Efficiency of Physical (Light) or Chemical (ABA, Tetracycline, $CuSO_4$ or 2-CBSU)-Stimulus-Dependent *gus* Gene Expression in Tobacco Cell Suspensions

H. Boetti,* L. Chevalier, L. A. Denmat, D. Thomas, B. Thomasset

Laboratoire de Technologie Enzymatique, UPRES A 6022 CNRS, Université de Technologie de Compiègne, BP 20529, 60205 Compiegne Cedex, France

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Abstract: In this study, the efficiency of inducible promoters to switch on gene expression in the presence of inducer or to switch it off in its absence was evaluated in tobacco cell suspensions transformed with the *gus* gene coding sequence. Either plant (*pats1A, pSalT, pIn2-2*) or microbial (*pMre, pTet*) inducible promoters were used to drive *gus* expression. The inducers were light, abscisic acid, 2-CBSU, $CuSO_4$, tetracycline, respectively. For each construct (inducible promoter-*gus* coding sequence), the optimal induction conditions were determined (inducer concentration, induction time, and age of cells in culture cycle before induction). The efficiency of the inducible promoter was then evaluated under optimal induction conditions. GUS-expression levels obtained under non-inducing and inducing conditons were systematically compared. Thirty or forty percent of the clones transformed with the *pSalT-gus* or *pTet-gus* construct, respectively, showed high induction rates (>1000) and GUS activities of the same order as those obtained with a constitutive system. However, basal GUS levels were always high for the *pTet-gus* cell lines. Seventy or eighty-five percent of the cell lines transformed with the *pMre-gus* or *pIn2-2-gus* construct, respectively, had induction rates of 1.5 to 1000. The *pats1A-gus* construct gave very low induction rates—55% of cell lines had induction rates less than 1.5. Only the *pSalt-gus* construct gave both the highest induction rates and basal GUS-levels equivalent to the endogenous GUS background. *Biotechnol Bioeng* **64**: 1—13, 1999.

Keywords: *Escherichia coli;* β-D-glucuronidase; inducible promoters; gene-expression control; *Nicotiana tabacum*

INTRODUCTION

Plants can provide food, fuel, and building materials and also serve as an indispensable source of other specialty products such as flavors, perfumes, insecticides, and pharmaceuticals (Duke et al., 1985; Kutchan, 1995; Rhodes, 1994). These last compounds belong to the broad category of secondary metabolites. Little is known about how plants synthesize these substances, and almost nothing is known about how this synthesis is regulated (Kutchan, 1995). No real progress was made in identifying secondary metabolite biosynthetic enzymes until the use of plant cell cultures as experimental systems was introduced in the 1970s. Since then, plant cell cultures are universally recognized as appropriate systems for use in investigating the molecular responses of plants to stimuli that affect growth (Dominov et al., 1992: Roitsch et al., 1995) and metabolic pathways (Brodelius and Pedersen, 1993). Plant cell cultures are also now considered as suitable systems in which the synthesis, transformation, and degradation of secondary metabolites can be studied. However, it seems that the biosynthesis of such molecules depends on the development of specific structures—there are a number of reports describing the loss of secondary productivity in undifferentiated cells (Brodelius and Pedersen, 1993). Many attempts have been made to manipulate the overall metabolism of cells to increase the total yield of the metabolites of interest or to stimulate metabolic excretion (Archambault et al., 1996; Constabel, 1988; Dörnenburg and Knorr, 1995; Goddijn and Pen, 1995; Williams et al., 1996). The introduction of foreign genes constitutes a widely used tool to study metabolite biosynthesis in response to stimulation of the endogenous gene expression in genetically engineered transgenic plants (Chavadej et al., 1994; Hashimoto et al., 1993; Songstad et al., 1990) and plant cell cultures (Berlin, 1997; Brodelius and Pedersen, 1993). However, overexpression of certain gene products can reduce cell viability and exhibit a number of morphological systems similar to those that occur in senescent plant tissues. Thus, the ability to control the expression of a gene via a highly specific mechanism such as an inducible promoter offers an unique opportunity to study the physiological functions of gene products at different stages of cell cultures. Ideally, an inducible promoter should give little or no basal gene expression prior to induction and a high level of gene expression in the induced state. Moreover, the inducing treatments must be specific to the inducible genes and not affect other cellular processes, not have physiological consequences for plant cells, and not be toxic

* *Present address:* Laboratoire de Biochimie Cellulaire: E.R-.R.M.E.C.E., Département de Biologie, Université de Cergy-Pontoise, 2, avenue Adolphe Chauvin, BP 222, 95 302 Cergy-Pontoise Cedex, France; fax: 33 1 34 25 65 52; e-mail:boetti@u-cergy.fr

Correspondence to: H. Boetti

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at the concentrations typically used for effective regulation of these promoters. Various inducible promoters including those regulated by heat shock (Ainley and Key, 1990), light (Kuhlemeier, 1989), wound (Firek et al., 1993), phytohormones (Li et al., 1991) or antioxidants (Hérouart et al., 1993) are usually used to control expression of transgenes. The availability of a broad spectrum of promoters that differ in their ability to regulate the temporal and spatial expression patterns, increases the application of transgenic technology. However, induction systems based on heat shock, environmental cues, or hormones are not very useful in this regard because of their pleiotropic effects on plant cells.

In this study, the inducible promoters chosen to drive *gus* expression were of either plant origin—*pats1A* of *Arabidopsis thaliana* (Krebbers et al., 1988), *pSalT* or *Oryza sativa* (Claes et al., 1991), *pIn2-2* of *Zea mays* (Hershey and Stoner, 1991), or came from microorganisms—*pMre* of *Neurospora crassa* (Mett et al., 1993) or *pTet* of *Escherichia coli* (Gatz et al., 1991). The inducers were of a physical stimulus, light (Krebbers et al., 1988), or chemical stimuli such as abscisic acid (ABA) (Claes et al., 1991), *N*-aminocarbonyl-2-chlorobenzenesulfonamide (2-CBSU; Hershey and Stoner, 1991), copper (Mett et al., 1993) and tetracycline (Gatz et al., 1991), respectively. These five inducers were chosen for their temporal transgene expression and for their different effects on plant cell metabolism.

A novel feature of this work is that these experiments were performed in liquid tobacco cultures for easy transgene induction. For each inducible system (inducible promoter-*gus* coding sequence), optimal induction conditions (inducer concentration, induction time, and age of the cells in the culture cycle before induction) were determined. The efficiency of the promoters to control foreign gene expression was evaluated by measuring GUS activity before and after treatment. The results are discussed as a function of the type of promoter and inducer. A statistical analysis of interclonal variability allowed us to identify the most-efficient inducible system.

MATERIAL AND METHODS

Plant Material and Cultre

The calli of *Nicotiana tabacum* var. Petit havana SRI derived from leaf protoplasts were kept in a growth chamber at 24°C under periodic light (16 h light, 8 h darkness, 33 μmol.m^{-2} s^{-1} irradiance). They were cultured in solid Linsmaïer and Skoog medium (LS medium) containing 2 mg.L^{-1} NAA, 0.3 mg.L^{-1} kinetin, 30 g.L^{-1} sucrose in the presence or absence of 9 g.L^{-1} agar, pH 5.7 (Linsmaïer and Skoog, 1965).

Cell suspensions were initiated from protoplasts-derived calli after filtration through 1000 μm–50 μm sieves. They were cultured in liquid LS medium (6%, w/v) under continuous shaking (110 rpm) at 24°C in the dark. Tobacco cells transformed with the constructs *pSalT-gus* or *pTet-gus* were grown in modified LS medium containing hygromycin (25 μg ml^{-1}) while those transformed with the constructs *pMre-gus* or *pats1A-gus* were grown in modified LS medium containing kanamycin (100 μg ml^{-1}). Tobacco cells transformed with the construct *pIn2-2-gus* were grown in modified LS medium containing streptomycin (100 μg.ml^{-1}).

To evaluate the increase of biomass in the cell cultures, the growth ratio was evaluated on liquid cell cultures and was presented by the ratio between the increase of the biomass (final biomass at x − initial biomass at 0) on the initial biomass.

Strains and Plasmids

Escherichia coli strains were grown in LB medium and *Agrobacterium tumefaciens* strains in YEB medium. Plasmid constructions were performed to *Escherichia coli* MC1061 by electroporation (Van Lÿsebettens et al., 1986).

The *pMre* is the promoter of the gene encoding metallothioneins and has been isolated from *Neurospera crassa* (Mett et al., 1993). The system consists of two elements:the yeast *ace1* (activating copper MT expression) gene encoding a transcription factor under the control of the *CaMV 35S* promoter and a gene under the control of a chimeric promoter consisting of the 90 bp domain of the Cauliflower Mosaïc Virus *CaMV 35S* promoter linked to the transcription binding site (Mett et al., 1993). The T-DNA of the *pMre-gus* system was kindly provided by Dr. Reynolds (The Horticulture and Food Research Institute of New Zealand Ltd). The *pMre* promoter fused with the *gus* coding sequence was digested with *Eco*RI and cloned into a binary vector (Mett et al., 1993; Fig. 1a, *pMre-gus*). The pMre promoter responds to high copper-ion concentration.

The *pTet-gus* system provided by the group of Dr. Gatz use the *Tn10*-encoded Tet repressor-operator system (Gatz et al., 1991). The *Tn10* encoded Tet repressor can regulate the activity of a plant promoter containing two *Tet* operators. The pTet-1 plasmid containing the coding sequence of the *Tet R* gene was first integrated into tobacco cells via *Agrobacterium tumefaciens* and the cell lines were selected for their high levels of Tet repressor (Gatz et al., 1991). The pAT2 plasmid consisting of the *CaMV 35S* (*tet*) promoter containing the two *Escherichia coli Tet* operators fused with the *gus* coding sequence was digested with *Bam*H1-*Hind*III and cloned into a binary vector (Gatz et al., 1991; Fig. 1b, *pTet-gus*).

The *pIn2-2* promoter is the promoter of the *In2-2* gene of *Zea mays* highly regulated by chemical compounds called safeners (Hershey and Stoner, 1991). The T-DNA of the pGSIns binary vector containing the *pIn2-2-gus* coding sequence construct was supplied by the group of Dr. Hershey (Fig. 1c; *pIn2-2-gus*).

The *pats1A* is the promoter of the *ats*1 gene encoding the small subunit polypeptides of ribulose 1,5 bisphosphate carboxylase of the *Arabidopsis thaliana* (Krebbers et al., 1988). The expression of the gene is regulated by light and

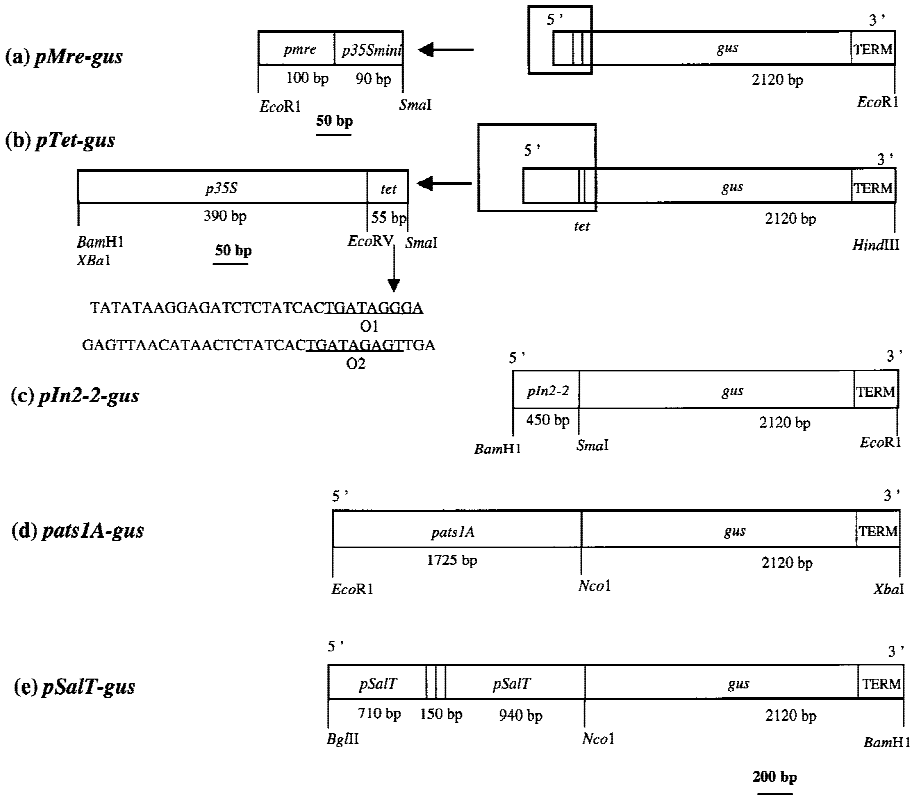

Figure 1. Promoter-gus constructs. gus: β-D-glucuronidase, *p35S:* promoter of *CaMV 35S* (Cauliflower Mosaïc Virus) gene, *pmini35S:* domain A (90 pb) of *pCaMV 35S, mre:* promoter of *Neurospora crassa* metallothionein gene, *Tet: Escherichia coli* promoter containing the operators O_1 and O_2 essential to the tetracycline inducible response.

is under phytochrome control (Krebbers et al., 1988). The *pats1A* promoter (1725 kb *Eco*RI/*Nco*I fragment) fused with the *gus* coding sequence was kindly provided in the pWb ss1 plasmid by the group of the Professor Van Montagu (Laboratorium Genetika, Ghent, Belgium). The *Eco*RI/*Xba*I fragment containing the *pats1A-gus* construct was cloned into this binary vector (Krebbers et al., 1988; Fig. 1d, *pats1A-gus*).

The *pSalT* is the promoter of a gene from rice designated *SalT* that is highly induced when rice plants grown under osmotic conditions as well as by exogenous application of ABA (Claes et al., 1991). The pGVB 310 binary vector containing the 1.8 kb *Bam*HI/*Bgl*II*pSalT-gus* fragment was kindly provided by Professor Van Montagu. The fragment *Bgl*II/*Bam*HI was transfered in the binary vector (Claes et al., 1991; Fig. 1e, *pSalT-gus*).

Mobilization of Plasmid from *Escherichia coli* to *Agrobacterium tumefaciens*

All the binary plasmids containing the constructs (*pSalT-gus, pTet-gus, pMre-gus, pIn2-2-gus,* or *pats1A-gus*) were transferred into *Agrobacterium tumefaciens* C58C1Rif by triparental conjugation, using the helper plasmid pRK2013 (Deblaere et al., 1985).

Preparation of Protoplasts

Protoplasts from *Nicotiana tabacum* were prepared as described by Van Lÿsebettens et al. (1986). About 2 g tobacco leaf material was cut into pieces and incubated in an enzyme mixture containing 0.2% Macerozyme R10 (Sigma, Saint Quentin Fallavier, France) and 0.5% cellulase R10 (Serva), in the dark at 24°C for 12 h. After filtration and centrifugation, the final purified protoplasts were resuspended in K_3^1 medium (Nagy and Maliga, 1976) so that the density of the protoplasts was $1.8/10^5$/mL. The protoplasts were then incubated in the dark at 24°C for 2 d, with no shaking, to regenerate their cell wall.

Plant Cell Transformation

Tobacco cell lines were obtained after 3 d cocultivation of protoplasts with transformed *Agrobacterium tumefaciens* harboring the binary T-DNA vectors. The protoplasts were then diluted twofold in medium containing cefotaxime (500 μg/mL) and plated into 0.7% (w/v) agarose (Seaplaque: Fluka, Saint Quentin Fallavier, France) before their division (Van Lÿsebettens et al., 1986). By using this protocol, each protoplast provided a callus (Van Lÿsebettens et al., 1986). The solidified agarose discs were transferred to 7 mL K_3^- culture medium (Nagy and Maliga, 1976) supplemented with kanamycin (100 $\mu g/mL^{-1}$) or streptomycin (100 $\mu g/mL^{-1}$) or hygromycin (25 $\mu g/mL^{-1}$) as selection agents. The first cell division occurred after 7–10 d, and small colonies were observed microscopically after 2–3 weeks of cultures. The individual microcolonies proliferated on or in the agarose, and every week some were subcultured for the additional period of

4–6 weeks. Six or eight weeks after plating, macroscopically visible microcalli were transferred onto 0.8% agar containing LS medium (Linsmaïer and Skoog, 1965) and the corresponding antibiotics.

Induction Steps

All experiments were performed immediately after subculturing except for studies on GUS activity changes as a function of the state of cell cultures. To determine optimal induction conditions for each system, the cell suspensions (containing the *pSalT-gus, pTet-gus, pMre-gus, pIn2-2-gus,* or *pats1A-gus* construct) were incubated with different inducer concentrations (ABA: 0 to 40 μ*M;* tetracycline: 0 to 40 μ*M;* $CuSO_4$: 0 to 300 μ*M;* 2-CBSU: 0 to 500 μ*M;* light: 0 to 53.1 W/m^2, respectively). Cell cultures were then treated with the corresponding optimal concentration for different times prior to GUS assays (0, 2, 4, 6, 8, 10, or 12 incubation days for chemical induction; 0 to 72 h for light induction). Finally, they were incubated with the previously determined optimal inducer concentration for the respective optimal time as a function of the age of the cells in the culture cycle before induction (0, 2, 4, 6, 8, 10, or 12 d of culture). In the determined induction optimal conditions, the induction rate was evaluated as the ratio between the value of GUS activity determined in a cell suspension treated with inducer and its value determined in the same untreated cell suspension. Toxicity was evaluated as the ratio between the value of the growth ratio for an untreated cell suspension and its value for the same treated cell suspension. Cell suspensions transformed with the *pats1A-gus* construct were treated with infrared emission for 5 mn before the induction experiments.

For each construct (*pSalT-gus, pTet-gus, pMre-gus, pIn2-2-gus,* or *pats1A-gus* construct) and for each tested condition (concentration, time, age of cell cultures), all experiments were systematically performed on three independent cell lines. For each tested condition and for each cell line, the measurements were replicated five times.

Protein and GUS Assays

As described by Jefferson et al. (1987), 0.8 g of tobacco cells were ground in a mortar in 3 mL of extraction buffer containing 50 m*M* Na_3PO_4 buffer pH 7, 1 m*M* EDTA, 0.1% Triton X-100, and 10 m*M* β-mercaptoethanol. After centrifugation, the supernatant was purified by passage through a G-25 spin column (Boehringer-Mannheim, Meylan, France) prior to assay (Thomasset et al., 1996). Protein concentration in plant cell extracts was determined by the method of Bradford (1976) using Biorad reagent and bovine serum albumin as standard. GUS activity was measured by the spectrofluorimetric method (Perkin-Elmer LS 5: excitation at 365 nm and emission at 455 nm) using 3 m*M* 4-methylumbelliferyl β-D-glucuronic acid (4 MUG) in extraction buffer (Jefferson et al., 1987). For each experiment, the spectrofluorimeter was calibrated with freshly prepared 4-methylumbelliferone (MU) standards (1 n*M* to 50 n*M*) in the same buffer. GUS activity is expressed as pmol of 4-methylumbelliferone per h and per μg of proteins (pmol MU $h^{-1}.\mu g^{-1}$ of proteins). All experiments were performed at 37°C for 90 mn with 15 to 30 μg of purified proteins.

RESULTS

The bacterial *gus* coding sequence was fused with different regulatory sequences allowing inducible *gus* expression in callus cultures or cell suspensions (Fig. 1). In our experiments, the T-DNA containing the *gus* coding sequence under the control of an inducible promoter was introduced via *Agrobacterium tumefaciens* into tobacco protoplasts according to the method described by Van Lÿsebettens et al. (1986). The transgenic cells were then embedded in agarose. In this way, each transformed cell resulting from a single transformation event can be collected separately, and numerous cell lines were thus obtained.

The integration of the transgene was confirmed by PCR analysis using oligonucleotides specific to the *gus* gene in all tested cell lines (data not shown: Boetti, 1997). Moreover, genomic DNA from ten 3-week-old calli was used for Southern blot analyses to determine if the antibiotic-resistant tobacco cell lines contained the inducible *gus* gene at different integration sites. The tested cell lines presented different patterns of DNA hybridization with *gus* probe (*gus* fragment of 775 bp; data not shown: Boetti, 1997).

Each cell line was then quantitatively analyzed for *gus* expression. Around 50 calli were first tested for each promoter construct (*pSalT-gus, pTet-gus, pMre-gus, pIn2-2-gus,* or *pats1A-gus*) by using induction conditions described in the original articles first using these promoters (Krebbers et al., 1988; Gatz et al., 1991; Mett et al., 1993; Hershey and Stoner, 1991; Claes et al., 1991). For each inducible system, three representative cell lines were then chosen for their best GUS activity and quantitatively analyzed for each induction condition (inducer concentration, induction time and age of the cells). For all these studies, we checked that the initial cell growth and the growth ratio at the end of the culture were not dependent on the integrated promoter construct (data not shown: Boetti, 1997). Moreover, the presence of the inducer did not affect the growth of the untransformed cell lines (data not shown: Boetti, 1997).

In this study, we compared the induction efficiency of five different promoters in tobacco cells using the *gus* gene as a reporter. The *gus* gene encodes β-D-glucuronidase, whose reaction with different substrates can be easily quantified by either spectrophotometry or spectrofluorimetry (Jefferson et al., 1987). The detection threshold with the spectrofluorimetric method is 100 to 1000 times more sensitive than that obtained in spectrophotometry (Jefferson et al., 1987), allowing GUS activity to be measured over a wide range.

Determination of Optimal Induction Conditions in Our Protocol

Optimal Inducer Concentration for gus *Expression*

Different inducer concentrations (ABA, tetracycline, $CuSO_4$, 2-CBSU, or different light treatments) were tested to determine the optimal concentrations required to obtain the highest GUS activity in the transgenic cell lines (respectively transformed with *pSalT-gus, pTet-gus, pMre-gus, pIn2-2-gus,* or *pats1A-gus* construct). Three cell lines were systematically analyzed and the results showed that they were induced by the same optimal induction concentration. GUS-activity levels were different for the three cell lines. However, GUS activity and growth ratio measured as a function of the inducer concentration presented equivalent patterns (data not shown: Boetti, 1997). Therefore, the results obtained from data of five independently repeated experiments were presented for only one cell line (Fig. II).

GUS activity (0.3 pmol MU $h^{-1}.\mu g^{-1}$ of proteins) in cell-line 306 containing the *pSalT-gus* construct and cultured without ABA in the medium was equivalent to the endogenous GUS background measured in untransformed cells [0.28 pmol MU $h^{-1}.\mu g^{-1}$ of proteins (Thomasset et al., 1996)] indicating that the endogenous ABA concentration was not sufficient to activate the *pSalT* promoter. Addition of ABA to the culture medium at concentrations up to 20 μM resulted in a concentration-dependent increase in GUS activity, but at higher ABA concentrations the GUS activity decreased and growth ratios of less than 1 were observed above 25 μM ABA (Fig. 2A). The highest GUS activity (45.0 pmol MU $h^{-1}.\mu g^{-1}$ of proteins) was obtained with 20 μM ABA (Fig. 2A) with a growth ratio of 1.5, indicating that cells still divided. Therefore, 20 μM ABA was used for future induction experiments in cell suspensions containing the *pSalT-gus* construct.

In cell-line 21 containing the *pTet-gus* construct, GUS activity increased as a function of tetracycline concentration up to 20 μM and then decreased at higher concentrations (Fig. 2B), while biomass decreased at tetracycline concentrations greater than 15 μM. Indeed, the growth ratio decreased from 2.8 to 1.3, respectively, with 5 μM and 40 μM of tetracycline, illustrating the toxicity of this inducer. Optimal induction was obtained at 20 μM tetracycline with a corresponding growth ratio and GUS activity of 1.7 and 20180.0 pmol of MU $h^{-1}.\mu g^{-1}$ of proteins, respectively. This concentration was used in subsequent experiments.

For cell-lines 22 or 20 containing the *pMre-gus* or *pIn2-2-gus* construct, respectively, the highest GUS activities (334.0 or 14900.0 pmol MU $h^{-1}.\mu g^{-1}$ of proteins, respectively) were obtained with 100 μM $CuSO_4$ or with 200 μM 2-CBSU (Figs. 2C,D). The growth ratios of the treated *pMre-gus* cell suspension remained stable whatever the $CuSO_4$ concentration (1.7 and 1.5 at 0 and 300 μM $CuSO_4$, respectively), showing that $CuSO_4$ was not toxic for the plant cells (Fig. 2C). On the other hand, the growth ratios of the *pIn2-2-gus* cell suspensions treated with the increasing 2-CBSU concentrations (0 to 100 μM) increased regularly to 2.7 and then decreased above 200 μM 2-CBSU, indicating that the inducer was toxic (Fig. 2D). Subsequent induction experiments from cell suspensions containing the *pMre-gus* or *pIn2-2-gus* construct used 100 μM $CuSO_4$ or 200 μM 2-CBSU, respectively, with corresponding growth ratio of 1.8 or 2.1.

For all *pats1A-gus* experiments, the cells were exposed for 12 h to different light intensities. Cell-line 29 containing the *pats1A-gus* construct treated with a light intensity of 3.4 Wm^{-2} had the highest GUS activity (18 480.0 pmol MU $h^{-1}.\mu g^{-1}$ of proteins; Fig. 2E). The growth ratios were not affected by such a treatment because the induction time was very short (data not shown). Therefore, a light intensity of 3.4 Wm^{-2} was used for subsequent inductions.

GUS expression in all the transgenic cell lines was always higher in the presence of the chosen optimal inducer concentration than in its absence, demonstrating that these five systems are efficient to switch on/off GUS expression.

Optimal Induction Time for gus *Expression*

The induction optimal time was determined by incubating each transgenic cell line (containing the *pSalT-gus, pTet-gus, pMre-gus, pIn2-2-gus,* or *pats1A-gus* construct) with the optimal inducer concentration (20 μM ABA, 20 μM tetracycline, 100 μM $CuSO_4$, 200 μM 2-CBSU, or 3.4 Wm^2) for different periods (2, 4, 6, 8, or 10 d of treatment). It was evaluated from three cell lines for each inducible system. As the recorded GUS activity and biomass increase patterns were equivalent (data not shown: Boetti, 1997), the results obtained from compiled data of five independent experiments were only presented for one cell line (Fig. 3).

For the cell lines containing the *pSalT-gus* (number 306), *pTet-gus* (number 21), or *pIn2-2-gus* (number 20) construct, the highest GUS activities (50.0, 178 100.0, or 4469.5 pmol MU $h^{-1}.\mu g^{-1}$ of proteins, respectively) were obtained after 4 d of treatment with the optimal concentrations of ABA, tetracycline, or 2-CBSU, respectively. The growth ratios of the corresponding cell suspensions were 1.9 (*pSalT-gus*), 3.2 (*pTet-gus*), or 2.2 (*pIn2-2-gus*), showing that the cells were growing (Figs. 3A,B,D).

The highest GUS activity (240 pmol MU $h^{-1}.\mu g^{-1}$ of proteins) was obtained with the *pMre-gus* cells (number 22) treated with 100 μM $CuSO_4$ for 6 d with a growth ratio of 2.6 (Fig. 3C).

After 24 or 30 h of light induction (3.4 Wm^{-2}), the *pats1A-gus* cells (number 29) had the highest GUS activities (12950.0 and 16950.0 pmol MU $h^{-1}.\mu g^{-1}$ of proteins, respectively; Fig. 3E) with corresponding growth ratios of 0.1 and 0.3. In subsequent experiments, these cells were exposed to light for 24 h.

Cell Suspension Age for Optimal gus *Expression*

Using transgenic cells (containing the *pSalT-gus, pTet-gus, pMre-gus, pIn2-2-gus,* or *pats1A-gus* construct) cultured

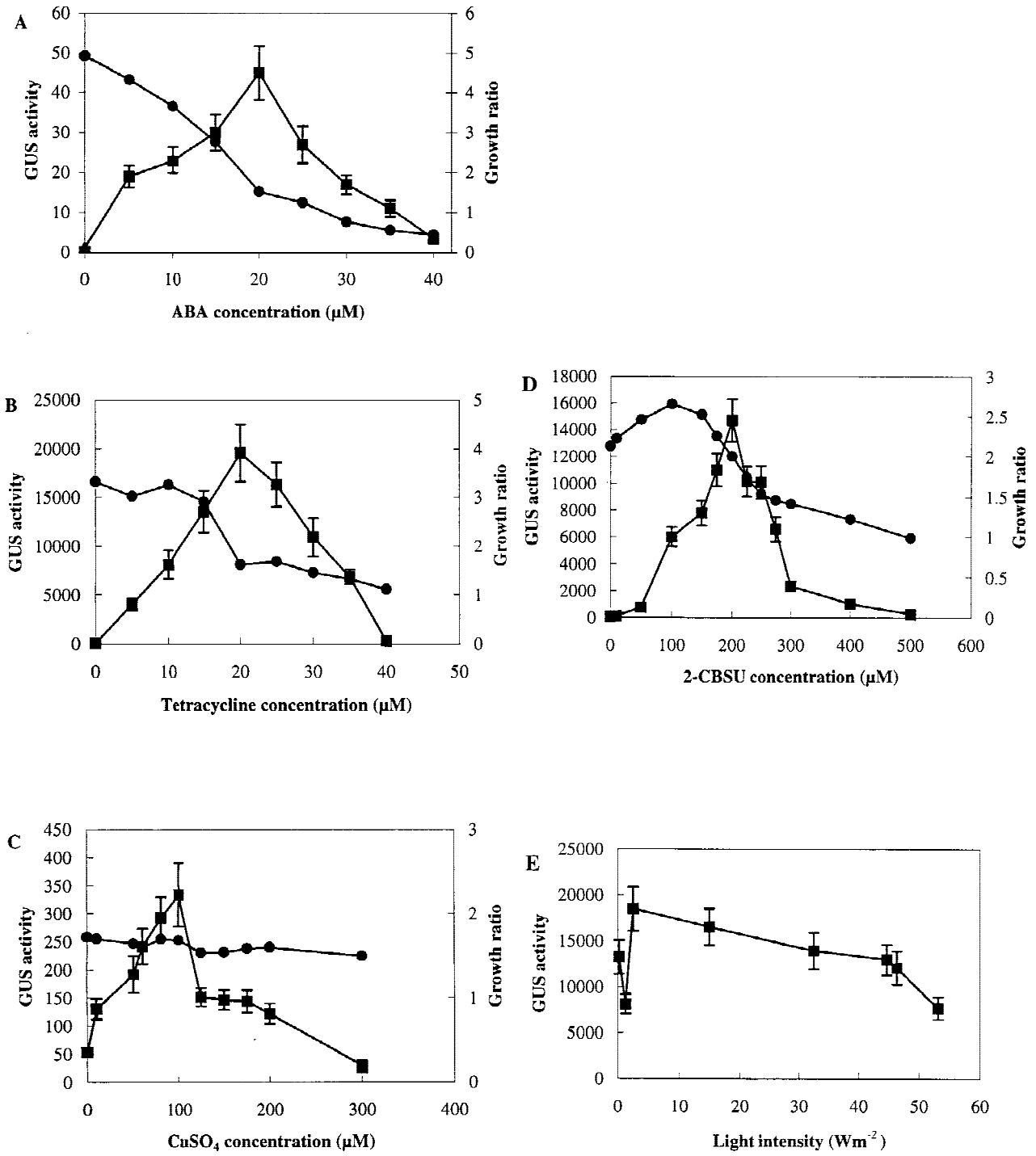

Figure 2. GUS activity and growth ratio as a function of inducer concentration. GUS activity (■) and growth ratio (●) were evaluated as a function of ABA **(A),** tetracycline **(B),** $CuSO_4$ **(C),** 2-CBSU **(D)** concentration or light intensity **(E).** GUS activity was expressed in pmol MU h^{-1} μg^{-1} of proteins. The growth ratio was presented by the ratio between the increase of the biomass (final biomass at x − initial biomass at 0 d) on the initial biomass. The experiments were performed with the cell suspensions initiating from the cell lines 306, 21, 22, 20, or 29 containing the *pSalT-gus, pTet-gus, pMre-gus, pIn2-2-gus,* or *pats1A-gus* construct, respectively. Cell-line 306 was incubated with ABA for 7 d, cell-lines 21, 22, or 20 were incubated with tetracycline, $CuSO_4$, or 2-CBSU, respectively, for 2 d and cell-line 29 was exposed to light for 12 h. Each point represents the average of five independent measurements. For each growth ratio value, the standard deviations were comprised between 5% and 15%. For GUS activity values, standard deviations (±SD) are shown.

with the corresponding optimal inducer concentration (20 µ*M* ABA, 20 µ*M* tetracycline, 100 µ*M* $CuSO_4$, 200 µ*M* 2-CBSU, or 3.4 Wm^{-2} light, respectively) and time (4 d, 4 d, 6 d, 4 d, 24 h, respectively), we determined the state of the culture required to obtain the optimal induction. The results were presented for only one cell line (Fig. 4). Indeed, the experiments performed from three cell lines gave equivalent patterns (data not shown).

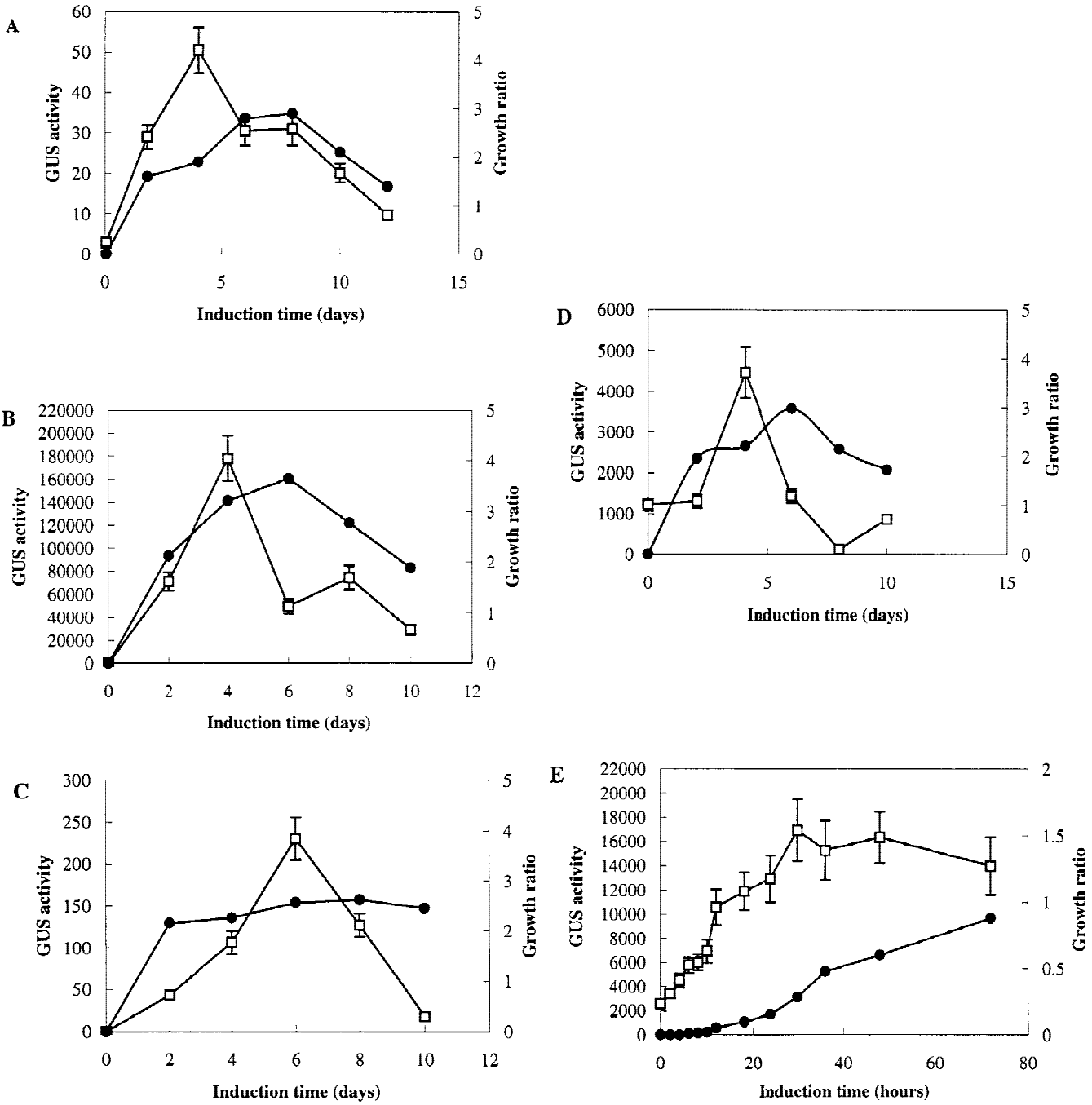

Figure 3. GUS activity and growth ratio as a function of induction time. GUS activity (□) and growth ratio (●) were evaluated in cell-lines 306 **(A),** 21 **(B),** 22 **(C),** 20 **(D),** or 29 **(E)** containing the *pSalT-gus, pTet-gus, pMre-gus, pIn2-2-gus,* or *pats1A-gus* construct, respectively. GUS activity was expressed in pmol MU h^{-1} μg^{-1} of proteins and represented as the difference between the activity measured in treated cell suspensions and that in untreated cell suspensions (□). The growth ratio was presented by the ratio between the increase of the biomass (final biomass at x d − initial biomass at 0 d) on the initial biomass. Cell-lines 306, 21, 22, 20, or 29 were treated with 20 μM ABA, 20 μM tetracycline, 100 μM $CuSO_4$, 200 μM 2-CBSU, or exposed to 3.4 Wm^{-2} light intensity, respectively. Each point represents the average of five measurements. For each growth ratio value, the standard deviations were comprised between 5% and 15%. For GUS activity values, standard deviations (±SD) are shown.

The cells containing the *pSalT-gus* or *pMre-gus* construct cultured for 8 d prior to induction displayed the highest GUS activities: 18.8 or 390.0 pmol MU h^{-1} μg^{-1} of proteins, respectively (Figs. 4A,C). In *pTet-gus* cells (Fig. 4B), maximal *gus* expression (145,000 pmol MU h^{-1} μg^{-1} of proteins) was observed in the cells cultured for 10 d prior to the induction step. For the *pSalT-gus, pTet-gus* or *pMre-gus* cells, the growth ratios were 3.1, 1.9, or 3.0, respectively, indicating that the cells were still growing. The *pIn2-2-gus* cells (Fig. 4D) had to be cultured for 6 d before 2-CBSU induction for maximal GUS activity (16,980.0 pmol MU h^{-1} μg^{-1} of proteins). Optimal GUS expression (13,500

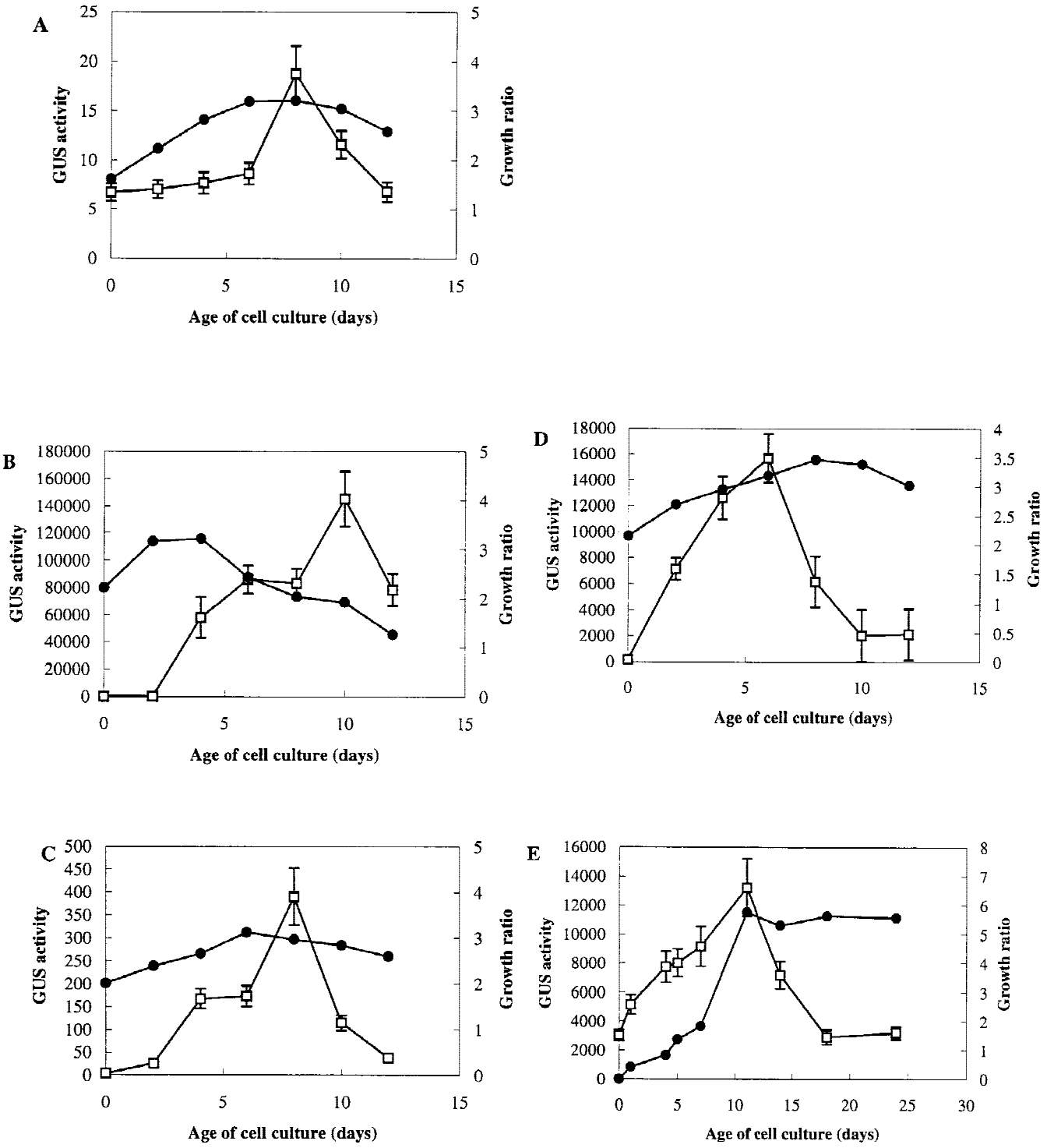

Figure 4. GUS activity and growth ratio as a function of cell age during the culture. GUS activity (□) and growth ratio (●) were evaluated in cell suspensions 306 **(A),** 21 **(B),** 22 **(C),** 20 **(D),** 29 **(E)** containing the *pSalT-gus*, *pTet-gus*, *pMre-gus*, *pIn2-2-gus*, or *pats1A-gus* construct, respectively. GUS activity was expressed in pmol MU h^{-1} μg^{-1} of proteins and represented as the difference between the activity measured in treated cell suspensions and that in untreated cell suspensions (□). The growth ratio was presented by the ratio between the increase of the biomass (final biomass at x d − initial biomass at 0 d) on the initial biomass. Cell-lines 306, 21, 22, 20, or 29 were treated with 20 μM ABA for 4 d, 20 μM tetracycline for 4 d, 100 μM $CuSO_4$ for 6 d, 200 μM 2-CBSU for 4 d, or exposed to 3.4 Wm^{-2} light intensity for 24 h respectively. Each point represents the average of five measurements. For each growth ratio value, the standard deviations were comprised between 5% and 15%. For GUS activity values, standard deviations (±SD) are shown.

pmol MU h^{-1} μg^{-1} of proteins) measured with the *pats1A-gus* construct was observed after induction in a 12-d-old cell culture. For the *pIn2-2-gus* and *pats1A-gus* cells, the growth ratios were 3.2 and 5.8, respectively (Figs. 4D,E).

In these experiments, we also tested inducer toxicity during culture under the chosen optimal induction conditions. ABA was more toxic at the beginning and at the end of the cell culture, with toxicity values of 1.6 and 1.4, respectively,

than during the exponential stage (Table I). Tetracycline and 2-CBSU were weakly toxic for the *pTet-gus* and *pIn-2-2-gus* cells, respectively, with toxicity values between 1.0 and 1.2 (Table I). $CuSO_4$ was toxic for cells during the exponential and stationary stages (maximal toxicity of 1.8 was observed in 10-d-old cells; Table I). Light, an essential factor for cell growth, was not toxic in our experiment. Indeed, the growth ratios in cells treated with light were equivalent to those obtained with untreated cells (data not shown).

Under the optimal induction conditions, the inducers ABA, tetracycline, $CuSO_4$, and 2-CBSU were not toxic enough to cause cell death. Therefore, the cells could be used for repeated induction after filtration and medium change.

GUS Expression Levels as a Function of the Promoter

For each inducible system, several independent transgenic cell lines were then tested under our chosen optimal induction conditions to evaluate interclonal variability. We systematically compared the GUS expression level under inducing and non-inducing conditions for several different cell lines for each construct. These results allowed calculation of the induction rate used to measured the efficiency of the different systems. The induction rate was evaluated as the ratio between the value of GUS activity determined in a cell suspension treated with inducer and its value determined in the same untreated cell suspension.

for the *pSalT-gus* cells, induction rate was 1.0 to 14,143.0. Basal *gus* expression measured without induction was usually higher than that measured in untransformed cells (Fig. 5A: compare the italic numbers above bars with that of the control). Indeed, most of the cell lines (227, 247, 249, 300, 301, 305, 306, and 309) displayed a 10- to 30-fold higher activity than the endogenous GUS background of the control in the absence of induction (Fig. 5A), indicating that the promoter was partially turned on. Only two cell lines (308 and 311) had basal GUS expression that was 1400 to 27,000-fold higher than the control endogenous GUS background (Fig. 5A). After addition of ABA to the culture medium, GUS activity increased in almost all cases. In these experiments, the maximal GUS activities were about 177,600.0 and 60,610.0-fold higher than the endogenous GUS background of the control measured with ABA in cell lines 311 and 247, respectively. So, maximal GUS activity obtained with a constitutive promoter *CaMV 35S-gus* [54 400.0 pmol MU h^{-1} μg^{-1} of proteins; (Thomasset et al., 1996)] was lower than that observed in cell lines 247 and 311 (Fig. 5A).

Basal GUS activity measured in clones transformed with the *pTet-gus* construct were 180- to 141,000-fold higher than the endogenous GUS background of the control obtained with tetracycline (Fig. 5B). GUS activities determined after tetracycline treatment were also very high, and greater than those obtained with a constitutive promoter, for all tested cell lines except for cell-line 22. The induction rate was 2.2 to 28,036.0.

For all tested clones containing the *pMre-gus* or *pIn2-2-gus* construct, basal GUS expression was 14- to 440- or 7- to 395-fold higher than the endogenous GUS background of the controls obtained with $CuSO_4$ or 2-CBSU, respectively (Figs. 5C,D). GUS activities after induction were lower than the constitutive level. So, $CuSO_4$ or 2-CBSU gave only very low induction rates of 1.2 to 4.6 or 1.3 to 488.2 for *pMre-gus* or *pIn2-2-gus* cells, respectively (Figs. 5C,D).

Clones transformed with the *pats1A-gus* construct had low induction rates of 1.0 to 30.8 (Fig. 5E). Basal and induced GUS activities were very low with the exception of the clone number 9, for which both values were 14,100 pmol MU h^{-1} μg^{-1} of proteins, as if the *gus* expressions were driven by a constitutive promoter.

Maximal induction was obtained in approximately 30% or 40% of the cell lines containing the *pSalT-gus* or *pTet-gus* construct, respectively (Table II), with induction rates greater than 1000. Induction rates in the majority of clones containing the *pMre-gus* (70%) or *pIn2-2-gus* (85%) construct were between 1.5 and 1000 (Table II), while those measured in the *pats1A-gus* clones were very low (55% of the clones are not stimulated after induction; Table II).

These results show that the promoters with the best potential for future metabolic production in cell suspensions appear to be *pSalT* and *pTet.*

DISCUSSION

Development of a practical system to externally control the expression of selected genes would be useful, both for studying the mechanisms underlying basic biological processes and for commercial applications. The inducer of such

Table I. Estimation of inducer toxicity. ABA, tetracycline, $CuSO_4$ or 2-CBSU toxicity was evaluated from the cell lines containing the *pSalT-gus, pTet-gus, pMre-gus* or *pIn2-2-gus* construct, respectively. Cell cultures were incubated with 20 μM ABA or tetracycline for 4 d, 100 μM $CuSO_4$ for 6 d, or 200 μM 2-CBSU for 4 d, respectively. Toxicity was expressed as the ratio between the growth ratio in an untreated cell suspension and that in the same induced cell suspension. The results showed the average (±SD) obtained from five separate measurements.

Age of Culture Before the Induction Step (Days)	0	2	4	6	8	10	12
ABA toxicity	1.6 ± 0.4	1.2 ± 0.4	1.2 ± 0.3	1.0 ± 0.2	1.2 ± 0.3	1.2 ± 0.3	1.4 ± 0.3
Tetracycline toxicity	1.1 ± 0.2	1.0 ± 0.2	1.2 ± 0.2	1.0 ± 0.2	1.0 ± 0.3	1.2 ± 0.3	1.0 ± 0.2
$CuSO_4$ toxicity	1.1 ± 0.2	1.1 ± 0.1	1.5 ± 0.4	1.3 ± 0.3	1.6 ± 0.4	1.8 ± 0.5	1.2 ± 0.2
2-CBSU toxicity	1.0 ± 0.2	1.1 ± 0.2	1.2 ± 0.2	1.2 ± 0.2	1.1 ± 0.2	1.1 ± 0.2	1.2 ± 0.3

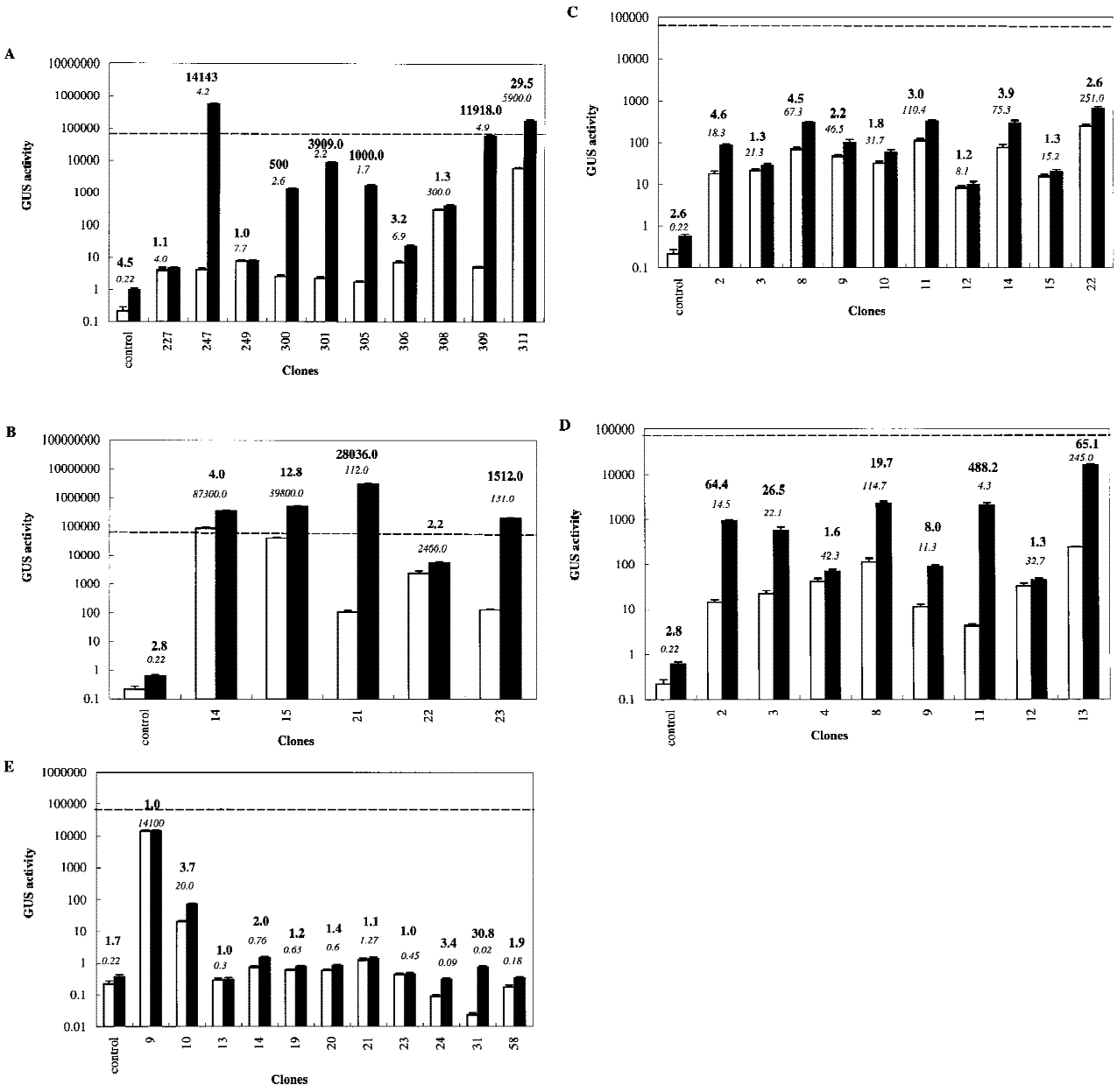

Figure 5. Induction step in several individual transgenic cell lines. GUS activity (pmol MU h^{-1} μg^{-1} of proteins) was measured before (white bars) and after (black bars) treatment with ABA **(A),** tetracycline **(B),** $CuSO_4$ **(C),** 2-CBSU **(D),** or light **(E)** under the optimal induction conditions from the cell suspensions transformed with the *pSalT-gus*, *pTet-gus*, *pMre-gus*, *pIn2-2-gus*, or *pats1A-gus* construct, respectively. The broken line represented GUS activity (54,400 pmol MU h^{-1} μg^{-1} of proteins) obtained with the *pCaMV 35S-gus* construct. The bold numbers above bars indicate the induction rate for each cell line expressed as the ratio between the GUS activity in the induced cell suspension and that in the same untreated cell suspension. The italic numbers above bars indicate the basal GUS activities for each cell line. The control bars represent GUS activity measured in untransformed cell suspensions. The results show the average (±SD) obtained from five separate measurements.

a system should have minimal toxicity, should be easily applied during the cell cultures, and should be efficacious at a low-use rate.

Induction Steps and Inducer Toxicity

In all systems tested (*pSalT-gus*, *pTet-gus*, *pMre-gus*, *pIn2-2-gus*, or *pats1A-gus*), the promoter was turned on by the corresponding chemical or physical stimulus and the optimal induction conditions were determined. Maximal GUS activities were observed in the cells cultured for 8 to 12 d prior to a 4 to 6 d induction step (24 h for the cells containing the *pats1A-gus* construct).

We noted that the *pats1A-gus* cells had the shortest induction time. One possible explanation is that the light receptors are more sensitive than those controlled by chemical compounds such as ABA, tetracycline, copper, or 2-CBSU. In fact, light is a stimulus that regulates numerous developmental and metabolic processes and the variable responses of the plant depend upon the quality and quantity of the

Table II. Estimation of expression variability between the different inducible systems. This study was performed from cell suspensions transformed with the *pSaIT-gus, pTet-gus, pMre-gus, pIn2-2-gus,* or *pats1A-gus* construct. The results were expressed in % of cell lines calculated from the results shown in Figure 5.

		Construct				
		pSaIT-gus	*pTet-gus*	*pMre-gus*	*pIn2-2-gus*	*pats1-gus*
% of clones with induction rates	>1000	30	40	0	0	0
	between 1.5 and 1000	40	60	70	85	45
	<1.5	30	0	30	15	55

light. Indeed, in photosynthetic organisms, solar energy is rapidly collected by specific photoreceptors and subsequently transferred to the photosynthetic reaction centers (Zuber et al., 1987), while the chemical compounds must physically reach the nucleus to turn on the inducible promoter.

Our results show that ABA and copper were more toxic for cells than tetracycline and 2-CBSU (Table I). ABA is a growth regulator involved in the regulation of cellular differentiation and division and has been identified as an agent in the stress perception-response pathways (Zeevaart and Creelman, 1988). Its concentration must, therefore, be precisely regulated in cells, and a small increase (above 25 μM) can be toxic (growth rate less than 1; Table I). Copper is also implicated in cellular metabolism as a cofactor of oxido-reductases, and a slight variation in its concentration might disturb the regulation of their activity (Clarkson and Hanson, 1980). Although both copper and ABA are involved in plant metabolism, optimal induction with copper occurs at much higher concentrations of the former (100 μM vs. 20 μM ABA). Silverberg et al. (1976) have shown that a significant fraction of copper is sequestered in the wall of root cells before accumulating in the nucleus while Kubota et al. (1988) have demonstrated that copper is trapped in the cytoplasm. Cell suspensions cultured in liquid medium might be able to develop a similar system to sequester copper (Kubota et al., 1988). Tetracycline and 2-CBSU, which are not synthesized by plant cells, were shown to be toxic, but they did not affect cell growth under our optimal induction conditions (Table I). These molecules would be considered as foreign by the cultured plant cells, and would probably be sequestered in vacuoles.

Comparison of Different Inducible Systems for the Expression of *gus* Gene

After determining the optimal induction conditions, GUS expression under inducing and non-inducing conditions was evaluated in several cell lines of each construct. In our study, we used plant cell suspensions to test expression of a reporter gene driven by different inducible promoters. In our work, an inducible system is considered as an efficient system to control the transgene expression when: (1) the expression is close to the level of the endogenous GUS background (0.28 ± 0.03 pmol MU h^{-1} μg^{-1} of proteins) in the absence of inducer, preventing gene activation in the uninduced state; and (2) the expression is of the same level as those recorded with a constitutive system (such as a *CaMV 35S-gus* system: 54400 ± 800 pmol MU $h^{-1}/\mu g^{-1}$ of proteins) when the inducer is present. Moreover, the toxicity of the corresponding inducer is evaluated by the ratio between the value of the growth ratio for an untreated cell suspension and its value for the same treated cell suspension. An inducer is considered as a non-toxic stimulus when it does not affect the cell growth. In this case, this ratio is around 1.

Numerous clones transformed with the *pSalT-gus* construct presented important induction rates (Fig. 5A) and the basal GUS activities were relatively low. So, the induction response was homogeneous and ABA, in our induction conditions, was not toxic for tobacco cells. The *pMre-gus* cell lines had a very low induction rate (between 1.2 and 4.6; Fig. 5C). The *pMre* promoter may be less efficient in undifferentiated cell cultures than in tissues or whole plants (Mett et al., 1993). Indeed, Odell et al. (1985) have studied the same promoter with *cat* gene (chloramphenicol acetyltransferase), the −90Δ *CaMV 35S-cat* system in tobacco plants and shown that CAT expression occurs specifically in the roots. Moreover, the structure of this promoter can explain why high basal GUS levels and consequently, low induction rates were obtained (Fig. 5C). The *pMre* promoter is composed of two elements: the *Neurospora crassa mre* region belonging to the gene encoding the metallothioneins, and the −90Δ *CaMV 35S* sequence, a region of the Cauliflower Mosaïc Virus *CaMV 35S* promoter that can drive transcription in prokaryotic and eukaryotic cells (Mett et al., 1993; Odell et al., 1985). This chimeric promoter elaborating with the −90Δ *CaMV 35S* sequence might drive *gus* expression in absence of copper leading to high basal GUS levels. The *pTet* promoter is also composed of a modified *CaMV 35S* promoter and despite the elevated basal GUS levels obtained in the *pTet-gus* cell lines, the induction rates could be very high (Fig. 5B). Thus, the nature of the inducer and not only the structure of the promoter can explain the differences in induction between the *pTet-gus* and *pMre-gus* clones (Gatz et al., 1991; Mett et al., 1993). Copper ions might be rapidly sequestered in cell vacuoles or cytoplasm as shown by Kubota et al. (1988) in transgenic tobacco plants, and nuclear concentrations might be insufficient to induce the *pMre-gus* construct. Moreover, copper ions are involved in several metabolic reactions such as oxido-

reductase activation, and only a much lower effective concentration should be available to stimulate the *pMre-gus* construct. Tetracycline is not synthesized by the plant cells and not involved in plant metabolism. Even if certain tetracycline molecules are sequestered in a cell compartment, the others could efficiently stimulate the *pTet-gus* construct. This hypothesis might explain the differences between the higher induction rates observed in the *pTet-gus* vs. the *pMre-gus* cell lines. Likewise, 2-CBSU is also not synthesized by plant cells and the induction rates in the *pIn2-2-gus* cell lines are higher than in the *pMre-gus* cell lines. The induction rates in the *pats1A-gus* clones were lower than those observed with the other constructs. The *pats1A* is the promoter of a gene encoding the RUBISCO SSU from *Arabidopsis thaliana* (Krebbers et al., 1988). Berry et al. (1990) have shown that SSU synthesis was only 20-fold higher after light induction than in darkness. Light has been shown to slightly induce *ssu* gene expression in plants, which might explain the low induction rates obtained with the *pats1A-gus* construct. However, Herrera-Hestrella (1984) studied CAT expression driven by the *prbc 3A* promoter, which also belongs to a gene encoding the SSU. They have shown that the chimeric *prbc 3A-cat* gene is not expressed in tissues lacking chloroplasts. In our experiments, the undifferentiated cells cultured in the dark might not possess differentiated chloroplasts and thus, *pats1A-gus* inducible system might not be efficiently expressed.

By using independently transformed cell lines for each construct, the observed variation of GUS expression may be due to the copy number. However, while some authors found the transgene copy number to be positively correlated to the expression rate (Gendloff et al., 1990; Hobbs et al., 1993), others found a negative correlation (Hobbs et al., 1993; Kilby et al., 1992) or no correlation at all (Hobbs et al., 1990). Therefore, the copy number seems to be a secondary parameter for foreign gene expression.

CONCLUSIONS

This work aimed to identify specific promoters that can efficiently switch off GUS expression in the absence of inducer and strongly switch it on in the presence of inducer at selected times in the tobacco culture. The *pSalT-gus* and *pTet-gus* constructs were the most promising systems: 30% and 40% of the cell lines have induction rates higher than 1000, and GUS activities after induction are equivalent to those obtained with a constitutive system (Table II and Figs. 5A,B). However, many *pTet-gus* transgenic lines present a high-expression level when they are not induced. These elevated basal GUS activities obtained with the *pTet-gus* cell lines might be decreased by modification of the promoter structure. The link between repressor and operators might be improved if other *Tn10* region operators could be added within the *pTet* promoter. The use of tetracycline analogs might also increase the induction efficiency. Indeed, according to Lederer et al. (1996), the affinity between the repressor and tetracycline is greater when tetracycline molecules substituted in position 7 or 8 are used to induce the *pTet-gus* system in bacteria. These substituted molecules might be tested in plant cells.

The novel feature of this comparative study of five inducible systems is that it used cell suspensions rather than whole plants. Indeed, the advantages of cell suspensions (shorter biosynthetic cycle, higher metabolic activity, controlled environmental conditions) can facilitate regulatory studies or improve and control secondary metabolite productions. The tests with these chosen inducible promoters have been performed to use these systems for future studies in cell suspensions. We show that the *pSalT* promoter has the best characteristics to be used for these studies. The on/off response in cell suspensions can now be further improved by the development of other inducible systems, which should be finely adapted to plant cells, such as those inducible by glucocorticoids (Aoyama and Chua, 1997).

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