CO-EXPRESSION OF FEEDBACK RESISTANT ENZYMES IN PHENYLALANINE BIOSYNTHESIS PATHWAY TO INCREASE PHENYLALANINE PRODUCTION



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	PHENYLALANINE PRODUCTION
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ชรินทร์ทิพย์ เย็นยุวดี : การแสดงออกร่วมของเอนไซม์ที่ต้านทานการเกิดการยับยั้งแบบ ย้อนกลับในวิถีการสังเคราะห์ฟีนิลอะลานีนเพื่อเพิ่มการผลิตฟีนิลอะลานีน. (CO-EXPRESSION OF FEEDBACK RESISTANT ENZYMES IN PHENYLALANINE BIOSYNTHESIS PATHWAY TO INCREASE PHENYLALANINE PRODUCTION) อ.ที่ ปรึกษาหลัก : รศ. ดร.กนกทิพย์ ภักดีบำรุง

แอล-ฟีนิลอะลานีน (L-Phe) เป็นกรดอะมิโนจำเป็นที่มีความสำคัญเชิงพาณิชย์ที่ถูกนำมาใช้อย่าง แพร่หลายในอตสาหกรรมอาหารและยา ในปัจจบันมีความต้องการของแอล-ฟีนิลอะลานีนสงขึ้นตามความ ต้องการในการผลิตแอสปาแตมซึ่งเป็นสารให้ความหวานแทนน้ำตาลที่มีแคลอรี่ต่ำ ใน Escherichia coli การ สังเคราะห์แอล-ฟีนิลอะลานีนถูกควบคุมหลายลำดับขั้น ไอโซฟอร์ม AroG ของ 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase (DAHP synthase) และ chorismate mutase/prephenate dehydratase (PheA) เป็นเอนไซม์ที่สำคัญ 2 ชนิด ที่ถูกยับยั้งแบบย้อนกลับได้โดย L-Phe การแสดงออกร่วม ของ pheA ที่ต่อต้านการยับยั้งแบบย้อนกลับ (pheA^{L359D}) กับยืนอื่น ๆ ที่สำคัญในวิถีการสังเคราะห์ L-Phe ได้แก่ *aroB, aroL, phedh, tktA, aroG, pheA, yddG* และ *glpF* ใน pRSFDuet-1 (pPTFBLYA^{L359D}) สามารถ เพิ่มการผลิต L-Phe ได้เป็น 3.78 เท่า เมื่อเทียบกับโคลนที่มี pheA^{wt} (pPTFBLYA^{wt}) ในงานวิจัยนี้ยืน aroG^{wt} และ aroG ที่ต้านทานการควบคมแบบย้อนกลับ (aroG^{L175D}, aroG^{Q151L}, aroG^{Q151A} และ aroG^{Q151N}) ได้ถก โคลนเข้า pRSFDuet-1 แล้วทรานส์ฟอร์มเข้า *E. coli* BL21(DE3) พบว่าโคลน AroG^{0151N} มีแอคติวิตีจำเพาะของ DAHP synthase สูงสุดในภาวะที่มี L-Phe 20 มิลลิโมลาร์ ดังนั้นจึงได้ทำการสร้างโคลนของ E. coli BL21(DE3) ที่มี pBLPTA^{L359D}G^{wt} & pYF และ pBLPTA^{L359D}G^{Q151N} & pYF แล้วทำการตรวจวัดการผลิต L-Phe ใน minimum medium ที่มีกลีเซอรอลร้อยละ 6 เทียบกับโคลนที่มี pBLPT & pYF พบว่าโคลน pBLPTA^{L359D}G^{0151N} & pYF มีการผลิต L-Phe สูงขึ้น 8.7 เท่า และโคลนที่มี AroG^{0151N} ผลิต L-Phe ได้มากกว่า โคลนที่มี Arog^{wt} 1.2 เท่า จุฬาลงกรณั้มหาวิทยาลัย

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PHENYLALANINE BIOSYNTHESIS PATHWAY TO INCREASE PHENYLALANINE PRODUCTION. Advisor: Assoc. Prof. KANOKTIP PACKDIBAMRUNG, Ph.D.

L-Phenylalanine (L-Phe) is an important commercial amino acid. It is widely used in food and pharmaceutical industries. Currently, the requirement of L-Phe is increased according to the great demand for the low-calorie sweetener, aspartame. In Escherichia coli, the synthesis of L-Phe is controlled by the multi-hierarchical regulations. AroG isoform of 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAHP synthase) and chorismate mutase/prephenate dehydratase (PheA), two important enzymes, are feedback inhibited by L-Phe. Co-expression of feedback-resistant pheA (pheA^{L359D}) with other pivotal genes in L-Phe biosynthesis pathway: aroB, aroL, phedh, tktA, aroG, pheA, yddG, and glpF in pRSFDuet-1 (pPTFBLYA^{L359D}) elevated L-Phe production of *E. coli* BL21(DE3) 3.78 fold in comparison to that of wildtype (pPTFBLYA^{wt}). In this research, wildtype *aroG* (*aroG^{wt}*) and feedback resistant *aroG* genes (aroG^{L175D}, aroG^{Q151L}, aroG^{Q151A} and aroG^{Q151N}) were cloned into pRSFDuet-1 and then transformed into *E. coli* BL21(DE3). AroG^{Q151N} clone gave the highest specific activity of DAHP synthase in the presence of 20 mM L-Phe. Therefore, E. coli BL21(DE3) containing pBLPTA^{L359D}G^{wt} & pYF and pBLPTA^{L359D}G^{Q151N} & pYF were constructed and their production of L-Phe in 6% glycerol medium were determined in comparison to pBLPT & pYF clone. The presence of PheA^{L359D} and AroG^{Q151N} elevated L-Phe production 8.7 fold while the clone containing AroG^{Q151N} produced L-Phe 1.2 fold higher than AroG^{wt} clone.

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LIST OF ABBREVIATIONS

Ala	alanine
aroB	3-dehydroquinate synthase gene
AroG	L-phenylalanine sensitive isoform of 3-deoxy-D-arabinoheptulosonate- 7-phosphate synthase
AroG ^{fbr}	L-phenylalanine feedback resistant AroG
aroG	L-phenylalanine sensitive isoform of 3-deoxy-D-arabinoheptulosonate- 7-phosphate synthase gene
aroG ^{fbr}	L-phenylalanine-feedback resistant 3-deoxy-D-arabinoheptulosonate- 7-phosphate synthase gene
aroL	shikimate kinase II gene
Asn	asparagine
Asp	aspartate
bp	base pairs
BSA	bovine serum albumin
°C	degree celsius
CMPD	chorismate mutase/ prephenate dehydratase
DAHP	3-deoxy-D-arabinoheptulosonate-7-phosphate synthase
DNA	deoxyribonucleic acid
dNTP	2'-deoxynucleoside 5'-triphosphate
EDTA	ethylene diamine tetraacetic acid
E4P	erythrose 4-phosphate

fbr	feedback resistant
glpF	glycerol facilitator gene
Gln	glutamine
h	hour
HPLC	high-performance liquid chromatography
IPTG	isopropyl-β-D-thiogalactoside
kb	kilobase pairs
kDa	kilodalton
L	liter
L-Phe	L-phenylalanine
L-Trp	L-tryptophan
L-Tyr	L-tyrosine
LB	Luria-Bertani
Leu	leusineจุฬาลงกรณ์มหาวิทยาลัย
μ_{g}	CHULALONGKORN UNIVERSIT
μL	microliter
μM	micromolar
Μ	mole per liter (molar)
mA	milliampere
mg	milligram
min	minute

mL	milliliter
mМ	millimolar
MW	molecular weight
ng	nanogram
nm	nanometer
OD	optical density
PCR	polymerase chain reaction
PEP	phosphoenolpyruvate
PheA	chorismate mutase/ prephenate dehydratase
pheA	chorismate mutase/ prephenate dehydratase gene
pheA ^{fbr}	L-phenylalanine feedback resistant chorismate mutase/ prephenate
	dehydratase gene
phedh	phenylalanine dehydrogenase gene
RNase	ribonuclease
rpm	revolution per minute ORN UNIVERSITY
S	second
tktA	transketolase gene
Tm	melting temperature
U	unit (µ mol/min)
UV	ultraviolet
Val	valine

v/v volume by volume

wt wildtype

yddG aromatic amino acid exporter gene



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CHAPTER I

Introductions

1.1 Aromatic amino acid

Aromatic amino acids are amino acids that have an aromatic ring in the side chain. Among the 20 standard amino acids, the following are aromatic: L-tryptophan (L-Trp), L-phenylalanine (L-Phe) and L-tyrosine (L-Tyr). Aromatic amino acids are the building blocks of proteins. Normally, they constitute less than 10% of the protein. Ltryptophan is the largest and the rarest of the amino acids in proteins [1].

The aromatic amino acids are produced from the shikimate pathway which is found in bacteria, fungi, plants, and some protists. In the three aromatic amino acids metabolism, L-Phe has the highest carbon flux because up to 30% of organic matter constitutes from L-Phe-derived compounds. Plant and microbe can synthesize their own aromatic amino acids to make proteins. In plants, the aromatic amino acids also use as precursors of plant natural products that play a key role in plant growth, development, reproduction, defense and environmental responses. L-Trp is used as a precursor of alkaloids, phytoalexins, indole glucosinolates and auxin. L-Tyr is used as a precursor of isoquinoline alkaloids, pigment betalains and quinones while L-Phe is also used as a precursor of numerous phenolic compounds including flavonoids, tannins, lignans, lignin, and phenylpropanoid/benzenoid volatiles (Figure 1) [2]. For animals, L-Phe and L-Trp are essential amino acids. However, animals have lost the metabolic pathway for aromatic amino acid. They must derive these amino acids through their food and L-Phe can be converted to L-Tyr by Phe hydroxylase. In animals and humans, the aromatic amino acids are used as precursors for the synthesis of biologically and neurologically active compounds that are essential for maintaining biological functions. Nowadays, the aromatic amino acids are important examples of chemical products that can be produced by renewable raw material in microorganisms such as glucose or glycerol [3].



Figure 1. The aromatic amino acid pathways support the formation of numerous natural products in plants. The shikimate pathway (shown in green) produces chorismate, a common precursor for the Trp pathway (blue), the Phe/Tyr pathways (red), and the pathways leading to folate, phylloquinone, and salicylate. Trp, Phe, and Tyr are further converted to a diverse array of plant natural products that play crucial roles in plant physiology, some of which are essential nutrients in human diets (bold). Abbreviations: ADCS, aminodeoxychorismate synthase; AS, anthranilate synthase; CM, chorismate mutase; CoA, coenzyme A; ICS, isochorismate synthase [2].

1.2 L-Phenylalanine

L-Phenylalanine ($C_9H_{11}NO_2$) is an essential hydrophobic aromatic amino acid. It has a benzyl group as a side chain as shown in Figure 2. It is classified as nonpolar and neutral because of the hydrophobic nature and inert of the side chain. L-Phe is also one of the most important commercial aromatic amino acid for humans and animals [4, 5] . It is provided from diet such as meat, cottage cheese, and wheat germ. In human brain, L-Phe is converted to L-Tyr which is used as a precursor to produce catecholamines such as tyramine, dopamine, epinephrine, and norepinephrine. The neurotransmitter catecholamines act like adrenalin substances that transmit signals between nerve cells and the brain for keeping us awake and alert, reduce hunger pains, function as an antidepressant and help improvement of memory [6-8].

In pharmaceutical industries, L-Phe is used for chemical synthesis of pharmaceutically active compounds like HIV protease inhibitor, anti-inflammatory drugs [9], phenylethylamine, catecholamines [10] and combination with UVA therapy for the treatment of vitiligo [11]. Moreover, L-Phe also used in several psychotropic drugs (mescaline, morphine, codeine, and papaverine). In food industries, L-Phe is used as a supplementary food and a precursor for low calorie sweetener, aspartame (L-aspartyl-L-phenylalanine methyl ester) which is 160–180 times sweeter than sucrose [12]. Currently, the requirement of L-Phe is increased according to the great demand for the low-calorie sweetener, aspartame which approximate the world market of US \$1.5 billion.



Figure 2. Structure of L-phenylalanine [7].

1.3 L-Phenylalanine production

The L-Phe can be produced by chemical synthesis and bioprocessing such as enzymatic transformation or microbial process [13]. In early industrial process of L-Phe, it was mainly produced by chemical process. However, the chemical synthesis of L-Phe has many disadvantages for example, chemical synthesis uses toxic raw materials that are nonrenewable and generate racemic mixtures of D and L Phe isomers which make it difficult for the purification processes. Furthermore, the process has a high cost and various problems. Therefore, the L-Phe biosynthesis is an attractive alternative since it is a clean technology and uses renewable simple carbohydrates that generates less environmental pollution [14, 15] . More recently, metabolic engineering in *Escherichia coli* (*E. coli*) has been focused because the main metabolisms of *E. coli* have enabled the introduction of such genetic modifications [16]. Furthermore, researchers can use a genetic engineering technique to enhance L-Phe yield [17].

1.4 L-Phenylalanine biosynthesis pathway in E. coli

When *E. coli* cell was cultured in medium containing carbon sources such as glucose or glycerol, the carbon sources can be converted to phosphoenolpyruvate (PEP) by glycolysis pathway and erythrose 4-phosphate (E4P) by pentose phosphate pathway. The biosynthesis of aromatic amino acids is started from the condensation of PEP and E4P to D-arabinoheptulosonate7-phosphate (DAHP) by DAHP synthase (EC 2.5.1.54). This step is tightly regulated by its final product, phenylalanine, tyrosine and tryptophan to convert the carbon flow into the shikimate pathway. There are three isozymes of DAHP synthase encoded by *aroF* (L-Tyr-sensitive), *aroH* (L-Trp-sensitive) and *aroG* (L-Phe-sensitive) [18-20].

In the second step of the pathway (shikimate pathway), there are 2 rate limiting enzymes including 3-dehydroquinate (DHQ) synthase encoded by *aroB* and

shikimate kinase II, which is encoded by *aroL*. At chorismite (CHA), the pathways of each aromatic amino acid are separated. To produce L-Phe, chorismate is converted to phenyl pyruvate (PPA) and then PPA is changed to phenylpyruvate (PPY) by bifunctional enzyme chorismate mutase/prephenate dehydratase (CMPD) encoded by *pheA*. This enzyme is feedback regulated by L-Phe. Finally, PPY is converted to L-Phe by amino transferase. The excess L-Phe is excreted by aromatic amino acid exporter encoded by *yddG*. The aromatic amino acid biosynthesis pathway is shown in Figure 3 [21].



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Figure 3. Pathway of aromatic amino acid biosynthesis and its regulation in *E. coli*. To indicate the type of regulation, different types of lines are used: – – –, transcriptional and allosteric control exerted by the aromatic amino acid end products; · · · , allosteric control only; —, transcriptional control only. Abbreviations used: ANTA, anthranilate; aKG, a-ketoglutarate; CDRP, 1-(o-carboxyphenylamino)-1-deoxyribulose 5-phosphate; CHA, chorismate; DAHP, 3-deoxy-d-arobino-heptulosonate 7-phosphate; DHQ, 3-dehydroquinate; DHS, 3-dehydroshikimate; EPSP, 5 enolpyruvoylshikimate 3-phosphate; E4P, erythrose 4-phosphate; GA3P, glyceraldehyde 3-phosphate; HPP, 4-hydroxyphenlypyruvate, I3GP, indole 3-glycerolphosphate; IND, indole; L-Gln, L-glutamine; L-Glu, L-glutamate; L-Phe, L-phenylalanine; L-Ser, L-serine; L-Trp, L-tryptophan; L-Tyr, L-tyrosine; PEP, phosphoenolpyruvate; PPA, prephenate; PPY, phenylpyruvate; PRAA, phosphoribosyl anthranilate; PRPP, 5-phosphate [21].

1.5 L-Phe sensitive DAHP synthase

In *E. coli*, the three isozymes of DAHP synthase are L-Phe sensitive (AroG), L-Tyr sensitive (AroF) and L-Trp sensitive (AroH) which are encoded by *aroG*, *aroF* and *aroH*, respectively [22]. About 80% of total DHAPS activity is made up by AroG while AroF and AroH share 20% and 1% of total activity, respectively [20]. The structure of AroG is a homotetramer whereas AroH and AroF are homodimer. Specific activities of AroG varied widely with different metal ions as follows; $Mn^{2+} > Cd^{2+}$, $Fe^{2+} > Co^{2+} >$ Ni²⁺, Cu^{2+} , $Zn^{2+} > Ca^{2+}$. Moreover, metal variation significantly affectes the apparent affinity for the substrate, E4P, but not for the second substrate, PEP or for feedback inhibition, L-Phe [23].

The first reported 3D structure of DAHP synthase is the crystal structure of AroG complexed with PEP and Pb²⁺ [24]. The tetramer consists of two tight dimers. The monomers of the tight dimer are coupled by interactions including a pair of three stranded intersubunit β -sheets. The monomer is a (β/α)₈ barrel with several additional β strands and α helices. The PEP and Pb²⁺ are at the C-ends of the β strands of the barrel. Mutations that reduce feedback inhibition cluster 15 Å from the active site, indicating the location of a separate regulatory site (Figure 4).

In 2002, crystal structure of AroG in complex with it inhibitor, L-Phe, PEP and metal ion cofactor, Mn²⁺ was determined to 2.8 Å resolution [25]. L-Phe binds in a cavity formed residues of two adjacent subunits and is located about 20 Å from the closet active site. The mechanism of allosteric mechanism was derived from conformational difference between Phe-bond and Phe-free structures. The inhibitory signal is transmitted from Phe-binding site to the active site of AroG by two interrelated paths. The first path is transmission within a single subunit of clone segments of the protein. The second involves alternative in the contact between subunits. On binding of Phe, AroG loses binding ability to E4P and binds PEP in a flipped orientation.







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Hu and coworker (2005) investigated the feedback inhibition site of AroG using 3D structure of AroG co-crystallized with PEP. Phe141, Leu175, Leu179, Phe209, Trp215 and Val221 was replaced by using site-directed mutagenesis. The DAHP synthase activity in the presence of L-Phe from 0 mM to 3 mM showed that L175D was mostly resistant to feedback inhibition. The specific enzymatic activity of L175D at 0 mM of L-Phe increased significantly about 4.46 U/mg when compared with that of wild-type AroG and the relative enzymatic activity remained at 1 mM of L-Phe is about 83.5% [26].

Kikuchi and colleagues also studied the Phe-binding site of AroG. They performed hydroxamine mutagenesis on *aroG*. The mutant A220T, D146N and M147I were partially resistant to Phe inhibition [22]. From this result, Ding and coworker integrated different combinations of two mutation sites into the *aroG*, generating three double-site mutants. The *E. coli* clone possessing AroG^{A202T/D146N} showed the highest enzymatic activity and greatest resistant to feedback inhibition. The relative enzymatic activity of AroG^{A202T/D146N} remained at 20 mM of L-Phe was 96.66% [27].



1.6 Chorismite mutase and prephenate dehydratase (CM-PDT)

The chorismite mutase (CM) and prephenate dehydratase (PDT) (EC 5.4.99.5/4.2.1.51) is one of the allosteric enzymes in the aromatic amino acid biosynthesis pathway. CM-PDT which is encoded by *pheA* catalyzes the second committed step of L-Phe biosynthesis. It can be feedback inhibited by L-Phe. CM-PDT contains 386 amino acids with a molecular mass of 43 kDa. It is a bifunctional enzyme which contains two catalytic domains chorismite mutase domain (residues 1-109) and prephenate dehydratase domain (residues 101-285) and one regulatory-domain (residues 286-386) for L-Phe inhibitor binding site [28-30]. The feedback inhibition regulation of CM-PDT is mediated through allosteric binding of L-Phe which contributes a shift in the aggregation state of the enzyme from an active dimer to

less active tetrameric and octameric species [29]. Inhibition of the prephenate dehydratase activity at 1mM of L-Phe showed that almost total activity was inhibited (85% inhibition). In contrast, chorismate mutase activity was inhibited only 55% [31]. Overexpression of the CM-PDT domain of PheA could improve the metabolic influx to overproduce L-Phe and improve the survival ability under *m*-fluoro-DL-phenylalanine (an analog of Phe) stress [32].

To investigate L-Phe binding site, Nelms and coworker (1992) constructed four mutants which located within codons 304 to 310 of the *pheA* and measured the enzyme activity at various the L-Phe concentrations. They suggested that the recombinant *E. coli* harboring *pheA*^{W339P} displayed almost complete resistance to feedback inhibition of prephenate dehydratase by L-Phe concentrations up to 200 mM [29].

1.7 Glycerol as a carbon source

Biofuels, such as ethanol and biodiesel, are among the most promising source for the substitution of fossil. About 10% (w/w) glycerol as a main by-product is generated in biodiesel production. The excess glycerol may become an environment problem. The market price of crude glycerol is low with the price of US\$ 0.13-0.24 per kilogram [33]. Therefore, glycerol has been considered as a feedstock for new industrial fermentations [34]. Compared to the conventionally fermentation used glucose and sucrose, glycerol is efficient low-cost carbon source. The initial step of glycerol utilization in *E. coli* is the uptake of glycerol molecule into the cytoplasm via protein-mediated glycerol facilitator (GlpF) encoded by *glpF*. Glycerol is trapped by an ATP-dependent glycerol kinase (GlpK) to yield glycerol- 3-phosphate (G3P) which is then oxidized by a membrane-bound ubiquinone-8(UQ8)-dependent G3P dehydrogenase (GlpD) to dihydroxyacetone phosphate (DHAP) that enters glycolysis [35-37].

1.8 Our previous works

Thongchuang (2011) cloned *glpF*, *tktA*, *aroG*, *aroB*, *aroL*, *yddG* from *E. coli* and phenylalanine dehydrogenase gene (*phedh*) from *Bacillus lentus*. Each gene was regulated under T7 promoter in pRSFduet-1. The clone harboring pPTFBLY which contained *phedh*, *tktA*, *glpF*, *aroB*, *aroL* and *yddG* produced high level of L-Phe about 429 mg/L when it was cultured in minimum medium containing 3% glycerol for 240 h. The disadvantage of this clone were slowly growth rate and the colonies changed into flat shape when it was grown on the agar plate because of overexpression of membrane proteins under the regulation of T7 promoter [38]. To solve the problem, Ratchaneeladdajit (2014) applied the dual plasmid system. *phedh*, *tktA*, *aroB*, and *aroL* under T7 promoter was cloned into pRSFduet-1 (pBLPT) while *glpF* and *yddG* were expressed under ara promoter of pBad-33 (pYF). The ara promoter is tight regulated promoter and it uses arabinose as an inducer. After that, these two plasmids were co-transformed into *E. coli* BL21(DE3). The high level of L-Phe production at 746 mg/L was found when the medium was optimized (3.1% glycerol) [39]

Naksusuk (2015) improved the production of L-Phe by overexpression of phenylalanine feedback resistant PheA (PheA^{fbr}). Five Leu/Met residues in L-Phe binding pocket were selected for site-directed mutagenesis. The *pheA^{fbr}* was co-expressed with *phedh*, *tktA*, *aroB*, *aroL*, *glpF* and *yddG* in pRSFDuet-1. Among these mutated clones, pPTFBLYA^{L359D} produced the highest concentration of L-Phe at 135 mg/L which was 3.8 fold of that of wildtype PheA clone (pPTFBLYA^{wt}) [40] when the clones were cultured in glycerol medium formulated for pBLPT & pYF clone. All genes that were overexpressed in *E. coli* BL21(DE3) for L-Phe production are shown in Figure 5.

Kanoksinwuttipong analysed the amino acid residues that interact with L-Phe in the regulatory site of AroG using the crystal structure of AroG complex with Mn²⁺, PEP and Phe (code 1KFL in protein Data Bank). The structure was displayed by UCSF chimera program (Figure 6). Q151 was selected for substitution by Leu, Ala and Asn. The these mutated genes were cloned into pRSFDuet-1 and transformed into *E. coli* BL21(DE3) on phenylalanine feedback inhibition L175D was used as a control [41].

Therefore, the objectives of this research are

- 1. To determine the expression of PheA and AroG.
- 2. To co-express *pheA^{L359D}* and *aroG^{fbr}* with *phedh*, *tktA*, *aroB*, *aroL*, *glpF* and *yddG* using dual plasmid system of pRSFDuet-1 and pBad33 in order to produce high quantity of L-Phe.





Figure 5. All important genes in this study. Abbreviations used: F6P, fructose 6-phosphate; G3P, glycerol 3-phosphate; GA3P, glyceraldehyde 3-phosphate; *glpF*, glycerol facilitator; *phedh*, phenylalanine dehydrogenase, *yddG*, aromatic amino acid exporter

Source: modified from Bongaert, et al 2001 [21]



Figure 6. The 3D structure of AroG co-crystallized with inhibitor L-Phe [41].



CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipments

Autoclave: MLS-3020, SANYO Electric Co, Ltd., Japan

Autopipette: Pipetman, Gilson, France

Analytical Balance: AB135-S/FACT, Mettler Toledo, Germany

Balance: GB1501-S, Mettler Toledo, Germany

Benchtop centrifuge: SorvallBiofuge Primo, Kendro Laboratory Products L.P., USA Centrifuge, refrigerated centrifuge: Sorvall Legend XTR, Thermo Scientific, USA

Centrifuge, microcentrifuge: Microfuge 22R, Beckman Instrument Inc., USA

Dry bath incubator: MD-01N, Major Science, USA

Electrophoresis unit: Minis-150, Major Science, USA

Electroporator: MicroPulserTM electrophoretor, Bio-Rad Laboratories Inc., USA Electroporation cuvette: Gene Pulser[®]/E. coli Pulser[®] Cuvettes, Bio-Rad, USA Gel documentation instrument: BioDoc-ItTm Imaging system, UVP, USA High Performance Liquid Chromatography (HPLC): UFLC, Shimadzu, Japan HPLC column: Chirex[®] Chiral 3126 (D)-penicillamine, Phenomenex, USA Incubator Shaker: Model E24R, New Brunswick Scientific, USA Incubator Shaker: InnovaTM4000, New Brunwick Scientific, USA Incubator oven: Series04067, Contherm Scientific., Ltd., New Zewland Laminar flow: HT123, ISSCO, USA Magnetic stirrer: Model Cerastir CH-1 series, Nickel-electro., Ltd., UK

Membrane filter: 0.45 μ m Nylon Membrane Disc, Gs-Tek, USA

Microcentrifuge tube: 1.5 mL, Nest biotechnology, China

Microwave oven: GX-2021M, Galaxy, Korea

Mini personal centrifuge: Model microONE, Tomy Digital Biology Co., Ltd., Japan

PCR tube: thin-well dome-cap PCR tube, MCT-150, Axygen Inc., USA

pH meter: Model S200, Mettler ToledoCo., Ltd., Switzerland

Pipette: Labwarehouse, New Zealand

Pipette tip: Axygen Inc., USA

Sonicator: Vibra cellTm, SONICS & MATERIALS, Inc., USA

Spectrophotometer: BioSpectrometer[®] kinetic, Eppendorf, Germany

Syringe: 3 mL, 5 mL, 10mL, 20 mL latex free disposable syringe, Nipro Co., Ltd., Thailand

Syringe membrane filter: 0.2 μ m Supor[®] Membrane Acrodisc[®], PALL, USA

Thermal Cycler: T100[™], Bio-Rad, USA

UV Transilluminator: MacroVueTM UV-25, Hoefer Inc., USA

Vacuum pump: Model number. WP6111560, Millipore Inc., USA

Vortex mixer: TopMix FP15024, Fisher Scientific, USA

2.1.2 Chemicals

Acrylamide: Sigma, USA

Agar: Himedia Laboratories Pvt. Ltd., India

Agarose: SERVA Electrophoresis GmbH, Germany

Agarose: ISC BioExpress, USA

Aluminium sulfate: Univar, USA

Ammonium sulphate: Carlo Erba Reagents, Italy

Boric acid: Merck, Germany

Bovine serum albumin: Sigma, USA

Bromophenol blue: Merck, Germany

Calcium chloride: Scharlau Chemie S.A., Spain

Cobalt sulphate: Sigma, USA

Copper sulfate: Carlo Erba, Italy

Coomassie brilliant blue R-250: Sigma, USA

D-Erythrose 4-phosphate sodium salt: Sigma, USA

Di-sodium hydrogen arsenate: Sigma, USA

6X DNA Loading Dye: Thermo Fisher Scientific Inc., USA

dNTP: Biotechrabbit, Germany

Ethanol (Absolute): RCI Labscan Limited, Thailand

Ethylene diaminetetraacetic acid di-sodium salt (EDTA): Scharlau Chemie S.A., Spain

Glacial acetic acid: Carlo ErbaReagenti, Italy
Glycerol: Analytical Univar Reagent, Ajax finechem, Australia

Glycine: BDH, England

Hydrochloric acid: Carlo ErbaReagenti, Italy

Isopropylthio-β-D-galactosidase (IPTG): Serva, Germany

Iron (II) sulfate heptahydrate: Sigma, USA

β- mercaptoethanol: Acros Organics, USA

Maganese sulfate heptahydrate: Carlo Erba Reagents, Italy

Magnesium sulfate heptahydrate: Carlo Erba Reagents, Italy

Methanol (HPLC grade): RCI Labscan, Thailand

N,N'-methylene-bis-acrylamide: Sigma, USA

N,N,N',N'-tetramethyl-1, 2-diaminoethane (TEMED): Carlo Erba Reagents, Italy

Nickel(II) sulfate hexahydrate: Carlo Erba Reagents, Italy

Pancreatic digest of casein: Criterion, USA

10X pfu buffer with MgSO₄: Biolabs, England Phenol reagent: Sigma, USA

L-Phenylalanine: Sigma, USA

Phosphoenol pyruvate: Sigma, USA

Potassium di-hydrogen phosphate: Carlo Erba Reagents, Italy

di-Potassium hydrogen phosphate: Carlo Erba Reagents, Italy

RedSafe[™]: Intron Biotechnology, Hongkong

Sodium (meta) arsenite: Sigma, USA

Sodium chloride: Univar, New Zealand

Sodium citrate: Carlo Erba Reagents, Italy

Sodium hydroxy: Carlo Erba Reagents, Italy

Sodium molybdate dihydrate: Carlo Erba Reagents, Italy

Sodium periodate: Sigma, USA

Thiamine hydrochloride: Sigma, USA

2-Thiobarbituric acid: Sigma, USA

Trichloroacetic acid: Sigma, USA

Tris(hydroxymethyl)-aminomethane: Carlo Erba Reagents, Italy

Yeast Extract: Scharlau Chemie S.A., Spain

Zinc sulfate heptahydrate: Carlo Erba Reagents, Italy

2.1.3 Antibiotics

Chloramphenicol: Sigma, USA

Kanamycin: Sigma, USA

2.1.4 Markers

100 base pair DNA ladder: Fermentas Inc., USA

GeneRuler 1 kb DNA Ladder: #SM0311, ThermoFisher Scienctific, Inc., USA

Lamda (λ) DNA /*Hin*dIII: #SM0102, BioLabs, Inc., USA

TriColor protein ladder: Biotechrabbit, Germany

2.1.5 Kits

GenepHlow™ Gel/PCR Kit: Geneaid Biotech Ltd, Taiwan

Presto™ Mini Plasmid Kit: Geneaid Biotech Ltd, Taiwan

2.1.6 Enzymes and restriction enzymes

Pfu DNA polymerase: Biotechrabbit, Germany

Phusion High-Fidelity DNA Polymerase: Thermo Scientific, USA

Restriction enzymes: New England BioLabs, Inc., USA

T4 DNA ligase: Biotechrabbit, Germany

Taq DNA polymerase: Apsalagen, Thailand

2.1.7 Oligonucleotide primers

The oligonucleotide primers were synthesized by Integrated DNA Technologies, Singapore. The oligonucleotide primers used in this study are described in Table 1.

2.1.8 Bacterial strains

E. coli Top10, genotype: F⁻ mcrA Δ (mrr-hsdRMS-mcrBC) Φ 80lacZ Δ M15 Δ lacX74 recA1 araD139 Δ (ara-leu)7697 galU galK λ ⁻ rpsL(Str^R) endA1 nupG, was used for cloning and plasmid preparation.

E. coli BL21(DE3), genotype: $F^- ompT hsdS_B$ (r_B^- , m_B^-) gal dcm (DE3), was used as an expression host for the overexpression of all genes.

2.1.9 Plasmids HULALONGKORN UNIVERSITY

pRSFDuet-1 was used for cloning and expression of *aroB*, *aroL*, *tktA*, *phedh*, *aroG* and *pheA* under T7 promoter (Appendix A).

pBAD33 was used for cloning and expression of *glpF* and *yddG* under arabinose pBAD promoter (Appendix B).

All plasmids used in this study are shown in Table 2.

Primer	Sequence	T _m (°C)
For PCR amplifica	tion	
aroG_Pacl_F	5'-CC <u>TTAATTAA</u> TCCCTTATGCGACTCCTGCATTAGG-3'	63.3
aroG_AvrII_R	5'-ACT <u>CCTAGG</u> TTACCCGCGACGCGCTTTCACT-3'	67.0
F_AroG _Ncol	5'-CATG <u>CCATGG</u> TGTATCAGAACGACGATTTACGCATCAA	65.0
	AGAA ATC-3'	
R_AroG_HindIII	5'-CC <u>AAGCTT</u> TTACCCGCGACGCGCTTTCACTGC-3'	62.6
PheA_F2	5'-ATAAGAAT <u>GCGGCCGC</u> CGATCCCGCGAAATTAA-3'	61.4
PheA_R	5'-TGA <u>TGTACA</u> TCAGGTTGGATCAACAGGCA-3'	67.2
For DNA sequenci	ing	
ACYCDuetUP1	5'-GGATCTCGACGCTCTCCCT-3'	60.0
DuetDown1	5'-GATTATGCGGCCGTGTACAA-3'	57.3
F_tktA_aroG_Int	5'-GCTATCGTCGGTATGACCACCTTCGGTGAAT-3'	63.9
T7 terminator	5'-GCTAGTTATTGCTCAGCGG-3'	57.0
Seqduet_R	5'-CGCTTATGTCTATTGCTGGTTTACCGG-3'	59.4

Table 1. The oligonucleotide primers for PCR amplification and DNA sequencing usedin this study.

Table 2. Plasmids used in this study.

Plasmids	Characteristics	Source or reference
pAroG	pRSFDuet-1 inserted with <i>aroG^{wt}</i> at <i>Nco</i> I and	This study
	HindIII sites.	
pAroG ^{fbr}	pRSFDuet-1 inserted with <i>aroG^{fbr}</i> (L175D,	This study
	Q151L, Q151A and Q151N) at <i>Nco</i> I and <i>Hin</i> dIII	
	sites.	
pBLPT	pRSFDuet-1 inserted with aroB, aroL, phedh	Ratchaneeladdajit,
	and tktA.	2014
pBLPTA ^{L359D} G ^{wt}	pRSFDuet-1 inserted with aroB, aroL, phedh,	This study
	tktA, phe A^{L359D} and $aroG^{wt}$.	
pBLPTA ^{L359D} G ^{Q151N}	pRSFDuet-1 inserted with aroB, aroL, phedh,	This study
	$tktA$, $pheA^{L359D}$ and $aroG^{Q151N}$.	
pBLPTG ^{Q151L}	pRSFDuet-1 inserted with aroB, aroL, phedh,	Ulfah, 2018
	tktA and aroG ^{Q151L} .	
pBLPTG ^{Q151L} A ^{wt}	pRSFDuet-1 inserted with aroB, aroL, phedh,	This study
	$tktA, aroG^{Q151L}$ and $pheA^{wt}$.	
pBLPTG ^{Q151L} A ^{L359D}	pRSFDuet-1 inserted with aroB, aroL, phedh,	This study
	$tktA, aroG^{Q151L}$ and $pheA^{L359D}$.	
pDuet_AroG	pRSFDuet-1 inserted with <i>aroG^{wt}</i> at <i>Bam</i> HI	Kanoksinwuttipong,
	and HindIII sites.	2015
pDuet_AroG ^{fbr}	pRSFDuet-1 inserted with <i>aroG^{fbr}</i> (L175D,	Kanoksinwuttipong,
	Q151L, Q151A and Q151N) at BamHI and	2015
	HindIII sites.	
pDuet_pheA ^{wt}	pRSFDuet-1 inserted with <i>pheA^{wt}</i> at <i>Hin</i> dIII and	This study
	AflII sites.	
pDuet_pheA ^{L359D}	pRSFDuet-1 inserted with <i>pheA^{L359D}</i> at <i>Hin</i> dIII	This study
	and AflII sites.	
pPheA ^{wt}	pRSFDuet-1 inserted with <i>pheA^{wt}</i> at <i>Not</i> I and	This study
	<i>Bsr</i> GI sites.	
pPheA ^{L359D}	pRSFDuet-1 inserted with <i>pheA^{L359D}</i> at <i>Not</i> I	This study
	and BsrGI sites.	
pYF	pBAD33 inserted with <i>yddG</i> and <i>glpF</i> .	Ratchaneeladdajit,
		2014

2.2 Methods

2.2.1 Construction of pPheA^{wt} and pPheA^{L359D}

To clone *pheA* from pDuet_pheA into pBLPTG^{Q151L}, the restriction sites at 5' and 3' ends of *pheA* have to change from *Hin*dIII and *Afl*II to *Not*I and *Bsr*GI because there are no *Hin*dIII and *Afl*II sites on pBLPTG^{Q151L}

2.2.1.1 Plasmid preparation

The single colony of each *E. coli* BL21(DE3) clones harboring pDuet_pheA^{wt} and pDuet_pheA^{L359D} was cultured in 5 mL LB medium (Appendix C) containing 30 mg/mL of kanamycin and incubated at 37 °C, 250 rpm for 16-18 h. After that, the cell pellet was harvested by centrifugation at 5,000xg, 3 min. The plasmid was extracted using Presto[™] Mini Plasmid Kit as described in Appendix E.

2.2.1.2 Agarose gel electrophoresis

pDuet_pheA^{wt} and pDuet_pheA^{L359D} were analyzed by agarose gel electrophoresis. To prepare agarose gel, 0.8% (w/v) agarose in 1x TBE buffer containing 89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA, pH 8.0 was melted in microwave oven. After the gel solution was cooled to 50 - 60 °C, RedSafeTM was added to make 5% (v/v) of final concentration and then poured into the tray. The DNA samples were mixed with 6x DNA loading dye and then loaded into the well of gel. The electrophoresis was set at 100 volts for 40 min. The DNA bands were detected by UV light of gel documentation instrument. The intensity and size of DNA samples were compared with DNA marker (GeneRuler 1 kb DNA ladder and λ DNA /*Hin*dIII).

2.2.1.3 PCR amplification of pheA^{wt} and pheA^{L359D}

 $pheA^{wt}$ and $pheA^{L359D}$ (1,161 bp) were amplified from pDuet_pheA^{wt} and pDuet_pheA^{L359D}, respectively, using PheA_F2 forward primer containing *Not*l site and PheA_R reverse primer containing *Bsr*Gl site as listed in Table 2. To increase the level of gene expression, the PheA_F2 primer was designed from 5' end of T7 promoter. The 50 µL of PCR reaction mixture contained 1x *Pfu* reaction

buffer with MgSO₄, 1x PCR enhancer, 200 μ M of dNTP mix, 1 μ M of forward and reverse primer, 1.25 U of *Pfu* polymerase, 1 ng of DNA template and nuclease free water. The PCR condition consisted of initial activation at 95 °C for 2 min, 32 cycles of denaturation at 95 °C for 30 s, annealing at 62 °C for 45s, extension at 72 °C for 2 min and final extension at 72 °C for 5 min. The PCR products were cleaned by using GenepHlowTM Gel/PCR Kit (Appendix F). After that, the PCR fragments were separated by agarose gel electrophoresis.

2.2.1.4 Cloning of *pheA^{wt}* and *pheA^{L359D}*2.2.1.4.1 Preparation of inserts

Each $pheA^{wt}$ and $pheA^{L359D}$ fragments from section 2.2.1.3 was digested with *Bsr*Gl. The 25 µL of digestion mixture contained 1x NEBufferTM 2.1, 10 U of *Bsr*Gl and 200 ng of DNA fragment. The digestion mixture was incubated at 37 °C for 16 h. After cleaning by GenepHlowTM Gel/PCR Kit, each DNA fragment was checked by agarose gel electrophoresis. Next step, *Bsr*Gl digested *pheA^{wt}* and *pheA^{L359D}* fragments were used as DNA templates for digestion with *Not*I in 1x CutSmart® Buffer. After incubation at 37 °C for 16 h, purified DNA fragments were confirmed by agarose gel electrophoresis.

2.2.1.4.2 Preparation of vector

CHULA pRSFDuet-1 was extracted by using the method described in section 2.2.1.1. The single digestions with *Bsr*GI and *Not*I of pRSFDuet-1 were performed as described in section 2.2.1.4.1. After incubation, linear form of pRSFDuet-1 was confirmed by agarose gel electrophoresis.

2.2.1.4.3 Ligation of inserts and vector

Each purified DNA fragment (section 2.2.1.4.1) was ligated into pRSFDuet-1 (2.2.1.4.2) with vector to insert ratio of 3:1. The 20 µL of ligation mixture contained 89 ng of DNA fragment, 100 ng of vector DNA, 1x T4 DNA ligase buffer and 30 U of T4 DNA ligase. The ligation reaction was incubated at 16 °C for 16 h. After incubation, the ligation reaction was purified by GenepHlow[™] Gel/PCR Kit. The recombinant plasmids from ligation reaction were called pPheA^{wt} and pPheA^{L359D}.

2.2.1.5 Transformation of recombinant plasmid

pPheA^{wt} and pPheA^{L359D} obtained from section 2.2.1.4.3 were transformed into *E. coli* Top10 by electroporation. In electroporation step, each 5 μ L (5 ng) of pPheA^{wt} and pPheA^{L359D} was mixed with 50 μ L of competent *E. coli* Top10 cells (Appendix D) and chilled on ice. Each of reaction mixture was transferred to a cold electroporation cuvette. After that, the electroporation cuvette was placed in the chamber and applied for one pulse by electroporator. Five hundred μ L of LB medium was added in cuvette to resuspend the transformant cell and transferred to 1.5 mL microcentrifuge tube. After that, the transformant was incubated at 37 °C, 250 rpm for 1 h. Two hundred μ L of transformant was spread on LB agar plate that contained 30 mg/mL of kanamycin and then incubated at 37 °C for 16-18 h. The construction of pPheA^{wt} and pPheA^{L359D} are shown in Figure 7.

2.2.1.6 Confirmation of recombinant plasmid

The plasmids of transformants from section 2.2.1.5 were identified by double digestion with *Not*I and *Bsr*GI. Each single colony of pPheA^{wt} and pPheA^{L359D} clones was picked up to culture in 5 mL of LB broth containing 30 mg/mL of kanamycin and incubated at 37 °C with shaking at 250 rpm for 16-18 h. Then recombinant plasmids were extracted by PrestoTM Mini Plasmid Kit. Each recombinant plasmid was digested with *Not*I and *Bsr*GI by the method described in section 2.2.1.4.1. The DNA fragments were identified by agarose gel electrophoresis. The recombinant plasmids with correct size were sent to perform DNA sequencing.



Figure 7. Construction of pPheA^{wt} and pPheA^{L359D}

2.2.1.7 Nucleotide sequencing

The DNA sequencing of recombinant plasmids were performed by Bioneer, Korean Korean using ACYCDuetUP1 as a forward primer and T7 terminator as a reverse primer. The obtained DNA sequences were compared with wild type *pheA* in NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and translated to protein sequence using Genetyx-Win program.

2.2.1.8 Expression of PheA^{wt}

The single colony of pPheA^{wt} clone was cultured in 5 mL LB medium containing 30 mg/mL of kanamycin and incubated at 37 °C, 250 rpm for 16-18 h. The cell culture was inoculated into 100 mL of the same medium and cultured in the same conditions. When OD_{600} reached log phase ($OD_{600} = 0.6$), the expression was induced with 1 mM IPTG for 1-6 h. 1.5 mL of whole cells were harvested at 10,000xg for 3 min and kept for SDS-PAGE analysis.

2.2.1.9 SDS-PAGE analysis

The expression of PheA was confirmed by SDS-PAGE analysis. The SDS-PAGE analysis was performed by the method of Bollag et al.,1996 [42] (Appendix G). The slab gel solution consisted of 12.5% separating gel and 5% stacking gel. Tris-glycine buffer (Appendix G) at pH 8.3 was used for running buffer. For protein loading preparation, the whole cells from section 2.2.1.8 were mixed with 5x sample buffer (Appendix G) and boiled for 15 min. After that, the cell pellets were eliminated by centrifugation at 10,000xg for 15 min. The supernatants were loaded into the gel. Tri-color protein color was used for protein molecular weight marker. The electrophoresis was set at 20 mA per slab gel for 40 min. After running the gel, the SDS gel was stained by the destaining solution (Appendix H) with shaking for 30 min. After that, the destaining solution in SDS gel was changed and shaken for overnight. The molecular weight of each protein was analyzed by comparing the band with the protein marker.

2.2.2 Construction of $pBLPTG^{Q151L}A^{wt}$ and $pBLPTG^{Q151L}A^{L359D}$ 2.2.2.1 $pBLPTG^{Q151L}$ preparation

The pBLPTG^{Q151L} containing *aroB*, *aroL*, *phedh*, *tktA* and *aroG^{Q151L}* was extracted using the method in section 2.2.1.1. The single digestions with *Not*I and *Bsr*GI of pBLPTG^{Q151L} were performed, respectively as described in section 2.2.1.4.1. After incubation, purified linear form of pBLPTG^{Q151L} was confirmed by agarose gel electrophoresis.

2.2.2.2 pheA^{wt} and pheA^{L359D} preparation

The pPheA^{wt} and pPheA^{L359D} from section 2.2.1.6 were digested with *Not*I and *Bsr*GI as described in section 2.2.1.4.1. After incubation, the *pheA^{wt}* and *pheA^{L359D}* fragments were collected from agarose gel using GenepHlowTM Gel/PCR Kit.

2.2.2.3 Cloning of *pheA^{wt}* and *pheA^{L359D}* into pBLPTG^{Q151L}

After digestion with *Not*I and *Bsr*GI, each *pheA*^{wt} and *pheA*^{L359D} fragment was ligated with pBLPTG^{Q151L} using the method described in section 2.2.1.4.3. After that, the ligation reactions were transformed into *E. coli* BL21(DE3) by electroporation method as described in section 2.2.1.5. The construction of $pBLPTG^{Q151L}A^{wt}$ and $pBLPTG^{Q151L}A^{L359D}$ are shown in Figure 8.

2.2.2.4 Confirmation of pBLPTG^{Q151L}A^{wt} and pBLPTG^{Q151L}A^{L359D}

The single colonies of pBLPTG^{0151L}A^{wt} and pBLPTG^{0151L}A^{L359D} from section 2.2.2.3 were picked up to culture in 5 mL of LB broth containing 30 mg/mL of kanamycin and incubated at 37 °C with shaking at 250 rpm for 16-18 h. Each of recombinant plasmid was extracted by Presto[™] Mini Plasmid Kit. The recombinant plasmids were digested with *Xho*I by the method described in section 2.2.1.4.1. The recombinant plasmids with correct size were identified by agarose gel electrophoresis.



Figure 8. Construction of $pBLPTG^{Q151L}A^{wt}$ and $pBLPTG^{Q151L}A^{L359D}$

2.2.3 Expression of AroG

2.2.3.1 Thiobarbiturate assay

DAHP synthase activity was determined by the thiobarbiturate assay method modified from Schoner, 1976 [43] and Liu, 2008 [44]. Each single colony of pAroG^{wt} and pAroG^{fbr} (pAroG^{L175D}, pAroG^{Q151L}, pAroG^{Q151A} and pAroG^{Q151N}) was cultured in 5 mL LB medium containing 30 mg/mL of kanamycin and then incubated with shaking at 250 rpm, 37 °C for 16-18 h. 5% (v/v) of each culture was inoculated into 50 mL of the same medium and cultured in the conditions. For shake flask cultivation, the 5% (v/v) of starters were cultured into 200 mL of LB medium and incubated with shaking at 250 rpm, 37 °C. After the OD₆₀₀ reached 0.6 (log phase), the expression was induced with 1 mM IPTG for 2 h. The cells of each clone were collected by centrifugation at 8,000xg for 10 min and washed with resuspend buffer (0.1 mM KPB, pH 6.5, 200 µM PEP, 0.5 mM DTT, 0.1 mM PMSF and 10 mM EDTA) The cell pellet of each clone was dissolved in resuspend buffer and then broken by ultrasonic cell disruption. The crude extracts were centrifuged at 10,000xg for 20 min to collect the supernatants for dialysis. After dialysis, 1.5 mL of each crude extract was centrifuged at 10,000xg for 20 min to collect the supernatants for assay of enzyme activity.

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C In thiobarbiturate assay method, the reaction mixture contained 50 mM potassium phosphate, pH 6.5, 5 mM PEP, 2 mM E4P, 0-20 mM L-Phe, 30 μ M MnCl₂, crude enzyme and H₂O in a total volume of 33.75 μ L. The mixture was incubated at 30 °C for 10 min. The reaction was initiated when the enzyme was added and stopped by addition of 180 μ L of 10% (w/v) trichloroacetic acid. After that, the 45 μ L of mL of 25 mM NalO₄ in 62.5 mM H₂SO₄ was added in the mixture and incubated at 37 °C for 30 min. Then, the 45 μ L of 2% (w/v) Na₂SO₄ in 0.5 M HCl was rapidly mixed for stopped the reaction and 450 μ L of 0.36% (w/v) thiobarbituric acid was added and mixed. The reaction mixture was boiled for 20 min and then cooled in room temperature. The absorbance at a wavelength 549 nm was measured by spectrophotometer.

2.2.3.2 Protein measurement

The protein concentration of crude extracts were measured using Lowry's method [45]. The 250 μ L of crude extract was mixed with 50 μ L of solution A and 2.5 mL of solution B and incubated at 30 °C for 10 min. After incubation, 250 μ L of solution C was added and rapidly mixed and then incubated at room temperature for 20 min. The absorbance of protein was detected by spectrophotometer at a wavelength 610 nm. The protein concentration was calculated using the standard curve of BSA. All solutions were prepared as described in Appendix I. The expression of AroG^{wt} and AroG^{fbr} (L175D, Q151L, Q151A and Q151N) under T7 promoter were detected by SDS-PAGE analysis using the method described in section 2.2.1.9.

2.2.4 Reconstruction of pAroG and pAroG^{fbr}

2.2.4.1 Plasmid extraction

The single colonies of *E. coli* Top10 clones harboring pDuet_AroG^{wt} and pDuet_AroG^{fbr} from Kanoksinwutthipong were cultured in 5 mL LB medium containing 30 mg/mL of kanamycin and incubated at 37 °C, with shaking for 16-18 h. After that, the cell pellets were harvested by centrifugation at 5,000xg for 3 min. The plasmids were extracted using Presto[™] Mini Plasmid Kit.

2.2.4.2 Forward primer design

The forward primer containing *Nco*I site at 5' end of *aroG* (5' F AroG NcoI) was designed. The ATG of *Nco*I site was used as a start codon of *aroG*.

2.2.4.3 PCR amplification of *aroG^{wt}* and *aroG^{fbr}*

To construct the pBLPTA^{L359D}G, restriction sites at 5' and 3' end of inserted *aroG^{wt}* and *aroG^{fbr}* from pDuet_AroG^{wt} and pDuet_AroG^{fbr} have to change to *Nco*I and *Hind*III, respectively. For PCR amplification of *aroG^{wt}* and *aroG^{fbr}* (1,053 bp), pDuet AroG^{wt} and pDuet AroG^{fbr} were used as DNA templates, respectively Forward primer (F_AroG_Ncol) containing *Nco*I site and reverse primer (R_AroG_HindIII) containing *Hin*dIII site as listed in Table 2 were used. The 50 µL of PCR reaction mixture contained 1x Phusion HF buffer, 1 U Phusion DNA polymerase, 3% DMSO, 200 µM of dNTP mix, 1 µM of forward and reverse primers, 1 ng of DNA template and nuclease free water. The PCR condition consisted of initial activation at 98 °C for 30 s, 32 cycles of denaturation at 98 °C for 10 s, annealing at 62 °C for 10 s, extension at 72 °C for 1 min and final extension at 72 °C for 10 min. The PCR products were cleaned by using GenepHlow[™] Gel/PCR Kit. After that, the PCR fragments were separated by agarose gel electrophoresis.

2.2.4.4 Preparation of inserts and vector

The $aroG^{wt}$ and $aroG^{br}$ fragments from section 2.2.1.3 and pRSFDuet-1 were digested with *Hind*III. The 25 µL of digestion mixture contained 1x NEBufferTM 2.1, 10 U of *Hind*III and 200 ng of DNA template. The digestion mixture was incubated at 37 °C for 16 h. After cleaning by GenepHlowTM Gel/PCR Kit, each DNA fragment was checked by agarose gel electrophoresis. Next step, $aroG^{wt}$, $aroG^{fbr}$ and pRSFDuet-1 fragments digested with *Hind*III were used as DNA template for digestion with *Nco*I using 1x CutSmart[®] Buffer. After digestion, DNA fragments were confirmed by agarose gel electrophoresis.

2.2.4.5 Ligation and transformation

After digestion with *Ncol* and *Hin*dIII, the *aroG^{wt}* and *aroG^{fbr}* fragments were ligated with pRSFDuet-1 using the method described in section 2.2.1.4.3. After that, the ligation reactions were used for transformation into *E. coli* BL21(DE3) by electroporation method as described in section 2.2.1.5. The construction of pAroG^{wt} and pAroG^{fbr} are shown in Figure 9.

2.2.4.6 Confirmation of recombinant plasmid

The plasmids of transformants from section 2.2.4.5 were identified by double digestion with *Nco*I and *Hin*dIII. Each single colony of pAroG^{wt} and pAroG^{fbr} clones was picked up to culture in 5 mL of LB broth containing



Figure 9. Construction of pAroG^{wt} and pAroG^{fbr}

30 mg/mL of kanamycin and incubated at 37 °C with shaking at 250 rpm for 16-18 h. Then recombinant plasmids were extracted by Presto[™] Mini Plasmid Kit. Each of recombinant plasmid was digested with *Nco*I and *Hin*dIII by the method described in section 2.2.1.4.1. The DNA fragments were identified by agarose gel electrophoresis. The recombinant plasmids with the correct size was sent to perform DNA sequencing by Bioneer, Korean using ACYCDuetUP1 as a forward primer and DuetDown1 as a reverse primer as described in section 2.2.1.7.

2.2.5 Construction of pBLPTA^{L359D}G^{wt} and pBLPTA^{L359D}G^{Q151N} 2.2.5.1 pBLPTA^{L359D} preparation

The recombinant pBLPTGA^{L359D} containing *aroB*, *aroL*, *phedh*, *tktA*, *aroG* and *pheA* from section 2.2.2.4 was double digested with *PacI* and *AvrII*. The 25 µL of digestion mixture contained 1x CutSmart® Buffer, 10 U of *PacI* and *AvrII* and 200 ng of DNA template. The digestion mixture was incubated at 37 °C for 16 h. After incubation, linear fragment of pBLPTA^{L359D} was separated by agarose gel electrophoresis. The linear form of pBLPTA^{L359D} was purified by GenepHlow[™] Gel/PCR Kit. The size of pBLPTA^{L359D} was confirmed by agarose gel electrophoresis.

2.2.5.2 T7_aroG^{wt} and T7_aroG^{Q151N} preparation

2.2.5.2.1 PCR amplification of T7_aroG^{wt} and T7_aroG^{Q151N}

CHULALTO construct pBLPTA^{L359D}G, T7_*aroG* fragments were amplified using forward primer containing *Pac*I and reverse primer containing *Avr*II site at their 5' end. For PCR amplifications, $aroG^{wt}$ and $aroG^{Q151N}$ (1,053 bp) were amplified from pAroG^{wt} and pAroG^{Q151N}, respectively, using forward primer (aroG_PacI_F) and reverse primer (aroG_AvrII_R). To increase the level of gene expression, the aroG_PacI_F primer was designed from 5' end of T7 promoter. The 50 µL of PCR reaction mixture contained 1x *Pfu* reaction buffer with MgSO₄, 1x PCR enhancer, 200 µM of dNTP mix, 1 µM of forward and reverse primers, 1.25 U of *Pfu* polymerase, 1 ng of DNA template and nuclease free water. The PCR condition consisted of initial activation at 95 °C for 2 min, 32 cycles of denaturation at 95 °C for 30 s, annealing at 62 °C for 45s, extension at 72 °C for 2 min and the last step of final extension at 72 °C for 5 min. The PCR products were cleaned by using GenepHlow[™] Gel/PCR Kit. After that, the PCR fragments were separated by agarose gel electrophoresis.

2.2.5.2.2 Double digestion with restriction enzymes

Each *aroG* PCR fragment was double digested with *Pac*I and *Avr*II using the method described in section 2.2.5.1.

2.2.5.3 Ligation and transformation

After digestion with *Pacl* and *AvrII*, each *aroG*^{wt} and *aroG*^{Q151N} fragments was ligated with pBLPTA^{L359D} vector using the method described in section 2.2.1.4.3. After that, each ligation reaction was transformed into *E. coli* BL21(DE3) by electroporation method as described in section 2.2.1.5. The construction of pBLPTA^{L359D}G^{Wt} and pBLPTA^{L359D}G^{Q151N} are shown in Figure 10.

2.2.5.4 Confirmation of pBLPTA^{L359D}G^{wt} and pBLPTA^{L359D}G^{Q151N}

The transformants from section 2.2.5.3 were identified by digestion of their plasmid with *Xhol*. The single colonies of pBLPTA^{L359D}G^{wt} and pBLPTA^{L359D}G^{Q151N} were picked up to culture in 5 mL of LB broth containing 30 mg/mL of kanamycin and incubated at 37 °C with shaking at 250 rpm for 16-18 h. Then recombinant plasmids were extracted by PrestoTM Mini Plasmid Kit. Each of recombinant plasmid was digested with *Xhol* by the method in section 2.2.5.1. The DNA fragments were identified by agarose gel electrophoresis. The recombinant plasmids with correct size were sent to sequence by Bioneer, Korean using F_tktA_aroG_Int as a forward primer and Seqduet_R as a reverse primer as described in section 2.2.1.7.



Figure 10. Construction of $pBLPTA^{L359D}G^{wt}$ and $pBLPTA^{L359D}G^{Q151N}$

2.2.6 Co-transformation of pBLPTA^{L359D}G^{wt} and pBLPTA^{L359D}G^{Q151N} with pYF into *E. coli* BL21(DE3)

The pBLPTA^{L359D}G^{wt} and pBLPTA^{L359D}G^{Q151N} were co-transformed with pYF into *E. coli* BL21(DE3) competent cell using method described in section 2.2.1.5. The transformants were spreaded on LB agar plate containing 30 mg/mL of kanamycin and 10 mg/mL of chloramphenicol and then incubated at 37 °C for 16-18 h. The growing colonies of pBLPTA^{L359D}G^{wt} & pYF and pBLPTA^{L359D}G^{Q151N} & pYF were picked up to culture in 5 mL LB broth that contained 30 mg/mL of kanamycin and 10 mg/mL of chloramphenicol and incubated at 37 °C with shaking at 250 for 16-18 h. The pBLPTA^{L359D}G^{wt} & pYF and pBLPTA^{L359D}G^{Q151N} & pYF were extracted by PrestoTM Mini Plasmid Kit. The recombinant plasmids were confirmed by restriction enzyme digestion. Each of recombinant plasmid was digested with *Bam*HI using method in section 2.2.5.1. The size of pBLPTA^{L359D}G^{wt} & pYF and pBLPTA^{L359D}G^{Q151N} & pYF were identified by agarose gel electrophoresis. The colonies containing pBLPTA^{L359D}G^{wt} & pYF and pBLPTA^{L359D}G^{Q151N} & pYF were collected to determine of L-Phe production. The construction of pBLPTA^{L359D}G^{wt} & pYF and pBLPTA^{L359D}G^{Q151N} & pYF are shown in Figure 11.



Figure 11. Construction of $pBLPTA^{L359D}G^{Wt} \& pYF$ and $pBLPTA^{L359D}G^{Q151N} \& pYF$

2.2.7 Determination of L-Phe production by HPLC

Each single colony of pBLPT & pYF, pBLPTA^{L359D}G^{wt} & pYF and pBLPTA^{L359D}G^{Q151N} & pYF clones from section 2.2.6 was cultured in 5 mL LB medium containing 30 mg/mL of kanamycin and 10 mg/mL of chloramphenicol and then incubated at 37 °C, 250 rpm for 16-18 h. 5% (v/v) of each culture was inoculated into 50 mL of the same medium and cultured of the same conditions. For shake flask cultivation, the 5% (v/v) of starters were separately cultured into 200 mL of minimum medium containing (g/L): 60 glycerol, 42.5 (NH_4)₂SO₄, 0.3 MgSO₄·7H₂O, 0.075 FeSO₄·7H₂O, 0.015 CaCl₂·2H₂O, 12 K₂HPO₄, 3 KH₂PO₄, 1 NaCl, 1 Na-citrate, 0.0075 thiamine-HCl and 1.5 mL of trace elements solution contained (g/L): 2.0 Al₂(SO₄)₃·18H₂O, 3.0 Na₂MoO₄·2H₂O, 0.75 CoSO₄·7H₂O, 15 ZnSO₄·7H₂O, 2.5 CuSO₄·5H₂O, 0.5 H₃BO₃, 24 MnSO₄·7H₂O and 2.5 NiSO₄·6H₂O at pH 7.0. The cultures were shaken at 37 °C, 250 rpm. After the OD₆₀₀ reached 0.6 (log phase), the expression of yddG and glpF under ara promoter of pBAD33 were induced with 0.02% arabinose. The 1.5 mL of each sample were collected every 24 h for 8 days to measure cell density (OD_{600}) and L-Phe production. The supernatants of each sample were filtrated through 0.22 µm nylon syringe filter. The L-Phe production was measured by HPLC method using Chirex 3126 (D)-penicillamine column. The ratio of 75:25 of 2 mM copper sulfate and methanol was used as a mobile phase and the flow rate was 0.7 mL/min. The peak of L-Phe was detected at wavelength 254 nm. The concentrations of L-Phe were estimated from the standard curve of L-Phe.

CHAPTER III

RESULTS AND DISCUSSIONS

3.1 Construction of pPheA^{wt} and pPheA^{L359D}

3.1.1 Plasmid extraction

pDuet_pheA^{wt} and pDuet_pheA^{L359D} (5,078 bp) were extracted from *E. coli* Top10. The agarose gel electrophoresis was shown in Figure 12. Then, the pDuet_pheA^{wt} and pDuet_pheA^{L359D} were confirmed by digested with *Afl*II and *Bam*HI. From the result of agarose gel electrophoresis in Figure 13, the DNA fragments were observed around 3.4 kb and 1.2 kb. Therefore, pDuet_pheA^{wt} No.1 and pDuet_pheA^{L359D} No.1 were used for *pheA* amplification.

3.1.2 Amplification of pheA^{wt} and pheA^{L359D}

The pheA^{wt} and pheA^{L359D} were amplified from pDuet_pheA^{wt} and pDuet_pheA^{L359D}, respectively, using forward primer containing *Not*l site and reverse primer containing *Bsr*Gl site. After cleaning by GenepHlow^M Gel/PCR Kit, the PCR fragments were separated by agarose gel electrophoresis. The size of *pheA^{wt}* and *pheA^{L359D}* fragments were detected around 1.2 kb as shown in Figure 14.

3.1.3 Cloning of pheA^{wt} and pheA^{L359D}

The PCR products of *pheA^{wt}* and *pheA^{L359D}* from section 3.1.2 and pRSFDuet-1 vector were digested with *Not*I and *Bsr*GI. The results of *pheA^{wt}* and *pheA^{L359D}* fragments (1.2 kb) and pRSFDuet-1 linear vector (3.9 kb) after digestion are shown in Figure 15. After that, *pheA^{wt}* and *pheA^{L359D}* fragments were ligated with pRSFDuet-1 linear vector and then transformed into *E. coli* Top10 by electroporation. Four colonies of pPheA^{wt} and five colonies of pPheA^{L359D} transformants were randomly picked and cultured in 5 mL of LB broth containing 30 mg/mL of kanamycin. Each recombinant plasmid was extracted and then detected by agarose gel electrophoresis. From Figure 16, the plasmid from pPheA^{wt} transformant No.1, 3 and 4 and pPheA^{L359D} transformant No.1, 2 and 4 moved slower than pRSFDuet-1. Thus, the pPheA^{wt} No.1, 3 and 4 and pPheA^{L359D} No.1, 2 and 4 might harbor the inserted genes. These six plasmids were confirmed by digestion with *Not*I and *Bsr*GI.

From digestion pattern, each recombinant plasmid gave two bands of DNA fragments around 3.9 kb and 1.2 kb as shown in Figure 17. This result confirmed that *pheA^{wt}* and *pheA^{L359D}* were successfully inserted into pRSFDuet-1. After that, the nucleotide sequences of the inserts were checked by Bioneer Inc. (Korea).

3.1.4 Nucleotide sequencing

To verify the nucleotide sequence of *pheA^{wt}* and *pheA^{L359D}* genes, the DNA sequencing of recombinant plasmids were performed by Bioneer, Korean using ACYCDuetUP1 as a forward primer and T7 terminator as a reverse primer. The obtained DNA sequences were compared with wild type *pheA* reported by Naksusuk in 2015 using nucleotide blast tools in NCBI and then translated to protein sequence using Genetyx-Win program.

From Figure 18 - 19, nucleotide sequences of $pheA^{wt}$ and $pheA^{L359D}$ are similar to that of reference nucleotides [40]. Only nucleotide sequence at restriction sites were changed from *Afl*II to *Not*I and *Bam*HI to *Bsr*GI. The correct $pheA^{wt}$ and $pheA^{L359D}$ were used in the further experiment.

3.1.5 Expression of PheA^{wt}

The expression of PheA^{wt} under T7 promoter was evaluated by SDS-PAGE analysis. The *E. coli* BL21(DE3) harboring pPheA^{wt} from section 3.1.4 was cultured in LB medium containing 30 mg/mL of kanamycin. After cell culture reached log phase, IPTG was added to 1 mM to induce *pheA* expression. The whole cells of *E. coli* BL21(DE3) harboring pPheA^{wt} were mixed with 5x sample buffer. The supernatants were loaded into the gel. Tri-color protein color was used as protein molecular weight marker. The protein of *E. coli* BL21(DE3) and *E. coli* BL21(DE3) harboring pRSFDuet-1 were used as a control in lane 1 and 2, respectively. The protein band of chorismite mutase/prephenate dehydratase was detected after 1 mM IPTG induction for 1-6 h in lane 3-8, respectively. The size of recombinant protein was approximately 43 kDa as shown in Figure 20.



Figure 12. Electrophoretic patterns of pPheA^{wt} and pPheA^{L359D}

Lane M1 : λ DNA /HindIII marker Lane 1 : pRSFDuet-1 Lane 2-4 : pPheA^{wt} No.1-3, respectively Lane 5-7 : pPheA^{L359D} No.1-3, respectively Lane M2 : Gene Ruler 1 kb DNA ladder

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Lane M1	: Gene Ruler 1 kb DNA ladder			
Lane 1	: uncut pRSFDuet-1			
Lane 2	: AflII/HindIII digested pRSFDuet-1			
Lane 3, 5, 7	: uncut pPheA ^{wt} No.1-3 , respectively			
Lane 4, 6, 8	: <i>Afl</i> II/ <i>Hin</i> dIII digested pPheA ^{wt} No.1-3, respectively			
Lane 9, 11, 13 : uncut pPheA ^{L359D} No.1-3, respectively				
Lane 10, 12, 1	.4 : <i>AflII/HindIII</i> digested pPheA ^{L359D} No.1-3, respectively			
Lane M2	: \ DNA / <i>Hin</i> dIII marker			
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Figure 15. Notl and BsrGI digestion patterns of PCR products and pRSFDuet-1.

Lane M : Gene Ruler 1 kb DNA ladder

Lane 1 : uncut pRSFDuet-1

Lane 2 : Notl and BsrGI digested pRSFDuet-1

Lane 3 : uncut PCR product of *pheA^{wt}*

- Lane 4 : Notl and BsrGI digested PCR product of pheA^{wt}
- Lane 5 : Notl and BsrGI digested PCR product of pheA^{L359D}

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Figure 16. Electrophoretic pattern of plasmid from pPheA transformants.

Lane M : Gene Ruler 1 kb DNA ladder

Lane 1 : pRSFDuet-1

Lane 2-5 : pPheA^{wt} from transformant No.1-4, respectively

Lane 6-10 : pPheA^{L359D} from transformant No.1-5, respectively

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Figure 17. Notl and BsrGl digestion patterns of pPheA^{wt} and pPheA^{L359D}.

Lane M : Gene Ruler 1 kb DNA ladder

- Lane 1 : uncut pRSFDuet-1
- Lane 2 : pRSFDuet-1 digested with Notl and BsrGl
- Lane 3 : uncut pPheA^{wt} transformant No.1
- Lane 4 : Notl and BsrGl digested pPheA^{wt} from transformant No.1
- Lane 5 : Notl and BsrGI digested pPheA^{wt} from transformant No.3
- Lane 6 : Notl and BsrGI digested pPheA^{wt} from transformant No.4
- Lane 7 : uncut pPheA^{L359D} transformant No.1
- Lane 8 : Notl and BsrGl digested pPheA^{L359D} from transformant No.1
- Lane 9 : Notl and BsrGI digested pPheA^{L359D} from transformant No.2
- Lane 10 : Notl and BsrGI digested pPheA^{L359D} from transformant No.4

core 145 bits(1161	Expect	Identities 1161/1161(100%)	Gaps 0/1161(0%)	Strand Plus/Plus
uery 1	ATGACATCGGAAA	ACCCGTTACTGGCGCTGC	GAGAGAAAATCAGCGCG	CTGGATGAAAAA
bjct 1	ATGACATCGGAAA	ACCCGTTACTGGCGCTGC	GAGAGAAAATCAGCGCG	CTGGATGAAAAA
uery 61	TTATTAGCGTTAC	TGGCAGAACGGCGCGAAC	TGGCCGTCGAGGTGGGA	AAAGCCAAACTG
bjct 61	TTATTAGCGTTAC	TGGCAGAACGGCGCGAAC	I I I I I I I I I I I I I I I I I I I	AAAGCCAAACTG
uery 121	CTCTCGCATCGCC	CGGTACGTGATATTGATC	GTGAACGCGATTTGCTG	GAAAGATTAATT
bjct 121	CTCTCGCATCGCC	CGGTACGTGATATTGATC		GAAAGATTAATT
uery 181	ACGCTCGGTAAAG	CGCACCATCTGGACGCCC.	ATTACATTACTCGCCTG	TTCCAGCTCATC
bjct 181	ACGCTCGGTAAAG	CGCACCATCTGGACGCCC	ATTACATTACTCGCCTG	TTCCAGCTCATC
uery 241	ATTGAAGATTCCG	TATTAACTCAGCAGGCTT	TGCTCCAACAACATCTC	AATAAAATTAAT
bjct 241	ATTGAAGATTCCG	TATTAACTCAGCAGGCTT	IIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII	 AATAAAATTAAT
uery 301	CCGCACTCAGCAC	GCATCGCTTTTCTCGGCC	CCAAAGGTTCTTATTCC	CATCTTGCGGCG
bjct 301	CCGCACTCAGCAC	GCATCGCTTTTCTCGGCC	CCAAAGGTTCTTATTCC	CATCTTGCGGCG
uery 361	CGCCAGTATGCTG	CCCGTCACTTGAGCAAT	TCATTGAAAGTGGCTGC	GCCAAATTTGCC
bjct 361	CGCCAGTATGCTG	CCCGTCACTTTGAGCAAT		GCCAAATTTGCC
uery 421	GATATTTTTAATC	AGGTGGAAACCGGCCAGG	CCGACTATGCCGTCGTA	CCGATTGAAAAT
bjct 421	GATATTTTTAATC	AGGTGGAAACCGGCCAGG	CCGACTATGCCGTCGTA	CCGATTGAAAAT
uery 481	ACCAGCTCCGGTG	CCATAAACGACGTTTACG.	ATCTGCTGCAACATACC	AGCTTGTCGATT
bjct 481	ACCAGCTCCGGTG	CCATAAACGACGTTTACG	ATCTGCTGCAACATACC	AGCTTGTCGATI
uery 541	GTTGGCGAGATGA	CGTTAACTATCGACCATT	GTTTGTTGGTCTCCGGC	ACTACTGATTTA
bjct 541	GTTGGCGAGATGA	CGTTAACTATCGACCATT	GTTTGTTGGTCTCCGGC	ACTACTGATTTA
uery 601	TCCACCATCAATA	CGGTCTACAGCCATCCGC	AGCCATTCCAGCAATGC	AGCAAATTCCTT
bjct 601	TCCACCATCAATA	CGGTCTACAGCCATCCGC	AGCCATTCCAGCAATGC	AGCAAATTCCTT
uery 661	AATCGTTATCCGC	ACTGGAAGATTGAATATA	CCGAAAGTACGTCTGCG	GCAATGGAAAAG
bjct 661	AATCGTTATCCGC	ACTGGAAGATTGAATATA	CCGAAAGTACGTCTGCG	GCAATGGAAAAG
uery 721	GTTGCACAGGCAA	AATCACCGCATGTTGCTG	CGTTGGGAAGCGAAGCT	GGCGGCACTTTG
bjct 721	GTTGCACAGGCAA	AATCACCGCATGTTGCTG	CGTTGGGAAGCGAAGCT	GGCGGCACTTTG
uery 781	TACGGTTTGCAGG	TACTGGAGCGTATTGAAG	CAAATCAGCGACAAAAC	TTCACCCGATTT
bjct 781	TACGGTTTGCAGG	TACTGGAGCGTATTGAAG	CAAATCAGCGACAAAAC	TTCACCCGATTT
uery 841	GTGGTGTTGGCGC	GTAAAGCCATTAACGTGT	CTGATCAGGTTCCGGCG	AAAACCACGTTG
bjct 841	GTGGTGTTGGCGC	GTAAAGCCATTAACGTGT	CTGATCAGGTTCCGGCG	AAAACCACGTTG
uery 901	TTAATGGCGACCG	GGCAACAAGCCGGTGCGC	TGGTTGAAGCGTTGCTG	GTACTGCGCAAC
bjct 901	TTAATGGCGACCG	GGCAACAAGCCGGTGCGC	TGGTTGAAGCGTTGCTG	GTACTGCGCAAC
uery 961	CACAATCTGATTA	TGACCCGTCTGGAATCAC	GCCCGATTCACGGTAAT	CCATGGGAAGAG
bjct 961	CACAATCTGATTA	TGACCCGTCTGGAATCAC	GCCCGATTCACGGTAAI	CCATGGGAAGAG
uery 1021	ATGTTCTATCTGG	ATATTCAGGCCAATCTTG	AATCAGCGGAAATGCAA	AAAGCATTGAAA
bjct 1021	ATGTTCTATCTGG	ATATTCAGGCCAATCTTG	AATCAGCGGAAATGCAA	AAAGCATTGAAA
uery 1081	GAGTTAGGGGAAA	TCACCCGTTCAATGAAGG	TATTGGGCTGTTACCCA	AGTGAGAACGTA
bjct 1081	GAGTTAGGGGAAA	TCACCCGTTCAATGAAGG	TATTGGGCTGTTACCCA	AGTGAGAACGTA
uery 1141	GTGCCTGTTGATC	CAACCTGA 1161		
bjct 1141	GTGCCTGTTGATC	CAACCTGA 1161		

Figure 18. Nucleotide sequence of *pheA*^{wt}

Query represents the nucleotide sequence of *pheA^{wt}* in this work. Sbjct represents the nucleotide sequence of *pheA^{wt}* reference [40]. The chromatogram of *pheA^{wt}* is shown in Appendix N.

guery 1 AFGACATCCGAAAACCCGTTACTGGCGTCGCAGACAAAATCAGCGCGCTGGATAAAAA sbjet 1 AFGACATCCGAAAACCGGTTACTGGCGTGGCAGACGGCGCGGGATAAAATCAGCGCGGCGGATGGAAAAAA Guery 61 TTATTACGGTTACTGGCGAAACGGCCGGAACGGCCGGAGGGAAAAAGCGAAACGG Sbjet 61 TTATTAGCGTTACTGGCGGAACGGCCGGAACGGCCGGAGGGGAAAAAGCGAAACGG Guery 211 CCCCCGGTCGCCGGAGGGAGATGTGGTCGGACGGACTGGCGAAACGGCCGATTAGGTTGCGGAAGGGCCAACTGCGAGTGGCGAAACGGCCGCGATGGGGAGGACGATTGCGTGCG	Score 2145 b	its(1161	L)	Expect 0.0	Identities 1161/1161(100%)	Gaps 0/1161(0%)	Strand Plus/Plus	
sbjct 1 ATERCATCGGGAAAACCGCUTHACTGGCGGATGGGCGAGATGGGCGGCUTGGAAAGCGCUGGGGAAAAGCGCUGGAGATGGGCGGAAAGGCUGGUGGAAAAGCGCUGGAGATGGGCGGGGGAAAAGCGGCUGAAAGCG guery 11 TTATTAGCGTTACTGGCAGAACGGCUGGAACGGCUGGAGTGGGAAAAGCCAAACTG guery 121 CTCTCGCCATCGCUGGGGAACGGCUGAACGGCUGAGTGGGAAAAGCCAAACTG guery 121 CTCTCGCCATCGCUGGGTAGTGGAAACGGCUGATTGGTCGAACGCUGATTGGTGGAAAGACTAATT sbjct 121 CTCTCGCCATCGCUGGTAGTAGTGGACACTGGUCGUCATTACGTGGAAGACTAATT guery 121 CTCTCGCATCGCUGGTATTACGGGACGCUTTGGTCCAACGACTTGCTGCGCTGTCTCGAGCGCTGATC guery 241 ATTGGAAGATTCGGTATTACTCGGCGCCATAGCATTGCCTCCAACAACATCTCAATGAAATTAAT gbjct 241 ATTGGAAGATTCGGTATTACTCAGCAGCATTGCTCGCCCAACATCTCAATGAAATTAAT gbjct 241 ATTGGAAGATTCGGTATTACTCAGCAGATTGCTCGCCCAACATCCACTCAATGAAATTAAT gbjct 241 ATTGGAAGGCCCACATCGCCTTTTCTGGGCCCAAAAGCACTCCACTCAACTCACATGCGGCGGGGGGGG	Query	1	ATGACA	TCGGAAA	ACCCGTTACT	GCGCTGCGAGA	GAAAATCAGCGCG	CTGGATGAAAAA	6
Query 61 TERTFAGGETERCEGGEAAAGGEGGEGEAACTGECGETEGGAAAAGGEGAAAAGCCAAACTG Sbjet 61 TEATTAGEGTEACTGEGAGAACGGEGGAACTGECGTEGGAAAAGCCAAAACTG Query 121 CTCTCGCATCGCCCGGTAGTGETAATTATTCATCATGAACGCGATTGEGTGGAAAAGCCAAACTG Sbjet 121 CTCTCGCATCGCCCGGTAGTGETAATTGATCGTGAACGCGATTGEGTGGAAAAGCCAAACTG Sbjet 121 CTCTCGCATCGCCCGGTACGTCGGCGCCATTGCTGCAACGCGATTGCCCGGGCGCGCACATCGCGGCGCATTACTCGCGGGTTGGCCCGACGCCGGCGCCATAACGGCGCCTTTGCGCCGGCGGCGGCTTGCGCCGGCGGCGCGCGTGCGCGGCG	Sbjct	1	ATGACA	TCGGAAA	ACCCGTTACT	GCGCTGCGAGA	AGAAAATCAGCGCG	CTGGATGAAAAA	6
sbjet 61 TTATTAGECGTTACTGGCLGGALCGGGCGGALCTGGCLGGGGGGTTGGCTGGLALAGCCGALARCTG Query 121 CTCTCGGCATCGCCCGGTAGGGCGGCATTGGCTGGLAGGGGATTGGCTGGLALAGATTAATT Sbjet 121 CTCTCGGCATCGCCCGGTAGGGCGCCATTGGALAGGGGATTGGCTGGLALAGATTAATT Guery 181 ACGCTCGGTALAGGCGCACCATCGGGAGGCCCATTAGCATTACTGGCCGTGTTGCCAGCTCATC Sbjet 181 ACGCTCGGTALAGGCGCACCATCGGGAGGCCCATTAGCATTACTGGCCGTGTTGCCGCACCATCAC Query 241 ATTGGALGATTCGGTGTTTGCTGGGCCCCALAGGTTGTGTCCGACCATCAC Sbjet 201 CCGCCACTCAGCACCGCGTCGTTTGCTGGGCCCCALAGGTTGTTTCTCGGGGC Sbjet 361 CGCCACGTAGGCCGGTCGCTTTGCTGGGCCCCALAGGTTGTTTTCTCGGGCGCALAGTTGTTGCTGGGCGCCALAGTTGCGGCGCCALAGTTGCGGCGCCACATTTGCGGCGCCALAGTTGGCGCGCGCGACATTTGCGGCGCCACATTGCGGCACATTGGCGGCGCGCACTTGCGGCACATTGGCGGCGCACTATGCGGCACATTGGGCGGCGCACTATGCGGCGCACTATGCGGCGCACTATGCGGCGCACTATGCGGCGCACTATGGCGGGCG	Query	61	TTATTA	GCGTTAC	TGGCAGAACGO	CGCGAACTGGC	CGTCGAGGTGGG	AAAGCCAAACTG	1
Query 121 CTCTCSCATCSCCCGGTACGTGATATTGATCGTGAACGCGATTGCTGGAAGATTAATT Sbjet 121 CTCTCGCATCGCCCGGTACGTGATTGATCGTGAACGCGTATTGCTGGAAGATTAATT Sbjet 121 ACCCTCGGTAAAGGCGCACCATCGTGGACCCCATTACATTACTGCCCTGTTCCAGCTATC Sbjet 181 ACCCTCGGTAAAGGCGACCATCGTGGACCCCATTACATTACTGCCCTGTTCCAGCTATC Query 241 ATTGAAGATTCCGTATTAATCCAGCGCTTTGCTCCCAACACATCTCAATAAAATTAAT Sbjet 241 ATTGAAGATTCCGTATTAATCCAGCAGGCTTTGCTCCCAACACATCTCAATAAAATTAAT Query 261 CCGCACTCAGCCACCACCGCGTTTTGCTGCCCCAAAGGTTCTATTCCAACAATCTGCGGGGG Sbjet 261 CGCCAGTATCGCCGCTCACTTGGGCCGACTTGCGTCTAATCGCAGCGAATTGGCGGGGGGGG	Sbjct	61	TTATT	GCGTTAC	TGGCAGAACGO	GCGCGAACTGGC	CGTCGAGGTGGG	AAAGCCAAACTG	1
Sbjet 121 CTCTCGCATCGCCCGGTACGTGALATTGALCGTGALGGGGATTGGTGGGALGAGTATAATT Query 181 ACGCTCGGTAAAGCGCACCATCTGGACGCCCCATACATTACTGCCGCTGTTCCAGCTCATC Sbjet 181 ACGCTCGGTAAAGCGCACCATCTGGACGCCCCATACATAC	Query	121	CTCTCO	CATCGCC	CGGTACGTGAI	ATTGATCGTGA	ACGCGATTTGCTG	GAAAGATTAATT	1
Query 181 ACGCTCGGTAAAGCGCACCATCTGGACGCCCATTACATTACTCGCCGTGTCCAGCTCATC Sbjet 181 ACGCTCGGTAAAGCGCCATCTGGACGCCATTACATTACTCGCCGCCAGTCACAACCATCTCAAATAAAT	Sbjct	121	CTCTCG	CATCGCC	CGGTACGTGAT	PATTGATCGTGA	ACGCGATTTGCTG	GAAAGATTAATT	1
Sbjet 181 ACCETCGGTHARAGEGEACCATTEGEAGGECEATTACATTACTECCONTECTACCONTECTACT Query 241 ATTGARGATTECCGTATACCTCGCAGGECTTGECCCAACAACATCTCAATAANAATTAT Sbjet 241 ATTGARGATTECCGTATACTCAGEAGGECTTGECCCAACAACATCTCAATAANAATTAT Sbjet 241 ATTGARGATTECCGTATAAACTCAGEAGGECTTGECCCAACAACATCTCAATAANAATTAAT Guery 301 CCGCCACTCAGEAGGECTTTTETEGGGCCCAAAGGTTETATTECCACTTTGEGGGG Sbjet 301 CCGCCACTCAGEAGEGCTTTTETEGGGCCCAAAGGTTETATTECCAACATTGAAAATTGEGC Sbjet 311 CCGCCACTCAGEAGEGCGGCACGCCCAAAGGCCGACTATGCGGTCGACCGATTGAAAAT Sbjet 312 CGCCCACTCAGECAGGCGGCAAGCCGACCAATGCCGTCGCACCGATTGAAAAT Sbjet 313 CCGCCACTTTECAGGTGGAAACCGGCCAGGCCGACTATGCCGTCGCACCGATTGAAAAT Sbjet 421 GATATTTTTAATCAGGTGGAAACCGGCCAGGCCGACTATGCGCTCGACCGATTGCAGGTAAAAT Sbjet 421 GATATTTTTAACAGGTGGAAACCGGCCACGCCGACCACTTGCGCGACCTACCGATTGAAAAT Sbjet 421 GATATTTTTAACCGGCTGGAACCGGCCGACCTTTGGCGCGACCTACCGGATGGAAAAT Query 421 GATATTTTTAACCGGCCATATGCAGCCCACCTTGGCGGCGCCTACTGGGCGCACTTCG Sbjet 421 GATGGTTACCGGGTGCACTACGGCCCATTGCGGCGACTTCGCGGCACATCGGGCGACATTCA Sbjet 541 GTTGGCGG	Query	181	ACGCTO	GGTAAAG	CGCACCATCTO	GACGCCCATTA	CATTACTCGCCTC	TTCCAGCTCATC	2
Query 241 ATTGAAGATTCCGTATTAACTCAGCAGGCTTTGCTCCAACAACATCTCAATAAAATTAAT Sbjet 241 ATTGAAGATTCCGTATTAACTCAGCAGGCTTTGCTCCAACAACATCTCAATAAAATTAAT Query 301 CCGCACTCAGCACGCATCGCTTTGCTCGGCCCAAAGGTTCTTATTCCCAATAAAATTAAT Query 301 CCGCACTCAGCACGCATCGCTTTGCTGCGGCCCAAAGGTTCTTATTCCCAATAAAATTAAT Query 301 CCGCACTCAGCACGCATCGCTTTGCGGCCCAAAGGTTCTTATTCCCAATAAAATTAAT Sbjet 301 CCGCCACTAGCTGCCCGTCACTTGCGGCCCAAAGGTTCCAATGAAAGTGCCCCACATTGCGGCCCAAATTGCCC Sbjet 361 CGCCACTATGCTGCCCGTCACTTGGGCCCAGCCGACTATGCCGTCGCCCCAAATTGCC Sbjet 361 CGCCACTAGCTGCGCCGTCACTTGAGCAATCCATGCAGCTTGCCGGCCG	Sbjct	181	ACGCTO	GGTAAAG	CGCACCATCT	GACGCCCATTA	CATTACTCGCCTG	TTCCAGCTCATC	2
Sbjet 241 ARTGARAGATTCOGTATTAACTCAGCAGGCTTTGCTCCAACAACATCTCATTAAAATTAAT Query 301 CCGCACTCAGCACGCATCGCTTTGCTGCGCCCAAAGGTTCTATTCCCATCTGCGGCG Sbjet 301 CCGCCACTCAGCACGCATCGCTTTGCTGCGCCCCAAAGGTTCTATTCCCATCTGCGGCGG Sbjet 301 CCGCCACTCAGCACGCATCGCTTTGCGGCCCCAAAGGTTCCTATTCCCATCTGCGGCGG Sbjet 301 CCGCCACTATGCTGCCCGTCACTTGAGCCATTCATGAAGGTCTGCCCCACATTGAAATTGCC Sbjet 301 CCGCCACTATGCTGCCCGTCACTTGAGCAATTCATTGAAGGTGCGCCCAAATTGCC Sbjet 301 CCGCCACTATGCGTGCCATCATTGAGCATTCATTGAAGGTGCGCCCAAATTGCC Sbjet 421 GATATTTTTAATCAGGTGGAAACCGGCCAGCCGACTATGCCGTCGCACCGATTGCAGTT Sbjet 421 GATATTTTTAATCAGGTGGAAACCGGCCAGCCGACTATGCCGCCGCCATGCGGCAATTGCATT Sbjet 421 GATATTTTTAATCAGGTGGCAAACGGCCGACCGATCGCGCGCCGTCGCGCACTGCGGCACTTGGTGGGGCGATTACTATCAGGGCGCGCGC	Query	241	ATTGA	GATTCCG	TATTAACTCAG	CAGGCTTTGCI	CCAACAACATCTC	AATAAAATTAAT	3
Query 301 CCGCACTCAGCACGCATCGCTTTCCCGCGCCCAAAGGTTCTATTCCCATCTTGCGGCG Sbjet 301 CCGCCACTCAGCACGCATCGCTTTTCCGGCCCCAAAGGTTCTATTCCCATCTTGCGGCG Query 361 CGCCCAGTATGCTGCCCGTCACTTTGAGCAATTCATTGAAAGGTGGCTGCGCCCAAATTTGCC Sbjet 361 CGCCCAGTATGCTGCCCGTCACTTTGAGCAATTCATTGAAAGGTGGCTGCGCCCAAATTTGCC Query 421 GATATTTTTAATCAGGTGGAAACCGGCCAGGCCGACTATGCCGTCGCACCGATTGAAAAT Sbjet 421 GATATTTTTAATCAGGTGGAAACCGGCCAGGCCGACTATGCGCGCCACCGACTGACGATGCAGTTGGAT Query 481 ACCAGCTCCGGTGCCATAAACGACGTTAACGACCTATGCGTGCG	Sbjct	241	ATTGAA	GATTCCG	TATTAACTCAG	CAGGCTTTGCI	CCAACAACATCTC	AATAAAATTAAT	3
Sbjet 301 CCGCACTCAGGACCGARCGCTTTTCTCGGGCCCCAAAGGTTCTTATTCCCATCTTGGGGG Guery 361 CGCCAGTATGCTGCCCGTCACTTTGAGCATTCATTGAAAGTGGCTGCGCCAAATTTGCC Sbjet 361 CGCCAGTATGCTGCCCGTCACTTTGAGCATTCATTGAAAGTGGCTGCGCCAAATTTGCC Guery 421 GATATTTTTAATCAGGTGGAAACCGGCCAGCCGACTATGCCGTGCGCCGACTAGGCGTGCGACCGATTGAAAAT Sbjet 421 GATATTTTTAATCAGGTGGAAACGGCCAGCCGACTATGCCGTCGGACCGACTTGAAAAT Query 481 ACCAGCTCCGGTGCCATAAACGACGTTACGACTGCGCGCACTATGCCGGCACTATCCAGCTTGTGGGATT Sbjet 481 ACCAGCTCCGGTGCCATAACGACGTTACCAACTGCGCGCGC	Query	301	CCGCAC	TCAGCAC	GCATCGCTTT	CTCGGCCCCAP	AGGTTCTTATTCC	CATCTTGCGGCG	3
Query 361 CGCCAGTATGCTGCCCGTCACTTTGAGCAATTCATTGAAAGTGGCTGCGCCCAAATTTGCC Sbjet 361 CGCCAGTATGCTGCCCGTCACTTGGACAATTCATTGAAAGTGGCTGCGCCGCCAAATTTGCC Query 421 GATATTTTTAATCAGGTGGAAACCGGCCAGCCGACTATGCCGTCGTACCGATTGAAAAT bjet 421 GATATTTTTAATCAGGTGGAAACGGCCAGCGACTATGCCGTCGTACCGACTTGCGATTGAAAAT Query 481 ACCAGCTCCGGTGCCATAAACGACGTTTACGATCTGCTGCGACATACCAGCTTGTCGGATT bjet 481 ACCAGCTCCGGTGCCATAACGACGTTTACGATCTGCTGCGAACATACCAGCTTGTGGATT bjet 541 GTTGGGGAGATGACGGTTAACTATCGACCATTGTTGTTGGTGCTCCGGCACTAGCTGAGTATTA query 601 TCCACCATCAATAGCGTCTAACGACCATCCGCAGCCATTGCGACAATGCAACGAAATTCCTT juliiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiii	Sbjct	301	CCGCAC	TCAGCAC	GCATCGCTTT	CTCGGCCCCAA	AGGTTCTTATTCC	CATCTTGCGGCG	3
Sbjet 361 CGCCAGATTATGCTGCCGTCGTTCTGGCAGCGACTATGCCGTCGTGCGCAAAAATTTGCC Query 421 GATATTTTTAATCAGGTGGAAACCGGCCAGCCGACTATGCCGTCGTACCGATTGAAAAT sbjet 421 GATATTTTTAATCAGGTGGAAACGGCCAGCGGCCAGCTATGCCGTCGTGCGATAGAAAAT Query 481 ACCAGCTCCGGTGCCATAAACGACGTTTACGATCTGCTGCAACATACCAGCTTGTCGATT Sbjet 481 ACCAGCTCCGGTGCCATAACGACGTTTACGATCTGCTGCGAACATACCAGCTTGTCGATT Sbjet 541 GTTGGCGAGATGACGTTAACTATCGACCATTGTTGTTGGTGCTCCGGCACTACTGATTAA Query 601 TCCACCATCAATACGGTCTAACGACCATCCGCAGCCATTGCTGCGGCAATGCAACGAAATTCCTT Juliiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiii	Query	361	CGCCAG	TATGCTG	CCCGTCACTT	GAGCAATTCAT	TGAAAGTGGCTGC	GCCAAATTTGCC	4
Query 421 GATATTTTAATCAGGTGGAAACCGGCCGAGCTATGCCGTCGTACCGATTGAAAAT Sbjet 421 GATATTTTAATCAGGTGGAAACCGGCCGAGGCCGACTATGCCGTCGTACCGATTGAAAAT Query 481 ACCAGCTCCGGTGCCATAAACGACGTTTACGATCGGCTGCAACATACCAGGCTGTGCGAAT Query 541 GTTGGCGAGATGACGTTAACGACGTTACGACCTGTGTGGCAACATACCAGGCTGTCGGGCAATTA Query 541 GTTGGCGAGATGACGTTAACTATCGACCATTGTTGTTGGTCTCCGGCCATGGATTA Query 541 GTTGGCGAGATGACGTTAACTATCGACCATTGTTGTTGGTCTCCGGCCATGCAATGTAATTA Query 601 TCCACCATCAATACGGTCTACAGCATCGCACGCCATTCCAGCAATGCAGGCAATTCCTT Sbjet 601 TCCACCATCAATACGGTCTACAGCCATCCGCAGCCATTCCAGCAATGCAGGCAAATTCCTT Query 601 TCCACCATCAATACGGTCTACAGCCATGGCAGCCATTCCGAGCAATGCAGGCAAATCCTT Sbjet 601 TCCACCATCAATACGGTCTACAGCCATGCGCGCACTTCCGGCAATGGAAAAG Query 601 AATCGTTATCCGCACTGGAAGCTGGCGCTGGTGGGAAGGTGGCGACGCGCCGCCGCATGGGAAAGCACGGCGCCGCGCGCAAAGCACGGCGCAAAACCACGGCGCACAACGCCGGCCACAAACCACGTCGGCGCACAAACCACCGCGGCACAACGCGCGGCAAAGCACGGCGG	Sbjct	361	CGCCAG	TATGCTG	CCCGTCACTT	GAGCAATTCAT	TGAAAGTGGCTGC	GCCAAATTTGCC	4
Sbjet 421 GATATTATAACAGSTGGAAACCGGCCAGGCCGACTATGGCCGTCGTACCGATTGAAAAT Query 481 ACCAGCTCCGGTGCCATAAACGACGTTTACGATCTGCTGCAACATACCAGCTGTGCGATT Sbjet 481 ACCAGCTCCGGTGCCATAAACGACGTTTACGATCTGCTGCAACATACCAGCTGTGCGATT Query 541 GTTGGCGAGATGACGTTAACTATCGACCATTGTTGTTGGTCTCCGGCACTACGAATTA Sbjet 541 GTTGGCGAGATGACGTTAACTATCGACCATTGTTGTTGGTCTCCGGCCATTGCAGCTACTGATTTA Query 601 TCCCACCATCAATACGGTCTACAGCCATCGCCAGCCATTCCAGCAAGGCGAAATCCTT Sbjet 601 TCCCACCATCAATACGGTCTACAGCCATCGCCGCCCATTCCAGCAATGCAGGCAAATCCTT Query 601 TCCCACCATCAATACGGTCTACAGCCATCGCGCGCCATTCCGGCAATGGAAAAG Sbjet 601 TCCCACCATCAATACGGTCTACAGCCATCGCGCGCATTCCGGCAATGGAAAAAG Sbjet 601 AATCGTTATCCGCACTGGAAGATTGAATATACCGAAAGTACGTCTGCGGCAATGGAAAAG Sbjet 601 AATCGTTATCCGCACTGGAAGCTGGCGCTGGTGGGAAAGCAGCGGCGCAATGGAAAAG Query 721 GTTGCACAGGCAAAATCACCCGCATTGTCGGCTGGTGGGACAAACCTCACCGGATT Sbjet 781 TACGGTTTGCCAGGTAATGGCGTATTGAAGCAATTAACGAGCAAAACCACGGCGAAAACCACCGATT Query 901 TTAATGGCGACCGGCCAACAAGCCGGTGGTGGTGGTGGTGGACAAAACCACCGGTGGCAAACCACGGCGAAACCACGGCGCAAACCACGGCCGGAATCCAGGGCGAAATCCACGGCGGAAACCACGGCGGAAACCACGGCGGAAATCACCGCGGTATCACGCGGAAATCCACGGGGAAAACCACGGCGAAAGCACGGCGGAAATCACCCGGTCGGAGAATCACGCCGAT	Query	421	GATATI	TTTAATC	AGGTGGAAACO	GGCCAGGCCGA	CTATGCCGTCGT	CCGATTGAAAAT	4
Query 481 ACCAGCTCCGGTGCCATAAACGACGTTTACGATCTGCTGCAACATACCAGCTTGTCGATT sbjet 481 ACCAGCTCCGGTGCCATAAACGACGTTACGATCTGCTGCGAACATACCAGCTTGTCGATT Query 541 GTTGGCGAGATGACGTTAACGACCATGTTGTTGTTGGTGCGCCCGGCACTACTGATTA guery 601 TCCACCATCAATACGGTCTACAGCCATCCGCAGCCATTCCAGCAATGCAGCAATCCTT guery 601 TCCACCATCAATACGGTCTACAGCCATCCGCAGCCATTCCAGCAATGCAGCAATGCAGAAAAG sbjet 601 TCCACCATCAATACGGTCTACAGCCATCCGCAGCCATTCCAGCAATGCAGAAAAG sbjet 601 TCCACCATCAATACGGTCTACAGCCATCCGCAGCGCATTCCAGCGACATGGAAAAG guery 661 AATCGTTATCCGCACTGGAAGATTGAATATACCGAAAGTACGTCTGCGGCGCAATGGAAAAG guery 721 GTTGCACAGGCAAAATCACCGCATGTGTGCTGCGGTGGGAAGCGAAGCTGGCGGCACTTG guery 721 GTTGCACAGGCAAAATCACCGCATGTGTGCTGCGGTGGGAAGCGAAACTTCACCCGATT guery 781 TACGGTTTGCAGGTACTGGAGCGTATTGAAGCAAATCAGCGGACAAAACTTCACCGGATT guery 841 GTGGTGTTGGCGCGTAAAGCCATTAACGTGTCTGATCAGGGTACTGGCGAAAACCACGGTG guery 901 TTAATGGCGACCGGGCAACAAGCCGGTGCGCTGGTGTGAAGGGTACTGCGGCAAA guery 901 TTAATGGCGACCGGGCAACAAGCCGGTCGGCGCTGGTTGACGGGGAAATCCACGGTG guery 901 TTAATGGCGACCGGGCAACAAGCCGGTCGGCGCGGGTGCGCGGTGCGCGGAAAACCACGGTG s	Sbjct	421	GATATI	TTTAATC	AGGTGGAAACO	GGCCAGGCCGA	CTATGCCGTCGT	CCGATTGAAAAT	4
Sbjet 481 ACCAGETCCGGEGCCATAAACGACGTTACGATCGCTGCTGCAACATACCAGCTTGTCGGATTA Query 541 GTTGGCGAGATGACGTTAACTATCGACCATTGTTGGTGGTCTCCGGCACTACTGATTA guery 601 TCCACCATCAATACGGTCTACAGCCATCGCAGCCATTCCAGCAATGCGACAAATCCCTT guery 601 TCCACCATCAATACGGTCTACAGCCATCCGCAGCCATTCCAGCAATGCGACAAATCCCTT guery 601 TCCACCATCAATACGGTCTACAGCCATCCGCAGCCATTCCAGCAATGCGACAAATCCCTT guery 661 AATCGTTATCCGCACTGGAAGATTGAATATACCGAAAGTACGTCTGCGGCCAATGGAAAAG guery 721 GTTGCACAGGCAAAATCACCGCATGTGCTGCGGTGGGAAGCGGAGCGGGGCACTTG sbjet 721 GTTGCACAGGCAAAATCACCGCCATGTGGTGCGGTGGGAAGCGGAAGCTGCCGGGCACTTG guery 781 TACGGTTTGCAGGTACTGGAGCGTATTGAAGCAAATCAGCGACAAACTCCACCGGATT guery 781 TACGGTTTGCCAGGTACTGGAGCGTATTGAAGCAAATCAGCGACAAACCACGTTG guery 841 GTGGTGTTGGCGCGTAAAGCCGATCAGGTGCTGGATCAGGGTACTCCGGCAAACCACGTTG guery 901 TTAATGGCACCGGGCCAACAAGCCGGTCGGTGGTGAAGCGGTTGCTGGTACTGCGGCAAC sbjet 901 TTAATGGCACCGGGCAACAAGCCGGTCGGTGGTGTGAAGCGGTACTCAGGGAAAGCACGGTG sbjet 901 TTAATGGCGACCGGCCGACCAAGCCGGTCGGCGGGTGTACGGGGAAATCCACGGGAACACCACGAAG guery 901 TTAATGGCGACCGGGCCGACAAGCCGGTCGGTGGGCGGGGGTGTACCGGGGAAACCACGGGAAGGAA	Query	481	ACCAGO	TCCGGTG	CCATAAACGAC	GTTTACGATCI	GCTGCAACATACO	AGCTTGTCGATT	
Query 541 GTTGGCGAGATGACGTTAACTATCGACCATTGTTGTTGGTCTCCGGCACTACTGATTTA Sbjet 541 GTTGGCGAGATGACGTTAACTATCGACCATTGTTTGTTGGTCTCCGGCACTACTGATTTA Query 601 TCCACCATCATACGGTCTACAGCCATCGCAGCCATTCCAGCAATGCAGCAAATTCCTT Query 661 AATCGTTATCCGCACTGGAAGATTGAATATACCGAAAGTACGTCTGCGGCAATGGAAAAG Sbjet 661 AATCGTTATCCGCACTGGAAGATTGAATATACCGAAAGTACGTCTGCGGCAATGGAAAAG Query 721 GTTGCACAGGCAAAATCACCGCATGTTGCTGCGGTTGGGAAGCGGAAGCTGGCGGCACTTTG Sbjet 721 GTTGCACAGGCAAAATCACCGCATGTTGCTGCGTTGGGAAGCGAAGCTGGCGGCACTTTG Query 721 GTTGCACAGGCAAAATCACCGCATGTTGCTGCGGTTGGGAAGCGAAACTGGCGCGCACTTTG Guery 781 TACGGTTTGCAGGGCATATGGAGCGTATGAAGCAAATCAGCGACAAACTCACCCGATT Sbjet 781 TACGGTTGGCGCGTAAAGCCATTAACGTGTCTGATCAGGTTCCGGCGGAAAACCACGTTG Sbjet 841 GTGGTGTTGGCGCGGTAAAGCCATTAACGTGTCTGATCAGGTTCCGGCGGAAAACCACGTTG Sbjet 841 GTGGTGTTGGCGCGGTAAAGCCGGTCGGTGGTGAAGAGCTGGTGGTAACCACGGTGG Sbjet 901 TTAATGGCGACCGGCCAACAAGCCGGTCGGTGGTGGAAGCGTAATCCATGGGAAAGCACCAGTGG Sbjet 901 TTAATGGCGACCGGCCAACAAGCCGGTCGGTGGGATCACGCGGAAATCCACGGGAAAGCACACGGAA Sbjet 901 TTAATGGCGACCGGCCACCGGCTGGAATCCACGCGGGAATCCACGGGAAAGCCCGGAAGAGCGAACGAA	Sbjct	481	ACCAGO	TCCGGTG	CCATAAACGAC	GTTTACGATCI	GCTGCAACATAC	AGCTTGTCGATT	-
Sbjet 541 GTTGGCGAGATGACGGTTAACTATCGACCATTGTTGTTGGTCTCCGGCACTACTGATTTA Query 601 TCCACCATCATACGGTCTACAGCCATCCGCAGCCATTCCAGCAATGCAAGCAA	Query	541	GTTGGC	GAGATGA	CGTTAACTATC	GACCATTGTTI	GTTGGTCTCCGGC	ACTACTGATTTA	e
Query 601 TCCACCATCAATACGGTCTACAGCCATCCGCAGCCATTCCAGCAATGCAGCAAATTCCTT Sbjet 601 TCCACCATCAATACGGTCTACAGCCATCCGCAGCCATTCCAGCAATGCAGCAAATTCCTT Query 661 AATCGTTATCCGCACTGGAAGATGAATATACCGAAAGTACGTCTGCGGCAATGGAAAAG Sbjet 661 AATCGTTATCCGCACTGGAAGATGAATATACCGAAAGTACGTCTGCGGCAATGGAAAAG Query 721 GTTGCACAGGCAAAATCACCGCATGTGCTGCGGTGGGAAGCGAAGCTGGCGGCACTTG Sbjet 721 GTTGCACAGGCAAAATCACCGCATGTGCTGCGTGGGAAGCGAAGCTGGCGGCGCATTG Sbjet 721 GTTGCACAGGCAAAATCACCGCATGTGCTGCGTGGGAAGCGAACTGCACCGGCGCATTG Sbjet 721 GTTGCACAGGCAAAATCACCGCATGTGCTGCGGTGGGAAGCGAACTCACCCGATTT Sbjet 721 GTTGCACAGGCAAAATCACCGCATGGAGCGATATGAAGCAAAACTCACCCGATTT Sbjet 781 TACGGTTTGCAGGTACTGGAGCGTATAGAGCAAAATCAGCGGCAAAAACTTCACCCGATTT Guery 841 GTGGTGTTGGCGCCTAAAGCCATTAACGTGTCTGACAGGTTCCAGGGAAAACCACCGTTG Sbjet 901 TTAATGGCGACCGGGCAACAAGCCGGTGGTGCGCTGGTTGAAGCGTGCTGGTAATCCATGGGCAAC Query 901 TTAATGGCGACCGGGCAACAAGCCGGTGGTGGCGCGTGTTCCAGGGTAATCCATGGGGAAAGAG Sbjet 901 TTAATGGCGATATTCAGGCCGATCTGGAATCACGCCGGTAATCCATGGGAAAGCAGAA Sbjet 901 TAGTGTTATGACCCGGTCGGAACCAGCCGGTCGGTGCGGCGAATCCACGGGGAAAGAG <td< td=""><td>Sbjct</td><td>541</td><td>GTTGGC</td><td>GAGATGA</td><td>CGTTAACTATC</td><td>GACCATTGTTT</td><td>GTTGGTCTCCGGC</td><td> ACTACTGATTTA</td><td>6</td></td<>	Sbjct	541	GTTGGC	GAGATGA	CGTTAACTATC	GACCATTGTTT	GTTGGTCTCCGGC	 ACTACTGATTTA	6
Sbjet 601 TCCACCATCAATACGGTCTACAGCCATCCGCAGCAGCAGCGCAATGCAGCAATTCCTT Query 661 AATCGTTATCCGCACTGGAAGATTGAATATACCGAAGTACGTCTGCGGCAAATGGAAAAG Sbjet 661 AATCGTTATCCGCACTGGAAGATTGAATATACCGAAGTACGTCTGCGGCGAATGGAAAAG Query 721 GTTGCACAGGCAAAATCACCGCATGTGCTGCGGCAGAGCTGGCGGCACTTG Sbjet 721 GTTGCACAGGCAAAATCACCGCATGTGCTGCGCGTGGGAAGCTGGCGGCACTTG Query 781 TACGGTTTGCAGGTACTGGAGCGTATTGAAGCAAATCAGCGACAAAACTCACCCGATTT Sbjet 781 TACGGTTTGCAGGTACTGGAGCGTATTGAAGCAAATCAGCGACAAAACTTCACCCGATTT Query 841 GTGGTGTTGGCGCGTAAAGCCATTAACGTGCTGGATCAGGGTCCGGCGAAAACCACCGTTG Query 901 TTAATGGCGACCGGCAACAAGCCGGTGCGTGGTGAAGCGGTTGCTGGGTGACTGCGGCAAC Sbjet 901 TTAATGGCGACCGGCCAACAAGCCGGTGGGTGGATTCACGGTTGCTGGTACTGCGGCAAC Query 961 CACAATCTGATTATGACCCGTCTGGAATCACGCCCGATTCACGGTAATCCATGGGAAGA Sbjet 901 TTAATGGCGACCGGGCAACAAGCCGGTGGGCGGCGGTGGTTGAAGCGTATCCATGGGAAGAG Sbjet 901 TTAATGACCGGTCTGGAATCACGCCCGATTCACGGTAATCCATGGGAAGAG Sbjet 901 TTAATGACCGGTCTGGAATCACGCCCGATTCACGGTAATCCATGGGAAGAG Sbjet 901 TTAATGGCGACCGGCCAACAAGCCGGTCGGTGGCGCGGTGTTCACGGTAATCCATGGGAAGAG Sbjet <td< td=""><td>Query</td><td>601</td><td>TCCACC</td><td>ATCAATA</td><td>CGGTCTACAGO</td><td>CATCCGCAGCO</td><td>ATTCCAGCAATGC</td><td>AGCAAATTCCTT</td><td>6</td></td<>	Query	601	TCCACC	ATCAATA	CGGTCTACAGO	CATCCGCAGCO	ATTCCAGCAATGC	AGCAAATTCCTT	6
Query 661 AATCGTTATCCGCACTGGAAGATTGAATATACCGAAGTACGTCTGCGGCAATGGAAAAG Sbjet 661 AATCGTTATCCGCACTGGAAGATTGAATATACCGAAGTACGTCTGCGGCGACATGGAAAAG Query 721 GTTGCACAGGCAAAATCACCGCATGTGCTGCGGTGGGAAGCGGAGCGGCGCACTTG Sbjet 721 GTTGCACAGGCAAAATCACCGCATGTGCTGCGGCTGGGAAGCGAAGCTGGCGGCACTTG Query 781 TACGGTTTGCAGGGTACTGGAGCGTATTGAAGCAAATCAGCGACAAAACTTCACCCGATTT Sbjet 781 TACGGTTTGCGGGCGTAAAGCCAATAACAGCGGACACAGCGGCGAAAACTCACCCGATT Query 781 TACGGTGTTGGCGCGTAAAGCCATTAACGTGTCTGATCAGGTCCGGCGAAAACTCACCGGTG Sbjet 781 TACGGTGTTGGCGCGGTAAAGCCATTAACGTGTCTGATCAGGTTCCGGCGAAAACCACGTTG Query 841 GTGGTGTTGGCGCGGTAAAGCCATTAACGTGTCTGATCAGGTTCCGGGCGAAAACCACGTTG Sbjet 901 TTAATGGCGACCGGGCAACAAGCCGGTGGCTGGTTGAAGCGTTGCTGGTACTGCGCAAC Sbjet 901 TTAATGGCGACCGGGCAACAAGCCGGTGGCTGGTTGAAGCGTTGCTGGTACTCACGGGAAGAG Sbjet 901 TTAATGGCGACCGGGCAACAAGCCGGTGGCGCGTGGTTGAAGCGTAATCCATGGGAAAGC Sbjet 901 TTAATGGCGAACCAGCGTGTGGGAATCACGCCGATTCACGGTAATCCATGGGAAAGCAGAAG Query 961 CACAATCTGATTATGACCCGTCTGGAATCACGCCGATTCACGGTAATCCATGGGAAAGCAAGAGC Sbjet 1021 ATGTTCTATCTGGATATTCAGGCCAATCTTGAATCACGCGGAAATCCACGGGAAAGCAAAGCCGAA	Sbjct	601	TCCACC	ATCAATA	CGGTCTACAGO	CATCCGCAGCO	ATTCCAGCAATGC	AGCAAATTCCTT	6
 sbjet 661 AATCGTTATCCGCACTGGAAGATGGAATGACCGCGAAAGGAGCGGCGCGCAATGGGAAAAG Query 721 GTTGCACAGGCAAAATCACCGCATGTTGCGGCGAAGGAGCGGAGCGGCGCGCACTTG sbjet 721 GTTGCACAGGCAAAATCACCGCATGTGCGGCGTAGGGGAAGCGGCGAACTCGCGCGCACTTG Query 781 TACGGTTTGCAGGTACTGGAGCGTATGAAGCAAATCACCGCGACAAACCTCCCCCGATT ALGCGTTTGCAGGTACTGGAGCGTATGAAGCAATCACCGCGCAAAACCTCCCCCGATT Query 841 GTGGTGTTGGCGCGTAAAGCCATTAACGTGTCTGATCAGGTTCCGGCGGAAAACCACGTTG ybjet 841 GTGGTGTTGGCGCGGTAAAGCCAATAACGTGTCTGATCAGGTTCCGGCGGAAAACCACGTTG Query 901 TTAATGGCGACCGGGCAACAAGCCGGTGGTGGAGCGTTGCTGGGCGGTAACGCGGCCAACAAGCCGGTGGTGAAGCCGTTGCTGGGTGGTGGTGGTGGTGGTGGTGGTGGTGGT	Query	661	AATCGT	TATCCGC	ACTGGAAGATI	GAATATACCGA	AAGTACGTCTGCG	GCAATGGAAAAG	7
Query 721 GTTGCACAGGCAAAATCACCGCATGTTGCTGCGGTTGGGAAGCGAAGCTGGCGGCACTTTG Sbjet 721 GTTGCACAGGCAAAATCACCGCATGTTGCTGCGGTTGGGAAGCGAAGCTGGCGGCACTTTG Query 781 TACGGTTTGCAGGTACTGGAGCGTATTGAAGCAAATCAGCGGCAAAACTCACCCGATTT Sbjet 781 TACGGTTTGCAGGTACTGGAGCGTATTGAAGCAAATCAGCGGCGAAAACTCACCCGATTT Query 781 TACGGTTGGCGCGTAAAGCCATTAGAGCAAATCAGCGGCGAAAACTCACCCGATT Query 841 GTGGTGTTGGCGCCGTAAAGCCATTAACGTGTCTGATCAGGTTCCGGCGGAAAACCACGTTG sbjet 841 GTGGTGTTGGCGCGGTAAAGCCATTAACGTGTCTGATCAGGTTCCGGCGGAAAACCACGTTG guery 901 TTAATGGCGACCGGCCAACAAGCCGGTGGTTGAAGCGTTGCTGGTAACTGCGCAAC sbjet 901 TTAATGGCGACCGGCCAACAAGCCGGTCGGTGGTTGAAGCGTAATCCAGGGAAACCACGTTG guery 961 CACAATCTGATATGACCGGTCTGGAATCACCGCCGGATTCACGGTAATCCATGGGAAAGG sbjet 961 CACAATCTGATATGACCGGTCTGGAATCACGCCCGATTCACGGTAATCCATGGGAAAGG sbjet 1021 ATGTTCTATCTGGATATTCAGGCCAATCTTGAATCACGCGGAAATCCACGGGAAAGCGAAGGAA sbjet 1021 ATGTTCTATCTGGATATTCAGGCCAATCTTGAATCAGCGGAAATCACCCGAGCGAAAGCGAAGGAAG	Sbjct	661	AATCGT	TATCCGC	ACTGGAAGATI	GAATATACCGA	AAGTACGTCTGCG		7
sbjet 721 GTTGCACAGGCAAAATCACCGCATGTGCGCGTGGGAAGCTGGCGAACTTGGCGCGCACTTG Query 781 TACGGTTTGCAGGTACTGGAGCGTATTGAAGCAAATCAGCGGACAAAACTTCACCCGATTT sbjet 781 TACGGTTTGCAGGTACTGGAGCGTATTGAAGCAAATCAGCGGACAAAACTTCACCCGATTT Query 781 TACGGTTTGCAGGTACTGGAGCGTATTGAAGCAAATCAGCGGACAAAACTTCACCCGATTT Query 841 GTGGTGTGGCGCGTAAAGCCATTAACGTGTCGATCAGGTTCCGGCGGAAAACCACGTTG sbjet 841 GTGGTGTGGCGCGTAAAGCCGTGGTGGTGGTGGTGCAGGTCGGCGGAAAACCACGTTG Query 901 TTAATGGCGGACCGGGCAACAAGCCGGTGGCTGGTTGAAGCGTTGCTGGTACTGCGCAAC Sbjet 901 TTAATGGCGGCCGGCAACAAGCCGGTGGTGGTGGTGGTGCTGGTACTGCGGAAACCACGTTG Query 961 CACAATCTGATTATGACCCGTCTGGAATCACGCCCGATTCACGGTAATCCATGGGAAAGA Sbjet 961 CACAATCTGATATTAGACCCGTCTGGAATCACGCCCGATTCACGGTAATCCATGGGAAAGA Sbjet 1021 ATGTTCTATCTGGATATTCAGGCCAATCTTGAATCAGCGGAAATGCAAAAAGC GACAAA sbjet 1021 ATGTTCTATCTGGATATTCAGGCCAATCTTGAATCAGCGGAAATGCAAAAGC GACAAA sbjet 1021 ATGTTCTATCTGGATATTCAGGCCAATCTTGAATGAGCGGAAATGCAAAAGC GACAAA sbjet 1081 GAGTTAGGGGAAATCACCCGTTCAATGAAGGTATTGGGCTGTTACCCAAGTGAGAACGTA Query 1081 GAGTTAGGGGAAATCACCCGTCCAATGAAGGTATTGGGCTGTTACCCAAGTGAGAACGTA	Query	721	GTTGCA	CAGGCAA	AATCACCGCAI	GTTGCTGCGTI	GGGAAGCGAAGCI	GGCGGCACTTTG	7
Query 781 TACGGTTTGCAGGTACTGGAGCGTATTGAAGCAAATCAGCGACAAAACTTCACCCGATTT Sbjet 781 TACGGTTTGCAGGTACTGGAGCGTATTGAAGCAAATCAGCGACAAAACTTCACCCGATTT Query 841 GTGGTGTTGGCGCGTAAAGCCATTAACGTGTCTGATCAGGGTCCGGCGAAAACCACCGTG Sbjet 841 GTGGTGTTGGCGCGTAAAGCCATTAACGTGTCTGATCAGGTTCCGGCGAAAACCACCGTG Query 901 TTAATGGCGACCGGGCAACAAGCCGGTGGCTGGTTGAAGCGTTGCTGGTACTGCGCAAC Sbjet 901 TTAATGGCGACCGGGCAACAAGCCGGTGGCTGGTTGAAGCGTTGCTGGTACTGCGCGAAC Query 901 TTAATGGCGACCGGGCAACAAGCCGGTGGGTGGATGCACGGTAATCCATGGGAACGAG Sbjet 901 TTAATGGCGACCGGGCAACAAGCCGGTGGCTGGTTGAAGCGTAATCCATGGGAACGACCACGTG Query 901 TTAATGGCGACCGGCCAGGTCGGAATCACCGCCGATTCACGGTAATCCATGGGAAAGG Sbjet 901 TTAATGGCGACCGGCCAGTTGGAATCACGCCGGTTGAAGCGGAAATGCAAGAAGC Sbjet 901 TTAATGGCGACCGGCCAGTCTGGAATCACGCCGGTTAACCAGGGGAAAGCAAGC	Sbjct	721	GTTGCA	CAGGCAA	AATCACCGCAT	GTTGCTGCGTT			-
Sbjet 781 TACGGTTTGCAGGTACTGGAGCGTATTGAAGCAAATCAGCGACAAAACTTCACCCGATTT Query 841 GTGGTGTTGGCGCGTAAAGCCATTAACGTGTCTGATCAGGTCCGGCGAAAACCACGTTG Sbjet 841 GTGGTGTTGGCGCGTAAAGCCATTAACGTGTCTGATCAGGTTCCGGCGAAAACCACGTTG Query 901 TTAATGGCGACCGGGCAACAAGCCGGTGCGCTGGTTGAAGCGTTGCTGGTACTGCGCAAC sbjet 901 TTAATGGCGACCGGGCAACAAGCCGGTGGCGCTGGTTGAAGCGTTGCTGGTACTGCGCAAC sbjet 901 TTAATGGCGACCGGGCACCAAGCAGCGGTGGCGCTGGTTGAAGCGTGGTGGTACTGCGCCAAC Query 961 CACAATCTGATTATGACCCGTCTGGAATCACGCCCGATTCACGGTAATCCATGGGAAGAG Sbjet 961 CACAATCTGATTATGACCCGTCTGGAATCACGCCGATTCACGGTAATCCATGGGAAGAG Query 1021 ATGTTCTATCTGGATATTCAGGCCAATCTTGAATCACGCGGAAATGCAAAAAGCGGACGAA sbjet 1021 ATGTTCTATCTGGATATTCAGGCCAATCTTGAATCACGCGGAAATGCAAAAAGCGGAAACCATA HIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII	Query	781	TACGGT	TTGCAGG	FACTGGAGCGI	ATTGAAGCAAA	TCAGCGACAAAAC	TTCACCCGATTT	8
Query 841 GTGGTGTTGGCGCGTAAAGCCATTAACGTGTCGATCAGGTTCCGGCGAAAACCACGTTG sbjet 841 GTGGTGTTGGCGCGGTAAAGCCATTAACGTGTCTGATCAGGTTCCGGCGAAAACCACGTTG Query 901 TTAATGGCGACCGGGCAACAAGCCGGTGCGTGGTGAAGCGGTTGCTGGTACTGCGGCAAC Sbjet 901 TTAATGGCGACCGGGCAACAAGCCGGTGGCTGGTTGAAGCGTTGCTGGTACTGCGCAAC Query 901 TTAATGGCGACCGGGCAACAAGCCGGTGGCTGGTGAAGCGTTGCTGGTACTGCGCAAC Query 961 CACAATCTGATTATGACCCGTCTGGAATCACGCCGATTCACGGTAATCCATGGGAAGAG Sbjet 961 CACAATCTGATTATGACCCGTCTGGAATCACGCCGATTCACGGTAATCCATGGGAAGAG Query 1021 ATGTTCTATCTGGATATTCAGGCCAATCTTGAATCACGCGGAAATGCAAAAAGC GACGAA Sbjet 1021 ATGTTCTATCTGGATATTCAGGCCAATCTTGAATCACGCGGAAATGCAAAAAGC GACGAA Query 1081 GAGTTAGGGGAAATCACCCGTTCAATGAAGGTATTGGGCTGTTACCCAAGTGAGAACGTA Sbjet 1081 GAGTTAGGGGAAATCACCCGTTCAATGAAGGTATTGGGCTGTTACCCAAGTGAGAACGTA Query 1141 GTGCCTGTTGATCCAACCTGA 1161 Sbjet 1141 GTGCCTGTTGATCCAACCTGAA 1161	Sbjct	781	TACGGT	TTGCAGG	IIIIIIIIIII FACTGGAGCGI	ATTGAAGCAAA	TCAGCGACAAAAC	TTCACCCGATTT	8
sbjet 841 GTGGTGTGGCGCGTAAAGCCAATTAACGTGTCTGATCAGGTTCCGGCGGAAAACCACGTTG Query 901 TTAATGGCGACCGGGCAACAAGCCGGTGGTGGTGAAGGCGTTGCTGGTACTGCGCCAAC sbjet 901 TTAATGGCGACCGGGCAACAAGCCGGTGGCTGGTTGAAGCGTTGCTGGTACTGCGCCAAC Query 901 TTAATGGCGACCGGGCAACAAGCCGGTGGCTGGTTGAAGCGTTGCTGGTACTGCGCCAAC Query 901 CACAATCTGATTATGACCCGTCTGGAATCACGCCCGATTCACGGTAATCCATGGGAAGAG Sbjet 961 CACAATCTGATTATGACCCGTCTGGAATCACGCCCGATTCACGGTAATCCATGGGAAGAG Query 1021 ATGTTCTATCTGGATATTCAGGCCAATCTTGAATCAGCGGAAATGCAAAAAGC GACTAA sbjet 1021 ATGTTCTATCTGGATATCAGGCCAATCTTGAATCAGCGGGAAATGCAAAAAGC GACTAA Query 1081 GAGTTAGGGGAAATCACCCGTTCAATGAAGGTATTGGGCTGTTACCCAAGTGAGAACGTA Sbjet 1081 GAGTTAGGGGAAATCACCCGTTCAATGAAGGTATTGGGCTGTTACCCAAGTGAGAACGTA Query 1141 GTGCCTGTTGATCCAACCTGA Sbjet 1141 GTGCCTGTTGATCCAACCTGA Sbjet 1141 GTGCCTGTTGATCCAACCTGA Sbjet 1141 GTGCCTGTTGATCCAACCTGA Sbjet 1141 GTGCCTGTTGATCCAACCTGA	Query	841	GTGGTG	TTGGCGC	STAAAGCCATI	AACGTGTCTGA	TCAGGTTCCGGCG	AAAACCACGTTG	9
Query 901 TTAATGGCGACCGGGCAACAAGCCGGTGGTTGAAGCGTTGCTGGTACTGCGCAAC Sbjet 901 TTAATGGCGACCGGGCAACAAGCCGGTGGTGGTGAAGCGTTGCTGGTACTGCGCAAC Query 961 CACAATCTGATTATGACCCGTCTGGAATCACGCCCGATTCACGGTAATCCATGGGAAGAG Sbjet 961 CACAATCTGATTATGACCCGTCTGGAATCACGCCCGATTCACGGTAATCCATGGGAAAGAG Query 1021 ATGTTCTATCTGGATATTCAGGCCAATCTTGAATCAGCGGAAATGCAAAAAGC GACAAA Sbjet 1021 ATGTTCTATCTGGATATTCAGGCCAATCTTGAATCAGCGGAAATGCAAAAAGC GACAAA Query 1081 GAGTTAGGGGAAATCACCCGTTCAATGTAGAGGTATTGGGCTGTTACCCAAGTGAGAACGTA Sbjet 1081 GAGTTAGGGGAAATCACCCGTTCAATGAAGGTATTGGGCTGTTACCCAAGTGAGAACGTA Query 1081 GAGTTAGGGGAAATCACCCGTTCAATGAAGGTATTGGGCTGTTACCCAAGTGAGAACGTA Query 1141 GTGCCTGTTGATCCAACCTGA 1161	Sbjct	841	GTGGTG	TTGGCGC	JTAAAGCCATI	AACGTGTCTGA	TCAGGTTCCGGCG	AAAACCACGTTG	-
sbjet 901 TTAATGGCGACCGGGCAACAAGCGGTGGCGGTGTGAAGCGTGTGAGGCGTACTGGGCAACA Query 961 CACAATCTGATTATGACCCGTCGGAATCACCGCCGATTCACGGTAATCCATGGGAAGAG Sbjet 961 CACAATCTGATTATGACCCGTCTGGAATCACCGCCGATTCACGGTAATCCATGGGAAGAG Query 1021 ATGTTCTATCTGGATATTCAGGCCAATCTTGAATCACGCGGAAATGCAAAAAGC GACAAA Sbjet 1021 ATGTTCTATCTGGATATTCAGGCCAATCTTGAATCAGCGGAAATGCAAAAAGC GACAAA Query 1081 GAGTTAGGGGAAATCACCCGTTCAATGGAGGTATTGGGCTGTTACCCAAGTGGAAAGCA Sbjet 1081 GAGTTAGGGGAAATCACCCGTTCAATGAAGGTATTGGGCTGTTACCCAAGTGAGAACGTA Uuery 1081 GAGTTAGGGGAAATCACCCGTTCAATGAAGGTATTGGGCTGTTACCCAAGTGAGGAACGTA Uuery 1081 GAGTTAGGGGAAATCACCCGTTCAATGAAGGTATTGGGCTGTTACCCAAGTGAGGAACGTA Sbjet 1081 GAGTTAGGGGAAATCACCCGTTCAATGAAGGTATTGGGCTGTTACCCAAGTGAGAACGTA Query 1141 GTGCCTGTTGATCCAACCTGA 1161 Sbjet 1141 GTGCCTGTTGATCCAACCTGA 1161	Query	901	TTAATG	GCGACCG	GCAACAAGCO	GGTGCGCTGGI	TGAAGCGTTGCTG	GTACTGCGCAAC	9
Query 961 CACAATCTGATTATGACCCGTCTGGAATCACGCCCGATTCACGGTAATCCATGGGAAGAG Sbjet 961 CACAATCTGATTATGACCCGTCTGGAATCACGCCGCATTCACGGTAATCCATGGGAAGAG Query 1021 ATGTTCTATCTGGATATCAGGCCAATCTTGAATCACGCGGAAATGCAAAAGC,GACAAA Sbjet 1021 ATGTTCTATCTGGATATCAGGCCAATCTTGAATCAGCGGAAATGCAAAAGC,GACAAA Query 1081 GAGTTAGGGGAAATCACCCGTTCAATGAAGGTATTGGGCTGTTACCCAAGTGAGAACGTA Sbjet 1081 GAGTTAGGGGAAATCACCCGTTCAATGAAGGTATTGGGCTGTTACCCAAGTGAGAACGTA Query 1081 GAGTTAGGGGAAATCACCCGTTCAATGAAGGTATTGGGCTGTTACCCAAGTGAGAACGTA Query 1081 GAGTTAGGGGAAATCACCCGTTCAATGAAGGTATTGGGCTGTTACCCAAGTGAGAACGTA Query 1141 GTGCCTGTTGATCCAACCTGA 1161 Sbjet 1141 GTGCCTGTTGATCCAACCTGA 1161	Sbjct	901	TTAATG	GCGACCG	GCAACAAGCO	GGTGCGCTGGT	TGAAGCGTTGCTG	GTACTGCGCAAC	9
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Query 1021 ATGTTCTATCTGGATATTCAGGCCAATCTTGAATCAGCGGAAATGCAAAAAGCAGACAAA Sbjct 1021 ATGTTCTATCTGGATATTCAGGCCAATCTTGAATCAGCGGAAATGCAAAAAGCAGACAAA Query 1081 GAGTTAGGGGAAATCCACCGTTCAATGAAGGTATTGGGCTGTTACCCAAGTGAGAACGTA Sbjct 1081 GAGTTAGGGGAAATCACCCGTTCAATGAAGGTATTGGGCTGTTACCCAAGTGAGAACGTA Query 1181 GAGTTAGGGGAAATCACCCGTTCAATGAAGGTATTGGGCTGTTACCCAAGTGAGAACGTA Query 1141 GTGCCTGTTGATCCAACCTGA 1161 Sbjct 1141 GTGCCTGTTGATCCAACCTGA 1161	Sbjct	961	CACAAT	CTGATTA	TGACCCGTCTG	GAATCACGCCC	GATTCACGGTAAT	CCATGGGAAGAG	1
Sbjet 1021 ATGTTCTATCTGGATATTCAGGCCAATCTGGACGGAAATGCAAAAGCAGACAAA Query 1081 GAGTTAGGGGAAATCACCCGTTCAATGAAGGTATTGGGCTGTTACCCAAGTGAGAACGTA Sbjet 1081 GAGTTAGGGGAAATCACCCGTTCAATGAAGGTATTGGGCTGTTACCCAAGTGAGAACGTA Query 1141 GTGCCTGTTGATCCAACCTGA 1161 Sbjet 1141 GTGCCTGTTGATCCAACCTGA 1161	Query	1021	ATGTTC	TATCTGG	ATATTCAGGCC	AATCTTGAATC	AGCGGAAATGCAA	AAAGCAGACAAA	1
Query 1081 GAGTTAGGGGAAATCACCCGTTCAATGAAGGTATTGGGCTGTTACCCAAGTGAAAGGTA Sbjct 1081 GAGTTAGGGGAAATCACCCGTTCAATGAAGGTATTGGGCTGTTACCCAAGTGAGAACGTA Query 1141 GTGCCTGTTGATCCAACCTGA 1161 Sbjct 1141 GTGCCTGTTGATCCAACCTGA 1161	Sbjct	1021	ATGTTC	TATCTGG	ATATTCAGGCC	AATCTTGAATC	AGCGGAAATGCAA	AAAGCAGACIAA	1
Sbjet 1081 GAGTTAGGGGAAATCACCCGTTCAATGAAGGTATTGGGCTGTTACCCAAGTGAGAACGTA Query 1141 GTGCCTGTTGATCCAACCTGA 1161 	Query	1081	GAGTTA	GGGGAAA	FCACCCGTTCA	ATGAAGGTATI	GGGCTGTTACCCA	AGTGAGAACGTA	1
Query 1141 GTGCCTGTTGATCCAACCTGA 1161 	Sbjct	1081	GAGTTA	GGGGAAA'	IIIIIIIIII FCACCCGTTCA	ATGAAGGTATT	GGGCTGTTACCCA	AGTGAGAACGTA	1
Sbjet 1141 GTGCCTGTTGATCCAACCTGA 1161	Query	1141	GTGCCT	GTTGATCO	CAACCTGA 1	161			
	Sbjct	1141	GTGCCT	GTTGATCO	CAACCTGA 1	161			

Figure 19. Nucleotide sequence of *pheA*^{L359D}

Query represents the nucleotide sequence of *pheA^{L359D}* in this work. Sbjct represents the nucleotide sequence of *pheA^{L359D}* reference [40]. Red box represents the mutation of Leu359Asp. The chromatogram of *pheA^{L359D}* is shown in Appendix O.





Lane M : TriColor Protein Ladder (10-180 kDa)

Lane 1 : E. coli BL21(DE3) after IPTG induction for 1 h

Lane 2 : E. coli BL21(DE3) harboring pRSFDuet-1 after IPTG induction for 1 h

Lane 3 : *E. coli* BL21(DE3) harboring pPheA^{wt} after IPTG induction for 1 h

Lane 4 : E. coli BL21(DE3) harboring pPheA^{wt} after IPTG induction for 2 h

Lane 5 : E. coli BL21(DE3) harboring pPheA^{wt} after IPTG induction for 3 h

Lane 6 : E. coli BL21(DE3) harboring pPheA^{wt} after IPTG induction for 4 h

Lane 7 : E. coli BL21(DE3) harboring pPheA^{wt} after IPTG induction for 5 h

Lane 8 : E. coli BL21(DE3) harboring pPheA^{wt} after IPTG induction for 6 h

3.2 Construction of $pBLPTG^{{\tt Q151L}}A^{{\tt wt}}$ and $pBLPTG^{{\tt Q151L}}A^{{\tt L359D}}$

The pPheA^{wt} and pPheA^{L3590} from section 3.1.4. were digested with *Bsr*GI and *Not*I. After that, the *pheA^{wt}* and *pheA^{L3590}* fragments were purified from agarose gel using GenepHlowTM Gel/PCR Kit. The pBLPTG^{Q151L} was digested with the same restriction enzymes and purified using same method. From Figure 21, the linear form of pBLPTG^{Q151L} was shown at size around 9.9 kb in lane 3. For *pheA^{wt}* and *pheA^{L359D}* fragments, the DNA bands were obtained at size around 1.2 kb as shown in Figure 22 in lane 3 and 6, respectively. Then, *pheA^{wt}* and *pheA^{L359D}* fragments were ligated with linear pBLPTG^{Q151L} vector and transformed into *E. coli* BL21(DE3). The single colonies of transformants were randomly picked up and cultured in LB broth containing 30 mg/mL of kanamycin. After extraction, the recombinant plasmids were digested with *XhoI* to confirm the positive plasmids of pBLPTG^{Q151L}A^{wt} and pBLPTG^{Q151L}A^{L359D} with *XhoI* gave two DNA bands around 9.9 kb and 1.2 kb as shown in lane 2, 4, 6, 8 and 10. This result showed that the pBLPTG^{Q151L}A^{wt} and pBLPTG^{Q151L}A^{L359D} were successfully constructed.

3.3 Expression of AroG^{wt}

The expression of AroG^{wt} under T7 promoter was evaluated by SDS-PAGE analysis. The *E. coli* BL21(DE3) harboring pDuet_AroG^{wt} from Kanoksinwutthipong was cultured in LB medium containing 30 mg/mL of kanamycin. After cell culture reached log phase, IPTG was added to 1 mM to induce *aroG* expression. The whole cells of *E. coli* BL21(DE3) harboring pDuet_AroG^{wt} were mixed with 5x sample buffer. The supernatants were loaded into the gel. Tri-color protein color was used as protein molecular weight marker. The whole cell extract of *E. coli* BL21(DE3) harboring pRSFDuet-1 was used as a control in lane 1. The protein bands of *E. coli* BL21(DE3) harboring number of after 1 mM IPTG induction for 1-6 h are shown in lane 3-8, respectively (Figure 24).



Figure 21. Notl and BsrGI digestion pattern of pBLPTG^{Q151L}.

Lane M : Gene Ruler 1 kb DNA ladder

Lane 1 : uncut pBLPTG^{Q151L}

Lane 2 : *Not*I digested pBLPTG^{Q151L}

Lane 3 : Notl and BsrGl digested pBLPTG^{Q151L}







Lane M : Gene Ruler 1 kb DNA ladder

Lane 1 : uncut pPheA^{wt}

Lane 2 : NotI digested pPheA^{wt}

- Lane 3 : Notl and BsrGI digested pPheA^{wt}
- Lane 4 : uncut pPheA^{L359D}
- Lane 5 : NotI digested pPheA^{L359D}
- Lane 6 : *Not*I and *Bsr*GI digested pPheA^{L359D}



- **Figure 23.** *Xho*I digestion patterns of pBLPTG^{Q151L}A^{wt} and pBLPTG^{Q151L}A^{L359D}. Lane M : Gene Ruler 1 kb DNA ladder
 - Lane 1 : uncut pBLPTG^{Q151L}A^{wt} from transformant No.1
 - Lane 2 : *Xho*I digested pBLPTG^{Q151L}A^{wt} from transformant No.1
 - Lane 3 : uncut pBLPTG^{Q151L}A^{wt} from transformant No.2
 - Lane 4 : *Xho*I digested pBLPTG^{Q151L}A^{wt} from transformant No.2
 - Lane 5 : uncut pBLPTG^{Q151L}A^{L359D} from transformant No.1
 - Lane 6 : *Xho*I digested pBLPTG^{Q151L}A^{L359D} from transformant No.1
 - Lane 7 : uncut $pBLPTG^{Q151L}A^{L359D}$ from transformant No.2
 - Lane 8 : *Xho*I digested pBLPTG^{Q151L}A^{L359D} from transformant No.2
 - Lane 9 : uncut pBLPTG^{Q151L}A^{L359D} from transformant No.3
 - Lane 10 : *Xho*I digested pBLPTG^{Q151L}A^{L359D} from transformant No.3




Lane M : TriColor Protein Ladder (10-180 kDa)

Lane 1 : *E. coli* BL21(DE3) harboring pRSFDuet-1 after IPTG induction for 1 h Lane 2 : *E. coli* BL21(DE3) harboring pDuet_AroG^{wt} after IPTG induction for 1 h Lane 3 : *E. coli* BL21(DE3) harboring pDuet_AroG^{wt} after IPTG induction for 2 h Lane 4 : *E. coli* BL21(DE3) harboring pDuet_AroG^{wt} after IPTG induction for 3 h Lane 5 : *E. coli* BL21(DE3) harboring pDuet_AroG^{wt} after IPTG induction for 4 h Lane 6 : *E. coli* BL21(DE3) harboring pDuet_AroG^{wt} after IPTG induction for 5 h Lane 7 : *E. coli* BL21(DE3) harboring pDuet_AroG^{wt} after IPTG induction for 6 h In our previous work, we paid a lot of attempt to determine the expression of AroG by SDS-PAGE analysis and DAHP synthase activity assay. The AroG band could not be observed on SDS-PAGE. Moreover, DAHP synthase activity of the recombinant clone was not differ from that of *E. coli* host cell. At first, we suspected that there were some defects on T7 promoter of pRSFDuet-1 vector used in our laboratory, so *aroG* was subcloned under T7 promoter of pET-28b and again the protein band and activity of AroG could not be detected.

In this research, the sequence of pDuet_AroG was rechecked. The *aroG* was cloned into pRSFDuet-1 between *Bam*HI and *Hin*dIII sites. As shown in Figure 25, the sequence of forward primer was 5'-CGGGATCCATGAATTATCAGA ACGACGATTTACGC-3'. *Bam*HI site is shown in blue and start codon of *aroG* is shown in red. Translation of the gene inserted under T7 promoter-1 is started from Met of His-tag (shown in red box). When *aroG* was inserted, the translation frame of *aroG* was one base shifted and translation was stopped at TGA (shown in green box). Thus, AroG could not be synthesized.

3.4 Reconstruction of pAroG and pAroG^{fbr}

In this part, new forward primer was designed and used for cloning *aroG* into pRSFDuet-1.

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3.4.1 Plasmid extraction

pDuet_AroG^{wt} and pDuet_AroG^{fbr} (5,039 bp) were extracted from *E. coli* Top10. The agarose gel electrophoresis is shown in Figure 26. Each recombinant plasmid was used as a template for *aroG* amplification.

Α.		Pfol	ACYCDuetUP1 Primer #71178-3		T7 pror	noter-1
GCCA	TACCGCGAAAGGTTTTGCGG	CATTCGATGGTGTCCGGG	ATCTCGACGCTCTC	CCTTATGCGACTCCTGC	ATTAGGAAATTAATACGA	CTCACTATA
T7 tr	anscription start-1 lac operator			rbs Nee+	His	s-Tag
GGGG	AATTGTGAGCGGATAACAAT	ETCCCCTGTAGAAATAATT BspM I	TTGTTTAACTTTAA	TAAGGAGATATAC ATG	GCAGCAGCCATCACCAT LySerSerHisHisHis	CATCACCAC HisHisHis
4000	EcolCR I BamH I EcoR I Sac I	Asci Psti Sall	Hind III Not I	Afill		at I DuetUP2 Prime
SerG	IP1 Primer	AlaArgLeuGlnValAsp	LysLeuAlaAlaAla	aEnd	Di	#71179-3
GCAT/ DuetD	AATCGAAATTAATACGACTC OOWN1 Primer #71179-3		Bae I	CCCATCTTAGTATATAG	rbs	Nde I ATATACAT
Nde I ATGG MetA	<u>Bg/ll Mfel EcoR V</u> CAGATCTCAATTGGATATCO laAspLeuAsnTrpIleSer	Fsel AsiSI GCCGGCCACGCGATCGCT AlaGlyHisAlaIleAla	Aat II Acc65 I X GACGTCGGTACCCT AspValGlyThrLe	<u>hol</u> CGAGTCTGGTAAAGAAAG uGluSerGlyLysGluTH	S•Tag CCGCTGCTGCGAAATTTG/ hrAlaAlaAlaLysPheG	AACGCCAG LuArgGln
CACAT	S·Tag TGGACTCGTCTACTAGCGC/ etAspSerSerThrSerAla	<u>Pacl</u> <u>AvrII</u> AGCTTAATTAACCTAGGCT AAlaEnd	GCTGCCACCGCTGA	GCAATAACTAGCATAACO minator Primer #69337-3	Eco0109 I CCCTTGGGGCCTCTAAACO	GGGTCTTG
в.	BamHI <u>CAG GAT CCA</u> T	GA ATTATCAGAAC	GACGATTTACC	CATCAAAGAAAT	CAAAGAGTTACTT	сстсстб
	Stop	codon	111			

Figure 25. Frameshift mutation of aroG

- A. pRSFDuet-1 vector, red box shows start codon of the recombinant protein.
- B. Sequence of inserted *aroG*, *Bam*HI site blue letter

start codon of *aroG* – red letter

stop codon – green box

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Figure 26. Electrophoretic patterns of pDuet_AroG^{wt} and pDuet_AroG^{fbr}



3.4.2 Primer design at 5'end of aroG

The new forward primer containing *Nco*I at 5'end was designed. The sequence of new forward primer F_AroG_NcoI is

5'-CATGCCATGGTGTATCAGAACGACGATTTACGCATCAAAGAAATC-3'

Blue letter indicates Ncol sites while blue underline shows start codon of aroG.

3.4.3 PCR amplification of *aroG^{wt}* and *aroG^{fbr}*

The $aroG^{wt}$ and $aroG^{fbr}$ were amplified from pDuet_AroG^{wt} and pDuet_AroG^{fbr} using forward primer containing *Nco*I site and reverse primer containing *Hind*III site. After cleaning by GenepHlowTM Gel/PCR Kit, the PCR fragments were separated by agarose gel electrophoresis. From the result shown in Figure 27, the size of $aroG^{wt}$ and $aroG^{fbr}$ fragments were detected around 1.1 kb.

3.4.4 Digestion of *aroG^{wt}* and *aroG^{fbr}* fragments and pRSFDuet-1

The *aroG^{wt} and aroG^{fbr}* fragments from section 3.4.3 and pRSFDuet-1 vector were digested with *Ncol* and *Hin*dIII. The linear pRSFDuet-1 (3.9 kb) and *aroG^{wt}* and *aroG^{fbr}* fragments (1.1 kb) after digestion are shown in Figure 28-29, respectively. After that, *aroG^{wt}* and *aroG^{fbr}* fragments were ligated to pRSFDuet-1 linear vector and then transformed into *E. coli* Top10 by electroporation. The pAroG^{wt} and pAroG^{fbr} transformants were randomly picked and cultured in 5 mL of LB broth containing 30 mg/mL of kanamycin. Each recombinant plasmid was extracted and then detected by agarose gel electrophoresis. The pAroG^{wt} from transformant No.1, 3, 4, 5 and 6, pAroG^{L175D} from transformant No.2 - 6 (Figure 30), pAroG^{Q151L} from transformant No.3 and pAroG^{Q151A} from transformant No.4 (Figure 31) as well as pAroG^{Q151N} from transformant No.6 (Figure 32) moved slower than that of pRSFDuet-1.





Figure 28. Ncol and HindIII digestion pattern of pRSFDuet-1.

Lane M : Gene Ruler 1 kb DNA ladder

Lane 1 : uncut pRSFDuet-1

Lane 2 : Ncol and HindIII digested pRSFDuet-1





Figure 29. Ncol and HindIII digestion pattern of PCR products.

Lane M : Gene Ruler 1 kb DNA ladder

Lane 1 : uncut PCR product of aroG^{wt}

Lane 2 : Ncol and Hindlll digested PCR product of aroG^{wt}

Lane 3 : uncut PCR product of aroG^{L175D}

Lane 4 : Ncol and HindIII digested PCR product of aroG^{L175D}

Lane 5 : uncut PCR product of aroG^{Q151L}

Lane 6 : Ncol and HindIII digested PCR product of aroGQ151L

Lane 7 : uncut PCR product of aroG $^{\rm Q151A}$

Lane 8 : Ncol and HindIII digested PCR product of aroG^{Q151A}

Lane 9 : uncut PCR product of $aroG^{Q151N}$

Lane 10 : Ncol and HindIII digested PCR product of aroG^{Q151N}



Figure 30. Electrophoretic pattern of plasmid from pAroG^{wt} and pAroG^{L175D} transformants.

Lane M	: Gene Ruler 1 kb DNA ladder
Lane 1	: pRSFDuet-1
Lane 2-7	: pAroG ^{wt} from transformant No.1-6, respectively
Lane 8-13	3 : pAroG ^{L175D} from transformant No.1-6, respectively

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Figure 31. Electrophoretic pattern of plasmid from pAroG^{Q151L} and pAroG^{Q151A} transformants.

Lane M	: Gene Ruler 1 kb DNA ladder
Lane 1	: pRSFDuet-1
Lane 2-6	: pAroG ^{Q151L} from transformant No.1-5, respectively
Lane 6-11	: pAroG ^{Q151A} from transformant No.1-5, respectively
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Figure 32. Electrophoretic pattern of plasmid from pAroG^{Q151N} transformants.

- Lane M : Gene Ruler 1 kb DNA ladder
- Lane 1 : pRSFDuet-1

Lane 2-7 : pAroG^{Q151N} from transformant No.1-6, respectively

Thus, the transformant No.1 from pAroG^{wt}, transformant No.2 from pAroG^{L175D}, transformant No.3 from pAroG^{Q151L}, transformant No.4 from pAroG^{Q151A} and transformant No.6 from pAroG^{Q151L} were confirmed by digested with *Nco*I and *Hin*dIII. From digestion pattern, each transformant gave two bands of DNA fragments around 3.9 kb and 1.1 kb as shown in Figure 33. This result confirmed that *aroG* genes were inserted into pRSFDuet-1. After that, the nucleotide sequences of the inserts were checked by Bioneer Inc. (Korea).

3.4.5 Nucleotide sequence of aroG genes

To verify the nucleotide sequences of *aroG^{wt}* and *aroG^{fbr}*, the DNA sequencing of recombinant plasmids were performed by Bioneer, Korean using F_tktA_aroG_Int as a forward primer and Seqduet_R as a reverse primer. The obtained DNA sequences were compared with wild type *aroG* reported by Kanoksinwutthipong in 2014. Genetyx-Win program was used to translate protein sequence [41].

The all of nucleotide sequence of $aroG^{wt}$ and $aroG^{fbr}$ were changed only at the mutated sites (Figure 34 - 38). The $aroG^{wt}$ and $aroG^{fbr}$ were used in the further experiment.

3.4.6 Expression of AroG^{wt} and AroG^{fbr} 3.4.6.1 Protein expression

The expression of *aroG^{wt}* and *aroG^{fbr}* under T7 promoter were evaluated by SDS-PAGE analysis. The crude enzyme of AroG^{wt} and AroG^{fbr} were mixed with 5x sample buffer. The supernatants were loaded into the gel. Tri-color protein color was used for protein molecular weight marker. Crude extracts of *E. coli* BL21(DE3) and *E. coli* BL21(DE3) harboring pRSFDuet-1 were used as controls in lane 1 and 2. The protein bands of AroG^{wt} and AroG^{fbr} were detected after 1 mM IPTG induction for 2 h in lane 3-7, respectively. The sizes of recombinant proteins were approximately 38 kDa as shown in Figure 39.



Figure 33. Ncol and HindIII digestion patterns of pAroG^{wt} and pAroG^{fbr}.

Lane M1 : Gene Ruler 1 kb DNA ladder

- Lane 1 : uncut pRSFDuet-1
- Lane 2 : Ncol and HindIII digested pRSFDuet-1
- Lane 3 : uncut PCR product of *aroG^{wt}*
- Lane 4 : Ncol and HindIII digested PCR product of aroG^{wt}
- Lane 5 : uncut pAroG^{wt} from transformant No.1
- Lane 6 : Ncol and HindIII digested pAroG^{wt} from transformant No.1
- Lane 7 : uncut pAroG^{L175D} from transformant No.2
- Lane 8 : Ncol and HindIII digested pAroG^{L175D} from transformant No.2
- Lane 9 : uncut pAroG^{Q151L} from transformant No.3
- Lane 10 : Ncol and HindIII digested pAroG^{Q151L} from transformant No.3
- Lane 11 : uncut pAroG^{Q151A} from transformant No.4
- Lane 12 : Ncol and HindIII digested pAroG^{Q151A} from transformant No.4
- Lane 13 : uncut pAroG^{Q151N} from transformant No.6
- Lane 14 : Ncol and HindIII digested pAroG^{Q151N} from transformant No.6

10 20 30 40 50 60 TTATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTGTAGAAATAATTT 70 80 90 100 110 120 TGTTTAACTTTAATAAGGAGATATACCATGGTGTATCAGAACGACGATTTACGCATCAAA M V Y Q N D D L R I K 0 150 160 170 180 140 130 GAAATCAAAGAGTTACTTCCTCCTGTCGCATTGCTGGAAAAATTCCCCCGCTACTGAAAAT E I K E L L P P V A L L E K F P A T E N 190 200 210 220 230 240 GCCGCGAATACGGTTGCCCATGCCCGAAAAGCGATCCATAAGATCCTGAAAGGTAATGAT A A N T V A H A R K A I H K I L K G N D 250 260 270 280 290 300 GATCGCCTGTTGGTTGTGATTGGCCCATGCTCAATTCATGATCCTGTCGCGGCAAAAGAG D R L L V V I G P C S I H D P V A A K E 310 320 330 340 350 360 TATGCCACTCGCTTGCTGGCGCTGCGTGAAGAGCTGAAAGATGAGCTGGAAATCGTAATG Y A T R L L A L R E E L K D E L E I V M 370 380 390 400 410 420 CGCGTCTATTTTGAAAAGCCGCGTACCACGGTGGGCTGGAAAGGGCTGATTAACGATCCG R V Y F E K P R T T V G W K G L I N D P 430 440 450 460 470 480 480 CATATGGATAATAGCTTCCAGATCAACGACGGTCTGCGTATAGCCCGTAAATTGCTGCTT H M D N S F Q I N D G L R I A R K L L L 490 500 510 520 530 540 GATATTAACGACAGCGGTCTGCCAGCGGCAGGTGAGTTTCTCGATATGATCACCCCACAA D I N D S G L P A A G E F L D M I T P Q 550 560 570 580 590 600 600 TATCTCGCTGACCTGATGAGCTGGGGCGCAATTGGCGCACGTACCACCGAATCGCAGGTG Y L A D L M S W G A I G A R T T E S Q V 610 620 630 640 650 660 CACCGCGAACTGGCATCAGGGCTTTCTTGTCCGGTCGGCTTCAAAAATGGCACCGACGGT H R E L A S G L S C P V G F K N G T D G 670 680 690 700 710 720 ACGATTAAAGTGGCTATCGATGCCATTAATGCCGCCGGTGCGCCGCACTGCTTCCTGTCC T I K V A I D A I N A A G A P H C F L S 730 740 750 760 770 780 GTAACGAAATGGGGGGCATTCGGCGATTGTGAATACCAGCGGTAACGGCGATTGCCATATC V T K W G H S A I V N T S G N G D C H I 790 800 810 820 830 840 ATTCTGCGCGGCGGTAAAGAGCCTAACTACAGCGCGAAGCACGTTGCTGAAGTGAAAGAA I L R G G K E P N Y S A K H V A E V K E 850 860 870 880 890 900 GGGCTGAACAAAGCAGGCCTGCCAGCACAGGTGATGATCGATTTCAGCCATGCTAACTCG G L N K A G L P A Q V M I D F S H A N S 910 920 930 940 950 960 TCCAAACAATTCAAAAAGCAGATGGATGTTTGTGCTGACGTTTGCCAGCAGATTGCCGGT S K Q F K K Q M D V C A D V C Q Q I A G 970 980 990 1000 1010 1020 GGCGAAAAGGCCATTATTGGCGTGATGGTGGAAAGCCATCTGGTGGAAGGCAATCAGAGC G E K A I I G V M V E S H L V E G N Q S 1030 1040 1050 1060 1070 1080 CTCGAGAGCGGGGGGGCCCTGGCCTACGGTAAGAGCATCACCGATGCCTGCATCGGCTGG L E S G E P L A Y G K S I T D A C I G W 1090 1100 1110 1120 1130 1140 1140 GAAGATACCGATGCTCTGTTACGTCAACTGGCGAATGCAGTGAAAGCGCGTCGCGGGTAA E D T D A L L R Q L A N A V K A R R G * 1150 AAGCTT

Figure 34. Nucleotide sequence of *aroG^{wt}*

Orange and blue underlines represent the restriction site of *Nco*I and *Hin*dIII, respectively.

The chromatogram of $aroG^{wt}$ is shown in Appendix P.

10 20 30 40 50 60 TTATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCCTGTAGAAATAATTT 70 80 90 100 110 120 TGTTTAACTTTAATAAGGAGATATACCATGGTGTATCAGAACGACGATTTACGCATCAAA M V Y Q N D D L R I K 150 160 170 160 170 180 140 130 GAAATCAAAGAGTTACTTCCTCCTGTCGCATTGCTGGAAAAATTCCCCCGCTACTGAAAAT E I K E L L P P V A L L E K F P A T E N 190 200 210 220 230 240 GCCGCGAATACGGTTGCCCATGCCCGAAAAGCGATCCATAAGATCCTGAAAGGTAATGAT A A N T V A H A R K A I H K I L K G N D 250 260 270 280 290 300 300 GATCGCCTGTTGGTTGTGATTGGCCCATGCTCAATTCATGATCCTGTCGCGGCAAAAGAG D R L L V V I G P C S I H D P V A A K E 310 320 330 340 350 360 360 TATGCCACTCGCTTGCTGGCGCTGCGTGAAGAGCTGAAAGATGAGCTGGAAATCGTAATG Y A T R L L A L R E E L K D E L E I V M 370 380 390 400 410 420 420 ${\tt CGCGTCTATTTTGAAAAGCCGCGTACCACGGTGGGCTGGAAAGGGCTGATTAACGATCCG}$ R V Y F E K P R T T V G W K G L I N D P 430 440 450 460 470 480 480 CATATGGATAATAGCTTCCAGATCAACGACGGTCTGCGTATAGCCCGTAAATTGCTGCTT H M D N S F Q I N D G L R I A R K L L L 490 500 510 520 530 540 540 ${\tt GATATTAACGACAGCGGTCTGCCAGCGGCAGGTGAGTTTCTCGATATGATCACCCCACAA}$ D I N D S G L P A A G E F L D M I T P Q 550 560 570 580 590 600 600 TATCTCGCTGACCTGATGAGCTGGGGGCGCAATTGGCGCACGTACCACCGAATCGCAGGTG Y L A D L M S W G A I G A R T T E S Q V 6100 620 630 640 650 660 61<mark>0</mark> 660 CACCGCGA/GAT GCGTCTGGTCTTCTTGTCCGGTCGGCTTCAAAAATGGCACCGACGGT H R E D A S G L S C P V G F K N G T D G 670 680 690 700 710 720 720 ${\tt ACGATTAAAGTGGCTATCGATGCCATTAATGCCGCCGGTGCGCCGCACTGCTTCCTGTCC}$ T I K V A I D A I N A A G A P H C F L S 730 740 750 760 770 780 GTAACGAAATGGGGGGCATTCGGCGATTGTGAATACCAGCGGTAACGGCGATTGCCATATC V T K W G H S A I V N T S G N G D C H I 790 800 810 820 830 840 840 ATTCTGCGCGGCGGTAAAGAGCCTAACTACAGCGCGAAGCACGTTGCTGAAGTGAAAGAA I L R G G K E P N Y S A K H V A E V K E 850 860 870 880 890 900 900 GGGCTGAACAAAGCAGGCCTGCCAGCACAGGTGATGATCGATTTCAGCCATGCTAACTCG G L N K A G L P A Q V M I D F S H A N S 910 920 930 940 950 960 TCCAAACAATTCAAAAAGCAGATGGATGTTTGTGCTGACGTTTGCCAGCAGATTGCCGGT S K Q F K K Q M D V C A D V C Q Q I A G 970 980 990 1000 1010 1020 1020 GGCGAAAAGGCCATTATTGGCGTGATGGTGGAAAGCCATCTGGTGGAAGGCAATCAGAGC G E K A I I G V M V E S H L V E G N Q S 1030 1040 1050 1060 1070 1080 1080 CTCGAGAGCGGGGGGGGCCCTGGCCTACGGTAAGAGCATCACCGATGCCTGCATCGGCTGG L E S G E P L A Y G K S I T D A C I G W 1090 1100 1110 1120 1130 1140 1130 1140 GAAGATACCGATGCTCTGTTACGTCAACTGGCGAATGCAGTGAAAGCGCGTCGCGGGTAA E D T D A L L R Q L A N A V K A R R G * 1150 AAGCTT

Figure 35. Nucleotide sequence of aroG^{L175D}

Orange and blue underlines represent the restriction site of *Nco*I and *Hin*dIII, respectively.

Red box represents the mutation of Leu175Asp (CTG to GAT).

The chromatogram of $aroG^{L175D}$ is shown in Appendix Q.

10 20 30 40 50 60 TTATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCCTGTAGAAATAATTT 80 70 90 100 110 120 TGTTTAACTTTAATAAGGAGATATACCATGGTGTATCAGAACGACGATTTACGCATCAAA M V Y Q N D D L R I K 150 160 170 180 L I K 14 X 9 160 130 140 150 170 180 ${\tt GAAATCAAAGAGTTACTTCCTCCTGTCGCATTGCTGGAAAAATTCCCCCGCTACTGAAAAT$ E I K E L L P P V A L L E K F P A T E N 190 200 210 220 230 240 GCCGCGAATACGGTTGCCCATGCCCGAAAAGCGATCCATAAGATCCTGAAAGGTAATGAT A A N T V A H A R K A I H K I L K G N D 250 260 270 280 290 300 260 280 300 GATCGCCTGTTGGTTGTGATTGGCCCATGCTCAATTCATGATCCTGTCGCGGCAAAAGAG D R L L V V I G P C S I H D P V A A K E 310 320 330 340 350 360 360 TATGCCACTCGCTTGCTGGCGCTGCGTGAAGAGCTGAAAGATGAGCTGGAAATCGTAATG Y A T R L L A L R E E L K D E L E I V M 370 380 390 400 410 420 420 ${\tt CGCGTCTATTTTGAAAAGCCGCGTACCACGGTGGGCTGGAAAGGGCTGATTAACGATCCG}$ R V Y F E K P R T T V G W K G L I N D P 430 440 450 460 470 480 480 CATATGGATAATAGCTTCCAGATCAACGACGGTCTGCGTATAGCCCGTAAATTGCTGCTT H M D N S F Q I N D G L R I A R K L L L 490 500 510 520 530 540 GATATTAACGACAGCGGTCTGCCAGCGGCAGGTGAATTCCTCGATATGATCACTCCT D I N D S G L P A A G E F L D M I T P 550 560 570 580 590 600 TATCTCGCTGACCTGATGAGCTGGGGGCGCAATTGGCGCACGTACCACCGAATCGCAGGTG Y L A D L M S W G A I G A R T T E S Q V 610 620 630 640 650 660 660 H R E L A S G L S C P V G F K N G T D G 670 680 690 700 710 720 720 ${\tt ACGATTAAAGTGGCTATCGATGCCATTAATGCCGCCGGTGCGCCGCACTGCTTCCTGTCC}$ T I K V A I D A I N A A G A P H C F L S 730 740 750 760 770 780 GTAACGAAATGGGGGGCATTCGGCGATTGTGAATACCAGCGGTAACGGCGATTGCCATATC V T K W G H S A I V N T S G N G D C H I 790 800 810 820 830 840 840 ATTCTGCGCGGCGGTAAAGAGCCTAACTACAGCGCGAAGCACGTTGCTGAAGTGAAAGAA I L R G G K E P N Y S A K H V A E V K E 850 860 870 880 890 900 900 ${\tt GGGCTGAACAAAGCAGGCCTGCCAGCACAGGTGATGATCGATTTCAGCCATGCTAACTCG}$ G L N K A G L P A Q V M I D F S H A N S 910 920 930 940 950 960 960 TCCAAACAATTCAAAAAGCAGATGGATGTTTGTGCTGACGTTTGCCAGCAGATTGCCGGT S K Q F K K Q M D V C A D V C Q Q I A G 970 980 990 1000 1010 1020 ${\tt GGCGAAAAGGCCATTATTGGCGTGATGGTGGAAAGCCATCTGGTGGAAGGCAATCAGAGC}$ G E K A I I G V M V E S H L V E G N Q S 1030 1040 1050 1060 1070 1080 1080 CTCGAGAGCGGGGGGGGCCCGCTGGCCTACGGTAAGAGCATCACCGATGCCTGCATCGGCTGG L E S G E P L A Y G K S I T D A C I G W 1090 1100 1110 1120 1130 1140 1140 GAAGATACCGATGCTCTGTTACGTCAACTGGCGAATGCAGTGAAAGCGCGTCGCGGGTAA E D T D A L L R Q L A N A V K A R R G * 1150 AAGCTT

Figure 36. Nucleotide sequence of aroG^{Q151L}

Orange and blue underlines represent the restriction site of *Nco*I and *Hin*dIII, respectively.

Red box represents the point mutation of Gln151Leu (CCA to CTG).

The chromatogram of $aroG^{Q151L}$ is shown in Appendix R.

30 10 20 40 50 TTATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTGTAGAAATAATTT 70 80 90 100 110 120 TGTTTAACTTTAATAAGGAGATATA<u>CCATGG</u>TGTATCAGAACGACGATTTACGCATCAAA MVYQNDDLRIK 150 160 170 180 130 140 180 ${\tt GAAATCAAAGAGTTACTTCCTCCTGTCGCATTGCTGGAAAAATTCCCCCGCTACTGAAAAT$ E I K E L L P P V A L L E K F P A T E N 190 200 210 220 230 240 240 GCCGCGAATACGGTTGCCCATGCCCGAAAAGCGATCCATAAGATCCTGAAAGGTAATGAT A A N T V A H A R K A I H K I L K G N D 250 260 270 280 290 300 300 ${\tt GATCGCCTGTTGGTTGTGATTGGCCCATGCTCAATTCATGATCCTGTCGCGGCAAAAGAG$ D R L L V V I G P C S I H D P V A A K E 310 320 330 340 350 360 360 TATGCCACTCGCTTGCTGGCGCTGCGTGAAGAGCTGAAAGATGAGCTGGAAATCGTAATG Y A T R L L A L R E E L K D E L E I V M 370 380 390 400 410 420 ${\tt CGCGTCTATTTTGAAAAGCCGCGTACCACGGTGGGCTGGAAAGGGCTGATTAACGATCCG}$ R V Y F E K P R T T V G W K G L I N D P 430 440 450 460 470 480 480 CATATGGATAATAGCTTCCAGATCAACGACGGTCTGCGTATAGCCCGTAAATTGCTGCTT H M D N S F Q I N D G L R I A R K L L L 490 500 510 520 530 540 490 500 510 520 530 540 GATATTAACGACAGCGGTCTGCCAGCGGCAGGTGAATTCCTCGATATGATCACTCCTGCC D I N D S G L P A A G E F L D M I T P 550 560 570 580 590 600 TATCTCGCTGACCTGATGAGCTGGGGCGCAATTGGCGCACGTACCACCGAATCGCAGGTG Y L A D L M S W G A I G A R T T E S Q V 610 620 630 640 650 66 660 ${\tt CACCGCGAACTGGCATCAGGGCTTTCTTGTCCGGTCGGCTTCAAAAATGGCACCGACGGT}$ H R E L A S G L S C P V G F K N G T D G 670 680 690 700 710 720 ${\tt ACGATTAAAGTGGCTATCGATGCCATTAATGCCGCCGGTGCGCCGCACTGCTTCCTGTCC}$ T I K V A I D A I N A A G A P H C F L S 730 740 750 760 770 780 780 ${\tt GTAACGAAATGGGGGGCATTCGGCGATTGTGAATACCAGCGGTAACGGCGATTGCCATATC}$ V T K W G H S A I V N T S G N G D C H I 790 800 810 820 830 840 840 ATTCTGCGCGGCGGTAAAGAGCCTAACTACAGCGCGAAGCACGTTGCTGAAGTGAAAGAA I L R G G K E P N Y S A K H V A E V K E 850 860 870 880 890 900 GGGCTGAACAAAGCAGGCCTGCCAGCACAGGTGATGATCGATTTCAGCCATGCTAACTCG G L N K A G L P A Q V M I D F S H A N S 910 920 930 940 950 960 960 ${\tt TCCAAACAATTCAAAAAGCAGATGGATGTTTGTGCTGACGTTTGCCAGCAGATTGCCGGT$ S K Q F K K Q M D V C A D V C Q Q I A G 970 980 990 1000 1010 1020 1020 GGCGAAAAGGCCATTATTGGCGTGATGGTGGAAAGCCATCTGGTGGAAGGCAATCAGAGC G E K A I I G V M V E S H L V E G N Q S 1030 1040 1050 1060 1070 1080 1080 ${\tt CTCGAGAGCGGGGGGGGGGGCCTGGCCTACGGTAAGAGCATCACCGATGCCTGCATCGGCTGG$ L E S G E P L A Y G K S I T D A C I G W 1090 1100 1110 1120 1130 1140 1140 GAAGATACCGATGCTCTGTTACGTCAACTGGCGAATGCAGTGAAAGCGCGTCGCGGGTAA DTDALLRQLANAVKARRG* 1150 AAGCTT

Figure 37. Nucleotide sequence of *aroG^{Q151A}*

Orange and blue underlines represent the restriction site of *Nco*I and *Hin*dIII, respectively.

Red box represents the point mutation of Gln151Ala (CCA to GCC).

The chromatogram of $aroG^{Q151A}$ is shown in Appendix S.

30 10 20 40 50 TTATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCCTGTAGAAATAATTT 70 80 90 100 110 120 TGTTTAACTTTAATAAGGAGATATACCATGGTGTATCAGAACGACGATTTACGCATCAAA MVYQNDDLRIK 150 160 170 180 130 140 GAAATCAAAGAGTTACTTCCTCCTGTCGCATTGCTGGAAAAATTCCCCCGCTACTGAAAAT E I K E L L P P V A L L E K F P A T E N 190 200 210 220 230 240 GCCGCGAATACGGTTGCCCATGCCCGAAAAGCGATCCATAAGATCCTGAAAGGTAATGAT A A N T V A H A R K A I H K I L K G N D 250 260 270 280 290 300 GATCGCCTGTTGGTTGTGATTGGCCCATGCTCAATTCATGATCCTGTCGCGGCAAAAGAG D R L L V V I G P C S I H D P V A A K E 310 320 330 340 350 360 360 TATGCCACTCGCTTGCTGGCGCTGCGTGAAGAGCTGAAAGATGAGCTGGAAATCGTAATG Y A T R L L A L R E E L K D E L E I V M 370 380 390 400 410 420 420 CGCGTCTATTTTGAAAAGCCGCGTACCACGGTGGGCTGGAAAGGGCTGATTAACGATCCG R V Y F E K P R T T V G W K G L I N D P 430 440 450 460 470 480 480 CATATGGATAATAGCTTCCAGATCAACGACGGTCTGCGTATAGCCCGTAAATTGCTGCTT H M D N S F Q I N D G L R I A R K L L L 490 500 510 520 530 540 540 GATATTAACGACAGCGGTCTGCCAGCGGCAGGTGAATTCCTCGATATGATCACTCCTAAT D I N D S G L P A A G E F L D M I T P N 550 560 570 580 590 600 TATCTCGCTGACCTGATGAGCTGGGGGGGCGCAATTGGCGCACGTACCACCGAATCGCAGGTG Y L A D L M S W G A I G A R T T E S Q V 610 620 630 640 650 660 660 CACCGCGAACTGGCATCAGGGCTTTCTTGTCCGGTCGGCTTCAAAAATGGCACCGACGGT H R E L A S G L S C P V G F K N G T D G 670 680 690 700 710 720 720 ACGATTAAAGTGGCTATCGATGCCATTAATGCCGCCGGTGCGCCGCACTGCTTCCTGTCC T I K V A I D A I N A A G A P H C F L S 730 740 750 760 770 780 GTAACGAAATGGGGGGCATTCGGCGATTGTGAATACCAGCGGTAACGGCGATTGCCATATC V T K W G H S A I V N T S G N G D C H I 790 800 810 820 830 840 840 ATTCTGCGCGGCGGTAAAGAGCCTAACTACAGCGCGAAGCACGTTGCTGAAGTGAAAGAA I L R G G K E P N Y S A K H V A E V K E 850 860 870 880 890 900 900 GGGCTGAACAAAGCAGGCCTGCCAGCACAGGTGATGATCGATTTCAGCCATGCTAACTCG GLNKAGLPAQVMIDFSHANS 910 920 930 940 950 960 960 TCCAAACAATTCAAAAAGCAGATGGATGTTTGTGCTGACGTTTGCCAGCAGATTGCCGGT S K Q F K K Q M D V C A D V C Q Q I A G 970 980 990 1000 1010 1020 GGCGAAAAGGCCATTATTGGCGTGATGGTGGAAAGCCATCTGGTGGAAGGCAATCAGAGC KAIIGVMVESHLVEGNQS 1030 1040 1050 1060 1070 1080 GEK 1080 ${\tt CTCGAGAGCGGGGGGGGCCCTGGCCTACGGTAAGAGCATCACCGATGCCTGCATCGGCTGG$ L E S G E P L A Y G K S I T D A C I G W 1090 1100 1110 1120 1130 1140 GAAGATACCGATGCTCTGTTACGTCAACTGGCGAATGCAGTGAAAGCGCGTCGCGGGTAA E D T D A L L R Q L A N A V K A R R G * 1150 AAGCTT

Figure 38. Nucleotide sequence of *aroG^{Q151N}*

Orange and blue underlines represent the restriction site of *Nco*I and *Hin*dIII, respectively.

Red box represents the point mutation of Gln151Asn (CCA to AAT).

The chromatogram of $aroG^{Q151N}$ was shown in Appendix T.



Figure 39. SDS-PAGE of crude extract of aroG clones.

- lane M : TriColor Protein Ladder (10-180 kDa)
- Lane 1 : E. coli BL21(DE3)
- Lane 2 : E. coli BL21(DE3) harboring pRSFDuet-1
- Lane 3 : E. coli BL21(DE3) harboring pAroG^{wt}
- Lane 4 : E. coli BL21(DE3) harboring pAroG^{L175D}
- Lane 5 : E. coli BL21(DE3) harboring pAroG^{Q151L}
- Lane 6 : E. coli BL21(DE3) harboring pAroG^{Q151A}
- Lane 7 : *E. coli* BL21(DE3) harboring pAroG^{Q151N}

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3.4.6.2 DAHP synthase activity

In previous studies, Hu and coworkers investigated the feedback inhibition site of AroG using the 3D structure of AroG co-crystallized with PEP. The amino acids were replaced at Phe144, Pro150, Leu175, Leu179, Phe209, Trp215 and Val221. DAHP synthase activity in crude extract of each clone was measured in the presence of L-Phe from 0 mM to 3 mM. The results showed that the mutant at position L175D was mostly resistant to feedback inhibition. L175D enzyme elevated specific enzyme activity at 0 mM phenylalanine from 2.70 U/mg of wild type to 4.46 U/mg and increased of relative enzymatic activity at 1 mM phenylalanine from 8.2% to 83.5% [26]. In 2014, Ding and coworkers constructed three single-site mutant and combined to generate three double-site *aroG*^{fbr} mutant alleles. They analyzed enzymatic activity in all of mutants. The results showed that AroG8/15 had high level of feedback resistance to L-Phe at 20 mM of L-Phe. The relative enzymatic activity of AroG8/15 remained at 20 mM of L-Phe was 96.66% [27].

The amino acid residues that interact with phenylalanine at the regulatory site of AroG are displayed in Figure 40 using Discovery Studio 2020 program. Van der Waal interaction was found between Leu175 and phenylalanine. Among Gln151 as well as Asp6 and Asp7 of the companion tight subunit which form one H-bonding with phenylalanine, we interested in Gln151 since the pocket accommodative the aromatic ring of phenylalanine is formed by hydrophobic sidechains including that of Gln151. To investigate this amino acid residue, structure of AroG when Gln151 was substituted by Ala, Asn and Leu were simulated. All replaced amino acid cannot form H-bonding with phenylalanine (Figure 40B-40D). Differ from Ala and Asn, hydrophobic interaction between Leu151 and phenylalanine was detected.



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DAHP synthase activity of crude extract from AroG clones were assayed for sensitivity to feedback inhibition by L-Phe at a concentration from 0 mM to 20 mM. The results are shown in Figure 41. In the absent L-Phe, all mutated AroG at Gln151 exhibited higher specific activities than AroG^{wt} (1.88 U/mg). In contradiction to the result of Hu and co-worker [26], the control AroG^{L175D} mutant showed lower specific activity (0.82 U/mg) than AroG^{wt} (1.88 U/mg). The activity of all recombinant enzymes were decreased in the same pattern when L-Phe was added. Moreover, all AroG mutants at Gln151 showed greater resistance to feedback inhibition when compared with AroG^{wt} and AroG^{L175D}. AroG^{Q151N} gave the greatest inhibition pattern at the concentration of L-Phe at 0 - 20 mM. % inhibitions by 20 mM phenylalanine were decreased from 51% of wild type to 12, 16 and 27% for Q151L, Q151N and Q151A, respectively (Table 3). Destruction of two H-bonding between Ser180 and phenylalanine by substitution with Phe (S180F) was reported to decline % inhibition by phenylalanine at concentration of 20 mM from 58% to 7.4% [46]. The result indicated that H-bonding between Gln151 of AroG and the inhibitor, phenylalanine, had a high impact on phenylalanine feedback inhibition. Then, the aroG^{Q151N} gene was used for combination with other genes.

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The data were received from three independent experiments.

(opened diamonds: *E.coli* BL21(DE3), opened triangles: *E.coli* BL21(DE3) harboring pRSFDuet-1, closed diamonds: *E.coli* BL21(DE3) harboring pAroG^{wt}, crosses: *E.coli* BL21(DE3) harboring pAroG^{L175D}, closed boxes: *E.coli* BL21(DE3) harboring pAroG^{Q151L}, closed triangles: *E.coli* BL21(DE3) harboring pAroG^{Q151A} and closed circles: *E.coli* BL21(DE3) harboring pAroG^{Q151N})

Mutant	Specific ac	%inhibitor	
	0 mM L-Phe	20 mM L-Phe	
E. coli BL21(DE3)	0.31 ± 0.04	0.18 ± 0.05	42
pRSFDuet-1	0.40 ± 0.07	0.24 ± 0.04	41
Arog ^{wt}	1.89 ± 0.39	0.93 ± 0.45	51
AroG	0.82 ± 0.40	0.25 ± 0.09	69
AroG	2.15 ± 0.11	1.90 ± 0.05	12
AroG ^{Q151A}	2.11 ± 0.75	1.55 ± 0.41	27
AroG Q151N	2.26 ± 0.06	1.90 ± 0.06	16

 Table 3 DAHP synthase activities and feedback inhibition of various AroG clones.



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3.5 Construction of $pBLPTA^{L359D}G^{wt}$ and $pBLPTA^{L359D}G^{Q151N}$

3.5.1 pBLPTA^{L359D} preparation

The pBLPTG^{Q151L}A^{L359D} containing *aroB*, *aroL*, *phedh*, *tktA*, *aroG* and *pheA* was double digested with *Pac*I and *Avr*II. After digestion, linear fragment of pBLPTA^{L359D} was separated by agarose gel electrophoresis and purified by GenepHlowTM Gel/PCR Kit. From Figure 42, the linear form of pBLPTA^{L359D} vector around 9.9 kb was detected (lane 3).

3.5.2 Amplification of T7 aroG^{wt} and T7 aroG^{Q151N}

 $T7_aroG^{wt}$ and $T7_aroG^{O151N}$ fragments were amplified from pAroG^{wt} and pAroG^{O151N} using forward primer containing *PacI* site and reverse primer containing *AvrII* site. After cleaning, the PCR fragments were separated by agarose gel electrophoresis. The size of $T7_aroG^{wt}$ and $T7_aroG^{O151N}$ fragments were detected around 1.2 kb as shown in Figure 43. Then, PCR fragments were double digested with *PacI* and *AvrII*. After digestion, $T7_aroG^{wt}$ and $T7_aroG^{O151N}$ fragments were purified by GenepHlowTM Gel/PCR Kit. The size of $T7_aroG^{wt}$ and $T7_aroG^{O151N}$ fragments were confirmed by agarose gel electrophoresis. From Figure 44, size of $T7_aroG^{wt}$ and $T7_aroG^{O151N}$ fragments were around 1.2 kb in lane 2 and 4, respectively.

3.5.3 Cloning of $pBLPTA^{L359D}G^{wt}$ and $pBLPTA^{L359D}G^{Q151N}$

The *aroG^{wt}* and *aroG^{O151N}* fragments were ligated into pBLPTA^{L359D} linear vector and then transformed into *E. coli* BL21(DE3) by electroporation. The single colonies of pBLPTA^{L359D}G^{wt} and pBLPTA^{L359D}G^{O151N} transformants were randomly picked and cultured in 5 mL of LB broth containing 30 mg/mL of kanamycin. Each recombinant plasmid was extracted, digested with *Xho*I and then detected by agarose gel electrophoresis. From digestion pattern, each recombinant plasmid gave two DNA bands around 10.0 kb and 1.1 kb as shown in Figure 45. This result confirmed that *aroG* genes were inserted into pBLPTA^{L359D}. After that, the nucleotide sequences of the inserts were checked by Bioneer Inc. (Korea).





Lane M1 : Gene Ruler 1 kb DNA ladder

Lane 1 : pBLPTA^{L359D} uncut

Lane 2 : Pacl and AvrII digested pBLPTA^{L359D}





Lane 2 : PCR product of T7_*aroG*^{Q151N}



Figure 44. *Pacl* and *Avr*II digestion patterns of PCR products of *T7_aroG^{wt}* and *T7_aroG^{Q151N}*.

Lane M : Gene Ruler 1 kb DNA ladder

Lane 1 : uncut PCR product of T7_aroG^{wt}

- Lane 2 : Pacl and AvrII digested PCR product of T7_aroG^{wt}
- Lane 3 : uncut PCR product of T7 aroG^{Q151N}
- Lane 4 : Pacl and AvrII digested PCR product of T7_aroG^{Q151N}

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Figure 45. Xhol digestion patterns of pBLPTA^{L359D}G^{wt} and pBLPTA^{L359D}G^{Q151N}.

- Lane M : Gene Ruler 1 kb DNA ladder
- Lane 1 : uncut pBLPTA^{L359D}G^{wt}
- Lane 2 : Xhol digested pBLPTA^{L359D}G^{wt}
- Lane 3 : uncut pBLPTA^{L359D}G^{Q151N}
- Lane 4 : Xhol digested pBLPTA^{L359D}G^{Q151N}



3.5.4 Nucleotide sequencing

To verify the nucleotide sequences of *aroG^{wt}* and *aroG^{Q151N}*, the DNA sequencing of recombinant plasmids were performed by Bioneer, Korean using ACYCDuet1 as a forward primer and DuetDown1 as a reverse primer. The obtained DNA sequences were compared with *aroG* sequence from section 3.4.4 by nucleotide blast tools in NCBI.

The nucleotide sequence of $aroG^{Wt}$ and $aroG^{Q151N}$ showed 100% similarity to these of aroG in pAroG (Figure 46 - 47). The correct $aroG^{Wt}$ and $aroG^{Q151N}$ genes were used in the further experiment.

3.6 Co-transformation of pBLPTA^{L359D}G^{wt} and pBLPTA^{L359D}G^{Q151N} with pYF into *E. coli* BL21(DE3)

The correct pBLPTA^{L359D}G^{wt} and pBLPTA^{L359D}G^{Q151N} from section 3.2.3.4 were co-transformed with pYF into *E. coli* BL21(DE3) competent cell using electroporation. The growing transformants of pBLPTA^{L359D}G^{wt} & pYF and pBLPTA^{L359D}G^{Q151N} & pYF clones were picked up to culture in 5 mL LB broth that contained 30 mg/mL of kanamycin and 10 mg/mL of chloramphenicol. After extraction, the pBLPTA^{L359D}G^{wt} & pYF and pBLPTA^{L359D}G^{Wt} & pYF and pBLPTA^{L359D}G^{Wt} & pYF and pBLPTA^{L359D}G^{Q151N} & pYF were confirmed by digestion with *Bam*HI. From Figure 48, pBLPTA^{L359D}G^{Wt} & pYF digested with *Bam*HI in lane 10 gave three bands at 10.0 kb, 6.0 kb and 1.1 kb which were same size as pBLPTA^{L359D}G^{Wt} (10.0 kb) in lane 2 and pYF (6.0 and 1.1 kb) in lane 6. pBLPTA^{L359D}G^{Q151N} & pYF also gave the same result (lane 12). Then, pBLPTA^{L359D}G^{Wt} & pYF in lane 8 and pBLPTA^{L359D}G^{Q151N} & pYF were used to determine L-Phe production.

1949 b	its(1055	Expect Ider) 0.0 105	5/1055(100%)	Gaps 0/1055(0%)	Strand Plus/Plus
Query Sbjct	123 1	CCATGGTGTATCAGAAC	GACGATTTACGCATCAAA GACGATTTACGCATCAAA	AGAAATCAAAGAG AGAAATCAAAGAG	TTACTTCCTCCTG
Query	183	TCGCATTGCTGGAAAAA	TTCCCCGCTACTGAAAAI	GCCGCGAATACG	GTTGCCCATGCCC
Sbjct	61	TCGCATTGCTGGAAAAA	TTCCCCGCTACTGAAAAI	GCCGCGAATACG	GTTGCCCATGCCC
Query	243	GAAAAGCGATCCATAAG	ATCCTGAAAGGTAATGAI	TGATCGCCTGTTG	GTTGTGATTGGCC
Sbjct	121	GAAAAGCGATCCATAAG	ATCCTGAAAGGTAATGAI	GATCGCCTGTTG	GTTGTGATTGGCC
Query	303	CATGCTCAATTCATGAT	CCTGTCGCGGCAAAAGAG	TATGCCACTCGC	TTGCTGGCGCTGC
Sbjct	181	CATGCTCAATTCATGAT	CCTGTCGCGGCAAAAGAG	TATGCCACTCGC	TTGCTGGCGCTGC
Query	363	GTGAAGAGCTGAAAGAT	GAGCTGGAAATCGTAATG	CGCGTCTATTTT	GAAAAGCCGCGTA
Sbjct	241	GTGAAGAGCTGAAAGAT	GAGCTGGAAATCGTAATG	CGCGTCTATTTT	GAAAAGCCGCGTA
Query	423	CCACGGTGGGCTGGAAA	GGGCTGATTAACGATCCG	CATATGGATAAT	AGCTTCCAGATCA
Sbjct	301	CCACGGTGGGCTGGAAA	GGGCTGATTAACGATCCG	CATATGGATAAT	AGCTTCCAGATCA
Query	483	ACGACGGTCTGCGTATA	GCCCGTAAATTGCTGCTI	GATATTAACGAC	AGCGGTCTGCCAG
Sbjct	361	ACGACGGTCTGCGTATA	GCCCGTAAATTGCTGCTT	GATATTAACGAC	AGCGGTCTGCCAG
Query	543	CGGCAGGTGAGTTTCTC	GATATGATCACCCCACAA	ATATCTCGCTGAC	CTGATGAGCTGGG
Sbjct	421	CGGCAGGTGAGTTTCTC	GATATGATCACCCCACAA	TATCTCGCTGAC	CTGATGAGCTGGG
Query	603	GCGCAATTGGCGCACGI	ACCACCGAATCGCAGGT	SCACCGCGAACTG	GCATCAGGGCTTT
Sbjct	481	GCGCAATTGGCGCACGI	ACCACCGAATCGCAGGTG	CACCGCGAACTG	GCATCAGGGCTTT
Shict	541		AAAAATGGCACCGACGGT		CCTATCGATGCCA
Ouery	723	TTAATGCCGCCGGTGCG	CCGCACTGCTTCCTGTCC	CETALCEALTER	GGGCATTCGGCGA
Shict	601	TTAATGCCGCCGGTGCG		GTAACGAAATGG	GGGCATTCGGCGA
Query	783	TTGTGAATACCAGCGGI	AACGGCGATTGCCATATO	CATTCTGCGCGGC	GGTAAAGAGCCTA
Sbjct	661	TTGTGAATACCAGCGGI	ACGGCGATTGCCATAT	CATTCTGCGCGGC	GGTAAAGAGCCTA
Query	843	ACTACAGCGCGAAGCAC	GTTGCTGAAGTGAAAGAA	AGGGCTGAACAAA	GCAGGCCTGCCAG
Sbjct	721	ACTACAGCGCGAAGCAC	GTTGCTGAAGTGAAAGA	AGGGCTGAACAAA	GCAGGCCTGCCAG
Query	903	CACAGGTGATGATCGAT	TTCAGCCATGCTAACTCO	STCCAAACAATTC	AAAAAGCAGATGG
Sbjct	781	CACAGGTGATGATCGAT	TTCAGCCATGCTAACTC	JIIIIIIIIIIIII GTCCAAACAATTC	AAAAAGCAGATGG
Query	963	ATGTTTGTGCTGACGTI	TGCCAGCAGATTGCCGGI	IGGCGAAAAGGCC	ATTATTGGCGTGA
Sbjct	841	ATGTTTGTGCTGACGTI	TGCCAGCAGATTGCCGG	rggcgaaaaggcc	ATTATTGGCGTGA
Query	1023	TGGTGGAAAGCCATCTG	GTGGAAGGCAATCAGAG	CTCGAGAGCGGG	GAGCCGCTGGCCT
Sbjct	901	TGGTGGAAAGCCATCTG	GTGGAAGGCAATCAGAGC	CTCGAGAGCGGG	GAGCCGCTGGCCT
Query	1083	ACGGTAAGAGCATCACC	GATGCCTGCATCGGCTGG	GAAGATACCGAT	GCTCTGTTACGTC
	961	ACGGTAAGAGCATCACC	GATGCCTGCATCGGCTG	GAAGATACCGAT	GCTCTGTTACGTC
Sbjct					
Sbjct Query	1143	AACTGGCGAATGCAGTG	AAAGCGCGTCGCGGGTA	A 1177	

Figure 46. Nucleotide sequence of $aroG^{wt}$ in pBLPTA^{L359D}G^{wt}

Query represented the nucleotide sequence of $aroG^{wt}$ in pBLPTA^{L359D}G^{wt}.

Sbjct represented the nucleotide sequence of in $\mathsf{pAroG}^{\mathsf{wt}}$

Score 1949 b	its(105	Ex 5) 0.0	pect 0	Identities 1055/1055(100%)		Gaps 0/1055(0%)	Strand Plus/Plus	
Query	123	CCATGGTG	TATCAG	AACGACGATTTACGC	ATCAAA	GAAATCAAAG	AGTTACTTCCTCC	CTG 182
Sbjct	1	CCATGGTG	TATCAG	AACGACGATTTACGC	ATCAAA	GAAATCAAAG.	AGTTACTTCCTCC	TG 60
Query	183	TCGCATTG	CTGGAA	AAATTCCCCGCTACT	GAAAAT	GCCGCGAATA	CGGTTGCCCATGO	CC 242
Sbjct	61	TCGCATTG	CTGGAA	AAATTCCCCGCTACT	GAAAAT	GCCGCGAATA	CGGTTGCCCATGO	CC 120
Query	243	GAAAAGCG	ATCCAT	AAGATCCTGAAAGGT	AATGAT	GATCGCCTGT	TGGTTGTGATTGG	CC 302
Sbjct	121	GAAAAGCG.	ATCCAT	PAAGATCCTGAAAGGT	AATGAT	GATCGCCTGT	TGGTTGTGATTGG	SCC 180
Query	303	CATGCTCA	ATTCAT	GATCCTGTCGCGGCA	AAAGAG	TATGCCACTC	GCTTGCTGGCGCI	IGC 362
Sbjct	181	CATGCTCA	ATTCAT	GATCCTGTCGCGGCA	AAAGAG	TATGCCACTC	GCTTGCTGGCGCI	CGC 240
Query	363	GTGAAGAG	CTGAAA	GATGAGCTGGAAATC	GTAATG	CGCGTCTATT	TTGAAAAGCCGCG	TA 422
Sbjct	241	GTGAAGAG	CTGAAA	GATGAGCTGGAAATC	GTAATG	CGCGTCTATT	TTGAAAAGCCGCG	TA 300
Query	423	CCACGGTG	GGCTGG	AAAGGGCTGATTAAC	GATCCG	CATATGGATA	ATAGCTTCCAGAT	CA 482
Sbjct	301	CCACGGTG	GGCTGG	AAAGGGCTGATTAAC	GATCCG	CATATGGATA	ATAGCTTCCAGAI	CA 360
Query	483	ACGACGGT	CTGCGT	ATAGCCCGTAAATTG	CTGCTT	GATATTAACG	ACAGCGGTCTGCC	AG 542
Sbjct	361	ACGACGGT	CTGCGT	ATAGCCCGTAAATTG	CTGCTT	GATATTAACG	ACAGCGGTCTGCC	AG 420
Query	543	CGGCAGGT	GAATTC	CTCGATATGATCACT	CCTAAT	TATCTCGCTG	ACCTGATGAGCTG	GG 602
Sbjct	421	CGGCAGGT	GAATTC	CTCGATATGATCACT	CCTAAT	TATCTCGCTG	ACCTGATGAGCTG	GG 480
Query	603	GCGCAATT	GGCGCA	CGTACCACCGAATCG	CAGGTG	CACCGCGAAC	TGGCATCAGGGCI	CTT 662
Sbjct	481	GCGCAATT	GGCGCA	CGTACCACCGAATCG	CAGGTG	CACCGCGAAC	TGGCATCAGGGCI	TT 540
Query	663	CTTGTCCG	GTCGGC	TTCAAAAATGGCACC	GACGGT	ACGATTAAAG	IGGCTATCGATGC	CA 722
Sbjct	541	CTTGTCCG	GTCGGC	TTCAAAAATGGCACCO	GACGGT	ACGATTAAAG	TGGCTATCGATGC	CA 600
Query	723	TTAATGCC	GCCGGT	GCGCCGCACTGCTTC	CTGTCC	GTAACGAAAT	GGGGGCATTCGGC	GA 782
Sbjct	601	TTAATGCC	GCCGGT	GCGCCGCACTGCTTC	CTGTCC	GTAACGAAAT	GGGGGCATTCGGC	GA 660
Query	783	TTGTGAAT	ACCAGO	GGTAACGGCGATTGC	CATATC	ATTCTGCGCG	GCGGTAAAGAGCC	TA 842
Sbjct	661	TTGTGAAT	ACCAGO	GGTAACGGCGATTGC	CATATC	ATTCTGCGCG	GCGGTAAAGAGCC	TA 720
Query	843	ACTACAGC	GCGAAG	CACGTTGCTGAAGTG	AAAGAA	GGGCTGAACA	AAGCAGGCCTGCC	AG 902
Sbjct	721	ACTACAGC	GCGAAG	CACGTTGCTGAAGTG	AAAGAA	GGGCTGAACA	AAGCAGGCCTGCC	AG 780
Query	903	CACAGGTG	ATGATC	GATTTCAGCCATGCT	AACTCG	ICCAAACAAT	TCAAAAAGCAGAT	GG 962
Sbjct	781	CACAGGTG	ATGATC	GATTTCAGCCATGCT	AACTCG	ICCAAACAAT	TCAAAAAGCAGAT	GG 840
Query	963	ATGTTTGT	GCTGAC	GTTTGCCAGCAGATTO	GCCGGT	GCGAAAAGG	CCATTATTGGCGT	GA 1022
Sbjct	841	ATGTTTGT	GCTGAC	GTTTGCCAGCAGATTO	SCCGGT	GCGAAAAGG	CCATTATTGGCGI	GA 900
Query	1023	TGGTGGAA	AGCCAT	CTGGTGGAAGGCAAT	CAGAGC	CTCGAGAGCG	GGGAGCCGCTGGC	CT 1082
Sbjct	901	TGGTGGAA	AGCCAT	CTGGTGGAAGGCAAT	CAGAGC	CTCGAGAGCG	GGGAGCCGCTGGC	CT 960
Query	1083	ACGGTAAG	AGCATC	ACCGATGCCTGCATCO	GCTGG	GAAGATACCG	ATGCTCTGTTACG	TC 1142
Sbjct	961	ACGGTAAG	AGCATC	ACCGATGCCTGCATC	GCTGG	GAAGATACCG	ATGCTCTGTTACG	TC 1020
Query	1143	AACTGGCG	AATGCA	GTGAAAGCGCGTCGC	GGTAA	1177		
Sbjct	1021	AACTGGCG	AATGCA	GTGAAAGCGCGTCGC	GGTAA	1055		

Figure 47. Nucleotide sequence of $aroG^{Q151N}$ in pBLPTA^{L359D}G^{Q151N}

Query represented the nucleotide sequence of $aroG^{Q151N}$ in pBLPTA^{L359D}G^{Q151N}. Sbjct represented the nucleotide sequence of $aroG^{Q151N}$ in pAroG^{Q151N}



Figure 48. *Bam*HI digestion patterns of pBLPTA^{L359D}G^{wt} & pYF and pBLPTA^{L359D}G^{Q151N} & pYF.

Lane M : Gene Ruler 1 kb DNA ladder

Lane 1 : uncut pBLPTA^{L359D}G^{wt}

Lane 2 : BamHI digested pBLPTA^{L359D}G^{wt}

Lane 3 : uncut pBLPTA^{L359D}G^{Q151N}

Lane 4 : BamHI digested pBLPTA^{L359D}G^{Q151N}

Lane 5 : uncut pYF

Lane 6 : BamHI digested pYF

Lane 7 : uncut pBLPT & pYF

Lane 8 : BamHI digested pBLPT & pYF

Lane 9 : uncut $pBLPTA^{L359D}G^{wt} & pYF$

Lane 10 : BamHI digested pBLPTA $^{L359D}G^{wt}$ & pYF

Lane 11 : uncut $pBLPTA^{L359D}G^{Q151N}$ & pYF

Lane 12 : BamHI digested pBLPTA^{L359D}G^{Q151N} & pYF

3.7 Production of L-Phe

Each recombinant clone was cultured in 200 mL of minimum medium containing 6% glycerol as a carbon source (Ulfah, 2018). Membrane protein genes (glpF and yddG) under the tight regulation of ara promoter were induced by 0.02% arabinose. The growth profile measured at a wavelength of 600 nm showed that growth of pBLPTA^{L359D}G^{wt} & pYF and pBLPTA^{L359D}G^{Q151N} & pYF were not significantly different and higher than that of pBLPT & pYF. Cell growth of each clone exhibited the exponential phase until 72 h (Figure 49). L-Phenylalanine production was correlated with phase of cell growth. At 192 h, the recombinant <code>pBLPTAL359D</code>G Q151N & pYF clone gave the highest L-Phe production at 1.95 g/L that was 8.7 and 1.2 fold of that obtained from pBLPT & pYF (0.224 g/L) and pBLPTA^{L359D}G^{wt} & pYF (1.61 g/L), respectively (Figure 50). Addition of recombinant phenylalanine feedback resistant PheA clearly showed to have more impact on L-Phe production than the addition of feedback resistant AroG. PheA (chorismite mutase/prephenate dehydratase) catalyzes a conversion of chorismite to phenylpyruvate through prephenate intermediate that is the key step in determining the L-Phe production while AroG, a main isoform of DAHP synthase, catalyzes the first step of aromatic amino acid biosynthesis pathway. DAHP, the product of AroG can be used not only in the biosynthesis of phenylalanine but also in the syntheses of tyrosine and tryptophan. The cultivating conditions and medium used in this experiment was optimized by Ulfah (2018) for pBLPT & pYF clone. Thus, the composition of glycerol medium as well as culture conditions should be adjusted to improve yield of L-Phe.



Figure 49. Growth curve of recombinant clones in minimum medium.

The data were received from three independent experiments.



Figure 50. L-Phe production of recombinant clones in minimum medium. The data were received from three independent experiments.

CHAPTER IV

CONCLUSIONS

- 1. The expression of PheA in pRSFDuet-1 (pPheA) was confirmed by the appearance of a high intensity protein band around 43 kDa in SDS-gel.
- Frame shift mutation of *aroG* inserted at *Bam*HI site of pRSFDuet-1 was found. Therefore, pAroG^{wt} and pAroG^{fbr} (pRSFDuet-1 harboring *aroG^{wt}* and *aroG^{fbr}*, respectively) were reconstructed.
- 3. All obtained *aroG* clones gave the protein band approximately 38 kDa on SDS-gel. The mutated enzymes exhibited slightly higher DAHP synthase activity to the wildtype enzyme and %inhibitions by 20 mM L-Phe were decreased from 51% to 12 27%. Thus, H-bonding between Gln151 of AroG and the inhibitor, phenylalanine, has a high impact on phenylalanine feedback inhibition.
- 4. The recombinant *E. coli* BL21(DE3) clones containing *aroB, aroL, phedh, tktA, aroG, glpF, yddG* and *pheA* was successfully constructed. After 8 days of fermentation in 6% glycerol medium, pBLPTA^{L359D}G^{Q151N} & pYF clone gave the highest L-phenylalanine production (1.95 g/L) that was 8.7 and 1.2 fold of that obtained from pBLPT & pYF and pBLPTA^{L359D}G^{wt} & pYF clones, respectively. The result revealed that feedback resistant PheA and AroG could elevate L-phenylalanine production.

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Appendix A

Map of pRSFDuet-1



pRSFDuet-1 cloning/expression regions

Appendix B

Map of pBAD-33



Appendix C

Preparation of Luria-Bertani (LB) broth

Luria-Bertani (LB) broth is used for medium growth of *E. coli*. LB medium contained 1% pancreatic digestion of casein, 0.5% NaCl and 0.5% yeast extract. For agar plate, the LB medium is supplemented with 1.5% (w/v) agar. The medium was sterilized by autoclave for 15 minutes at 121 °C, 15 psi. Then, antibiotic drug (kanamycin, ampicillin or chloramphenicol) was added for selection.



Appendix D

Preparation of competent cell

In this study, we used *E. coli* Top10 and *E. coli* BL21(DE3) as competent cells. Each single colony of *E. coli* Top10 and *E. coli* BL21(DE3) was picked up to culture in 5 ml of LB broth and incubated at 37 °C with shaking at 250 rpm for 16-18 h. 5% of starter was inoculated into 50 ml of LB broth. The cultures were incubated at 37 °C with shaking at 250 rpm for 16-18 h. The 5% of each starter was inoculated into 200 ml of LB broth and grown at 37 °C with shaking at 250 rpm until OD_{600} reached 0.3-0.4. The cultures were chilled on ice and centrifuged at 4°C, 3,000xg for 10 min. The supernatants were removed and the cell pellets were washed with 2 volume of cool sterilized DI water for 2 times. After that, the cell pellets were washed with 20 ml of cool sterilized 10% glycerol and centrifuged at 4 °C, 3,000xg for 10 min. Then, the cell pellets were resuspended with cool sterilized 10% glycerol to the final volume 2 ml. Finally, 50 µl of competent cell was aliquoted into microcentrifuge tube and stored at -80 °C.

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Appendix E

Protocol of Presto™ Mini Plasmid Kit

Plasmid extraction was performed using Presto[™] Mini Plasmid Kit (Geneaid).

1. Harvesting

The 1.5 ml of cultured bacterial cells was transferred to a microcentrifuge tube and centrifuged at 5,000 x g for 2 min at room temperature. Then, the supernatant was discarded. The harvesting step was repeated using the same 1.5 ml microcentrifuge tube.

2. Resuspension

Two hundred μ l of PD1 buffer contained RNaseA was added to the tube containing the cell pellet and then mixed by vortex.

3. Cell Lysis

Two hundred μ l of PD2 buffer was added to lyse the cell, mixed gently by inverting the tube and incubated at room temperature for 2 min.

4. Neutralization

Three hundred μ l of PD3 buffer was added to neutralize the reaction and then mixed immediately by inverting the tube. After that the supernatant was separated by centrifugation at 10,000 x g for 15 min at room temperature.

5. DNA Binding

All the supernatant was transferred to the PDH column and centrifuged at $10,000 \times g$ for 2 min at room temperature and the flow-through was discarded.

6. Wash

Four hundred μ l of W1 Buffer was added into the PDH column. The column was taken centrifuged at 10,000 x g for 2 min to discard the flow-through. After that, 600 μ l of wash buffer containing absolute ethanol was added into the PDH column. Centrifugation was performed at 10,000 x g for 2 min at room temperature to discard the flow through follow by centrifugation at 10,000 x g for 3 min at room

temperature to dry the column matrix. The dried PDH column was transferred to a new microcentrifuge tube.

7. Elution

fifty μ l of water was added into the center of the column matrix and stood for at least 2 min. Centrifugation was performed at 10,000 x g for 3 min at room temperature to elute the purified DNA.



Appendix F

Protocol of GenepHlow™ Gel/PCR Kit

Purification of DNA fragment using Gel/PCR DNA Fragments Extraction Kit (Geneaid) was performed following these steps:

1. Sample preparation

Gel Dissociation

The 300 mg of agarose gel slice containing relevant DNA fragments was cut and transferred into a microcentrifuge tube. Five hundred μ l of Gel/PCR Buffer was added to the sample then mix by vortex. The sample was incubated at 55-60°C for 10-15 min to completely dissolved the gel. After incubation, cool the dissolved sample mixture was cooled to room temperature.

PCR reaction

The 5 volumes of Gel/PCR Buffer was added to the PCR reaction and mixed.

2. DNA Binding

The sample mixture was transferred to the DFH column and centrifuged at $10,000 \times g$ for 2 min. The flow-through was discarded then place the DFH column back in the 2 ml collection tube.

3. Wash

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Four hundred μ l of W1 buffer was added into the DFH column and centrifuged at 10,000 x g for 2 min and discarded the flow-through. After that, 600 μ l of wash buffer contained absolute ethanol was added into the DFH column and stood for 1 min. Centrifugation at 10,000 x g for 2 min was performed to discard the flow-through followed by centrifugation at 10,000 x g for 3 min at room temperature to dry the column matrix. The dried DFH column was transferred to a new microcentrifuge tube.

4. Elution

The dried DFH column was transferred to a new microcentrifuge tube. 20-50 μ l of water was added into the center of the column matrix and stood for at least 2 min. The column was centrifuged at 10,000 x g for 3 min at room temperature to elute the purified DNA.



Appendix G

Preparation for SDS-PAGE analysis 1. Stock solution 2 M Tris-HCl (pH 8.8) Tris (hydroxymethyl)-aminomethane 24.2 g Adjusted pH to 8.8 with 1 N HCl and adjusted volume to 100 ml with distilled water. 1 M Tris-HCl (pH 6.8) Tris (hydroxymethyl)-aminomethane 12.1 g Adjusted pH to 6.8 with 1 N HCl and adjusted volume to 100 ml with distilled water. 10% (w/v) SDS Sodium dodecyl sulfate (SDS) 10 g Dissolved in distilled water to a total volume of 100 ml. 50% (w/v) Glycerol 100% Glycerol 50 ml Dissolved in distilled water to a total volume of 100 ml. 1% (w/v) Bromophenol blue Bromophenol blue 100 mg Brought to 10 ml with distilled water and stirred until dissolved.

The aggregated dye was removed by filtration.

Appendix G (continued)

2. Working solutions

Solution A (30% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide)

Acrylamide	29.2	g
N, N'-methylene-bis-acrylamide	0.8	g

Adjusted volume to 100 ml with distilled water.

Solution B (1.5 M Tris-HCl, pH 8.8 and 0.4% SDS)

2 M Tris-HCl (pH 8.8)	75	ml
10% (w/v) SDS	4	ml
Distilled water	21	ml
Solution C (0.5 M Tris-HCl, pH 6.8, 0.4% SDS)		
1 M Tris-HCl (pH 6.8)	50	ml
10% (w/v) SDS	4	ml
Distilled water จุฬาลงกรณ์มหาวิทยาลัย	46	ml
10% (w/v) Ammonium persulfate		
Ammonium persulfate	0.5	g
Distilled water	5.0	ml

Appendix G (continued)

Electrophoresis buffer (25 mM Tris, 192 mM glycine and 0.1% (w/v) SDS)

Tris (hydroxymethyl)-aminomethane	3.0	g
Glycine	14.4	ml
SDS	1	g

Dissolved and adjusted to total volume to 1 liter with distilled water

(final pH should be approximately 8.3)

5x Sample buffer (312.5 mM Tris-HCl pH 6.8, 50% (v/v) glycerol, 1% (w/v)				
bromophenol blue)				
1 M Tris-HCl (pH 6.8)	0.6	ml		
50% (v/v) Glycerol	5.0	ml		
10% (w/v) SDS	2	ml		
1% (w/v) Bromophenol blue	1	ml		
β-Mercaptoethanol จุฬาลงกรณ์มหาวิทยาลัย	0.5	ml		
Distilled water GHULALONGKORN UNIVERSITY	1.4	ml		

Appendix G (continued)

3. SDS-PAGE

12.5% Separating gel

Solution A	4.2	ml
Solution B	2.5	ml
Distilled water	3.3	ml
10% (w/v) Ammonium persulfate	50	μι
TEMED	5	μι
5.0% Stacking gel		
Solution A	0.67	ml
Solution C	1.0	ml
Distilled water	2.3	ml
10% (w/v) Ammonium persulfate	30	μι
า TEMED จุฬาลงกรณ์มหาวิทยาลัย CHULALONGKORN UNIVERSITY	5	μι

Preparation for protein staining solution		
Staining solution, 1 liter		
Coomassie brilliant blue R-250	1.0	ml
Methanol	450	ml
Distilled water	450	ml
Destaining solution, 1 liter		
Methanol	100	ml
Glacial acetic acid	100	ml
Distilled water	800	ml
จุหาลงกรณ์มหาวิทยาลัย		

Appendix H

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Appendix I

Preparation for Lowry's method solution

Solution A (0.5% copper sulfate and 1% potassium tartate, pH 7.0)

Copper sulfate	0.5	g
Potassium tartate	1	g

Adjusted pH to 7.0 and adjusted to total volume to 100 ml.

Solution B (2% sodium carbonate and 1 N sodium hydroxide)

Sodium carbonate	20	g
Sodium hydroxide	4	g
Dissolved in distilled water to a total volume of 1 liter.		

Solution C (phenol reagent)

Folin-Ciocalteu phenol reagent : distilled water is 1:1

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Appendix J

Preparation of 10X TBE for agarose gel electrophoresis

Electrophoresis buffer (10X TBE)

Tris (hydroxymethyl)-aminomethane	54	g
Boric acid	27.5	g
Ethylenediaminetetraacetic acid, disodium salt	9.3	g

Adjust volume to 1 liter with deionized water



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Standard curve for protein determination by Lowry's method



Appendix L

Standard curve for L-Phe determination by HPLC

==== Shimadzu LabSolutions Calibration Curve ====



Appendix M

HPLC Chromatogram of L-Phenylalanine

- 1. L-Phe Standard
 - A) L-Phe at 0.2 g/L





E) L-Phe at 1.0 g/L



Appendix N

The sequencing chromatogram of *pheA^{wt}* in pRSFDuet-1 using ACYCDuetUP1 (A) and T7terminator (B) primers

(A) Bioneer pheA_wt6-ACYCDuetUP1	Bioneer pheA_wt6-ACYCDuetUP1
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www.www.www.white www.	MMMMMMMMMMMMMMMMMMMMMMMMMMMMMMMMMMMMMM
Bioneer pheA_wt6-ACYCDuetUP1	Bioneer pheA_wt6-ACYCDuetUP1
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Appendix N (continued)

(B)

Bioneer pheA_wt6-T7terminator-

M Bioneer pheA_wt6-T7ter MMMMMMMMMM MM Man Man Man Marken M Mentaland

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Appendix O

The sequencing chromatogram of *pheA^{L359D}* in pRSFDuet-1 using ACYCDuetUP1 (A) and T7terminator (B) primers

(A)	
Bioneer pheA_D1.ACYCDuetUP1	
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	MMMMMMMMMMMMMMMMMMMMMMMMMMMMMMMMMMMMMM
Bioneer phea_D1-ACYCDuetUP1	Bioneer pheA_D1-ACYCDuetUP1
Bioneer phea_D1-ACYCDuetUP1	Bioneer phea_D1-ACYCDuetUP1
	Bioneer Pheal D1-ACYCDuetUP1
	Bioneer phea_D1-ACYCDuetUP1

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Appendix O (continued)

(B)

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Appendix P

The sequencing chromatogram of *aroG^{wt}* in pRSFDuet-1 using ACYCDuetUP1 (A) and DuetDown1 (B) primers

(A)					
ABB Applied Biosystems	121140-88-003-1-end w4-4CYCDuetU1.ab1 121140-88-003-1-end w4-4CYCDuetU1.ab1 CH18 VVH4 Film Name.200354-4	KB 1424 KB top KB_3730_POPT_BDT+3 meb T5:41 CRL:1106_CV20+1136	AB Applied Biosystems	121140-84-003-1-em05 with ACYCDuet30 1 ab1 121140-844-003-1-em05 with ACYCDuet30 1 CH 18 VYHF Plate Name 200255-4	K81424 K8bop K8_3730_POP7_80Tv3.meb T641 CRL1106 CV20+:1136
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Appendix P (continued)

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Applied	121140-BA-004-1-aroG.std-DuatDown1.ab1	KB1424 KB.bop
S Biosystems	121140-BA-004-1-aroG.wt4-DuatDown1	KB_3730_POP7_EDTv3.mob
8: G 3279 A 2528 T 3635 C 4095 Aug Sig: 3384	C#.47 W:A5 Plate Name 200325-4	TS:40 CRL:1068 QV20+:1121

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Appendix Q

The sequencing chromatogram of *aroG^{L175D}* in pRSFDuet-1 using ACYCDuetUP1 (A) and DuetDown1 (B) primers

ABB Applied Biosystems	121145-84-005-1 web D2-ACYCDwelle1 at 1 121145-84-055-1 web D2-ACYCDwelle1 C#45 W 85 Pate Nene 20026-4	KB 14.2.4 KB logs KB_3730_POPT_BDTv3 mob T5-48 CRL1100 GV20+1091	AB Applied Biosystems	121145-8A-005-1-exil D2-ACYCDuet(p1.eb1 121145-8A-005-1-exil D2-ACYCDuet(p1 0F45 W 85 Piste Nene 20025-4	KB 1424 KB bap KB_3750_PCPT_BCTV3 web T5 48 CRL1100 CV20+1091
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CCTGTCGCATTGCTGGAAAA 160 165 170 175	ATTCCCCCCCTACTGAAAATGCCCCCCAATACGGTTGCC 180 185 180 185 200 205 210 215	LAT G C C C G A A A A G C B A T C CA TA A G 220 225 250 255	ATTOCTOCTTOATATTAACGA 400 405 500 505	CA & C & O T C T O C CA & C & O C A & O T O A & T T C T C O. 810 815 820 825 820 826 840	ATATOATCACCCCACAATATCTCOCTOACC 545 550 555 560 585
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605 000 005 870	0 0 TA COATTAAA GTO COATT COATO COATTAAT BCCGC 875 680 885 890 895 700 705 710	20010C0CC0CAC10C11CC101C 715 720 725 730 735	TTATT 00 C 01 GAT 0 A0 00 AA AG 995 1000 1005 1010	CCATCT 00 T 0 0 AA 00 C AAT CA 0A 0 CCT C A 0A A C 1015 1020 1025 1030 1035 1040 104	0 0 0 A 0 00 0 0 0 0 0 0 0 0 0 0 0 0 0
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Appendix Q (continued)

(B)

AB Applied Biosystems	121140-84-005-1-eroS 202-DawDown1 ex1 121140-84-005-1-eroS 202-OweDown1 C#43 19208 Pale Name 2000254	KB 1.424 KB.log KB_3130_POPT_BOT-0.mm T0.30 OKL411 0/00-495 50	AB Applied Biosystems	121140-86-000-1 errig 32 DaxCevent akt 121140-86-000-1 errig 32 DaxCevent C# 43 11105 Pass Nerre 200354	KB 1424 KB kep KB_5755_POPT_BOT-5 mit TB 38_ORL913_QVD+965
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AB Applied					

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Appendix R

The sequencing chromatogram of *aroG^{Q151L}* in pRSFDuet-1 using ACYCDuetUP1 (A) and DuetDown1 (B) primers

(A)

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Appendix R (continued)

(B)					
Applied Biosystems	121140-84-012-1-and LI-DuadDevril ab 1 121140-84-010-1-and LI-DuadDevril (2143-94-010-1-and LI-DuadDevril (2143-95-44) Paies Name 200231-4	KB 1424 KB key KB_3102_FOPT_ROT43 met T141 OR_1137 0/20-1141	Applied Biosystems	121140-88-012-1-am/3.1.8-DaxDown1.ak1 121140-88-012-1-am/3.1.8-DaxDown1 (24:48.11):40.Fees tame 200254	88 1424 K8 kep 88,3730,9597,8574 kep 7841,684,1137 0/26+1141
10 A AT AC TT T C TOOT T COACT T A O 5 10 15 20 25	CATTATOCOGCOCAA0CTTTTACCCOCOACOCO 30 28 40 45 50 55 60 0	TTCACTO CATTCOCCAGTTOACO	226 340 346 346 300 30	TCA BCA A COT BCTTC BC BC T BT A GTTA BBCT CTTTA C 5 380 365 370 375 360 386 380	200 COCOCCAGAAT GATAT GOCAAT 205 400 405 410 415
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AB Applied Biosystems	121142-84-012-1-and L&DowDownt e81 121142-84-012-1-and L&DowDownt	KB 1424 KB log KB_3730_POPT_B074 mit	AB Applied Biosystems	121145-84-012-1 and L9-DaeDown1 ab1 121145-84-012-1 and L9-DaeDown1	881424 88 kep 88_3735_POPT_8074.mitk
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Appendix S

The sequencing chromatogram of *aroG^{Q151A}* in pRSFDuet-1 using ACYCDuetUP1 (A) and DuetDown1 (B) primers

Appendix S (continued)

(B)

AB Applied Biosystems	121140-8A-014-1-areG.A3-QuelDown1 ab1 121140-8A-014-1-areG.A3-QuelDown1	KB 1.4.2.4 KB bop KB_3730_POP7_BOTv3 mith	AB Applied Biosystems	121140-84-014-1-estG.A3-DueDown1-et1 121140-84-014-1-estG.A3-DueDown1	KB 1424 KB kep KB_3730_POP7_B01%1 mit
G ATCT 11 TCT 0 TC 0 ACT 4	0 CATTATOCO OCCO CAAO C TITTACCCO CO ACO CO C 25 30 35 40 41 50 83	TT TCAC TO CATTCOC CAO TTO ACOT 40 65 70 75 40	111 GTTCA GCCCTTCTTCACT 205 340 545 350	CRA4 WC6 PAGE Nere 200254	TE 40 CM, 1131 GV20-1148 C G C C G C G C A A A T G A T A T G G C A A T C C 50 285 400 405 410
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AB Applied	121140-8A-014-1-extG-A3-QueDeen1.ab1 (13140-8A-014-1-extG-A3-QueDeen1	KB1424 KBing	AB Applied	121140-84-014-1-emG-A3-DuedDown1.eb1	KB 1424 KB kp
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(11)
Appendix T

The sequencing chromatogram of *aroG^{Q151N}* in pRSFDuet-1 using ACYCDuetUP1 (A) and DuetDown1 (B) primers

(A	I)
v .	

Appendix T (continued)

(B)

Applied	121140-88-005-1-ave5 165-DueDeve1.ab1	KB1424 KB3up	AS Applied	121140-84-020-1-evG.16-DueDown1.ab1	X81424 X8 http
8011 2007 BIOSYSTEMS Bigwi 0.2014A 1983 T2298 C2687 Aug8g 2148	1211420 BA-020-1 areG 38 CustDown1 CR 63 W A7 Plate Name 200325-4	X8_3730_PCPT_80743.mill T8.42_CRL.1138_0V25+1145	Bigwil 0.2014A 1453 T2298 C 2557 AvgBig 2145	121142-64-620-1-eroG365-CuerDown1 Cit 63 19-47 Flate Name 200326-4	KB_3730_POP7_BOT43 meb 75.42 CRL1135 GV29+1145
6 AM CCC TTT CT 00TTC 0AC TT 5 10 18 20	A O CATTATO CO OCCO CAAO CTTTTACCCO CO ACO CO 25 50 55 42 45 50 55 6	0 85 78 75 80	335 340 345 350 5	IS SHE 245 270 275 260 365 260	200 400 408 410 415
	Margan M.				
A A CA O A O CATCO O TATCTTCCO 80 85 100 108	CA GC CG A T G C A G G C A T C G G T G A T G C T C T T A C G T A G 116 118 120 128 138 138 140	145 150 154 160 165	000 0TT ACCOCTO 0TATT CA CA 420 428 430 438	ATCOCCGAATOCCCCCATTTCOTTACGOACAGOAAO 40 445 450 455 460 468 479 471	CA OT OC OOC OC A CC O OC O C A TT A A T O 480 485 480 485 800
286 280 246 270 400-AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	276 260 266 260 266 200 300 306 1882.488.48.48.48.48.48.48.48.48.48.48.48.48	200 205 220 226 220 14. 14. 14. 1. 1. 14. 14. 1. 1. 1. 1. 1. 1. 1. 1. 1. 1. 1. 1. 1.	400 805 800 805	010 015 020 020 020 030 030 040 MAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	440 460 488 440 448 AXAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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AB Applied Biosystems	121140-8A-020-1-ere0.186-Due/Devrit.ak1	881424 KBiep	Applied	121140-88-020-1-exp0-16-DueDown1.eb1	881424 SB3ep
BigHel Q2074A 1653 T2200 C2567 AugBig 2145	121140-84-020-1 ext6 36:0uetDown1 C8:62 W.A7 Piete Nerve 200325-4	K8_3730_POPT_807v8.meb 75.42 ORL 1136 0V20+1146	Biosystems	121145-8A-025-1-ex03.14E-Due/Down1 CIF43 W/A7 Pate Nervs 200325-4	K8_3730_POP7_80713.mi8 75.42 CRL1136 QVD1-1148
Egnal 0.30744 1663 T2289 0.2567 Aug Sp 3145	121140 BA 4001 Hasto BH O battowell CBRS WAT Pelo Name 2000554 IT OT COTTAATATCAASCASCAATTTACSGSCTATACG 696 700 706 719 716 720 728 1	Kg_2720_007_0070.0014.mis TE42 CRL 1136 0X26-3148 CLARACCGTCGTTCATCTGGAAGCTA 720 746 746 746 750	Bio Bio Systems Bytel 0.2014A 1683 7.2019 C.2687 Aug Big 2148 TTT CA 07 A 0C 00 00 AA TTTTT 1010 1018 1020 1025	121140-84-420-1 4e0 348-0440-04400481 CHB 104-37 Files Netro 200203-4 CCA 65 A4 T 0 0 54 CA 604 60 AA 67 AA 67 CA T T 0 ATT 0 1020 1028 1040 1048 1088 1088 1088 1088	KE_2720_POPT_ROTG.nuk T5.42 CRL1135 Q/02H-1145 TT GAT GCGTAAATCGCCGTTCTGATA 6 1075 1075 1080 1088 1090
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	Kanoktip Packdibamrung. Effect of Gln151 on L-
	phenylalanine feedback resistance of AroG isoform of
	DAHP synthase in Escherichia coli. ScienceAsia. (summitted
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