Chapter 4

Reporter Genes and Transgenic Plants to Study Response to Environmental Signals

Stanton B. Gelvin[1] and Susan J. Karcher[2]

Department of Biological Sciences, Purdue University
West Lafayette, IN 47907
[1]Telephone: (317) 494-4939; Fax: (317) 496-1496; e-mail: gelvin@bilbo.bio.purdue.edu
[2]Telephone: (317) 494-8083; Fax: (317) 494-0876; e-mail: suek@bilbo.bio.purdue.edu

Stanton Gelvin received an A.B in Biology from Columbia University (1970), a M.Phil. in Molecular Biophysics and Biochemistry from Yale University (1973), and a Ph.D. in Biology from the University of California, San Diego (1977). Following postdoctoral traineeships at the University of California, San Diego (1977–78) and the University of Washington (1978–81), where he was a Damon Runyon–Walter Winchell postdoctoral fellow, Dr. Gelvin assumed a position as Assistant Professor of Biological Sciences at Purdue University in 1981, where he is currently a Professor. Dr. Gelvin was a National Science Foundation Presidential Young Investigator, has served on the editorial boards of Plant Molecular Biology and Molecular Plant-Microbe Interactions (of which he is now Editor-in-Chief), and served on numerous grant panels for the USDA and NIH. His research interests include *Agrobacterium tumefaciens* and crown gall tumorigenesis, plant genetic engineering, and plant molecular biology. He has taught undergraduate and graduate level lecture and laboratory courses in plant molecular biology, cell biology, and genetics.

Susan Karcher received an A.B. (1972) in Biochemistry from the University of Wisconsin, Madison, and a M.S. (1974) and Ph.D. (1979) in Chemistry from the University of California, San Diego. She received postdoctoral training at the University of Washington (1979–1981). Dr. Karcher is an instructor in the Department of Biological Sciences at Purdue University, where she teaches both introductory and upper level genetics and molecular biology laboratory courses. Her research interests include plant molecular biology, interactions between pathogenic microbes and their host plants, and *Agrobacterium tumefaciens*. She hosted the 17th annual ABLE workshop/conference.

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Experiment I. Induction of *Agrobacterium tumefaciens* Genes by Plant Chemical Signals

Introduction

Many microorganisms that are associated with plants interact with them in complex ways. The plants and microorganisms are constantly exchanging chemical signals and thereby monitoring events and responses important to these interactions. For example, root cells of many leguminous plants secrete flavone and flavanone compounds that serve either to activate or repress genes in *Rhizobium* that are involved in nodulation. Similarly, many wound-induced phenolic compounds serve as defense response chemicals that allow plants to resist invasion by bacterial and fungal pathogens (see Peters and Verma, 1990, for a mini-review). In this laboratory, we shall investigate the responses of *Agrobacterium tumefaciens* genes to two different plant-derived chemical signals. One of these signals is a polygalacturonic acid-like molecule, most probably derived from the pectic portion of the plant cell wall, that activates a chromosomal gene, *picA*, of unknown function. The other chemical is acetosyringone, a phenolic molecule, that activates the *vir* genes of the Ti-plasmid.

Agrobacterium tumefaciens is a Gram-negative soil bacterium that causes the disease crown gall on many species of plants. Virulent strains of the bacterium bind to wounded plant cells and eventually cause the production of tumors (crown gall tumors) at the site of infection. The molecular mechanism of tumorigenesis involves the processing of a piece of DNA (the T-DNA, or transferred DNA) from a bacterial plasmid (the Ti-, or tumor inducing, plasmid) and the transfer of the T-DNA to the plant cell as a DNA-protein complex. This complex finds its way to the plant nucleus, following which the T-DNA covalently integrates into the plant chromosomes. Expression of genes carried by the T-DNA results in the over-production of the phytohormones auxin and cytokinin, resulting in uncontrolled plant cell growth, hence tumors. “Disarming” the T-DNA (i.e., deleting the hormone-producing oncogenes) has resulted in the development of the *Agrobacterium* system for the

genetic engineering of many plant species. This is the only known natural system in which genetic material is efficiently transferred from a prokaryotic organism (a bacterium) to an eukaryote (in this case, a plant).

The processing of the T-DNA from the Ti-plasmid and its transfer to plant cells is a complex process that is highly regulated genetically. Virulence (*vir*) genes found on the Ti-plasmid control these processes. The *vir* genes are not themselves transferred to the plant (i.e., they are not in the T-DNA region of the Ti-plasmid). Two *vir* proteins (VirA and VirG) compose a two-component sensing/response regulating system that responds to phenolic defense compounds synthesized by wounded plant cells. The VirA protein is located in the bacterial inner (periplasmic) membrane and senses the presence of wound-induced phenolic compounds such as acetosyringone. The VirA protein then transfers a phosphate group to an inactive VirG protein, thereby activating the VirG protein. Activated VirG protein is a transcriptional regulator that binds to DNA sequences ("*vir* box" sequences) preceding each *vir* operon, thus activating the expression of these genes. These remaining *vir* genes are involved in the processing of the T-DNA and its transfer to the plant cell. Thus, T-DNA processing and transfer do not occur until *Agrobacterium* detects a wounded and therefore potentially susceptible plant host.

Genes involved in bacterial pathogenesis and response to plant chemical signals are also located on the *Agrobacterium* chromosome. Some of these genes are involved in producing bacterial exopolysaccharides that are important for the binding of the bacteria to wounded plant cells. Other genes have unknown functions, but respond to various compounds produced by plant cells. *picA* (plant-inducible chromosomal gene A) is one such gene. This gene responds to pectic polysaccharides that probably derive from the cell walls of wounded plants. It is known that such oligosaccharides can regulate certain developmental and defense responses in plants. The finding that these pectic compounds at very low concentrations induce *Agrobacterium* genes implies that they form part of a chemical signaling system between the plant and the bacterium.

Materials

Quantities given are per pair of students, unless otherwise specified.

Bacterial culture tubes (sterile) and racks (12 tubes, 1 rack)
AB minimal media, pH 7.0 and 5.6 (5 ml each medium)
Acetosyringone induction medium (50–100 ml per class)
Acetosyringone (See recipe below)
Carrot root extract (50 ml per class)
Microcentrifuge (1 per 4–6 students)
Microcentrifuge tubes (14)
Vortex mixer (1)
30°C water bath (1 per 4–6 students)
Bacterial inoculation loops (1)
Z-buffer (50 ml)
ONPG (See recipe below)
1 M Na_2CO_3 (10 ml)
0.1% SDS (5 ml)
Chloroform (2 ml)
Klett-Somerson (or comparable) spectrophotometer (1 per class)
Sterile side-arm (Klett) flasks for bacterial growth (6 per class)
Spectronic 20 (or comparable) spectrophotometer (1)
7 ml tubes (or comparable) for the Spectronic 20 (or comparable) spectrophotometer (14)
1, 5, and 10 ml graduated pipettes (clean, but not necessarily sterile) (20)
25°C and 30°C incubator-shaker (you can use one shaker for both temperatures) (1 per class)

Student Outline

Goals

In this experiment, students will investigate the induction of *Agrobacterium tumefaciens* chromosomal and Ti-plasmid genes by various plant-derived chemicals. They will additionally determine under what conditions (e.g., pH) each gene responds to these chemicals.

Background

In the laboratory of the authors, we have isolated an *Agrobacterium tumefaciens* strain harboring a MudI-1681 insertion in a gene that is inducible by extracts from carrot roots and by polygalacturonic acid. MudI-1681 is a mini-Mu transposon harboring a kanamycin-resistance gene and a promoterless *lacZYA* operon. This operon is positioned just inside the Mu terminal inverted repeat sequence and, when inserted into an active gene in the correct orientation, serves as a reporter of gene activity by synthesizing β-galactosidase. We initially screened several thousand kanamycin-resistant *Agrobacterium* transconjugants, harboring MudI-1681 insertions in different chromosomal loci, on medium containing kanamycin and X-gal and either containing or lacking carrot root extract. Several colonies that were darker blue on the medium containing the carrot extract than on the medium lacking the extract were rescreened using ONPG as the substrate for β-galactosidase in a more quantitative assay. One strain, At156, consistently showed 10–50 fold higher β-galactosidase activity in the presence of carrot extract, or 1% polygalacturonic acid, than in the absence of these compounds. Thus, the gene into which MudI-1681 inserted is inducible by pectic substances.

In this laboratory, you will assay a derivative of At156, called At489, grown in the presence or absence of carrot root extract. In addition, because we shall be inducing another *Agrobacterium* strain (At41) with acetosyringone to monitor *vir* gene induction, we shall additionally test the effect of acetosyringone on strain At489.

Stachel et al. (1985a) constructed a *lacZ* fusion reporter cassette based upon Tn*3*, called Tn*3*-HoHo1. This transposon contains a β–lactamase gene (ampicillin or carbenicillin resistance) and a promoterless *lacZ* gene placed just inside the Tn*3* border repeat sequences. It is an extremely useful construction for the mutagenesis of DNA fragments cloned in a vector that can replicate in *E. coli.* Using this system, Stachel and Nester (1986) generated and tested a number of Tn*3*-HoHo1 insertional mutations in the *vir* region of the Ti-plasmid pTiA6 of *A. tumefaciens*. They discovered that *Agrobacterium* cells harboring certain of these fusions, when incubated in the presence of tobacco protoplasts (co-cultivation), showed greatly enhanced β–galactosidase activity relative to bacteria incubated in bacterial growth medium. Fractionation of the plant extracts identified a compound, acetosyringone, that induces the *vir* genes under the appropriate conditions (low pH, presence of a carbon source such as glucose, etc.) (Stachel et al., 1985b). Subsequently, others have shown that a number of related phenolic compounds can also induce the *vir* genes, sometimes to a greater extent than can acetosyringone.

Procedure

The strain that you will use to monitor *vir* gene induction, At41 (A348mx219), harbors a Tn*3*-HoHo1 insertion into the gene *pinF* (also called *virH*). This gene is greatly induced by acetosyringone at low pH (5.0–5.8) at temperatures less than 30°C. You will use this strain to monitor *pinF* induction when the bacteria are grown in various media.

Both *Agrobacterium* strains (At41 and At489) will be induced for approximately 24 hours in various media.

Grow precultures of these strains in AB minimal medium plus 0.5% sucrose (and kanamycin for strain At489 or carbenicillin for strain At41) at 30°C. Dilute *A. tumefaciens* At41 in AB-sucrose medium and grow to a Klett of 100 (overnight), pellet the cells, and resuspend them at a Klett of 50 in 2 ml of media #3, #4, #5, and #6 described below. Incubate them at room temperature overnight with shaking at 250 rpm. The next day, assay aliquots of the cells for β-galactosidase activity as described below. For *A. tumefaciens* strain At489, on the day before the assays, dilute these strains 1:100 into 2 ml of media #1 and #2 described below. Incubate them at room temperature overnight with shaking at 250 rpm.

The next day, assay aliquots of the cells for β-galactosidase activity as described in the Appendix.

The various induction (and control) media are:

1. AB minimal medium + 0.5% sucrose (pH 7)
2. AB minimal medium + 0.5% sucrose (pH 7) plus carrot root extract
3. Modified AB minimal medium + 0.5% glucose (pH 7)
4. Modified AB minimal medium + 0.5% glucose (pH 7) plus 100 μg/ml acetosyringone
5. Modified AB minimal medium + 0.5% glucose (pH 5.6)
6. Modified AB minimal medium + 0.5% glucose (pH 5.6) plus 100 μg/ml acetosyringone

Notes for the Instructor

1. The instructor may wish to set up the bacterial pre-cultures and induction cultures for the students. Streak fresh cultures of the bacterial strains on AB minimal medium plus 0.5% sucrose plus the appropriate antibiotics. Grow the cultures 2–3 days at 30°C until single colonies form. Use a single colony for inoculation of AB minimal growth medium. Growth in this medium should take approximately 1 day at 30°C. For *A. tumefaciens* At41, an additional overnight period of growth will be needed to grow the bacteria to a Klett of 100. Thus, one day more growth will be required for *A. tumefaciens* At41 than for At489. Induction of the bacteria can be set up before going home in the evening one day, and the bacteria assayed the next day. Thus, 3 days of preparation (growth and induction) will be required for *A. tumefaciens* At41, and 2 days for *A. tumefaciens* At489.
2. *A. tumefaciens* At41 may show some relatively low level of β-galactosidase activity when induced with acetosyringone at pH 7.0. This is because as the bacteria grow, they acidify the medium, sometimes overwhelming the buffering capacity of the MES buffer in the induction medium.
3. When incubated in some induction media, a particular strain of *A. tumefaciens* may not induce the *picA*::*lacZ* or *pinF*::*lacZ* fusion at all. Students should terminate the reaction after 30 minutes if little yellow color develops. On the other hand, following incubation in some induction media,

some *A. tumefaciens* strains may show high levels of β-galactosidase activity. Students should be ready, in these instances, to stop the reaction in 1–2 minutes.

4. The A_{550} and A_{600} readings, taken during the β-galactosidase assays, represent scattering by the cells (A_{600}) or by cellular debris after bacterial lysis (A_{550}). These readings, therefore, roughly estimate the concentration of bacterial cells in the assay tube.
5. *A. tumefaciens* strains used in these experiments can be obtained from the authors.

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APPENDIX I.

β-Galactosidase Activity Assay:

1. Spin down an aliquot (approximately 0.5 ml) of bacterial cells for 1 minute in Eppendorf microfuge tubes. Resuspend the bacteria in a final volume of 4 ml Z buffer in the tubes used in the spectrophotometer.
2. Adjust the concentration of cells such that the A_{600} is 0.1–0.25. Note this reading. Take out 2 mls of cells on which to perform the assays.
3. Add 2 drops of 0.1% SDS and 4 drops of chloroform.
4. Vortex.
5. Incubate the tubes in a 30°C water bath for 10 minutes.
6. Add 400 μl ONPG (*o*-nitrophenyl-β-D-galactoside; 4 mg/ml in Z buffer), vortex, and start timing. Incubate the reaction at 30°C. Do not let the color turn a very deep yellow, because the reaction is no longer linear at this stage. The color will deepen when adding sodium carbonate.
7. Terminate the reaction by the addition of 1 ml 1.0 M Na_2CO_3.

8. Read the absorption at both 420 nm and at 550 nm.
   During the assay, ONPG will be cleaved by β-galactosidase, yielding a yellow color that absorbs at 420 nm. What do the readings at 550 and 600 nm represent? (See Notes for the Instructor).
   Calculate β-galactosidase activity as follows:

$$\text{Units} = \frac{1000 \infty (A_{420} - 1.75 \infty A_{550})}{\text{time (min)} \infty A_{600}}$$

ONPG (*o*-nitrophenyl-β-D-galactoside) can be purchased inexpensively from:
Research Organics Inc.
1-800-321-0570
Catalogue number 0429N. Price is: $12.50 per gram (1995-1996 catalogue)

Z Buffer: (per liter)

$Na_2HPO_4 \cdot H_2O$	16.1 g
$NaH_2PO_4 \cdot H_2O$	5.5 g
KCl	0.75 g
$MgSO_4 \cdot 7H_2O$	0.246 g
β-mercaptoethanol	2.7 ml

Do not autoclave. Adjust pH to 7.0.

Media

AB minimal medium (per liter):

0.5% sucrose or 0.5% glucose	900 ml
20∞ AB salts	50 ml
20∞ AB buffer	50 ml

20∞ AB salts (per liter):

NH_4Cl	20 g
$MgSO_4 \cdot 7H_2O$	6 g
KCl	3 g
$CaCl_2 \cdot H_2O$	0.26 g
$FeSO_4 \cdot 7H_2O$	0.05 g

20∞ AB buffer (per liter):

$K_2HPO_4 \cdot 3H_20$	78.6 g
$NaH_2PO_4 \cdot H_2O$	23 g

This is for pH 7.0
For pH 5.6, adjust the concentrations of the two phosphate salts accordingly.

The modified AB induction medium contains AB salts, 0.5% glucose, 2 mM phosphate, and 20 mM MES (pH 5.6).

Acetosyringone (3',5'-dimethoxy-4'-hydroxyacetophenone) can be purchased from Aldrich Chemical Co. 1001 West Saint Paul Ave., Milwaukee, WI 53233 (1-800-558-9160). It is catalogue number D13,440-6 (1 g is $18.40 in the 1994-1995 catalogue). It should be stored in the freezer. It may lose activity over the course of a year or two, especially if it gets wet. Make stock solutions fresh just before use by dissolving in dimethylsulfoxide (DMSO).

Making carrot extract for *picA* gene induction:

1. Get fresh carrots (with the greens still attached) from a local market. Wash thoroughly, and cut off both ends.
2. Place approximately 3–5 g carrots in a blender with 10 ml AB-sucrose medium. Blend well.
3. Incubate with shaking at room temperature for 6–8 hours.
4. Pour through a Buchner funnel (do not use filter paper) to eliminate large clumps, then filter the liquid three times through one layer of Whatman 3 MM filter paper.
5. Mix the extract with 9 volumes of acetone, and let sit overnight at 4°C (in a refrigerator).
6. Gently decant off the supernatant solution, leaving the precipitate in the flask. Evaporate off the remaining acetone in a fume hood.
7. Suspend the precipitate in 5 ml 10 mM sodium phosphate (pH 7.0) and dialyze the extract extensively against several changes of 10 mM sodium phosphate (pH 7.0) in a cold room for 24 hours. Centrifuge the extract at 8,000 rpm for 10 minutes, discard the pellet, and store the supernatant solution frozen at –20°C. This extract is stable to multiple cycles of freezing and thawing.

Experiment II. Transgenic Plants to Investigate Development and Responses to the Environment

Introduction

Plant biology is frequently an area that is neglected in the teaching of biological sciences, yet plants display unique characteristics in their developmental and physiological processes that set them apart from their animal and microbial counterparts. In addition to these unique biological characteristics is the ease with which plants can be cultured, manipulated, and stored (as seeds)—aspects that make them ideally suited as experimental systems for large-scale undergraduate teaching laboratories.

It has often been said that "plants cannot run away from their problems; they must deal with them." Plants have therefore developed unique and interesting mechanisms to deal with problems of predation, environmental stresses (such as heat and cold, salinity, heavy metal toxicity, shading), and biotic stresses (such as pathogen attack). In addition, plants, unlike their animal counterparts, take many of their developmental cues from the environment. Plant cell walls limit cellular movement, and plants therefore do not show significant cellular migration during development. Finally, developmental processes originate from specialized meristematic zones, and many aspects of plant development occur continuously throughout the life cycle. A ramification of this type of development is the totipotency of many plant cells not commonly found in animal cells. In order better to appreciate how organisms interact with each other and with the environment, it is important for undergraduate students to understand the unique cellular and physiological processes plants display.

Plants possess other important benefits for use in the teaching laboratory: (1) no specialized facilities are required for growth and seed storage; (2) many plants, such as *Arabidopsis thaliana*, require very limited space for propagation; (3) many plants undergo a rapid life cycle under defined growth conditions; (4) plants undergo highly predictable patterns of growth and differentiation; (5) many plants can be genetically transformed quite easily with "reporter genes." These plants are ideal for use in demonstrating important principles of both physiology and gene regulation during development and adaptation to stress. We have recently initiated a project to develop for use in the undergraduate laboratory a series of transgenic plants that contain chimaeric reporter genes. These genes are regulated by plant promoters (DNA sequences that control the transcription of genes) that demonstrate interesting developmental or environmental responses. Most of these changes in gene activity can subsequently be assayed by simple color changes resulting from the induction of activity of the reporter gene.

Reporter genes are those that encode enzymes with easily assayable activities. These reporter genes can, by recombinant DNA manipulations, be placed under the transcriptional regulation of promoters that show interesting developmental and/or stress response characteristics. A widely used reporter gene in plants is the *uidA*, or *gusA*, gene that encodes the enzyme β-glucuronidase (GUS). This enzyme can cleave the chromogenic substrate X-gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronic acid), resulting in the production of a blue color in those plant cells displaying GUS activity (Jefferson, 1987; Jefferson et al., 1987; Jefferson and Wilson, 1991). Plant cells do not contain endogenous GUS activity; therefore, the production of a blue color in particular cells is indicative of the activity of the promoter that drives the transcription of the *gusA*-chimaeric gene in that particular cell. The assay is easy to perform, sensitive, relatively inexpensive (especially when large quantities of X-gluc are purchased at a time), highly reliable, safe, requires no specialized equipment, and is highly visual, all characteristics important for use in large-scale undergraduate laboratories.

The general methodology for the production of transgenic plants is similar for all the experiments described below. Briefly, promoter sequences from genes of interest are linked, as transcriptional or translational fusions, to cassettes carrying the *gusA* gene and a polyadenylation signal sequence. These cassettes are contained within the border sequences of the *Agrobacterium tumefaciens* T-DNA, along with a selectable marker (generally an antibiotic resistance gene) used to select for transformed plant cells. This entire "T-DNA/GUS cassette" is maintained on a plasmid that is simple to manipulate both in *E. coli* (where the initial recombinant constructions are made) and in *A. tumefaciens*. An example of such a widely used vector having these characteristics is the plasmid pBI101 (Jefferson et al., 1987). *A. tumefaciens* harboring plasmids containing various promoter-GUS fusion genes are used to infect plant tissue (*Nicotiana tabacum* [tobacco] and *Arabidopsis thaliana* will be used for the experiments described below) and kanamycin-resistant transgenic plants are selected in tissue culture. The plants are regenerated, self-pollinated, and seed of this R_0 generation germinated on tissue culture medium containing kanamycin. Only plants that have subsequently been shown to be homozygous for the inserted T-DNA (containing the *gusA* gene) are used for these experiments.

Materials

Quantities given are per pair of students, unless otherwise specified.

Transgenic plants (grown as described below) (1–2)
X-gluc staining solution (5–10 ml)
70% ethanol (10–20 ml)
Microcentrifuge tubes (As required per experiment)
4 ml capped plastic tubes (As required per experiment)
Forceps (1)
Refrigerator (or refrigerated controlled temperature chamber) (1 per class)
Light source (1)
37°C water bath (1 per class)
Single-edge razor blades (1)

Student Outline

Goals

In these experiments, students will investigate the induction of different plant promoters by enviromental stresses or developmental cues. Below are listed a number of specific experiments that students can perform. It is easy, however, to make these experiments "investigative" by having students alter the protocols and environmental perturbations. At the end of each section, we make suggestions for student-initiated experiments. Students may, of course, devise other experiments using these plants.

Experiments with Specific Transgenic Plant Lines:

A. Response to Cold Stress

BACKGROUND: 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 and cryobehavior. There is, in addition, an alteration in the activity of many genes in response to low temperatures. These genes are known as *co*ld-*r*egulated, or *cor* genes. Most *cor* genes also respond to drought and the exogenous application of the phytohormone abscisic acid (ABA). These responses involve a large increase in gene activity, generally at the level of transcription. One *cor* gene that has been isolated from *Arabidopsis thaliana* is known as *cor15a* (Hajela et al., 1990). *cor15a* is a nuclear gene that encodes a 15 kDa polypeptide. The function of this protein is not known, although it is ultimately translocated to the chloroplasts and may be involved in cryoprotection. Baker et al. (1994) isolated a DNA fragment containing the promoter region and the first few amino acids from the *cor15a* gene and affixed it, in the correct translational reading frame, to a *gusA* gene, thus generating a *cor15a-gusA* translational fusion. This chimaeric reporter gene can be used to monitor the response of the *cor15a* gene in various plant tissues.

PROCEDURE: To investigate the induction of the *cor15a* gene by low temperatures, seeds of transgenic *Arabidopsis thaliana* plants harboring a *cor15a-gusA* chimaeric gene are first germinated in soil at room temperature (approximately 14 hours light–10 hours dark) until the plants are approximately 1 inch tall (1–2 weeks). Plants serving as controls are then stained immediately in X-gluc staining solution. Be sure, when taking the plants from the soil, to be gentle so as not to break off the roots because you will be examining them. The plants can be gently washed in water before staining.

Place experimental plants, still in soil, in a refrigerator for 48 hours. Stain and then gently wash them in the same manner as the control plants.

Because X-gluc is somewhat expensive, staining should be done in as small a volume as possible. Staining can be done in a 1.5 ml plastic Eppendorf microcentrifuge tube, or in a small volume of staining solution in a 4 ml plastic tube. Staining should be performed for 2 hours at 37°C, or overnight at room temperature. Under these conditions, the leaves, stems, and roots of the plants will all turn blue, whereas the tissues of the control plants should not turn blue. If the plants are incubated in staining solution at 37°C overnight, some light blue staining of the control plants may also occur (indicating that, even at room temperature, the *cor15a* gene is transcriptionally active to a low extent).

In order better to visualize the blue staining of the plant tissues, incubate the tissues in several changes of 70% ethanol. Chlorophyll is extracted into the ethanol, allowing better visualization of the blue color against a light green or clear background of plant tissues. The blue color is often more difficult to visualize against the dark green background of fully mature leaves and stems.

Suggestions for Student-Initiated Experiments:

1. What are the kinetics of induction of the *cor15a-gusA* fusion gene in the cold?
2. Which temperatures best induce the gene? At what temperature can you first observe gene induction?
3. The *cor15a* gene is also induced by drought. Stop watering the plants. How many days does it take to induce the *cor15a-gusA* fusion gene?
4. The *cor15a* gene is also induced by the hormone abscisic acid (ABA). What concentration of ABA is necessary for this induction? What are the kinetics of induction? Is the *cor15a-gusA* fusion gene induced by ABA better in some plant tissues than other tissues?

B. Developmental Auxin Gradients

BACKGROUND: The phytohormone auxin controls a large number of plant developmental and growth characteristics. Many plant genes respond rapidly to increased levels of auxin; these genes may be involved in mediating growth and developmental phenomena. One group of genes that respond very rapidly (within a few minutes) to auxin are the SAUR (small auxin up RNAs) genes (Hagen and Guilfoyle, 1985; McClure and Guilfoyle, 1987; McClure et al., 1989). The promoter from the soybean SAUR gene GH3 has been isolated and fused to a *gusA* reporter gene. This chimaeric gene has been used to investigate a number of auxin-stimulated phenomena in transgenic tobacco plants (Li et al., 1991; Liu et al., 1994).

In plants, auxin is synthesized in the upper regions of the plant and is transported downward. The roots of plants serve as an auxin “sink”; thus, root tips (the apical portions of the root) have a higher

level of auxin than do the more basal regions of the roots. This phenomenon can be seen by staining the roots of transgenic tobacco plants, harboring a SAUR GH3-*gusA* fusion, in X-gluc staining solution.

PROCEDURE: Germinate seeds of transgenic tobacco plants in soil and grow (either room temperature or 25°C, 14 hour light–10 hour dark photoperiod) until the plants are approximately 1 inch tall (3–4 weeks, depending upon the germination time). Pull plants gently from the soil, being careful not to damage the roots. Wash the roots gently in water to remove excess soil, and then stain overnight in X-gluc staining solution. Students will observe that the tips of the roots stain dark blue, whereas the more basal regions (the cell elongation and maturation zones) remain either unstained or lightly stained.

Suggestions for Student-Initiated Experiments:

1. What other tissues in the transgenic plant contain high levels of auxin that induce the GH3-*gusA* chimaeric gene?
2. How does the transgenic plant respond to the exogenous application of various concentrations of the auxin indole-3-acetic acid (IAA)? In which tissues is the GH3 promoter activated?
3. How does the transgenic plant respond to the exogenous application of various concentrations of synthetic auxins such as naphthalene acetic acid (NAA), indole-3-butyric acid (IBA), and 2,4-dichlorophenoxyacetic acid (2,4-D)?
4. How do the transgenic plant and the chimaeric gene respond to the exogenous application of other plant hormones, such as cytokinins and ethylene?

C. Auxin Gradients in the Stems of Transgenic Tobacco Undergoing Gravitropic Curvature

BACKGROUND: When plants are shaded, they tend to bend toward a source of light (phototropism). Similarly, plant stems tend to grow straight up. When a plant is placed such that the stem is horizontal to the surface, the stem will bend and grow up (gravitropism). Because the phytohormone auxin causes plant cell elongation, it has been proposed that, during phototropic or gravitropic bending, an auxin gradient is set up across the stem. The auxin concentration on the outer side of the bend becomes greater than the concentration on the inner side of the bend. The cells on the outer side of the stem thus elongate faster, causing the bending.

Because the SAUR GH3 promoter is very sensitive to auxin concentrations, the gene should be more active in parts of the plant containing higher levels of auxin. This auxin gradient may be detected by the use of transgenic plants harboring a SAUR GH3-*gusA* chimaeric reporter gene.

PROCEDURE: Grow transgenic tobacco plants in soil in individual pots (either room temperature or 25°C, 14 hour light–10 hour dark photoperiod) until the plants are approximately 4 inches tall (1–2 months). Move the plants into total darkness for 24 hours. Place plants on their sides so that the plant stems are horizontal. After 4–6 hours, the stems have started to bend upward. At this time, cut the stem sections from the plant, bisect the stems longitudinally with a razor blade, and stain the tissue overnight at room temperature in X-gluc staining solution (if the stem sections are placed in staining solution overnight at 37°C, they become soft and mushy and difficult to manipulate). Clear the stem sections of chlorophyll by repeated incubations in 70% ethanol

overnight. According to Li et al. (1991), the cells on the outer side of the bend stain more intensely blue than do the cells on the inner side of the bend. We have seen this also, although there is frequently interference by strong staining of stem cells directly adjacent to where leaf petioles emerge from the stems. This occurs regardless of whether the leaves emerge from the inner or outer side of the bent stem. Thus, you may wish to perform this experiment with several transgenic plants; some will show the staining (auxin) gradient, whereas other plants may have this gradient obscured by high GUS activity in regions of the stem from which leaves emanate.

Suggestions for Student-Initiated Experiments:

1. How do the plant and the chimaeric gene respond to bending resulting from a unidirectional light source (phototropism)?

Notes for the Instructor

1. Seeds for these transgenic plants can be obtained from the authors.
2. We are currently developing, with the help of other laboratories, transgenic plants containing various promoters that direct the expression of GUS activity. These promoters are regulated in various ways, either developmentally or in response to specific biotic or environmental stresses.

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Appendix II.

X-GLUC Staining Solution:

1–2 mM X-Gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronic acid)
50 mM Na_2HPO_4, pH 7.0
0.1% Triton X-100

Store solution in the dark in a refrigerator.

X-gluc is very expensive. The least expensive supplier that I have been able to find is:

Rose Scientific
4027 97th St.
Edmonton, Alberta
CANADA T6E 5Y5
Telephone: 1–800–661–9288

Catalogue number ES 1007–001
1 g is $220 (1994 catalogue)