Characterization of a Tobacco Bright Yellow 2 Cell Line Expressing the Tetracycline Repressor at a High Level for Strict Regulation of Transgene Expression[1]

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Manipulating the expression of a transgene in transient and stable transformed cells is a requirement for many functional analyses. We have investigated the use of the tetracycline-dependent gene expression system developed by Gatz et al. (1992) in tobacco (*Nicotiana tabacum* L. cv Bright Yellow 2 [BY2]) cells, the most widely used plant cell culture. We have selected a BY2 cell line, named BY2-tetracycline repressor (tetR) 17, which expresses the tetR at a high level, and have evaluated the capacity of this cell line to suppress the expression of a green fluorescent protein reporter gene under the control of the "Triple-Op" promoter in the absence of tetracycline in a large number of independent transformants. The ability to induce the expression of green fluorescent protein after treatment by anhydrotetracycline in the same transformants was also analyzed. BY2-tetR17 cells were demonstrated to be excellent recipient cells for recovery of clonal cell lines with a highly controlled regulation of the introduced transgene.

Established cell lines such as Hela cells have played an important role in the basic understanding of the molecular and cellular biology of mammalian cells. The establishment of cell lines from plant tissues is relatively easy and numerous cell lines have been obtained from various tissues and species of higher plants. Among these, the tobacco (*Nicotiana tabacum* L. cv Bright Yellow 2 [BY2]) cell line, isolated by Kato and coworkers (1972), is rather unique and is well characterized (Nagata et al., 1992). This cell line is highly homogeneous and shows an exceptionally high growth rate, multiplying 80- to 100-fold in 1 week. After treatment with aphidicholin, a high cell cycle synchrony can be obtained. As a consequence, the BY2 line has emerged as a model system for the study of the plant cell cycle (Combettes et al., 1999). In addition, BY2 cells can be easily transformed without the need for protoplast preparation and stable transgenic calli and suspension-cultured cells are easily obtained. These features make this cell line a powerful tool for exploring the molecular and cellular biology of plant cells.

A regulatory system is often desirable to induce transgene expression at defined time points and is particularly important if the gene product is toxic for the plant cell. A number of plant promoters regulated by light (Kuhlemeier et al., 1989), heat (Ainley and Key, 1990), wounding (Firek et al., 1993), phytohormones (Li et al., 1991), or antioxidants (Hérouart et al., 1993) are available for the controlled expression of a transgene. However, these systems all suffer from the disadvantage that the inducing conditions influence a variety of nonspecific responses in the plant. Therefore, Gatz and collaborators have developed a tetracycline-specific derepressible expression system (Gatz and Quail, 1988). This system was first used in plant protoplasts and since has been successfully used for the inducible expression of *rolB* (Röder et al., 1994), *rolC* (Faiss et al., 1996), *ipt* (Faiss et al., 1997), a dominant negative mutant of the bZIP transcription factor *PG13* (Rieping et al., 1994), as well as an Arg decarboxylase (Masgrau et al., 1997).

We have brought together the advantages of the BY2 cell line and the tetracycline derepressible system. We report here on the generation and characterization of a BY2 cell line expressing the tetracycline repressor (tetR) at a high level and its use to completely suppress the expression of a transgene in the absence of inducers and to obtain high level of expression after anhydrotetracycline (Ahtc) treatment.

RESULTS AND DISCUSSION

The objective of this work was to generate a BY2 cell line allowing a controlled regulation of the expression of a gene of interest. The tetracycline derepressible system developed by Gatz et al. (1992) was selected because it was demonstrated to be an efficient system in tobacco plants. The main constraint of this system is the preliminary selection of material expressing the tetR at a high level, a prerequisite for strict control of the expression of a gene under the control of the "*triple-Op*" promoter (Gatz et al., 1992). We used the *pBinTet1* vector, containing *tetR* under the control of the *cauliflower mosaic virus* (*CaMV*)*-35S* promoter, to transform BY2 cells. Clonal and stable

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transformants, named BY2-tetR, were selected on kanamycin medium.

To screen for calli expressing high levels of functional repressor, transient transformations were performed on 40 independent calli with *pTX-Gus-int*, a vector containing β-glucuronidase (Gus) under the control of the "*Triple-Op*" promoter coupled with *CaMV 35S* (Gatz et al., 1992). The transformed BY2-tetR cells were analyzed for Gus after 2 d of induction with or without 5 μg mL^{-1} AhTc. Gus activity was followed over 4 h by measurement of fluorescence in cell extracts of each BY2-tetR cell line and in a control cell line corresponding to wild-type BY2 cells transiently transformed with *pTX-Gus-int vector* (Fig. 1).

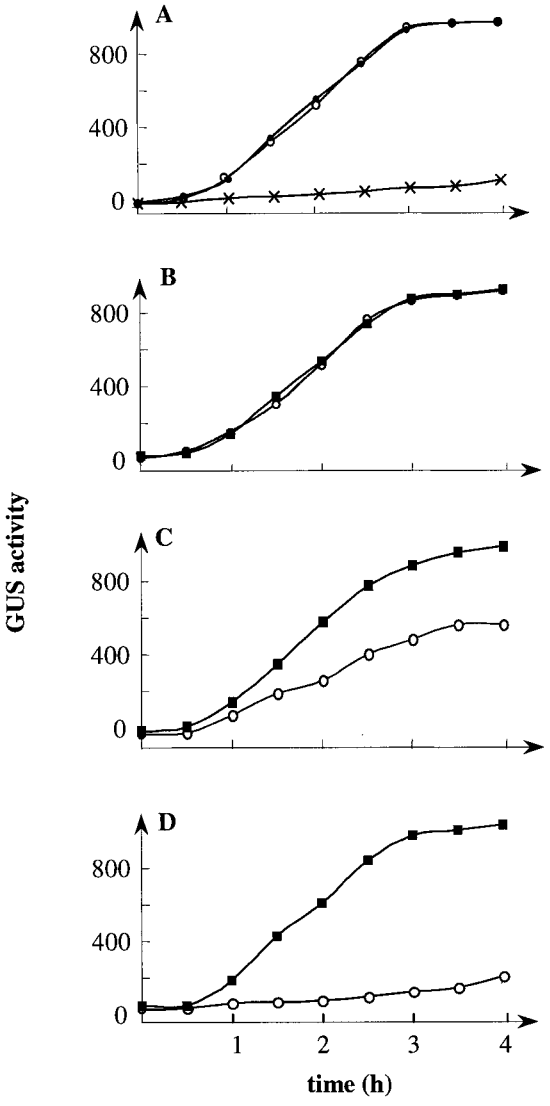

Figure 1. Induction of GUS activity by Ahtc in transient assays. Measurements of Gus activity in wild-type BY2 cells (A) and three distinct BY2-R clones R13 (B), R20 (C), and R17 (D), transformed with *pTX-GUS-int* reporter plasmid and incubated in the presence (■) or absence (○) of 5 μg mL^{-1} Ahtc. Untransformed BY2 cells (X) were used as a control. GUS activity is expressed in pmol 4 MU min^{-1} μg^{-1} proteins.

In a wild-type background, the *CaMV-35S* promoter conferred a high constitutive expression of Gus and no difference was observed between Ahtc-treated and non-treated cells. In both cases, maximal activity of about 1,000 pmol 4-methylumbelliferone (4 MU) min^{-1} μg^{-1} was reached after 3 h (Fig. 1A). Untransformed cells were used as a negative control to evaluate the fluorescence background of cell extracts (Fig. 1A). Although Gus activity reached similar levels in all Ahtc-treated BY2-tetR cell lines, Gus activity measured in the absence of inducers differed among the clones. Ten of the cell lines did not show any difference in the presence or in the absence of Ahtc treatment as illustrated by the BY2-tetR13 cell line (Fig. 1B). Gus activity kinetics in BY2-tetR13 were similar to the control (Fig. 1A), indicating that the expression of Gus was not repressed in the absence of the inducer. The other 30 BY2-tetR cell lines conversely showed an Ahtc-dependent Gus activity (Fig. 1, C and D). Among these, a large number of cell lines showed an approximately 2-fold increase in Gus activity following Ahtc treatment, as illustrated for BY2-tetR20 (Fig. 1C). The background of Gus activity in untreated cells suggests that the tetR repression system is leaky in these cells, at least in the experimental conditions of transient assays. Figure 1D, corresponding to BY2-tetR17, illustrates the results obtained with the only three BY2-tetR cell lines. In these cells, Gus activity in untreated cells remains similar to the fluorescence background of untransformed BY2 cells, suggesting an efficient repression of the reporter gene transcription by interaction of tetR with the Triple-Operator sequences. After Ahtc treatment, an approximate 10-fold increase in Gus activity was measured, reaching a level similar to that of the 35S CaMV::Gus positive control. Differences observed between the different BY2-tetR lines could reflect differences in the expression of tetR in the respective lines or differences in transformation efficiencies in the transient assay.

To further characterize the different BY2-tetR cell lines, the expression of tetR was investigated at the RNA and protein levels (Fig. 2). Results are illustrated for the same cell lines as shown in Figure 1, i.e. BY2-tetR13, 20, and 17. A good correlation was observed between *tetR* RNA accumulation (Fig. 2A) and tetR protein levels (Fig. 2B). The highest accumulation of tetR RNA and the highest immunodetection of the tetR protein were observed in BY2-tetR17, which showed no Gus expression in the absence of Ahtc and a strong activity after treatment with Ahtc (Fig. 1D). Lower levels were observed in BY2-tetR20 extracts and only a very weak signal was detected in BY2-tetR13. These results demonstrate that high steady-state expression of tetR ensures an efficient repression of the "*Triple-Op*" promoter. The tetR expressed in the BY2-tetR17 line is functional and able to interact with the "*Triple-Op*" promoter to block transcription but can also dissociate from operons after bind-

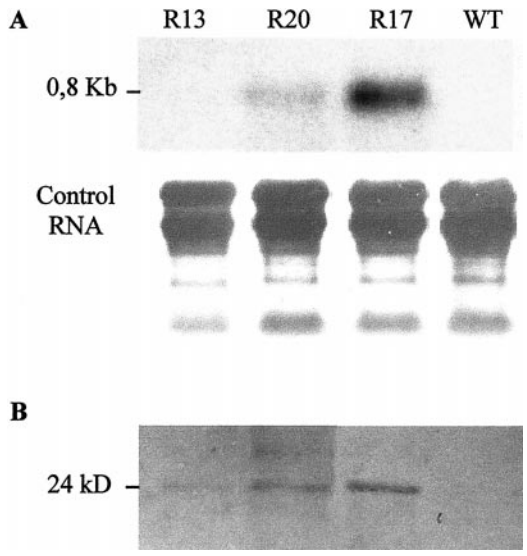

Figure 2. Tet R expression levels of different BY2-tetR cell lines. A, Northern hybridization analysis of *tetR* expression in three independent BY2-tetR cell lines (R13, R20, and R17) and in untransformed BY2 cells (WT). Ten micrograms of total RNA was loaded on each lane. The methylene blue staining of total RNA after blotting onto membrane is presented (control RNA). B, Western-blot analysis of the tetR in the same cell lines using monoclonal antibodies against tetR. About 5 μg of protein extracts was loaded.

ing of Ahtc to allow expression. Our results show that BY2-tetR cell lines expressing tetR at a high level allow the production of a foreign protein in the cell under conditions of transient expression. However, the ability to control the expression of a transgene in stable transformants is also of great interest for functional studies in plant cells.

On the basis of the results obtained in transient transformation, the BY2-tetR17 line was chosen for recipient cells. Stable transformants were generated in the BY2-tetR17 background with either *pTX-Gus-int* or *pGFPHyg-TX* vectors. Wild-type BY2 cells were also transformed with the same constructs to generate stable transformants expressing Gus or green fluorescent protein (GFP) constitutively for use as positive controls.

A large number of independent calli issuing from selection on kanamycin and hygromycin medium were obtained after transformation. Interclonal variability was studied by measuring Gus activity or GFP fluorescence in at least 24 independent clones for each, with or without Ahtc treatment. We have developed a simple procedure for growing small amounts of cells on solid medium in the presence or absence of the Ahtc inducer. Small growing calli were first resuspended in 200 μL of modified MS liquid medium, one-half of which was transferred in the well of a 24-well plate containing modified MS agar medium with 5 μg mL^{-1} Ahtc. The second half was transferred to a medium without Ahtc. Measured Gus activity in individual transformants ranged from 2,000 to 3,500 pmol 4 MU min^{-1} μg^{-1} after 3 d of treatment, whereas the background measured in untreated cells was approximately 50 pmol 4 MU min^{-1} μg^{-1} (data not shown).

For the GFP construct, expression in the different clones was first followed over time by observing GFP accumulation fluorescence by microscopy. In most cells, GFP fluorescence was detected 24 h after addition of Ahtc and reached a maximum after 3 d. Because Ahtc was not renewed in the culture medium, GFP fluorescence decreased in the following days. Only five clones out of 24 exhibited strong GFP fluorescence in the absence of Ahtc in the medium. In each case, the fluorescence was lower than for the same cells growing on Ahtc. Figure 3 illustrates the fluorescence observed with the clone 10 grown in the absence of inducer (Fig. 3B) and after 1 and 3 d on Ahtc medium (Fig. 3, C and D), respectively. In the absence of Ahtc, no fluorescence was detected, suggesting an efficient repression of the transcription of the transgene by tetR. After Ahtc treatment, the fluorescence was observed mainly in the cytosol and the nuclei. A similar distribution of the GFP fluorescence was observed in the positive control cell line expressing GFP under the control of the 35S-CaMV promoter (not shown). The presence of Ahtc in plant cells did not obviously interfere with the observation of GFP in the experimental conditions used.

A quantitative analysis of the fluorescence was performed to further characterize individual transformants. Results corresponding to 24 independent stable transformants are reported in Figure 4 together with a negative control corresponding to BY2-tetR17 cells (sample 1) and the BY2 cells expressing GFP under the control of the 35S-CaMV promoter as a positive control (sample 2). Apart from clone 16, which resembled the positive control, all the other independent transformants displayed an increased fluorescence in the Ahtc-treated cells. A number of clones exhibited a very high GFP fluorescence, some-

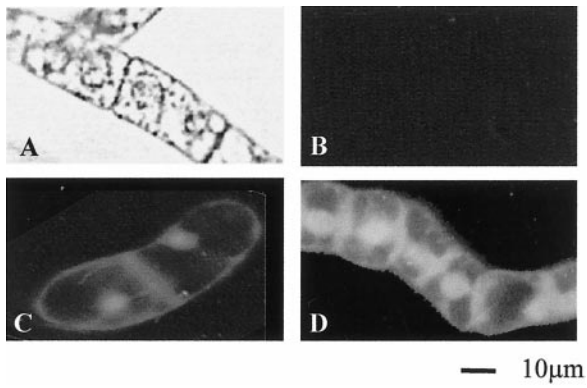

Figure 3. Detection of GFP fluorescence in the transgenic cell line BY2-tetR17 expressing *GFP* under the control of the "*Triple-Op*" promoter. Light and fluorescent microscope observations of the stable transformant (clone 10) generated in the BY2-tetR17 background using the *pGFPHyg-TX* vector. Untreated cells (A and B) and cells treated with 5 μg mL^{-1} Ahtc after 1 (C) or 3 (D) d.

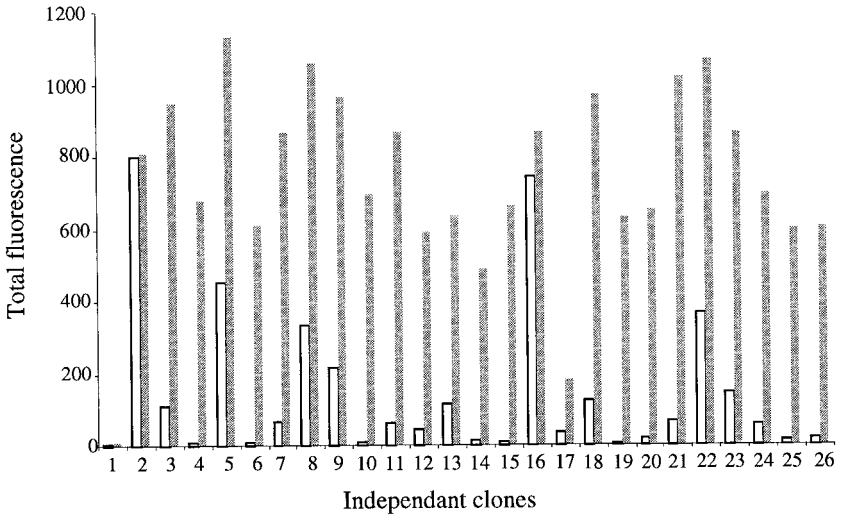

Figure 4. Quantitative GFP analysis of distinct BY2-tetR17-GFP stable cell lines. Proteins were extracted from calli treated (black) or not (white) with Ahtc. Thirty micrograms of proteins was used for fluorimetric analysis. Measurements corresponding to 24 individual clones of BY2-tetR17-GFP (3–26) are plotted. BY2 untransformed cells (lane1) and BY2 cells expressing *GFP* under the control of the *35S-CaMV* promoter (lane 2) were used as internal references. For each sample, GFP fluorescence was measured at 510 nm with an excitation wavelength of 480 nm.

times higher than in the positive control, but in these transformants GFP could also be detected in the cells in the absence of Ahtc (for example, clones 5, 8, or 22). For about one-half of the clones analyzed, the GFP fluorescence was undetectable or very low in the absence of Ahtc confirming an efficient repression of the transcription by tetR (for example, clones 4, 10, 19, or 25). For some of these clones, the difference of fluorescence between untreated and Ahtc-treated cells can reach a factor of 50 to 100. Given the sensitivity of the method used to detect the expression of the transgene (GFP) in stable transformants, these results demonstrate that it is possible to generate stable cell lines with no leak in the control of the expression of the transgene. To investigate whether differences measured between stable transformants were correlated or not to the number of copies of the transgene integrated in the genome, we have studied the different clones by Southern blot. Profiles observed for the clones 6, 8, and 16, which exhibited distinct regulation, are shown in Figure 5. No obvious correlation has been observed between the apparent number of copies and the relative suppression of expression in the absence of Ahtc.

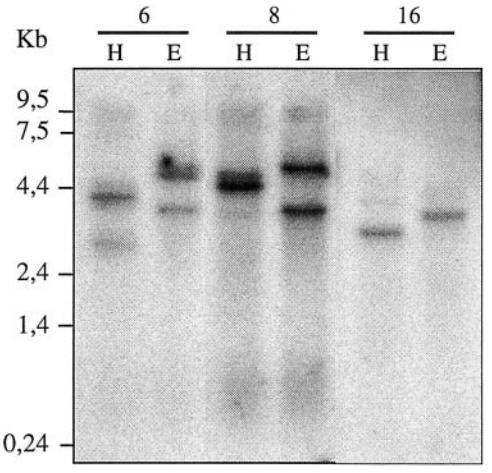

Figure 5. Southern-blot analysis of three BY2-tetR clones transformed with GFP. Total DNA (10 μg) was isolated from three BY2-tetR17 clones transformed with *pGFPHyg-TX* (clones 6, 8, and 16). Genomic DNA was digested with *Eco*RI (E) and *Hpa*I (H) and subjected to Southern-blot analysis using the *HPTII* gene as probe.

In conclusion, we have demonstrated that the tetracycline derepressible system developed by Gatz and coworkers (1992) is applicable for the control of transgene expression in tobacco BY2 cells. Efficient repression, in the absence of the inducer Ahtc, requires a high steady-state level of tetR expression in the cell line used as a recipient. We characterized such a BY2 cell line, named BY2-tetR17, and have shown that it can be used to follow the expression of a transgene in transient assays as well as for generating stable transformants. An efficiently controlled expression system with undetectable expression in the absence of inducer is of great interest for addressing many questions in cell biology. It is also of special importance for the expression of gene products that interfere with cell growth and, more specifically, with the regulation of the cell cycle. The BY2-tetR17 cell line is available for studies in a large range of potential applications.

MATERIALS AND METHODS

Plant and Bacterial Strains

Suspension-cultured tobacco (*Nicotiana tabacum* L. cv BY2) cells (Kato et al., 1972) were grown in the dark at 26°C on a rotary shaker (130 rpm) in modified MS, a modified Murashige-Skoog basal medium (Sigma, St-Quentin Fallavier, France), supplemented with 1.5 mM KH2PO4, 3 μM thiamine, 0.55 mM inositol, 87 mM Suc, and 1 μM 2,4 dichlorophenoxy acetic acid. Cells were subcultured every 7 d by transferring 2 mL into 100 mL of fresh medium. *Agrobacterium tumefaciens* strain LB4404 was cultivated in yeast extract bactotryptone medium and transformed according to Höfgen and Willmitzer (1988).

Constructs

pBinTet1 contains the *tetR* coding region under the control of the *CaMV-35S* promoter (Gatz et al., 1991). The octopine synthase (*ocs*) polyadenylation signal is used as transcriptional terminator. This vector confers kanamycin resistance to the plant. *pTX-Gus-int* (Gatz et al., 1992) is a plasmid containing the β-glucuronidase (*gus*) gene coding region inserted into the *pBinHygTX* vector. The latter contains a cassette with the "Triple-Op" promoter, a combination of three *tet* operators with the *CaMV-35S* promoter (Gatz et al., 1992), and confers hygromycin resistance to plants. *pGFPHyg-Tx* has been constructed by insertion of a 940-bp *Xba*I/EcoRV fragment, excised from *pMON30049*, encoding a modified version of a GFP gene (*gfp*; Pang et al., 1996) into *Xba*I/*Sal*I sites of *pBinHygTX*.

Cell Transformation

Co-incubations of 4 mL of BY2 cell suspension at an exponential phase of growth (3–4 d after subculture) and 100 μL of a 24-h *A. tumefaciens* preculture were performed in small petri dishes in the dark for 48 h without agitation. The BY2 cells were then washed two to three times with 15 mL of fresh culture medium. To obtain stable and independent transformants, cells were plated at low density onto agar-MSST medium containing 500 μg mL^{-1} cefotaxime and the selective antibiotic (100 μg mL^{-1} Kanamycin for the tetR lines or 100 μg mL^{-1} Kanamycin and 30 μg mL^{-1} Hygromycin for the double transformants). After 4 weeks, isolated calli were picked and transferred onto new plates. Individual calli were then maintained on agar medium or resuspended in liquid medium to obtain cell suspensions of transformed cells.

Transient Expression

After cell transformation (2 × 4 mL), cells were resuspended in 30 mL of liquid MSST medium and one-half of the cells were treated with 5 μg mL^{-1} of Ahtc (ACROS, Geel, Belgium). The cells were harvested by filtration 48 h after treatment.

Assays for GUS Activity

For fluorimetric GUS assays, proteins were extracted from BY2 cells in extraction buffer [50 mM (NaH_2/Na_2H)PO_4 (pH 7), 10 mM Suc, 10 mM β-mercapto-ethanol, 0.1% (w/v) sodium laurylsarcosine, and 0.1% (w/v) Triton X-100] as described by Jefferson et al. (1987). Protein concentration in cell extracts was determined according to Bradford (1976). Total protein (30 μg) was incubated with 2 mM of the substrate 4-methylumbelliferyl β-D-GlcUA (4-methyl umbellyferyl β-D-glucuronide) at 37°C. Gus activity was measured by a spectrofluorimeter (Fluoroskan II, Labsystem, Franklin, MA; excitation at 365 nm and emission at 455 nm) in the extraction buffer. For each experiment, the spectrofluorimeter was calibrated with freshly prepared 4-methylumbelliferone (4 MU) standards (1–500 nM) in the same buffer. GUS activity is expressed as pmol of 4-methylumbelliferone per h and per μg of proteins (pmol 4 MU min^{-1} μg^{-1} of proteins).

Detection of GFP by Fluorescence Microscopy and Spectroscopy

For fluorescence spectroscopy, BY2 calli (300 mg) were ground in 300 μL of 100 mM Tris-HCl (pH7.5), 100 mM NaCl, 1 mM $MgCl_2$, and 10 mM dithiothreitol (Chalfie et al., 1994). Fluorescence of GFP at 510 nm in the cleared supernatants was analyzed with excitation at 480 nm in a luminescence spectrometer (Kontron SFM25, Watford, UK).

For microscopic analysis, BY2 cells were observed using an epifluorescence microscope (Reichert-Jung, Polyvar, Paris) equipped with B4 interferential filters (excitation band pass 475–495 nm, emission BP 520–560). No marked autofluorescence was seen using this filter combination.

DNA and RNA Analysis

Genomic DNA was extracted from BY2 cells as described by Dellaporta et al. (1983) and 10 μg digested to completion with the restriction enzymes indicated in Figure 5. The DNA fragments were separated by electrophoresis in 0.6% (w/v) agarose gel in Tris-buffer EDTA and were blotted onto Hybond-N+ membrane (Amersham Pharmacia Biotech, Saclay, France) in alkaline conditions. Total RNA was prepared from BY2 calli as described by Logemann et al. (1987). RNA (10 μg) was electrophoresed through 1% (w/v) agarose gels containing 6% (w/v) formaldehyde. The gels were blotted onto nylon membranes (Hybond-N, Amersham Pharmacia Biotech) in 10× SSC. Southern and northern hybridizations were performed under similar conditions. After 2 h of prehybridization in a solution consisting of 5× SSC, 20 mM NaH2PO4/Na2HPO4 (pH 6.8), 10× Denhardt's solution, 7% (w/v) SDS, and 150 μg mL^{-1} sonicated, denatured salmon sperm DNA, hybridizations were carried out in the same solution with α-^{32}P-dCTP-labeled probe at 62°C. The blots were subsequently washed under stringent conditions (0.5× SSC and 0.5% [w/v] SDS at 62°C).

Southern blots were hybridized with an *hpt II* fragment, whereas northern blots were hybridized with the *tetR* gene as a probe.

Western Blot

Total protein extracted from BY2 calli were separated by SDS-PAGE analysis on 12.5% (w/v) polyacrylamide gels and transferred electrophoretically to nitrocellulose membranes (Hybond C, Amersham Pharmacia Biotech). After blocking with 5% (w/v) fat-free milk, the membranes were incubated with mouse anti-tetR antibodies (Mobitec, Göttingen, France) diluted at 1/2,000 for 1 h in Tris-buffered saline with tween (250 mM NaCl, 50 mM Tris-HCl [pH 7.6], and 0.1% [w/v] Tween 20). After washing in Tris-buffered saline with tween, antibodies were detected by goat anti-mouse IgG-alkaline phosphatase conjugate (diluted at 1/1,000, Biosys, Compiègne, France). Membranes were

then transferred in 50 mM Tris acetate (pH 9.7), 10 mM magnesium acetate, and the enzymatic activity was revealed using 5-bromo-4-chloro-3-indoyl phosphate/nitroblue tetrazolium chloride (Bio-Rad, Marnes-la-Coquette, France) as substrates.

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