas9 gene editing tools using bioinformatics and genetics, their ability to employ elements of experimental design, and improved science communication skills. They also felt a stronger connection to their scientific education and were more likely to continue on a STEM career path. Overall, this project can be used to introduce CRISPR-Cas9 technology to undergraduates using plants in a single-semester laboratory course.

INTRODUCTION

Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) RNA and the nuclease enzyme Cas9 are components of a highly-specific, targeted microbial pathogen defense system (1). Recently, biotechnological advances have allowed research scientists to use CRISPR-Cas9 as a genome-modifying molecular tool in other living systems. This technology can be used in many ways, including editing genes and controlling gene expression (2), and its applications have the potential to affect all of human society, from how we grow our crops (3) to treating diseases (4). A public discourse on this technology is essential for a broader societal understanding of its potential ramifications given its rapid development and the profound ethical concerns

it raises (5). For many, this discussion begins in the college classroom, where students can learn the molecular and genetic underpinnings of the technology, how it is done in a research lab, and its practical applications (6). Because CRISPR-Cas9 technology is new, only a relatively few examples exist of laboratory modules that introduce its concepts to undergraduates. Bhatt and Challa (7) used the course-based undergraduate research experience (CURE) format (8) to study gene function in *Danio rerio* (zebrafish), taking students through the cloning, transformation, and genotyping steps. Anderson (9) applied CRISPR-Cas9 to mammalian cell cultures in a 15-week undergraduate cell biology class, with demonstrated pedagogical success and gene editing efficacy. Similarly, Adame *et al.* (10) created gene deletion lines in *Drosophila melanogaster* (fruit fly) during a one-semester genome engineering laboratory. The students associated with this course were given autonomy to create a target sequence (i.e., a site where the CRISPR-Cas9 system would potentially create a mutation) in genes for which no known mutant *D. melanogaster* existed, introduce the CRISPR-Cas9 gene editing plasmid into the flies, and go through several generational crosses to isolate the mutants. As a class, these students were able to successfully isolate

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three new mutant lines. All of these examples demonstrate that CRISPR-Cas9 technology can be taught within the framework of an undergraduate laboratory course.

With respect to plant applications, CRISPR-Cas9 has been used to improve crop yield, architecture manipulation, modified nutrient usage, disease resistance, and adaptation to stress, as well as in basic science research on *Arabidopsis thaliana* (thale cress) (11). *A. thaliana* is used as a model organism in the research laboratory due to its relatively small size and simple genome and has been utilized for undergraduate course-based projects in molecular biology and genetics (12). However, genetic manipulation experiments in *A. thaliana*, including CRISPR-Cas9 projects, have traditionally been a significant challenge in classroom settings due to their prolonged incubation requirements relative to other non-plant models. Also, CRISPR-Cas9 gene editing-derived phenotypes are not typically robust or fully penetrant until the second transgenic (T_2) generation (11). Although plant growth and seed acquisition speed can be experimentally manipulated, the procedures used to generate T_2 *A. thaliana* plants often takes longer than the standard undergraduate semester. Thus, there is a need to develop engaging plant-based projects for students that enable them to effectively experience the use of CRISPR-Cas9 technology over the course of a single semester.

In this project, our goal was to create a protocol for student-driven genetic manipulation of *A. thaliana* in a single-semester undergraduate laboratory class. This required our schedule to follow a non-linear timeline relative to that in a research setting so that students would obtain T_2 plants before the end of a 14-week (13 lab period), 200-level *Genetics* class (Fig. 1). We evaluated mutational phenotypes that were observable early in the plant life cycle (i.e., the seedling or juvenile phase) so that T_2 plants could be assessed soon after germination. We focused on six *A. thaliana* genes—*Glabrous1*, *Too Many Mouths*, *Hypocotyl5*, *Phosphoglucosyltransferase*, *Scarecrow*, and *Werewolf*—all of which fit this criteria (Table 1). Our analysis of student learning gains revealed modest improvement on the nuts-and-bolts processes of CRISPR-Cas9 but

substantial improvements in general scientific skills, including experimental design and communication/dissemination, as well as overall scientific identity and interest in future additional work in the STEM disciplines.

Intended audience

This project is appropriate for a mid- to upper-level undergraduate laboratory course in genetics, molecular genetics, genetic engineering, or plant physiology.

Learning time

As described, this project occurred over a 14-week semester, consisting of 13 two-and-a-half- to three-hour laboratory periods and a one-week mid-semester break (Fig. 1). Alternative modular schedules can be developed that vary the amount of student engagement depending on instructor goals and time limitations. For example, a three- to five-week project could be developed if the instructors design and create T_2 generation seeds to give to the students at the outset. This method would eliminate the student DNA transformation procedures and T_1 growth time, allowing the class to focus on CRISPR-Cas9-derived phenotypes and inheritance patterns.

Prerequisite student knowledge

The students should have prior exposure to a number of genetic principles often introduced in undergraduate prerequisite courses, including content knowledge such as DNA and RNA structure, the regulation of gene expression, mutation, genetic inheritance patterns, and genetic analysis techniques. It is not necessary for students to have knowledge of the CRISPR-Cas9 system, plant physiology or propagation methods, or genetic transformation principles. They should have some experience with experimental design, such as developing hypotheses, setting up an appropriate experiment, and evaluation of qualitative and

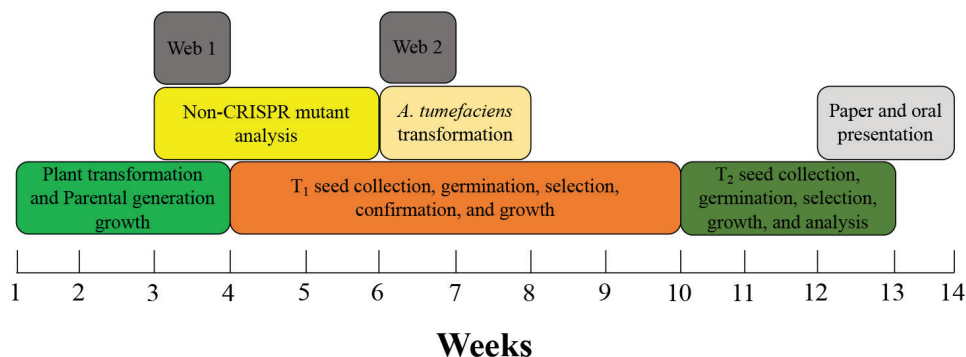


FIGURE 1. The workflow for each weekly unit during a 14-week semester. The two bioinformatic exercises (Appendices 4 and 5) are labeled as Web 1 and Web 2. Our spring break occurred during the 8th week when T_1 transgenic plants were growing. CRISPR = clustered regularly interspaced short palindromic repeat.

quantitative data. Although laboratory experience with practices such as sterile technique and pipet usage are ideal, these techniques can be easily incorporated into class laboratory instruction. Finally, we expected students to read and comprehend the primary literature at a basic level so that they could develop testable hypotheses on the function of their gene. Literature analysis is a skill introduced during our first-year coursework; however, instructors could modify this reliance depending on institutional curricular setup by providing the students more information on gene function (see references in Table 1).

Learning objectives

The following learning objectives (LOs) and their measurable assessments are listed in Table 2. Upon completion of this activity, students will:

1. Demonstrate understanding of the mechanisms of CRISPR-Cas9 gene-editing technology
2. Apply their knowledge of CRISPR-Cas9 to the *A. thaliana* genome using bioinformatic and genetic tools
3. Generate testable hypotheses and utilize them in an experimental system
4. Communicate their scientific findings both orally and in writing
5. Progress in their ability to read and comprehend the primary literature
6. Improve their scientific engagement through several opportunities to experience science identity, project ownership, and resilience (13, 14).

PROCEDURE

This was a multi-unit project occurring once a week for two and a half to three hours over a 14-week semester. Figure 1 details the workflow for each weekly unit. The general instructor materials and those needed to complete each unit separately are provided in Appendix I. The student handouts detailing laboratory procedures are provided as Appendices 3–8.

Instructions for faculty and students

I. Pre-semester instructor preparation. Plants to be used for student transformation in Weeks 1 and 2 were germinated six to eight weeks prior to the start of the semester. We grew an excess number of plants, staggering germination by three to four days so that at least one set of plants would be flowering during the first lab period.

Six *A. thaliana* target genes were selected by the instructors in advance of the semester based on previously published research indicating a seedling- and/or juvenile-stage mutant phenotype that could be assessed by eye or with a microscope (Table 1). The goal was for T₂ analysis to be achieved within one to two weeks of seed germination, coinciding with a period near the end of the semester (Fig. 1). The full-length gene sequences were determined by using The Arabidopsis Information Resource (TAIR) (21). Within the gene sequences, the guide RNA (gRNA) DNA complement (i.e., the DNA sequence expected to be altered in transgenic plants) and protospacer-adjacent motif (PAM) sequences were determined by using the CRISPR-PLANT website according to portal instructions (Table 3) (22). We ordered six unique CRISPR-Cas9 plasmids based on this analysis from the Sigma Aldrich CRISPR Plant division (<https://www.sigmaaldrich.com/catalog/product/sigma/crisprpl?lang=en®ion=US>), each containing one of the unique gRNA/PAM combination sequences. Each plasmid was designed for compatibility with *A. tumefaciens*-mediated transformation of *A. thaliana*, using dicot promoter sequences, bacterial kanamycin selection, and glufosinate-ammonium selection in plants. *A. tumefaciens* transformation of the six plasmids was completed according to Weigel and Glazebrook (23), with transformant confirmation assessed by colony PCR.

2. Plant transformations. *A. tumefaciens*-mediated transformation of *A. thaliana* was done by a floral 'dip' protocol (24) during the first class laboratory period (Appendix 2). These steps were repeated the following week on the same plants. The floral dip transformation integrates plasmid DNA from *A. tumefaciens* into the plant germline genome, where it can then be heritably transferred into subsequent

TABLE 1.
A. thaliana gene targets.

| Gene name | Mutant Phenotype | ABRC Stock | TAIR Gene Annotation | Article Reference |
|---|------------------------|------------|----------------------|-------------------|
| <i>Too Many Mouths (TMM)</i> | Clustered stomata | CS6140 | At1g80080 | 15 |
| <i>Glabrous1 (GL1)</i> | Trichomes absent | CS3126 | At3g27920 | 16 |
| <i>Scarecrow (SCR)</i> | Short root | CS8539 | At3g54220 | 17 |
| <i>Hypocotyl5 (HY5)</i> | Elongated hypocotyl | CS71 | At5g11260 | 18 |
| <i>Werewolf (WER)</i> | Excess root hairs | CS6349 | At5g14750 | 19 |
| <i>Phosphoglucosyltransferase (PGM)</i> | Reduced starch content | n/a | At5g51820 | 20 |

ABRC = Arabidopsis Biological Resource Center; TAIR = The Arabidopsis Information Resource.

TABLE 2.
Course learning objectives and assessments.

| Learning Objective | Assessment |
|---|--|
| 1 Students will demonstrate understanding of the mechanisms of CRISPR-Cas9 gene-editing technology | Pre- and post-content quiz (Appendix 12) |
| 2 Students will apply their knowledge of CRISPR-Cas9 to the <i>A. thaliana</i> genome using bioinformatic and genetic tools | Bioinformatic exercises (Appendices 5 and 6); Final written report and oral presentation (Appendices 10 and 11) |
| 3 Students will generate testable hypotheses and utilize them in an experimental system | Final written report and oral presentation (Appendices 10 and 11); Student identity, resilience, and ownership survey (Fig. 6) |
| 4 Students will communicate their scientific findings both orally and in writing | Final written report and oral presentation (Appendices 10 and 11) |
| 5 Students will progress in their ability to read and comprehend the primary literature | Student identity, resilience, and ownership survey (Fig. 5) |
| 6 Students will develop scientific engagement by emphasizing science identity, project ownership, and resilience | Student identity, resilience, and ownership survey (Figs. 7 and 8) |

CRISPR = clustered regularly interspaced short palindromic repeat.

TABLE 3.
gRNA complement and PAM sequences for each of the six target genes.

| Gene Name | gRNA Sequence (5' to 3') | PAM Sequence |
|-----------|--------------------------|--------------|
| TMM | TCTTCCGGCGCTGAGCGTTC | TGG |
| GLI | ATGGACTATGTTCTTAATCA | TGG |
| SCR | CACGTGCGACTCACGGGACT | TGG |
| HY5 | GATCGGCGACCGGTCAGGAG | CGG |
| WER | ATTAGGCTCCACAAGTTGCT | TGG |
| PGM | TTTCCGTGCTGGTCCTAAGG | TGG |

gRNA = guide RNA; PAM = protospacer-adjacent motif.

generations. The parental 'dipped' plants were then grown to maturity for approximately three additional weeks, after which the seeds were collected.

3. Transgenic plant selection, propagation, and confirmation. Transgenic T₁ and T₂ plants were selected by spraying every other day with 300 μM glufosinate-ammonium (Sigma Aldrich) beginning two weeks after germination on soil (25) (Fig. 2). The presence of transgenic DNA in plants that survived the spray was confirmed by PCR analysis (Appendix 3).

4. Mutant phenotype analysis. The students became more familiar with their predicted mutant phenotype by growing and analyzing other, publicly-available, *A. thaliana* plants with a mutation in their assigned gene (Fig. 1 and Table 1). These steps began in the third laboratory period after the students had read and presented to classmates on the original primary literature article describing the mutant (see references in Table 1), and continued for up to three weeks depending on phenotype. None of these publicly-available



FIGURE 2. Image of TMM T₁ transgenic plant. Note that most of the T₁ generation plants were susceptible to the glufosinate-ammonium spray. Scale equals 1 cm.

lines were generated using CRISPR-Cas9. By doing this early in the semester, each student group could develop a testable hypothesis and experimental procedure to distinguish their mutant phenotype from wild-type. Ultimately, the 'non-CRISPR' mutants served as a basis of comparison for the student-generated T₂ CRISPR mutants, which were isolated at the end of the semester.

5. CRISPR-Cas9 bioinformatic analyses. The students performed two bioinformatic exercises meant to familiarize them with their assigned gene, other genes that have a similar molecular role, and how CRISPR sequences

are identified and targeted (Appendices 4 and 5). The first exercise is done in parallel with their non-CRISPR mutant analysis, whereas the second is done after it is completed (Fig. 1).

6. A. *tumefaciens* transformation. The CRISPR-Cas9 plasmids were transformed into *A. tumefaciens* by the instructors prior to the beginning of the semester; however, we had our students repeat this activity to gain a better hands-on understanding of the transformational process (Appendix 7). The student transgenic colonies were not subsequently used for plant transformation. Colony PCR techniques were performed to confirm plasmid integration into *A. tumefaciens* (Appendix 8).

7. Written and oral communication. A) Each student wrote an individual full-length laboratory report based on their group analysis. An example student submission is provided in Appendix 9, and the instructor grading rubric in Appendix 10. The report included a Title, Introduction, Materials and Methods, Results, Discussion, Tables and Figures, and References section. B) Each group was assigned either a poster or oral presentation during an end-of-semester campus-wide research showcase. The presentation grading rubric (Appendix 11) can be used for either of these formats.

Suggestions for determining student learning

We used several methods to assess student comprehension of project content. A baseline understanding of genetic principles and CRISPR-Cas9 mechanisms was assessed with an in-class content quiz (Appendix 12) administered in the first laboratory period; the same quiz was re-administered in the last laboratory to measure content learning gains. The bioinformatic exercises (Appendices 4 and 5) evaluated the students' ability to comprehend and apply CRISPR-Cas9 technology to a living system. Student comprehension of genetic principles was assessed in the final written and oral communication deliverables (Appendices 10 and 11). Both communication venues also enabled us to measure student understanding of experimental design principles (e.g., developing and testing hypotheses) and their ability to search, read, and comprehend the primary literature.

Another central goal of this module was to provide an engaging and meaningful scientific research experience that allows students to develop scientific identity, project ownership, and resilience. As these traits are vital to producing students who will become the next generation of scientists, we felt that measuring these values was of equal importance to the content-based assessments mentioned above (14). The students were given a pre- and post-project survey based on assessment instruments previously developed and validated as effective in these measures (13, 14). The authors received Institutional Review Board approval from their respective institutions prior to collecting student data.

Safety issues

A. tumefaciens is a BSL1 plant pathogen, and transgenic *A. thaliana* is at a BSL-1P biosafety level. Laboratory biosafety guidelines were followed at the BSL1 level as according to the American Society for Microbiology Guidelines for Biosafety in the Teaching Laboratory (<https://www.asm.org/Guideline/ASM-Guidelines-for-Biosafety-in-Teaching-Laborator>). The MSDS sheets for each of the main chemicals used can be found by searching for the Sigma Aldrich catalog numbers provided in Appendix 1.

DISCUSSION

Field testing

This project was taught concurrently at two primarily undergraduate institutions (PUI). It has now been implemented for two years at each institution in a 200-level *Genetics* course. Between the two institutions, a total of 94 students have participated, in the spring semesters of 2017 and 2018. For the most part, the reactions from students and instructors were typical for a long-term authentic research experience such as the one presented here. Many students were initially overwhelmed by the scope and open-ended nature of the project but were soon able to become more engaged in the material as they appreciated the scaffolded nature of the assignments over the course of the semester and took ownership of their work on a particular mutant set. Since a subset of the class was responsible for the outcomes with each mutant, the students working on each particular mutant were responsible for delivering their portion of the overall data set and thus became more excited as they worked on the project. Instructors, meanwhile, could become overwhelmed by the demands of different student groups simultaneously performing different tasks and having unique questions about their particular mutant line. Like the students, however, the instructors became accustomed to the demands of the project and were able to rely on student lab assistants to help with many of the session-to-session needs of the students.

Evidence of student learning

The content quizzes (Appendix 12) produced a slight improvement in performance between pre-content and post-content administration (Fig. 3), which suggests that our mechanisms of improving student understanding of CRISPR-Cas9 technology (LO 1, Table 2) are modestly effective. In our offerings of this module, we elected to emphasize the development and implementation of long-term research projects over the nuts-and-bolts mechanisms of CRISPR-Cas9. Clearly, this does not need to be a zero-sum choice, and in future offerings, we will devote time in the lecture portions of the courses to provide companion foundational material that will likely further improve the students'

understanding of CRISPR-Cas9 mechanics. Importantly, the student deliverables did provide evidence that they were successful in developing and testing hypotheses after reading the primary literature (LOs 3 and 5, Table 2). For example, students studying the *Hypocotyl5* gene hypothesized that the mutant would have a longer hypocotyl when grown in the light, and they developed a protocol to compare its length in seven-day-old wild-type and CS71 plants (Fig. 4). Another objective of the laboratory unit was for students to successfully complete a genome analysis exercise. Appendix 6 illustrates sample data generated during this exercise and demonstrates students' ability to carry out bioinformatic analyses (LO 2, Table 2). Finally, our project emphasized communication in written and oral formats (LO 4, Table 2). Appendix 9 demonstrates a student's ability to synthesize the entirety of this project into a culminating document.

In contrast to the content assessment, students showed intriguing learning gains as measured by the student identity, ownership, and resilience surveys (LO 6, Table 2) (13, 14). We were interested in monitoring and measuring these characteristics among our students since these factors are

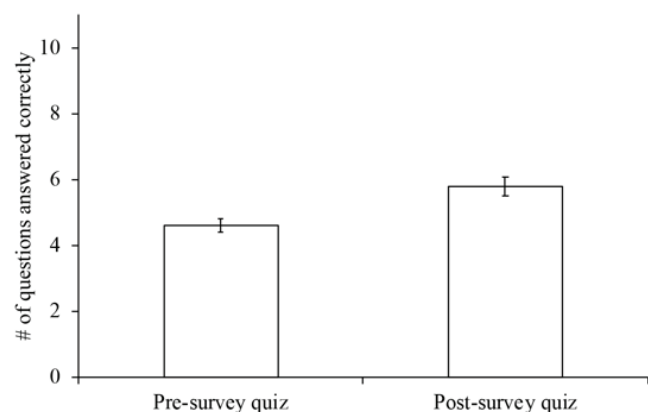


FIGURE 3. Pre- and posttest content assessment results. The quiz had 11 questions (Appendix 12) and was administered in the first and final laboratory periods. Student scores improved from an average of 4.61 ± 0.19 to 5.8 ± 0.29 (by Student's *t*-test, $p < 0.01$, $n = 41$). Bars represent standard error.

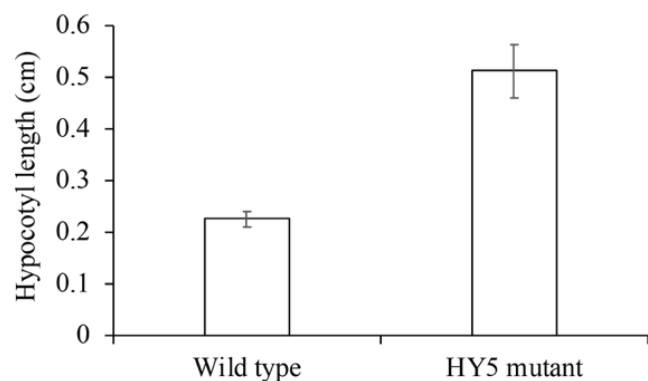


FIGURE 4. Hypocotyl length of seven-day-old wild-type *A. thaliana* and the HY5 mutant (ABRC CS71) plants.

often indicative of whether students will elect to remain in the STEM disciplines in the long term or move on to other interests while still undergraduates (14). As previously mentioned, the students who participated in this project were all in 200-level courses and were thus a self-selected group interested in potential careers in the life or health sciences. However, the surveys revealed notable increases in student confidence in their abilities to engage in such critical scientific tasks as using the primary literature to guide experimental design and implementation (Fig. 5) as well as integrating multiple ideas into central testable hypotheses (Fig. 6). Perhaps most importantly, students also showed gains in their overall sense of belonging in a scientific career (Fig. 7) and in their intentions to ultimately pursue a science-related career (Fig. 8). Thus, the project showed critical gains among student participants in their engagement and enthusiasm for “doing science.” As such engagement is crucial for promoting retention of top scientific talent in STEM career pathways, it is exciting to report that this project provides an authentic scientific experience that can deliver this experience.

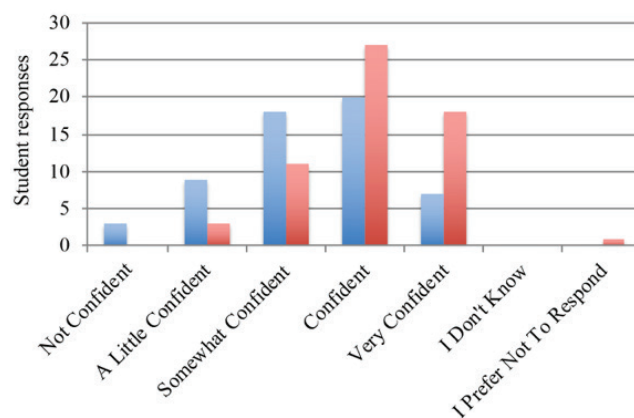


FIGURE 5. Student responses to the question “How confident are you in your ability to use scientific literature and/or reports to guide research?” Pre-assessment results are in blue; post-assessment results are in red.

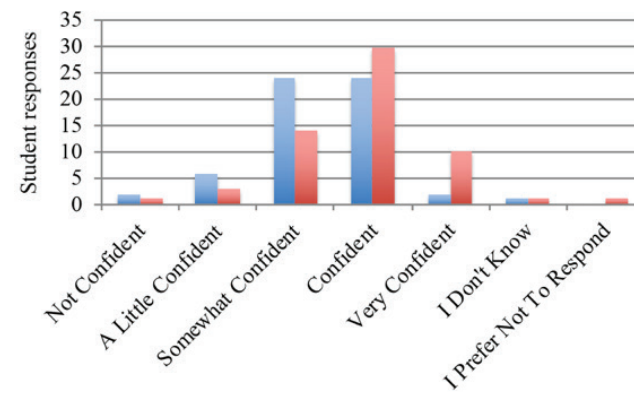


FIGURE 6. Student responses to the question “How confident are you in your ability to develop theories (integrate and coordinate results from multiple studies)?” Pre-assessment results are in blue; post-assessment results are in red.

Possible modifications

The module presented is driven by the use of *A. thaliana* genes with obvious visual phenotypes. It is thus a great way to expose students to CRISPR-Cas9 as a molecular biology tool but, as written, is not a full-fledged CURE. However, the module could easily be revised to allow the students the latitude to select their own targets to manipulate by CRISPR-Cas9 for use in modifying *A. thaliana*. While doing this may be impossible from a time perspective in a single semester, the “labor” could be divided between parallel sections or classes of Genetics/Plant Physiology/etc. or a system could be established in which the molecular constructs of one semester’s class become the material used for *A. thaliana* by another semester’s class. In short, there is great potential to introduce more unknown variables into this module.

As previously stated, alternative schedules can be developed that vary the amount of student engagement. For example, a shorter, non-CURE project could be developed if the instructors design and create T₂ generation seeds on

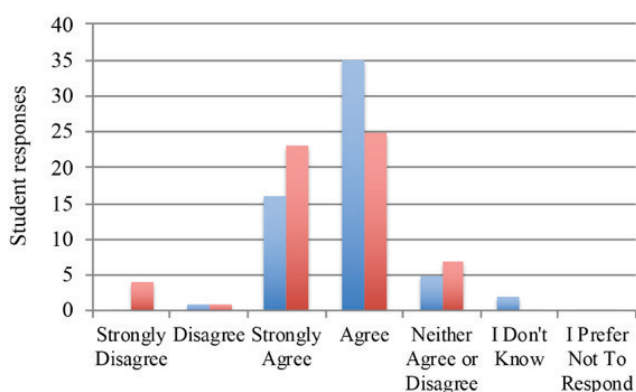


FIGURE 7. Student responses to the statement “Please indicate the extent to which you agree with the following statement: I feel like I belong in the field of science.” Pre-assessment results are in blue; post-assessment results are in red.

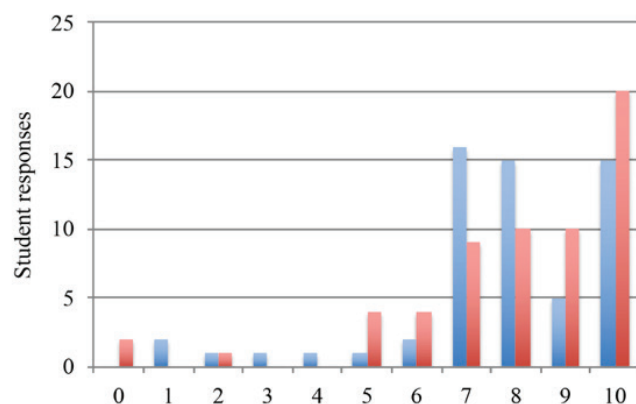


FIGURE 8. Student responses to the question “On a scale from 0 (least) to 10 (most), to what extent do you intend to pursue a science-related career?” Pre-assessment results are in blue; post-assessment results are in red.

their own to give to the students at the outset. This method would eliminate the student DNA transformation procedures and T₁ growth time, allowing the class to focus on CRISPR-Cas9-derived phenotypes and inheritance patterns. This would also afford the students more time to confirm mutations in the target genes by sequencing.

Our future goal is to expand student involvement in the gene target development process, making this project more wholly a CURE. We concede that many materials were developed pre-semester and given to the students, which is a reality of working in a plant model. Recent evidence suggests that T₁ plants can be monitored for mutational phenotypes if grown under variable heat stress (26). By doing so, the students could have a larger role in CRISPR-Cas9 target choice and development, as well as in plasmid construction and transformation. These elements could be done in the first half of the semester, with plant transformation near the mid-point and T₁ plant genetic analysis at the end.

SUPPLEMENTAL MATERIALS

- Appendix 1: Materials
- Appendix 2: Plant transformation protocol
- Appendix 3: Plant DNA isolation and PCR amplification protocol
- Appendix 4: Bioinformatics exercise I
- Appendix 5: Bioinformatics exercise II
- Appendix 6: Bioinformatics exercise I – student example
- Appendix 7: *Agrobacterium tumefaciens* transformation protocol
- Appendix 8: *Agrobacterium tumefaciens* colony PCR protocol
- Appendix 9: Final written paper – student example
- Appendix 10: Final written paper rubric
- Appendix 11: Oral presentation rubric
- Appendix 12: Pre- and post-content assessment survey

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