Heat-inducible production of β -glucuronidase in tobacco hairy root cultures

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Abstract The production of β -glucuronidase (GUS) driven by the *Arabidopsis* small heat shock protein 18.2 promoter in liquid cultures of transgenic tobacco (*Nicotiana tabacum*) hairy roots is reported. Clone GD-3, showing high GUS heat induction and a moderate growth rate, was selected from 436 clones for study. Treatment of GD-3 with heat shock at 36–42°C for 2 h then recovery at 27°C resulted in an increase in GUS specific activity, while higher heat-shock temperatures led to a decline. These results were in accordance with the change in esterase activity, a measure of tissue viability. Using 2 h of 42°C heat shock and a recovery phase at 27°C, GUS specific activity increased rapidly and reached a maximum of

267.6 nmol 4-methylumbelliferyl β -D-glucuronic acid (MU) min⁻¹ mg⁻¹ protein at 24 h of recovery. When tissues were continuously heated at 42°C and tested without a recovery period, GUS mRNA was detectable at 2 h and peaked at 5 h, but GUS activity was not seen until 10 h and did not peak until 28 h; in addition, the maximum activity was lower than that seen after heat shock for only 30 min or 2 h, followed by recovery. This shows that recovery at normal temperature is crucial for the heat-inducible heterogeneous expression system of transgenic hairy roots. Multiple heat-shock treatments showed that this system was heat reinducible, although a gradual decline in GUS specific activity was seen in the second and third cycles.

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Introduction

For decades, plant cell cultures have been successfully used as chemical factories producing secondary metabolites (Rao and Ravishankar 2002). Commercial protein production has traditionally relied on microbial fermentation and mammalian cell lines. Compared to these traditional expression systems, the use of plant cells results in the folding, assembly, and modification of proteins, as with animal cells, and the cells are inexpensive to grow and maintain, as with microbes (Hellwig et al. 2004). In addition, there is no risk of contamination with oncogenes, prions, mammalian viruses, or mycotoxins or endotoxins from microbes. It has been suggested that they may be useful systems for expressing foreign proteins for commercial applications

(Su and Aris 2003; Fisher et al. 2004). Although reports of *Agrobacterium rhizogenes*-mediated transformation for foreign protein production are limited, the hairy roots induced by *A. rhizogenes* are thought to be an ideal system for plant metabolic engineering and an alternative to plant cell suspension cultures for specialty chemical production (Doran 2000). Hairy roots show genetic and biochemical stability, which is lacking in suspension cell cultures, and have a growth rate similar to that of suspension cells (Shanks and Morgan 1999).

The small heat shock protein (sHSP) 18.2 promoter from *Arabidopsis thaliana*, driving the expression of an 18.2-kD heat shock protein (HSP), was first cloned in 1989 (Takahashi and Komeda 1989). In a previous study (Moriwaki et al. 1999a), the sHSP18.2 promoter was used as a tool to investigate the response to heat shock in transgenic tobacco plants. In gently agitated liquid cultures, the temperature can be controlled more precisely to evaluate the efficiency of the sHSP18.2 promoter in switching on gene expression. In this study, a heat-inducible expression model system of tobacco transgenic hairy roots was established for the production of β -glucuronidase (GUS) and was found to be a useful system for studying the expression of recombinant proteins.

Materials and methods

The chimeric gene

The binary vector, pHS/GUS, consisting of the *Arabidopsis* sHSP18.2 promoter, containing heat-shock elements and 77 bp of the sHSP18.2 coding sequence, fused to *gusA* and which were inserted into pBI101.2, was kindly provided by Prof. Komeda (Takahashi et al. 1992). After confirming the sequences of fused fragments of sHSP18.2 promoter and *gusA* gene, the plasmid pHS/GUS was transformed into the plasmid carrier host, *Escherichia coli* strain JM109. Purified binary vector pHS/GUS was prepared to transform *A. rhizogenes* strain 1724 by electroporation; a cuvette with a 1.0-mm-gap chamber for 2.5 kV was used as the conditions. The strain was subsequently screened for kanamycin resistance.

Plant material and liquid cultures of transformed roots

Five-week-old leaf stalks of sterile tobacco explants, *Nicotiana tabacum* var. TT5 (native fluecured tobacco var. Taiwan tobacco 5; P₂, wide oval leaf), were used to carry out the downstream of *A. rhizogenes*-mediated transformation. *Agrobacterium rhizogenes* was applied to the cut edges of tobacco leaf discs (ca. 1 cm²) and the leaf

discs were placed on a fresh half-strength Murashige and Skoog (MS) agar medium for 4 days at 22°C in the dark. Cocultured leaf discs were then transferred to agar media containing 300 mg L⁻¹ cefotaxime to facilitate the sterile cultivation, which lasted 21 days at 27°C. Induced transgenic hairy roots with a rapid growth rate and without residual *A. rhizogenes* were established as clones. The *rolC* and HSP/GUS PCR fragments were sequenced to ensure successful *A. rhizogenes*-mediated transformation.

Liquid cultures of transformed roots were cultivated at 100 rpm at 27°C in the dark, and were regularly subcultured every 14 days. One hundred milligrams fresh weight of tissue was used to inoculate a 250-ml flask containing 50 ml of liquid half-strength MS medium supplemented with 3% sucrose. Growth was measured by weighing the fresh biomass after 21 days of culture (unless otherwise stated) and expressed as the final mass/initial mass.

Heat-shock treatments and GUS specific activity and tissue viability assays

Thirty-day-old (unless otherwise stated) transformed root cultures were subjected to heat shock. Flasks of root cultures were submerged in a water-bath shaker for the indicated period of time at the indicated temperature. Except in the study of the effect of continuous treatment at 42°C, they were then allowed to recover by culture at 100 rpm at 27°C for the indicated time, and then samples were taken to evaluate the heat-induced expression of GUS specific activity and tissue viability.

A total protein extract was prepared by freezing samples in liquid nitrogen and grinding them in a chilled mortar, lyophilizing the tissue, homogenizing it on ice in 0.5 mL of extraction buffer (50 mM Na-PO₄, 10 mM dithiothreitol, 1 mM Na₂EDTA, 0.1% Triton X-100, pH 7.0 at 4°C), centrifuging the homogenate at 12,000×g for 15 min at 4°C, and taking the supernatant. The protein concentration was measured using Bradford's reagent (Biorad) with bovine serum albumin as the standard. This extract was used to measure GUS specific activity by the MU method (Jefferson et al. 1987) (expressed as nmol MU mg protein⁻¹ min⁻¹). Tissue viability of hairy roots was estimated using fluorescein diacetate (FDA) as substrate to measure cytoplasmic esterase activity (Steward et al. 1999).

DNA extraction, PCR, and probe synthesis

Genomic DNA was extracted from hairy roots using a Plant DNeasy kit (Qiagen, Hilden, Germany). The digoxigenin (DIG)-labeled 401-bp *gusA* DNA probe was prepared by PCR using pBI101.2 as the template and DIG-labeled

nucleotides (Roche, Mannheim, Germany). The oligonucleotide primers 5'-ATGTTACGTCCTGTAGAAAC-3' and 5'-ACACAAACGGTGATACGTAC-3' were used with 35 cycles of 1 min at 94°C, 1 min at 55°C and 30 s at 72°C. The PCR product (401 bp) was analyzed by electrophoresis on a 1% agarose gel.

RNA extraction and Northern hybridization

Total RNA from hairy roots was isolated using the Trizol® method as described by the manufacturer (Invitrogen, Carlsbad, CA, USA). Ten micrograms of total RNA was separated on a 1.2% denaturing agarose gel (50 V, 60 min), then the gel was immersed in 20×saline-sodium citrate (SSC) buffer for 20 min for equilibrium and blotted onto a nylon membrane (NY⁺ membrane, Millipore, Billerica, MA, USA) overnight at room temperature, then cross-linking was performed for 3 min at 254 nm using a Biolinker BLX-E254 UV cross linker (Vilber Lourmat, Marne-la-Vallée, France). The membrane was prehybridized in hybridization buffer at 45°C for 1 h, then hybridized with the DIG-labeled probe (100 ng ml⁻¹) in the same hybridization buffer for 18 h at 48°C, and the membrane was washed with 1×SSC containing 0.1% sodium dodecyl sulfate (SDS) for 10 min at room temperature, followed by 0.1×SSC containing 0.1% SDS for 30 min at 65°C. The hybridized probe signal was detected using alkaline phosphatase-conjugated anti-DIG antibody (Roche) according to the manufacturer's instructions, and the image was captured using an imaging system (Autochemi, UVP, Upland, CA, USA).

Results

Establishment of transgenic hairy roots and characterization of clone GD-3

In the establishment of transgenic hairy roots, 436 clones were obtained and 10 completely sterilized clones with high biomass accumulation in flask cultures were finally selected. The *rolC* and HSP/GUS gene fragments were successfully detected in these 10 clones by PCR (data not shown). For clone selection, the biomasses of these 10 clones and their GUS specific activities at 21 days after heat shock at 40°C for 2 h were compared. Biomass accumulation ranged from 26- to 115-fold and the GUS specific activity ranged from 27.7 to 227.9 nmol MU min⁻¹ mg protein⁻¹. A tendency was observed for clones with higher accumulated biomass (more than 80-fold) to express lower heat-inducible GUS activity (data not shown). We finally chose clone GD-3 as the research material, as it showed the highest GUS specific activity and a moderate growth rate.

Growth of GD-3 in a 250-ml flask is shown in Fig. 1. After inoculation with 100 mg of fresh tissues, the transgenic hairy roots grew rapidly from days 14 to 28, and then entered a stationary phase. The fresh weights of GD-3 on days 14, 25, 28, and 30 were 0.3, 1.6, 2.7, and 2.9 g, respectively. The relationship between tissue age and heat-inducible GUS specific activity was then investigated. When transgenic hairy roots were cultured for 25, 28, or 30 days, then underwent heat shock at 40°C for 2 h and recovery for 24 h at 27°C, the GUS specific activities were 125.3, 198.7, and 278.5 nmol MU min⁻¹ mg protein⁻¹, respectively, showing that the more mature the tissue, the higher the GUS specific activity induced.

GUS gene expression and specific activity after different heat-shock treatments

The effect of temperatures from 33 to 48°C on tissue viability and GUS specific activity was investigated. The intracellular esterase activity in hairy roots was used as a measure of the viability of the tissue. After 2 h heat shock at 33, 36, 39, or 42°C and 24 h at 27°C (recovery), GUS specific activity increased with temperature to 21.8, 75.3, 105.6, or 219.8 nmol MU min⁻¹ mg protein⁻¹, respectively; however, at higher temperatures (45 and 48°C), it declined (Fig. 2a). In parallel, the tissue viability of transgenic hairy roots subjected to 2 h of heat shock at 36 or 42°C was maintained for at least 5 days in recovery cultures, whereas heat shock at 45°C for 2 h resulted in a rapid increase in esterase activity on day 1, followed by a dramatic decrease (Fig. 2b).

Heat induction of GUS activity was then investigated in transgenic hairy roots subjected to heat shock at 42°C for

Fig. 1 Growth and GUS specific activity after heat treatment of GD-3 hairy root cultures of different ages. The biomass (black circles) and heat-induced GUS specific activity (gray bars) in 25-, 28-, and 30-day cultures subjected to heat shock at 40°C for 2 h was measured after recovery at 27°C for 24 h. The means and standard deviations for triplet experiments are shown

Fig. 2 Effect of temperature on GUS expression and tissue viability of GD-3 transgenic hairy root cultures. **a** GUS expression; **b** tissue viability. Thirty-day-old GD-3 root cultures were subjected to different temperatures for 2 h, then allowed to recover at 27°C for 24 h (**a**) or for 0–5 days (**b**). The total protein extracts were assayed for GUS (**a**) or esterase activity (**b**), as described in the “Materials and methods.” Tissue viability is expressed as a percentage of that of hairy roots with no heat shock. Filled circles, open circles, and triangles represent heat shock at 36, 42, and 45°C, respectively. The means and standard deviations for triplet experiments are shown

different times. Regarding the heat shock applied for 30 min and 2 h, transcription of recombinant *gusA* was observed in the recovery phase at 27°C, reaching the maximum at 2 and 0 h, respectively, and then decreasing, but remaining detectable at 5 h (Fig. 3a). As shown in Fig. 3b, in the case of heat shock for 2 h, GUS specific activity in transgenic hairy roots during the recovery phase reached a maximum of 267.6 nmol MU min⁻¹ mg protein⁻¹ within 24 h, then decreased up to 100 h. Using 30 min of heat shock, GUS specific activity reached a maximum of 115.5 nmol MU min⁻¹ mg protein⁻¹ after 5 h of recovery, which was maintained up to 24 h, then gradually decreased.

Fig. 3 Time-course of GUS mRNA accumulation and GUS specific activity in transgenic hairy roots subjected to heat-shock at 42°C for 30 min or 2 h, then allowed to recover at 27°C for the indicated time. **a** Northern blots of GUS mRNA. Total RNA was used as the internal control; C is the control (GUS mRNA before heat-shock). **b** GUS specific activity during the recovery phase at 27°C after heat shock for 2 h (filled circles) or 30 min (open circles). The means and standard deviations for triplet experiments are shown

In theory (Moriwaki et al. 1999a), HSPs and their mRNAs are produced during the heat-shock period, and their levels decrease following a return to normal temperatures. However, in this study, maximum GUS specific activity was seen during recovery; a similar result of maximum GUS specific activity at 6 h during the recovery phase was seen when transgenic *Arabidopsis* plants were exposed to heat shock at 35°C for 1 h (Takahashi et al. 1992).

The effect of continuous heat treatment at 42°C with no recovery period on tissue viability, GUS mRNA levels, and GUS specific activity was investigated. As shown in Fig. 4a, transcription was detected within 2 h, reached a maximum at 5 h, then decreased, but was still detectable at 34 h. In contrast, as shown in Fig. 4b, an increase in GUS specific activity was not seen until 10 h after the start of heat treatment, and the peak GUS specific activity, seen at 28 h, was only 59.4 nmol MU min⁻¹ mg protein⁻¹, i.e., only 25% of the maximum response seen using 2 h of heat shock at 42°C followed by recovery. In accordance with the low GUS specific activity, a decline in tissue viability was seen after 5 h of heat shock (Fig. 4b). Heat damage of

Fig. 4 Time-course of GUS mRNA accumulation and GUS specific activity in transgenic hairy roots subjected to continuous 42°C heat-shock with no recovery period. **a** Northern blots of GUS mRNA. Total RNA was used as the internal control. **b** Heat-induced GUS specific activity (filled circles) and esterase activity (open circles). Tissue viability without heat shock was set as 100%. The means and standard deviations for triplet experiments are shown

the translation system might be responsible for the observed delayed and low GUS specific activity.

Repeated heat shock treatments

The effect of three 2-h/42°C heat-shock treatments separated by 48 h of recovery at 27°C on induction of GUS activity was investigated. The induced GUS specific activities measured 24 h after the end of each of the three heat shocks (i.e., at 26, 76, and 126 h) were 233.3, 212.3, and 170.3 nmol MU min⁻¹ mg protein⁻¹, respectively (Fig. 5). No change in tissue esterase activity was seen following multiple treatments (data not shown). Our results show that, using the optimum heat shock conditions for single treatment (2 h at 42°C), heat-induction could be performed several times and without tissue damage, although there was a gradual decline in GUS activity induction in the second and third cycles compared to the first.

Discussion

For efficient expressions of foreign proteins with high yield, a good expression system using an appropriate promoter is needed. However, overexpression of certain gene products may reduce cell vitality and result in a number of morphological features similar to those seen in

Fig. 5 GUS specific activity in transgenic hairy roots in response to multiple heat shocks. Thirty-day-old GD-3 root cultures were treated for three cycles of 42°C for 2 h and recovery for 48 h at 27°C; then, GUS specific activity was measured. The arrows indicate heat shock at 0–2, 50–52, and 100–102 h. The means and standard deviations for triplet experiments are shown

senescent plant tissues. A two-stage culture system consisting of a growth stage and an induction stage is desirable. To date, numerous inducible systems that respond to chemical or physical stimuli have been developed in plant cells, e.g., using wounding (Firek et al. 1993), phytohormones (Li et al. 1991), antioxidants (Herouart et al. 1993), chemicals (Boetti et al. 1999; also see review by Padidam 2003), heat (Yoshida et al. 1995), or light (Shimizu-Sato et al. 2002). However, the use of a chemical, such as salicylic acid, may result in many unexpected physiological manifestations (Shimizu-Sato et al. 2002), and the cost of the inducers, toxicity to the host, or possible induction of unexpected plant physiology-related metabolic pathways should also be concerned. The use of a physical stimulus, such as heat, gives more precise and easy regulation of gene expression.

Cell death in plant cell cultures is usually estimated by coloration methods or specific enzymatic tests (Vaňková et al. 2001). Steward et al. (1999) reported that the in vitro measurement of FDA fluorescence after extraction of the intracellular esterases from *Medicago sativa* L. (Alfafa) suspension cells could be used to assess cell viability. Using this method, we showed that cytoplasmic esterase activity in transgenic hairy roots decreased at a 45°C temperature (Fig. 2b) and caused a low response of the *Arabidopsis* SHSP18.2 promoter to GUS activity (Fig. 2a). The transient increase in esterase seen at 45°C in transgenic hairy roots (Fig. 2b) might also be a response to the heat stress, which then triggered the death mechanism. Our data showed that the FDA method is a simple and efficient way of monitoring tissue viability of transgenic hairy roots in vitro and that, for transgenic tobacco hairy roots, 42°C was the optimum temperature for induction of GUS activity without compromising tissue viability.

A previous study, in which an increase in specific activity was not seen until the recovery period, also showed that a recovery period is crucial to the expression of GUS protein (Takahashi et al. 1992). In contrast to using the *Ha hsp* 17.7 G4 promoter with no N-terminal hsp sequence, immediate expression of GUS mRNA and GUS activity were seen in *Helianthus annuus* (sunflower) when heat shock was applied, using the *Ha hsp* 17.7 G4 promoter and an N-terminal hsp sequence, a strong induction of *gus* gene expression but a delayed increase in GUS activity were seen (Almoguera et al. 2002), as in our study (Fig. 3). This difference on onset of the GUS activity increase may be due to the additional N-terminal sequence or to other factors inhibiting GUS activity in vivo, or protein–protein interactions might occur during heat stress. In addition, coaggregation of endogenous and heterologous sHSPs might occur (Kirschner et al. 2000) during the heat shock period.

In *A. thaliana*, the sHSP18.2 promoter shows highest activity at 35°C, while maximal activity in petunia is seen at 37–38°C (Takahashi et al. 1992). However, the optimum temperature for the sHSP18.2 promoter in transgenic *Nicotiana plumbaginifolia* explants is 42°C (Moriwaki et al. 1999a). This shows that the thermosensitivity of the sHSP18.2 promoter is species-sensitive. In previous studies, maximal expression activity in transgenic *A. thaliana* was seen 6 h after a 1-h heat-shock treatment at 35°C (Takahashi et al. 1992), while the maximum in regenerated tobacco explants was seen at 12 h after a 2-h treatment at 42°C (Moriwaki et al. 1999b) and, in both cases, the GUS specific activity remained high for at least 48 h. However, in this study (Fig. 3), GUS specific activity increased rapidly in 12 h, peaked at 24 h, and then declined. The peak of induced GUS specific activity regulated by the same sHSP18.2 promoter in transgenic *N. tabacum* hairy roots (this study) occurred later than in transgenic *N. plumbaginifolia* (Moriwaki et al. 1999b) and *A. thaliana* (Takahashi et al. 1992). However, the maximal level of 267.6 nmol MU min⁻¹ mg protein⁻¹ induced in our tobacco transgenic hairy root system was 6-fold higher than that in tobacco cells (Yoshida et al. 1995), 18-fold higher than that in tobacco plants (Moriwaki et al. 1999b), and 5-fold higher than that in transgenic *Arabidopsis* plants (Takahashi et al. 1992). Some possible explanations for the highly expressive activity in *A. rhizogenes*-transformed *N. tabacum* roots are listed below.

- (1) The sHSP18.2 promoter has higher expression efficiency in root tissue. The heat-induced GUS specific activity driven by the sHSP18.2 promoter in tobacco BY-2 cells (Yoshida et al. 1995) was lower than that found in *A. rhizogenes*-transformed roots (this study). Furthermore, Moriwaki et al. (1999b) detected a

higher expression activity of the sHSP18.2 promoter in roots than in other organs in *N. plumbaginifolia* explants. These results suggest that root-differentiated tissues might show better expression. In addition, Sharp and Doran (1999) found that recombinant protein levels were significantly higher in hairy roots than in plant cell suspensions; this higher expression might be due to better recombinant protein expression in *A. rhizogenes*-transformed roots.

- (2) The 5' untranslated region (UTR) of sHSP18.2 may contain sequences that regulate translation efficiency or mRNA stability. In our study, the sHSP18.2 promoter with the 5' UTR and 77 bp of the sHSP18.2 coding sequence was fused to the N-terminal of *gusA*, while Dansako et al. (2003) reported that the 5' UTR of the sHSP18.2 gene contributes to efficient translation in plant cells. Thus, 5' UTR/N-terminal-mediated translational and/or posttranslational regulation might affect the detected GUS activity.

In this study, a heat-inducible system for exogenous protein expression using tobacco transgenic hairy root was established. This not only provides an alternative way to investigate the physiology of heat shock in plants, but it is also a useful system for target gene expression that is rapid, noninvasive, nontoxic, and easily controlled by heat regulation.

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