EXPRESSION OF SIX CHLOROPLAST DNA GENES IN *Jatropha curcas* CALLUS UNDER LIGHT AND DARK CONDITIONS

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DECLARATION

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ABSTRACT

EXPRESSION OF SIX CHLOROPLAST DNA GENES IN *Jatropha curcas* CALLUS UNDER LIGHT AND DARK CONDITIONS

The expression of genes encoded in the open reading frames of chloroplast genomes have been posited to be induced by light. The current study focused on the induction of ORFs encoded in the chloroplast genome of *Jatropha curcas* (Accession number FJ695500). *J. curcas* is an important non-edible oil seed crop, which produces oil with high calorific value and is regarded as a potential fuel substitute. By understanding the gene expression of chloroplast DNA under light stress, potential genes candidate can be used for callus transformation. Gene induction was characterized in leaves of the plant, green callus and white callus cultivated under condition of light and darkness. A total of six ORFs representing the genes *YCF* 1, *YCF* 2, *psbD* (photosystem II), *rbcL* (Rubisco), *matK* (Maturase K) and *rpoC1* (RNA polymerase) were targeted by designing specific primers for their characterization. Specificity of primers was tested against the genomic DNA. Transcripts of six targeted genes were detected in all three replicates of the green and white callus under light and dark, except for *ycf* 2 gene in green callus under light. A part of gene *ycf* 2 was no transcribed using reverse transcription PCR, was then validated using real-time PCR assay. The reverse transcription PCR did not detect the gene amplified by YCFD primer in the green callus under photoperiod treatment. The *ycf* 2 gene located in the region of region 94050 to 95483 was post-transcriptional modified. In the real-time PCR assay, the amplification curves were produced from the amplification of all callus under different treatments, except for green callus under light.

ABSTRAK

Ekspresi gen dikodkan dalam 'opening reading frames' (ORFs) dari genom kloroplas telah posited diinduksi oleh cahaya. Penyelidikan ini memfokuskan pada induksi ORFs dalam genom kloroplas Jatropha curcas (Aksesi nombor FJ695500). J. curcas adalah non-minyak biji tanaman yang penting dan menghasilkan minyak dengan kalori tinggi dan juga dianggap sebagai pengganti bahan bakar yang berpotensi. Dengan memahami gen DNA kloroplas di bawah tekanan ringan, potensi gen calon boleh digunakan untuk transformasi kalus. Gene induksi dipercirikan dalam daun tanaman, kalus putih dan kalus hijau ditanamkan dalam keadaan cahaya dan kegelapan. Sebanyak enam ORFs mewakili gen YCF 1, YCF 2, psbD (fotosistem II), rbcL (Rubisco), matK (Maturase K) and rpoC1 (RNA polymerase) manjadi sasaran untuk merancang primer khusus untuk ciri-ciri gen tersebut. Spesifisitas primer diuji terhadap DNA genom. Transkrip enam gen yang disasarkan telah dikesan di semua kalus hijau dan putih di bawah terang dan gelap, kecuali bagi kalus hijau di bawah cahaya.Sebahagian dari gen YCF 2 yang diuji dengan PCR konvensional telah 'swith off', kemudian disahkan menggunakan PCR 'real-time'. PCR konvensional tidak mengesankan gen diamplifikasi dengan primer YCFD dalam kalus hijau bawah fotoperiodik. Transkripsi berbalik PCR tidak mengesan gen yang dikuatkan oleh primer YCFD dalam kalus hijau di bawah rawatan photoperiod. Ycf 2 gen yang terletak di rantau rantau 94050 untuk 95483 pasca-transkripsi diubahsuai.Dalam uji PCR real-time, kurva amplifikasi dihasilkan dari amplifikasi di semua kalus bawah perlakuan yang berbeza, kecuali untuk kalus hijau di bawah fotoperiode.

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%	percent
°C	degree of Celsius
µg/ml	microgram per milliliter
µl	microliter
BLASTn	basic local alignment search tool for nucleotide
bp	base pair
$CaCl_2$	calcium chloride
cDNA	complementary DNA
dNTP	deoxynucleoside-5'-triphosphate
E. coli	*Escherichia coli*
g	gram
h	hour
HCN	hydrogen cyanide
kb	kilo base
kg	kilogram
LB	Luria Bertani
matK	maturase
M	molar
mg/ml	milligram per milliliter
mg	milligram
$MgCl_2$	magnesium chloride
ml	milliliter
mm	millimeter
mM	millimolar
ORF	open reading frame
PCR	polymerase chain reaction
pmol/µl	picamole per microliter
RNA	ribonucleic acid
rbcl	rubisco
rcf	g-force
rpoC	RNA polymerase beta' chain
rpm	resolution per minute
s	seconds
SDS	sodium dodecyl sulphate
Ta	annealing temperature
TE	tris-ethylenediaminetetraacetic acid buffer
V	voltage
xg	times gravity
ycf	hypothetical chloroplast open reading frame

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CHAPTER 1

INTRODUCTION

1.1 Introduction to *Jatropha*

Jatropha is a genus of approximately 175 succulent plants, shrubs and trees, from the family Euphorbiaceae. The *Jatropha* species includes *Jatropha cuneata, Jatropha curcas, Jatropha gossypiifolia, Jatropha podagrica* and others. Recently, Goldman Sachs cited *J. curcas* as one of the best candidates for future biodiesel production (Barta, 2007). It is resistant to drought and pests, and produces seeds containing 27 to 40% oil. Unlike other biodiesel crops, *Jatropha* can be grown almost anywhere, including deserts, trash dumps, and rock piles. It does not need much water or fertilizer, and it is not edible.

The fact that *Jatropha* oil cannot be used for nutritional purposes without detoxification makes its use as energy or fuel source very attractive as biodiesel. In Madagascar, Cape Verde and Benin, *Jatropha* oil is used as a mineral diesel substitute during the Second World War (Akbar *et al.*, 2009).

In 2007, the Malaysian government cooperated with the Plantation Industries and Commodities Ministry and launched a *Jatropha* pilot project in Kota Marudu, Sabah. The ministry pledged to expand cultivation, especially in poorer areas, if the crops proved to be viable. Besides Malaysia, *Jatropha* projects have also been piloted in China, the Philippines, Rwanda, and the United State of America. In addition, the Indian Government implements the cultivation of *Jatropha* plants as the increased of *Jatropha* oil production delivers economic benefits to India at the macroeconomic or national levels as it reduces the nation's fuel import bill for diesel production.

1.1.1 Challenges for Commercial Scale *Jatropha* Plantation

The cultivation of *Jatropha* faces several challenges. *Jatropha* encounter various disease problems, such as the spider mite, white fly and the mealy bugs, especially on the surface of the leaves. It is reported that, in some areas of Zimbabwe, the golden flee beetle (*Podagrica* spp) can harm *Jatropha* and plays host to the 'frog eyes' fungus (*Cercospera* spp) (Openshaw, 2000).

Beside disease, toxicity of *Jatropha* is another challenge faced. The main toxic compounds are curcin, a protein that synthesis inhibitor, and phorbol ester (cancer promoting compound). Phorbol esters usually are ubiquitous while curcin stays mostly in seeds (Rakshit *et al.*, 2008). However, this is not a significant problem in traditional planting and uses. More concerns should be emphasized for large scale farming, for example the safety of workers, safety of grazing animals, possible adulteration of edible oil and other ecological concerns.

The key challenge for building *Jatropha* oil markets is coordinating feedstock supply and demand when neither exists for lack of the other. The smallholder farmers are unwilling to take risk of paying for, planting, and maintaining *Jatropha* trees. Nonetheless, the refiners are unwilling to make longer-term investments in refining capacity unless they have a secure source of adequate supply. Therefore, by developing institutional innovation, including contractual arrangements, fiscal and other incentives, blend ratio requirements, and other policy tools can overcome the obstacles in *Jatropha* biodiesel markets. Institutions will also be the key in determining the fairness of and allocation of risk in these markets (Weyerhaeuser *et al.*, 2007).

Uncertainty in how much *Jatropha* will cost to grow and the process of *Jatropha* into biodiesel seems to become another challenge for the exploitation of *Jatropha*. Some of this uncertainty is undoubtedly related to scale; once *Jatropha* growing begins on a commercial scale, costs are likely to fall at some level. However, increasing *Jatropha* acreage does not guarantee improvements in oil content and seed yields. If these remain low, unit production costs for *Jatropha* and thus the subsidies required to make *Jatropha* biodiesel cost competitive will remain high (Weyerhaeuser *et al.*, 2007).

1.1.2 Future Perspective of *Jatropha curcas*

The development of *J. curcas* into a high-yielding and efficient new biofuel source is still at a relatively early stage. Therefore, in order to ensure the market of *J. curcas* moves forward, some tactical strategies need to be taken. For example, enhance priority in breeding either the traditional or molecular and distribution of uniform and superior planting materials. One of the key short to medium term research objectives is to search superior species aimed at higher overall yields of *Jatropha*. This will be achieved through systematic seed selection from different regions and supported by a scientific seed-breeding programme (Achten *et al.*, 2010). Other than that, more researches need to be carried out to address ecological concerns. Tree propagation techniques for specific climates and a wide variety of environmental conditions have to be refined (Vries, 2007).

1.2 Significance of Study

Now, not many studies have been done on the expression of genes encoded in the open reading frames of *J. curcas* chloroplast genome. In order to carry out preliminary study on the differential expression of chloroplast genes under condition of photoperiod and darkness, a total of seven ORFs representing the genes *ycf* 1, *ycf* 2, *psaB* (photosystem I), *psbD* (photosystem II), *rbcL*(RubisCO), *rpoC1* (RNA polymerase) and *matK* (Maturase K) were targeted by designing specific primers for their characterization. These primers were tested against transcriptome under different environment conditions. The molecular perspective of understanding the photosynthesis of *J. curcas*, is the key to productivity, hence, the regulatory mechanism of the gene responsible for photosynthesis is vital for future research work involving *Jatropha*. In addition, by studying the gene expression of chloroplast DNA of *J. curcas*, potential genes response to light stress can be potential candidate for callus transformation and become molecular marker for callus regeneration.

1.3 Objectives

The objectives of this study are:

a. To identify the chloroplast genes which are induced under conditions of light and complete darkness in *Jatropha curcas.*

b. To validate expression of genes using real-time PCR.

1.4 Research Approach

The research approach involves the callus culture conditions, RNA extraction, amplification and gel extraction or cloning. One of the approaches, which is through the design of conserved or gene specific primers which will target the transcripts to be induced by light and dark condition. Validation of gene expression was carried out using quantitative real-time PCR.

CHAPTER 2

LITERATURE REVIEW

2.1 *Jatropha curcas*

J. curcas (Figure 2.1), which belongs to the Family Euphorbiaceae have different local names, for example Barbados Nut, Jarak pagar (Indonesia), Physic Nut and Puring Nut. The genus name of Jatropha derives from the Greek *jatrós* (doctor), *trophé* (food), which implies medical uses. Curcas is the common name for physic nut in Malabar, India (Hanna-Jones and Csurches, 2008).

Figure 2.1: *Jatropha curcas* plant (source:http://www.lijunoilacnepimple.com /lijun01/jatrophaE.htm

2.1.1 Habitat and Ecology

The plant is native to the American tropics, mostly in Mexico and Central America. Currently it is cultivated in almost all tropical and subtropical countries as protection hedges gardens and fields (Verm and Gaur, 2009).

Jatropha is a poisonous bush or small tree, which can reach the height of five meters and grows well with more than 600 mm of rainfall per year and withstands long drought periods (Verm and Gaur, 2009).

2.1.2 Botanical Characteristics

J. curcas's branches exude whitish colored watery latex, upon cut. It has yellow-green flowers and large heart-shaped (pale) green leaves, arranged alternately. The inflorescence is formed in the leaf axel while the flowers are formed terminally, individually, with female flowers usually slightly larger (Verma and Gaur, 2009).

After pollination, a trilocular ellipsoidal fruit is formed. The exocarp remains fleshy until the seeds are mature. The seeds are black and in the average 18 mm long and 10 mm wide. The seed weight (per 1000) is about 727 g, this are 1375 seeds per kg in the average (Verma and Gaur, 2009). Different plant parts of *J. curcas* are shown in Figure 2.2.

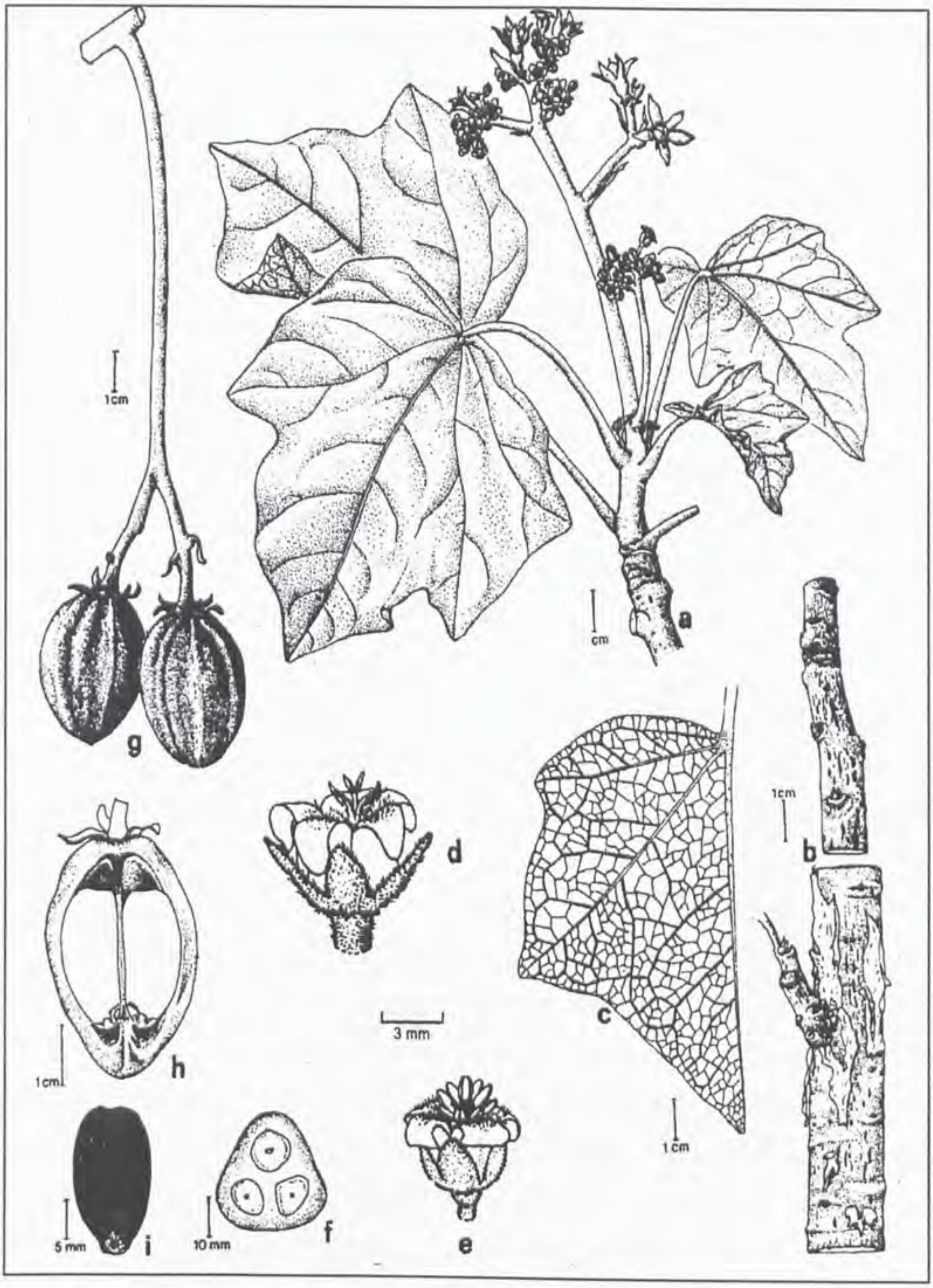

Figure 2.2: Different plant parts of *Jatropha curcas*; (a) whole plant, (b) bark, (c) leaf, (d) flower, (e) flower bud, (f) internal of fruit (g) fruits
(Source: Verma and Gaur, 2009)

2.1.3 Importance of *J. curcas*

J. curcas is considered as an ideal plant for wetland and its non-edible oil is a prime choice for biodiesel production. Different parts of *J. curcas* have traditionally been used for various purposes and have considerable potential. The importance of *Jatropha* can be categorized into two major parts which are fuel use and non-fuel use.

The oil from *Jatropha* is regarded as a potential fuel substitute, with a hydrocarbon of 16 to 18 carbon atoms per molecule. Compared with diesel oil, *Jatropha* biodiesel molecules are simple hydrocarbon chains, containing no sulphur, or aromatic substances associated with fossil fuels. They contain higher amount of oxygen (up to 10%) that ensures more complete combustion of hydrocarbons. The first successful trial run of a passenger train was conducted on December 31, 2002 when the Delhi-Amritsar Shatabdi Express used 5% biodiesel as fuel (Pahl, 2008).

The fuels can be obtained directly from different parts of *Jatropha* plant, such as wood, the whole fruit and parts of the fruit burnt separately or in combination, namely the exocarp (coat), the nut shell and the kernel (Openshaw, 2000).

Also, *Jatropha* could be used as a substitute for wire fencing for posts round fields, along roadsides and railway tracks. In countries like Zimbabwe, Mali, *Jatropha* is mostly used by farmers as fences around their homesteads and gardens, sometimes also around their fields, to protect crops against roaming animals. In addition, by planting *J. curcas,* it functions to reclaim eroded land and *Jatropha* hedges and shelterbelts can assist other crops on all land types by keeping out animals, improving the microclimate and providing humus to the soil (Openshaw, 2000).

Jatropha seeds contain fatty acid or viscous oil and one of its uses is as a raw material for soap making in cosmetic industries. In India, it is used by a large industry (Hindustan Lever). In Zimbabwee, there is a high demand of *Jatropha* oil although soap is produced by small informal industries in rural areas (Openshaw, 2000).

According to Osoniyi and Onajobi (2003), *J. curcas* is a medicinal plant, traditionally used as a haemostatic. Some of the ethnomedical uses of the extracts of the leaves and roots are remedy for cancer, as an abortifacient, antiseptic, diuretic, and purgative. The nut of plants has also been used traditionally for the treatment of many ailments including burns, convulsions, fever and inflammation. Esimone *et al.* (2009) demonstrated a significant wound-healing activity in *J. curcas* extracts by applying the latex directly, which is an alkaloid known as jatrophine, or the crushed leaf of the plant to cuts and bleeding wounds.

The ashes of *J. curcas* can also be used as salt substitute, whereas the bark which contains HCN, can be used as fish poison. Besides that, the latex can strongly inhibit the watermelon mosaic virus, which is a kind of virus that can infect all commercial cucurbit crops. In Mexico, the people grow the shrub as a host for the lac insect, which is used in medicine as hepatoprotective and antiobesity drug and also for erosion control (Tee, 2009).

2.2 Callus

Evans *et al.* (2003) defined callus as an amorphous mass of unorganized thin-walled parenchyma cells. Callus is formed at the cut surfaces when the plant is wounded. It is thought to be a protective response by the plant to seal off damaged tissue. According to Purohit (2003), callus is the results when a cell from any part of the plant, such as shoot apex, bud, leaf, mesophyll cells, epidermis, cambium, anthers, pollen or fruit is inoculated in a suitable medium under aseptic laboratory conditions. They are able to differentiate and survive.

Callus varies widely in its general appearance and in other physical features. The variation usually depends on the parent tissue, the age of the callus and the growth conditions. Callus can be in white, green or highly coloured due to the presence of anthocyanin pigments (Evans *et al.,* 2003).

2.2.1 Media and Culture Environment of Callus

Culture media used for the *in vitro* cultivation of plant tissues are composed of three basic components: (i) essential elements, or mineral ions, supplied as a complex mixture of salts; (ii) an organic supplement supplying vitamins and/or amino acids; and (iii) a source of fixed carbon which is usually supplied as the sugar sucrose. The composition of the nutrient solution plays a key role in plant nutrition. A defined nutrient medium consists of inorganic salts, a carbon source, vitamins and growth regulators. Generally, the media can be divided into micronutrients and macronutrients which are necessary for plant tissue or cell culture.

The MS medium was published in 1962 (Murashige and Skoog, 1962) and was used to support rapid growth of tobacco tissue culture and it is the most widely used salt composition, especially in procedures where plant regeneration is the objective. There are over 20 variants based on MS basic medium embracing a large number of specialty cultures (Evans *et al.* 2003). The B5, N6, Nitsch and Nitsch, and derivatives of these media have been applied widely for many plant species and for different objectives.

When cultured *in vitro*, all the needs, both chemical and physical, of the plants cells have to be met by the culture vessel, the growth medium has to supply all the essential mineral ions required for growth and development. In many cases, it must also supply additional organic supplement such as amino acid and vitamins. Many plant cell cultures, as they are not photosynthetic, also require the addition of a fixed carbon source in the form of sugar, for example sucrose. Physical factors, such as temperature, pH, the gaseous environment, light in the term of quality and duration, and osmotic pressure, also have to be maintained within acceptable limits (Vasil, 1994).

2.2.2 Advantages of Callus Culture

The callus culture is a technique of tissue culture, which is usually carried out on solidified gel medium in the presence of growth regulators and initiated by inoculation of small explants. Callus cultures are being widely used for study of expression of race-specific and non-host resistance and offer several advantages

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