COMMUNICATION TO THE EDITOR

BIOTECHNOLOGY and BIOENGINEERING

Five Year Maintenance of the Inducible Expression of Anthranilate Synthase in *Catharanthus roseus* Hairy Roots

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ABSTRACT: Transgenic hairy root cultures have the potential to be an industrial production platform for a variety of chemicals. This report demonstrates the long-term stability of a transgenic *Catharanthus roseus* hairy root line containing the inducible expression of a feedback-insensitive anthranilate synthase (AS). After 5 years in liquid culture, the presence of the inserted AS gene was confirmed by genomic PCR. The inducible expression of AS was confirmed by enzyme assay and by changes in terpenoid indole alkaloid concentrations. This report also demonstrates that it may take as long as 2 years for the metabolite profile to stabilize.

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KEYWORDS: plant metabolic engineering; inducible promoter; terpenoid indole alkaloid; cell factories

Introduction

It is well established that plants produce numerous metabolites that have economical value in a wide variety of industries ranging from fuels and lubricants to pharmaceuticals and human health. The complexity of many of these metabolites limits their production to plant systems. This coupled with low yields leads to the engineering of plant systems for increased productivity and yield. Plants can also be engineered to produce novel compounds from the introduction of non-native pathways or through the engineering of enzymes to increase metabolite diversity. While traditional agriculture has been primarily used for production of these metabolites, the use of plant cell and tissue culture is becoming a feasible production alternative (Georgiev et al., 2007; Guillon et al., 2006a; Guillon et al., 2006b; Sivakumar, 2006; Srivastava and Srivastava, 2007). In particular, hairy root cultures are an attractive platform because they produce many metabolites on the same order of magnitude as the whole plant. To be a viable industrial production platform, the long-term stability of transgenic hairy root cultures needs to be established. In this paper, transgenic hairy root cultures are defined as cultures containing an inserted gene of interest in addition to the genes required for hairy root formation. In a recent report, it was shown that after 4.5 years a *Catharanthus roseus* hairy root line transgenic for inducible GFP expression maintained inducible expression of GFP without selective pressure (Peebles et al., 2007). This paper builds upon that work by tracking the terpenoid indole alkaloid metabolite levels in response to the inducible expression of a pathway gene, anthranilate synthase (AS), in *C. roseus* hairy roots at different time points over 5 years. These results show that it can take up to 2 years for the metabolite profiles to stabilize.

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Materials and Methods

Culture Conditions

The creation of the hairy root line ASαβ-1 has been previously described (Hong et al., 2006). The line contains an ASα feedback insensitive subunit under the control of a glucocorticoid inducible promoter system along with the constitutive expression of the ASβ subunit. Hairy root cultures of ASαβ-1 were initiated by placing five root tips approximately 3 cm long in 50 mL of culture media in a 250 mL Erlenmeyer flask. The culture media consisted of a filter-sterilized (0.22 μm filter) solution of 30 g/L sucrose (Sigma, St. Louis, MO), half-strength Gamborg's B5 salts (Sigma), and full-strength Gamborg's vitamins (Sigma) with

the pH adjusted to 5.7. These cultures were grown in the dark at 26°C at 100 rpm and were maintained by serial sub-culturing every 3 weeks. This line was maintained in parallel cultures by two different research groups starting at 2.25 years after liquid adaptation.

Genomic PCR

Genomic DNA was extracted from hairy root cultures of ASαβ-1 using the GenElute Plant Genomic DNA Miniprep Kit (Sigma) according to the manufacturer's instructions. One primer specific to the inducible promoter region outside the gene and one primer specific to a region inside the gene were used to confirm the insertion of ASα into the genome: 5′-AGCTTGCATGCCGGTCGACTCTAGAG-GATCC-3′ and 5′-TCACAAATGCAGATTCAGCCAAGTC-GATGGCTCGAG-3′. The expected PCR band size is ~2.1 kb. Taq polymerase was used to perform the PCR with the following conditions: 95°C for 1 min followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min with a final extension time of 10 min at 72°C.

Induction of ASαβ Activity and Alkaloid Extraction and Detection

Triplicate cultures of ASαβ-1 were treated with 0.2 μM dexamethasone (induced cultures) or with an equal volume of ethanol (un-induced cultures). Cultures were fed on day 18 and harvested 72 h later on day 21. The cultures were then frozen at −80°C until they were lyophilized. Approximately 50 mg dry weight of the ground tissue was extracted in 30 mL of methanol by ultrasonication for 10 min (Model W-225, Heat Systems. Ultrasonics Inc., USA). The extracts were clarified by centrifugation at 1,300 g for 15 min at 4°C. The supernatants were concentrated to 2 mL using a vortex evaporator and passed through a 0.22 μm nylon filter (13 mm). Ten μL of each samples was analyzed for the following terpenoid indole alkaloids: tryptophan, tryptamine, ajmalicine, serpentine, catharanthine, hörhammericine, lochnericine, and tabersonine on a HPLC as previously described (Peebles et al., 2005).

Anthranilate Synthase Enzyme Assay

Anthranilate synthase enzyme assay was performed as previously reported (Li and Last, 1996). After a 72 h induction with dexamethasone or ethanol, 1 gm of fresh weight tissue was ground in 3 mL of grinding buffer (200 mM Tris-HCl (pH 7.5), 0.2 mM EDTA, 8 mM $MgCl_2$, 0.2 mM DTT, and 60% glycerol). The supernatant was clarified by centrifugation and desalted on a Bio-Rad (Hercules, CA) Econo-Pac l0DG column pre-equilibrated in column buffer (50 mM Tris-HCl pH 7.5, 0.05 mM EDTA, 2 mM $MgCl_2$, 0.05 mM DTT, 5% glycerol). The crude protein concentration for each sample was determined by a Bradford assay (Sigma). Anthranilate synthase activity was measured in a 2 mL reaction with 1 mL column buffer plus 0.05 mM chorismic acid, 10 mM glutamine, 1 mM MgC12, 12.5 mM Tris-HCl (pH 8), and 20 μg crude protein. To test the inhibitory effects of tryptophan, 0, 1, 3, 10, or 30 μM tryptophan was added to each reaction. The reaction was terminated after 30 min at 30°C by adding 0.2 mL 1 M HCl. Anthranilate was extracted in 3.5 mL of ethyl acetate and the fluorescence of each sample was determined on a Shimadzu RF-10AXL fluorescence detector with the excitation set at 340 nm and the emission set at 400 nm. A standard curve for anthranilate was produced following the above reaction procedure without crude protein.

Results and Discussion

The *C. roseus* hairy root line ASαβ-1 has been continuously maintained by transferring five root tips into fresh liquid media every 3 weeks for the past 5 years as previously described (Hong et al., 2006). This line was maintained in parallel cultures by two separate research groups starting 2.25 years after liquid adaptation. Five years after being transferred to liquid media, the presence of the inserted ASα gene under the control of the glucocorticoid inducible promoter was confirmed in ASαβ-1 using genomic PCR (Fig. 1). The primers were designed so that the 5′ primer is specific to the inducible promoter region and the 3′ primer is specific to the ASα gene. This primer design ensures that only the inserted ASα gene is amplified and not the native gene.

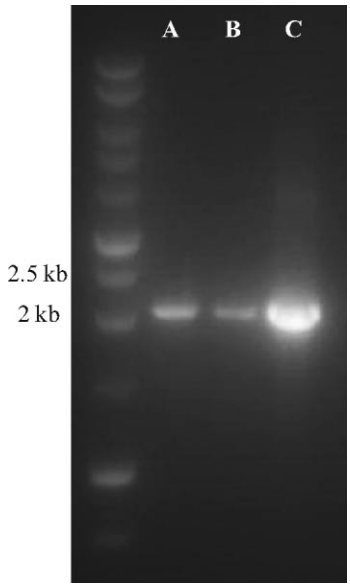

Figure 1. Genomic PCR confirmation of the ASα insert in *C. roseus* hairy root line ASαβ-1 at 5 years. The left lane is a 1 kb DNA ladder (Promega). Lanes A and B are the PCR product from the genomic DNA extraction. Lane C is a control reaction using PCR product from the original plasmid construct containing ASαβ.

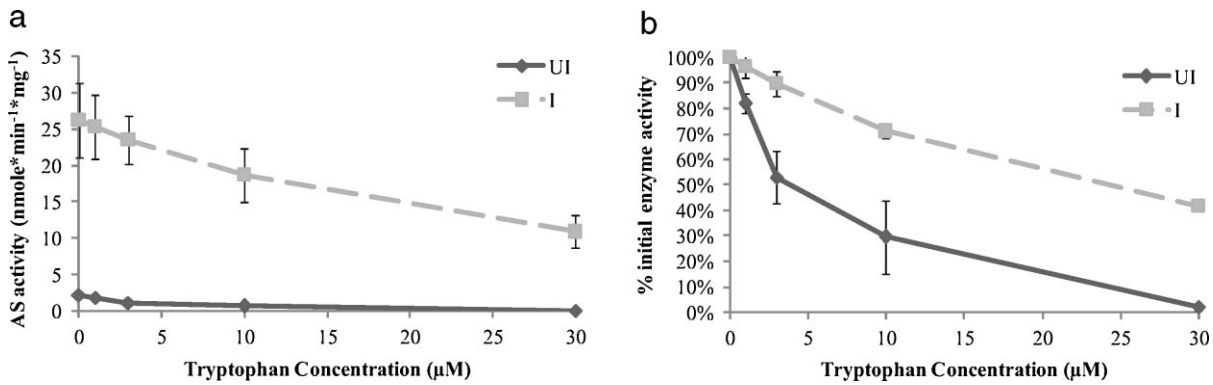

Figure 2. Enzyme activity of anthranilate synthase (AS) 5 years after first being transferred to liquid media. Triplicate cultures were induced with 0.2 μM dexamethasone (I) or an equal volume of ethanol (UI) and harvested 72 h later. Part (a) reports the AS enzyme activity (nmole*min^{-1}*mg^{-1}) while (b) reports the % of initial enzyme activity seen as tryptophan concentrations increase. Error bars represent the standard deviation of triplicate samples.

The AS enzyme activity was also determined for both the un-induced and induced (overexpressing ASα) conditions at 5 yrs. Figure 2 shows that ASαβ-1 exhibits inducible expression of AS and that the induced state exhibits an increase in feedback insensitive ASα. The original report of AS activity for ASαβ-1 was 0.77 nmole min^{-1} mg^{-1} for the un-induced state and 1.7 nmole min^{-1} mg^{-1} for the induced state, a 2 fold difference (Hong et al., 2006). After 5 years, the AS activity is 2.2 nmole min^{-1} mg^{-1} for the un-induced state and 26.2 nmole min^{-1} mg^{-1} for the induced state, a 12 fold difference.

An induction study was performed at various time points over the 5 year period to track the effect of overexpressing AS on the terpenoid indole alkaloid concentrations (Figs. 3 and 4). The indole precursor's tryptophan and tryptamine were analyzed along with the following alkaloids: ajmalicine and serpentine from the Corynanthe family, catharanthine from the Iboga family, and tabersonine, hörhammericine, and lochnericine from the Aspidosperma family. Vindoline, an important precursor to the anticancer drugs vinblastine and vincristine, has not been detected within these hairy root cultures (Shanks et al., 1998). A more detailed discussion of the effect of overexpressing AS on the terpenoid indole alkaloid concentrations can be found in Hong et al. (2006) and Peebles (2008). The first two time points, at 14 and 16 months after transfer to liquid media, have been previously reported (Peebles et al., 2005, 2006). The 3.6 year time point was independently determined. Figures 3 and 4 suggest that the cell line may take up to 2 years for the metabolite profile to stabilize. While the response may be transient over time, the overall trends were consistent at each time point showing that it is not necessary to wait for the line to stabilize before determining the general effect of overexpressing a gene of interest. Practically this means if you want to test the line under a variety of conditions and compare the results, it is best to do it all at once or wait until the line stabilizes. The parallel cultivation of the ASαβ-1 line starting at 2.25 years after liquid adaptation and the stabilized metabolite profile from 2–5 years provides additional evidence that the line has become stable and is insensitive to differences in sub-culturing techniques. The increase in enzyme activity reported at

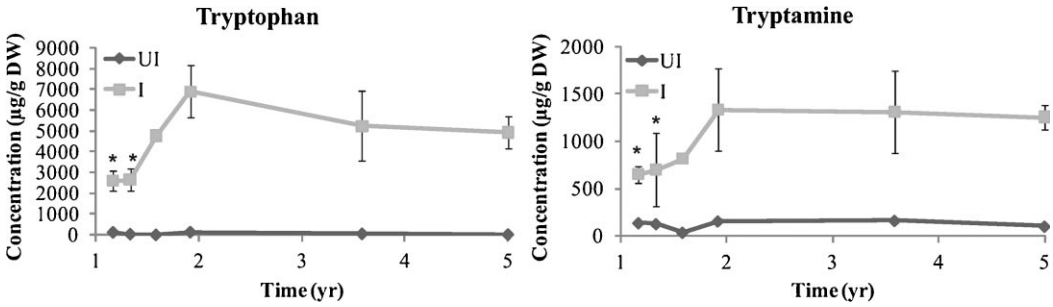

Figure 3. The change in tryptophan and tryptamine concentrations over time in liquid culture. Each data point represents the results of a 72 h induction study performed in triplicate. (*) has been adapted from Peebles et al., 2005, 2006.

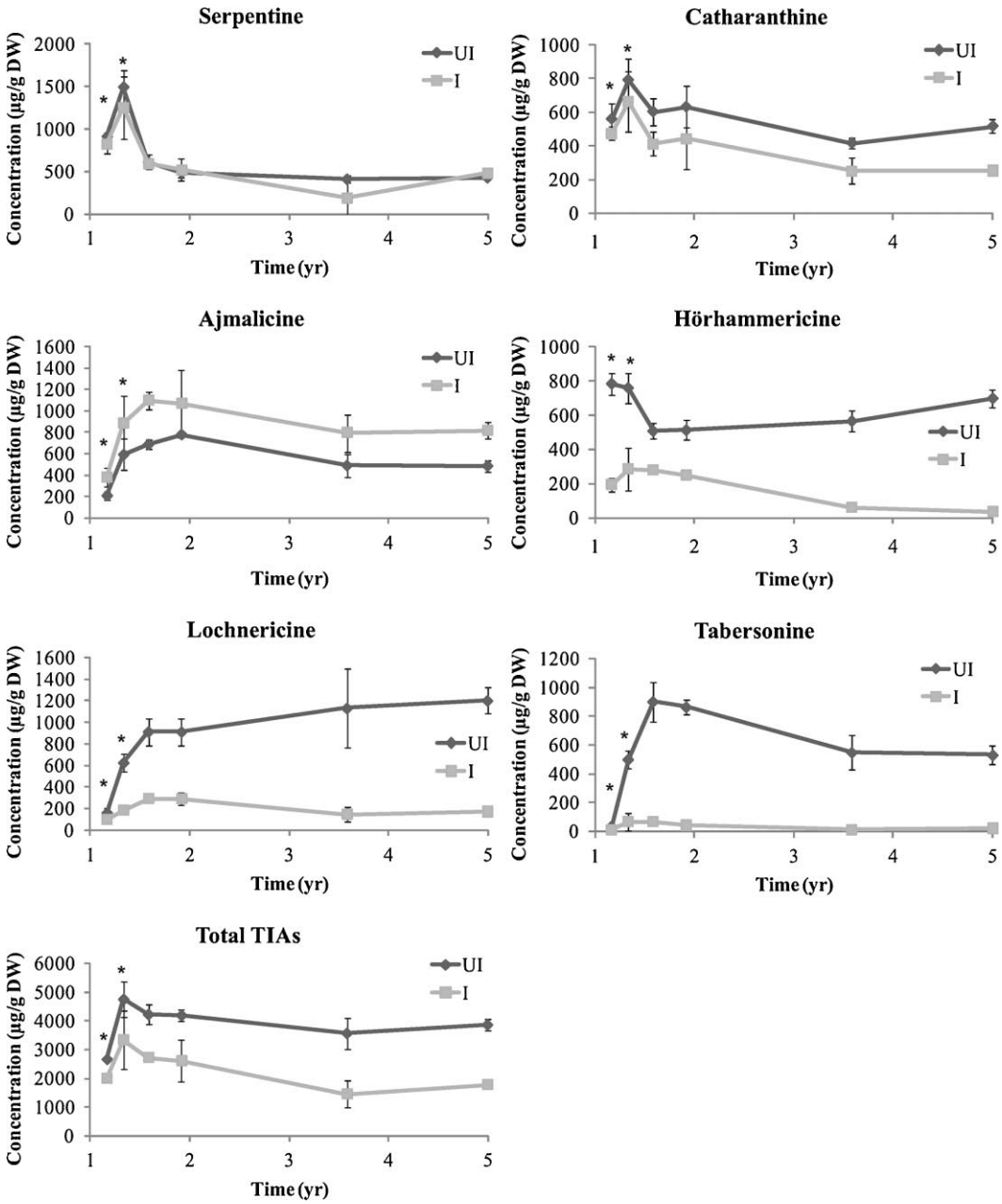

Figure 4. The change in terpenoid indole alkaloid (TIA) concentrations over time in liquid culture. Each data point represents the results of a 72 h induction study performed in triplicate. (*) has been adapted from Peebles et al., 2005, 2006.

5 years may explain why there is an observed change in metabolite levels over the first 2 years (Figs. 3 and 4). These results also suggest that the ASαβ-1 line maintains the inducible feedback resistant expression of AS over the 5 year time period.

The initial transient profile may arise from the way in which the hairy root line was generated. Initially a number of hairy roots grow from one site of infection on the plant. It is possible that each hairy root represents a different infection event and thus contains a different genetic or metabolic background. We have found that selecting for the whole mass of hairy roots from the infection site is more successful in generating hairy root cultures than selecting for each individual hairy root. Once on the selection plate, the faster growing hairy roots are further adapted to solid culture and subsequently liquid media. At this point, a mixture of hairy roots containing different backgrounds may still exist. Over time as the five best growing hairy root tips are chosen for

sub-culturing, we maybe selecting for a faster growing, more homogeneous culture. We are currently using a modified procedure in which each selection is based on a single root tip from the infected plant. This should reduce the time for a stable hairy root culture to emerge.

This study shows that a transgenic hairy root line can be stably maintained for 5 years while retaining the inducible feedback resistant expression of an inserted gene. It also indicates that the hairy root cultures may go through an initial transient stage before the metabolite profile reaches a stabilized state. These observations have important implications for the future use of transgenic hairy roots in industrial applications.

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