Curcin gene promoters in Jatropha (*Jatropha curcas L.*): sequence analysis, prediction of *cis*-regulatory motifs, and studies on amplicon profile

A Dissertation

Submitted in partial fulfillment of the requirement for the award of degree of

Masters of Technology
in
Biotechnology

By

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July 2016

CANDIDATE'S DECLARATION

I, hereby declare that the work which is being presented in the thesis entitled, "Curcin gene promoters in Jatropha (*Jatropha curcas L.*): sequence analysis, prediction of *cis*-regulatory motifs, and studies on amplicon profile" in the partial fulfillment of the requirement for the award of degree of Master of Technology in Biotechnology, Thapar University, Patiala, is an authentic record of my own research work carried out under the guidance and supervision of **Dr. N. Das**, Professor, Department of Biotechnology, Thapar University, Patiala, India. The matter embodied in this dissertation has not been submitted to any other university or institute for award of any other degree.

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Date: 15th July, 2016

CERTIFICATE

This is to certify that the dissertation entitled "Curcin gene promoters in Jatropha (*Jatropha curcas L.*): sequence analysis, prediction of *cis*-regulatory motifs, and studies on amplicon profile" submitted by **Preeti Khokhar** (Regd. No. 601404016) in partial fulfillment of the requirement for the award of the degree of Master of Technology in Biotechnology, to Thapar University is a record of student's own work carried out by her under our guidance and supervision. The report has not been submitted for the award of any other degree or certificate in this or any other university or institute.

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ABBREVIATIONS

Abbreviations	Name
bp	Base-pair
B20	20 % blend of biodiesel with fossil diesel
BSA	Bovine serum albumin
dNTP	2′-deoxynucleoside-5′-triphosphate
EDTA	Ethylenediamine-tetra acetic acid
HeLa	Human cervical cancer cell line
IU/mL	International unit per mL
kb	Kilo base
kDa	Kilo-daltons
M	Molar
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyl tetrazolium bromide
mM	Milli-molar
nm	nanometer
N	Normal
O.D	Optical Density
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
Pmoles	picomoles
RIPs	Ribosome inactivating proteins
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TE	Tris EDTA
UTR	Untranslated region
µL	Microlitre
µg	Microgram
U/mL	Unit per mL

ABSTRACT

Jatropha (*Jatropha curcas* L.) as a renewable energy source is the best solution to the problems associated with production of biofuels, as it is very easy to grow. It is a multipurpose shrub of significant economic importance because of its several potential industrial and medicinal uses. Apart from a number of advantages, presence of toxic substances particularly in the seeds, like curcin and phorbol esters limits its application. Here we chose eight accessions of *Jatropha curcas* namely TJS 17#03, TJS 42 #04, TJS 35#01, TJS 19#17, TJS 06#24 , TJS 01#03, TJS 01#04 and TJS 46#04. Our work is mainly restricted to the promoter region of the curcin2A gene of *Jatropha curcas.* First, Quality and quantity of the genomic DNA preparations were checked by nanodrop spectrophotometry. BLAST and multiple sequence alignment revealed that the promoter region of the curcin gene showed considerable sequence divergence if compared with the other members of this family. Multiple sequence alignment was made using different forms of curcin namely curcin precursor, curcin2A, curcin-L precursor and ribosome inactivating protein (RIP). This exercise revealed both sequence identity and divergence between them. PCR were carried out using the specific primer pairs. Some forward and reverse primers were made based on the Curcin2A gene sequence as available in the database. Most of the forward primers correspond to the promoter region, and the reverse primers are from the coding region. The purpose was to amplify the curcin genes with varying upstream (promoter) regions. The amplicons corresponding to the individual PCR were noted in order to analyse and compare between the *Jatropha* accessions. Apart from the expected sizes, some amplicons of varying sizes were also found. All the promising amplicons i.e., PCR products as reported in the study need to be further studied at molecular level.

CHAPTER-1 INTRODUCTION

1.1 *Jatropha curcas*: a renewable energy source (petrocrop)

Energy input is very essential for human development and civilization. The rapid depletion of non-renewable sources brought about a situation of energy crisis, thus forcing the scientists to search for the renewable sources. Bio-fuels and bio-energy are being used as an alternative to non-renewable hydrocarbons. Till today, biodiesel derived from *Jatropha curcas* seed oil is the best alternative to fossil fuels, as it possesses the desired physiochemical characteristics, and after suitable modifications it can even beat the conventional petroleum products.

Jatropha is a genus of flowering plants of *Euphorbiaceae* family and contains approx. 170 known species. Among the several *Jatropha* species *Jatropha curcas, Jatropha integerrima, Jatropha glandulifera, Jatropha nana, Jatropha gossypifolia, Jatropha multifida* are cultivated in India. The name of the genus *Jatropha* derived from the two greek words i.e *iatros* (doctor) and *trophe* (food). *Jatropha curcas* is a multipurpose plant with many characteristics and significant botanical features. Common names of *Jatropha curcas* include Barbados nut, purging nut, physic nut, and JCL (Linnaeus). *J.curcas* is a poisonous shrub or small tree with a height of upto 6 m (20 ft.). It can be grown in tropical or sub tropical region, roughly between 30°north and 35°south. It occurs mainly at lower altitudes (0-500 m) in areas with average annual temperatures above 20^0 C and annual rainfall of 300-1000 mm but can also grow at higher altitudes and tolerates slight frost. Besides biodiesel from seed, the plant produces several useful products that also have commercial value. Hardiness, rapid and easy propagation, short gestation period, wide adaptation, and optimum plant size combine to make this species suitable for cultivation on wastelands. It is a drought resistant crop and also has a potential to grow on any type of soil like sandy, gravel, saline, stony and eroded. It is well adapted to marginal soils with low nutrient content. The pH of soil should range between 6-8.5. The centre of origin is still uncertain, but it is believed to be Mexico and central America. Burkill (1966) assumes that the Portuguese brought the *Jatropha curcas* to Asia. Cultivation of *Jatropha* is very simple. *Jatropha curcas* can be propagated by two methods: germination propagation and vegetative propagation. Germination propagation is by seeds and vegetative propagation is by cuttings. *Jatropha* propagation is generally by seeds. The whole genome was sequenced by *Kazusa DNA Research Institute*, Chiba Japan in October 2010. The genomic sequence and linkage map provide a valuable resource not only for fundamental and applied research on physic nut but also for evolutionary and comparative genomics analysis, particularly in the Euphorbiaceae. Different parts of *Jatropha curcas* are shown in Fig.1.

Fig. 1 Different parts of *Jatropha* plant

1.2 Scientific classification of *Jatropha*

Table 1 Taxonomic hierarchy of *Jatropha curcas*

Kingdom	*Plantae*
Division	*Tracheophyta*
Class	*Mangnoliopsida*
Order	*Malpighiales*
Family	*Euphorbiaceae*
Genus	*Jatropha*
Species	*Curcas*

1.3 Biofuel from *Jatropha*

Recognition of the limitations of world oil resources, special interest has been shown in the cultivation of *Jatropha curcas*. The oil extracted from this plant is further transesterified to obtain biodiesel in a usable form. Various steps are shown in Fig. 2. The biodiesel so obtained can be used in existing diesel engines upto 20% mix generally termed as B20. Biodiesel does not produce Sulphur and carbon monoxide on burning. Hence, reduces green house gas emission upto 50%. Also, it has lesser mutagenic potency than commercial diesel.

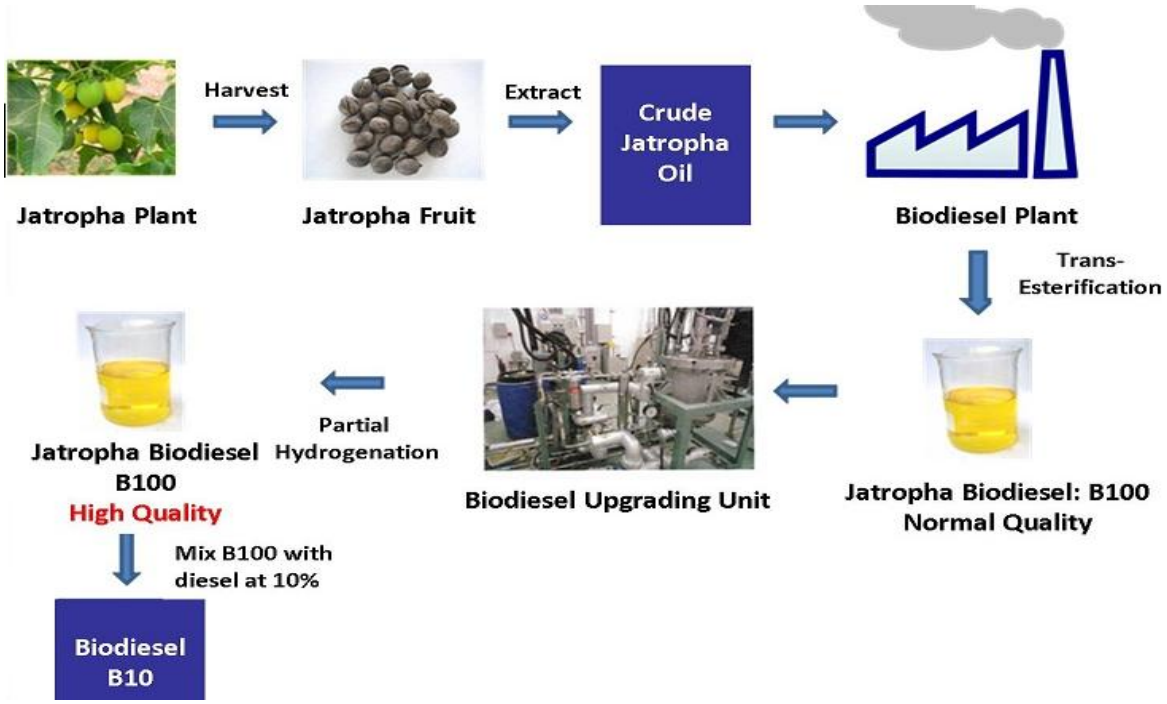


Fig. 2 Biodiesel production from *Jatropha* seeds

1.4 Other uses of *Jatropha*

Apart from production of biodiesel, various tissues of *Jatropha* are used for a variety of commercial purposes as shown in Fig. 3.

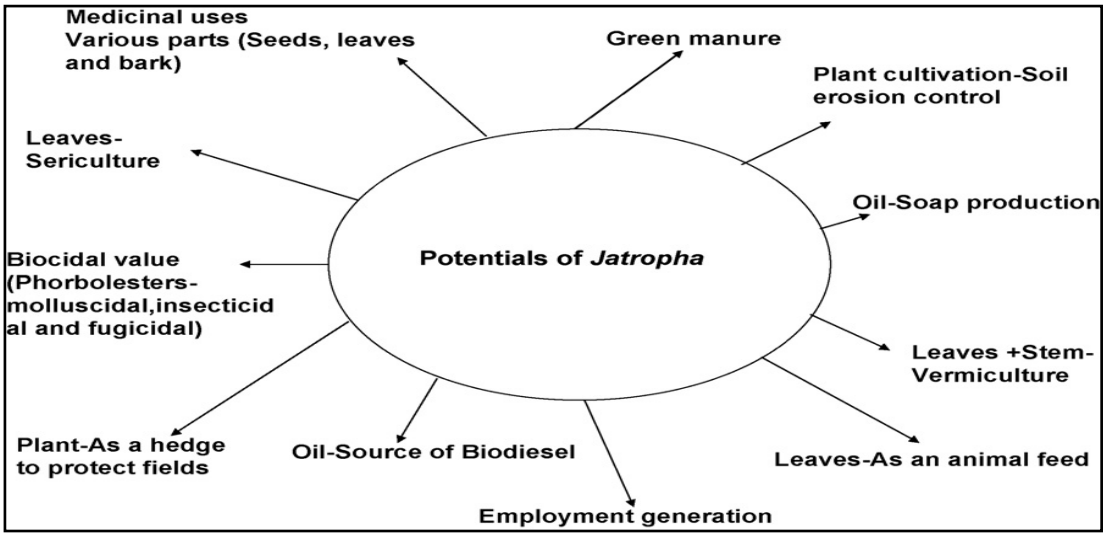


Fig. 3 Various commercial uses of *Jatropha*

1.5 Various toxins in *Jatropha*

Due to the presence of some toxic proteins and non-proteins, the seeds, press cake and oil of *Jatropha* cannot be used for human or animal consumption (Sujatha, 2009). All parts of plant are toxic but seeds are more toxic in nature.

Lectins: Lectins are basically proteins, chiefly of plant origin, that bind specifically to carbohydrate moiety (Goldstein and Hayes 1978). It has been shown that lectin is not the major toxic component in *Jatropha* meal (Aderibigbe et al. (1997) and Aregheore et al. (1998). Lectins are heat labile and their activity can be decreased by heat treatment (Pusztai 1991; Liener 1994).

Phorbol esters: Phorbol esters are the tetracyclic diterpenoids present in almost every part of plant. These are classified as main toxic agent of *Jatropha* curcas seed cake due to which seed cake limits its use as human or animal food (Becker et al, 2006). The toxicity of phorbol esters affect humans and animals by causing tumor promotion, cell proliferation, erythema of the skin, blood platelet activation, lymphocyte mitogenesis, prostaglandin production, sand stimulation of degranulation of neutrophils (Aitken A, 2006). Phorbol ester act as a anologue for DAG which hyperactivate PKC and trigger cell proliferation which results in the tumour promotion.

Ribosome Inactivating Proteins (RIPs): Ribosome inactivating proteins are the inhibitor of protein synthesis that acts on ribosome. These are N-glycosidases which enzymatically cleave the glycosidic bond of adenine of 28S rRNA, irreversibly blocking the protein synthesis as RIP prevent the binding of 28S rRNA to elongation factor (Peumnas et al. 2001). RIPs results in apoptotic and necrotic lesions, induce production of cytokines which is responsible for inflammation. RIPs have been classified into two sub-categories i.e. type 1 RIPs and type 2 RIPs (Stirpe F, 2006). Type 1 RIPs are monomeric proteins of approximately 30 kDa which possess RNA *N*-glycosidase enzymatic activity. Type 2 RIPs consist of two subunits i.e A-chain and B-chain. A-chain with RNA *N*-glycosidase activity and B-chain is a lectin like peptide which has a high affinity for sugar moiety present on the surface of receptor and promote the translocation through plasma membrane (reviews by Van Damme et al. 2001; Girbés et al. 2004; Stirpe and Battelli 2006; Puri et al. 2012).

Phytate: It is a saturated cyclic acid, which is the principal storage form of phosphorous, especially in the seeds of many plant tissues. Phytate is heat stable, antimetabolic and metal chelating factor reported in *J.curcas* meal (Makkar, Aderibigbe and Becker 1998). Phytate have the ability to bind to

essential minerals such as copper, magnesium, zinc etc. in the digestive tract and inhibit their absorption by the body.

Curcin: Curcin is a major toxic protein in *Jatropha* which was first isolated from the seeds of *Jatropha curcas* by Felke (1914). It is a toxalbumin which is mainly found in the endosperm of the seeds of *Jatropha* (Mourgue et al. 1961). Curcin is a type 1 ribosome inactivating protein (RIP) which inhibit the activity of prokaryotic and eukaryotic ribosome by specific modification of the larger rRNA (Barbieri et al. 1993). Recent advances on curcin research are discussed in the Review of Literature section (Chapter-2).

CHAPTER-2 REVIEW OF LITERATURE & OBJECTIVES OF THIS STUDY

Curcin is a major toxic protein in *Jatropha* which was first isolated from the seeds of *Jatropha curcas* by Felke (1914). It is a toxalbumin which is mainly found in the endosperm of the seeds of *Jatropha* (Mourgue et al.1961). Curcin is a type 1 Ribosome inactivating protein (RIP) which inhibits the activity of prokaryotic and eukaryotic ribosome by specific modification of the larger rRNA (Barbieri et al. 1993). It is a single chain protein with molecular mass 28.1 kDa. In NCBI database, different forms of curcin genes are reported which are, i) Curcin2A gene (Accession no. GQ925453), ii) Curcin-L precursor (Accession no. EU195892), iii) Curcin gene (Accession no. EU395775), iv) RIP partial *Jatropha curcas* gene (Accession no. AY435214), v) Curcin precursor gene (Accession no. AF469003). According to in vitro studies, it is found to be 1000 times less toxic than ricin (phytotoxin found in the castor bean) (Stripe et al. 1976). Crude curcin was extracted from seeds of *Jatropha curcas* by solvent extraction method using hexane as a solvent and Phosphate buffer saline as an extracting buffer (Jummai et al. 2014). Curcin remains in the seed after the oil has been extracted. It has pharmacological importance as described in the following section.

2.1 Pharmacological importance of curcin

Curcin acts as a potential anthelmintic: Helminth infections are one of the most widespread infections in humans, affecting the huge population of the world. The majority of helminth infections are limited to tropical regions. It causes a massive hazard to the health and adds to the already prevailing diseases like undernourishment, anaemia, eosinophilia and pneumonia (Bundy, 1994). Nirmal et al. (2007) and Ravindra et al. (2007) investigated the anthelmintic effect. According to their procedure, five groups of fifteen earthworms were treated with normal saline (control), Niclosamide (15 mg/ml), and crude curcin (10, 25 and 50 mg/ml). The observations were made for the time of paralysis of individual worms and paralysis assumed to occur when cells were not able to revive themselves even in normal saline. Ahirrao et al. (2011) discovered that as *J.curcas* are rich in alkaloids and saponins which further minimizes the helminthes effect by diminishing the support of glucose to helminthes. The data in Table 2 clearly shows that the anthelmintic activity of crude curcin is more potent than the presently used niclosamide.

Table 2 Anthelmintic activity

Group	Treatment	Conc.(mg/ml)	*Pheretima posthuma* (Earthworm)	
			Time taken for paralysis (min)	Time taken for death (min)
I	Normal saline		-	-
II	Niclosamide	15	23 ± 1.15	61 ± 0.88
III	Crude curcin	10	13 ± 0.41	34 ± 1.20
		20	10 ± 0.46	25 ± 1.18
		50	04 ± 0.58	15 ± 1.36

Curcin as Immunotoxin: Immunotoxin is a type of hybrid molecule consisting of toxic peptide chain linked to an antibody. According to Frankel et al. 1986, the intense activity of crude curcin can be attributed its use as a component of immunotoxin. Curcin contains one cysteine residue at 209th position. Thus, curcin may directly form a disulphide linkage with an activated antibody (Juan et al. 2003)**.** However, there are some problems in the use of immunotoxins, such as poor stability, immunogenicity and promotion of vascular leak syndrome, which would raise serious questions on their application (Kreitman et al. 1999).

Anti-tumour activity of curcin: Mohamed et al. (2014) made a significant contribution in the area of curcin research. They showed cytological and subcellular response of cells exposed to type 1 RIP curcin by exposing normal and cancerous cell lines with a curcin, type-1 RIP (100 µg/ml) for 72 hr. They worked on six mammalian cell lines which are, L929 (normal), HCN-1A (normal), HuVEC (normal), glioma cells (cancerous), MCF-7 (cancerous), MDA-MB-453 (cancerous). According to their study, cancerous cell lines are more sensitive than normal cell lines (Table 3 and Table 4). With respect to the cell lines under study, cytocidal rather than cytostatic effects of curcin were observed. Viability rate of different cell lines was measured at highest concentration of curcin (100 µg/ml).

Table 3 Viability of normal cell lines

Sr No.	Cell line	Viability rate
1.	L929	70%
2.	HCN-1A	35%
3.	HuVEC	60%

Table 4 Viability of cancerous cell lines

Sr No.	Cell line	Viability rate
1.	Glioma cells	< 20%
2.	MCF-7	30%
3.	MDA-MB-453	45%

Cells toxicity due to curcin is dose dependent i.e. the severity increases with increase in curcin concentration. RIP exposure results in the various damages in cell, i) Prominent mitochondrial dysfunction, ii) Elevated reactive oxygen species (ROS) levels, iii) Nuclear degeneration, iv) Structural/mechanical destabilization and suppression of defense mechanisms were imminent with the RIP treated cells, v) Expression levels of nuclear factor kB (NF-kB), cytoskeletal focal adhesion kinases (FAK) and vinculin were significantly diminished, vi) Vital cellular organelles such as nucleus, mitochondria and actin were severely incapacitated on RIP exposure resulting in multimodal apoptosis and necrosis.

2.2 Salient features of a typical eukaryotic promoter

Promoter is a region of DNA located in the 5' region adjacent to the transcriptional start site that initiates transcription of a particular gene. Promoters can be around 100-1000 bp long. In eukaryotes, transcription is RNA polymerase II mediated. Promoters for different RNA polymerases are different in nature. A typical eukaryotic promoter sequence (as shown in Fig. 4) comprises of some *cis*-regulatory motifs positioned at specific sites relative to the transcription start site (TSS). The structure of eukaryotic promoters is generally more complex than prokaryotic promoters and they have several different regulatory motifs, such as TATA box (Hogness box), INR box, BRE, CCAAT-box and GC-box which helps in the regulation of transcription. Based on the analysis of a huge number of promoters, these regulatory motifs represent consensus sequences. Promoters consist of major two elements, i) core element ii) regulatory element. Core element of promoter consists of TATA box and DPE (downstream core promoter element), whereas regulatory element comprises of CAAT box and GC box.

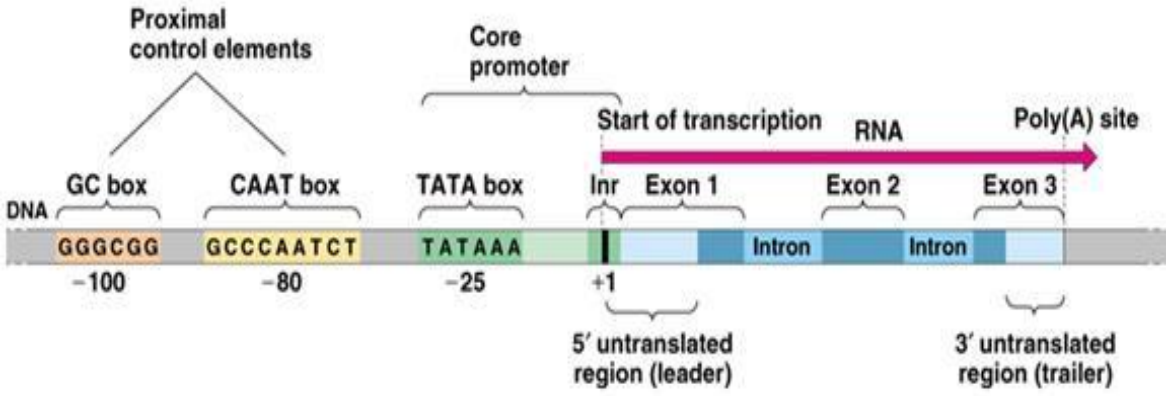


Fig. 4 Salient features of a typical eukaryotic promoter

The TATA box was the first eukaryotic core promoter motif to be identified (Goldberg 1979; Breathnach and Chambon 1981). The TATA box resembles to the −10 region (Pribnow box) of prokaryotic promoters. TATA element with consensus sequence TATAAA, is recognized by the general transcription factor TATA-binding protein (TBP).

2.3 Differential expression of curcin in *Jatropha*

Expression of curcin is tissue-specific which is due to the presence of different *cis*-elements in the promoter region of the gene. Expression and function of gene is promoter-dependent. Qin et al. (2009), clearly demonstrated that expression profiles of different curcin forms vary as promoter of each form have their unique *cis*-regulatory elements. To date, expression profile of two types of ribosome-inactivating proteins (RIPs) have been known in *Jatropha*. One is curcin, which has been isolated from the endosperm (seed), and the other is curcin-L, which is expressed in leaves only upon stress treatment. Analysis and comparison of the *cis*-regulatory elements in the 5′ flanking region of curcin and curcin-L revealed that curcin-L has three major inserted fragments, which are not present in the corresponding region of curcin.

Curcin gene promoters: Qin et al. (2009) made a significant contribution on curcin gene promoter from *Jatropha curcas L.* The aim of this study is to investigate the regions required for promoter and tissue specific activity of curcin in *Jatropha curcas.* For this study, a 620-bp 5′ flanking region (CP1) preceding a curcin gene has been isolated and characterized. Here, β-glucuronidase (GUS) is used as a reporter gene and analysis of GUS activity was investigated in transgenic tobacco plant. Analysis of GUS activity showed that, this 620-bp fragment of curcin (promoter, CP1) is enough to drive the

expression of GUS in transgenic tobacco seed. It also clearly showed that expression of gene is fully dependent on promoter. The Histochemical analysis of GUS activity indicated that the promoter of curcin gene was specifically active in the endosperm tissue of tobacco embryo. This shows that, probably, these are seed specific curcin gene form.

The activity of this flanking fragment was analyzed at different stages of seed development. But studies indicate that expression of this curcin promoter begin only after a certain stage of seed development in transgenic tobacco. A comparison of promoter activity of CP1 and CaMV 35S clearly indicates that the former expression is higher in seeds and on the other hand, CaMV 35S can activate GUS expression in most of tissues and organs of transgenic tobacco plant. Qin et al. also demonstrated that the endosperm itself has some regulatory motifs that may contribute to the above results. With the help of PLACE database, they identified that endosperm responsive region contains four DOFCORE, one GT-1 binding site, one E box and one W box motifs. For example, in the endosperm of maize Dof proteins are present which binds to the DOFCORE motif and enhance transcription.

Curcin-L promoter: Qin et al. (2009) also studied the stress induced expression of curcin-L promoter in the leaves of *Jatropha curcas*. The RNA blot showed that probe could detect the mRNA of curcin-L in leaves with stress treatments, but could not without the treatment. Curcin-L is a type 1 RIP found in the leaves of *J.curcas* under stress-induction conditions. The expression of curcin-L could be activated in leaves by treatment with abscisic acid, salicylic acid, polyethylene glycol, low temperature (4^0 C), high temperature (45^0 C) and ultraviolet light. For this study, a 654 bp 5′ flanking region of curcin-L gene (CP2) was isolated from *J.curcas* and analysis was done via the expression of the beta-glucuronidase (GUS) reporter gene in transgenic tobacco plant. They examined the inducible expression of curcin-L under six different conditions. The results suggested that gene transcripts were observed in leaves, but were not detected in roots, stems, and seeds. PEG6000 induced a high level of GUS activity in the mature leaves of *Jatropha curcas*, whereas other five stresses induced low level of GUS activity. Analysis of a series of 5′ deletions of the CP2 suggested that several regulatory motifs were necessary to respond to environmental stresses. These regulatory motifs were identified with the help of PLACE database.

Several putative regulatory motifs, which were homologous to the *cis-acting elements involved in the activation of the defense* genes in plants, were: i) One typical TATA boxes ii) four CAAT boxes iii) one ABRE, iv) Two MYBCORE, v) three GT1 binding sites, vi) five DOFCORE, vii) one I box, viii) one Prolamin box, ix) three E box, x) three W box elements within CP2, xi) one MYB binding sites .

2.4 Rationale behind the present study

Jatropha curcas is mainly known for its biodiesel producing quality all over the world. Most of the researchers also focused their work towards the processing of the oil of *Jatropha* so that they could easily replace the" petroleum products". In India, from the last two decades, both government and non-government agencies are promoting this biodiesel crop. In our country, significant progress has been made on the cultivation of *Jatropha* in the field, clonal propagation of *Jatropha* germplasm and importantly biodiesel production through transesterification. Though, less progress has been made in studying the promoters of toxic proteins such as curcin, RIPs etc present in the seeds of *Jatropha*. The level of above toxic proteins could vary between the *Jatropha* tissues and also between the varieties because of difference in promoter functionalities. One can develop a transgenic *Jatropha* with low curcin content by gene silencing approaches for the purpose of consumption by humans or animals. On the other hand, by gene manipulation techniques we can develop a novel *Jatropha* plant with high curcin content in the seeds for its medicinal potential. Research on curcin gene promoters has immense potential. Therefore, in-depth understanding of the structure and function of promoters of different forms of curcin genes and their expression patterns in the *Jatropha* varieties is a prerequisite for possible biotechnological manipulations. A number of Jatropha accessions collected from different regions of Punjab are being maintained in our university campus. Earlier, in our laboratory the focus was mainly on micropropagation of *Jatropha* plant through plant tissue culture technique. At present, we need to focus on curcin gene promoters for possible biotechnological manipulations. Keeping the above points in view, the following objectives were framed in this study.

2.4.1 Objectives of the present study

The following objectives are framed based on the literature survey and background work in our laboratory:

- Sequence analysis, comparison and prediction of *cis*-regulatory motifs in the available sequences of curcin gene promoters in *Jatropha*
- Isolation and purification of genomic DNA from some of the *Jatropha* accessions collected in the state of Punjab
- PCR amplifications using curcin gene promoter-specific primers, and analyses of the amplicon profile between the *Jatropha* accessions

CHAPTER 3 MATERIALS AND METHODS

3.1 Procurement of *Jatropha curcas* L. plant material

For DNA isolation, leaf samples of *Jatropha* plant were collected from the different *Jatropha* cultivars such as TJS 17#03, TJS 42#04, TJS 35#01 and TJS 01#03 maintained at *Jatropha curcas* L. germplasm bank, COS-complex, Thapar University, Patiala.

3.2 Other materials

Various enzymes used were purchased from Bangalore Genei Pvt. Ltd., Bangalore. The chemicals required were bought from Siga-aldrich India Pvt. Ltd., and Himedia Pvt. Ltd, Mumbai. Primers used were synthesized by Eurofilms Genomic Pvt. Ltd., Bangalore. The gel extraction Qiagen kit was purchased from Genetix.

Buffers and other chemicals and Enzymes

- *Gel loading buffer (5X)*

  Sucrose - 35 % (w/v)
  EDTA - 50 mM (pH 8.0)
  Tris – 25 mM
  Bromophenol blue - 0.2 % (w/v)

- *TBE (5X) buffer*

  Tris Base - 54 gL^{-1}

  Boric acid - 28 gL^{-1}

  EDTA - 3.8 gL^{-1}

  The pH of the buffer was set at 8.0

- *TE buffer*

  Tris.HCl - 10 mM (pH 8.0)

  EDTA - 1 mM (pH 8.0)

- *Extraction buffer*

  50 mM Tris-HCL (pH8.0)

  50 mM EDTA

  250 mM Nacl

  15 % Sucrose

- Ethanol

- Isopropanol
- 5 M potassium acetate solution
- Sodium acetate
- RNase
- Alcohol
- Chloroform

3.3 Methods

3.3.1 Isolation of Genomic DNA from different varieties of *Jatropha curcas*

Procedure: Genomic DNA was isolated from the *Jatropha* leaves by the protocol described by Kumari et al. (2012). Leaf samples of *Jatropha* were washed in tap water followed by sterile distilled water. Removal of excess water from the leaves was done by using blotting filter paper. 0.7 g of plant material was weighed and fine powder was made in the presence of liquid nitrogen by using mortar and pestle. The fine powder was then transferred to 20 mL tube containing 5 mL of extraction buffer and 0.5 % SDS (250 µL of SDS) maintained at 65°C. Contents were mixed properly with intermittent gentle shaking and incubated at 65°C for 15 min. The solution was centrifuged at 5500 rpm for 10 minutes. Then 170 µL of 5.0 M potassium acetate solution was added, mixed vigorously and incubated further on ice for 20 min and solution was centrifuged at 5500 rpm at 4°C for 15 min. The supernatant was filtered through a fine muslin cloth and equal volume of isopropanol was added, mixed gently and incubated at -20°C overnight. DNA was extracted by centrifugation at 12,000 rpm at 4°C for 10 min. The crude DNA pellet was washed with ice cold 70% ethanol, air dried and suspended in 50 µL of TE buffer and stored at -20°C.

3.3.2 Purification of *Jatropha* DNA sample

Materials required:

- Sterile water
- DNase-free RNA enzyme
- 0.3 M sodium acetate (pH 5.5)
- Ethanol
- TE Buffer
- Extraction buffer
- 8 M Licl
- DEPC-treated water

Procedure: After isolation, purification of DNA was done by treatment with DNase–free RNase. 350 µL of sterile water was added into eppendrof containing DNA sample to make the total volume 400 µL. Then, 3µL of DNase–free RNase enzyme was added into solution. The solution was incubated for 45 minutes at 37° C. Equal volume of phenol and chloroform (200 µL each) was added into the solution and mixed properly for 10-15 minutes by gently inversion mixing. After this, the sample was centrifuged at 8000 srpm for 10 minutes. DNA was precipitated using $1/10^{th}$ (0.1) volume of 3.0 M sodium acetate (pH 5.5) and double the volume of ethanol. DNA was finally recovered by dissolving the pellet in 50 µL of TE buffer and stored at (-20°C). The quality and quantity of DNA was checked spectrophotometrically by measuring the A_{260}/A_{280} ratio.

3.3.3 RNA isolation from different varieties of *Jatropha*

Materials required:

Components of extraction buffer

Solution	***Stock Solution***	***Working Solution***
Tris Buffer	0.5M (pH 8.0)	100mM (pH 8.0)
LiCl	8M	100mM
EDTA	0.5M (pH 8.0)	10mM (pH 8.0)
SDS	10%	1%
B-mercaptoethanol	0.2%	0.1m**l**

Procedure: Plant tissues contain high amount of nucleases, polysaccharides, phenolics and other storage material. Therefore, RNA isolation from plant materials is relatively difficult in terms of intactness and quality. For that, number of methods are reported in literature. Here we used, SDS-Phenol method by Gilman (1987) which was used as such or with modifications depending upon the plant material.

The plant material (0.2-1.0 g) was frozen and pulverized in liquid nitrogen with the help of mortar and pestle to a fine powder. The contents were mixed in a extraction buffer followed by direct extraction with phenol choloroform(1:1). Under ice cold conditions,$1/3^{rd}$ volume of 8M Licl was added for the precipitation of RNA. The crude RNA was further purified by RNase-free DNase treatment followed by solvent extraction and ethanol precipitation. After that RNA was dissolved in RNase-free deionized water and kept in aliquots at -70°C for further use.

3.3.4 Designing of Curcin2A gene-specific oligonucleotide primers

The oligonucleotide primers were designed based on the available genome sequence of Curcin2A gene in GenBank database (GenBank ID: GQ925453). Curcin2A gene sequence is of 3748-bp length comprising of 5' flanking region (including promoter) upto 1790-bp. The transcriptional start site is located at the base 1791 corresponding to first exon and the translational start site is present at the base 2085. In curcin2A gene, the ORF of polypeptide spans from the base 2085 to 3014. In this thesis work, our main emphasis is on promoter region.

3.3.5 Polymerase Chain Reaction (PCR)

PCR is a molecular technique used to amplify specific DNA sequence in an easy and automated manner using forward and reverse primer. PCR is a cycle of three steps : heat denaturation of template DNA (94°C); annealing of primers to the complementary sequences in template DNA (55°C); extension of annealed primers by a thermo stable DNA polymerase (72°C).

Materials required

Total reaction volume for PCR is: 50 µL

Composition of PCR reaction:

	Concentration	volume taken
Template DNA	3µL	3 µL
Buffer 10X	5µL	5 µL
Forward primer	10 pmoles	2.5 µL
Reverse primer	10 pmoles	2.5 µL
dNTPs	2.5 mM	3 µL
Sterile deionized water	volume made up to 50 µL	~33 µL
Taq DNA polymerase	3 U/µL	0.7 µL

Procedure:

The thermal cycling parameters were as given below:

Step	*Temperature*	*Time*
Denaturation	94°C	1 min
Annealing	55 °C	2 min
Polymerization	72°C	2 min

The reaction was carried out for 30 cycles with final extension at 72°C for 5 min.

3.3.6 Agarose gel electrophoresis

Materials required:

- Agarose (Himedia)
- 0.5 X TBE buffer
- Ethidium bromide dye (0.5 μg mL^{-1})
- Sterile water
- DNA samples and amplicons
- Bromophenol blue dye
- Gel Electrophoresis instrument
- UV Transilluminator
- Gel documentation system (BIO-RAD)

Procedure: Agarose gel electrophoresis was performed using standard methods (Sambrook- a laboratory manual). 0.8 % agarose gel was made in 0.5X TBE buffer and ethidium bromide dye (0.5 μgmL^{-1}) was added to it. Gel was then casted into the casting tray. After solidification of gel, the DNA samples were loaded in the wells. Electrophoresis was carried out in 0.5X TBE (running buffer) at 2 – 5 Volt per cm till the tracking dye covered 75% of the gel length. Finally, the DNA bands were visualized under UV light.

CHAPTER-4 RESULTS AND DISCUSSION

4.1 Sequence analysis, comparison and searching of *cis*-regulatory motifs

4.1.1 Salient sequence features of Curcin2A gene from *Jatropha*

For this thesis work, we followed curcin2A gene of *Jatropha curcas* with GenBankID: GQ925453. It is of 3748 bp in length encoding curcin2A protein consisting of 309 amino acids. This is an example of ribosome inactivating protein (RIP). This 3748-bp curcin2A gene consists of 1790-bp 5′ flanking region (promoter), two exons i.e. from 1791 to 1845 & 2074 to 3194, one intron i.e from 1846 to 2073 and 3′ flanking region i.e from 3195 to 3748 bp. The length of first exon is 55 bp, second exon is 1121-bp and the length of only one intron is 228 bp. The length of 5′UTR is equal to 294 bp (including intron) and 3′ UTR of Curcin2A gene is equal to180 bp. The ORF length of curcin2A gene is 930 bp (3014-2084) which is equal to 310 amino acid (codons) including stop codon. Hence, the Curcin2A protein consists of 309 amino acids.

4.1.2 BLASTn

Blastn is used for finding the regions of similarity between nucleotide sequences. For this thesis work, curcin2A gene (GenBankID: GQ925453) is used as a query sequence. We used the 5′flanking region of the curcin2A gene along with some part of coding region (query sequence: 1-2100 ntds). Coding region is used just for identification of the gene. For our study, main emphasis is on promoter region of curcin 2A gene. Sequence identity values of the different curcin sequences are: 99% (GenBank ID: EU195892), 99% (GenBank ID: EF612740), 99% (GenBank ID: EU395775), 97% (GenBank ID: EF612741), 97% (GenBank ID: AF469003), 97% (GenBank ID: EF612739). Query coverage ranged from 77% to 25 % in the BLAST search data for these sequences. 5′flanking region of the Curcin2A gene was not found 100% identical with any other Curcin gene reported till date. Blastn results of curcin2A gene are shown in Table 5.

Table 5 Blastn results

Sr. No.	Accession number	Size (bp)	Tentative size of promoter	Size of CDS (bp)	Number of amino acids
1	GQ925453	3748	1790	930	309
2	EU195892	1827	470	930	309
3	EF612740	654	654	-	-
4	EU395775	1522	291	930	309
5	EF612741	489	489	-	-
6	AF469003	1802	435	882	293
7	EF612739	620	620	-	-

4.1.3 Different *cis*-regulatory motifs in the 5′ flanking region of curcin2A gene

The sequence of curcin2A gene was carefully examined for prediction of *cis*-regulatory motifs that included basal promoter elements and enhancers/silencers (Fig. 4). Most of these sequence motifs were identical/nearly identical to a number of well-characterized *cis*-regulatory sequence motifs present in many plant genes reported earlier.

```
agttagagttagggtataattaaaactactcgcaaaatttggatatttttaacgattta    60
accattctttgagtattgctttttgtggaaatataaaatcaatgaggaaaaaaaatgata   120
ttaagaactagattacaagagtagcttttatacagaattatgaataaaaaatatctagct   180
attatatcttcgagccactttctattacactttgtagttgcttctatattatccaaaaca   240
tacaaaccatggaaaagataagggagccattgcacttcacaagtagtagaagcaagtgca   300
atgctttctgcttttgatgaggaacgtaaaaaagtctgactgtttgttaagttctagcag   360
actaattacattatctaaggtaaacacaataacttgtaatagatttgtgtgtgtctaaac   420
agccggcccagtcgtaaccattagatgctttaagctaaaagaaaaattagatgggaaaaa   480
taatctagcatctagggcatccttaatgtagtgaagagcatgattaggatttttttttt   540
ccatcggttttctttatctttatttagttttctttctagagtaggatcgtttaaatttat   600
ctttatttacttttctttctagagcaggatcgtttaaaacaaatcttagttttttattttt   660
cggatctatcatagttaatagaccttcgatgttttgatacctcctagtggggttgtcc   720
aattttgatttgttacgttttctctcatttgatgaagttatttctcattattaaagagtt   780
tttacgtgatttgtgatttttctttcttaaaagtctaaaatataagatgaatcatttatt   840
gattgcagtggagacttttaattcttcgaatgctgataatttctttttcaatagatcttg   900
gtagcaccaccttcctaagttaaaatttgataatttcaaactcttctataggaggtaaca   960
agttttctcaaggacaaagttcaagacactagttcaaaaaatttattttatcattctatt  1020
tgaaaatagattggtctttacctagaagattaatgtaccaaatcatacattggttatgga  1080
aatatttatgacctacttaagctataatctttattaaaggcttgagaagtttgtaaaaaaa  1140
taaaaataaaaataaataaataaataaataaatcaatactaagcttaagatgagcgttag  1200
aaaaactagatgatttgagtttgagtttagtttgttggtttgataatgctaatttttaat  1260
aaatttaatttttaaaaaattatagtttccttttattattttccctttttacaaataa  1320
atgttggaatagaaagactttgttttcttttctttcattacttttttttttttggttac  1380
atttctttcattatttttcatctaaggtttcaccaataaaaaaataataataaaaaaaat  1440
tataaacgtgtcatatttctcgtttgtcttttatcaatttttgtttttttctaatcaacgg  1500
tcatcaaatcagcgccccacacccgctatgacgcggcatttatcaatttttgttttttct  1560
ccttggattttcaataattctttttcattgggatttgttattgtttgtctttctttttt  1620
tttttttttgacagttgttattgtttgtctttatttttacatcttatgaaactgtcgtt  1680
tggtagctaataatcaaaataataataaaaaataataataataaagaaaaaaaattgac  1740
aaaataaagggcagtttcccctataaagcaggtgatgggggaaggcaaaa           1790
```

Fig. 4 Prediction of *cis*-regulatory motifs in curcin2A gene promoter (GenBank ID: GQ925453)

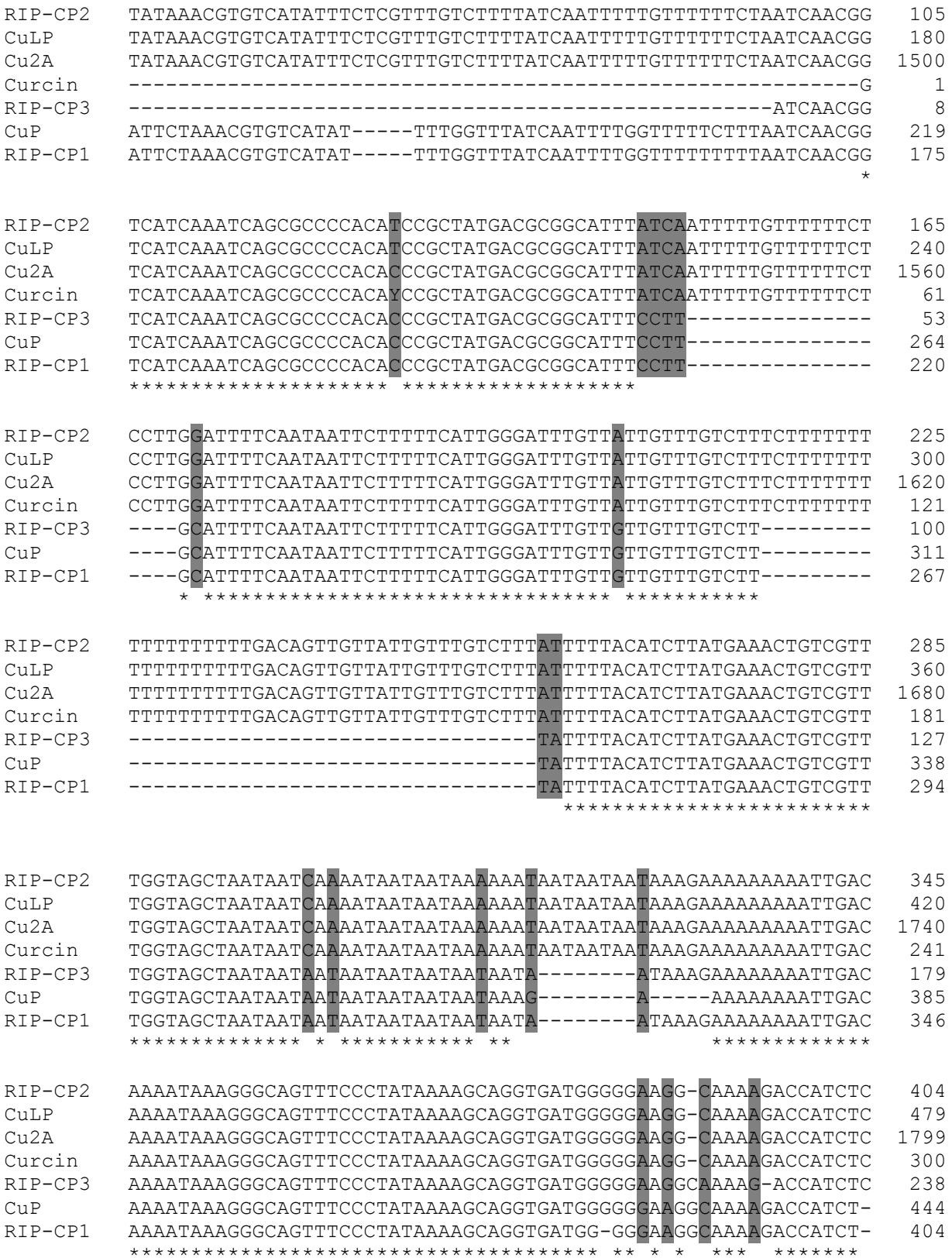
Table 6 Showing *cis*-regulatory motifs and their position in the promoter of curcin2A gene

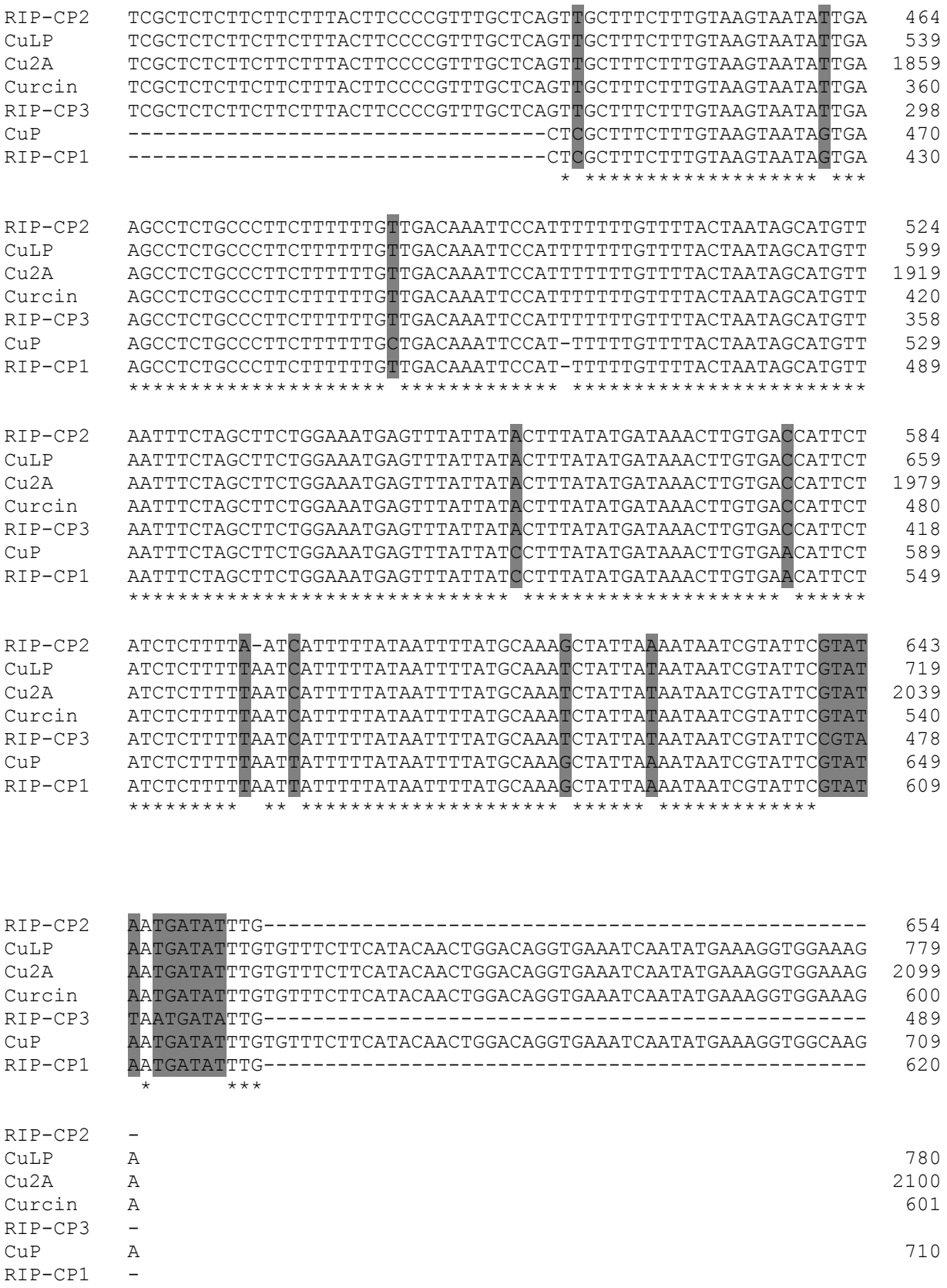
Sr. No	*cis*-element	Consensus sequence	Motifs predicted	Motifs position	References
1	TATA box	TATAAA	TATAAA	91,1441,1761	Forde el al.(1985), Joshi (1987)
2	Auxin/tissue specificity	ACTTTA	ACTTTA	854	Baumann et al. (1999)
3	Putative enhancer	ATTTTTA	ATTTTTA	1252,1654	Allen et al.(1989)
4	Light response elements	AACCAA	ACCAAT	1412	Degenhardt and Tobin (1996)
5	CCAAT Box	CCAAT	CCAAT	1413	Forde el al.(1985)
6	GATA Box	GATA	GATA	43,117,257,697,874, 929,1242	Lam and Chua (1989)
7	W-Box	TTGAC	TTGAC	1629,1736	Eulgem et al. (2000)
8	T-Box	ACTTTG	ACTTTG	209,1336	Chan et al. (2001)
9	TAAAG motif	TAAAG	TAAAG	1722,1745	Plesch et al. (2001)
10	Root motif	ATATT	ATATT	46,226,1082,1453	Elmayan and Tepfer (1995)
11	Pyrimidine Box	CCTTTT	CCTTTT	1290,1306	Morita et al. (1998)
12	Box II	ATAGAA	ATAGAA	1328	Kapoor and Sugiura (1999)
13	ABRE element	ACGTG	ACGTG	1446, 784	Nakashima et al.(2006); Simpson et al. (2003)
14	I Box	GATAAG	GATAAG	257	Donald and Cashmore(1990)
15	SURE core	GAGAC	GAGAC	851	Maruyama-Nakashita et al. (2005)
16	E-Box	CANNTG	CATTTG	746	Stalberg et al. (1996)

4.1.4 Multiple sequence alignment (MSA) of different curcins from *Jatropha*

A total of seven curcin gene sequence from Jatropha, i.e., Curcin 2A gene (Cu2A, GQ925453), Curcin-L precursor (CuLP, EU195892), clone CP2 curcin RIP (RIP-CP2, EF612740), curcin gene (curcin, EU395775), clone CP3 curcin RIP (RIP-CP3, EF612741), curcin precursor (CuP, AF469003) and clone CP1 curcin RIP (RIP-CP1, EF612739) were included in the multiple sequence alignment (Fig. 5). This study was done to examine sequence similarities and divergence, the amino acid substitutions, insertions and deletions between the seven different forms of curcin gene.

```
Cu2A      AGTTAGAGTTAGGGTATAATTAAAACTACTCGCAAAATTTGGGATATTTTTAACGATTTA    60

Cu2A      ACCATTCTTTGAGTATTGCTTTTTGTGGAAATATAAAATCAATGAGGAAAAAAAATGATA   120

Cu2A      TTAAGAACTAGATTACAAGAGTAGCTTTTATACAGAATTATGAATAAAAAATATCTAGCT   180

Cu2A      ATTATATCTTCGAGCCACTTTCTATTACACTTTGTAGTTGCTTCTATATTATCCAAAACA   240

Cu2A      TACAAACCATGGAAAAGATAAGGGAGCCATTGCACTTCACAAGTAGTAGAAGCAAGTGCA   300

Cu2A      ATGCTTTCTGCTTTTGATGAGGAACGTAAAAAAGTCTGACTGTTTGTTAAGTTCTAGCAG   360

Cu2A      ACTAATTACATTATCTAAGGTAAACACAATAACTTGTAATAGATTTGTGTGTGTCTAAAC   420

Cu2A      AGCCGGCCCAGTCGTAACCATTAGATGCTTTAAGCTAAAAGAAAAATTAGATGGGAAAAA   480

Cu2A      TAATCTAGCATCTAGGGCATCCTTAATGTAGTGAAGAGCATGATTAGGATTTTTTTTTT   540

Cu2A      CCATCGGTTTTCTTTATCTTTATTTAGTTTTCTTTCTAGAGTAGGATCGTTTAAATTTAT   600

Cu2A      CTTTATTTACTTTTCTTTCTAGAGCAGGATCGTTTAAAACAAATCTTAGTTTTTATTTTT   660

Cu2A      CGGATCTATCATAGTTAATAGACCTTCGATGTTTTTGATACCCTCCTAGTGGGGTTGTCC   720

Cu2A      AATTTTGATTTGTTACGTTTTCTCTCATTTGATGAAGTTATTTCTCATTATTAAAGAGTT   780

Cu2A      TTTACGTGATTTGTGATTTTTCTTTCTTAAAAGTCTAAAATATAAGATGAATCATTTATT   840

Cu2A      GATTGCAGTGGAGACTTTAATTCTTCGAATGCTGATAATTTCTTTTTTCAATAGATCTTG   900

Cu2A      GTAGCACCACCTTCCTAAGTTAAAATTTGATAATTTCAAACTCTTCTATAGGAGGTAACA   960

Cu2A      AGTTTTCTCAAGGACAAAGTTCAAGACACTAGTTCAAAAAATTTATTTTATCATTCTATT  1020

Cu2A      TGAAAATAGATTGGTCTTTACCTAGAAGATTAATGTACCAAATCATACATTGGTTATGGA  1080

Cu2A      AATATTATGACCTACTTAAGCTATAATCTTTATTAAAGGCTTGAGAAGTTTGTAAAAAAA  1140

Cu2A      TAAAAATAAAAATAAATAAATAAATAAATAAATCAATACTAAGCTTAAGATGAGCGTTAG  1200

Cu2A      AAAAACTAGATGATTTGAGTTTGAGTTTAGTTTGTTGGTTTGATAATGCTAATTTTTAAT  1260

CuLP      ------------------------------------------------------------A     1
Cu2A      AAATTTAATTTTTTAAAAAATTATAGTTTCCTTTTTATTATTTTCCCTTTTTACAAATAA  1320
CuP       ---------------AAAAAATATAGTTTCCTTTTTATTATTTTCCCTTTTTACAAATAA    45
RIP-CP1   ------------------------------------------------------------A     1

CuLP      ATATTGGAATAGAAGACTTTGTTTTCTTTTCTTTCATTACTTTT-TTTTTTTGGGGTTAC    60
Cu2A      ATGTTGGAATAGAAGACTTTGTTTTCTTTTCTTTCATTACTTTTTTTTTTTTTTGGTTAC  1380
CuP       ATATTGGAATAGAAGACTTTGTTTTCTTTTCTTTCATTACTTTTTTT-TTTTGGGGTTAC   104
RIP-CP1   ATATTGGAATAGAAGACTTTGTTTTCTTTTCCTTCATTACTTTTTTT-TTTTGGGGTTAC    60


RIP-CP2   ---------------TTTCATCTAAGGTTTCACCAATAAAAAAATAATAATAAAAAAAAT    45
CuLP      ATTTCTTTCATTATTTTTCATCTAAGGTTTCACCAATAAAAAAATAATAATAAAAAAAAT   120
Cu2A      ATTTCTTTCATTATTTTTCATCTAAGGTTTCACCAATAAAAAAATAATAATAAAAAAAAT  1440
CuP       ATTTCTTTCATTATTTTTCATCTAAGGTTTCACCAATAAAAAAATAATAATAAAAAAAAA   164
RIP-CP1   ATTTCTTTCATTATTTTTCATCTAAGGTTTCACCAATAAAAAAATAATAATAAAAAAAAA   120
```


RIP-CP2 TATAAACGTGTCATATTTCTCGTTTGTCTTTTATCAATTTTTGTTTTTTCTAATCAACGG 105
CuLP TATAAACGTGTCATATTTCTCGTTTGTCTTTTATCAATTTTTGTTTTTTCTAATCAACGG 180
Cu2A TATAAACGTGTCATATTTCTCGTTTGTCTTTTATCAATTTTTGTTTTTTCTAATCAACGG 1500
Curcin ----------G 1
RIP-CP3 ----------ATCAACGG 8
CuP ATTCTAAACGTGTCATAT-----TTTGGTTTATCAATTTTGGTTTTTCTTTAATCAACGG 219
RIP-CP1 ATTCTAAACGTGTCATAT-----TTTGGTTTATCAATTTTGGTTTTTTTTTAATCAACGG 175
RIP-CP2 TCATCAAATCAGCGCCCCACATCCGCTATGACGCGGCATTTATCAATTTTTGTTTTTTCT 165
CuLP TCATCAAATCAGCGCCCCACATCCGCTATGACGCGGCATTTATCAATTTTTGTTTTTTCT 240
Cu2A TCATCAAATCAGCGCCCCACACCGCTATGACGCGGCATTTATCAATTTTTGTTTTTTCT 1560
Curcin TCATCAAATCAGCGCCCCACAYCCGCTATGACGCGGCATTTATCAATTTTTGTTTTTTCT 61
RIP-CP3 TCATCAAATCAGCGCCCCACACCGCTATGACGCGGCATTTCCTT---------- 53
CuP TCATCAAATCAGCGCCCCACACCGCTATGACGCGGCATTTCCTT---------- 264
RIP-CP1 TCATCAAATCAGCGCCCCACACCGCTATGACGCGGCATTTCCTT---------- 220
RIP-CP2 CCTTGGATTTTCAATAATTCTTTTTCATTGGGATTTGTTATTGTTTGTCTTTCTTTTTTT 225
CuLP CCTTGGATTTTCAATAATTCTTTTTCATTGGGATTTGTTATTGTTTGTCTTTCTTTTTTT 300
Cu2A CCTTGGATTTTCAATAATTCTTTTTCATTGGGATTTGTTATTGTTTGTCTTTCTTTTTTT 1620
Curcin CCTTGGATTTTCAATAATTCTTTTTCATTGGGATTTGTTATTGTTTGTCTTTCTTTTTTT 121
RIP-CP3 ----GCATTTTCAATAATTCTTTTTCATTGGGATTTGTTGTTGTTTGTCTT--------- 100
CuP ----GCATTTTCAATAATTCTTTTTCATTGGGATTTGTTGTTGTTTGTCTT--------- 311
RIP-CP1 ----GCATTTTCAATAATTCTTTTTCATTGGGATTTGTTGTTGTTTGTCTT--------- 267
RIP-CP2 TTTTTTTTTTGACAGTTGTTATTGTTTGTCTTTATTTTTACATCTTATGAAACTGTCGTT 285
CuLP TTTTTTTTTTGACAGTTGTTATTGTTTGTCTTTATTTTTACATCTTATGAAACTGTCGTT 360
Cu2A TTTTTTTTTTGACAGTTGTTATTGTTTGTCTTTATTTTTACATCTTATGAAACTGTCGTT 1680
Curcin TTTTTTTTTTGACAGTTGTTATTGTTTGTCTTTATTTTTACATCTTATGAAACTGTCGTT 181
RIP-CP3 ----------TATTTTACATCTTATGAAACTGTCGTT 127
CuP ----------TATTTTACATCTTATGAAACTGTCGTT 338
RIP-CP1 ----------TATTTTACATCTTATGAAACTGTCGTT 294
RIP-CP2 TGGTAGCTAATAATCAAAATAATAATAAAAAATAATAATAATAAAGAAAAAAAAAATTGAC 345
CuLP TGGTAGCTAATAATCAAAATAATAATAAAAAATAATAATAATAAAGAAAAAAAAAATTGAC 420
Cu2A TGGTAGCTAATAATCAAAATAATAATAAAAAATAATAATAATAAAGAAAAAAAAAATTGAC 1740
Curcin TGGTAGCTAATAATCAAAATAATAATAAAAAATAATAATAATAAAGAAAAAAAAAATTGAC 241
RIP-CP3 TGGTAGCTAATAATAATAATAATAATAATAATA--------ATAAAGAAAAAAAAATTGAC 179
CuP TGGTAGCTAATAATAATAATAATAATAATAAAG--------A-----AAAAAAAATTGAC 385
RIP-CP1 TGGTAGCTAATAATAATAATAATAATAATAATA--------ATAAAGAAAAAAAAATTGAC 346
RIP-CP2 AAAATAAAGGGCAGTTTCCCTATAAAAGCAGGTGATGGGGGAAGG-CAAAAGACCATCTC 404
CuLP AAAATAAAGGGCAGTTTCCCTATAAAAGCAGGTGATGGGGGAAGG-CAAAAGACCATCTC 479
Cu2A AAAATAAAGGGCAGTTTCCCTATAAAAGCAGGTGATGGGGGAAGG-CAAAAGACCATCTC 1799
Curcin AAAATAAAGGGCAGTTTCCCTATAAAAGCAGGTGATGGGGGAAGG-CAAAAGACCATCTC 300
RIP-CP3 AAAATAAAGGGCAGTTTCCCTATAAAAGCAGGTGATGGGGGAAGGCAAAAG-ACCATCTC 238
CuP AAAATAAAGGGCAGTTTCCCTATAAAAGCAGGTGATGGGGGGAAGGCAAAAGACCATCT- 444
RIP-CP1 AAAATAAAGGGCAGTTTCCCTATAAAAGCAGGTGATGG-GGGAAGGCAAAAGACCATCT- 404



See the legend to **Fig. 5** in the next page.

Fig. 5 Multiple sequence alignment of the nucleotide sequences of seven different forms of Curcin gene from the Jatropha cultivars. This alignment is based on Clustal omega tool. The star symbols denote the conserved sequences between all the seven forms of curcin gene studied. Alignment of Curcin gene showed some divergence with respect to the other six forms of curcin gene which are highlighted grey.

Multiple alignment sequence shows that there is no match of the query sequence i.e. curcin2A gene with other six sequences upto 1275-bp. Highlighted area showing the non-conserved area among the seven different nucleotide sequences and non-highlighted is the conserved area. It revealed that the promoter region of the curcin gene showed considerable sequence divergence if compared with the other members of this family. However, their coding regions are mostly conserved.

4.2 Analysis of amplicon profile in various *Jatropha* cultivars using genomic DNA

4.2.1 Isolation of genomic DNA from *Jatropha* accessions

Total genomic DNA was isolated from *Jatropha* cultivars namely TJS 17#03, TJS 42 #04, TJS 35#01, TJS 19#17, TJS 06#24 , TJS 01#03, TJS 01#04 and TJS 46#04 from the *Jatropha* leaf samples collected from the field grown plant. The quality of genomic DNA was checked by agarose gel electrophoresis as shown in Fig. 6.

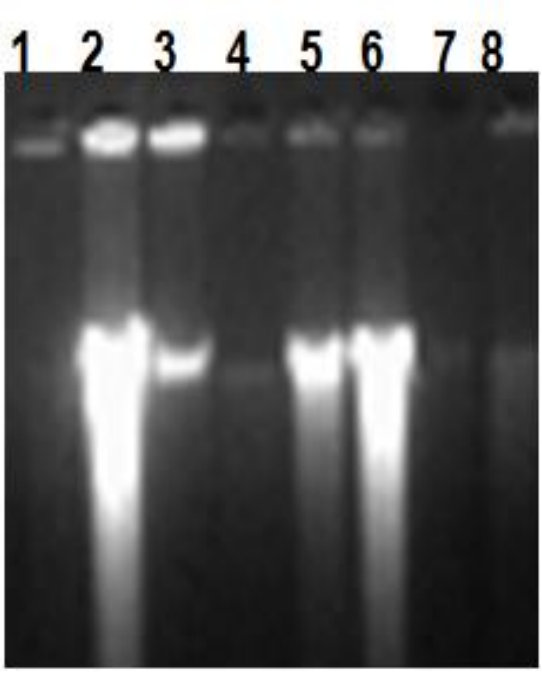


Fig .6 Genomic DNA isolation from Jatropha accessions, Lane 1- 01#13; Lane 2-TJS 17#03, Lane 3- TJS 42#04 , Lane 4- TJS 46#04 ,Lane 5- TJS 35#01, Lane 6- TJS 19#17, Lane 7- TJS 06#24, Lane 8- TJS 35#01

The quality and quantity of DNA was checked spectrophotometrically by measuring the A_{260}/A_{280} ratio respectively (Table 7),

Table 7 Quantification of *Jatropha* DNA using nanodrop spectrophotometer

Jatropha Cultivar	Amount of Material (g)	Volume of DNA soln. (µL)	Absorb. Ratio (A260/A280)	Conc. (ng/µL)
TJS17#03	0.7	50	1.23	920
TJS04#42	0.7	50	1.09	420
TJS35#01	0.7	50	1.00	810
TJS01#03	0.7	50	1.04	290
TJS19#17	0.7	50	1.00	810
TJS06#24	0.7	50	1.04	620
TJS46#01	0.7	50	1.35	600

4.2.2 Isolation of RNA from *Jatropha* accessions

RNA was isolated from *Jatropha* cultivar namely TJS 01#04, from the various *Jatropha* tissues i.e. leaf, seed kernel, seed pericarp and stem samples collected from the field grown plant (see section 3.2 of 'Materials & Methods'). The quality of RNA was checked by agarose gel electrophoresis as shown in Fig.7.

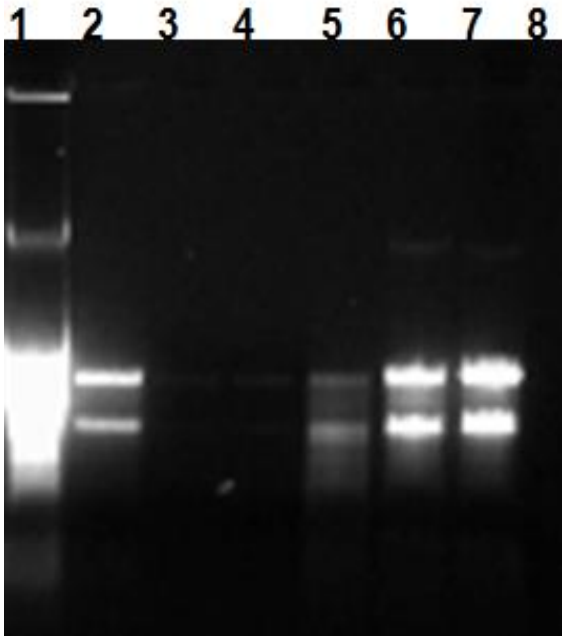


Fig.7 RNA isolation from various *Jatropha* tissues lane wise data is given as: Lane 1- Leaf ; Lane 2-seed kernel 1, Lane 3-seed kernel 2, Lane 4- seed kernel 3 ,Lane 5-stem, Lane 6-seed pericarp 1, Lane 7-seed pericarp 2, Lane 8- seed pericarp 3

4.2.3 Some details of Curcin2A gene specific primers as used in the study:

For our thesis work, we used three forward primers and two reverse primers corresponding to curcin2A gene. The features of these primers are discussed below:

Forward primers

- F1-CU126 is a forward primer that corresponds to bases 126-145 of the genome sequence i.e., 5′-AACTAGATTACAAGAGTAGC-3′
- F2-CU1318 is forward primer that corresponds to the bases 1318-1337 of the genome sequence i.e., 5′-TAAATGTTGGAATAGAAGAC-3′
- F3-CU1526 is forward primer that corresponds to the bases 1526-1545 of the genome sequence i.e., 5′-CTATGACGCGGCATTTATCA-3′

As the transcription start site is located at 2084 base, all three forward primers belong exclusively to extended promoter region of the gene.

Reverse Primers

- R1-CU2258 is a reverse primer that is complementary to the bases 2239-2258 of the genome sequence i.e., 5′-ATCAGTAGTAGCGTCATAAG-3′
- JCu2A-R1812 is a reverse primer that is complementary to the base 1793-1812 of curcin2A i.e., 5′- AAGAAGAGAGCGAGAGATGG -3′

The gene sequence corresponding to the reverse primers lies in the coding region encoding the polypeptide of the Curcin2A gene i.e., 309-aa peptide. The purpose was to see whether the sequences are conserved in the coding region in curcin gene family members. To ensure minimum chances of non-specific amplification, important factors kept in consideration while designing the primers were:

- PCR primers should be 15-20 nucleotides long.
- GC content should be 40-60%.
- The primer should not be self-complementary or complementary to any other primer to form primer-dimer or hairpin.
- Melting temperatures of primer pairs should not differ by more than 5º C, so the GC content and length must be chosen accordingly.
- The annealing temperature should be about 5°C lower than the melting temperature.
- Stretches of a particular nucleotide sequence is avoided.
- Primers with significant structures are avoided.

PCR using Curcin2A gene-specific primer pairs under different conditions

One of the major objective of the study is to amplify curcin gene(s) preferably with the upstream regions from a few *Jatropha* accessions through PCR approach under different conditions using

specific primer pairs. For this purpose, genomic DNA preparations from the following Jatropha accessions namely TJS17#03, TJS42#04,TJS19#17, TJS06#24, TJS46#01 TJS35#01 and TJS01#03 were used during PCR. For each specific primer pair, PCR was carried out under annealing temperature 55°C. Individual primer pair-specific amplicon profiles are shown in the following sections.

The following primer pairs were used to carry out individual PCR:

- F2-CU1318 and R1-CU2258
- F2-CU1318 and JCU2A-R1812
- F3-CU1526 and JCU2A-R1812
- F1-CU126 and JCU2A-R1812

i) Results of PCR using the primer pair: F2-CU1318 and R1-CU2258

Here Fig. 8 represents PCR amplification results of genomic DNA of different *Jatropha* cultivars using the primer set, F2-CU1318 and R1-CU2258. The sizes of the amplicons are shown in Table 8.

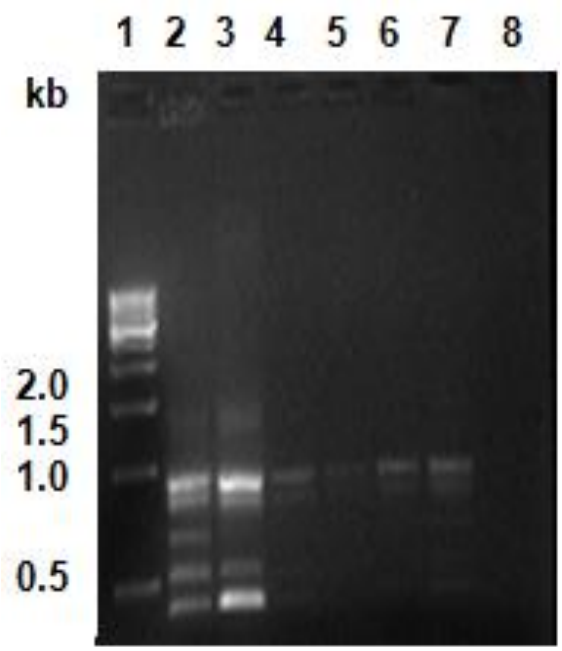


Fig. 8 PCR amplified products using gene specific primer pairs (F2-CU1318 & R1-CU2258) Annealing temp. 55°C *Lane* 1- 500 bp ladder*; Lane* 2- PCR amplified bands of TJS 42#04; *Lane* 3- PCR amplified band of TJS 17#03; *Lane* 4- PCR amplified band of TJS 01#03; *Lane* 5- PCR amplified band of TJS 06#24; *Lane* 6- PCR amplified band of TJS 35#01; *Lane* 7-- PCR amplified band of TJS 19#17; *Lane* 8-- PCR amplified band of TJS 17#03

Table 8 F2-CU1318 and R1-CU2258 primer pair-specific amplicons

Jatropha Accessions	Size of amplicons (annealing temp. 55°C)
TJS 42#04	~1.0 kb, ~0.9 kb, ~0.7 kb
TJS 17#03	~1.0 kb, ~0.9 kb
TJS 01#03	~1.0 kb, ~0.9 kb
TJS06#24	~1.0 kb, ~0.9 kb
TJS 35#01	~1.0 kb, ~0.9 kb
TJS19#17	~1.0 kb, ~0.9 kb
TJS17#03	-

For F2-CU1318 & R1-CU2258 primer pairs, expected size is ~1.0 kb. Moreover, 0.9 kb band was also found in most of the cultivars. In case of TJS 42#04, a prominent band of 0.7 kb was also found. No amplicons was found in TJS 17#03. At higher annealing temperature i.e. 55°C amplicons of 1.0 kb size was found in all *Jatropha* accessions, suggesting that this region of curcin2A gene is almost conserved in different Jatropha germplasm; However there may be some sequence heterogeneity.

ii) Results of PCR using the primer pair: F2-CU1318 and JCu2A-R1812

Here Fig. 9 represent PCR amplification results of genomic DNA of different *Jatropha* cultivars using the primer set, F2-CU1318 and JCu2A-R1812. The sizes of the amplicons are shown in Table 9.

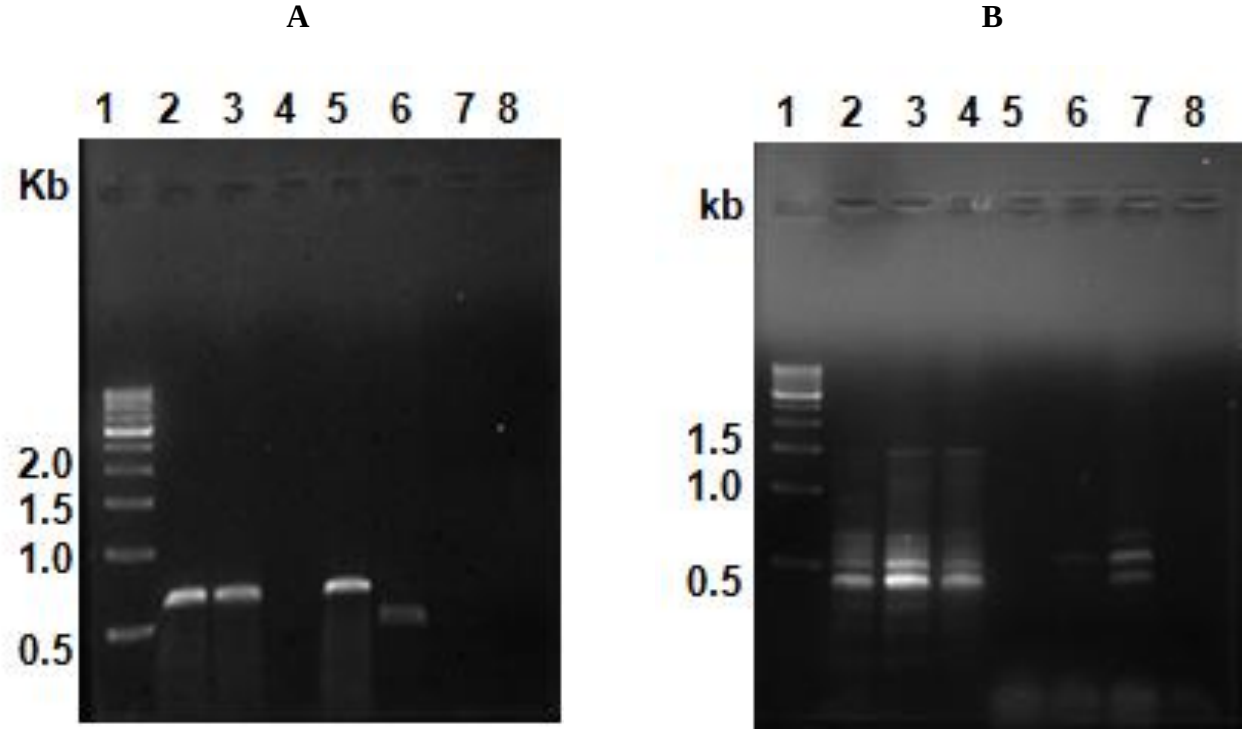


Fig. 9 PCR amplified products using gene specific primer pairs (F2-Cu1318 , JCu2A-R1812) **A** annealing temperature **55°C** and **B** annealing temperature **50°C** *Lane Lane* 1- 500 bp ladder*; Lane* 2- PCR amplified bands of TJS 17#03; *Lane* 3- PCR amplified band of TJS 04#42; *Lane* 4- PCR amplified band of TJS 01#03; *Lane* 5- PCR amplified band of TJS 06#24; *Lane* 6- PCR amplified band of TJS 35#01.; *Lane* 7-- PCR amplified band of TJS19#17; *Lane* 8-- PCR amplified band of TJS 17#03. Order of cultivars remains unchanged in both the figures.

Table 9 F2-Cu1318 & JCu2A-R1812 primer pair-specific amplicons at varying temperatures

Jatropha Accessions	Size of amplicons (annealing temp. 55°C)	Size of amplicons (annealing temp. 50°C
TJS 17#03	~0.7	~0.5
TJS 04#42	~0.7	~0.5
TJS 01#03	No band	~0.5
TJS 06#24	~0.7	No band
TJS 35#01	~0.6	~0.5
TJS 19#17	No band	~0.5
TJS17#03	No band	No band

For F2-CU1318 & JCu2A-1812 primer pair, expected size of amplicon is ~0.5 kb. At 55° C, the amplicon size was found to be slightly higher than the expected size in TJS17#03, TJS 04#42, TJS 06#24 and TJS35#01. However, the amplicon size at 50° C is nearly as expected size in the cases of TJS 17#03, TJS 04#42, TJS 01#03, TJS 35#01, TJS 19#17. Such differences in the amplicon size under varying annealing temperatures need to be understood.

iii) Results of PCR using the primer pair: F1-CU126 and JCu2A-R1812

Here Fig. 10 represent PCR amplification results of genomic DNA of different *Jatropha* cultivars using the primer set, F1-CU126 and JCu2A-R1812. The sizes of the amplicons are shown in Table 10.

In this case, the size of amplicon was found to be ~0.5 kb. This data is not consistent according to the expected size. This data clearly shows significant sequence divergence in the upstream promoter region which explains this forward primer failed to anneal with the template.

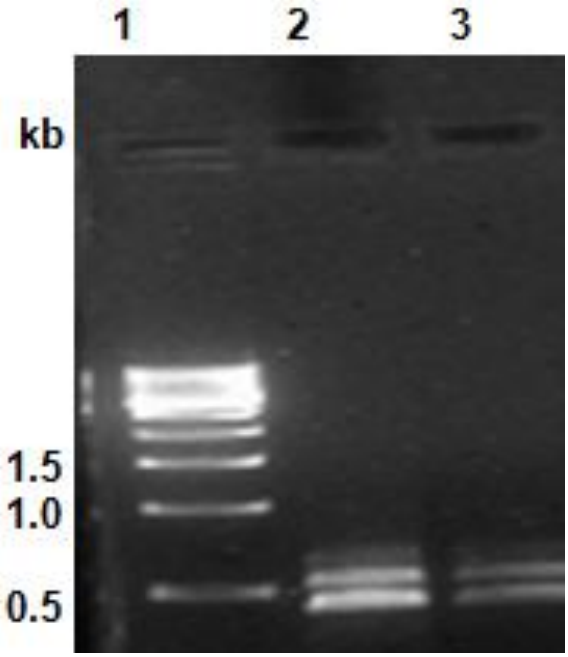


Fig.10 PCR amplified products using gene specific primer pairs (F1-Cu126 , JCu2A-R1812) Annealing temp. 55°C *Lane* 1- 500 bp ladder*; Lane* 2- PCR amplified bands of TJS 17#03; *Lane* 3- PCR amplified band of TJS 04#42

Table 10 F1-CU126 & JCu2A-R1812 primer pair-specific amplicons

Jatropha Accessions	**Size of amplicons (annealing temp. 55°C)**
TJS 17#03	~0.5 kb
TJS 04#42	~0.5 kb

. **iv) Results of PCR using the primer pair: F3-CU1526 and JCu2A-R1812**

Here Fig. 11 represent PCR amplification results of genomic DNA of different *Jatropha* cultivars using the primer set, F3-CU1526 and JCu2A-R1812. The size of the amplicons is shown in Table 11.

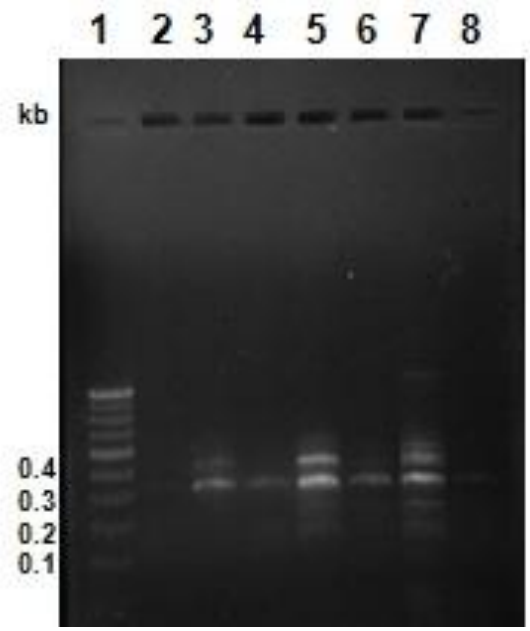


Fig.11 PCR amplified products using gene specific primer pairs (F3-Cu1526 , JCu2A-R1812) Annealing temp. 55°C *Lane* 1- 100 bp ladder*; Lane* 2- PCR amplified bands of TJS 42#04; *Lane* 3- PCR amplified band of TJS 17#03; *Lane* 4- PCR amplified band of TJS 01#03; *Lane* 5- PCR amplified band of TJS 06#24; *Lane* 6- PCR amplified band of TJS 19#17.; *Lane* 7-- PCR amplified band of TJS 35#01; *Lane* 8-- PCR amplified band of TJS 46#01

Table 11 F3-CU1526 & JCu2A-R1812 primer pair-specific amplicons

Jatropha Accessions	**Size of amplicons (annealing temp. 55°C)**
TJS 42#04	~0.3
TJS 17#03	~0.3
TJS 01#03	~0.3
TJS 06#24	~0.3
TJS 19#17	~0.3
TJS 35#01	~0.3
TJS46#01	~0.3

For F3-Cu1526 & JCu2A-R1812 primer pairs, expected size is ~0.3 kb. At higher annealing temperature i.e. 55°C amplicons of 0.3 kb size was found in all *Jatropha* accessions, suggesting that this region of curcin2A gene is almost conserved in different Jatropha germplasm; however there may be some sequence heterogeneity.

Usually, the coding regions remain more or less conserved between the members of a gene family. Considerable divergence occurs in their upstream regions which in turn control the overall functionality of the individual promoters. A number of forward primers were designed based on the upstream promoter sequence; whereas the reverse primers were based on the coding regions. PCR was carried out using different primer pairs. The purpose was to amplify the curcin gene along with the

promoters of varying length. Most of the amplicons as found in the study are quite consistent in terms of the size. However, some amplicons were found which could be promising. Some of the additional amplicons may represent the allelic variants of curcin gene having distinct promoters. Therefore, it is apparent that different amplicons including the expected ones need to be cloned and sequenced for further molecular study. Multiple allelism is a common feature in eukaryotic genes. The amplicon profiles as evident in this study also suggest that some of the *Jatropha* accessions have genotypic/allelic variations. Therefore, in-depth molecular studies are required to understand the variations in the curcin gene promoters.

CONCLUSION

This report made a consolidated base for further studying the structure, functions of curcin genes in *Jatropha*. Moreover this study would be very helpful for transgenic work in order to improve *Jatropha* accessions. The thesis work could be summarized as below:

- Sequence comparison revealed that the promoter regions of the curcin genes in *Jatropha* have considerable variations. However the coding regions of the individual isoforms are mostly conserved.
- Diverse *cis*-regulatory motifs were predicted in the 5′ flanking region of curcin2A gene which were not documented earlier.
- Multiple sequence alignment of the promoter regions of different forms of curcin such as curcin, curcin-L precursor, curcin2A, RIP clearly revealed that the proximal promoter regions are highly conserved as compared to their upstream regions. Therefore, it is very likely that various curcin forms differ with regard to their overall expression patterns.
- Genomic DNA was isolated from Eight *Jatropha* cultivars namely TJS 17#03, TJS 42 #04, TJS 35#01, TJS 19#17, TJS 06#24 , TJS 01#03, TJS 01#04 and TJS 46#04 and their quality checked through agarose gel electrophoresis. The DNA samples were used as template in PCR.
- A total of five oligonucleotides (20-mer each) namely F1-CU126, F2-CU1318, F3-CU1526 (forward) along with R1-CU2258, JCu2A-R1812 (reverse) were designed based on the Curcin2A gene sequence available in GenBank database (Accession no. GQ925453). Three forward primers were designed exclusively from the Curcin2A promoter region whereas, two reverse primers were designed from coding region. PCR was carried out using different combinations of primers.
- Some amplicons were found as expected. However, additional amplicons were also noticed in some of the *Jatropha* accessions. The results possibly explain allelic variants of curcin genes which needs to be understood.

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