Hairy Root as a Model System for Undergraduate Laboratory Curriculum and Research

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Abstract: Hairy root transformation has been widely adapted in plant laboratories to rapidly generate transgenic roots for biochemical and molecular analysis. We present hairy root transformations as a versatile and adaptable model system for a wide variety of undergraduate laboratory courses and research. This technique is easy, efficient, and fast making it an ideal tool for undergraduate teaching. Students in a biotechnology course successfully transformed soybean cotyledons with *Agrobacterium rhizogenes* strain K599 during laboratory sessions. The students introduced the green fluorescent protein (GFP) gene into soybean and observed hairy roots regeneration. After two weeks, 45% of the cotyledons developed roots. Of the roots that appeared after transformation, 55% expressed the GFP protein.

Keywords*:* hairy root, *Agrobacterium rhizogenes*, transformation, cotyledons

Introduction

Currently in undergraduate teaching laboratories, plant transformation experiments are demonstrated mostly with *Arabidopsis*, using the floral dip method (Clough and Bent, 1998), or tobacco leaf disc transformation (Fraley et. al., 1983). However, both transformations suffer one major limitation. These techniques are time consuming. Both experiments take at least 6 weeks to prepare, which is needed to bring the plants to maturity. After the transformation, both experiments take another 5 or 6 weeks to harvest the transgenic material. The considerable waiting time reduces student interest in the subject significantly. For trimester universities, the entire term is only 10 weeks long, making *Arabidopsis* and tobacco transformation experiments difficult to set up. In this article, we present the classroom use of a unique, rapid, and reliable method to genetically modify the roots of a wide variety of plant species. This study relies on the hairy root plant transformation system.

Hairy roots originate from a plant disease caused by the Gram-negative soil bacterium *Agrobacterium rhizogenes*. Hairy root tumors are characterized by a proliferation of adventitious roots at the bacterial infection site. The genetic determinant of hairy root disease is a large plasmid called the root-inducing (Ri)-plasmid which is carried by virulent strains of *A. rhizogenes* (Chilton et. al., 1982). The Ri plasmid is similar to the tumor-inducing (Ti) plasmid found in *Agrobacterium tumefaciens*, which is the causative agent of Crown Gall tumors in many dicotyledonous plants. Both the Ri and Ti plasmids transfer a segment of the plasmid called transfer DNA or T-DNA to the plant genome during infection. Transfer of the T-DNA to the plant genome is directed by a different region of the plasmid called the virulence (*vir*) region. The T-DNA contains genes that encode enzymes responsible for the biosynthesis of plant hormones, such as auxin and cytokinin. Upon transformation, these genes are expressed resulting in tumor-like growth. In addition, the T-DNA has genes controlling the production of various opines which serve as carbon, nitrogen, and energy sources for the infecting bacterium.

The Ti and Ri plasmids can be engineered to insert novel genes into the T-DNA for the introduction of genes-of-interest in various plant species. A more common approach is the use of a binary vector system where the *vir* genes reside in one plasmid (i.e. Ti or Ri plasmid) and the T-DNA in a second plasmid. The use of disarmed strains of *A. tumefaciens*, where the tumor causing genes have been removed from the Ti plasmid, results in non-tumorous transgenic tissue. These strains are commonly used for plant transformation. Reporter gene(s) to detect the transformation event may include antibiotic resistance genes, Beta-

Glucuronidase [GUS] (Li and Leung, 2003) or the Green Fluorescent Protein (GFP) (Hughes et al., 2002). The GFP gene was originally isolated from fluorescent jelly fish. It emits green light when activated by UV irradiation. Various forms of GFP have been widely used as non-destructive visible markers in biological research.

While *A. tumefaciens* produces transgenic tumors, *A. rhizogenes* produces transgenic roots. This provides a major advantage for root-related studies since it eliminates the lengthy and expensive process of regenerating whole transgenic plants. As an additional advantage, these roots have the ability to grow in culture when exogenous sugar is supplied. This system has been broadly utilized by biologists to genetically modify roots of a wide range of plant species for basic and applied research. Originally, hairy roots were thought to be a disease limited to dicotyledonous plants (De Cleene and De Ley, 1981). After further research, *A. rhizogenes* root induction was demonstrated on monocots (Porter, 1991) and in gymnosperms such as radiata pine [*Pinus radiata*] and larch [*Larix*] (McAfee, et. al., 1993; Li and Leung, 2003). The hairy root transformation system is adaptable to a very broad range of plant species, which is important for teaching different subjects to suit various curricula.

Hairy root cultures are potentially useful for the production of a large number of foreign proteins and secondary metabolites. The transformed roots are genetically stable and exhibit faster growth rates than normal roots (Hu and Du, 2006). For example, hairy root cultures of red beet (Beta vulgaris) have shown promise for the commercial production of peroxidase (Rudrappa et al., 2005). Brigham et al. (1999) demonstrated that hairy root cultures of *Lithospermum erythrorhizon*, a member of the Boraginaceae plant family, manufacture shikonins at an elevated level. Shikonins exhibit varying degrees of antimicrobial activity against a wide range of bacteria and fungi. Hakkinen et. al. (2005) showed that hairy root cultures of *Nicotiana tabacum* expressing the *h6h* gene from *Hyoscyamus niger* had an enhanced secretion of the alkaloid scopolamine. This alkaloid compound has pharmaceutical significance as an anticholinergic agent.

In addition to metabolic engineering, hairy root cultures have proved to be a powerful tool for gene expression and gene silencing studies. Kumagai and Kouchi (2003) used hairy root cultures to introduce hairpin RNA (hpRNA) sequences complementary to the GUS gene in *Lotus japonicus*. They were successful in silencing GUS gene activity in the hairy roots and on symbiotic nodules formed on the hairy roots. Subramanian et al. (2005) silenced isoflavone synthase (IFS) genes in soybean cotyledons by introducing hairy root cultures containing RNAi silencing constructs. Hairy root cultures exhibiting IFS silencing were more susceptible to the plant pathogen *Phytophthora sojae* than control cultures suggesting a protective role for isoflavones. The above study used a non-tissue culture based method to generate hairy roots.

In this study, eight undergraduate students representing an entire biotechnology class started hairy root cultures for research projects. This biotechnology course is offered at a sophomore level and was an elective for the majority of the students. A diverse cross-section of students were involved in this study and included the following majors: Pre-Medical, Clinical Laboratory Science, and Biology. Hairy root cultures were used to demonstrate plant genetic transformation and expression of the GFP protein. The short and engaging experiences of hairy root transformation improved the teaching results significantly.

Materials and Methods

Students were prepared for the exercise by reading assignments of literature on hairy root cultures. The literature included three research journal articles and an overview of the process in their lab manual. The instructor gave a short lecture on *A. rhizogenes* and plant root transformation. Reading, lectures, and student discussion on the hairy root transformation system occurred prior to the laboratory exercise. Due to time limitations, the instructor planted the soybean seed and prepared bacterial cultures for the class.

<u>Materials that are needed for transformation:</u>

Soybean seedlings—one 6" pot can supply enough cotyledons for an entire lab section.
24 hour *A. rhizogenes* cultures (containing a binary plasmid of interest)
Sterile Whatman filter paper
Sterile Paper towels
Sterile 1/4 strength Murashige and Skoog (MS) salts (no sucrose) or 10mM MgSO4.
Micropipettes
70% ethanol
Sterile surgical steel blades

<u>Equipment:</u>

Clean bench (optional)
Centrifuge to pellet bacterial cells
Spectrophotometer (optional)

Protocol for Transforming Soybean cotyledons using *A. rhizogenes*
(Subramanian et al., 2005)

The soybean seeds (variety Williams 82) were planted in an artificial soil mix (Superfine germinating mix, Fafard, New Jersey) and placed in a mist chamber. If a mist chamber is not available, the seedlings need to be sprayed on a daily basis with a mist-sprayer. This helps dislodge the seed coat and results in high quality cotyledons. The cotyledons were ready to harvest 5 days after planting. Younger or older cotyledons may not yield good results.

Overnight cultures of *A. rhizogenes* strain K599 were prepared the day before inoculation. The K599 strain that was used contains the GFP gene as a visible marker. This gene is located in a binary vector (pCAM-sUbi:GFP) published previously (Subramanian et al, 2005, 2006). Cultures were started in 10 mL of Luria Broth with 50 µg mL^{-1} kanamycin added. *A. rhizogenes* can be maintained in 25% glycerol at –80°C for several years. Fresh cultures were spun down at 8°C for 10 minutes at 5,000 rpm. Pellets appeared pinkish-brown as expected. The pellets were drained and resuspended in the original volume (10 ml) of 1/4 strength MS medium. Cells were diluted to an OD_{600} of approximately 0.3 for the inoculation of cotyledons. If a spectrophotometer is not available, overnight (16h) cultures can be diluted five times in 1/4 strength MS medium.

Solutions and materials were gathered while the cells were being centrifuged. Optimally, the transformation procedure should be finished within 2 hours after the cell resuspension. It took our class approximately 2 hours to finish the transformation process. Although a laminar flow hood or clean bench is optional, we performed the transformation steps and transfer in a clean box. Sterile 9 cm Whatman filter paper was added to the sterile Petri dishes. The filter papers were moistened with sterile 1/4 strength MS salts. Excess fluid was poured off the plates.

Cotyledons were carefully inspected by the students. As instructed, they used only cotyledons that were disease-free and have fully opened, exposing the first true leaves (fig. 1A). Cotyledons were gently twisted off the plant and surface sterilized in 70% ethanol. After surface sterilization, the cotyledons were promptly blotted in sterile paper towels to remove the excess ethanol. Cotyledons were placed on sterile filter papers soaked in 1/4 strength MS medium until the students were ready for wounding infection. Students wiped their hands with 70% ethanol prior to the wounding process. Cotyledons were wiped on both sides with 70% ethanol using a cotton swab.

A sterile surgical steel blade was used to cut a diamond shaped wound on the abaxial side of the cotyledon close to the petiole end. Students were instructed to make the slice deep enough to expose the midrib, but not slice through it. The wounded cotyledon was transferred to the filter paper lined Petri dish. Six cotyledons were placed on each plate. Plates were labeled with the date and student initials. Students added 20 µl of the *A. rhizogenes* suspension to each wound (fig. 1B). The Petri plates were promptly covered with aluminum foil as darkness provides better results. Plates were placed in a 22°C growth chamber.

The plates were checked weekly. Sterile water was added as necessary to maintain moisture on the filter paper. Every other week, the plates were watered with 1/4 strength MS medium. Plates were kept moist, but not excessively wet. Hairy root cultures were viewed under the fluorescent microscope. Other constructs and reporter genes (e.g. GUS) can be used if a fluorescent microscope is not available. The experiment was duplicated during the semester.

Results and Discussion

Students were successful in obtaining hairy root cultures. After 4 weeks, only 25% of the plates produced hairy roots in the first trial. Most of the plates were contaminated and disposed of. Of the plates that produced callus and roots, 75% of the cotyledons produced roots and 57% of the roots were transgenic (GFP positive). The experiment progressed more rapidly in the second trial and 100% of the plates produced callus and hairy roots. After two days, the area that was inoculated appeared necrotic. Callus tissue appeared in 6-7 days (fig. 1C). After 2 weeks, roots appeared from the callus site (fig. 1D). Of the cotyledons that were inoculated, 45% produced roots. After transgenic roots appeared, the GFP protein was expressed in the transgenic tissue when viewed under a fluorescent microscope. Of the roots that were induced, 55% were transgenic roots expressing the GFP protein (fig. 1 F, G). This procedure was concluded in five weeks, but could easily be adapted for a semester project depending upon the goals of the research. As shown in fig. 1E, the cultures will continue to grow and produce additional roots for 4-5 weeks.

This system is very adaptable, but conditions for the hairy root culture system may vary depending upon the plant species that will be studied. One must determine the appropriate bacterial strain of *A. rhizogenes*, the proper antibiotic to control

bacterial growth after inoculation, the best plant tissue to use, and a suitable culture medium. Protocols are published in the literature for such model plants as *Medicago truncatula* (Chabaud et al. 2006) and *Lycopersicon esculentum* (Collier et. al., 2005). Due to the high rate of transformation, hairy root transformation does not use antibiotic resistance genes for selection. This might be an advantage in a classroom setting since it minimizes biosafety issues for disposal.

Soybean cotyledons are easy to work with, but a great variety of plant materials can be used. Hypocotyl, leaf, stem, stalk, petiole, shoot tip, protoplast, storage root, tuber, and cotyledons have been used successfully (Hu and Du, 2006). When adapting this protocol, the proper explant material may vary by species. Typically, juvenile material will give the best results.

Figure 1. The outline of hairy root transformation procedures(see p 84).

a) 5 day old soybean seedlings ready for hairy root inoculation
b) A student inoculating cotyledons with *A. rhizogenes*
c) 6 day old cultures showing browning and callus
d) 15 day old cultures showing callus formation
e) 30 day old cultures showing root development
f) Transgenic root using visible light
g) expression of GFP in adventitious hairy roots

Conclusions

A written report including an abstract, review of literature, objectives, materials and methods, results, discussion, and conclusions were required of all students in the course. The written report required interpretation of the data obtained by the entire class. Assessments of the written reports were based on the student's ability to demonstrate an understanding of the technique and root transformation process through their writing. All of the papers were well written and indicated that students gained valuable insights from the project. Students were further asked to evaluate this experiment by completing the following survey and commenting on the value of the exercise. Responses were strong for all of the questions (Table 1). One student commented that this lab exercise was "the most interesting lab exercise that I have done". Another student suggested that this experiment "made us feel like real scientists and was both a fascinating and highly rewarding experiment". Other students in the course stated that the contaminated plates and slow callus growth in the first trial were actually a plus demonstrating some of the realities of conducting laboratory research.

Hairy root culture systems can easily be adapted for use in classroom laboratories or for undergraduate research. Historically, root systems have been very difficult to study. Hairy root culture systems offer an extremely versatile tool that can be used on a wide variety of plant species to answer a range of biological questions. Classroom studies could be designed for secondary plant metabolism in biochemistry, plant transformation studies in biotechnology, root morphology in botany, symbiosis between plant and bacteria, fungi, or parasitic plants in ecology, genetic knockouts of genes involved in root development for genetics, gene silencing of transcription factors involved in root development for gene expression, antimicrobial activity of plant compounds in microbiology, potential for drug development in pharmacology, study of pest resistance genes in plant pathology, or auxin insensitivity in hairy roots for plant physiology courses. Students could be asked to design their own experiments and large scale screening would be possible with this system. This system ideally lends itself to investigative teaching in the laboratory.

Classroom laboratory exercises utilizing hairy roots could be very simple as in this situation or very sophisticated. We have demonstrated that this system can be used in small universities or colleges with limited resources. All of the work in this paper, with the exception of the fluorescent microscopy, was conducted at Maryville University. Maryville's biology department is located in a small, liberal arts college which has very limited laboratory resources. The fluorescent microscopy was conducted as a part of a field trip to the Donald Danforth Plant Science Center. Other reporter genes are available (e.g. GUS) that would not require specialized techniques or equipment to detect transformation/ gene expression, making the hairy root system useful for small institutions with limited resources.

Table 1 –Student survey on hairy root transformation study.

5= Strongly agree
4= Agree
3= Neutral
2=Disagree
1=Strongly disagree

1. This laboratory exercise increased my appreciation for sterile technique.

1 2 3 4 5—Average response = 5; SD=0

2. This laboratory exercise increased my curiousity concerning genetic transformation systems.

1 2 2 4 5—Average response = 5; SD=0

3. This laboratory exercise increased my understanding of genetic transformation systems.

1 2 3 4 5—Average response = 5; SD=0

4. This laboratory exercise increased my understanding of the use of marker genes in genetics transformation systems.

1 2 3 4 5—Average response = 4.8; SD= 0.35

5. This laboratory exercise increased my confidence in my ability to undertake independent research.

1 2 3 4 5—Average response = 4.6; SD= 0.5

6. I think that this exercise is a useful supplement to a biotechnology laboratory experience.

1 2 3 4 5—Average response = 5; SD=0

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NOTE: Figure 1 of this article is displayed in its entirety on page 84 of this issue. Our apologies to the authors for this omission.

Figure 1. from Keyes et al. (see page 9 for complete legend)