FEBS Letters 535 (2003) 136–140 FEBS 26918

In plants the *alc* gene expression system responds more rapidly following induction with acetaldehyde than with ethanol

Björn H. Junker[a], Chengcai Chu[b,c], Uwe Sonnewald[b], Lothar Willmitzer[a], Alisdair R. Fernie[a,*]

[a] *Max-Planck-Institut für Molekulare Pflanzenphysiologie, Am Mühlenberg 1, 14476 Golm, Germany*
[b] *Institut für Pflanzengenetik und Kulturpflanzenforschung, Correnstrasse 3, 06466 Gatersleben, Germany*
[c] *Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, 100101 Beijing, China*

Received 6 December 2002; accepted 16 December 2002

First published online 8 January 2003

Edited by Ulf-Ingo Flügge

Abstract **It has recently been proposed that acetaldehyde is the physiological inducer of the *alc* gene system and hence indirectly the activator of the AlcA promoter in *Aspergillus nidulans*. Here we show that this chemical induces expression of a GUS (β-D-glucuronidase) reporter under the control of the *alc* gene system in transgenic potato tubers more rapidly than ethanol allowing tighter control of transgene expression. Furthermore by analysis of metabolite levels we demonstrate that the application of inducer has few effects on metabolism. We propose that this system is therefore ideal for the temporal regulation of important metabolic enzyme activities.**

Key words: Chemical inducer; Gene expression; Metabolism; Ethanol; Acetaldehyde

1. Introduction

A variety of gene expression systems that allow temporal control of transgene expression by environmental or chemical means have been recently characterized (for detailed reviews see [1–3]). It was envisaged that such systems would both avoid lethality problems that can be associated with constitutive overexpression and furthermore allow a more direct analysis of the primary effect of introducing a foreign protein into a cell. Although promoters such as the dexamethasone inducible promoter [4] have been employed to great effect in the delineation of the immediate targets of the transcription factors CONSTANS and APETALA3/PISTILLATA in *Arabidopsis* [5,6], very little use of these promoters has as yet been made in the understanding of metabolism.

One exception to this is the expression of a yeast invertase in tobacco under the control of the two-component ethanol inducible system: the *alc*R encoded transcription factor (ALCR) and a promoter derived from the *alc*A promoter of *Aspergillus nidulans* [7,8]. The authors demonstrated that using this promoter high levels of invertase expression were recorded following induction (but not in the absence of inducer). They demonstrated the advantages that such an approach has over constitutive manipulation of enzyme activities as it allows the consequences of a manipulation at a specific point in time. Since this initial publication several further characterizations of the utility of the *alc* gene expression system in plants have been carried out. These studies demonstrated the efficiency of this inducible system in a wide range of plant hosts including *Arabidopsis thaliana*, *Brassica napus*, *Nicotiana tabacum* and in detached tubers of potato [8–10]. In all instances the induction of the *alc* gene system has been documented following application of ethanol supplied to the plants by drenching the roots in ethanol solution [7–9] or exposing the plants or plant parts to ethanol vapor [10]. However, since it has recently been demonstrated by elegant, yet indirect experiments in *A. nidulans* that acetaldehyde is the sole physiological inducer of the *alc* gene system [11], it is conceivable that this is also a better inducer of plant transgene expression under the control of this system.

Here we demonstrate, for the developing potato tuber, that the *alc* gene switch is induced more rapidly with low concentrations of acetaldehyde than with the corresponding concentrations of ethanol. Furthermore we illustrate that the application of the inducers to wild type tissues has minimal effects on the metabolic complements of this tissue suggesting the great potential of the use of this system in studying temporal aspects of metabolism. However observations of the pattern of expression following induction of B33-Alc-GUS transgenic potato lines indicate that expression of the transgene is not uniform across this bulky tissue suggesting the need for great caution in planning and interpreting the results following the use of this promoter.

2. Materials and methods

2.1. Material

Solanum tuberosum L. cv. Desirée was supplied by Saatzucht Lange AG (Bad Schwartau, Germany). All enzymes were obtained from Roche Diagnostics (Mannheim, Germany). Acetaldehyde was purchased from Fluka Chemie GmbH (Buchs, Switzerland), all other chemicals were obtained either from Sigma-Aldrich (Taufkirchen, Germany) or Merck (Darmstadt, Germany).

2.2. Generation, growth and maintenance of transgenic plants

The plasmid pB33-Alc-GUS was produced by exchanging the 35S promoter from the vector p35S:*alcR*,p*alcA*:GUS-in-pBin19 [10] with the tuber-specific B33 patatin promoter [12]. Transformation of potato (*S. tuberosum* cv. Desirée) with the construct pB33-Alc-GUS was performed as described in [13]. Transgenic plants were selected on kanamycin-containing media [13] and maintained in tissue culture with a 16 h light, 8 h dark regime on MS medium [14], which contained 2% sucrose. In the greenhouse, plants were grown under the

*Corresponding author. Fax: (49)-331-5678408.
E-mail address: fernie@mpimp-golm.mpg.de (A.R. Fernie).

0014-5793/03/$22.00
doi:10.1016/S0014-5793(02)03889-9

same light regime with a minimum of 250 μmol photons m^{-2} s^{-1} at 22°C. Initial screening of 74 lines was carried out by pouring 150 ml 0.2% ethanol twice daily for 3 days to the soil, followed by the sampling of a 10 mm diameter, 1 mm thick tuber disk from the area directly under the skin of a developing tuber of a 10-week-old plant and staining for GUS activity in an 1.5 ml Eppendorf tube containing 0.5 ml GUS staining solution [15]. A second screen was then performed in the same manner, except this time staining latitudinal cross sections from tubers of two plants per line for three pre-selected lines in the greenhouse. In this paper, the term *developing tubers* is used for tubers (over 10 g FW) harvested from healthy 10-week-old plants; *mature tubers* is used to refer to tubers harvested from senescent plants.

2.3. Analysis of GUS expression

For the GUS expression pattern experiment, a whole tuber was sliced into 1 mm thick slices from top to base, which then were stained for GUS activity in six-well macrotiter plates containing GUS staining buffer [15] overnight at 37°C.

For time course and dose dependence experiment a tuber disk (10 by 1.5 mm) was cut from directly underneath the region of stolon attachment and snap-frozen in a 1.5 ml Eppendorf tube in liquid nitrogen. The samples were homogenized with 500 μl GUS extraction buffer [16] using a RZR 2040 grinder (Heidolph Instruments, Schwabach, Germany). Samples were then centrifuged 10 min at 4°C at $20\,000 \times g$ and the resultant supernatant was assayed for total protein [17] and GUS activity by fluorometry [16].

2.4. Biochemical analysis

The relative levels of a wide range of metabolites were determined and quantified following the protocols detailed in [18].

2.5. Statistical analysis

The *t*-tests have been performed using the algorithm embedded into Microsoft Excel (Microsoft Corporation, Seattle, WA, USA). The term significant is used in the text only when the change in question has been confirmed to be significant ($P < 0.05$) with the *t*-test.

3. Results

3.1. Preparation and selection of transgenic lines

The aim of this work was to find a suitable and rapid mechanism of controlled gene expression in a bulky, hypoxic system such as the potato tuber. For this purpose we chose to express the two-component *alc* gene system from *A. nidulans* under the control of the class I patatin promoter [12] in order to additionally confer tissue-specific expression. A total of 74 independently transformed potato lines were generated following an *Agrobacterium*-mediated gene transfer protocol [13] that contained the construct B33-Alc-GUS. These lines were initially selected on kanamycin and subsequently transferred to the greenhouse. Tubers were formed in 2.5 l pots and after 10 weeks of plant growth 7% showed observable staining for β-glucuronidase (GUS) following application of 150 ml 0.2% ethanol (v/v) twice daily to the soil for 4 days. Surprisingly, the induction of GUS expression was not uniform across the tissue in these transformants. At the developmental stage used in these experiments no tubers yielded detectable GUS expression in the absence of the inducer, however mature tubers (> 100 g in weight) yielded very strong expression of GUS in the absence of inducer, interestingly the pattern of staining was the opposite of that observed following chemical induction being strongest in the center of the tuber (data not shown). This is best explained by the fact that hypoxic conditions develop in larger tubers [19]. From the initial screening three B33-Alc-GUS lines were selected, amplified in tissue culture, and the resultant clones grown in the greenhouse for further study.

3.2. Pattern of gene expression following induction of the alc gene system

To further investigate the non-uniform expression observed in the preliminary characterization of these lines we performed a larger experiment in which 10-week-old potato plants were induced with 0.2% (v/v) ethanol for 24 h or 96 h. In addition, driven by the recent finding that acetaldehyde is the physiological inducer of the *alc* gene system in situ [11] we evaluated the patterns of expression following induction under identical conditions. Fig. 1 shows representative photographs of the patterns of expression observed under the above-mentioned induction conditions. In all cases our initial observation that the pattern of expression following induction was non-uniform was confirmed with no expression observable in the center of the tubers. Interestingly in all cases the highest staining was observed in the stolon and apex regions of the tuber. In addition, as best illustrated by the acetaldehyde-induced samples, expression of GUS is prominent in patches close to the tuber skin. Close scrutiny revealed that these patches were adjacent to the tuber lenticles. Despite the lack of uniformity in expression it is very clear from these

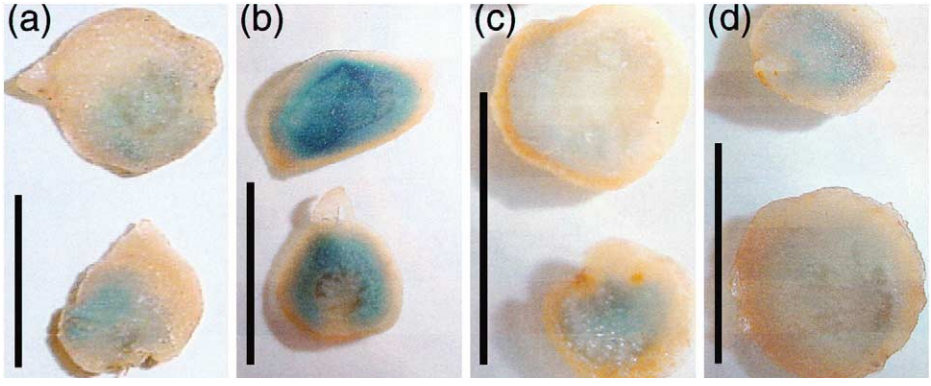

Fig. 1. Detailed comparison of GUS expression following induction with a range of solvents. Histochemical staining of 1 mm thick tuber slices was carried out on tubers isolated from 10-week-old soil grown plants from the transgenic line B33-Alc-GUS 10. Staining of wild type and non-induced tubers from lines B33-Alc-GUS 7, 10 and 13 did not reveal any GUS expression. Induction was achieved by soil drenching with 150 ml 0.2% solvent for 96 h (application twice daily) before analysis. Representative staining of slices from the cap of the tuber is presented for the following solvents: ethanol (a); acetaldehyde (b); 2-butanone (c); acetone (d). Scale bars = 1 cm.

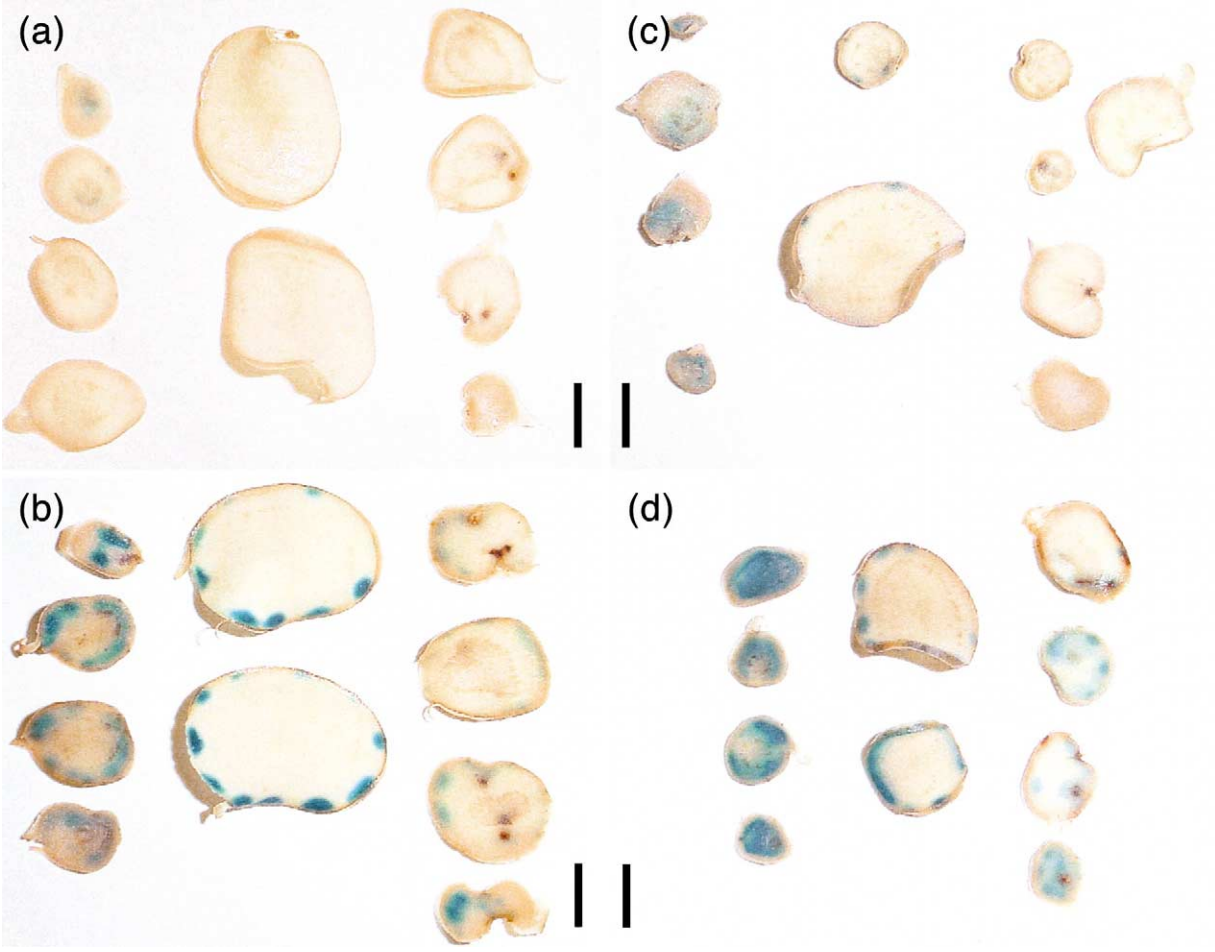

Fig. 2. Inducible GUS expression in B33-Alc-GUS plants. Histochemical staining of 1 mm thick tuber slices was carried out on tubers isolated from 10-week-old soil grown plants from the transgenic line B33-Alc-GUS 10. Staining of wild type and non-induced tubers from lines B33-Alc-GUS 7, 10 and 13 did not reveal any GUS expression. Induction was achieved by soil drenching with 150 ml 0.2% (v/v) solvent for the stated time interval (twice daily) before analysis. Representative staining patterns are presented for the following treatments: ethanol, 24 h (a); acetaldehyde, 24 h (b); ethanol, 96 h (c); acetaldehyde, 96 h (d). The photographs show the expression pattern down the tuber following the stolon to apex axis (every second slice is shown). Scale bars = 1 cm.

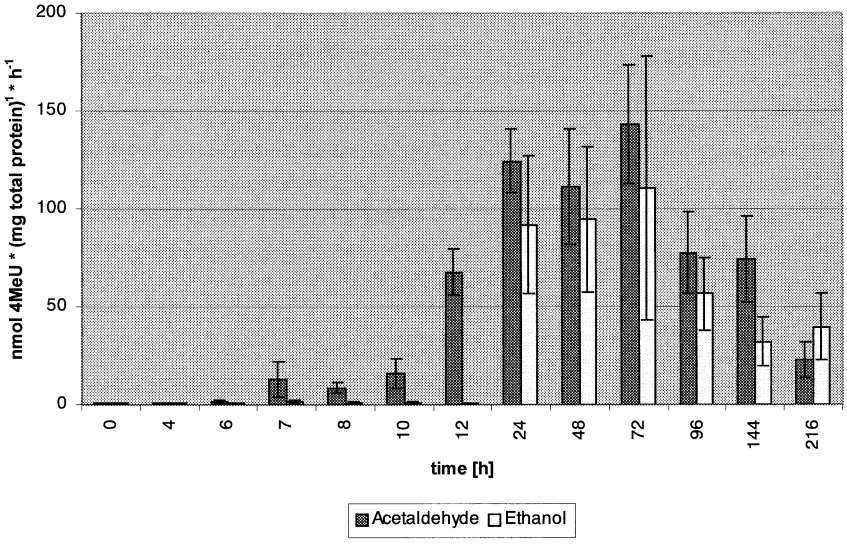

Fig. 3. Ethanol and acetaldehyde induction of reporter gene expression with time. GUS activity was determined using fluorometry; the values are means ± S.E.M. of six independent determinants. Plants were left untreated (control watering only) in a separate growing area or induced by soil drenching with 150 ml of 0.2% solvent at $t = 0$ and 6 h. A disk was taken from the region directly under the stolon from a total of six tubers from two plants and GUS activity was determined in protein extracts by fluorometry. GUS activity was undetectable in untreated samples. 4MeU: 4-methylumbelliferone.

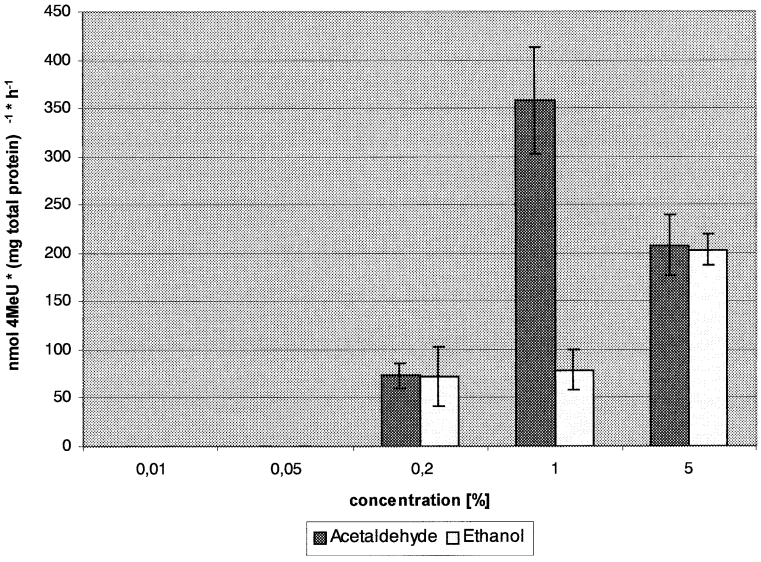

Fig. 4. Ethanol and acetaldehyde induction of reporter gene expression with concentration. GUS activity was determined using fluorometry; the values are means ± S.E.M. of six independent determinants. Plants were left untreated (control watering only) in a separate growing area or induced by soil drenching with various concentrations of solvent at $t = 0$ and 6 h. After 24 h a disk was taken from the region directly under the stolon from six tubers from one plant and GUS activity was determined in protein extracts by fluorometry. GUS activity was undetectable in untreated samples. 4MeU: 4-methylumbelliferone.

qualitative data that induction of the *alc* gene system by acetaldehyde is effective in plant systems.

3.3. Comparison of induction following the application of different chemical inducers

Having established the effectiveness of acetaldehyde for the induction of the *alc* system in potato tubers we next decided to evaluate the possibility of inducing the system with 2-butanone (EMK), which has been demonstrated to be the best (non-lethal) inducer of the system in *A. nidulans*, and with acetone which showed close to 80% of inducing activity in *A. nidulans* compared to EMK [20]. Whilst histochemical staining of tissue from line 10 allowed visualization of GUS expression, the pattern of expression with EMK as inducer was similar to that observed with ethanol and acetaldehyde and the level of expression was less intense (Fig. 2). The situation observed following induction with acetone was even less promising with very little expression visible even 96 h following the initial induction. Given these results we decided to concentrate subsequent experiments on the quantitative comparison of the relative efficacy of induction by ethanol and acetaldehyde.

3.4. Time course of induction

A time course of induction with either acetaldehyde or ethanol was carried out on tubers from line 10 by assessing GUS enzyme activity, from tissue extracted from directly under the stolon, at regular intervals over a period of 9 days (Fig. 3). GUS activity was first detected at appreciable levels 7 h after application of acetaldehyde peaking at 24 h after the initial application, the enzyme then remained present at stable activity for 2 days before slowly decreasing. In keeping both with our earlier qualitative interpretation and with the findings described in [10] the activity of GUS was not detectable in the ethanol-treated samples until 24 h after the initial application, thereafter it remained stable for 3 days before decreasing. This observation was reproduced in three independent experiments. GUS activity in the untreated transformant control was negligible in all instances. Interestingly the mean coefficient of variation was lower for the samples induced with acetaldehyde than with ethanol (0.81 and 0.99, respectively) across all three experiments indicating that the reproducibility of induction may be slightly higher in response to acetaldehyde.

3.5. Dose dependency of induction

In a separate experiment we quantified the levels of GUS enzyme activity following induction by soil drenching with different concentrations of inducer for a 24 h period (Fig. 4). When the results are analyzed it is clear both for acetaldehyde and ethanol that induction is negligible in concentrations below 0.05% (v/v) inducer. Furthermore, in both instances the expression is significantly higher following induction with 5.0% (v/v) inducer than 0.2% (v/v) inducer. Surprisingly, the highest GUS enzyme activity recorded following acetaldehyde induction occurred at the intermediate concentration of 1.0% (v/v). With the exception of this concentration GUS activities are comparable between the two treatments.

3.6. Effect of soil drenching on metabolism in the potato tubers

Having established that, at least in the case of the region below the stolon, high levels of expression could be achieved following induction in 0.2% (v/v) ethanol or acetaldehyde for 24 h we next decided to investigate the effect of these treatments on potato tuber metabolism. For this purpose we soil drenched untransformed wild type plants with 0.2% (v/v) of each of the inducer species or with water and took sub-stolon tuber tissue samples after 24 h. This tissue was rapidly extracted and frozen in liquid nitrogen prior to metabolite analysis using a recently established gas chromatography-mass spectrometry (GC-MS) protocol [18]. This analysis allowed us to identify and quantify more than 60 metabolites of primary metabolism including sugars, sugar alcohols, amino acids and organic acids: the full data set being available for

scrutiny on our website (www.mpimp-golm.mpg.de). Surprisingly there was very little change in the metabolite complements following exposure to either of these chemicals. This was particularly noticeable in the case of acetaldehyde in which the levels of only three metabolites significantly altered with respect to the control watering (alanine decreased, whilst aspartate and inositol increased). Tuber tissue that was exposed to ethanol solutions exhibited far greater a number of significant changes (13 in all) including a general decrease in the levels of sugars coupled to a large increase in several organic and amino acids, notably succinate and alanine.

4. Discussion

We attempted this strategy in order to characterize the utility of the inducible *alc* gene system in a tissue-specific manner. The aims of this study were two-fold, firstly to develop an expression system that allowed modulation of potato tuber metabolism independently of leaf metabolism and secondly to establish the optimal conditions for the induction of this system for studies of metabolism within the growing potato tuber. The expression of a GUS reporter construct under the control of the *alc* gene system and the B33 patatin promoter demonstrates that this strategy was, at least to a limited extent, successful. Expression was, however, not uniform across the tuber with high-level expression limited to the stolon and apex of the tuber and to parenchyma tissue adjacent to the lenticles. Two different explanations can be put forward to explain this pattern of expression – it could be due either to the activity of the patatin promoter or to accessibility of cells to the inducing agent. It seems more likely that the later hypothesis is correct for several reasons. First, previous GUS reporter expression studies carried out with the patatin promoter expressed constitutively reveal that the promoter is active in all regions of the tuber [12]. Secondly, induction studies with ethanol vapor on detached tubers expressing the *alc* gene system under the control of the CaMV 35S promoter revealed that expression was also considerably higher on the outside of the tuber [10]. Thirdly, in the current study we observed that large tubers from 16-week-old plants auto-induce expression of GUS even in the absence of inducer. Under these growth conditions the pattern of GUS expression is completely different with expression throughout the tuber but maximal in its center.

Despite the fact that high-level induction is confined to certain regions of the tuber providing the experiments are carried out with caution this system is clearly capable of conferring tissue-specific inducible expression. In order to optimize the induction of this system we applied a range of potential inducers to 10-week-old plants. These experiments demonstrated that the *alc* gene system is sensitive to soil drenchings of low concentrations of EMK, acetaldehyde and to a lesser extent to acetone as well as the previously reported effects of ethanol [7–10]. In concordance with these previous studies *alc* background activity was negligible in all our tests. As was previously observed in detached tubers [10] the response of the *alc* system to ethanol is much slower for potato tubers than for leaves from *Arabidopsis*, rapeseed or tobacco [7–9]. However, in the potato tuber the *alc* system responds to acetaldehyde within 7 h. Recent experiments in *A. nidulans* have revealed that this is the true physiological inducer in the fungus [11]. Given that we also demonstrated that its application to wild type tubers had little effect on metabolism (in sharp contrast to ethanol which resulted in a large decrease in sugar content and an induction of anaerobic metabolism), we propose that this chemical should routinely be used for induction in bulky systems.

In conclusion, when used in conjuncture with the B33 patatin promoter the *alc* gene expression system is a very effective way of expressing inducible transgenes in a tissue-specific manner. The induction of this system in the potato tuber is much more rapid following the application of acetaldehyde than that of ethanol. Moreover, application of acetaldehyde to potato tubers produces far fewer changes in the metabolite complement than does that of ethanol. When taken together we believe that these data indicate that acetaldehyde is a promising method by which to induce the *alc* gene system particularly in metabolic studies.

Acknowledgements: We thank Syngenta Limited for the generous gift of the plasmid containing the *alc* system. We are indebted to Helga Kulka and Karin Köhl for excellent supervision of the plants. We are also grateful to Romy Baran for performing the potato transformation, and to Joachim Kopka and Dirk Steinhauser for critically reading the manuscript.

References

[1] Gatz, C. (1997) Annu. Rev. Plant Physiol. Plant Mol. Biol. 48, 89–109.
[2] Jepson, I., Martinez, A. and Sweetman, J.P. (1998) Pestic. Sci. 54, 360–367.
[3] Zuo, J. and Chua, N.H. (2000) Curr. Opin. Biotechnol. 11, 146–151.
[4] Aoyama, T. and Chua, N.H. (1997) Plant J. 11, 605–612.
[5] Sablowski, R.W.M. and Meyerowitz, E.M. (1999) Cell 92, 93–103.
[6] Samach, A., Onouchi, H., Gold, S.E., Ditta, G.S., Schwarz-Sommer, Z., Yanofsky, M.F. and Coupland, G. (2000) Science 288, 1613–1616.
[7] Caddick, M.X., Greenland, A.J., Jepson, I., Krause, K.P., Qu, N., Riddell, K.V., Salter, M.G., Schuch, W., Sonnewald, U. and Tomsett, A.B. (1998) Nat. Biotechnol. 16, 177–180.
[8] Salter, M.G., Paine, J.A., Riddell, K.V., Jepson, I., Greenland, A.J., Caddick, M.X. and Tomsett, A.B. (1998) Plant J. 16, 127–132.
[9] Roslan, H.A., Salter, M.G., Wood, C.D., White, M.R.H., Croft, K.P., Robson, F., Coupland, G., Doonan, J., Laufs, P., Tomsett, A.B. and Caddick, M.X. (2001) Plant J. 28, 225–235.
[10] Sweetman, J.P., Chu, C., Qu, N., Greenland, A.J., Sonnewald, U. and Jepson, I. (2002) Plant Physiol. 129, 943–948.
[11] Flipphi, M., Mathieu, M., Cirpus, I., Panozzo, C. and Felenbok, B. (2001) J. Biol. Chem. 267, 6950–6958.
[12] Rocha-Sosa, M., Sonnewald, U., Frommer, W., Stratmann, M., Schell, J. and Willmitzer, L. (1989) EMBO J. 8, 23–29.
[13] Dietze, J., Blau, A. and Willmitzer, L. (1995) in: Gene Transfer to Plants xxii (Potrykus, I. and Spangenberg, G., Eds.), pp. 24–29, Springer, Berlin.
[14] Murashige, T. and Skoog, F. (1962) Physiol. Plant 15, 473–497.
[15] Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. (1987) EMBO J. 6, 3901–3907.
[16] Rao, A.G. and Flynn, P. (1992) in: GUS Protocols (Gallagher, S.R., Ed.), pp. 89–99, Academic Press, San Diego, CA.
[17] Bradford, M. (1976) Anal. Biochem. 72, 248–254.
[18] Roessner, U., Luedemann, A., Brust, D., Fiehn, O., Linke, T., Willmitzer, L. and Fernie, A.R. (2001) Plant Cell 13, 11–29.
[19] Geigenberger, P., Fernie, A.R., Gibon, Y., Christ, M. and Stitt, M. (2000) Biol. Chem. 381, 723–740.
[20] Creaser, E.H., Porter, R.L., Britt, K.A., Pateman, J.A. and Doy, C.H. (1985) Biochem. J. 225, 449–454.