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Enhanced luciferin entry causes rapid wound-induced light emission in plants expressing high levels of luciferase

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Abstract. In-vivo imaging of transgenic tobacco plants (*Nicotiana tobacum* L.) expressing firefly luciferase under the control of the *Arabidopsis* phenylalanine ammonia-lyase 1 (PAL1)-promoter showed that luciferase-catalyzed light emission began immediately after the substrate luciferin was sprayed onto the leaves and reached a plateau phase after approximately 60 min. This luminescence could easily be detected for up to 24 h after luciferin application although the light intensity declined continuously during this period. A strong and rapid increase in light emission was observed within the first minutes after wounding of luciferin-sprayed leaves. However, these data did not correlate with luciferase activity analysed by an in-vitro enzyme assay. In addition, *Arabidopsis* plants expressing luciferase under the control of the constitutive 35S-promoter showed similar wound-induced light emission. In experiments in which only parts of the leaves were sprayed with luciferin solutions, it was shown that increased uptake of luciferin at the wound site and its transport through vascular tissue were the main reasons for the rapid burst of light produced by preformed luciferase activity. These data demonstrate that there are barriers that restrict luciferin entry into adult plants, and that luciferin availability can be a limiting factor in non-invasive luciferase assays.

Key words: *Arabidopsis* (wounding) – Firefly-luciferase – *Nicotiana* (wounding) – Phenylalanine ammonia-lyase – Reporter gene – Wounding

Introduction

Reporter-gene constructs are frequently employed for analyzing gene expression in tissues and at the cellular level. Particularly in plant biology, the genes encoding β-glucuronidase (GUS), green fluorescent protein (GFP) and firefly luciferase are widely used. Whereas GUS represents a reporter that can only be assayed in vitro by either histochemical staining or enzyme assays, GFP and the different luciferases represent systems that allow the non-invasive in-vivo observation of gene activity. However, for continuous observation of promoter-driven reporter-gene activity, GFP has the disadvantage that the high light intensities required for excitation of its fluorescence might interfere with biological processes such as UV- or blue-light-dependent gene activation. In contrast, as no appreciable toxic side effects for firefly luciferin have been reported thus far, the firefly luciferase system seems to be well suited for continuous detection of reporter-gene activity. As an additional advantage, firefly luciferase has a short half-life of about 3 h in cells (Thompson et al. 1991; Worley et al. 2000), which is further reduced by the presence of the substrate. Thus, if luciferin is present in saturating amounts, the luciferase catalyzed light emission directly reports the de-novo synthesis of luciferase as the enzyme does not accumulate in significant amounts. Obviously, it is a prerequisite for continuous measurements that luciferase is supplied with saturating amounts of luciferin.

Several studies using firefly luciferase catalyzed luminescence from plant tissue have been conducted (e.g., Millar et al. 1995; Ishitani et al. 1997). All such studies reported fast entry of luciferin into the tissues leading to immediate light emission (Ow et al. 1986; Kost et al. 1995). However, most of these studies could not completely exclude a possible contribution of substrate accessibility to the obtained images (Schneider et al. 1990).

Surprisingly, only one study (van der Krol et al. 1999) employing continuous luciferase imaging after invasive treatments of adult plants expressing luciferase under the control of a stress-regulated promoter has been published so far.

We are studying gene activation processes underlying pathogen resistance in plants. As tools, we have generated a set of transgenic plants expressing firefly luciferase

Abbreviations: GUS = β-glucuronidase; PAL = phenylalanine ammonia-lyase

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under the control of a variety of pathogen-inducible promoters. One of these pathogen-responsive promoters was from the phenylalanine ammonia-lyase 1 (PAL1) gene of *Arabidopsis* which has been characterized by several groups before (Ohl et al. 1990; Wanner et al. 1995). In these studies, the PAL1 promoter was fused to either GUS (Ohl et al. 1990) or bacterial luciferases (Giacomin and Szalay 1996). Based on these studies, we expected that the regulatory elements present in this promoter region were sufficient to confer responsiveness to various stimuli in transgenic tobacco. Here, we demonstrate that continuous in-planta imaging of luciferase activity is drastically affected by invasive treatments that alter the availability of luciferin to the cells.

Materials and methods

Plant transformation

The PAL1 promoter fragments were amplified from genomic DNA [*Arabidopsis thaliana* (L.) Heynh. ecotype Columbia] using the primers pal1 (CATTGCCTTTGTCATGCAAACG position 800–821) and pal2 (TGTCTTGATGTCTCCGCCGC position 2019–2000) provided by MWG-Biotech (Ebersberg, Germany). The DNA fragments were cloned into the pCRII-vector (Clontech, Palo Alto, Calif., USA) and sequenced. Plasmids were sequenced using a Licor 4000L DNA-sequencer and the thermosequenase-cycle sequencing kit provided by Amersham-Pharmacia-Biotech. An individual clone showing no deviations from the published sequence was chosen for further construction of the reporter-gene transformation vector. The promoter fragment was excised by *Bgl*II and *Sna*BI digestion and ligated into the pGEM-luciferase vector (Promega) that had been cut by *Not*I, filled with the Klenow fragment of DNA polymerase, and was further cleaved by *Bam*HI. From this vector the promoter – reporter fusion cassette was removed by digestion with *Hind*III and *Ecl*136II and ligated into the binary transformation vector pBI101 (Clontech) that had been cut by the same enzymes to remove the GUS reporter gene. The resulting vector pBIPALLUC was used for *Agrobacterium* (GV3101)-mediated transformation of tobacco (*Nicotiana tabacum* L. SamsungNN) leaf disks (Horsch et al. 1985). Resulting primary transformed plants (PL-tobacco) were screened for luciferase activity and selfed. Individual plants from the T1 generation with considerable reporter gene activity were selected and their offspring (T2) used for the experiments described in this paper.

Luciferase measurements

Plants were sprayed with 2.5 mM Na-luciferin (Molecular Probes, PoortGebouw, The Netherlands) solutions with or without 0.01% Triton X-100. Luminescence was measured using a Hamamatsu photon-counting video system controlled by either Argus50 or HiPic5.1 software. In the case of uptake studies, quantitative analysis was performed on images taken in "centre of gravity" mode in which a detected photon is represented by a single pixel. For general imaging, the "slice"-mode, in which a photon corresponds to an area rather than a pixel, was used. Quantitative image analysis was performed using the ImagePro-software (Media Cybernetics). Luciferase assays were carried out as previously described (Luehrsen et al. 1992; Hartmann et al. 1998). Briefly, plant material was homogenized in an Eppendorf-reaction tube in extraction buffer (100 mM potassium phosphate, pH 7.5; 5 mM EDTA; 10 mM 2-mercaptoethanol) with a plastic cone and quartz-sand. Extracts were centrifuged and the supernatant used for luciferase assays and protein determination. The protein content was estimated using the Bradford protein assay reagent (BioRad) according to the manufacturer's protocol. Luciferase activity assays were analysed using a Luminoskan Ascent plate-reader (Labsystems, Frankfurt, Germany). Fifty or 100 µl of assay mix (20 mM Tricine, 3.74 mM $MgCl_2$, 270 µM coenzyme A, 530 µM ATP, 470 µM luciferin, 33.3 mM dithioetythritol, 0.1 mM EDTA, pH 8.0) were injected into 5 or 10 µl of extract, respectively, and light emission measured for 1 s. Specific luciferase activity was defined as light units per well divided by protein content in µg/µl.

Treatment of plants

Plants were wounded by crushing the central vein of a leaf using either a hemostat or forceps. Alternatively, leaf disks were excised using a cork borer. For cycloheximide treatment, leaves were removed using a sharp blade and the petiole immersed into either water (control) or cycloheximide (10 µg/ml). Leaves were then sprayed by luciferin and observed for 4–12 h before wounding.

Results and discussion

During this study we generated 15 primary transgenic tobacco plants carrying the *pal1-luc*-fusion construct. All of these plants expressed luciferase, as determined by light emission after application of luciferin by spraying. Constant light emission from leaves was reached 40–60 min after spraying (Fig. 1a). Light was emitted from stems, leaves and flowers as expected for luciferase expression under the control of the PAL1 promoter (Ohl et al. 1990; data not shown). Light emission decayed slowly after the initial saturation phase (Fig. 1b).

All plants tested showed a strong induction of light emission after wounding of the leaves by either cork borer, forceps, or hemostat; these treatments are frequently used in studies analyzing wound-responses in plants. This wound-induced light emission began within the first minute after treatment, reached a maximum at about 60 min after treatment (Fig. 1b) and was strongest in the veins of the wounded leaf (Fig. 2). As we were intrigued by this apparently rapid gene activation, which would require completion of signal transduction, transcription and translation of luciferase within less than a minute, we attempted to confirm the response by in-vitro measurements of luciferase activity in wounded leaves 30 min after treatment (Fig. 3). To our surprise, these in-vitro data did not correlate with the strong induction observed with the photon-counting video system in planta. Only a weak local increase in luciferase activity could occasionally be observed. We also tried to visualize the assumed increase of luciferase protein by immunofluorescence, but no substantial change in luciferase amounts or distribution was detected (U. z. Nieden, IPB, Halle, Germany, personel communication).

To establish further whether light emission was due to the activity of preformed or de-novo-synthesized luciferase, we conducted a series of experiments in which we attempted to deplete the plants of accumulated enzyme. One strategy employed in many laboratories is to preincubate the plants with luciferin which destabilizes luciferase activity. Indeed, light emission of our transgenic plants decreased with time (Fig. 1b).

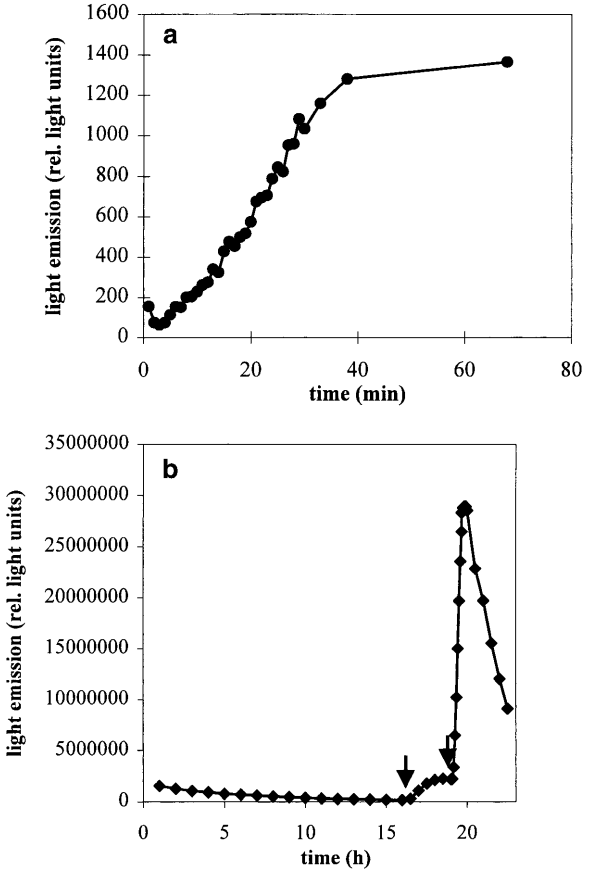

Fig. 1. Light emission from a leaf of a transgenic tobacco plant carrying the PAL1:luciferase reporter cassette after initial spraying with substrate solution (**a**) and 16 h following this saturation phase (**b**). After this 16-h observation, the leaf was re-sprayed with luciferin solution (*first arrow*), and then crushed using a hemostat (*second arrow*). Light emission was integrated for 1 min each using the photon-counting video system. Pixel intensities were calculated and are represented as relative light units

However, re-spraying such pretreated plants with luciferin-solutions still led to increased luminescence, which indicated that substrate, not enzyme, was limiting after overnight incubation. Furthermore, we observed rapid wound-induced light emission without correlation to the length of the preincubation period. Treatment of leaves with the translation inhibitor cycloheximide led to reduction of total light emission; however, the pattern and timing of the rapid wound response remained unchanged (Fig. 2E–H). The careful removal of leaves for such experiments did not cause a significant response. However, the interpretation of experiments using cycloheximide is problematic. Luciferase is an unstable enzyme with a half-life of approx. 3 h and can only accumulate to high levels if it is continuously produced. If protein synthesis is inhibited by cycloheximide, luciferase activity will decline according to its half-life. Therefore, determining if the wound-induced light emission depends on preformed enzyme or on de-novo synthesis might not be possible by using cycloheximide as this inhibitor has the potential to block both. In order to analyze whether increased access to the substrate luciferin could be the reason for the increased light emission we analyzed uptake and distribution of luciferin in more detail. After local spotting of luciferin solutions, treated areas started to emit photons within the first minute; however, light production did not extend to untreated parts of the leaf. In another set of experiments, only half of the leaf area was sprayed, and light emission was analyzed before and after wounding (Fig. 2A–D). Light emission was observed from the sprayed parts of the leaf within minutes and this pattern remained stable for several hours with little light emission from untreated parts of the leaf. When leaves were wounded within the sprayed area we observed the expected wound response (Fig. 2A–D) followed by the rapid spread of light emission into leaf areas that had not been sprayed. This indicates that luciferin is transported via the vascular system into the untreated tissue. Conversely, when the untreated part was wounded no increase in light emission was found, indicating that no increased luciferin transport from unwounded tissue into the wounded area took place. In a third set of experiments, similar to those reported by Barnes (1990), we excised leaves from the plants with a sharp blade and applied luciferin by immersing the petiole in substrate solutions. In this case, light emission started from the petiole and moved towards the leaf tip with a speed and intensity that was comparable to that observed after wounding. When such leaves were wounded no rapid burst of light emission was observed. However, the expected activation of the PAL1 promoter by wounding caused a weak increase in luciferase activity about 1 h after treatment as visualized in planta by the photon-counting camera system (data not shown).

To obtain further evidence for the hypothesis that wounding causes enhanced entry and transport of luciferin, we analyzed transgenic *Arabidopsis* plants expressing luciferase under the control of the strong and constitutive 35S-promoter (I. Hofmann and B. Tschiersch, IPB, Halle, Germany) with respect to wound-induced light emission. Consistent with our earlier results, a burst of light emission was induced by wounding these plants (Fig. 2I–J).

In summary, these data clearly demonstrate that spraying leaves with substrate did not result in saturation of the tissue with luciferin, which is a key presumption for kinetic studies of luciferase expression studies in vivo. We assume that in plants that constitutively express substantial amounts of luciferase activity, luciferin sprayed onto the leaf surface will be consumed as soon as it has entered the plant and, therefore, a saturation of more-distant cells and tissue can not be achieved. By damaging the leaf surface, luciferin that has accumulated on the cuticle will then be able to enter the tissue without any significant barrier. As soon as it has reached vascular tissue by diffusion, it will be distributed throughout the whole leaf where it becomes accessible to preformed luciferase.

As we have observed wound-induced uptake of luciferin in two different species with two different promoters, we expect that the restricted access to luciferin is evident in a variety of systems, although

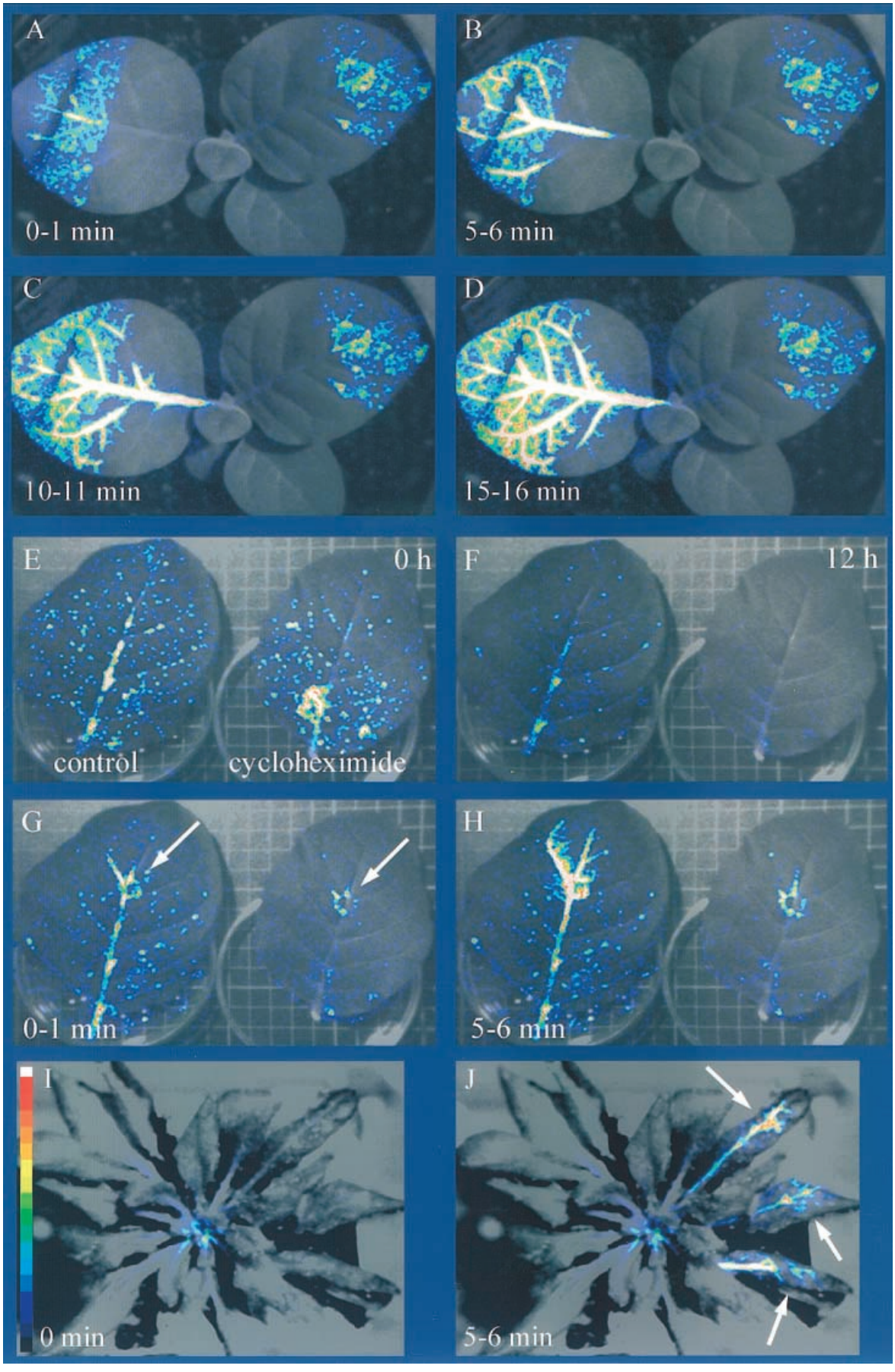
A
0-1 min
B
5-6 min
C
10-11 min
D
15-16 min
E
0 h
control
cycloheximide
F
12 h
G
0-1 min
H
5-6 min
I
0 min
J
5-6 min

Fig. 2A–J. *In-planta*-imaging of light emission from luciferase-expressing plants. Light emission is represented in false colors (see **I** for color scale) overlayed onto greyscale images of the illuminated plants. **A–D** Light emission of partially sprayed leaves of a PL1-tobacco plant. The distal halves of the leaves had been sprayed with luciferin 1 h before the left leaf was crushed using a hemostat (visible as dark mark on the background greyscale image) and light emission was integrated for intervals indicated. **E–H** Light emission from control and cycloheximide-treated leaves of PL-1-tobacco. Leaves were removed from the plant and the petiole immersed in either water (control) or cycloheximide solution (10 μg/ml). Light emission was recorded for 1 min immediately after the onset of cycloheximide treatment (**E**) and 12 h later (**F**). After these 12 h the leaves were wounded using a cork borer (*arrows*) and the light emission recorded immediately (**G**, 0–1 min) and 5 min after wounding (**H**). **I–J** Light emission of an *Arabidopsis thaliana* plant expressing luciferase under the control of the constitutive 35S-promoter. The plant had been sprayed with luciferin 1 h before three leaves were wounded using a hemostat (indicated by *arrows*). Light emission was integrated for 1 min immediately before (**I**) and 5–6 min after wounding (**J**)

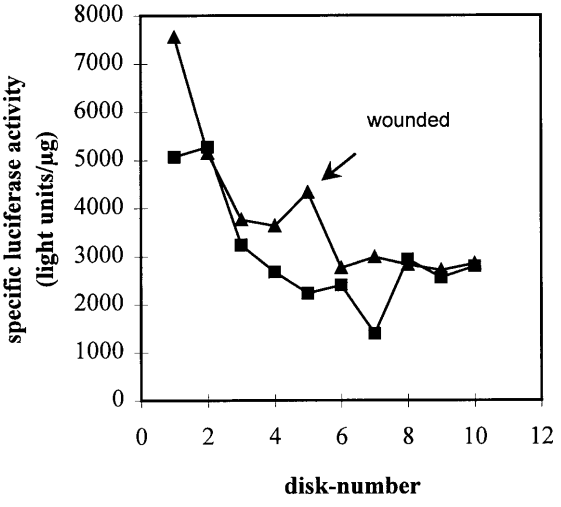

Fig. 3. In-vitro assay of specific luciferase activity in wounded and control leaves of a PAL1: luciferase tobacco plant. The central veins were dissected from base (disk no. 1) to tip (disk no. 10) and each of these leaf disks extracted for in-vitro luciferase measurements. Leaf disk no. 5 contained the wounded area. *Triangles*, wounded leaf; *squares*, untreated control leaf

the observed effects will depend on the physiological conditions of a plant, particularly how strongly luciferin uptake via the cuticle is restricted. Also, the expression pattern of the particular transgene will influence luciferin uptake as high expression of luciferase in epidermal tissue or cells surrounding the vascular bundles would be expected to limit luciferin transport into the more-distal parts of the plant since luciferin will be consumed before it reaches other tissues.

Earlier studies that successfully used continuous measurement of light emission from luciferase-expressing plants mainly focused on in-vitro-grown seedlings where uptake via roots and leaves was obviously not restricted. In such an experimental system, effects of light or hormone treatment on luciferase activity could easily be visualized using continuous observation. Additionally, in these studies no treatment that could alter the plants' surface, and which could alter luciferin uptake, was needed. In a recent study published by van Der Krol et al. (1999), continuous imaging was successfully employed to analyse luciferase expression under the control of the ZPT2-2 promoter in petunia. Here, in contrast to our experiments, the constitutive expression of luciferase activity was very low (20 min integration time compared with less than 1 min in our experiments). The fact that these authors could not detect a rapid burst of light production after wounding is consistent with our hypothesis that high levels of luciferase activity restrict luciferin distribution in plants.

Despite these findings, in-planta imaging of luciferase has become, and will remain a versatile tool in gene-expression studies as long as one is aware of, and carefully controls the associated technical problems described herein. Appropriate controls, such as in-vitro measurements of luciferase activity or RNA-blot analysis should always be conducted while establishing an experimental system based on luciferase activity imaging. Treatments that involve invasive techniques such as infiltration or application of pressure should be interpreted with special care. If possible, plants can be saturated by means other than spraying, such as the application via the transpiration stream described here; nonetheless, as this is still an invasive method and the influence of such a treatment should always be analyzed in detail.

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