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The Use of the Luciferase Reporter System for *in Planta* Gene Expression Studies

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Abstract. The properties of the firefly luciferase (LUC) make it a very good non-destructive reporter to quantify and image transgene promoter activity in plants. The short half-life of the LUC mRNA and protein, and the very limited regeneration of the LUC protein after reacting with luciferin, enables monitoring of changes in gene activity with a high time resolution. However, the ease at which luciferase activity is measured *in planta*, using a light sensitive camera system (2D-luminometer), contrasts sharply with the complications that arise from interpreting the results. A variegated pattern of luciferase activity, that is often observed in *in planta* measurements, might either be caused by differences in influx, availability of the substrates (luciferin, oxygen, ATP) or by local differences in reporter gene activity. Here we tested the possible contribution of differences in the availability of each substrate to the variegated *in planta* luciferase activity, and we show when *in planta* luciferase activity is measured under substrate equilibrium conditions and can be related to the promoter activity of the reporter gene. Furthermore, we demonstrate the effects of protein stability, apparent half-life of luciferase activity, regeneration of luciferase and pH on the *in vivo* and *in vitro* luciferase measurements. The combined results give the prerequisites for the correct utilisation of the luciferase reporter system, especially for *in vivo* gene expression studies in plant research.

Key words: Cauliflower Mosaic Virus 35S promoter, luciferase reporter system, *Petunia hybrida*, reporter gene, transgene expression, variegation

Abbreviations: FW, fresh weight; LUC, luciferase; rlu, relative light units.

Introduction

The luciferase gene from the North American firefly *Photinus pyralis* has emerged as a popular choice for *in vitro* and *in vivo* reporting of transcriptional activity in eukaryotic cells. Since the cloning of the cDNA encoding the enzyme

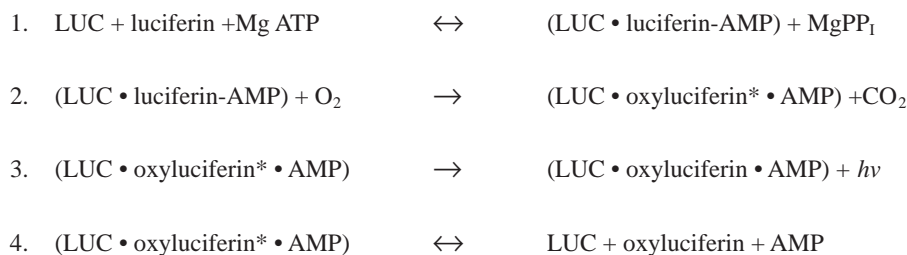*Author for correspondence. e-mail: Wessel.vanLeeuwen@Algem.PF.WAU.NL; fax: +31 317 484740; ph: +31 317 482822.

1. LUC + luciferin +Mg ATP $\leftrightarrow$ (LUC • luciferin-AMP) + MgPP$_I$

2. (LUC • luciferin-AMP) + O$_2$ $\rightarrow$ (LUC • oxyluciferin* • AMP) +CO$_2$

3. (LUC • oxyluciferin* • AMP) $\rightarrow$ (LUC • oxyluciferin • AMP) + *hv*

4. (LUC • oxyluciferin* • AMP) $\leftrightarrow$ LUC + oxyluciferin + AMP

Figure 1. The luciferase reaction (Aflalo, 1991). Brackets and bullets indicate the complexes formed. Step 1 is a fast equilibrium reaction. Step 2 is the oxidative decarboxylation, in which the oxylucife-rin is excited. Step 3 is the fast photon emission at 562 nm. Step 4 is the very slow release of product from the active site of the LUC protein.

luciferase (LUC) by DeWet et al. in 1985, the luciferase gene has been expressed in tobacco and carrot plants (Ow et al., 1986), mammalian cells (DeWet et al., 1987) and zebrafish (Mayerhofer et al., 1995) and *Drosophila* (Brandes et al., 1996). In firefly, the LUC protein is targeted to the peroxisomes and the C-terminal peroxisome import signal was shown to function in plants as well. For enhanced expression in mammalian cells and plants, the luciferase coding sequence was modified and the peroxisomal import sequence (*luc*[+], Promega) was removed (Sherf and Wood, 1994).

Luciferase catalyses the oxidative decarboxylation of the substrate (firefly) luciferin (Figure 1). The reaction causes the release of a photon at 562 nm in 90% of the catalytic cycles with the substrates luciferin, Mg^{2+}-ATP and oxygen (DeLuca et al., 1974; Aflalo, 1991). The luciferase enzyme is only slowly regenerated after reacting with the substrate, because the end product of the reaction, oxyluciferin, is only slowly released from the Luciferase ● Oxyluciferin -complex (Figure 1, step 4, Denburg et al., 1969). *In vitro* in the presence of high ATP concentrations, Coenzyme A enhances the light production through removal of oxyluciferin from luciferase resulting in a nearly constant production of light (Ford et al., 1995). We will discuss whether the enhanced regeneration of luciferase by the presence of Coenzyme A occurs *in vivo*.

This slow regeneration in combination with the short half-life of luciferase (Nguyen et al., 1989; Thompson et al., 1991), implies that in the presence of all substrates, each luciferase molecule can only react once and emit one photon. In the presence of all substrates the LUC protein will therefore not accumulate *in vivo*. Luciferase as a reporter gene thus represents gene expression as the flux of protein molecules (LUC) made in the cell (Δ LUC/sec), while more stable reporter genes only show the accumulation of protein molecules as an indication of gene expression (total amount of reporter protein in the cell at any given time point). Therefore, luciferase can be used as a non-invasive reporter in plants to accurately mark changes in transgene expression.

After the plant tissue has been provided with luciferin, the only substrate that is not naturally present in the plant cell, *in planta* luciferase activity can be monitored with a 2D-luminometer. However, in order to relate the changes in luciferase activity to changes in transgene expression, the availability of each of the substrates (luciferin, oxygen and ATP) should remain constant during the period

over which the luciferase activity is monitored. In order to relate differences in luciferase activity within a tissue to local differences in transgene expression, the availability of each of the substrates should also be similar in different parts of the tissue.

When we used the luciferase reporter system to measure gene expression *in vivo* in *Petunia* leaves, we noticed a high degree of variation in light emission within each leaf (variegation) as was observed before by Schneider et al. (1990) and Quandt et al. (1992). In this article we studied the possible contribution of the different substrates to differences and changes in *in planta* luciferase activity. We discuss what precautions have to be taken when the luciferase reporter system is used in plant research and under which circumstances the observed light production directly reflects luciferase gene expression.

Materials and Methods

Luc reporter gene constructs

Agrobacterium tumefaciens (*A. tum.* strain ABI) was transformed with the binary vector pMON721 containing either a CaMV 35S promoter - *luc* construct (pGM46) or a CaMV m35S promoter - *luc+* construct (pGM107). The CaMV promoter used in our constructs consists of the -343 to +8 sequence (Gardner et al., 1981; Benfey et al., 1989). The modified CaMV 35S (m35S) promoter, contains the -90 to +8 fragment of the CaMV 35S promoter, with four copies of the B3 domain and four copies of an optimised AS-1 binding site placed upstream (van der Krol et al., 1993), thereby increasing potential binding of B-ZIP transcription factors. The *luc* gene that is used in the pGM46 construct is the original luciferase coding sequence cloned by deWet et al. (1985, 1987). For the pGM107 construct a modified firefly luciferase gene (*luc+*, Promega) was used (Sherf and Wood, 1994). In the *luc+* gene the peroxisomal translocation sequence is removed, as well as several restriction sites. Codon usage is improved for mammalian cells and consensus glycosylation sites, and consensus sequences for transcription factor binding sites were eliminated (Sherf and Wood, 1994). It was shown that *luc+* had a 10-100 times higher expression than *luc* in mammalian cells (Groskreutz et al., 1995). In tobacco no significant effect on expression was found, but in maize and wheat a 20- and 55-fold increase in activity was obtained respectively (Lonsdale et al., 1998). In the pGM46 and pGM107 constructs an N-terminal SV40 Nuclear Localisation Signal (van der Krol and Chua, 1991) was present in front of the *luc* coding sequence, which had no apparent effect on its activity.

Plant material

Petunia hybrida (Vilm.) plants (cv. V26) were transformed by *A. tum.* clones containing either pGM46 or pGM107, and transformed shoots were, after rooting, transferred to soil and grown in growth chambers with a 16 h light period (30 W m^{-2}, 22°C, and 70% RH) and an 8 h dark period (20°C, and 65% RH). For the experiments shown here the F_1 progeny plants of a back-cross with wild-type V26 were used.

Petunia cell suspensions were made by using seedlings of the back-cross from the 35S-*luc Petunia* plants. Seedlings were grown in 250 mL Erlenmeyer flasks on a rotary shaker at 100 rpm in 60 mL MS medium (Murashige and Skoog, 1962) supplemented with sucrose (30 g/L) and 2,4-D (1 mg/L). The suspension was sub-cultured every 10-12 d (10 mL culture with 50 mL fresh medium). After several weeks the cell suspension was sieved (< 120 μm). The sub-culturing resulted in a homogenous cell suspension after several months.

Tobacco plants containing a 35S-*luc* construct were kindly provided by Dr. Nap, Wageningen University and Research centre –Plant Research International.

In vivo luciferase activity measurement with the 2D-luminometer

Petunia or tobacco *luc* reporter plants were sprayed with a luciferin solution (1 mM firefly D-luciferin, sodium-salt, Duchefa, 0.01% Tween 80) by using an air-brush dispenser to obtain a fine mist, 24 h, 16 h and 2 h before measurement. Cell suspensions derived from the *Petunia luc* reporter plants were treated with 0.5 mM luciferin 2 h before measurement. Luciferase activity was imaged with a 2D-luminometer, consisting of an intensified CCD camera (C2400-77, Hamamatsu Photonics, Japan), or with a liquid nitrogen cooled slow-scan CCD camera (512-TKB, Princeton, Trenton, NJ, USA). Photon emission by *luc*-expressing plants (or suspensions) was quantified by computer (Argus-50 Image Processor, Hamamatsu Photonics, Japan). Luciferase activity is shown in relative light units per pixel (rlu/pixel). Integration intervals varied from 2 to 30 min. Images of luciferase activity are depicted with false colour scales (blue indicating low activity, red indicating high activity) or grey scales (dark grey indicating low activity, white indicating high activity).

LUC protein extraction

Leaf parts up to 100 mg, frozen in liquid nitrogen were ground and suspended in 100 μL luciferase extraction buffer (25 mM Tris H_2PO_4, 2 mM EDTA, 10% Glycerol, 1% Triton X-100, 2 mM DTT, pH 7.8). Cell fragments were removed by 4-10 min centrifugation at 16,000 g (14,000 rpm, Eppendorf centrifuge 5414C) and the supernatant was frozen in liquid nitrogen and stored at -80°C, until use in an *in vitro* luciferase activity assay.

In vitro luciferase flash-assay

For measurement of luciferase activity the frozen luciferase extract was thawed on ice and a 5 μL aliquot was pipetted in a 96-wells microtiter plate and measured in a Labsystem Luminoskan DS luminometer by addition of 100 μL flash-assay buffer (20 mM Tricine, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 2 mM DTT, 470 μM D-Luciferin, 5 mM ATP (pH 7.8)). Two seconds of light production of the initial flash (caused by the rapid single use of the LUC protein after which a complex is formed with oxyluciferin) was quantified and shown as relative light units (rlu) as measured in two seconds. A dilution of luciferase (Boehringer) in extraction buffer was used for calibration (0.1 U/mL – 200 U/mL).

In vitro luciferase assay with Coenzyme A

The luciferase extract can also be measured with a luciferase assay buffer containing Coenzyme A (CoA), which will prolong the light production (Ford et al., 1995). The steady state light production can then be quantified for 5 s after a 10 s interval directly after addition of the CoA-assay buffer (20 mM Tricine, 2.67 mM MgSO$_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 μM CoA, 470 μM D-Luciferin, 530 μM ATP (pH 7.8), Luehrsen and Walbot, 1993) and is shown as relative light units (rlu) averaged over 2 s (for easier comparison with flash-assay).

For the CoA experiments, shown in Table 2 the following buffers used were all containing 20 mM Tricine, 2.67 mM MgSO$_4$, 0.1 mM EDTA with a final pH of 7.8 and one of the following:

a) Buffer: without extra additions;
b) Flash2: (=flash-assay buffer) containing 2 mM DTT, 470 μM D-Luciferin, 5 mM ATP;
c) CoAdil. (dilution): buffer with 270 μM CoA;
d) CoA33: (=CoA-assay buffer) containing 33.3 mM DTT, 270 μM CoA, 470 μM D-Luciferin, 530 μM ATP;
e) CoA2: buffer with 2 mM DTT, 270 μM CoA, 470 μM Luciferin, 530 μM ATP.

Oxygen determination in Petunia cell suspension

Oxygen levels were measured with a Clark oxygen monitor at 25°C in a stirred *Petunia* cell suspension during the *in vivo* luciferase activity measurement with the 2D luminometer.

Determination of stomatal aperture by silicone rubber imprints

Silicone rubber imprints were made from a *Petunia* leaf surface to determine the stomatal aperture of the leaf. Two parts of silicon rubber (Xantopren light body, thin flowing silicone precision impression material; ADA specification No.19, type II, low viscosity, Bayer Dental) were mixed with one part accelerator and subsequently mixed thoroughly for 30 s, without introduction of air bubbles in the mixture. An imprint of the leaf should be made within 2 min, after which an additional 2 min are required for polymerisation. By making two silicone-rubber imprints on the same location, a cleaner imprint can be acquired. Approximately 3 g polystyrene (Mw ~100,000, BDH Chem. Ltd.) was dissolved in 12 mL toluol (Merck) at a temperature of 45°C. The polystyrene solution was applied to the surface of the rubber imprint as thinly and evenly as possible, with a brush. The polystyrene film was carefully removed by gentle bending of the rubber replica after 3 min and laid upside down on a glass slide. The replica was covered with a cover glass, which was subsequently fixed by tape. Stomatal aperture in the preparation could now be examined through a microscope.
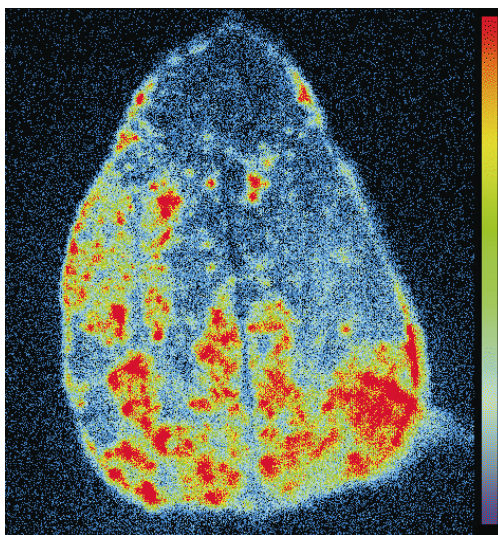
Figure 2. A 35S-*luc Petunia* leaf showing variegated patterns of light emission (luciferase activity measured for 5 min). Scale on the right indicates the colour scale used to represent the luciferase activity. The size of the leaf is approximately 13 by 18 mm.

Results and Discussion

Imaging of *in planta* luciferase activity in transgenic *Petunia* leaves expressing the 35S- or m35S-luciferase reporter gene shows different patterns of luciferase activity that can vary up to 16-fold within a leaf (variegation, Figure 2). In order to distinguish between variegated luciferase reporter gene expression and a variegated distribution of one or more of the substrates (luciferin, Mg^{2+}-ATP or oxygen), we tested the possible contribution of differences in the availability of each of the substrates to the variegated *in planta* luciferase activity. As ATP and oxygen are present within plant cells, only the substrate luciferin needs to be applied from the outside. We first investigated whether local differences in the penetration of luciferin into plant cells may be the cause of the variegated luciferase activity pattern in leaves.

Effects of luciferin on in planta luciferase measurements

Luciferin (in aqueous solution) can be applied either by repeated spraying on the plant or by uptake through the roots and vascular tissue. In order to optimise the distribution of luciferin over the leaf surface we used for spraying an air-brush dispenser to create a fine mist of luciferin and we used 0.01% Tween 80 as a surface-active agent (especially necessary when applied to hairy plant structures, like leaf surfaces or roots).

The solution of the luciferin as described by Millar et al. (1992) contains 0.01% Triton X-100 as a surfactant. However, Triton X-100 may cause necrosis of the leaf after prolonged application. We tested the possible necrotic effect of prolonged application of different surfactants on leaves. When *Petunia* leaves were put in a petri-dish, on a solution with different concentrations of either Tween 20,
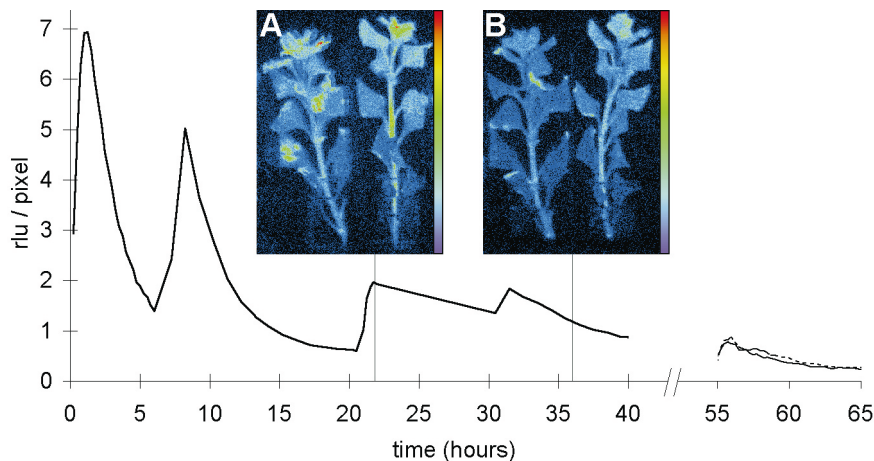
Figure 3. Luciferase activity of a 35S-*luc Petunia* branch as measured in 15 min (rlu/pixel) plotted against time (h). The measurement is continuously repeated for 40 h. The branch is put in water at t=0 h and sprayed at t=0 h, t=7 h, t=20 h and t=30 h with a 1 mM luciferin 0.01%Tween-80 solution. Another branch from the same 35S-*luc Petunia* plant is put in 1 mM luciferin at t=0 h. The luciferase activity reaches the same equilibrium after 20 to 30 h as is shown in the inserted panel A (22 h) and panel B (36 h). In panels A and B, the left branch is put in 1 mM luciferin at t=0 h, the right branch is the branch described above (put in water at t=0 h and sprayed with luciferin). Scale on the right in panels A and B indicates the colour scale used to represent the luciferase activity. After 55 h both branches are sprayed with a 1 mM luciferin 0.01% Tween-80 solution and measured for 10 h. The black line represents the luciferase activity in the branch put on water at t=0 h, the dashed line represents the luciferase activity in the branch put on 1 mM luciferin at t=0 h.

Tween 80, Non-Idet P40 or Triton X-100, severe necrosis was observed with Triton X-100 (1%) after a few hours or, with 0.01%, after a few days. The severity of necrosis was with Triton X-100> Non-Idet P40> Tween 20> Tween 80. Prolonged application of a 0.01% solution of Tween 80 had no visible necrotic effect on leaves (equal to water, data not shown). Therefore, in all experiments in which plants were sprayed with a luciferin solution, 0.01% Tween 80 was used as a surfactant.

Luciferin readily penetrates most plant tissues when applied by spraying. *In planta* luciferase activity can be imaged within seconds after spraying when the luciferase reporter gene is expressed in epidermal cells. With vascular uptake, luciferin is transported through the plant within minutes. We compared the effect of luciferin application, by repeated spraying with 1 mM luciferin and through vascular uptake of a 1 mM luciferin solution, on *in planta* luciferase activity in two branches from the same 35S-luciferase *Petunia* plant. In Figure 3 the quantified luciferase activity of the sprayed branch is shown in time. Spraying of the *Petunia* branch with 1 mM luciferin at t=0, 7, 20 and 30 h, results in an increase of luciferase activity at 0 and 7 h, but has almost no effect at 20 and 30 h. The panels inserted in Figure 3 show the two branches at t=22 h (panel A) and t=36 h (panel B). With both types of luciferin application, a similar variegated pattern of *in planta* luciferase activity emerges. When after 55 h both branches are sprayed with 1 mM luciferin, both branches show a comparable and only small increase in luciferase activity, indicating that for both ways of luciferin application an

equilibrium is reached in luciferin influx (Figure 3). The slow overall decrease of luciferase activity after 30 h might reflect a decrease in luciferase gene expression, due to the prolonged absence of light during measurement under the luminometer. These results indicate that a continuous application of luciferin is not required, once equilibrium between luciferase activity and luciferin inflow is reached. Intermittent spraying of luciferin twice a day is sufficient to keep the luciferase activity at the same level as continuous application through the vascular feeding. The substrate luciferin itself is very stable in plant cells, because luciferase-expressing plants that previously have been sprayed with luciferin can still show luciferase activity after 7-10 d without further addition of luciferin.

Spraying the plants requires less luciferin than application of luciferin by watering, which induces patterns caused by vascular luciferin uptake when the plants are imaged too soon (Schneider et al., 1990; Quandt et al., 1992). However, some plant structures will not take up luciferin, either when applied from the outside or through the vascular system. For instance, the locules of stamen or developing seeds initiate a dehydration program at a certain stage of their development, which will block an influx of water and consequently an influx of luciferin. A luciferase reporter gene that is expressed in these tissues will only show *in planta* luciferase activity when luciferin is applied at an early stage of development, when the structure is still in contact with the vascular system of the entire plant, or when the mature tissue is damaged to facilitate substrate penetration.

The luciferase substrate luciferin may have an adverse effect on plant cells when used at high concentrations (> 10 mM). Repeated spraying of plants with a 1 mM solution (e.g. daily for several weeks) does not markedly inhibit *Petunia* or tobacco plant growth or reproduction. Sensitive cell systems like tobacco suspension cells or protoplasts can survive in luciferin concentrations of up to 80 μM, but concentrations > 400 μM luciferin were found to kill the tobacco suspension cells (Ow et al., 1986). A comparable toxic effect on somatic carrot embryo development at > 400 μM luciferin was also found by Toonen et al. (1997). In *Petunia* cell suspensions, we found no toxic effect (within the 10 d subculture) when we used 500 μM luciferin, which was enough to bring the luciferase reaction in the cell suspensions to an equilibrium (raising the concentration to 1.0 mM or 1.5 mM luciferin had no effect on the level of light produced by the cells, data not shown).

In conclusion, different ways of luciferin application have no effect on the variegated pattern of luciferase activity in plants. In plants pre-sprayed 3x with luciferin, additional re-spraying does not significantly influence the level and pattern of luciferase activity. We therefore conclude that in our experimental set-up the observed differences in luciferase activity (Figure 2) were not caused by differences in luciferin availability.

Effects of oxygen on in planta luciferase measurements

The luciferase reaction is dependent on oxygen. When the oxygen availability within a leaf is decreased (e.g. by submergence in water) light production as a result of luciferase activity drops to zero within 15-20 min and is immediately restored after re-exposure to air (data not shown). By measurement of luciferase
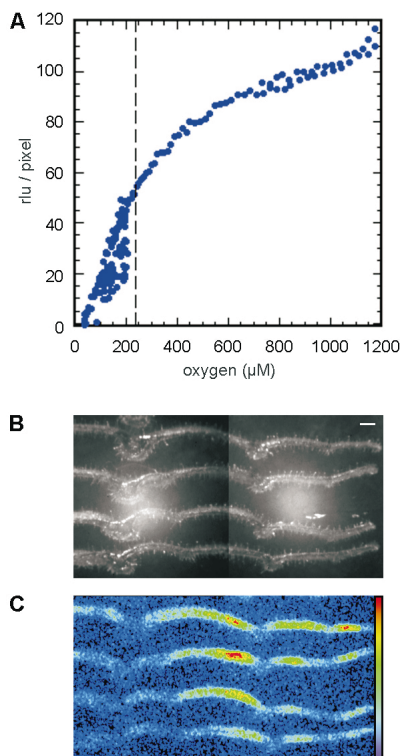
Figure 4. Panel A. Oxygen dependence of luciferase mediated light production in a 35S-*luc Petunia* cell suspension. Plotted are relative light units (rlu) per pixel versus oxygen concentration (μM). The dashed line shows the maximum level of oxygen when the suspension is oxygenated with air. Panel B. Four cross sections of a variegated m35S-*luc Petunia* leaf. The main vein (left) and a lateral vein are visible. The scale bar represents 1 mm. Panel C. Variegated luciferase activity in the cross sections shown in panel B. Scale on the right indicates the colour scale used to represent the luciferase activity.

activity in a *Petunia* cell suspension at different levels of oxygenation, the dependence on oxygen of the luciferase reaction is illustrated (Figure 4A).

We investigated whether possible local variations in oxygen concentration within a leaf contribute to the observed variegated patterns of luciferase activity. Cross sections were made of an excised *Petunia* leaf with different levels of luciferase activity within the leaf. In these cross sections (Figure 4B) the luciferase activity remains variegated (Figure 4C), indicating that variable oxygen availability within the leaf is not the cause for the variegated luciferase activity pattern.

Local oxygen levels may however vary depending on the opening of stomata and photosynthetic activity of the leaf. It has been shown before that stomatal aperture may vary within a leaf (Laisk et al., 1980, 1983). A variegated stomatal aperture may actually cause or contribute to the variegated luciferase activity that is observed in 35S-*luc* transgenic leaves (Figure 2). We therefore tested whether the stomatal aperture can influence the luciferase activity in 35S-*luc Petunia* plants. Three genetically identical plants with comparable luciferase activity

Table 1. The effects of stomatal aperture on the level of luciferase activity in 35S-*luc Petunia* plants[*].

Treatment	luciferase activity (rlu/pixel)	Aperture stomata (ratio open/closed (n))
Light	3.5	6.7 (115)
Dark	3.3	0.26 (122)
ABA	3.5	0.21 (104)

[*]Three genetically identical plants with comparable luciferase activity were kept under greenhouse conditions (light), kept in the dark (dark) or treated with $10 \mu M$ ABA (ABA) for 24 h. The average luciferase activity in the first expanded leaf after 24 h treatment is shown (rlu/pixel). The aperture of the stomata in these leaves is subsequently measured under the microscope (shown as ratio open/closed). The number of stomata used to calculate this ratio is shown between brackets (n).

were either kept under greenhouse conditions for 24 h, kept in the dark for 24 h, or treated with 10 μM ABA (sprayed three times in 24 h). One leaf of each plant was then measured in close-up with the luminometer and stomatal aperture was determined in this leaf by microscopic analysis (Table 1). The analysis shows that both the dark treatment and the ABA treatment resulted in a similar ratio of open to closed stomata, which was 3-4 % of that in control leaves. However, the luciferase activity in these leaves was not significantly affected by stomatal aperture, indicating that variegated stomatal aperture within a leaf does not contribute to the variegated luciferase activity in leaves.

We therefore conclude from these experiments that the variegated luciferase activity in *Petunia* leaves is not caused by local differences in oxygen availability between the cells within the leaf.

Availability of ATP in in planta luciferase measurements

Because of the direct relation between ATP and the photon production of the luciferase reaction, and the high sensitivity at which photons can be detected, the luciferase reaction is often used to quantify ATP in plant extracts (Malik and Thimann, 1980). It can be concluded from these experiments by Malik and Thimann (1980) that the cellular steady state concentration of ATP in plant leaf cells under normal physiological conditions is in the range of 100-200 pmol/mg fresh weight (FW). We determined the ATP level that is required for the reaction of a high amount of luciferase that can be present in a plant with high expression of a luciferase reporter gene. We first used an *in vitro* luciferase flash assay in which different ATP dilutions were added to a fixed amount of LUC protein (20,000 U). Light production was detectable above 10 fmol ATP added (Figure 5). The resulting light production from a fixed amount of ATP can now be compared with the light production we normally obtain in leaf extracts per mg FW (0.2-20 rlu/mg FW, grey area Figure 5). Apparently, the amount of luciferase present in our *Petunia* leaves requires 20 fmol ATP/mg FW to a maximum of 2 pmol ATP/mg FW. This is 2 to 4 orders of magnitude below the ATP concentration in leaves (Malik and Thimann, 1980).
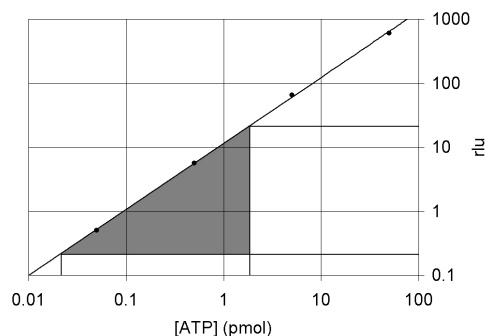
Figure 5. ATP dependence of the luciferase reaction as determined in an *in vitro* luciferase flash assay. Plotted are the relative light units (rlu) versus the ATP levels added (pmol). Points are means of duplicate determinations. The linear regression line has an R^2 of 0.999. The grey area shows the range of rlu we generally obtain in luciferase extracts from transgenic leaves and the corresponding picomoles of ATP required.

Assuming that 10% of the fresh weight consists of cytoplasm, 100 pmol ATP/mg FW would correspond to a concentration of approximately 1 mM ATP, which is far above the Km of luciferase for ATP (50 μM) (Lembert and Idahl, 1995). The variegated luciferase activity that we observe in 35S-*luc Petunia* leaves (Figure 2) utilises at maximum only 1-2% of the available ATP pool (provided that no regeneration of luciferase activity takes place). We conclude from these experiments that the luciferase activity probably has no disturbing effect on the intercellular ATP levels and that the observed variegated pattern of luciferase activity in *Petunia* leaves is not caused by local differences in ATP availability.

Local luciferase activity corresponds to local LUC protein and mRNA levels

The aforementioned experiments make it unlikely that there are local differences in the availability of each of the substrates of the luciferase reaction. We therefore conclude that the observed local luciferase activity is related to the local protein production rate and thus to the local expression of the luciferase transgene. When LUC protein is extracted from patches in leaves with either low or high luciferase activity, the *in vitro* quantification of luciferase activity shows a similar low/high distribution (data not shown). Preliminary results with RT-PCR also showed that the observed differences in luciferase activity correlated with differences in *luc* mRNA levels.

Luciferase signal/light penetration

Plant cell pigmentation and structure may quench or divert some of the light produced by the luciferase activity in cell layers below the imaged surface. To investigate this, a simple experiment can be done as is shown in Figure 6. Two 35S-*luc* tobacco leaves (pre-sprayed with luciferin) were measured for 5 min. Figure 6A and 6B show that the activity in both leaves is comparable. The leaves were subsequently covered with a wild-type (non light-producing) tobacco leaf (Figure 6C and 6D) and again measured for 5 min. The measured light production (of the luciferase activity in the leaf) dropped in the covered leaf to approximately 20-25%
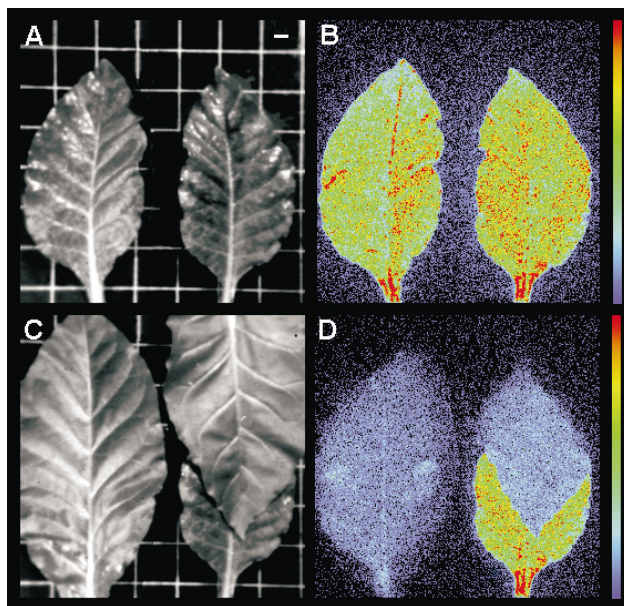
Figure 6. Penetration of the light emitted by luciferase from two 35S-*luc* tobacco leaves. Panel A shows these leaves (bar represents 1 cm). Panel B shows the acquired photon image of 5 min measurement of the leaves in panel A. Scale on the right indicates the colour scale used to represent the luciferase activity. Panel C shows these same leaves covered (completely, left and partly, right) by a wild-type tobacco leaf. Panel D shows the acquired photon image of 5 min measurement of the leaves as shown in panel C. Scale on the right indicates the colour scale used to represent the luciferase activity.

of the light production of the non-covered leaf. This light-transmission-efficiency may vary depending on the plant organ, age and plant species. The structure of a leaf may result in scattering of light, thereby blurring distinct patterns of luciferase activity. However, some patterns of activity (e.g. vein patterns in left leaf) are still visible when the leaf is covered with an additional 10 cell layers. Although light is most effectively measured when emitted by the epidermal cell layer, these results show that all cell layers contribute (although to different degrees) to the observed luciferase activity in whole plants. The variegated pattern of luciferase activity in leaves is also maintained after sectioning (Figure 4B), which illustrates that local high luciferase activity in leaves is present in all cell layers, i.e. locally all cell layers contribute to patches of high activity in the leaf (note that the sections of the leaf in Figure 4B are facing the camera). Therefore, the leaf structure does not seem to contribute to the variegated pattern of luciferase activity in transgenic leaves (Figure 2).

Effect of pH on the detection of luciferase activity in plant tissue

Like most enzymes the activity of firefly luciferase is optimal at neutral pH and activity declines towards lower or higher pH values. However, the bioluminescence spectrum might also be changed by perturbations of the chemical environment. The yellow-green luminescence of most firefly luciferases (562 nm) can be changed to

red by lowering the pH below 7 (Selinger and McElroy, 1964). This shift to longer wavelength is caused by the fact that the substrate luciferin changes from a di-anion to a mono-anion at lower pH (DeLuca and McElroy, 1976). The enol configuration of oxyluciferin results in yellow-green light emission, while the keto configuration results in red light emission (Aflalo, 1991).

The shift in wavelength can be important in relation to the sensitivity and spectral characteristics of the camera that is used to measure the luminescence. A camera optimised for measuring yellow-green light (e.g. an intensified CCD camera) can show a large decrease in sensitivity towards the red part of the spectrum in comparison to, for example, a cooled slow-scan CCD camera (Figure 7A).

In order to demonstrate the effect of pH on the detection of luciferase activity, we measured luciferase activity at different pH values *in vitro,* using an intensified camera, with and without a long pass filter (100% relative transmission red light, 10% relative transmission blue-green light). When *in vitro* luciferase (Boehringer, 20U/mL) is measured at pH 5.8, luminescence drops below 20% of the luminescence measured at pH 7.6 (black line, Figure 7B). With a long pass filter (dashed line, Figure 7B), the activity measured at pH 5.8 is approximately 60% of the luminescence measured at pH 7.6. This decrease is due to the decrease in luciferase activity. The additional 40% drop to 20% of the luminescence measured at pH 7.6 without a long pass filter (black line) is probably due to the relatively higher emission of red light and the decrease in sensitivity of the intensified camera. Changes in observed luminescence related to pH changes (either cellular pH *in vivo* or pH in extracts *in vitro*), might thus be caused by a real change in luciferase activity or might reflect a decrease in sensitivity of the camera.

However, this decrease in sensitivity towards the red side of the spectrum, makes an intensified CCD camera also less sensitive for delayed luminescence of chlorophyll (Hideg et al., 1992). In the first few minutes after the light is switched off, this light emission in the red part of the spectrum, can be as strong as the *in planta* luciferase activity. In contrast to an intensified CCD camera, a cooled CCD camera is very sensitive towards the red side of the spectrum. When this type of camera is used for the detection of *in planta* luciferase activity, the delayed luminescence of chlorophyll can be a serious problem. In Figure 7 C-E three wild-type leaves (top) and three 35S-*luc* transgenic leaves (bottom) are measured with a cooled CCD camera for 5 min, directly after the light is switched off. Panel C shows the luminescence of these leaves without a DT Green filter (Image Optics Components Ltd.), depicted with a greyscale of 0 to 400 rlu. Panels D and E show the luminescence of these leaves with a filter, depicted with either a greyscale of 0 to 400 rlu (panel D) or 0 to 100 rlu (panel E). Note that the filter reduces the yellow-green light transmission of the luciferase to 25%, but also effectively blocks the red light of the delayed luminescence of chlorophyll. After 5-10 min, the delayed luminescence is almost undetectable with a cooled CCD camera without a filter (data not shown). With a cooled CCD camera, imaging of plant material should therefore either be delayed for at least 10 min after placing the plant in the dark, or appropriate filters should be used that effectively block chlorophyll luminescence.

As is visible in Figure 7C-E, we still observe variegated patterns of luciferase activity in *Petunia* leaves with a cooled CCD camera, which is not sensitive to
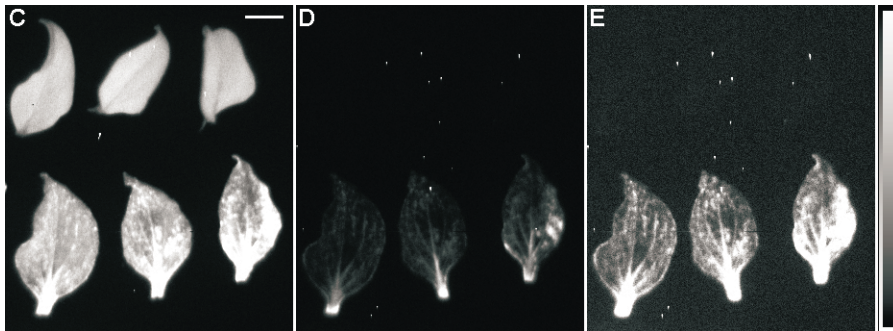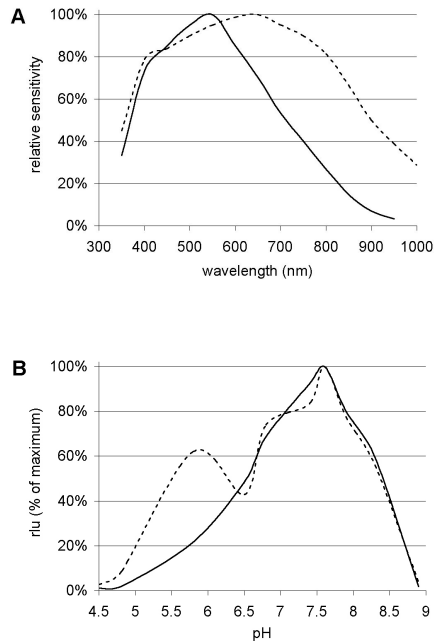
Figure 7. The effect of "red-shift" of the light emission by the luciferase due to pH differences. Panel A shows the drop in sensitivity of an intensified luminometer (black line) towards the red part of the spectrum (above a wavelength of 650 nm), compared to the sensitivity of a cooled slow-scan lumino-meter (dashed line). Panel B shows the relative light emission of luciferase at different pH levels as a percentage of the maximum light emission at pH 7.6. The black line represents a measurement with the intensified luminometer under normal circumstances (with no filter), while the dashed line shows the effect of a long pass filter (allowing 100% relative transmission of red light and 10 % relative transmission of shorter wavelength light). Panels C-E show the effect of delayed luminescence of chlorophyll in three wild-type *Petunia* leaves (top) and three 35S-*luc* transgenic *Petunia* leaves (bot-tom), measured with a cooled slow-scan CCD camera for 5 min, directly after the light is switched off (bar represents 1 cm). Panel C shows the luminescence of these leaves without a filter, depicted with a greyscale of 0 to 400 rlu. Panels D and E show the luminescence of these leaves with a filter, depicted with either a greyscale of 0 to 400 rlu (panel D) or 0 to 100 rlu (panel E). Scale on the right indicates the greyscale used to represent the luciferase activity. Random white pixels are caused by cosmic rays.

spectral shifts in the light emission of the luciferase. We therefore conclude that the observed variegated pattern of luciferase activity in 35S- luciferase *Petunia* leaves (Figure 2) is not caused by possible intracellular differences in pH between cells of a leaf.

In vitro quantification of luciferase activity in plant extracts

Luciferase activity can be measured *in vitro* in extracts from transgenic plant material with either a flash assay or a Coenzyme A (CoA) assay. When (commercial) pure LUC protein is used in a calibration curve, the quantified light from both assays is proportional to the LUC protein concentration over three orders of magnitude (data not shown). In the presence of high ATP concentrations, Coenzyme A enhances the light production through removal of oxyluciferin from luciferase resulting in a nearly constant production of light (Ford et al., 1995). The LUC protein thus shows standard enzymatic behaviour in the presence of Coenzyme A. The CoA reaction yields approximately three times more light than the flash reaction (relative light units, rlu, per second). The light production of the flash reaction (single use of LUC) and the CoA reaction ("enzymatic" use of LUC) are linearly related to each other (R^2=0.993) over three orders of magnitude (0.1 to 200 Units LUC/mL, data not shown).

The fact that CoA can prolong light production of the luciferase by releasing the luciferase from the Luciferase $\bullet$ Oxyluciferin complex, could indicate that a flash-assay would measure the total amount of non-reacted luciferase in a leaf-extract, while a CoA-assay would measure the total amount of non-reacted luciferase and of previously reacted luciferase. It was investigated whether an inactive pool of luciferase (in a complex with oxyluciferin) could be regenerated by CoA after the initial flash reaction, when CoA is not present during the reaction. A flash assay was performed with commercial luciferase (t=0 min, rlu set to 100% in Table 2), followed by addition of 100 μL of different buffers after 5 min. Light emission was measured for 2 s, directly before addition (t=5 min) and 2 min after addition (t=7 min). In a control flash reaction without extra additions (column 1) light production after 5-7 min is still 40% of the light production during the flash. Addition of buffer without ATP or luciferin (column 2) or addition of flash-buffer (column 3) showed a small extra decrease of light production after the flash at t= 7 min. However, addition of Coenzyme A (in different buffers, column 4-6), was able to increase light production up to 89-111% of the initial flash. Row 4 in Table 2 shows the effect of buffer addition (t=7/t=5), while row 5 shows these levels of light production after buffer addition as a percentage of the level of light production obtained by a normal CoA assay at t=0 min. (i.e. with the same amount of luciferase, without a prior flash assay when CoA is present from the start, t=7 as % of CoA33).

These experiments show that CoA needs to be present during the Luciferase-Luciferin reaction and does not release luciferase from the Luciferase $\bullet$ Oxyluciferin complex. 5 min after application of assay buffer (t=5 min), the light production of a CoA assay is still at 75% (230%/306% in Table 2, column 7), while the light production of a flash assay is at 35-40% (Table 2). When the luciferase in the complex with oxyluciferin can be regenerated, addition of CoA should produce more than the 300% of the light that is always produced by a CoA

Table 2. Regeneration of luciferase activity by Coenzyme A after a flash assay[*].

| | Addition at t=0 min | | | | | | |
	1) Flash2	**2**) Flash2	**3**) Flash2	**4**) Flash2	**5**) Flash2	**6**) Flash2	**7**) CoA33
t=0 min	100%	100%	100%	100%	100%	100%	306%
t=5 min	40.8%	40.6%	36.1%	38.7%	36.7%	37.0%	230%
	Addition at t=5 min						
	—	Buffer	Flash2	CoAdil	CoA33	CoA2	—
t=7 min	40.0%	34.4%	29.7%	88.9%	76.8%	111%	219%
t=7/t=5 (%)	97.9%	84.7%	82.4%	230%	209%	300%	95.2%
t=7 (% of CoA33)	13.1%	11.2%	9.7%	27.6%	26.0%	36.8%	71.6%

*Shown are the effects of addition of different buffers 5 min after a normal flash reaction (flash2), as a percentage of the initial flash. The average light production (per 2 s) of six determinations is shown at 1) t=0 min, the initial flash set to 100%), t=5 min, (directly before addition of the different buffers) and t=7 min, (2 min after buffer addition). Shown are in column 1) a flash assay without extra additions at t=5 min, in column 2 to 6) the effects of addition of a) tricine buffer (Buffer), b) flash buffer (Flash2), c) CoA dilution in tricine buffer (CoAdil), d) CoA buffer (CoA33) and e) CoA buffer (with 2 mM DTT= CoA2). For comparison the light production of a normal CoA assay is also shown in column 7 (CoA buffer with 33 mM DTT= CoA33, present at t=0, averaged over 2 s) as a percentage of the flash assay at t=0. In row 4 the light production after buffer addition (t=7 min) is shown as a percentage of the light production directly before buffer addition (t=5 min), i.e. the effect of buffer addition. Shown in row 5 is the light production after buffer addition (t=7 min) as a percentage of the light production of a CoA assay at t=0.

assay (Table 2, column 7, t=0 min, 306% of the light of a flash assay). Addition of CoA at t=5 min however, produces maximal 300% of the light emitted without extra addition of CoA (Table 2, column 6, t=7/t=5, 300% of the amount of light prior to buffer addition). Note that the amount of luciferase at t=5 min produces 37.0% of the light in the initial flash assay (Table 2, column 6) as well as 36.8% of the light of a comparable CoA assay (Table 2, column 6, t=7 (% of CoA33)), truly indicating that only the remaining (non-reacted) luciferase (which is 37%, 5 min after a flash assay) can produce light and that the pool of reacted luciferase can not be regenerated by CoA.

When extracts from leaves are measured *in vitro*, after these leaves are measured *in vivo*, no discrepancy will be found between a flash and a CoA measurement (data not shown). Although the leaf has been pre-sprayed with luciferin for the *in vivo* measurement and thus contains both reacted luciferase and non-reacted luciferase (related to the luciferase production in the leaf), in both assays the same pool of luciferase is measured *in vitro* (luciferase which has not reacted *in planta* yet). In samples of non-luciferin-pre-treated leaves the luciferase activity represents all the LUC protein that has accumulated over time, while in samples from luciferin pre-treated plants, the luciferase activity represents the fraction of protein that has not reacted with the luciferin *in vivo*. Since after prolonged pre-treatment with luciferin the previously accumulated luciferase has formed a complex with the oxyluciferin, the fraction of free LUC protein in these plants mainly arises from ongoing transcriptional and translational activity. The observation that CoA does not affect the regeneration of luciferase from previously

formed luciferase-oxyluciferin complexes is also important for the interpretation of changes in gene expression *in vivo*.

Although changes in the availability of CoA in cells may affect the efficiency of the reaction of free luciferase, the large pool of inactive luciferase-oxyluciferin complexes that has accumulated during pre-treatment with luciferin can not be regenerated into active LUC protein by a sudden increase in cellular CoA concentration.

*Stability of LUC protein and luciferase activity*

In order to monitor rapid changes in gene expression, a high turnover of both mRNA and protein is required. The half-life of LUC mRNA is about 45 min (Gallie et al., 1991), but is also dependent on 5'-leader and 3'-tail sequences that have been added or deleted during construction of the reporter gene - without a poly-A tail the LUC mRNA half-life is 24 min (Gallie et al., 1991).

It was shown before that the half-life of LUC protein in mammalian cells was approximately 200 min (Nguyen et al., 1989; Thompson et al., 1991) providing a reporter system capable of reacting to rapid changes in gene expression. We tested the half-life of LUC protein in a 35S-*luc Petunia* cell suspension. Samples were taken from a) a non-treated cell suspension, b) a cell suspension with 10 μg/mL cycloheximide added at t=0 (to block translation), and c) a cell suspension with 0.5 mM luciferin added at t=0. Samples taken at different time points were frozen in liquid nitrogen and luciferase was extracted with luciferase extraction buffer, and subsequently quantified *in vitro* (shown in Figure 8A as a % of rlu at t=0). The luciferase activity of the non-treated cell suspension does not change in time. The cycloheximide treated cell suspension shows a decrease of luciferase activity, coinciding with the degradation of the LUC protein and the luciferin treated cell suspension shows a very rapid decay of luciferase activity, caused by the rapid single use of the accumulated LUC protein after which a complex is formed with oxyluciferin. Figure 8B, shows this decrease of luciferase activity as the treated/non-treated ratio on a logarithmic scale with regression lines indicating the half-life. The half-life of LUC protein in a 35S-*luc Petunia* cell suspension is 155 min, but the apparent half-life of luciferase activity when luciferin is added is 10 times faster at 15.3 min.

This apparent half-life of luciferase activity can reveal decreases in reporter protein content within minutes. Such rapid changes would remain undetectable, when the reporter gene product accumulates. *In vitro*, a flash of light is detected directly after luciferin addition, with an even more rapid apparent half-life of luciferase activity. The apparent half-life of luciferase activity *in vivo* is therefore possibly limited by the influx of luciferin in the *Petunia* cells and the reaction rate of luciferase. The rapid decrease in luciferase activity *in planta* after the first application of luciferin indicates that the LUC protein is not regenerated *in vivo* (in plant cells).

Conclusions

Luciferase activity can easily be monitored and quantified *in vivo* and *in vitro*. Prerequisite for the application of the luciferase reporter system in plants, is the continuous availability of all substrates. Luciferin is sufficiently available in intact
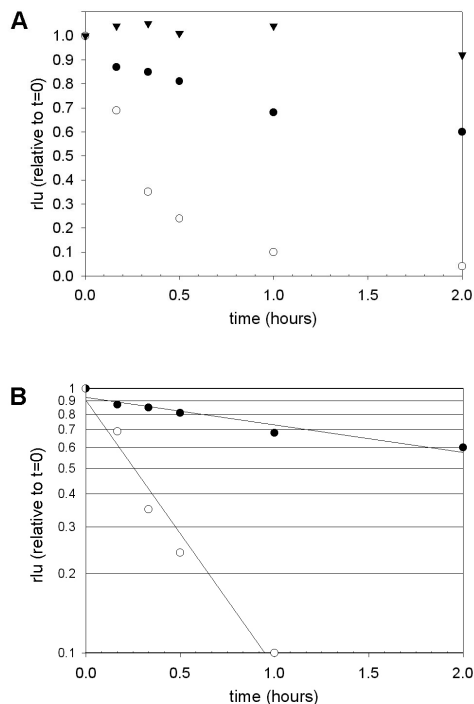
Figure 8. Half-life of luciferase and luciferase activity *in vitro* in extracts of *Petunia* cell suspensions. In panel A the average luciferase activity of a *Petunia* 35S-*luc* cell suspension as determined in extracts in an *in vitro* flash assay is shown, plotted as a percentage of luciferase activity at t=0 min. Samples were taken at t=0, 10, 20, 30, 60 and 120 min from a non-treated cell culture (▼), a cell culture treated with cycloheximide (10 μg/mL) at t=0 (●), and a cell culture treated with luciferin (0.5 mM, ○) at t=0. Points are means of triplicate *in vitro* determinations of triplicate samples. Panel B shows in a logarithmic plot that the half-life for the LUC protein (with cycloheximide, without luciferin addition, closed symbols) as determined from panel A is 155 min ($R^2 = 0.920$). The apparent half-life of luciferase activity with the addition of luciferin (open symbols), as determined from panel A is 15.3 min ($R^2 = 0.971$).

plants when these plants are pre-sprayed 3x with a 1 mM luciferin solution in 0.01% Tween 80. Oxygen is also sufficiently available in intact plants, although in liquid environments (e.g. cell suspensions) the oxygen levels should be closely monitored. Steady state ATP levels in intact plant cells under normal physiological circumstances are 2 to 4 orders of magnitude higher than the ATP levels required for the luciferase reaction.

The relative instability of the luciferase mRNA and protein and the lack of regeneration of luciferase after the reaction with luciferin (*in vivo*, in the absence of CoA), make the luciferase ideal for monitoring rapid changes in gene activity (flux in luciferase production). However, the luciferase activity that is measured *in planta* immediately after application of the luciferin is both the result of previously accumulated LUC protein and LUC protein made by ongoing transcription and translation during the interval of the photon counting. In order to relate the *in planta* luciferase activity to ongoing transcription and translation activity, changes

in *in planta* luciferase activity of different luciferase reporter genes can be compared under similar conditions and/or changes in substrate influx need to be excluded, which can be ensured by measuring under substrate equilibrium conditions (as is shown in this paper).

Acknowledgements

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