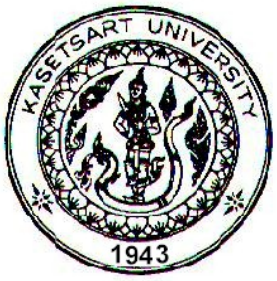

THESIS

TRANSFORMATION OF *α−amylase* GENE INTO SILKWORM (*Bombyx mori* L.) BY USING A *piggyBac* VECTOR

NIPAPORN NGERNYUANG

GRADUATE SCHOOL, KASETSART UNIVERSITY

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THESIS APPROVAL

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THESIS

TRANSFORMATION OF *α-amylase* GENE INTO SILKWORM (*Bombyx mori* L.) BY USING A *piggyBac* VECTOR

NIPAPORN NGERNYUANG

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Master of Science (Genetics)
Graduate School, Kasetsart University
2007

Nipaporn Ngernyuang 2007: Transformation of *α-amylase* Gene into Silkworm (*Bombyx mori* L.) by Using a *piggyBac* Vector. Master of Science (Genetics), Major Field: Genetics, Department of Genetics. Thesis Advisor: Assistant Professor Lertluk Ngernsiri, Ph.D. 140 pages.

α-amylase is commonly enzyme for starch hydrolysis. In the silkworm, *Bombyx mori*, α-amylase is found in both digestive fluid and hemolymph. *α-amylase* genomic DNA of *B. mori,* Nanglai strain (multivoltine strain), was 6,942 bp long and showed 97% identity with *α-amylase* of *B. mori*, p50 strain, in database (nscaf2827.1 Data of NIAS). A 1,503 bp full length ORF sequence was cloned by using Rapid Amplification of cDNA Ends (5' and 3' RACEs) strategy. Six clones were randomly selected and subjected to nucleotide sequencing. Four predicted deduced amino acid sequences, 500 residues each, sharing 99% homology to each other. One of these clones, AMYT-1, provided the sequence homology to α-*amylase* of *B. mori* in database with 97-99 % and to that of other insects, *Ostrinia nubilalis*, *Spodoptera frugiperda*, *Diatraea saccharalis* and *Ceratitis capitata* with 78%, 81%, 79% and 60 % respectively. Comparison of the nucleotide sequence between cDNA and genomic DNA of Nanglai's *α-amylase* showed that the *α-amylase* gene consists of 9 exons. *α-amylase* gene expression was detected by RT-PCR method in silkworm strains, Nanglai, C108 and w1-pnd and found that the *α-amylase* gene was expressed only in gut tissue of the Nanglai and w1-pnd strains. Then, the *α-amylase* gene was cloned into two *piggyBac* vectors, *pBac*[A3-AMY1-A3UTR, 3xP3-DsRed2] and *pBac*[A3-AMY1-SV40UTR, A3-EGFP]. Each recombinant vector was injected into silkworm eggs to produce transgenic silkworms. Two transgenic lines harboring *pBac*[A3-AMY1-A3UTR, 3xP3-DsRed2] were obtained. The expression of *α-amylase* transgene in the transgenic silkworms could express in several tissues.

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Student's signature Thesis Advisor's signature

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Special appreciation is extending to Dr. Isao Kobayashi, who taught me many laboratory techniques and all unnamed kindly friends and office staffs of Transgenic Silkworm Research Center for their support and warm hospitality during my stay in Tsukuba.

Sincere thanks are also extending to all my Thai friends in Tsukuba. The sincere thanks are also extending to all my lovely friends in Thailand for their cooperation and friendship.

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Nipaporn Ngernyuang

October, 2007

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LIST OF ABBREVIATIONS

bp	base pair
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EtBr	ethidium bromide
GFP	green fluorescent protein
GMO	genetically modified organism
hr	hour
ITR	inverted terminal repeat
kb	kilobasepair (1,000 base pair)
LTR	long terminal repeat
nt	nucleotide
ORF	open reading frame
RNA	ribonucleic acid
rpm	rotations per minute
RT-PCR	reverse transcription polymerase chain reaction

TRANSFORMATION OF *α-amylase* GENE INTO SILKWORM (*Bombyx mori* L.) BY USING A *piggyBac* VECTOR

INTRODUCTION

The silkworm, *Bombyx mori*, is the most important economy insect in sericulture industry of Thailand. The main product of sericulture is raw silk yarn, which is the raw material for silk fabric production. Three strains of silkworm, polyvoltine strain, bivotine strain and hybrid strain, were reared in sericulture industry of Thailand. The polyvotine strain can be reared through out the year, in general, approximately six generations. It hardly resists to disease and environment. Unfortunately, the polyvoltine strain produces low yield per unit quantity of eggs. Whereas, the yield produced by bivoltine strain is better than that of polyvoltine strain but the bivotine strain is susceptible to diseases when it is reared in tropical environment. And the hybrid strain is more resist to disease and environment than the bivoltine strain but the silk quality is lower than that of the bivoltine strain.

The most important disease of silkworm is grasserie disease. The disease is caused by an infection of the nuclear polyhedrosis virus (NPV), the most harmful virus in the sericulture industry. Therefore, intensive efforts have been consistently investigated to improve the breeding lines for grasserie disease resistance in addition to the high yield character. The desired lines have not been successfully achieved so far.

α-amylase enzymes play a central role in carbohydrate metabolism so organisms with a starch-rich diet depending on the effectiveness of their amylases for survival. In the silkworm *B. mori*, there are two types of amylase activities in digestive fluid and haemolymph. Interestingly, the activity of amylases in non dipausing strains is higher than that of diapausing strains. The increased enzyme activity and efficient starch digestion might have adaptive significance in the non-diapausing strain which is known to survive better under tropical condition than the diapausing strains. The *α-amylase* gene in the silkworm has been studied and submitted in Genbank but it is only a truncated *α-amylase* gene not full length sequence.

In this study is the first report about a complete nucleotide sequence of *α-amylase* gene from a native Thai silkworm, Nanglai strain. Then, the *α-amylase* gene will be constructed into a *piggyBac* vector for generating a transgenics silkworm having an exogenous *α-amylase* gene.

OBJECTIVES

1. To clone genomic DNA and cDNA sequence of *α-amylase* gene from a native Thai silkworm (multivoltine strain), Nanglai strain.

2. To construct *piggyBac* expression vectors for injection. The vectors contain *α-amylase* gene under the control of *Actin3* promoter.

3. To generate transgenic silkworms having exogenous *α-amylase* gene and detect its expression by RT-PCR method.

LITERATURE REVIEW

History of sericulture

Man has reared beneficial animals since ages for his variety of needs and benefits. Mulberry silkworm, *Bombyx mori* Linneous has also been domesticated for production very precious and beautiful fabric ever since it was discovered by Chinese more than 4,000 years ago. During the long process of domestication, the silkworms lost the ability to search food and also the mates so their survivals are entirely dependant upon their rears. Meanwhile, their physiological, behavioral and genetical processes were greatly influenced to obtain a more desirable type of insect architecture for satisfying the needs of mankind. Now, there are three types of silkworm rearing around the world, univoltine, biovoltine and polyvoltine, each having several varieties (Kamili *et al.*, 2000).

Sericulture began in the north China along the bank of the Hwang Ho River and it expanded outside China, as mulberry seeds and silkworm eggs were smuggled out. Thereafter, the secrets of silkworm rearing and silk production spread to neighboring countries and to the rest of the world through the “silk road”, leading to the establishment of a sericulture industry in many countries. The sericulture was introduced in Thailand more than 100 years ago. At present, Thailand is famous for hand-woven silks, commonly known as “Thai Silks” (Kamili *et al*., 2000).

Biology of silkworm

Phylum Arthropoda
Class Hexpoda or Insecta
Sub - Class Pterygota
Division Endopterygota
Order Lepidoptera
Family Bombycidae
Genus *Bombyx*
Species *Bombyx mori*

The mulberry silkworm, *B. mori*, is a holometabolous insects, it passes through complete metamorphosis from egg, larva and pupa to adult. However, the silkworm feeds and grows rapidly in only the larval stage, whereas all other stags are non-feeding and are therefore, not accompanied with increase in body weight and size.

Life cycle of silkworm

The duration of the whole life cycle of the silkworm is about 55-60 days at temperatures of 23-25 ºC. The silkworm life cycle are separated into 4 stages (Figure 1) as follows:

Egg stage: There are two types of eggs, namely the diapause and the non-diapause type. Silkworm races indigenous to temperate regions usually lay diapause eggs, while those of subtropical regions such as native varieties of Thailand lay mostly non- diapause eggs. The eggs of the diapause type aestivate and hibernate and hatch out in early May. The eggs of the diapause will hatch in 11-14 days if kept at a temperature of 23-25 ºC., whereas the incubation period of the non-diapause type eggs is about 9-12 days. However, the invention of an artificial hatching method can make the diapuase type eggs hatching in about the same time. The method is very

simple. Eggs at 20 hours after they have been laid are immersed in hot hydrochloric acid for 3-5 minutes to break this hibernation.

Larva stage: The newly hatched larvae are black, about 3 mm long, weigh about 0.45 mg, and are usually called “ants”. The larvae start eating all day and night and will stop eating during molting periods. During larval stage, larvae have 4 molting times in 25 days when reared at 25 ºC. Their body weigh is about 10,000 times as large as that of newly hatched worm. The larvae reach the maximum weigh one or one and half days before spinning cocoons. When the larvae are fully grown and are ready to spin cocoons, they are called “ripe” or “mature”. They stop eating and become very restless, raising their heads in search of support.

Pupa stage: It takes about 4-5 days for the pupa stage. The silk glands of the mature larva are large, their weigh amounts to about 40% of the body weight. The larvae are transferred to a spinning nest, which is usually made of straw or cardboard, and this procedure is called “mounting”.

Moth stage: The moths emerge from cocoons after developing in the papa stage for 9 to 14 days, which usually takes place in the morning. Soon after that male and female moths mate. The mating ability of male moths can deteriorate very quickly. To prolong their sexual ability for a week, the male moths can be kept in a refrigerator at 10 ºC. The female moths lay eggs shortly after the separation from the males and lays continuously for several hours until almost all eggs are deposited (Tazima, 1962).

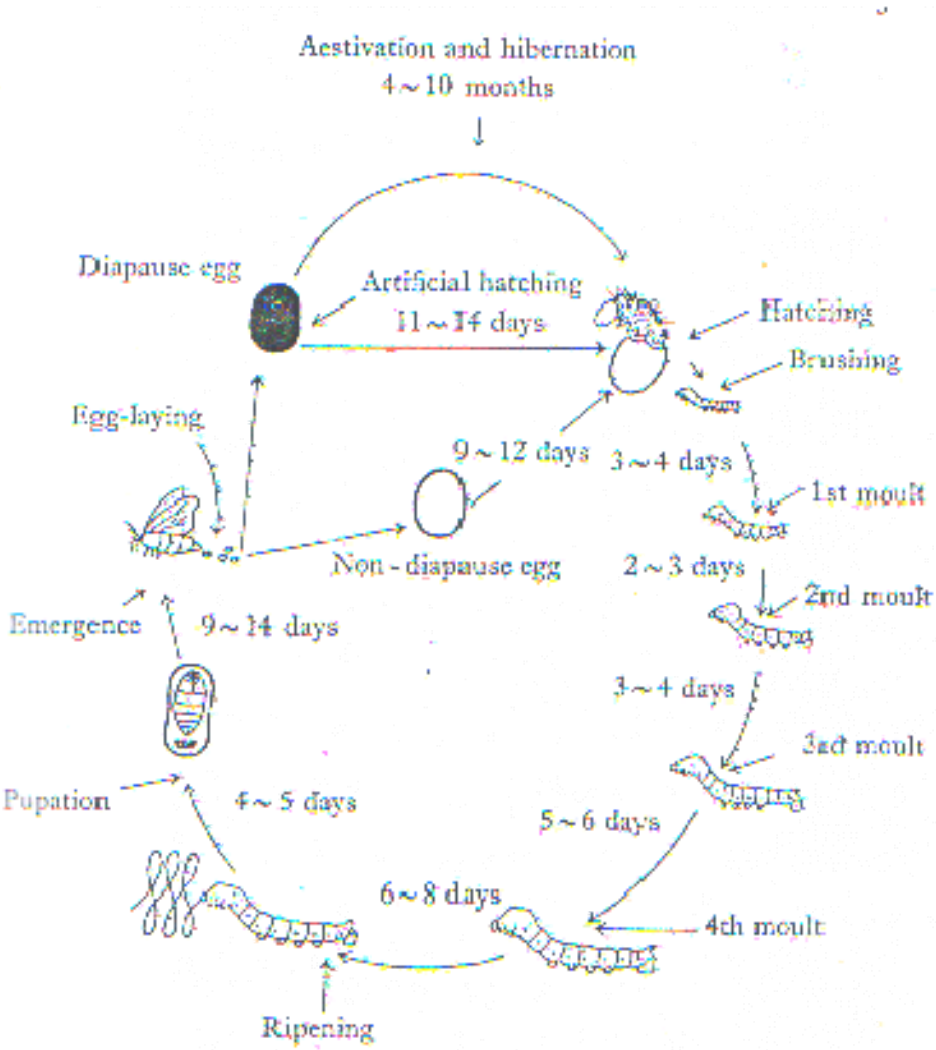


Figure 1 Life cycle of the silkworm
Source: Tazima (1978)

Nanglai strain is tropical silkworm of Thailand. They have zebra lines on their skin and the producing is yellow cocoon. The zebra line was abnormal marking of the 3rd chromosome at the position Ze, 3-1.5. The Nanglai strain can be reared through out the year. They hardly resist to disease and high temperature. Unfortunately, the Nanglai strain produces low quantity and short length silk fiber.

General Morphology

Regions of the larva's body are divided into 3 regions, namely, the head, the thorax and the abdomen. The head has two sets of 6 ocelli which situated just behind and downward on the left and right face. Antennae are situated a little under the ocelli. The antenna is composed of three short segments. The mouth parts are located downward and in front of the face and are composed of a pair of mandibles and maxillae with labrum and labium. The mandibles are used for mastication and consist of two hard pieces. The maxilla consists of a single maxillary lobe and the palpi, which is made up of three segments. The labrum hangs down from the frontal portion to form a flap of the mouth. The labium is situated on the ventral region of the head and has a pair of labial palps which form a sensory organ. A spinneret is located at the proximal position between the two labial palpi. The thorax comprises three segments with one pair of spiracles and three pairs of thoracic legs. The abdomen is composed of 11 segments with 8 pairs of spiracles, 4 pairs of abdominal legs, 1 pair of caudal legs and 1 caudal horn (Tazima *et al.*, 1978).

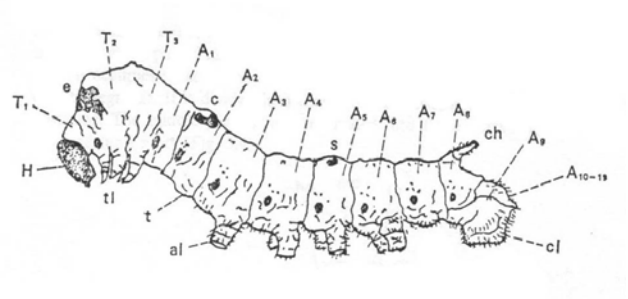


Figure 2 Fifth instar larva just after molting. A1-11, Abdominal segments; H, head; T1-3, thoracic segments; al, abdominal legs; c, crescents; ch, caudal horn; cl, caudal legs; e, eye spots; s, star sots; t, spiracles; tl, thoracic legs.
Source: Tazima *et al.* (1978)

Since *B. mori* is the most important economy insect, a numerous researches on silkworm have been conducted in both applied and basic science. Now silkworm is become one of the important insect models for Lepidopteran genomics and genetics. Therefore, study of silkworm will help further elucidate the function of gene

homologs and facilitate studies of insect domestication, morphogenesis, endocrinology, reproduction, behavior and immunity (Wang *et al.*, 2005).

Virus diseases

The silkworm, *B. mori* is susceptible to various disease and pest that cause dramatically economic loss in sericulture industry of many countries. The most important disease of silkworm is caused by nucleopolyhedrosis virus. The virus is a most harmful virus in the sericulture industry. The *B. mori* nucleopolyhedrosis virus (*Bm*NPV), a genus of the family Baculoviridae, is characterized by circular double stranded DNA genomes (Kamita and Maeda, 1993). The diseased larvae are appeared pale yellow color and it is commonly called as Jaundice, Grassery disease or nuclear polyhedrosis. BmNPV attacks the advance stage worms after fourth molt (Kamili *et al.*, 2000).

The infection cycle of BmNPV is mediated by two phenotypically different viral particles: the occlusion-derived virus (ODV) and the budded virus (BV). Occlusion bodies consisting of a crystalline matrix of polyhedron proteins contain ODV particles. When the occlusion bodies are ingested by *B. mori* larvae, they are dissolved by the alkaline gut juice. The enveloped virions are released and then initiate infection in the midgut columnar epithelial cells. In contrast to ODV, the BV particle consists of a single nucleocapsid surrounded by an envelope acquired as it buds from the plasma membrane of an infected cell and spreads beyond the midgut through the tracheae. Studies on antiviral immunity in insects are still in their infancy, and defense mechanisms at an early stage of viral infection in the alimentary canal remain unknown (Ponnuvel *et al.*, 2003).

There are some researches investigating on an antiviral protein, Lipases and serine proteases (Ponnuvel *et al.*, 2003; Nakazawa *et al.*, 2004). Lipases are likely to contribute immune defenses, conceivably acting directly against invading microorganisms. Bmlipase-1, a lipase purified from the digestive juice of *B. mori* larvae proved to have a strong antiviral activity against *Bm*NPV and showed 56% homology with *Drosophilla melanogaster* lipase and 21% homology with human

lipase. It has also been confirmed that the Bmlipase-1 gene is expressed only in the midgut tissue, but not in other tissues. When the fifth instar larvae of silkworm were orally inoculated with pre-treated *Bm*NPV-ODV (ODV incubated with Bmlipase); the larvae showed resistance to viral infection and successfully entered the pupal stage, thereby indicating the suppression of viral proliferation by midgut *Bm*lipase1 (Ponnuvel *et al*., 2003). In *D. melanogaster*, four lipase genes were found to be induced upon immune challenge. The inhibition of equine and porcine rotavirus by alpha-amylase and lipase also was observed (de Gregorio *et al*., 2002).

Serine proteases are among the group of proteins that regulate several invertebrate defense responses including haemolymph coagulation, antimicrobial peptide synthesis and melanization of pathogen surfaces (Gorman and Paskewitz, 2001). Nakazawa *et al*. (2004) showed that the presence of serine protease in the digestive juice of silkworm larvae has strong antiviral activity against *Bm*NPV. The molecular mass and partial N-terminal sequence was also determined. Since, the deduced amino acid sequence of the cDNA showed 94% homology with *B*. *mori* serine protease, they designated it as BmSP-2, and reported that there may be about five serine protease isoforms including *Bm*SP-2 (Nakazawa *et. al*., 2004). The haemolymph of mosquito, *Anopheles gambiae* also contains five serine proteases, which play an important role in insect immunity (Gorman and Paskewitz, 2001).

Enzyme α-amylases

α-amylases (α-1, 4-glucan-4-glucanohydrolases) are a family of enzymes that catalyze the hydrolysis of the α- (1, 4) glycosidic linkages in starch and related compounds. α-Amylases from insects and mammals have been characterized in both biochemical and molecular levels. α-amylases play a central role in carbohydrate metabolism so organisms with a starch-rich diet depend on the effectiveness of their amylases for survival (Titarenko and Chrispeels, 2000). Amylase enzymes from different origins have been characterized. In the silkworm *B. mori*, there are two types of amylase activities in digestive fluid and haemolymph. Silkworm strains of tropical

countries like Thailand lay non-diapausing eggs, breed throughout the year (polyvoltine). These native varieties are very strong, high survival rate and short rearing time. However, they produce short larval span with small quantity of silk fiber, while those of temperate countries like Japan lay diapausing eggs, undergo two generations in a year (bivoltine), secrete longer silk fiber but have poor survival rate under tropical conditions (Murakami, 1989).

Biochemical differences between diapausing and non-diapausing strains are not well understood (Gamo, 1983). However, different amylase enzymes were identified by analysis of digestive fluid and haemolymph in diapausing and non-diapausing strains of larvae silkworms. The activity of haemolymph amylase was not different between nondiapausing and diapausing strains, whereas, the activity of amylase in digestive fluid of nondiapausing strain was higher than that of the diapausing strain (Abraham *et al.*, 1992). The increased enzyme activity and efficient starch digestion may have adaptive significance in the non-diapausing strain which is known to survive better under tropical condition than the diapausing strains (Abraham *et al.*, 1992). *α-amylase* encodes the digestive enzyme amylase in insects. The expression of *α-amylase* gene of silkworm, *B. mori* (polyvoltine race, Pure Mysore) was restricted to the salivary gland by using anti-sense *α-amylase* RNA probe for whole mount in situ hybridization (Parthasarathy and Gopinathan, 2004).

The amylase activity of Thai native silkworm (polyvoltine strain) was studied and also found that its activity in different tissues was different. The amylase activity in digestive fluid (from gut) was higher than amylase activity from heamolymph. The optimal pH for amylase of digestive fluid from gut was pH 9.8, whereas the optimal pH for heamolymph was pH 6.5 (Promboon *et al.*, 1993).

Recently, they were some reports about digestive enzymes involving in *Bombyx mori* immunity, such as serine proteases and lipases (Nakazawa *et al*., 2004; Ponnuvel *et al*., 2003). However, amylase is a major digestive enzymes but no report about its property to enhance antiviral infection in silkworm. While in Drosophila melanogaster, the ability of *α-amylase* to inhibit the infection of equine and porcine

rotavirus was reported (Yao *et al*., 2006). Therefore, the activity of amylase in enhance silkworm immunity to viral infection should be studied in the future.

Transgenic Silkworm

Gene transformation is a powerful methodological tool to elucidate gene function and regulation. Transgenic organisms are tools for the analysis gene function and regulation. In insects, germ-line transformation was first reported 20 years ago in *D. melanogaster* by using the transposable *P* elements (Rubin and Spradling, 1982). *P* elements based gene vectors for stable transgenesis of *D. melanogaster* do not function in insects from other genera. Later, other transposon else such as *hermes*, *hobo*, *mariner, minos* and *piggyBac* were developed to be a vector for transformation (Handler, 2001).

The *piggyBac* element (originally called IFP2) was identified by its association with a mutation, but in this instance the element was the causative agent of FP (fewpolyhedra) mutations in a baculovirus passed through the *Trichoplusia ni* TN-368 cell line. It is a Class II transposon, 2.5 kb in length, having 13 bp inverted terminal repeat sequences and a 2.1 kb open reading frame, and is part of a subclass of elements originally found in lepidopterans that insert exclusively into TTAA target sites. Upon insertion, the target site is duplicated with excision occurring only in a precise fashion, restoring the insertion site. Beyond this functional similarity, the TTAA elements share no apparent structural identities (Handler, 2001). The *piggyBac* element was first used to transform the medfly, *Ceratitis capitata*, using the medfly *white* marked vector and a self-regulated *piggyBac* transposase helper. Transformation frequencies were relatively low (3–5% per fertile G0), but notably, these experiments demonstrated *piggyBac* transcriptional and enzymatic function in an insect order different from the original host (Handler *et al.*, 1998).

The vector *piggyBac* has also been tested in several other insect species. In 2000, Tamura and colleagues developed a germ line transformation method for the silkworm using the transposable element *piggyBac* vector. The transformation

constructs consist of the *piggyBac* inverted terminal repeats flanking a fusion of the *B. mori* cytoplasmic actin gene *BmA3* promoter and the *green fluorescent protein* (*GFP*). A nonautonomous helper plasmid encodes the *piggyBac* transposase. The reporter gene construct was coinjected into preblastoderm eggs of two strains of *B. mori*. Approximately 2% of the individuals in the G1 broods expressed *GFP*. Southern blot analysis confirmed that the transgene was integrated into the *B. mori* chromosomes (Tamura *et al.*, 2000). Later, many research used this method for construction transgenics silkworm. Thomas and colleagues used *piggyBac* vector which contained *3xP3-EGFP* marker for the screening of transgenic *B. mori*. The position of injection, anterior and posterior of eggs was tested in this research. The result show the position of injection, anterior and posterior of eggs had effected for percent transformation, approximately 3.4 and 0.95% of the G0 broods positive *GFP*, respectively were expressed the EGFP in the stemmata of embryos (Thomas *et al.*, 2002).

The silkworm could be a host for mass-production of recombinant proteins because of a high protein biosynthetic activity of its silk glands. Tomita and colleagues generated transgenic silkworms that produce cocoons containing recombinant human collagen sequences (Tomita *et al.*, 2003). A fusion cDNA was constructed and used as a transgene, which comprises fibroin L-chain, human type III mini-collagen, a fibroin L-chain and EGFP under the control of fibroin L-chain promoter sequence. This promoter drove the expression of the cDNA in a PSG cell-specific manner in the transgenic silkworms. The synthesized fusion proteins were secreted to the PSG-lumen, incorporated into the fibroin layer, and spun into cocoons through the MSG- and the ASG-lumen. The concentration of fusion proteins extracted from the cocoon was 36.7 μg/mg of total extracted protein or 8.4 μg/mg of dried cocoon. Percent of G1 broods with positive larvae were 18.3 to 27.6 % (Tomita *et al.*, 2003). Recently, the L-chain-GFP fusion gene was introduced into the Nd-s^D mutant to assess its ability to produce recombinant protein. The L-chain– GFP fusion gene was expressed in the PSG of the mutant transgenic silkworm; large amounts of recombinant protein were secreted into the lumen and spun into the cocoon. The Nd-s^D phenotype, which consisted of an immature PSG and thin cocoon, was rescued dramatically and the cocoon was almost indistinguishable from those of normal

silkworms. A single cocoon contained about 8.6 mg dry weight (16.6 mmol) of L-chain-GFP per dried cocoon fusion protein (78.4 mg) (Inoue *et al.*, 2005). In the other hand, Kurihara *et al.* (2007) used the fibroin H-chain expression system for the production of recombinant feline interferon (FeIFN) protein in the cocoon of transgenics silkworm. Using this H-chain expression system, the targeted gene was successfully expressed in the cocoon of transgenics silkworm and the active pharmaceutical protein was obtained from the extracts of cocoons after protease treatment. The yield, 2.8 mg of 99% pure rHSA were obtained from 2 g of cocoons (Kurihara *et al.*, 2007).

Recently, there are some reports for developing a transgenic silkworm that synthesizes recombinant proteins in middle silk gland cells and secretes the proteins into the sericin layer (Ogawa *et al*., 2007; Tomita *et al.*, 2007). The transgenic silkworm could secrete recombinant human serum albumin (rHSA) into the sericin layer of silk fiber. In construction the vector, a *Ser*1 promoter was inserted into a *piggyBac*-based vector at an upstream region of HAS cDNA and at a downstream region of *B. mori* nucleopolyhedrosis virus (BmNPV) enhancer, *hr*3. However, the yield of recombinant proteins in the sericin layer was not high enough for industrial purposes (Ogawa *et al*., 2007). Later, Tomita *et al.* (2007) generated a piggyBac, in which EGFP or secretory EGFP (sEGFP) cDNA was placed as a reporter gene under the control of hr3-linked sericin-1 gene (*ser1*) promoter and *iel*. The transgenic silkworm could secrete a recombinant protein in the sericin layer of its cocoon.

In the other hand, Imamura and colleagues used the *Gal4/UAS* system in combination with the *piggyBac* transposon vector in the silkworm, by using the GFP gene for screening the progeny of crosses between the *Gal4* and *UAS-GFP* lines. The result showed approximate 2.5% of percent transformation in G0 of *Gal4* crosses *UAS-GFP* lines expressed GFP positive (Imamura *et al.*, 2003)

The silkworm is only domesticated insect, which completely controlled by humans (Veda *et al.*, 1997). Moreover, the adult moths are unable to fly. Therefore,

the sensitive issue of releasing genes from genetically modified organisms (GMO) into the environment does not seem to be relevant for this species.

MATERIALS AND METHODS

Materials

1. Silkworm strains

1.1 Nanglai strain for molecular cloning

1.2 w1-pnd strain for microinjection

1.3 Nanglai, w1-pnd and C108 strain for detection of gene expression

2. Bacterial strain

Echerichia coli strain DH5α (TOYOBO, Japan)

Echerichia coli strain JM109 (Promega, USA)

3. Type of vector

BmA3 vector (vector from Dr. Toshiki Tamura, NAIS, Japan)

*pBac*MCS(A3-SV40, A3-EGFP)R, which contained EGFP reporter gene (vector from Dr. Toshiki Tamura, NAIS, Japan).

pBac(3xP3-DsRedaf), which contained *DsRed* reporter gene (a gift from Prof. Stafan Baumgartner, BMC, Lund University, Sweden)

pGEM-T® easy vectors (Promega, USA)

pTA2 vector (TOYOBO, Japan)

4. Chemicals and Reagents

4.1 General Chemicals and Reagents

Absolute ethanol (C_2H_5OH) (Merck, Germany)

Acetic acid glacial (CH_3COOH) (BDH, UK)

Ammonium acetate (Merck, Germany)
Bromophenolblue (Merck, Germany)
Calcium chloride (Sigma, USA)
Chloroform (Merck, Germany)
Formaldehyde (BDH, Germany)
Glucose (Sigma, USA)
Glycerol (BDH, UK)
Hydrochloric acid (Merck, Germany)
Isoamyl alcohol (Merck, Germany)
Isopropanol (Merck, Germany)
2-mercaptoethanol (Sigma, USA)
Polyvinylpyrrolidone (Sigma, USA)
Potassium chloride (Sigma, USA)
Sodium acetate (Merck, Germany)
Sodium chloride (Merck, Germany)
Sodium dodecyl sulfate (SDS) (Merck, Germany)
Sodium hydroxide (BDH, UK)
Xylene cyanol FF (Sigma, USA)

4.2 Chemicals for Bacterial Culture

Bacto agar (Difco, USA)
Bacto tryptone (Difco, USA)
Tryptone (Difco, USA)
Yeast extract (Difco, USA)

4.3 Chemicals and Reagent for Molecular Cloning

Agarose (Sekem)
Ampicillin (Sigma, USA)
5-bromo-4-chloro-3-indoyl-ß-D-galactopyranoside (X-gal) (Sigma, USA)
Chloroform (Merck, Germany)

Dextran sulphate (Sigma, USA)

Diethyl pyrocarbohydrate (DEPC)

100 mM dATP, dCTP, dGTP, and dTTP (Promega)

DNA marker: lampda DNA, 1 Kb plus DNA ladder and 100 bp ladder (Gibco, USA)

Ethanol, absolute (Merck, Germany)

Ethidium bromide (EtBr) (Sigma, USA)

Ethylene diamine tetraacetic acid, disodium salt dihydrate (EDTA) (Merck, Germany)

Formaldehyde (BDH, UK)

Formamide (BDH, UK)

Isopropyl-ß-D-thiogalactoside (IPTG) (Sigma, USA)

Phenol (Sigma, USA)

Tris-(hydroxy methyl)-aminomethane (Sigma, USA)

4.4 Reagent Kit for Molecular Cloning

A-attachment kit (TOYOBO, Japan)

FirstChoice® RLM-RACE (Ambion, USA)

ImProm-II™ Reverse Transcription System (Promega, USA)

Marathon cDNA Amplfication Kit (Clontech, USA)

NeucleoSpin® Extract II gel extraction kit (MACHEREY – NAGEL, Germany)

NeucleoSpin Plasmid Extraction Kit (MACHEREY – NAGEL, Germany)

pGEM-T® easy vectors Systems (Promega, USA)

Qiaquick Gel Extraction Kit (Qiagen, Germany)

Reverse transcription System Kit (Promega, USA)

TArget Clone™ -Plus- (TOYOBO, Japan)

TRIzol reagent (Gibco BRL, USA)

4.5 Enzyme and Restriction enzymes

Advantage® 2 PCR Enzyme System (Clontech, USA)

BIO-X-ACT Short DNA polymerase (Bioline, USA)

KOD plus DNA polymerase (TOYOBO, Japan)

Restriction enzymes *Apa* I, *Asc*I, *Bnl*I, *Eco*RI, *Eco*RV, *Fse*I, *Sac* I, *Xho*I and *Xba*I (BioLabs, USA)

RNase A (Sigma, USA)

5. Equipments for analysis

Autoclave: Model HA-300M

Autopipette: Pipetteman (Gilson, France)

Centrifuge, refrigerated centrifuge: Model BR 4i (JOUAN SA)

Centrifuge, microcentrifuge: Model Spectrafuge 16M

Electrophoresis unit: Submerged Agarose Gel Electrophoresis System

Incubator: Model IPR 150.XX2.C

Incubator shaker: Model IOC400.XX2.C (GALLENKAMP PLC)

Incubator water bath: Model INNOVA 3100 (New Brunswick Scientific)

Larminar flow: BH-120 (GelmanScience)

Leica MZ16FA for screening

Magnetic stirrer

Microwave

Nikon SMZ1500 for injection

Olympus SZX16 for screening

pH meter

Thermal Cycler (Px2Thermal cycle: Thermo hybrid)

UV Visible spectrophotometer (Beckman)

Vortex mixer

Methods

1. *B. mori* RNA Isolation

1.1 Total RNA isolation from head of *B.mori*

Total RNA was extracted from the head of the mid 5th instar larva of *Bombyx mori*, Nanglai strain, using TRIzol reagent (Gibco BRL, USA). Fifty microgram of the head was homogenized by a pestle in 300 µl TRIzol reagent and incubated at room temperature for 5 min to dissociate nucleoprotein complex. The mixture was centrifuged at 12,000 rpm for 10 min at 4 °C. Supernatant was removed into a new microcentrifuge tube and 200 µl of chloroform was added into that tube, shake the tube vigorously by hand for 15 sec and then incubated for 3 min at room temperature for precipitation proteins. The mixture was centrifuged at 12,000 rpm at 4 °C for 15 min. Following centrifugation, the mixture separated into a lower red phase, phenol-chloroform phase and a colorless upper phase. The upper aqueous phase was transferred to a new microcentrifuge tube. The RNA was precipitated by mixing with 150 µl of isopropanol. The sample was incubated at -20 °C for 1 hr. Total RNA was precipitated by centrifugation at 12,000 rpm at 4 °C for 10 min. After the supernatant was removed, the RNA pellet was washed twice with 75% (v/v) ethanol 300 µl and centrifuged at 7,500 rpm at 4 °C for 5 min. The washed RNA pellet was air-dried until ethanol evaporated completely and dissolved in RNase-free water (DEPC-treated water) 30 µl.

1.2 Determination of RNA quality and concentration

1.2.1 Spectrophotometric determination

RNA concentration was estimated by UV spectrophotometer at the absorbance 260 nm (A_{260}). An absorbance of 1.0 corresponds to 40 µg/ml of RNA.

The RNA concentration of each sample was calculated in µg/ml by the following equations:

$$[RNA] = A_{260} \times \text{dilution factor} \times 40\ \mu g/ml$$

Quality of the purified total RNA was determined by calculating the A_{260} /A_{280} ratio. The ratio between 1.8 and 2.0 indicated good quality of the RNA. The RNA was stored at -20 ºC. For long term storage, the total RNA was precipitated and kept in absolute ethanol at -70 ºC.

1.2.2 Agarose gel electrophoresis

Agarose was mixed with 1X Tris-acetate EDTA (TAE) buffer and heat in a microwave until complete solubilization. An appropriate comb was selected for forming the sample slot in the gel. The warm agarose (55 °C) was poured into the casting tray. After the gel setting completely, the comb was removed carefully. The gel is placed in an electrophoresis chamber containing TAE buffer.

The RNA sample was mixed with 6X gel-loading buffer and the sample mixture was loaded slowly into the slots of the submerged 1% agarose gel. The lid of the gel chamber was closed and the electrophoresis was carried out in 1X TAE running buffer. The RNA should migrate toward the positive anode at 100 volts and the bromophenol blue should migrate from the wells into the body of the gel within a few min. The gel was run until the bromophenol blue and xylene cyanol FF have migrated to an appropriate distance through the gel. After finishing, the gel was stained in 0.1 mg/ml ethidium bromide (EtBr) solution for 30 min and destained by submerging in an excessive amount of distilled water for 2 min. The nucleic acid bands were visualized under UV transilluminator.

2. Amplification and sequencing *α-amylase* gene

2.1 Synthesis the first strand cDNA

Total RNA (from section 1.1) was incubated at 65 ºC for 10 min and put on ice for 2 min respectively. First strand cDNA was synthesized with 1 µg of total RNA of Nanglai strain using Reverse transcription System Kit (Promega). The reaction mixture of 20 µl contained 1 µl of total RNA, 4 µl of Improm II™ 5x buffer, 1 µl of 10 mM dNTP mixture, 1.6 µl of 25 mM $MgCl_2$, 0.5 of 40 unit/ µl Recombinant RNasin ®Ribonuclease Inhibitor, 1 µl of Improm II™ reverse transcriptase, 1 µl of 0.5 µg oligo(dT)15 primer and sterile deionized water was added to the final volume. The solution reaction was mixed and incubated at room temperature for 5 min and at 42 ºC for 1 hr. After incubation, the reaction mixture was heated at 70 ºC for 5 min and incubated on ice for 5 min.

2.2 Oligonucleotide primers design

Oligonucleotide primers were designed based on the cDNA sequences of *Bombyx mori* truncated *alpha-amylase* (*amy*) gene (GenBank Accession No.U07847) as shown in Figure 3. The oligonucleotide primers were as followed:

AmyF (sense)

5'- ATGTTTCGGTACATCCTTCTACT -3' [nt 1- nt 23]

AmyR (antisense)

5'- GTCAGCACCCCTATGGATAG -3' [nt 1,165 – nt 1,184]

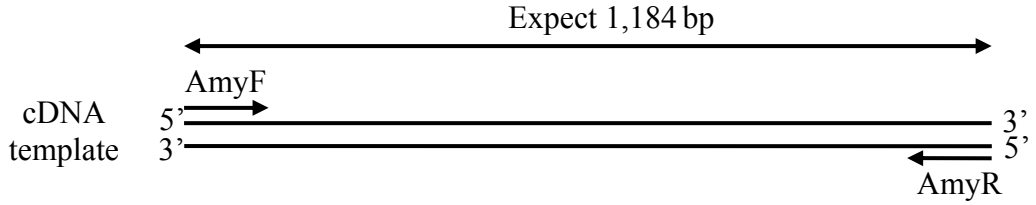


Figure 3 Diagram shows the cDNA template and position of the primers used in *α-amylase* PCR.

2.3 PCR amplification

The *α-amylase* gene of Nanglai strain was amplified by polymerase chain reaction (PCR), using the first strand cDNA as a template and AmyF primer and AmyR primer. The amplified reaction of 25 µl contained 1 µl of the first-stranded cDNA template, 2.5 µl of 10x OptiBuffer, 4 µl of 10 mM dNTP mixture, 1.25 µl of 50 mM $MgCl_2$, 0.5 µl of 4U/ µl Bio-X-ACT Short DNA Polymerase, nuclease-free water 13.75 µl , Primer sense (AmyF 10 pmol/ µl) and Primer antisens (AmyR 10 pmol/ µl). The reaction mixture was amplified in a Px2 Thermal cycle (Thermo Hybrid). PCR procedure was as followed: preheated at 95 ºC for 3 min, followed by 20 cycles of denaturing at 94 ºC for 1 min, annealing at 60 ºC for 30 sec and extension at 70 ºC for 2 min. The final extension was carried out at 70 ºC for 10 min.

2.4 Determination of PCR product quality

The PCR product was mixed with 6X gel-loading buffer and the sample mixture was loaded slowly into the slots of the submerged 2% agarose gel. The lid of the gel chamber was closed and the electrophoresis was carried out in 1X TAE running buffer. The RNA should migrate toward the positive anode at 100 volts and the bromophenol blue should migrate from the wells into the body of the gel within a few min. The gel was run until the bromophenol blue and xylene cyanol FF have migrated to an appropriate distance through the gel. After finishing, the gel was stained in 0.1 mg/ml ethidium bromide (EtBr) solution for 30 min and destained by

submerging in an excessive amount of distilled water for 2 min. The nucleic acid bands were visualized under UV transilluminator.

2.5 Purification of DNA fragment from agarose gel

Purification of DNA fragment from agarose gel was performed according to PCR clean-up gel extraction (NucleoSpin® Extract II). In brief, the desired DNA fragment on agarose gel (from section 2.4) was cut and transferred to a microcentrifuge tube. Two hundred microliters of NT buffer was added to the agarose gel. The sample was incubated at 50 ºC for 10 min or until the agarose gel was completely dissolved. The sample was transferred to a spin column. The column was centrifuged at 11,000 rpm at room temperature for 1 min. The spin column was removed from the collection tube and discarded the liquid inside the tube. The spin column was placed back into the tube and added 600 µl of NT3 buffer. The sample was centrifuged at 11,000 rpm for 1 min at room temperature. The flow-through was discarded. The spin column was placed back into the tube and centrifuge for an additional 1 min at 11,000 rpm. The spin column was placed into a new microcentrifuge tube. The DNA fragment was eluted by adding 20 µl of NE buffer and incubated for 1 min at room temperature. After incubation, the column was centrifuge at 11,000 rpm for 1 min. The purified DNA fragment was determined its concentration by gel electrophoresis and stored at –20 ºC until use.

2.6 DNA ligation

The purified DNA fragment from section 2.5 was ligated into pGEM-T® easy vectors (Promega) which possessed a single 3’ deoxythimidine (T) overhanged at both ends (Figure 2). This vector allowed easy cloning of PCR product based on the fact that Bio-X-ACT Short DNA Polymerase used in PCR tend to add an additional nucleotide, usually a deoxyadenosine (A) to the 3’-end of each strand that it synthesized. Therefore, a double-stranded PCR product has a single adenosine nucleotide overhang. The molar ratio of the insert DNA to the vectors is 3:1. The

amount of inserted DNA depended on the length of the inserted DNA fragment. The quantity of inserted DNA was estimated in ng by the following equation:

$$\text{x ng of insert DNA} = \frac{\text{(50 ng of pGEM-T vector)(y bp of insert DNA)}}{\text{3000 bp of pGEM-T vector}} \times \text{insert : vector molar ratio}$$

Where x was the amount of insert DNA of y bp to be ligated for 3:1 (insert: vector molar ratio). The 10 µl of each ligation reaction composed of inserted DNA, 1 µl of pGEM-T vector (50 ng), 5 µl of 2X T4 DNA ligase buffer, 1 µl of 3 unit/µl of T4 DNA ligase and sterile deionized water was added to the final volume of 10 µl. Each mixture was gently mixed by pipetting and then incubated at 25 ºC for 3 hr.

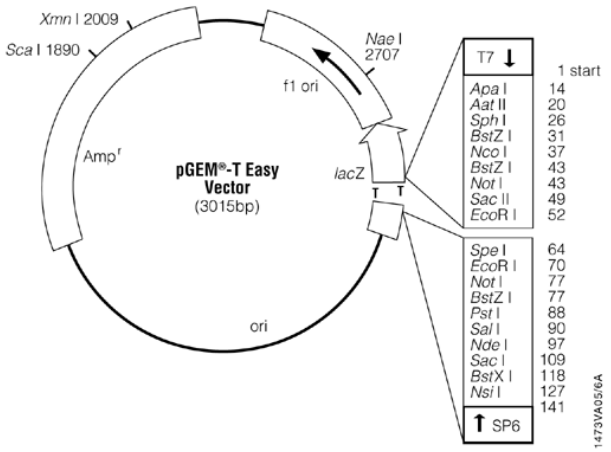


Figure 4 Diagram shows structure of pGEM-T® easy vectors
Source: Promega (2003)

2.7 Transformation into *E.coli* using heat shock transformation

Fifteen microlitres of the *E.coli* JM109 High Efficiency Completent Cells (Promega, USA) was mixed with 3 µl of ligation mixture. The tube was placed on ice for 30 min. The contents in the tube was heat-shocked in a water bath at exactly 42 ºC (do not shake) for 2 min and then placed immediately on ice for 2 min. The tube containing cells transformed with ligation reactions was added with 500 µl of room

temperature LB broth (see Appendix) and incubated at 37 ºC for 1 hr with shaking at 250 rpm. After incubation, the tube was centrifuge at 10,000 rpm for 1 min. Supernatant was discarded and added 100 µl of room temperature LB broth into the tube. Before the transformed mixture was plated on LB ampicillin agar plates (see Appendix), 100 µl of 100 mM Isopropylthio- ß-D-galactoside (IPTG) and 20 µl of 50 mg/ml 5–Bromo-4-chloro-3-indoly-ß-D-galactosidase (X-Gal) were spread over the surface of an LB ampicillin plate and allowed to absorb at 37 ºC for 30 min prior to use. The plates were incubated at 37 ºC overnight (12-16 hr). Recombinant clones were analyzed by blue/white screening. The transformant colonies were counted and selected randomly to check the transformation of the inserted DNA by restriction analysis.

2.8 Extract plasmid DNA by alkaline method

A single white colony of X-gal-IPTG-ampicillin plate was cultured in 3 ml of LB broth containing 50 µg/ml ampicillin and incubated at 37 ºC with shaking at 250 rpm overnight (12-16 hr). The culture was transferred into 1.5 ml microcentrifuge tube and centrifuged at 12,000 rpm at 4 ºC for 30 sec. After centrifugation, the medium was removed by aspiration, leaving the bacterial pellet as dry as possible. The bacterial pellet was resuspended in 100 µl of alkaline lysis solution I (50 mM glucose, 25 mM Tris-HCl pH 8.0 and 10 mM EDTA pH 8.0) by vigorous vortexing. Two hundred microlitres of freshly prepared alkaline lysis solution II (0.2 M NaOH + 1% SDS = 1:1) was added to each bacterial suspension and mixed the contents by inverting the tube rapidly five times. After incubating the tube on ice for 3 min, 150 µl of alkaline lysis solution III (5M potassium acetate, glacial acetic acid and water) was added and mixed gently by inverting the tube 5 times. The tube was stored on ice for 5 min and then centrifuged at 12,000 rpm at 4 ºC for 5 min to separate cell debris. The supernatant was transferred into a microcentrifuge tube. An equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1, v/v) was added to the supernatant and mixed by vortexing. The emulsion was centrifuged at 12,000 rpm at 4 ºC for 10 min and the upper aqueous phase was transferred to a new tube. Plasmid DNA was precipitated by adding 2 volumes of absolute ethanol then mixed the solution by

vortexing. The plasmid DNA was collected by centrifugation at 12,000 rpm at 4 ºC for 10 min. The pellet was washed with 70% (v/v) ethanol and inverted the close tube several times. The plasmid DNA was recovered by centrifugation at 12,000 rpm at 4 ºC for 5 min. The supernatant was removed by gentle aspiration. The open tube was stored at room temperature until the ethanol evaporated completely then the DNA was dissolved in 30 µl of TE buffer. The extracted plasmid DNA was kept at –20 ºC for further analysis.

2.9 Detection of DNA insertion by restriction digestion

The digestion reaction of 15 µl contained approximately 1 µg (spectrophotometric determination) of the purified plasmid DNA of each clone, 1 U of *Apa*I, 1 Uof *Sac*I, 0.15 µl of 100x BSA for *Apa*I and sterile deionized water was added to the final volume. The digestion reaction was incubated at 37 ºC for 3 hr. The digestion reaction was detected by loading onto 1% agarose gel and electrophoresed at 100 volts. The size of inserted DNA was compared with 1.5 kb.

2.10 Extraction of plasmid DNA by neucleoSpin plasmid extraction kit (MACHEREY – NAGEL, Germany)

A single white colony of X-gal-IPTG-ampicillin plate was cultured in 3 ml of LB broth containing 50 µg/ml ampicillin and incubated at 37 ºC with shaking at 250 rpm overnight (12-16 hr). The culture was transferred into 1.5 ml microcentrifuge tube and centrifuged at 11,000 rpm for 1 min. The supernatant was discarded. The 250 µl buffer A1 was added into the tube. The cell pellet was resuspended by vigorous vortexing. The 250 µl buffer A2 was added and mix gently by inverting the tube 6-8 times. The tube was incubated at room temperature for a 5 min. The 300 µl buffer A3 was added and mix gently by inverting the tube 6-8 times. The tube was centrifuged at 11,000 rpm at room temperature for 5 min. NucleoSpin Plasmid column was placed into a 2 ml collecting tube and transferred the supernatant into the column. The column was centrifuged at 11,000 rpm for 10 min. The flow-through was discarded. The NucleoSpin Plasmid column was placed back into the 2 ml collecting

tube and added 500 µl buffer prewarm AW. The NucleoSpin Plasmid column was incubated at room temperature for 1 min and centrifuged at 11,000 rpm at room temperature for 1 min. The flow-through was discarded. The NucleoSpin Plasmid column was placed back into the 2 ml collecting tube and added 600 µl buffer A4 (with ethanol). The NucleoSpin Plasmid column was centrifuged at 11,000 rpm at room temperature for 3 min. The flow-through was discarded. NucleoSpin Plasmid column was placed into a 1.5 ml microcentrifuge tube and added 40 µl buffer AE. The NucleoSpin Plasmid column was incubated at room temperature for 1 min. The NucleoSpin Plasmid column was centrifuged at 11,000 rpm at room temperature for 1 min. The plasmid DNA was detected by loaded onto 1% agarose gel and electrophoresed at 100 volts.

3. Cloning and sequencing cDNAs using Rapid Amplification of cDNA Ends (3'-RACE)

3'- RACE were performed to generate the full-length *Amylase* cDNAs using the Marathon cDNA Amplfication Kit (Clontech, USA). The overview of full-length ds cDNA synthesis was shown in Figure 5.

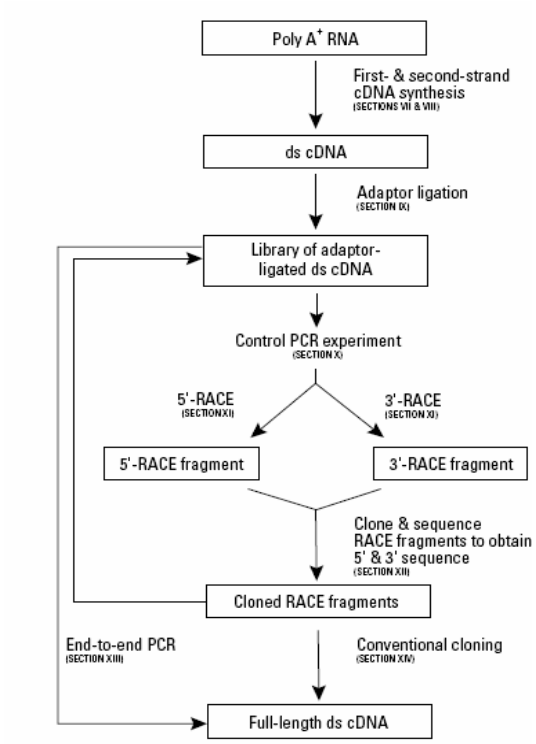


Figure 5 Full-length double strand cDNA synthesis flow chart of Marathon cDNA Amplfication Kit

Source: Clontech (2001)

3.1 Second-strand cDNA synthesis

The second-strand cDNA was synthesized from the first-strand cDNA produced in the 10 µl first-strand reaction. The Second-Strand Enzyme Cocktail contains RNase H, *E. coli* DNA polymerase I, and *E. coli* DNA ligase. These enzymes degrade the RNA and synthesize the second cDNA strand. The action of T4 DNA Polymerase creates blunt ends on the ds cDNA. All components and reaction vessels should be prechilled on ice. The following premix was prepared for second-strand cDNA synthesis: 48.4 µl of sterile water, 16 µl of 5X Second-Strand Buffer, 1.6 µl of dNTP Mix (10 mM), 4 µl of 20X Second-Strand Enzyme Cocktail [*E. coli* DNA polymerase I (6 U/µl), *E. coli* DNA ligase (1.2 U/µl) and *E. coli* RNase H (0.25 U/µl)]. The 70 µl of premix was added to 10 µl of first-strand and mixed contents thoroughly with gentle pipetting. The tube was incubated at 16 ºC for 45 min. After

incubation, 4 µl of EDTA/glycogen (0.2 M EDTA and 2 mg/ml glycogen) was added to terminate second-strand cDNA synthesis. One hundred microlitres of solution contains phenol: chloroform: isoamyl alcohol (25: 24: 1, v/v) were added. The tube was mixed thoroughly and centrifuged at 12,000 rpm for 10 min at 4 ºC to separate phases. The upper aqueous phase was carefully transferred to a new 0.5 microcentrifuge tube. One hundred microlitres of chloroform: isoamyl alcohol (24: 1, v/v) was added and mixed thoroughly. The microcentrifuge tube was centrifuged at 12,000 rpm for 10 min and upper phase was transferred to a new microcentrifuge tube. One-half volume of 4 M ammonium acetate and 2.5 volumes of 95% (v/v) ethanol were added to the upper phase. The tube was mixed thoroughly then immediately centrifuged at 12,000 rpm for 20 min at room temperature. The supernatant was removed carefully and the pellet was gently overlaid with 300 µl of 80% (v/v) ethanol. Double strand cDNA pellet was collected by centrifugation at 12,000 rpm for 10 min at room temperature, air-dried, and then dissolved in 10 µl of water and detected by loaded onto 1.2% agarose gel and electrophoresis at 100 volts.

3.2 Preparation of adaptor ligated double strand cDNA

An adaptor was ligated to the double strand cDNA obtained from section 3.1. The 5X DNA ligation buffer used in adaptor ligation reaction was allowed to completely thaw and placed at room temperature for 30 min before use. The following reagents were combined in a 0.5 ml microcentrifuge tube at room temperature and in order shown: 5 µl double strand cDNA in a reaction containing 2 µl of 10 µM Marathon cDNA adaptor, 2 µl of 5X DNA ligation buffer, 1 µl of T4 DNA ligase (400 U/µl) in a final volume of 10 µl. After mixing, the tube was incubated at 23 ºC for 3 hr and the ligase was inactivated by heating at 70 ºC for 5 min. The resulting adaptor-ligate double strand cDNA was used as a template for 3'- RACE or stored at –20 ºC until use. The adaptor-ligated double strand cDNA was diluted to a concentration with Tricine-EDTA buffer. Dilute 1 µl of the adaptor-ligated double strand cDNA reaction mixture with 250 µl of Tricine-EDTA Buffer. Heat the diluted ds cDNA at 94 ºC for 2 min to denature the ds cDNA. Cool the tube on ice for 2 min.

The tube was briefly spun in a microcentrifuge to collect the contents in the bottom of the tube. Store at –20 ºC.

3.3 Rapid Amplification of *α-amylase* cDNA Ends (3'- RACE) Fragments

3.3.1 Oligonucleotide primers design

Gene-specific primer was designed based on the sequences of cDNA of *Bombyx mori* truncated *alpha-amylase* (*amy*) gene (GenBank Accession No.U07847). The gene-specific primer (GSP) was as followed:

5' GSP (AmyF) 5'- ATGTTTCGGTACATCCTTCTACT -3'

AP1 primer 5'–CCATCCTAATACGACTCACTATAGGGC–3'

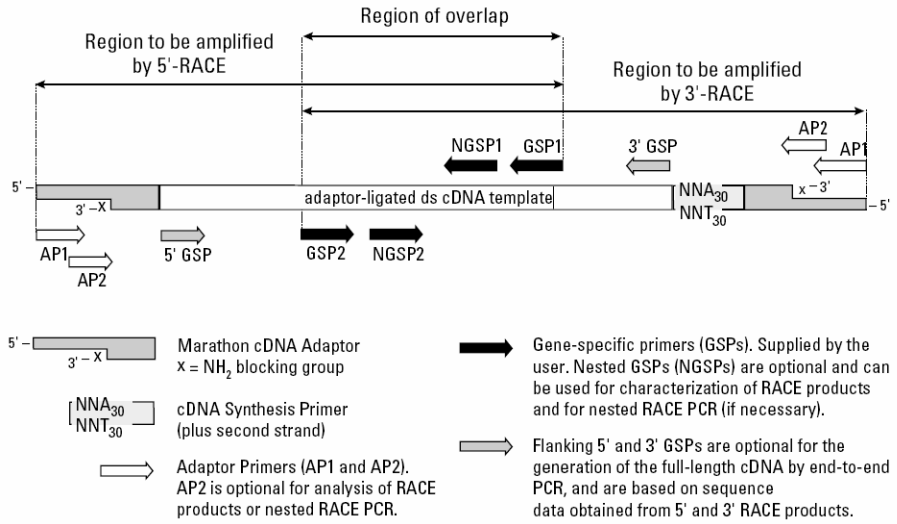


Figure 6 Template and primers used in RACE reactions.
Source: Clontech (2001)

3.3.2 PCR amplification

PCR master mix was performed in a 43 µl volume containing 5 µl of 10X cDNA PCR reaction buffer, 1 µl of 10 mM dNTP mixture, 1 µl of 50X Advantage 2 polymerase mix and 36 µl of Nuclease-free water. 3'-RACE reaction was performed in a 7 µl volume containing 5 µl of diluted adaptor-ligated ds-cDNA, 1 µl of Adaptor Primer 1 (AP1 primer) (10 µM), 1 µl of 5' GSP primer (AmyF 10 µM) 3'-RACE reaction was mixed with 43 µl of PCR master mix. Reaction mixtures was preheated at 94 ºC for 1 min, followed by five cycles of denaturing at 94 ºC for 30 sec, annealing/extension at 72 ºC for 4 min; five cycles of denaturing at 94 ºC for 30 sec, annealing/extension at 70 ºC for 4 min and twenty cycles of denaturing at 94 ºC for 20 sec, annealing/extension at 68 ºC for 4 min. PCR products of 3'- RACE fragments was detected by 1.2% agarose gel and purified from agarose gel using NucleoSpin® Extract II. cloned into pGEM-T® easy vector and transformed into *E coli* JM109 by heat shock transformation, extracted plasmid as described in section 2.4-2.10 and sequenced.

4. *B. mori* DNA isolation

4.1 *B. mori* DNA isolation

Genomic DNA was extracted from 1st larvae of Nanglai strain. The larvae were homogenized by a pestle in 300 µl of DDW water and add 500 TE µl buffer. Add 250 µl of 10% SDS into the tube, shaked the tube vigorously by hand and incubated at 60 °C for overnight. Add 400 µl of chloroform, add 200 µl of chloroform and shaked the tube vigorously by hand. The mixture was centrifuged at 12,000 rpm at room temperature for 5 min. The mixture was removed into a new microcentrifuge tube and add phenol: chloroform: isoamyl alcohol (25: 24: 1) and shake the tube vigorously by hand. The mixture was centrifuged at 12,000 rpm at room temperature for 5 min. The mixture was removed into a new microcentrifuge tube. The 500 µl of chloroform was added in to the tube and shaked the tube vigorously by hand. The 30 µl of potassium acetate and 700 µl of absolute ethanol wear added into the mixture. The mixture was centrifuged at 12,000 rpm at room temperature for 10 min. After the

supernatant was removed, the DNA pellet was washed twice with 75% (v/v) ethanol 300 µl and centrifuged at 12,000 rpm at room temperature for 5 min. The washed DNA pellet was air-dried until ethanol evaporated completely and dissolved in TE buffer 50 µl.

4.2 Determination of quality and concentration of DNA

4.2.1 Spectrophotometric determination

DNA concentration was estimated by UV spectrophotometer at the absorbance 260 nm (A_{260}). An absorbance of 1.0 corresponded to 50 µg/ml of RNA. The RNA concentration of each sample was calculated in µg/ml by the following equations:

$$[DNA] = A_{260} \times \text{dilution factor} \times 50\ \mu g/ml$$

Quality of the purified total DNA was determined by calculating the A_{260} /A_{280} ratio. The ratio between 1.8 and 2.0 indicated good quality of the DNA. The DNA was stored at -20 ºC.

5. Amplification and sequencing *α-amylase* genomic DNA

5.1 Oligonucleotide primers design

Oligonucleotide primers were designed based on the sequences of genomic DNA of *Bombyx mori* p50 strain (Data of NIAS) as shown in figure 7. The oligonucleotide primers were as followed:

Sense primers
1038U 5'- GTATTTTGGGCAGATCTCATG -3' [nt 1,038 – nt 1,058]
3805U 5'- AGCGCTATCAACCAATCTCC -3' [nt 3,805 – nt 3,824]
4590U 5'- CCCGTATGCCCGTCTGCCTACA -3' [nt 4,590 – nt4,611]

4836U 5’- GACGCCATCATCAACCACAT -3’ [nt 4,836 – nt 4,855]
5600U 5’- CCGGTGCTCGTCCCTACATC -3’ [nt 5,600 – nt 5,619]
6270U 5’- TGCGGCAATGGTTGGATATG -3’ [nt 6,270 – nt 6,289]
7042U 5’- CATCCACCGCAGCAACGAGT -3’ [nt 7,042 – nt 7,061]
7859U 5’- GGGAACCGTACTGGCAAATA -3’ [nt 7,859 – nt 7,878]

Antisense primers
3886L 5’- AAACCTGACGCCAACATTGT -3’ [nt 3,886 – nt 3,905]
4884L 5’- TCGGCTGTGCTTCCACCTGT -3’ [nt 4,884 – nt 4,903]
5062L 5’- AAACTTAGTTCCCGTAAAGCTACA -3’ [nt 5,062– nt5,085]
5858L 5’- TCGGGATTGCTTGTATGTCA -3’ [nt 5,858 - nt 5,877]
6323L 5’- CAGCGGTGTTCCTGAAGACA -3’ [nt 6,323 – nt 6,342]
7882L 5’- TTTCAGATTTCCAGTCTTCA -3’ [nt 7,882 – nt 7,901]
8456L 5’- TTAACCCGGTTTCCAATCGTTAGC -3’ [nt8,456 - nt 8,479]

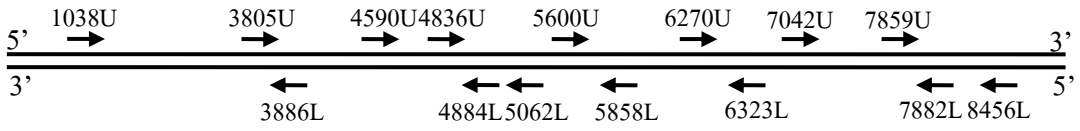


Figure 7 Diagram shows the DNA template and position of the primers used in sequencing.

5.2 PCR amplification

The *α-amylase* genomic DNA of Nanglai silkworm were amplified. Fragment 1, 2 and 3 were amplified with AmyF primer+AmyR primer, 1038U primer+3886L primer and 5600U primer+8456L primer respectively. The amplified reaction of 10 µl contained 1 µg of the plasmid AMYT-1 template, 1 µl of 5% DMSO, 1 µl of 0.2 mM each dNTPs, 10x KOD Buffer, 0.6 µl of 25 mM $MgCl_2$, 0.2 µl of 1 unit/ µl KOD Plus DNA polymerase, 0.25 µl of 10 µM Primer sense, 0.25 µl of 10 µM Primer antisense and adjusted by nuclease-free water. The reaction mixture was amplified in an ICycler Thermal cycler (BIO-RAD). PCR procedure was as

followed: preheated at 95 ºC for 2 min, followed by 35 cycles of denaturing at 95 ºC for 30 sec, gradient annealing at 64.2, 61.7, 60.0, 58.0 ºC for 1 min and extension at 68 ºC for 1 min. The final extension was carried out at 68 ºC for 5 min. PCR products was detected by 1% agarose gel and purified from agarose gel using QIAquick Gel Extration Kit.

5.3 Purification of DNA fragment from agarose gel

Purification of DNA fragment from agarose gel was performed according to QIAquick Gel Extration Kit (Qiagen, Germany). Agarose gel containing the band was transferred to a microcentrifuge tube. One hundred microliters of QG buffer was added to the agarose gel. The sample was incubated at 50 ºC for 10 min or until the agarose gel was completely dissolved. The 350 µl isopropanol was added into the tube, mixed and transferred the mixture into a spin column. The column was centrifuged at 12,000 rpm at room temperature for 1 min. The spin column was removed from the collection tube and discarded the liquid inside the tube. The spin column was placed back into the tube and added 500 µl PE buffer. The sample was centrifuged at 12,000 rpm for 2 min at room temperature. The flow-through was discarded. The spin column was placed back into the tube and centrifuge for an additional 2 min at 12,000 rpm. The spin column was placed into a new microcentrifuge tube. The DNA fragment was eluted by adding 20 µl of EB buffer and centrifuge at 12,000 rpm for 2 min. The purified DNA fragment was determined its concentration by gel electrophoresis.

5.4 Preparation of purified DNA fragment for ligation

The purified DNA fragment was prepared before ligation. Add A-overhang into the ends of the purified DNA fragment by used A-attachment reagent (TOYOBO, Japan). The reaction of 20 µl contained 14.8 µl of PCR product, 2 µM of 2 mM dNTP mixture, 1.2 µl of 25 mM $MgCl_2$ and 2 µl of 10X KOD buffer. The reaction was mixed. Used 9 µl of reaction mixed with 1 µl of 10X A-attachment Mix and incubated at 60 ºC for 10 min. Cloned 3 µl of the DNA fragment into 1 µl of

pTA2 vector (TOYOBO, Japan) by T4 DNA ligase and transformed into *E. coli* DH5α (TOYOBO, Japan) by heat shock transformation, extracted plasmid as described in section 2.7-2.10 and sequenced.

6. *B. mori* RNA isolation

6.1 *B. mori* RNA isolation

Total RNA was extracted from head of the mid 5th instar larva of *B. mori* Nanglai strain using Isogen reagent. The head was homogenized by a pestle in 300 µl Isogen reagent and incubated at room temperature for 5 min to dissociate nucleoprotein complex. Add 300 µl of DEPC water, add 100 µl of chloroform, shake the tube vigorously by hand and then centrifuged at 12,000 rpm for 10 min at 4 °C. Remove supernatant into a new microcentrifuge tube, add 100 µl of Isogen reagent again and shake the tube vigorously by hand. The mixture was centrifuged at 12,000 rpm at 4 °C for 10 min. Remove supernatant into a new microcentrifuge tube, add 100 µl of chloroform and shake the tube vigorously by hand. The mixture was centrifuged at 12,000 rpm at 4 °C for 10 min. The RNA was precipitated by mixing with 40 µl of 3M potassium acetic acid and 800 µl of absolute ethanol. The sample was incubated on ice for 10 min. Total RNA was precipitated by centrifugation at 12,000 rpm at 4 °C for 10 min. After the supernatant was removed, the RNA pellet was washed twice with 70% (v/v) ethanol 200 µl and centrifuged at 12,000 rpm at 4 °C for 1 min. The washed RNA pellet was air-dried until ethanol evaporated completely and dissolved in RNase-free water (DEPC-treated water) 30 µl.

6.2 Determination of quality and concentration of RNA

RNA concentration was estimated by UV spectrophotometer at the absorbance 260 nm (A_{260}) as described in section 1.2.

7. Cloning and sequence *α-amylase* cDNAs using (5'-RACE)

5'- RACE were performed to generate the full-length *α-amylase* cDNAs using the FirstChoice® RLM-RACE Kit (Ambion, USA). The overview of full-length ds cDNA synthesis was shown in Figure 8.

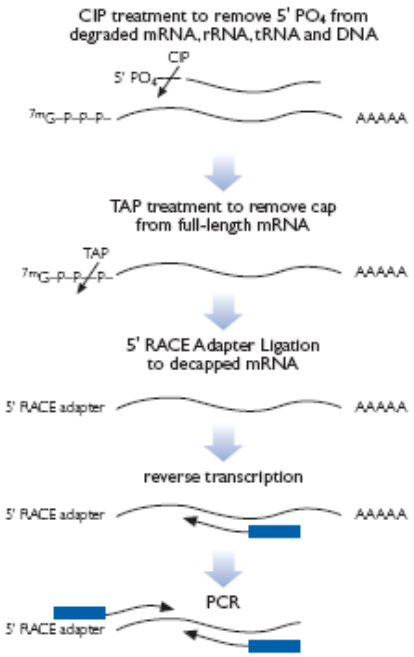


Figure 8 Full-length double strand cDNA synthesis flow chart of FirstChoice® RLM-RACE

Source: Ambion (2007)

7.1 RNA processing

7.1.1 Calf intestine alkaline phosphatase (CIP) treatment

Total RNA was treated with Calf Intestine Alkaline Phosphatase (CIP) to remove free 5'-phosphates from molecules such as ribosomal RNA, fragmented mRNA, tRNA, and contaminating genomic DNA. The cap structure

found on intact 5' ends of mRNA is not affected by CIP. The mixture was prepared for CIP treatment: total RNA 10 µg, 2 µl of 10X CIP buffer, 2 µl of Calf Intestine Alkaline Phosphatase (CIP) and adjust to 20 µl by using Nuclease-free Water. Mixed gently, spin briefly and incubated at 37°C for one hour. Added 15 µl Ammonium Acetate Solution, 115 µl Nuclease-free water and 150 µl phenol: chloroform for terminate CIP reaction. The tube was vortexed thoroughly and centrifuged at 12,000 rpm at room temperature for 5 mins. Transfer aqueous phase (top layer) to a new tube. The mixture was added 150 µl chloroform, vortex thoroughly and centrifuged at 12,000 rpm at room temperature for 5 min. The aqueous phase (top layer) was transferred into a new tube. The tube was added 150 µl isopropanol and vortex thoroughly. The tube was chilled on ice for 10 min and centrifuged at 12,000 rpm at room temperature for 20 min. The pellet was washed with 500 µl cold 70% (v/v) ethanol and centrifuged at 12,000 rpm at room temperature for 5 min. The washed pellet was air-dried until ethanol evaporated completely and dissolved in RNase-free water 11 µl.

7.1.2 Tobacco acid pyrophosphatase (TAP) treatment

The RNA is then treated with Tobacco Acid Pyrophosphatase (TAP) to remove the cap structure from full-length mRNA, leaving a 5'-monophosphate. The mixture was prepared for TAP treatment: 5 µl of CIP'd RNA (from section 1), 1 µl of 10X TAP buffer, 2 µl of Tobacco Acid Pyrophosphatase, 2 µl of Nuclease-free Water. The mixture was mixed gently, span briefly and incubated at 37°C for one hour.

7.1.3 5' RACE adapter ligation

A 45 base RNA Adapter oligonucleotide was ligated to the RNA population using T4 RNA ligase. The adapter cannot ligate to dephosphorylated RNA because these molecules lack the 5'-phosphate necessary for ligation. During the ligation reaction, the majority of the full length decapped mRNA acquires the adapter sequence as its 5' end. An adaptor was ligated to the CIP/TAP-treated RNA obtained from section 2. The following premix was prepared for 5' RACE Adapter Ligation: 2

µl of CIP/TAP-treated RNA, 1 µl of 5' RACE Adapter (5'-GCUGAUGGCGAUGAAUGAACACUG CGUUUGCUGGCUUUGAUGAAA-3'), 1 µl of 10X RNA Ligase Buffer, 2 µl of T4 RNA Ligase (2.5 U/µl) and 4 µl of Nuclease-free Water. The solution was mixed gently, briefly spined and incubated at 37°C for one hour.

7.2 Reverse transcription

The reaction mixture of 20 µl contained 2 µl of Ligated RNA (from section 3), 4 µl of dNTP Mix, 2 µl Random Decamers, 2 µl of 10X RT Buffer, 1 µl of RNase Inhibitor, 1 µl of M-MLV Reverse Transcriptase and 8 µl of Nuclease-free Water. The solution was mixed gently, briefly span and incubated at 42°C for one hour.

7.3 Nested PCR for 5' RLM-RACE

7.3.1 Oligonucleotide primers design

The sequences of the 5' RACE Outer primer and 5' RACE inner primer were as followed: 5' RACE Outer Primer 5'-GCTGATGGCGATGAATGAACACTG-3' and 5' RACE Inner Primer 5'-CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG-3'. The 5' RACE Primers work well in PCR using an annealing temperature from 55–65°C. The 5' RACE gene-specific outer primer and 5' RACE gene specific inner primer were designed based on the cDNA sequences of Nanglai. They were as followed: 5' RACE gene-specific outer primer 5'-TTGCGGGACCAGATTACCAAATTCTCGT-3' and 5' RACE gene specific inner primer 5'-GGCCGTGCTTCCACCTGTACCAAC-3'. The position of primer as showed in Figure 9.

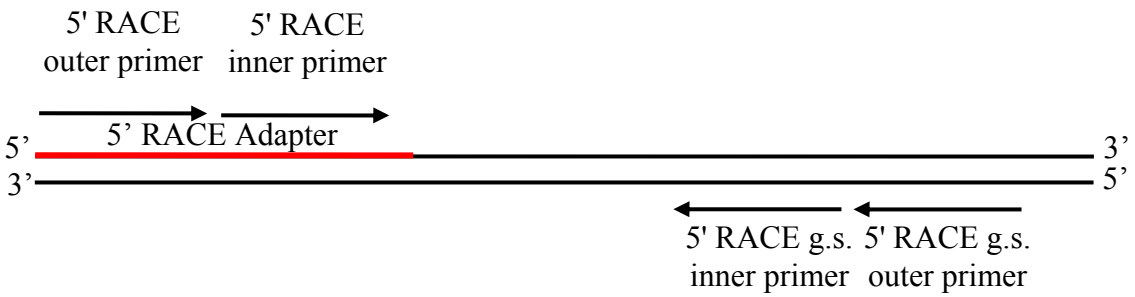


Figure 9 Diagram shows the cDNA template and position of the primers used in 5’ RACE.

7.3.2 Outer 5' RLM-RACE PCR

The reaction mixture of 50 µl contained 1 µl RT reaction (from section B), 10x KOD Buffer, 0.2 Units of KOD Plus DNA polymerase, 4 µl dNTP Mix, 2 µl 5' RACE gene-specific outer primer (10 µM), 2 µl 5' RACE Outer Primer and adjust by using Nuclease-free Water into 50 µl. The reaction mixture was amplified in a ICycler Thermal cycler (BIO-RAD). PCR procedure was as followed: preheated at 95 ºC for 2 min, followed by 35 cycles of denaturing at 95 ºC for 30 sec, annealing at 60 ºC for 30 sec and extension at 68 ºC for 1 min. The final extension was carried out at 68 ºC for 5 min.

7.3.3 Inner 5' RLM-RACE PCR

The reaction mixture of 50 µl contained 1 µl Outer PCR, 10x KOD Buffer, 0.2 Units of KOD Plus DNA polymerase, 4 µl dNTP Mix, 2 µl 5' RACE gene specific inner primer (10 µM), 2 µl 5' RACE Inner Primer and adjust by using Nuclease-free Water into 50 µl. The reaction mixture was amplified in a ICycler Thermal cycler (BIO-RAD). PCR procedure was as followed: preheated at 95 ºC for 2 min, followed by 35 cycles of denaturing at 95 ºC for 30 sec, annealing at 60 ºC for 30 sec and extension at 68 ºC for 1 min. The final extension was carried out at 68 ºC for 5 min.

PCR products of Inner 5' RLM-RACE PCR fragments was detected by 1% agarlose gel and purified from agarose gel using QIAquick Gel Extration Kit. Add A-overhang into the ends of the DNA fragment by used A-attachment reagent (TOYOBO, Japan). Cloned DNA fragment into pTA2 vector (TOYOBO, Japan) by T4 DNA ligase and transformed into *E. coli* DH5α by heat shock transformation, extracted and sequenced.

8. Construction of vectors

Two type of plasmids, *pBac*[A3-AMY1-A3UTR, 3xP3-DsRed2] and *pBac*[A3-AMY1-SV40UTR, A3-EGFP] were used.

8.1 Construction of *pBac*[A3-AMY1-A3UTR, 3xP3-DsRed2**]**

pBac[A3-AMY1-A3UTR, 3xP3-DsRed2] was constructed from *pBac*(3xP3-DsRedaf), which contained *DsRed* reporter gene. This vector contained *α-amylase* cDNA under the control of the *Actin3* promoter and *Actin3* terminator sequence in piggyBac vector. The *Actin3* promoter and *Actin3* terminator was PCR-amplified from plasmid BmA3 [vector from Dr. Tamura]. *α-amylase* gene was PCR-amplified from AMYT-1 plasmid.

8.1.1 Amplification and Sequencing of promoter and terminator of *Acin3* gene

A. Oligonucleotide primers design

Oligonucleotide primers were designed based on the sequences of *Bombyx mori* cytoplasmic *Actin* (*A3*) gene [GenBank Accession No.U49854] as shown in Figure 8. The oligonucleotide primers were as followed:

Oligonucleotide primers for *Actin3* promoter were as followed: A3FselIF (sense) (F1) 5’- GGCCGGCCTGAGTCAGCCCGCGATTGGTG -3’,

which contained *Fse*I site. A3DSXF (antisense) (R1) 5'-CGAAACATTGCCCGTACGAGTCCTTCT -3', which contained 19 nucleotides of 3' promoter sequence connected to 8 nucleotides of 5' *α-amylase* cDNA.

Oligonucleotide primers for *Actin*3 terminator were as followed: FAcPAF (sense) (F3) 5'- CTGTGCGATGGCCTCGAGAAGTCTTACGAAC -3' and AcPAR4 (antisense) (R3) 5'- GGCGCGCCACGCAATGACGTTGACAAGTC -3', which contained *Asc*I site.

B. PCR Amplification

The promoter and terminator of *Actin3* gene were amplified by PCR, using the plasmid A3 (vector from Dr. Tamura) as a template and two sets of primers, A3FselIF primer (F1) and A3DSXF primer (R1), FAcPAF primer (F3) and AcPAR4 primer (R3) respectively.

The amplified reaction of 25 µl contained 1 µl of the plasmid A3 template (vector from Dr. Tamura), 10x OptiBuffer, 5 mM dNTP mixture, 50 mM $MgCl_2$, 1 U Bio-X-ACT Short DNA Polymerase, nuclease-free water17.75 µl, Primer sense (10 µM of A3FselIF for amplified promoter, 10 µM of FAcPAF for amplified terminator) and Primer antisense (10 µM of FAcPAF for amplified promoter, 10 µM of AcPAR4 for amplified promoter). The reaction mixture was amplified in a Px2 Thermal cycle (Thermo Hybrid). PCR procedure was as followed: preheated at 94 ºC for 3 min, followed by 15 cycles of denaturing at 96 ºC for 30 sec, annealing at 55 ºC for 1 min and extension at 70 ºC for 2 min. The final extension was carried out at 70 ºC for 10 min.

PCR products was detected by 1.2% agarose gel and purified from agarose gel using (NucleoSpin® Extract II). The DNA was ligated into pGEM-T® easy vector, transformed into *E coli* JM109 by heat shock transformation, extracted plasmid as described in section 2.4-2.10 and sequenced.

C. Connection of *Actin3* promoter, *α-amylase* cDNA and *Actin3* terminator into single fragment

Plasmid of *Actin3* promoter was amplified by PCR with forward primer A3FselIF (F1) and reverse primer A3DSXF (R1). Plasmid of cDNA of *α-amylase* gene from 3'-RACE (AMYT-1) was amplified by PCR with forward primer AmyA3DSXF (F2) and reverse primer AmyRACE (R2). Plasmid of *Actin3* terminator was amplified by PCR with forward primer FAcPAF (F3) and reverse primer AcPAR4 (R3) (Figure 10).

Each PCR products were removed A-overhang at the ends of PCR products by used Klenow fragment enzyme: PCR products 5 µl, 40 mM dNTP mixture, 0.4 µl of Klenow fragment enzyme. Each the solutions were mixed and incubated at 37 ºC for 10 min. Each the solutions 1 µl were used as the template for join each fragments into single fragment (Figure 11). The amplified reaction of 25 µl contained 1 µl of each the solution fragments, 10x OptiBuffer, 5 mM dNTP mixture, 50 mM $MgCl_2$, 1 Unit Bio-X-ACT Short DNA polymerase, 17.75 µl of nuclease-free water, 10 µM of Primer sense A3FselIF (F1) and 10 µM of Primer antisense AcPAR4 (R3). The reaction mixture was amplified in a Px2 Thermal cycle (Thermo Hybaid). PCR procedure was as followed: preheated at 95 ºC for 3 min, followed by 15 cycles of denaturing at 94 ºC for 1 min, annealing at 60 ºC for 30 sec and extension at 70 ºC for 2 min. The final extension was carried out at 70 ºC for 10 min.

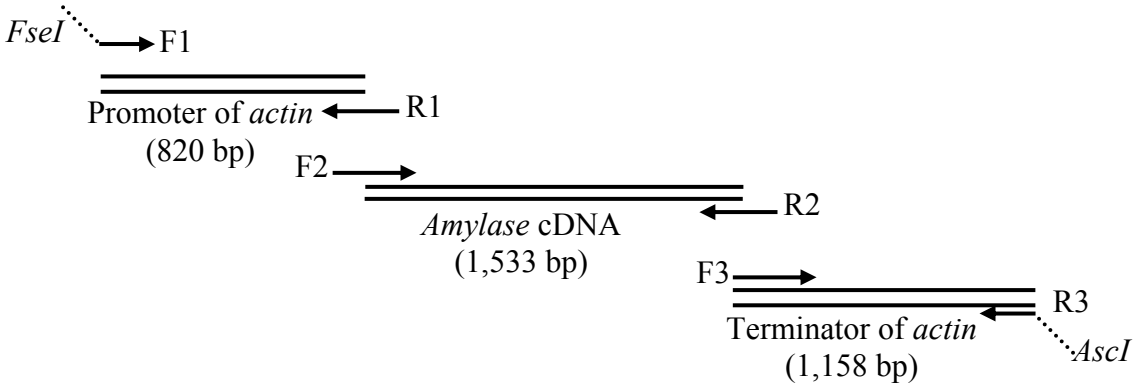


Figure 10 Structure of each DNA fragments, *Actin3* promoter, *α-amylase* cDNA and *Actin3* terminator, respectively.

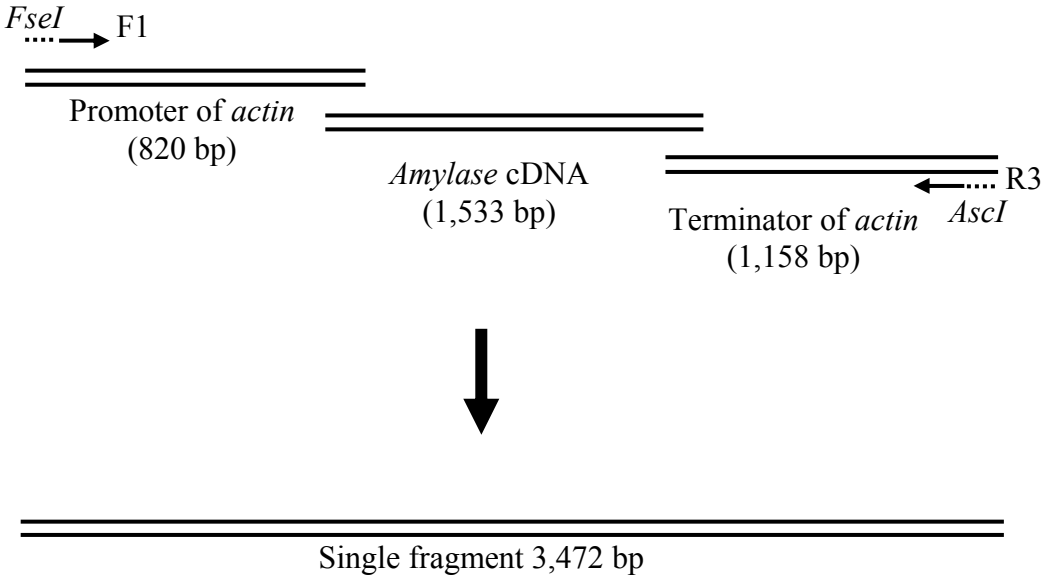


Figure 11 Structure of single DNA fragment *Actin3* promoter - *α-amylase* cDNA - *Actin3* terminator.

PCR products was detected by 1.2% agarose gel and purified from agarose gel using (NucleoSpin® Extract II). cloned into pGEM-T® easy vector and transformed into *E coli* JM109 by heat shock transformation, extracted plasmid as described in section 2.2-2.10 and sequenced.

D. Ligation of the single DNA fragment into *pBac*(3xP3-DsRedaf) vector

Plasmid of the single DNA fragment was used as a template and used condition in C section. The PCR product was detected by 1.2% agarose gel and purified from agarose gel using NucleoSpin® Extract II. The single DNA fragment was double digested with *Fse*I and *Asc*I and purified from agarose gel.

The *Fse*I/*Asc*I fragment of single DNA fragment was inserted into *Fse*I/*Asc*I fragment of *pBac*(3xP3-DsRedaf) vector. The ligation reaction of 10 µl contained: 3 µl of *Fse*I/*Asc*I fragment of single DNA fragment, 0.5 µl of *pBac*(3xP3-DsRedaf), 5X rapid ligation buffer, 1 µl of T4 DNA ligase and adjusted by deionized water. The ligation reaction was mixed and incubated at 25 ºC for 3 hrs. The resulting plasmid was name *pBac*(A3-AMY1-A3UTR, 3xP3-DsRed2). This plasmid was transformed into *E. coli* JM109 by heat shock transformation, extracted plasmid as described in section 2.4-2.10 and sequenced.

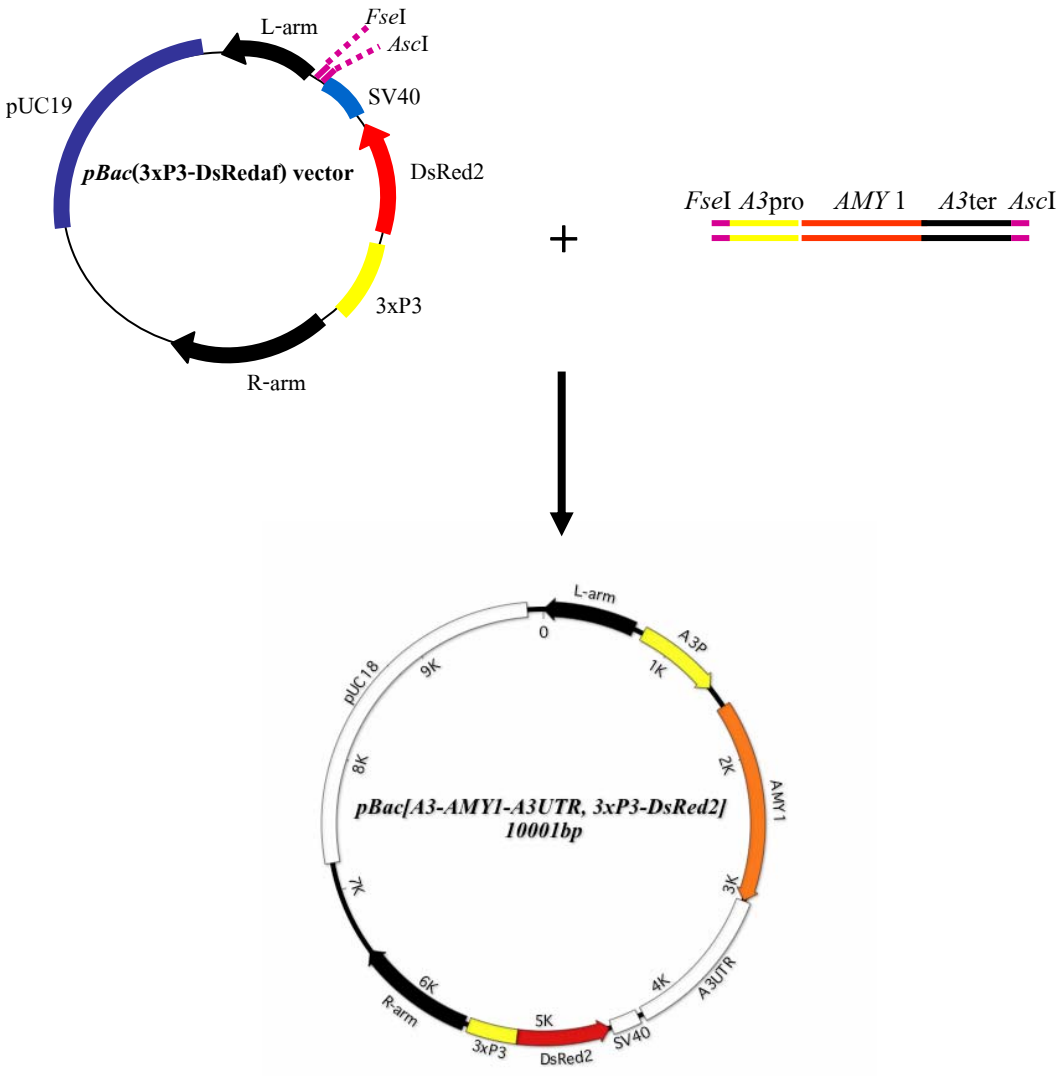


Figure 12 Construction of *pBac*(A3-AMY1-A3UTR, 3xP3-DsRed2) vector.

8.2 Construction of *pBac*[A3-AMY1-SV40UTR, A3-EGFP]

pBac[A3-AMY1-SV40UTR, A3-EGFP] was constructed from *pBac*MCS(A3-SV40, A3-EGFP)R, which contained *EGFP* reporter gene. This vector contained *α-amylase* cDNA under the control of the *Actin3* promoter and SV40 terminator sequence in *piggyBac* vector. The *α-amylase* cDNA was PCR-amplified from AMYT-1 plasmid.

8.2.1 Amplification and sequencing of cDNA of *α-amylase* gene

A. Oligonucleotide primers design

Oligonucleotide primers were designed based on the sequences of *α-amylase* cDNA from 3'-RACE. The oligonucleotide primers were as followed: AMY-XbaI.U (sense) (F4) 5'-TCTAGAATGTTTCGGTACATCCTTCT-3' and AMY-XbaI.L (antisense) (R4) 5'-TCTAGATTACAATCTCGAGTCAGCAC-3'. Single *Xba*I sites were added to both primers.

B. PCR amplification

The *α-amylase* cDNA was amplified by PCR, using the plasmid AMYT-1 as a template for amplified with AMY-XbaI.U primer (F4) and AMY-XbaI.L primer (R4).

The amplified reaction of 10 µl contained 0.2 µl of the plasmid AMYT-1 template, 5% DMSO, 0.2 mM of each dNTPs, 10x KOD Buffer, 1.5 mM $MgCl_2$, 0.2 Units of KOD Plus DNA polymerase, 0.25 µM Primer sense AMY-XbaI.U, 0.25 µM Primer antisens AMY-XbaI.L and adjusted by nuclease-free water. The reaction mixture was amplified in a ICycler Thermal cycler (BIO-RAD). PCR procedure was as followed: preheated at 95 ºC for 2 min, followed by 35 cycles of denaturing at 95 ºC for 30 sec, gradient annealing at 64.2, 61.7, 60.0, 58.0 ºC for 1 min and extension at 68 ºC for 1 min. The final extension was carried out at 68 ºC for 5 min. PCR products was detected by 0.8% agarose gel.

C. Purification of DNA fragment from agarose gel

Purification of DNA fragment from agarose gel was performed according to QIAquick Gel Extration Kit. Agarose gel containing the band was transferred to a microcentrifuge tube. One hundred microliters of QG buffer was added to the agarose gel. The sample was incubated at 50 ºC for 10 min or until the

agarose gel was completely dissolved. The 350 µl isopropanol was added into the tube. The tube was mixed and transferred the mixture into a spin column. The column was centrifuged at 12,000 rpm at room temperature for 1 min. The spin column was removed from the collection tube and discarded the liquid inside the tube. The spin column was placed back into the tube and added 500 µl PE buffer. The sample was centrifuged at 12,000 rpm for 2 min at room temperature. The flow-through was discarded. The spin column was placed back into the tube and centrifuge for an additional 2 min at 12,000 rpm. The spin column was placed into a new microcentrifuge tube. The DNA fragment was eluted by adding 20 µl of EB buffer and centrifuge at 12,000 rpm for 2 min. The purified DNA fragment was determined its concentration by gel electrophoresis.

D. Preparation of purified DNA fragment for ligation

The purified DNA fragment was prepared before ligation. Add A-overhang into the ends of the purified DNA fragment by used A-attachment reagent (TOYOBO, Japan). The reaction of 20 µl contained 14.8 µl of PCR product, 4 mM dNTP mixture, 30 mM $MgCl_2$ and 10X KOD buffer. The reaction was mixed. Used 9 µl of reaction mixed with 1 µl of 10X A-attachment Mix and incubated at 60 ºC for 10 min. Cloned 3 µl of the DNA fragment into 1 µl of pTA2 vector (TOYOBO, Japan) by T4 DNA ligase and transformed into *E. coli* DH5α by heat shock transformation, extracted plasmid as described in section 2.7-2.10 and sequenced.

E. Ligation of the single DNA fragment into *pBac*[A3-SV40, A3-EGFP]R vector

Plasmid of *α-amylase* cDNA in D section was digested with *Xba*I. The DNA fragment was detected by 0.8% agarose gel and purified from agarose gel using QIAquick Gel Extration Kit.

pBac[A3-SV40, A3-EGFP]R vector was digested with *Bnl*I. The DNA fragment was detected by 0.8% agarose gel and purified from agarose gel using QIAquick Gel Extration Kit.

The *Xba*I fragment of *α-amylase* cDNA was inserted into *Bnl*I fragment of *pBac*[A3-SV40, A3-EGFP]R vector. The ligation reaction of 8 µl contained: 2 µl of *Xba*I fragment of *α-amylase* cDNA, 2 µl of *pBac*[A3-SV40, A3-EGFP]R vector and 4 µl ligation mix. The ligation reaction was mixed and incubated at 37ºC for 1 hr. The resulting plasmid was name *pBac*[A3-AMY1-SV40UTR, A3-EGFP]. This plasmid was transformed into *E. coli* DH5α by heat shock transformation, extracted plasmid as described in section 2.7-2.10 and sequenced.

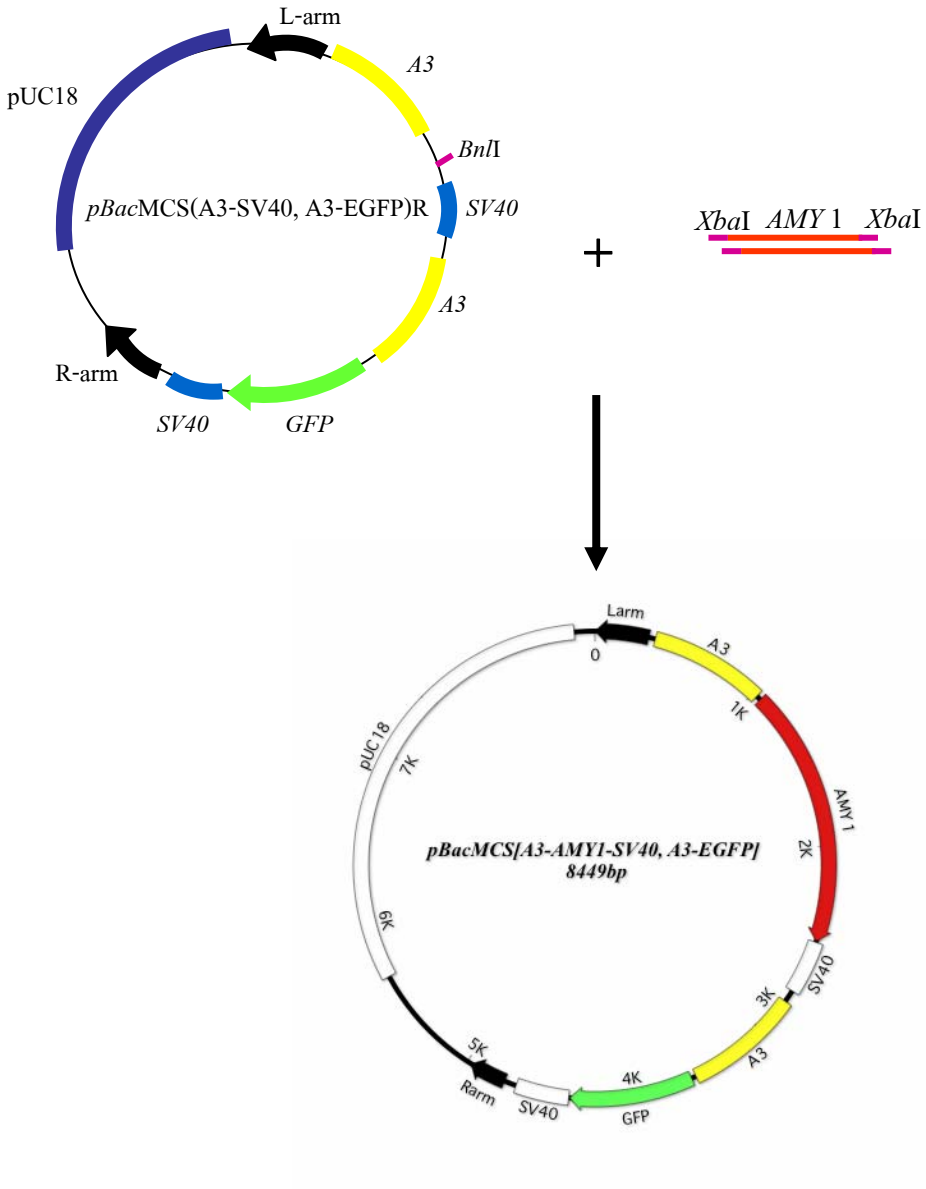


Figure 13 Construction of *pBac*[A3-AMY1-SV40UTR, A3-EGFP] vector.

9. Microinjection

Plasmid recombinant was injected into eggs, according to the method of Dr. Tamura (Transgenic silkworm Research Center, National Institute of Agrobiological Sciences, Japan).

9.1 Preparation of eggs for injection

The *B. mori*, pnd-W1 strain was used for the experiment. Newly emerged male and female moths allowed to mating at 25 ºC for at least 3 hr. The mating moths were stored at 5 ºC for 1-2 nights and then separated. The female moths were transferred to paper covered in glue and stored in dark boxes at 25 ºC. The female moths began to lay eggs. The portion of paper-laid eggs were sterilized by used 3% formalin gas at plastic box for 1 min, washed with tap water, immersed in distilled water for 3 min. The eggs were transferred to glass slide, aligned in the same direction under a binocular microscope (8 rows, 6 columns) and dried by put on the glass slide at room temperature for 10 min. The glass slides were sterilized by used 3% formalin gas at plastic box for 3 min.

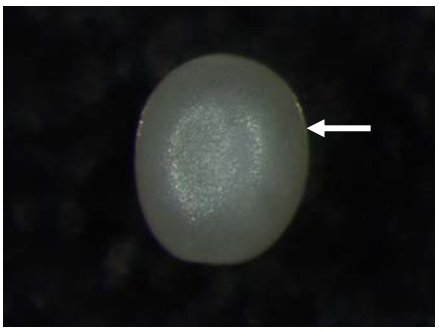
A.  B. 

Figure 14 A glass slide of eggs silkworm for injection. Panel A: alignment of eggs on the glass slide. Panel B: position of eggs on the glass slide. An arrow points at injected position.

9.2 Preparation of plasmid DNA for injection

Helper plasmid pHA3PIG (Tamura *et al.*, 2000) and two type of vector plasmid *pBac*[A3-AMY1-A3UTR, 3xP3-DsRed2] and *pBac*[A3-SV40, A3-EGFP]R were used in experiment. The plasmid DNA were purified by using Hispeed® plasmid midi kit (QIAGEN, Japan)

A single white colony of X-gal-IPTG-ampicillin plate was cultured in 3 ml of LB broth containing 50 μg/ml ampicillin and incubated at 37 ºC with shaking at 250 rpm overnight (12-16 hr). One microliter of the starter culture was transferred into 50 ml LB broth and incubated at 37 ºC with shaking at 250 rpm overnight (12-16 hr). The supernatant was discarded. The culture was transferred into tube and centrifuged at 10,000 rpm for 3 min. The bacterial pellet was resuspended in 6 ml Buffer P1 by vigorous vortexing. The 6 ml buffer P2 was added into the tube and mixed thoroughly by vigorously inverting the sealed tube 4–6 times. The incubation prepared the QIAfilter Cartridge: Screw the cap onto the outlet nozzle of the QIAfilter Midi Cartridge. The QIAfilter Cartridge was placed into a QIArack. The 6 ml chilled Buffer P3 was added and mixed immediately and thoroughly by vigorously inverting 4–6 times. The lysate was poured into the barrel of the QIAfilter Cartridge. The QIAfilter Cartridge was incubated at room temperature for 10 min. A HiSpeed Midi Tip was equilibrated by applying 4 ml Buffer QBT and allowed the column to empty by gravity flow. The cap was removed from the QIAfilter outlet nozzle. The plunger was gently inserted into the QIAfilter Midi Cartridge and filtered the cell lysate into the previously equilibrated HiSpeed Tip. Allow the cleared lysate to enter the resin by gravity flow. The HiSpeed Midi Tip was washed with 20 ml or Buffer QC. The DNA was eluted with 5 ml of Buffer QF. Precipitate DNA by added 3.5 ml isopropanol to the eluted DNA. The solution was mixed and incubated at room temperature for 5 min. During the incubation, the plunger was removed from a 20 ml syringe and attached the QIAprecipitator Midi Module onto the outlet nozzle. The QIAprecipitator was placed over a waste bottle, transfered the eluate/isopropanol mixture into the 20 ml syringe, and inserted the plunger. Filter the eluate/isopropanol mixture through the QIAprecipitator using constant pressure. The QIAprecipitator from the 20 ml syringe was removeed and pulled out the plunger. The QIAprecipitator was re-attached and added 2 ml 70% ethanol to the syringe. The DNA was washed by inserting the plunger and pressing the ethanol through the QIAprecipitator using constant pressure.The QIAprecipitator was removed from the 20 ml syringe and pull out the plunger. The QIAprecipitator was attached to the 20 ml syringe again and inserted the plunger, and dry the membrane by pressing air through the QIAprecipitator quickly and forcefully. Repeat this step. The outlet nozzle of the QIAprecipitator was dry with

absorbent paper to prevent ethanol carryover. The plunger from a new 5 ml syringe was removed and attached the QIAprecipitator onto the outlet nozzle. The 5 ml of Buffer TE was added to the 5 ml syringe. The plunger and elute the DNA wera inserted into the collection tube using constant pressure. The QIAprecipitator was removed from the 5 ml syringe, pull out the plunger and reattached the QIAprecipitator to the 5 ml syringe. One milliliter of phenol: chloroform: isoamyl alcohol (25: 24: 1, v/v) was added to the supernatant and mixed by vortexing. The emulsion was centrifuged at 10,000 rpm at room temperature for 10 min and the upper aqueous phase was transferred to a new tube. One microliter of chloroform was added into the tube. The tube was centrifuged at 10,000 rpm at room temperature for 10 min. The upper aqueous phase was transferred to a new tube, added 1 ml potassium acetic acid and 6 ml absolute ethanol into the tube. The tube was incubated the tube at room temperature for 10 min. The mixture was centrifuged at 10,000 rpm at room temperature for 10 min. The supernatant was removed and added 6 ml absolute ethanol and centrifuged at 10,000 rpm at room temperature for 10 min. The pellet was washed with 70% (v/v) ethanol and inverted the close tube several times. The plasmid DNA was recovered by centrifugation at 10,000 rpm at room temperature for 3 min. The supernatant was removed by gentle aspiration. The open tube was stored at room temperature until the ethanol evaporated completely then the DNA was dissolved in an injection buffer (0.5 mM phosphase buffer (pH 7.0) and 5 mM KCl) at a concentration plasmid.

9.3 Injection and screening of transformed silkworms

Vector and helper plasmid pHA3PIG (each 200 μg/ml) were injected into eggs. GFP and DsRed fluorescence was observed under a fluorescence microscope that was equipped with filter sets for GFP2 and DsRed, respectively. Transient expression of the injected DNA was observed in the G0 eggs 8 days after injection.

10. Detection the expression of *α-amylase* gene by RT-PCR

10.1 *B. mori* RNA isolation

Total RNA was extracted from the mid 5th instar larva of *B. mori* Nanglai, C108, w1-pnd, transgenic Y1.1 and transgenic Y1mix tissues including heamolymph, salivary gland, malphighian tube, silk gland, gut and fat body. Total RNA was extracted and measured concentration of RNA, as described in section 6.1-6.2.

10.2 One step RT-PCR Amplication

10.2.1 Oligonucleotide Primers Design

Oligonucleotide primers for RT-PCR amplification *α-amylase* gene were designed based on the clone of *α-amylase* cDNA sequences of Nanglai strain (AMYT-1) as shown in Figure 10. The oligonucleotide primers were as followed:

Primer set 1
AMY1 (RT) 452U (sense)
5'- ACTGGCCTCATTGTGTCATTACTG -3' [nt 452- nt 475]

AMY1 (RT) 758L (antisense)
5'- TGTATTCGTTGCGACTGATAGCTT -3' [nt 758- nt 781]

Primer set 2
AMY1 (RT) 15U (sense)
5'- CCTTCTACTTTCGGCCGTGACT -3' [nt 15- nt 36]

AMY1 (RT) 378L (antisense)
5'- GGCCGTGCTTCCACCTGTACCAAC -3' [nt 376 – nt 385]

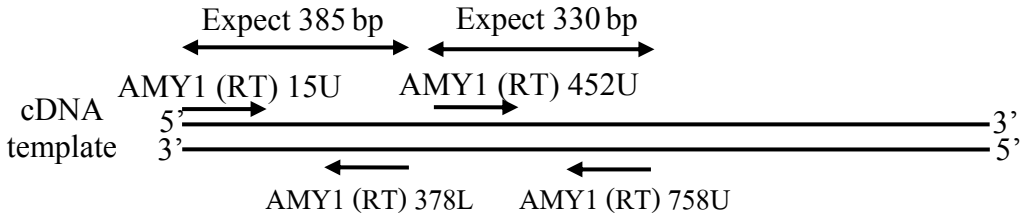


Figure 15 Diagram shows the cDNA template and position of the primers used in RT-PCR.

Oligonucleotide primers for RT-PCR amplification *Actin3* gene were designed based on the sequences of *Bombyx mori* cytoplasmic *Actin* (*A3*) gene [GenBank Accession No. U49854]. The oligonucleotide primers were as followed:

actin.341U28 (RT) (sense)
5'-AGGCCAACAGAGAGAAGATGACCCAGAT-3'

actin.721L24 (RT) (antisense)
5'-GACCTGACCGTCGGGAAGTTCGTA-3'

Total RNA (1 µg) was used as a template for amplified partial 1 and 2 of *α-amylase* and *Actin3* gene with primer set 1 (AMY1(RT)452U+AMY1(RT) 758L), primer set 2 (AMY1 (RT) 15U + AMY1 (RT) 378L) and actin.341U28(RT) + actin.721L24(RT), respectively. The reaction mixture of 10 µl contained 1 µg of total RNA, 5x Reaction Buffer, 0.2 mM dNTP mixture, 25 mM $Mn(OAc)_2$, 0.4U rT*th*DNA Polymerase, RNase Inhibitor, Primer sense (AMY1 (RT) 15U; 10 pmol/ µl), Primer antisense (AMY1 (RT) 378L; 10 pmol/ µl) and adjust volume by using nuclease-free water into 10 µl. The reaction mixture was amplified in an ICycler Thermal cycler (BIORAD). PCR procedure was as followed: incubate at 60 ºC for 2 min, 40 ºC for 10 min, 50 ºC for 10 min, preheat at 95 ºC for 2 min, followed by 25 cycles of denaturing at 95 ºC for 15 sec, annealing at 64 ºC for 1 min and extension at 60 ºC for 5 min. PCR products was detected by 1% agarose gel.

RESULTS AND DISCUSSIONS

Results

1. Total RNA isolation from head of Nanglai strain

Total RNA was isolated from the head of the mid 5th instar larva of Nanglai strain by using TRIzol reagent. The isolated total RNA had an A 260/A280 ratio of, 3.40, 2.14, 2.21 and 1.90 suggesting that the RNA had a little protein contamination. Yields of the RNA were 1800, 2520, 3140 and 3840 ng/µl. The qualities of total RNA were detected by 1% agarose gel. The total RNA not degraded (data not show).

2. PCR-Amplification, cloning and sequencing *α-amylase* gene

2.1 PCR Amplification

The total RNA was used as a template to synthesis the 1st strand using RT-PCR method of Improm II^{TM} Reverse Transcriptase System. Then, the 1st strand was used as a template for amplification *α-amylase* cDNA. Two primers, AmyF (sense) and AmyR (antisense) were used in amplification of *α-amylase* cDNA fragment. These primers were designed based on the sequences of cDNA of *Bombyx mori* truncated *alpha-amylase* (*amy*) gene (GenBank Accession No. U07847). The PCR product, approximately 1,500 bp long (Figure 16), was purified from agarose gel and ligated into pGEM-T® easy vectors. Then, the recombinant vectors were transformed into *E. coli* JM109 by heat shock transformation.

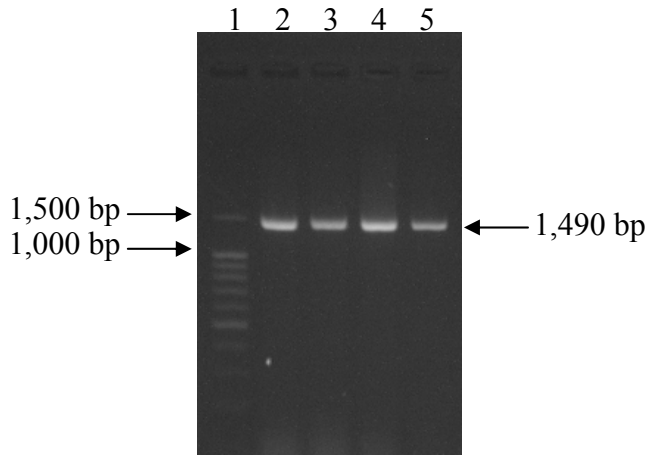


Figure 16 Agarose gel electrophoresis of PCR products of *α-amylase* cDNA. Lane 1: 100 bp + 1.5 kb DNA ladder, Lane 2-4: PCR products of *α-amylase* cDNA.

2.2 Detection of DNA Insertion by Restriction Digestion

Recombinant plasmids containing the *α-amylase* cDNA were screened by the formation of blue/white colonies on LB ampicillin plate. Two white colonies were randomly selected for plasmid isolation and analyzed by cutting with *Apa*I and *Sac*I to get fragment with 3,000 bp for DNA vector and 1,500 bp long for the *α-amylase* cDNA fragment (data not show). One colony containing the 1,500 bp of *α-amylase* cDNA fragment was selected for sequencing. The sequence of *α-amylase* cDNA clone was 1,491 bp long (Figure 17).

The *α-amylase* cDNA from Nanglai strain differed from nucleotide sequence of *Bombyx mori* truncated *alpha-amylase* (*amy*) gene (GenBank Accession No. U078470). The nucleotide sequence of *α-amylase* cDNA from Nanglai strain has 2 extra regions. One region composes of the nucleotide at position 323 to 620 and the other one covers the nucleotide at position 1,014 to 1,023 (Figure 18).

```
5′   1  ATGTTTCGGTACATCCTTCTACTTTCGGCCGTGACTCTGGCGTTGGCCTATAAAAACCCTCACTATGCAT   70
    71  CGGGTCGCACCACCATGGTACATCTCTTCGAATGGAAGTGGGATGACATCGCTGCTGAATGCGAAAGGTT  140
   141  CCTTGGACCCCGAGGATTCGGTGGTATTCAGGTTTCGCCACCAAACGAGAATTTGGTAATCTGGTCCCGC  210
   211  AACCGTCCTTGGTGGGAGCGCTATCAACCAATCTCCTACCGTCTAGTAACAAGATCTGGAAATGAAAATC  280
   281  AATTTTCGAATATGGTGCGTCGCTGCAACAATGTTGGCGTCAGGATTTATGTGGACGCCATCATCAACCA  350
   351  CATGACTGGAACTTGGAATGAGAATGTTGGTACAGGTGGAAGCACAGCCAATTTCGGAAACTGGCACTAT  420
   421  CCTGCTGTTCCTTACGGCAGGAATGACTTCAACTGGCCTCATTGTGTCATTACTGGCAACGACTACAACT  490
   491  GCTGCCCTGACAGAGTGCGCAACTGTGAATTGTCTGGTCTGAAAGACTTGAACCAAGGCTCTGATTATGT  560
   561  TAGACAGCAGATTCTAAATTATATGAACCGTCTTATTGACATGGGTGTTGCTGGTTTCAGAATTGATGCT  630
   631  GCAAAGCACATGTGGCCTCACGATTTGCGAGTGATCTACGATCGTCTCCGCAATTTAAATACCGCCCACG  700
   701  GTTTCCCATCCGGTGCTCGTCCCTACATCTACCAAGAAGTCATCGACCTCGGTGGTGAAGCTATCAGTCG  770
   771  CAACGAATACACTCCACTTGCTGCCGTTACTGAGTTCAGATTTGGGCTGGAACTCAGTCAAGCTTTCCAA  840
   841  AGAAGAAACCAGCTTAGATGGTTGGTTAACTGGGGTCCACAGTGGGGCCTTCTCGCTTCTGGAGACGCTT  910
   911  TGACTTTCATTGACAACCACGACAACCAGAGAGGTCATGGCGCTGGTGGAAACATCTTGATATACAAGCA  980
   981  ATCCCGACAATACAAGGGCGCTATCGCTTTCATGTTGGCACATCCTTATGGCTACCCTCAATTGATGAGT 1050
  1051  AGTTTCGCCTTCACGGACACCGAAGCTGGACCTCCAATGAACAGCAGGGGTGATATCACCTCTCCTACTA 1120
  1121  TCAACGCTGATAATTCTTGCGGCAATGGTTGGATATGCGAGCACCGTTGGCGTCAGATCCACAACATGGT 1190
  1191  TGTCTTCAGGAACACCGCTGGTAACGGTGCCCTTACCAACTGGTGGGACAACGGTAGCAACCAGATTGCT 1260
  1261  TTCTGCCGCGGCAACCAGGCCCTCATTGCTTTCAACAACGATGCATGGGACATGGACCAGACTCTTCAGA 1330
  1331  CTTGTCTCCCCGCCGGAACATACTGCGATATTATTTCCGGCGCCAGGTCTGGTAACCGCTGCACTGGAAA 1400
  1401  ATCTATTGTAGTAGGCAGCGATGGTCGCGCTCGTATCATCCACCGCAGCAACGAGTATGACATGATGGTT 1470
  1471  GCTATCCATAGGGGTGCTGAC                                                  1491 3′
```

Figure 17 The 1,491 bp of *α-amylase* cDNA sequence from Nanglai strain.

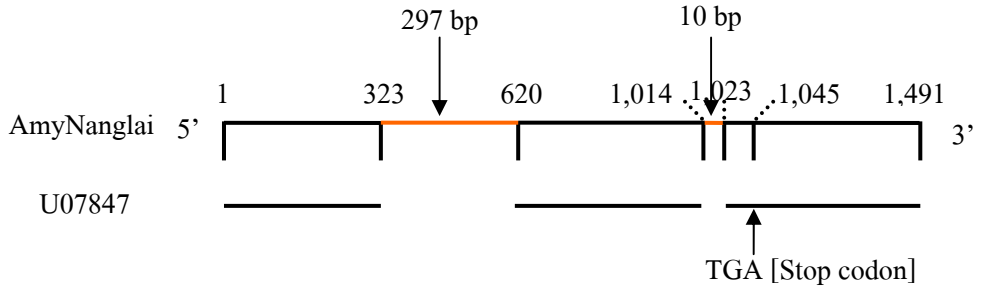


Figure 18 Alignment of the nucleotide sequences of *α-amylase* cDNA from Nanglai strain showed 99% homology to the cDNA of *Bombyx mori* truncated *alpha-amylase* (*amy*) gene (GenBank Accession No. U078470)

To confirm the nucleotide sequence of *α-amylase* cDNA of Nanglai strain is true, Total RNA was isolated from head of other silkworm of Nanglai strain in next generation and another strain w1-pnd, which is bivoltine strain but it has some characters as found in the polyvoltine strain.

2.4 Total RNA isolation of the 2nd experiment from head of Nanglai and w1-pnd strain.

Total RNA was isolated by using same method as described above. The RNA yields of Nanglai and w1-pnd strains were 2,300 and 7,900 ng/µl respectively. Total RNA of pnd-w1 strain had an A260/A280 ratio of 1.76 and 1.66 suggesting that the RNA had a little protein contamination. The RNA yields were 5,200 and 5,520 ng/µl. The qualities of total RNA were detected by 1% agarose gel. The total RNA not degraded (data not show).

2.5 PCR amplification

Total RNA isolation from Nanglai and w1-pnd strains were used as templates to synthesize the 1st strand using the method described above. The 1st strand was used as a template to amplify *α-amylase* cDNA. The PCR products obtained from both templates were approximately 1,500 bp long (Figure 19 and Figure 20, respectively). The DNA fragments were purified from agarose gel, ligated into pGEM-T® easy vectors, transformed into *E. coli* JM109 by heat shock transformation.

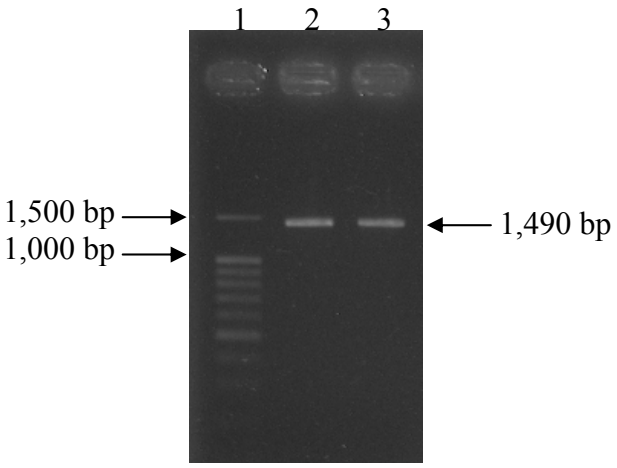


Figure 19 Agarose gel electrophoresis of *α-amylase* cDNA PCR products of the 2nd experiment of Nanglai strain. Lane 1: 100 bp + 1.5 kb DNA ladder, Lane 2-4: PCR products of *α-amylase* cDNA.

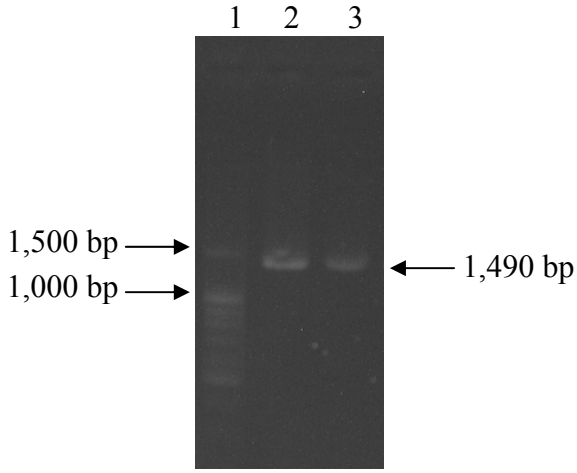


Figure 20 Agarose gel electrophoresis of the PCR products from *α-amylase* cDNA of w1-pnd strain. Lane 1: 100 bp + 1.5 kb DNA ladder, Lane 2-4: PCR products of *α-amylase* cDNA.

2.6 Detection of DNA insertion by restriction digestion

Recombinant plasmids containing the *α-amylase* cDNA from Nanglai and pnd-w1 strains were randomly selected and analyzed by cutting with *Apa*I and *Sac*I to get fragment with 3,000 bp for DNA vector and 1,500 bp long for the *α-amylase* cDNA fragment (data not show). One colony of each silkworm strains containing the 1,500 bp of *α-amylase* cDNA fragment was selected for sequencing. The sequence of *α-amylase* cDNA each clone was 1,491 bp long (Figure 21 and 22).

```
5′   1  ATGTTTCGGTACATCCTTCTACTTTCGGCCGTGACTCTGGCGTTGGCCTATAAAAACCCTCACTATGCAT   70
    71  CGGGTCGCACCACCATGGTACATCTCTTCGAATGGAAGTGGGATGACATCGCTGCTGAATGCGAAAGGTT  140
   141  CCTTGGACCCCGAGGATTCGGTGGTATTCAGGTTTCGCCACCAAACGAGAATTTGGTAATCTGGTCCCGC  210
   211  AACCGTCCTTGGTGGGAGCGCTATCAACCAATCTCCTACCGTCTAGTAACAAGATCTGGAAATGAAAATC  280
   281  AATTTTCGAATATGGTGCGTCGCTGCAACAATATTGGCGTCAGGATTTATGTGGACGCCATCATCAACCA  350
   351  CATGACTGGAACTTGGAATGAGAATGTTGGTACAGGTGGAAGCACAGCCAATTTCGAAAACTGGCACTAT  420
   421  CCTGCTGTTCCTTACGGCAGGAATGACTTCAACTGGCCTCATTGTGTCATTACTGGCAACGACTACAACT  490
   491  GCTGCCCTGACAGAGTGCGCAACTGTGAATTGTCTGGTCTGAAAGACTTGAACCAAGGCTCTGATTATGT  560
   561  TAGACAGCAGATTCTAAATTATATGAACCGTCTTATTGACATGGGTGTTGCTGGTTTCAGAATTGATGCT  630
   631  GCAAAGCACATGTGGCCTCACGATTTGCGAGTGATCTACGATCGTCTCCGCAATTTAAATACCGCCCACG  700
   701  GTTTCCCATCCGGTGCTCGTCCCTACATCTACCAAGAAGTCATCGACCTCGGTGGTGAAGCTATCAGTCG  770
   771  CAACGAATACACTCCACTTGCTGCCGTTACTGAGTTCAGATTTGGACTGGAACTCAGTCAAGCTTTCCAA  840
   841  AGAAGAAACCAGCTTAGATGGTTGGTTAACTGGGGTCCACAGTGGGGCCTTCTCGCTTCTGGAGACGCTT  910
   911  TGACTTTCATTGACAACCACGACAACCAGAGAGGTCATGGCGCTGGTGGAAACATCTTGACATACAAGCA  980
   981  ATCCCGACAATACAAGGGCGCTATCGCTTTCATGTTGGCACATCCTTATGGCTACCCTCAATTGATGAGT 1050
  1051  AGTTTCGCCTTCACGGACACCGAAGCTGGACCTCCAATGAACAGCAGGGGTGATATCACCTCTCCTACTA 1120
  1121  TCAACGCTGATAATTCTTGCGGCAATGGTTGGATATGCGAGCACCGTTGGCGTCAGATCCACAACATGGT 1190
  1191  TGTCTTCAGGAACACCGCTGGTAACGGTGCCCTTACCAACTGGTGGGACAACGGTAGCAACCAGATTGCT 1260
  1261  TTCTGCCGCGGCAACCAGGCCTTCATTGCTTTCAACAACGATGCATGGGACATGGACCAGACTCTTCAGA 1330
  1331  CTTGTCTCCCCGCCGGAACATACTGCGATATTATTTCCGGCGCCAGGTCTGGTAACCGCTGCACTGGAAA 1400
  1401  ATCTATTGTAGTAGGCAGCGATGGTCGCGCTCGTATCATCCACCGCAGCAACGAGTATGACATGATGGTT 1470
  1471  GCTATCCATAGGGGTGCTGAC                                                  1491 3′
```

Figure 21 The 2nd experiment of *α-amylase* cDNA from Nanglai strain

```
5′   1  ATGTTTCGGTACATCCTTCTACTTTCGGCCGTGACTCTGGCGTTGGCCTATAAAAACCCTCACTATGCAT   70
    71  CGGGTCGCACCACCATGGTACATCTCTTCGAATGGAAGTGGGATGACATCGCTGCTGAATGCGAAAGGTT  140
   141  CCTTGGACCCCGAGGATTCGGTGGTATTCAGGTTTCGCCACCAAACGAGAATTTGGTAATCTGGTCCCGC  210
   211  AACCGTCCTTGGTGGGAGCGCTATCAACCAATCTCCTACCGTCTAGTAACAAGATCTGGAAATGAAAATC  280
   281  AATTTTCGAATATGGTGCGTCGCTGCAACAATGTTGGCGTCAGGATTTATGTGGACGCCATCATCAACCA  350
   351  CATGACTGGAACTTGGAATGAGAATGTTGGTACAGGTGGAAGCACAGCCAATTTCGGAAACTGGCACTAT  420
   421  CCTGCTGTTCCTTACGGCAGGAATGACTTCAACTGGCCTCATTGTGTCATTACTGGCAACGACTACAACT  490
   491  GCTGCCCTGACAGAGTGCGCAACTGTGAATTGTCTGGTCTGAAAGACTTGAACCAAGGCTCTGATTATGT  560
   561  TAGACAGCAGATTCTAAATTATATGAACCGTCTTATTGACATGGGTGTTGCTGGTTTCAGAATTGATGCT  630
   631  GCAAAGCACATGTGGCCTCACGATTTGCGAGTGATCTACGATCGTCTCCGCAATTTAAATACCGCCCACG  700
   701  GTTTCCCATCCGGTGCTCGTCCCTACATCTACCAAGAAGTCATCGACCTCGGTGGTGAAGCTATCAGTCG  770
   771  CAACGAATACACTCCACTTGCTGCCGTTACTGAGTTCAGATTTGGACTGGAACTCAGTCAAGCTTTCCAA  840
   841  AGAAGAAACCAGCTTAGATGGTTGGTTAACTGGGGTCCACAGTGGGGCCTTCTCGCTTCTGGAGACGCTT  910
   911  TGACTTTCATTGACAACCACGACAACCAGAGAGGTCATGGCGCTGGTGGAAACATCTTGACATACAAGCA  980
   981  ATCCCGACAATACAAGGGCGCTATCGCTTTCATGTTGGCACATCCTTATGGCTACCCTCAATTGATGAGT 1050
  1051  AGTTTCGCCTTCACGGACACCGAAGCTGGACCTCCAATGAACAGCAGGGGTGATATCACCTCTCCTACTA 1120
  1121  TCAACGCTGATAATTCTTGCGGCAATGGTTGGATATGCGAGCACCGTTGGCGTCAGATCCACAACATGGT 1190
  1191  TGTCTTCAGGAACACCGCTGGTAACGGTGCCCTTACCAACTGGTGGGACAACGGTAGCAACCAGATTGCT 1260
  1261  TTCTGCCGCGGCAACCAGGCCTTCATTGCTTTCAACAACGATGCATGGGACATGGACCAGACTCTTCAGA 1330
  1331  CTTGTCTCCCCGCCGGAACATACTGCGATATTATTTCCGGCGCCAGGTCTGGTAACCGCTGCACTGGAAA 1400
  1401  ATCTATTGTAGTAGGCAGCGATGGTCGCGCTCGTATCATCCACCGCAGCAACGAGTATGACATGATGGTT 1470
  1471  GCTATCCATAGGGGTGCTGAC                                                  1491 3′
```

Figure 22 The *α-amylase* cDNA sequence from w1-pnd strain

3. Cloning and sequencing of the 3' untranslated of *α-amylase* cDNAs using 3'-Rapid Amplification of cDNA End (3'-RACE)

Since the sequence of *α-amylase* gene had no stop codon, the 3'-RACE method was performed to find the stop codon.

3.1 Total RNA isolation from salivary gland of Nanglai strain

Total RNA was isolated from head of the mid 5th instar larva of Nanglai strain by using TRIzol reagent. The isolated total RNA had an A 260/A280 ratio of 1.63 and 2.14 suggesting that the RNA had a little protein contamination. The RNA yields were 5,220 and 7,980 ng/µl. One microgram of total RNA was used as a template for synthesized the 1st strand DNA using RT-PCR by Improm II™ Reverse Transcriptase System. The 1st strand was used as a template for synthesized the 2nd strand DNA by using the Marathon cDNA Amplification Kit. The 2nd strand DNA was run on 1% agarose gel and the smear of them were observed (Figure 23).

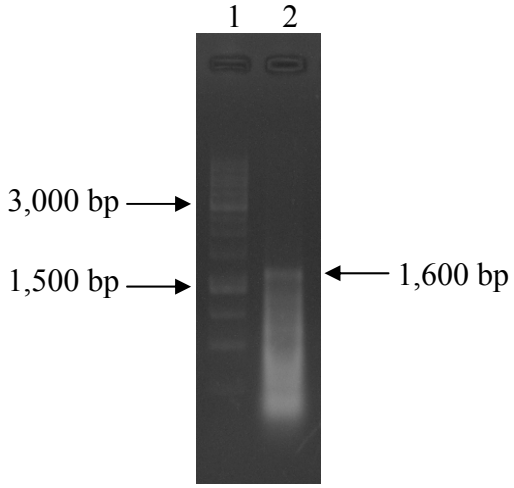


Figure 23 The smear of the 2nd strand DNA. Lane 1: DNA marker 1 kb, Lane 2: The smear of the 2nd strand DNA.

The 2nd strand DNA was used as a template for creation of blunt ends with a T4 DNA polymerase, a cDNA adaptor provided within the kit was ligated to both ends of the cDNA by a T4 DNA ligase. The adaptor-ligated cDNA library was used as a template for 3'-RACE reaction. The primer GSP and AP1 primer were used to amplify an adaptor-ligated double strand cDNA yielding the 3'-end of *α-amylase* cDNA sequence. A 1.5% agarose gel electrophoresis was performed in order to visualize bands of the PCR products. The length of amplified products of 3'-RACE1 was approximately 1,500 bp (Figure 24). The 3'-RACE product was purified from

agarose gel and cloned into pGEM-T® easy vectors, transformed into *E. coli* JM109 by heat shock transformation and sequenced.

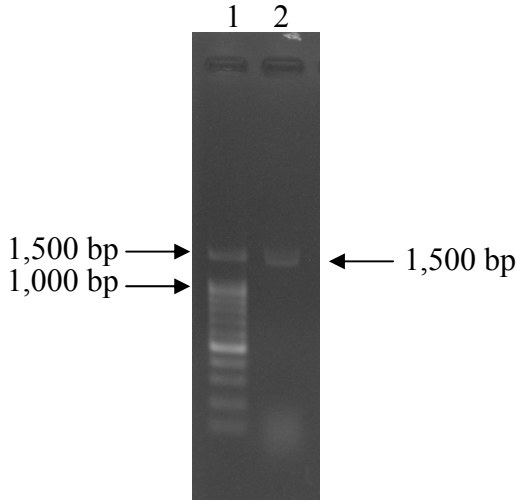


Figure 24 Agarose gel electrophoresis of 3'-RACE PCR products of *α-amylase* cDNA. Lane 1: 100 bp + 1.5 kb DNA ladder, Lane 2: The 1,500 bp PCR product from 3'-RACE reaction of *α-amylase* coda

3.2 Detection of DNA insertion by PCR amplification

Six white colonies were randomly selected to detect the insertion of 3'-RACE DNA product using PCR method. In the PCR method, forward primer (AmyF) and reverse primer (AmyR) were used to amplify the expected 1,500 bp DNA fragment (Figure 25).

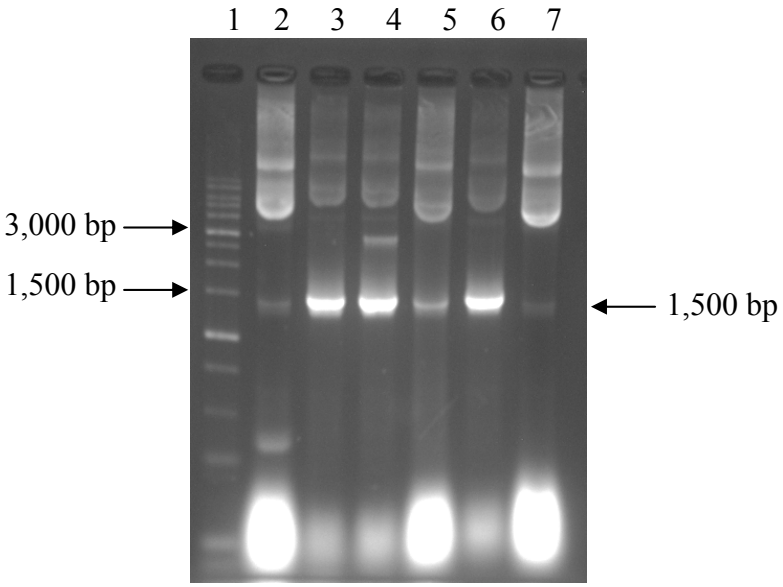


Figure 25 Gel electrophoresis patterns of full-length *α-amylase* cDNA inserted clones. Six clones were selected to examine the size of inserted cDNA by PCR amplification with AmyF and AmyR primers. Lane 1: 1 kb DNA ladder, Lane 2-7: inserted cDNA from six clones.

3.3 Sequence analysis of 3'-RACE clones

The inserting DNA of those six colonies, namely AMYT-1, AMYT-2, AMYT-3, AMYT-4, AMYT-5 and AMYT-6 were sequenced. Nucleotide sequences and deduced amino acid sequences of six clones were shown in Figure 26 to Figure 31.

Nucleotide sequences excluding a poly(A) tail of AMYT-1, AMYT-2, AMYT-3, AMYT-4, AMYT-5 and AMYT-6 were 1603 bp, 1603 bp, 1695 bp, 1694 bp, 1694 bp and 1599 bp respectively. In these six clones, their ORF were equal in size which was 1,503 bp long. All the start and stop codon of these six ORF clones were ATG and TAA, respectively. The nucleotide sequence and deduce amino acid sequence at some position were obtained (Figure 32 and Figure 33, respectively). They shared 99-100% sequence homology (Table 1 and Table 2, respectively).

The AMY-1 sequence was used for further alignment. Alignment of the nucleotide sequence of AMY-1 showed 97% and 99 % sequence homology to ORF and mRNA of *Bombyx mori* truncated *alpha-amylase* (*amy*) gene (Genbank Accession No.U07847), respectively (Figure 34). Compared with nucleotide sequence of ORF of *Bombyx mori* truncated *alpha-amylase* (*amy*) gene (Genbank Accession No. U07847), the ORF of *α-amylase* gene of Nanglai strain had three extra regions, at a position 324-620 (297 bp in length), position 1,014-1,023 (10 bp in length) and position 1,046-1,503 (99 bp in length) respectively.

The deduce amino acid from the full-length cDNA of AMYT-1 ORF consisted of 500 residues and showed 99 % sequence homology to *Bombyx mori* truncated *alpha-amylase* (*amy*) gene (Genbank Accession No.U07847) (Figure 35). However, the deduce amino acid of AMYT-1 ORF had the extra sequences at a position 108-206 (98 residues in length) and the deduce amino acid from the 239 position to the end of sequence were different from that of *Bombyx mori* truncated *alpha-amylase* (*amy*) gene (Genbank Accession No. U07847). When compare the deduced amino acid sequence of AMYT-1 with other insects, *Ostrinia nubilalis* (AAA03715.1), *Diatraea saccharalis* (AAP92665.1) , *Spodoptera frugiperda* (AF280891_1) and *Ceratitis capitata* (AAO13691.1), the alignment showed 78%, 79%, 81% and 60% sequence homology, respectively (Figure 36 and Table 3).

```
5′ 1   atgtttcggtacatccttctactttcggccgtgactctggcgttggcctataaaaaccct   60
        M  F  R  Y  I  L  L  L  S  A  V  T  L  A  L  A  Y  K  N  P
  61   cactatgcatcgggtcgcaccaccatggtacatctcttcgaatggaagtgggatgacatc   120
        H  Y  A  S  G  R  T  T  M  V  H  L  F  E  W  K  W  D  D  I
 121   gctgctgaatgcgaaaggttccttggaccccgaggattcggtggtattcaggtttcgcca   180
        A  A  E  C  E  R  F  L  G  P  R  G  F  G  G  I  Q  V  S  P
 181   ccaaacgagaatttggtaatctggtcccgcaaccgtccttggtgggagcgctatcaacca   240
        P  N  E  N  L  V  I  W  S  R  N  R  P  W  W  E  R  Y  Q  P
 241   atctcctaccgtctagtaacaagatctggaaatgaaaatcaattttcgaatatggtgcgt   300
        I  S  Y  R  L  V  T  R  S  G  N  E  N  Q  F  S  N  M  V  R
 301   cgctgcaacaatgttggcgtcaggatttatgtggacgccatcatcaaccacatgactgga   360
        R  C  N  N  V  G  V  R  I  Y  V  D  A  I  I  N  H  M  T  G
 361   acttggaatgagaatgttggtacaggtggaagcacggccaatttcgaaaactggcactat   420
        T  W  N  E  N  V  G  T  G  G  S  T  A  N  F  E  N  W  H  Y
 421   cctgctgttccttacggcaggaatgacttcaactggcctcattgtgtcattactggcagc   480
        P  A  V  P  Y  G  R  N  D  F  N  W  P  H  C  V  I  T  G  S
 481   gactacaactgctgccctgacagagtgcgcaactgtgaattgtctggtctgaaagacttg   540
        D  Y  N  C  C  P  D  R  V  R  N  C  E  L  S  G  L  K  D  L
 541   aaccaaggctctgattatgttagacagcagattctaaattatatgaaccgtcttattgac   600
        N  Q  G  S  D  Y  V  R  Q  Q  I  L  N  Y  M  N  R  L  I  D
 601   atgggtgttgctggtttcagaattgatgctgcaaagcacatgtggcctcacgatttgcga   660
        M  G  V  A  G  F  R  I  D  A  A  K  H  M  W  P  H  D  L  R
 661   gtgatctacgatcgtctccgcaatttaaataccgcccacggtttcccatccggtgctcgt   720
        V  I  Y  D  R  L  R  N  L  N  T  A  H  G  F  P  S  G  A  R
 721   ccctacatctaccaagaagtcatcgacctcggtggtgaagctatcagtcgcaacgaatac   780
        P  Y  I  Y  Q  E  V  I  D  L  G  G  E  A  I  S  R  N  E  Y
 781   actccacttgctgccgttactgagttcagatttggactggaactcagtcaagctttccaa   840
        T  P  L  A  A  V  T  E  F  R  F  G  L  E  L  S  Q  A  F  Q
 841   agaagaaaccagcttagatggttggttaactggggtccacagtggggccttctcgcttct   900
        R  R  N  Q  L  R  W  L  V  N  W  G  P  Q  W  G  L  L  A  S
 901   ggagacgctttgactttcattgacaaccacgacaaccagagaggtcatggcgctggtgga   960
        G  D  A  L  T  F  I  D  N  H  D  N  Q  R  G  H  G  A  G  G
 961   aacatcttgacatacaagcaatcccgacaatacaagggcgctatcgctttcatgttggca   1020
        N  I  L  T  Y  K  Q  S  R  Q  Y  K  G  A  I  A  F  M  L  A
1021   catccttatggctaccctcaattgatgagtagtttcgccttcacggacaccgaagctgga   1080
        H  P  Y  G  Y  P  Q  L  M  S  S  F  A  F  T  D  T  E  A  G
1081   cctccaatgaacagcaggggtgatatcacctctcctactatcaacgctgataattcttgc   1140
        P  P  M  N  S  R  G  D  I  T  S  P  T  I  N  A  D  N  S  C
1141   ggcaatggttggatatgcgagcaccgttggcgtcagatccacaacatggttgtcttcagg   1200
        G  N  G  W  I  C  E  H  R  W  R  Q  I  H  N  M  V  V  F  R
1201   aacaccgctggtaacggtgcccttaccaactggtgggacaacggtagcaaccagattgct   1260
        N  T  A  G  N  G  A  L  T  N  W  W  D  N  G  S  N  Q  I  A
1261   ttctgccgcggcaaccaggccttcattgctttcaacaacgatgcatgggacatggaccag   1320
        F  C  R  G  N  Q  A  F  I  A  F  N  N  D  A  W  D  M  D  Q
1321   actcttcagacttgtctccccgccggaacatactgcgatattatttccggcgccaggtct   1380
        T  L  Q  T  C  L  P  A  G  T  Y  C  D  I  I  S  G  A  R  S
1381   ggtaaccgctgcactggaaaatctattgtagtaggcagcgatggtcgcgctcttatcatc   1440
        G  N  R  C  T  G  K  S  I  V  V  G  S  D  G  R  A  L  I  I
1441   caccgcagcaacgagtatgacatgatggttgctatccataggggtgctgactcgagattg   1500
        H  R  S  N  E  Y  D  M  M  V  A  I  H  R  G  A  D  S  R  L
1501   taaaacgggaaccgtactggcaaatattttgaagactggaaatctgaaaattgcaaatat   1560
        -
1561   taattgtgaaatacatttatgtctttcttaaaaaaaaaaaaaa                   1603  3′
```

Figure 26 Nucleotide and deduced amino acid sequence of AMYT-1. Initial codon ATG and stop codon TAA are in bold and underline.

```
5′   1  atgtttcggtacatccttctactttcggccgtgactctggcgttggcctataaaaaccct   60
         M  F  R  Y  I  L  L  L  S  A  V  T  L  A  L  A  Y  K  N  P
    61  cactatgcatcgggtcgcaccaccatggtacatctcttcgaatggaagtgggatgacatc  120
         H  Y  A  S  G  R  T  T  M  V  H  L  F  E  W  K  W  D  D  I
   121  gctgctgaatgcgaaaggttccttggaccccgaggattcggtggtattcaggtttcgcca  180
         A  A  E  C  E  R  F  L  G  P  R  G  F  G  G  I  Q  V  S  P
   181  ccaaacgagaatttggtaatctggtcccgcaaccgtccttggtgggagcgctatcaacca  240
         P  N  E  N  L  V  I  W  S  R  N  R  P  W  W  E  R  Y  Q  P
   241  atctcctaccgtctagtaacaagatctggaaatgaaaatcaattttcgaatatggtgcgt  300
         I  S  Y  R  L  V  T  R  S  G  N  E  N  Q  F  S  N  M  V  R
   301  cgctgcaacaatgttggcgtcaggatttatgtggacgccatcatcaaccacatgactgga  360
         R  C  N  N  V  G  V  R  I  Y  V  D  A  I  I  N  H  M  T  G
   361  acttggaatgagaatgttggtacaggtggaagcacggccaatttcgaaaactggcactat  420
         T  W  N  E  N  V  G  T  G  G  S  T  A  N  F  E  N  W  H  Y
   421  cctgctgttccttacggcaggaatgacttcaactggcctcattgtgtcattactggcagc  480
         P  A  V  P  Y  G  R  N  D  F  N  W  P  H  C  V  I  T  G  S
   481  gactacaactgctgccctgacagagtgcgcaactgtgaattgtctggtctgaaagacttg  540
         D  Y  N  C  C  P  D  R  V  R  N  C  E  L  S  G  L  K  D  L
   541  aaccaaggctctgattatgttagacagcagattctaaattatatgaaccgtcttattgac  600
         N  Q  G  S  D  Y  V  R  Q  Q  I  L  N  Y  M  N  R  L  I  D
   601  atgggtgttgctggtttcagaattgatgctgcaaagcacatgtggcctcacgatttgcga  660
         M  G  V  A  G  F  R  I  D  A  A  K  H  M  W  P  H  D  L  R
   661  gtgatctacgatcgtctccgcaatttaaataccgcccacggtttcccatccggtgctcgt  720
         V  I  Y  D  R  L  R  N  L  N  T  A  H  G  F  P  S  G  A  R
   721  ccctacatctaccaagaagtcatcgacctcggtggtgaagctatcagtcgcaacgaatac  780
         P  Y  I  Y  Q  E  V  I  D  L  G  G  E  A  I  S  R  N  E  Y
   781  actccacttgctgccgttactgagttcagatttggactggaactcagtcaagctttccaa  840
         T  P  L  A  A  V  T  E  F  R  F  G  L  E  L  S  Q  A  F  Q
   841  agaagaaaccagcttagatggttggttaactggggtccacagtggggccttctcgcttct  900
         R  R  N  Q  L  R  W  L  V  N  W  G  P  Q  W  G  L  L  A  S
   901  ggagacgctttgactttcattgacaaccacgacaaccagagaggtcatggcgctggtgga  960
         G  D  A  L  T  F  I  D  N  H  D  N  Q  R  G  H  G  A  G  G
   961  aacatcttgacatacaagcaatcccgacaatacaagggcgctatcgctttcatgttggca  1020
         N  I  L  T  Y  K  Q  S  R  Q  Y  K  G  A  I  A  F  M  L  A
  1021  catccttatggctaccctcaattgatgagtagtttcgccttcacggacaccgaagctgga  1080
         H  P  Y  G  Y  P  Q  L  M  S  S  F  A  F  T  D  T  E  A  G
  1081  cctccaatgaacagcaggggtgatatcacctctcctactatcaacgctgataattcttgc  1140
         P  P  M  N  S  R  G  D  I  T  S  P  T  I  N  A  D  N  S  C
  1141  ggcaatggttggatatgcgagcaccgttggcgtcagatccacaacatggttgtcttcagg  1200
         G  N  G  W  I  C  E  H  R  W  R  Q  I  H  N  M  V  V  F  R
  1201  aacaccgctggtaacggtgcccttaccaactggtgggacaacggtagcaaccagattgct  1260
         N  T  A  G  N  G  A  L  T  N  W  W  D  N  G  S  N  Q  I  A
  1261  ttctgccgcggcaaccaggccttcattgctttcaacaacgatgcatgggacatggaccag  1320
         F  C  R  G  N  Q  A  F  I  A  F  N  N  D  A  W  D  M  D  Q
  1321  actcttcagacttgtctccccgccggaacatactgcgatattatttccggcgccaggtct  1380
         T  L  Q  T  C  L  P  A  G  T  Y  C  D  I  I  S  G  A  R  S
  1381  ggtaaccgctgcactggaaaatctattgtagtaggcagcgatggtcgcgctcttatcatc  1440
         G  N  R  C  T  G  K  S  I  V  V  G  S  D  G  R  A  L  I  I
  1441  caccgcagcaacgagtatgacatgatggttgctatccatagggtgctgactcgagattg  1500
         H  R  S  N  E  Y  D  M  M  V  A  I  H  R  G  A  D  S  R  L
  1501  taaaacgggaaccgtactggcaaatattttgaagactggaaatctgaaaattgcaaatat  1560
        -
  1561  taattgtgaaatacatttatgtctttcttaaaaaaaaaaaaaa                  1603 3′
```

Figure 37 Nucleotide and deduced amino acid sequence of AMYT-2. Initial codon ATG and stop codon TAA are in bold and underline.

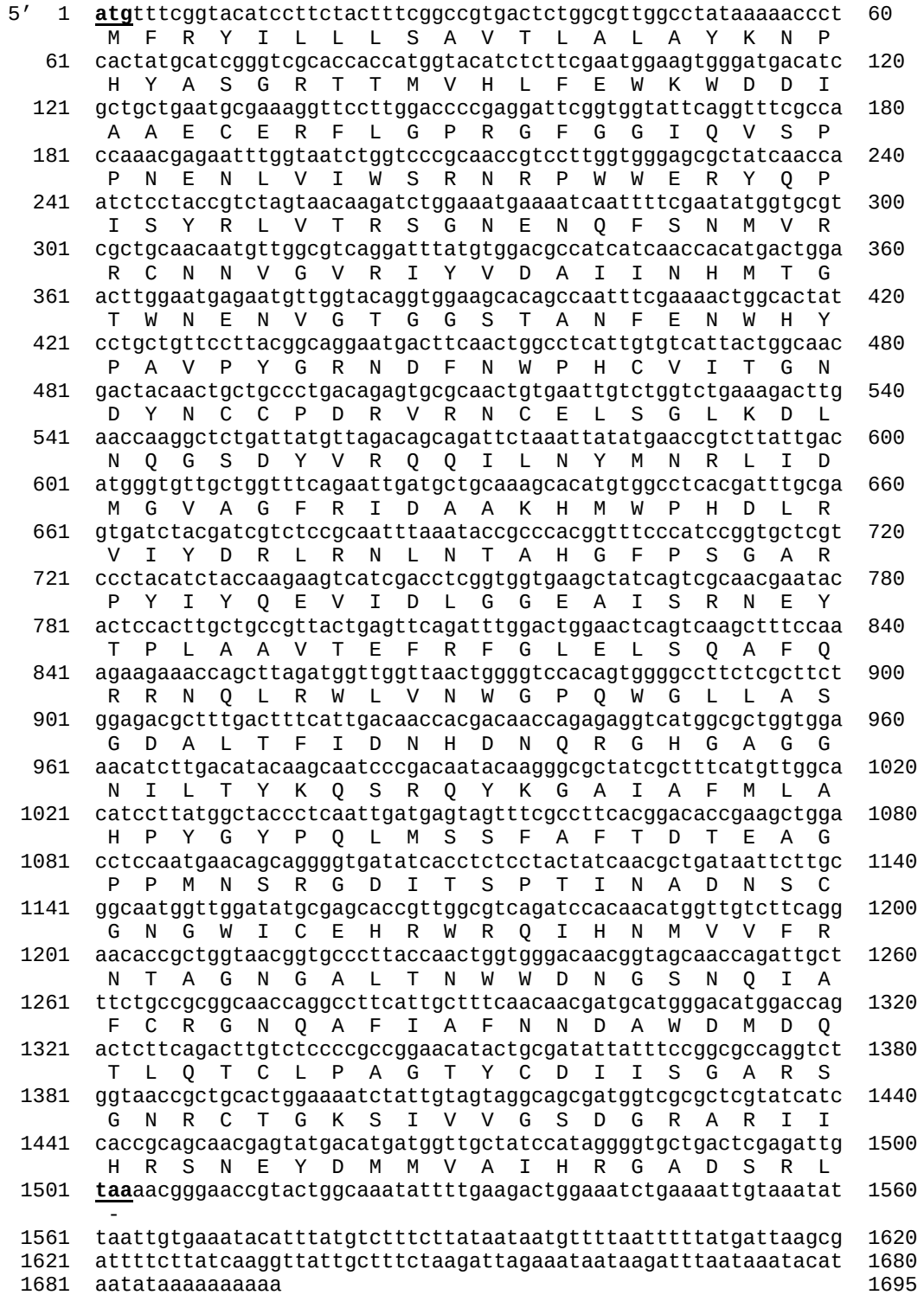


Figure 28 Nucleotide and deduced amino acid sequence of AMYT-3. Initial codon ATG and stop codon TAA are in bold and italics.

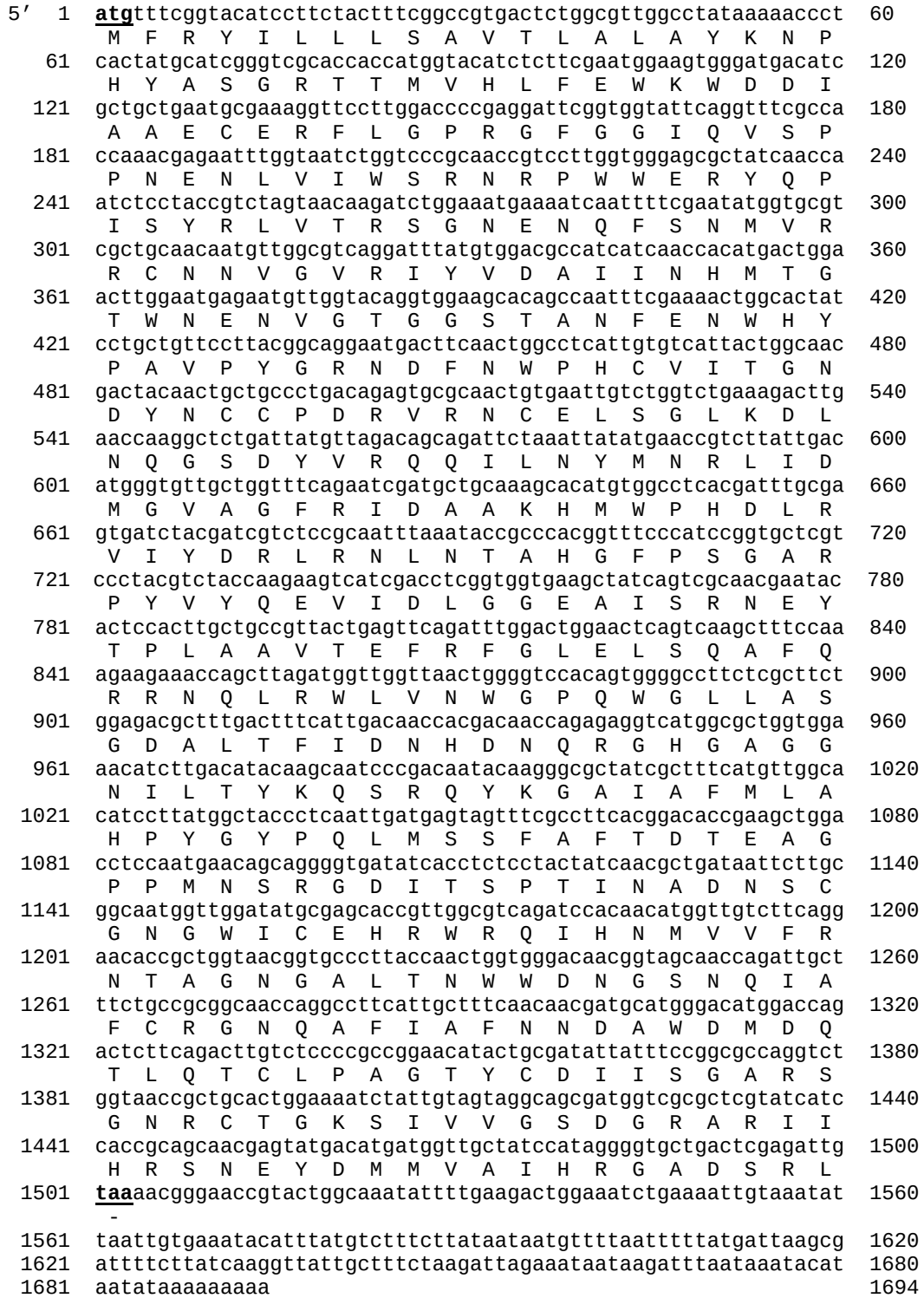


Figure 29 Nucleotide and deduced amino acid sequence of AMYT-4. Initial codon ATG and stop codon TAA are in bold and underline.

```
5′   1  atgtttcggtacatccttctactttcggccgtgactctggcgttggcctataaaaaccct  60
         M  F  R  Y  I  L  L  L  S  A  V  T  L  A  L  A  Y  K  N  P
    61  cactatgcatcgggtcgcaccaccatggtacatctcttcgaatggaagtgggatgacatc  120
         H  Y  A  S  G  R  T  T  M  V  H  L  F  E  W  K  W  D  D  I
   121  gctgctgaatgcgaaaggttccttggaccccgaggattcggtggtattcaggtttcgcca  180
         A  A  E  C  E  R  F  L  G  P  R  G  F  G  G  I  Q  V  S  P
   181  ccaaacgagaatttggtaatctggtcccgcaaccgtccttggtgggagcgctatcaacca  240
         P  N  E  N  L  V  I  W  S  R  N  R  P  W  W  E  R  Y  Q  P
   241  atctcctaccgtctagtaacaagatctggaaatgaaaatcaattttcgaatatggtgcgt  300
         I  S  Y  R  L  V  T  R  S  G  N  E  N  Q  F  S  N  M  V  R
   301  cgctgcaacaatgttggcgtcaggatttatgtggacgccatcatcaaccacatgactgga  360
         R  C  N  N  V  G  V  R  I  Y  V  D  A  I  I  N  H  M  T  G
   361  acttggaatgagaatgttggtacaggtggaagcacagccaatttcgaaaactggcactat  420
         T  W  N  E  N  V  G  T  G  G  S  T  A  N  F  E  N  W  H  Y
   421  cctgctgttccttacggcaggaatgacttcaactggcctcattgtgtcattactggcaac  480
         P  A  V  P  Y  G  R  N  D  F  N  W  P  H  C  V  I  T  G  N
   481  gactacaactgctgccctgacagagtgcgcaactgtgaattgtctggtctgaaagacttg  540
         D  Y  N  C  C  P  D  R  V  R  N  C  E  L  S  G  L  K  D  L
   541  aaccaaggctctgattatgttagacagcagattctaaattatatgaaccgtcttattgac  600
         N  Q  G  S  D  Y  V  R  Q  Q  I  L  N  Y  M  N  R  L  I  D
   601  atgggtgttgctggtttcagaatcgatgctgcaaagcacatgtggcctcacgatttgcga  660
         M  G  V  A  G  F  R  I  D  A  A  K  H  M  W  P  H  D  L  R
   661  gtgatctacgatcgtctccgcaatttaaataccgcccacggtttcccatccggtgctcgt  720
         V  I  Y  D  R  L  R  N  L  N  T  A  H  G  F  P  S  G  A  R
   721  ccctacgtctaccaagaagtcatcgacctcggtggtgaagctatcagtcgcaacgaatac  780
         P  Y  V  Y  Q  E  V  I  D  L  G  G  E  A  I  S  R  N  E  Y
   781  actccacttgctgccgttactgagttcagatttggactggaactcagtcaagctttccaa  840
         T  P  L  A  A  V  T  E  F  R  F  G  L  E  L  S  Q  A  F  Q
   841  agaagaaaccagcttagatggttggttaactggggtccacagtggggccttctcgcttct  900
         R  R  N  Q  L  R  W  L  V  N  W  G  P  Q  W  G  L  L  A  S
   901  ggagacgctttgactttcattgacaaccacgacaaccagagaggtcatggcgctggtgga  960
         G  D  A  L  T  F  I  D  N  H  D  N  Q  R  G  H  G  A  G  G
   961  aacatcttgacatacaagcaatcccgacaatacaagggcgctatcgctttcatgttggca  1020
         N  I  L  T  Y  K  Q  S  R  Q  Y  K  G  A  I  A  F  M  L  A
  1021  catccttatggctaccctcaattgatgagtagtttcgccttcacggacaccgaagctgga  1080
         H  P  Y  G  Y  P  Q  L  M  S  S  F  A  F  T  D  T  E  A  G
  1081  cctccaatgaacagcaggggtgatatcacctctcctactatcaacgctgataattcttgc  1140
         P  P  M  N  S  R  G  D  I  T  S  P  T  I  N  A  D  N  S  C
  1141  ggcaatggttggatatgcgagcaccgttggcgtcagatccacaacatggttgtcttcagg  1200
         G  N  G  W  I  C  E  H  R  W  R  Q  I  H  N  M  V  V  F  R
  1201  aacaccgctggtaacggtgcccttaccaactggtgggacaacggtagcaaccagattgct  1260
         N  T  A  G  N  G  A  L  T  N  W  W  D  N  G  S  N  Q  I  A
  1261  ttctgccgcggcaaccaggccttcattgctttcaacaacgatgcatgggacatggaccag  1320
         F  C  R  G  N  Q  A  F  I  A  F  N  N  D  A  W  D  M  D  Q
  1321  actcttcagacttgtctccccgccggaacatactgcgatattatttccggcgccaggtct  1380
         T  L  Q  T  C  L  P  A  G  T  Y  C  D  I  I  S  G  A  R  S
  1381  ggtaaccgctgcactggaaaatctattgtagtaggcagcgatggtcgcgctcgtatcatc  1440
         G  N  R  C  T  G  K  S  I  V  V  G  S  D  G  R  A  R  I  I
  1441  caccgcagcaacgagtatgacatgatggttgctatccataggggtgctgactcgagattg  1500
         H  R  S  N  E  Y  D  M  M  V  A  I  H  R  G  A  D  S  R  L
  1501  taaaacgggaaccgtactggcaaatattttgaagactggaaatctgaaaattgtaaatat  1560
         -
  1561  taattgtgaaatacatttatgtctttcttataataatgttttaatttttatgattaagcg  1620
  1621  attttcttatcaaggttattgctttctaagattagaaataataagatttaataaatacat  1680
  1681  aatataaaaaaaaaa                                               1694
```

Figure 30 Nucleotide and deduced amino acid sequence of AMYT-5. Initial codon ATG and stop codon TAA are in bold and underline.

```
5′   1  atgtttcggtacatccttctactttcggccgtgactctggcgttggcctataaaaaccct  60
         M  F  R  Y  I  L  L  L  S  A  V  T  L  A  L  A  Y  K  N  P
    61  cactatgcatcgggtcgcaccaccatggtacatctcttcgaatggaagtgggatgacatc  120
         H  Y  A  S  G  R  T  T  M  V  H  L  F  E  W  K  W  D  D  I
   121  gctgctgaatgcgaaaggttccttggaccccgaggattcggtggtattcaggtttcgcca  180
         A  A  E  C  E  R  F  L  G  P  R  G  F  G  G  I  Q  V  S  P
   181  ccaaacgagaatttggtaatctggtcccgcaaccgtccttggtgggagcgctatcaacca  240
         P  N  E  N  L  V  I  W  S  R  N  R  P  W  W  E  R  Y  Q  P
   241  atctcctaccgtctagtaacaagatctggaaatgaaaatcaattttcgaatatggtgcgt  300
         I  S  Y  R  L  V  T  R  S  G  N  E  N  Q  F  S  N  M  V  R
   301  cgctgcaacaatgttggcgtcaggatttatgtggacgccatcatcaaccacatgactgga  360
         R  C  N  N  V  G  V  R  I  Y  V  D  A  I  I  N  H  M  T  G
   361  acttggaatgagaatgttggtacaggtggaagcacagccaatttcgaaaacaggcactat  420
         T  W  N  E  N  V  G  T  G  G  S  T  A  N  F  E  N  R  H  Y
   421  cctgctgttccttacggcaggaatgacttcaactggcctcattgtgtcattactggcaac  480
         P  A  V  P  Y  G  R  N  D  F  N  W  P  H  C  V  I  T  G  N
   481  gactacaactgctgccctgacagagtgcgcaactgtgaattgtctggtctgaaagacttg  540
         D  Y  N  C  C  P  D  R  V  R  N  C  E  L  S  G  L  K  D  L
   541  aaccaaggctctgattatgttagacagcagattctaaattatatgaaccgtcttattgac  600
         N  Q  G  S  D  Y  V  R  Q  Q  I  L  N  Y  M  N  R  L  I  D
   601  atgggtgttgctggtttcagaattgatgctgcaaagcacatgtggcctcacgatttgcga  660
         M  G  V  A  G  F  R  I  D  A  A  K  H  M  W  P  H  D  L  R
   661  gtgatctacgatcgtctccgcaatttaaataccgcccacggtttcccatccggtgctcgt  720
         V  I  Y  D  R  L  R  N  L  N  T  A  H  G  F  P  S  G  A  R
   721  ccctacatctaccaagaagtcatcgacctcggtggtgaagctatcagtcgcaacgaatac  780
         P  Y  I  Y  Q  E  V  I  D  L  G  G  E  A  I  S  R  N  E  Y
   781  actccacttgctgccgttactgagttcagatttggactggaactcagtcaagctttccaa  840
         T  P  L  A  A  V  T  E  F  R  F  G  L  E  L  S  Q  A  F  Q
   841  agaagaaaccagcttagatggttggttaactggggtccacagtggggccttctcgcttct  900
         R  R  N  Q  L  R  W  L  V  N  W  G  P  Q  W  G  L  L  A  S
   901  ggagacgctttgactttcattgacaaccacgacaaccagagaggtcatggcgctggtgga  960
         G  D  A  L  T  F  I  D  N  H  D  N  Q  R  G  H  G  A  G  G
   961  aacatcttgacatacaagcaatcccgacaatacaagggcgctatcgctttcatgttggca  1020
         N  I  L  T  Y  K  Q  S  R  Q  Y  K  G  A  I  A  F  M  L  A
  1021  catccttatggctaccctcaattgatgagtagtttcgccttcacggacaccgaagctgga  1080
         H  P  Y  G  Y  P  Q  L  M  S  S  F  A  F  T  D  T  E  A  G
  1081  cctccaatgaacagcaggggtgatatcacctctcctactatcaacgctgataattcttgc  1140
         P  P  M  N  S  R  G  D  I  T  S  P  T  I  N  A  D  N  S  C
  1141  ggcaatggttggatatgcgagcaccgttggcgtcagatccacaacatggttgtcttcagg  1200
         G  N  G  W  I  C  E  H  R  W  R  Q  I  H  N  M  V  V  F  R
  1201  aacaccgctggtaacggtgcccttaccaactggtgggacaacggtagcaaccagattgct  1260
         N  T  A  G  N  G  A  L  T  N  W  W  D  N  G  S  N  Q  I  A
  1261  ttctgccgcggcaaccaggccttcattgctttcaacaacgatgcatgggacatggaccag  1320
         F  C  R  G  N  Q  A  F  I  A  F  N  N  D  A  W  D  M  D  Q
  1321  actcttcagacttgtctccccgccggaacatactgcgatattatttccggcgccaggtct  1380
         T  L  Q  T  C  L  P  A  G  T  Y  C  D  I  I  S  G  A  R  S
  1381  ggtaaccgctgcactggaaaatctattgtagtaggcagcgatggtcgcgctcgtatcatc  1440
         G  N  R  C  T  G  K  S  I  V  V  G  S  D  G  R  A  R  I  I
  1441  caccgcaacaacgagtatgacatgatggttgctatccataggggtgctgactcgagattg  1500
         H  R  N  N  E  Y  D  M  M  V  A  I  H  R  G  A  D  S  R  L
  1501  taaaacgggaaccgtactggcaaatattttgaagactggaaatctgaaaattgtatatat  1560
        -
  1561  taattgtgaaatacatttatgtctttcttaaaaaaaaaa                      1599
```

Figure 31 Nucleotide and deduced amino acid sequence of AMYT-6. Initial codon ATG and stop codon TAA are in bold and underline.

```
AMYT-4          ATGTTTCGGTACATCCTTCTACTTTCGGCCGTGACTCTGGCGTTGGCCTATAAAAACCCT 60
AMYT-5          ATGTTTCGGTACATCCTTCTACTTTCGGCCGTGACTCTGGCGTTGGCCTATAAAAACCCT 60
AMYT-3          ATGTTTCGGTACATCCTTCTACTTTCGGCCGTGACTCTGGCGTTGGCCTATAAAAACCCT 60
AMYT-6          ATGTTTCGGTACATCCTTCTACTTTCGGCCGTGACTCTGGCGTTGGCCTATAAAAACCCT 60
AMYT-2          ATGTTTCGGTACATCCTTCTACTTTCGGCCGTGACTCTGGCGTTGGCCTATAAAAACCCT 60
AMYT-1          ATGTTTCGGTACATCCTTCTACTTTCGGCCGTGACTCTGGCGTTGGCCTATAAAAACCCT 60
                ************************************************************

AMYT-4          CACTATGCATCGGGTCGCACCACCATGGTACATCTCTTCGAATGGAAGTGGGATGACATC 120
AMYT-5          CACTATGCATCGGGTCGCACCACCATGGTACATCTCTTCGAATGGAAGTGGGATGACATC 120
AMYT-3          CACTATGCATCGGGTCGCACCACCATGGTACATCTCTTCGAATGGAAGTGGGATGACATC 120
AMYT-6          CACTATGCATCGGGTCGCACCACCATGGTACATCTCTTCGAATGGAAGTGGGATGACATC 120
AMYT-2          CACTATGCATCGGGTCGCACCACCATGGTACATCTCTTCGAATGGAAGTGGGATGACATC 120
AMYT-1          CACTATGCATCGGGTCGCACCACCATGGTACATCTCTTCGAATGGAAGTGGGATGACATC 120
                ************************************************************

AMYT-4          GCTGCTGAATGCGAAAGGTTCCTTGGACCCCGAGGATTCGGTGGTATTCAGGTTTCGCCA 180
AMYT-5          GCTGCTGAATGCGAAAGGTTCCTTGGACCCCGAGGATTCGGTGGTATTCAGGTTTCGCCA 180
AMYT-3          GCTGCTGAATGCGAAAGGTTCCTTGGACCCCGAGGATTCGGTGGTATTCAGGTTTCGCCA 180
AMYT-6          GCTGCTGAATGCGAAAGGTTCCTTGGACCCCGAGGATTCGGTGGTATTCAGGTTTCGCCA 180
AMYT-2          GCTGCTGAATGCGAAAGGTTCCTTGGACCCCGAGGATTCGGTGGTATTCAGGTTTCGCCA 180
AMYT-1          GCTGCTGAATGCGAAAGGTTCCTTGGACCCCGAGGATTCGGTGGTATTCAGGTTTCGCCA 180
                ************************************************************

AMYT-4          CCAAACGAGAATTTGGTAATCTGGTCCCGCAACCGTCCTTGGTGGGAGCGCTATCAACCA 240
AMYT-5          CCAAACGAGAATTTGGTAATCTGGTCCCGCAACCGTCCTTGGTGGGAGCGCTATCAACCA 240
AMYT-3          CCAAACGAGAATTTGGTAATCTGGTCCCGCAACCGTCCTTGGTGGGAGCGCTATCAACCA 240
AMYT-6          CCAAACGAGAATTTGGTAATCTGGTCCCGCAACCGTCCTTGGTGGGAGCGCTATCAACCA 240
AMYT-2          CCAAACGAGAATTTGGTAATCTGGTCCCGCAACCGTCCTTGGTGGGAGCGCTATCAACCA 240
AMYT-1          CCAAACGAGAATTTGGTAATCTGGTCCCGCAACCGTCCTTGGTGGGAGCGCTATCAACCA 240
                ************************************************************

AMYT-4          ATCTCCTACCGTCTAGTAACAAGATCTGGAAATGAAAATCAATTTTCGAATATGGTGCGT 300
AMYT-5          ATCTCCTACCGTCTAGTAACAAGATCTGGAAATGAAAATCAATTTTCGAATATGGTGCGT 300
AMYT-3          ATCTCCTACCGTCTAGTAACAAGATCTGGAAATGAAAATCAATTTTCGAATATGGTGCGT 300
AMYT-6          ATCTCCTACCGTCTAGTAACAAGATCTGGAAATGAAAATCAATTTTCGAATATGGTGCGT 300
AMYT-2          ATCTCCTACCGTCTAGTAACAAGATCTGGAAATGAAAATCAATTTTCGAATATGGTGCGT 300
AMYT-1          ATCTCCTACCGTCTAGTAACAAGATCTGGAAATGAAAATCAATTTTCGAATATGGTGCGT 300
                ************************************************************

AMYT-4          CGCTGCAACAATGTTGGCGTCAGGATTTATGTGGACGCCATCATCAACCACATGACTGGA 360
AMYT-5          CGCTGCAACAATGTTGGCGTCAGGATTTATGTGGACGCCATCATCAACCACATGACTGGA 360
AMYT-3          CGCTGCAACAATGTTGGCGTCAGGATTTATGTGGACGCCATCATCAACCACATGACTGGA 360
AMYT-6          CGCTGCAACAATGTTGGCGTCAGGATTTATGTGGACGCCATCATCAACCACATGACTGGA 360
AMYT-2          CGCTGCAACAATGTTGGCGTCAGGATTTATGTGGACGCCATCATCAACCACATGACTGGA 360
AMYT-1          CGCTGCAACAATGTTGGCGTCAGGATTTATGTGGACGCCATCATCAACCACATGACTGGA 360
                ************************************************************

AMYT-4          ACTTGGAATGAGAATGTTGGTACAGGTGGAAGCACAGCCAATTTCGAAAACTGGCACTAT 420
AMYT-5          ACTTGGAATGAGAATGTTGGTACAGGTGGAAGCACAGCCAATTTCGAAAACTGGCACTAT 420
AMYT-3          ACTTGGAATGAGAATGTTGGTACAGGTGGAAGCACAGCCAATTTCGAAAACTGGCACTAT 420
AMYT-6          ACTTGGAATGAGAATGTTGGTACAGGTGGAAGCACAGCCAATTTCGAAAACAGGCACTAT 420
AMYT-2          ACTTGGAATGAGAATGTTGGTACAGGTGGAAGCACGGCCAATTTCGAAAACTGGCACTAT 420
AMYT-1          ACTTGGAATGAGAATGTTGGTACAGGTGGAAGCACGGCCAATTTCGAAAACTGGCACTAT 420
                ********************************** *************** ********

AMYT-4          CCTGCTGTTCCTTACGGCAGGAATGACTTCAACTGGCCTCATTGTGTCATTACTGGCAAC 480
AMYT-5          CCTGCTGTTCCTTACGGCAGGAATGACTTCAACTGGCCTCATTGTGTCATTACTGGCAAC 480
AMYT-3          CCTGCTGTTCCTTACGGCAGGAATGACTTCAACTGGCCTCATTGTGTCATTACTGGCAAC 480
AMYT-6          CCTGCTGTTCCTTACGGCAGGAATGACTTCAACTGGCCTCATTGTGTCATTACTGGCAAC 480
AMYT-2          CCTGCTGTTCCTTACGGCAGGAATGACTTCAACTGGCCTCATTGTGTCATTACTGGCAGC 480
AMYT-1          CCTGCTGTTCCTTACGGCAGGAATGACTTCAACTGGCCTCATTGTGTCATTACTGGCAGC 480
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Figure 32 Multiple alignments of the nucleotide sequences of Nanglai *α-amylase* cDNA from six clones.

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AMYT-4          GACTACAACTGCTGCCCTGACAGAGTGCGCAACTGTGAATTGTCTGGTCTGAAAGACTTG 540
AMYT-5          GACTACAACTGCTGCCCTGACAGAGTGCGCAACTGTGAATTGTCTGGTCTGAAAGACTTG 540
AMYT-3          GACTACAACTGCTGCCCTGACAGAGTGCGCAACTGTGAATTGTCTGGTCTGAAAGACTTG 540
AMYT-6          GACTACAACTGCTGCCCTGACAGAGTGCGCAACTGTGAATTGTCTGGTCTGAAAGACTTG 540
AMYT-2          GACTACAACTGCTGCCCTGACAGAGTGCGCAACTGTGAATTGTCTGGTCTGAAAGACTTG 540
AMYT-1          GACTACAACTGCTGCCCTGACAGAGTGCGCAACTGTGAATTGTCTGGTCTGAAAGACTTG 540
                ************************************************************

AMYT-4          AACCAAGGCTCTGATTATGTTAGACAGCAGATTCTAAATTATATGAACCGTCTTATTGAC 600
AMYT-5          AACCAAGGCTCTGATTATGTTAGACAGCAGATTCTAAATTATATGAACCGTCTTATTGAC 600
AMYT-3          AACCAAGGCTCTGATTATGTTAGACAGCAGATTCTAAATTATATGAACCGTCTTATTGAC 600
AMYT-6          AACCAAGGCTCTGATTATGTTAGACAGCAGATTCTAAATTATATGAACCGTCTTATTGAC 600
AMYT-2          AACCAAGGCTCTGATTATGTTAGACAGCAGATTCTAAATTATATGAACCGTCTTATTGAC 600
AMYT-1          AACCAAGGCTCTGATTATGTTAGACAGCAGATTCTAAATTATATGAACCGTCTTATTGAC 600
                ************************************************************

AMYT-4          ATGGGTGTTGCTGGTTTCAGAATCGATGCTGCAAAGCACATGTGGCCTCACGATTTGCGA 660
AMYT-5          ATGGGTGTTGCTGGTTTCAGAATCGATGCTGCAAAGCACATGTGGCCTCACGATTTGCGA 660
AMYT-3          ATGGGTGTTGCTGGTTTCAGAATTGATGCTGCAAAGCACATGTGGCCTCACGATTTGCGA 660
AMYT-6          ATGGGTGTTGCTGGTTTCAGAATTGATGCTGCAAAGCACATGTGGCCTCACGATTTGCGA 660
AMYT-2          ATGGGTGTTGCTGGTTTCAGAATTGATGCTGCAAAGCACATGTGGCCTCACGATTTGCGA 660
AMYT-1          ATGGGTGTTGCTGGTTTCAGAATTGATGCTGCAAAGCACATGTGGCCTCACGATTTGCGA 660
                ********************** ***********************************

AMYT-4          GTGATCTACGATCGTCTCCGCAATTTAAATACCGCCCACGGTTTCCCATCCGGTGCTCGT 720
AMYT-5          GTGATCTACGATCGTCTCCGCAATTTAAATACCGCCCACGGTTTCCCATCCGGTGCTCGT 720
AMYT-3          GTGATCTACGATCGTCTCCGCAATTTAAATACCGCCCACGGTTTCCCATCCGGTGCTCGT 720
AMYT-6          GTGATCTACGATCGTCTCCGCAATTTAAATACCGCCCACGGTTTCCCATCCGGTGCTCGT 720
AMYT-2          GTGATCTACGATCGTCTCCGCAATTTAAATACCGCCCACGGTTTCCCATCCGGTGCTCGT 720
AMYT-1          GTGATCTACGATCGTCTCCGCAATTTAAATACCGCCCACGGTTTCCCATCCGGTGCTCGT 720
                ************************************************************

AMYT-4          CCCTACGTCTACCAAGAAGTCATCGACCTCGGTGGTGAAGCTATCAGTCGCAACGAATAC 780
AMYT-5          CCCTACGTCTACCAAGAAGTCATCGACCTCGGTGGTGAAGCTATCAGTCGCAACGAATAC 780
AMYT-3          CCCTACATCTACCAAGAAGTCATCGACCTCGGTGGTGAAGCTATCAGTCGCAACGAATAC 780
AMYT-6          CCCTACATCTACCAAGAAGTCATCGACCTCGGTGGTGAAGCTATCAGTCGCAACGAATAC 780
AMYT-2          CCCTACATCTACCAAGAAGTCATCGACCTCGGTGGTGAAGCTATCAGTCGCAACGAATAC 780
AMYT-1          CCCTACATCTACCAAGAAGTCATCGACCTCGGTGGTGAAGCTATCAGTCGCAACGAATAC 780
                ****** ***************************************************

AMYT-4          ACTCCACTTGCTGCCGTTACTGAGTTCAGATTTGGACTGGAACTCAGTCAAGCTTTCCAA 840
AMYT-5          ACTCCACTTGCTGCCGTTACTGAGTTCAGATTTGGACTGGAACTCAGTCAAGCTTTCCAA 840
AMYT-3          ACTCCACTTGCTGCCGTTACTGAGTTCAGATTTGGACTGGAACTCAGTCAAGCTTTCCAA 840
AMYT-6          ACTCCACTTGCTGCCGTTACTGAGTTCAGATTTGGACTGGAACTCAGTCAAGCTTTCCAA 840
AMYT-2          ACTCCACTTGCTGCCGTTACTGAGTTCAGATTTGGACTGGAACTCAGTCAAGCTTTCCAA 840
AMYT-1          ACTCCACTTGCTGCCGTTACTGAGTTCAGATTTGGACTGGAACTCAGTCAAGCTTTCCAA 840
                ************************************************************

AMYT-4          AGAAGAAACCAGCTTAGATGGTTGGTTAACTGGGGTCCACAGTGGGGCCTTCTCGCTTCT 900
AMYT-5          AGAAGAAACCAGCTTAGATGGTTGGTTAACTGGGGTCCACAGTGGGGCCTTCTCGCTTCT 900
AMYT-3          AGAAGAAACCAGCTTAGATGGTTGGTTAACTGGGGTCCACAGTGGGGCCTTCTCGCTTCT 900
AMYT-6          AGAAGAAACCAGCTTAGATGGTTGGTTAACTGGGGTCCACAGTGGGGCCTTCTCGCTTCT 900
AMYT-2          AGAAGAAACCAGCTTAGATGGTTGGTTAACTGGGGTCCACAGTGGGGCCTTCTCGCTTCT 900
AMYT-1          AGAAGAAACCAGCTTAGATGGTTGGTTAACTGGGGTCCACAGTGGGGCCTTCTCGCTTCT 900
                ************************************************************

AMYT-4          GGAGACGCTTTGACTTTCATTGACAACCACGACAACCAGAGAGGTCATGGCGCTGGTGGA 960
AMYT-5          GGAGACGCTTTGACTTTCATTGACAACCACGACAACCAGAGAGGTCATGGCGCTGGTGGA 960
AMYT-3          GGAGACGCTTTGACTTTCATTGACAACCACGACAACCAGAGAGGTCATGGCGCTGGTGGA 960
AMYT-6          GGAGACGCTTTGACTTTCATTGACAACCACGACAACCAGAGAGGTCATGGCGCTGGTGGA 960
AMYT-2          GGAGACGCTTTGACTTTCATTGACAACCACGACAACCAGAGAGGTCATGGCGCTGGTGGA 960
AMYT-1          GGAGACGCTTTGACTTTCATTGACAACCACGACAACCAGAGAGGTCATGGCGCTGGTGGA 960
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Figure 32 (Continued) Multiple alignments of the nucleotide sequences of Nanglai *α-amylase* cDNA from six clones.

```
AMYT-4          AACATCTTGACATACAAGCAATCCCGACAATACAAGGGCGCTATCGCTTTCATGTTGGCA 1020
AMYT-5          AACATCTTGACATACAAGCAATCCCGACAATACAAGGGCGCTATCGCTTTCATGTTGGCA 1020
AMYT-3          AACATCTTGACATACAAGCAATCCCGACAATACAAGGGCGCTATCGCTTTCATGTTGGCA 1020
AMYT-6          AACATCTTGACATACAAGCAATCCCGACAATACAAGGGCGCTATCGCTTTCATGTTGGCA 1020
AMYT-2          AACATCTTGACATACAAGCAATCCCGACAATACAAGGGCGCTATCGCTTTCATGTTGGCA 1020
AMYT-1          AACATCTTGACATACAAGCAATCCCGACAATACAAGGGCGCTATCGCTTTCATGTTGGCA 1020
                ************************************************************

AMYT-4          CATCCTTATGGCTACCCTCAATTGATGAGTAGTTTCGCCTTCACGGACACCGAAGCTGGA 1080
AMYT-5          CATCCTTATGGCTACCCTCAATTGATGAGTAGTTTCGCCTTCACGGACACCGAAGCTGGA 1080
AMYT-3          CATCCTTATGGCTACCCTCAATTGATGAGTAGTTTCGCCTTCACGGACACCGAAGCTGGA 1080
AMYT-6          CATCCTTATGGCTACCCTCAATTGATGAGTAGTTTCGCCTTCACGGACACCGAAGCTGGA 1080
AMYT-2          CATCCTTATGGCTACCCTCAATTGATGAGTAGTTTCGCCTTCACGGACACCGAAGCTGGA 1080
AMYT-1          CATCCTTATGGCTACCCTCAATTGATGAGTAGTTTCGCCTTCACGGACACCGAAGCTGGA 1080
                ************************************************************

AMYT-4          CCTCCAATGAACAGCAGGGGTGATATCACCTCTCCTACTATCAACGCTGATAATTCTTGC 1140
AMYT-5          CCTCCAATGAACAGCAGGGGTGATATCACCTCTCCTACTATCAACGCTGATAATTCTTGC 1140
AMYT-3          CCTCCAATGAACAGCAGGGGTGATATCACCTCTCCTACTATCAACGCTGATAATTCTTGC 1140
AMYT-6          CCTCCAATGAACAGCAGGGGTGATATCACCTCTCCTACTATCAACGCTGATAATTCTTGC 1140
AMYT-2          CCTCCAATGAACAGCAGGGGTGATATCACCTCTCCTACTATCAACGCTGATAATTCTTGC 1140
AMYT-1          CCTCCAATGAACAGCAGGGGTGATATCACCTCTCCTACTATCAACGCTGATAATTCTTGC 1140
                ************************************************************

AMYT-4          GGCAATGGTTGGATATGCGAGCACCGTTGGCGTCAGATCCACAACATGGTTGTCTTCAGG 1200
AMYT-5          GGCAATGGTTGGATATGCGAGCACCGTTGGCGTCAGATCCACAACATGGTTGTCTTCAGG 1200
AMYT-3          GGCAATGGTTGGATATGCGAGCACCGTTGGCGTCAGATCCACAACATGGTTGTCTTCAGG 1200
AMYT-6          GGCAATGGTTGGATATGCGAGCACCGTTGGCGTCAGATCCACAACATGGTTGTCTTCAGG 1200
AMYT-2          GGCAATGGTTGGATATGCGAGCACCGTTGGCGTCAGATCCACAACATGGTTGTCTTCAGG 1200
AMYT-1          GGCAATGGTTGGATATGCGAGCACCGTTGGCGTCAGATCCACAACATGGTTGTCTTCAGG 1200
                ************************************************************

AMYT-4          AACACCGCTGGTAACGGTGCCCTTACCAACTGGTGGGACAACGGTAGCAACCAGATTGCT 1260
AMYT-5          AACACCGCTGGTAACGGTGCCCTTACCAACTGGTGGGACAACGGTAGCAACCAGATTGCT 1260
AMYT-3          AACACCGCTGGTAACGGTGCCCTTACCAACTGGTGGGACAACGGTAGCAACCAGATTGCT 1260
AMYT-6          AACACCGCTGGTAACGGTGCCCTTACCAACTGGTGGGACAACGGTAGCAACCAGATTGCT 1260
AMYT-2          AACACCGCTGGTAACGGTGCCCTTACCAACTGGTGGGACAACGGTAGCAACCAGATTGCT 1260
AMYT-1          AACACCGCTGGTAACGGTGCCCTTACCAACTGGTGGGACAACGGTAGCAACCAGATTGCT 1260
                ************************************************************

AMYT-4          TTCTGCCGCGGCAACCAGGCCTTCATTGCTTTCAACAACGATGCATGGGACATGGACCAG 1320
AMYT-5          TTCTGCCGCGGCAACCAGGCCTTCATTGCTTTCAACAACGATGCATGGGACATGGACCAG 1320
AMYT-3          TTCTGCCGCGGCAACCAGGCCTTCATTGCTTTCAACAACGATGCATGGGACATGGACCAG 1320
AMYT-6          TTCTGCCGCGGCAACCAGGCCTTCATTGCTTTCAACAACGATGCATGGGACATGGACCAG 1320
AMYT-2          TTCTGCCGCGGCAACCAGGCCTTCATTGCTTTCAACAACGATGCATGGGACATGGACCAG 1320
AMYT-1          TTCTGCCGCGGCAACCAGGCCTTCATTGCTTTCAACAACGATGCATGGGACATGGACCAG 1320
                ************************************************************

AMYT-4          ACTCTTCAGACTTGTCTCCCCGCCGGAACATACTGCGATATTATTTCCGGCGCCAGGTCT 1380
AMYT-5          ACTCTTCAGACTTGTCTCCCCGCCGGAACATACTGCGATATTATTTCCGGCGCCAGGTCT 1380
AMYT-3          ACTCTTCAGACTTGTCTCCCCGCCGGAACATACTGCGATATTATTTCCGGCGCCAGGTCT 1380
AMYT-6          ACTCTTCAGACTTGTCTCCCCGCCGGAACATACTGCGATATTATTTCCGGCGCCAGGTCT 1380
AMYT-2          ACTCTTCAGACTTGTCTCCCCGCCGGAACATACTGCGATATTATTTCCGGCGCCAGGTCT 1380
AMYT-1          ACTCTTCAGACTTGTCTCCCCGCCGGAACATACTGCGATATTATTTCCGGCGCCAGGTCT 1380
                ************************************************************

AMYT-4          GGTAACCGCTGCACTGGAAAATCTATTGTAGTAGGCAGCGATGGTCGCGCTCGTATCATC 1440
AMYT-5          GGTAACCGCTGCACTGGAAAATCTATTGTAGTAGGCAGCGATGGTCGCGCTCGTATCATC 1440
AMYT-3          GGTAACCGCTGCACTGGAAAATCTATTGTAGTAGGCAGCGATGGTCGCGCTCGTATCATC 1440
AMYT-6          GGTAACCGCTGCACTGGAAAATCTATTGTAGTAGGCAGCGATGGTCGCGCTCGTATCATC 1440
AMYT-2          GGTAACCGCTGCACTGGAAAATCTATTGTAGTAGGCAGCGATGGTCGCGCTCTTATCATC 1440
AMYT-1          GGTAACCGCTGCACTGGAAAATCTATTGTAGTAGGCAGCGATGGTCGCGCTCTTATCATC 1440
                ************************************************** *******
```

Figure 32 (Continued) Multiple alignments of the nucleotide sequences of Nanglai *α-amylase* cDNA from six clones.

```
AMYT-4          CACCGCAGCAACGAGTATGACATGATGGTTGCTATCCATAGGGGTGCTGACTCGAGATTG 1500
AMYT-5          CACCGCAGCAACGAGTATGACATGATGGTTGCTATCCATAGGGGTGCTGACTCGAGATTG 1500
AMYT-3          CACCGCAGCAACGAGTATGACATGATGGTTGCTATCCATAGGGGTGCTGACTCGAGATTG 1500
AMYT-6          CACCGCAACAACGAGTATGACATGATGGTTGCTATCCATAGGGGTGCTGACTCGAGATTG 1500
AMYT-2          CACCGCAGCAACGAGTATGACATGATGGTTGCTATCCATAGGGGTGCTGACTCGAGATTG 1500
AMYT-1          CACCGCAGCAACGAGTATGACATGATGGTTGCTATCCATAGGGGTGCTGACTCGAGATTG 1500
                ******* **************************************************

AMYT-4          TAA 1503
AMYT-5          TAA 1503
AMYT-3          TAA 1503
AMYT-6          TAA 1503
AMYT-2          TAA 1503
AMYT-1          TAA 1503
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```

Figure 32 (Continued) Multiple alignments of the nucleotide sequences of Nanglai *α-amylase* cDNA from six clones.

```
AMYT-4          MFRYILLLSAVTLALAYKNPHYASGRTTMVHLFEWKWDDIAAECERFLGPRGFGGIQVSP 60
AMYT-5          MFRYILLLSAVTLALAYKNPHYASGRTTMVHLFEWKWDDIAAECERFLGPRGFGGIQVSP 60
AMYT-3          MFRYILLLSAVTLALAYKNPHYASGRTTMVHLFEWKWDDIAAECERFLGPRGFGGIQVSP 60
AMYT-6          MFRYILLLSAVTLALAYKNPHYASGRTTMVHLFEWKWDDIAAECERFLGPRGFGGIQVSP 60
AMYT-2          MFRYILLLSAVTLALAYKNPHYASGRTTMVHLFEWKWDDIAAECERFLGPRGFGGIQVSP 60
AMYT-1          MFRYILLLSAVTLALAYKNPHYASGRTTMVHLFEWKWDDIAAECERFLGPRGFGGIQVSP 60
                ************************************************************

AMYT-4          PNENLVIWSRNRPWWERYQPISYRLVTRSGNENQFSNMVRRCNNVGVRIYVDAIINHMTG 120
AMYT-5          PNENLVIWSRNRPWWERYQPISYRLVTRSGNENQFSNMVRRCNNVGVRIYVDAIINHMTG 120
AMYT-3          PNENLVIWSRNRPWWERYQPISYRLVTRSGNENQFSNMVRRCNNVGVRIYVDAIINHMTG 120
AMYT-6          PNENLVIWSRNRPWWERYQPISYRLVTRSGNENQFSNMVRRCNNVGVRIYVDAIINHMTG 120
AMYT-2          PNENLVIWSRNRPWWERYQPISYRLVTRSGNENQFSNMVRRCNNVGVRIYVDAIINHMTG 120
AMYT-1          PNENLVIWSRNRPWWERYQPISYRLVTRSGNENQFSNMVRRCNNVGVRIYVDAIINHMTG 120
                ************************************************************

AMYT-4          TWNENVGTGGSTANFENWHYPAVPYGRNDFNWPHCVITGNDYNCCPDRVRNCELSGLKDL 180
AMYT-5          TWNENVGTGGSTANFENWHYPAVPYGRNDFNWPHCVITGNDYNCCPDRVRNCELSGLKDL 180
AMYT-3          TWNENVGTGGSTANFENWHYPAVPYGRNDFNWPHCVITGNDYNCCPDRVRNCELSGLKDL 180
AMYT-6          TWNENVGTGGSTANFENRHYPAVPYGRNDFNWPHCVITGNDYNCCPDRVRNCELSGLKDL 180
AMYT-2          TWNENVGTGGSTANFENWHYPAVPYGRNDFNWPHCVITGSDYNCCPDRVRNCELSGLKDL 180
AMYT-1          TWNENVGTGGSTANFENWHYPAVPYGRNDFNWPHCVITGSDYNCCPDRVRNCELSGLKDL 180
                **************** ********************.********************

AMYT-4          NQGSDYVRQQILNYMNRLIDMGVAGFRIDAAKHMWPHDLRVIYDRLRNLNTAHGFPSGAR 240
AMYT-5          NQGSDYVRQQILNYMNRLIDMGVAGFRIDAAKHMWPHDLRVIYDRLRNLNTAHGFPSGAR 240
AMYT-3          NQGSDYVRQQILNYMNRLIDMGVAGFRIDAAKHMWPHDLRVIYDRLRNLNTAHGFPSGAR 240
AMYT-6          NQGSDYVRQQILNYMNRLIDMGVAGFRIDAAKHMWPHDLRVIYDRLRNLNTAHGFPSGAR 240
AMYT-2          NQGSDYVRQQILNYMNRLIDMGVAGFRIDAAKHMWPHDLRVIYDRLRNLNTAHGFPSGAR 240
AMYT-1          NQGSDYVRQQILNYMNRLIDMGVAGFRIDAAKHMWPHDLRVIYDRLRNLNTAHGFPSGAR 240
                ************************************************************

AMYT-4          PYVYQEVIDLGGEAISRNEYTPLAAVTEFRFGLELSQAFQRRNQLRWLVNWGPQWGLLAS 300
AMYT-5          PYVYQEVIDLGGEAISRNEYTPLAAVTEFRFGLELSQAFQRRNQLRWLVNWGPQWGLLAS 300
AMYT-3          PYIYQEVIDLGGEAISRNEYTPLAAVTEFRFGLELSQAFQRRNQLRWLVNWGPQWGLLAS 300
AMYT-6          PYIYQEVIDLGGEAISRNEYTPLAAVTEFRFGLELSQAFQRRNQLRWLVNWGPQWGLLAS 300
AMYT-2          PYIYQEVIDLGGEAISRNEYTPLAAVTEFRFGLELSQAFQRRNQLRWLVNWGPQWGLLAS 300
AMYT-1          PYIYQEVIDLGGEAISRNEYTPLAAVTEFRFGLELSQAFQRRNQLRWLVNWGPQWGLLAS 300
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Figture 33 Multiple alignment of the deduce amino acid sequences from Nanglai *α-amylase* cDNA of six clones.

```
AMYT-4          GDALTFIDNHDNQRGHGAGGNILTYKQSRQYKGAIAFMLAHPYGYPQLMSSFAFTDTEAG 360
AMYT-5          GDALTFIDNHDNQRGHGAGGNILTYKQSRQYKGAIAFMLAHPYGYPQLMSSFAFTDTEAG 360
AMYT-3          GDALTFIDNHDNQRGHGAGGNILTYKQSRQYKGAIAFMLAHPYGYPQLMSSFAFTDTEAG 360
AMYT-6          GDALTFIDNHDNQRGHGAGGNILTYKQSRQYKGAIAFMLAHPYGYPQLMSSFAFTDTEAG 360
AMYT-2          GDALTFIDNHDNQRGHGAGGNILTYKQSRQYKGAIAFMLAHPYGYPQLMSSFAFTDTEAG 360
AMYT-1          GDALTFIDNHDNQRGHGAGGNILTYKQSRQYKGAIAFMLAHPYGYPQLMSSFAFTDTEAG 360
                ************************************************************

AMYT-4          PPMNSRGDITSPTINADNSCGNGWICEHRWRQIHNMVVFRNTAGNGALTNWWDNGSNQIA 420
AMYT-5          PPMNSRGDITSPTINADNSCGNGWICEHRWRQIHNMVVFRNTAGNGALTNWWDNGSNQIA 420
AMYT-3          PPMNSRGDITSPTINADNSCGNGWICEHRWRQIHNMVVFRNTAGNGALTNWWDNGSNQIA 420
AMYT-6          PPMNSRGDITSPTINADNSCGNGWICEHRWRQIHNMVVFRNTAGNGALTNWWDNGSNQIA 420
AMYT-2          PPMNSRGDITSPTINADNSCGNGWICEHRWRQIHNMVVFRNTAGNGALTNWWDNGSNQIA 420
AMYT-1          PPMNSRGDITSPTINADNSCGNGWICEHRWRQIHNMVVFRNTAGNGALTNWWDNGSNQIA 420
                ************************************************************

AMYT-4          FCRGNQAFIAFNNDAWDMDQTLQTCLPAGTYCDIISGARSGNRCTGKSIVVGSDGRARII 480
AMYT-5          FCRGNQAFIAFNNDAWDMDQTLQTCLPAGTYCDIISGARSGNRCTGKSIVVGSDGRARII 480
AMYT-3          FCRGNQAFIAFNNDAWDMDQTLQTCLPAGTYCDIISGARSGNRCTGKSIVVGSDGRARII 480
AMYT-6          FCRGNQAFIAFNNDAWDMDQTLQTCLPAGTYCDIISGARSGNRCTGKSIVVGSDGRARII 480
AMYT-2          FCRGNQAFIAFNNDAWDMDQTLQTCLPAGTYCDIISGARSGNRCTGKSIVVGSDGRALII 480
AMYT-1          FCRGNQAFIAFNNDAWDMDQTLQTCLPAGTYCDIISGARSGNRCTGKSIVVGSDGRALII 480
                ************************************************************ **

AMYT-4          HRSNEYDMMVAIHRGADSRL- 500
AMYT-5          HRSNEYDMMVAIHRGADSRL- 500
AMYT-3          HRSNEYDMMVAIHRGADSRL- 500
AMYT-6          HRNNEYDMMVAIHRGADSRL- 500
AMYT-2          HRSNEYDMMVAIHRGADSRL- 500
AMYT-1          HRSNEYDMMVAIHRGADSRL- 500
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Figure 33 (Continued) Multiple alignment of the deduce amino acid sequences from Nanglai *α-amylase* cDNA of six clones.

Table 1 Comparison of the ORF sequences of Nanglai *α-amylase* cDNA from six clones.

	Percent nucleotide identity					
	AMYT-1	AMYT-2	AMYT-3	AMYT-4	AMYT-5	AMYT-6
AMYT-1	100	100	99	99	99	99
AMYT-2		100	99	99	99	99
AMYT-3			100	99	99	99
AMYT-4				100	99	99
AMYT-5					100	99
AMYT-6						100

Table 2 Deduce amino acid sequence shown identity of full-length cDNA with ORF of Nanglai *α-amylase* cDNA. Deduce amino acid sequence of each clone was compared.

	Percent deduce amino acid identity					
	AMYT-1	AMYT-2	AMYT-3	AMYT-4	AMYT-5	AMYT-6
AMYT-1	100	100	99	99	99	99
AMYT-2		100	99	99	99	99
AMYT-3			100	99	99	99
AMYT-4				100	99	99
AMYT-5					100	99
AMYT-6						100

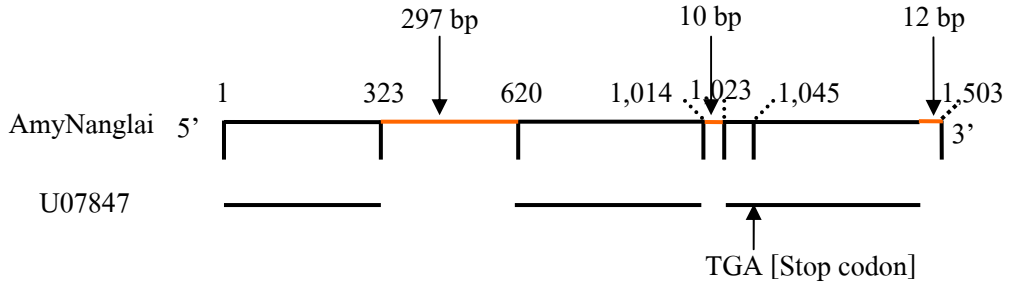


Figure 34 Sequence alignment of Nanglai *α-amylase* ORF to cDNA of *Bombyx mori* truncated *alpha-amylase* (*amy*) gene (Genbank Accession No. U07847).

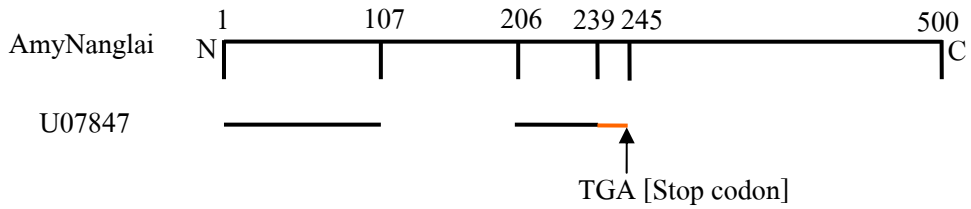
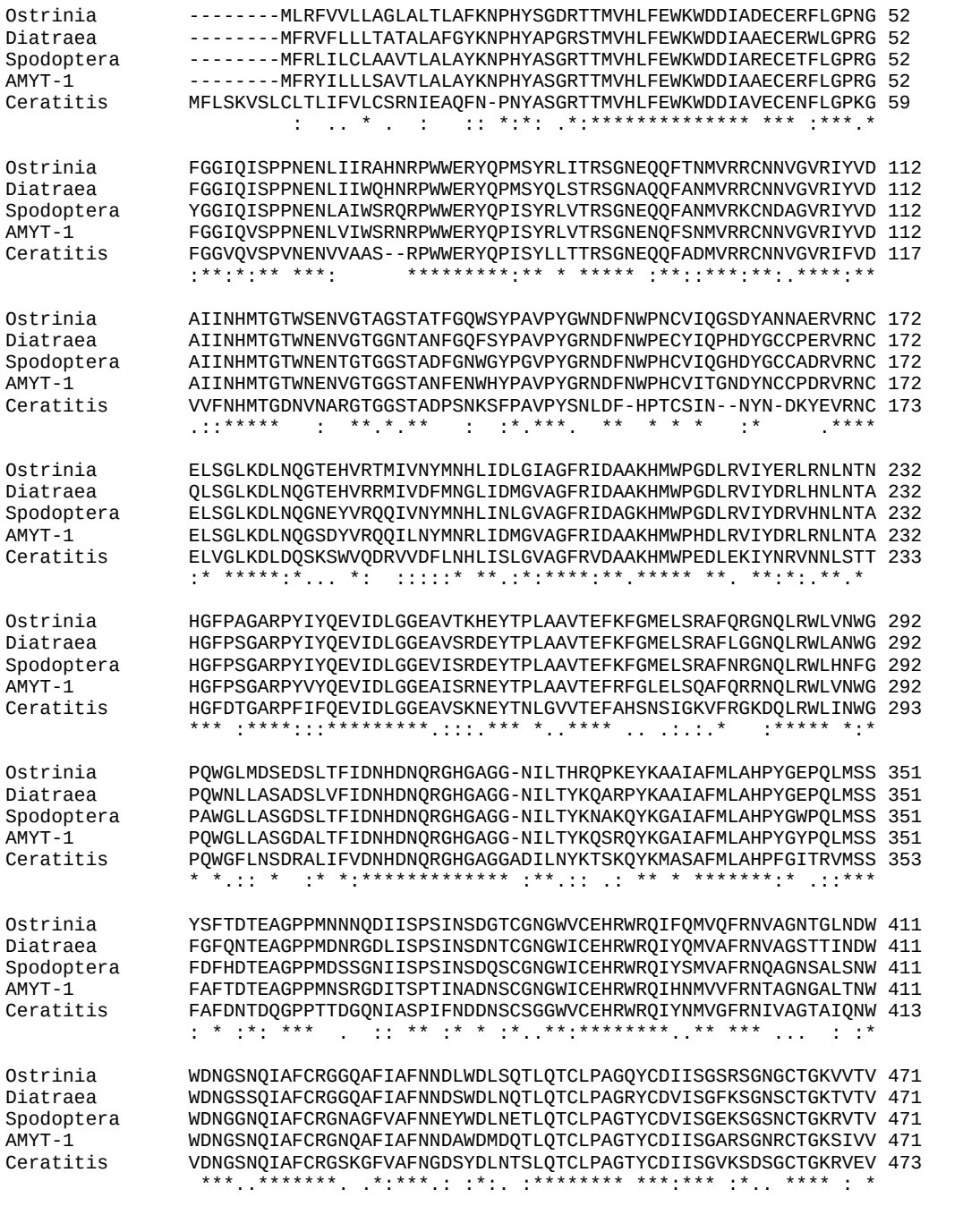


Figure 35 Deduced amino acid sequence alignment of Nanglai *α-amylase* to *Bombyx mori* truncated *alpha-amylase* (*amy*) gene (Genbank Accession No. U07847).



Figture 36 Deduced amino acid sequence alignment of AMYT-1 with other insects. AMYT-1 provided the sequence homology to *Ostrinia nubilalis* (AAA03715.1), *Diatraea saccharalis* (AAP92665.1), *Spodoptera frugiperda* (AF280891_1) and *Ceratitis capitata* (AAO13691.1) with 78, 79, 81 and 60% respectively.

```
Ostrinia         GNDGRAHISVGANEYDMMLAIHVGTQ-------- 497
Diatraea         GNDGRAHISVGANDYDMMLAIHRGDQSRL----- 500
Spodoptera       GGDGRAHISLGANEFDMVLAIHTGPEVRIFVALS 505
AMYT-1           GSDGRARIIHRSNEYDMMVAIHRGADSRL----- 500
Ceratitis        GSDGRATISITQAEEDGTLAIHFESKL------- 500
                 *.**** *     : *  :***   .
```

Figture 36 (Continued) Deduced amino acid sequence alignment of AMYT-1 with other insects. AMYT-1 provided the sequence homology to *Ostrinia nubilalis* (AAA03715.1), *Diatraea saccharalis* (AAP92665.1), *Spodoptera frugiperda* (AF280891_1) and *Ceratitis capitata* (AAO13691.1) with 78, 79, 81 and 60% respectively.

Table 3 Deduce amino acid sequence shown identity of *α-amylase* cDNA (AMYT-1) of Nanglai strain with other insects. Deduce amino acid sequence of each clone was compared.

Percent nucleotide identity					
	AMYT-1	*Ostrinia*	*Diatraea*	*Spodoptera*	*Ceratitis*
AMYT-1	100	78	79	81	60
Ostrinia		100	81	76	59
Diatraea			100	79	59
Spodoptera				100	60
Ceratitis					100

4. *B. mori* DNA isolation

Genomic DNA was extracted from 1st instar larvae of *B. mori*, Nanglai strain. The isolated genomic DNA had an A 260/A280 ratio of 2.075 suggesting that the genomic DNA had a little protein contamination. Yield of the DNA was 2,679 ng/µl. Genomic DNA was run on 1% agarose gel. The DNA not degraded (Figure 37).

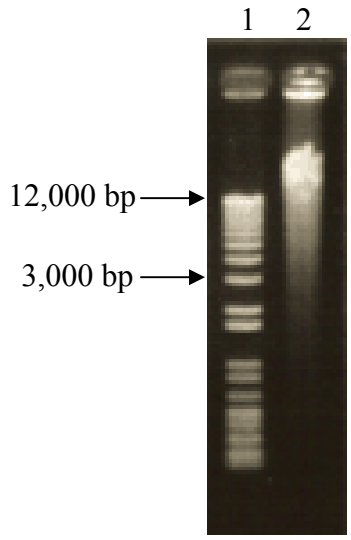


Figure 37 Agarose gel electrophoresis of genomic DNA from the 1st instar larvae of Nanglai strain.

5. Amplification and Sequencing genomic DNA of *α-amylase* gene

5.1 PCR Amplification

Genomic DNA isolation was used as a template for amplification the *α-amylase* genomic DNA. Three pairs of primers, AmyF primer+AmyR primer, 1038U primer+3886L primer and 5600U primer+8456L primer, were designed based on the sequences of cDNA of *Bombyx mori* truncated *alpha-amylase* (*amy*) gene (GenBank Accession No. U07847). The three PCR products with approximately 4,000 bp (fragment 1, Figure 38), 3,000 bp (fragment 2, Figure 39) and 3,000 bp long (fragment 3, Figure 39), were obtained. These PCR products were purified from agarose gel, added A overhang into the ends of the DNA fragment by used A-attachment reagent before ligated into pTA2 vector. The vectors were transformed into *E. coli* DH5α by heat shock transformation. Therefore, three types of recombinant plasmids, each carrying one of the three fragments, were obtained. Then, their sequences were analyzed by used the primer as described in section 6.3.1 of the part of methods.

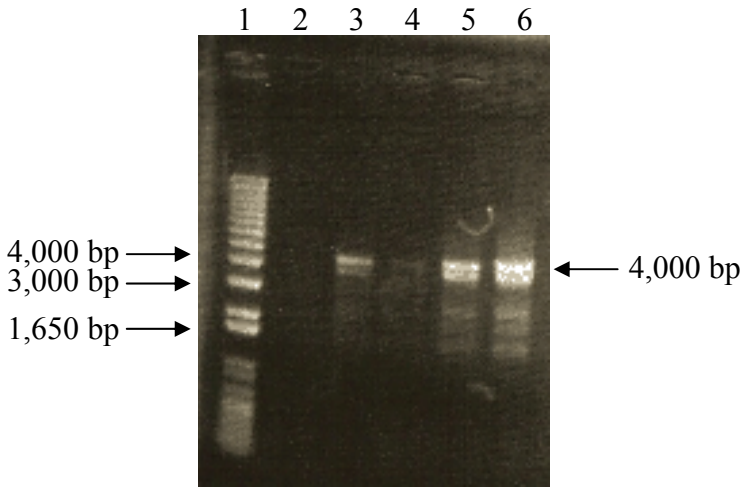


Figure 38 Agarose gel electrophoresis of PCR products of *α-amylase* genomic DNA fragment 1. Lane 1: 100 bp + 1.5 kb DNA ladder and Lane 2-6: PCR products of genomic DNA of *α-amylase* fragment 1.

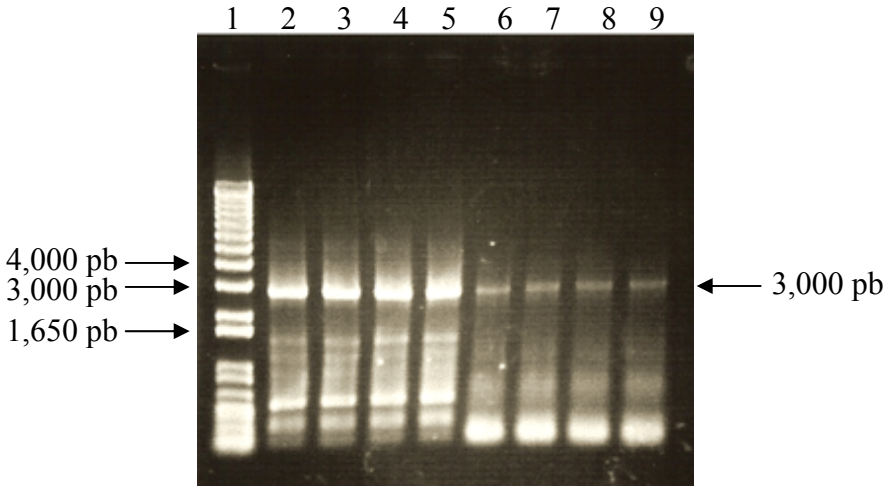


Figure 39 Agarose gel electrophoresis of PCR products of *α-amylase* genomic DNA fragment 2 and fragment 3. Lane 1: 100 bp + 1.5 kb DNA ladder, Lane 2-5: PCR products of genomic DNA of *α-amylase* fragment 2 and Lane 6-9: PCR products of genomic DNA of *α-amylase* fragment 3.

5.2 Detection of DNA insertion by restriction digestion

The fragments of *α-amylase* genomic DNA in three types of recombinant plasmids were selected by the formation of blue/white colonies on LB ampicillin plate. White colonies containing fragment 1, 2 and 3 were randomly selected for plasmid isolation and restriction analysis with restriction enzymes (Figure 40, Figure 41 and Figure 42). The DNA fragment from each clone was sequenced. The genomic DNA sequences of fragment 1, 2 and 3 of were shown in Figure 43, Figure 44 and Figure 45. The inserted fragment length of fragment 1, 2 and 3 was 4,110 bp, 2,772 bp and 2,931 bp, respectively. Therefore, nucleotide sequence of *α-amylase* genomic DNA of Nanglai strain consisted of 6,941 bp (Figure 46). Alignment of the nucleotide sequence of *α-amylase* genomic DNA showed 97 % sequence homology to the nucleotide sequence of genomic DNA of *B. mori*, p50 strain (Data of NIAS).

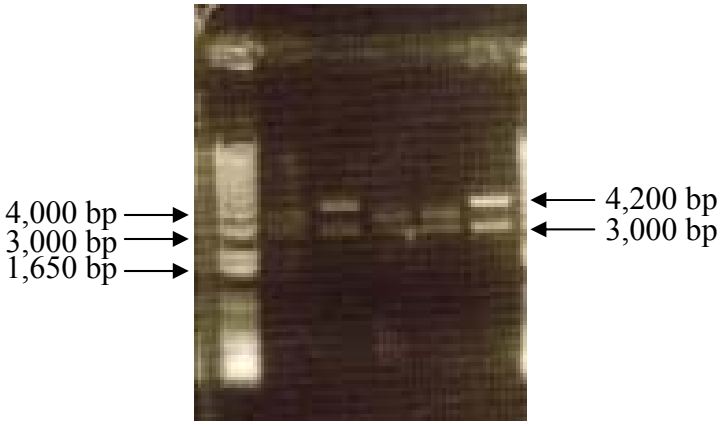


Figure 40 Agarose gel electrophoresis of recombinant vectors digested with *Eco*RI to select the expected inserted fragment 1. Lane 1: 100 bp + 1.5 kb DNA ladder and Lane 2-11: *Eco*RI digested recombinant plasmid clones.

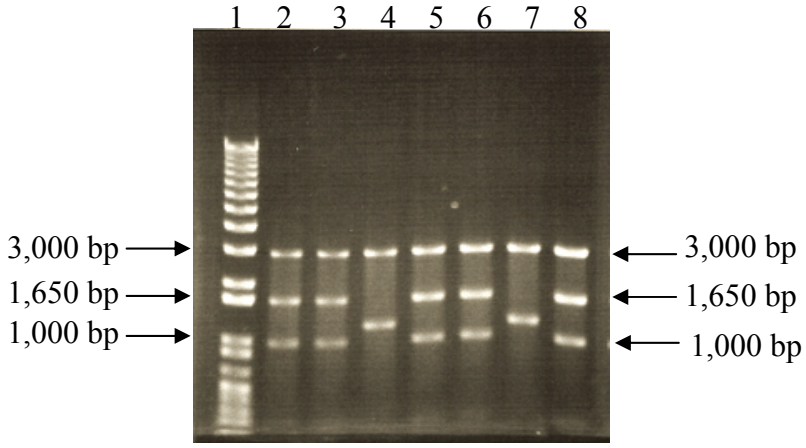


Figure 41 Agarose gel electrophoresis of recombinant vectors digested with *Eco*RI to select the expected inserted fragment 2. Lane 1: 100 bp + 1.5 kb DNA ladder and Lane 2-7: *Eco*RI digested recombinant plasmid clones.

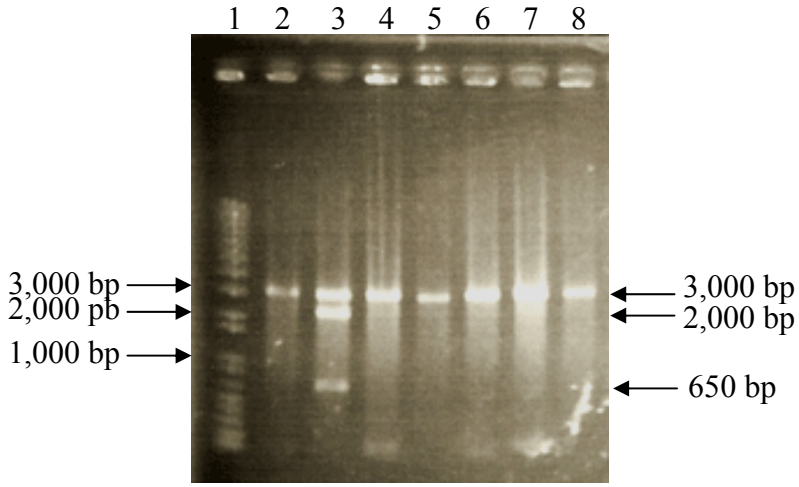


Figure 42 Agarose gel electrophoresis of recombinant vectors digested with *Eco*RI and *Pst*I to select the expected inserted fragment 3. Lane 1: 100 bp + 1.5 kb DNA ladder and Lane 2-7: *Eco*RI and *Pst*I digested recombinant plasmid clones.

```
5′   1  ATGTTTCGGTACATCCTTCTACTTTCGGCCGTGACTCTGGCGTTGGCCTATAAAAACCCTCACTATGCAT   70
    71  CGGGTCGCACCACCATGGTACATCTCTTCGAATGGAAGTGGGATGACATCGCTGCTGAATGCGAAAGGTT  140
   141  CCTTGGACCCCGAGGATTCGGTGGTATTCAGGTAAGCAATGACAAACACTATGAATTCATAAATTCAAAA  210
   211  TTTAAAAACCGTGCACGTCACATTAAATCTAAATTAAAAAATGATGACGTTCTTATTATTCGTCTTATCA  280
   281  TTCATCAATATCATTTCTGTAATTATCATAATAATGCATCCGCCGTATAGTTCTGACTCCTGACCTTGCA  350
   351  CCTTCAGATTTCTACCCGTTTCGGTCTCTTTAGAATTCTTTAGGCAGTGTAAGGTTAACATTACGAGAGG  420
   421  AATGCCAAAACCAATTGTCACGGTATTTTTATCAGAAGCCCCAAAATTTCTATAGCAATGGGATCATGTT  490
   491  CCTACCTACAAGATGATAAAAAGCTATCGAACAAAATGGTACCTAGATACTTAAGCTAAATGTAAATAAA  560
   561  CTACATTAAAAAATGTTGTGAATTTTCTTAAAAAATGCGAAGAAACTTTTCCCCAACCTATTAAAAAGGC  630
   631  AGATGATTTAAATCAGTGGATCCCGTAAGCACCATAAAGTATAATCCATTATCCAAATGTCTTTGTGGGA  700
   701  CTCTTTAATAAGCTCTTTTTTTGAAACGGCTCTCCTACCGTTTGAGTCATTAACCACTACTTCGCTGCGG  770
   771  AAACAAAACCATGATACTAATTAATGTCGGCTCATAGCCTGTCTTATCACCAGTACGTTCAAGTTCTAGT  840
   841  TATAATATGGATATATGAGTATATCGTAGCTGAGGAATGTCGTCATAAAGATCAAAACCTTCTCTTCAAT  910
   911  ATTCCCTTGTAAGACAGTTATAGATAGTTTTGGTGGTAGGACCTCTTGTGAGTCCGCGCGGGTCTGTACC  980
   981  ACCGCCCTGCCTATTTCTGCCGTGAAGCAGTAATGCGTTTTGGTTTGAAGTGTGGGGCAGCCGTTGTAAC 1050
  1051  TATACTTGAGATCTTAGAACTTATATCTCAAGGTGGGTGGCACATTTACTTTTTAGATGTCCATGGGCTC 1120
  1121  CAGTAACTACTTAACACCAGGTCGGCTGTGAGCTCGTCCACCCATCTAAGCAATAAAGAAAAAAGTTCAT 1190
  1191  TCAATATTTTTTGATCTTCATTTTCATAGGTTTCGCCACCAAACGAGAATTTGGTAATCTGGTCCCGCAA 1260
  1261  CCGTCCTTGGTGGGAGCGCTATCAACCAATCTCCTACCGTCTAGTAACAAGATCTGGAAATGAAAATCAA 1330
  1331  TTTTCGAATATGGTGCGTCGCTGCAACAATGTTGGCGTCAGGTTTGTTGCATTACGACTTTGGTTCAAAA 1400
  1401  TTTTGTAATTTAGTAATTGATAGAATTTATAAGACACCGTAGAGATGGCCTAAAATACAAATCATAGATA 1470
  1471  TAAAAAATATAATAGCCAGCTCATCAATTGAAGTTTTTTTTGCTAGATAGATCAATGTGGTCACAGACTA 1540
  1541  TCTGCTCCCAAATTTTTTTTTTTTTTATTGCCCTTGTCGGCAGACGAGCCTACGGCCCACCATCAGGTGG 1610
  1611  GCCGTATGCCGGTCTGCCTACAAGGGCAATAAAAAAAAAGAAGCGAATAACATTGTGTATTATGCACTTA 1680
  1681  GGAAATTGTTTCTATTAAATACGAAAAAAGGAGTATTATAGGGTACTACTCATATCTTACCGTAAACCTC 1750
  1751  AAACGCGCGTTCTAGGTTTTAAAGTACTCATGGAAAAACGAATGTTACGGAAAGTTCGGCTTCCACTTTT 1820
  1821  CATTACGCTTTGACTTTCAGGATTTATGTGGACGCCATCATCAACCACATGACTGGAACTTGGAATGAGA 1890
  1891  ATGTTGGTACAGGTGGAAGCACGGCCAATTTCGAAAACTGGCACTATCCTGCTGTTCCTTACGGCAGGAA 1960
  1961  TGACTTCAACTGGCCTCATTGTGTCATTACTGGCAGCGACTACAACTGCTGCCCTGACAGAGTACGAGCA 2030
  2031  TCTTATGTTATTTCTGTAGTGATGTTTGGGAAAACGCTTAAATTTATGTAGCTTTACGGGAACTAAGTTT 2100
  2101  TATGGGAATCAAAATTAAATAATCCCAAATTAATTTGATTTGATTTGATTTTAGGTGCGCAACTGTGAAT 2170
  2171  TGTCTGGTCTGAAAGACTTGAACCAAGGCTCTGATTATGTTAGACAGCAGATTCTAAATTATATGAACCG 2240
  2241  TCTTATTGACATGGGTGTTGCTGGTTTCAGTATGATAGTGACTTTTTTTTTATTGATTAGTTGGGTGGAT 2310
  2311  GAGCTCACTTTGAGATGTAAGTTCTAAGATCTCAGTATAGTTACAACGGCTGCCCTACCCTTCAGACCGA 2380
  2381  AACGCATTATTGCTTCACGGCAGAAATAGGTAAGGCGGTGGTACCTACCCGTGCGGACTCACAATAGATC 2450
  2451  CTACCACCAGTTGTTAGTTTGTTATAACTTACCCTCCTCAACTTTACTGGTTTAAATAAATTATGGTTTC 2520
  2521  TCCAGAATTGATGCTGCAAAGCACATGTGGCCTCACGATTTGCGAGTGATCTACGATCGTCTCCGCAATT 2590
  2591  TAAATACCGCCCACGGTTTCCCATCCGGTGCTCGTCCCTACATCTACCAAGAAGTCATCGACCTCGGTGG 2660
  2661  TGAAGCTATCAGTCGCAACGAATACACTCCACTTGCTGCCGTTACTGAGTTCAGATTTGGACTGGAACTC 2730
  2731  AGTCAAGCTTTCCAAAGAAGAAACCAGCTTAGATGGTTGGTTAACTGGGGTCCACAGTGGGGCCTTCTCG 2800
  2801  CTTCTGGAGACGCTTTGACTTTCATTGACAACCACGACAACCAGAGAGGTCATGGCGCTGGTGGAAACAT 2870
  2871  CTTGACATACAAGCAATCCCGACAATACAAGGGCGCTATCGCTTTCATGTTGGCACATCCTTATGGCTAC 2940
  2941  CCTCAATTGATGAGTAGTTTCGCCTTCACGGACACCGAAGCTGGACCTCCAATGAACAGCAGGGGTGATA 3010
  3011  TCACCTCTCCTACTATCAACGCTGTATGTATTGACATGACATTATGTAATCAAGTCCATTTCGCTTTTCA 3080
  3081  AAGGTTAATTTGCATTTATTCAACAGATAATTGTGCTATCGTTCGATTAGATAAAGTTAATATAGAATGT 3150
  3151  TTGTATTATTCTTGATCTGTTTTGTACCTACCAAGAAATAAATCAATTATATCGCGTTGTCGATCTAGTG 3220
  3221  CTTAGTGACCAACTATTTTACAACGGGTTATTTGGTATTTTTTTATCCGAAACAGGATAATTCTTGCGGC 3290
  3291  AATGGTTGGATATGCGAGCACCGTTGGCGTCAGATCCACAACATGGTTGTCTTCAGGAACACCGCTGGTA 3360
  3361  ACGGTGCCCTTACCAACTGGTGGGACAACGGTAGCAACCAGATTGCTTTCTGCCGCGGCAACCAGGCCTT 3430
  3431  CATTGCTTTCAACAACGATGCATGGGACATGGACCAGACTCTTCAGGTTTTTATTTTATTTTTATTAGGC 3500
  3501  AAATGAGTGATATCGGTTTGATAGATATATTAAGTATGATGAGAATTGACCAAATCATTTTGATTTTAAG 3570
  3571  CTATTTAACACGCTAGCATCGGAGTGGATAAGACAGTTGAACTTGTTTTTAATAGTGCATGGTATCAGTA 3640
  3641  GTTATAAATATCTTATGAACAGATATGAATAGAAGGGTTATTTTGATAATATCCTGAAAAGCAGTTTTTT 3710
  3711  CAATAAAATCATTTTTTCTTACACTATTTTTAATTCAAATTGATTGCAATTTATTTTCTATTTTTTAGTT 3780
  3781  GGATTTCCGATAAAAGCGTATTATCTTTGTTTTTTTTTTAAAGCTATTATTTATTGTAAAAAAAAATGTT 3850
  3851  TTGCTTTCATCATTCGTTTTAGAAGGAATCACCTAAAAATTAAACCTCATAGATTATTTTACGTGCCGGT 3920
  3921  AAAGCATAACTCAATGTCTCCGTTTCAGACTTGTCTCCCCGCCGGAACATACTGCGATATTATTTCCGGC 3990
  3991  GCCAGGTCTGGTAACCGCTGCACTGGAAAATCTATTGTAGTAGGCAGCGATGGTCGCGCTCTTATCATCC 4060
  4061  ACCGCAGCAACGAGTATGACATGATGGTTGCTATCCATAGGGGTGCTGAC                     4110 3′
```

Figure 43 Nucleotide sequences of *α-amylase* genomic DNA fragment 1 consisted of 4,110 bp.

```
5′   1  GGCGCGCCCGAGCACGAAGAAATATGAATAGCAAAATTAAAAATAACAAATTTAATAAAATCAATTAGTA   70
    71  GGATTATTTATAAGCAAGAAGTTAACATTCCAACGCTGTGAAAAGCCATTTTTTTTTTAAAAACAGTTCC  140
   141  TTCAAATAACCGCTTTCAAAACACAAATGATTTCACAAATGAATCATTTGGCTGAAATAATGTGTTTTAG  210
   211  GTGTGAAAATTTGATTTTTATCGTACAAAATCATCTTGGATACGGAGCGCCGAATATTTATCAATGTTTT  280
   281  TTTGAACTTTTATCGAACGATTATGTTCATTATGTCGATAAATTTCGTGTATCGCAACACAGACCGAGAT  350
   351  TTTATCTTCAAGTATATATAACTTGGGTACGGGAGGCTGTTATCAGATTTATTTAATTATATCACACGAC  420
   421  AGGAGGTCCAGTATATTAGATAAGGTGAGCAATTTAAATTATAAATATTATTTAATTCCATGGACTAAGC  490
   491  TCTCATATAATTATAGAGAAAATGTGCGCACATAAAAATCAATAAATTATCATGTATCGGATTTGGAATC  560
   561  GTAATTTATTCGTGGTTGCAGCTAGTTTTATAAATTTCACTTATTAGCCGAAAAGCGCTTTTAAATTGTA  630
   631  AGTGTGAAACTGTGTGACTTAAGACTAATTTCATCAAGTACACTAAATGCGCTCACTCACTTTCGCATTC  700
   701  TCAATGATCACAAATAAATATACGCATTGGATAAATAATTTCTTCTTTATAAGGAGATTAAAATTGAATA  770
   771  AAAATTGATATTAAAATGAAGAAGTATTTGCATCTGTGTATACTATGTATGAGTAATAATTTTTAAAATG  840
   841  ACGGATTTTTTTCATAAGACCAGAACATTCAGGGCTTCGGCTAAACATCTCGTTTATATTATAATACAAA  910
   911  TATTTTTCTCTTGATTTGGGACATGCTTACAGGCTGAAGAATTAGTTCTCAGGTTTTATGAAAAGAATTA  980
   981  TATTACTCATGCTAGTATGCTTAGTAATAGGTATAGTTATGTTGATTGCTTAAAGTTATATTATTGAAAA 1050
  1051  AAAGTGTATATTATACTAAGGAAATTATTAAAAAATAATTATAACTAAGCTACTGAGACTAGTTCTAGAT 1120
  1121  TAGGCACTTTATTCATGCGCTATTTTTTGTATGGAAATGGTAGTGCAAGGTAGAGGGGGAAGAGGTCGAC 1190
  1191  CGAAGAAGACATGGATGGAGTGTGTGAATAACGATATGAGAGAGAGAGAGGAGTGAGTGTTGAGATGACG 1260
  1261  GCTGATAGAAGAGAATGGAAGAGAAAAATTAGCTGTGCCGACCCCACCTAGTGGGATAAGGTGGAGAAAA 1330
  1331  AGAAGAAGCACAAGTACATATTATTATATTATACTACTAAACCTTTCAAAACTTTCGCTTTGTTAGGATG 1400
  1401  TTTCGGTACATCCTTCTACTTTCGGCCGTGACTCTGGCGTTGGCCTATAAAAACCCTCACTATGCATCGG 1470
  1471  GTCGCACCACCATGGTACATCTCTTCGAATGGAAGTGGGATGACATCGCTGCTGAATGCGAAAGGTTCCT 1540
  1541  TGGACCCCGAGGATTCGGTGGTATTCAGGTAAGCAATGACAAACACTATGAATTCATAAATTCAAAATTT 1610
  1611  AAAAACCGTGCACGTCACATTAAATCTAAATTAAAAAATGATGACGTTCTTATTATTCGTCTTATCATTC 1680
  1681  ATCAATATCATTTCTGTAATTATCATAATAATGCATCCGCCGTATAGTTCTGACTCCTGACCTTGCACCT 1750
  1751  TCAGATTTCTACCCGTTTCGGTCTCTTTAGAATTCTTTAGGCAGTGTAAGGTTAACATTACGAGAGGAAT 1820
  1821  GCCAAAACCAATTGTCACGGTATTTTTATCAGAAGCCCCAAAATTTCTATAGCAATGGGATCATGTTCCT 1890
  1891  ACCTACAAGATGATAAAAAGCTATCGAACAAAATGGTACCTAGATACTTAAGCTAAATGTAAATAAACTA 1960
  1961  CATTAAAAAATGTTGTGAATTTTCTTAAAAAATGCGAAGAAACTTTTCCCCAACCTATTAAAAAGGCAGA 2030
  2031  TGATTTAAATCAGTGGATCCCGTAAGCACCATAAAGTATAATCCATTATCCAAATGTCTTTGTGGGACTC 2100
  2101  TTTAATAAGCTCTTTTTTTGAAACGGCTCTCCTACCGTTTGAGTCATTAACCACTACTTCGCTGCGGAAA 2170
  2171  CAAAACCATGATACTAATTAATGTCGGCTCATAGCCTGTCTTATCACCAGTACGTTCAAGTTCTAGTTAT 2240
  2241  AATATGGATATATGAGTATATCGTAGCTGAGGAATGTCGTCATAAAGATCAAAACCTTCTCTTCAATATT 2310
  2311  CCCTTGTAAGACAGTTATAGATAGTTTTGGTGGTAGGACCTCTTGTGAGTCCGCGCGGGTCTGTACCACC 2380
  2381  GCCCTGCCTATTTCTGCCGTGAAGCAGTAATGCGTTTTGGTTTGAAGTGTGGGGCAGCCGTTGTAACTAT 2450
  2451  ACTTGAGATCTTAGAACTTATATCTCAAGGTGGGTGGCACATTTACTTTTTAGATGTCCATGGGCTCCAG 2520
  2521  TAACTACTTAACACCAGGTCGGCTGTGAGCTCGTCCACCCATCTAAGCAATAAAGAAAAAAGTTCATTCA 2590
  2591  ATATTTTTTGATCTTCATTTTCATAGGTTTCGCCACCAAACGAGAATTTGGTAATCTGGTCCCGCAACCG 2660
  2661  TCCTTGGTGGGAGCGCTATCAACCAATCTCCTACCGTCTAGTAACAAGATCTGGAAATGAAAATCAATTT 2730
  2731  TCGAATATGGTGCGTCGCTGCAACAATGTTGGCGTCAGGTTT                              2772 3′
```

Figure 44 Nucleotide sequences of *α-amylase* genomic DNA fragment 2 consisted of 2,772 bp.

```
5′   1  CCGGTGCTCGTCCCTACATCTACCAAGAAGTCATCGACCTCGGTGGTGAAGCTATCAGTCGCAACGAATA   70
    71  CACTCCACTTGCTGCCGTTACTGAGTTCAGATTTGGACTGGAACTCAGTCAAGCTTTCCAAAGAAGAAAC  140
   141  CAGCTTAGATGGTTGGTTAACTGGGGTCCACAGTGGGGCCTTCTCGCTTCTGGAGACGCTTTGACTTTCA  210
   211  TTGACAACCACGACAACCAGAGAGGTCATGGCGCTGGTGGAAACATCTTGACATACAAGCAATCCCGACA  280
   281  ATACAAGGGCGCTATCGCTTTCATGTTGGCACATCCTTATGGCTACCCTCAATTGATGAGTAGTTTCGCC  350
   351  TTCACGGACACCGAAGCTGGACCTCCAATGAACAGCAGGGGTGATATCACCTCTCCTACTATCAACGCTG  420
   421  TATGTATTGACATGACATTATGTAATCAAGTCCATTTCGCTTTTCAAAGGTTAATTTGCATTTATTCAAC  490
   491  AGATAATTGTGCTATCGTTCGATTAGATAAAGTTAATATAGAATGTTTGTATTATTCTTGATCTGTTTTG  560
   561  TACCTACCAAGAAATAAATCAATTATATCGCGTTGTCGATCTAGTGCTTAGTGACCAACTATTTTACAAC  630
   631  GGGTTATTTGGTATTTTTTTATCCGAAACAGGATAATTCTTGCGGCAATGGTTGGATATGCGAGCACCGT  700
   701  TGGCGTCAGATCCACAACATGGTTGTCTTCAGGAACACCGCTGGTAACGGTGCCCTTACCAACTGGTGGG  770
   771  ACAACGGTAGCAACCAGATTGCTTTCTGCCGCGGCAACCAGGCCTTCATTGCTTTCAACAACGATGCATG  840
   841  GGACATGGACCAGACTCTTCAGGTTTTTATTTTATTTTTATTAGGCAAATGAGTGATATCGGTTTGATAG  910
   911  ATATATTAAGTATGATGAGAATTGACCAAATCATTTTGATTTTAAGCTATTTAACACGCTAGCATCGGAG  980
   981  TGGATAAGACAGTTGAACTTGTTTTTAATAGTGCATGGTATCAGTAGTTATAAATATCTTATGAACAGAT 1050
  1051  ATGAATAGAAGGGTTATTTTGATAATATCCTGAAAAGCAGTTTTTTCAATAAAATCATTTTTTCTTACAC 1120
  1121  TATTTTTAATTCAAATTGATTGCAATTTATTTTCTATTTTTTAGTTGGATTTCCGATAAAAGCGTATTAT 1190
  1191  CTTTGTTTTTTTTTTAAAGCTATTATTTATTGTAAAAAAAAATGTTTTGCTTTCATCATTCGTTTTAGAA 1260
  1261  GGAATCACCTAAAAATTAAACCTCATAGATTATTTTACGTGCCGGTAAAGCATAACTCAATGTCTCCGTT 1330
  1331  TCAGACTTGTCTCCCCGCCGGAACATACTGCGATATTATTTCCGGCGCCAGGTCTGGTAACCGCTGCACT 1400
  1401  GGAAAATCTATTGTAGTAGGCAGCGATGGTCGCGCTCTTATCATCCACCGCAGCAACGAGTATGACATGA 1470
  1471  TGGTTGCTATCCATAGGGGTGCTGACGTAAGTATTTTTTATTGACTTAAATCTACTTCTTTGTCCTAAGT 1540
  1541  CTCAAAAGCATAATATAAAAATAATCTTAAGTATAGTTGTTGCAAAGTACGACTGCCTTCCGCCCTTCGT 1610
  1611  TGTGTGAGTGACCAATCTGTATACGCGTCAATATAGGCTATATGTAGCTAATGTAGCTACTGACTGCTCA 1680
  1681  CATACCCGATCCTGTAGCCCTAATGGTAAACAGTCGACGTCGTCCTAAACACGTCATTACGGATCCTCCC 1750
  1751  GATCCACTAACGGTGCTTTTAAGTAACTCAAGCACCGGTCAGCGTCCTGGTGAAACCCGTCGCTTGCGAG 1820
  1821  GATGGGCTTGACGAGTGAATTAACCCATAGACACAGATGGTAGGACCTCTTGGGAGTCCGCGCGGGTAGG 1890
  1891  TACCACCGCCCTGCCTATTTCTGCCGTGAAGCAGTAATGCGTTTCGGTTTGAAGGGTAGGGCAGTCGTTG 1960
  1961  TGACTATACTGAGATCTTAGAACTTATATCTCAAGGTGGGATCCGCTGAGAAGATCCGGCGAGAAACTCA 2030
  2031  GTGGATTTTTTTTTAAAATTTTCTTAGATCGATGGACGAACTCACAGCCCACCTGATGTTACTGGAGCCT 2100
  2101  ATAGACATCTACAACGTAAATGCGCCACCCAGCTTGAGATATAAGTTCTAAGGTCTCAAGTATAGTTACA 2170
  2171  ATAGGTGTTAAGTGCTTACAAGTATAGTTGTCTGTGTCTGCAGAATATTTAATGCTCAAAGCAATTGAAT 2240
  2241  AGATTTTTTTAAGCATTAATTATTTTAATTATTAATTATTTCTTATTTTTTACAGTCGAGATTGTAAAAC 2310
  2311  GGGAACCGTACTGGCAAATATTTTGAAGACTGGAAATCTGAAAATTGCAAATATTAATTGTGAAATACAT 2380
  2381  TTATGTCTTTCTTATAATAATGTTTTAATTTTTATGATTAAGCGATTTTCTTATCAAGGTTATTGCTTTC 2450
  2451  TAAGATTAGAAATAATAAGATTTAATAAATACATAATATAAATATGAAATTTTTTTTTTAGAAAATATTG 2520
  2521  AAATTTAATAAAAGTCTATGGACTCCGGTAACCACTTCGAACCAGGTGGGCCGTGAGCTCGTCCACCCAT 2590
  2591  TAAAGCAATAAAAAAAATCTTTTAAAGGTTTTAGTGAGGTTTCTGAGGAAATTTAAATTCTTTTCTTGGT 2660
  2661  AAGCAAGTGTTCATATAGAATTTGTATAGCACATACGAACGTTGACGTCACTATACCTATACAATGTATT 2730
  2731  GTAACTCTCCTACATGAAACTGAAACACTTACCACTTCACATCTGGATATATTTCAATATTATTACAAAT 2800
  2801  CGATTTATTAAAGAAATATAATACTTTATCAACTTATTTGCCGATGATAGCATCATAATATTGCGTACCA 2870
  2871  GATTTTATGATAACCACAACAACAAACAGAATTCATTGCTAACGATTGGAAACCGGGTTAA          2931 3′
```

Figure 45 Nucleotide sequences of *α-amylase* genomic DNA fragment 3 consisted of 2,931 bp.

```
5′   1  GGCGCGCCCGAGCACGAAGAAATATGAATAGCAAAATTAAAAATAACAAATTTAATAAAATCAATTAGTA   70
    71  GGATTATTTATAAGCAAGAAGTTAACATTCCAACGCTGTGAAAAGCCATTTTTTTTTTAAAAACAGTTCC  140
   141  TTCAAATAACCGCTTTCAAAACACAAATGATTTCACAAATGAATCATTTGGCTGAAATAATGTGTTTTAG  210
   211  GTGTGAAAATTTGATTTTTATCGTACAAAATCATCTTGGATACGGAGCGCCGAATATTTATCAATGTTTT  280
   281  TTTGAACTTTTATCGAACGATTATGTTCATTATGTCGATAAATTTCGTGTATCGCAACACAGACCGAGAT  350
   351  TTTATCTTCAAGTATATATAACTTGGGTACGGGAGGCTGTTATCAGATTTATTTAATTATATCACACGAC  420
   421  AGGAGGTCCAGTATATTAGATAAGGTGAGCAATTTAAATTATAAATATTATTTAATTCCATGGACTAAGC  490
   491  TCTCATATAATTATAGAGAAAATGTGCGCACATAAAAATCAATAAATTATCATGTATCGGATTTGGAATC  560
   561  GTAATTTATTCGTGGTTGCAGCTAGTTTTATAAATTTCACTTATTAGCCGAAAAGCGCTTTTAAATTGTA  630
   631  AGTGTGAAACTGTGTGACTTAAGACTAATTTCATCAAGTACACTAAATGCGCTCACTCACTTTCGCATTC  700
   701  TCAATGATCACAAATAAATATACGCATTGGATAAATAATTTCTTCTTTATAAGGAGATTAAAATTGAATA  770
   771  AAAATTGATATTAAAATGAAGAAGTATTTGCATCTGTGTATACTATGTATGAGTAATAATTTTTAAAATG  840
   841  ACGGATTTTTTTCATAAGACCAGAACATTCAGGGCTTCGGCTAAACATCTCGTTTATATTATAATACAAA  910
   911  TATTTTTCTCTTGATTTGGGACATGCTTACAGGCTGAAGAATTAGTTCTCAGGTTTTATGAAAAGAATTA  980
   981  TATTACTCATGCTAGTATGCTTAGTAATAGGTATAGTTATGTTGATTGCTTAAAGTTATATTATTGAAAA 1050
  1051  AAAGTGTATATTATACTAAGGAAATTATTAAAAAATAATTATAACTAAGCTACTGAGACTAGTTCTAGAT 1120
  1121  TAGGCACTTTATTCATGCGCTATTTTTTGTATGGAAATGGTAGTGCAAGGTAGAGGGGGAAGAGGTCGAC 1190
  1191  CGAAGAAGACATGGATGGAGTGTGTGAATAACGATATGAGAGAGAGAGAGGAGTGAGTGTTGAGATGACG 1260
  1261  GCTGATAGAAGAGAATGGAAGAGAAAAATTAGCTGTGCCGACCCCACCTAGTGGGATAAGGTGGAGAAAA 1330
  1331  AGAAGAAGCACAAGTACATATTATTATATTATACTACTAAACCTTTCAAAACTTTCGCTTTGTTAGGATG 1400
  1401  TTTCGGTACATCCTTCTACTTTCGGCCGTGACTCTGGCGTTGGCCTATAAAAACCCTCACTATGCATCGG 1470
  1471  GTCGCACCACCATGGTACATCTCTTCGAATGGAAGTGGGATGACATCGCTGCTGAATGCGAAAGGTTCCT 1540
  1541  TGGACCCCGAGGATTCGGTGGTATTCAGGTAAGCAATGACAAACACTATGAATTCATAAATTCAAAATTT 1610
  1611  AAAAACCGTGCACGTCACATTAAATCTAAATTAAAAAATGATGACGTTCTTATTATTCGTCTTATCATTC 1680
  1681  ATCAATATCATTTCTGTAATTATCATAATAATGCATCCGCCGTATAGTTCTGACTCCTGACCTTGCACCT 1750
  1751  TCAGATTTCTACCCGTTTCGGTCTCTTTAGAATTCTTTAGGCAGTGTAAGGTTAACATTACGAGAGGAAT 1820
  1821  GCCAAAACCAATTGTCACGGTATTTTTATCAGAAGCCCCAAAATTTCTATAGCAATGGGATCATGTTCCT 1890
  1891  ACCTACAAGATGATAAAAAGCTATCGAACAAAATGGTACCTAGATACTTAAGCTAAATGTAAATAAACTA 1960
  1961  CATTAAAAAATGTTGTGAATTTTCTTAAAAAATGCGAAGAAACTTTTCCCCAACCTATTAAAAAGGCAGA 2030
  2031  TGATTTAAATCAGTGGATCCCGTAAGCACCATAAAGTATAATCCATTATCCAAATGTCTTTGTGGGACTC 2100
  2101  TTTAATAAGCTCTTTTTTTGAAACGGCTCTCCTACCGTTTGAGTCATTAACCACTACTTCGCTGCGGAAA 2170
  2171  CAAAACCATGATACTAATTAATGTCGGCTCATAGCCTGTCTTATCACCAGTACGTTCAAGTTCTAGTTAT 2240
  2241  AATATGGATATATGAGTATATCGTAGCTGAGGAATGTCGTCATAAAGATCAAAACCTTCTCTTCAATATT 2310
  2311  CCCTTGTAAGACAGTTATAGATAGTTTTGGTGGTAGGACCTCTTGTGAGTCCGCGCGGGTCTGTACCACC 2380
  2381  GCCCTGCCTATTTCTGCCGTGAAGCAGTAATGCGTTTTGGTTTGAAGTGTGGGGCAGCCGTTGTAACTAT 2450
  2451  ACTTGAGATCTTAGAACTTATATCTCAAGGTGGGTGGCACATTTACTTTTTAGATGTCCATGGGCTCCAG 2520
  2521  TAACTACTTAACACCAGGTCGGCTGTGAGCTCGTCCACCCATCTAAGCAATAAAGAAAAAAGTTCATTCA 2590
  2591  ATATTTTTTGATCTTCATTTTCATAGGTTTCGCCACCAAACGAGAATTTGGTAATCTGGTCCCGCAACCG 2660
  2661  TCCTTGGTGGGAGCGCTATCAACCAATCTCCTACCGTCTAGTAACAAGATCTGGAAATGAAAATCAATTT 2730
  2731  TCGAATATGGTGCGTCGCTGCAACAATGTTGGCGTCAGGTTTGTTGCATTACGACTTTGGTTCAAAATTT 2800
  2801  TGTAATTTAGTAATTGATAGAATTTATAAGACACCGTAGAGATGGCCTAAAATACAAATCATAGATATAA 2870
  2871  AAAATATAATAGCCAGCTCATCAATTGAAGTTTTTTTTGCTAGATAGATCAATGTGGTCACAGACTATCT 2940
  2941  GCTCCCAAATTTTTTTTTTTTTTATTGCCCTTGTCGGCAGACGAGCCTACGGCCCACCATCAGGTGGGCC 3010
  3011  GTATGCCGGTCTGCCTACAAGGGCAATAAAAAAAAAGAAGCGAATAACATTGTGTATTATGCACTTAGGA 3080
  3081  AATTGTTTCTATTAAATACGAAAAAAGGAGTATTATAGGGTACTACTCATATCTTACCGTAAACCTCAAA 3150
  3151  CGCGCGTTCTAGGTTTTAAAGTACTCATGGAAAAACGAATGTTACGGAAAGTTCGGCTTCCACTTTTCAT 3220
  3221  TACGCTTTGACTTTCAGGATTTATGTGGACGCCATCATCAACCACATGACTGGAACTTGGAATGAGAATG 3290
  3291  TTGGTACAGGTGGAAGCACGGCCAATTTCGAAAACTGGCACTATCCTGCTGTTCCTTACGGCAGGAATGA 3360
  3361  CTTCAACTGGCCTCATTGTGTCATTACTGGCAGCGACTACAACTGCTGCCCTGACAGAGTACGAGCATCT 3430
  3431  TATGTTATTTCTGTAGTGATGTTTGGGAAAACGCTTAAATTTATGTAGCTTTACGGGAACTAAGTTTTAT 3500
  3501  GGGAATCAAAATTAAATAATCCCAAATTAATTTGATTTGATTTGATTTTAGGTGCGCAACTGTGAATTGT 3570
  3571  CTGGTCTGAAAGACTTGAACCAAGGCTCTGATTATGTTAGACAGCAGATTCTAAATTATATGAACCGTCT 3640
  3641  TATTGACATGGGTGTTGCTGGTTTCAGTATGATAGTGACTTTTTTTTTATTGATTAGTTGGGTGGATGAG 3710
  3711  CTCACTTTGAGATGTAAGTTCTAAGATCTCAGTATAGTTACAACGGCTGCCCTACCCTTCAGACCGAAAC 3780
  3781  GCATTATTGCTTCACGGCAGAAATAGGTAAGGCGGTGGTACCTACCCGTGCGGACTCACAATAGATCCTA 3850
  3851  CCACCAGTTGTTAGTTTGTTATAACTTACCCTCCTCAACTTTACTGGTTTAAATAAATTATGGTTTCTCC 3920
  3921  AGAATTGATGCTGCAAAGCACATGTGGCCTCACGATTTGCGAGTGATCTACGATCGTCTCCGCAATTTAA 3990
  3991  ATACCGCCCACGGTTTCCCATCCGGTGCTCGTCCCTACATCTACCAAGAAGTCATCGACCTCGGTGGTGA 4060
  4061  AGCTATCAGTCGCAACGAATACACTCCACTTGCTGCCGTTACTGAGTTCAGATTTGGACTGGAACTCAGT 4130
  4131  CAAGCTTTCCAAAGAAGAAACCAGCTTAGATGGTTGGTTAACTGGGGTCCACAGTGGGGCCTTCTCGCTT 4200
  4201  CTGGAGACGCTTTGACTTTCATTGACAACCACGACAACCAGAGAGGTCATGGCGCTGGTGGAAACATCTT 4270
  4271  GACATACAAGCAATCCCGACAATACAAGGGCGCTATCGCTTTCATGTTGGCACATCCTTATGGCTACCCT 4340
  4341  CAATTGATGAGTAGTTTCGCCTTCACGGACACCGAAGCTGGACCTCCAATGAACAGCAGGGGTGATATCA 4410
  4411  CCTCTCCTACTATCAACGCTGTATGTATTGACATGACATTATGTAATCAAGTCCATTTCGCTTTTCAAAG 4480
  4481  GTTAATTTGCATTTATTCAACAGATAATTGTGCTATCGTTCGATTAGATAAAGTTAATATAGAATGTTTG 4550
```

Figure 46 Nucleotide sequences of *α-amylase* genomic DNA of Nanglai consisted of 6,941 bp.

```
4551  TATTATTCTTGATCTGTTTTGTACCTACCAAGAAATAAATCAATTATATCGCGTTGTCGATCTAGTGCTT  4620
4621  AGTGACCAACTATTTTACAACGGGTTATTTGGTATTTTTTTATCCGAAACAGGATAATTCTTGCGGCAAT  4690
4691  GGTTGGATATGCGAGCACCGTTGGCGTCAGATCCACAACATGGTTGTCTTCAGGAACACCGCTGGTAACG  4760
4761  GTGCCCTTACCAACTGGTGGGACAACGGTAGCAACCAGATTGCTTTCTGCCGCGGCAACCAGGCCTTCAT  4830
4831  TGCTTTCAACAACGATGCATGGGACATGGACCAGACTCTTCAGGTTTTTATTTTATTTTTATTAGGCAAA  4900
4901  TGAGTGATATCGGTTTGATAGATATATTAAGTATGATGAGAATTGACCAAATCATTTTGATTTTAAGCTA  4970
4971  TTTAACACGCTAGCATCGGAGTGGATAAGACAGTTGAACTTGTTTTTAATAGTGCATGGTATCAGTAGTT  5040
5041  ATAAATATCTTATGAACAGATATGAATAGAAGGGTTATTTTGATAATATCCTGAAAAGCAGTTTTTTCAA  5110
5111  TAAAATCATTTTTTCTTACACTATTTTTAATTCAAATTGATTGCAATTTATTTTCTATTTTTTAGTTGGA  5180
5181  TTTCCGATAAAAGCGTATTATCTTTGTTTTTTTTTTAAAGCTATTATTTATTGTAAAAAAAAATGTTTTG  5250
5251  CTTTCATCATTCGTTTTAGAAGGAATCACCTAAAAATTAAACCTCATAGATTATTTTACGTGCCGGTAAA  5320
5321  GCATAACTCAATGTCTCCGTTTCAGACTTGTCTCCCCGCCGGAACATACTGCGATATTATTTCCGGCGCC  5390
5391  AGGTCTGGTAACCGCTGCACTGGAAAATCTATTGTAGTAGGCAGCGATGGTCGCGCTCTTATCATCCACC  5460
5461  GCAGCAACGAGTATGACATGATGGTTGCTATCCATAGGGGTGCTGACGTAAGTATTTTTTATTGACTTAA  5530
5531  ATCTACTTCTTTGTCCTAAGTCTCAAAAGCATAATATAAAAATAATCTTAAGTATAGTTGTTGCAAAGTA  5600
5601  CGACTGCCTTCCGCCCTTCGTTGTGTGAGTGACCAATCTGTATACGCGTCAATATAGGCTATATGTAGCT  5670
5671  AATGTAGCTACTGACTGCTCACATACCCGATCCTGTAGCCCTAATGGTAAACAGTCGACGTCGTCCTAAA  5740
5741  CACGTCATTACGGATCCTCCCGATCCACTAACGGTGCTTTTAAGTAACTCAAGCACCGGTCAGCGTCCTG  5810
5811  GTGAAACCCGTCGCTTGCGAGGATGGGCTTGACGAGTGAATTAACCCATAGACACAGATGGTAGGACCTC  5880
5881  TTGGGAGTCCGCGCGGGTAGGTACCACCGCCCTGCCTATTTCTGCCGTGAAGCATAATGCGTTTCGGTTT  5950
5950  GAAGGGTAGGGCAGTCGTTGTGACTATACTGAGATCTTAGAACTTATATCTCAAGGTGGGATCCGCTGAG  6020
6021  AAGATCCGGCGAGAAACTCAGTGGATTTTTTTTTAAAATTTTCTTAGATCGATGGACGAACTCACAGCCC  6090
6091  ACCTGATGTTACTGGAGCCTATAGACATCTACAACGTAAATGCGCCACCCAGCTTGAGATATAAGTTCTA  6160
6230  AGGTCTCAAGTATAGTTACAATAGGTGTTAAGTGCTTACAAGTATAGTTGTCTGTGTCTGCAGAATATTT  6230
6301  AATGCTCAAAGCAATTGAATAGATTTTTTTAAGCATTAATTATTTTAATTATTAATTATTTCTTATTTTT  6300
6371  TACAGTCGAGATTGTAAAACGGGAACCGTACTGGCAAATATTTTGAAGACTGGAAATCTGAAAATTGCAA  6370
6441  ATATTAATTGTGAAATACATTTATGTCTTTCTTATAATAATGTTTTAATTTTTATGATTAAGCGATTTTC  6440
6511  TTATCAAGGTTATTGCTTTCTAAGATTAGAAATAATAAGATTTAATAAATACATAATATAAATATGAAAT  6510
6581  TTTTTTTTTAGAAAATATTGAAATTTAATAAAAGTCTATGGACTCCGGTAACCACTTCGAACCAGGTGGG  6580
6651  CCGTGAGCTCGTCCACCCATTAAAGCAATAAAAAAAATCTTTTAAAGGTTTTAGTGAGGTTTCTGAGGAA  6650
6721  ATTTAAATTCTTTTCTTGGTAAGCAAGTGTTCATATAGAATTTGTATAGCACATACGAACGTTGACGTCA  6720
6791  CTATACCTATACAATGTATTGTAACTCTCCTACATGAAACTGAAACACTTACCACTTCACATCTGGATAT  6790
6861  ATTTCAATATTATTACAAATCGATTTATTAAAGAAATATAATACTTTATCAACTTATTTGCCGATGATAG  6860
6861  CATCATAATATTGCGTACCAGATTTTATGATAACCACAACAACAAACAGAATTCATTGCTAACGATTGGA  6930
6931  AACCGGGTTAA                                                            6941 3′
```

Figure 46 (Continued) Nucleotide sequences of *α-amylase* genomic DNA of Nanglai consisted of 6,941 bp.

6. Cloning and sequencing of the 5'- end *α-amylase* cDNA (5'-RACE)

6.1 *B. mori* RNA isolation

Total RNA from head of the mid 5th instar larva of Nanglai strain was extracted by using Isogen reagent. The isolated total RNA had an A 260/A280 ratio of 1.7029 suggesting that the RNA had a little protein contamination. The RNA yield was 5,037 ng/μl.

6.2 PCR Amplification

5'-RACE were performed to generate the full-length *α-amylase* cDNAs using the FirstChoice® RLM-RACE Kit (Ambion, USA). Total RNA was treated with

Calf Intestine Alkaline Phosphatase (CIP) to remove free 5'-phosphates from molecules such as ribosomal RNA, fragmented mRNA, tRNA, and contaminating genomic DNA. The CIP'd RNAs were treated with Tobacco Acid Pyrophosphatase (TAP) to remove the cap structure from full-length mRNAs, leaving 5'-monophosphate mRNAs. The 45 base RNA adapter oligonucleotides (5'-GCUGAUGGCGAUGAAUGAACACUGCGUUUGCUGGCUUUGAUGAAA-3') were ligated to the CIP/TAP-treated RNAs using T4 RNA ligase. During the ligation reaction, the majority of the full length decapped mRNAs acquire the adapter sequence as their 5' end. The *α-amylase* cDNA was amplified by RT-PCR with M-MLV Reverse Transcriptase enzyme. 5' RLM-RACE was amplified by using nested PCR with two pairs of primers. The first pair for amplication the outer 5'RLM-RACE was 5' RACE outer primer and 5' RACE gene-specific primer and the second amplifying the inner 5'RLM-RACE was 5' RACE inner primer and 5' RACE gene specific inner primer. The PCR product of Inner 5' RLM-RACE PCR was approximately 400 bp long (Figure 50). The Inner 5' RLM-RACE PCR fragments was detected by 1% agarlose gel and purified from agarose gel using QIAquick Gel Extration Kit. Then, the A-overhangs were added into the ends of these DNA fragments by used A-attachment reagent. The A-overhanged DNA fragments were cloned into pTA2 vector by T4 DNA ligase and transformed into *E. coli* DH5α by heat shock transformation.

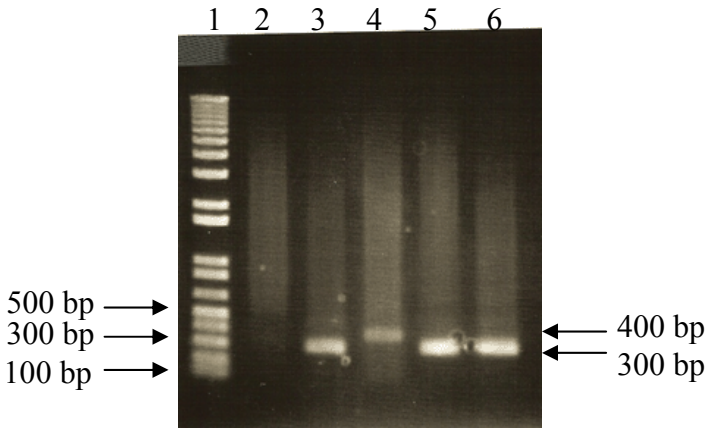


Figure 47 Agarose gel electrophoresis of the inner 5' RLM-RACE PCR fragments. Lane 1: 100 bp + 1.5 kb DNA ladder and Lane 3, 5 and 6: Inner 5' RLM-RACE PCR fragments. Lane 2 and 4: non-specific band.

6.3 Detection of DNA insertion by restriction digestion

Recombinant plasmids containing the inner 5' RLM-RACE PCR fragments of *α-amylase* gene were selected by the formation of blue/white colonies on LB ampicillin plate. White colonies having Inner 5' RLM-RACE PCR fragments were randomly selected for plasmid isolation and restriction analysis with *EcoR*I (Figure 51). The 453 bp sequence of the inner 5' RLM-RACE PCR fragment was shown in Figure 52.

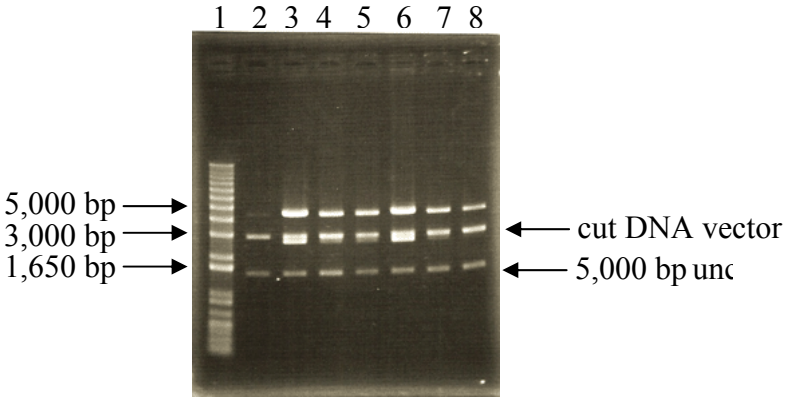


Figure 48 Agarose gel electrophoresis of recombinant vectors digested with *Eco*RI to select the expected inserted the inner 5' RLM-RACE PCR fragments. Lane 1: 100 bp + 1.5 kb DNA ladder and Lane 2-9: *Eco*RI digested recombinant plasmid clones.

```
5′   1  ATCAGATTTATTTAATTATATCACACGACAGGAGGTCCAGTATATTAGATAAGGATGTTTCGGTACATCC  70
    61  TTCTACTTTCGGCCGTGACTCTGGCGTTGGCCTATAAAAACCCTCACTATGCATCGGGTCGCACCACCAT  140
   141  GGTACATCTCTTCGAATGGAAGTGGGATGACATCGCTGCTGAATGCGAAAGGTTCCTTGGACCCCGAGGA  210
   211  TTCGGTGGTATTCAGGTTTCGCCACCAAACGAGAATTTGGTAATCTGGTCCCGCAACCGTCCTTGGTGGG  280
   281  AGCGCTATCAACCAATCTCCTACCGTCTAGTAACAAGATCTGGAAATGAAAATCAATTTTCGAATATGGT  350
   351  GCGTCGCTGCAACAATGTTGGCGTCAGGATTTATGTGGACGCCATCATCAACCACATGACTGGAACTTGG  420
   421  AATGAGAATGTTGGTACAGGTGGAAGCACGGCC                                        453  3′
```

Figure 49 Nucleotide sequences of the inner 5' RLM-RACE PCR fragments of *α-amylase* gene consisted of 453 bp. Initial codon ATG is in bold and underline.

6.4 Structure of *α-amylase* gene

Nucleotide sequence of *α-amylase* cDNA of Nanglai strain was expected by assembly the nucleotide sequence of 3'RACE (AMYT1) and the 5' RLM-RACE. Therefore, the putative *α-amylase* cDNA sequence of Nanglai strain was 1,678 bp long (Figure 50). The *α-amylase* cDNA sequence was compared to the *α-amylase* genomic DNA sequence. The result showed that the *α-amylase* gene consisted of 9 exon, at position nt 392- nt 444, nt 1,397- nt 1,568, nt 2,617- nt 2,768, nt 3,238- nt 3,418, nt 3,552- nt 3,667, nt 3,923- nt 4,431, nt 4,674- nt 4,874, nt 5,347- nt 5,508 and

nt 6,308-nt 6,406 respectively. The start codon ATG and stop codon TAA was located at the nt 1,398- nt 1,400 and the nt 6,317- nt 6,319 respectively (Figure 51).

```
5′   1  ATCAGATTTATTTAATTATATCACACGACAGGAGGTCCAGTATATTAGATAAGGATGTTTCGGTACATCC   70
    70  TTCTACTTTCGGCCGTGACTCTGGCGTTGGCCTATAAAAACCCTCACTATGCATCGGGTCGCACCACCAT  140
   141  GGTACATCTCTTCGAATGGAAGTGGGATGACATCGCTGCTGAATGCGAAAGGTTCCTTGGACCCCGAGGA  210
   211  TTCGGTGGTATTCAGGTTTCGCCACCAAACGAGAATTTGGTAATCTGGTCCCGCAACCGTCCTTGGTGGG  280
   281  AGCGCTATCAACCAATCTCCTACCGTCTAGTAACAAGATCTGGAAATGAAAATCAATTTTCGAATATGGT  350
   351  GCGTCGCTGCAACAATGTTGGCGTCAGGATTTATGTGGACGCCATCATCAACCACATGACTGGAACTTGG  420
   421  AATGAGAATGTTGGTACAGGTGGAAGCACGGCCAATTTCGAAAACTGGCACTATCCTGCTGTTCCTTACG  490
   491  GCAGGAATGACTTCAACTGGCCTCATTGTGTCATTACTGGCAGCGACTACAACTGCTGCCCTGACAGAGT  560
   561  GCGCAACTGTGAATTGTCTGGTCTGAAAGACTTGAACCAAGGCTCTGATTATGTTAGACAGCAGATTCTA  630
   631  AATTATATGAACCGTCTTATTGACATGGGTGTTGCTGGTTTCAGAATTGATGCTGCAAAGCACATGTGGC  700
   701  CTCACGATTTGCGAGTGATCTACGATCGTCTCCGCAATTTAAATACCGCCCACGGTTTCCCATCCGGTGC  770
   771  TCGTCCCTACATCTACCAAGAAGTCATCGACCTCGGTGGTGAAGCTATCAGTCGCAACGAATACACTCCA  840
   841  CTTGCTGCCGTTACTGAGTTCAGATTTGGACTGGAACTCAGTCAAGCTTTCCAAAGAAGAAACCAGCTTA  910
   911  GATGGTTGGTTAACTGGGGTCCACAGTGGGGCCTTCTCGCTTCTGGAGACGCTTTGACTTTCATTGACAA  980
   981  CCACGACAACCAGAGAGGTCATGGCGCTGGTGGAAACATCTTGACATACAAGCAATCCCGACAATACAAG 1050
  1051  GGCGCTATCGCTTTCATGTTGGCACATCCTTATGGCTACCCTCAATTGATGAGTAGTTTCGCCTTCACGG 1120
  1121  ACACCGAAGCTGGACCTCCAATGAACAGCAGGGGTGATATCACCTCTCCTACTATCAACGCTGATAATTC 1190
  1191  TTGCGGCAATGGTTGGATATGCGAGCACCGTTGGCGTCAGATCCACAACATGGTTGTCTTCAGGAACACC 1260
  1261  GCTGGTAACGGTGCCCTTACCAACTGGTGGGACAACGGTAGCAACCAGATTGCTTTCTGCCGCGGCAACC 1330
  1331  AGGCCTTCATTGCTTTCAACAACGATGCATGGGACATGGACCAGACTCTTCAGACTTGTCTCCCCGCCGG 1400
  1401  AACATACTGCGATATTATTTCCGGCGCCAGGTCTGGTAACCGCTGCACTGGAAAATCTATTGTAGTAGGC 1470
  1471  AGCGATGGTCGCGCTCTTATCATCCACCGCAGCAACGAGTATGACATGATGGTTGCTATCCATAGGGGTG 1540
  1541  CTGACTCGAGATTGTAAAACGGGAACCGTACTGGCAAATATTTTGAAGACTGGAAATCTGAAAATTGCAA 1610
  1611  ATATTAATTGTGAAATACATTTATGTCTTTCTTAAAAAAAAAAAAAAACCTGCCCGGGCGGCCGCTCGA   1678
```

Figure 50 Nucleotide sequences of Nanglai *α-amylase* cDNA consisted of 1,678 bp. Initial codon ATG and stop codon TAA are in bold and underline.

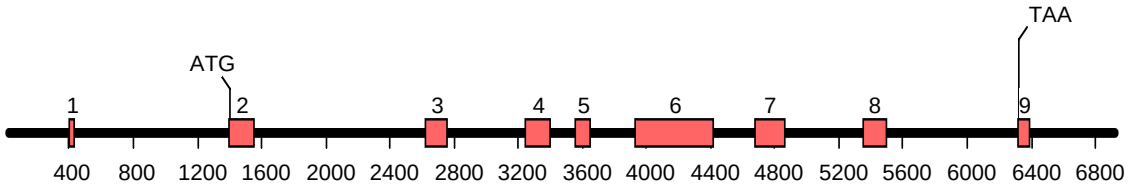


Figure 51 The putative *α-amylase* genomic DNA sequence of Nanglai strain.The red boxes are exon.

7. Construction of vectors

The full length cDNA of *α-amylase* gene was cloned into two plasmids, *pBac*[A3-AMY1-A3UTR, 3xP3-DsRed2] and *pBac*[A3-AMY1-SV40UTR, A3-EGFP].

7.1 Construction of *pBac*[A3-AMY1-A3UTR, 3xP3-DsRed2]

pBac[A3-AMY1-A3UTR, 3xP3-DsRed2] was constructed from *pBac*(3xP3-DsRedaf), containing *DsRed* reporter gene. In the *piggyBac* vector *α-amylase* cDNA was under the control of an *Actin3* promoter and *Actin3* terminator sequence. The *Actin3* promoter and *Actin3* terminator were amplified from the plasmid A3 (kindly provided by Dr.Tamura) and *α-amylase* gene was amplified from the AMYT-1 plasmid.

7.1.1 Amplification and sequencing of *Acin3* promoter and *Acin3* terminator

Oligonucleotide primers were designed based on the sequences of *Bombyx mori* cytoplasmic *Actin* (A3) gene [GenBank Accession No. U49854]. The promoter and terminator of *Actin3* gene were amplified by PCR. The plasmid A3 was used as a template, the primers A3FselIF primer (F1) and A3DSXF primer (R1) for amplified promoter and the primers FAcPAF primer (F3) and AcPAR4 primer (R3) for amplified terminator of *Actin3* gene to get approximately 800 bp and 1,000 bp, respectively (Figure 52). The two PCR products was purified from agarose gel, ligated into pGEM-T® easy vectors and transformed into *E. coli* JM109 by heat shock transformation.

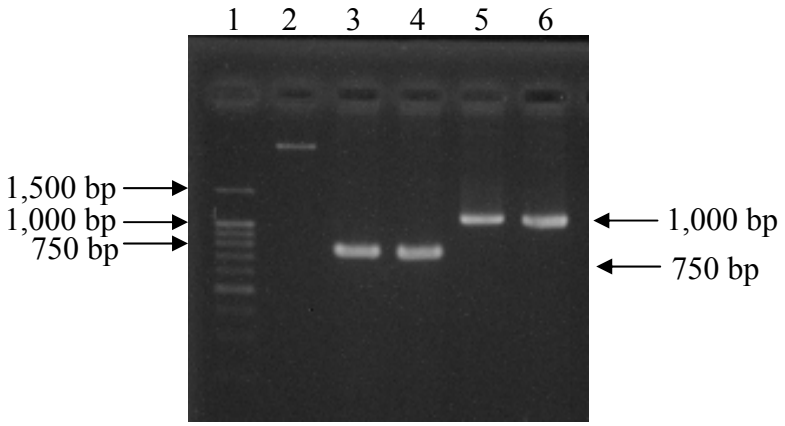


Figure 52 PCR products of *Actin3* promoter and terminator. Lane 1: 100 bp + 1.5 kb DNA ladder, Lane 2: Lamda marker 200 ng, Lane 3-4: PCR products of *Actin3* promoter and Lane 5-6: PCR products of *Actin3* terminator.

7.1.2 Detection of DNA Insertion by Restriction Digestion

Recombinant plasmids containing either the *Actin3* promoter fragment or *Actin3* terminator fragment were selected and examined by restriction analysis with *Apa*I and *Sac*I to get approximately 800 bp and 1,000 bp fragments, respectively (data not show). The fragments were then sequenced to get exactly size. The result showed that the *Actin3* promoter and *Actin3* terminator fragments was 820 bp and 1,158 bp long respectively (Figure 53 and Figure 54).

```
5′   1  GGCCGGCCTGAGTCAGCCCGCGATTGGTGGAAAAACAAACTGGAGCCGATACTGTGTAAATTGTGATAAC  70
    71  GGCTCTTTTATATAGTTTATCCTCACGAGTCGGTTCTCATTTACTAAGGTGTGCTCGAACAGTGCGCATT  140
   141  CGCATCTACGTACTTGTCACTTATTTAATAATACTATGTAAGTTTTAATTTTAAAATTGCGAAAGAAAAA  210
   211  AAACATATTTATTTATTTGTAAAATTTGAATTTCGAAGGTTCTCCGTCCCTTTACCTTTAAGTATTACAT  280
   281  ATGTTTGAGTGTTTTTTTTTTTTAATAATACGCTAATGATAACGTGTTACGTTACATAATTGTTGCATAA  350
   351  CTAGTGAAGTGAAATTTTTTATAAAAAAAACAGTTTTCGGAATTAGTGTACTGCAGATGTTAATAAACAC  420
   421  TACTAAATAAGAAATAAGTTTATTGGACGCACATTTCAAAGTGTCCACTCGCATCGATCAATTCGGAAAC  490
   491  AGAAATTGGGAACAGTGAATTATGAATCTTATACAGTTTTCTTTAACGTCACTAAATAGATGGACGCAAA  560
   561  TAAATTTGTCGTTTACTTAGTATAATGTATGGAATGAGAATGTAGTTTGAATTGTTTTTTTTCTTTTCTT  630
   631  GCAGACTAATTCAAGATGTGCGACGAAGAAGTTGCCGCGTTGGTAGTAGACAATGGCTCCAGTATGTGCA  700
   701  AGGCCGGTTTCGCAGGAGATGATGCTCCTCGCGCCGTGTTCCCCTCGATCGTCGGAAGGCCCCGCCATCA  770
   771  GGGCGTGATGGTCGGCATGGGACAGAAGGACTCGTACGGGCAATGTTTCG                       820  3′
```

Figure 53 Nucleotide sequences of *Actin3* promoter consisted of 820 bp.

```
5′   1  CTGTGCGATGGCCTCGAGAAGTCTTACGAACTTCCCGACGGTCAGGTCATCACCATCGGAAACGAAAGAT   70
    71  TCCGTTGCCCAGAGGCCCTCTTCCAACCCTCGTTCTTGGGTATGGAAGCCAACGGCATCCACGAAACCAC  140
   141  ATACAACTCCATCATGAAGTGCGACGTGGACATCCGTAAGGACTTGTACGCCAACACCGTATTGTCCGGT  210
   211  GGTACCACCATGTACCCTGGAATCGCCGACCGTATGCAAAAGGAAATCACAGCTCTCGCCCCATCGACAA  280
   281  TGAAGATTAAGATCATCGCTCCCCCAGAGAGGAAGTACTCCGTATGGATCGGTGGATCGATCCTCGCCTC  350
   351  CCTCTCTACCTTCCAACAGATGTGGATCTCGAAACAGGAGTACGACGAGTCTGGTCCCTCCATTGTACAC  420
   421  AGGAAGTGCTTCTAAGCGTTGAGACTTTAAGTTATGATGCCCTACAGCAGAACCTCAAGAGGGTGGCTCA  490
   491  AATTACGCTTGTGATCTTGTAAATAAATTCAGTATTTAATGTAGGTTGTAAGGTATTGTAATATGCATAT  560
   561  TACGTAAAACGAACGGAATGTTGTTGTTGCCGTTTTTTTTTTGACAAAGATTTTTATTTATTAAAGTTAC  630
   631  TAACCCCAAAACTTTTTAATAAAATAAATTTATATACCGGTATAATAACTGACGTTTTTCACTTGCTGTC  700
   701  CCCGCTCCCGACTAACAGTACGTCGTGTGCACCGAAATTACCGATTTCGTACACCGTTTGAGACAGTTAC  770
   771  GCTAGGAGCACAAATCTCCCAGCTGCATACCGTTGTTTACTGCAGTCTTTAATTGGAATGCGAGTCGTTG  840
   841  ACCGCTTAATACGAAACATTCTAAAATTCGCATCGTAACGCCATTAAAAATGCAAAGGAAACTGGTTCTG  910
   911  TACTTTCTACCTTTCAAAAGATCTGATTCACCAAATTAATTTTATGCGGACTCACTAATTCCGTAGAAAT  980
   981  CTGTGTTCGTTAATAATGAATCAGAGGTACCCAGGTTACGCTTAGGCATAAGATGACTGTTCGCGTTTTT 1050
  1051  ACAATACATACGAGCAGGTTACACACAAGATGAACATCCTTTGATGCGTCTGTGTCTTGAACCCGTCTGA 1120
  1121  GATTTGAGTGACTTGTCAACGTCATTGCGTGGCGCGCC                                 1158 3′
```

Figure 54 Nucleotide sequences of *Actin3* terminator consisted of 1,158 bp.

7.1.3 Assembly of *Actin3* promoter, *α-amylase* cDNA and *Actin3* terminator into a single DNA fragment

The *BmA3* plasmid was used as a template and the forward primer A3FselIF (F1) and reverse primer A3DSXF (R1) were used for amplification the 820 bp *Actin3* promoter fragment and the forward primer A3FselIF (F1) and reverse primer A3DSXF (R1) were used for amplification the 1,158 bp *Actin3* terminator fragment. Whereas, the *α-amylase* cDNA sequence was amplified from the 3'-RACE clone (AMYT-1) using forward primer AmyA3DSXF (F2) and reverse primer AmyRACE (R2) to get a 1,533 bp fragment (Figure 55).

The A-overhang at the ends of each three fragments was removed by using Klenow fragment enzyme. Then, the three PCR fragments were used as the template for establishment a 3,000 bp single fragment with forward primer A3FselIF (F1) and reverse primer AcPAR4 (R3). The approximately 3,000 bp PCR product (Figure 60) was purified from agarose gel, ligated into pGEM-T® easy vectors and transformed into *E. coli* JM109 by heat shock transformation. Recombinant plasmids containing the long DNA fragment (*Actin3* promoter - *α-amylase* cDNA - *Actin3* terminator) were selected and detected with *Apa*I and *Sac*I (Figure 56). The single DNA fragment was sequenced and found that its length was 3,472 bp (Figure 57).

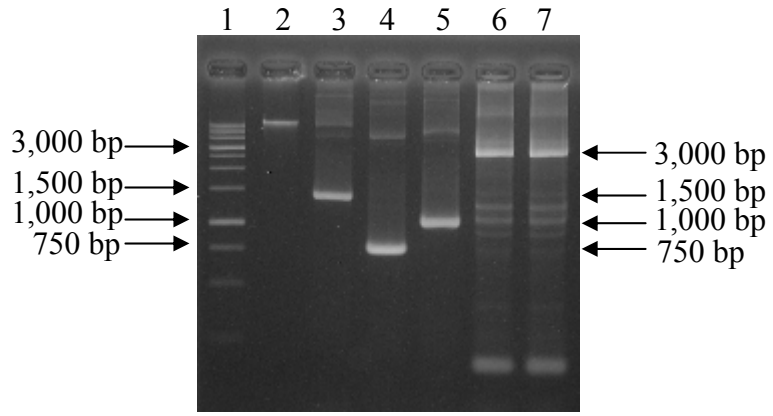


Figure 55 Agarose gel electrophoresis of PCR products of single DNA fragment (*Actin3* promoter - *α-amylase* cDNA - *Actin3* terminator). Lane 1: 100 bp + 1.5 kb DNA ladder, Lane 2: Lamda marker 200 ng, Lane 3: PCR products of *α-amylase* cDNA, Lane 4: PCR products of *Actin3* promoter, Lane 5: PCR products of *Actin3* terminator and Lane 6-7: PCR products of single DNA fragment (*Actin3* promoter - *α-amylase* cDNA - *Actin3* terminator).

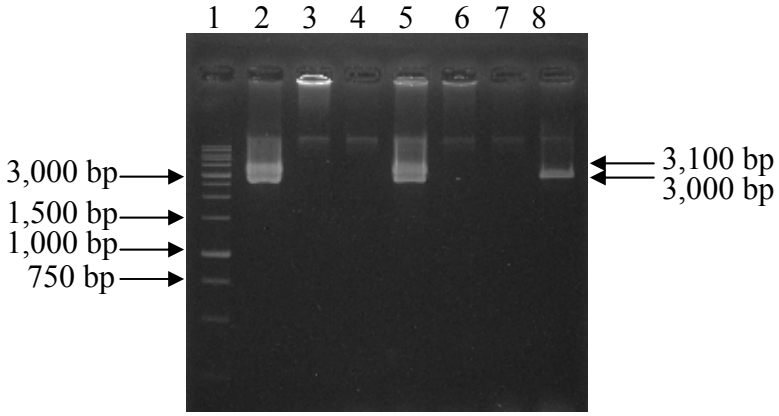


Figure 56 Agarose gel electrophoresis of recombinant vectors containing a single DNA fragment (*Actin3* promoter - *α-amylase* cDNA - *Actin3* terminator) were digested with *Apa*I and *Sac*I to select the expected inserted fragment. Lane 1: 100 bp + 1.5 kb DNA ladder, Lane 2-7: recombinant plasmid clones digested with *Apa*I and *Sac*I and Lane 8: control plasmid clones digested with *Apa*I and *Sac*I (blue colony).

```
5′   1  GGCCGGCCTGAGTCAGCCCGCGATTGGTGGAAAAACAAACTGGAGCCGATACTGTGTAAATTGTGATAAC   70
    71  GGCTCTTTTATATAGTTTATCCTCACGAGTCGGTTCTCATTTACTAAGGTGTGCTCGAACAGTGCGCATT  140
   141  CGCATCTACGTACTTGTCACTTATTTAATAATACTATGTAAGTTTTAATTTTAAAATTGCGAAAGAAAAA  210
   211  AAACATATTTATTTATTTGTAAAATTTGAATTTCGAAGGTTCTCCGTCCCTTTACCTTTAAGTATTACAT  280
   281  ATGTTTGAGTGTTTTTTTTTTTTAATAATACGCTAATGATAACGTGTTACGTTACATAATTGTTGCATAA  350
   351  CTAGTGAAGTGAAATTTTTTATAAAAAAAACAGTTTTCGGAATTAGTGTACTGCAGATGTTAATAAACAC  420
   421  TACTAAATAAGAAATAAGTTTATTGGACGCACATTTCAAAGTGTCCACTCGCATCGATCAATTCGGAAAC  490
   491  AGAAATTGGGAACAGTGAATTATGAATCTTATACAGTTTTCTTTAACGTCACTAAATAGATGGACGCAAA  560
   561  TAAATTTGTCGTTTACTTAGTATAATGTATGGAATGAGAATGTAGTTTGAATTGTTTTTTTTCTTTTCTT  630
   631  GCAGACTAATTCAAGATGTGCGACGAAGAAGTTGCCGCGTTGGTAGTAGACAATGGCTCCAGTATGTGCA  700
   701  AGGCCGGTTTCGCAGGAGATGATGCTCCTCGCGCCGTGTTCCCCTCGATCGTCGGAAGGCCCCGCCATCA  770
   771  GGGCGTGATGGTCGGCATGGGACAGAAGGACTCGTACGGGCAATGTTTCGGTACATCCTTCTACTTTCGG  840
   841  CCGTGACTCTGGCGTTGGCCTATAAAAACCCTCACTATGCATCGGGTCGCACCACCATGGTACATCTCTT  910
   911  CGAATGGAAGTGGGATGACATCGCTGCTGAATGCGAAAGGTTCCTTGGACCCCGAGGATTCGGTGGTATT  980
   981  CAGGTTTCGCCACCAAACGAGAATTTGGTAATCTGGTCCCGCAACCGTCCTTGGTGGGAGCGCTATCAAC 1050
  1051  CAATCTCCTACCGTCTAGTAACAAGATCTGGAAATGAAAATCAATTTTCGAATATGGTGCGTCGCTGCAA 1120
  1121  CAATGTTGGCGTCAGGATTTATGTGGACGCCATCATCAACCACATGACTGGAACTTGGAATGAGAATGTT 1190
  1191  GGTACAGGTGGAAGCACGGCCAATTTCGAAAACTGGCACTATCCTGCTGTTCCTTACGGCAGGAATGACT 1260
  1261  TCAACTGGCCTCATTGTGTCATTACTGGCAGCGACTACAACTGCTGCCCTGACAGAGTGCGCAACTGTGA 1330
  1331  ATTGTCTGGTCTGAAAGACTTGAACCAAGGCTCTGATTATGTTAGACAGCAGATTCTAAATTATATGAAC 1400
  1401  CGTCTTATTGACATGGGTGTTGCTGGTTTCAGAATTGATGCTGCAAAGCACATGTGGCCTCACGATTTGC 1470
  1471  GAGTGATCTACGATCGTCTCCGCAATTTAAATACCGCCCACGGTTTCCCATCCGGTGCTCGTCCCTACAT 1540
  1541  CTACCAAGAAGTCATCGACCTCGGTGGTGAAGCTATCAGTCGCAACGAATACACTCCACTTGCTGCCGTT 1610
  1611  ACTGAGTTCAGATTTGGACTGGAACTCAGTCAAGCTTTCCAAAGAAGAAACCAGCTTAGATGGTTGGTTA 1680
  1681  ACTGGGGTCCACAGTGGGGCCTTCTCGCTTCTGGAGACGCTTTGACTTTCATTGACAACCACGACAACCA 1750
  1751  GAGAGGTCATGGCGCTGGTGGAAACATCTTGACATACAAGCAATCCCGACAATACAAGGGCGCTATCGCT 1820
  1821  TTCATGTTGGCACATCCTTATGGCTACCCTCAATTGATGAGTAGTTTCGCCTTCACGGACACCGAAGCTG 1890
  1891  GACCTCCAATGAACAGCAGGGGTGATATCACCTCTCCTACTATCAACGCTGATAATTCTTGCGGCAATGG 1960
  1961  TTGGATATGCGAGCACCGTTGGCGTCAGATCCACAACATGGTTGTCTTCAGGAACACCGCTGGTAACGGT 2030
  2031  GCCCTTACCAACTGGTGGGACAACGGTAGCAACCAGATTGCTTTCTGCCGCGGCAACCAGGCCTTCATTG 2100
  2101  CTTTCAACAACGATGCATGGGACATGGACCAGACTCTTCAGACTTGTCTCCCCGCCGGAACATACTGCGA 2170
  2171  TATTATTTCCGGCGCCAGGTCTGGTAACCGCTGCACTGGAAAATCTATTGTAGTAGGCAGCGATGGTCGC 2240
  2241  GCTCTTATCATCCACCGCAGCAACGAGTATGACATGATGGTTGCTATCCATAGGGGTGCTGACTCGAGAT 2310
  2311  TGTAATGTGCGATGGCCTCGAGAAGTCTTACGAACTTCCCGACGGTCAGGTCATCACCATCGGAAACGAA 2380
  2381  AGATTCCGTTGCCCAGAGGCCCTCTTCCAACCCTCGTTCTTGGGTATGGAAGCCAACGGCATCCACGAAA 2450
  2451  CCACATACAACTCCATCATGAAGTGCGACGTGGACATCCGTAAGGACTTGTACGCCAACACCGTATTGTC 2520
  2521  CGGTGGTACCACCATGTACCCTGGAATCGCCGACCGTATGCAAAAGGAAATCACAGCTCTCGCCCCATCG 2590
  2591  ACAATGAAGATTAAGATCATCGCTCCCCCAGAGAGGAAGTACTCCGTATGGATCGGTGGATCGATCCTCG 2660
  2661  CCTCCCTCTCTACCTTCCAACAGATGTGGATCTCGAAACAGGAGTACGACGAGTCTGGTCCCTCCATTGT 2730
  2731  ACACAGGAAGTGCTTCTAAGCGTTGAGACTTTAAGTTATGATGCCCTACAGCAGAACCTCAAGAGGGTGG 2800
  2801  CTCAAATTACGCTTGTGATCTTGTAAATAAATTCAGTATTTAATGTAGGTTGTAAGGTATTGTAATATGC 2870
  2871  ATATTACGTAAAACGAACGGAATGTTGTTGTTGCCGTTTTTTTTTTGACAAAGATTTTTATTTATTAAAG 2940
  2941  TTACTAACCCCAAAACTTTTTAATAAAATAAATTTATATACCGGTATAATAACTGACGTTTTTCACTTGC 3010
  3011  TGTCCCCGCTCCCGACTAACAGTACGTCGTGTGCACCGAAATTACCGATTTCGTACACCGTTTGAGACAG 3080
  3081  TTACGCTAGGAGCACAAATCTCCCAGCTGCATACCGTTGTTTACTGCAGTCTTTAATTGGAATGCGAGTC 3150
  3151  GTTGACCGCTTAATACGAAACATTCTAAAATTCGCATCGTAACGCCATTAAAAATGCAAAGGAAACTGGT 3220
  3221  TCTGTACTTTCTACCTTTCAAAAGATCTGATTCACCAAATTAATTTTATGCGGACTCACTAATTCCGTAG 3290
  3291  AAATCTGTGTTCGTTAATAATGAATCAGAGGTACCCAGGTTACGCTTAGGCATAAGATGACTGTTCGCGT 3360
  3361  TTTTACAATACATACGAGCAGGTTACACACAAGATGAACATCCTTTGATGCGTCTGTGTCTTGAACCCGT 3430
  3431  CTGAGATTTGAGTGACTTGTCAACGTCATTGCGTGGCGCGCC                              3472
```

Figure 57 Nucleotide sequences of single DNA fragment (*Actin3* promoter - *α-amylase* cDNA - *Actin3* terminator) consisted of 3,472 bp.

7.1.5 Ligation of the single DNA fragment into *pBac*(3xP3-DsRedaf) vector

The single long DNA fragment was amplified from the plasmid and cut with *Fse*I/*Asc*I. The cut DNA fragment was inserted into *Fse*I/*Asc*I site of *pBac*(3xP3-DsRedaf) vector by T4 ligase enzyme yielding *pBac*(A3-AMY1-A3UTR,

3xP3-DsRed2) (Figure 58). This plasmid was transformed into *E. coli* JM109 by heat shock transformation. The recombinant plasmids containing the single DNA fragment were selected. The DNA insertion was detected by PCR using forward primer A3FselIF (F1) and reverse primer AcPAR4 (R3) and its sequence was examine to confirm that it was still the 3,472 bp fragment (Figure 59).

Figure 58 Structure of *pBac*(A3-AMY1-A3UTR, 3xP3-DsRed2) vector.

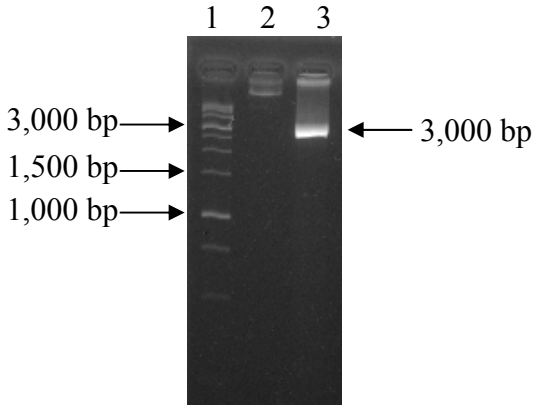


Figure 59 Agarose gel electrophoresis of recombinant *pBac*(A3-AMY1-A3UTR, 3xP3-DsRed2). Two clones were selected to examine the size of inserted DNA fragment by PCR using forward primer A3FselIF (F1) and reverse primer AcPAR4 (R3). Lane 1: 1 kb DNA ladder, Lane 2: no insert fragment and Lane 3: the 3,000 bp inserted fragment.

7.2 Constrution of *pBac*[A3-AMY1-SV40UTR, A3-EGFP]

pBac[A3-AMY1-SV40UTR, A3-EGFP] was constructed from *pBac*MCS(A3-SV40, A3-EGFP)R, which contained EGFP reporter gene. In this piggyBac vector, *α-amylase* cDNA was under the control of *Actin3* promoter and SV40 terminator.

7.2.1 Amplification and sequencing of *α-amylase* cDNA

Oligonucleotide primers were designed based on the *α-amylase* cDNA sequences from 3'-RACE. The *α-amylase* cDNA was amplified by using the plasmid AMYT-1 as a template and primers, AMY-XbaI.U primer (F4) and AMY-XbaI.L primer (R4). The approximately 1,500 bp PCR (Figure 60) was purified from agarose gel and the A-overhang was added into its ends using A-attachment reagent before ligation into pTA2 vector and transformation into *E. coli* DH5α by heat shock. Recombinant plasmids were randomly selected and detected with *Xba*I (Figure 61). The inserted single DNA fragment was 1,515 bp long (Figure 62).

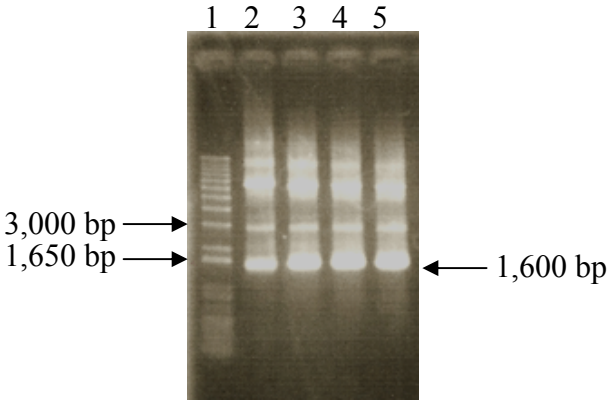


Figure 60 Agarose gel electrophoresis of PCR products from *α-amylase* cDNA in plasmid AMYT-1. Lane 1: 100 pb + 1 kb DNA ladder and Lane 2-5: PCR products of *α-amylase* cDNA from plasmid AMYT-1.

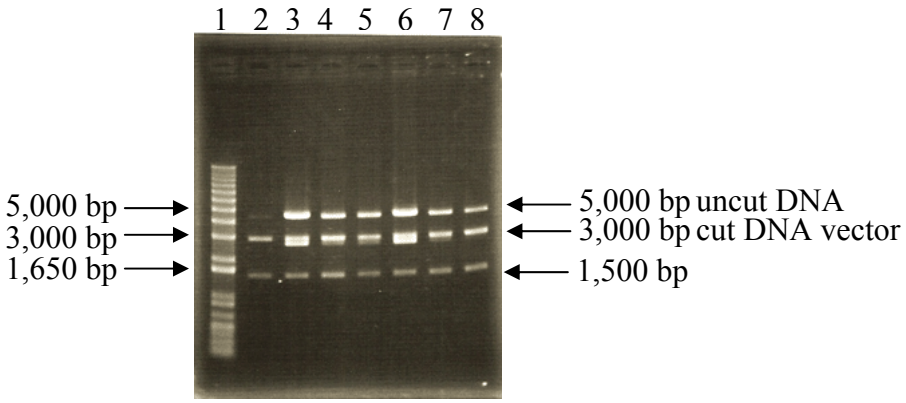


Figure 61 Agarose gel electrophoresis of recombinant vectors of *α-amylase* cDNA digested with *Xpa*I to select the expected inserted fragment. Lane 1: 100 pb + 1 kb DNA ladder and Lane 2-8: recombinant plasmid containing *α-amylase* cDNA digested with *Xba*I (white colonies).

```
5′   1  TCTAGAATGTTTCGGTACATCCTTCTACTTTCGGCCGTGACTCTGGCGTTGGCCTATAAAAACCCTCACT  70
    71  ATGCATCGGGTCGCACCACCATGGTACATCTCTTCGAATGGAAGTGGGATGACATCGCTGCTGAATGCGA  140
   141  AAGGTTCCTTGGACCCCGAGGATTCGGTGGTATTCAGGTTTCGCCACCAAACGAGAATTTGGTAATCTGG  210
   211  TCCCGCAACCGTCCTTGGTGGGAGCGCTATCAACCAATCTCCTACCGTCTAGTAACAAGATCTGGAAATG  280
   281  AAAATCAATTTTCGAATATGGTGCGTCGCTGCAACAATGTTGGCGTCAGGATTTATGTGGACGCCATCAT  350
   351  CAACCACATGACTGGAACTTGGAATGAGAATGTTGGTACAGGTGGAAGCACGGCCAATTTCGAAAACTGG  420
   421  CACTATCCTGCTGTTCCTTACGGCAGGAATGACTTCAACTGGCCTCATTGTGTCATTACTGGCAGCGACT  490
   491  ACAACTGCTGCCCTGACAGAGTGCGCAACTGTGAATTGTCTGGTCTGAAAGACTTGAACCAAGGCTCTGA  560
   561  TTATGTTAGACAGCAGATTCTAAATTATATGAACCGTCTTATTGACATGGGTGTTGCTGGTTTCAGAATT  630
   631  GATGCTGCAAAGCACATGTGGCCTCACGATTTGCGAGTGATCTACGATCGTCTCCGCAATTTAAATACCG  700
   701  CCCACGGTTTCCCATCCGGTGCTCGTCCCTACATCTACCAAGAAGTCATCGACCTCGGTGGTGAAGCTAT  770
   771  CAGTCGCAACGAATACACTCCACTTGCTGCCGTTACTGAGTTCAGATTTGGACTGGAACTCAGTCAAGCT  840
   841  TTCCAAAGAAGAAACCAGCTTAGATGGTTGGTTAACTGGGGTCCACAGTGGGGCCTTCTCGCTTCTGGAG  910
   911  ACGCTTTGACTTTCATTGACAACCACGACAACCAGAGAGGTCATGGCGCTGGTGGAAACATCTTGACATA  980
   981  CAAGCAATCCCGACAATACAAGGGCGCTATCGCTTTCATGTTGGCACATCCTTATGGCTACCCTCAATTG 1050
  1051  ATGAGTAGTTTCGCCTTCACGGACACCGAAGCTGGACCTCCAATGAACAGCAGGGGTGATATCACCTCTC 1120
  1121  CTACTATCAACGCTGATAATTCTTGCGGCAATGGTTGGATATGCGAGCACCGTTGGCGTCAGATCCACAA 1190
  1190  CATGGTTGTCTTCAGGAACACCGCTGGTAACGGTGCCCTTACCAACTGGTGGGACAACGGTAGCAACCAG 1260
  1261  ATTGCTTTCTGCCGCGGCAACCAGGCCTTCATTGCTTTCAACAACGATGCATGGGACATGGACCAGACTC 1330
  1331  TTCAGACTTGTCTCCCCGCCGGAACATACTGCGATATTATTTCCGGCGCCAGGTCTGGTAACCGCTGCAC 1400
  1401  TGGAAAATCTATTGTAGTAGGCAGCGATGGTCGCGCTCTTATCATCCACCGCAGCAACGAGTATGACATG 1470
  1471  ATGGTTGCTATCCATAGGGGTGCTGACTCGAGATTGTAATCTAGA                           1515 3′
```

Figure 62 Nucleotide sequences of *α-amylase* cDNA consisted of 1,515 bp.

7.2.3 Ligation of the single DNA fragment into *pBac*[A3-SV40, A3-EGFP]R vector

The *α-amylase* cDNA was cut by *Xba*I and cloned into *Xba*I site of *pBac*[A3-SV40, A3-EGFP]R vector using T4 ligase enzyme. The recombinant plasmid was name *pBac*[A3-SV40, A3-EGFP]R vector (Figure 63). The plasmid was

transformed into *E. coli* DH5α by heat shock. Recombinant plasmids containing *α-amylase* cDNA fragment were randomly selected and detected with *EcoR*V. The three fragments obtained from digestion the correct recombinant plasmids with *EcoR*V were 1,800 bp, 3,000 bp and 3,600 bp long (Figure 64). The *α-amylase* cDNA sequences cloned in *pBac*[A3-AMY1-SV40UTR, A3-EGFP] vector was confirmed and got the same 1,515 bp nucleotide (Figure 62).

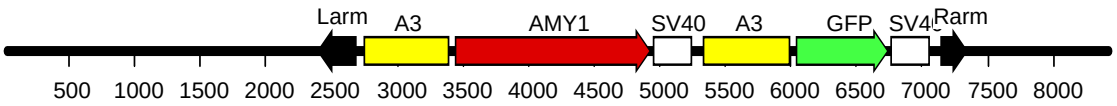


Figure 63 Stucture of *pBac*[A3-AMY1-SV40UTR, A3-EGFP] vector.

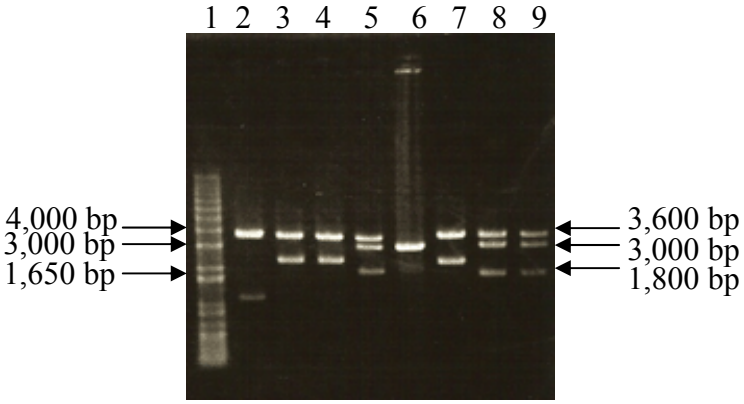


Figure 64 Agarose gel electrophoresis of recombinant *pBac*[A3-AMY1-SV40UTR, A3-EGFP] vector digested with *EcoR*V to select the expected inserted fragment. Lane 1: 100 pb + 1 kb DNA ladder and Lane 2-9: recombinant *pBac*[A3-AMY1-SV40UTR, A3-EGFP] vector digested with *EcoR*V. Lane 5, 8 and 9: the correct recombinant *pBac*[A3-AMY1-SV40UTR, A3-EGFP] vector.

8. Silkworm transformation

8.1 Embryonic microinjection and screening of transformed silkworm

The *pBac*[A3-AMY1-A3UTR, 3xP3-DsRed2] and *pBac*[A3-AMY1-SV40UTR, A3-EGFP] vector was contained *DsRed* fluorescence and GFP fluorescence as a reporter gene respectively. Each *piggyBac* vector, *pBac*[A3-AMY1-A3UTR, 3xP3-DsRed2] or *pBac*[A3-AMY1-SV40UTR, A3-EGFP] was mixed with the helper plasmid pHA3PIG and injected into pre-blastoderm silkworm embryos (3 hours after laid eggs). Embryos hatching from the injected eggs with *pBac*[A3-AMY1-A3UTR, 3xP3-DsRed2] and with *pBac*[A3-AMY1-SV40UTR, A3-EGFP] were 22.21% and 25.4% . All transient moths (G0) of the both vectors were sibling mated and backcrosses mated (Table 4 and Table 5 respectively) to get the G1 eggs.

The G1 broods were screened under a Leika MZFL III fluorescence microscope (Leika, Japan). Fifty nine broods transgenic silkworms (G1) of *pBac*[A3-AMY1-A3UTR, 3xP3-DsRed2] were observed by screening for *DsRed* expression in the stemmata of embryos and in the compound eyes of moths (Figure 65). Moths (G1) expressing *DsRed* were sibling mated and backcrosses mated. The transgenic silkworms G2 were used for detected expression of *α-amylase* gene (section 12). The transgenic lines contained *pBac*[A3-AMY1-A3UTR, 3xP3-DsRed2] was named Y1.1 for the first detected brood of *DsRed* positive and Y1 mix for the remaining positive broods mixed together. Whereas, seventy five broods transgenic silkworms (G1) from the G0 injected with *pBac*[A3-AMY1-SV40UTR, A3-EGFP] could not seen the GFP expression in the whole body of embryos.

Table 4 Injection of *pBac*[A3-AMY1-A3UTR, 3xP3-DsRed2] vectors in silkworm embryos of the strain pnd-wl.

No. of injection	Number of injected embryos	Number of hatched embryos	Number of fertile moths	Number of single-pair mated	Number of backcrosses mated	Number of total G1 broods	Number of G1 broods with *DsRed* positive(%)
1st	561	123	77	28	9	37	2 (5.4)
2nd	443	103	60	21	0	21	0 (0.0)

Table 5 Injection of *pBac*[A3-AMY1-SV40UTR, A3-EGFP] vectors in silkworm embryos of the strain pnd-wl.

No. of injection	Number of injected embryos	Number of hatched embryos	Number of fertile moths	Number of single-pair mated	Number of backcrosses mated	Number of total G1 broods	Number of G1 broods with *DsRed* positive(%)
1st	574	78	50	21	5	26	0 (0.0)
2nd	443	180	128	49	0	49	0 (0.0)

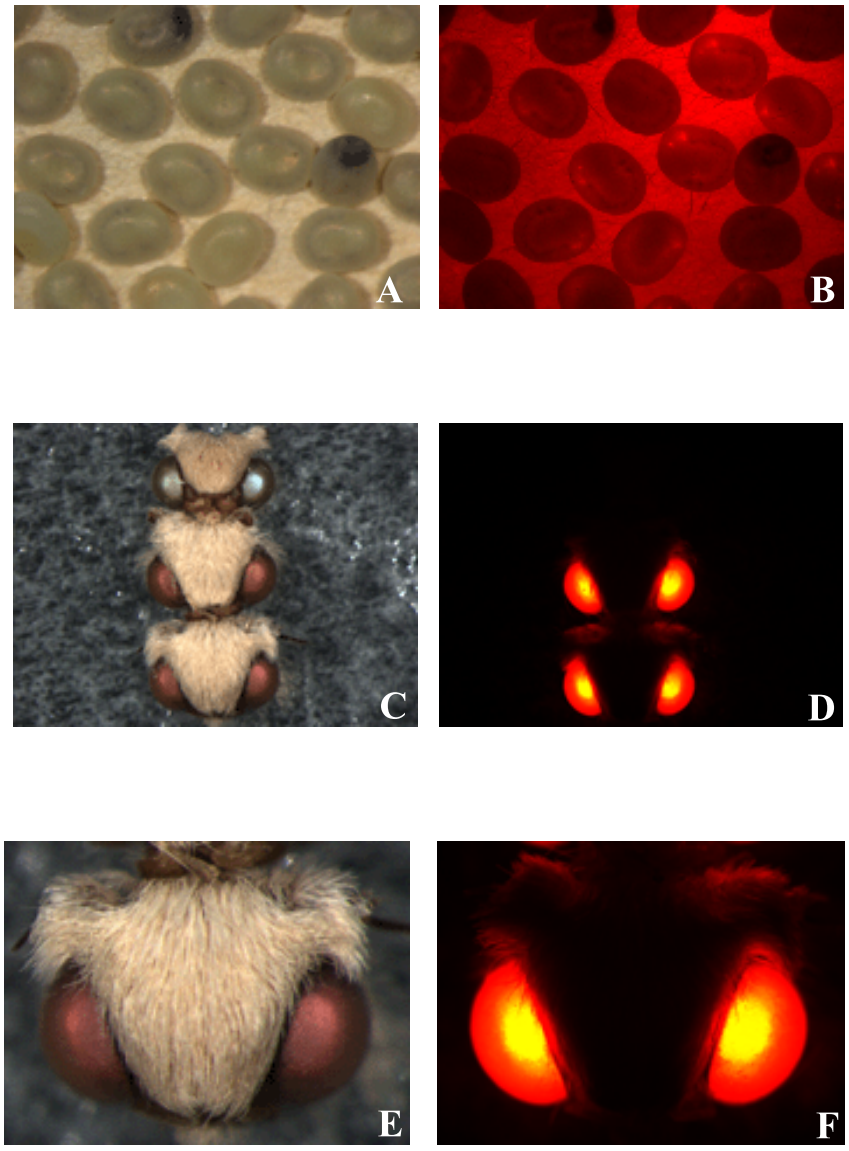


Figure 65 Transgenic silkworms bearing pBac [A3-AMY1-A3UTR, 3xP3-DsRed2]**.** G1 broods with *DsRed* positive embryos at the eighth day of embryonic development were observed under white light (A). Transformed eggs displayed *DsRed* positive at stemmata (arrows), compared with non transgenic eggs (B). Head of adults wild type (upper) and transgenic (lower) silkworms were observed under white light (C) and under light of the *DsRed* fluorescence (D). Panel E and F show a magnification of a *DsRed* positive head of adult in panel C and D.

9. Detection the *α-amylase* expression by RT-PCR

9.1 *B. mori* RNA Isolation

Total RNA was extracted from several tissues of the mid 5th instar larva of *B. mori*, Nanglai and C108 strains including heamolymph, salivary gland, malphighian gland, silk gland, gut and fat body and yielded 810, 2,560, 5,544, 4,109, 4,286 and 1,389 ng/µl respectively for Nanglai, and 1,425, 786, 10,081, 9,253, 11,990 and 8,272 ng/µl respectively for C108.

9.2 One step RT-PCR amplification

Oligonucleotide primers for RT-PCR amplification the *α-amylase* and *Actin3* genes were designed based on the sequences of *α-amylase* cDNA (AMYT-1) and the sequences of *Bombyx mori* cytoplasmic *Actin* (A3) gene [GenBank Accession No.U49854], respectively. The total RNA were used as templates for amplification the partial 1 and 2 of *α-amylase* with primer set 1 (AMY1(RT) 452U + AMY1(RT) 758L), primer set 2 (AMY1 (RT) 15U primer+AMY1 (RT) 378L primer) and partial *Actin3* gene with the primers actin.341U28 (RT) and actin.721L24(RT). The PCR products were 330 bp, 385 bp and 404 bp long respectively. The results showed that the two partials of *α-amylase* gene were amplified only from gut tissues of Nanglai strain, whereas no PCR product was amplified from C108 strain (Figure 66, panel A and B). In contrast, the partial *Actin3* gene was amplified from all tissues of both silkworm strains (Figure 66, panel C).

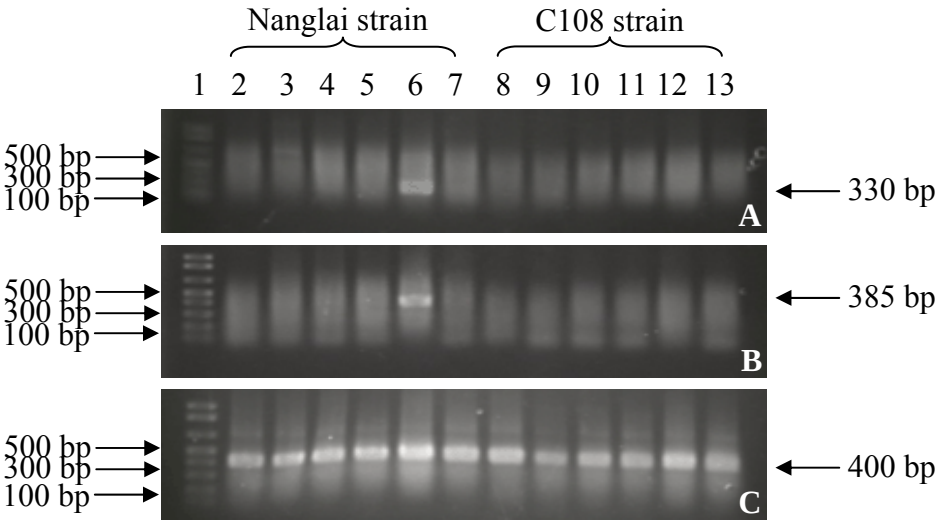


Figure 66 RT-PCR products of *α-amylase* amplified by primer set 1 and primer set 2 (panel A and B, respectively) and partial *Actin3* gene (panel C) from difference tissues of Nanglai and C108 strains. Lane 1: 100 pb + 1 kb DNA ladder, Lane 2-7: Tissues of Nanglai strain, Lane 2: heamolymph, Lane 3: salivary gland, Lane 4: malpigion tube, Lane 5: silk gland, Lane 6: gut, Lane 7: fat body. Lane 8-13: Tissues of C108 strain, Lane 8: heamolymph, Lane 9: salivary gland, Lane 10: malpigion tube, Lane 11: silk gland, Lane 12: gut, Lane 13: fat body.

9.3 RNA isolation from non transgenic w1-pnd, transgenic Y1.1 and Y1 mix strain

Total RNA was extracted from gut, fat body and silk gland of the mid 5th instar larva of non transgenic, transgenic Y1.1 and Y1 mix strains.

9.4 One step RT-PCR Amplification

The total RNAs were used as templates for amplified the partial 1 of *α-amylase* gene with primer set 2 AMY1(RT)15U + AMY1(RT)37L (385 bp). The PCR products were 385 bp long. The result showed that the partial 2 of *α-amylase* gene

was amplified from gut of non transgenic strain (Figure 67, Panel A) but could not be amplified from fat body and silk gland tissues (Figure 67, Panel B and C, respectively). While, in transgenic Y1.1and Y1 mix, the partial 2 of *α-amylase* gene could be amplified from all tissues (Figure 67, Panel B and C, respectively).

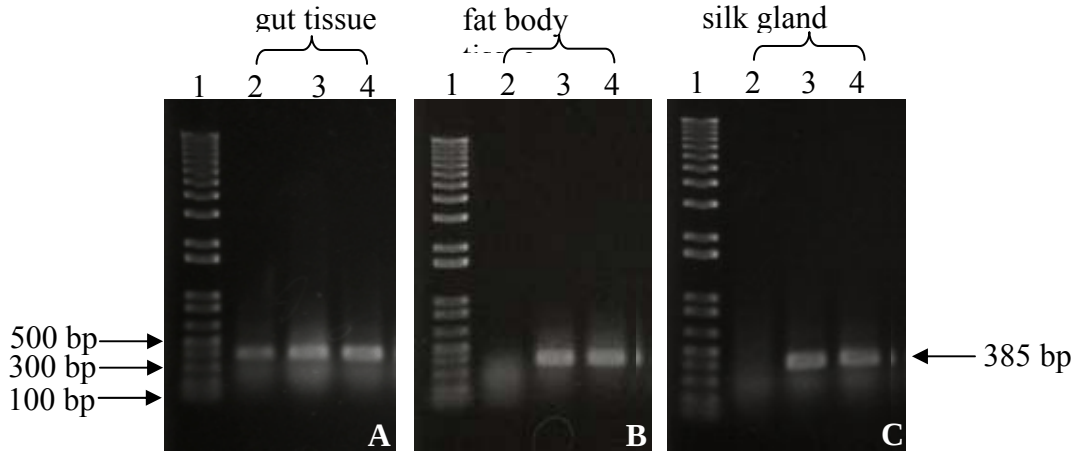


Figure 67 RT-PCR products of *α-amylase* amplified by primer set 2 from difference tissues of non transgenic, transgenic Y1.1 and Y1 mix strains. Panel A: RT-PCR products from gut tissue, Panel B: RT-PCR products from fat body tissue, Panel C: RT-PCR products from silk gland tissue, Lane 1: DNA marker 100 bp + 1 kb, Lane 2: RT-PCR product of non transgenic, Lane 3: RT-PCR product of transgenic Y1.1 and Lane 4: RT-PCR product of transgenic Y1 mix.

Discussion

Amylases are enzymes that occur in all organisms; catalyze the hydrolysis of α-1, 4 glycoside bonds in starch and glycogen. In silkworm, amylase enzyme was found in digestive fluid and haemolymph (Tazima, 1962; Promboon *et al.*, 1993). Interestingly, the activity of amylase in non dipausing strains is higher than that of diapausing strains (Abbraham *et al.*, 1992). The advantages of non diapause strains are very strong, high survival rate and short rearing time. However, they produce short larval span with small quantity of silk fiber (Murakami, 1989). The *α-amylase* gene in the silkworm has been studied and submitted in Genbank (Foster *et al.*,

unpublished) but it is a truncated *α-amylase* gene not full length. This study is the first report about complete nucleotide sequence of *α-amylase* gene from a native Thai silkworm multivotine strain, Nanglai strain. The Nanglai *α-amylase* genes composed of 1,503 bp long encoding for a predited 500 amino acid residue. The nucleotide sequence of *α-amylase* cDNA of Nanglai strain was alignment with the *α-amylase* genomic DNA sequence, the result showed that the *α-amylase* cDNA composed of 9 exons, at a position nt 392- nt 444, nt 1,397- nt 1,568, nt 2,617- nt 2,768, nt 3,238- nt 3,418, nt 3,552- nt 3,667, nt 3,923- nt 4,431, nt 4,674- nt 4,874, nt 5,347- nt 5,508 and nt 6,308-nt 6,406 respectively. The start codon ATG and stop codon TAA were a position nt 1,398- nt 1,400 and nt 6,317- nt 6,319, respectively.

The sequence of Nanglai *α-amylase* cDNA showed 97% and 99 % sequence homology to ORF and mRNA of *Bombyx mori* truncated *alpha-amylase* (*amy*) gene (Genbank Accession No.U07847), respectively. Compared with nucleotide sequence of ORF of *Bombyx mori* truncated *alpha-amylase* (*amy*) gene (Genbank Accession No. U07847), the ORF of Nanglai *α-amylase* gene had three extra regions, at a position 324-620 (297 bp in length), position 1,014-1,023 (10 bp in length) and position 1,046-1,503 (99 bp in length) respectively. The difference might be due to they came from different silkworm strains. However, I could not conclude that why they are different since the data base did not report which silkworm strain that the *Bombyx mori* truncated *alpha-amylase* (*amy*) gene was original. Moreover, no reports about *alpha-amylase* of bivoltine strain. Multiple sequence alignment of the α-Amylase deduced amino acid sequence of Nanglai strain to α-Amylase of *Ostrinia nubilalis* (AAA03715.1) (Foster *et al.*, unplublished), *Diatraea saccharalis* (AAP92665.1) (Guerra *et al.*, unplublished), *Spodoptera frugiperda* (AF280891_1) (Da Lage *et al*., 2002) and *Ceratitis capitata* (AAO13691.1) (Da Lage *et al*., 2002) showed 78%, 79%, 81% and 60% identity, respectively. In other hand, the *α-amylase* genomic DNA sequence of Nanglai consisted 6,942 bp and showed 97% identity with the sequences of genomic DNA of *B. mori* p50 strain (nscaf2827.1 Data of NIAS) (data not showed).

In insect, *α-amylase* is expressed in difference tissues. In the *D. ananassae* (Da Lage *et al.*, 2003) and *Phaedon cochleariae* (Girard *et al.*, 1999), *α-amylase* gene is expressed in the gut. In *Lutzomyia longipalpis*, *α-amylase* gene was also found in the crop and midgut (Hill *et al.*, 2005). In *Blattella germanica* (L.), a tergal gland-secreted alpha-amylase, insect tergal glands are specialized exocrine glands (Saltzmann et al., 2006). In *Apis mellifera* L., *α-amylase* is expressed in the hypopharyngeal gland of forager bees. Hypopharyngeal gland believed to synthesize bee milk, is well developed in the nurse bee but shrinks in the forager, which then develops the ability to hydrolyze the sucrose of nectar into glucose and fructose (Ohashi, 1999). In the silkworm, *B. mori*, expression of *α-amylase* was analyzed by whole mount in situ hybridization by using anti-sense RNA probe of Amylase. The hybridization was detected using anti-DIG antibody conjugated to fluorescein, the result showed that *α-amylase* is expressed in the salivary gland (Parthasarathy and Gopinathan, 2005). However, they did not detect the *α-amylase* expression in gut. In this study, *α-amylase* expression was detected only in gut tissue of Nanglai and w1-pnd strains. Whereas, in the silkworm C108 strain, a biovotine strain, the expression of this gene could not be detected in this study. It is likely that the *α-amylase* gene of bivoltine strains might be different from that of polyvoltine strains in both structure and nucleotide sequence. They are some reports about the difference of amylase enzyme activity in digestive fluid and haemolymph of diapausing and nondiapausing strains (Abraham *et al.*, 1992).

In the transgenic silkworm, the *actin A3* (*BmA3*) and artificial *3xP3* promoters had been used to drive the expression of the *GFP* gene (Tamura *et al*., 2000; Thomas *et al.*, 2002). The *Bm-Actin3EGFP* maker was used for screening of the G1 transgenic individuals, such screening was possible at the larval, pupal and adult tissue but could not detect at embryonic stage (Tamura *et al.*, 2000). This screening was difficult because it was necessary to check moving larvae fed on the artificial diet. Later, the *3xP3-EGFP* reporter was developed. The *3xP3-EGFP* marker could be expressed in embryonic stemmata of insects (Horn *et al*., 2000). The artificial *3xP3* promoter, containing three optimal binding sites for Pax 6 homodimers, drives the tissue specific

expression of the *GFP* gene in the stemmata of embryos. These results are consistent with those obtained in *D. melanogaster* (Horn *et al*., 2000) and *Musca domestica* (Hediger *et al.*, 2001). In 2002, Thomas and colleagues used *3xP3-EGFP* maker gene for screening transgenic silkworm, the result showed the *3xP3-EGFP* maker was very likely to be expressed in the stemmata of *B. mori* embryos. The *EGFP* expression was detected from the fifth day of embryonic development until the imago stage (Thomas *et al.*, 2002).

This study used two type of *piggyBac* vectors, *pBac*[A3-AMY1-A3UTR, 3xP3-DsRed2] and *pBac*[A3-AMY1-SV40UTR, A3-EGFP]. These vectors were constructed by using *Actin3* promoter for drives *α-amylase* gene of Nanglai but different in the marker gene and region of *Actin3* promoter, the *Actin3* promoter of *pBac*[A3-AMY1-A3UTR, 3xP3-DsRed2] longer than *Actin3* promoter of *pBac*[A3-AMY1-SV40UTR, A3-EGFP]. The *pBac*[A3-AMY1-A3UTR, 3xP3-DsRed2] contained *α-amylase* cDNA under the control of the *Actin3* promoter and *Actin3* terminator sequence in *piggyBac* vector. This vector was constructed from *pBac*(3xP3-DsRedaf), in which the gene for the red fluorescent protein (*DsRed*) was introduced as a marker gene under the eyes and nervous tissue-specific promoter *3xP3* (Tomita *et al.*, 2003). In this study, the transgenic silkworms (G1) were detected by screening for *DsRed* expression in the stemmata of embryos and in the compound eyes of moths. The result showed low number of *DsRed* positive embryos. In other hand, the *pBac*[A3-AMY1-SV40UTR, A3-EGFP] was constructed from *pBac*MCS(A3-SV40, A3-EGFP)R, which contained EGFP reporter gene. This vector contained *α-amylase* cDNA under the control of the *Actin3* promoter and SV40 terminator sequence in *piggyBac* vector. The result showed the transgenic silkworms (G1) of *pBac*[A3-AMY1-SV40UTR, A3-EGFP] were not observed by screening for *GFP* expression in the whole body of embryos. This result might be come from the effect of difference in lengh of *Actin3* promoter.

The percentage of silkworm transformation using microinjection technique in the first report was 2% of the individuals in the G1 broods expressing *GFP* (Tamura *et al.*, 2000). The later percent of transformation were increased to 18.3, 25.8 and 27.6

% (Tomita *et al.*, 2003). In this study, the percent transformation of the *pBac*[A3-AMY1-A3UTR, 3xP3-DsRed2] was approximately 5.4%. The low percentage of this transformation might be that the silkworm eggs used for injection were not healthy enough so that many the injected eggs could not hatch.

The expression of *α-amylase* in non transgenic silkworm could be detected in only gut tissue by using RT-PCR. While, in transgenic strains, Y1.1 and Y1 mix, the *α-amylase* was expressed in several tissues including, gut tissue, fat body tissue and silk gland tissue. The expression of *α-amylase* gene in those tissues of the transformation larvae is in agreement with the ubiquitous activity of the driving actin *BmA3* promoter (Tamura *et al.*, 2000). This transgenic silkworm should produce α-amylase enzymes more than non transgenic strain.

There are some reports studied about antiviral activity of lipases and serine proteases enzymes from digestive juice, the role of both digestive enzymes is food digestion (Ponnuvel *et al.*, 2003; Nakazawa *et al.*, 2004). In the case of α-amylase enzymes, one of the main digestive enzymes, the increase of α-amylase enzymes in the transgenic silkworm might enhance the silkworm resistance to virus disease.

For the future prospects of this work, I think it would be useful to perform an experiment to test the viral disease resistance of the transgenic silkworm. If the increase of the α-amylase enzymes providing the resitance to viral disease, it would be very beneficial to silkworm rearing farmers and Thai industry sericulture.

CONCLUSION

1. The *α-amylase* genomic DNA and cDNA sequence of Nanglai were 6,942 bp and 1,678 bp long, respectively. The Nanglai *α-amylase* cDNA consists of 9 exons, the start codon was ATG and the stop codon was TAA. The 1,503 bp open reading frame (ORF) encodes a predicted protein of 500 amino acids.

2. Two types of *piggyBac* vectors, *pBac*[A3-AMY1-A3UTR, 3xP3-DsRed2] and *pBac*[A3-AMY1-SV40UTR, A3-EGFP] were successfully constructed and introduced to silkworm eggs. In the *pBac*[A3-AMY1-A3UTR, 3xP3-DsRed2], *α-amylase* cDNA was under the control of the *Actin3* promoter and *Actin3* terminator sequence. While, the *pBac*[A3-AMY1-SV40UTR, A3-EGFP] contained *α-amylase* cDNA under the control of the *Actin3* promoter and *SV40* terminator sequence.

3. Two transgenic lines, Y1.1 and Y1 mix, containing the *pBac*[A3-AMY1-A3UTR, 3xP3-DsRed2] were successfully generated.

4. Detection of *α-amylase* gene expression in silkworm, *B. mori*, Nanglai, C108 and w1-pnd strains by using RT-PCR method, the result showed the *α-amylase* gene could expressed in only gut tissue of Nanglai and w1-pnd strains. In the case of C108 strain could not expressed this gene. While, in the case of the transgenic silkworm (Y1.1 and Y1 mix), the *α-amylase* transgene could expressed in the several tissues including, gut tissue, fat body tissue and silk gland tissue.

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APPENDICES

Appendix A

Silkworm raring

Silkworm rearing

1. Eggs are incubated in dark and enough humidity at 28 ^{o}C
2. After 10-14 days of incubation, a black dot spot which is usually called “eye spotting” is observed near the polar end of the eggs. These dots correspond to the pigmented larvae heads and are the sign that the eggs are to hatch after 3 days.
3. When the whole egg color turns into pale gray, the eggs will hatch within 1-2 days.
4. If an enough number/percentage of the larvae hatch, remove them to a fresh young mulberry leaf sliced into small pieces on at sheet of paper at the bottom of a plastic box.
5. For the young (1st to 3rd larva) silkworm, giving mulberry leaf, which sliced at 2-5 mm (approximately to the length of the instar). Rear at 27-28 ^{o}C
6. For the 4th and 5th larva, rear at 25-26^{o}C on plastic-mesh pacer..
7. Between each stage of instar, there is ecdysis, stop feeding and wait until most of the larva complete ecdysis (newly ecdysed larvae are resistant to starvation). Give new mulberry leaf
8. The end stage of 5th larva, they will spin silk fiber and become cocoons. Cut to open a cocoon after one week of spinning, and remove the pupa.
9. Determine the sex of pupa, keep in separately box.
10. When the moths hatch; males moths are usually small and active, while female moths are big and behave quietly, mate each couple in separated space for 4 hours.
11. Let the female moth lay eggs on the wax paper in a oviposition chamber (cylinder).

Appendix B

Reagent preparation

General reagent

0.5 M EDTA, pH 8.0

EDTA 18.61 g

The chemical was dissolved in distilled water, adjusted to pH 8.0 with 1 M NaOH. Distilled water was added to the final volume 100 ml. The solution was sterilized by autoclaving for 15 minutes at 121 °C, 15 lb/square inches.

1.0 M $CaCl_2$

Dissolved 54 g of $CaCl_2 \cdot 6H_2O$ in 200 ml of distilled water.

Sterized the solution by pass through a 0.22 μm filter. Store at –20 °C until use.

$MgCl_2$-$CaCl_2$ solution

80 mM $MgCl_2$

20 mM $CaCl_2$

Dispensed into aliquots and sterilized by autoclaving.

5 M NaCl

NaCl 29.2 g

Distilled water was added to final volume 100 ml

Dispensed into aliquots and sterilized by autoclaving.

10 % SDS

Sodium dodecyl sulfate 10 g

Distilled water was added to final volume 90 ml

The chemical was dissolved at 68 °C and adjusted to pH 7.2 by adding 2-3 drops of concentrated HCl. The solution was adjusted to the volume 100 ml.

2 M Mg_2^+ stock

$MgCl_2 \cdot 6H_2O$ 20.33 g

$MgSO_4 \cdot 7H_2O$	24.65 g

The chemicals were dissolved by adding 100 ml of distilled water and then filtered through a 0.22 µm filter.

Reagent for molecular cloning

DNA extraction buffer

100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, pH 8.0, 2 % CTAB, 0.3 % v/v ß mercaptoethanol

10X MOPS buffer

0.4 M Morpholinopropane sulfonic acid	83.70 g
0.1 M sodium acetate·$3H_2O$	13.60 g
10 mM EDTA·Na·$2H_2O$	1.87 g

Adjust to pH 7.2 with 1 M NaOH

Add distilled water to make 1 l

TE, pH 8.0

1 M Tris-HCl, pH 8.0	1.0 ml
1 mM EDTA, pH 8.0	0.2 ml

The mixture was mixed thoroughly and adjusted to the final volume of 100 ml with distilled water.

3.0 M Sodium acetate, pH 5.2

Sodium acetate	24.2 g

The chemical was dissolved in distilled water, adjusted to pH 5.2 with glacial acetic acid. The final volume was adjusted to 100 ml with distilled water.

50X TAE buffer (stock)

Tris	240.2 g
Glacial acetic acid	57.0 ml

0.5 M EDTA (pH 8.0) 100.0 ml

The chemicals were dissolved in distilled water, and adjust the final volume to 1 liter with distilled water. (working solution is 1 X)

Chloroform : Isoamyl alcohol (24 : 1, v/v)

Chloroform (Merck) 24.0 ml

Isoamyl alcohol 1.0 ml

Both reagents were mixed together and stored in a dark bottle at room temperature.

1.0 M IPTG

Isopropylthio-ß-D-galactoside 2.38 g

Distilled water 100 ml

The solution was sterilized by filtration through a 0.2 µm filter and dispensed the solution into 1 ml aliquot tube and stored at –20 °C.

X-gal

5-bormo-4-chloro-3-indolyl-ß-D-galactoside 100 mg

The chemical was dissolved in 2 ml of dimethyl-formamide. The solution was stored in a tube covered with aluminum foil and stored at -20°C.

Ethidium bromide (10 mg/ml)

Ethidium bromide 1.0 g

Distilled water 10.0 ml

The solution was stored in a dark bottle at room temperature.

6X gel-loading dye buffer

0.25 % bromophenol blue

0.25 % xylene cyanol FF

30 % glycerol in water

The chemicals were dissolved and adjusted the final volume with distilled

water.

Proteinase K (20 mg/ml)

Proteinase K	0.2 g
Distilled water	10.0 ml

The solution was stored at –20 °C.

RNase A (10 mg/ml)

RNaseA	10.0 mg

RNase was dissolved in 10 mM Tris-HCl (pH 7.5), 15 mM NaCl and stored at -20°C.

Reagent for alkaline lysis method

Solution I (50 mM glucose, 25 mM Tris-Cl, and 10 mM EDTA, pH 8.0)

1.0 M glucose	5.0 ml
1.0 M Tris-HCl (pH 8.0)	2.5 ml
0.5 M EDTA (pH 8.0)	2.0 ml
Distilled water	90.5 ml

The mixture was sterilized by autoclaving for 15 minutes and stored at 4 °C.

Solution II (0.2 M NaOH, 1 % SDS)

1.0 M NaOH	2.0 ml
10 % SDS	1.0 ml
distilled water	7.0 ml

Solution III (3 M Potassium acetate, Glacial acetic acid)

5 M Potassium acetate	90.0 ml
Glacial acetic acid	11.5 ml
Distilled water	28.5 ml

Media for bacterial culture

Luria-Bertani medium (LB medium per liter)

Tryptone (Difco)	10 g
Yeast extract (Difco)	5.0 g
NaCl	10 g

Adjust pH to 7.0 with NaOH. Then the solution was adjusted to the final volume of 1000 ml with distilled water and sterilized by autoclaving.

LB plates with ampicillin

Fifteen grams of agar was added to 1 l of LB medium then the media was sterilized by autoclaving. The medium was allowed to cool to 50°C before adding ampicillin to a final concentration of 100 µg/ml. The medium (30-35 ml) was poured into 85 mm petri dishes. The agar was allowed to harden. Agar plates were stored at 4°C for up to 1 month or room temperature for up to 1 week.

LB plates with ampicillin / IPTG / X-gal

The LB with ampicillin was prepared then supplement with 0.5 mM IPTG and 80 µg/ml X-Gal and pour the plates. Alternatively, 100 µl of 100 mM IPTG and 20 µl of 50 mg/ml X-gal may be spread over the surface of an LB ampicillin plate and allowed to absorb for 30 minutes at 37°C prior to use.

CURRICULUM VITAE

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