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Luciferase as a Reporter of Gene Activity in Plants

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ABSTRACT

Reporter gene systems based upon modified luciferase genes isolated from organisms, ranging from bacteria to insects, have proven to be important tools for plant molecular studies. The biochemical characteristics of these genes combine very high sensitivity with the ability to determine reporter activity non-destructively *in vivo*, allowing many applications in plants that cannot be accomplished using any other single reporter system. The relative ease of *in situ* detection of the firefly luciferase has made it an especially successful reporter for screening mutants in the model plant genetic system, *Arabidopsis thaliana*. The rapid turnover rate of luciferase has aided the characterization of promoters and elements that are influenced by time-sensitive factors such as circardian rhythm (diurunal cycles), gene silencing, and environmental stresses. The high sensitivity of luciferase as a reporter has facilitated the analysis and development of synthetic promoters for plant gene expression. Additionally, the biochemical characteristics of different luciferases have allowed their use as *in situ* indicators of metabolic activity and oxygen levels, as well as direct indicators of *in vivo* protein-protein interaction. The various applications of luciferase-based reporter systems in plants will be the subject of this review.

Keywords: luminescence, reporter gene, transgenic

Abbreviations: 35S, the 35S promoter from cauliflower mosaic virus (CaMV); **BiFC**, bimolecular fluorescence; **BRET**, bioluminescence resonance energy transfer; **CbLUC**, click beetle luciferase (from *luc* gene, *Pyrophorus plagiophthalamus*); **FiLUC**, plant intron-containing firefly luciferase gene; **FLUC**, firefly luciferase (from *luc* gene, *Photinus pyralis*); **FRET**, fluorescence resonance energy transfer; **GFP**, green fluorescent protein (from *Aequorea victoria*); **GUS**, β-glucuronidase (from *gusA* gene, *Escherichia coli*); **HcPro**, helper component protein (viral suppressor of silencing from potato virus Y) ; **LUX**, Bacteria luciferase (from *luxA* and *luxB* genes, *Vibrio harveyi*); **PTGS**, post-transcriptional gene silencing; **RiLUC**, plant intron-containing Renilla luciferase gene; **RLUC**, Renilla luciferase (from *ruc* gene, *Renilla reniformis*); **RNAi**, RNA interference; **SuPro**, modified agropine synthase promoter (SuperPromoter)

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INTRODUCTION

Since the development and introduction of reporter gene technology in the early days of plant genetic engineering, reporter genes have established a proven track record as effective tools for exploring the molecular underpinnings of gene regulation. When driven by appropriate genetic control systems (e.g. transcriptional promoters), an archetype reporter gene produces a product that is easily, accurately, and uniquely assayable within diverse biochemical environments. Moreover, a reporter's signal should precisely reflect the current level of gene expression, accurately indicating the tissue and/or cellular location, as well as any developmental or chronological changes in gene activity (de Ruijter *et al.* 2003). To date, several reporter systems have been found that meet the majority of these criteria and serve as the primary workhorses for plant reporter gene research.

The β-glucuronidase gene (*gusA*) from *Escherichia coli*,

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Invited Review

Table 1 Luciferases commonly used in plant research.

Enzyme	Standard substrates	Emission Max	Genetic modifications to luciferases
Bacterial luciferase (*Vibrio harveyi*)	$H_2C—(CHOH)_3—CH_2—(PO_4)^{-2}$ (structure) Reduced riboflavin phosphate Also requires O_2 and a long chain aldehyde	490 nm	Plant expression: Koncz *et al.* 1987 *luxAB*-subunit fusion: Olsson *et al.* 1989 Codon usage: Mayfield and Schultz 2004
Firefly luciferase (*Photinus pyralis*)	Firefly luciferin Also requires O_2 and ATP	562 nm	Plant expression: Ow *et al.* 1986 Plant intron: Luehrsen and Walbot 1991; Mankin *et al.* 1997 Codon usage: Lonsdale *et al.* 1998 Protein fusions: Zenser *et al.* 2003; Koo *et al.* 2007 Subcellular targeting: Gnanasambandam and Birch 2004 Thermal stablility and shifted emission: Branchini *et al.* 2007
Renilla luciferase (*Renilla reniformis*)	Coelenterazine Also requires O_2	480 nm	Plant expression: Mayerhofer *et al.* 1995 Protein fusions: Minko *et al.* 1999; Wang *et al.* 2002; Subramanian *et al.* 2006 Plant intron: Cazzonelli and Velten 2003 Codon usage: Fuhrmann *et al.* 2004 Split gene protein-protein interaction: Fujikawa and Kato 2007

and of late from *Staphylococcus* (Jefferson 1987; Jefferson 1989; Hull and Devic 1995; Broothaerts *et al.* 2005) has proven to be a sensitive indicator of transgene activity and has been very effective at identifying which tissues and cells actively express the reporter construct being tested. However, analysis using the GUS systems nearly always results in the death and/or destruction of test tissues or organisms, which for some applications limits the reporter's utility (Kirchner *et al.* 1993).

More recently, intrinsically fluorescent proteins, typified by the green fluorescent protein (GFP) from the marine jellyfish, *Aequorea victoria*, have been adapted to serve as non-destructive reporters, allowing 'real time' characterization of gene activity within living tissues subjected to different biological or environmental treatments (March *et al*. 2003; Dixit *et al*. 2006; Haseloff and Siemering 2006; Hraska *et al*. 2006). Reporters based upon GFP are more difficult to accurately quantify than GUS, but have proven extremely effective at localizing expression to tissue, cellular and sub-cellular levels. In the green tissues of plants GFP detection is negatively impacted by the intrinsic fluorescence of chlorophylls, although this has been somewhat attenuated through the use of dedicated optics and synthetic GFP mutants (such as red or yellow fluorescent forms), or related natural proteins, that display altered fluorescence spectra (Haseloff and Siemering 2006).

A third class of reporters that make use of bioluminescent luciferases has found broad use for both qualitative and quantitative tracking of gene activity in plants (**Table 1**). Biological systems for the generation of biochemical chemiluminescence, or bioluminescence, have evolved multiple times, producing several independent enzymatic systems that show almost no DNA sequence similarity [see (Hastings 1995; Wilson and Hastings 1998; Greer and Szalay 2002; Viviani 2002; Roda *et al*. 2004) for reviews]. The firefly luciferase from *Photinus pyralis* and the bacterial *lux* genes from *Vibrio harveyi* were the first to be adapted for use in plants, and were introduced at about the same time as the *gusA* gene from *E. coli*. The collection of plant-functional luciferases was supplemented a bit later through addition of the "sea pansy" or *Renilla reniformis* enzyme (Ow *et al*. 1986; Koncz *et al*. 1987; Howell *et al*. 1989; Schneider *et al*. 1990; Millar *et al*. 1992b; Luehrsen and Walbot 1993; Mayerhofer *et al*. 1995; Baruah-Wolff *et al*. 1999; Greer and Szalay 2002; Cazzonelli and Velten 2006; Southern *et al*. 2006). Luciferase reporters combine the high sensitivity and accurate quantification of GUS with the non-destructive delectability of GFP. The rapid turnover and relative ease of *in situ* detection of firefly luciferase made it an especially successful tool for mutant screening in the model plant genetic system, *Arabidopsis* (**Table 2**). Additionally, the biochemical characteristics of different luciferases [see **Table 1** and review articles (Hastings 1995; Wilson and Hastings 1998; Greer and Szalay 2002; Viviani 2002)] have allowed their use as *in situ* indicators of metabolic activity and oxygen levels, as well as direct indicators of *in vivo* protein-protein interaction (**Table 2**).

An expanded understanding of bioluminescent proteins and their genes, coupled with extensive applied research, has generated an impressive set of comprehensive protocols for monitoring luciferase activity in plants [cited throughout this review and in (Howell *et al*. 1989; Millar *et al*. 1992b; Luehrsen and Walbot 1993; Van Leeuwen *et al*. 2000; Southern *et al*. 2006)]. This account represents the first broad overview into the numerous applications of luciferase-based reporter systems to plant biological research and biotechnological innovation.

Table 2 Various plant species in which luciferase reporters have been successfully utilized. The list is not intended to be comprehensive and we apologize to anyone who may have been omitted. Additional applications are cited in the text and by (Greer and Szalay 2002).

Gene	Plant Species	Application	Reference
FLUC	*Arabidopsis thaliana*	Function and imaging	Chinnusamy *et al.* 2002
		Recombination	Jelesko *et al.* 1999
		Gene trapping	Alvarado *et al.* 2004; Calderon-Villalobos *et al.* 2006
		ABA and stress	Christmann *et al.* 2005
		Hormone biosynthesis	Castle *et al.* 2005; Bancos *et al.* 2006
		Photo-oxidative stress and systemic signalling of high light stress	Karpinski *et al.* 1999; Fryer *et al.* 2002; Rossel *et al.* 2007
		Gene silencing	Naumann *et al.* 2005; Wielopolska *et al.* 2005; Fischer *et al.* 2006; Zhu *et al.* 2007
		Cold stress	Chinnusamy *et al.* 2003
		Osmotic stress	Ishitani *et al.* 1997
		Drought and high light stress	Ball *et al.* 2004; Rossel *et al.* 2006
		Circadian rhythm	Onai *et al.* 2004
		RNA stability	Lee *et al.* 2006
		UV signal transduction	Ulm and Nagy 2005
	Nicotiana tabacum	Function and imaging	Ow *et al.* 1986; Barnes 1990
		Promoter analysis	Arpat *et al.* 2004; Ono *et al.* 2004; Cazzonelli *et al.* 2005a; Velten *et al.* 2005; Cazzonelli and Velten 2007
		Recombination	Ilnytskyy *et al.* 2004
		Ribozyme substrate	Ando *et al.* 2006
		Gene silencing	Kasim *et al.* 2003; Cazzonelli and Velten 2006
		mRNA function	Gallie *et al.* 1991
		Codon usage	Lonsdale *et al.* 1998
	Nicotiana benthamiana	Gene silencing	Hellens *et al.* 2005; Cazzonelli and Velten 2006
	Medicago truncatula	Extracellular ATP	Kim *et al.* 2006
	Petunia hybrida	*In vivo* quantification	Van Leeuwen *et al.* 2000
	Beta vulgaris	Promoter analysis	Schmidt *et al.* 2004
	Zea maize	Altered codon usage	Lonsdale *et al.* 1998
	Triticum aestivum	Altered codon usage	Lonsdale *et al.* 1998
	Saccharum officinarum	Vacuoular targeting	Gnanasambandam and Birch 2004a
	Hordeum vulgare	ATP gradients (*in situ*)	Rolletschek *et al.* 2004
	Vicia faba	ATP levels (*in situ*)	Borisjuk *et al.* 2003
	Physcomitrella patens	Circadian rhythm	Aoki *et al.* 2004
	Oryza sativa	Gene silencing	Bart *et al.* 2006
	in vitro	Heat shock protein assay	Lee *et al.* 1997
RLUC	*Arabidopsis thaliana*	Protein-protein interaction	Subramanian *et al.* 2006; Fujikawa and Kato 2007
	Nicotiana tabacum	Function and imaging	Mayerhofer *et al.* 1995; Cazzonelli and Velten 2007
		Promoter analysis	Cazzonelli *et al.* 2005b
		Gene silencing	Cazzonelli and Velten 2006
		Intron function	Cazzonelli and Velten 2003
	Nicotiana benthamiana	Promoter analysis	Hellens *et al.* 2005
		Gene silencing	Cazzonelli and Velten 2006
	Medicago sativa	Function and imaging	Mayerhofer *et al.* 1995; Hellens *et al.* 2005
	Lycopersicon esculentum	Function and imaging	Mayerhofer *et al.* 1995
	Solanum tuberosum	Function and imaging	Mayerhofer *et al.* 1995
	Chlamydomonas reinhardtii	Promoter Analysis	Fuhrmann *et al.* 2004
	Allium cepa	Protein-protein interaction	Subramanian *et al.* 2004
luxAB	*Arabidopsis thaliana*	Promoter analysis and pathogen response	Greer and Szalay 2002
	Nicotiana tabacum	Function and imaging	Koncz *et al.* 1987
		Promoter analysis,	Langridge *et al.* 1989
		Pathogen response	Greer and Szalay 2002
	Glycine max	Root nodulation	Langridge *et al.* 1994
	Lycopersicon esculentum	Function and imaging	Langridge *et al.* 1994
	Solanum tuberosum	Function, imaging and pathogen response	Langridge *et al.* 1994
	Datura stranibuyn	Function and imaging	Langridge *et al.* 1994
	Populus (hybrid)	Promoter analysis	Nilsson *et al.* 1992; Johansson *et al.* 2003
	Daucus carota	Gene fusion	Koncz *et al.* 1987
	Chlamydomonas reinhardtii	Promoter analysis	Mayfield and Schultz 2004

LUCIFERASE REPORTERS UTILIZED IN PLANT RESEARCH

The majority of publications describing the use of luciferase reporters in plants have made use of genes from three sources; insect (*Photinus pyralis* or firefly), coelenterate (*Renilla reniformis* or sea pansy) and bacteria (*Vibrio harveyi* and *Vibrio fisheri*) [see **Table 1** and Greer and Szalay (2002)]. The firefly (*luc*), Renilla (*ruc*) and the two-subunit bacterial (*luxA* and *luxB*) luciferase genes, each evolved independently and do not share common DNA sequence or overall protein structure. They, however, do share the use of molecular oxygen as a substrate for the highly energetic reactions required for light production. As with all recombinant genes that originate in a different species, some engineering of the original luciferase regulatory and coding sequences has proven necessary in order to optimize these enzymes for use in plants (examples listed in **Table 1**). Each luciferase has unique biochemical properties that dictate which enzyme is the best reporter for addressing specific experimental questions within plant systems. These characteristics have been presented in previous reports (Wilson

and Hastings 1998; Greer and Szalay 2002) and will only be described briefly in the context of the specific application being discussed.

Firefly luciferase (FLUC)

Firefly (*Photinus pyralis*) luciferase is the best characterized bioluminescent reporter and continues to be the benchmark for imaging bioluminescence in transgenic plants (Travis and McElroy 1966; DeLuca 1969; Gates and DeLuca 1975). The cDNA encoding firefly luciferase was originally cloned from *P. pyralis* and functionally expressed in *E. coli* (De Wet *et al.* 1985). The luciferase enzyme is a monomeric protein (62 Kda) which can generate yellow-green light through mono-oxygenation of beetle luciferin substrate (Wood *et al.* 1984). The luciferin substrate is a benzothiazole (structure shown in **Table 1**) found exclusively in fireflies (*P. pyralis* and *Luciola*) and in the presence of oxygen undergoes a Mg^{2+} and ATP-dependent reaction to produce dehydroluciferin, CO_2 and a photon of light (Hastings 1998). The wavelength of photons emitted by FLUC centers around 560 nm and observed alterations in emission spectrum associated with specific changes in luciferase structure have been well documented (Seliger and McElroy 1964; Hastings 1995).

The ability to exploit the enzymatic properties and sensitivity of FLUC was demonstrated over 40 years ago when the first luciferin-based ATP assay was developed for eukaryotic systems (Neufeld *et al.* 1975). It was subsequently shown that the FLUC reporter has superior qualities as a non-invasive and non-destructive reporter for monitoring gene expression *in vivo* when it was used to characterize the expression pattern of the "gold standard" cauliflower mosaic virus 35S promoter in tissues of *Nicotiana tabacum* (Ow *et al.* 1986; Howell *et al.* 1989). FLUC has since gained widespread use as a tool for monitoring gene expression changes in whole plants, protoplasts and detached tissues (Millar *et al.* 1992b; Van Leeuwen *et al.* 2000; Southern *et al.* 2006).

Despite the initial and early success of luciferase as a reporter, it had naturally evolved for the nocturnal mating behavior of beetles and not as a tool for plant molecular research. Thus, substantial research effort has been aimed at optimizing the firefly reporter to facilitate its application in a wide variety of host organisms. The original FLUC protein contains a C-terminal tri-peptide targeting signal that directs protein import into sub-cellular organelles such as the peroxisome, a cellular mechanism hat has been conserved between yeast, plants, mammals and insects (Gould *et al.* 1990). The targeting of large amounts of luciferase to the peroxisome has the potential of negatively impacting normal cellular function, and may also interfere with the *in vivo* performance of the reporter assay, depending on how stable FLUC is within the peroxisome and how effectively the FLUC substrate can reach the interior of that organelle. The peroxisomal translocation sequence, potential glycosylation sites and putative regulatory motifs were all removed from the native FLUC protein to create Luc+ (Sherf and Wood 1994). In this modified FLUC gene, codon usage was also altered, producing an improved form of luciferase that has been used to great effect by many researchers (Sherf and Wood 1994; Lonsdale *et al.* 1998).

The introduction of a plant intron into the Luc+ gene has extended its utility to research using *Agrobacterium tumefaciens*-based gene expression assays in plants (Mankin *et al.* 1997). The intron containing FLUC reporter eliminates background due to production of active luciferase within *A. tumefaciens*, but is efficiently removed during splicing in plant cells, providing an accurate tool to monitor reporter activity during and after T-DNA transformation events (Mankin *et al.* 1997; Cazzonelli and Velten 2006).

Other insect luciferases are currently being adapted for monitoring plant gene regulation (Viviani 2002) and will provide a new set of luciferase reporters with unique emission spectra and substrate specificities. Using the new luciferase reporters scientists will be able to multiplex reporters and develop new tools for plant molecular research.

Sea pansy luciferase (RLUC)

Sea pansy luciferase from the anthozan coelenterate, *Renilla reniformis* – a bioluminescent soft coral, or sea pansy – has served as a valuable alternative to firefly luciferase and has been indispensable in the development of a dual-luciferase reporter assay for plant systems (Matsuo *et al.* 2001; Cazzonelli and Velten 2006). The RLUC enzyme exists in its active form as a nearly spherical single polypeptide monomer of 35 kDa (Matthews *et al.* 1977). The cDNA was later isolated and functionally expressed in *E. coli*, *N. tabacum* and the chloroplast of *Chlamydomonas reinhardtii* (Lorenz *et al.* 1991; Mayerhofer *et al.* 1995; Minko *et al.* 1999). Renilla luciferase catalyzes the oxidative decarboxylation of coelenterazine substrates producing blue-green bioluminescence, oxyluciferin and CO_2 (Matthews *et al.* 1977). The coelenterazine substrate, although chemically distinct from the FLUC substrate, is also referred to as luciferin. However, coelenterazine is only found in a range of salt water organisms such as sea urchins, shrimp and certain fish taxa [reviewed by (Greer and Szalay 2002)]. The emission spectra of RLUC peaks around 480 nm (Matthews *et al.* 1977), which is a significant shift into the blue light region when compared to that of FLUC.

Like its predecessor FLUC, there are several Renilla variants with redesigned coding sequences that include modifications such as deletion of the peroximal targeting peptide, removal of potential transcriptional element binding sites and optimization of codon usage (Zhuang *et al.* 2001; Fuhrmann *et al.* 2004; Loening *et al.* 2007). The modified RLUC reporters show improved sensitivity and enhanced reliability in mammalian cells (Zhuang *et al.* 2001) and chloroplasts of *Chlamydomonas reinhardtii* (Fuhrmann *et al.* 2004), although there are no reported comparative studies in plants to confirm the effectiveness of these improvements. The insertion of a plant intron into a modified RLUC (pRL-null; Promega, www.promega.com) has provided a useful co-reporter for examining changes in gene expression and intron splicing events when using *Agrobacteria*-mediated transformation (Cazzonelli and Velten 2003; Cazzonelli *et al.* 2005b).

The RLUC reporter was successfully used to report promoter activity in alfalfa protoplasts, tobacco, tomato and potato transgenic tissues and surpassed both the bacterial and firefly luciferase reporters in terms of overall sensitivity (Mayerhofer *et al.* 1995). The higher sensitivity of RLUC was also confirmed in transient co-transfection assays of mammalian cell lines, showing up to a 100-fold higher signal when compared to FLUC (Behre *et al.* 1998). RLUC has since been developed as the reporter of choice for monitoring chloroplast gene expression in *C. reinhardtii* (Minko *et al.* 1999; Fuhrmann *et al.* 2004) and for imaging cell surface receptors in living organisms (Venisnik *et al.* 2006). Despite the high sensitivity and efficiency of the RLUC reporter, it has not gained the same popularity as FLUC when investigating gene regulation in plants. Instead, RLUC has served primarily as a normalizer, or control, for promoter::FLUC quantification in dual-reporter assays (Frey *et al.* 2001; Matsuo *et al.* 2001). This may be in part due to a noted inefficiency of coelenterazine substrate penetration into intact plant tissues (Mayerhofer *et al.* 1995; Cazzonelli and Velten 2003). Nonetheless, when the RLUC reporter is combined with firefly luciferase for dual reporter applications, highly accurate quantification of gene expression can be obtained (Matsuo *et al.* 2001; Cazzonelli and Velten 2006). Furthermore, it displays unique properties that make it superior for some experimental systems, including a high substrate specificity and relatively simple assay conditions, requiring only dissolved oxygen and coelenterazine as substrates.

Bacterial luciferase (luxAB)

Luminescent marine bacteria (*Vibrio harveyi and Vibrio fisheri*) have provided an additional source of bioluminescent genes that were first characterized around the same time as firefly luciferase (Baldwin *et al*. 1984; Engebrecht and Silverman 1984; Cohn *et al*. 1985). The bacterial luciferase enzyme is a dimeric protein encoded by the *Lux*A and *Lux*B genes (Cohn *et al*. 1985; Johnston *et al*. 1986) and problems associated with reconstitution of the dimer in eukaryotes were overcome with the development of a *lux*AB gene fusion (Olsson *et al*. 1989). Bacterial luciferase oxidizes its substrate luciferin, which is a reduced riboflavin phosphate (FMNH2), in association with a long chain aldehyde and oxygen molecule [see **Table 1**, and Hastings *et al*. (1978)]. Bacterial luciferin has been identified in free-living bacteria and in association with pyrosomes, as well as some squid and fish [reviewed by (Greer and Szalay 2002)]. The emission spectra from *luxAB* luminescence peaks in the blue-green region (490 nm).

Bacterial luciferase was initially ectopically expressed in *E. coli* (Baldwin *et al*. 1984) and later shown to serve as a useful reporter of promoter function in several plant systems, including tobacco, carrot and populus (Koncz *et al*. 1987; Langridge *et al*. 1989; Langridge *et al*. 1994; Johansson *et al*. 2003). While the *luxAB* reporter system has proven effective for *in vitro* assays in bacteria and plants, it has not gained widespread use for *in vivo* imaging in plants. This is in part due to the insensitivity of the *in vivo luxAB* assay, but there are also problems with decanal substrate toxicity and non-specific chemiluminescence (Mudge *et al*. 1996). For these reasons and the successful applicability of the FLUC reporter for *in vivo* imaging in plants, the *luxAB* reporter system is rarely utilized for plant imaging.

Click beetle luciferase (CbLUC)

The luminous click beetle *Pyrophorus plagiophthalamus* harbors a luciferase that is related to the firefly and has emerged as a new alternative for co-reporter assays. CbLUC bioluminescence is unusual in that individual beetle specimens contain two sets of light-emitting organs that can differ in the color of light emitted. Four CbLUC cDNAs were cloned from the ventral light organ and shown to be 95-99% identical, with only a few variations in their amino acid sequence. The altered amino acids were found to be responsible for the shift in emission spectra from green to orange (544 to 593 nm). The CbLUC genes were subsequently engineered to remove the perioximal target peptide, optimize codon usage, and shift emission to the green (CBG68luc and CBG99luc) or red (CBRluc) regions of the visible spectrum (537 to 613 nm) (Almond *et al*. 2003). The green and red click beetle luciferases share a common substrate, but have emission spectral maxima separated by ~75 nm. This allows light from the two reporters to be independently quantified using filters that discriminate between the two emission maxima. Application of these unique luciferase reporters for investigating gene expression changes in plants is still in its infancy.

LUCIFERASE ASSAYS IN PLANTS

There are several well established protocols for monitoring luminesence in intact plants and for quantifying luciferase activity in tissue extracts (**Tables 1, 2**). As with all assays, substrate concentration and, in the case of *in vivo* assays, substrate availability are important considerations. In addition to molecular oxygen and the corresponding luciferin, FLUC requires ATP, while LUX uses a long-chain aldehyde for light production (see **Table 1**). RLUC is distinct in that it requires only oxygen and coelenterazine for bioluminescence, making it more suitable for experiments where the other substrates of FLUC or LUX may to be limiting and/or unavailable. Furthermore, with photons being the quantifiable output, signal attenuation through absorption or scattering by plant tissues must be taken into consideration. This section highlights a few key advantages and disadvantages of each luciferase assay technology as applied to plant research, specifically: 1) quantification of reporter activity and 2) imaging luminescence in intact plant tissues.

Luciferase imaging of intact plant tissues

Highly sensitive light detection systems allow luciferase activity to be semi-quantitatively assessed *in vivo* from intact living plant tissues that can be supplied with the appropriate luciferase substrates (Greer and Szalay 2002; Cazzonelli and Velten 2006; Southern *et al*. 2006). High sensitivity combined with a relatively short enzyme half-life, have made luciferase reporters indispensable for research addressing temporally sensitive plant functions such as environmental stress response and circadian rhythm (see next section). Although a completely uniform distribution of all the different luciferase substrates within intact living plants is nearly impossible it is important to optimize substrate dispersal as much as possible. In the case of FLUC, RLUC and LUX, the luciferin substrates have proven to be both membrane permeable and relatively non-toxic, facilitating non-destructive *in vivo* detection of activity. For FLUC *in vivo* luminescence relies primarily on the ability of luciferin to diffuse through cellular membranes, since the intracellular availability of ATP, magnesium and O_2 are generally sufficient to saturate the enzyme (Kost *et al*. 1995). However, luciferin penetrates some tissues poorly, making the LUC assay more difficult on intact plants, especially those with hydrophobic outer layers (Ow *et al*. 1986). In addition, luciferin is a weak acid carrying a negative charge at physiological pH and is not expected to enter living cells efficiently. In order to facilitate uptake, luciferin solutions are normally buffered with 100 mM sodium citrate (pH ~5.5) which has been reported to enhance luminescence (Kost *et al*. 1995; Mudge *et al*. 1996).

In contrast, the volatile aldehyde substrate of bacterial luciferase penetrates tissues rapidly and reportedly without damage to treated plants (Mudge *et al*. 1996). However, the substrate *n*-decanal has been shown to be toxic when applied for long periods, limiting extended measurements in eukaryotic systems (Hollis 2001; Fuhrmann 2004). The RLUC reporter has not gained widespread use as a reporter for monitoring luminescence *in vivo*, partially because the coelenterazine substrate is photo-labile, restricting its use to protocols where material is maintained primarily in the dark (Southern *et al*. 2006).

Artifacts associated with substrate penetration of plant tissues can be reduced by altering the method of substrate application. For instance, luciferin applied through the roots is effectively absorbed and transported via the vascular system. Alternatively, the RLUC substrate, coelenterazine, appears to be poorly distributed via root uptake, at least in some plant species (Cazzonelli and Velten 2003). Firefly luciferin can also be sprayed onto intact plant tissues, or applied to plant material partially submerged in a luciferin solution [**Fig. 1**, Cazzonelli and Velten 2006]. However, the effectiveness of this method is in general reduced in the absence of some form of tissue wounding which facilitates diffusion/transport throughout the tissues. An effective, though destructive, strategy that promotes more uniform substrate uptake is to dissect tissues partially submerged within a luciferin solution. Even though this method is destructive of the excised tissues (but not necessarily lethal to the source plant), it provides the substrate with more effective and uniform access to vascular and associated tissues (Cazzonelli and Velten 2006). Alternative methods of substrate application involve direct infiltration into the intercellular spaces of intact leaf tissues using a blunt ended syringe (**Fig. 2**), direct uptake via submerged cut petioles, or injection of substrate into an intact vascular system using a needle and syringe.

FLUC has the advantage of a relatively short protein half-life *in vivo* (approximately 2-4 hrs) making it suitable

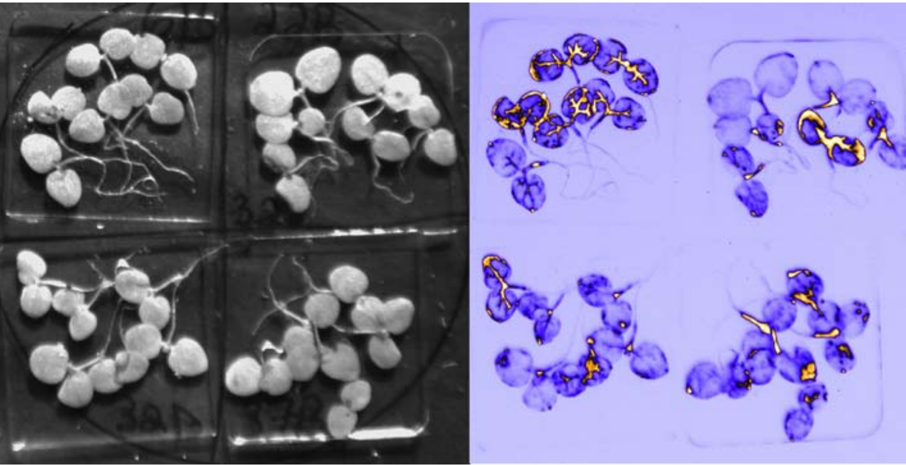


Fig. 1 ***In vivo*** **expression of FLUC in tobacco seedlings from transgenic lines containing different promoter::FiLUC constructs**. Left, reflected light image of FLUC positive seedlings partially submerged in a luciferin buffer. Right, false colorized image of the same seedlings using captured bioluminescence.

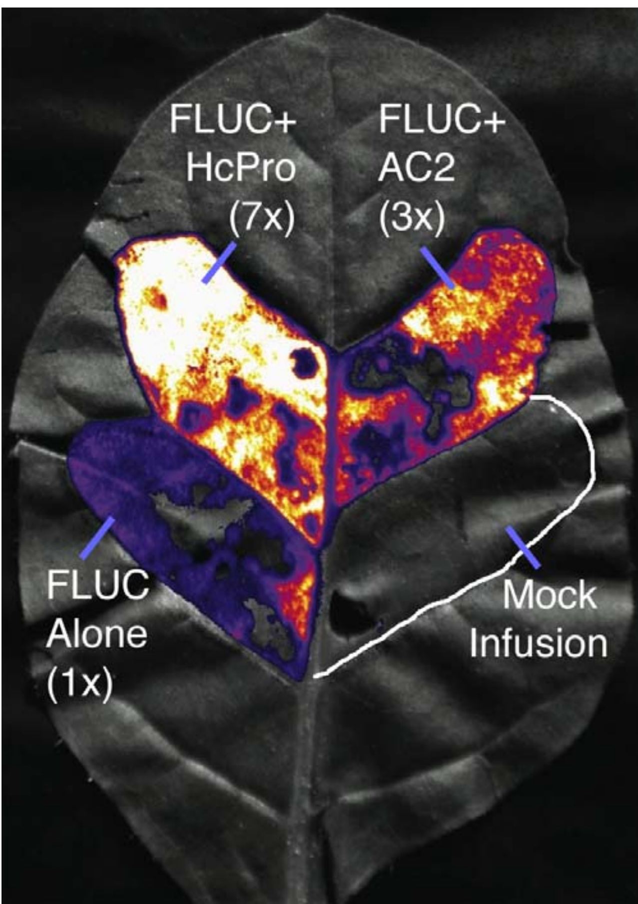


Fig. 2 Viral suppressor activity can be directly assessed within tobacco leaf infiltrations. A 35S::FiLUC construct was co-infiltrated with chimeric genes expressing two different viral suppressors of PTGS, HcPro and AC2. Silencing of FiLUC expression within the Agro-infiltrated leaf tissues (FiLUC alone) is indicated by increased light emission (after re-infiltration with the luciferin substrate) in the presence of co-expressed viral suppressors of silencing; HcPro (a stronger suppressor, 7X FLUC alone) and AC2 (a weaker suppressor, 3X FLUC alone (Cazzonelli *et al*. 2005b)).

for kinetic investigations of the effects of biotic and abiotic stimuli on gene expression. When luciferase and luciferin react, the product (oxyluciferin) inhibits luciferase catalysis and further light production (Matthews *et al*. 1977), providing a real time measure of the current enzyme level. These characteristics extend the use of luciferase reporters into imaging reporter changes in real time and with the development of highly sensitive cameras and stronger optical magnification, it may become possible to one day monitor luminescence at the cellular and possibly sub-cellular levels (see technical references below under Bioluminescence resonance energy transfer (BRET).

Quantification of luciferase activity

A major advantage of using bioluminescent reporters for *in vitro* assays is their extreme sensitivity, being several orders of magnitude more sensitive then available colorimetric, radiometric and fluorescent assays. Most luciferase assays use relative crude protein extracts added to reaction buffers containing the appropriate substrates and buffers. Resulting bioluminescence is quantified via luminometry and reported as relative light units emitted per milligram of extracted protein or per gram fresh weight of tissue used. Kits are commercially available which provide all necessary substrates and reaction buffers, although these can also be easily prepared following well established protocols (Cazzonelli and Velten 2006; Cazzonelli and Velten 2008). Both the FLUC and RLUC reporters are routinely used to quantify gene expression levels in plant systems, with the RLUC reporter having a higher overall sensitivity when compared to FLUC or LUX (Mayerhofer *et al*. 1995; Cazzonelli and Velten 2003). The unique chemistries of FLUC and RLUC have also allowed the development of quantitative dual-reporter assays that provide accurate quantification of both FLUC and RLUC using the same assay sample (Sherf *et al*. 1996).

Leaf disk assays can be used to quantify both FLUC and RLUC activities without protein extraction by measuring light emission from excised disks floating on reaction buffers tailored for each luciferase (Cazzonelli and Velten 2006). These "*in vivo*" assays generate results comparable to those obtained using commercially available kits. Application of the *in vivo* assay to quantification of both transient and stably integrated reporter gene expression was successful in determining the strength of constitutive promoters (**Fig. 3**) and the effects of viral suppressors of post transcriptional gene silencing (PSTG, **Fig. 4**). The assay relies upon consistent penetration of the substrate into excised leaf discs and assay buffers were optimized for efficient penetration and maximum luminescence (Cazzonelli and Velten 2006). FLUC light emission from leaf disks peaks after ~60 minutes of incubation, while RLUC bioluminescence peaks after ~11 minutes in coelenterazine buffer (**Fig. 5**). Avoiding the labor and time associated with protein extraction makes the leaf disk assay highly efficient and readily scalable, significantly reducing cost in comparison to other assay systems.

The extreme sensitivity and broad range of luciferase assays using both *in vivo* and *in vitro* systems has allowed these reporters to be used to examine the earliest stages or gene induction and/or silencing, as well as intracellular protein-protein interactions. The details of how luciferase reporters have been used for these and other applications will be the next topic of this review.

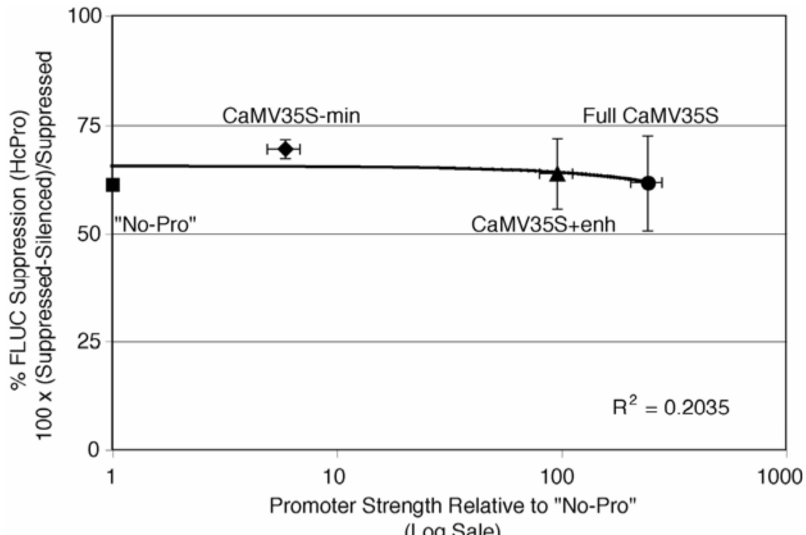
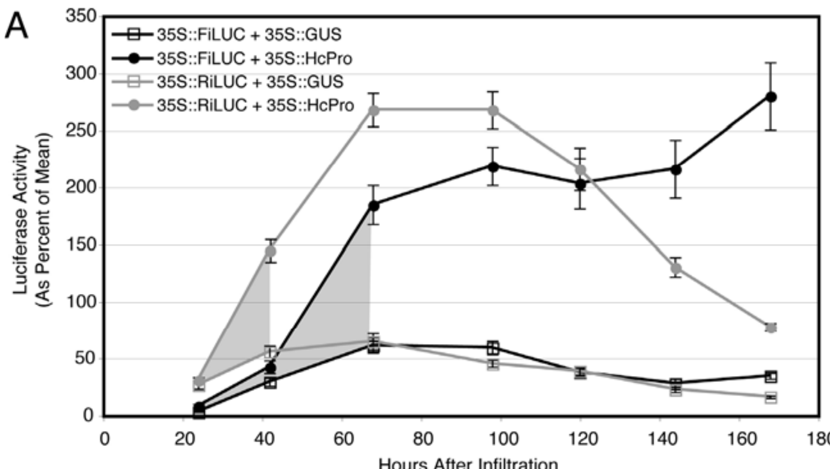


Fig. 3 Analysis of PTGS across a range of promoter strengths. Tobacco leaves were separately infiltrated with mixed *A. tumefaciens* cultures containing a test promoter::FLUC construct and either the 35S::HcPro suppressor construct (suppressed) or a 35S::*gusA* negative (silenced) control. The four different promoter::FLUC constructs cover a wide range of transcriptional activities; "No-Pro", containing no defined plant promoter (read-through from T-DNA sequences, produces very low FLUC activity); CaMV35S-min, containing a minimal CaMV promoter (TATA-box to transcription start of CaMV35S); CaMV35S+enh, containing CaMV35S-min with the 35S enhancer region added; and the full CaMV35S promoter. To allow comparison of suppression over the full range of activities, FLUC activity is plotted as a percentage of HcPro-induced suppression (100 x [RLUpHcPro–RLUpIG121]/RLUpHcPro), verses relative promoter strength (log scale). Error bars represent the standard error from two time point measurements and two assays (*n*=4).



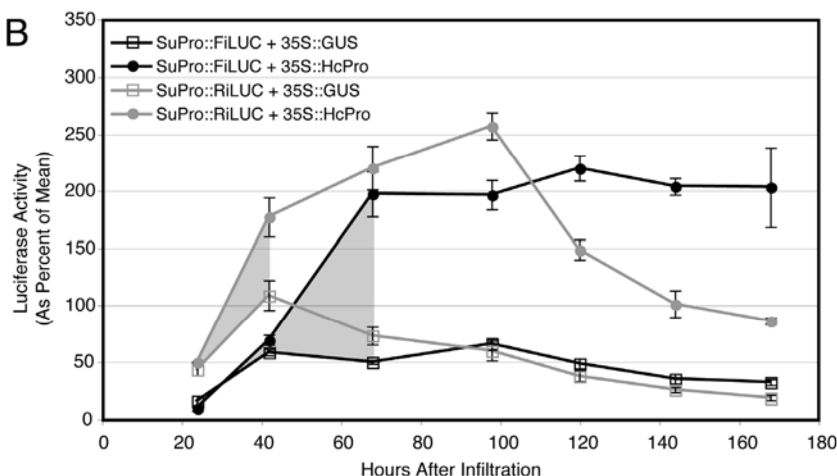


Fig. 4 Time-course of luciferase activity from Agro-infiltrated tobacco leaves. Emission is plotted as percent of the mean value for each dataset (n=4), standard error indicated. The SuPro (super-promoter) constructs use a RK2 plasmid replication origin, while the 35S (CaMV-35S promoter) plasmids contain a compatible VS1 origin, allowing both T-DNA containing binary vectors to co-exist within the same *Agrobacteria* strain (EHA105). Shaded area indicates the earliest measured difference between suppressed (co-expressed with HcPro) and unsuppressed (co-expressed with a control GUS construct) assays, an indicator of PTGS. Silencing appears to initiate earlier against RiLUC (between 24 h and 44 h) than against FiLUC (between 44h and 68h), independent of the promoter used to express the reporters. (**A**) CaMV 35S constructs, (**B**) SuPro constructs.

SELECTED RESEARCH APPLICATIONS OF LUCIFERASE REPORTERS IN PLANTS

Promoter structure/function analysis

Plant genes are normally expressed in a highly regulated manner, with the level of expression precisely delineated in response to metabolic, developmental and environmental cues. Bioluminescence has proven very effective as a reporter of promoter activity in plants, with all three luciferases being used to investigate the regulation of gene expression in multiple plant species (see listings in **Table 2**). The high sensitivity and non-destructive nature of the FLUC and RLUC assays have made these genes very effective tools for examining plant promoter strength, tissue specificity, temporal regulation and developmental control (e.g. **Fig. 1**).

In addition to contributing to detailed analysis of numerous natural plant promoters [e.g. (Baier Stroher and Dietz 2004; Castle *et al*. 2005; Cazzonelli *et al*. 2005a; Christmann *et al*. 2005; Frey *et al*., 2001b; Harmer and Kay 2005; Hellens *et al*. 2005; Langridge *et al*. 1989; Maxwell *et al*. 2003; Remy *et al*. 2005; Schmidt *et al*. 2004; Schreiber *et al*. 2004; Siefritz *et al*. 2004; Tsukagoshi *et al*. 2005)], the firefly luciferase gene has also served as a valuable reporter for identifying and characterizing novel transcriptional enhancer elements and understanding how these elements interact to define plant promoter function. For example, the sensitivity and high throughput of a FLUC *in vivo* transient assay system: allowed screening of a large number of short inverted and directly repeated viral sequence ele-

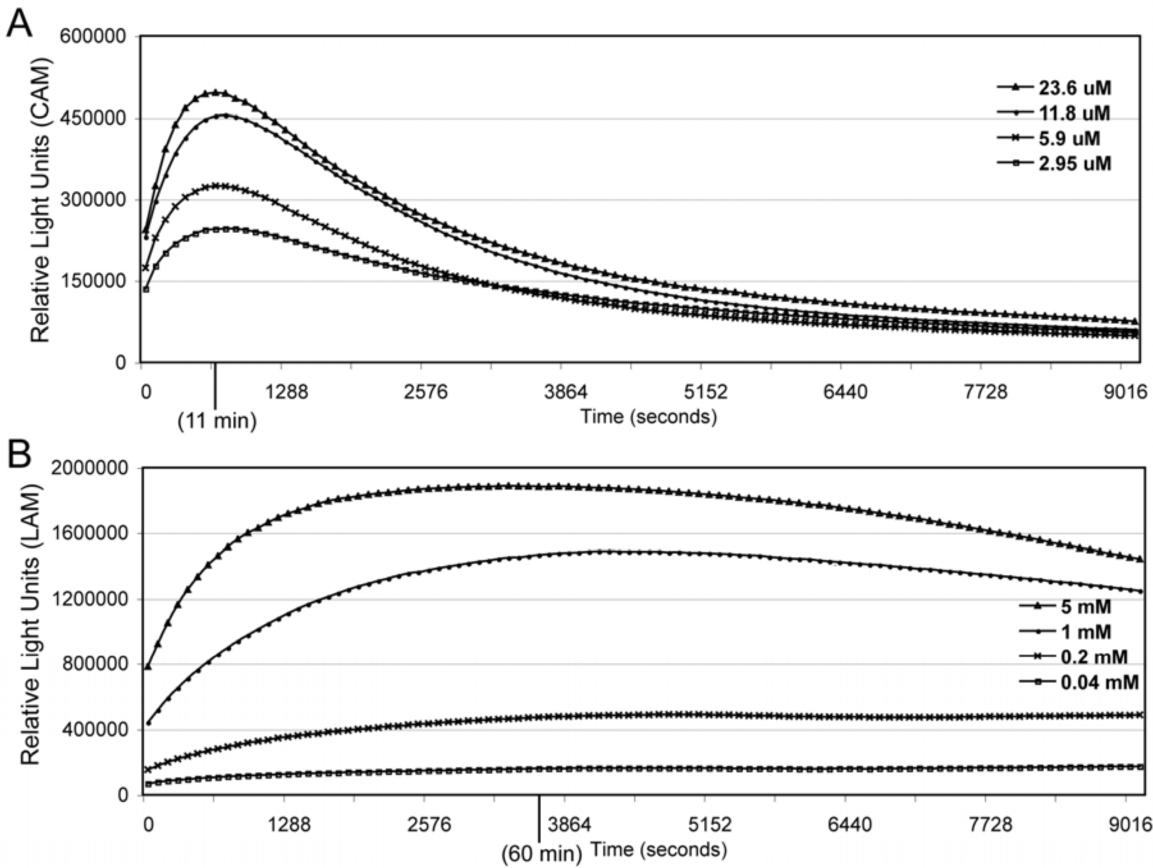


Fig. 5 Titration analysis of substrates used to measure firefly and sea pansy luciferase activities *in vivo*. *N. tabacum* leaf tissues were infiltrated with *A. tumefaciens* harboring pTm35enh and pE1778-SUPER-R (SR) binary vectors. 92 hrs after Agro-infiltration four leaf discs were incubated on different substrate concentrations in duplicate and light emission monitored over time. (**A**) Titration curves of four concentrations of coelenterazine used to detect sea pansy (RiLUC) luciferase activity in SR infiltrated tobacco leaf tissues. (**B**) Titration curves for four concentrations of beetle luciferin used to detect firefly (FiLUC) luciferase activity in pTm35enh infiltrated tobacco leaf tissues.

ments for transcriptional enhancement (Velten *et al*. 2005); was instrumental in the dissection of the contribution of individual nucleotides to the function of a small 'conserved late element' (CLE) from the geminivirus family of DNA plant viruses (Cazzonelli *et al*. 2005b; Velten *et al*. 2005); and permitted a detailed and quantitative *in vivo* analysis of the functional interactions between several short enhancer sequence elements (Cazzonelli and Velten 2008). Such findings have provided new insight into the structure/function relationship of plant promoters.

Abiotic stress response and signal transduction

The use of luciferase reporters linked to promoters that respond to environmental stresses has proven very successful at studying the regulation of stress responsive genes. For example, systemic signaling of high light stress (Karpinski *et al*. 1999; Rossel *et al*. 2007), where *in vivo* assay of luciferase activity has provided spatial and tissue-specific information on gene regulation (Mullineaux *et al*. 2006). Here we will focus on the use of luciferease in identifying components of signal transduction pathways integral to stress response in plants (Xiong *et al*. 1999; Chinnusamy *et al*. 2002; Murray *et al*. 2005). Mutagenesis of transgenic plants containing promoter:luciferase transgenes by either chemical (ethylmethanesulfonate, EMS) or gene-tagging strategies (transposon and/ T-DNA), followed by screening of the mutant populations for altered luciferase activity, has identified key regulators and components of plant stress signaling networks. Descriptions of such screens, and the steps for generating and recovering mutants, have been reported (Ishitani *et al*. 1997; Rossel *et al*. 2004). One of the most effective FLUC-based screens for signal transduction mutants looked at genes involved in the molecular response of *Arabidopsis* to salt, cold, osmotic or ABA stresses using plants transformed with the stress-inducible RD29A promoter fused to FLUC (Ishitani *et al*. 1997). Screens employed using the RD29::FLUC transgene include have identified: salt overly sensitive (*sos*) mutants; constitutive expression of osmotically responsive genes (*cos*); low expression of osmotically responsive genes (*los*); high expression of osmotically responsive genes (*hos*); and fiery (*fry*) mutants (Ishitani *et al*. 1997). On the order of a dozen genes involved in stress signaling networks have been identified to date [for a review of some of these see (Yamaguchi-Shinozaki and Shinozaki 2006)]. These screens have paid off in numerous, and sometimes surprising, ways [e.g. identification of the contribution of ROS1 to transcriptional gene silencing (Zhu *et al*. 2007)]. Cold stress screens using the CBF promoter has identified inducer of CBF expression 1 (*ice1*) mutants (Chinnusamy *et al*. 2003). Another group screened for genes not regulated by CBF using RCI2A::FLUC fusions (Medina *et al*. 2005). High light and oxidative stress-inducible promoters have also been used, such as *AOX1a*, which is targeted to mitochondia and the promoter of the cytosolic-localized APX2 (Mullineaux *et al*. 2000; Rossel *et al*. 2004; Zarkovic *et al*. 2005). The APX2::FLUC screens have been used to implicate glutathione in chloroplast-nuclear signaling (Ball *et al*. 2004) and to identify high light and drought tolerance mutants (Rossel *et al*. 2006; Wilson and Pogson, pers. comm.). Interestingly, some independent screens have isolated the same genes, genetically demonstrating their importance in stress signaling. For example, the nucleotidase/phosphatase, SAL1, has been isolated as *fry1* (Xiong *et al*. 2001), *hos2* (Xiong *et al*. 2004) and *alx8* (Wilson and Pogson, pers. comm.) in 3 different screens.

Circadian rhythm in plants

The relatively rapid turnover of both mRNA and protein compared to other reporter genes (Van Leeuwen *et al*. 2000) has made FLUC very useful for the analysis of time-dependent processes. One of the clearest examples of this type of research in plants has been the role played by FLUC reporter systems in the identification of factors that impact plant circadian rhythm. Recent advances in our understanding of plant circadian rhythm are already well documented in the literature and will not be further discussed here (Millar *et al*. 1992a; Millar *et al*. 1995; Nakamichi *et al*. 2004; Harmer and Kay 2005; Welsh *et al*. 2005; Welsh and Kay 2005; Darrah *et al*. 2006; Hall and Brown 2007).

Protein-protein interaction in plants

Bioluminescence resonance energy transfer (BRET) is a luciferase-based alternative to fluorescence resonance energy transfer (FRET) that allows confirmation and quantification of protein-protein interactions *in vivo* [see (Xu *et al*. 1999; Gorbunova *et al*. 2000; Issad and Jockers 2006; Pfleger and Eidne 2006; Pfleger *et al*. 2006; Prinz *et al*. 2006; Subramanian *et al*. 2006; Xu *et al*. 2007)]. Using BRET, energy in the form of bioluminescence is transferred from an active luciferase-protein fusion (emitter) to a second fusion protein containing a functional fluorescent component (acceptor, e.g. GFP). The resonance transfer is strictly dependent upon the proximity of the two fusion proteins ('bait' and 'prey'), directly indicating the close association of the fused target protein domains within either *in vivo* or *in vitro* assays. Although BRET is currently not as sensitive as FRET, which depends upon an external light source to induce fluorescence in the emitter half of the paired proteins, recent advances have broadened the applicability of BRET in plants (Xu *et al*. 2007).

More recently 'split-enzyme' luciferase complementation imaging (LCI) systems [e.g. (Massoud *et al*. 2007; Paulmurugan and Gambhir 2007; Villalobos *et al*. 2007)], a modification of the fluorescence technique called bimolecular fluorescence complementation (BiFC) (Hu *et al*. 2002), have made use of *trans*-complementing luciferase gene fragments fused to bait and pray proteins to examine protein-protein interactions in plants (Remy and Michnick 2006; Fujikawa and Kato 2007; Chen *et al.* 2008). Catalytically inactive subunits of a luciferase enzyme are separately joined to two proteins suspected of *in vivo* interaction. Essentially no light is generated from the luciferase unless the bait and prey components bind (either directly to each other or via a larger protein complex), bringing the two parts of the luciferase together to produce an active, *trans*-complementing, enzyme. As very little light is generated until the split-gene components come into proximity of each other, the background to signal ratio is low relative to BRET or fluorescence-based assays.

Homologous recombination in plants

An unrelated version of a split gene assay makes use of overlapping luciferase gene segments to quantify the rate of homologous recombination occurring within plant tissues (Ilnytskyy *et al*. 2004). The simplicity and sensitivity of the assay has allowed quantification of recombination rate changes in response to various treatments (e.g. radiation, stress, virus infection) (Lucht *et al*. 2002; Kovalchuk *et al*. 2003) and has allowed identification of genetic loci and physiological factors that influence homologous recombination in plants (Filkowski *et al*. 2004; Boyko *et al*. 2006a, 2006b).

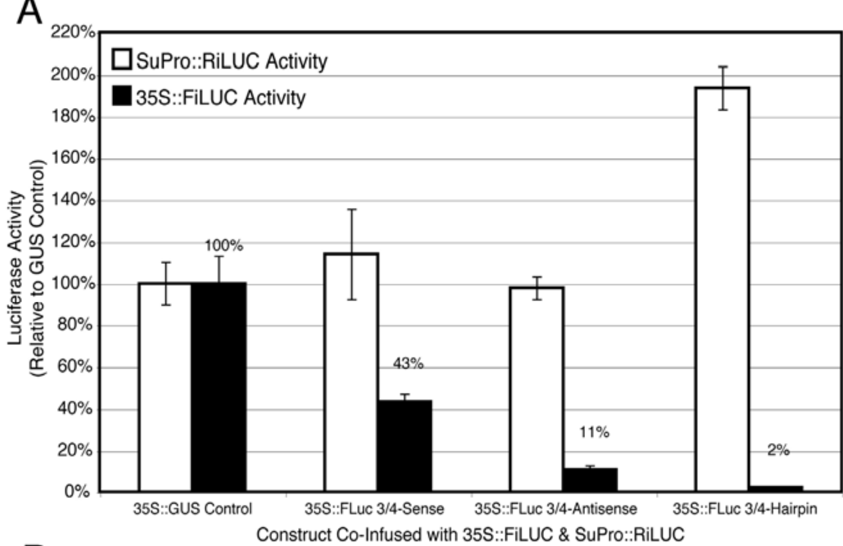
Gene silencing in plants

As mentioned in the section on plant stress and signal transduction mutations, one mutation discovered during a screen for cold-response control genes was not stress-related but instead appears to encode a DNA glycosylase associated with altering DNA methylation and gene silencing in *Arabidopsis* (Gong *et al*. 2002; Agius *et al*. 2006; Zhu *et al*. 2007). Other contributions by luciferase reporters to plant gene silencing research include: viral induced gene silencing against FLUC (Kjemtrup *et al*. 1998); the impact of cell proliferation on transgene silencing (Mitsuhara *et al*. 2002); specific changes in histone modifications identified at a FLUC locus in *Arabidopsis* (Naumann *et al*. 2005; Fischer *et al*. 2006); and the impact of siRNA targeting luciferase expression in rice protoplasts (Bart *et al*. 2006).

Our understanding of RNA-based gene silencing in plants is still in its infancy (Brodersen and Voinnet 2006; Vaucheret 2006) and to better understand the characteristics of how PTGS targets different genes within alternative plant species, our work has examined silencing of FLUC and RLUC expression within tobacco leaf cells using an *Agrobacteria*-infiltration (Agro-infiltration) based transient assay system (Cazzonelli and Velten 2006). Before discussing these results it must be noted that multiple pathways of RNA-based gene silencing exist in plants (Rana 2007) and it is possible that the form of RNAi observed within Agro-infiltrations may not be fully representative of how plant PTGS works in general (Dunoyer *et al*. 2006). The assay used to determine the degree of silencing within infiltrated plant tissues requires co-expression of a viral suppressor of silencing (HcPro, from *Potato virus Y*) with the targeted reporter (Cazzonelli and Velten 2006). If the luciferase reporter is subject to PTGS, HcPro suppression produces a reduction in silencing that is indicated by a corresponding increase in reporter activity relative to that of the reporter alone (see **Fig. 2**). In the absence of reporter silencing (e.g. in stably transformed tobacco plants expressing luciferase), HcPro infiltration of leaves does not produce any significant change in light emission (Cazzonelli and Velten 2008).

One model for how some transgenes are able to trigger PTGS in plants hypothesizes that high level expression of the introduced gene is somehow recognized by the plant, initiating the process of PTGS (Mallory *et al.* 2002; Watson *et al.* 2005; Lakatos *et al.* 2006; Rana 2007). The high sensitivity of the FLUC assay was used to test this theory in tobacco leaves infiltrated with *A. tumefaciens* containing constructs that express an intron-containing FLUC (FiLUC, the intron prevents background expression of FLUC within *A. tumefaciens*) over a wide range of expression levels [see **Fig. 3** and (Cazzonelli and Velten 2006)]. The weakest (barely detectable) FiLUC expression resulted from 'read-through' in a 'no promoter' construct in which the FiLUC coding region is downstream from DNA that contains no known plant promoter sequences. The strongest expression level tested (~700 times the "no promoter" value) came from a 35S::FiLUC fusion. As measured by HcPro suppression, the level of silencing (% suppression: 100 x [Suppressed activity - Silenced activity] / Suppressed activity) was essentially the same at all levels of FLUC expression. Despite the high sensitivity of the FLUC assay, it was impossible to measure any expression level that did NOT appear to be enhanced by HcPro suppression (i.e. to initiate PTGS).

The high sensitivity of the luciferase assays also allowed examination of the very early stages of reporter expression within the Agro-infiltrations, identifying the transition from un-silenced to silenced expression [see **Fig. 4** and (Cazzonelli and Velten 2006)]. At the earliest time point at which FLUC and RLUC activities could be detected (24 h post infiltration) there was no significant difference between suppressed (co-expression of HcPro and luciferase) and unsuppressed (luciferase alone). Twenty hours later there is a clear separation between the suppressed and unsuppressed RLUC activities (indicating silencing has initiated) while FLUC levels remain essentially identical between the two assays. Only after an additional 24 hours does the suppressed FLUC assay jump dramatically, indicate silencing of that reporter. These data clearly indicate that under identical conditions the two luciferase genes are per-



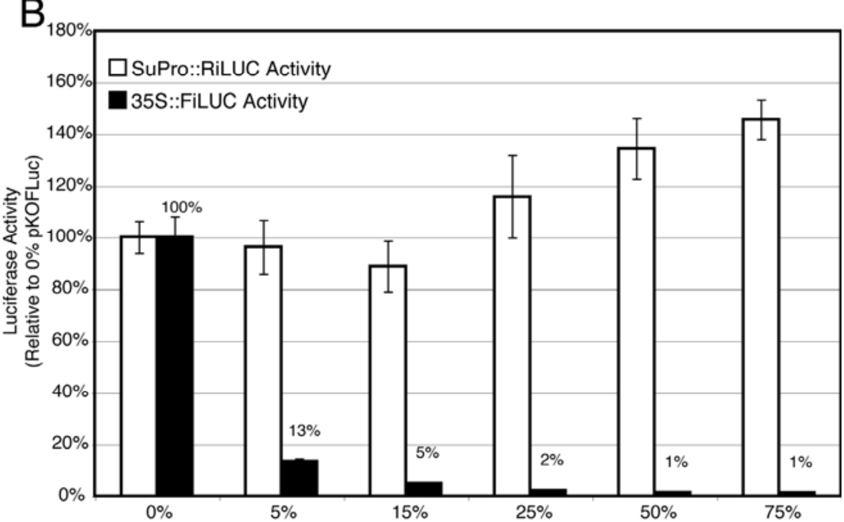


Fig. 6 Silencing cross-talk between targeted (35S::FiLUC) and non-targeted (SuPro::RiLUC) reporter activities within tobacco leaves. *N. tabacum* leaves were infiltrated with *Agrobacteria* cultures containing T-DNA constructs that co-express both luciferase reporters **plus** either: 35S::FLUC 3/4-Sense, 35S::FLUC 3/4-antisense **or** 35S::FLUC 3/4-hairpin (3/4 indicates the 3'-most 73% of the FLUC coding sequence). (**A**) Activity from the non-targeted RiLUC reporter is consistently elevated within 35S::FLUC 3/4-hairpin (pKOFLUC) co-infiltrations, relative to co-expression of the 35S::*gusA* control, 35S::FLUC 3/4-sense or 35S::FLUC 3/4-antisense. Each infiltration consisted of 25% (by bacterial density) of [35S::FiLUC plus SuPro::RiLUC] co-infiltrated with 75% of the indicated test *Agrobacteria* line. Reported are mean values (n=3) of relative luciferase activities measured at 72 h and 114 h post infiltration, with standard error bars indicated. The RLUC activity in the FLUC-Hairpin co-infultration is significantly greater than that in the GUS control co-infiltration (Student's T-test, $p<0.01$) while the RLUC signals from the Sense and Antisense FLUC co-infiltrations s did not differ statistically from the GUS control. (**B**) Cross-talk is dosage dependent. Reported are mean values (n=2) of relative luciferase activities measured at 66 h and 136 h post infiltration, with standard error bars indicated.. Each infiltration consisted of 25% (by *Agrobacteria* density) of 35S::FiLUC plus SuPro::RiLUC co-infiltrated with the indicated percent bacterial density of the 35S::FLUC ¾-Hairpin *Agrobacteria* line (diluted with 35S::*gusA* bacteria to make up to 75% total bacterial density). The 75% RLUC signal is significantly greater than the 0% control, Student's T-test - $p<0.01$; with the 50% signal calculated at - $p<0.07$. None of the other dilutions were statistically different from the 0% control.

ceived differently by the system tobacco uses to initiate PTGS.

A standard technique for producing PTGS targeting specific mRNA in plants is to introduce constructs designed to produce double stranded hairpin transcripts (hpRNA) (Watson *et al*. 2005). One such construct, pKOFLUC (35S::FLUC ¾-hairpin, which generates dsRNA using the 3′-most 73% of the FLUC coding region) was tested and found to efficiently silence co-infiltrated 35S::FiLUC expression (Cazzonelli and Velten 2004). Surprisingly, it was determined that when hpRNA was used to create a high level of PTGS against FLUC, activity of the co-expressed SuPro::RiLUC reporter [intron containing RLUC construct (Cazzonelli and Velten 2003; Lee *et al*. 2007)] appeared to be consistently elevated above control levels (see **Fig. 6A**). This was not anticipated since FiLUC and RiLUC have no significant sequence homology and one would not expect PTGS targeting FLUC to have any impact on RLUC expression and/or silencing. From previous data it was already known that even in that absence of hpRNA transcription, both luciferase reporters are significantly silenced within Agro-infiltrated tissues (e.g. **Fig. 3**). The simplest explanation for the observed rise in RLUC activity is that activation of excessive silencing against FLUC, initiated by expression of the pKOFLUC hpRNA construct, is able to titrate one or more essential components of the PTGS machinery and thus relieve some of the pre-existing silencing of the co-expressed RLUC reporter (**Fig. 6A**). This theory was further tested by assaying a pKOFLUC dilution series for dosage-dependence of the observed cross-talk. Mixed *A. tumefaciens* cultures in which a constant 25% of the bacterial density infiltrated is a FiLUC + RiLUC line (35S::FiLUC + SuPro::RiLUC in the same bacterial line), while the remainder of the mixture contains a range of pKOFLUC bacterial densities mixed with a filler line (harboring a 35S::*gusA* construct that does not directly impact lucifrase expression or silencing) combined to create a constant bacterial density between each infiltration (**Fig. 6B**). The *Agrobacteria* culture mixture that contained 15% of the pKOFLUC line reduced FLUC acivity by 95%, but produced no discernable cross-talk (**Fig. 6B**). However, when the infiltrated mixture consisted of between 50% to 75% pKOFLUC, a statistically significant ($p=0.07$ and <0.01, respectively), enhancement of RLUC activity was observed (**Fig. 6B**). This observed cross-talk will be the subject of additional research into the mechanisms of PTGS in *N. tabacum*.

CONCLUSION

Luciferase reporters have proven to be very successful research tools in plants, especially for exploring different aspects of gene regulation and signal transduction. With the development of new and more sensitive luciferase assays, and continuing breakthroughs in the use of split luciferases for examining protein-protein interactions, it is fully expected the these versatile reporters will remain important players in plant molecular and cellular research.

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