Free-Roaming Cat Management Through the Combination of Trap-Neuter-Return and Cat Sedative: Nepetalactone Produced by Engineered *Escherichia coli*

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Abstract

The number of free-roaming cats in Shenzhen and other Chinese metropolises has increased steadily for the past decade due to cats' rapid reproductivity, abundant food source, and lack of population control. An accepted solution to the overpopulation is Trap-Neuter-Return (TNR), which is considered more humane, successful, and effective than its alternatives; however, TNR is not widely practiced. The program sometimes requires contact with aggressive free-roaming cats, which are likely to perform stress behaviors. In attempts to relax cats and encourage citizens, we designed a Catnip Electronic Sensor Trap, utilizing liquid extract of a non-addictive, harmless herb called catnip and establishing an electronic thermal sensor that texts conductors when a cat is trapped. We chose catnip as a cat attractant and sedative because previous researches show that its essential oil nepetalactone, a bicyclic monoterpenoid, is responsible for felines' pleasurable and relaxing behaviors. In this project, we aimed to construct a complete engineering metabolic pathway on engineered Escherichia coli BL21 (DE3) strain that would produce nepetalactone. The production of nepetalactone was achieved by conversion of geraniol to nepetalactol, followed by conversion of nepetalactol to our target product nepetalactone. We introduced three cytochrome P450 enzymes: 8-hydroxylase (G8H), geraniol oxidoreductase (GOR), and iridoid synthase (ISY), P450 reductase enzyme (CPR), and two nepetalactol-related short-chain reductases: NESP1 and NEPS3A to our host. We expected biosynthesized nepetalactone to be a helpful attractant and sedative utilized in TNR programs. In a practice test, our Catnip Electronic Sensor Trap successfully attracted a community cat and sent a SMS message to the conductor's phone number when the cat is trapped. In addition, entertaining behaviors were exhibited when the cat sniffed catnips.

Keywords

Nepetalactone; Catnip; *E. coli*; Synthetic Biology; TNR; Engineering.

1. Introduction

Overpopulation of stray and ferry cats has been a societal problem in many Chinese metropolises, including Shenzhen. It is common to see tens of homeless cats roaming in neighborhoods. According to a wildlife organization, an unspayed female cat, her mate, and their following offspring can produce about 12 cats in a year and 376 cats in 3 years [1]. The current approach to control the cats' population is Trap-Neuter-Return (TNR) (Fig. 1), which is considered an effective, humane alternative to the lethal management of free-roaming cats. Shenzhen Cat Organization reported that in the year of 2020, 3,863 free-roaming cats were trapped, neutered, and returned in Shenzhen and two adjacent cities [2]. Although efforts have been made, the number of neutered free-roaming cats is still relatively insufficient compared to the estimated thousands of stray and ferry cats in the cities. Reasons behind inability to widely conduct TNR include time consumption and concerns about cats' aggressive behaviors and stress responses.

In an interview with Shenzhen Cat Organization, a 5-year volunteer said that currently, only approximately 1,000 citizens are willing to practice TNR in Shenzhen and two other cities because traditional trapping method involves occasional close interaction with aggressive cats. A potential

solution to the concern is catnip, a non-addictive, harmless sedative herb. When exposed to stuffed toys sprayed with catnip extract, two-thirds of domestic cats exhibit pleasurable behaviors in a short time, such as sniffing, licking and chewing with head shaking, chin and cheek rubbing, and head-over roll and body rubbing [3-6]. Because of its entertaining effects, we consider catnip an effective attractant and sedative for free-roaming cats in TNR programs. Usage of catnip would reduce the possibility of cats' overreaction, making TNR a safer and easier practice and ultimately encouraging more citizens to engage in the program.

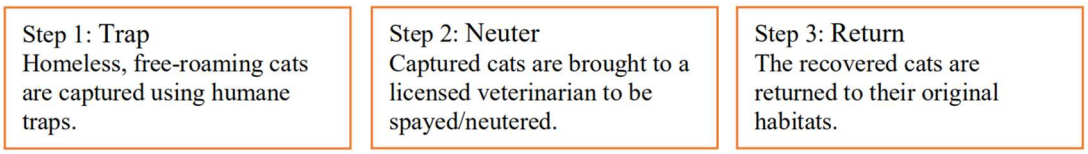


Fig. 1 Trap-Neuter-Return Method. TNR is a management technique in which homeless, free-roaming cats are humanely trapped, evaluated, spayed/neutered (sometimes also vaccinated), and returned to original territory.

The idea of constructing an electronic sensor on the trap came from personal observation of TNR programs. Conductors usually are not present when a cat is trapped because simultaneously, other traps and situations need to be supervised and handled. Trapped cats, therefore, have to wait for minutes before they can be transferred into more comfortable, private cages. When free-roaming cats are in temporary traps, it is highly possible that they get terrified and perform stress responses. To minimize time consumption, we constructed an electronic sensor, which monitors cat presence. The sensor functions similarly to a heat detector: it signals thermal changes present in 5 cm^2. A SMS message is sent when a body of temperature higher than surrounding atmosphere is detected. Along with catnip extract, our trap is expected to facilitate conductors in trap stages of TNR practices.

The magic of catnip lies in its essential oil nepetalactones (Fig. 2). Current production of commercial nepetalactone extracts depends on natural cultivation of catnip-related plants, such as *Nepeta cataria*, *Catharanthus roseus*, and *Arabidopsis thaliana*. A drawback with the method is its productivity: the natural yield of nepetalactones by plants is extremely low [7]. It is also difficult to chemically synthesize catnip compounds due to their structural complexity [8]. Synthetic biology offers a potential solution. In 2018, an iGEM team established geraniol production and converted geraniol to nepetalactol; however, they only produced the intermediate nepetalactol instead of nepetalactone and their metabolic pathway was split on two hosts: *E. coli* and *S. cerevisiae.*

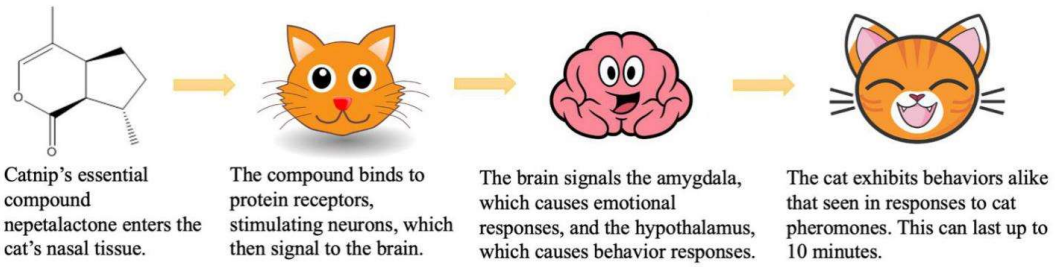


Fig. 2 The Nepetalactone Response. The compound's effects on cats act similarly to those of cats' pheromones, causing the binding of nepetalactones to specific olfactory receptors at the olfactory epithelium of cats, which is hypothesized to stimulate the medial amygdala and medial preoptic area associated with sexual behaviors [9].

Nepetalactone is an iridoid monoterpene with a broad range of biological activities produced by plants in the Nepeta genus [10]. Previous studies have shown that iridoid biosynthesis begins with geraniol synthase (GES) catalyzing geranyl pyrophosphate (GPP) to transfer to geraniol (Fig. 3) [11].

Conversion of geraniol to nepetalactol involves three cytochrome P450 enzymes: geraniol 8-hydroxylase (G8H), 8-hydroxygeraniol oxidoreductase (HGO), and iridoid synthase (ISY) (Fig. 3) [12-14]. In May 2020, it was further identified that different combinations of nepetalactol-related short-chain reductases/dehydrogenases (NEPSs) and MLP-like (MLPL) act in partnership with ISY and 8OG to control profiles of three distinct nepetalactone stereoisomers (Fig. 3) [15]. Though iridoid pathway and formation of nepetalactone biosynthesis were studied, there have not been experiments that produced nepetalactone in bacteria.

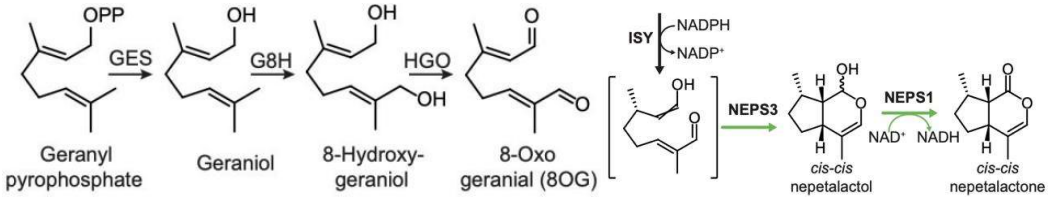


Fig. 3 Biosynthesis Pathways of Iridoids and Nepetalactones. Iridoid biosynthesis begins with hydrolysis of GPP, catalyzed by GES, followed by oxidation, catalyzed by G8H and HGO, yielding 8OG. ISY then catalyzes 8OG into nepetalactol. Because of ISY and 8OG, NEPSs can eventually lead to the formation of nepetalactone stereoisomers [11-15].

In this project, we constructed a complete pathway in *E. coli* BL21 (DE3) that produced nepetalactone. We chose *E. coli* BL21 (DE3) as our host because previous studies show that it serves as a promising chassis for heterologous biosynthesis of complex terpenoids highly modified by cytochrome P450 enzymes [16] and exhibited much better efficiency in production of intermediates, such as geraniols [17]. Based on previous studies on iridoid pathway and nepetalactone biosynthesis, we divided the construction of the whole pathway into four mains steps: 1) introducing the MVA pathway and GES into *E. coli* BL21 (DE3) to produce geraniol; 2) introducing cytochrome P450 enzymes to catalyze geraniol to produce nepetalactol; 3) introducing NEPS1 and NEPS3A to convert nepetalactol to nepetalactone; 4) culturing nepetalactone through fermentation. Our project involved participations of cytochrome P450 reductase enzyme (CPR), 8-hydroxylase (G8H), geraniol oxidoreductase (GOR), iridoid synthase (ISY), and two nepetalactol-related short-chain reductase/ dehydrogenases NEPS1 and NEPS3A.

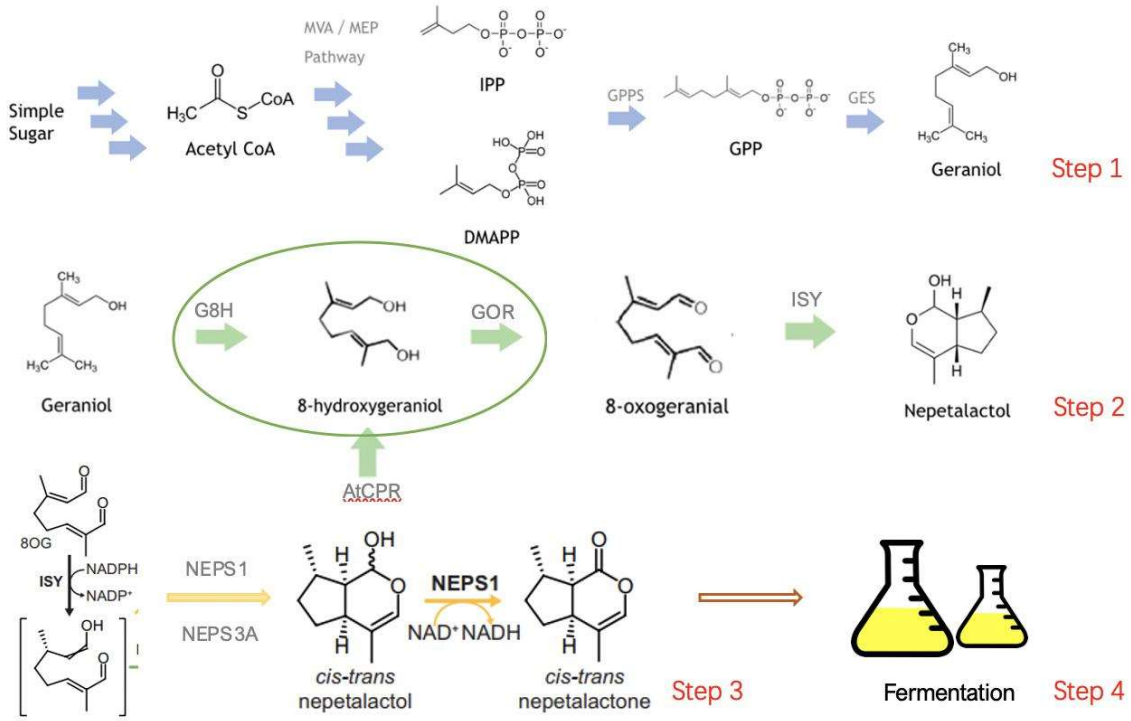
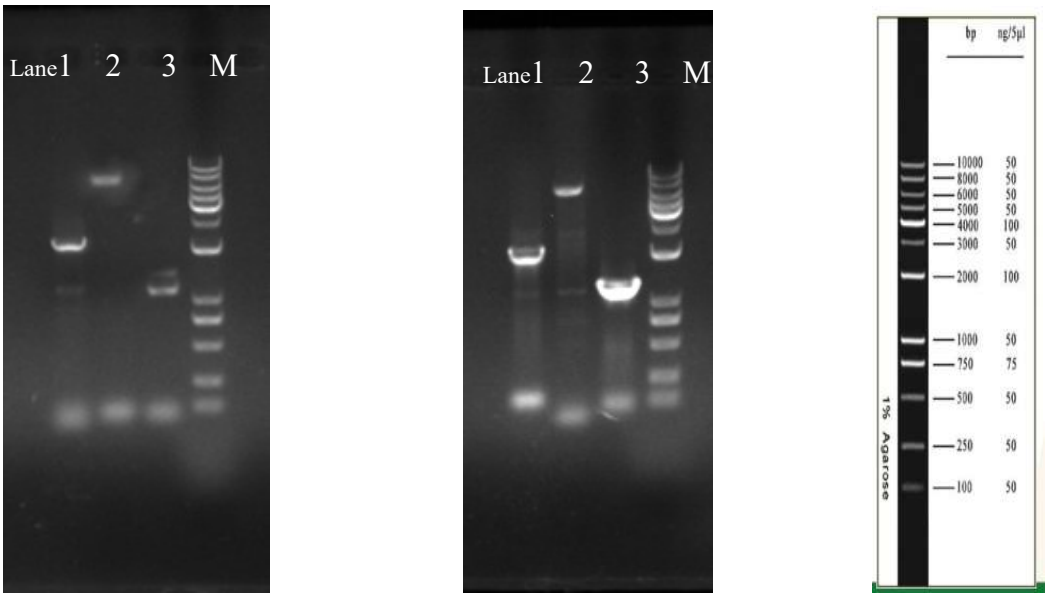


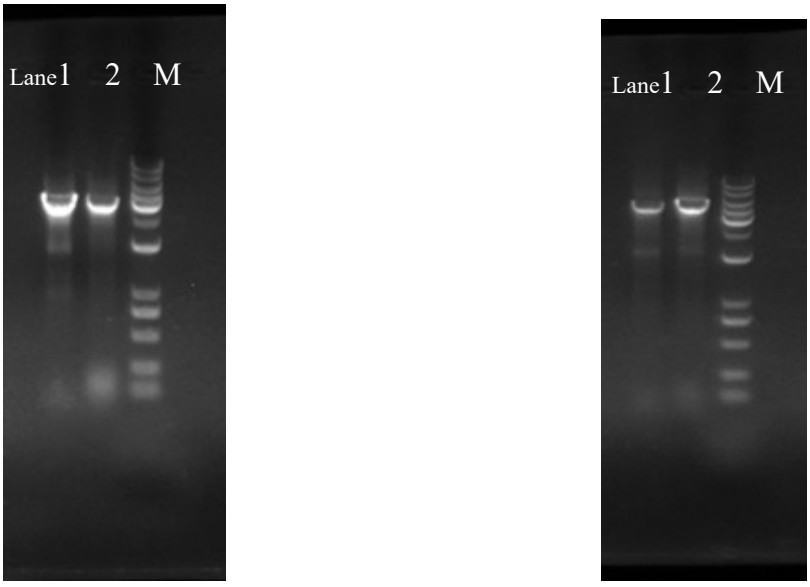
Fig. 4 The Main Steps for Constructing Nepetalactone Biosynthesis Pathway.



(A) (B) (C)

Lane 1: AtCPR; Lane 2: V-CrG8H-pET28a; Lane 3: CrGOR/V-NcG8H-pET28a-F; Lane M: Marker

Fig. 5 The First Electropherogram of PCR. (A) Sizes of gene segments shown in the first electropherogram corresponded with those of the standard gene segments: the standard gene segment of CPR is 2079 bp, that of V-CrG8H-pET28a-R was 6763 bp, and that of CrGOR was 1143 bp. PCR successfully developed the expected gene segments. (B) Sizes of gene segments shown in the figure corresponded with those of the standard gene segments: the standard gene segment of CPR is 2079 bp, that of V-CrG8H-pET28a-R was 6761 bp, and that of CrGOR was 1137 bp. PCR successfully developed the expected gene segments. (C) DNA Marker



(A) pET28a-CrG8H-AtCPR-CrGOR (B) pET28a-NcG8H-AtCPR-NcGOR

Lane 1: Codon 1; Lane 2: Codon 2; Lane M: Marker

Fig. 6 The Second Electropherogram of PCR. (A) and (B) showed that sizes of both tested gene segments were approximately 5100 bp, indicating that they corresponded with those of the standard gene segments, and the Gibson assembly successfully developed the carriers of the two previously stated genes.

2. Results

2.1 Construction of pET28a-CrG8H-AtCPR-CrGOR and pET28a-NcG8H-AtCPR-NcGOR

A polymerase chain reaction (PCR) and a gel electrophoresis were conducted to detect presence of introduced genes AtCPR, V-CrG8H-pET28a, CrGOR, and V-NcG8H-pET28a-F in our engineered strain. The report of the electropherogram was shown below in Fig. 5. After a DNA purification, a Gibson assembly was conducted to complete assembly of genes pET28a-CrG8H-AtCPR, pET28a-CrG8H-AtCPR-CrGOR, pET28a-NcG8H-AtCPR, and pET28a-NcG8H-AtCPR-NcGOR. The Gibson assembly transferred previously stated genes into DH5α cells. The successfully transferred strains were then reserved -80°C refrigerator. Since the strains were guaranteed to be successfully constructed and transferred by the company, their plasmids were extracted out and transferred into the *E. coli* BL21 (DE3). After eight hours of culture, a PCR was conducted on the grown-up bacterial colonies, and a gel electrophoresis was applied and verified presence of the assembled genes (Fig. 6).

2.2 Fermentation Experiment and GC of Engineered Strains

The two engineered strains pET28a-CrG8H-AtCPR-CrGOR + pRG-ISY-NESP1-NESP3A/BL21 and pRG-ISY-NESP1-NESP3A + pET28a-NcG8H-AtCPR-NcGOR/BL21 were the experimental groups of the fermentation experiment. One of the control groups was a plasmid-free *E. coli* BL21 (DE3) strain added with geraniol as substrate; the other control group plasmid-free *E. coli* BL21 (DE3) strain shared same conditions but did not carry any geraniol substrate.

The first set of GCs was conducted to test standard nepetalactone and commercial catnip oil. Their peak graphs indicated that they had the same peak retention time (Fig. 7). To demonstrate the relation between peak area and concentration of nepetalactone, a curve graph was also made. Followed by the fermentation experiment described in the previous paragraph, a second set of GCs was conducted to detect and compare peak graphs of fermented samples and standard nepetalactone. Peak graphs of two fermented samples were also shown in Fig. 9.

We compared peak graphs of our fermented samples and those of standard nepetalactone. The result was that our fermented samples showed a peak retention time of 4.82 minute, while the standard nepetalactone showed a peak retention time of 5.22 minute.

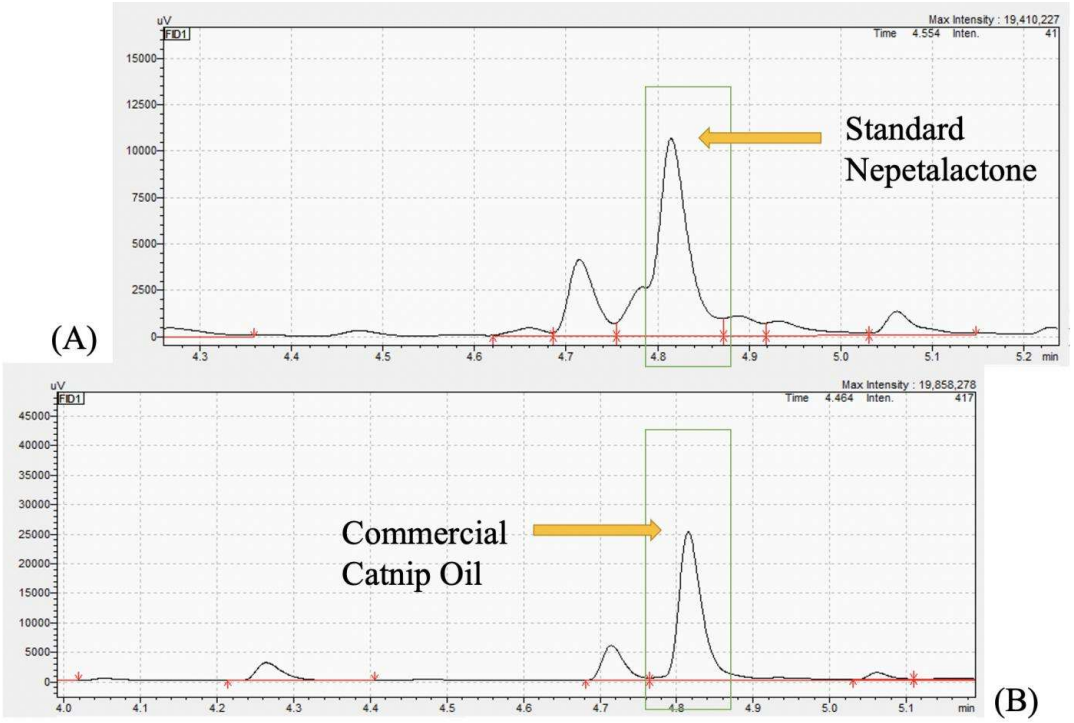


Fig. 7 The First Set of GC Peak Graphs. (A) and (B) showed that standard nepetalactone and commercial catnip shared a same peak retention time at 4.82 minute.

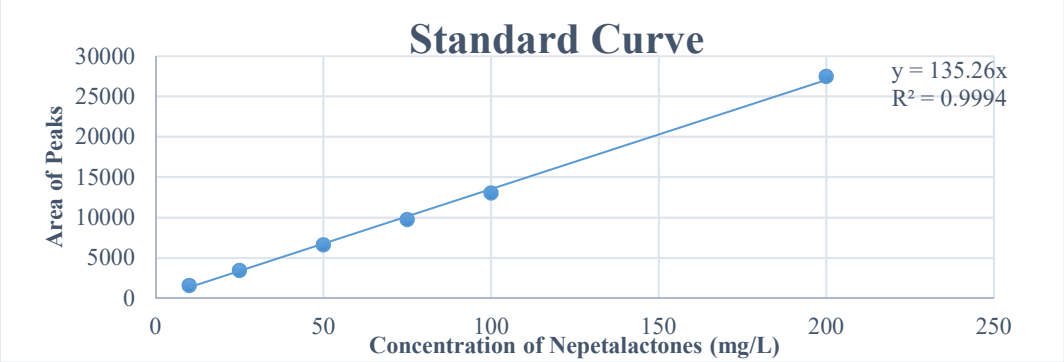


Fig. 8 Standard Curve

Fig. 8 Standard Curve showed the relation between peak areas presented in the first set of GC peak graphs and concentration of nepetalactones. As concentration of nepetalactones increased, peak areas increased. Based on their ratio and equation of the standard curve, concentration of nepetalactones could be calculated when peak area is given.

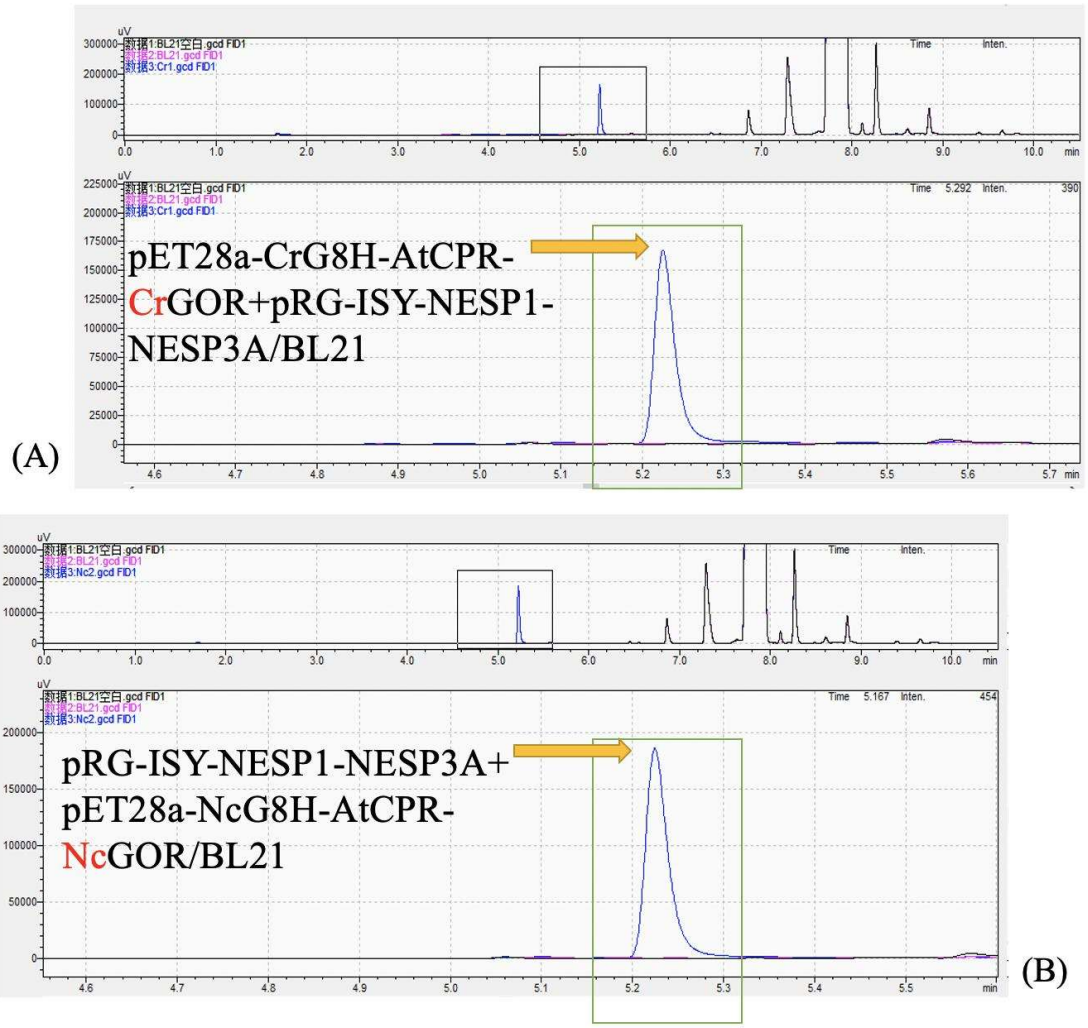


Fig. 9 The Second Set of GC Peak Graphs. (A) and (B) showed that two fermented samples (pRG-ISY-NESP1-NESP3A + pET28a-NcG8H-AtCPR-NcGOR/BL21 and pRG-ISY-NESP1-NESP3A + pET28a-NcG8H-AtCPR-NcGOR/BL21) shared a peak retention time of 5.22 minute. The first plasmid contained genes from plant *Catharanthus* roseus; the second one contained genes from plant *Nepeta cataria*.

2.3 Catnip Electronic Sensor Trap

As stated in Abstract and Background, the purposes of utilizing catnip extract were to attract and appease cats. When a catnip oil extract of 50mg/mL was emanated, my Chinese Dragon Li cat Zima exhibited pleasurable behaviors shown in previous research. In a mock test for our Catnip Electronic Sensor Trap, we applied the same catnip oil extract liquid extract on the trap. Firstly, he was attracted by the odor and approached to the liquid. He then started frequently sniffing the extract, and few seconds later, he rolled over and over on the floor, a behavior indicated cats' happiness. Zima's response to nepetalactone liquid extract proved the oil's effects (Fig. 10).

In a TNR practice in my neighborhood, our Catnip Electronic Sensor Trap was proved to be affective and helpful. The process and mechanism were the same as they were in the mock test described above. A volunteer at Shenzhen Cat Organization said that our design saved a lot of time and should be widely utilized in future TNR programs.

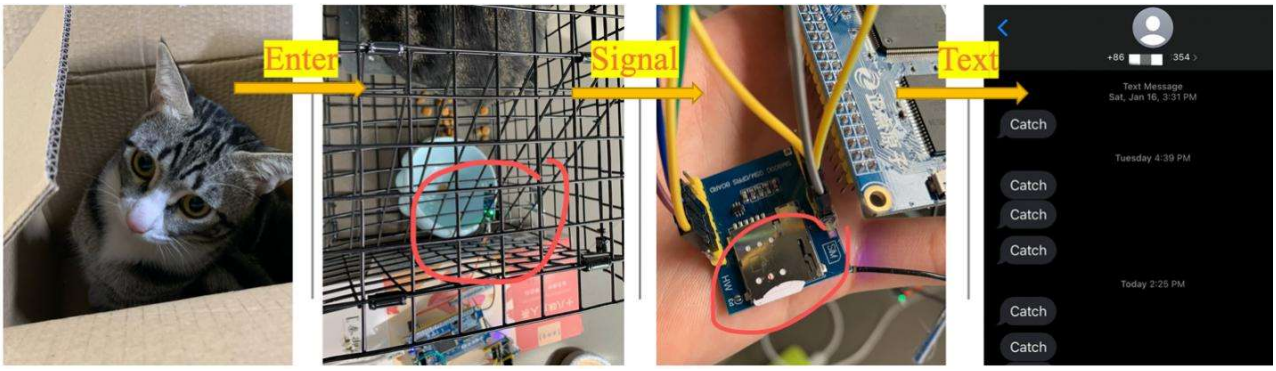


Fig. 10 Steps of Catnip Electronic Sensor Trap. Zima smelt something special. He approached the odor and was attracted by the liquid extract and food in the trap. He was very timid and vigilant: he observed and roamed around the trap for about 2 minutes. However, he could not resist the temptation and entered the Catnip Electronic Sensor Trap. After he activated the closing of the trap's entrance, a text message saying "Catch" was received on my phone. Catnip Electronic Sensor Trap qualified our expectations.

3. Discussion

3.1 Production of Nepetalactone

The overall goal of our experiments was to produce nepetalactone on engineered *E. coli* BL21 (DE3) strain. We first catalyzed geraniol to produce the intermediate nepetalactol, followed by conversion of nepetalactol to our target nepetalactone and fermentation. The MVA pathway, three cytochrome P450 genes and their related enzyme, and two nepetalactol-related short-chain reductases/ dehydrogenases were key participants in the catalysis process.

To detect production of nepetalactone, we conducted two sets of gas chromatology tests. Our fermented samples and two other control samples revealed a difference in peak retention times. We composed two hypotheses to explain the difference. The first hypothesis was that our fermented samples produced nepetalactones that shared different stereoisomers with the standard nepetalactone. It was identified that nepetalactones are stereoisomers, indicating that different structures may result in different peak retention times. Based on our Standard Curve, we plugged peak areas of our fermented samples into the equation and calculated their corresponding concentrations of nepetalactones. Fermented sample pET28a-CrG8H-AtCPR-CrGOR+pRG-ISY-NESP1-NESP3A/ BL21 was expected to have a peak area of 338.15 and contain nepetalactones at a concentration of 2.5mg/L; fermented sample pRG-ISY-NESP1-NESP3A+pET28a-NcG8H-AtCPR-NcGOR/BL21 was expected to have a peak area of 352.56 and contain nepetalactones at a concentration of 2.6mg/L. These concentration levels were lower than what we expected, but productivity may be improved if

using other nepetalactone-related genes. The second hypothesis was that the fermented samples were not nepetalactones; they might still be the intermediates nepetalactols or even products of a new metabolic substance. Future research should aim to establish a more sophisticated, stable metabolic pathway to produce nepetalactone in *E. coli* and increase its productivity and efficiency. We suggested future researchers to introduce other nepetalactone-related genes into *E. coli* or construct the metabolic pathway in a new bacterial host.

3.2 Application of Catnip Electronic Sensor Trap

Supported by the result of our mock test, Catnip Electronic Sensor Trap was feasible and effective. We, thus, recommended conducting our trap in TNR programs. SMS message notifications would be very helpful to voluntary conductors who cannot be always around traps. On average, it took the sensor 5 seconds to send a text message when a cat enters the trap. When text message is received, conductors can pick the trapped cat up and take him/her to registered animal hospital. Catnip Electronic Sensor Trap benefits both the voluntary conductors and trapped free-roaming cats: it enables more TNR practices to be conducted in a given time period and minimizes frequency of cat overreacting and exhibiting stress responses.

4. Materials and Methods

4.1 Construction pET28a-CrG8H-AtCPR-CrGOR and pET28a-NcG8H-AtCPR-NcGOR

Since geraniol could produce nepetalactol, related genes geraniol 8-hydroxylase (G8H), P450 reductase enzyme (CPR), and geraniol oxidoreductase (GOR) were introduced into our engineered strain; this process was achieved by the following steps. Firstly, protein sequences of genes CrG8H, CrGOR (from *Catharanthus roseus*), NcG8H, NcGOR (from *Nepeta cataria*), and AtCPR (from *Arabidopsis thaliana*) were procured from NCBI. Codons in *E. coli* BL21 (DE3) were optimized by using an online codon optimizer tool. Plasmid profiles of genes AtCPR-pET21a, CrG8H-pET28, CrGOR-pET28a, NcG8H-pET28a, and NcGOR-pET28a were drawn on SnapGene (Fig. 11).

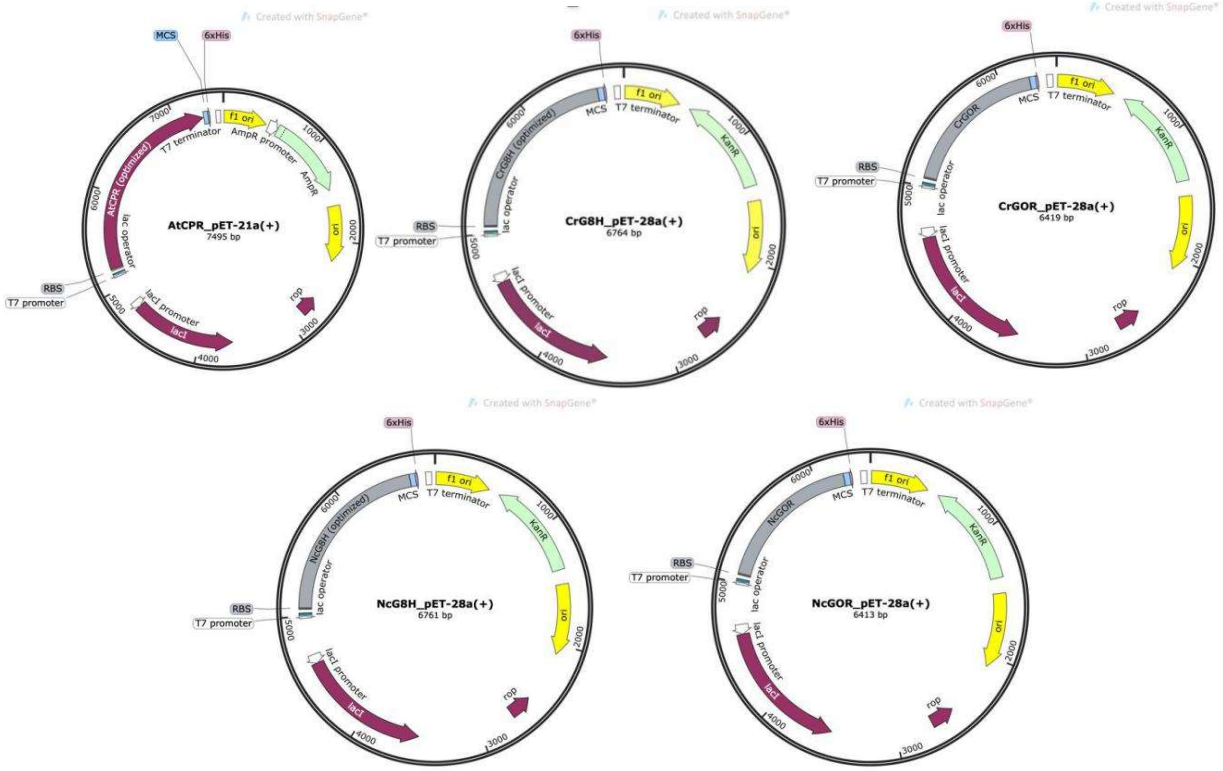
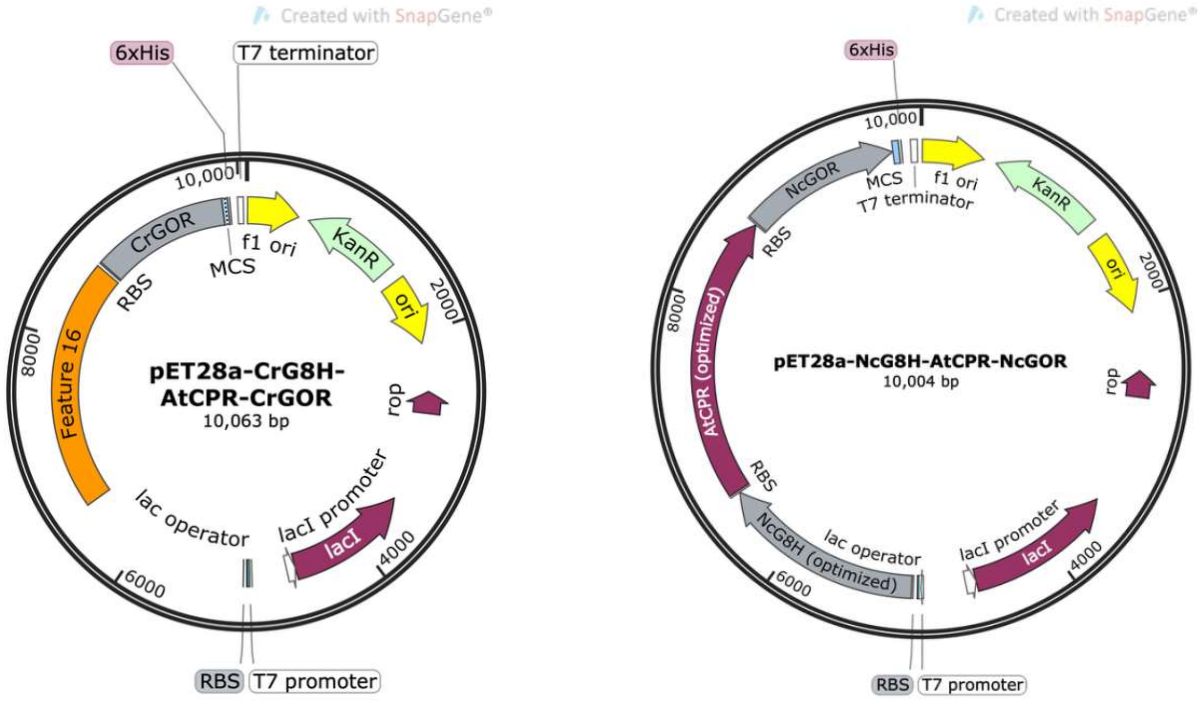


Fig. 11 Plasmid Maps of AtCPR-pET21a, CrG8H-pET28, CrGOR-pET28a, NcG8H-pET28a, and NcGOR-pET28a. Those graphics were generated by SnapGene.

Table 2. DNA Sequences of Introduced Genes

Genes	DNA Sequences
AtCPR-F	CTGTTCCGAGCACGCTTTAAACTAGAAATAATTTTGTTTAAC
AtCPR-R	CTCGAGTGCGGCCGCAAGCTTGTCGACGGAGCTCGAA
V-CrG8H-pET28a-R	TTAAACAAAATTATTTCTAGTTTAAAGCGTGCTCGGAACAG
V-CrG8H-pET28a-F	GAATTCGAGCTCCGTCGACAAGCTTGCGGCCGCACTCGAG
CrGOR-F	CCTGCGTGACGTTTGGTAAACTAGAAATAATTTTGTTTAAC
CrGOR-R	CTCGAGTGCGGCCGCAAGCTTGTCGACGGAGCTCGAATTC
AtCPR-F(Nc)	CAGTGCCGATTGCGTTGTAAAAGAAGGAGATATACATATGACATCAGC
AtCPR-R(Nc)	CCATGGTATATCTCCTTCTTTTACCAAACGTCACGCAGGTA
NcGOR-F	ACCTGCGTGACGTTTGGTAAAAGAAGGAGATATACCATGGCTG
NcGOR-R	AGCAGCCAACTCAGCTTCCTTTCGGGCTTTGTTAGCAGCC
V-NcG8H-pET28a-F	GGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGC
V-NcG8H-pET28a-R	CATATGTATATCTCCTTCTTTTACAACGCAATCGGCACT

The designed plasmids were sent to a commercial company which optimized their codons and completed synthesis. Gene fragments of synthesized V-Cr/NcG8H-pET28a-R/F and their corresponding genes AtCPR-R/F and Cr/NcGOR-R/F were amplified through a polymerase chain reaction (PCR). A gel electrophoresis was then conducted to test expression of the plasmids. After it was ensured that they were successfully expressed, A DNA purification and a Gibson Assembly were conducted to preserve and fully connect genes in the plasmids (Fig. 12), which were then transferred into DH5αcells of an experimental *E. coli* BL21 (DE3) strain. To detect presence of gene expressions in the cells, a PCR test was conducted; the expressed plasmids were sent to a commercial company to be sequenced and their carrier bacterial strains were mostly reserved in a -80°C refrigerator. Some expressed strains were cultured in cuvettes, and their plasmids were transferred into our expressed host *E. coli* BL21 (DE3). Another PCR test was conducted to detect presence of gene expression. Strains carried the expressed plasmids were cultures and reserved in a -80°C refrigerator.



(A) pET28a-NcG8H-AtCPR-NcGOR (B) pET28a-CrG8H-AtCPR-CrGOR

Fig. 12 Plasmid Profiles of pET28a-CrG8H-AtCPR-CrGOR and pET28a-NcG8H-AtCPR-NcGOR. (A) was the plasmid profile of pET28a-NcG8H-AtCPR-NcGOR presented by SnapGene. (B) was the plasmid profile of pET28a-NcG8H-AtCPR-CrGOR presented by SnapGene.

4.2 Synthesis of ISY-NEPS1-NEPS3A

DNA sequences of ISY (from *Catharanthus roseus*), NEPS1, and NEPS3A (from *Nepeta cataria*) were procured from NCBI; their codons were displayed and designed by using a codon OPTIMIZER. Plasmid profile of gene pRG-ISY-NEPS1-NEPS3A was drawn on SnapGene and sent to a commercial company to be optimized; it was then separately transferred into engineered strains pET28a-CrG8H-AtCPR-CrGOR/BL21 and pET28a-NcG8H-AtCPR-NcGOR\BL21. Construction of our ultimate engineered strain was completed.

4.3 Fermentation Experiment and Gas Chromatography

A fermentation experiment was applied to catalyze production of our target product – nepetalactone. Groups in the experiments were presented below.

Experimental Groups:

1.pET28a-CrG8H-AtCPR-CrGOR + pRG-ISY-NESP1-NESP3A/BL21

2.pRG-ISY-NESP1-NESP3A + pET28a-NcG8H-AtCPR-NcGOR/BL21

Note: "Cr" indicated that the engineered strain contained genes from *Catharanthus roseus*; "Nc" indicated that the genes in the strain were from *Nepeta cataria* source.

Control Groups:

1. *E. coli* BL21 (DE3) strain added with geraniol (plasmid-free)

2. Empty *E. coli* BL21 (DE3) strain (plasmid-free)

Note: The two control groups shared all other conditions but addition of geraniol.

After the fermentation experiment, two sets of gas chromatography were conducted to detect presence of nepetalactones.

5. Some Specific Methods

5.1 PCR Test

a. Prepare the components according to Table 2. (Note: Table 3 is conducted in a scenario in which the total volume is 50 μL.)

Table 3. Materials for PCR Test

Components	Volume (μL)	Final Concentration
Template	-	<1,000 ng
Forward Primer	1	0.2 μM
Reverse Primer	1	0.2 μM
5×TransStart FastPfu Buffer	10	1×
2.5 mM dNTPs	4	0.2 mM
TransStart FastPfu DNA polymerase	1	2.5 units
ddH_2O (sterilized)	32	-
Total Volume	50	-

Table 4. PCR Test Program

Steps	Temperature (℃)	Time	Cycle (#)
Initial Denaturation	95	2 min	1
Denaturation	95	20 sec	25-30
Renaturation	56	20 sec	
Extension	72	4 kb/min (targets ≤ 1 kb) 2-4 kb/min (targets >1 kb)	
Final Extension	72	5 min	1
Hold	12	∞	-

b. Gently and fully mix the system. Centrifugation can be applied to ensure the system is concentrated at the bottom of the tube.

c. Follow and apply the PCR test program according to Table 4.

5.2 Gibson Assembly

a. Prepare and label a PCR tube that already contained 5 μL of Gibson Assembly Mix.

b. According to Table 5, add corresponding amounts of Backbone, Fragments, ddH_2O to the system.

Table 5. Gibson Assembly System

Components	Volume (μL)
V-Cr/NcG8H-pET28a-R/F	1
AtCPR-R/F	2
Cr/NcGOR-R/F	2
Gibson Assembly Mix	5
ddH_2O (sterilized)	10

c. Gently and fully mix the system. Centrifugation can be applied to ensure the system is concentrated at the bottom of the tube.

d. Follow and apply the Gibson program presented in Table 6.

Table 6. Gibson Program

Step	Temperature	Time	Cycle
Digestion and Ligation	50 ℃	15 min	1 cycle
Hold	12 ℃	∞	-

5.3 Nepetalactone Extract

a. Prepare a mother solution which contain 200 mg/mL of nepetalactone liquid extract and a isopropyl myristate solution.

b. Take 5.682 μL of the mother solution. Add isopropyl myristate to achieve a volume of 5 mL. Label the final solution as Solution 1.

c. Take 3 mL of Solution 1 and add 3 mL of isopropyl myristate into it. Label the final solution as Solution 2.

d. Take 2.25 mL of Solution 2 and add 0.75 mL of isopropyl myristate into it. Label the final solution as Solution 3.

e. Take 1.5 mL of Solution 3 and add 0.5 mL of isopropyl myristate into it. Label the final solution as Solution 4.

f. Take 1 mL of Solution 4 and add 1 mL of isopropyl myristate into it. Label the final solution as Solution 5.

g. Take 0.5 mL of Solution 5 and add 2 mL of isopropyl myristate into it. Label the final solution as Solution 6.

Table 7. Concentrations of Solutions

Solution #	1	2	3	4	5	6
Concentration of Solution (mg/mL)	200	100	75	50	25	10

h. Filtrate the solutions and prepare them into GC tubes.

i. Conduct a gas chromatography to all solutions.

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