Chapter 3

Blue Plants: Transgenic Plants With The Gus Reporter Gene

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Reprinted From: Karcher, S. J. 2002. Blue plants: Transgenic plants with the GUS reporter gene. Pages 29-42, in Tested studies for laboratory teaching, Volume 23 (M. A. O'Donnell, Editor). Proceedings of the 23rd Workshop/Conference of the Association for Biology Laboratory Education (ABLE), 392 pages.

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Introduction

The investigative laboratory exercise described in this chapter is used as an independent project in a sophomore level laboratory in genetics and molecular biology. The transgenic *Arabidopsis* plants we use contain the GUS reporter gene under the control of the *cor*15a gene promoter, which responds to cold stress. Following induction by cold or other environmental signals, the gusA gene will respond by producing the enzyme beta-glucuronidase (GUS). When plant tissue is incubated with the chromogenic substrate X-gluc, those tissues that produce GUS turn blue.

This is a large class of about 200 students taught in 12 laboratory sections. For this experiment, students work in groups of two. Some students work in larger groups of their own choosing so that they will have more plants to work with to test the effects of a larger range of variables or treatments. Students are given the material (background on transgenic plants, reporter genes, and the beta-glucuronidase assay) provided below in the laboratory manual. Before the laboratory time for this experiment, students do the Independent Project Plan (provided below) that asks them basic questions about the system and asks them to plan an experiment that they will then carry out themselves. The instructors approve the experimental plan the students design. Typically the students design a protocol that examines the effects of other stressors on the *cor*15a promoter. Such stressors include drought, high salt, elevated temperatures and reduced temperatures. For this large class, the instructors grow the transgenic plants so they will be ready for the experimental treatments by a certain date. Students could be asked to grow their own plants from seed. Students are given their plants (at least six - eight individual plants per student). Students have two or three weeks to conduct their experiments and assay for beta-glucuronidase activity. The students then prepare a poster summarizing their experimental design and results. The class concludes with a poster session and brief oral presentation by each group of students.

Student Outline

Objectives

- To understand how reporter genes work.
- To understand applications of reporter genes.
- To learn about transgenic plants.
- To gain experience in designing and setting up a protocol with appropriate controls.
- To prepare a virtual poster describing the experiment's results.

- Using the Transgenic Blue Plants System.
  Formulate a hypothesis to test.
  Devise a method to test it.
  For this independent project, students may work in larger groups of 4 or 6 if they prefer.
  Submit a detailed list of materials needed.
  Prepare the Transgenic Blue Plants for the experiment.
  Assay the GUS reporter gene in the Transgenic Blue Plants.

Pre-lab Planning Questions

Read the independent project description in the lab manual. Note we are restricting the independent project to work with the Transgenic Blue Plant System -- cor15a-GUS fusion *Arabidopsis* plants. You and your partner will determine a question to ask about the plants. You will generate a list of materials you will need and indicate the number of plants you plan to test. You will be conducting a small pilot study (please limit plant use to 6 per group). You will receive feedback about your project before you begin it.

Note:

Pre-treat your plants. See the schedule to know when plants will be available.

Answer the following.

This may be handwritten. Hand your answers to your TA.

1. What is a reporter gene?
2. What is X-Gluc?
3. What is T-DNA?
4. What is the cor15a gene?
5. After reading the lab manual, consult with your partner to determine a question you would like to ask about the cor15a-GUS fusion *Arabidopsis* plants we have available.

Formulate a hypothesis to test.
State your hypothesis.

6. Write a list of materials you will need to test your hypothesis. Be specific about the condition or conditions you plan to test. Please be practical about the needs. You may be asked to supply some of the materials yourself.

Overview of Independent Project

Students will formulate a hypothesis, design, and execute a protocol using transgenic plants containing a *uid*A (*gus* A) reporter gene under the control of the *cor15a* gene promoter, which responds to cold stress. Following induction by these environmental or developmental signals, the *gus*A gene will respond by producing the enzyme β-glucuronidase (GUS). When plant tissue is stained with the chromogenic compound X-gluc, those tissues that produce GUS will turn blue.

Introduction

Plant Biology

Plants have developed unique and interesting mechanisms to deal with problems of predation, environmental stresses (such as heat and cold, salinity, heavy metal toxicity, lack of light) and biotic stresses (such as pathogen attack). Some of these mechanisms are very rapid (*i.e.,* the response occurs within seconds or a few minutes of the stimulus) while some responses occur only after many minutes or hours. In some instances, (*i.e.,* pathogen attack) secondary responses can occur in remote portions of the plant not under attack a day or two after the initial inducing insult. These latter responses generally result from changes in gene activity, and lead to the production of proteins not normally made by the plant, or normally made only in small quantities in specific organs. In addition, plants take many of their developmental cues form their environment.

If one has isolated a gene that is induced in response to a specific stimulus; if one has developed an assay for the activity of an induced enzyme, or if one has raised an antibody to an induced protein, one can follow the induction of that specific gene by using RNA or Western blot analysis, *in situ* RNA or protein visualization (tissue printing), or enzymatic assays for the activity or the specific gene. However, because these techniques and assays are cumbersome and time-consuming, researchers often turn to following "reporter gene" activity to monitor the response of genes to specific stimuli.

Reporter Genes

A reporter gene encodes an enzyme with an easily assayable activity that is used to report on the transcriptional activity of a gene of interest. Using recombinant DNA methods, the original promoter of the reporter gene is removed and replaced by the promoter of the gene to be studied. The new chimeric gene (Fig. 1) is introduced into an organism, and the expression of the gene of interest is monitored by assaying for the reporter gene product. A reporter gene allows the study of expression of a gene for which the gene product is not known or is not easy to identify. To determine the patterns of expression of environmentally or developmentally regulated genes, reporter genes are placed under the transcriptional regulation of promoters that show interesting developmental and/or stress responses. In bacteria, the *lac*Z gene from *E. coli*, that encodes β-galactosidase, as a reporter of gene activity. *lac*Z can be used as a reporter in bacteria that are naturally lac^-, or that are lac^- due to a mutation. This gene can also be used in many animal systems that lack endogenous β-galactosidase activity. Other reporter genes often used in bacteria and animals include *cat*

(encoding the enzyme chloramphenical acetyl transferase), *fus* (encoding the jellyfish green fluorescent protein), and *lux* (encoding the enzyme firefly luciferase). Plants contain endogenous β-galactosidase activity, so *lac*Z is not generally a useful reporter gene for plants. A widely used reporter gene in plants is the *uidA*, or *gus*A, gene that encodes the enzyme β-glucuronidase (GUS). This enzyme can cleave the chromogenic (color-generating) substrate X-gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronic acid; Fig. 2), resulting in the production of an insoluble blue color in those plant cells displaying GUS activity. Plant cells themselves do not contain any GUS activity, so the production of a blue color when stained with X-gluc in particular cells indicates the activity of the promoter that drives the transcription of the *gus*A-chimeric gene in that particular cell. The GUS assay is easy to perform, sensitive, relatively inexpensive, highly reliable, safe, requires no specialized equipment, and is highly visual (Jefferson 1987; Jefferson *et al.* 1987; Jefferson and Wilson 1991).

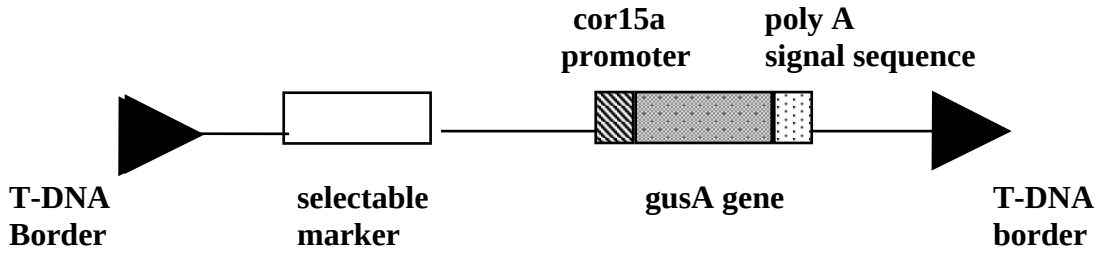

Figure 1. The T-DNA promoter/gus fusion. This represents the T-DNA region of an *Agrobacterium tumefaciens* vector to make transgenic plants containing promoter-*gusA* fusions. The T-DNA borders are processed out of the vector and delimit the DNA that will be transferred to the plant cell. The selectable marker (kanamycin-resistance, in this case) is used to select plant cells that contain this T-DNA region. The fusion gene is made of the promoter of interest added to create a translational fusion to the *gusA* gene and a polyadenylation signal sequence. This fusion gene will express GUS activity in appropriate cell types under the appropriate environmental conditions.

GUS reporter gene fusions used in plants include genes that respond to the phytohormone auxin, that have been studied by Hagen and Guilfoyle (1985). They identified genes (the SAURs or small auxin up RNAs) that respond very rapidly to auxin (McClure and Guilfoyle 1987; McClure *et al.* 1989). Li *et al.* (1991) and Liu *et al.* (1994) have used SAUR gene/*gusA* reporter gene fusions to investigate auxin-stimulated events in transgenic tobacco plants.

Transgenic Plants

In order to use GUS as a reporter of promoter activity, a transgenic plant containing the promoter-*gus*A chimeric gene is made. (A transgenic organism has added foreign DNA.) There are several ways to generate transgenic plants. For many plant species, the easiest method is *Agrobacterium*-mediated gene transfer. When *Agrobacterium* infects a host plant, a part of the Ti (tumor-inducing) plasmid of the bacterium is transferred from the bacterium to the plant. The transferred DNA (called the T-DNA) is integrated into the plant nuclear DNA (Binns and Thomashow 1988; Watson 1992). The normal T-DNA contains genes that encode plant growth hormones and cause the production of a plant tumor called a crown gall. None of the T-DNA genes are necessary for T-DNA transfer from the bacterium to the plant

cell. Other genes (the virulence, or *vir* genes) on the Ti-plasmid are necessary for transfer. *Vir* genes are not normally transferred to the plant, but encode proteins needed for the processing of the T-DNA from the Ti-plasmid, proteins that form the channels in bacterial walls through which T-DNA exits to the plant, and proteins that accompany the T-DNA to the plant cell, target it to the nucleus, protect it from nuclease digestion, and perhaps aid in the integration of the T-DNA into the plant chromosome. Any DNA within the T-DNA will be transferred to the plant and integrated into the plant nuclear DNA. Using recombinant DNA methods, the tumor causing genes were deleted from the T-DNA and any DNA of interest can be inserted. In addition to the gene of interest, the gene for a selectable marker is put between the T-DNA borders. This allows the selection of plant cells that have been genetically transformed. Selectable markers used include antibiotic resistance genes and herbicide resistance genes. The modified T-DNA is put back into *Agrobacterium* and is transferred to the plant by the normal infection process. Intact plants are produced (Fig. 3). Plants are regenerated, self-pollinated, and seed from these plants is germinated on the selective media. Only plants that have been shown to be homozygous for the inserted T-DNA (containing the *gus*A gene) are used for these laboratory experiments.

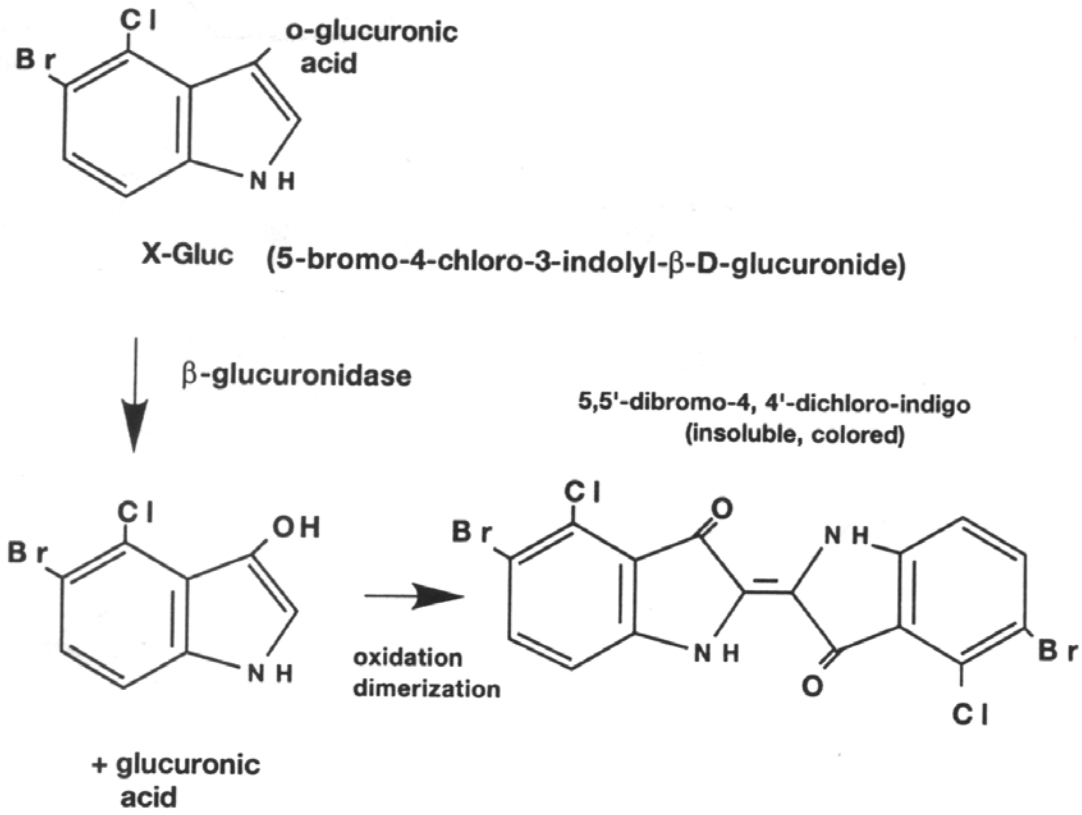

Figure 2. X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide), a substrate of β-glucuronidase, is cleaved to produce glucuronic acid and chloro-bromoindigo. When oxidized, chloro-bromoindigo dimerizes to produce the insoluble blue precipitate dichloro-dibromoindigo.

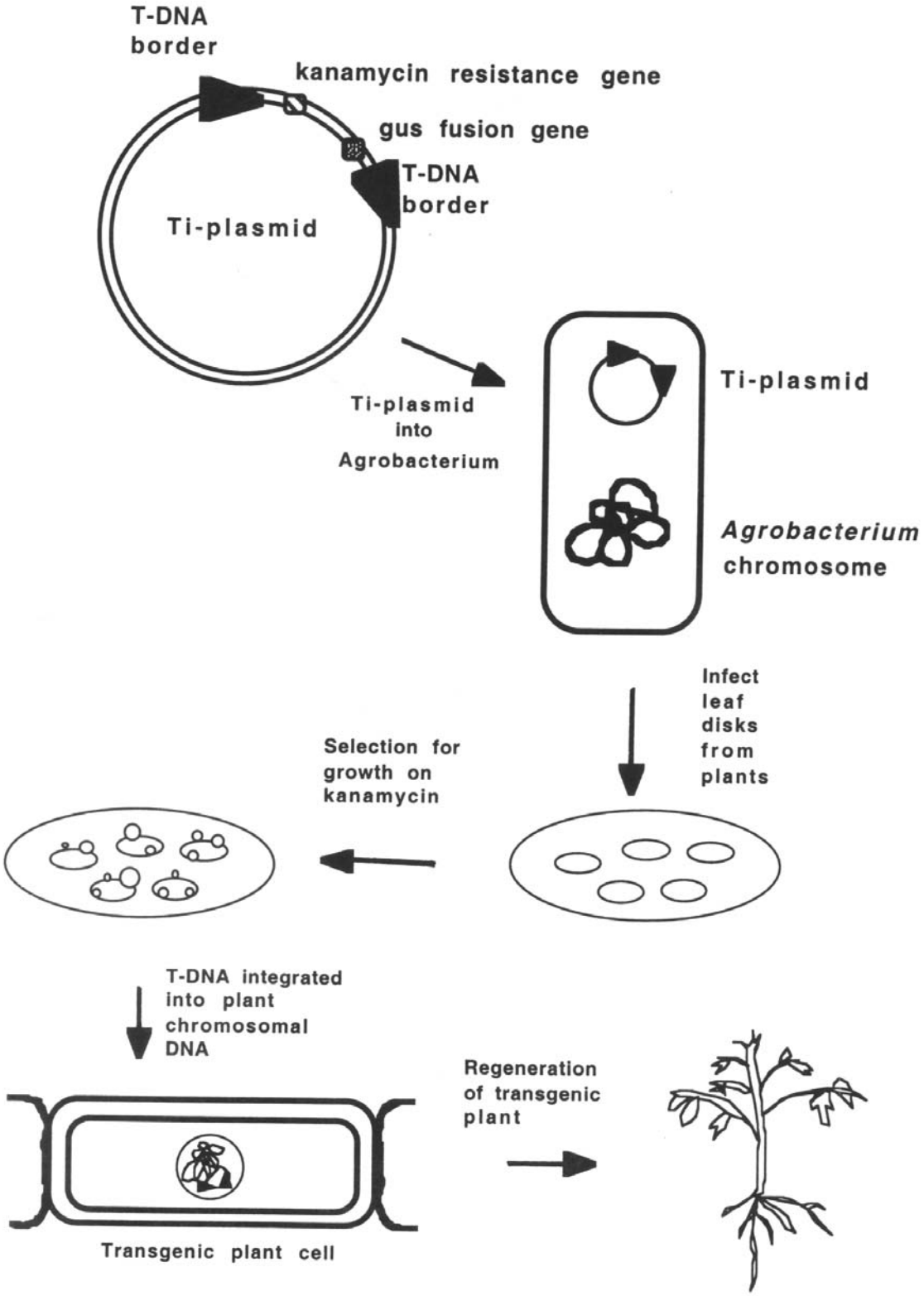

Figure 3. T-DNA vector to insert foreign DNA into plants.

cor or Cold-Regulated Genes

Many plants respond to low, nonfreezing temperatures by changing the intracellular concentrations of carbohydrates and free amino acids, their isozyme patterns, and their membrane composition. In addition, there is an alteration in the activity of many genes in response to low temperatures. Dr. Michael Thomashow of Michigan State University studies how plants respond to cold temperatures. He has examined cold-induced genes in the plant *Arabidopsis* and cloned *cor* (cold related) genes. The response of one of these genes, *cor15a* will be used in the laboratory experiments presented in this paper. The *cor15a* gene is induced by cold temperatures, drought, and the hormone abscisic acid. *cor15a* is a nuclear gene that encodes a 15 kDa protein (Baker *et al*. 1994; Hajela *et al*. 1990). The function of this protein is not known. The protein is translocated to the chloroplasts and may have a role in protecting plants from cold. Baker *et al.* (1994) isolated a DNA fragment containing the promoter region and the first few amino acids from the *cor15a* gene and joined it, in the correct translational reading frame, to the gusA gene, generating a *cor15a-gusA* translational fusion that was introduced into *Arabidopsis* plants using *Agrobacterium.* This chimeric reporter gene can be used to monitor the response of the *cor15a* gene in various plant tissues.

In this laboratory investigation, transgenic *Arabidopsis* plants that contain the *cor*15a/GUS fusion gene are used. Students can investigate the expression of the *cor* gene under different conditions by assaying for GUS activity. Procedures to examine the cold response of the *cor* gene are given in detail.

The expected results for cold response are that plants from the cold will show blue indicating GUS activity in the leaves, stems, and roots while the control plants will not be blue. If the plants are incubated in X-gluc for more than 16 hours, some light blue staining of the control plants may also occur because the *cor15a* gene is transcriptionally active to a low level at room temperature.

Procedure

Source of Seeds

Starter seeds of the *cor15a Arabidopsis* transgenic plants may be obtained from the author.

Germination of seeds

1. Seeds of transgenic *Arabidopsis thaliana* plants harboring a *cor15a-gusA* chimeric gene are germinated in sterile soil at room temperature (approximately 14 hours light-10 hours dark) until the plants are approximately 1 inch tall (2-3 weeks).
2. Seeds need to be refrigerated for 12-24 hours before planting to improve the germination rate. Seeds may be stored in the refrigerator indefinitely, so that seeds are ready to plant at any time.
3. Place sterile soil in pots or trays that have holes in the bottom of the pots or trays. Scatter the seeds onto the soil. The seeds are very small—somewhat larger than the size of the period at the end of this sentence. One easy way to distribute seeds evenly is to place the seeds on a piece of white paper and tap the paper to knock seeds off onto the soil. Seeds are not covered with soil. Place the pots or tray in larger trays. Fill the larger trays with enough water to let the soil get wet. Note that the soil is only watered from beneath, not from above. Cover the pots or trays with plastic wrap to keep the soil moist until germination occurs.

Note: Do not allow the soil to dry out because desiccation stress can induce the expression of the *cor15a* gene.

Placing plants under cold stress

1. Response to cold stress. Plants, still in soil, are placed in a plastic bag (so they do not dry out) and refrigerated for 24 to 48 hours.
2. Control plants are kept at room temperature**.**
3. Assay the plants for Gus activity.

Expected results for cold stressed plants

The expected results are that the leaves and stems of the plants incubated in the refrigerator will all turn blue, whereas the tissues of the control plants should not turn blue. If the plants are incubated in the assay solution for more than 16 hours, some light blue staining of the control plants may also occur, indicating that, even at room temperature, the *cor15a* gene is transcriptionally active at a low level.

GUS Assay

1. Gently remove plants from the soil. Be careful not to break off the roots when removing the plants from the soil, because one will want to examine the roots. Gently wash the plants in water.
2. Place the plants in a small 1.5-ml plastic microcentrifuge tube containing the GUS assay solution. (Four ml plastic test tubes with caps containing a small volume of the assay solution can also be used.) Be sure to include a positive control to monitor the extent of reaction. For example, it may be necessary to leave the plants in the assay solution at 37^{o}C for 30-45 minutes to overnight or at room temperature for 8 to 12 hours to 24 hours.
3. If the blue color is difficult to visualize against the dark green background of mature leaves, take the plants out of the GUS assay solution and place them in 70% ethanol. (Make 70% ethanol by mixing 30 ml distilled water and 70 ml 100% ethanol.) The chlorophyll is extracted into the ethanol making it easier to see the blue staining of the plant tissue. Move the plants to fresh 70% ethanol several times, if necessary, until the plant tissues are light green or clear.

Preparation of GUS assay solution.

X-gluc solution to assay for GUS activity:

1 mM X-Gluc (5-bromo-4-choloro-3-indolyl) β-D-glucuronic acid in 50 mM Na_2HPO_4, pH 7.0 and 0.1% Triton X-100. Store aliquots of the solution in the dark in a refrigerator or freezer. [Supplier of X-Gluc: Rose Scientific; 4027 97th St.; Edmonton, Alberta; CANADA T6E 5Y5; phone 1-800-661-9288]

To make 100 ml of X-Gluc solution:
Weigh out 0.052 g of X-Gluc. Dissolve the X-Gluc in about 50 ml of sterile, distilled water. Add 5 ml of 1 M sodium phosphate stock solution and 0.1 ml of the detergent Trition X-100. Mix. Bring the volume up to 100 ml with distilled water.
Store the solution in the dark in the refrigerator. Solutions of X-Gluc can be prepared, aliquoted, and stored frozen until needed.

To make 100 ml of 1M sodium phosphate stock solution:

Dissolve 14.2 g of Na_2HPO_4 (dibasic sodium phosphate) and 13.8 g of $NaH_2PO_4 \cdot H_2O$ (monobasic sodium phosphate) in approximately 90 ml of sterile, distilled water. Adjust the pH to 7.0 using NaOH. If necessary to reduce the pH, use phosphoric acid. Bring the volume to 100 ml with distilled water.

Suggestions for investigations
Using this system, the student design and carry out a set of experiments to answer his/her own questions about these transgenic plants. For example, some questions might include:

1. What is the shortest time in the cold that is needed to induce the *cor15a* gene?
2. What temperature induces the gene best? At what temperature is gene induction first observed? Do high temperatures as well as cold temperatures induce the gene?
3. Drought and the hormone abscisic acid also induce the *cor15a* gene. How many days does it take to induce the *cor15a-gusA* fusion gene after watering is stopped the plants?
4. Is the gene fusion induced by abscisic acid to a greater extent in some plant tissues than in other tissues?
5. Other questions???

Notes for the Instructor

Answers to Pre-lab Planning Questions

1. A reporter gene codes for an easily assayable enzyme that is used to report on the transcriptional activity of the promoter of the gene of interest. The original promoter of the reporter gene is removed and replaced by the promoter of the gene to be studied.
2. X-gluc is 5-bromo-4-chloro-3-indolyl beta-D-glucuronic acid, which is a color-generating substrate for the enzyme beta-glucuronidase.
3. T-DNA is the region of the *Agrobacterium tumefaciens* Tumor Inducing plasmid that is excised from the plasmid and transferred to the infected plant cell where the T-DNA is integrated into the nuclear DNA. A gene of interest can be inserted into the T-DNA and then introduced into a host plant cell.
4. The cor15a gene is a gene identified from *Arabidopsis,* the expression of which is induced by cold.

Materials needed for the Blue Plants Experiment

- A room (kept at room temperature) with a rack of lights to use for germination of seeds.
- Trays, pots, sterile soil.
- A refrigerator (14 ºC) to use to cold stress plants.
- Incubators or other refrigerators to provide other temperatures students may propose to test.
- Water bath set at 37 ºC. The water bath should have a few racks in it to hold microfuge samples, about six tubes per group.
- If possible, having a few dissecting microscopes (1 per every 4 people) available is useful. This is not absolutely necessary, however.
- Several pints of 70% ethanol.
- A white light box and Saran wrap. The light box is to display plants on.
- The following materials are needed per pair of students:
  tweezers or forceps
  scalpel
  dissecting needle
  about 6-10 sterile 1.5 ml microfuge tubes
  rack for microfuge tubes
  sterile 5 ml pipettes (about 5-10 per group) and pipette aids--black bulb or a Pi-pump
  Parafilm

Acknowledgments

This exercise is used as an independent project in Biology 242, Laboratory in Genetics and Molecular Biology at Purdue University, a sophomore level course for biology majors.

Thanks to Dr. Stanton B. Gelvin of Purdue University for his leadership role in developing these plant exercises for the teaching laboratory. Thanks to Dr. Michael Thomashow of Michigan State University for providing the first seeds for the *cor15a Arabidopsis* transgenic plants. Development of Transgenic Blue Plants was funded by National Science Foundation grant #9354721. Transgenic Blue Plants are also a part of Research Link 2000 *(http://www.researchlink.ferris.edu/).*

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