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Notes

Still stable after 11 years: A *Catharanthus roseus* hairy root line maintains inducible expression of anthranilate synthase

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Short running title: Hairy roots still stable after 11 years

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Abstract

Hairy root cultures generated using *Agrobacterium rhizogenes* are an extensively investigated system for the overproduction of various secondary metabolite based pharmaceuticals and chemicals. This study demonstrated a transgenic *Catharanthus roseus* hairy root line carrying a feedback-insensitive anthranilate synthase (AS) maintained chemical and genetic stability for 11 years. The AS gene was originally inserted in the hairy root genome under the control of a glucocorticoid inducible promoter. After 11 years continuous maintenance of this hairy root line, genomic PCR of the ASA gene showed the presence of ASA gene in the genome. The mRNA level of AS was induced to 52 fold after feeding the inducer compared to the un-induced control. The AS enzyme activity was 18.4 nmol/(min*mg) in the induced roots compared to 2.1 nmol/(min*mg) in the control. In addition, the changes in terpenoid indole alkaloid concentrations after overexpressing AS were tracked over 11 years. The major alkaloid levels in induced and control roots at 11 years are comparable with the metabolite levels at 5 years. This study demonstrates the long term genetic and biochemical stability of hairy root lines which has important implications for industrial scale applications.

Key Words: plant metabolic engineering, inducible promoter, terpenoid indole alkaloid, cell factories

1. Introduction

Plants are a rich source of industrial chemicals ranging from fuels and cosmetics to pharmaceuticals and health supplements. Many medicinal plants produce a broad spectrum of economically valuable alkaloids being extensively used in the clinic.[1] The low yield of these alkaloids and the complex chemical structures make metabolic engineering of the plant a

potential strategy to enhance the production of the chemicals in industry.[2] Hairy root culture is an attractive biological system for the production of alkaloids and other plant derived compounds due to the ease of genetic engineering, to the rapid growth in hormone-free liquid medium and to the production of physiological levels of secondary metabolites.[3] Hairy roots can be grown and scaled in bioreactors allowing for the establishment of a continuous production system.[2] More importantly, the long term stability of genetic engineering in a hairy root culture is a crucial criterion for viable industrial production. Transgenic plant cell lines usually lose the expression of the modified gene after several generations.[4] A transgenic *Catharanthus roseus* plant obtained from the *Agrobacterium tumefaciens* lost the engineered GUS gene expression after five years.[5] The short term genetic stability of maintaining a transgenic gene in hairy root cultures has been demonstrated in two species. *Datura stramonium* hairy roots showed stable alkaloid production for 5 years.[6] *C. roseus* hairy roots have been reported to maintain the stability of an inducible GFP gene for 4.5 years [7] and an inducible anthranilate synthase (AS) gene for 5 years. [8] This paper further demonstrates that *C. roseus* hairy root lines can maintain inducible expression of a gene for 11 years. This study provides information on the long term chemical and genetic stability of a transgenic hairy root which provides critical information for industrial scale applications.

2. Materials and Methods

2. 1 Culture Conditions

The generation of the hairy root line ASαβ-1 has been previously described. [9] Briefly, the construct pASαβ (Figure1a) carrying *Arabidopsis thaliana* ASA1 (feedback-resistant α subunit) and *Catharanthus roseus* ASB gene was transformed into *C. roseus* seedlings by wounding with

Agrobacterium rhizogenes 15834 containing the plasmid. The hairy roots were harvested, selected on the plates and adapted to the liquid medium as previously described. [9] Hairy root cultures of ASαβ-1 were maintained by placing five 2-3 inch root tips 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), half-strength Gamborg's B5 salts (Sigma), and full-strength Gamborg's vitamins (Sigma) at a pH of 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.

2.2 Genomic PCR

Genomic DNA was extracted from hairy root cultures of ASαβ-1 using the GenElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich). Genomic PCR was performed with one primer specific to the inducible promoter region outside the gene and one primer specific to a region inside the gene (Figure 1a) to confirm the insertion of ASα into the genome: 5'-AGCTTGCATGCCGGTCGACTCTAGAGGATCC-3' and 5'-TCACAAATGCAGATTCAGCCAAGTCGATGGCTCGAG-3'. The expected PCR product size is ~2.1 kb.

2.3 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 hrs later on day 21. The cultures were then frozen at -80 °C until they were lyophilized. Tryptophan (Sigma Aldrich), tryptamine (Sigma Aldrich), ajmalicine (AvaChem Scientific), serpentine (AvaChem Scientific), catharanthine (Qventas), hörhammericine (gift from Dr. Jacqueline V. Shanks at Iowa State University), lochnericine (gift from Dr. Jacqueline V. Shanks at Iowa State University) and tabersonine (AvaChem Scientific) were extracted and was quantified by a previously reported HPLC based protocol. [8]

2.4 cDNA synthesis and RT-qPCR

The total RNA was extracted and reversely transcribed to cDNA as previously described.[10] qPCR amplification was carried out following the protocol in a previous paper.[11] The AS primer was specific to the engineered ASA rather than the native one.[10] The relative transcription of measured gene in the induced hairy roots were shown by $2^{(-\Delta\Delta C_T)}$ normalizing to the control gene (40S ribosomal protein S9) and the transcription level in the uninduced control.[12]

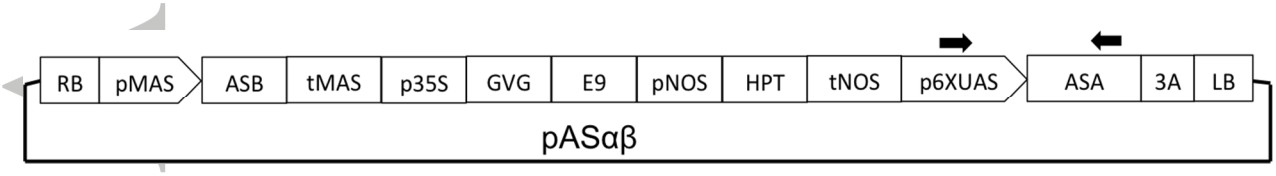
2.5 Anthranilate Synthase Enzyme Assay

Anthranilate synthase enzyme assay was performed as previously described[8] with Synergy H1 Multi-Mode Reader (BioTek) used as a fluorescence detector with the excitation set at 340 nm and the emission set at 400 nm.

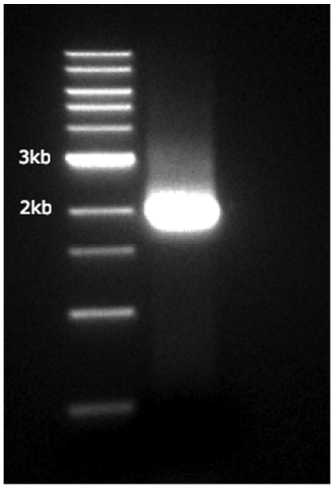
3. Results and discussion

3.1 The AS expression cassettes is still stable after 11 years

The *C. roseus* hairy root line ASαβ-1 has been continuously maintained in liquid media by sub-culturing every 3 weeks. Eleven years after this transgenic hairy root line was established in liquid media, the genomic presence of the inserted feedback-insensitive gene ASA under control of a glucocorticoid inducible promoter was confirmed by genomic PCR (Figure 1b). The primers used were specific to the inducible promoter and to the inserted ASA which sequence is originally from *Arabidopsis* (Figure1a). This primer design ensures that only the inserted ASA is amplified and not the native gene.



(a)



(b)

Figure 1. (a) ASαβ gene expression cassettes under the dexamethasone-inducible promoter. p35S, cauliflower mosaic virus 35S promoter; GVG, hybrid transcription factor; E9, pea rbcs-E9 gene terminator; pNOS, nopaline synthase gene promoter; *HPT*, hygromycin resistance gene; tNOS, nopaline synthase gene terminator; p6XUAS, dexamethasone-inducible promoter; 3A, pea rbcs-3A gene terminator; RB, T-DNA right border; LB, T-DNA left border. tMAS and pMAS indicate terminator and promoter region of the mannopine synthase gene, respectively. *ASA* denotes the cDNA of the *Arabidopsis* feedback-insensitive anthranilate synthase α subunit gene. *ASB* denotes the cDNA of the *Catharanthus roseus* anthranilate synthase β subunit gene. Arrows represent the primer pair used for genomic PCR.
(b) Genomic PCR confirmation of the ASA insert in *C. roseus* hairy root line ASαβ-1 at 11 years. The left lane is a 1 kb DNA ladder (NEB). The right lane is the PCR product from the genomic DNA extraction. The expected size of the PCR product is ~2.1 kb.

The mRNA levels of ASA in the 72 h induced hairy roots and uninduced control were measured by RT-qPCR. 51.8 ± 8.4 fold change of ASA mRNA levels were detected in the induced hairy roots compared to the control, which demonstrated the engineered gene ASA was still controlled by the glucocorticoid inducible promoter system.

Next the enzyme activity of AS was tested, three biological replicates of ASαβ-1 were treated with 0.2 μM dexamethasone (induced cultures) or with an equal volume of ethanol (uninduced cultures) for 72 h. Figure 2 showed that ASα enzyme activity was higher in the induced cultures than the uninduced cultures. In the control hairy roots, the native AS enzyme activity was strongly feedback inhibited by the increased concentration of its catalytic product tryptophan. While in the AS induced hairy roots, both native AS and the induced Arabidopsis

feedback resistant AS contribute the total AS activity. Thus, AS activity was only partially inhibited in the induced hairy roots when fed with highest level of tryptophan. In addition, the non-inhibited enzyme activity was tracked for 11 years (Figure 3). The induced AS activity was increased to 2.2 times of the control when it was first generated. While after being maintained for 5 or 11 years, AS activity increased 11 fold and 9 fold in the induced hairy roots compared to the control, respectively. The difference in the fold increase between year 5 and 11 are not significant. The results are likely due to a heterogeneous culture during the initial stages of hairy root culture generation and maintenance. As time progressed, the fastest growing roots were likely selected for sub-culture which resulted in a homogeneous culture that was stable by 5 years and has been maintained thru year 11. This stabilization of the characteristics of the culture was also seen in the metabolite data described below. This indicates that once the transgenic hairy root line was stabilized, AS can be reliably induced to consistent concentrations over time.

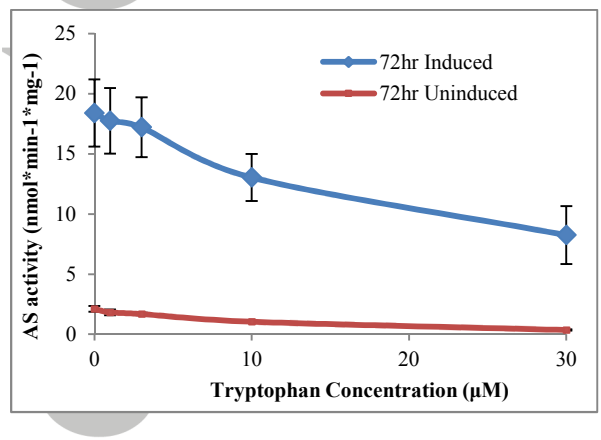


Figure 2. Enzyme activity of anthranilate synthase (AS) and inhibition study 11 years after first being transferred to liquid media. Triplicate cultures were induced with 0.2 µM dexamethasone (induced) or an equal volume of ethanol (uninduced) and harvested 72 h later. Error bars represent the standard deviation of triplicate samples.

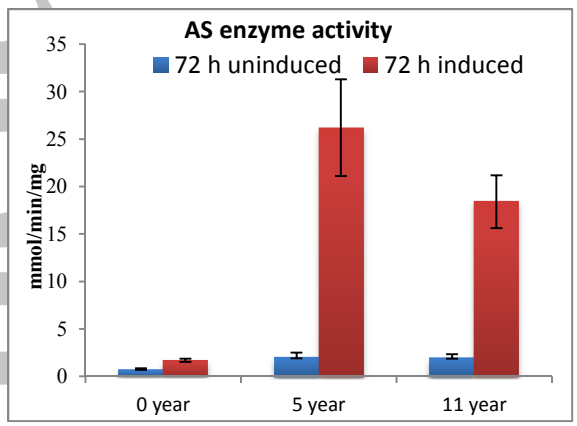


Figure 3. Enzyme activity of anthranilate synthase (AS) during 11 years after first being transferred to liquid media. Triplicate cultures were induced with 0.2 μM dexamethasone (induced) or an equal volume of ethanol (uninduced) and harvested 72 h later. Error bars represent the standard deviation of triplicate samples.

3.2 The alkaloids levels maintained stable after 11 years

Additionally, the effect of overexpressing AS on metabolite concentrations was investigated at various time points throughout the 11-year period. Overexpressing AS caused a large increase in tryptophan and tryptamine concentrations compared to the control (Figure 4) throughout the past 11 years with the change in concentration remaining stable since the 5 year study.[13] Terpenoid indole alkaloids concentrations including ajmalicine, serpentine catharanthine, tabersonine, lochnericine and hörhammericine showed complex results after overexpression AS which was previously discussed.[13] From figure 5, the effect of overexpressing AS fluctuated in the first two years, which may due to the heterogeneous background. After 2 years, the TIA levels maintained stable changes after the induction of AS and follow the same trend through year 11.[13]

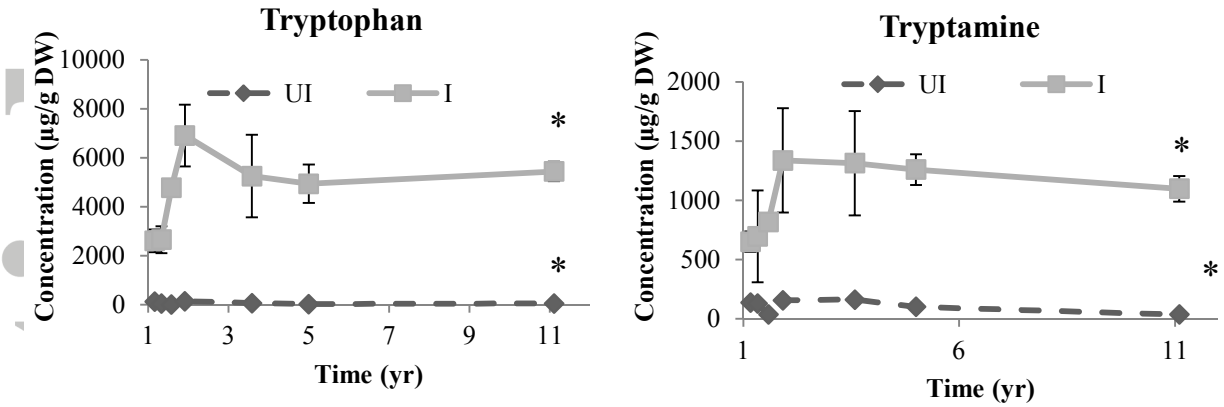


Figure 4. The change in tryptophan and tryptamine concentrations of 72 h induced/uninduced cultures over time. Each data point represents the results of a 72 h induction study performed in triplicate. Error bars represent the standard deviation of triplicate samples. (*) represents data that has not been previously published [8,13,14].

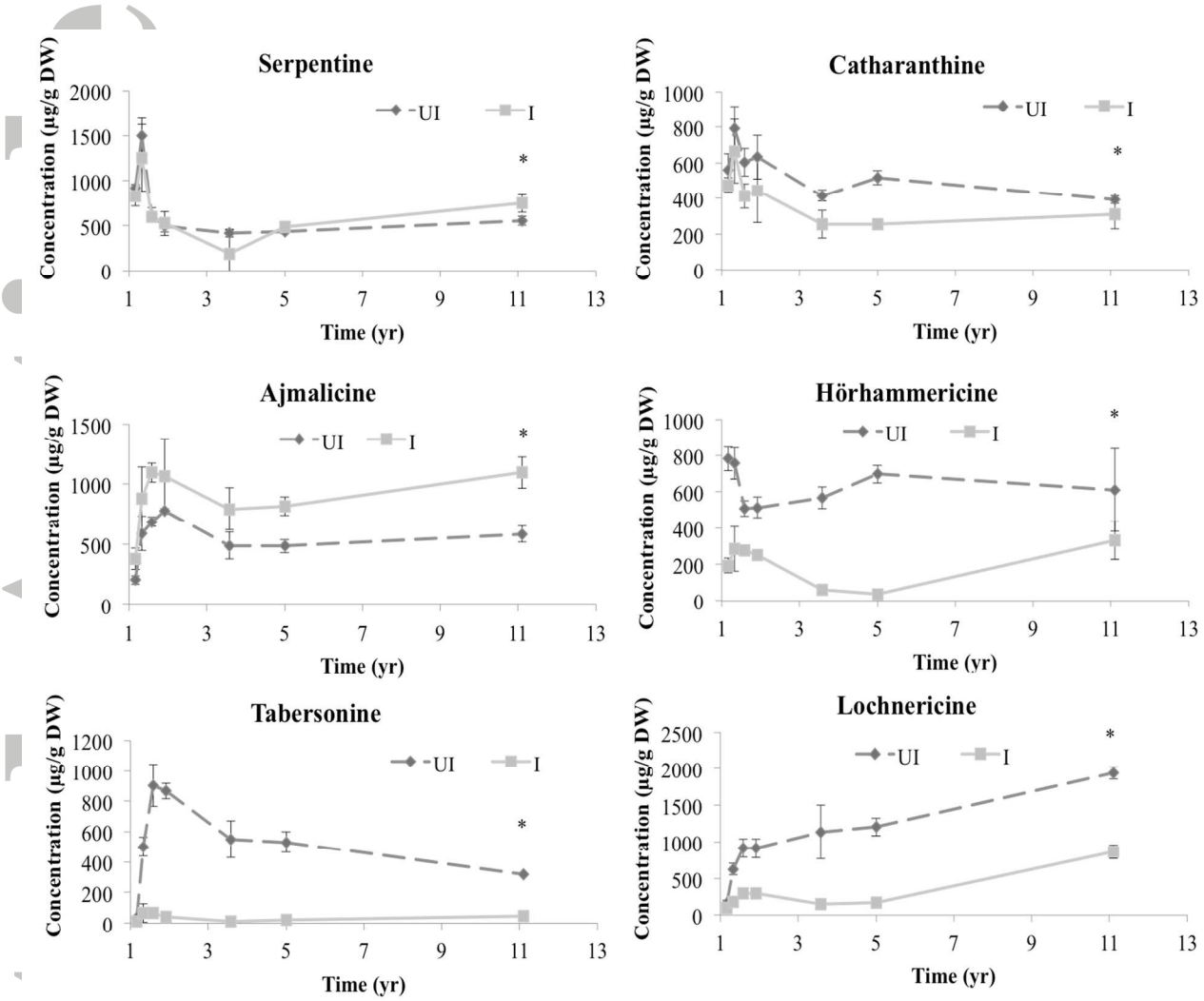


Figure 5. The change in terpenoid indole alkaloid (TIA) concentrations of 72 hr induced/uninduced cultures over time. Each data point represents the results of a 72 h induction study performed in triplicate. Error bars represent the standard deviation of triplicate samples. (*) represents data that has not been previously published. [8,13,14]

This study shows that the inducible expression of AS in the transgenic hairy root line ASαβ-1 can be stably maintained for up to 11 years. The long-term stability of the transgenic hairy root line suggests its great potential for future use as a viable industry production platform.

Conflicts of Interest.

The authors declare no conflicts of interest.

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