Characterization of the ethanol-inducible *alc* gene-expression system in *Arabidopsis thaliana*

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Summary

Controlled expression of transgenes in plants is key to the characterization of gene function and the regulated manipulation of growth and development. The *alc* gene-expression system, derived from the filamentous fungus *Aspergillus nidulans*, has previously been used successfully in both tobacco and potato, and has potential for use in agriculture. Its value to fundamental research is largely dependent on its utility in *Arabidopsis thaliana*. We have undertaken a detailed function analysis of the *alc* regulon in *A. thaliana*. By linking the *alcA* promoter to β-glucuronidase (GUS), luciferase (LUC) and green fluorescent protein (GFP) genes, we demonstrate that *alcR*-mediated expression occurs throughout the plant in a highly responsive manner. Induction occurs within one hour and is dose-dependent, with negligible activity in the absence of the exogenous inducer for soil-grown plants. Direct application of ethanol or exposure of whole plants to ethanol vapour are equally effective means of induction. Maximal expression using soil-grown plants occurred after 5 days of induction. In the majority of transgenics, expression is tightly regulated and reversible. We describe optimal strategies for utilizing the *alc* system in *A. thaliana*.

Keywords: *Arabidopsis thaliana*, ethanol, chemically inducible expression, plant-expression system, *Aspergillus nidulans*.

Introduction

A key tool in plant molecular biology is the development of effective gene-expression systems. The expression of a transgene can be achieved by using constitutive promoters such as the viral CaMV35S promoter (Odel et al., 1985). However, a constitutive promoter is unsuitable when dealing with genes for which inappropriate expression is either highly deleterious or lethal. Additionally, such expression systems are unsuitable for studies where precise temporal regulation is required, for example, where expression of a gene is desired at a specific stage of plant development, or for analysis of mRNA decay properties. Biotechnological application of a regulated expression system may also be important for crop plants; for instance, the conditional expression of pesticides or herbicide resistance; the induction of synchronous flowering of plants; and the production of a conditional male sterility system. In such cases an effective regulated gene-expression system is required.

The optimal system would employ an inexpensive, non-toxic inducer whose application can be fully controlled, and would lead to a reversibly dose-dependent expression with the potential to achieve high levels of gene expression but with negligible basal activity. To these ends, a number of regulated gene-expression systems have been...
developed for work in plants (Aoyama and Chua, 1997; Bohner et al., 1999; Bruce et al., 2000; Gatz et al., 1992; Martinez et al., 1999; Weismann et al., 1994; Zuo et al., 2000). Of those previously reported, the alc regualon which is derived from the filamentous fungus Aspergillus nidulans has a number of features that give it great potential as a gene switch (Caddick et al., 1998; Salter et al., 1998). Firstly, it is relatively simple. It is composed of two elements: the alcR encoded transcription factor (ALCR); and a promoter derived from alcA. In A. nidulans, alcR controls the activation of a number of structural genes, such as alcA and aldA, which encode alcohol dehydrogenase I (ADHI) and aldehyde dehydrogenase (AldDH), respectively (Gwynne et al., 1987; Lockington et al., 1985; Pickett et al., 1987; Sealy-Lewis and Lockington, 1984). For use in plants, the alcR cDNA was expressed from the constitutively expressing CaMV35S promoter. A region of the alcA promoter, including two ALCR binding sites, was fused to the CaMV35S minimal promoter at the TATA sequence. This chimeric promoter was then used successfully to drive inducible gene expression in transgenic tobacco and potato (Caddick et al., 1998; Salter et al., 1998). Secondly, when used in tobacco and potato the alc system exhibited negligible basal expression and rapid induction, resulting in high levels of transgene expression throughout the plant. Thirdly, the system responds to a number of inducers which are cheap, non-toxic at effective concentrations, and suitable for use both in the laboratory and in the field. The chemicals used successfully as inducers for tobacco included threonine, ethylamine, propan-1-ol and butan-2-ol, but ethanol consistently gave the highest levels of transgene expression (Caddick et al., 1998; Salter et al., 1998). It is now known that ethanol is likely to act as an inducer after metabolism to acetaldehyde (Flipphi et al., 2000).

In this study we characterize in detail the efficacy of the alc system in Arabidopsis thaliana. Transgenic plants were produced which included alcR alcA::reporter constructs, and expression was monitored under a variety of regimes.

Results

Preliminary characterization of alc-mediated expression.

A total of 30 independent transformed Columbia and 106 Landsberg erecta (Ler) plants were generated containing the alcR alcA::GUS construct (Figure 1). Of the 106 lines in Ler, 63 soil-grown plants showed induction after the application of 1% ethanol (v/v) by root drenching; of these, 65% showed good general staining for β-glucuronidase (GUS) activity only in induced plants. 13% of the transgenics had detectable GUS expression in the absence of the inducer, but none appeared fully constitutive. 27% failed to stain for GUS activity under either induced or uninduced conditions (data not shown). Of the six Columbia lines assayed quantitatively, all exhibited negligible levels of GUS expression in the absence of inducer. Induction with 1% ethanol for 16 h, which was subsequently found to be suboptimal (see below), resulted in levels of expression up to 60% of that observed in a highly expressing CaMV35S::GUS transgenic line.

Both alc::GUS and alc::GFP constructs were used to examine uniformity of expression. Generally, GUS was found throughout the plants, with no specific tissue consistently either failing to express the gene or revealing constitutive expression (Figure 2). However, staining in certain lines did reveal specific patterns consistent with position effects, for example, one Gus line (Ler 13.1) showed GUS expression in young leaves and meristem, but not in cotyledons. In other cases, expression was discontinuous.

Homozygous lines (AGS1-3, 2-5, 3-2 and 5-1) were generated from four of the Columbia alc::GUS transformants in order to facilitate more detailed characterization.

Dose–response

To determine the optimal ethanol concentration for induction, 2-week-old soil-grown seedlings from the four homozygous alcA::GUS lines were induced by root drenching with a range of ethanol concentrations (Figure 3). After addition of the inducer, the plants were incubated in a propagator for 16 h before quantifying GUS activity using a fluorometric assay. Optimal GUS expression was observed with 2% ethanol (v/v) in all four AGS lines tested. Significant GUS activity was detectable after induction with 0.01% ethanol, with levels of GUS expression in the absence of ethanol being indistinguishable from the background readings detected using untransformed A. thaliana. With the exception of the 1% ethanol treatment in one line (AGS2-5), all the lines showed a continuous rise in GUS activity with increasing ethanol concentrations from 0.01 to 2%, with the AGS3-2 line giving the highest levels of activity. At ethanol concentrations higher than 2%, the level of transgene expression

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decreased significantly. At ethanol concentrations above 2%, the seedlings exhibited signs of stress (e.g. discoloration and curling of leaves), probably resulting from ethanol toxicity.

**Time course**

The optimal ethanol concentration of 2% (v/v) was subsequently used for further characterization of the four homozygous GUS lines (AGS1-3, AGS2-5, AGS3-2 and AGS5-1). Expression was monitored over an 11-day time course, as indicated in Figure 4. A dramatic increase in GUS expression was detected after 4 h induction, but in three of the four lines a marginal increase in activity was detected after just 1 h. With one exception (96 h), GUS levels showed a stable increase, reaching a peak at 120 h. Compared to basal expression levels (Figure 4; Table 1) this represents an approximately 2000-fold increase in gene expression. The GUS level began to decrease after 120 h, and at 360 h GUS activity was equivalent to that seen 6 h after induction. Each line exhibited distinct attributes: AGS3-2 exhibited the highest mean levels of GUS expression, but the highest level of variation between seedlings. AGS1-3 showed maximal expression at day 6, and low levels of variation between seedlings. The AGS1-3 line also differed from other lines, with optimal expression being detected 144 h after induction.

**Vapour response**

It has been reported previously that in tobacco the alc system has the potential to be induced by ethanol vapour (Salter et al., 1998). To investigate the ability of ethanol vapour to induce the system, 5 ml of 100% ethanol, in a
glass universal, was placed inside a covered propagator (48 cm long × 30 cm wide × 20 cm high) containing 2-week-old soil-grown seedlings. GUS expression was monitored over a 5-day time course (Figure 5). The highest levels of GUS were observed after 96 h, and subsequently these decreased. The level of GUS expression observed in response to vapour was higher than that obtained by soil drenching. However, the same general trend of GUS expression was observed with induction in both experiments.

Continuous induction–response

Various expression profiles might be expected if ethanol is applied continuously, for example: will the gene continue to be expressed linearly with time; will the system shut down after prolonged induction; will gene expression reach a stable equilibrium or will the expression fluctuate upon each addition of the inducer? To study this, ethanol was added by root drenching with 2% ethanol at 4-day intervals over a 15-day time course. After the initial induction, the level of GUS activity increased over the first 3 days and then stabilized (Figure 6). While the initial induction resulted in a relatively low level of expression in this experiment, the second application at day 4 increased expression to a high level, which was maintained throughout the remaining 16 days of the experiment; further applications of ethanol occurring on day 8 and day 12. Therefore using repeated application of ethanol, extended periods of high expression can be maintained.

Real-time bioluminescence imaging

Expression analysis using GUS has three limitations. First, it is a destructive assay, and therefore expression in an individual plant cannot be monitored across the full time course. Second, the stability of the GUS product distorts the expression profile – more specifically, a decrease in de novo synthesis is observed only after a substantial lag. Third, GUS cannot be used simultaneously to monitor expression both quantitatively and spatially. In order to supplement the GUS data, we employed the LUC reporter gene to visualize and quantify expression in real-time. This is a non-destructive assay, luciferase activity being observed as bioluminescence. In addition, the luciferase activity does not persist in the cell in the presence of substrate (Millar et al., 1992; White et al., 1996). This combined with the fact that, where analysed, both the

Table 1. GUS expression in agar-grown and soil-grown transgenic plants

<table>
<thead>
<tr>
<th>Plant lines</th>
<th>Agar-grown Uninduced</th>
<th>Agar-grown Induced</th>
<th>Soil-grown Uninduced</th>
<th>Soil-grown Induced</th>
</tr>
</thead>
<tbody>
<tr>
<td>35SGUS</td>
<td>501 ± 38.3</td>
<td>822 ± 118</td>
<td>501 ± 38.3</td>
<td>822 ± 118</td>
</tr>
<tr>
<td>AGS-3</td>
<td>26.1 ± 5.5</td>
<td>480 ± 223</td>
<td>1.5 ± 0.9</td>
<td>508 ± 45.8</td>
</tr>
<tr>
<td>AGS-4</td>
<td>1.8 ± 0.8</td>
<td>63 ± 18.9</td>
<td>0.7 ± 0.1</td>
<td>78 ± 9.3</td>
</tr>
<tr>
<td>AGS-5</td>
<td>20.6 ± 8.2</td>
<td>270 ± 111</td>
<td>0.9 ± 0.3</td>
<td>498 ± 61.8</td>
</tr>
<tr>
<td>AGS-6</td>
<td>4.8 ± 2.1</td>
<td>112 ± 33.4</td>
<td>0.5 ± 0.2</td>
<td>181 ± 34.2</td>
</tr>
<tr>
<td>Wild type</td>
<td>0.4 ± 0.1</td>
<td>0.3 ± 0.01</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
</tbody>
</table>

alcR alc::GUS seedlings from four lines (AGS-3, -4, -5 and -6) were grown on agar and soil. Two-week-old T1 generation seedlings were used. The plants were induced by root drenching with 1% (v/v) ethanol, covered using a plant propagator, and subsequently incubated for 17 h before assaying for GUS activity. The values represent nm 4MU h⁻¹ mg⁻¹ total protein. Each data point represents the mean of five replicates ± SE. A constitutively expressing GUS line (35SGUS) and wild-type plants were used as controls.

Figure 4. Time course of ethanol induction by root drenching.

Two-week-old soil-grown seedlings from the four homozygous lines were induced at t₀ by the application of 2% ethanol to the roots, and GUS activity was monitored over a 360 h time course (as indicated). After induction the plants were covered using a plant propagator for 6 days, at which point the lid was removed. The values represent GUS activity (nm 4MU h⁻¹ mg⁻¹ total protein) of the mean of five seedlings (±SE) from each independent line.
LUC transcript and protein have relatively short half-lives (Farfan, 1999): in *Petunia* cells a half life of 15.3 min has been reported for luciferase in the presence of luciferin (van Leeuwen et al., 2000), thus it is an ideal reporter to study the kinetics of induction. These half-lives are considerably shorter than that of either GUS or the chloramphenicol acetyl transferase (CAT) gene, which have RNA and protein half-lives of more than 40 h (Bronstein et al., 1994; Thompson et al., 1991).

Four-week-old soil-grown transgenic seedlings, carrying the *alcR alc::LUC* construct, were removed from the soil and used for bioluminescent imaging for 13 h post-induction (Figure 7). Luciferase activity was quantified as photons detected per 10 min throughout the imaging process (unpublished data). Induction was achieved by placing 100 μl of 2% ethanol (v/v) containing 0.5 mM D-luciferin at the root tip. Wild-type and *alc::GUS* transgenic *Arabidopsis* was imaged at the same time as controls. Bioluminescence was initially detected in the roots within 1 h, and this continued to intensify for up to 8 h. Expression in the apical meristem was detectable at 3 h. Leaf expression was also detectable at 3 h and spread throughout the leaves by 6 h. By 8 h all seedling tissues were expressing maximally. In both the wild-type and transgenic *alcR alc::GUS* control plants, no bioluminescence was detected throughout the experiment.

### Basal expression levels in agar-grown plants

Many experiments using *A. thaliana* employ agar as a growth medium. It was therefore important to test the behaviour of the *alc* system under these growth conditions. From this analysis, relatively high basal (uninduced) GUS activity was observed in most seedlings grown on
Figure 7. Real-time bioluminescence imaging.
(a) Four-week-old soil-grown seedlings from a homologous line LUC1-12 (thick arrow) were imaged alongside a wild-type seedling (thin arrow).
(b) A photon-counting image taken at the time of induction which indicates no luciferase activity above background of the camera. Induction was achieved by adding 100 μl of a solution containing 2% ethanol and 0.5 mM D-luciferin.
(c–j) A selection of the images taken at hourly intervals over a 13 h time course. Bioluminescence was detected at 1 h post-induction in the transgenic plants, but no background activity was observed in the wild-type control throughout the full time course.
agar plates when compared to those grown on soil (Table 1). The expression detected ranged from four to 60 times the background values seen in wild-type, uninduced A. thaliana seedlings. To characterize further the relatively high basal level of expression observed in agar grown plants, two sets of alcR alc::LUC seedlings, one grown on agar and one on soil, were monitored simultaneously (Figure 8). Luciferase expression was detected in agar-grown plants prior to induction. The bioluminescence observed was not limited to tissues imbedded in the agar, but included stems and leaves (data not shown). In contrast, soil-grown plants showed no bioluminescence. Two hours after induction by the application of 2% (v/v) ethanol; plants were left uncovered for the duration of the experiment. Wild-type plants were monitored in parallel, and no activity was observed throughout the time course. The high level of photon emissions at T2 represents the luciferase that had accumulated in the seedlings prior to the addition of substrate.

Expression was also monitored in 4-week-old, soil-grown, alcR alc::LUC seedlings which were subject to long-term induction by 2% ethanol (v/v). Ethanol was added at the beginning of the time course by root drenching and re-applied 16 and 44 h later. After an initial peak of activity at 8 h, a sustained level of luminescence was obtained for the remainder of the experiment (50 h). These results are consistent with those obtained using the GUS reporter system (Figure 5).

Stability of the system
Silencing has been observed for both the alcR alc::GUS and alcR alc::GFP lines. The frequency and extent varies
both between and within lines. One alcR alc::GUS homo-
zygous line (AGS1-3) showed about 40% of the progeny
with significantly reduced GUS staining after three suc-
cessive back-crosses; however, the seed resulting after an
additional round of back-crossing showed no silencing.
For a second line (AGS4-2), 10% of the progeny from a
back-cross showed reduced GUS expression. Where
examined by Northern analysis, the levels of alcR expres-
sion are significantly reduced in the majority of, but not all,
plants displaying silencing (data not shown). One possible
cause of the silencing observed may be the duplication of
the CaMV35S minimal promoter, which is present in the
alcR-derived promoter as well as the full promoter driving
alcR.

Discussion
The data presented clearly demonstrate that the alc
regulon is functional and tightly regulated in A. thaliana.
In soil-grown transgenic plants, the level of uninduced
expression mediated by the alcR regulator is negligible, in
some transgenic lines being undetectable on the basis of
alc-mediated GUS expression. On application of the
inducer, alc-mediated expression was rapid, being detec-
ted within 1 h. Where residual uninduced expression could
be detected on soil-grown plants, optimal induction con-
ditions resulted in up to a 2000-fold increase in expression,
the induced expression levels achieved being in a similar
range to constitutive expression mediated by the
CaMV35S promoter. Induction was shown to be reversible,
but it can be maintained via repeated application of the
inducer. The system is very sensitive to ethanol, induction
being observed on application of 0.01% ethanol. The level
of expression is proportional to the concentration of
ethanol applied, potentially providing a means for con-
trolling the level of alc-mediated expression. In addition,
ethanol vapour is an effective means of induction. GUS
staining and both GFP and luciferase imaging have
provided visual confirmation of gene expression. Using
these approaches we observed alc-mediated expression in
a majority of plant cells, with no specific tissue appearing
to be consistently non-expressing or deregulated.

The level of uninduced, basal expression is fundamental
to the utility of any inducible expression system. Soil-
grown plants from the four alcR alcA::GUS homozygous
lines did not express detectable levels of GUS in the
absence of exogenous inducer, on the basis of either
histochemical staining or the fluorimetric assay. This is an
important attribute, which may be essential where low
levels of inappropriate expression may prove lethal. Plants
are known to express alcohol dehydrogenase (Dolferus
et al., 1994; Sachs et al., 1980), and the roots are the
primary organ where anaerobic conditions are likely to
pertain, leading to fermentative respiration. The end
product of this mode of respiration is ethanol. Potentially,
accumulation of ethanol and its metabolism in the roots may induce the alc system in the absence of
exogenous inducer. Previous work on tobacco grown in an
artificially induced anaerobic condition has shown that the
alc system was activated only under very severe condi-
tions (Salter et al., 1998). Arabidopsis grown on agar,
where oxygen diffusion is limited, shows increased levels
of anaerobic respiration in the roots (Chung and Ferl,
1999). Under similar growth conditions we have observed
a significant level of basal expression mediated by alc in
the absence of exogenous inducer. It is therefore likely that
anaerobic respiration in agarose-grown seedlings is result-
ing in the production of an endogenous inducer or
inducers at a sufficient concentration to partially induce
the system.

As with the majority of transgenic systems (De Wilde
et al., 2000), particularly where they are highly expressed,
we have observed some gene silencing in certain batches
of seed from specific lines which have undergone a series
of back-crosses. The problem of silencing may be exacer-
bated by the design of the constructs, which include a
repeat of CaMV35S promoter sequences within a single
vector. The homologous repeats extend for only 91 bp, but
this may be sufficient to promote silencing, particularly as
a significant proportion is included in the transcript
(Metzlaff et al., 1997). Recent work in tobacco has involved
replacing the CaMV35S promoter sequence in the alc
promoter (unpublished results). The modified promoter
appears to be more robust, reducing variation between
transgenic lines in tobacco. It will be interesting to
determine if this also reduces the occurrence of silencing.

An additional development, which may be advantageous
in this respect, would be the use of a less active promoter to
drive alcR. We are currently developing systems that
utilize tissue-specific promoters for alcR, potentially lead-
ing to the development of a series of expression cassettes
that will provide both temporal and spatial regulation.

An important consideration in the experimental use of
this system is the background expression observed in the
absence of exogenous inducer in seedlings grown on agar.
This could be a significant limitation if alcR-containing
transgenics are selected on such media where expression
of the transgene is deleterious. This could bias the range,
reduce the frequency, or even prevent the selection of
viable transgenics. Several strategies can minimize poten-
tial difficulties. For example, the transgene, driven by the
alc promoter, is completely silent when in plants in the
absence of alcR (Roslan, 1999). Such transgenics can
subsequently be crossed with an alcR alc::reporter line in
order to express the transgene. The presence of the
alc::reporter construct is not essential, but it provides a
means of demonstrating that alcR is fully functional in any
given plant and that the induction regime is effective. This strategy has proved successful in our hands.

The induction strategy used will vary with the types of experiment being pursued, both vapour and direct application of ethanol being effective. High levels of ethanol are toxic and prolonged application may also be damaging. The age and general health of the plants affects their sensitivity to ethanol, so care must be taken in developing the optimal conditions. For these reasons we recommend the initial use of 0.5–1% ethanol for root drenching and leaving the plants uncovered. The use of ethanol vapour in a covered plant propagator provides a very good method for a short-period induction of the system, having the ability to produce maximal expression while minimizing the effects of ethanol toxicity, but cannot be used for periods of more than 2 days without damaging the plants. As has been demonstrated in tobacco (Salter et al., 1998), there are other potentially useful inducers which may be used if ethanol is not suitable. The sensitivity of the system also demands that care is taken to ensure induction is not triggered inadvertently by the presence of inducers such as ethanol vapour or solvents used in media preparation.

Comparison of data from different experiments (e.g. Table 1 compared with Figures 3 and 8) demonstrates that even in soil-grown plants, low levels of basal expression are occasionally, but not always, observed and that background activity is more prominent in certain lines. This variability should be taken into account when choosing lines for experimentation, and low-level basal expression should always be considered a possibility.

In conclusion, the alc expression system is very effective in Arabidopsis, as in other species, leading to high levels of inducible expression. We envisage its use will contribute significantly to the characterization and manipulation of gene function.

Experimental procedures

Transgenic plants, growth and maintenance

Arabidopsis thaliana ecotype Columbia was used in all the experiments unless stated otherwise. For growth on both agar and soil, plants were propagated in a controlled growth room at 22°C/18°C (day/night) and a 12 h photoperiod. The light intensity in the growth room was 2200 lux (Philips TLD36W/89). For agar-grown plants, 1 × MS medium was used supplemented with 0.2 × B5 vitamins, 2% sucrose and 0.4% agar (Phytagel, Sigma-Aldrich Company Ltd, Dorset, UK) pH 5.8. After autoclaving, nystatin was added to a final concentration of 10 µg ml⁻¹ to reduce fungal contamination. Hygromycin B was added for selection of transformants (35 µg ml⁻¹). The autoclaved agar medium was poured into square (12 × 12 × 1 cm) plastic Petri dishes. An overlay media was prepared using the same ingredients but with 0.05% phytagel. The overlay was used to assist in spreading the sterilized seeds on agar plates during screening for the transformants. Agar plates sowed with Arabidopsis seeds were sealed using parafilm, and incubated vertically to limit the effects of condensation and minimize root penetration into the media, thus minimizing endogenous ethanol production.

For soil-grown plants a mixture of Levington Fission M3, John Innes No. 3 and perlite in a 3 : 3 : 1 ratio was used. The plants were grown either in 10 cm (diameter) plastic pots or square (25 × 20 × 5 cm) plastic multi-well trays. When covered during induction, these were held in plant propagators (48 × 30 × 20 cm).

Seed sterilization and stratification

Seeds were sterilized for 5 min in 100% isopropanol, followed by 1% virkon (w/v) for an additional 10 min. The seeds were then rinsed thoroughly with sterile distilled water at least five times. The seeds were then either plated individually on agar using a fine sterile paintbrush or mixed with an overlay agar and spread on agar plates.

For soil-grown plants, the seeds were mixed with sand at a sand-to-seed ratio of 5 : 1 (v/v), vortexed for a few seconds, and then sprinkled onto the soil. To ensure uniform germination, both agar and soil-sown seeds were vernalized at 6°C for 3 days before transfer to the growth room.

Transformation protocols: vacuum infiltration and silwet-mediated infiltration

Arabidopsis were transformed using two methods: the vacuum infiltration method (Bechtold et al., 1993); and silwet-mediated infiltration (Clough and Bent, 1998). The Agrobacterium strain (pGV2260) was transformed with the appropriate plant transformation plasmids (pGPTV-series vector) containing the GUS and luc reporter constructs. Agrobacterium was subsequently transformed into Arabidopsis. The plant was then left to senesce, and seeds were collected and screened on agar containing hygromycin B (35 µg ml⁻¹). Stably transformed plants were distinguishable from non-transformants after 2 weeks with the formation of green cotyledons and extensive root formation. The transformants were subsequently transferred to soil after 4 weeks.

Homozygous line selection

The T₁ generation was analysed by segregation analysis to determine the T-DNA copy number in each independent transformant. Transformants that resulted in segregation of the hygromycin-resistant marker, consistent with the expected Mendelian ratio of 3 : 1 for a single integration event, were chosen for further analysis. The seeds from individual plants were collected separately. From these, each line was tested for hygromycin resistance and non-segregating T₂ progeny were selected for further characterization.

Induction conditions

Standardization of the volume of the inducer used was essential to make the experiments comparable. The establishment of an optimal ethanol concentration that will maximally express the reporter gene without affecting plant growth is crucial. Unless stated otherwise, 5 ml ethanol at the appropriate concentration was added per 100 ml soil by root drenching, to minimize the

variations and ensure the inducing conditions are comparable for each experiment. For agar-grown plants, 1 ml 2% (v/v) ethanol solution was used per plate (12 × 12 × 1 cm). The ethanol solution was applied directly to the roots of each seedling.

**Protein and GUS quantification**

Protein was extracted from 2-week-old seedlings, unless stated otherwise, and the total soluble protein was determined as described by Bradford (1976). The GUS activity was determined as described by Jefferson et al. (1987). Whole seedlings were homogenized in GUS extraction buffer (50 mM NaH₂PO₄, 10 mM EDTA, 0.1% Triton X-100, 1.0 g l⁻¹ Sarcosyl). A fluorometric assay was conducted using 4-methylumbelliferyl α-D-glucuronidase as a substrate. GUS activity was quantified using a Perkin-Elmer (Perkin-Elmer Analytical Instruments UK, Beaconsfield, UK) Luminescence Spectrometer LS30 and read at the 365 nm excitation and 455 nm emission wavelengths. The assays were quantified using 100 nmol 4-methylumbelliferyl (4MU) as standard.

**GUS histochemical staining**

Plant tissue explants and whole seedlings were stained according to Jefferson et al. (1987). The samples were treated in chloroform to remove the cuticle, placed in a polypropylene desicator containing phosphate buffer (50 mM NaPO₄, 2.6 mg potassium ferricyanide, 2.1 mg potassium ferrocyanide, 1 mM EDTA, 26 mg X-gluc) and a vacuum was applied until the liquid bubbled. The samples were incubated in a sealed container, immersed in phosphate buffer and incubated at 37°C for 16 h. Upon completion, the plant was boiled in 70% ethanol for 5 min to remove the chlorophyll and enhance the blue coloration.

**Bioluminescence imaging**

The bioluminescence produced by luciferase enzyme activity requires the presence of Mg²⁺, ATP, O₂ and ð-luciferin, and releases photons (560 nm), PP₃ AMP and oxyluciferin. The bioluminescence was detected using an intensified photon-counting VIM camera, C2400-20H (Hamamatsu Photonics, Japan) dark-box and sprayed with the substrate, D-luciferin (0.5 mM), containing phosphate buffer (50 mM NaPO₄, 2.6 mg potassium ferricyanide, 2.1 mg potassium ferrocyanide, 1 mM EDTA, 26 mg X-gluc) and a vacuum was applied until the liquid bubbled. The production of bioluminescence requires the presence of Mg²⁺, ATP, O₂ and ð-luciferin, and releases photons (560 nm), PP₃ AMP and oxyluciferin. The bioluminescence was detected using an intensified photon-counting VIM camera, C2400-20H (Hamamatsu Photonics, Japan) dark-box and sprayed with the substrate, D-luciferin (0.5 mM), containing phosphate buffer (50 mM NaPO₄, 2.6 mg potassium ferricyanide, 2.1 mg potassium ferrocyanide, 1 mM EDTA, 26 mg X-gluc) and a vacuum was applied until the liquid bubbled. The bioluminescence was detected using an intensified photon-counting VIM camera, C2400-20H (Hamamatsu Photonics, Japan) dark-box and sprayed with the substrate, D-luciferin (0.5 mM), containing phosphate buffer (50 mM NaPO₄, 2.6 mg potassium ferricyanide, 2.1 mg potassium ferrocyanide, 1 mM EDTA, 26 mg X-gluc) and a vacuum was applied until the liquid bubbled. The bioluminescence was detected using an intensified photon-counting VIM camera, C2400-20H (Hamamatsu Photonics, Japan) dark-box and sprayed with the substrate, D-luciferin (0.5 mM), containing phosphate buffer (50 mM NaPO₄, 2.6 mg potassium ferricyanide, 2.1 mg potassium ferrocyanide, 1 mM EDTA, 26 mg X-gluc) and a vacuum was applied until the liquid bubbled.

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**References**


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