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Laboratory Exercises

Transformation of a Dwarf *Arabidopsis* Mutant Illustrates Gibberellin Hormone Physiology and the Function of a Green Revolution Gene*

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Isabel Molina‡, Katrin Weber, Déborah Y. Alves Cursino dos Santos, and John Ohlrogge

From the Department of Plant Biology, Michigan State University, East Lansing, Michigan 48824

The introduction of dwarfing traits into crops was a major factor in increased grain yields during the "Green Revolution." In most cases those traits were the consequence of altered synthesis or response to the gibberellin (GA) plant hormones. Our current understanding of GA synthesis and physiology has been facilitated by the characterization of mutants. To introduce concepts about GA hormone physiology and plant transformation in an undergraduate laboratory course we have used *ga5*, a semi-dwarf *Arabidopsis* mutant with reduced activity of GA 20-oxidase. In this laboratory exercise, *Arabidopsis ga5* mutant plants are transformed by the floral-dip method using *Agrobacterium tumefaciens* carrying plasmid constructs conferring kanamycin resistance and containing the *GA5* gene. Within 4 weeks, seeds of transformed plants can be easily screened by antibiotic resistance on plates. After transfer to soil the dwarf mutant plants transformed with a wild-type version of the gene show normal size. In addition to offering a visual understanding of the effect of GA on stem elongation, students learn additional techniques in this experiment, including PCR and agarose gel electrophoresis. This experiment is cost effective and can be completed within a 4-month term.

Keywords: Gibberellin, *Arabidopsis* transformation, dwarf mutant, Green Revolution.

The term "Green Revolution" refers to the major increases in crop yields that were achieved in the second half of the 20th century through improved crop varieties and the application of modern agricultural practices. The development of high-yielding varieties of rice, wheat and other grains has been particularly important. The average yield of grains per area more than doubled between 1960 and 1985 and the increases were particularly significant for developing countries. These major increases allowed food production to more than keep up with population growth [1, 2].

Much of the improvement of yield of rice and wheat, which are the food crops most consumed by humans in the world, is the result of introduction of dwarf or semi-dwarf traits. These varieties devote less photosynthate to the stalk, more to the grain and have much reduced losses from "lodging" (the tendency of grain crops to fall over in the field, making harvest difficult). Plant breeders first identified dwarf lines of certain rice or wheat populations and were able to introduce this useful trait into a wide variety of important commercial cultivars.

Only in the last 10 years has the underlying biochemistry and genetics of the Green Revolution become well understood. For both rice and wheat, the key genes responsible for the dwarf trait are involved in gibberellin (GA) biosynthesis [3–5] or perception [6]. Gibberellins are a group of phytohormones that are involved in stem elongation, control of flowering, mobilization of seed reserves, and other processes [7]. After the identification of the genes involved it has become possible to extend the semi-dwarf trait to more rice varieties by genetic engineering using specific gene-targeted strategies that yield crops with reduced height and normal reproductive development [8–10].

Gibberellins are synthesized by the isoprenoid pathway in three stages [11]. In higher plants, the third biosynthetic stage involves a series of oxidations where the multifunctional enzyme GA 20-oxidase plays a critical role in the production of active GA molecules (Fig. 1). Our current understanding of GA synthesis and physiology has been facilitated by the characterization of GA-responsive *Arabidopsis* mutants [12]. One of these mutants, *ga5,* is a semi-dwarf and the *GA5* locus has been shown to encode the GA 20-oxidase [13]. Although in previous studies the *ga5* mutant was not transformed with the wild-type copy of the *GA5* gene, we designed a laboratory exercise to perform such complementation and we demonstrated that transgenic semi-dwarf *ga5* mutants expressing a wild-type copy of the enzyme recover normal wild-type stature.

The dual goals of this exercise are to introduce a series of biotechnological techniques commonly used in plant genetic engineering research (*Agrobacterium* transformation, PCR, gel electrophoresis) and for students to learn some basics of gibberellin hormone physiology.

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‡To whom correspondence should be addressed: 362 Plant Biology, East Lansing, Michigan 48824. Tel: 517-353-9399; Fax: 517-353-1926. E-mail: molinam3@msu.edu.

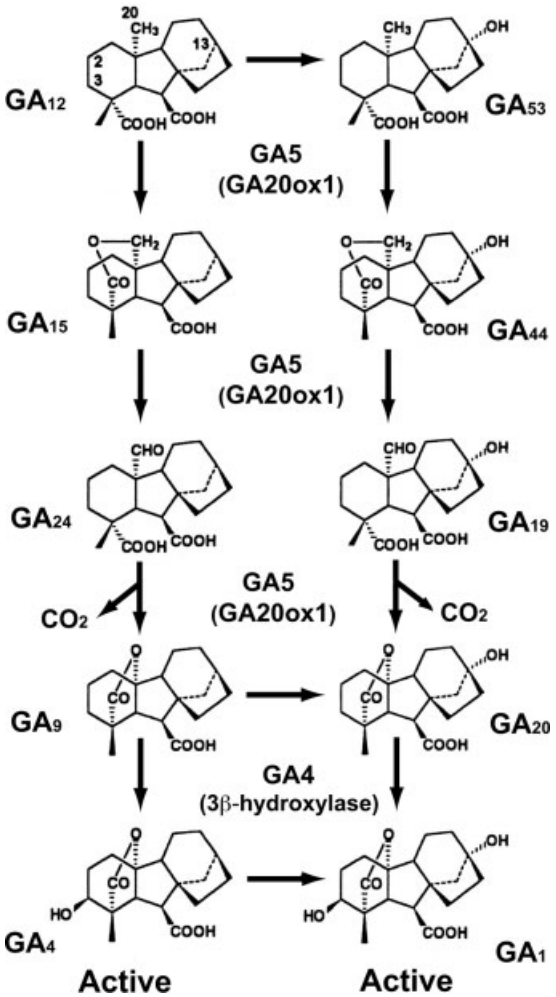

FIG. 1. **Gibberellin metabolism**. GA 20-oxidase (GA5) is a multifunctional enzyme expressed in *Arabidopsis* stems. It catalyzes a series of oxidation and elimination reactions. These reactions convert inactive forms of GA to GA_4 and GA_1 (Figure adapted from ref. [13]).

Underlying these goals is the introduction of a series of important concepts: the link between genotype and phenotype, the use of mutants as a tool to infer biological reactions *in planta*, and the difference between forward and reverse genetic approaches. This exercise was included as a part of an undergraduate course (Experiments in Plant Biology), and the results shown herein are actual results that the students have obtained in class. The course consisted of two lab sessions per week, covering a total of 7 hr/wk. This experiment can be adapted according to the number and length of labs the students have each week.

EXPERIMENTAL PROCEDURES

Materials

All chemical reagents were purchased from Sigma, unless otherwise specified.

Equipment and Supplies

1) Room or chamber suitable to grow *Arabidopsis* plants (or bench top with appropriate white fluorescent lights controlled by timer), plastic trays and pots
2) Centrifuge with GSA rotor or equivalent, centrifuge bottles
3) Vessels for plant dipping
4) Spray bottles
5) Digital camera
6) Bench microcentrifuge
7) Thermal cycler for PCR
8) Pipettors (e.g. Pipetman)
9) Gel electrophoresis chambers, gel casting trays, sample combs
10) Power supplies
11) Microwave oven
12) UV transilluminator
13) Sterile hood
14) Sterilized microcentrifuge tubes (1.5 mL), PCR tubes (0.2 mL)
15) Sterilized tips (0.2 mL, 1 mL)
16) Disposable sterile Petri dishes
17) Safety supplies: gloves, goggles, lab coats

Construct Design

The full length *GA5* cDNA was amplified by PCR using the following primers: GA5For: 5′ cacactctagaATGGCCGTAAGTTTCGTAAC 3′ (*Xba*I restriction site underlined) and GA5Rev: 5′cacacg agctcTTAGATGGGTTTGGTGAGCC 3′ (*Sac*I restriction site underlined). Amplification products were analyzed on 1% agarose gels and purified using the QIAquick Gel Isolation Kit (Qiagen, Valencia, CA). PCR conditions were as follows: 95 °C for 5 min, followed by 30 cycles of 30 seconds at 95 °C, 30 seconds at 55 °C, 2 min at 72 °C, with a final extension step at 72 °C for 10 min. Amplified DNA fragments were subcloned into pGEM®-T Easy vector (Promega Corporation, Madison, WI) using T4 DNA ligase supplied with the kit. The ligation reactions were incubated overnight at 10 °C, after which 10 μL of each were used to transform 50 μL DH5α competent cells. Transformation was performed by heat shock and bacteria were plated onto Luria-Bertani medium (LB)/100 μg/mL Ampicillin/0.5 mM IPTG/80 μg/mL X-Gal plates. White colonies were picked and grown overnight at 37 °C with agitation in 5 mL LB (1% Bacto tryptone, 0.5% Bacto® yeast extract, 1% NaCl, 1.5% Bacto agar, pH 7) supplemented with 100 μg/mL ampicillin (from stock solution 100 mg/mL). Recombinant plasmids were purified using Wizard® Plus SV Minipreps DNA purification system (Promega). Insertion of the PCR product into the plasmid was verified by digestion of 1 μL of recombinant plasmid followed by 1% agarose gel electrophoresis. DNA inserts in recombinant plasmids were sequenced on both strands using Applied Biosystems cycle sequencing technology (Applied Biosystems, Foster City, CA), and sequences were determined on an BABI PRISM® 3100 Genetic AnalyzerB. Primers used for sequencing were Sp6 (5′-ATTTAGGTGACACTATAG-3′) and T7 (5′-TAATACGACT CACTATAGGG-3′). Sequence data were analyzed using Clustal W program. The *GA5* cDNA fragment was released from pGEM®-T Easy by *Xba*I/ *Sac*I digestion and introduced into pBI121 binary vector (Clontech, Palo Alto, CA), which contained the 35S promoter. The *GUS* sequence was released using *Xba*I and *Sac*I restriction sites and replaced by ligation of the above *GA5* to create pBI121-*GA5*. Constructs for *Arabidopsis* transformation are shown schematically in Fig. 2.

Safety

Agrobacterium tumefaciens is a microorganism and, therefore, a potential pathogen. In addition, Silwet L-77 may be harmful by inhalation, ingestion, or contact. Safety goggles, gloves, and labcoats must be used at all times when performing

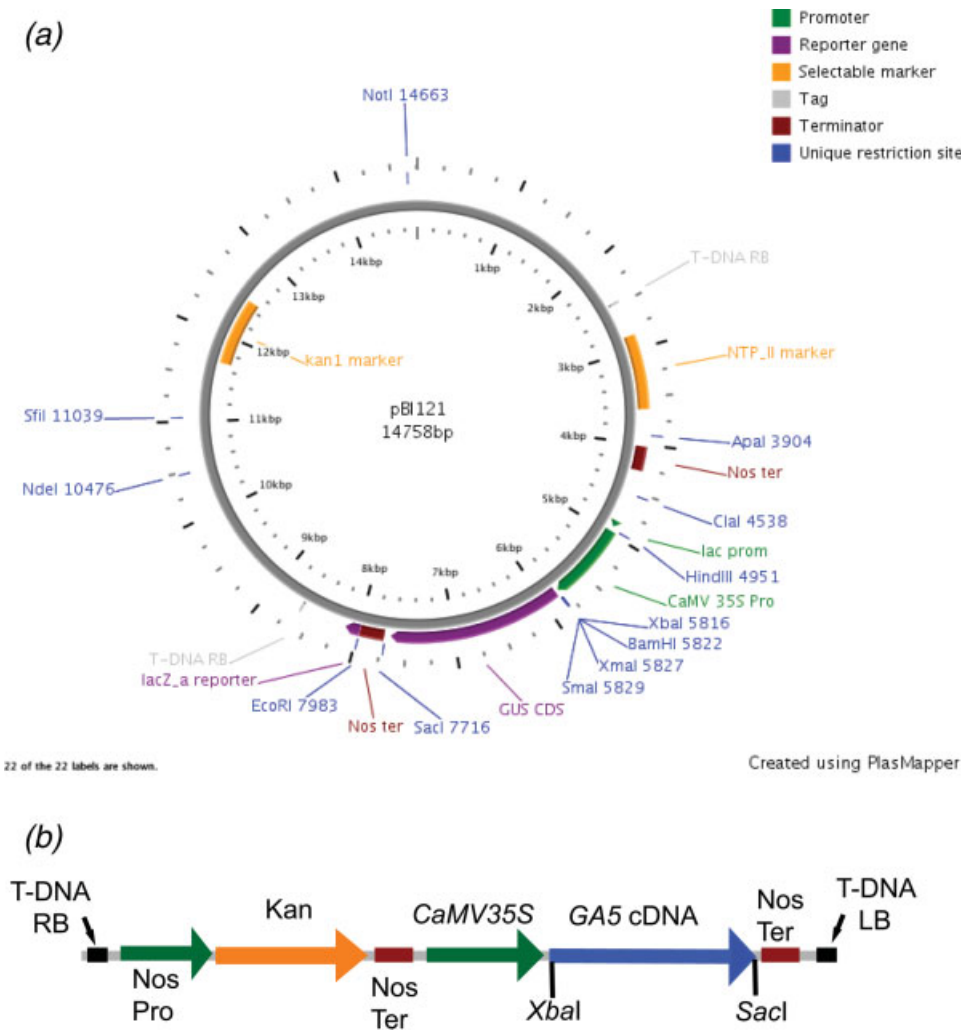

FIG. 2. **Structure of the pBI-*GA5* vector used for transformation of *Arabidopsis ga5* plants** (*a*) Empty plant transformation vector pBI121 (Clontech, USA). (*b*) Scheme of the construct used for expression of AtGA 20-oxidase (the *GUS* gene was replaced by *GA5* cDNA).

transformation. *Agrobacterium* waste must be properly disposed in biohazard bags. Ethidium bromide (EtBr) is carcinogen and always must be handled by wearing gloves. Containers for EtBr waste must be provided to the students for discarding contaminated gels, gloves, and tips. Alternatively, SYBR® Safe DNA gel stain (Molecular Probes, Invitrogen) can be used to visualize DNA bands. This stain is not classified as hazardous.

RESULTS AND DISCUSSION

Arabidopsis thaliana is the most widely used model plant in basic plant science research. Transformation has become a routine method in thousands of plant science labs. This model plant has a small genome (approximately 120 million base pairs encoding approximately 28,000 genes) that has been completely sequenced, allowing for many types of sophisticated genetic analyses. Almost all *Arabidopsis* genes have homologues in crop plants [14], and thus, knowledge of *Arabidopsis* biology can be transferred to major agricultural systems. Transformation via *Agrobacterium* using the "floral dip" method [15] is the most frequently used method to introduce genes into the *A. thaliana* genome and does not require tissue culture under aseptic conditions. This easy method of generating transgenic plants is performed in thousands of laboratories worldwide and can be conducted by undergraduate students even if more sophisticated plant transformation facilities are not available.

The involvement of the *GA5* (At4G25420) gene product, GA 20-oxidase, in the synthesis of active GA molecules in *Arabidopsis* has been shown by means of forward genetic approaches [12, 13]. In those studies, however, the complementation of the *Arabidopsis ga5-1* mutant with the wild-type copy of the *GA5* gene has not been reported. In the present work we show that semidwarf *ga5* mutants ectopically expressing a wild-type copy of the enzyme recover the normal size of wild-type plants. Binary constructs designed in this work (Fig. 2), *ga5-1* mutants, *ga5*-transgenic and wild-type *A. thaliana* ecotype Landsverg-*erecta* (L*er*) seeds are accessible to the community from our laboratory upon request, allowing instructors to skip the cloning steps. Transgenic *Agrobacterium* cultures carrying the pBI-*GA5* or empty vector constructs are also available. The rest of the equipment used in this exercise is common in a standard molecular biology classroom.

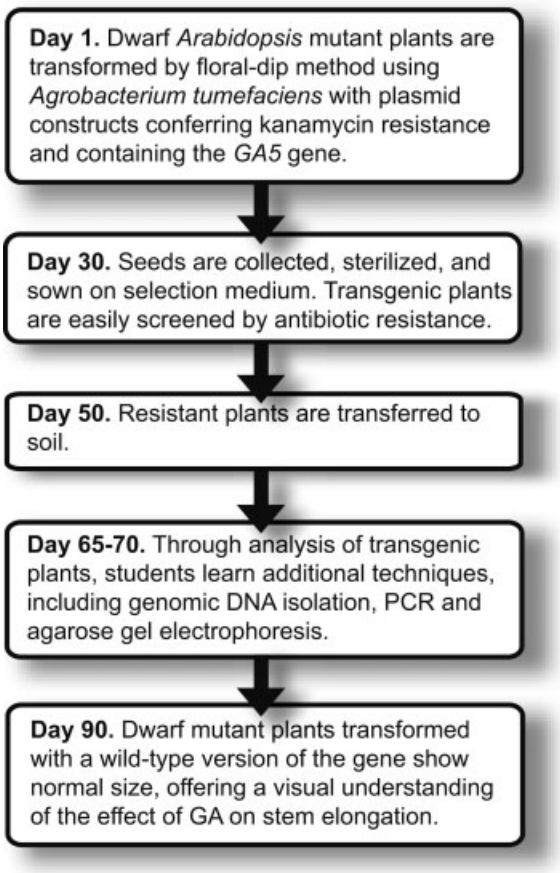

FIG. 3. **Summary of the laboratory exercise.** Dates are approximate and will vary with growth conditions and teaching schedules.

A summary of the main steps involved in this experiment is illustrated in Fig. 3. *Ga5* plants, which had been regularly sprayed with GA by the instructor and therefore showed wild-type height, were transformed during the first and third lab sessions. In addition, nontransformed *ga5* mutants were sprayed with GA or water (controls), and plants were observed in subsequent lab sessions. The fact that the semi-dwarf mutants that were sprayed with the hormone showed a normal stature compared with the controls sprayed with water, confirms that the mutation is at the level of hormone production, rather than affecting hormone perception [12]. Mature, dry seeds from transformed plants were harvested, sterilized, and germinated on Murashige Skoog (MS) plates in the presence of antibiotic. As shown in Fig. 4, green transgenic seedlings are easily distinguished from nontransgenic ones, which show white cotyledons.

PCR Analysis of Transgenics—The analysis of the transgenic plants by PCR allows the students to confirm the transformation and provides experience with this important technique. However, if time is limiting and/or this procedure is covered in other experiments, it is not essential and can be skipped because the selection medium allows only transformants to grow.

Further analysis of the transgenic plants involves PCR and gel electrophoresis methods widely applied in biotechnology. Students perform a crude DNA isolation from leaves, by means of a simple, inexpensive protocol that uses a buffer containing detergent and EDTA to break cells and inactivate DNA-degrading enzymes. Further precipitation with alcohol (isopropanol) eliminates other contaminants. After DNA isolation from several transgenic and wild-type plants, the presence of the transgene is confirmed by PCR. The *Arabidopsis* genomic DNA is the template, and the enzyme Taq DNA polymerase makes copies of the DNA sequence between two single-stranded DNAs (primers). A thermal cycler is used to repeatedly carry out a sequence of different temperature reactions required for the amplification. A better understanding of this technique and for other methods frequently used in molecular biology can be facilitated for students by simulations available on the internet [16].

PCR products are visualized by agarose gel electrophoresis (Fig. 5). The principle of this technique relies on DNA molecules (negatively charged because of their phosphate backbone) moving through an agarose gel when an electric field is applied, with the smaller molecules running faster. The size of the fragments is estimated by adding a reference "ladder" of defined DNA size fragments in one of the wells. The detection of the bands is possible because a dye (ethidium bromide) is added to the gel matrix. When the fluorescent dye is intercalated between DNA bases, the bands are visible upon UV illumination. Several interactive animations are found on the internet for this and other molecular techniques (e.g. ref. [17]).

As observed in Fig. 5, the selected primer pair allows for specific detection of the transgene by amplifying the fragment comprised by the *CaMV35S* promoter fused to the *GA5* cDNA (1.9-Kb band). As expected, the 1.9-Kb band is absent in wild-type L*er* and *ga5* mutant plants, and only present in transgenic lines L1–L5. Another primer set can also be used to amplify only the *GA5* sequence (data not shown; described below under day

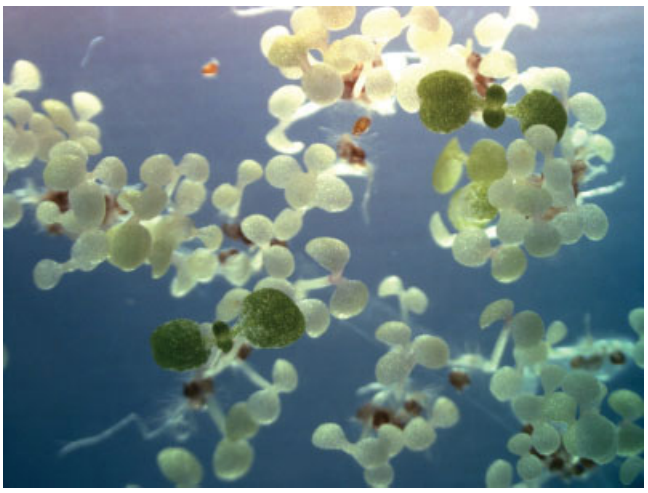

FIG. 4. **Selection of transgenic seedlings on kanamycin MS plates.** After 10 days, seeds carrying the kanamycin-resistance gene develop green-cotyledon seedlings with long roots. By comparison, nontransformants remain small, with short roots and pale coloration.

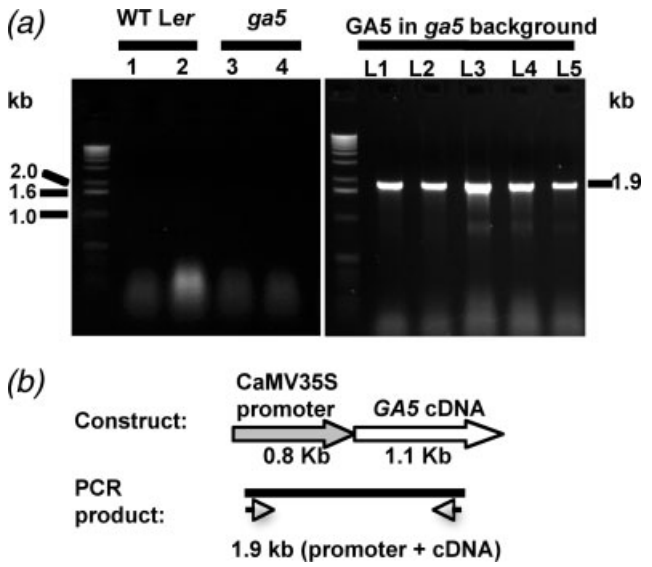

FIG. 5. **Screening of transgenic plants by PCR.** DNA was extracted from leaves of wild-type L*er*, *ga5-1* KO mutant and *ga5-1* lines transformed with *GA5* cDNA (L1-L5). (*a*) PCR reactions performed using a primer pair that amplifies the *CaMV35S* promoter sequence upstream of the *GA5* cDNA sequence, as illustrated in (*b*). Bands are absent in wild-type L*er* (lanes 1–2) and *ga5* KO (lanes 3–4), and present in *ga5* complemented lines (L1–L5).

65). In this case, PCR products are obtained in all samples including *ga5* controls and wild-type plants. This is expected, as this is an endogenous gene, and because the *ga5-1* is a probable point mutation resulting in a base substitution that cannot be seen unless the amplified fragment is sequenced. However, the bands differ in their size: the 1.4-Kb band observed in all samples corresponds to the length of the full genomic sequence, which contains the 190-bp intron, whereas the smaller 1.2-Kb band corresponds to the cDNA sequence and is only present in the transgenic plant. The size difference between cDNA and the genomic amplified fragment provides the basis to introduce the concept of introns, exons, coding regions and messenger RNA (mRNA).

The success of the genetic complementation approach described in this laboratory exercise is illustrated in Fig. 6. Dwarf mutants expressing the inactive GA 20-oxidase protein (Fig. 6, center) are efficiently rescued by the introduction of a wild-type copy of the gene (Fig. 6, right). The transgenic plants show the same size as wild-type control plants (Fig. 6, left).

Suggested extensions—Depending on the scope of the course and time availability, further extensions can be applied to this experiment. For instance, a simple bioinformatics exercise where students are given the wild-type and the mutated gene sequences, would consist of finding the base mutation by sequence alignment, and further identifying the amino acid change by aligning the translated protein sequences.

Possible extensions of this experiment if time allows could include the following

1) Crossing the transformed plants with *ga5* mutants to observe the segregation of the transgene. Depending on the number of transgene insertions in the line, students should observe 3:1 or higher ratios of normal:dwarf. Explaining these ratios would be an interesting exercise (PCR on some of the offspring should confirm the genetics).
2) Crossing over several generations should result in suppression of the transgene and reversion to dwarf phenotype. This would be detectable by PCR (dwarves which have the transgene).

SCHEDULING

Before the plant transformation laboratory, introductory lectures are offered, which include background on *Agrobacterium* infection of plants, plant transformation, and gibberellin hormone physiology. Additional introductory lectures can be presented at the beginning of each lab session.

Student Schedule

Day 1. Transform Plants by Floral Dip (I)—Students are organized in teams of two members. Each team is provided four pots of *ga5* mutant plants that were previously sprayed with active gibberellin (GA_4) to overcome dwarfing and ensure fertility. Two pots per team are transformed with the pBI-*GA5* construct, and two with pBI121 empty vector (control). To minimize the number of untransformed seeds, the TA should clip off the siliques the day before the transformation. The *Agrobacterium* inoculum is poured into a beaker and pots are inverted to dip plants into the suspension. After 5 min dipping, plants are removed, placed horizontally on their side on a tray, and covered with plastic wrap until the next day. The floral dip method is described in detail in ref. [15], with a simplified version being available online [18], and it is also clearly illustrated at http://www.bioinformatics.vg/Methods/arabitransformationf.htm.

FIG. 6. **Comparison of 6-week-old plants grown in soil.**

To chemically rescue the wild-type phenotype, dwarf *ga5* plants are sprayed with a 10^{-4} M solution of GA_4, whereas controls are sprayed with water. These plants, and wild-type L*er*, are grown side by side in a growth chamber and students should make weekly observations as described below. Note: this step can be omitted if plant growth facilities are limited.

Day 2—Transformed plants are uncovered, set upright, and grown under the same conditions described in TA schedule (see below).

Day 7. Transform plants (II)—Observe and photograph images of WT, *ga5* mutants and *ga5* mutants sprayed with GA_4.

Plants that were sprayed with GA and controls (*ga5* sprayed with water and wild-type) are measured and photographed. Students will compare the size of wild-type, mutants, and GA-sprayed mutants. Pictures and plant height data are recorded for presentation in the final report.

The transformation step is repeated (as explained in Day 1) on the same plants. The instructor needs to prepare a second *Agrobacterium* suspension and the transformation procedure described is again performed. Although this second transformation step can be omitted, we encourage instructors to repeat it because transformation efficiency is considerably improved. After dipping, plants are again placed horizontally on plastic trays and covered with plastic wrap.

Day 8—Transformed plants are uncovered, set upright, and grown under the same conditions as described above for approximately 4 weeks. Watering is stopped when siliques begin to turn yellow.

Day 30–35. Harvest, sterilize and plate seeds from transformed plants

Harvest seeds—Approximately one month after transformation, plants should be ready for seed harvesting. It is convenient that stems and leaves are not completely dried because these brittle tissues produce small fragments that make seed cleaning difficult. Seeds from transformed plants are collected by shaking the plants on a clean piece of paper (e.g. newspaper sheet). To separate the very small seeds from other plant debris, the plant material is threshed with a hand sieve (mesh size No. 40) although other methods, such as machine threshing, are available. Ideally, a second sieve (mesh size No. 180) can be used to retain seeds and remove residual particles that are smaller than the seeds, although this last step can be skipped. Clean seeds are placed into 1.5 mL microfuge tubes. A typical yield is 20,000 seeds per plant if grown under optimal conditions [19].

Selecting transformed seeds by germination on antibiotics—Typically, about 0.1–1% of the seeds that are produced by plants dipped in the *Agrobacterium* solution will be transformed [20]. Transformation efficiency improves with the application of the vacuum infiltration method [21]. However, it varies among *Arabidopsis* strains, with Columbia 10-fold more efficiently transformed by this method than Landsberg *erecta*.

To identify transgenic seeds, the seeds collected above are germinated on sterile MS plates containing the selection antibiotic (kanamycin) and a bacteriostatic agent to prevent *Agrobacterium* growth (carbenicillin). The following steps are performed under a simple sterile transfer hood such as typically used in microbiological labs. Before or during this lab, students are taught how to work using sterile conditions. However, as undergraduate students are often not skilled in working in sterile conditions, plates frequently show contamination after a few days. To overcome this problem, the solid media can be prepared without sucrose, although growth is delayed by several days under these conditions. Seeds are sterilized by adding a 50% aqueous solution of commercial bleach with 0.1% Triton X100 and shaking for 15 min. After three to four rinses with sterile distilled water, seeds are resuspended in a 0.1% agarose sterile solution and evenly placed on antibiotic selection plates using a pipettor (about 100 seeds/plate). Wild-type control seeds are sterilized and sown on MS plates without antibiotic. Plates are placed 3–5 days in the dark at 4 °C to break seed dormancy and then incubated in a growth room/chamber for 2 weeks (Photoperiod: 18-hr-light/6-hr-dark, temperature: 20–22 °C). Alternatively, grow on bench top under white fluorescent light (80–100 μE m-2 s-1) in the same photoperiod conditions at room temperature. If a sterile transfer hood is not available, use of plates without sucrose and attention to standard microbiological procedures for transfer of organisms under aseptic conditions will allow success with the experiment.

Day 50. Transfer resistant plants to soil—Seedlings that are sensitive to kanamycin show arrested root growth, and do not develop green cotyledons. Resistant seedlings, which have a normal green phenotype, are carefully transplanted to soil using forceps, avoiding root damage, and placing five seedlings/pot. Because in the agar plate the humidity is nearly 100%, the soil must be well-moistened, and transplanted seedlings must be covered with plastic wrap or a plastic dome for a few days.

Day 65. Extract DNA and perform PCR—The next steps consist of confirming the presence of the transgene by PCR. Genomic DNA is extracted from a small leaf disk for PCR amplification [22]. Samples are taken from transgenic and control plants by pinching out a disc of leaf with the lid of a 1.5-mL microfuge tube. Material is quickly ground with a small pestle and 400 μL of extraction buffer (TA must prepare this in advance) are added. After 1 hour incubation at room temperature (preferably with agitation), samples are centrifuged at full speed, and supernatant is transferred into a clean 1.5 mL tube. DNA is precipitated by adding 300 μL isopropanol, mixing, and incubating 2 min at room temperature. DNA is pelleted by centrifuging 5 min at full speed in a microcentrifuge. After supernatant removal, the DNA sample is resuspended in 100 μL TE.

After preparing the DNA templates as above, we used a primer set consisting of GA5Rev (used for cloning and described in "construct design" section) as reverse primer with the forward primer annealing to a sequence in the 35S promoter (35SFor: 5′ cacacTTAGAGAGGCTTACGCAGCA 3′). Each PCR reaction contains 1 μL DNA template, 1 μL each primer, 2 μL 10X buffer Taq, 2 μL dNTPs (10 mM stock), 0.5 μL Taq polymerase and water to make a 20 μL final volume. Optionally, premixed PCR

reactions can be purchased (e.g. Go Taq, Promega). PCR conditions are set as follows: 95 °C for 5 min (denaturation), followed by 30 cycles of 30 seconds at 95 °C (annealing), 30 seconds at 55 °C (synthesis), 2 min at 72 °C, with a final extension step at 72 °C for 10 min. Samples may be removed by the instructor when the PCR sequence is done and placed at −20 °C until the next lab.

Note: PCR can also be done with *GA5* cDNA specific primers used for cloning (see "construct design" for primer sequences).

Day 67. Analyze PCR Products by Agarose Gel Electrophoresis—To visualize the amplification products, a 1% agarose gel is prepared by heating 1 g agarose powder and 100 mL buffer tris-borate EDTA (TBE, made by the TA) in a microwave until the agarose is completely melted. At this point, ethidium bromide is added (to a concentration of 1 μg/mL) and, when the solution reaches a temperature of about 60 °C, it is poured into a casting tray with a comb to make the sample wells. Upon solidification, the comb is removed and the gel is placed into the electrophoresis chamber. TBE buffer is added and samples are loaded together with a reference ladder. Usually, electrophoresis is performed at 90 mV for about 45 min. Finally, DNA bands are visualized by placing the gel on an UV transilluminator, and a picture is taken using a digital camera.

Day 90. Compare Transgenic Plants—Transgenic and control plants are observed, their size is measured, and photos are taken. These results are included in a written final report.

ASSESSMENT

Because it is fundamental that students understand the experiment before they begin, each lab session they are asked to prepare a protocol and a preabstract which explains the objectives of the experiments. They must have a notebook were they take lecture notes, and write working protocols, raw data, data analysis and conclusions. Short (5 min) quizzes are also given at the beginning of most lab periods to test student comprehension of the experiment before starting it.

As part of the course requirements, students produce a final report, which ideally should be written in the format of a short scientific publication. When the course starts they are given a handout with guidelines on how to write abstracts and how to prepare the final report. Special emphasis is made on plagiarism. They need to be aware that their written work must be always properly credited and they cannot report the work of others as if it were their own (http://www.msu.edu/unit/ombud/plagiarism.html).

CONCLUSIONS

This laboratory exercise allows teachers to integrate plant transformation biotechnology in their classrooms, illustrating the application of basic knowledge to modify plant traits that have applications to global food production. It provides an early training for students interested in plant biology, as these techniques are broadly used in plant research. It also stimulates and facilitates discussions on contemporary issues, such as application of biotechnology to food production, and help students understand the procedures behind biotechnological manipulations, allowing them to more objectively evaluate potential risks and benefits related to these procedures. By means of a simple transformation assay, students learn about gibberellin hormone physiology while acquiring hands on experience on plant research methods.

Instructor/TA Schedule

Prepare Plant Material—A detailed explanation of *Arabidopsis* growth conditions and care can be found at The Arabidopsis Information Resource online database [23]. It is recommended to start planting at least 30–40 days before the course begins. Seeds of wild-type *Arabidopsis* (ecotype Landsberg *erecta*) and *ga5-1* mutants (in L*er* background) are sown on a mixture of soil:vermiculite:perlite (1:1:1 v/v/v) and stratified in a cold room or refrigerator for 4 days at 4 °C. After planting, it is helpful to cover the soil in the pots with a nylon window screen mesh (available from hardware stores) to hold the soil during the plant dipping procedure. Plants are grown under white fluorescent light (80–100 $\mu E/m^{-2}/s^{-1}$) in a 18-hr-light/6-hr-dark photoperiod at 20–22 °C and relative humidity at 60–70%. Starting at 4-weeks and continuing weekly, plants that are to be transformed are sprayed with an aqueous solution of 10^{-4} M solution of GA_4, containing 5% (v/v) ethanol [12]. Because *ga5* mutants grow very slowly unless sprayed regularly with GA, the hormone-spraying step is essential for the project to work in the allotted time. Reserve some pots without spraying as a control so that students can observe the size of the mutant.

Transform and Grow Agrobacterium—Start at least 1 week before plant transformation (transformation is Day 1 in student schedule). The DNA construct or empty vector (1 μL of 0.2 μg/mL) is introduced into electro-competent *Agrobacterium tumefaciens* strain C58CI (50 μL aliquot) by electroporation. Alternatively, if an electroporator is not available, use the freeze-thaw transformation method [20, 24]. After adding 1 mL sterile YEP[a] liquid medium (without antibiotics), cultures are grown for 4–6 hours at 28 °C with shaking. To select transformed *Agrobacterium*, plate 10–25 μL of 1:10 and 1:100 dilutions of liquid culture on YEP[b] plates containing 50 μg/mL rifampicin, 25 μg/mL gentamycin, and 50 μg/mL kanamycin and grow at 28 °C for 2 days. Isolate an individual colony, streak onto a fresh plate, and allow growing for 2 days in the same conditions. Use colonies from this plate to start 3 mL-YEP liquid cultures (for both *GA5* construct and control) 2 days before the plant transformation. Grow overnight at 28 °C with shaking. Inoculate 2 × 250 mL YEP in 1L flasks (plus antibiotics) with 1 mL of the ON culture. Grow 20–24 hour.

[a]YEP medium: 1% Bacto® tryptone, 1% Bacto® yeast extract, 0.5% NaCl, pH 7.0. Sterilize in autoclave for 20 min.

[b]YEP-solid medium: add 1.5% Bacto agar before autoclaving.

Prepare Agrobacterium for Plant Transformation—Pour the above 250 mL *Agrobacterium* cultures into 250 mL centrifuge bottles, and centrifuge at 6,000 rpm for 15 min at 4 °C. Resuspend the bacterial pellet in about 600 mL of infiltration media. The infiltration media is prepared as follows (1.5 L): 3.3 g MS salts (available from Research Products international Corp, IL, or can be prepared according to ref. [25]), 1.5 mL B5 vitamins 1000X, 75 g sucrose, 0.75 g MES, 4.95 μL BAP (benzylaminopurine) 13.3 mM. After autoclaving for 20 min, add 300 μL Silwet L-77 (surfactant purchased from Lehle Seeds, TX). The 1000X B5 vitamin solution (10 mL) contains 1 g Myo-inositol, 0.1 g Thiamine, 10 mg Nicotinic Acid, and 10 mg Pyridoxine. Steps 2 and 3 are required for Day 1 and Day 7 in student schedule.

Prepare Media and Buffers—MS plates are used to grow transgenic and control plants after seed harvesting (Day 30). For 50 plates, 1 L medium is prepared containing 3.3 g MS salt, 5 g sucrose, 7 g Phytagar (Gibco), and 1 mL B5 vitamins 1000X (use 1 M KOH to adjust to pH 5.7). After autoclaving, about 20 mL per petri dish is poured; these plates are used to germinate and grow wild-type seedlings. For selection of plants that are transformed, 50 μg/mL kanamycin (from 50 mg/mL stock stored at −20 °C) and 200 μg/mL carbenicillin (from 200 mg/mL freshly prepared stock) are added to media before pouring. Also prepare a solution of 0.1% agarose in distilled water and autoclave; this will be used to spread the seeds easily on the plates. DNA extraction buffer and TE are prepared for use about Day 65, when students perform DNA extractions for PCR. DNA extraction buffer has the following composition: 200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS and must be autoclaved. Buffer TE consists of 10 mM Tris and 1 mM EDTA, pH 8.0, and is prepared from 1 M Tris and 0.5 M EDTA stock solutions in sterile water. TBE buffer is used for agarose gel electrophoresis and is needed to check PCR products. Make 500 mL of 10X TBE, autoclave, and dilute 1 to 10 for the gels at the start of the lab period (10X TBE: 54 g Tris base, 27.5 g boric acid, 20 mL 0.5 M EDTA pH8.0, and distilled water up to 500 mL).

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