

Exploring Grass Morphology & Mutant Phenotypes Using *Setaria viridis*

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ABSTRACT

Globally, most human caloric intake is from crops that belong to the grass family (Poaceae), including sugarcane (*Saccharum spp.*), rice (*Oryza sativa*), maize (or corn, *Zea mays*), and wheat (*Triticum aestivum*). The grasses have a unique morphology and inflorescence architecture, and some have also evolved an uncommon photosynthesis pathway that confers drought and heat tolerance, the C_4 pathway. Most secondary-level students are unaware of the global value of these crops and are unfamiliar with plant science fundamentals such as grass architecture and the genetic concepts of genotype and phenotype. Green foxtail millet (*Setaria viridis*) is a model organism for C_4 plants and a close relative of globally important grasses, including sugarcane. It is ideal for teaching about grass morphology, the economic value of grasses, and the C_4 photosynthetic pathway. This article details a teaching module that uses *S. viridis* to engage entire classrooms of students in authentic research through a laboratory investigation of grass morphology, growth cycle, and genetics. This module includes protocols and assignments to guide students through the process of growing one generation of *S. viridis* mutants and reference wild-type plants from seed to seed, taking measurements, making critical observations of mutant phenotypes, and discussing their physiological implications.

Key Words: *Setaria viridis*; authentic research; grass morphology; mutations; phenotype screening; model organisms.

○ Introduction

More than half of human dietary calories, globally, come from grasses (Poaceae) such as rice (*Oryza sativa*), wheat (*Triticum aestivum*), sugarcane (*Saccharum officinarum*), and maize (*Zea mays*). Moreover, grasses are major ingredients of livestock feeds, the basis for many biofuels, and dominate both rural and urban landscapes (Kellogg, 2001; FAO, 2019). Most middle and high school students and educators are, however, unaware of the global economic value of the grasses and know little about plant-science fundamentals such as grass architecture, the unique inflorescence that determines seed yield, and how to conceptualize a plant's genotype and phenotype.

Furthermore, even in agriculturally dependent “Bio-Belt” states of the Midwest, such as Missouri, minimal teaching on plant biology and agriculture occurs in secondary-level classrooms, having been significantly downplayed in state-defined curricula, classroom learning expectations, and associated testing (Missouri Department of Elementary and Secondary Education, 2016). The Donald Danforth Plant Science Center (DDPSC) launched the Mutant Millets Science Education and Outreach program in 2013 to take hands-on inquiry-based learning and cutting-edge agriculture research into high school classrooms using a weedy grass.

Green foxtail millet or green millet (*Setaria viridis*) is a common weed of roadsides, sidewalk cracks, and abandoned lots in urban areas. It is classified in the same group of grasses that includes sorghum (*Sorghum bicolor*), maize, sugarcane, and the domesticated foxtail millet (*Setaria italica*), all of which deploy the C_4 pathway for photosynthesis (Figure 1; Brutnell et al., 2010; Li & Brutnell, 2011). *Setaria viridis* currently serves as a model plant for improving genetically related C_4 crops – such as maize, pearl millet (*Pennisetum glaucum*) and other edible millets, and sorghum – due to its rapid life cycle, small growth stature, simple growth requirements,

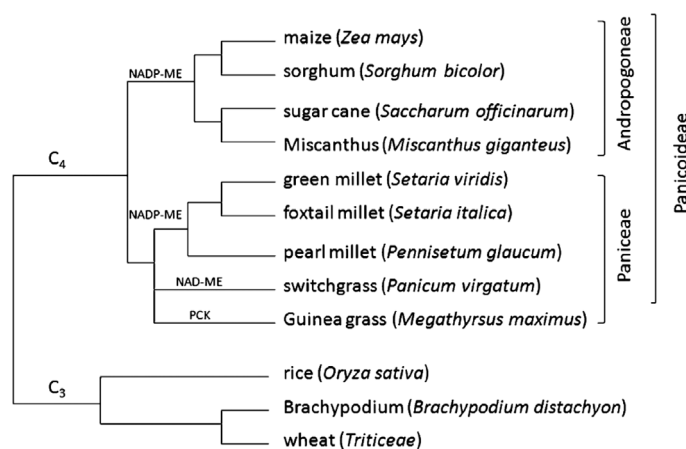


Figure 1. Phylogenetic relationships of grass family members (from Li & Brutnell, 2011).

and a fully sequenced genome (Brutnell et al., 2010; Bennetzen et al., 2012; Huang et al., 2016, 2017).

The Mutant Millets program is an authentic research project that provides preselected mutant populations of *Setaria viridis* to engage entire classrooms of students in scientific practices, including data collection, communication, and the use of scientific tools to monitor plant phenomena. Given the unpredictability of mutagenesis, students participate in true scientific discovery, conducting experiments in which the outcome is not known fully by screening for plant mutant phenotypes. Experiences conducting research greatly contribute to academic achievement, future career interests, and increased scientific self-efficacy of students (Auchincloss et al., 2014; Ballen et al., 2017). Despite such benefits, these research experiences have historically been unavailable to entire classrooms of students (Harrison et al., 2011; Kloser et al., 2013). The Mutant Millets program provides lab instructions, equipment, and mutant and wild-type *S. viridis* seed to interested high school educators who have participated in a training workshop on conducting phenotypic/genetic screens of mutant lines of *S. viridis* in the classroom. High school teachers express interest in the program through the project's website (<https://mutantmillets.org>) and are then invited to a free training workshop at DDPSC in St. Louis, Missouri. Teachers are provided with resources to enrich their teaching of biology, while students experience investigative scientific research that enhances their knowledge and improves appreciation of plant science, communication, and data sharing through the project's website. The development of hands-on research skills in the classroom fosters interest in plants and opens the door to discussions of advanced agriculture, sustainable energy, and food security. Furthermore, the program's activities expose students to plant fundamentals, such as germination date and flowering date, that inform agronomic decisions like cultivar selection by growers in the crop production industry. Screening of mutants under controlled environmental conditions like classrooms provides students with access to authentic science experiences critical to learning of life science disciplines like agriculture, and eliminates barriers such as transportation to crop field trials. Moreover, research findings generated from studies under controlled environments are known to be useful for guiding field trials (Gil-Loaiza et al., 2016; Telfer et al., 2018).

Additionally, the program helps teach required topics in the state biology curriculum such as photosynthesis, and project activities are aligned with numerous *Next Generation Science Standards* (NGSS) on growth and development of organisms (LS1); ecosystems, interactions, energy, and dynamics (LS2); and inheritance of traits (LS3), as detailed below. The program also fits into AP Biology curriculum units on Cellular Energetics, Heredity, and Gene Expression and Regulation.

○ Lesson Details

The activities described here can be used to engage students in planting, nurturing, and screening of a diverse panel of preselected mutant families of *Setaria viridis*. Students observe, measure, and document plant morphology, inflorescence architecture, growth-stage progression, and mutant phenotypes and report findings through the project's website. Students also collect data on light intensity, humidity, temperature, and photoperiod in the plants' growth environment throughout the growth cycle, to assess their

effects on plant phenotypes and phenology (see Supplemental Material). Students can also engage in scientific writing exercises to document and report findings. However, the requirement of a summative paper should be at the discretion of the instructor, given the learning already achieved by students in the hands-on experiences. These activities are designed for use with classrooms of high school students and provide a rich, hands-on laboratory experience. The full laboratory course, comprising Activities 1–3 (see below), lasts 13–15 weeks, with weekly observations that each take about 15–20 minutes of class time (see Table 1). In school systems that grade quarterly, the activities can be carried over all of the semester's quarters, especially if the teacher has the same students enrolled throughout. Furthermore, teachers can assign a grade to certain parts of the investigation and then final data can be assessed in another quarter. Teachers could also make assignments for the first quarter (e.g., research on plant anatomy), with students planting and collecting data on plants. In another quarter, the final data and a summative paper could be written and assessed.

○ Mutant Population Background

The *Setaria viridis* seeds used in this program were generated by a laboratory at the DDPSC using mutagenesis techniques described by Jiang et al. (2016) and Huang et al. (2017). The result was ~20,000 mutant families of plants with a median of 66 homozygous non-synonymous mutations per mutant family (Jiang et al., 2016). In the second generation after mutagenesis (M2 generation), several visible aboveground mutant phenotypes were obtained that are available for use in this lesson. These include albino, dwarf, bushy, fat panicle, yellow tie dye, yellow leaf, and sparse panicle (*spp*), some of which have been characterized by scientists at the DDPSC using the forward genetics technique (Figure 2; Huang et al., 2017; Yang et al., 2018). Mutant phenotypes like dwarf, fat panicle, and high tillering/bushy and large seed size are agronomically relevant for contributions to crop improvement efforts. Mutant dwarfing alleles at the *Reduced height-1* (*Rht-1*) loci in cereals like wheat are a hallmark of the green revolution, having contributed to significantly higher grain yields (Börner et al., 1996; Peng et al., 1999). Large panicle size is a selectable trait for increasing grain yield in breeding of grasses like rice (Peng et al., 2008). High tillering/bushy mutants are useful for bioenergy grass breeding efforts, as *S. viridis* is closely related to the bioenergy feedstock switchgrass (*Panicum virgatum*; Doust et al., 2009).

○ Lesson Preparation

In the summer and fall of each academic year, the project receives requests from high school teachers interested in participating for a full semester through the project's website. Teachers are invited to a half-day training workshop, after which interested teachers are recruited as partners in the Mutant Millets program and provided with mutant seed populations of *S. viridis* (M3+ generations), plus all supplies, documentation, and equipment required to grow and nurture plants in their classrooms to physiological maturity. The ideal growth environment for *S. viridis* includes a 16 hour/8 hour (day/night) photoperiod, temperature of 31°C/22°C (day/night), relative humidity 30%, and light intensity approximately 250–400 micromoles/m²/sec (Jiang et al., 2013).

Table 1. Schedule of procedures and assignments for Activities 1 and 2.

Week	Activity/Event	Estimated Time	Learning Objective
1	Plant seeds Seedlings appear Water plants Assignment 1: Observe and record germination Assignment 2: Compare growth rates Assignment 3: Compare phenotypes	30–40 minutes (prep) 30–40 minutes (plant) 20 minutes 30 minutes per week	1, 2
2–4*	Tillers appear Assignments 2 and 3: Continued	30 minutes per week	1, 2
6–8*	First panicles appear Assignments 2 and 3: Continued	30 minutes per week	1, 2
9–10*	Flowers mature Plant self-pollinate, fertilization occurs Assignments 2 and 3: Continued	30 minutes per week	1, 2, 3
11–12	Seeds form Bag plants, Assignments 2 and 3: Continued	1 hour	1, 2
13	Flag leaves senesce Stop watering, let plants dry out Assignment 3: Continued	30 minutes	2
14–15	Harvest seeds Assignment 3: Continued	1 hour	2
15	Student lab notebook data compilation and transfer to the project's website https://mutantmillets.org/	1 hour	4

* Water plants and apply fertilizer.



Figure 2. Mutant phenotypes commonly observed by high school students (A10.1 is the wild-type reference): (A) spindly, non-tillering; (B) tall; (C) bushy; (D) sparse panicle; (E) deformed leaves; (F) yellow tie dye; (G) dwarf; (H) fat panicle; (I) larger seed size; (J) Albino; and (K) high-biomass mutant.

○ Activity 1: Observation of Growth, Development & Morphology of *Setaria viridis* Plants

This activity allows students to observe and analyze the unique morphology of grass plants, and specifically the vegetative and reproductive growth stages of *S. viridis* mutant and wild-type plants.

Learning Objectives & NGSS Alignment

1. Identify and describe the growth stages of mutant and wild-type *S. viridis* plants. (HS-LS1-2: Develop and use a model to illustrate the hierarchical organization of interacting systems that provide specific functions within multicellular organisms.)
2. Create a model with labels of the anatomy of a mature grass plant and explain the roles of panicles, spikelets, and the flag leaf. (HS-LS1-2: Develop and use a model to illustrate the hierarchical organization of interacting systems that provide specific functions within multicellular organisms.)
3. Evaluate the growth rates of mutant and wild-type plants by creating a graph of height data and number of tillers. (Science and Engineering Practices, Constructing Explanations and Designing Solutions: Make a quantitative and/or qualitative claim regarding the relationship between dependent and independent variables.)

Materials

- *S. viridis* wild-type and mutated seed
- Pots
- Trays and lids (2 ft²)
- Metro-Mix 360 potting soil
- Spray bottle
- Light bank and light bulbs (four or eight fluorescent T-8 bulbs)
- Jack's Classic fertilizer (20:20:20) (nitrogen: phosphorus: potassium)
- Timers
- Light meter
- Labels and permanent markers
- Waste bags
- Empty seed collection envelopes
- Twist ties
- Forceps
- Plastic beaker
- Lab notebooks
- Dissecting microscopes or magnifying glasses

Procedure 1: Plant Mutant & Wild-Type Families

Students will plant a diverse panel of three or six mutant families of *S. viridis* that include homozygous and heterozygous mutants with segregating populations as well as wild-type plants:

1. Fill clean pots with Metro-Mix 360 potting soil. Place these in a tray, leaving one space in the far right corner of the tray empty for watering.

2. Plant one tray with wild-type seeds (A10.1). Additional trays should be planted with one mutant family each of *S. viridis*. The number of trays will depend on classroom space (see Figure 3).
3. Label pots with *S. viridis*, plant family number, generation after mutagenesis (will start with an "M," for mutant families only), the date of planting, and your group/class name. Using plant tags, label each pot 1–17 to track individual plants throughout the experiment.
4. Using the empty spot, add water and let the trays stand for 20–30 minutes until all the water is absorbed by the soil. The pots are ready for planting when the soil on top looks and feels wet.
5. Use forceps to plant two seeds in the soil at a depth of a pencil eraser or 0.5–1 cm. Use only the viable (black or gray) seeds, not the empty tan/white ones.
6. Using a plastic beaker, sprinkle a light layer of soil over the seeds. Mist the pots with water, taking care not to displace soil or the seeds.
7. When all pots have been planted, add extra water, using the empty spot, till the water level is about <1 cm from the bottom of the tray.
8. Set up the light banks by inserting the light bulbs. Set the timer on the lights to 16 hours light/8 hours darkness. Place the trays under the light source or light bank.
9. To keep the humidity high, cover the trays with a clear plastic lid until the seedlings emerge from the soil. After the plants have emerged, remove the clear lid.
10. After plants emerge above the soil, uproot all extra plants, so that only one remains per pot. You may replant an "extra" plant in the empty pot lacking a plant.
11. Add 1.5 L of water to the bottom of the tray, using the empty spot. After watering, wait about one hour and then pour off any excess water. Do not overwater, as *S. viridis* prefers dry soil.
12. After germination, all watering events should include fertilizer application. To fertilize, mix ½ tsp. of fertilizer (20:20:20) per 1.5 L of water. Use this fertilized water to supply fertilizer to the plants once every other week.

Assignment 1

Observe *Setaria viridis* morphology, growth, and development.

Students should check their plants daily after planting to record the date of germination and watch for short-lived mutant phenotypes such as the albino. After the first week, students should observe plants weekly, take the measurements listed below, and record them in their lab notebooks.

1. Measure the height of the plants in centimeters each week, from the soil to the apical bud of the plant (Figure 4). When students have several data points, they can plot the data on a graph to see how much their plant has grown over time.
2. Use plant height data to compare the heights of mutant and wild-type plants.
3. Count the number of tillers coming from the base of each plant every week and construct a graph showing the number of tillers over time.

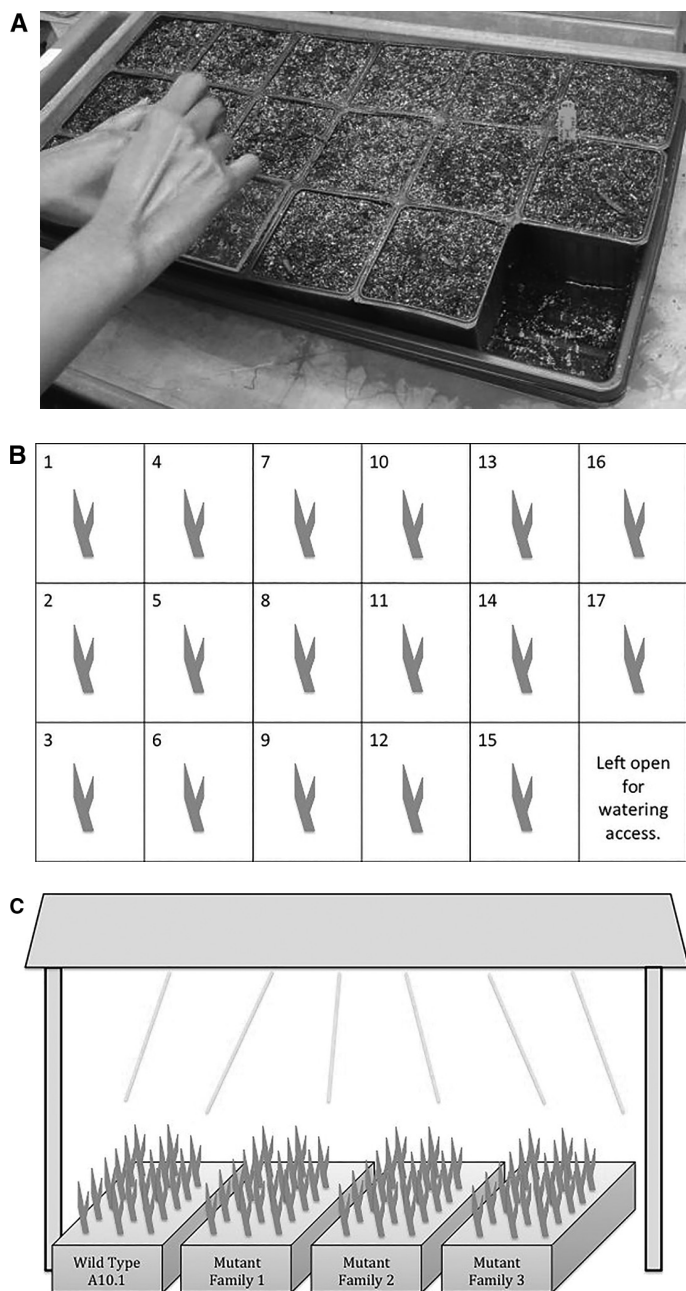


Figure 3. (A) Planting. (B) Arrangement of pots in a tray. (C) Tray layout under the light bank.

- Look at the classroom data set. Is the number of tillers different between plants? Calculate the average number of tillers per mature plant. Does this differ between mutant and wild-type plants?
- Teachers should pool all data and student observations and initiate a discussion on population genetics.

Assignment 2

Describe *S. viridis* morphology and inflorescence architecture; compare and contrast these features between mutants and wild-type plants.

The conversion of the shoot apical meristem to an inflorescence meristem marks the onset of reproduction in the grasses, where the

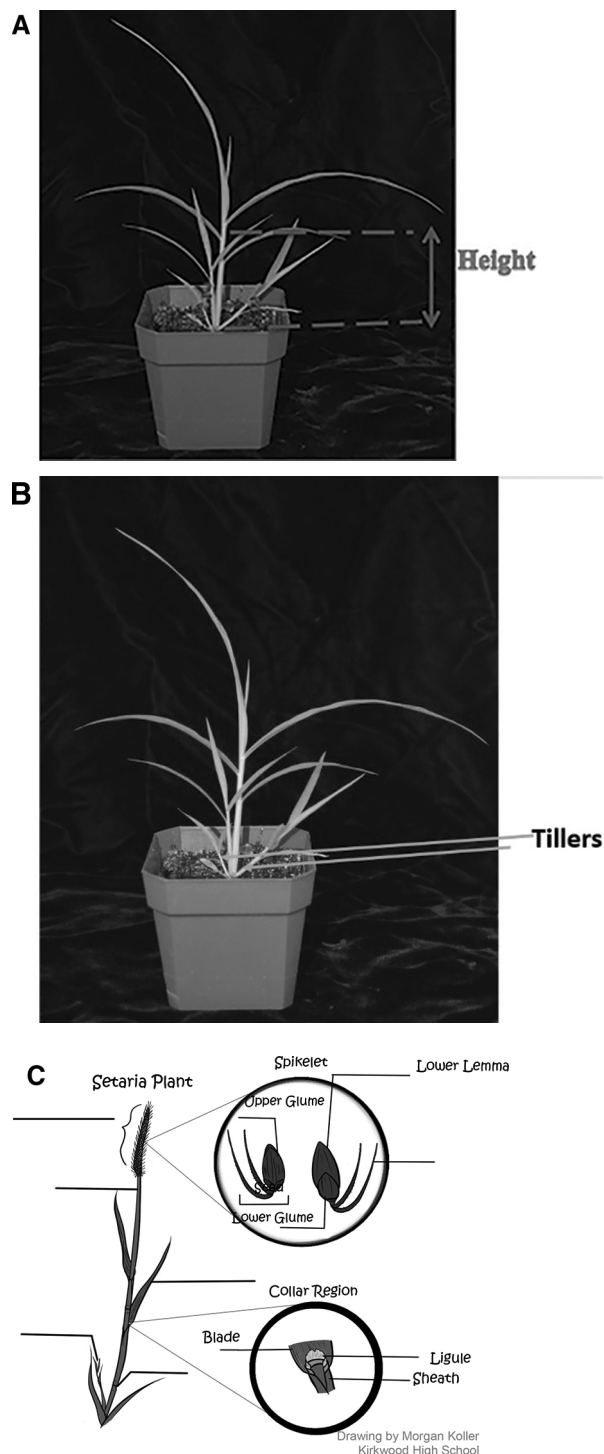


Figure 4. (A) Measuring plant height. (B) Counting tillers. (C) Examining *S. viridis* morphology.

plants switch from producing leaves to floral structures (Kellogg et al., 2013). In *S. viridis*, the inflorescence meristem produces spikelet meristems that produce glumes (bracts) and flowers in tiny spikelets (Kellogg et al., 2013); panicles undergoing flowering will have slightly opened spikelets (Figure 5).

In this assignment, students will learn about grass inflorescence architecture by examining the *S. viridis* plants as they transition from vegetative to reproductive stage (panicle formation) using the questions listed below, which are aligned with the NGSS (HS-LS1-2:

Develop and use a model to illustrate the hierarchical organization of interacting systems that provide specific functions within multicellular organisms; HS-LS2-8: Evaluate evidence for the role of group behavior on individual and species' chances to survive and reproduce).

1. How many days did it take mutant and wild-type plants to produce their first panicle? To flower?
2. Can you see any flowering occurring in the spikelets (use a dissecting microscope or hand lens)? If so, pry open spikelets and draw, label, and describe the flower structure (Figure 5).
3. How many panicles develop per plant? Does this vary across your plants? Create a graph showing the relationship between plant height and the number of panicles. Describe this relationship.
4. Construct a graph showing the relationship between the number of panicles and the number of tillers. Describe this relationship.
5. Draw a *S. viridis* plant at the flowering stage and label all aboveground structures (see Figure 4).
6. When are seeds evident in the panicles? How does the panicle change over time? Describe what you observe.

○ Activity 2: Screen Mutant Families for Visible Phenotypes & Their Implications for Physiological Functions Like Photosynthesis

In this activity, students will see what happens to the plant's phenotype when its genotype is altered by a chemical mutagen. Students will screen a population of *S. viridis* mutant plants for mutant phenotypes. Students will observe each plant in the mutant families provided from germination to physiological maturity and record all unusual phenotypes displayed on all plant structures and in growth cycle. Additionally, students will engage in discussions about mutations, forward genetics, photosynthesis, the C_3 and C_4 pathways, and the physiological significance of observed mutant phenotypes.

Learning Objectives & NGSS Alignment

1. Contrast shoots of mutant and wild-type plants to identify visible mutant phenotypes. (HS-LS3-2: Make and defend a claim based on evidence that inheritable genetic variations may result from (1) new genetic combinations through meiosis, (2) viable errors occurring during replication, and/or (3) mutations caused by environmental factors.)
2. Evaluate the impact of the mutations on plant structure, reproductive fitness, growth rate, inheritance patterns, and physiological function through analysis and interpretation of the data collected. (HS-LS3-1: Ask questions to clarify relationships about the role of DNA and chromosomes in coding the instructions for characteristic traits passed from parents to offspring. HS-LS4-3: Apply concepts of statistics and probability to support explanations that organisms with an advantageous heritable trait tend to increase in proportion to organisms lacking this trait.)

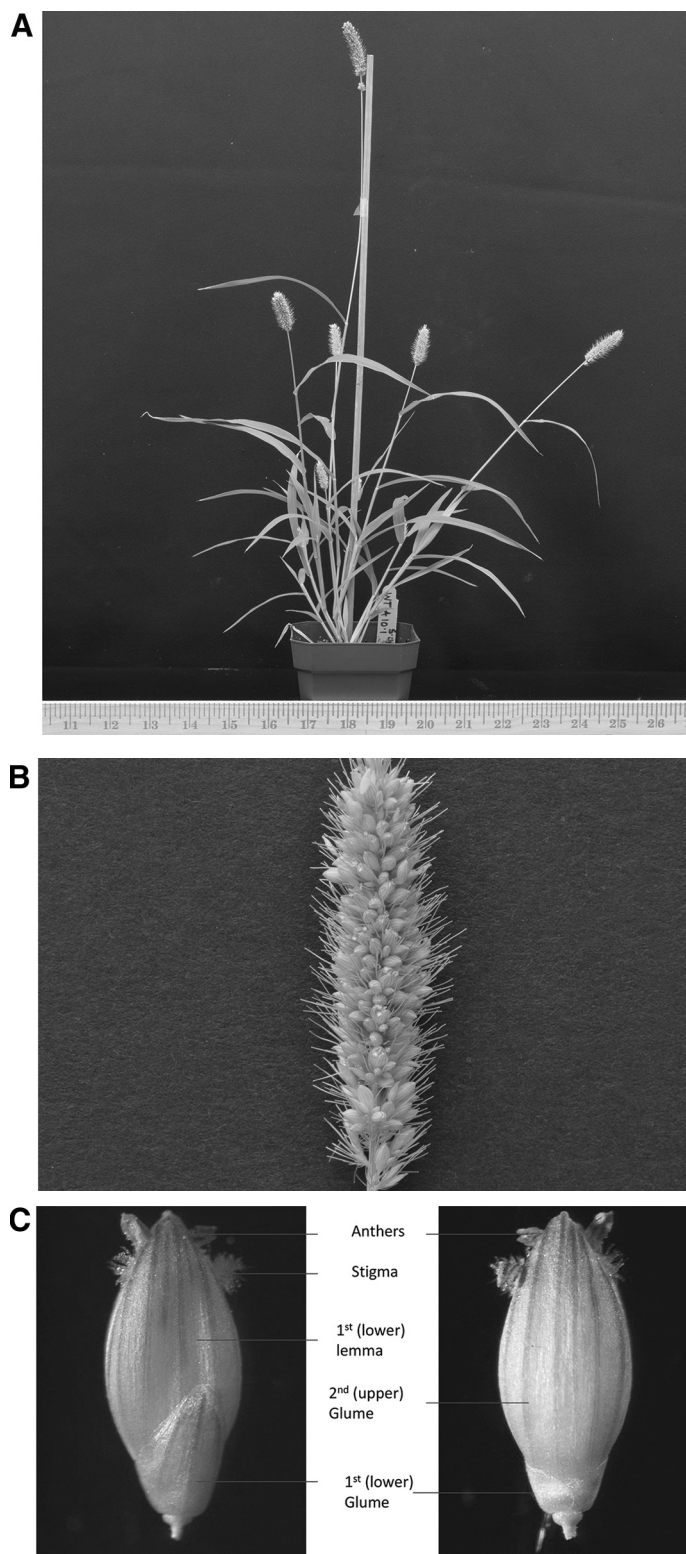


Figure 5. (A) A *Setaria viridis* plant. (B) A single panicle (inflorescence) at flowering, with open spikelets and exposed anthers. (C) A spikelet, viewed from two sides.

3. Summarize the use of mutagenesis for gene discovery in plants. (HS-LS4-2: Construct an explanation based on evidence that the process of evolution primarily results from four factors: [1] the potential for a species to increase in number, [2] the heritable genetic variation of individuals

in a species due to mutation and sexual reproduction, [3] competition for limited resources, and [4] the proliferation of those organisms that are better able to survive and reproduce in the environment.)

4. Explain the C_4 photosynthetic pathway and how it differs from the C_3 pathway, using examples of C_4 and C_3 crops as support. (HS-LS1-5: Use a model to illustrate how photosynthesis transforms light energy into stored chemical energy. HS-LS2-5: Develop a model to illustrate the role of photosynthesis and cellular respiration in the cycling of carbon among the biosphere, atmosphere, hydrosphere, and geosphere.)
5. Construct a scientific argument around mutation and plant development by writing a scientific manuscript from the Mutant Millets lab. (HS-LS3-2: Make and defend a claim based on evidence that inheritable genetic variations may result from [1] new genetic combinations through meiosis, [2] viable errors occurring during replication, and/or [3] mutations caused by environmental factors.)

Materials

- Three or six trays containing mutant plants and one or two trays containing wild-type *S. viridis* A10.1 plants from Activity 1
- Poster with commonly observed mutant phenotypes of *S. viridis*
- An infographic of photosynthesis, distinguishing the C_3 and C_4 photosynthesis pathways

Assignment 1

Students observe plants daily for the first two weeks, recording any mutant phenotypes, then weekly. Some phenotypes are obvious right after the seeds germinate, and others may not appear until later. Students should use the sample data sheet in Figure 6 to record data for each plant in the mutant family tray.

Assignment 2

Discuss mutations and the functional physiological significance of observed mutant phenotypes. Mutant phenotypes that might relate

to photosynthesis include pale green leaves or albino plants. Students should review the topics listed below in a classroom discussion led by a scientist from DDPSC or their teacher.

- I. Mutations, forward genetics, genotypes and phenotypes
- II. Photosynthesis, the C_3 and C_4 pathways
- III. Plant tolerance mechanisms to heat and drought, coping mechanisms unique to C_4 crops
- IV. Physiological significance of mutant phenotypes such as albino, yellow leaf, dwarf

Assignment 3

Students practice scientific communication by producing a manuscript that discusses the importance of the Mutant Millets lab, characterizations of mutants, data and discussion using measurements, qualitative images, drawings, and written observations. This assignment requires students to organize all study information into a logical and standardized format so that results can be communicated to an external audience. Guidelines for each section of the manuscript are detailed in Table 2.

Activity 3

See the Supplemental Material available with the online version of this article.

Conclusions

This module demonstrates ways in which the C_4 model plant *Setaria viridis* can be utilized for hands-on investigative teaching to expose high school students to grass morphology, growth cycle, and concepts in genetics, including genotype and phenotype, to reinforce learning of plant biology. Through this module, students engage in scientific research and share their knowledge via the project's website with scientists at DDPSC to aid efforts in gene discovery for crop improvement. The module lends itself to students working in groups, helping to build teamwork, and shows

Planting date:	7-10 days after planting				14 days after planting					21-23 days after planting		28 days after planting		40 days after planting		harvesting		
Label	germination Yes/No	Albino	Pale green	other notes	small	dwarf	pale green	virescent	Other notes	# few tillers	other notes	# few tillers	Other notes	# few tillers	other notes	late senescence	shattering	sterile
Family:																		
1																		
2																		
3																		
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Figure 6. Phenotype scoring chart for mutant plants.

Table 2. Guide for students' writing exercise, using the argument-driven inquiry method of communicating science.

Section	Content
Introduction	- Statement on the purpose of the study – what is the research question being investigated? - What is the impact of mutagenesis for gene discovery in plants? - Background about <i>Setaria</i> that includes - Vegetative stages of Poaceae <i>Setaria</i> plant architecture - C₄ photosynthesis - Value of <i>Setaria viridis</i> as a model organism - Scientific claim: Summarize the use and impact of mutagenesis for gene discovery in plants.
Evidence	Graphs and descriptions from Activity 1: Assignments 1 and 2 Descriptions of mutants from Activity 2: Assignment 1 All figures should be an appropriate representation of the data type and should have titles, axis labels, and written captions to explain how each component is related to the study.
Justification	- Evaluate the impact of the mutations on plant structure, reproductive fitness, growth rate, inheritance patterns, and physiological function using the evidence presented above. - Appraise the significance of any mutants observed (e.g., increased biomass accumulation, increased grain production, increased growth, increase or decrease of plant life cycle). - Justify how classroom characterizations of mutants add value to the science community.

students the collaborative nature of scientific research. This module is in alignment with NGSS for K–12 and can be successfully integrated into various high school courses, including AP Biology, Genetics, Honors Biology, Biology, Biotechnology, and Agricultural Sciences.

○ Glossary

albino: No color, white.

awn/bristle: A slender, hair-like structure found on the spikelets of many grasses.

culm: The stem of a grass.

florets: A small flower.

genotype: The two specific alleles an individual has for a trait.

glume: One of a pair of dry membranous bracts at the base of the spikelet of grasses.

inflorescence: The part of a plant that consists of a cluster of flower-bearing stalks.

internode: The part of a plant stem between two nodes.

lemma: The outer bract that encloses the flower in a grass spikelet.

ligule: The structure that clasps the stem at the junction of blade and sheath.

model organism: An organism chosen by scientists for study, usually based on characteristics such as short generation time, high reproductive rates, easily observed characteristics, and a close relationship to other organisms of interest.

mutagenic agent: Any substance that can change DNA, including UV radiation, asbestos, and certain chemicals.

mutation: A change in DNA base pairs.

node: The point on a plant stem from which the leaves or lateral branches grow.

palea: The inner bract that encloses the flower in a grass spikelet.

panicle: A branched cluster of flowers in which the branches are racemes.

plant architecture: Structures of the plant, leaves, stems, roots, and the patterns they take.

raceme: A type of inflorescence that is unbranched and bears flowers having short stalks along the axis.

sheath: The tubular portion of the leaf, which wraps around or encloses the stem.

silent mutation: A change in DNA that does not produce a change in the phenotype.

spike: An inflorescence consisting of a raceme of flowers growing directly from the stem.

spikelet: A flowering structure common to grasses, in which a reduced flower is encased in several protective structures.

tiller: A shoot that arises from the base of the stem in grasses.

virescent: Not fully green as compared to the wild type.

wild type (n.), *wild-type (adj.)*: Considered the typical phenotype for the species; considered “normal” when compared to new, possibly mutant traits.

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