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Controlled expression of recombinant proteins in *Physcomitrella patens* by a conditional heat-shock promoter: a tool for plant research and biotechnology

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Abstract

The ability to express tightly controlled amounts of endogenous and recombinant proteins in plant cells is an essential tool for research and biotechnology. Here, the inducibility of the soybean heat-shock *Gmhsp17.3B* promoter was addressed in the moss *Physcomitrella patens*, using β -glucuronidase (GUS) and an F-actin marker (GFP-talin) as reporter proteins. In stably transformed moss lines, *Gmhsp17.3B*-driven GUS expression was extremely low at 25 °C. In contrast, a short non-damaging heat-treatment at 38 °C rapidly induced reporter expression over three orders of magnitude, enabling GUS accumulation and the labelling of F-actin cytoskeleton in all cell types and tissues. Induction levels were tightly proportional to the temperature and duration of the heat treatment, allowing fine-tuning of protein expression. Repeated heating/cooling cycles led to the massive GUS accumulation, up to 2.3% of the total soluble proteins. The anti-inflammatory drug acetyl salicylic acid (ASA) and the membrane-fluidiser benzyl alcohol (BA) also induced GUS expression at 25 °C, allowing the production of recombinant proteins without heat-treatment. The *Gmhsp17.3B* promoter thus provides a reliable versatile conditional promoter for the controlled expression of recombinant proteins in the moss *P. patens*.

Abbreviations: ASA, acetyl salicylic acid; BA, benzyl alcohol; GFP, green fluorescent protein; GT, GFP-talin; GUS, β -glucuronidase; Fv/Fm, the maximal photochemical efficiency of PSII; MU, 4-methylumbelliferone; MUG, 4-methylumbelliferyl- β -D-glucuronide; PSII, photosystem II; SA, specific activity; sHSP, small heat-shock protein; TSP, total soluble proteins; Ubi, ubiquitin; X-Gluc, 5-bromo-4-chloro-3-indolyl- β -D-glucuronide

Introduction

The use of inducible protein-expression systems in model multi-cellular organisms is central to research in molecular genetics. The ability to induce and regulate the expression of recombinant proteins at will, is a valuable tool to

correlate protein levels with biological functions, to localize proteins in the cell and to visualize protein dynamics *in vivo* at various stages of development. This approach is exemplified by the wide use of conditional promoters such as *lacZ* in *Escherichia coli* (Donovan *et al.*, 1996), *GAL4* in budding yeast (Johnston, 1987) and *nmt1* in

fission yeast (Maundrell, 1993). Efficient high protein expression is also central to the production of recombinant proteins for biotechnology and pharmaceuticals.

Ideal inducible gene-expression systems should meet the following requirements: (1) without induction, expression should be the least leaky possible. (2) Induction should be dose-dependent and induction levels should span over at least three orders of magnitude. (3) The pattern of protein expression should be homogenous in all tissue of the organism and (4) inducing conditions should bare minimal consequences on the organism's physiology, fitness and viability (Gatz, 1997). In plants, prevalent conditional gene-expression systems use chemically regulated heterologous promoters (reviewed in (Padidam, 2003)) with chemical inducers such as tetracycline (Gatz, 1997), steroids (Aoyama and Chua, 1997; Zuo *et al.*, 2000) and ethanol (Caddick *et al.*, 1998; Roslan *et al.*, 2001). These systems may reach up to a few hundred folds of induction, but there are major limitations such as promoter leakiness, non-uniform uptake and toxic effects of the inducing chemicals (reviewed in details in (Padidam, 2003)).

Plants are sessile organisms with outstanding abilities to withstand temperature stresses. Gene expression in plants can therefore be induced by a mild heat-shock with minor impact on the fitness, using expression cassettes driven by heat-regulated promoters (Ainley and Key, 1990; Schöffl *et al.*, 1989). Plant heat-inducible promoters are well known for their strong, rapid and short lasting transcriptional response to heat stress (Vierling and Kimpel, 1992; Nover *et al.*, 2001). The plant promoters from the small heat shock protein (sHSP) gene family provide highly sensitive responses to mild temperature variations (Sun *et al.*, 2002). Thus, in *Nicotiana tabacum* the promoter *hsp17.6L* from soybean can control antibiotic resistance in a temperature-dependent manner (Severin and Schöffl, 1990) as well as the expression of the FLP recombinase in maize (Lyznik *et al.*, 1995) and *Arabidopsis thaliana* (Kilby *et al.*, 2000). Similarly, the *A. thaliana hsp18.2* promoter was successfully used as a heat-inducible expression system in tobacco BY2 cells (Yoshida *et al.*, 1995), and to achieve heat-inducible RNAi expression in *A. thaliana* (Masclaux *et al.*,

2004). Recently, using the soybean *Gmhsp17.3B* promoter, inducible insertional mutagenesis, based on *Ac-Ds* transposons, was developed in *A. thaliana* (Nishal *et al.*, 2005).

The moss *Physcomitrella patens* is emerging as a powerful model system for plant functional genomics. It shares many biological features with other land plants, in terms of gene conservation, physiology and development (Reski, 1998). At the same time *P. patens* offers the unique opportunity to perform targeted mutagenesis in a complex multi-cellular land plant by efficient homologous recombination (Schaefer and Zryd, 1997; Schaefer, 2002). Moreover, genetic studies in haploid mosses such as *P. patens* are much simpler than in diploid plants (Cove *et al.*, 1997; Schaefer and Zryd, 2001). Molecular tools (Schaefer and Zryd, 2004) and genetic information (Rensing *et al.*, 2002; Nishiyama *et al.*, 2003) are rapidly developing for *P. patens*. However, one tool still in need to be developed is a versatile conditional gene-expression system allowing to generate conditional phenotypes and circumvent lethality in mutations of essential genes. A tetracycline-repressible gene-expression system has been reported in *P. patens* (Zeidler *et al.*, 1996), but it requires continuous growth on tetracycline, which is detrimental to the moss (Laloue M., personal communication). Abscisic acid or auxin-induced gene expression have also been shown using, respectively, the wheat *Em* gene promoter (Knight *et al.*, 1995) or the soybean *GH3* gene promoter (Bierfreund *et al.*, 2003). However, the use of phyto-hormone-regulated gene expression may interfere with the plant physiology and remain unfit for a general versatile conditional gene-expression system in plants.

Here, we describe a highly efficient inducible protein-expression system in the moss *P. patens*, using the *hsp17.3B* promoter of a soybean class I sHSP (*Gmhsp17.3B* gene, SwissProt P02519, EMBL X011041 (Schöffl *et al.*, 1984)). Heat-induced expression of a soluble reporter enzyme (GUS) and of a cytoskeletal marker (GFP-talin) was characterized. A short heat-treatment under non-damaging conditions rapidly induced dose-dependent protein accumulation, in the whole plant. At 25 °C, protein expression remained tightly repressed, unless plants were exposed to specific chemical inducers, such as benzyl alcohol (BA) or acetyl salicylic acid (ASA).

Materials and methods

Plasmids

pHSP-GUS-AH108 and pHSP-GT-AH108

Plasmid pHsp-2 contains the promoter region (–512 to –5 bp) from the soybean *hsp17.3B* gene (Schöffl *et al.*, 1984) cloned as an XbaI-BamHI fragment in pBSKS II (kind gift from Eric Lam, Rutgers University). pHSP-GUS contains the BamHI/KpnI GUS-CaMVter fragment from pNco-GUS (Bonneville *et al.*, 1989) cloned into the corresponding sites of pHsp-2. pHSP-GT was constructed by replacing the NcoI – EcoRI GUS-CaMVter fragment of pHSP-GUS with a NcoI – EcoRI GFP-talin-CaMVter fragment from pYSC14 (Kost *et al.*, 1998). Finally, a 4.7 kb KpnI fragment from pBS-BAMH108 vector (A. Finka, unpublished) containing a hygromycin resistance cassette driven by the rice *actin-1* gene promoter (McElroy *et al.*, 1991) and a 1.9 kb of PP-108 genomic locus as targeting fragment (Schaefer and Zryd, 1997), was inserted in the KpnI restriction site of both vectors to create the pHSP-GUS-AH108 and pHSP-GT-AH108 targeting vectors used in moss transformation.

pUBI-GUS-MU108H₂

pUbi-GUS (Schweizer *et al.*, 1999) was digested by HindIII, to release a 4.2 Kb fragment containing *ubiquitin-1* promoter from maize, the *uidA* gene and 35S terminator. This fragment was cloned in the HindIII digested pMU108H₂ transformation vector (M. Uzé, unpublished) carrying the PP-108 genomic locus (Schaefer and Zryd, 1997) and a hygromycin resistance cassette driven by 35S promoter (Details provided upon request).

Plant material and protoplast transformation

Physcomitrella patens was grown on agar containing minimal medium (Ashton *et al.*, 1979) overlaid with a cellophane disk, as previously described (Schaefer and Zryd, 1997). Isolation of protoplasts, polyethylene glycol-mediated transformation, regeneration and selection of hygromycin-resistant transgenic moss strains was performed as described earlier (Schaefer and Zryd, 1997). Protoplasts were transformed with circular pHSP-GUS-AH108, pHSP-GT-AH108

and pUBI-GUS-MU108H₂, generating HSP::GUS, HSP::GT and UBI::GUS lines, respectively.

Heat treatment

Six days old *P. patens* protonemata or 8 weeks old gametophores were transferred to liquid minimal medium and liquid-adapted at 25 °C for 1 h. Heat treatment was then performed in a temperature-controlled water bath at the indicated temperatures and durations. Unless indicated otherwise, heat-treated mosses were tested for GUS activity or fixed for histochemical staining after 16 h recovery at 25 °C. For HSP::GT lines, heat treatments were performed by transferring moss tissue on preheated agar plates containing minimal medium and incubated in temperature-controlled chamber. We did not observe differences between heat treatments on solid and in liquid media.

Chemical treatments

For BA treatment, 6 days old *P. patens* protonemata were transferred 1-h prior treatment to liquid medium to which was added BA (Fluka), or ethanol (Fluka) to the indicated final concentrations. Following 1 h treatment at 25 °C, mosses were washed 3 times with minimal medium and incubated without inducer at 25 °C for 16 h, then tested for GUS activity.

For ASA treatment, protonemata were transferred to a solid medium supplemented with O-Acetyl salicylic acid (ASA) (Fluka) at the indicated concentrations. ASA was added from a (1 M) stock solution in dimethylsulphoxide (DMSO) to the sterilized medium. DMSO was added at equivalent concentrations as control. Plates were then placed in the growth chamber at 25 °C for 1 h. After ASA treatment, mosses were returned to the original ASA-less medium and, after 16 h recovery at 25 °C, tested for GUS activity or GFP-talin labelling. No significant change in the pH of the medium was detected in the presence of the highest ASA concentration.

GUS assay

GUS activity was determined according to (Jefferson *et al.*, 1987), using 2 mM 4-methylum-

belliferyl- β -D-glucuronide (MUG) as substrate in 110 μ l final reaction volume. Production of 4-methylumbelliferone (MU) was monitored kinetically on a microtiter plate Fluoroscan reader, using fresh serial dilutions of MU as standards (excitation wave length 365 nm and emission wave length 450 nm). Protein concentrations in the extracts were determined by the Bradford method (BioRad). GUS specific activity (SA) were expressed as nmol MU produced per minute per mg protein. Extracts with high GUS specific activities were diluted ten folds in extraction buffer with 1 g/l BSA, to stay in the linear measuring rate of the Fluoroscan.

Histochemical assays were as in (Nishiyama *et al.*, 2000) with small modifications. Plant tissues were fixed in a solution containing 0.3% (v/v) formalin, 5.45% (w/v) mannitol and 0.2% (w/v) MES-KOH (pH 5.6) during 30 min at room temperature, washed 3 times with 50 mM NaH_2PO_4 (pH 7.0). After a brief vacuum infiltration, tissues were incubated at 37 °C in a 500 μ l X-glu solution (1 mM 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (Duchefa Biochemie B.V. Netherlands), 50 mM NaH_2PO_4 (pH 7.0), 0.05% Triton X-100), and coloration was followed in time. Tissues were fixed 10 min in 5% formalin, and 10 min in 5% acetic acid. Tissue pigments were removed by serial ethanol incubation (30%, 50%, 70% and 95%). The stained tissues were observed under a light microscope and images were acquired using a LEICA DC 200 camera.

To correlate specific GUS activity with known amounts of GUS protein, the GUS enzyme was purified to at least 95% from heat-induced total moss protein extracts, by ion exchange chromatography (on ResourceQ, Pharmacia), using a linear gradient of 50–500 mM KCl. The GUS activity of the purified fraction was correlated on SDS-PAGE with a standard curve of Coomassie blue-stained GUS bands and of a known amount of the G6PDH enzyme (Diamant *et al.*, 2000).

Western blotting

Twenty microgram of moss crude soluble protein extracts were fractioned on 10% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane (Bio-Rad) by electroblotting and incubated with a rabbit-derived polyclonal antibody (Molec-

ular probes) against GUS and diluted 1/500 (v/v). The blot was then incubated with HRP-conjugated anti-rabbit IgG (Promega) diluted 1/2500 (v/v). Immune complexes were visualized using the chemiluminescent Immunstar™ Kit (Bio-Rad) according to manufacturer's instructions.

Measurement of PSII activity

Chlorophyll *a* fluorescence measurements were made with the Plant Efficiency Analyser PEA® from Hansatech Instruments Ltd, UK as detailed by (Strasser *et al.*, 1995). To measure chlorophyll *a* fluorescence, a small sample of moss protonema was placed on the cellophane disk and surrounded by the 5 mm diameter plastic clip provided by the instrument. Measurements were taken following a brief (2s) strong illumination of protonemata that have been previously dark-adapted for 2 min. Illumination red light of 3000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (peak at 650 nm) was provided by the PEA sensor unit. The following parameters were assessed: F_0 , F_m and F_v (Strasser *et al.*, 1995). The F_v/F_m ratio is the maximal photochemical efficiency of Photosystem II (PSII), with $F_v = F_m - F_0$ where F_0 and F_m are respectively, minimum and maximum fluorescence of dark-adapted protonema.

Live cell microscopy and image analysis

For confocal microscopy, excised pieces of cellophane carrying undamaged moss tissue were transferred into the glass slide chamber (Lab Tek II, Nunc) in inverted position and covered with a block of solid medium. Confocal microscopy was performed on a LEICA TCS SP2 system, using an inverted microscope (Leica-DMRE). A krypton-argon laser (488 nm line) was used for excitation. The bandwidth mirror settings for discriminating between the GFP fluorescence and red auto-fluorescence of chloroplasts were 504/531 for GFP and 634/696 for TRITC (chloroplasts). The two channels were allocated false green (GFP) and red (TRITC) colours. The step size between image slices was 0.65 μm . The obtained images represent orthogonal projections of 150 slices in the image stack. These projections were processed using Photoshop 6.0 software (Adobe Systems, Mountain View, CA).

Results

Generation of transgenic HSP::GUS, HSP::GT and UBI::GUS moss lines

In this study, we used three transformation vectors, all carrying a selectable hygromycin resistance gene, 2Kb of the genomic PP-108 locus as targeting sequence (Schaefer and Zryd, 1997) and the reporter expression cassettes HSP::GUS, HSP::GT (GFP-talin) or UBI::GUS (see Materials and methods). Targeted integration of the tested cassettes in the PP-108 locus of the moss chromosome considerably reduces the variability between different transgenic lines and allows a precise comparison between the activities of different reporter genes integrated at the same chromosomal location. Following direct transformation of *P. patens* protoplasts, stable hygromycin-resistant plants were regenerated: six lines with HSP::GUS, thirteen with HSP::GT and four with UBI::GUS. Southern blot analysis confirmed the stable integration of 5–10 direct repeats of the transforming plasmids in the PP-108 locus (data not shown).

At 25 °C, a very weak GUS activity was detected in HSP::GUS lines (specific activity (SA) of 0.59 ± 0.17 nmol MU/min mg averaged from six different lines). Expectedly, in the UBI::GUS lines, the GUS activity was over 100-fold higher (SA 73.3 ± 14 nmol MU/min mg averaged from four different lines) than in HSP::GUS lines (Figure 1A). Unless otherwise stated, one representative of each stably transformed line was randomly chosen for further studies.

In the HSP::GUS line, GUS is strongly induced by heat treatment

One-week-old protonemata from the HSP::GUS and UBI::GUS lines were treated for 60 min at increasing temperatures. Following 16 h recovery at 25 °C, GUS activities were measured in crude extracts. In the HSP::GUS strain, the GUS activity displayed a remarkable temperature-dependent response (Figure 1A). Compared to 25 °C, 1 h at 32 °C induced GUS expression 24-fold (SA 13.7 ± 9.6 nmol MU/min mg), at 34 °C more than 100-fold (SA 66.8 ± 31 nmol MU/min mg) and at 38 °C, up to 1500-fold (SA 854 ± 31 nmol MU/min mg). Above 40 °C, sub-maximal GUS activities were observed,

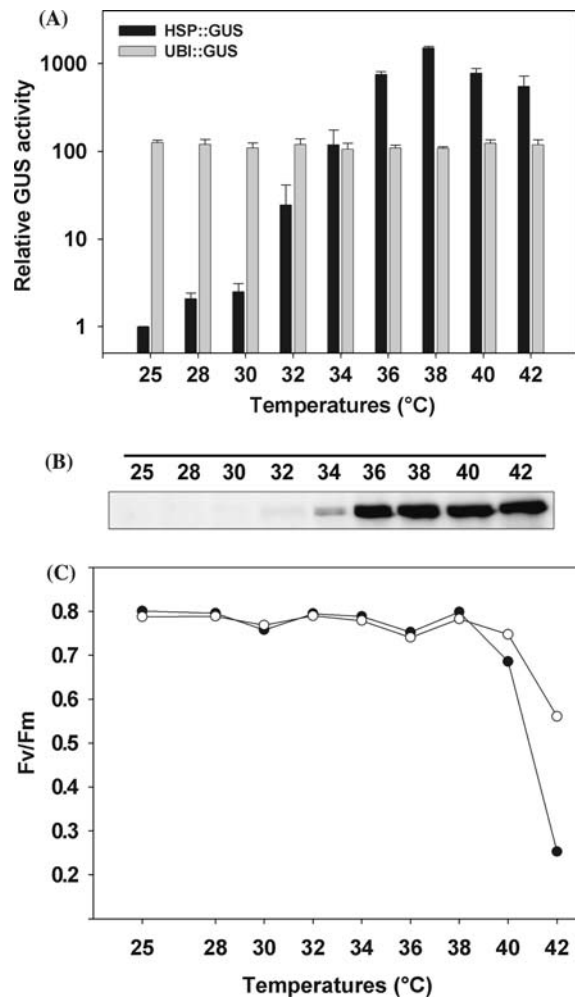


Figure 1. Heat-induction of the GUS enzyme. (A) GUS expression levels after heat treatment at different temperatures. *P. patens* protonemata from HSP::GUS (plain bars) and UBI::GUS (grey bars) lines were heat-treated for 1 h at indicated temperatures. GUS activities were measured after 16 h of recovery at 25 °C. After 1 h at 25 °C, GUS SA was 0.5 ± 0.12 and 62.7 ± 4.47 nmol MU/min mg in HSP::GUS and UBI::GUS line respectively. After 1 h at 38 °C, GUS SA was 854.29 ± 31 nmol MU/min mg in HSP::GUS line. GUS activities were determined from three independent experiments and were expressed relatively to the level of expression in HSP::GUS line at 25 °C, which was arbitrary fixed to 1. (B) Western blot analysis of GUS amounts as in a. Equal amounts of crude soluble protein extracts (20 μ g) were loaded and fractionated by 10% SDS-PAGE, blotted, and probed using an anti-GUS antibody. (C) Effect of increasing temperatures on the Fv/Fm in *P. patens*. Chlorophyll a fluorescence of HSP::GUS protonema was measured immediately after 1 h heat treatment (●) and following 16 h of recovery at 25 °C (○).

suggesting some heat damages. The temperature-dependent pattern of GUS expression was confirmed by western-blot (Figure 1B). In contrast, the maize ubiquitin (*Ubi-1*) promoter was found to drive a constant, strong GUS expression, which remained unaffected by the various heat-treatments (Figure 1A). This observation confirms that the *Ubi-1* promoter is not responsive to heat-treatment and that 60 min exposure at 42 °C does not significantly affect the stability of the GUS enzyme already present in *P. patens* cells. Noticeably, GUS expression levels in the UBI::GUS line were at least 10-fold lower than the maximal GUS expression in heat-induced HSP::GUS. Five minutes heat-shock at 45 °C is optimal for moss protoplast transformation (Schaefer and Zryd, 1997). We found that this strong but short heat-shock induced the same optimal high level of GUS expression as mild 60 min at 38 °C (data not shown).

The specificity of the heat-response in the HSP::GUS strain was demonstrated with other stresses: cold shock (60 min at 4–15 °C), salt (20 hours on NaCl 50–250 mM at 25 °C) and osmotic stress (20 h on 0.48 M mannitol at 25 °C) induced very low GUS expression, comparable to that observed at 25 °C (data not shown).

Optimal heat inducing treatments cause no apparent damages

Photosynthetic activity is among the most sensitive functions of plant cells. PSII becomes readily damaged by various stresses (Krueger *et al.*, 1997; Lu *et al.*, 2003), in particular by heat-stress (Havaux, 1992; Srivastava *et al.*, 1997). The maximal photochemical efficiency of PSII corresponding to Fv/Fm was measured in moss tissues immediately and 16 h after the heat-treatment (see Materials and methods). As shown in Figure 1C, 60 min at up to 38 °C caused no measurable damage in the photochemical efficiency of PSII. However, exposure to 40 °C resulted in a 10% decrease of Fv/Fm, which was fully restored during the 16 h recovery period. Expectedly, exposure to 42 °C caused more severe damage (70% decrease of Fv/Fm), which was only partially restored during recovery (Figure 1C). These partial damages correlate with the sub-optimal GUS activities we measured following 1-h induction at 40 and 42 °C. We conclude that optimal conditions for maximal

protein induction (1 h at 38 °C) have no significant deleterious effect on the moss physiology. Thus, an essential criterion of quality for a new inducible gene-expression system in *P. patens* is fulfilled.

Induction levels can be tightly controlled by the duration of the heat-treatment

Heat-induced expression of sHSP is one of the most sensitive, rapid and short lasting molecular responses in plants (Merquiol *et al.*, 2002; Sun *et al.*, 2002; Rizhsky *et al.*, 2004). To determine when the heat-response becomes saturated and how accurate is the control of expression by the duration of the heat-treatment, we measured the GUS activity resulting from increasing periods of heat-exposure at 38 °C. Confirming the temperature independent nature of the *Ubi-1* promoter, two hours exposure at 38 °C did not affect the constant high level of *Ubi-1*-expressed GUS (Figure 2A). By contrast, a tight, reproducible correlation was observed between the duration of heat-induction and the level of *hsp17.3B*-expressed

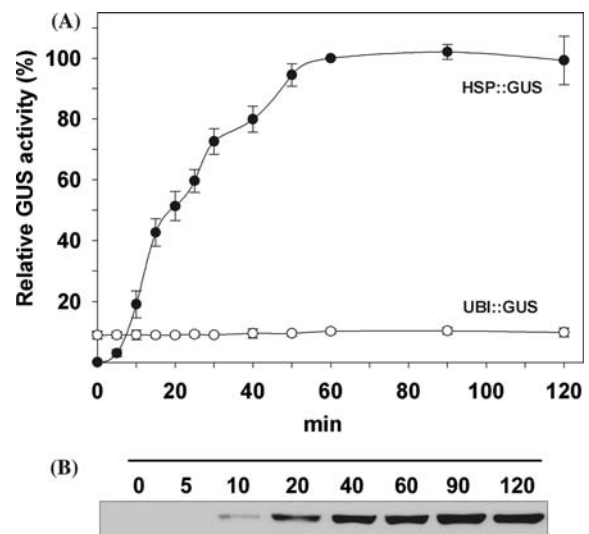


Figure 2. Time-dependent induction of GUS expression at 38 °C. (A) Time-dependent induction of GUS expression at 38 °C. Protonemata from HSP::GUS (●) and UBI::GUS (○) strains were heat treated for the indicated durations. GUS activities were measured 16 h after heat-treatment. 60 min at 38 °C, generated 860 ± 85.29 nmol MU/min mg in HSP::GUS strain, normalised to 100% induction. Mean values were established from six independent experiments. (B) Western blot analysis of GUS accumulation, performed as in Figure 1.

GUS (measured 16 h later). The half optimal induction time (at 38 °C) was 20 min. Optimal induction time was 60 min, producing over 1500-fold more GUS activity than at 25 °C (Figure 2A). Prolonged incubation beyond 60 min did not affect the high level of GUS activity. This gradual accumulation of GUS protein was confirmed by western blot analysis as shown in Figure 2B. The small standard errors, obtained with independent measurements using different HSP::GUS lines, demonstrate the tight reproducible control over this *hsp17.3B* promoter.

The kinetics of heat-induced gene expression

To further characterise the *hsp17.3B* promoter in moss, we measured the kinetics of GUS expression at 25 °C, following 1 h induction at 38 °C. Remarkably, 15% of the optimal GUS expression was already synthesised and active at the end of the heat-treatment (Figure 3A, inset, time 0). This confirms that both transcriptional and translational machineries are functional during the heat-shock. During the subsequent incubation at 25 °C, half maximal GUS expression was reached 4 h after heat-treatment and maximal GUS expression after 8–10 h (Figure 3A, inset). Maximal GUS levels remained stable during the 24 h post-induction and then gradually degraded at a rate of 10–15% per day (Figure 3). The GUS protein is highly stable in plant cells (Jefferson *et al.*, 1987) and expectedly, 1 week following induction, a third of the maximal GUS amounts was still present in the plant cells. Immunoblot analysis showed the rapid GUS accumulation during and after heat-induction, followed by a much slower rate of degradation (Figure 3B).

The pattern of hsp17.3B-mediated protein expression is uniform

To define the pattern of *hsp17.3B*-driven gene expression in different tissues, and to test its suitability for *in vivo* labelling approaches in moss, we performed histochemical staining of protonemata and differentiated leafy shoots from the HSP::GUS line, and a microscopic analysis of the actin network in the HSP::GT tissues. As expected, no X-gluc staining was detected in protonemata or gametophores of the non-induced HSP::GUS line, even following 12 h incubation in revealing buffer

(Figure 4A, D). This confirms that without heat-treatment, there is no detectable cell-specific expression of *hsp17.3B*-driven gene in the moss gametophyte. In contrast, a strong GUS staining was observed following heat-treatment in nearly all cells of HSP::GUS protonema and gametophore (Figure 4B, E). This histochemical staining was homogenous in all tissues. Noticeably, the pattern of *Ubi-1*-mediated expression was heterogeneous. GUS staining in the UBI::GUS line was stronger in the older cells of the protonema and gametophore (Figure 4C, F). This is consistent with the constitutive nature of the *Ubi-1* promoter, allowing a progressive accumulation of the stable GUS enzyme in older cells.

The expression of green fluorescent protein (GFP) fused to other proteins is widely used as a tool to investigate the protein sub-cellular localization and to observe structural protein complexes *in vivo*. The GFP-talin fusion allows the non-destructive labelling of F-actin filaments in higher plants (Kost *et al.*, 1998). Here we used the *in vivo*

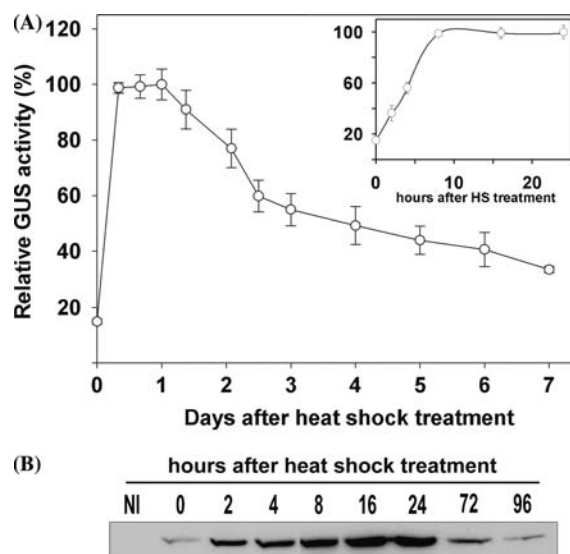


Figure 3. Time-dependent accumulation and degradation of GUS after induction. (A) One week old protonemata from the HSP::GUS strain were heat treated 1h at 38 °C and subsequently grown at 25 °C. GUS activities were measured in samples collected at the indicated time up to 7 days after heat treatment. GUS activities were represented as percentage of the maximal expression level. Inset, GUS activity during the first 24 h following heat-treatment (HS). Mean values were established from 3 independent experiments. (B) Immunoblot analysis of GUS amounts as in a. NI: total proteins extracted before heat treatment. 0: total proteins extracted immediately after heat treatment.

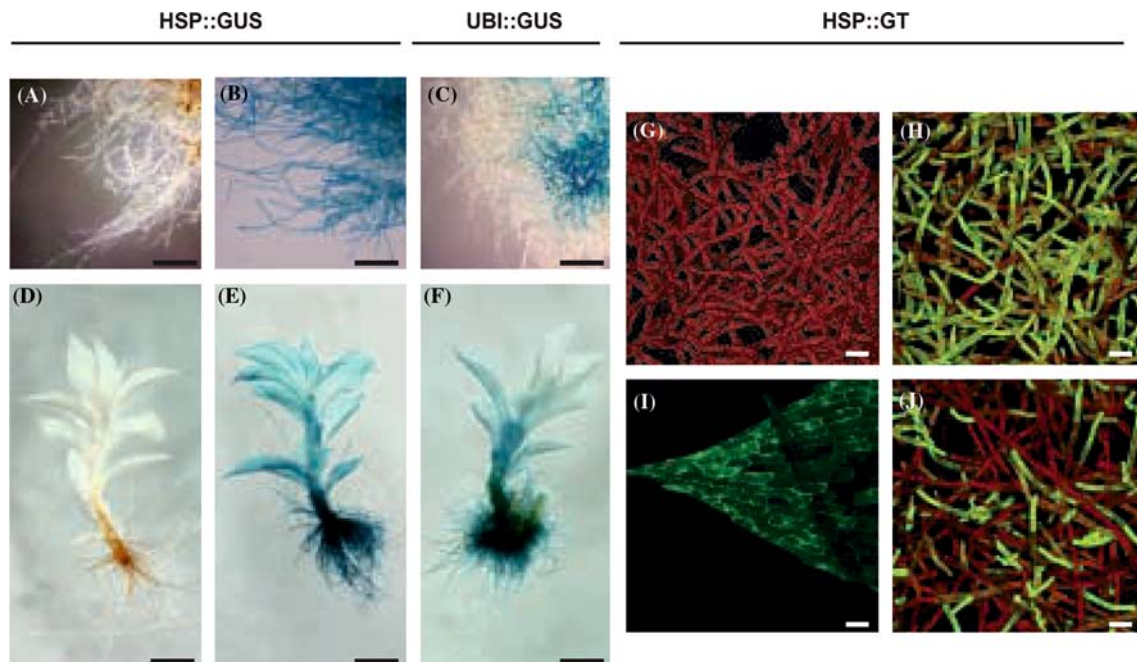


Figure 4. Expression pattern of the *hsp17.3B* and the ubiquitin promoter. (A–F) Histochemical localization of GUS expression driven by the *hsp17.3B* (A, B, D and E) or the *Ubi-1* (C and F) promoter. Protonema from non-induced HSP::GUS (A), heat-treated (1 h, 38 °C) HSP::GUS (B), and untreated UBI::GUS (C) strains. Gametophores from non-induced HSP::GUS (D), heat-treated HSP::GUS (E) and untreated UBI::GUS (F) strains. The staining pattern in UBI::GUS was identical with or without heat treatment (data not shown). Tissues were incubated 12 h (A, D), 1 h (B, C) and 4 h (E, F) in X-gluc solution at 37 °C. Bars: 500 μ m. G–J: Confocal projections of HSP::GT protonema and leaf tissue. (G) Untreated protonema displaying chlorophyll autofluorescence but no GFP fluorescence, (H) heat-treated (1 h, 38 °C) protonema displaying chlorophyll autofluorescence and strong GFP in every cell, (I) GFP labelled actin structures in an induced leaf, (J) protonema treated with ASA displaying GFP labelling in young cells. Bars: 100 μ m.

labelling of the *P. patens* actin network with the GFP-talin marker (GT) to evaluate the potential of the *hsp17.3B* promoter to monitor the dynamics of protein complexes in moss. As with HSP::GUS line, GFP decorated actin structures were not observed in non-treated HSP::GT plants (Figure 4G). Dose-response experiments showed that optimal conditions for heat induction of GT were similar to those described for HSP::GUS lines, i.e. one hour at 38 °C, for optimal labelling observed 16 h later (data not shown). In heat-treated plants, all protonema and gametophore cells displayed clear GFP-labelled actin structures (Figure 4H, I). Cortical actin cables, a fine meshwork of actin around the nucleus and the accumulation of actin at active growth sites were clearly detectable (A. Finka, manuscript in preparation). Similar actin structures have been described in *P. patens* cells following histochemical staining with rhodamin-phalloidin (Doonan and Duckett, 1988).

Over-production of recombinant proteins by 'in physco' thermocycling

Induction being exhausted beyond one hour at 38 °C, we addressed the possibility to increase the yields of expressed GUS protein by iterative thermocycling treatment of the moss. HSP::GUS protonemata were submitted to three consecutive cycles of 1 h at 38 °C and 4 h at 25 °C. After one, two and three such '*in physco*' thermocycles, GUS accumulated respectively to 1% (SA 697.3 nmol MU/min mg), 1.7% (SA 1264 nmol MU/min mg) and 2.3% (SA 1709 nmol MU/min mg) of the total soluble proteins (TSP). This suggests that after the first heat-shock, a short incubation at 25 °C, during which partial GUS synthesis is achieved, can reset the heat-shock induction machinery and initiate synthesis of additional mRNA and GUS protein during in the next cycles. These high amounts was readily detectable by

coomassie blue staining on SDS-polyacrylamide gel (Figure 5, line 1, 2, 3). As expected, no GUS was detected in extracts of heat-treated wild type (WT) and untreated HSP::GUS line (NI) (Figure 5). The maximal yield obtained here corresponds to 3.5 mg GUS protein per gram dry weight.

Chemical induction of the hsp17.3B promoter in P. patens

At non-inducing temperatures, the heat-shock response can be induced in bacteria and animal cells by specific chemicals affecting membrane fluidity (Vigh *et al.*, 1998) and/or protein stability (Jurivich *et al.*, 1992). The membrane fluidiser benzyl alcohol BA can optimally induce the heat-shock response in *E. coli* and cyanobacteria (Shigapova *et al.*, 2005; Vigh *et al.*, 1998). We found here that the *hsp17.3B* promoter was proportionally induced by 1 h incubation with increasing amounts of BA at 25 °C, to reach a maximal 70-fold induction with 64 mM BA (SA 35.2 ± 4.6 nmol MU/min mg) (Figure 6A). At this concentration, the Fv/Fm ratio was decreased by 8%, but was fully recovered 16 h post-induction. Higher BA concentrations caused a severe

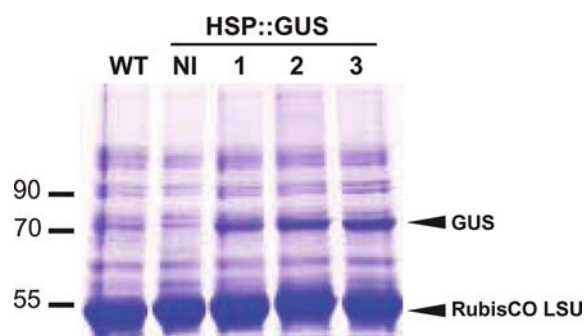


Figure 5. Coomassie blue detectable amounts of GUS after repetitive heat-induction. 50 μ g of total proteins prepared from WT or HSP::GUS protonema were separated by 8% SDS-PAGE and stained with Coomassie brilliant blue R250. (WT) Protein extract from wild type after 1h heat treatment at 38 °C and 4 h recovery at 25 °C. (NI) Protein extract from untreated HSP::GUS protonema (SA = 0.54 nmol MU/min mg). Protein extract from HSP::GUS protonema after the first induction cycle (SA = 697.35 nmol MU/min mg) (1), after the second induction cycle (SA = 1264 nmol MU/min mg) (2) and after the third induction cycle (SA = 1709 nmol MU/min mg) (3). One cycle = 1 h at 38 °C and 4 h recovery at 25 °C. The arrowheads indicate the position of GUS protein and RubisCO LSU.

decrease and only a partial recovery of Fv/Fm (Figure 6C).

Also significant, was the effect of acetyl salicylic acid ASA, a non-steroid anti-inflammatory drug affecting heat-shock signalling in animals (Amici *et al.*, 1995). When protonemata from the HSP::GUS line were treated (at 25 °C) for 1 h with increasing ASA concentrations, a significant dose-dependent induction of the GUS activity was observed. A maximum induction of 125-fold was reached at 0.9 mM ASA (SA 100 ± 12 nmol MU/min mg). This maximal GUS expression was 8-fold lower than optimal heat-induction, but 1.5-fold higher than *Ubi-1*-mediated expression (Figure 6B). As with heat-treatment, constant GUS activity was observed in the UBI::GUS line when treated with increasing concentrations of ASA (Figure 6B). Measurements of chlorophyll *a* fluorescence confirmed that ASA concentrations above 0.3 mM reduced, albeit reversibly, the photochemical efficiency of PSII (Figure 6D). Exposures for more than 1 h to 0.8 mM ASA resulted in a lower GUS expression, likely because of irreversible damages (data not shown).

Acetyl salicylic acid induction was confirmed with the HSP::GT strain. Whereas 1 h exposure at 38 °C resulted in a uniform GFP-talin labelling of a majority of *P. patens* cells, treatment with 0.8 mM ASA resulted in a strong labelling but only in the youngest apical and sub-apical cells. Thus, unlike heat-induction, reporter protein expression in differentiated old cells was poorly inducible by ASA treatment (Figure 4J). Similar results were observed with histochemical staining of ASA-treated HSP::GUS protonema (data not shown). This effect is possibly due to the physical barriers, such as thicker cell walls in older tissues that limit the accessibility of ASA and BA. Despite the non-homogenous expression, actin filaments were clearly observed in ASA-induced young cells and were indistinguishable from those in heat-treated young cells (data not shown).

Discussion

Here, we describe a highly efficient inducible protein-expression system in the moss *P. patens*, using a highly sensitive heat-inducible promoter from soybean. A detailed analysis of regulatory elements in the *hsp17.3B* promoter identifies

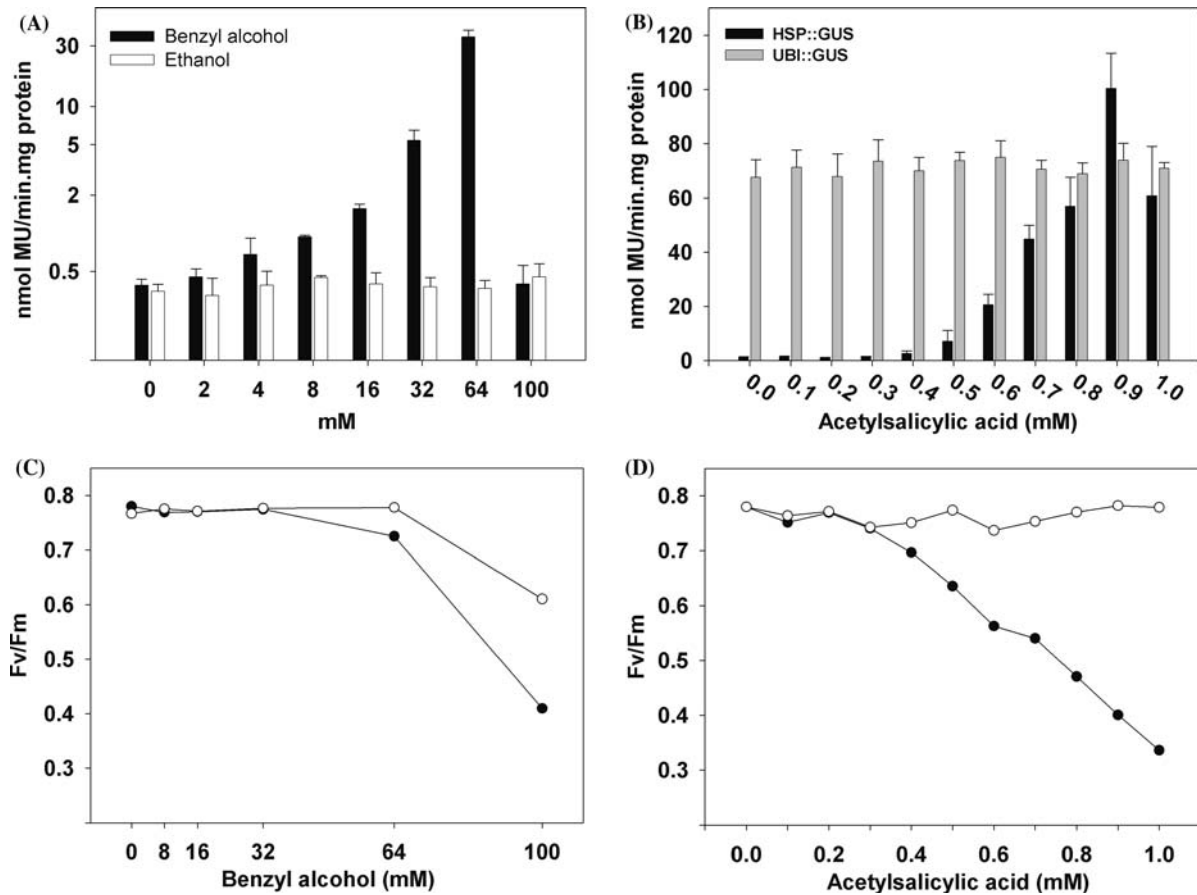


Figure 6. Chemical induction of GUS expression at 25 °C. (A) GUS expression levels induced by benzyl alcohol (BA) treatment. Protonemata of the HSP::GUS line were incubated in a minimal medium supplemented with different concentrations of benzyl alcohol. As a control, protonemata were also incubated in a medium supplemented with equivalent concentrations of ethanol. (B) GUS induction levels after 1 h acetyl salicylic acid (ASA) treatment at different concentrations. Protonemata of the HSP::GUS or the UBI::GUS strain were transferred to a minimal medium supplemented with ASA for 1 h at 25 °C. GUS activities were measured 16 h after each chemical treatment and are expressed as nmol MU/min mg. (C, D) Changes in the maximum efficiency of PSII photochemistry (Fv/Fm) after treatment with BA (C) or ASA (D). Measures of PSII activity were determined immediately after the chemical treatment (●) and after 16 h recovery at 25 °C (○).

several heat shock regulating elements located between -321 and -12 bp and an additional enhancer element located between -593 and -312 bp (Rieping and Schoffl, 1992). The promoter we used covers the 5'UTS of the *hsp17.3B* promoter from -512 to -5 , containing the enhancer element. The data presented demonstrate that the molecular mechanism of the heat-shock response is highly conserved between bryophytes and spermatophytes. For promoters of sHSPs, optimal induction temperatures, dose-dependent responses and kinetics of protein expression were similar in *P. patens*, *A. thaliana* and Tomato (Baniwal *et al.*, 2004) (for a review see (Nover

et al., 2001)). In Table 1, the reporter activities driven by the soybean *hsp17.3B* promoter were compared to that driven by other constitutive and inducible promoters tested in *P. patens*. Under non-inducing condition, *hsp17.3B*-mediated GUS expression was extremely low: about 6–8-fold lower than that observed with a 35S-GUS construct carrying an optimised translation initiation context (pNcoGUS), 50-fold lower than that driven by the rice *actin-1* (*act-1*) promoter and at least 100-fold lower than that driven by the maize *Ubi-1* promoter. Using a dual luciferase assay, a comparison of the relative strength of the 35S and *act-1* promoters in moss provided similar values

Table 1. GUS or Luciferase activities with constitutive and inducible promoters in *P. patens*.

Plasmid (a)		Test (b)	GUS (c)	GUS normalised (d)	Luciferase normalised values	
Constitutive promoters						
CaMV 35S	pNcoGUS (1)	T	3	1.0		
	pNcoGUS (2)	S	3	1.0		
	35S-LUC (3)	T/DF			1	
Rice <i>act1</i>	Act1-F (1)	T	25	8.3		
	(3)	T/DF			10 ± 5.5	
Maize <i>Ubi-1</i>	Ubi-GUS (4)	T	50	16.7		
	pUBI-GUS-MU108H ₂ *	S	70	23.3		
Plasmid (a)		Test (b)	Treatment (f)	GUS (c)	GUS normalised (d)	Induction factor (e)
Inducible promoters						
wheat <i>Em</i>	BM 113 (5)	S	H ₂ O	20	6.7	1
			Mannitol	93	31.0	6
			ABA	550	183.3	30
Glucocorticoid inducible	Act-GVG + UAS GUS (1)	T	- Dex	2	0.7	1
			+ Dex	35	11.7	15
Ethanol inducible	35S AlcR + Alc-GUS (4)	T	- EtOH	12	4.0	1
			+ EtOH	14	4.7	1.2
			+ Tc	> 0.05	0.02	1
Tetracycline repressible <i>hsp17.3B</i>	ptTA + pTop10 (2)	T/S	- Tc	4	1.3	65
			25 °C	0.5	0.16	1
			38 °C	850	283	1500
			ASA	100	33	125
			BA	35	12	70

Notes: (a) References for these values are (1) (Chakhparonian, 2001), (2) (Zeidler *et al.*, 1996; Zeidler *et al.*, 1999), (3) (Horstmann *et al.*, 2004), (4) Schaefer and Finka, unpublished data, (5) (Knight *et al.*, 1995), * this work. (b) T transient gene expression, S stable transgenic lines, DF dual luciferase assay. (c) GUS specific activities are given in nmol MU/min mg protein. The values obtained in reference (1) were averaged from at least 6 independent transformations and standard deviations between transformations never exceeded 25%. (d) GUS activities were normalised to the value of the 35S promoter, to enable comparison with the dual luciferase assay. (e) Determined relatively to the non-induced GUS level obtained with the same system. (f) ABA: abscisic acid, Dex: dexamethasone, Et OH: ethanol, Tc: tetracycline, BA: benzyl alcohol, ASA: acetyl salicylic acid.

(Horstmann *et al.*, 2004). Finally, this low basal level was about 4–6-fold lower than the basal level measured in *P. patens* for the glucocorticoid-inducible GVG system (Chakhparonian, 2001). The *hsp17.3B* expression system in the moss thus satisfies the quality requirement for minimal promoter leakiness.

A tight dose-response relationship was established between the level of induced gene-expression and the temperature (Figure 1) and/or duration (Figure 2) of the heat induction. Gene expression was induced accurately over three orders of magnitude and maximal induction was achieved following one hour at 38 °C. This condition was not associated with apparent heat damages and maximal GUS activity corresponded to about 1% GUS protein of TSP. This activity is at least 10-fold higher than that driven by the strong maize

Ubi-1 promoter. Kinetic experiments showed that maximal induction was rapidly achieved (within 1 h), transient and lasted up to 8 h post-induction. The *hsp17.3B* promoter thus enables the fine-tuning of transient gene expression in moss, which satisfies the requirement for a reproducible, dose-dependent, inducible gene-expression system.

Noticeably, a low activity of the *hsp17.3B* promoter has been reported in developing tobacco seeds without heat shock (Prandl *et al.*, 1995). In contrast, histochemical and *in vivo* labelling showed that the *hsp17.3B* promoter had no tissue-specific pattern of expression in *P. patens*. Heat-induced GUS was uniformly expressed in most cells of the plant. By contrast, in UBI::GUS line, GUS molecules accumulated more in aging cells than in younger cells. In HSP::GT line, a uniform labelling of actin structures was also

observed *in vivo* (Figure 4). This allowed for the first time to clearly visualize different actin structures at all stages of cellular differentiation in various tissues. Interestingly, transgenic *A. thaliana* constitutively expressing 35S-mediated GFP-talin displayed labelled actin filaments only in developed trichomes, some leaf cells and root hairs (Mathur *et al.*, 1999). Moreover, constitutive over-expression of GFP-talin in pollen tubes inhibited pollen germination (Kost *et al.*, 1998). Thus, by controlling the precise timing of expression and the cellular concentrations of recombinant proteins, an *hsp17.3B*-based conditional expression system may circumvent dominant-negative effects, such as cell growth inhibition by excess GFP-talin. We conclude that the *hsp17.3B* promoter fulfils all the methodological criteria for a most reliable, non-leaky, controllable, inducible gene-expression system in *P. patens*. The other inducible gene-expression systems developed for plants and tested in *P. patens* do not fully satisfy such criteria. Many of them have significantly leakier promoters, drive lower induction levels and/or necessitate the use of toxic chemical inducers (see Table 1).

Chemical induction of the heat shock promoter

In plants, little is known about chemicals that can specifically induce the heat-shock response. We have shown here that BA, affecting membrane fluidity in *E. coli* and cyanobacteria (Vigh *et al.*, 1998), or ASA, affecting the activity of heat shock factors in mammals (Amici *et al.*, 1995), can induce *hsp17.3B*-mediated GUS expression at room temperature (Figure 6). In both cases, induction factors were proportional to the concentration of the chemicals and maximal induction was at comparable concentrations as observed in cyanobacteria with BA and in mammalian cells with ASA. Yet, maximal GUS activities were respectively, 8 and 25-folds lower for ASA and BA, than with optimal heat-induction. This is because of the uneven expression pattern that we observed using HSP::GT line (Figure 4H/4J). Permeability differences in the cell walls of young and old cells may determine the accessibility and efficacy of chemical inducers, especially hydrophobic ones. Yet, this is the first report of BA and ASA-induced activation of a heat-shock promoter

in plants. Chemical induction of the *hsp17.3B* promoter provides an alternative to heat-induction, when heat-treatments are to be avoided, as in the case of expressing a heat-sensitive protein.

Biotechnological applications

Technical advances and limitations of plant-based protein production systems have been recently detailed in (Fischer *et al.*, 2004). We showed here a high yield (2.3% TSP) obtained with a cytosolic protein-expression system. This yield corresponds to 3.5 mg GUS produced per gram dry weight and is two orders of magnitude higher than that obtained for a recombinant human growth factor expressed in *P. patens* (10–13 µg/g dry weight (Jost *et al.*, 2005)). *P. patens* suspension culture can be grown in fermentors (Decker and Reski, 2004) and can therefore be used for the industrial production of recombinant proteins. In addition, secretion signals are highly conserved between moss and other eukaryotes and can thus be used to target produced proteins to various cellular compartments and to the secretory pathway for collection in the extracellular medium (Decker and Reski, 2004). The major advantage of *P. patens* is its exceptional amenability to targeted gene-mutagenesis. This allows inactivation of specific functions, such as proteases that may degrade recombinant proteins, or enzymes involved in immunogenic post-translational glycosylations (Koprivova *et al.*, 2004). Finally, the high rate at which high amounts of *hsp17.3B*-mediated recombinant proteins can accumulate in moss (2.3% of TSP in half a day) is of significant biotechnological interest. Higher yields have been reported using a chloroplast transgenic system in tobacco (31.1% TSP) (Molina *et al.*, 2004) and a seed specific promoter in *A. thaliana* (36.5% TSP) (De Jaeger *et al.*, 2002). However, the time required to achieve such amounts was 60 and at least 40 days, respectively.

In conclusion, our findings have three major applications. (1) The versatility of the system enables the fine-tuning of transient gene expression over three orders of magnitude in all cells and tissues, in a dose dependent manner, with very low promoter leakiness. This would enable correlation studies between expression levels and biological function of given proteins. (2) The behaviour of this promoter in response to non-damaging heat treatment in *P. patens* is identical to that

previously described in tobacco and *A. thaliana*. Thus, further functional studies of the plant heat-shock response can be advantageously complemented in *P. patens*. (3) The yield of protein production and the rate of protein accumulation in the moss after iterative heat induction cycles is remarkably high. This can serve as efficient biotechnological tool for controlled production of industrial recombinant proteins.

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