GENOMICS OF FEMALE FLOWERING AND SEED YIELD IN *JATROPHA CURCAS* L.

Thesis submitted in fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

By

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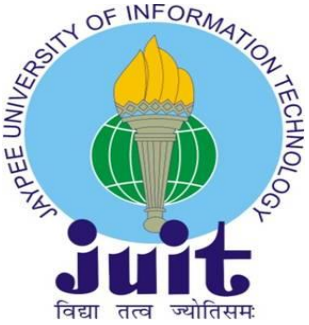

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ABSTRACT

Increased demand of fossil fuels has raised an interest towards culling an alternate and promising renewable source of energy. *Jatropha curcas* L., a non-edible energy crop of the family euphorbiaceae, has showed potential to provide feedstock for biodiesel due to its high oil content (42%) in the seed. The stumbling roadblock for commercialization of *Jatropha* based biodiesel production is low seed yield per inflorescence which is governed by low ratio of female to male flowers (1:25-30). On the other hand, exogenous application of cytokinin was done on floral meristems to increase the female to male flower ratio and seed yield. It resulted in increase in number of female flowers (~8-fold) and number of fruits (~3-fold) however, decrease in fruiting rate (number of fruits formed in comparison to increase in female flowers), and weight and size of seed was reduced. However, molecular mechanism associated to female to male flower ratio upon cytokinin treatment affecting their yield is not clear. The current study was under taken with the following objectives; (1) deciphering molecular cues for floral transitions and female flowering in *Jatropha curcas* through comparative genomics and gene transcript profiling; (2) deciphering molecular components of cytokinin on flowering and source-sink relationship of *Jatropha curcas* L.

Using comparative genomic approach, flowering related genes were selected from related species available in literature. Gene expression profiling of selected forty-two genes was carried out at six different floral developmental stages (vegetative to reproductive, differentiation of floral buds into male/female and transition towards female flowering) of *J. curcas*. *CRYPTOCHROME 2* (*CRY2*), *CUP-SHAPED COTYLEDON 2* (*CUC2*), *PINFORMED* (*PIN1*) *Tryptophan-pyruvate aminotransferase 1* (*TAA1*) and *Cytokinin oxidase 1* (*CKX1*) genes were identified for their role in female flowering and *SUPERMAN* (*SUP*) gene was observed for its role in the transition of female flower. Further, c*is-regulatory* elements for selected genes like GAREAT, UP2ATMSD and MYB1AT were observed for female flowering. Furthermore, to understand the molecular mechanisms underlying carbon assimilation response to cytokinin application, RNA-seq based comprehensive transcriptome sequencing of inflorescence meristems (treated with cytokinin) and control (untreated inflorescence meristems) at time-intervals of 15 and 30 days, was performed. Functional annotation through KEGG based *in*

silico approach, metabolic pathways associated with carbon channeling were observed. Pathways were mapped to photosynthesis, carbon fixation, carbohydrate metabolism and nitrogen metabolism. These pathways were upregulated post cytokinin treatment (15 days) however, after 30 days, genes involved in these pathways were observed to be downregulated. We observed significant downregulation of genes such as fructose-1,6-bisphosphatase I (*FBP*), sedoheptulose-bisphosphatase (*SBP*), glutamine synthetase (*GS*), glutamate dehydrogenase (*GDH*) and ADP glucose pyrophosphorylase (*AGPase*) downregulated post 30 days of cytokinin treatment. Temporal data suggested that there is a shift in the metabolic process majorly to carbon channeling that possibly cause the reduction in fruiting in Jatropha.

The current study provides a repertoire of genes/transcripts profile which can be explored further to increase the feedstock yield of Jatropha through transgenic or molecular breeding approaches. Further, present study provides information on how genetic factors allow differentiation of male/female flower and transitions towards female flowers as well as female to male flower ratio in Jatropha. The present study also shed lights on the metabolic pathways affected by cytokinin treatment thereby, altering source to sink ratio, in turn, the feedstock yield of Jatropha.

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All may not be mentioned, but no one is forgotten.

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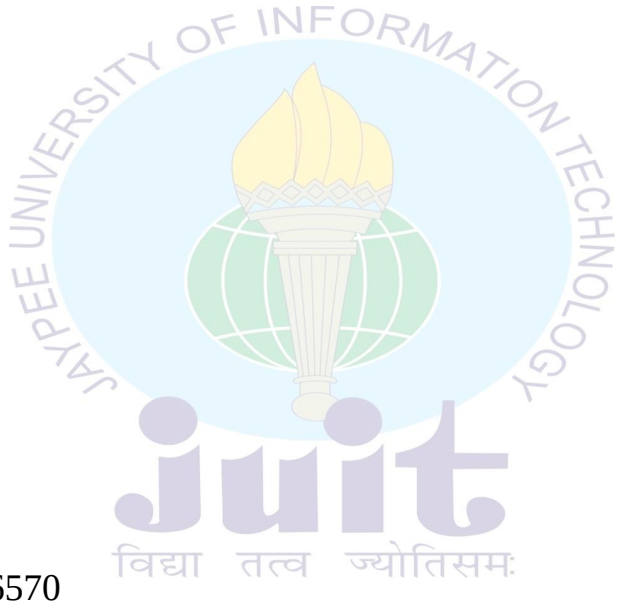
DECLARATION BY THE SCHOLAR

I hereby declare that the work reported in the Ph.D. thesis entitled **"Genomics of female flowering and seed yield in *Jatropha curcas* L"** submitted at **Jaypee University of Information Technology, Waknaghat, India,** is an authentic record of my work carried out under the supervision of **Dr. Jata Shankar** and co-supervision of **Dr. RS Chauhan**. I have not submitted this work elsewhere for any other degree or diploma. I am fully responsible for the contents of my Ph.D. Thesis.



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SUPERVISOR'S CERTIFICATE

This is to certify that the work reported in the Ph.D. thesis entitled **"Genomics of female flowering and seed yield in *Jatropha curcas* L"** submitted by **Ms. Manali Gangwar** at **Jaypee University of Information Technology, Waknaghat, India,** is a bonafide record of his original work carried out under my supervision. This work has not been submitted elsewhere for any other degree or diploma.

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LIST OF ABBREVIATIONS

ABA	Abscisic acid
ACLY	ATP citrate (pro-S)-lyase
ACO2	1-aminocyclopropane-1-carboxylic acid oxidase 2
ACS1	1-aminocyclopropane-1-carboxylate synthase1
ACS7	1-aminocyclopropane-1-carboxylate synthase 7
AG	Agamous
AGL-20	Agamous-Like 20
AHK2	Histidine kinase 2
ALDO	Aldolase
AMS	Aborted microspores
AMYA	Alpha-amylase
AMYB	Beta-amylase
AN1	Anther ear 1
AP1	Apetala 1
ARF2	Auxin response factor 2
ATP	Adenosine tri phosphate
AUX1	Auxin transporter protein 1
BA	6-Benzladenine
BBX19	B-box motif
BEL1	BELL1
BIF1/ BIF2	Barren Inflorescence 1 & 2
BIN1/2	B*rassinosteroid-insensitive*
BR	Brassinosteroids
BSK	BR-signaling kinases
BSR	Brassinosteroid resistant
CA	Carbonic anhydrase
CAL	Cauliflower

cDNA	Complementary Deoxy ribonucleic acid
CEN1	CentroradIalis 1
CHS A	Chalcone synthase A
CISZOG	Cis-zeatin O-glucosyltransferase
CK	Cytokinin
CKI1	Cytokinin-independent 1
CKX1	Cytokinin dehydrogenase/oxidase 1
CKX5	Cytokinin dehydrogenase/oxidase 5
CLV1	Clavata1
CRC	CRABS CLAW
CRE1	Cytokinin response 1
CRY1	Cryptochrome 1
CRY2	Cryptochrome 2
Ct	Cycle threshold
CTAB	Cetyl trimethylammonium bromide
CTR1	Constitutive Triple Response1
CUC1	Cup-Shaped Cotyledon 1
CUC2	Cup-Shaped Cotyledon 2
CycA3;2	cyclin-dependent protein kinase 3;2
CycD3;1/2	Cyclin-D3-1
CYP450	Cytochrome P450
CYP735A	Cytochrome P735
DAD1	Defective in anther dehiscence1
EBS	Early bolting in short days
EIN2	Ehylene-insensitive protein 2
ERS	Ethylene response sensor 1
ETR1	Ethylene receptor 1
FAME	Fatty acid methyl esters
FBP	Fructose-1,6-bisphosphatase I
FBP11	Floral Binding Protein 11

FCA	Flowering locus C
FD	Flowering Locus D
FLC	Flowering Locus C
FLO	Floricaula
FPA	Flowering time control protein FPA
FPGS1	Folylpolyglutamate synthetase
FPKM	Fragments per kilobase of transcripts per million mapped reads
FT	Flowering Locus T
FUL	Fruitfull
GA	Gibberellic acids
GAOX	Gibberellin *Oxidase*
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GASA4	Gibberellin-regulated protein 4 precursor
GDH	Glutamate dehydrogenase
GI	Gigantea
GID1	Gibberellin receptor protein
GLGA	Starch synthase
GLGB	1,4-alpha-glucan branching enzyme
GLGC	Glucose-1-phosphate adenylyltransferase
GO	Gene ontology
GPI	Glucose-6-phosphate isomerase
GPI	Glucose-6-phosphate isomerase
GS	Glutamine synthetase
HEC2	Hectate 2
HK	Hexokinase
IAA	Indole-3-acetic acid
IDH	Isocitrate dehydrogenase
IPT1	Adenylate isopentenyltransferase
IPT2	tRNA dimethylallyltransferase
IPT3	Adenylate isopentenyltransferase

IPT9	tRNA dimethylallyltransferase
JS	Jasmonic acid
KAAS	KEGG Automatic Annotation Server
KEGG	Kyoto encyclopedia of genes and genomes
LAP3	Less adherent pollen
LHC	Light-harvesting complex
LOG	Lonely Guy
m asl	Meters above sea level
MED13	Mediator complex subunit 13
mRNA	Messenger ribonucleic acid
MSI4	Multicopy suppressor of Ira4
MYB98	Myeloblastosis98
N3	Nodulin
ng	Nanogram
ng	Nanogram
NR	Nitrate reductase
PAN	Periantha
PCA	Principal Component Analysis
PCR	Polymerase chain reaction
PDH	Pyruvate dehydrogenase
PEBP	Phosphatidylethanolamine-binding protein
PFK	6-phosphofructokinase 1
PGK	Phosphoglycerate kinase
PGL1	Polygalacturonase 1
PGM	Phosphoglucomutase
PI	Pistillata
PIN1	Pinformed
PLACE	Plant cis-acting regulatory DNA elements
PPR	Pentatricopeptide repeat-containing protein
PRK	Phosphoribulokinase

PSI/II	PhotosystemI/II
PVP	Polyvinylpyrrolidone
RBCL	Ribulose-bisphosphate carboxylase large chain
REV	Revoluta
RGL	Repressor of ga1-3-LIKE protein
RL1	Radialis-like 1
RNA	Ribonucleic acid
RPIA	Ribose 5-phosphate isomerase A
rRNA	Ribosomal RNA
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
SAM	Shoot apical meristem
SAUR	Small Auxin-Up RNA
SBP	Squamosa Binding Protein
SIN1	Short Integument 1
SOC1	Suppressor of overexpression of Constans 1
SPA	Spatula
SPA1	Suppressor of phyA-105
SPL	Squamosa promoter binding protein-like
SPS	Sucrose phosphate synthase
STY	Stylosa
SUP	Superman
TAA1	L-tryptophan-pyruvate aminotransferase
TDF1	Defective in Tapetal development and function 1
TDZ	Thidiazuron
TFL1	Terminal Flower 1
TFL1	Terminal Flower 1
TIR1	TRANSPORT INHIBITOR RESPONSE1
TK	Transketolase
TLP	TUBBY-like protein
TR15	Treated inflorescence post 15 days of BA treatment

TR30	Treated inflorescence post 30 days of BA treatment
TS2	Tasselseed 2
TSS	Translational start site
TYPA1	Tyrosine phosphorylated protein A
µg	Microgram
µl	Microlitre
VEG	Vegetative
WEPA	World environmental protection agency
WUS	Wuschel

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CHAPTER 1

INTRODUCTION

Rising demand for fossil fuels has attracted the attention of researchers towards biofuels due to gradual exhaustion of fossil fuels and increased pollution, inflicting global warming. The global oil consumption is about 11 billion tons and ~4 billion tons of crude oil is vanishing every year. With this rate of oil consumption our known oil deposits will not last for longer time. Also, combustion of fuels are the major contributors to global warming [1]. To keep a check on global temperature, a treaty has been signed in Paris agreement to keep the increase in temperature below 2°C. To achieve this, we will have to leave 70-80% of fossil fuels untouched [2]. Thus, an alternate source of energy is needed which is sustainable and eco-friendly. Biofuel such as bioethanol and biodiesel has already providing as an alternative source to fossil fuel and have an advantage in terms of renewability and environment friendly. Biodiesel from plants is an excellent substitute for fossil fuels as it is non-toxic, biodegradable and the amount of carbon monoxides and hydrocarbons emitted is lower than that from petro-diesel [3]. An important component of plant seed oils is triacylglycerols has similarity to fossil fuel material thus, an excellent precursor for producing biodiesel. Transesterification of triacylglycerols in plant seed oils with methanol in presence of an alkali or acid forms biodiesel chemically known as fatty acid methyl esters (FAMEs) [4]. The efficacy of biodiesel relies on the composition of fatty acids in the seed oil. Five types of fatty acids are present in plant oils which are Palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3). Earlier, edible crop plants like soybean, canola oil, sunflower etc. were used for biodiesel production which caused the scarcity of overall food supply and agricultural lands. Thus, for sustainable biodiesel production, non-edible crops have gained importance due to non-competition with food security and soil with food crops. Ideal biodiesel crop can be grown in wastelands thus no competition for agricultural land for food crops as well as cost-effective [5].

Of many energy plants, *Jatropha* (*Jatropha curcas* L) has emerged as a potential bioenergy plant and considered as an economically important plant attributed to its high seed oil content (45-50%). *Jatropha* is a perennial shrub or a small tree which belongs to family euphorbiaceae. It can grow upto a height of 6 m and have 40-60 years of life expectancy.

Oil can be extracted from *Jatropha* seeds after 2-5 years depending upon the climatic conditions. Being a monoecious plant i.e. male and female flowers grow on the same plant. Flowers are either unisexual or hermaphrodite and are pollinated by bees and moths [6]. Morphologically diverse genus *Jatropha* comprised of more than 200 species which are dispersed primarily in dry tropical areas of America. *Jatropha*, primarily from Central America, has spread into tropical and subtropical areas. Now *Jatropha* is cultivated globally as a biodiesel crop [7]. It is introduced in India in the16th century by Portuguese settlers. About 18 species of *Jatropha* are found in India and are scattered across various states [8]. *Jatropha* can easily grow in extreme climatic conditions such as in temperate and semi-arid climates, tropical savannah and monsoon climates, without any requirement of the special nutritive regime [9]. Another factor for popularity of *Jatropha* oil is the higher content of unsaturated fatty acids and high oil content (50%) and it is a non-edible crop for human being, thus no competition with food security (Table 1.1). *Jatropha* has been listed as a fuel and fuel additive with the world environmental protection agency (WEPA) [10]. *Jatropha* has gained prominence over other oil seed plants because of its added features like excellent adaptability to various habitats, rapid growth, easy propagation, wide adaptability, larger fruits and seeds, drought hardiness, soil conservation capabilities, small gestation period, thriving well as a live fence and can easily be grown in wastelands. *Jatropha* seeds are toxic because of presence of phorbol esters and curcin thus, mainly used for biodiesel purpose. Even though it's a potent biodiesel crop and toxic in nature, it has a medicinal value. Most of the parts of *Jatropha* is used as an industrial raw material for making insecticides, soaps, cosmetics etc. and a source of green [11]. Though numerous efforts have been made to develop *Jatropha* as an industrial crop, the scant information on its agronomic practices and lack of improved genotypes and cultivars are the major bottlenecks in its full exploitation as a potential bioenergy crop.

Table 1.1: Fatty acid composition and oil content of major oil plants [12]

Fatty acids (%)	***Jatropha***	**Castor bean**	**Sunflower**	**Soybean**
Palmitic acid	10	3	10	10
Stearic acid	10	2	5	5
Oleic acid	45	10	30	35
Linoleic acid	35	10	50	45
Linolenic acid	1	-	5	5
Ricinoleic acid	-	75	-	-
Total oil content	25-50	40-45	25-35	20-25

Due to various constraints like low seed yield, unreliable flowering and fruiting, non-availability of sufficient feedstock, limited availability of wasteland and high plantation maintenance cost limits the commercialization of this plant as a source of biodiesel.

Jatropha feedstock is highly affected by seed oil content, number of branches per plant, number of fruits per bunch, number of seeds per fruit and seed weight/size etc. The seed yield of *Jatropha* majorly depends on a number of female flowers per inflorescence. At each inflorescence, 10-12 female flowers are formed out of ~300 present at each inflorescence. This results in only 8-10 ovoid fruits and the number is quite low compared to the number of flowers present at the inflorescence [13]. Thus, increasing the number of female flowers could increase the overall yield of *Jatropha*.

Phenotypic study on floral development of *Jatropha* was conducted to observe how sex differentiation occurs during flowering. When male and female start to differentiate, the top of female elongates whereas, no such development occurs in male flowers. Female flowers are present in a bisexual stage until sixth phase of development. As sexual differentiation commences, abortion of male occurs in female flowers and traces of aborted stamens could be found in mature females. However, the development of male flower is unisexual right from the beginning and no traces of females are present. When abortion of male tissues does not occur in female flowers, then, they develop as males at the female flowering site. Such inflorescence is called as middle type inflorescence with either female/male flowers at an inflorescence. These middle type inflorescences showed

variation in a total number of female flowers at each inflorescence. When statistical analysis of *Jatropha* inflorescences was done, it was observed that middle type inflorescences constitute ~75% whereas, females type inflorescences represents only 0.09% at the inflorescence. The study also demonstrated that out of 18 female sites only 7 female flowers are formed (Figure 1.1) [14, 15]. Thus, these might have important role in increasing the female to male flower ratio. Apart from male/females, hermaphroditic flowers were also reported in *Jatropha* [15-17]. Structurally, they are similar to females having diffused stamens [18]. Through these observations, transforming the male-type inflorescence either into the middle-type or the female-type inflorescence can be targeted to increase the number of female flowers.

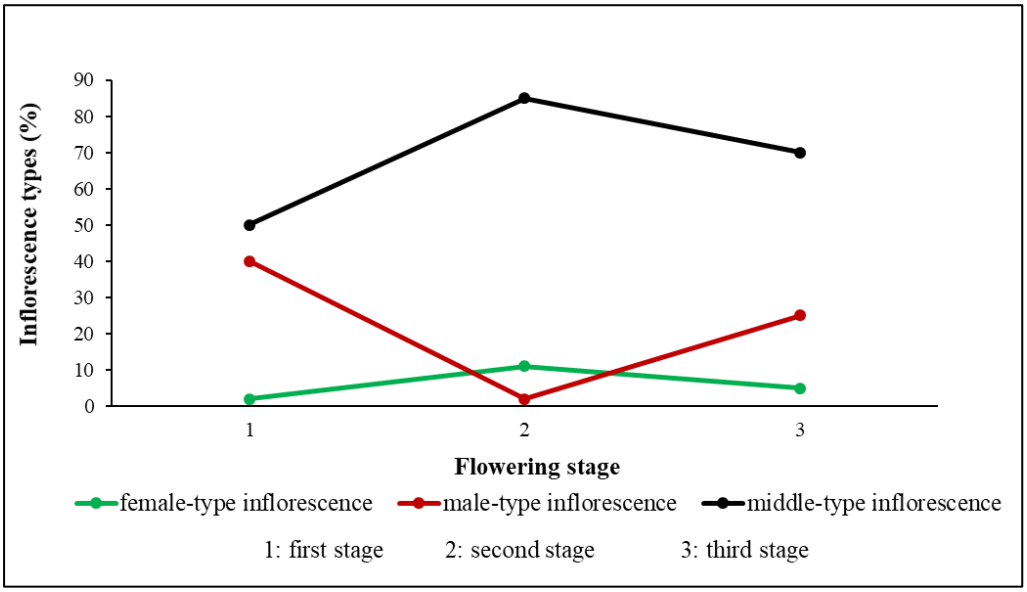

Figure 1.1: Inflorescence types at different flowering stages of *Jatropha* [15].

Jatropha has been studied at the molecular level to illustrate the mechanism of oil biosynthesis and other yield traits. *Jatropha* genome has been sequenced by Sato et al. [19] in 2011 and has been made publicly available. The size of *Jatropha* genome is ~410Mb and estimated number of assigned protein encoding genes is 40,929 and transposon-related genes are 16,447 [19]. Transcription analysis of staminate and in-staminate flowers of *Jatropha* did shed light on the role of hormones in development of floral organs. Through transcriptomics, genes such as *CUP-SHAPED COTYLEDON 1* (*CUC1*), *LEAFY* (*LFY*), *GIGANTEA* (*GI*) and *SUPPRESSOR OF CONSTANS1* (*SOC1*) were found to activate

flowering signals [20-22]. Gene encoding Gibberellin-regulated protein 4 precursor (GASA4) and AMP-activated protein kinase were observed to be associated with stamen differentiation. Gene encoding pentatricopeptide repeat-containing (PPR) proteins found to restore the cytoplasmic male sterility during gametogenesis [20]. A recent study on sex differentiation of *Jatropha* identified that cytokinin activates the formation of female floral primordia and flavonoid biosynthesis activate male development [23]. Other phytohormones such as brassinosteroids (BR) signaling, jasmonic acid (JA) signaling and abscisic acid (ABA) signaling promotes the female floral development [23]. Despite these studies, information on molecular mechanisms that are associated with male and female floral determination is limited. The ratio of female to male flowers vary in different *Jatropha* genotypes with respect to season, climatic conditions (India, Mozambique, Ethiopia, Mexico, Brazil, Honduras) and nutrition [14, 24]. Genetic factors contributing to these changes in female to male flower ratio is not available. Furthermore, molecular mechanisms underlying during female flower transition is not studied yet. Thus, the study was conducted to identify genes associated with sex determination and abortion of male tissues which can be targeted for genetic engineering in *Jatropha* for enhanced yield.

Increase in a number of flowers (females) with a subsequent increase in number of fruits has been demonstrated by exogenous treatment of growth hormones like gibberellic acids, cytokinins and brassinosteroids etc. [25, 26]. Of all the phytohormones, cytokinins have demonstrated as a most promising growth regulator for improving the number of female flowers and seed yield. In *Jatropha*, benzyl adenine (BA) and thidiazuron (TDZ) treatments resulted in a radical increase in total floral buds, female flowers and induced bisexual flowers [25, 27]. Applications of cytokinins did increase the number of fruits i.e. 2-3-fold, however when the fruiting rate was compared to increase in female flowers (9-10 fold), it was relatively low. Also, in cytokinin treated fruits, reduction in 100 seed weight was observed [21, 27]. This reduction in seed yield was also observed in other plants such as jojoba, lupin and soybean in response to cytokinin application [28-30]. In cytokinin treated seeds, only 1% increase in oil content was observed by Pan and Xu [25] whereas, Chan et al. [21] observed decrease in seed oil content. Further studies need to be conducted to observe the effect of cytokinin on seed oil content.

Differential transcriptional profiling of cytokinin treated *Jatropha* inflorescence shed light on molecular insights of the increased number of flowers by delaying floral organ forming genes and induced the expression of flowering genes [21, 22]. Thus, the correlation of

fruiting rate and seed yield (weight and size) at an inflorescence, treated with cytokinin, needs to be explored. Further, molecular insights of central carbon channeling towards biomass (florets) and overall yield due to foliar phytohormone application, is not identified in *Jatropha*. Furthermore, it is unclear how exogenous application of hormone such as cytokinin may affect physiology and molecular precursors. Thus, to gain insight into genetic factors that determines male/female flowering and transition towards female flowering, and how the application of hormone could affect the molecular precursors to increases the biomass (florets) and seed yield, following objectives were taken:

1. Dissecting the molecular mechanism underlying during floral transitions and female flowering in *Jatropha curcas* through comparative genomics and transcript profiling
2. Deciphering the molecular components associated with carbon channeling in cytokinin treated inflorescences affecting biomass and seed yield in *Jatropha curcas*

CHAPTER 2

REVIEW OF LITERATURE

2.1 Biodiesel

In 1890s Rudolph Diesel, the inventor of diesel engine designed engine that could be powered by vegetable oils for remote areas. First public demonstration of this engine was done in 1900 World's fair. Shortly after his death, fossil fuels (petro-diesel) become widely available and the engines were modified for modern petro-diesel. Transesterification of vegetable oils for producing biodiesel was proposed in 1937 [31]. Biodiesel, an alternative for conventional or 'fossil' diesel, produced from vegetable oil, animal fats and waste cooking oil through transesterification process [32]. It is an eco-friendly and a renewable source of energy. There is no net carbon produced through burning of biofuels as oil-crops absorb carbon at the same rate as it is released through combustion of biodiesel [33]. Transesterification is the process of converting triacylglycerols present in fatty acids to form glycerol and alkyl ester in presence of alcohol (Figure 2.1) [34].

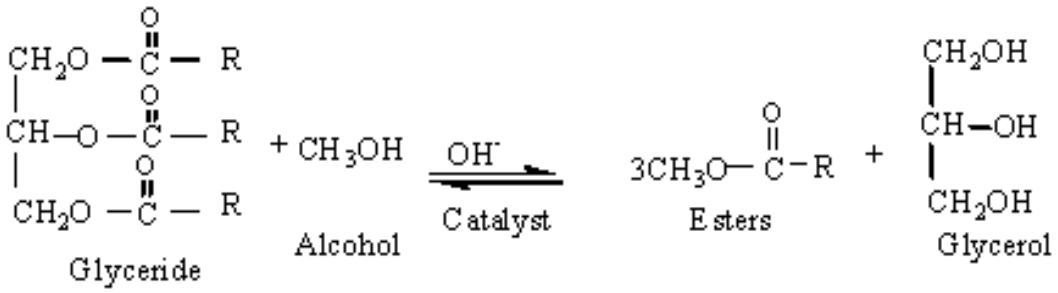

Figure 2.1: Transesterification reaction [34]. R represents fatty acid chains.

2.2 Feedstocks of Biodiesel

Plants and algae are the potential sources of biodiesel. Various oil-crops such as soybean, canola, rapeseed, sunflower, palm kernels and cotton seed have been used as a feedstock for commercial production of biodiesel (Table 2.1) [35]. Being edible crops, a competition with food security as well as agricultural land occurs, hence non-edible oil-crops could be the better source for biodiesel feedstock. Thus, non-edible oil from *Jatropha curcas* (*Jatropha*), is a potential feedstock for biodiesel production in tropical areas such as India

and Africa [36]. Algae, another potential feedstock for biodiesel have low monounsaturated fatty acids and high cost of biodiesel production. Thus, *Jatropha* has gained an economic value for biodiesel production.

Table 2.1: Comparison of different feedstocks for biodiesel [35]

Source	Oil yield (kg/ha)	Crop
Azadirachta indica	2670	Medicinal used
Jatropha curcas	1900–2500	Non-edible
Pongamia pinnata	225–2250	Food-crop
Ricinus communis	450	Food-crop
Zea mays	172	Food-crop
Cocos nucifera	2686	Food-crop
Elaeis guineensis	5989	Food-crop
Brassica napus	1190	Food-crop

2.3 Taxonomy classification and origin of *Jatropha curcas*

Kingdom Plantae
Subkingdom Tracheobionta
Division Magnoliophyta
Sub division Spermatophytina
Class Magnoliopsida
Subclass Rosidae
Order Euphorbiales
Family Euphorbiaceae
Genus *Jatropha*
Species *J.* curcas

Jatropha curcas belongs to the spurge family, Euphorbiaceae. The genus *Jatropha* is related to tribe Joannesieae of the subfamily Crotonoideae [37]. In 1753 Linnaeus named *Jatropha curcas,* a physic nut. Genus '*Jatropha*' is originated from a Greek word jatr´os which stands for 'doctor' and troph´e for 'food', suggesting its medicinal value [38]. The origin of *Jatropha* is Central America and is confined to the tropical regions. *Jatropha* was also found in the coastal areas of Mexico. It was then introduced into Africa and Asia, as a hedge plant [38-40]. About 175 species of *Jatropha* are known, native to South America and about 17 species are found in India. Subdivision made by Pax in 1910 was revised and two distinguish subgenera **(***Jatropha* and Curcas**)** of the genus *Jatropha* was introduced [41]. They have 10 sections and 10 subsections for the accommodation of the old and new species in genus *Jatropha*. Now, about 200 species of *Jatropha* are known. Dehgan and Webster [37] postulated that physic nut i.e. *Jatropha curcas*. Other species are evolved from the ancestral form with changes in habitat. The genetic fidelity of *Jatropha* genus is questionable as the species easily forms artificial and natural hybrid complexes [42].

2.4 Morphological features of *Jatropha curcas*

The height of *Jatropha* plant is ~5-6 meters with a life expectancy of 40-50 years. *Jatropha* plant is easily identified by its dense leaves, smooth gray bark and strong branches (Figure 2.2). When the bark is cut, white colored latex oozes out. Leaves are glossy and glabrous in texture and pale green in color [43]. Leaf of a *Jatropha* plant is large and 3-5 lobed with spiral phyllotaxis. *Jatropha* is a monoecious plant having both male and female flowers on the same plant and on same inflorescence. Flowers are unisexual and greenish yellow/dark green in color. At the inflorescence, higher number of male flowers are present as compared to females [42]. The inflorescence forms in the leaf axile at branch terminal [43]. Fruits are capsulated and ovoid with length and width of 4-5 cm and 2-3 cm, respectively. When formed, the fruit is green in color which when ripe, it turns into yellow in color and dark brown colored at the mature stage. A single fruit contains 3 elliptical, black colored seeds [43].

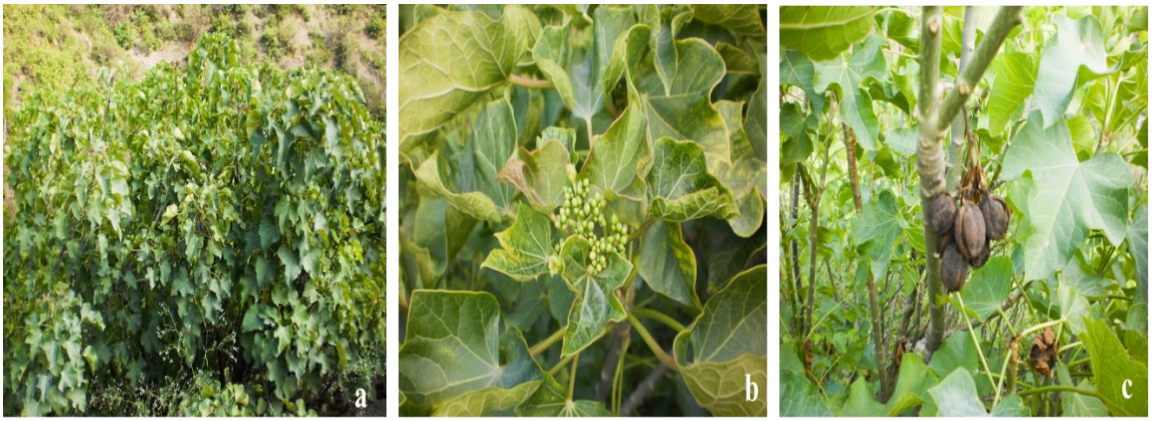

Figure 2.2: Morphology of *Jatropha curcas* (a) *Jatropha* plant (b) *Jatropha* plant with inflorescence (c) *Jatropha* plant with fruits.

2.5 Application of *Jatropha curcas*

2.5.1 Fuel uses

One of the most important use of *Jatropha* is to produce a high-quality biodiesel. Seed oil is transformed into biodiesel through transesterification process. The characteristics and physicochemical properties are similar to that of diesel **(**Table 2.2**)** [44, 45]. With diesel, up to 40% of Jatropha biodiesel can be blended. This diesel mix can be used in the existing combustion engines of cars. Crude *Jatropha* oil can be used as an illuminant as well as for cooking in place of kerosene because it does not produce smoke when burns. After oil extraction from seed, waste material like press cake, fruit exocarp, seed shell and pruning materials can be used for biogas production [13]. Nutshells and woody parts and nutshells can be transformed into charcoal.

Table 2.2: Comparison of properties of *Jatropha* oil vs Diesel [44, 45]

Properties	**Diesel**	***Jatropha curcas* oil**
Density**(**gm/cc**)** at 30 °C	0.836–0.85	0.93292
Kinematic viscosity **(**cSt**)** at 30 °C	4-8	37-52.76
Pour point **(**°C**)**	-20	-3
Cloud point **(**°C**)**	-15- -5	2

Flash point **(**°C**)**	68	210-240
Calorific value	42–46	38.20
Cetane No.	38	40–55
Sulphur %	2	-
Iodine No	-	94.00
Saponification value	-	198.00

2.5.2 Non-fuels use

Other plant parts such as leaves, stem, flowers etc. have many used. Flavonoids, cyanogenic glycosides, alkaloids, phlobatannins, cardiac glycosides, tannins and saponins have been identified in leaves of *Jatropha* have antioxidants and antileukemic properties making its potential medicinal plant [46]. Leaves of *Jatropha* can be used to treat strained muscles. Stem bark of *Jatropha* contains tannins which are used to make blue dyes. Ointment made from the leaf and bark extracts accelerates the healing process. Leaves are also used as a feedstock for silkworms [47]. The latex also contains flavonoid and saponin which has antioxidant, anticancer and anti-inflammatory activities [48]. The press cake is rich in proteins, carbohydrates and essential minerals like nitrogen, phosphorous and potassium making it an excellent fertilizer. Due to high saponification, *Jatropha* oil is also used in soap industry. It is also used as a lubricant for machinery [49]. This plant is also used as a hedge plant as animals do not feed on *Jatropha*. This plant also helps to reduce erosion due to water and/or wind (Table 2.3) [13].

Table 2.3: Uses of different plant parts of *Jatropha*

Plant parts	**Medicinal Uses**	**Other Uses**	**References**
Leaves	Treat strained muscles	Feedstock of silkworms	[47, 50]
Stem	Guinea worm infection, tumors, syphilis, skin infestation, abortifacient	Cooking fuel, illuminant	[51]
Bark	Muscular pain, Diabetes, Sore mouth	Make dark blue dyes	[47, 52, 53]
Latex	Heartburn and heartache, scabies, burn,	-	[47, 54]
Roots	Anthelminthic, Dysentery, Gonorrhea, Dressing wound and sores		[55]
Nuts	Contraceptive	Cooking fuel, illuminant	[32]
Seeds	Treating arthritis and jaundice. Used as contraceptives	Biodiesel, lubricating machinery, soap industry	[49, 56]
Press cake	-	Fertilizer and animal feed	[57]
Plant extract	Allergies, burns, cuts and wounds	-	[58]
Plant sap	Dermatomucosal diseases	-	[58]

2.6 Floral Biology of *Jatropha curcas*

2.6.1 Morphology

Jatropha is a monoecious plant bearing both male and female flowers at each inflorescence. It has racemose inflorescence in a dichasial cyme pattern. Inflorescences are formed at the terminal or axis of the branches with higher number observed in the areas exposed to sun [59, 60]. Inflorescences are formed by the grouping of 6 or 10 individual cymes, resulting in secondary/tertiary inflorescence present at the base of the main inflorescence with tertiary inflorescences attached to it [61]. Length of the inflorescences varies from 5 to 25. *Jatropha* flowers **(**both male and female**)** measure around 0.75cm to 0.9cm in length and 0.3cm to 0.4cm in width. Flowers have five sepals and petals [14].

At the inflorescence, single female flower is present at the top surrounded by male flowers. At the first sub-branch, female flower may be present at the joint of the nodes of a dichotomous branch [14]. There are three types of flowering sites in the inflorescence:

female flowering sites where female flower forms, male flowering sites where only male flowers are formed and the intermediate flowerings sites where both male and female flowers are formed. *Jatropha* is a male dominant plant with a ratio of male to female flower 25:1. Males can develop at the female flowering sites whereas, the possibility of development of females at the male sites is zero. Female flowers consist of three stigmas, three styles and a glabrous ovary, which are green in color **(**Figure 2.3a**)**. The floral base consists of five flat square yellow glands [14]. Male flowers are greenish yellow in color having 10 diadelphous **(**fused by filaments in two separate bundles**)** stamens **(**Figure 2.3b**)**. Stamens are dicyclic i.e. present in two whirls and has four microsporangia [17]. Anthers are ten in number and are dithecous (two anther lobes), dorsifixed (attached dorsally to the filament), and dehisce by the longitudinal slits. At the floral base of male flowers, five oval-shaped yellow glands are present [14, 37]. Pollen are globular and binucleate which becomes nonviable after 48 hours [14, 17]. Presence of hermaphrodite flower in the *Jatropha* has also been reported, which is similar to female flowers with 8-10 stamens. Pollens of hermaphrodite are weak and less viable with lower germination rates [16].

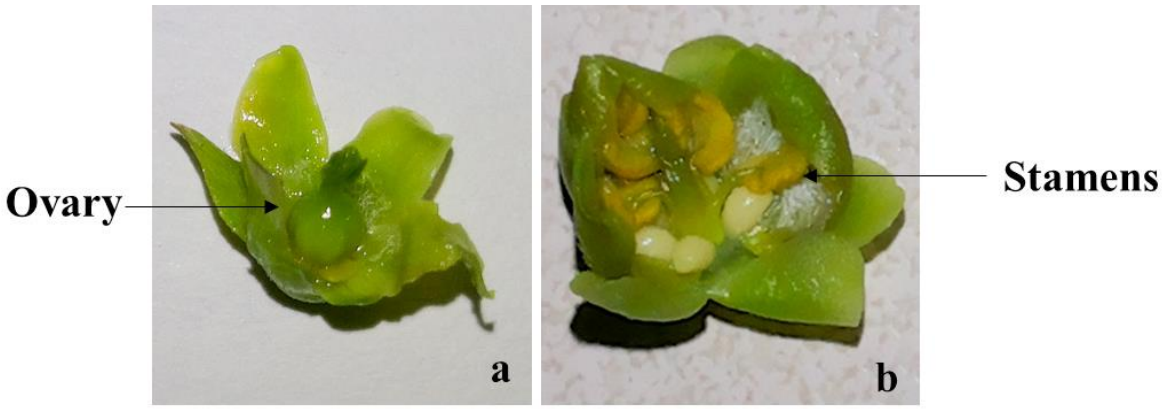

Figure 2.3: Morphology of *Jatropha* flowers (a) Female flowers (b) Male flowers.

2.6.2 Biology of sex differentiation

The floral development of *Jatropha* has been divided into 12 phases starting with vegetative to reproductive transition forming inflorescence meristem. During the first five phases no sexual differentiation occurs, and the females are present as bisexual tissue. Further development causes the abortion of male tissue allowing the development of female flower. No traces of female tissues were found in the male flowers. Thus, there are

two modes of development in *Jatropha*: formation of female flowers after the abortion of male tissues and the other is the formation of male flowers with early adolescence and no occurrence of female primordia [15, 62]. As male can occupy female flowering site, causing the decreased ratio of female to male flowers. The inflorescence has less number of female flower **(**average 8-10**)** despite of having 18 female flowering sites due to 0.09% of female type inflorescences compared to 75% middle-type inflorescence [15]. Thus, to raise the female flower number, the most effective approach is either to transform male type inflorescences to middle of intermediate type or by increasing the male abortion rate, allowing female flowers to develop. This would only be possible by having the knowledge of genetic switches causing the transition towards female flowers. This is one of the method to increase seed yield, as fruits are formed only by female flowers.

2.7 Molecular cues for flowering

Floral initiation, a process in which shoot apical meristem develops into the inflorescence meristem which forms reproductive organs. The process is controlled at environmental and genetic level, regulating various genes associated with triggering floral pathways. Flowering is initiated by vernalization, photoperiod, hormones, autonomous and age-related pathways. These signals initiate the reproductive phase by inducing meristem identity genes, which directs the groups of cells of SAM to differentiate into floral meristems in an irreversible manner [63]. The floral induction signaling is mediated via floral integrator genes *FLOWERING LOCUS T* (*FT*), *FLOWERING LOCUS C* (*FLC*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) which are activated in response to various genetic pathways. These integrator genes further, activate the floral meristem identity genes which initiates flowering at the apical meristem. *FT, a* mobile flowering signal (floreign) which induces transition towards reproductive phase. Activity of *FT* is induced by *CONSTANS* (*CO*) and *GIGENTEA* (*GI*) which are circadian clock–controlled genes, promoting photoperiod mediated flowering [64-66]. *FT* is expressed in leaf phloem once activated and moves to the shoot apex and forms a complex with *FLOWERING LOCUS D* (*FD*), a basic domain/leucine zipper protein. *FT/FD* heterodimer further activates *APETALA1* (*AP1*) and *SOC1*, a floral meristem identity gene and a floral promoter respectively [67, 68]. *AP1, FRUITFULL* (*FUL*) and *CAULIFLOWER* (*CAL*) genes regulates the flower meristem identity and inflorescence architecture by regulating *LFY* and *TERMINAL FLOWER 1* (*TFL1*) genes [69, 70]. *FT* & *LFY* genes have been

isolated and characterized in *Jatropha*. *FT* is a member of *FT*/*TFL1* family that have phosphatidylethanolamine-binding protein (PEBP) domain. *FT* and *TFL1* acts antagonistically in which *TFL1* suppresses flowering. In *Jatropha FT* was expressed mainly in the reproductive organs whereas, *TFL1* in roots. Overexpression of *FT* & *LFY* induced early flowering in *Jatropha* [71, 72]. Co-suppression of *LFY* in *Jatropha* resulted in delayed flowering with more number of secondary inflorescence branches and abnormal flowers [73]. Though, these studies shed light on genetic factors for flowering in *Jatropha*. However, flowering induction along with sex determination could be explored at different stages to identify key genes which can be targeted to increase the number of female flowers by altering the architecture of inflorescence or any abnormality in the reproductive organs.

2.8 Molecular basis of sex determination

In plants, sex determination is a process through which unisexual flowers are formed. There are two dominant ways of unisexual flower development: One is the emergence of only one type of sex organ without formation of any bisexual tissue at any stage floral development. Whereas in other; there is initiation of a bisexual floral meristem with both stamens and pistils followed by a developmental arrest or abortion of one sex with only the stamens or the carpels attaining functional maturity. The step impeding the development of floral sex organs is at an immature stage well prior to reaching sexual maturity [74, 75]. Many monoecious species advances through an initial androgynous stage to differentiated **(**androecia or gynoecia) stages by aborting or arresting either of the sexual organs later in floral development [75, 76]. *Jatropha* being a monoecious plant, sexual differentiation occurs by abortion of stamens, allowing the female flowers to develop. No remnants of female tissues were found in fully developed male flowers however, in fully developed female flowers; remains of male tissues (aborted stamens) were found at the bottom of the female flower. Transcriptome analysis of *Jatropha* floral buds identifies *TASSELSEED 2* **(***TS2***)** gene which is required for stamen development and its reduced expression promotes the carpel development by aborting male tissues. Recently, transcriptome analysis of male and female floral buds at different developmental stages of *Jatropha* identifies *CRABS CLAW* (*CRC*) gene for sex differentiation. They have also identified an ATP-binding protein promotes stamen degeneration in female flower at later stage of development. Chlorophyll A/B-binding protein, inorganic phosphate transporter and ubiquitin carboxyl-

terminal hydrolase contribute to the female organ development whereas cytochrome C oxidase subunit 1, gibberellin-regulated protein 4-like protein and AMP-activated protein kinase genes involved in stamens development. *AGAMOUS-LIKE-2* (*AGL*-2), *CLAVATA1* (*CLV1*), auxin-induced protein 22D, RING-H2 finger protein ATL3J, and transcription factor r2r3-myb contribute to embryo sac development in female flowers. Gene encoding ARP1 (Auxin repressed protein), GID1 (gibberellin receptor protein) and X10A (auxin induced protein) were expressed in both male and female flowers [20]. Functional study of *JcFT*, a floreign and a key regulator of flowering pathway showed highest expression level in female flowers [71]. These studies identify genes for sex determination however, genetics factors contributing to the differentiation of male, female and intermediate flowers, and the transition towards female flowering and higher female to male flower ratio in *Jatropha* is still lacking

Studies on other monoecious plants have been conducted for sex determination through genomics and transcriptomic analysis. Transcriptome analysis of inflorescence of *Plukenetia volubilis, a seed oil bearing* monoecious plants identified genes *CRC*, *HECTATE 2* (*HEC2*), *RADIALIS*-like 1/2 (*RL1/2*), *SUPERMAN* (*SUP*) and *WUS*-related homeobox 9 for females as they expressed at higher rate in female flower buds. *Polygalacturonase 1 (PGL1),* Endo-1,3(*4*)-beta-*glucanase and Cytochrome P450 (CYP450)* were identified in *Quercus sober* for female flowering and for pollen development. *Chalcone synthase A* (*CHS A*), *Defective in anther dehiscence1* (*DAD1*) and *4-Coumarate-CoA ligase like 1* were identified in males [77]. Endo-1,3(*4*)-beta-*glucanase* inhibits male tissues from developing in female flowers and mutation in *DAD1* suppresses anther dehiscence and pollen maturation [78]**.** In *Ricinus communis* PDC related genes (cysteine protease) identified for female development with higher expression level at the stage of anther abortion [79]**.**

In cucumber, sex differentiation has been studied extensively and is genetically controlled by F locus (for females) and M locus (for male). *Aminocyclopropane-1-carboxylic acid synthases* (*ACS1 & ACS2*), *Ethylene receptor2* (*ETR2*) and *Ethylene response sensor 1* (*ERS*) genes associated with ethylene biosynthesis and signaling pathways were involved in the process of sex determination. *ACS1 & ACS2* promotes gynoecia development by inhibiting male reproductive organs [80, 81]. *ETR2* and *ERSI* are ethylene receptors which were accumulated in gynoecia, thus promoting female development [82]. A MADS-box protein *ERAF-17* in cucumber induces female flowering. *CONSTITUTIVE TRIPLE*

RESPONSE (*CTR1* and *CTR2*) *g*enes encodes CTR1-like kinase proteins, negative regulators of ethylene signaling promotes male flower development by lowering ethylene accumulation, as males are sensitive for ethylene. *WIP1* gene in cucumber also promotes male flowers. Transcriptome analysis of cucumber identifies genes encoding Indoleacetic acid-induced protein 2 (*IAA2*), Auxin transporter protein 1 (*AUX1*) and TUBBY-like protein (*TLP*) along with EREBP-9, a transcription factor associated with phytohormones and playing a critical role in determining sex and organ development [83]. Through transgenic approach; suppression of *LESS ADHERENT POLLEN* (*LAP3*) and *Nodulin MtN3* resulted in sterile pollen and their abortion in female flowers in *Vitis vinifera I* and rice [84, 85]. In *Medicago truncatula, N3* (*Xa1*) when suppressed, resulted in small anthers and reduced fertility due to abortive pollen [85]**.** In *Pisum sativum* L carpel senescence is associated with high levels of lipoxygenase gene expression [86]. Pentatricopeptide repeat-containing genes found to restores the cytoplasmic male sterility in rice and petunia [87, 88]. These proteins have also been reported in *Jatropha* where they are involved in differentiation of stamen and carpel and in later stages they are active in embryo sac of females. These studies shed light on how differentiation of male and female occurs in monoecious plants and the possible genetic factors contributing to this. However, limited information on genetic cues for female flower transition and sex determination in *Jatropha* is the reason that it is further needed to be studied. Furthermore, molecular mechanisms for transitions of intermediates into female flowering and males to intermediate types is important and possibly targeted for increasing the female flower number further the seed yield of *Jatropha*.

ABCDE model elucidates the role of floral homeotic genes in sex determination and floral development. Genes of A-class specifies sepal formation and A- and B-class genes together specifies petals development. For stamen development, expression of both B- and C-class genes is required. Determination of carpel development specifies by C-class genes and for ovules activity of D-class genes is necessary. Recently, E-class genes have been added based on their role in determination of the corolla, androecium and gynoecium [89, 90] (Figure 2.4). B- and C- class genes belong to the MADS-box family, which are highly important transcription factors with its role in floral organogenesis. The highly conserved DNA binding domain (MADS box) and moderately conserved domain (K box) are identified in these transcription factors [91]. *PERIANTHA* (*PAN*), a bZIP transcription factor activates *AG*, a C-class MADS box protein further regulates floral organ number and

patterning of whorls [92]. In *Elaeis guineensis* mutation in *AP3* and *PISTILLATA* (*PI*) suppressed male tissues, allowing females to develop. In cucumber, *FLORAL BINDING PROTEIN 11* **(***FBP11***)**, a D-class gene determines ovule formation [93]. *AG2* has mixed C/D function gene as it is expressed only in ovule primordia and carpel in *Arabidopsis* and *Elaeis guineensis* [93, 94]. *Liquidambar styraciflua* L and *Rumex acetosa* L are monoecious plants in which C gene expression arrests sexual organs [76]**.** In *Populus trichocarpa*, a genetic switch at a sex locus controls expression of B and C-class genes, thereby controlling the development of male or female flowers [95]. In *Jatropha PI* and *AP3*, a B-class gene were identified for stamen development whereas, *SEEDSTICK 1* (*STKI*) and *AG*, a D-class gene for carpel development [23]. Thus, study of ABCDE model genes shed some light on floral differentiation in *Jatropha*. These genes can be further studied for identifying the molecular cues for sex determination and arresting of stamens in female flowers further, increasing the female flower ratio hence seed yield.

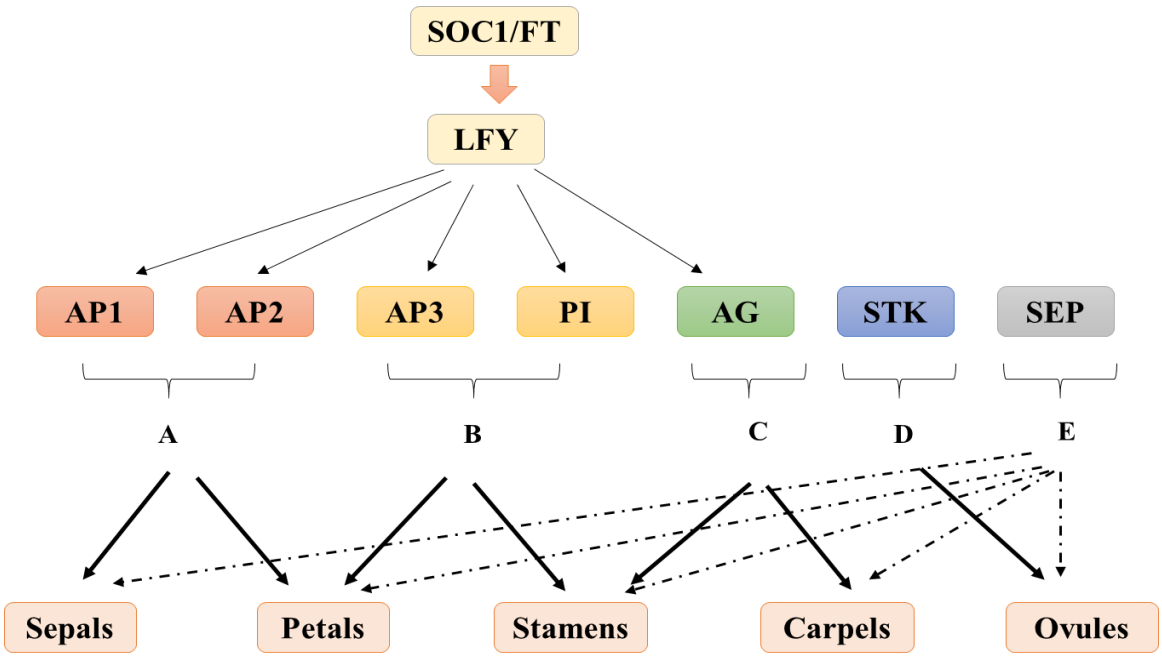

Figure 2.4: ABCDE model for floral development by floral homeotic genes (*LFY*-Leafy; *SOC1*- Suppressor of overexpression of constans1; *FT*-Flowering Locus T; *AP1*-Apetala1; *AP2*-Apetala2; *AP3*-Apetala3; *PI*-Pistillata; *AG*-Agamous; *STK*-Seedstick; *SEP*-Sepalata) [23].

2.9 Role of hormones in sex determination and yield

Flower development and sex determination is highly regulated by crosslink of endogenous hormones. Auxin plays a pivotal role in regulation of plant growth such as embryogenesis, organogenesis, flower and fruit development as well as patterning of organ. Application of IAA in *Jatropha* improved female to male ratio from 1:27 to 1:23 and 3-fold increase in seed weight [96]. Transcriptome analysis of *Jatropha* identifies genes associated with auxin biosynthesis and signaling such as *AUX1,* Transport inhibitor response 1 (*TIR1) and ARFs* of which *AUX1* is responsible for sex determination. Trp-dependent auxin biosynthesis is the main source of auxin production for formation reproductive organ and patterning of embryos [97]. In this pathway, *YUCCA* (*YUC*), a flavin-dependent monooxygenase produces IAA from indole-3-pyruvic acid [98]**.** *YUC1* and *YUC4* produces auxin during formation of stamen primordia and at the later stage of stamen development, *YUC2* and *YUC6* gets activated [99, 100]. *YUC4* and *YUC8* were expressed in the style and *YUC2* in carpel valve tissues of mature gynoecia [99, 101]. *TAA1* is associated with early gynoecial development and localized within the medial domain of the gynoecia [98]. Increased expression of *ARF 10/16/17/18* causes organ abortion and abnormality in females resulting in less seeds set in rice [102]. *PINFORMED* **(***PIN1***)**, an auxin efflux carrier protein has been studied in Arabidopsis for its role in ovule formation. Mutation in *Barren Inflorescence 1 & 2* **(***BIF1/ BIF2***)** in maize resulted in reduced number of spikelets/florets and less kernels in maize [103, 104]. Thus, auxin signaling plays an important role in development of floral organs at different stages and effects the plant yield.
Cytokinin plays a significant role in regulating shoot apical meristem. Many studies on cytokinin application have been conducted and overproduction in floral tissues was observed. Thus, cytokinin influences the number of flowers through regulating floral meristem size [105]. Through transgenics, it is observed that plants with overexpression of cytokinin, resulted in surplus flower primordia along with the development of extra organs per-whorl. Cytokinin regulates *CLV1***,** a meristem maintenance gene which in turn regulates the floral primordia and organ development [106]. Recent studies have observed the role of cytokinins in gynoecia formation and fruit morphogenesis and patterning as well as on seed number [107, 108].
Gibberellic acids **(**GAs**)** plays an important role in flower sex differentiation, especially promoting the development of stamens in monoecious plants. Exogenous treatment of GA on *Jatropha* inflorescence increased the number of female flowers by 2-fold. No increase

in inflorescence branches was observed and with higher GA concentration withering of floral buds occurred. There was no significant increase in fruits/inflorescence, fruit weight, seed rate, 100-seeds weight however, seed oil content was reduced after GA application [109]. In *Jatropha*, *Gibberellin Oxidase* **(***GA20OX* and *GA3OX***)** were identified for promoting stamen development and the exogenous GA3 treatment resulted in arrest of pistil development in females, allowing the male to develop. GA induced stamens in gynoecia cucumber plants. In other monoecious plants, GA treatment induced bisexual flowers by promoting the development of stamens in monoecious female. *GID1*, a positive regulator of GA-signaling pathway participates in the embryo sac development in female flowers whereas GASA4 protein functions in stamen differentiation in *Jatropha* [110, 111]. However, in maize plant GAS promotes carpel development by arresting stamens. *Anther ear 1* **(***AN1***)** gene is crucial for ent-kaurene synthesis, a precursor of GA and mutation in this gene results in bisexual flowers in ears [112]. GA deficiency greatly affects male flowers by causing partial or complete male sterile plants. Thus, GA promotes the development of stamens with no effect on female flowers with no significant change in plant yield.

Jasmonic acid and Brassinosteroids are associated with floral development along with stamen formation, pollen maturation and male fertility [78, 113, 114]. Brassinosteroids also results in abortion of pistils in staminate maize flowers. *AG*, a floral organ identity gene controls the stamen maturation through regulation of a jamonate biosynthesis late developmental stages in Arabidopsis [115]. Mutants defective in synthesis or signaling of jasmonates and brassinosteroid signaling results in male sterility in Arabidopsis, maize and tomato [114, 116]. B*rassinosteroid-insensitive1/2* (*bin1/2*) mutants have defective pollens with reduced viability and some mutants are male sterile [117]. BRs control male fertility through regulation of *SPL/NZZ, Defective in Tapetal development and function 1* (*TDF1*), *Aborted microspores* (*AMS*) genes which are essential for anther and pollen development [114]. Thus, these two hormones promote the male organs development.

Ethylene promotes female flowering in cucumber, Arabidopsis and tobacco. 1-aminocyclopropane-1-carboxylic acid oxidase 2 (*ACO1*) oxidizes ethylene intermediates to form ethylene. Transgenics studies showed that overexpression of *ACO2* suppressed the development of stamens resulting in male sterility [118, 119]. *ETR1*, an ethylene receptor gene when down-regulated, it represses ETR1-interacting kinase CTR1 further, repressing the ethylene signaling thereby, enhancing female flowers development [120]. Role of

ethylene was observed development of the inflorescence, floral meristems, pistils, stigma, style, and ovary however, no or less activity has been observed during development of pollen and anthers in Arabidopsis, tomato and tobacco [121-123]. Thus, these studies implicated the importance of ethylene towards feminism.

2.10 Effect of cytokinin application on *Jatropha curcas* yield

2.10.1 Flowering and sex determination

Cytokinin has been well documented for its feminizing effects in many plant species such as *Mercurialis annua*, *Luffa cylindrica, Pinus densiflora* and *Vitis vinifera* [124-126]. Wu et al. [15] demonstrated that, female flowers have remains non-functional male organ or stamen whereas male flowers does not have any rudiment of female organs during developmental process. This phenomenon is important for alteration of sex ratio after hormonal application. Recently, Pan et al. [27] showed that thidiazuron **(**TDZ**)**, a cytokinin in different concentrations **(**25/50/225 µM TDZ**)** alters the sex expression in *Jatropha*. TDZ induced pistil development which resulted in increased female flower number (~40 female flowers per inflorescence) along with induction of bisexual flowers by reversing stamens with 225 µM TDZ concentration. Studies by Pan and Xu [25] and Gouveia et al. [127] showed the effect of cytokinin BA **(**6-benzyladenine**)** treatment on the floral number and female flowering. Pan and Xu [25] used different concentrations of BA **(**80mg/l, 160mg/l & 320 mg/l of BA**)** on *Jatropha* inflorescence and found a significant increase in flower number (3.6 fold) as compared to untreated inflorescence. Cytokinin promotes female flowering in *Mercurialis annua* of euphorbiaceae family, by endorsing the development of floral primordia into carpels [124]. BA application also increased the number of female flower of up to 4.3-fold as compared to control. TDZ & BA treatments also induced the bisexual flowers in the single inflorescence. BA treatment increased the female flower number upto 5-fold in *P. volubilis*, a seed oil promising plant on which only 1–2 female flowers are present on control inflorescences [128]. Apart from foliar application, increase of cytokinin by overexpression of *IPT4* gene (encoding cytokinin biosynthesis enzyme), under *AP1* promoter resulted in 3-fold increase in number of flowers in the transgenic plants [129]. BA-application also induced the number of branches which bear flowers along with increase in floral buds [68, 130]. However, after several days flower abortion occurred post BA application. Possible cause for this abortion is the availability of sucrose, reduced light intensity, reduced source and sink strength and

hormones [131], heat stress [29]. Thus, the effect of cytokinin for this abortion needs to be studied as less information is available on the possible molecular cause of it.

2.10.2 Seed yield

Jatropha inflorescence treated with BA showed an increase of 4.5fold in fruit number as compared to control [25]. This treatment also produced more four-seed fruits (2.0% of total fruits) which is rare phenomenon as under normal condition *Jatropha* fruit has three seeds. However, in some Mexican genotypes, fruits with 4-seeds was observed under normal growth conditions [131]. In contrast, Abdelgadir et al. [16] defined no change in number of seeds per fruit. Pan and Xu [25] also found one- or two-seed fruits after BA-application. Due to higher number of one-seed and two-seed fruits, BA-treated inflorescences have slightly lower number of seeds per fruit as that of untreated inflorescences. Although, after BA treatment results in higher number of flowers and fruits, however, the fruiting rate, 100 seed weight, seed size and seed oil content are lower than those from control. Also, there was no significant change in oil yield per tree [14, 21, 22, 27]. Even TDZ treatment could not improve the yield and resulted in premature fruiting without any noteworthy change in seed weight and seed oil content. This might be due to the allocation of photosynthetic products. Further studies are necessary for the molecular insights of lower yield **(**weight of 100 seeds, seed size**)** and photosynthetic products allocation after cytokinin application which may identify the key gene/pathway to be targeted for increasing the yield through genetic engineering.

2.11 Molecular insights of cytokinin treatment on flowering and sex determination

Transcriptomic studies conducted on cytokinin treated inflorescences of *Jatropha* at different time intervals to identify the change in expression status of flowering genes [21, 22]. They observed that genes *GI, SOC1, LFY,* which controls the flowering initiation were induced by cytokinin application. Expression of *CYP89A5,* a member of the *P450* gene family found to be associated with inflorescence development was induced after BA application. The expression of A-function genes *AP1,2,* B-function gene *PI,* C-function gene *AG* and E-function gene *SEP1,2,3* were reduced, delaying the formation of floral organs. This expression status allowed more time to generate floral primordia. During this event, expression of *CUC1 was increased,* resulting in substantial increase in flower number in *Jatropha*. After BA application, rate of cell division has been increased at the

inflorescence meristem, indicated by the upregulation *Cyclin-dependent protein kinase (CycA3;2)* and *Cyclin-D3-1 (CycD3;1/2) genes* possibly resulting in increased flower number. However, these studies were conducted after 4 &12 hours of BA application and no phenotypic change was occurred. Increased expression of CK biosynthetic gene **(***AtIPT4***)** in transgenic Arabidopsis resulted in increased flowers number with enlarged inflorescences and flower meristems [129]. However, overexpression of *CKX* gene, a CK-degrading enzyme, reduced the flower number [132, 133]. Functional mutation of *LONELY GUY* (*LOG*) gene encoding a CK-activating enzyme for the final step of bioactive cytokinin synthesis caused a reduction in floral organ numbers [134]. *Gn1a*, a quantitative trait locus identified for governing number of grains in rice which encodes CK oxidase/dehydrogenase. Reduced expression of *CKX2* either by mutations or through antisense inhibition, accumulated CK in the inflorescence meristem resulted in increased grain number. Whereas, overexpression of *CKX2* reduced the numbers of grains in transgenic plants [135]. A similar effect on plant productivity was obtained in barley with the silencing of the *CKX1* gene [136]. Mutation in *CKX* genes produced larger inflorescence meristem resulting in higher flower number, 40% more siliques, and 2-fold increase in ovules per gynoecium as compared to wild type [105]. Chen et al. [21] identified that BA treatment decreased the expression of *TS2* which arrest the carpel development in maize [137]. Thus, its decreased expression in *Jatropha* allowed the development of female flowers hence increased female to male flower ratio. In another study, reduced expression of CK-synthesizing enzyme isopentenyl transferase **(***IPT***)** gene reversed the aborted pistil of the lower floret in a female maize inflorescence [138]. In *Jatropha*, application of BA on inflorescence helps to increase the seed number however, the yield was compromised due to reduced seed weight and size. There was no significant change in seed oil content. Lack of information on molecular mechanism of increase in seed number and reduced yield. The proposed hypothesis is due to source-sink interaction.

2.12 Cytokinins and Carbon flux to seed yield

Source-sink relation plays an important role at different stages of growth and development of plants. Source is the plant organ **(**leaves**)** where the assimilation of carbon through photosynthesis occurs which is transported to the sink tissues like shoot apex or fruits. This source-sink relation determines the yield of the crop as the number of sink organs compete with the common pool of assimilates from the single source organ. In source organ, carbon

is fixed for carbohydrate synthesis through photosynthesis. Under ideal conditions, source organ supply photoassmilates at optimum rate to the sink tissues achieving maximum yield [139, 140]. However, when the demand of sink increases from which the plant source can supply resulting the compromised yield. Studies have been done in many plants to understand the relation between carbon allocation and sink strength affecting their growth and yield. Through photosynthesis atmospheric carbon is fixed to form triose phosphate which serves as a precursor starch and sucrose. Starch **(**non-mobile**)** and sucrose **(**mobile**)** are the major form of carbohydrates in many plants [141, 142]. Along with the carbon availability, nitrogen availability and carbon to nitrogen ratio determines the photosynthetic activity. Nitrogen is required for producing proteins for plant growth. Leaves have high demand of nitrogen where photosynthetic machinery is assembled and maintained. Rubisco enzyme requires upto 30% of the leaf nitrogen [143, 144]. Rubisco catalyzes the first major step of fixing the inorganic carbon into energy rich compounds. Thus, nitrogen and carbon co-ordinate intrinsically to regulate source-sink interaction.

Through various approaches like environmental modifications, molecular interventions and transcriptome analysis effect of photosynthesis level on source to sink relation and yield parameters have been studied in many plant species (Table 2.4). By increasing the atmospheric CO_2 levels, increase in seed number was observed in soybean [145]. This is due to the increased photosynthetic activity. However, reducing the assimilate supply by defoliation or decreasing CO_2 levels reduction in seed size and number was observed. Transcriptome analysis identifies the increase in expression of major enzymes associated with electron transport and ATP synthesis as well as the regulatory enzyme of carbon metabolism. Under elevated CO_2, the activity of Rubisco enzyme was increased upto 25% [146, 147].

Table 2.4: Modifications in different plant species to increase photosynthesis

Plant sps	Manipulation	Activity	Reference
Eucalyptus globulus	Defoliation /Debudding	Increases in photosynthesis	[148]
Lolium perenne	Elevated CO_2	Increase in photosynthesis	[149]
Lolium perenne	Low nitrogen	Decrease in Photosynthesis rate	[150]

Phaseolus vulgaris	Reduced light	Photosynthetic rate decreased	[151]
Arabidopsis thaliana	Genetic manipulation of T6P/ SnRK1 signaling pathway	Affecting sucrose levels and growth	[152]
Glycine max	Elevated CO_2	Reduced sink capacity and decreased photosynthesis	[145]
Solanum tuberosum	Transgenic reduction of ADP glucose pyrophosphorylase	Reduction in starch synthesis	[153]
Triticum aestivum	High nutrients /Elevated CO_2	Increase in photosynthesis and growth	[154]

Plant growth regulators improves the physiological efficacy of plants. Change in photosynthesis rate due to hormonal treatment have been studies in many plant species **(**Table 2.5**)**. Cytokinins help to augment the source activity by enhancing and regulating photosynthesis and strengthening the sink [155-157]. Many reports show that cytokinins upregulate the expression of light harvesting chlorophyll a/b protein, carbonic anhydrase, Rubisco and phosphoenolpyruvate carboxylase thus, enhancing photosynthesis and sink strength [158]. In oil plants, CK improves the flow of assimilates from source organs to regulate source-sink relations [25, 159]. However, decrease in overall yield and the specific oil gravity was observed. In *Jatropha* decrease in seed size and weight was noticed after CK treatment. Thus, identifying the molecular cue for this phenomenon needs to be studied to shed light on the effect on pathways associated with energy metabolism after a span of Cytokinin application. This information might provide suitable targets for genetic intervention in *Jatropha* to develop a high yielding variety.

Table 2.5: Phytohormonal treatment to increase photosynthesis

Plant sps	Phytohormones	Activity	Reference
Glycine max	GA3, ethylene	Increase in Photosynthesis	[160]
Oryza sativa	GA3	Increase in Photosynthesis	[161]
Phaseolus vulgaris	GA3	Increase in Rubisco activity	[162]
Zea mays	IAA, Cytokinin	Increase in photosynthesis by increase in activity of carbonic anhydrase and phophoenol pyruvate	[163]
Linum usitatissimum L	Cytokinin	Improved photosynthesis	[164]
Triticum aestivum	GA3, Ethylene	Increase in photosynthesis	[165]
Hordeum vulgare	Cytokinin	Increases photosynthesis by accumulating photosynthetic machinery	[166]
Nicotiana tabacum	Cytokinin	Increases photosynthetic capacity	[167]
Arabidopsis thaliana	Cytokinin	Increases photosynthetic capacity	[168]

The following research gaps was defined after comprehensive review of literature on bioenergy crop, *J. curcas*:

(1) Lack of information on genetic factors contributing towards higher female to male flowering and transition towards female flowering in *Jatropha curcas*

(2) Lack of information on molecular mechanisms underlying central carbon metabolism affecting floral biomass and seed yield in *Jatropha curcas*

CHAPTER 3

MATERIAL AND METHODS

3.1 Selection of high and low female to male flower ratio accession

For identification of high (IC561335; 1:8) and low (IC561235; 1:25-30) female flowers accession of *Jatropha*, total number of flowers were counted along with male and female flowers present at each plant (Table 3.1). High ratio accession was then selected for conducting various experiments studies.

Table 3.1: *Jatropha* accessions with high/low female to male ratio

***Jatropha* accessions**	**Branches/ plant (No.)**	**Inflorescences/ plant (No.)**	**Female/male flowers (No.)**	**Female to male flowers (ratio)**	**Number of fruits per plant**
IC561335	60	55	160/1106	1:7	156
	45	30	94/802	1:8	90
	13	8	39/330	1:8	36
	21	07	41/204	1:5	41
IC561235	49	46	60/1502	1:25	81
	26	20	48/1104	1:25	44
	37	35	35/959	1:30	32
	57	31	33/961	1:28	28

3.2 Plant material

Shoot apical meristems with inflorescences and without inflorescence bifurcating from same node, floral buds were collected from high (IC561335) and low (IC561235) female flower ratio accessions of *Jatropha*. These accessions were planted at experimental farm of the Himalayan Forest Research Institute (HFRI) at Sultanpur (altitude 1400m asl), HP, India. Different stages of floral development were identified for the study and classified as **VS** (vegetative stage): shoot apex without inflorescence having no floral buds (shoot apex not primed for flowering); **RSI** (reproductive stage I): shoot apex with inflorescence and floral buds were removed (shoot apex primed for flowering); **RSII** (reproductive stage II): initial undifferentiated floral buds; Differentiated floral bud stages (**RSIII**; **RSIV** & **RSV**)-

RSIII (reproductive stage III): intermediate buds; **RSIV** (reproductive stage IV) : male flower buds; **RSV** (reproductive stage V): female flower buds (Figure 3.1). Floral buds were differentiated into male, female and intermediates after flower dissection. After collecting the plant material, samples were immediately frozen in liquid nitrogen and stored at -80°C.

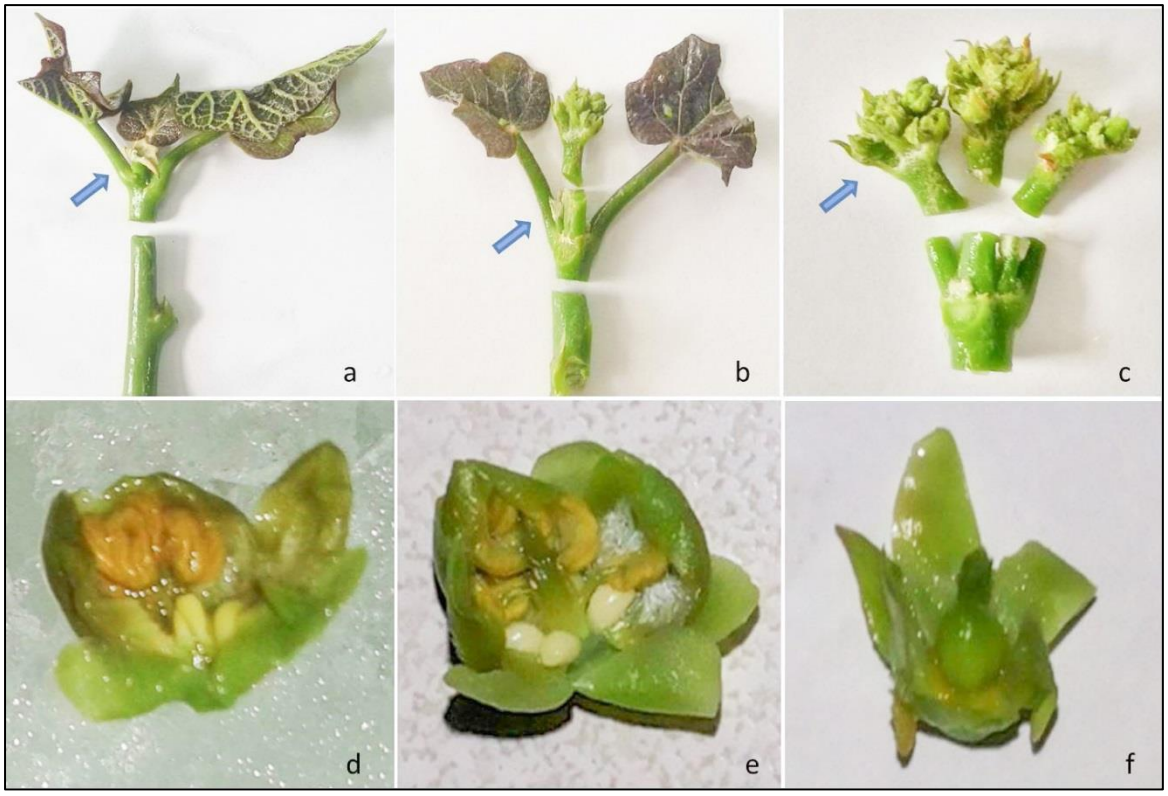

Figure 3.1: Different floral developmental stages of *J. curcas*; (a) Vegetative Stage: VS-Shoot apex without floral buds (b) Reproductive Stage I: RSI-Shoot apex with floral buds (c) Reproductive Stage II: RSII-Initial bud stage (d) Reproductive Stage III: RSIII-Intermediate floral bud € Reproductive Stage IV: RSIV-Male floral bud (f) Reproductive Stage V: RSV-Female floral bud. Arrow indicates the tissue section used for RNA extraction.

3.3 Identifying floral genes from different plant species and retrieving their sequences

Through literature survey, biological information of genes identified in different plant species for flowering were collected. All genes were classified in four different categories according to their function in floral development: floral organ identity genes, flowering transition genes, female flowering genes and genes associated with hormonal regulation

(Table 3.2). Nucleotide sequences for all genes were retrieved from their respective plant species and their putative orthologous sequences were identified in *J. curcas* genome (https://www.kazusa.or.jp/*Jatropha*/) [169]. The *J. curcas* genome sequence contigs showing highest identity were selected. In these contigs, genes were annotated through FGENESH (http://www.softberry.com/) (Table 3.3). Sequence for coding region of genes were identified and used for primer designing by using an online tool Primer3Plus (https://primer3plus.com/) to amplify up to 300bp fragments (Figure 3.2).

Table 3.2: Genes classified according to their role in floral development

Category	**Genes**
Flowering transition genes	*ACS1, AHK2, AG, AP1, ARF2, BEL1, CEN1, CLV1, CRE1, CRY1, CRY2, CKI1, CKX1, DYAD, ETR1, FLO, FT, IPT2, IPT3, MYB98, REV, RGL, SBP, SIN1, STY, TFL1, WUS*
Floral organ identity genes	*ACS1, ACS7, AG, AP1, CUC2, DAD1, DYAD, ERAF17, ETR1, FLO, REV, SIN1, SPA, SUP, TYPA1, WUS*
Female flowering genes	*ACS1, ACS7, AG, AHK1, ARF2, BEL1, CKI1, CRE1, DYAD, ERAF17, ETR1, PIN1, REV, RGL, SIN1, TypA1, WUS*
Gene associated with hormonal regulation	*AHK2, ARF2, CKI1, CKX1, CKX5, CRE1, CTR1, CYP735A, BEL1, EIN1, ERAF17, ETR1, IPT1, IPT2, IPT3, IPT9, PIN1, RGL, TAA1*

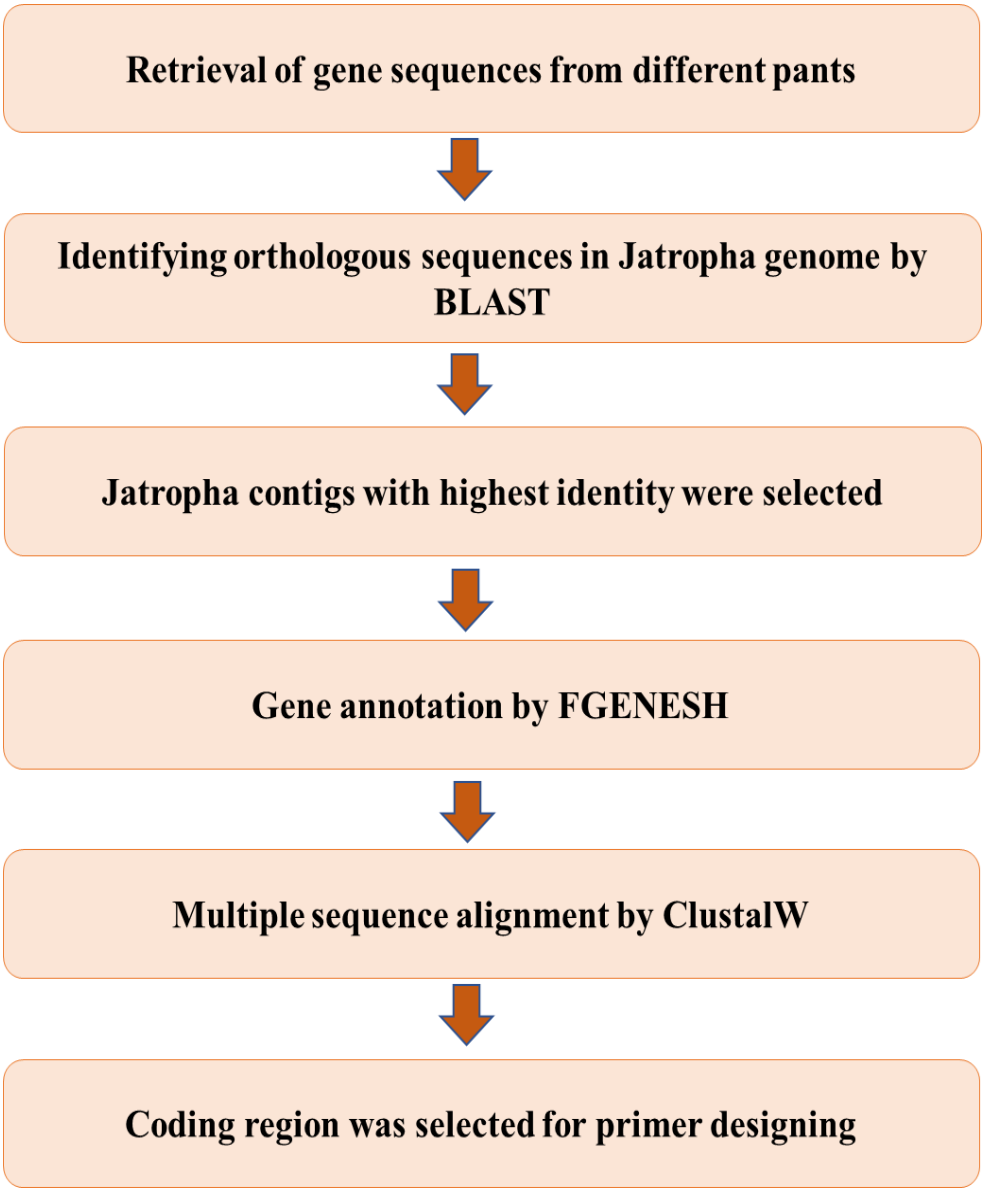

Figure 3.2: Methodology for identifying floral genes through comparative genomics in *Jatropha curcas*.

Table 3.3: Retrieving orthologous sequences from *Jatropha* genome

Gene	Plant spp.	Gene product	Pathway/Association	Biological function	Sequence identity to *J. curcas* orthologue	Accession No.	Contig ID (*Jatropha* genome)	References
ACS1	*Cucumis sativus*	1-aminocyclopropane-1-carboxylate synthase1	Ethylene biosynthesis	Gynoecium development and Sex determination.	81%	XM_012231434	Jcr4S00535.100	[170]
ACS7	*Cucumis sativus*	1-aminocyclopropane-1-carboxylate synthase 7	Ethylene biosynthesis	Gynoecium development and sex differentiation	85%	XM_012222130.1	Jcr4S00752.130	[81]
AG	*Populus trichocarpa*	MADS-box transcription factor	MADS-box regulators of differentiation, Homeotic genes	Ovule development	89%	XM_012218118.1	Jcr4S01776.10	[171]
AHK2	*Arabidopsis thaliana*	Histidine kinase 2 (cytokinin receptor)	Cytokinin signaling	Organ development	86%	XM_012226041.1	Jcr4S00179.130	[172]
AP1	*Arabidopsis thaliana*	MADS-box transcription factor	MADS-box regulators of differentiation, Homeotic genes	Identity of floral meristem	89%	XM_012212431.1	Jcr4S08113.10	[173]
ARF2	*Arabidopsis thaliana*	AUXIN RESPONSE FACTORS	Auxin-mediated transcriptional activation/repression	Pleotropic developmental regulator	82%	XM_012235209.1	Jcr4S07213.40	[174]
BELL1	*Arabidopsis thaliana*	HOMEOBOX family transcription factor	Cytokinin and auxin polarity	Ovule development	82%	XM_012223721.1	Jcr4S00622.70	[175]
CEN1	*Antirrihinum majus*	TCP (TEOSINTE BRANCHED1, CYCLOIDEA, PCF) family of transcription factors.	Florigen signaling	Flowering transition	82%	NM_001306038.1	Jcr4S01285.20	[132]
CKI1	*Arabidopsis thaliana*	CYTOKININ-INDEPENDENT1, protein histidine kinase	Cytokinin regulation	Gynoecium development	80%	XM_012228411	Jcr4S00788.100	[174]
CKX1	*Arabidopsis thaliana*	Cytokinin dehydrogenase	Cytokinin degradation	Young floral parts Development	83%	XM_012230843	Jcr4S01482.30	[174]

CKX5	*Arabidopsis thaliana*	Cytokinin dehydrogenase	Cytokinin degradation	Mature floral parts development	81%	XM_012216552	Jcr4S00296.200	[105]
CLV1	*Arabidopsis thaliana*	Receptor protein kinase	Peptide-receptor signaling	Floral meristem identity	86%	XM_012217630.1	Jcr4S00356.90	[176]
CRE1	*Arabidopsis thaliana*	Histidine kinase 4 (cytokinin receptor)	Cytokinin signaling	Ovule development	84%	XM_012216971.1	Jcr4S00216.30	[172]
CRY1	*Arabidopsis thaliana*	A flavin-type blue-light photoreceptor with ATP binding and autophosphorylation activity	Circadian rhythm pathway	Inducing flowering and lateral organ formation	87%	XM_012212486.1	Jcr4S10731.20	[177]
CRY2	*Arabidopsis thaliana*	Flavin-type blue-light photoreceptor with ATP binding and autophosphorylation activity	Circadian rhythm pathway	Inducing flowering and lateral organ formation	82%	XM_012214439.1	Jcr4S01716.30	[177]
CTR1	*Arabidopsis thaliana*	Serine/threonine-protein kinase	Ethylene response pathway (negative regulator)	Floral organ development	88%	XM_012234542.1	Jcr4S02794.60	[78]
CUC2	*Arabidopsis thaliana*	A NAC family transcription factor	Cytokinin signaling	Organ boundary specification	87%	XM_012220920.1	Jcr4S14571.10	[178]
CYP735A	*Arabidopsis thaliana*	Cytokinin trans-hydroxylase	Cytokinin biosynthesis	Petal differentiation and expansion	49%	XM_012222581	Jcr4S00742.20	[179]
DAD1	*Arabidopsis thaliana*	Phospholipase A1	Jasmonic acid biosynthesis	Anther dehiscence, pollen maturation	87%	XM_012228472.1	Jcr4S11690.10	[78]
DYAD	*Arabidopsis thaliana*	Chromatin modification factor	Female meiotic progression	Female gametophyte development	78%	XM_012235377.1	Jcr4S00435.120	[180]

EIN2	*Cucumis sativus*	Ehylene-insensitive protein 2	Ethylene signalling	Positively regulates ethylene responses.	84%	XM_012226983.1	Jcr4S03599.50	[181]
ERAF17	*Cucumis sativus*	MADS-box transcription factor	MADS-box regulators of differentiation, Homeotic genes	Expressed strongly in the pedicel of female flowers.	71%	XM_012211153.1	Jcr4S01776	[182]
ETR1	*Cucumis sativus*	Protein-histidine kinases (ethylene receptor)	Ethylene signaling	Organ development and flowering	81%	XM_012222480.1	Jcr4S09468.10	[181]
FLO	*Arabidopsis thaliana*	Meristem specific transcription factor	Regulate floral homeotic genes	Floral meristem identity gene	83%	XM_012235184.1	Jcr4S02370.80	[182]
FT	*Arabidopsis thaliana*	TCP (TEOSINTE BRANCHED1, CYCLOIDEA, PCF) family of transcription factors.	Florigen signaling	Flowering induction	80%	KJ130139.1	Jcr4S07821	[71]
IPT1	*Arabidopsis thaliana*	Adenylate isopentenyltransferase (cytokinin synthase)	Cytokinin biosynthesis	Floral organ development	84%	XM_012226712	Jcr4U31121.10	[183]
IPT3	*Arabidopsis thaliana*	Adenylate isopentenyltransferase (cytokinin synthase)	Cytokinin biosynthesis	Floral organs development	85%	XM_012237097	Jcr4S00053.80	[183]
IPT2	*Arabidopsis thaliana*	tRNA dimethylallyl transferase	Cytokinin biosynthesis	Floral development	84%	XM_012220475	Jcr4S04080.10	[183]
IPT9	*Arabidopsis thaliana*	tRNA dimethylallyl transferase	Cytokinin biosynthesis	Floral organs development	83%	XM_012223167	Jcr4S01802.10	[183]
MYB98	*Arabidopsis thaliana*	MYB protein	Tryptophan clusters Myb, Myb-factors	A member of the R2R3-MYB gene family and involved in the synergid cells	82%	XM_012225140.1	Jcr4S00700.100	[184]
PIN1	*Arabidopsis thaliana*	Transporters	Auxin efflux carrier family	Ovule development	89%	XM_012228517.1	Jcr4S10204.10	[98]

REV	*Arabidopsis thaliana*	Homeo domain only, HD-ZIP	Homeobox-leucine zipper protein	Sex determination and regulation of meristem formation	87%	XM_012209426.1	Jcr4S05250.20	[185]
RGL	*Arabidopsis thaliana*	DELLA regulators	Encodes a negative regulator of gibberellin	Carpel, petals and cotyledon formation	87%	XM_012224505.1	Jcr4S00100.100	[186]
SBP	*Antirrihinum majus*	MADS-box transcription factor	Regulating Homeotic genes	Flowering initiation	81%	XM_012218150.1	Jcr4S00002.200	[187]
SIN1	*Arabidopsis thaliana*	DICER	Chromosomal segregation and gene silencing	Embryo development	85%	XM_012227694.1	Jcr4S00011.190	[188]
SPA	*Arabidopsis thaliana*	Encodes a bHLH transcription factor	Regulation of auxin accumulation or transport	Female flowering	87%	XM_012212930.1	Jcr4S01784.30	[189]
STY	*Antirrihinum majus*	Regulatory component of floral organogenesis	Regulates floral homeotic meristem-and organ-identity genes	Homeotic regulation of floral organogenesis	69%	AJ620905	Jcr4S05372.10	[190]
SUP	*Arabidopsis thaliana*	Encodes transcription factor (C2H2 type zinc finger protein)	Cytokinin signal mediated boundary formation between stamen and carpel	Gynoecia development	88%	XM_012214547.1	Jcr4S00294.10	[191]
TAA1	*Vitis vinifera*	L-tryptophan-pyruvate aminotransferase	Auxin biosynthesis	Ovule development	74%	XM_012230067.1	Jcr4S01001.20	[98]
TYPA1	*Cucumis sativus*	GTP-binding protein	Phytohormone signaling	Sex determination and ovule development	89%	NM_105724	Jcr4S02582	[192]
TFL1	*Arabidopsis thaliana*	TCP (TEOSINTE BRANCHED1, CYCLOIDEA, PCF) family of transcription factors.	Florigen signaling	Flowering induction	80%	KF944350.1	Jcr4S10517.10	[193]
WUS	*Arabidopsis thaliana*	HOMEOBOX family transcription factor	Maintains stem cell in shoot meristems	Organ development	85%	XM_012233154.1	Jcr4S08740.10	[194]

3.4 RNA isolation and cDNA synthesis

Floral buds were dissected to differentiate between male, female and intermediate flowers of both genotypes of *Jatropha*. Total RNA was extracted from identified floral developmental stages of *Jatropha* by fusing CTAB with 2% PVP and RNeasy plant purification kit [195]. In extraction buffer 2% PVP was used to remove the inhibitory components followed by phase separation using chloroform/isoamyl alcohol in 24:1 ratio, supernatant obtained after centrifugation (12,000rpm) was transferred to spin column followed by washing and elution of RNA as per kit's protocol. RNA quality as well as quantity, is measured by taking absorbance at 230, 260 and 280nm followed by calculating the 260/280 and 260/230 ratios by using a NanoDrop spectrophotometer (Thermo Scientific). Integrity of RNA was checked on 1% (w/v) ethidium bromide-stained agarose gel. First-strand cDNA synthesis was done form 1µg total RNA using Verso cDNA synthesis kit (Thermo Scientific, USA) as per manufacturer's protocol. Concentration of each cDNA sample was adjusted to 100ng/µl for RT-qPCR.

3.5 Expression analysis using quantitative real time PCR (RT-qPCR)

Expression analysis of 42 genes at different floral developmental stages of *Jatropha* (Figure 3.1) was investigated by RT-qPCR. 100ng of cDNA of each sample was taken. For amplification of single product was checked by standard PCR using gene specific primers on cDNA. Quantitative real time PCR was performed in triplicates by using gene specific primers (Table A1) on a CFX96 system (Bio-Rad Laboratories; Hercules, CA) with the iScript one step RT-PCR kit (Bio-Rad). Quantitative real time PCR was conducted for two biological replicates. The PCR protocol was as follows: denaturation for 3min at 95°C, followed by 39 cycles each of denaturation for 10sec at 95°C, annealing for 30sec at 50–65°C, and elongation for 30sec at 73°C. In the final step, melt curve analysis was done at 65-95°C with 0.5°C increment at each 0.05sec to verify amplification of a single product. For normalization of gene expression, housekeeping genes 26S rRNA and GAPDH were used as internal controls. Relative fold changes were determined using the comparative Ct ($\Delta\Delta$Ct) method. Comparative expression analysis of genes with significant higher expression for female flowering in IC561335 was compared with low female flower ratio accessions in male, female and intermediate buds to validate the genes playing significant role in female flower transition and development.

3.6 Statistical analysis by Principal component analysis (PCA)

PCA recognizes the patterns in data, highlighting the similarities and differences in it. PCA generates the graph and represents data in different co-ordinates. We performed PCA to correlate the expression data of floral genes at different stages by using XLSTAT. The PCA for four developmental stages generated the plot between variability, eigen-values and principal components. To govern relative expression of genes for sex determination, plot was generated for initial floral buds, male floral buds, female floral buds and intermediate buds.

3.7 Analysis of promoter regions

For promoter analysis genes with higher expression at different floral stages were selected and *in-silico* analysis was performed to identify the putative regulatory elements for floral transitions and female flowering. Their genomic sequences were extracted from *Jatropha* genome. Potential promoter regions (1.5 kb upstream region) of the translational start site (TSS) were retrieved from *Jatropha* genome. i*n silico* promoter region was identified by TSPP. For *in silico* detection of *cis* regulatory elements, database PLACE (http://www.dna.affrc.go.jp/PLACE/) and Plant Care (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) were used. Biological function of all cis-regulatory elements was identified to understand the regulatory control of genes (commonality or differences).

3.8 Benzyl adenine treatment

Jatropha accession IC561235, planted at the experimental farm of Himalayan Forest Research Institute at Sultanpur (altitude 1400 m asl), HP, India were selected for cytokinin application. These seed propagated plants were 7 years old at the time of cytokinin treatment. For BA application, 5 inflorescences of 5 plants were treated with three different concentrations of BA (80mg/l, 160mg/l & 320mg/l). We selected 5 plants for cytokinin application and sprayed 160mg/l BA on 18-20 inflorescences of each plant, next year. For 160mg/l BA concentration 0.5mM of BA is dissolved in Tween-20 (0.05%v/v). Foliar BA was applied on inflorescence meristem along with surrounding leaves using a hand sprayer. Three- four count of inflorescence meristems were sprayed with Tween-20 (0.05% v/v) in distilled water for control. Each inflorescence was sprayed 3 times in every 2 days. We collected shoot apices i.e. vegetative state of floral transition (not primed to flowering),

from TR15 (fifteen days BA treatment) & TR30 (thirty days BA treatment) and untreated CONTROL (Figure 3.3). All four of the tissues i.e. vegetative, TR15, TR30 and untreated CONTROL were immediately frozen in liquid N_2 (-80°C).

3.9 Isolation of total RNA

We isolated total RNA from the collected shoot apices (vegetative, TR15, TR30 and CONTROL) by using RaFlex Total RNA isolation Kit (Genei) in two biological replicates. RNA quality as well as quantity, is measured by taking absorbance at 230, 260 and 280nm followed by calculating the 260/280 and 260/230 ratios by using Qubit RNA BR kit (fluorometer).

Figure 3.3: *Jatropha* tissues for transcriptome analysis. (a) Vegetative stage (VS): Shoot apex without floral buds (b) CONTROL: Shoot inflorescence meristem without BA application (c) TR15: Inflorescence meristem after 15 days of BA treatment, with increased floral buds (biomass) (d) TR30: Inflorescence meristem after 30 days of BA treatment, with less number of floral buds (biomass) as compared to TR15.

3.10 Illumina NextSeq library preparation and sequencing

Paired-end cDNA sequencing libraries were prepared in duplicates by using Illumina TruSeq stranded total RNA Library Preparation Kit as per the protocol described (Illumina). rRNA was depleted from total RNA followed by fragmentation. First-strand cDNA was synthesized from fragmented rRNA depleted RNA, followed by second-strand cDNA generation, A-tailing, adapter ligation and finally ended by index PCR amplification of adaptor-ligated library. For PCR amplification, paired end adaptor ligated fragments were used. For quantification and validation of library High Sensitivity Assay Kits and Qubit dsDNA HS kits were used. The mean size of the fragment distribution ranged from 550-700 bp.

3.10.1 Data generation on NextSeq

The raw reads of *Jatropha* tissue samples were generated on NextSeq. These raw reads were filtered using trimmomatic (v 0.30) along with QV (quality value) greater than 20. Following parameters were used for filtration: 1. Trimming/cutting adapter (20 bp) 2. Leading (which means cutting the bases from the start when average quality once with in the window drops below a threshold (preset to 30 bp) 3. Trailing (which means cutting the bases of the ends of a read when window drops below the threshold (preset 30 bp)) 4. Sliding window (which means cutting the bases when average quality with in the window drops below the threshold (preset to 20 bp)) 5. Minimum length at 40 bp. The reference genome of *J. curcas* for annotation was downloaded from the link ftp://anonymous@ftp.kazusa.or.jp/pub/*Jatropha*/. In *Jatropha* reference genome ~57,437 coding sequences (CDS) were present. The Illumina NextSeq transcriptome data for three tissues: CONTROL, TR15 & TR30 were mapped separately to the reference genome by using default parameters of BWA version 0.7.5a (http://bio-bwa.sourceforge.net/) (Figure 3.4).

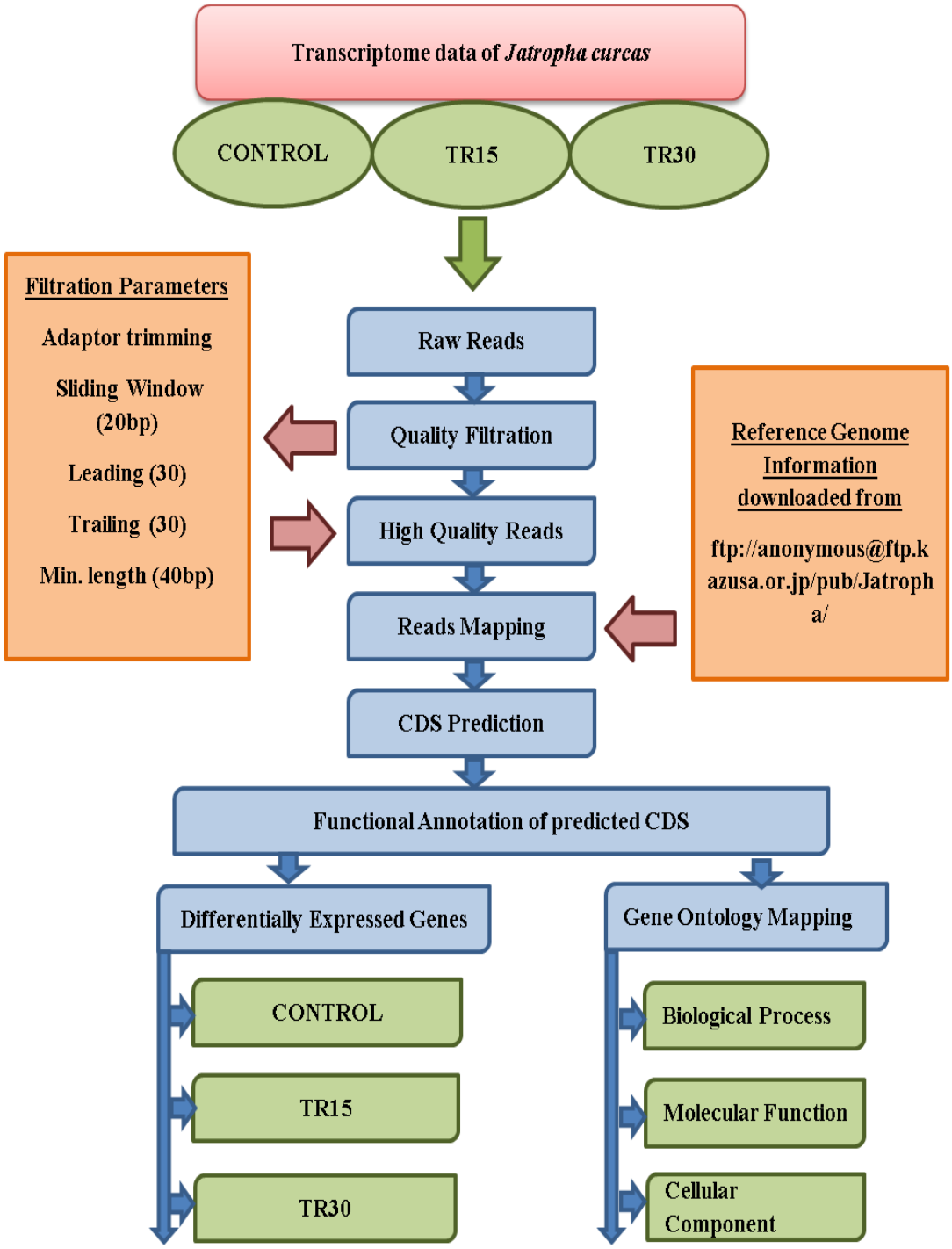

Figure 3.4: Methodology for bioinformatics approach for transcriptome analysis.

3.10.2 Differential gene expression analysis

Abundance of gene transcripts of mapped CDS was calculated in terms of FPKM (Fragment per kilobase per million mapped reads). The FPKM values for each gene were calculated for the grouping of control and treated samples with DESeq package. Log fold change [log2 (FPKM_Treated/FPKM_Control)] change was determined between control and treated samples for commonly expressed genes. Screening of differentially expressed genes (DEG) was done by using p-value < 0.05 and absolute log2fold change (Log2fold value > 0.5 for up regulation and Log2fold value < 0.5 down regulation). Genes were further categorized as up-regulated genes and down-regulated genes according to their relative transcript abundance in the treated samples when compared to control.

3.10.3 Heat map analysis

Heat map was generated for top 100 highly differentially expressed genes in VEG Vs CONTROL and treated conditions (TR15 & TR30) Vs CONTROL for hierarchical clustering by using multiple experiment viewer (MEV v4.9). Clustering was done by considering two parameters i.e. log2 ratio and normalized value of genes. For normalized value of genes, Pearson's uncentered correlation distance and complete linkage method were used.

3.10.4 Gene ontology (GO) analysis

The functions of predicted CDS were classified by using GO analysis. For functional annotation of the predicted CDS in all four tissues, BLAST2GO program was used with default parameters to retrieve GO annotation. Main GO categories were determined after the genes were further analyzed for BLAST, gene mapping and annotation. BLASTx resulted in accession IDs which were directly searched in the gene product table of GO database**.** GO mapping groups the annotated nodes into three main domains: biological process, molecular function and cellular component.

3.10.5 Pathway analysis

Functional annotation of mapped CDS for untreated (CONTROL) & treated (TR15 & TR30) samples was done by using KAAS (KEGG Automatic Annotation Server). KAAS annotate pathways through BLAST comparisons against KEGG GENES database by BBH (Bi-directional best hit) which assigns KO terms. KEGG Orthology database (http://www.genome.jp/kegg/ko.html) was applied for pathway mapping.

3.10.6 RT-qPCR based experimental validation

The expression status of 31 selected genes was checked via RT-qPCR for validating transcript abundance. These genes were shortlisted based on their transcript abundance and biological function. First-strand cDNA synthesis was done by using Verso cDNA synthesis kit (Thermo Scientific, USA) from total RNA (2µg) as per manufacturer's instructions, in two biological replicates. With gene specific primers (Table A2), RT-qPCR was performed in triplicates on CFX96 system (Bio-Rad, USA) with iScript One-Step RT PCR kit (Bio-Rad, USA). The RT-qPCR cycle as follows: denaturation at 94°C for 5min followed by 40 cycles of denaturation at 94°C for 20s and then annealing at 50-55°C for 30s followed by one elongation step of 20 s at 72°C. 26S rRNA and beta-actin were used as an internal reference for data normalization.

CHAPTER 4

RESULTS

Results obtained from objective 1:

4.1 Dissection of floral buds

After dissecting the flower buds, it was observed that both male and female flowers have peculiarly well-developed organs whereas, intermediates have fused organs (Figure 4.1). Females consisted of green colored well-developed ovary with three bifid stigma and styles. Male flowers have fully formed 10 yellow stamens arranged in a diadelphous manner. In male flowers, five oval shapes light yellow-colored glands were present at the base of the flower whereas, in females five square shaped glands of honey dew color were present. Intermediates were present at the middle type inflorescence site, where both males and females can develop. We observed fused stamens (retarded stamens) with flat yellow-colored glands similar to the glands present in female flowers in intermediate. Higher number of distinctively developed female flowers were observed at the terminal position of sub-branches of the inflorescence in high female to male flower ratio accession, whereas terminal position was occupied by the intermediate types (fused) in low ratio accession.

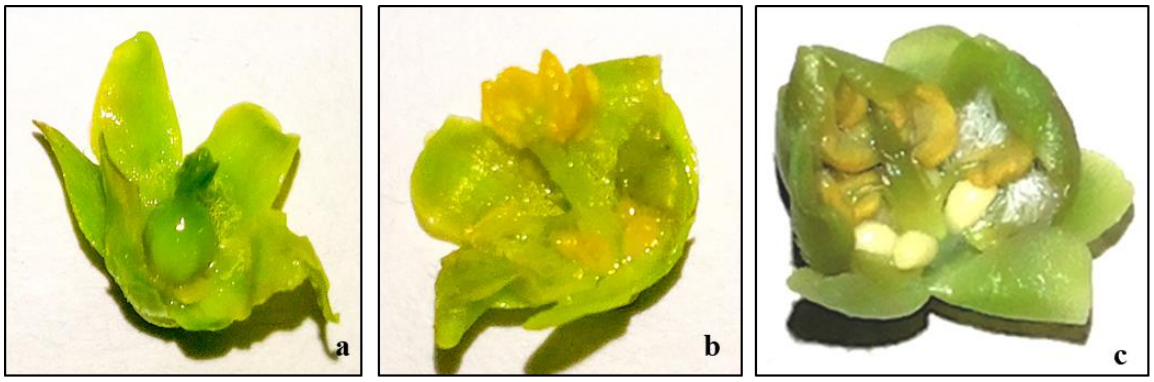

Figure 4.1: Dissected floral buds of *J. curcas* (a) Female floral buds with globular ovary with flat yellow glands (b) Intermediate floral buds with diffused stamens and yellow colored glands (c) Male floral buds with stamens and cream-colored oval glands.

4.2 Expression profiling of floral genes at different floral developmental stages

Expression profiling of 42 genes (Table 3.3) at six floral development stages beginning with induction of flowering (vegetative to reproductive), initial undifferentiated buds and floral buds differentiating into male or female flowers and intermediate types showed an overall ~1-1953-fold increase in transcripts.

4.2.1 Vegetative (VS) and reproductive stage I (RSI) stages

Shoot apical meristem (SAM) progresses into an inflorescence meristem (reproductive phase) when molecular/environmental signals are transmitted to it. Gene transcripts of *SUP*, *TFL1* and *AP1* was increased up to 426-fold. Expression of *CUC2*, *CRY2*, *PIN1* and *TAA1* genes was upregulated up to ~5 fold at RSI as compared to VS (Figure 4.2).

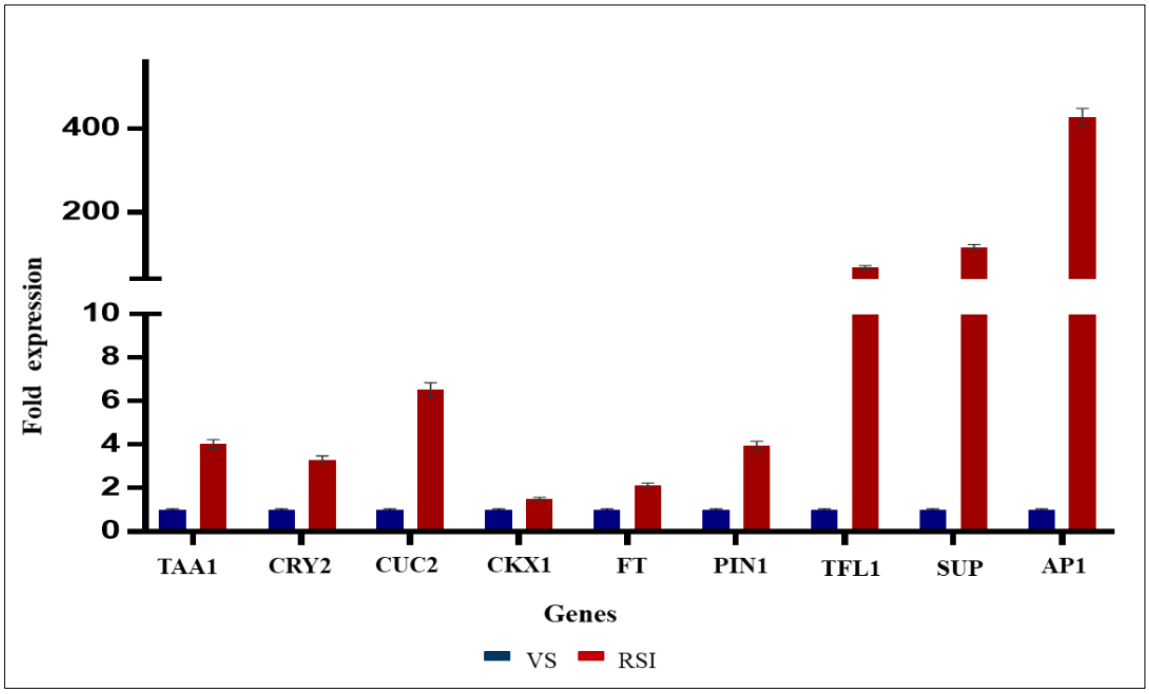

Figure 4.2: Relative expression of genes associated with vegetative to reproductive phase transition. (*TAA1*-Tryptophan-pyruvate aminotransferase 1; *CRY2*-Cryptochrome 2; *CUC2*-Cup-Shaped Cotyledon2; *FT*-Flowering Locus T; *PIN1*-Pinformed 1; *TFL1*-Terminal Flower 1; *SUP*-Superman; *AP1*-Apetala1).

4.2.2 RSI (shoot apical meristem primed for flowering) and RSII (undifferentiated floral buds) stages

After transition of SAM to reproductive phase, initial buds are formed. Thus, at this stage, reproductive organs commence to form. 21 genes were upregulated in RSII stage. Genes contributing in this phase transition are *IPT2*, *AP1*, *RGL*, *CUC2*, *EIN2*, *PIN1* and *TYPA1*. *AP1*, *PIN1*, and *TYPA1* showed a significant increase in expression of about ~1953-fold at RSII followed by ~13-fold increase in expression of genes *CUC2*, *RGL*, *IPT2* and *EIN2* respectively. *CEN1*, *BEL1*, *CRY1*, *CYP735A*, *TAA1*, *WUS*, *STY*, *FLO*, *FT*, *REV*, *AHK2*, *CRE1*, *CKX1*, *CTR1* and *ACS1* showed an upregulation of upto 8-fold higher in RSII (Figure 4.3).

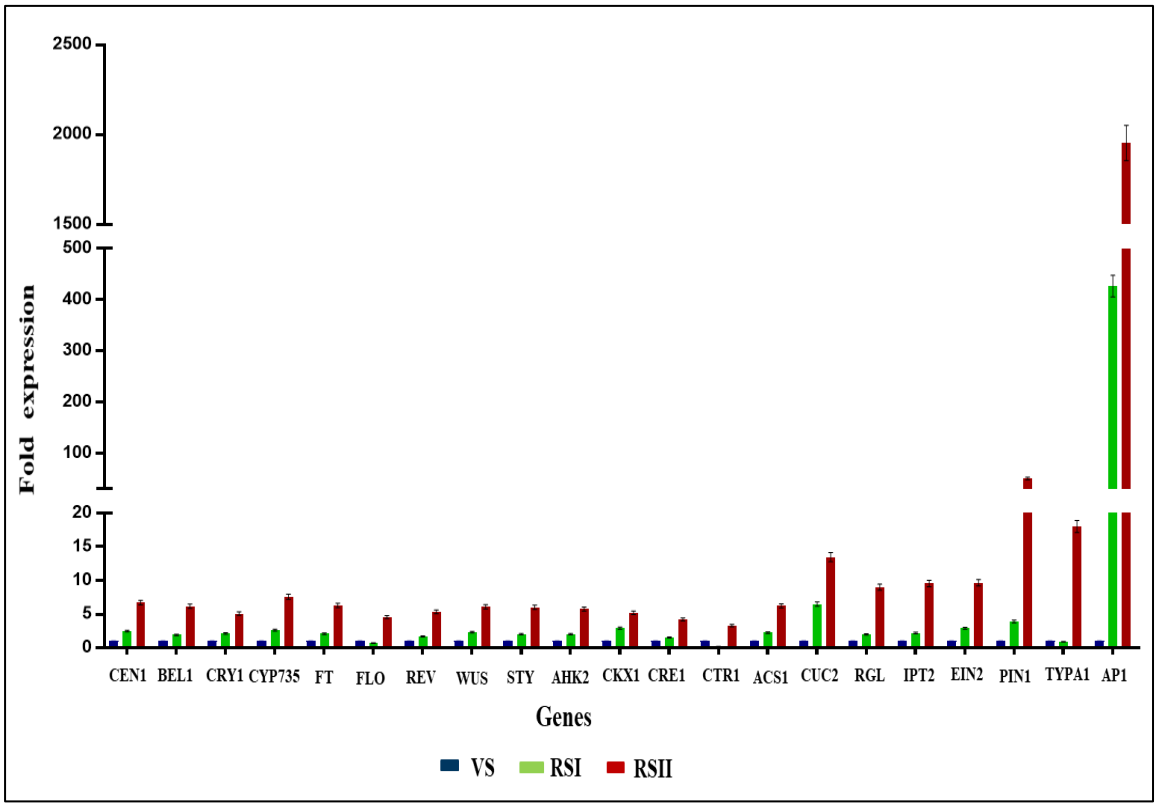

Figure 4.3: Relative expression of genes associated with floral organ formation. (*CEN1*-Centroradalis 1; *BEL1*-Bell 1; *CRY1*-Cryptochrome1; *CYP735*-Cytochrome P735; *FT*- Flowering Locus T; *FLO*- Floricaula; *REV*- Revoluta; *WUS*-Wuschel; *STY*-Stylosa; *AHK2*-Histidine Kinase 2; *CKX1*-Cytokinin oxidase 1; *CRE1*-Cytokinin response 1; *CTR1*- Constitutive triple responsive 1; *ACS1*-1-aminocyclopropane-1-carboxylate synthase; *CUC2*-Cup-Shaped Cotyledon2; *RGL*-Repressor of ga1-3-LIKE protein; *IPT2*-tRNAisopentenyltransferase 2; *EIN2*-Ethylene insensitive 2; *PIN1*-Pinformed 1; *TYPA1*-Tyrosine phosphorylated protein A; *AP1*-Apetala1).

4.2.3 RSIII (intermediate floral buds), RSIV (male floral buds) and RSV (female floral buds) stages

Sex determination of initial buds occurs, forming male or female or intermediate flowers. *CRY2*, *TAA1*, *CUC2*, *CKX1*, *PIN1*, *FT*, and *SUP* gene might be contributing in development of female flower. *CUC2* gene showed ~59-fold, ~18-fold, ~ 3-fold increase in transcript level at RSV, RSIII and RSIV, respectively. *SUP* gene, encoding a zinc finger protein was highly expressed at RSIII with ~43-fold followed by RSV (~29-fold) and RSIV (~2-fold). Expression of *CRY2* gene was higher in RSIII (~8-fold) and RSV (~7.5-fold) as compared to RSIV (~3.5-fold). Expression of genes *IPT1*, *IPT2*, *IPT3*, and *CKI1* showed ~2-fold higher in RSV as compared to RSIV (Figure 4.4).

TFL1, *AP1* and *TYPA1* might be contributing in development of male flowers. *TFL1* gene expressed highest in RSIII (~81-fold) followed by RSIV (~67-fold) and RSV (~63-fold). Expression of *AP1* and *TYPA1* genes was highest at RSIV (~1607-fold & ~8-fold) followed by RSIII (~1520-fold & ~6-fold) and lowest at RSV (~709-fold & ~2-fold), respectively.

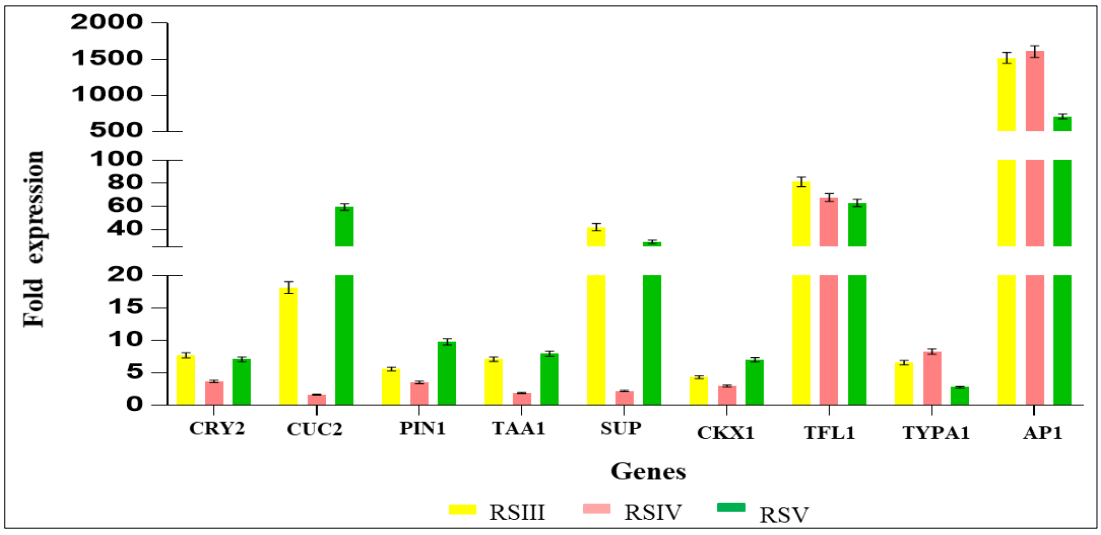

Figure 4.4: Relative expression of genes associated with female flowering and transition. (*CRY2*- Cryptochrome 2; *CUC2*- Cup-Shaped Cotyledon 2; *PIN1*-Pinformed 1; *TAA1*- Tryptophan-pyruvate aminotransferase 1; *SUP*- Superman; *CKX1*- Cytokinin oxidase 1; *TFL1*- Terminal Flower 1; *TYPA1*- Tyrosine phosphorylated protein A; *AP1*-Apetala1).

4.3 Differential expression profiling of female flowering genes high versus low female to male flower ratio accession

PIN1, *SUP*, *CRY2*, *TAA1*, *CKX1* and *CUC2* genes were shortlisted by their expression status at different developmental stages. The relative expression of these genes was higher in female floral buds in high female to male flower ratio accession. Their expression status was studied in RSIII, RSIV and RSV in low female flower ratio accession. *SUP* gene showed ~7-fold increased expression at RSIII stage which was lowered to ~0.1-fold at RSIV with no significant change at RSV. Expression of *CUC2* gene was ~10-fold higher in RSV with relatively same expression status at RSIII and RSIV in accession of high female flower ratio. Expression of genes *CRY2*, *TAA1*, and *CKX1* was increased to ~2.5-fold at RSIII. They showed similar expression at RSV whereas relative expression at RSIV was significantly decreased in low ratio accession. *TYPA1* and *AP1* genes were expressed ~2.5-fold higher at RSIII and RSIV. The relative expression status of genes showed genes *TAA1*, *SUP*, *CRY2* and *CKX1* might be involved in transition towards female flowering. *CUC2* gene showed its association with female flower development and *TYPA1* & *AP1* genes in male flower development (Figure 4.5).

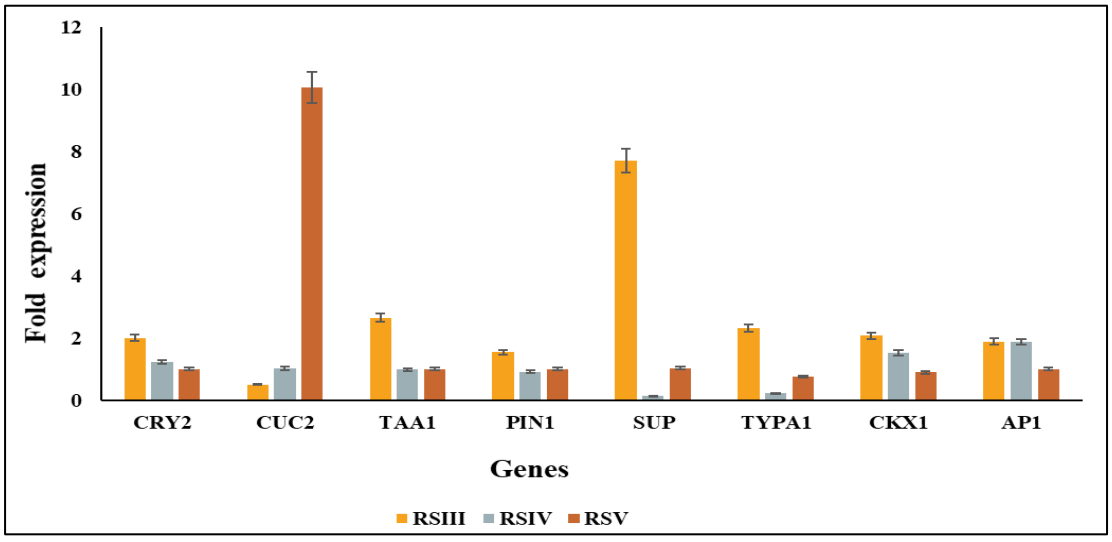

Figure 4.5: Expression status of key floral genes in high (Acc. No. IC561235) versus low (Acc. No. IC561225) female to male flower ratio accessions of *Jatropha*. (C*RY2*- Cryptochrome 2; *CUC2*- Cup-Shaped Cotyledon 2; *PIN1*-Pinformed 1; *TAA1*- Tryptophan-pyruvate aminotransferase 1; *SUP*- Superman; *CKX1*-Cytokinin oxidase 1; *TFL1*- Terminal Flower 1; *TYPA1*- Tyrosine phosphorylated protein A; *AP1*-Apetala1).

4.4 Correlation of gene expression in transition of female flowering

IPT1, *IPT3*, *IPT9*, *AHK2* and *AHK4* are involved in cytokinin biosynthesis and signaling showed higher expression in female flower buds. The expression of *AHK2* and *AHK4* was highest in RSII followed by RSV, indicating their role in reproductive organ development and then in female flowering. *AHK2* activates a transcription factor *CUC2* which is highly expressed in RSV. *CUC2* in turn regulates *PIN1* activity, which is expressed highest in RSII and then in RSV. The expression pattern of *PIN1* and *CKI1* genes was alike in all the developmental stages. *AHK4/CRE1*, a cytokinin binding receptor activates *BEL1* expressed higher in RSII followed by RSV (Figure 4.6). *SUP* gene expressed highest in RSIII followed by RSV which blocks the B-class genes expression necessary for development of stamens. Circadian rhythm pathway might be regulating female flowering as *FT*, *CRY1* & *CRY2* genes were shoed higher transcript abundance in RSV compared to RSIV (Figure 4.6).

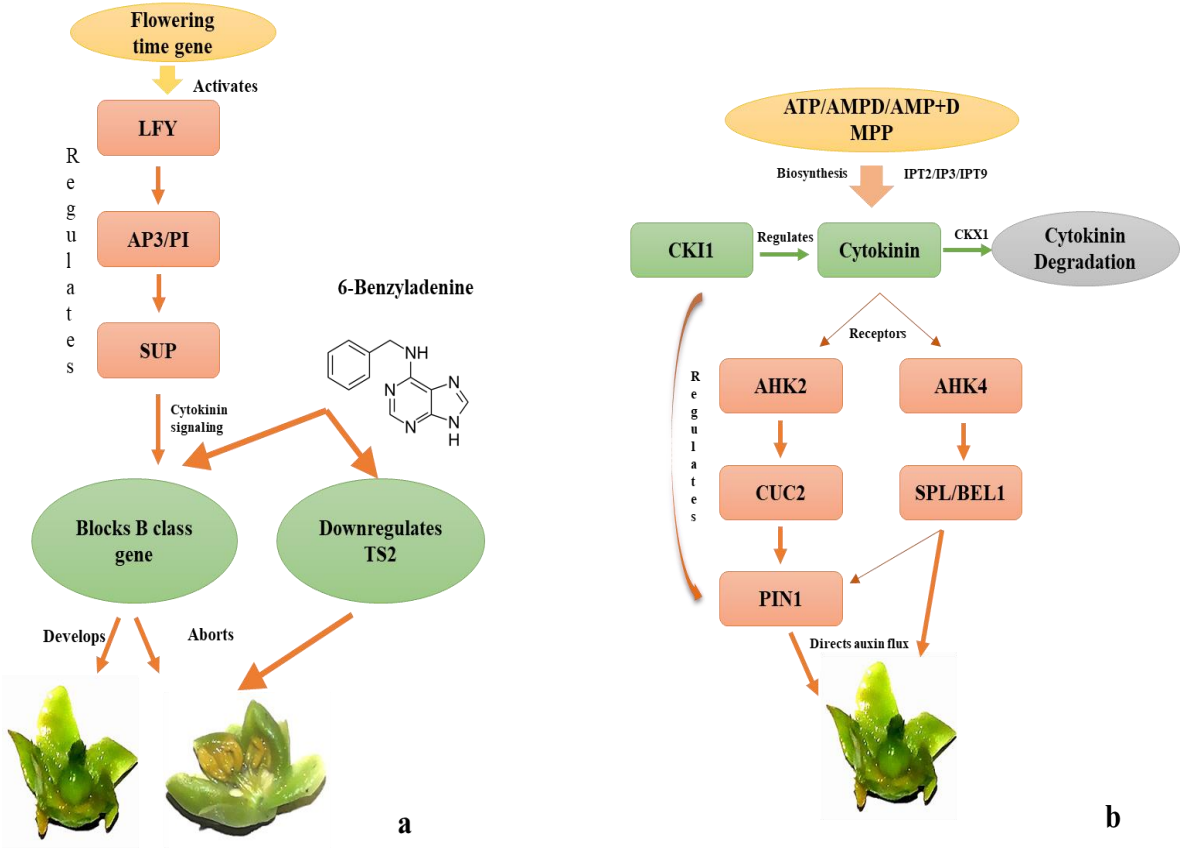

Figure 4.6: Proposed model for female flowering **a.** Transition to female flowering**.** *LFY* activates SUP by *AP3/PI*-dependent pathways which represses Class B floral homeotic genes thereby, arresting pistil primordia in male flowers **b.** Development of female flowers in *Jatopha curcas***.** ITP1/2/3/9 synthesizes cytokinin which transduces signals by binding to receptors *AHK2* and *AHK4/CRE1*. *AHK2* then interacts with *CUC2* which regulates PIN1. *BEL1* is regulated by cytokinin signaling mediated through *CRE1*

receptor. *BEL1*/*SPL* modulates auxin fluxes by regulating PIN1 for ovule development (LFY-Leafy; *AP3*-Apetala3; *PI*-Pistillata; *SUP*-Superman; *TS2*-Tasselseed2; *CKI*- Cytokinin-independent 1; *AHK2/4*-Histidine kinase; *CUC2*- Cup-Shaped Cotyledon 2; *PIN1*-Pinformed 1; SPL- Squamosa promoter binding protein-like; *BEL1*-Bell1)

4.5 Principal Component Analysis

PCA was done for 4 developmental stages vis-à-vis expression status of 42 genes. PCA clustered genes into two coordinates (F1 & F2). The major variance generated by principle components F1 and F2 where in positive F1 co-ordinate genes highly associated to initial stage were clustered and in negative F1 co-ordinate clustering of genes occurred of those involved in intermediate stage. Positive F2 co-ordinate represented cluster of genes for female flowering whereas the negative F2 co-ordinate represented the gene cluster of male floral buds. PCA showed that genes *TAA1*, *CUC2*, *CRY2*, *SPA*, *IPT1*, *SUP*, *CKX1*, *MYB98*, *SBP*, *ARF2*, and *CKI1* were highly correlated to the female flowering. These results are in correlated with the expression analysis data, where these genes showed significant increase in transcripts at RSV stage (female floral buds). These all genes were in a quadrant where F2 is positive. *TYPA1*, *AP1*, and *EIN2* genes were grouped in negative F2 quadrant and were observed for development of male flowers (Figure 4.7).

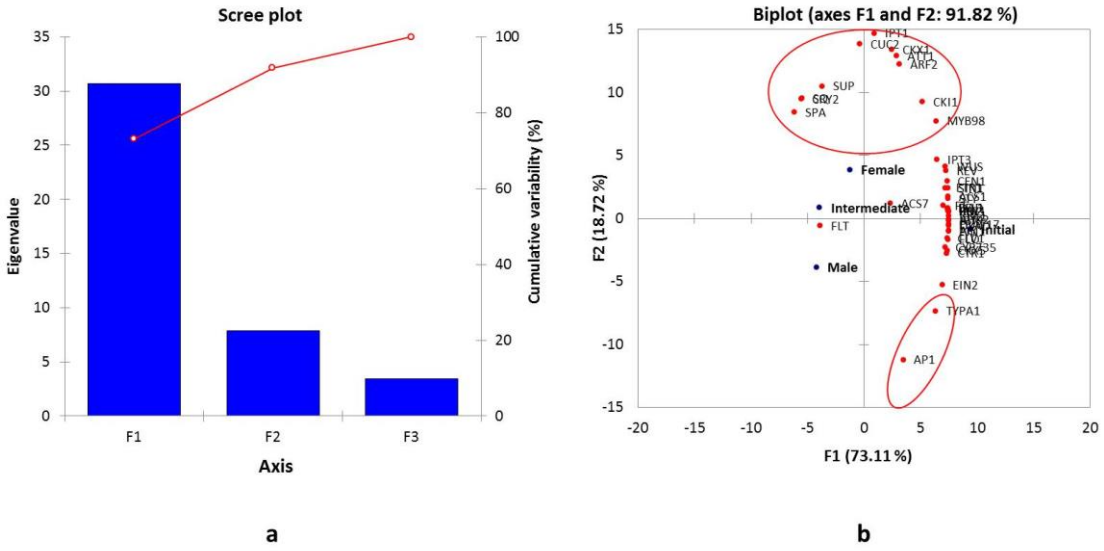

Figure 4.7: PCA **(a)** Scree plot showing variability between factors F1 and F2 **(b)** Biplot showing gene distribution among factors F1 and F2 where factors F1 is discriminating between initial and intermediate stages whereas F2 discriminating between male and female flowering stages.

4.6 *in silico* promoter analysis

The promoter regions of genes showing higher expression in transition to reproductive phase and female flowering were further analyzed to identify regulatory elements. Through *in silico* analysis 194 motifs were identified from which 34 were common motifs. The identified motifs have been implicated in light responsiveness, basal transcription, low temperature responsiveness, hormones (gibberellins, abscisic acid, cytokinins, and auxin) responsiveness, tissue specificity (leaf, flower, meristems, seed and root), etc. (Table 4.1). For floral transition, common elements ARR1AT, MYB1AT, BIHD1OS, POLLEN1LELAT52 and WRKY71OS were found which were associated with gibberellins, cytokinins, auxin pathways, abscisic acid and pollen development. GARE2OSREP1 and CARGATCONSENSUS were unique elements identified in promoter of genes involved in both transitions i.e. vegetative to reproductive phase and female flower transition in *Jatropha*. In *TAA1*, *CKX1*, and *PIN1* gens UP2ATMSD, GAREAT and MYB1AT respectively were uniquely present.

Table 4.1: *cis* regulatory elements identified in the promoter regions of *Jatropha* genes for female flowering

Regulatory elements	**Biological Function**
-10PEHVPSBD	Chloroplast psbD light-responsive promoter
ANAERO1CONSENSUS	Related to fermentative pathway
ARR1AT	Cytokinin-regulated transcription factor
BOXIINTPATPB	NCII regulation
BIHD1OS	BELL homeodomain transcription factor
CAATBOX1	CAAT promoter consensus sequence
CACTFTPPCA1	C4 phosphoenolpyruvate carboxylase gene.
CANBNNAPA	Embryo and endosperm-specific transcription factor
CARGATCONSENSUS	Regulates flowering time
DOFCOREZM	Dof protein
EBOXBNNAPA	E-box of napA storage-protein gene
EECCRCAH1	Binding site of Myb transcription factor LCR2
GAREAT	Gibberellin biosynthesis and response
GATABOX	Light responsive factor
GARE2OSREP1	Gibberellin-upregulated proteinase expression
GT1CONSENSUS	Light-regulated transcription
GT1GMSCAM4	Pathogen- and salt-induced SCaM-4 gene expression
GTGANTG10	cis-regulatory elements for pollen gene g10
IBOXCORE	Light-regulated transcription factor
INRNTPSADB	Light-responsive transcription factor

MYB1AT	Transcriptional activators in abscisic acid signaling
MYB2AT	Induced in draught stress
MYBST1	DNA binding protein
NODCON2GM	Regulating lbc3 and N23 gene promoters.
OSE1ROOTNODULE	Regulation in infected cells of root nodules
OSE2ROOTNODULE	Regulation in infected cells of root nodules
POLASIG1	poly A signal
POLLEN1LELAT52	Regulating anther and pollen development.
PREATPRODH	Hypo-osmolarity-responsive expression of the ProDH
RAV1AAT	DNA binding domain
ROOTMOTIFTAPOX1	Motif found both in promoters of rolD
SEF4MOTIFGM7S	beta-conglycinin enhancer
TAAAGSTKST1	Guards cell-specific gene expression
TATABOX3	TATA box found in the 5'upstream region
TATABOX5	5'upstream region of glutamine synthetase gene
WBOXATNPR1	WRKY *Sup*er family of plant transcription factors.
WBOXNTERF3	Activation of transcription of the ERF3 gene
WRKY71OS	Repressor of gibberellin signaling pathway
UP2ATMSD	Regulates gene expression during axillary bud growth

Results obtained from objective 2:

Through first objective we identified gene might be playing role in sex differentiation. To increase the number of female flowers, application of cytokinin on inflorescence meristem was done. Further, study was carried out to observe the effect of cytokinin on plant hormone signaling as well as genetic factors for increase in female flowers as well as floral buds. Furthermore, when the seed yield parameters were observed, we found increase in number of seed however, fruiting rate, seed size/weight was compromised. We hypothesized that increase in floral buds (biomass) and reduction in fruiting rate, seed weight/size might be the resultant of alteration in central carbon channeling.

4.7 Cytokinin (6-Benzladenine) application

Field experiment was conducted to study the effect of different concentrations (80mg/l, 160mg/l & 320mg/l) of BA (6-Benzyladenine) on *Jatropha* yield (Table 4.2). Out of three BA concentrations, 160mg/l of BA showed a significant increase i.e. ~3-fold in seed numbers and 80mg/l showed 2-fold increase in seed number. No significant change was observed in seed numbers at 320mg/l of BA application. Change in morphology of inflorescence meristem was observed at every week after 160mg/l of BA application (Figure 4.8).

Table 4.2: Seed numbers at different BA concentrations

Benzyl adenine (BA) Conc.	**Fruits/inflorescence (no.)**
Control	5
80 mg/l	10.2
160 mg/l	16
320 mg/l	6.9

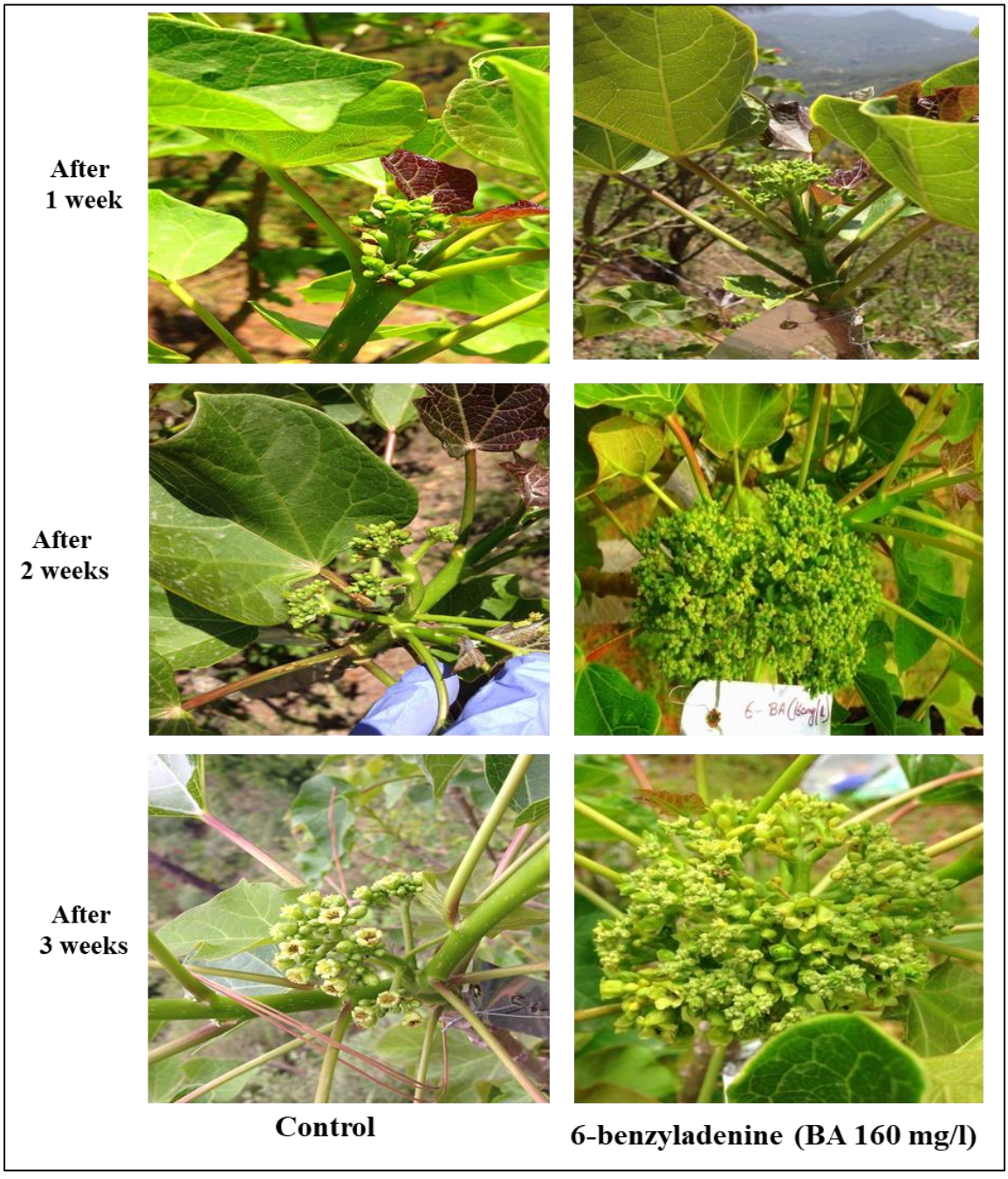

Figure 4.8: Morphological changes in inflorescence meristem after BA (160mg/l) treatment.

4.8 Effect of cytokinin treatment on biomass and yield

BA treated inflorescence showed ~7 to 8-fold increase of in floral buds (biomass) after 15 days and reduced to reduce to ~3 to 4-fold after 30 days of application (Table 4.3). No significant change was seen in number of leaves around the inflorescence post BA application. Further, post 80 days of treatment, yield parameters such as seed number, seed

size and seed weight were recorded (Table 4.4). Data showed the decrease in seed yield parameters (seed size & seed weight) in cytokinin treated inflorescences as compared to control however, increase in seed number was observed BA application (Figure 4.9, 4.10). These results showed compromised in overall fruiting rate and seed yield (weight & size) after application of cytokinin.

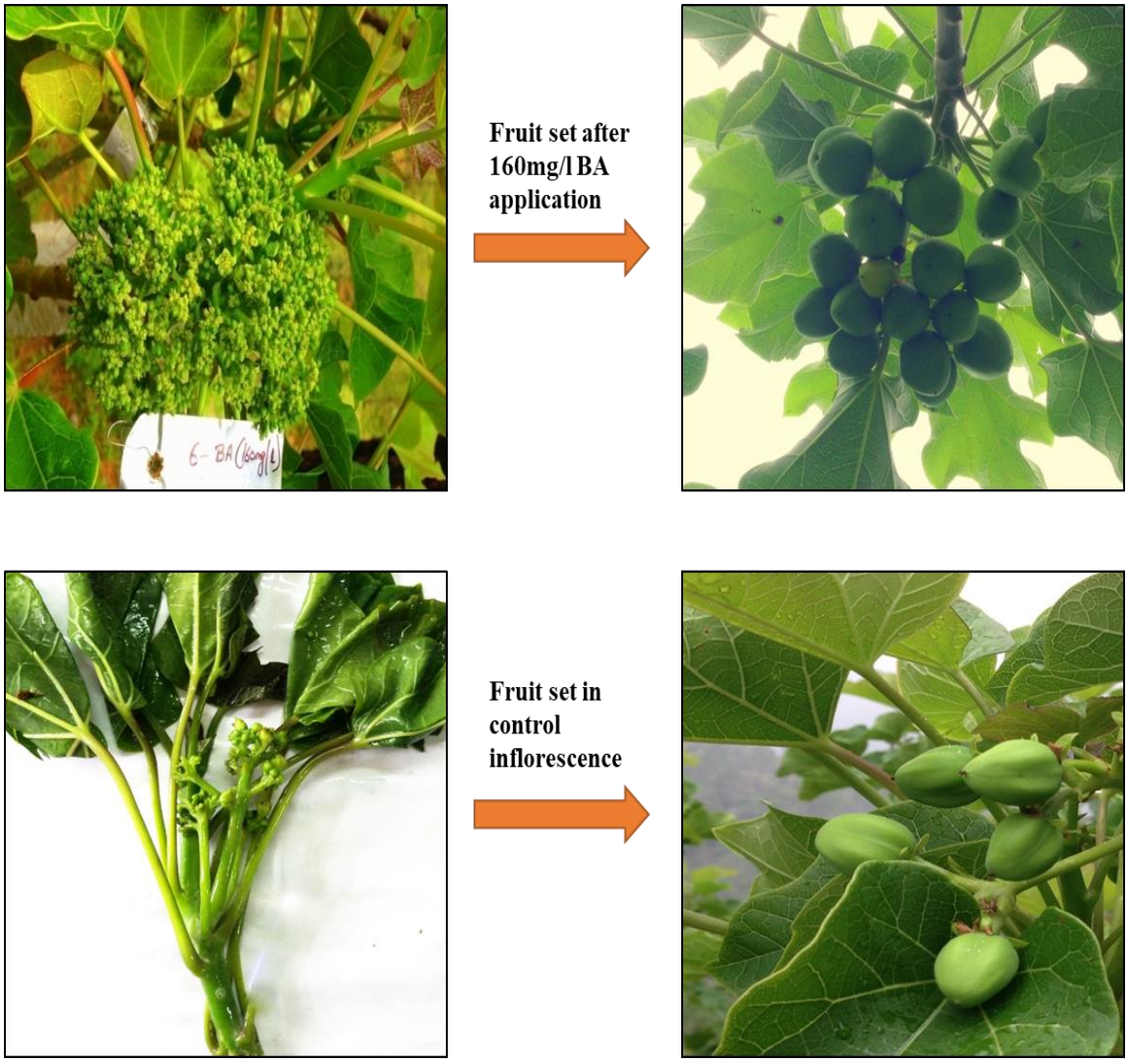

Figure 4.9: Fruit setting in BA-treated and control inflorescences.

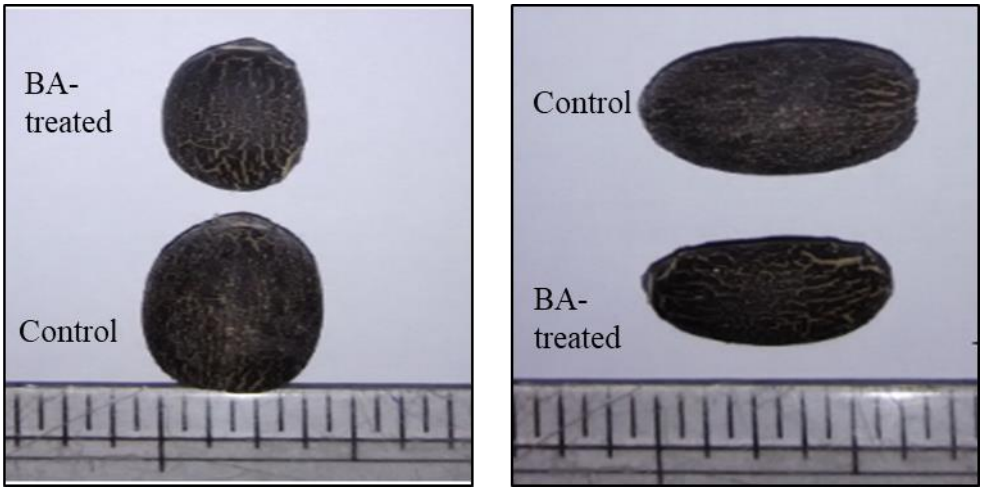

Figure 4.10: Morphology of seeds collected from Control and BA treated inflorescence (Scale bar – 1.27 cm).

Table 4.3: Effect of BA treatment on floral buds at different time intervals

Parameter	Control	TR15	TR30
Number of floral buds	150	1050	500
Number of female flowers	10	-*	55-60

(*No differentiation between female and male flowers in TR15)

Table 4.4: Seed yield parameters after BA treatment as compared to control

Yield Parameters	Control	BA treated
Seed size	Length -1.8 ± 0.02cm Diameter - 0.9 ± 0.012cm	Length - 1.4 ± 0.03cm Diameter - 0.7 ± 0.01cm
Seed weight	0.78 ± 0.02 g	0.48 ± 0.01 g
100 seed weight	65-67 g	50-52 g
Seed number	21-23	48-50

4.9 Transcriptome sequencing and data generation

cDNA libraries of VS, CONTROL, TR15 and TR30 tissues were sequenced on NextSeq 500 platform of Illumina using 2x150 PE Chemistry. The average coverage of transcriptome on NextSeq500 platform was ~200x. Raw reads were filtered by using Trimmomatic v0.30 to screen out the adaptor contamination and low quality (reads with

QV<20) reads. We got a total of 11,608,257, 12,184,924, 11,811,964 and 10,592,622 high quality reads in VS, CONTROL, TR15 and TR30, respectively (Table 4.5).

Table 4.5: Statistics of generated reads

Sample	Number of reads	Number of high quality reads
Vegetative	22,735,557	11,608,257
Control	22,707,846	12,184,924
TR15	22,988,570	11,811,964
TR30	20,562,981	10,592,622

4.10 Differential gene expression analysis

Upon annotation, based on FPKM value, a total of 55,755 transcripts were expressed in CONTROL, TR15 and were associated with multiple pathways. Expression of 253, 510 and 444 transcripts were found exclusively in CONTROL, TR15 and TR30, respectively. About 1350 transcripts were upregulated and 1236 transcripts were down regulated in TR15 as compared to CONTROL. In comparison with TR30, 3192 transcripts were upregulated, and 375 transcripts were down regulated in TR15. When transcript abundance was compared between VS & CONTROL tissues, 3486 transcripts were upregulated, and 191 transcripts were down regulated CONTROL. 413 transcripts were uniquely expressed in VS and 419 transcripts in CONTROL (Figure 4.11).

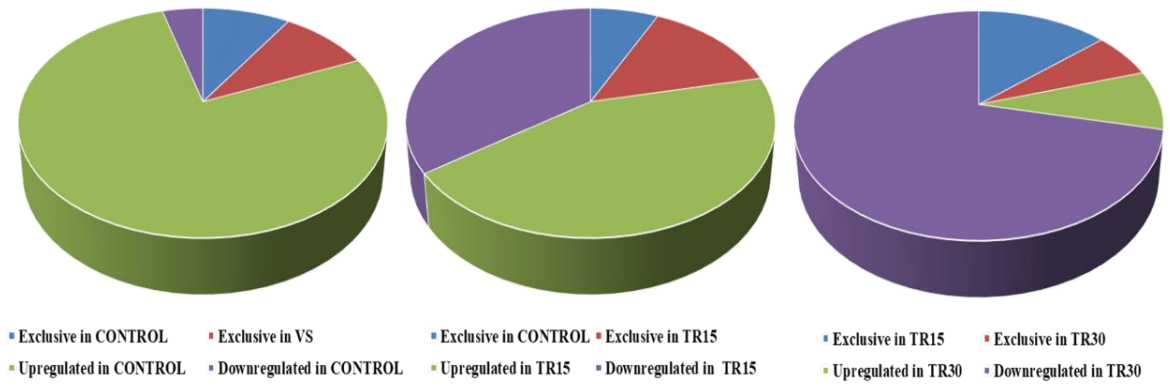

Figure 4.11: Distribution of genes expressed (a) Vegetative stage (VS) vs CONTROL, (b) CONTROL vs TR15, (c) TR15 vs TR30.

4.11 Gene ontology (GO) mapping and CDS distribution

The GO analysis categorized GO assignments majorly in 'biological process' (15,834 in CONTROL, 12,752 in TR15 & 15,584 in TR30) followed by 'molecular function' (12,073 in CONTROL, 11,945 in TR15 & 14,423 in TR30) and 'cellular component' (3592 in CONTROL, 2380 in TR15 & 3218 in TR30) in CONTROL, TR15 and TR30 (Figure 4.12). Highly represented process in biological process were single-organism process metabolic process, cellular process, biological regulation, localization, and response. Transporter activity, nucleic acid binding transcription factor, binding, structural molecule activity and catalytic activity were highly represented in molecular function. In cellular component category, highly represented components were; cell, membrane, organelle and macromolecular complex. Overall analysis revealed that in TR30, metabolic processes were significantly repressed as compared to TR15.

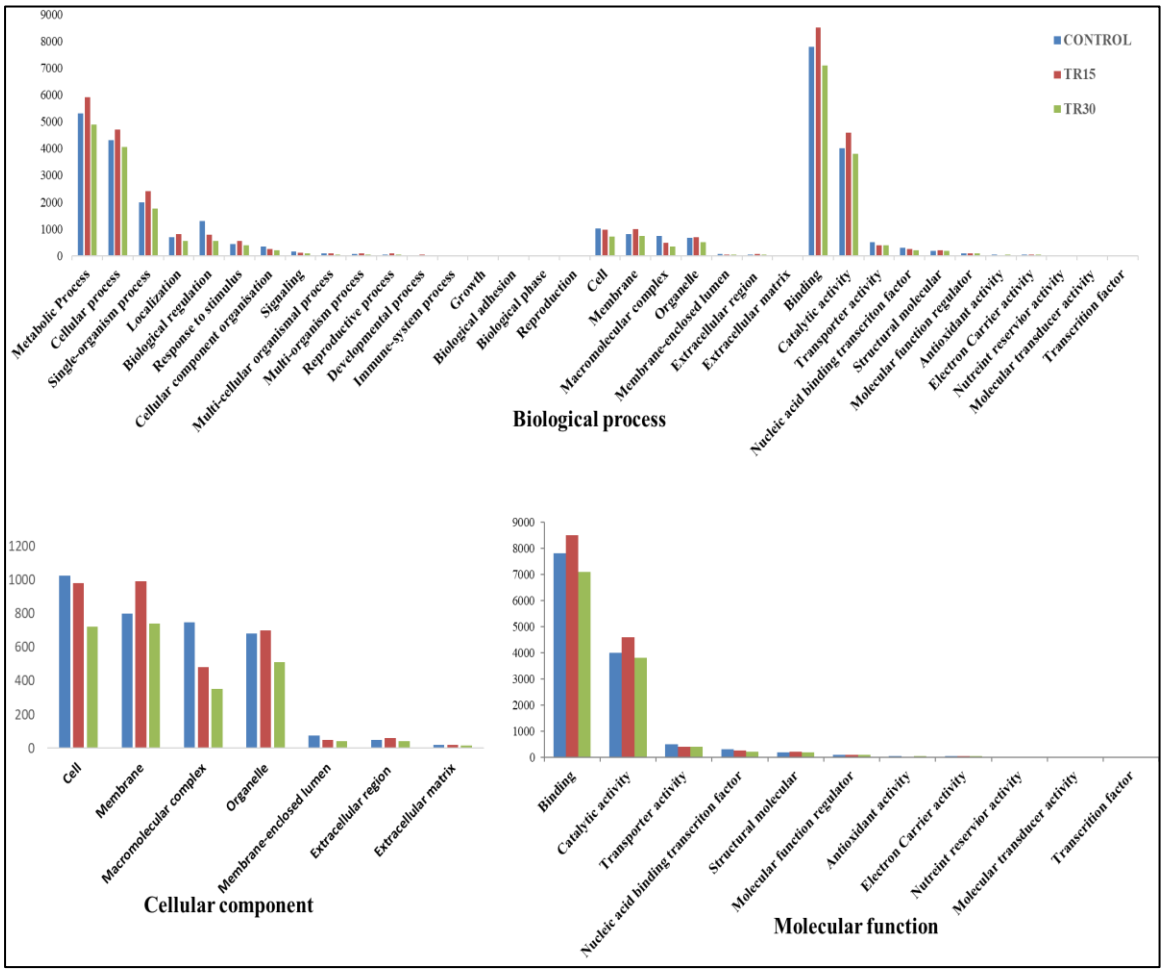

Figure 4.12: GO classification and distribution of GO annotated genes in CONTROL, TR15 & TR30 tissues of *Jatropha curcas*.

4.12 Vegetative to reproductive phase transition

We identified transcriptome profiling of vegetative tissues and control tissues for transition to reproductive phase. *MED13*, *EBS*, *FPGS1*, *REV*, *MSI4*, *MAF5*, *SPA1*, *SPA2*, *FPA*, *MED12*, *FCA*, *PIN1* and *BBX19* gene transcripts were found exclusively in the CONTROL indicating their association with reproductive phase transition. Transcript abundance of gene *AGL12*, *AGL17*, *AGL24*, *CRY1*, *CRY2 SPL3*, *SPL5* and *SPL15* was higher in CONTROL as compared to VS, inferring their role in transition towards flowering.

4.13 Induction of female flowering after BA-application

Transcriptome profiling identified genes that promotes feminism in the inflorescence in cytokinin treated inflorescence. In TR15, transcript level of *CKI1*, *SUP* and *PIN1* genes was higher as compared to control and TR30. These genes were found to be associated with female flowering. *TYPA1* gene showed lower transcripts in TR15 as compared to CONTROL and TR30 was identified for male flowering. Transcripts encoding *TCP18*, a *CYC* subfamily, known for stamen repression has been identified in *Jatropha*. Increased abundance of *TCP18* in TR15 indicated increase in rate of stamen abortion thus, promoting female flowering.

4.14 BA effects on endogenous cytokinin metabolism

Transcriptome analysis through comparative approach identified 11 transcripts associated with genes involved in cytokinin metabolism. These genes encode isopentenyl transferases homologs (*IPT1*, *IPT3*, *IPT5*, *IPT6*), cytokinin oxidase/dehydrogenase homologs (*CKX1*, *CKX3*, *CKX5*, *CKX6*, *CKX7*), cis-zeatin O-glucosyltransferase (*CISZOG*) and cytokinin hydroxylase (*CYP735A2*). *CISZOG*, *IPT3*, *IPT5*, *IPT6* and *CYP735A* showed higher gene transcript level in TR15 then TR 30 and CONTROL. Increase in transcript abundance of *CKX5* and *CKX7* genes in TR15 due to the induced *CKX* activity resulted by the elevated cytokinin levels in TR15.

4.15 BA application and effect on other phytohormones

33 gene transcripts associated with phytohormones (abscisic acids, brassinosteroids, ethylene and jasmonic acids) were identified through comparative transcriptome analysis. Gene transcripts of Ethylene -insensitive protein 3 and EIN3-binding F-box protein (*EIN3*) were upregulated in TR15 and are the positive regulators of ethylene signaling. On the other hand, in TR15, *ETR* gene which regulates ethylene signaling negatively was downregulated. Transcripts of genes encoding auxin-responsive protein *IAA*, *SAUR* family protein, auxin responsive *GH3* gene family associated with auxin signaling were identified. *SAUR* and *IAA* gene induces auxin and were upregulated by BA treatment in TR15 whereas, gene encoding auxin response factors were downregulated in TR15. Transcript abundance of abscisic acid receptor (*PYL* family) and *PP2C* was higher in TR15 whereas, transcripts of ABA responsive element binding factor decreased in TR15 as compared to CONTROL. *GID1* transcript level was reduced in TR15 which is a positive GA regulator. BR-signaling kinases (*BSK*) gene transcripts were increased in TR15 and those of brassinosteroid resistant ½ (*BSR*), a transcriptional repressor was decreased in TR15 as compared to CONTROL (Table 4.6).

Table 4.6: Differentially expressed genes of phytohormones

Sequence ID	**Enzyme name/number**	**Log2 fold change**	
		CN vs TR15	**TR15 vs TR30**
Jcr4S00177_82998-86435	EIN3-binding F-box protein	0.56	-1.12
Jcr4S01759_11162-18682	Ethylene receptor [EC:2.7.13.1]	-0.66	0.26
Jcr4S02323_6624-8483	Ethylene-insensitive protein 3	0.8	0.34
Jcr4S00049_111184-111600	SAUR family protein	2.84	-1.76
Jcr4S00458_50619-53558	Auxin-responsive protein IAA	1.24	-1.5
Jcr4S02357_942-3048	Auxin responsive GH3 gene family	0.6	-0.38
Jcr4S00497_63734-67994	Auxin response factor	-0.79	0.3
Jcr4S03612_13164-17862	BR-signaling kinase [EC:2.7.11.1]	0.82	-1.03
Jcr4S01477_53915-55411	Brassinosteroid resistant 1/2	-0.92	0.93
Jcr4S00168_90949-91494	Jasmonate ZIM domain-containing protein	2.12	-3.23
Jcr4S03704_890-2365	Jasmonic acid-amino synthetase	2.19	-2.84

Jcr4S00137_90631-91284	Abscisic acid receptor PYR/PYL family	0.83	-0.73
Jcr4S02550_1115-4019	Protein phosphatase 2C [EC:3.1.3.16]	0.75	-1.02
Jcr4S01146_27749-28276	F-box protein GID1	-0.72	-0.3

4.16 Pathway analysis

KEGG annotation mapped transcripts to pathways associated with carbon fixation and carbon flow. Out of these, majority of genes mapped to pathways such as photosynthesis, Calvin cycle, oxidative phosphorylation, carbohydrate metabolism, nitrogen metabolism and glycolysis.

4.17 BA application modulating pathways of carbon fixation and carbon flow

Plant feedstock is largely affected by photosynthesis rate, carbon fixation and carbon/nitrogen ratio. These pathways provide carbon skeleton, which is a backbone for synthesis of compounds involved in numerous biological activities. The effect of exogenous cytokinin treatment was studied on pathways/genes contributing to photosynthesis, citrate cycle, pentose phosphate pathway, oxidative phosphorylation, pyruvate metabolism, starch metabolism, nitrogen metabolism at different time intervals affecting the overall feedstock yield in *Jatropha* (Figure 4.13). Differential gene expression of genes associated with these pathways was done in CONTROL, TR15, TR30 stages of *Jatropha*.

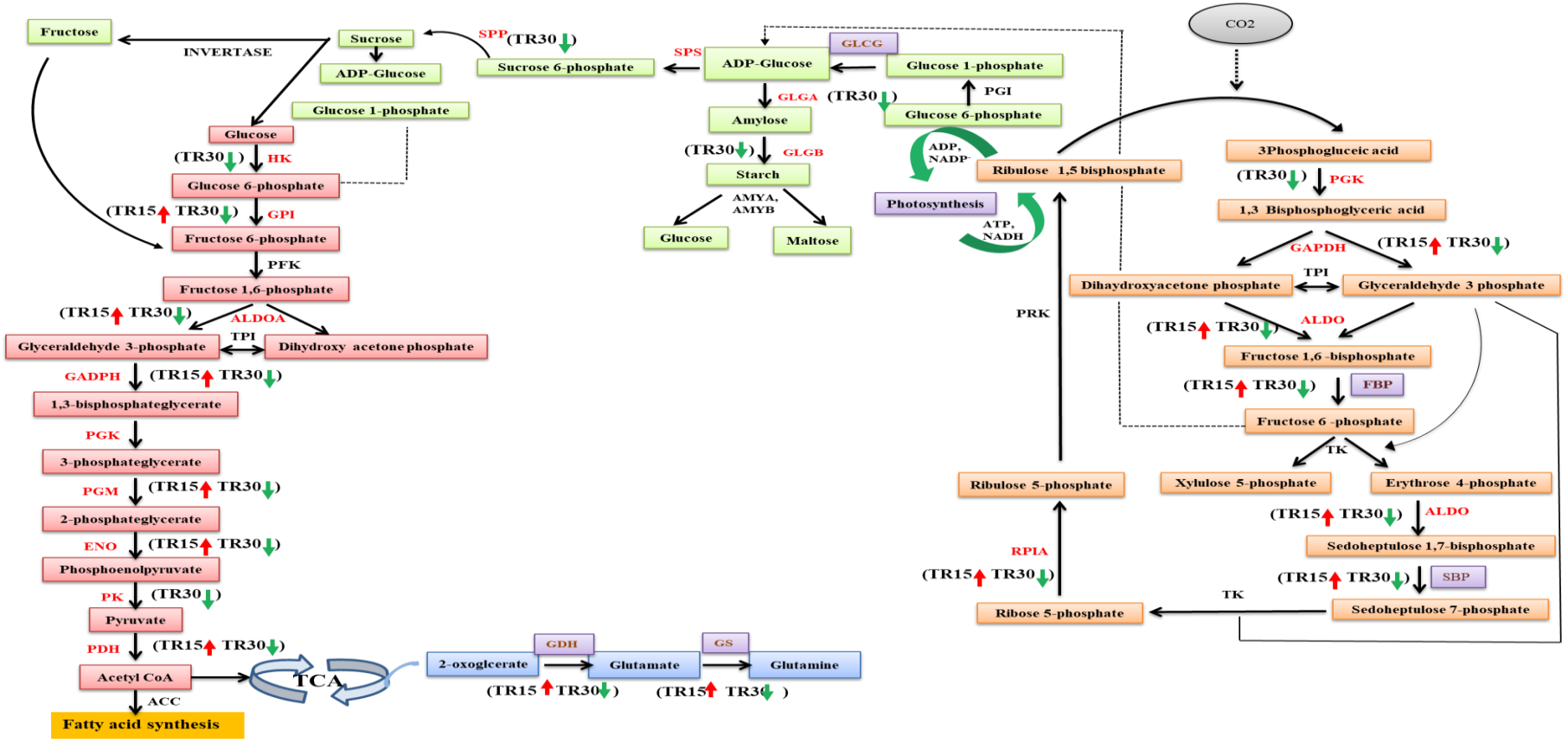

Figure 4.13: Representation of enzymes upregulated and downregulated in metabolic pathways associated with carbon flux in *Jatropha* post 15 days (TR15) & TR30 days (TR30) of cytokinin treatment. Enzymes are shown in red and black colors, Red color represents differential expression status of genes, Black color represents no significant change in expression status. (↑Arrow represents upregulation &↓Arrow represents downregulation). (RBCL- Ribulose-bisphosphate carboxylase large chain; PGK- Phosphoglycerate kinase; GAPDH- Glyceraldehyde 3-phosphate dehydrogenase; ALDO- aldolase, class I; FBP- Fructose-1,6-bisphosphatase I; TK- Transketolase; SBP- Sedoheptulose-bisphosphatase; PRK- Phosphoribulokinase; RPIA- Ribose 5-phosphate isomerase A; GPI- Glucose-6-phosphate isomerase; PGM-Phosphoglucomutase; GLGC- Glucose-1-phosphate adenylyltransferase; GLGA-Starch synthase; GLGB- 1,4-alpha-glucan branching enzyme; AMYA- Alpha-amylase; AMYB- Beta-amylase; HK- Hexokinase; GPI- Glucose-6-phosphate isomerase; PFK- 6-phosphofructokinase 1; PDH-Pyruvate dehydrogenase).

4.17.1 Photosynthesis

Photosynthesis, a tightly controlled process captures light energy and convert it into ATP and NADPH. Upon comparative transcriptome analysis, 30 genes were identified for to be involved in photosynthesis pathway. Genes encoding photosystem *PSI* & *PSII*, light harvesting complex *LHCI* & *LHCII* and F-type H+-transporting ATPase were upregulated in TR15 as compared to control. However, these genes were whereas showed downregulation in TR30 as compared to CONTROL and TR15. Transcripts of genes encoding plastocyanin, F-type H+-transporting ATPase and ferredoxin-NADP+ reductase was decreased in TR30 compared to TR15. No significant difference in transcript abundance of these genes was observed in TR15 and CONTROL (Table 4.7).

Table 4.7: Differentially expressed genes of photosynthesis

Sequence ID	**Enzyme (name/number)**	**Log2 (fold change)**	
		CN vs TR15	**TR15 vs TR30**
Jcr4S00004_59679-60185	Plastocyanin	-0.005	-0.7
Jcr4S00006_31263-33359	Ferredoxin--NADP+ reductase [EC:1.18.1.2]	0.25	-0.8
Jcr4S00013_121663-122920	Photosystem II oxygen-evolving enhancer protein 1	1.16	-0.99
Jcr4S00033_37885-38319	Ferredoxin	0.65	-1.42
Jcr4S00209_18779-19955	Photosystem II 13kDa protein	0.56	-0.9
Jcr4S00072_51797-52989	Photosystem I subunit X	1.03	-1.36
Jcr4S00476_10364-11288	Photosystem II oxygen-evolving enhancer protein 3	0.5	-0.84
Jcr4S00520_62094-62844	Photosystem II PsbW protein	0.5	-1.14
Jcr4S00559_5842-7233	F-type H+-transporting ATPase subunit beta [EC:3.6.3.14]	0.0	-0.95
Jcr4S01334_19013-19681	Photosystem I subunit IV	1.9	-0.83
Jcr4S01478_30103-31246	Photosystem I subunit VI	0.3	-0.6
Jcr4S02812_132-9944	Photosystem II PsbK protein	0.11	-0.98
Jcr4S00002_84410-87459	Light-harvesting complex II chlorophyll a/b binding protein 7	0.33	-1.44

Jcr4S00132_96270-97760	Light-harvesting complex I chlorophyll a/b binding protein 2	0.17	-1.7
Jcr4S00152_27207-28163	Light-harvesting complex II chlorophyll a/b binding protein 4	0.6	-2.79
Jcr4S00217_73371-74542	Light-harvesting complex I chlorophyll a/b binding protein 3	1.22	-0.87
Jcr4S00313_61165-62078	Light-harvesting complex I chlorophyll a/b binding protein 4	0.44	-1.88
Jcr4S01794_19047-20251	Light-harvesting complex II chlorophyll a/b binding protein 3	0.6	-1.83

4.17.2 Carbon fixation

ATP and NADPH, the products of light-dependent reaction (photosynthesis), are used in carbon fixation in Calvin cycle. Transcripts for 18 genes were identified for the calvin cycle using *Jatropha* transcriptome. Enzymes such as 6-phosphogluconolactonase, ribulose-bisphosphate carboxylase small chain, fructose-1,6-bisphosphatase I and glyceraldehyde-3-phosphate dehydrogenase genes shoed increase in transcript abundance in TR15 compared to CONTROL and TR30 whereas enzymes such as phosphoglycerate kinase, phosphoglucomutase, fructose-bisphosphate aldolase (*FBP*), ribose 5-phosphate isomerase A, ribulose-phosphate 3-epimerase & transaldolase, sedoheptulose-bisphosphatase (*SBP*) showed higher transcript abundance in TR15 compared to TR30 with no significant change with respect to CONTROL. Genes encoding enzymes glutamate—glyoxylateaminotransferase, phosphoenolpyruvate carboxykinase, malate dehydrogenase, pyruvate orthophosphate dikinase, acetyl-CoA carboxylase carboxyl transferase subunit alpha, ATP citrate (pro-S)-lyase, methylenetetrahydrofolate reductase (*NADPH*), isocitrate dehydrogenase, acetyl-CoA carboxylase aconitate hydratase, were also involved in carbon fixation showing higher transcript abundance in TR15 as compared to both CONTROL and TR30 (Table 4.8).

Table 4.8: Differentially expressed genes of carbon fixation

Sequence ID	Enzyme (name/number)	Log2 (fold change)	
		CN vs TR15	TR15 vs TR30
Jcr4S00445_53788-60856	Phosphoglucomutase [EC:5.4.2.2]	-0.11	-0.48
Jcr4S00240_41352-43682	Glyceraldehyde-3-phosphate dehydrogenase (NADP+) (phosphorylating) [EC:1.2.1.13]	1.17	-2.04
Jcr4S00484_15852-19652	Fructose-bisphosphate aldolase, class I [EC:4.1.2.13]	0.13	-0.51
Jcr4S00669_31271-32080	Ribose 5-phosphate isomerase A [EC:5.3.1.6]	0.44	-1.48
Jcr4S01203_22526-25157	Ribulose-phosphate 3-epimerase [EC:5.1.3.1]	-0.11	-0.54
Jcr4S01363_41711-42271	Ribulose-bisphosphate carboxylase small chain [EC:4.1.1.39]	1.16	-1.88
Jcr4S02321_22180-23477	6-phosphogluconolactonase [EC:3.1.1.31]	1.12	-1.49
Jcr4S00615_6656-13321	Phosphoglycerate kinase [EC:2.7.2.3]	0.05	-1.25
Jcr4S01914_9435-11836	Sedoheptulose-bisphosphatase [EC:3.1.3.37]	0.34	-1.13
Jcr4S02543_11726-16502	Phosphoenolpyruvate carboxykinase (ATP) [EC:4.1.1.49]	0.63	-0.7
Jcr4S00219_29945-34033	Glutamate--glyoxylate aminotransferase [EC:2.6.1.4]	0.36	-0.9
Jcr4S00007_152737-158948	Pyruvate, orthophosphate dikinase [EC:2.7.9.1]	0.23	-0.6
Jcr4S01192_1754-6509	Methylenetetrahydrofolate reductase (NADPH) [EC:1.5.1.20]	0.07	-0.7
Jcr4S03343_8480-10365	Fructose-1,6-bisphosphatase I [EC:3.1.3.11]	0.78	-1.3
Jcr4S00736 7917-16674	Aconitate hydratase [EC:4.2.1.3]	0.58	-0.78

4.17.3 Oxidative phosphorylation

ATPs for carbon fixation, are also generated by oxidation of NADH and FADH2 through oxidative phosphorylation process. About 69 transcripts were identified for oxidative phosphorylation in *Jatropha* transcriptome. Genes encoding inorganic pyrophosphatase, NAD(P)H-quinone oxidoreductase subunit 5, NADH dehydrogenase, cytochrome c oxidase subunit 6a, ubiquinol-cytochrome c reductase and H+-transporting ATPase showed higher transcript abundance in TR15 compared to CONTROL and TR30. Genes encoding, F-type H+-transporting ATPase subunit beta, F-type H+-transporting ATPase subunit g, V-type H+-transporting ATPase, succinate dehydrogenase showed lower transcripts level in TR30 as compared to TR15 (Table 4.9).

Table 4.9: Differentially expressed genes of oxidative phosphorylation

Sequence ID	**Enzyme (name/number)**	**Log2 (fold change)**	
		CN vs TR15	**TR15 vs TR30**
Jcr4S00049_59951-67112	V-type H+-transporting ATPase subunit A [EC:3.6.3.14]	0.92	-0.7
Jcr4S00059_85242-85580	F-type H+-transporting ATPase subunit delta	0.98	-1.32
Jcr4S00087_92804-94332	NADH dehydrogenase (ubiquinone) Fe-S protein 7 [EC:1.6.5.3 1.6.99.3]	1	-1.37
Jcr4S00095_68085-68393	NADH dehydrogenase (ubiquinone) 1 beta subcomplex subunit 7	0.21	-0.86
Jcr4S00169_55484-58425	NADH dehydrogenase (ubiquinone) Fe-S protein 4	1.2	-0.62
Jcr4S00202_25627-27992	NADH dehydrogenase (ubiquinone) Fe-S protein 5	0.4	-0.69
Jcr4S00609_17304-25164	Cytochrome c oxidase subunit 6b	0.24	-0.57
Jcr4S00626_18589-18963	NAD(P)H-quinone oxidoreductase subunit 5 [EC:1.6.5.3]	-0.21	-1.03
Jcr4S00665_23824-25477	V-type H+-transporting ATPase 21kDa proteolipid subunit	-0.17	-0.76
Jcr4S00742_13156-13951	NADH dehydrogenase (ubiquinone) 1 alpha/beta subcomplex 1	0.9	-1.28
Jcr4S00752_66768-68242	F-type H+-transporting ATPase subunit g	-0.4	-0.87
Jcr4S00785_3706-10081	H+-transporting ATPase [EC:3.6.3.6]	0.22	0.71

Jcr4S00914_32001-34845	V-type H+-transporting ATPase subunit E	-0.07	-0.68
Jcr4S01070_21212-21905	Cytochrome c oxidase subunit 6a	0.49	-2.01
Jcr4S01215_55453-57814	Ubiquinol-cytochrome c reductase subunit 7	1.21	-1.25
Jcr4S01331_10546-12857	Ubiquinol-cytochrome c reductase cytochrome c1 subunit	0.67	-1.46
Jcr4S02161_2440-3638	Succinate dehydrogenase (ubiquinone) iron-sulfur subunit [EC:1.3.5.1]	-0.1	-1.17
Jcr4S02700_14652-17508	Inorganic pyrophosphatase [EC:3.6.1.1]	0.75	-1.11
Jcr4S02741_27535-28962	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex subunit 6	1.38	-1.26
Jcr4S04536_15700-18333	NADH dehydrogenase (ubiquinone) 1 beta subcomplex subunit 9	0.89	-1.28

4.17.4 Carbohydrate metabolism

Carbon assimilated through Calvin cycle is utilized for carbohydrate synthesis. Starch and sucrose serve as a major carbohydrate biomolecule in plants. Based on KEGG pathway assignments, 23 genes were identified for starch and sucrose metabolism and transcriptome showed upregulation of 18 genes in TR15 then TR30. Transcript abundance showed higher expression of genes encoding enzymes pectinesterase, sucrose synthase, endoglucanase, trehalose 6-phosphate phosphatase, beta-glucosidase, polygalacturonase, beta-fructofuranosidase, beta-D-xylosidase 4 and alpha-1, 4-galacturonosyltransferase in TR15 as compared to CONTROL and TR30. Transcript abundance of genes encoding enzymes 4-alpha-glucanotransferase, hexokinase, trehalose 6-phosphate synthase/phosphatase, 1, 4-alpha-glucan branching enzyme, phosphoglucomutase (*PGM*), sucrose-phosphate synthase and glucose-1-phosphate adenylyltransferase (*AGPase*) increased in TR15 with respect to TR30 showing no significant difference with CONTROL.

During night, the stored carbon (starch) is mobilized as sucrose in the cytoplasm. Triose phosphate form fructose1, 6-bisphosphate is converted to fructose-6 phosphate by fructose-1, 6-bisphosphatase (*FBP*). Transcript abundance of *FBP* regulating sucrose metabolism pathway was increased in TR15 as compared to CONTROL and TR30. Reduction in transcript abundance of gene encoding Sucrose phosphate synthase (*SPS*) occurred in TR30 as compared to TR15. (Table 4.10).

Table 4.10: Differentially expressed genes in carbohydrate metabolism

Sequence ID	Enzyme (name/number)	Log2 (fold change)	
		CN vs TR15	TR15 vs TR30
Jcr4S00004_187681-189573	Pectinesterase [EC:3.1.1.11]	0.93	-1.67
Jcr4S00093_62597-69132	Sucrose synthase [EC:2.4.1.13]	0.88	-1.09
Jcr4S00103_22259-24324	Trehalose 6-phosphate phosphatase [EC:3.1.3.12]	1.97	0.66
Jcr4S00282_52772-59164	Beta-glucosidase [EC:3.2.1.21]	0.67	-1.22
Jcr4S00335_29091-31621	Beta-fructofuranosidase [EC:3.2.1.26]	0.68	-1.2
Jcr4S00241_82269-83743	Polygalacturonase [EC:3.2.1.15]	1.28	-2.68
Jcr4S01628_3966-7157	Endoglucanase [EC:3.2.1.4]	0.73	-0.88
Jcr4S03568_15969-20860	Trehalose 6-phosphate synthase/phosphatase [EC:2.4.1.15 3.1.3.12]	0.26	-1.12
Jcr4S03968_5690-9225	Beta-D-xylosidase 4 [EC:3.2.1.37]	0.62	-1
Jcr4S04515_637-3529	4-alpha-glucanotransferase [EC:2.4.1.25]	0.46	-0.7
Jcr4S00476_22059-25615	Alpha-1,4-galacturonosyltransferase [EC:2.4.1.43]	0.79	-0.98
Jcr4S00594_60398-65518	Sucrose-phosphate synthase [EC:2.4.1.14]	-0.1	-1.42
Jcr4S00679_7418-10356	Hexokinase [EC:2.7.1.1]	0.11	-0.47
Jcr4S01214_7963-15059	1,4-alpha-glucan branching enzyme [EC:2.4.1.18]	0.12	-0.7
Jcr4S01990_32377-36466	Glucose-1-phosphate adenylyltransferase [EC:2.7.7.27]	0.04	-0.93
Jcr4S00075_66135-70136	Acetyl-CoA carboxylase carboxyl transferase subunit alpha [EC:6.4.1.2]	0.20	-1.24
Jcr4S01952_11474-15725	Isocitrate dehydrogenase [EC:1.1.1.42]	0.29	-0.77
Jcr4S00265_43297-46308	ATP citrate (pro-S)-lyase [EC:2.3.3.8]	0.57	-1.67
Jcr4S03343_8480-10365	Fructose-1,6-bisphosphatase I [EC:3.1.3.11]	0.78	-1.38

4.17.5 Citric acid cycle

Starch phosphorylase transfers glucose from nonreducing end of α-1, 4-linked glucan to orthophosphate. This mechanism generates glucose 1-phosphate and was downregulated in TR30. This glucose 1-phosphate then enters glycolysis for sugar breakdown. Citric acid cycle (TCA) then use pyruvate as precursors for carbon skeleton formed at the end of glycolysis. 15 transcripts were identified for citric acid cycle of which 7 genes were differentially expressed. Genes for citrate synthase, pyruvate dehydrogenase E2 component, ATP citrate (pro-S)-lyase, phosphoenolpyruvate carboxykinase (ATP) and aconitate hydratase were upregulated in TR15. Genes for dihydrolipoamide dehydrogenase, pyruvate dehydrogenase E1 component beta subunit and succinate dehydrogenase (ubiquinone) iron-sulfur subunit were downregulated in TR30 as compared to control (Table 4.11).

Table 4.11: Differentially expressed genes in citric acid cycle

Sequence ID	**Enzyme (name/number)**	**Log2 (fold change)**	
		CN vs TR15	**TR15 vs TR30**
Jcr4S00215_70324-75209	Citrate synthase [EC:2.3.3.1]	0.57	-1.48
Jcr4S00306_73221-80332	Pyruvate dehydrogenase E2 component (dihydrolipoamide acetyltransferase) [EC:2.3.1.12]	0.58	-0.85
Jcr4S00312_24824-27164	Pyruvate dehydrogenase E1 component alpha subunit [EC:1.2.4.1]	0.48	-0.48
Jcr4S00014_57901-60369	Dihydrolipoamide dehydrogenase [EC:1.8.1.4]	0.002	-0.84

4.17.6 Nitrogen assimilation and carbon to nitrogen (C/N) ratio

Transcripts for 9 genes associated with nitrogen metabolism were identified, of which 6 were differentially expressed. Transcript abundance of genes encoding enzymes such as carbonic anhydrase, glutamine synthetase, formamidase, nitrate reductase and nitrate/nitrite transporters were decreased in TR30 (Table 4.12) showing less rate of nitrogen metabolism.

Table 4.12: Differentially expressed genes in nitrogen metabolism

Sequence ID	Enzyme (name/number)	Log2 (fold change)	
		CN vs TR15	TR15 vs TR30
Jcr4S00031_72882-74985	Glutamate dehydrogenase (NAD(P)+) [EC:1.4.1.3]	0.57	-1.48
Jcr4S00507_2229-5065	Glutamine synthetase [EC:6.3.1.2]	0.06	-0.78
Jcr4S00587_33470-36783	Carbonic anhydrase [EC:4.2.1.1]	0.84	-1
Jcr4S01404_28574-31709	Formamidase [EC:3.5.1.49]	0.16	-0.55
Jcr4S02321_3916-8442	Nitrate reductase (NAD(P)H) [EC:1.7.1.1 1.7.1.2 1.7.1.3]	0.7	-0.77
Jcr4S03942_2293-4082	MFS transporter, NNP family, nitrate/nitrite transporter	0.09	-1.23

4.18 Heat map of differentially expressed genes

Differentially expressed genes in Vegetative, Control, TR15 & TR30 were analyzed by hierarchical clustering. A heat map was constructed by using log-transformed and normalized value of genes based on Pearson's uncentered correlation distance as well as based on complete linkage method (Figure 4.14).

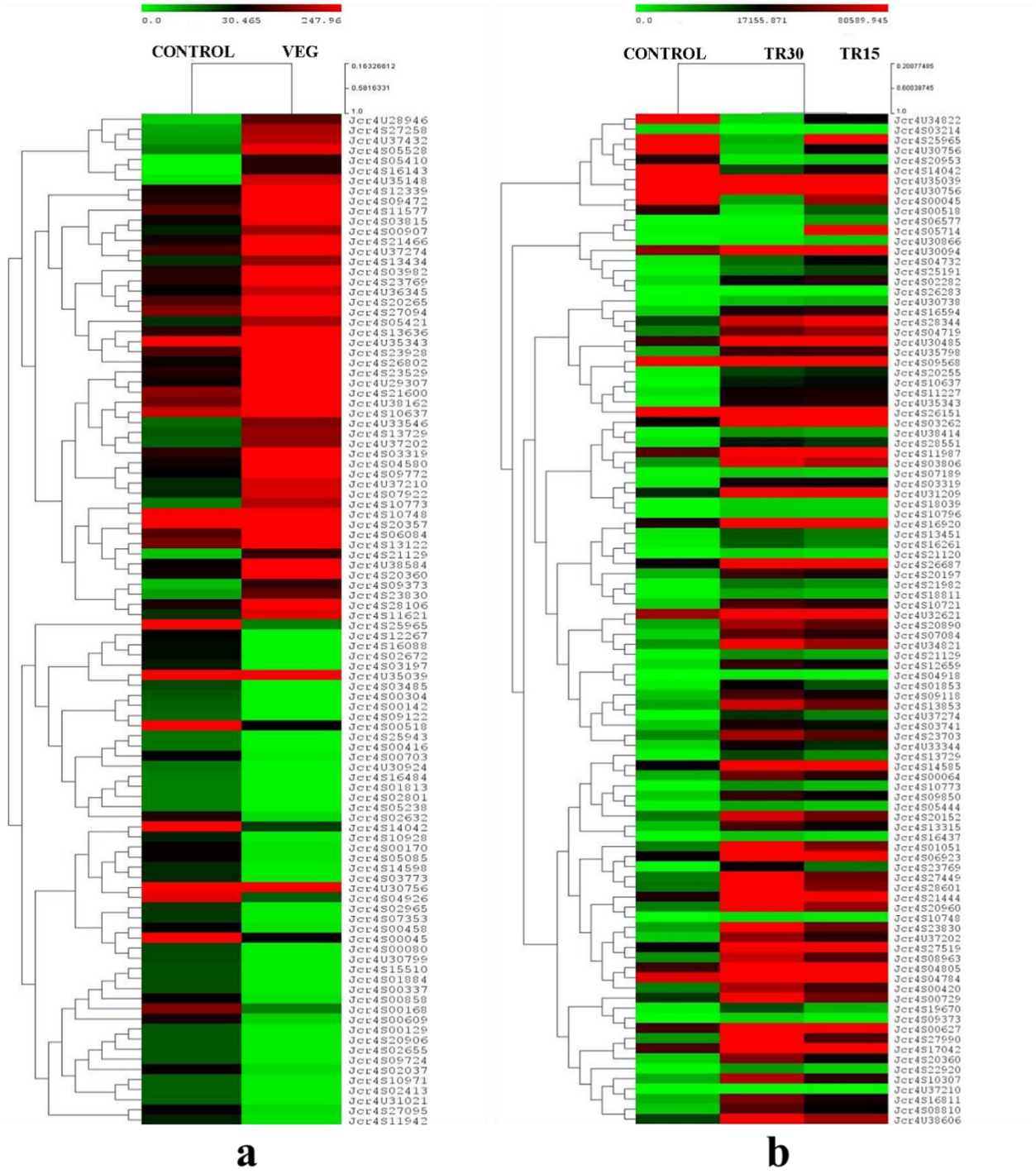

Figure 4.14: Heat-map representing differentially expressed genes at (a) Vegetative vs Control, (b) Control vs TR15 & TR30 (MultiRankSeq differentially expressed CDS based on DESeq2 rank<=200).

4.19 RT-qPCR based experimental validation

Transcript abundance of genes involved in photosynthesis (6), carbon fixation (9), starch metabolism (3) nitrogen metabolism (4), glycolysis (2) and cytokinin metabolism (5), were validated through RT-qPCR. *SBP*, *FBP*, *GDH*, *GS*, *RBCL*, *RPIA* and *GLGC* were upregulated upto ~4-fold in TR15 as compared to CONTROL and ~0.03-fold of downregulation in TR30 as compared to TR15 (Figure 4.15). Expression status of flowering genes and female flowering genes was also checked in CONTROL and TR15 (Figure 4.16). Thus, a positive correlation was observed between transcriptome (RNA-seq data) and experimental validation (RT-qPCR).

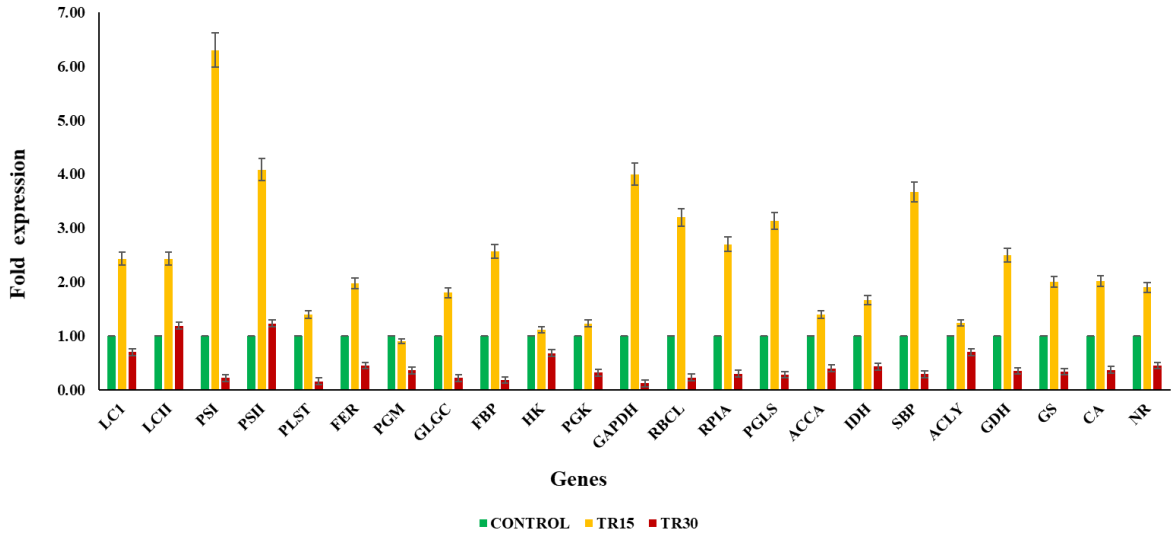

Figure 4.15: RT-qPCR-based expression pattern of genes involved in photosynthesis, carbon fixation, starch and nitrogen metabolism and glycolysis. (*LCI*- light-harvesting complex I; *LCII*- light-harvesting complex I; *PSI*- photosystem I; *PSII*- photosystem II; *PLST*- plastocyanin; *FER*- ferredoxin ; *PGM*-phosphoglucomutase; *GLGC*-glucose-1-phosphate adenylyltransferase; *FBP*- fructose-1,6-bisphosphatase; *G6PE1*- glucose-6-phosphate 1-epimerase; *HK*- hexokinase; *PGK*- phosphoglycerate kinase; *GAPDH*-Glyceraldehyde 3-phosphate dehydrogenase; *RBCL*- Ribulose-bisphosphate carboxylase large chain; *RPIA*-Ribose 5-phosphate isomerase A; *PGLS*- 6-phosphogluconolactonase; *ACCA*-acetyl-CoA carboxylase carboxyl transferase subunit alpha; *IDH*- isocitrate dehydrogenase; *SBP*- sedoheptulose-bisphosphatase; *ACLY*- ATP citrate (pro-S)-lyase; *GDH*- glutamate dehydrogenase ; *GS*- glutamine synthetase; *CA*-carbonic anhydrase; *NR*- nitrate reductase).

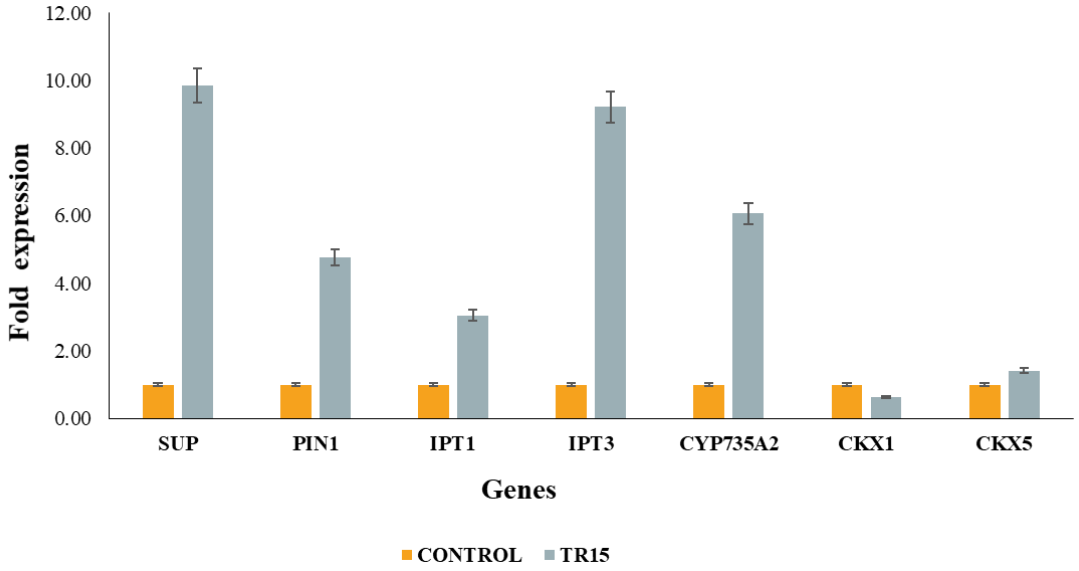

Figure 4.16: RT-qPCR-based expression pattern of flowering genes and female flowering genes. (SUP-Superman; PIN1-Pinformed1; *IPT2*-tRNAisopentenyltransferase; *IPT3*-Adenylate isopentenyltransferase; *CYP735*-Cytochrome P735; *CKX1*-Cytokinin oxidase 1; *CKX5*-Cytokinin oxidase 5)

CHAPTER 5
DISCUSSION

Overall increase in oil production is the long-term objective for plant-based biodiesel. *J. curcas* due to its various merits is considered as a potential source of biodiesel. However, some major constraints like variation in oil content among accessions, low female flower ratio, low productivity in terms of yield, susceptibility to various biotic stresses and non-availability of sufficient feedstock have been limiting factors in this plant as a feasible source for biodiesel production. As *Jatropha* has lower female to male flower ratio, which in turn reduces seed yield and overall seed oil. Thus, identification of molecular machinery to comprehend female flowering and its transition is crucial. Also, there is a need to identify molecular components associated with yield in response to cytokinin treatment in *J. curcas*. In our study, we treated *Jatropha* inflorescence with 6-Benzyladenine (BA). After BA application there was no significant increase in seed yield (fruiting rate, seed size and seed weight). Therefore, the present study was carried out with an aim of elucidating molecular basis of female flowering and its transition as well as understanding molecular mechanisms underlying carbon capture and flux in response to cytokinin application in *J. curcas* to identify the factors affecting the overall yield. This research work has provided leads which can be taken forward for the enhancement of seed yield in *J. curcas* using gene engineering tools.

The results obtained to achieve the defined objectives are discussed as under:

5.1 Molecular cues for transition from vegetative to reproductive phase

Floral cycle of plants is largely influenced by meristem behavior, producing two types of meristems: meristem which does produce any reproductive organs (vegetative) and other is the inflorescence meristem (reproductive) having organ primordia and floral organs. When flowering signal is triggered, *FT/FD* heterodimer moves to the shoot apex and activates *AP1* resulting in induction of expression floral organ genes which in turn initiates phase transition by initiation flowering. *AP1*, a floral meristem identity gene belongs to MADS-box family transcription factor, In *Jatropha*, *AP1*, a floral meristem identity gene of MADS-box family, acts a phase activator or switch for transition to reproductive phase

showed ~426-fold increase in RSI stage (SAM with emerging floral buds) and initiates the floral development by patterning, integrating growth, and hormonal pathways [196-199]. The promoter of *AP1* gene is a flower-specific promoter which directs the expression of *SUP* gene. *SUP*, a cadastral gene and C2H2 type zinc finger protein family transcription factor, defines the boundary of floral organs and the expression of organ identity genes [200]. *TFL1,* a TCP transcription factors showed ~ 65-fold higher expression in RSI suggesting its role in floral transition and maintaining inflorescence meristem. Li et al. [71] also reported that *JcTFL1b* was highly expressed in reproductive phase in *Jatropha* and maintains the inflorescence meristem, floral buds and reproductive growth [193]. Apart from floral homeotic genes, circadian rhythms also play an important rale in phase transition. *CRY2* encodes a cryptochrome protein which was expressed ~3-fold higher in reproductive phase. Cryptochrome protein interacts with circadian clock and promotes flowering by inducing the expression of *CO* gene which then activates floreign. This activates the floral meristem identity genes resulting in floral transition [201]. Thus, the expression pattern of *SUP*, *TFL1*, *CRY2*, *AP1*, *CUC2*, *PIN1* and *TAA1* indicated their involvement in vegetative to reproductive phase transition. Transcriptome profiling of vegetative tissues with respect to control (shoot apices with floral buds) identified genes *MED13*, *EBS*, *FPGS1*, *REV*, *MSI4*, *MAF5*, *SPA1*, *SPA2*, *FPA*, *MED12*, *FCA*, *PIN1* and *BBX19* to be associated with the reproductive phase transition. *BBX19* regulates the *FT* via circadian rhythm promoting reproductive phase change. *MED12*, *MED13*, *FPA*, *MSI4*, and *FCA* genes represses *FLC* thereby, regulating flowering [202-206]. *MSI4* interacts with *CLF-PRC2* complex to epigenetically regulates flowering time in Arabidopsis [204]. *SPA* protein interacts with a floral inducer *CONSTANS* to regulate photoperiodic flowering [207]. *MAF5*, induces flowering by repressing *FLC* [208]. *AGL12*, *AGL17*, *AGL24* genes regulates flowering transitions where A*GL12* acts by up-regulating *FT*, *SOC*, and *LFY*; *AGL17* regulates *LFY* and *AP1* via *FT*- independent pathway and *AGL24* acts via regulation of *SOC1* and *FUL* in *Arabidopsis* [209-211].

5.2 Formation of floral organs

Hormonal crosstalk plays a major role in commitment to reproductive phase, where floral organs (undifferentiated buds) are formed. Expression of *PIN1*, *TYPA1* and *AP1* was ~1953-fold higher and genes *CUC2*, *IPT2*, *RGL* and *EIN2* was ~13-fold higher at RSII. Along with development of floral organs *AP1* gene is involved in organ patterning [212].

EIN2 is involved in vegetative to reproductive phase transition and *RGL* protein modulates floral development [179]. Genes *CYP735A*, *AHK2*, *IPT2*, *CRE1*, *CUC2* are associated with cytokinin biosynthesis and signaling and *PIN1* and *TAA1* with auxin signaling are actively involved in floral organ formation. Auxin hormone initiates organ development in peripheral zone of shoot apical meristem forming organ primordia [213-215].

5.3 Genetic mechanism of male and female differentiation in *Jatropha curcas*

Through expression analysis at male, female and intermediate floral buds, we observed genes *AP1* and *TYPA1* are possibly involved in development of male flower and *CKX1*, *CUC2* & *TAA1* genes in female flower development. In present study, *TFL1* gene was expressed in order intermediate>male>female signifying its role in floral transition and development of males as well. *SUP* and *CRY2* genes were expressed in order intermediate>female>male thus, we infer that these genes might be causing female flower transition. *CUC2*, *TAA1* and *CKX1* genes might be involve in floral organ formation and female flowering as depicted by their expression pattern as female>initial reproductive stage>vegetative stage. *CUC2*, a NAC transcription factor has been reported for role in organ separation in *Arabidopsis* by expressing between the boundary of meristems and organ primordia [196]. Kamiuchi et al. [216] and Zluvova et al. [217] showed the role *CUC2* in development of female organs by controlling meristematic activity in *Arabidopsis* and *Silene latifolia* respectively. Stepanova et al. [98] reported that *TAA1* produces local auxin production thereby, have tissue-specific ethylene effects playing an important role in organ development and female flower development. Increased *CKX1* accumulation resulted in male-sterility in transgenic maize [218]. Expression pattern of *PIN1* in *Jatropha* reflected its role in formation of initial bud formation and then in female flowering. *CUC1* and *CUC2* regulates the activity of *PIN1* further, regulating the development of ovule primordia in *Arabidopsis* [186]. *AP1* is responsible for patterning of floral organs [217]. In cucumber, *CsTYPA1* is expressed differentially during sex determination, expressing higher in ovary whereas it is expressed ~5.5-fold higher in male floral buds in Jatropha as compared to female flower buds [192].

5.4 Transition towards female flowering

Since the aim of study was to identify genetic factors that may increase female flower number. Through expression pattern of genes, we observed the involvement of endogenous cytokinin signaling in female flowering [219]. Isopentenyltransferase–encoding gene *IPT1* was expressed in ovules of *Arabidopsis thaliana* [183]. In our study, it was observed that genes *IPT3* and *IPT9* expressed higher in female floral buds. *AHK2* and *AHK4/CRE1* are histidine kinase receptors mediating cytokinin signaling. *AHK2* signaling in floral development is mediated by the effector gene *CUC2* and both are active in female flowering showing higher expression in RSV [129]. *CUC1* and *CUC2* may increase CKs which regulates *PIN1* whose expression needed for primordia formation [183]. *CKI1* might be regulating female flowering through regulating auxin flux (directly or indirectly) indicated by the same pattern of *CKI1* and *PIN1*. This also suggests the correlation between cytokinins and auxin flux. *CRE1* gene encodes receptor for cytokinin signaling which regulates *BEL1* activity. *BEL1* and *SPL* further modulates auxin fluxes via regulation of *PIN1* expression thereby, regulating ovule development [220].

SUP regulates the process of flowering transition by cytokinin signaling. According to previous studies *SUP* causes the abortion of stamens during sex differentiation by blocking the expression of B class floral identity genes thus, promoting gynoecia development. *LFY,* a floral meristem identity gene activates *SUP* through *AP3/PI*-dependent and -independent pathways [221]. Upregulated *AP3*, suppressed expression of *TS2* & B floral homeotic genes increased the arrest of pistil primordia resulting in more females in response to BA application [21]. Through these studies and our observations suggested role of *SUP* in flower induction and female flower transition. Thus, circadian rhythms and hormones (auxin and cytokinin) signaling regulates sex determination in *Jatropha*.

5.5 Molecular basis of high female flowering in *Jatropha curcas*

To nullify the effect of environment effect on variation in accessions (female flower number) plants were grown at same location (environmental conditions), thus, the change was a result of molecular effects. At an inflorescence, number of female flowers present at the at the apex of each sub branches was higher in high ratio accession whereas, only intermediate buds were present in low ratio accession. For validation, relative expression genes *CRY2*, *SUP*, *CKX1*, *PIN1*, *TAA1*, *TYPA1*, *CUC2*, and *AP1* at female, male and

intermediate tissues in high female flower ratio accession was compared with low accessions. After expression analysis it was observed that molecular signals at intermediate buds might be the reason of change in female to male flower ratio. Expression of *SUP* gene was increased upto 7-fold in intermediate buds in high female flower ratio and was considerably reduced in males when compared with low accessions. This significant increase in *SUP* gene might cause high stamen abortion rate allowing more female flowers to develop. After dissection, higher number female flowers at female flowering sites accession were observed which would otherwise occupied by occupied by intermediates with fused stamens in low ratio accessions. Our results are in accordance with other studies which reported the role of *SUP* in floral patterning, specification of male–female boundary, carpel compartmentalization and female flower development in *Arabidopsis*, cucumber and *Silene latifolia* [191, 222, 223]. The increased expression of *SUP* resulted in suppression of stamens in *SUP Silene latifolia*. *CUC2* expressed 10-fold higher in female flower buds in high ratio accession when compared with low female flower accession. The same result was observed in cucumber by Rocheta et al. [77]. Both *SUP* and *CUC2* genes differentiates floral organs by forming a boundary between them [216, 222]. *SUP* gene is a potential candidate to increase female flower number in *Jatropha* through genetic modification after functional validation.

5.6 Regulatory elements in promoter region of flowering genes

Genes *SUP*, *AP1*, *FLT*, *CUC2*, *CRY2*, *CKX1*, *TAA1* and *PIN1* expressing higher in reproductive phase transition and in female flowering might be regulated commonly by ARR1AT, WRKY71OS, MYB1AT, POLLEN1LELAT52 and BIHD1OS. ARR1AT, a cis-regulatory cytokinin response motif is reported in promoter of genes involved in meristem formation [175]. BIHD1OS, a BELL homeodomain transcription factor encodes a protein for patterning of ovule primordia in *Arabidopsis* [224]. POLLEN1LELAT52 element is required for pollen specific expression in tomato [225]. *WRKY71*, a transcriptional repressor binds at WRKY71OS suppressing gibberellin signaling [226]. Female flowering might be regulated by elements GAREAT, UP2ATMSD and MYB1AT in genes *SUP*, *CUC2*, *TAA1*, *CRY2* and *PIN1*. During the initiation of axillary bud growth, UP2ATMSD, a *cis*-regulatory element regulates this phenomenon by regulation of gene expression in *Arabidopsis* [227]. GAREAT & MYB1AT regulates gibberellin biosynthesis and signaling and abscisic acid signaling respectively [228, 229]. Of all the genes

GARE2OSREP1 and CARGATCONSENSUS were present only in promoter region of CRY2 gene, possibly regulating reproductive development and female flowering transition. CARGATCONSENSUS regulated flowering time genes and floral homeotic genes in *Arabidopsis* [230]. GARE2OSREP1 regulates gibberellin signaling in rice [231].

5.7 Cytokinin application increases biomass (florets) and seed number

BA treated inflorescence showed ~7 to 8-fold increase of in floral buds (biomass) after 15 days which was ~3 to 4-fold after 30 days of application This might be the consequence of reduced in photoassimilates/photosynthates accumulation due causing abortion of flowers [22, 27]. No significant change was seen in number of leaves around the inflorescence post BA application which might be credited to the fact that the surrounding leaves were mature at the time of BA treatment and it has been reported that cytokinins are most effective when applied at early developmental stage [232]. In *Jatropha*, post cytokinin treatment a reduction in parameters like seed size and seed weight was observed, however, seed number was increased [21, 25]. Above findings deduce that the seed yield was affected due to downregulation of *AP1*, *AP2* and *LEAFY* which regulates floral biomass, seed size and seed size/weight, proteins and seed oil content respectively [233-234].

5.8 Cytokinin application promotes female flowering

In *Jatropha* application of BA has increased upto 8-fold female flowers. BA has been reported to induce female flowers in other plants like *Plukenetia volubilis*, *Mercurialis annua* and *Vitis vinfera* etc. [124, 125, 128]. In TR15, increased transcript level of *CKI1*, *SUP*, and *PIN1* indicated augmentation of female flowering. *TYPA1* gene identified for male flowering was downregulated in TR15. *TCP18*, a *CYC* subfamily represses stamens and its transcripts level have increased in TR15 indicated augmented stamen abortion rate in *Jatropha* [234]. Recently, Xu et al. [20] reported ATP-binding protein which degenerates stamens at later developmental stages. Thus, these observations implied that the cytokinin treatment either promotes the female flower development or augment the stamen abortion rate thereby, increase in female flowers [22].

5.9 Cytokinin application increases endogenous cytokinin metabolism

Cytokinins plays important role in the formation and maintenance of shoot apical meristem (SAM) [105, 133]. The rate limiting step of cytokinin biosynthesis is catalyzed by *IPT*. Increase in transcript levels of *IPTs* (*IPT2*, *IPT3*, *IPT9)* and *CYP735A2* genes in augmented

the development of floral organ thereby, increasing number of floral buds (biomass) by accumulating cytokinin [135; 235]. Increase in transcript abundance of *CKX5* and *CKX7* genes in TR15 due to the induced *CKX* activity resulted by the elevated cytokinin levels in TR15 to maintain cytokinin homeostasis [236, 237]. Thus, there's is a positive correlation between exogenous and endogenous cytokinin levels, well related to growth and development [237].

5.10 Exogenous cytokinin affecting metabolism and signaling of phytohormones

In cytokinin treated inflorescence change in expression of genes associated with brassinosteroids, ethylene, jasmonic acids and abscisic acids signaling was observed. Cytokinin may have a positive effect on ethylene signaling as the positive regulators of ethylene were upregulated in TR15. Ethylene is involved in floral organogenesis and carpel development by regulating meristem identity genes hence in TR15 the increased expression of ethylene genes probably causes the increase of floral buds [238, 239]. Cytokinin affects auxin synthesis and signaling and their interaction regulates meristem development. After BA treatment in TR15 genes encoding auxin responsive *GH3* gene family, auxin-responsive protein *IAA* and *SAUR* family protein were upregulated. Auxins triggers organ initiation in TR15 stage by accumulating at the peripheral zone of the shoot meristem as well as facilitating the efflux/influx of carriers to organ initiation sites at shoot meristem [213]. Cytokinin probably affected the BR and JS signaling positively whereas, antagonistically effected GA signaling according to the expression pattern of genes associated with them. The crosstalk of these hormones promotes female flowers and floral organogenesis.

5.11 Temporal effect of cytokinin on central carbon channeling

Photosynthesis rate, carbon fixation and metabolism as well as carbon to nitrogen ratio determines the availability of carbon skeleton for synthesis of varied compounds for numerous biological activities affecting plant feedstock [240]. Various studies reported the positive correlation photosynthesis and carbon channeling with biomass and yield in tomato, sorghum, rice wheat and maize etc. [241-243] Thus, temporal effect of exogenous cytokinin treatment was studies on pathways/genes contributing to photosynthesis, pentose phosphate pathway, oxidative phosphorylation, citrate cycle, pyruvate metabolism, starch

metabolism, nitrogen metabolism which might affect the feedstock yield in *Jatropha* (Figure 5.1).

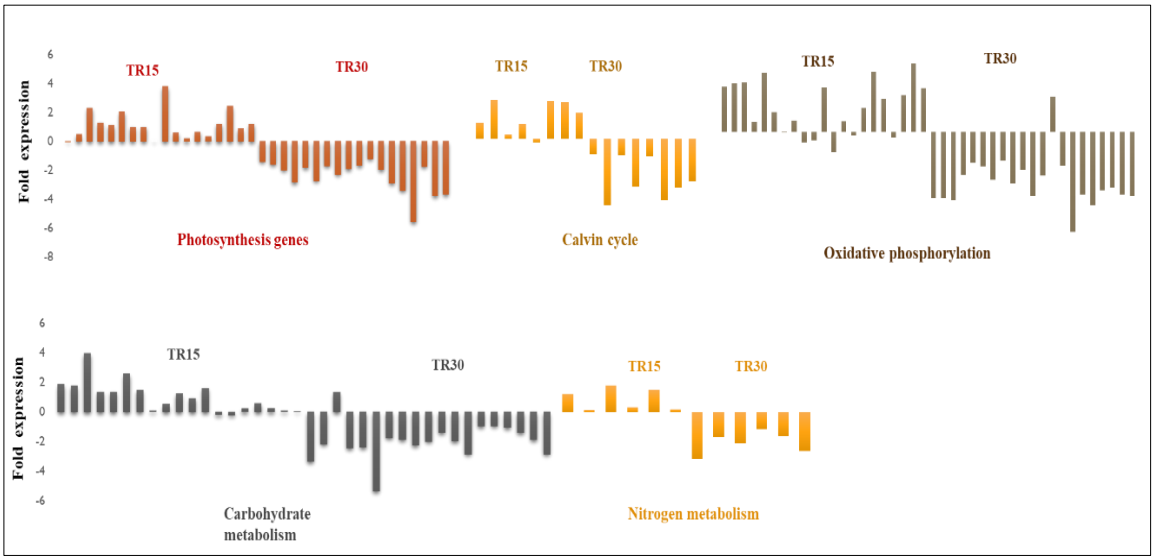

Figure 5.1: Status of central carbon metabolic pathways at different time interval of cytokinin application.

5.11.1 Photosynthesis

Photosynthesis is a tightly controlled process, in which light energy is captured and converted into ATP and NADPH. Photosystems (*PSI* and *PSII*) regulates the primary photochemistry of photosynthesis, transfer of energy and light absorption [244]. Photosynthetic pigments determine the photosynthetic capacity and was increased in response to cytokinin application after which photosynthesis genes were induced through increase in transcript levels of PSI and PSII genes in TR15. This might increase the photosynthetic pigment complexes after 15 day. However, after 30 days their activity was reduced, leading to reduction of floral buds due to increased abortion rate and can be correlated with reduced photosynthetic activity [245]. The correlation of cytokinin level with photosynthetic activity has been reported by Liu et al. [246] in transgenic rice. These observations showed that photosynthetic activity was induced at first post cytokinin application, but its prolonged impact was ascertained. Further, it couldn't keep pace with increased floral mass weakening the source to sink ratio which was inflicted with abortion/abscission of floral buds in TR30 further, eventually decreasing the fruiting rate. Photosynthetic rate is also affected by RuBP synthesis, ATP synthesis, and electron transfer [240]. Gene encoding enzyme NADP+ reductase was downregulated in TR30

which might have reduced the photosynthetic electron flow limiting ATPs required for carbon fixation [247]. Thus, decrease in overall photosynthetic capacity or photosynthates were observed in TR30, which is required by increased floral mass for their proper development consequently for forming fruits [248].

5.11.2 Carbon fixation

Carbon fixation through Calvin cycle by utilizing ATP and NADPH generated through photosynthesis. The decrease in transcripts of genes encoding enzymes involved in carbon fixation after 30 days reduced supply of the carbon skeletons to keep pace with the demand of increased sink tissues (floral buds). In Calvin cycle, *FBP* and *SBP* are the major control points for CO_2 fixation. Previous studies reported an increase photosynthetic CO_2 fixation and accumulation of carbohydrate (starch and sucrose) by overexpressing *SBP/FBP* genes [249-251]. In *Jatropha*, downregulation of *SBP* in TR30 probably reduced the floral biomass [252]. Lawson et al. [249] also reported that decreased SBP activity resulted in decreased floral biomass with the possibility of decreased carbon assimilation and carbohydrate supply to the shoot apical meristem in transgenic tobacco. Recently, a study conducted on *Jatropha*, where yield is increased when plants grown under elevated CO2 enhanced production of carbon intermediates for carbon fixation resulting in increased photosynthetic rates, biomass accumulation and reproductive yields [146]. Thus, by increasing the carbon flux, biomass and seed yield can be increased in *Jatropha*. Thus, from our observations, after 15 days carbon fixation was increased however, reduction in expression of genes for carbon fixation after 30 days of BA treatment might have reduced the carbon availability which affected the floral biomass and the seed yield. Above findings are in accordance with different studies on rice, tobacco and *Arabidopsis* [253-255].

5.11.3 Carbohydrate metabolism

Carbohydrates are synthesized by using carbon fixed in Calvin cycle and majorly stored as starch and sucrose in plants [256, 257]. The expression of *PGM* was reduced in TR30 could have reduced the rate of starch accumulation [258, 259]. *AGPase* regulated the rate of starch biosynthesis by catalyzing an irreversible reaction. *AGPase* activity is regulated by *3-PGA* (an allosteric activator) & orthophosphate (an allosteric inhibitor) as well as under the redox control [260]. *AGPase* activity could determine the carbon flux into starch and as its reduced expression in TR30 might have limited the carbon availability for starch synthesis [261]. Thus, in TR30, decreased floral biomass might be the result of reduced

carbohydrate synthesis and its supply needed by the developing reproductive organs [70]. Aside reduction in floral biomass in TR30, decrease in seed size and weight may be because of less carbohydrate reserves. Additionally, translocation of carbohydrates to the reproductive sink tissue affected the seed yield as the development advances. These observations suggested that cytokinin application at first increased carbon flux causing increase in biomass. However, the required demand of carbon flux couldn't be achieved due to reduced availability of triose phosphate required for carbohydrate synthesis [262].

During night, Starch is mobilized, and the is carbon stored in the form sucrose. Triose phosphate form fructose1, 6-bisphosphate which is then converted into fructose-6 phosphate by fructose-1, 6-bisphosphatase (*FBP*). *FBP* regulates sucrose metabolism and was upregulated in TR15. Sucrose phosphate synthase (*SPS*) converts UDP-glucose and D-fructose-6-phosphate into sucrose phosphate and regulates the partitioning of carbon into starch production and carbohydrate accumulation [263]. These observations indicated reduced carbohydrate synthesis and flow to the sink organs (florets), thus, weakened sink strength [264, 265]. Glucose is transferred from a-1, 4-linked glucan, to orthophosphate by starch phosphorylase which forms glucose 1-phosphate. This glucose 1-phosphate enters glycolysis, and this does breakdown sugar. After this pyruvate which is the end product of the reaction, enters cycle of citric acid (TCA). This provides the precursors for C skeleton [256]

Carbon flow was also reduced due decrease in gene encoding enzyme ATP citrate lyase of TCA in TR30 [266]. The decreased TCA activity may have influenced the biomass following 30 days of treatment because trade off in proficient use of carbohydrates [267]. Overall, increase in photoassimilates i.e. carbon gain and carbon assimilation was observed at first, but exhaustion of carbohydrate reserves may be increased the starvation leading to floral abortion further affecting yield [268]. Through these results it is observed that there might be a shift in metabolic pathways of carbon assimilation and flow, affecting carbon precursors for carbohydrates which might affect the fatty acids metabolism further altering seed oil content.

5.11.4 Nitrogen assimilation and carbon to nitrogen (C/N) ratio

2-oxoglutarate and ATP acts as precursor of carbon, required for assimilation of nitrogen which depends on the co-ordination between nitrogen and carbon [269]. Sucrose and amino acids play a vital role regulating enzymes for nitrogen assimilation. Nitrogen significant

for formatting compounds needed in cellular activities. Glutamine synthetase (*GS*) assimilates inorganic nitrogen and its decreased transcript level in TR30 might resulted in less nitrogen availability [270]. Glutamate dehydrogenase (*GDH*) acts as a shunt of glutamate synthase cycle and under low C/N ratios it reduces the release of carbon from amino compounds as keto-acids and its activity was decreased from TR15 to TR30 [271]. In glutamate synthesis, *GDH* catalyzes the amination of 2-oxoglutarate. Thus, according to obtained observations availability of carbon was reduced due to repressed *GDH* activity thereby, reducing floral biomass in TR30 and yield. *GS* transformation resulted in increased biomass and yield [272-274]. The assimilate partitioning was affected between vegetative and reproductive organs because of reduced nitrogen availability post 30 days of cytokinin treatment (TR30) resulted in decreased biomass [240, 275-277]. Thus, nitrogen availability can increase total yield and *GS* and *GDH* can be the potential targets in *Jatropha* [276, 277].

SUMMARY

Understanding of molecular mechanisms of floral development, sex determination and floral transitions (vegetative to reproductive, differentiation of floral buds into male/female and transition towards female flowering) and the alteration in source to sink interaction after cytokinin application are important for instigating the genetic intervention strategy to increase feedstock yield of *Jatropha*. Current study provided the genetic differences contributing towards female flowering between high vs low ratio accession and key genes associated with female flower development. Also, through comparative transcriptomics, molecular mechanisms unveiled the cause of compromised yield in response to cytokinin application.

Through comparative genomics, floral genes were identified, and their relative expression status was studies at different floral developmental stages. Gene identified for vegetative to reproductive phase transition in *Jatropha* were *SUP*, *TFL1*, *AP1 CUC2*, *CRY2*, *PIN1* and *TAA1* and showed a relative increase in expression of ~426-fold. For development of floral organ genes *AP1*, *CUC2*, *RGL*, *EIN2*, *IPT2*, *TYPA1*, *PIN1* were identified from which *PIN1*, *AP1* and *TypA1* showed a significant increase in expression of about ~1953-fold and others showed ~13-foldincrease in expression at initial floral buds. Gene for sex determination like *CRY2*, *TAA1*, *CUC2*, *PIN1*, *FT*, *CKX1*, *SUP*, *TFL1*, *AP1* and *TYPA1* were identified in *Jatropha*. From these, *SUP* and *CRY2* genes showed ~59-fold increase in expression level in intermediates followed by ~18-fold in female floral buds. They were found to be associated with transition towards female flowering by suppressing the stamen development, allowing females to develop. *TAA1*, *CUC2*, *PIN1*, *FT*, *CKX1* showed higher expression in female flowers whereas *TFL1*, *AP1* and *TYPA1* were expressed higher in male floral buds. Further on comparing expression in male, female and intermediates between high and low female to male flower ratio genotypes, it was observed that *SUP* gene was upregulated in intermediate stage with ~7-fold when compared with low ratio genotype. *CUC2* gene in female floral buds expressed ~10-fold higher in high female flower ratio genotype. These results showed that the female development was strong in high ratio genotype along with increase abortion rate of stamens. By correlating the expression of these genes possible interactions between them and the pathways might be

contributing in development of females and their transition were also predicted. PCA analysis proved useful in correlating the data of expression analysis with male and female florals buds. The current study provided a repertoire of key genes for female flowering which can be further considered as suitable candidates enhancing seed yield by increasing number of female flowers. *In-silico* analysis of promoter regions of key genes revealed the presence of putative regulatory elements associated with floral transition and associated pathways were ARR1AT, BIHD1OS, MYB1AT, POLLEN1LELAT52, and WRKY71OS. They were found to be associated with gibberellins, cytokinins, abscisic acid, and auxin pathways as well as pollen development. GARE2OSREP1 and CARGATCONSENSUS were unique elements found to be associated with genes involved in female flowering. Overall, these findings together with the previous information provided a more comprehensive understanding on mechanism of sex determination in *J. curcas*.

Comparative transcriptome-based analysis of inflorescences treated with cytokinin and untreated inflorescences provided details on molecular components associated with compromised yield in *Jatropha*. The current study revealed that the carbon fixation and its allocation was affected by BA application. Through GO and KEGG based functional annotation, it was observed that processes like photosynthesis, carbon accumulation and flow were downregulated post cytokinin application affecting source-sink balance further affecting floral biomass, fruiting rate and seed size/weight. Key genes *FBP*, *SBP*, *GS*, *GDH* and *AGPase* were identified to be significantly downregulated post 30 days of cytokinin application and are associated with biomass and yield. These results would be critical for understanding the molecular dynamic of source-sink interaction in response to cytokinin application in *Jatropha*.

Transcriptome sequences and data generated was submitted on a public domain (Link: http://14.139.240.55/NGS/download.php).

FUTURE PROSPECTS

- ✓ Sequences of key genes of female flowering can be used to scan for SNPs for developing gene markers associated with high female flowering in *J. curcas*
- ✓ Functional validation of genes associated with female flowering and transition to female flowering or suppressing development of male.
- ✓ Gene controlling key steps in biological processes related to carbon capture, carbon fixation and carbon flow as well nitrogen metabolism can be targeted through metabolic engineering to increase the yield.

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APPENDICES

Table A1: Gene specific primers used for RT-qPCR analysis

Genes	Forward Primer	Reverse Primer	Tm (°C)
ACS1	TGGAAAGCCTGGTGAATACG	ATAAGGGGACACAGCAAAGC	55
ACS7	AGACGACGAAGTAAAGAAAGAGTTG	TCTGGTTTTGGTGGACAAGG	58
AG	GCAAGAGGAGAAATGGACTGTT	AGGGTGAAGAGAAGTGAAGTTG	55
AHK2	TAGTTCATTCGGAGCCAGTG	CGTCTCTTCCCTTCTCAGCA	54
AP1	CAAGAAACAACCAACTGATGC	GCACAAGGAAGGGTGACATA	58
ARF2	GGCAAGAGACGCTGAAACTG	GCCACCTGATTTGTTGACG	57
BEL1	TAATGCGAGGGTGAGGTTGT	AGTAGGCGTTGGTGTCTGTG	56
CEN1	TGGCAAAGAAGTGGTGAGC	AAAGAAGACAGCAGCAACAGG	57
CKI1	ACGCTGTTGCTATGGATGG	TTTGGGTGTCTTCAGGATTTG	55
CKX1	GATTACCTTCACCTCACCATTG	CCTCCAAGAACACCATAAAAGA	56
CKX5	ACGGAAACCCATCTACCCAA	TCACGACAGATTTGCCTTAT	53.5
CLV1	GATGAGATGCCAAGACACCA	CCAACCACCCATTTGAGATT	56
CRE1	TGGTATCGTGGCTGAAGTTG	GTCCGTTCTGTTTCCAGTCC	48
CRY1	ACTGGTTGGTTGCATGATCG	ACCCGAAATGTACTGCCAAC	55.5
CRY2	GGCATTTCGGTGCAAAGC	TTCTCAACAGCTCCTGCAAC	55.5
CTR1	AACGCCTACGACATCCAAAC	CTCAACCTTCGCCTTTCATC	55.5
CUC2	CCCATTTACCTCCTGGCTTT	TCCCATTTTCGCTTTATCAG	56
CYP735A	ATCCTGACAGGTTTGCTTCG	AACAACAACAGGAGCATGGC	55.5
DAD1	CGATACGGAGGATTTGTTGAC	CCAGATGTAGCACGGAGGTT	55
DYAD	CAAAGACCCACCTTCCTTCA	ATGTAGTGCCAATGCTGCTG	60
EIN2	CCGCCATCATTTAGGAGTCT	TTGCTTCTTGAGTTGCTTGC	55
ERAF17	GGGAAGAGGGAAAGTTGA	TGGGCATCACAAAGAACAGA	54
ETR1	CTGGGTAGGACATTGGCATT	GCACGGTTGCTGCTGAATAC	58
FLO	GCTATGCCCTTCACTGTCTTG	ATGGCTAAACGAGGATGTGC	56
FT	TGATGACAGACCCTGATGCT	CTTTCCCTTTGGTAGGATTT	52
IPT1	CTCCCATTACATTTGCCACA	GCTCCCATAACAACGACGAT	56
IPT2	TTATCAGGGAAAGGCCGCAG	ACCGAGCAATCCAGCATCAA	55.5
IPT3	TCGATCCGAGGCTTACTTCC	ACCATTTCATCGACTCGCTT	55.5
IPT9	GTCTTGATGTTGGGTCTGC	ATCTCGTGTGGTTTGCCTTG	56.5
MYB98	AGCAAACCAGCCACAGAAGA	CATCAAGAAGCGAATCAAAGC	56

PIN1	TGGTTCTGTGAAATGGTGGA	TTGGAAGCGTGGAAAGAGA	56
REV	GTTCCTCCTGCTGTTCTCGT	ATGATTTGGCTCCCAGTGAA	57
RGL	ATTGAAGAGGCTGAGGAAGGTG	TGCTACACGCTGGAAAGAAG	55.5
SIN1	TGGCAATCTGGCTACTGTTG	TCCCATTCTCATCACCCTTT	57
SPA	CCGATTCTTCACCAGCAACT	GCACCCTTCTCGCTATCACA	62
SBP	GGAACTGAGATGGGACAACC	GCAAGAACAGGCAGACACAA	58
STY	GGACATAACAAGCCAATCAGC	GGAAAACACAGGAGCGGAAT	58
SUP	GCTGTAAAAGGCCAAAGACG	CTACTTCACTTTTGGTGGCTCTC	55
TAA1	GATGGCTGAAAGATGGGAGA	GCAAAGGCAGGATGTGATTC	56
TFL	TGGCAAAGAAGTGGTGAGC	AAGAAGACAGCAGCAACAGG	54.5
TypA1	CGAGGGATTACGATACTGAGC	AACCTTGTCTGTGGCATTGG	60
WUS	CCGTCACTTCCTCCTTCAAA	GCTGCTCTGGTGTTGGATTC	60
26S rRNA	CACAATGATAGGAAGAGCCGAC	CAAGGGAACGGGCTTGGCAGAATC	58
ACTIN	GAGAGAGGGTACATGTTCAC	AGCTCGTAGTTCTTCTCAAC	46

Table A2: Primer used for validation through qRT-PCR

Gene	Jatropha Sequence ID	FORWARD PRIMER	REVERSE PRIMER	Tm (°C)
LC1	Jcr4S00217_73371-74542	ATCCTTCACTTGGGGCTGAA	ATCCTTCACTTGGGGCTGAA	56
LCII	Jcr4S01794_19047-20251	GGAATTTCGTCCTCGTCAAA	CGTGGGTGTTGCTGTTGTAG	56
PSI	Jcr4S00072_51797-52989	TCCCATTGGCCAGTAGTGTT	GCTCCATGTCAAGCCTTCTC	56
PSII	Jcr4S00520_62094-62844	GAGAGAAGCACCCACAGGAG	GTGGGAAAATCGGTGCTTTA	56
PLST	Jcr4S00004_59679-60185	GGAATTTCGTCCTCGTCAAA	CGTGGGTGTTGCTGTTGTAG	56
PGM	Jcr4S00445_53788-60856	TGATGGATGCAGGACTGTGT	GATAGCCAAGCCAGAACTGC	56
GLGC	Jcr4S01990_32377-36466	GCTCGCTCATCATCAAGACC	GCTGAGAAACCAAAAGGCGA	56
FBP	Jcr4S03343_8480-10365	GGCCAAAGTGAAGAAGAGGC	TTTGCACCTCACCAAGAACG	56
HK	Jcr4S00679_7418-10356	GGTGTCTTCATGTGCCTGTG	ACGGACATCTTTGGACCTGT	56
PGK	Jcr4S00615_6656-13321	GCAGAACCCAGTTGAAAGCA	AGGCATCTTGGACACTTGGT	56
GAPDH	Jcr4S00240_41352-43682	ACTACGAACTGTCTGGCTCC	CGGTGTGAAGCATCCAAGAG	56
RBCL	Jcr4S01363_41711-42271	CACCATTCACAGGCCTCAAG	GGAGAGAGGTGGCAGGTATG	56
RPIA	Jcr4S00669_31271-32080	ATCGACGTTGGGGTGAGAAT	AGTCTGGTATGGTTCTCGGC	56
PGLS	Jcr4S02321_22180-23477	TGGTGATCAAGTCCGTTCGA	GGCCTCTACCACCATAACCA	56
ACCA	Jcr4S00075_66135-70136	AGGACAGGAATCGGAAGTGG	GAAGCGTCACTGGTTTTGGT	56
IDH	Jcr4S01952_11474-15725	GTGAAACCAGCACCAACAGT	TTGTCGTCCAACTTAGCCCT	56

SBP	Jcr4S01914_9435-11836	GCAAGACAAGCACTCGATCA	GATGATCTTGGCACCAACCG	56
ACLY	Jcr4S00265_43297-46308	ACCTCCAGCTACCATTGTCC	TGTGCATCAGGTGGGGTAAT	56
FER	Jcr4S00033_37885-38319	ATGCCAAAAGATCCAAATGC	ACAAGAATGAGCGGAAAGGA	56
GDH	Jcr4S00031_72882-74985	TTGGCAATGTGGGTTCTTGG	TCCAAGAGCAGCTGGAATGA	57
GS	Jcr4S00507_2229-5065	GTCACGGCCAACACGAATAG	AGAAGATTGACAGGTCGCCA	58
CA	Jcr4S00587_33470-36783	GCGAGAATCTGAGCAAGCAA	ATCCAGCGAATCAAGTGGGG	58
NR	Jcr4S02321_3916-8442	TGTATCATAGGTGGTGGCCC	TATGCGAACCGGACAGAAGA	56

LIST OF PUBLICATIONS

Research papers

1. **M Gangwar**, H Sood and RS Chauhan (2016). Multiple genes associate with transition to flowering and female flower development in a high female to male flower ratio genotype of *Jatropha curcas* L. **Molecular Biology Reports;** 43(4): 305-22. **(Scopus), UGC Journal list No. 31535**
2. **M Gangwar**, A Sood, A Bansal and RS Chauhan (2018). Comparative transcriptomics reveals a reduction in carbon capture and flux between source and sink in cytokinin treated inflorescences of *Jatropha curcas* L. **3 Biotech**; 8:64. **(Scopus), UGC Journal list No. 99**
3. **M Gangwar**, S Sharma, H Sood H and R.S. Chauhan (2015). Indirect shoot organogenesis in *Jatropha curcas* for in vitro propagation. **Indian Journal of Research**; 4(12): 56-8.

Conferences

1. **M. Gangwar** and R.S. Chauhan. Dissecting the molecular basis of female flower in *Jatropha curcas*. Proceedings of the National Symposium on Advances in Biotechnology for Crop Improvement [Eternal University, Badu Sahib, HP: July 12, 2014]

2. R.S. Chauhan, A. Sood and **M. Gangwar**. Genomics-Assisted Genetic Improvement of *Jatropha curcas*. *Jatropha* Updates 2014. [TERI, New Delhi: October 13, 2014]