Overexpression of *AtDGAT1* and metabolome analysis of *Jatropha curcas* L. for enhanced oil in seeds and leaves

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By

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STATEMENT

I do hereby declare that the matter embodied in this thesis entitled "**Overexpression of *AtDGAT1* and metabolome analysis of *Jatropha curcas* L. for enhanced oil in seeds and leaves"** is the result of investigations carried out by me in the Centre for Energy, Indian Institute of Technology Guwahati, India, under the guidance of **Prof. Lingaraj Sahoo** and **Prof. Vaibhav V Goud**.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators.

December, 2016

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CERTIFICATE

It is certified that the work described in this thesis, entitled "**Overexpression of *AtDGAT1* and metabolome analysis of *Jatropha curcas* L. for enhanced oil in seeds and leaves**", done by **MR. Devendra Kumar Maravi** [Roll No. 11615104] for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under my supervision in the Centre for energy, Indian Institute of Technology Guwahati, India, and this work has not been submitted elsewhere for a degree.

December, 2016

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Dedicated to my beloved parents

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ABSTRACT

The diminishing fossil fuel stock and soaring international crude oil price have renewed the interest in the alternative source of fuels. Oil from oilseed crops that are largely in the form of triacylglycerol (TAG) are the promising source of renewable supply of fuels in the form of biodiesel. *Jatropha curcas* L is an important non-edible oilseed crop which received worldwide attention as a biodiesel feedstock. Despite the significance of Jatropha seed oil as a potential source of biodiesel, not much research efforts have been made through breeding or transgenic approaches to improve its seed oil quality for sustainable biodiesel production. Transgenic approaches offer immense opportunities to improve oil content and quality through manipulation of oil biosynthetic pathway in both seed and leaves. Since DGAT1 is the only enzyme that is exclusively committed to TAG biosynthesis in Kennedy pathway, direct up-regulation of Kennedy pathway through overexpression of the DGAT1 enzyme responsible for the last and only committed step in seed TAG biosynthesis is promising. In the present study, transgenic Jatropha plants were developed that accumulates a high level of TAGs in seeds as well as in leaves by the overexpression of Arabidopsis DGAT1. In this work, the CaMV35S promoter was used because of its strong and constitutive nature in regulating transgene expression to enable ectopic overexpression of AtDGAT1 in leaves as well as in seeds. The enhanced TAG accumulation in transgenic Jatropha lines had no penalty on the growth rates, growth patterns, leaf number, and leaf size of plants. Enhanced expression of AtDGAT1, in transgenic Jatropha lines, appears to have increased the total lipid content by 1.5 to 2 fold in leaves and 20-30 % in seed kernels. In the transgenic line (TR1), enhancement in DGAT1 activity resulted in an increase in total lipid content by two-fold in leaves and 30% in seed kernels. This increment was

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accompanied with no significant change in protein content in leaves, but a minor increase in protein content in seed kernels was found in the transgenic plants. In contrast to proteins in leaves and seeds, a significant increase in carbohydrate content in leaves and a marked decrease in carbohydrate content in seeds of transgenic lines were observed. These results suggest that increased accumulation of total sugar may have contributed to the reallocation of precursor for enhanced TAG synthesis in transgenic leaves. Carbohydrates are important osmotic solutes in leaves and seeds and are potentially involved in the carbon source transformation to lipids. Previous studies suggested that carbohydrate content in seed was correlated with seed oil contents. However, the reallocation of precursors for photosynthesis to TAG biosynthesis is more in leaves than seeds of transgenic AtDGAT1 Jatropha lines, and possibly the contribution to TAG biosynthesis in these transgenic lines by leaves are more than seeds. The mechanisms underlying these changes need to be addressed in future investigations.

More importantly, ectopic over-expression of AtDGAT1 in Jatropha resulted in an increase in oil content, average plant height, seeds per tree, average 100-seed weight, and seed length and breadth. Thus, there was no penalty in 100-seed weight caused by the increase in oil content, and as the result being an increase in total oil on a per seed basis of between 20-30% more in the AtDGAT1 transgenic lines, thus indicated a 20-30% net overall oil increase when compared with the wild-type plants. Additionally, AtDGAT1 overexpression lines of Jatropha also exhibited an increase in oil contents by 1.5 to 2 fold in leaves. This distinct difference between AtDGAT1 transgenics and wild-type plants with respect to effects on seed weight, seed length and breadth, and plant height is unclear and suggests a more complex interaction between the traits of oil increase and seed traits than is currently understood.

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The second phase of investigation involved comprehensive metabolite profiling by LC-FT- ICR-MS used to study the consequences of the *AtDGAT1* gene transfer on Jatropha metabolites. The metabolite analysis allowed a complete assessment of the plants metabolic status following genetic manipulations. It was found that sucrose concentration was decreased in all transgenic lines, which indicates towards utilization of carbon source was significantly enhanced in transgenic lines. Several lipids such as fatty acyls, glycophospholipids, glycolipids, sterol lipids, sphingolipids, PI, PE, and apart from that many falvonoids, alkaloids, and other secondary metabolites were identified. The abundance of these lipid derivatives indicated significant changes in metabolic profiles between the transgenic lines and wild-type Jatropha curcas. Characterization of these metabolic patterns also emphasized several interesting differences among the transgenic lines.

The vast array of lipids and their derivatives, flavonoids, and other secondary metabolites tentatively identified in this study suggest an important role for the metabolite class in Jatropha oil biosynthesis pathways.

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ABBREVIATIONS

g	Gram
mg	Milli gram
µg	Microgram
µl	Micro litter
cDNA	Complementary DNA
LB	Luria-Bertini
dNTP	Deoxyribonucleotide triphosphate
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
bp	Base pair
kb	Kelobase pair
L	Liter
ml	Milli liter
M	Molar
nm	Nano meter
mM	Milli molar
SDS	Sodium dodecyl sulphate
PCR	Polymerase chain reaction
mg/L	Miligram per litre
rpm	Revolution per minute
v/v	Volume/volume
w/v	Weight/volume
MS	Murashige and Skoog's medium
LCM	Liquid co-cultivation media
BAP	6-Benzulaminopurine
IBA	Indole-3-butyric acid
NPTII	Neomycin phosphotransferase
GUS	β-glucoronidase
DIG	Digoxygenin
CTAB	Cetylrmethyl ammonium bromide
CaMV	Cauliflower mosaic virus
OD	Optical density
SSC	Sodium chloride and sodium citrare buffer
T-DNA	Transferred-DNA
FAME	Fatty acid methyl esters
FA	Fatty acid
TLC	Thin layer chromatography
LC	Liquid chromatography
MS	Mass spectroscopy
FT	Furier transform

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ICR	Ion cyclotron resonance
TR	Transgenic
TAG	Triacylglycerol
DGAT	Diacylglycerol acyltransferase
PI	Phosphatidylinositol
DAG	Diacylglycerol
PG	Phosphatidylglycerol
PC	Phosphatidylcholine
PA	Phosphatidic acid
PCA	Principle component analysis

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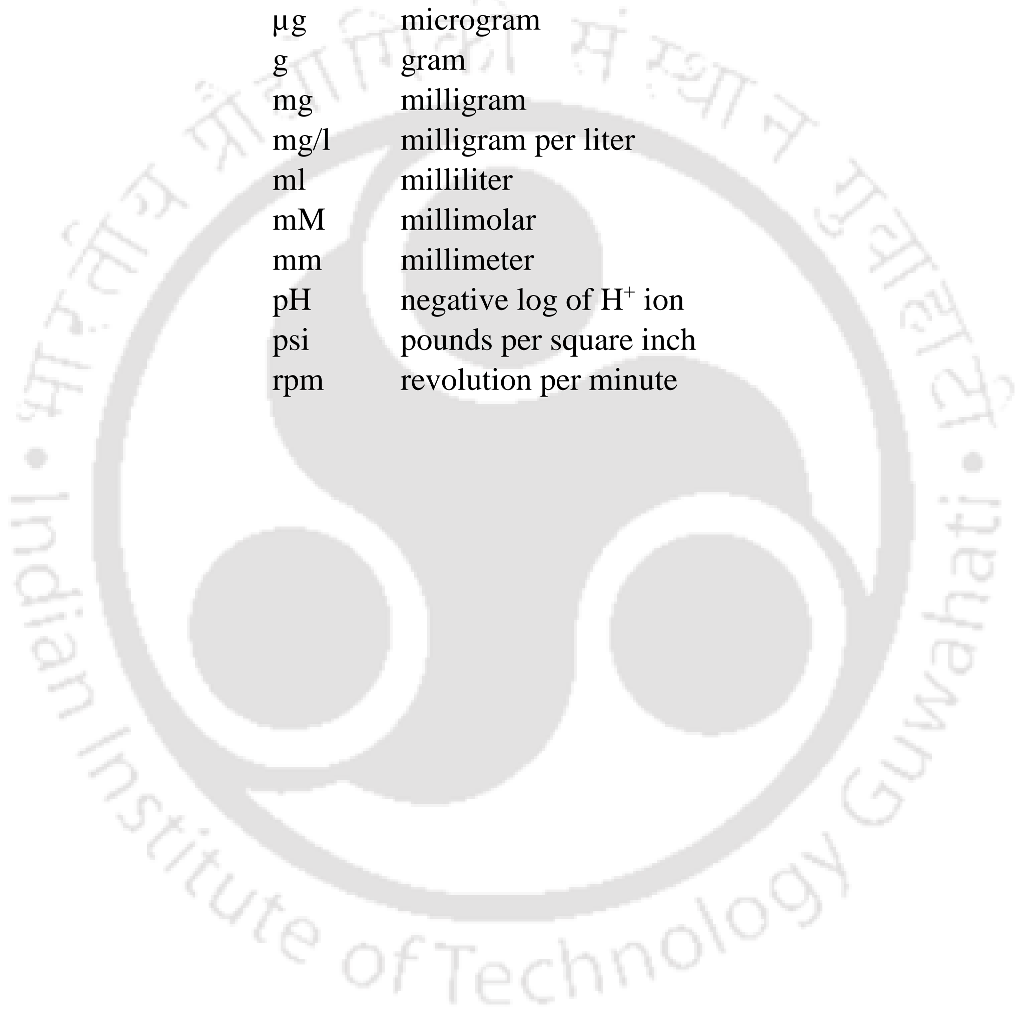
UNITS

^{0}C	degree celsius
µl	microliter
µM	micromolar
bp	base pair
kb	kilo basepair
cm	centimeter
µg	microgram
g	gram
mg	milligram
mg/l	milligram per liter
ml	milliliter
mM	millimolar
mm	millimeter
pH	negative log of H^+ ion
psi	pounds per square inch
rpm	revolution per minute



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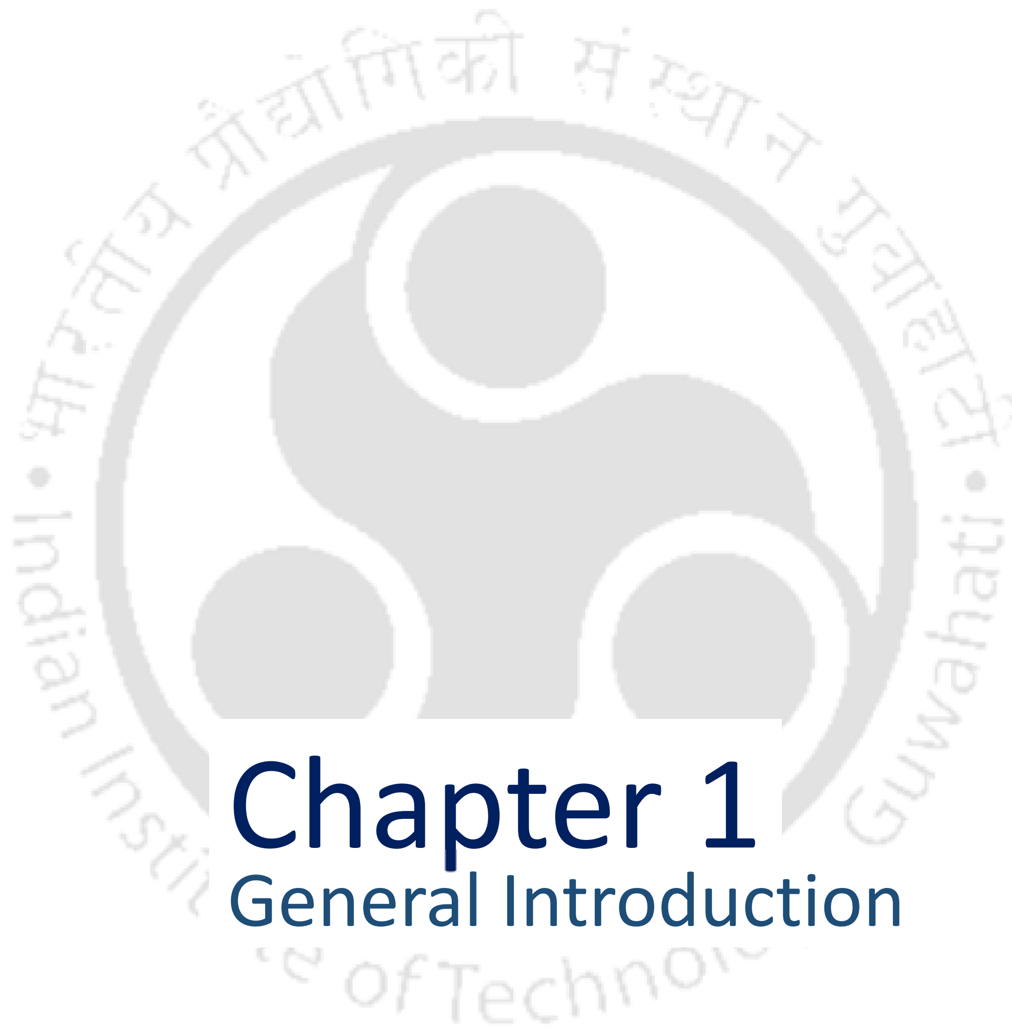

Chapter 1
General Introduction

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1.1 Introduction

Limited fossil fuel reserves, increasing fuel price and environmental concerns for reducing greenhouse gases have driven the search for alternative renewable fuel sources. Biodiesel derived from plant seed feedstock is gaining wider attention for its potential to replenish partially the petroleum diesel, reducing pollution and creating socioeconomic benefits to farmers. Biodiesel is renewable, non-toxic, bio-degradable nature with similar combustion properties as that of petroleum-based diesel. Biofuel oil is usually associated not with green biomass, leaves and stems, but with seeds which accumulate oil in the form of triacylglycerols as storage reserves. Despite accumulation in seeds, primary oil synthesis occurs in green photosynthetic tissues, and leaf oil deposits in the form of oil bodies have been reported for many plant species (Lersten *et al.*, 2006). A number of recent studies show that gene manipulations enable the relocation or elevation of oil storage in alternative plant organs such as roots, stems or leaves making the green biomass a plausible system for manufacturing biodiesel (Durrett *et al.*, 2008). Accumulation of oil bodies in both seeds and green tissue biomass is envisaged to be most benefiting for sustainable biofuel production.

Jatropha curcas a non-edible oilseed plant belonging to the family *Euphorbiaceae* has emerged as an attractive source for seed-based biofuel production. Seeds of Jatropha contain 35-42% oil with high percentage of polyunsaturated fatty acids and high calorific value which makes it the most suitable feedstock for biodiesel production (Achten et al., 2008; Devappa et al., 2010). Its ability to grow on degraded land, fast growth, short gestation period and adaptation to diverse environmental conditions further adds benefits for selection of Jatropha as biofuel feedstock plantation in non-agricultural lands. Jatropha seed derived oil is non-edible and hence does not compete with the production of edible oils. Despite being a potential feedstock for biodiesel production, no genetic improvement has been made for oil enhancement

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in Jatropha through conventional breeding. Furthermore, limited research has been conducted for oil enhancement through biotechnological interventions (Bhering et al., 2013).

Genetic engineering over the years has emerged as powerful tool to complement conventional breeding for improvement of crops. It is now well documented that enhanced expression of some enzymes involved in lipid metabolism can lead to increased oil accumulation in different plant organs (Thelen and Ohlrogge, 2002; Baud *et al.*, 2007; Vigeolas *et al.*, 2007). Particularly, over-expression in plants of a key enzyme of triacylglycerols (TAG) biosynthesis, diacylglycerol acyltransferase (DGAT), was shown to increase the accumulation of triacylglycerols in seeds (Jako *et al.*, 2001), tubers (Klaus *et al.*, 2004) and leaves (Zou *et al.*, 1997; Bouvier-Nave *et al.*, 2000). Recent advance in our understanding of genetic regulation of key metabolic pathway for TAG biosynthesis generate opportunity for manipulation of candidate genes for enhanced oil accumulation in plants. This may help surpass existing limitations of the breeding programmes and help to minimize the time required to generate those improved varieties.

Both forward and reverse genetics have shown that DGATs are key targets for enhancing oil content in seed and leaf biomass. An EMS-induced mutation of *DGAT1* in *Arabidopsis thaliana* (AS11) resulted in reduced seed oil content and delayed seed maturation (Zou et al., 1999). Similar findings were obtained from another *A. thaliana* mutant ABX45 caused by frame shift mutation of the gene (Routaboul et al., 1999). In addition, overexpression of *DGAT1* orthologues from plant sources including *A. thaliana* (Jako et al., 2001), *B. napus* (Weselake et al., 2008), and *Tropaeolum majus* (Xu et al., 2008) have been successfully used for enhancing overall oil production in *A. thaliana* and *B. napus*. Lardizabal et al. (2008) reported that overexpression of *DGAT2* from the fungus *U. ramanniana* led to an increase of

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seed oil content in soybean (*Glycine max*), indicating heterologous expression of fungal *DGAT* orthologues in plants could be also feasible for increasing overall oil production.

Development of an efficient plant regeneration system amenable to genetic transformation is however, key to successful generation of transgenic Jatropha. Although an *Agrobacterium*-mediated transformation system in *Jatropha* using cotyledonary leaf explants has been reported earlier (Li et al. 2007), the protocol was found either inefficient or difficult to reproduce, a proposition attributed to the inappropriate age of the explants used. An efficient regeneration system amenable to genetic transformation using explants of appropriate age and selecting their orientation for regeneration has been established in our laboratory by previous researchers (Mazumdar et al., 2010).

The present study was undertaken with the objectives to overexpress *Arabidopsis DGAT1* under constitutive CaMV35S promoter to develop transgenic Jatropha plants for enhanced accumulation of TAG in seeds and leaves and carry out non-targeted metabolomics analysis to investigate the unintended effects of *AtDGAT1* overexpression on other plant traits and metabolisms in transgenic Jatropha.

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1.2 OBJECTIVES

The present investigation was carried out with the broad objective to develop transgenic *Jatrophacurcas* overexpressing *Arabidopsis thaliana diacyl glycerol acyl transferase* (*AtDGAT1*) and non-targeted metabolomics analysis of transgenic Jatropha. The salient objectives outlined as:

- Cloning of *Arabidopsis thaliana Diacyl glycerol acyltransferase* 1 (*AtDGAT1*) in a plant binary vector and mobilization to *Agrobacterium tumefaciens*.
- *Agrobacterium* mediated Jatropha transformation and generation of transgenic CaMV35S::*AtDGAT1* Jatropha lines.
- Molecular analysis for presence, integration and expression of *AtDGAT1*.
- Characterization of leaf biomass of transgenics for enhanced TAG accumulation for biodiesel production.
- Non-targeted metabolomics analysis of transgenic lines with enhanced oil accumulation.

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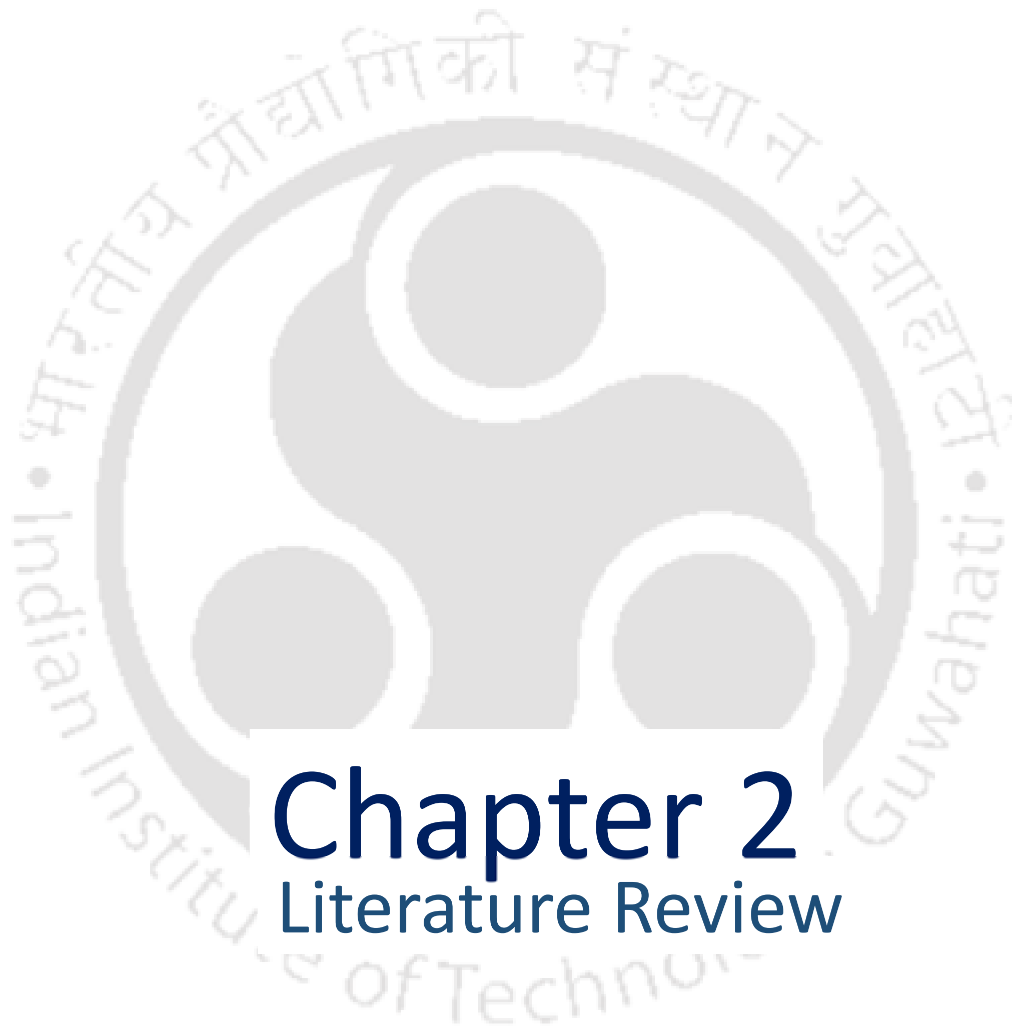

Chapter 2
Literature Review

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Biodiesel derived from transesterified animal fats or vegetable oils has emerged as the most viable alternative for mitigating the rising global energy crisis and depleting non-renewable fossil fuel. It offers the unique opportunity for generation of sustainable, environment friendly energy with distinct advantages of easy availability and renewability, low emission values of greenhouse gases, high biodegradability rate and good lubricant properties. The upsurge in biofuel production has triggered the demand of feedstock for source materials. Edible feedstock seed resource including rape seed, soybean, sunflower, corn, palm oil and others currently used for biofuel production (**Figure 2.1**) meet only 5-7% of world's energy demands. Furthermore, use of edible seed derived oil for biofuel production has serious consequences on food commodity price. Non-edible oil seeds obtained from *Ricinus communis* (castor), *Pongamia pinnata* (karanj), *Jatroph curcas* (ratanjyot) are potential feedstock for biodiesel production in tropical and tropical climatic developing countries. Among them, Jatropha is highly promising owing to several advantages discussed in the next section.

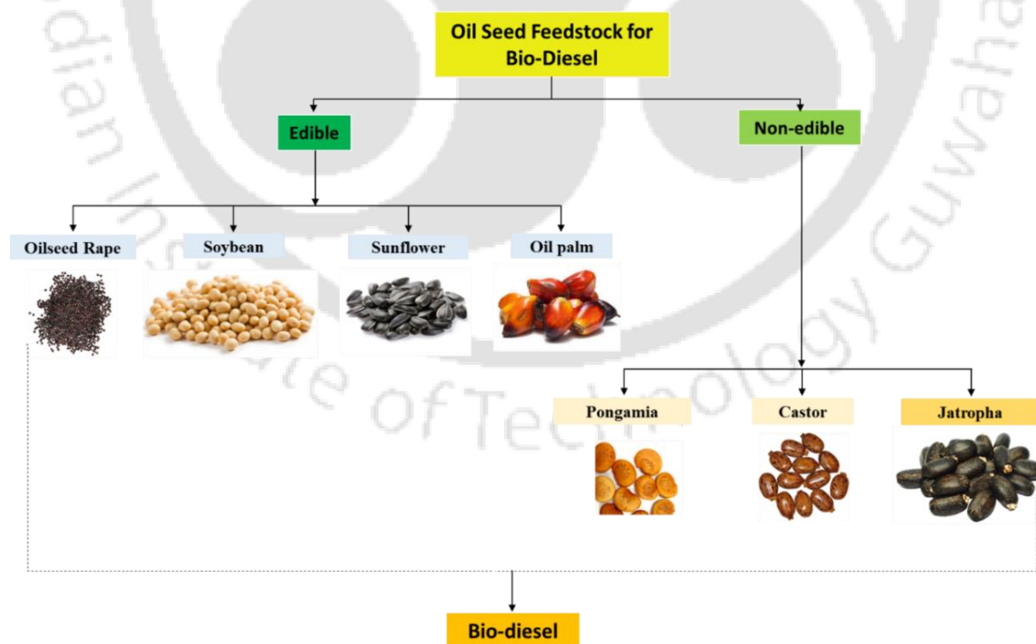

Figure 2.1 Feedstock's for biodiesel production

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2.1 *Jatropha curcas*: a biofuel crop

Jatropha is considered as one of the most promising potential feedstock for biodiesel production in India owing to its several advantages:

- Easy propagation from plant cutting or seeds, faster growth and high seed yield in semi-arid on degraded and saline soils.
- Robust varietal improvement program and established good farming practices in the tropical and subtropical environments.
- Used for halting and reversing land degradation and periodic leaf shedding facilitates nutrient recycling.
- Leaves are unpalatable to grazing livestock, hence used as hedge to protect crops.
- Seed oil has physical and chemical properties best suitable for biodiesel. Even the seed oil can be used directly in suitable diesel engines, lamps and cooking stoves.
- The by-products such as nitrogen rich seed cake are excellent organic manure while the seed husk used for gasification (Vyas and Singh 2007), animal feed (non-toxic varieties) or biogas, and fruit shells and seed husks for biogas and combustion.
- Oil has diverse medicinal uses, insecticidal, molluscidal, fungicidal and nematicidal properties.

2.2 Botanical description

The genus Jatropha belongs to tribe Joannesieae of subfamily Crotonoideae in the *Euphorbiaceae* family. Jatropha is morphologically diverse and containing approximately 175 known species. Linnaeus (1753) was the first to name the *Jatropha curcas* L as physic nut. The genus name Jatropha derives from the Greek word jatrós (doctor) and trophé (food), which implies its medicinal uses (Kumar et al., 2008). Jatropha is a perennial deciduous shrub to small

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tree with height of three to five meters with articulated growth with a morphological discontinuity at each internode and dormancy is induced by rainfall and temperature variations. Flowering in Jatropha occurs during the wet season and two flowering peaks are often seen, i.e. during summer and autumn. The plant is monocious and flower s are unisexual with occasional occurrence of hermaphrodite flowers (Dehgan and Webster 1979). The flowering season, flowering flushes and floral sex ratio of Jatropha depend largely upon temperature moisture level and fertility of soil (Kant and Wu 2011). The flowers are entemophilous. Each inflorescence yields a bunch of approximately 10 or more ovoid trilobed fruits with seed inside. The fruits mature 2-4 months after flowering. Mature Jatropha seeds contain about 30-42% oil with high percentage of unsaturated fatty acids (oelic acid and linoleic acid) which make Jatropha oil for suitable feedstock for biodiesel production (Pramanik 2003). Jatropha seeds also contain a wide range of phytochemicals some of which are toxic to human and animals. Phorbol esters, a group of tetracyclic diterpenoids, have been identified as the main agent responsible for Jatropha toxicity (Makkar et al., 1997). Different developmental stages of *Jatropha curcas* plants are illustrated in **Figure 2.2**.

2.3 Origin and distribution

Jatropha is native to Central America (Belize, Costa Rica, El Salvador, Guatemala, Nicaragua and Panama) and but is now found abundantly in many tropical and sub-tropical regions of Asia and Africa (Heller, 1996). It grows pantropically, from Brazil to the tropical island of Fiji. Jatropha have been well adapted in different eco-geographical conditions of India. It is widely spread in wild and semi wild to hedge plant, road side and forest-ecosystems in various states of India (Sunil et al., 2012).

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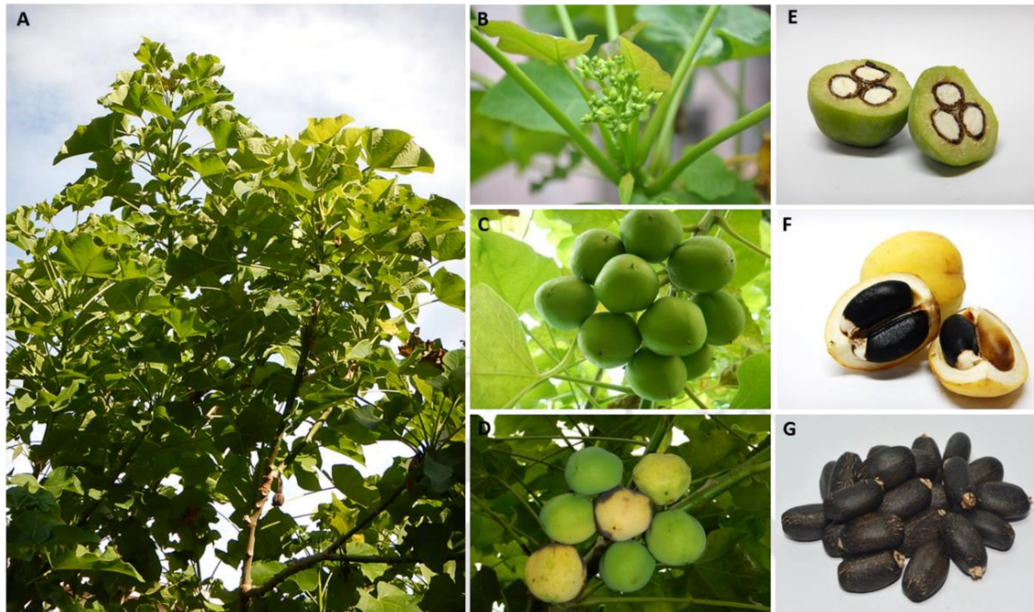

Figure 2.2 *Jatropha curcas* plant (A) mature plant (B) Axillary inflorescence of *J. curcas* (C) Jatropha plant bearing fruits (D) Jatropha fruits with different stages (E, F) cross section of Jatropha seed pod containing three developing seeds (G) Mature seeds of *J. curcas*.

2.4 Agronomic requirements

Jatropha can thrive well in hot conditions, survive in lower temperature and withstand a light frost. It can thrive under a wide range of rainfall ranging from 250-3000 mm per annum (Foidl et al., 1996). In arid and semi-arid areas and in prolonged rainless periods, the plant sheds its leaves to reduce transpiration loss of water. It grows on well-drained soil with good aeration, however it is also well adapted to marginal soils with low nutrient content as well as moderately sodic and saline soil (Benerji et al., 1985). The plant seedling biomass allocation and root/shoot were significantly influenced by drought (Achten et al., 2010). It is suitable for eco-restoration and mitigating soil erosion.

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2.5 Diversity assessment in Jatropha

Jatropha being an exotic species is well naturalized among the flora of diverse landform of India, so there is high probability of existence of considerable amount of genetic variations. The physical characteristics of Jatropha seeds varies depending on their geographical distribution and climatic conditions. Generally, seed weight varies from 0.4 to 0.7 g and seed dimension vary with length and width from 15-17 mm and 7-10 mm respectively (Martinez-Herrera et al., 2006). Oil content of seed is in the range of 30-42%. These observations clearly indicate the prominent role of habitat and environmental conditions over genotype in determining phenotypic variations in *Jatropha curcas*. Some varieties of *Jatropha curcas* recognized in the world based upon their phenotypic traits or the toxicity of seed, but this classification is not realistic, factor of unpredictability is more. For example, three varieties, Cape Verde variety that has spread all over the world, the Nicaraguan variety with few but larger fruits and a non-toxic Mexican variety that has only traces of phorbol esters in the fruit (Heller, 1996; Henning, 1997; Sujatha et al., 2005). Furthermore, germplasm characterization will assist in broadening Jatropha genetic resource through introduction of diverse accessions. Studies on morphometric, molecular and phytochemical analyses of Jatropha germplasm have been carried out randomly throughout the world.

2.6 Seed oil and biodiesel characteristics

Seeds are the main source of oil from Jatropha. Each mature tree on average produce about four kilogram of seed per year when cultivated under optimal condition (Tamalampundi et al., 2008). Mature Jatropha plantations yields 4 and 5 tonnes of seed per hectare with a long productive period of around 30 - 50 years, which equate to approximately 1.5 tonnes of oil per

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hectare (Matsuno et al., 1984; Foidl et al., 1996). Jatropha seeds is good candidate for non-edible oilseed crop as its seed contain 30-42% oil with a high percentage of unsaturated fatty acids (78-84%). The oil in Jatropha is relatively rich in unsaturated fatty acids such as oleic acid (18:1; 34.3–45.8%) and linoleic acid (18:2; 29.0–44.2%) while saturated FAs like palmitic acid (16:0; 4.1–15.3%) and stearic acid (18:0; 3.7–9.8%) account for a smaller fraction (Misra et al., 2013). The elevated levels of oleic and linoleic acids make the respective FAMEs suitable for biodiesel. Reksowardojo et al., (2006) compared five diesel types–petrodiesel, Jatropha B10, B100 and palm oil B10 and B100 and found the biodiesels to be more efficient in direct injection engines. Martnez-Herrera et al., (2006) have characterized four provenances of *J. curcas* from different agro-climatic regions of Mexico (Castillo de Teayo, Pueblillo, Coatzacoalcos and Yautepec) and showed that seed kernels were rich in crude protein (31–34.5%) and lipid (55–58%).

Seed oil of Jatropha is easily convertible to biodiesel. Biodiesel is monoalkyl esters of fatty acid prepared by transesterification of oil. The transesterification reaction consists of transforming triglycerides into fatty acid alkyl esters in presence of catalyst (Palligarnai et al., 2008) (**Figure 2.3**). This process consisted of three consecutive reversible reactions, where in, oil was successively converted into diglyceride and monoglyceride, and then into glycerine and FAMEs (fatty acid methyl esters).

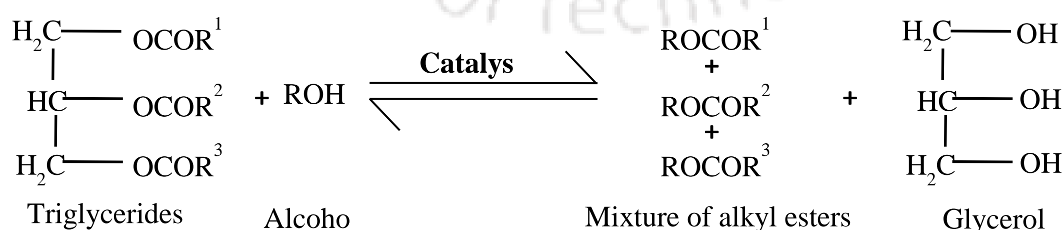

Figure 2.3 Transesterification process of TAG for methyl ester (biodiesel) production.

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Furthermore, Foidl et al., (1996) showed that both methyl and ethyl esters of Jatropha fatty acids could be used without engine modification. Physiochemical properties of Jatropha biodiesel are similar conventional fossil fuel as shown in table 1. Previously, physio-chemical properties of Jatropha biodiesel were compared with fossil fuel in our laboratory (Mazumdar et al., 2013). However, the use of pure Jatropha biodiesel (B100) is contested (Wood, 2008) due to high NOx emissions (Reksowardojo et al., 2006) and it has been shown that engine performance can be improved with petro- and biodiesel blends (Pramanik, 2003). Blending with mixes of biodiesel e.g. from Jatropha and palm oil showed better stability at low temperature and also improves oxidation stability compared to the use of either Jatropha or palm oil biodiesel alone (Sarin et al., 2007). Corrosion tests on engine parts and emissions analysis showed that both Jatropha and palm oil biodiesel make acceptable substitutes for petro-diesel (Kaul et al., 2007; Reksowardojo et al., 2006). The effects of using biofuels in internal combustion engines have recently been reviewed by Agarwal (2006), while Rao et al., (2007) specifically compared diesel to Jatropha biodiesel and its blends with diesel. Their results indicated that ignition delay, maximum heat release rate and combustion duration were lower for Jatropha derived biodiesel and its blends compared to diesel. Jatropha had lower tail pipe emissions than diesel except for nitrogen oxides (Reksowardojo et al., 2006). Pradeep and Sharma (2007) recently addressed the latter with Jatropha derived biodiesel and concluded that hot exhaust gas recirculation (EGR) was an effective solution to reducing nitrous oxides. Many reports suggest for effective routes to obtain Jatropha derived biodiesel with minimal free fatty acids (FFA; Berchmans and Hirata, 2008; Tiwari et al., 2007) so that seed oil from varieties with relatively large FFAs can also be used as long as the C16:C18 ratio is acceptable.

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Table 2.1 Comparison of properties of diesel and Jatropha biodiesel (Mazumdar et al., 2013)

Parameters	Diesel	Jatropha oil	Jatropha biodiesel
Calorific value (MJ/kg)	42.21	37.07-38.49	39.65-41.63
Specific gravity	0.846	0.912-0.922	0.868-0.884
Flash point (^{0}C)	52	225	175-189
Cetane No.	46	34.11-35.37	50.74-53.27
Acid value (mg KOH/g)	0.35	25-28	0.41-0.43
Diesel index	50	35.24	56.59-59.41
Kinematic viscosity (mm^2/s)	2.6	20.5-22	2.35-2.47

2.7 Potential co-product of Jatropha

Co-product produced during and after the extraction of and conversion of oil, provides added value to the use of Jatropha as bioenergy crop. It has diverse uses ranging from biodiesel production, traditional medicine for common human and animal ailments; protection against land erosion, boundary fence or live hedge to production of valuable product in chemical and cosmetic industries (Openshaw, 2000). Besides, fruit husk is used as feedstock for open core down draft gasifiers (Vyas et al., 2007) for wood gas, glycerine by-product used to convert it to a number of value added products (Pagliaro et al, 2007). The seed cake provides a rich source of protein when detoxified of its toxins, co-carcinogens and anti-nutrients factors providing an excellent animal feed (Devappa and Swamylingappa, 2008). Mahanta et al., (2008) have demonstrated a more high-value use of the seed cake, without the need for detoxification, using solid-state fermentation with *Pseudomonas aeruginosa* to produce enzymes such as proteases and lipases (**Table 2.2**).

In the seed cake, the co-carcinogenic compounds, diterpene phorbol esters are a major cause of concern limiting the wide-spread commercialization of Jatropha. However, some diterpenes are known for their antimicrobial and antitumor activities such as jatropholone A and B (Ravindranath et al., 2004) which have been recently shown to have gastro-protective and

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cytotoxic effects (Pertino et al., 2007). Other terpenoids such as Jatrophatrione and acetylaleuritolic acid obtained from other species of Jatropha have also been shown to have antitumor activities (Torrance et al., 1977).

Table 2.2 Showing the list of various uses of the Jatropha

Plant parts	Applications	References
Leaf/extract	Feed for silkworms, larvicidal, Bactericidal, Fungicidal	Rahuman et al., 2008; Eshilokun et al., 2007; Onuh et al., 2008
Stem/Bark	Dark blue dye, tannins and waxes, Jatrophone	Srivastava et al., 2008; Burkill 1985; Biehl and Hecker 1986
Stem latex	Nanoparticle synthesis, antimicrobial, anticancerous activity	Maravi et al., 2014
Root	Antibacterial diterpenoids	Alyelaagbe et al., 2007
Seeds	Seed diterpenes and toxines as antimicrobial, Antitumour, insecticidal, Jatrophone for gastric lessions, Seed coat copper biosorption for waste water	Goel et al., 2007; Luo et al., 2007; Liu et al., 1997; Pertino et al., 2007; Jain et al., 2008
Seed oil	Biodiesel, Resins, varnishes, soap, lubricant, illuminant, Antimicrobial, Biopesticide	Kaushik et al., 2007; Patel et al., 2008;
Seed cake	Protein rich feed, organic fertilizer, lipase and protease production, activated carbon	Devappa and Swamylingappa, 2008; Mendoza et al., 2007; Mahanta et al., 2008; Sricharoenchaikul et al., 2008
Fruit husk	Fuel through combustion, pyrolysed for biogas	Singh et al., 2008; Vyas and Singh, 2007

2.8 In vitro plant regeneration studies in Jatropha

Jatropha has received considerable attention as a potential source of non-edible vegetable oil which is eminently suitable for production of liquid biofuel (Azam et al., 2005; Tiwari et al., 2007). This biofuel crop grows in diverse eco-physiological regions with minimum agronomic input, with a life span up to 50 years (Dong et al., 2009; Behera et al., 2010). However, the

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major limitation in large-scale cultivation of this bioenergy crop is the low and inconsistent seed yield due to heterozygous nature of plants and traditional propagation through stem cuttings is seasonal, prone to diseases and easy uprooting of established plants hamper the practical utility of this propagation method (Heller, 1996; Sujatha et al., 2005). Therefore, development of an efficient and reproducible micro-propagation system is expected to boost the mass propagation of this biofuel plant (**Table 2.3**).

Several protocols have been published in literature for plant regeneration of Jatropha using both direct and callus-mediated process, using shoot (Rajore et al., 2005; Purkayastha et al., 2010) and nodal meristems (Kalimuthu et al., 2007; Sharma et al., 2010), cotyledonary leaf (Li et al., 2008; Mazumdar et al., 2010; Li et al., 2012; Khemkaladngoen et al., 2011) and leaf (Sujatha et al., 1996; Sujatha et al., 2005; Deore et al., 2008; Khurana-Kaul et al., 2010; Kumar et al., 2010; Jha et al., 2007), hypocotyls (Sujatha et al., 1996; Kaewpoo et al., 2010; Sahoo et al., 2011; Li et al., 2012) and epicotyls segments (Qin et al., 2004; Kaewpoo et al., 2010) as well as mature (Shrivastava et al., 2009) and immature zygotic embryos (Varshney et al., 2010).

2.9 Current status of transgenic research in Jatropha

Development of genetic transformation system in Jatropha has become very necessary for trait improvement of this biofuel crop as well as for understanding functional genomics of plant. Several researchers have succeeded in developing different transformation methods for Jatropha (**Table 2.4**).

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Table 2.3 Currents status on regeneration studies of Jatropha

Explant used	Mode of regeneration	References
Hypocotyl, petiole and leaf	Regeneration via adventitious shoots, rooted in vitro	Sujatha and Mukta 1996
Leaf	Somatic embryogenesis	Sardana et al., 2000
Axillary node and leaf	Direct adventitious shoot regeneration	Sujatha et al., 2005
Leaf	Direct adventitious shoots	Deore and Johnson 2008
Leaf	Direct organogenesis	Khurana-Kaul et al., 2010
Leaf	Direct organogenesis	Shrawan Kumar et al., 2010
Leaf	Somatic embryogenesis	Jha et al., 2007
Leaf and hypocotyl explants	Callus and suspension culture	Soomro and Memon 2007
Petiole	Direct organogenesis	Kumar and Reddy 2010a
Petiole	Direct organogenesis	Kumar et al., 2010b
Petiole, Hypocotyl	Indirect organogenesis	Li et al., 2012
Nodal	Direct organogenesis	Datta et al., 2007
Nodal segment	Somatic embryogenesis	Kalimuthu et al., 2007
Nodal segment	Direct organogenesis	Sharma et al., 2010
Axillary node	Direct shoot regeneration	Shrivastava and Banerjee 2008
Axillary bud	Multiple shoot induction	Thepsammran et al., 2007
Stem	Direct organogenesis	Singh et al., 2010
Leaf	Direct and indirect organogenesis	Misra et al., 2010
Shoot tips	Direct organogenesis	Rajore and Batra 2005
Shoot tips	Direct organogenesis	Purkayastha et al., 2010
Hypocotyl and epicotyl	Direct and indirect organogenesis	Kaewpoo and Te-chato 2010
Hypocotyl	Direct organogenesis	Sahoo et al., 2011
Epicotyl	Direct organogenesis	Qin et al., 2004
Cotyledon (14 days)	Indirect organogenesis	Li et al., 2008
Embryogenic cotyledons	Indirect organogenesis	Mazumdar et al., 2010
Cotyledon	Direct shoot regeneration	Kumar et al., 2010c
Juvenile cotyledon	Direct organogenesis	Khemkaladngoen et al., 2011
Embryo	Direct organogenesis	Shrivastava et al., 2009
Immature embryo	In direct organogenesis	Varshney et al., 2010
Microshoot	Direct organogenesis	Shah et al., 2010

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2.9.1 Genetic transformation of Jatropha by micro-projectile bombardment:

Our lab reported for the first time gene transfer by particle bombardment in Jatropha (Purkayastha et al., 2010) using shoot apices as explants. Explants bombarded with particles coated with plasmid pBI426 with a GUS-NPT II fusion protein under the control of a double 35S cauliflower mosaic virus (CaMV) promoter showed β-glucuronidase (GUS) activity in bombarded shoot apices and the GUS activity was significantly affected by the gold particle size, bombardment pressure, and target distance, macro carrier travel distance, number of bombardments, and type and duration of osmotic pre-treatment.

Preculture of shoot apices on 0.2 M mannitol (osmotic treatment) for 4 h prior to bombardment was found to improved transient GUS expression The highest frequency of transient GUS expression was observed when explants were bombarded at 1100 psi with a target distance 9 cm. Joshi et al., (2011) reported genetic transformation of Jatropha by micro-projectile bombardment using embryo axes and they found that, microcarrier size, helium pressure and target distance had significant influence on transformation efficiency. Among different variables evaluated, microcarrier size 1 µm, He pressure 1100 and 1350 psi with a target distance of 9 and 12 cm respectively were found to show high GUS expression and survival of putative transformants. Although frequency of transient GUS expression increases with increase in helium pressure and microcarrier size but there was a reduction in the frequency of regeneration due to tissue damage. The result obtained from the experiment showed the possibility of stable transformation of Jatropha through direct gene transfer method with 44.7% transformation efficiency.

2.9.2 *Agrobacterium* mediated genetic transformation in Jatropha

Agrobacterium tumefaciens mediated transformation is most preferred method of gene delivery, due to its simplicity, cost effectiveness, defined integration of transgene in to the host

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genome (Koncz et al., 1989; Hamilton et al., 1996; Ingelbrecht et al., 1991). Li et al., (2008) reported *Agrobacterium* mediated genetic transformation first time in Jatropha, in Chinese accessions using cotyledonary disc as explants. Transformation was carried out with Agrobacterium cells harbouring p3301-BI121-*SaDREB1* plasmid, which contain *SaDREB1* gene and bar gene for selection on phosphinothricin and β-glucuronidase (gus) as reporter (cloned from PBI121) gene and pCAMBIA1301 carrying both gus and the selectable marker gene for *hygromycin phosphotransferase* (*hpt*). The result obtained from the experiments revealed significance influence of *Agrobacterium* strain (LBA4404 and EHA105), selection agent (phosphinothricin, hygromycin and kanamycin) and, bacterial incubation time (5–30 min) on transformation efficiency in Jatropha. Maximum transformation efficiencies were achieved with LBA 4404 strain (OD600 of 0.4–0.5) with bacterial incubation period of 10 min at 28°C, 3 days of co-cultivation at 25°C in a co-cultivation medium containing 20 mg/L acetosyringone, followed by selection on phosphinothricin and hygromycin. Presence of the gene in primary transformants was confirmed through PCR and Southern analysis. The overall transformation efficiency was achieved around 13%. Trivedi et al., (2009) also reported an *Agrobacterium*-mediated genetic transformation system in Jatropha using cotyledonary explants, however the study was only confined to callus. The major focus of the work was to study the Agro-infection to leaf explants from cotyledons and integration of transgene in the callus. Transformation was carried out with Agrobacterium LBA4404 strain harbouring pCAMBIA1301S-*DREB2A* containing *hpt* gene, as selectable marker and *gus* as reporter gene. Sensitivity of cotyledons and callus to antibiotics was tested and 5 mg/L of hygromycin were found optimum for selection of transgenic. Effect of different concentration of Acetosyringone (50, 100, 200 and 400 μM), dipping time of cotyledonary leaf explants in Agrobacterium suspension was also studied. They observed in all concentration of Acetosyringone and dipping

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time 6h, 100% infection was visible in explants. Whereas, in case of 12h dipping, it was found lethal for all explants. Li et al., (2008) established an Agrobacterium transformation system in Jatropha using 14 days cotyledonary explants. Transformation was carried out with Agrobacterium LBA4404 strain harbouring p3301-BI121-SaDREB1 containing bar gene, as selectable marker and *gus* as reporter gene. The age of the explant is a crucial aspect in transformation experiment and therefore, appropriate biological condition of the explant is vital for optimal infection and T-DNA transfer by *Agrobacterium tumefaciens* (Purkayastha et al., 2010). Therefore, the effect of the age of the cotyledonary leaf explants on Agrobacterium-mediated transformation has been evaluated (Mazumdar et al., 2010). Transformation was carried out with EHA105 *Agrobacterium* strain harbouring pCAMBIA2301 containing neomycin phosphotransferase (*nptII*) gene, conferring kanamycin resistance and gus reporter gene. Cotyledonary leaf segment, prepared from freshly germinated seeds were found to be most amenable to *Agrobacterium*-mediated transformation as compared to older explants. Later on, Zong et al., (2010) reported a transformation method, using young leaf as explants. Based on that procedure the lateral shoot inducing factor (LIF) from Petunia has been introduced in to Jatropha. Ttransformation was carried out with LBA4404 strain harbouring PBI121-LIF recombinant plasmid, containing gus as reporter gene and neomycin phosphotransferase II (*nptII*), as selectable marker. LIF is a zinc finger protein isolated from Petunia and overexpression of this protein in petunia, tobacco and Arabidopsis resulted dramatic increased in lateral shoot and reduced height (Nakagawa et al., 2005). Contrary to the previous report, they observed LBA4404 and EHA105 had the same virulence on young leaf of Jatropha. The sensitivity of Jatropha tissues to cefotaxime (300 mg/L) and the selection agent kanamycin (40 mg/L) was optimized. Maximum transformation efficiencies were achieved with bacterial incubation period of 10 min, 5 days of co-cultivation followed by

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selection on kanamycin. Presence of the gene in primary transformants was confirmed through PCR and Southern analysis. The overall transformation efficiency was achieved around 23.91±5.78%. Pan et al., (2010) reported an *Agrobacterium*- mediated transformation protocol using cotyledonary explants. *Agrobacterium* strain LBA4404 harbouring the binary vector pCAMBIA2301, which contains *nptII* and *gus* gene was used for transformation experiments. Kanamycin was observed to inhibit callus induction. Therefore, kanamycin was not included in callus induction medium. 20 mg/L kanamycin and 100 mg/L cefotaxime was found optimum for selection of transgenic shoots from callus. PCR and Southern analysis was performed to confirm the presence of transgene. Transformation parameter like, incubation period of 20 min with agitation at 200 rpm (at room temperature) followed by co- cultivation of 3 days at 26±2°C was found optimum for successful transformation.

Khemkladngoen et al., (2011) investigated 1-min sonication treatment of cotyledonary explants followed by 9 min shaking in Agrobacterium suspension is an effective protocol for obtaining the transformation of Jatropha with a high stable transformation efficiency (approx. 53%). LBA4404 containing the binary vector pBI121 containing *nptII* and *gus* gene was used for transformation treatments. Stable transformation and gene integration was confirmed by PCR and southern hybridization. Wounding of the tissues by sonication and sand-vortexing in addition to shaking were found to facilitate efficient *Agrobacterium* infection which is contrary to the report of Kumar et al., (2010), where they showed experimentally, wounding treatments by glass beads and hand pricking with needle reduces transformation efficiency.

However, stable transformation efficiency (approx. 53%) was found much higher compared to Kumar et al., 2009 (29%) and Li et al., 2007 (13%). Misra et al., (2012) reported an *Agrobacterium* mediated transformation protocol for *J. curcas* using in-vitro Leaf and

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hypocotyl segments. Transformation of Jatropha studies using both of these explants has not been reported earlier, as all previous studies in Jatropha were done using cotyledonary and leaf explants, as it was found most permissible explant for their adaptation to gene delivery methods. *Agrobacterium* strain EHA101, harboring the binary vector pIG121Hm containing *nptII* as selectable marker and gus as reporter gene were used for all transformation studies. The super virulent strain EHA101 was found very effective with distinct blue visible spots in both the explants, whereas, LBA4404 did not show any GUS expression, which is contrary to the previous published report (Li et al., 2008). Both types of explants showed similar transient GUS expression after 3, 4 or 5 days of co-cultivation. However, 5 days led to overgrowth of *A. tumefaciens*, killing the explants, whereas, after 3rd day 80% of the explants survived after co-cultivation. Screening of transgenic was done by PCR of gus reporter gene and formation of transscipt was confirmed by RT-PCR of gus gene. Stable transformation efficiency was found 5% in case of leaf explants and whereas 4% in hypocotyl explants, which was found very low compared to previous published reports.

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Table 2.4 Transformation studies in *Jatropha curcas*

Explants Used	DNA Delivery method	Strain/Vector/Construct	Selection	Transformation Efficiency (%)	References
14 days old cotyledon	*Agrobacterium* mediated transformation	LBA4404 PCAMBIA 3301-BI121-SADREB1	hptII	13	Li et al., 2008
cotyledon	*Agrobacterium* mediated transformation	LBA4404 PCAMBIA 1301S-DREB2A	hptII	-	Trivedi et al., 2009
Young leaf	*Agrobacterium* mediated transformation	LBA4404 PBI121-LIF	nptII	23.91	Zong et al., 2010
Cotyledon	*Agrobacterium* mediated transformation	LBA4404 PCAMBIA 2301	nptII	-	Pan et al., 2010
Juvenile cotyledons	*Agrobacterium* mediated transformation	LBA4404 PBI121	nptII	53	Khemkladngoen et al., 2011
Leaf	*Agrobacterium* mediated transformation	LBA4404 PCAMBIA 1304-*S-DREB2A*	hptII	29	Kumar et al., 2010
Cotyledon	*Agrobacterium* mediated	LBA4404, EHA105 pBI121:: *LIF*	nptII	-	Zong et al., 2010
Cotyledon	*Agrobacterium* mediated	EHA105 pCAMBIA2301	nptII	-	Mazumdar et al., 2010
Shoot apices	Biolistic gun	pBI426	nptII	-	Purkayastha et al., 2010
Embryo axes	Biolistic gun	PCAMBIA 1301	hptII	44.7	Joshi et al., 2011
Cotyledon	*Agrobacterium* mediated transformation	PCAMBIA 1300 JcFAD2-1 RNAi	hptII -	-	Qu et al., 2012
Primary shoot	*Agrobacterium* mediated	EHA105	nptII	-	Jaganath et al., 2014
Cotyledon	*Agrobacterium* mediated	EHA105 pCAMBIA2301 35S::*AtFT*	nptII	-	Fu et al., 2015

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Most of the reports are based on optimization of *Agrobacterium* mediated transformation in Jatropha. To date, only one report is available on generation of stable transgenic plants using candidate genes (Qu et al., 2012). Qu et al., (2012) developed marker free transgenic Jatropha plant with increased levels of seed oleic acid. *JcFAD2*-1, a delta-12 fatty acid desaturase gene in Jatropha was down regulated in a seed-specific manner by RNA interference technology. To silence JcFAD2-1 expression by RNAi technology, 862 bp coding region that shares 72.9% identity with JcFAD2-2 was targeted. The resulting JcFAD2-1 RNA interference transgenic plants showed a dramatic increase of oleic acid ($> 78\%$) and a corresponding reduction in polyunsaturated fatty acids ($< 3\%$) in its seed oil, whereas, control Jatropha had around 37% oleic acid and 41% polyunsaturated fatty acids. Due to the changes in the fatty acids profile, the oil of the JcFAD2-1 RNA interference seed was estimated to yield a cetane number as high as 60.2, which is similar to the required cetane number for conventional premium diesel fuels (60) in Europe. For obtaining transgenic plants free of an antibiotic selection marker, chemical inducible Cre-lox-mediated site-specific recombination system was used. After obtaining the hygromycin-resistant regenerated shoot, they were immediately transferred to b-estradiol induction medium without hygromycin to induce marker excision. After two weeks of induction, shoots were transferred to regeneration medium without hygromycin antibiotic. This is the first successful report on genetic modification of key agronomic traits in Jatropha.

2.10 Triacylglycerol biosynthesis in plants

Increasing demand for plant oils as a resource for food, fodder and fuel has renewed researchers interest in understanding lipid biosynthesis in various plant tissues in addition to seeds. Lipids are essential component of all living cells and biological membranes. It serves

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as energy reserve and secondary messengers. Lipids include oil, fats and wax that are glycerol esters of fatty acid (TAG) and mainly derived from plants and animal sources. Plant lipids are categorized into two broad classes, simple and complex lipids. Lipids that yield not more than two types of products per mole by hydrolysis are referred to as simple, while complex lipids are those that yield more than three types of products per mole (Christie and Han 1982). Complex lipids are major component of the cell membrane and can be distinguished into phospholipids and glycolipids. Phospholipids are composed of glycerol backbone attached to a hydrophilic phosphate head group and hydrophobic fatty alcohols or fatty chains. Glycolipids contain different types of sugars attached to the glycerol backbone and they provide energy and as well act as cellular recognition markers (Buchanan et al.,2000). Simple lipids on the other hand consist of one, two, or three fatty acids (FA) attached to the glycerol backbone generating mono, di, or tri acylglycerols (**Figure 2.3**), respectively or long-chain fatty alcohols that form wax esters. Wax esters may store energy but they mostly serve as waterproof barrier on plant surfaces (Kolattukudy 1970). Triacylglycerols, on the other hand, are accumulated as major component of storage lipids, ranging from 20 to 90% oil, by dry weight, in various plant tissues (Bewley and Black 1994). Although the biochemical pathway for FA synthesis and TAG assembly is highly conserved among plants (**Figures 2.4** and **2.5**), the specific mechanisms by which the TAG content and composition is regulated in various plants and tissue types remain to be elucidated (Ohlrogge and Browse 1995).

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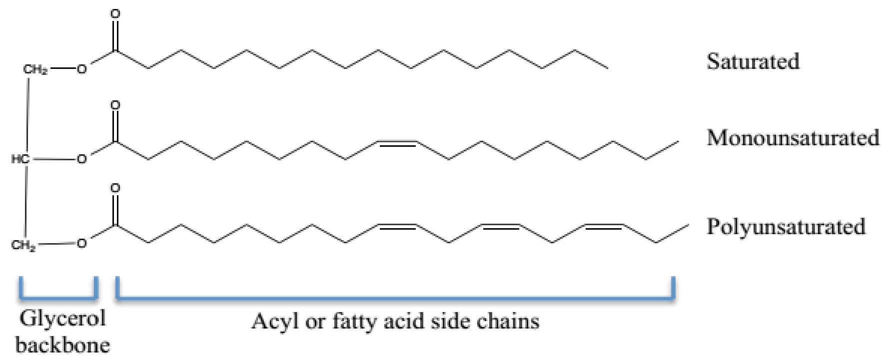

Figure 2.4. Structure of Triacylglycerol.
Three fatty acyl groups are esterified to a glycerol backbone at the sn-1, sn-2, and sn-3 positions to form a molecule of triacylglycerol. The acyl chains may be saturated with no double bond or unsaturated with one (mono) or more than one double bond (poly).

TAG biosynthesis has been extensively studied since the 1950s and may involve several pathways (Dahlqvist et al., 2000; Sorger and Daum, 2003; Yen et al., 2002). The classical pathway leading to TAG formation is known as the Kennedy or *sn*-glycerol-3-phosphate (G3P) pathway (Weiss and Kennedy, 1956), which has been described in various organisms including, animals, plants, fungi and bacteria (Alvarez and Steinbuchel, 2002, Athenstaedt and Daum, 2006).

2.10.1 Fatty Acid Biosynthesis

In plants, FA biosynthesis takes place in the plastid and the necessary substrates are either supplied from cytosol and/or generated within the plastid (Figure 4). Pyruvate, the end product of cytosolic and/or plastidial glycolysis of the photosynthate, is converted to acetyl-CoA, the primary substrate for de novo FA synthesis, by plastidial pyruvate dehydrogenase (Ohlrogge and Browse 1995). The pyruvate dehydrogenase provides most of the acetyl-CoA (30-50 µM) that is needed for FA synthesis (Post-Beitten miller et al., 1992). The first committed and thus regulated step, however, in de novo FA synthesis is the formation of

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malonyl-CoA from acetyl-CoA, which is catalyzed by acetyl-CoA carboxylase (Ohlrogge and Browse 1995; Sasaki and Nagano 2004). The acetyl-CoA carboxylase in plants exists in two distinct molecular forms- multi protein complex and multifunctional protein. The plastid localized isoform is a multi-enzyme complex and soluble Class 1 biotin-containing enzyme that uses ATP and bicarbonate to carboxylate a biotin prosthetic group that is attached to the flexible arm of biotin carboxyl carrier protein via a lysine residue. The initial partial carboxylation of the biotin prosthetic group is catalysed by biotin carboxylase and completed by carboxyl transferase.

A type II multi protein complex, fatty acid synthase, carries out the remaining reactions of FA synthesis (**Figure 2.4**). While β-ketoacyl-ACP synthase (KAS) III catalyzes the first condensation reaction, using the acetyl-CoA and malonyl-ACP substrates, the successive addition of two-carbon units is accomplished sequentially by four enzymes- a condensing enzyme KAS I, first reductase (β-ketoacyl-ACP reductase), dehydrase (β-hydroxyacyl-ACP dehydrase) and a second reductase (enoyl-ACP reductase). Addition of two-carbon units is continued until generation of palmitoyl-ACP (Figure 4) and then the final reaction between palmitoyl-ACP and malonyl-ACP is catalyzed by KAS II to produce stearoyl-ACP (PostBeittenmiller et al.,1992; Ohlrogge and Browse 1995). Finally, the acyl-ACP thioesterases hydrolyze the acyl moiety from ACP, preventing the extension of acyl groups and targets them for export out of the plastids (**Figure 4**). Although, the FA synthesis produces saturated FAs, over 75% of the FAs in plants are unsaturated (Mattson and Volpenhein 1963) by desaturases prior to hydrolysis by thioesterases. Short chain fatty acids with 10-12C atoms are water-soluble, while long-chain FAs are less water-soluble (Kikuta and Erickson 1968; Requejo-Tapia et al., 1999). The number of carbon atoms in the acyl chain and double bonds

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are typically denoted in representing a FA. For example, a representation of 18:1 indicates an 18C chain length and the presence of one double bond in oleic acid (Davidson and Cantrill 1985). The hydrolyzed FAs in the plastid are exported as acyl-CoAs to the endoplasmic reticulum (ER) where TAG assembly occurs (Ohlrogge and Browse 1995; Lacey and Hills 1996; Buchanan et al., 2000).

2.10.2 Triacylglycerol Biosynthesis and Assembly

An acyl-CoA synthetase catalyzes the formation of acyl-CoA thioester that is exported to the ER for glycerolipid synthesis, including TAG assembly (Kennedy 1961; Huang 1992). In most organisms, the conventional Kennedy pathway for synthesis of glycerolipids in the ER is expected to play a role in the synthesis of TAGs (**Figure 2.5**). This pathway involves sequential acylation of the sn-1, sn-2 and sn-3 positions of glycerol-3-phosphate with acyl-CoA to finally yield TAG. The first acylation is catalyzed by glycerol-3-phosphate acyltransferase and the lysophosphatidic acid generated in the first step is converted to phosphatidic acid by lysophosphatidic acid acyltransferase, which is further hydrolyzed by phosphatidic acid phosphatase to yield diacylglycerol (DAG). Because phosphatidic acid and DAG are also precursors of membrane glycerolipids, the acylation of sn-3 position of DAG, to yield TAG, is considered as the crucial and rate-determining step in TAG biosynthesis. This terminal acylation step in TAG biosynthesis in Kennedy pathway is catalyzed by diacylglycerol acyltransferase (DGAT). Since the acyl-CoAs imported to the ER are directly incorporated to generate TAG by the three acyltransferases, these enzymes are described as acyl-CoA dependent (Weiss et al., 1960; Kennedy 1961; Ohlrogge and Browse 1995; Lung and Weselake 2006). Alternatively, several lines of evidence have indicated occurrence of a DGAT-independent mechanism for TAG biosynthesis (Stymne and Stobart 1987).

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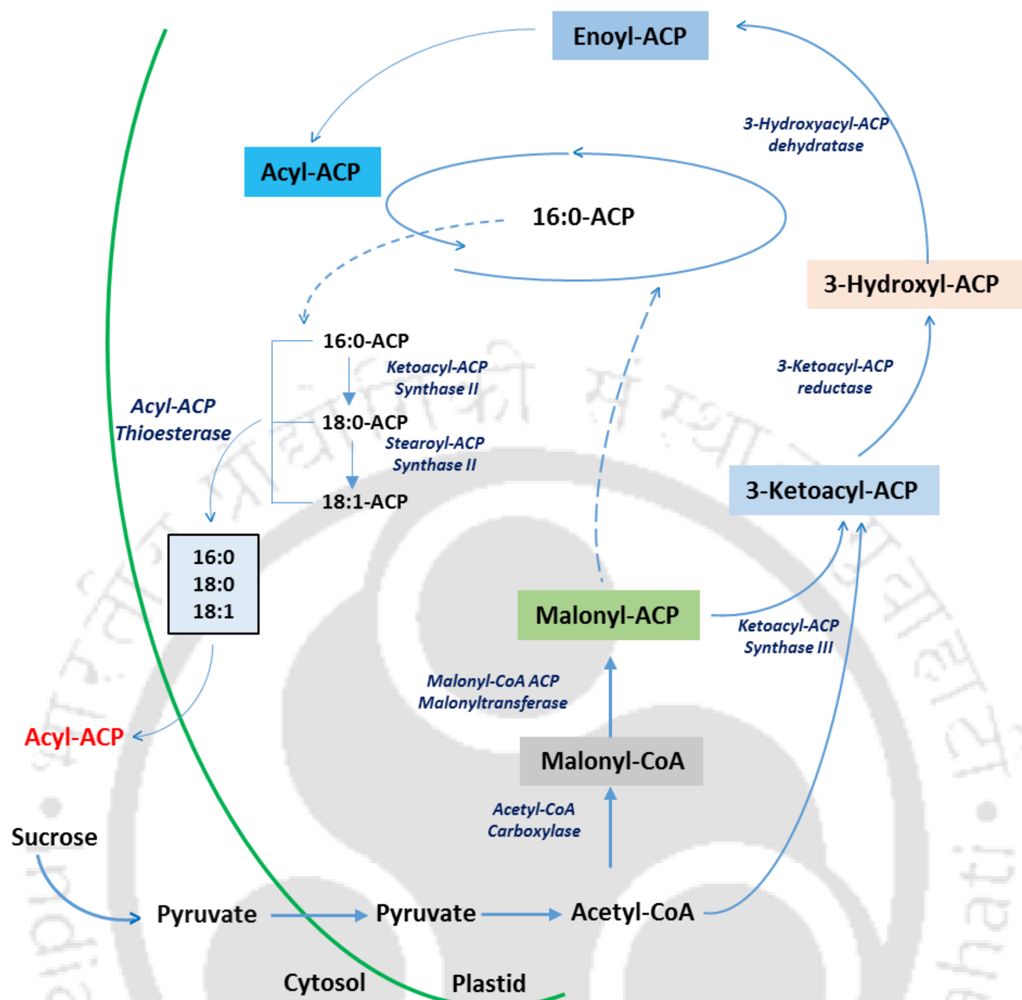

Figure 2.5 Fatty acid biosynthesis pathway in plants. Sucrose generated from photosynthesis is converted to pyruvate via glycolysis.

Series of enzymes are involved in converting pyruvate to acetyl-CoA, malonyl-CoA and acyl carrier proteins (ACP) in the plastid. Acyl-ACP thioesterases terminate fatty acid synthesis and the free fatty acids are exported to cytosol and imported to the ER as acylCoAs.

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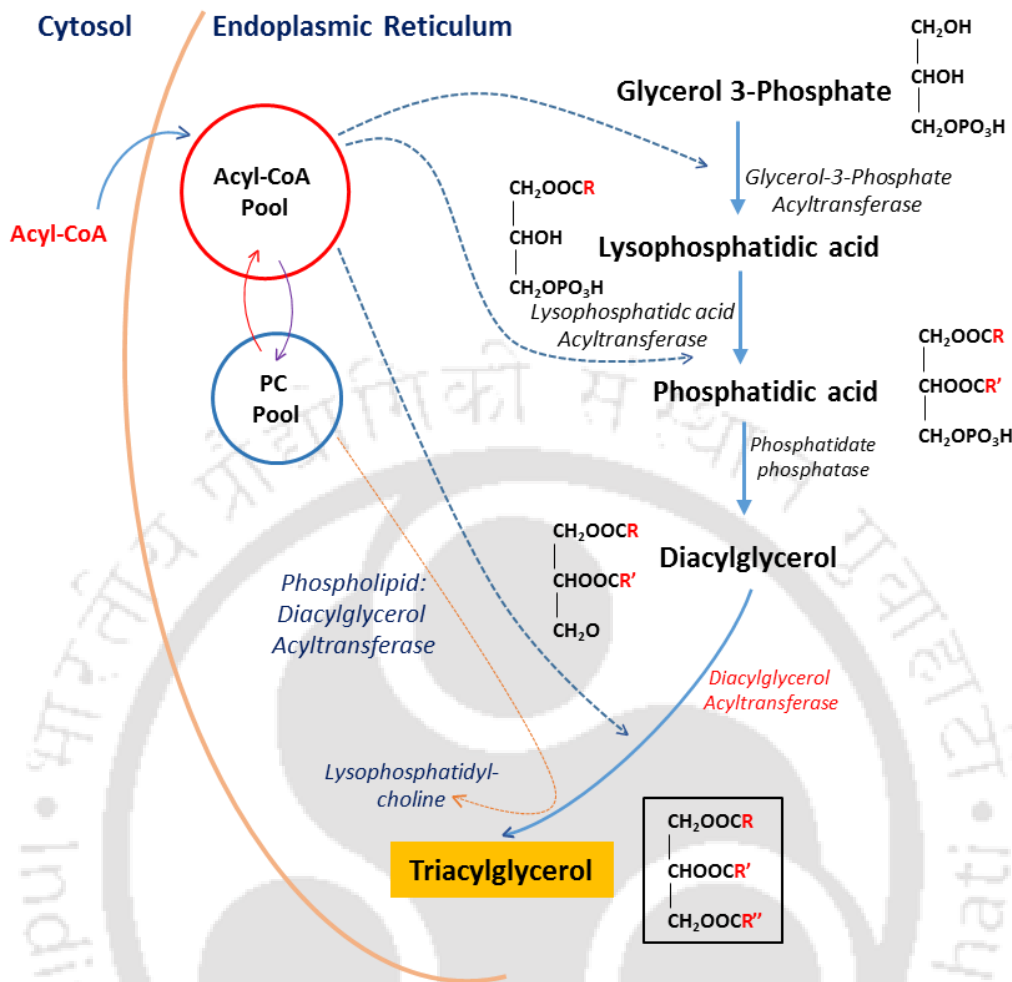

Figure 2.6 Triacylglycerol assembly in plants.

Acyl-CoAs imported to the endoplasmic reticulum are sequentially acylated to glycerol-3-phosphate to generate lysophosphatidic acid, phosphatidic acid, diacylglycerol and finally triacylglycerol. This terminal step in TAG synthesis via Kennedy pathway is catalyzed by diacylglycerol acyltransferase. Alternatively, acyl-CoA incorporated into phosphatidylcholine (PC) may also provide the acyl group to the DAG to generate TAG, which is catalyzed by phospholipid: diacylglycerol acyltransferase, an acyl-CoA independent enzyme. R, R' and R" represent the acyl/fatty acid side chain.

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To this extent, a phospholipid:DAG acyltransferase (PDAT) was identified and characterized as an acyl-CoA-independent transacylase that synthesizes TAG from phosphatidylcholine (PC) and DAG, also yielding lyso-PC (**Figure 2.6**) (Dahlqvist et al., 2000). Furthermore, another DAG-utilizing enzyme activity, a DAG acyltransferase that forms TAG and monoacylglycerol has also been detected in vitro (Stahl et al., 2004), but its identity remains to be characterized. Finally, the TAG that is synthesized in the ER, particularly in seed tissues, as revealed by electron microscopic studies, is surrounded by a monolayer embedded with unique proteins called oleosins (Murphy, 1999; Siloto et al.,2006; Robnek et al., 2006). It has been suggested that oleosins maintain oil bodies as small single units preventing coalescence during desiccation and thus far, high expression levels for oleosin proteins have been reported only in oil-rich seeds and pollen but not in mesocarp or other nonseed tissues (Huang 1992; Bourgis et al., 2011).

2.10.2.1 Acyl-CoA Dependent TAG Synthesis

Three unrelated gene families of DGAT, referred to as DGAT1, DGAT2 and DGAT3 have been identified in various plants. Among the three, DGAT1 and DGAT2 were shown to be involved in TAG synthesis (Ichihara et al., 1988) but do not share any significant homology with each other (Kalscheuer and Steinbüchel 2003). In Arabidopsis, DGAT1 (AT2G19450.1) and DGAT2 (AT3g51520.1) were found to be approximately 59 and 36 KDa, respectively. Several studies suggested that DGATs are membrane localized and associated with oil bodies (Gurr 1974) and/or ER (Stymne and Stobart 1987; Frentzen 1993; Lacey and Hills 1996; Lung and Weselake 2006). The mechanism by which the DGATs are localized in the oil bodies is debatable. A generally accepted theory is that the oil bodies are generated from the ER and imported to the cytoplasm, with DGAT in the membrane of the oil bodies (Wanner and Theimer

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1981; Huang 1992). On the other hand, some studies have hypothesized that oil bodies are not associated with the ER and when nascent oil bodies (without oleosins and phospholipids) are export from the ER, they are surrounded by oil body components by other factors, in which case DGATs would not be present in their membranes (Bergfeld et al., 1978; Ichihara 1982; Murphy and Kang 1989). These observations varied with plant species and more studies are required to understand the activity of DGAT, particularly in those species where oil bodies protected by oleosins are not reported. The third member DGAT3, on the other hand, discovered in peanut, was found to be soluble, cytosolic protein and distinct from the membrane localized DGAT1 and 2 (Saha et al., 2006). Unlike DGAT 1 & 2, the soluble DGAT is barely expressed in seed plants (Cases et al.,1998; Oelkers 1998; Routaboul 1999; Zou et al., 1999; Saha et al., 2006). Both DGAT1 and DGAT2 have been shown to be involved in TAG biosynthesis in several seed and nonseed tissues; while in most tissues one of the enzyme may play a predominant role, in several others they may function together in an overlapping manner. In several seed tissues, including embryos of Arabidopsis, rapeseed and nasturtium, the transcript levels for DGAT1 were abundant and DGAT2 was scarcely expressed ((Li et al., 2010; Troncoso-Ponce et al., 2011). The role of DGAT1 in TAG accumulation in Arabidopsis seeds was further confirmed by overexpression of DGAT1 in seed tissues, which increased TAG content but its disruption resulted in only 20 to 40% reduction in oil content (Bouvier-Navé et al., 2000; Andrianov et al., 2010). Lack of incomplete abolition of TAG synthesis in dgat1$^{-/-}$ mutants of Arabidopsis suggested that alternate pathways and enzymes are capable of compensating for each other. Castor seed endosperm, on the other hand, expressed relatively higher DGAT2 levels than other acyltransferases, and since then DGAT2 was considered to play an important role for the selective accumulation of unusual FAs (Li et al., 2010). More recent studies have, however, indicated otherwise. For example, transcript levels for DGAT2

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were shown to be associated with oil accumulation in olive and oil palm mesocarp tissues, which do not accumulate unusual FAs (Banilas et al., 2011; Bourgis et al., 2011). Furthermore, the expression patterns for DGAT1 and DGAT2 were overlapping, in the mesocarp tissue of olive (Banilas et al., 2011). Further studies that determine the localization, regulation, and specificity of DGAT enzymes may lead to deeper understanding of their role in TAG synthesis.

2.10.2.2 Acyl-CoA Independent TAG Synthesis

An acyl-CoA independent PDAT enzyme was first identified in yeast and increase in TAG content was noticed with its overexpression (Dahlqvist et al., 2000). While six PDAT like genes were identified in Arabidopsis, only PDAT1 accounted for most of its PDAT activity (Dahlqvist et al., 2000; Stahl et al., 2004). However, disruption or overexpression of PDAT1 did not significantly affect the TAG accumulation in Arabidopsis seeds (Stahl et al., 2004; Mhaske et al., 2005). Nevertheless, when PDAT1 expression was silenced in a dgat1-1$^{-/-}$ mutant background, TAG content in seeds was reduced by 70–80% and both seed and pollen development was disrupted (Zhang et al., 2009). These data suggest that PDAT1 is likely compensating for the loss of DGAT1 and vice versa and that the acyl-CoA-dependent and independent pathways are likely to cooperate in TAG synthesis, in the majority of oil storing tissues of plants. More recent studies with non-seed tissues have also suggested that PDAT may also play an overlapping role with DGATs in accumulating oil. In mesocarp tissues of oil palm, which accumulates up to 90% oil by tissue weight, PDAT1 expression was predominant throughout its development while DGAT2 was noted during the later stages of development (Bourgis et al., 2011). In olive, however, DGAT1 expression was predominant in the early stages and DGAT2 in the later stages of mesocarp development (Banilas et al., 2010). Although both species contain high amount of oil in mesocarp, the acyltransferases that were

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preferentially expressed and their temporal pattern was different, like in oil-rich seeds (Troncoso-Ponce et al., 2011).

As the enzyme catalyzing the terminal and committed step in the acylCoA-dependent synthesis of TAG, DGAT has been proposed to play a key role in determining the carbon flux into TAG. For instance, Stals et al. (1994) observed that the rate of TAG synthesis in rat hepatocytes is controlled by the affinity of DGAT for acyl-CoA. Similarly, a close correlation between DGAT activity and oil accumulation has been reported in oilseed crops such as canola (*Brassica napus*) (Perry et al., 1999). Therefore, increasing our understanding of the molecular mechanisms involved in DGAT catalysis could lead to a development of novel strategies for manipulation of TAG assembly in different organisms. In plants, DGAT is an attractive tool in developing new strategies to produce oils enriched in desirable FAs in oleaginous plant species (Lung and Weselake, 2006; Snyder et al., 2009).

2.11 Genetic engineering approaches for enhanced oil production

Genetic engineering over the years has emerged as powerful tool to complement conventional breeding for improvement of crops. An efficient and reproducible genetic transformation system would provide a valuable tool for unravelling the function of genes in plants wherein candidate gene involved mainly in fatty acid biosynthesis as well as triacylglycerol biosynthesis, regulation of secondary metabolites is considered as breeding target have been identified from whole genome sequencing analysis. Genetic transformation through genetic engineering techniques needs an efficient in vitro regeneration system which will be rapid, reproducible, and applicable to a wide-ranging range of genotypes (Mukharjee et al., 2011). Efficient Genetic transformation procedure are now accessible (**Table 2.5**). These techniques

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help to modify genetically and subsequent in vitro multiplication of cultivars for improved oil quality and composition.

It will also be necessary to determine the relative contributions of different enzymes specialized (**Table 2.5**) for these pathways in the native species and to possibly down-regulate non-productive, competing pathways in seeds of host oilseeds. The integrated approach of engineering transcription factors that up regulate fatty acid synthesis and overexpression of TAG biosynthetic enzymes to sequester the enhanced fatty acid production coupled with downregulation of TAG catabolic enzymes is proving to be an effective strategy for generating substantial levels of oil in leaves of model plants. Successful translation of these strategies in existing biomass crops such as sweet sorghum will likely also require the selection of promoters for transgenes that allow the persistence of accumulated oil through leaf senescence. Future success of metabolic engineering of specialty oil traits will likely rely on more predictability of genetic modifications on fatty acid and oil metabolism in seeds and other target tissues of crop hosts by use of techniques, such as mass spectrometry based lipidomics that was essential for optimizing LCPUFA engineering in *camelina* seeds, as described by Ruiz et al., 2013. Similarly, emerging techniques such as matrix-assisted laser desorption/ionization-mass spectrometry imaging (MALDI-MSI) as applied recently to engineering of oil pathways in *Camelina* seeds (Horn et al., 2013) and tobacco leaves (Vanhercke et al., 2013) are providing insights into spatial heterogeneity of fatty acid compositions in specific lipid classes among cell types in target tissues to enable for more informed metabolic engineering. Eventually, the task of integrating a small number of transgene-derived activities with a much greater number of endogenous metabolic processes still remains an exciting challenge.

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Table 2.5 Enzyme related to Fatty acid synthesis, TAG biosynthesis and breakdown in seeds (Costa et al., 2010)

SN	Symbol	Enzyme
		Fatty Acid Biosynthesis
1	FatA	Acyl-ACP thioesterase A
2	FatB	Acyl-ACP thioesterase B
3	ACC	Acetyl-CoA carboxylase
4	EAR	Enoyl-ACP reductase
5	HAD	Hydroxyacyl-ACP dehydrase
6	KAR	Ketoacyl-ACP reductase
7	KAS I	Ketoacyl-ACP synthase I
8	KAS II	Ketoacyl-ACP synthase II
9	KAS III	Ketoacyl-ACP synthase III
10	MAT	Malonyl-CoA ACP transacyclase
11	FAD2	Oleoyl-ACP desaturase
12	PCH	Palmitoyl-CoA hydrolase
13	SAD	Stearoyl-Acp desaturase
		Fatty Acid Degradation
1	FADA	Acetyl-CoA acyltransferase
2	ATOB	Acetyl-CoA C-acyltransferase
3	ADH	Alcohol dehydrogenase
4	ACADM	Acyl-CoA dehydrogenase
5	ACOX	Acyl-CoA oxygenase
6	ALDH3A2	Aldehyde dehydrogenase
7	DCR	Dienoyl CoA reductase
8	PAAG	Enoyl CoA hydratase
9	ACSL	Longchain acyl-CoA synthase
		Triacylglycerol Biosynthesis
1	LAT	Acylglycerol-3-phosphate-O-acyltransferase
2	DGAT	Acyl-CoA:diacylglycerol acyltransferase
3	GPAT	Glycerol-3-phosphate acyltransferase
4	PDAT	Phospholipid :diacylglycerol acyltransferase
5	PP	Phosphotidate phosphatase
6	OLE	Oleosin
		Triacylglycerol Degradation
1	ML	Monoacylglycerol lipase
2	PLAS	Peroxysomal long chain acyl CoA synthetase
3	PFAT	Peroxysomal fatty acid /acyl-CoA transporter
4	TL	Triacylglycerol Lipase

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2.11.1 Overexpression of fatty acid synthesis pathway enzymes

Different plant and different cultivars of the same species have highly variable accumulation and their composition of fatty acids in seeds and vegetative tissues and no considerable differences in their physiology. Manipulation of oil content and composition should not alter the physiology of engineered plants. Plant accumulates oil with different FA compositions, genetics and regulation of FA and TAG biosynthesis have been extensively studied (Snyder et al., 2009; Weselake et al., 2009; Baud and Lepiniec, 2010; Beisson et al., 2010; Lu et al., 2011; Chapman and Ohlrogge, 2012). Overexpression of FA biosynthesis gene will be the possible target to increase oil content and compositions in seeds as well as vegetative part. To develop economically viable oilseed crops with modified fatty acid profiles, there is a requirement to manipulate the gene expression of relevant key enzymes in the modification pathways (**Table 2.6**).

Acetyl-CoA carboxylase (ACCase) catalyses the first committed step of the fatty acid synthetic pathway. It has been overexpressed in different species for enhance oil production. The cytosolic ACC from Arabidopsis was overexpressed in rapeseed with 6% increment of fatty acid and ACC activity was just about 2 fold than the control (Roesler et al., 1997). Decreased level of saturated and polyunsaturated fatty acids while increasing the a mount of monounsaturated fatty acids, like oleic acid and palmitic acid are important goal for biodiesel production (Durret et al., 2008). This approach can be achieved with manipulation of TAG biosynthesis. Reduced content of the saturated fatty acid palmitate in soybean were obtained by the down-regulation of acyl-ACP thioesterase (*FATB*) and accumulation of oleic acid were achieved up to 85% from 18% in the control (Buhr et al., 2002).

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Table 2.6 Modification of fatty acids in oil crops.

Fatty acid modification	Gene engineered	Regulation	References
Palmitic acid	acyl-ACP thioesterase	overexpression	Jones et al., 1995
Lauric acid	acyl-ACP thioesterase	overexpression	Voelker et al., 1992
Caprylic acid	acyl-ACP thioesterase	overexpression	Dahesh et al., 1996
Oleic acid	oleoyl desaturase	down regulation	Stoutjesdijk et al., 2000
Saturated FA	palmitoyl acyl carrier	overexpression	Bondaruk et al., 2007
Stearic acid	Steroyl ACP delta 9 desaturase	down regulation	Liu et al., 2000
Oleic acid	delta 12 fatty acid desaturase	overexpression	Buhr et al., 2002
ϒ Linolenic aacid	delta 6 desaturase	overexpression	Sato et al., 2004
Arachidnic accid	Δ-5 Desaturase, Δ-6 desaturase	overexpression	Chen et al., 2006
Stearidonic acid	Δ-15 desaturase	overexpression	Eckert et al., 2006
Long chain PUFA	Δ-6 Desaturase, Δ-6 elongase,	overexpression	Kajikawa et al., 2008
Increase oil content	diacylglycerol acyltransferase	overexpression	Lardizabal et al., 2008; Li et al., 2010
Improved oil composition	fatty acid omega 3 desaturase	overexpression	Li et al., 2010

A *Jatropha* cDNA encoding FATB homolog *JcFATB* has been recently cloned (Wu et al., 2002). Once CoA is produced by ACC, the FAS transfers the malonyl moiety to acyl carrier protein (ACP) to maintain the carbon flux for the synthesis of long chain fatty acids, likely 16:0 and 18:0. C2 addition in each cycle of reaction is catalysed by 3-ketoacyl-ACP synthase (KAS) and participates in condaensation process of malonyl-ACP with acyl acceptor (Jaworski et al., 1989; Dahesh et al., 2001). A group of KAS family (KASI, KASII, KASII and KASIV) catalyses the further condensation reaction (Dahesh et al., 1998). Observations indicates the KASI catalyses the majority of condensations using acyl-ACP up to 14:0, whereas KASII functions primarily in steric acid synthesis and active 14:0 – 16:0 substrates (Shimakata and Stumpf, 1982). Overexpression of KASII enzymes resulted in an increased level of 16:0 fatty acids (30%) in tobacco leaves indicates the role of this enzyme in fatty acid biosynthesis (Dahesh et al., 2001).

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2.11.2 Overexpression of TAG biosynthesis enzymes

The first step in the biosynthesis of membrane phospholipids and storage triacylglycerols is catalyzed by a glycerol-3-phosphate acyltransferase (GPAT). Alteration in carbon flux to TAG biosynthesis by affecting the glycerol 3-phosphate is another substrate in addition to fatty acid in the ER is an alternative way of increasing oil accumulation in plants seeds. Overexpression of the yeast glycerol 3-phosphate dehydrogenase (*ghpd1*) gene in canola seeds increased the lipid content by 40% (Vigeolas et al., 2007).

Transformation of rapeseed with a putative sn-2-acyl-transferase gene from the yeast *saccharomyces cerevisiae* was carried out by Zou et al.,(1997), leading to overexpression of seed lysophosphatidate acyl-transferase (LPAT) activity. This enzyme is involved in TAG formation and its overexpression led to increases from 8% to 48% seed oil content on the seed dry weight basis. However, it was advised that the steady-state level of diacylglycerol could be distressed by an increase of LPAT activity in developing seeds.

In plants, the major seed storage material is TAG located in oil bodies of seeds. DGAT, is a transmembrane enzyme that catalyses the final step of Kennedy pathway for TAG biosynthesis (Kennedy, 1961). It has been proposed to as rate limiting enzyme for accumulation of storage lipid in plants (Perry et al., 1993) due to their committed role in TAG biosynthesis (Stymne et al., 1987; Lu et al., 2002; Kaup et al., 2002). Arabidopsis mutant with DGAT activity indicates the reduced of TAG content, abnormalities in seed development and alteration in fatty acid compositions therefore decreased storage lipid content (Katavic et al., 1995; Zou et al., 1999; Routaboul et al., 1999), however over expression of DGAT in Arabidopsis, shows increased lipid deposition in seed and enhanced seed weight by 10-70% (Jako et al., 2001). Two classes of membrane bound DGAT designated as DGAT1 and DGAT2

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in plants have been identified (Adelsberger, 2004; Cases et al., 2001; Shockey et al., 2006) and DGAT encoding genes from several plant have been cloned and characterized for their role for TAG biosynthesis (Hobbs et al., 1999; He et al., 2004; Kroon et al., 2006; Manas-Fernandez et al., 2009). Acyl-CoA: diacylglycerol acyltransferases (DGATs) functionally characterized in recombinant organisms which are summarized in **table 2.7**. DGAT1 transcripts are detected in most plant tissues such as roots, leaves, stems, petals, flowers, anthers, developing siliques, young seedlings and germinating seeds (Hobbs et al., 1999; Zou et al., 1999; Kaup et al., 2002; Lu et al., 2003). Up-regulation of DGAT1 during senescence of Arabidopsis leaves indicates that enhanced level of TAG (Kaup et al., 2002).

Overexpression of fungal DGAT2 enzyme in soybean seeds leads to a 1.5% increase in oil content (Ladizabal et al., 2008). Similar observation was indicated in Arabidopsis by overexpression a DGAT cDNA (Jaco et al., 2001). Over-expression of the Arabidopsis DGAT1gene in tobacco leaves enhanced TAG accumulation (Bouvier-Nave et al., 2001; Nookaraju et al., 2014). In leave TAG accumulation is also reported in TGD1 mutated Arabidopsis which encodes a plastid lipid permease (Xu et al., 2005). TAG biosynthesis and accumulation in plant has been described and noted that accumulation of mono unsaturated FA (18:1) were significantly enhanced while PUFA (18:2, 18:3) is decreased when WR1 and DGAT1 co-expressed in tissues (Vanherche et al., 2013). Upregulation of FA synthesis and TAG biosynthesis genes with synergetic effect of transcription factor has great potential to enhance TAG accumulation in plants.

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Table 2.7 Acyl-CoA:diacylglycerol acyltransferases (DGATs) functionally characterized in recombinant organisms.

Organisms	cDNA	Host species	References
A.thaliana	*DGAT1*	*Spodoptera frugiperda*	Hobbs et al., 1999
		S. cerevisiae	Zou et al., 1999
		B.napus	Weselake et al., 2008
		S. cerevisiae	Bouvier-Nave et al., 2000
		A. thaliana AS11	Jako et al., 2001
		J. curcas	Maravi et al., 2016
B. napus	*DGAT1*	*P. pastoris*	Nykiforuk et al., 2002
		B. napus	Weselake et al., 2008
T. majus	*TmDGAT1*	*B. napus*	Xu et al., 2008
Z. mays	*ZmDGAT1*	*Z. mays*	Zheng et al., 2008
V. galamensis	*VgDGAT1*	*S. cerevisiae*	Yu et al., 2008
V. fordii	*VfDGAT1*	*S. cerevisiae*	Shockey et al., 2006
R. communis	*RcDGAT1*	*S. cerevisiae*	He et al., 2004
T. gondii	*TgDGAT1*	*S. cerevisiae*	Quittnat et al., 2004
N. tobaccum	*NtDGAT1*	*S. cerevisiae*	Bouvier-Nave et al., 2000
M. musculus	*MmDGAT1*	*Spodoptera frugiperda* Sf9	Cases et al., 2001
	EzDGAT1	*S. cerevisiae* H1266	Milcamps et al., 2005
J. curcas	*JcDGAT1*	*A. thaliana*	Misra et al., 2014
H. sapiens	*HsDGAT2*	*S. frugiperda Sf9*	Cases et al., 2001
		S. cerevisiae 12501	Inokoshi et al., 2009
M. musculus	*MmDGAT2*	*S. frugiperda Sf9*	Cases et al., 2001
R. communis	RcDGAT2	*S. cerevisiae*	Kroon et al., 2006
		A. thaliana	Burgal et al., 2008
Vernicia fordii	VfDGAT2	*S. cerevisiae*	Shockey et al., 2006
S. cerevisiae	ScDGAT2	*S. frugiperda*	Lardizabal et al., 2001
U. romanniana	UrDGAT2	*G. max*	Lardizabal et al., 2008
S. pombe	SpDGAT2	*S. pombe*	Zhang et al., 2003

2.11.3 Targeted gene inactivation

TAG degradation is critical to many plants for which lipids rather than carbohydrates are the major seed storage reserves. In the majority of reports suggest that the occurrence of TAG is carbohydrate dependent (Carnac and Benning 2004; Casson and Lindsey, 2006; Tsukogoshi et al., 2007). The breakdown of the seed storage lipid provides carbon skeletons and energy that

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can drive post-germinative plant growth before the plant root system and photosynthetic capacity are fully established (Eastmond 2006, Kim et al., 2013, Schmidt et al., 2013). TAG is further converted in sugar through the TAG lipase, peroxisomal fatty acid β- oxidation (Ramli et al., 2002; Shen and Burger, 2008), the glyoxylate cycle and gluconeogenesis (Graham, 2008). Therefore, it is possible to enhance the TAG production by disruption of a number of gene involved in this process (Graham, 2008). These genes include *COMATOSE (CST),* ACX encoding the acyl Co-A oxidase that catalyses the first step of β-oxidation (Pinfield et al., 2005) and *CGI-58* homologue, disruption of this gene in *Arabidopsis thaliana* resulted in the accumulation of neutral lipid droplets in mature leaves (Christopher et al., 2010). The homeobox transcriptional factor required for leaf and root epidermal cell fates and have an inhibitory effect on seed oil content (Shen et al., 2006).

2.11.4 Transcription factor approach

Transcriptional factor plays important role in regulating seed oil accumulations and offers a further opportunity to engineer oil production in non-oil seed tissues (Slocombe et al., 2009). A number of transcription factors have been identified that play major role in storage oil accumulation in different plant species including LEAFY COTYLEDON1 (*LEC1),* LEAFY COTYLEDON2 (*LEC2)* encodes an NF-YB TF, enhances expression of several lipid synthesis-related genes and increases the lipid content of leaves in *Arabidopsis* (Santos-Mendoza et al., 2005; Mu et al., 2008; Tan et al., 2011). Overexpression of *AtLEC1and* its orthologs in canola (Brassica napus), *BnLEC1and BnL1L*, causes an increased fatty acid up to 20% in transgenic *Arabidopsis* plants. *FUS3* is AP2/B3-like transcriptional factor family protein, *FUS3* specifies cotyledon identity. Regulator of gene expression during late embryogenesis. Full length FUS3 protein binds to the highly conserved RY DNA motif

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[CATGCA(TG)] present in many seed-specific promoters, and the B3 domains of this transcription factor is necessary for the specific interaction with the RY element (Stone et al., 2001; Lee et al., 2003). It involved in the control foliar organ identity in Arabidopsis by regulating the synthesis of two hormones, abscisic acid and gibberellin. FUS3 together with LEC1 positively regulate the abundance of the ABI3 protein in the seed (Wang et al., 2007). WRINKLED1 (*WRI1*) encodes transcription factor of the AP2/ERWEBP class and involved in the control of storage compound biosynthesis in Arabidopsis. Mutant show wrinkled phenotype and 80% reduction of TAG in seeds compared with wild-type plants (Cornac and Benning, 2004, Baud et al., 2007). Transcription factor promoting multiple gene expression in the ACCase and FAS complex to influence FA accumulation. For example, overexpression of *WRI1* upregulated a set of genes involved in FA synthesis in plastids, including BIOTIN CARBOXYL CARRIER PROTEIN 2 (*BCCP2)*, ACYL CARRIER PROTEIN 1 (*ACP1)*, and *KAS1* (Baud *et al*., 2007). Seed storage reserve accumulation has also been reported in non-seed tissues of Arabidopsis mutants disrupted in chromatin remodelling factor CHD3 (*PICLE*) or in both of HIGH LEVEL EXPRESSION SUGAR-I (*HSI2* and *HSL1*) gene whose products repress embryonic traits. Overexpression of two soybean transcription factors in *Arabidopsis*, *Dof4* and *Dof11* increases the total fatty acid and lipid in seed oil (Wang et al., 2007). Both enzymes were involved in fatty acid biosynthesis in Arabidopsis by activating the acetyl CoA carboxylase and long acyl CoA synthase. In Soybean (*Glycin max*) bZIP123 transcription factors possess the transcription activity to promote expression of target genes (Hanson et al., 2008, Ma et al., 2011) is responsible for the expression of sucrose transporter gene (*SUC1* and *SUC5*) and three cell wall invertase genes (*cwINV1*, *cwINV3* and *cwINV6*) by attaching directly to the promoters of these genes (Song et al., 2013). bZIP123 may promotes the flow of carbon to oil through modification of transport machinery in the cell wall with the activation of sugar

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transport but not genes in involved in fatty acid synthesis (Song et al., 2013). Various set of transcription factors activates oil and protein pathway genes. Among all transcription factors that regulate storage genes ABI3, AtbZIP10 AtbZIP25 activate storage protein transcription, WRI1 promotes oil accumulation by promoting the carbon flux through glycolysis (Focks and Benning 1998; Lara et al., 2003; Cernac and Benning 2004).

2.12 DGATs in oilseed engineering

As crude oil resources decline, vegetable oils produced by oil-bearing crops are gaining growing interest as sustainable replacements for petroleum derived chemical feedstocks for industrial applications. Thus, recent oilseed biotechnology has set out to expand the potential of increasing overall oil accumulation and producing desirable FA compositions in the seed oil. Both forward and reverse genetics have proven DGATs to be essential targets for such applications as summarized in Table 2.2. For example, an EMS-induced mutation of *DGAT1* in *A. thaliana* (AS11) resulted in reduced seed oil content and delayed seed maturation (Zou et al., 1999). Similar findings were obtained from another *A. thaliana* mutant ABX45 caused by frame shift mutation of the gene (Routaboul et al., 1999). In addition, overexpression of *DGAT1* orthologues from plant sources including *A. thaliana* (Jako et al., 2001), *B.napus* (Weselake et al., 2008), and *Tropaeolum majus* (Xu et al., 2008) have been successfully used for boosting overall oil production in *A. thaliana* and *B. napus*. Also, Lardizabal et al., (2008) reported that overexpression of *DGAT2* from the fungus *U. ramanniana* also led to an increase of seed oil content in soybean (*Glycine max*), signifying that heterologous expression of fungal *DGAT* orthologues in plants could be also feasible for increasing overall oil production. Plant DGAT2 has been demonstrated to be selective for unusual FAs (Kroon et al., 2006; Shockey et al., 2006), providing important tools for producing industrially useful oils by coexpression

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of *DGAT2* and an enzyme responsible for the synthesis of an unusual FA from the same species in a transgenic host (Shockey et al., 2006).

This hypothesis was subsequently confirmed in a study by Burgal et al., (2008) in which coexpression of FA hydroxylase (*FAH12*) and *DGAT2* from castor bean doubled the hydroxy FA content of the oil produced in transgenic *A. thaliana* compared to expression of *FAH12* alone. Recently, Mavraganis et al., (2010) eported coexpression in yeast of *FAH* and ricinoleoyl-specific *DGAT2* from *C. purpurea*. As expected, their coexpression in yeast led to an elevated level of ricinoleic acid in the oil compared to expression of *FAH* alone. This finding suggests that fungal *DGAT2*s also represent attractive gene candidates for producing value-added novel oils in crops.

It is noteworthy that *DGAT1* could also play a role in channelling certain FAs into TAG. In *Arabidopsis*, knock out of *DGAT1* has been shown to affect FA composition resulting a reduction in monounsaturated FAs (eg., 18:1 and 20:1) and enrichment in the polyunsaturated FA, α-18:3 (Katavic et al., 1995; Zou et al., 1999). This phenotype was reversed by restoring DGAT1 activity (Jako et al., 2001). Further understanding of the mechanism by which DGAT1 is involved in channelling these FAs into TAG fatty acids will likely provide additional insight into the malleability of TAG biosynthesis processes in oilseeds. Overexpression of *DGAT* orthologues in transgenic plants has been successfully used to increase seed oil production with concomitant production of desirable FAs. In the longer term, approaches to enhance the overall catalytic efficiency and modulate substrate selectivity of DGATs are necessary to further raise the level of desired FA in the seed oil. Currently, molecular genetic approaches, such as SDM and directed evolution, are being explored as tools for these types of studies (Siloto et al., 2009b; Xu et al., 2008). Recently, full length cDNA of *JcDGAT1*, a key enzyme involved in

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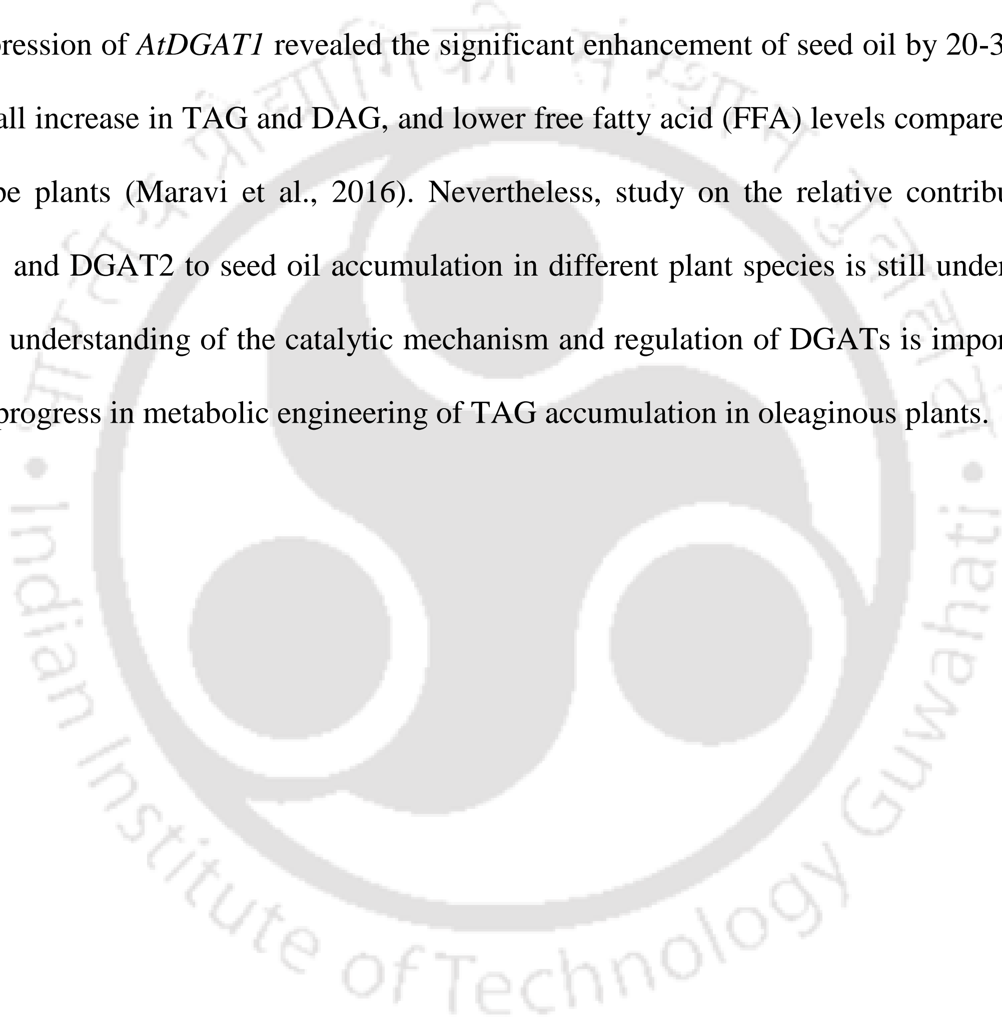
oil biosynthesis, from Jatropha has been cloned in Arabidopsis (Misra et al., 2014). Transcription analysis of *JcDGAT1* reveals a gradual increase from early seed development to its maturation. Homozygous transgenic Arabidopsis lines expressing *JcDGAT1* both under CaMV35S promoter and a seed specific promoter showed enhanced level of total oil content by 30-41% in seeds without any phenotypic differences (Misra et al., 2014). Similarly, we have cloned DGAT1 from *Arabidopsis* into Jatropha under the CaMV35S promoter. Ectopic overexpression of *AtDGAT1* revealed the significant enhancement of seed oil by 20-30% and an overall increase in TAG and DAG, and lower free fatty acid (FFA) levels compared to the wild-type plants (Maravi et al., 2016). Nevertheless, study on the relative contribution of DGAT1 and DGAT2 to seed oil accumulation in different plant species is still underway. A detailed understanding of the catalytic mechanism and regulation of DGATs is important for further progress in metabolic engineering of TAG accumulation in oleaginous plants.



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2.13 Plant metabolic engineering

Plant metabolomics is a particularly interesting field of research not least because plants have the ability to synthesize an enormous diversity of secondary metabolites; current estimation of the number of different compounds present in plant kingdom is 200.000 or higher. Behind the diversity of plant chemicals is the fact that plants are, among all living organisms on earth, unique in several aspects; they are sessile autotrophs that are obliged to respond chemically to survive in continually changing and often hostile environment, as well as to allure pollinators and seed dispersals for their reproduction (Saito et al., 2006; Hall, 2006). Further interest in plant metabolomics arises from the fact that several important attributes significant for human health also originate from plant metabolites, such as food quality (e.g., carotenoid content; Fraser and Bramley, 2006) and drug production (e.g., taxol-producing suspension cultures; Ketchum and Croteau, 2006).

Metabolomics is currently one of the fastest growing areas in plant science, and is most likely also the field where major scientific breakthroughs remain to be made. Perspective to this can be drawn from the fact that even though *Arabidopsis thaliana* genome has been completely sequenced, over 30% of its genes still lack functional classification, and metabolomics is expected to play a key role in the elucidation of the whole molecular phenotype (Fiehn, 2002). Plant kingdom still holds unexplored resources, which can be revealed by “bioprospecting” or pharmaceutically active compounds from wild endemic species. In order to be able to exploit the natural resources efficiently but soundly, a comprehensive picture of plant metabolism is needed, implying a better coverage of pant metabolome as well as a better understanding of its function; these are tasks where metabolomics plays an essential role (Fernie, 2007).

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Particular lipid biosynthetic pathways with fixed end product fluxes are not always enough for dietary or industrial fulfilments. The identification of such pathways and isolation of the enzyme coding genes to overexpress them not at the cost of other pathways, in order to derive a genetically modified product with desired phenotype, is called metabolic engineering. The designing of genetically modified transgenic plants has boosted the opportunities to produce industrially useful fatty acids (FAs) in oilseed crops.

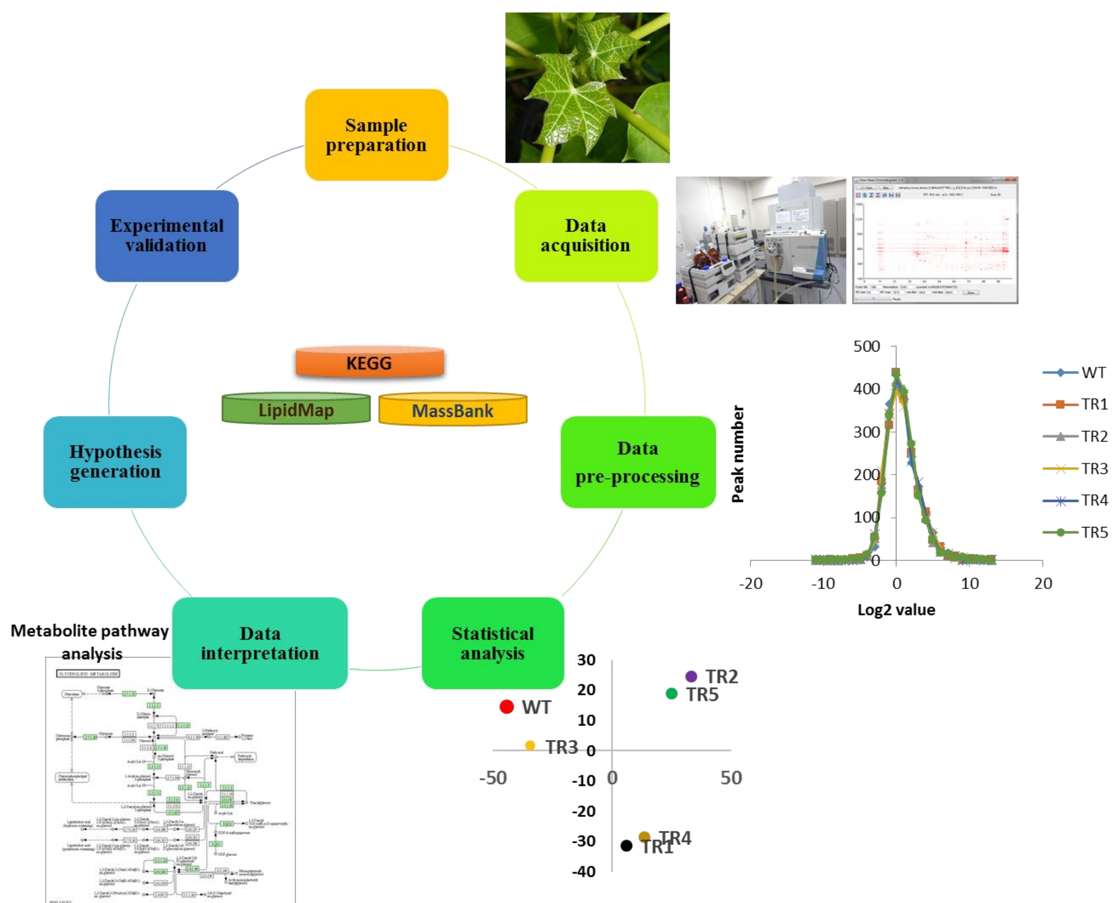

Figure 2.7 Schematic representation of outline of metabolomics study.

A transgenic plant generally consists of a heterologous polynucleotide sequence usually attained by genetic engineering. This has been posed as the central role of green chemistry within the context of lipid metabolic engineering in plants. Scientists have identified several

genes encoding suitable FA-modifying enzymes from available wild species (Table). Still the major limiting factor in the improvement in this field has been the low yields of the desired products in plants. Major constituents of plant oils are unsaturated FAs. Corn oil contains 86% unsaturated FAs and 14% saturated FAs. A recent experiment produced an insufficient 17% of hydroxyl fatty acids (HFAs), when the *Ricinus communis* fatty acid hydroxylase 12 (FAH12) was expressed in Arabidopsis (Burgal et al., 2008).

There are two major approaches in metabolomics, targeted and untargeted. The attempt to measure as many metabolites as possible is defined as untargeted analysis and the selection and accurate quantification of a subset of metabolites of interest is called the targeted approach. There are advantages and disadvantages for both approaches. Thousands of metabolites can now be analysed simultaneously by using LC-MS (Callahan et al., 2009). The resulting data matrices are highly multidimensional and require sophisticated statistical and multivariate analysis techniques to make comparisons between stages and identify the differences and similarities between those stages. One difficulty in the interpretation of data resulting from untargeted analysis is the sheer number of peaks determined that cannot be related to a particular chemical structure, and therefore cannot be related to a particular biochemical pathway. The major advantage of targeted analysis, on the other hand, is that the user knows what is being measured which gives better interpretable results in a biological context. The actual concentrations of the metabolites targeted are determined, which may indicate importance and function of the metabolites in a rather biochemical context. Targeted analysis is generally characterized by a much higher accuracy, greater selectivity and, most important, a greater sensitivity compared to untargeted analysis (Kitteringham et al., 2009; Wei and Seymour, 2010) since the metabolites of interest are separated from the crude extract using specialized extraction procedures or via specific targeting in the mass spectrometer. In metabolomics, the technique

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is very comparable, the molecular ion of compound is first selected and further fragmented. One specific fragment is then selected monitored and the number of both the mother and fragment ion is counted. When compared to standard calibration curve it is then possible to estimate the concentration of the compound in the extract. Using this physical instrumentation development thus far have allowed the quantification of several metabolites simultaneously. However, the greatest limitation is the availability of authentic standards required for the establishment of calibration curves.

2.13.1 Analytical technologies in plant metabolomics

2.13.1.1 Sample preparation

Delicate sample preparation is the most important step in metabolite analysis. Sample types encountered in plant metabolite research include versatile types of tissues extending from soft fruit to rigid root material.,Like the sample type, also the compounds to be analysed and the analytical tools of choice determine the extraction method and the solvent to be used, and need to be adapted on a case-by-case basis (Seger and Sturm, 2007). Factors having the most significant effect on metabolite profiling with gas-chromatography mass-spectrometry (GC-MS) were evaluated on sample preparation of potato tubers (Shepherd et al., 2007). In addition to reaction conditions used to prepare the GC-MS-detectable derivatives, also sampling methods had effect on the results. The use of freeze-dried material gave highly linear and repeatable results

2.13.1.2 Analytical platforms

A number of analytical platforms have to be used in a complementary manner to measure the huge chemical diversity. The most common metabolite detection technologies, often referred as “work horses” are mass spectrometry (MS) and NMR. Recent advances made in the tools

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applied for metabolite analysis has paved the way for simultaneous monitoring of multitude of targets and enabled the development of the metabolomics concept. Sufficient number of detailed reviews on various aspects of metabolite analysis is available (Seger and Sturm, 2007; Dettmer et al., 2007; Glinski and Weckwerth, 2006; Dunn et al., 2005; Villas-Boas et al., 2005). Complex metabolite mixtures extracted from biological fluids and tissues often requires a separation step before detection particularly when MS detection system is used. Gas chromatography (GC), liquid chromatography (LC) and capillary electrophoresis (CE) are the most common separation techniques coupled with MS. Different mass-spectrometric (MS) technologies are currently the key detection methods for small molecules (Dunn et al., 2005). Critical instrumental parameters are 1) the ionization technology, i.e., electron ionization (EI), chemical ionization (CI), electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), matrix-assisted laser desorption/ionization (MALDI) or fast atom/ion bombardment (FAB); and 2) the type of the mass analyzer, i.e. quadrupole, triple-quadrupole, ion-trap or time of flight (TOF) (Villas-Boas et al., 2005). The most common analyser and their modes of ion separation are listed in table 8. The samples can be analysed directly by MS or may be separated by different chromatographic techniques; the choice of method depends on the metabolites of interest. GC-MS has the longest history in metabolite detection, providing the advantage of well-established protocols and instrumental set-up as well as chromatogram evaluation and interpretation (Fernie et al., 2004). GC-MS has a very high separation efficiency that can resolve complex biological volatile mixtures. Sample preparation for GC-MS may involve solvent-free preparation by, e.g., headspace sampling, or requires volatile solvent and derivatization techniques to increase analyte volatility (Seger and Sturm, 2007; Dettmer et al., 2007). Liquid chromatography coupled MS (LC-MS) allows the separation and detection of different groups of metabolites according to the column and eluent applied, and thus enables

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to study versatile metabolite classes in single analysis. When accompanied by targeted purification and chromatographic optimization it is suitable for accurate measurement and classification of a particular metabolite class present even in a very complex matrix (Dunn et al., 2005). The column technology for the liquid phase separation is also rapidly evolving, and the performance of the columns is moving from the high (HPLC) to ultra-high (UPLC) level. The advantage of UPLC is the enhanced retention time reproducibility and better separation performance accompanied with faster chromatographic runs.

Capillary-electrophoresis-mass spectrometry (CE-MS) is a highly sensitive method that can detect low abundance metabolites with good analyte separation, and potentially will be applied in metabolomics studies in the future; until now the research with CE-MS has focused on the optimization of operational parameters (Villas Boas et al., 2007; Dunn et al., 2005). Fourier-transform-ion-cyclotron resonance-MS (FTICR-MS) is a mass spectrometric method that relies solely on extremely high mass accuracy detection, allowing the determination of empirical formulae for thousands of metabolites. The pitfall of the method is, however, the lack of chromatographic separation, which renders it incapable of discriminating between metabolite isomers (Fernie et al., 2004). Nuclear magnetic-resonance spectroscopy (NMR) is one of the most widely applied methods to study organic molecules, and has been used for over 20 years for metabolomics research. It is a non-destructive method and offers a variety of detection schemes, being highly versatile and adjustable technique for any chemical analysis. NMR is the ultimate tool to unravel the identity of a compound (Ward et al., 2007; Krishnan et al., 2005; Dunn et al., 2005).

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Table 2.8 List of common mass analyser, their principle of mass separation and detection efficiency of mass resolution

Analyzers	Symbol	Principle of mass separation	Resolution
Nuclear magnetic resonance	NMR	magnetic field	Low
Quadruple	Q	m/z (trajectory stability)	Low
Ion trap	IT	m/z (resonance frequency)	Low
Time of flight	TOF	Velocity (flight time)	High
Fourier transform ion cyclotron resonance	FTICR	m/z (resonance frequency)	Very high
Fourier transform orbitrap	FT-OT	m/z (resonance frequency)	Very high

In spite of the extensive development of analytical methods for metabolome research, the ultimate goal of plant metabolomics, i.e. gaining a complete overview of the metabolite complement of a plant in one or small series of analyses is currently inconceivable. Sample preparation technologies and analytical tools do not yet meet the demands, but continuous improvement in the technologies, including combined platforms of NMR and GC or LC-MS, should bring further advances in the field (Hall, 2006; Ryan and Robards, 2006).

2.13.1.3 Data processing

Multiple experimental platforms applied in metabolomics research generate complex data matrices which involve laborious handling and have great impact in the extent and quality of interpretation of the analytical results (Katajamaa and Oresic, 2007). A typical LC-MS raw dataset contains information about recorded histograms from the mass detector, including retention time, *m/z* value and ion intensity for each signal.,Several processing steps such as filtering, feature detection, alignment and normalization need to be applied to purify and condense the data so that they are accessible for data mining tools and multivariate analyses such as clustering, or for discovering important differences between groups of samples.

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Bioinformatics and biostatistics are among the most essential disciplines in metabolome research, and automated computational techniques and algorithms are currently being developed to aid in metabolite identification, quantification and differential profiling. Furthermore, as increasing amounts of data accumulate, there is a need for standards in order to keep the data accessible and comparable between the laboratories. A metabolomics standards initiative has been taken by the metabolomics society, and minimal reporting standards have been recently proposed on metabolomics data (Fiehn et al., 2007). The accumulating information on plant metabolomics also necessitates the organization of metabolic information into currently accessible databases, and initiatives are taken both by individual laboratories as well as broader scientific community, including the KEGG (Kanehisa et al., 2002), KNApSAcK (Shinbo et al., 2006) and MoTo databases (Moco et al., 2006).

2.13.2 Metabolomics in the context of transgenic plants

Targeted analysis is usually the method of choice in the characterization of transgenic plants, as the direct effect of the transgene is studied at the metabolite, RNA and/or protein level. Application of metabolomics in the context of genetically modified crops analysis are listed in table 9. A targeted metabolite analysis was carried out on transgenic potato tubers that had been altered in primary carbohydrate metabolism, polyamine biosynthesis and glycoprotein processing (Shepherd et al., 2006). No consistent differences were revealed between GM and parental lines, when soluble carbohydrates, glycoalkaloids, vitamin C, total nitrogen, fatty acids and trypsin inhibitor were assayed. Results on non-targeted metabolite profiling of GM plants are also emerging. Roessner et al., (2001) showed that metabolic profiling may be used together with other profiling techniques for genotyping of GM potato lines altered in sucrose metabolism. Defernez and co-workers (2004) described metabolite profiles of 40 GM potato

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lines modified in primary carbon metabolism, starch synthesis, glycoprotein processing or polyamine/ethylene metabolism. The main differences in NMR and HPLC-UV profiles between the GM and parental varieties were found in polyamine metabolism. Metabolite profiles of GM and parental wheat cultivars were analyzed by NMR and GC-MS in a study where substantial equivalence was determined (Baker et al., 2006). Metabolic changes in spermidine and spermine accumulating GM tomato line have been studied in extensive metabolite profiling by NMR (Mattoo et al., 2006). Also flavonoid enriched GM-tomatoes have been studied by LC/NMR and LC/MS metabolite profiling, revealing 10-fold increase in flavonoid glycosides in two GM lines (Le Gall et al., 2003).

Metabolite profiling may elucidate also differences in metabolome beyond those intended to be achieved by incorporation of the transgene, e.g. those resulting from integration site or somaclonal variation that takes place during the *in vitro*-regeneration process (Filipecky and Malepszy, 2006). Such variation has been demonstrated e.g. on potato tubers, as differences in individual compounds between parental and vector-control, as well as between non-transformed cell-culture-derived lines were found (Shepherd et al., 2006). Similarly, NMR metabolite profiling study of GM tomatoes revealed significant changes other than those anticipated from the gene transfer, although the changes were determined to be within natural variation of the field-grown crop (Le Gall et al., 2003). In addition to the integration site-specific changes and somaclonal variation, genetic modification often results in pleiotropic effects that limit the usefulness of the technology, and are encountered especially when secondary metabolism is modified (Filipecki & Malepszy, 2006). Pleiotropic effects shown so far include, e.g., silencing of the flavonoid pathway eventually leading to male sterility (Napoli et al., 1990, Van der Meer et al., 1992).

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Metabolite profiling can, however, facilitate the interpretation of the effects of the genetic modification, as has been demonstrated, e.g., in GM potato profiling (Defernez et al., 2004), in NMR analysis of GM peas (Charlton et al., 2004). One of the reasons for unexpected effects is that the modified pathways are still insufficiently characterised, which does not support predictive metabolic engineering (Sweeltove et al., 2003; Trethewey, 2004). Metabolite profiling has even been suggested as an integral part of the assessment of the safety of foods derived from GM crops (Kuiper et al., 2001) in addition to the extensive test regime which includes careful monitoring of the key nutrients, toxins, allergens, anti-nutrients and biologically active substances known to be associated with the crop (Konig et al., 2004). Several interesting modifications of lipid metabolism in transgenic plants have been performed in the last decade. These modifications led to the production of important oil products which possess a large share in the market even today. Medium chain lauric acid (12:0) is overproduced in coconut and palm-kernel to be used in soaps, detergents and surfactants. Conversion of 24% of total FA to laurate was facilitated when the *Umbellularia californica* lauryl-ACP thioesterase was expressed in Arabidopsis. The same thioesterase expressed in rapeseeds showed conversion of 58% of total FA to laureate. Cuphea hookeriana FatB1 thioesterase co-expressed with LPAT of Cocos nucifera in Brassica napus converted 67% of total FA to laurate (Yu et al., 2011).

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Table 2.9 Metabolomics study on genetically modified crops with respective phenotypes.

GM Crop	Tissue	Donor Species	Gentic Modification	Phenotype	Analytical Technique	Reference
Rice	seed	BT	Cry1Ab	Insect resistance	FTIR-MS, NMR	Keymanesh et al., 2009
	leaf	Z. mays	C1, R-S	Flavonoid production	LC-ESI-Q MS,	Shin et al., 2006
	leaf, seed, root	O. sativa	YK1	Stress tolerance	CE-ESI-Q MS	Takahashi et al., 2006
	seed	N. tobacum	ASA2	Nutritionally enahnced	LC-ESI-Q MS	Matsuda et al., 2010
	leaf, seed	E. coli	LysC, dapA	Nutritionally enahnced	LC-FTIR MS	Long et al., 2013
	seed	A. tumefaciens	Bar	Herbicide resistence	GC-EI-TOF MS	Kim et al., 2013
Maize	grain	BT	Cry1Ab	Insect resistance	NMR	Manetti et al., 2004
	grain	BT	Cry1Ab	Insect resistance	CE-ESI-TOF MS	Levandi et al., 2008
	grain	A. tumefaciens	CP4 EPSPS	Herbicide resistence	GC-EI-Q MS	Frank et al., 2012
	grain	Z. mays	mod. (Rpd3)	seed development	NMR	Piccioni et al., 2009
Soybean	seed	AT	CP4 EPSPS	Herbicide resistence	GC-EI-Q MS	Bernal et al., 2008
	seed	Agrobacterium	837ASDIS	Herbicide resistence	GC-EI-Q MS	Jimenez et al., 2009
	leaf, seed	N. tobacum	ASA2	Nutritionally enahnced	GC-EI-Q MS	Inaba et al., 2007
	seed	Avena spp	HPPD	Herbicide resistence	LC-ESI-Q MS,	Clarke et al., 2013
Alfalfa	stem	N. tobacum	PAL2	Nutritionally enahnced	LC-UV	Chen et al., 2003
	leaf	N. tobacum	PAL2	Nutritionally enahnced	LC-UV	Chen et al., 2003
Pea	leaf	S. hygroscopicus	bar	Herbicide resistence	NMR	Charlton et al., 2004
Wheat	leaf	U.maydis	Chit/Gluc, RIP, KP4	Antifungal	LC-ESI-Q MS	Shepherd et al., 2006
	seed	T. aestivum	gluc-A1, Gluc-D1	Nutritionally enahnced	NMR	Baker et al., 2006
Potato	leaf	S. cerevisiae	TPS1	Drought resistance	GC-EI-Q MS	Kondrak et al., 2012
	tuber	A. Thaliana	DREB1A	Stress tolerance	GC-EI-TOF MS,	Iwaki et al., 2013
	tuber	A.thaliana	Potato virus	Virus resistance	GE-ESI-IT-MS/MS	Bianco et al., 2003
	tuber	C.scolymus	1-SST, 1-FFT	Insulin synthesis	GC-EI-TOF MS,	Catchpole et al., 2005

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Tomato	leaf	A.thaliana	AtNHX1	cabohydrate metabolism	GC-EI-Q MS	Roessner et al., 2003
	fruit	Z. mays	LC1, C1	Increased flavonol content	NMR	Le Gall et al., 2003
	fruit	V. vinfera	Stilbense synthase	Reseratrol synthesis	LC-ESI-Q MS	Long et al., 2006
	fruit	R.dulcifica	miraculin	sweet flavor	GC-EI-TOF MS,	Kusano et al., 2011
Tobacco	leaf	E. coli	Ent/CpmsB	Salicylic acid producing plants	NMR	Choi et al., 2004
	leaf	A. rhizogenes	GR receptor and rol C	-	HPLC-HRMS	Scalabrin et al., 2015
Lettuce	leaf	E. coli	Asn A	Growth enhanced	NMR, GC-FID	Sobolev et al., 2010
	seed	A. thaliana	KNAT1		NMR	Sobolev et al., 2010
Tarley	seed	B. amyloliquefaciens	GluB, ChGP	Antifungal activity	LC-ESI-IT MS	Kogel et al., 2010
Grapevine	leaf	E. coli	Adh	abiotic stress	GC-EI-Q MS,	Tesniere et al., 2006
Grapevine	Fruit	A. majus	iaaM	indole-3-acetic acid	NMR	Picone et al., 2016
Strawberry	Flower			Phenolic cmpounds	UPLC-qTOF MS	Hanhineva et al., 2008

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The hydroxylipid, ricinoleic acid extracted from castor bean is used in coatings and lubricants. Trienoic lipid, linolenic acid overproduced in flax has been used in paints and varnishes. Vernolic acid has remained a low cost oil of high industrial importance. This is due to the possible conversion of this FA into polymers like PVC (polyvinyl chloride), paints, terpenes and lubricants. This epoxy lipid extracted from transgenic epoxidized soybean oil, is used as plasticizers.

2.13.3 Metabolic engineering of TAG as a potential source of biodiesel

Genetic modifications and designing of transgenic crops have opened the possibility of oil production in vegetative tissues in order to squeeze out the maximum energy from plant biomass. Reports have shown the capability of certain tissues to produce TAGs and lipid droplets which have been spotted in the cytoplasm of mesophyll cells in leaves (Lin et al., 2008). High accumulation of TAGs is found in senescent leaves experiencing stress. Still, the vegetative tissues have a low content of TAGs (Kelly et al., 2013; Vanhercke et al., 2013). Metabolic engineering can increase TAG accumulation. This has been attained by the ectopic expression of biosynthetic enzymes like acyl-CoA: diacylglycerol acyltransferase (DGAT) or monoacylglycerol acyltransferase (MGAT); genes such as LEAFY COTYLEDON1 (LEC1), LEC2 or WRINKLED1 (WR1) controlling seed development and maturation (Andrianov et al., 2010; Sanjaya et al., 2013). The TAG content can also be increased by mutating genes involved in FA turnover, like COMATOSE2 (CTS2), SUGAR DEPENDENT1 (SPD1) or COMPARATIVE GENE IDENTIFICATION-58 (CGI58) (Napier et al., 2014; Slocombe et al., 2009; James et al., 2010). Increased levels of storage lipids in leaf biomass can be achieved by re-orienting the carbon flux into TAG, while overexpressing LEC2 in the cts2 β-oxidation mutant (Vanhercke et al., 2014). Non-seed proteins which possess the inherent ability to bind

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and stabilize lipid-rich particles in the cytosol are becoming new targets of metabolic engineering (Vanhercke et al., 2014).

2.14 Conclusion and future prospective

A number of options are now available as potential sources of renewable bioenergy including a variety of routes to the production of biodiesel, bioethanol and biogas. Apart from the Brazilian model of bioethanol production, only biodiesel is applicable and cost effective over the short to medium term. For biodiesel the non-edible oilseed plants are favoured due to questions of whether respectable agricultural land should be used for food or fuel. As a non-edible oil crop Jatropha is a valuable multipurpose plant to alleviate soil degradation, deforestation and for bio-diesel production besides valuable by-products and environmental protection. Jatropha biodiesel is found applicable and cost-effective over available sources of renewable bioenergy including a variety of routes to the production of biodiesel. However, the unsubstantiated claims and known facts must be balanced to have an objective view on the chances of this plant delivering its perceived potential (Jongschaap et al., 2007). Evaluation of Jatropha accessions in terms of their agronomic performance and yield suggests that the optimization of simple agronomic practices and breeding can help to deliver quantifiable increases in yields. However, conventional plant breeding approaches are lack behind due to the unavailability of systematic survey on the genetic variability Jatropha accessions. Therefore, for improvement of this species widening the genetic base through the introduction of accessions with broad geographical background and assessment of fuel properties and selection of elite genotypes and application hybridization is necessary (Johnson et al.,2011; Mukherjee et al.,2011). However, to understand the full potential of Jatropha as a bioenergy crop requires the development of elite lines showing enhanced TAG accumulation with

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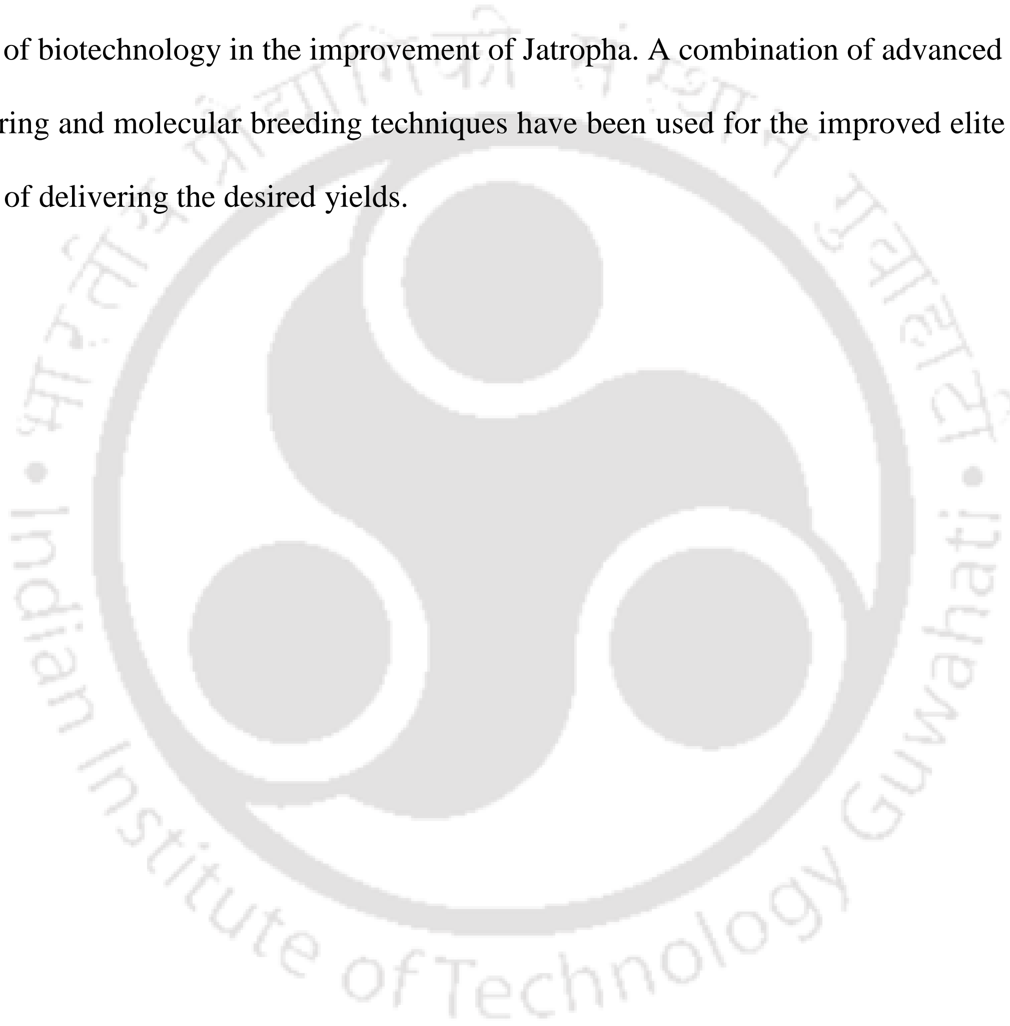
improved fatty acid composition. Thus, utilizing the genetic engineering tools present to manipulate the adaptive responses in Arabidopsis, a model system can be generated out of it to study seed oil evolution and the breeding of bioenergy crops such as Jatropha. Recently, a number of transformation protocols for gene delivery have been developed and optimized (Li et al.,2008; Purkayastha et al., 2010; Pan et al.,2010; Kumar et al., 2010; Mazumdar et al.,2011, Maravi et al., 2014). Recently, Sujatha et al., (2008) and Maghuly and Laimer, (2013) reviewed the role of biotechnology in the improvement of Jatropha. A combination of advanced genetic engineering and molecular breeding techniques have been used for the improved elite variety capable of delivering the desired yields.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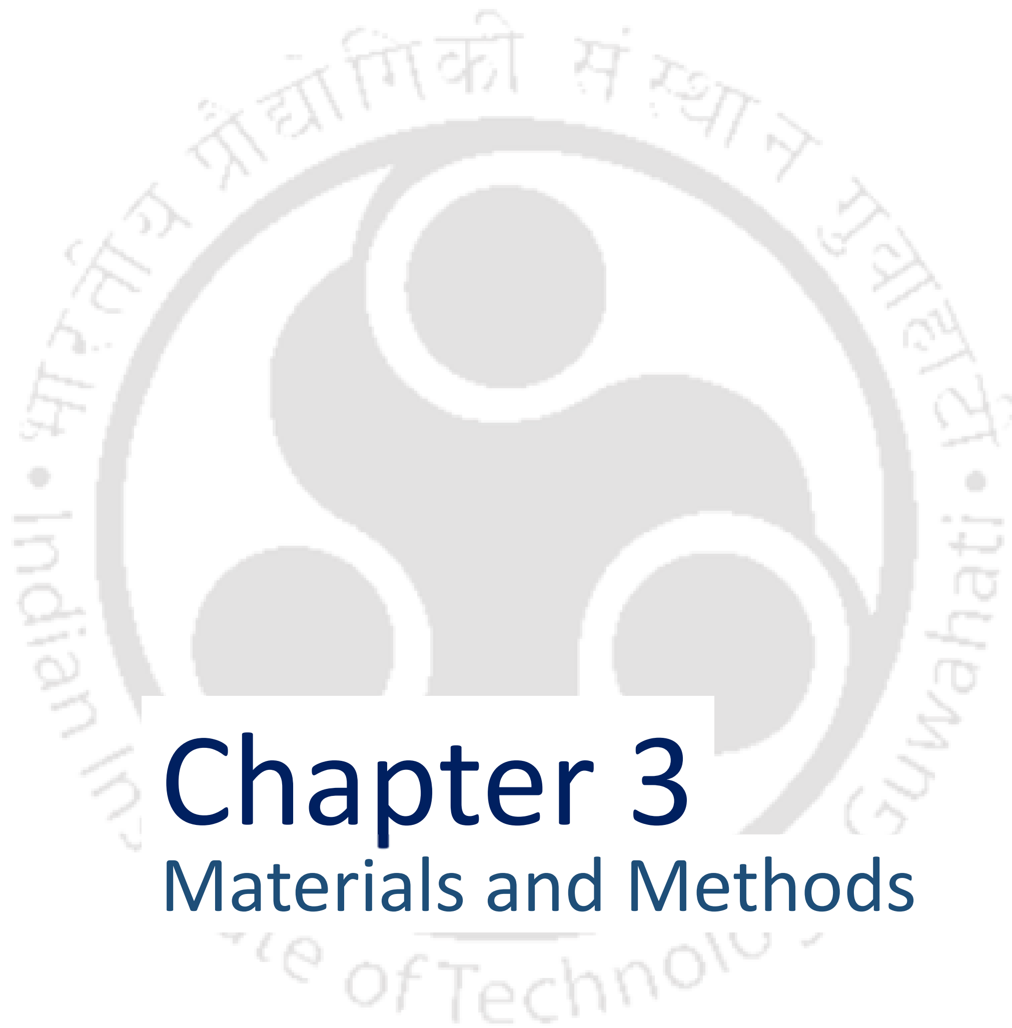

Chapter 3

Materials and Methods

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3.1 Construction of *DGAT1* expression vector

The cDNA encoding the full length *Arabidopsis thaliana* DGAT1 (Gene Bank: AF051849.1) was amplified with primers that contain *XbaI* (GCA TCT AGA ATG GCG ATT TTG GAT TC) and *BamHI* (GCA GGA TCC TGA CAT CGA TCC TTT TC) restriction sites and cloned in the pTZ57R/T vector. Cloning was confirmed by PCR amplification of *AtDGAT1* with *XbaI* and *BamHI* restriction sites. The AtDGAT1 insert was released from pTZ57R/T AtDGAT1 and cloned in *XbaI* and *BamHI* restriction sites fusion with GUS gene in plant binary vector pBI121.

3.2 Binary plasmid, *Agrobacterium* strain and culture conditions

The binary plasmid pBI12135S::*AtDGAT1* containing *AtDGAT1* cDNA driven by cauliflower mosaic virus (CaMV) 35S promoter **(Figure 4.1G)** was mobilized into the disarmed hypervirulent *Agrobacterium tumefaciens* strain EHA105 and used for transformation experiments. The T-DNA of pBI121 includes neomycin phosphotransferase gene (*npt*II) and β-glucuronidase gene (*gus*), both driven by the CaMV35S promoter. The *A. tumefaciens* strain harboring pBI12135S::*AtDGAT1* was maintained on solid LB medium supplemented with 50 mg/l of kanamycin and 10 mg/l of rifampicin. Single bacterial colony was inoculated into 25 ml of liquid AB minimal medium (Chilton et al 1974) with appropriate antibiotics and grown overnight at 28 ^{0}C on a rotary shaker at 180 rpm, until optical density at 600 nm reached to 0.8. The cells were collected by centrifuging at 5,000 rpm for 5 min, and then the pellet was resuspended in liquid co-cultivation medium, LCM (MS medium containing 6.66 µM of BAP and 0.24 µM of IBA, pH adjusted to 5.7) supplemented with 100 µM acetosyringone and used for inoculation.

3.3 Plant material and explant preparation

Seeds of *J. curcas* elite clones (Jc-19) were decoated and soaked in distilled water overnight at room temperature. The soaked decoated seeds were treated with a 0.1% sodium hypochlorite

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solution containing few drops of Tween-20, for 10 min followed by washing under tap water for 20 min. The decoated seeds were then surface sterilized with 70% alcohol for 5 min followed by with 0.2% mercuric chloride for 10 min, and finally rinsed four times with sterile double distilled water. After blot-dried on sterile filter paper, the endosperm was carefully dissected out to expose embryos with papery cotyledonary leaves. The papery cotylodonary leaves were germinated on MS basal medium. The cotyledonary leaves were separated out and cut into four segments (10 mm^2) with the edges removed and used as explants for *Agrobacterium*-mediated transformation experiments.

3.4 Plant transformation and recovery of transformants

Transformation of Jatropha and generation of the primary transformants were accomplished by an improved transformation procedure (Mazumdar et al., 2010). The juvenile cotyledonary leaf segment explants were inoculated in bacterial suspension at 25 ^{0}C for 30 min by occasional shaking in dark. The explants were then blotted on sterile filter paper and co-cultivated for 3 days under dark condition at 25 ^{0}C, in petri-dishes lined with filter paper moistened with LCM supplemented with 100 µM acetosyringone. After 3 days of co-cultivation, the explants were washed five to six times with sterile distilled water followed by rinsing with sterile distilled water containing 75mg/L meropenem. The explants were then blotted dry on sterile filter paper and transferred to callus induction medium (CI, MS medium supplemented with 6.66 µM BAP and 0.24 µM IBA) containing 500 mg/l cefotaxime and 75 mg/l meropenem) in dark condition for callus induction. The cultures were transferred to fresh callus induction medium at an interval of 5, 7 and 8 days. After 3 weeks of culture, the calli were transferred onto shoot regeneration medium (SR, MS medium supplemented with 6.66 µM BAP, 0.24 µM IBA, 1.44 µM GA3) containing 15 mg/L kanamycin, 500 mg/l cefotaxime, and incubated at 16 h photoperiod. The cultures were periodically transferred onto fresh selection medium. After 4 weeks of culture on selection, the regenerating kanamycin-resistant shoots were detached and

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transferred to shoot elongation medium (SE, MS medium supplemented with 1.0 µM GA3) containing 15 mg/L kanamycin and 400 mg/L cefotaxime. After a week, the elongated shoots were transferred to root induction medium (RI, ½ MS medium supplemented with 0.5µM IBA) and 500 mg/L cefotaxime. Well-rooted transformed plantlets were washed thoroughly in running tap water and acclimatized and maintained in greenhouse as per the procedure described earlier (Mazumdar et al., 2010).

3.5 Molecular analysis

3.5.1 Polymerase Chain Reaction (PCR) analysis

Genomic DNA was isolated from the young leaves of putative transformants and control *Jatropha* plants using the NucleoSpin Plant II Maxi kit (Macherey-Nagel, Duren Germany). PCR amplification was carried out with gene specific primers *AtDGAT1*, *nptII* and *gus* using gDNA from putative plants, non-transformant plants (negative control), and pBI21:*AtDGAT1* (positive control) as templates. The 384 bp region of *AtDGAT1*, 540 bp region for *nptII* and 400 bp region of *gus* were amplified by 20-mers (*AtDGAT1Fw*: TCT GCT GGC GTT ACT ACG GT; *AtDGAT1* Rv: CGG CAT GGC TCT GTT TGA AG, *nptII* Fw: GTG GAG AGG CTA TTC GGC TA; *nptII* Rv: CCA CCA TGA TAT TCG GCA AC, *gus* Fw: GGT GGG AAA GCG CGT TAC AAG; *gus* Rv: TGG ATT CCG GCA TAG TTA AA) oligonucleotide primers. The amplification reaction was carried out under following conditions: 95 ^{0}C for 5 min (1 cycle), 95 ^{0}C for 1 min (denaturation), 58 ^{0}C for 1 min (annealing), 72^{0}C for 1 min (extension) for 35 cycles followed by the final extension at 72 ^{0}C for 5 min (1 cycle). PCR amplified product were resolved by electrophoresis on 1% agarose gel and visualized by 10 mg/ml ethidium bromide staining (Sambrook et al. 1989).

3.5.2 Southern hybridization

Randomly selected PCR-positive T_0 transgenic Jatropha lines were analyzed by Southern hybridization for the integration of transgene. Genomic DNA was isolated from leaves of

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transgenic and control untransformed (UT) plants using the NucleoSpin Plant II Maxi kit (Macherey–Nagel, Duren, Germany). Sixty microgram of genomic DNA was digested with *BamHI* and separated on a 0.8 % agarose gel. The gel was processed and transferred to ZetaProbe nylon positively charged membrane (Bio-Rad, USA) following standard procedures. The blot was hybridized with DIG-labelled 0.54-kb *nptII PCR* amplified product as probe. Hybridization and detection of signals were carried according to the DIG Labelling and detection supplier instructions (Roche Diagnostics, Mannheim, Germany).

3.5.3 RNA isolation and Semi-quantitative RT PCR analysis

The total RNA was isolated from transgenic and wild type plant using RNA extraction kit (NucleoSpin RNA Plant, MN, Germany) and quantified with nanodrop spectrometer (Nanodrop, USA). The cDNA was prepared using 1 µg of total RNA using reverse transcription system kit according to the manufacturer's instructions (ThermoScientific). Semi-quantitative PCR was performed using (1µl) cDNA as template, AtDGAT1 gene as target and JcActin gene as an internal control using AtDGAT1 and *JcActin* specific primers (*AtDGAT1* Fw: GGTGGCGGAGAGTTCGTCGA, *AtDGAT1* Rv: TCTTCCTTCTCCGCCGCCTC and *JcActin* Fw: ATGAGCTTCGAGTTGCAC, *JcActin* Rv: ACCATCACCAGAATCCAG) for amplifying a 249-bp fragment of AtDGAT1 and a 590-bp of *JcActin* as an internal control to indicate the amount of starting RNA. The PCR condition was 95 ^{0}C for 10 min; 30 cycles of 95 ^{0}C for 30 sec, 58 ^{0}C for 30 sec and 72 ^{0}C for 30 sec and a final extension of 72 ^{0}C for 5 min. Semi quantitative PCR were amplified and amplification product were analyzed with 1% agarose gel, detected by ethidium bromide staining and photographed through GelDoc system (Bio-Rad, USA).

3.6 Histochemical *GUS* assay

Histochemical GUS assays were used to assess transient expression of the *gus* gene (Jefferson et al, 1987). Transient *gus* expression in leaf explants was scored after three days of co-

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cultivation and the number of explants showing transient *gus* expression at their edges were scored by immersing tissue materials in GUS substrate solution for 24 h at 37 ^{0}C. Following incubation, tissues were bleached with 100% ethanol and examined under microscope.

3.7 Biochemical Analysis

3.7.1 Estimation of soluble protein

Samples were prepared by grinding 200 mg leaf and seed tissue in 1.5 ml chilled phosphate buffer (0.1 M, pH 7.0) containing 50 mg insoluble PVP. Then centrifuged at 10000 rpm at 4^{0}C for 15 min. The fraction was pooled and the amount of protein was measured according to Bradford (1976) using three technical replications. Ready to use Bradford's reagent (Fermentas) is used for protein estimation. To 0.1 ml of diluted extract, 1.5 ml Bradford's reagent was added. Mixed thoroughly and absorbance was measured at 595 nm after 5 min. Standard curve was prepared using graded concentration of bovine serum albumin (BSA).

3.7.2 Estimation of carbohydrates

Analysis of total soluble carbohydrates was performed by using spectrophotometry according to the phenol-sulphuric acid method (Dubois et al., 1956). The extraction of soluble carbohydrates was done according to Barnett and Naylar (1966). Two hundred mg of fresh samples were homogenized in 80% ethanol (v/v) and the homogenates was placed in boiling water bath for 15 min. After cooling samples were centrifuged at 5000 rpm for 20 min. Supernatant was separated out and residue was further refluxed twice with 80% ethanol. The supernatant was pooled together and volume was made 10 ml with 80% ethanol and used for estimation of soluble carbohydrates. Absorbance was taken at 475 nm, D-glucose was used as standard, and data was expressed as mg carbohydrate g-^{1} tissue fresh weight.

3.7.3 Lipid extraction

Total lipids were extracted according to the method described by Bligh and Dyer (1959). Two hundred milligram of leaves and 500 mg seeds kernel of control and transgenic lines were

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homogenized in mortar-pestle and lipid were extracted from organic phase. Lipid fraction in bottom phase were collected in glass tube and evaporated in rotary evaporator. Total lipids were quantified after dryness in desiccator for 24 h. The weight of the total oil was determined gravimetrically and oil content was recorded as the ratio of lipid/oil to dried leaf sample and seed kernel weight.

3.8 Analysis of TAG by thin layer chromatography

Lipid were fractioned from neutral lipids by thin layer chromatography. On silica gel plate (TLC Silica Gel 60 F254, Merck), 150 µg of extracted lipid was spotted and resolved using solvent system of Hexane: diethyl ether: acetic acid (70:30:1, v/v/v). Triacylglycerol spots were revealed by staining with iodine vapour.

3.9 Fatty acid methyl ester preparation

About 20 mg of lipid was dissolved in methanol in a test tube, and 0.5 M Potassium hydroxide in anhydrous methanol was added with reaction volume of 20 ml. The solution was maintained at 60 ^{0}C for 30 min. The methyl esters were extracted with hexane (2 x 5 mL), and the organic phase was washed twice with MiliQ water to remove any aqueous impurities. Organic phase was collected in screw cap glass tube and solvent was removed in rotary evaporator, after filtration through a 0.2 µm filter, methyl esters were used for NMR and GC analysis.

3.10 ^{1}H-NMR spectroscopy

^{1}H-NMR spectrum of *J. curcas* oil and the fatty acid methyl esters were obtained on Bruker 600 MHz NMR spectrometer, Avance III HD using a 5 mm broad band inverse probe head, equipped with shielded z-gradient accessories. Samples were dissolved in 600 µl deuterated chloroform ($CdCl_3$) and transferred to the 5-mm NMR tube. The spectra were recorded in CDCl3 as the solvent at room temperature, TMS (tetramethylsilane sodium salt) served as internal standard (δ=0.00 ppm). Typical parameters used were: spectral width: 12019.23 Hz;

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time domain data points: 32 K; flip angle: 90°C; relaxation delay: 5 s; spectrum size: 32 K points; and line broadening for exponential window function: 0.3 Hz.

3.11 FAME analysis by Gas chromatography

FAME analysis was performed on Varian 450-GC (Varian Capillary Column CP-SiL8 CB, 30m 0.25mm i.d. 0.25 µm film thickness). FAMEs were separated and detected by flame ionization detector (FID). Nitrogen was used as carrier gas with 0.4ml s^{-1} at constant flow rate. The oven regime: 140 ^{0}C for 5 min, 180 to 240 ^{0}C at 3 ^{0}C/min and hold at 220 ^{0}C for 10 min. The injector and detector temperature was kept at 250 ^{0}C and 1 µl injection volume at split ratio of 1:20 was used for the analysis. Peak were identified based on their retention times compared with a FAME reference mixture (Supelco, Bellenfonte, PA, USA). Fatty acid composition was calculated based on the peak area percentage of total fatty acids.

3.12 Seed weight and size determination

Mature seeds were harvested from untransformed control (UT) and transgenic Jatropha lines grown under the same conditions. The seeds were then weighed carefully on analytical balance with sample size 50; values (n = 5) are given as mean ± SD. LIA image processing software (Nagoya University, Japan) was used to measure seed sizes. Values (n = 10) are given as mean ± SD. The moisture content was determined as the difference between the initial and dry weights divided by the initial seed weight and represented in percentage.

3.13 Microscopy analysis

Leaf section of transgenic and control Jatropha plants were stained with Nile red (Hi-Media, India), mounted in 70% glycerol and examined using a laser confocal scanning microscope. Oil droplet were observed at 570-630 nm emission following 559 nm excitation by solid state laser. Image were captured with LSM 510 META laser scanning microscope (Lieca, Germany).

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3.14 Metabolomics analysis of transgenic *Jatropha curcas*

3.14.1 Plant samples

Transgenic *Jatropha curacs* plant (*AtDGAT1*) were grown in a greenhouse at 65% humidity and a photoperiod of 16/8h light (200 µmol m^{-2} s^{-1}) at 25 °C and dark. Newly emerged Jatropha leaves were collected for metabolomic analysis. Plant handling and sample preparation were standardized under the same condition and same period to ensure a similar comparable metabolite level of the plants. Leaf samples were harvested, immediately frozen and pestle in a mortar with liquid nitrogen treatment to avoid unwanted enzymatic reaction and then lyophilized in MICROMODULYO, Thermo Scientific. Lyophilized plant samples were stored in -80°C till further analysis.

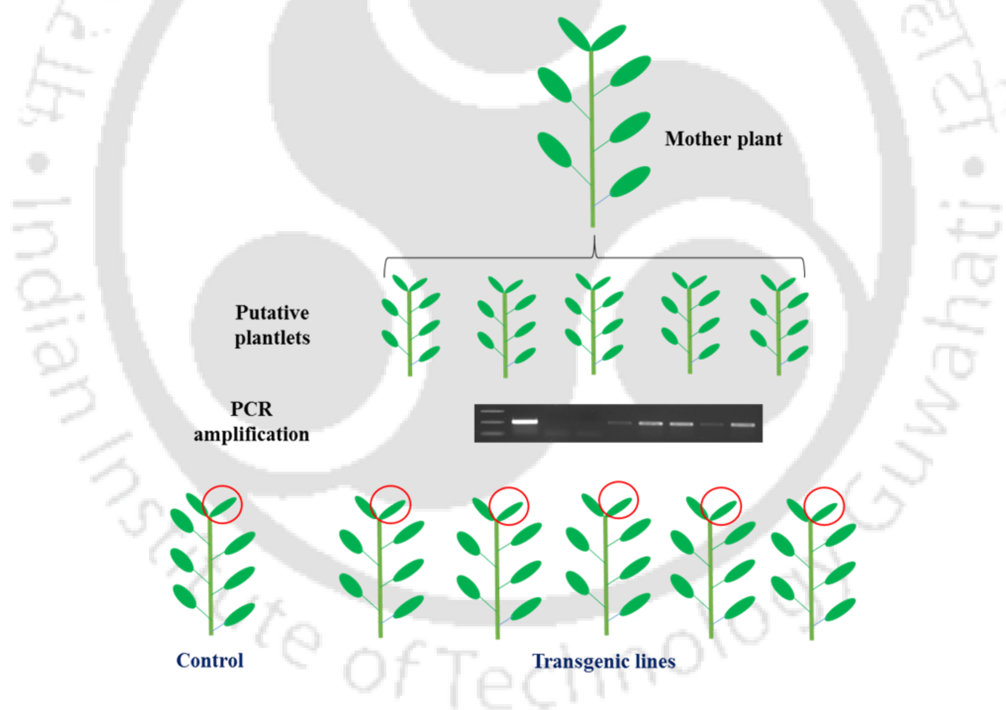

Figure 3.1 Sample collection for metabolomics analysis from *Jatropha curcas*.

Cotyledonory leaf used for transformation putative plantlets generated with kanamycin selection. Transgenic lines maintained in green condition. Newly emerged leaves sample were collected for Metabolome Analysis.

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3.14.2 Metabolite extraction from leaf samples

Thirty milligram of lyophilized powder of Jatropha leaves were extracted with 900 µl of methanol containing 25 µM of 7-hydroxy-5-methylflavone as an internal standard. After homogenization using a Mixer Mill MM 300 (Quiagen) at 25 Hz for 2 min twice, homogenates were centrifuged (17,400 x g, 5 min, and 4 ^{0}C). The supernatant was filtered through 0.2 µm PTFE membrane (Millipore). Hydrophobic compounds in the filtrate were removed by absorbing to C18 silica column (MonoSpin C18, GL Science, Tokyo, Japan). The elutes was used for LC-FT-ICR-MS analysis.

3.14.3 LC-FT-ICR-MS analysis

Metabolites were analyzed by an Agilent 1100 system (Agilent, Palo Alto, CA, USA) coupled to a Finnigan LTQ-FT (Thermo Fisher Scientific, Waltham, MA). Twenty µl of extract was applied to TSK-gel column ODS-100V (4.6 x 250 mm, 5 µm; TOSOH). The gradient was as follows: 3% B (0 min), 97% B (90 min), 97% B (100 min), 3% B (100.1), and 3% B (107 min). The flow rate was set to 0.25 ml/min (0-100 min) and 0.1 ml/min (100.1-107 min). The column oven temperature was set at 40 ^{0}C. Compounds are detected in ESI-positive mode in the range m/z 100-1500. Multistage MSn analyses were carried out using collision induced dissociation (CID) in a linear ion trap detector at a normalized collision energy of 35.0% and an isolation width of 4.0 (m/z), and monitored by both ion trap detector and FT-ICR detector at 100,000 (at m/z 400) mass resolution. To monitor HPLC elution, a photodiode array detector was used in the wavelength range 200–650 nm. The ESI setting was as follows: spray voltage 4.0 kV and capillary temperature 300 ^{0}C. Nitrogen sheath gas and auxiliary gas were set at 40 and 15 arbitrary units, respectively.

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Figure 3.2 LC-FT-ICR-MS

3.14.4 Data processing and statistical analysis

RAW MS data sets from FT-ICR/MS were acquired and browsed using Xcalibur software version 2.07 (Thermo Fisher Scientific). Integrated peak ion counts were used to compare relative abundance of metabolites in each sample. Missing values for a given metabolites were imputed with the observed minimum detected value for statistical analysis, assuming that they were below the limits of instrument detection sensitivity. Peak identification was performed by using in house tools and ion fragmentation were imported to Massbank (http://www.massbank.jp/) to further evaluate accurate mass. Accurate m/z values of the detected ions were searched by public metabolite databases such as KEGG (http://www.genome.jp/kegg/), LipidMaps (http://www.lipidmaps.org/) and KNapSAcK (http://kanaya.naist.jp/KNApSAcK/) respectively. Metabolites difference between transgenic and wild type were determined using principle component analysis (PCA), metabolite-metabolite correlations employing the mean profile values. The Hierarchical cluster analysis (HCA) was performed using the heatmap.2 function of R 3.3.1 software version (http://www.r-project.org/).

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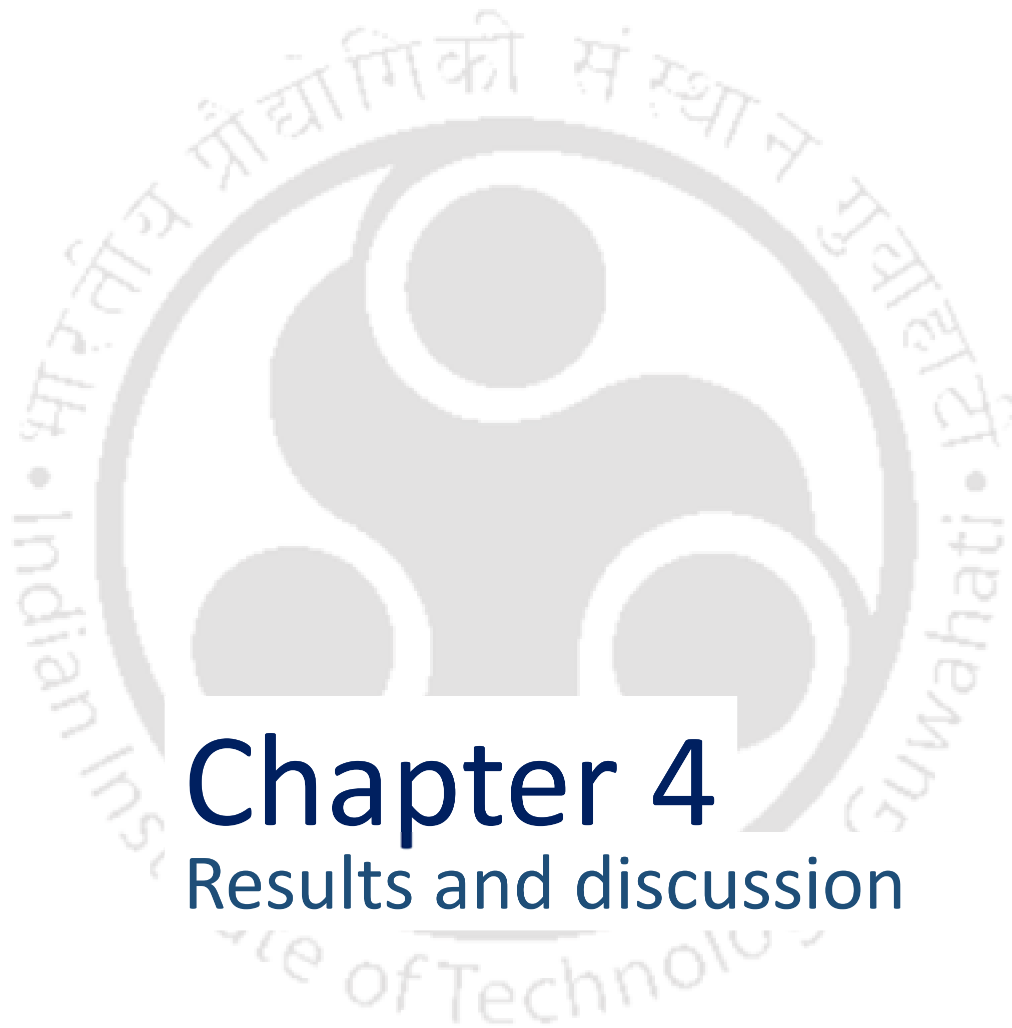

Chapter 4

Results and discussion

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4.1 Confirmation of *AtDGAT1* in plant binary construct

The pTZ57R/T AtDGAT1 was digested with *XbaI/ BamHI* to release 1.562 kb of AtDGAT1 gene which as used as insert for the cloning in plant binary vector pBI121 (14.752 kb) at *XbaI/BamHI* sites. After ligation, the recombinant clones were confirmed by PCR using primers specific to AtDGAT1 (**Figure 4.1**). The plant binary construct pBI121::AtDGAT1 was successfully mobilized to *Agrobacterium tumefacience* hypervirulent strain EHA105 and mobilization was confirmed by colony PCR. The amplification of 1.56 kb fragment from pBI121::*AtDGAT1* clones confirmed the presence of *AtDGAT1* gene (**Figure 4.2**).

4.2 Generation of transgenic *Jatropha curcus* plants

To investigate the impact of constitutive overexpression of *Arabidopsis DGAT1* cDNA on TAG accumulation in leaves and seeds of Jatropha, we prepared a 35S::*AtDGAT1* construct that consisted of *AtDGAT1* fused to *gus* reporter gene driven by CaMV35S promoter and *nptII* as plant selection marker (**Figure 4.1G**). Transgenic Jatropha plants harboring *AtDGAT1* were generated through *Agrobacterium*-mediated transformation of cotyledonary leaf segment explants and selection of transformed shoots through kanamycin-based selection. Putative transformed shoots were rooted on kanamycin-free rooting medium and successfully hardened and acclimatized in greenhouse (**Figure 4.3, a–g**). Stable GUS expression was checked randomly in the cultures, at various developmental stages for validating the strength of kanamycin selection in recovery of transgenic plants. Stable GUS expression was detected in transformed cotyledonary leaf segment explants, developing shoot buds from callus, and regenerated shoots, leaf, stem, roots, and seeds of transgenic plants (**Figure 4.4, a–m**).

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4.3 Molecular analysis of the transgenics

4.3.1 PCR analysis

The putative transformed plants were confirmed by genotyping through genomic DNA PCR using primers specific to *AtDGAT1*, *nptII*, and *gus*. Amplification of 384-bp fragment corresponding to the *AtDGAT1*, a 540-bp fragment internal to *nptII*, and 400-bp internal to *gus* confirmed the transgenic plants (**Figure 4.5, a-c**).

4.3.2 Southern hybridization

Five randomly selected PCR-positive independent T_0 transgenic Jatropha lines recovered on kanamycin selection medium were screened by Southern hybridization to confirm the integration of *nptII* gene using a 0.54 kb *nptII* probe. Two of the transgenic lines (TR3 and TR4) exhibited single copy integration events (**Figure 4.6**), whereas the three transgenic lines (TR1, TR2, and TR5) showed integration of two copies (**Figure 4.6**). No hybridization signal was detected in control WT plant (**Figure 4.6**).

4.3.3 Semi-quantitative PCR

RNA was extracted from randomly selected five *AtDGAT1* Jatropha transgenic lines, for expression analysis for semi-quantitative RT-PCR analysis which revealed abundance of AtDGAT1 mRNA in transgenic lines, albeit high expression in transgenic lines TR1, TR2, and TR3, and moderate expression in TR4 and TR5 in contrast to the wild type (**Figure 4.5d**). Several independent lines expressing *AtDGAT1* were generated in which the growth rates, growth patterns, leaf number, and leaf size were observed normal as compared to their counterpart wild type.

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4.4 Biochemical analysis

4.4.1 Estimation of protein and total carbohydrate content

The five *AtDGAT1* transgenic Jatropha lines were analyzed to find out the possible changes in protein and carbohydrate content at the expense of increased lipid accumulation in leaves and seed kernels, as the precursors of fatty acid biosynthesis in plants are derived from sugar during photosynthesis. The protein content in leaves of transgenic lines showed no significant change as compared to WT plants (**Figure** 4.**7a**). However, seed kernels of transgenic lines showed a minor increase in protein content except in transgenic line TR1 and TR2 that showed an insignificant decrease in protein content as compared to WT (**Figure 4.7b**). It was observed that the level of total soluble sugar in leaves was significantly increased (38– 112 %) in transgenic lines tested as compared to leaves of WT plants (**Figure 4.7c**). The transgenic lines had total soluble sugar in the range of 43.60–66.70 mg g-1 FW as compared to wild-type 31.45 mg g-1 FW (**Figure 4.7c**). These results suggest that increased accumulation of total soluble sugar may have contributed to reallocation of precursor for enhanced TAG synthesis in transgenic leaves (Cernac et al., 2004). However, the seed kernels of transgenic lines showed a corresponding decrease in sugar content as compared to wild-type plants (**Figure 4.7d**). These observations suggest that the reallocation of precursor of photosynthesis to TAG biosynthesis is more in leaves than seeds of transgenic AtDGAT1 Jatropha lines, and possibly the contribution to TAG biosynthesis in these transgenic lines by leaves is more than seeds which may be due to the lower expression of 35S-driven genes in the seed versus vegetative tissue. In previous studies, it has been found that 35S does not express as highly in *Arabidopsis* seed and germline tissue compared to vegetative tissue like leaves.

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4.4.2 Estimation of lipid and seed oil content

The AtDGAT1-transgenic Jatropha overexpressing AtDGAT1 were evaluated to see if AtDGAT1 overexpression lead to increased seed oil accumulation in leaf biomass and seed kernel. As we intended to specifically allow lipid accumulation in the leaves as well as in mature seeds, the *AtDGAT1* was placed under the control of the constitutive CaMV35S promoter. To investigate total lipid content in mature seeds and leaves of transgenic Jatropha plants, we harvested leaves, and mature seeds from five individual transgenic plants and separated the seed kernels for further studies. Compared to control (WT) plants, all tested transgenic lines showed significant increase (1.5 to 2 fold) in total lipid content in leaves (**Figure 4.8a**). The leaves of best transgenic line (TR1) accumulated 2 fold higher total lipid content than the control (WT) plants which represented a 100% increase of total lipid in transgenic line (TR1). The seed kernels of all the transgenic lines tested, showed enhancement of total lipid content by 20-30% on a relative basis as compared to WT plants (**Figure 4.8b**). The best transgenic line (TR1) showed 30% relative increase of total lipid as compared to WT plants (**Figure 4.8b**).

4.5 Thin layer chromatography

In order to characterize TAG accumulation in mature seed kernels, we used TLC on silica gel plates to analyze qualitatively the total neutral lipids from control (WT) plants and five transgenic lines. The TLC plates revealed an overall increase in TAG content in leaves and seeds of all *AtDGAT1* over expressing transgenic Jatropha lines as compared to control (WT) plants (**Figure 4.9**). Furthermore, the DAG content in seeds of transgenic lines showed a relative increase as compared to WT plants (**Figure 4.9B**). FFA was not detected in seeds of any of the transgenic lines except TR2 (Figure 3.8B). All the five transgenic lines showed a very similar level of TAG accumulation in their seeds (**Figure 4.9B**). These results suggest that

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AtDGAT1 encodes a functional protein capable for catalyzing the final rate limiting step of TAG biosynthesis in transgenic Jatropha lines expressing *ATDGAT1*.

4.6 ^{1}H NMR spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy was employed to check the conversion of oil to FAME in the transesterification reaction. The peaks of oxymethylic hydrogen in triacylglycerol and the methoxy group in the methyl esters were used to follow the reaction progress (**Figure 4.10, A-D**). In NMR spectrum of methyl ester, the characteristic peak of methoxy protons, which was attributed to methyl esters was observed as singlet at δ 3.65 ppm and this signal was absent in the NMR spectrum of TAG (**Figure 3.10, A and C**). ^{1}H NMR spectrum of oil, multiplet signals were recorded in the regions δ4.11, δ4.12, δ4.13, δ4.17 and δ4.27-4.29 ppm due to oxymethylic hydrogen that assigned of TAG (**Figure 4.10 B and D**). These NMR spectra of TAG and FAME confirmed the successfully conversion of Jatropha TAG into FAME.

4.7 FAME analysis by Gas chromatography

To determine and compare the FA profiles between the transgenic lines and WT plants, the fatty acids methyl esters resulting from transesterification of seed oil and leaf lipids were quantified by GC. A significant variation in composition of FAs was detected among the leaves of control and transgenic plants. The leaves of transgenic lines showed a relative increase of oleic acid (18:1) by 20–31 % as compared to WT plants (**Figure 4.11A**). In addition, the level of linolenic acid (18:3) showed a reduction from 18.17 to 5–8 % in transgenic lines as compared to WT plants. There were no marked changes observed in the level of palmitic (16:0), stearic (18:0), and linoleic (18:2) acid in leaves of transgenic lines as compared to WT plants (**Figure 4.11A, Table 4.1**). Increased TAG accumulation in seed kernels was accompanied by a profound change in fatty acid composition in TAG fraction (**Figure 4.11B**). GC analysis

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showed that oleic acid (18:1) accumulation in seed kernels of transgenic lines increased by 9–25 % as compared to WT. On the other hand, the level of linolenic acid (18:3) in seed kernels decreased by 12–35 % in transgenic lines as compared to WT (13.79 %) (Figure 3.11B). The linoleic acid (18:2) levels in seed kernels of transgenic lines showed the similar abundance to that of WT, and in contrast, the lines TR4 and TR5 showed the level of linoleic acid decreased by 9.94 and 10.04 %, respectively. The level of palmitic acid (16:0) in seed kernels decreased by 12–21 % in all transgenic lines as compared to WT (**Figure 4.11B)**. There were no changes in the stearic acid (18:0) levels in seed kernels of the transgenic lines (**Table 4.2**). Jako et al. (2001) and Xu et al. (2008) also reveal the expression of DGAT1 from *Arabidopsis* or *Trapaeolum majus* in Arabidopsis was able to increase the proportion of oleic acid and decrease the proportion of linolenic acid in seed oil.

4.8 Microscopic analysis

TAGs, the predominant plant storage neutral lipids with twice the energy density of cellulose, are used to generate biodiesel (**Hill et al., 2006)**. Increased demand to produce more energy from plant biomass has prompted means to produce oil in vegetative tissues, mostly in leaves. Therefore, we made an attempt to engineer Jatropha plants by overexpressing the *DGAT1* gene that codes for the enzyme responsible for the final and only committed step in TAG biosynthesis, to accumulate TAGs in leaves, in addition to seeds. Hand sections of control and transgenic petioles of Jatropha were examined for determining the intracellular localization of the TAG. Fresh samples from the fifth leaf from apical bud of wild-type and transgenic Jatropha plants were stained with lipid-specific fluorescent dye, Nile red, and observed under a confocal laser scanning microscope after excitation at 559 nm of light. The frequency of oil droplets was found increased in transgenic samples compared to the wild type, and oil droplets mostly distributed close to inner peripheral region of cells (**Figure 4.12 A, B**).

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4.9 Agronomic trait analysis

We investigated whether there were any off targets of TAG accumulation on agronomic traits in *AtDGAT1* overexpressing Jatropha lines under greenhouse conditions. Five transgenic lines with high TAG content and control WT plants were monitored for agronomic traits including time duration for flowering, plant height, number of primary branches after one trimming, secondary branches, seed number per tree after acclimatization in soil, seed length, seed width, seed breadth, and 10-seed weight (**Table 4.3**). The transgenic lines were morphologically no different from the wild type (**Figure 4.13**). The transgenic lines as well as the WT plants took approximately two and half years for flowering, and there were no obvious differences in time taken for inflorescence emergence. There was no significant difference in number of primary branches and secondary branches found among the transgenic lines and WT plants; however, some of the transgenic lines had a marked increase in plant height (**Table 4.3**). The transgenic lines had an average of 56.88 ± 7.6 seeds per tree with an average seed weight of 7.46 ± 0.03 g, and with increased average seed length, seed width, and seed breadth (**Table 4.3**). These data collected from transgenic lines under greenhouse conditions indicated that high TAG accumulation had no negative effects on important agronomic traits in Jatropha.

4.10 Correlation between oil content and physiological indexes

The results revealed that significant differences of physiological indexes exist between transgenic line and wild type. In order to investigate relations among the physiological factors with oil accumulation, a correlation analysis between transgenic and wild type plants were performed (**Figure 4.14**). The correlation analysis showed 132 unique profile and resulted in significant correlation coefficient. Out of 132 significant correlations, 62 were positive and 70 were negative. However, heat map represents the both positive and negative strong correlation with physiological parameters (**Figure 4.14**). Correlation analysis showed that seed oil

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positively correlated with seed weight. Total carbohydrates were significantly associated and negatively correlated with seed oil, this indicated that carbon flux in enhanced TAG biosynthesis occurred in seeds. Correlation analysis also revealed the physiological indexes were associated with TAG biosynthesis. These observations confirm the findings from multivariate statistical analysis showing that the insertion of the transgene caused a variation in the metabolites concentrations in transgenic plants compared to wild type.

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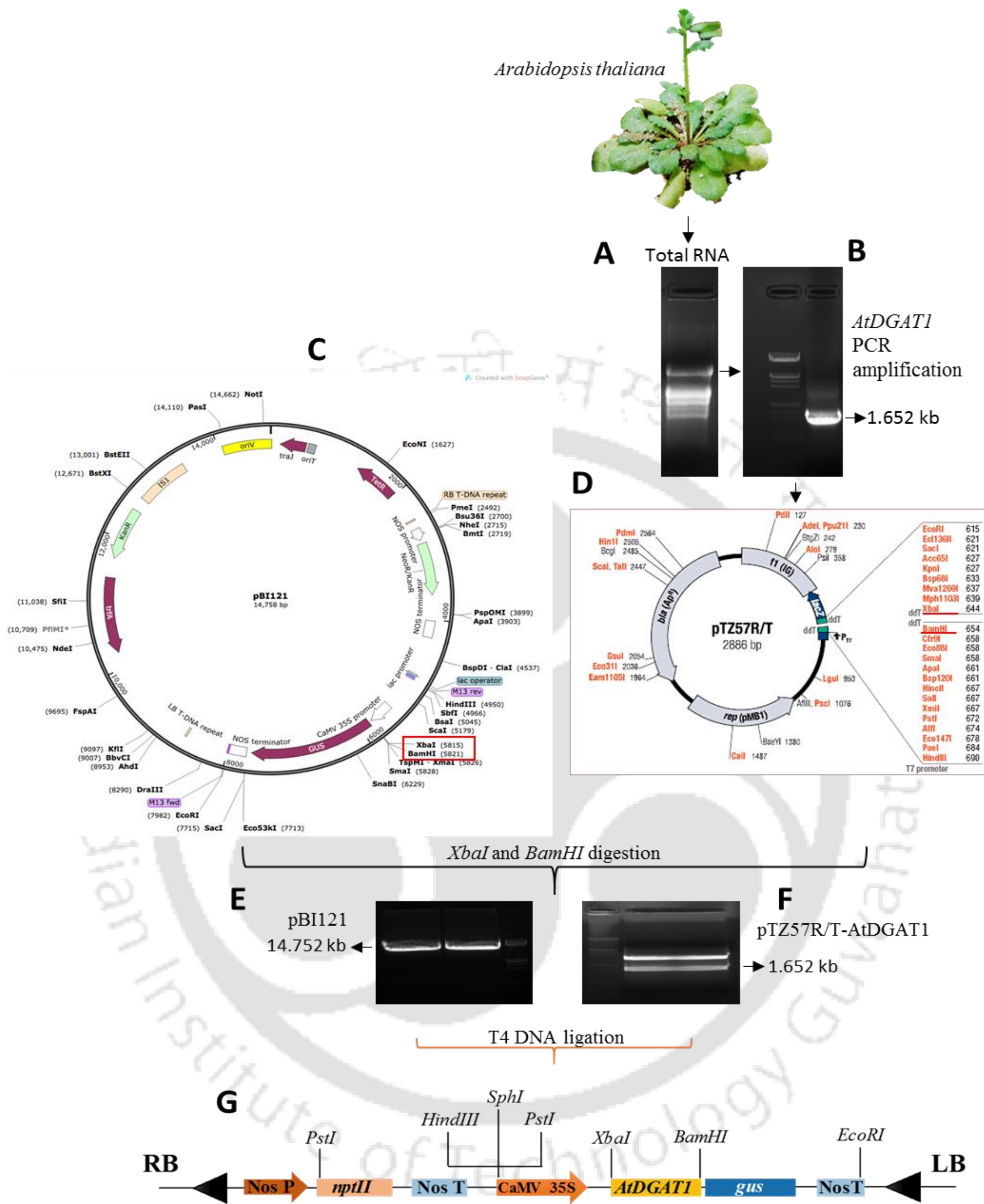

Figure 4.1 Cloning of *AtDGAT1* in plant binary vector.

Total RNA isolation from Arbidopsis thaliana (**A**), Amplification of 1.562 kb, AtDGAT1 cds with specific primer (**B**), pBI121 map (**C**), pTZ57R/T map (**D**), digested and purified plant binary vector pBI121 with XbaI/BamHI (**E**), Digested pTZ57R/T harbouring *AtDGAT1* cassette (**F**), T-DNA region (6.2kb) of pBI121CaMV35S:: *AtDGAT* plasmid**.** RB, right border; LB, left border; CaMV 35S promoter Nos P, Nopaline synthase promoter; Nos T, Nopaline synthase terminator; NptII, Neomycin phosphotransferase, ß-glucuronidase (**G**).

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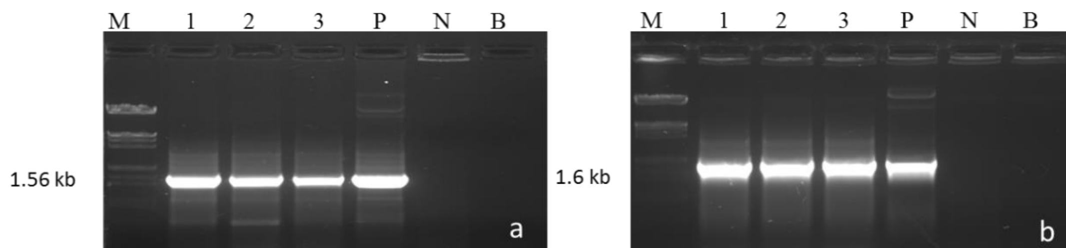

Figure 4.2 Mobilization of pBI121CaMV35S::*AtDGAT1* vector into *Agrobacterium tumefacians* EHA105.

a, PCR amplification of 1.56 kb *AtDGAT* gene and **b**, 1.6 kb *gus* gene. M, Marker; Lane 1-3, clone of *Agrobacterium tumefaciens* after triparental mating; P, *pBI121:AtDGAT*1 plasmid (positive control), N, *Agrobacterium* strain EHA105 (negative control); B, Blank.

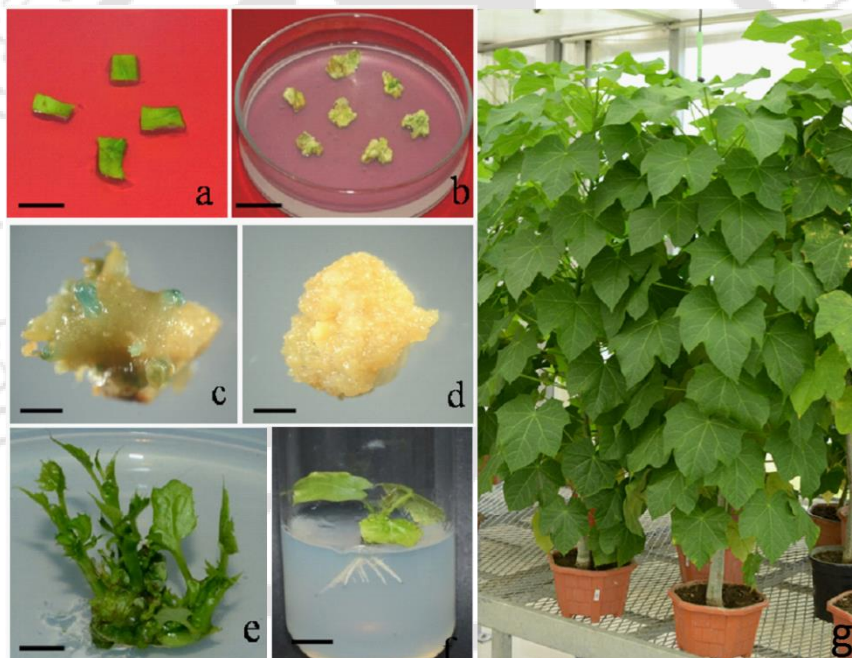

Figure 4.3 Agrobacterium-mediated genetic transformation of *Jatropha curcas* with 35S::*AtDGAT1* construct.

a, excised cotyledonary leaf explants subjected to *Agrobacterium tumefaciens* mediated transformation and cultured on kanamycin free callus induction medium (bar = 5 mm); **b**, formation of callus from explant (bar = 8 mm); **c**, Stable GUS expression in buds induced from transformed callus (bar = 8 mm); **d**, untransformed control callus (bar = 8 mm); **e**, elongated putative transformed shoots on kanamycin selection (bar = 1 cm); **f,** rooted transformed plantlet (bar = 1 cm); **g,** acclimatized transformed plant.

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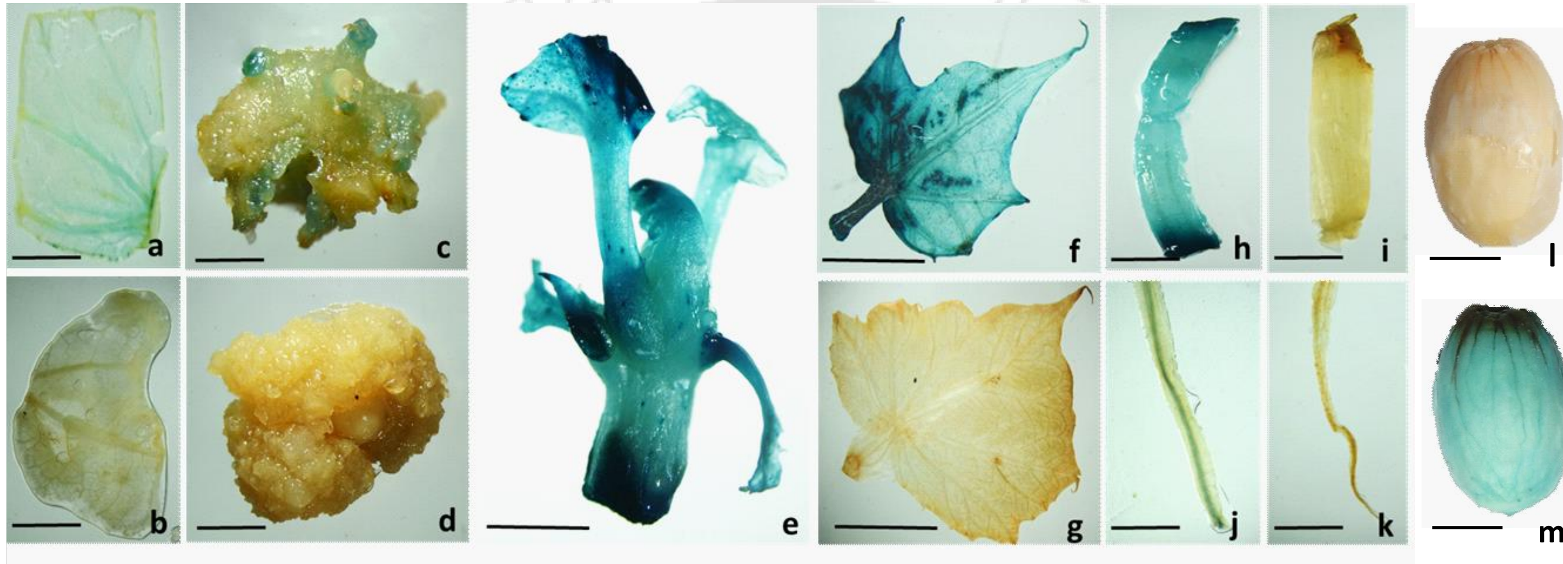

Figure 4.4 Stable *gus* expression in transgenic plant tissue parts.

a, transformed cotyledonary leaf segment; **b**, untransformed leaf segment; **c**, emerging shoots from callus showing gus expression; **d**, callus from untransformed tissues; **e**, gus expression in in-vitro shoot; **f**, gus expression in leaf; **g**, control leaf; h, gus expression in stem; **i**, control stem; j, gus expression in transgenic root and **k**, control root; **l**, control seed and **m**, transgenic seed. (Bar = 0.5 cm).

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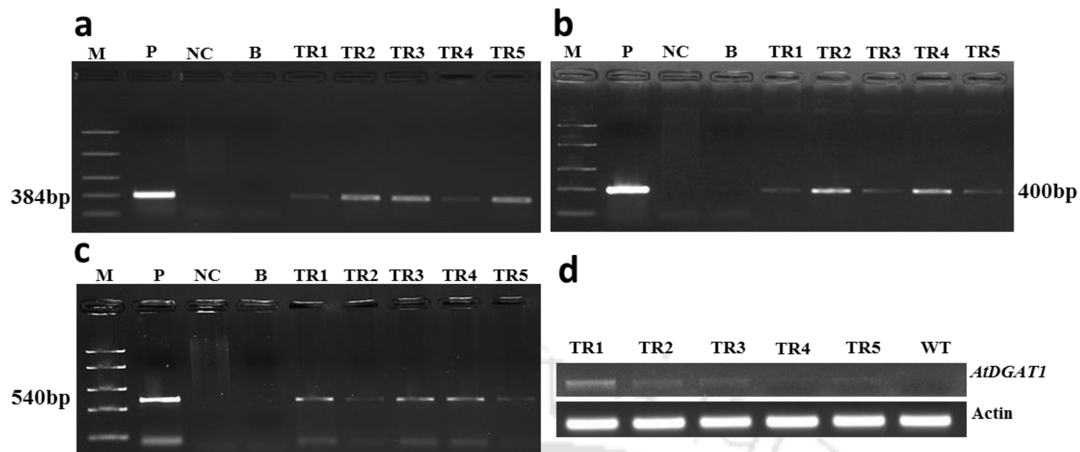

Figure 4.5 Molecular analysis of transgenic plants of *Jatropha curcas*.

a, PCR amplification of the 384 bp fragment of *AtDGAT* gene; **b**, PCR amplification of the 400 bp fragment of *gus* gene; **c**, PCR amplification of the 540 bp fragment of *npt II* gene. Lane M, Molecular marker; Lane TR1, TR2, TR3, TR4, TR5, genomic DNA from five transgenic plant Lane P, pBI121AtDGAT plasmid (positive control), Lane NC, DNA from untransformed plant (negative control) and Lane B, Blank; **d**, Transcript abundance of AtDGAT1 in transgenic line of *Jatropha curcas*. Expression analysis was carried out by semi-quantitative PCR using actin as an internal control.

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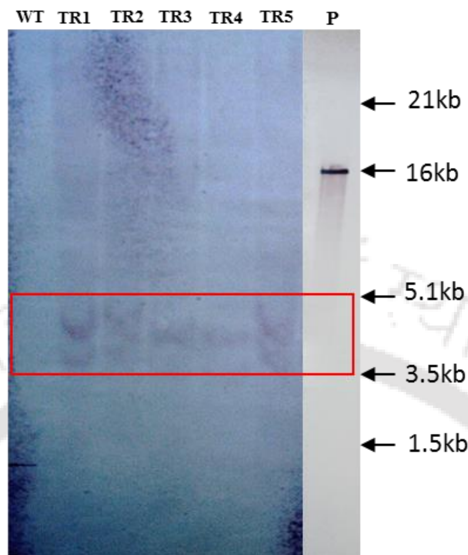

Figure 4.6 Southern blot hybridization analysis of five independently selected PCR positive T_0 lines.

The plasmid and 60 µg genomic DNA was digested with *BamHI*, and hybridized with PCR amplified *nptII* probe, lane WT genomic DNA from untransformed plant, Lane TR1-TR5 genomic DNA from transgenic lines, lane P *BamHI* digested pBI121::*AtDGAT1* plasmid.

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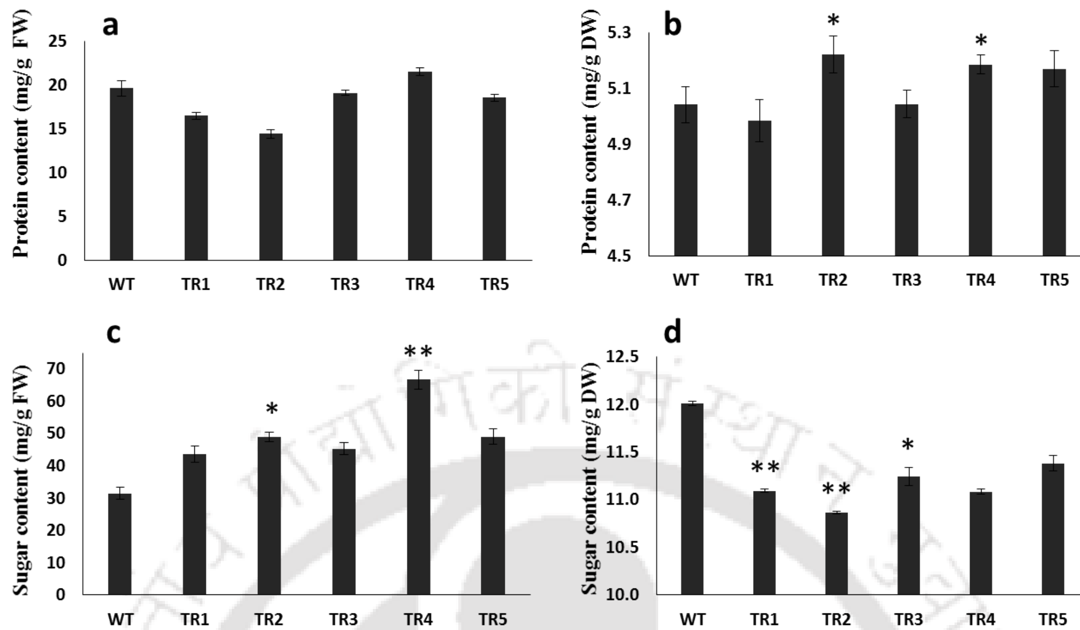

Figure 4.7 Variation in leaves and seed protein and sugar contents.

a, leaf protein content; **b**, seed protein content; **c**, leaf sugar content and **d,** seed sugar content analysis in wild-type and *AtDGAT1-Jatropha curcas* transgenic lines. Difference between untransformed (WT) and transgenic lines was significant at P < 0.05 (*), P < 0.01 (**) by Tukey test. Values are shown as mean ± SD (n = 3).

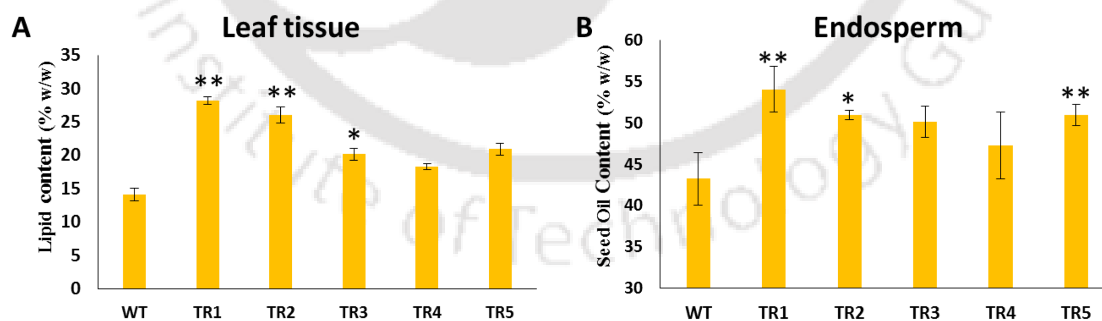

Figure 4.8 Variation in leaves and seed lipids contents.

A, Leaf lipid content and **B,** seed oil content analysis in wild-type and *AtDGAT1-Jatropha curcas* transgenic lines. Difference between untransformed (WT) and transgenic lines was significant at P < 0.05 (*), P < 0.01 (**) by Tukey test. Values are shown as mean ± SD (n = 3).

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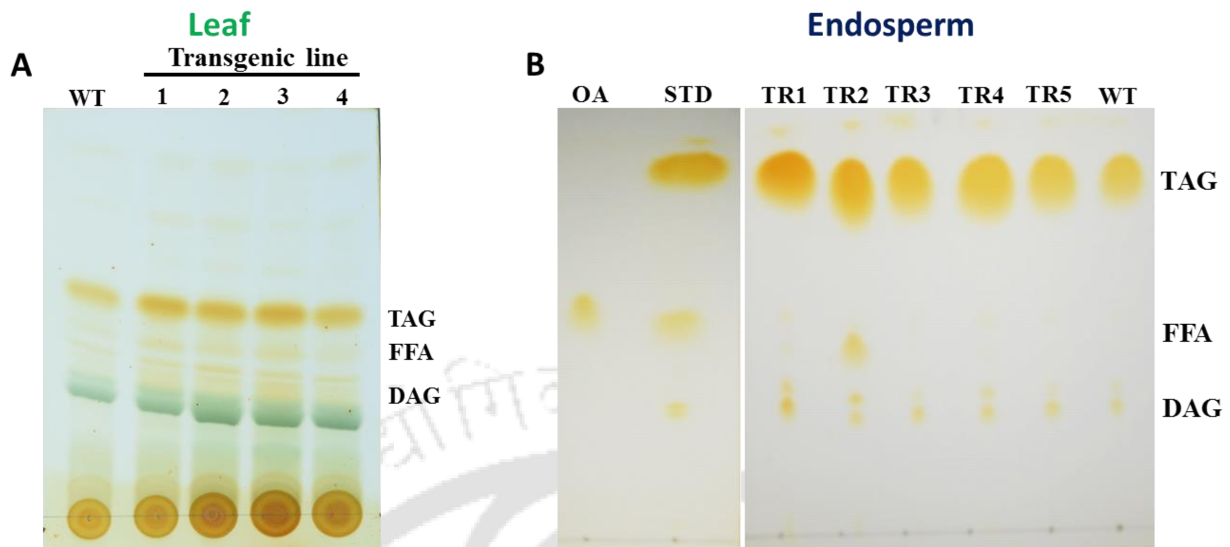

Figure 4.9 Triacylglycerol accumulation in transgenic *Jatropha curcas*.

TLC separation of neutral lipids from transgenic lines and wild type *Jatropha curcas;* 150 µg lipids were fractioned by Silica Gel plates, **A**, lipid from leaves; **B**, seed oil. Triacylglycerol (TAG), free fatty acids (FFA) and diacylglycerol (DAG), oleic acid (OA), standard (STD).

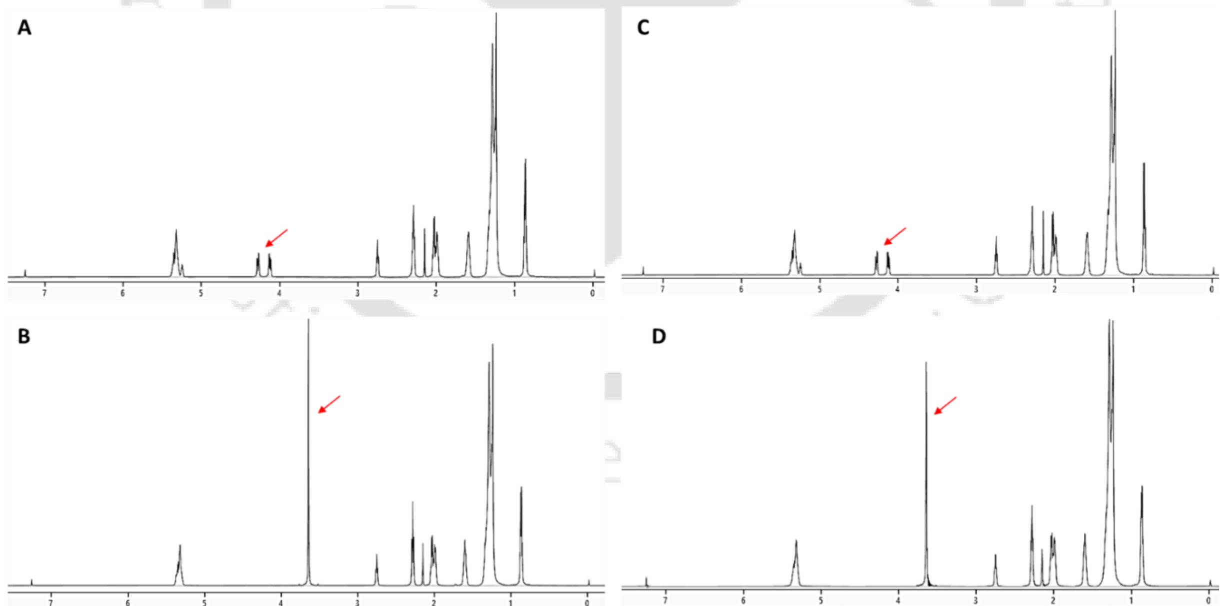

Figure 4.10 ^{1}H NMR spectrum of *Jatropha curcas* oil and methyl ester.

^{1}H NMR spectrum of transgenic Jatropha oil and methyl esters (**A, B**), ^{1}H NMR spectra of Wild type Jatropha oil and methyl ester (**C, D**).

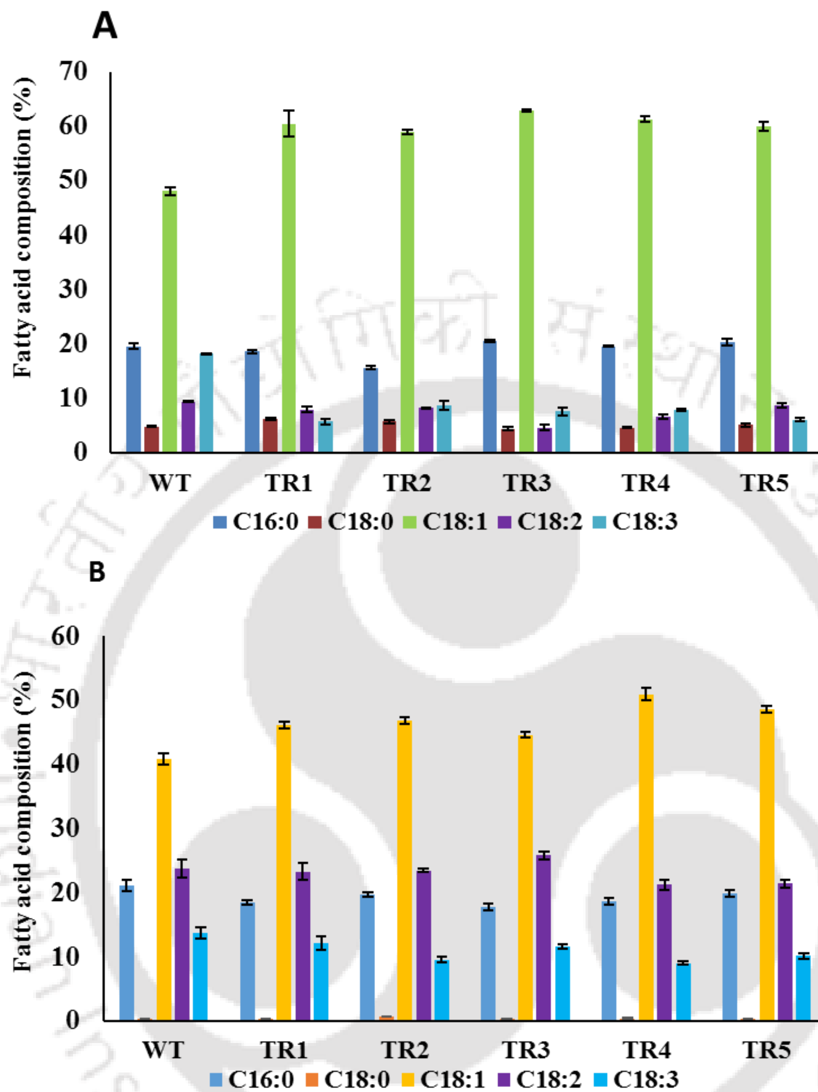

Figure 4.11 Fatty acid pprofiling by GC/MS.

The relative amount was calculated with comparing their peak areas and peak intensities. Each experiment was performed with 200 mg leaf tissue and 500 mg dried seed kernel per line with 3 biological replicates. Error bar shows standard deviation (n = 3).

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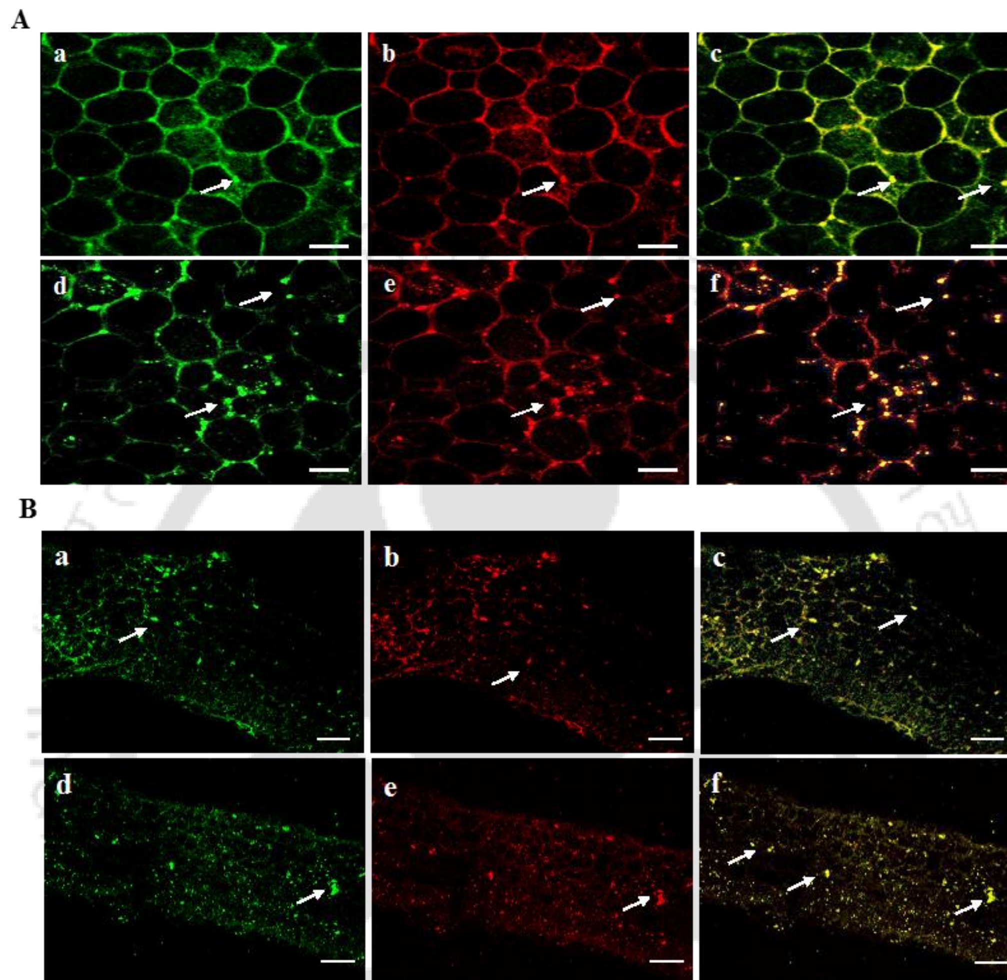

Figure 4.12 Oil droplets are abundant in leaf sections of *AtDAGT1* overexpressing transgenic lines.

Confocal fluorescence image of leaf petiole of wild type (**a-c**) and transgenic lines (**d-e**) showing oil droplets (arrow) and leaf cross section of wild type (**a-c**); transgenic line (**d-f**) stained with Nile red. Bar = 20 µm.

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Table 4.1 Fatty acid composition in leaves of wild type and transgenic line of *Jatropha curcas*.

	Fatty acid composition				
	C16:0	**C18:0**	**C18:1**	**C18:2**	**C18:3**
WT	19.56 ± 0.18	4.85 ± 0.04	48.07 ± 0.13	9.37 ± 0.15	18.17 ± 0.12
TR1	18.58 ± 0.29	6.16 ± 0.58	60.47 ± 2.38	7.95 ± 0.47	5.70 ± 0.26
TR2	17.48 ± 0.08	5.52 ± 0.16	60.64 ± 0.36	10.43 ± 0.21	5.93 ± 0.38
TR3	18.89 ± 0.2	6.75 ± 0.82	58.59 ± 1.62	8.48 ± 0.29	6.14 ± 0.09
TR4	15.59 ± 0.35	5.71 ± 0.86	58.85 ± 0.43	8.23 ± 0.05	8.7 ± 0.24
TR5	20.53 ± 0.28	4.41 ± 0.75	62.88 ± 0.28	4.59 ± 0.52	7.58 ± 0.36

Values are mean ± SE (n = 3).

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Table 4.2 Fatty acid composition in seed kernels of wild type and transgenic line of *Jatropha curcas*. Values are mean ± SE (n = 3).

			Transgenic lines				
		WT	**TR1**	**TR2**	**TR3**	**TR4**	**TR5**
Fatty acid composition	**C16:0**	21.04±0.89	18.43±0.39	19.64±0.36	17.76±0.45	18.65±0.54	19.91±0.49
	C18:0	0.3±0.026	0.31±0.26	0.67±0.65	0.36±0.034	0.37±0.042	0.35±0.025
	C18:1	40.81±0.85	**46.13±0.48**	**46.8±0.47**	**44.55±0.44**	**50.88±0.98**	**48.56±0.49**
	C18:2	23.77±1.37	23.23±1.31	23.46±0.25	25.81±0.79	21.24±0.79	21.39±0.63
	C18:3	13.78±.89	12.110.98	9.55±0.41	11.57±0.27	9.01±0.27	10.14±0.56

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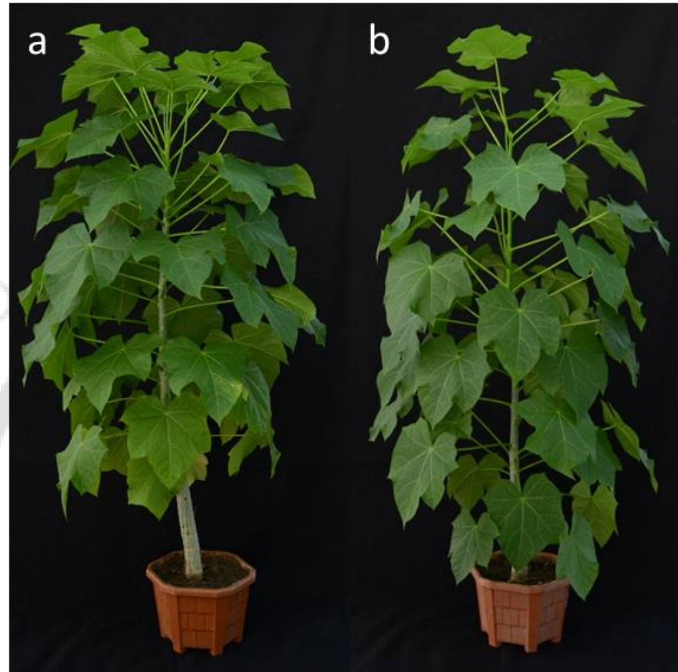

Figure 4.13 Growth comparison of AtDGAT1 transgenic line and wild type *Jatropha curcas*.

a *AtDGAT1* transgenic *J. curcas*; ***b*** wild type. Transgenic plants and wild-type Jatropha plants were planted in pots containing soil, manure, and sand in 1:1:1 ratio. Green house condition was maintained at 25 ± 2 ^{0}C, relative humidity 60 ± 5 %, and 16-h photoperiod. The light intensity was maintained at a photosynthetic photon flux density (PPFD) of 240 μM/m2/s provided by 40-W cool white fluorescent lamps.

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Table 4.3 Physiological parameter comparison between wild type and *AtDGAT1* transgenic lines

	Primary branch*	Secondary branches**	Seed number per tree	Seed length (mm)	Seed width (mm)	Seed breadth (mm)	10 seeds weight (g)
WT	4	4.5±0.6	76	18.35±0.21	10.65±0.15	8.95±0.29	7.13±0.21
TR1	3	8.6±0.7	47	18.36±0.12	**10.71±0.13**	**9.19±0.20**	**7.47±0.07**
TR2	3	5.3±2.7	83	**18.94±0.16**	10.90±0.13	8.94±0.18	7.39±0.06
TR3	4	4.6±1.2	43	18.38±0.23	10.72±0.17	**9.14±0.12**	**7.52±0.09**
TR4	3	6.0±0.6	46	**18.78±0.21**	**11.39±0.16**	8.64±0.23	**7.54±0.05**
TR5	3	4.3±0.7	65	18.35±0.14	10.51±0.14	8.83±0.14	7.43±0.11

* Number of branches after pruning

** Average secondary branches per primary branch

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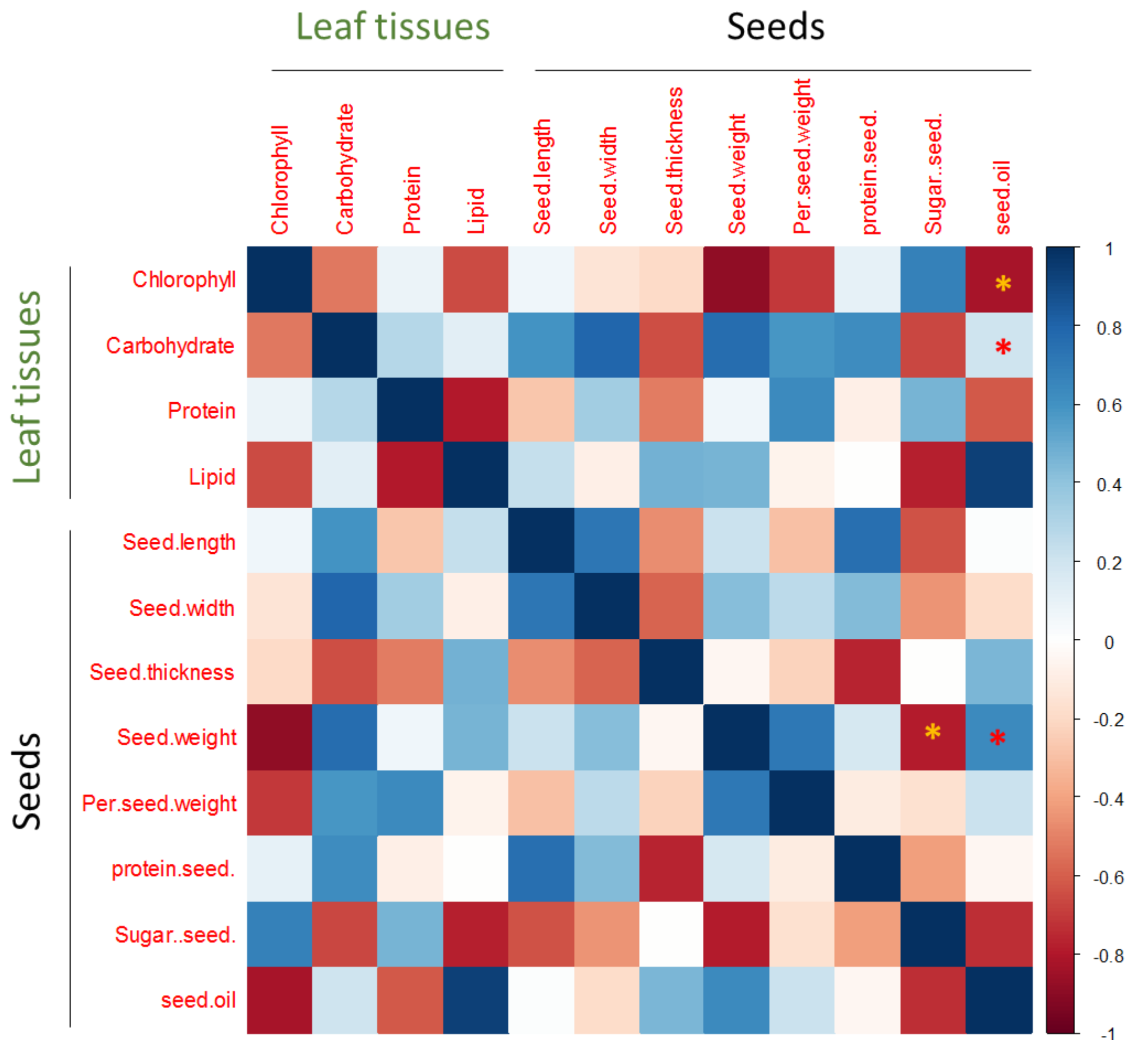

Figure 4.14 A colour heat map of the Pearson's correlation coefficients computed for the 12 physiological parameters observed in the leaf tissues and seeds from *AtDGAT1* overexpression lines of *Jatropha curcas*.

The colours refer to the pair-wise correlation coefficient (*p*-value) ranging from 1 (blue) to −1 (red). ($p \geq 0.05$).

4.11 Metabolomic analysis of *Jatropha curcas*

Liquid chromatography-FT-MS analysis was performed for evaluation of different secondary metabolites from various plant samples. Detected ions were identified and annotated by database and standard compound on the basis of accurate m/z ratio (Horai et al., 2010; Tohge et al., 2011). However, liquid chromatography-FT-ICR-MS is the most powerful tool among other MS instrument to acquire fingerprint complex samples, due to its extremely high resolution and high mass precision (Aharoni et al., 2002; Brown et al., 2005). Therefore, it has a huge potential in the screening of different samples. There are very limited studies that use FT-MS in plant metabolomics (Oikawa et al., 2006; Sano et al., 2012; Marques et al., 2016). Most of these studies are based on the analysis of a single extract from the plant material, either using a single solvent, normally methanol or acetonitrile, or a mixture of solvents, either methanol/water or methanol/water/chloroform. It has several advantages in metabolite extractions and reproducibility, by allowing the synchronized extraction of metabolites based on their polarity (Wu et al., 2008, Kim et al., 2010).

4.11.1 Metabolite identification of transgenic *Jatropha* leaves

Non-targeted metabolic profiling platform, FT-ICR-MS was used to identify *Jatropha* leaves metabolome. The amount of metabolites in *Jatropha* leaves were huge and belongs to various chemical families with multiple chemical properties. For peak identification m/z spectra and accurate mass were compared with a database (major procedures are illustrated in **Figure 4.15**). Missing MS/MS spectrum in the database were deduced with identified ion fragment of accurately matched metabolites. Deduced fragment ions from metabolite were same as candidate metabolites measured by MS/MS spectrum. Then metabolite feature intensities were transformed to $\log_2$ base scale to normalize the data distribution. Total **2021** compound feature obtained in metabolomics analysis in positive ion modes and **1183** compound features were

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annotated according to their m/z ratio and accurate mass (**Figure 4.16**). However, *Jatropha* transgenic lines were established with *AtDGAT1* which catalyzes the final rate limiting step of Kennedy pathway. Therefore, the present metabolomics analysis Lipids and its derivatives along with some other secondary metabolites were considered for further analysis. Similarly, genetically modified crops such as *cry1Ac* and *sck*- transgenic rice (Chang et al., 2014), transgenic maize seeds over-expressing the *Aspergillus niger phyA2* (Rao et al., 2016) and different rice cultivars (Hu et al., 2014) were evaluated by non-targeted metabolomics analysis. Since, huge members of metabolites have been obtained it is required to identify these compound features which has correlation with the expression of *AtDGAT1* and TAG biosynthesis. Among the identified lipids, it includes sterol lipids, phospholipids, sphingolipids, glycolipids, diacylglycerols and triacylglycerols which were annotated. Observed changes in Lipids and FAs of the transgenic lines can be easily explained by catalytic activity of *AtDGAT1*. This leads a characteristics accumulation of TAG and FAs precursor. Identified metabolites and their corresponded pathways were listed in **Table 4.4**. Among identified metabolites, there were 18 glycerophospholipids, 21 fatty acyls, 6 glycerolipids, 3 sterol lipids, 3 sphingolipids, 4 chlorophyll metabolic compounds, 4 flavonol epoxy and hydroxyl fatty acyls and sucrose based on accurate mass and m/z ratio (**Table 4.4**).

PCA (Unsupervised method) was performed for all 1183 metabolites for interpreting the relationship among samples and metabolites in the transgenic and wild type. PCA score plot of all the metabolites were taken together with PC1 explaining 68.55% of the variation and PC2 explaining 25.19% of the variation (**Figure 4.17**). Transgenic lines TR1, TR2, TR4 and TR5 were well separated from WT where TR3 did not show proper clustering with other transgenic lines and positioned in outliers. However, remaining transgenic lines revealed significant metabolic changes highlighting the separation based on the genetic modification. The heat map highlighted the total incidence of metabolites in high intensities in transgenic

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lines compared to wild type (**Figure 4.18**). Among all the metabolites, 69 of them have been identified as lipids and their derivatives, some secondary metabolites such as flavonoids, alkaloids and chlorophyll synthetic metabolites which were compared with the wild types. PCA was also performed for the identified metabolites which reveals the proper separation of identified metabolites due to the genetic manipulation. PCA score plot of the identified 69 metabolites also showed the proper separation based on the genetic manipulation event and PC1 and PC2 were able to explain 45.28%, 30.92% variations respectively (**Figure 4.19**). PCA loading plot explained the highly positive and negative correlation between the metabolites. In loading plot, ladderane-sn-glycerol showed positive and TAG (17:1/17:2/20:0) showed highly negative correlation with other metabolites (**Figure 4.20**). PCA output was used to construct the biplot for visualizing variation in relation to the principle component and show the how transgenic event separated the Jatropha transgenic line compared to wild type. Biplot helped in the identification of clusters of the metabolites that may be related by functions or regulations. From the PCA on metabolite data, biplot were constructed. Together, these first two PCs explained 87% of all the variation in the Jatropha leaf metabolite data (**Figure 4.21**).

4.11.2 Metabolomic variation in leaf samples

Interestingly, flavonoids were significantly reduced in all transgenic lines. These compounds are known for antioxidative properties and it was found been effective against cancer, coronary heart disease and other diseases in humans (Rahman 2007). In plants flavonoids play important roles in biological functions such as pigmentation, fertility, plant pathogen interaction, protection against pathogens and protection against ultra violet-B radiation (Falcone-Ferreyra et al., 2012; Rao et. al., 2016). In the transgenic lines some flavonoids like Quercetin β-D-glucoside, Pinocembrin 7-rhamnosylglucoside, Anhydroicaritin rhamnoside, Luteolin were down-regulated. Chlorophylls play important role in plant growth and help to synthesize

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carbohydrate by photosynthesis. Metabolomics study revealed that, Chlorophyll biosynthetic metabolites including Red chlorophyll catabolite, Heme-O, Chlorophyllide b were down regulated and Protochlorophyllides were significantly upregulated in transgenic lines compared to the wild type. Carotenoid biosynthetic compound Antheraxanthin was also detected which has a property of absorb light energy for photosynthesis, and also they protect, chlorophyll from photo damage. Sphingolipids act as a signalling compound and plays significant role in plant reproduction and development. Fumonisin C4, 3-Ketosphingosine, Phytosphingosine are the classes of sphingolipids which were greatly reduced in the transgenic lines. In case of sterol lipids, Cholic acid glucuronide was significantly decreased but 3alpha, 7-alpha-dihydroxy cholanate were upregulated in all transgenic lines. Sterol lipids has function in maintaining membrane fluidity and signal transduction (Lindsey et al., 2003). Hydroxylated fatty acids (hydroxylinoleic acid, dihydroxyoctadecenoic acid) epoxy fatty acid (Epoxyoctadeca dienoic acid) and archidonic acid (2, 3-dinor-8-iso PGF1alpha, 2, 3-dinor-8-iso PGF2alpha and hydroxyicosa-tetraenoic acid) were also observed and showed significantl changes in transgenic samples. Hydroxy fatty acids are main components of *Ricinus communi and its* TAG contained 85% of hydroxy fatty acid and membrane lipid contains about 5% of it (Millar et al., 2000).

Sucrose contents showed expressively reduction in all the transgenic lines which indicate towards utilization of carbon source which is much higher than wild types. Increased Carbon flux towards central metabolic pathway leads to enhanced fatty acid and TAG accumulation (Bates et. al., 2009, Weselake et al., 2009, Napier et al., 2014). Group of fatty acyls and their conjugated compounds with amino acids (N-palmitoyl alanine, N-stearoyl serine, N-linolenoyl-glutamine and N-stearoyl glutamic acid) were also detected in all the leave samples. Among the annotated compounds, N-oleoyl proline was uparegulated and N-

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palmitoyl threonine was dowregulated in the transgenic lines. Fatty acyl such as 10-nitro-9E-octadecenoic acid, Rhamno lipids, PGF2alpha-EA, PGH2-EA, N-stearoyl glutamic acid, N-oleoyl proline and N-hydroxy arachidonoyl amines accumulation were enhanced in the transgenic lines. Fatty acyls are the important component of unsaturated fatty acid synthesis which indicates a successful transgenic event. Diacylglycerol (DG, 13:0/20:4) content was significantly overexpressed in all transgenic lines compared to the wild type. Furthermore, in higher plants TAG biosynthesis take place in endoplasmic reticulum through metabolic pathway in which acyl groups in fatty acyl-COA are transferred to the hydroxyl group of glycerol-3-phosphate and diacylglycerol (Ohlrogge and Browse, 1995: Durrett et al., 2008, Bates et al., 2008). Triacylglycerol were produced by the sequential acylation of fatty acids. Our targeted gene (*AtDGAT1*) catalyzes the final sn-acylation step of TAG biosynthesis. In the present metabolomics analysis accumulation of triglycerides (TAGs) TG (17:1/17:2/20:0), TG (17:0/17:2/20:0) has been increased in all transgenic lines compared to wild type. Apart from these glycerolipids, glycerophospholipids, sphingolipids, sterol lipids also reveal their significant abundance in transgenic lines compared to control (**Figure 4.22, 23**).

4.11.3 Metabolite-metabolite correlation

Pearson correlation coefficient analysis was used to analyze the metabolite-metabolite correlation (Camacho et al., 2005; Blekhman et al., 2014; Rao et al., 2016) among 69 identified metabolites in transgenic and wild type *Jatropha curcas*. The results were visualized as heat map, which showed 4761 correlations ranging from -0.93 for sucrose and TG (17:1/17:2/20:0) to 0.98 for LTB-4 dimethyl amide and chlorophyllide. Out of these 4761 significant correlations 2295 were positive and 2466 were negative (**Figure 4.24**). The analysis allowed the identification of metabolites that relates to each other.

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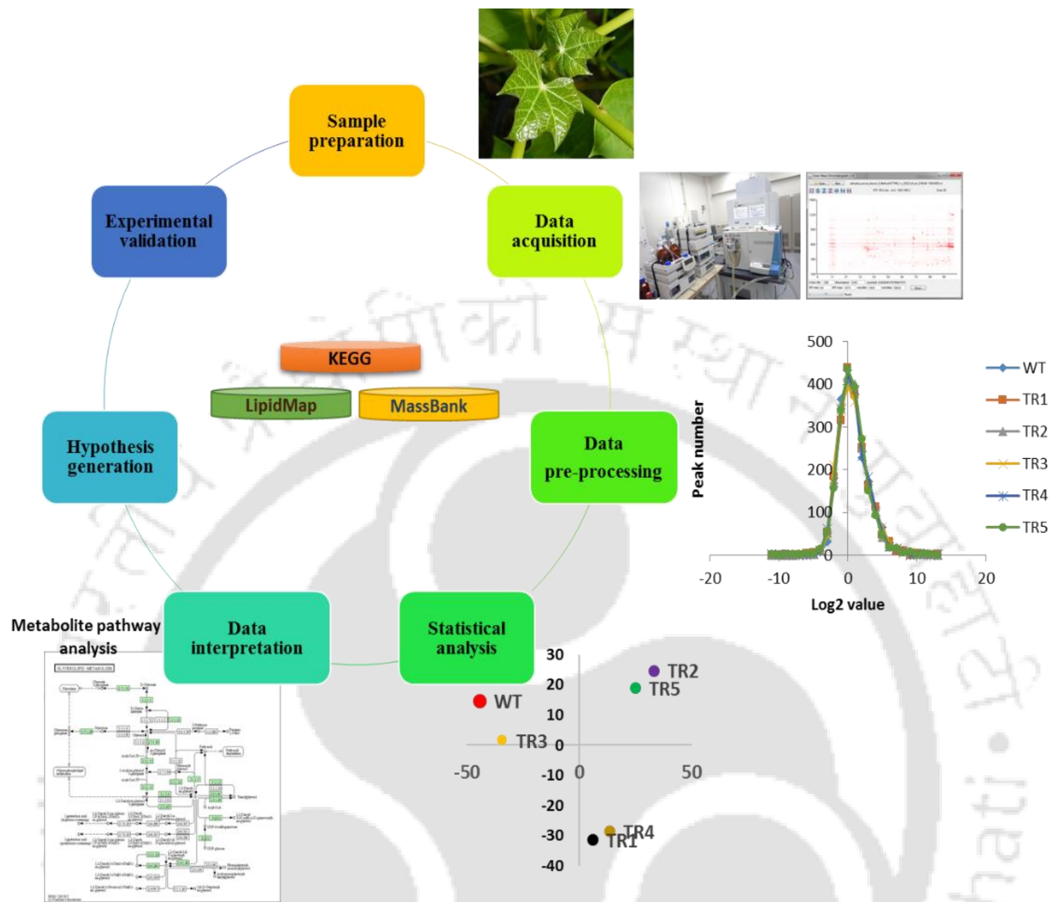

Figure 4.15 Outline of the metabolomic analysis of *AtDGAT1* overexpressing *Jatropha curcas*.

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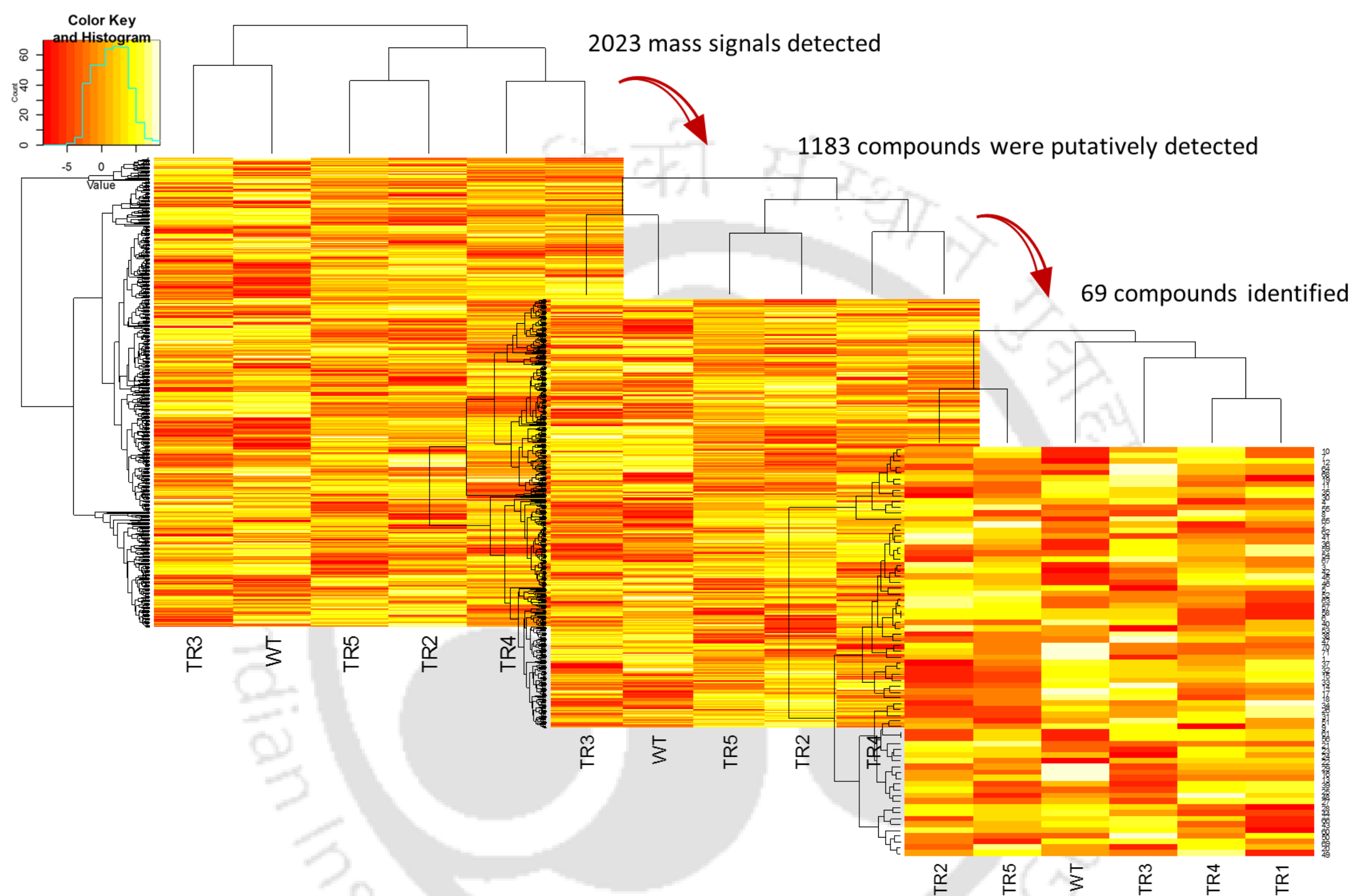

Figure 4.16 Heat map represents the successive metabolites selection from non-targeted metabolomics analysis. Total 69 metabolites were considered for further analysis which related to lipid metabolism and some secondary metabolites.

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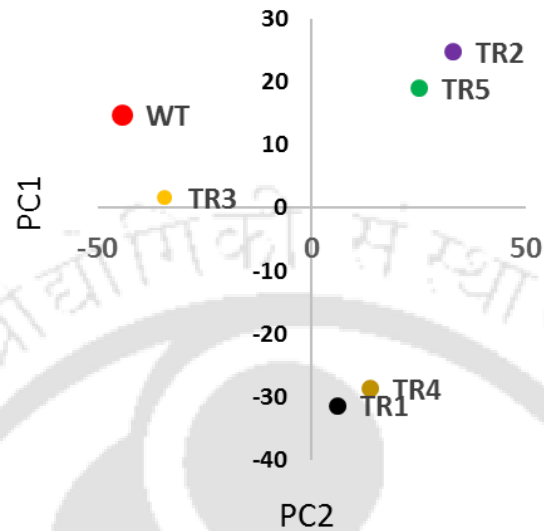

Figure 4.17 Score plot of Principle component analysis of the metabolites detected in *Jatropha curcas* leaves samples.

Analysis were carried out for all annotated 1183 metabolites. PCA score plot of all metabolites taken together with PC1 explaining 68.55% of the variation and PC2 explaining 25.19% of the variation.

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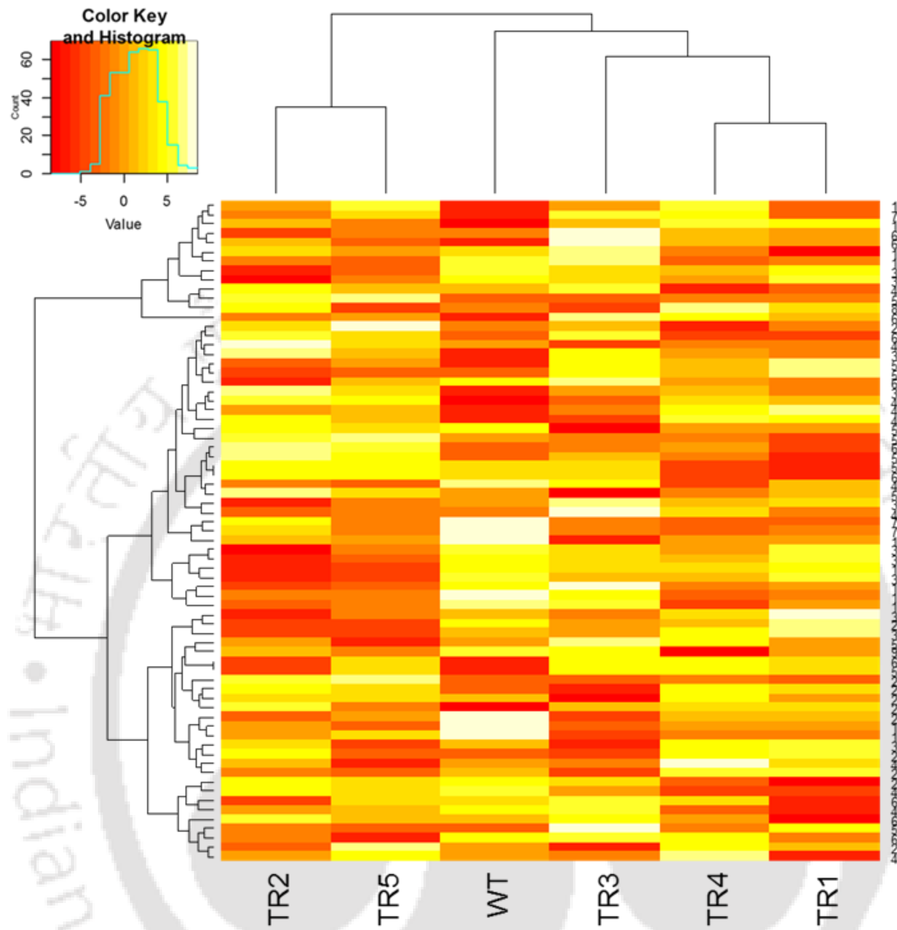

Figure 4.18 Heat map of the 69 metabolite detected in *Jatropha curcas* leaf samples.

A colour coded Matrix represents the intensities of annotated Metabolites in the transgenic and wild type *Jatropha curcas* plants. Values have been log2 transformed and normalized. Numerical represents the individual metabolites. Statistical analysis highlighted the most relevant compounds related to overexpression of *AtDGAT1* for each plants (TR)

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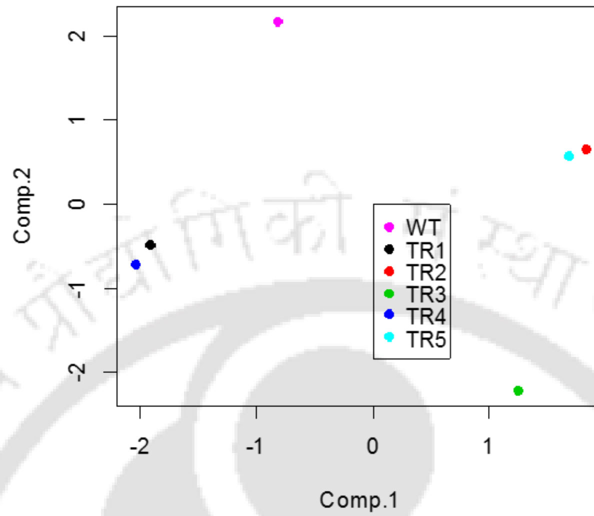

Figure 4.19 Score Plot of Principle component analysis of the metabolites detected in *Jatropha curcas* leaves samples.

Score plot clearly showed the separation of Wild type (WT) from transgenic lines (TR). Mean values are used for the analysis (n=3).

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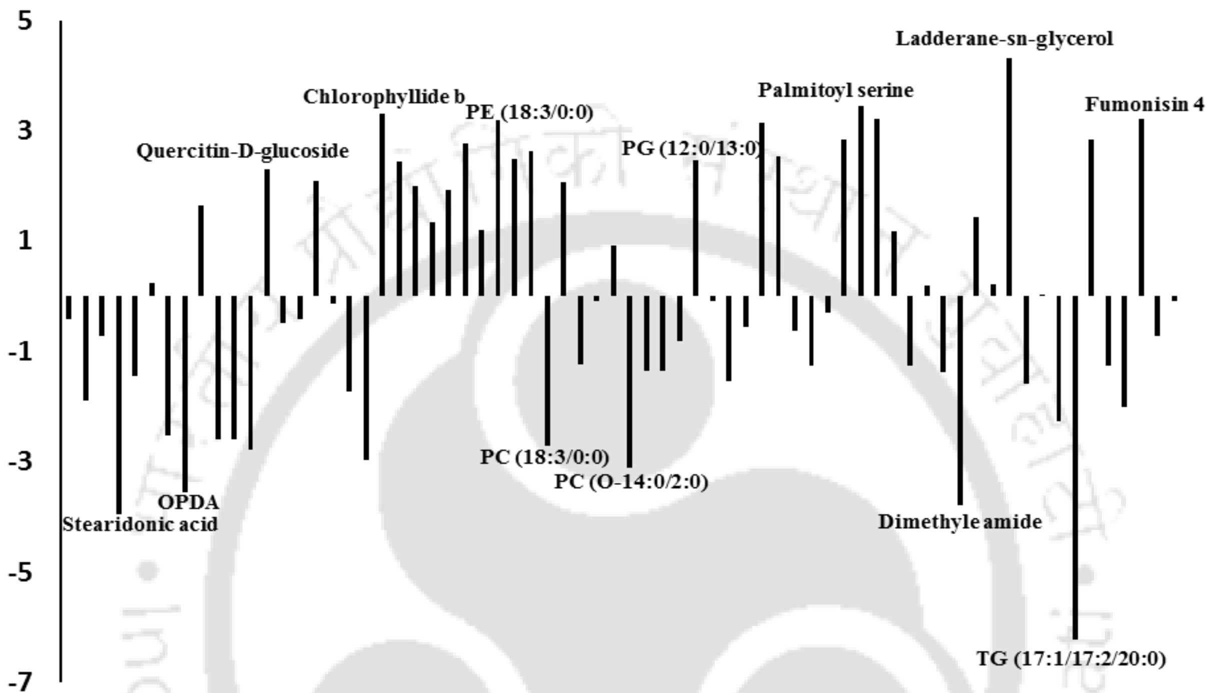

Figure 4.20 PCA loading plot explained the positively and negatively correlated metabolites.

In loading plot ladderane-sn-glycerol shows their positive and TG (17:1/17:2/20:0) showed highly negative correlation with other metabolites.

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Figure 4.21 A principle component analysis biplot diagram showing similarity relationship among identified metabolites.

The direction and length of arrow indicate how each sample contributes to first two components in biplot. These two components explained 80% and 7% of the variance respectively.

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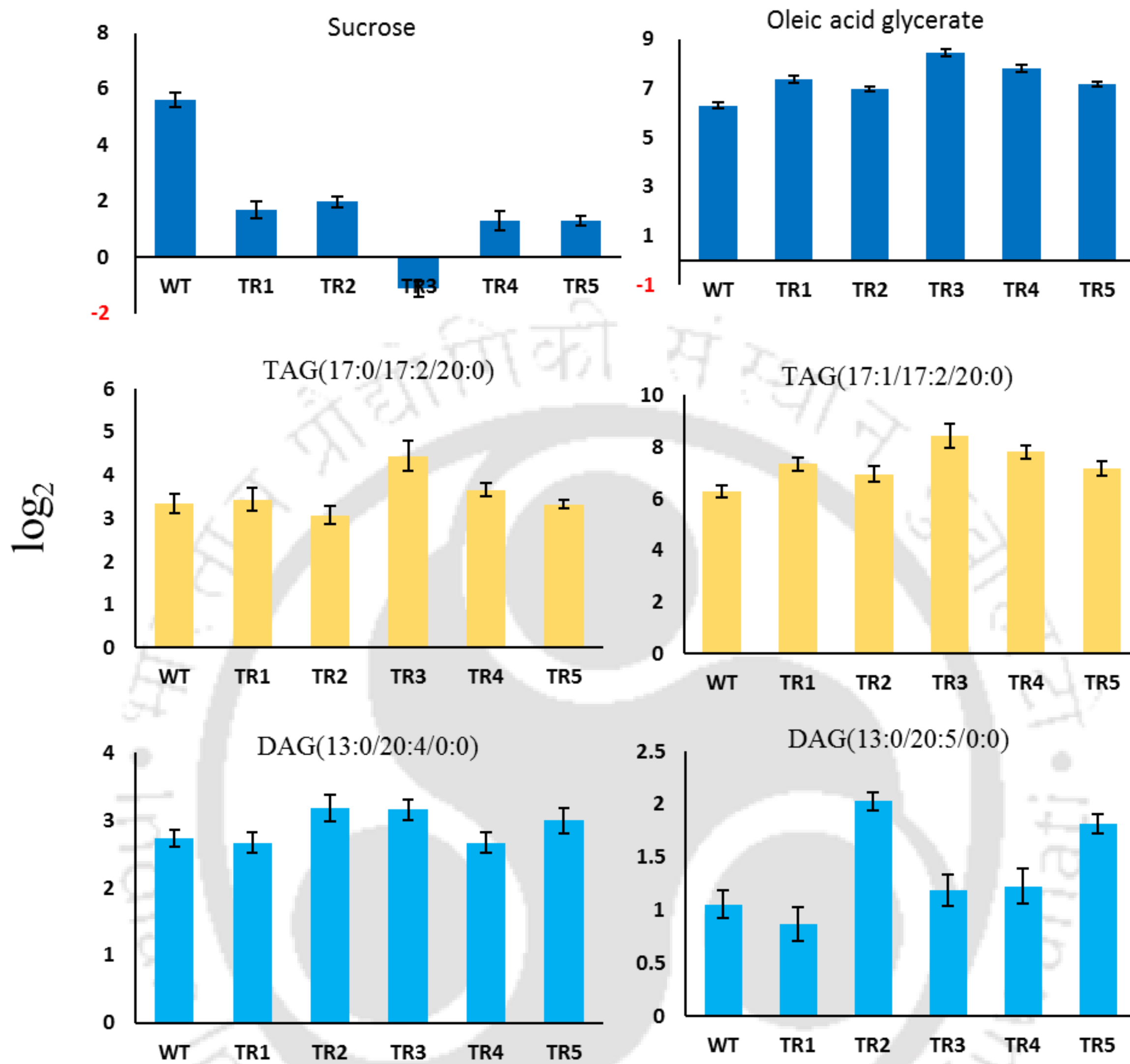

Figure 4.22 Metabolite changes between transgenic lines and wild type *Jatropha curcas*.

The ratio of the levels of these metabolites between transgenic lines and wild type.

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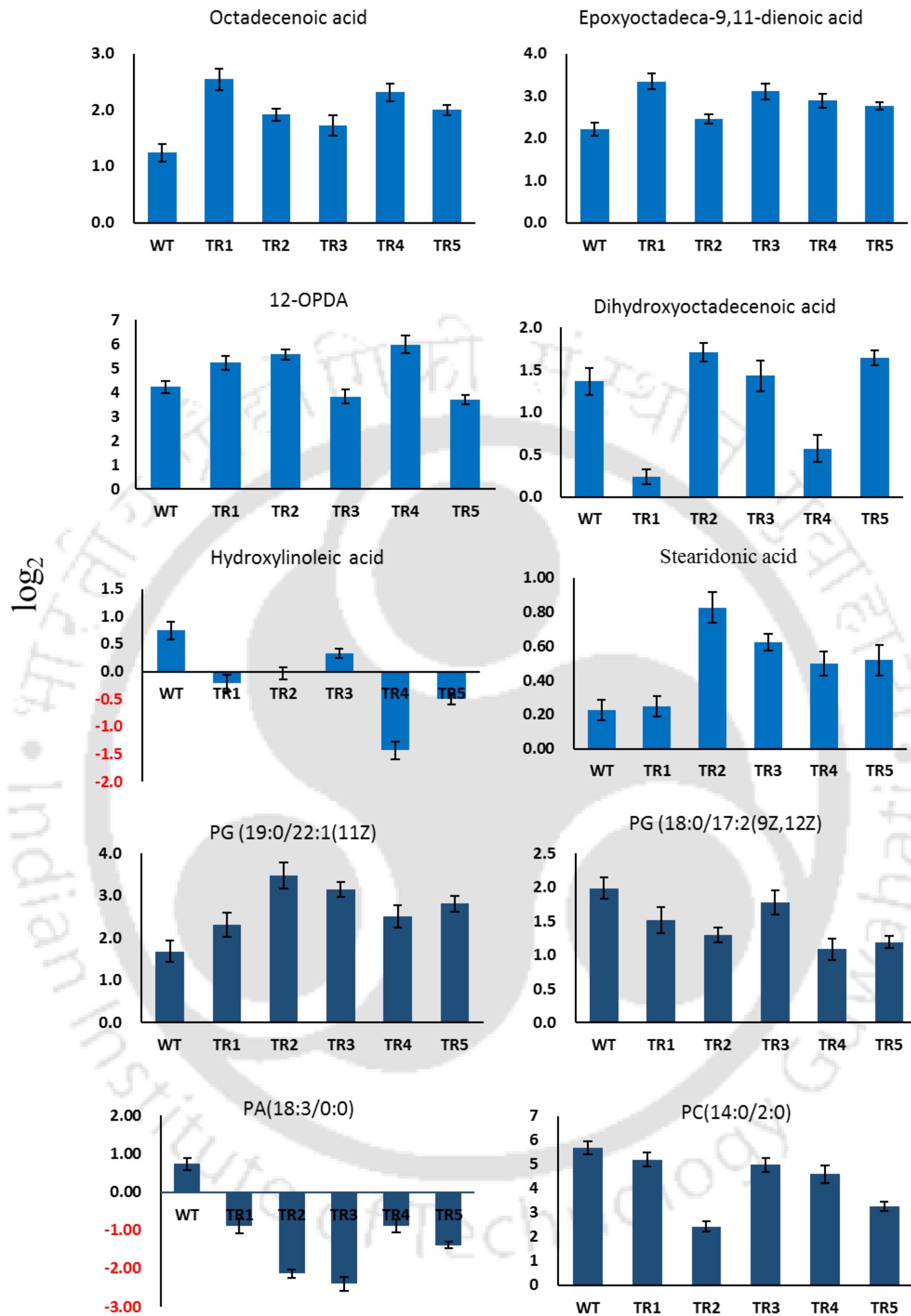

Figure 4.23 Metabolite changes between transgenic lines and wild type *Jatropha curcas*.

The ratio of the levels of these metabolites between transgenic lines and wild type. Lipid derivatives were presented.

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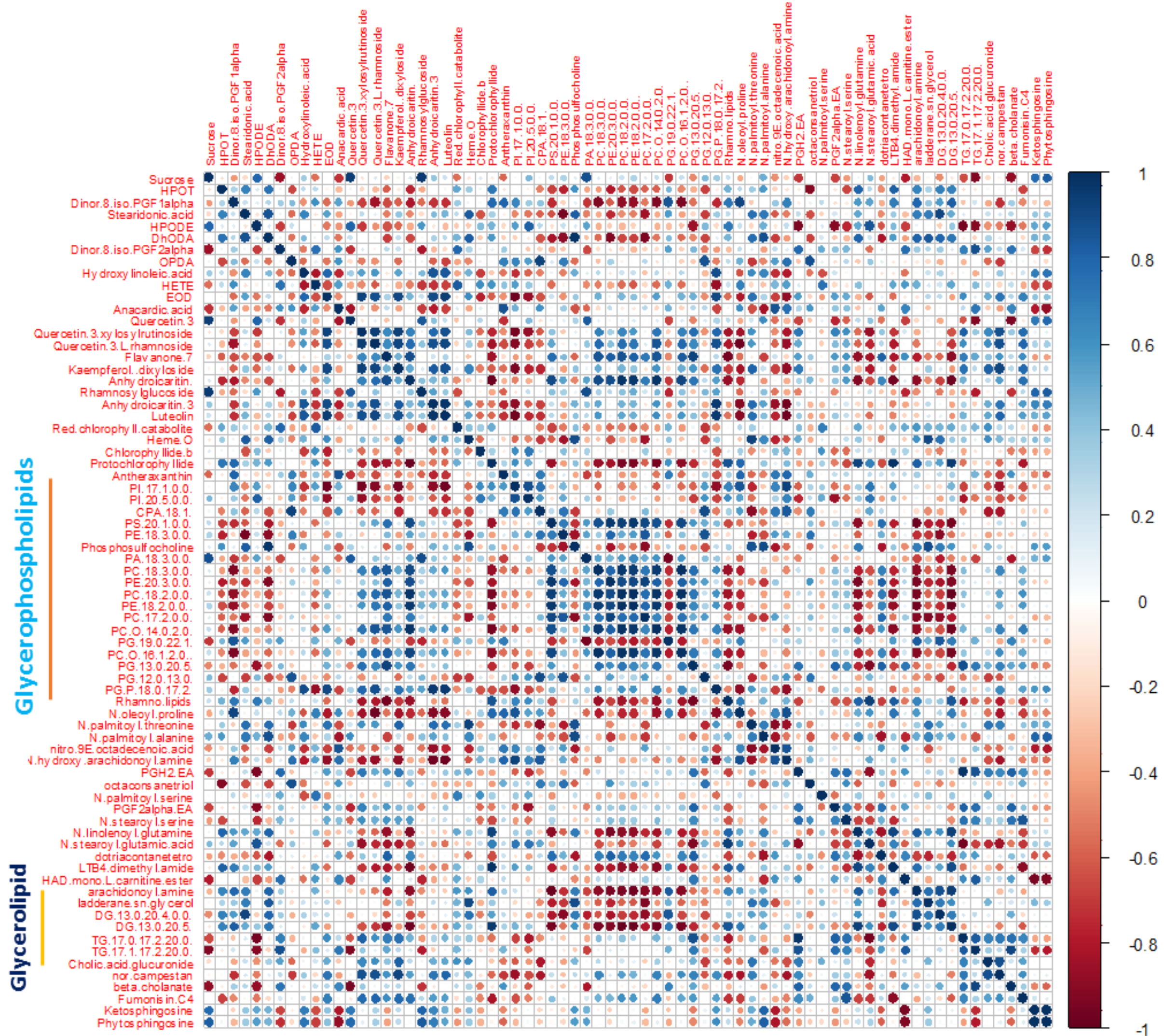

Figure 4.24 Metabolite-metabolite correlations.

Positive correlations are shown in blue; negative correlations are shown in red.

Table 4.4 Annotated metabolites by LC-FT-ICR-MS and fold of metabolites between transgenic *Jatropha curcas* and wild type.

SN	Metabolites	Mol formula	ID	Pathway	RT	m/z	fold (WT/Av TR)
1	Sucrose	$C_{12}H_{22}O_{11}$	C00089	D-Glucose metabolism	12.268	381.079	23.59
2	13(S)-HPOT	$C_{18}H_{30}O_4$	C04785	Alpha Linolenic acid metabolism	59.887	311.222	0.85
3	2,3-Dinor-8-iso PGF1alpha	$C_{18}H_{32}O5$	C14795	Arachidonic acid metabolism	59.892	621.436	0.57
4	Stearidonic acid	$C_{18}H_{28}O_2$	C16300	Alpha Linolenic acid metabolism	84.457	277.216	1.03
5	13(S)-HPODE	$C_{18}H_{32}O_4$	C04717	Linoleic acid metabolism	77.009	313.237	1.99
6	(9z)-(7S, 8S)-DhODA	$C_{18}H_{34}O_4$	C07355	Hydroxy Fatty acyls	79.249	315.253	1.19
7	2,3-Dinor-8-iso PGF2alpha	$C_{18}H_{30}O_5$	C14794	Arachidonic acid metabolism	83.156	327.216	0.52
8	12-OPDA	$C_{18}H_{28}O_3$	C01226	Alpha-Linolenic acid metabolism	84.146	293.211	0.64
9	8-Hydroxylinoleic acid	$C_{18}H_{32}O_3$	C08318	Hydroxy Fatty acyls	86.506	297.242	2.17
10	15(S)-HETE	$C_{20}H_{32}O_3$	C04742	Arachidonic acid metabolism	89.609	321.242	0.54
11	13(S)-EOD	$C_{18}H_{30}O_3$	C04594	Epoxy Fatty acids	92.248	295.227	1.72
12	Anacardic acid	$C_{22}H_{36}O_3$	C10759	-	96.203	349.274	0.27
13	Quercetin*	$C_{26}H_{28}O_{16}$	C12637	Flavonol biosynthesis	33.727	656.218	2.63
14	Quercetin 3-(2G-xylosylrutinoside)	$C_{32}H_{38}O_{20}$	C10175	Flavonol biosynthesis	34.369	743.203	2.43
15	Quercetin 3-L-rhamnoside	$C_{33}H_{40}O_{14}$	LMPK12112007	Flavonoids	40.687	449.107	4.76
16	Flavanone*	$C_{27}H_{32}O_{13}$	C09828	Flavonoids	46.983	549.196	1.05
17	Kaempferol 3,4'-dixyloside	$C_{33}H_{40}O_{14}$	LMPK12112007	Flavonoids	38.811	565.155	2.09
18	Red chlorophyll catabolite	$C_{15}H_{10}O_6$	C01514	Flavonoids	79.154	627.281	0.46
19	Heme O	$C_{49}H_{58}N_4O_5Fe_1$	C15672	Chlorophyll metabolism	78.448	839.384	1.24
20	Chlorophyllide b	$C_{35}H_{32}N_4O_6Mg_1$	C16541	Chlorophyll metabolism	55.705	629.224	0.93
21	Protochlorophyllide	$C_{35}H_{32}N_4O_5Mg_1$	C02880	Chlorophyll metabolism	60.173	613.227	0.58
22	Antheraxanthin	$C_{40}H_{56}O_3$	C08579	Carotenoid biosynthesis	98.327	585.431	0.42
23	PI (17:1(10Z)/0:0)	$C_{26}H_{49}O_{12}P_1$	LMGP06050003	Glycerophospholipids	49.677	585.303	0.6
24	PI (20:5(5Z,8Z,11Z,14Z,17Z)/0:0)	$C_{29}H_{47}O_{12}P_1$	LMGP06050026	Glycerophospholipids	50.905	619.287	1
25	CPA (18:1(9Z))	$C_{21}H_{39}O_6P_1$	LMGP00000056	Glycerophospholipids	51.282	419.254	0.53

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26	PS (20:1(11Z)/0:0))	$C_{26}H_{50}N_{1}O_{9}P_{1}$	LMGP03050020	Glycerophospholipids	58.98	552.33	1.84
27	PE (18:3(6Z,9Z,12Z)/0:0)	$C_{23}H_{42}N_{1}O_{7}P_{1}$	LMGP02050017	Glycerophospholipids	64.401	476.277	1.07
28	Phosphosulfocholine*	$C_{35}H_{69}O_{8}P_{1}S_{1}$	LMGP00000051	Glycerophospholipids	66.615	681.454	1.78
29	PA (18:3(6Z,9Z,12Z)/0:0)	$C_{21}H_{37}O_{7}P_{1}$	LMGP10050023	Glycerophospholipids	71.899	433.235	4.85
30	PC (18:3(9Z,12Z,15Z)/0:0)	$C_{26}H_{48}N_{1}O_{7}P_{1}$	LMGP01050038	Glycerophospholipids	75.39	518.324	3.56
31	PE (20:3(8Z,11Z,14Z)/0:0)	$C_{25}H_{46}N_{1}O_{7}P_{1}$	LMGP02050022	Glycerophospholipids	76.92	504.308	1.51
32	PC (18:2(2E,4E)/0:0)	$C_{26}H_{50}N_{1}O_{7}P_{1}$	LMGP01050034	Glycerophospholipids	78.828	520.339	3.15
33	PE (18:2(9Z,12Z)/0:0)	$C_{23}H_{44}N_{1}O_{7}P_{1}$	LMGP02050011	Glycerophospholipids	79.041	478.292	4
34	PC (17:2(9Z,12Z)/0:0)	$C_{25}H_{48}N_{1}O_{7}P_{1}$	LMGP01050127	Glycerophospholipids	81.866	506.324	1.11
35	PC (O-14:0/2:0))	$C_{24}H_{50}N_{1}O_{7}P_{1}$	LMGP01020019	Glycerophospholipids	83.987	496.34	3.03
36	PG (19:0/22:1(11Z))	$C_{47}H_{91}O_{10}P_{1}$	LMGP04010476	Glycerophospholipids	84.457	847.645	0.44
37	PC (O-16:1(11Z)/2:0)	$C_{26}H_{52}N_{1}O_{7}P_{1}$	LMGP01020147	Glycerophospholipids	86.478	522.355	3.62
38	PG (13:0/20:5(5Z,8Z,11Z,14Z,17Z)	$C_{39}H_{67}O_{10}P_{1}$	LMGP04010087	Glycerophospholipids	91.412	727.454	0.8
39	PG (12:0/13:0)	$C_{31}H_{61}O_{10}P_{1}$	LMGP04010001	Glycerophospholipids	97.834	625.407	1.2
40	PG (P-18:0/17:2(9Z,12Z)	$C_{41}H_{77}O_{9}P_{1}$	LMGP04030038	Glycerophospholipids	43.808	745.539	1.53
41	Rhamno lipids	$C_{36}H_{64}O_{10}$	LMFA13030004	Fatty acyls	64.233	657.457	0.86
42	N-oleoyl proline	$C_{23}H_{41}N_{1}O_{3}$	LMFA08020118	Fatty acyls	65.365	380.316	0.39
43	N-palmitoyl threonine	$C_{20}H_{39}N_{1}O_{4}$	LMFA08020107	Fatty acyls	68.618	358.295	2.83
44	N-palmitoyl alanine	$C_{19}H_{37}N_{1}O_{3}$	LMFA08020123	Fatty acyls	71.876	328.284	4.48
45	10-nitro-9E-octadecenoic acid	$C_{18}H_{33}N_{1}O_{4}$	LMFA01120003	Fatty acyls	75.985	328.248	0.55
46	N-hydroxy arachidonoyl amine	$C_{20}H_{33}N_{1}O_{2}$	LMFA08020029	Fatty acyls	74.488	320.258	0.26
47	PGH2-EA	$C_{23}H_{39}N_{1}O_{4}$	LMFA03010219	Fatty acyls	76.37	394.295	0.71
48	Octaconsanetriol*	$C_{34}H_{68}O_{8}$	LMFA13010009	Fatty acyls	78.084	605.496	0.78
49	N-palmitoyl serine	$C_{19}H_{37}N_{1}O_{4}$	LMFA08020101	Fatty acyls	78.966	344.279	0.95
50	PGF2alpha-EA	$C_{22}H_{39}N_{1}O_{5}$	LMFA03010075	Fatty acyls	82.401	398.29	0.67
51	N-stearoyl serine	$C_{21}H_{41}N_{1}O_{4}$	LMFA08020102	Fatty acyls	83.398	372.311	0.85
52	N-linolenoyl-glutamine	$C_{23}H_{38}N_{2}O_{4}$	LMFA00000004	Fatty acyls	83.656	407.29	0.79
53	N-stearoyl glutamic acid	$C_{23}H_{43}N_{1}O_{5}$	LMFA08020090	Fatty acyls	90.797	414.321	1
54	α-D-glucopyranosyl*	$C_{38}H_{76}O_{9}$	LMFA13010017	Fatty acyls	102.408	677.554	0.74
55	LTB4 dimethyl amide	$C_{22}H_{37}N_{1}O_{3}$	LMFA03020011	Fatty acyls	92.802	364.284	0.52

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56	HAD mono-L-carnitine ester	$C_{23}H_{43}N_1O_6$	LMFA07070007	Fatty acyls	94.083	430.316	0.24
57	Arachidonoyl amine*	$C_{24}H_{41}N_1O_3$	LMFA08020028	Fatty acyls	100.381	392.316	0.7
58	ladderane-octanyl-sn-glycerol*	$C_{41}H_{66}O_3$	LMGL02030029	Glycerolipids	81.446	607.511	1.15
59	DG(13:0/20:4(5Z,8Z,11Z,14Z)/0:0)	$C_{36}H_{62}O_5$	LMGL02010368	Glycerolipids	88.911	575.467	0.87
60	ladderane-octanyl-sn-glycerol*	$C_{41}H_{64}O_4$	LMGL02070007	Glycerolipids	91.44	621.49	0.24
61	DG(13:0/20:5(5Z,8Z,11Z,14Z,17Z)/0:0)	$C_{36}H_{60}O_5$	LMGL02010369	Glycerolipids	99.793	573.452	0.77
62	TG(17:0/17:2(9Z,12Z)/20:0)[iso6])	$C_{57}H_{106}O_6$	LMGL03010203	Glycerolipids	103.307	887.81	0.85
63	TG(17:1(9Z)/17:2(9Z,12Z)/20:0)	$C_{57}H_{104}O_6$	LMGL03010233	Glycerolipids	100.965	885.791	0.42
64	Cholic acid glucuronide	$C_{30}H_{48}O_{11}$	LMST05010044	Sterol Lipids	77.284	585.327	1.45
65	27-nor-campestan*	$C_{27}H_{48}O_9$	LMST01031078	Sterol Lipids	87.574	534.363	1.48
66	3,7alpha-Dihydroxy-5beta-cholanate	$C_{24}H_{38}O_5$	LMST04010176	Sterol Lipids	86.927	407.279	0.59
67	Fumonisin C4	$C_{33}H_{57}N_1O_{13}$	LMSP01080030	Sphingolipids	67.335	676.39	1.46
68	3-Ketosphingosine	$C_{18}H_{35}N_1O_2$	LMSP01010002	Sphingolipids	69.094	298.274	12.84
69	Phytosphingosine	$C_{18}H_{39}N_1O_3$	LMSP01030001	Sphingolipids	69.81	318.3	13.63

Quercetin* Quercetin 3-O-[beta-D-xylosyl-(1->2)-beta-D-glucoside]
Flavanone* Flavanone 7-O-[alpha-L-rhamnosyl-(1->2)-beta-D-glucoside]
Phosphosulfocholine* 1,2-ditetradecanoyl-sn-glycero-3-phosphosulfocholine
Octaconsanetriol* 1-(O-α-D-glucopyranosyl)-(1,3R,27R)-octaconsanetriol
α-D-glucopyranosyl* 1-(O-α-D-glucopyranosyl)-(1,3R,29S,31R)-dotriacontanetetro
Ladderane-octanyl-sn-glycerol* 1-(6-[5]-ladderane-hexanyl)-2-(8-[3]-ladderane-octanyl)-sn-glycerol
Arachidonoyl amine* N,N-(2,2-dihydroxy-ethyl) arachidonoyl amine
Ladderane-octanyl-sn-glycerol** 1-(6-[5]-ladderane-hexanoyl)-2-(8-[3]-ladderane-octanyl)-sn-glycerol
27-nor-campestan* 27-nor-campestan-3β,4β,5α,6α,7β,8β,14α,15α,24-nonol
3,7alpha-Dihydroxy-5beta-cholanate* 3alpha,7alpha-Dihydroxy-12-oxo-5beta-cholanate

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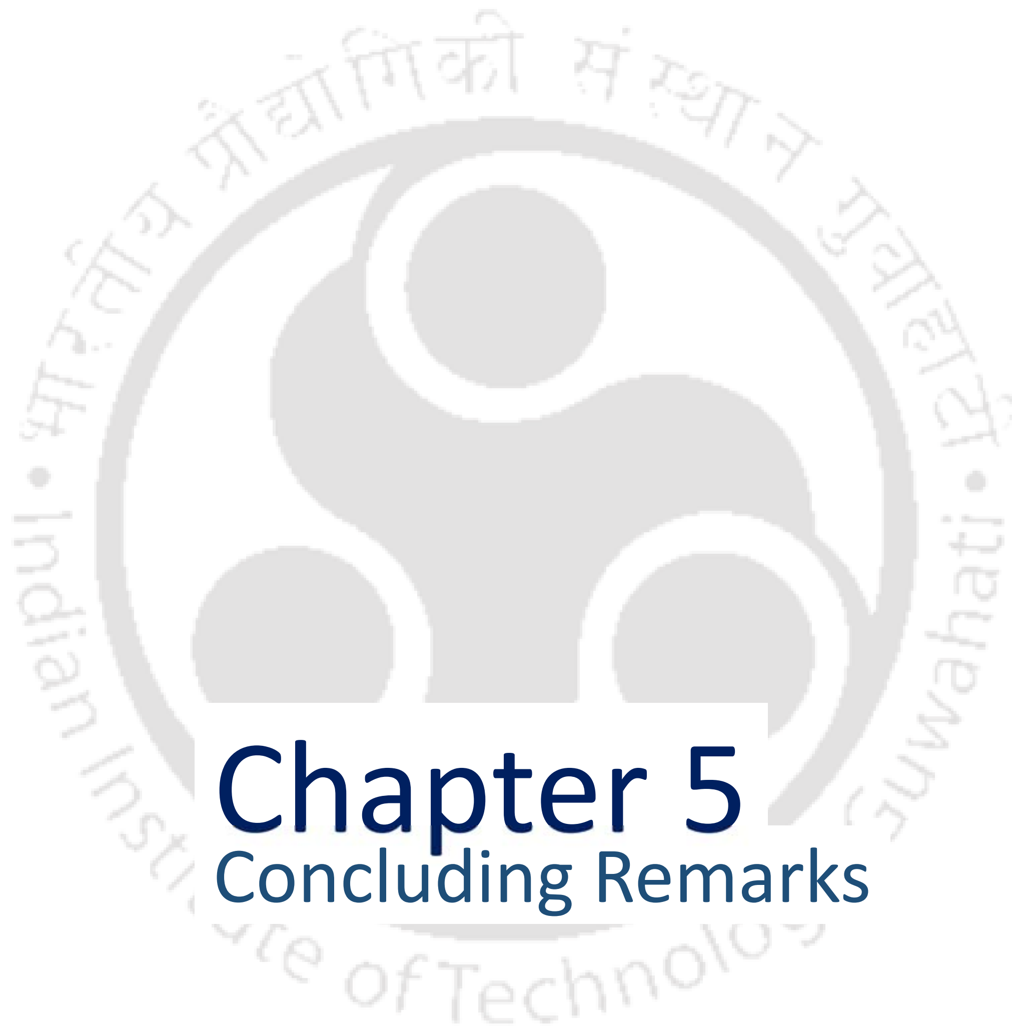

Chapter 5
Concluding Remarks

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5.1 Significance and salient features of the study

Jatropha curcas has attracted a great deal of global attention as drought resistant oil rich biodiesel plant. It is believed that *J. curcas* can be used to alleviate the energy crisis and is becoming one of the world's promising crop for biodiesel production. However, Jatropha must not directly compete with food crops due to possible food crisis and limited farmland in the world. Jatropha can be used to rehabilitate wastelands and improve the environment. However, the net financial gain from Jatropha is low as the plant often suffers from seed toxicity, poor and inconsistent seed and oil yield. Development of improved varieties of *J. curcas* using genetic engineering and molecular breeding tools is required for commercial exploitation of plant for enhance yield and improved fuel properties.

In the present study, we demonstrated that, the upregulation of TAG biosynthesis by ectopic overexpression of *AtDGAT1* in transgenic *J. curcas* plants leads to an enhanced accumulation of TAGs in leaves as well as seeds without compensating plant and seed traits. In principle, accumulation of oils in leaf foliage and seeds provides an opportunity to enhance the energy density of the biomass and thus have significant impact on biofuel production. This is the first report in Jatropha demonstrating enhanced oil accumulation in both seeds and vegetative tissues. These promising results are a first step towards making an economically viable biofuel crop through transgenic approach. Although a lot of future efforts are to be made to look at partitioning substantial carbon into TAG in vegetative tissues in addition to enhanced accumulation in seeds to make it highly sustainable, our results support the basic feasibility of a strategy to redirect carbon partitioning from starch to oil in plant biomass. The resulting seed oil content changes may have commercial significance in terms of increasing oil content for greater productivity.

Plant metabolomics is an emerging field in plant science and is applied to diverse plant systems in order to elucidate the regulation of growth and development. The aim in plant

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metabolomics is to analyze, identify and quantify all low molecular weight molecules of plants. In order to compare the metabolome of different plants in high through put approach, a number of biological analytical and data processing tools have to be performed. In the present thesis work high through-put non-targeted approach has been performed for routine analysis of plant metabolite profile using liquid chromatography-FT-ICR coupled with MS. However, it is the most powerful tool among other MS instrument to fingerprint complex samples, due to its extremely high resolution and high mass precision It was found that sucrose concentration was decreased in all transgenic lines, which indicates towards utilization of carbon source was significantly enhanced in transgenic lines. Several lipids such as, fatty acyls, glycophospholipids, glycolipids, sterol lipids, sphingolipids, PI, PE, apart from that many falvonoids, alakloids and other secondary metabolites were identified. Abundance of lipid derivatives indicating the significant changes between transgenic lines and wild type *Jatropha curcas*. In addition to the differences in metabolites composition observed between the parental and genetically modified Jatropha lines, significantly different metabolite profiles were characterized. The vast array of lipids and their derivative and flavonoids and other secondary metabolites tentatively identified in this study, suggest an important role for the metabolite class in Jatropha oil biosynthesis pathways.

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5.2 The salient features of the present study are summarized below:

- *Arabidopsis thaliana Diacyl glycerol acyltransferase* 1 (*AtDGAT1*) was successfully cloned into a plant binary expression vector and mobilized to *Agrobacterium tumefaciens*.
- *Agrobacterium* mediated Jatropha transformation was performed to develop *AtDGAT1* overexpression lines.
- Transgenic Jatropha plants overexpressing *AtDGAT1* lines developed and transgenic event were confirmed by molecular analysis as well as expression analysis.
- The leaf biomass and seeds were successfully characterized for enhanced TAG accumulation for biodiesel production.
- Non-targeted metabolomics analysis of transgenic lines was performed by LC-FT-ICR coupled with MS.

This study will provide guidelines for successful exploitation of *Jatropha curcas* for genetic improvement of the plant with enhanced oil content and improved oil properties for biodiesel production.

Future prospects

Jatropha curcas is an important biofuel plant due to the presence of high amount of oil in its seeds suitable for biodiesel production. Triacylglycerols (TAGs) are the most abundant form of storage oil in plants. Diacylglycerol *O*-acyltransferase (*DGAT1*) enzyme is responsible for the last and only committed step in seed TAG biosynthesis. Transgenic plants had reduced FFA content compared with control plants. Our strategy of increasing energy density by enhancing oil accumulation in both seeds and leaves in Jatropha would make it economically more sustainable for biofuel production. Direct upregulation of TAG biosynthesis in seeds and vegetative tissues through overexpression of the *DGAT1* could enhance the energy density of the biomass, making significant impact on biofuel production.

Thus it can be concluded that the studies reported here *can be used to cultivate J. curcas* improvement program and greater economic returns.

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List of Publications

Journals/Chapter/Published

1. **Maravi DK**, Kumar S, Sharma P, Kobayashi Y, Goud VV, Sakurai N, Koyama H, Sahoo L (2016) Ectopic expression of *AtDGAT1*, encoding diacylglycerol O-acyl transferase exclusively committed to TAG biosynthesis, enhances oil accumulation in seeds and leaves of Jatropha. **Biotechnology for Biofuels**, 9:226.
2. **Maravi DK**, Sahoo AK, Goswami U, Prasad DS, Sahoo L, Ghosh SS (2015) Phytogenic "Green synthesis" of silver nano particles with enhanced antibacterial and anti-cancer activity. **International J Pharma and Biosciences**, 6(4), 482-493.
3. **Maravi DK**, Alam S, Mazumdar P, Goud VV, Sahoo L, book chapter titled as *Jatropha curcas* L., in ***Agrobacterium* protocols** 'Volume 2 (Springer: ISBN:978-1-4939-1657-3).

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1. **Maravi DK**, Ksunoki K, Sharma P, Sakurai N, Goud VV, Koyama H, Sahoo L (2016) Non-targeted metabolomics analysis of Jatropha curcas overexpressing Arabidopsis thaliana DGAT1. **Metabolomics** (Communicated).

Conferences and workshop

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2. **Maravi DK**, Goud VV, Sahoo L (2015) Enhanced triacylglycerol (TAG) synthesis in Jatropha curcas leaves following overexpression of an Arabidopsis Diacylglycerol acyl transferase 1. National Conference on Biotechnology and Human Welfare: New Vistas, **21-22 March 2015**. VBSP University Jaunpur, UP.
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Biotechnology for Biofuels

RESEARCH Open Access

Ectopic expression of *AtDGAT1*, encoding diacylglycerol *O*-acyltransferase exclusively committed to TAG biosynthesis, enhances oil accumulation in seeds and leaves of Jatropha

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Abstract

Background: *Jatropha curcas* is an important biofuel crop due to the presence of high amount of oil in its seeds suitable for biodiesel production. Triacylglycerols (TAGs) are the most abundant form of storage oil in plants. Diacylglycerol *O*-acyltransferase (DGAT1) enzyme is responsible for the last and only committed step in seed TAG biosynthesis. Direct upregulation of TAG biosynthesis in seeds and vegetative tissues through overexpression of the *DGAT1* could enhance the energy density of the biomass, making significant impact on biofuel production.

Results: The enzyme diacylglycerol *O*-acyltransferase is the rate-limiting enzyme responsible for the TAG biosynthesis in seeds. We generated transgenic Jatropha ectopically expressing an *Arabidopsis DGAT1* gene through *Agrobacterium*-mediated transformation. The resulting *AtDGAT1* transgenic plants showed a dramatic increase in lipid content by 1.5- to 2 fold in leaves and 20–30 % in seeds, and an overall increase in TAG and DAG, and lower free fatty acid (FFA) levels compared to the wild-type plants. The increase in oil content in transgenic plants is accompanied with increase in average plant height, seeds per tree, average 100-seed weight, and seed length and breadth. The enhanced TAG accumulation in transgenic plants had no penalty on the growth rates, growth patterns, leaf number, and leaf size of plants.

Conclusions: In this study, we produced transgenic Jatropha ectopically expressing *AtDGAT1*. We successfully increased the oil content by 20–30 % in seeds and 1.5- to 2.0-fold in leaves of Jatropha through genetic engineering. Transgenic plants had reduced FFA content compared with control plants. Our strategy of increasing energy density by enhancing oil accumulation in both seeds and leaves in Jatropha would make it economically more sustainable for biofuel production.

Keywords: AtDGAT1, Biodiesel, Jatropha, Leaf, Seed oil, Transgenic, Triacylglycerols (TAGs)

Background

The diminishing fossil fuel stock and soaring international crude oil price have renewed the interest in alternative sources of fuels. Oils from oilseed crops that are largely in the form of triacylglycerols (TAG) are the promising source of renewable supply of fuels in the form of biodiesel [1, 2]. *Jatropha curcas* L. is an important non-edible oilseed crop which received worldwide attention as a biodiesel feedstock. It is a shrub grown in tropical and subtropical regions of the world. The seeds which contain 30–42 % of oil can be directly blended with diesel

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or transesterified for use as biodiesel. In addition to high oil content, favorable oil composition for biodiesel such as seed oil with approximately 75 % unsaturated fatty acids (FAs) [3, 4], and a high level (around 47 %) of linoleic acid (C18:2) [5], Jatropha plants have a short gestation period, easy adaptation to various agroclimatic conditions [6, 7], and ability to grow on marginal and semi-marginal lands, making this plant the most sought oilseed crop among the non-edible oil-yielding crops for biodiesel production [8].

Despite the significance of Jatropha seed oil as a potential source for biodiesel, not much research efforts have been made through breeding or transgenic approaches to improve its seed oil content and quality for sustainable biodiesel production. Transgenic approaches offer immense opportunities to improve oil content and quality through manipulation of oil biosynthetic pathway in both seed and leaves [9–11].

TAGs, which consist of three FA chains (usually C16 or C18) covalently linked to glycerol, serve as an energy reserve for the seed germination, and seedling growth and development. Depending on the source of plants, TAGs may contain FAs with different chain lengths and extent of saturation, and diverse modified FAs [10]. Plant TAGs are generally stored in small organelles, oil bodies, which are assembled in the developing seeds, flower petals, pollen grains, and fruits of a large number of plant species [12, 13]. A series of condensation, reduction, and dehydration reactions led to fatty acid synthesis in plastid, and the free fatty acids (FFAs) are transported to endoplasmic reticulum (ER). FFAs are then involved in sequential acylation of the sn-1, sn-2, and sn-3 positions of glycerol-3-phosphate with acyl-CoA to finally yield TAG through Kennedy pathway.

In the Kennedy pathway, diacylglycerol acyltransferase (DGAT), which catalyzes the terminal step, is the only enzyme that is exclusively committed to TAG biosynthesis using acyl-CoA as its acyl donor [14] and plays a vital role in diverting fatty acid flux towards the formation of TAGs [15, 16]. Two different DGAT gene family members, DGAT1 and DGAT2 that differ considerably in sequence, have been attributed to have a non-redundant role in TAG biosynthesis [17, 18]. However, the preferences for either of these two forms for TAG production and its accumulation during seed development have been found to be species specific [19]. Ever since *DGAT1* gene from *Arabidopsis* was identified simultaneously by three laboratories [20–22], its homologues were subsequently reported from several other plants including tobacco [22], canola [23], castor bean [24], burning bush [25], soybean [26], peanut [27], tung tree [18], *Tropaeolum majus* [13], *J. curcas* [28–30], *Populus trichocarpa* [31], and Indian mustard [32]. Therefore, manipulation of DGAT1 gene expression has a significant effect on the improvement of the oil content and alteration of the fatty acid composition. *Arabidopsis* lines lacking DGAT1 were found deficient in DGAT activity and accumulated less oil with decreased TAG/DAG ratios [20, 21, 33, 34], while RNAi suppression of DGAT1 in tobacco resulted in decreased seed oil content and an increase in protein and carbohydrate [35]. On the contrary, overexpression of DGAT1 has lead to the increase in levels of oil in *Arabidopsis* [36], *Brassica napus* [32, 37], tobacco [38], soybean [39, 40], castor [41], maize [42, 43], and Indian mustard [32]. Although several genetic transformation methods have been reported for *J. curcas* [44–46], this is the first report of using genetic engineering approach to improve its oil quantity and quality in seeds and leaves.

In the present study, we demonstrate the constitutive overexpression of *AtDGAT1* results in the enhanced accumulation of TAGs and better oil attributes, in both seeds and leaves of transgenic Jatropha without compromising the seed yield, and morphological and developmental features.

Results

Generation of *AtDGAT1* overexpressing transgenic Jatropha using a constitutive promoter and molecular characterization

To investigate the impact of constitutive overexpression of *Arabidopsis DGAT1* cDNA on TAG accumulation in leaves and seeds of Jatropha, we prepared a 35S::AtDGAT1 construct that consisted of *AtDGAT1* fused to *gus* reporter gene driven by CaMV35S promoter and *nptII* as plant selection marker (Fig. 1A). We generated transgenic Jatropha plants harboring *AtDGAT1* through *Agrobacterium*-mediated transformation of cotyledonary leaf segment explants and selection of transformed shoots through kanamycin-based selection. Putative transformed shoots were rooted on kanamycin-free rooting medium and successfully hardened and acclimatized in greenhouse (Fig. 1B, a–g). Stable GUS expression was checked randomly in the cultures, at various developmental stages for validating the strength of kanamycin selection in recovery of transgenic plants. Stable GUS expression was detected in transformed cotyledonary leaf segment explants, developing shoot buds from callus, and regenerated shoots, leaf, stem, roots, and seeds of transgenic plants (Fig. 1C, a–m). These plants were confirmed by genotyping through genomic DNA PCR using primers specific to *AtDGAT1*, *nptII*, and *gus*. Amplification of 384-bp fragment corresponding to the *AtDGAT1*, a 540-bp fragment internal to *nptII*, and 400-bp internal to *gus* confirmed the transgenic plants (Fig. 2a–c).

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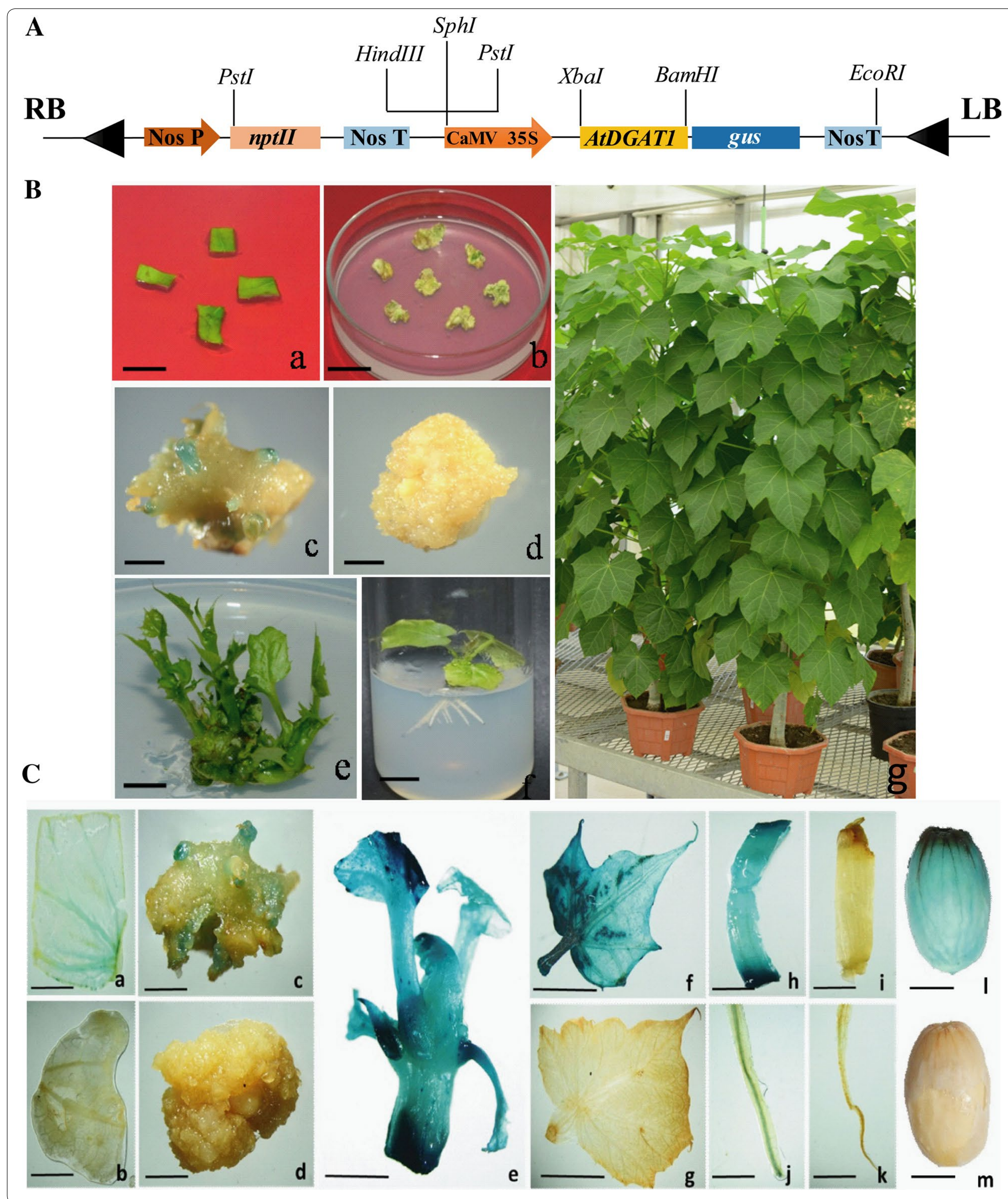

We randomly selected five *AtDGAT1* Jatropha transgenic lines and extracted RNA for expression analysis. Semi-quantitative RT-PCR analysis revealed abundance of AtDGAT1 mRNA in transgenic lines, albeit high expression in transgenic lines TR1, TR2, and TR3, and moderate expression in TR4 and TR5 in contrast to the

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Fig. 1 Development of transgenic *Jatropha curcas* plants expressing *AtDGAT1*. **A** T-DNA region (6.2 kb) of pBI121CaMV35S::*AtDGAT* plasmid. *RB* right border, *LB* left border, *CaMV 35S promoter Nos P* nopaline synthase promoter, *Nos T* nopaline synthase terminator, *NptII* neomycin phosphotransferase, ß-glucuronidase. **B** *Agrobacterium*-mediated genetic transformation of *Jatropha curcas* with 35S::*AtDGAT* construct: *a* excised cotyledonary leaf explants subjected to *Agrobacterium tumefaciens*-mediated transformation and cultured on kanamycin-free callus induction medium (*bar* 5 mm); *b* formation of callus from explant (*bar* 8 mm); *c* Stable GUS expression in buds induced from transformed callus (*bar* 8 mm); *d* untransformed control callus (*bar* 8 mm); *e* elongated putative transformed shoots on kanamycin selection (*bar* 1 cm); *f* rooted transformed plantlet (*bar* 1 cm); *g* acclimatized transformed plant. **C** Stable *gus* expression in transgenic plant tissues. *a* transformed cotyledonary leaf segment; *b* untransformed leaf segment; *c* emerging shoots from callus showing *gus* expression; *d* callus from untransformed tissues; *e gus* expression in in vitro shoot; *f gus* expression in leaf; *g* control leaf; *h gus* expression in stem; *i* control stem; *j* gus expression in transgenic root and *k* control root; *l gus* expression in transgenic seeds and *m*, control seed. (*Bar* 0.5 cm)

wild type (Fig. 2d). We generated several independent lines expressing *AtDGAT1* in which the growth rates, growth patterns, leaf number, and leaf size were all observed normal as compared to their counterpart wild type.

Five randomly selected PCR-positive independent T_0 transgenic Jatropha lines recovered on kanamycin selection medium were screened by Southern hybridization to confirm the integration of *nptII* gene using a 0.54 kb *nptII* probe. Two of the transgenic lines (TR3 and TR4) exhibited single copy integration events (Fig. 2e), whereas the three transgenic lines (TR1, TR2, and TR5) showed integration of two copies (Fig. 2e). No hybridization signal was detected in control WT plant (Fig. 2e).

AtDGAT1 overexpressing transgenic Jatropha plants accumulated enhanced levels of storage lipids in their leaves and seed kernels

We generated AtDGAT1 transgenic Jatropha plants to see if an overexpression of AtDGAT1 in Jatropha would

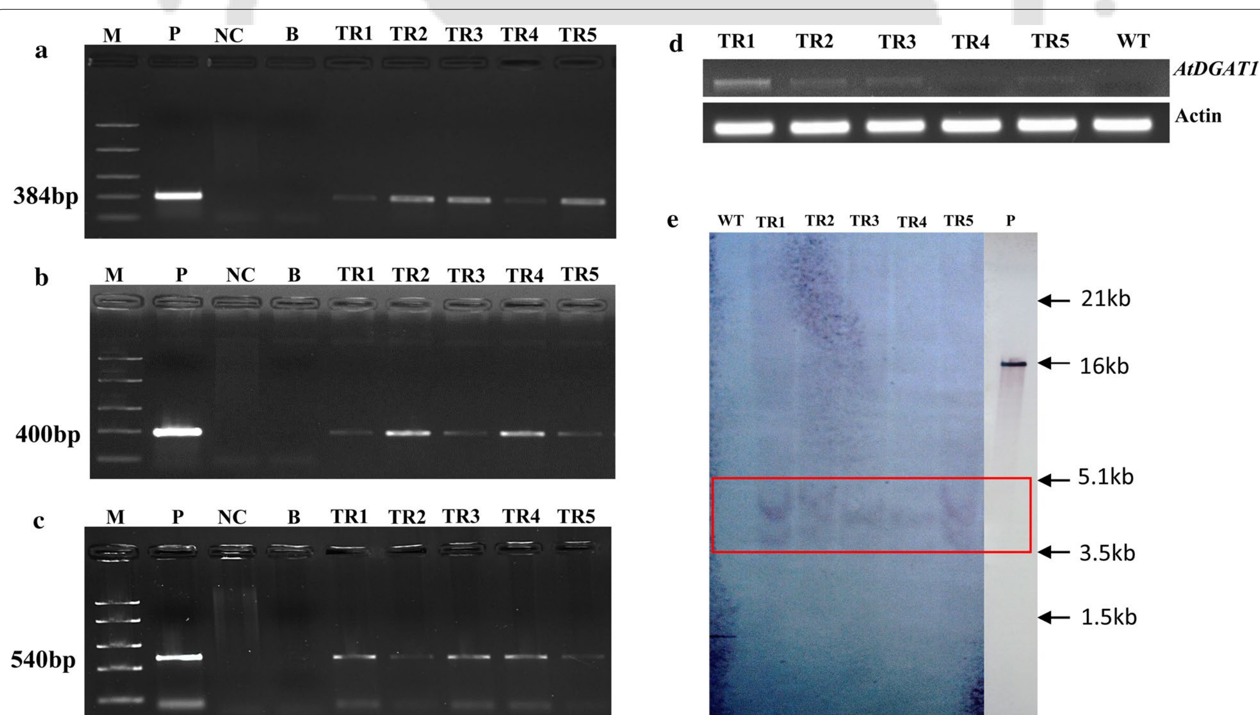

Fig. 2 Molecular analysis of transgenic plants of *Jatropha curcas*. **a** PCR amplification of the 384-bp fragment of *AtDGAT* gene; **b** PCR amplification of the 400-bp fragment of *gus* gene; **c** PCR amplification of the 540-bp fragment of *nptII* gene. *Lane* M molecular marker, *Lane* TR1, TR2, TR3, TR4, TR5, genomic DNA from five transgenic plant *Lane* P, pBI121::*AtDGAT1* plasmid (positive control), *Lane* NC, DNA from untransformed plant (negative control) and *Lane* B, blank; **d** Transcript abundance of *AtDGAT1* in transgenic line of *J. curcas*. Expression analysis was carried out by semi-quantitative PCR using *actin* as an internal control. **e** Southern blot analysis of transgenic plants expressing *AtDGAT1*,the plasmid and 60 µg genomic DNA was digested with *BamHI*, and hybridized with PCR-amplified *nptII* probe, lane WT genomic DNA from untransformed plant, *Lane* TR1–TR5 genomic DNA from transgenic lines, *Lane* P *BamHI* digested pBI121::*AtDGAT1* plasmid

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also lead to increased oil accumulation in leaf biomass and seed kernel. As we intended to specifically allow lipid accumulation in the leaves as well as in mature seeds, the *AtDGAT1* was placed under the control of the constitutive CaMV35S promoter.

To investigate total lipid content in mature seeds and leaves of transgenic Jatropha plants, we harvested leaves and mature seeds from five individual transgenic plants and separated the seed kernels for further studies. Compared to control (WT) plants, all tested transgenic lines showed significant increase (1.5- to 2-fold) in total lipid content in leaves (Fig. 3a). The leaves of best transgenic line (TR1) accumulated twofold higher total lipid content than the control (WT) plants which represented a 100 % increase of total lipid in transgenic line (TR1). The seed kernels of all the transgenic lines tested showed enhancement of total lipid content by 20–30 % on a relative basis as compared to WT plants (Fig. 3d). The best transgenic line (TR1) showed a 30 % relative increase of total lipid as compared to WT plants (Fig. 3d).

We analyzed the five *AtDGAT1* transgenic Jatropha lines to find out the possible changes in protein and carbohydrate content at the expense of increased lipid accumulation in leaves and seed kernels, as the precursors of fatty acid biosynthesis in plants are derived from sugar during photosynthesis. The protein content in leaves of transgenic lines showed no significant change as compared to WT plants (Fig. 3b). However, seed kernels of transgenic lines showed a minor increase in protein content except in transgenic line TR1 and TR2 that showed an insignificant decrease in protein content as compared to WT (Fig. 3e). It was observed that the level of total soluble sugar in leaves was significantly increased (38–112 %) in transgenic lines tested as compared to leaves of WT plants (Fig. 3c). The transgenic lines had total soluble sugar in the range of 43.60–66.70 mg g^{-1} FW as compared to wild-type 31.45 mg g^{-1} FW (Fig. 3c). These results suggest that increased accumulation of total soluble sugar may have contributed to reallocation of precursor for enhanced TAG synthesis in transgenic leaves [47]. However, the seed kernels of transgenic lines showed a corresponding decrease in sugar content as compared to wild-type plants (Fig. 3f). These observations suggest that the reallocation of precursor of photosynthesis to TAG

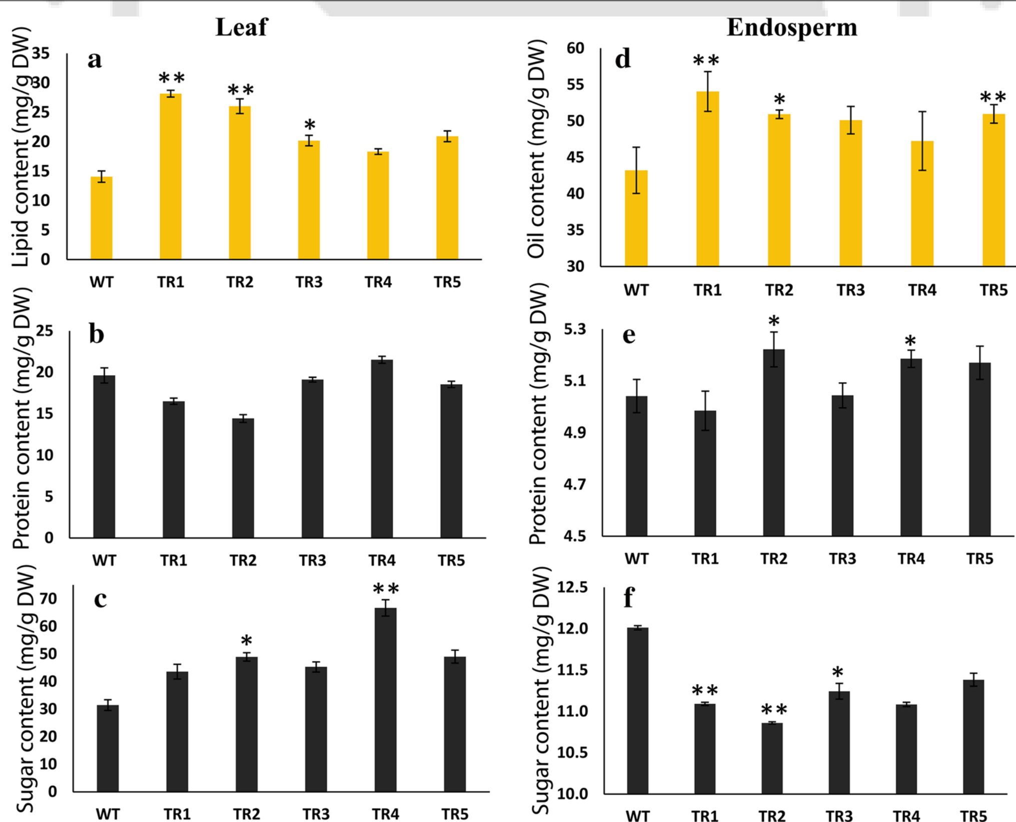

Fig. 3 Variation in leaves and seed lipids, protein, and sugar contents. **a** Leaf lipid content; **b** leaf protein content; **c** leaf sugar content analysis, and **d** seed oil; **e** seed protein content; **f** seed sugar content analysis in wild-type and *AtDGAT1-Jatropha curcas* transgenic lines. Difference between untransformed (WT) and transgenic lines was significant at $P < 0.05$ (*), $P < 0.01$ (**) by Tukey test. Values are shown as mean ± SD ($n = 3$)

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biosynthesis is more in leaves than seeds of transgenic AtDGAT1 Jatropha lines, and possibly the contribution to TAG biosynthesis in these transgenic lines by leaves is more than seeds which may be due to the lower expression of 35S-driven genes in the seed versus vegetative tissue. In previous studies, it has been found that 35S does not express as highly in *Arabidopsis* seed and germline tissue compared to vegetative tissue like leaves [48, 49].

In order to characterize TAG accumulation in mature seed kernels, we used TLC on silica gel plates to analyze qualitatively the total neutral lipids from control (WT) plants and five transgenic lines. The TLC plates revealed an overall increase in TAG content in seeds of all *AtDGAT1* over expressing transgenic Jatropha lines as compared to control (WT) plants (Fig. 4a). Furthermore, the DAG content in seeds of transgenic lines showed a relative increase as compared to WT plants (Fig. 4a). FFA was not detected in seeds of any of the transgenic lines except TR2 (Fig. 4a). All the five transgenic lines showed a very similar level of TAG accumulation in their seeds (Fig. 4a). These results suggest that *AtDGAT1* encodes a functional protein capable for catalyzing the final rate-limiting step of TAG biosynthesis in transgenic Jatropha lines expressing *ATDGAT1*.

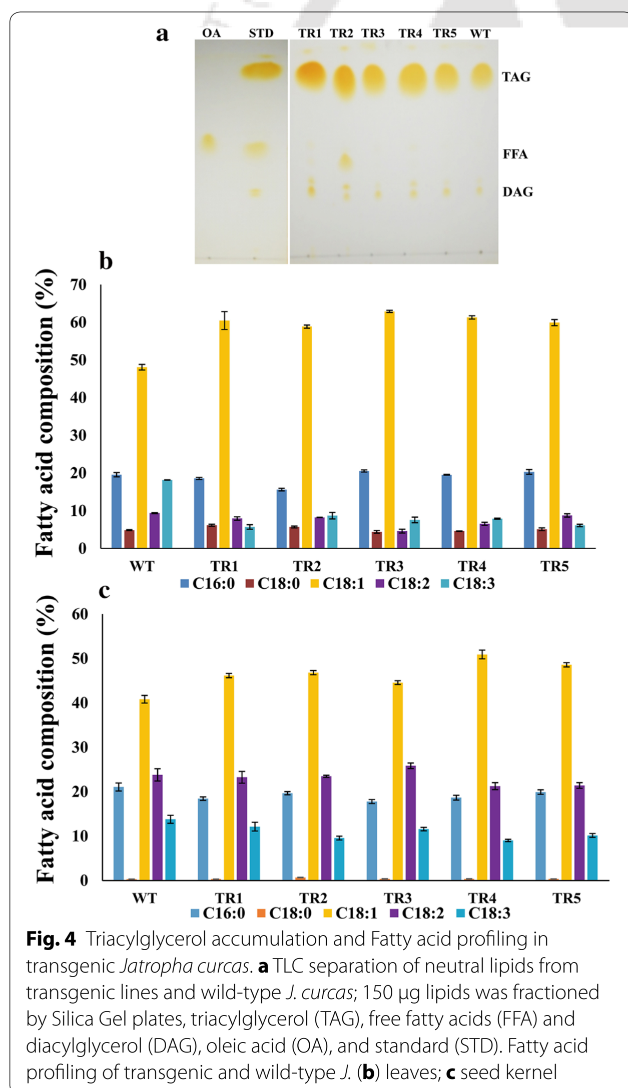

Fig. 4 Triacylglycerol accumulation and Fatty acid profiling in transgenic *Jatropha curcas*. **a** TLC separation of neutral lipids from transgenic lines and wild-type *J. curcas*; 150 µg lipids was fractioned by Silica Gel plates, triacylglycerol (TAG), free fatty acids (FFA) and diacylglycerol (DAG), oleic acid (OA), and standard (STD). Fatty acid profiling of transgenic and wild-type *J.* (**b**) leaves; **c** seed kernel

To determine and compare the FA profiles between the transgenic lines and WT plants, the fatty acids methyl esters resulting from transesterification of seed oil and leaf lipids were quantified by GC. A significant variation in composition of FAs was detected among the leaves of control and transgenic plants. The leaves of transgenic lines showed a relative increase of oleic acid (18:1) by 20–31 % as compared to WT plants (Fig. 4b). In addition, the level of linolenic acid (18:3) showed a reduction from 18.17 to 5–8 % in transgenic lines as compared to WT plants. There were no marked changes observed in the level of palmitic (16:0), stearic (18:0), and linoleic (18:2) acid in leaves of transgenic lines as compared to WT plants (Fig. 4b). Increased TAG accumulation in seed kernels was accompanied by a profound change in fatty acid composition in TAG fraction (Fig. 4c). GC analysis showed that oleic acid (18:1) accumulation in seed kernels of transgenic lines increased by 9–25 % as compared to WT. On the other hand, the level of linolenic acid (18:3) in seed kernels decreased by 12–35 % in transgenic lines as compared to WT (13.79 %) (Fig. 4c). The linoleic acid (18:2) levels in seed kernels of transgenic lines showed the similar abundance to that of WT, and in contrast, the lines TR4 and TR5 showed the level of linoleic acid decreased by 9.94 and 10.04 %, respectively (Fig. 4c). The level of palmitic acid (16:0) in seed kernels decreased by 12–21 % in all transgenic lines as compared to WT (Fig. 4c). There were no changes in the stearic acid (18:0) levels in seed kernels of the transgenic lines (Fig. 4c).

AtDGAT1 overexpressing transgenic Jatropha lines have increased number of oil bodies in leaves

TAGs, the predominant plant storage neutral lipids with twice the energy density of cellulose, are used to generate biodiesel [50]. Increased demand to produce more energy from plant biomass has prompted means to produce oil in vegetative tissues, mostly in leaves. Therefore, we made an attempt to engineer Jatropha plants by overexpressing the *DGAT1* gene that codes for the enzyme responsible for the final and only committed step in TAG biosynthesis, to accumulate TAGs in leaves, in addition to seeds. Hand sections of control and transgenic petioles of Jatropha were examined for determining the intracellular localization of the TAG. Fresh samples from the fifth leaf from apical bud of wild-type and transgenic Jatropha plants were stained with lipid-specific fluorescent

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dye, Nile red, and observed under a confocal laser scanning microscope after excitation at 559 nm of light. The frequency of oil droplets was found increased in transgenic samples compared to the wild type, and oil droplets mostly distributed close to inner peripheral region of cells (Fig. 5A, B).

Effect of enhanced TAG accumulation on agronomic traits in transgenic Jatropha lines

We investigated whether there were any negative effects of TAG accumulation on agronomic traits in ATDGAT1 overexpressing Jatropha lines under greenhouse conditions. Five transgenic lines with high TAG content and control WT plants were monitored for agronomic traits including time duration for flowering, plant height, number of primary branches after one trimming, secondary branches, seed number per tree after acclimatization in soil, seed length, seed width, seed breadth, and 10-seed weight (Table 1). The transgenic lines were morphologically no different from the wild type (Fig. 6). The transgenic lines as well as the WT plants took approximately two and half years for flowering, and there were no obvious differences in time taken for inflorescence emergence. There was no significant difference in number of

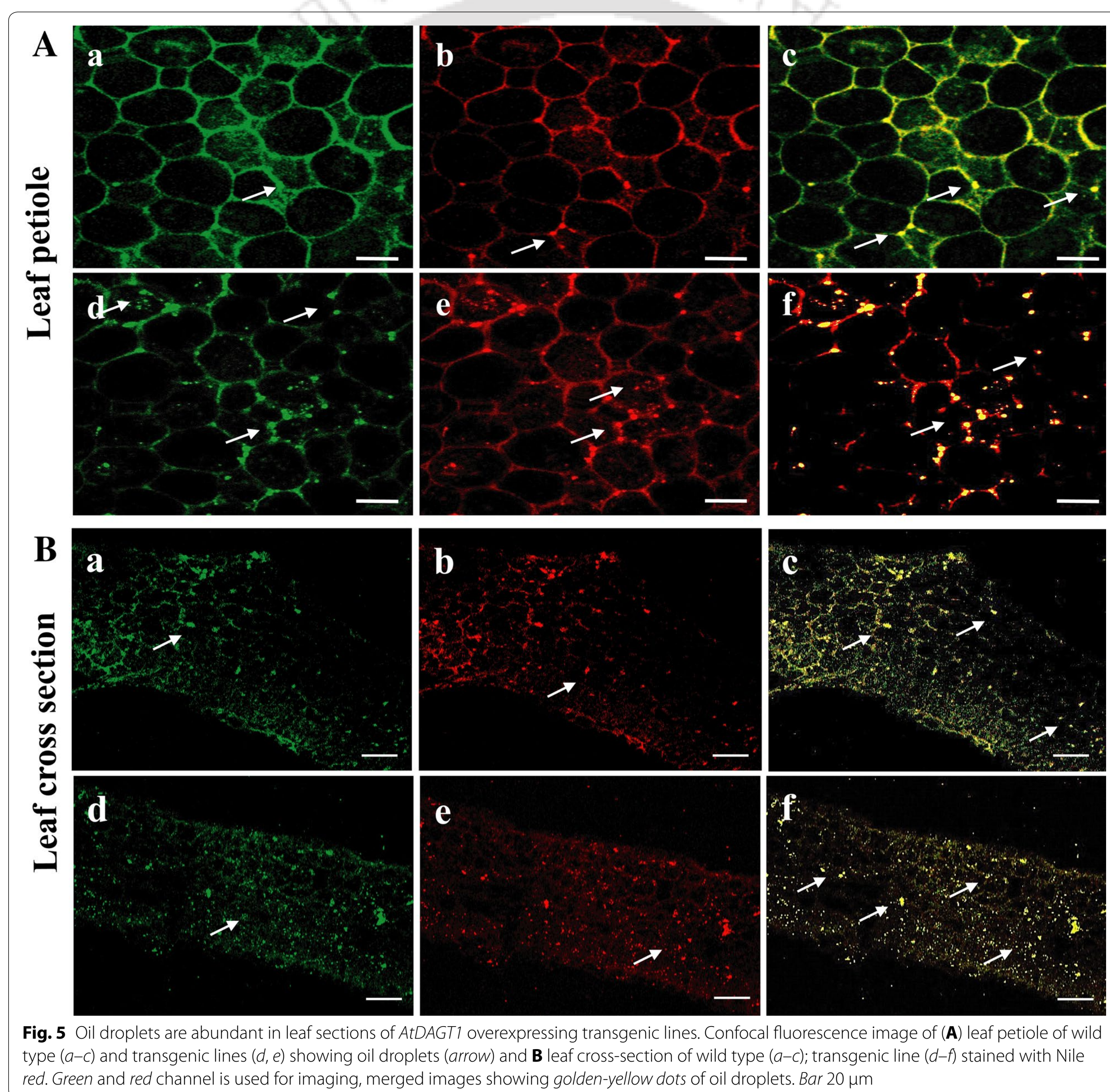

Fig. 5 Oil droplets are abundant in leaf sections of *AtDAGT1* overexpressing transgenic lines. Confocal fluorescence image of (**A**) leaf petiole of wild type (*a–c*) and transgenic lines (*d*, *e*) showing oil droplets (*arrow*) and **B** leaf cross-section of wild type (*a–c*); transgenic line (*d–f*) stained with Nile *red*. *Green* and *red* channel is used for imaging, merged images showing *golden-yellow dots* of oil droplets. *Bar* 20 µm

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Table 1 Physiological parameter comparison between wild type and *AtDGAT1* transgenic lines

	Plant height (cm)	Primary branch[a]	Secondary branches[b]	Seed number per tree	Seed length (mm)	Seed width (mm)	Seed breadth (mm)	10 seeds weight (g)
WT	169.47	4	4.5 ± 0.6	76	18.35 ± 0.21	10.65 ± 0.15	8.95 ± 0.29	7.13 ± 0.21
TR1	170.68	3	8.6 ± 0.7	47	18.36 ± 0.12	10.71 ± 0.13	9.19 ± 0.20	7.47 ± 0.07
TR2	179.83	3	5.3 ± 2.7	83	18.94 ± 0.16	10.90 ± 0.13	8.94 ± 0.18	7.39 ± 0.06
TR3	163.07	4	4.6 ± 1.2	43	18.38 ± 0.23	10.72 ± 0.17	9.137 ± 0.12	7.52 ± 0.07
TR4	167.34	3	6.0 ± 0.6	46	18.78 ± 0.21	11.39 ± 0.16	8.64 ± 0.23	7.54 ± 0.05
TR5	172.68	3	4.3 ± 0.7	65	18.35 ± 0.14	10.51 ± 0.14	8.83 ± 0.14	7.43 ± 0.11

[a] Number of branches after pruning

[b] Average secondary branches per primary branch (Sample size for measurement was $n = 10$)

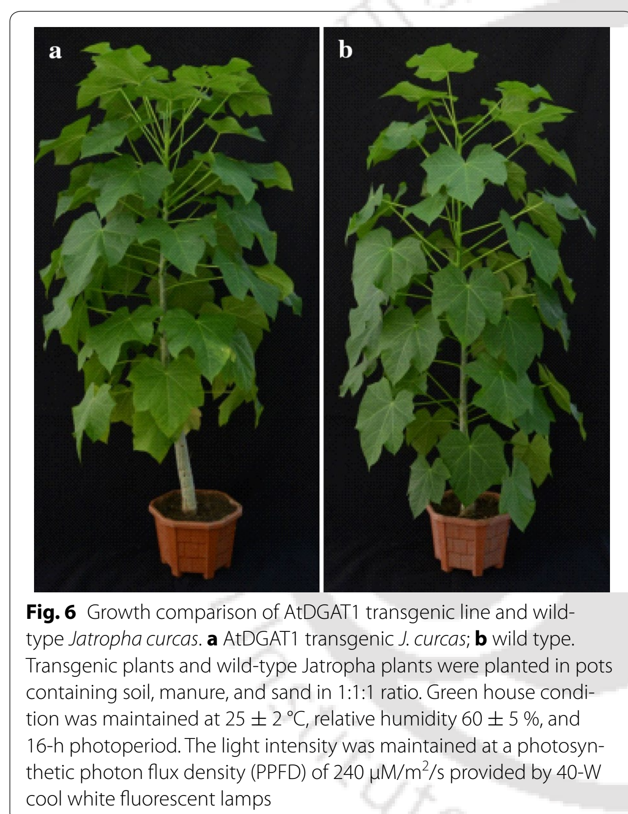

Fig. 6 Growth comparison of AtDGAT1 transgenic line and wild-type *Jatropha curcas*. **a** AtDGAT1 transgenic *J. curcas*; **b** wild type. Transgenic plants and wild-type Jatropha plants were planted in pots containing soil, manure, and sand in 1:1:1 ratio. Green house condition was maintained at 25 ± 2 °C, relative humidity 60 ± 5 %, and 16-h photoperiod. The light intensity was maintained at a photosynthetic photon flux density (PPFD) of 240 μM/m^2/s provided by 40-W cool white fluorescent lamps

primary branches and secondary branches found among the transgenic lines and WT plants; however, some of the transgenic lines had a marked increase in plant height (Table 1). The transgenic lines had an average of 56.88 ± 7.6 seeds per tree with an average seed weight of 7.46 ± 0.03 g, and with increased average seed length, seed width, and seed breadth (Table 1). These data collected from transgenic lines under greenhouse conditions indicated that high TAG accumulation had no negative effects on important agronomic traits in Jatropha.

Discussion

Seed-derived triacylglycerol (TAG), a neutral lipid with twice the energy density of cellulose, is the most suitable for generating high energy density desirable biodiesel with one of the simplest and most efficient manufacturing processes [51–57]. Increasing accumulation of TAGs in seeds as well as vegetative tissues is gaining momentum in order to make plant-derived biodiesel production economically more sustainable [38, 57]. Consequently, engineering plants to accumulate TAG in vegetative tissues and raising the TAG content in seeds are becoming the most potential strategy [54, 55, 58]. Since DGAT1 is the only enzyme that is exclusively committed to TAG biosynthesis in Kennedy pathway, direct upregulation of Kennedy pathway through overexpression of the DGAT1 enzyme responsible for the last and only committed step in seed TAG biosynthesis is most promising.

Here, we describe the generation of transgenic Jatropha plants that accumulates high level of TAGs in seeds as well as in leaves by the overexpression of *Arabidopsis DGAT1*. In this work, we used the CaMV35S promoter because of its strong and constitutive nature in regulating transgene expression to enable ectopic overexpression of *AtDGAT1* in leaves as well as seeds. The enhanced TAG accumulation in transgenic Jatropha lines had no penalty on the growth rates, growth patterns, leaf number, and leaf size of plants. Enhanced expression of AtDGAT1 in transgenic Jatropha lines appears to have increased the total lipid content by 1.5- to 2-fold increase in leaves and 20–30 % in seed kernels. In the best transgenic line (TR1), total lipid content was increased by twofold in leaves and 30 % in seed kernels. This increase was accompanied with no significant change in protein content in leaves, but a minor increase in protein content in seed kernels was found in transgenic plants. In contrast to proteins in leaves and seeds, significant increase in carbohydrate content in leaves and marked decrease in carbohydrate content in seeds of transgenic lines were

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observed. These results suggest that increased accumulation of total sugar may have contributed to reallocation of precursor for enhanced TAG synthesis in transgenic leaves. Carbohydrates were important osmotic solutes in leaves and seeds, and were potentially involved in the carbon source transformation into lipids. Previous studies suggested that carbohydrate content in seed was correlated with seed oil contents [59]. However, the reallocation of precursor of photosynthesis to TAG biosynthesis is more in leaves than seeds of transgenic AtDGAT1 Jatropha lines, and possibly the contribution to TAG biosynthesis in these transgenic lines by leaves is more than seeds. The mechanisms underlying these changes need to be addressed in future investigations.

We found that AtDGAT1 overexpressing transgenic lines contain in seeds an overall increase in TAG and DAG, and lower FFA levels compared with control wild-type Jatropha plants. Therefore, crude oil extracted from AtDGAT1 overexpressing Jatropha lines would be expected to be a better substrate compared to those of wild-type plants for alkaline transesterification in biodiesel production. Seed oil containing very low levels of FFA and moisture is ideal for biodiesel production as alkaline treatment process which is the preferred method for transesterification owing to its shorter reaction time and reduced energy consumption requires crude oil with very low levels of FFA and moisture [60, 61], as a high level of FFA and water can convert transesterification into saponification, leading to easy depletion of catalysts [62, 63]. However, a thorough analysis of alkaline-treated transesterification with crude oils from *AtDGAT1* overexpressing Jatropha lines should be performed in future studies. Overexpression of *AtDGAT1* in Jatropha resulted in increase of oleic acid in leaves by 20–31 % and in seeds by 9–25 %, and decrease of linoleic acid in both leaves and seeds without compromising its agronomic performance. Nevertheless, these high oleic acid level transgenic lines need to be further characterized for extensive analysis of their suitability for diesel engines. We observed distinct oil droplets in the leaf tissues as revealed by Nile red staining. These observations were consistent with increased oil accumulation in the leaves of *AtDGAT1* overexpressing lines. Intriguingly, the presence of oil bodies typically found only in seeds was detected in the vegetative tissue, suggesting that seed-like biosynthetic mechanisms were perhaps ectopically induced. These results are similar to those reported with the overexpression of LEC2 in senescing leaves [58].

However, more importantly, ectopic overexpression of *AtDGAT1* in Jatropha resulted in an increase in oil content, average plant height, seeds per tree, average 100-seed weight, and seed length and breadth. Thus, there was no penalty in 100-seed weight due to the oil content increase, the result being an increase in total oil on a per seed basis of between 20 and 30 % more in the *AtDGAT1* transgenic lines, thus indicating a 20–30 % net overall oil increase when compared with the wild-type plants [64]. Additionally, *AtDGAT1* overexpression lines of Jatropha also exhibited an oil content increase of 1.5- to 2-fold in leaves. This distinct difference between *AtDGAT1* transgenic and wild-type plants with respect to effects on seed weight, seed length and breadth, and plant height is unclear, and suggests a more complex interaction between the traits of oil increase and seed traits than is currently understood.

Conclusions

In this paper, we demonstrated that upregulation of TAG biosynthesis by ectopic overexpression of *AtDGAT1* in transgenic *J. curcas* plants leads to an enhanced accumulation of TAGs in leaves as well as seeds without compensating plant and seed traits. In principle, accumulation of oils in leaf foliage and seeds provides an opportunity to enhance the energy density of the biomass and thus have significant impact on biofuel production. This is the first report in Jatropha demonstrating enhanced oil accumulation in both seeds and vegetative tissues. These promising results are a first step towards making an economically viable biofuel crop through transgenic approach. Although a lot of future efforts are to be made to look at partitioning substantial carbon into TAG in vegetative tissues in addition to enhanced accumulation in seeds to make it highly sustainable, our results support the basic feasibility of a strategy to redirect carbon partitioning from starch to oil in plant biomass. The resulting seed oil content changes may have commercial significance in terms of increasing oil content for greater productivity.

Methods

Plant material and explant preparation for transformation

Seeds were collected from elite lines of *J. curcas* (IITG JC-19) and maintained in shade house of Indian Institute of Technology Guwahati [65]. The seeds were decoated and soaked in distilled water overnight at room temperature. The soaked decoated seeds were treated for 10 min with 0.1 % sodium hypochlorite solution containing 4–5 drops of Tween-20, followed by washing with distilled water for 20 min. The decoated seeds were then surface sterilized with 0.2 % mercuric chloride for 2 min and finally rinsed with sterile distilled water for 4–5 times. After blot drying on sterilized filter paper, the endosperm was dissected out carefully to expose embryos with papery cotyledonary leaves and germinated on Murashige

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and Skoog (MS) basal media [66]. The 4-day-old papery cotyledonary leaves were cut into four segments (8 mm^2) with the edges removed and used as explants for *Agrobacterium*-mediated transformation.

Construction of *DGAT1* expression construct

Full-length *Arabidopsis thaliana diacylglycerol acyltransferase 1* (DGAT1) (Gene Bank: AF051849.1) cDNA was PCR-amplified with *XbaI* and *BamHI* sites on the 5′- and 3′- ends, respectively, using the forward primer (GCA TCT AGA ATG GCG ATT TTG GAT TC) and reverse primer (GCA GGA TCC TGA CAT CGA TCC TTT TC), and the PCR product was cloned as *XbaI–BamHI* fragment and maintained in the intermediate vector, pTZ57R/T. The PCR fragment was inserted into the *XbaI/BamHI* sites of plant expression vector, pBI121 as C-terminal fusion to gus gene under the control of CaMV35S promoter and NOS terminator. The AtDGAT1 construct was mobilized into the disarmed *Agrobacterium tumefaciens* strain EHA105 and used for the transformation.

Jatropha transformation

Plant transformation of Jatropha was carried out using the protocol described earlier by our lab [40]. In brief, the protocol consisted of four steps: co-cultivation, shoot induction, shoot elongation, and rooting. The explants after inoculation with *Agrobacterium* suspension were co-cultivated for 3 days. After co-cultivation, the explants were transferred to callus induction medium (CI, MS medium supplemented with 6.66 μM BAP and 0.24 μM IBA) containing 500 mg/L cefotaxime and 400 mg/L augmentin in dark condition. The cultures were transferred to fresh CI medium at an interval of 5, 7, and 8 days. After 3 weeks of culture, the calli were transferred onto shoot regeneration medium (SR, MS medium supplemented with 6.66 μM BAP, 0.24 μM IBA, 1.44 μM GA_3) containing 50 mg/L kanamycin, 500 mg/L cefotaxime, and 400 mg/L augmentin, and incubated at 16 h photoperiod. The cultures were periodically transferred onto fresh selection medium. After 4 weeks of culture on selection, the regenerating kanamycin-resistant shoots were detached and transferred to shoot elongation medium (SE, MS medium supplemented with 1.0 μM GA_3) containing 15 mg/L kanamycin and 400 mg/L augmentin. After a week, the elongated shoots were shifted to root induction medium (RI, ½ MS medium supplemented with 0.5 μM IBA) and 400 mg/L augmentin. Well-rooted transformed plantlets were washed thoroughly in running tap water, and acclimatized and maintained in greenhouse as per our lab protocol described earlier [65].

Molecular analysis of transgenic Jatropha plants

Polymerase chain reaction (PCR) analysis

Genomic DNA was isolated from the untransformed (UT) and putative transformed Jatropha plants using the NucleoSpin Plant II Maxi kit (Macherey–Nagel, Duren, Germany). PCR was carried out to detect the presence of *AtDGAT1*, *nptII,* and *gus* in transformed Jatropha plants. The 384-bp region internal to *AtDGAT1*, 540-bp region internal to *nptII,* and 400-bp region internal to *gus* were amplified using primers (*AtDGAT1Fw*: TCT GCT GGC GTT ACT ACG GT and *AtDGAT1*Rv: CGG CAT GGC TCT GTT TGA AG; *nptII*Fw: GTG GAG AGG CTA TTC GGC TA and *nptII*Rv: CCA CCA TGA TAT TCG GCA AC; and *gus*Fw: GGT GGG AAA GCG CGT TAC AAG and *gus*Rv: TGG ATT CCG GCA TAG TTA AA) using the PCR conditions: 95 °C for 5 min (1 cycle), 95 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min for 35 cycles followed by a final extension of 72 °C for 5 min. The recombinant plasmid pBI21*AtDGAT1* was used as positive control. The PCR-amplified products were analyzed on 1 % agarose gel and visualized by ethidium bromide staining.

RNA isolation and semi-quantitative RT-PCR analysis

The total RNA was isolated from the PCR-positive transgenic Jatropha lines and wild-type untransformed plants using RNA extraction kit (NucleoSpin RNA Plant, MN, Germany) and quantified with nanodrop spectrometer (Nanodrop, USA). The cDNA was prepared using 1 μg of total RNA using reverse transcription kit (ThermoScientific, USA), according to its manufacturer's instructions. The semi-quantitative RT-PCR was performed using primers (*AtDGAT1*Fw: GGTGGCGGAGAGTTCGTCGA and *AtDGAT1*Rv: TCTTCCTTCTCCGCCGCCTC; *JcActin*Fw: ATGAGCTTCGAGTTGCAC and *JcActin*Rv: ACCATCACCAGAATCCAG) for amplifying a 249-bp fragment of *AtDGAT1* and a 590-bp of *JcActin* as an internal control to indicate the amount of starting RNA. Semi-quantitative PCR was amplified, and amplification products were analyzed with 1 % agarose gel, detected by ethidium bromide staining.

Southern blot analysis

Randomly selected PCR-positive T_0 transgenic Jatropha lines were analyzed by Southern hybridization for the integration of transgene. Genomic DNA was isolated from leaves of transgenic and control untransformed (UT) plants using the NucleoSpin Plant II Maxi kit (Macherey–Nagel, Duren, Germany). Sixty microgram of genomic DNA was digested with *BamHI* and separated

on a 0.8 % agarose gel. The gel was processed and transferred to ZetaProbe nylon positively charged membrane (Bio-Rad, USA) following standard procedures. The blot was hybridized with DIG-labeled 0.54-kb *nptII* PCR-amplified product as probe. Hybridization and detection of signals were carried according to the DIG Labeling and detection supplier instructions (Roche Diagnostics, Mannheim, Germany).

Stable *GUS* assay
Histochemical GUS assays were used to assess the stable GUS expression [67] in leaf explants after three days of co-cultivation, callus, stem, leaf, roots, and seeds of transgenic plants.

Fatty acid analysis in transgenic Jatropha
Total lipids were isolated following the method described by Bligh and Dyer [68]. Two hundred milligram of leaves and 500 mg seeds kernel of control untransformed and transgenic lines were homogenized in mortar-pestle, and lipids were extracted from organic phase. Lipid fraction in bottom phase was collected in glass tube and evaporated in rotary evaporator. Total lipids were quantified after drying in a desiccator for 24 h. The weight of the total oil was determined gravimetrically, and oil content was recorded as the ratio of lipid and oil to dried leaf sample and seed kernel weight.

Analysis of lipids by thin-layer chromatography
Lipids were fractioned from neutral lipids by thin-layer chromatography. On silica gel plate (TLC Silica Gel 60 F_{254}, Merck), 150 µg of extracted lipid was spotted and resolved using solvent system of Hexane: diethyl ether: acetic acid (70:30:1, v/v/v). Triacylglycerol spots were revealed by staining with iodine vapor.

Fatty acid methyl ester (FAME) preparation and analysis by Gas chromatography
We used GC to analyze the FA profile of transgenic Jatropha lines. About 20 mg of lipid was dissolved in methanol in a test tube, and 0.5 M potassium hydroxide in anhydrous methanol was added with reaction volume of 20 mL. The solution was maintained at 60 °C for 30 min. The methyl esters were extracted with hexane (2 × 5 mL), and the organic phase was washed twice with Milli-Q water to remove any aqueous impurities. Organic phase was collected in screw cap glass tube, and solvent was removed in rotary evaporator; after filtration through a 0.2-µm filter, methyl esters were used for GC analysis. FAME analysis was performed on Varian 450-GC (Varian Capillary Column CP-SiL8 CB, 30 m 0.25-mm i.d., 0.25-µm film thickness). FAMEs were separated and detected by flame ionization detector (FID). Nitrogen was used as carrier gas with 0.4 ml s^{-1} at constant flow rate. The oven regime: 140 °C for 5 min, 180–240 °C at 3 °C/min, and hold at 220 °C for 10 min. The injector and detector temperatures were kept at 250 °C, and 1 µL injection volume at split ratio of 1:20 was used for the analysis. Peaks were identified based on their retention times compared with a FAME reference mixture (Supelco, Bellenfonte, PA, USA). Fatty acid composition was calculated based on the peak area percentage of total fatty acids.

Protein and carbohydrate analysis
Protein content of Jatropha transgenic lines was determined as described by Focks and Benning [69] using 200 mg of each leaf and dried endosperm. Protein amounts were measured according to Bradford [70] using three technical replications using ready to use Bradford's reagent (Fermentas, USA). To analyze the carbohydrate content, 200 mg of each of leaf and dried endosperm were homogenized in 200 µL of assay buffer and centrifuged at full speed. The extracted supernatant was used for quantification of carbohydrate using a Total Carbohydrate Assay Kit (Sigma-Aldrich). D-glucose was used as a standard for calibration, and data were expressed as mg carbohydrate g^{-1} tissue fresh weight.

Seed weight and size determination
Mature seeds were harvested from untransformed control (UT) and transgenic Jatropha lines grown under the same conditions. The seeds were then weighed carefully on analytical balance with sample size 50; values ($n = 5$) are given as mean ± SD. LIA image processing software (Nagoya University, Japan) was used to measure seed sizes. Values ($n = 10$) are given as mean ± SD. The moisture content was determined as the difference between the initial and dry weights divided by the initial seed weight and represented in percentage.

Microscopy analysis
Leaf sections of transgenic and control Jatropha plants were stained with Nile red (HiMedia, India), mounted in 70 % glycerol, and visualized using a laser confocal scanning microscope. Oil droplets were observed at 570–630 nm emission following 559-nm excitation by solid-state laser. Images were captured with LSM 510 META laser scanning microscope (Lieca, Germany).

Abbreviations
TAG: triacylglycerol; DAG: diacylglycerol; DGAT: diacylglycerol acyltransferase; FFA: free fatty acid; FAME: fatty acid methyl esters; BAP: 6-benzylaminopurine; IBA: indole-3-butyric acid; GA3: gibberellic acid; GC: gas chromatography; PCR: polymerase chain reaction; bp: base pairs.

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Authors' contributions

LS conceived the work. LS, VVG, NS, and HK designed the experiments. DKM conducted the transformation experiments. DKM, SK, and YK performed the molecular analysis. DKM and PKS carried out the oil analysis. LS, NS, HK, and VVG critically analyzed the data. LS prepared the manuscript. NS and HK corrected the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Consent for publication

The authors hereby consent to publication of this work in Biotechnology for Biofuels.

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PHYTOGENIC 'GREEN SYNTHESIS' OF SILVER NANOPARTICLES WITH ENHANCED ANTIBACTERIAL AND ANTI-CANCER ACTIVITY

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ABSTRACT

Phytogenic 'green synthesis' of the Ag NPs offers huge promises as the process is environmentally benign, economical and scalable. Herein, we demonstrate an one step, easy and eco-friendly method for synthesis of Ag NPs-composites (Ag NPs-plant extract) by using the extract of *Jatropha curcas* as reducing as well as stabilizing agent. The composites were characterized by UV-Vis spectroscopy, transmission electron microscopy (TEM), selected area electron diffraction (SAED) and Fourier transform infrared spectroscopy (FTIR) analysis. The green fluorescent protein expressing *E.coli* was used as an exemplary to study the anti-bacterial activity and establish the mechanism of action of these nano-composites. Our results demonstrate that cell wall perforation is the primary reason of non-viability of the bacterial cells as there is no direct effect of Ag NPs-composite on cellular DNA. Further, the cytotoxicity test of Ag NPs-composites against the HeLa cells revealed the potential of these Ag NPs composites for use as cancer therapeutic agents.

KEYWORDS: **Green synthesis, Nano-composites, Antibacterial activity.**

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INTRODUCTION

The silver nanoparticles (Ag NPs) and their products have offered immense opportunity for a wide range of applications including development of biosensors, data storage, catalysis, cosmetics, renewable energy, nanomedicine and environmental treatments[1, 2]. Essentially, silver nanoparticle based products offer huge prospects as novel therapeutic agents with profound implications for healthcare. For example, emergence of antibiotic resistant bacterial strains with number of evolving mechanisms to combat against conventional antibiotics has become major concern for healthcare worldwide. This demands development of new efficient materials and/or economic process with enhanced anti-bacterial activity[3, 4]. The use of Ag NPs and their composites as an alternative of antibiotics would be promising, as they exert antibacterial activity against both Gram positive and Gram negative bacteria and till now there is no report showing the resistance of bacteria against Ag NPs[5]. Furthermore, Ag NPs are found to be potent anti-cancer agent, which trigger apoptosis mediated cell death to prevent the growth of tumour. Hence, current demand of Ag NPs in various applications has up-surged research efforts to develop simple and low cost methods for large scale production of Ag NPs. Several methods based on chemical reduction, electrochemical reduction, photochemical reduction, sonochemical, radiation, and microwave have been reported for synthesis of NPs with desired size and shape[6]. However, most of these methods employ high temperature, pH and chemicals, which are relatively of high cost. Moreover, the toxic reagents involved in the synthesis and by-products that are generated pose serious concerns for human health and environment. Use of plant extracts has emerged as the effective alternatives to conventional methods of synthesis of NPs enabling 'greensynthesis'. The plants are the predominant photosynthetic autotrophs present in the biosphere and serves as natural chemical laboratory as its extracts contain a wide range of metabolites (primary and secondary) which can act as a reducing as well as stabilizing agent for synthesis of NPs. The plant extracts are easy to obtain and can be used directly with minimum pre-treatment for downstream applications.The use of *Jatropha curcas* oil as biodiesel is promising and commercially viable alternative to diesel oil[7]. The various parts of this oil seed plant find their use in traditional folk medicine in many countries. The plant is a source of several secondary metabolites of medicinal importance. The leaf, fruits, latex and bark contain glycosides, tanins, phytosterols, flavonoids and steroidal sapogenins that exhibit wide ranging medicinal properties[8]. A decoction of the leaves is used against cough and as antiseptic, latex used to dress wounds and ulcer, root decoction as mouthwash for bleeding gums and toothache[9]. Branches are used as chewing sticks and for the treatment of preterm labour in nigeria[10]. The latex has been demonstrated to have antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, *Klebsiellapneumoniae*, *Streptococcouspyogenes* and *Candida albicans*[9, 11]. A methanol extracts of the leaves afforded moderate protection for cultured human lymphoblastoid cells against the cytopathic effects of human immunodeficiency virus[12]. Extracts of the fruit have been shown to have pregnancy terminating effects in rats[13]. The latex of *Jatropha* contains cucain (an enzyme) [14]curcacycline A (a cyclic octaoeotide) [15] and curcacycline B (a cyclic nanopeptide) [16].Keeping in view of the presence of several secondary metabolites of medicinal importance as well as bioactive peptides in *Jatropha* in therapeutic applications, we decided to use this plant extract for developing a 'green' route to synthesize silver nanoparticles. In the present study, we have established a 'green synthesis' method of Ag NP by using the latex of *Jatropha* as a reducing as well stabilizing agent. There is a report of nanoparticle synthesis from *Jatropha*, which takes longer time (4h) and high temperature (85^0C) for synthesis[17]. Therefore main advantages of the method we are reporting over the previous methods are that it takes 10 min for synthesis and can be performed at physiological condition (pH 7.4 and 25^0C) and thus holds promise for large scale production of the Ag NPs. Our results demonstrated that Ag NPs composites are more potent than Ag NPs in their antibacterial activity against GFP expressing recombinant

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E.coli at relatively low concentrations of silver. Cell wall destabilization was found to be the primary reason of the bacterial cell death. Further, cytotoxicity test carried out to examine the effect of the synthesized Ag NPs composites on the proliferation of HeLa (human cervical carcinoma) cells revealed that the synthesized Ag NPs promoted the apoptosis in HeLa cells via generation of reactive oxygen species (ROS) indicating its scope in biomedical applications.

MATERIALS AND METHODS

CHEMICALS

Luria Bertani (LB) medium (Hi-Media, Mumbai, and India), Dulbecco's modified Eagles medium, Dulbecco's modified Eagle's medium supplemented with L-glutamine (4 mM), Penicillin (50 units mL-1), Streptomycin (50 mg mL-1), and Fetal bovine serum (obtained from PAA Laboratories, Austria) Silver nitrate ($AgNO_3$) Merck India and Sodium hydroxide (NaOH) Merck India Ltd were used.

PREPARATION OF PLANT EXTRACT AND SYNTHESIS OF AG NPS

By making incisions of the green stems of *Jatropha curcas* the latex was collected and centrifuged at 6000 rpm for 5 minutes to remove insoluble matters. The supernatant was collected and used for AgNPs synthesis. For synthesis, 200 µl of latex and 100 µl of 10 mM Ag NO_3were taken in a beaker containing 10 ml of Millipore water. Then, 100 µl of 0.3N NaOH was added into the mixture to adjust the pH of the solution (pH approx. 7.5) and the mixture was stirred continuously, till the colour of the solution changed from colourless to yellow.

TRANSMISSION ELECTRON MICROSCOPY (TEM)

Transmission electron microscope (TEM; JEM 2100; Jeol, Peabody, MA, USA) operating at 200 KeV accelerating speed was used to study the nanocomposites. For that 8 µl of sample was dropped onto a carbon coated copper TEM grid followed by air drying used for analysis.

FLUORESCENCE ACTIVATED CELLS SORTER ANALYSIS (FACS)

Recombinant GFP expressing *E. coli* cells were incubated at physiological conditions in LB medium with composite as well as individual controls for 6 h. Then, 500 µL (10^6 cells / mL) bacterial cultured was centrifuged at 1000 rpm for 5 min to collect cell pallet, which was redispersed in 0.9 % NaCl and stained with 3 µL of 1 mg /mL of propidium iodide (PI) for 5 min before doing the FACS analysis (BD FACS calibur System, BD Biosciences, San Jose, CA).

CELL CULTURE AND CYTOTOXICITY STUDY

HeLa cells (human cervical carcinoma) were taken from National Centre for Cell Sciences (NCCS), Pune, India, and grown in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal bovine serum and is maintained in 5% CO_2 humidified incubator at 37 °C. To perform the cell viability assay, cells were quantified by using haemocytometer and were seeded in 96 well- plate such that each well contains 5×10^3 cells and grown for 12 h. Then, different amount of the Ag NPs composite was added into the cell and incubate for another 24 h followed by MTT (3-(4,5- dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay. The MTT leads to formation of insoluble purple colour formazan, which was further dissolved by adding DMSO, due to reduction of MTT by oxidoreductase enzyme of viable cells. The amount of formazan, hence, directly correlates the number of viable cells. The formazan product has an absorbance at 550 nm (A550) and background absorbance at 690 nm due to culture media. The percentage of viable cells was calculated as mentioned below.% of cell viability = {(A550-A690) of composite treated cells /(A550-A690) control cells} X100

RESULTS AND DISCUSSION

The nanoparticles are synthesized by reacting silver nitrate with latex of *Jatropha curcas* at pH 7.5 and room temperature. After about 2 min, the colourless solution turns into yellow colour, indicating the formation of silver nanoparticles (Ag NPs). The UV-Vis spectrum of the composite (Ag NPs- plant extract)

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showed the characteristic surface plasmon resonance (SPR) absorption band of Ag NPs at 415 nm (Figure 1A). The TEM analysis was carried out to find out the size and shape of the nanoparticles. The TEM images showed that NPs were polydisperse and spherical in shape, however, there is a wide distribution in the particle size (Figure 1B). In addition, the HRTEM was also performed, which exhibited the individual lattice planes of Ag NPs (Figure 1C). The average particle size of NPs was found to be 8.80 ± 4.4 nm (Figure 1D). During TEM, selected area electron diffraction (SAED) was also performed and Scherer ring patterns of Ag NPs were observed signifying that particles were polycrystalline (inset of the Figure 1C). The size of the NPs was reasonably good for antibacterial activity as smaller Ag NPs (< 10 nm) have better antibacterial efficacy. The powder X- ray diffraction (PXRD) patterns of the composite revealed three peaks at 38.30, 44.50 and 64.50 corresponding to diffraction from (111), (200) and (220) lattice planes, respectively of Ag (0) (Supplementary Information, Figure S1). Efficiently the composite NPs were stable and there was no substantial aggregation of particles even after 24 h of synthesis which was probed by UV-Vis spectroscopy (Supplementary Information, Figure S2). Further, to find out the role of the pH on the synthesis of the NPs, pH of the reaction was varied from 4.2 to pH 9.75 by keeping all other parameters and reactants concentration fixed. It was found that at basic pH (>7.0) the synthesis was better and there was a blue shift in the UV-Vis spectra from 425 nm to 410 nm when pH was increased from 4.2 to 9.75, respectively, indicating the variation in size of Ag NPs (Supplementary Information, Figure S3).

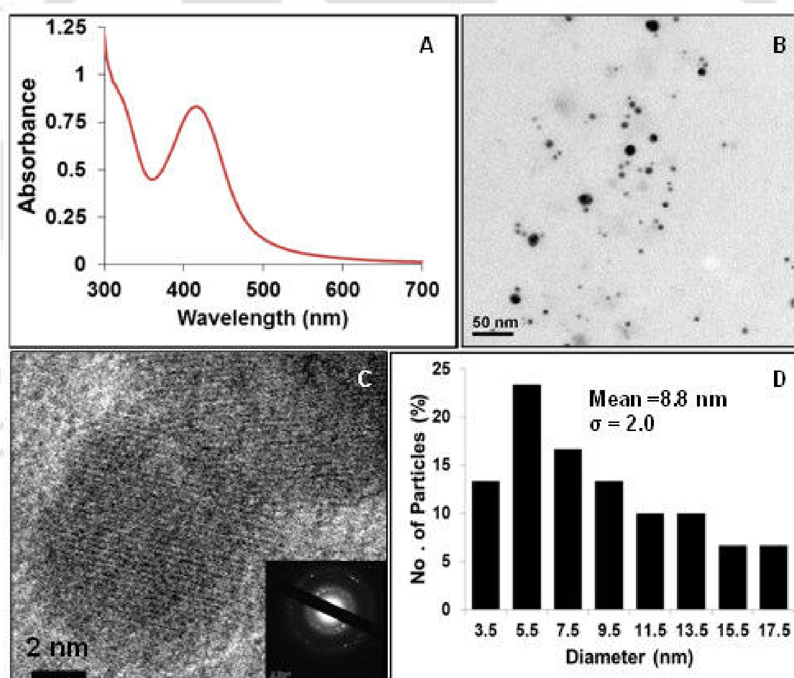

Figure 1
(A) UV-Vis spectra of the as synthesized Ag NPs-plant extract, showing peak at 415 nm (B) TEM image of the Ag NPs (C) HRTEM image of the Ag NPs. (D) Particle size distribution, calculated based on the TEM image.

However, all the characterizations and anti-bacterial experiments were performed by using the Ag NPs synthesized at pH 7.5, as it is close to the physiological pH. In order to address the functional groups involved in the stability of synthesized Ag NPs, FTIR analysis was performed. The major constituents of the latex of *Jatropha curcas* are curcacycline A (acyclic octapeptide), curcacycline B (acyclic nonapeptide) and curcain (an enzyme)[15].

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These peptides act as reducing and stabilizing agent for Ag NPs[18]. FTIR spectra revealed that, in case of the composite (Ag NPs-plant extract), all the corresponding FTIR peaks remained same as plant extracts but the peak of -NH stretching and C=O (-NHCOCH3) were shifted from 3390 cm^{-1} to 3454 cm^{-1} and 1610 cm^{-1} to 1627 cm^{-1} respectively (Supplementary Information, Figure S4). It can be inferred from the FTIR data that -NH and /or amide groups of plant extracts were involved in the synthesis as well as stabilization of Ag NPs.

ANTI-BACTERIAL ACTIVITY OF THE NANO-COMPOSITE

The antibacterial properties of the composite were tested against recombinant GFP-expressing *E. coli* (DH5α). For that, various amounts of nano-composites were added and time-dependent bacterial growth was monitored by measuring OD at 595 nm. It was found that with increasing concentration of composite, bacterial growth decreased. The lowest concentration of the composite, at which no visual turbidity was observed up to 12 hours, was considered as the minimum inhibitory concentration (MIC), which contained 3.1 ± 0.76 µg/ mL of silver. The minimal killing concentration (MKC) of the composite, which prevents growth of the bacterial cells, following reinoculation of MIC for another 12 h contained 4.4 ± 0.87 µg/ mL of silver. It is to be noted that respective amount of individual components of the composite i.e. Ag NPs and plant extract didn't show any significant growth inhibition. Thus, the overall results indicated that the composite (Ag NPs –plant extract) was more effective than its constituent components. Moreover, the MIC and MKC values of silver (Ag) obtained in our study are far less than only Ag NPs used in the previous studies[19, 20].

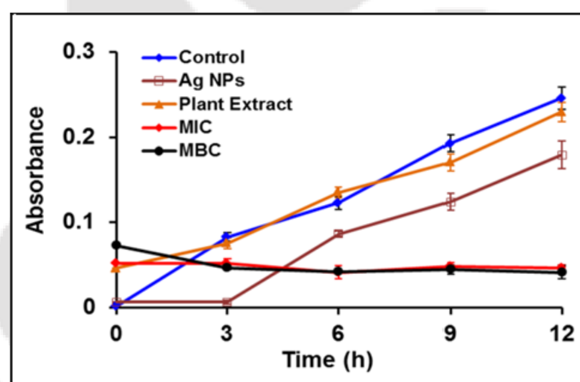

Figure 2
Growth curve of the GFP expressing E.coli in presence of the composite and various controls.

The GFP expressing *E.coli* was opted, as it is easy to get an idea about the number of viable cells by probing the fluorescence of GFP by using fluorescence microscopy after treatment with composite. The GFP expressing recombinant bacteria shows green fluorescence (510 nm) when excited with blue light. For microscopy, bacteria treated with MIC and MBC doses of composite along with the control was withdrawn at specific time intervals (6 and 12 h) and visualized under microscope. It was found that there was almost equal numbers of bacteria at 0 h in all three samples as shown in Figure 3. However, due to cell division the number of bacterial population increased with time in case of the control whereas no bacterial population was observed at 3 h and 6 h time points in case of composite treated cells (MIC and MBC). This is possibly due to inhibition of bacterial growth with the treatment of composite.

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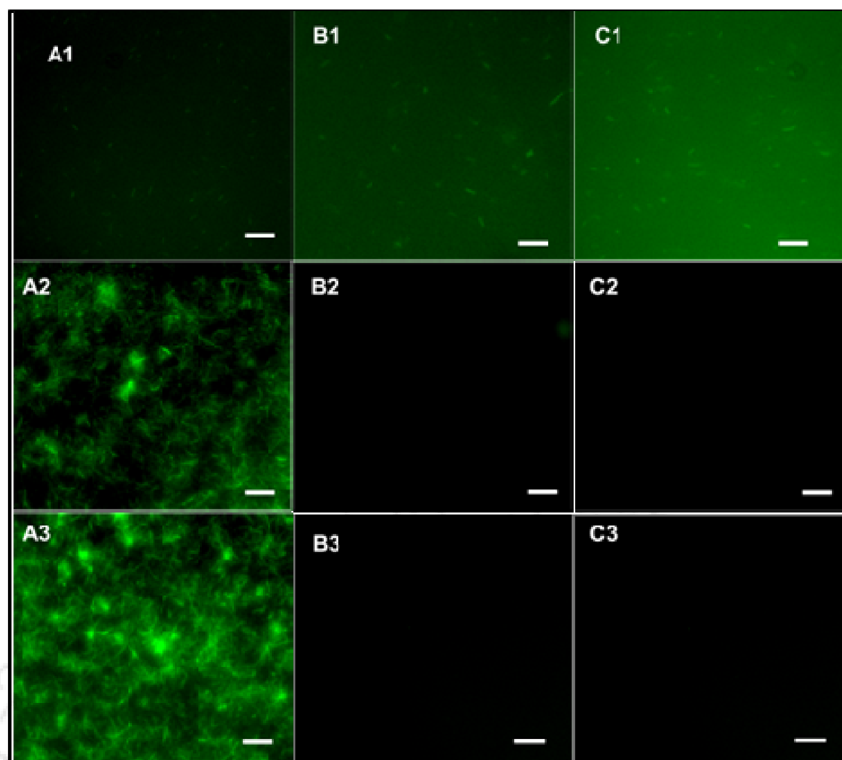

Figure 3
Fluorescence microscopy images of the GFP expressing E.coli in presence of the composite and various controls. (A-C) represents control, MIC and MBC treated bacterial cells respectively. 1, 2 and 3 represents the 0, 6 and 12 h time points. Bacterial cell number was increased with time in case of control, whereas no bacterial cells were observed after treatment with the composites after 12 h. Scale bar was 20 µm.

MECHANISM OF ANTI-BACTERIAL ACTIVITY

For development of novel antibacterial agents it is very important to find out the mechanism of cell death such that it helps to increase the efficiency of the materials. To probe the interaction of nano-composite with the bacterial cell surface, FESEM analysis was carried out, which revealed that sufficient amount of Ag NPs were attached with the bacterial cell surface in case of composite treated cells as compared to the control cells (Figure 4). It was also found the composite induced pores in the bacterial cell walls (marked with red circle in Figure 4B) possibly leading to cell death.

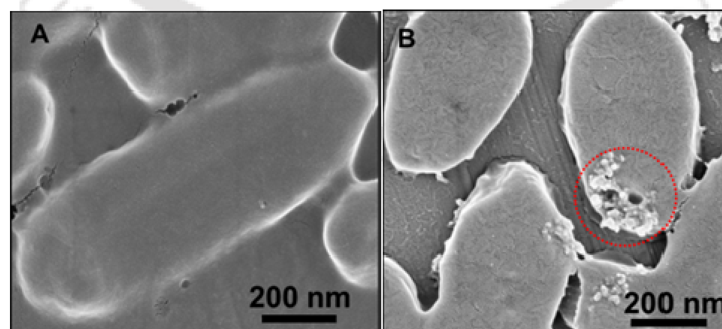

Figure 4
FESEM image (A) control (B) composite treated bacterial cells (MIC). Red circle marked damage portion of the bacterial cell wall due to effects of composite.

Further, to substantiate the above results, fluorescence activated cells sorter (FACS) analysis was performed by using propidium iodide (PI) staining method. The PI is a DNA

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binding dye gives red emission (620 nm). However, it cannot penetrate the normal intact cell wall, formation of a considerable number of pores in the cell wall facilities its cellular internalization and sequent binding with the DNA. Therefore, PI positive cells indicate that organisms are failed to maintain their membrane integrity and are therefore nonviable. In the FACS analysis PI and GFP emission were collected in FL2-H and FL1-H respectively. The results revealed that the number of bacterial cells in FL2-H channel (PI) was increased and the number was decreased in FL-1H (GFP) in case of composite treated cells due to leak out of intracellular materials including GFP through the damaged cell wall as compared to its respective controls, as depicted in Figure 5. Different stages of the cells such as live (only GFP emission), compromised cells (both GFP and PI emission) and dead cells (only PI emission) were illustrated in the Figure 5A. For example, the numbers of the compromised cells which showed the both emissions of PI and GFP and dead cells increased from 1.22 % to 60.73 % and from 0.11 % to 17.24 in case of control and at the MBC concentration of the composite, respectively. The detail results have been provided in the supplementary information (Table S1). The overall results depicted that cell wall damage was the primary reason of cell death in the present study.

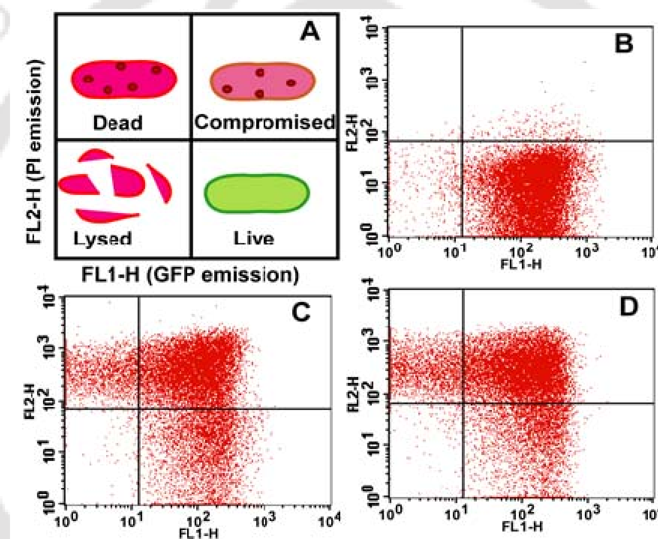

Figure 5

(A) Schematic representation of dot plots of the FACS results, showing the different cell viability stages, live cells (green fluorescence due to the GFP), compromised cells (double fluorescence of GFP and PI), dead cells (PI emission due leak out of the GFP) and lysed cells (cell debris, no fluorescence) (B) Control cells, (C) and (D) are the Ag NPs composite treated cell at the concentration of MIC and MBC respectively.

To find out the effects of Ag NPs at DNA level, bacteria were treated with MIC and MBC doses of the composite followed by isolation of GFP containing pDNA. It should be mentioned here that to obtain the sufficient amount of pDNA followed by treatment with composite, higher amount of bacteria culture was used for inoculation as compared to the standard (1 %). The gel electrophoresis results showed a decrease in the relative amount of plasmid DNA in the treated samples as compared to their respective controls, but the migration patterns of DNA remained unaltered (Supplementary Information, Figure S5). This result confirms that in the present case the Ag NPs composite led to bacterial cell death due to cell wall perforation as there was no direct effect on DNA level found.

ANTI-CANCER ACTIVITY OF THE AG NPS COMPOSITE

Various plant products and its metabolites are predominantly used as conventional medicines (quinine, digitoxin, aspirinetc) for a long time to cure several diseases including cancer which are killing many lives every day in all over the world. Importantly, it was found that different parts of Jatropha could be used

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to kill the cancer cells. Thus, to find out the effects of the Ag NPs composite, different concentrations of the composites were added into the HeLa cells followed by performed the MTT assay (3-(4, 5- dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide). The result demonstrated that with increased the amount of Ag NPs-composite the number of viable cells were decreased (Figure 6). The IC_{50}, the concentration of the drugs where 50 % cells dies, was found to be 4.58 µg/mL. It would be mentioned here that composite was found to be highly cytotoxic as compared to the previous studies[21]. Thus, it is essential to probe the reason of this high level of the cytotoxicity. Silver NPs were reported to increase the reactive oxygen species generation into the treated cells and leads to mitochondria depended apoptosis[22].

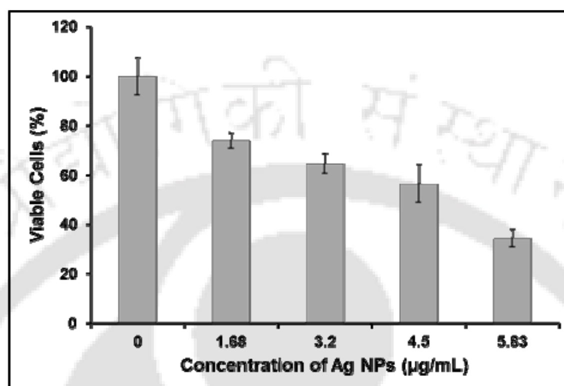

Figure 6
MTT assay showing cell viability after 24 h of treatment with the Ag NPs-composite.

ROS measurement was carried out by flowcytometry analysis after treatment with the IC50 dose of the composite. To measure the ROS, 2, 7-dichlorofluoresceindiacetate (DCFH-DA) was used, which is a non-fluorescent molecule. It diffuses into the cell through the plasma membrane where it is hydrolysed to DCFH by intracellular esterase enzyme and trapped inside the cells, which is further oxidized to fluorescent DCF (green emission) by intra cellular reactive oxygen species. Thus, the amount of fluoresce intensity is directly correlated with the amount of ROS production inside the cells, the fluorescent signal is collected in the FL1 channel of FACS. The results demonstrated the composite treated cells showed significant shifting of the fluorescence intensity in case of the composite treated as compared to the control due to ROS generation (Figure 7). Overall results imply that composite possibly leads to ROS mediated cells death.

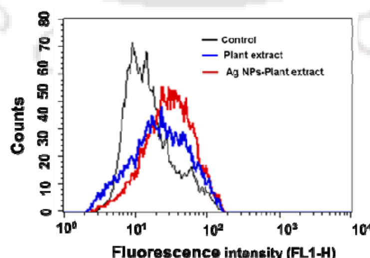

Figure 7
Reactive oxygen species (ROS) generation by treatment with the Ag NPs-plant extract. The fluorescence signal was collected in FL1-H corresponds to the green emission of the DCF.

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MATERIALS AND METHODS

ANALYTICAL MEASUREMENTS

UV–vis and fluorescence spectra were recorded by a Perkin–Elmer lambda 45 spectrophotometer (Perkin–Elmer, Fremont, CA) and Perkin–Elmer fluorescence spectrophotometer (model: LS 55) respectively.For XRD measurements, the as-synthesized composite (Ag NPs-plant extract) was evaporatedon a glass slide followed by recording of the diffraction using a Bruker Advance D8 XRD machine (with Cu α source at 1.54 A). The FTIR spectra were recorded with a lyophilized sample in Perkin–Elmer Spectrum One FTIR spectrometer, in the range of 4000–400 cm^{-1}. The KBr was used to make pellets requisite for the measurements.

RESULTS AND DISCUSSION

Figure S1
XRD spectra of the Ag NPs –composite.

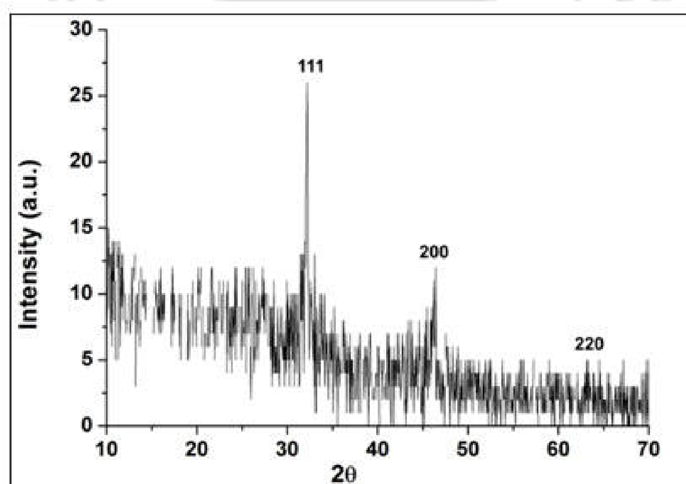

Figure S2
Time dependent UV-Vis Spectra of the composite.

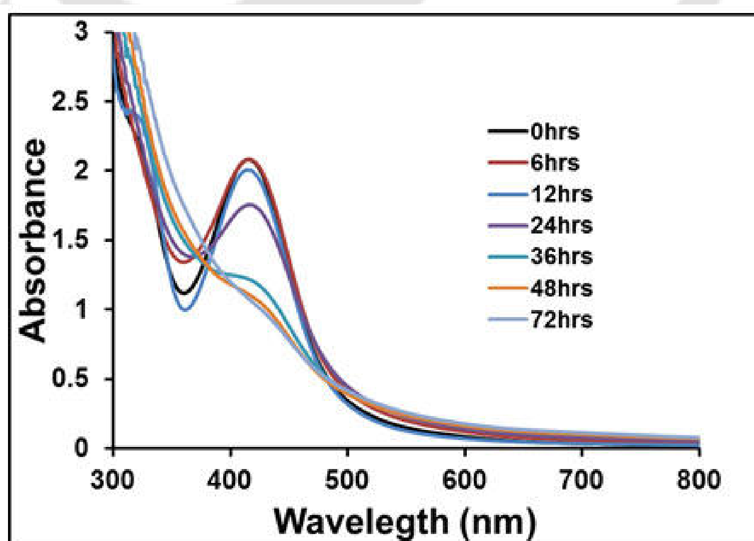

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Figure S3
UV-Vis spectra of the Ag NPs-composite at different pH

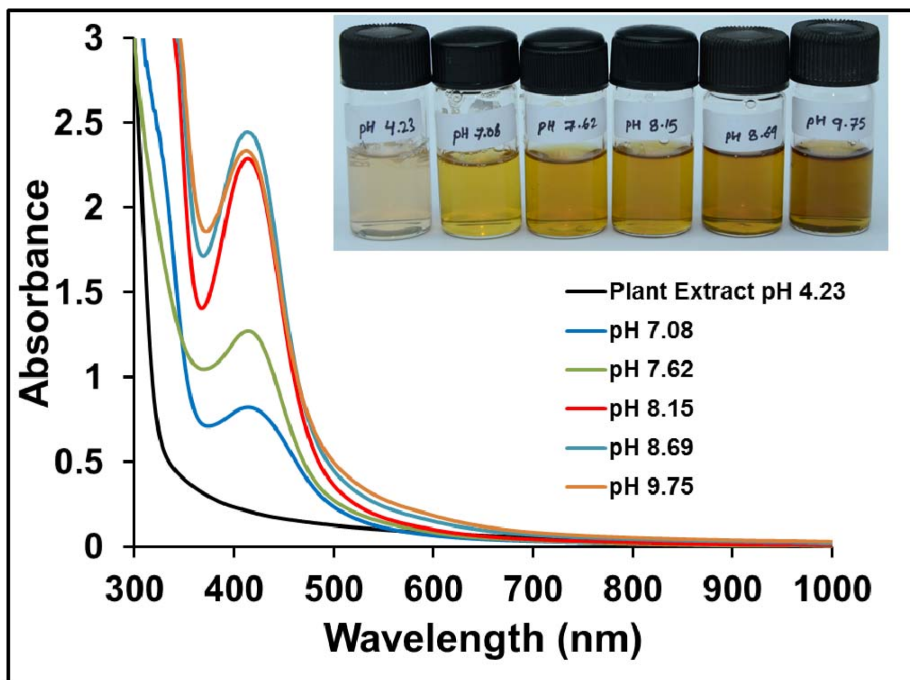

Figure S4
FTIR spectra of the (A) Plant extract (B) Ag NPs –plant extract

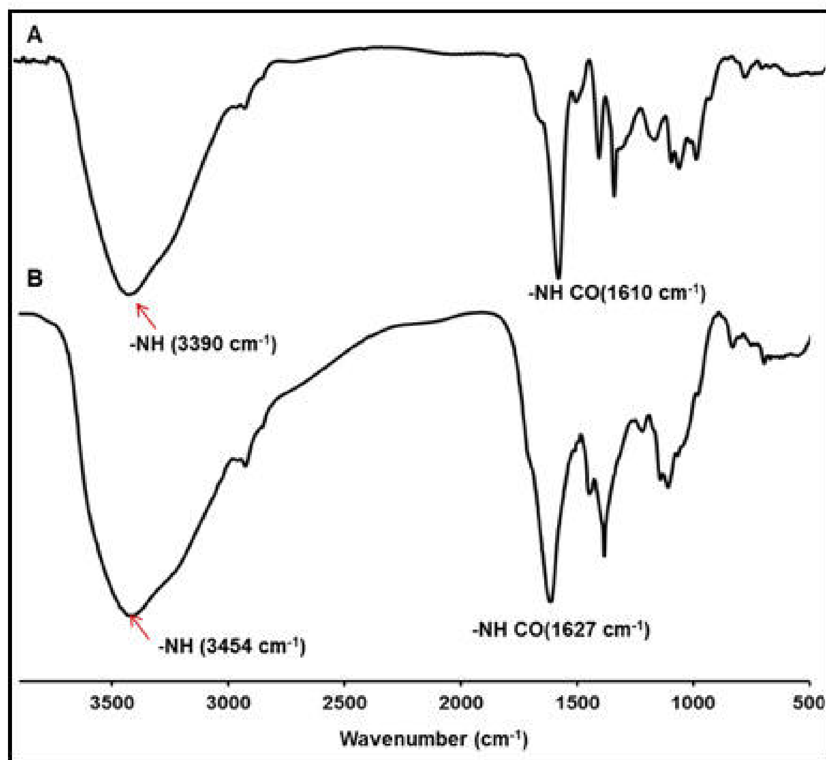

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Figure S5

Gel image of the plasmid DNA (GFP -plasmid) which was isolated from the composite treated cells. Lane 1: control, Lane 2: plant extract, lane 3: MIC and Lane 4: MBC treated cells.

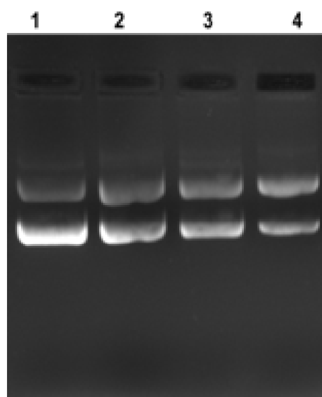

Table S1

FACS results demonstrating the number of the bacteria died after treatment with Ag NPs-plant extract.

	Control	MIC	MBC
Live (%)	96.63	26.03	21.30
Compromised (%)	1.22	60.31	60.73
Dead (%)	0.11	13.10	17.24
Lysed (%)	2.05	0.57	0.73

CONCLUSION

In brief, we have described a simple one step 'green syntheses' of Ag NPs from the latex of *Jatropha curcas* at physiological condition. The method minimizes the risk of the environmental pollution as well as on human health. The synthesized NPs exhibited excellent anti-bacterial activity against *E.coli* and cytotoxicity for cancer cell. Thus, the present method of synthesis of Ag NPs would have huge potential in the fields of pharmaceuticals and therapeutics.

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Chapter 3

Jatropha (*Jatropha curcas* L.)

Devendra Kumar Maravi, Purabi Mazumdar, Shamsher Alam, Vaibhav V. Goud, and Lingaraj Sahoo

Abstract

The seed oil of *Jatropha* (*Jatropha curcas* L.) as a source of biodiesel fuel is gaining worldwide importance. Commercial-scale exploration of *Jatropha* has not succeeded due to low and unstable seed yield in semiarid lands unsuitable for the food production and infestation to diseases. Genetic engineering is promising to improve various agronomic traits in *Jatropha* and to understand the molecular functions of key *Jatropha* genes for molecular breeding. We describe a protocol routinely followed in our laboratory for stable and efficient *Agrobacterium tumefaciens*-mediated transformation of *Jatropha* using cotyledonary leaf as explants. The 4-day-old explants are infected with *Agrobacterium tumefaciens* strain EHA105 harboring pBI121 plant binary vector, which contains *nptII* as plant selectable marker and *gus* as reporter. The putative transformed plants are selected on kanamycin, and stable integration of transgene(s) is confirmed by histochemical GUS assay, polymerase chain reaction, and Southern hybridization.

Key words *Agrobacterium tumefaciens*, Cotyledonary leaf, *Jatropha curcas*, Kanamycin resistance, Plant binary vector, Stable transformation

1 Introduction

Jatropha (*Jatropha curcas* L.) is an important nonedible oilseed crop receiving worldwide attention as a biodiesel feedstock. The seeds contain 40–50 % of oil, which can be blended directly with petro-diesel or transesterified for use as biodiesel. The short gestation period, drought endurance, low cost of seeds, high oil content, and easy adaptation on degraded soil unsuitable for food crops make Jatropha as the most sought oil seed crop among the nonedible oil-yielding crops for biodiesel production [1, 2]. The huge demand of Jatropha seeds is expected to be met through its large-scale cultivation and increasing its productivity. However, several undesirable traits of Jatropha such as low and unstable seed yield, dependent on the growth conditions and environment, limit its commercial use [3]. Furthermore, large-scale cultivation of Jatropha through monocrop plantation especially under

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humid conditions encounters devastation by insects [4–6] and begomoviruses epidemics [7–9]. Conventional breeding for the development of superior genotype of Jatropha with high and stable seed yields in degraded and marginal lands and resistant to insect pest and virus has not progressed due to the narrow genetic base and failure in wide hybridization. Genetic engineering offers immense opportunity to complement conventional breeding for the improvement of Jatropha for enhanced fatty acid biosynthesis, tolerance to biotic and abiotic stress, regulation of secondary metabolites and phorbol esters, and manipulation of female to male flower ratio. A reproducible and efficient protocol for the genetic manipulation of Jatropha will assist in its targeted improvement besides unraveling the functions of genes identified in this plant. Stable transformation of Jatropha has been reported by few laboratories including ours [10–14]. *Agrobacterium*-mediated transformation is preferred as it offers several advantages, such as the defined integration of transgenes, preferential integration into transcriptionally active regions of the chromosomes, and potentially single or low copy number with rearrangement being relatively rare [15, 16].

In this chapter, we provide details of an efficient protocol for *Agrobacterium tumefaciens*-mediated Jatropha transformation using cotyledonary leaf as explants. *Agrobacterium tumefaciens* strain EHA105 harboring plant binary vector, pBI121, containing *nptII* as a plant selectable marker and *gus* as reporter is used to infect the cotyledonary leaves followed by regeneration of stable transformed plants under kanamycin selection. The entire process of explant infection to transgenic plant establishment in greenhouse requires approximately 90 days (Fig. 1). The average transformation efficiency of our system is 1.5 % (defined as number of plants positive for plant selectable marker gene by polymerase chain reaction recovered from the total number of explants infected).

2 Materials

2.1 Plant Materials

Seeds of an elite accession of *Jatropha curcas*, IITGJC-19, were used for plant transformation. Jatropha seeds are collected from the shade house-grown plants (*see* **Note 1**).

2.2 Agrobacterium tumefaciens *Strain and Vector*

Agrobacterium tumefaciens strain EHA105 harboring the binary vector pBI121 is used for Jatropha transformation. The T-DNA of pBI121 includes *nptII* (neomycin phosphotransferase) driven by nopaline synthase (NOS) promoter and *gus* (β-glucuronidase) gene driven by CaMV35S promoter (*see* **Note 2**).

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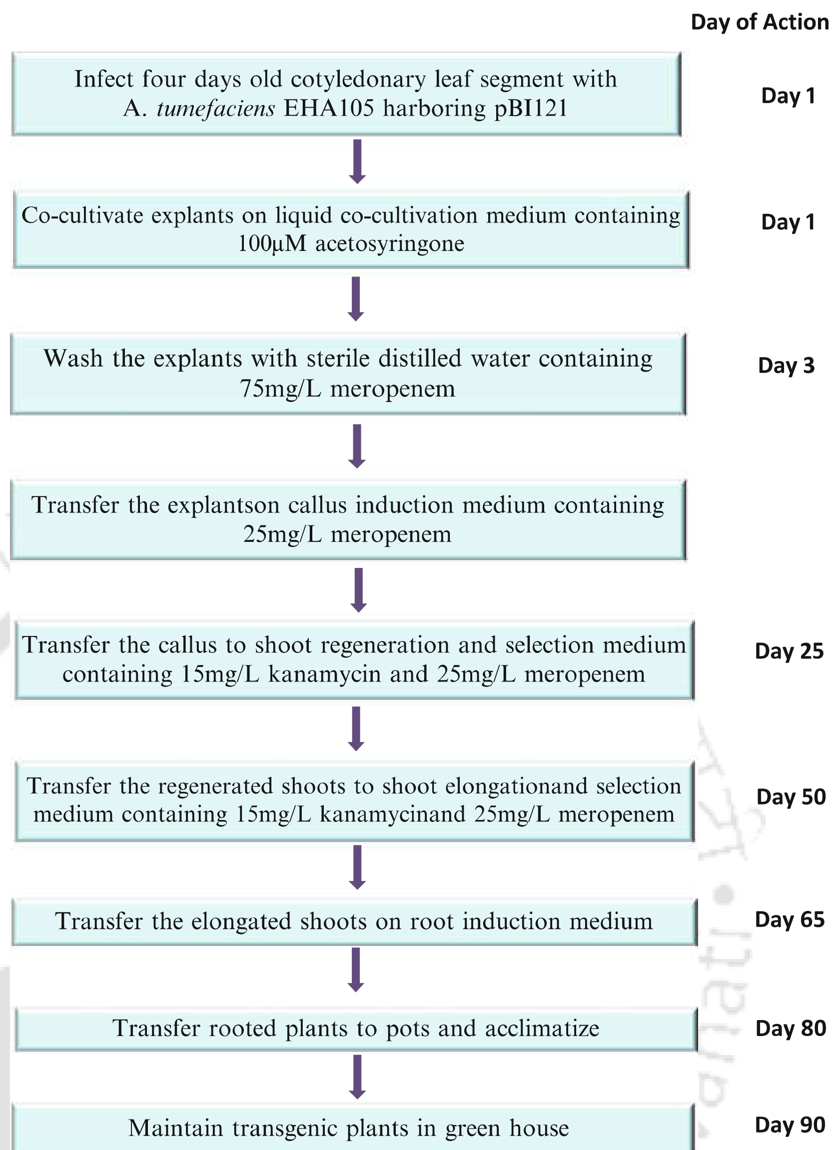

Fig. 1 Schematic representation of *Agrobacterium*-mediated transformation of *Jatropha curcas*

2.3 Stock Solutions

2.3.1 Plant Growth Hormones

1. 6-Benzylaminopurine (BAP): Prepare the stock solution of 1 mM by dissolving 22.52 mg of BAP salt in a few drops of 1 N NaOH and then make up the volume to 100 ml with dH_2O, filter sterilize (*see* **Note 3**), and store at –20 °C.
2. Indole-3-butyric acid (IBA): Prepare the stock of 1 mM by dissolving 20.04 mg of IBA salt in few drops of 1 N NaOH and then make up the volume to 100 ml with dH_2O, filter sterilize, and store at –20 °C.
3. Gibberellic acid (GA3): Prepare the stock of 1 mM by dissolving 34.6 mg of GA3 salt in few drops of 1 N NaOH and then make up the volume to 100 ml with distilled water, filter sterilize, and store at –20 °C.

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2.3.2 Antibiotics

1. Kanamycin sulfate: Prepare the stock of 100 mg/ml by dissolving 500 mg of kanamycin powder in 5 ml of sterile dH_2O, filter sterilize, and store at −20 °C (*see* **Note 4**).
2. Rifampicin: Prepare the stock of 10 mg/ml by dissolving 10 mg of rifampicin in few drops of dimethyl sulfoxide (DMSO) and then make up the volume by adding sterile dH_2O, filter sterilize, and store at −20 °C.
3. Meropenem: Prepare the stock of 50 mg/ml by dissolving 1 g of meropenem salt in 20-ml sterile distilled water, filter sterilize, and store at 4 °C.

2.3.3 Other Solution

1. Sterilization solution: 70 % (v/v) ethanol and 0.1 % (v/v) sodium hypochlorite, Tween 20 and 0.2 % (w/v) mercuric chloride (*see* **Note 5**).
2. Acetosyringone: Prepare the stock solution of 100-mM acetosyringone (3′,5′-dimethoxy-4′-hydroxyacetophenone) by dissolving 0.392-g acetosyringone salt in 10-ml DMSO, filter sterilize, and store at −20 °C.
3. ß-Glucuronidase: GUS assay buffer (50–100 μg/ml X-glcA in 20 mM $NaPO_4$, pH = 7.0).
4. DNA extraction buffer: 20-μM Tris–HCl, pH 7.5, 250-μM NaCl, 25-μM EDTA, pH 8.0, and 0.5 % sodium dodecyl sulfate (SDS).
5. High-salt TE buffer: 10-mM Tris–HCl, pH 8.0, and 1-mM EDTA, pH 8.0, 1 M NaCl.
6. Polymerase chain reaction (PCR) components: 10× Taq buffer, 10-mM dNTPs, 50 pmol/μl each of forward and reverse primer, 3 U/μl Taq polymerase, ~100-ng DNA template, and sterile Milli-Q water to make up the volume up to 25 μl.

2.3.4 Culture Medium Stock

All stock solutions are freshly prepared on a monthly basis and stored at 4 °C.

1. Murashige and Skoog (MS) major salts (10×): Dissolve 19.0 g/l KNO_3, 16.5 g/l NH_4NO_3, 3.7 g/l, $MgSO_4 \cdot 7H_2O$, 4.4 g/l $CaCl_2 \cdot 2H_2O$, and 1.7 g/l KH_2PO_4 [17].
2. MS minor salts (100×): Dissolve 2.2 g/l $MnSO_4 \cdot 4H_2O$, 83 mg/l KI, 620 mg/l H_3BO_4, 860 mg/l $ZnSO_4 \cdot 7H_2O$, 2.5 mg/l $CuSO_4 \cdot 5H_2O$, 25 mg/l $NaMoO_4 \cdot 2H_2O$, and 2.5 mg/l $CoCl_2 \cdot 6H_2O$ [17].
3. MS vitamin stock (200×): Dissolve 100 mg/l niacin, 100 mg/l pyridoxine HCl, 400 mg/l glycine, and 20 mg/l thiamine HCl [17].
4. Iron stock (200×): Dissolve 7.45 g/l of Na_2EDTA (ethylenediaminetetraacetic acid, disodium salt) in 500-ml dH_2O and 5.57 g/l of $FeSO_4 \cdot 7H_2O$ in 500-ml dH_2O separately. Boil the

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Na_2EDTA solution and add $FeSO_4$ solution to it, gently by stirring [17].

5. All culture medium stock is stored in amber-colored bottle at 4 °C.
6. *Agrobacterium* minimal medium (AB) buffer (20×): Dissolve 60 g/l K_2HPO_4 and 20 g/l NaH_2PO_4 in dH_2O, adjust pH to 7.0 with 1 N NaOH or 1 N HCl, as required, then autoclave (*see* **Note 6**) [18].
7. AB salts (20×): Dissolve 20 g/l NH_4Cl, 6 g/l $MgSO_4 \cdot 7H_2O$, 3 g/l KCl, 0.2 g/l $CaCl_2$, 50 mg/l $FeSO_4 \cdot 7H_2O$ in dH_2O then autoclave (*see* **Note 7**) [18].
8. D-glucose (0.5 %): Dissolve 5-g D-glucose in 1 l of dH_2O then autoclave [18].

2.3.5 Plant Culture Media

1. Murashige and Skoog medium (MS): MS medium (major salt 50 ml, minor salt 5 ml, vitamin 5 ml, iron 5 ml, and myoinositol 100 mg) supplemented with 3 % (w/v) sucrose, adjust pH 5.84 with 1 N NaOH or 1 N HCl. Add agar-agar (3.2 g/l) prior to autoclaving (Subheading 2.3.4).
2. Liquid cocultivation media (LCM): MS liquid medium containing 6.66-μM BAP and 0.24-μM IBA, adjust pH to 5.7, and supplement with 100-μM acetosyringone (*see* **Note 8**).
3. Callus induction medium (CI): MS medium supplemented with 6.66-μM BAP, 0.24-μM IBA, 3 % (w/v) sucrose, adjust pH to 5.84 with 1 N NaOH or 1 N HCl and solidified with 0.8 % (w/v) agar-agar, autoclave and add 25 mg/l meropenem, mix thoroughly, and dispense 25 ml into 90-mm sterile Petri dishes.
4. Shoot regeneration and selection medium (SR): MS medium supplemented with 6.66 μM BAP, 0.24 μM IBA, 1.5 μM GA3, 3 % (w/v) sucrose, adjust pH to 5.84 with 1 N NaOH or 1 N HCl, and solidified with 0.8 % (w/v) agar-agar. Autoclave and add 15 mg/l kanamycin and 25 mg/l meropenem during explant inoculation (*see* **Note 9**).
5. Shoot elongation and selection medium (SE): MS medium supplemented with 1-μM GA3, 3 % (w/v) sucrose, adjust pH to 5.84 with 1 N NaOH or 1 N HCl and solidify with 0.8 % (w/v) agar-agar, autoclave, and add 15 mg/l kanamycin and 25 mg/l meropenem during explant inoculation.
6. Root induction medium (RI): MS medium consisting of half strength of MS major and MS minor elements, full strength of vitamin, and 2 % (w/v) sucrose; adjust pH to 5.84 with 1 N NaOH or 1 N HCl, solidify with 0.6 % (w/v) agar, autoclave, and add 5-μM IBA and 25 mg/l meropenem during shoot transfer.
7. Sterilize all tissue culture media by autoclaving for 20 min at 121 °C and 15 psi.

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2.3.6 Culture Media for A. tumefaciens

1. Luria-Bertani (LB) agar medium: Dissolve 25 g of LB powder in 1 l of dH_2O. Add 15 g/l agar-agar, autoclave, cool to about 55 °C, and add 50 mg/l kanamycin, 10 mg/l rifampicin for the selection of *Agrobacterium tumefaciens* strain EHA105 harboring plant binary vector pBI121.
2. AB minimal media: Combine 5-ml sterile 20× AB buffer and 5-ml 20× AB salts in 90-ml sterile D-glucose (final concentration of D-glucose is 0.5 %).

3 Methods

3.1 Preparation of Explant for Inoculation

1. Remove the seed coat and soak in distilled water for overnight at room temperature.
2. Surface-sterilize the de-coated seeds with 0.1 % sodium hypochlorite solution supplemented with few drops of Tween 20 for 15 min and rinse with distilled water several times.
3. Surface-sterilize the seeds with 70 % ethanol for 5 min and rinse three times with sterilized distilled water.
4. Finally, sterilize with 0.2 % mercuric chloride for 2 min and rinse with sterile distilled water 3–4 times under the laminar air flow cabinet.
5. Blot dry the seeds using sterile filter paper.
6. Carefully dissect the endosperm to expose out embryos with papery cotyledonary leaves using forceps and scalpel.
7. Separate out the papery cotyledonary leaves and germinate on MS basal medium.
8. Cut the papery cotyledonary leaves into four segments (8 mm) with their edges removed and use as explant for *Agrobacterium-mediated* transformation.

3.2 Agrobacterium Culture for Infection

1. Streak *Agrobacterium tumefaciens* strain EHA105 on solid LB medium supplemented with 50 mg/l kanamycin and 10 mg/l rifampicin for 2 days at 28 °C (*see* **Note 10**).
2. Inoculate a single bacterial colony into 25 ml of liquid AB minimal medium with appropriate antibiotics to select the binary plasmid and agitate overnight at 28 °C on a rotary shaker at 180 rpm, until optical density at 600 nm reaches to 0.8.
3. Transfer the liquid culture to a centrifuge tube, and pellet the cells by spinning for 10–15 min at 4 °C for 4,025 × *g*. Decant the supernatant and resuspend the cell pellet in liquid cocultivation media (pH 5.7) supplemented with 100-μM acetosyringone and 0.24-μM IBA. Adjust the bacterial cell density, if necessary, by further dilution to achieve $OD_{600} = 0.6–0.8$. *Agrobacterium* suspension cultures are then ready to use for transformation experiment.

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3.3 Infection, Cocultivation, and Regeneration

1. Inoculate the explants in *Agrobacterium* suspension for 30 min with occasional shaking in the dark at 22 °C.
2. Decant the *Agrobacterium* suspension and blot dry the explants gently on sterile filter paper to remove excess *Agrobacterium*.
3. Cocultivate the explants in 90-mm sterile Petri dishes lined with filter paper, moistened with LCM, supplemented with 100-μM acetosyringone and 0.24-μM IBA. Seal the Petri dish with Parafilm and incubate at 22 °C for 3 days in dark condition (*see* **Note 11**).
4. After 3 days of cocultivation, wash the explants 4–5 times with sterile distilled water containing 75 mg/l meropenem.
5. Blot dry the explants on sterile filter paper and transfer to the callus induction medium containing 25 mg/l meropenem; incubate in dark condition for callus induction.
6. Transfer the cultures to fresh CI medium containing 25 mg/l meropenem at an interval of 12 days (*see* **Note 12**).
7. After 3 week of culture, transfer the calli to SR and selection medium containing 15 mg/l kanamycin and 25 mg/l meropenem, and incubate at 16-h photoperiod with a photosynthetic photon flux density of 35 μmol/m^2/s provided by cool white fluorescent tubes.
8. Transfer the cultures periodically onto fresh selection medium at an interval of 12 days.
9. After 3 week of culture on selection, detach the proliferating kanamycin-resistant shoots and transfer to SE medium containing 15-mg/l kanamycin and 25-mg/l meropenem.

3.4 Root Induction and Acclimatization

1. Transfer the elongated shoots after a week to the RI medium containing 25-mg/l meropenem (*see* **Note 13**).
2. Wash the well-rooted putative transformed plantlets to remove excess agar under running tap water.
3. Establish the plantlets into pots containing soil and vermicompost (1:1), and cover the pots with transparent plastic bags to maintain adequate moisture for 1–2 weeks (*see* **Note 14**).
4. Remove the plastic bags after 1–2 weeks and maintain the plants in greenhouse (*see* **Note 15**) plastic pots containing normal garden soil.
5. Seeds are ready for harvesting around 90 days after flowering when the fruits have changed color from green to yellow brown (*see* **Note 16**).

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3.5 GUS Histochemical Assay

1. In order to verify stable expression of *gus* gene in transgenic shoots [18], dip the callus and leaf into X-Gluc solution and incubate at 37 °C in the dark for 18–24 h.
2. After incubation, transfer the callus and leaf to 95 % ethanol, and incubate overnight to bleach the chlorophyll out from tissues (*see* **Note 17**).
3. The intensity of the blue color in the tissue should indicate stable expression of *gus* (*see* **Note 18**).

3.6 Genomic DNA Isolation and PCR Analysis

1. Take 100-mg leaf tissue, wash with sterile distilled water, and blot dry with tissue paper to remove water.
2. Place the tissue in a prechilled mortar and pestle and homogenate to powder with liquid nitrogen; transfer the powder to sterile microcentrifuge tube.
3. Add 700 μl of extraction buffer (preheated at 60 °C for 15 min) to homogenate, mix gently to avoid shearing of DNA, and incubate in water bath at 65 °C for 45 min.
4. Bring down the sample temperature to room temperature, add 700-μl chloroform-isoamyl alcohol (24:1), and mix gently by inverting for a period of 5 min.
5. Spin at 7,155 × *g* for 10 min at 25 °C; transfer the supernatant to a fresh microcentrifuge tube.
6. Add 150 μl of 5 M NaCl and mix properly.
7. Add 0.6 volume of cold isopropanol. Mix gently and allow the mixture to stand at 4 °C for 45 min (*see* **Note 19**).
8. After 60 min, spin the samples at 11,180 × *g* for 10 min at 25 °C, discard the supernatant, and wash the pellet with 80 % ethanol.
9. Dry the pellet in vacuum for 15 min and dissolve in 200 μl of high-salt TE buffer.
10. Add 5 μl of RNase and incubate at 37 °C for 45 min.
11. Extract with equal volume of chloroform-isoamyl alcohol (24:1), mix gently, and spin at 10,000 rpm for 10 min.
12. Transfer the aqueous layer to a fresh 1.5-ml centrifuge tube, and add 2 volume of cold ethanol; keep in −20 °C for 1 h.
13. Spin at 10,000 rpm at 4 °C for 10 min, perform ethanol (80 %) wash, and spin for 10 min at 10,000 rpm.
14. Dry the pellet and suspend in sterile double distilled water (*see* **Note 20**).
15. Measure the DNA concentration by running the sample on 0.8 % agarose gel or taking the absorbance at 260 nm (*see* **Note 21**).
16. PCR reaction mixture: in an Eppendorf tube, add 2.5-μl 10× Taq buffer, 0.5-μl 10-mM dNTPs, 1-μl forward primer

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(50 pmol/μl), 1-μl reverse primer (50 pmol/μl) for nptII and gus gene, respectively (*see* **Note 22**), 0.5-μl Taq polymerase (5 U/μl), ~100-ng DNA template, then make up the final volume up to 25 μl.

17. PCR condition: 95 °C for 5 min (1 cycle), 95 °C for 1 min (denaturation), 58 °C for 1 min (annealing), 72 °C for 1 min (extension) for 35 cycles, followed by the final extension at 72 °C for 5 min (1 cycle).
18. Resolve the PCR-amplified product by electrophoresis on 1 % agarose gel and visualize with ethidium bromide staining under UV transilluminator and document in gel documentation system (Bio-Rad Laboratories).

4 Notes

1. The seeds are collected from the shade house-grown *Jatropha curcas* of Indian Institute of Technology Guwahati.
2. Selectable marker neomycin phosphotransferase encoding gene II (*nptII*) confers kanamycin resistance to transformed plant cells.
3. Antibiotic stocks are aliquots in 1.5-ml sterile Eppendorf tubes and stored at −20 °C.
4. All filter sterilization is carried out with 0.22-mm syringe filter.
5. Mercuric chloride ($HgCl_2$) is a highly effective surface sterilant and extremely toxic and must be disposed off according to safety regulations in the laboratory. It may be necessary to use a specially designated sink for toxic chemicals for the washing step.
6. Prepare KH_2PO_4 and $NaH_2PO_4 \cdot 2H_2O$ separately and then mix and bring the final volume up to 100 ml with distilled water.
7. AB salt solution may show yellow precipitates after autoclaving which is dissolved by shaking vigorously just before use.
8. Increased concentration of acetosyringone up to 100 μM enhances the transient transformation efficiency and decreases with further increases in concentration.
9. The 15-mg/l kanamycin is the optimal concentration to select transformed explants, and 25-mg/l meropenem is used to eliminate the growth of *Agrobacterium*.
10. *Agrobacterium* culture lose their viability when kept in LB + Rif + Kan plate at 4 °C for more than 3 weeks; therefore, subculture the plate in regular intervals.
11. A 3-day cocultivation period is optimum for transient transformation experiment. Cocultivation period longer than 3-day

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reduces the transformation efficiency and results in *Agrobacterium* overgrowth which causes detrimental effect on regeneration of explants.

12. The subculture is performed at the interval of 12 days in fresh medium of the same compositions to avoid drying of the tissues.
13. Kanamycin inhibits the root formation; therefore, elongated shoots are transferred to kanamycin-free rooting medium.
14. Plastic bags are used to maintain adequate moisture and to prevent wilting of plantlets.
15. Green house is maintained at 25 ± 2 °C, relative humidity 60 ± 5 %, and 16-h photoperiod. The light intensity is maintained at a photosynthetic photon flux density (PPFD) of 240 μM/m^2/s provided by 40 W cool white fluorescent lamps.
16. Gestation period of *Jatropha curcas* is 2–3 years, and each fruiting body contains three seeds.
17. Bleaching of explants is performed with 99.5 % ethanol to remove the chlorophyll content and explants are observed under the microscope.
18. GUS-positive plantlets are used for the molecular analysis to confirm the *gus* and *npt*II transgenes.
19. After 45-min incubation, slow and careful mixing results in floating of fibrous nucleic acid, which can be scooped off into fresh microcentrifuge tube.
20. Pellet may be air-dried for 10 min.
21. The nptII and gus are amplified using respective 20 mers primers (nptII Fw, CCACCATGATATTCGGCAAC; Rv, GTGGAGAGGCTATTCGGCTA) and 24 mers (gus Fw, TAACCTTCACCCGGTTGCCAGAGG; Rv, CCTTAACTA AGCCGGAATCCATCG).
22. To ensure the isolated DNA is of high quality, the DNA sample is examined by running 1 % agarose gel electrophoresis.

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Appendix

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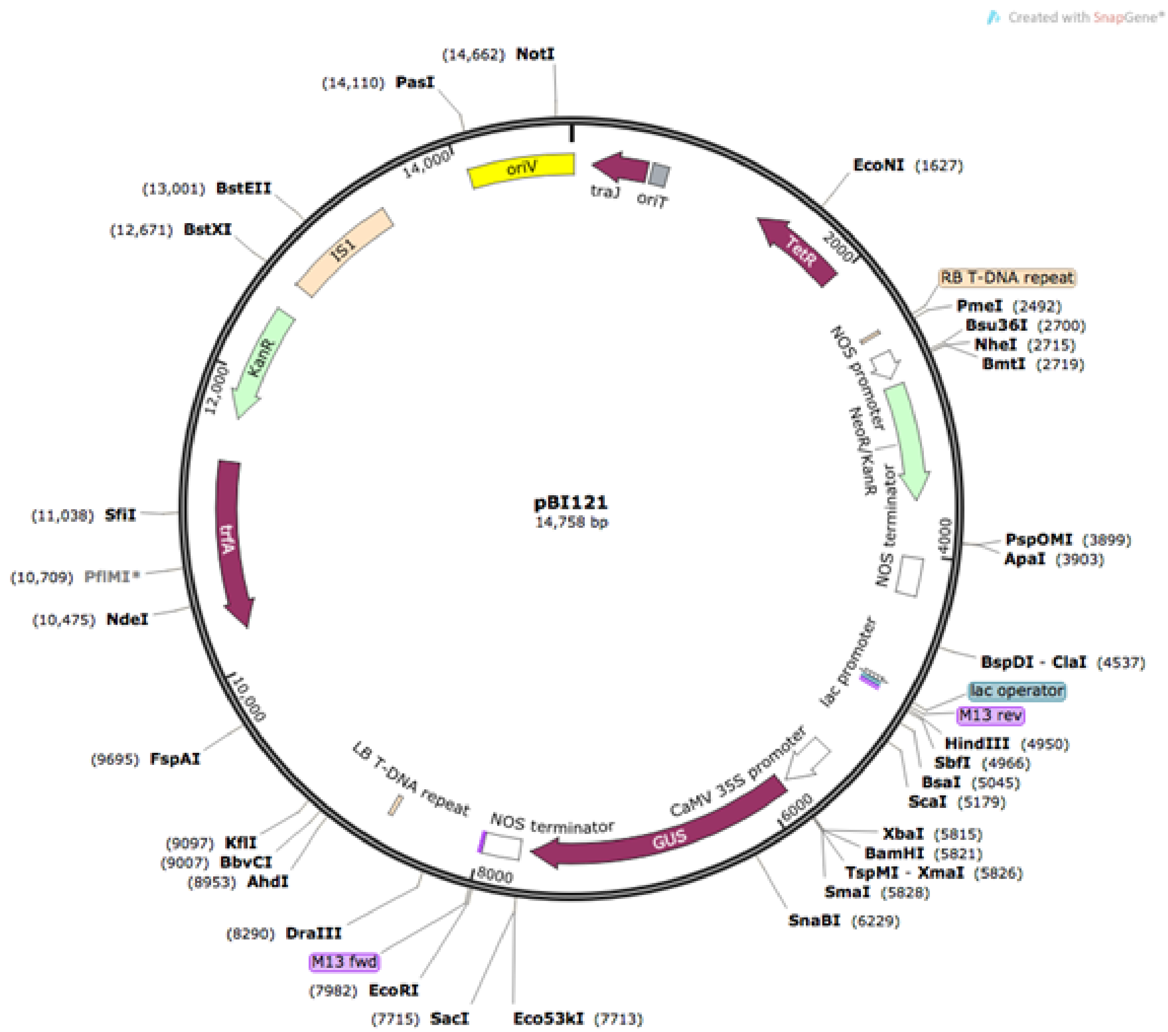
Vactor map: pBI121

(Source: http://www.snapgene.com/resources/plasmid_files/plant_vectors/pBI121/)



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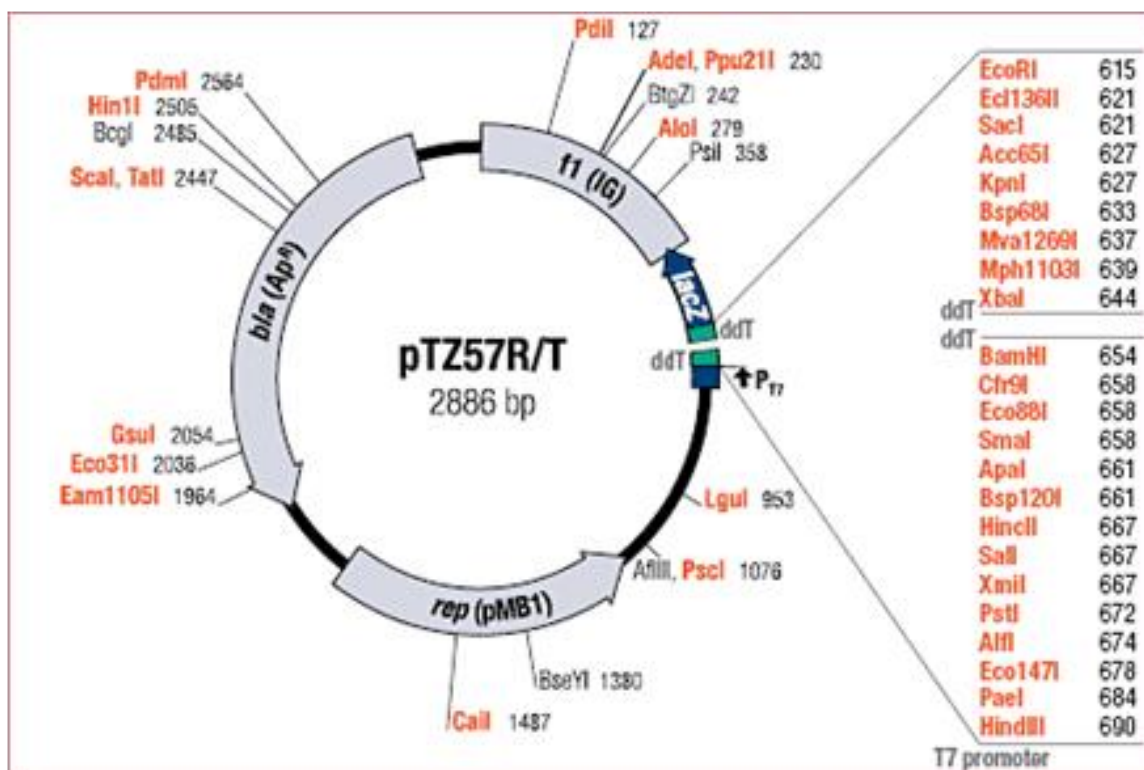
Vactor map: pTZ57R/T

(Source: https://www.thermofisher.com/order/catalog/product/K1213)



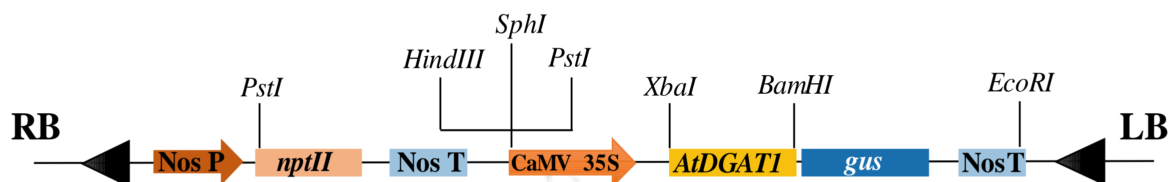


Figure 1 T-DNA region (6.2kb) of pBI121CaMV35S::*AtDGAT* plasmid. RB, right border; LB, left border; CaMV 35S promoter Nos P, Nopaline synthase promoter; Nos T, Nopaline synthase terminator; NptII, Neomycin phosphotransferase, ß-glucuronidase.

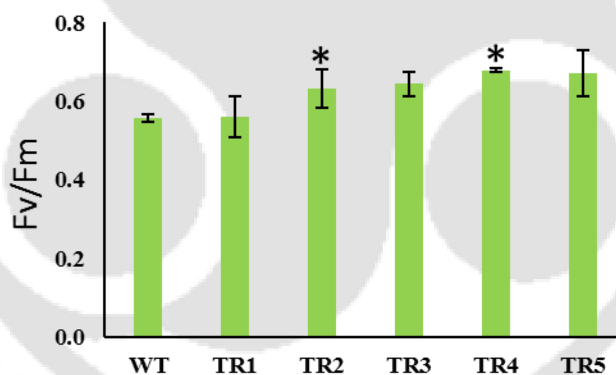


Figure 2 Photosynthetic efficiency of photosystem II (Fv/Fm) of *Jatropha curcas* overexpressing *AtDGAT1*.

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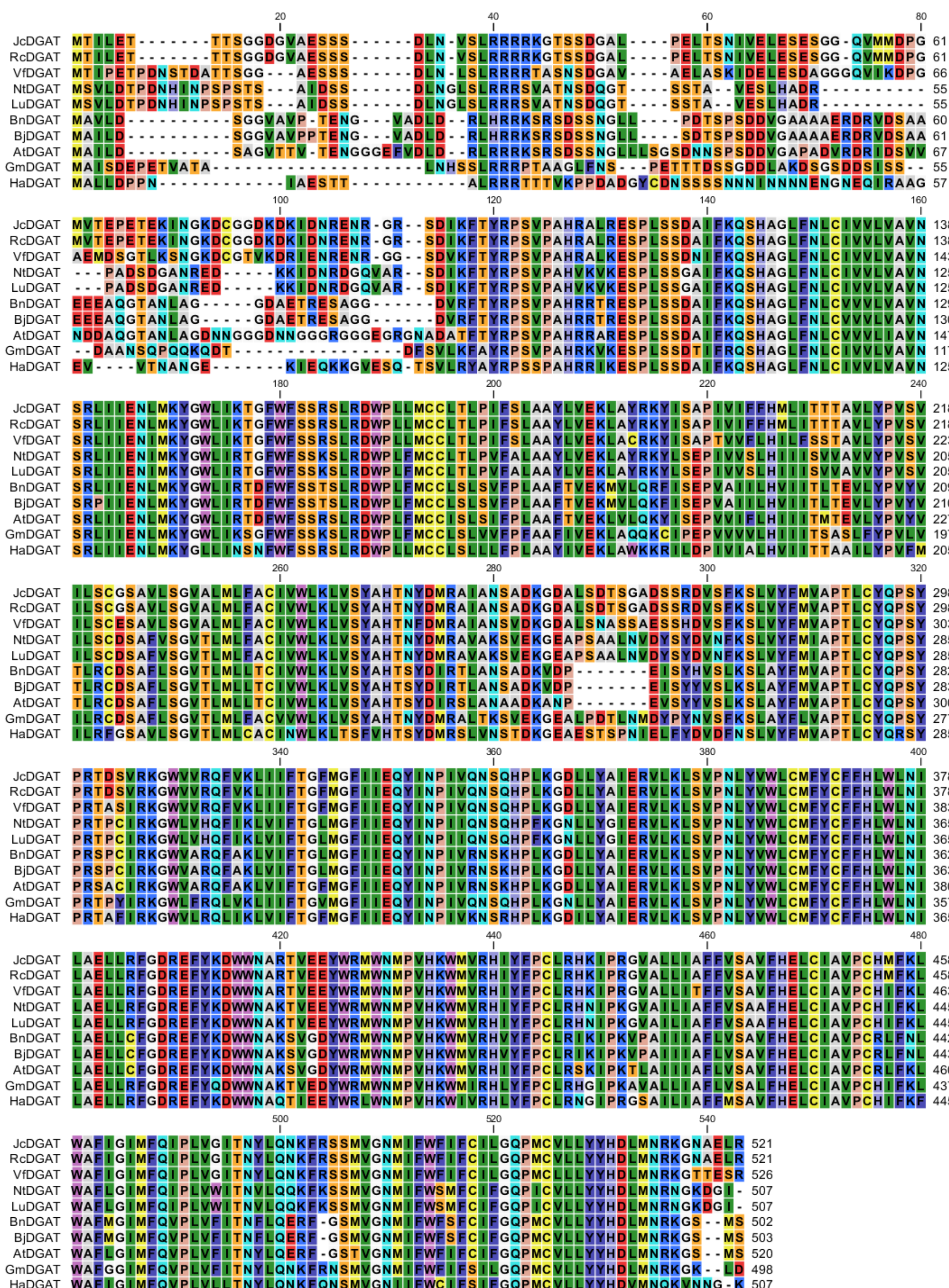

Figure 3 Multiple alignment of the amino acid sequences of *DGAT1* and homologous proteins from other plant species.

Jatropha curcas (ACA49853.1), *Ricinus communis* (ACB30543.1), *Vernicia fordii* (ABC94472.1), *Nicotiana tobacum* (AAF19345.1), *Linum usitatissimum* (AHA57450.1), *Brassica napus* (AIA67019.1), *Brasica juncea* (AAY40784.1), *Arabidopsis thaliana* (AAF19262.1), *Glycine max* (NP_001237289.1), *Halianthus annus* (ADT91687.1)

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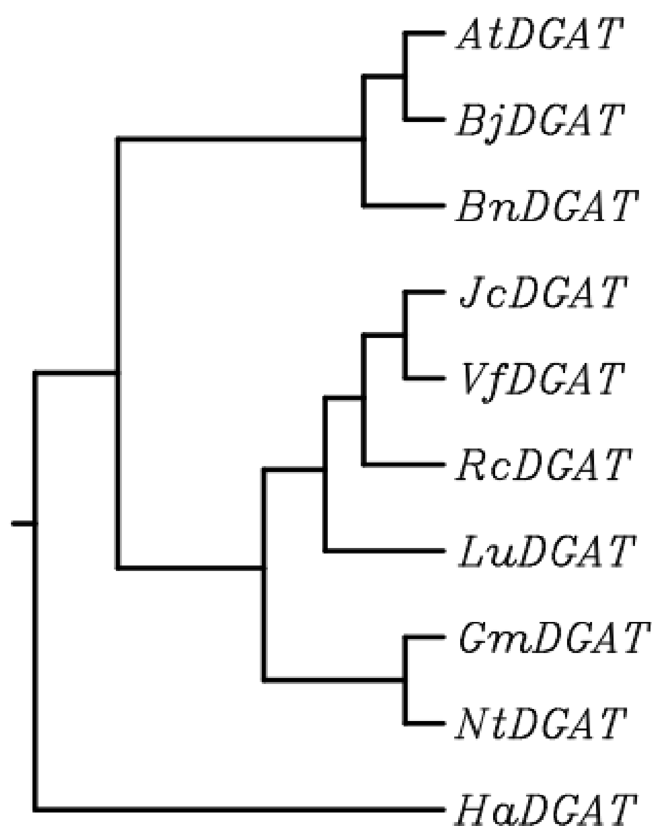

Figure 4 Phylogenetic tree of proteins of *DGAT1* in oil bearing higher plants.

The analysis was performed with CLC Sequence Viewer version 6.3 software, using the neighbour joining method. The tree was constructed from a sequence alignment of the following proteins: *Jatropha curcas* (ACA49853.1), *Ricinus communis* (ACB30543.1), *Vernicia fordii* (ABC94472.1), *Nicotiana tobacum* (AAF19345.1), *Linum usitatissimum* (AHA57450.1), *Brassica napus* (AIA67019.1), *Brasica juncea* (AAY40784.1), *Arabidopsis thaliana* (AAF19262.1), *Glycine max* (NP_001237289.1), *Halianthus annus* (ADT91687.1)

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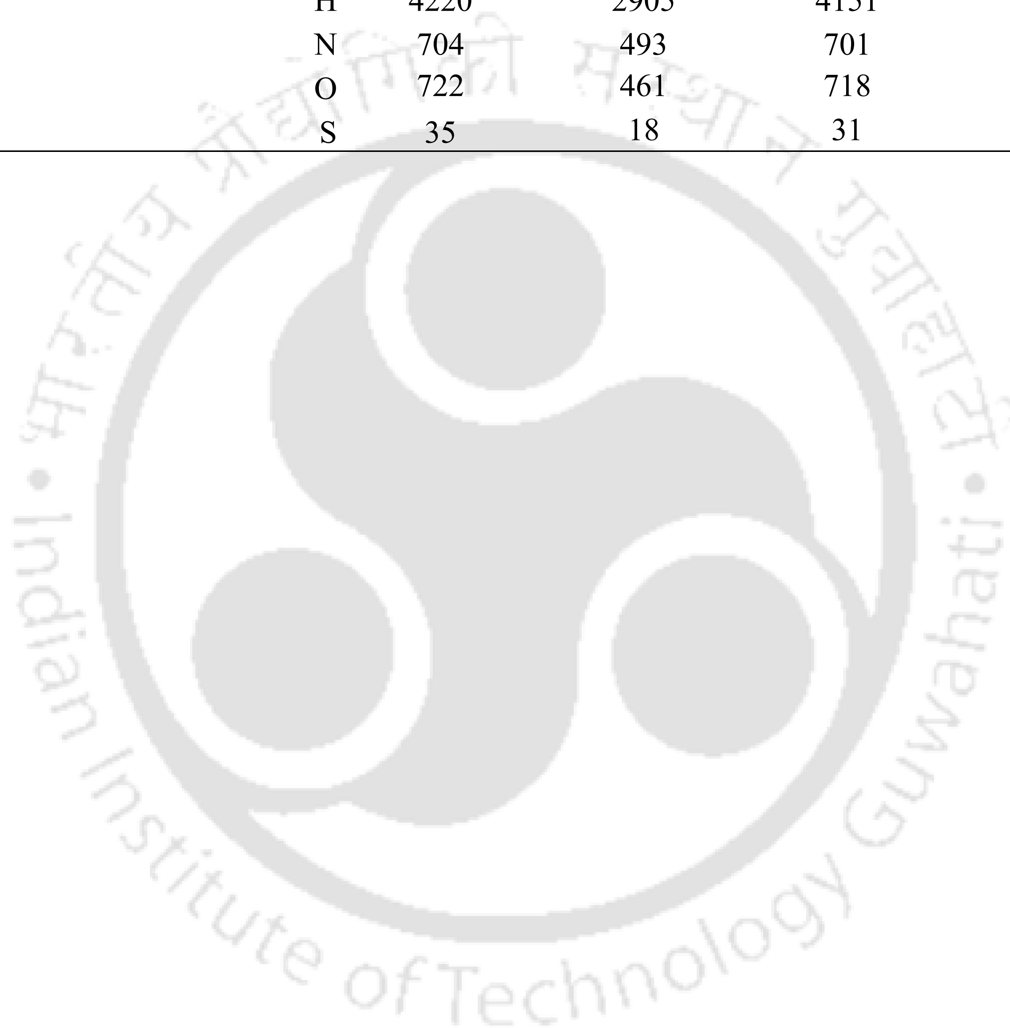
Table 5. Amino acid composition of the Jatropha DGATs and Arabidopsis DGATs.

		JcDGAT_1	JcDGAT_2	AtDGAT_1	AtDGAT_2
Amino acid residues		521	352	520	314
Molecular weight		59602	40283	58986	35854
Isoelectric point (PI)		8.93	9.84	8.85	9
Aliphatic index		99.33	95.23	99.35	97.77
Hydropathicity (GRAVY)		0.215	0.09	0.242	0.19
	C	2732	1873	2706	1663
	H	4220	2905	4151	2540
	N	704	493	701	430
	O	722	461	718	424
	S	35	18	31	16



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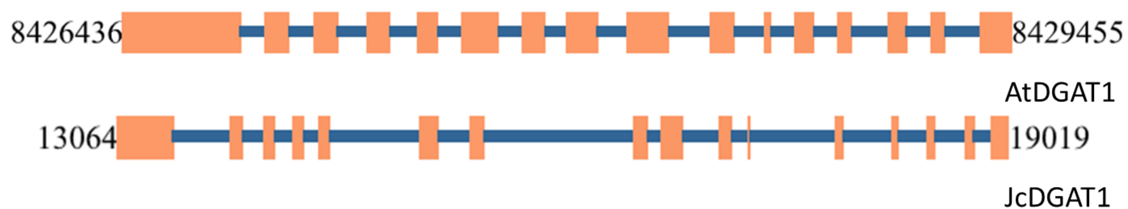

Figure 5 Exon and Intron structure of Jatropha *DGAT1* and Arabidopsis *DGAT1* comparison

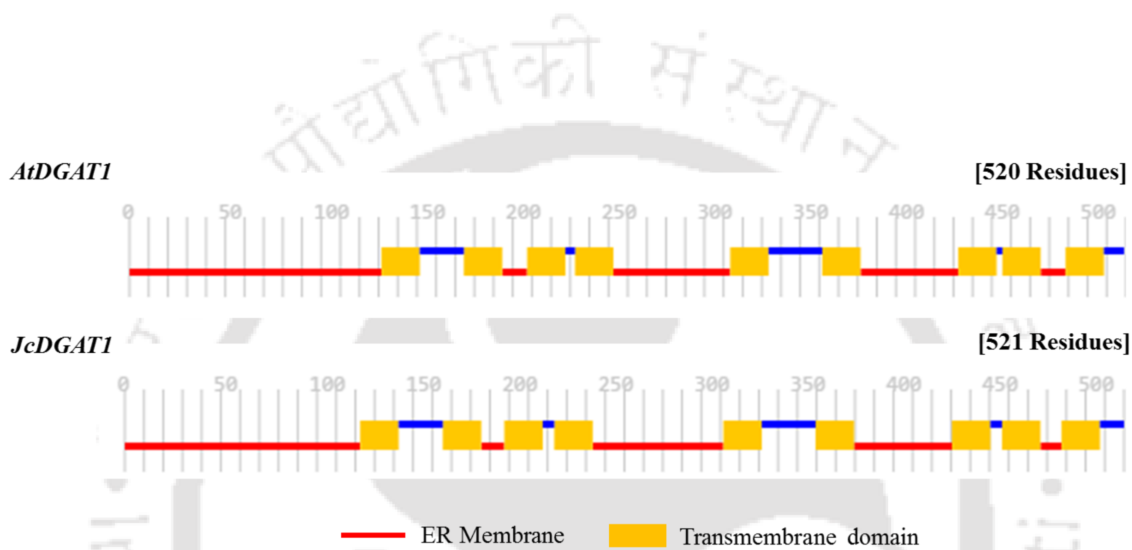

Figure 6. Putative transmenbrane domains in *AtDGAT1* and *JcDGAT1* proteins.

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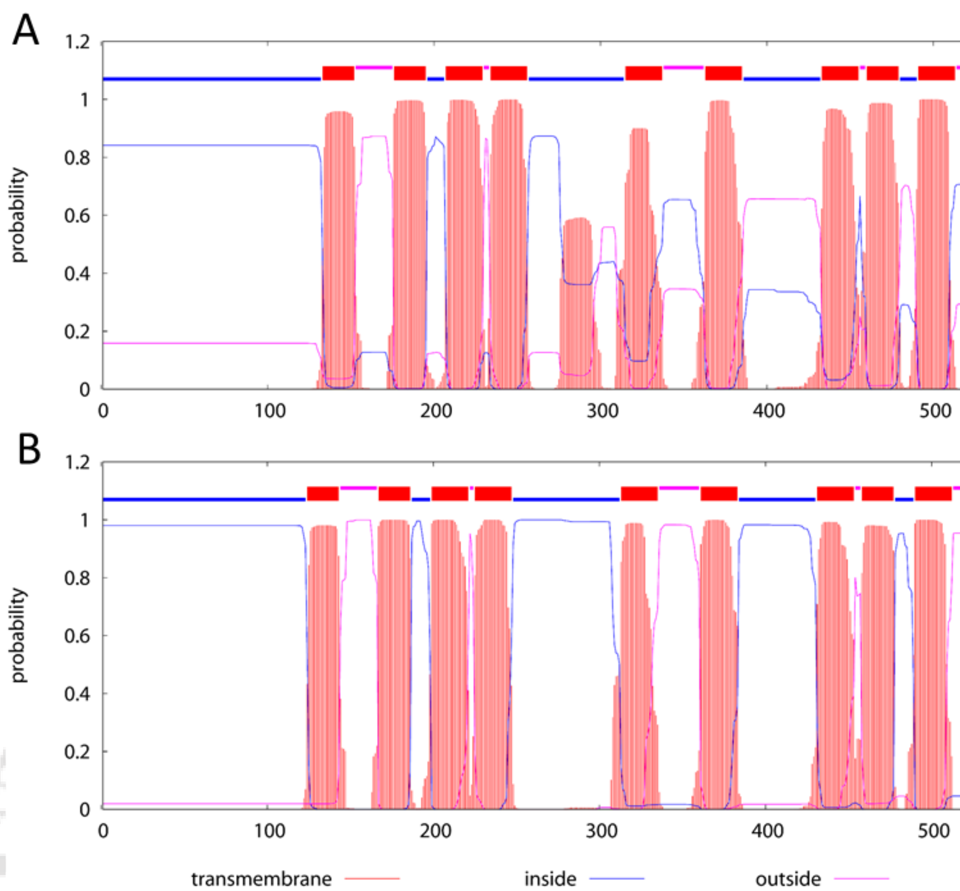

Figure 7. Transmembrane helix prediction of the *Arabidopsis thaliana DGAT1* (**A**) and Jatropha curcas *DGAT1* (**B**) using TMHMM2.0.

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Function of TAG biosynthesis genes

GPAT (Glycerol 3-phosphate acyltransferase)
Orthologue: 9

- Cutin biosynthesis
- Flower development
- Suberin biosynthesis
- Regulation of meristem growth
- TAG biosynthesis process
- Post replication repair
- Pollen sperm cell differentiation
- Response to karrikin
- Cutin development
- Cuticle development
- Regulation of cell size

LPAT (Lysophasphatidic acyltransferase)
Orthologue: 5

- Ultra-fatty acid biosynthesis process
- Iron sulfur cluster assembly
- Carotenoid biosynthesis
- Chlorophyll biosynthesis process
- Systemic acquired resistance, salicylic acid mediated signalling pathway

DGAT (TAG1)
Diacylglycerol acyl transferase

- Regulation of embryonic development
- Regulation of seed germination
- Positive regulation of seed germination

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Table 1. List of commercial kits

Name	Use	Supplier
NucleoSpin Plasmid	Plasmid Isolation	MN, Germany
NucleoSpin Gel and PCR clean up	Purification	MN, Germany
NucleoSpin RNA Plant	RNA isolation	MN, Germany
NucleoSpin Plant II Maxi	Genomic DNA isolation	MN, Germany
DIG Labelling and detection	Southern Hybridization	Roche Diagnostic, Mannheim, Germany

Table 2. List of plasmid vectors

Plasmid		Promoter	Selection gene
pTZ57R/T	T/A cloning		AmpR
pRT101	Plant expression	CaMV35S	AmpR
pBI121	Plnat expression	CaMV35S	KanR

Table 3. List of commercially available media

Media	Constituents	Concentration	pH
Luria Broth	Bactotryptone	1.0%	7.2
	Yeast extract	0.5	
	NaCl	1.0%	
Luria Agar	LB media		7.2
	Agar	1.5%	

Table. 4 Murashige and Skoog media composition

Salt and vitamins	mg/l medium	500 ml stock (20X)
Major salt		
NH_4NO_3	1650 mg	16.5 gm
KNO_3	1900 mg	19 gm
$CaCl_3.2H_2O$	440 mg	4.4 gm
$MgSO_4.7H_2O$	370 mg	3.7 gm
KH_2PO_4	170 mg	1.7 gm
Minor Salt	**mg/l**	**500 mlstock (200X)**
H_3BO_3	6.2 mg	620 mg
$MnSO_4.4H_2O$	22.3 mg	2230 mg
$ZnSO_4.4H_2O$	8.6 mg	860 mg
KI	0.83 mg	83 mg
$NaMoO_4.2H_2O$	0.25 mg	25 mg
$CoCl_2.6H_2O$	0.025 mg	2.5 mg
$CuSO_4.5H_2O$	0.025 mg	
Vitamins	**mg/l**	**500 ml stock (200X)**
Thiamine (HCl)	0.1 mg	10 mg
Niacine	0.5 mg	50 mg
Glycine	2 mg	20 mg
Pyrodoxine (HCl)	0.5 mg	50 mg

Iron, 500 ml stock (200X)
Dissolve 3.725 gm Na_2EDTA in 250 ml dH_2O. Dissolve 2.785 gm of $FeSO_4.7H_2O$ in 250 ml dH_2O, boil Na_2EDTA solution and add to it $FeSO_4$ solution gently by stirring.

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