Transgene expression in plants

position-induced spatial and temporal variations of luciferase expression

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Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus,
van Wageningen Universiteit,
prof. dr. ir. L. Speelman
in het openbaar te verdedigen
op woensdag 21 maart 2001
des namiddags te half twee in de aula

The work presented in this thesis was performed at the Wageningen University, Department of Plant Sciences, Laboratory of Plant Physiology, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands.

This research was financially supported by the Foundation for Life Sciences (SLW), part of the Dutch Organisation for Scientific Research (NWO).

Cover illustration: A false-colour image showing variegated firefly luciferase activity in a petunia leaf. The back cover illustrates the temporal variation of luciferase activity in six transgenic petunia lines (from left to right: 46-2b4; 46-5s3; 520-7; 520-9; 107-13; 107-3) in one -growing- leaf, measured on subsequent days (from top to bottom: day 1, 2, 3, 4, 5, 6, 7, 8, & 15) shown in false colours (see also chapter 3, figure 5, page 56). Leaves were originally imaged by Tom Ruttink.

CIP-data Koninklijke Bibliotheek Den Haag

Van Leeuwen, Wessel

Transgene expression in plants: Position-induced
spatial and temporal variations of luciferase expression.
PhD thesis of Wageningen University – With summary in Dutch.

Subject headings: Cauliflower Mosaic Virus 35S promoter, luciferase reporter system, *Petunia hybrida*, position effect, reporter gene, transgene expression, variegation.

ISBN 90-5808-344-6

Voorwoord

Voilá! Dit is het dan: 120 bladzijden als representatie van 5 jaar werk… Beginnend in 1995 kwam ik er al gauw achter dat het luciferase-reportersysteem, zoals dat bij de vakgroep plantenfysiologie werd gebruikt, voor grote hoeveelheden data zorgt, doordat er de mogelijkheid is om hele planten te "filmen" met de lichtgevoelige camera (de luminometer). Dit resulteert dus in zo'n 20 CD's met data, waaruit wel weer blijkt dat een groot deel van dit werk bestaat uit data-reductie / -verwerking / -manipulatie… Uiteraard is het niet mogelijk om al dit werk alleen voor elkaar te krijgen, vandaar dat ik hier graag een aantal mensen voor wil bedanken.

Nadat ik net mijn eerste lichtgevende petunia's had gecreëerd, kreeg ik het genoegen mijn eerste student te begeleiden. Tom, het werk wat jij in mijn eerste jaar hebt gedaan (een groot deel van hoofdstuk 3), heeft toch tot 1998-'99 voor het nodige analyse-werk gezorgd en heeft (samen met onze veelvuldige discussies) behoorlijk bijgedragen aan de gedachtevorming over hoe we nou met het luciferase-reportersysteem moesten omgaan; Bedankt! Om maar meteen verder te gaan met de discussie / gedachtevorming; onze samenwerking met het CPRO was hierin ook zeer nuttig en, Jan-Peter, ik heb het erg gewaardeerd dat je hier de tijd voor hebt genomen. Ik ben dan ook blij dat deze samenwerking heeft geleid tot hoofdstuk 4.

Verder bladerend door het proefschrift… Al deze discussie heeft uiteindelijk geleid tot hoofdstuk 2, mede dankzij de brainstorm- en experimentele inbreng van Marc: 't was fantastisch om met jou samen te werken! Alle mogelijke experimenten die we samen bedacht en gedaan hebben, zijn ook verder nog gedeeltelijk terug te vinden in hoofdstuk 6. Veel van dit werk was uiteraard niet mogelijk zonder de goede zorgen voor en de creatie van de celsuspensies door Diaan. Bedankt voor al je tijd; volgens mij mag je die suspensies nou echt weggooien… Experimenteel gezien natuurlijk ook een woord van dank voor Tanja: met name voor het RNA / RT-PCR werk en voor de vele kruisingen was je inbreng onmisbaar. Verder is al dit bovenstaande ook uitsluitend mogelijk gemaakt door de zorgen van Unifarm voor de petuniaplanten en dan met name de zorgen van Leen en Aart: bedankt.

Als laatste in dit rijtje mensen hoort uiteraard ook Sander: bedankt voor alle discussie en (overweldigende) inbreng van nieuwe ideeën en experimenten. Ik ben blij dat we het uiteindelijk in deze vorm hebben kunnen krijgen. Waarschijnlijk een belangrijke regulerende factor hierin is ook Linus geweest: jullie beiden bedankt voor het (vooral in het laatste jaar) doorworstelen / corrigeren / (manipuleren) / moduleren van de vele versies van hetgeen nu hier voor jullie ligt!

Rest mij natuurlijk nog om alle andere mensen te bedanken die het leven in “de banaan” en met name de koffiehoek gezellig hebben gemaakt. Allereerst Marc en Diaan als kamergenoot en vervolgens de koffiehoekgenoten. Beginnend bij Patrick: het was toch altijd fijn dat er nog iemand om half negen koffie kwam drinken. Dat was toch wel anders toen Ronny, Janhendrik, Juul, Marc of zelfs Maarten er nog was. Jullie worden gemist… Alleen de “laatste(n) der Mohikanen” zijn er nog (met de lunch): Margo en John, jullie waren vooral het laatste half jaar regelmatig reden genoeg voor mij om weer even op de vakgroep te komen lunchen.

De laatste alinea wilde ik graag gebruiken om mijn ouders te bedanken; voor steun, interesse, vertrouwen… gewoon voor alles: bedankt. En als allerlaatste natuurlijk Annelies: bedankt voor de nodige afleiding en voor de tijd om dit werk mogelijk te maken; je bent geweldig.

Wessel

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Abbreviations

ACC	1-aminocyclopropane 1-carboxylate
CaMV	Cauliflower Mosaic Virus
CV	coefficient of variation
FW	fresh weight
GUS	ß-glucuronidase
JA	jasmonic acid
LUC	luciferase (protein)
luc	luciferase (gene)
LTP	*Arabidopsis thaliana* Lipid Transfer Protein
MAR	matrix associated regions
max/avg	maximum luciferase activity / average luciferase activity
MCP	1-methylcyclopropene
MJA	methyl jasmonic acid
m35S	modified CaMV 35S
rlu	relative light units
SA	salicylic acid

Some genes used in chapter **6**:

aco	L. ethylene forming enzyme, ACC oxidase
acs	1-aminocyclopropane 1-carboxylate synthase, ACC synthase
gapdh	glyceraldehyde-3-phosphate dehydrogenase
hsp70	heat shock protein 70
myb	myb.Ph3 gene encoding protein 1
nia	nitrate reductase apoenzyme
pr-p	pathogenesis-related protein P, PR-3a
rbcS	ribulose 1,5-bisphosphate carboxylase small subunit (ssu8)
sod	chloroplast superoxide dismutase
yrps	ribosomal protein S3 gene

1 General introduction

With the development of molecular biology and biotechnology, it became possible to introduce and express foreign genes in an organism. Initially starting with bacteria and yeasts, transgenes were eventually introduced into multi-cellular organisms, like animals and plants. To study the expression pattern of the introduced transgenes, the promoter of the gene under study could be fused to the coding sequence of an enzyme or protein of which the activity can easily be measured, a so-called reporter gene. The use of reporter genes in (trans)gene expression studies revealed that independent transformed lines containing the same (number of) transgene(s) generally show different levels of reporter protein activity. In independent transformants the transgene is inserted at different positions in the genome. Therefore, the quantitative differences in transgene expression level are attributed to varying effects of chromosomal DNA, flanking the inserted transgene. These quantitative differences are referred to as the position effect.

In this thesis it is shown that the position effect does not only result in quantitative differences between independent transformants. The reporter gene used in this thesis (firefly luciferase) can visualise transgene promoter activity in whole tissues and plants. Therefore, qualitative differences (spatial and temporal variation) between independent transformants can be shown. To obtain high expression levels and expression throughout the organism, viral promoters (*e.g.* the Cauliflower Mosaic Virus -CaMV- 35S promoter in plant cells) are most commonly used to drive transgene expression at the moment. In this thesis the CaMV 35S promoter (*e.g.* Benfey *et al.,* 1989), a modified CaMV 35S promoter (m35S, van der Krol *et al.*, 1993) and an *Arabidopsis thaliana* lipid-transfer-protein promoter (LTP, Toonen *et al.*, 1997) are studied. The results in this thesis are the first detailed description of both the spatial and temporal aspects of transgene activity in plants in multiple, independent transformants. These results give a basis for the origin of the position effect (variations in transgene expression between independent lines), and may have consequences for our view on gene expression in multi-cellular tissues in general.

The use of reporter genes in gene expression studies

In order to characterise the expression pattern of a gene and specifically of a promoter, several reporter genes are available, which can be fused to the promoter region under study. The lacZ gene -from the Lac operon in *Escherichia coli*- encoding ß-galactosidase, was introduced as a reporter for transgene expression several decades ago (Gilbert and Muller-Hill, 1967). Galactosidase activity can be scored by a colour assay (blue stain) and is successfully used in the mammalian field as a marker of gene expression. The ß-galactosidase turned out to be less suitable as a reporter in plants due to the presence of galactosidase activity in most plant tissues, resulting in a high background activity (Helmer *et al*., 1984). ChlorAmphenicol Transferase (CAT), another reporter gene which was frequently used in the animal field, did proof to function in the quantification of gene expression in plants (Herrera-Estrella *et al*., 1988). Together with nopaline synthase (nos) and octopine synthase (ocs) from *Agrobacterium*, these were the first reporters to quantify gene expression in plants (Herrera-Estrella *et al*., 1988). Although nos, ocs and CAT allowed the quantification of gene expression in transgenic plants, they did not allow for the characterisation of the spatial distribution of the reporter gene activity. For this purpose the ß-glucuronidase (GUS) was introduced as a reporter gene in plants (Jefferson, 1987), which lacked background activity in most plant tissues. The GUS enzyme assay allowed for easy quantification of expression levels and gene expression could be localised to a cellular level in a histochemical staining assay in tissue samples.

The initial advantage of the GUS assay (its stability in plant cells and robustness under varying assay conditions) became a disadvantage when the scientific focus shifted from gene expression localisation studies to expression dynamic analysis. The histochemical staining to localise the reporter gene activity with GUS is both destructive and permanent. With the isolation of the luciferase gene (*luc*) from the North American firefly (DeWet *et al*., 1985), a non-destructive reporter became available, of which the activity could be monitored by light emission (Ow *et al*., 1986). The green-fluorescent protein (GFP) from jellyfish *Aequorea victoria* also developed in the last decade as a light emitting (fluorescent) reporter protein (Chalfie *et al*., 1994), although the protein is too stable to monitor swift changes in gene expression.

The history of luciferase as a reporter gene

The light reaction in the American firefly (*Photinus pyralis*) has intrigued people for a long time. Already in 1668 Robert Boyle discovered that light is emitted by the firefly with no perceptible heat in dependence of air (oxygen). Applying emerging biochemical approaches, Raphael Dubois (1885 and 1887) first demonstrated the involvement of organic compounds in light-emitting reactions in the firefly and the clam. He was able to restore light production by mixing two crude extracts from the same organism, and concluded that a heat-stable component (*luciferin*) served as a substrate to a heat-labile catalytic component (*luciferase*) in the luminescent reactions occurring in each organism (Aflalo, 1991). The biochemical properties and kinetics of firefly luciferase (LUC) were further analysed in the 1950-1970's (*e.g.* Green and McElroy, 1956; Denburg *et al.*, 1969; DeLuca and McElroy, 1974).

Luciferase catalyses the oxidative decarboxylation of firefly luciferin. A photon is released at 562 nm (yellow-green light) in 90% of the catalytic cycles with the substrate luciferin, Mg^{2+}-ATP and oxygen. During this reaction a complex is formed between luciferase and oxyluciferin. The LUC protein is only very slowly regenerated after reacting with the substrate, because the end-product, oxyluciferin, is only slowly released from the enzyme-complex. An advantage of firefly luciferase is that it only needs 1 ATP molecule to produce a photon, in contrast to *e.g.* bacterial luciferase (*lux*) derived from *Vibrio* and *Photabacterium* spp. which needs approximately 60 ATP molecules per emitted photon (Koncz *et al*., 1990). Firefly luciferase is therefore often used in an *in vitro* assay as a detector of ATP, in plants, animals, bacteria, soil, or *e.g.* to detect an increase in bacterial bio-mass in food products, like milk or meat (Orth and Steigert, 1996).

For all this research both luciferase and luciferin initially had to be extracted from the firefly itself, until in 1985 the firefly luciferase cDNA was cloned in *Escherichia coli* by DeWet *et al.* and shown to be expressed in an active form. In the years following, the luciferase gene was expressed in both plants (in tobacco and carrot, Ow *et al.*, 1986) as well as in mammalian cells (DeWet *et al.*, 1987). Limitations in the application of the LUC reporter gene initially were the high costs of the substrate luciferin (12 kfl/gram), but the price dropped substantially when it became possible to

produce it synthetically (2.5 kfl/gram when bought in bulk quantities at the moment). This strongly increased the use of firefly luciferase as a reporter for gene expression.

The original luciferase protein is targeted to the peroxisomes, both in the firefly as well as in plants. The peroxisomal import sequence was removed and experiments showed that the protein could function, when targeted to either the chloroplast (Schneider *et al.*, 1990) or the nucleus (Van der Krol, unpublished). For improved genetic reporting in non-insect hosts, the luciferase gene was altered (*luc*$^+$, Promega). In the *luc*$^+$ gene the peroxisomal translocation sequence is removed, as well as several restriction sites. Codon usage is improved for expression in 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 however, 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).

The advantages of luciferase as a reporter gene

The very short half-life of the luciferase protein allows the monitoring of rapid changes in gene expression. In the absence of luciferin, the luciferase protein has *in vivo* a half-life of only 3 hrs in mammalian cells (Nguyen *et al.*, 1989) and 2.5 hrs in petunia cells (Chapter 2, Van Leeuwen *et al*., 2000). It is important to realise that GUS reporter activity can only be quantified in an *in vitro* assay. In such *in vitro* GUS measurements changes in gene expression can be measured, but the protein will have a half-life of approximately 150 hours. When LUC activity is quantified in an *in vitro* assay, such changes in gene expression can be measured with a LUC protein half-life of only 2.5 hours. The large advantage of the firefly luciferase as a reporter of promoter activity *in vivo* however, is that (in the absence of CoenzymeA) the protein is used only once, due to the very slow release of oxyluciferin from the luciferase-oxyluciferin complex. Therefore, whenever all its substrates are abundantly available (under substrate equilibrium conditions), firefly luciferase is very suitable to monitor changes in gene expression (each LUC protein molecule is reported by only 1 photon). Especially rapid down-regulation of promoter activity can be monitored with LUC, in contrast to when more stable reporter proteins like GUS and GFP are used. With *in vivo* LUC

measurements rapid down regulation of gene expression can thus be measured within seconds to minutes, only limited by the synthesis rate of the LUC protein, the rate of the luciferase reaction and the sensitivity of the photon-detecting equipment.

The capability to measure spatial distribution and to follow temporal changes

The aforementioned properties of luciferase as a reporter gene, (especially the slow regeneration of the luciferase activity) not only allows accurate quantification of ongoing gene expression, but with *in vivo* imaging of luciferase activity also the spatial distribution as well as the temporal changes of gene expression with a time resolution of seconds can be measured.

In this thesis the spatial and temporal distribution of (trans)gene expression within a plant and between individual transformed lines is described and was shown to vary dramatically for promoters that are active throughout a tissue (*e.g.* the CaMV 35S promoter). To unravel the cause for these variations, we first had to ensure that the variations we measured in luciferase activity were no artefacts of the luciferase reporter system (caused by a variation in any of its substrates). In chapter 2 other possible causes for the variation in luciferase activity than a variation in gene expression are characterised and eliminated. We then could use the luciferase reporter gene to examine position induced quantitative differences in gene expression (caused by the different integration sites of the transgene into the plant genome, known as the position effect; Dean *et al.*, 1988) and determine the contribution of temporal and spatial variation of transgene expression to this position effect.

Different levels of spatial variation

When luciferase activity was imaged in transgenic plants, different levels of spatial variation were observed, illustrated in figure 1. We noticed quantitative differences between different transformants (level I), differences in luciferase activity between leaves of the same plants (level II) and differences in luciferase activity between cells in the same tissue (as shown in a leaf, level III). This variation within a leaf is referred to as variegation. As a final level of variation, not only the *level* of gene expression varies between the leaves (level II), also the (pattern of) variegation (level III) can vary

between these leaves. Furthermore, all these different variations differed between individual transformants; *i.e.* each transformant was differently variegated.

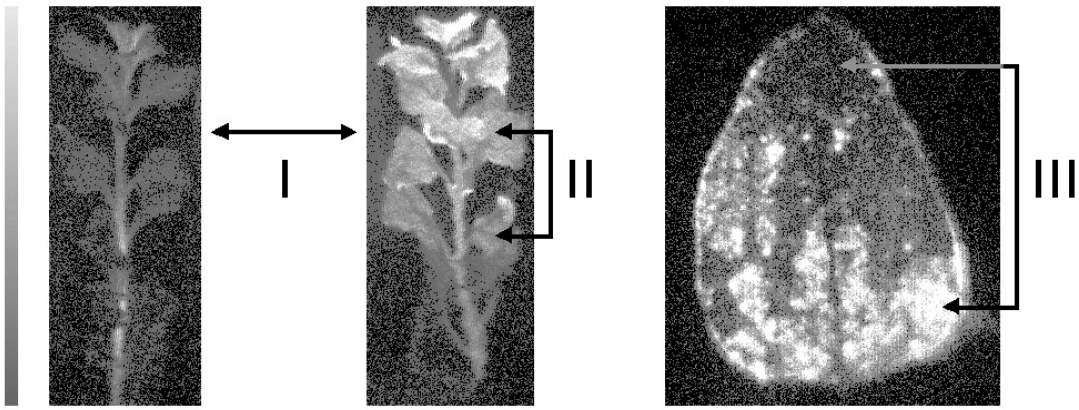


Figure 1. Different levels of spatial variation. Level I: between individual transformants. Level II: between leaves within a transformant. Level III: between cells within a leaf (variegation). Images are shown with a false grey-scale shown on the left (dark grey = low activity, white = high activity).

Variegated gene expression is not noted with stable gene products or large samples

Variations in transgene promoter activity are often not noted, due to the stability of the gene product. The effects of a temporal variation in transgene promoter activity, gene product stability and spatial variation on the quantification of gene expression (either by LUC or more stable reporter genes) are illustrated in the following examples. When a reporter gene product has a very long half life (*e.g.* several days like GUS), temporal changes, occurring within days or even faster, will only lead to minor fluctuations in the accumulated protein pool. When expression varies from day to day (as shown in the representation of a leaf in figure 2, black is gene expression, white is no gene expression) the accumulated result, which will be measured with reporter genes that are stable for weeks, will not show any differences (right leaf, figure 2). Because the luciferase protein is unstable these differences will be noted using the luciferase reporter system. Because ongoing gene expression can be measured with luciferase, even changes within minutes or seconds will be noted with luciferase as a reporter gene.

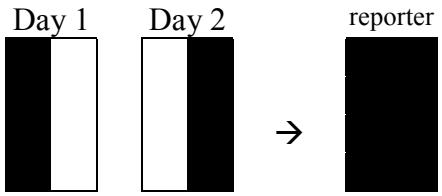


Figure 2. The effect of day-to-day changes in gene expression pattern on the measurement of a stable reporter protein (white = no expression; black = high expression).

Variation in gene expression will also be undetected, when instead of imaging reporter gene activity *in vivo*, whole leaves are taken to isolate and determine mRNA levels. When again several leaves are shown (figure 3), in which white is no gene expression (0 mRNA molecules) and black is high gene expression (*e.g.* 10 mRNA molecules), the quantified mRNA levels will be comparable as is shown below these leaves (figure 3, 5 mRNA molecules). An even distribution of medium gene expression (figure 3, grey in leaf 6, 5 mRNA molecules) will of course result in a comparable quantification of gene expression in this leaf. These spatial differences in figure 3 will thus not be noted in *in vitro* mRNA quantifications, but will be noted in *in vivo* luciferase activity measurements.

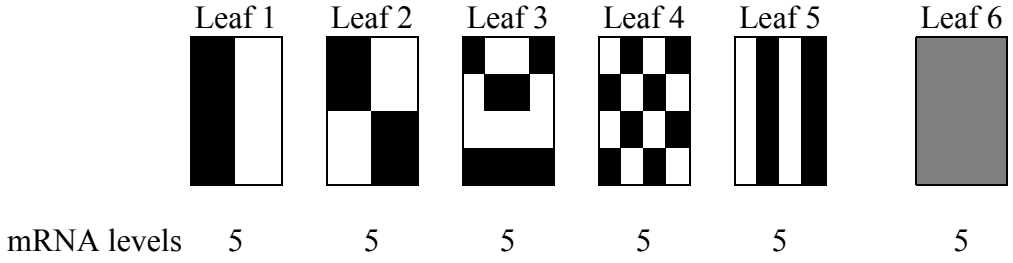
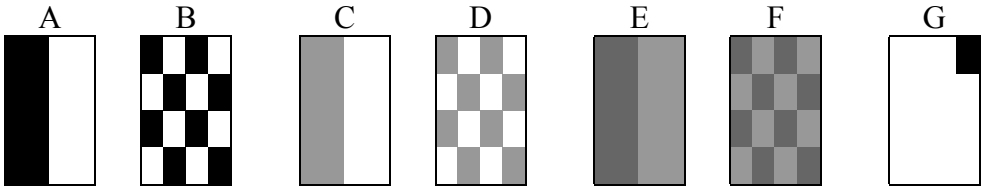


Figure 3. The effect of sample size on quantification of gene expression by mRNA levels. Six different leaves are shown with different levels (white=0; grey=5; black=10) and patterns of gene expression. When the whole leaf is sampled for determination of mRNA levels, each leaf will show an expression level of 5.

Quantification and interpretation of variegation

Variation within a leaf resulting in patterns is referred to as variegation. Variegation can be characterised in different ways. The level of variegation can be indicated by the standard deviation as a measure for the different levels of (luciferase) activity that occur

within a plant. However, the standard deviation in (luciferase) activity cannot be used to compare variegation between different independent transformants, because of differences in the average activity level. When the standard deviation as a percentage of the average activity of the whole leaf (standard deviation/average x100%; coefficient of variation or CV) is used as a measure for variegation, differences in variegation between leaves with different levels of activity can be compared. Both the standard deviation and CV do not give information about how these levels of activity are distributed within the leaf, as is shown in figure 4. The examples shown in figure 4



	A	B	C	D	E	F	G
Average	5	5	2	2	5	5	0.6
StDev.	5	5	2	2	1	1	2.5
CV (%)	100	100	100	100	20	20	417
Max/Avg	2	2	2	2	1.2	1.2	16

Figure 4. The effect of variegation on average activity and standard deviation (StDev.) and different ways to quantify the variegation. CV: Coefficient of Variation = StDev. / average x100%. Max/Avg: Maximum activity / Average activity. Seven different leaves are shown. Panels A-B: activity of white cells =0, black cells =10; C-D: white =0, light grey =4; E-F: light grey =4, dark grey =6; G: black=10.

A-B and E-F all have the same average activity of 5. Leaves A-B have a standard deviation of 5 and thus a CV of 100%, while leaves E-F have a standard deviation of 1 and thus a CV of 20%. Panels C-D indicate that although the standard deviation is lower than in panels A-B, the CV shows that in these panels (A-B and C-D) the variegation is comparable. The difference in variegation between leaves A-D and leaves E-F in figure 4 as quantified by he CV, will also be seen when "the maximum luciferase activity in a leaf compared to the average luciferase activity in a leaf" is used as a measure for variegation. However, with this method (maximum/average or max/avg ratio), the presence of one very high active pixel might significantly increase the calculated variegation (figure 4G), while this would have less effect on the coefficient of variation as a measure for variegation (compare A/B vs. G).

The effect of sample size on the interpretation of variegation

As mentioned above, sampling might obscure spatial differences within a leaf, when whole leaves are taken as a sample as ***e.g.*** shown in figure 3. However, often samples are taken from a leaf (***e.g.*** for GUS activity determinations). When samples of ***e.g.*** a quarter of a leaf are taken of leaves A and B in figure 4, the chance increases in leaf B that two samples with similar activity are taken from one leaf, when gene expression is quantified *in vitro*. Measurement *in vitro* of the activity in a sample of leaf B, will therefore more accurately represent the average activity of the whole leaf than measurement *in vitro* of the activity in a sample of leaf A. If the variegation is reduced by showing more comparable levels within a leaf (as shown in figure 4 in leaves E-F), the sample taken from leaf E will more accurately represent the average activity of the whole leaf, than would have been the case in a sample taken from leaf A.

All these effects of variegation (and the effects of the calculation of the variegation) have to be taken into account when the spatial variation in luciferase gene expression is monitored and quantified. Most of the examples / problems will not be noted when *in vivo* luciferase activity is measured. These problems are however important when levels of luciferase activity are compared with known levels of gene expression (or gene product activity) or when different samples within a leaf are compared (***e.g.*** one sample for protein activity and one sample for mRNA levels). For quantification of the level of variegation, mostly the CV will be used in this thesis, sometimes compared to the max/avg ratio in order to provide a better insight in the variegation. We realise that this will not allow us to distinguish between differences in variegation as shown in figure 3 leaf 1-5 or figure 4 leaf A-B, but this would require complex mathematical calculations, not fitting in the scope of this thesis.

Characterisation of temporal variation

The observed differences in luciferase activity between different leaves of a plant imply that the level of gene expression varies during the development and growth of the plant. When luciferase activity in the same leaf was imaged on different days, we could indeed show that both the level and variegation of luciferase activity differs from day to day (chapter 3). We also noted that there are different types of temporal variation in transgene activity in different lines carrying the same transgene construct. In chapter 4

we examine whether this variation between lines might be limited when a transgene is flanked by matrix-associated regions (MARs), *i.e.* whether the temporal variation does no longer differ between different lines. Three examples of temporal variation of luciferase activity (plotted in successive leaves) are shown in figure 5. Plant A shows little temporal variation, while plant B and C show more temporal variation (the level of gene expression varies more from day to day -or from leaf to leaf-). When different plants show a comparable temporal variation, this means that the relative changes from

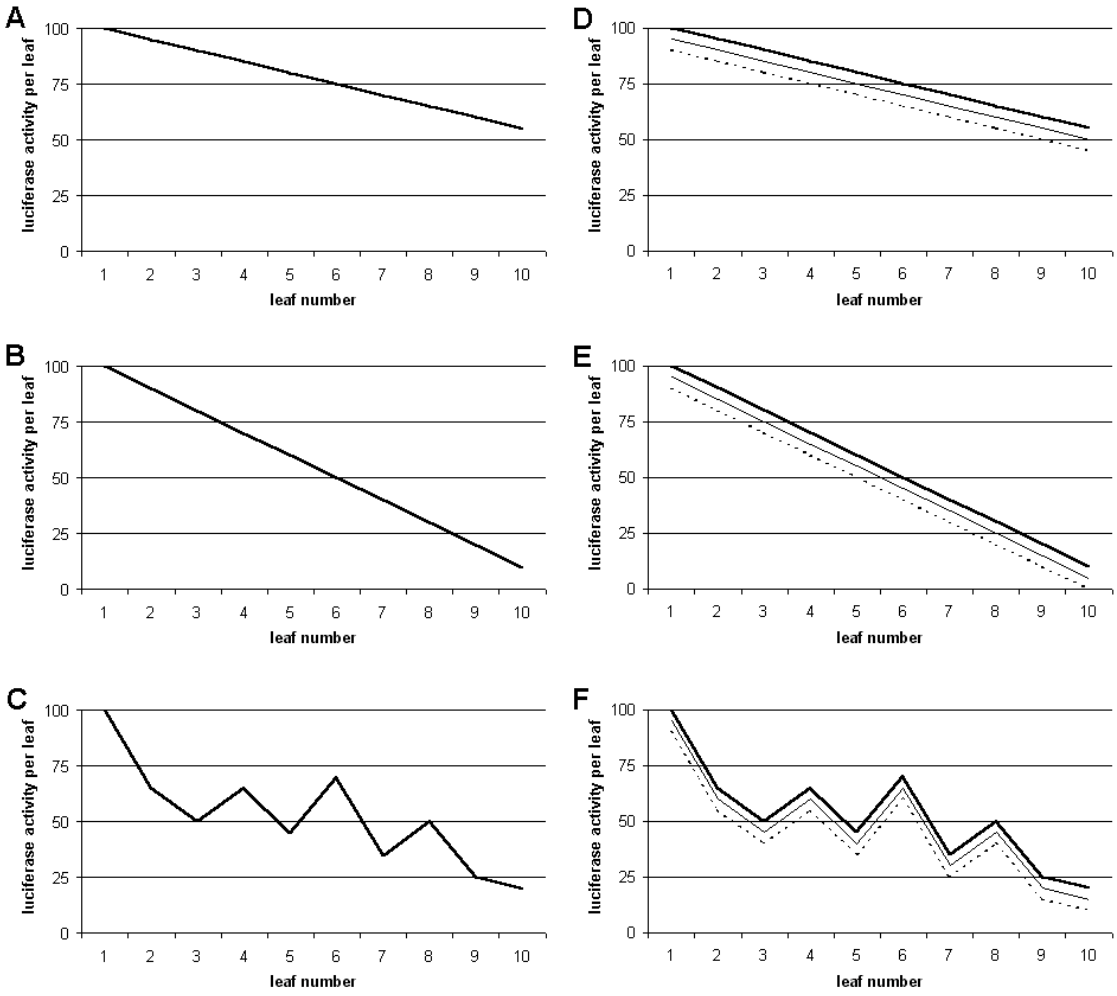


Figure 5. Differences in temporal variation. Luciferase activity per leaf is plotted for 10 successive leaves. Panels A-C show three different plants with a different temporal variation. Panels D-F show three populations (of 3 plants). Within each population, each plant has a comparable temporal variation. This temporal variation however, clearly differs between the populations.

day to day -or from leaf to leaf- are comparable (see three plants in each panel, D-F). This means that the plants within panel D, E and F show mutually less *difference* in temporal variation, but it also means that each plant in panel D still has less temporal variation than each plant in panel E. This figure should be kept in mind when in chapter 4 temporal variation is discussed and especially the difference between more or less temporal variation and more or less difference (or variation) in temporal variation.

Scope of this thesis.

The effect of spatial and temporal variation of transgene expression on the position effect is described in this thesis using firefly luciferase. The possible causes for this phenomenon are discussed.

2 In *chapter 2* the technical aspects of the luciferase reporter system are described. More specifically, the correct use of the luciferase reporter system for *in planta* gene expression studies is investigated. We discuss all the advantages (high spatial and temporal resolution and sensitivity) and disadvantages (three different substrates might be limiting) of luciferase as a reporter system.

3 In *chapter 3* we show that luciferase activity varies between plants, between leaves within a plant, between cells within a leaf and within a leaf between different days. All these variations are transformant specific and thus position induced. It is further shown that the variation of luciferase activity (both between leaves as well as within a leaf) correlates to a variation in luciferase mRNA levels. We conclude that differences in spatial and temporal variation of transgene promoter activity clearly contribute to position induced quantitative differences in transgene expression.

4 In *chapter 4* it is shown that MAR elements do not reduce position induced quantitative differences in luciferase gene expression -although this was shown before for GUS gene expression-, and do not reduce the level of variegation. We speculate whether differences in temporal variation as we can observe with the luciferase reporter system, might contribute to quantitative differences in gene expression levels when a stable reporter like GUS is used. MAR elements might than

change the temporal variation of gene expression (which is invisible when measured on a single time-point with a real-time monitoring reporter gene like luciferase).

5 In *chapter 5* the effect of wounding on luciferase gene expression will be shown as well as the roles different hormones may play in this wound response. This wound response is discussed for the three promoters used in this thesis. These promoters were shown to be wound-responsive, with a specific response per promoter, while furthermore the wound response was characteristic for each individual transformant (*i.e.* there was an additional position induced effect on the wound response). When excised leaves are measured (as *e.g.* in chapter 3) one clearly has to take this wound response into account.

6 In *chapter 6* we analyse the cause for the variegated gene expression and examine whether endogenous genes are also variegated expressed. The possible contributions of variegated levels of transcription factors (TFs), variegated sensitivity of the transgenes to TFs or a variegated hormonal regulation are discussed.

References

Aflalo C (1991) Biologically localized firefly luciferase: A tool to study cellular processes. Int Rev Cytol 130: 267-323.

Benfey PN, Ren L, Chua N-H (1989) The CaMV 35S enhancer contains at least two domains which can confer different developmental and tissue-specific expression patterns. EMBO J 8: 2195-2202.

Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC (1994) Green Fluorescent Protein as a marker for gene expression. Science 263: 802-805.

Dean C, Jones J, Favreau M, Dunsmuir P, Bedbrook J (1988) Influence of flanking sequences on variability in expression levels of an introduced gene in transgenic tobacco plants. Nucl Acids Res 16: 9267-9283.

DeLuca M, McElroy WD (1974) Kinetics of the firefly luciferase catalyzed reactions. Biochem. 13: 921-925.

Denburg JL, Lee RT, McElroy WD (1969) Substrate-binding properties of firefly luciferase. Arch Biochem Biophys 134: 381-394.

DeWet JR, Wood KV, Helinski DR, DeLuca M (1985) Cloning of firefly luciferase cDNA and the expression of active luciferase in *Escherichia coli*. Proc Natl Acad Sci 82: 7870-7873.

DeWet JR, Wood KV, DeLuca M, Helinski DR, Subramani S (1987) Firefly luciferase gene: structure and expression in mammalian cells. Mol and Cell Biol 7 (2): 725-737.

Gilbert W, Muller-Hill B (1967) The lac operator is DNA. Proc Natl Acad Sci 58 (6): 2415-2421.

Green AA, McElroy WD (1956) Crystalline firefly luciferase. Biochim Biophys Acta 20: 170-178.

Groskreutz DJ, Sherf BA, Wood KV, Schenborn ET (1995) Increased expression and convenience with the new pGL3 luciferase reporter vectors. Promega notes 50: 2-8.

Helmer G, Casadaban M, Bevan M, Kayes L, Chilton MD (1984) A new chimeric gene as a marker for plant transformation: the expression of Escherichia coli beta-galactosidase in sunflower and tobacco cells. Bio-Technology 2 (6): 520-527.

Herrera-Estrella L, Leon P, Olsson O, Teeri TH (1988) Reporter genes for plants. In Plant Molecular Biology Manual, Volume B1, Kluwer Academic Publishers: 1-22.

Jefferson RA (1987) The GUS reporter gene system. Nature 342, 837-838.

Koncz C, Langridge WHR, Olsson O, Schell J, Szalay AA (1990) Bacterial and firefly luciferase genes in transgenic plants: advantages and disadvantages of a reporter gene. Dev Genet 11: 224-232.

Lonsdale DM, Moisan LJ, Harvey AJ (1998) The effect of altered codon usage on luciferase activity in tobacco maize and wheat. Plant Cell Rep 17: 396-399.

Nguyen VT, Morange M, Bensaude O (1989) Protein denaturation during heat shock and related stress. J of Biol Chem 264 (18): 10487-10492.

Orth R, Steigert M (1996) Hygiene monitoring - Practical experience in the ATP-bioluminescence measuring method to control hygiene after cleaning of a meat processing plant. Fleishwirtschaft 76 (11): 1143-1144.

Ow DW, Wood KV, DeLuca M, DeWey JR, Helinski DR, Howell SH (1986) Transient and stable expression of the firefly luciferase gene in plant cells and transgenic plants. Proc Natl Acad Sci 84: 4870-4874.

Schneider M, Ow DW, Howell SH (1990) The *in vivo* pattern of firefly luciferase expression in transgenic plants. Plant Mol Biol 14: 935-947.

Sherf BA, Wood KV (1994) Firefly luciferase engineered for improved genetic reporting. Promega notes 49: 14-21.

Toonen MAJ, Verhees JA, Schmidt EDL, van Kammen A, de Vries SC (1997) *AtLTP1* luciferase expression during carrot somatic embryogenesis. Plant J 12: 1213-1221.

Van der Krol AR, Brunelle A, Tsuchimoto S, Chua N-H (1993) Functional analysis of petunia floral homeotic MADSbox gene pMADS1. Genes and Dev 7: 1214-1228.

Van Leeuwen W, Hagendoorn MJM, Ruttink T, Van Poecke R, Van Der Plas LHW, Van Der Krol AR (2000) The use of the luciferase reporter system for in planta gene expression studies. Plant Mol Biol Reptr 18 (2): 143a-143t.

2

The use of the luciferase reporter system for *in planta* gene expression studies

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Published in Plant Molecular Biology Reporter (2000), 18 (2): 143a-143t
Full text (in colour): http://www.nrc.ca/cisti/journals/ispmb/ispmb18/R00-022.pdf

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 or 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 of the substrates to the variegated *in planta* luciferase activity and 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.

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 luciferase (LUC) by DeWet *et al*. in 1985, the luciferase gene has been expressed in plants (tobacco and carrot, Ow *et al.*, 1986), mammalian cells (DeWet *et al.*, 1987) as well as in *e.g.* 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 was removed (*luc*$^{+}$, Promega, 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 (trans)gene expression.

$$\text{LUC} + \text{luciferin} + \text{Mg ATP} \leftrightarrow (\text{LUC} \bullet \text{luciferin-AMP}) + \text{MgPP}_I$$

$$(\text{LUC} \bullet \text{luciferin-AMP}) + O_2 \rightarrow (\text{LUC} \bullet \text{oxyluciferin}^* \bullet \text{AMP}) + CO_2$$

$$(\text{LUC} \bullet \text{oxyluciferin}^* \bullet \text{AMP}) \rightarrow (\text{LUC} \bullet \text{oxyluciferin} \bullet \text{AMP}) + h\nu$$

$$(\text{LUC} \bullet \text{oxyluciferin}^* \bullet \text{AMP}) \leftrightarrow \text{LUC} + \text{oxyluciferin} + \text{AMP}$$

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 oxyluciferin 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.

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 (trans)gene 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 (trans)gene 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 *e.g.* 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 was used (*luc*$^+$, Promega, 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 of 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 days (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 two hours 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 Instruments, 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 minutes. Images of luciferase activity are depicted with false 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 micro-titerplate 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 five seconds after a 10 seconds 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 two seconds (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:

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

Oxygen determination in a 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 seconds, without introduction of air bubbles in the mixture. An imprint of the leaf should be made within two minutes, after which two additional minutes are required for polymerisation. By making two silicone-rubber imprints on the same location, a cleaner imprint can be acquired. Approximately 3 gram 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 three minutes 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.

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.

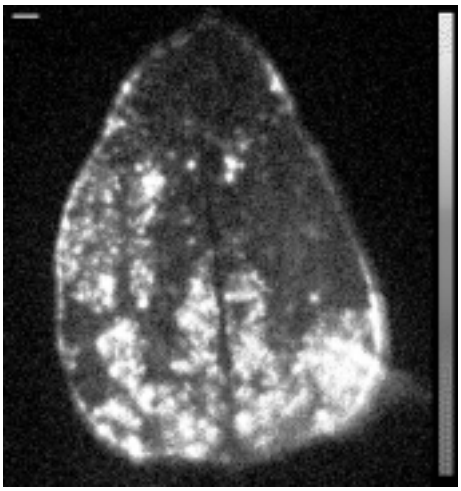

Figure 2. A 35S-*luc Petunia* leaf showing variegated patterns of light emission (luciferase activity measured for 5 minutes). Scale on the right indicates the grey scale used to represent the luciferase activity. The size of the leaf is approximately 13 by 18 mm (bar is 1 mm).

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, 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 hours, results in an increase of luciferase activity at 0 and 7 hours, but has almost no effect at 20 and 30 hours. The panels inserted in figure 3 show the two branches at t=22h (panel A) and t=36h (panel B). With both types of luciferin application, a similar variegated pattern of *in planta* luciferase activity emerges. When after 55 hours 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 hours 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 an equilibrium between luciferase activity and luciferin inflow is reached. Intermittent spraying of luciferin twice a day is sufficient to keep the luciferase

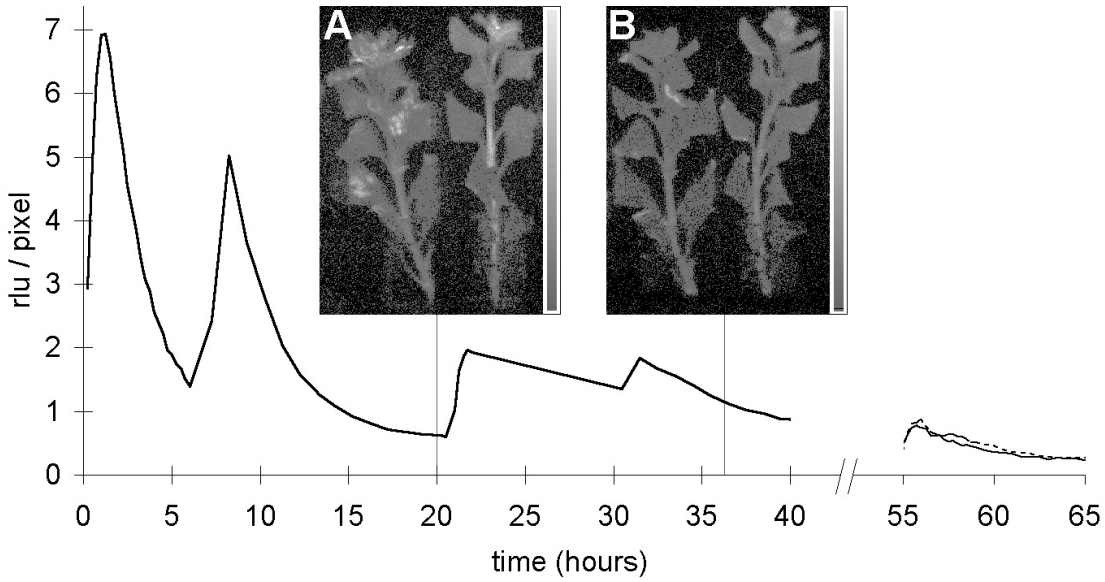

Figure 3. Luciferase activity of a 35S-*luc Petunia* branch as measured in 15 minutes (rlu / pixel) plotted against time (hours). The measurement is continuously repeated for 40 hours. The branch is put in water at t=0h and sprayed at t=0h, t=7h, t=20h and t=30h with a 1 mM luciferin 0.01%Tween-80 solution. Another branch from the same 35S-*luc Petunia* plant is put in 1 mM luciferin at t=0h. The luciferase activity reaches the same equilibrium after 20 to 30 hours as is shown in the inserted panel A (22 hours) and panel B (36 hours). In panels A and B, the left branch is put in 1 mM luciferin at t=0h, the right branch is the branch described above (put in water at t=0h and sprayed with luciferin). Scale on the right in panels A and B indicates the grey scale used to represent the luciferase activity. After 55 hours both branches are sprayed with a 1 mM luciferin 0.01% Tween-80 solution and measured for 10 hours. The black line represents the luciferase activity in the branch put on water at t=0h, the dashed line represents the luciferase activity in the branch put on 1 mM luciferin at t=0h.

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 days 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 (see *e.g.* 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 days-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 minutes and is immediately restored after re-exposure to air (data not shown). By measurement of luciferase activity in a *Petunia* cell suspension at different levels of oxygenation, the dependence on oxygen of the luciferase reaction is illustrated (figure 4A).

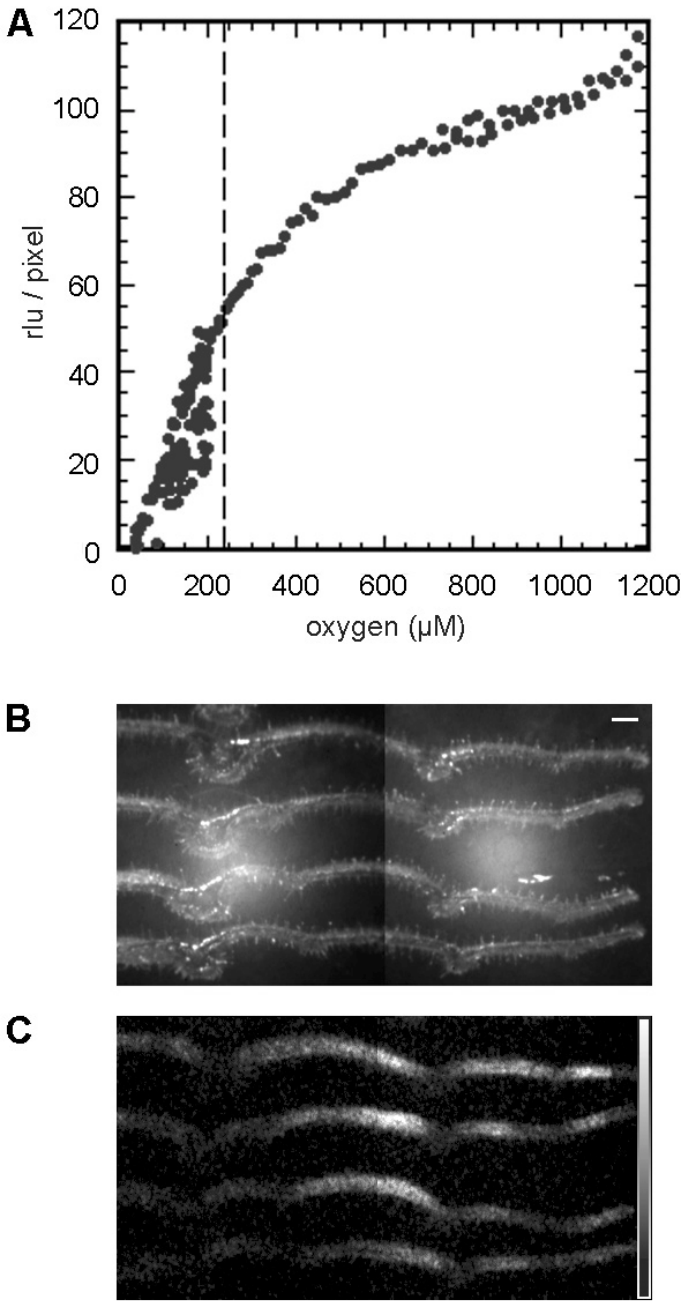

Figure 4. panel A. Oxygen dependence of luciferase mediated light production in a 35S-*luc Petunia* cell suspension. Plotted are relative light units (rlu) per pixel versus oxygen concentration (µM). The dashed line shows the maximum level of oxygen when the suspension is oxygenated with air. Panel B. 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 grey scale used to represent the luciferase activity.

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 were either kept under greenhouse conditions for 24 hours, kept in the dark for 24 hours or treated with 10 μM ABA (sprayed three times in 24 hours). 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).

	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)

Table 1. The effects of stomatal aperture on the level of luciferase activity in 35S-*luc* *Petunia* plants. Three genetically identical plants with comparable luciferase activity were kept under greenhouse conditions (light), kept in the dark (dark) or treated with 10 μM ABA (ABA) for 24 hours. The average luciferase activity in the first expanded leaf after 24 hours 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).

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

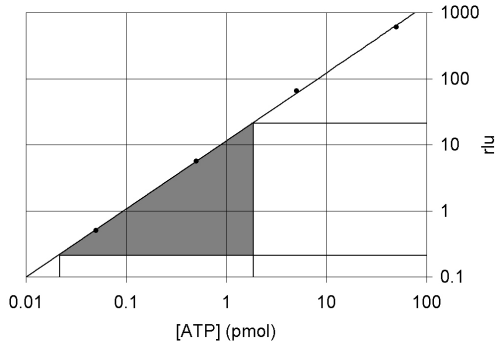

Figure 5. ATP dependence of the luciferase reaction as determined in an *in vitro* luciferase flash assay. Plotted are the relative light units (rlu) versus the ATP levels added (pmol). Points are means of duplicate determinations. The linear regression line has an R^2 of 0.999. The grey area shows the range of rlu we in general obtain in luciferase extracts from transgenic leaves and the corresponding picomoles of ATP required.

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). 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 five minutes. 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 five minutes. The measured light production (of the luciferase activity in the leaf) dropped in the covered leaf to approximately 20-25% of the light production of

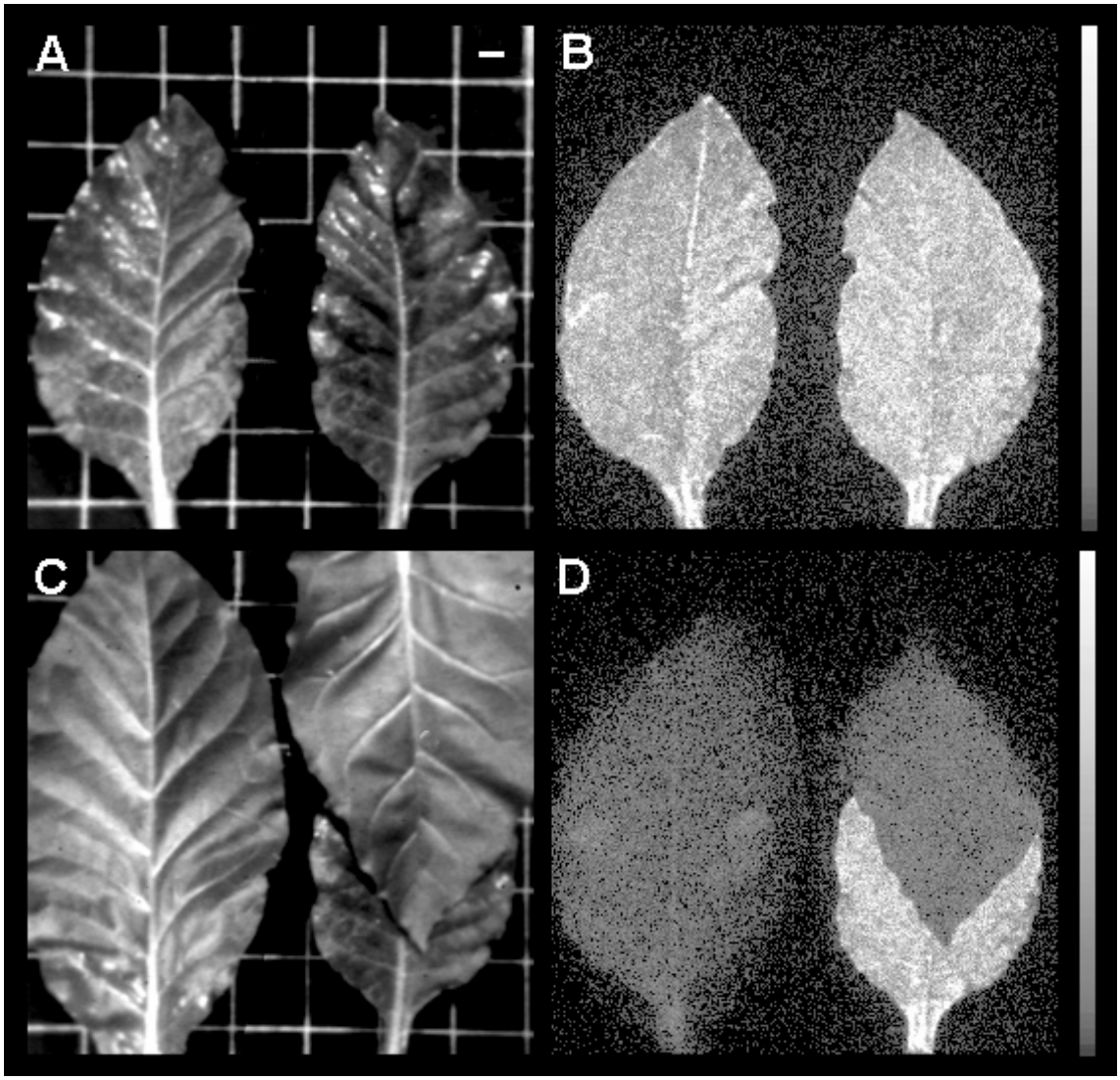

Figure 6. Penetration of the light emitted by luciferase from two 35S-*luc* tobacco leaves. Panel A shows these leaves (bar represents 1 cm). Panel B shows the acquired photon image of five minutes measurement of the leaves in panel A. Scale on the right indicates the grey scale used to represent the 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 five minutes measurement of the leaves as shown in panel C. Scale on the right indicates the grey scale used to represent the luciferase activity.

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 *e.g.* 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 *e.g.* 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

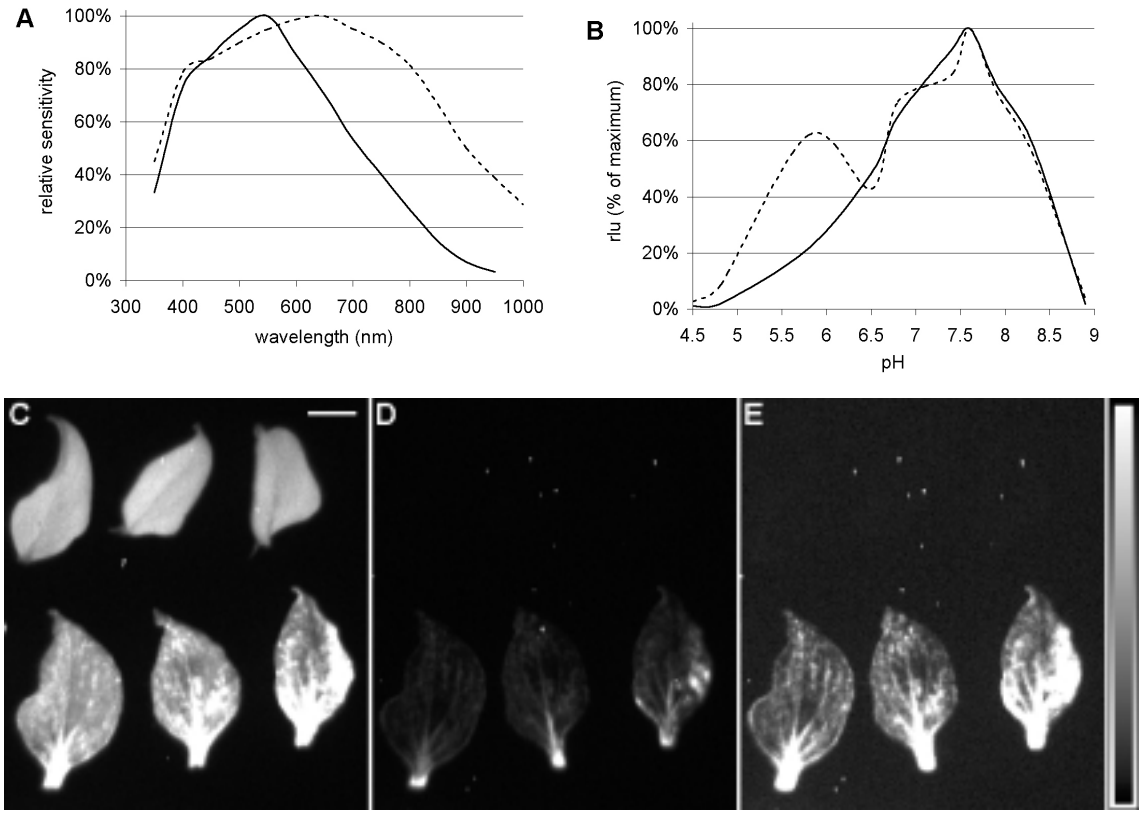

Figure 7. The effect of "red-shift" of the light emission by the luciferase due to pH differences. Panel A shows the drop in sensitivity of an intensified luminometer (black line) towards the red part of the spectrum (above a wavelength of 650 nm), compared to the sensitivity of a cooled slow-scan luminometer (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 luminescece of chlorophyll in three wild-type *Petunia* leaves (top) and three 35S-*luc* transgenic *Petunia* leaves (bottom), measured with a cooled slow-scan CCD camera for five minutes, 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.

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 five minutes, 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 five to 10 minutes, 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 minutes 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 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 • 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 five minutes. Light emission was measured for 2 seconds, directly before addition (t=5 min) and two minutes after addition (t=7 min). In a control flash reaction without extra additions (column 1) light production after five to seven minutes 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).

	Addition at t=0min						
	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=5min						
	---	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%

Table 2. Regeneration of luciferase activity by Coenzyme A after a flash assay. 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 seconds) of six determinations is shown at t=0 min (the initial flash set to 100%), t=5 min (directly before addition of the different buffers) and t=7 min (two 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 seconds) 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.

These experiments show that CoA needs to be present during the Luciferase-Luciferin reaction and does not release luciferase from the Luciferase • Oxyluciferin complex. Five minutes 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 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%, five minutes 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 minutes (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 (*e.g.* without a poly-A tail the LUC mRNA half life is 24 minutes, Gallie *et al*., 1991).

It was shown before that the half life of LUC protein in mammalian cells was approximately 200 minutes (Nguyen *et al*., 1989 and 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 the ratio of rlu relative to 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

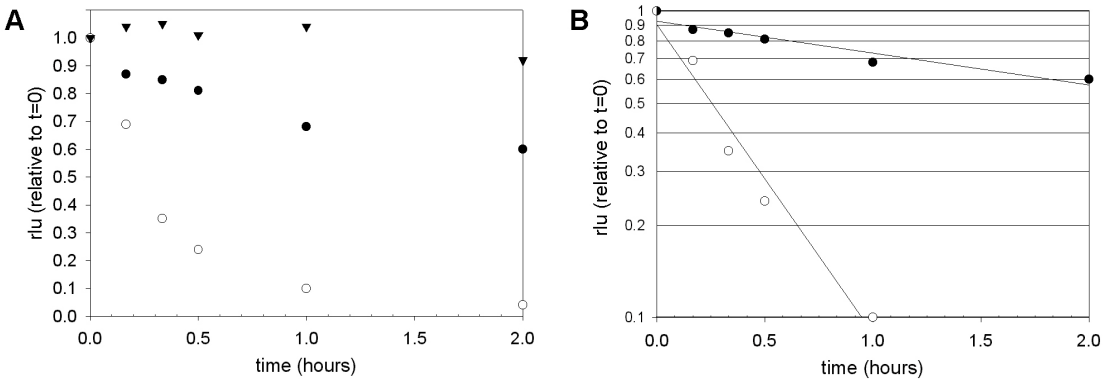

Figure 8. Half life of luciferase and luciferase activity *in vitro* in extracts of *Petunia* cell suspensions. In panel A the average luciferase activity of a *Petunia* 35S-*luc* cell suspension as determined in extracts in an *in vitro* flash assay is shown, plotted as a percentage of luciferase activity at t= 0 min Samples were taken at t=0, 10, 20, 30, 60 and 120 minutes 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 minutes (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 minutes (R^2 = 0.971).

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 minutes, but the apparent half life of luciferase activity when luciferin is added is 10 times faster (*i.e.* 15.3 minutes).

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 plants when these plants are three-times pre-sprayed 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

We would like to thank Dorien Postma-Haarsma and René Kuijpers for creating the 35S-*luc* and m35S-*luc* constructs used to transform *Petunia*, Ludmilla Mlynárová for cloning of the 35S-*luc* construct and transformation of the tobacco plants, Diaan Jamar for creation and maintenance of the *Petunia* cell suspensions and Ria van den Noort for technical assistance with the determination of stomatal aperture. The 35S-*luc* tobacco plants were kindly provided by Dr. Jan-Peter Nap. This work is financially supported by the Dutch Organisation for Scientific Research (NWO).

References

Aflalo C (1991) Biologically localized firefly luciferase: A tool to study cellular processes. Int Rev Cytol 130: 267-323.

Benfey PN, Ren L, Chua, N-H (1989) The CaMV 35S enhancer contains at least two domains which can confer different developmental and tissue-specific expression patterns. EMBO J 8: 2195-2202.

Brandes C, Plautz JD, Stanewsky R, Jamison CF, Straume M, Wood KV, Kay SA, Hall JC (1996) Novel features of Drosophila period transcription revealed by real-time luciferase reporting. Neuron 16 (4): 687-692.

DeLuca M, McElroy WD (1974) Kinetics of the firefly luciferase catalyzed reactions. Biochemistry 13: 921-925.

DeLuca M, McElroy WD (1976) Firefly luciferase. Adv Enzymol 44: 37-68.

Denburg JL, Lee RT, McElroy WD (1969) Substrate-binding properties of firefly luciferase. Arch Biochem Biophys 134: 381-394.

DeWet JR, Wood KV, Helinski DR, DeLuca M (1985) Cloning of firefly luciferase cDNA and the expression of active luciferase in *Escherichia coli*. Proc Natl Acad Sci 82: 7870-7873.

DeWet JR, Wood KV, DeLuca M, Helinski DR, Subramani S (1987) Firefly luciferase gene: structure and expression in mammalian cells. Mol Cell Biol 7 (2): 725-737.

Ford SR, Buck LM, Leach FR (1995) Does the sulfhydryl or the adenine moiety of CoA enhance firefly luciferase activity? Biochim Biophys Acta 1252: 180-184.

Gallie DR, Feder JN, Schimke RT, Walbot V (1991) Post-transcriptional regulation in higher eukaryotes: The role of the reporter gene in controlling expression. Mol Gen Genet 228: 258-264.

Gardner RC, Howarth AJ, Hahn P, Brown-Luedi M, Shepherd RJ, Messing J (1981) The complete nucleotide sequence of an infectious clone of cauliflower mosaic virus by M13mp7 shotgun sequencing. Nucl Acids Res 9: 1871-1880.

Groskreutz DJ, Sherf BA, Wood KV, Schenborn ET (1995) Increased expression and convenience with the new pGL3 luciferase reporter vectors. Promega notes 50: 2-8.

Hideg E, Kobayashi M, Inaba H (1992) Delayed fluorescence and ultraweak light emission from isolated chloroplasts (comparison of emission spectra and concentration dependence). Plant Cell Physiol 33 (6): 689-693.

Laisk A, Oja V, Kull K (1980) Statistical distribution of stomatal apertures of Vicia faba and Hordeum vulgare and the spannungsphase of stomatal opening. J Exp Bot 31: 49-58.

Laisk A (1983) Calculation of leaf photosynthetic parameters considering the statistical distribution of stomatal apertures. J Exp Bot 34: 1627-1635.

Lembert N, Idahl L (1995) Regulatory effects of ATP and luciferin on firefly luciferase activity. Biochem J 305: 929-933.

Lonsdale DM, Moisan LJ, Harvey AJ (1998a) The effect of altered codon usage on luciferase activity in tobacco maize and wheat. Plant Cell Rep 17: 396-399.

Luehrsen KR, Walbot V (1993) Firefly luciferase as a reporter for plant gene expression. Promega notes 44: 24-29.

Malik NSA, Thimann KV (1980) Metabolism of oat leaves during senescence. Changes in ATP levels. Plant Physiol 65: 855-858.

Mayerhofer R, Araki K, Szalay AA (1995) Monitoring of spatial expression of firefly luciferase in transformed zebrafish. J Biolum Chemilum 10 (5): 271-275.

Michels AA, Nguyen VT, Konings AWT, Kampinga HH, Bensaude O (1995) Thermostability of a nuclear-targeted luciferase expressed in mammalian cells. Destabilizing influence of the intranuclear microenvironment. Eur J Biochem 234: 382-389.

Millar AJ, Short SR, Hiratsuka K, Chua N-H, Kay SA (1992) Firefly luciferase as a reporter of regulated gene expression in higher plants. Plant Mol Biol Reptr 10 (4): 324-337.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473-479.

Nguyen VT, Morange M, Bensaude O (1989) Protein denaturation during heat shock and related stress. J Biol Chem 264 (18): 10487-10492.

Ow DW, Wood KV, DeLuca M, DeWey JR Helinski DR, Howell SH (1986) Transient and stable expression of the firefly luciferase gene in plant cells and transgenic plants. Proc Natl Acad Sci 84: 4870-4874.

Quandt H-J, Broer I, Pühler A (1992) Tissue-specific activity and light-dependent regulation of a soybean *rbcS* promoter in transgenic tobacco plants monitored with the firefly luciferase gene. Plant Sci 82: 59-70.

Schneider M, Ow DW, Howell SH (1990) The *in vivo* pattern of firefly luciferase expression in transgenic plants. Plant Mol Biol 14: 935-947.

Selinger HH, McElroy WD (1964) The colors of firefly luminescence: enzyme configuration and species specifity. Proc Natl Acad Sci 52: 75-81.

Sherf BA, Wood KV (1994) Firefly luciferase engineered for improved genetic reporting. Promega notes 49: 14-21.

Thompson JF, Hayes LS, Lloyd DB (1991) Modulation of firefly luciferase stability and impact on studies of gene regulation. Gene 103: 171-177.

Toonen MAJ, Verhees JA, Schmidt EDL, van Kammen A, de Vries SC (1997) *AtLTP1* luciferase expression during carrot somatic embryogenesis. Plant J 12 (5): 1213-1221.

Van der Krol AR, Chua N-H (1991) The basic domain of plant B-Zip proteins facilitates import of a reporter protein into plant nuclei. Plant Cell 3: 667-675.

Van der Krol AR, Brunelle A, Tsuchimoto S, Chua N-H (1993) Functional analysis of petunia floral homeotic MADSbox gene pMADS1. Genes and Development 7: 1214-1228.

3

Characterisation of position induced spatial and temporal regulation of transgene promoter activity in plants

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Journal of Experimental Botany (2001), in press

Abstract. Quantitative differences in transgene expression between independent transformants generally are ascribed to different integration sites of the transgene (position effect). We characterised the contribution of spatial and temporal changes in transgene promoter activity to these position induced differences in transgene expression *in planta,* using the firefly luciferase (*luc*) reporter system. The activity of three different promoters (Cauliflower Mosaic Virus (CaMV) 35S, modified CaMV 35S and the promoter of an *Arabidopsis thaliana* Lipid Transfer Protein gene) was shown to vary not only among independent transformants, but also between leaves on the same plant and within a leaf. The differences in local LUC activity between leaves and within a leaf correlated with differences in local *luc* mRNA steady state levels. Imaging of LUC activity in the same leaves over a 50-day period, shows that individual transformants can show different types of temporal regulation. Both the spatial and the temporal type of *luc* transgene expression pattern are inherited to the next generation. We conclude that previously reported position induced quantitative differences in transgene expression are probably an accumulated effect of differences in spatial and temporal regulation of transgene promoter activity.

Introduction

The expression of plant genes is usually characterised by quantifying mRNA or protein steady state levels in different tissues. Gene expression analysis was greatly facilitated by the use of plant transformation techniques and the introduction of reporter genes like Chloramphenicol Acetyl Transferase (*CAT*, Gorman *et al*., 1982), ß-Glucuronidase (*GUS*, Jefferson *et al*., 1987), Green Fluorescent Protein (*GFP*, Niedz *et al*., 1995) and firefly luciferase (*luc*, Ow *et al.*, 1986). However, it has been shown that the level of transgene expression varies among individual transformants with the same transgene copy number. Apparently this quantitative variation depends on the site of integration and it is referred to as the 'position effect' (Blundy *et al*., 1991; Dean *et al*., 1988; Mlynárová *et al.*, 1994; Peach and Velten, 1991). When the character of the promoter driving the transgene expression is known (*e.g.* its tissue specificity), often no detailed information is available on the distribution of transgene expression throughout a plant, the distribution of transgene activity within a tissue, the distribution of transgene activity in the same tissue over prolonged periods of time, or possible differences in these distributions between independent transformants. There are several possible origins of the position dependent quantitative differences in transgene expression. Independent transgenic lines can show (1) differences in the level of promoter activity, but the same spatial and / or developmental regulation or (2) the same level of promoter activity, but differences in spatial and / or developmental regulation or (3) a combination of these two possibilities. With the introduction of the firefly *luc* reporter gene all these aspects of transgene expression can now be imaged *in planta* (Gould and Subramani, 1988).

The *luc* gene encodes a protein that catalyses the oxidative decarboxylation of firefly luciferin using Mg^{2+}-ATP and oxygen. A photon (562 nm) is released in 90% of the catalytic cycles (DeLuca and McElroy, 1974). The substrate luciferin is an amphipathic molecule that easily penetrates most plant tissues. Therefore a transgenic *luc* plant, sprayed with luciferin will emit photons where and when a *luc* reporter gene is active. These photons can be visualised with a sensitive CCD camera (2D-luminometer). The *luc* transgene expression can be monitored *in vivo* in the same tissue throughout plant development and under different physiological conditions. The LUC enzyme activity is

only very slowly regenerated after reacting with the substrates, because the product of the reaction, oxyluciferin, is only very slowly released from the AMP-oxyluciferin-luciferase complex (Denburg *et al*., 1969). Therefore, after pre-incubation with luciferin, continuous light production *in vivo* is mostly caused by newly synthesised LUC and not by previously accumulated LUC. Under these conditions, luciferase activity is closely related to the promoter activity of the reporter gene. This feature allows for the identification of changes in *luc* transgene activity within a tissue, enhancing the temporal resolution of the gene expression study. An extensive report on the features of luciferase activity measurements *in planta* has recently been published (Van Leeuwen *et al*., 2000).

Here, we have compared the spatial and temporal aspects of gene expression among individual transgenic lines, carrying the same *luc* reporter gene construct. For these studies we used the Cauliflower Mosaic Virus (CaMV) 35S promoter, a modified CaMV 35S promoter (m35S) and the promoter of an *A. thaliana* Lipid Transfer Protein gene (*LTP1*; Thoma *et al.*, 1994) to drive *luc* expression in transgenic *Petunia hybrida* (Vilm.) plants. The CaMV 35S promoter is often used as a 'constitutively active' promoter for ectopic expression of foreign genes (Benfey *et al.*, 1989). The m35S promoter was designed to increase the level of transgene expression by optimisation and multimerisation of DNA binding-sites within the CaMV 35S promoter and has been used for ectopic expression of floral homeotic genes (van der Krol *et al.*, 1993). The LTP promoter has been shown to be active in the L1-layer, both in *Arabidopsis* (Thoma *et al.*, 1994) as well as in *Daucus carota* (Toonen *et al.*, 1997).

Our analyses show for all three *luc* reporter gene constructs, (1) that the LUC activity is variegated, occasionally showing a more than 100-fold difference within a leaf tissue, (2) that the type of variegated LUC activity differs between transformants carrying the same reporter construct, indicating that the pattern of variegation is not related to the developmental stages of the cells within a leaf, (3) that a different temporal regulation might occur in different transformants carrying the same transgene, and (4) that the variegated LUC activity correlates with variegated *luc* mRNA steady state levels. The differences in variegation and in temporal regulation of transgene promoter activity, can account for the previously reported position dependent quantitative differences in transgene expression (Dean *et al*., 1988). We speculate on the factors contributing to

this variegated transgene promoter activity and discuss the implications for gene expression studies.

Materials and Methods

Introduction of luc reporter gene constructs in Petunia hybrida *plants*

Agrobacterium tumefaciens (*A. tum.* strain ABI) was transformed with the binary vector pMON721 containing one of the following gene constructs:

CaMV 35S promoter - *luc* (pGM46)

CaMV m35S promoter - *luc*$^+$ (pGM107)

LTP promoter - *luc* (pMT520)

The CaMV promoter used in our constructs consists of the -343 to +8 sequence (Benfey *et al.*, 1989; Gardner *et al*., 1981). 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 luciferase gene that is used in the pGM46 and in the pMT520 construct is the original luciferase coding sequence cloned by deWet *et al*. (1985). For the pGM107 construct a modified firefly luciferase gene was used (*luc*$^+$, without the peroxisomal protein import signal, Promega, Madison, WI, USA, Sherf and Wood, 1994), which shows increased expression in plant cells (Lonsdale *et al*., 1998). In the pGM46 and pGM107 constructs an N-terminal SV40 Nuclear Localisation Signal (NLS) was present in front of the *luc* coding sequence, which had no apparent effect on its activity (van der Krol and Chua, 1991). *Petunia hybrida* (Vilm.) plants (cv. V26) were transformed by *A. tum.* clones containing either pGM46, pGM107 or pMT520, and grown on Murashige and Skoog (1962) agar plates containing selective antibiotics (100 µg mL^{-1} kanamycin to select for the transformed shoots). Transformed shoots were, after rooting, transferred to soil and grown in growth chambers with a 16 h light period (50 W m^{-2}, 22°C, and 70% RH) and an 8 h dark period (20°C, and 65% RH). For the analyses of the pGM46 transformed plants, the F_1 progeny plants of a back-cross with wild-type V26 were used. These plants are coded as: 35S-"primary transformant code"b"F_1 progeny code" (*e.g.* 35S-1b4).

In vivo *luciferase activity measurement with the 2D-luminometer*

Petunia *luc* reporter plants were sprayed with a luciferin solution (1 mM firefly D-luciferin, sodium-salt, Molecular Probes, Eugene, OR, USA, 0.01% Tween 80) 48h, 40h, 24 h, 16 h and 2 h before measurement as described in van Leeuwen *et al.* (2000). Plants were analysed by measuring the excised leaves of the main shoot. Plants or excised leaves were imaged with a 2D-luminometer, consisting of an intensified CCD camera (C2400-77, Hamamatsu Photonics, Japan). Photon emission by *luc*-expressing plants was quantified by computer (shown as

relative light units per pixel (rlu pixel^{-1}), Argus-50 Image Processor, Hamamatsu Photonics, Japan) and depicted with false grey scales (dark grey indicating low activity, white indicating high activity). Integration intervals varied from 5 to 30 min.

RNA isolation

Petunia leaf material was ground in a liquid nitrogen cooled 2.2 mL microtube containing two 0.25 inch vanadium bullets in a Braun Biotech Micro-dismembrator for 90 s at 1600 rpm. Subsequently, 300 µL RNA extraction buffer (4M guanidinthiocyanate (GuSCN), 25 mM sodium citrate, 0.5% lauroyl sarcosine) per 100 mg sample was added and the samples were thawed on ice. After addition of 0.1 volume 2M NaAc, 1 volume acidic phenol and 0.2 volume chloroform / isoamylalcohol (24:1), the mixture was vortexed vigorously for at least 1 min and, subsequently put on ice for 5-15 min. After vortexing the mixture was centrifuged for 20 min at *16,000 g* at 4°C. RNaid MATRIX glass beads (BIO 101 inc., Carlsbad, CA, USA) were, subsequently added to the aqueous phase (20 µL per 100 mg leaf material) and mixed and incubated for 10-15 min at RT. After 30 s centrifugation at *16,000 g* at RT, the pelleted glass beads were resuspended in 1 mL 6M GuSCN. This step was repeated and the beads were again centrifuged for 30 s at *16,000 g* at RT. The pellet was now washed 2-3 times with 0.5-0.8 mL 60% ethanol-$T_{10}E_1$ by resuspending and centrifuging. The pellet was resuspended in RNase free water in a volume equal to the original volume of beads added and incubated at 60-65°C for 5 min to wash the RNA from the RNaid beads. The mixture was centrifuged for 2 min at maximum speed and the supernatant was again centrifuged. These two steps were repeated to elute another 10% of the RNA from the beads. The RNA was quantified in a GeneQuant RNA/DNA Calculator (Pharmacia, Peapack, NJ, USA / LKB Biochrom Ltd. model 80-2103-98) and stored at -80°C.

Reverse transcriptase PCR and hybridisation

Ten microgram total RNA was DNase treated in 60 µL with 2U DNase (Boehringer-Mannheim, Germany) and 20 U RNAsin (Gibco BRL, Paisley, UK) (Sambrook *et al*., 1989). One microgram was again quantified with the GeneQuant as well as on 1.5% agarose formaldehyde gel. First strand cDNA was then synthesised of 2.5 µg RNA using reverse transcriptase with Oligo(dT) primers (Superscript™ Preamplification System, Gibco BRL, Paisley, UK). Two µL of the obtained 20 µL was then used in a PCR, using ubiquitin specific primers (UBIQ-f and UBIQ-r, Geurts *et al*., 1997). When the ubiquitin PCR showed comparable levels of total RNA as determined after 25 cycles on a 1.0% w/v agarose (Ethidium Bromide stained, 150 µg L^{-1}) 1x TAE gel, two µL of the cDNA was used in a PCR using *luc* specific primers. Of each *luc* PCR a 5 µL sample was taken after 16, 18, 20, 22 and 24 cycles. Five µL of the *luc* samples was size-fractionated by electrophoresis through a 1.0% w/v agarose gel and transferred to positively charged nylon membrane according to manufacturers instructions (Genescreen Plus, NEN™ Life Science Products, Boston, MA, USA). Blots were pre-hybridised in 1% w/v BSA, 1 mM EDTA, 0.5 M $NaHPO_4$, pH 7.2, 7% SDS at 60°C for 90 min. Hybridisation was carried out in the same mixture in the hybridisation oven at 60°C for 16 h, after addition of approximately 100

ng $\gamma[^{32}P]$dATP (Amersham, Didcot, UK) radiolabeled probe prepared by random priming (Boehringer-Mannheim, Germany) of gel-purified DNA or PCR-products. Filters were washed with 2x SSC, 0.1% SDS at 60°C and exposed to Kodak X-Omat AR films at -70°C with intensifying screens or exposed to a Molecular Dynamics Phosphor Screen and subsequently, scanned with a Phosphor Imager (Molecular Dynamics, Sunnyvale, CA, USA). Intensity of the bands was quantified with the ImageQuant program (Molecular Dynamics, Sunnyvale, CA, USA).

Primers used for RT-PCR

Ubiquitin primers:	UBIQ-f:	ATG CAG ATY TTT GTG AAG AC
	UBIQ-r:	ACC ACC ACG RAG ACG GAG
Luciferase primers:	SK333:	ATG GAA GAC GCC AAA AAC ATA AAG
	SK305:	GGC GGA TCC TAT ATG AGG ATC TCT CTG ATT TTT C

Results and Discussion

Differences in the level of luciferase activity between independent transgenic lines

Petunia hybrida (Vilm.) plants (cv. V26) were transformed by *Agrobacterium* with a luciferase (*luc*) reporter gene, driven by either the viral CaMV 35S promoter, a modified version of the CaMV 35S-promoter (m35S), or the *A. thaliana* Lipid Transfer Protein (LTP) promoter. For each of the chimeric genes, several independent transformants were obtained which contained one to eight copies of the transgene (as determined by Southern analysis, data not shown).

For each *luc* reporter gene construct, the individual transformants were analysed for *in planta* LUC activity in leaf tissue, after equilibration with luciferin. The average LUC activity per petunia plant was calculated by quantifying LUC activity in excised, fully expanded leaves 5 through 11 of the main shoot (numbering starting at the first visible leaf at the apex). For the CaMV 35S promoter the average transgene activity in leaves (averaged per total leaf area) varied between plants from 0.1 to 61 rlu pixel^{-1}, for the m35S promoter the transgene activity varied from 0.1 to 51 rlu pixel^{-1} and for the LTP promoter from 0.5 to 27 rlu pixel^{-1}. Relative differences between single copy transformants were as large as between multiple copy transformants (*e.g.* in seven single copy m35S plants LUC activity varied from 0.3 to 51, while six m35S plants with 3 copies showed LUC activity varying between 0.1 and 11). These results confirm previous reports, which show that the level of expression of a transgene can vary among independent transformants (Dean *et al*., 1988).

Differences in the level of luciferase activity between leaves on the same plant

For each *luc* reporter gene construct, we quantified the LUC activity in individual excised, fully expanded leaves from the main shoot of three independent single locus transgenic lines. Figure 1 shows the average LUC activity per leaf measured in 30 min in leaves 5 through 11 (rlu pixel^{-1}), in three single locus plants. In general, the LUC activity decreases upon ageing of the leaf. However, in *e.g.* line 35S-2b10, line m35S-6 and line LTP-7, the highest average LUC activity occurs in leaf 9, leaf 9 and leaf 10, respectively, instead of in leaf 5. The differences in LUC activity between the leaves of

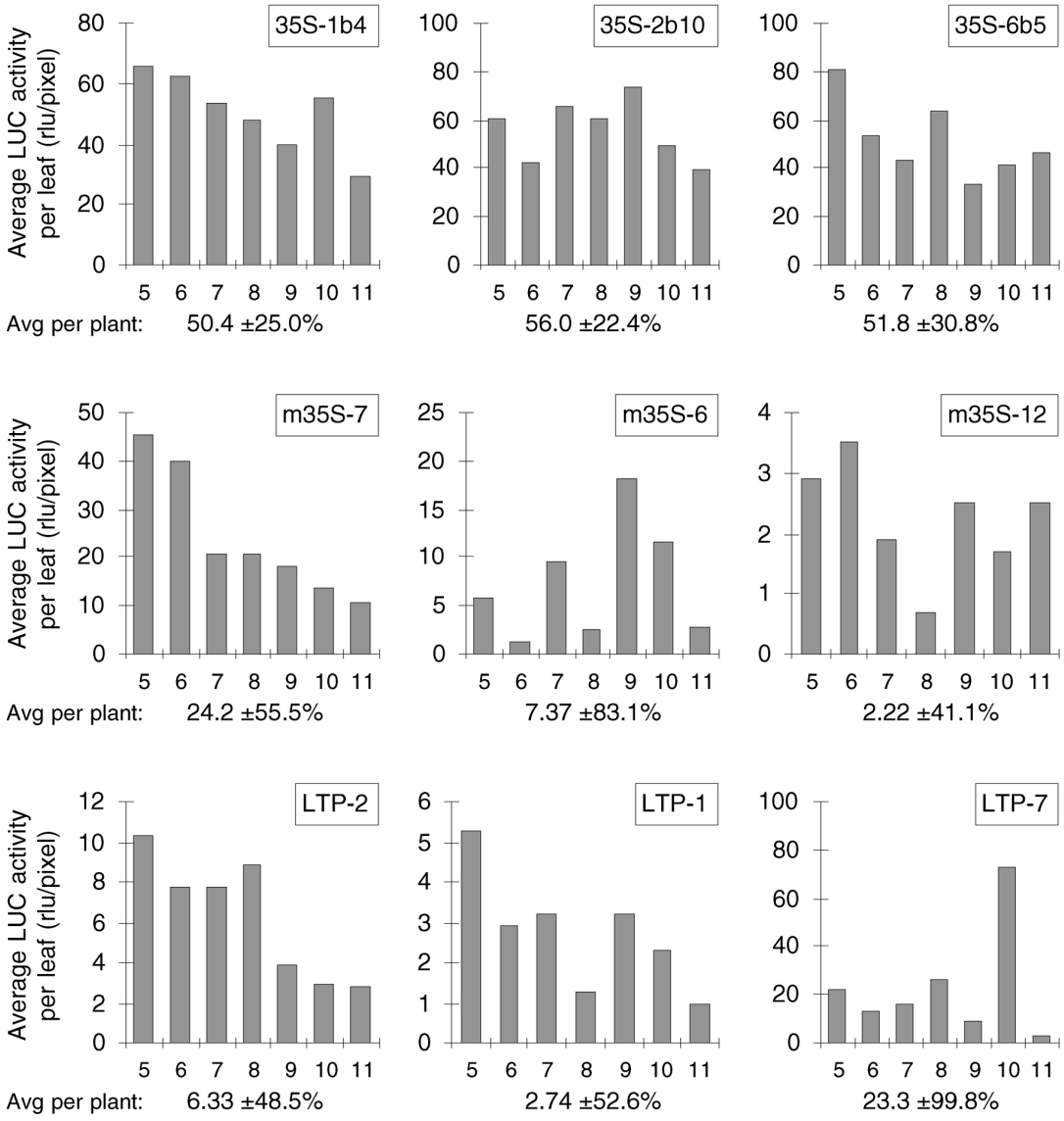

Figure 1. The average luciferase activity per leaf measured in 30 min (rlu pixel^{-1}) of excised, fully expanded leaves 5-11 of different primary petunia transformants. Three independent single locus transformants of each promoter-*luc* construct are shown. The leaf number is shown on the x-axis. The average LUC activity per plant (avg) is shown below the graphs (rlu pixel^{-1}) with the CV (%). The code of the transformant is shown in each graph.

a single plant can range from a factor 1.9 (in line 35S-2b10) to a factor 22 (in line LTP-7). The variation in average expression level of a plant can be characterised by the coefficient of variation (CV = [standard deviation / average] x 100%) of the average LUC activity in leaves 5 to 11. The average LUC activity and CV are shown for each individual transformant below each graph in figure 1. The CV for these seven

subsequent leaves in all analysed plants varies per plant from 17 to 49 % in the 35S-*luc* plants (n=12), from 34 to 169 % in the m35S-*luc* plants (n=16) and from 23 to 100 % in the LTP-*luc* plants (n=8).

Although there are differences in the range of CV per plant between the different transgenes, we conclude that for each of the three different transgenes the change in average LUC activity in leaves from one plant does not seem to follow a distinct pattern that can be related to either the intrinsic properties of the transgene promoter or to the developmental stage of the leaves.

The average in vivo *LUC activity correlates with the average* luc *mRNA steady state levels in leaves*

Under our conditions, the observed variation in LUC activity is not related to differences in substrate availability (described in van Leeuwen *et al*., 2000). The alternative explanation for the observed variation is that there is local variation in the amount of LUC protein. This can be caused by variations either in translation efficiency or by differential promoter activity within a leaf. We therefore verified whether the (average) photon production in leaves relates to the steady state level of *luc* mRNA in these leaves.

We sampled a low, a medium, and a high LUC active leaf from plant 35S-1b4. Total RNA was extracted from each leaf and the amount of *luc* mRNA in each pool was semi-quantified by reverse transcriptase PCR, using ubiquitin expression levels as an internal control (figure 2). Figure 2A shows the result of the reverse transcriptase PCR reaction, which was quantified after 24 cycles and plotted against the average LUC activity per leaf (figure 2B). The additional lower band that is visible in figure 2A is caused by a small percentage of single stranded DNA in each sample. This percentage is the same in each sample. Figure 2C-E show that for all of the three different reporter constructs the imaged *in vivo* LUC activity in individual leaves correlates with the relative *luc* mRNA levels. The lower values of average luciferase activity per leaf in figure 2 (compared to figure 1; *e.g*. 35S-1b4) are caused by the shorter measuring time and by the fact that older leaves are used (leaf 8 and higher). The data in figure 2 indicate that the observed differences in average LUC activity in leaves as measured by photon production, are a true reflection of differences in average transgene transcription rate.

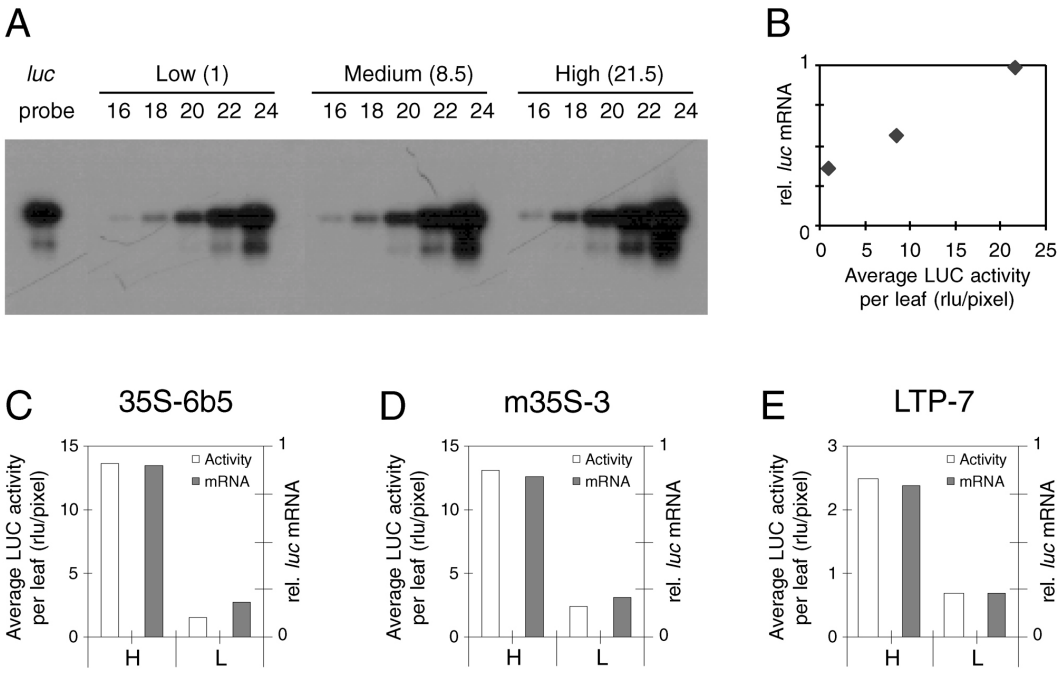

Figure 2. The relation between *in planta* luciferase activity in leaves and luciferase mRNA steady state levels. Shown are the luciferase reverse transcriptase PCR products after 16, 18, 20, 22 and 24 cycles from three samples derived from a low luciferase active leaf (Low), a medium luciferase active leaf (Medium) and a high luciferase active leaf (High), as measured in a single 35S-*luc* plant (35S-1b4) in 15 min. The average LUC activity (rlu pixel^{-1}) is shown between brackets. The luciferase probe is blotted as a control. Ubiquitin expression levels were used as an internal control. Panel **B**: The relative *luc* mRNA levels (quantified from the hybridised RT-PCR products after 24 cycles) are plotted against the average LUC activity per leaf measured in 15 min (rlu pixel^{-1}). In Panels **C**, **D** and **E**, high (H) and low (L) LUC active leaves of lines 35S-6b5, m35S-3 and LTP-7 respectively, were quantified for *in vivo* LUC activity measured in 15 min (white bars, rlu pixel^{-1}) and samples were taken for RNA extraction and semi-quantified from the blot containing the luciferase reverse transcriptase PCR products (dark bars, relative *luc* mRNA levels). The code of the transformants is shown above each graph.

Different variegated patterns of LUC activity between transformants and between leaves of the same plant

We observed a large variation in luciferase activity within a single leaf (referred to as variegation) for each construct. In figure 3, the seventh, excised leaf of three different single locus lines for each of the three *luc* reporter constructs is shown as an example of this variegation. Similar variations were also observed in other plant species transformed with these and other *luc* constructs (*e.g.* tobacco, *Arabidopsis*, tomato and

potato, data not shown), indicating that variegated promoter activity in leaves is intrinsic to many plant gene promoters. An indication of the degree of variegation can be obtained by calculating the CV of the LUC activity within each leaf. Below each image in figure 3, the average LUC activity and CV of the leaf are shown, as well as the maximum LUC activity (measured in 30 min, rlu pixel^{-1}). When the CV of all fully expanded leaves (5 to 12) of all independent transformants is compared we see that the leaves of the 35S plants have an average CV of 89%, the leaves of the m35S plants have an average CV of 162% and the leaves of the LTP plants have an average CV of 99%. The percentage of leaves with a CV below 100% is for the 35S, the m35S and the LTP population, 81%, 31% and 69% respectively.

In figure 1 we showed that the average level of LUC activity varies in different ways between individual leaves of a shoot. In figure 3 we showed differences in the type and level of variegation between leaves (in the same developmental stage) of *different* plants. We therefore examined whether there are only differences in the *level* of gene expression between leaves of the *same* shoot, or whether there are also differences in the degree of variegation in those leaves.

Like the average LUC activity (figure 1), the leaf to leaf variation of the maximum LUC activity per leaf does not seem to follow a distinct pattern that can be related to either the transgene promoter or to the developmental stage of the leaves (data not shown). Differences in average LUC activity per leaf are therefore not caused by a different number of "cells with maximum activity" that are active within a leaf. The ratio of the maximum luciferase activity over the average luciferase activity (max/avg) can also be used to characterise the degree of variegation within a leaf. A linear relation is observed when the CV is plotted against the max/avg value (data not shown). In table 1 we show this max/avg ratio for each leaf of three single locus lines per construct. In leaves with a more or less even distribution of LUC activity, the maximum activity that was measured within a leaf was no more than 2-3 times the average value within that leaf. In highly variegated leaves this ratio can be as large as 40 (*e.g.* line m35S-6, table 1). The degree of variegation differed between the three reporter gene constructs. The max/avg ratio for leaves 5 to 11 varied in 35S-*luc* plants from 2 to 8, in m35S-*luc* plants this ratio varied from 7 to 41 and in LTP *luc* plant the ratio varied from 3 to 14 (table 1).

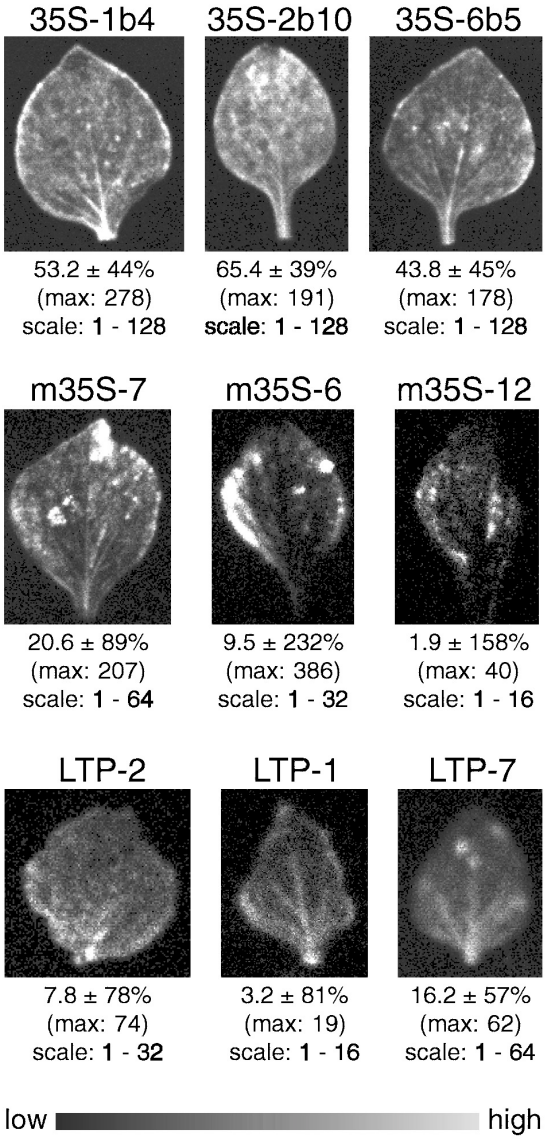

Figure 3. Examples of several spatial distributions of luciferase activity *in planta* in the seventh leaf of different petunia transformants containing either the 35S, the m35S or the LTP construct. The average luciferase activity of the leaf measured in 30 min is shown (rlu pixel^{-1}) as well as the CV within that leaf (%) as an indication of the degree of variegation. For this purpose, also the maximum activity of the leaf measured in 30 min is shown between brackets (rlu pixel^{-1}). The values (rlu pixel^{-1}) that are represented by the used grey scale (bottom) vary in the images. The range of values represented by the grey scale is shown below each image. In all images, a black pixel represents zero rlu pixel^{-1}, a dark grey pixel represents one rlu pixel^{-1} and a white pixel represents all values between the maximum value of the grey scale (16 to 128) and the maximum value of the luciferase image (max) to optimally show the differences in the leaf. The number of pixels quantified in the leaves varies between 5925 and 13366. The code of the transformant is shown above each image.

	Max/Avg in leaves 5 - 11						
	5	**6**	**7**	**8**	**9**	**10**	**11**
35S-1b4	4	4	5	4	5	7	8
35S-2b10	4	7	3	6	5	2	3
35S-6b5	4	6	4	8	6	8	7
m35S-7	7	9	10	10	8	7	14
m35S-6	8	11	41	9	9	11	27
m35S-12	17	8	21	20	27	16	20
LTP-2	7	9	9	7	7	13	8
LTP-1	5	10	6	10	11	9	14
LTP-7	3	3	4	3	6	3	5

Table 1. Characterisation of the degree of variegation of LUC activity in excised leaves. Three different single locus primary *luc* petunia transformants were analysed for each construct. The ratio of the maximum value over the average value (as measured in 30 min) of the subsequent leaves 5-11 is shown as an indication of the degree of variegation. The code of the transformant is indicated in the first column.

Although both the average luciferase activity (figure 1) as well as the maximum luciferase activity vary from leaf to leaf (and therefore the variegation varies, table 1), there seems to be no obvious relation between these two quantitative aspects of transgene activity. This is illustrated *e.g.* in leaf 8 and 11 of plant m35S-6 which have comparable average activity (figure 1), but have a max/avg ratio of 9 and 27 respectively (table 1). The substantial differences in the degree of variegation that can occur between subsequent leaves on the same plant make it difficult to assign a single pattern of expression to a plant. This complicates the comparison of transgene expression between individual transformants or between primary transformants and progeny plants.

In order to investigate whether the local luciferase light emission within a leaf correlates with local mRNA steady state levels, several leaves with highly variegated luciferase activity were analysed. Figure 4 gives an example of this analysis for a highly variegated m35S-*luc* leaf. RNA was isolated from the low and high LUC active half of

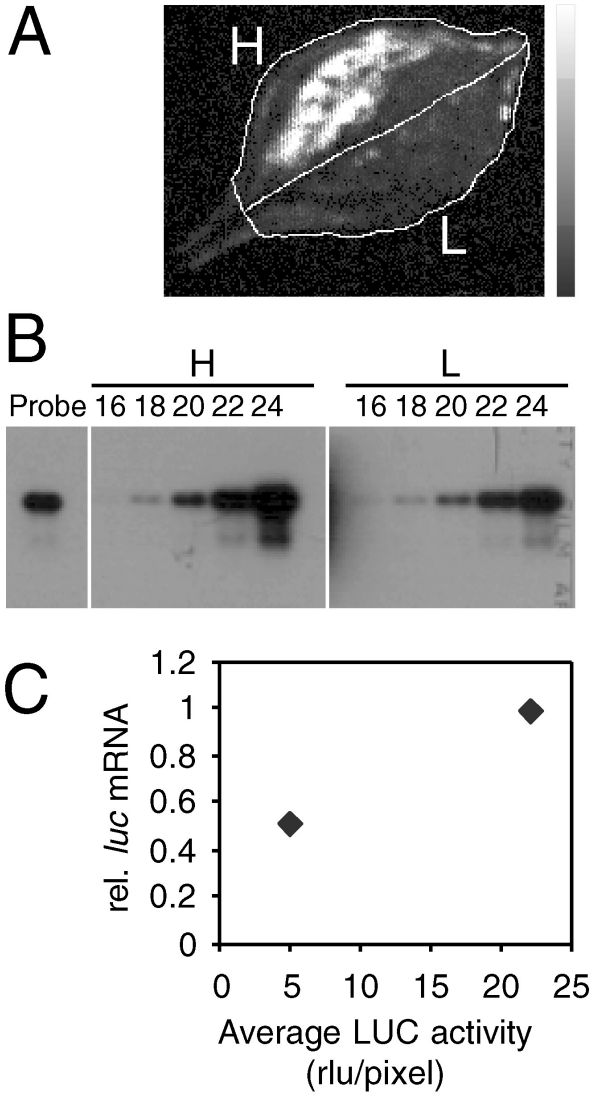

Figure 4. Local LUC activity within a leaf correlates with local *luc* mRNA steady state levels. From a leaf of plant m35S-3, showing both high (H) and low (L) luciferase activity (**A**, LUC activity is represented by the grey scale, right), the outlined parts were used for RNA extraction and reverse transcriptase PCR. The luciferase reverse transcriptase PCR products on the hybridised blot (**B**) also show differences in *luc* mRNA levels within this leaf. Ubiquitin expression levels were used as an internal control. Panel **C** shows the relative *luc* mRNA levels (quantified from the hybridised RT-PCR products after 24 cycles) plotted against the average LUC activity per leaf measured in 15 min (rlu pixel^{-1}).

the leaf (as shown in figure 4A) and *luc* mRNA levels were semi-quantified by RT-PCR (figure 4B). Figure 4C shows that the *luc* mRNA level in the higher active part was higher than the *luc* mRNA level in the lower active part. Small deviations in the mRNA

isolation of the small leaf samples as well as in the cDNA synthesis, RT-PCR and background of hybridisation, might disturb the absolute correlation between LUC activity and *luc* mRNA. This figure indicates however that not only between leaves but also within leaves, the results from imaging LUC activity *in vivo* does give information about the local activity of the promoter driving the luciferase transgene.

Developmental regulation of variegated luc transgene activity in leaves

To examine the temporal variation in the pattern of luciferase expression during the development of a single leaf, we imaged the *luc* transgene activity in the same leaf over a period of 50 days. The imaging started at a developmental stage where the size of the leaf was only a few millimetres and continued up to the stage of a fully expanded leaf. Several leaves of petunia plants containing either one of the three promoter driven *luc* constructs were imaged every day *in planta.* The LUC activity images of line m35S-3 are shown as an example in figure 5 from day 1 to 43. As an indication of the degree of variegation, the max/avg ratio for two lines of each promoter-*luc* construct is shown in table 2. With the continuous application of luciferin, a renewed application of luciferin directly after the measurement had no effect on the variegated pattern of LUC activity (Van Leeuwen *et al*., 2000). From figure 5 and table 2, it can be seen that the degree of variegation within a leaf is not the same, but varies between subsequent days. Although the activity generally decreases after several weeks, the degree of variegation still can be very high. Eventually the leaves senesced and at the same time showed extinction of luciferase activity, mostly between 44 and 50 days (data not shown).

Since individual transgenic lines show distinct and different types of variegated expression patterns, it is unlikely that the variegated patterns of LUC activity are strictly related to different developmental stages of the leaf. It would then be expected that clonal patterns related to leaf development would appear more often (Poethig and Sussex, 1985). The pattern of LUC activity within a leaf is not stable, but slowly changes from day to day (figure 5). This indicates that there is a temporal regulation to the spatial distribution of transgene promoter activity. These day-to-day variations in gene expression within a leaf may also contribute to the observed variation in gene expression between leaves of the same shoot (figure 1).

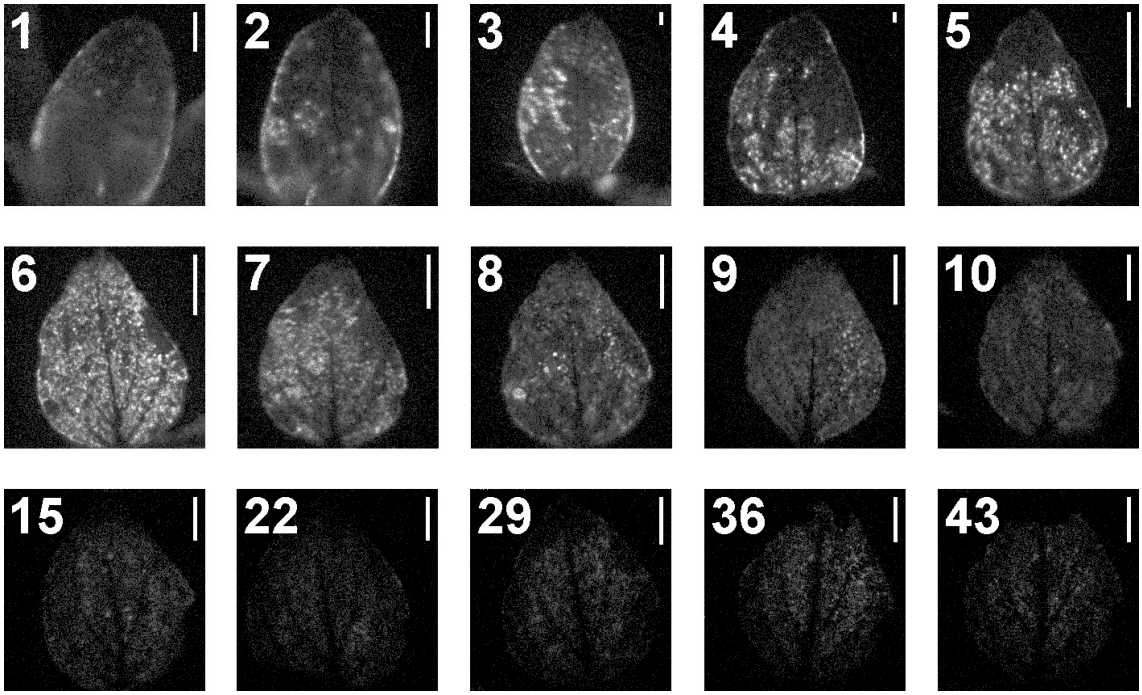

Figure 5. Temporal regulation of *in planta* LUC activity. Luciferase activity images of one leaf of plant m35S-3 followed through time. The numbers in each image indicate the day of measurement. Leaves were saturated with luciferin by daily spraying and imaged daily for 5 min. The size of the leaf is indicated in each image (bar on day 1-4 represents 1 mm; bar on day 5-43 represents 1 cm). Because of the varying size of the leaves, (and therefore height of the camera) the levels of luciferase expression should not be compared.

	Max/Avg in the same leaf on day 1 - 43														
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**15**	**22**	**29**	**36**	**43**
35S-5s3	9	17	27	13	21	13	13	15	9	10	15	11	17	11	20
35S-2b4	33	78	31	26	31	21	23	17	26	13	20	17	22	12	19
m35S-3	12	13	8	16	14	8	9	28	12	19	19	23	36	28	46
m35S-13	5	9	18	24	15	11	18	16	25	14	51	21	24	19	28
LTP-7	10	22	56	30	31	18	33	21	24	24	22	27	26	21	43
LTP-9	11	16	21	13	17	11	14	13	8	12	19	16	25	25	20

Table 2. Variegation in a single petunia leaf, as measured daily over a 50 day period. The max/avg ratio is shown as an indication for this variegation for two examples of each promoter-*luc* construct. The code of the transformant is indicated in the first column.

Stable inheritance of the patterns of LUC activity

We compared the luciferase activity pattern in progeny plants of transformants carrying a single locus of the *luc* reporter gene. The primary transformants were back-crossed with wild-type petunia plants and F_1 progeny plants were selected on basis of LUC activity. In genetically identical F_1 progeny plants, approximately the same global level of LUC activity was observed, as shown in figure 6A for three progeny plants for each of the three *luc* reporter constructs. The inheritance of easily recognisable developmental patterns of LUC activity (different levels between leaves) can also be shown. In LTP line 7 both the primary transformant as well as the genetically identical progeny plants showed a striking increase in LUC activity in the seventh leaf from the top of the shoot (figure 6B), directly followed by a decrease in LUC activity in older leaves (figure 6B). The temporal regulation of the level of LUC activity is thus also inherited. Inheritance of the spatial distribution of LUC activity (within a leaf) is difficult to examine, because variegation may vary within a plant from leaf to leaf or within a leaf from day to day (see figure 5). However, figure 6C shows three leaves from m35S line 3 (primary transformant and two back-cross progeny plants, respectively) with a comparable pattern of variegation. Specific for these plants is the presence of higher LUC activity at the edges of the leaf. Since the different types of spatial expression patterns can be stably inherited to progeny plants, variegation can not only be caused by local physiological conditions, but must also be determined by the integration site of the transgene.

Factors that possibly cause variegated luciferase activity

The observed variegated LUC activity might be intrinsic to the used transgene promoters. However, each of the three reporter gene constructs shows a variety of different variegated expression patterns in individual transformed lines. The m35S promoter was designed to increase the level of expression. Although very high active patches of luciferase gene expression exist in m35S leaves (figure 3), the overall level of average LUC activity per plant was (in the population of 16 independent transformants: 9.3 ± 14.1 rlu pixel^{-1}) lower than that of the 35S-*luc* population (12 plants: 24.0 ± 22.1 rlu pixel^{-1}), but clearly higher than that of the LTP-*luc* population (8 plants: 5.8 ± 7.2 rlu pixel^{-1}).

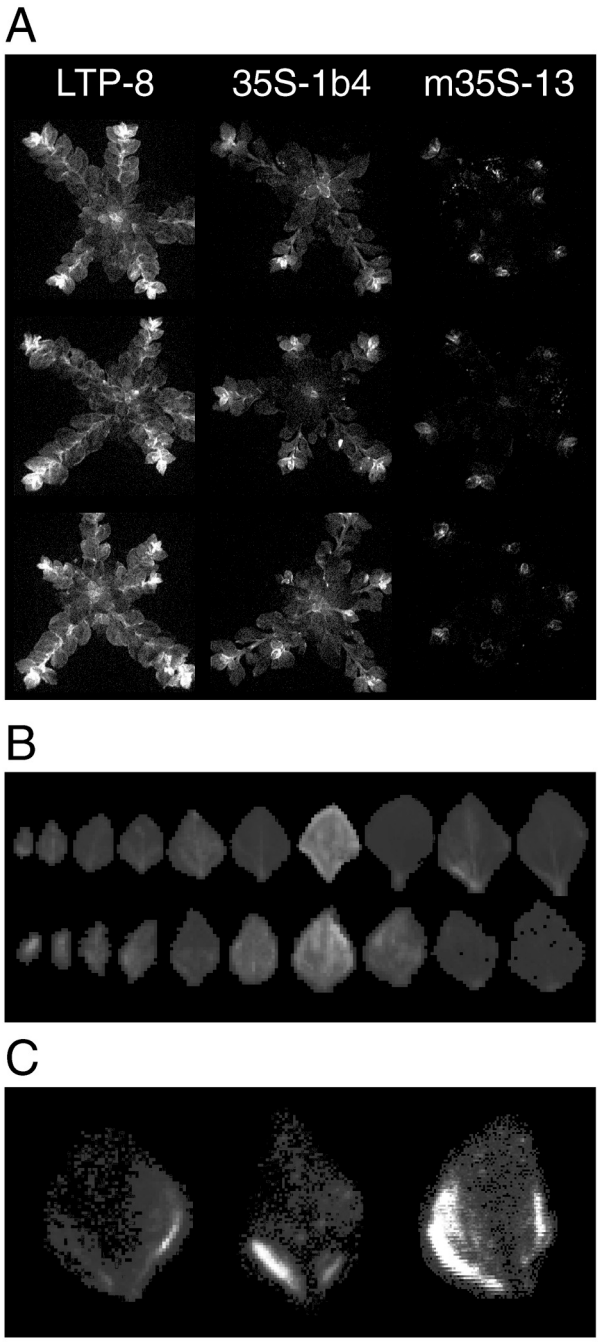

Figure 6. Inheritance of the LUC expression patterns. Panel **A:** Three progeny plants of different petunia lines (LTP-8, 35S-1b4 and m35S-13 respectively) with different levels of LUC activity (measured in 5 min), showing comparable activity within the progeny. Whole petunia plants are shown from above, each with five to six shoots Panel **B:** Luciferase activity measured for 15 min in ten subsequent leaves of a shoot of primary petunia transformant LTP-7 (top) and ten subsequent leaves of its back-cross progeny (bottom). Panel **C:** Variegation in leaves of primary petunia transformant m35S-3 (left) and comparable variegation in the leaves of two back-cross progeny plants (middle, right), all measured for 15 min.

The LTP promoter is active in the L1 cell layer (epidermis) of the shoot. We noted that in all our LTP-*luc* transformants this general tissue and cell layer specificity was retained (data not shown). Apparently, within this tissue and cell layer specific expression an extensive variation in spatial and temporal regulation of transgene expression still occurred. Imaging of leaf sections has shown that the 35S promoter and m35S promoter both are active in all cell layers of a leaf (Van Leeuwen *et al.*, 2000). Sectioning through high and low active LUC patches in leaves of plants expressing a 35S or m35S *luc* gene showed that the observed local LUC activity was present in all cell layers (either all cell layers high or all cell layers low, Van Leeuwen *et al.*, 2000). In all plants variegated LUC activity is thus caused by variegated promoter activity that locally extends to all cell layers of the leaf tissue.

Variegated gene expression has been observed and described before in relation to gene silencing phenomena (Depicker and Van Montagu, 1997; Flavell, 1994; Matzke and Matzke, 1998; Stam *et al.*, 1997). It has also been shown that gene silencing can occur in distinct different patterns within a tissue (Jorgensen *et al.*, 1996; Van der Krol *et al.*, 1988). However, gene silencing phenomena rarely show a range of levels of gene expression, but only “on” or “off” gene expression (Jorgensen *et al.*, 1998). Patterns caused by (trans-) gene silencing are fixed within the tissue and only may undergo reversion in newly synthesised organs. The observed day-to-day varying patterns of LUC activity in the same leaf (figure 5) in our experiments are therefore different from such gene silencing phenomena.

The chromatin structure around each transgene locus may differ and may result in a variable accessibility for transcription factors (resulting in the position effect, Dean *et al.*, 1988). Our results would then indicate that this DNA accessibility might not only vary quantitatively between individual transformants, but also may vary differently within a plant in time and place. In that case, a variegated transgene expression pattern might occur with an even distribution of transcription factors; such a variegated pattern will not (or not necessarily) occur for endogenous gene expression.

Alternatively, the variegated transgene promoter activity may be caused by true local differences in amount and / or activity of transcription factors within cells of a tissue. Since these transcription factors also act on endogenous plant genes, the prediction would be that some plant genes also would show variegated expression patterns. Such a

heterogeneous promoter activity of *e.g.* the endogenous chalcone synthase (chs) gene has already been reported in *in situ* experiments by Nick *et al*. (1993). Our results indicate that at least for the ubiquitin gene, expression does not seem to be comparably variegated within a leaf (the *luc* mRNA levels were plotted relative to comparable ubiquitin mRNA levels).

We have established that the variegation of LUC activity in plants can be attributed to differences in local mRNA steady state levels, and we are currently investigating whether this variegation is a feature of only the transgene(s) or whether some endogenous plant genes also show such varied patterns of promoter activity, possibly related to local differences in transcription factor availability and hormone signalling.

Conclusions

Transgene promoter activity can only be characterised by the distribution of the different expression levels within a plant, each level occurring with its own frequency. Sampling of single leaves might lead to different conclusions about the level of gene expression per plant (compare *e.g.* leaf 6 of plant m35S-6 and m35S-12 in figure 1). From figure 3 it is also clear that sampling leaf disks in order to compare the level of gene expression per plant, is even more imprecise, because the variations within a leaf can be as large as the variations between plants. Therefore, instead of describing the level of gene expression as one value per plant, one has to describe the range and frequency of gene expression levels per leaf and per plant.

Only the general spatial and temporal expression features of a transgene in different independent transgenic lines must therefore be intrinsic to the transgene promoter. Since every independent transformant shows minor or major differences in spatial and temporal regulation of the transgene, apparently in every transformant there is a different influence from flanking plant DNA sequences. Our results show that the promoter driving the transgene specifies the cell type(s) in which the transgene is expressed and defines the global temporal regulation of the transgene promoter activity within these cells. Superimposed on this are the effects of differences in transgene integration site, which may result in different local modulations of the temporal regulation of transgene activity. Local differences in temporal regulation may thus result in different variegated patterns of transgene activity (both within a leaf as well as

between leaves), as were observed by the imaging of *in planta* transgene (luciferase) activity.

Acknowledgements

We would like to thank D. Postma-Haarsma and R. Kuijpers for creating the 35S-*luc* and m35S-*luc* constructs, M. Toonen for providing the LTP-*luc* construct and R. Geurts for providing the *ubiquitin* primers. We thank O. Vorst for his contributions to the initial set-up of the luciferase reporter system in our lab. This work is supported by the Dutch Organisation for Scientific Research (NWO).

References

Benfey PN, Ren L, Chua N-H. (1989) The CaMV 35S enhancer contains at least two domains which can confer different developmental and tissue-specific expression patterns. European Molecular Biology Organisation Journal 8, 2195-2202.

Blundy KS, Blundy MAC, Carter D, Wilson F, Park WD, Burell MM. (1991) The expression of class I patatin gene fusions in transgenic potato varies with both gene and cultivar. Plant Molecular Biology 16, 153-160.

Dean C, Jones J, Favreau M, Dunsmuir P, Bedbrook J. (1988) Influence of flanking sequences on variability in expression levels of an introduced gene in transgenic tobacco plants. Nucleic Acids Research 16, 9267-9283.

DeLuca M, McElroy D. (1974) Kinetics of the firefly luciferase catalyzed reactions. Biochemistry 13, 921-925.

Denburg JL, Lee RT, McElroy WD. (1969) Substrate-binding properties of firefly luciferase. Archives of Biochemistry and Biophysics 134, 381-394.

Depicker A, Van Montagu M. (1997) Post-transcriptional gene silencing in plants. Current Opinion in Cell Biology 9, 373-382.

DeWet JR, Wood KV, Helinski DR, DeLuca M. (1985) Cloning of firefly luciferase cDNA and the expression of active luciferase in *Escherichia coli*. Proceedings of the National Academy of Science 82, 7870-7873.

Flavell RB. (1994) Inactivation of gene expression in plants as a consequence of specific gene duplication. Proceedings of the National Academy of Science 91, 3490-3496.

Gardner RC, Howarth AJ, Hahn P, Brown-Luedi M, Shepherd RJ, Messing J. (1981) The complete nucleotide sequence of an infectious clone of cauliflower mosaic virus by M13mp7 shotgun sequencing. Nucleic Acids Research 9, 1871-1880.

Geurts R, Heidstra R, Hadri Az-E, Downie JA, Franssen H, van Kammen A, Bisseling T. (1997) Sym2 of pea is involved in a nodulation factor-perception mechanism that controls the infection process in the epidermis. Plant Physiology 115, 351-359.

Gorman CM, Moffat LF, Howard BH. (1982) Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Molecular and Cellular Biology 2, 1044-1051.

Gould SJ, Subramani S. (1988) Firefly luciferase as a tool in molecular and cell biology. Analytical Biochemistry 175, 5-13.

Jefferson RA, Kavanagh TA, Bevan MW. (1987) GUS fusions: ß-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. European Molecular Biology Organisation Journal 6, 3901-3907.

Jorgensen RA, Cluster PD, English J, Que Q, Napoli CA. (1996) Chalcone synthase co-suppression in petunia flowers: comparison of sense vs. antisense constructs and single-copy vs. complex T-DNA sequences. Plant Molecular Biology 31, 957-973.

Jorgensen RA, Atkinson RG, Forster RLS, Lucas WJ. (1998) An RNA-based information superhighway in plants. Science 279, 1486-1487.

Lonsdale DM, Moisan LJ, Harvey AJ. (1998) The effect of altered codon usage on luciferase activity in tobacco: maize and wheat. Plant Cell Reports 17, 396-399.

Matzke AJM, Matzke MA. (1998) Position effects and epigenetic silencing of plant transgenes. Current Opinion in Plant Biology 1, 142-148.

Mlynárová L, Loonen A, Heldens J, Jansen RC, Keizer P, Stiekema WJ, Nap J-P. (1994) Reduced position effect in mature transgenic plants conferred by the chicken lysozyme matrix-associated region. Plant Cell 6, 417-426.

Murashige T, Skoog F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15, 473-479.

Nick P, Ehmann B, Furuya M, Schäfer E. (1993) Cell communication, stochastic cell responses, and anthocyanin pattern in mustard cotyledons. Plant Cell **5**, 541-552.

Niedz RP, Sussman MR, Satterlee JS. (1995) Green Fluorescent Protein: An in vivo reporter of plant gene expression. Plant Cell Reports 14, 403-406.

Ow DW, Wood KV, DeLuca M, DeWet JR, Helinski DR, Howell SH. (1986) Transient and stable expression of the firefly luciferase gene in plant cells and transgenic plants. Proceedings of the National Academy of Science 84, 4870-4874.

Peach C, Velten J. (1991) Transgene expression variability position effect. of CAT and GUS reporter genes driven by linked divergent T-DNA promoters. Plant Molecular Biology 17, 49-60.

Poethig RS, Sussex IM. (1985) The cellular parameters of leaf development in tobacco: a clonal analysis. Planta 165, 170-184.

Sambrook J, Fritsch EF, Maniatis T. (1989) Molecular cloning: A laboratory manual. Second edition: Cold Spring Harbor Laboratory, Cold Spring Harbor.

Sherf BA, Wood KV. (1994) Firefly luciferase engineered for improved genetic reporting. Promega Notes 49, 14-21.

Stam M, Mol JNM, Kooter JM. (1997) The silence of genes in transgenic plants. Annals of Botany 79, 3-12.

Thoma S, Hecht U, Kippers A, Botella J, De Vries S, Sommerville C. (1994) Tissue-specific expression of a gene encoding a cell wall-localized Lipid Transfer Protein from *Arabidopsis*. Plant Physiology 105, 35-45.

Toonen MAJ, Verhees JA, Schmidt EDL, van Kammen A, de Vries SC. (1997) *AtLTP1* luciferase expression during carrot somatic embryogenesis. Plant Journal 12, 1213-1221.

Van der Krol AR, Lenting PE, Veenstra J, van der Meer IM, Koes RE, Gerats AGM, Mol JNM, Stuitje AR. (1988) An antisense chalcone synthase gene in transgenic plants inhibits flower pigmentation. Nature 333, 866-869.

Van der Krol AR, Chua N-H. (1991) The basic domain of plant B-Zip proteins facilitates import of a reporter protein into plant nuclei. Plant Cell 3, 667-675.

Van der Krol AR, Brunelle A, Tsuchimoto S, Chua N-H. (1993) Functional analysis of petunia floral homeotic MADSbox gene pMADS1. Genes and Development 7, 1214-1228.

Van Leeuwen W, Hagendoorn MJM, Ruttink T, Van Poecke R, Van der Plas LHW, Van der Krol AR. (2000) The use of the luciferase reporter system for in planta gene expression studies. Plant Molecular Biology Reporter 18 (2), 143a-143t.

4

The effect of MAR elements on variation in spatial and temporal regulation of transgene expression

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Submitted

Abstract. The firefly luciferase (*luc*) as a reporter gene allows the monitoring of real-time gene expression *in vivo*. Imaging of *in planta* LUC activity has revealed a large variation of level, spatial distribution and temporal regulation of gene activity within a transgenic plant, as well as between individual transformants. These position induced quantitative differences between individual transformants were shown to be reduced for ß-glucuronidase (GUS) reporter gene expression by the introduction of matrix associated regions (MAR elements) on the T-DNA. Using the stable GUS reporter gene it can not be determined however whether position induced quantitative differences arise from differences in the level, spatial distribution or temporal regulation in activity. We have analysed the influence of MAR elements on the position induced quantitative differences in *luc* reporter gene expression, to investigate whether the level, spatial distribution or temporal regulation of the transcriptional activity of luciferase is influenced by the presence of MAR elements around the gene.

Imaging of *in planta* LUC activity in populations of approximately 30 *luc*-containing tobacco plants showed that the presence of MAR elements does not reduce the variation in the average level of *in vivo luc* expression. Also no reduction of the variation in spatial patterns of *in vivo* LUC activity within a plant was observed. However, MAR elements do seem to affect the variation in temporal regulation of transgene expression. The differences between the *in vivo* LUC data and previously obtained GUS data are probably due to differences in the relative stability of these two reporter proteins. The potential effects of MAR elements on the variability of transgene expression and the relationship of this effect to the relative stability of the encoded protein is discussed.

Introduction

The introduction of transgenes into plants by *Agrobacterium*-mediated cell transformation results in a stable random integration of one or a few copies of a well-defined T-DNA region (Gheysen *et al.*, 1989). Due to differences in the integration site of the newly introduced DNA, differences in T-DNA copy number and/or T-DNA organisation (Breyne *et al.* 1992; Hobbs *et al.*, 1993), or epigenetic effects (Matzke and Matzke, 1998), the individual transformants often show very different levels of transgene expression (referred to as the position effect, Dean *et al.*, 1988; Peach and Velten, 1991).

In eukaryotes, genes are thought to be organised in chromatin loops that vary in size from 5 to 200 kb (Bonifer, 1990). These loops are attached to the proteinaceous nuclear matrix at locations in the DNA, known as matrix associated regions (MARs, Getzenberg, 1991; Boulikas, 1993). MAR elements are thought to form the boundaries of DNA loops with independent transcription activity in the interphase nucleus. The inclusion of MAR elements at the borders of the *Agrobacterium* T-DNA is thus predicted to insulate the inserted transgene within the loop from the influences of surrounding chromatin (Bonifer, 1990; Laemmli *et al.*, 1992).

One of the best characterised MAR elements is the chicken lysozyme A element, which is localised far upstream of the chicken lysozyme gene (Phi-Van and Strätling, 1988). In a population of independent transformed animal cell lines this MAR element has been shown to increase the overall average level of transgene expression approximately 10-fold and to decrease the position-dependent variability in transgene expression when placed around a reporter gene, possibly by allowing transcription factors better access to the reporter gene (Stief *et al.*, 1989, Phi-Van *et al.*, 1990).

The chicken lysozyme A element has specific affinity for the tobacco nuclear matrix in an *in vitro* binding assay (Mlynárová *et al.*, 1994). Positioning the A element just inside the T-DNA borders, resulted in a significant reduction of the position induced variability of ß-glucuronidase (GUS) activity levels in leaves of mature transgenic tobacco plants in a population of independently transformed plants. This reduction was independent of the promoter driving the GUS gene (Mlynárová *et al.*, 1994, 1995). The presence of MAR elements reduced the variation of *in vitro* GUS activity between

individual transformants to the level of variation in. genetically identical plants (Mlynárová *et al.,* 1996).

In contrast to GUS (which accumulates and can only be measured *in vitro*), firefly luciferase allows the monitoring of real-time gene expression *in vivo* (Van Leeuwen *et al.*, 2000). We have previously shown with the luciferase reporter gene in petunia, that position induced quantitative differences in transgene activity between independent transformants can be attributed to an accumulative effect of differences in both spatial as well as temporal regulation of the transgene (Van Leeuwen *et al.*, 2001). Within each independent transformant, different levels of *luc* gene expression were observed between different leaves as well as between different parts of each leaf (*i.e.* between cells within the leaf, referred to as variegation). Each of these differences was shown to differ between individual transformants. With more stable reporter genes (*e.g.* GUS or GFP) these differences cannot be noted and it cannot be determined whether position induced quantitative differences arise from differences in the level, spatial distribution or temporal regulation in activity. When gene expression is quantified by mRNA extraction often whole leaves are sampled, which also obscures the variegation present in each leaf.

The LUC protein catalyses the oxidative decarboxylation of luciferin, which causes the release of a photon at 562 nm (yellow-green light) in 90% of the catalytic cycles (DeLuca and McElroy, 1974, Aflalo, 1991). After this reaction the luciferase protein is only slowly regenerated, because the end product of the reaction, oxyluciferin, is only very slowly released from the luciferase • oxyluciferin -complex (Denburg *et al.*, 1969). This slow regeneration of LUC protein activity implies that in the presence of all substrates, each luciferase molecule can only react once to emit one photon. In the continuous presence of all substrates, the active LUC protein will therefore not accumulate. Luciferase activity measured *in vivo* under these conditions thus represents the flux of protein molecules (LUC) made in the cell [Δ LUC / sec). In luciferin pre-sprayed plants, luciferase can therefore be used as a non-invasive reporter in plants to accurately follow changes in (trans)gene expression (Van Leeuwen *et al.*, 2000), in contrast to more stable reporter proteins (such as GUS) that only show the accumulated total amount of reporter protein in the cell at any given time point.

In this paper we investigate whether MAR elements affect the variation in level, in spatial regulation or in temporal regulation of *in vivo* luciferase transgene expression between transformants. We discuss a possible mechanism for the reduction of position effect variation by MAR elements as previously shown with the GUS reporter gene.

Materials and Methods

Constructs

Three constructs were used (pAHLGA, pAHGLA and pHGL; figure 1), all containing two selection genes (neomycin phosphotransferase II -NPTII- and hygromycin phosphotransferase -HYG-) as well as two reporter genes (GUS and *luc*). The *luc* reporter gene consists of the cauliflower mosaic virus 35S promoter (as described in Van Leeuwen *et al.*, 2000) driving the *luc* gene (as cloned by DeWet *et al.*, 1985) with a nuclear localisation signal as described in Van Leeuwen *et al.* (2000). Details on the construction of these vectors will be presented elsewhere (Mlynarova *et al*., in preparation). The MAR element used in the pAHLGA and pAHGLA constructs consist of the chicken lysozyme A element as described in Mlynárová *et al.* (1994). In the pAHLGA construct the *luc* reporter gene is in the centre of the presumed MAR-loop, while in the pAHGLA construct the *luc* reporter gene is next to the MAR element near the left border of the T-DNA. In the pHGL construct no MAR elements are present (figure 1). Populations are named according to the T-DNA vector used.

Plant material

Transgenic tobacco (*Nicotiana tabacum* cv Petit Havana SR1) plants carrying at least one intact copy of the T-DNA were used. Details on the transformation and analysis of tobacco plants will be presented elsewhere (Mlynarova *et al.*, in preparation). F1 progeny plants obtained by self-pollination of single-copy primary transformants -used for measurement of temporal regulation- were grown in a growth chamber 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).

Quantitative in vivo *luc reporter gene activity measurement*

Tobacco reporter plants were pre-sprayed with a luciferin solution (1 mM firefly D-luciferin, sodium-salt, Duchefa, 0.01% Tween 80), using an air-brush dispenser at 24 h, 16 h and 2 h before measurement. Luciferase activity was visualised in excised leaves with a 2D-luminometer, consisting of an intensified CCD camera (C2400-77, Hamamatsu Photonics, Japan) or a liquid nitrogen cooled slow-scan CCD camera (512-TKB, Princeton Instruments, Trenton NJ, US). Photon emission of whole leaves was quantified by computer (Argus-50 Image Processor, Argus 3.43, Hamamatsu Photonics, Japan or MetaMorph 4.1, Universal

Imaging Corp., US, respectively). Average luciferase activity was calculated by dividing the total luciferase activity per leaf by the area of the leaf. Luciferase activity is shown in relative light units per pixel (rlu / pixel) per 5 minutes. When using the liquid nitrogen cooled slow-scan CCD camera, luciferase activity is measured twice in order to eliminate spikes caused by cosmic rays from the image by a 'minimum calculation' of the two images (MetaMorph 4.1, Universal Imaging Corp., US). Luciferase activity as determined with the liquid nitrogen cooled slow-scan CCD camera was corrected for background caused by the read-out of the luminometer chip. Images of luciferase activity are depicted in false grey scales (dark grey indicating low activity, white indicating high activity).

Statistical analysis and characterisation of variegation

Analyses of the expression data of the experiments containing a population of one leaf of each primary tobacco transformant are based on the natural logarithm (ln) of the respective activity to give a normal distribution (Nap *et al*., 1993a). Statistical analysis was performed using Microsoft Excel 97. Normal distribution was decided on basis of a normal probability plot. The significance of the difference in variance between AHLGA vs. HGL and AHGLA vs. HGL was determined by the F-test for homogeneity of variances. The significance of the difference in mean activity between AHLGA vs. HGL and AHGLA vs. HGL was determined by a t-test assuming equal or unequal variances depending on the F test.

In order to characterise the variegation within each leaf, we show the (non-ln transformed) ratio of the standard deviation over the mean x 100% (this is referred to as the coefficient of variation or CV).

Results

In vivo *LUC activity measurements*

In order to investigate whether MAR elements affect the variation in level, in spatial regulation or in temporal regulation of *in vivo* luciferase transgene expression between transformants, three different T-DNA vectors were constructed for *Agrobacterium* mediated transformation of tobacco (figure 1). Two constructs each contain two MAR elements (the chicken lysozyme A element), in identical orientation (figure 1, pAHLGA and pAHGLA), creating a loop that could act as a transcriptional unit of gene regulation. In the pAHLGA construct, the *luc* reporter gene is in the middle of the loop. In the pAHGLA construct the *luc* gene is next to the MAR element near the left border of the T-DNA. The pHGL construct contains no MAR elements and is used to generate a control population for the effects of the MAR elements (figure 1). For each of these three vectors (pAHLGA, pAHGLA and pHGL) about 30 independent tobacco transformants containing at least one intact copy of the T-DNA were analysed for *in vivo* LUC activity.

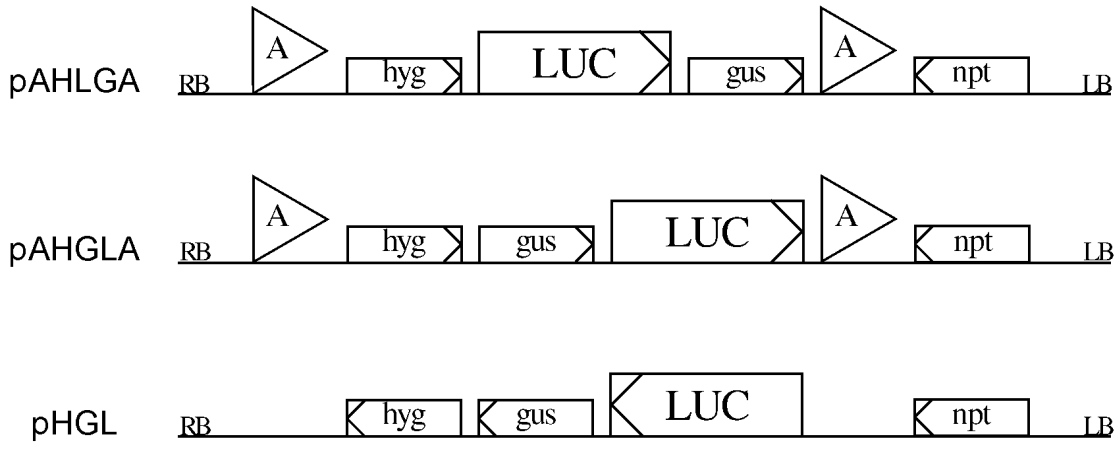

Figure 1. Structure of the three T-DNA vectors used for the plant transformation. A= Chicken Lysozyme A element; hyg = nopaline synthase promoter driven hygromycin gene; gus = *Lhca3.St.1* promoter driven ß-glucuronidase gene; LUC = double cauliflower mosaic virus 35S promoter driven luciferase gene; npt = nopaline synthase promoter driven neomycin phosphotransferase II gene; > indicates the 5'-3' orientation of the promoter-reporter gene construct. The constructs are shown from Right border (RB) to left border (LB).

The third leaf from the top (with a size of approximately 9 cm) from each plant of the three populations was pre-sprayed with luciferin. *In vivo* luciferase activity was subsequently measured in each excised leaf for five minutes with an intensified 2D-luminometer. The average LUC activity per leaf (rlu per pixel per 5 min) was obtained by image analysis. The distribution of transgene activity in a population of first-generation transgenic plants can be markedly skewed. For a proper statistical analysis of the expression levels, a logarithmic transformation may be required in order to yield an approximately normal distribution of gene activity (Nap *et al*., 1993a). A normal probability plot of the *in vivo* LUC data indeed showed that a natural logarithmic (ln) transformation was required to obtain a normal distribution of the average luciferase activity in each of the three populations (data not shown).

The ln transformed *in vivo* LUC activity data is shown for all transformants in figure 2. The mean and variance of the data on the logarithmic scale with the statistical test results are shown in table 1. The mean luciferase activity is calculated on a logarithmic scale and subsequently re-transformed to the normal scale. The mean LUC activity in table 1 is thus shown on the normal scale (rlu per pixel per 5 min). Residual analysis indicated that the low active HGL plant was an outlier. The analysis of the HGL population including the outlier is shown in the values between brackets (table 1). Analysis of the low expresser in the AHGLA population (figure 2) indicated that this plant is not an outlier. The significance of the difference in mean LUC activity and variance in LUC activity, of the MAR containing populations compared to the HGL population, was calculated without the outlier in the HGL population. The population without MAR elements (HGL) has the highest mean LUC activity and the lowest variance in LUC activity. Only the AHGLA population had a significantly lower mean LUC activity and a significantly higher variance in LUC activity than the HGL population.

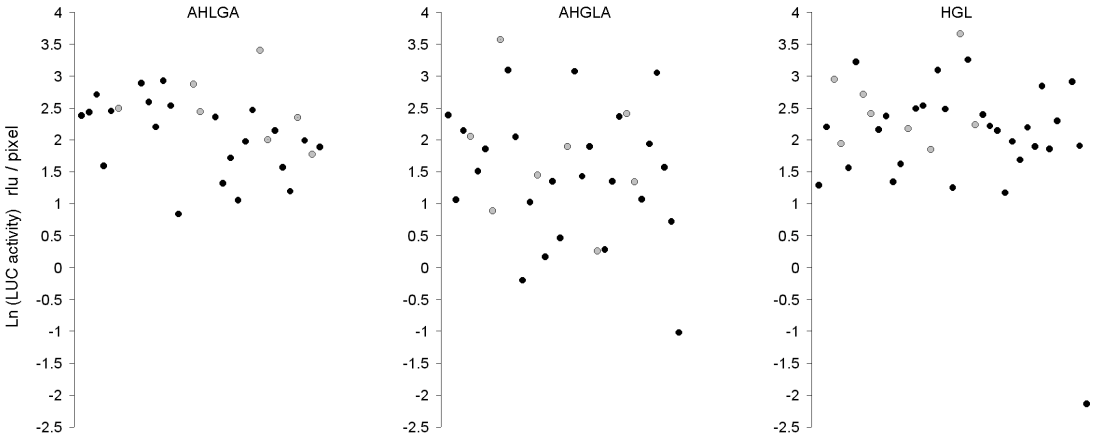

Figure 2. Distribution of the natural logarithm transformed *in vivo* LUC activity (rlu per pixel per 5 min) in different transformants. LUC activity was measured in the third leaf from the top of each transformant in the AHLGA (*luc* in centre of loop), AHGLA (GUS in centre of loop) and HGL population. Each circle represents the activity of one leaf per one individual primary transformant. The very low LUC activity in one HGL plant could only reliably be determined when the luciferase activity was measured for 30 minutes instead of 5 minutes. The statistical analysis is shown in table 1. The grey (open) circles represent the leaves used as an example of variegation in leaves in figure 3.

Population	LUC mean	t-test	LUC variance	F-test	n
AHLGA	8.62	ns	0.39	ns	27
AHGLA	4.62	P<0.01	0.94	P<0.001	31
HGL	9.36 (8.29)		0.36 (0.88)		35 (36)

Table 1. Mean activity and variance of *in vivo* LUC activity in the third leaf from the top in each plant of the AHLGA (*luc* in centre of loop), AHGLA (GUS in centre of loop) and HGL population. LUC mean: the re-transformed mean natural logarithm of LUC activity (rlu / pixel as measured in 5 minutes). t-test: the probability that the mean LUC activity of the populations differs from the HGL population; ns not significant. LUC variance: the variance of the natural logarithm of LUC activity. F-test: the probability that the LUC variance of the populations differs from the HGL population; ns not significant. n: number of transformants measured. Values between brackets indicate the values with the inclusion of one putative outlier, see also figure 2.

Analysis of variegation of in vivo *LUC activity*

In vivo LUC activity usually varies within a leaf (referred to as variegation; ***e.g.*** when driven by the 35S promoter; Van Leeuwen ***et al***., 2001). An example of this variegation in *in vivo* luciferase activity is given in excised leaves of eight different plants for each population (figure 3). The average luciferase activity in the leaves shown in figure 3 are represented in figure 2 by the grey circles.

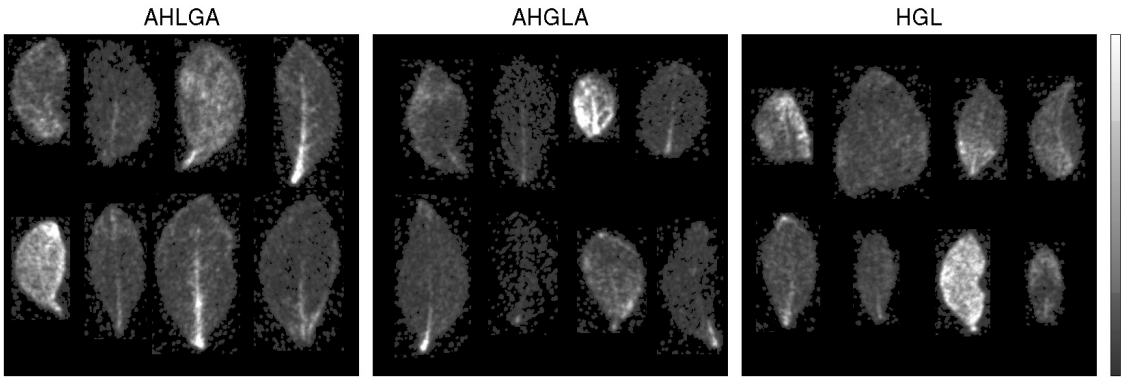

Figure 3. Variegation in luciferase activity. Luciferase activity images showing an example of eight individual leaves (third leaves) derived from eight different primary transformants, as analysed in figure 2. The variegation of luciferase activity in these leaves is shown for the AHLGA (*luc* in centre of loop), AHGLA (GUS in centre of loop) and HGL population. Luciferase activity is shown with false shades of grey as shown by the scale on the right (dark grey indicating low activity, white indicating high activity).

In order to examine the effect of MAR elements on variegation, the level of variegation was quantified. We use the coefficient of variation (CV) as a measure for variegation ***i.e.*** the standard deviation as a percentage of the average LUC activity of the leaf. The CV of the luciferase activity in a single leaf (the third leaf, as examined in figure 2) from all plants in the three populations is shown in figure 4. The CV values show a normal distribution and do not require a transformation for statistical analysis, as determined by a normal probability plot analysis (data not shown). The figure shows that the AHGLA population has the widest distribution of average CV values in the population.

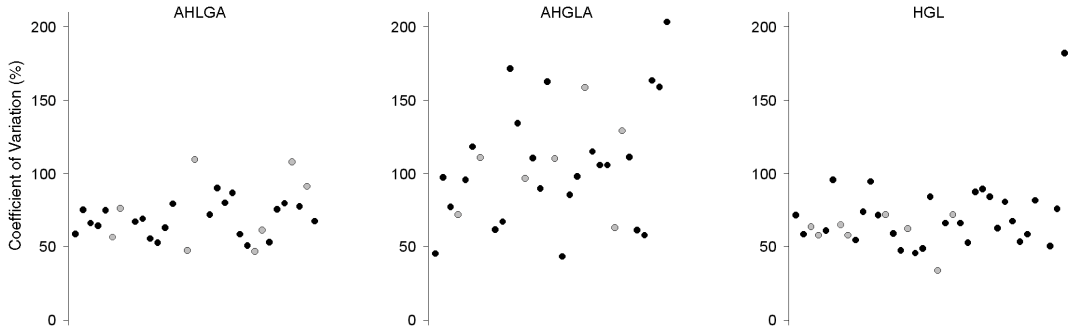

Figure 4. Variegation distribution in the AHLGA, AHGLA and HGL population. The CV of the third leaf in each plant of the AHLGA (*luc* in centre of loop), AHGLA (GUS in centre of loop) and HGL population is shown. Each circle represents one individual primary transformant. The grey (open) circles represent the leaves as shown in figure 3.

In table 2 the mean CV of *in vivo* LUC activity (determined for the third leaf from each plant) is given for each population. The luciferase activity in the leaves of the plants in the AHGLA population show the highest average level of variegation, which was significantly higher than the average CV of 35S driven LUC activity in leaves of the HGL or AHLGA populations.

Population	mean CV	t-test	n
AHLGA	69 %	ns	27
AHGLA	106 %	P<0.001	31
HGL	66 % (70 %)		35 (36)

Table 2. Variegation per population as characterised by the mean CV of *in vivo* LUC activity (rlu per pixel as measured in 5 minutes) of the third fully expanded leaf in each plant of the AHLGA (*luc* in centre of loop), AHGLA (GUS in centre of loop) and HGL population. t-test: the probability that the mean CV value in the populations differs from the HGL population (ns: not significant). n: the number of transformants measured. Values between brackets indicate the values with the inclusion of one putative outlier for average LUC activity, see also figure 2 and table 1.

Analysis of temporal regulation of in vivo LUC activity in successive leaves

The *in vivo* luciferase activity in petunia leaves from the same shoot can differ dramatically in time. This difference in time is referred to as temporal regulation. The

temporal regulation in transgene expression also differs between individual plants (Van Leeuwen *et al*., 2001). In order to measure the temporal regulation of *in vivo* luciferase activity, LUC activity in a single leaf can be followed *in planta* for several weeks. However, successive leaves on a shoot of a plant represent a set of progressing developmental stages. The LUC activity in successive leaves on a shoot at one time point can thus also be used as a measure of temporal control of (trans)gene activity. We analysed the developmental regulation by measuring *in vivo* LUC activity in successive leaves of F_1 progeny plants, of four to five single copy transformants from each population, as a measure for temporal regulation. For each line, LUC activity was quantified in seven to eight successive excised, luciferin pre-sprayed tobacco leaves per plant (figure 5). In order to compare the individual transformants, the average LUC activity of each leaf was set relative to the LUC activity of the most active leaf in each developmental series (set to 1; figure 5). The plants in the AHLGA population show the smallest variation in temporal regulation of the LUC reporter gene activity (smallest grey area), while the HGL population shows the widest distribution in temporal regulation.

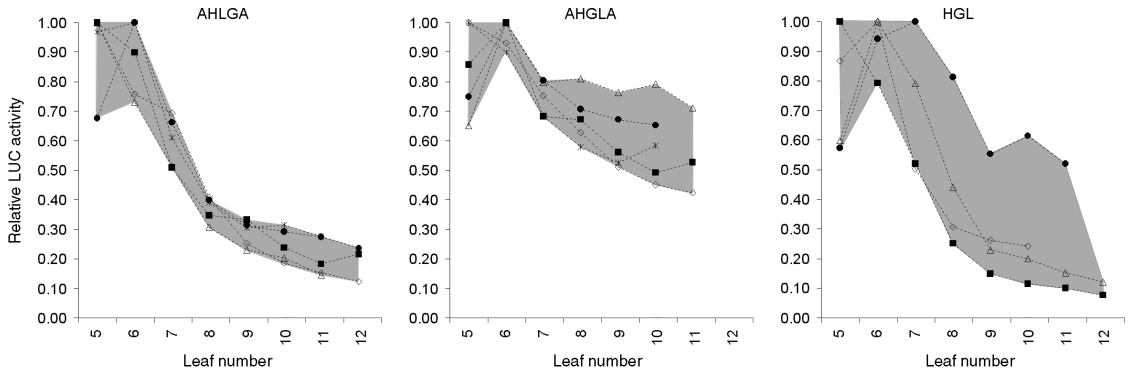

Figure 5. Relative *in vivo* LUC activity (based on average LUC activity per leaf) in seven to eight successive leaves (starting with the first full grown leaf with a size of approx. 125 mm, which is leaf five; successive leaves are numbered from top to bottom; the youngest visible leaf is leaf 1) of progeny plants (derived by self-pollination of the primary transformants) of 5 AHLGA, 5 AHGLA and 4 HGL primary transformed tobacco plants as measured with a liquid nitrogen cooled CCD camera. The leaf with the highest average activity in each plant is set to 1 to show a possible relative difference in decrease in LUC activity (*i.e.* in ageing leaves). Note that the leaves shown in this figure (starting with leaf 5) are older (and more expanded) than the leaves used in figure 2.

Consequences of differences between LUC and GUS activity measurements (a theoretical simulation)

A reduction in position induced variation of transgene expression in plants by MAR elements has been demonstrated using a GUS reporter gene (Mlynárová *et al.*, 1994, 1995). However, this reduction is not observed when *in vivo* LUC activity was measured (figure 2, table 1). Any swift temporal changes in promoter activity (with or without MAR elements) cannot be noted with GUS as a reporter, due to the stability of the GUS protein (*e.g.* Thompson *et al*., 1991). The reduction in position induced variation of GUS expression by MAR elements could be the result of a reduction in the (long-term) variation in temporal regulation in transgene expression. In that case, this reduction will not be noted when *luc* expression is measured on a single time point (as in figure 2), but may result in a smaller variation in accumulated GUS reporter protein levels and activity between plants.

To illustrate this principle we show in figure 6 the effects of 4 different theoretical temporal regulations (panels A-D) of promoter activity (1) over a period of 24 days. In each panel the effect on calculated *in vivo* LUC activity (2; directly linked to the promoter activity) and on calculated GUS activity is shown (3; with a GUS protein half life of 6 days, Nap *et al*., 1993b). Promoter activity, LUC activity and GUS activity are shown in the same arbitrary units, assuming a 1 to 1 transcription and translation rate. The difference between the black and grey lines represents the maximum possible difference that would occur within a non-synchronous population of plants (all slightly shifted in phase). Because the activities in this population are normally distributed, no (logarithmic) transformation is necessary. The resulting average LUC (*in planta*) and GUS (*in vitro* assay) activity for these theoretical promoter activities are shown in table 3. The accumulative result for GUS approaches a plateau value only after 24 days and is therefore determined at the end of the period. In the presence of luciferin, the LUC activity directly follows the promoter activity and can be determined at any time-point. Furthermore, the range of reporter gene activity values in these virtual populations of plants is shown as well as the variation in reporter gene activity (table 3, variation). Standard deviation will show comparable differences between the virtual populations of plants, while the variance will square the difference as the standard deviation is the square-root of variance. In figure 6 the effects of a reduction in frequency (panel B), an

increase in amplitude (panel C) and an increase in level (panel D) of promoter activity are shown. The bottom 3 rows of table 3 compare the four temporal representations of figure 6, *i.e.* the ratio is given of the effect of a change in frequency (B compared to A; two times reduced frequency), the effect of a change in amplitude (C compared to A; two times increased amplitude) or the effect of a change in level (D compared to A, two

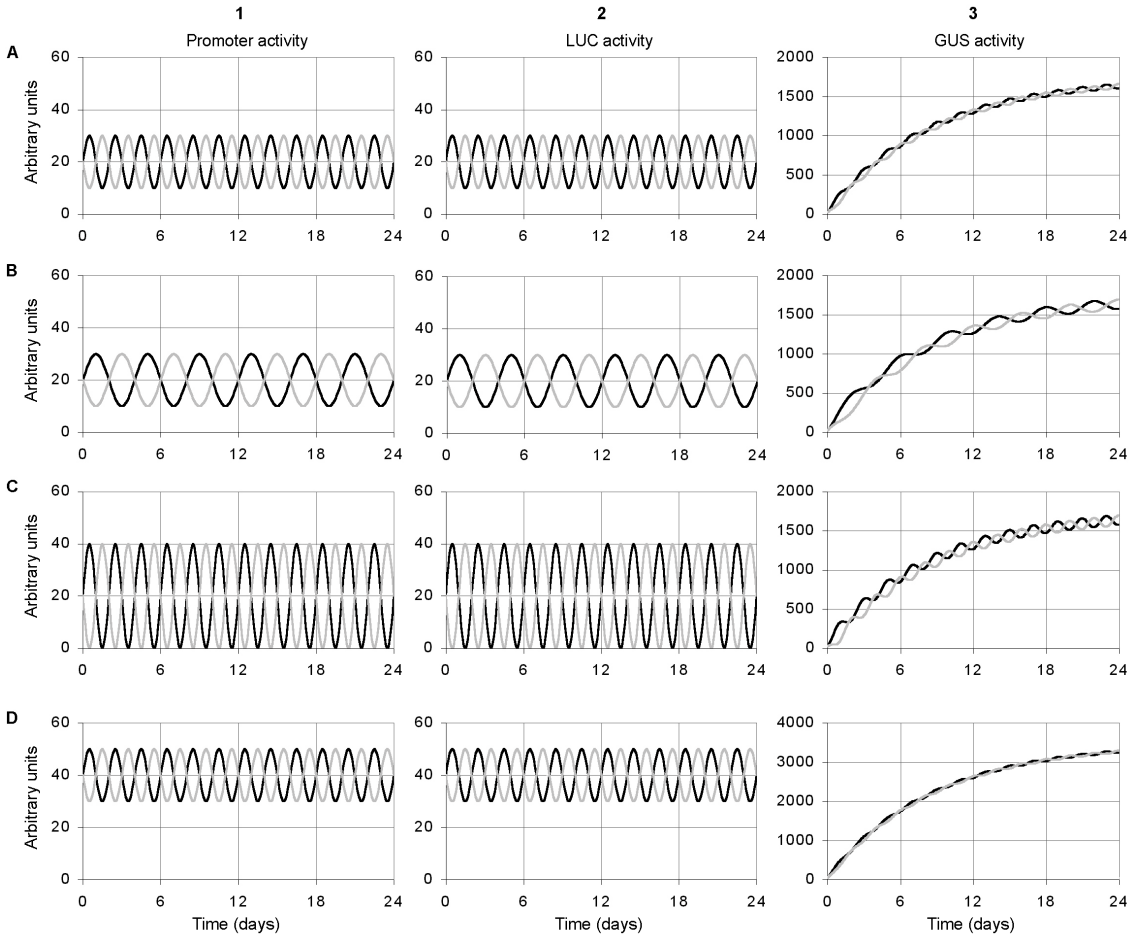

Figure 6. The effect of a variation in promoter activity on the measurement of LUC activity and GUS activity, plotted for 24 subsequent days. The difference between the black and grey lines represents the maximum possible difference that would occur within a non-synchronous population of plants (all slightly shifted in phase). Panel A shows a theoretical variation in promoter activity (in arbitrary units, 1) and the consequently calculated LUC activity (2) and GUS activity (3). Panel B shows the effect of a comparable variation in promoter activity with a two times lower frequency (higher wavelength). Panel C shows the effect of a comparable variation in promoter activity with the same frequency as panel A, but with a two times higher amplitude. Panel D shows the effect of a comparable variation in promoter activity with the same frequency and amplitude as panel A, but at a two times higher level.

times higher average). Especially the effect of a change in frequency (B / A) shows a clear difference between the observed variation of LUC activity (no effect) and GUS activity (two times higher variation, equal to the reduction in frequency).The effect on variance is similar to the effect on variation, indicated in this example (data not shown).

	LUC			GUS		
	average	range	variation	average	range	variation
A	20	10-30	20	1634	1604-1663	59
B	20	10-30	20	1634	1575-1693	118
C	20	0-40	40	1634	1575-1693	118
D	40	30-50	20	3267	3238-3297	59
	Δ avg		Δ var	Δ avg		Δ var
B/A	1		1	1		2
C/A	1		2	1		2
D/A	2		1	2		1

Table 3. The effect of frequency (B), amplitude (C) and level (D) on temporal variation of promoter activity (A). The effect is shown for LUC activity and GUS activity. For GUS (half life of 6 days) the values are determined after 24 days. average: average value within a non-synchronous population of plants (all slightly shifted in phase), shown in figure 6. range: range of values within this population. variation: difference within this population.
In the bottom 3 rows the four temporal representations of figure 6 are compared, *i.e.* the ratio is given of values of respectively B, C and D compared to A. Δ avg: difference in average activity. Δ var: difference in variation.

These examples give us an indication that a reduction in variance of GUS reporter protein activity between plants (as in figure 6; A vs. B) not necessarily results in a change in variance of LUC reporter protein activity, due to the accumulative nature of GUS protein activity. A reduction in variance of GUS activity, induced by MAR elements, might thus have no effect on the variance of LUC activity.

Discussion

We have used the luciferase reporter gene to analyse the effect of MAR elements on the spatial and temporal regulation of transgene expression in plants. The luciferase reporter system enables a direct analysis of promoter strength, and spatial and temporal regulation of transgene activity *in planta* (Van Leeuwen *et al*., 2000). Tobacco plants were transformed with three different constructs, two with MAR elements (pAHLGA and pAHGLA) and one without MAR elements (pHGL, figure 1). In the pAHLGA construct, the *luc* reporter gene is in the middle of the loop. In the pAHGLA construct the *luc* gene is directly flanking the MAR element near the left border of the T-DNA.

In vivo *LUC activity measurement in single leaves*

From each plant of the three populations, *in vivo* LUC activity in the third excised leaf was quantified (comparable to the leaf analysed in previous MAR studies with the GUS reporter gene, Mlynárová *et al.*, 1994, 1995, 1996). The distribution of the ln transformed average LUC activity in leaves (figure 2) and the statistical analysis presented in table 1 show that the variance in *in vivo* LUC activity is the lowest in the HGL population (with the exclusion of one putative outlier). For the AHGLA population the variance in LUC activity was significantly higher than the HGL population (without the outlier), while the mean LUC activity was significantly lower than the HGL population (without the outlier). The AHLGA and HGL population were not significantly different in mean and variance of *in vivo* LUC activity. In contrast to the reported effect of MAR elements on GUS reporter activity, MAR elements seem to have no effect on increasing the average level of LUC activity or decreasing the variation of *luc* gene expression in a transgenic population, although a comparable promoter was used to drive transgene expression in both experiments. The 35S promoter used here and the double 35S promoter used by Mlynárová *et al.* (1995) consist of the same domains and could be equally responsive to the effects of MAR elements. This different effect of MAR elements on GUS and LUC activity might be attributed to the different aspects of transgene activity that are measured with each reporter protein; *in vivo* LUC activity is directly related to the ongoing promoter activity (Van Leeuwen *et al*., 2000, 2001), while *in vitro* GUS activity is the accumulated result

of promoter activity over a prolonged period of time (see below). Copy numbers or epigenetic effects may also influence the effect of MAR elements, but this will affect both GUS and *luc* reporter gene activities.

The level of variegation in in vivo *LUC activity within a leaf*

The *in vivo* LUC activity in leaves of the 35S-*luc* tobacco plants from the three populations showed variegated patterns (figure 3). Such variegated patterns of 35S driven luciferase activity were observed before in transgenic petunia plants (Van Leeuwen *et al*., 2001). We observe up to 15-fold difference between the average LUC activity and the maximum LUC activity in tobacco leaves. The level of variegated LUC activity was quantified by calculating the CV for each leaf (figure 4). These CV values for 35S driven LUC activity were comparable to CV values previously observed in petunia plants (for 35S and other promoters, Van Leeuwen *et al.*, 2001). Variegation can be quantified in different ways. Each method has its limitations and none can fully account for all the differences in spatial distribution within a leaf. However, characterisation of the variegation by *e.g.* the maximum activity / average activity ratio (Van Leeuwen *et al*., 2001) shows comparable differences between the 3 populations (data not shown). There seems to be no reduction in the level of variegation in the plant populations containing MAR elements, compared to the plant population without MAR elements (compare AHLGA and HGL). The AHGLA population even shows the highest mean CV value as well as the highest variation in variegation (widest distribution of data points, figure 4). However, the variegation level for each plant was characterised by the CV in only a single leaf, while variegation significantly differs between different leaves of the same plant (Van Leeuwen *et al.*, 2001). The variation in CV levels of LUC activity between leaves of the same plant (in these tobacco plants with a standard deviation of 50 to 60 %; data not shown), exceeds the variation in CV levels between the measured leaves of individual transformants (standard deviation of the CV values shown in figure 4 does not exceed 40%; data not shown).

Because all plants in all three populations show comparable levels of variegation, we conclude that MAR elements do not reduce the level of variegation. The close presence of a MAR element (for *luc* in the AHGLA population) even results in a wider variation and a higher level of CV values.

Temporal regulation of luciferase activity in tobacco leaves

To examine the variation in temporal regulation of (trans)gene expression, we analysed the *in vivo* LUC activity in seven to eight excised, luciferin pre-sprayed tobacco leaves from one plant (figure 5). These successive leaves of a shoot of a plant have an increasing age (a different developmental stage) and thus luciferase activity in these leaves represents the temporal regulation of gene expression in a single plant. We analysed four to five plants of each population. In general, the LUC activity decreased in older tobacco leaves in a much more orderly manner than we observed in 35S-*luc* petunia plants (without MAR elements), *i.e.* there are no sudden increases in activity in some of the older leaves (Van Leeuwen *et al*., 2001). This indicates that the variation in temporal regulation of transgene activity probably depends both on the transgene promoter as well as on plant species. The variation in temporal regulation is indicated by the width of the grey area in figure 5. The plants in the HGL population show a large relative variation in LUC activity between plants, indicating a large variation in temporal regulation. The LUC activity in leaves of the AHLGA plants show the smallest variation in temporal regulation (figure 5). These results indicate that MAR elements affect the temporal regulation of transgene activity.

A change in variation of temporal regulation of transgene activity may have a long-term effect on the accumulation of gene products, depending on the stability of the gene product. In case of GUS the product is very stable resulting in a high accumulation, while the active LUC protein in the presence of luciferin is very unstable and does not accumulate. Previous results showed a reduction in the variance in GUS activity, when the GUS transgene is flanked by MAR elements (Mlynárová *et al.*, 1994, 1995). However, when a comparable promoter drives *luc* transgene expression, no reduction in variation in LUC activity in individual leaves between plants is observed. This seemingly contrasting result may be explained by considering the differences in stability between GUS and LUC protein activity, combined with the effect of MAR elements on temporal regulation.

A reduction in variation in temporal regulation can be obtained in many different ways. Figure 6 illustrates the effects of a reduction in a theoretical temporal variation by a reduction in the frequency of transgene expression (panel 6B) or by an increase in the

amplitude of transgene expression (panel 6C). Furthermore the effect is shown of an increase in the level of transgene expression without changing the temporal regulation (panel 6D). This figure shows that not all changes in temporal control have the same effect on the variation of measured LUC activity and GUS activity. Most changes in temporal regulation of gene expression will be measured comparably when either *luc* or GUS is used as a reporter gene (*e.g.* panel 6C and 6D, table 3). An increase in amplitude has no effect on the average activity, but does have an effect on the variation as would be expected (two lines are further apart in figure 6C, table 3). An increase in level has only an effect on the average activity, but does not affect the variation (figure 6D, note higher Y-scale for GUS (3); table 3).

When the frequency is changed of the temporal regulation of promoter activity (as in panel 6B), no effect is visible on the average activity and variation of luciferase activity or on the average activity of accumulated GUS protein (table 3). However, in this case there is an effect on the variation in GUS activity, while this has no effect on the variation in LUC activity. This is shown in the virtual population of plants in panel 6B (difference between black and grey lines). A two times lower frequency of the temporal regulation of promoter activity results in a two times higher variation of measured GUS activity.

A reduction in the variation of GUS activity measured in a population can thus be accomplished by changing the variation in temporal regulation. This change would not be visible when the transgene activity is determined on a single time point using the firefly luciferase as a reporter gene. Measurement of the temporal regulation of luciferase transgene activity however revealed that there are differences in this temporal regulation between a population with MAR elements and a population without MAR elements.

We therefore conclude that MAR elements probably reduce variations in transgene activity by reducing the variation in temporal regulation of transgenes between transformants. This effect of MAR elements affects only stable gene products (*e.g.* GUS) and not the unstable LUC activity (related to ongoing gene expression), when measured on a single time-point. The reduction of position induced quantitative differences in reporter gene expression by MAR elements will thus be mainly effective on (or detectable by) stable transgene products.

Acknowledgements

We would like to thank Annelies Loonen and Ela Mietkiewska for cloning and analysis. This work is financially supported by the Dutch Organisation for Scientific Research (NWO).

References

Aflalo C. (1991) Biologically localized firefly luciferase: A tool to study cellular processes. Int Rev Cytol 130: 267-323.

Bonifer C. (1990) Tissue specific and position independent expression of the complete gene domain for chicken lysozyme in transgenic mice. EMBO J 9: 2843-2848.

Boulikas T. (1993) Nature of DNA sequences at the attachment regions of genes to the nuclear matrix. J Cell Biochem 52: 14-22.

Breyne P, Gheysen G, Jacobs A, Van Montagu M, DePicker A. (1992) Effect of T-DNA configuration on transgene expression. Mol Gen Genet 235: 389-396.

Dean C, Jones J, Favreau M, Dunsmuir P, Bedbrook J. (1988) Influence of flanking sequences on variability in expression levels of an introduced gene in transgenic tobacco plants. Nucl Acids Res 16: 9267-9283.

DeLuca M, McElroy WD. (1974) Kinetics of the firefly luciferase catalyzed reactions. Biochemistry 13: 921-925.

Denburg JL, Lee RT, McElroy WD. (1969) Substrate-binding properties of firefly luciferase. Arch Biochem Biophys 134: 381-394.

DeWet JR, Wood KV, Helinski DR, DeLuca M. (1985) Cloning of firefly luciferase cDNA and the expression of active luciferase in *Escherichia coli*. Proc Natl Acad Sci 82: 7870-7873.

Getzenberg RH. (1991) Nuclear structure and the three-dimensional organization of DNA J. Cell Biochem 47: 289-299.

Gheysen G, Herman L, Breyne P, Van Montagu M, Depicker A. (1989) Agrobacterium tumefaciens as a tool for the genetic transformation of plants. In Butler LO, Harwood C, Mosely BEB, (eds) Genetic transformation and expression. Intercept, Andover: 151-174.

Hobbs SLA, Warkentin TD, DeLong CMO. (1993) Transgene copy number can be positively or negatively associated with transgene activity. Plant Mol Biol 21: 17-26.

Laemmli UK, Käs E, Poljak L, Adachi Y. (1992) Scaffold associated regions: cis-Acting determinants of chromatin structural loops and functional domains. Curr Opinion Gen Dev 2: 275-285.

Mlynárová L, Loonen A, Heidens J, Jansen RC, Keizer PC, Stiekema WJ, Nap J-P. (1994) Reduced position effect in mature transgenic plants conferred by the chicken lysozyme matrix-associated region. Plant Cell 6: 417-426.

Mlynárová L, Jansen RC, Conner AJ, Stiekema WJ, Nap JP. (1995) The MAR-mediated reduction in position effect can be uncoupled from copy number-dependent expression in transgenic plants. Plant Cell 7: 599-609.

Mlynárová L, Keizer LPC, Stiekema WJ, Nap J-P. (1996) Approaching the lower limits of transgene variability. Plant Cell 8: 1589-1599.

Nap J-P, Keizer P, Jansen R. (1993a) First-generation transgenic plants and statistics. Plant Mol Biol Reptr 11: 156-164.

Nap J-P, van Spanje M, Dirkse WG, Baarda G, Mlynárová L, Loonen A, Grondhuis P, Stiekema WJ. (1993b) Activity of the promoter of the Lhca3.St.1 gene encoding the potato apoprotein 2 of the light-harvesting complex of photosystem I, in transgenic potato and tobacco plants. Plant Mol. Biol. 23: 605-612.

Peach C, Velten J. (1991) Transgene expression variability (position effect) of CAT and GUS reporter genes driven by linked divergent T-DNA promoters. Plant Mol Biol 17: 49-60.

Phi-Van L, Strätling WH. (1988) The matrix attachment regions of the chicken lysozyme gene co-map with the boundaries of the chromatin domain. EMBO J: 655-664.

Phi-Van L, Von Kries JP, Ostertag W, Strätling WH. (1990) The chicken lysozyme 5' matrix attachment region increases transcription from a heterologous promoter in heterologous cells and dampens position effects on the expression of transfected genes. Mol Cell Biol 10: 2302-2307.

Stief A, Winter DM, Strätling WH, Sippel AE. (1989) A nuclear DNA attachment element mediated elevated and position-independent gene activity. Nature 341: 343-345.

Thompson JF, Hayes LS, Lloyd DB. (1991) Modulation of firefly luciferase stability and impact on studies of gene regulation. Gene 103: 171-177.

Van Leeuwen W, Hagendoorn MJM, Ruttink T, Van Poecke R, Van Der Plas LHW, Van Der Krol AR. (2000) The use of the luciferase reporter system for in planta gene expression studies. Plant Mol Biol Reptr 18: 143a-143t.

Van Leeuwen W, Ruttink T, Borst-Vrenssen AWM, Van Der Plas LHW, Van Der Krol AR. (2001) Characterisation of position induced spatial and temporal regulation of transgene promoter activity in plants. J Exp Bot, in press.

5

Position induced differences in dynamic transgene promoter activity in response to wounding

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Abstract. The reporter gene firefly luciferase allows the real-time monitoring of changes in transgene expression. Upon wounding of a plant, several metabolic changes occur. In transgenic petunia plants we noted a transient increase in luciferase activity upon wounding. We characterised this wound response in small leaf discs for three promoters; the Cauliflower Mosaic Virus (CaMV) 35S promoter, a modified CaMV 35S promoter (m35S) and an *Arabidopsis thaliana* lipid transfer protein (LTP). We also investigated the role of ethylene and jasmonic acid in this response. Our results show that the dynamics of the wound response is different for each promoter, but is also slightly different in independent transformed lines. This shows that the response of the transgene to wounding and thus the consequent responsiveness to ethylene and jasmonic acid are position dependent.

Introduction

Petunia plants transformed with firefly luciferase (*luc*) constructs driven by the Cauliflower Mosaic Virus (CaMV) 35S promoter, a modified CaMV 35S promoter (m35S) or an *Arabidopsis thaliana* lipid transfer protein (LTP) promoter show, beside position induced quantitative differences, also spatial and (long-term) temporal differences of gene expression between independent lines with the same transgene construct (Van Leeuwen *et al*., 2001). Luciferase allows the monitoring of ongoing gene expression (Van Leeuwen *et al*., 2000) and can thus be used to monitor gene expression over a prolonged period of time. During wounding of a plant, there are rapid metabolic changes in the wounded tissue. The elevated wound respiration reaches a plateau within 15 minutes of excision (MacNicol, 1976). Also the expression of several genes increases. Because the level of expression of transgenes also increases, luciferase is the ideal reporter gene to monitor the dynamics of gene expression during the wound response. Here we investigated the differences in short-term temporal regulation of transgene activity during the transient response to wounding in leaves in independent transformants. Because wounding is accompanied by transient changes in endogenous hormone levels, changes in *luc* reporter gene activity could be related to differences in hormone responsiveness of the transgene.

The wound response pathway can be triggered by mechanical wounding or insect feeding and results in the induction of several genes (Ryan, 1990; Bowles, 1998). Although the CaMV 35S promoter has been used in many gene expression studies, a direct effect of wounding on 35S promoter activity has not been studied extensively. Because this viral promoter uses plant cell transcription factors under stress conditions, it is likely to respond also to wounding. For the *Arabidopsis thaliana* LTP promoter, the wound response was also not previously studied, although for a *Brassica napus* orthologous LTP promoter no increase in expression was observed in response to wounding (Sohal *et al*., 1999).

Wounding rapidly and transiently induces the accumulation of the plant hormones jasmonate (JA) and methyl jasmonate (MJA) (Creelman *et al*., 1992). Jasmonic acid has been proposed as an essential component in regulating plant responses to wounding (Hildmann *et al*., 1992), since the expression of many wound responsive genes is also

JA-inducible (reviewed by Wasternack and Parthier, 1997). The accumulation of salicylic acid (SA) during the wound response may be responsible for the transient nature of the JA dependent induction of gene expression by wounding, as SA inhibits JA synthesis (Doares *et al*., 1995). Another hormone that is involved in the wound response in plants is ethylene. Ethylene levels rise quickly upon wounding, often peaking within an hour. Upon wounding ethylene regulates endogenous JA levels and application of exogenous JA induces ethylene biosynthesis (O'Donnell *et al*., 1996). It has also been shown that JA and ethylene act synergistically in the induction of pathogenesis related (PR) genes (Xu *et al*., 1994).

The molecular response to wounding does not only occur at the wound site, but can also occur systemically. Tomato cells respond to wounding / herbivore attack by releasing a highly mobile octadecameric polypeptide termed systemin, the first peptide hormone found in the plant kingdom, required for the systemic induction of the wound response (Pearce *et al*., 1991). This polypeptide moved throughout the leaf within 30 min and was identified in the phloem exudate within 1-2 hours (Pearce *et al*., 1991). Although oligo-galacturonides (OGA) also play a role in the wound response, they are not mobile and do not move when applied to wounds on tomato plants (Baydoun and Fry, 1985). In addition to these signals, abscisic acid (ABA) may also play a role in the wound response. Exogenous application of ABA has been shown to induce a systemic pattern of proteinase inhibitor mRNA accumulation, identical to that induced by mechanical wounding (Hildmann *et al*., 1992). Endogenous levels of ABA increase, both locally and systemically, in wild-type plants upon wounding (Peña-Cortés *et al*., 1989). This induction may be related to the induced local water stress by wounding as well as to systemin production. An alternative systemic induction may be caused by the volatile nature of the hormones (or hormone derivatives) involved in the wound response (ethylene, methyl-JA and methyl-SA). Probably all the wound signal are transduced to JA, which is the key hormone in wound induced gene expression (Seo *et al*., 1997).

The wound response of different promoter-*luc* reporter constructs in transgenic petunia plants was characterised by measuring luciferase activity in freshly made 3 mm leaf discs. Our experiments show that all three promoters show a different response to wounding, indicating that the overall response is promoter specific. The dynamics of

this response in independent lines with the same reporter gene construct also differs slightly and is characteristic for each independent transformant. This indicates that the short-term temporal response to transient signals during wounding is dependent on the position of the transgene. We were not able to resolve the action of the endogenously regulated hormones that occur in response to wounding (ethylene, JA). The wound response was not consistently altered by either saturating the ethylene or jasmonic acid signalling or blocking ethylene synthesis or the ethylene receptor prior to wounding.

Materials and Methods

LUC reporter gene constructs and plant material

A Cauliflower mosaic virus (CaMV) 35S promoter - *luc* construct (pGM46) or a CaMV m35S promoter - *luc*$^+$ construct (pGM107) was used as described in Van Leeuwen *et al*. (2000). In addition a Lipid Transfer Protein (LTP, Thoma *et al.*, 1994) promoter - *luc* construct (pMT520) was used, kindly provided by Dr Toonen.

Petunia hybrida (Vilm.) plants (cv. V26) were transformed by *A. tum.* clones containing either pGM46, pGM107 or pMT520 as described before (Van Leeuwen *et al*., 2000, 2001).Plants were grown in growth chambers with a 16 h light period (50 W m^{-2}, 22°C, and 70% RH) and an 8 h dark period (20°C, and 65% RH). For the analyses of the pGM46 transformed plants, the F_1 progeny plants of a back-cross with wild-type V26 were used. These plants are coded as: 46-"primary transformant code"b"F_1 progeny code" (*e.g.* 46-1b4).

In vivo *luciferase activity measurement with the 2D-luminometer*

Petunia *luc* reporter plants were sprayed with a luciferin solution (1 mM firefly D-luciferin, sodium-salt, Molecular Probes, Eugene, OR, USA, 0.01% Tween 80) 24 h, 16 h and 2 h before measurement as described in van Leeuwen *et al.* (2000). Plants or leaf discs were imaged with a 2D-luminometer, consisting of an intensified CCD camera (C2400-77, Hamamatsu Photonics, Japan) or a liquid nitrogen cooled slow-scan CCD camera (512-TKB, Princeton Instruments, Trenton NJ, US). Photon emission by *luc*-expressing plants or leaf discs was quantified by computer (Argus-50 Image Processor, Argus 3.43, Hamamatsu Photonics, Japan or MetaMorph 4.1, Universal Imaging Corp., US, respectively). Luciferase activity is quantified in relative light units per pixel (rlu pixel^{-1}). Integration intervals varied from 2 to 15 min.

Measurement of a wound response in 3 mm leaf discs of a transgenic petunia leaf

Two 3-mm leaf discs were taken from a single petunia leaf (between the visible veins) and put in a 3 cm petri-dish with two Whatman 3 filters wetted with 600 mL 0.5 mM luciferin. Petri-dishes were sealed with parafilm and measured overnight with the luminometer.

Results and Discussion

The wound response in planta

The wound response of the different promoter driven luciferase reporter genes was initially studied by wounding of a single leaf of a vegetative petunia plant. These experiments showed that the induced changes in luciferase reporter gene expression depended partly on the way of wounding as shown in a 35S-*luc* petunia plant in figure 1. In figure 1 A-F the effect of an incision through the main vein is shown in a luciferin-pre-sprayed 35S-*luc* petunia leaf *in planta*. Within 2 minutes after the incision, an increase in luciferase activity was observed, originating at the wound and progressing through the veins. Because evaporation continues in the leaf after the wounding, the veinal fluid will be transported from the wounding site to the first and second order veins in the same leaf, resulting in spreading of the activity through the whole leaf within 2-12 minutes. The increased luciferase activity however does not progress to the other leaves of the plant. When the veins of the leaf are not cut, but two 3 mm leaf discs are taken between the major veins as in panels G-I, the fast transport of the wound response through the veins does not occur (figure 1 G-I). Within the 15 minutes interval of measurement, the activity increase is only seen around the wounded area. *In planta* the peak of luciferase activity thus occurs in the first image after wounding *i.e.* within 15 minutes (panel H). This response *in planta* (increased activity directly around the wound, peaking in the first 15 minutes) is comparable for all promoters tested, *i.e.* the 35S, m35S and LTP promoter. *In planta* this wound response swiftly decays, especially in the case of leaf discs taken between the main veins (figure 1 G-I). In contrast, the wound response *in vitro* in leaf discs mostly peaks between 2 and 4 hours (see below). Because of the variable and irreproducible response to wounding in whole plants, it was not possible to compare and distinguish significant differences in the wound response between lines with the same *luc* reporter gene construct. Therefore, the wound response was further characterised in leaf discs.

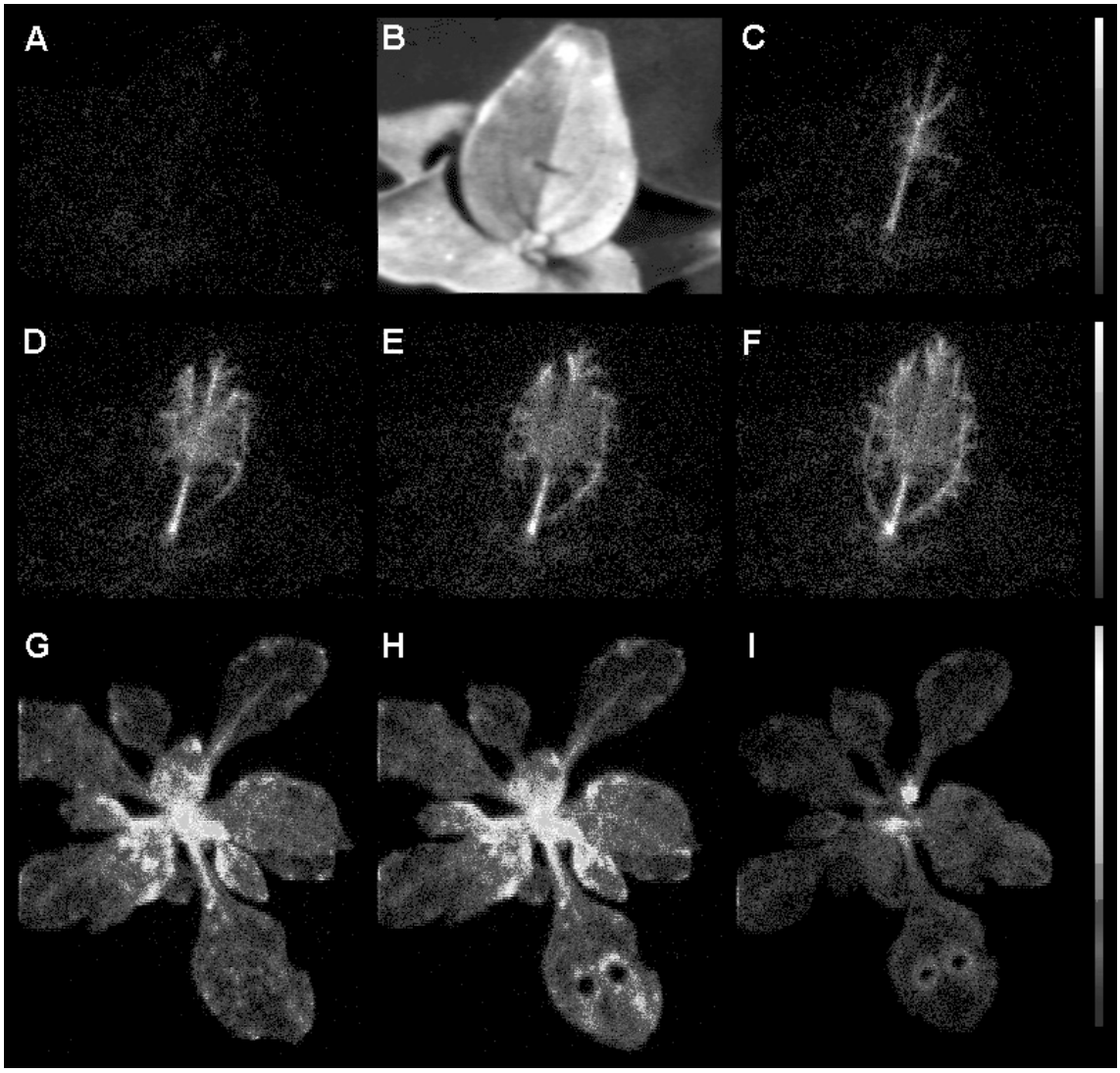

Figure 1. *In planta* wound response in a 35S-*luc* petunia leaf, as shown by luciferase activity (panels A, C, D, E and F) in subsequent images at different time points. Panel A: t= -2 min, B: light image at t= 0 showing incision through main vein, C: t= +2 min, D: t= +4 min, E: t= +6 min, F: t= +12 min).

Panels G to I show another 35S-*luc* petunia plants with a variegated pattern of luciferase activity. Panel G shows the luciferase activity as measured for 15 minutes, after which 2 leaf discs were taken from this plant (bottom right leaf). The plant was measured again directly after leaf disc excision for 15 minutes and the luciferase activity image is shown in panel H. Panel I shows this plant 10 hours after wounding. The grey scale that is used to represent the luciferase activity is shown on the right

Characterisation of a transient wound response in leaf discs

In order to study the wound responsiveness of the different promoters and to compare expression dynamics in independent lines with the same transgene, the response of the luciferase transgene to wounding in leaf discs was studied. The changes in (35S, m35S or LTP driven) luciferase activity in isolated leaf discs were highly variable when the leaf discs had a diameter of 5 mm or larger. This was probably due to the variable amount of vascular tissue in leaf discs of this size (data not shown). In order to get a reproducible wound response, we used 3 mm leaf discs, taken between major veins, consisting of uniform mesophyll cells. In these discs luciferase activity was measured for 18 hours (Van der Krol *et al.,* 1999). The average response (change in luciferase activity) of two times two leaf discs (taken from two leaves) is shown in the experiments below.

The wound response is characterised by an increase of luciferase activity in the first hours after the initial wounding, followed by a decrease resulting in a steady-state level of luciferase activity after overnight incubation (16 to 18 hours). This wound response is shown in figure 2 for four 35S-*luc* transformants (coded 46, panels A-B), three m35S-*luc* transformants (coded 107, panels C-D) and three LTP-*luc* transformants (coded 520, panels E-F). In order to compare transformants with different levels of luciferase transgene expression, the luciferase activity is set to 1, either relatively to t=0 (figure 2A, C, E) or relatively to the maximum peak height (figure 2B, D, F). Although within each transformant, each leaf has a different level of activity, the dynamics of the wound response is reproducible and characteristic for each transformant when plot relatively (to either the activity at t=0 or to the activity at the peak, data not shown, see also Van der Krol *et al*., 1999). The change in LUC activity in response to wounding is different for each of the different promoters. The 35S promoter shows a peak response between 2 and 3 hours; the m35S promoter shows an immediate peak response (within 15 to 30 minutes), while the LTP promoter shows a peak response between 1.5 and 2 hours. Although the timing of this peak resembles that of the 35S promoter, the decay in luciferase activity is much slower in case of the LTP promoter. Because the overall dynamics of LUC activity is different between 35S-*luc*, m35S-*luc* and LTP-*luc* lines (for identically wounded leaf discs), the differences in the dynamics of LUC activity

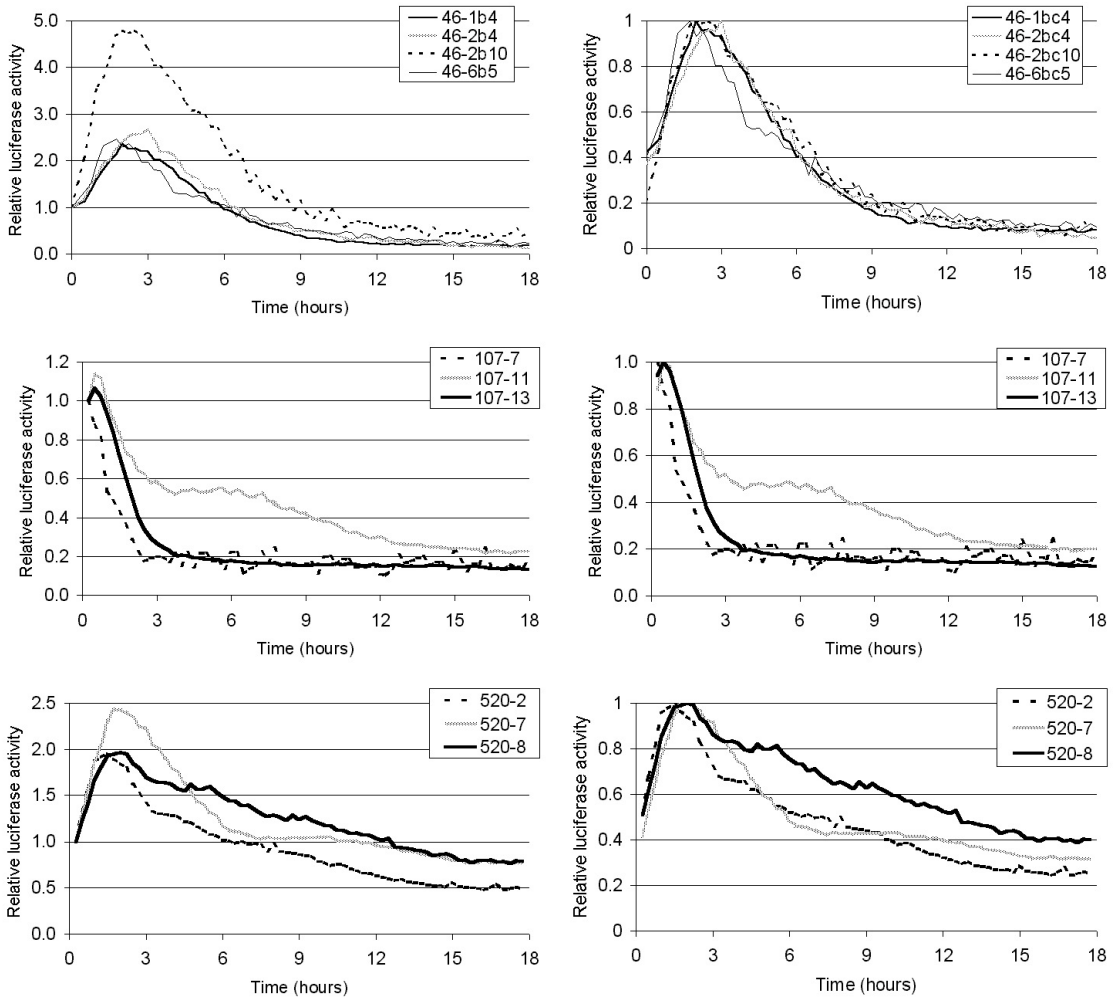

Figure 2. The wound response in excised leaf discs is shown for four 35S-*luc* transformants (panels A,B), three m35S- *luc* transformants (panels C,D) and three LTP-*luc* transformants (panels E,F) as characterised by luciferase activity. Lines 46-2b4 and 46-2b10 are two different segregated progeny plants from the same primary transformant, which contained 2 copies of the 35S-*luc* transgene. In panels A, C and E luciferase activity is plotted relative to t=0 (set to 1). In panels B, D and F luciferase activity is plotted relative to the peak height (set to 1).

can be attributed to a regulation of promoter activity and not to a general physiological effect on LUC activity as a result of the wounding. Although the overall response dynamics is similar for a specific promoter in independent lines, there are also consistent differences between independent lines with the same transgene. For instance, induction in response to wounding is 5-fold for the 46-2b10 line, while only 2-fold in the other 35S-*luc* lines. The relative expression of the m35S-*luc* line 107-11 is also clearly higher between 3 and 12 hours after wounding than the other m35S-*luc* lines.

Similarly, line 520-8 has a slower relative decrease than the other LTP-*luc* lines. In case of the very wound-responsive ZPT2-2 promoter, the increase can be around a factor 30, as was also confirmed by RT-PCR of endogenous ZPT2-2 mRNA steady state levels (Van der Krol *et al.*, 1999). Combined these results show that part of the wound response is influenced by the position of the transgene in the different transformants.

The effect of hormones on an induced wound response in leaf discs

The involvement of ethylene and jasmonic acid during the wound response is previously described (O'Donnell *et al*., 1996; Creelman *et al*., 1992; Seo *et al*., 1997; Bowles, 1998). We investigated whether the changes in LUC activity in response to wounding for all three promoters could be influenced by the addition of hormones or inhibitors of hormone action. Two separate effects could be distinguished: an effect on the peak height and/or on the long-term (overnight) steady state level. We added the hormones or hormone inhibitors either directly after the leaf discs were taken, or we pre-treated whole plants for 24 hours and then took the leaf discs.

Addition of AVG (inhibition of ethylene synthesis) to the leaf discs at t=0 results in a slightly higher peak level and a lower overnight steady-state level than the control for all promoters (data not shown). This suggests that ethylene synthesis during the wound response (O'Donnell *et al*., 1996) initially slightly inhibits the response (possibly induced by JA, Hildmann *et al*.,1992), but after several hours is required to keep the wound induced at a higher level (of gene expression as measured here by luciferase activity), because inhibition of ethylene synthesis results in a lower overnight steady-state level.

Petunia plants were also pre-incubated with either 1-methylcyclopropene (MCP), ethylene or MJA for 24 hours. After this pre-incubation, leaf discs were taken from these plants and the wound response was analysed. For both the 35S-*luc* and LTP-*luc* transgenic petunia's manipulation of ethylene signalling had no or little effect on the wound response, when compared to non-treated plants. MJA pre-treated plants however mostly showed the lowest peak level of the treated plants. This suggests that pre-treatment of the plants with jasmonic acid induces a higher steady state equilibrium of intracellular JA levels, making the plants less responsive to the wound signal (increase in JA, Hildmann *et al*.,1992) upon wounding (faster negative feedback). The reduced

sensitivity to JA thus results in a lower increase in gene expression than the increase in gene expression in non-treated plants, as measured by luciferase activity in the leaf discs. Because addition of ethylene or blocking of the ethylene receptor (MCP) had little effect on the wound response, ethylene effects should probably be studied in synergistic action with JA during the wound response.

Conclusions

The different promoters show different dynamics during the wound response, due to different *cis* elements in the promoters. Different promoters have different binding sites for different transcription factors, which may all be differently regulated by hormones released during the wound response. Further the independent transformants showed a distinctively different characteristic wound response (figure 2) indicating that part of this wound response is influenced by the chromatin surrounding the transgene (position effect).

Saturating the ethylene or jasmonic acid signalling prior to wounding or blocking ethylene synthesis or the ethylene receptor, could not prevent the wound response, nor alleviate the differences in wound response between independent transgenic lines. Although the role these hormones play in the wound response has been established, the overall wound response is probably governed by many other factors involved.

Acknowledgements

We would like to thank Ad Vrolijk and Jessica van Dun for their experimental work in characterising the wound response and the effects of hormones thereon. This work is financially supported by the Dutch Organisation for Scientific Research (NWO).

References

Baydoun EA-H, Fry S (1985) The immobility of pectic substances in injured tomato leaves and its bearing on the identity of the wound hormone. Planta 165: 269-276.

Bowles D (1998) Signal transduction in the wound response of tomato plants. Phil Trans R Soc Lond B 353: 1495-1510.

Creelman RA, Tierney ML, Mullet JE (1992) Jasmonic acid/methyl jasmonate accumulate in wounded soybean hypocotyls and modulate wound gene expression. Proc Natl Acad Sci 89: 4938-4941.

Doares SH, Narváez-Vásquez J, Conconi A, Ryan CA (1995) Salicylic acid inhibits synthesis of proteinase inhibitors in tomato leaves induced by systemin and jasmonic acid. Plant Physiol 108: 1741-1746.

Hildmann T, Ebneth M, Peña Cortés H, Sánchez-Serrano JJ, Willmitzer L, Prat S (1992) General roles of abscisic and jasmonic acids in gene activation as a result of mechanical wounding. Plant Cell 4: 1157-1170.

MacNicol PK (1976) Rapid metabolic changes in the wounding response of leaf discs following excision. Plant Physiol 57: 80-84.

O'Donnell PJ, Calvert C, Atzorn R, Wasternack C, Leyser HMO, Bowles DJ (1996) Ethylene as a signal mediating the wound response of tomato plants. Science 274: 1914-1917.

Pearce G, Strydom D, Johnson S, Ryan CA (1991) A polypeptide from tomato leaves induces wound-inducible proteinase-inhibitor proteins. Science 253: 895-898.

Peña-Cortés H, Sánchez Serrano J, Mertens R, Willmitzer L, Prat S (1989) Abscisic acid is involved in the wound-induced expression of the proteinase inhibitor II gene in potato and tomato. Proc Natl Acad Sci 86: 9851-9855.

Ryan CA (1990) Protease inhibitors in plants: genes for improving defenses against insects and pathogens Ann Rev Phytopathol 28: 425-429.

Seo S, Sano H, Ohashi Y (1997) Jasmonic acid in wound signal transduction pathways. Phys Plant 101 (4): 740-745.

Sohal AK, Pallas JA, Jenkins GI (1999) The promoter of a Brassica napus lipid transfer protein gene is active in a range of tissues and stimulated by light and viral infection in transgenic Arabidopsis. Plant Mol Biol 41: 75-87.

Thoma S, Hecht U, Kippers A, Botella J, De Vries S, Sommerville C 1994. Tissue-specific expression of a gene encoding a cell wall-localized Lipid Transfer Protein from *Arabidopsis*. Plant Physiol 105: 35-45.

Van der Krol AR, Van Poecke RMP, Vorst OFJ, Voogt C, Van Leeuwen W, Borst-Vrenssen AWM, Takatsuji H, Van der Plas LHW (1999) Developmental and wound-, cold-, desiccation-, ultraviolet-B-stress-induced modulations in the expression of the petunia zinc finger transcription factor gene zpt2-2. Plant Physiol 121: 1153-1162.

Van Leeuwen W, Hagendoorn MJM, Ruttink T, Van Poecke R, Van der Plas LHW, Van der Krol AR (2000) The use of the luciferase reporter system for in planta gene expression studies. Plant Mol Biol Reptr 18 (2): 143a-143t.

Van Leeuwen W, Ruttink T, Borst-Vrenssen AWM, Van der Plas LHW, Van der Krol AR (2001) Characterisation of position induced spatial and temporal regulation of transgene promoter activity in plants. J Exp Bot: in press.

Wasternack C, Parthier B (1997) Jasmonate-signalled plant gene expression. Trends in Plant Sci 2 (8): 302-307.

Xu Y, Chang PFL, Liu D, Narasimhan ML, Raghothama KG, Hasegawa PM, Bressan RA (1994) Plant defense genes are synergistically induced by ethylene and methyl jasmonate. Plant Cell 6: 1077-1085.

6

Variegated transgene expression is not related to local endogenous gene expression or to local ethylene or jasmonic acid signalling

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Linus H. W. van der Plas and Alexander R. van der Krol

Abstract. We have shown that many different promoters, which are active throughout leaf tissue, show different patterns of variegation when driving luciferase (*luc*) as a reporter gene. Analysis of local *luc* mRNA levels confirmed that variegated LUC activity in leaves was related to variegated luciferase transgene transcription. In this paper we investigated whether variegated LUC reporter gene activity reflects variation in endogenous transcription factor activity. We also investigated whether local differences in *in planta* LUC activity are related to local hormone signalling. For this purpose we determined the responsiveness of the Cauliflower Mosaic Virus (CaMV) 35S and lipid transfer protein (LTP) promoter to ethylene, jasmonic acid (JA) and salicylic acid (SA). We also tested the effect of saturating the ethylene or jasmonic acid signalling or blocking the ethylene receptor in intact petunia plants, in order to change the variegation of luciferase expression driven by either the 35S, a modified 35S (m35S) or LTP promoter.

Introduction

Quantitative differences between transgenic lines have been observed before and are attributed to the position effect (Dean *et al.*, 1988). Differences in spatial and temporal regulation of transgene promoter activity between individual transformed plants however contribute to these position induced quantitative differences in transgene expression (Van Leeuwen *et al.*, 2001). This was shown by luciferase (*luc*) gene expression driven by promoters known to be active throughout a tissue (*e.g.* Cauliflower Mosaic Virus -CaMV- 35S, lipid transfer protein -LTP-, ZPT), in transgenic petunia plants. *In planta* LUC activity in leaves often shows a variegated pattern with differences in luciferase activity up to a factor 300. Similarly, in transgenic potatoes containing the luciferase reporter gene driven by either a cell division related promoter -CDC2a-, a cyclin promoter -cycB1;1-, an AGPaseS promoter or a patatin promoter -λpat21-, variegated patterns are also observed within different tissues (leaf, stolon, tuber, Verhees, personal communication).

Different independently transformed lines also show differences in the type of variegated luciferase activity. This indicated that the variegated patterns of luciferase activity within a leaf are not exclusively developmentally regulated. Furthermore, we demonstrated that the variegated patterns of luciferase activity in leaves reflect local differences in transgene mRNA steady state levels, suggesting a difference in transgene promoter activity (Van Leeuwen *et al.*, 2001). We examined two hypotheses which may explain the occurrence of variegated patterns of transgene promoter activity within a tissue.

(1) Variable accessibility: Each randomly integrated transgene has different flanking plant DNA sequences, which may have different chromatin structures (*e.g.* euchromatin or heterochromatin). This different chromatin structure around each transgene may result in different accessibility for transcription factors in cells at different positions within the leaf. It has been suggested before that quantitative differences in transgene mRNA steady state levels in independent transformants may be caused by differences in local chromatin structure around the transgene (Dean *et al.*, 1988). The variegated luciferase activity within a leaf would then indicate that this DNA accessibility may not only vary between individual transformants, but also within a plant. Due to the

disruption of the local chromatin structure by the transgene, the accessibility of the transgene promoter may vary in time and place. In this case, an even distribution of transcription factor activity within a tissue would still result in a variegated expression pattern of the transgene. This pattern would then only apply to expression of the transgene and not to that of endogenous genes. The pattern would also be characteristic for each individual transformant.

(2) Variable transcription factor activity: Alternatively, the variegated transgene promoter activity may be caused by true local differences in amount and / or activity of transcription factors that act on the transgene promoter. Since these transcription factors also act on endogenous plant genes, the prediction would be that some of the plant genes also show variegated expression patterns, similar to that of the luciferase reporter gene.

In order to distinguish between these two hypotheses, the mRNA steady state level of a selected group of endogenous plant genes was quantified in leaf tissue samples - sampled by high or low LUC activity- using RT-PCR. As an extension to the second hypothesis, we can speculate on the cause of such variegated transcription factor activity. We previously noted that the CaMV 35S promoter, a modified 35S promoter (m35S) and an LTP promoter are all induced by wounding, indicating a responsiveness to ethylene or jasmonic acid. Such a hormone responsiveness can partly be caused by *cis* elements within the promoter sequence, but we also showed that part of this responsiveness is imposed by endogenous DNA flanking the transgene (position dependent). The observed variegated luciferase activity in petunia plants may then be caused by a variation in local hormone concentrations within a leaf. These variegated hormone concentrations will then result in variegated levels of (hormone responsive) transcription factors and thus (hormone responsive) gene expression. When the variegated LUC activity in leaves is related to local hormone signalling, we would predict that equalising the signalling reduces the variegation. We therefore investigated whether saturation of the plants with methyl-jasmonic acid (MJA) or ethylene or inhibition of the ethylene receptors with 1-methylcyclopropene (MCP) could reduce or change the variegation patterns in transgenic petunia plants.

Materials and Methods

LUC reporter gene constructs and plant material

A Cauliflower mosaic virus (CaMV) 35S promoter - *luc* construct (pGM46) or a CaMV m35S promoter - *luc*$^+$ construct (pGM107) was used as described in Van Leeuwen *et al*. (2000). A Lipid Transfer Protein (LTP, Thoma *et al.*, 1994) promoter - *luc* construct (pMT520) was used, kindly provided by Dr Toonen.

Petunia hybrida (Vilm.) plants (cv. V26) were transformed by *A. tum.* clones containing either pGM46, pGM107 or pMT520, and grown as described in Van Leeuwen *et al*. (2000). For the analyses of the pGM46 transformed plants, the F_1 progeny plants of a back-cross with wild-type V26 were used. These plants are coded as: 46-"primary transformant code"b"F_1 progeny code" (*e.g.* 46-1b4).

Petunia cell suspensions were made by using seedlings of F_1 35S-*luc* or LTP-*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 days (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.

RNA isolation and RT-PCR

RNA was isolated from leaf tissue samples, sampled by high or low LUC activity, as previously described in Van Leeuwen *et al.* (2001). Subsequently, cDNA was synthesised from 2.5 µgram total RNA and an RT-PCR was performed on 1 µL (of 20µL) cDNA, as previously described in Van Leeuwen *et al.* (2001). Intensity of the bands was quantified with the ImageQuant program (Molecular Dynamics, Sunnyvale, CA, USA). Comparison between samples were made after calculation of the amount of amplified cDNA (in pg) that was hybridised on the blot, compared to 20 pg of probe loaded on each blot. The level of ubiquitin mRNA in each sample showed the least variation and all samples were quantified relative to the level of ubiquitin mRNA in each sample.

Used primers and probes

Primers for RT-PCR were designed on the genes mentioned below. The primers on these genes were tested on cDNA derived from wild-type petunia leaves. The amplified DNA fragments (with the expected size) were isolated by agarose gel electrophoresis and were cloned in the pGEM-T easy vector (Promega, Madison, WI, USA). After confirmation of the DNA sequence of the clones, these cloned fragments were used as a probe. The blots containing the RT-PCR fragments, amplified from the RNA samples derived from the high and low luciferase active parts of petunia leaves, were hybridised with these probes.

Function and general expression of genes used for RT-PCR

Gene	Description	GenBank Accession number
luc	firefly luciferase	(M15077)
gapdh	glyceraldehyde-3-phosphate dehydrogenase	(X60346)
yrps	ribosomal protein S3 gene	(U56910)
rbcS	ribulose 1,5-bisphosphate carboxylase small subunit (ssu8)	(X03820)
hsp70	heat shock protein 70	(X13301)
nia	nitrate reductase apoenzyme	(L11563)
myb	myb.Ph3 gene encoding protein 1	(Z13996)
sod	chloroplast superoxide dismutase (EC 1.15.1.1)	(X14352, M20792)
pr-p	pathogenesis-related protein P, PR-3a	(M29869)
aco	L. ethylene forming enzyme, ACC oxidase	(M90294)
acs	1-aminocyclopropane 1-carboxylate synthase, ACC synthase	(Z18953)

All genes (except *luc*)were originally isolated from *Petunia hybrida*. The *gapdh*, *rbcS*, *hsp70*, *nia* and *pr-p* genes are expressed in leaves. The *sod* gene is expressed in chloroplasts, the *yrps* gene is expressed in mitochondria. The *acs* gene is expressed in petals, the *aco* gene is expressed in corolla and the *myb ph3* gene is expressed in flowers. These expression profiles are derived from the references as mentioned in the text below.

Gapdh is a highly conserved glycolytic enzyme often used as a constitutively expressed gene to correlate total mRNA levels between samples (Martin *et al*., 1989). Ribosomal *yrps* can also be used to correlate mRNA levels (Yesodi *et al*., 1997). RbcS catalyses the first reaction in photosynthetic carbon dioxide fixation. Small subunit 8 (ssu8) of the *rbcS* gene family, is expressed at high levels in petunia leaves (4-5 % of the total transcripts, Tumer *et al.*, 1986). Heat shock proteins (*hsp*) are generally thought to act as molecular chaperones in preventing the aggregation of non-native polypeptides and in aiding their correct folding. Because *hsp70* is evolutionary conserved and members of the *hsp70* family in petunia are stress inducible, this gene is also examined (Winter *et al*., 1988). The nitrate reductase apoenzyme (*nia*) was shown to be regulated in petunia by the nitrogen source and to be under the control of the circadian rhythm (Salamoubat and Budang, 1993). The *myb*.Ph3 gene encoding protein 1 has a possible role as a transcriptional activator, although only detected by Avila *et al*. in flowers (1993). Superoxide dismutase (*sod*, EC 1.15.1.1) catalyses the disproportionation of a superoxide anion O_2^- to H_2O_2 and O_2, providing an important defence against oxygen toxicity (Tepperman and Dunsmuir, 1988). We noted before that luciferase gene activity was up-regulated by *e.g*. JA and SA. Because during pathogen attack these hormones are also up-regulated (Maleck and Dietrich, 1999), the pathogenesis-related protein P (*pr-p*), an acid chitinase (originally cloned from tobacco leaf), was also studied. The deduced amino acid sequence of tobacco *pr-p* is 93% identical to the petunia enzyme (Linthorst *et al*., 1990). Two ethylene related genes were also

studied: ACC synthase (*acs*; Michael *et al*., 1992) and ACC oxidase (*aco*; Wang and Woodson, 1992, Tang *et al*., 1993). These are the two final enzymes (in this order) required for the synthesis of ethylene. These genes were studied, because a change in ethylene synthesis can influence (trans)gene expression.

In vivo *luciferase activity measurement with the 2D-luminometer*
Petunia *luc* reporter plants were sprayed with a luciferin solution (1 mM firefly D-luciferin, sodium-salt, Molecular Probes, Eugene, OR, USA, 0.01% Tween 80) 24 h, 16 h and 2 h before measurement as described in van Leeuwen *et al.* (2000). Cell suspensions derived from the Petunia *luc* reporter plants were treated with 0.5 mM luciferin two hours before measurement. Cell suspensions or plants were imaged for 15 minutes 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 Instruments, Trenton NJ, US). Photon emission by *luc*-expressing plants or cell suspensions was quantified by computer (Argus-50 Image Processor, Argus 3.43, Hamamatsu Photonics, Japan or MetaMorph 4.1, Universal Imaging Corp., US, respectively).

*Measurement of the effect of hormones on a transgenic 35S-*luc *or LTP-*luc *petunia cell suspension*
Cell suspensions were equilibrated with 0.5 mM luciferin two hours before hormone addition. 2 mL samples were measured in small containers shaken at 100 rpm or 6 mL samples were measured in a very thin layer in a petri-dish. Hormones were added in different concentrations. The volume of the added hormones was 0.5 to 1% of the total cell suspension volume. The effect on luciferase activity was measured in sequential images overnight.

Hormone treatment of intact transgenic petunia plants
Petunia plants were pre-sprayed with luciferin as described above. Four genetically identical petunia plants were subsequently measured with the luminometer and put in a transparent air-tight container (by water-lock) with an air-volume of 500 mL. One plant was subsequently used as a control, one plant was treated with ethylene (30 μL L^{-1}), one plant was treated with 1-methylcyclopropene (MCP, 100 nL L^{-1}) and the fourth plant was treated with methyljasmonate (MJA). Because the optimum concentration of MJA (with a maximum effect, *e.g.* Xu *et al*., 1994) is approximately 50 μM, we applied pure liquid MJA (Duchefa) to a small (1 cm) disc of filter paper and placed this disc inside the container. Because the MJA rapidly evaporated, 5.5 μL liquid MJA in 500 mL air corresponds to approximately 50 μM MJA. The four containers were put back to the growth chambers for 24 hours. Luciferase activity in the plants was measured after removal of the containers and 15 minutes dispersal of the accumulated volatiles in the headspace.

Results and Discussion

Expression of endogenous plant genes in leaf samples with high or low LUC activity

We analysed variegated *in planta* LUC activity either driven by the 35S, m35S or LTP promoter in leaves and we sampled several high and low luciferase active parts from these leaves for RNA isolation. From each isolated RNA sample, cDNA was synthesised, which was used for RT-PCR using specific primers. Luciferase mRNA levels were tested, as well as mRNA levels of several endogenous genes. For the quantification of endogenous gene expression, genes from different endogenous pathways were selected, as summarised in materials and methods.

The RT-PCR products were detected by hybridisation to specific probes and all samples were quantified relative to the level of ubiquitin mRNA in each sample (Van Leeuwen *et al.*, 2001). The relative luciferase mRNA levels correlated to the *in planta* luciferase activity, although in two sample pairs the ratio between the two samples was smaller than 1.5. In table 1 the results are shown for seven sample pairs (containing a high and a low luciferase active sample): three sample pairs of 35S-*luc* petunia leaves, 1 sample pair of an m35S-*luc* petunia leaf and 3 sample pairs of LTP-*luc* petunia leaves. Ratios within a sample pair were rounded to integer numbers (*i.e.* with a ratio smaller than 1.5, the samples are considered to be similar). A repeat of the RT-PCR for *yrps* showed that the quantification of the RT-PCR was reproducible for all samples (data not shown). In some of the sample pairs the expression of the endogenous genes was differential, similar to luciferase expression (also high/low with a ratio larger than 1.5). In other sample pairs the expression of the endogenous genes was also differential, but opposite to the luciferase activity and expression, *i.e.* high luciferase active part was low in endogenous gene activity and *vice versa*. In these samples the high/low ratio of endogenous gene expression was smaller than 0.67, *i.e.* 1/1.5. Finally, in some sample pairs the mRNA level of the endogenous gene was similar in the two samples.

When the expression data of the endogenous genes in the seven sample pairs are plotted against the luciferase expression in each sample (and not compared in pairs) little or no correlation could be found between luciferase mRNA levels and that of endogenous plant genes ($R^2 < 0.25$). The apparent comparable variegation of *acs* with

	+	0	-	nd
luc	5	2	-	-
gapdh	2	2	3	-
yrps	1	3	3	-
rbcs	1	2	2	2
hsp70	4	1	1	1
nia	4	2	1	-
myb	1	2	4	-
sod	1	4	2	-
pr-p	3	2	2	-
aco	3	-	4	-
acs	5	1	-	1

Table 1. The number of sample pairs for which the difference in gene expression corresponds to the difference in luciferase activity. All samples were compared relative to ubiquitin mRNA levels. Shown in the first three columns are the sample pairs (consisting of a high luciferase active sample and a low luciferase active sample) for which the expression of the genes (high / low) was either corresponding to the measured luciferase activity (+; high/low in LUC activity = high/low in gene expression; ratio>1.5), not different in the two samples (0; 0.67<ratio<1.5) or opposite to the measured luciferase activity (-; high/low in luc expression = low/high in gene expression; ratio < 0.67-*i.e.* smaller than 1/1.5-). In the fourth column the number of sample pairs are shown in which gene expression was not detectable in both samples (column 4, nd).

luciferase activity in 5 sample pairs in table 1 is caused because *acs* was not detectable in the low luciferase expressing sample in 4 of these 5 sample pairs. This lack of correlation means that the observed variegation in luciferase activity is specific for the transgene and that endogenous genes are differently expressed in these samples than luciferase. The data presented in table 1 indicates then, that the examined genes are all regulated by different transcription factors than those acting on the promoter-*luc* transgenes (concluded on the basis of the lack of correlation). The expression of the *hsp70* gene shows the highest (though still very low) correlation with the expression of *luc* (R^2 of 0.25, not shown). The expression of the *yrps* gene has the lowest correlation with the expression of *luc* (R^2 of 0.0003) due to little variation in mRNA levels between the samples, as would be expected of a constitutively expressed gene. Obviously, the samples were small enough to find differences of endogenous gene expression.

Apparently the level of variegation in these random samples is rather high. If there was little variegation, the ratio of expression in the random samples would be near 1 (*i.e.* all in column 0).

The expression of some endogenous genes shows a comparable pattern of variegation (in the sample pairs) and these expression patterns are thus correlated to each other. The R-square values are shown for some genes in table 2. The expression of these genes is more correlated to each other than to the expression of *luc*. R-square values of genes possibly regulated by the same transcription factor are grouped. It is striking that the expression of the *gapdh* gene is also differentially expressed relative to ubiquitin expression (often only a ratio of 2 to 4 was observed, but also a ratio of 34 was observed in one sample pair), while this gene is often used as a constitutively expressed gene to correlate total mRNA levels between samples. The *myb* and *gapdh* genes may be regulated by the same transcription factor (correlation with an R^2 of 0.97). This same transcription factor may also regulate the *sod* and *nia* genes (see R^2 values in table **2**). The *acs* and *aco* gene are also correlated to each other (R^2 of 0.58), as would be expected because they encode enzymes that are part of the same biosynthetic pathway (leading to ethylene). Obviously the *pr-p* is regulated by the same transcription factors acting on *acs* and *aco* (R^2 of 0.80 and 0.78 respectively). That the *yrps* and *rbcs* genes also correlate, indicates that *rbcs* gene expression is also not very variegated. The number of sample pairs was not high enough to distinguish between transcription factors working on the 35S (and m35S) promoter and the LTP promoter.

	myb	*sod*	*nia*
gapdh	0.97	0.80	0.87
nia	0.79	0.55	
sod	0.87		

	pr-p	*acs*
aco	0.78	0.58
acs	0.80	

	rbcs
yrps	0.76

Table 2. R^2 values of the expression levels of several endogenous genes in the seven examined sample pairs plotted against each other.

We conclude that the observed luciferase variegation is specific for the transgene, but that probably most of the examined endogenous genes show some form of variegation. Local differences in amount and / or activity of transcription factors thus probably contribute to the observed variegation in luciferase gene expression. The expression of the endogenous genes is also variegated, albeit not correlated to the *luc* expression. Furthermore, some of the studied endogenous plant genes may be regulated by the same transcription factors.

The effect of hormones on a transgenic petunia cell suspension

To investigate the general sensitivity of the studied promoters to plant hormones in a non-tissue specific situation, petunia cell suspensions were used. Different hormones (or hormone precursors) were added to a cell suspension of transgenic petunia cells and the effect on 35S driven LUC activity was monitored over a period of 18 hours. An important prerequisite for the interpretation of the luciferase reporter activity in cell suspensions is to ensure a constant (high) oxygen pressure in the cell suspension. Luciferase activity in cell suspensions was therefore either measured while shaking at 100 rpm or measured in a very thin layer in a petri-dish. Cell suspensions, were equilibrated with 0.5 mM luciferin two hours before hormone addition, although simultaneous addition of luciferin and hormones did not result in a different response to the added hormone.

In figure 1 the effects of different concentrations of the ethylene precursor ACC (panel A), different concentrations of jasmonic acid (JA, panel B) and different concentrations of salicylic acid (SA, panel C) are shown. The luciferase activity is shown relative to the water treated control (black line). The effects of hormone addition are comparable between a shaken cell suspension in an open container and a cell suspension in a petri-dish, although the "amplitude" of the effect is smaller in an open shaking container due to the dilution of gaseous hormones in the air. The addition of *e.g.* ACC to a cell suspension in an open environment has less effect, probably because of only limited ethylene accumulation under these conditions (even 5 mM ACC only slightly increased luciferase activity, data not shown). An LTP-*luc* containing petunia cell suspension shows a comparable though less severe response than the 35S-*luc*

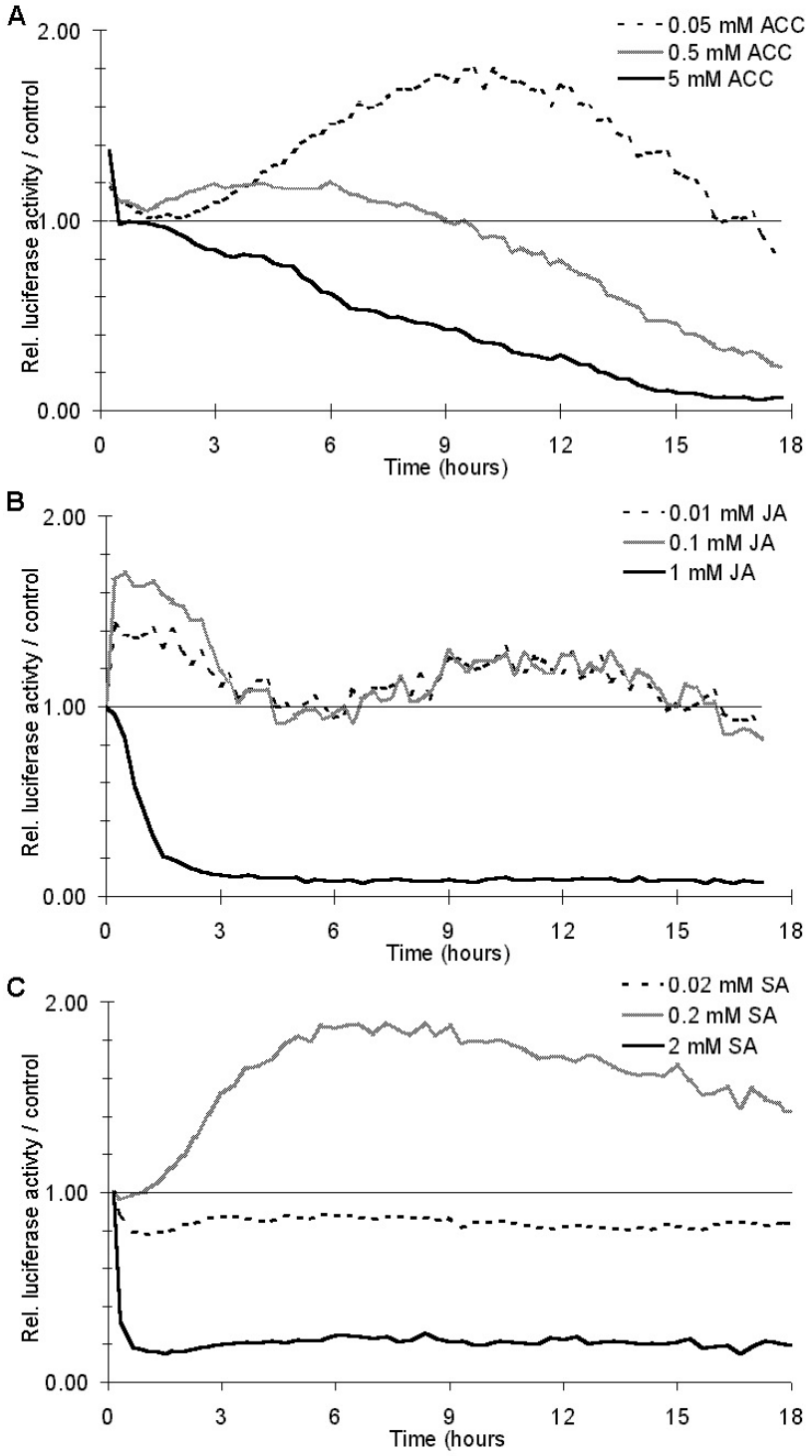

Figure 1. The effect of addition of different concentrations of different hormones to a 35S-*luc* petunia cell suspension. Panel A shows the effects of ACC addition. Panel B shows the effects of jasmonic acid (JA) addition. Panel C shows the effects of salicylic acid (SA) addition. Panel A & C were measured in 6 mL cell suspension in a petri-dish; panel B was measured in 2 mL cell suspension in an open container at 100 rpm.

containing petunia cell suspension to these hormones. Both comparable concentrations that induce gene expression as well as comparable concentrations that reduce gene expression were found (data not shown).

The effect of ACC addition on the luciferase activity of the cell suspension (figure 1A) shows that high levels of ACC (5 mM in a closed environment) result in a decrease in luciferase activity. The addition of 0.5 mM ACC results in a slight increase of luciferase activity (20% increase compared to the control) for several hours, followed by a decrease after 6 hours possibly as a consequence of ethylene accumulation in the sample. The addition of low concentrations of ACC (50 µM) results in a stronger increase of luciferase activity (80% increase compared to the control) for several hours, only decreasing after 11 hours after which probably the ethylene concentration in the sample became inhibiting for the luciferase gene expression.

The effect of jasmonic acid (JA) addition on the luciferase activity of the cell suspension (figure 1B) shows that high concentrations of JA (1 mM) result in a reduction of luciferase activity, while lower concentrations (0.01-0.1 mM) quickly and transiently increase the luciferase activity (to 40-60% increase compared to the control) resulting in a luciferase activity level comparable to the control after 5 hours. Addition of methyl-JA [MJA] to the cell suspension has a comparable effect as JA (data not shown). An optimum / maximum effect on increasing the gene expression of 50 µM has been described for MJA (in tobacco seedlings, Xu *et al*., 1994), corresponding to the results shown in figure 1B.

Salicylic acid (SA) addition to the cell suspension (figure 1C) shows that high concentrations (2 mM) quickly decrease the luciferase activity, that low concentrations (0.02 mM) have no effect on the luciferase activity (comparable to control), but that 0.2 mM SA increases the luciferase activity after a one hour lag. After 6-8 hours the luciferase activity slowly decreases again, probably due the inhibiting effect of accumulated hormones (possibly ethylene or JA). Even with the high levels of hormones added in this experiment (5 mM ACC, 1 mM JA or 2 mM SA) the cells still look viable after overnight measurement. Only the luciferase activity is strongly inhibited. The data shown in figure 1 indicate that the 35S promoter is sensitive to ACC, JA and SA in cell suspensions. The *Arabidopsis thaliana* LTP promoter is comparably, but slightly less sensitive.

The effect of hormones on the level and variegation of in planta *luciferase activity*

We observed several effects of hormone addition on the level of luciferase activity in cell suspensions as well as effects on the wound response in leaf discs (chapter 5). It could therefore be speculated that different levels of hormones or sensitivity to hormones contribute to the different levels of luciferase activity in a leaf (Van Leeuwen *et al.*, 2001). We investigated whether MCP, ethylene or MJA could influence this pattern and / or the level of luciferase activity *in planta*. After 24 hours treatment there was no clear effect of the hormone treatment on the level or on the variegation of luciferase activity. The level of variegation in the control plant differed more from day to day (as was also shown in Van Leeuwen *et al.*, 2001), than that the level of variegation changed in the hormone treated plants (which was mostly between 80 and 120 % of the level of variegation of the control plant). Although these hormones have no clear effect on the level of variegation, it can not be excluded that other hormones may have such an effect.

Conclusions

Position induced quantitative differences in transgene expression are often found between individual transformed plants and are mostly accompanied by a variation in spatial and temporal regulation of the transgene promoter activity. We have shown that the variegated LUC activity patterns observed in transgenic luciferase petunia plants are specific for the transgene. The expression of several endogenous genes was examined and found not to correlate to the LUC activity or *luc* expression in the samples. However, in the examined samples the expression of the studied endogenous plant genes also varied, although different than the luciferase gene expression. Probably the amount and / or activity of different transcription factors, acting on the different genes - including luciferase-, all vary differently. The first hypothesis mentioned in the introduction could only be proven, when the endogenous genes were not variegated (which is not the case). The second hypothesis mentioned in the introduction may thus be correct, although the action of different transcription factors complicates the elucidation of the phenomenon variegation.

Because different hormones and hormone levels have an effect on gene expression, local differences in hormone signalling may cause local differences in transcription

factor activity. We showed in a 35S-l*uc* petunia cell suspension that ethylene, jasmonic acid and salicylic acid indeed can increase the luciferase gene expression without wounding. Because the 35S, m35S and the LTP promoters are all responsive to ethylene and jasmonate signals, local differences in hormone concentrations (or sensitivity) within a leaf may cause the variegated gene expression within a leaf. These differences in hormone concentrations or hormone sensitivity may be caused by micro-wounds (by insect or even bacteria) and / or minor-scale pathogen-attack. The effect of flanking plant DNA sequences (for the luciferase gene different in each transformant) could then be a modulation of the hormone sensitivity of the gene. However, for the hormones studied here, we were not able to eliminate such a proposed local hormone signalling by the use of inhibitors (*e.g.* MCP) or by saturating the plant with a hormone signal (JA or ethylene), *i.e.* the variegation did not clearly change. Thus local ethylene or JA hormone signalling does not seem to be involved in establishing different patterns of transgene activity in independent transformants. We have previously also shown (Van Leeuwen *et al.*, 2000) that treatment of petunia plants with 10 µM ABA had no visible effect on the level or pattern of variegation.

Obviously, the hormones studied in this thesis are not directly responsible for the variegation in luciferase activity as we observe it in our petunia plants.

Acknowledgements

We would like to thank Diaan Jamar for creation and maintenance of the *Petunia* cell suspensions and Jessica van Dun for her work on the effect of hormone treatment on the level or on the variegation of luciferase activity. This work is financially supported by the Dutch Organisation for Scientific Research (NWO).

References

Avila J, Nieto C, Canas L, Benito MJ, Paz-Ares J (1993) Petunia hybrida genes related to the maize regulatory C1 gene and to animal myb proto-oncogenes. Plant J 3 (4): 553-562.

Dean C, Jones J, Favreau M, Dunsmuir P,. Bedbrook J (1988) Influence of flanking sequences on variability in expression levels of an introduced gene in transgenic tobacco plants. Nucl Acids Res 16: 9267-9283.

Linthorst HJM, van Loon LC, van Rossum CMA, Mayer A, Bol JF, van Roekel JC, Meulenhoff EJS, Cornelissen BJC (1990) Analysis of acidic and basic chitinases from tobacco and petunia and their constitutive expression in tobacco. Mol. Plant-Micr Int 3 (4): 252-258.

Maleck K, Dietrich RA (1999) Defense on multiple fronts: how do plants cope with diverse enemies ? Trends in Plant Sci 4 (6): 215-219.

Martin W, Gierl A, Saedler H (1989) Molecular evidence for pre-Cretaceous angiosperm origins. Nature 339: 46-48.

Michael MZ, Baudinette SC, Savin KW, Cobbett CS, Cornish EC (1992) Isolation of petal senescence-associated cDNA clones encoding 1-aminocyclopropane-1-carboxylate synthase from Petunia hybrida and Dianthus caryophyllus. Unpublished.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473-479.

Salamoubat M, Budang HD (1993) Analysis of the petunia nitrate reductase apoenzyme-encoding gene: A first step for sequence modification analysis. Gene, 128: 2, 147-154.

Tang X, Wang H, Brandt AS, Woodson WR (1993) Organization and structure of the 1-aminocyclopropane-1-carboxylate oxidase gene family from Petunia hybrida. Plant Mol Biol 23: 1151-1164.

Tepperman J, Dunsmuir P (1988) Cloning and nucleotide sequence of a petunia gene encoding a chloroplast-localized superoxide dismutase. Plant Mol Biol 11: 871-872.

Thoma S, Hecht U, Kippers A, Botella J, De Vries S, Sommerville C (1994) Tissue-specific expression of a gene encoding a cell wall-localized Lipid Transfer Protein from *Arabidopsis*. Plant Phys 105: 35-45.

Tumer NE, Clark WG, Tabor GJ, Hironaka CM, Fraley RT, Shah DM (1986) The genes encoding the small subunit of ribulose-1,5-bisphosphate carboxylase are expressed differentially in petunia leaves. Nucl Acids Res 14 (8): 3325-3342.

Van Leeuwen W, Hagendoorn MJM, Ruttink T, Van Poecke R, Van der Plas LHW, Van der Krol AR (2000) The use of the luciferase reporter system for in planta gene expression studies. Plant Mol Biol Reptr 18 (2): 143a-143t.

Van Leeuwen W, Ruttink T, Borst-Vrenssen AWM, Van der Plas LHW, Van der Krol AR (2001) Characterisation of position induced spatial and temporal regulation of transgene promoter activity in plants. J Exp Bot, in press.

Wang H, Woodson WR (1992) Nucleotide sequence of a cDNA encoding the ethylene forming enzyme from petunia corollas. Unpublished.

Winter J, Wright R, Duck N, Gasser C, Fraley R, Shah D (1988) The inhibition of petunia hsp 70 mRNA processing during CdCl(2) stress. Mol Gen Genet 211: 315-319.

Yesodi V, Izhar S, Hauschner H, Tabib Y, Firon N (1997) Homologous recombination involving cox2 is responsible for a mutation in the cmS-specific mitochondrial locus of Petunia. Mol Gen Genet 255 (1): 106-114.

Xu Y, Chang PFL, Liu D, Narasimhan ML, Raghothama KG, Hasegawa PM, Bressan RA (1994) Plant defense genes are synergistically induced by ethylene and methyl jasmonate. Plant Cell 6: 1077-1085.

7 Summarising conclusions

In this thesis we have examined the spatial and temporal aspects of gene expression and the position induced differences in transgene expression between individual transformants. For this purpose we imaged luciferase (*luc*) gene expression driven by three different promoters that are active throughout a leaf tissue of transgenic petunia plants. The Cauliflower Mosaic Virus (CaMV) 35S promoter, a modified CaMV 35S promoter (m35S) and an *Arabidopsis thaliana* lipid transfer protein (LTP) were studied. We observed differences in *in planta* luciferase activity between individual transformants, but also between leaves within a plant and within a single leaf. The luciferase activity within a leaf of a single transformant could differ up to a factor 300. Moreover, the way in which *in planta* luciferase activity varies within a plant is different in independent transformants expressing the same transgene.

In chapter **2** we describe experiments from which it can be concluded that the local differences in *in planta* luciferase activity cannot be attributed to possible artefacts of the reporter system. Local limitations of luciferin, ATP or oxygen, which are all required for the luciferase reaction, might contribute to these artefacts. Pre-spraying the plants three to five times with luciferin is sufficient to obtain a steady state light production in "undisturbed" luciferase reporter plants, similar to feeding luciferin through the vascular system. We calculated that the ATP consumed by the luciferase reaction *in planta* is very small compared to the ATP steady state levels within leaf cells, while the cellular ATP concentration is far above the Km of luciferase for ATP. Furthermore, it was shown that oxygen levels could not be limited physiologically, resulting in changes in the local differences in luciferase activity within a leaf. We showed that the *in planta* activity of accumulated luciferase is rapidly declining after the first application of luciferin, indicating that *in planta* either no or very little regeneration of luciferase (activity) occurs due to a possible co-reaction with CoA. We also showed that the half-life of the luciferase protein itself is 2.5 hours *in planta* and

that the half life of the active luciferase protein in the presence of luciferin is only 15 minutes. We thus conclude that, under the condition of repeated pre-treatment with luciferin, the *in planta* luciferase activity is closely related to on-going promoter and translational activity. The differences in luciferase activity that we observed within a plant thus truly reflect differences in luciferase gene expression.

In chapter **3** we thoroughly characterised the spatial differences of luciferase gene expression within a leaf and show that the variegated luciferase activity within a leaf may also vary from day to day. Both the type of spatial expression pattern and the type of temporal expression pattern differed between independent transformants with the same promoter-luciferase construct. This indicates that these differences are related to the different sites of integration in independent transformants. Analysis of local luciferase mRNA levels showed a good correlation with local *in planta* luciferase activity, indicating that *in planta* luciferase activity is directly related to luciferase transgene expression.

The influence of matrix-associated-regions (MAR elements) on reducing the position induced variation was already known from literature. In plants it had been shown that MAR elements can reduce the variation in activity of the stable ß-glucuronidase (GUS) in a population of independent transformants. The luciferase reporter gene allowed a much more precise study of the effect of MAR elements, especially on the spatial and temporal aspects of transgene expression. In chapter **4** we show experiments in which we analysed the effect of MAR elements on position induced differences in luciferase transgene expression. We noted no change in the level of variegation within leaves, in a population of transformants with a luciferase reporter gene flanked by MAR elements compared to a population of transformants with a luciferase reporter gene not flanked by MAR elements. Also, the presence of MAR elements did not seem to result in a reduction in the variation of *in planta* luciferase activity within this plant population. The effect of MAR elements on *in planta* luciferase activity is thus different from that on GUS activity. We concluded that MAR elements probably reduce the position effect of stable gene products by reducing the long-term temporal variation of gene expression. This effect is not visible when real-time reporter genes like luciferase are

used. The complicated relation between gene product stability, a reduction in temporal regulation in gene expression by MAR elements, and the potential effects on reducing variation in gene expression were discussed using a model. Analysis of the model system shows that indeed some changes in gene regulation would affect the variance in stable transgene expression with no effect on the variance in unstable transgene expression.

Apart from the differences in day-to-day temporal variations in luciferase activity between independent transformants (discussed in chapter **3**), the variation in short-term temporal regulation of luciferase activity was also investigated. In chapter **5** we investigated whether and how the position effect influences the changes in transgene expression in response to wounding. Each of the three promoters that were tested (35S, m35S and LTP) showed a different response to wounding. Although the overall response per promoter type was characteristic, there were also differences in wound response dynamics between independent transformants, carrying the same luciferase construct. This indicated that also the short-term dynamics in transgene expression, like that during the wound response, is affected by the site of integration of the transgene. As it was known from literature that ethylene and jasmonic acid play a role in the wound response, we investigated the effects of inhibition of ethylene synthesis, blocking of ethylene receptors, addition of ethylene, or addition of jasmonic acid on the wound response. None of these treatments showed a consistent and dramatic effect on the wound response.

The central question now is: What is the origin of the variegated expression patterns that were observed with *in planta* activity of luciferase reporter genes? We showed that MAR elements did not reduce the level of variegation of 35S-luciferase activity in leaves, indicating that the variegated luciferase activity is not influenced by the chromosomal organisation of transgene DNA in defined loops of transcriptional activity. The question remained whether the spatial and temporal variation of transgene expression, as shown throughout this thesis, are specific for just the luciferase transgene, or also apply to some endogenous plant genes. In chapter **6** we speculated on the possible causes for variegated transgene activity: cell-to-cell differences in

chromatin structure, local differences in the level of transcription factor activity or in sensitivity to transcription factors. We showed that the expression of some endogenous plant genes is also variegated in leaves, albeit this variegated activity was different from or opposite to the luciferase expression. As endogenous gene expression does show signs of variegated activity it is not likely that variegated transgene activity is caused by local differences in transgene accessibility for transcription factors and that this is specific for the transgene only. One might further hypothesise, assuming that hormones can influence the level of transcription factor activity, that local differences within a leaf in level or in sensitivity to hormones can result in variegated patterns of gene expression. However, we showed in chapter **6** that equalising hormone signalling, either by blocking of ethylene receptors, or exogenous application of ethylene or jasmonic acid, had no dramatic effect on either the 35S-LUC, m35S-LUC or LTP-LUC activity in leaves. These hormone treatments neither resulted in a reduction in the level of variegated *in planta* luciferase activity, indicating that at least these hormones are not (solely) responsible for the observed variegated patterns.

The results presented in this thesis show that the luciferase reporter gene allows a much broader analysis of the position induced differences in gene expression than previously shown with more stable reporter genes, like GUS. The results also show that gene expression in a tissue or in a whole plant is more complex than we anticipated, *i.e.* there are very complex patterns in local promoter activity, both in space and in time.

8

Samenvatting

In dit proefschrift zijn de temporele en spatiële aspecten van (trans)genexpressie beschreven. De verschillen in transgenexpressie tussen individuele transformanten, veroorzaakt door het positie-effect, zijn onderzocht door gebruik te maken van het luciferase-gen (*luc*) uit de Amerikaanse vuurvlieg (*Photinus pyralis*). Luciferase-expressie is onderzocht in transgene petuniaplanten (*Petunia hybrida*), waarin het luciferase-gen werd aangestuurd door drie verschillende promotoren, te weten: de Cauliflower Mosaic Virus (CaMV) 35S promoter, een aangepaste CaMV 35S promoter (m35S) of een "lipid transfer protein" (LTP) promoter uit *Arabidopsis thaliana*. Deze drie promotoren zijn allen actief in de bladeren van transgene petuniaplanten en hun expressie in het vegetatieve deel van de plant is hier onderzocht.

De *in planta* luciferase-activiteit verschilde in niveau tussen individuele transformanten (het traditionele positie-effect), maar er werden ook verschillen in luciferase-activiteit opgemerkt tussen de individuele bladeren van een plant. Zelfs binnen elk blad van elke transformant varieerde de luciferase-activiteit (soms wel tot een factor 300). De manier waarop de luciferase-activiteit varieerde binnen een plant (en binnen een blad) was verschillend tussen en karakteristiek voor individuele transformanten, zelfs als de transformanten een zelfde promoter-luciferase-construct bevatten.

In hoofdstuk **2** worden de experimenten beschreven die aantonen dat de geobserveerde lokale verschillen in *in planta* luciferase-activiteit niet worden veroorzaakt door mogelijke artefacten van het luciferase-reportersysteem. Een lokale beperking in de hoeveelheid luciferine, ATP of zuurstof (de drie substraten die nodig zijn voor de luciferase-reactie) zou tot een dergelijk artefact kunnen leiden. Door de te meten planten drie tot vijf maal voor te bespuiten met 1 mM luciferine wordt een steady-state lichtproductie verkregen, die vergelijkbaar is met de lichtproductie in petuniastengels die in luciferine staan (vasculaire opname). Verder is er berekend dat de hoeveelheid ATP, die verbruikt wordt door de luciferase-reactie *in planta*, 100 tot

10,000 keer kleiner is dan de ATP steady-state niveaus in bladcellen en dat dit niveau ver boven de Km van luciferase voor ATP ligt. Een fysiologische reductie van de zuurstofniveaus binnen in de plant, door sluiting van de huidmondjes, resulteerde ook niet in (lokale) veranderingen in luciferase-activiteit binnen een plant of in een blad. Aangezien de *in planta* activiteit van opgehoopt luciferase snel afneemt na de eerste keer toedienen van luciferine, is het waarschijnlijk dat er weinig of geen regeneratie van luciferase is *in planta* door een mogelijke co-reactie met co-enzym A. De halfwaardetijd van het actieve luciferase-eiwit is in de aanwezigheid van luciferine slechts 15 minuten, terwijl het luciferase-eiwit zelf (in de afwezigheid van luciferine) een halfwaardetijd heeft van 2.5 uur *in planta*. Onder de in dit proefschrift beschreven condities van herhaaldelijke voorbehandeling van de planten met luciferine, is de *in planta* luciferase-activiteit dus zeer nauw gerelateerd aan de ”live” transcriptie en translatie van luciferase. De verschillen in luciferase-activiteit, die hier beschreven zijn binnen de plant, representeren dus werkelijke verschillen in luciferase-genexpressie.

In hoofdstuk **3** zijn de spatiële verschillen in luciferase-genexpressie binnen een blad gekarakteriseerd en wordt getoond dat deze gevariëgeerde luciferase-activiteit binnen een blad ook van dag tot dag verschilt. Zowel het spatiële expressiepatroon als het temporele expressiepatroon verschilt tussen individuele transformanten met hetzelfde promoter-luciferase-construct. Dit duidt erop dat deze verschillen worden veroorzaakt door verschillen in integratieplaats van het transgen tussen individuele transformanten. Analyse van de lokale luciferase-mRNA niveaus resulteerde in een goede correlatie met *in planta* luciferase-activiteit. Dit toont aan dat *in planta* luciferase-activiteit direct gerelateerd is aan luciferase-transgenexpressie.

Nucleaire matrix-geassocieerde DNA-sequenties (zogenaamde “matrix-associated-regions” of MAR-elementen) kunnen de positie-geïnduceerde variatie in transgenexpressie reduceren, zoals al beschreven in de literatuur. In planten is aangetoond dat MAR-elementen de variatie kunnen verminderen in de activiteit van het stabiele ß-glucuronidase (GUS) in een populatie van onafhankelijke transformanten. Het luciferase-reportergen gaf de mogelijkheid om het effect van MAR-elementen veel nauwkeuriger te bestuderen, met name het effect op de spatiële en temporele aspecten van transgenexpressie. In hoofdstuk **4** worden de experimenten beschreven waarin we het effect van MAR-elementen analyseren op positie geïnduceerde variatie in luciferase-transgenexpressie. Twee populaties transformanten werden vergeleken: één met een luciferase-reportergen geflankeerd door MAR-elementen en één met een

luciferase-reportergen niet geflankeerd door MAR-elementen. We zagen geen reductie in het niveau van variegatie binnen bladeren, noch een reductie in het niveau van variatie van *in planta* luciferase-activiteit tussen planten. Het effect van MAR-elementen op *in planta* luciferase-activiteit is dus anders dan het effect op GUS activiteit. We kwamen tot de conclusie dat MAR-elementen waarschijnlijk het positie-effect van stabiele genproducten reduceert door de lange-termijn temporele variatie van genexpressie te verminderen. Dit effect is niet zichtbaar wanneer "live" reportergenen zoals luciferase worden gebruikt. De ingewikkelde relatie tussen de stabiliteit van het genproduct, een reductie in temporele regulatie van genexpressie door MAR-elementen, en de mogelijke effecten hiervan op een reductie in de variatie van genexpressie, worden bediscussieerd in een model. De analyse van dit modelsysteem toont aan dat er inderdaad veranderingen in genregulatie zijn, die wel een effect hebben op de variantie van stabiele genproducten maar geen effect hebben op de variantie van instabiele genproducten.

Behalve de verschillen van dag tot dag in temporele variatie van luciferase-activiteit tussen onafhankelijke transformanten (hoofdstuk **3**), hebben we ook de korte-termijn temporele variatie in luciferase-activiteit onderzocht. In hoofdstuk **5** wordt beschreven of en hoe het positie-effect de verschillen in transgenexpressie als gevolg van verwonding beïnvloedt. Elk van de drie promotoren hier getest (35S, m35S en LTP) vertoont een verschillende wondrespons. Hoewel de algemene respons karakteristiek was voor elke promoter, waren er ook duidelijk verschillen in wondresponsdynamiek tussen onafhankelijke transformanten met hetzelfde luciferase-construct. Dit geeft een indicatie dat ook de korte-termijndynamiek van genexpressie, zoals tijdens een wondrespons, beïnvloed wordt door de plaats van integratie van het transgen. Zoals bekend uit de literatuur, spelen ethyleen en jasmonzuur een rol in de wondrespons. We hebben daarom onderzocht wat het effect van remming van de ethyleensynthese, blokkeren van de ethyleenreceptoren, of toevoeging van ethyleen of jasmonzuur op de wondrespons was. Geen van deze behandelingen resulteerde in een consistent en duidelijk effect op de wondrespons.

De centrale vraag blijft nu: Wat is de oorzaak van de gevariëgeerde expressie patronen, zoals die zijn geobserveerd bij *in planta* activiteit van luciferase-reportergenen? MAR-elementen reduceerden niet het variëgatieniveau van 35S-luciferase-activiteit in bladeren. Dit geeft aan dat de gevariëgeerde luciferase-activiteit

niet beïnvloed wordt door de chromosomale organisatie van het transgen-DNA in afgebakende "lussen" van transcriptionele activiteit. De vraag bleef echter of de spatiële en temporele variatie van transgenexpressie, zoals getoond in dit proefschrift, specifiek is voor alleen het luciferase-transgen, of ook van toepassing is op endogene plantgenen. In hoofdstuk **6** speculeren we over de mogelijke oorzaken van gevariëgeerde transgenactiviteit: intercellulaire verschillen in chromatine structuur, lokale verschillen in het niveau van transcriptiefactoren of in de gevoeligheid voor transcriptiefactoren. We laten zien dat de expressie van bepaalde endogene plantgenen ook gevariëgeerd is in bladeren, alhoewel deze gevariëgeerde activiteit anders was dan of zelfs tegenovergesteld was aan de luciferase-expressie. Omdat endogene genexpressie ook gevariëgeerde activiteit vertoont, is het niet waarschijnlijk dat gevariëgeerde transgenactiviteit veroorzaakt wordt door specifieke lokale verschillen in transgentoegankelijkheid voor transcriptiefactoren en dat dit specifiek is voor het transgen. Aangezien de hier gebruikte promotoren allen gevoelig zijn voor bepaalde hormonen (hoofdstuk **6**), zou een verdere hypothese over de mogelijke oorzaak van gevariëgeerde genexpressie kunnen zijn, dat lokale verschillen in niveau van of in gevoeligheid voor bepaalde hormonen resulteren in een gevariëgeerd patroon van genexpressie. Door echter het hormoonsignaal te egaliseren (door blokkeren van de ethyleen receptoren, of exogene toediening van ethyleen of jasmonzuur) zagen we geen duidelijk effect op de 35S-LUC, m35S-LUC of LTP-LUC activiteit in bladeren. Deze hormoonbehandelingen resulteerden ook niet in een reductie in het niveau van gevariëgeerde *in planta* luciferase-activiteit. Dit geeft een indicatie dat deze hormonen niet (alleen) verantwoordelijk zijn voor de geobserveerde gevariëgeerde patronen.

Door een beter inzicht te krijgen in genexpressie, zal het mogelijk zijn om transgenen effectiever en meer gecontroleerd tot expressie te brengen in planten. De resultaten in dit proefschrift laten zien, dat het luciferase-reportergen (uit de vuurvlieg) een veel bredere analyse van positie geïnduceerde verschillen in genexpressie mogelijk maakt, dan eerder getoond met stabielere reportergenen, zoals bijvoorbeeld GUS. De resultaten tonen tevens dat genexpressie in een weefsel of in een hele plant veel complexer is dan we verwachtten. Er zijn zeer complexe patronen in lokale promoteractiviteit (genexpressie), die zowel in niveau als in ruimte en tijd variëren.