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RESEARCH PAPER

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

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Abstract

The efficacy of the ethanol-inducible *alc* transgene expression system, derived from the filamentous fungus *Aspergillus nidulans*, has been demonstrated in transgenic tomato. Two direct comparisons have been made. First, this study has utilized two transgenic lines carrying distinct reporter genes (chloramphenicol acetyltransferase and β-glucuronidase) to distinguish aspects of induction determined by the nature of the gene/gene product rather than that of the plant. Second, comparisons have been made to data generated in other species in order to identify any species-specific effects. The induction profiles for different genes in different species have shown remarkable similarity indicating the broad applicability of this gene switch. While there are minor differences observed between species, these probably arise from diversity in their metabolism. A series of potential alternative inducers have also been tested, revealing that ethanol (through metabolism to acetaldehyde) is better than other alcohols and ketones included in this study. Expression driven by *alc* was demonstrated to vary spatially, the upper younger leaves having higher activity than the lower older leaves; this will be important for some applications, and for experimental design. The highest levels of activity from ethanol-inducible transgene expression were determined to be the equivalent of those from the constitutive Cauliflower Mosaic Virus 35S promoter. This suggests that the *alc* system could be an important tool for plant functional genomics.

Key words: *Aspergillus nidulans*, chemically inducible expression, ethanol, plant expression system, tomato.

Introduction

With the elucidation of the complete genome sequence of *Arabidopsis thaliana* and rice, new opportunities and new challenges have arisen for the study of plant genes and their functions. The classification or 'taxonomy' of genes is largely by comparison to previously ascribed genes/proteins; this is reinforced by high throughput screening using transcriptomics and proteomics that attempt to correlate expression to function. Ultimately, functional genomics will require the definition of function through the analysis of phenotype *in planta*. This process has been the cornerstone of genetics, in which screens of mutant phenotypes would lead to the identification of the mutant gene. More recently, molecular methods have enhanced such analyses through the screening of transposon-tagged populations of plants, or by the transformation of cloned genes into mutant plants to observe the reversion of phenotype. The large-scale analysis of plant phenotypes has been termed phenomics (Boyes *et al.*, 2001; Holtorf *et al.*, 2002).

The annotation of genome databases has, however, delivered new challenges. Many genes remain unknown because no clues can be found from their sequence. Even for genes that are recognizably similar to previously described sequences, the multiplicity of paralogues can lead to challenges of unravelling the exact roles in the determination of phenotype throughout development. In these cases, function can be studied directly by transformation of the

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Abbreviations: CAT, chloramphenicol acetyltransferase; GUS, β-glucuronidase; Kan, kanamycin; LeCAT5, *Lycopersicon esculentum* transformed with the alcR;alcA:CAT T-DNA line 5; LeGUS20, *Lycopersicon esculentum* transformed with the alcR;alcA:GUS T-DNA line 20; MS, Murashige and Skoog; PCR, polymerase chain reaction; RNAi, RNA interference technology.

gene into a plant: over-expression of sense in the host, or in a heterologous plant, can cause novel phenotypes; antisense/RNAi silencing can lead to a reduction/elimination of product, again producing a modified phenotype.

Most studies to date have utilized a strong constitutive promoter to over-express the gene of study in a transgenic line. However, inducible promoter systems offer considerable advantages. First, transgenic plants can be recovered even if over-expression of the transgene leads to a highly deleterious or lethal phenotype. Second, expression can be induced at specific times in development, or to coincide with other stimuli applied to the plant. Third, it may be possible to modulate the expression and hence determine the response of the plant to differing levels of gene product. Fourth, it may be useful to reveal the immediate molecular consequences of transgene expression. Finally, the expression can be used to determine whether the phenotypic effect is reversible, or fixed at specific stages in development. It is this latter aspect that makes the use of inducible promoters such a powerful tool for functional genomics.

A number of inducible promoter systems have been described for use 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; Wilde *et al.*, 1992; Zuo *et al.*, 2000; and see reviews by Padidam, 2003; Wang *et al.*, 2003). A system based upon the *alc* regulon from the filamentous fungus *Aspergillus nidulans* has previously been described (Caddick *et al.*, 1998; Salter *et al.*, 1998). It is a relatively simple two-component system: AlcR, a transcription factor encoded by the *alcR* gene; and a promoter derived from the *alcA* gene. Activation has been achieved by application of ethanol to the plant, however, it has recently been shown that ethanol is metabolized to acetaldehyde, the physiological inducer in both species (Flipphi *et al.*, 2002; Junker *et al.*, 2003). The use of this gene switch has previously been described for *Nicotiana tabacum* and *A. thaliana* (Caddick *et al.*, 1998; Salter *et al.*, 1998; Roslan *et al.*, 2001), and recently Sweetman *et al.* (2002) have reported data from tobacco, potato (*Solanum tuberosum*), and oil-seed rape (*Brassica napus*). In this study, its use in tomato (*Lycopersicon esculentum*) is characterized with two different reporter genes, and parallels have been drawn with previous studies.

Materials and methods

Plant material and transformation

Four tissues (stem, leaf, cotyledon, and hypocotyl) of cultivated tomato (*Lycopersicon esculentum* L. var. Ailsa Craig Mill.) were used for transformation using *Agrobacterium*-mediated gene transfer with two binary vectors, pSRN::pACN and pSRN::pAGS; both contain a kanamycin gene as selectable marker, the *AlcR* gene expressed from the CaMV 35S promoter (SRN) and either the chloramphenicol acetyltransferase gene (CAT) or β-glucuronidase (GUS) as reporter gene expressed from the *alcA* promoter (pACN and pAGS, respectively) (Caddick *et al.*, 1998; Roslan *et al.*, 2001). Stem, hypocotyl, and leaf tissues from 4–6-week-old sterile plants, and cotyledons from 8–10-d-old seedlings, were transformed as described previously (Bird *et al.*, 1988; McCormick *et al.*, 1986; Shahin *et al.*, 1986). Initial screening of transformants was carried out on kanamycin selective media, followed by PCR tests for the presence of *alcR* and CAT/GUS genes. Subsequently, reporter gene expression was tested. After preliminary characterization, one CAT line (LeCAT5), and one GUS line (LeGUS20) were selected for detailed and comparative analysis. Sterile plants and seedlings were established in MS medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose, and soil-grown plants were grown in pots containing compost in a plant growth room at 60% relative humidity in a cycle of 16 h light (300 μmol m^{-2} s^{-1}) at 25±2 °C and 8 h dark at 16±1.5 °C. Tissue cultures were maintained in similar conditions except at a low light intensity (35–50 μmol m^{-2} s^{-1}). For induction experiments, seedlings were carefully removed from the soil, rinsed to remove any remaining soil particles, and placed in 120 ml of 0.05% (w/v) Miracle-Gro™ containing the required concentration of ethanol (% v/v) in a 400 ml Magenta pot.

Ethanol dose–response in seedlings

Soil-grown seedlings (15-d-old [LeCAT5] and 18-d-old [LeGUS20]) were transferred to hydroponics containing the required concentration of ethanol (% v/v). One pot of seedlings (four to five per container for CAT and GUS, respectively) was used for each ethanol concentration. Each pot was incubated at 25 °C until harvest (48 h for CAT and 28.5 h for GUS), when all the seedlings were harvested and assayed. Each seedling was extracted separately and assayed for the appropriate reporter gene expression.

Ethanol dose–response of mature plants

LeCAT5 plants were propagated in soil in 5-inch pots until mature (with at least six leaves). Plants were separately exposed (to avoid cross-induction) to various concentrations of ethanol by the addition of the appropriate solution to the soil (100 ml per pot). The top leaf for each plant was removed after 72 h and assayed to detect CAT expression (expressed as ng CAT mg^{-1} total protein).

Time-course of ethanol induction

Seedlings of LeCAT5 plants and LeGUS20 plants were propagated in soil for 15 d and 20 d, respectively, before transfer to hydroponics containing 0.1% (v/v) ethanol. One pot of seedlings (five to eight per container) was used for each time point. Each pot was incubated at 25 °C for the appropriate time, when all the seedlings were harvested and assayed.

Induction by alternative inducers

A series of potential inducers were individually tested for their ability to induce the *alc* system: acetone, acetophenone, acetyl acetone, butan-1-ol, butan-2-ol, diacetone alcohol, di-isobutyl ketone, fenchone, glycerol, polyethylene glycol, propan-1-ol, and propan-2-ol. Each was added to the growth medium at a final concentration of 17 mM, equivalent to 0.1% ethanol (v/v). Soil-grown seedlings (20-d-old) of LeCAT5 were transferred to hydroponics containing each test compound in a 400 ml Magenta pot. One pot of seedlings (three per container) was used for each compound. Each pot was incubated at 25 °C for 20 h until harvest, when all the seedlings were assayed individually to detect CAT expression.

Spatial distribution of activity in seedlings after pulsed induction

Seedlings of LeGUS20 plants were propagated in soil for 18 d, until the first pair of true leaves had developed, before transfer to

hydroponics. The seedlings were induced with 0.1% (v/v) ethanol for 1 h, after which the hydroponic solution was replaced with identical growth media but lacking the inducer. After 18 h, the roots, hypocotyls, cotyledons, epicotyls, and leaves of the seedlings were harvested and assayed for GUS activity.

Spatial distribution of activity in mature plants

LeCAT5 and LeGUS20 plants were propagated in soil in 5-inch pots for 35–40 d until eight leaves had formed. Plants were induced by the addition of 100 ml of 2% (v/v) ethanol per pot under growth room conditions. After 48 h induction, the terminal leaflet of each of the eight leaves of each plant was removed and assayed to detect CAT expression or GUS activity.

Comparison of 35S-GUS and alc-GUS

Seedlings from homozygous CaMV35S-GUS, hemizygous LeGUS20, and wild type were propagated on soil before being induced in 120 ml of 0.05% (w/v) Miracle-Gro with or without 0.1% (v/v) ethanol in 400 ml Magenta pot. Containers were incubated for either 6 h or 120 h in a growth room prior to assay.

Protein, CAT and GUS quantification

CAT protein was measured using an ELISA assay method (Roche, Lewes, UK). GUS activity was determined fluorometrically as described previously (Jefferson *et al.*, 1987; Roslan *et al.*, 2001). The total soluble protein was determined as described by Bradford (1976).

Results and discussion

Generation and preliminary characterization of transgenic plants

Two constructs were used in this study, alcR;alcA:CAT and alcR;alcA:GUS, both of which have been described previously (Caddick *et al.*, 1998; Roslan *et al.*, 2001). These were transformed separately into tomato. For the chloramphenicol acetyltransferase (CAT) construct, 902 tomato explants generated 41 T_0 plants in soil. For the β-glucuronidase (GUS) construct, 1111 explants yielded 30 T_0 plants in soil. The plants were subjected to a rapid screen by the addition of 7% (v/v) ethanol as a soil root drench for 24 h. Only three of the CAT lines and six of the GUS lines showed no reporter gene activity as shown by ELISA (for CAT expression) and β-glucuronidase enzyme assays. Eight CAT lines and seven GUS lines were identified as having high reporter gene activity, and these were subjected to preliminary characterization by PCR for the presence of the transgenes, and further reporter gene assays to check for inducibility (data not shown).

After preliminary characterization, one CAT line (LeCAT5), and one GUS line (LeGUS20), were selected for detailed and comparative analysis. Each was selfed, and 26 and 51 seedlings, respectively, were tested for segregation of reporter gene and T-DNA selectable marker (kan) by PCR, as well as reporter gene activity. GUS was tested by β-glucuronidase assays and histochemical staining. In all cases, segregation was consistent with a 3:1 ratio indicating the presence of a single T-DNA insert (data not shown).

Dose–response

The concentration of ethanol required to give optimal induction was tested in both seedlings and mature plants in soil. Seedlings of LeCAT5 and LeGUS20 were grown in soil for 15 d and 18 d, respectively, before transfer to a nutrient solution containing the required concentration of ethanol. After 48 h (LeCAT5) and 28.5 h (LeGUS20) induction, whole seedlings were used to make protein extracts: three to five individual seedlings were used to determine expression. After ELISA (CAT) or fluorometric assay (GUS), the specific activity was calculated and expressed as percentages of the maximum activity detected (Fig. 1A). Only background activity was detected in uninduced control seedlings, but at 0.05% ethanol significant expression was measured. Optimal activity was detected in both transgenic lines at 0.1% ethanol, with a decline to approximately 60% of the maximum by 0.5%

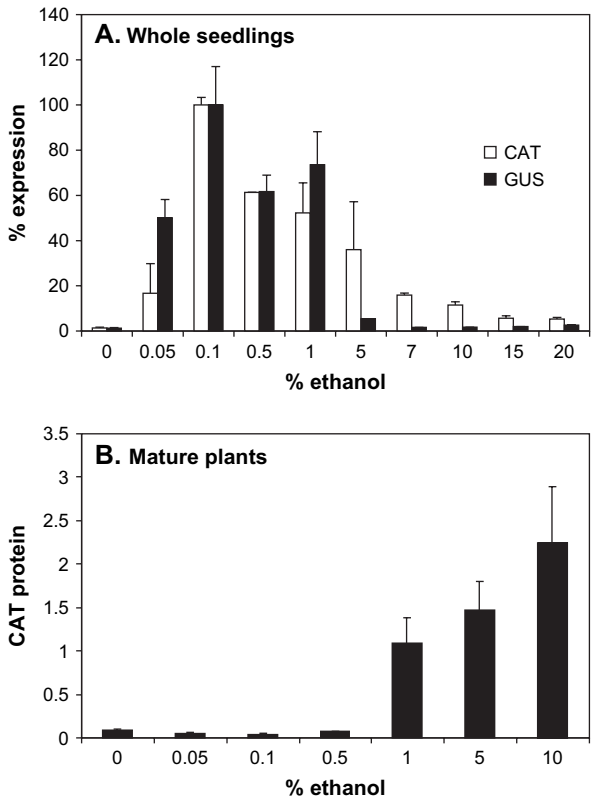

Fig. 1. Effect of increasing concentrations of ethanol on induction. (A) Seedlings of LeCAT5 and LeGUS20 were incubated in hydroponic solutions containing ethanol. Expression is shown as a percentage of the maximum observed: 100% represents 12.6 ng CAT mg^{-1} total protein, or for GUS, 191 nmol 4-MU $h^{-1}mg^{-1}$ total protein. (B) Mature LeCAT5 plants were propagated in soil and separately exposed to ethanol by addition of the appropriate solution to the soil. The top leaf was removed after 72 h and assayed to detect CAT expression (expressed as ng CAT mg^{-1} total protein).

ethanol. Similar activity was measured at 1% ethanol, but concentrations greater than this resulted in serious reductions in expression, presumably through toxicity.

The similarity of the profiles from two distinct lines incorporating two different reporter genes demonstrated that the dynamics of induction were similar, and hence the magnitude of expression could be modulated to differing degrees by the concentration of ethanol applied. Of course both CAT and GUS are relatively stable proteins, and hence a more labile protein may produce a different profile. Nevertheless, one might anticipate that the process of induction in tomato would be similar for any gene, only the accumulation of product would differ through increased/decreased lability of transcript/polypeptide product. In similar assays of tobacco in hydroponics (Salter *et al.*, 1998), 0.1% was also shown to be the ethanol concentration for optimal expression of CAT, however, the plants were assayed 17 h after induction and so the absolute levels of activity were higher.

LeCAT5 was used to determine the concentration of ethanol required for induction of mature plants (Fig. 1B). Only background levels of CAT expression were detectable using concentrations up to 0.5%, and increasing levels of expression were observed with 1, 5, and 10% ethanol after 72 h. While the higher concentrations yielded higher levels of CAT protein, high amounts of ethanol appeared to stress the plants and so concentrations in the 1–2% range appeared optimal. This is directly comparable to findings in soil-grown *A. thaliana* seedlings (Roslan *et al.*, 2001), but 0.5% ethanol was shown to be the most effective in tobacco (Salter *et al.*, 1998).

Taken together, these data suggest that the physiological processes underpinning ethanol-inducible gene transcription from the *alc* promoter are remarkably similar across several plant species when the inducer is supplied via the plant roots. However, it was shown that there was considerable variation in response when ethanol was applied via foliar spray, both in terms of toxicity and magnitude of expression. In tobacco, Salter *et al.* (1998) showed that similar levels of CAT expression could be achieved by a 5% ethanol foliar spray as obtained with a 0.5% root drench in soil. In this study of tomato, a direct comparison was made between the application of 7% ethanol as a foliar spray and a soil root drench. The magnitude of induction was 10–100-fold higher from root application compared with leaf spray, and this concentration of ethanol killed some of the plants when sprayed onto leaves (data not shown). This variability from plant-to-plant and species-to-species presumably reflects differences in leaf morphology (density of stomata, epicuticular wax, etc.), whereas it would appear that roots respond in remarkably similar ways. Inevitably, any requirement for modulation of expression from *alc* to deliver defined levels of gene product will require an appropriate choice of ethanol concentration that takes account of differences in product accumulation (which is dependent on the stability of the product of the transgene).

Time course

Using the optimal ethanol concentration (0.1% v/v), the rate and duration of ethanol-induced expression was tested over a 5 d time period (Fig. 2). In both transgenic lines, activity was detectable after 4 h (7% of peak expression in LeCAT5; 2% of peak in LeGUS20). The LeCAT5 plants rose to a maximal activity at 60 h, after which activity declined. Optimal activity was detected between 3 d and 5 d in LeGUS20 (96–100%) and did not decline over the time period of the experiment. These data were comparable to previous reports in which optimal expression appears to be detectable with these and another gene at 3, 4, or 5 d after induction in tobacco, *A. thaliana* and potato (invertase, Caddick *et al.*, 1998; CAT, Salter *et al.*, 1998; GUS, Roslan *et al.*, 2001; GUS, Junker *et al.*, 2003). Of course as described above, the differences in the stability of the gene product and the metabolism of individual species will combine to affect the speed, level, and duration of expression in any transgenic line.

Alternative inducers

While modulation of *alc*-directed transgene expression could occur by adjustment of ethanol concentration or the time of induction, or some combination of the two, an attempt was made to establish whether it could also be effected by using alternative inducers. Ethanol may not be the optimal inducer in plant cells, and equally there may be compounds that could lead to reproducibly lower induction for certain experimental protocols.

The ability of a range of chemicals to induce expression of the *alcA* and *aldA* genes in *A. nidulans* has been tested previously (Creaser *et al.*, 1985). In that study, good

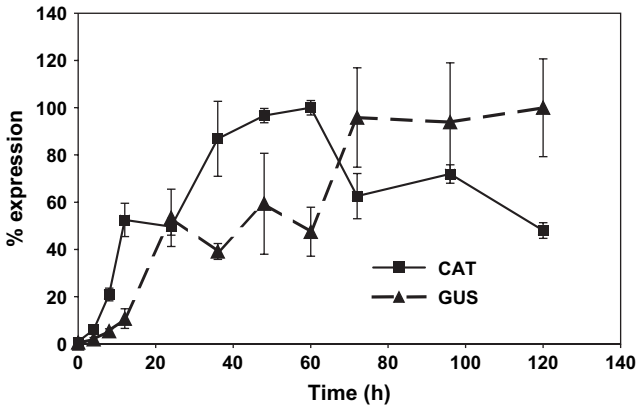

Fig. 2. Time-course of ethanol induction. Seedlings of LeCAT5 plants and LeGUS20 plants were incubated in hydroponic solution containing 0.1% (v/v) ethanol for the required period. Expression is shown as a percentage of the maximum observed: 100% represents 25 ng CAT mg^{-1} total protein, or for GUS, 338 nmol 4-MU h^{-1} mg^{-1} total protein.

induction (>10% of the best inducer) was obtained from 3-hydroxybutan-2-one (34%), threonine (35%), propan-2-ol (45%), 3-oxobutyric acid (52%), butan-2-ol (56%), acetone (78%), cyclohexanone (98%) and butan-2-one (100%). Each chemical was added as 50 mM of the growth media, and all other chemicals tested showed less than 10% of the maximal inducer, including ethanol (6%), or were inhibitory to growth of the fungus at this concentration.

Analysis of the response of LeCAT5 seedlings to 17 mM (equivalent to 0.1% v/v ethanol) of a range of chemicals revealed that, of those tested, ethanol gave the maximal activity (Fig. 3). Only two others had significant inducing ability, propan-1-ol and acetone that represent 83% and 62% of ethanol, respectively. Other chemicals tested were toxic to tomato seedlings at that concentration. In a parallel analysis of the LeGUS20 line, only ethanol gave significant induction (data not shown), propan-1-ol and acetone generating just 4.5% and 3.4% of the maximally induced ethanol value. On this basis, it would appear that reliable induction in tomato only results from the use of ethanol. In a parallel experiment using tobacco, it was found that ethanol was also the best inducer, but that cyclohexanone and acetone gave 53% and 25% of the ethanol induction (Salter, 1997).

Recently, analysis of induction in *A. nidulans* has revealed that acetaldehyde (rather than ethanol) is the physiological inducer in this fungus, and that other inducers act because their breakdown results in the production of inducing aliphatic aldehydes (Flipphi *et al.*, 2001, 2002). Unfortunately, acetaldehyde was not tested as part of this study's experimental series in either tomato or tobacco, but it has been recently reported from a study of potato that acetaldehyde induces *alc*-directed expression more rapidly than ethanol, and with fewer effects on metabolism (Junker *et al.*, 2003).

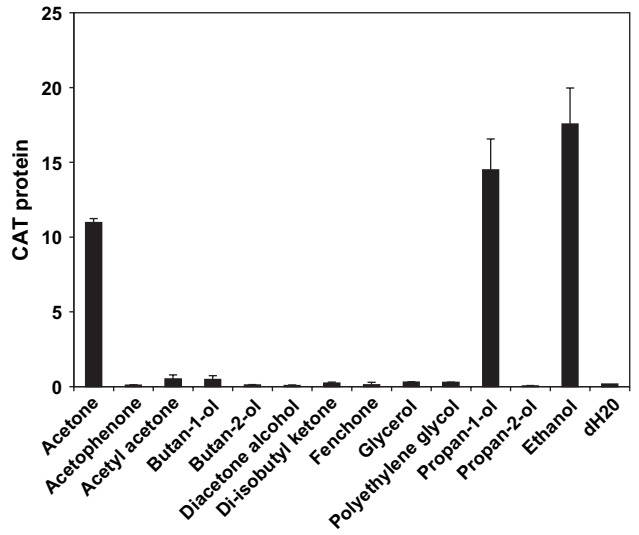

Fig. 3. Induction by alternative inducers. LeCAT5 seedlings were incubated in hydroponic solution containing 17 mM of the test compound for 20 h. Values are for CAT protein (expressed as ng CAT mg^{-1} total protein).

Spatial distribution of ethanol-induced activity

Imaging has revealed previously that ethanol-inducible transgene expression was systemic throughout seedlings of transformed lines (Roslan *et al.*, 2001), and multiple studies have shown that *alc* directs expression throughout mature plants. However, it is known that metabolism changes throughout development (for example see Roessner-Tunali *et al.*, 2003), and even within a given organ type that there are both spatial and temporal changes arising from metabolic control (Stitt and Sonnewald, 1995). Given that transport of the inducer is necessary to any given cell from either the roots or from vapour entering leaves, such differences in metabolism could greatly influence the magnitude of the induction in leaves of different ages, particularly if ethanol is applied exogenously and requires conversion to acetaldehyde. This has been addressed in two ways.

Firstly, seedlings of the LeGUS20 line were subjected to a pulsed induction by the addition of ethanol for 1 h, after which they were incubated for 18 h in identical growth medium without ethanol. The seedlings were each divided into five parts: root, hypocotyl, cotyledon, epicotyl, and first leaves. Each section was assayed separately to determine GUS activity (Fig. 4A). The highest levels of activity were found in the root with lower amounts in the aerial parts of the plant. Furthermore, it would appear that there was a decreasing gradient of induction up to the first leaves. This could be explained in one of three ways: (a) the primary source of induction was ethanol transport from the root to the leaves, and that because the induction was only a short pulse, ethanol concentrations were limited in the aerial parts of the plant; (b) induction in the aerial tissues was from ethanol vapour from the growth medium, and thus was less efficient than direct entry into the root; or (c) some combination of these. In any case, the data suggest that the concentration of the inducer was lower in aerial tissues limiting the expression of GUS.

Secondly, leaves of mature tomato plants from both the LeCAT5 and LeGUS20 lines were tested to establish whether there were spatial differences in expression between different leaves following root application of ethanol (Fig. 4B). In contrast to the seedling data, the highest levels of expression were found in the youngest leaves at the top of the plant, and there was a steady gradient of activity to the lowest, oldest leaves. This could also arise by one or a combination of several means. Younger leaves could generally have a higher metabolic activity or greater gene expression. Alternatively, it could also relate to higher concentrations of the inducer in the top leaves, for example, more transport of inducer (ethanol/acetaldehyde) to the top leaves, or a higher conversion of ethanol to acetaldehyde in

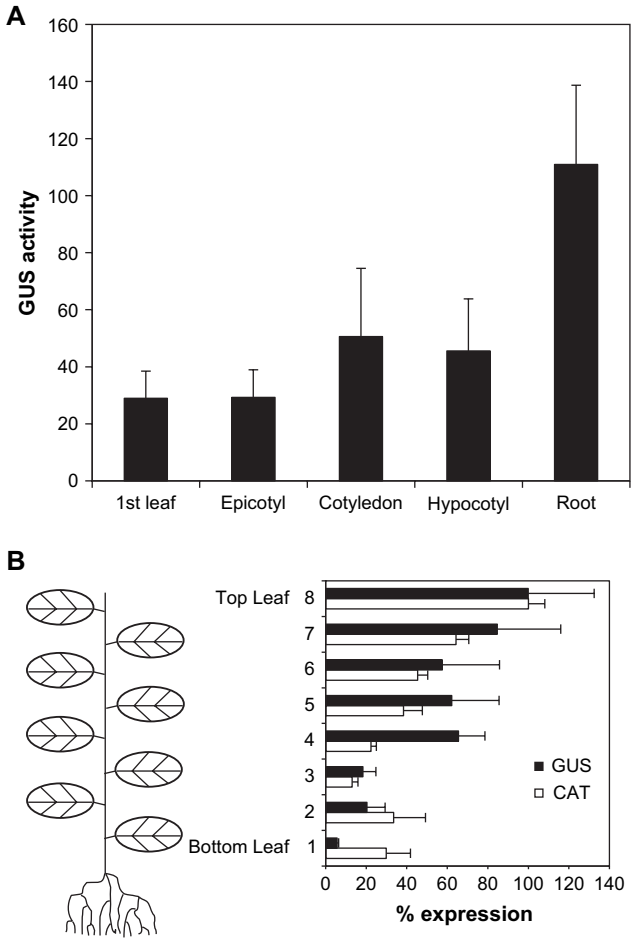

Fig. 4. Spatial distribution of activity in plants. (A) Seedlings of LeGUS20 plants were incubated in hydroponic solution with ethanol for 1 h, after which the solution was removed and replaced with identical growth medium lacking inducer. After a further 18 h incubation, organs were removed and assayed to detect GUS activity (nmol 4-MU h^{-1} mg^{-1} total protein). (B) LeCAT5 and LeGUS20 plants were induced with 2% (v/v) ethanol for 48 h after which the terminal leaflet of each of the eight leaves of each plant was removed and assayed to detect reporter gene activity. Expression is shown as a percentage of the maximum observed: 100% represents 4 ng CAT mg^{-1} total protein, or for GUS, 527 nmol 4-MU h^{-1} mg^{-1} total protein.

these tissues, or a slower breakdown of acetaldehyde. However, it could also be an artefact of the efficiency of extraction of protein from young versus older leaves.

These data are in contrast to an experiment reported by Sweetman *et al*. (2002) who showed that smaller (and presumably younger) leaves tended to have the same or lower amounts of activity as larger (and presumably older) leaves. They also clearly demonstrated that exposure of a single attached leaf to ethanol results in activation of *alc*-directed expression in that leaf, but not in adjacent leaves. Furthermore by using ^{14}C-labelled ethanol, it was shown that the ethanol does not translocate to other parts of the plant. This would suggest that the inducer is not readily transported around the plant, an observation supported by other studies (MacDonald and Kimmerer, 1993), and hence that some of the systemic induction may arise from ethanol vapour inducing the different parts of the plant independently, as well as from transpiration. Induction by ethanol vapour of whole soil-grown *Arabidopsis* was clearly demonstrated to be as effective as root drenching since the time-courses of expression were remarkably similar (Roslan *et al.*, 2001). However, imaging of ethanol-inducible luciferase expression clearly followed a pattern consistent with transpiration, in that expression started in the root, proceeded to the shoot meristem, and only subsequently appeared in the rosette leaves (Roslan *et al.*, 2001). Ethanol vapour must have been present in this experiment, but had it been the primary route of induction one would have anticipated that development of expression would have happened simultaneously through all tissues in the plant.

There are clearly a complex combination of factors affecting the speed and efficiency of induction in a defined set of tissues of any given species, and hence it is essential that experiments using the *alc* system should be carefully controlled to ensure that equivalent tissues are being sampled.

The alc *system*

The *alc* system is simple and easy to use, requires low concentrations of inducer that can be delivered through the roots or by vapour, gives good expression within sensible time periods for experimental design, and is not at serious risk of inadvertent induction. The maximal levels of activity are extremely high: analyses of the highest expressing line after transformation with *CaMV 35S:GUS* and the highest from these *alcR;alc:GUS* populations are comparable (Table 1). Furthermore, the uninduced background level of activity was negligible, and equivalent to an untransformed wild-type control. It has recently been used to deliver tissue-specific expression by coupling the *alcR* gene to appropriate promoters (Deveaux *et al.*, 2003; Maizel and Weigel, 2004), and gene silencing by the inducible expression of double-stranded RNA (Chen *et al.*, 2003). It has previously been demonstrated to be effective in a range of dicotyledonous species, and in this report its efficacy in regulating transgene expression in tomato has been described.

Table 1. *Comparison of GUS gene expression from 35S-GUS and* alc-*GUS*

Seedlings were incubated with or without 0.1% (v/v) ethanol in hydroponics for either 6 h or 120 h in a growth room. Values represent nmol 4-MU $h^{-1}mg^{-1}$ total protein ±SE. nd, not determined.

Line	6 h		120 h	
	Induced	Uninduced	Induced	Uninduced
35S-GUS	2305±6	2208±3	1810±17	1971±229
alc-GUS	81±6	1±0.4	1890±340	1±0.1
Wild type	2±0.1	1±0.2	nd	nd

In conclusion, the *alc* transgene expression system is a versatile tool for functional genomics. Many genes are currently only annotated within databases through the comparison of the primary sequence of their predicted protein product with other known genes. Given the degree of redundancy within genomes, gene function will need to be confirmed experimentally, and an inducible expression system should aid this process for many genes.

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