Effect of Sodium Nitroprusside on Growth and Terpenoid Indole Alkaloid Production in *Catharanthus roseus* Hairy Root Cultures

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Nitric oxide (NO) is known as a signaling molecule involved in elicitor-induced defense responses of plants. Sodium nitroprusside (SNP), a donor of NO, stimulates catharanthine formation in Catharanthus roseus cells.[1] Two important terpenoid indole alkaloids produced in small quantities within C. roseus are vinblastine and vincristine which are being used clinically as anticancer drugs. We are interested in engineering C. roseus hairy roots to increase the production of the TIAs. The present work investigates the effects of treating different concentrations of SNP to the hairy root cultures from line LBE-6-1. The alkaloid concentrations were analyzed 9, 14, 17, 20, 23, 26, and 30 days after treatment of SNP on day 0. We also studied the transient effects of SNP treatment during the exponential phase in C. roseus hairy roots. Analysis of the results showed that treatment of 0.1-mM SNP did not affect the growth of hairy roots, whereas 1-mM SNP suppressed the growth significantly, and 10-mM SNP almost completely inhibited the growth of hairy roots. 0.1-mM SNP treatment on day 0 caused a significant increase in the concentration of serpentine, catharanthine, ajmalicine, lochnericine and tabersonine production. SNP treatment on day 12 stimulated the formation of serpentine, catharanthine, ajmalicine, hörhammericine, lochnericine and tabersonine by day 21. After the initial stimulation, serpentine, horhammericine and lochnericine concentrations returned to the basal level by day 28. Treatment of 0.1-mM SNP on day 0 caused significant decrease in the mRNA levels for TDC, ASA, STR, ORCA3, ZCT1, and Crgbf1 on day 23. Treating 0.1-mM SNP on day 12 caused decreases in the expression levels of STR, ORCA3, ZCT1, and Crgbf1 on day 21 and day 28. Compared with day 28, the mRNA transcript of ZCT1 on day 21 is about twofold higher. Expression levels of G10H increased significantly. © 2011 American Institute of Chemical Engineers *Biotechnol. Prog.,* 27: 625–630, 2011
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Introduction

Because of potent biological activity of alkaloids, many of the alkaloids have been exploited as pharmaceuticals, stimulants, narcotics, and poisons.[2] A number of important pharmaceutically valuable terpenoid indole alkaloids (TIAs) are produced in *Catharanthus roseus*. Vincristine and vinblastine are used as antineoplastic agents, ajmalicine is an antiarrhythmic drug, and serpentine can lower blood pressure. The importance of *C. roseus* as a source of medicines promoted the research of alkaloid biosynthesis in this plant.[3] Metabolic engineering is used as an efficient way to increase the alkaloid production of cell suspension cultures and hairy roots. For hairy roots, alkaloid production was investigated by feeding precursors,[4] by overexpression of TIA pathway genes,[5–7] and by overexpression of transcriptional regulators.[8] For the *C. roseus* cell suspension cultures, similar metabolic engineering studies have been performed to investigate alkaloid production.[9–15]

Alkaloid synthesis in plants is induced by various elicitors and hormones. Nitric oxide (NO) is a plant signaling molecule which has been implicated in bewildering array of processes related to plant growth, development, function, and response to abiotic and biotic stresses.[16] Sodium nitroprusside (SNP), a

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donor of NO, stimulated production of catharanthine and ajmalicine in *C. roseus* cell suspension cultures.[1] However, the role of NO in the biosynthesis of TIA and the effects of NO on the metabolic network in hairy roots are not well established.

This study explores the capability of NO to induce the TIA pathway in *C. roseus* hairy roots. This effect was studied by SNP treatment at various concentrations to the hairy root cultures and by monitoring the concentration of TIAs, the mRNA levels of specific TIA genes, and the specific yields of several TIAs in response to SNP. The results showed that low concentrations of SNP did not affect the growth of hairy roots and enhanced the production of select alkaloids. The examination of mRNA levels also indicated SNP altered the expression of some TIA genes.

Materials and Methods

Chemicals

SNP (Sigma) was first dissolved in MilliQ water to make a 0.5 M of SNP solution and then filter-sterilized through a 0.22-μm filter before adding to the culture media.[17] Serpentine (Sigma), ajmalicine (Sigma), catharanthine (Qventas, Bradford, CT), and tabersonine (a gift from Dr. Hamada, Okayama University, Japan) were dissolved in methanol and used as high performance liquid chromatography (HPLC) standards.

Culture conditions and measurements of hairy root growth

The culture media for the hairy roots consisted of a filter-sterilized (0.22-μm filter) solution of 30-g/L sucrose (Sigma), half-strength Gamborg's B5 salts (Sigma), and Gamborg's vitamins (Sigma). pH of the media was adjusted to 5.7 with 1-M NaOH solution.

C. roseus hairy root cultures LBE-6-1[18] were initiated by placing five 3-cm long root tips in 50 mL of the culture media in a 250-mL Erlenmeyer flask. These cultures were grown in the dark at 26°C at 100 rpm. Hairy roots growth was described by fresh weight and dry weight. The fresh weight of the harvested hairy root cultures were measured after the cultures were blotted to remove excess media. The cultures were then frozen at −80°C. The dry weight was measured after lyophilization.

SNP treatment

The effects of NO were studied by treating the hairy roots cultures with SNP. In this study, 0.1-, 1-, and 10-mM SNP were fed on day 0 to study the effects of SNP on the hairy roots growth and alkaloids production. The samples were harvested on day 9, 14, 17, 20, 23, 26, and 30. Furthermore, to study the effects of SNP on the alkaloids production in the exponential phase, 0.1-mM SNP was fed on day 12, and samples were harvested on day 21 and 28. The unfed cultures were used as a control. Each experiment was repeated in triplicate.

Alkaloid extraction and HPLC detection

Approximately fifty milligram dry weight of the ground tissue was extracted in 25 mL of methanol by ultrasonication for 10 min (Model W-225, Heat System. Ultrasonics). 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 microliters of each sample was analyzed for the following TIAs: serpentine, catharanthine, ajmalicine, hörhammericine, lochnericine, and tabersonine on the HPLC as previously described.[5]

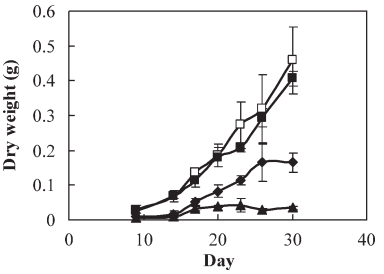

Figure 1. Effect of SNP on the growth of LBE-6-1 line *C. roseus* hairy roots. □, 0-mM SNP; ■, 0.1-mM SNP; ◆, 1-mM SNP; ▲, 10-mM SNP.

mRNA extraction, cDNA synthesis, and Q-PCR amplification

Approximately 0.3 g of hairy root tissues were frozen and grounded in liquid nitrogen. RNA isolation, DNA treatment, reverse transcription, and Q-PCR amplification were carried out as described previously.[8] The primers used for the genes: *G10H* (geraniol 10-hydroxylase), *TDC* (tryptophan decarboxylase), *ASα* (anthranilate synthase), *ORCA3* (AP2-domain DNA-binding protein), *Crgbf1* (G-box binding factors), *STR* (strictosidine synthase), and *ZCT1* (zinc finger DNA-binding protein) were listed in a previous article.[8] The reaction mixture was incubated for 2 min at 50°C, 10 min at 95°C, and for 40 cycles of 15 s at 95°C, and 1 min at 60°C. The relative gene expression was quantified by using the comparative C_T (threshold cycle) method as previously described.[19] The 40S ribosomal protein S9 (*rsp9*) was used as a control gene.[20]

Statistical analysis

The statistical analysis was performed using analysis of variance (ANOVA) or student *t*-test. In figures, the stars denote statistically significant difference between the data points: $*P < 0.05$, $**P < 0.01$, $***P < 0.005$.

Results

Effect of SNP concentrations on the growth of LBE-6-1 line C. roseus *hairy roots*

The effects of SNP at different concentration on the growth of LBE-6-1 hairy roots were studied by treating the root cultures with 0, 0.1, 1, and 10-mM SNP on the day when they were inoculated (day 0). In this study, the fresh weight and dry weight of the root tissue was used to characterize the growth of hairy roots, which were shown in Figure 1 and supplementary figure. Examination of the growth of the hairy roots showed that 0.1-mM SNP did not suppress the growth, whereas 1-mM SNP repressed the growth of hairy roots significantly. Ten millimolar SNP almost completely repressed the growth of the hairy roots (Figure 1). As 0.1-mM SNP did not affect the growth of hairy roots, this concentration was chosen for the time profile of alkaloid concentration study.

Effect of SNP on the alkaloid production in LBE-6-1 line C. roseus *hairy roots*

The effects of SNP on TIA production were evaluated by measuring the specific yields of serpentine, catharanthine,

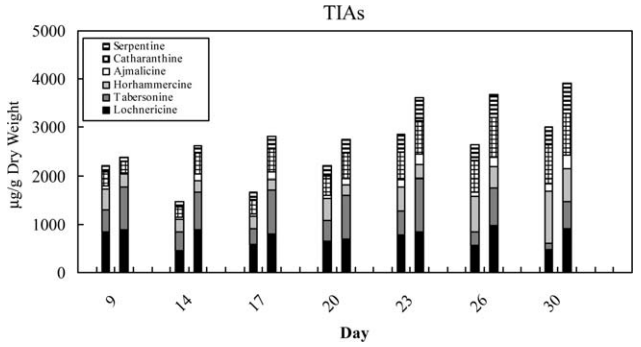

Figure 2. Effect of SNP on the alkaloid production of *C. roseus* hairy roots when 0.1-mM SNP is fed on day 0.

Each group of stacked bars: left bar represents control experiment; right bar represents 0.1-mM SNP treatment experiment.

ajmalicine, hörhammericine, lochnericine, and tabersonine, as shown in Figure 2. The alkaloid levels were analyzed by sampling on day 9, 14, 17, 20, 23, 26, and 30 days after treating of SNP on day 0. The alkaloid levels results were compared between SNP treatment and control experiments at the same time point.

C. roseus hairy root cultures fed with 0.1-mM SNP on day 0 showed significant increases in specific yields of most TIAs measured, except for hörhammericine. Enhanced production was observed for serpentine ($P < 0.05$) after day 17, ajmalicine ($P < 0.005$, lochnericine ($P < 0.005$) and tabersonine ($P < 0.001$), and the total TIAs ($P < 0.001$) over time after day 9. The temporal profiles of the specific yields of ajmalicine and serpentine showed very similar patterns. Both of them showed a gradual increase over time, and the difference between fed and unfed cultures reached the largest value on day 30 ($P < 0.05$). Catharanthine first showed signs of enhanced specific yields 14 days after treating with SNP, but returned to the level of unfed cultures at day 30. Hörhammericine specific yields were found to decrease ($P < 0.05$) in the fed cultures.

To investigate the effect of SNP treatment during exponential growth phase on TIA accumulation, hairy roots were cultivated without SNP at the beginning, and then were treated with 0.1-mM SNP on day 12, which is in the early exponential phase. The alkaloids specific yields showed significant increases in hörhammericine and lochnericine ($P < 0.05$) accumulation 9 days after treatment with SNP (Figure 3). The concentrations of hörhammericine and lochnericine tended to return to the basal levels 16 days after treatment, whereas ajmalicine, catharanthine, and tabersonine specific yields showed significant increase up to 16 days ($P < 0.05$ for ajmalicine and tabersonine, $P < 0.001$ for catharanthine) after treatment. A treatment of 0.1-mM SNP in the exponential phase resulted in a significant increase of total TIAs at both time points ($P < 0.05$).

Effect of SNP on mRNA transcript levels for TIA pathway genes

The comparative C_T method was used to analyze changes in mRNA transcript levels in the fed cultures relative to unfed cultures in this study. Gene expression levels of TIA genes such as *ASA*, *TDC*, *G10H*, *STR*, *ORCA3*, *ZCT1*, and *Crgbf1* for the different conditions are shown in Figures 4 and 5.

Treatment of 0.1-mM SNP on day 0 caused significant decrease in the mRNA levels for TDC ($P < 0.05$), ASA

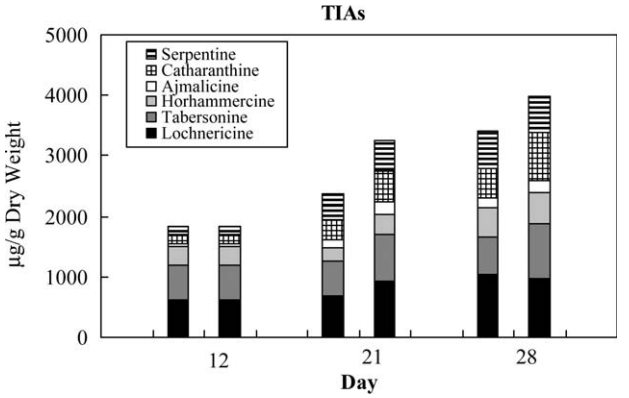

Figure 3. Effect of SNP on the alkaloid production of *C. roseus* hairy roots when 0.1-mM SNP is fed on day 12.

Each group of stacked bars: left bar represents control experiment; right bar represents 0.1-mM SNP treatment experiment.

($P < 0.005$), STR ($P < 0.01$), ORCA3 ($P < 0.01$), ZCT1 ($P < 0.005$), and Crgbf1 ($P < 0.001$) on day 23, and the mRNA levels for STR and Crgbf1 returned to the similar levels for both feeding conditions. Expression levels of G10H remained unchanged.

Many of the trends observed with SNP treatment at day 0 were observed with treatment of SNP on day 12. Treating SNP on day 12 caused decreases in the expression levels of STR ($P < 0.05$), ORCA3 ($P < 0.01$ on both day 21 and $P < 0.005$ on day 28), ZCT1 ($P < 0.005$ on day 21 and $P < 0.05$ on day 28) and Crgbf1 ($P < 0.001$ on both day 21 and 28) on day 21 and day 28. Especially, ORCA3 transcript was reduced to $1/7^{th}$ of its original value 9 days after treatment of 0.1-mM SNP on day 12, which indicated that ORCA3 may play in being a transcriptional regulator. Compared with day 28, the mRNA transcript of ZCT1 on day 21 is about twofold higher. Expression levels of G10H increased significantly on day 21.

Discussion

To investigate the effect of NO on the growth, TIA formation and the regulation of NO dependent genes in *C. roseus* hairy roots, the strategy we used is to stimulate the NO burst. Treatment with one of the NO donors, SNP, is a feasible strategy of NO simulation. SNP is a spontaneous NO donor, releasing NO upon dissolution in aqueous solvents not requiring enzymatic reduction or hydrolysis for this process. SNP has been used in numerous studies as a donor of NO, which includes studies with roots[21–23] and hairy root cultures.[24–26] In this study, the effect of SNP was examined by treating different amount of SNP in the hairy roots culture during different growth phase.

As alkaloids in plants can help protect plants against an adverse environment, their synthesis and accumulation are frequently induced in response to environment change. In this study, the TIA results showed that treating a certain amount of SNP increased the alkaloid production in LBE-6-1 line *C. roseus* hairy roots. SNP was found toxic to the *C. roseus* hairy roots at high concentration (1 and 10 mM), and could induce the roots to produce more alkaloids at low concentration (0.1 mM). Treating 1-mM SNP in the exponential phase (day 12) also caused increase in lochnericine, tabersonine and total TIA specific yields (data not shown). Because of toxicity of SNP at higher concentration (1 mM), the increase in total TIA

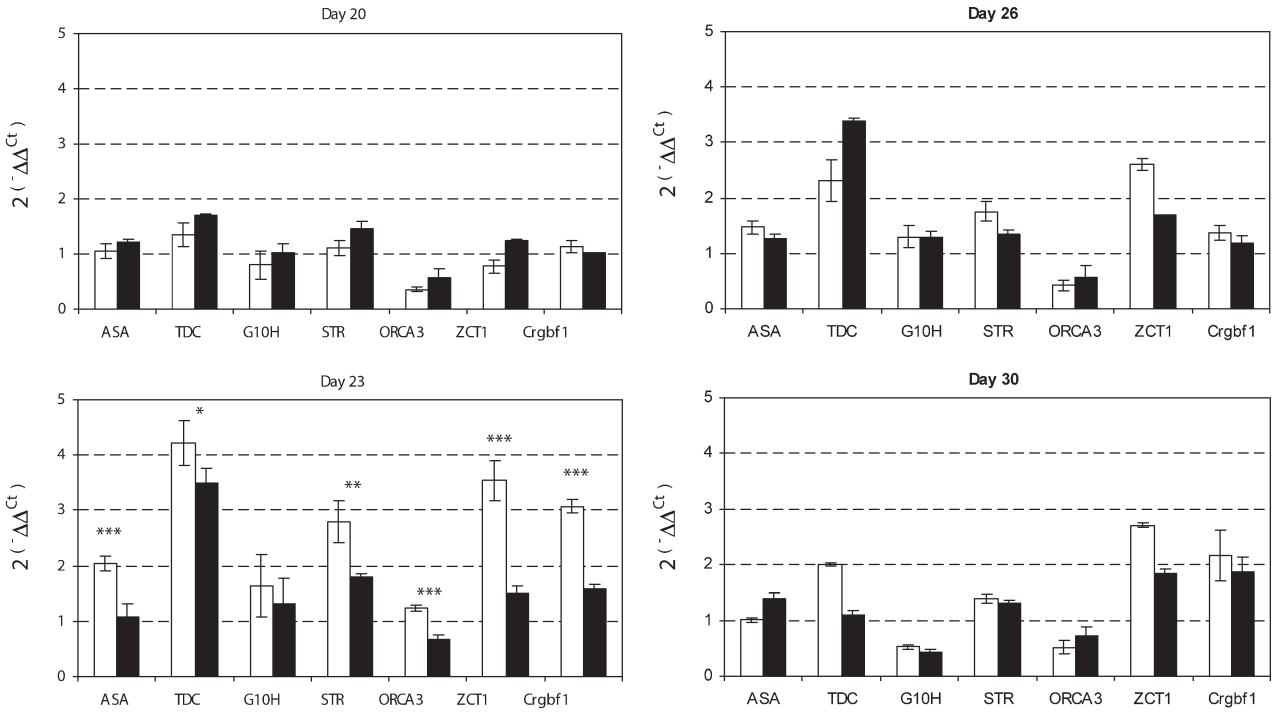

Figure 4. Effect of SNP on the related gene expression of *C. roseus* hairy roots when 0.1-mM SNP is fed on day 0. Symbols: □, 0-mM SNP; ■, 0.1-mM SNP: stars indicate the statistically significant data: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$. G10H (geraniol 10-hydroxylase), TDC (tryptophan decarboxylase), ASα (anthranilate synthase), ORCA3 (AP2-domain DNA-binding protein, Crgbf1 (G-box binding factors), STR (strictosidine synthase), and ZCT1 (zinc finger DNA-binding protein).

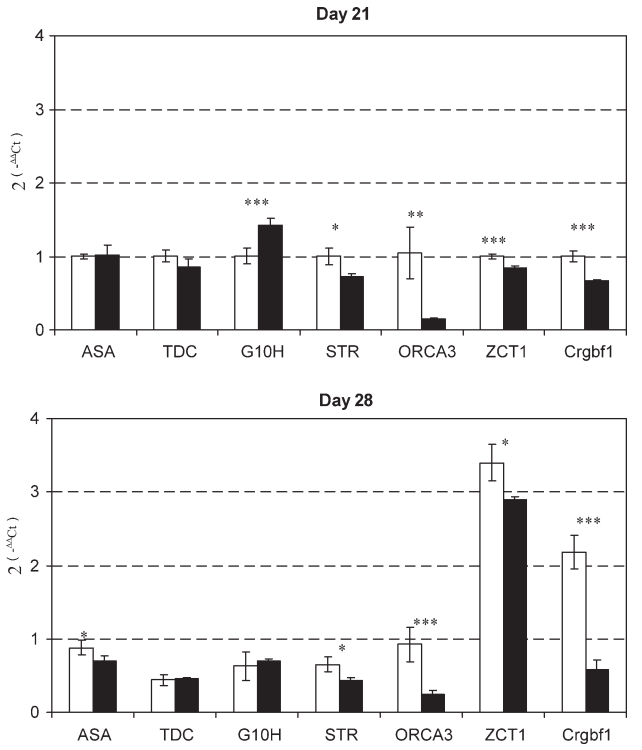

Figure 5. Effect of SNP on the related gene expression of *C. roseus* hairy roots when SNP is fed on day 12. Symbols: □, 0-mM SNP; ■, 0.1-mM SNP: stars indicate the statistically significant data: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$. G10H (geraniol 10-hydroxylase), TDC (tryptophan decarboxylase), ASα (anthranilate synthase), ORCA3 (AP2-domain DNA-binding protein, Crgbf1 (G-box binding factors), STR (strictosidine synthase), and ZCT1 (zinc finger DNA-binding protein)

concentration was not as high as that from low concentration (0.1 mM) SNP experiment (data not shown).

It is important to note that the results in cell cultures may not necessarily be the same for hairy roots as these cells are differentiated. In contrast to the previous study on the effect of SNP on *C. roseus* cell suspension culture, low concentration of SNP (0.1 mM) did not affect the growth of *C. roseus* hairy roots, and high concentration of SNP (10 mM) almost completely inhibited the growth of *C. roseus* hairy roots, whereas low concentration of SNP (0.1 and 0.5 mM) enhanced the growth of *C. roseus* cells, and high concentration of SNP (10 and 20 mM) inhibited the growth of *C. roseus* cells.[1,27] It was found that SNP at high concentrations (10 and 20 mM) stimulated the formation of ajmalicine, catharanthine, and total alkaloids of *C. roseus* cells, and low concentration of SNP (0.1mM) had no effect on catharanthine synthesis. Compared with the *C. roseus* cell cultures results, low concentration of SNP (0.1 mM) stimulated the alkaloids formation in *C. roseus* hairy roots either treating SNP at the very beginning of the cultivation or treating during the exponential phase.

The RT-PCR results indicate that SNP caused a decrease in mRNA transcripts of TIAs pathway genes. It was reported that ORCA3 overexpression led to an increase in ASA and STR mRNA transcripts,[8,15] which could explain the decrease in the ASA and STR mRNA transcripts on the SNP treatment experiment. It was also found that the zinc finger binding proteins ZCT1, 2, 3 (members of the transcription factor IIIA zinc finger family) can bind to the promoters of STR and TDC, which repressed the activities of STR and TDC.[28] Crgbf1 was also identified to show the repression on the transcription of STR in *C. roseus*.[29] The decrease of ZCT1 and Crgbf1 expression could negate the activation of STR and TDC caused by ORCA3. However, the combined

function of ORCA3, ZCT1, and Crgbf1 led to the decrease of mRNA transcripts level of STR in both treatment conditions. Furthermore, the accumulation of ajmalicine and catharanthine could be another reason for the down regulation of STR mRNA transcripts.[30]

It is interesting to compare the trend for the TIA metabolites and the TIA transcripts treated with 0.1-mM SNP. The metabolites increase with treatment whereas the transcripts decrease with treatment compared with the control. These results are counterintuitive. An explanation for this phenomenon is unavailable at this time. Further investigation into this phenomenon is needed to reveal the complex regulation networks that control the TIA pathway and the production of its metabolites.

It appears that both growth phases (very beginning of the cultivation and exponential phase) of *C. roseus* hairy roots exposed to SNP have an increase in almost all the TIAs concentration. Different from the former, the TIAs concentrations of the hairy roots of exponential phase exposed to SNP returned to the same levels as control (without SNP elicitation). The reason for this difference is unclear, however, it is possible that *C. roseus* hairy roots in exponential growth phase able to adapt to the changes in the culture environment. It is interesting that there is a difference in hörhammericine formation between treating at day 0 and day 12. It shows that SNP treatment at day 0 causes a decline of hörhammericine concentration in *C. roseus* hairy roots. The gene regulation mechanism of some TIAs genes on the different growth phase possibly causes this difference.

Results from this study suggest that SNP plays a role in triggering the production of some secondary metabolites such as TIAs in *C. roseus* hairy roots. It is also interesting to note that SNP caused an increase in most of the TIAs concentration but a decrease in mRNA transcripts of TIAs pathway genes. There might be regulations on other levels such as protein translation and enzymatic kinetics.

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