GENE EXPRESSION RELATED TO FREE FATTY ACID SYNTHESIS IN PHYSIC NUT *Jatropha curcas* L. KERNEL BY PROTEOMICS TECHNIQUE ANALYSIS

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การแสดงออกของยีนที่เกี่ยวข้องกับการสังเคราะห์กรดไขมันอิสระในเนื้อเมล็ดสบู่ดำ *Jatropha curcas* L. โดยการวิเคราะห์ด้วยเทคนิคโปรติโอมิกส์

นางสาว ฐิติพร บูรณะศรีศักดิ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	GENE EXPRESSION RELATED TO FREE FATTY ACID SYNTHESIS IN PHYSIC NUT *Jatropha curcas* L. KERNEL BY PROTEOMICS TECHNIQUE ANALYSIS
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ฐิติพร บูรณะศรีศักดิ์ : การแสดงออกของยีนที่เกี่ยวข้องกับการสังเคราะห์กรดไขมันอิสระในเนื้อเมล็ดสบู่ดำ *Jatropha curcas* L. โดยการวิเคราะห์ด้วยเทคนิคโปรติโอมิกส์. (GENE EXPRESSION RELATED TO FREE FATTY ACID SYNTHESIS IN PHYSIC NUT *Jatropha curcas* L. KERNEL BY PROTEOMICS TECHNIQUE ANALYSIS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รองศาสตราจารย์ ดร.วรวุฒิ จุฬาลักษณานุกูล, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : ดร. สิทธิรักษ์ รอยตระกูล 134 หน้า.

สบู่ดำ (*Jatropha curcas* L.) เป็นพืชเศรษฐกิจที่สำคัญชนิดหนึ่ง เนื่องจากน้ำมันจากเนื้อเมล็ดสามารถนำมาใช้เป็นไบโอดีเซลได้ ผู้วิจัยจึงสนใจศึกษาชนิดและปริมาณของกรดไขมันอิสระและโปรตีนที่แสดงออกในแต่ละระยะการเจริญของเมล็ด โดยแบ่งการเจริญของผลสบู่ดำเป็น 8 ระยะจากลักษณะภายนอกของผล แล้วนำมาศึกษาทั้งชนิดและปริมาณของกรดไขมันและโปรตีน ตลอดการเจริญของเมล็ดสบู่ดำตั้งแต่ผลอ่อนจนถึงผลแก่ จากการตรวจวิเคราะห์ด้วยเครื่องแก๊ซโครมาโตกราฟฟีพบว่า ปริมาณกรดไขมันอิสระในเนื้อเมล็ดมีการเปลี่ยนแปลงจากระยะที่ 1 ถึง ระยะที่ 8 กรดไขมันหลักที่พบคือ กรดโอเลอิก ส่วนกรดไขมันที่พบรองลงมาคือ กรดไลโนเลอิก, กรดปาล์มมิติก และกรดสเตียริก ตามลำดับ นอกจากนี้จากการศึกษาการแสดงออกของโปรตีนในเนื้อเมล็ดสบู่ดำด้วยเทคนิคโปรติโอมิกส์พบว่า มีโปรตีนทั้งหมด 245 ชนิดที่มีการแสดงออกเปลี่ยนแปลงตลอดการเจริญของเมล็ดสบู่ดำ เช่น โปรตีนที่เกี่ยวข้องกับการเจริญของเมล็ด (เช่น AP2 domain-containing protein และ late embryogenesis abundant protein) โปรตีนที่เกี่ยวข้องกับการสังเคราะห์กรดไขมัน (เช่น acetyl CoA carboxylase และ phosphoenolpyruvate carboxylase) โปรตีนที่เกี่ยวข้องกับการสุกแก่ (เช่น ACC oxidase และ ACC deaminase) นอกจากนี้ยังพบโปรตีนพิษ เคอร์ซิน (curcin) ซึ่งเป็นโปรตีนที่พบเฉพาะในสบู่ดำอีกด้วย

เมื่อศึกษาการแสดงออกของยีนที่เกี่ยวข้องกับการสังเคราะห์กรดไขมันจำนวน 4 ชนิด ได้แก่ *acetyl CoA carboxylase* (*ACCase*), *phosphoenolpyruvate carboxylase* (*PEPC*), *mercaptopyruvate sulfurtransferase* (*MST*) และ *4-coumarate: coenzyme A ligase* (*CMCoA*) ด้วยเทคนิค Real-time RT-PCR พบว่า การแสดงออกในระดับ mRNA ของแต่ละยีน มีแนวโน้มของการเปลี่ยนแปลงไปในทางเดียวกันกับปริมาณของกรดไขมันอิสระส่วนใหญ่ในแต่ละระยะของการเจริญ แสดงว่า ยีนที่นำมาศึกษามีส่วนเกี่ยวข้องกับการสังเคราะห์กรดไขมันอิสระในเนื้อเมล็ดสบู่ดำ

นอกจากนี้ยังพบว่าการลดลงของกรดไขมันอิสระในระยะที่ 4 ถึงระยะที่ 6 ซึ่งมีผลสีเหลืองนั้น มีสาเหตุมาจากการสลายไขมันในกระบวนการ β-oxidation เพื่อนำพลังงานไปใช้ในกระบวนการเมตาบอลิสมอื่นๆ นอกจากนี้สารตัวกลางจากกระบวนการ β-oxidation อาจนำไปใช้ในกระบวนการสังเคราะห์สารประกอบฟีนอลิก flavonoid

ภาควิชา…พฤกษศาสตร์……..ลายมือชื่อนิสิต.....

สาขาวิชา..พันธุศาสตร์…….ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา..2552…… ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

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Jatropha curcas L. is one of economic important crops used for its seed oil which can substitute for diesel. Free fatty acid profiles and proteins profiles of *J. curcas* kernel during development were studied. Developmental seeds were divided into 8 stages based on morphology. Free fatty acid was analyzed using gas chromatography. The results suggested that free fatty acid levels were changed at all stages of development from stage I to stage VIII. The major free fatty acid was oleic acid and the minor free fatty acids included linoleic acid, palmitic acid, and stearic acid. Furthermore, the protein profiles determined by proteomics technique indicated that 245 proteins were differentially expressed during the seed development such as proteins related to seed development (AP2 domain-containing protein and late embryogenesis abundant protein), proteins related to free fatty acid synthesis (acetyl CoA carboxylase and phosphoenolpyruvate carboxylase), and proteins related to fruit ripening (ACC oxidase and ACC deaminase). Moreover, a protein related to toxicity (curcin), that was only found in *J. curcas* was discovered.

Real-time RT PCR was employed in a study of proteins involved in the biosynthesis of free fatty acids. These proteins included *acetyl CoA carboxylase* (*ACCase*), *phosphoenolpyruvate carboxylase* (*PEPC*), *mercaptopyruvate sulfurtransferase* (*MST*) and *4-coumarate: coenzyme A ligase* (*CMCoA*). The results revealed that the relative mRNA expression level of each gene was altered in a similar pattern with regard to the amount of most free fatty acids.

The decreased level of free fatty acids from stage IV to stage VI may caused by β-oxidation metabolism to generate energy for other metabolism. Additionally, intermediate substances from β-oxidation process may be used in flavonoid biosynthesis.

Department : Botany	Student's Signature .....
Field of Study : Genetics	Advisor's Signature .....
Academic Year : 2009	Co-Advisor's Signature .....

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LIST OF ABBREVIATIONS

bp	base pair
°C	degree celsius
DEPC	diethylpyrocarbonate
DTT	dithiothreitol
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
DNA	deoxyribonucleic acid
et al.	*et. alli.* (Latin), and others
FAME	fatty acid methyl ester
FFA	free fatty acid
g	gram
h	hour (s)
IAA	iodoacetamide
IPTG	isopropyl-thiogalactoside
Kb	kilobase
kDa	kilo Dalton
l	Litre

LC-MS/MS	liquid chromatography tandem mass spectrometry
M	molarity (concentration)
µg	microgram
mg	milligram
ml	millilitre
mM	millimolar
ng	nanogram
OD	optical density
PCR	polymerase chain reaction
RNA	ribonucleic acid
RNase A	ribonuclease A
rpm	revolution per minute
RT	reverse transcription
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Tris	tris (hydroxyl methyl) aminomethane
µg	microgram
µl	microlitre
µM	micromolar
UV	ultraviolet

CHAPTER I

INTRODUCTION

The seed is the main form of dissemination and is the reproductive organ of higher plants. During development of seed plant, the quiescent kernel tissue shift into a metabolically active state in which complex biochemical and physiological changes occur. In the past decades, many studies have been carried out on seed development, mainly through physiological and transcriptomic analysis (Liu *et al*., 2009). These previous studies have provided robust information about several aspects during seed development, such as the role of ethylene in ripening fruits and mobilization of energy reserves. An overview of related gene in the seed development has been provided by transcriptomic analyses in some plant species, such as avocado and tomato (Buse and Laties, 1993).

However, proteome study in plant seed development is very limited at present especially in *Jatropha curcas*. Only descriptions of European beech and *Prunus campanulata* were presented recently (Pawlowski, 2007; Lee *et al*., 2006). Their studies were mainly focused on lipid, protein, and starch in the embryos and endosperm. In oil seeds, the major storage reserve is lipid which is stored in small spherical organelles called oil bodies. Knowledge of the underlying biochemistry and metabolism is essential for the study of proteins related to seed development that are not only accumulated in high levels of the desired oil, but also efficiently support vigorous seed development.

Jatropha curcas is a plant with many attributes, multiple uses and considerable potential. Its seed has a high content of oil that can be reformed as biodiesel which is becoming increasingly important as an alternative fuel for diesel engines (Openshaw, 2000).

In *J. curcas* seed, Most of the foregoing researches focused on single or few genes, so the results were still far from comprehensively elucidating the mechanisms of the seed development and fatty acid biosynthesis during developmental stages. In this study, a proteomic approach, in combination with the SDS-PAGE observation and LC-MS/MS technique will help to understanding related mechanisms. The proteomics will illustrate in the protein expression pattern associated with developmental stages of *J. curcas* seeds.

CHAPTER II

LITERATURE REVIEW

2.1 Introduction of *Jatropha curcas* L.

2.1.1 Source and Growing Condition of *J. curcas* L.

The genus *Jatropha* belongs to the tribe *Jatropheae* in the Euphorbiaceae family and contains approximately 170 known species. *Euphorbiaceae* is an ancient and diverse family in the large rosid order Malpighiales and includes in addition to Jatropha such familiar members as rubber, cassava, castor bean, poinsettia, and leafy spurge (Wurdack, 2008). *Jatropha curcas* is a native of tropical America, but now thrives in many parts of the tropics and sub-tropics in Africa/Asia (Openshaw, 2000). It grow in condition of annual rainfall above 944 mm per year and an average minimum temperature of the coldest month (T_{min}) above 10.5 ˚C. The mean annual temperature range was 19.3–27.2 ˚C. The observed precipitation preferences indicate that jatropha is not common in regions with arid and semi-arid climates. Plantations in arid and semi-arid areas hold the risk of low productivity or irrigation requirement. Plantations in regions with frost risk hold the risk of damage due to frost (Maes *et al*., 2009). *J. cucas* is widely grown in Maxico, Nicaragua, Thailand, Brazil and in parts of India (Martı´nez-Herrera *et al*., 2006; Foidl *et al*., 1996; Winayanuwattikun *et al*., 2008; Lapola *et al*., 2009).

Jatropha curcas or “physic nuts”, is a small tree or large shrub that normally reaches a height of 3-5 m, but can reach a height of 8-10 m under favorable conditions. The plant root system proceeds through the development of a main taproot and four shallow lateral roots. The leaves are smooth with five lobes spanning 10-15 cm and may fall once a year, depending on their genotype. The plant is monoecious and the terminal inflorescences contain unisexual flowers on the same inflorescence (raceme). The inflorescence is a panicle, with the female flowers (about 10-20%) at

the apices of the main stem and branches of the inflorescence. Male flowers are more numerous (about 80-90 %) and occupy subordinate positions on the inflorescence (Raju and Ezradanam, 2002). Male flowers open for a period of 8-10 days, whereas female flowers open for 2-4 days only. The continuous flowering results in a sequence of reproductive development stages from yellow mature fruits at the base of the branch, to green fruits in the middle, and flowers at the top. After pollination, the inflorescences form grapes of 10 green fruits, 2-3 cm long with an ovoid shape. Each fruit typically has three carpels and the potential for two seeds per carpel (Kochhar *et al*., 2008). Under optimal conditions, flowering and fruiting start 4-5 months after transplantation and the first crop occurs about 7 months after transplantation, but may last into the second year if the plant is pruned to increase production. Pruning is recommended for building tree architecture (Openshaw, 2000).

2.1.2 Usefulness of *J. curcas*

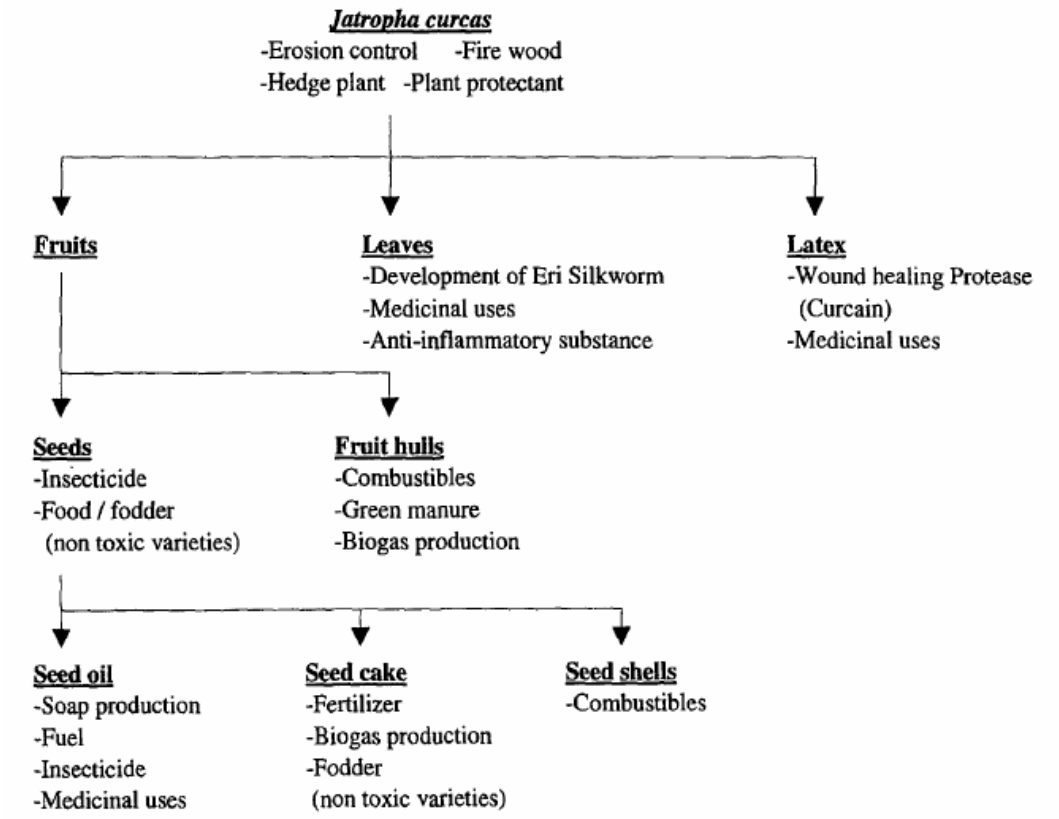
Jatropha curcas is a plant with many attributes, multiple uses and considerable potential. The plant can be used to prevent and/or control erosion, to reclaim land, grown as a live fence, especially to contain or exclude farm animals and be planted as a commercial crop. Various parts of the plant are of medicinal value, its bark contains tannin that can used to treat leather, the leaves are a feedstock for silk worms, the flowers attract bees and thus the plant has a honey production potential; its wood and fruit can be used for numerous purposes including fuel. Of particular importance, the fruit of jatropha contain viscous oil that can be used for soap making, in the cosmetics industry and as a diesel/kerosene substitute or extender (Openshaw, 2000). The utilization of various parts of *Jatropha curcas* is shown in Figure 2.1.

2.1.3 Physical Properties of *J. curcas* Fruits, Nuts and Kernels

The hull of the fruit had very high moisture content compared to nut shell and kernel. The whole fruit contained 77.03% moisture content. The sphericity values indicated that fruit shape (0.95) is close to a sphere compared to nut (0.64) and kernel (0.68), both of which are close to an ellipsoid. Bulk densities of fruits, nuts and kernels were 0.47, 0.45 and 0.42 g/ cm^3, the corresponding solid densities were 0.95,

1.04 and 1.02 g/cm^3, and the corresponding porosities were 50.53%, 56.73% and 58.82%, respectively. The surface area of fruit was larger than those of nut and kernel, by 5.88% and 10.24%, respectively. The static coefficient of friction and angle of repose of kernels on all surfaces studied (plywood, steel, and stainless steel) were the highest as the surface is viscous and hardness is less. Rupture force, hardness and toughness of fruit, nut and kernel were 135.39, 146.63 and 67.72 N; 30.58, 69.98 and 38.52 N/mm and 300.88, 124.44 and 51.61Nmm, respectively (Sirisomboon *et al*., 2007).

Figure 2.1 The utilization of various parts of *Jatropha curcas* L (Gübitz, Mittelbach and Trabi, 1999)



2.1.4 Fruit Harvesting and Oil Yield

Fruits are harvested at seed maturity, which occurs 40 days after flowering and then dried to < 8% humidity for 2 days in the sun. Understanding the physical properties of the *Jatropha* fruit is essential to design equipment for harvesting, drying, cleaning, grading, decorticating, and storing them. When dried, the fruits are about

400-425 per kg, and composed of 35-40% shell and 60-65% seeds (Pradhan *et al*., 2008; Sirisomboon *et al*., 2007).

Laboratory experiments have reported that the rate of oil extraction carried out by a mechanical press is 40-55% for hulled seeds, 70-75% for kernel (Willems *et al*., 2008 a, b) and by manual ram presses is 60-65% (Beerens, 2007). Solvent (*n*-hexane) extraction of the kernel alone yields 60% oil (Veronique *et al*., 2002). An enzymatic method of extraction is also available (Shah *et al*., 2004). It gives essentially the same rate of oil extraction as that obtained with hexane, but is about 12 times faster. In industrial operating conditions, the rate of oil extraction can be much lower and is typically 35% by mechanic extraction or 40% by solvent extraction of the hulled seed.

The oil yield from the crops is always the key factor to decide the suitability of a feedstock for biodiesel production. Oil crops with higher oil yield are more preferable in the biodiesel industry because it can reduce the production cost. Generally the cost of raw materials accounts about 70–80% of the total production cost of biodiesel. Table 2.1 shows the oil yield in terms of kg/ha and wt% for various types of edible and non-edible oils in the world. Palm oil was found to give the highest oil yield with 5000 kg oil per hectare; this value is far higher than other oils which are only in the range of hundreds to 2000 kg oil per hectare. On the other hand, among the various non-edible oils shown in Table 2, *Jatropha* was found to give the highest yield. This is followed by *Psoralea pinnata* and castor (Gui *et al*., 2008). More than 95% of biodiesel is made from edible oil. By converting edible oils into biodiesel, food resources are actually being converted into automotive fuels. It is believed that large-scale production of biodiesel from edible oils may bring global imbalance to food supply and demand (Kumar and Sharma, 2008).

Table 2.1 Oil yield for major non-edible and edible oil sources. (Adapted from Gui *et al*., 2008)

Type of oil	Oil yield (kg oil/ha)	Oil yield (%wt)
Non-edible oil		
Jatropha	1590	Seed: 35-40 Kernel: 50-60
Rubber seed	80-120	40-50
Castor	1188	53

Type of oil	Oil yield (kg oil/ha)	Oil yield (%wt)
Pongamia pinnata	225-2250	30-40
Sea mango	N/A	54
Edible oil		
Soybean	375	20
Palm	5000	20
Rapeseed	1000	37-50

2.1.5 Bio-fuel Uses of *J. curcas*

In the present investigation *J. curcas* oil, a non-edible vegetable oil which has been considered as a potential alternative fuel for compression ignition engines (diesel engines), has been chosen to find out its suitability for use as fuel oil. The *J. curcas* oil is slowly-drying oil which is odourless and colourless when fresh but becomes yellow on standing when store for a long time. The oil content of *J. curcas* seed ranges from 30 to 50% by weight and the kernel itself ranges from 45 to 60% (Pramanik, 2003). Various vegetable oils such as palm oil, soybean oil, sunflower oil, rapeseed oil, and canola oil have been used to produce biodiesel fuel and lubricants (Demirbas, 2003) The oil compares well against other vegetable oils and more importantly to diesel itself in terms of its fuel rating per kilogram or hectare of oil produced (Pramanik, 2003). The study of the physical and chemical properties of *J. curcas* oil is shown in Table 2.2 Its crude oil consists of both saturated (14.1-15.3% palmitic acid and 3.7-9.8% stearic acid) and unsaturated fatty acid (34.3-45.8% oleic acid and 29.0-44.2 % linoleic acid) (Berchmans and Hirata, 2008). Ideally the vegetable oil should have low saturation and low polyunsaturation but high in monounsaturated fatty acid (Gunstone, 2004). That properties of plant indicated that *J. curcas* is suitable as biodiesel.

The main components of plant oils are fatty acids and their derivatives; mono-, di-, and tri-acylglycerides. The triacylglycerides (TAGs) are esters formed by fatty acid condensation with tri-alcohol glycerol (propanetriol). Depending on the number of fatty acids fixed on the glycerol molecule, one can have mono-, di-, or tri-

acylglycerides. Of course, these fatty acids can be the same or different. Biodiesel can be obtained by esterification and transesterification.

Esterification is the process by which a fatty acid reacts with a mono-alcohol to form an ester. Esterification reactions are catalyzed by acids. Esterification is commonly used as a step in the process of biodiesel fabrication to eliminate free fatty acids (FFAs) from low quality oil with high acid content.

Transesterification, or alcoholysis, is the displacement of alcohol from an ester by another alcohol in a process similar to hydrolysis. This process has been widely used to reduce the viscosity of TAGs. The transesterification reaction is represented by the general equation RCOOR' + R"OH → RCOOR" + R'OH. This stepwise reaction occurs through the successive formation of di- and mono-glycerides as intermediate products. Theoretically, transesterification needs three alcohol molecules for one of TAG, however, an excess of alcohol is necessary because the three intermediate reactions are reversible (Freedman *et al*., 1986; Marchetti *et al*., 2007). After the reaction period, the glycerol-rich phase is separated from the ester layer either by decantation or centrifugation. In the traditional way of performing the transesterification, the catalyst can be a base such as NaOH, KOH, and $NaOCH_3$ or an acid such as H_2SO_4. The alkaline catalysis is, however, much faster than the acid catalysis. Low cost and favorable kinetics turned NaOH into the most-used catalyst in industry (Schwab *et al*., 1987). Transesterification is typically obtained with a molar ratio of alcohol to oil of 6:1 (10 wt%), a reaction temperature near the boiling point of alcohol (60-70ºC at atmospheric pressure), and NaOH or KOH catalysts at concentrations <1.0 wt% of the oil. In these conditions, the reaction should yield >95% alkyl ester and 10 wt% glycerol. The drawback of acid catalysis is that it is about 400 times slower than alkaline catalysis by conventional technologies (Al-Zuhair *et al*., 2007; Schuchardt *et al*., 1998).

Table 2.2 Chemical and physical properties of *J. curcas* oil. (Akbar *et al*., 2009)

Parameter	Value
% FFA as oleic acid	2.23± 0.02
Iodine value	103.62 ± 0.07
Saponification value	193.55 ± 0.61
Peroxide value	1.93 ± 0.012
Percentage oil content (kernel)	63.16 ± 0.35
Density at 20ºC (g/ml)	0.90317
Viscosity at room temperature (cp)	42.88
Physical state at room temperature	Liquid

Values are mean ± standard deviation of triplicate determinations

2.1.6 Toxification of *J. curcas*

The term 'phorbol esters' is used to describe a naturally occurring family of compounds that can be referred to as tigliane diterpenes (Evans, 1986) Phorbol esters are defined as "polycyclic compounds in which two hydroxyl groups on neighboring carbon atoms are esterified to fatty acids". Several plants, such as *Saponin indicum, S. japonicum, Euphorbia frankiana, E. cocrulescene, E. ticulli, Croton spareiflorus, C. tigilium, C. ciliatoglandulifer, Jatropha curcas, Exoecaria agallocha,* and *Homalanthus nutans,* are reported to contain the toxic phorbols (Beutler *et al*., 1989). Among these plants, *J. curcas* kernels contain at least four different phorbol esters (Hirota *et al*., 1988). The major phorbol ester is phorbol-12-myristate-13-acetate (Makkar *et al*., 1997). The oil from the nontoxic Mexican varieties of *Jatropha* was reported to have negligible or low amount of phorbol esters (0.27 mg/ml of oil), where as the toxic varieties were found to contain 2.49 mg/ml of oil. Recently, Hass *et al.* (2002) have determined the common diterpene 12-deoxy-16-hydroxyphorbol in six different diterpene esters from the *J. curcas* oil using HPLC method. They named the isolated fraction as *Jatropha* factors C1 to C6. There are several other plants that contain different derivatives of phorbol and diterpenes, such as crotonogyne, crytogonone, dimorphocalyx, duvigneaudia, fahrenheitia, maprounea, and plagiostyles (Beutler *et al*., 1989).

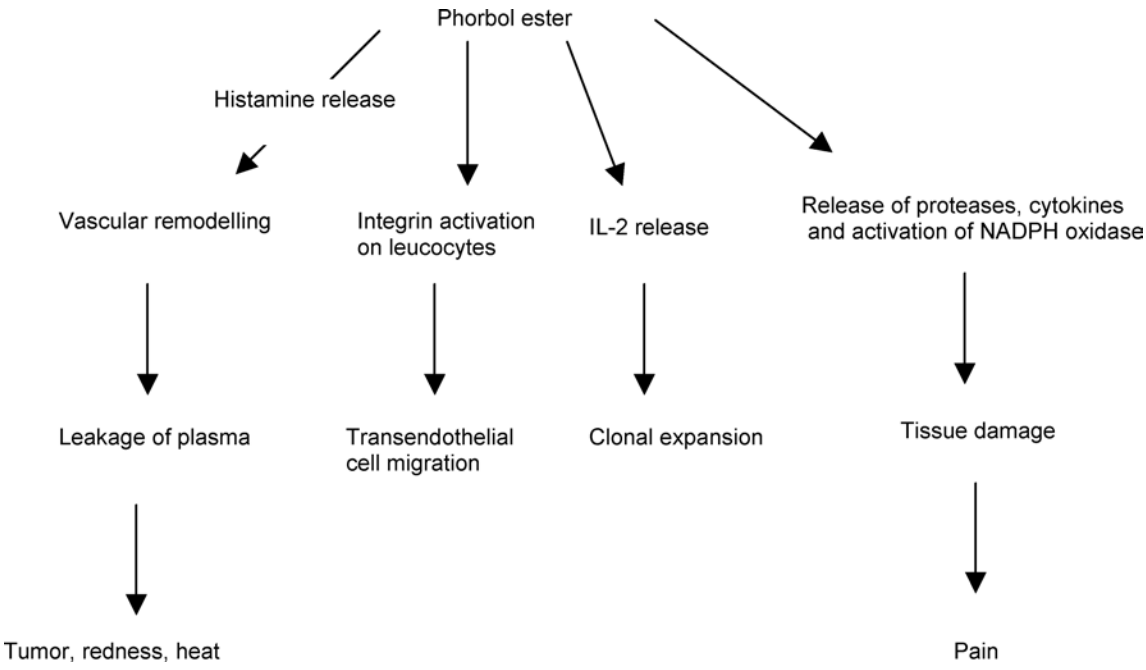
The phorbols and their derivatives are reported to be potent tumor promoters. In addition to this effect, these induce a remarkable diversity of other biological

effects at exceptionally low concentration. These are responsible for skin irritant effects and tumor promotion because they stimulate protein kinase (PKC), which is involved in signal transduction and developmental processes of most of the cells and tissues, producing a variety of biological effects in a wide range of organisms. The inflammatory responses induced by phorbol esters are summarized in Figure 2.2 (Goel *et al.*, 2007). The feasible routes for detoxification are deacidification with sodium hydroxide and potassium hydroxide and bleaching with different agents. That processes could reduce the content of phorbol esters level to 55% (Hass and Mittelbach, 2000).

Furthurmore, *J. curcas* has also been reported to posses other potential toxic compounds such as curcin similar to ricin from castor bean that is capable of inhibiting protein synthesis (Stirpe *et al*., 1976).

If it can be detoxified cheaply, or the oil can be extracted from free varieties, it could be used in food preparation and the seed cake used as animal feed.

Figure 2.2 Inflammatory responses induced by phorbol ester.



2.1.7 Lipid Profiles of *J. curcas*

Recently, lipid profiling of developing *J. curcas* seeds was studied using NMR (Annarao *et al*., 2008). They found that oil content, lipid profile and concentration of sterols at various stages of development, starting from one week after fertilization and in an interval of five days thereafter till maturity (stage I to stage VII) were different. Moisture content of the seeds ranged from 8.8 to 90.3%; the lowest in mature seeds in stage VII and highest in stage I. The seed area increased as the seed grew from stage I to stage VI (0.2–10.2 mm^2 per seed), however, the seed area shrunk at stage VII. Increase in seed area corresponded to increase in fresh weight of the seeds. 1H NMR spectroscopy of hexane extracts examined at different stages of seed development revealed the presence of FFAs, methyl esters of fatty acids (FAMEs) and TAGs, along with small quantity of sterols. The young seeds synthesized predominantly polar lipids. Lipid synthesis was noticed nearly three weeks after fertilization. From the fourth week the seeds actively synthesized TAGs. Stage III is a turning point in seed development since at this stage, the concentration of sterols decreased to negligible, there was very little FAME formation, accumulation of TAGs increased substantially, and there was a sudden decrease in FFA concentration. The findings can be helpful in understanding the biosynthesis and in efforts to improve biosynthesis of TAGs and reduce FFA content in the mature seeds.

2.2 Proteomics Study

2.2.1 Proteomics Technology

'Proteomics' is about 10 years old since it was initially proposed in 1994 at the Conference on Genome and Protein Maps (Siena, Italy) as the "PROTEin complement expressed by a genOME" (Wilkins *et al*., 1995). Therefore a proteome study is expected to represent a comprehensive survey of all proteins expressed at a given time, in given conditions, in a given organism. The major adventage of proteomics is that it focuses on the functional translated portion of the genome (Komatsu and Ahsan., 2009).

Mass spectrometry-based methods for the identification of proteins have become a standard platform in proteomics. The most popular MS-based strategies rely on proteolytic digestion of proteins into peptides before introduction into the mass spectrometer. Digestion of proteins into similar sized peptides helps to overcome the solubility and handling problems associated with proteins and creates peptide fragments which are easily ionized in the mass spectrometer. Peptide ions are first measured as intact fragment ions, then selected based on their ion mass to charge ratio (m/z) and subject to collisionally induced dissociation (CID), in a process known as tandem mass spectrometry (MS/MS) (Harrison and Cotter, 1990; McLuckey, 1992). Computer algorithms use the CID fragmentation patterns of sample peptides to determine the sequence of the peptide, and this sequence information is used to search against theoretical spectra generated from protein and nucleotide databases. Protein identifications are made by finding the best correlation between experimentally derived sequence information and sequences in the database.

However, if the mixture of peptides is highly complex, it is advantageous to use a separation step prior to analysis to limit the number of peptides. The method most commonly used to reduce sample complexity prior to introduction into the mass spectrometer is the separation of sample proteins by gel electrophoresis followed by excision of the individual protein spots from the gel and in-gel digestion with a protease (i.e., trypsin) (Delahunty and Yates, 2005).

To date, comparative plant proteomics studies are still predominantly performed employing traditional two dimensional polyacrylamide gel electrophoresis (2-D PAGE) with isoelectric focusing in the first and SDS-PAGE in the second dimension. Limitations of this method regarding sensitivity, dynamic range and reproducibility of protein quantification could be improved significantly by introducing the differential gel electrophoresis (DIGE) technology employing spectrally resolvable fluorescent dyes to differentially label proteins prior to separation by 2-D PAGE. One-dimensional (1D) gels, which separate proteins based on molecular mass, provide a low-resolution separation of proteins, but when coupled

with tandem mass spectrometry can be used to identify proteins in moderately complex mixtures.

In plant, proteome analysis is a powerful tool for determining the roles and functions of individual proteins. Several proteomics studies about the embryo and endosperm of seeds in *Arabidopsis* (Gallardo *et al*., 2002), rice (Woo *et al*., 2002; Yang *et al.*, 2007), wheat (Vensel *et al*., 2005), maize (Fasoli *et al*., 2009), barley (Funnie and Svensson, 2009), peanut (Chassaigne *et al*., 2009), soybean (Komatsu and Ahsan, 2009) and strawberry (Bianco *et al*., 2009) have been carried out.

2.2.2 Proteomics in *J. curcas*

Proteomics in *J.curcas* has been studied for recently years such as analysis of oil mobilization in seed germination and postgermination development using 2D-PAGE, HPLC and LC-MS/MS (Yang *et al*., 2009), the results showed that the oil mobilization was initiated during germination, and then the oil was consumed for early seedling development. The significant change in abundance of 50 protein spots during germination indicated that several pathways including β-oxidation, glyoxilate cycle, glycolysis, citric acid cycle, gluconeogenesis, and pentose phosphate pathway were involved in the oil mobilization, shown in Figure 2.3. Another proteomics study, comparative analysis of embryo and endosperm proteome from seeds of *J. curcas* using 2D-PAGE and LC-ESI-Q/TOF MS/MS) (Liu *et al*., 2009), and found that embryo and endosperm had similar distribution patterns for protein spots (shown in Figure 2.4). However, there is a need for more sensitive, reproducible, and high-throughput proteomics methodology. To improve analysis of complex protein mixtures in proteomics, the direct analysis of proteins in SDS-PAGE gel bands prior to LC-MS/MS is considered.

Mobilization of storage oil during germination and postgermination is illustrated in Figure 2.3. The TAGs in the oil bodies are hydrolyzed under catalyzation of lipases into fatty acids and glycerol (a), or are oxidated under catalyzation of lipoxygenase into hydroperoxides first (b-1) and then hydrolyzed under catalyzation of lipases into fatty acids and glycerol (b-2). The glycerol can be

fed into the gluconeogenesis pathway after oxidation to dehydroxy acetone (c). Fatty acids are activated in the glyoxysomes as CoA-thioesters and degraded by oxidation into acetyl CoA (d). From two molecules of acetyl CoA, the glyoxylate cycle forms one molecule of succinate (e), which is converted by the citric acid cycle in the mitochondria to malate (f). Phosphoenolpyruvate formed from malate in the cytosol is a precursor for the synthesis of hexoses via the gluconeogenesis pathway (g). The intermediates formed in pentose phosphate pathway (h) can be used for biosyntheses of other compounds. The identified protein spots related to oil mobilization, pathways involved in oil mobilization, and key steps of the oil mobilization are highlighted by red, green, and blue, respectively**.**

Figure 2.3 Mobilization of storage oil during germination and postgermination.

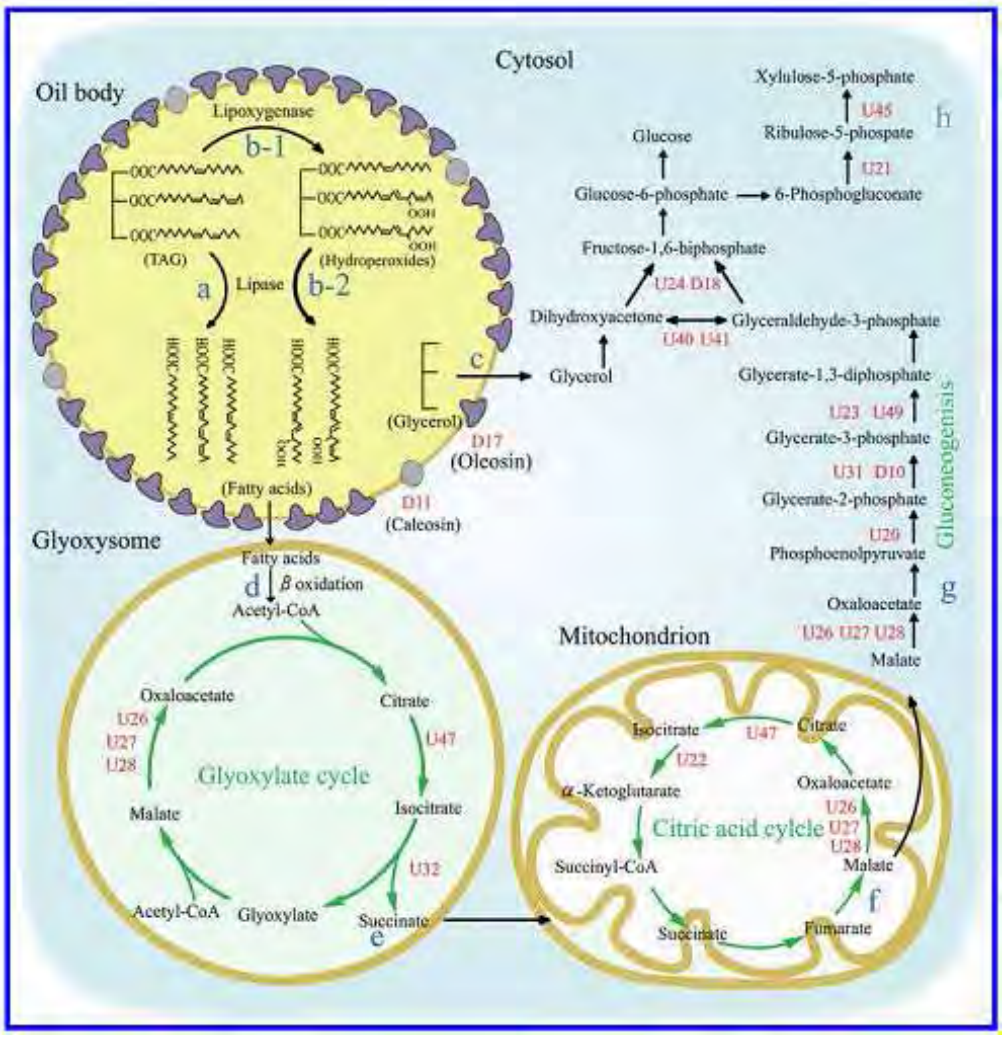
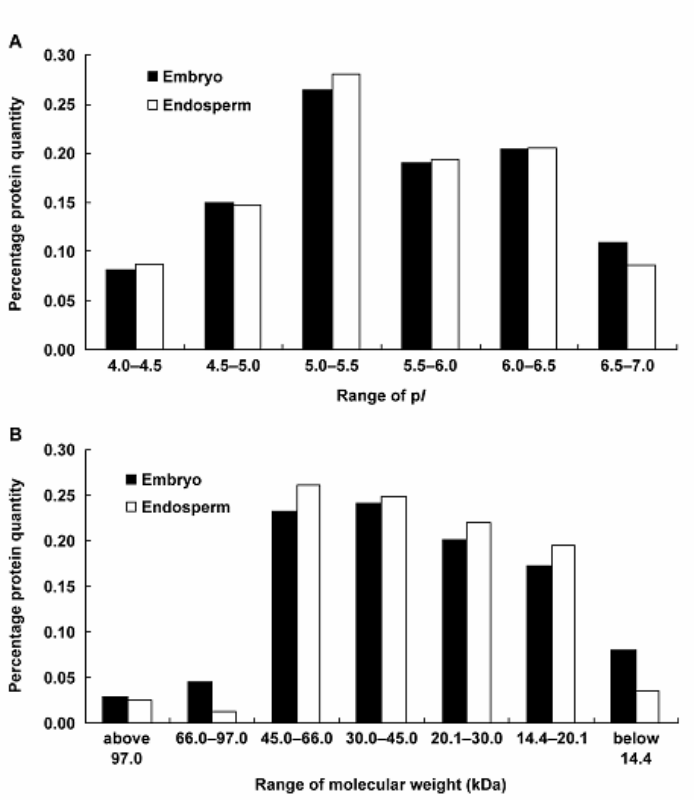


Figure 2.4 The protein distribution pattern of the embryo and endosperm (A) The distribution of protein number (%) according to pI (B) The distribution of protein number (%) according to molecular weight.



2.3 Fatty Acid Biosynthesis

In biochemistry, fatty acids are aliphatic monocarboxylic acids derived from animal or vegetable fat, oil, or wax. Fatty acids commonly have a chain of four to 28 carbons, which is either saturated or unsaturated. The input to fatty acid biosynthesis is acetyl-CoA, which is carboxylated to malonyl-CoA by acetyl-CoA carboxylase (ACCase). Both acetyl-CoA and malonyl-CoA are also the immediate precursors for condensation reaction and formation the fatty acid chain by fatty acid synthase complex. In plants and bacteria, the seven active sites for fatty acid biosynthesis (6 enzymes and acyl carrier protein) reside in seven separate polypeptides. Proteins of the fatty acid synthase complex and their roles are shown in Table 2.3. In these complexes, each enzyme is positioned with its active site near that of the proceeding and succeeding enzymes of the sequence. The flexible pantetheine arm of ACP can

reach all of the active sites, and it carries the growing fatty acyl chain from one site to the next; the intermediates are not released from the enzyme complex until the finished product is obtained.

Table 2.3 Proteins of the fatty acid synthase complex and their roles in fatty acid biosynthesis pathway (Nelson and Cox, 2000)

Protein	Role
Acyl carrier protein (ACP)	Carries acyl groups in thioester linkage
Acetyl-CoA-ACP transacetylase (AT)	Transfers acyl group from CoA to Cys residue of KS
β-Ketoacyl-ACP synthase (KS)	Condenses acyl and malonyl groups (there are at least 3 isozymes of KS)
Malonyl-CoA-ACP transferase (MT)	Transfers malonyl group from CoA to ACP
β-Ketoacyl-ACP reductase (KR)	Reduces β-Keto group to β-hydroxy group
β-Hydroxyacyl-ACP dehydratase (DH)	Removes H_2O from β-hydroxyacyl-ACP, creating double bond
Enoyl-ACP eductase (ER)	Reduces double bond, forming saturated acyl-ACP

Palmitate (C16:0) is the precursor of stearate and longer-chain saturated fatty acids, as well as the monounsaturated acids palmitoleate and oleate. Polyunsaturated fatty acids and eicosanoids are converted from linoleate by desaturase enzyme.

In *J. cucas*, several enzymes were studied such as acetyl-CoA carboxylase (beta-ketoacyl-ACP synthase III (Li *et al*., 2008), stearoyl-ACP-thioesterase (Dani *et al*., 2009) and stearoyl- ACP desaturase (Tong *et al*., 2006).

2.4 Protein Related to Fruit Ripening

Ethylene is considered a plant hormone influencing many aspects of growth, development, and senescence of higher plants. The enzyme1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) catalyzes the first regulatory step in the ethylene biosynthesis pathway, conversion of S-adenosyl-l-Met into ACC, whereas ACC oxidase (ACO) catalyzes the final step, conversion of ACC into ethylene (Yang and Hoff-man, 1984; Kende, 1993). Both ACS and ACO are encoded by multigene families (Fluhr and Mattoo, 1996). In tomato, *ACS* (*LeACS*) genes characterized so far

have differences in their tissue-specific expression patterns, developmental control, and kinetics of ethylene induction (Vander Straeten *et al*., 1990; Olson *et al*., 1991; Rottmann *et al*., 1991; Yip *et al.*, 1992; Spanu *et al*., 1993; Terai, 1993; Nakatsuka *et al*., 1998) but it is not yet known how or if they interact with auxin to regulate fruit development.

In tomato, two systems have been proposed to explain the regulation of ethylene during plant development (Lelievre *et al*., 1997). System 1 provides for the basal level of ethylene present in vegetative tissues and preclimateric and non-climateric fruits, whereas system 2 is responsible for the high levels of ethylene production associated with ripening of climacteric fruits and flower senescence (Oetiker and Yang, 1995). Specific members of the *LeACS* and *LeACO* gene families are proposed to regulate the transition from system 1 to 2 ethylene production. Based on their gene expression patterns, as well as their regulation by ethylene, LeACS2 and 4 are proposed to mediate system 2 ethylene production, whereas LeACS1A and 6 function in system 1 in green fruit and vegetative tissue (Nakatsuka *et al*., 1998; Barry *et al*., 2000).

2.5 Proteins Related to Seed Development

Plant seed development and germination are under strict temporal and spatial regulation, and transcription factors play important roles in this regulation (Mei *et al*., 2008).

2.5.1 Phenolic Compound

Phenolic compounds are widely distributed in plants. They are particularly important in fruits and vegetables, to which they contribute color and flavor. Polyphenols are involved in astringent and bitter tastes, which contribute to “the overall mouthfeel” of fresh fruits (Macheix *et al*., 1990). Their contribution during food processing, particularly in browning phenomena (enzymatic or non enzymatic), has been demonstrated by several authors (Romeyer *et al*., 1985). Changes in phenolic compounds during fruit maturation have been reported for apple (CoSeteng

and Lee, 1987; Murata *et al*., 1995), grape (Lee and Jaworski, 1988), peach (Lee *et al*., 1990), pear (Amiot *et al*., 1995), and plum (Raynal *et al*., 1989).

Phenolics play an important role in plant metabolism, but also protect the plant against stresses. For instance, it has been recently shown that flavonoids, such as catechin, regulate the auxin transport in plants, and, therefore, play an important role in plant development (Brown *et al*., 2001).

Flavonoids sensu latu constitute the largest class of phenolic compounds with more than 3000 structures, possessing in common a flavylium unit (C -C - C) 6 3 6 (Iacobucci and Sweeny, 1983). Sorghum contains flavonoids such as flavanols (flavan-3-ols, flavan-4-ols, etc.), flavanones, flavones and anthocyanins (Haslam, 1998).

2.5.2 APETALA (AP2) Domain

Previous studies have suggested that AP2 functions as a nuclear transcription factor in plant cells. DNA sequence analysis showed that *AP2* encodes a theoretical polypeptide of 432 amino acids that is distinct from known fungal and animal regulatory proteins. One important feature of the AP2 protein is a novel 68 amino acid repeated motif called the AP2 domain. The AP2 domain has been shown to be essential for AP2 functions and contains an 18-amino acid core region that helix. The AP2-domain transcription factors were named after the original gene AP2, which functions in flower organ development (Jofuku *et al*., 1994).

2.5.3 Late Embryogenesis Abundant Proteins (LEA)

LEA proteins have first been described about 25 years ago as accumulating late in plant seed development. They were later found in vegetative plant tissues following environmental stress and also in desiccation tolerant bacteria and invertebrates. Although they are widely assumed to play crucial roles in cellular dehydration tolerance, their physiological and biochemical functions are largely unknown. LEA proteins are classified in at least five groups by virtue of similarity in their amino acid sequences (Cuming, 1999; Wise, 2003). Most genes encoding LEA

proteins had abscisic acid response (ABRE) and/or low temperature response (LTRE) elements in their promoters and many genes containing the respective promoter elements were induced by abscisic acid, cold or drought (Hundertmark and Hincha, 2008). LEA proteins were first found in cotton (*Gossypium hirsutum*) seeds, accumulating late in embryogenesis (Dure *et al*., 1981). They were subsequently found in the seeds of many other plants, but also in vegetative organs, especially under stress conditions such as cold, drought, or high salinity (Ingram and Bartels, 1996; Thomashow, 1999).

2.6 Proteins Related to Fatty Acid Biosynthesis

2.6.1 Acetyl CoA Carboxylase (ACCase)

ACCase is a biotin-containing enzyme that catalyzes the ATP-dependent carboxylation of acetyl-CoA to form malonyl-COA. This is the first committed reaction of de novo fatty acid biosynthesis and is a major determinant of the flux through that pathway (Vagelos, 1971; Lane *et al*., 1974; Wakil *et al*., 1983). This reaction is believed to be a key regulatory step in fatty acid biosynthesis in animals, bacteria, and plants (Kim *et al*., 1989; Jackowski *et al*., 1991). Similarly, Ohlrogge and Jaworski (1997) reported in biochemical studies that the acetyl-CoA carboxylase gene product may be involved in the control of the lipid accumulation process.

In the alga *Cyclotella cryptica*, the *ACCase* gene with encodes a polypeptide is predicted to be composed of 2089 amino acids and to have a molecular mass of 230 kDa. The deduced amino acid sequence exhibits strong similarity to the sequences of animal and yeast ACCases in the biotin carboxylase and carboxyltransferase domains. There is less sequence similarity in the biotin carboxyl carrier protein domain, although the highly conserved Met-Lys-Met of the biotin binding site is present (Roessler and Ohlrogge, 1993).

In plants, the malonyl-CoA produced by ACCase is involved in several biochemical reactions and pathways, including fatty acid synthesis and elongation, flavonoid synthesis and malonation of the ethylene precursor aminocyclopropane-1-

carboxylate, and malonation of amino acids and glycosides (Dixon and Bendall, 1978; Klejdus *et al*., 2005; Post-Beittenmiller *et al*., 1991; Post-Beittenmiller *et al*., 1992).

2.6.2 Phosphoenol Pyruvate Carboxylase (PEPC)

Many studies had already demonstrated that phosphoenol pyruvate carboxylase (PEPC) is a ubiquitous plant cytosolic enzyme catalyzes the irreversible of PEP to yield oxaloacetate and Pi and then oxaloacetate converted to malate. (Andreo *et al*., 1987; Hudspecth *et al*., 1986; Leary 1982).

In castor oil seed, Smith and coworkers (1992) recently report that malate supports very high rates of fatty acid synthesis by isolated leucoplasts prepared from developing castor oil seed. Based on the measurement of leucoplast-localized $NADP^+$-malic enzyme, they proposed that malate may be a key carbon source for fatty acid synthesis *in vivo*. Similarity to the observation of Sangwan *et al*. (1992) that PEPC of castor oil seed plays a fundamental role *in vivo* in the cytosolic production of an important substrate (malate) for fatty acid biosynthesis by developing castor oil seed leucoplast.

Recently years, PEPC activity was monitored during seed maturation of two varieties (Hybridol and Pactol) of rapeseed (*Brassica napus* L.) (Sebei *et al*., 2006). They discovered that in the Pactol variety, the evolution of PEPC activity showed a classical curve, i.e. an increase during the most active phase of lipid accumulation in maturating seeds, followed by a rapid decrease until the end of seed maturation. Moreover they studied about the evolution pattern of fatty acids and TAGs contents, and found that it was similar to that of PEPC activity. Taken together, their findings suggest that PEPC may be involved in fatty acid and triacylglycerol biosynthesis during seed maturation of rapeseed.

2.6.3 Mercaptopyruvate Sulfurtransferase (MST)

Sulfurtransferases are involved in biosynthesis of some sulfur-containing cofactors. The enzymology of C-S bond forming reactions and genetics involved in the biosynthesis of biotin, lipoic acid, thiamin, and molybdopterin. Biotin (vitamin H)

is a water-soluble vitamin that can be produced by bacteria, plants and a few fungi. Biotin plays a very important physiological role as the prosthetic group of several carboxylases involved in central metabolism, including gluconeogenesis and fatty acid biosynthesis, and also in secondary metabolism.

The MST (mercaptopyruvate sulfurtransferase) enzyme catalyzes the conversion of 3-mercaptopyruvate to pyruvate and a reduced sulfur species. In 1996, rat liver MST was purified to homogeneity by Nagahara and Nishino. They reported that MST is very similar to rhodanese (thiosulfate sulfurtransferase) in physicochemical properties. Sequence identity in cDNA is 65%. Both MST and rhodanese possess MST and rhodanese activities, although the ratio of rhodanese to MST activity is low in MST and high in rhodanese. There is a strong possibility that Cys247 is a catalytic site of MST. Arg187 is suggested to be a binding site of both mercaptopyruvate and thiosulfate in MST. Arg196, which is missed in rhodanese, is important for catalysis in MST. On the other hand, the substitution of Arg for Gly248 or Lys for Ser249 facilitates catalysis of thiosulfate in MST.

2.6.4 Coenzyme A Ligase

The formation of acyl-coenzymes (Co) A occurs as an obligatory step in the metabolism of a variety of endogenous substrates, including fatty acids. The reaction is catalysed by ATP-dependent acid: CoA ligases, classified on the basis of their ability to conjugate saturated fatty acids of differing chain lengths, short (C2-C4), medium (C4-C12) and long (C10-C22). The enzymes are located in various cell compartments (cytosol, smooth endoplasmic reticulum, mitochondria and peroxisomes) and exhibit wide tissue distribution (Knights, 1998).

2.7 Analysis of Relative Gene Expression by Real-time RT-PCR

2.7.1 Real-time PCR Technique

Recent research has shown that genetic components in gene transcription as well as genetic variability in coding sequences are of evolutionary importance (Oleksiak *et al*., 2002; Wegner *et al*., 2006). Quantitative reverse transcription

polymerase chain reaction (qRT-PCR) is a technique for the quantification of gene expression at the mRNA level that combines the advantages of specificity, sensitivity, speed, throughput and reproducibility. Therefore it is a powerful tool in experimental research (Haller *et al*., 2004).

Nowadays real-time RT-PCR is widely and increasingly used. It is the most sensitive method for the detection and quantification of gene expression levels, in parcicular for low abundance mRNA, in tissues with low concentrations of mRNA (e.g. bone marrow, fatty tissues), from limited tissue samples (e.g. biopsies, single cells) (Lockey *et al*., 1998; Steuerwald *et al*., 1999) and to elucidate small changes in mRNA expression levels (Wittwer *et al*., 1997). However, it is a very complex technique with various substantial problems associated with its true sensitivity, reproducibility and specificity and as a fully quantitative methodology, it suffers from the problems inherent in real-time RT-PCR.

The relative expression is based on the expression ratio from a target gene versus a reference gene and is adequate for most purposes to investigate physiological changes in gene expression levels (Pfaffl, 2001).

2.7.2 Housekeeping Gene: Ubiquitin

The utilization of qPCR for gene expression analysis could be a powerful tool for investigating the expression levels, interactions, and regulation of embryo-related genes. However, in order to obtain accurate results when using qPCR to study gene expression, several factors must be considered: the quantity of the initial material, the quality of the RNA, the efficiency of cDNA synthesis, primer performance, selection of reference genes, and the methods to be used for statistical analysis (Vandesompele *et al*., 2002; Gonzalez-Verdejoa *et al*., 2008; Jorge *et al*., 2008; Udvardi *et al*., 2008; Bustin *et al*., 2009). Moreover, the mRNA level itself is not only influenced by regulation of gene expression. Many other conditions, e.g. nutrition, differences in size and components of the tissue, can influence the mRNA level of the target gene. To obtain accurate data, one or several internal control genes for normalization are needed. At present, potential housekeeping genes are used as references for gene

expression analysis; however, many studies have shown that their expression often is affected by different experimental parameters and conditions. Therefore, to obtain meaningful results, the stability of expression of specific reference genes under different experimental conditions or developmental stages needs to be determined in qPCR assays.

Until recently, the housekeeping genes involved in basic cellular processes such as 18S rRNA, ubiquitin (UBQ), actin (ACT), b-tubulin (TUB), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as internal controls for gene expression analysis as they were supposed to have a uniform expression. (Thellin *et al*., 1999; Suzuki *et al*., 2000; Lee *et al*., 2002; Bustin, 2002; Czechowsk *et al*., 2005).

In plant, UBQ expression has been found that stable across different tissue samples examined (Jain *et al*., 2006).

Ubiquitin (UBQ) is a highly conserved 76 amino acid protein found in all eukaryotic organisms that plays an essential role in intracellular turn over of proteins (Jentsch, 1992; Hershko and Ciechanover, 1992; Hershko and Ciechanover, 1998; Hochstrasser, 1996; Yamao, 1999). The covalent ligation of UBQ to various acceptor proteins in eukaryotic cells participates in or regulates a number of cellular processes, such as selective protein degradation, DNA repair, progression through the cell cycle, signal transduction, transcriptional regulation, the nuclear transport process, receptor control by endocytosis, the processing of antigens in the immune system, pathological alterations and programmed cell death (Schwartz and Ciechanover, 1999; Kato, 1999). A major function of UBQ is to label proteins destined for selective elimination.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant Material Preparation

The 8 developmental stages of *Jatropha curcas* fruit (Figure 3.1 and detail was shown in Table 3.1) were collected from Biofuel Production by Biocatalyst in Faculty of Sciences, Chulalongkorn University, Bankok, Thailand. All fruits were stored at -80ºC until used.

Figure 3.1 8 development stages of *J. curcas* fruit

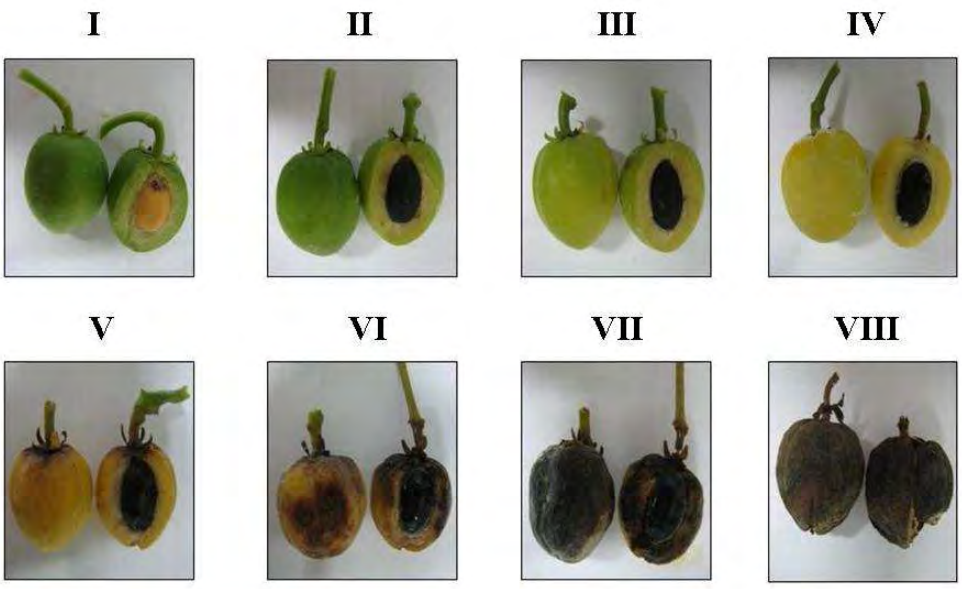
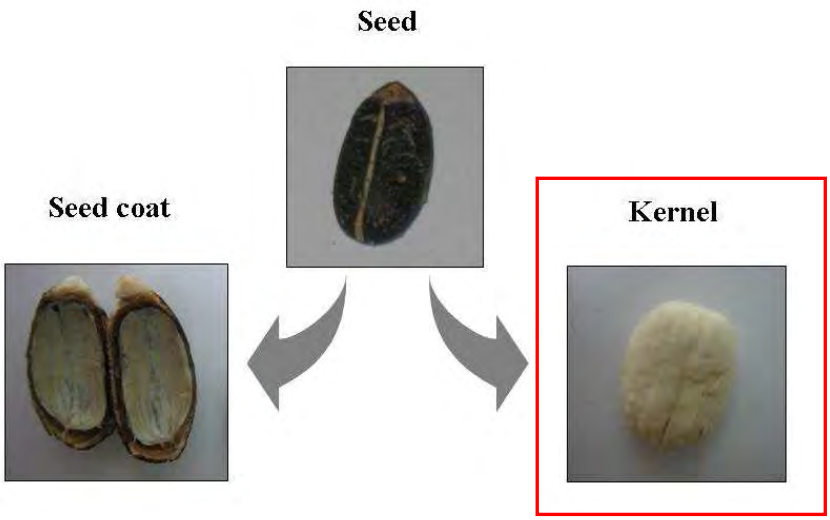


Table 3.1 The characteristics of 8 developmental stages of *J. curcas* fruit

Developmental stages	Age (Days after fertilized)	Fruit color	Seed color	Fruit stalk color	Fruit texture
I	14	Green	Brown	Green	Hard
II	19	Green	Black	Green	Hard
III	24	Greenish yellow	Black	Green	Hard
IV	29	Yellow	Black	Green	Hard
V	34	Yellowish black	Black	Green	Hard
VI	39	Blackish yellow	Black	Green	Soft
VII	44	Black	Black	Green	Soft
VIII	50 up	Black	Black	Black	Hard, dry

The seeds from 8 developmental stages of *J. curcas* fruits were cracked, the seed coats were carefully removed and the white seed kernels were collected. The *J. curcas* seed, seed coat and kernel were shown in Figure 3.2.

Figure 3.2 Seed components: seed, seed coat and kernel



3.1.2 Chemicals and Reagents

Acrylamide, bis-N,N'-acrylamide, ammonium persulfate, N,N',N'',N'''-tetramethylethylenediamine (TEMED), sodium dodecyl sulfate (SDS) were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Ammonium sulfate, bromphenol blue, Folin reagent, chloroform, copper sulfate, sodium hydroxide, methanol, ethanol, sodium bicarbonate, sodium dihydrogen phosphate, glacial acetic acid, hydrochloric acid, and disodium ethylenediamine tetraacetate (EDTA) were purchased from Carlo ERBA (Rodano, Milano, Italy). Bacteriological tryptone, yeast extract, and agar were purchased from DIFCO (Grayson, USA). Sodium chloride, phenylmethylsulfonyl fluoride (PMSF), diethyl pyrocarbonate, and magnesium chloride were purchased from Fluka (Steinheim, Swizerland). Boric acid, Bovine serum albumin (BSA), isopropyl-β-D-thiogaltopyranoside (IPTG) were purchased from Merck (Damsladt, Germany). Amplicilin and ethidium bromide (EtBr) were purchased from Sigma (St. Louis, USA). Agarose, deoxyribonuclotide (dATP, dCTP, dGTP and dTTP) and X-Gal were purchased from Promega (Madison, USA). Other chemicals and solvents used but not listed here were purchased from variety of suppliers.

3.1.3 Oligonucleotide primers

All oligonucleotide primers were designed and ordered from BioDesign (Pathumthani, Thailand)

3.2 Methods

3.2.1 The Study of Fatty Acid Profiles by Gas Chromatography

3.2.1.1 Oil Extraction

Jatropha curcas seeds were cracked, seed coats were carefully removed, white seed kernel tissues from 8 developmental stages were milled using laboratory mortar and pestle. *Jatropha curcas* oil was extracted using hexane with a ratio of 500 µl hexane per 500 mg seed kernel and leaved the seed kernel to vortex overnight. After

centrifugation (using centrifuge, Spectrafuge^{TM} 16M, Labnet, USA) at 10,000xg for 10 min to precipitate tissues and cell debris, the supernatant (oil and solvent) were collected. Solvents were removed from oil by incubating in desiccator at room temperature for 6-8 h.

3.2.1.2 Transesterification

Jatropha curcas oil were changed to FAME or biodiesel by transesterification reaction. Methanol mixture (1:9 mol of *J. curcas* oil: alcohol) was mixed with NaOH (1% wt/wt) and incubated at 60 ºC for 3 h. After reaction was completed, two phases will be observed. Upper phase was methyl ester and lower part was glycerin. The glycerin was precipitated by centrifugation (using centrifuge, Spectrafuge^{TM} 16M, Labnet, USA) at 10,000xg for 10 min and the methyl ester was collected.

3.2.1.3 Gas Chromatography

Fatty acid composition was determined using gas chromatography (GC-2010A, Shimadzu, Japan) with a capillary column (Omegawax^{TM} 320, 30 m length x 0.32 mm inner diameter x 0.25 µm film thickness, Sigma-Aldrich, USA), and flame-ionization detector. Linear velocity was 55.0 cm/sec and the split ratio was 20.0. Hydrogen and air at flow rates of 40 and 400 ml/min was used. The sample (1 µl) was injected into the system at 230ºC injector temperature. The oven temperature was kept at 185 ºC and then it was gradually increased at 5.0 ºC/min up to 240 ºC and a total analytical time was 12 min. The methyl ester was identified by comparing its retention time to the retention time of standard methyl ester of fatty acid.

3.2.2 The Study of Protein Expression by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

3.2.2.1 Total Protein Extraction

Seed kernel tissues from 8 developmental stages were milled using laboratory mortar and pestle and 250 mg fine powder was then transferred into the microcentrifuge tube. Each sample was added with 1 ml of 0.1% SDS, vortexed for 1

h and centrifuged at 10,000xg for 15 min. The supernatant was transferred to a new tube, mixed well with 4 volumes of cold acetone, and incubated overnight at -20ºC. The mixture was centrifuged at 10,000xg for 15 min and the supernatant was discarded. The protein pellet was resuspended with 0.15% sodium deoxycholic acid (DOC).

3.2.2.2 Protein Concentration Determination

Concentration of protein in each sample was determined by Lowry method (Lowry *et al*., 1951) using bovine serum albumin (BSA) as standard. The absorbance at 750 nm (OD_{750}) was determined and the protein concentration was calculated using the standard curve, plotted between OD_{750} on Y-axis and BSA concentration (µg/ml) on X-axis.

3.2.2.3 Optimization of Sample Concentration

The optimal protein concentration for each developmental stages (between 22, 5, 10, 20, 30, 50 and 60 µg) was examined using SDS-PAGE. The protein content that yield more bands and clear results were selected for further experiments.

3.2.2.4 Preparation of Sample for SDS-PAGE Analysis

The appropriated protein concentration of all sample and protein standard marker (Low Molecular Weight SDS Marker Kit, GE Healthcare Bio-Sciences AB, Sweden) were mixed with 5 µl of 5x loading buffer (0.125M Tris-HCl pH 6.8, 20% glycerol, 4% SDS, 0.2M DTT, 0.02% bromophenol blue) and deionized distilled water until the final volume was 25 µl. The mixture was heated at 95ºC for 10 min before loaded onto 15% SDS-PAGE.

3.2.2.5 Preparation of SDS-PAGE Slab Gel

Whole proteins were separated on SDS-PAGE mini slab gel (miniPAGE chamber AE-6530, ATTO, Japan). The polyacrylamide gel was prepared according to the method of Laemmli (1970). The gel used for separating of soluble protein

contained 15% acrylamide. The separating and stacking gels were prepared according to Table 3.2.

Table 3.2 Preparation of SDS-PAGE separating and stacking gel for 90 x 80 x 1mm mini-slab format gel size

Reagent	**Separating gel (15%)**	**Stacking gel (4%)**
40% acrylamide (ml)	3.7605	0.3
1.5 M Tris-HCl pH 8.8 (ml)	2.5	-
0.5 M Tris-HCl pH 6.8 (ml)	-	0.742
10% SDS (µl)	125	30
Deionized distilled water (ml)	3.589	1.916
10% ammonium persulfate (µl)	50	23
TEMED (µl)	3.3	1.7

3.2.2.6 Running Condition

SDS-PAGE was carried out at 30 Volts for stacking gel and 50 Volts for separating gel using an electrophoresis power supply (Electrophoresis Power Supply, GE Healthcare Bio-Sciences AB, Sweden) until blue line of loading dye reached the bottom of the gel.

3.2.2.7 Silver Staining

The advantage of silver staining is high sensitivity, approximately 1 ng of protein in a single band is detected. The gel was stained according to Blum *et al*. (1987) as shown in Table 3.3.

Table 3.3 Silver staining procedure for 8x9 cm gel

Step	**Solution**	**Time**
Fixation	50% Methanol 12% Acetic acid 0.0185% Formaldehyde	Overnight
Washing	35% Ethanol	2x5 min

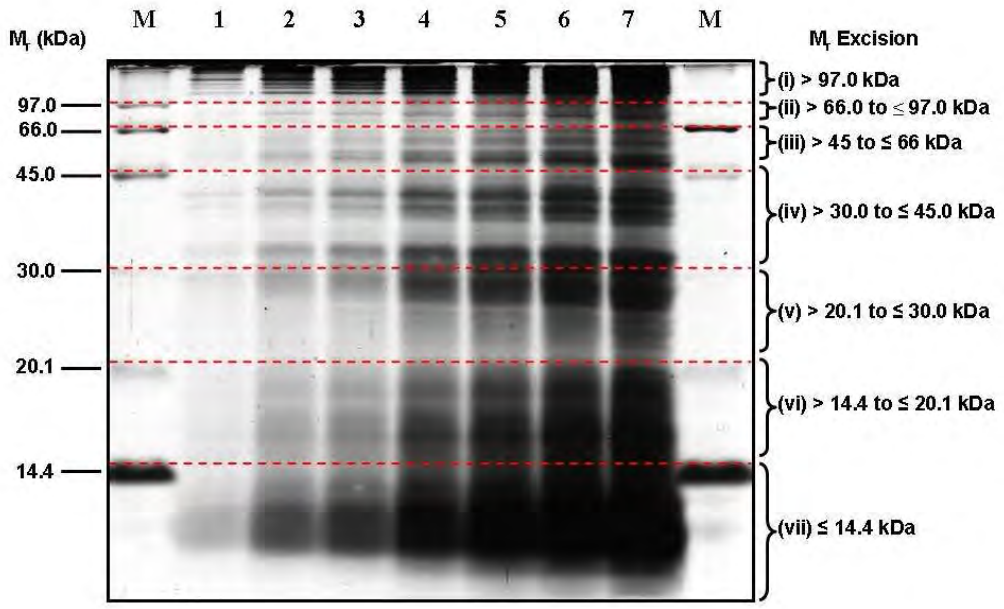
Step	Solution	Time
Sensitizing	0.02% Sodium thiosulfate	2 min
Washing	Distilled water	2x5 min
Staining	0.2% Silver nitrate	20 min
Washing	Distilled water	1 min
Developing	6% Sodium carbonate 0.0004% Sodium thiosulfate 0.0185% Formaldehyde	The reaction can be stopped when all bands of protein marker appear
Stopping	0.46% EDTA	20 min
Storing	0.1% Acetic acid	Until used

3.2.3 Protein Identification by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)

3.2.3.1 Excision of Protein Bands

Protein bands from the silver stained SDS-PAGE gel were excised according to molecular mass range of low molecular weight protein standard marker as shown in Figure 3.3. The molecular mass contains 7 ranges which are (i) higher than 97 kDa, (ii) 66-97 kDa, (iii) 45-66 kDa, (iv) 30-45 kDa, (v) 20.1-30 kDa, (vi)14.4-20.1 kDa and (vii) lower than 14.4 kDa. Protein bands were cut manually into small pieces about 1x1x1 mm^3 using scalpel and forcep. The gel pieces were placed in 96-well-microtiter plate (10 pieces per well) and washed with sterile nanopure water until ready for the next analysis.

Figure 3.3 Molecular mass range of low molecular weight protein standard marker**.** Lane M represents standard molecular weight marker; Lane 1 represents 2 µg protein; Lane 2 represents 5 µg protein; Lane 3 represents 10 µg protein; Lane 4 represents 20 µg protein; Lane 5 represents 30 µg protein; Lane 6 represents 50 µg protein and Lane 7 represents 60 µg protein of *J. curcas* kernel in stage I .



3.2.3.2 In-Gel Digestion

The gel pieces were in-gel digested using an in-house protocol developed in Proteomics Laboratory, Genome Institute, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Thailand. Firstly, the gel pieces in each well were dehydrated with 200 µl of 100% acetonitrile (ACN) for 5 min in a shaker (Mx2, Micromixer, Korea). The proteins in gel plugs were reduced with 30 µl of 10 mM Dithiotreitol (DTT) in 10 mM ammonium bicarbonate at room temperature for 1 h and alkylated at room temperature for 1 h in the dark in the presence of 100 mM iodoacetamide (IAA) in 10 mM ammonium bicarbonate. After alkylation, the gel pieces were dehydrated twice with 200 µl of 100% ACN, each for 5 min. Then, 10 µl of trypsin solution (10 ng/µl trypsin in 50% ACN/10mM ammonium bicarbonate) was added followed by

incubation at room temperature for 20 min, and 20 µl of 30% ACN was added to keep the gels immersed throughout digestion. The gels were incubated at 37ºC for a few hours or overnight. To extract peptide digestion products, 30 µl of 50% ACN in 0.1% formic acid (FA) was added into the gel plugs, and then the gel plugs were incubated at room temperature for 10 min in a shaker. Extracted peptides were collected and pooled together. The peptides extraction step was performed for three times. The pooled extracted peptides were dried at 40 ºC in an incubator for 4 h and kept at -80 ºC for further LC-MS/MS analysis.

3.2.3.3 LC-MS/MS Analysis

Before injection, extracted peptides were resuspended with 0.1% FA and centrifuged at 10,000xg for 5 min. The protein digests were injected to Ultimate 3000 LC system (Dionex) coupled to ESI-Ion Trap MS (HCT ultra PTM Discovery System, Bruker Daltonik) with electrospray at a flow rate of 20 µL/min to µ-precolumn (Monolithic Trap Column, 200 µm i.d. x 5 cm). The sample was separated on a nano column (Monolithic Nano Column, 100 µm i.d. x 5 cm) at a flow rate of 1 µL/min . A solvent gradient (solvent A: H_2O, 0.1% formic acid; solvent B: 5$_0$% H_2O, 50% ACN, 0.1% formic acid) was run in 20 min.

3.2.3.4 MS/MS Spectra Differential Analysis

As mentioned above in section 3.2.3, the protein bands of each sample were excised into 7 molecular mass ranges, the MS/MS spectra of all ranges were analyzed by DeCyder MS 2.0 differential analysis software (GE Healthcare). The software consists of two analysis features: Peptide Detection with the PepDetect module and Run-to-Run Matching with the PepMatch module. PepDetect module provides consistent and accurate peptide detection, background subtraction, isotope and charge-state deconvolution, as well as peak volume calculations using novel imaging algorithms. The software's visualization tools offer the user new capabilities to optimize chromatography and MS/MS settings. Besides, Pepmatch module supports a wide range of experimental designs, such as control/treated experiments as well as

time-dose studies, it detects small quantitative differences between peptides across multiple runs with high statistical confidence at p value < 0.05.

3.2.3.5 Identification of Protein Using Database Search

Differential tandem mass spectra of peptides from 3.2.3.4 were identified by Mascot (Matrix Science Ltd., London, U.K.), the search engine that uses mass spectrometry data to identify proteins from primary sequence databases. The database obtained from the NCBI (http:/www.ncbi.nlm.gov). Viridiplantae (Green plants) was choosen and the conditions were fixed at carbamidomethyl (C) and oxidation (M), while peptide tolerance and MS/MS tolerance was 1.2 Da. All proteins were functional identified by Gocat (http://eagl.unige.ch/GOCat).

3.2.4 The Study of RNA Expression by Real-time PCR

3.2.4.1 Total RNA Isolation

Frozen *J. curcas* seeds were ground in liquid nitrogen and extracted with the Hao Yu buffer (Yu and Goh, 2000) containing 2% (v/v) □-mercaptoethanol, 2% (w/v) hexadecyltrimethyl-ammonium bromide, 100mM Tris [tris (hydroxymethyl)-aminomethane]-HCl (pH7.5), 20mM EDTA, 2M NaCl, and 1% (w/v) polyvinylpyrrolidone. The homogenate was incubated in a water bath at 60ºC for 15 min with occasional shaking and then centrifuged at 7,500xg for 15 min at 4ºC. After centrifugation, the aqueous phase was extracted at least twice with an equal volume of chloroform: isoamyl alcohol (24:1, v/v). The mixture was mixed vigorously by shaking for 5-10 min and then centrifuged at 7,500xg for 15 min at 4ºC. Supernatant was kept and extracted with chloroform:isoamyl alcohol (24:1, v/v) for four times. Nucleic acid was precipitated with 0.9 volume of supernatant, on ice for 10 min and centrifuged at 7,500xg for 30 min at 4ºC. The RNA pellet was resuspended with diethylpyrocarbonate-treated water. Total RNA was precipitated by adding 0.25 volume of 10M lithium chloride and kept overnight at -20ºC. The pellet was washed with 2.5M lithium chloride and washed again with 70% (v/v) ethanol, dried, and dissolved in diethylpyrocarbonate-treated water.

If solution was turbid, recentrifugation at 13,000xg for 1 min at 4ºC was performed. The supernatant was kept and the RNA was reprecipitated by 10M lithium chloride and left overnight at -20ºC again. RNA purity and concentration were determined by nanodrop (NanoDrop ND-1000 Spectrophotometer, USA). The integrity of RNA was evaluated by separation on a denaturing gel electrophoresis (according to Table 3.4) by mixing RNA with 2 volumes of RNA loading dye (according to table 3.5), incubated the mixture at 65ºC for 10 min and then left on ice for 2 min before run. The RNA solution was kept at -80ºC until used.

Table 3.4 Preparation of 1.2% denaturing gel

Chemicals	**Quatitation**
Agarose	0.54 g
DEPC water	39.15 ml
10X MOPS	4.5 ml
Formaldehyde	1.35 ml

Table 3.5 Prepration of RNA loading dye

Chemicals	**Volume (µl)**			
Formamide	12.5	25	50	100
Formaldehyde	4.15	8.3	16.6	33.2
10X MOPS	2.5	5	10	20
2.5% Bromophenol blue	0.5	1	2	4
10 mg/ml Ethidium bromide	0.5	1	2	4
50% Glycerol	2.5	5	10	20
DEPC water	2.35	4.7	9.4	18.8
Total volume	**25**	**50**	**100**	**200**

3.2.4.2 cDNA Synthesis

Total RNA extract was used as the template for the synthesis of first-strand cDNA using *RevertAid*™ First Strand cDNA Synthesis Kits (Fermentas). The 10 µl reaction mixture was composed of 1 µg total RNA, 0.5 µg Oligo (dT) 12-18 mers and

sterile distilled water. The mixture was incubated at 70ºC for 5 min and quick chilled on ice. The 4 µl of 5X reaction buffer, 2 µl of dNTPs mixture, 1 µl of RNase inhibitor were added. The contents of the tube were mixed gently and incubated at 37ºC for 5 min. After adding 1 µl of RevertAID RT followed by incubation at 42ºC for 60 min, the reaction was stopped by heating at 70ºC for 10 min and chilled on ice. The cDNA was used as a template for amplification by PCR.

3.2.4.3 DNA Amplification

The reaction mixture was composed of 1 µl cDNA, 1 µl 10 pmol forward primer, 1 µl 10 pmol reverse primer and 5 µl of 2X GeneJET™ Fast PCR Master Mix (0.05 units/µl Taq DNA polymerase in reaction buffer, 4 mM $MgCl_2$, 0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dTTP and 0.4 mM dGTP) and distilled water to make the volume up to 10 µl. PCR condition were used as shown in the following Table 3.6.

Table 3.6 PCR condition for amplification of target genes

Cycle 1	1x	95 ºC	5 min
Cycle 2	30x	95 ºC	30 sec
		50-60 ºC (up to primer)	30 sec
		72 ºC	30 sec
Cycle 4	1x	72 ºC	10 min
Store	forever	4 ºC	forever

3.2.4.4 DNA Analysis (Gel Electrophoresis)

The amplification PCR products were analyzed by gel electrophoresis on 1% agarose gel in 0.5X TBE buffer. DNA sample were mixed with 6X loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol). The samples in loading dye and the DNA marker, were applied to the gel wells. Electrophoresis was performed at constant 50V for 30 min and the gel was stained with 10 µg/ml of ethidium bromide solution for 15 min and destained with distilled water. The DNA bands in gel were visualized by UV transilluminator.

3.2.4.5 DNA Purification from Agarose Gel

The DNA bands in agarose gel were extracted using the Gel/PCR DNA Fragments Extraction Kit (BIO101, Vista, CA). The DNA bands visualized under UV light were cut out of the gel with laboratory blade and transferred into a 1.5-ml microcentrifuge tube. The agarose was dissolved by adding 500 µl of DF buffer and incubated at 60ºC for 10-15 min. During incubation, the tube was inverted every 2-3 min, and the dissolved sample mixture was left to room temperature. The mixture was transferred to the column and centrifuged at 13,000xg for 30 sec. The flow-through was discarded and the column was placed back in the 2 ml collection tube. Then 600 µl of wash buffer was added into the column and left standing for 1 min. After centrifugation for 30 sec the flow-trough was discarded and centrifuged again for 3 min to dry the column matrix. Finally, the bound DNA was eluted with 15 µl of sterile distilled water and then centrifuged at 13,000xg for 1 min. The supernatant containing the eluted DNA was removed and transferred to a new tube.

3.2.4.6 Ligation of DNA Fragment into Vector

The gel-purified PCR products were ligated into pTZ57R/T vector (Figure 3.4) using InsT/Aclone™ PCR Product Cloning Kit. When a PCR fragment with 3'-dA overhangs is ligated into the vector, a circular molecule with two nicks is produced as shown in Figure 3.5.

The reaction mixture (15 µl) was composed of 0.5 µl of pTZ57R/T (50 ng/µl), 11 µl of eluted DNA fragment (10-50 ng/µl), 3 µl of 5x T4 DNA ligase buffer and 0.5 µl of T4 DNA ligase. The mixture solution was incubated at 4ºC for overnight.

Figure 3.4 pTZ57R/T plasmid vector

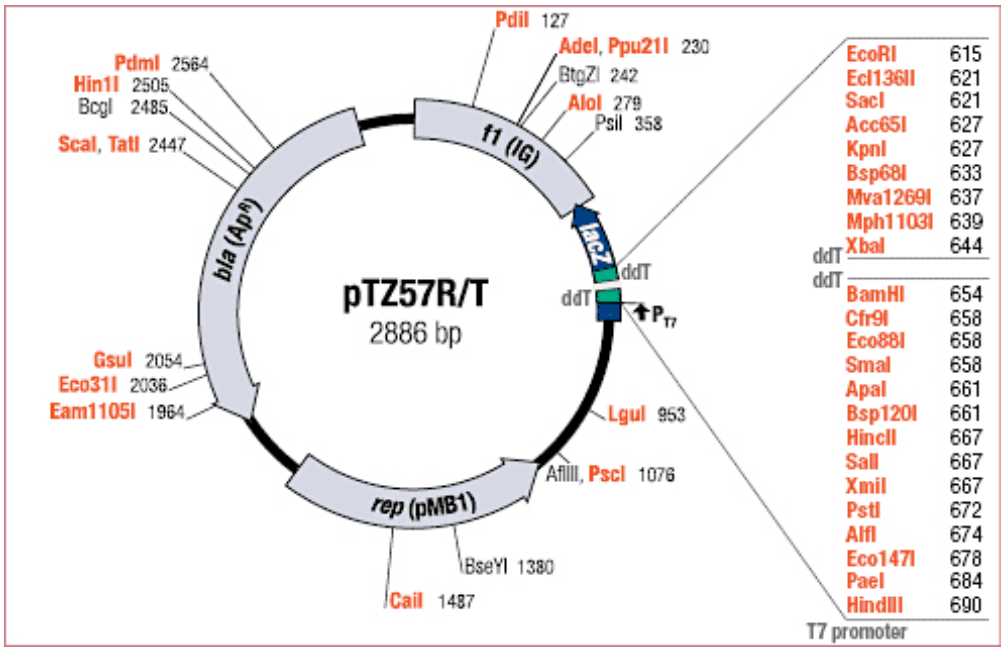
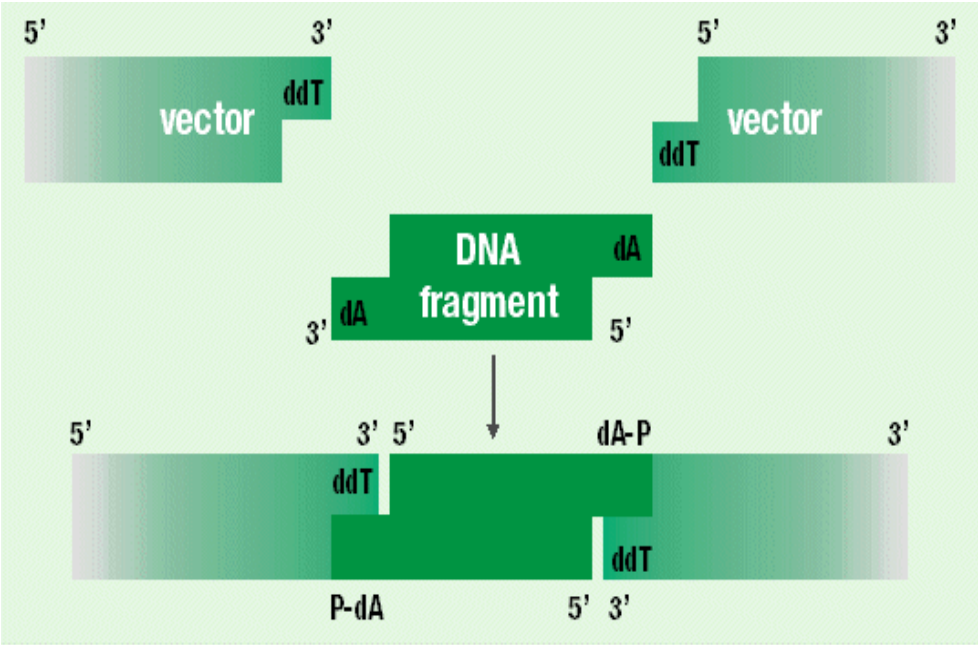


Figure 3.5 Ligation of a PCR fragment into the pTZ57R/T vector



3.2.4.7 Competent Cell Preparation

Competent *E.coli* DH5α cells were prepared using the $CaCl_2$ method (Sambrook *et al*., 1989). A single colony of *E.coli* was inoculated into 100 ml of LB broth and incubated for 3 h at 37 ºC with vigorous shaking until the optical density (OD) at 600 nm was approximately 0.3-0.4. The cells were transferred into sterile disposable, ice cold 50 ml polypropylene tube, chilled on ice for 10 min and the cells were recovered by centrifugation at 4000xg for 10 min at 4 ºC. The media was decanted from the cell pellet, and each pellet was resuspended in 10 ml of ice cold 0.1 M $CaCl_2$ and stored on ice. The supernatant from cell pellet was decanted, and the tube was placed in an inverted position for 1 min to allow the last of fluid to drain away. Each pellet was resuspended in 2 ml of ice cold 0.1 M $CaCl_2$ for each 50 ml of original culture. The suspension of competent cells was transferred to 50 µl aliquots to sterile microcentrifuge tubes. These competent cells were transformed immediately or kept at -80ºC.

3.2.4.8 Transformation and Selection

An aliquot of frozen competent cells was thawed on ice. The ligation mixtrure was added to the thawed competent cells, mixed gently by swirling and stored the tube on ice for 30 min. The cells were heat shocked at 42ºC for 90 sec and the tube was rapidly transferred to ice for 2 min. The transformed cells were grown by adding 800 µl of SOC medium and the culture was incubated at 37ºC for 1h. This culture was spread on LB plate containing 100 µg/ml ampicilin along with 100 µl of 100 mM IPTG and 20 µl of 5 mg/ml X-Gal and incubated at 37ºC overnight. The recombinant clones were identified as white colonies, while colonies containing nonrecombinant plasmid were blue. The recombinant clones were confirmed by colony PCR amplification using M13 forward and M13 reverse primer which present in plasmid vector.

3.2.4.9 Recombinant Plasmid Isolation

White colonies in LB agar plate with amplicilin were picked up and cultured in LB broth at 37 ºC overnight. Plasmid was isolated using Plasmid DNA Purification Kit (Nucleospin® Plasmid, Germany). The 1-5 ml of a saturated *E. coli* LB culture was pelleted in a standard benchtop microcentrifuge for 30 sec at 11,000g. The supernatant was discarded and the liquid was removed as much as possible. The cell pellet was completely resuspended in 250 µl of buffer A1 by vortexing or pipetting up and down. Then the *E. coli* cells were lysed by adding 250 µl of buffer A2, mixed gently by inverting tube 6-8 times and incubated at room temperature for up to 5 min or until lysate appeared clear. Then, pH of the mixture was neutralized by 300 µl of buffer A3. The clarification of lysate by centrifugation for 5 min at 11,000xg at room temperature. A Nucleospin® Plasmid column was placed in a collection tube (2 ml) and loaded with 750 µl of the supernatant. After centrifugation at 11,000xg for 1 min, the flow-through was discarded and the Nucleospin® Plasmid column was placed back into the collection tube (2 ml). The silica membrane was washed by 600 µl of buffer A4 (supplemented with ethanol). The washing step was repeated once. Finally, highly pure DNA was eluted by 50 µl of buffer AE. DNA concentration was determined by nanodrop (NanoDrop ND-1000 Spectrophotometer, USA) before determination of the nucleotide sequences of the insert DNA fragment.

3.2.4.10 Analysis by Real-time PCR

3.2.4.10.1 Primers and Construction of the Standard Curve

The SYBR Green real-time RT-PCR was performed using iCycler iQ Real-time PCR System (Bio-Rad). The experimental cycling profile was set as recommended in the manufacturer's manual for iQ™ SYBR® Green Supermix (Bio-Rad). The solution mixture contained 100 mM KCl, 40 mM Tris-HCI, pH 8.4, 0.4 mM of each dNTPs, 50 units/ml iTaq DNA polymerase, 6 mM $MgCl_2$, 20 nM SYBR Green I, and stabilizers. Primers for target genes were applied for real-time PCR analysis. For construction of the standard curve of each gene, the PCR product of the target genes and ubiquitin was amplified according to the thermal profile as shown in

table 3.7, whereas recombinant plasmid (pTZ57R/T vector with target gene) were extracted and used as the template for construction of the standard curve. Plasmid DNA templates of each gene and ubiquitin were diluted covering 10^1–10^9 copy numbers. Real-time RT-PCR was carried out and each standard point was run in duplicate.

3.2.4.10.2 Quantitative Real-time PCR

The first strand cDNA was reverse-transcribed. The target transcript and internal control (ubiquitin) of each sample were amplified in reaction volume of 10 µl containing 5 µl of iQ™ SYBR® Green Supermix. The specific primer pairs were used at a final concentration of 10 pmol, amplified under thermal profile as shown in Table 3.7.

A graph plotted between cycle threshold and total copy number was constructed and used as a standard curve to calculate the copy number of each target gene.

Table 3.7 The thermal profile for real-time analysis

Cycle 1	1X	95 ºC	2 min 30 sec
Cycle 2	40X	95 ºC	30 sec
		60 ºC	30 sec
		72 ºC	30 sec
Data collection and real-time analysis enabled			
Cycle 3	1X	95 ºC	1 min
Cycle 4	1X	60 ºC	1 min
Cycle 5	40X	60 ºC	30 sec
Increase set point temperature after cycle 2 by 1 ºC. Melting curve data collection and analysis enabled.			
Cycle 3	1X	25 ºC	Hold

CHAPTER IV

RESULTS

Not only differences in morphology of 8 developmental stages of *J. curcas* but also changes in oil contents, fatty acid profiles, protein expression profiles and mRNA expression were observed. The other details were shown below.

4.1 Lipid

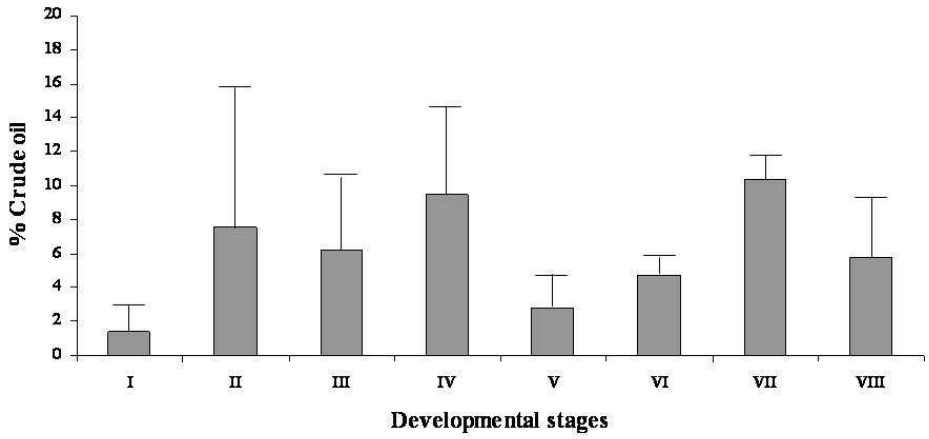
4.1.1 Oil Contents

Oil content and fatty acid profiles of *J. curcas* seeds were found to vary among the developmental stages. The average percentage of extracted oil and its standard deviation using hexane were shown in Table 4.1. Figure 4.1 showed the bar graph of average crude oil in 8 developmental stages. It was noticed that oil content gradually increased from stage I (1.44%) to IV (9.50%), decreased in stage V (2.82%) and raised again in stage VI (4.74%) and up to 10.32% in stage VII. Finally it was decreased again in stage VIII to 5.80%.

Table 4.1 Percentage of average crude oil in 8 developmental stages and their standard deviation. The data were average values among triplicate experiments.

Developmental stage	% crude oil	Standard deviation
I	1.44	1.51
II	7.57	9.90
III	6.24	4.71
IV	9.50	5.05
V	2.82	2.21
VI	4.74	0.99
VII	10.32	1.78
VIII	5.80	4.09

Figure 4.1 Changes in level of crude oil from 8 developmental stages of *J. curcas* seeds.



4.1.2 Fatty Acid Profiles

The fatty acid profiles of all developmental stages of *J. curcas* seeds were monitored by GC. The obtained chromatograms were compared to the known 21 standard fatty acids. Due to high concentration of all samples, they were diluted to 500 folds before injected to GC.

According to Table 4.2, 12 fatty acids were observed. The major fatty acid was oleic acid and the minor fatty acids were linoleic acid, palmitic acid and stearic acid. There were 2 fatty acids, vaccinate and eicosapentaenoate, found only in the third stage at very low level, 23.35 and 13.75 µg/ml FAME, respectively. The stages that contained the most types of fatty acid and the highest concentration of fatty acids were stages III and VII. On the contrary, the stages that had the lowest concentration of fatty acid were stages I and VIII.

Figure 4.2 represents the alteration of fatty acids concentration in 8 developmental stages of *J. curcas* seeds. Most fatty acids gradually increased from stage I to III but decreased in stage IV to VI and increased again in stage VII. Finally, they decreased again in stage VIII.

The ratio of each fatty acid concentration between all stages to stage I was shown in Figure 4.3. Stearic acid, oleic acid and arachidic acid significantly increased to 7-8 folds in stage III. Subsequently, all fatty acid contents decreased in stage IV and significantly raised again in stage VII, especially oleic acid content increased up to 6 folds comparing to stage I.

Table 4.2 Fatty acid composition in 8 developmental stages of *Jatropha curcas* seeds (µg/ml FAME). The data were average values ± standard deviation. All experiments were done in triplicate.

Fatty acid	I	II	III	IV	V	VI	VII	VIII
myristate	8.17±0.16	12.55±0.07	24.62±0.14	11.12±0.05	12.47±0.07	9.32±0.17	17.03±0.01	8.63±0.02
palmitate	2042.70±35.77	3762.83±21.94	7773.38±50.56	3465.88±31.45	3886.17±23.91	3492.60±33.61	5356.23±4.65	2269.45±16.88
palmitoleate	143.05±2.50	214.42±1.13	285.92±5.41	179.28±1.20	197.10±1.20	191.23±1.89	241.53±0.18	144.98±1.24
stearate	666.38±10.91	2078.95±12.77	5052.02±28.27	1817.90±16.38	2450.32±15.95	1918.77±19.00	3295.03±4.41	1115.35±7.70
oleate	3456.65±61.83	12424.98±72.04	27338.47±197.46	12514.58±143.72	16447.23±101.64	12666.22±120.26	22152.58±21.07	7821.92±72.94
vaccenate	0.00	0.00	23.35±0.81	0.00	0.00	0.00	0.00	0.00
linoleate	4135.67±72.41	7765.15±44.77	12709.25±79.46	7495.20±84.15	6690.75±40.72	6223.97±60.31	11818.60±27.04	4319.47±41.68
linolenate	44.33±0.90	75.62±0.41	143.10±0.87	51.15±0.19	64.08±0.38	64.35±0.64	80.47±0.17	43.77±0.19
arachidate	22.48±0.44	60.65±0.38	151.55±0.85	48.97±0.31	72.77±0.48	61.52±0.59	96.90±0.19	19.12±0.34
11-eicosenoate	0.00	8.75±0.15	35.18±0.36	11.60±0.21	12.50±0.22	9.78±0.18	23.65±0.05	0.00
eicosapentaenoate	0.00	0.00	13.75±0.24	0.00	0.00	0.00	0.00	0.00
docosahexaenoate	0.00	0.00	0.00	7.62±0.26	0.00	18.63±0.64	0.00	0.00

Figure 4.2 Fatty acid content in 8 developmental stages of *Jatropha curcas* seeds (µg/ml FAME).

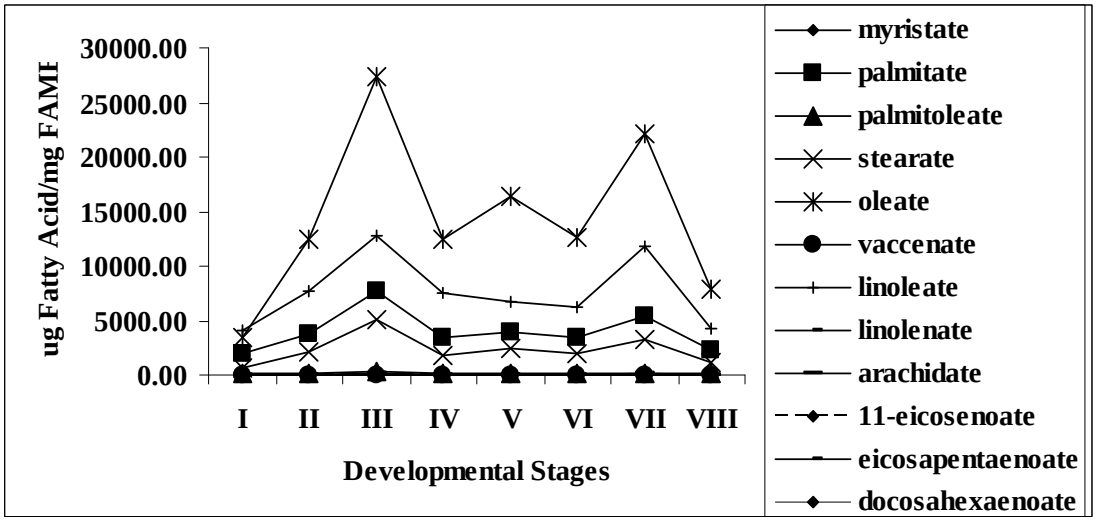
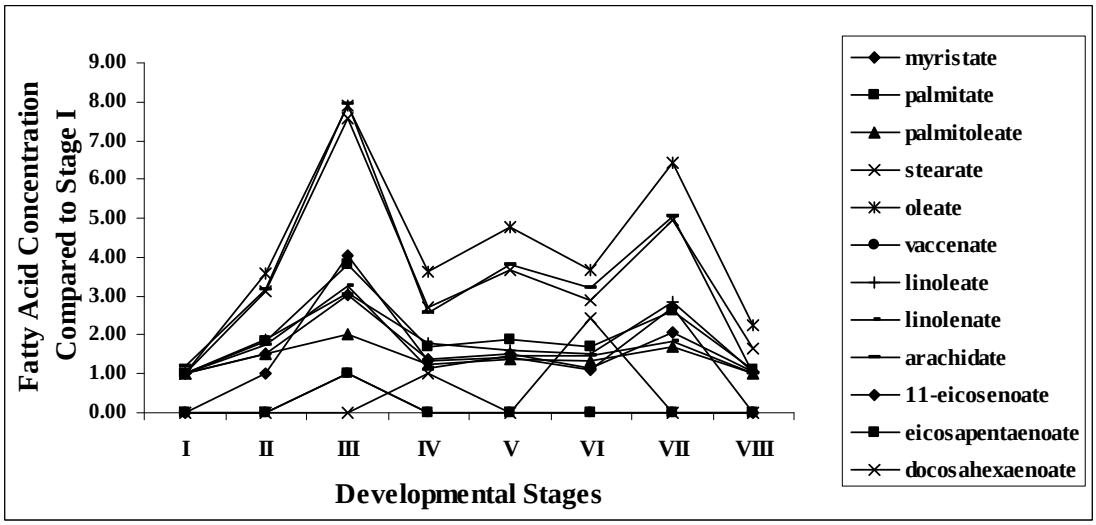


Figure 4.3 Fatty acid content of 8 developmental stages compared to stage I



4.2 Protein

The total protein was extracted from 500 mg of kernel powder and the protein concentration was determined according to the method of Lowry. BSA standard curve, plotted between OD_{750} on Y-axis and BSA concentration (µg/ml) on X-axis has R^2 = 0.9859, was shown in Figure 4.4. Protein concentration (µg protein/µl) of 8 developmental stages of *Jatropha curcas* seeds were demonstrated in Table 4.3

Figure 4.4 The BSA standard curve**.** All experiments were done in triplicate.

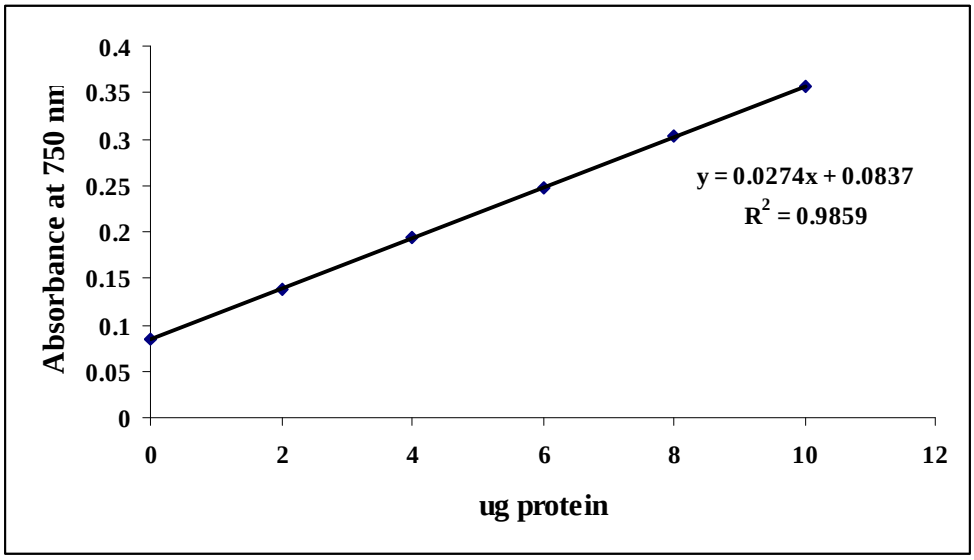


Table 4.3 Protein concentration of 8 developmental stages of *Jatropha curcas* seeds. All experiments were done in triplicate.

Developmental stage	µg protein/µl
I	2.3487
II	3.3255
III	3.2793
IV	2.8365
V	3.3759
VI	3.2100
VII	3.6109
VIII	3.9263

4.2.1 Optimization of Protein Loading for SDS-PAGE Analysis

After protein concentration was determined by Lowry assay, the appropriate amount of protein for all sample were optimized. Protein amount started from 2, 5, 10, 20, 30, 50 and 60 µg of stage I were used. The suitable protein concentration loaded for SDS-PAGE analysis was 10 µg because this concentration showed the best separated protein pattern, as shown in Figure 4.5. Therefore, the 10 µg of all protein samples were used for further experiments.

4.2.2 SDS-PAGE Analysis

The SDS-PAGE analysis was used to observe the protein patterns of total protein extracted from 8 developmental stages of *Jatropha curcas* seeds followed by LC-MS/MS analysis for protein quantification and identification. The protein samples of 8 developmental stages were analyzed by 15% gel SDS-PAGE, and the gel was visualized by silver staining as shown in Figure 4.6. When SDS-PAGE gels were observed by eye, it was found that the intensity of many protein bands within a range of 20.1-30 kDa had increased (up regulated) but the intensity of some protein bands with molecular weight lower than 14.4 kDa had decreased (down regulated).

Figure 4.5 Pattern of total protein extracted from Stage I in kernel. The protein concentrations were varied as 2, 5, 10, 20, 30, 50 and 60 µg.

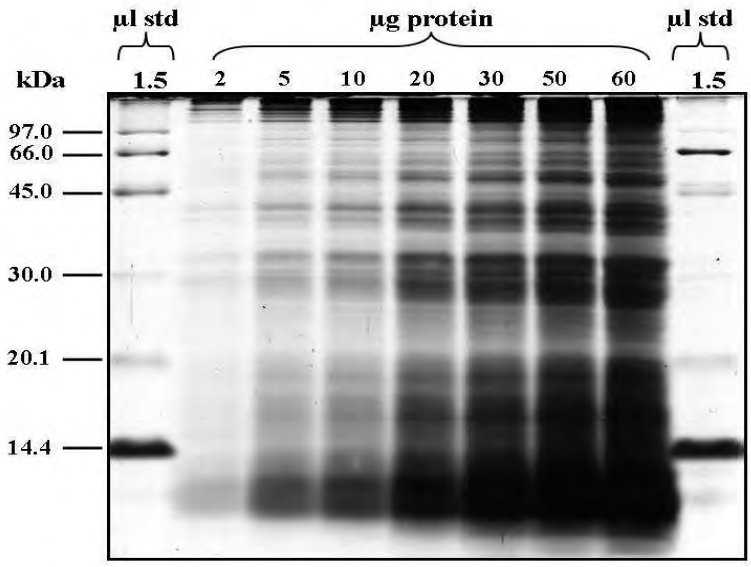
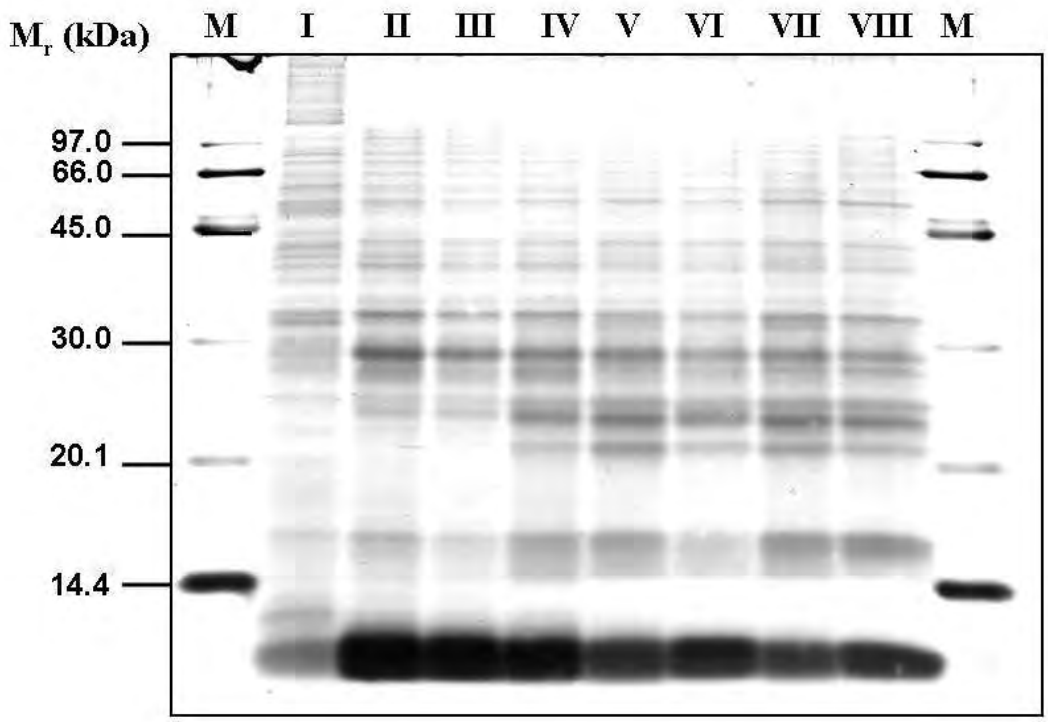


Figure 4.6 The 15% SDS-PAGE of 10 µg protein of 8 developmental stages of *Jatropha curcas* seeds**.**



4.2.3 LC-MS/MS

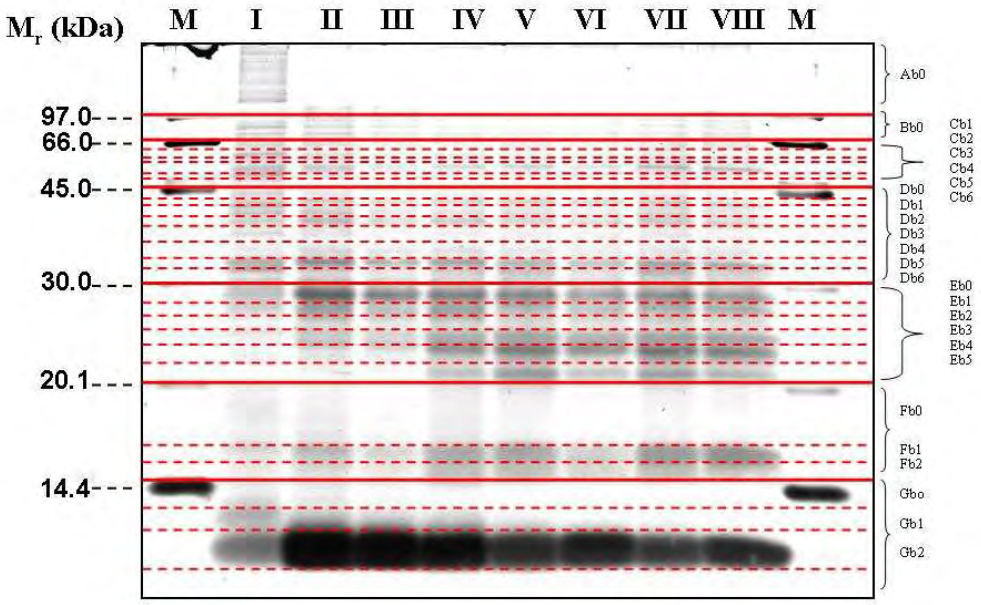
Gel bands were excised according to the molecular mass range of protein standard markers (as described in section 3.2.3.1 of chapter III). Moreover, for better results, each protein band in molecular mass range was excised separately as shown in Figure 4.7. After that, the tryptic in-gel digestion was performed. All extracted peptides of each molecular mass range sample were individually injected to LC-MS/MS.

After analysis by DecyderMS software, the MS/MS data of the peptides showing differentially high expression among all 8 developmental stages of *J. curcas* seeds were exported and further identified by Mascot software using protein and DNA databases available in the NCBI. There are 245 identified proteins as shown in Table 4.4. Several differentially expressed proteins identified in this study were related to

transcription, translation, photosynthesis and respiratory system, seed development and fatty acid biosynthesis, as shown in Figure 4.8.

Furthermore, the relative expression level of proteins related to ripening process, seed development and fatty acid synthesis were shown in Table 4.5, 4.6, 4.7 and in Figures 4.9, 4.10 and 4.11, respectively.

Figure 4.7 The 27 excised ranges of (follow the lines).



A note : A-G represents the mass ranges (upper 97 kDa to lower 14 kDa)

b represents the protein band (such as b1)

b0 represents no band

Table 4.4 Identified proteins in each protein band using peptide tolerance of 1.2 Da and p value < 0.05.

Protein band	Accession No.	Name of identified protein	Organism	MOWSE score	Peptide sequence
Ab0	gi|22093783	putative oxidoreductase, FAD-binding	*Oryza sativa*	28	R.LPAGSVRLNSR.A
	gi|118152387	photosystem II subunit	*Trithuria submersa*	20	MATQMAEGGSR.F + 2 Oxidation (M)
	gi|76446070	trypsin proteinase inhibitor precursor	*Nicotiana rustica*	20	K.SKVAVI.-
	gi|26225031	self-incompatibility ribonuclease	*Petunia axillaris*	19	K.YVNFEGDIK.S
	gi|18406846	SIN3 associated polypeptide P18	*Arabidopsis thaliana*	18	K.DEVQIYTWK.D
	gi|255615159	mandelate racemase, putative	*Ricinus communis*	18	K.MNVRVVR.V + Oxidation (M)
	gi|15232678	ubiquitin protein ligase binding	*Arabidopsis thaliana*	16	K.LLSIILQLIR.D
	gi|27807565	phytochrome 3	*Onoclea sensibilis*	16	K.EVTPLPHSK.I
	gi|108864642	kinesin motor protein, putative	*Oryza sativa*	16	R.MLVQTMLSDK.K + Oxidation (M)
	gi|1161926	alpha-carboxyltransferase aCT-1 precursor	*Glycine max*	15	K.ILQLKLEVPK.A
	gi|255563064	hypothetical protein RCOM_1013810	*Ricinus communis*	15	K.ESSKGDSSSGNHR.G
Bb0	gi|255554873	beta-1,3-galactosyltransferase sqv-2	*Ricinus communis*	25	K.GQPAVQTLDNK.I
	gi|116061081	lipase	*Ostreococcus tauri*	25	R.VDAVTFGQPR.V
	gi|77553249	hAT dimerisation domain-containing	*Oryza sativa*	23	R.FLDLQHIER.C
	gi|14589378	putative gag-pol polyprotein	*Oryza sativa*	22	K.DEMKSMSTNK.V
	gi|15232121	MBD9; DNA / methyl-CpG binding	*Arabidopsis thaliana*	20	K.KDDSTLSAK.R
	gi|116000579	putative mitochondrial carrier protein	*Ostreococcus tauri*	17	K.EAGVDLKSCR.R

Protein band	Accession No.	Name of identified protein	Organism	MOWSE score	Peptide sequence
Bb0	gi|195622258	CTD small phosphatase-like protein	*Zea mays*	15	K.DAEGKQK.V
	gi|255602105	FMN-dependent NADH-azoreductase	*Ricinus communis*	13	K.KVIMASTR.G
	gi|47848031	putative tocopherol cyclase	*Oryza sativa*	13	R.NDGGKGK.M
	gi|81051961	dna-directed rna polymerase ii - related	*Asparagus officinalis*	13	K.LTLEPSASK.L
Cb1	gi|108707211	myb family transcription factor	*Oryza sativa*	14	R.QSRANGPQT.-
	gi|255577062	mom(plant), putative	*Ricinus communis*	14	K.KQSQNWPKPK.S
	gi|37518772	maturase-like protein	*Diplotropis martiusii*	13	K.KSISTLSR.S
	gi|111608874	plastid triose phosphate isomerase	***Polytomella parva***	13	K.TLVEDLNK.G
	gi|255553075	Monothiol glutaredoxin-4	*Ricinus communis*	13	K.DVVEQDVK.D
	gi|398997	ACC oxidase	*Arabidopsis thaliana*	12	R.KSTMELIK.D
	gi|224141325	oxidoreductase, 2OG-Fe(II) oxygenase family protein	*Populus trichocarpa*	12	K.KSVFDPTR.V
	gi|255073339	dynein heavy chain	*Micromonas sp. RCC299*	11	R.FGVMLVGPTGGAKTTNYQILK.S + Oxidation (M)
	gi|27545461	S-related kinase 10.1A	*Arabidopsis lyrata*	11	K.QLWYTPK.D
	gi|92870993	protein kinase	*Medicago truncatula*	11	K.QSPGSPPSGER.V
	gi|24061807	transferrin-like protein IDI-100	*Dunaliella salina*	10	R.ENANSGCGK.T
	gi|253825549	dioscorin precursor	*Dioscorea japonica*	10	K.SKLGII.-
	gi|15231038	meprin and TRAF homology	*Arabidopsis thaliana*	10	K.ADFVASK.T
	gi|1769897	lectin receptor kinase	*Arabidopsis thaliana*	10	R.GEFEALQMK.E + Oxidation (M)
	gi|171921109	matrixin family protein	*Brassica oleracea*	10	K.SVFSRAFVR.W

Protein band	Accession No.	Name of identified protein	Organism	MOWSE score	Peptide sequence
Cb1	gi|15225304	1-phosphatidylinositol-4-phosphate 5-kinase 3	*Arabidopsis thaliana*	10	K.ASGKGR.F
Cb2	gi|15226197	leucine-rich repeat transmembrane protein kinase	*Arabidopsis thaliana*	28	R.VVSLSIPR.K
	gi|255545954	mads box protein, putative	*Ricinus communis*	27	K.MLESGLTRVLQTK.G
	gi|159470791	exostosin-like glycosyltransferase	*Chlamydomonas renhardtii*	21	R.VAEADIPR.L
	gi|53793437	peptidyl prolyl cis trans isomerase-like	*Oryza sativa*	21	R.FLKGEAPDEQLTK.E
	gi|255571911	arginyl-tRNA-protein transferase	*Ricinus communis*	21	K.ESAAGCKR.K
	gi|162462751	mitochondrial F-1-ATPase subunit 2	*Zea mays*	18	R.EMIESGVIKLDDK.Q
	gi|109892850	cytochrome c oxidase subunit II PS17	*Pinus strobus*	15	R.VVEALSPR.-
	gi|74273641	tryptophan decarboxylase	*Gossypium hirsutum*	14	K.QSESDSVVDFK.D
	gi|77557109	transposon protein, Mutator sub-class	*Oryza sativa*	13	K.ECGGFGHRDK.N
	gi|224089239	rna-dependent RNA polymerase	*Populus trichocarpa*	13	K.VEFLVRDINEIK.Q
Cb3	gi|255573870	DNA (cytosine-5)-methyltransferase	*Ricinus communis*	21	R.LNLSSDSFVYK.G
	gi|37779744	NADH dehydrogenase subunit F	*Aponogeton elongates*	19	K.QSPKLVK.R
	gi|255626179	unknown	*Glycine max*	15	K.QVQTQMR.S + Oxidation (M)
	gi|18401449	F-box family protein	*Arabidopsis thaliana*	15	K.KSYDSYK.I

Protein band	Accession No.	Name of identified protein	Organism	MOWSE score	Peptide sequence
Cb3	gi|58257481	DRP3 protein	*Triticum turgidum*	15	K.AQMDARSK.G
	gi|108706991	branched-chain-amino-acid aminotransferase 5, putative	*Oryza sativa*	15	K.AQMDAKSR.G
	gi|11994276	alpha 1,2-mannosidase-like protein	*Arabidopsis thaliana*	13	K.QSNKVLLR.K
	gi|5231145	xyloglucan fucosyltransferase	*Arabidopsis thaliana*	13	K.YLSSGTMKLTR.T + Oxidation (M)
	gi|116000741	FOG: PPR repeat	*Ostreococcus tauri*	13	K.ALADNDMGK.R + Oxidation (M)
Cb4	gi|3342148	myosin heavy chain	*Chlamydomonas reinhardtii*	20	R.MTASEEAAK.A + Oxidation (M)
	gi|8071634	pumilio domain-containing protein	*Populus tremula*	16	-.MATESPMR.M + 2 Oxidation (M)
	gi|15231611	glycosyl hydrolase family 38 protein	*Arabidopsis thaliana*	14	K.RGEAVDAEK.L
	gi|6714418	NAM-like protein (no apical meristem)	*Arabidopsis thaliana*	13	K.KIVIEAKPR.D
	gi|84794464	flavonol synthase	*Vitis vinifera*	13	K.LINEKNPPK.Y
	gi|255541112	chaperone protein dnaJ, putative	*Ricinus communis*	12	R.VIEIILPTR.L
	gi|15236175	arabinogalactan protein 13	*Arabidopsis thaliana*	12	-.MEAMKMR.L + 2 Oxidation (M)
	gi|171853016	flavanone 3-hydroxylase	*Triticum aestivum*	12	-.LPAEDKVR.Y
	gi|32401868	small ribosomal protein 4	*Amentotaxus formosana*	12	-.NSNAGQSGK.K

Protein band	Accession No.	Name of identified protein	Organism	MOWSE score	Peptide sequence
	gi|162461137	chlorophyll a/b-binding apoprotein	*Zea mays*	11	R.YATAAPTAGAQK.I
Cb4	gi|84794468	flavonol synthase	*Vitis vinifera*	11	K.LINEENPPK.Y
	gi|62732784	transposon protein, putative, CACTA, En/Spm sub-class	*Oryza sativa*	19	K.VASGMAIDSHY.-
Cb5	gi|15241987	cold acclimation protein	*Spinacia oleracea*	16	R.TDTGGLNK.S
	gi|189172009	WRKY16 transcription factor	*Triticum aestivum*	13	K.MRAEMFK.R + 2 Oxidation (M
	gi|105923026	TIR-NBS type disease resistance protein	*Populus trichocarpa*	13	R.DMGREIAR.Q
	gi|61656097	1-deoxy-D-xylulose 5-phosphate synthase	*Zea mays*	36	K.GRILLEGDR.V
	gi|157325573	hypothetical chloroplast RF21	*Ipomoea purpurea*	23	K.DDPDLDSSNK.I
	gi|2145479	phosphoenolpyruvate carboxylase	*Vanilla aphylla*	21	R.KSAAELVK.L
	gi|255083080	chloroplast envelope protein translocase	*Micromonas sp.*	21	R.AGSGTSMGAGIK.L + Oxidation (M)
Cb6	gi|18395236	kelch repeat-containing F-box protein	*Arabidopsis thaliana*	21	R.NNLSFLSTSKY.-
	gi|255573870	DNA (cytosine-5)-methyltransferase	*Ricinus communis*	19	R.LNLSSDSFVYK.G
	gi|50878368	N'-5'-phosphoribosyl-formimino-5-aminoimidazole-4- carboxamide ribonucleotide isomerase	*Oryza sativa Japonica Group*	18	R.LVLDLSCRK.K
	gi|119358861	TyrS Tyrosyl-tRNA synthetase	*Ostreococcus tauri*	18	R.NITLLDFLR.D
	gi|1262752	lamin	*Arabidopsis thaliana*	17	R.DIVPLYETR.R
	gi|242910198	RNA polymerase b-subunit	*Alsophila spinulosa*	17	MNLGVILGGDRK.T

Protein band	Accession No.	Name of identified protein	Organism	MOWSE score	Peptide sequence
Cb6	gi|157325572	ribosomal protein L23	*Ipomoea purpurea*	17	K.IKNFNQNT
	gi|116078099	MADS-box protein	*Citrus unshiu*	16	K.LLSQEAQCR.E
	gi|224815362	flavonoid 3'-hydroxylase	*Echinops bannaticus*	16	R.ALVGAGESTVK.L
	gi|5231145	xyloglucan fucosyltransferase	*Arabidopsis thaliana*	16	K.YLSSGTMKLTR.T + Oxidation (M)
	gi|14009640	cytosolic 6-phosphogluconate dehydrogenase	*Spinacia oleracea*	15	R.AMFLDRIK.K + Oxidation (M)
Db0	gi|145347091	predicted protein	*Ostreococcus lucimarinus*	22	R.LLLETCR.N
	gi|56606540	dormancy-associated protein	*Codonopsis lanceolata*	16	K.VVTGEGSGGK.F
	gi|15240591	pentatricopeptide (PPR) repeat-containing protein	*Arabidopsis thaliana*	16	K.LFDEMSELNAVSR.T + Oxidation (M)
	gi|255552313	kinase associated protein phosphatase	*Ricinus communis*	15	R.ILSDSLTR.E
	gi|18394738	dehydration-responsive protein-related	*Arabidopsis thaliana*	15	K.LLLEMNR.I + Oxidation (M)
	gi|29150369	putative AAA-type ATPase	*Oryza sativa*	14	K.LLIQTTSK.S
	gi|15232927	mannose-6-phosphate isomerase	*Arabidopsis thaliana*	14	MEIATVVK.A + Oxidation (M)
	gi|56783732	alpha-glucosidase -like	*Oryza sativa*	13	R.VQRSSGGSK.K
	gi|62733693	retrotransposon protein, putative	*Oryza sativa*	13	K.VKENLMR.E + Oxidation (M)

Protein band	Accession No.	Name of identified protein	Organism	MOWSE score	Peptide sequence
	gi|168040014	ATP-binding cassette transporter, subfamily D	*Physcomitrella patens subsp. patens*	13	K.VQGWLFR.A
	gi|124360513	Prefoldin	*Medicago truncatula*	13	K.LLECIEK.R
	gi|162461519	respiratory burst oxidase protein B	*Zea mays*	13	R.LINSSNEK.Y
	gi|81051845	sucrose transporter	*Asparagus officinalis*	13	R.WPNISVSQTHNS
Db0	gi|124359897	F-box protein interaction domain	*Medicago truncatula*	13	R.KLEYDIPLNHK.D
	gi|56236708	ribosomal protein L2	*Widdringtonia cedarbergensis*	13	R.GIMIGDTILSGSR.A + Oxidation (M)
	gi|255607747	succinate semialdehyde dehydrogenase	*Ricinus communis*	13	R.MGALRAGDP + Oxidation (M)
	gi|108796705	P700 apoprotein A2 of photosystem I	*Zygnema circumcarinatum*	12	K.SNVLAR.M
	gi|116056636	origin recognition complex, subunit 5	*Ostreococcus tauri*	12	R.SNVIAR.A
	gi|159482152	elongation factor-like protein	*Chlamydomonas reinhardtii*	20	R.LTFVAPSR.G
	gi|258649739	vernalization protein	*Triticum aestivum*	17	R.QVAFSK.R
Db1	gi|19920111	putative Ras-related protein Rab	*Oryza sativa*	16	K.KNMAADPK.A + Oxidation (M)
	gi|168057891	SNF2 family chromodomain-helicase	*Physcomitrella patens subsp. patens*	14	R.QVSSMK.Q
	gi|33337835	cytochrome P450	*Triticum aestivum*	13	R.EVMSSK.L
	gi|116060125	animal-type fatty acid synthase	*Ostreococcus tauri*	13	R.TSGCEK.G

Protein band	Accession No.	Name of identified protein	Organism	MOWSE score	Peptide sequence
Db1	gi|54291440	pr1-like protein	*Oryza sativa*	13	R.SGAVDGVGGNTAK.R
	gi|50252221	putative far-red impaired response	*Oryza sativa*	12	R.EGFKAPDK.R
Db2	gi|20372934	FtsZ	*Chlamydomonas reinhardtii*	23	K.RSASASSSASR.V
	gi|116000242	PAB-dependent poly(A) ribonuclease, subunit PAN2	*Ostreococcus tauri*	19	K.KVTHGMSVPR.G + Oxidation (M)
	gi|159474642	double stranded RNA-binding protein	*Chlamydomonas reinhardtii*	17	K.ERGSGGGGDR.Q
	gi|38261538	self-incompatibility locus-linked putative F-box protein S1-A134	*Petunia integrifolia subsp. inflata*	14	R.ESLTAIPR.N
	gi|224179485	CF1 alpha subunit of ATP synthase	*Monomastix sp. OKE-1*	14	K.QSQSAPLR.V
	gi|23617201	putative sphingosine kinase	*Oryza sativa*	13	R.VDGRALR.V
	gi|60656567	putative endo-1,4-beta-xylanase	*Populus tremula x Populus tremuloides*	13	K.VKQTQNSFPFGSCMSR.M + Oxidation (M)
	gi|1644484	NADH dehydrogenase subunit	*Lycianthes heteroclita*	12	R.AYVDAFYTR.F
	gi|159479570	mitochondrial inner membrane translocase	*Chlamydomonas reinhardtii*	12	K.QQATGWQK.V
	gi|255556920	metal ion binding protein, putative	*Ricinus communis*	11	K.KEGGGGGGEAK.T
	gi|94466659	ALY protein	*Nicotiana benthamiana*	10	R.GRGGSFR.G
	gi|25992001	lecithine cholesterol acyltransferase	*Medicago*	10	K.LTFETAFK.L

Protein band	Accession No.	Name of identified protein	Organism	MOWSE score	Peptide sequence
Db3	gi|19847822	isoflavone reductase-like protein CJP-6	*Cryptomeria japonica*	14	MGGSR.V + Oxidation (M)
	gi|255552914	taxadien-5-alpha-ol O-acetyltransferase, putative	*Ricinus communis*	13	K.SKMSEEYIR.S + Oxidation (M)
	gi|46254823	allene oxide synthase	*Hevea brasiliensis*	11	MVSLLSVPSMIDLIISINK.A + Oxidation (M)
	gi|224579301	mercaptopyruvate sulfurtransferase	*Solanum lycopersicum*	10	K.LVASVTCR.M
	gi|13129429	similar to Zea mays retrotransposon	*Oryza sativa*	10	K.TLDDTQIPR.T
	gi|255619956	inosine-5-monophosphate dehydrogenase, putative	*Ricinus communis*	9	R.GHRSTMCR.L + Oxidation (M)
	gi|1705810	endochitinase 4	*Solanum tuberosum*	9	R.GSDSR.V
	gi|2541938	putative ethylene receptor	*Dianthus caryophyllus*	9	K.SSSDK.L
	gi|3775985	RNA helicase	*Arabidopsis thaliana*	9	R.SGDSR.V
	gi|2244959	reverse transcriptase like protein	*Arabidopsis thaliana*	8	R.FGGSR.G
	gi|18411268	DNA-binding storekeeper protein	*Arabidopsis thaliana*	7	K.DPVTVPSSK.T
	gi|256010126	dirigent-like protein 2	*Gossypium hirsutum*	7	R.VIGGSGLFR.F
Db4	gi|81051961	dna-directed rna polymerase ii - related	*Asparagus officinalis*	26	K.LTLEPSASK.L
	gi|32483754	maturase	*Ambavia gerrardii*	17	R.NPLVVR.S

Protein band	Accession No.	Name of identified protein	Organism	MOWSE score	Peptide sequence
	gi|197360149	maturase K	*Encholirium horridum*	17	R.NPLVVR.N
	gi|212525423	subtilisin-like protein	*Nicotiana benthamiana*	16	K.QSLSYEISLKSK.K
	gi|63078419	maturase K	*Quesnelia edmundoi*	15	R.NPLVVR.N
	gi|51038135	putative CTP synthase	*Oryza sativa*	14	R.TIIVALCR.R
	gi|56202024	epstein-Barr virus EBNA-1-like protein	*Oryza sativa*	14	R.QGEEKESEGK.G
Db4	gi|4775494	putative coat protein	*Nicotiana tabacum*	13	K.ITNRNLMTK.L + Oxidation (M)
	gi|15232213	autophagy 4b (APG4b)	*Arabidopsis thaliana*	12	K.TASMASGAIRR.F
	gi|6224758	maturase K	*Leptospermum madidum*	12	K.KSSSIIVK.R
	gi|156619094	chloroplast envelope membrane protein	*Floydiella terrestris*	12	K.QSSSLLVK.E
	gi|225468863	PREDICTED: similar to starch synthase	*Vitis vinifera*	11	K.LVIVGKYR.E
	gi|27464263	biogenesis protein	*Medicago truncatula*	11	R.TINSHGKR.I
	gi|255548952	atpase n2b, putative	*Ricinus communis*	10	K.NVTSGLLAR.Y
	gi|195647316	EH-domain-containing protein 1	*Zea mays*	10	K.KEMPAVMGK.A + Oxidation (M)
Db5	gi|124359852	polynucleotidyl transferase	*Medicago truncatula*	20	K.DSAIVR.I

Protein band	Accession No.	Name of identified protein	Organism	MOWSE score	Peptide sequence
Db5	gi|224080536	SAUR family protein	*Populus trichocarpa*	20	R.LSAIVR.A
	gi|11935156	FLORICAULA/LEAFY-like protein	*Lolium temulentum*	18	R.GRLMALHGGR.H + Oxidation (M)
	gi|15242262	AP2 domain-containing protein	*Arabidopsis thaliana*	18	K.QPDQGGDEK.V
	gi|109138543	trehalase	*Saccharum officinarum*	15	SKVNSMAAK.E + Oxidation (M)
	gi|246880753	cell division protein	*Dunaliella salina*	14	K.SQVTNIKTK.N
	gi|226500352	ATP binding protein	*Zea mays*	12	.KSVGGNYVK.M
	gi|45735845	putative chloroplast nucleoid DNA-binding protein cnd41	*Oryza sativa Japonica Group*	12	R.QSGPVFNPK.S
	gi|255567144	transcription elongation factor s-II	*Ricinus communis*	12	K.YRSLLFNIK.D
	gi|237682390	branched-chain aminotransferase 4	*Brassica rapa*	11	K.KWIPPPGR.G
	gi|145332395	(S)-2-hydroxy-acid oxidase,	*Arabidopsis thaliana*	10	K.TNGLFKR.T
Db6	gi|3608364	high-affinity nitrate transporter ACH2	*Arabidopsis thaliana*	18	K.SKLFADGAK.T
	gi|15224946	calcium-dependent phospholipid binding	*Arabidopsis thaliana*	16	K.DDSAKSDAK.I
	gi|15233479	late embryogenesis abundant domain-containing protein (LEA)	*Arabidopsis thaliana*	14	MAAMQLTR.T + Oxidation (M)
	gi|2586125	β-keto acyl reductase	*Arabidopsis thaliana*	12	K.DVSDSIR.S

Protein band	Accession No.	Name of identified protein	Organism	MOWSE score	Peptide sequence
Db6	gi|18033230	UDP-glucosyltransferase BX9	*Zea mays*	12	R.GFESGALPDGVEDEVRGR.G
	gi|242090467	thioredoxin-related:thioredoxin domain 2	*Ostreococcus tauri*	11	R.LTSALSNVVNDR.S
	gi|15230137	ROPGEF8; Rho guanyl-nucleotide exchange factor	*Arabidopsis thaliana*	10	K.VPPGGLSEPSRR.M
	gi|71040661	lea protein 2	*Arachis hypogaea*	10	K.LSNMVSAAK.E + Oxidation (M)
	gi|99079639	acetolactate synthase	*Oryza sativa Indica Group*	9	K.KSLVDIDPAEIGK.N
	gi|255547283	ATP binding protein, putative	*Ricinus communis*	8	K.SDQKMESDSSSK.G
Eb1	gi|18091793	curcin precursor	*Jatropha curcas*	96	K.VGGTSYFFNDPESLADAK.T
	gi|18394269	kelch repeat-containing F-box family protein	*Arabidopsis thaliana*	20	R.RDLMMMK.D + Oxidation (M)
	gi|255540551	disease resistance protein RPP13, putative	*Ricinus communis*	20	R.LSSNTIEGPK.E
	gi|166244912	ribosomal protein L22	*Scirpus ternatanus*	18	K.AEVNRSTIR.K
	gi|226502889	ankyrin-like protein	*Zea mays*	13	R.YIDFIQEAK.K
	gi|255548263	10-deacetylbaccatin III 10-O-acetyltransferase, putative	*Ricinus communis*	13	R.QVLDPPLPK.G
	gi|241911785	pyrophosphate-dependent phosphofructokinase	*Camellia sinensis*	13	K.NVSDYIADVICK.R
	gi|23429644	endoxylanase	*Carica papaya*	18	R.LLLIKV

Protein band	Accession No.	Name of identified protein	Organism	MOWSE score	Peptide sequence
	gi|31558995	phospholipase D	*Arachis hypogaea*	18	K.EPINPK.L
	gi|255080050	serine acetyl transferase	*Micromonas sp. RCC299*	17	R.DHSLVK.K
Eb2	gi|87162766	late embryogenesis abundant protein	*Medicago truncatula*	16	R.LIQVPK.S
	gi|255568954	endonuclease, putative	*Ricinus communis*	16	K.LIKVPK.A
	gi|148597393	RNA polymerase beta subunit	*Aphanorrhegma serratum*	16	K.LIKPVK.T
	gi|1561694	ACC synthase	*Solanum lycopersycum*	15	R.HAMLVK.G
	gi|226461153	WD40 repeat protein	*Micromonas pusilla CCMP1545*	14	MAGLSIDAKAK.V + Oxidation (M)
	gi|8118512	seed storage protein	*Ricinus communis*	25	K.ASNEGLEWVSFK.T
	gi|112253755	NADH-plastoquinone oxidoreductase subunit K	*Piper cenocladum*	19	K.YKSLVPNYDMN + Oxidation (M)
Eb3	gi|572606	acetyl-CoA carboxylase	*Brassica napus*	18	R.KIIIVANDVTFK.A
	gi|11181943	acetyl coa carboxylase pRS1	*Brassica napus*	18	R.KLLIVANDVTFK.A
	gi|11869927	acetyl-CoA carboxylase 1	*Arabidopsis thaliana*	18	R.KLLVIANDVTFK.A
	gi|188532141	RNA polymerase II largest subunit	*Beta vulgaris*	17	K.ILADEEDHKFK.Q
	gi|62734123	Ulp1 protease family, C-terminal catalytic domain, putative	*Oryza sativa Japonica Group*	16	K.ALKAMK.D + Oxidation (M)
	gi|116060500	GTP-binding protein-like	*Ostreococcus tauri*	16	R.ALQAMK.R + Oxidation (M)
Eb4	gi|198279421	RNA polymerase beta subunit	*Pinus oocarpa*	29	K.GIISI
	gi|38228683	receptor-like protein kinase	*Fagus sylvatica*	25	K.GLNAK.I

Protein band	Accession No.	Name of identified protein	Organism	MOWSE score	Peptide sequence
Eb4	gi|21553535	ras-GTPase-activating protein	*Arabidopsis thaliana*	14	R.GGGSS
	gi|108796840	beta'' subunit of RNA polymerase	*Staurastrum punctulatum*	13	R.ASIAPLK.E
	gi|255085658	SWI/SNF and RSC chromatin remodeling complex protein	*Micromonas sp. RCC299*	22	R.DIAIVR.N
Eb5	gi|14030932	NADH dehydrogenase F	*Mesosetum chaseae*	22	K.IKPNPK.K
	gi|255545954	mads box protein, putative	*Ricinus communis*	21	K.MLESGLTRVLQTK.G
	gi|116055378	glycosyl transferases, related to UDP-glucuronosyltransferase	*Ostreococcus tauri*	20	R.DGGAIVR.F
	gi|20066304	symbiosis-related disease resistance	*Daucus carota*	20	K.GPNSR.Q
	gi|226453610	transposase	*Micromonas pusilla CCMP1545*	18	R.VNKLMVVK.R
	gi|255599474	conserved hypothetical protein	*Ricinus communis*	17	R.VGHGADR.Q
Fb0	gi|255614689	conserved hypothetical protein	*Ricinus communis*	25	R.RAVTEAPR.F
	gi|31558995	phospholipase D	*Arachis hypogaea*	21	K.EPINPK.L
	gi|168028893	PsaH photosystem I reaction center subunit	*Physcomitrella patens subsp. Patens*	19	K.QVPVMGPR.G + Oxidation (M)
	gi|1561694	ACC synthase	*Solanum lycopersicum*	18	R.HAMLVK.G
Fb1	gi|255606236	amidase, putative	*Ricinus communis*	31	K.QSLKPGVMA + Oxidation (M)
	gi|159467413	DNA-directed RNA polymerase II, largest subunit	*Chlamydomonas reinhardtii*	20	R.EVVVGPRAMSGR.H + Oxidation (M)
	gi|162949342	4-coumarate:coenzyme A ligase 1	*Physcomitrella sp.*	12	K.KSLQSEVR.G

Protein band	Accession No.	Name of identified protein	Organism	MOWSE score	Peptide sequence
Fb1	gi|148466444	40S ribosomal protein S7-like protein	*Paeonia suffruticosa*	12	K.DVVFEYPITDA
	gi|255547946	steroid dehydrogenase, putative	*Ricinus communis*	11	K.KSQLQASQG
	gi|255581313	protein-tyrosine phosphatase mitochondrial 1, putative	*Ricinus communis*	11	K.QSSTEDSYSIR.A
	gi|50058577	F1-ATPase alpha subunit	*Convolvulus assyricus*	9	K.AMLSR.V + Oxidation (M)
	gi|34393323	putative cell death associated protein	*Oryza sativa Japonica Group*	9	K.DVLSERGR.R
Fb2	gi|255604374	conserved hypothetical protein	*Ricinus communis*	24	R.GASAKPMRR.R
	gi|116055441	putative short chain alcohol dehydrogenase	*Ostreococcus tauri*	16	R.AVVDR.T
	gi|2190980	minus dominance gene product	*Chlamydomonas incerta*	13	R.LGVDR.W
	gi|7637533	photosystem protein	*Pisum sativum*	13	K.IGVDR.D
	gi|13096474	chain A, photosystem Ii D1 C-terminal processing protease	*Scenedesmus obliquus*	13	R.GLVDR.G
	gi|15218407	UDP-glucose:sterol glucosyltransferase, putative	*Arabidopsis thaliana*	13	R.GIVDR.G
	gi|255547387	pentatricopeptide repeat-containing	*Ricinus communis*	13	K.GIVSIA.-
Fb2	gi|15208461	kinesin heavy chain	*Zea mays*	12	K.KDNGPETGR.T
	gi|15229715	ATP binding / kinase/serine/ kinase	*Arabidopsis thaliana*	11	R.VDGDLTALRK.S
	gi|34761721	gag/pol polyprotein	*Pisum sativum*	10	R.QRMPDR.R
	gi|255581821	conserved hypothetical protein	*Ricinus communis*	26	MDGLDDK.L

Protein band	Accession No.	Name of identified protein	Organism	MOWSE score	Peptide sequence
Gb0	gi|9294271	mitochondrial protein-like	*Arabidopsis thaliana*	16	K.DEIFR.D
	gi|55773657	always early 1 protein -like	*Oryza sativa*	16	K.DELMR.F + Oxidation (M)
	gi|255541818	histone deacetylase hda1, putative	*Ricinus communis*	16	R.DAGLMR.Q + Oxidation (M)
	gi|255538608	conserved hypothetical protein	*Ricinus communis*	16	R.MATGLGAR.D + Oxidation (M)
	gi|124359694	helix-loop-helix DNA-binding	*Medicago truncatula*	14	R.NQKMGLK.S + Oxidation (M)
	gi|6136830	aux/IAA protein	*Cucumis sativus*	13	R.KVDLGMVK.G + Oxidation (M)
Gb1	gi|13430684	putative MATE efflux family protein	*Arabidopsis thaliana*	17	K.QSQDEDK.E
	gi|167745172	FD-like 2 protein	*Triticum aestivum*	16	R.GASDGVTDK.V
Gb2	gi|255551479	conserved hypothetical protein	*Ricinus communis*	30	R.AVAETVPR.L
	gi|62734422	O-methyltransferase	*Oryza sativa*	22	R.AIAKAYPHVK.C
	gi|255557619	conserved hypothetical protein	*Ricinus communis*	21	K.LGREDPR.R
	gi|255549226	conserved hypothetical protein	*Ricinus communis*	14	R.GASVGHGTR.D
	gi|2981439	syntaxin of plants 31	*Arabidopsis thaliana*	13	K.QSTIFNDR.T
	gi|255588479	conserved hypothetical protein	*Ricinus communis*	12	R.SCRALSQGT
	gi|232041	ACC oxidase 1	*Malus x domestica*	11	R.AATLEK.I

A note : There is no match No. in Eb0

Figure 4.8 Functional classification percentages of total 245 identified proteins

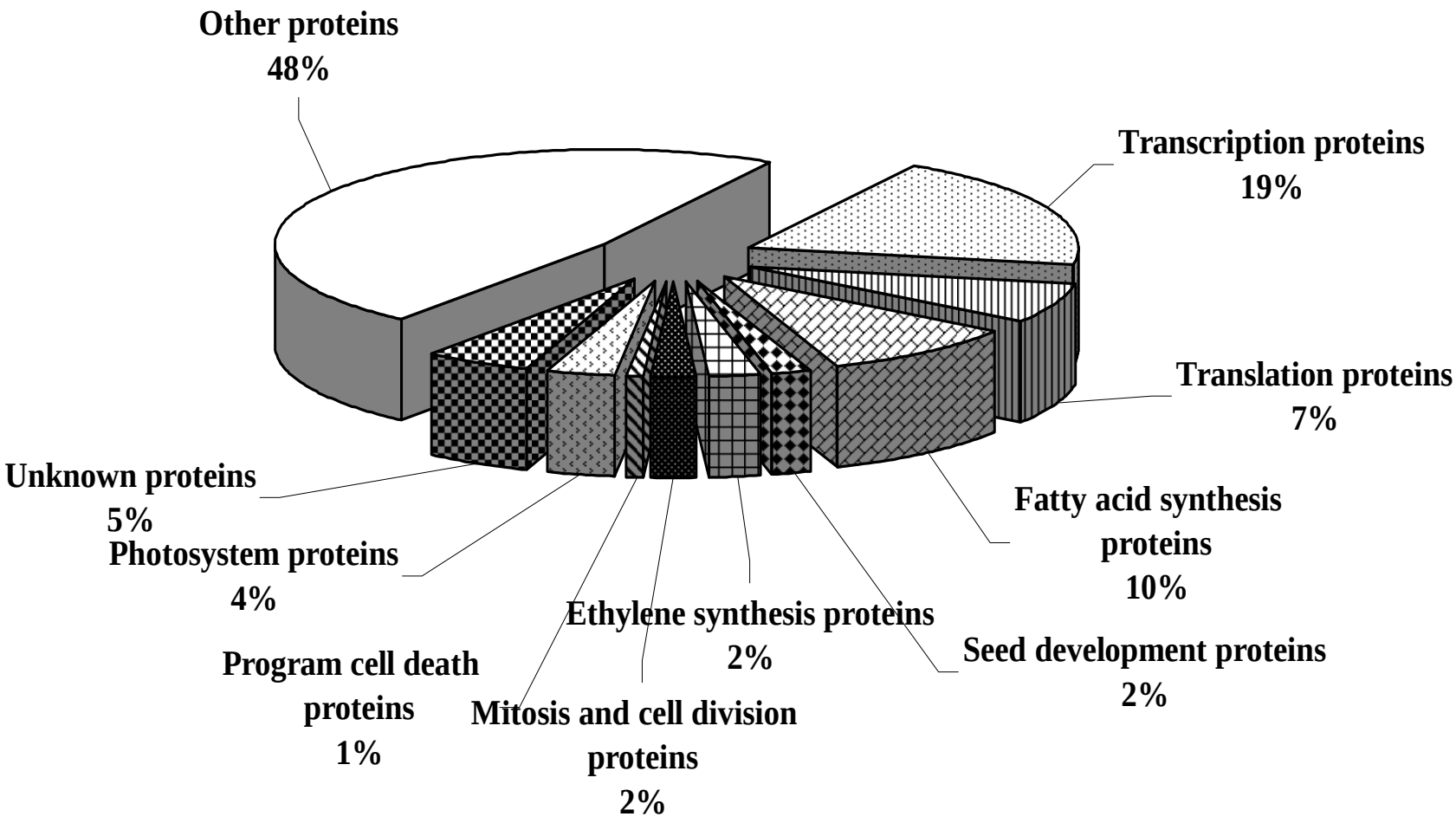


Table 4.5 Proteins related to ethylene synthesis and their relative expression levels compared to stage I

Accession No.	Name of identified proteins	Developmental stages							
		I	II	III	IV	V	VI	VII	VIII
gi|398997	ACC oxidase 1 (ACO1)	1.00	1.02	1.09	1.07	1.08	1.06	1.04	1.10
gi|2541938	Ethylene receptor	1.00	0.93	0.91	0.96	0.88	0.92	0.97	0.94
gi|1561694	ACC synthase 1 (ACS1)	1.00	1.01	0.79	1.00	0.99	0.88	1.00	0.90
gi|1561694	ACC synthase 2 (ACS2)	1.00	1.08	1.07	1.16	1.09	1.17	1.13	1.18
gi|116059648	ACC deaminase (ACD)	1.00	1.12	1.06	1.14	1.17	1.13	1.09	1.11
gi|232041	ACC oxidase 2 (ACO2)	1.00	1.45	1.20	1.15	1.24	1.23	1.29	1.20

Table 4.6 Proteins related to seed development and their relative expression levels compared to stage I (ND= Not detected)

Accession No.	Name of identified proteins	Developmental stages							
		I	II	III	IV	V	VI	VII	VIII
gi|84794464	flavonol synthase	1.00	1.02	1.04	1.11	1.09	1.06	1.05	1.06
gi|56606540	dormancy-associated protein	1.00	0.79	ND	0.85	0.76	0.82	0.91	0.91
gi|4775494	putative coat protein	1.00	0.92	0.89	0.93	0.90	0.91	0.93	0.93
gi|15242262	AP2 domain-containing protein	1.00	1.04	1.08	1.09	1.08	1.11	1.16	1.12
gi|15233479	late embryogenesis abundant protein 1 (LEA1)	1.00	1.04	1.08	1.11	1.10	1.08	1.05	1.02
gi|87162766	late embryogenesis abundant protein 2 (LEA2)	1.00	1.01	0.79	1.00	0.99	0.88	1.00	0.90
gi|8118512	seed storage protein	1.00	0.64	0.62	0.83	ND	0.81	0.74	0.69
gi|226453610	transposase	1.00	1.08	1.16	1.12	1.19	1.13	0.00	1.18

Figure 4.9 Relative protein expression levels of proteins related to ripening process compared to stage I. (ACS=ACC synthase; ACO=ACC oxidase; ACD=ACC deaminase)

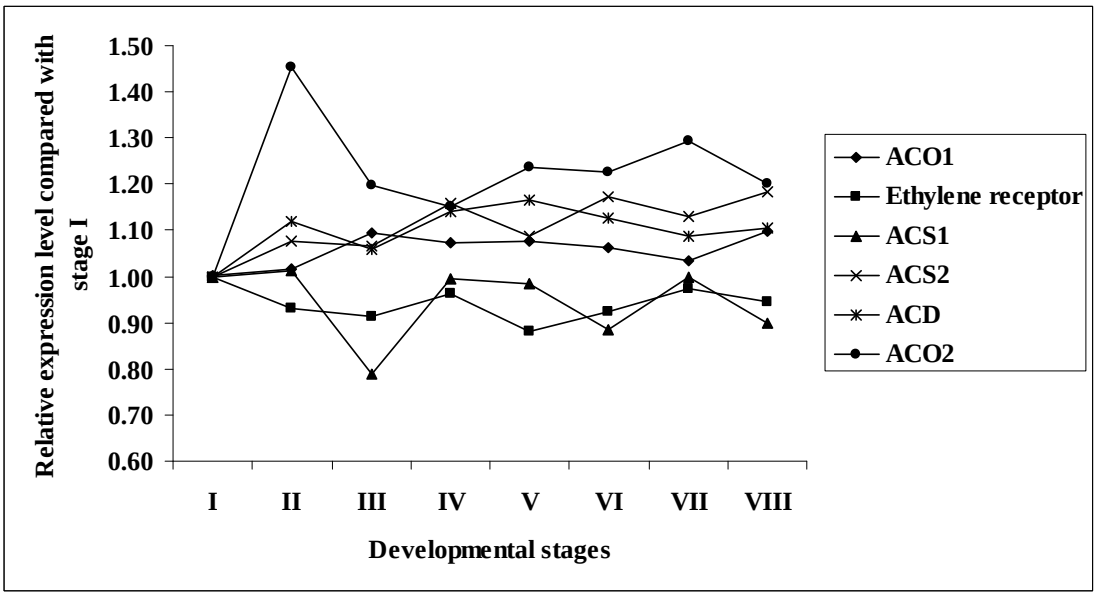


Figure 4.10 Relative protein expression levels of proteins related to seed development compared to stage I (AP2= APETALA2 domain; LEA= Late Embryogenesis Abundant protein)

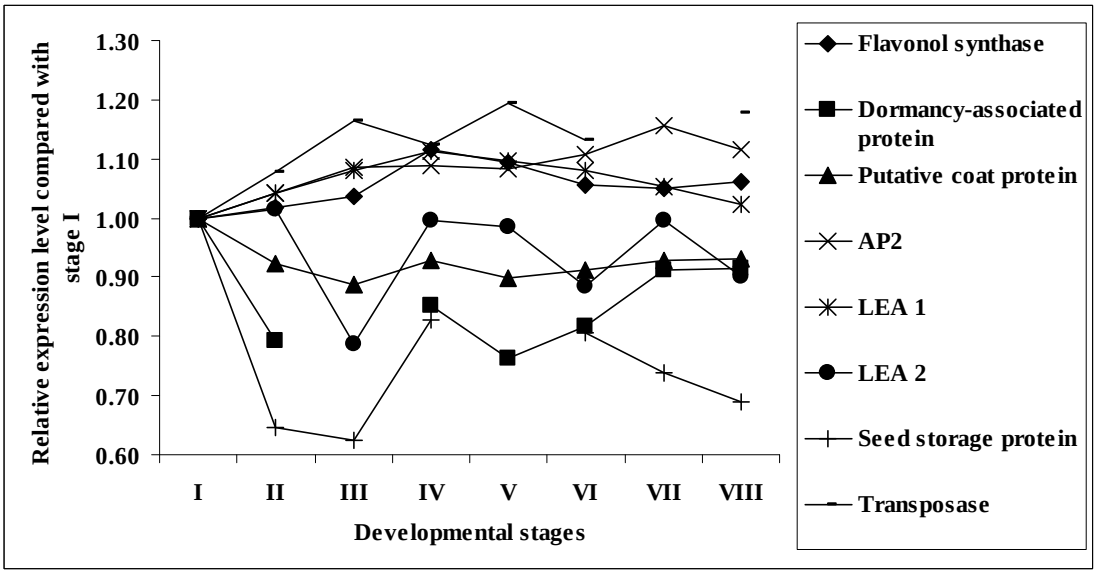
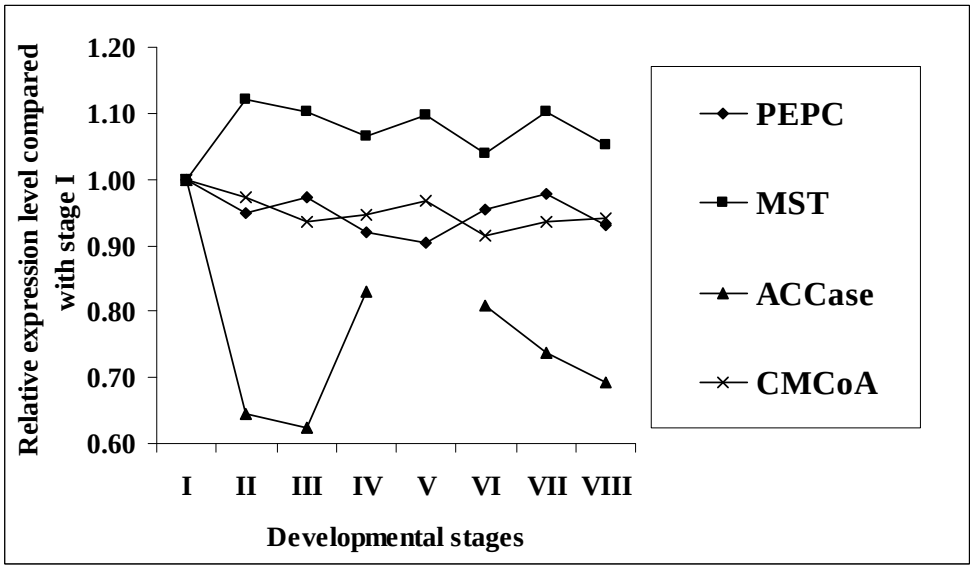


Table 4.7 Proteins related to fatty acid biosynthesis and their relative expression levels compared to stage I (ND=Not detected)

Accession No.	Name of identified proteins	Developmental stages							
		I	II	III	IV	V	VI	VII	VIII
gi|116061081	lipase	1.00	0.94	0.94	0.88	0.93	0.90	0.91	0.92
gi|116000579	putative mitochondrial carrier protein	1.00	1.11	1.13	1.04	1.04	1.04	1.10	1.15
gi|27545461	S-related kinase 10.1A	1.00	1.11	1.06	1.09	1.08	1.04	1.01	1.08
gi|108706991	branched-chain-amino-acid aminotransferase 5, putative	1.00	1.13	1.11	1.05	1.14	1.06	1.09	1.14
gi|15231611	glycosyl hydrolase family 38 protein	1.00	1.12	1.06	1.14	1.17	1.13	1.09	1.11
gi|2145479	phosphoenolpyruvate carboxylase	1.00	0.95	0.97	0.92	0.90	0.95	0.98	0.93
gi|56606540	dormancy-associated protein	1.00	0.79	ND	0.85	0.76	0.82	0.91	0.92
gi|81051845	sucrose transporter	1.00	1.13	1.13	1.15	1.19	1.05	1.12	1.13
gi|116060125	animal-type fatty acid synthase and related proteins	1.00	0.85	ND	0.90	ND	0.86	0.86	ND
gi|54291440	pr1-like protein	1.00	1.03	1.11	1.14	1.09	1.09	1.03	1.12
gi|25992001	lecithine cholesterol acyltransferase	1.00	1.07	1.03	1.17	1.10	1.08	1.13	1.11
gi|224579301	mercaptopyruvate sulfurtransferase	1.00	1.12	1.10	1.06	1.10	1.04	1.10	1.05
gi|237682390	branched-chain aminotransferase 4	1.00	0.92	0.88	0.89	0.87	0.87	0.95	0.89

Accession No.	Name of identified proteins	Developmental stages							
		I	II	III	IV	V	VI	VII	VIII
gi|2586125	□-keto acyl reductase	1.00	1.04	1.11	ND	1.03	1.10	1.11	1.11
gi|255080050	serine acetyl transferase	1.00	1.02	0.79	0.99	0.98	0.88	0.99	0.90
gi|572606	acetyl-CoA carboxylase	1.00	0.65	0.63	0.83	ND	0.81	0.74	0.69
gi|11181943	acetyl CoA carboxylase pRS1	1.00	0.65	0.63	0.83	ND	0.81	0.74	0.69
gi|11869927	acetyl-CoA carboxylase 1	1.00	0.65	0.63	0.83	ND	0.81	0.74	0.69
gi|38228683	receptor-like protein kinase	1.00	1.15	1.19	1.34	1.40	1.16	1.45	1.49
gi|21553535	ras-GTPase-activating protein SH3-domain binding protein	1.00	0.95	0.92	0.90	0.84	0.86	0.84	0.80
gi|162949342	4-coumarate:coenzyme A ligase 1	1.00	0.97	0.94	0.95	0.97	0.91	0.94	0.94
gi|116055441	putative short chain alcohol dehydrogenase	1.00	1.22	1.30	1.20	1.23	1.25	1.26	1.25

Figure 4.11 Relative protein expression levels of four target proteins related to fatty acid biosynthesis compared to stage I



4.2.4 Target Proteins

There were 22 identified proteins that related to fatty acid biosynthesis as shown in Table 4.7. Only 4 proteins, for instance, acetyl CoA carboxylase (ACCase), phosphoenol pyruvate carboxylase (PEPC), mercaptopyruvate sulfertransferase (MST) and 4-coumarate: coenzyme A ligase (CMCoA) were selected for studying the mRNA expression level. They may play a major role in fatty acid biosynthesis of *J. curcas* seed as mentioned in chapter II.

4.3 mRNA Expression

4.3.1 Specific Primers Designing

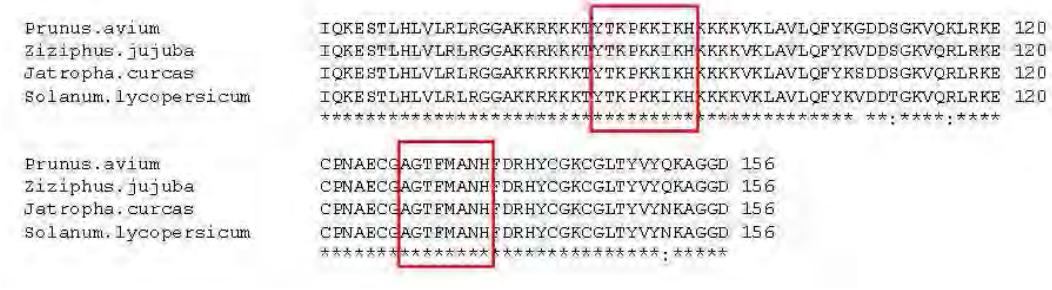
Specific primers for PCR amplification in *J. curcas* samples were designed by searching for gene(s) encoding for interesting enzymes from plants available in GenBank (http://ncbi.nlm.nih.gov/).

Ubiquitin (*UBQ*), a housekeeping gene, was chosen to be a reference for quantitative Real-time PCR. Primers for *ubiquitin* sequences were designed based on the ubiquitin gene from various plant available in ncbi database as shown in Figure 4.12.

The specific primers of 4 target genes were designed by the same criterior as *ubiquitin*, and the alignment results were shown in Figure 4.13-4.16.

Figure 4.12 (A). Alignment of *ubiquitin* (*UBQ*) and (B). Conserve sequence in the boxed used for design primers.

A.



B.

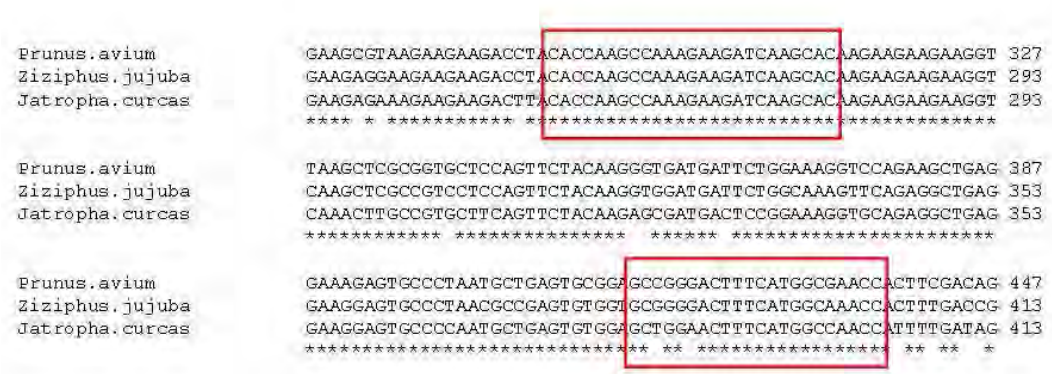
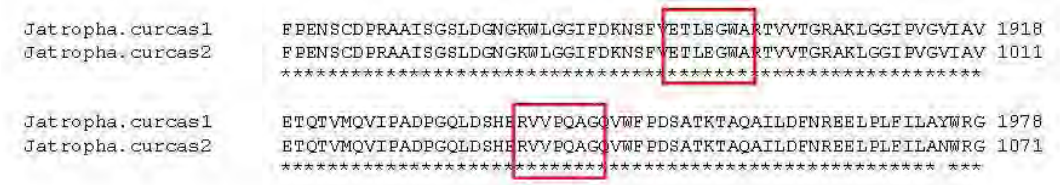


Figure 4.13 (A). Alignment of *acetyl CoA carboxylase* (ACCase) and (B). Conserve sequence in the boxed used for design primers.

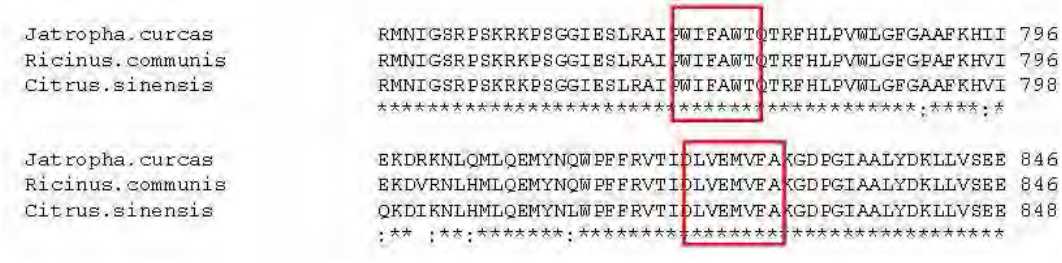
A.



B.

Figure 4.14 (A). Alignment of *phosphoenol pyruvate carboxylase* (*PEPC*) and (B). Conserve sequence in the boxed used for design primers.

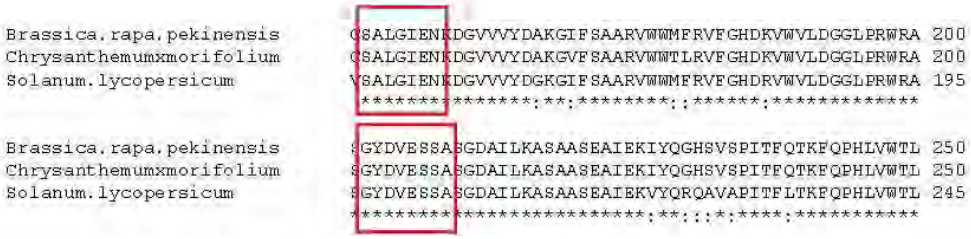
A.



B.

Figure 4.15 (A). Alignment of *mercaptopyruvate sulfertransferase* (*MST*) and (B). Conserve sequence in the boxed used for design primers.

A.

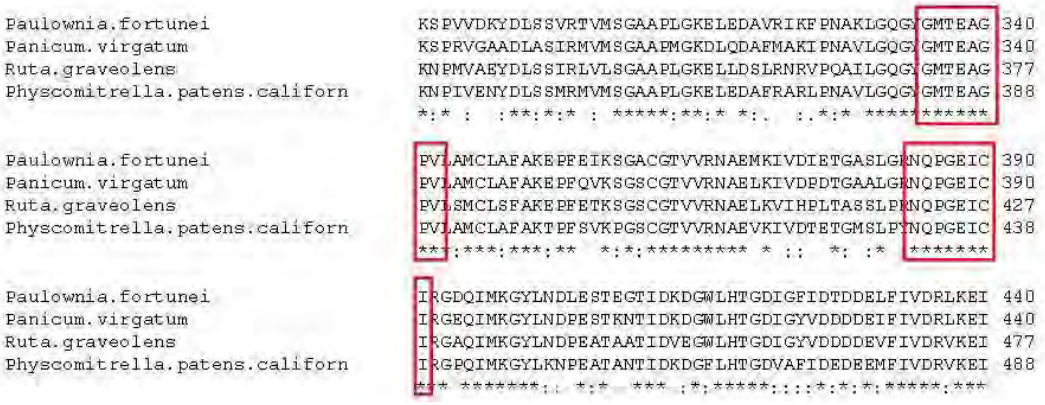


B.

Figure 4.16 (A). Alignment of *4-Coumarate: CoenzymeA ligase* (*CMCoA*) and (B). Conserve sequence in the boxed used for design primers.

A.

B.



The 5 PCR primers were used to amplify a reference gene (ubiquitin) and 4 other target genes that involved in fatty acid biosynthesis. All PCR products were expected to have sizes ranging from 148 to 174 bp according to Table 4.8.

Table 4.8 Oligonucleotide primers used to amplify target genes amplification

Gene	size	Location	Oligonucleotide primers (5'-3')	Tm (ºC)	GC
UBQ	148	F	CAC CAA GCC AAA GAA GAT CAA GCA C	48	58
		R	GGT TGG CCA TGA AAG TTC CAG C	55	57
ACCase	162	F	GAG ACA CTG GAA GGC TGG GC	65	58
		R	GCC AGC CTG AGG AAC AAC CC	65	58
PEPC	157	F	CCC TGG ATC TTC GCA TGG AC	56	60
		R	GGC AAA CAC CAT CTC AAC CAA GTC	57	50
MST	174	F	TCT GCT CTT GGA ATT GAG AAC A	51	41
		R	TGC ACT AGA TTC AAC ATC ATA TCC	52	38
CMCoA	170	F	GGA TGA CAG AAG CAG GGC CA	56	60
		R	GAT GCA AAT TTC ACC AGG CTG ATT	54	42

Note

UBQ = Ubiquitin (positive control)
ACCase = Acetyl CoA carboxylase
PEPC = Phosphoenolpyruvate carboxylase
MST = Mercaptopyruvate sulfurtransferase
CMCoA= 4-Coumarate: coenzyme A ligase

4.3.2 Total RNA Extraction

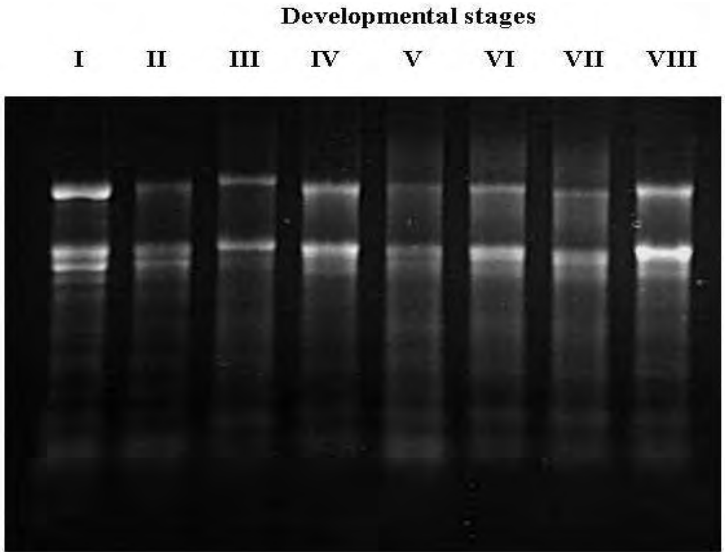
After total RNA extraction by Hao Yu buffer, RNA was resuspended in DEPC water to inhibit RNase activity. The ratio of $OD_{260/280}$ and $OD_{260/230}$ in all stages were determined, and found that the ratios were about 1.3 to 1.9. The results showed that the quality of RNA was not good enough for further experiment. Thus, RNA precipitation by 10M lithium chloride was performed again. After re-precipitation, the

ratios of purified RNA were 1.7-2.3 as shown in Table 4.9, implying that the quality of extracted RNA was acceptable for further experiments. Thus, 1 µg of total RNA of all stages were loaded into denatured gel electrophoresis to show the primary structure of RNA. Two obviously high intensity bands were detected, 28S and 18S rRNA, as shown in Figure 4.17. Total RNA were kept at -80ºC prior to use.

Table 4.9 RNA concentration, the ratio of $OD_{260/280}$ and $OD_{260/230}$ by Nanodrop spectrophotometer

Developmental stages	Concentration (ng/µl)	$OD_{260/280}$	$OD_{260/230}$
I	2903.55	2.06	2.21
II	2163.56	2.14	2.22
III	1173.92	2.25	2.20
IV	2413.94	2.16	2.28
V	1051.76	2.23	2.16
VI	1443.43	2.16	2.07
VII	1347.21	2.08	1.72
VIII	1123.67	2.22	2.23

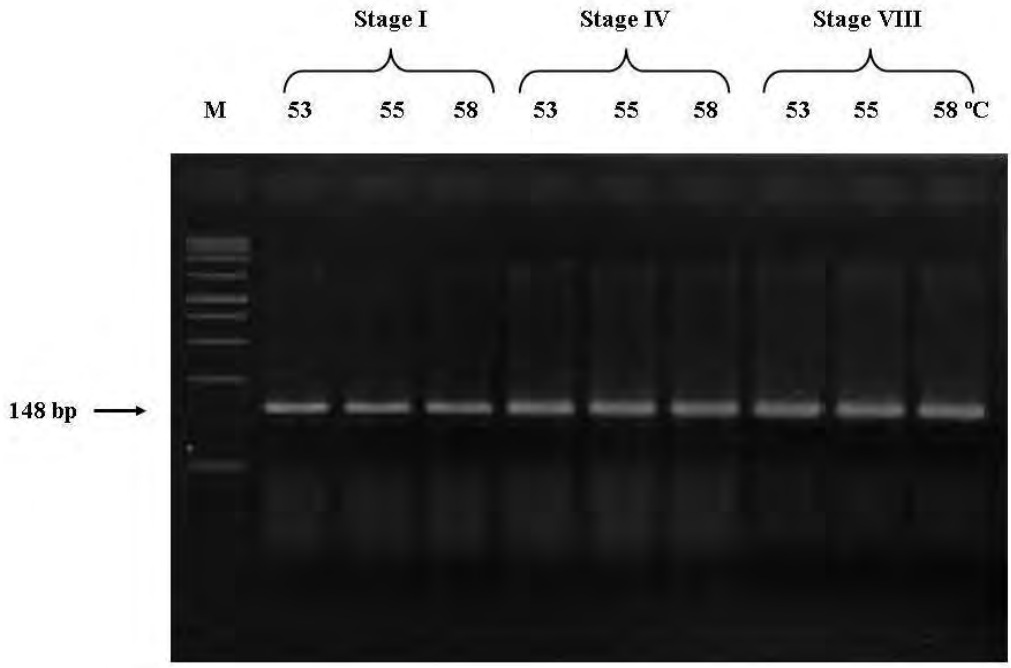
Figure 4.17 The ethidium bromide stained 1.2% denaturing agarose gel electrophoresis showing the quality of total RNA extracted from eight developmental stages of *J. curcas* seeds.



4.3.3 Optimization of Ubiquitin (UBQ) Amplification to Use as a Reference Gene

Three PCR conditions (annealing temperature variation: 55, 58 and 60 ºC) were tested to amplify the housekeeping gene, UBQ, whereas 1 µl of cDNA of stages I, IV and VIII were used as templates. As shown in Figure 4.18, the sizes of PCR products of different stages and templates appeared in the same size with nearly the same intensity.

Figure 4.18 The ethidium bromide stained 1.2% agarose gel electrophoresis showing the optimization of PCR condition for amplification of ubiquitin cDNA (M=1 kb plus ladder).



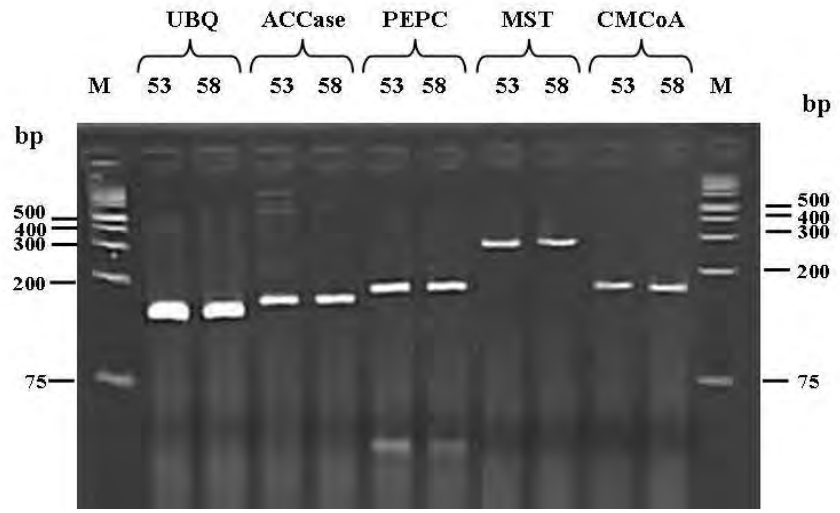
4.3.4 Optimization of PCR Condition of the Target Genes

RT-PCR of the target genes (Table 4.5) were amplified in a 10 µl reaction volume, the reaction was the same as mentioned in section 3.2.4.3 (chapter III). Annealing temperature was optimized between 53 and 58ºC. After optimization, it was noticed that PCR products appeared as clearly bands. When compared by eye, the

intensity of PCR product bands revealed no difference between annealing temperature at 53 and 58 ºC.

It was observed that PCR product of MST cDNA had sizes between 200-300 base pairs and the PEPC cDNA reaction contained a small fragment of non-specific bands as demonstrated in Figure 4.19. According to the results, annealing temperature used was increased until 60ºC in order to decrease non-specific bands in real-time PCR condition

Figure 4.19 PCR products from amplification of UBQ and 4 other target genes using annealing temperature at 53 and 58ºC (M=1 kb plus ladder).



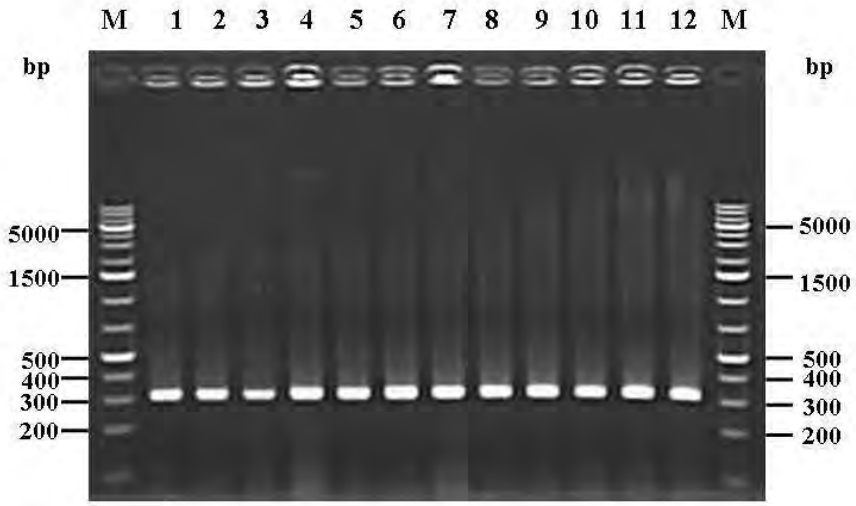
4.3.5 Cloning of PCR Products and Selection of the Recombinant Plasmid for Nucleotide Sequencing

After RT-PCR, target bands were cut and DNA fragments were extracted from agarose gel. Each eluted DNA sample was cloned into pTZ57R/T and transformed into *E. coli* DH5α. Most of white colonies were picked up and cultured in amplicilin-LB broth. The same white colonies were used as DNA templates to amplify with specific M13 primers and their PCR products were run in agarose gel electrophoresis to check their sizes. PCR product size of non-recombinant plasmid (only pTZ57R/T) was about 200 bp. In consequence, recombinant plasmid sizes must be approximately

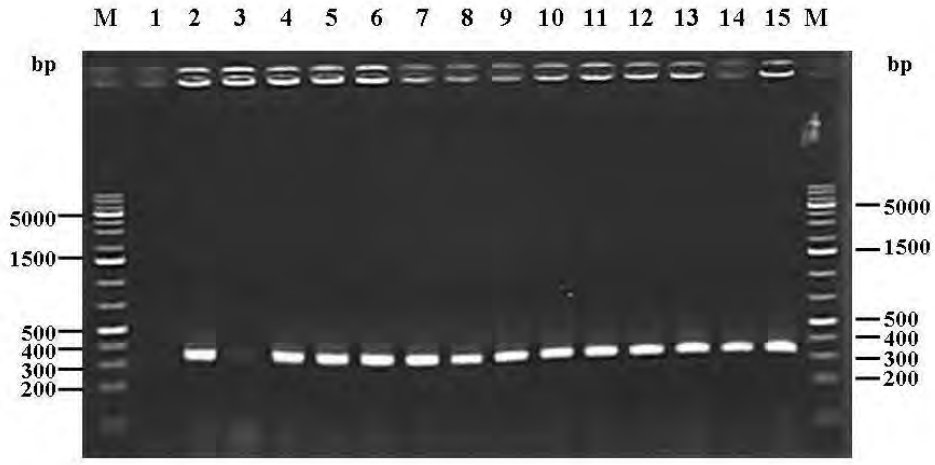
350 bp because PCR products of target genes were about 150 bp. The gel electrophoresis results were shown in Figure 4.20.

Figure 4.20 PCR products obtained with M13 primers using recombinant plasmids of target genes as templates (A) *Ubiquitin* (B) *Acetyl CoA carboxylase* (C) *Phosphoenolpyruvate carboxylase* (D) *Mercaptopyruvate sulfurtransferase* (E) *4-Coumarate: coenzyme A ligase.*

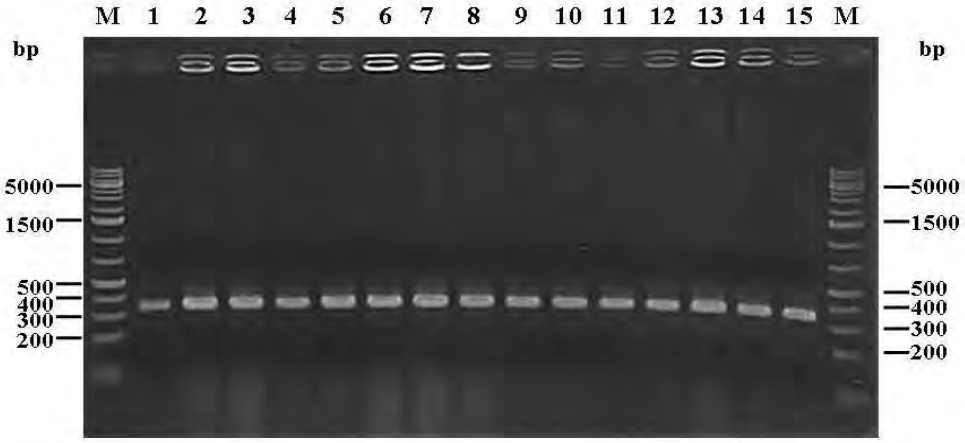
A.



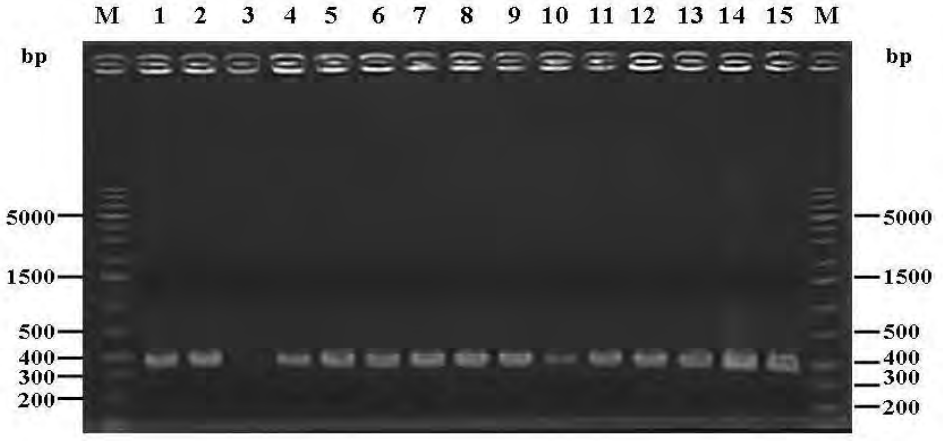
B.



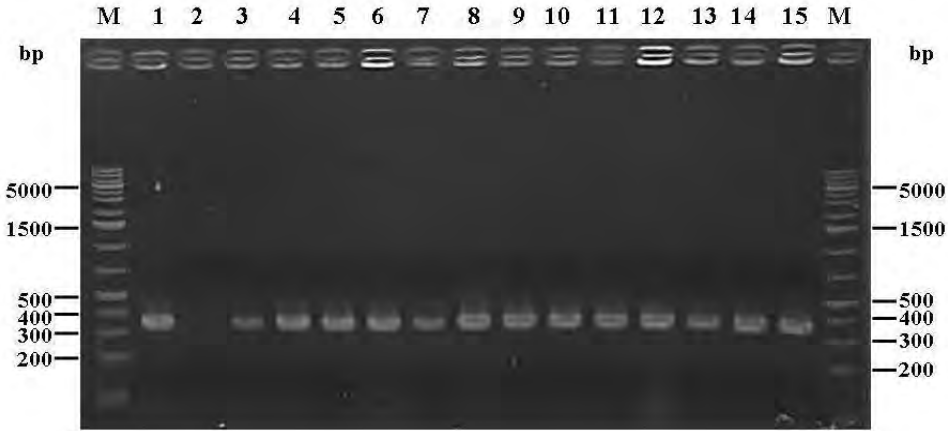
C.


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 M
bp
bp
5000
1500
500
400
300
200
5000
1500
500
400
300
200

D.


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 M
bp
bp
5000
1500
500
400
300
200
5000
1500
500
400
300
200

E.


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 M
bp
bp
5000
1500
500
400
300
200
5000
1500
500
400
300
200

Five of each recombinant plasmid which their PCR product sizes about 350 bp were extracted by Plasmid DNA Purification Kit according to section 3.2.4.9 in chapter III before determination of the nucleotide sequences.

4.3.6 Nucleotide Sequence Analysis

All of the recombinant plasmids were sequenced using M13 forward primer. After removing the DNA sequence of pTZ57R/T by VecScreen software (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html), it was found that the length of the target genes were approximately 150 to 200 bp as designed in Table 4.6 except MST which was about 300 bp. The sequences of these DNA fragments were compared to the nucleotide databases in GenBank database using blast (http://blast.ncbi.nlm.nih.gov/) and found that they showed similarity to ubiquitin, acetyl CoA carboxylase and phosphoenolpyruvate carboxylase in *J. curcas*. However, the other two DNA fragments showed high similarity to the DNA sequences of thiosulfate sulfertansferase and AMP dependent CoA ligase in *Ricinus communicus* as demonstrated in Table 4.10.

All minus strands were reversed complementary into plus strands by reverse complement program (http://www.bioinformatics.org/sms/rev_comp.html) before compaing to genes retrieved from GenBank database by ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

It was observed that most of the sequences of each gene were similar. However, some sequences with low quality had to be trimmed before alignment. Consequently, alignment report displayed the highly conserved sequences as shown in Figure 4.21.

Table 4.10 Detail of the first sequences in ncbi database with the highest similarity to known sequences of five genes.

Gene	Accession No.	Organism	% Identity
Ubiquitin	gi|256427001	*Jatropha curcas*	98
Acetyl-CoA carboxylase	gi|281428050	*Jatropha curcas*	100
Phosphoenolpyruvate carboxylase	gi|155967406	*Jatropha curcas*	99
Thiosulfate sulfurtansferase	gi|255544613	*Ricinus communis*	89
AMP dependent CoA ligase	gi|255544613	*Ricinus communis*	83

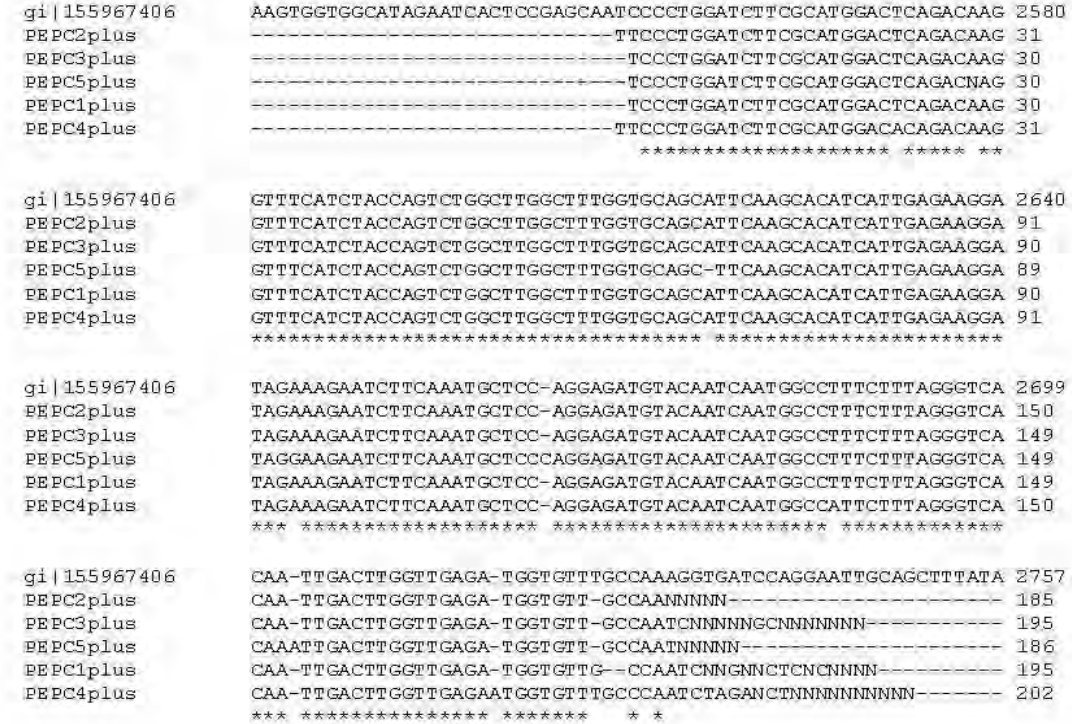
Figure 4.21 Alignment report of sequences from recombinant plasmids and sequences in ncbi database (A) *Ubiquitin* (B) *Acetyl CoA carboxylase* (C) *Phosphoenolpyruvate carboxylase* (D) *Mercaptopyruvate sulfurtransferase* (E) *4-Coumarate: coenzyme A ligase.*

A.

B.


gi|281428050 TTTTTGACAAGAAATAGTTTTGTTGAGACACTGGAAGGCTGGGCAAGGACAGTTGTGACA 240
ACCase5plus ----------TAGACACTGGAAGGCTGGGCAAGGACAGTTGTGACA 36
ACCase1plus ----------TGAGACACTGGAAGGCTGGGCAAGGACAGTTGTGACA 37
ACCase3plus ----------TGAGACACTGGAAGGCTGGGCAAGGACAGTTGTGACA 37
ACCase2plus ----------TGAGACACTGGAAGGCTGGGCAAGGACAGTTGTGACA 37
gi|281428050 GGAAGGGCAAAGCTCGGAGGAATCCCTGTTGGAGTAATAGCTGTTGAAACTCAAACTGTG 300
ACCase5plus GGAAGGGCAAAGCTCGGAGGAATCCCTGTTGGAGTAATAGCTGTTGAAACTCAAACTGTG 96
ACCase1plus GGAAGGGCAAAGCTCGGAGGAATCCCTGTTGGAGTAATAGCTGTTGAAACTCAAACTGTG 97
ACCase3plus GGAAGGGCAAAGCTCGGAGGAATCCCTGTTGGAGTAATAGCTGTTGAAACTCAAACTGTG 97
ACCase2plus GGAAGGGCAAAGCTCGGAGGAATCCCTGTTGGAGTAATAGCTGTTGAAACTCAAACTGTG 97
gi|281428050 ATGCAGGTGATTCCTGCTGACCCAGGACAGCTCGATTCTCATGAGAGGGTTGTTCCTCAG 360
ACCase5plus ATGCAGGTGATTCCTGCTGACCCAGGACAGCTCGATTCTCATGAGAGGGTTGTTCCTCAG 156
ACCase1plus ATGCAGGTGATTCCTGCTGACCCAGGACAGCTCGATTCTCATGAGAGGGTTGTTCCTCAG 156
ACCase3plus ATGCAGGTGATTCCTGCTGACCCAGGACAGCTCGATTCTCATGAGAGGGTTGTTCCTCAG 156
ACCase2plus ATGCAGGTGATTCCTGCTGACCCAGGACAGCTCGATTCTCATGAGAGGGTTGTTCCTCAG 156
gi|281428050 GCTGGCCAAGTATGGTTTCCAGATTCTGCAACCAAAACAGCTCAAGCTATATTGGATTTC 420
ACCase5plus GCTGGCAN---------- 164
ACCase1plus GCTGGCANNCNNGNNNNNNNNNNN---------- 182
ACCase3plus GCTGGCAATCNNGNNCNNNNNNNNNN---------- 184
ACCase2plus GCTGGCAATCNNNNNNNNN---------- 176

C.


gi|155967406 AAGTGGTGGCATAGAATCACTCCGAGCAATCCCCTGGATCTTCGCATGGACTCAGACAAG 2580
PEPC2plus ----------TTCCCTGGATCTTCGCATGGACTCAGACAAG 31
PEPC3plus ----------TCCCTGGATCTTCGCATGGACTCAGACAAG 30
PEPC5plus ----------TCCCTGGATCTTCGCATGGACTCAGACNAG 30
PEPC1plus ----------TCCCTGGATCTTCGCATGGACTCAGACAAG 30
PEPC4plus ----------TTCCCTGGATCTTCGCATGGACACAGACAAG 31
gi|155967406 GTTTCATCTACCAGTCTGGCTTGGCTTTGGTGCAGCATTCAAGCACATCATTGAGAAGGA 2640
PEPC2plus GTTTCATCTACCAGTCTGGCTTGGCTTTGGTGCAGCATTCAAGCACATCATTGAGAAGGA 91
PEPC3plus GTTTCATCTACCAGTCTGGCTTGGCTTTGGTGCAGCATTCAAGCACATCATTGAGAAGGA 90
PEPC5plus GTTTCATCTACCAGTCTGGCTTGGCTTTGGTGCAGC-TTCAAGCACATCATTGAGAAGGA 89
PEPC1plus GTTTCATCTACCAGTCTGGCTTGGCTTTGGTGCAGCATTCAAGCACATCATTGAGAAGGA 90
PEPC4plus GTTTCATCTACCAGTCTGGCTTGGCTTTGGTGCAGCATTCAAGCACATCATTGAGAAGGA 91
gi|155967406 TAGAAAGAATCTTCAAATGCTCC-AGGAGATGTACAATCAATGGCCTTTCTTTAGGGTCA 2699
PEPC2plus TAGAAAGAATCTTCAAATGCTCC-AGGAGATGTACAATCAATGGCCTTTCTTTAGGGTCA 150
PEPC3plus TAGAAAGAATCTTCAAATGCTCC-AGGAGATGTACAATCAATGGCCTTTCTTTAGGGTCA 149
PEPC5plus TAGGAAGAATCTTCAAATGCTCCCAGGAGATGTACAATCAATGGCCTTTCTTTAGGGTCA 149
PEPC1plus TAGAAAGAATCTTCAAATGCTCC-AGGAGATGTACAATCAATGGCCTTTCTTTAGGGTCA 149
PEPC4plus TAGAAAGAATCTTCAAATGCTCC-AGGAGATGTACAATCAATGGCCATTCTTTAGGGTCA 150
gi|155967406 CAA-TTGACTTGGTTGAGA-TGGTGTTTGCCAAAGGTGATCCAGGAATTGCAGCTTTATA 2757
PEPC2plus CAA-TTGACTTGGTTGAGA-TGGTGTT-GCCAANNNNN---------- 185
PEPC3plus CAA-TTGACTTGGTTGAGA-TGGTGTT-GCCAATCNNNNNGCNNNNNNN---------- 195
PEPC5plus CAAATTGACTTGGTTGAGA-TGGTGTT-GCCAATNNNNN---------- 186
PEPC1plus CAA-TTGACTTGGTTGAGA-TGGTGTTG--CCAATCNNGNNCTCNCNNNN---------- 195
PEPC4plus CAA-TTGACTTGGTTGAGAATGGTGTTTGCCCAATCTAGANCTNNNNNNNNNN------- 202

D.

```
MST2plus          ----------------------------------NNNNNNNNNGNATCTAGATTTCTGC 25
MST3plus          ----------------------------------NNNNNNNGNNTNT-GNTTTNTGC 22
MST4plus          ----------------------------------NNNNNCNTNNNGNNCNNGATTTCTGC 26
gi|255544613      GCATCCTGGTACATGCCAGACGAACAGAGGAATCCAATTCAAGAGTATCAGGTTGCTCAC 360
                                                                  * *     *

MST2plus          TTTCGGGATTGAGAGCAAGAT--GGATNGGTGTTTCG--GAATGGGAGGGGGTATTTTTG 81
MST3plus          TCTTGGAATTGAGNNCAAGAT--GGATGGGTTGTTTAT-GAATGGGGATGGGTATTTTTN 79
MST4plus          TCTTGGAATTGAGAACAAAGAT-GGATTGGTTGTTTAT-GATGGGAAGGG--TATTTTTA 82
gi|255544613      ATTCCTGGT-GCCCTTTTCTTTGATGTAGATGGGATATCAGATCGATCTACAAATTTGCC 419
                    *     * *               * * *           *        ****

MST2plus          GCGCA-GCTCGGGTC-GGTGGTGAGTAGGTTGACGTATTTCAAATTTTTGTGATTCTATG 139
MST3plus          GCGCA-GCTCGTGTCTGGTGGTGAGTAG-TTGACGTATTTCAAATTTTTGTGATTCTATG 137
MST4plus          GCGCA-GCTCGTGTCTGGTGGTGAGTAG-TTGACGTATTTCAAATTTTTGTGATTCTATG 140
gi|255544613      ACACATGCTGCCATCAGAGGAAGCTTTTTCAGCTGCTGTTTCAGCTCTTGGGATTGAGAA 479
                   * ** ***    ** *  *  *  *     *  *   **  *  * * * ****

MST2plus          CAAAATTGGTATTTTATGGTTAACCATCTAATATTTTTCCTTTGTTCTTTCATCAAATGA 199
MST3plus          CAAAATTGGTATTTTATG-TTAACCATCTAATATTTTTCCTTTGTTCTTTCATCAAATGA 196
MST4plus          CAAAATTGGTATTTTATG-TTAACCATCTAATATTTTTCCTTTGTTCTTTCATCAAATGA 199
gi|255544613      TAAAGATGG-ATTGGTTG-TTTATGATGGGAAAGGGATTTTTAGTGCAGC--TCGTGTCT 535
                   ***   ** ***   ** ** *  **   * *    *  ** ** *     **   *

MST2plus          GAAGGATGTTCCGAGTCTTTGGGCATGATAAAGTTTGGGTGTTGGATGGAGGCTTACCTA 259
MST3plus          GAAGGATGTTCCGAGTCTTTGGGCATGATAAAGTTTGGGTGTTGGATGGAGGCTTACCTA 256
MST4plus          GAAGGATGTTCCGAGTCTTTGGGCATGATAAAGTTTGGGTGTTGGATGGAGGCTTACCTA 259
gi|255544613      GGTGGATGTTTCGAGTCTTTGGACATGAAAAAGTTTGGGTGTTGGATGGAGGCCTGCCAA 595
                  *  ******* *********** ***** ********************** * ** *

MST2plus          GATGGCGTGCATCAGGATATGATGTTGAATCTAGTGCAA---------------------- 298
MST3plus          GATGGCGTGCATCAGGATATGATGTTGAATCTAGTGCA----------------------- 294
MST4plus          GATGGCGTGCATCAGGATATGATGTTGAATCTAGTGCAA---------------------- 298
gi|255544613      GATGGCGTGCATCAGGATATGATGTTGAATCTAGTGCTTCTGGTGATGCCATCTTGAAAG 655
                  ************************************
```

E.

```
CMCoA2plus        -------TGGATGACAGAAGCAGGGCCAGTTCTATCAATGGGCTTGGGGTTTGCAAAGCA 53
CMCoA3plus        -------TGGATGACAGAAGCAGGGCCAGTTCTATCAATGGGCTTGGGGTTTGCAAAGCA 53
CMCoA4plus        -------TGGATGACAGAAGCAGGGCCAGTTCTATCAATGGGCTTGGGGTTTGCAAAGCA 53
CMCoA5plus        -------TGGATGACAGAAGCAGGGCCAGTTCTATCAATGGGCTTGGGGTTTGCAAAGCA 53
gi|255557998      GGGGTATGGGATGACAGAAGCAGGGCCAGTGCTATCAATGTGCTTAGGATTTGCAAAGCA 1200
                         ********************** ********* **** ** ***********

CMCoA2plus        GCCAATCCCAGCTAAGTCAGGCTCATGTGGAACTGTGGTTAGAAATGCAGAGCTCAAAGT 113
CMCoA3plus        GCCAATCCCAGCTAAGTCAGGCTCATGTGGAACTGTGGTTAGAAATGCAGAGCTCAAAGT 113
CMCoA4plus        GCCAATCCCAGCTAAGTCAGGCTCATGTGGAACTGTGGTTAGAAATGCAGAGCTCAAAGT 113
CMCoA5plus        GCCAATCCCAGCTAAGTCAGGCTCATGTGGAACTGTGGTTAGAAATGCAGAGCTCAAAGT 113
gi|255557998      ACCTTTCCCAACAAAATCAGGTTCATGTGGAACAGTGGTCAGAAATGCAGAGCTCAAGGT 1260
                   **  ***** * ** ***** ********** ***** ***************** **

CMCoA2plus        TATTGACCCTGAAAC-TGGTTCATC-TCNTGGCTACAATTCAGCCTGGTGAAATTTGCAT 171
CMCoA3plus        TATTGACCCTGAAAC-TGGTTCATC-NNN-GGCTACAAT-CAGCCTGGTGAAATTTGCAT 169
CMCoA4plus        TATTGACCCTGAAAC-TGGTTCATC-TCTTGGCTACAAT-CAGCCTGGTGAAATT-GCAT 169
CMCoA5plus        TATTGACCCTGAAACCTGGTTCATCCTCTTGGCTACAATTCAGCCCGGTGAAATTTGCAT 173
gi|255557998      CATCGACCCCGAAAC-CGGTTGCTC-CCTTGGCTACAAT-CAGCCTGGTGAAATTTGCAT 1317
                   ** ***** *****  ****  **     ********** *************** ****

CMCoA2plus        CNNNCNNNNNNNNNNNTGG------------------------------------------ 190
CMCoA3plus        CNNNNNNN---------------------------------------------------- 177
CMCoA4plus        CNANNNNG---------------------------------------------------- 177
CMCoA5plus        CCAATCTAGATNCNNNNNNNNN-------------------------------------- 195
gi|255557998      CAGAGGACCTCAAATAATGAAAGGATATTTGAATGATCCGGAGGCCACAGCAAATACCAT 1377
                  *
```

4.3.7 Realtime PCR Analysis

Real-time PCR condition according to Table 3.7 in chapter III was used to quantify the specific transcripts in each sample. As demonstrated in Figure 4.22, the diluted series of recombinant plasmid templates yielded a linear graph. Some of Ct values were cut off for given trustworthy R-squared value (more than 0.98).

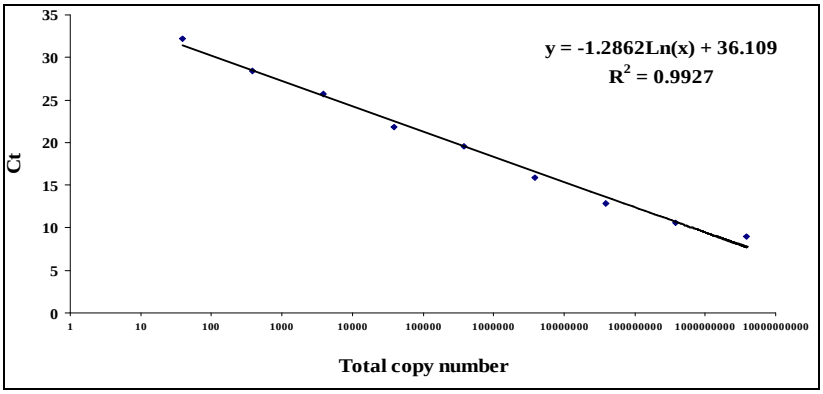
According to Figure 4.23, the expression levels of all genes were changed through eight developmental stages.

Similar expression patterns of other genes were observed. Accumulation of transcripts in stage I was moderate and they reached the maximum expression level in stages II and V. On the other hand, minimum expression level was observed in stages IV and VIII.

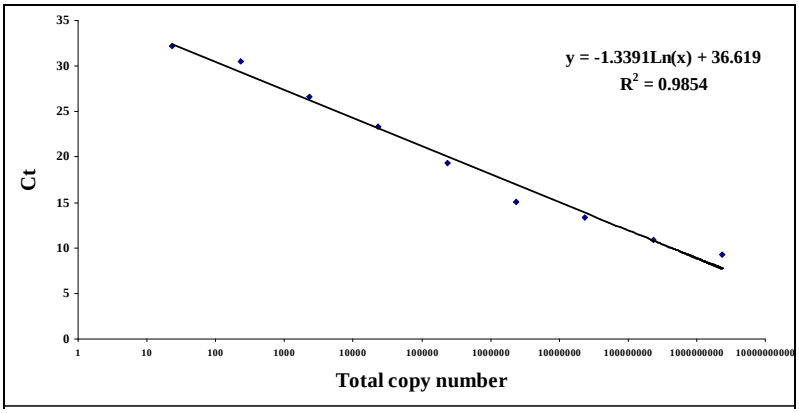
A summary of relative expression level of all genes was in Figure 4.24.

Figure 4.22 Standard logarithmic graph displayed equation and R-squared value for real-time PCR analysis: (A) Ubiquitin (B) Acetyl CoA carboxylase (C) Phosphoenolpyruvate carboxylase (D) Mercaptopyruvate sulfurtransferase (E) 4-Coumarate (coenzyme A ligase).

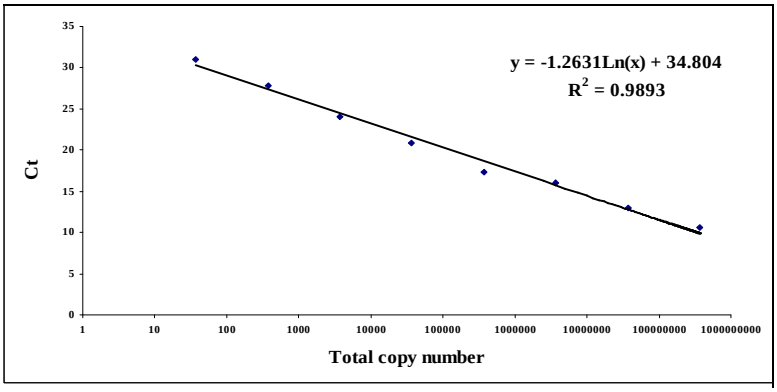
A.



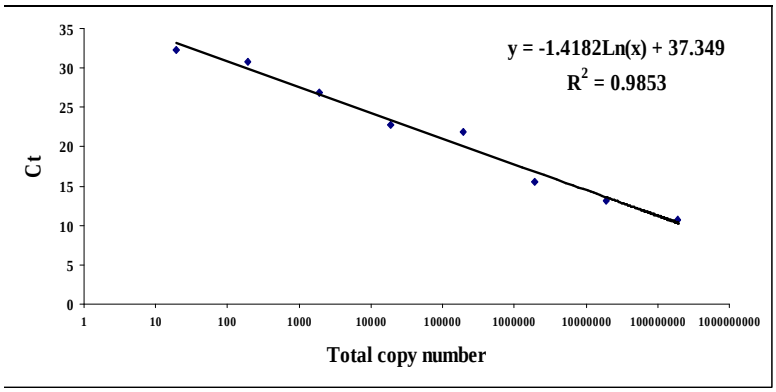
B.


y = -1.3391Ln(x) + 36.619
R2 = 0.9854
Ct
Total copy number

C.


y = -1.2631Ln(x) + 34.804
R2 = 0.9893
Ct
Total copy number

D.


y = -1.4182Ln(x) + 37.349
R2 = 0.9853
Ct
Total copy number

E.

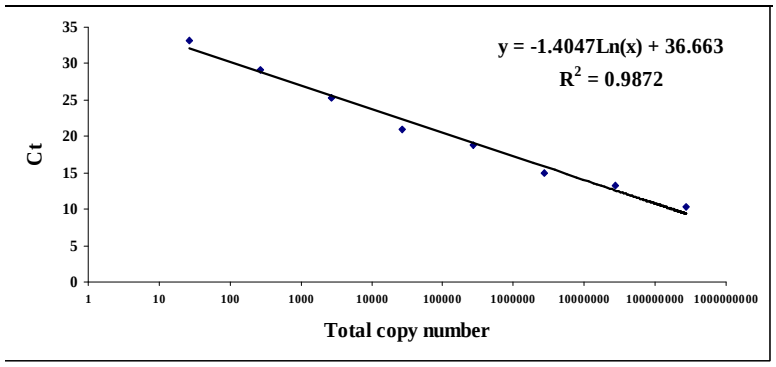
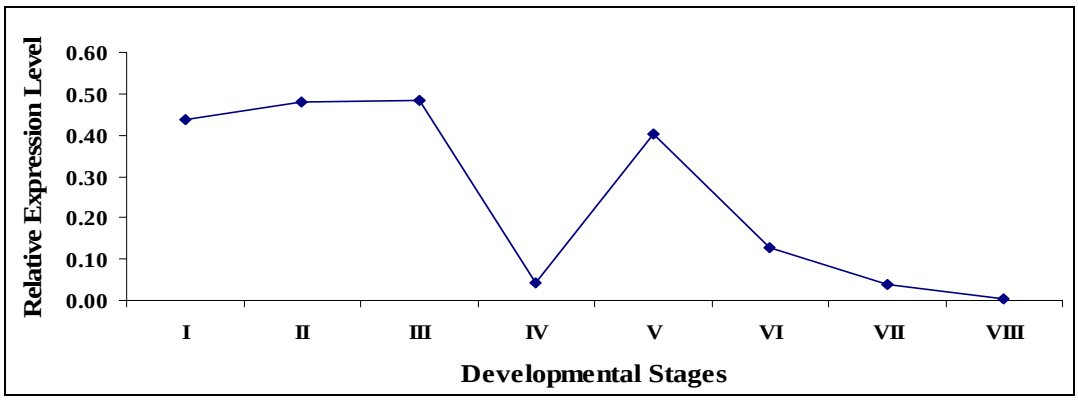
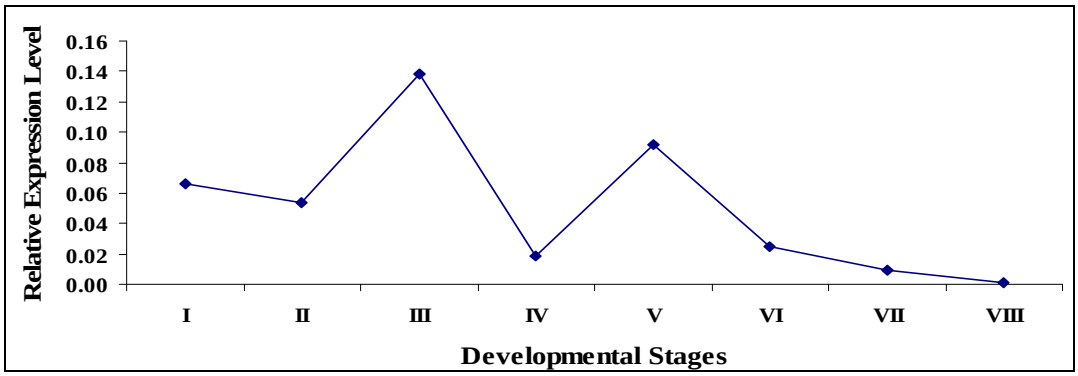


Figure 4.23 Relative expression level of four target genes compared to ubiquitin: (A) Acetyl CoA carboxylase (B) Phosphoenolpyruvate carboxylase (C) Mercaptopyruvate sulfurtransferase (D) 4-Coumarate: coenz A ligase.

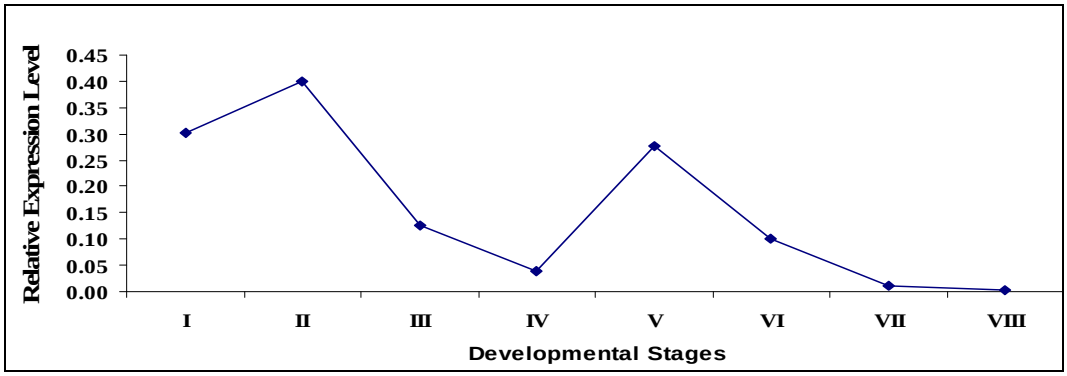
A.



B.



C.


Relative Expression Level
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
I
II
III
IV
V
VI
VII
VIII
Developmental Stages

D.

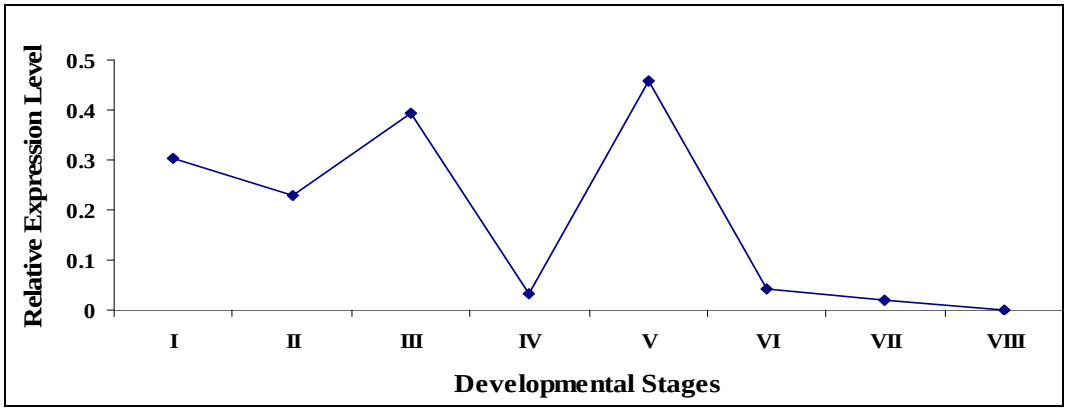
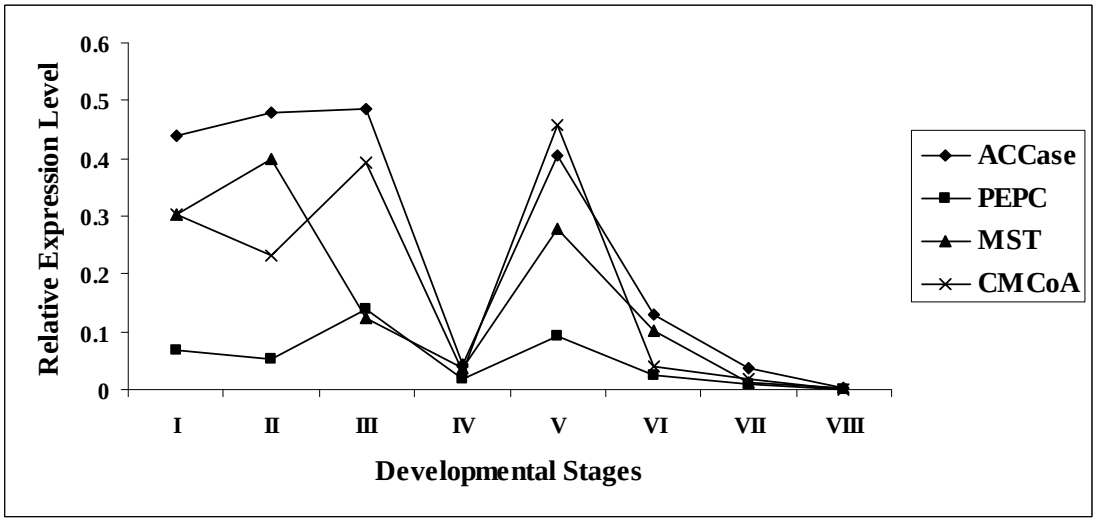

Relative Expression Level
0.5
0.4
0.3
0.2
0.1
0
I
II
III
IV
V
VI
VII
VIII
Developmental Stages

Figure 4.24 Comparison of relative expression level of four target genes



CHAPTER V

DISCUSSION

5.1 Fatty Acid Profiles Throughout the Eight Developmental Stages of *Jatropha curcas* Seed Kernel

According to Table 4.2, 12 free fatty acids including myristic, palmitic, palmitoleic, stearic, oleic, vaccinic, linoleic, linolenic, arachidic, 11-eicosenoic, eicosapentaenoic, and docosahexaenoic acids were found. Similar to the observations of Berchmans and Hirata (2008), *J. curcas* crude oil was consisted of both saturated fatty acids (14.1-15.3% palmitic acid and 3.7-9.8% stearic acid) and unsaturated fatty acids (34.3-45.8% oleic acid and 29.0-44.2 % linoleic acid).

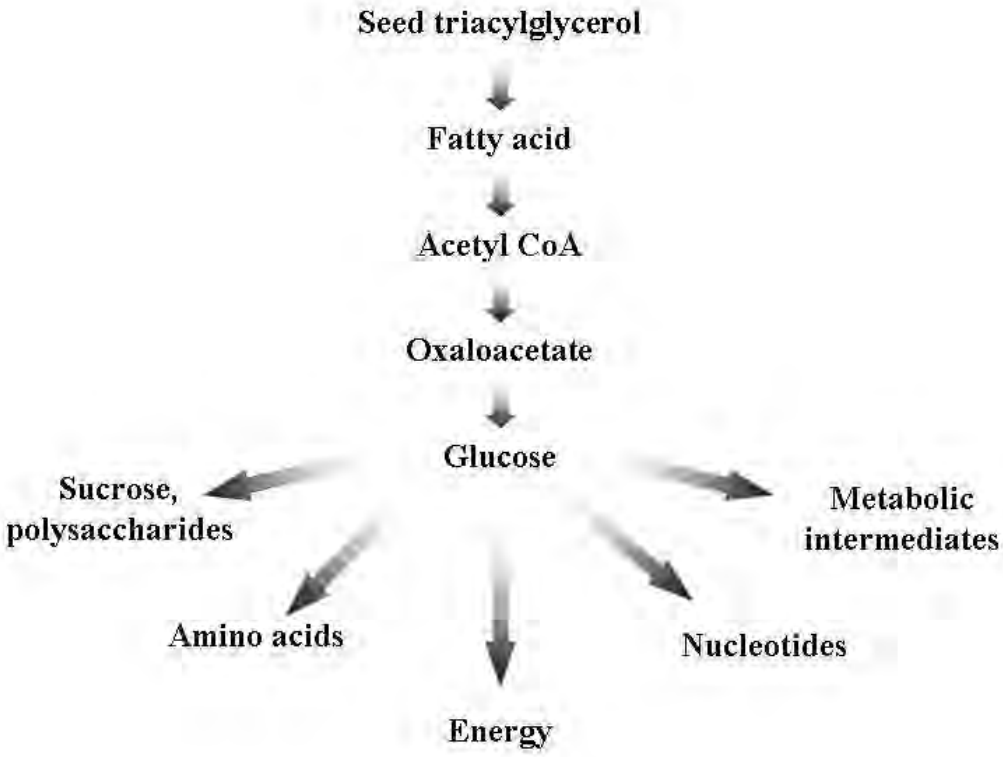
The major fatty acid was oleic acid and the minor fatty acid included linoleic acid, palmitic acid and stearic acid. Gunstone (2004) revealed that ideally the vegetable oil for used as biodiesel should have both low saturated and polyunsaturated fatty acids but high in monounsaturated fatty acid. This report implied that the properties of *J. curcas* seed oil in this study are suitable for biodiesel.

Annarao *et al* (2008) studied the lipid profiling of developing *J. curcas* seeds using NMR. They found that oil content and lipid profile at various stages of seed development starting from one week after fertilization and in an interval of five days thereafter till maturity were different. NMR spectroscopy of hexane extracts made at different stages of seed development revealed the presence of methyl esters of fatty acids (FAME). The young seeds synthesized predominantly polar lipids. Most of fatty acid content gradually increased from stage I to stage III (14-24 days after fertilization) but FAME formation decreased from stage IV to stage VI and increased again in stage VII, finally they decreased again in stage VIII. After three weeks of fertilization a turning point in seed development occurred because at this stage, there was a sudden decrease in free fatty acid concentration until maturity.

According to the result in Figure 5.1, the turning point of free fatty acid accumulation in our study was at stage IV because of a significant decrease of all free fatty acid content. It implied that at the stage IV of seed development, plant peroxisomes and glyoxysomes used acetyl CoA from β-oxidation as a biosynthesis precursor to synthesize glucose, sucrose and a wide variety of essential metabolites (Nelson and Cox, 2000.).

Based on the reason mentioned above, the best developmental stages for harvesting the oil product were stages III and VII because of high accumualtion of oleate and linoleate, the suitable fatty acids for biodiesel.

Figure 5.1 Role of β-oxidation in the conversion of stored triacylglycerols to glucose in plant seeds.



5.2 Protein Expression Pattern by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE is an alternative technique and the most widely used analytical method to separate components of a protein mixture. It is almost obligatory to access the purity of a protein through an electrophoretic method. SDS-PAGE simultaneously exploits differences in molecular sizes to resolve protein differing by as little as 1% in their electrophoretic mobility through the gel matrix (Scopes, 1994).

Before loaded into SDS-PAGE, protein concentration of each sample was determined by Lowry assay. This procedure is reliable, but it requires a lot of pipetting work and many buffer components can interfere (Larson *et al*., 1986; Legler *et al*., 1985; Markwell *et al*., 1978). Furthermore, color development reaches a maximum in 20-30 min, after which there is a gradual loss of signal at about 1% per hour (Peterson, 1977). Thus, preparation of all steps in this study was made in the same day to decrease errors.

Since separating protein was performed on mini-gel, protein pattern showed very closely spaced protein bands. One protein band could consist of many proteins because they were separated only by molecular sizes.

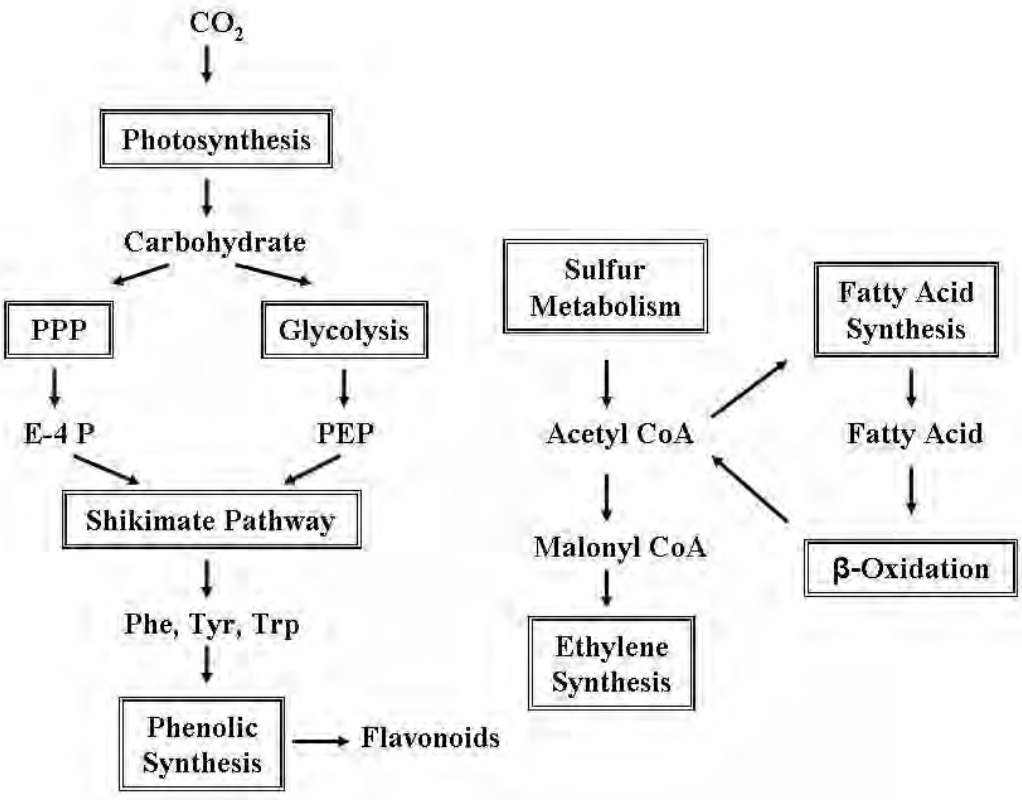
In addition, approximately 5 ng protein can be detected per band by silver staining (Merril *et al*., 1981). The Ag^{+} ion forms complexes with Glu, Asp and Cys residues of proteins. Alkaline formaldehyde reduces the Ag^{+} of the complexes to Ag (Moreno *et al*., 1984). The details of this reaction are unknown. However, this technique can be used to reduce the complexity of the proteins in samples before subjected to LC-MS/MS.

5.3 Protein Identification by LC-MS/MS

In this study, many high abundant protein bands from *J. curcas* seed kernel were present in the SDS-PAGE. The accuracy of protein identification by LC-

MS/MS depended on the availability of the protein or EST database. The incomplete nucleotide and protein databases of the *J. curcas* database would limit the success to identify *J. curcas* proteome. In this study, there were 245 differential expressed proteins that detectable by LC-MS/MS as demonstrated in Table 4.4. The overall metabolic processes during *J. curcas* seed development were concluded in sketch map as shown in Figure 5.2

Figure 5.2 A sketch map of the metabolic processes during seed development

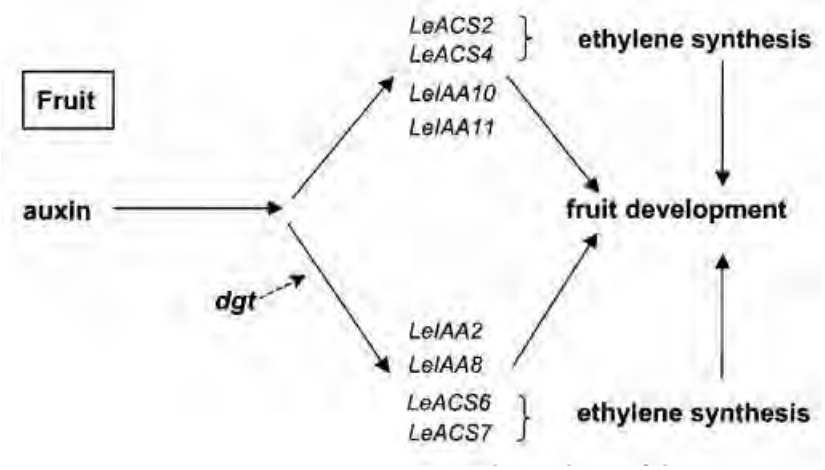


5.3.1 Ethylene Synthesis Related Proteins

According to the results in Table 4.5 and Figure 4.9, the differential expression level of ACC synthase 1 and 2 (ACS1 and ACS2) during seed development was observed. This result was similar to the report of Barry *et al*. (2000) showing the positive correlation between alteration of the specific members of the ACS and ACO gene families and the change of green to red color in tomato fruit. Based on their gene expression patterns, as well as their regulation by ethylene, the

ACS2 and ACS4 isoforms were proposed to mediate ethylene production through fruit ripening, whereas ACS6 and ACS7 functioned in green fruit and vegetative tissue (Nakatsuka *et al*., 1998). Therefore, ACS1 and ACS2 isoforms in our study may involve in ethylene production associated with ripening of *J. curcas* fruit because of high expression level in green fruit. The diagram of gene expression of *ACS* was shown in Figure 5.3

Figure 5.3 Diagram of gene expression of members of *IAA* and *ACS* (ACC synthase) gene families in tomato seedlings and fruit during fruit development (Balbi and Lomax, 2003).



5.3.2 Seed Development Related Proteins

Seed development in general is governed not only by a complex network of transcription factors that integrate external cues, e.g. light, but also internal signals such as the concentration of the plant growth factor abscissic acid or sugars (Brocard-Gifford *et al*., 2003; Finkelstein and Gibson, 2002; Finkelstein *et al*., 2002; Rolland *et al*., 2002). In this study, several differentially expressed proteins were found to relate to seed development (as shown in Table 4.6).

Flavonoids, the largest class of phenolic compounds that play an important role in plant metabolisms, regulated toxin transport and plant development (Brown *et al*., 2001).

According to Figure 4.2 and 4.10, it was noticed that the increase of protein related to flavonoid metabolism was related to the reduction of free fatty acid accumulation along seed development. These results were similar to the observations of Jun *et al*. (2009) that flavonoids have inhibitory effect on fatty acid synthase (FAS) activity in citrus fruit. Likely, the darkening (color change from green-yellow to brown or even black during fruit development) of olive fruit was related to the formation of polymers from the abundant natural phenolic compounds of olives (Panizzi *et al*., 1960).

Moreover, enzymatic modification of flavonoids and flavonoid glycosides were acylated by vinyl esters of fatty acids (Gao *et al*., 2001; Enaud *et al*., 2004). Being particularly interested in the regioselective enzymatic reparation of high value products, the application of these enzymes is expanded further so as to perform synthetic reactions (Coulon *et al*., 1996; Chamouleau *et al*., 2001) of compounds currently receiving much attention as “nutraceutical” components of the diet. It should be noted that, in all cases referred until now, reactions involved in the enzymatic acylation of flavonoids exclusively with saturated fatty acids as acyl donors.

The alteration of APETALA-containing domain (AP2) expression level in *J. curcas* seed kernel may indicate an involvement of AP2 in seed development as reported by many groups. Jofuku *et al*. (1994) reported that AP2 is expressed throughout flower development, in immature floral buds, in all four types of flower organ primordia, and in developing ovules and seeds. Many evidences showed that AP2 is also required for normal ovule and seed development (Leon-Kloosterziel *et al*., 1994; Modrusan *et al*., 1994). Moreover, Okamuro *et al*. (1997) reported that AP2 plays an important role in the control of *Arabidopsis* flower and seed development.

The Late Embryogenesis Abundant (LEA) proteins are the overwhelmingly hydrophilic proteins that accumulate to high level in the later stages of seed maturation and disappear following germination (Galau *et al*., 1986). While almost ubiquitous in the plant kingdom, data mining has revealed the widespread occurrence of LEA proteins in prokaryotes and eukaryotes (Arroyo *et al*., 2000). It has been

documented that a class of hydrophilic proteins expressed in late seed development, the so-called late embryo genesis abundant (LEA) proteins have been consistently associated with desiccation tolerance because of their expression profile and their hydrophilicity (Cuming, 1999). LEA proteins are classified in at least five groups by virtue of similarity in their amino acid sequences (Cuming 1999; Wise 2003). According to Table 4.6 and Figure 4.10, 2 isoforms of LEA proteins were found to be differentially expressed during seed development.

Another example was seed storage proteins which are about 50% of the total protein in mature seed. There are many kinds of storage proteins such as globulin, prolamin and plant hormones; gibberellin (GA) and abscissic acid (ABA) (Shewry and Halford, 2002).

5.3.3 Free Fatty Acid Biosynthesis Related Proteins

Jatropha curcas is a plant producing oil which is suitable for biodiesel. The proteins related to free fatty acid biosynthesis were of interest. In this study, many differential expressed proteins known to involve in free fatty acid biosynthesis were obtained. These included acetyl CoA carboxylase (ACCase), phosphoenol pyruvate carboxylase (PEPC), mercaptopyruvate sulfertransferase (MST) and 4-coumarate: coenzyme A ligase (CMCoA).

In the first step of fatty acid biosynthesis, the important input is acetyl-CoA, which is carboxylated to malonyl-CoA by acetyl-CoA carboxylase (ACCase). This reaction is believed to be a key regulatory step in fatty acid biosynthesis in animals, bacteria, and plants (Kim *et al*., 1989; Jackowski *et al*., 1991). Likewise, the expression level of ACCase was altered throughout seed development of *J. curcas*.

Furthermore, protein expression pattern of PEPC in *J. curcas* seed kernel was similar to a recent report of Sebei *et al*. (2006). The PEPC activity was monitored during seed maturation of rapeseed (*Brassica napus* L.). They discovered that the evolution of PEPC activity showed a classical curve, i.e. an increase during the most active phase of lipid accumulation in maturating seeds, followed by a rapid decrease

until the end of seed maturation. Moreover, the fatty acid profile was similar to that of PEPC activity. Taken together, their findings suggested that PEPC may involve in fatty acid biosynthesis during seed maturation of rapeseed.

MST and other sulfurtransferases are involved in biosynthesis of some sulfur-containing cofactors. The enzymology of C-S bond forming reactions involved in the biosynthesis of biotin, lipoic acid, thiamin, and molybdopterin. Biotin (vitamin H) is a water-soluble vitamin that can be produced by bacteria, plants and a few fungi. Biotin plays a very important physiological role as the prosthetic group of several carboxylases involved in central metabolism, including gluconeogenesis, fatty acid biosynthesis, and secondary metabolism (Nagahara and Nishino, 1996).

Another interesting protein is CoA ligase. Knights (1998) reported that the formation of acyl-coenzymes (Co) A occurs as an obligatory step in the metabolism of a variety of endogenous substrates, including fatty acids. The reaction is catalyzed by ATP-dependent acid: CoA ligases, classified on the basis of their ability to conjugate saturated fatty acids of differing chain lengths, short (C2-C4), medium (C4-C12) and long (C10-C22). The enzymes are located in various cell compartments (cytosol, smooth endoplasmic reticulum, mitochondria and peroxisomes) and exhibit wide tissue distribution.

5.3.4 Toxicity Related Protein

In addition to seed development and free fatty acid biosynthesis related proteins, an important toxicity related protein, curcin was also found to be expressed throughout developmental stages. Furthurmore, in *J. curcas* it has also been reported that the activity of curcin was similar to ricin, the specific toxic protein from castor bean that is capable of inhibiting protein synthesis (Stirpe *et al*., 1976).

5.4 DNA Sequence of Target Genes

According to Table 4.10, it was observed that the DNA sequences of UBQ, ACCase and PEPC genes that has been cloned in this experiment were nearly the same as *J. curcas* gene deposited in NCBI database (98, 100, 99 % identity, respectively).

Nevertheless, the *MST* showed high similarity to *Thiosulfate sulfurtansferase* (*TST*) of *Ricinus communicus* with the identity of 89%. This result was similar to Nagahara and Nishino (1996) which reported that the nucleotide sequence of rat liver *mercaptopyruvate sulfurtransferase* (*MST*) was very similar to rhodanese (*Thiosulfate sulfurtansferase*) in physicochemical properties with low identity (65%).

5.5 mRNA Expression and Free Fatty Acid Biosynthesis along Eight Developmental Stages of *Jatropha cucas* Seed Kernel

According to Figures 4.2 and 4.21, most of fatty acid production such as oleic acid, linoleic acid, palmitic acid and stearic acid were related to the expression level of all target genes; *acetyl CoA carboxylase* (*ACCase*), *phosphoenolpyruvate carboxylase* (*PEPC*), *mercaptopyruvate sulfurtransferase* (*MST*) and *4-coumarate: coenzyme A ligase* (*CMCoA*). Up-regulation of these genes at the early stages, down-regulation at stage IV, and up-regulation again at stage VIII were observed.

Figure 4.2 Free fatty acid concentration along developmental stages.

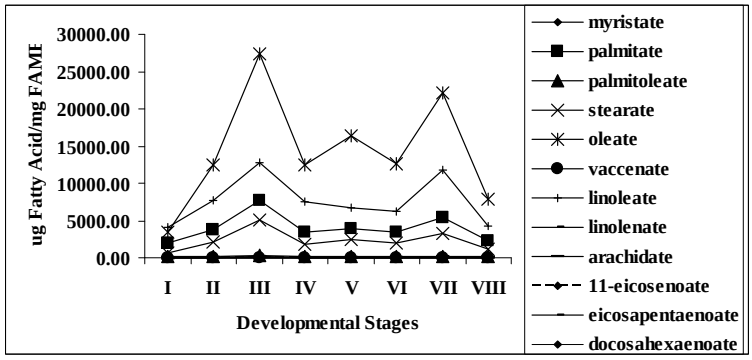
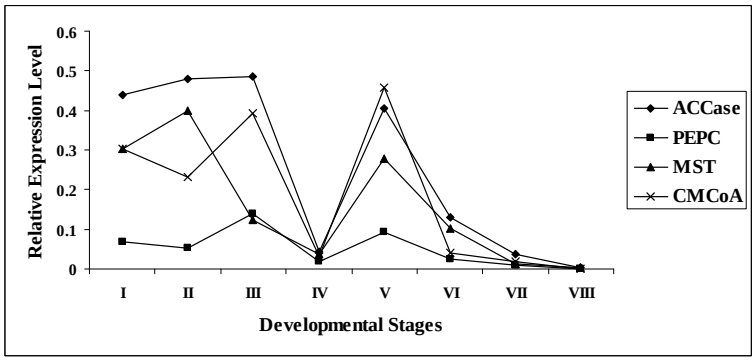
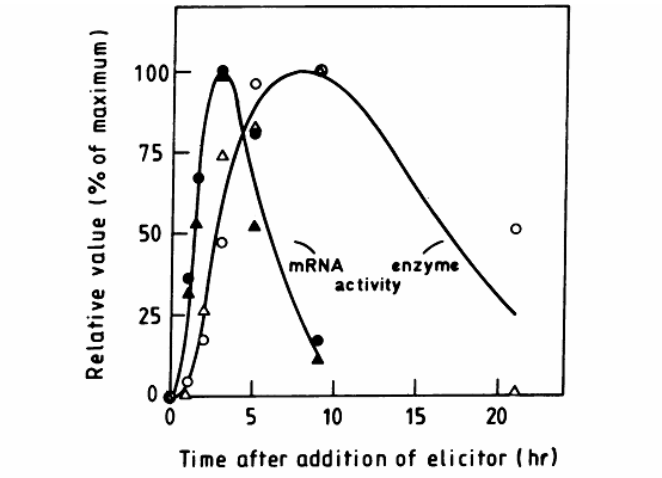


Figure 4.21 Comparison of relative expression level of four target genes.



Furthermore, according to the report of Kuhn *et al*. (1984), the kinetics of the relative changes in PAL (phenylalanine ammonia-lyase) and CMCoA (4-coumarate: CoA ligase) mRNA activities were identical within experimental error and coincided with previously determined changes in the rates of PAL synthesis *in vivo*, as measured by immuno-precipitation from extracts of pulse-labeled cells. The rapid changes in mRNA activity could account for the slower changes in PAL and CMCoA enzyme activities, as shown in Figure 5.4.

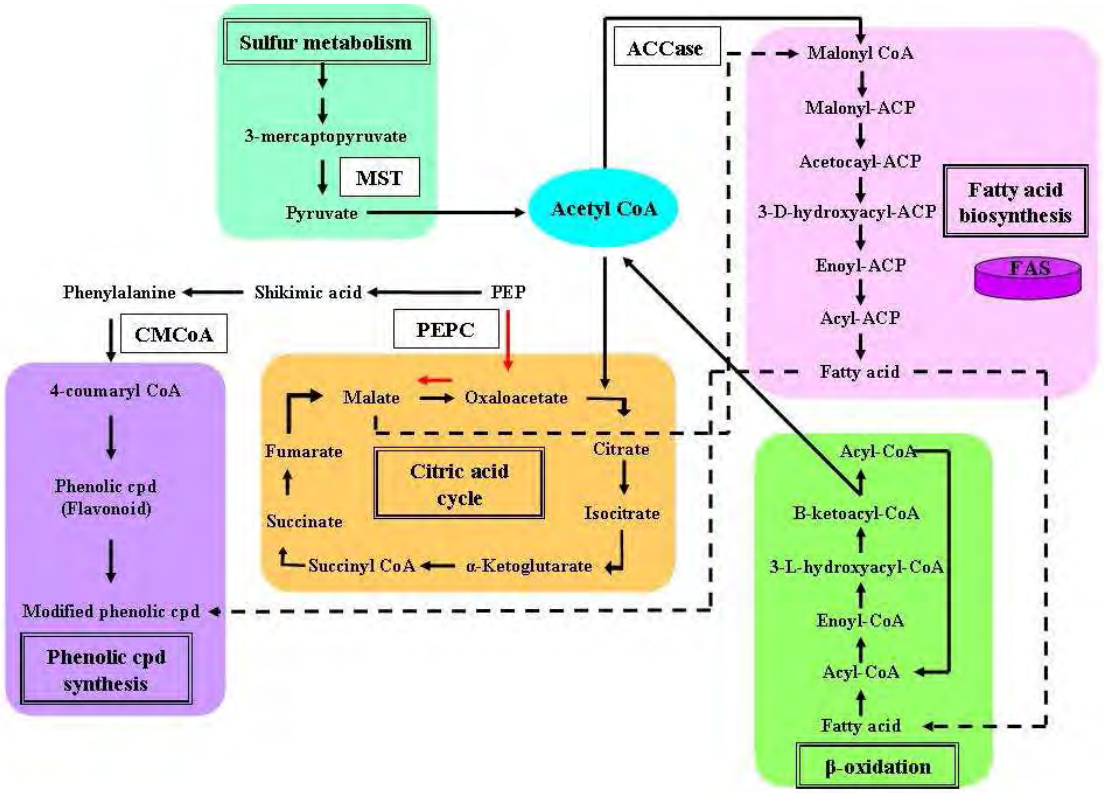
Figure 5.4 Elicitor-induced changes in enzyme and mRNA translational activities *in vivo* for PAL (○ , ●) and CMCoA (△, ▲). Cells were harvested at the indicated times. Data were plotted relative to the highest value for each mRNA or enzyme activity.



In our study, the rapid increase of most free fatty acids in Jatropha oil among eight developmental stages were associated with an increase in the accumulation of ACCase, PEPC, MST and CMCoA mRNAs. On the basis of these data, it can be concluded that the major control point in fatty acid accumulation during seed development is the transient increase in the amounts of mRNAs coding for the enzymes involved in fatty acid biosynthesis. Evidences suggested that these changes in mRNA amounts were due to increased rates of transcription of the genes encoding these enzymes.

Finally, on the basis of results in this study, a sketch map of the metabolic processes of fatty acid biosynthesis and other related metabolisms is shown in figure 5.5

Figure 5.5 A sketch map of the metabolic processes of fatty acid biosynthesis and other related metabolisms



CHAPTER VI

CONCLUSION

1. In *J. curcas* oil, maior fatty acid was oleic acid and minor fatty acids were linoleic acid and palmitic acid.

2. Free fatty acid profiles determined by GC-FID showed that the content of oleic acid and palmitic acid reached maximum at stage III and stage VII. Because dry weight of *Jatropha curcas* seed kernel increased along the seed maturation, stage VII was therefore the best for harvesting.

3. The 245 differentially expressed protein during 8 developmental stages of *Jatropha curcas* seed were identified and found to involve in several cellular processes including transcription, translation, glycolysis, ethylene metabolism, and free fatty acid biosynthesis.

4. The 22 differentially expressed protein during maturation of *Jatropha curcas* seed found to involve in free fatty acid biosynthesis were lipase, mitochondrial carrier protein, s-related kinase, branched-chain-amino acid aminotransferase, glycosyl hydrolase, phosphoenolpyruvate carboxylase, dormancy-associated protein, sucrose transpoter, animal-type fatty acid synthase, pr1-like protein, lecithine cholesterol acyltransferase, mercaptopyruvate sulfurtransferase, beta-keto acyl reductase, serine acetyl transferase, acetyl coenzyme A carboxylase, receptor-like protein kinase, ras-GTPase-activating protein SH3 domain, 4-coumarate: coenzymeA ligase, and putative short chain alcohol dehydrogenase.

5. Quantitative Real-time RT-PCR of genes involved in the biosynthesis of free fatty acids including included acetyl CoA carboxylase (ACCase), phosphoenolpyruvate carboxylase (PEPC), mercaptopyruvate sulfurtransferase (MST) and 4-coumarate: coenzyme A ligase (CMCoA) revealed that the relative mRNA expression level of

each gene was altered in a similar pattern with regard to the amount of most free fatty acids in *Jatropha curcas* seed kernel.

.6. The decreased level of free fatty acids from stage IV to stage VI may caused by β-oxidation metabolism to generate energy for other metabolisms. Additionally, intermediate substances from β-oxidation process may be used in flavonoid biosynthesis.

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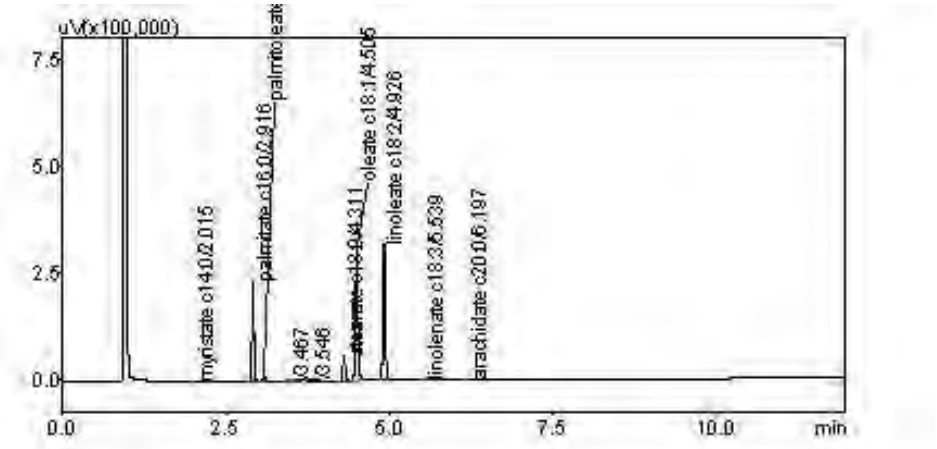
APPENDICES

Appendix A

Fatty Acid Profiles from GC

Stage I



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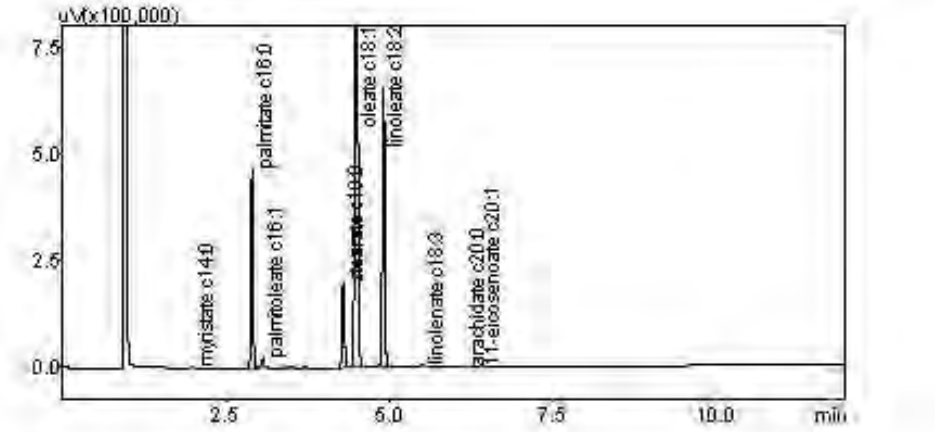

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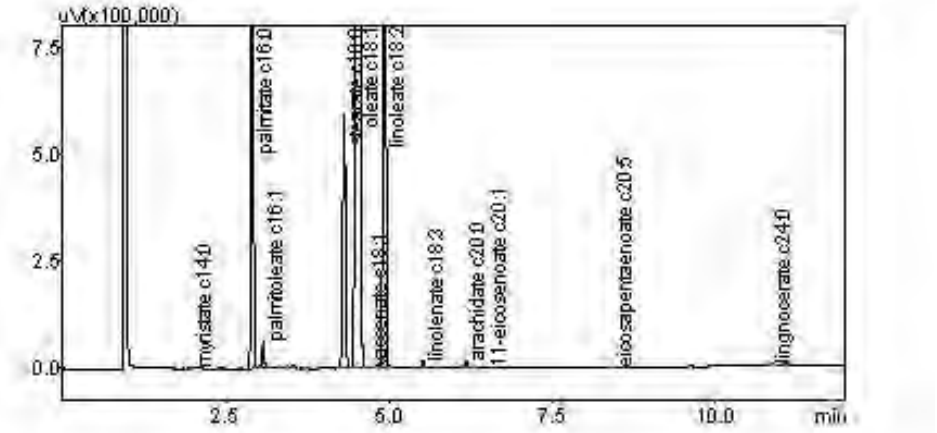

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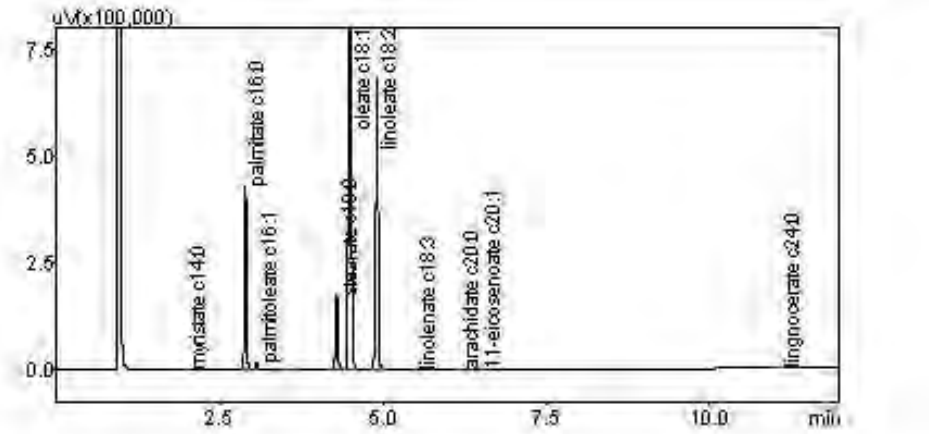

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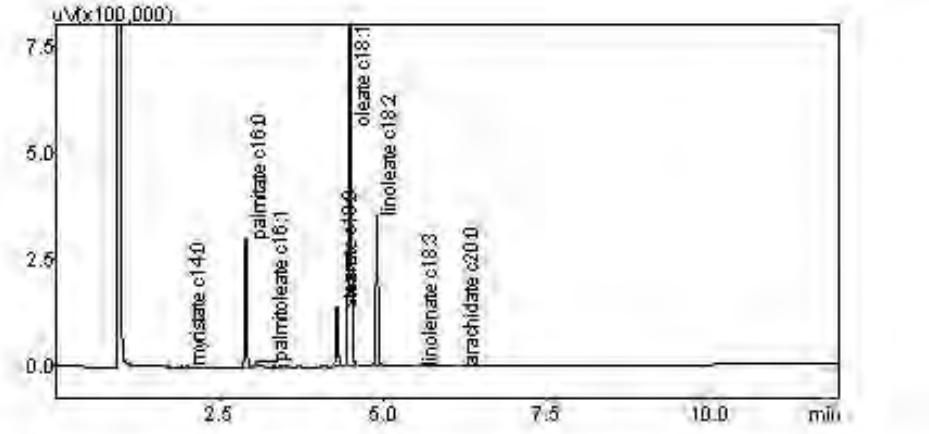

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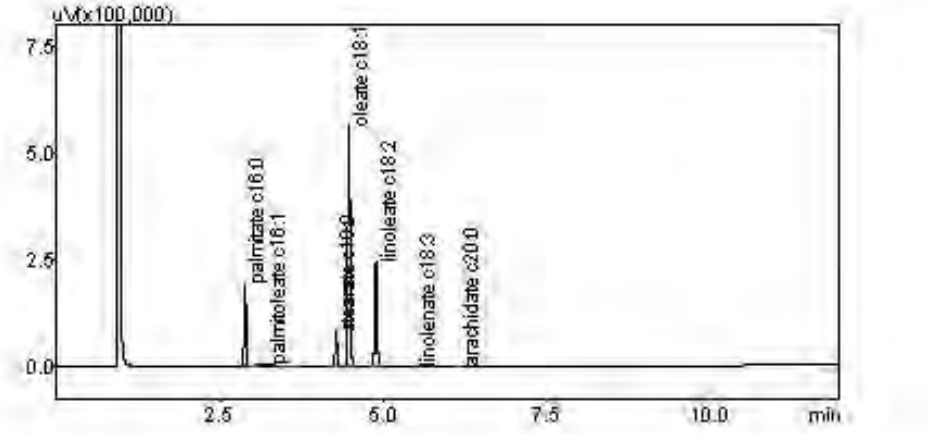
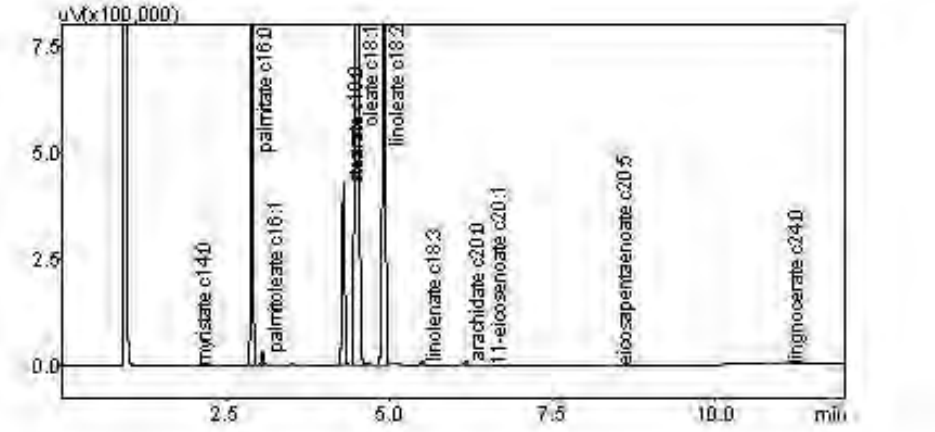
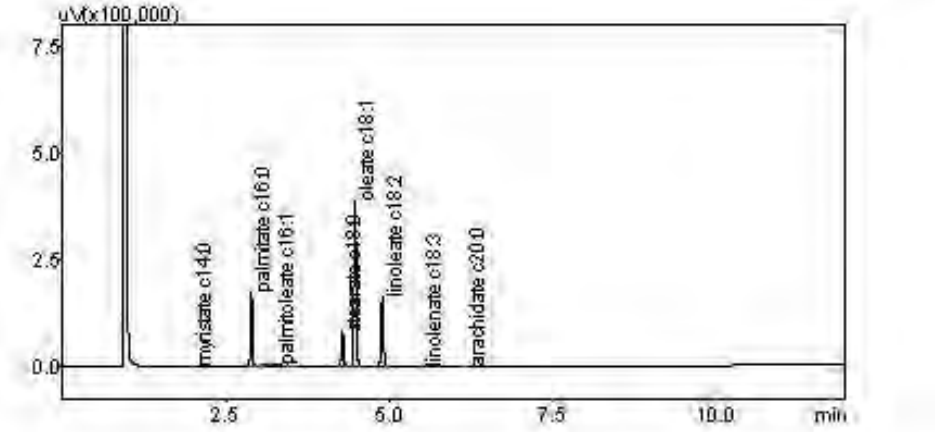


Stage VII



Stage VIII



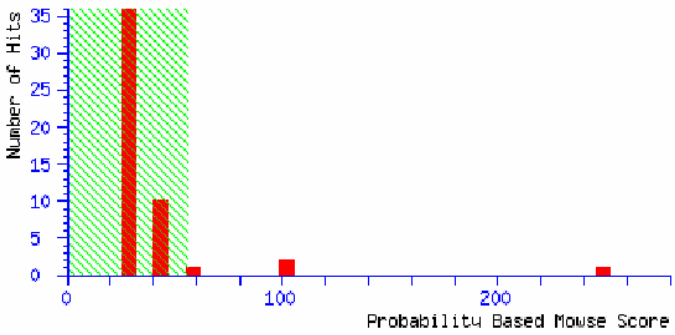
Appendix B

Identification of BSA Standard from LC-MS/MS Analysis

The average MOWSE Score of BSA standard is 238.75

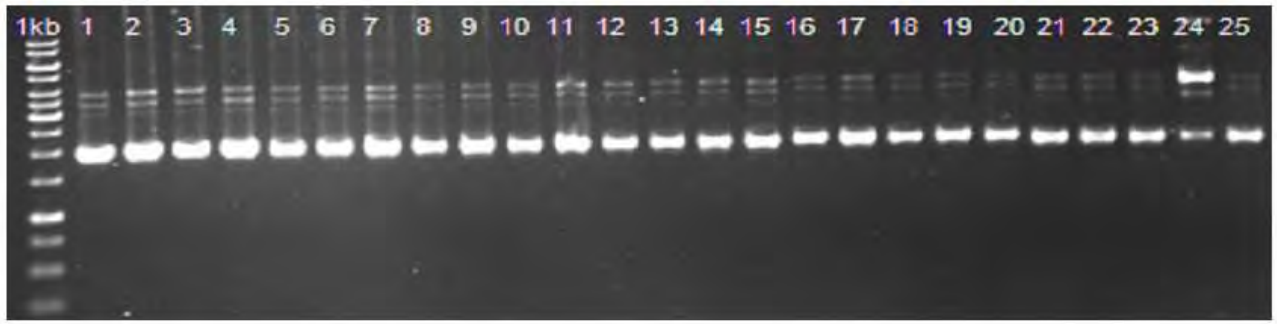
Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 56 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



Appendix C

The Ethidium Bromide Stained 1%Agarose Gel Electrophoresis for Quantification of Recombinant Plasmids before Nucleotide Sequencing



Volume of DNA ladder/ sample loaded per lane: 1uL each

1kb DNA Ladder (bp): 250, 500, 750, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 5000, 6000, 8000, 10000

1kb DNA Ladder (ng/0.5ug): 25, 25, 25, 60, 25, 25, 25, 70, 30, 30, 30, 70, 30, 30, 60, 70, 70

Note: The DNA ladder is not applicable for sizing comparison of non-linear DNA samples (e.g. plasmid DNA)

Lane 1-5 were Ubiquitin (UBQ) recombinant plasmid

Lane 6-10 were Acetyl CoA carboxylase (ACCase) recombinant plasmid

Lane 11-15 were Phosphoenol pyruvate carboxylase (PEPC) recombinant plasmid

Lane 16-20 were Mercaptopyruvate sulfertransferase (MST) recombinant plasmid

Lane 21-25 were 4-Coumarate: Coenzyme A ligase (CMCoA) recombinant plasmid

Appendix D

Copy Numbers Calculation of Real-time PCR

A serial dilution of linearized plasmid DNA is used to generate a standard curve for Real-time PCR. The size of the plasmid that contains the gene of interest can be used to calculate the number of grams/molecule also known as copy number as follows:

Weight in Dalton (g/mol) = (bp size of plasmid + insert)(330 Dax2 nucleotide/bp)

Ex. 3927000 g/mol / Avogadro's number (6.02×10^{23}) = 6.52×10^{-18} g/molecule

The copy number of plasmid and the concentration of the plasmid added to each PCR reaction can be used to determined the precise number of molecule in that reaction as follow:

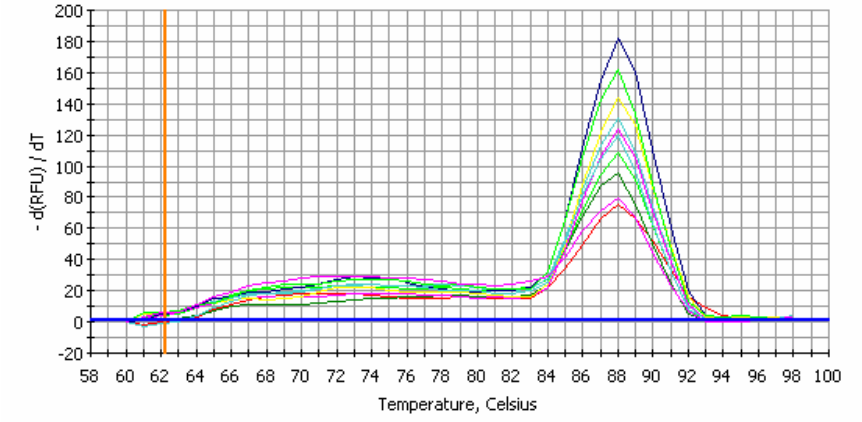
Concentration of plasmid (g/µl) / copy number

Ex. (3×10^{-7} g/µl) / (6.52×10^{-18} g/molecule) = 4.6×10^{10} molecule/ µl

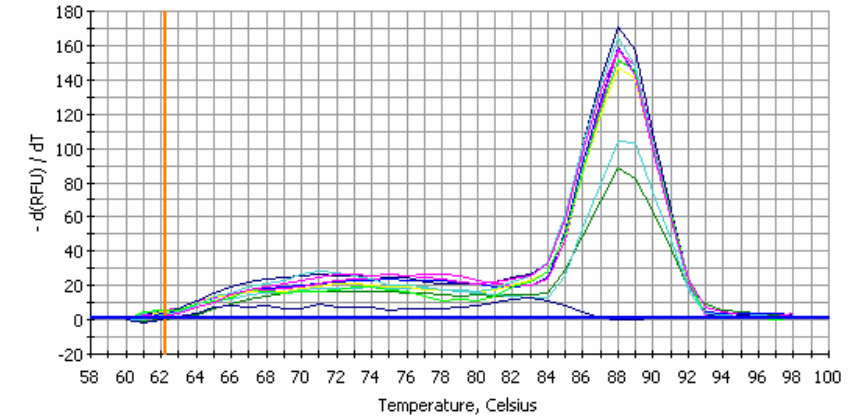
The number of molecules in one µl of linearized plasmid, a series of dilutions can be made for subsequent amplification allowing one to generate a standard curve. For the standard curve, the copy number of the unknown samples can be derived.

Appendix E

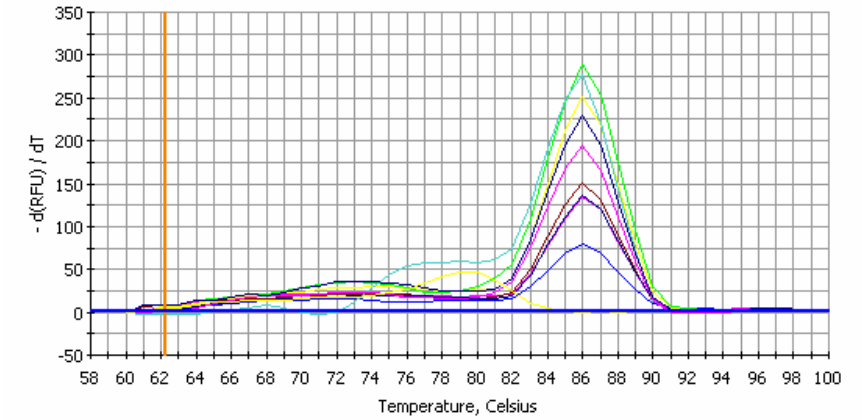
Melting curve graph of UBQ standard



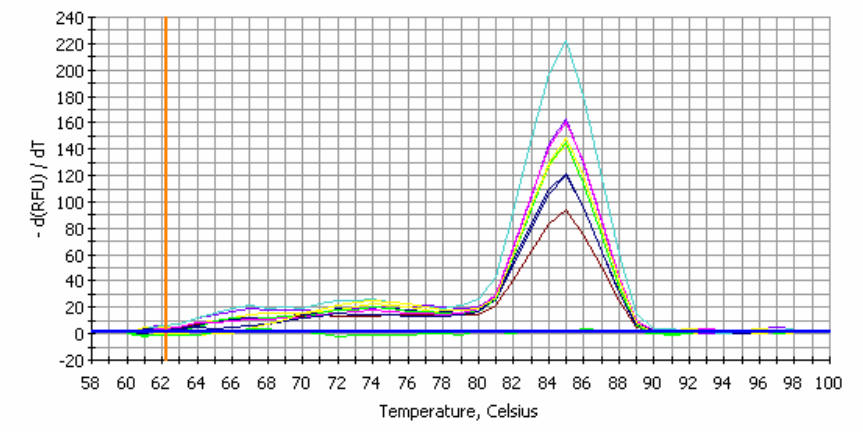
Melting curve graph of ACCase standard



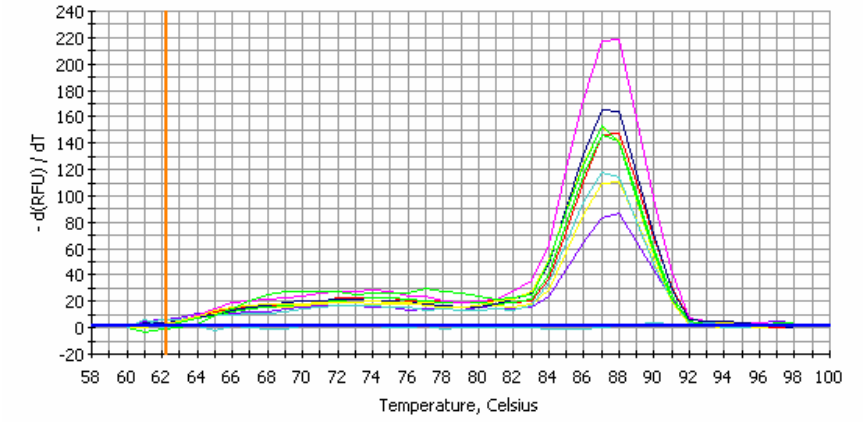
Melting curve graph of PEPC standard



Melting curve graph of MST standard



Melting curve graph of CMCoA standard



BIOGRAPHY

Miss Thitiporn Booranasrisak was born on May 13, 1984 in Chachoengsao. She graduated with the degree of Bachelor of Science from the Department of Genetics, Kasetsart University in 2005. She has enrolled a Master degree program at the Program in Genetics, Chulalongkorn University since 2008.

Publications related with this thesis

Booranasrisak, T., Roytrakul, S. and Chulalaksananukul, W. (2009) Study of fatty acid and protein profile in developmental stages of *Jatropha curcas* seeds. The 3rd Conference on Creative Economy, 28-29 January 2009, Silpakorn University, Nakhon Pathom, Thailand (Poster presentation).