Chapter 7

Plant Transformation by Lux+ *Agrobacterium*

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Introduction

One of the basic techniques used in biotechnology is the transfer of genetic information from one organism into another, to affect the production of recombinant products or to generate an organism with new recombinant properties. While modern science has only been able to do this recently, species of the soil bacterium *Agrobacterium* have been involved in the genetic engineering of plants for millions of years.

Agrobacterium tumefaciens is a plant pathogen that causes crown gall disease. The crown gall is a tumor-like growth of undifferentiated plant tissue. This altered growth of the plant is caused by the transfer of DNA (T-DNA or transfer DNA) from the bacterium into the plant nucleus where it becomes integrated into a plant chromosome. The T-DNA encodes the *onc* genes, that encode enzymes that synthesize plant hormones and cause tumor growth, and the *ops* genes for the synthesis of opines, which are modified amino acids (e.g. octopine or nopaline). The plant expresses the bacterial genes (is transgenic) and secretes opines into the soil, where they serve as a unique C- and N-source for *Agrobacterium* use.

This natural infection system can be modified to introduce foreign genes into a plant host. The T-DNA is a small part of the Ti-plasmid (tumor-inducing plasmid), which also encodes virulence (*vir*) genes needed for DNA transfer to the plant. The T-DNA is delimited by the T-DNA borders which are 25 bp imperfect direct repeats. In the normal infection process, the DNA between the borders is transferred to the plant and integrated into the plant chromosomal DNA. The T-DNA can be isolated from the Ti-plasmid, then cut at specific sites with restriction endonucleases to open up the T-DNA or to delete fragments of it. In this way the *onc* or *ops* genes can be modified or deleted to allow transformation of the plant without tumor or opine formation. Foreign genes can be inserted into these deleted regions. One of the first demonstrations of this technology involved the insertion of a gene from *Salmonella*, that determined resistance to the herbicide glyphosate, into the T-DNA. Transformed plants grew normally but were resistant to the herbicide, while non-transformed plants were killed if the herbicide was present. This allows the application of herbicides during the plant growing season when there is the greatest competition with weeds. See Madigan et al (1997) for a review.

Transformation with recombinant T-DNA also can be used to learn more about a plant or its interaction with its environment. For example, how would you determine which plant genes are activated upon challenge by an insect pathogen, or which plant genes are turned on in the flower or in a seed pod, or which plant genes confer cold-tolerance and thereby extend the growing season? In these studies, you need to genetically engineer the plant so that it will show you when a gene is turned on or turned off. This is reporter gene technology. (See Madigan et al 1997.) A variety of reporter systems has been developed, but in all cases, the activity of the reporter is generally easier to measure than the activity of the gene of interest. It is important at this point to remember that a promoter sequence upstream of the start of a gene is necessary for RNA polymerase binding and transcription of that gene. If the reporter gene with its own promoter deleted were placed

downstream of the promoter (e.g. it could be inserted into the gene of interest), then it will "report" when the promoter is active and the gene is turned on (transcribed).

Materials and Equipment

(numbers needed per group indicated in parentheses)

1. Plant transformation (work in groups of 4)

Jar of sterile 6-8 week old *Nicotiana tabacum* vr. SR1 tobacco plants (4 plants/jar)
Sterile small disposable Petri dishes for infection (1)
Sterile blunt end forceps (1)
Sterile scalpel (1)
Sterile 13x100 mm metal test tube cap wrapped in foil (1)
Sterile filter paper in glass Petri dish (10)
10 ml *Agrobacterium tumefaciens* (strain GV3101 pMP90RK pPCV701 luxAB) broth culture grown in Luria Bertani (LB) medium with selection [Rifampin (100μg/ml); Carbenicillin (100μg/ml); Kanamycin (50μg/ml); and Gentamycin (25 μg/ml).] (Cells are resuspended in LB medium with no selection for transformation.)
Murashige and Skoog (MS) agar plates for tissue culture (2)
MS agar plates with hormones [auxin: α-naphthaleneacetic acid (0.1μg/ml) and cytokinin: 6-benzylaminopurine (Sigma; 0.5μg/ml) and selection [Kanamycin (100μg/ml) and Cefotaxin (500μg/ml)] (2)
Disposal for contaminated material
Sharps disposal
Foil for wrapping plates
Parafilm to seal plates

2. Lux (LU) activity (sample preparation and reading; work in groups of 4)

50 mg regenerated calli from student's plate to take LU measurements
2 ml Lux buffer
Micropipettor 100-1000μl
Micropipettor tips 100-1000μl
Microcentrifuge tubes 1.5 ml (4)
Microcentrifuge tube rack
Plastic homogenizer (1)
Disposable mixer/Plastic Homogenizer--Kontes Pellet Pestle, 2 3/4 inch (Supplier: Fisher Scientific; phone 1-800-234-7437; http://www.fishersci.ca; Catalog number K749520-0000) (1)
Disposable scalpel blade (1) and holder for the blade
Blunt end forceps (1)
n-Decyl Aldehyde diluted with dH_2O (1:10000) and sonicated (20 μl / reading) (3)
Flavin Mononucleotide, sodium salt (FMN); Sigma F-2253 (0.8ml) reduced by light on the day of the assay ($FMNH_2$) in 1ml syringes fitted with 25G needles (3)
Disposable cuvettes (3)
Discard for liquid and solid waste

Luminometer, such as:

1. LKB-Wallac 1250 Luminometer (Manufacturer: Wallac Oy, P.O. Box 10, 20101 Turku 10 Finland; phone (921) 359-300; Telex 62333)
2. TD-20/20 Luminometer (Turner Designs, 845 W. Maude Avenue, Sunnyvale, CA 94086; phone (408) 749-0994; Fax (408) 749-0998)
3. Lumat LB 9507 (EG&G Berthold; distributed by Fisher Scientific; phone 1-800-234-7437.)

3. Bradford Dye-Binding Protein Assay

Homogenate (supernatant fluid) from transgenic callus (~50 µL)
dH_2O
1 ml pipettes
Pipette bulb
Micropipettors and tips
LKB Novaspec
Polystyrene cuvettes for Spectrophotometer (Fisher Scientific, Catalog number 14-385-942) (1)
Bradford dye (2.5 ml) (Bio-Rad, 1414 Harbour Way South, Richmond, CA 94804; phone (415) 232-7000)

Student Outline

Introduction

In this exercise, we will transform tobacco leaves with *A. tumefaciens* strain GV3101 which contains a disarmed Ti plasmid (pMP90RK) and the plant expression vector pPCV701. The T-DNA in the Ti-plasmid has been deleted and replaced with a rifampin-resistance gene, hence the engineered Ti plasmid is given the new name = pMP90RK. The pPCV701 vector is a chimeric plasmid that contains the left and right borders of the *Agrobacterium* T-DNA (B_L and B_R) inserted into an *Escherichia coli* plasmid. B_L and B_R are needed to direct insertion of the T-DNA into the plant genome, but the *onc* and *ops* genes are not needed and have been deleted. Hence the T-DNA is disabled and will not cause a tumor or form opines. The bacterial luciferase genes (*luxA* and *luxB* from *Vibrio harveyi*), under the control of the auxin-inducible plant promoters P1 and P2, have been placed between B_L and B_R of the T-DNA to act as a reporter. Genes for ampicillin-resistance (Amp^r) and neomycin phosphotransferase activity (NPTII) which confers resistance to aminoglycoside antibiotics (e.g. kanamycin) also are present in the recombinant T-DNA. These antibiotic-resistance genes allow positive antibiotic-selection to be used to maintain pPCV701 in *Agrobacterium* (Ampicillin present in the bacterial growth medium) or the T-DNA in the plant (Kanamycin present in the plant growth medium). In addition, there is an origin of replication (OriV) for replication of the plasmid in the bacterium and a wide-host-range origin of transfer (OriT) for recombinant DNA work. The *vir* genes on the disarmed Ti plasmid (pMP90RK) will mobilize the T-DNA from pPCV701, form the conjugation bridge between the bacterium and the plant, then direct the T-DNA to the plant nucleus.

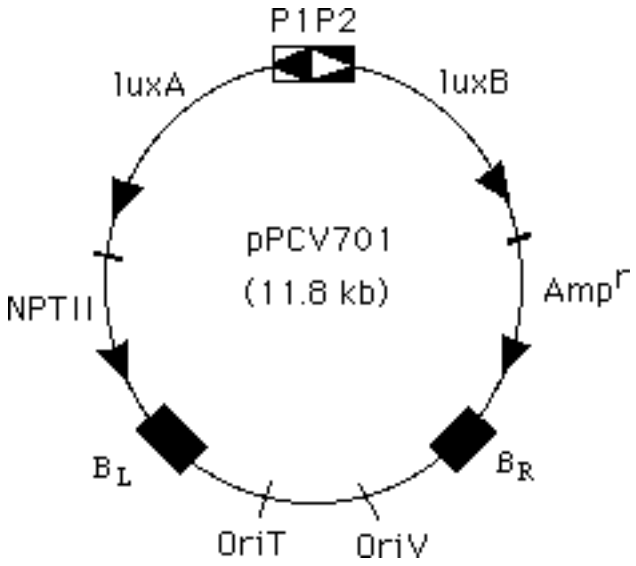

Figure 7.1. pPCV 701 Lux A and B plant expression vector containing the bacterial luciferase A and B genes cloned behind the auxin regulated P1 and P2 promoters.

The *lux* genes on pPCV701 will be used as reporters of plant hormone activity. The two *lux* genes are under the transcriptional control of plant promoters (P1 and P2, arranged in opposite polarity). Transcription from these plant promoters, P1 and P2, is activated by the plant hormone auxin. The gene products LuxA and LuxB must both be produced and combine to form an active bacterial luciferase, which carries out the reaction:

$$FMNH_2 + RCHO + O_2 \text{ ----------> } FMN + RCOOH + H_2O + light$$

Reduced flavin mononucleotide ($FMNH_2$) comes from the plant cytosol and you will add the long-chain aliphatic aldehyde (RCHO) decanal in the lab. The reaction results in bioluminescence in the tissue which can be detected and measured by light sensitive equipment. This is a non-destructive assay for gene activity inside a whole, living organism. This is an extremely powerful technique that can be used to study a myriad of problems in animal, plant or microbial physiology.

The amount of light produced by the plant will be dependent on the amount of tissue present (and its thickness) and the amount of LuxAB formed, which is in turn determined by the concentration of auxin in the tissue. Thus high levels of light will correspond to high levels of auxin. Auxin is associated with enlargement and lengthening of plant cells during their growth and differentiation. Therefore, we would expect to see a localized activity of Lux in a whole plant, in regions of auxin-promoted cell growth.

The amount of light formed is usually too small to be seen by eye. Therefore, more sensitive detectors (luminometers) are needed. We will use two different luminometers in this lab: a low light video imager and a luminescence photometer. Both instruments work on basically the same principle, whereby the small amount of light produced is collected in a darkened chamber over time, amplified by a photomultiplier, then processed into numeric or visual data by a computer and printer. In the photometer, only numeric data is produced. In the imager, a video camera and image processor are used to construct pictures of light production localized within tissues or

structures. Currently, there is a great deal of commercial interest in producing relatively inexpensive luminometers ($3,000-6,000), so that bioluminescence technology can be used more widely in different applications.

Procedure

A. Cutting leaf discs

For leaf explant transformation, you will use young leaves from sterile *Nicotiana tabacum* (cv SR1) grown in 475 ml Mason jars for 2 months at 26 °C with a 12 h/day photoperiod.

1. Detach several leaves from the young plants and place them in a sterile Petri dish containing a sterile filter paper base. Use a sterile Pasteur pipette to moisten the filter paper with sterile water
2. Carefully unwrap a metal test-tube-cap (that fits a 13x100 mm test tube) and use it to cut about 9 discs out of the leaves.
3. Using sterile forceps, carefully pick up a leaf disc and place it upside-down on a plate of MS medium. Do this with four leaf discs. This will be the uninfected control plate. Seal the plate with Parafilm.

B. Infection of leaf discs

- You are provided with a culture of *A. tumefaciens* GV3101 (pMP90RK, pPCV701) which has been grown overnight in LB broth with antibiotic selection (Rif, 100 µg/ml; Kan, 50 µg/ml; Gentamycin, 25 µg/ml; Carbenicillin, 100 µg/ml). The bacteria have been collected and washed free of antibiotics to prevent inhibition of plant growth.

1. With sterile forceps, carefully pick up a leaf disc and submerge it in the suspension of *A. tumefaciens* for 5 min. Do this with five leaf discs.
2. Use sterile forceps to remove the individual infected leaf discs from the bacterial suspension. Remove excess *Agrobacteria* by touching the leaf disc to a sterile filter paper in another Petri plate.
3. Place the leaf disc upside-down on MS medium. Space the leaves evenly around the plate. Seal the plate with Parafilm.
4. Wrap your 2 plates (infected leaves and control leaves) in aluminum. Incubate these plates for 2 days at 26 °C.

- During this time the bacteria and plant tissue are co-cultured without any selection, the bacteria bind to the plant cells on the cut edge of the leaf and T-DNA transfer occurs.

C. Growth of transformed plant callus

- This phase of this experiment will take 5 weeks to complete. During this time, you must transfer your leaf discs to new media at least once a week to encourage the growth of plant callus and to select against *Agrobacterium* and contaminants - notably fungi. In order for this section to work, you must treat these leaf discs with tender-loving-care to help them survive the trauma that you have just inflicted on them.

1. Aseptically transfer the leaf discs (still upside-down) to shooter generation medium (CGM), placing the discs evenly around the agar surface. This medium contains 0.1 mg/L NAA (α-naphthaleneacetic acid is an auxin) and 0.5 mg/L BAP (6-benzylaminopurine is a cytokinin) to promote undifferentiated plant cell (callus) growth, Kan (100 µg/ml) to select transformed plant tissue and sodium cefotaxime (Claforan) (500 µg/ml) to select against *Agrobacterium.*

2. Seal the plates with Parafilm and incubate them in the light at 26 °C (12 h photoperiod).

3. Aseptically transfer the leaf discs to fresh CGM medium at approximately every 7 day intervals.

- The CGM agar will turn yellowish as the Claforan in the medium degrades. When this happens there will be no more selection against *Agrobacterium*, so the leaf discs need to be transferred as soon as possible. Watch for fungi growing in the agar. Transfer the discs to new media immediately if fungi are detected. Do not transfer discs that have fungi touching them. If fungi have taken over a plate, do not open it -- discard it immediately -- all is lost! A happy leaf disc (:-) will appear fresh and green, hopefully with bumpy callus growth along its edge.

D. Demonstration of plant regeneration

- A set of leaf disks will be infected with *Agrobacterium* and grown to the callus stage as in Sections A & B. Callus will be excised and placed on "shooter" plates containing selective antibiotics and the correct ratio of the plant hormones auxin and cytokinin to promote shoot formation. When the shoots are about 1 cm high (3 to 4 weeks), they are excised and plated on MS medium with antibiotic selection and no hormones. This promotes root formation. After 2 to 3 weeks the plantlets may be transferred to soil or sterile medium to generate a recombinant transgenic plant (about 2 months total incubation).

- These regenerated transgenic plants will be used in the video imager to detect tissue specific Lux activity.

E. Lux activity measured in the Luminescence Photometer

1. Grind about 50 mg of callus (a lump about 5 mm in diameter) in 1.0 ml of Lux buffer in a 1.5 ml microcentrifuge tube. Use a blue plastic homogenizer to break up the tissue. (Do not throw this unit away.)

2. Using an micropipettor, carefully transfer the supernatant fluid from the mashed plant material into three luminometer cuvettes and dilute according to the table below:

Table 7.1. Dilution of samples used for Luminometric readings

Tube #	µL of Supernatant	µL Lux Buffer	Dilution
1	500	0	Undiluted
2	250	250	1/2
3	100	400	1/5

3. Place the cuvette tubes into the luminometer.

4. When your turn to measure Lux activity comes, take a 1.0 mL syringe (fitted with a 22 gauge needle) that has been filled with 0.8 ml of $FMNH_2$. Load 20 µL of decanal into the tip of the syringe, taking care not to mix it into the $FMNH_2$.
   • Do not use the $FMNH_2$ if it is yellow - this means that it has oxidized and needs to be reduced again.
5. Inject the substrates ($FMNH_2$ and decanal) into the sample tubes in the luminometer as demonstrated. The light will be collected for 10 sec after a 5 sec delay.

6. Record the amount of light collected as mV (milli-Volts).

7. Lux activity should be expressed as a Specific Activity corrected for the amount of material that was sampled (e.g. mV per min. per mg callus).

F. Bradford dye-binding protein assay

The Specific Activity also can be calculated based on the amount of soluble cell protein, which may give a more accurate measure of how much luciferase was liberated by the grinding procedure and available in the soluble extract.

In this assay, soluble protein is mixed with reagent containing Coomassie Brilliant Blue G-250**.** The protein will bind dye and the resulting blue color (A_{595}) will increase in proportion to protein concentration. This assay is very sensitive and can be used in the presence of reducing agents (e.g. mercaptoethanol) which interfere with other protein assay procedures.

Bradford dye-binding protein assay procedure:

1. A sample of the homogenate supernatant fluid from Step E2 is transferred to a clean microcentrifuge tube and sufficient distilled water is added to make 0.8 mL total volume. How much sample to add will depend on the amount of callus that was sampled and the efficiency of breakage of this material. A good place to start is 10 µL or 20 µL of extract.

2. The "blank" for zeroing the spectrophotometer consists of 800 µL distilled water in another microcentrifuge tube.

3. The reagent is added (0.2 mL) to both tubes and the closed tubes are mixed by inversion.

4. The A_{595} is read in a spectrophotometer that will accept 1 cm light path, polystyrene cuvettes that will hold a 1 mL sample in the light path of the photometer (e.g. LKB Novaspec). The use of this instrument will be demonstrated to you.

5. Since Coomassie Blue sticks to everything (it particularly likes skin!), you will need to wash the cuvette between the blank and samples with 70% ethanol.

6. Compare the A_{595} of your sample with the Standard Curve data provided.

Table 7.2. Protein assay data sheet for BSA standard and plant tissue samples.

µL BSA† Standard (0.1 mg/mL)	µL Distilled Water	Protein µg per assay tube	Absorbance 595 nm
0	800	0	
15	785	1.5	
50	750	5.0	
100	700	10	
150	650	15	
200	600	20	
250	550	25	
Samples			

† BSA = Bovine Serum Albumin

7. Calculate Lux Specific Activity as mV per min. per mg cell protein.

Notes for the Instructor

Assay Reagents and Media

1. Bacterial Culture media
Grow *Agrobacterium tumefaciens* strain GV3101 containing pMP90RK plasmid and pPCV701 lux A and B plant expression vector in LB media. For 1 Liter media use 10g Bactotryptone, 5g Yeast Extract, 10g NaCl. Add selection (antibiotics) after autoclaving: 100 mg Rifampicin (The *vir* genes necessary for plant transformation are placed on a separate plasmid and maintained in *Agrobacterium* by the selection on Rif.), 100 mg Carbenicillin (Ampicillin or Carbenicillin allow selection in E. coli and selects for conjugants after transfer to *Agrobacterium*), 50 mg Kanamycin (The selectable marker for a transformed plant.), 25 mg Gentamicin (The plant binary plasmid vector is maintained in Agrobacterium with this selection. The original vector did not have the NPT II (kan) gene.) All antibiotics are to insure that all the resistance genes are present and working in *Agrobacterium* and to insure that rearrangements or loss of markers is unlikely to occur.) Wash and resuspend *Agrobacterium tumefaciens* strain GV3101 containing pMP90RK plasmid and pPCV 701 lux A and B plant expression vector for transformation in LB media with no selection.

2. Tissue Culture media
Make Murashige and Skoog Basal Medium (MS) for plant growth in jars (100 ml/jars). For 1 Liter media add 30g Sucrose, 2.2g Gelrite to MS Basal Medium. Adjust to pH 5.7 before adding Gelrite. Autoclave for 25 minutes. Make MS media for incubation of leaf discs after transformation in 15x100 mm Petri dishes. Make MS media with antibiotic selection and hormones for regeneration of transgenic tissue in 20x100 mm Petri dishes. Add (antibiotics and hormones) 0.1 mg α-Naphthalleneacetic acid (NAA), 0.5 mg 6-Benzylaminopurine, 100 mg Kanamycin, 500 mg Sodium Cefotaxime (Claforan) after autoclaving and cooling the media. Store plates right side up in the refrigerator.

3. Lux Assay reagents
Make Lux Buffer by adding 50 mM $Na_2HPO_4 \cdot 7H_2O$ (1.34 g), 50 mM Mercaptoethanol (0.347 mL) and 0.4 M Sucrose (13.7 g) to 100 mL dH_2O. Prepare FMN Solution by mixing 10 mL of 1 mM FMN with 90 mL of 200 mM Tricine buffer solution, pH 7.0. Store in the -20°C freezer. FMN is photo-reduced on the day of the assay by placing it on or near a fluorescent light source. Sonicate 1 µL n-Decyl Aldehyde with 10 mL dH_20 (1:10000) for a few seconds.

4. Bradford Dye-Binding Protein Assay
Use commercial dye reagent from Bio-Rad prepared according to manufacturer's instructions. Set up a standard curve by preparing several dilutions of Bovine Serum Albumin from 1 to 25 µg/mL. A standard curve should be set up each time the assay is performed. Place 0.8 mL of the appropriately diluted sample not clean test tubes. Place 0.8 mL of the buffer used for the dilution into a test tube. (This will be the "blank".) Add 0.2 mL concentrated dye reagent to each tube, vortex, and measure the absorbency at 595 nm. Use the blank to zero the Spectrophotometer. Plot the Optical Density (absorbance) at 595 nm versus the concentration of standards.

Check out these Web sites:

http://www.euro.promega.com/pnotes/44/luerhsen/luerhsen.html
http://biochem-int.comvos.de/products/1669893.html
http://www.labsystems.fi/instru/manuals/applicat/an301.htm
http://vectordb.atcg.com/vector_descrip/PGL3ENHA...
http://biog-101-104.bio.cornell.edu/BioG101_104/explorati...
http://ss.cgk.affrc.go.jp/sakukai/ikukou/seika/mstransg-e...
http://sun1.ub.uni-freiburg.de/Flybrain/html/poster/regen...

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