

Tracking of Transgene Expression in Soybean using Robotics and GFP

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With the increased planting of genetically modified crops in the world over the past 10 years, it is important to develop a good understanding of the expression of introduced transgenes in plants. This is especially critical for soybean, which clearly dominates the other crops for the amount of land planted with transgenics. Most of the transgenic soybean grown world-wide contains the Roundup Ready® trait, which provides soybean containing the trait with resistance to the herbicide, Roundup®. Although this transgene is extremely well-characterized and represents a single, successful gene introduction from almost 15 years ago, efforts continue in other university, federal and industrial laboratories to introduce other genes of interest, into this valuable crop plant.

In order to study gene expression in soybean, tissues and plants are most-often extracted and the resultant products are then analyzed. Although this is necessary and useful for the final analysis, this clearly results in the destruction of the tissue and only single time point determinations can be performed. On the other hand, gene expression tracking using the *green fluorescent protein* (*gfp*) allows observations to be made, over time, from the same piece of tissue. GFP is easily visualized within living plant tissue and is therefore an extremely valuable tool for understanding and following gene expression. Visualization of GFP does require illumination of the subject material with the proper wavelength of light and use of GFP-specific filters but, up to this point, the instrumentation required for automated, continual tracking of gene expression at the macroscopic level was not available.

In our laboratory, we have developed an automated GFP image collection system that is composed of a 2-dimensional robotics platform, a Leica MZFLIII fluorescence dissecting microscope, a CCD digital camera, all under computer control (Figure 1; Buenrostro-Nava *et al*, 2005). This automated system is housed in a laminar flow hood, designed to provide a vibration-free work surface. Samples of tissue that express the *gfp* gene are placed on various media in Petri dishes, which are mounted on the robotics platform. The platform contains docking centers for 16 Petri dishes, which are secured to the platform using locking screws. Petri dishes are placed in the focal plane of the microscope using leveling screws, which are used to lower the dishes as the tissues enlarge over time. At pre-determined time points, the Petri dishes are moved below the microscope's single objective (using the robotics platform) and images are collected by the camera and stored on the computer. The image series are subsequently analyzed using a image analysis software and GFP intensity and area are nondestructively quantified as a measure of gene expression. In addition, image series can be assembled to generate time-lapse animations of *gfp* gene expression, providing yet more information on gene expression in plant tissues. Time-lapse animations are both striking and relatively easy to interpret, and movies can be used to make observations and conclusions, not possible with both tissue extraction analysis and single time point determinations.

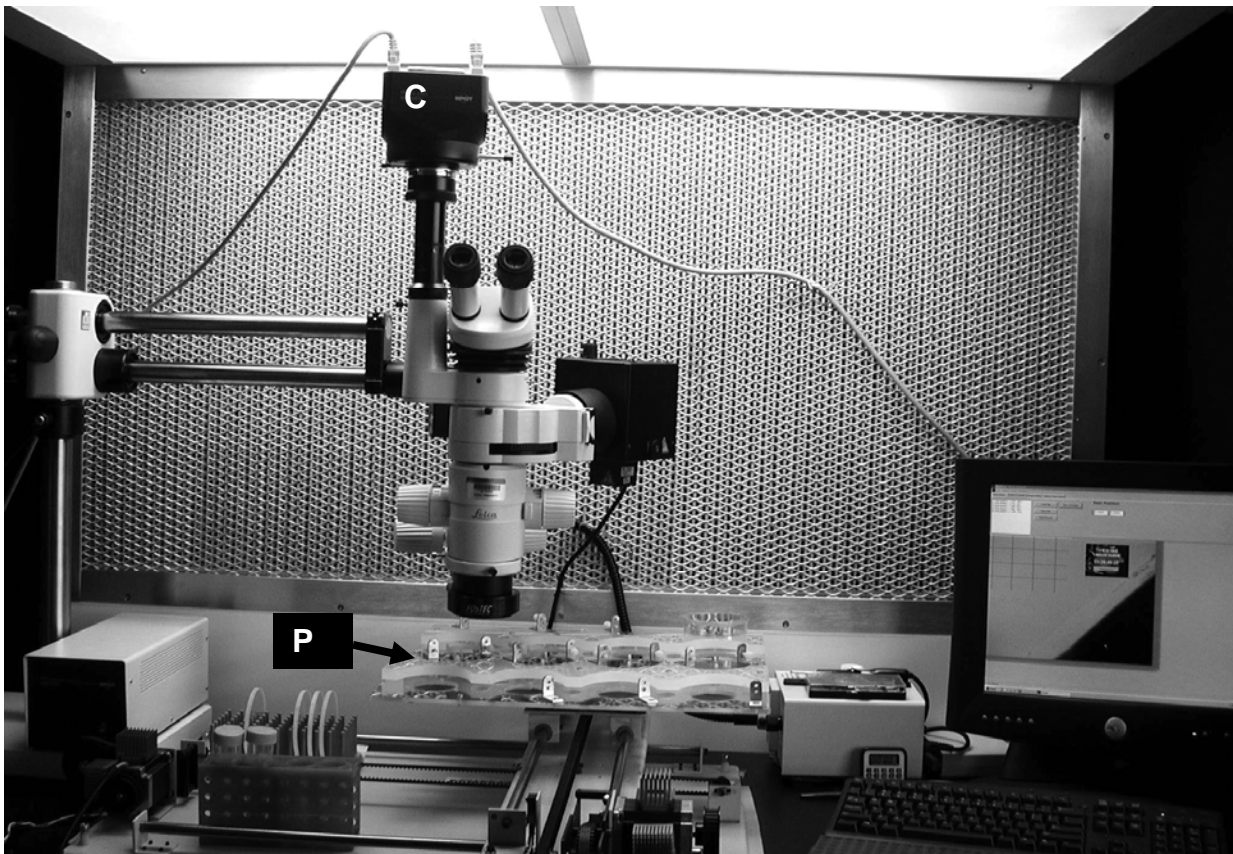


Figure 1. Robotics system used for automated image collection. C; Digital Camera, P with arrow; robotics Platform.

Although the robotics system hold tremendous potential for analysis of GFP expression, the main limitation of the system is the high cost, rapid degeneration and short life of the light source, needed to excite GFP to fluoresce. The mercury light bulbs, used for illumination for GFP detection, degenerate to 30% of the original intensity over their life of 300 hours. Compensation for low GFP intensity reading from the reduction in illumination intensity is overcome using image analysis standards but the bulb life restricts simple experiments to those that can be completed within 300 hours. Longer-lasting illuminators are available but these have not been thoroughly evaluated and it is unclear if image quality is comparable to images collected using mercury bulbs. As a result of the constraints from limited bulb life, experiments which can be completed within 300 hours have received the most attention. These experiments include mostly short-term tracking of GFP expression in stably-transformed tissue (Buenrostro-Nava *et al*, 2006) and transient expression studies with a variety of target tissue (Finer *et al*, 2006). Transient expression analyses are very useful for rapid evaluation of various promoters and factors which could potentially enhance or stabilize gene expression. To take advantage of the promoter evaluation capabilities of the robotic system, efforts have been placed to isolate, introduce and characterize numerous soybean promoters over the past year.

Tracking of GFP Expression in Stably Transformed Tissues

One of the original reasons for wishing to track GFP expression was to determine which cells within a target tissue had the ability to take up and integrate foreign DNA. The idea was to identify transformation competency by reverse tracking of GFP-expressing clumps of tissue back to the single cells, which gave rise to those events. It was envisioned that many cells express the *gfp* transgene transiently but only a few lead to stable transformation events. If the cells that gave those events were sufficiently unique, they could be identified and enriched, leading to higher transformation rates. After extensive efforts and, in spite of other reports of success in following stable transformation events from single GFP-expressing cells (El-Shemy *et al*, 2004; Kaeppler *et al*, 2000), it was determined that GFP alone could not be used as the visual selective agent. It became clear over the past 7 years (Ponappa *et al*, 1999) that GFP expression was extremely variable in stably transformed tissue culture cells and that a single transient GFP-expressing cell could not be followed to a stable event. In reality, we were able to document on numerous occasions, the “appearance” of GFP in stably-transformed soybean tissue (Figure 2) that was not accountable for expression in new growth. There appeared to be a “spread” or “induction” of GFP expression within that tissue, that often originated in only a portion of the stably-transformed clump.

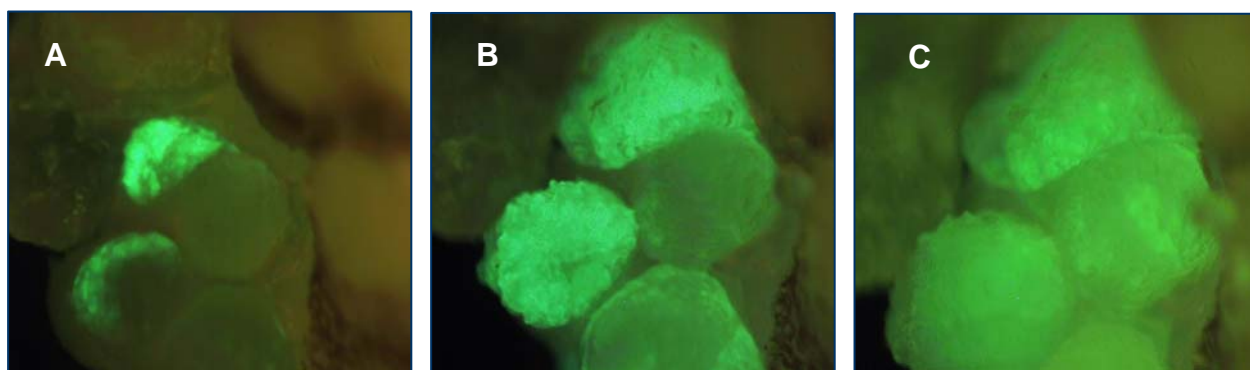


Figure 2. GFP expression in newly-selected, stably transformed soybean tissue, over the course of 14 days. From “A” to “B”; 7 days. From “B” to “C”; 7 days. Note the spread of GFP within the tissue, that is unaccountable for, based only on tissue growth.

More extensive analysis of GFP expression in stably-transformed tissues confirmed the variable nature of GFP expression in tissue culture (GFP expression in regenerated plant tissues do not appear to display the same level of variability in expression). Time-lapse animations of tissue cultured tissues showed areas of tissues that increased and then decreased in expression levels. Animations also revealed unusual surges and fluxes in expression patterns that were just not observable with single time point determinations. It is anticipated that the robotics system, together with the ability to generate time-lapse animations, will allow the discovery of unique expression pattern profiles, that would not otherwise be detectable.

Transient GFP Expression

For transient expression studies, the *gfp* gene can be introduced into a variety of different target tissues, resulting in the appearance of GFP in as little as 1.5 hours with a peak expression after 24 hours (Ponappa *et al*, 1999). The rapid appearance of the protein makes GFP well-suited for fast evaluation of factors that may influence gene expression. Although enhancers and stabilizers are needed and useful for fine control and optimization of transgene expression, little work has

been done on evaluation of promoters, which are the primary regulators of introduced transgenes. For soybean, very few soybean promoters have been characterized with most of the emphasis placed on seed-specific promoters (Chen *et al*, 1986; Cho *et al*, 1995) and one report of an extensin root specific promoter (Farah and Singh, 2002). But, with the wealth of information generated from genomics and microarrays (Thibaud-Nissen *et al*, 2003), isolation of additional promoters from soybean is relatively straightforward.

A number of additional soybean promoters have been isolated and characterized (Table 1). Many of these promoters were generated using 5' sequences from ESTs that have been shown from microarray data, to express during early embryo induction. Introduction of promoter:gfp constructions into stably-transformed soybean tissues has been ongoing but it is labor intensive and time-consuming. Rapid evaluation of promoters and promoter deletions using transient expression is much more feasible if quantification of transient expression using robotics and image analysis was utilized.

Soybean Promoters (except where noted)	Notes on expression in soybean
CaMV35S (not soybean-derived)	High, constitutive
Ubiquitin	Very high, constitutive
Actin	Early embryo specific
Extensin	Low root specific
HSP90-like	Early embryo specific
Actin	Early embryo specific
Phytase	Developing embryo hypocotyl
S11	Early embryo specific

Table 1. Soybean promoters (and CaMV35S) isolated and characterized using GFP analysis.

A new model has been developed for transient expression analysis of promoter:gfp constructions. DNAs are introduced into cotyledons of lima beans (*Phaseolus lunatus* cv Henderson bush) using particle bombardment (Finer *et al*, 1992). Lima bean seeds are sterilized and placed on filter paper under sterile conditions for imbibition and early germination. After 3-5 days, the cotyledons are excised and the adaxial surface of the cotyledons are bombarded. Lima bean cotyledons are ideally suited for these types of studies as they are flat, large and do not contain high levels of chlorophyll, which can interfere with GFP detection. In addition, lima bean seeds represent a consistent source of target tissues and these tissues are extremely receptive to transient GFP expression (Figure 3a).

Image analysis of transient GFP expression in lima bean cotyledons (Figure 3b) show that it is possible to quantify differences in expression levels using image analysis. Expression levels are influenced by the length of the promoter. For the soybean HSP90-like promoter (Figure 3b, left), truncation of the promoter did not yield a gradual decline in expression but showed a stepwise decline, suggesting that the sequence between bases 231 and 443 possessed critical elements in this promoter. It appears that the relatively small 443 bp HSP90-like promoter yielded high GFP expression levels. For the soybean ubiquitin promoter (Figure 3b, right), inclusion of the intron yielded very high transient expression levels. Although introns have been shown to be important in stabilizing transgene expression in monocot tissues (Vain *et al*, 1996), intron enhancement of transgene expression has not been previously demonstrated in dicots. It is also interesting to note that the soybean promoters gave much higher transient

expression than the CaMV35S promoter, which is believed to be a very strong constitutive promoters and is in use by numerous laboratories.

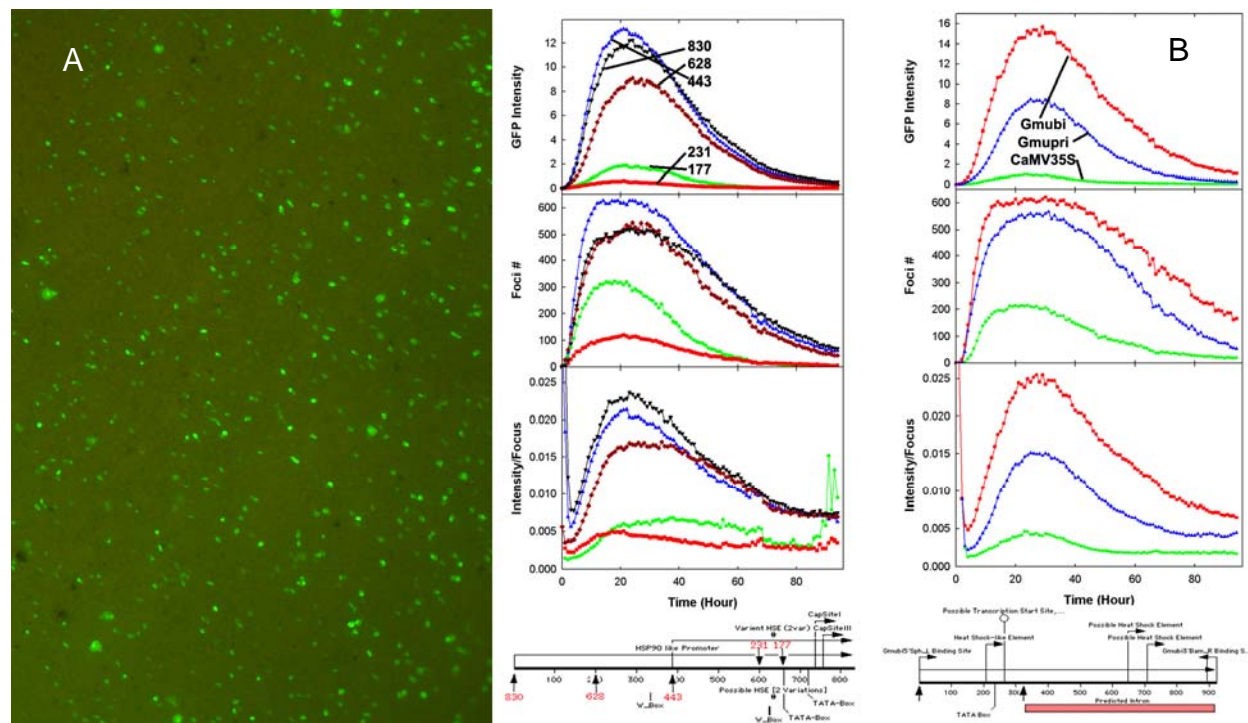


Figure 3. Transient expression analyses in cotyledons of lima beans. A) GFP expression foci after 24 hours. B) Graphs representing GFP intensity using the HSP90-like (left graph and map) and ubiquitin/35S (right graph and map) promoters. In the right bottom map, the full length ubiquitin promoter (Gmubi) is represented by the left upright arrow while the “pre-intronic” version (Gmupri) is represented as the region between the two upright arrows.

Additional Promoter/Stabilizer Studies with GFP

Promoter analysis has proceeded very rapidly with the use of the automated image collection system for GFP detection. Evaluation of the animations generated from the types of experiments summarized in Figure 3 show additional features, that are not visible from single time point determinations and from the graphical data in Figure 3b. The most interesting observation is the sudden appearance and disappearance of GFP expressing foci. Images are collected from numerous samples on an hourly basis and often, a cell expressing GFP will show great reductions in expression levels from one time point to the next (1 hr). These changes occur at low frequency and represent a very small percentage of expressing cells. As a result, the reduction in GFP expression does not alter the overall data on GFP expression intensity but it is an interesting phenomenon, nonetheless. It is unclear the rapid decline in GFP intensity occurs as a result of cell death, rapid message instability or silencing of the transgene. An understanding of the early events in transgene expression may give clues on how to enhance or stabilize transgene expression and transformation rates.

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