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Encephalomyocarditis (EMCV) Viral Internal Ribosome Entry Site Mediated Expression of Multiple Transgenes in Tobacco and *Arabidopsis thaliana*

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College Scholars Senior Thesis

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I. Abstract

Internal ribosome entry site (IRES) sequences provide a potentially useful method for bicistronic and coordinated transgene expression in plants. In mammalian cells infected with certain viruses, translation of proteins is initiated in a cap-independent manner via IRES. Ribosomes are able to bind internally at an initiating AUG start codon without scanning the 5' untranslated region of the transcript. Whereas IRES sequences have been shown to be of limited success in plant transgene expression experiments, my experiments attempting to over express two transgenes with a single promoter with IRES sequences in between the transgenes were not effective. The second transgene and IRES sequence was truncated in northern blot analyses for one set of transgenes. In another set, there was evidence of low and coordinated expression of both genes. These latter experiments demonstrated that perhaps lux genes for bioluminescence might be expressed in a coordinated fashion using a single promoter and IRES sequence cloned between genes of interest

II. Introduction

In order to create transgenic plants, foreign DNA from other species of plants, animals, or bacteria have to be inserted into the plant's genome. Transforming a plant's genome can be a long and tedious process, especially when multiple genes must be added to the genome together to produce a functional trait. Using conventional methods of plant breeding, two plants of the same species possessing desirable traits, such as fitness or crop production, would be selected for cross breeding. The progeny of this crossing would then be screened for possession of the two desirable traits from the paternal plants. These plants would then be crossed, screened and selected based upon trait expression and then used for further development.

The same basic principles can be applied to transgenic plants. In order to create a transgenic plant containing multiple foreign genes, two different genes would have to be inserted into two different T_0 (first generation) plants using *Agrobacterium tumefacians* mediated transformation. The progeny of the T_0 generation would then be crossed to form F_1 hybrid plants. After the F_1 plants mature, genetic analysis would have to be performed in order to recover plants that contained the two foreign genes from the T_0 generation. This is a very time consuming process; therefore, any method where multiple genes could be added from one bicistronic (two genes controlled by a single promoter) vector would greatly expedite the transformation process. Plants, like all eukaryotic organisms, have no internal mechanisms that can be utilized for multi-gene insertion. Therefore they are classified as monocistronic organisms. A monocistronic is an organism that contains only one open reading frame (ORF) in their RNA transcripts. In order for an RNA transcript to be translated by the plant cell's translation machinery, it must contain several key genetic sequences: a promoter, mRNA template and a terminator sequence; these three sequences make up a "gene" but only one coding sequence or ORF can be controlled by each promoter sequence.. The promoter regulates transcription and can be turned "on" or "off" due to intracellular or intercellular signals. The RNA template possesses a codon sequence that is the foundation for the protein's (or other molecule) primary structure. The terminator sequence signals the completion mRNA's transcriptional activity.

In contrast, most prokaryotes are classified as polycistronic organisms, meaning that their mRNA transcripts can contain multiple ORFs. One example of a polycistronic mRNA transcript can be found in the bacterial *lac* operon. The *lac* operon contains three tandem genes, *Lac Z, Lac Y* and *Lac A,* which are expressed in bacteria cells when the energy or glucose levels in the cell

are low. These signals initiate a cascade of events resulting in a break down of lactose into galactose and glucose, which can then be utilized for cellular energy. Since plants are monocistronic and not polycistronic organisms, researchers have to devise ways to insert multiple genes into the plant's genome to be expressed in a coordinated fashion.

There are currently two methods being applied to co-express heterologous gene products in a single vector: 1. independent promoters in a single plasmid or 2. internal ribosome entry sites (IRES) derived from mammalian viruses. It must also be stated that heterologous gene products can also be expressed by alternative splicing or reinitiation of translation in a limited number of cases (Mizuguchi et al, 2000). The first method to introduce multiple genes in a single transformation event uses multiple promoter-cDNA-terminator cassettes contained in a single expression vector. An example of this type of transformation was performed in 1999 with the formation of the pSAM12 binary expression vector which contained the selectable marker gene *mGFP5er* and *Bt cry1Ac,* an insecticidal gene (Harper et al. 1999). The expression of each gene was controlled by separate strong 35S constitutive promoter derived from the cauliflower mosaic virus (CaMV) (Harper et al. 1999) and the gene expression for each was correlated. However, in other studies it has been shown that promoter interference sometimes occurs with the use of two different or two strong promoters, i.e., transcription from one promoter suppresses transcription from another (Mizuguchi et al, 2000) resulting in gene silencing, which can be a problem when strong expression of each transgene is desired.

The second method for coordinate expression has been used only sporadically in plants, which employs an internal ribosome entry site (IRES) in between the two genes to be introduced into the plant's genome. By using the IRES method, two genes can be expressed from the same promoter, therefore eliminating the problem of silencing due to heterologous promoters.

Ribosomes can recognize the IRES sequence and bind internally at the AUG start codon of the second gene in the sequence without having to scan the 5' untranslated region (UTR) of the mRNA transcript.

The function of the IRES was first characterized in the 5' noncoding region of poliovirus RNA expressed in mammalian cells (Pelletier et al. 1988). It has also been demonstrated in picornavirus [*Encephalomyocarditis* virus (EMCV)] RNAs and other viral messages (Mizuguchi et al, 2000). IRES sequences taken from the 5' nontranslated regions of the EMCV genome are most widely used in gene therapy and gene transfer experiments in animals. It has also been shown that compared to other IRES sequences, including those from the hepatitis A and C, poliovirus, human rhinovirus, and foot-and-mouth disease virus, the IRES sequence derived from the EMCV virus has a higher translational efficiency (Mizuguchi et al, 2000). The EMCV IRES element has been extensively characterized in animal cell systems both *in vitro* and *in vivo*. EMCV IRES activity had not been used in plant cells until Urwin (2000) showed that the insertion of the EMCV IRES element between two open reading frames (ORFs) of a plant's bicistronic transcript could mediate translation of the second ORF.

The genes that were the subject of our experiments were *mGFP5er* and *Bt cry1Ac* fusions, and luxA and luxB fusions. We created constructs that contained GFP and Bt linked by the IRES, and we created constructs that contained luxA and luxB linked by the IRES sequence. Green fluorescent protein (GFP) is modified protein derived from the jellyfish *Aequorea victoria.* The *mGFP5er* gene is targeted to the endoplasmic reticulum of the plant cell by polypeptide fusion. It has been modified for cryptic intron mis-splicing and codon usage (Harper et al. 1999). GFP is a widely used marker for monitoring transgenic plants because it fluoresces green when excited with ultraviolet of blue light without the addition of substrates or cofactors

(Harper et al. 1999).Bt is an insecticidal protein derived from a naturally occurring soil bacterium called *Bacillus thuringiensis.* Synthetic *Bt cry1Ac,* patterned after the *Bt* ssp *kurstaki cry1Ac* sequence, was designed for high expression in plants (Stewart et al 1996). One advantage of using *Bt* genes in transgenic plants for insect control is that specific to certain orders of insects. For example, *Bt cry1Ac* is specifically toxic to the order Lepidoptera, and therefore not harmful to other insect, birds and mammals (Stewart et al. 1996). To date, there has been much success in cloning *Bt* into transgenic crops, which makes it an obvious candidate for IRES cloning. LuxA and luxB are derived from the luminescent bacteria *Photorhabdus luminscens*. The gene products luxA and luxB must both be produced and combine to form a heterodimer resulting in an active bacterial luciferase, which carries out the reaction:

$$FMNH_2 + RCHO + O_2 \rightarrow FMN + RCOOH + H_2O + \text{light } (\lambda\ 490nm)$$

The long-chained aliphatic aldehyde (RCHO) decanal was used as a substrate (Szenthe et al. 1998). The reaction results in bioluminescence in the tissues extract samples which can be detected and measured by a luminometer (Szenthe et al 1998, Koncz et al. 1987).

The objective of this study was to determine if IRES-mediated gene expression would be effective in expressing a small gene (GFP) followed by a large gene (Bt), and vice versa, compared with two relatively small genes in tandem (lux A and lux B) in two different plant species: *Nicotiana tabacum* (tobacco) and *Arabidopsis thaliana*.

III. Materials and Methods

Plasmid Construction

The coding mGFP5er gene was obtained from the pBIN-mGFP5-ER plasmid (Haseloff et al. 1997; Harper et al. 1999) using restriction enzyme digestion. The *Bt cry1Ac* gene was

obtained from the pH602.SBt (Singsit et al. 1997).The *luxA* and *luxB* genes were obtained from genes codon optimized for mammalian cell expression (Patterson et al. 2005). pIRES mammalian cloning vector was obtained from Clontech (Mountain View, CA).

The pMDC30 and pMDC32 binary vectors were developed by Dr. Mark D. Curtis and colleagues with the aim of providing Gateway-compatible *Agrobacterium* sp. binary vector system that facilitates fast and reliable DNA cloning. The pMDC30 vector contains a heat shock inducible promoter from the Gmhsp17.3B promoter fragment from the SHS3252 plasmid (Curtis et. al, 2003). The pMDC32 binary vector contains a dual 35S CaMV promoter which is highly active in transgenic plants. Both vectors contain a hygromycin selection cassette between the T-DNA borders and a nos terminator. The genes of interest were PCR amplified and then inserted into the different MCS that flanked the IRES sequence in the EMCV pIRES plasmid.

BtCry1Ac, mGFP5er, *luxA* and *luxB* were amplified from source template using Hotstart *Pfu* polymerase (Stratagene, La Jolla, CA) according to manufacturer's protocol. PCR products (see Table 1) were amplified. The genes of interest were PCR amplified and then inserted into the different MCS that flanked the IRES sequence in the pIRES plasmid through restriction digests. pIRES constructs containing both transgenes were used as a template for amplification of an attB flanked PCR product. attB flanked products (see Table 2) (luxA-IRES-luxBattB, luxB-IRES-luxAattB, mGFP5er-IRES-Btcry1AcattB, and Btcry1Ac-IRES-mGFP5er) were recombined into pDONR/Zeo vectors utilizing BP Clonase (Invitrogen, Carlsbad CA) according to manufacturer's specifications. The resulting entry vectors (pENTR-Zeo-luxAIluxB, pENTR-Zeo-luxBIluxA, pENTR-Zeo-mGFP5erIBt ry1Ac, and pENTR-Zeo-Btcry1AcImGFP5er) were sequence verified by DNA sequencing. Binary vectors were created by LR Clonase (Invitrogen) mediated recombination between IRES containing entry vectors and the binary destination

vectors pMDC30 and pMDC32 (see table 3). Binary vectors were transferred into *Agrobacterium tumefaciens* GV3850 and resulting cultures were used for plant transformation.

Plants

Two plant species were used: *Nicotiana tabacum* cv Xanthi and *Arabidopsis thaliana* ecotype Columbia.

Transformation and Plant Regeneration

Tobacco seeds were surface sterilized (10% commercial chlorine bleach and 0.001% Tween 20 solution) for 2.5 minutes. Seeds were washed with 70% ethanol solution for 1 minute and then rinsed with sterile water two times and allowed to air dry. The tobacco seeds were plated in Magenta GA7 boxes containing MSO media using the same method described in Harper et al. (1999). All plant media used 0.2% Gelrite gellan gum (Sigma, St. Louis, MO) as a solidifying agent. With the exception of the antibiotics that were filter sterilized and added after the media had cooled to 60ºC (prevents inactivation of the antibiotics) prior to pouring, all media components were autoclaved prior to pouring the media.

Leaves from the tobacco grown aseptically at the 8-12 leaf stage were cut into 1 cm^2 sections, which served as the explant material for *Agrobacterium tumefacians* transformation. The tobacco explants were co-incubated with *Agrobacterium tumefacians* strain GV3850 harboring the binary vectors. Tobacco explants were placed on DBI medium (DeVerna and Collins 1984). The media contained 1mg/L of indole acetic acid and 2mg/L kinetin in 100 mm Petri dishes for three days to initiate shoot organogenesis. Explants were moved to DBI with antibiotics (200 mg/L hygromycin (Sigma?) and 400 mg/l timentin (GlaxoSmithKline, Research

Triangle Park, NC) to select for transformed tobacco cells and against *Agrobacterium* growth. Explants were transferred to fresh media every 14 days until shoots were excised. Developing shoots were excised from developing callus and placed on MSO medium plus antibiotics. Rooted shoots were placed in soil-less potting media and moved to the greenhouse and allowed to set seed. T_1 seeds from individual plants were collected; each represented a discrete transformation event.

Arabidopsis was transformed by the floral dip method to transform ova in developing flowers directly on the plant using the Clough and Bent (REF year) method. The *Arabidopsis* seeds were plated in soil-less potting media to create highly dense populations within a single pot. Individual pots were dipped into a *Agrobacterium* culture before flowers opened. *Arabidopsis* seeds from dipped plants were collected when mature and surface sterilized (20% commercial chlorine bleach and 0.001% Tween 20) for 2.5 minutes. Seeds were washed with 70% ethanol solution for 1 minute and then rinsed with sterile water two times and allowed to air dry. The seeds were then plated on MS media containing antibiotics 200 mg/L hygromycin (Sigma) and 400 mg/L timentin (GlaxoSmithKline, Research Triangle Park, NC) to select for transformed *Arabidopsis* plants and against *Agrobacterium* growth.

GFP *fluorescence screening of T_1 plants*

T_1 Tobacco plants, containing the GFP/BT constructs, were grown on square grid MSO media plates containing antibiotics 200 mg/L hygromycin (Sigma) and 400 mg/L timentin. Ten lines from independent transformation events from constructs 17.15, 17.16, 17.17, 17.18, 17.19, and 17.20 were plated with 36 seeds per plate. Plants at the three week stage were examined using a hand-held UV fluorescent light. Plants were also examined using a Fluoromax-2 fluorescence

spectrophotometer (Jobin Yvon & Glen Spectra, Edison, NJ) using DataMax and GRAMS/386 software (Galatic Industries Corporation, Salem, NH) was used to quantify GFP fluorescence in leaf tissue. A negative non-transgenic control plant was used as well as a positive control plant containing the pBIN-mGFP5er plasmid. The youngest leaves were excited at 385 nm, and emission spectra was scanned and recorded from 420 nm to 600 nm. A fiber optic cable provided the excitation light to leaf tissue *in vivo,* and then collected the emission with no damage to the leaf tissue. Intensity was measured at 508 nm (green light) in counts per second (cps).

Luciferase Assay

T1 tobacco and *Arabidopsis* lines containing the *lux* genes (17.15 and 17.16) were screened for luminescence. The activity of the bacterial luciferase genes (LuxA and LuxB) was measured by a luminometer, using the total light produced during the first 10 seconds of enzymatic reaction (Szenthe et al 1998). Tissue samples were homogenized in 1.6 ml tubes in a solution containing 50 mM $Na_2HPO_4.7H_2O$, 50 mM BME, and 0.4 mM sucrose. 250 uL of tissue extract was mixed with 400 uL FMN in 1 mM Tricine Buffer pH 7 and 10 uL decanal (1:10,000) (Szenthe et al 1998) and then measured. Tissue extracts, with and without substrate, were measured for luciferase activity.

Southern blot analysis of Arabidopsis *plants*

Southern blot analysis was performed to assay for transgenicity. Genomic DNA was extracted from young tissue of five Arabidopsis samples: 5.2.8 A, 5.2.8 B, 5.2.1.5 A, 5.2.1.5 B, 5.2.1.5 C using a CTAB based procedure (Dellaporta et al 1983). After digestion with *EcoR*I, which restricts once within the T-DNA, genomic fragments were purified with QIAquick PCR

purification columns (QIAGEN, Valencia, CA) prior to electrophoretic separation on a 1% agarose gel. Fragments were transferred to Zeta-Probe GT membrane (Bio-Rad, Hercules, CA) and probed with PCR product contain the full-length open reading frame of LuxA and LuxB. PCR products were radiolabeled with α-^{32}P dCTP using Prime-It II Random Primers Labeling Kit (Stratagen, La Jolla, CA). Labeled probe was purified using mini Quick Spin DNA Columns (Rouche Diagnostics, Indianapolis, IN). Southern blots were hybridized with labeled probe overnight at 42ºC in ULTRAhyb hybridization buffer (Ambion, Austin, TX) and washed according to manufacturer's protocol. Blots were visualized by exposure to phosphor-imaging screens (Storage Phosphor Screen GP, Eastman Kodak, Rochester, NY) and scanned using Personal FX phosphoimager (Bio-Rad). Image analysis was performed using Quantity One software (Bio-Rad).

Northern Hybridization

T_1 Tobacco plants lines 17.17, 17.18, 17.19, and 17.20 were screened using northern hybridization. RNA was extracted from the developing plants using TRI REAGENT according to manufacturer's protocol (Molecular Research Center, Inc. Cincinnati OH). RNA was separated by formaldehyde agarose gel electrophoresis (Brown et al. 2004) prior to transfer to nylon membranes. The blots were probed with mGFP5er coding region or a 101bp fragment of the tobacco actin gene and washed as described above for Southern hybridization. Detection of hybridizing RNA bands was performed using the Personal FX phosphoimager (Bio-Rad).

IV. **Results**

GFP Fluorescence Screening of T_1 Tobacco Plants

When viewing the plants that contained the GFP-IRES-Bt and Bt-IRES-GFP insert (pMCD30-GFP-IRES-Bt, pMDC32-GFP-IRES-Bt, pMDC30- Bt-IRES-GFP, and pMDC32-Bt-IRES-GFP) under a hand-held UV light, GFP expression was not seen. The plants that contained the heat shock promoter (pMDC30 constructs) were heat shocked at 37°C for 18 hours prior to screening. When our homozygous GFP control (Xanthi + pBIN-mGFP5er) was screened, GFP expression was evident in the leaves. GFP expression was also not evident when the plants were screened using the Fluoromax-2 fluorescence spectrophotometer (Jobin Yvon & Glen Spectra, Edison, NJ).

Luciferase Assay

Luciferase activity was detected in one construct line (17.15.10) in both tobacco and *Arabidopsis.* Luciferase activity was low but present (average 0.063 photons/s). Samples were tested with and without substrate.

Southern Blot

Southern Blot analysis was performed on *Arabidopsis* construct lines 5.1 and 5.2 (pMDC30 luxA-IRES-luxB and pMDC30 luxA-IRES-luxB). Single band products of the expected size were observed when digested with *EcoR*I. However, this does not necessarily mean that the gene-IRES-gene fragment was inserted once within the genome. The gene fragment could have been inserted multiple times meaning that the single band observed on the Southern Blot was a result of multiple fragments of the same size.

Northern Bolt Analysis

Three total membranes were radiolabled with three different probes: mGFP5er probe, *Bt cry1Ac* probe (not shown), and an Actin probe. When GFP was inserted into the MCS before the IRES sequence, the protein product appeared to have been truncated. The truncated product was the same size as the GFP control (Figure 1). When GFP cloned after the IRES sequence, the band appeared to be the expected size. No GFP transcript was observed in the northern blot from the plant RNA in which the heat-shock promoter was used. RNA was extracted four days post heat-shock, and by then the promoter had already been deactivated. A separate membrane, containing the same RNA samples, was probed with an actin-specific probe to show that the RNA was intact on the membrane and that the probes were binding properly.

V. Discussion

Plasmid Construction

The Gateway® system (Invitrogen) was used to create the binary vectors. The Gateway® system was chosen because it eliminates the use of restriction enzymes, gel purification, and ligations, and speeds vector construction. Our gene-IRES-gene fragment was fairly large (# of bases), and contained several internal restriction site. Consequently, using restriction enzyme digestion to excise our gene-IRES-gene fragment and then recombine that fragment with a binary vector was not a viable option. Instead, we used the Gateway® system because it is highly efficient, with nearly 99% desired clones post reaction. Also, the reading frame and orientation of our transgenes and IRES sequence are maintained during transfer (http://www.invitrogen.com).

Northern Blot Analysis

It is not entirely clear why there were complications with the GFP-IRES-Bt and Bt-IRES-GFP insertions. The IRES sequence was shown to function with GFP and firefly luciferase (Urwin et. al 2000) and also in our experiment using luxA and luxB (northern data not shown). Compared to our tobacco GFP control plants, which contained the pBIN-mGFP5er insert and was grown under the same conditions as our IRES containing plant, the protein level was extremely low. This suggests that there might be aberrations during the transcription process in plant cells since transcripts were either truncated in some circumstances and low abundance in others. The IRES sequence is derived from a mammalian virus therefore, the plant transcriptional and translational machinery may be such that it cannot efficiently process the IRES sequence when large genes such as like *Bt cry1Ac* are inserted into the IRES vector multiple cloning sites.

We had to expose our northern for 3 day on phosphor-imaging screens (Storage Phosphor Screen GP, Eastman Kodak, Rochester, NY). This resulted in an over exposure of our GFP control bands. However, if we had not exposed our northern blot for 3 days, than the banding patterns for the IRES sequences would have been difficult to distinguish. An ELISA or a western blot might have been beneficial to determine actual protein levels of the Bt and GFP. However, such analyses were outside the scope of this project

Other Bicistronic Transformation Options

Although IRES mediated expression of transgenes have been shown to be functional in plants, in our hands the low expression of transgenes does not make it a feasible option for plant bicistronic vector construction. Other options for heterologous gene expression include:

continued use of multiple promoters and just hope that gene silencing does not occur or experimenting with the 2A region from the foot-and-mouth disease. The foot-and mouth-disease contains an IRES sequence like the *Encephalomyocarditis* virus. However, it also contains a sequence that is located in the 2A oligopeptide region. The 2A oligopeptide is emerging as a highly effective new tool for the co-expression of multiple proteins in a single transformation step (e.g. a geneencoding multiple proteins, linked by 2A sequences, is transcribed from a single promoter). The polyprotein self-processes co-translationally such that each constituent protein is generated as a discrete translation product (Felipe et al. 2006) So far the 2A oligopeptide has been shown to function is all of the eukaryotic systems that it has been tested in, including plants.

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Table 1. PCR products and primers utilized to amplify lux, GFP and BtCry1Ac fragments for use in cloning into IRES containing vectors. The sequence fragments in bold type represent the recognition site, and the underlined sequence represents the primer's start codon.

PCR Product	Size BP	Template	UP Primer	Sequence	DWN Primer	Sequence
Humanized luxA XbaI SalI	1103	pPA2	luxA:XbaUP	GCA**TCTAGA**CATGAAGTTCGGCAACTT	luxA:SalDWN	TTAT**GTCGAC**CTAGTACAGGAGGCTGCG
Humanized luxA NheI MluI	1098	pPA2	luxA:NheUP	T**GCTAGC**CATGAAGTTCGGCAACTT	luxA:MluDWN	T**ACGCGT**CTAGTACAGGAGGCTGCGT
Humanized luxB XbaI SalI	1003	pPB2	luxB: XbaUP	GCA**TCTAGA**CATGAAGTTCGGCAACTT	luxB:SalDWN	TTAT**GTCGAC**TTAGGTGTACTCCATGTG
Humanized luxB NheI MluI	998	pPB2	luxB: NheUP	T**GCTAGC**ATGAAGTTCGGACTGTTC	luxB:MluDWN	T**ACGCGT**TTAGGTGTACTCCATGTG
mGFP5er XbaI SalI	811	pBINmGFP5er	GFP: XbaUP	GCA**TCTAGA**ATGAAGACTAATCTTTTTC	GFP:SalDWN	TTAT**GTCGAC**TTAAAGCTCATCATGTTT
mGFP5er NheI MluI	806	pBINmGFP5er	GFP: NheUp	T**GCTAGC**ATGAAGACTAATCTTTTTCTC	GFP:MluDWN	T**ACGCGTTTA**AAGCTCATCATGTTT
BtCry1Ac SalI NotI	1862	pH603S.Bt	Cry1Ac: SalUP	CGC**GTCGAC**ATGGACAACAATCCCAACAT	Cry1Ac:NotDWN	CCGG**CGGCCGC**TTACTCGAGCGTTGCAGTAA
BtCry1Ac NheI MluI	1857	pH603S.Bt	Cry1Ac: NheUP	T**GCTAGCC**ATGGACAACAATCCCAACAT	Cry1Ac:MluDWN	T**ACGCGT**TTACTCGAGCGTTGCAGTAA

Table 2. Gateway Specific Primers: pIRES constructs containing both transgenes were used as a template for amplification of an attB flanked PCR product. attB flanked products (luxA-IRES-luxBattB, luxB-IRES-luxAattB, mGFP5er-IRES-Btcry1AcattB, and Btcry1Ac-IRES-mGFP5er) were recombined into pDONR/Zeo vectors utilizing BP Clonase. The sequence fragments in bold type represent the primer's ecognition site, and the underlined sequence represents the primer's start codon.

Working Name	*Full sequence (Gateway specific – start&stop codons – gene specific portion)*
luxAGUP	**GGGGACAAGTTTGTACAAAAAAGCAGGCT**ATGAAGTTCGGCAACTTCCTG
luxAGDWNstop	**GGGGACCACTTTGTACAAGAAAGCTGGGT**CTAGTACAGGAGGCTGCGTTG
luxBGUP	**GGGGACAAGTTTGTACAAAAAAGCAGGCT**ATGAAGTTCGGACTGTTC
luxBGDWN	**GGGGACCACTTTGTACAAGAAAGCTGGGT**TTAGGTGTACTCCATGTG
gfpGUP	**GGGGACAAGTTTGTACAAAAAAGCAGGCT**ATGAAGACTAATCTTTTT
gfpGDWN	**GGGGACCACTTTGTACAAGAAAGCTGGGT**TTAAAGCTCATCATGTTT
BtCry1AcGUP	**GGGGACAAGTTTGTACAAAAAAGCAGGCT**ATGGACAACAATCCCAAC
BtCry1AcGDWN	**GGGGACCACTTTGTACAAGAAAGCTGGGT**TTACTCGAGCGTTGCAGT

Table 3. Different constructs were created that contained alternating gene-IRES-gene sequences controlled by either a heat-shock inducible promoter or a constitutive 35S promoter. Construct name was used for differentiation purposes during tissue growth and development. Each numerical construct name corresponds to the specific binary vector that was inserted into *Agrobacterium* for tobacco and *Arabidopsis* transformation.

Construct Name	Binary Vector
5.1	pMDC30-LuxA-IRES-LuxB
5.2	pMDC32-LuxA-IRES-LuxB
17.15	pMDC30-LuxB-IRES-LuxA
17.16	pMDC32-LuxB-IRES-LuxA
17.17	pMDC30-mGFP5er-IRES-Bt cry1Ac
17.18	pMDC32-mGFP5er-IRES-Bt cry1Ac
17.19	pMDC30-Bt cry1Ac-IRES-mGFP5er
17.20	pMDC32-mGFP5er-IRES-Bt cry1Ac

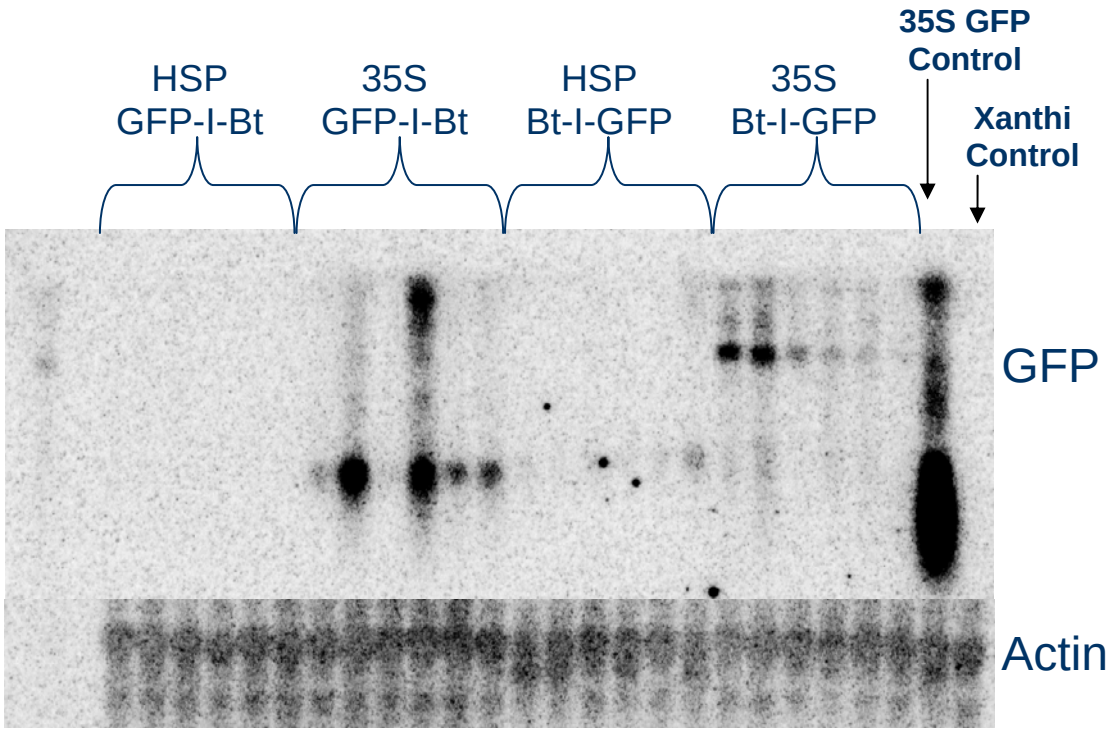

Figure 1. T1 Tobacco RNA Northern: When GFP was inserted into the MCS before the IRES sequence, the protein product appeared to have been truncated. The truncated product was the same size as the GFP control. When GFP cloned after the IRES sequence, the band appeared to be the expected size.