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Mutant Millets: Phenotype to genotype using the green foxtail millet (*Setaria viridis*)

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Abstract

The Mutant Millets program takes authentic, discovery research in modern agriculture into undergraduate laboratories using a phenotyping platform of mutant green foxtail millet (*Setaria viridis*) families. Green foxtail millet is a small, fast-growing grass related to several economically important cereals such as corn, sorghum, and sugarcane. Currently, it is being used as a model plant for engineering C_4 photosynthetic traits into C_3 plants to improve photosynthesis, carbon allocation, biomass gains, and eventually, enhancement of biofuel production. Through the Mutant Millets CURE (Course-based Undergraduate Research Experience) program, students partner with Danforth Plant Science Center (DDPSC) scientists to enrich their scientific knowledge, phenotyping and research skills by engaging in cutting edge plant science research. Students are provided with seed from wild type and a chemically mutagenized population of *S. viridis* at the M2 generation, with unknown mutations. Students grow the plants from seed to seed during the course of a semester in order to conduct genetic screens for mutations affecting plant morphology, part of gene discovery using forward genetic screens. Students are challenged to apply their learning to the process of mutant identification as they collect and synthesize data on plant traits such as seed germination, plant height, tillering, panicle emergence, leaf color, seed set, and seed quality. Environmental growth conditions, such as temperature, relative humidity, light intensity, and watering frequency, are recorded to emphasize the importance of favorable and standardized growth conditions. Once students identify a mutant with a visible phenotype, they can proceed to elucidate the inheritance and segregation patterns of the causative gene for the identified mutant, a critical step towards gene mapping. These data are shared through the Mutant Millets website and used by scientists to further our understanding of plant biology and to advance crop improvement efforts.

Keywords: CURE, Phenotyping, Gene Discovery, *Setaria viridis*, Mutations

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INTRODUCTION

Current efforts to improve undergraduate STEM education include the adoption of Course-based Undergraduate Research Experiences (CUREs) that have been inserted into college curricula and designed to successfully engage large numbers of undergraduate students in scientific research. CUREs are an evidence-based pedagogy that engages students in the use of scientific practices, collaboration, iteration, discovery, and research that has relevance outside of the classroom (Auchincloss et al. 2014; Provost et al. 2019). The benefits of CUREs for undergraduate students are well documented and include, stimulated interest in STEM fields and careers (Siritunga et al. 2011; Shaffer et al. 2014; Kerr et al. 2016; Mraz-Craig et al. 2018), STEM career interest clarification and retention (Shelby 2019; Ward et al. 2014). CUREs are also known to contribute to students developing and maintaining a science and STEM identity (Mraz-Craig et al. 2018), as well as gains in knowledge of STEM (Shaffer et al. 2010; Siritunga et al. 2011; Wolkow et al. 2014; 2019). Undergraduate students engaged in CUREs have contributed to the scientific enterprise while gaining skills and scientific competence (Shelby, 2019; Ward et al. 2014; Sweat et al. 2018), as well as improved science practice competencies and experimental design skills (Shelby, 2019; Ward et al. 2014; Peteroy-Kelley et al. 2017; Laungani et al. 2018).

CUREs also benefit instructors by increasing their academic research productivity through student-initiated research and student-authored manuscripts (Kowalski *et al.*, 2016; Sweat *et al.*, 2018). Moreover, they have also been reported to enrich faculty research programs' broader impacts, providing opportunities to obtain grant funding (Shortlidge *et al.*, 2016; Cascella and Jez, 2018).

In this paper, we present the Mutant Millets CURE, developed to provide undergraduate students with the opportunities to engage in authentic research and use scientific practices to make new discoveries in plant biology that are relevant to current research in the broad scientific community. Scientists at the Donald Danforth Plant Science Center (DDPSC) and elsewhere are using the green foxtail millet (*Setaria viridis*), the C_4 photosynthetic pathway model plant, to advance scientific discovery and crop improvement efforts in genetically related C_4 crops, such as maize (Huang et al 2017; Yang et al 2018). *Setaria viridis* is popular because of its small stature, low chromosome number, recently sequenced genome and the availability of new genetic resources such as a NMU mutant population (Brutnell et al., 2010; Bennetzen et al., 2012; Huang et al., 2016). The DDPSC has a rich resource of over 15,000 mutant families at the M2 generation that have yet to be screened for phenotypes. The Mutant Millets project provides faculty with training and resources at no cost to conduct forward genetic screens of mutant *Setaria viridis* families in their laboratories with undergraduates, to identify above ground phenotypes of interest to current scientific research advances as detailed in this paper. Participant undergraduate students also have the opportunity to determine the causative genes of their newly identified mutant phenotypes using protocols such as bulked segregant analysis (Abe et al. 2012).

The Mutant Millets CURE is adaptable to many different undergraduate courses including introduction to biology, botany, and genetics and to diverse majors in the life sciences such as biology, ecology and environmental science. The instructor is able to emphasize aspects of the protocol to meet the needs of their curriculum. The time commitment also has flexibility. The screening of the M2 generation of *Setaria viridis* is a semester-long commitment. If undergraduates take the opportunity to determine the causative genes of the newly identified mutant phenotype, the time commitment would be extended to multiple semesters. In that case, the instructor can choose to adapt the Mutant Millets CURE through multiple courses. Before starting the Mutant Millets CURE, the instructor and students should be familiar with the concept and goals of a CURE and how it is different than other research projects they might have conducted in undergraduate courses. In addition, they should understand the importance of research with *Setaria viridis* not only for classroom purposes but within the plant science field as well. The DDPSC is supportive of the Mutant Millets CURE and provides most of the materials and resources required for screening of the M2 generation to participating teachers and students.

STUDENT OUTLINE

Objectives

- Conduct genetic screens for mutations affecting plant morphology (mutant phenotypes) of *Setaria viridis* as part of gene discovery using forward genetics and apply the process of mutant identification to synthesize data on plant traits and plant physiology.
- Determine the inheritance pattern of the causative gene for the identified mutants using the principles of Mendelian inheritance.

Introduction

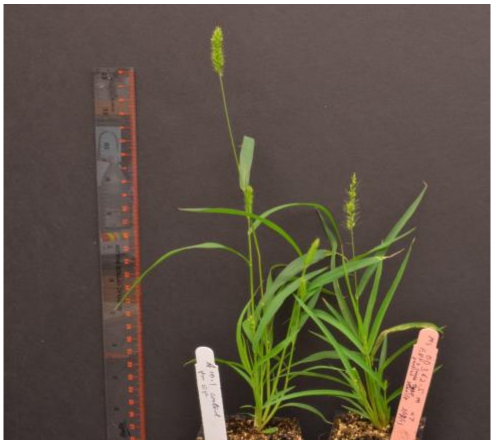

Figure 1: *Setaria viridis* A10.1 the wild-type reference (left) and mutant (right)

In this project, we will use the green foxtail millet (*Setaria viridis*) (Figure 1) as a model plant for studying important food crops such as maize, sorghum and sugar cane and potential biofuel grasses, as well as for the development of new plant photosynthesis technologies. *Setaria viridis* is native to China, with naturalized phenotypically diverse populations widely distributed in North America (Huang and Feldman, 2016). *Setaria viridis* is a good model grass species for plant research because:

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- It is very easy to grow and study in the lab(drought tolerant).
- It is a self-pollinating annual, does not require a pollinator to produce seeds- it can self-fertilize.
- It is related to economically important C_4 plants like corn and sugarcane (food crops) and switch grass (biofuel crop).
- It has a short generation time (Seed to seed in 16 weeks or less)
- Each plant produces a lot of seeds (~13,000 seeds per plant)
- Entire genome is sequenced, hence good for doing comparative genomics with other cereal grasses

We want to see what happens to this plant's phenotype (the observable characteristics or traits of an organism) when its genetic material is altered. You have the opportunity to grow and screen a population of *Setaria* plants for mutant phenotypes (variation from the normal phenotype of the plant), and share it with other scientists engaged in plant science research with *S. viridis*. In the lab at the Donald Danforth Plant Science Center, wild type *S. viridis* A10.1 seeds were treated with the mutagenic chemical N-nitroso-N-methylurea (NMU) at different concentrations (20mM or 30mM) and varied durations, 2h, 3h, 4h, respectively (Jiang, Huang and Brutnell 2017). NMU is an alkylating agent that transfers its methyl group to nucleic acids, which can lead to AT to GC transition mutations in the genome (Figure 2).

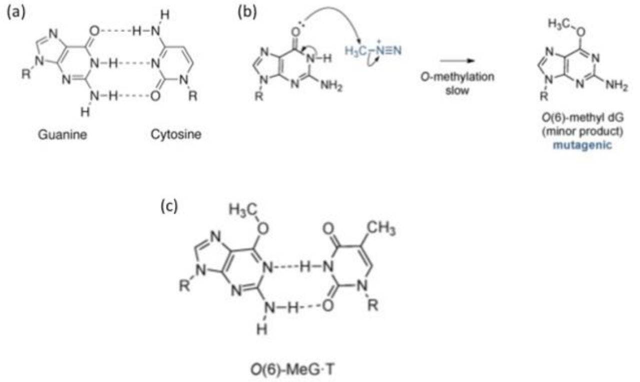


Figure 2. (a) Normal G-C base pairing. Note three hydrogen bonds between the bases (represented by dotted lines). (b) When treated with NMU, the NMU may transfer a methyl group (CH_3) to the oxygen atom in guanine. This prevents the oxygen atom from forming a hydrogen bond with its complementary base, cytosine. (c) Therefore, guanine mistakenly forms two hydrogen bonds with a complementary base, pairing with thymine instead.

Where this mutation occurs in the genome of the treated seed is random, and may occur anywhere in the 423+ million base pairs of the *Setaria* genome. The mutation may occur in non-essential genes, in redundant genes, or in a gene that works with other genes (e.g., part of a polygenic trait that is controlled by multiple genes) and result in a silent mutation (no observable change in phenotype). Alternatively, the mutation could result in a new phenotype by occurring in an important place in a gene.

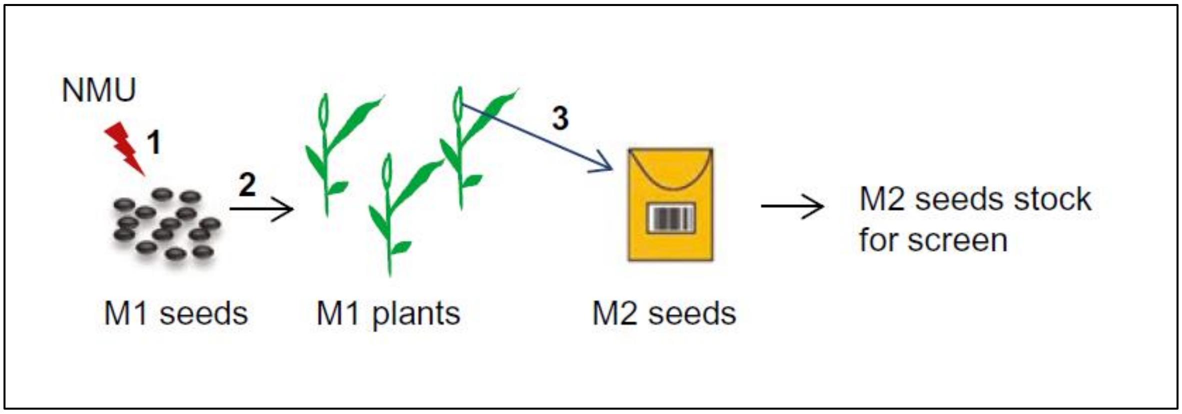


Figure 3. Process of creating the NMU mutant seed stock you will be planting (Adapted from Jiang, Huang and Brutnell 2017). M1 generation = The plants grown from the mutagenized seeds, which will be heterogeneous and chimeric for induced mutations; M2= The second generation of plants after the mutagenesis event (grown from the seeds harvested off of the M1 plants)

Many different mutant phenotypes can be interesting to scientists. Changes in pigmentation (light green or albino plants) may relate to altered photosynthetic pathways, and these mutants may be used to identify genes underlying the pathway of C_4 photosynthesis. A mutant that has an increased number of tillers ("heavy tillering") may result in increased biomass of the plant, which has potential applications to biofuel development. Eventually it is hoped that better food crops and biofuel sources will be developed from what we learn from this plant.

The <u>genotype</u> of an organism is the inherited instructions it carries within its genetic code. Not all organisms with the same genotype look or act the same way because appearance and behavior are influenced by environmental and developmental conditions. Likewise, not all organisms that look alike necessarily have the same genotype.

Phenotypic variation (due to underlying heritable genetic variation) is a fundamental prerequisite for evolution by natural selection. It is the living organism as a whole that contributes (or not) to the next generation, so natural selection affects the genetic structure of a population indirectly via the contribution of phenotypes. Discuss how the interaction between genotype and phenotype has often been conceptualized by the following relationship:

genotype (G) + environment (E) + genotype & environment interactions (GE) → phenotype (P)

Methods and Data Collection

Screening M2 plants for mutations affecting plant morphology using forward genetics

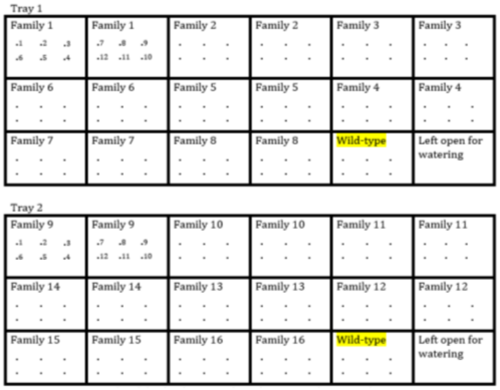
Materials required

- Trays ($2f^2$) with built-in cells
- Metro Mix 360 Potting Soil

- Spray Mister/Spray Bottle
- Seeds (in an envelope, they are exact numbers, no extras)
- Light Bank & Light Bulbs
- Thermometer
- Hydrometer
- Light Meter
- Jack's Classic Fertilizer
- Water Jug
- Timers
- Labels and Permanent Marker Pens
- Bags
- Empty Envelopes (for seed collection)
- Twist Ties
- Forceps
- Plastic Beaker
- Plastic Lid

Planting

1. To prepare trays for planting *S .viridis*, fill all cells within trays with a growth media such as Metro Mix 360 potting soil leaving one in the far right corner of the tray empty for watering. Each tray will have plants from eight M2 families of *S. viridis*. In each tray, one cell will be planted with wild type plants, A10.1, and the cell next to it will be left unplanted for watering (see figure 4). One forward genetics screen of 16 M2 families will be composed of two trays with eight families in each tray. The number of trays you have will depend on your space provisions.
2. For each of the sixteen M2 mutant families, 12 seeds will be planted and each seed numbered according to the lay out in figure 4. Each mutant family will be allocated 2 cells, and each cell planted with 6 seeds (2 cells x 6 seeds each = 12 seeds). Since each tray consists of 16 cells allocated to 8 mutant families and 1 cell allocated to wild type; two trays will contain (12 seeds x 16 families) =192 mutant plants and 6 seeds x 2 cells = 12 wild type plants (figure 4).



Tray 1

Family 1 .1 .2 .3 .6 .5 .4	Family 1 .7 .8 .9 .12 .11 .10	Family 2	Family 2	Family 3	Family 3
Family 6	Family 6	Family 5	Family 5	Family 4	Family 4
Family 7	Family 7	Family 8	Family 8	Wild-type	Left open for watering

Tray 2

Family 9 .1 .2 .3 .6 .5 .4	Family 9 .7 .8 .9 .12 .11 .10	Family 10	Family 10	Family 11	Family 11
Family 14	Family 14	Family 13	Family 13	Family 12	Family 12
Family 15	Family 15	Family 16	Family 16	Wild-type	Left open for watering

Figure 4: An example of the final set up after planting in the 2-tray layout

3. Using tags label tray cells with the mutant family number or A10.1 the wild type, the generation (will start with an "M," for mutant families only), the date of planting and your team/class name. Assign a pair of same color tags to a single family since one family is spread across two cells in each tray as shown (figure 5).
4. Using the empty spot, bottom water the trays with a sufficient amount of water. The cells are ready for planting when the soil on top appears wet/moist. Germinating seeds require wet/moist soil.
5. Examine the seeds prior to planting and be sure to only use the viable (black/grey) seeds, not the empty tan/white colored ones. Using a 6-hole stencil, make 6 holes per cell with a toothpick. Use forceps to plant 1 seed per hole at a depth equal to a pencil eraser. Using a plastic beaker, sprinkle a light layer of soil (about 0.5 mm) over the seeds. Mist the cells with water so that you do not displace soil or the seeds.

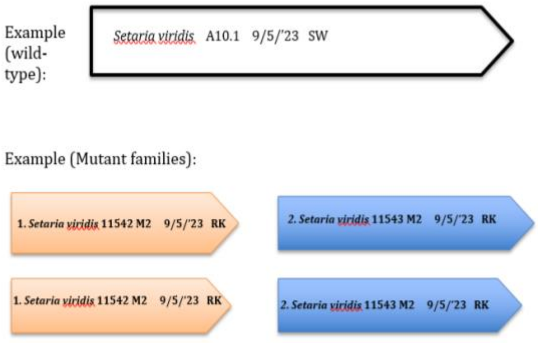
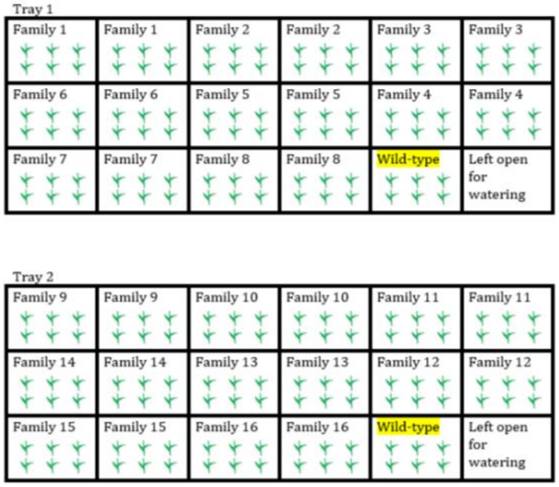


Figure 5: Sample labels

6. When the entire tray of cells has been planted, add extra water (if the tray doesn't have any standing water) to the bottom of the tray, using the empty spot, so that the water level is just below the "ribs" on the bottom of the tray (less than 1 cm of water). You want the cells to have plenty of moisture for germination. **Do not** supply too much water into the planting trays as *Setaria* prefers pretty dry soil and warm temperatures.
7. *Setaria viridis* has different light requirements than other plants, so make sure to use light bulbs providing >8000 lux light intensity. Set the lights on a timer. You should use 16 hours of light/8 hours of darkness. Determine a weekly schedule for recording of environmental variables (light, humidity, temperature).



Tray 1

Family 1	Family 1	Family 2	Family 2	Family 3	Family 3
Family 6	Family 6	Family 5	Family 5	Family 4	Family 4
Family 7	Family 7	Family 8	Family 8	Wild-type	Left open for watering

Tray 2

Family 9	Family 9	Family 10	Family 10	Family 11	Family 11
Family 14	Family 14	Family 13	Family 13	Family 12	Family 12
Family 15	Family 15	Family 16	Family 16	Wild-type	Left open for watering

Figure 6:A sample set up of plants per tray in the 2-tray layout with 100% germination

8. Cover the trays with clear plastic lids until germinating seedlings emerge from the soil. The lid helps keep the humidity high to encourage germination. After the plants have emerged, loosen the lid at one end and slide the dome about 3 inches

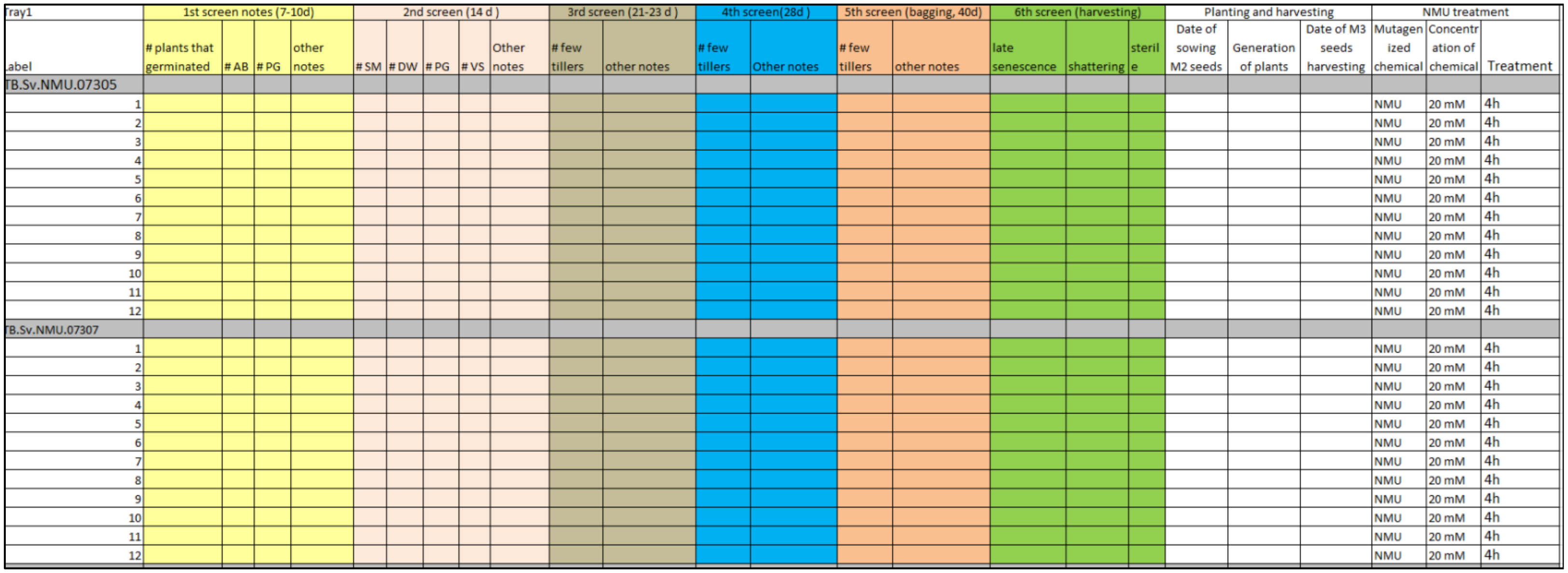
to *gradually* expose the plants to your growth room environment for one day, remove the clear dome entirely the following day.

9. Each cell will have a maximum of 6 plants if the germination rate is 100%. Low germination rates are common in M2 screens. Germination must be recorded for all 192 seeds planted in the 2-tray layout.
10. Determine a watering and fertilizing schedule, Plan out with your students when the plants will be watered and who will do this. Make sure to account for weekends and holidays. To fertilize, you should mix ½ tsp of Jack's Classic Fertilizer (20:20:20 NPK) per gallon of water (0.2 tsp per 1.5 L of water). Use this fertilized water to water the trays once every other week. Help your students create a fertilizing schedule.

Phenotypic evaluation and recording

Record the phenotypes indicated on the data collection sheet (figure 8) for each individual plant from each M2 family planted, at the various growth stages indicated between sowing to harvesting. All phenotype observed will be compared with the A10.1 in each tray (See figure 7 for albino mutant). After germination, observe plants weekly, students can fill out the data sheet (see figure 8), or create a lab journal. It may take up to 15 weeks before seed collection.

Figure 7: M2 families with albino mutant plants in a sample screen



Tray1	1st screen notes (7-10d)				2nd screen (14 d)					3rd screen (21-23 d)		4th screen(28d)		5th screen (bagging, 40d)		6th screen (harvesting)			Planting and harvesting			NMU treatment		
abel	# plants that germinated	# AB	# PG	other notes	# SM	# DW	# PG	# VS	Other notes	# few tillers	other notes	# few tillers	Other notes	# few tillers	other notes	late senescence	shattering	sterile	Date of sowing M2 seeds	Generation of plants	Date of M3 seeds harvesting	Mutagenized chemical	Concentration of chemical	Treatment
TB.Sv.NMU.07305																								
1																						NMU	20 mM	4h
2																						NMU	20 mM	4h
3																						NMU	20 mM	4h
4																						NMU	20 mM	4h
5																						NMU	20 mM	4h
6																						NMU	20 mM	4h
7																						NMU	20 mM	4h
8																						NMU	20 mM	4h
9																						NMU	20 mM	4h
10																						NMU	20 mM	4h
11																						NMU	20 mM	4h
12																						NMU	20 mM	4h
TB.Sv.NMU.07307																								
1																						NMU	20 mM	4h
2																						NMU	20 mM	4h
3																						NMU	20 mM	4h
4																						NMU	20 mM	4h
5																						NMU	20 mM	4h
6																						NMU	20 mM	4h
7																						NMU	20 mM	4h
8																						NMU	20 mM	4h
9																						NMU	20 mM	4h
10																						NMU	20 mM	4h
11																						NMU	20 mM	4h
12																						NMU	20 mM	4h

Figure 7: Sample data collection sheet for scoring phenotypes of M2 families
PG= Pale green, AB =Albino, d=days, SM= small, DW=dwarf, PG=pale green, VS= Virescent

Table 1: Data collection

Timing	**Activity**
3-5 days after planting	Check on plants daily and record the date of germination. For the first week after the plants emerge, try to observe them daily for mutations such as albino color.
3 weeks after planting (*Time to flowering may vary due to different classroom conditions*).	Flag individual mutants that need to be harvested with below information: 1. Name: Mutant family number e.g. 012345.3m;4m, 2. Mutant phenotype of the individual
4 -5weeks after planting	Start bagging individual mutant plants when needed Score panicle phenotype
Harvesting: 6-8 weeks after planting:	Score shattering at seed harvesting Score late senescence phenotype
Seed harvesting:	Label the M3 seeds as ● i. NMU_00001.2P: seeds from pool of individuals that don’t have obvious phenotypes in each family ● ii. NMU_00001.3m: seeds from one individual that has a phenotype of interest ● iii. NMU_00001.4m: seeds from one individual that has another phenotype of interest that was different from .3m ● iv. NMU_00001.3mp: seeds from a pool of individuals that have the same mutant phenotype as .3m.
Post harvest	Pool and discuss all student observations and recorded data. Initiate a discussion on plant physiology, environmental factors, population genetics and variability.

Flowering and seed collection

I. *S. viridis* plants need to be bagged two weeks after flowering to prevent seed loss due to shattering. Look for a few dark seeds to appear on the tallest panicle to start bagging plants. To bag the plants, invert a porous bread bag over each plant, so that the closed end is on top, leaving extra space at the top of the bag so the plant has room to grow. Put the individual tags with family number and plant number into the bag with the plant so they do not get lost. Use a twist tie to secure the bag around the plant near the soil line.

II. When the plants have matured and begin to undergo senescence (the flag leaf /uppermost leaf on the culm, begins to yellow or brown), you should stop watering and let the plants dry down. When they are dry and mostly brown you are ready to collect seeds.

III. To collect seeds, cut the plant at its base beneath the twist tie and roll the bag between your hands and using fingers to break apart the panicles, to release the seeds. You will be left with a large bag with a lot of tiny seeds at the bottom. Place the seeds into a clean seed envelope and label using the guide in Table 1. For mutant plants, include the generation. This should start with an M, and the number should be 1 greater than the seeds you planted, since this is the next generation. (i.e. if you planted M2 seeds, then on your seed packet of seeds collected from that plant, you would write M3). **The seeds cannot be used if you forget this step!** When you are finished collecting seeds, all of your *Setaria* plant biomass should be autoclaved before discarding.

Synthesis and Communication of results

Upon identification and documentation of mutant phenotypes, students are encouraged to engage in a synthesis discussion of plant traits and plant physiology for example an observation of albino mutants and their short-lived nature could lead to a discussion of leaf color, chlorophyll biosynthesis, photosynthesis and seed physiology. Students can present their new discoveries from the genetic and phenotypic screens of mutant families by preparing a poster presentation, lab reports, data write-ups, or through oral presentations (see samples in the appendix).

Once the mutant plants have been identified, to further understand what gene (a segment of DNA that might determine a phenotype), or genes, might be important to this mutant phenotype, will necessitate an examination of how the genotype of the mutant plant differs from the genotype of the wild type plant, a determination of the segregation patterns of the gene and conducting of experiments to determine what roles those gene(s) may play as described below.

Segregation ratios of the phenotypes in the identified mutant

List of equipment and supplies needed for a class of 25 students.

- Two sq ft trays with built-in cells x 8
- Metro Mix 360 Potting Soil
- Four Spray Mister/Spray Bottles
- Seeds in an envelope individually packed by family (12 seeds per family x 64 families, exact numbers, no extras)
- Two Light Banks & Eight Light Bulbs (Each light bank should supply a light intensity ≥ 8000 lux)
- Thermometer (1or 2)
- Hydrometer (1or 2)
- Light Meter (1or2)
- 10lbs Jack's Classic Fertilizer (20:20:20 NPK)
- Water Jug (1or2)
- Two Timers
- Labels and Permanent Marker Pens
- Plant waste Bags
- Empty Envelopes (for seed collection) x 70
- Twist Ties
- Forceps
- Plastic Beaker
- Plastic Lids
- Bread bags x 70

1. Plant M3 seeds of the **identified mutant of interest** on growth media such as Metro-Mix360 (Hummert International, Earth City, MO) at 31 °C/day, 22 °C/night with 50% relative humidity under 16-hr light/8-hr dark along with *Setaria viridis* A10.1 (*S. viridis* A10.1).

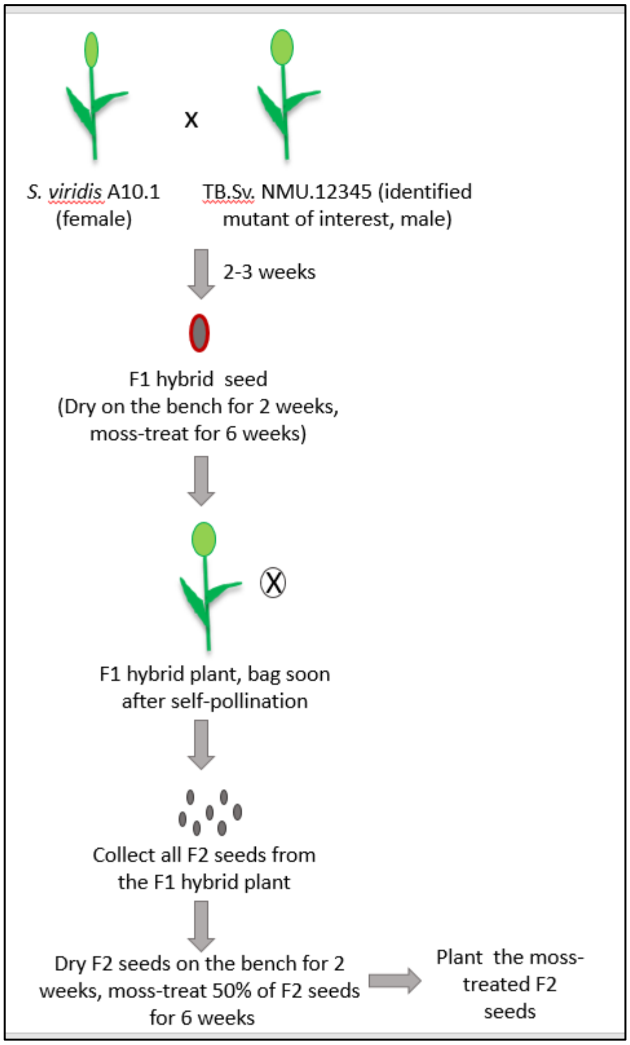


Figure 5: Determining the segregation pattern for the identified mutant phenotype of interest (moss treatment helps break seed dormancy)

2. Score each plant for germination, days to tiller, days to panicle emergence and panicle type.
3. Identified mutant plants with the phenotype of interest will be used as the pollen donor in an outcross with *S. viridis* A10.1 using established protocols (Jiang et al.2013).
4. F1 hybrid seeds will be harvested and planted, the F1 plant phenotype will be recorded (the F1 phenotype will indicate whether the gene underlying the trait of interest is dominant or recessive.
5. The F1 plants will be selfed (self-pollinated) to generate F2 seeds (See figure 5).
6. Harvested F2 seeds will serve as the F2 segregating population (See figure 5).
7. 300 F2 seeds of *Setaria viridis* A10.1 X identified mutant genetic outcross will be sown on growth media such as Metro-Mix360 (Hummert International, Earth City, MO) at 31 °C/day, 22 °C/night with 50% relative humidity under 16-hr light/8-hr dark.
8. Record the total number of seeds planted.
9. Record germination at 7 days post-sowing, record the total number of seeds germinated.
10. For each F2 plant, score days to germinate (DTG), days to tiller (DTT), days to panicle emergence (DPE) and panicle type/phenotype (PT)
11. Calculate the percentage of plants with Wild type like (WT-like), and those with the identified mutant phenotypes using the total number of seeds germinated as the denominator.
12. The ratios of segregating phenotypes of the identified mutant of interest versus wild type should indicate if the phenotype is controlled by one gene or two genes (For one gene, 3:1 or for two genes 9:3:3:1)
13. If the above ratios are not observed in the F2 population, it is likely that the identified mutant phenotype of interest is due to dosage effect.

14. To determine the causative gene of the identified mutant phenotype the molecular protocols such as below can be undertaken.
    I. Quantification and pooling of DNA samples
    II. DNA-seq library construction and sequencing
    III. DNA sequence data analysis and SNP mapping

Assessment of student outcomes:

Several diverse tools can be utilized to assess student outcomes from the Mutant Millets-Phenotype to genotype using the green foxtail millet (*Setaria viridis*) CURE, they include

- Writing assignments (See Appendix 1)
- Poster presentations (Appendix 2)
- The Laboratory Course Assessment Survey (LCAS) (Corwin et al. 2015), a 17-item survey instrument, that measures students' perceptions of three design features of biology lab courses: 1) collaboration, 2) discovery and relevance, and 3) iteration. (Appendix 3)
- The Project ownership Survey an instrument designed to measure students' perceptions and experiences of elements of their undergraduate research experiences (Hanauer and Dolan, 2014)

Discussion

The Mutant Millets laboratory is a great opportunity for students to participate in cutting edge research and authentic science, in partnership with the largest Plant Science research facility in the world, the Donald Danforth Plant Science Center. Scientists at various institutions including DDPSC are currently utilizing the C4 photosynthesis model plant green foxtail millet (*Setaria. viridis*), related to multiple important cereal crops including maize (*Zea mays*), sorghum (*Sorghum bicolor*), foxtail millet (*S. italica*) to advance crop improvement efforts in photosynthesis, biofuel production, plant adaptation to stress. These efforts require large data sets of known mutants that can be characterized to hasten gene discovery for crop improvement and advance our understanding of gene function. Undergraduate students can contribute to these efforts by partnering with scientific researchers to screen and phenotype chemically mutagenized families of *Setaria viridis* at the M2 generation, available at the Donald Danforth Plant Science Center, to reveal new mutants and potential new information about known and unknown causative genes. *Setaria viridis* is a small, fast growing annual, with a fully sequenced genome, and a 12 to15 week long growth cycle under classroom conditions, making it ideal for undergraduate students' semester long laboratory classes. Through the Mutant Millets CURE undergraduate students have opportunities to apply concepts acquired from multiple courses including soils, botany, genetics, biology, statistics and plant science to hands-on research with *S.*

viridis as they plant, observe, nurture plants, collect data, analyze, compare data and characterize their observed mutants for their physiological significance. Students have opportunities for authentic research, discovery of new knowledge, iteration, and collaboration and can design their own questions to further investigations of their newly discovered mutants, conduct additional experiments to characterize their mutants and to map their causative genes.

Multiple undergraduate institutions have successfully implemented the Mutant Millets CURE since its launch in Spring 2016 and continue to express interest in the opportunity. Student outcomes measured using the Laboratory Course Assessments Survey (Corwin 2015) have included students having the opportunity to collaborate through reflecting on learning and troubleshooting challenges collaboratively (see appendix 3). Students also reported having opportunities for discovery through formulating their own research questions and hypothesis as well as generating novel results that are of interest to the scientific community. Finally, students reported having opportunities to practice iteration through collecting and analyzing data to address new questions and changing the methods of an investigation (appendix 3).

NOTES FOR THE INSTRUCTOR

When screening M2 mutant families, only 1 seed is planted per hole. This is because planting multiple seeds per hole will require thinning out of extra seedlings. Since the genetic screen is based on visible phenotypes, thinning out biases the genetic screen. It also skews the ratio between plants with phenotypes of interest and plants with no phenotype within a particular M2 family. Therefore, when you are screening M2 mutant families, it is strongly advised that you plant one seed per hole. It is very likely that you may come across poor germination or no germination at all in certain M2 mutant families. This is a common observation and is acceptable in screening of M2 seeds. Please take records of the M2 families with poor/no germination. This is valuable information.

How you divide the plants between your students is up to you and will depend on the number of trays you have space for, and the number of students participating. It is not recommended to assign a student or student pair to one mutant family. Many students will feel disappointed if the mutant family they are responsible for dies, and this is more likely to happen with mutant families with seedling lethal mutations. For example, a mutation you may observe is an albino plant. These plants will die a few days after emerging, and the student will no longer have a plant, when other students do. Students in other classes could also observe the same mutant family, doubling up on data collection for that family. Alternatively, student groups could be assigned ½ of a mutant family tray and can compare and compile their data with the group responsible for the other ½ of the tray. This would reduce the amount that the plants are handled. All students should be observing the wild type plants too, so they know what to compare their mutant plants to.

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About the Authors

Ruth Kaggwa PhD. is a Senior Research Scientist and Principal Investigator at the Donald Danforth Plant Science Center and has been involved in the Mutant Millets CURE project since its inception, training instructors and students to conduct forward genetics screens with *Setaria viridis*.

Ashley Kass is the project manager of the Authentic Research Experience program at the Donald Danforth which includes the administration of the Mutant Millets CURE project.

Alisa Hove Ph.D is an Associate Professor of Biology at Warren Wilson College, where she teaches a variety of plant science courses, including General Botany, Plant Ecology, and Plant Physiology. Her research focuses on ecological genetics in wild plant and animal populations and developing effective ways to integrate authentic research into undergraduate classrooms. Alisa implemented the MutantMillets CURE with her undergraduate class during the Fall 2020 semester.

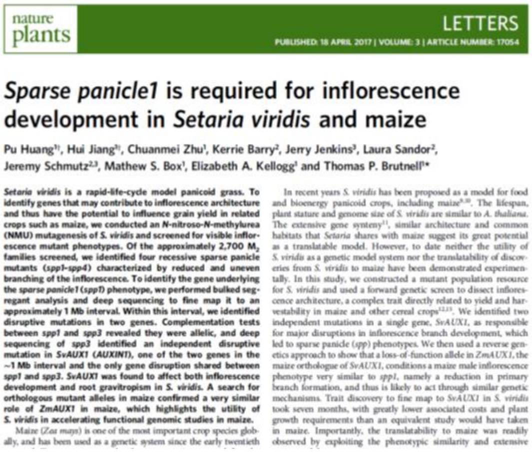
Hui Jiang PHD. has been involved with the Mutant Millets project since its inception, contributing greatly to the genetic resources' methodologies and protocols. She currently works on a project using photosynthesis, as well as mapping genes that are involved into C4 photosynthesis, using *Setaria viridis* as a model.

APPENDIX A

Paper Summary Writing Assignment designed by Co-author A. Hove

Students completed this assignment in advance of a journal article discussion that occurred in class.

Assignment: Please read the paper by Huang et al. (2017). This is a primary article, which means that the authors are reporting new data for the first time and sharing their research methods. Primary articles are generally aimed at an audience of experts and often contain a lot of discipline-specific terminology. This paper is a direct product of the Danforth Plant Science Center’s Mutant Millets Project and includes a great deal molecular genetics and genomics vocabulary.



nature plants

LETTERS

PUBLISHED: 18 APRIL 2017 | VOLUME: 3 | ARTICLE NUMBER: 17054

Sparse panicle1 is required for inflorescence development in *Setaria viridis* and maize

Pu Huang[1†], Hui Jiang[1†], Chuanmei Zhu[1], Kerrie Barry[2], Jerry Jenkins[3], Laura Sandor[2], Jeremy Schmutz[2,3], Mathew S. Box[1], Elizabeth A. Kellogg[1] and Thomas P. Brutnell[1*]

Setaria viridis **is a rapid-life-cycle model panicoid grass. To identify genes that may contribute to inflorescence architecture and thus have the potential to influence grain yield in related crops such as maize, we conducted an *N*-nitroso-*N*-methylurea (NMU) mutagenesis of *S. viridis* and screened for visible inflorescence mutant phenotypes. Of the approximately 2,700 M_2 families screened, we identified four recessive sparse panicle mutants (*spp1*-*spp4*) characterized by reduced and uneven branching of the inflorescence. To identify the gene underlying the *sparse panicle1* (*spp1*) phenotype, we performed bulked segregant analysis and deep sequencing to fine map it to an approximately 1 Mb interval. Within this interval, we identified disruptive mutations in two genes. Complementation tests between *spp1* and *spp3* revealed they were allelic, and deep sequencing of *spp3* identified an independent disruptive mutation in *SvAUX1* (*AUXIN1*), one of the two genes in the ~1 Mb interval and the only gene disruption shared between *spp1* and *spp3*. *SvAUX1* was found to affect both inflorescence development and root gravitropism in *S. viridis*. A search for orthologous mutant alleles in maize confirmed a very similar role of *ZmAUX1* in maize, which highlights the utility of *S. viridis* in accelerating functional genomic studies in maize.**

Maize (*Zea mays*) is one of the most important crop species globally, and has been used as a genetic system since the early twentieth

In recent years *S. viridis* has been proposed as a model for food and bioenergy panicoid crops, including maize[9,10]. The lifespan, plant stature and genome size of *S. viridis* are similar to *A. thaliana*. The extensive gene synteny[11], similar architecture and common habitats that *Setaria* shares with maize suggest its great potential as a translatable model. However, to date neither the utility of *S. viridis* as a genetic model system nor the translatability of discoveries from *S. viridis* to maize have been demonstrated experimentally. In this study, we constructed a mutant population resource for *S. viridis* and used a forward genetic screen to dissect inflorescence architecture, a complex trait directly related to yield and harvestability in maize and other cereal crops[12,13]. We identified two independent mutations in a single gene, *SvAUX1*, as responsible for major disruptions in inflorescence branch development, which led to sparse panicle (*spp*) phenotypes. We then used a reverse genetics approach to show that a loss-of-function allele in *ZmAUX1*, the maize orthologue of *SvAUX1*, conditions a maize male inflorescence phenotype very similar to *spp1*, namely a reduction in primary branch formation, and thus is likely to act through similar genetic mechanisms. Trait discovery to fine map to *SvAUX1* in *S. viridis* took seven months, with greatly lower associated costs and plant growth requirements than an equivalent study would have taken in maize. Importantly, the translatability to maize was readily observed by exploiting the phenotypic similarity and extensive

Learning goal: Your learning target here is NOT comprehensive plant genetics knowledge. Instead, your goal should be gaining an understanding of how the phenotypic data we are collecting in *Setaria virdis* connects to broader research efforts in gene discovery. Below are some key definitions to help you as you read this paper and ask you to answer some questions. Please focus your effort on the main text of the paper, NOT the Methods section.

Key Vocabulary

Inflorescence: a cluster of flowers that arises from a terminal and axillary buds

Panicle: a branched inflorescence with indeterminate growth that can continue producing flowers as long as environmental conditions are favorable. This inflorescence type occurs in *S. viridis* and other cereal crops (see Fig. 1a-1d). The size and branching of panicles impacts yield.

Gene: a sequence of DNA that provides a set of instructions for protein synthesis. Genes are transcribed into RNAs. mRNAs are translated into polypeptide chains.

Homologous genes: genes that arise from a common ancestor

Orthologous genes: homologous genes that diverged after evolution gives rise to a new species. The genes generally maintain a similar function to that of the ancestral gene that they evolved from.

Bulked segregant analysis (BSA): a technique used in gene discovery. Gene pools of Individuals that display opposing phenotypes (ex. sparse panicles vs dense panicles) are crossed and sequenced separately and then compared to identify alleles associated with the mutant phenotype.

Root gravitropism: a plant growth response that directs roots to grow downward (see Fig. 1e-1g)

Key Genes and Phenotypes

spp1-spp4 phenotypes: plants that show the loose, sparse panicles phenotype the researchers are trying to characterize genetically

wildtype phenotype: plants that show the regular dense panicles

***svAUX1* (*AUXIN1*) gene:** a gene that showed two different mutations (*spp1* and *spp4*) that were associated with sparse panicles. The gene plays a role in the development and branching of panicles. Auxin is a plant hormone that helps regulate the dormancy and growth of meristems inside of buds.

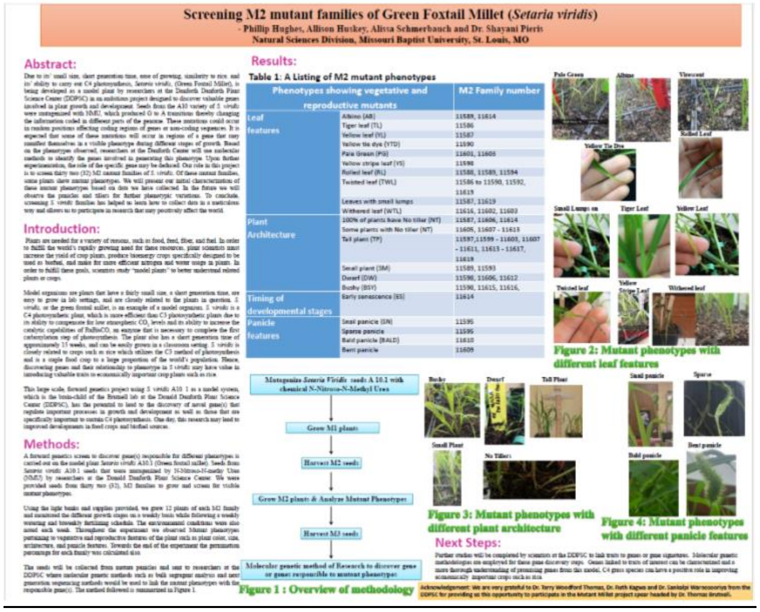
Summary Questions:

Please answer the questions below. For each question, please organize your thoughts into 1-2 paragraphs, each with a topic sentence.

- Question #1: The big picture: What are the research objectives of this study and how might these relate to the overall goal of increasing the efficiency of crops, such as maize?
- Question #2: Genotypes versus phenotypes. What is the difference between a genotype and a phenotype? Please explain this using both the wildtype and mutant genotypes and phenotypes presented in the paper.
- Question #3: Interpreting data. The research team argues that the mutations they observed are recessive alleles. What is a recessive allele? How does the information presented in Table 1 support the authors' argument? (hint: think about Mendel's peas).
- Question #4: Working with the scholarly literature. Scientific papers (including this one) are full of jargon and often quite dry to read. What did you think of this paper compared to the one we read about plant breeding earlier this semester? Why do you think scientists communicate their work in this style?

APPENDIX B

Sample poster presentation produced by undergraduates enrolled in the MutantMillets: Phenotype to genotype using the green foxtail millet (*Setaria viridis*) CURE at Missouri Baptist University, MO



Screening M2 mutant families of Green Foxtail Millet (*Setaria viridis*)

- Phillip Hughes, Allison Huskey, Alissa Schmerbauch and Dr. Shayani Pieris
Natural Sciences Division, Missouri Baptist University, St. Louis, MO

Abstract:

Introduction:

Methods:

Results:

Table 1: A Listing of M2 mutant phenotypes

Phenotypes showing vegetative and reproductive mutants		M2 Family number
Leaf features	Albino (AB)	11589, 11614
	Tiger leaf (TL)	11586
	Yellow leaf (YL)	11587
	Yellow tie dye (YTD)	11590
	Pale Green (PG)	11601, 11603
	Yellow stripe leaf (YS)	11598
	Rolled leaf (RL)	11588, 11589, 11594
	Twisted leaf (TWL)	11586 to 11590, 11592, 11619
	Leaves with small lumps	11587, 11619
	Withered leaf (WTL)	11616, 11602, 11603
Plant Architecture	100% of plants have No tiller (NT)	11587, 11606, 11614
	Some plants with No tiller (NT)	11605, 11607 - 11613
	Tall plant (TP)	11597,11599 - 11603, 11607 - 11611, 11613 - 11617, 11619
	Small plant (SM)	11589, 11593
	Dwarf (DW)	11596, 11606, 11612
	Bushy (BSY)	11590, 11615, 11616,
Timing of developmental stages	Early senescence (ES)	11614
Panicle features	Snail panicle (SN)	11595
	Sparse panicle	11596
	Bald panicle (BALD)	11610
	Bent panicle	11609

Figure 2: Mutant phenotypes with different leaf features

Mutagenize *Setaria Viridis* seeds A 10.1 with chemical N-Nitroso-N-Methyl Urea

Grow M1 plants

Harvest M2 seeds

Grow M2 plants & Analyze Mutant Phenotypes

Harvest M3 seeds

Molecular genetic method of Research to discover gene or genes responsible to mutant phenotypes

Figure 1 : Overview of methodology

Figure 3: Mutant phenotypes with different plant architecture

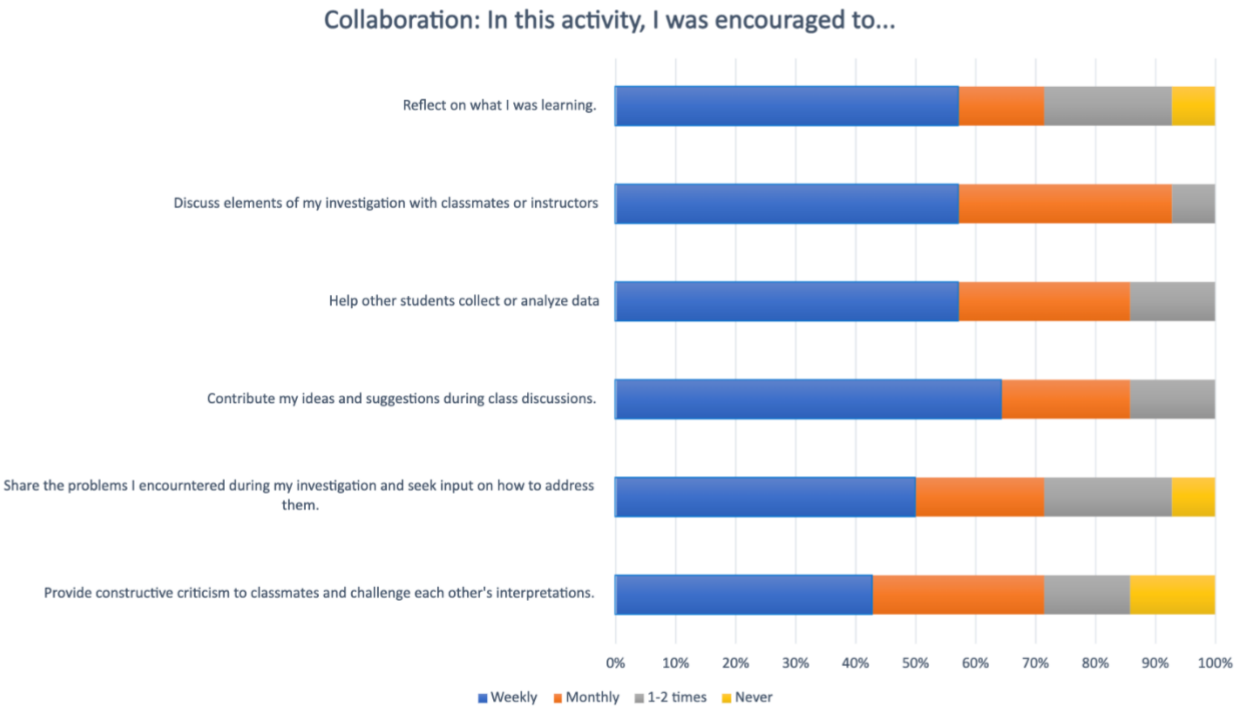
Figure 4: Mutant phenotypes with different panicle features

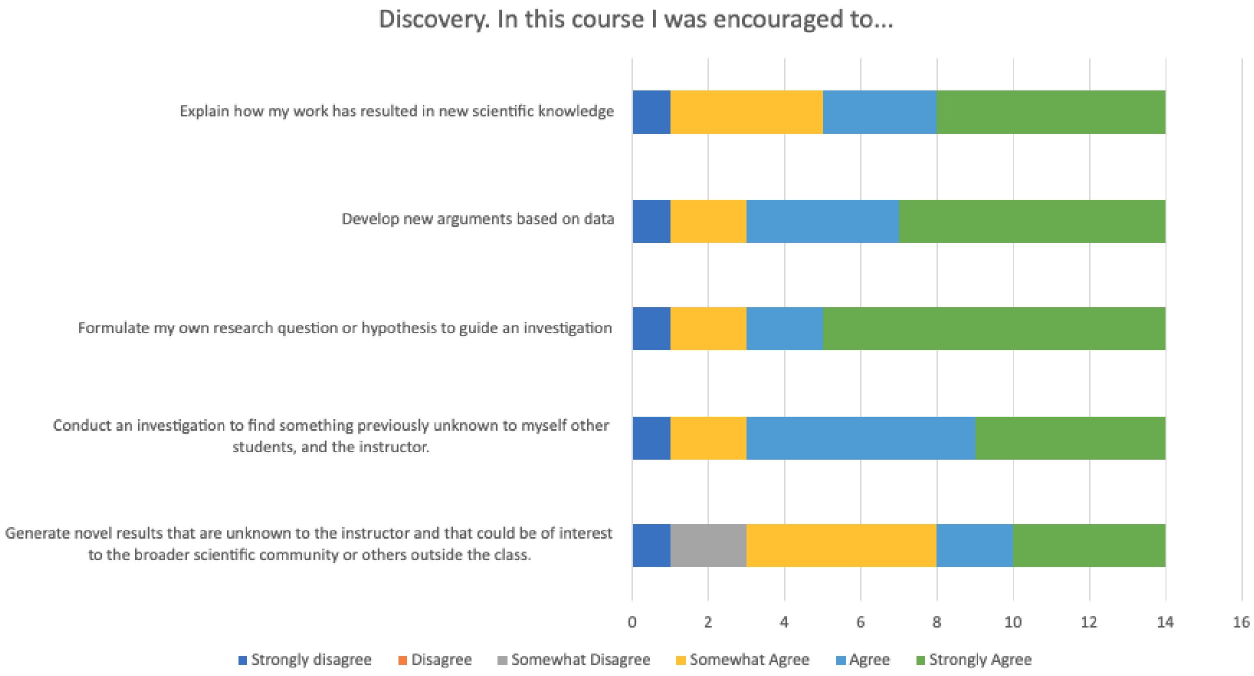
Next Steps:

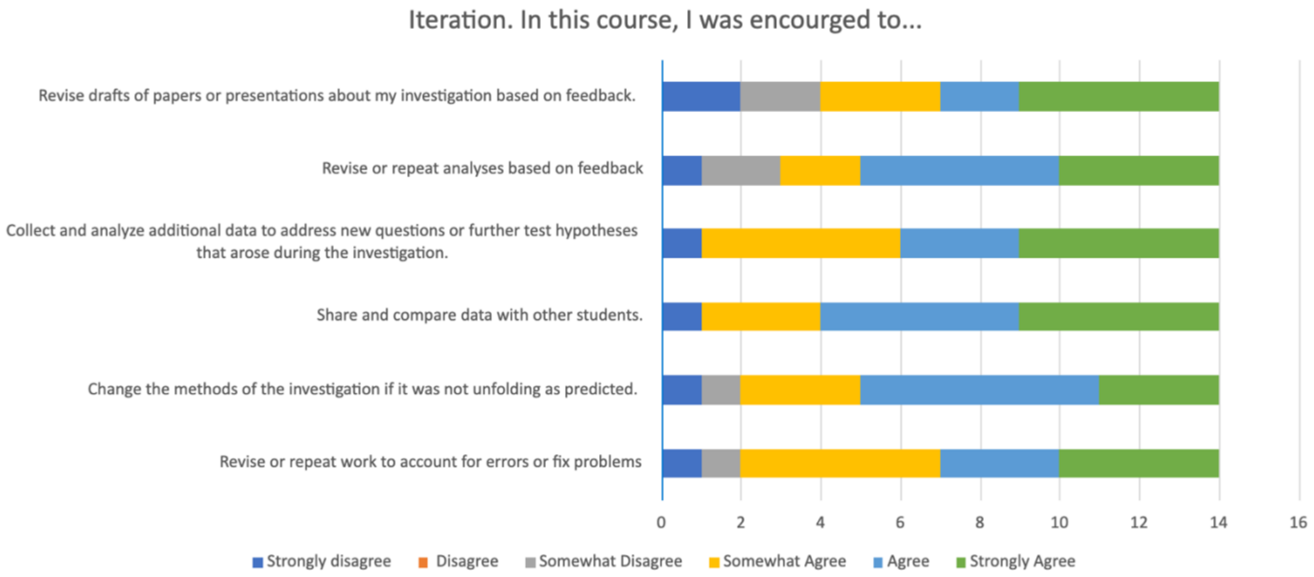
Acknowledgement: We are very grateful to Dr. Terry Woodford Thomas, Dr. Ruth Kagwa and Dr. Sankalpi Warnasooriya from the DDPSC for providing us this opportunity to participate in the Mutant Millet project spear headed by Dr. Thomas Brutnell.

APPENDIX C

Sample data using the Laboratory Course Assessment on undergraduate student outcomes from participation in the Mutant Millets: Phenotype to genotype using the green foxtail millet (*Setaria viridis*) CURE in 2022 at Pacific Lutheran University, (n= 14).







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