The Plant Journal (2001) **28**(2), 225–235

TECHNICAL ADVANCE

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; Weinmann *et al.*, 1994; Zuo *et al.*, 2000). Of those previously reported, the *alc* regulon 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* (L*er*) plants were generated containing the *alcR alcA::GUS* construct (Figure 1). Of the 106 lines in L*er,* 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.

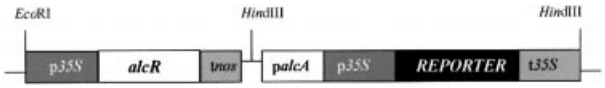

Figure 1. Construct containing the *alc*-derived gene-expression system. The construct included the full *CaMV35S* promoter (*p35S*) driving the *alcR* cDNA which was positioned upstream of the *nos* terminator (*tnos*). The three reporter genes used, GUS, *GFP* and *LUC*, were placed downstream from the chimeric promoter (*palcA p35S*) which consists of the *CaMV35S* minimal promoter (–31 to +5) fused at the TATA box to the upstream promoter sequences of *alcA* (Caddick *et al.*, 1998). The *CaMV35S* terminator (*t35S*) was placed downstream from the reporter gene. These constructs were introduced into the plant transformation vector pGPTVhyg (Becker *et al.*, 1992).

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 (L*er* 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

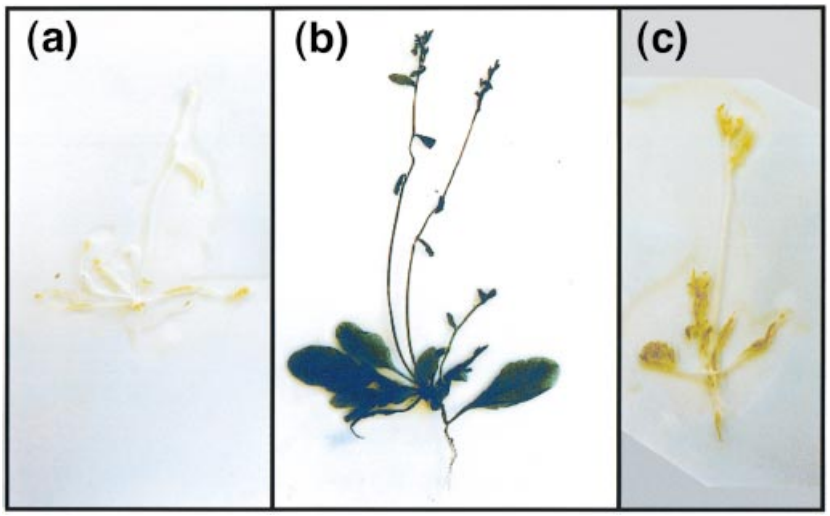

Figure 2. Inducible GUS expression in *alcR alc::GUS* plants.
Histochemical staining of whole plants for GUS activity was conducted using 4-week-old soil-grown wild type (a) and transgenic plants from the line AGS1-3 (b). Induction was achieved by root drenching with 1% ethanol 24 h before analysis. In the absence of inducer (c) the transgenic plants did not reveal any GUS expression. Ethanol-dependent expression is observed throughout all tissues in the AGS plants.

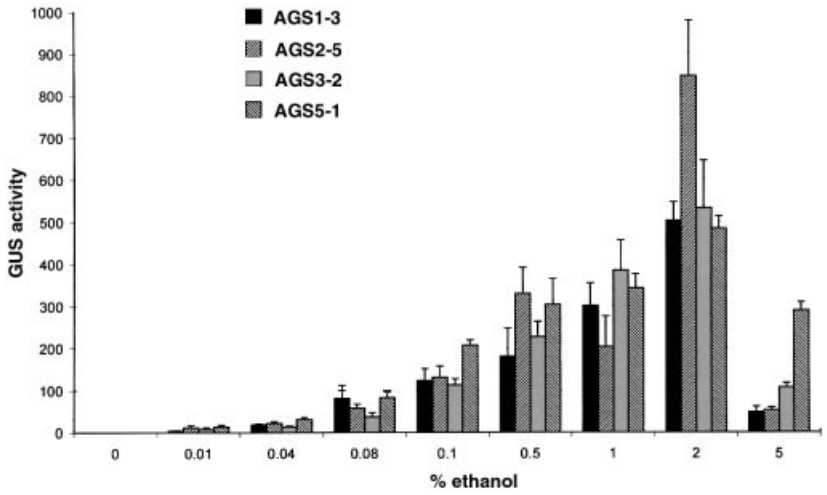

Figure 3. Ethanol dose–response in *alcR alc::GUS* lines.
Two-week-old, soil-grown seedlings from four homozygous *alcR alc::GUS* lines were monitored for GUS activity 17 h after root drenching. A range of ethanol concentrations were applied (as indicated), and subsequently the plants were covered using a plant propagator. The values represent GUS activity (nm 4MU h^{-1} mg^{-1} total protein) of the mean of five seedlings (±SE) from each independent line.

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

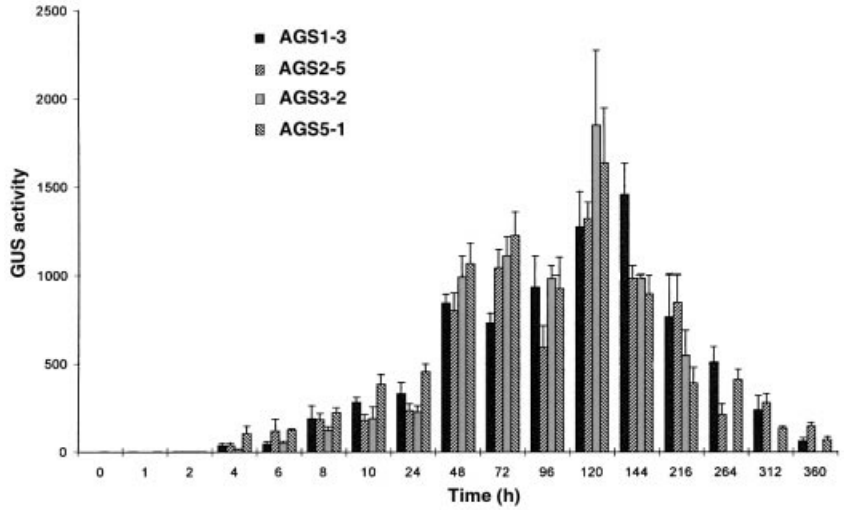

Figure 4. Time course of ethanol induction by root drenching.
Two-week-old soil-grown seedlings from the four homozygous lines were induced at t_0 by the application of 2% ethanol to the roots, and GUS activity was monitored over a 360 h time course (as indicated). After induction the plants were covered 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.

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

	nm 4MU h^{-1} mg^{-1} total protein			
	Agar-grown		Soil-grown	
Plant lines	Uninduced	Induced	Uninduced	Induced
35SGUS	501 ± 38.3	822 ± 118	501 ± 38.3	822 ± 118
AGS-3	26.1 ± 5.5	480 ± 223	1.5 ± 0.9	508 ± 45.8
AGS-4	1.8 ± 0.8	63 ± 18.9	0.7 ± 0.1	78 ± 9.3
AGS-5	20.6 ± 8.2	270 ± 111	0.9 ± 0.3	498 ± 61.8
AGS-6	4.8 ± 2.1	112 ± 33.4	0.5 ± 0.2	181 ± 34.2
Wild type	0.4 ± 0.1	0.3 ± 0.01	0.2 ± 0.1	0.2 ± 0.1

alcR alc::GUS seedlings from four lines (AGS-3, -4, -5 and -6) were grown on agar and soil. Two-week-old T_1 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.

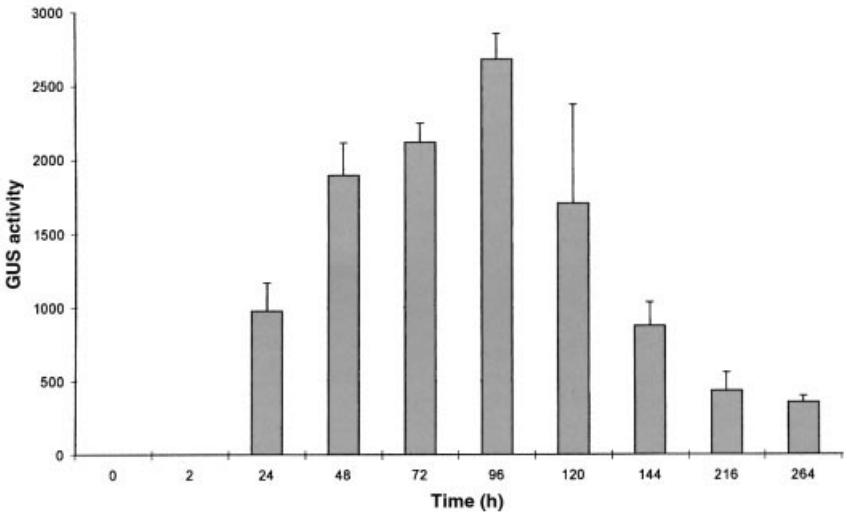

Figure 5. Effect of ethanol vapour on induction.
The *alcR alc::GUS* homozygous line AGS 3-2 was used to assess induction by ethanol vapour. Two-week-old soil-grown seedlings were used. Induction was achieved by placing 5 ml 100% ethanol in a glass universal and placed in the corner of an enclosed propagator, and taking samples for GUS assays at the time points indicated. The data represent mean GUS activity (nm 4MU h^{-1} mg^{-1} total protein) of five replicates (±SE). The lid of the propagator was taken off after 96 h and therefore represents the removal of the inducer. GUS activity was assayed for a further 11 days after induction. Wild-type plants were monitored in parallel, and no activity was observed in these throughout the time course.

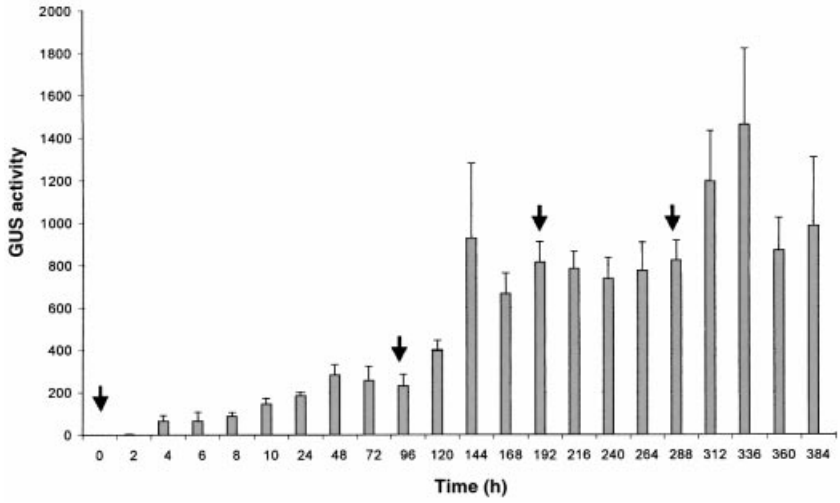

Figure 6. Effect of continuous ethanol induction.
Four-week-old soil-grown seedlings from the homozygous line AGS 3-2 were used. Ethanol (2% v/v) was applied at to the roots at time 0 (t_0) and subsequently every 4 days (arrows). The plants were covered for the first 2 days after each induction in a propagator. GUS activity (nm 4MU h^{-1} mg^{-1} total protein) was monitored over the 384 h time course as indicated. Each data point represents the mean of five replicates (±SE). Wild-type plants were monitored in parallel, and no activity was observed in these throughout the time course.

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

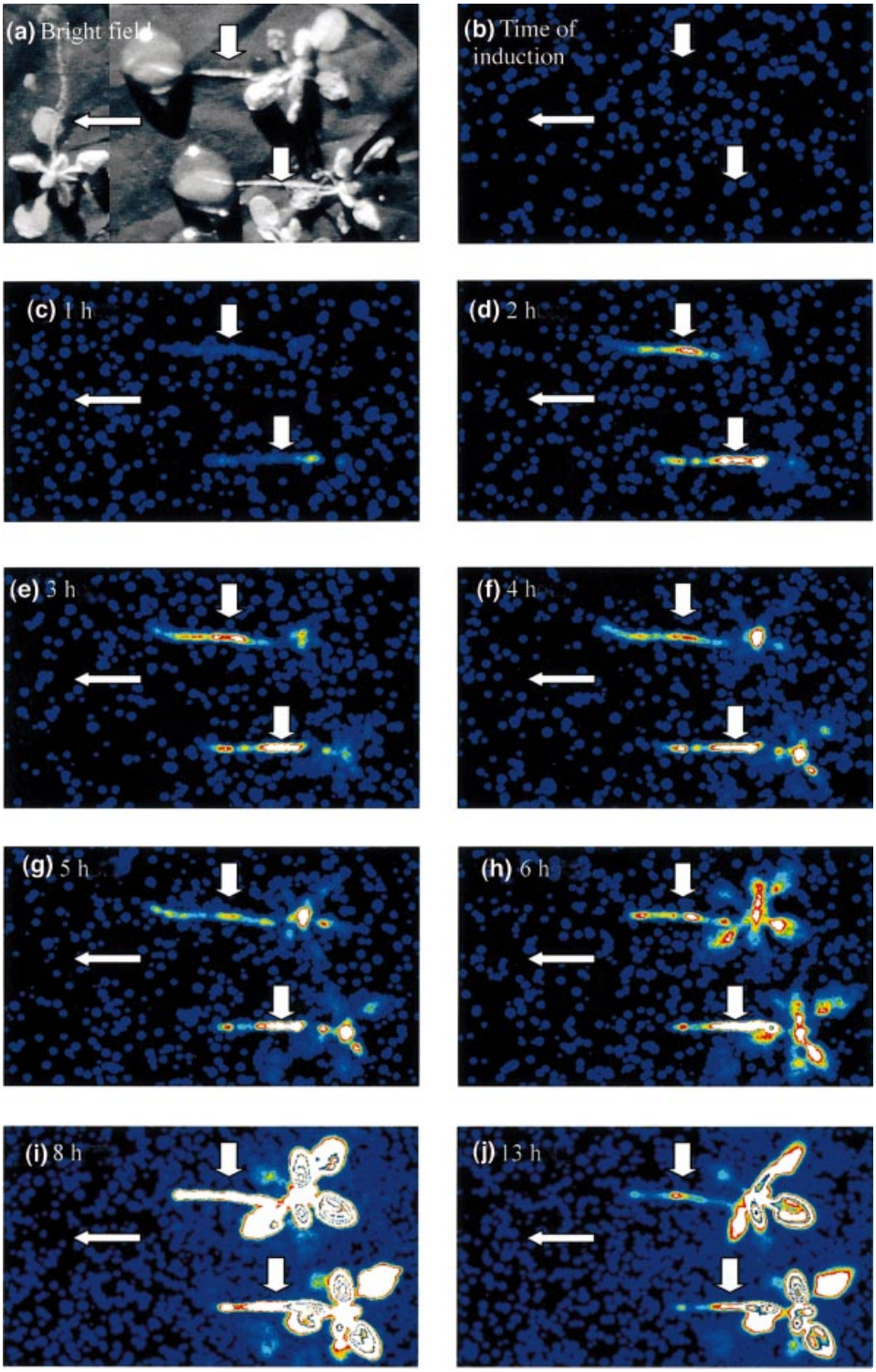

Figure 7. Real-time bioluminescence imaging.
(a) Four-week-old soil-grown seedlings from a homologous line LUC1-12 (thick arrow) were imaged alongside a wild-type seedling (thin arrow).
(b) A photon-counting image taken at the time of induction which indicates no luciferase activity above background 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.

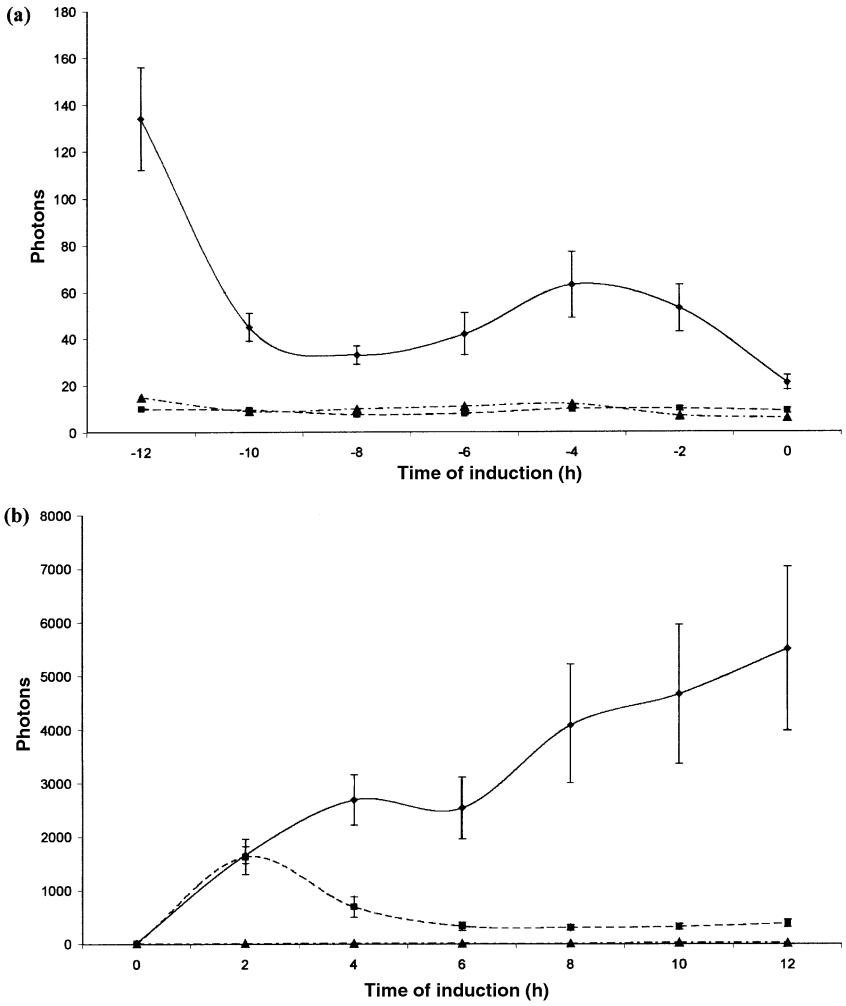

Figure 8. Comparison of agar-grown and soil-grown seedlings using the *LUC* reporter gene.
The data represent photons detected per 10 min, and are means of 10 seedlings (±SE). The LUC1-12 homozygous line seedlings were grown on agar (●) and soil (■) in square Petri dishes. Two-week-old seedlings were placed in a dark-box and imaged using an intensified photon-counting camera. Expression (a) before and (b) after induction. The plants were imaged for 24 h. Induction was achieved by root drenching the seedlings with 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 t_{12} represents the luciferase that had accumulated in the seedlings prior to the addition of substrate.

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% ethanol (v/v) to the roots, both sets of plants showed a significant increase in bioluminescence. For soil-grown plants, the photon emission reached a peak 2 h after induction. However, for agar-grown plants photon emission continued to increase over the 14 h time course, reaching 5500 photons per 10 min. The cause of the prolonged increase and higher expression levels achieved by agar-grown plants was not determined. These may be a consequence of the higher basal level of ethanol, possibly due to the production of endogenous ethanol, greater accumulation of inducer in these plants, and/or ethanol being absorbed into the agar and providing a continuous source for induction.

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* homozygous line (AGS1-3) showed about 40% of the progeny with significantly reduced GUS staining after three successive 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* expression 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 *alcA*-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 *alc* regulon 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 detected within 1 h. Where residual uninduced expression could be detected on soil-grown plants, optimal induction conditions 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 controlling 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 conditions (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 resulting 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 exacerbated 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 leading 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 potential 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 *A. thaliana*, 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 T_1 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 T_2 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 NaH_2PO_4, 10 mM EDTA, 0.1% Triton X-100, 1.0 g l^{-1} Sarcosyl). A fluorimetric assay was conducted using 4-methylumbelliferyl β-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 mM 4-methylumbelliferone (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 desiccator containing phosphate buffer (50 mM $NaPO_4$, 2.6 mg potassium ferricyanide, 2.1 mg potassium ferrocyanide, 1 M 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 Mg^{2+}, ATP, O_2 and D-luciferin, and releases photons (560 nm), PP_i, AMP and oxyluciferin. The bioluminescence was detected using an intensified photon-counting VIM camera, C2400-20H (Hayakawa *et al.*, 1986), mounted on top of a dark-box. The plants were placed in the dark-box and sprayed with the substrate, D-luciferin (0.5 mM), prior to imaging. Images were taken at 2 h intervals unless stated otherwise, and the data were processed using a photon-counting image processor, ARGUS-50 (Hamamatsu Photonics System, Hamamatsu Photonics UK Ltd, Hertfordshire, UK).

Plasmid constructions and plant transformations

Various plasmid constructs were generated using standard cloning techniques (Sambrook *et al.*, 1989). The plasmids were verified by restriction analysis, PCR and DNA sequencing. Constitutive expression of the *alcR* gene was directed by a CaMV35S promoter, obtained as a 2.7 Kb *Eco*RI/*Hin*dIII fragment from a derivative of p35S:*alcR* (Caddick *et al.*, 1998) pSRN4 (Roslan, 1999), and this includes a polyadenylation signal from *Agrobacterium tumefaciens nos* gene (Bevan *et al.*, 1983). The reporter constructs were first cloned into a pUC-based plasmid producing the pAGS2 and pALS plasmids. The *alcR* constructs containing *GUS* (Salter *et al.*, 1998) and *LUC* (pACT-LUC) cDNA, respectively, were cloned into the pGPTV binary vector into the *Hin*dIII site downstream of the *alcR* construct. The diagrams of the plant transformation vectors containing the GUS, *LUC* and *GFP* reporter genes are given in Figure 1. The constructs described in the paper are available for academic research purposes subject to satisfactory completion of a material transfer agreement with Syngenta. For further information contact andy.greenland@syngenta.com.

Acknowledgements

We thank Will Rowe and Angela Tregova for technical assistance, Andrew Millar for advice on bioluminescence imaging, Hamamatsu Photonics k.k. for the long-term loan of the photon-counting camera, Alberto Martinez for critical reading of the manuscript, BBSRC for support (Grant No. 26/P10302 to M.X.C., J.D. and A.B.T. and ROPA Grant No. C9M07229 to A.B.T.), and the Malaysian Government and the University of Malaysia Sarawak for a studentship (H.R.).

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