Genetic Modification of Soybean [*Glycine max* (L.) Merrill] with Delta-6 Desaturase Gene for Gamma Linolenic Acid Production

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In fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY
in Biotechnology

by

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July 2009

Dedicated to,

My Dear Parents

&

Sisters

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DECLARATION

I hereby declare that this thesis entitled **"Genetic Modification of Soybean [*Glycine max* (L.) Merrill] with Delta-6 Desaturase Gene for Gamma Linolenic Acid Production"** submitted to the **UNIVERSITY OF MYSORE**, for the award of the degree of **DOCTOR OF PHILOSOPHY** in **BIOTECHNOLOGY**, is the result of research work carried out by me in the **Plant Cell Biotechnology Department**, **Central Food Technological Research Institute, Mysore** under the guidance of **Dr. G. A. Ravishankar** during the period May, 2004 to April, 2009. I further declare that the work embodied in this thesis is original and has not been submitted previously for the award of any degree, diploma or any other similar title.

Place: Mysore (**Sakthivelu, G.)**

Date:

Dr. G. A. RAVISHANKAR
Scientist 'G' & Head
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CERTIFICATE

This is to certify that the thesis entitled **"Genetic Modification of Soybean [*Glycine max* (L.) Merrill] with Delta-6 Desaturase Gene for Gamma Linolenic Acid Production"** submitted by **Mr. Sakthivelu, G.,** to the **University of Mysore** for the award of the degree of **Doctor of Philosophy** in **Biotechnology**, is the result of work carried out by him in **Plant Cell Biotechnology Department, Central Food Technological Research Institute, Mysore** under my guidance during the period May, 2004 to April, 2009.

Place: Mysore (**G. A. Ravishankar)**
Date: Research Supervisor

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(Sakthivelu, G.)

ABSTRACT

Engineering of fatty acid pathway in higher plants for enhancing their polyunsaturated fatty acid (PUFA) content is gaining importance in recent years. Gamma linolenic acid (GLA), a long chain omega-6 polyunsaturated fatty acid has been reported to be efficacious in treating nastalgia, atopic eczema, cancer and diabetic neuropathy and the sources are very meager. In the present investigation, studies were undertaken to establish a genetic transformation protocol for soybean and to express the delta-6 desaturase gene in soybean for the production of gamma linolenic acid. *In vitro* regeneration of soybean was best achieved with cotyledonary nodal explants which gave a maximum of 8 shoots/ explant, under the influence of TDZ. Also addition of triacontanol along with BAP during multiplication and rooting phase increased the number of shoots to 14 per explant, indicating the synergistic effect between them. Embryonic axis served as a better explant for *Agrobacterium* mediated genetic transformation of soybean resulting in a transformation efficiency of 7.4%, compared to cotyledonary nodes. The full length gene sequence of delta-6 desaturase was isolated from *Spirulina platensis* (strain Sp-6) and cloned into the binary vector pCAMBIA1305.2 to generate the recombinant plasmid pCAMBIAD6d, under the control of CaMV35S constitutive promoter and 35S poly A terminator. The isolated delta-6 desaturase gene sequence exhibited 98% similarity with the target protein and showed three histidine rich motifs, which may act as an active site for the enzyme activity. *Agrobacterium* mediated genetic transformation of soybean cv. JS 335 with the recombinant plasmid (pCAMBIAD6d) resulted in the formation of GLA (3.8%) and octadecatetraenoic acid (6.2%) in contrast to their absence in control plants. The results from the present study suggests that delta-6 desaturase gene from *Spirulina platensis* is quite potential for producing GLA in oilseed crops. Further selection and manipulation of these delta-6 desaturase expressing soybean lines for accumulation of GLA in seeds to a greater extent, may make them nutritionally a more potential oilseed crop.

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LIST OF ABBREVIATIONS

Symbol	Abbreviation
α	Alpha
β	Beta
γ	Gamma
µ	Micro
µg l^{-1}	Microgram per litre
mg l^{-1}	Milligram per litre
°C	Degree centigrade
mM	Millimolar
rpm	Revolutions per minute
SD	Standard deviation
OD	Optical density
CN	Cotyledonary node
MS	Murashige & Skoog
BAP	N^6-benzylaminopurine
IBA	Indole-3- butyric acid
GA_3	Gibberllic acid 3
TDZ	Thidiazuron
TRIA	Triacontanol
T-DNA	Transfer DNA
GUS	β-glucuronidase
PUFA	Polyunsaturated fatty acid
LCPUFA	Long chain polyunsaturated fatty acid
D6d	Delta-6 desaturase
LA	Linoleic acid
ALA	Alpha linolenic acid
GLA	Gamma linolenic acid
OTA	Octadecatetraenoic acid
DHGLA	Dihomo gamma linolenic acid
AA	Arachidonic acid
EPA	Eicosapentaenoic acid
DHA	Docosahexaenoic acid
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass spectrophotometry
HPLC	High Performance Liquid Chromatography

Introduction & Review of literature

1.1. Soybean

1.1.1. Origin and history of soybean

Soybean [*Glycine max (L.)* Merill] is an annual crop and belongs to the family Fabaceae. The origin and history of soybean plant is obscure and ancient Chinese literature points out that as early as in 2853 BC, the Emperor Sheng-Nung of China named soybean as one of the five sacred grains (Hymowitz, 1970). The crop was first domesticated during the Shang dynasty in the eastern half of north China, which is believed to be the center of origin for soybean. Thus, soybean has been cultivated in China for more than 4,000 years. It is believed that with the development of sea and land trades, soybean moved out of China between the first century AD and 1100 AD to nearby countries such as Burma, Japan, India, Indonesia, Malaysia, Nepal, Philippines, Thailand and Vietnam. However, it remained a minor crop everywhere except in China, until its introduction into USA in the 18th century (Singh and Hymowitz, 1999).

1.1.2. Area and cultivation of soybean

Systematic breeding of soybean in USA (in the 1940s and 1950s) transformed the crop from an inefficient fodder type to a highly productive erect plant type and virtually USA became the largest producer of soybean in the world (Hymowitz and Harlan, 1983). Soybean has now become the largest source of vegetable oil and protein in the world and its large scale cultivation is concentrated in a few countries such as USA, Brazil, Argentina, China, India, Paraguay and Canada which together produce about 96% of the world's total production (Figure 1). The world's average soybean yield has also increased from less than 1 tonne/ ha to 2.3 tonnes/ ha.

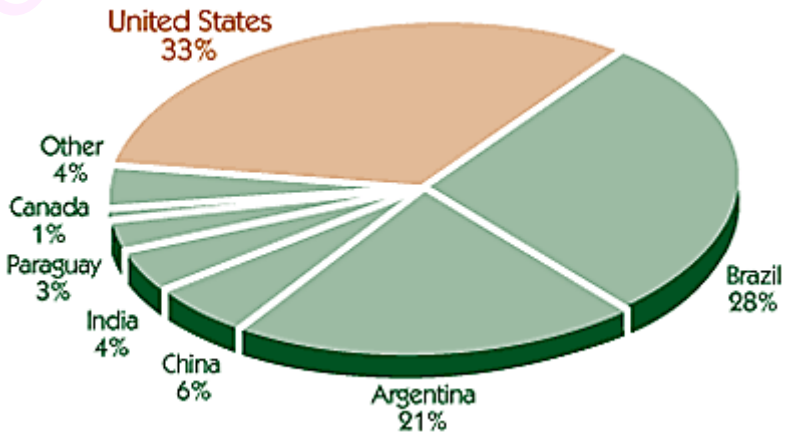

Figure 1. World Soybean production – 2008 (source: www.soystats.com)

In India, soybean cultivation was negligible until 1970; thereafter it has increased manifold as compared to any other oilseed crop and stands next only to groundnut. At present soybean stands as one of the most important agricultural commodity with a steady increase in annual production (Table 1). This has made India the 5th largest producer of soybean in the world (Figure 1) and sixth in terms of leading soybean consuming countries. In India, Soybean production is mainly confined to Madhya Pradesh (also known as soybean bowl of India), Maharashtra, Rajasthan, Andhra Pradesh, Karnataka, Uttar Pradesh and Chhattisgarh (Figure 2).

Table 1. Soybean production from India and world (1981-2008)

Year	World total production (Million Metric Tonnes)	Total production in India (Million Metric Tonnes)
1981-1982	86.67	0.466
1986-1987	98.01	0.858
1991-1992	107.38	2.549
1996-1997	132.22	4.028
2001-2002	185.09	5.400
2006-2007	235.77	7.150
2007-2008	220.81	9.473

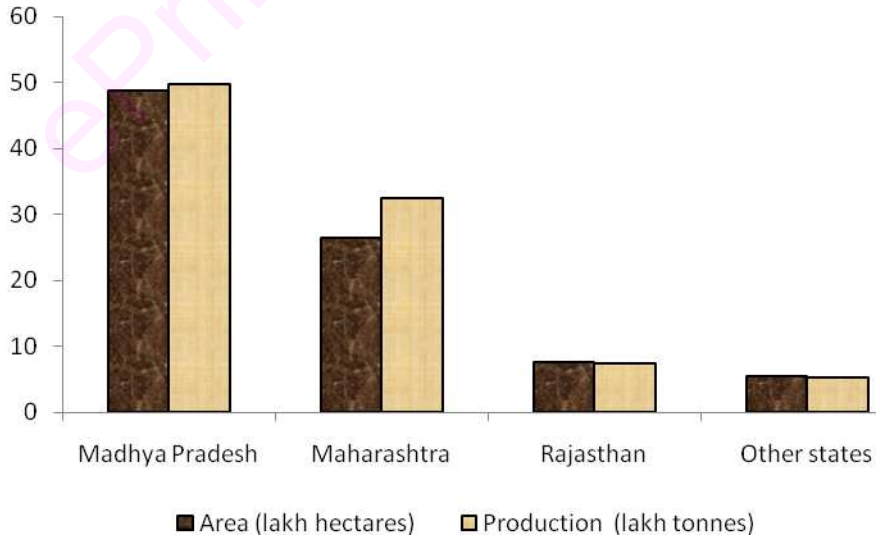

Figure 2. Statewise area and production of soybean in India (2007-08)

Source: The Soybean Processors Association of India (SOPA), 2008. [www.sopa.org]

India produces 5-7 million tonnes of beans, 1 million tonne of oil and 3-5 million tonnes of soy meal in a normal year (Source: The Soybean Processors Association of India). Out of the total production, 10 to 12% is directly consumed and the rest is used for processing. India is one of the major exporters of soy meal to the Asian countries *viz.*, South Korea, Thailand, Philippines and Japan. India typically exports around 65% of the country's soy meal production currently, which accounts for 84% of the total edible oilseed meal exports from the country.

1.1.3. Soybean classification

The genus *Glycine* is divided into two subgenera: *Glycine* and *Soja*. The former consists of perennial wild species (including *Glycine canescens* and *G. tomentella* Hayata) primarily from Australia and Europe, whereas the latter consists of three annual species from Asia *viz*., *Glycine max* Merrill, *Glycine soja* Sieb. & Zucc., and *Glycine gracilis* Skvortz (Hymowitz and Newell, 1980). *Glycine max* is the cultivated form of soybean grown worldwide, whereas *Glycine soja* is the wild ancestor of the cultivated soybean grown widely in China, Japan, Korea, Taiwan and Russia and *Glycine gracilis* is the weedy form of cultivated soybean, found only in China (Hymowitz and Singh, 1987; Lackey, 1981).

Scientific classification:

Kingdom	:	Plantae
Phylum	:	Magnoliophyta
Class	:	Magnoliopsida
Order	:	Fabales
Family	:	Fabaceae
Subfamily	:	Faboideae
Genus	:	*Glycine*
Species	:	*max*
Binomial name	:	*Glycine max* (L.) Merrill

1.1.4. Morphology and physical characteristics

The cultivated form [*Glycine max* (L.) Merrill] grows annually, with variation in growth, habit and height. It grows prostrate with pods, stems and leaves covered by fine brown or grey hairs. Leaves are trifoliate, having 3 to 4 leaflets per leaf and fall before the seeds mature (Lersten and Carlson, 1987). Flowers are standard papilionaceous type, borne on the axil of the leaf and are white, pink to purple in color. Anthers mature in the bud and shed their pollen directly onto the stigma of the same flower, thus ensuring a

high degree of self-pollination (Carlson and Lersten, 1987). The fruit is a hairy pod that grows in clusters of 3–5 and usually contains 2–4 seeds. Seeds occur in various sizes and in many seed coat colors, including black, brown, blue, yellow and green.

1.1.5. Uses of soybean

Soybean is known worldwide for its high quality protein and oil content. The crop has several favorable features which include its adaptability to a wide range of soils and climates, ability to fix nitrogen, capacity to produce more edible protein per acre of land than any other known crop and its versatile end uses as human food, animal feed and industrial material. Currently, the majority of annual soybean production is crushed into oil, for use in foods and food processing and defatted meal, for use as animal feed. Only a small fraction is processed into whole bean foods for direct human consumption (Soyatech, 2004).

Soy protein contains all the essential amino acids, required by humans. Soy oil contains a high proportion of unsaturated fatty acids and essential fatty acids including oleic, linoleic and linolenic acids (Messina et al., 1994). Extensive clinical research has shown the role of soybean in preventing and treating chronic diseases such as cancer, heart and bone diseases. This has led to a worldwide interest in using soybean for food and nutraceutical products. A number of studies both *in vivo* and *in vitro*, with animals and human subjects have shown that soybeans and soy components have many health benefits, including hypocholesterolemic, anticancer and antioxidant effects (Carroll and Kurowska, 1995; Anthony, 2000). Regular consumption of soybean and soy products are reported to reduce heart disease, prevent breast and prostate cancers, improve bone health and memory and alleviate menopausal symptoms in women (Wang and Wixon, 1999). Many types of biologically active components such as isoflavones, lecithin, saponins, lectins, oligosaccharides and trypsin inhibitors have been shown to be responsible for these effects. Among them, isoflavones have been recognized as the key components responsible for the above mentioned health promoting effects though other bioactive components are also of interest. These components are present in minor quantities as compared with protein and oil and still they can exert some unique health benefits for humans. The general nutrient contents as well as some phytochemicals present in soybean are listed in Table 2 (Liu, 2004).

Table 2. General concentrations of nutrients and phytochemicals in soybean (Dry weight basis)

Components	Range
Protein (%)	30-50
Amino acid composition (g/100 g seed)	
Non-essential	
Alanine	1.49–1.87
Arginine	2.45–3.49
Aspartic acid	3.87–4.98
Glutamic acid	6.10–8.72
Glycine	1.88–2.02
Cysteine	0.56–0.66
Proline	1.88–2.61
Serine	1.81–2.32
Essential	
Histidine	0.89–1.08
Isoleucine	1.46–2.12
Leucine	2.71–3.20
Lysine	2.35–2.86
Methionine	0.49–0.66
Phenyalanine	1.70–2.08
Threonine	1.33–1.79
Tryptophan	0.47–0.54
Tyrosine	1.12–1.62
Valine	1.52–2.24
Oil (%)	12–30
Fatty acid composition (% of total oil content)	
Palmitic acid	4–23
Stearic acid	3–30
Oleic acid	25–86
Linoleic acid	25–60
Linolenic acid	1–15
Carbohydrates (%)	26–38
Sucrose	2.5–8.2
Raffinose	0.1–0.9
Stachyose	1.4–4.1
Ash %	4.6–5.9
Vitamins (µg/g)	
Thiamine	6.26–6.85
Riboflavin	0.92–1.19
Vitamin E	
α-tocopherol	10.9–28.4
τ-tocopherol	150–190
δ-tocopherol	24.6–72.5

Components	Range
Isoflavones (%)	0.1–0.4
Saponins (%)	0.1–0.3
Phytate (%)	1.0–1.5
Phytosterols (mg/g)	0.3–0.6
Trypsin inhibitors (mg/g)	16.7–27.2
Lectin HU*/mg protein	1.2–6.0
Lunasin (% defatted flour)	0.33–0.95

*HU = Hemagglutinin unit.

Source: USDA nutrient database, 2008.

1.2. Genetic modification of soybean

Soybean is grown in more areas worldwide than any other dicotyledonous crop and is recognized as the most economical source of food protein. Hence improvement and optimization of soybean characteristics such as pest resistance, stress tolerance, lowering allergenic protein levels, improving the nutraceutical components and altering the fatty acid composition of seeds for human nutrition are therefore desirable (Kaneda et al., 1997). In attaining this goal, we owe much to the breeding techniques. Soybean is a self-pollinated legume with natural outcrossing of < 0.5 to approximately 1% (Carlson and Lersten, 1987). As a result of its self-pollinating reproductive behaviour, plant breeding procedures such as backcrossing, single pod descent, pedigree breeding and bulk population breeding are some of the more common procedures used to develop improved varieties of soybean (Poehlman and Sleper, 1995). All of these procedures involve making crosses or hybrids by hand pollination followed by selection, testing and ultimately release of a superior soybean variety, which is laborious and time consuming. In such situations biotechnological techniques such as tissue culture and genetic engineering can be a handy tool to enhance and improve the efficiency of plant breeding.

The progress of plant genomic research has reached a stage where transformation has become a basic technology for both analysis and verification of the function of isolated genes in crops as well as for the production of varieties engineered with desirable traits. The utilization of genetic transformation techniques to introduce useful or novel gene(s) into soybean requires an efficient method of transgene integration and regeneration of transformed plants. While transgenic soybeans have been already

produced using several transformation techniques that are either *Agrobacterium*-mediated or involve particle bombardment, the transformation technique itself is yet to become a practical breeding method.

1.2.1. *In vitro* regeneration of soybean

A number of successful regeneration protocols have been developed for soybean especially with a view to facilitate genetic transformation. However, the frequency of plant regeneration reported thus far does not seem high enough to be applied easily to soybean transformation. An improvement in the regeneration frequency would greatly contribute to an increase in the production of transgenic soybeans. Many reports have covered somatic embryogenesis and shoot organogenesis in soybean, which will be briefed in the following sections.

1.2.1.1. Somatic embryogenesis

Somatic embryogenesis in soybean was first achieved by Christianson et al., (1983). The process involves the induction of proliferating embryogenic tissues from immature cotyledons cultured on medium containing moderately high concentrations of auxin, either 2, 4-dichlorophenoxy acetic acid or α-naphthaleneacetic acid (Ranch et al., 1985; Lazzeri et al., 1985; Finer and Nagasawa, 1988; Parrott et al., 1988). Adventitious somatic embryos were allowed to develop from the embryogenic cultures, which germinated into a whole plant after maturation in a hormone free medium. The formation of proliferative embryonic tissue is dependent on genotype, although the effects of genotype have been partially overcome by modification of tissue culture protocols (Bailey et al., 1993). These successes have been limited to those genotypes that are amenable to embryogenic culture induction in tissue culture. Tomlin et al., (2002) have reported that somatic embryogenesis is positively associated with lower maturity groups of soybean. Various studies have been reported on the influence of factors such as auxin (Lazzeri et al., 1987; Komatsuda and Ohyama, 1988), sucrose (Lazzeri et al., 1987; Komatsuda et al., 1992), pH (Lazzeri et al., 1987; Santarem and Finer, 1999) and genotype (Komatsuda and Ohyama, 1988; Parrott et al., 1989; Tomlin et al., 2002) on somatic embryogenesis in soybean. Soybean somatic embryos were first used as a target for *Agrobacterium* mediated transformation (Parrott et al., 1989) and somatic embryogenic tissue was subsequently found to be amenable to transformation by particle bombardment (Finer and McMullen, 1991). Though somatic embryogenesis is an efficient system to produce genetically modified plants, its genotype specificity and high level of somaclonal variation in the regenerated plants needs to be addressed before it can

be used to regenerate transformed plants (Finer and Nagasawa, 1988; Parrott et al., 1989; Finer and McMullen, 1991).

1.2.1.2. Shoot organogenesis

Earlier reports on soybean organogenesis focused on callus regeneration. It was considered as advantageous over direct regeneration for transformation because of its efficiency in selection of transgenic cells. Christou et al., (1988) could effectively select transgenic calli after bombarding protoplasts, but they failed to produce transgenic plants. According to Hu and Wang (1999) the efforts made to regenerate plants from callus have yielded poor results since plants could not be regenerated from any type of soybean callus. An adventitious organogenic regeneration procedure from seedling hypocotyl sections was reported by Dan and Reichert, (1998). Later Reichert et al., (2003) improved the hypocotyl based organogenic regeneration system of soybean that was truly adventitious in nature and showed that it is independent of genotype.

Direct shoot organogenesis is less genotype dependent and has become a routine *in vitro* technique for regeneration of transformed plants. Recent reports on soybean shoot organogenesis focused on the utilization of cotyledonary node (Hu and Wang, 1999), which is based on the proliferation of meristems in the cotyledonary node. Shan et al., (2005) suggested that the efficiency of *in vitro* organogenesis for genetic modification can be improved if the number of shoots per explant is increased or by increasing the number of meristematic cells with the ability to regenerate. The same authors reported the formation of multiple shoot buds and their proliferation by sub-culturing cotyledonary nodes on thidiazuron (0.1 mg l^{-1}) supplemented medium. Similarly Franklin et al., (2004) reported the formation of green organogenic nodules induced at the proximal end of mature and immature cotyledons by combination of TDZ and BAP which gave 19 shoots per explants. The efficiency of TDZ in inducing multiple shoot buds was further demonstrated by Kaneda et al., (1997) using cotyledonary nodes and hypocotyl explants. They also suggested that by reducing the concentration of salts in the media, frequency of adventitious shoot buds can be increased. Later Sairam et al., (2003) used the proximal end of cotyledons with 2, 4-D and BAP to regenerate *in vitro* shoots. They also observed that among the carbon sources sorbitol is best for callus induction and maltose for plant regeneration. Even though plants have been successfully transformed by using cotyledonary nodal explants, the recovery of transgenic plants capable of transmitting the target gene(s) is very low (Christou et al., 1990). This is primarily due to pre-existing

meristematic shoot buds, which continue to grow effectively on a medium containing a selectable agent like antibiotic or herbicide (Meurer et al., 1998).

1.2.2. Genetic transformation studies on soybean

Early attempts in genetic engineering of soybean focused on regeneration of protoplast and embryonic suspension cultures. However despite some initial successes, progress was very slow and the prospects for recovering transgenic soybean remained a distant and elusive objective. Soybean genetic engineering became a reality following the invention and optimization of the technique of particle bombardment (Klein et al., 1987; Christou et al., 1990). In fact soybean was used as a model system to develop the technology for a large number of crop species that were shown to be extremely recalcitrant to genetic manipulation (Christou et al., 1988; McCabe et al., 1988). There are two modes of DNA delivery that are currently utilized by most researchers to transform soybean. One method utilizes particle bombardment of shoot meristems and embryogenic tissue with DNA coated carrier particles of inert materials (Hadi et al., 1996; Santarem and Finer, 1999; Droste et al., 2002). This technique often requires a prolonged tissue culture period to prepare target tissue. The other method involves *Agrobacterium*-mediated transformation of plant tissue such as embryonic axis, immature cotyledons or cotyledonary tissue from germinated seedlings (Hinchee et al., 1988; Parrott et al., 1989; Somers et al., 2003; Paz et al., 2004).

1.2.2.1. *Agrobacterium* mediated gene transfer

Agrobacterium tumefaciens provides a reliable and well documented means for introducing foreign DNA into plant cells. *A. tumefaciens* mediated delivery of foreign genes was first demonstrated by Horsch et al., (1985). Since then its utility has been extensively studied in numerous plant species with subsequent documentation by other workers (Wordragen and Dons, 1992; Fisk and Dandekar, 1993; Hiei et al., 1994; Ishida et al., 1996). Initial studies on *Agrobacterium*-mediated transformation of soybean revealed a highly variable response of the crop to *Agrobacterium spp.* Owens and Cress, (1985), using only a single strain of *A. tumefaciens*, demonstrated both significant genotypic and seedling age effects on the tumorigenic response of cut stems. Byrne et al., (1987) in their evaluation of multiple *Glycine* and *Agrobacterium* species identified significant genotype x strain interaction. They next evaluated eleven *A. tumefaciens* strains on the most susceptible soybean genotype, 'Peking' and found significant differences in tumor formation induced by different *A. tumefaciens* strains. Delzer et al., (1990) found that a significant strain x genotype interaction existed when they evaluated

10 early-maturity genotypes with 3 strains of *A. tumefaciens* for tumorigenicity of wounded cotyledons, a non-organogenic tissue. Similarly Mauro et al., (1995) and Bailey et al., (1994) identified genotypes divergent for *Agrobacterium*-induced tumor formation and have made crosses between these genotypes in order to study the heritability of this trait. Both groups determined this trait to be quantitative in nature with sufficient genetic variance to permit improvement by selection. The above studies suggest that tumor formation is a complex process that could be controlled by factors such as tissue growth stage, ability of transformed tissue to proliferate under *in vitro* conditions and also in the presence of bacteria and other factors.

The first successful recovery of transformed soybean plants using *Agrobacterium* was reported by Hinchee et al., (1988). The procedure relied on shoot organogenesis from cotyledons of soybean genotype (cultivar Peking) selected for its susceptibility to *Agrobacterium* infection. Later Parrott et al., (1989) used immature seeds to obtain cotyledonary tissues that were macerated on nylon or steel mesh, infected with *Agrobacterium* and placed on culture medium to generate somatic embryos. Later researchers concentrated on cotyledonary node (CN) method for soybean transformation, which offers one of the reliable methods for regeneration of fertile soybean plants due to a short seed-to-seed generation time and non requirement of the maintenance of parental donor plants or long term cultures (Paz et al., 2006). The efficiency of this transformation system remains low because of infrequent T-DNA delivery to cells (axillary meristems) in the cotyledonary node, ineffective wounding of explants prior to infection, inefficient selection of transgenic cells that give rise to shoot meristems and low rates of transgenic shoot regeneration and plant establishment (Olhoft and Somers, 2001). Improvements to this CN protocol have been actively pursued in the past one decade to increase its efficiency, which is summarized in Table 3.

The CN system involves wounding of explants derived from 5–7 day old seedlings by making accurate incisions on the adaxial side using a surgical blade. This wounding procedure requires precise cutting of the explant prior to infection. Discrepancies in transformation efficiency *via* the *Agrobacterium* method have been partially attributed to non-reproducibility of CN wounding procedures. Other soybean transformation approaches involving some forms of deliberate wounding on the explant have also been explored resulting in different transformation efficiencies. For example, sonication was used to assist *Agrobacterium*-mediated transformation (SAAT) of both CN explants (Meurer et al., 1998) and immature cotyledons (Santarem et al., 1998; Finer

and Finer, 2000). Biolistic treatment was used to wound embryogenic tissue derived from cotyledonary explants followed by *Agrobacterium* inoculation of the tissue (Droste et al., 2000).

Table 3. Improvements of the cotyledonary nodal transformation system in soybean

Improvements	Reference
Improved selection and regeneration of transformed plants	Zhang et al., 1999; Olhoft et al., 2003.
Increased *Agrobacterium* virulence by improving *vir* gene constructs and using chemical inducers of *vir* genes	Bolton et al., 1986; Dye et al., 1997; Hansen et al., 1994; Palanichelvam et al., 2000.
Identification and selection of susceptible soybean genotypes	Byrne et al., 1987; Delzer et al., 1990; Meurer et al., 1998; Cho et al., 2000.
Facilitating the gene delivery through wounding of explants/ microprojectile bombardment/ sonication.	Santarem et al., 1998; Meurer et al., 1998; Finer and Finer, 2000; Droste et al., 2000; Ko et al., 2003; Yan et al., 2000.

Explant wounding of excised embryonic axes from immature seeds using a multi-needle wounding prong (Ko et al., 2003) and cotyledons using forceps (Yan et al., 2000) have also been reported. Although some of these wounding treatments resulted in enhanced transient expression of marker genes, they did not imply the improvement of stable transgenic plant recovery, which has been corroborated using other plant systems (Wroblewski et al., 2005). While these studies represent substantial progress towards improving the cotyledonary node transformation system, the production of transgenic soybean plants remains inefficient.

Olhoft and Somers (2001) reported that application of thiol compounds (cysteine, dithiothreitol and sodium thiosulfate) during co-cultivation results in a significantly increased transformation rate using the cotyledonary node tissues. Based on this protocol coupled with hygromycin B as a selective agent, efficient stable transformation (16%) has been achieved (Olhoft et al., 2003). However, it was suggested that efficacy of transformant selection with hygromycin B might be genotype dependent (Zeng et al., 2004). Recently Liu et al., (2008) reported increased transformation efficiencies of soybean cotyledonary node by using 0.02% (v/v) surfactant (Silwet L-77, Arabidopsis Inc., USA) along with the *Agrobacterium* inoculums. The application of Silwet L-77 (0.02%) during infection and L-cysteine (600 mg l^{-1}) during co-cultivation resulted in more significantly improved transformation efficiency than each of the two factors alone.

They also optimized the selection of transgenic shoots by gradually increasing the concentration of hygromycin (from 0- 8 mg l^{-1} in 27 days) to regenerate more number of transgenic plants. Alternative to this CN system, Liu et al., (2004) reported the use of embryonic tip as explants for *Agrobacterium* mediated transformation of soybean. They pointed out the use of fewer and readily available explants, simple protocol and shorter time period to generate transgenic soybeans as the advantages of this system. The above literatures point to the extensive studies on *Agrobacterium* mediated transformation of soybean, which is well established now with only a few bottlenecks (like genotype specificity and transformation efficiency) to be overcome.

1.3. Genetic engineering of oilseed plants

1.3.1. Polyunsaturated fatty acids

Polyunsaturated fatty acids (PUFAs) are of 18 carbons or more in length with two or more methylene interrupted double bonds in the *cis* position. PUFAs can be grouped into two main families ω-6 (or n-6) and ω-3 (or n-3), depending on the position of the first double bond proximate to the methyl end of fatty acids. For example: linoleic acid, in the ω-6 family, is designated as C18:2 ω-6 to indicate that it has 18 carbons and 2 double bonds, with the first double bond at the sixth carbon atom starting from the methyl end. Very long chain polyunsaturated fatty acids (LCPUFA) have 20 or 22 carbon atoms and 4, 5 or 6 methylene interrupted *cis* double bonds in ω-3 or ω-6 arrangements (Huang and Ziboh, 2000). The nutritionally most important PUFAs include linoleic acid (LA), γ-linolenic acid (GLA) and α-linolenic acid (ALA), whereas the LCPUFA includes arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Alonso and Maroto, 2000).

1.3.2. Uses of PUFAs

Dietary polyunsaturated fatty acids (PUFA) have effects on diverse physiological processes impacting normal health and chronic disease, such as the regulation of plasma lipid levels, cardiovascular and immune function, insulin action, neuronal development and visual function (Table 4). Ingestion of PUFA will lead to their distribution to virtually every cell in the body with effects on membrane composition and function, eicosanoid synthesis and signaling as well as the regulation of gene expression. Cell specific lipid metabolism, as well as the expression of fatty acid regulated transcription factors is likely

to play an important role in determining cellular response to changes in PUFA composition (Alonso and Maroto, 2000).

Table 4. Polyunsaturated fatty acids and their putative role

PUFA	Formula	Metabolic role	Reference
Linoleic acid (LA)	18:2n-6	Essential fatty acid	WHO/FAO, 1977.
γ-linolenic acid (GLA)	18:3n-6	Essential for diet, Skin diseases and others	WHO/FAO, 1977; Horrobin, 1992.
α-linolenic acid (ALA)	18:3n-3	Essential fatty acid	WHO/FAO, 1977.
Octadecatetraenoic acid (OTA)	18:4n-3	Anti-inflammatory	Coupland et al., 1996.
Dihomo γ-linolenic acid (DHGLA)	20:3n-6	Virus infections, cancer, inflammatory diseases, and atopy of the skin and mucosa.	Jareonkitmongkol et al., 1993.
Arachidonic acid (AA)	20:4n-6	Essential for diet, Eicosanoid precursor, Development of the nervous system	WHO/FAO, 1977; Singh and Chandra, 1988; Innis, 1991.
Eicosapentaenoic acid (EPA)	20:5n-3	Eicosanoid precursor, Circulatory system, Anti-inflammatory, Anti-tumoral	Dyerberg, 1986; Singh and Chandra, 1988; Innis, 1991; Wingmore et al., 1996; Simonopoulos, 1989.
Docosahexaenoic acid (DHA)	22:6n-3	Development of the nervous system and retina	Singh and Chandra, 1988; Innis, 1991.

GLA plays a significant role in the prevention of skin diseases, diabetes and reproductive disorders (Gunstone, 1992, 1998; Horrobin, 1992). AA and DHA are found in high proportions in neuronal tissues such as brain, retina and testis (Singh and Chandra, 1988; Innis, 1991). DHA acts as a ligand for the retinoid receptor and is involved in the development of retina (Brown, 1994). Inclusion of AA and DHA in infant formula has also been shown to be beneficial to the growth and visual development in pre-term infants (Gill and Valivety, 1997). The role of EPA in the proper functioning of the circulatory system and cardiovascular diseases is now well supported (Dyerberg, 1986; Iacono and Dougherty, 1993; Simonopoulos, 1991; Wingmore et al., 1996). PUFAs also serve as a precursor for a number of biologically active molecules such as eicosanoids, growth

regulators and hormones. Eicosanoids include tromboxanes, leukotrienes and prostaglandins which are physiologically active compounds and play diverse roles in human metabolism (Gill and Valivety, 1997; Innis, 1991; Parent et al., 1992; Peck, 1994).

Because of their profound effects on human health, many PUFAs and PUFA-based products are now commercially available in the market (Table 5). Production of some PUFAs is relatively well established and intense research is underway to develop commercially viable production methods for others. The market demand of GLA is estimated to be about 2000 tonnes/year (Gunstone, 1998) and for EPA it is 125 tonnes/ year in Japan alone. GLA is priced at < \$100 kg^{-1} of crude oil, whereas EPA sells for < \$600 kg^{-1} of pure oil. EPA has proved beneficial in treatment of several important ailments and its demand is expected to increase rapidly in the near term (Belarbi et al., 2000).

Table 5. Commercially available PUFA and PUFA products

PUFA	Source	Company	Product
GLA	*Oenothera sp*.	Clover Co., Australia	Milkarra
	Oenothera sp.	Croda Oleochemicals, England	Crossesential EPO
	Borago officinalis	Scotia Lipids, Scotland	Crossesential GLA
	Oenothera sp.	Scotia Lipids, Scotland	Efamol®
OTA	*Trichodesma zeylanicum*	Croda Oleochemicals, England	Crossesential OTA
	Echium plantagineum		
AA	Fermentation	Suntory Ltd., Japan	SUN-TGA
	Single-cell oil	Hoffmann-La Roche Ltd, Switzerland.	ROPUFA
	Mortierella alpina	Gist-brocades, Holland	ARASCO
EPA	Fish oil	Croda Oleochemicals, England	Incromega
		Scotia Lipids, Scotland	Hi-EPA Oil
		BASF, Denmark	Dry n-3®
		PRONOVA, Norway	EPAX 0626 TG
DHA	Tuna	Clover Co., Australia	Milkarra™
		Hoffmann-La Roche Ltd., Switzerland	ROPUFA
	Fish oil	Croda Oleochemicals, England	Incromega
	Fish oil	Scotia Lipids, Scotland	Hi-DHA Oil
	Crypthecodinium cohnii	Martek Biosciences Co.	DHASCO
	Fish oil	BASF, Denmark	Dry n-3®
	Fish oil	PRONOVA, Norway	EPAX 0626 TG

1.3.3. Current sources and potential sources of PUFAs

PUFAs are currently obtained from a number of sources including higher plants, animal viscera (brain) and oily fish (Table 6). The predominant sources of ω-3 fatty acids are vegetable oils and fish. Vegetable oils are the major sources of ALA, which is also found in the chloroplast of green leafy vegetables such as purslane and spinach. Purslane (*Portulaca oleracea*), is the richest source of ALA and is also one of the few plants known to be a source of EPA (Simonopoulos, 1989). Canola oil (*Brassica napus*) contains appreciable levels of PUFA (22% LA and 11% ALA). Other sources include nuts and seeds, vegetables and some fruits, egg yolk, poultry and meat, all of which collectively contribute minor quantities of ω-3 fatty acids to the diet. Oils extracted from marine fish such as mackerel, herring, salmon and sardines serve as the richest source of EPA and DHA (Huang et al., 2004). Commercially, fish oils are available in the form of gelatin capsules or oily preparations, which contain 20% to 30% EPA and DHA (Trautwein, 2001). Fishes obtain their long chain PUFAs (LCPUFAs) by consuming the LCPUFA rich microalage and phytoplanktons. EPA and DHA oils can also be obtained from single cell organisms like microalgae and fungi. Diatoms such as *Nitzschia*, are good producers of EPA, and dinoflagellates such as *Crypthecodinium cohnii* are used for commercial production of DHA (Barclay et al., 1994). Marine protists such as the *Thraustochytrids* are a potential source of DHA for human consumption (Barclay et al., 1994; Bajpai et al., 1991).

Vegetables are the main sources of ω-6 fatty acids. The most important ω-6 fatty acid, LA, is found in large amounts in corn oil, safflower oil, sunflower oil and soybean oil (Adam, 1989). It is very plentiful in nature and found practically in all plant seeds with the exception of palm and cocoa. GLA is found in plant oils derived from borage, evening primrose and black currant (Barre, 2001). AA is commercially produced by fermentation of *Mortierella alpina* and *Porphyridium sp.* which are rich sources of AA (Kendrick and Ratledge, 1992; Streekstra, 1997).

Table 6. Current and alternative sources of PUFAs

PUFA	Sources
GLA	Plants: *Oenothera, Borago, Ribes, Macaronesian, Echium* Fungi: *Mucor javanicus*, *Mortierella isabelina* Microalgae: *Spirulina*
ALA	Plants: *Glycine*, *Linum*, (many others)
AA	Animal viscera (brain) Fungi: *Mortierella alpina* Microalgae: *Porphyridium cruentum*
EPA	Oil fish: *Sardina, Engraulis*, etc. Fungi: *M. alpina, Saprolegnia* sp. Microalgae: *Phaeodactylum tricornutum, Monodus subterraneous* Mosses: *Phytium irregulare* Bacteria: *Shewanella* sp.
DHA	Oil fish: *Sardina, Engraulis*, etc. Fungi: *Traustochitrids* Microalgae: Dynophyceae

1.3.4. Genetic engineering of oilseed crops for the production of PUFAs

PUFA production from the above mentioned sources are inadequate for meeting the expanding PUFA market (Napier et al., 1999). Additional and significant problems of the current sources are: (a) seasonal and climatic variation in oil composition, which can result in inconsistencies in oil supply and quality, (b) low productivity of the plant sources (c) complex downstream processing, (d) high production and purification costs and (e) unpleasant taste/odour of oils from single cell sources (Alonso and Maroto, 2000). Although strain improvement and cultivation optimization may raise the levels of the desired fatty acids in the oils, these processes are time consuming and labour demanding. The above mentioned drawbacks and the increasing demand have raised the interest in obtaining these PUFAs from alternate sources that are more economical and sustainable. One attractive option is to genetically engineer the oilseed crops like soybean, canola, sunflower and others to produce PUFAs such as GLA, AA, EPA and DHA. These oleaginous plants are attractive because of their oil productivity, well established agronomic practices and processing techniques for oil recovery. In addition, the knowledge of pathway engineering is advancing rapidly and has opened up new possibilities for production of PUFAs in plants. During the past few years, genes encoding the enzymes involved in biosynthesis of PUFAs, such as desaturases and

elongases have been successfully isolated (Budziszewski et al., 1996; Lassner, 1997) from PUFA rich organisms and transgenically expressed in oilseed crops.

In the last few years reviews have appeared on the possibilities of engineering oilseed crops to produce 'designer' oils by genetic transformation. There are several basic requirements for engineering oleaginous plants for producing PUFAs: (a) understanding of the metabolic pathways and the enzymes/genes involved, (b) cloning of the critical genes involved in the synthesis of a specific PUFA, (c) availability of suitable *cis*-regulatory elements (i.e. promoters) to achieve expression in the appropriate tissue and (d) availability of transformation methods for the oleaginous plants and stability of the trait (Budziszweski et al., 1996). A brief literature review of the above requirements is given below,

1.3.4.1. Metabolic pathways

The knowledge of lipids and fatty acid biosynthesis in plants has greatly expanded in the last decade, as summarized in several good reviews (Browse and Somerville, 1991; Harwood, 1996; Murphy, 1993; Ohlrogge and Jaworsky, 1997; Slabas and Fawcett, 1992; Somerville and Browse, 1991). Plant fatty acid biosynthesis occurs almost exclusively in the plastid (Somerville and Browse, 1991) and is carried out by the fatty acid synthetase complex (Harwood, 1988). Fatty acids are then used directly in the plastid for the production of plastid glycerolipids (prokaryotic pathway) or exported to the cytoplasm to produce other glycerolipids (eukaryotic pathway) *via* endoplasmic reticulum processing (Browse and Somerville, 1991). Desaturation reactions take place in both cellular compartments with the fatty acids in the form of acyl lipids (linked to the glycerol backbone), except for the desaturation of 18:0 to 18:1ω-9 (Harwood, 1996). The enzymes involved in the desaturation steps in plants are well known (Figure 3). Each desaturase enzyme is usually present in at least two isoforms; one in the plastid and another one in the microsomes (Harwood, 1996; Heppard et al., 1996). Plant desaturases seem to exhibit low activity on C_{20} fatty acids (Millar et al., 1998; Spychalla et al., 1997) and therefore, very long chain PUFAs are not synthesized despite a substantial accumulation in plants. Some fungi, mosses and microalgae have the ability to synthesize these very long chain PUFAs by pathways that are poorly understood (Figure 3) (Arao et al., 1994; Arao and Yamada, 1994; Bajpai and Bajpai, 1993; Girke et al., 1998). These organisms contain all desaturases found in higher plants and several additional desaturases, which can specifically desaturate C_{20} and C_{22} fatty acids.

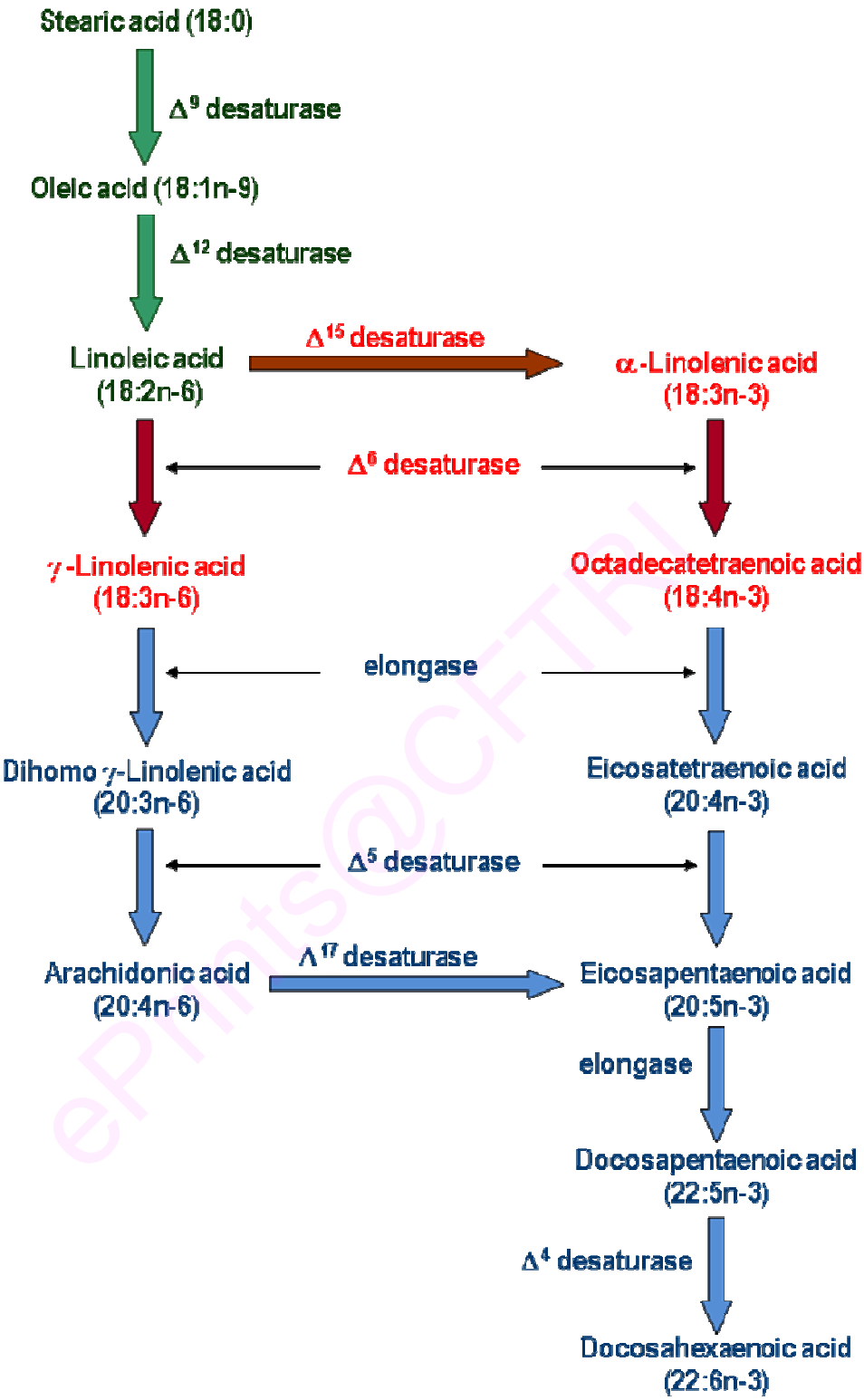

Figure 3. The general biochemical pathway of *in vivo* synthesis of PUFAs in various organisms. Green arrows represent reactions that are very common in higher plants; Red arrows represent reactions that occur only in a few plants; Blue arrows represent reactions that occur only in some lower organisms. (Source: Alonso and Maroto, 2000).

Both the prokaryotic and the eukaryotic pathways - also known as 'chloroplastic' and 'extra chloroplastic' pathways, respectively (Harwood, 1996) serve the cellular needs for structural lipids (i.e. membrane lipids). In addition, the extra chloroplastic pathway is the major route for the storage lipids, mainly triacylglycerols (TAG). Storage TAGs are synthesized by the Kennedy pathway in developing seeds (Gurr and Harwood, 1991; Murphy, 1993), which requires the enzyme diacylglycerol acyltransferase. This enzyme appears to have wide fatty acid specificity (Gurr and Harwood, 1991).

1.3.4.2. Gene cloning

Recent years have witnessed advances in the cloning of many genes involved in the biosynthesis of lipids, especially of the PUFA pathway which are listed in Table 7.

Table 7. List of the desaturase genes with potential biotechnological applications that have been cloned

Gene/Enzyme	Biological source	Reference
Δ^9-desaturase	*Arabidopsis thaliana*	Fukuchi et al., 1995
	Rosa hybrida	Fukuchi et al., 1995
	Anabaena variabilis	Sakamoto et al., 1994
	Synechocystis sp.	Sakamoto et al., 1994
n-3 desaturase (microsomal)	*Arabidopsis thaliana*	Yadav et al., 1993
	Glycine max	Yadav et al., 1993
	Brassica napus	Yadav et al., 1993
	Limnanthes douglasii	Bhella and Mackenzie, 1995
	Nicotiana tobaccum	Hamada et al., 1994
	Triticum aestivum	Horiguchi et al., 1998
	Perilla frutescens	Chung et al., 1999
n-3 desaturase (plastidial)	*Arabidopsis thaliana (FAD-7)*	Yadav et al., 1993
	Arabidopsis thaliana (FAD-8)	Gibson et al., 1994
	Glycine max	Yadav et al., 1993
	Brassica napus	Yadav et al., 1993
	Nicotiana tabaccum	Hamada et al., 1994
	Triticum aestivum	Horiguchi et al., 1998
Δ^{12}-desaturase (microsomal)	*Arabidopsis thaliana*	Okuley et al., 1994
	Glycine max	Heppard et al., 1996

Δ^{12}-desaturase (plastidial)	*Arabidopsis thaliana* *Spinacia oleracea*	Falcone et al., 1994 Schmidt et al., 1994
Δ^{6}-desaturase	*Borago officinalis* *Pythium irregulare* *Synechocystis* sp. *Mortierella alpina* *Mucor circinelloides* *Echium sp.*	Sayanova et al., 1997 Hong et al., 2002 Reddy et al., 1993 Huang et al., 1999 Hao et al., 2008 Marotoa et al., 2002
Δ^{5}-desaturase	*Mortierella alpina*	Michaelson et al., 1998 Knutzon et al., 1998

1.3.4.3. Transformation

Wada et al., (1990) reported the first case of lipid alteration by genetic engineering in cyanobacteria, which proved the feasibility of this approach in modifying the lipid composition. Initially the focus has been on non-oleaginous model plants such as *Arabidopsis* (Millar et al., 1998; Qi et al., 2004) and tobacco (Nunberg et al., 1994; Marotoa et al., 2002), which are of little direct practical value for agronomic based oil production. Currently efficient transformation methods are already available for several oilseed crops *viz*., soybean, canola, maize, etc (Olhoft et al., 2003; Liu et al., 2008; Frame et al., 2002), and the range of oilseed crops amenable to genetic manipulation is set to increase. The stability of the transformed trait seems not to be a problem in transgenic plants and there are many reports of extended expression of the transgene in modified plants. Friedt and Luhs, (1998) transformed rapeseed for high lauric acid content which is currently cultivated in the United States.

1.3.4.4. Appropriate expression

The expression of the transgene in the appropriate tissue is of paramount importance. The constitutive expression of some fatty acid transgenes has proven detrimental for the performance of the organism, especially under certain conditions (Kinney, 1994; Millar et al., 1998; Miquel and Browse, 1994). Other characteristics are sometimes poorly expressed or not expressed in seeds (Reddy and Thomas, 1996; Sayanova et al., 1999) where the transgene is designed for expression in the oilseed plant. These circumstances can invalidate the usefulness of the transgenic plant as production vehicle and greater effort is necessary for assuring the correct expression (i.e. in the

seeds) without other adverse effects (Budziszewski et al., 1996). Several reports of seed-specific promoters that work satisfactorily when used with heterologous genes were reported by Falco et al., (1995), Sarmiento et al., (1997) and Heppard et al., (1996).

1.3.5. Studies on transgenic oilseed plants as a source of PUFA

The rapid pace of genetic manipulation and regulation techniques have aided in utilizing the oilseed crops for PUFA production (Budziszewski et al., 1996). The following sections briefly review the achievements made in engineering oilseeds crops for the production of some specific PUFAs (GLA, AA, EPA, and DHA) that are commercially the most attractive.

1.3.5.1. Transgenic production of GLA

GLA can be produced from its precursor LA by the action of a Δ^6-desaturase (Figure 3). Potentially, GLA can be produced economically from oilseed crops by a relatively simple transformation of a single Δ^6-desaturase gene, providing that the gene is expressed stably in seeds and at a high rate and the transformed plants are easy to cultivate. A detailed review of the same will be dealt under section 1.4.8 at a later stage of this chapter.

1.3.5.2. Transgenic production of AA

The production of AA from LA involves the desaturation of LA to GLA, followed by the elongation of GLA to DHGLA and a subsequent desaturation of DHGLA to AA (Figure 3). This process takes place only in some fungi and microalgae because DHGLA is uniquely present in these organisms. Three major enzymes are involved in this process *viz*., a Δ^6-desaturase, a C18-PUFA specific elongase and a Δ^5-desaturase. Although transformation of a single plant with several different genes is still not routine, there are good expectations using the *Agrobacterium tumefaciens* system. Co-transformation of *Arabidopsis* using two different binary vectors each containing two genes on a single plasmid has been possible (Poirier et al., 2000).

The first C18-PUFA specific elongase was isolated and identified from the AA rich fungus, *Mortierella alpina* (Knutzon et al., 1998). This enzyme when tested in bakers' yeast, specifically recognized and elongated the n-6 and n-3 C18-PUFA substrates, GLA and OTA (C18:4n-3), respectively, whereas it demonstrated no activity towards monounsaturated or saturated fatty acid substrates (Parker et al., 2000; Das et al., 2000a, b; Das et al., 2002). Enzymes with similar elongating activity have also been isolated from *Caenorhabditis elegans* (Beaudoin et al., 2000), *Physcomitrella patens* (Zank et al., 2000), the marine protist, *Thraustochytrium* sp. (Heinz et al., 2001), algae,

Ostreococcus tauri and *Thalassiosira pseudonada* (Meyer et al., 2004), and from mammalian sources (Meyer et al., 2004; Leonard et al., 2000a; Agaba et al., 2004). These C18-PUFA specific elongases are thought to recognize CoA linked substrates (Domergue et al., 2003) and are different from the acyl carrier protein (ACP) linked substrate specific plant elongases that are involved in elongation of very long chain saturated and monounsaturated fatty acids, but not PUFAs (Cassagne et al., 1994; Von et al., 2001). An alternative pathway for the biosynthesis of DHGLA is also found in some organisms (Hulanicka et al., 1964; Lees and Korn, 1966; Ulsamer et al., 1969; Wallis and Browse, 1999). In these organisms, LA is first elongated to form eicosadienoic acid (EDA, C20:2n-6), and subsequently desaturated at the Δ^8-position to yield DHGLA. The PUFA-specific elongase involved in this pathway has been identified from the marine algae, *Isochrysis* (Qi et al., 2004) and a Δ^8-desaturase gene has been isolated from the DHA-producing marine protist, *Euglena gracilis* (Wallis and Browse, 1999).

The final step in the production of AA is the introduction of a double bond at the Δ^5-position of the DHGLA to generate AA. Delta-5 desaturase genes have been identified from fungi and algae such as *Mortierella alpina* (Knutzon et al., 1998; Michaelson et al., 1998), *Thraustochytrium* sp. (Qiu et al., 2002) and *Phaeodactylum tricornutum* (Domergue et al., 2002) and have been functionally characterized in yeast. Additional Δ^5-desaturases have been identified from *Caenorhabditis elegans* (Watts, and Browse, 1999), human (Leonard et al., 2000b) and rat (Zolfaghari et al., 2001). When introduced into a low linolenic variety of *Brassica napus*, the *Mortierella alpina* Δ^5-desaturase was capable of desaturating oleic acid (OA, 18:1n-9) to taxoleic acid ($\Delta^{5,\ 9}$ 18:2), and LA to pinolenic acid $\Delta^{5,9,12}$ 18:3) (Knutzon et al., 1998). Similarly co-expression of Δ^9-specific elongase from *Isochrysis galbana* (Qi et al., 2004), Δ^8-desaturase from *Euglena gracilis* (Wallis and Browse, 1999) and a Δ^5-desaturase from *Mortierella alpina* demonstrated the production of low amounts of AA in *Arabidopsis*.

1.3.5.3. Transgenic production of EPA

EPA is naturally found in some microalgae, fungi and mosses (Table 6). Fishes accumulate EPA and DHA obtained in their food chain, but do not synthesize these fatty acids. In micro-organisms, EPA is synthesized either from ALA (the n-3 pathway) or from AA (the n-6 pathway) (Figure 3). The production of EPA from ALA involves the desaturation of ALA to OTA by a Δ^6-desaturase, which is further elongated to eicosatrienoic acid (ETA, C20:4n-3) and is subsequently desaturated at the Δ^5-position to form EPA. Since the three enzymes involved in this process, i.e. Δ^6-desaturase, C_{18}-

PUFA specific elongase and Δ^5-desaturase function on both n-3 and n-6 pathways, and plant oils rich in ALA contains significant levels of LA, the transgenic expression of these three genes in an ALA rich plant would result in synthesis of both AA and EPA. To minimize the production of AA during EPA biosynthesis, one approach involves the shunting of the n-6 PUFA metabolites to their n-3 counterparts by the action of a group of enzymes designated as the ω^3-desaturases (Figure 3). These enzymes introduce a double bond at the third carbon atom counted from the methyl (ω-) end of the fatty acyl chain and are found in some plants, lower eukaryotes and cyanobacteria (Tocher et al., 1998). All plant and cyanobacterial ω^3-desaturases act exclusively on the C18-PUFA substrate LA, converting it to ALA.

However, these enzymes do not efficiently convert LA to ALA as shown by a high LA to ALA ratio in their seed oils (Padley et al., 1994). A novel ω^3-desaturase capable of recognizing multiple n-6 PUFA substrates, including the LA, GLA and DHGLA, was identified from *Caenorhabditis elegans* (Meesapyodsuk et al., 2000) and shown to be functional in plants (Spychalla et al., 1997). Pereira et al., (2003) successfully identified and expressed a novel fungal ω^3-desaturase that could specifically convert AA to EPA in an oil-seed crop. Thus, these ω^3-desaturases have potential applications for transgenic production of EPA by catalyzing the conversion of n-6 to n-3 pathway. In earlier reports, the yields of AA or EPA obtained by coexpression of the PUFA biosynthesis genes in yeast or plants have been poor (Domergue et al., 2002; Qi et al., 2004). These coexpression studies revealed an accumulation of the Δ^6-desaturated fatty acids in the glycerolipid fractions and almost none in the acyl-CoA pool and thus a decrease in flux through the pathway. This results from the fact that the fungal and algal Δ^6-desaturase function on phospholipid linked (mainly phosphatidylcholine linked) LA or ALA substrates, whereas the PUFA specific elongase requires its substrates to be present in the acyl-CoA pool. In the plant or yeast model systems tested, there appears to be an inefficient transfer of the Δ^6-desaturated products from the phospholipids to the acyl-CoA pool due to lack of suitable acyl transferase in these organisms (Domergue et al., 2003). Hence additional research work is needed to identify enzymes that are involved in the transfer of phospholipid linked PUFAs to the acyl-CoA pools.

An early report of recreating the microbial synthesis of n-3 LCPUFAs in plant was described by Qi et al. (2004). The genes for this pathway were obtained from micro-organisms, including the microalga *Isochrysis galbana* (Δ^9-elongase), the protist *Euglena gracilis* (Δ^8-desatuarase), and the microbial fungus *Mortierella alpina* (Δ^5-desaturase).

The pathway was expressed in non-seed tissue of the model plant *Arabidopsis* and EPA content in leaves of about 3% was observed. This study was a path-breaking first step towards producing EPA in plants. But the low content of EPA is hardly a viable alternative to fish oil and also the AA and by-product content needs to be reduced for commercial food oil. Kinney et al., (2004) filed a patent for the heterologous expression of an EPA pathway in soybean seeds. They expressed a gene set representing the Δ^6-fatty acid pathway from *Mortierella* along with ω^3-desaturases from *Arabidopsis* (Yadav et al., 1993) and freshwater mould *Saprolegnia diclina* (Pereira et al., 2004) in soybean embryos. The authors observed EPA content as high as 10% in seeds, which could be increased to 20% by replacing the Δ^6-desaturase gene from *Mortierella* with one from *Saprolegnia*. To date, this is the highest content of EPA achieved in any plant tissue. These transgenic soybean seeds contained only trace amounts of AA due to the presence of the highly efficient *Saprolegnia* ω^3-desaturase that has a substrate preference for AA, converting it to EPA (Pereira et al., 2004).

1.3.5.4. Transgenic production of DHA

In lower eukaryotes, DHA can be synthesized from EPA in a two-step process that involves (a) the elongation of EPA to docosapentaenoic acid (DPA) and (b) the desaturation of DPA by a Δ^4-desaturase to generate DHA (Figure 3). Thus the production of DHA in plant seed oils would involve coexpressing the C20-PUFA specific elongase and Δ^4-desaturase in addition to the previously described genes needed for EPA production. A number of mammalian elongases have been identified that can recognize and elongate C20-PUFAs (Meyer et al., 2004; Agaba et al., 2004; Leonard et al., 2000a). Expression of these genes in bakers yeast showed that these enzymes can recognize multiple chain length PUFAs, such as C18, C20 and C22-PUFAs (Leonard et al., 2004). Elongases specific for C20-PUFAs have been identified from lower eukaryotes and demonstrated to function in production of DHA (Meyer et al., 2004; Pereira et al., 2004).

The first Δ^4-desaturase was identified from the marine protist *Thraustochytrium*, which is capable of introducing a double bond at 4^{th} carbon of ω^3-DPA to form DHA, and of adrenic acid (ADA, C22:4n-6) to generate ω^6-DPA (C22:5n-6). Qiu et al. (2001) have expressed this Δ^4-desaturase gene in *Brassica juncea*, in the presence of exogenously supplied ω^3-DPA substrate and showed a production of 3–6% DHA in the leaves, stems and roots of the transgenic *Brassica*. Currently, Δ^4-desaturase genes were identified from *Euglena gracilis* (Meyer et al., 2003), *Pavlova* (Tonon et al., 2003) and *Isochrysis* (Pereira et al., 2004). Although feasibility of production of DHA in yeast model system has

been demonstrated by co-expression of the C18-elongase, Δ^5-desaturase, C20-elongase and a Δ^4-desaturase (Meyer et al., 2004), similar studies have not been done in the plant system.

1.3.6. Soybean Oil

Soybeans store their lipids mainly in the form of triglycerides in organelles known as oil bodies. Soybean lipids contain triglycerides (or triacylglycerols), phospholipids, tocopherols, phytosterols and free fatty acids. Among them triglycerides are the major components and account for nearly 80% of the total lipids. Triglycerides are neutral lipids, each consisting of three fatty acids linked to one glycerol moiety. Plant breeding and biotechnology has resulted in a wide range of genetic variation in fatty acid composition among the soybean germplasm. The typical soybean oil has a fatty acid composition of C16:0 (11%), C18:0 (4%), C18:1 (24%), C18:2 (53%) and C18:3α (7%).

Soybean is one of the few plant sources for the essential fatty acids, linoleic acid and α-linolenic acid. According to Chapkin (1992) many populations have diets low in omega-3 fatty acids and soy oil has significant amounts of omega-3 fatty acids in the form of α-linolenic acid. Since natural soy oil (without hydrogenation) is low in cholesterol and saturated fatty acids (about 15% total), and high in unsaturated fatty acids (about 85% total), it is considered as a healthy oil.

1.3.6.1. Genetic modification of soybean oil

Soybean is one of the most extensively genetically engineered oilseed crops for production of PUFAs and alteration of saturated and unsaturated fatty acid contents. A lot of research work has been carried out in soybean to modify its oil content as per human requirements for better nutrition. Kinney and Knowlton, (1998) first reported the efficacy of seed specific Δ^2-deaturase gene silencing to increase seed oleic acid content in soybean. High oleic soybean oil also has the added health benefit of having low saturated fatty acid content by 20% (Buhr et al., 2002). Similarly silencing of Δ^9-desaturase genes expressed in their seeds (Booth et al., 2006) and expression of a thioesterase gene with a high specificity for stearic acid (Kridl, 2002) resulted in soy oils containing 20 to 30% stearic acid when compared with just a few percent in commodity soy. The introduction of modified seed oils such as high oleic/stearic acid soybean oils will go a long way to remove the undesirable saturated fatty acids and trans fatty acids from the human diet (Lichtenstein et al., 2006). It is conceivable that soybean lines with very low saturated fatty acids and very high oleic acid contents could become the next generation of commodity oil.

Among the oilseed crops, genetic transformation protocol has been well studied in soybean and hence it has been used as a model crop for the production of PUFAs. Seed

specific expression of Δ^6-desaturase genes from borage, *Pythium irregulare* and *Saprolegnia diclina* resulted in GLA contents as high as 40% of total fatty acids in soybean seeds (Hong et al., 2002; Qiu et al., 2002; Sato et al., 2004; Knauf et al., 2006). Similarly expression of Δ^6-desaturase gene along with ω^3-desaturase gene resulted in an OTA content of 20-30% in soybean seeds (Eckert et al., 2006). As mentioned earlier the first demonstration of EPA and DHA production in plant seed oil was shown in soybean (Mukerji et al., 2002; Kinney et al., 2004). When a Δ^4-desaturase gene from *Schizochytrium aggregatum* was added to this pathway in soybean embryos, over 3% DHA was observed in the embryo oil. This is the highest DHA content seen in any plant oil till date. Soybean seeds expressing ω^3-desaturase from *Fusarium moniliforme* have been shown to have a total n-3 fatty acid content of over 70% (Damude et al., 2006). An attempt by Chen et al., (2006) in expressing the *Mortierella* Δ^6-desaturase pathway in soybean seed to produce AA (the endogenous n-3 desaturase was down regulated in these experiments) resulted in only trace amounts of AA (0.5–0.8% of total fatty acids). The authors hypothesized the use of same promoter multiple times, as the reason for this poor expression. However using the same *Mortierella* Δ^6-pathway, Kinney et al., (2004) reported AA contents of up to 20% in transgenic soybean seeds.

After many years of development and safety testing, two independently modified soybean transgencis/ transgenic products are expected to be commercialized soon to directly benefit the consumers; (i) one is the high oleic acid soybean line and (ii) the other one is the soybean oil containing OTA (Monsanto, 2008).

1.4. Gamma linolenic acid

Gamma linolenic acid (GLA) a trienoic C_{18} fatty acid is a member of the n-6 class of essential fatty acids (EFA) (Gill and Valivety, 1997; Broun et al., 1999) and is the first intermediate in the conversion of linoleic acid to arachidonic acid. GLA is a long chain polyunsaturated fatty acid with 18 carbon atoms and three methylene interrupted double bonds in the molecule. It is synthesized by the action of Δ^6 -desaturase enzyme on linoleic acid. The desaturation process is a rate determining step due to the impairment of the desaturase activity or the imbalance in the intake of ω-6 and ω-3 fatty acids in humans. An exogenous supply of γ-linolenic acid (GLA) can effectively overcome this rate limiting desaturation step. This possibility forms the basis for the current interest in this acid and in those oils in which it occurs.

1.4.1. History

GLA was first isolated from evening primrose (*Oenothera biennis*), an herbal plant that was traditionally used by Native Americans for treating the swellings of the body. Its use was learned by the early European settlers and was introduced in the seventeenth century to Europe where evening primrose became a popular folk remedy. Because of its wide range of uses as a general vulnerary and for treatment, it earned the name "king's cure all". However, despite its popularity, its active ingredient was known only in 1919, when Heiduschka and Luft (1919) extracted the oil from evening primrose seeds and reported the presence of an unusual linolenic acid. This particular acid yielded a hexabromide derivative of linolenic acid with very different physical characterisrtics from the other two common forms (α- and β- isomers) found in linseed oil. Thus they named it γ-isomer or GLA. The exact chemical structure was later characterized and confirmed by Riley, (1949).

Coincidentally, another GLA rich plant Borage (*Borago officinalis* L.) was also widely used as a folk medicine in early times. Its leaves and flowers were useful in treating putrid and pestitential fever, combating the venom of serpents, jaundice, consumption, sore throat and rheumatism, and increasing breast milk. Anectodal stories about borage include its reputation for instilling courage and producing absolute forgetfulness when steeped in wine (Awang, 1990). However, it was not until 1960 that its richness in GLA was reported (Miwa et al., 1960).

1.4.2. Nomenclature of GLA

GLA refers to a fatty acid that has an 18 carbon chain length and three double bonds attached to the 6^{th}, 9^{th} and 12^{th} carbons counting from the carbonyl end (Figure 4). According to the modified Geneva system, GLA is named as "$\Delta^{6,9,12}$ octadecatrienoic acid." This can be simplified to $\Delta^{6,9,12}$ 18:3, in which 18 represents the carbon chain length and 3 represents the number of double bonds. Since the naturally occurring fatty acids are mostly of the *cis* configuration, to distinguish them from fatty acids with double bonds in *trans* form, geometric configuration is often indicated. Thus GLA is *cis*-6, *cis*-9, *cis*-12 18:3 (or simply c6, c9, c12 -18:3). In literature, a more simplified shorthand name is often used in which GLA is designated as 18:3n-6 (or 18:3ω-6), where n-6 (or ω-6) indicates the position of the first double bond beginning from methyl end (CH_3-).

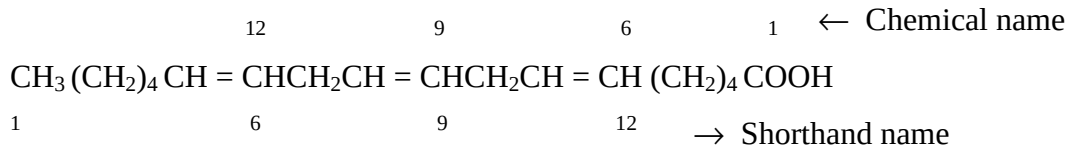

Figure 4. Molecular structure of gamma linolenic acid

1.4.3. Stereochemistry of GLA

The stereospecificity of GLA varies from source to source. In evening primrose oil and black currant oil GLA is concentrated in the *sn-3* position, while in borage oil it is concentrated in the *sn-2* position. GLA is concentrated in both the *sn-2* and *sn-3* positions of fungal oils (GLA monograph, 2004).

1.4.4. Biosynthesis and metabolism of GLA

GLA is not normally found in the diet, but is synthesized from the dietary linoleic acid (18:2n-6) by a desaturation reaction catalyzed by the enzyme Δ^6-desaturase. GLA represents the first product on the n-6 polyunsaturated fatty acid pathway (Figure 3). Once formed it is rapidly elongated to DHGLA (20:3n-6), by the activity of a polyunsaturated fatty acid specific elongase (Parker et al., 2000). DHGLA can be converted to arachidonic acid (AA; $\Delta^{5,8,11,14}$ 20:4) by the action of Δ^5-desaturase (Willis, 1981). Both DHGLA and AA can be metabolized to form eicosanoids (and on to prostaglandins and related compounds).

1.4.5. Delta-6 desaturase and gamma linolenic acid biosynthesis

The key enzyme necessary for the biosynthesis of GLA is the Δ^6-desaturase, which is a subclass of the microsomal fatty acid "front end" desaturases. These desaturases catalyze the introduction of a double bond between the pre-existing double bonds and the carboxyl group (the front end or Δ-end) of the fatty acid molecule (Napier et al., 1999). The desaturase reaction occurs under aerobic conditions and hence requires the presence of oxygen (Shanklin and Cahoon, 1998). These desaturases are membrane bound enzymes and because of the technical difficulties in obtaining large quantities of purified membrane protein, progress in understanding the structure-function of these desaturases has lagged behind that of a soluble class (acyl-ACP desaturases). Many "front end" desaturase genes from various organisms have been identified, which has significantly increased the scope for further investigation of the precise mechanism of desaturation and the enzymes involved in polyunsaturated fatty acid biosynthesis.

These front end desaturases use fatty acids either esterified to glycerolipid or to coenzyme A (CoA) as substrates, depending on the availability of fatty acid substrates in different organisms (Tocher et al., 1998; Los and Murata, 1998). In higher plants, the Δ^6 desaturase operates in the endoplasmic reticulum only on C18 chains in *sn-1* and *sn-2* of mainly phosphatidylcholine (PC), while in animals, Δ^6-desaturase generally utilizes CoA-linked substrates in the endoplasmic reticulum (Tocher et al., 1998). In lower eukaryotes like fungi, the fatty acids linked to phospholipids are believed to be the substrates for Δ^6 desaturase enzyme (Los and Murata, 1998). In group 3 (*Synechocystis* spp. PC 6714, *Spirulina platensis*) and group 4, (*Synechocystis* spp. 6803) cyanobacteria, the Δ^6 desaturase is believed to operate on C18 at *sn-1* in the thylakoid membrane and the desaturation occurs in fatty acids linked to monogalactosyl diacylglycerol (Tocher et al., 1998).

1.4.6. Sources of GLA

GLA is obtained mainly from three different sources *viz.* plant, cyanobacterial and fungal sources. Among the plants only a few higher plants *viz*., Borage, Evening primrose and Black currant are known to have GLA.

(i) Evening primrose:

Evening primrose is grown in many countries in Europe, and in Australia, Newzealand, Israel and the USA. GLA usually represents 8-10% of the triacylglycerols. Evening primrose oil is very rich in linoleic acid and contains only very little α-linolenic acid and fatty acids with more than 18 carbon atoms.

(ii) Borage

Borage oil is a richer source of GLA than evening primrose oil. The seeds contain 29-30% of oil with a GLA content of 20-25%. The level of GLA in seed is about three times that of evening primrose. Linoleic acid (35-40%) is lower than in evening primrose oil and there is an inverse ratio between oleic acid and GLA.

(iii) Black currant and other *Ribes* species

Black currant seeds contain 30% oil, which differs from evening primrose and borage oils in that it contains two n-3 acids *viz*., α-linolenic (18:3) and octadecatetraenoic acid (18:4). GLA is present at lower levels in other *Ribes* species such as redcurrant and gooseberry (Traitler et al., 1984, 1988).

(iv) Microbial sources

Several microorganisms produce GLA in their lipids and much effort have been put into studying them. Moulds belonging to the *Mucorales* family produce GLA as the sole 18:3 acid rather than the more usual α-isomer and commercial processes have been developed for *Mucor javanicus* and *Mortierella isabellina.* They differ from the seed oils in their higher levels of saturated and monoene acids and their lower level of linoleic acid. The consequences of varying growth conditions in an attempt to optimize the production of GLA are described in several papers (Table 8). The cyanobacteria *Spirulina platensis* and *Spirulina maxima* are potential sources of GLA. However GLA is present in various galatoacyl-diacylglycerols or in phosphatidic acids with triacylglycerols only a minor component. *S. platensis* contains about 1.2% of GLA on dry weight basis.

1.4.7. Microbial production of GLA

An overview of process investigations related to microbial production of GLA is presented in Table 8. Researchers have demonstrated the potential to produce GLA at concentrations of 15-25% of total fatty acids in oils produced by lower fungi from the order *Mucorales*, especially from *Mortierella, Mucor* and *Cunninghamella* species. It is possible to achieve biomass densities of > 50 g l^{-1} in submerged culture in culture periods of typically 10 days with productivities of about 0.6 g/l/day. In solid state media productivities of 1.3 g GLA/kg substrate/day were reported. High concentrations were obtained in a two stage process, whereby harvested mycelium was disrupted and incubated under defined conditions at 5 °C for 15 days. GLA (15%, w/w), was the first single cell oil (SCO) to be produced commercially from *Mucor circinelloides* (Kennedy et al., 1993). This fungus is used in oriental food fermentations and hence the oil was approved for human consumption. However, prices of the latter plant oils remained stable or even decreased, such that the SCO-GLA preparation becomes uncompetitive.

1.4.8. Production of GLA in transgenic plants

In order to modify the fatty acid biosynthetic pathways by genetic manipulation to produce GLA in an oilseed crop, the identification and isolation of the gene of the required enzyme Δ^6-desaturase is a prerequisite. Furthermore, GLA is not a common fatty acid in plants, since only a small number of plants posses this enzyme, which converts LA to GLA in seeds. For this reason many laboratories have extended the search of this gene to microorganisms.

Table 8. Production of GLA from different microbial sources

Sl. No.	Strategy	Organism	Reference
1.	Development of a high GLA producing mutant with resistance to low temperature	*Mortierella ramannia*	Hiruta et al., 1996
2.	Optimization of GLA production in hexadeconal media.	*Mortierella isabellina*	Xian et al., 2001
3.	Media optimization for GLA production	*Mucor rouxii* *Mucor circinelloides*	Mamata et al., 2008 Preez et al., 1997
4.	Investigation of low temperature incubation of disrupted mycelia	*Mucor circinelloides*	Kennedy et al., 1993
5.	Screening mucorales for highest GLA production	*Mucor mucedo*	Certik et al., 1997
6.	Effect of carbon and nitrogen source on GLA accumulation	*Rhizopus nigricans*	Bandyopadhyay et al., 2001
7.	Effect of metal ion concentration on GLA production	*Cunninghamella sp.*	Muhid et al., 2008
8.	Studies in solid state culture	*Cunninghamella echinulata*	Conti et al., 2001

In the past decade, significant progress has been made; the gene encoding the enzyme Δ^6-desaturase has been successfully identified and isolated from borage (Sayanova et al., 1997), *Mortierella alpina* (Huang et al., 1999), *Mucor rouxii* (Laoten et al., 2000), *Pythium irregulare* (Hong et al., 2002), *Physcomitrella patens* (Girke et al., 1998), *Echium sp*. (Marotoa et al., 2002), *Primula sp*. (Sayanova et al., 2003) and *Synechocystis* (Reddy et al., 1993). Generally, the Δ^6-desaturases isolated from the microbes and plants act exclusively on the phospholipid linked LA substrate (Domergue et al., 2003), whereas those from mammalian sources recognize CoA linked LA substrates (Okayasu et al., 1981). To date, most of the Δ^6-desaturases have been cloned and functionally expressed in yeast, tobacco and some have also been tested in oilseed crop plants (Table 9).

The first demonstration of the production of GLA in transgenic plants was reported by Reddy and Thomas (1996). In their study, the cyanobacterial (*Synechocystis sp*.) desD was sub cloned. Since in higher plants the fatty acid desaturation occurs in chloroplast and endoplasmic reticulum, the authors used two separate constructs containing either endoplasmic reticulum or chloroplast signal peptide sequence in order to target the Δ^6-desaturase to either endoplasmic reticulum or chloroplast respectively. However it is important to mention that in cyanobacteria the Δ^6-desaturation of fatty acids closely follows the plastid pathway, not the endoplasmic pathway. The analysis of fatty acids extracted from the transgenic tobacco showed low levels of GLA or OTA ranging from 1-3% of the total C18 fatty acid content (Reddy and Thomas, 1996). No significant amounts of GLA and OTA were detected in seeds of transgenic tobacco plants expressing cyanobacterial Δ^6-desaturase. Furthermore, targeting the Δ^6-desaturase to chloroplast did not increase the production of GLA despite the fact that cyanobacterial Δ^6-desaturase follows the plastid pathway.

Table 9. List of desaturases cloned and their expression levels

Biological source	Expressed in	% of GLA production	Reference
Borago officinalis	Tobacco	13.2	Sayanova et al., 1997
	Glycine max	17.0	Sato et al., 2004
Physcomitrella patens	Tobacco	12.3	Girke et al., 1998
Synechocystis sp.	Tobacco	1.2	Reddy et al., 1993
	Brassica juncea	15.0	Das et al., 2006
Helianthus annus	Tobacco	12.0	Sperling et al., 1995
Mortierella alpina	*Saccharomyces cerevisiae*	30.6	Huang et al., 1999
Pythium irregulare	*Brassica juncea*	25.5	Hong et al., 2002

Later Sayanova et al., (1997) taking a similar approach expressed the borage Δ^6-desaturase in transgenic tobacco plants. The authors did not use signal peptide sequence for targeted expression in their construct. Fatty acid analysis indicates that GLA and OTA accounted for about 13 and 10%, respectively in the transgenic leaf lipid and were absent in the control leaf lipid. The enzymatic activity of borage Δ^6-desaturase in

transgenic tobacco appeared to be much higher than that of cyanobacterial Δ^6-desaturase. This could be due to the fact that the cyanobacterial Δ^6-desaturase differs from the endoplasmic reticulum located plant desaturases in using ferredoxin rather than cytochrome b^5 as cofactor (Sayanova et al., 1997). Nonetheless, both examples clearly demonstrated that the transgenic expression of Δ^6-desaturase was the first step in engineering the production of GLA or LCPUFA in the transgenic oilseed crop.

Huang et al., (2001) have co-expressed the *Mortierella alpina* Δ^6-desaturase and Δ^{12}-desaturase genes in a low linolenic acid variety of *Brassica napus* resulting in the generation of GLA at a level of greater than 40%. Similarly Hong et al., (2002) transformed *Brassica juncea* with Δ^6-desaturase gene from *P. irregulare* to generate 25–40% GLA in the transgenic seeds. However, due to the high initial content of ALA in this plant, 2–10% octadecatetraenoic acid (OTA, 18:4n-3) was also reported to be formed.

The use of seed specific promoters resulted in more amounts of modified fatty acids in the seed oil. Δ^6-desaturase genes from borage, *Pythium irregulare* and *Saprolegnia diclina* have been seed specifically expressed in soybean, *Brassicca juncea* and safflower (*Carthamus tinctorius*), with resulting GLA contents in the seed oil as high as 70% of total fatty acids (Hong et al., 2002; Qiu et al., 2002; Sato et al., 2004; Knauf et al., 2006). Similarly Das et al., (2006) reported 15% accumulation of GLA in *B. juncea* by the seed specific expression of *Synechocystis* sp. PCC6803 Δ^6-desaturase.

1.5. Importance of the proposed work in the present scenario

In mammals, GLA is synthesized from dietary linoleic acid by the action of a rate limiting enzyme, Δ^6-desaturase. GLA represents the first product on the omega-6 polyunsaturated fatty acid pathway. Once formed it is rapidly elongated to DHGLA, converted to AA and finally metabolized to form eicosanoids through a series of elongation and desaturation reactions. Many physiological and pathological conditions have been shown to depress the Δ6-desaturation step. Decreased activity of this key enzyme was observed in case of ageing, stress, excessive alcohol consumption, diabetes, atopic dermatitis, premenstrual syndrome, rheumatid arthritis, eczema, cancer and cardiovascular diseases. Clinical studies have shown that dietary supplementation with GLA, bypassing the delta-6 desaturation step is effective in treating a number of such conditions and hence oils containing GLA are widely used as a general health supplement. This has prompted GLA rich oils to become a much demanded commodity.

GLA is an uncommon fatty acid in plant kingdom. Only a small number of higher plant species synthesize GLA. The sources of GLA are seed oils from Evening primrose (*Oenothera sp.*), Borage (*Borago officinalis*) and Black currant (*Ribes nigrum*), *Spirulina* and certain fungi such as *Rhizopus, Mucor* and *Mortierella.* The commercial sources of GLA include seed oils of evening primrose (7-10%) and borage (18-26%). Unfortunately, production of these oils is generally limited by low yield and poor agronomic performance of these crops. GLA from microorganisms such as *Spirulina*, *Mortierella sp.* and *Mucor sp*. are also found to be unsuitable for dietary supplementation due to their high production/purification costs, unpleasant odour and safety concerns. Thus there is a tremendous opportunity to produce GLA in conventional oilseed crops by cloning Δ6-desaturase gene, which will provide new dietary sources of GLA. Also from the brief literature survey mentioned earlier it can be noted that the introduction and expression of Δ^6-desaturase enzyme is the first and foremost step for the production of PUFAs and LCPUFAs in transgenic oilseed crops.

Hence the present investigation focused on the cloning of Δ^6-desaturase gene from the cyanobacterial source (*Spirulina platensis*) and its expression in soybean (*Glycine max*) a conventional oilseed crop. With this background, the following objectives were framed for the research work.

1.6. Objectives:

1. Genetic transformation of soybean using selectable marker genes.
2. Cloning of Δ^6-desaturase gene from *Spirulina platensis* and its expression in soybean for the production of gamma linolenic acid.

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Materials & Methods

SECTION I

IN VITRO REGENERATION OF SOYBEAN

2.1. Materials

Soybean genotypes (viz., Hardee and JS 335) were obtained from Gandhi Krishi Vigyan Kendra (GKVK), University of Agricultural Sciences, Bangalore, Karnataka, India. The characteristics of the procured soybean seeds were presented in Table 10. All the salts and chemicals that are used in the experimental study were obtained from Hi-Media, Mumbai; the hormones and plant growth regulators from Sigma-Aldrich, USA; antibiotics (Cefotaxime, kanamycin, hygromycin, etc.,) from Duchefa, Germany; and glasswares from Borosil, Mumbai, India, unless or otherwise mentioned.

Table 10. Description of Indian soybean varieties used in the present study

Soybean genotype	Year of release/ notification	Area of adaptability	Maturation duration (days)	Yield (q/ha)	Salient features
Hardee	1976	Southern zone	105-110	15-20	White flowers, grey pubescence, yellow seed coat, rosy hilum, determinate and suitable for intercropping
JS 335	636(E)/2.9.94	Central zone	95-100	25-30	Purple flowers, semi-determinate, resistant to shattering, black hilum. Performs well in Eastern and Southern states

2.2. Methodology

2.2.1 Sterilization of culture media and culture conditions

MS medium (Murashige and Skoog, 1962) with sucrose (30 g l^{-1}) and *myo*-Inositol (0.10 g l^{-1}) was used as the basal medium (unless otherwise stated) and was supplemented with different concentrations and combinations of plant growth regulators for different types of response as per the requirement. MS salts and vitamins were prepared as stocks (Solution A to G) and stored under refrigerated conditions for its use on requirement. Stock solutions were prepared as reported by Helgeson (1979) and Dodds and Roberts (1982) with minor modifications as given in the Table 11.

From the stocks, MS basal medium was prepared as mentioned in Table 11, and the required hormones and growth regulators at desired concentrations and combinations were added to the MS basal medium. The pH of the medium was set to 5.6-5.8 (Mann *et al*., 1982) using a pH meter (Control Dynamics Digital pH meter, APX 175). For solid medium, tissue culture grade agar (Hi-Media, Mumbai) at 0.8% (w/v) was added as a gelling agent, boiled and dispensed into 150 ml Erlenmeyer flasks or test tubes or tissue culture bottles and sterilized at 121 °C with a pressure of 1.05 kg cm^{-2} for 20 min (George, 1993). Hormones or plant growth regulators that are heat labile are filter sterilized using a 0.22 μm filters (Sartorius, Mumbai, India) and added to the sterilized medium under sterile conditions in a laminar air flow chamber. The cultures were maintained in a 16/8 h (light/dark) photoperiod using the fluorescent lights (Philips India Ltd., Mumbai, India) at 45 μmol m^{-2} s^{-1} and the temperature was maintained at 24 ± 2 °C.

2.2.2. Seed germination

Matured and uniform sized soybean seeds of cv. Hardee and JS 335 were handpicked and surface sterilized in 70% ethanol for 30 seconds followed by 0.2% carbendazim (w/v) for 5 min and 0.1% mercuric chloride (w/v) for 1 min. The surface sterilized seeds were rinsed for four to six times in sterile distilled water and cultured in petridishes containing MS basal medium for germination.

Table 11. Components of MS basal medium with minor modifications (Murashige and Skoog, 1962; Helgeson, 1979; Dodds and Roberts, 1982).

Stocks	**Chemical**	**Stock (g l^{-1})**	**Quantity required/ litre**
A (50X)	Ammonium nitrate	82.50	20.0 ml
B (50X)	Potassium nitrate	95.00	20.0 ml
C (200X)	Boric acid Potassium dihydrogen phosphate Potassium iodide Sodium molybdate dihydrate Cobalt chloride hexahydrate	1.24 34.00 0.166 0.050 0.005	5.0 ml
D (200X)	Calcium chloride dihydrate	66.40	5.0 ml
E (200X)	Magnesium sulfate heptahydrate Manganese sulfate monohydrate Zinc sulfate heptahydrate Copper sulfate pentahydrate	74.0 3.38 1.725 0.005	5.0 ml
F (200X)	EDTA disodium salt dihydrate Ferrous sulfate heptahydrate	7.46 5.56	5.0 ml
G (200X)	Glycine Nicotinic acid Pyridoxine-HCl Thiamine-HCl	2.0 0.5 0.5 0.1	5.0 ml
	Sucrose		30.0 g
	myo-Inositol		0.10 g

2.2.3. Effect of cytokinin and explants on *in vitro* regeneration

Seven days after seed germination, cotyledonary nodes and upper hypocotyl segments about 1 cm long were excised from the seedlings. The explants (cotyledonary node and hypocotyl) were then cultured on media containing MS basal medium with varying concentrations of thidiazuron (TDZ) at 0.2, 0.5, 1.0 and 2.0 mg l^{-1} or N^6-benzylaminopurine (BAP) at 0.5 and 1.0 mg l^{-1} for shoot bud tissue formation. Ten explants were placed in each petridish and the experiments were repeated thrice. After 6

weeks of culture in the above media combinations, the number of explants forming adventitious shoot bud tissues was recorded. For regeneration or elongation, the shoot bud tissues were placed in media containing MS basal medium supplemented with Gibberellic acid (GA_3) at 0.1, 0.2, 0.5 and 1.0 mg l^{-1} or BAP at 0.2 and 0.5 mg l^{-1}. The number of elongated shoots was recorded after 8 weeks of culture. The elongated shoots were further transferred to MS basal medium supplemented with IBA (0.5 mg l^{-1}) for rooting. After 4 weeks of culture, the rooted plantlets were transferred to pots containing the mixture of peat, sand and red soil (1:1:2) for hardening in green house and allowed to set seeds.

2.2.4. Effect of Triacontanol (TRIA) on *in vitro* shoot multiplication

Triacontanol [$CH_3 (CH_2)_{28} CH_2OH$] stock solution was prepared by dissolving 1 mg of TRIA in 0.75 ml of $CHCl_3$ containing one drop of Tween 20 and was gradually diluted with distilled H_2O to a final volume of 200 ml (Giridhar et al., 2004). The explants (cotyledonary nodes and shoot tips) were placed in the MS basal medium supplemented with varying concentrations of TRIA (0, 2, 5 and 10 µg l^{-1}) along with Indole-3-butyric acid (IBA) at 0.2 mg l^{-1} and BAP (0.2 mg l^{-1}) or GA_3 (50 µg l^{-1}) for adventitious shoot bud formation. The explants were sub-cultured in fresh medium once in two weeks. After 4 weeks, the newly formed shoots were transferred to MS basal medium with varying concentrations of TRIA (0, 2, 5 and 10 µg l^{-1}) for rooting. The rooted plantlets were transplanted to pots in green house for hardening and allowed to set seeds.

2.2.5. Regeneration from embryonic axis

Mature soybean seeds were surface sterilized as mentioned above in section 2.2.2. The sterilized seeds were soaked in distilled water for 24 h in the dark at 24 °C. The embryonic axis was separated and primary leaves on the embryonic axis were excised to expose the meristem. The explants were placed (apical regions directed upwards) in MSB_5 medium [MSB_5 medium contains MS macro and micronutrients (Murashige and Skoog, 1962) with B_5 vitamins (Gamborg et al., 1968)] supplemented with 4 mg l^{-1} BAP. After 48 h, these explants were transferred to the above mentioned medium supplemented with 0.2 mg l^{-1} BAP and 0.2 mg l^{-1} IBA. The explants were sub-cultured afresh onto the same medium at biweekly intervals. The number of adventitious shoots was recorded after 6 weeks and the regeneration frequencies were determined. Adventitious shoots of 4-6 cm were excised from the explant and transferred to rooting medium (½ MS basal medium devoid of any growth regulators). After 4 weeks in the rooting medium, the rooted plantlets were grown to maturity in pots under green house conditions.

2.2.6. Statistical analysis

All the experiments were based on a randomized block design with three replications for each treatment. The data were statistically analyzed using the AGRES 7.01 software. Significant difference between the treatments was determined using Analysis of variance (ANOVA) test and the means were compared using the Duncan's New Multiple Range Test at a significance level of $P < 0.05$.

SECTION II

AGROBACTERIUM MEDIATED TRANSFORMATION OF SOYBEAN

2.3. Materials

Soybean genotype JS 335 was obtained from Gandhi Krishi Vigyan Kendra (GKVK), University of Agricultural Sciences, Bangalore, Karnataka, India and its characteristics are presented in Table 10. The binary vector pCAMBIA 1305.2 and *Agrobacterium tumefaciens* strain EHA 105 maintained by Plant Cell Biotechnology Department, Central Food Technological Research Institute, Mysore was used for the experiments. All the salts and chemicals that are used in the experimental study were obtained from Hi-Media, Mumbai; the plant growth regulators from Sigma-Aldrich, USA; antibiotics (Cefotaxime, kanamycin, hygromycin, etc.,) from Duchefa, Germany; and glasswares from Borosil, Mumbai, India, unless or otherwise mentioned.

2.4. Methodology

2.4.1. System sensitivity to hygromycin B

The explants cotyledonary nodes and embryonic axis of soybean cv. JS 335 were prepared from matured seeds as mentioned under sections 2.2.3 and 2.2.5 respectively. The explants were cultured on regeneration medium supplemented with various concentrations of hygromycin B from 0 to 50 mg l^{-1}. After 4 weeks, the number of explants that survived hygromycin B levels was documented.

2.4.2. *Agrobacterium* strains and vector

The plasmid pCAMBIA 1305.2 (CAMBIA, Canberra, Australia), a compact binary vector (11.6 kb) was used in this study. This plasmid contains the *uidA* gene, designated as GUSplus™ with secretion signal peptide [a glycine rich protein signal peptide sequence (GRP) from rice for rapid *in vivo gus* assays], an intron from the castor bean catalase gene to confirm detection of plant specific β-glucuronidase (GUS) expression and a hygromycin resistance gene (*hptII*) for selection (Figure 5). The plasmid was mobilized into the *Agrobacterium tumefaciens* strain EHA 105 by freeze thawing method (Appendix G) and maintained on solid Luria Bertanii (LB) media (Appendix A) supplemented with 20 mg l^{-1} of rifampicin and 50 mg l^{-1} of kanamycin.

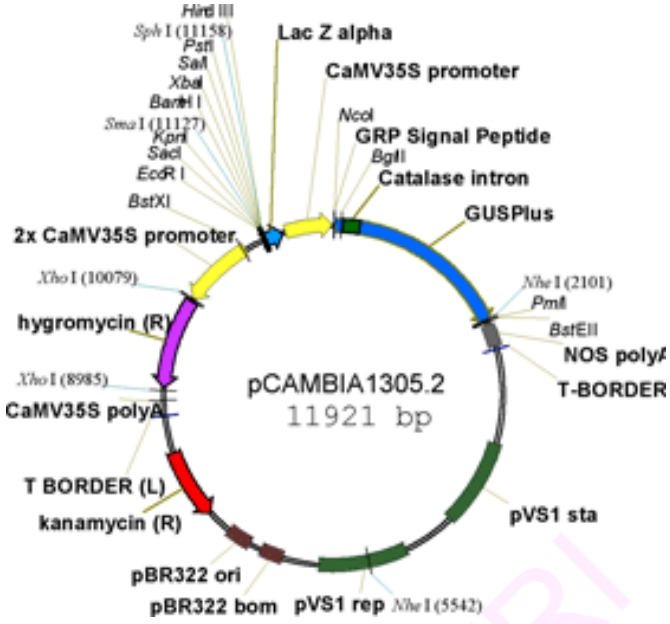

Figure 5. Diagrammatic representation of the binary vector pCAMBIA 1305.2

2.4.3. Bacterial infection and co-cultivation

Single bacterial colony was transferred to 5 ml of liquid LB medium with 50 mg l^{-1} kanamycin and grown overnight at 28 ºC in an incubatory shaker (BEST shaker, LSI 50) at 120 rpm. Overnight grown culture was used to inoculate 25 ml of LB broth containing 50 mg l^{-1} kanamycin and kept at 28 °C with 120 rpm until the absorbance value reaches 1.0-1.2 at 600 nm. The resulting bacterial culture was pelleted by centrifuging at 3,000 rpm for 10 min and resuspended in liquid co-cultivation medium (CCM) to an OD_{600} of 0.5. The components of the CCM are presented in Table 12, in which the thiol compound L-Cysteine was added in varying concentrations (200 to 1000 mg l^{-1}) to study its effect on gene transformation. The explants cotyledonary node and embryonic axis of soybean cv. JS 335 were prepared as mentioned under sections 2.2.3 and 2.2.5 respectively. The explants were then immersed in the bacterial suspension for 30 min, blotted dry in filter paper and plated on a petridish (90 mm diameter × 15 mm deep) containing solid CCM overlaid with a piece of Whatman no. 42 filter paper. The Petri dishes holding ten explants each were wrapped with paraffin film and incubated in the dark for 5 days.

2.4.4. Selection and plant regeneration

After five days in dark, the excess *A. tumefaciens* was briefly washed in liquid shoot induction medium (SIM) and the explants were subsequently embedded in the

semi-solid SIM. During shoot induction different selection strategies with hygromycin B as a selective agent were evaluated. The explants were cultured without hygromycin B for the first 2 weeks. Later the explants were transferred to the same medium supplemented with 5 mg l^{-1} hygromycin B for further 4 weeks with biweekly subculture into fresh medium. After 4 weeks, the differentiating tissues were transferred to the shoot elongation medium (SEM). The explants were sub-cultured every 2 weeks into fresh SEM until the shoot buds elongate. Elongated shoots (4-6 cm) were excised and transferred to rooting medium (RM).

Table 12. Media components during different stages of soybean transformation with binary vector pCAMBIA 1305.2

Components	Co cultivation medium (CCM)	Shoot induction medium (SIM)	Shoot elongation medium (SEM)	Rooting medium (RM)
MS	½ MS	½ MS	½ MS	½ MS
Sucrose	3%	3%	3%	3%
Purified agar	0.6%	0.8%	0.8%	0.8%
MES	4 g l^{-1}	0.5 g l^{-1}	0.5 g l^{-1}	0.5 g l^{-1}
L-Cysteine	0.2 to 1 g l^{-1}	0.2 to 1 g l^{-1}	-	-
BAP	2 mg l^{-1}	0.2 mg l^{-1}	0.2 mg l^{-1}	-
IBA	-	0.2 mg l^{-1}	-	1.0 mg l^{-1}
GA_3	-	-	0.05 mg l^{-1}	-
Acetosyringone	100 µm	-	-	-
Cefotaxime	-	300 mg l^{-1}	300 mg l^{-1}	-
Hygromycin B	-	0, 5 mg l^{-1}	10 mg l^{-1}	-

2.4.5. Histochemical assay for GUS activity

GUS activity was histochemically measured after 4 weeks in shoot elongation medium. Tissues were incubated for 24 h at 37 °C in a buffer consisting of 80 mM Na_2HPO_4 (pH 8.0), 10 mM Na_2EDTA, 0.5 mM $K_4Fe(CN)_6$, 0.1% (v/v) Triton-X, 1% (v/v) dimethyl sulfoxide, 20% (v/v) methanol and 0.1 mg l^{-1} 5-bromo-4-chloro-3-indolyl-β-D-glucuronide [X-Gluc; Sigma Aldrich, Bangalore]. Following incubation, the tissues were destained with 95% ethanol and then visually observed for blue staining. For transient GUS assay scores, the ranking system of Olhoft and Somers, (2001) was followed.

2.4.6. Transformation efficiencies

Transformants were defined as GUS-positive plants that also demonstrated resistance to hygromycin B and transformation efficiency as the number of both GUS positive and hygromycin B resistant plants divided by the total number of explants at the start of the experiment.

2.4.7. Biosafety measures

All the work that involves manipulation of biological materials was carried out according to the guidelines of IBSC (Institutional Biosafety Committee, CFTRI, Mysore). Transgenic work was carried out with permission from RCGM (Regulatory Committee for Genetic Modifications, Department of Biotechnology, Government of India). ISO 14001 guidelines of CFTRI were followed for the disposal of contaminants and transgenic wastes.

2.4.8. Statistical analysis

All the experiments were based on a randomized block design with three replications for each treatment. The data were statistically analyzed using the AGRES 7.01 software. Significant difference between the treatments was determined using Analysis of variance (ANOVA) test and the means were compared using the Duncan's New Multiple Range Test at a significance level of $P < 0.05$.

SECTION III

CLONING AND EXPRESSION OF DELTA-6 DESATURSASE GENE IN SOYBEAN

2.5. Materials

Spirulina platensis strain Sp-6, *Agrobacterium tumefaciens* strain EHA 105 and plasmid vectors pRT 100 and pCAMBIA 1305.2 maintained by Plant Cell Biotechnology Department, Central Food Technological Research Institute, Mysore was used for the experiments. Soybean genotype JS 335 obtained from Gandhi Krishi Vigyan Kendra (GKVK), University of Agricultural Sciences, Bangalore was used for transformation studies. All the enzymes, buffers and other molecular biological grade chemicals were procured from MBI Fermentas, Germany unless or otherwise mentioned.

2.6. Methodology

2.6.1. Maintenance of *Spirulina* cultures

The axenic cultures of *S. platensis* were maintained in Zarrouk's media (Zarrouk, 1996) with slight modification. Chemical composition of the medium is presented in appendix C. The pH was adjusted to 7.5 using a pH meter (Control Dynamics Digital pH meter, APX 175) prior to autoclaving at 121 ºC, for 20 min. The solid medium was prepared by gelling with 1.2% (w/v) tissue culture grade agar (Hi-media, Mumbai, India), in test tubes each containing 15 ml of the medium. The tubes and flasks were closed with cotton plugs. The slant and flasks were inoculated with *S. platensis* cultures, under aseptic conditions in laminar airflow cabinet (Airflow control systems, Bangalore, India). Axenic cultures were maintained in both solid as well as in liquid medium.

2.6.2. Isolation of delta-6 desaturase gene

Spirulina platensis strain Sp-6 was grown with continuous stirring in 2 l of Zarrouk's medium (Zarrouk, 1996) under illumination by fluorescent light at 100 $Em^{-2}s^{-1}$. The culture was grown to mid log phase (until the optical density at 560 nm reached 0.4) and the biomass was harvested by centrifuging at 10,000 rpm for 10 min in a centrifuge. Genomic DNA was isolated by using the Gen Elute Plant Genomic DNA isolation kit (Sigma, USA). The extracted DNA was tested by electrophoresing on a 0.8% agarose (Sisco Research Laboratories, Mumbai) gel using 1X Tris-acetate EDTA (TAE) buffer (Appendix B). Based on the nucleotide sequence available in the NCBI database

(Genbank accession number X87094), primers were designed for the isolation of Δ^6-desaturase (D6d) gene from *Spirulina platensis.* Three set of primers were designed with tagged restriction sites using Primer3 software (Rozen and Skaletsky, 2000) and their sequences are listed in the following Table 13.

Table 13. List of primers for amplification of D6d gene from *Spirulina platensis*

Sl. no.	Oligos name	5' — 3'	Length (bp)
1.	D6dF1	AGCAGTTATCGGCGCTTCTA	20
	D6dR1	ACTGCTCGATCGCTTTTGTT	20
2.	D6dF2	TGG*GGATCC*TTAATGACATCAACAAC (*BamHI*)	26
	D6dR2	GG*GAAG*CTTAAGGTTAGTGATGATT (*Hind III*)	25
3.	D6dF3	TGG*CTCGAG*GTTTAATGACATCAACAAC (*Xho I*)	28
	D6dR3	GGG*TCTAGA*AAGGTTAGTGATGATT (*Xba I*)	25

Of the three primers, the first set of primers was for partial amplification of the gene (to a size of ≈500 bp) and the other two primer sets was for full amplification of the gene (≈1200 bp), with different restriction sites as mentioned in the Table 13. Primers were synthesised from Sigma Aldrich, Bangalore. Polymerase chain reaction was performed using the above mentioned primers for amplification of D6d gene in a thermal cycler (Eppendorf Mastercycler personal, Germany). The PCR mixture (25 µl) contained 50 ng of *Spirulina platensis* DNA as the template, 1X PCR buffer, 2.5 mM of dNTPs and 1 unit of *Taq* DNA polymerase (MBI Fermentas), 25 pmoles of each primer (Genosys, Sigma USA). Amplification was performed with an initial denaturation at 94 ^{o}C for 4 min followed by 35 cycles of 1 min denaturation at 94 ^{o}C, 1 min annealing at different annealing temperatures and 1 min extension at 72 ^{o}C with a final extension of 72 ^{o}C for 10 min. An aliquot of 10 µl from each PCR reaction was fractionated on a 1.5% (w/v) agarose gel in Tris acetate EDTA (TAE) buffer.

2.6.3. Agarose gel electrophoresis

PCR product obtained were resolved using 1.2% agarose (Sigma-Aldrich, USA) gel at 100 V cm^{-1} using 1X TAE buffer prepared from the stock solution of 50X TAE buffer (Appendix B) with an electrophoresis unit (GeNeI, Bangalore). The size of amplicon was compared using a 3 kb DNA ladder (MBI fermentas, Germany). The gel was stained using ethidium bromide (Sigma-Aldrich, USA) solution of 0.5 µg ml^{-1} for 15

min, at room temperature. The gel was further destained in distilled water for 15 min and examined on a UV trans-illuminator and documented using Gel documentation unit (Herolab, Germany).

2.6.4. Construction of recombinant plasmid

The amplified fragment of the desired gene was purified using a Qiagen PCR purification kit (Qiagen, GmbH, Germany). The purified PCR product was subjected to double digestion with *Xho I* and *Xba I* enzymes using the following conditions (Table 14). Simultaneously the plant expression vector pRT100 (see appendix D for vector map of pRT 100), with the constitutive CaMV35S promoter and polyadenylation signal (poly A) of CaMV strain Cabb B-D with a host of restriction enzyme sites was also subjected to double digestion with the same restriction enzymes.

Table 14. Components of restriction enzyme digestion (Double digestion)

Components	Quantity (µl)	Quantity (µl)
10X tango buffer	30.0	30.0
D6d gene (PCR product)	5.0	---
Plasmid DNA (pRT 100)	---	5.0
Xho I	2.0	2.0
Xba I	3.0	3.0
dd H_2O	10.0	10.0
Total	50.0	50.0

The components were set up in ice and incubated at 37 °C in a water bath for overnight (~12 h). The digestion reaction was stopped by inactivating the enzymes at 80 °C for 20 min. The digested products were resolved in a 1.2% agarose gel and eluted from the gel matrix using Qiagen minelute gel elution kit (Qiagen, GmbH, Germany). The eluted products were stored at -20 °C for further use in ligation.

The digested plasmid vector (pRT 100) and D6d gene was ligated in a ligation reaction mixture as mentioned in Table15. To ensure optimal ligation, the ratio of vector to DNA insert was maintained at 1:3 molar ratios. The ligation reaction mixture was incubated at 16 °C for 6 h and stored at 4 °C until use.

1M DNA = DNA size (kb)*1000*average molecular weight of a nucleotide

1M plasmid DNA (pRT 100) = 3.3 * 1000 * 660

$= 2.178 * 10^6$

1M DNA insert (D6d gene) = 1.1 * 1000 * 660

$= 0.726 * 10^6$

Table 15. Components of ligation reaction

Components	Quantity (µl)
Plasmid DNA (pRT 100 @ 1 µg μl^{-1})	2
Insert DNA (D6d gene @ 1 µg μl^{-1})	2
10X ligation buffer	2
T_4 DNA ligase (2.8 Weiss units μl^{-1})	2
dd H_2O	12
Total	20

2.6.5. Transformation of recombinant pRT 100 plasmid into *Escherichia coli*

The ligated products were transformed into the chemically competent cells of *E. coli* strain DH5α and incubated in a 37 °C incubator for 12 to 16 h, for the transformed bacterial cells to grow and form colonies. Chemically competent cells of *E. coli* strain DH5α was prepared and transformed according to Sambrook and Russell, (2001), which is given in the appendix E and F, with slight modifications.

Each transformed colonies were inoculated into a 5 ml LB liquid broth (with 50 µg l^{-1} ampicillin) and incubated at 37 °C overnight with shaking at 200 rpm. Recombinant plasmids were isolated using miniprep plasmid isolation kit (Qiagen, GmbH, Germany) and were confirmed by agarose gel electrophoresis using a 0.8% agarose gel. The isolated plasmids were digested with the same restriction enzymes (*Xho I* and *Xba I*) as mentioned earlier in section 2.6.4. The digested products were run in 0.8% agarose gel and the fragments (plasmid vector and DNA insert) were verified using a 3 kb DNA ladder. The positive recombinant plasmids were outsourced for sequencing to MWG Biotech Pvt. Ltd., Bangalore, for further confirmation.

2.6.6. Multiple sequence analysis of the cloned D6d gene

The obtained sequences of the recombinant plasmid from MWG Pvt. Ltd., Bangalore were subjected to BLAST (Altschul *et al.*, 1990) using the BLAST program from the NCBI database for the confirmation of the gene and the vector backbone. Also, a restriction map for the gene sequence has been done by using NEB cutter 2.0 (Vincze *et al*., 2003). Alignment of amino acid sequences for the Δ^6-desaturase proteins was done using the program ClustalW v2 (Thompson et al, 1994) with the default settings. The whole alignment output was used to generate a phylogenetic tree based on the neighbor-joining algorithm of Saitou and Nei (1987) with the following parameters: The whole

amino acid sequence of the protein was considered, positions with gaps were not excluded and distances were not corrected for multiple substitutions. Bootstrap values over 1,000 replicates were also calculated using the same program. The resulting phenogram was drawn using the program Tree-view (Page, 1996).

2.6.7. Sub-cloning of the expression cassette into a binary vector

The term expression cassette refers to the DNA insert along with the constitutive CaMV35S promoter and polyadenylation (poly A) signal with a set of restriction enzymes attached to it. The expression cassette was isolated by digesting the recombinant plasmid with *Hind III* enzyme. Simultaneously the binary vector pCAMBIA 1305.2, maintained in *E.coli* DH5*α* strain was isolated using the miniprep plasmid isolation kit (Qiagen, GmbH, Germany). The recombinant pRT 100 and the binary vector were digested with the common enzyme *viz*., *Hind III* to release the expression cassette and to linearise the vector respectively (Table 16). The digestion conditions and the elution of digested products were carried out as mentioned under section 2.6.4.

The eluted products were stored at -20 °C for further use in ligation. The digested products were ligated in a ligation mixture of 20 µl containing 10X ligation buffer (2.0 µl); linearized pCAMBIA 1305.2 vector (7.0 µl); released gene cassette from clone 4 of pRT100 vector (7.0 µl); T_4 DNA Ligase (2.0 µl, 2.8 Weiss units μl^{-1}; TaKaRa Bio Inc., Japan) and sterile dd H_2O (2.0 µl). The ligation mixture was kept at room temperature (~25 °C) for overnight and the ligated products were transformed into *E coli* strain DH5α competent cells as described earlier under section 2.6.5.

Table 16. Components for restriction digestion of binary vector and recombinant pRT 100 vector

Components	Quantity (µl)	Quantity (µl)
10X Tango buffer	30.0	30.0
Recombinant pRT 100 vector	5.0	---
Binary plasmid pCAMBIA 1305.2	---	5.0
Hind III	2.0	2.0
dd H_2O	13.0	13.0
Total	50.0	50.0

The transformed colonies were identified using the blue-white selection (Ullmann *et al.*, 1967) by adding X-gal (40 ml of 2% w/v per plate for spreading) and IPTG (7 ml of 20% w/v per plate for spreading) onto the LB solid ampicillin plates (100 mg l^{-1}) and incubated at 37 °C for two hours before plating. Individual white colonies were selected and grown in LB broth with ampicillin (100 mg l^{-1}) for further confirmation of the recombinant clones. Isolated plasmids from the selected colonies were run on a 0.8% agarose gel and the positive clones were digested with the same restriction enzyme (*Hind III*) for the release of D6d expression cassette. The digested products along with the unligated pCAMBIA 1305.2 vector (as control) were verified in 0.8% agarose gel against a 10 kb DNA ladder and documented. The recombinant pCAMBIA 1305.2 plasmid containing the D6d gene cassette will be herein called as pCAMBIAD6d (14354 bp). Recombinant pCAMBIA1305.2 vector *viz*., pCAMBIAD6d containing the gene cassette was isolated using miniprep plasmid isolation kit (Qiagen, GmbH, Germany) from *E coli* DH5α strain and transformed into *Agrobacterium tumefaciens* strain EHA 105 by freeze thawing method as described in appendix G. The transformed white colonies were selected and confirmed for the presence of recombinant binary plasmid by SDS method as reported by Barnes, (1977) and by restriction enzyme digestion as described earlier in this section (section 2.6.7). Glycerol stocks were prepared for the confirmed colonies and stored at -70 °C for further use in plant transformation.

2.6.8. Transformation of soybean

2.6.8.1. *Agrobacterium* infection: strains and explant preparation

The experiments were performed as per the protocol described in section 2.4., with slight modifications. The *Agrobacterium tumefaciens* strain EHA 105 harbouring the recombinant binary plasmid pCAMBIAD6d having GUS as the reporter gene and hygromycin as the selection marker was used for transformation. Single bacterial colony was transferred to 5 ml of liquid LB medium with 100 mg l^{-1} kanamycin, grown overnight at 28 ºC. Overnight grown culture was used to inoculate 50 ml of liquid LB medium and kept at vigorous shaking at 28 ºC until the OD reaches 1.3-1.5 at 600 nm. Bacterial culture was centrifuged at 3,000 rpm for 10 min after which the pellet was resuspended in liquid co-cultivation medium (Table 17) to an OD_{600} of 0.5. The embryonic axis explants of soybean cv. JS 335, prepared as mentioned under section 2.2.5 were immersed in the bacterial suspension for 24 h, blotted dry in filter paper and plated on a petridish (90 mm diameter × 15 mm deep) containing solid CCM overlaid with a piece of Whatman no. 42

filter paper. The Petri dishes holding ten explants each were wrapped with paraffin film and incubated in the dark for 5 days.

2.6.8.2. Selection and screening of transformants

After five days in dark, the excess *A. tumefaciens* was briefly washed in liquid resting medium (ResM) and the explants were subsequently embedded in the semi-solid ResM for a week. Later the explants were transferred to the selection medium (SM) supplemented with 5 mg l^{-1} hygromycin B for 4 weeks with biweekly subculture into fresh medium. The differentiating tissues were transferred to fresh SM with 10 mg l^{-1} hygromycin B for further 4 weeks. The explants were sub-cultured every 2 weeks into fresh SM until the shoot buds elongate. Elongated shoots (4-6 cm) were excised and transferred to rooting medium (RtgM).

Table 17. Media components during different stages of soybean transformation with recombinant binary vector pCAMBIAD6d#

Components	Co-cultivation medium (CCM)	Resting medium (ResM)	Selection medium (SM)	Rooting medium (RtgM)
Basal medium	½ MSB_5	½ MSB_5	½ MSB_5	½ MSB_5
Sucrose	3%	3%	3%	3%
Purified agar	0.6%	0.6%	0.6%	0.6%
MES	4 g l^{-1}	0.5 g l^{-1}	0.5 g l^{-1}	0.5 g l^{-1}
L-Cysteine	1000 mg l^{-1}	1000 mg l^{-1}	-	-
BAP	6 mg l^{-1}	0.2 mg l^{-1}	0.2 mg l^{-1}	-
IBA	-	0.2 mg l^{-1}	0.2 mg l^{-1}	1.0 mg l^{-1}
Acetosyringone	100 µm	-	-	-
Cefotaxime	-	300 mg l^{-1}	300 mg l^{-1}	300 mg l^{-1}
Hygromycin	-	-	0,5,10 mg l^{-1}	10 mg l^{-1}

#MSB_5 medium contains MS macro and micronutrients (Murashige and Skoog, 1962) with B_5 vitamins (Gamborg et al., 1968)

Histochemical GUS assay using X-glucA was carried out to confirm the putative transformants which exhibits growth in the antibiotic selection medium. Well rooted transformed plants were transferred to green house and were allowed to set seeds. The transformed plants were subjected to molecular confirmation by PCR using *hptII* and D6d gene specific primers and the seeds harvested from them were analyzed for their fatty acid profile by GC and confirmed by GC-MS.

2.6.8.3. Histochemical GUS assay

GUS activity was histochemically measured by the method of Jefferson et *al*., (1987) in embryonic tips at 2 days post co-cultivation, after 5 days in resting medium and after 1 week in selection medium. Tissues were incubated for 24 hr at 37°C in a buffer consisting of 0.1 M $NaPO_4$ (pH 7.0), 0.1 M potassium ferricyanide, 0.1 M potassium ferrocyanide, 0.2 M EDTA (pH 7.0), 0.05% Triton X-100 with 500 mg l^{-1} of 5-bromo- 4-chloro-3- indolyl-β-D-glucuronide (X-gluc). Following incubation, tissues were destained with 95% ethanol as necessary and then visually observed for blue staining.

2.6.9. Analysis of transformants

2.6.9.1. Molecular confirmation of transformation using PCR

Genomic DNA was isolated from the putative transformants using Gen-elute genomic kit (Sigma, Bangalore). The genomic DNA was used for PCR analysis to confirm the presence of the T-DNA in the plant genome. Forward and reverse primers specific to hygromycin phosphotransferase (*hptII*) gene was designed using Primer3 software (Rozen and Skaletsky, 2000) and synthesized from Sigma-Aldrich, Bangalore. The primer sequences are presented in Table 18.

Table 18. List of forward and reverse primers for *hptII* and D6d gene

Oligos name	5' — 3'	Length (bp)	T_m (°C)
hptII F	GATGTTGGCGACCTCGTATT	20	50 - 55
hptII R	GTGTCACGTTGCAAGACCTG	20	
D6dF3	TGGCTCGAGGTTTAATGACATCAACAAC	28	55
D6dR3	GGGTCTAGAAAGGTTAGTGATGATT	25	

PCR analyses were performed using the primers mentioned above along with D6d gene specific primers. Amplification reactions were in volumes of 25.0 µl each containing 16.0 µl of sterilized double distilled water, 2.5 µl of 10X assay buffer with 15 mM $MgCl_2$, 1.0 µl of dNTP (2.5 mM each in the dNTP mix; to obtain 0.1 mM final concentration), 2.0 µl each of forward and reverse primer (0.08 mM each in final concentration) and 0.5 µl (~2.5 U) of *Taq* polymerase (GeNei, Bangalore, India) using 1.0 µl of the template DNA. Amplification were performed using 0.2 ml PCR tubes (Axygen Inc.,) in an Eppendorf mastercycler (Eppendorf, Germany) programmed for 30 cycles consisting of thirty seconds in 94 °C; thirty seconds in 55 °C and one minute thirty seconds in 72 °C

for denaturing, annealing and extension respectively. These cycles were preceded by a step for initial denaturation of two minutes at 94 °C and the cycles followed by a final extension of five minutes at 72 °C. Amplified products were resolved by agarose gel electrophoresis and documented.

2.6.9.2. Southern blot analysis

A portion of the hygromycin gene was amplified using plasmid DNA (pCAMBIA1305.2) as template and then purified using a Qiagen PCR purification kit (Qiagen GmpH, Germany). The fragment was labeled with psoralen-biotin labeling kit (from Ambion Inc, Texas, USA), according to the method prescribed by the manufacturer. The *hptII* labeled probe was used for the detection of T-DNA in the transformants. Aliquots of 25 µl of genomic DNA (~25 µg) from transformed and non-transformed plants were digested with *Cfr* 421 (*Sac II*) and *Xba I* enzymes separately as described by Sambrook and Russell, (2001). Digested fragments of DNA were separated on 0.8% agarose gel, transferred to positively charged nylon membrane (Brightstar^{TM} Plus, Ambion Inc, Texas, USA) and hybridized at 58 ^{0}C with biotin labeled *hptII* gene fragments. The probe hybridized fragments in the membrane was blocked with Strep-alkaline phosphates by incubation and then washed two to three times. The washed membrane was incubated in the detection buffer with NBT substrate for colour development.

2.6.9.3. Fatty acid analysis

Total lipid was extracted from matured seeds of transformed and non-transformed soybean plants by the method of Bligh and Dyer (1959). One gram of soybean seeds was homogenized with 25 ml of chloroform and methanol (2:1) and filtered. The process was repeated thrice and the filtrate was concentrated. Wash the filtrate with 2% KCl and hexane separately. The lipid fraction was recovered and stored at -20 °C for further analysis. Fatty acid methyl esters (FAME) were prepared from the lipid fraction by trans-esterification with 0.75 ml of 1N methanolic-HCl under reflux at 80 °C for 2 h, followed by the addition of 0.5 ml of 0.9% NaCl and 100 µl hexane (after cooling). After vortexing vigorously, 30 µl of the hexane fraction was transferred to a fresh tube and analyzed by GC.

2.6.9.3.1. Capillary gas-liquid chromatography (GC)

FAMEs were analyzed with a Perklin Elmer auto system XL Gas chromatograph fitted with a flame ionization detector (FID). The detector temperature was set at 240 °C. Samples (1 µl from 500 to 1000 µl) were injected at 120 °C onto a capillary column (SPB1; 50 m x 0.32 mm; i.d. 0.17 µm film thickness). Identification of peaks was based on the comparison of relative retention time data with standard FAMEs (FAME standard mix C8-C24, from SUPELCO) and confirmation by mass spectrometry.

2.6.9.3.2. Gas chromatography-mass spectrometry (GC-MS)

GC-MS was carried out on a Shimadzu, GC 17A (QP 5000) fitted with on column injection set. FAME's of the samples were injected onto a retention gap attached to an HP-5 ultra 2 bonded phase column (50 m x 0.32 mm; i.d. 0.17 µm film thickness). The mass spectra of the relevant peaks were compared to those of standards (GLA and ALA from Sigma-Aldrich, Bangalore) processed by the same equipment.

2.6.9.4. Isoflavone analysis

The total and individual isoflavones were extracted and analyzed according to Sakthivelu et al., (2008). Two grams of ground soybean seed with the seed coat was mixed with 2 ml of 0.1 N HCl and 10 ml of acetonitrile (ACN) in a 125 ml screw-top flask, stirred for 2 h at room temperature and filtered through Whatman no. 42 filter paper. The filtrate was dried under vacuum rotary evaporator at a temperature below -30 °C and then re-dissolved in 10 ml of 80% HPLC grade methanol in distilled water. The re-dissolved sample was filtered through a 0.45 µm filter unit (Cameo 13N syringe-filter, nylon) and then transferred to 1 ml vials.

The Shimadzu LC 10-AS high pressure liquid chromatograph equipped with a dual pump and a UV detector (Model SPD-10A) was used to separate, identify and quantify isoflavones. Separation of isoflavones was achieved by a Bondapak C_{18} reversed phase HPLC column (250 x 4.6 mm and 5 µm internal diameter). A linear HPLC gradient was used with solvent A (0.1% glacial acetic acid in distilled water) and solvent B (0.1% glacial acetic acid in acetonitrile). Following the injection of 20 µl of the sample, solvent B was increased from 15 to 35% for 50 min and then held at 35% for 10 min. The solvent flow rate was 1 ml/min. The wavelength of the UV detector was set at 256 nm.

Authentic standards of daidzein, genistin, genistein and glycitien were purchased from Sigma-Aldrich, Bangalore. Daidzin, glycitin, malonyl daidzin, malonyl genistin, malonyl glycitin, acetyl daidzin, acetyl genistin and acetyl glycitin were isolated and purified using the modified method of Wang and Murphy, (1994). All the 12 isoflavones

were identified by their retention times and by co-chromatography with standard compounds and the individual concentrations were calculated based on each peak area.

2.6.10. Biosafety measures

All the work that involves manipulation of biological materials was carried out according to the IBSC guidelines of the Institute (Institutional Bio-safety Committee, CFTRI). Transgenic work was carried out with permission from RCGM (Regulatory Committee for Genetic Modifications, Department of Biotechnology, Govt. of India) and ISO 14001 guidelines of the institute was followed for the disposal of contaminants and transgenic wastes.

2.6.11. Statistical analysis

All the experiments were based on a randomized block design with three replications for each treatment. Significance between the control and treatments for all the experiments in this study were tested using the one way ANOVA (Analysis of variance) test and values are presented in the tables with its mean separation either by LSD or by student's t-test. AGRES 7.01 software was used for all the statistical analysis.

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Results & Discussion

SECTION I

IN VITRO REGENERATION OF SOYBEAN

3.1. Background

In vitro techniques are important tools for plant improvement and the ability to regenerate fertile plants is crucial to the successful application of *in vitro* methods with regard to plant breeding and genetic modification (Fratini and Ruiz, 2003). The primary aim of *in vitro* regeneration is to produce as easily and as quickly as possible, a large number of regenerable cells that is accessible to gene transfer. The subsequent regeneration step is often the most difficult step in plant transformation studies. The improvement of soybean using genetic engineering necessitates the availability of a rapid and efficient regeneration system. The two major routes of soybean regeneration are somatic embryogenesis (Bailey et al., 1993; Kita et al., 2007) and shoot organogenesis (Hu and Wang, 1999; Franklin et al., 2004). Christianson et al., (1983) published the first report on successful soybean regeneration in which several somatic embryoids were produced. Somatic embryogenesis is an efficient system to produce genetically modified plants, however, it is genotype specific, requires long period of culture maintenance and is accompanied with a high level of somaclonal variation in the regenerated plants (Parrott et al., 1989; Finer and McMullen, 1991).

Organogenesis is less genotype dependent and has become routine *in vitro* technique for regeneration of transformed plants (Wright et al., 1986, 1987a, 1987b; Dan and Reichert, 1998). Adventitious soybean shoots have been induced from cotyledonary nodes (Barwale et al., 1986; Wright et al., 1986), primary leaves (Wright et al., 1987a) and epicotyls (Wright et al., 1987b), hypocotyl sections (Dan and Reichert, 1998; Reichert et al., 2003) and embryonic tips (Liu et al., 2004). Hypocotyls have a low regeneration frequency, genotype dependent (Dan and Reichert, 1998) and have not been combined successfully with genetic modification. Regeneration from cotyledons and embryonic axis has been combined successfully with genetic modification (Hinchee et al., 1988; Yan et al., 2000; Olhoft and Somers, 2001; Olhoft et al., 2003; Ko et al., 2003; Liu et al., 2004). The regeneration is based on the proliferation of meristems in both cotyledonary node and embryonic axis. However, the frequency of plant regeneration reported so far does not seem to be high enough to be applied to soybean transformation. The recovery of transgenic plants capable of transmitting the target genes in the

successive generation is also very low (Christou et al., 1990). Hence, improvement in the regeneration frequency would contribute to an increase in the production of transgenic soybeans. Shan et al., (2005) suggested that the efficiency of the organogenic system for genetic modification can be improved if the number of shoots per explant is increased or if the number of meristematic cells in explants is increased.

Thidiazuron (TDZ; N –phenyl, N 1, 2, 3 -thidiazol-5-yl urea) is a substituted phenyl-urea compound which was developed for mechanized harvesting of cotton bolls, has now emerged as a highly efficacious bio-regulant of morphogenesis in the tissue culture of many plant species (Tzitzikas et al., 2004). TDZ induces a diverse array of *in vitro* responses ranging from induction of callus to formation of somatic embryos. TDZ exhibits the unique property of mimicking both auxin and cytokinin effects on growth and differentiation of cultured explants although structurally it is different from either auxin or purine based cytokinins. A number of physiological and biochemical events in cells are likely to be influenced by TDZ, but these may or may not be directly related to the induction of morphogenic responses and hence the mode of action of TDZ is unknown. Various reports indicate that TDZ may act through modulation of the endogenous plant growth regulations, either directly or as a result of induced stress. The other possibilities include the modification in cell membranes, energy levels, nutrient uptake and nutrient assimilation. The positive influence of pretreatment of seeds with TDZ or BAP on regeneration of shoots has been reported in soybean (Wright et al., 1986; Shan et al., 2005) as well as in many other legumes such as pea (Tzitzikas et al., 2004), lentil (Malik and Saxena, 1992) and Azuki bean (Mohamed et al., 2006). In lentil Malik and Saxena, (1992) observed that pretreatment of seeds with TDZ saves time and results in more number of shoots compared to the initiation of shoots from isolated organs such as hypocotyls or cotyledonary nodes. The influence of salt concentrations of basal media and thidiazuron on the frequency of shoot organogenesis was reported by Kaneda et al., (1997) in soybean.

Triacontanol (TRIA) a 30 carbon primary alcohol, is a naturally occurring plant growth promoter (Ries and Houtz, 1983) and has been shown to increase the growth and yield of plants *in vivo* as well as *in vitro* (Stoutemyer and Cook, 1987; Ma et al., 1990; Tantos et al., 1999). TRIA is a component of the epicuticular waxes of alfalfa and many other plants (Chibnall et al., 1993; Azam et al., 1997). The plant growth stimulating property of TRIA was demonstrated for the first time by Crosby and Vlitos, (1959) and later by Stoutemyer, (1981) and Stoutemyer and Cook, (1987). There are many reports on

enhancement of crop yield by TRIA. Knight and Mitchell, (1987) reported the stimulating productivity of hydroponic lettuce in controlled environment with TRIA. Mahadevappa et al., (1989) studied the effect of TRIA on rice seedling weight and grain yield effect. Balyan et al., (1994) investigated the TRIA effect on growth and yield parameters of CKP-25 variety of lemon grass and found that plant height, number of leaves and leaf length were increased by 21% over control. TRIA has been shown to increase seed weight, chlorophyll content of leaves, enhance photosynthesis, branching, shoot length and rooting (Ries and Wert, 1988; Srivastava and Sharma, 1990; Kulandaivelu, 1996; Tantos et al., 1999). Ma et al., (1990) found that TRIA had a significant stimulating effect on the *in vitro* growth of the plant, *Malus domestica* CV 'Fuji'. Tantos et al., (1999) studied the effect of TRIA on *Melissa officinalis* and found that it supports shoot multiplication at 5 µg l^{-1} and rooting at 2 µg l^{-1}. Reddy et al., (2002) for the first time reported the effect of TRIA on micropropagation of *Capsicum frutescens* and *Decalepis hamiltonii*. Giridhar et al., (2004) reported the influence of TRIA on direct somatic embryogenesis from cultured leaf and stem tissues of *Coffea arabica* and *Coffea canephora*. The use of TDZ and Triacontanol has not been well documented in soybean. Hence in the present study the effects of both the growth regulators on *in vitro* regeneration of soybean were examined and the results were discussed in detail in this section.

3.2. Results

3.2.1. Effect of cytokinin and explants on *in vitro* regeneration

Adventitious shoot bud tissues were directly induced from cotyledonary nodes and from upper sections of the hypocotyl segments by both TDZ and BAP. Within 15 days, the explants bulged and formed deep green, shiny organogenic shoot bud tissues, which in turn, differentiated, into numerous shoot buds in 4 weeks (Figure 6). In contrast, hypocotyls took a longer time (6 weeks) for organogenic shoot bud formation and differentiation (Figure 7).

The percentage of explants forming shoot buds after 6 weeks of culture is presented in Table 19. The frequency of shoot bud formation was influenced by both the type of explant and the choice of cytokinin. Significant difference between and within the growth regulator concentrations were observed for both TDZ and BAP during shoot bud induction. Maximum frequency of shoot bud tissues was noticed in the MS basal

medium supplemented with 0.2 mg l^{-1} and 2.0 mg l^{-1} of TDZ for cotyledonary nodes and hypocotyls respectively, with cotyledonary nodes (68.3%) recording more response than hypocotyl (56.3%) for the soybean cv. Hardee. In case of BAP, the frequency of adventitious shoot bud formation was 63.7 and 45.7% for cotyledonary node and hypocotyls respectively (Table 19) for the cultivar JS 335. The induction of shoot bud tissues from cotyledonary nodal segments decreased with increasing concentration of TDZ whereas it was the reverse in case of hypocotyl explants in both the cultivars. Irrespective of the cultivars, the combination of TDZ and cotyledonary nodal segments induced shoot bud tissues most efficiently than the other combinations. No significant genotypic difference was observed between the soybean cultivars for shoot bud induction with TDZ and BAP.

Table 19. Response of explants on MS basal medium with TDZ and BAP

Plant growth regulators [mg l^{-1}]	Shoot bud formation (%)[#]			
	Hardee		JS 335	
	Cotyledonary node	Hypocotyl	Cotyledonary node	Hypocotyl
TDZ				
0.2	68.3^{a}	23.7^{d}	64.7^{a}	20.0^{d}
0.5	60.0^{b}	30.3^{cd}	56.3^{b}	31.7^{bc}
1.0	47.7^{c}	40.0^{bc}	48.0^{bc}	38.3^{b}
2.0	44.3^{cd}	56.3^{a}	40.0^{cd}	50.0^{a}
BAP				
0.5	51.7^{c}	37.7^{bc}	44.0^{c}	40.0^{b}
1.0	60.3^{b}	42.0^{bc}	63.7^{a}	45.7^{ab}

[#]Mean values with different alphabets are statistically significant at $P < 0.05$. Data recorded after 6 weeks of inoculation.

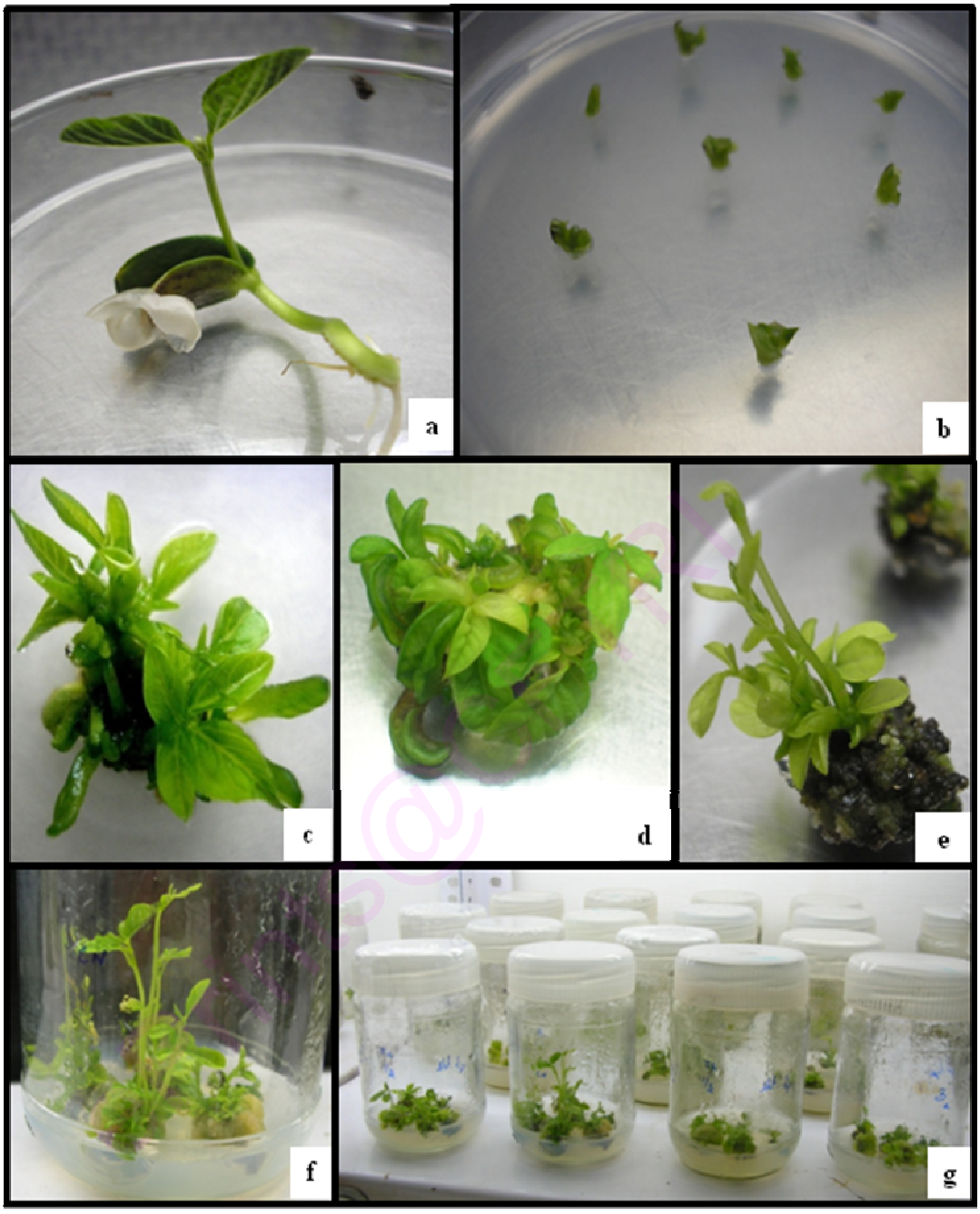


Figure 6. Effect of TDZ on i*n vitro* regeneration of soybean from cotyledonary nodal explants; (a) Soybean seedling (two weeks old); (b) Cotyledonary nodal explants; (c & d) adventitious shoots from cotyledonary nodal explants of soybean cv. Hardee; (e & f) adventitious shoots from cotyledonary nodal explants of soybean cv. JS 335; and (g) micropropagation of soybean cv. JS 335 in tissue culture bottles on MS basal medium supplemented with 0.2 mg l^{-1} BAP.

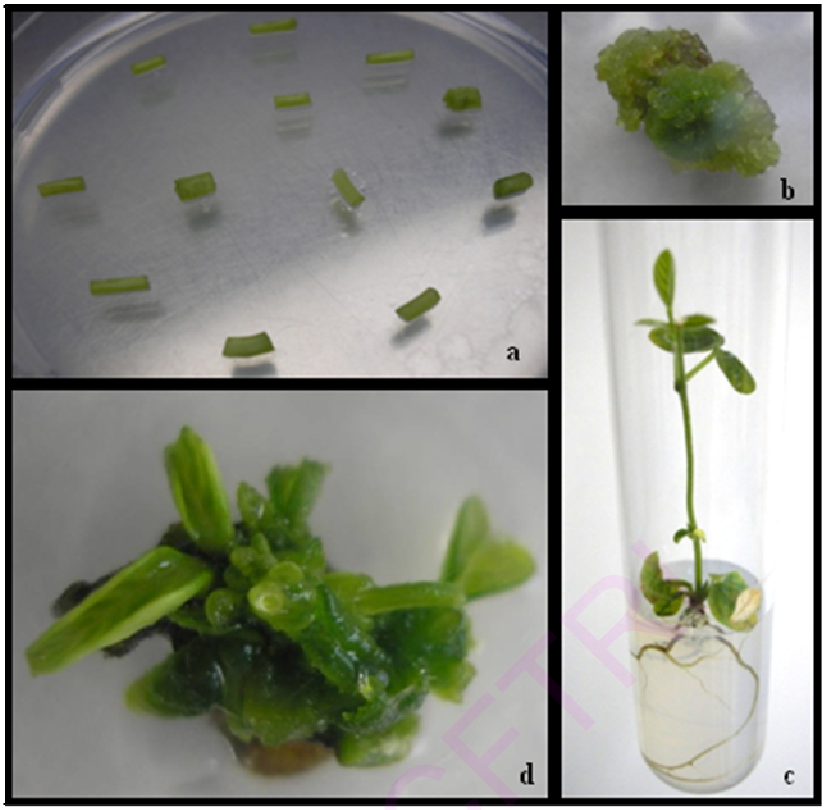


Figure 7. Effect of TDZ on i*n vitro* regeneration of soybean from hypocotyl explants; (a) hypocotyl explants; (b & d) adventitious shoots from hypocotyl explants on medium containing 0.2 mg l^{-1} BAP; and (c) rooting of plantlets in MS basal medium containing 0.5 mg l^{-1} IBA.

Table 20. Effect of GA_3 and BAP on shoot bud elongation*

Plant growth regulators [mg l^{-1}]	Hardee		JS 335	
	No. of shoots/ explant	Shoot length [cm]	No. of shoots/ explant	Shoot length [cm]
GA_3				
0.1	3.6 ± 0.8^{cd}	3.44 ± 0.86^{d}	3.0 ± 0.7^{cd}	4.14 ± 0.26^{cd}
0.2	5.2 ± 1.4^{b}	4.96 ± 0.30^{bc}	4.4 ± 1.5^{b}	4.84 ± 0.29^{c}
0.5	6.2 ± 1.1^{ab}	5.24 ± 0.62^{b}	5.4 ± 0.8^{a}	5.72 ± 0.62^{b}
1.0	4.3 ± 1.5^{c}	6.24 ± 0.42^{a}	3.6 ± 1.1^{c}	6.56 ± 0.38^{a}
BAP				
0.2	6.6 ± 1.1^{a}	5.04 ± 0.52^{bc}	5.4 ± 1.6^{a}	4.68 ± 0.55^{c}
0.5	5.0 ± 1.5^{bc}	4.66 ± 0.69^{c}	3.8 ± 1.3^{bc}	4.00 ± 0.38^{cd}

* Values are mean ± SD; Mean values with different alphabets are statistically significant at $P < 0.05$. Data recorded after 8 weeks in shoot elongation medium.

The shoot bud tissues were sub-cultured in elongation medium containing MS basal medium with varying concentration of GA_3 (0.1, 0.2, 0.5, 1.0 mg l^{-1}) and BAP (0.2 and 0.5 mg l^{-1}). After 8 weeks of culture, the number of elongated shoots were recorded and the same was presented in Table 20. Significant difference was observed for number of shoots and shoot length between the growth regulator concentrations of both TDZ and BAP during shoot bud elongation. Elongation of these shoot buds was best achieved in 0.5 mg l^{-1} GA_3 and 0.2 mg l^{-1} BAP, compared to other concentrations (Figure 6c, e). On medium containing 0.5 mg l^{-1} GA_3, the shoot buds showed good elongation with an average shoot number of 6.2 and 5.4 for the cultivar Hardee and JS 335 respectively (Figure 8a). The number of shoots increased with increase in the concentration of GA_3 upto 0.5 mg l^{-1} and slightly decreased at the higher concentration of 1 mg l^{-1}. BAP also induced equal number of shoots (6.6 and 5.4 respectively for Hardee and JS 335) at lower concentration of 0.2 mg l^{-1} compared to GA_3 at 0.5 mg l^{-1}. The shoot length was maximum at higher concentration of GA_3 (1 mg l^{-1}) and showed positive correlation with GA_3 concentration in both the cultivars (Table 20). Significant difference was observed for shoot length at higher concentrations of GA_3 and BAP, with the former recording maximum shoot length. The regenerated shoots were rooted on MS basal medium with IBA (0.5 mg l^{-1}), for a period of 4 weeks (Figure 8b). All the *in vitro* rooted plants survived the transfer to greenhouse and within a period of 8 weeks, they flowered and subsequently formed fertile seeds (Figure 8c, d).

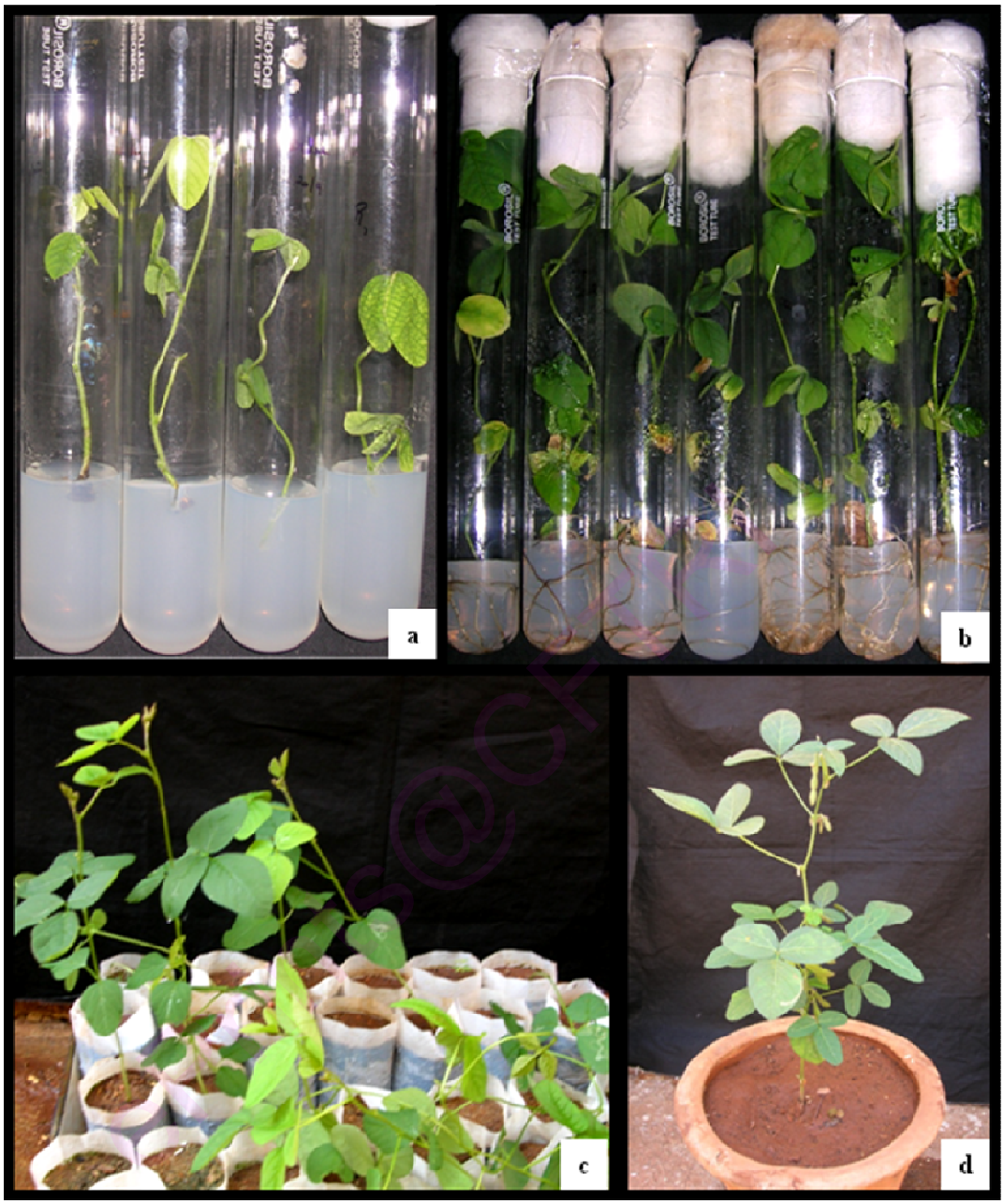


Figure 8. Rooting of *in vitro* grown soybean plantlets; (a) elongated soybean plantlets (cv. Hardee) in MS basal medium with 0.5 mg l^{-1} GA_3; (b) rooted soybean plantlets on MS medium with 0.5 mg l^{-1} IBA; (c) acclimatization of rooted plantlets of soybean cv. JS 335 in polybags; and (d) fully grown *in vitro* plant of soybean cv. Hardee with pods.

3.2.2. Effect of Triacontanol (TRIA) on *in vitro* regeneration

The effect of TRIA was evaluated in both the multiplication and rooting phase of soybean *in vitro* organogenesis. After 4 weeks of culture, the percentage of response (the ratio of number of explants cultured to the number of explants responded expressed in %), number of shoots per explant and shoot length were measured in the multiplication phase and the same was presented in Table 21 and 22.

Table 21. Influence of TRIA and IBA on adventitious shoot formation from cotyledonary node and shoot tip explants#

Explants	MSB+BAP (mg l^{-1})	TRIA (µg l^{-1})	IBA (mg l^{-1})	% of response	No. of shoots per explant*	Shoot length (cm)*
Cotyledonary node	0.2	-	-	50.0^{d}	4.0 ± 0.7^{cd}	0.60 ± 0.5^{d}
	0.2	2	-	75.0^{bc}	8.5 ± 0.7^{ab}	1.33 ± 0.5^{cd}
	0.2	5	-	100.0^{a}	9.3 ± 0.5^{a}	2.33 ± 0.5^{bc}
	0.2	10	-	83.3^{b}	6.3 ± 0.7^{c}	2.16 ± 0.2^{bc}
	0.2	2	0.2	75.0^{bc}	4.3 ± 1.2^{cd}	1.83 ± 0.7^{c}
	0.2	5	0.2	83.3^{b}	7.0 ± 0.5^{b}	3.83 ± 0.7^{a}
	0.2	10	0.2	100.0^{a}	3.5 ± 0.7^{cd}	2.50 ± 0.3^{bc}
Shoot tips	0.2	-	-	30.0d	2.0 ± 1.0^{d}	0.80 ± 0.4^{cd}
	0.2	2	-	50.0c	3.0 ± 1.0^{cd}	1.62 ± 0.6^{c}
	0.2	5	-	80.0a	5.3 ± 0.5^{b}	4.16 ± 0.7^{a}
	0.2	10	-	60.0b	3.6 ± 0.5^{c}	2.75 ± 0.3^{b}
	0.2	2	0.2	40.0cd	3.3 ± 1.2^{c}	3.50 ± 0.5^{ab}
	0.2	5	0.2	66.6b	6.8 ± 0.5^{a}	4.50 ± 0.5^{a}
	0.2	10	0.2	50.0c	2.7 ± 0.7^{cd}	1.25 ± 0.3^{cd}

* mean value ± SD; # mean values with different alphabets are statistically significant at $P < 0.05$. Data was recorded after 4 weeks in the triacontanol media.

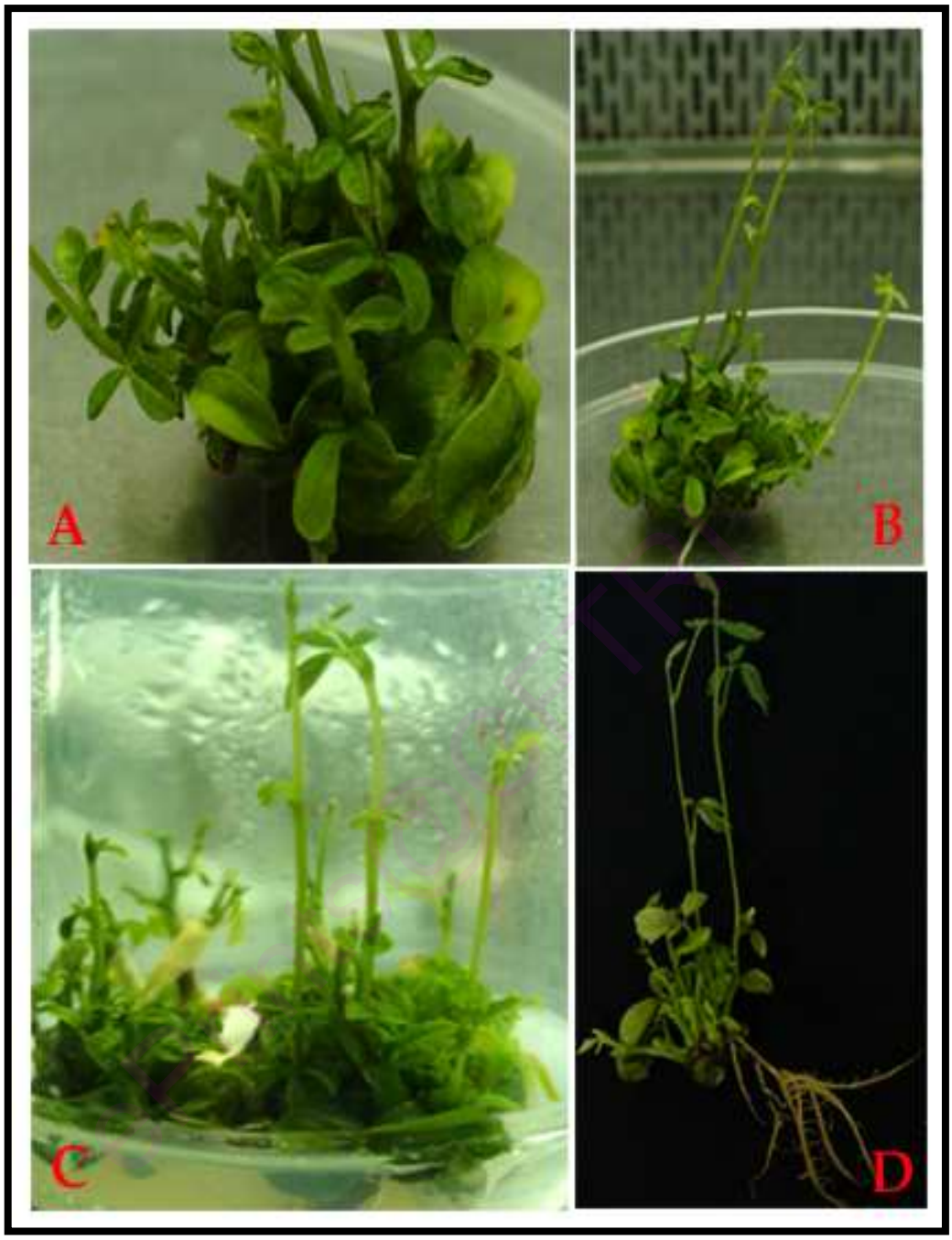


Figure 9. Effect of TRIA on *in vitro* shoot regeneration of soybean (cv. JS 335) from cotyledonary nodal explants; (A) Shoot multiplication in 0.2 mg l^{-1} BAP and 5.0 µg l^{-1} TRIA; (B & C) Shoot induction in 50 µg l^{-1} GA_3, 0.2 mg l^{-1} IBA and 5.0 µg l^{-1} TRIA; and (D) *In vitro* rooting of soybean plantlets in 5.0 µg l^{-1} TRIA.

In the multiplication phase, maximum response was obtained in the media containing 5 μg l^{-1} TRIA for both cotyledonary node (Figure 9) and shoot tip explants (Figure 10), with the former recording more response than the latter. As shown in Table 21and 22, even the lowest concentration of TRIA (2 μg l^{-1}) resulted in an increase in the number of shoots and shoot length of the explants. Significant increase in the number of shoots and shoot length was observed at lower concentration of TRIA (2, 5 μg l^{-1}) whereas higher TRIA concentration (10 μg l^{-1}) did not show any significant difference compared to the control. TRIA at 5 μg l^{-1} along with 0.2 mg l^{-1} BAP recorded the maximum number of shoots (9.3 and 5.3) and shoot length (2.33 and 4.16 cm) for cotyledonary node and shoot tip explants respectively. Addition of IBA (0.2 mg l^{-1}) to the tested concentrations of TRIA reduced shoot number and increased shoot length in case of cotyledonary nodal explants whereas, it significantly increased the number of shoots (6.8) and shoot length (4.5) in case of shoot tip explants.

Table 22. Influence of TRIA and GA_3 on adventitious shoot induction from cotyledonary node and shoot tip explants#

Explants	MSB+GA_3 (μg l^{-1})	IBA (mg l^{-1})	TRIA (μg l^{-1})	% of response	No. of shoots per explant*	Shoot length (cm)*
Cotyledonary node	50	0.2	-	30^{d}	4.2 ± 0.7^{d}	2.0 ± 0.5^{d}
	50	0.2	2	75^{ab}	9.3 ± 1.2^{bc}	3.2 ± 0.3^{cd}
	50	0.2	5	90^{a}	14.0 ± 0.5^{a}	4.6 ± 0.3^{c}
	50	0.2	10	65^{b}	8.5 ± 0.7^{bc}	2.5 ± 0.5^{d}
Shoot tips	50	0.2	-	40^{cd}	6.2 ± 0.7^{cd}	3.0 ± 0.5^{cd}
	50	0.2	2	54^{bc}	7.3 ± 1.2^{c}	6.3 ± 0.7^{bc}
	50	0.2	5	80^{ab}	9.0 ± 0.5^{bc}	10.0 ± 0.7^{a}
	50	0.2	10	50^{c}	6.5 ± 0.7^{d}	4.5 ± 0.3^{c}

*mean values ± SD; # mean values with different alphabets are significantly different at $P < 0.05$. Data was recorded after 4 weeks in the triacontanol media.

Similarly addition of GA_3 (50 μg l^{-1}) to the tested concentration of TRIA and IBA, resulted in 90% response for cotyledonary node and 80% for shoot tip explants (Table 22). Both the explants showed significant difference for number of shoots and shoot length for the TRIA concentrations compared with the control. Cotyledonary nodes recorded a maximum of 14 shoots/ explant with an average shoot length of 4.6 cm (Figure

10), whereas shoot tip explants recorded an average of 9 shoots/ explant with an average length of 10 cm (Figure 10b, c). The shoot length almost doubled with the addition of GA_3 along with TRIA and IBA in case of cotyledonary nodal explants, whereas the shoot tips exhibited a triple fold increase in shoot length, compared to the control.

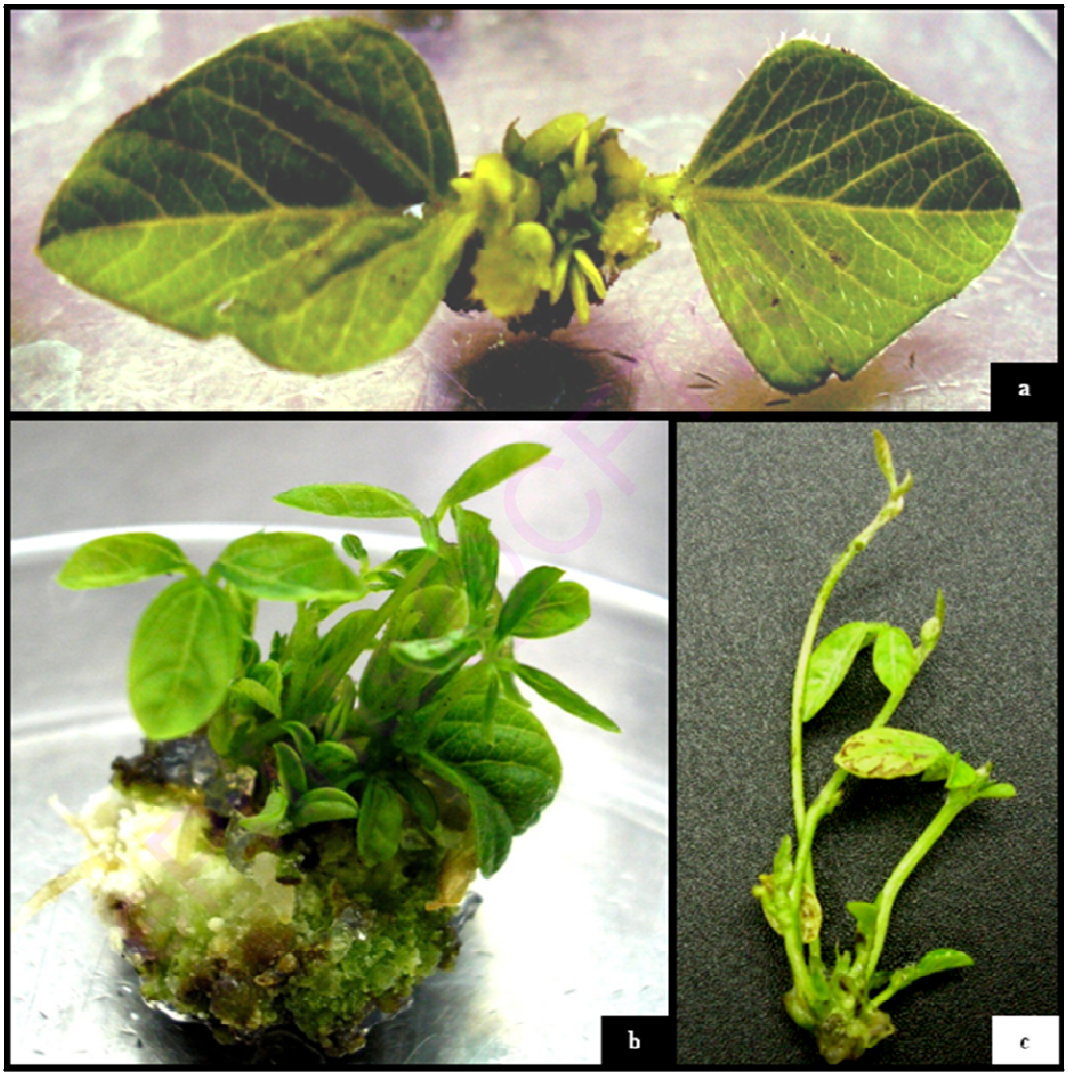


Figure 10. Effect of TRIA on *in vitro* shoot regeneration of shoot tip explants of soybean cv. JS 335; (a) adventitious shoot induction in 0.2 mg l^{-1} BAP and 5.0 µg l^{-1} TRIA; (b) shoot induction and elongation in 0.2 mg l^{-1} IBA, 5.0 µg l^{-1} TRIA and 50 µg l^{-1} GA_3; and (c) elongated soybean shoots in MS medium with 5.0 µg l^{-1} TRIA.

In the root induction phase, the parameters including root number, root length, node number, shoot length and fresh weight were recorded after 4 weeks and are presented in Figure 11 and 12. As shown in Figure 11, the number of roots and root length significantly increased with 2 and 5 µg l^{-1} triacontanol and decreased at higher concentration of 10 µg l^{-1}. Although triacontanol was found to be less effective at higher concentrations, it still induced more number of roots with increased root length than the control. Shoot growth was also evaluated in the rooting phase and similar pattern was found in both shoot length and number of nodes after triacontanol treatment (Figure 12). Triacontanol positively influenced shoot growth and increased the number of nodes, with reduced length of internodes. The maximum shoot length was recorded at 5 µg l^{-1} concentration, whereas the highest number of nodes was found at 10 µg l^{-1} with slightly reduced shoot length. The fresh weight of the plantlets was significantly higher at all concentrations of triacontanol than that of the control.

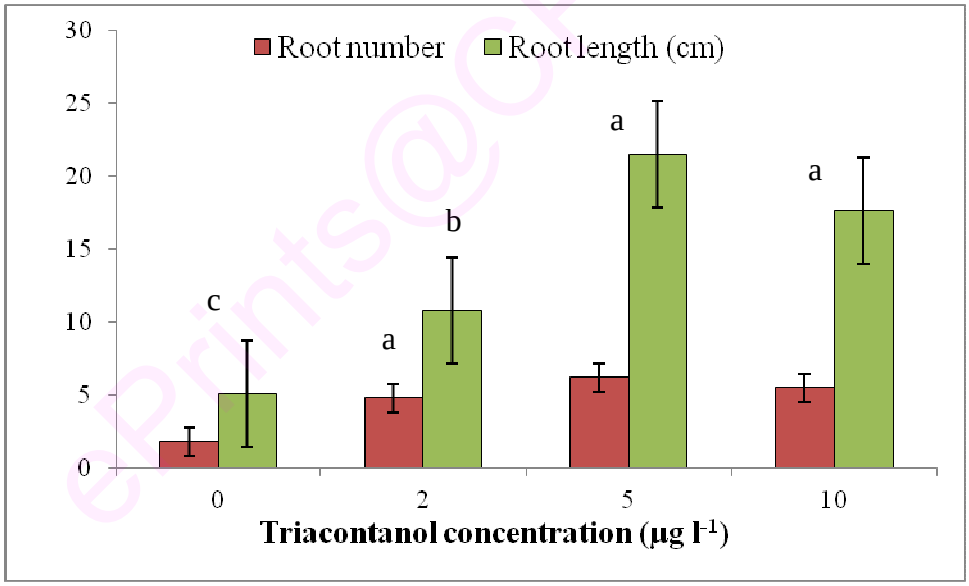


Figure 11. Effect of TRIA added during the root induction phase on number of roots and root length; values with different alphabets are significantly different at $P < 0.05$. Data was recorded after 4 weeks in the triacontanol media.

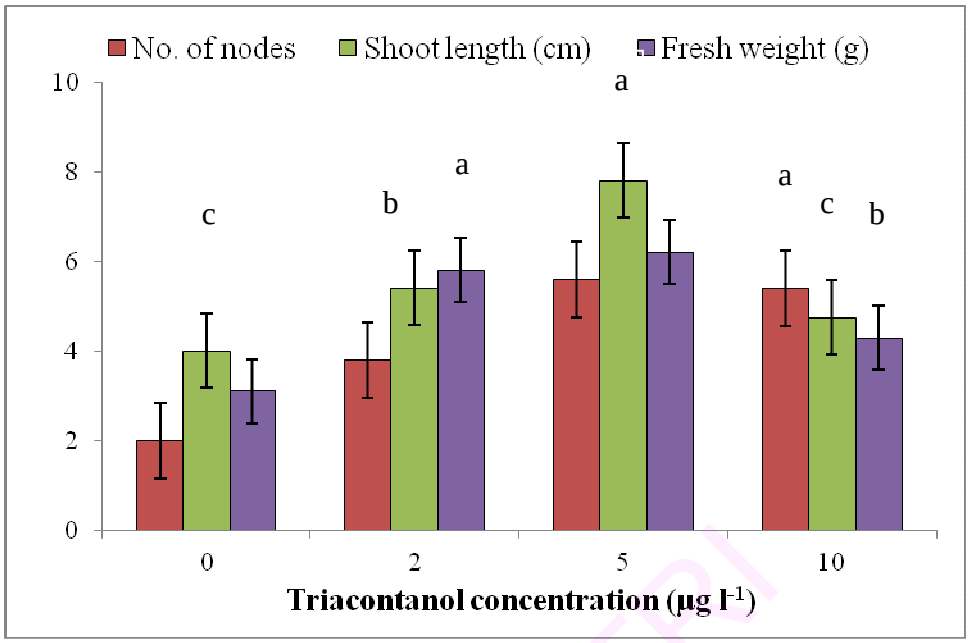


Figure 12. Effect of TRIA added during the root induction phase on number of nodes, shoot length and fresh weight; values with different alphabets are significantly different at $P < 0.05$. Data was recorded after 4 weeks in the triacontanol media.

3.3. Discussion

High frequency of regeneration is vital for efficient transformation and hence we first evaluated the different explants for their regeneration frequencies with selected growth regulator combinations. Several studies on soybean regeneration have shown the importance of specific plant growth regulators in determination of the morphogenic responses according to cultivar and tissue type. To date, BAP has been the most commonly tested cytokinin in soybean, while there have been few reports on the effects of TDZ on shoot regeneration from cotyledonary nodes and hypocotyl of few soybean genotypes (Passey et al., 2003; Shan et al., 2005).

3.3.1. Effect of cytokinins and explants on *in vitro* regeneration

The type of explant and the choice of growth regulator influenced the frequency of shoot bud formation and subsequent shoot regeneration. TDZ concentration in the medium, influenced shoot bud tissue formation differently in the soybean explants. The shoot bud formation and TDZ concentration was directly proportional in case of hypocotyl explants whereas it is inversely proportional in case of cotyledonary nodal explants in both the genotypes of soybean (Table 19). Kaneda et al., (1997) reported that frequency of shoot regeneration was reduced at lower concentration (2.24 μM) and inhibited at higher concentration (18.16 μM) of TDZ. Similar results on TDZ morphogenesis was earlier reported by Malik and Saxena, (1992) in common bean, and Gill and Saxena, (1992) in peanut. Collectively, these results suggest that the response of explants to shoot bud formation is dependent on growth regulator concentration and/or genotype. Earlier reports by Mok et al., (1982) and Thomas and Katterman, (1986) suggested that the high morphogenic activity of this substituted phenyl urea compound is due to its cytokinin like activity. It is plausible that TDZ has a higher cytokinin activity or a different action than other cytokinins influencing differentiation.

Apart from its cytokinin like activity, TDZ has been suggested to be a modulator of the endogenous hormone levels. Experimental evidence points out that TDZ stimulates *de novo* synthesis of auxins by increasing the levels of indole 3-acetic acid (IAA) and its precursor, tryptophan (Murthy et al., 1995). Increase in endogenous auxin, cytokinin and ethylene have been seen in response to TDZ treatment (Murthy et al., 1995; Yip andYang, 1986). As a consequence, TDZ has been shown to be useful for rapid plant regeneration in several species through organogenesis (Malik and Saxena, 1992) or somatic embryogenesis (Zhou et al., 1994). The above mentioned activities of TDZ to

plant cells could explain the high frequency of shoot regeneration observed in the present study. Also in our experiments, the shoots induced by TDZ often showed stunting, as has been reported in other plant species such as apple and *Rhododendron* (Van et al., 1986). The inhibition of shoot elongation may be due to the high cytokinin activity of TDZ. Cytokinins are commonly known to stimulate shoot proliferation while inhibiting their elongation (Huetteman and Preece, 1993).

In the above mentioned *in vitro* regeneration system, only the cotyledonary nodes with axillary buds gave 100% regeneration and produced shoot buds whereas those without axillary buds produced only excess callus. Wright et al., (1986) and Carmen et al., (2001) showed histologically that exogenously applied cytokinins altered the development of axillary meristems, promoted proliferation of the meristematic cells in the axillary buds and increased the number of bud primordia which originated from the existing axillary meristems. Shan et al., (2005) suggested that the structural integrity of axillary meristem might contribute to high efficiency of regeneration in soybean when pretreated with TDZ. Malik and Saxena (1992) hypothesized that the high level of regeneration through TDZ or BAP treatment of pea and common bean seeds was due to the morphological integrity of the seedlings. In our system, the shoots were regenerated from shoot buds, whose multiplication can be viewed as the proliferation of meristematic cells in the existing axillary buds.

3.3.2. Effect of Triacontanol (TRIA) on *in vitro* regeneration

At low concentrations, triacontanol promoted both shoot growth and rooting of plants, but at high concentrations it had a slight inhibitory effect. This may be due to the sensitivity of whole explants to extremely low doses of TRIA (Biernbaum et al., 1988). Corresponding data published in the literature also indicate that triacontanol has an inhibitory effect above certain concentrations (Somen and Seetalakshmi, 1991; Tantos et al., 1999; Reddy et al., 2002). The explants on medium containing 5 μg l^{-1} triacontanol had the most number of shoots with maximum shoot length. TRIA enhanced the shoot length and number of nodes with a reduced length of internodes. As reported by Hangarter et al., (1978), the increased growth caused by TRIA is not simply caused by water uptake and cell enlargement but rather by an increase in cell number. After initial application of TRIA, a metabolite of TRIA or a secondary messenger moves rapidly in plants and influences enzymes relating to carbohydrate metabolism (Ries and Houtz, 1983) and growth processes (Ries and Wert, 1988), which might be responsible for the high growth activity in plants.

The present work shows the stimulating effect of TRIA on shoot multiplication and rooting at very low concentrations, which clearly indicates that TRIA can be effectively used as a growth regulator for *in vitro* regeneration of soybean. This is in contrast with the results reported by Yun and Kim, (1986), who found no significant activity of this compound in *Oryza sativa* regeneration, but in agreement with the reports by Ma et al., (1990) in *Malus domestica* tissue culture, Tantos et al., (1999) in regeneration of *Melissa officinalis* and Reddy et al., (2002) in micropropagation of *Capsicum* and *Decalepis.*

3.4. Summary

Soybean genotypes (Hardee and JS 335) were subjected to *in vitro* regeneration with different plant hormones *viz*., TDZ, BAP and GA_3 against the explants hypocotyl and cotyledonary node. Maximum frequency of shoot buds was obtained in MS basal medium supplemented with 0.2 mg l^{-1} and 2.0 mg l^{-1} of TDZ for cotyledonary nodes and hypocotyls respectively, with cotyledonary nodes (68.3%) recording more response than hypocotyl (56.3%) for the soybean cv. Hardee. The type of explant and the choice of growth regulator influenced the frequency of shoot bud formation and subsequent shoot regeneration. Elongation of these shoot buds was best achieved in 0.5 mg l^{-1} GA_3 and 0.2 mg l^{-1} BAP with a maximum shoot length of 5.7 cm. Triacontanol at low concentration of 5 µg l^{-1} significantly increased the frequency of shoot buds for both cotyledonary node and shoot tip explants compared to the control. A maximum of 14 shoots/ explant was obtained from the cotyledonary nodal explants on media containing 50 µg l^{-1} GA_3, 5 µg l^{-1} TRIA and 0.2 mg l^{-1} IBA with an average shoot length of 4.6 cm. The results indicate that triacontanol can be successfully used as a growth regulator in both the multiplication and rooting phase of soybean micropropagation.

SECTION II

AGROBACTERIUM MEDIATED TRANSFORMATION OF SOYBEAN

3.5. Background

Developing an efficient transformation system should be a prerequisite for both improvement of cultivar quality and investigation of the functional genes in soybean (Liu et al., 2008). According to the United Soybean Board Production Committee's Soybean Genomics white paper, soybean transformation efficiency must be improved 5 to 10 fold so that one can produce upto 300 transgenic lines /year to meet the existing soybean genomics initiatives. Olhoft et al., (2003) stated that an ideal transformation method for soybean should (i) be simple, inexpensive and rapid; (ii) be successful with a wide range of cultivars, *Agrobacterium* strains and binary plasmids and (iii) provide efficient selection without production of non-transformed or chimeric plants. Transgenic soybeans are produced by different DNA delivery methods and plant tissues, including microprojectile bombardment of shoot meistems (McCabe et al., 1988) and embryogenic suspension cultures (Finer and McMullen, 1991) and *Agrobacterium* mediated T-DNA delivery into immature cotyledons (Parrott et al., 1989; Yan et al., 2000), embryogenic suspension cultures (Trick and Finer, 1998) and axillary meristematic tissue located in the seedling cotyledonary nodes (Hinchee et al., 1988). Among them the cotyledonary node based *Agrobacterium* mediated transformation has been a successful method with lot of improvements. However the production of transgenic plants remains low most likely due to (i) inefficient T-DNA delivery from *A. tumefaciens* into cells in the cotyledonary node region, (ii) inefficient selection of proliferating transgenic cells and regenerated shoots and (iii) difficulty with plant regeneration.

Various efforts have been made to overcome the above mentioned drawbacks. Olhoft and Somers, (2001) reported that the addition of thiol compounds during co-cultivation significantly increased the *A. tumefaciens* infection and T-DNA transfer into cotyledonary node cells. The effectiveness of tissue culture selection regimes is dependent on many factors including tissue type, size of explant, chemical properties and concentration of selective agent and the time of application (Bowen, 1993). Incomplete selection of transformed cells is reflected in some selection systems by the high percentage of escapes (Hinchee et al., 1988) or the production of chimeric plants (Di et al., 1996). Olhoft and Somers, (2001) observed 90-95% non-transgenic escapes despite

the presence of high levels of glufosinate as selective agent. Later Olhoft et al., 2003 developed an efficient hygromycin B based selection system for rapid selection of transgenic shoots with a very low frequency of escapes. However Zeng et al., (2004) suggested that the efficacy of transformant selection with hygromycin B might be genotype dependent. With all these improvements the cotyledonary node based regeneration system is still relatively inefficient, complicated and time consuming (Barwale et al., 1986; Shetty et al., 1992). Therefore, it will be interesting to test other explants like embryonic axis for their regeneration and transformation efficiency and also to examine some key parameters which have the potential to promote transformation that would be helpful for further optimization of soybean transformation system.

3.6. Results

3.6.1. Embryonic axis and cotyledonary node system

The soybean cultivar JS 335 was used to assay several factors which might affect transformation. In the study of using embryonic axis and cotyledonary node as explants for *Agrobacterium* mediated transformation of soybean, the former (Figure 13) showed highest frequency of regeneration (86.4%) compared to the latter (46.5%).

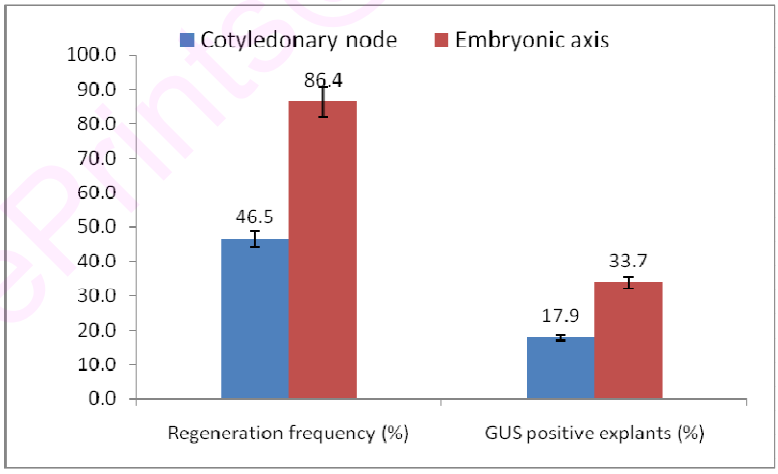


Figure 13. Efficiency of *Agrobacterium* mediated transformation of soybean (cv. JS 335) using cotyledonary node and embryonic axis explants.

For generation of fertile transgenic soybean plants using an organogenic regeneration system, it is important that the shoots should be regenerated in a relatively short time. In our experiments, the embryonic axis system regenerated adventitious shoots in relatively short period of time (4-6 weeks) with more uniform length than the cotyledonary node systems (Figure 14). Taking into account the time required for germination and preparation of explants, the cotyledonary node system is relatively slow and complicated in comparison to the embryonic axis system. The explants after inoculation with *Agrobacterium* were assayed for their transient GUS activity (one week after co-cultivation). Since the binary plasmid pCAMBIA 1305.2 contains an intron from the castor bean catalase gene to confirm detection of plant specific β-glucuronidase (GUS) activity, GUS staining is an indicative of the presence of GUS gene being expressed in plant cells. Based on GUS staining, 33.7% of embryonic axis derived explants and 17.9% of cotyledonary nodal explants were found to be GUS positive (Figure 13).

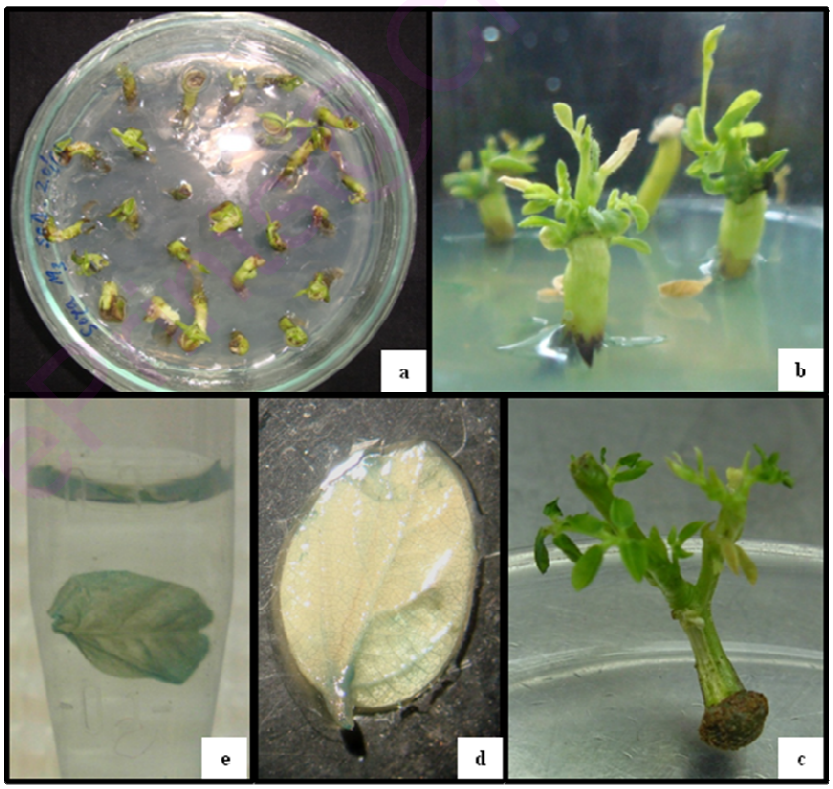


Figure 14. *Agrobacterium* mediated transformation of soybean cv. JS 335; (a) embryonic axis explants during co-cultivation; (b & c) adventitious shoots from embryonic axis explants and (d & e) visualization of GUS activity in leaf of transformed plants.

Another factor that affects the production of fertile transgenic plants is the sensitivity of explants to antibiotic selection. The explants cotyledonary node and embryonic axis were screened for their sensitivity over a wide range of hygromycin B concentration from 0 to 50 mg l^{-1} for a period of 4 weeks (Table 23). The embryonic axis were unable to form any shoots when treated with 5 mg l^{-1} or more and complete browning and death of the explants were observed at 10 mg l^{-1} within two weeks. In contrast the cotyledonary nodal explants were able to regenerate shoots even at 5 mg l^{-1} and the complete death occurred at 20 mg l^{-1} concentration. Based on the results of sensitivity studies, the embryonic tips were found to be more sensitive to antibiotic selection than the cotyledonary nodes.

Taken together these results suggest that the embryonic axis system may be useful for effective soybean transformation and generation of non-chimeric transgenic plants compared to the cotyledonary nodal system. Hence the embryonic axis was used for further studies on the role of L-cysteine and antibiotic selection schemes of *Agrobacterium* mediated genetic transformation of soybean.

Table 23. Screening of explants for their sensitivity to hygromycin B.

Concentration of hygromycin B (mg l^{-1})	No. of explants survived	
	Embryonic axis	Cotyledonary node
0	50	50
2	21	29
5	4	18
10	0	7
20	0	0
30	0	0
40	0	0
50	0	0

3.6.2. *Agrobacterium* infection and the role of L-cysteine

The explants were immersed in the bacterial suspension for 20 h, blotted dry on sterile tissue paper and placed in the solid co-cultivation medium (CCM) for 5 days in dark. The CCM was supplemented with different concentrations of L-cysteine (200, 400, 600, 800 and 1,000 mg l^{-1}) to investigate its effect on T-DNA delivery into the meristematic cells of embryonic axis by transient *Agrobacterium* infection assay (Meurer

et al., 1998). Following co-cultivation, T-DNA transfer to cells of embryonic axis was determined by transient GUS expression (GUS+) using GUS histochemical staining. The mean frequency of explants that contained at least a single focus of GUS staining cells (GUS+ focus) in the embryonic tip region was determined for each level of L-cysteine tested. The addition of L-cysteine to the solid co-cultivation medium increased the average frequency of explants containing a GUS positive focus from 0.8% in control to 6.1% in CCM containing 1,000 mg l^{-1} L-cysteine (Figure 15). L-Cysteine also enhanced the physical appearance of the explants with less browning on the cut surfaces of the explants. The above results demonstrated that L-cysteine significantly increased the number of GUS positive cells and thereby increased the frequency of T-DNA delivery, when the latter was expressed as a function of the numbers of GUS positive cells per explant.

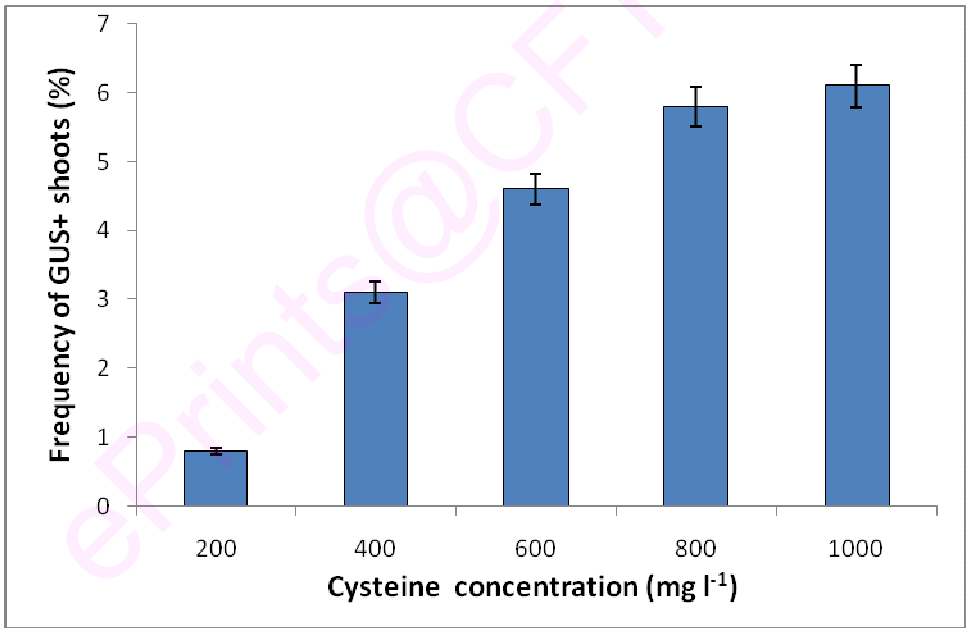


Figure 15. Effect of L-cysteine on the frequency of GUS positive shoots

3.6.3. Hygromycin B (Hyg B) selection of transformed cells

Different selection schemes (Table 24) during shoot induction was performed to optimize the selection of transgenic events. After co-cultivation the explants were either directly transferred to selection medium (SIM with 5 mg l^{-1} Hyg B) or after a resting period of 7 and 14 days in SIM without Hyg B. The frequency of the GUS positive shoots obtained from the three different selection schemes varied from 2 to 7.4% (Table 24).

Table 24. Effect of selection schemes on regeneration of transformed cells

Resting period	Total no. of explants infected	Regenerated plants	Recovered plants	GUS+ plants	Escape rate (%)	Transformation efficiency (%)
0 days	98	24	11	2	81.8	2.0[c]
7 days	108	72	9	8	11.1	7.4[a]
14 days	128	86	6	5	16.7	3.9[b]

The explants which were transferred onto the selection medium immediately after co-cultivation seemed to suffer from the combined stress of *Agrobacterium* and hygromycin B (5 mg l^{-1}) during shoot bud initiation phase and produced few GUS positive shoots. It was found that an appropriate duration of resting period, after *Agrobacterium* infection was helpful for efficient selection and regeneration of transformed cells. The results demonstrated that the 7 day resting period led to lower escape rate than 14 day resting period and the survival of transgenic shoots benefited from the gradually increased selective pressure after the 7 day resting period. Among the selection schemes tested, 7 day resting period was shown to be optimal, under which the production of the GUS positive shoots was consistent with an average frequency of 7.4%.

3.7. Discussion

3.7.1. Embryonic axis and cotyledonary node system

The embryonic axis regeneration system has many advantages over the cotyledonary node and hypocotyl segment systems: the starting material is easy to obtain, more shoots are regenerated in the same amount of time and the meristem shows greater division. The latter can be explained by the strong dividing ability of the promeristems and procambium in the embryonic axis. This characteristic likely improves the growth and division of transformed cells in the selection medium, as confirmed by our observations of high level transient GUS expression in highly dividing meristematic tissues. Because high regeneration frequency is desirable for transformation, we have used embryonic axis regeneration system (Liu et al., 2004) for *Agrobacterium* mediated soybean transformation compared to cotyledonary node system which has been previously used (Di et al., 1996; Meurer et al., 1998; Zhang et al., 1999; Donaldson and Simmonds, 2000).

During *A. tumefaciens* mediated transformation of soybean and other crops, it is uncommon for explants to be suspended for 20 h in liquid infection medium, as was done in the present study. In the cotyledonary node system, suspension time is limited by the pollution and necrosis caused by *A. tumefaciens*. However, the embryonic axis system responded well to *A. tumefaciens* infection and there was no obvious necrosis. This difference between the cotyledonary node and embryonic axis systems may relate to the smooth surface of the latter, which can be easily washed free of *A. tumefaciens,* or it may be associated with the greater growth potential of the explants. Thus, the extended suspension time is another aspect in which the embryonic axis system outperforms the cotyledonary node system in the *Agrobacterium* mediated transformation of soybean.

3.7.2. *Agrobacterium* infection and the role of L-cysteine

The addition of L-cysteine in the CCM demonstrated an increase in the frequency of T-DNA delivery, as analyzed by transient GUS assay, which is in line with the results of previous investigations (Olhoft and Somers, 2001; Liu et al., 2008). The addition of L-cysteine to the solid co-cultivation medium increased *Agrobacterium* mediated T-DNA delivery into the cells with a notable decrease in enzymatic browning on the explants. Since both *Agrobacterium* infection and tissue browning were affected, it is possible that L-cysteine interacts with the plant's response to wound and pathogen infection during co-cultivation, resulting in an increase in T-DNA delivery. However, high levels of L-

cysteine in the CCM might also have some negative impacts on *in vitro* plant morphogenesis. In the *Agrobacterium*-mediated transformation of maize, Frame et al., (2002) reported that the infected embryos as well as the non-infected ones treated with 400 mg l^{-1} L-cysteine exhibited significant decrease in giving rise to embryogenic callus. A similar negative effect of L-cysteine (80 mg l^{-1}) on embryogenesis in Japonica rice explants was reported by Obregon et al., (1999). Both Frame et al., (2002) and Obregon et al., (1999) opined that L-cysteine enhancement on *Agrobacterium*-mediated transformation might be a balanced consequence of its positive and negative roles.

3.7.3. Hygromycin B selection of transformed cells

An effective selection strategy is very important for developing an efficient plant transformation procedure. Olhoft et al., (2003) employed hygromycin B as a selective agent in the *Agrobacterium* mediated cotyledonary node transformation of soybean and achieved rapid production of multiple, transgenic shoots with considerable efficiencies. In the present study, based on the result of Olhoft et al., (2003), an improved selection strategy was developed by adjusting the resting period and hygromycin B levels in the shoot initiation and elongation phase. A resting step was usually applied to alleviate the suffering from the combined stress of *Agrobacterium* and selection agents. Zhao et al., (2001) reported that a 4 day resting period increased the callus transformation frequency by 2.7 times higher than the control (without resting) in maize. Olhoft et al., (2003) also obtained good results by eliminating the selection pressure during the first 14 days of shoot initiation. The time required for resting the culture in our system (7 days) was far shorter than the 14 days rest required by the cotyledonary node system (Olhoft et al., 2003). Within the 7 days resting period, the embryonic axis explants turned green and began growing quickly, whereupon we could successfully move them to the selection medium. The 7 day resting period was found adequate to reduce the combined stress of *Agrobacterium* and selection agents, and also helped to arrest the rapid growth of most non-transgenic shoot primordial cells with strong dividing ability, resulting in fewer escapes.

In summary, an improved soybean transformation system involving better explant with high regeneration frequency, optimized L-cysteine concentration and selection strategy was established. This system could provide a useful tool for both improvement of cultivar quality and investigations on the functional genes in soybean. The results indicated that the embryonic axis system yielded a transformation efficiency of 7.4 % on the soybean cv. JS 335 which is not an improvement over the 16.4% efficiency reported

for the cotyledonary node system (Olhoft et al., 2003). However, the embryonic axis system has many advantages compared with the cotyledonary nodal system. The embryonic axis system uses fewer and more easily available starting materials, involves a simple protocol, utilizes hygromycin B as an efficient selective agent, and takes less time to generate transgenic soybeans. Based on the above experiments, an efficient *Agrobacterium* mediated transformation system was established for the soybean cultivar JS 335 which is widely cultivated in north and central India.

3.8. Summary

The *Agrobacterium tumefaciens* strain EHA 105 harboring the binary plasmid pCAMBIA 1305.2 was used for transformation of the cotyledonary node and embryonic axis explants of soybean. The transformation efficiency as analyzed by transient GUS expression showed 33.7% for embryonic axis and 17.9% for cotyledonary node explants. Addition of L-cysteine during co-cultivation enhanced the T-DNA delivery into the explants with a notable decrease in enzymatic browning of the explants. Also a resting period of 7 days was found to improve the selection of transgenics by eliminating selection pressure in the early shoot induction phase. The embryonic axis transformation system has advantages in terms of easy handling and less time consuming as compared with cotyledonary nodal transformation system and hence can be effectively used for genetic improvement of soybean. The same was adopted for further studies on genetic transformation of soybean with the target gene delta-6 desaturase, for the production of gamma linolenic acid.

SECTION III

CLONING AND EXPRESSION OF DELTA-6 DESATURSASE GENE IN SOYBEAN

3.9. Background

Genetic manipulation of the oil yielding crop plants for better oil quality through biotechnological methods is an important aspect of crop improvement (Kinney, 1994; Cardoza and Stewart, 2004). In recent years, genetic modification of oilseed plant to synthesize nutritionally and industrially important fatty acids that are generally obtained from other sources, has gained considerable importance (Damude and Kinney, 2008; Singh et al., 2005). Gamma-linolenic acid (GLA), an unusual fatty acid in plant kingdom, is produced by specific desaturation of linoleic acid (C18:2). Dietary GLA has therapeutic benefits in being an active precursor of arachidonic acid (C20:4), which participates in the synthesis of prostaglandins in human beings (Napier et al., 1999). GLA has been reported to have anti-inflammatory and antitumoral effects (Goffman and Galletti, 2001). It improves dysregulation of inflammation and provides immunity in atopic eczema (Henz et al., 1999). Consumption of vegetable oils containing GLA is reported to be effective against hypercholesteromia and other related clinical disorders that provide susceptibility to coronary atherosclerosis (Barre, 2001). Therefore, production of oilseed crop rich in GLA is extremely desirable for the benefit of human race. In addition to the nutritional value of GLA, OTA produced from α-linolenic acid (ALA) by the same Δ^6-desaturase (D6d) has immense industrial applications, which include production of oil films, special waxes and plastics (Reddy and Thomas 1996).

In plants, synthesis of unsaturated fatty acids is initiated in the plastids through a series of condensation reactions which are continued either in the plastids or in the endoplasmic reticulum resulting in a pool of C18:2 and C18:3 unsaturated fatty acids. Acylation and hydrolysis of these precursors in the endoplasmic reticulum lead to the formation of triacylglycerol, the main storage lipid (Kinney, 1994). C18 desaturation pathway in higher plants involves desaturation at C9, C12 and C15, generating predominantly oleic (C18:1-Δ^9), linoleic (C18:2-$\Delta^{9,12}$) and ALA (C18:3-$\Delta^{9,12,15}$) acids. An additional C18 desaturation step is present in the cyanobacteria (e.g. *Synechocystis*, *Spirulina*) and in few higher plants (e.g. *Borago officinalis*, *Oenothera sp.*) where the desaturation proceeds towards production of γ-linolenic acid (GLA, C18:3-$\Delta^{6,9,12}$) and octadecatetraenoic acid (OTA, C18:4-$\Delta^{6,9,12,15}$) *via* a single D6d enzyme (Reddy and

Thomas, 1996). Transgenic higher plants expressing genes for alteration in fatty acid metabolic pathway to produce long chain polyunsaturated fatty acids, generally obtained from marine sources like microalgae and fish, are now being developed (Abbadi et al., 2004; Qi et al., 2004). Commercially grown oil seed crops like soybean, groundnut, rapeseed etc., are deficient in GLA because they inherently lack the D6d gene (Reddy and Thomas, 1996). Till date several attempts have been made by a number of researchers to explore the possibilities of altering the C18 desaturation pathway of plants for production of GLA (Knutzon et al., 1998; Sayanova et al., 1999; Qiu et al., 2002). Reddy and Thomas, (1996) first reported the production of GLA in transgenic tobacco plants after successful introgression of the D6d coding sequence from *Synechocystis*. Later, D6d gene(s) from several eukaryotic sources have been introgressed in different plants for production of GLA. Such attempts include transformation of *Lycopersicun esculentum* with borage D6d (Cook et al., 2002), *Brassica juncea* with *Pythium irregulare* D6d (Hong et al., 2002) and *Oenothera sp*. with borage D6d (Gyves et al., 2004).

Due to the inherent absence of the Δ^6-desaturase (D6d) function, soybean a pulse cum oilseed crop plant, is unable to synthesize γ-linolenic acid (GLA), a nutritionally important fatty acid, although the crop plant synthesizes the precursor fatty acids required for GLA production. *Spirulina platensis*, contains a high level of γ-linolenic acid (20% of total fatty acid content) and has been considered as one of the alternate source for γ-linolenic acid production (Hongsthong et al., 2004; Huang and Ziboh, 2000). Cyanobacterial Δ^6-desaturase introduces carbon–carbon double bond onto linoleic acid (C18:2) and α-linolenic acid (C18:3) by desaturation processes for production of GLA and OTA respectively. Cyanobacterial fatty acid desaturases are categorized as being part of the family of acyl-lipid desaturases or plant-type desaturases (Murata and Wada, 1995), which are membrane-bound enzymes (Mustady et al., 1996). Acyl-lipid desaturases introduce double bonds into fatty acids that have been esterified to glycerolipids (Schmidt et al., 1993). These membrane bound enzymes associate with endoplasmic reticulum and chloroplast membrane in plants, and thylakoid and plasma membranes in cyanobacteria (Murata and Wada, 1995; Hongsthong et al., 2003). Expression of a cyanobacterial D6d in oil seed crop such as soybean has not been reported so far. This section explores the possibilities of using a cyanobacterial (*Spirulina platensis*) D6d gene in soybean to modify the C18 desaturation pathway towards GLA production for obtaining nutritionally superior soybean oil.

Soybean seeds also contain isoflavones, which are bioactive molecules of low molecular weight, hydrophobic peptides or fatty acid components that are known to influence the physiological state in animals as well as in humans. Isoflavones are structurally similar to naturally occurring estrogens and show promise in protecting against hormone-dependent cancers (Sakthivelu et al., 2008). Epidemiological studies have shown that consumption of soybeans and soy products reduces the risk of human cancer, osteoporosis and cardiovascular diseases (Devi et al., 2009). In addition, isoflavones belong to a group of active plant defense compounds known as phytoalexins, and thereby acts as a repellant against insect feeding and pathogenic fungi (Dixon, 1993). Isoflavones are also involved in nodulation of leguminous plants by inducing the expression of nodulation genes in rhizobial bacteria during symbiosis (Pueppke, 1996). Considering the importance of soybean as an oilseed crop and also as a source of phytochemicals like isoflavones, the unintended effects of soybean transformation with delta-6 desaturase gene was also analyzed and discussed in this section with reference to isoflavone content.

3.10. Results

3.10.1. Isolation of D6d gene from *Spirulina*

All the three sets of primers were tested against the genomic DNA of the *Spirulina platensis* strain Sp-6 for the amplification of D6d gene. The internal primer set (*viz*., $D6dF_1$ and $D6dR_1$) gave good amplification (Figure 16A) of the expected size of 480 bp (a part of the Δ^6-desaturase gene). The annealing temperature was standardized at 50 °C for the partial primers. The remaining primer sets ($D6dF_2$ & R_2 and $D6dF_3$ & R_3) gave good amplification for the full length gene of 1.2 kb, from the genomic DNA of *Spirulina platensis* strain Sp-6 (Figure 16B & C). The annealing temperature was standardized at 50 and 55 °C respectively for $D6dF_2$ & R_2 and $D6dF_3$ & R_3 primers.

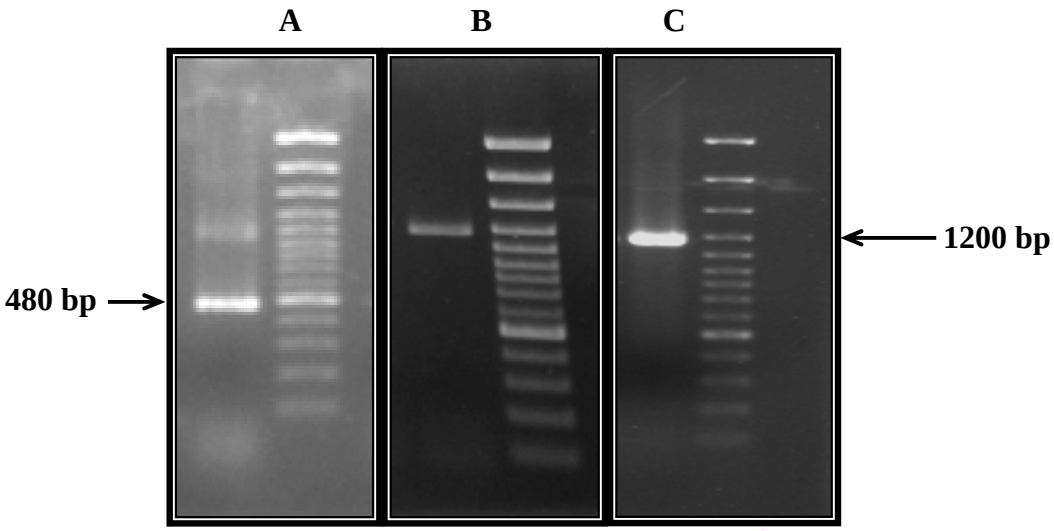


Figure 16. PCR amplification of delta-6 desaturase gene from *Spirulina platensis* strain Sp-6; (A) PCR amplicon of partial D6d gene (480 bp) using internal primers $D6dF_1$ and $D6dR_1$ with 3 kb DNA ladder; PCR amplicon of full length D6d gene (1.2 kb) using primers (B) $D6dF_2$ & $D6dR_2$, and (C) $D6dF_3$ & $D6dR_3$ with 3 kb DNA ladder.

3.10.2. Construction of recombinant plasmid with D6d gene

The plasmid pRT100 (3340 bp) with CaMV35S (cauliflower mosaic virus) promoter, poly A (polyadenylation signal of CaMV strain Cabb B-D) terminator, ampicillin resistance gene and a multicloning site with different restriction sites was used as the plant expression vector (Appendix D). The isolated coding sequence of the delta-6 desaturase gene (1.2 kb) was inserted in between the *Xho I* and *Xba I* site of pRT100 by directional cloning. The cloned plasmids were transformed to *E.coli* strain DH5α and the colonies were subjected to antibiotic (ampicillin) selection. Six individual recombinant colonies were obtained (Figure 17) and each of them was confirmed by restriction digestion and PCR with CaMV35S F and $D6dR_3$ as forward and reverse primers respectively. Upon digesting the recombinant plasmids with the same restriction enzymes *Xho I* and *Xba I*, the gene insert was released from the vector backbone as shown in Figure 18. After verification the positive recombinant plasmids were stored in deep freezer (-70°C) for further use.

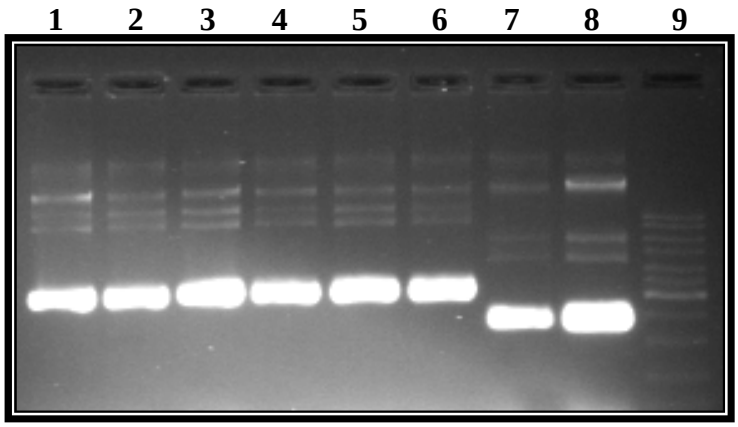


Figure 17. Recombinant plasmid of pRT 100 with delta-6 desaturase gene; Lane 1-6: Recombinant pRT100 plasmid clones (≈4500 bp); lane 7-8: wild pRT 100 plasmid (3340 bp); lane 9: 10 kb DNA ladder

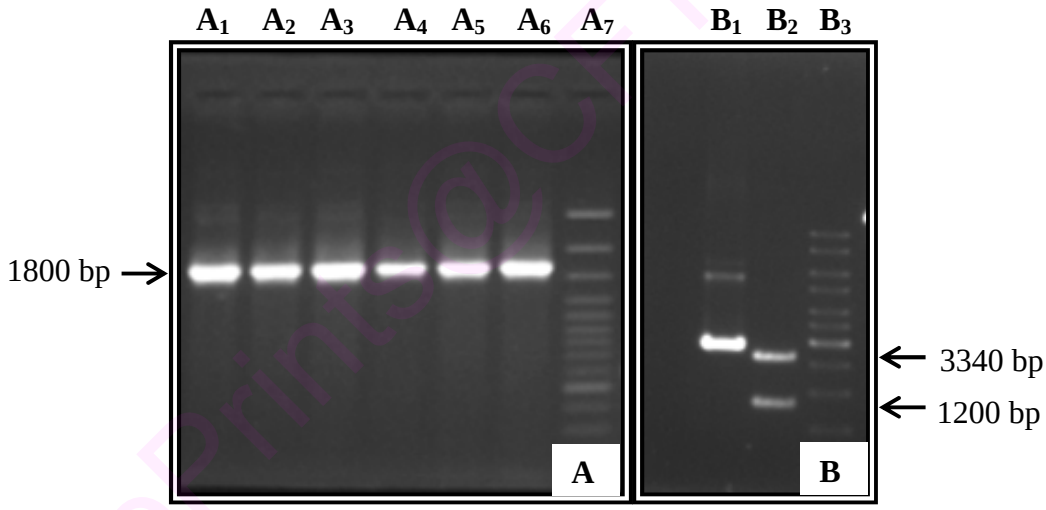


Figure 18. Confirmation of recombinant pRT 100 clones; (A) PCR confirmation of recombinant pRT 100 plasmid clones; Lane A_1-A_6 - PCR amplicon of D6d gene (≈1800 bp) from the recombinant pRT 100 plasmid clones using CaMV35S F & $D6dR_3$ as forward and reverse primers; Lane A_7 – 3 kb DNA ladder; (B) Restriction digestion confirmation of recombinant pRT 100 clone; Lane B_1 - wild pRT100 plasmid (3340 bp); lane B_2 – digested products of recombinant pRT 100 clone showing the release of gene insert (1200 bp) and vector backbone (3340 bp); Lane B_3 - 10 kb DNA ladder.

3.10.3. Multiple sequence analysis of the cloned delta-6 desaturase gene

The recombinant pRT100 plasmid was sequenced from MWG Biotech private limited, Bangalore, and the sequence was deposited in the NCBI (GenBank) database with the assigned accession number FJ752023 (Sakthivelu et al., 2009). The cloned gene is devoid of any intervening sequences and exhibited 98% identity at the DNA level, when aligned with the reported gene sequence of open reading frame (ORF) l106 bp through pairwise BLAST 2.0 analysis. The recombinant plasmid presented very similar open reading frames encoding proteins of the same length *viz*., 368 amino acids (Figure 20). Computational analysis of the deduced amino acids from the cloned gene sequence showed 98% similarity with the targeted protein sequence. The 2% deviation is due to the conserved substitution of amino acids, except for the amino acid proline (P) at the 63rd position which was replaced by Arginine (R) in the cloned sequence of *Spirulina platensis*. The influence of this replacement on the expression of the D6d gene in plants should be further investigated. Furthermore, the hydropathy profile (Figure 19) of amino acid sequences revealed the presence of two membrane-spanning domains, which have a similar characteristic to membrane-bound desaturases found in other organisms (Hong et al., 2002; Laoten et al., 2000).

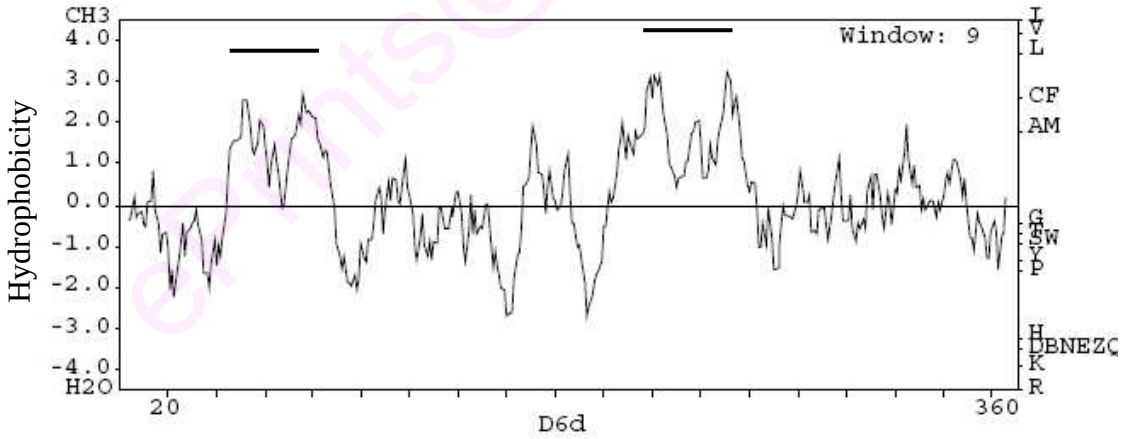
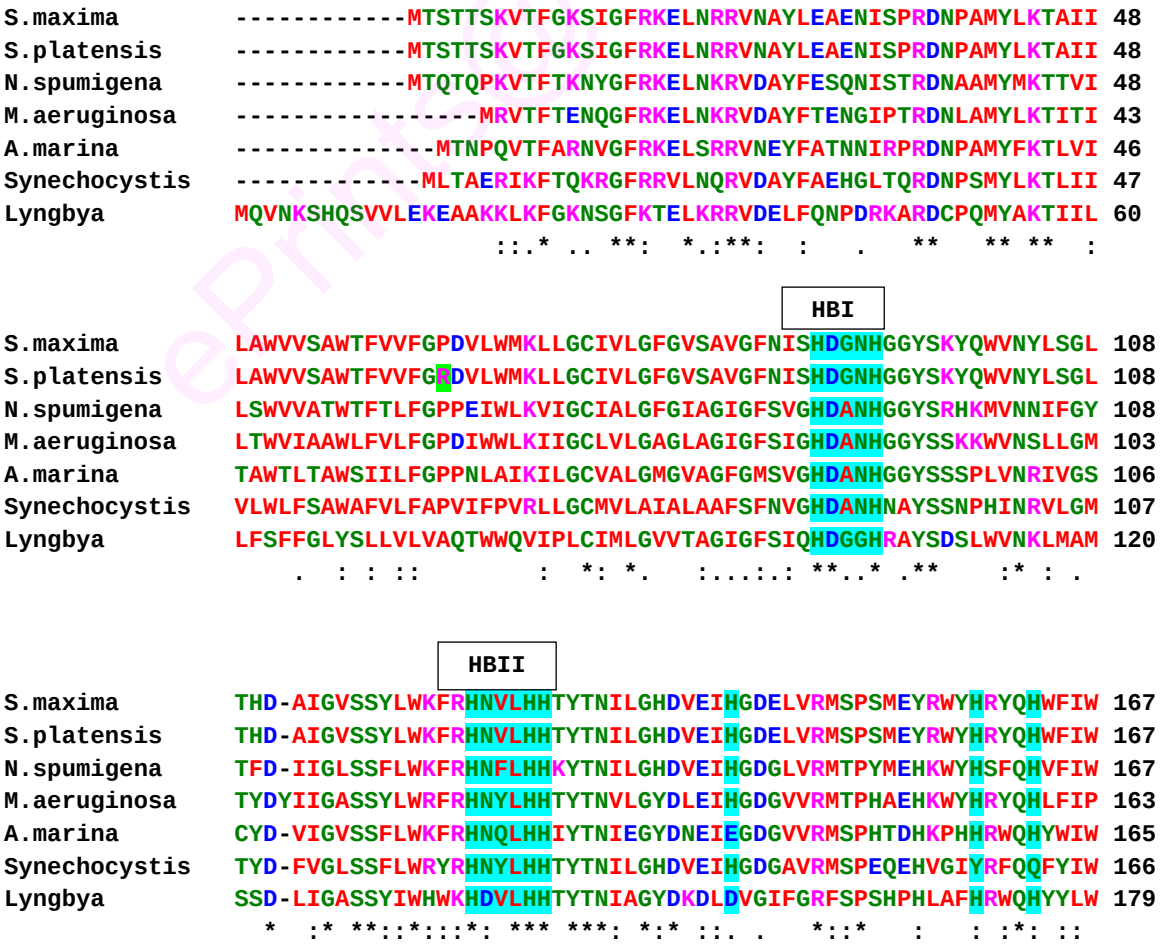


Figure 19. Hydropathy profile of the delta-6 fatty acid desaturase of *Spirulina platensis.* The plots were analyzed using the Kyte-Doolittle method (Kyte and Doolittle, 1982). Bars indicate the two hydrophobic domains

3.10.3.1. Multiple sequence alignment with other cyanobacterial delta-6 desaturases

The amino acid sequence alignment of cyanobacterial delta-6 desaturases (Figure 20) demonstrates the three histidine rich motifs at amino acid positions 89-93, 124-129

and 302-309 conserved among all the fatty acid desaturases, together with two histidine residues adjacent to the third motif (amino acid positions 313-315), which are conserved among the acyl-lipid desaturases of cyanobacteria. In total there are about 83 amino acid residues which are conserved among the compared cyanobacterial species. The two species from *Spirulina* genus *viz*., *S. maxima* and *S. platensis* encoded the same number of amino acids and showed 98% similarity (Table 25) with only four substituitions, out of which three are conserved. Followed by *S. maxima*, the cloned delta-6 desaturase of *S. platensis* showed 60% amino acid sequence identity to delta 6-desaturase of *Nodularia spumigena* followed by *Microcystis aeruginosa* (59%), *Acarychloris marina* (54%), *Synechocystis* (50%) and *Lyngbya sp* (35%) (Table 25). Phylogenetic analysis also demonstrated the homology between delta-6 fatty acid desaturase with other cyanobacterial D6d (Figure 21). The cloned delta-6 fatty acid desaturase is closely related with the other species of the same genus *S. maxima*. The analysis also revealed closer association with *Nodularia spumigena*, *Microcystis aeruginosa*, *Acarychloris marina* and *Synechocystis* (Figure 21).



```
S.maxima          FVYPFIPYYWSIADVQTMLFKRQYHDHEIPSPTWVDIATLLAFKAFGVAVFLIIPIAVGY 227
S.platensis       FVYPFIPYYWSIADVQTMLFKRQYHDHEIPPPTWVDIATLLAFKAFGVAVFLIIPIAVGY 227
N.spumigena       FIYPIIPLYWSFADVYLVMFKRKYHTYDIPKLKPLDLLVFFSGKLMWLGLFLGIPIAVGY 227
M.aeruginosa      ILYGIIPIYWSFSDVRSILFRHRFGEIKLPNPKAIDLFVLLSGKVVYLFWFIGIPLLVGY 223
A.marina          FIYPFIPIYWYFRDIQRFIRNTPYQTNTLPTRQPIDYVTFWVGRIIGVSFFMVTPYLVGY 225
Synechocystis     GLYLFIPFYWFLYDVYLVLNKGKYHDHKIPPFQPLELASLLGIKLLWLGYVFGLPLALGF 226
Lyngbya           LLYGLLVIKWHFYDDFYCLITGKIGDRRYPRPKSSNLVIFFGGKLVFFALAFAIPLQF-H 238
                   :* ::   * : *    :          *     :   :   : . .   :  *  . .

S.maxima          SPLEAVIGASIVYMTHGLVACVVFMLAHVIEPAEFLDPDN--LHIDDEWAIAQVKTTVDF 285
S.platensis       SPLEAVIGASIVYMTHGLIACVVFMLAHVIEPAEFLDPDN--LHIDDEWAIAQVKTTVDF 285
N.spumigena       SPIQAVVGFVITYMTYGLMICIIFMMAHVLEAAEFIEPNSDLQQVNDEWAIFQIKTTVDF 287
M.aeruginosa      SPLEIAIGFLIVFMTYGVLACHVFMLAHVLEPAEFIQPSAT-NQIEDEWAIFQVRTTVDF 282
A.marina          SPLQIVLGFCIAYWTYGAIVCEIFMLAHVMEGIDFPKPDPESNQIEDEWAIFQLRTTADF 285
Synechocystis     SIPEVLIGASVTYMTYGIVVCTIFMLAHVLESTEFLTPDGESGAIDDEWAICQIRTTANF 286
Lyngbya           ALSQVLASYGLVAFTWGVVLSIVFQLAHVVEEADFPLPIAELDLIEKDWAVHQIETTVNF 298
                  :  :   .  :.  * * : . :* :***:*  :*  *      ::.:**: *:.**.:*

                                  HBIII     HBIV
S.maxima          APNNPIINWYVGGLNYQTVHHLFPHICHIHYPKIAPILAEVCEEFGVNYAVHQTFFGALA 345
S.platensis       APKNPIINWYVGGLNYQTVHHLFPHICHIHYPKIAPILAEVCEEFGVNYAVHQTFFGALA 345
N.spumigena       APKNQFLNWYLGGLNYQVVHHLFPNICHIHYPQLAKILADVCEDFGVKYNVCETFTEALA 347
M.aeruginosa      APKNIFLNWYLGGLNYQVVHHLFPQICHIHYPKIAPILAEVCQEFGVNYAVYPTFWGALT 342
A.marina          APRNPFLNWYVGGLNYQAVHHLFPQICHIHYPQIAPIVAEVCQEFGVNYMVYPTFAKAIA 345
Synechocystis     ATNNPFWNWFCGGLNHQVTHHLFPNICHIHYPQLENIIKDVCQEFGVEYKVYPTFKAAIA 346
Lyngbya           SRNNHLMTWFLGGLNFQVEHHLFPNICHINYPELSKVVEQTCREYGVRYNQHSSFWSGLS 358
                  : .* : .*: ****.*. *****:****:**::  :: :.*.::**.*    :*  .::

S.maxima          ANYSWLKKMSINPETKAIEQLTV- 368
S.platensis       ANYSWLKKMSINPETKAIEQLTV- 368
N.spumigena       SNYRWLKLMGSAPNLE-------- 363
M.aeruginosa      YNYRWLRQLGNKQSNFDLKLAS-- 364
A.marina          SNYRWLRLMAIGEEEVSYSQATTS 369
Synechocystis     SNYRWLEAMGKAS----------- 359
Lyngbya           SHYRWLRKMGTTV----------- 371
                   :* **. :.
```

Figure 20. Comparison of the deduced amino acid sequence of delta-6 desaturase from *Spirulina platensis* (FJ752023) with other cyanobacteria *viz*., *Spirulina maxima* (B5W883), *Nodularia spumigena* (AOZMU7), *Microcystis aeruginosa* (A8YCB7), *Acarychloris marina* (BOCIU6), *Synechocystis sp.* (LLCDSYNY3) and *Lyngbya sp.* (AOYJL7). The Histidine rich motifs are represented as HBI, HBII, HBIII and HBIV. The symbol at the bottom of the alignment denotes the degree of conservation observed in each column: "*" means that the amino acid residues in that column are identical in all sequences in the alignment; ":" means that conserved substitutions have been observed and "." means that semi-conserved substitutions are observed.

Table 25. Percentage sequence identity of delta-6 desaturase among the cyanobacteria

Cyanobacteria	*S. maxima*	*S. platensis*	*N. spumigena*	*M. aeruginosa*	*A. marina*	*Synechocystis*	*Lyngbya*
S. maxima	100	98	60	59	54	51	35
S. platensis		100	60	59	54	50	35
N. spumigena			100	61	56	52	36
M. aeruginosa				100	56	52	39
A. marina					100	49	34
Synechocystis						100	36
Lyngbya							100

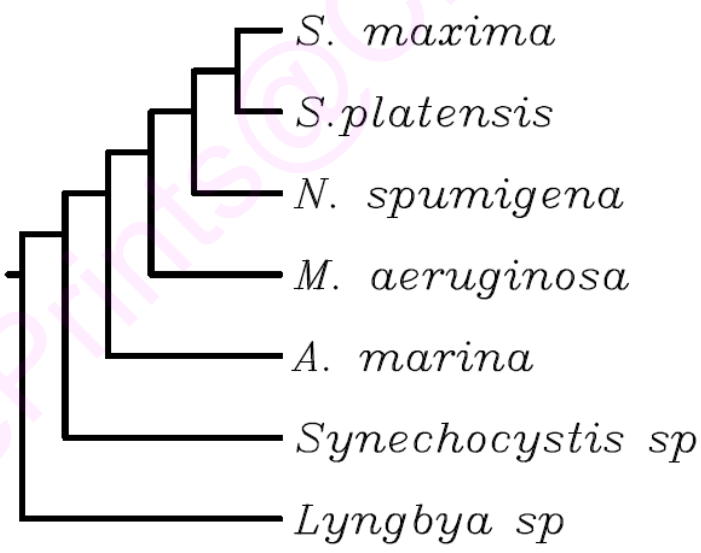


Figure 21. Phylogenetic relationship between delta-6 desaturase of *Spirulina platensis* (FJ752023) with other cyanobacteria *viz*., *Spirulina maxima* (B5W883), *Nodularia spumigena* (AOZMU7), *Microcystis aeruginosa* (A8YCB7), *Acarychloris marina* (BOCIU6), *Synechocystis sp.* (LLCDSYNY3), and *Lyngbya sp.* (AOYJL7).

3.10.3.2. Sequence comparison of delta-6 desaturase with other organisms

The multiple sequence alignment of the deduced amino acid sequence of delta-6 desaturase (D6d) gene with delta-6 desaturase from other organisms revealed three conserved histidine rich motifs at amino acid positions 89-93, 124-129 and 302-306, and hydrophobic regions known to all membrane bound desaturases (Figure 19 & 22). Alignment and analysis of those homologous sequences indicated that the homology occurs mainly in the three conserved histidine rich motif areas. The cloned delta-6 fatty acid desaturase showed 50% amino acid sequence identity to delta-6 desaturases of *Synechocystis,* followed by the fungal and plant delta-6 fatty acid desaturases (Table 26). Phylogenetic analysis also revealed the homology between delta-6 fatty acid desaturase of *S. platensis* with other organisms (Figure 23). The cloned *S. platensis* delta-6 fatty acid desaturase was closely related with the cyanobacterial delta-6 desaturase from *Synechocystis.* Similarly the plant and fungal D6d are clustered together along with *Caenorhabditis elegans*. The cyanobacterial D6d showed almost equal identity of 14% with the plant and fungal delta-6 desaturases (Table 26). The human and cyanobacterial D6d are distantly related compared with the plant and fungal D6d. All these features mentioned under section 3.10.3 suggests that the cloned gene encodes for a putative delta-6 desaturase that is involved in the synthesis of GLA in *S. platensis.*

```
Borago          ----MAAQIKKYITSDELK--------NHDKPGDLWISIQGKAYDVSDWVKDHPGGSFPL 48
Echium          ----MANAIKKYITAEELK--------KHDKAGDLWISIQGKIYDVSDWLKDHPGGNFPL 48
Mucor           MSSDVGATVPHFYTRAELADIHQDVL-DKKPEARKLIVVENKVYDITDFVFDHPGGERVL 59
Rhizopus        MSTSDRQSV---FTLKELELINQKHR-DGDKSAMKFIIIDRKVYDVTEFLEDHPGGAQVL 56
Mortierella     MAAAPSVRT---FTRAEVLNAEALNEGKKDAEAPFLMIIDNKVYDVREFVPDHPGGS-VI 56
Pythium         -MVDLKPGVKRLVSWKEIR--------EHATPATAWIVIHHKVYDISKWDS-HPGGS-VM 49
Synechocystis   ------------------------------------------------------------
S.platensis     ------------------------------------------------------------
C.elegans       --MVVDKNASGLRMKVDGKWLYLSEELVKKHPGGAVIEQYRNSDATHIFHAFHEGSSQAY 58
H.sapiens       MGKGGNQGEGAAEREVSVPTFSWEEIQKHNLRTDRWLVIDRKVYNITKWSIQHPGGQRVI 60

Borago          KSLAGQEVTDAFVAFHPASTWK--NLDKFFTGYYL-----------------KDYSVSE 88
Echium          LSLAGQEVTDAFVAFHSGTTWK--LLEKFFTGYYL-----------------KDYSVSE 88
Mucor           LTQEGRDATDVFHEMHPPSAYE--LLANCYVGDCEPK--------LPIDSTDKKALNSAA 109
Rhizopus        LTHVGKDASDVFHAMHPESAYE--ILNNYFVGDVK-------------DAHVKETPSAQ 100
Mortierella     LTHVGKDGTDVFDTFHPEAAWE--TLANFYVGDID-------------ESDRDIKNDD 99
Pythium         LTQAGEDATDAFAVFHPSSALK--LLEQFYVGDVDETSKAEIEGEPASDEERARRERINE 107
Synechocystis   -------------------ML--TAERIKFTQKR-----------------------G 14
S.platensis     ------------------MTS--TTSKVTFGKSI-----------------------G 15
C.elegans       KQLDLLKKHGEHDEFLEKQLEK--RLDKVDINVSAYD--------------VSVAQEKK 101
H.sapiens       GHYAGEDATDAFRAFHPDLEFVGKFLKPLLIGELAPE-----------EPSQDHGKNSK 108

Borago          VSKDYRKLVFEFSKMGLYD--KKGHIMFATLCFIAMLFAMSVYGVLFCEGVLVHLFSGCL 146
Echium          VSKDYRKLVFEFNKMGLFD--KKGHIVLVTVLFIAMLFGMSVYGVLFCEGVLVHLLAGGL 146
Mucor           FAQEIRDLRDKLEKQGYFDASTGFYIYKVSTTLLVCIVGLAILKAWGRESTLAVFIAASL 169
Rhizopus        FASEMRQLRDQLKKEGYFHSSKAYYVYKVLSTLALCAAGLTLLYAYGHTSTLAVVASAII 160
Mortierella     FAAEVRKLRTLFQSLGYYDSSKAYYAFKVSFNLCIWGLSTVIVAKWGQTSTLANVLSAAL 159
Pythium         FIASYRRLRVKVKGMGLYDASALYYAWKLVSTFGIAVLSMAICFFFN--SFAMYMVAGVI 165
Synechocystis   FRRVLNQRVDAYFAEHGLTQRDNPSMYLKTLIIVLWLFSAWAFVLFAPVIFPVRLLGCMV 74
S.platensis     FRKELNRRVNAYLEAENISPRDNPAMYLKTAIILAWVVSAWTFVVFGRDVLWMKLLGCIV 75
C.elegans       MVESFEKLRQKLHDDGLMKANETYFLFKAISTLSIMAF------AFYLQYLGWYITSACL 155
H.sapiens       ITEDFRALRKTAEDMNLFKTNHVFFLLLLAHIIALESIAWFTVFYFG-NGWIPTLITAFV 167
                .    .                          :                    .    :

                          HBI                                  HBII
Borago          MGFLWIQSGW-IGHDAGHYMVVSDSRLNKFMGIFAANCLSGISIGWWKWNHN-AHHIACN 204
Echium          MGFVWIQSGW-IGHDAGHYIVMPDARLNKLMGIVAANCLSGISIGWWKWNHN-AHHIACN 204
Mucor           VGLFWQQCGW-LAHDYAHYQVIKDPNVNNLFLVTFGNLVQGFSLSWWKNKHN-THHASTN 227
Rhizopus        VGIFWQQCGW-LAHDFGHHQCFEDRSWNDVLVVFLGNFCQGFSLSWWKNKHN-THHASTN 218
Mortierella     LGLFWQQCGW-LAHDFLHHQVFQDRFWGDLFGAFLGGVCQGFSSSWWKDKHN-THHAAPN 217
Pythium         MGLFYQQSGW-LAHDFLHNQVCENRTLGNLIGCLVGNAWQGFSVQWWKNKHN-LHHAVPN 223
Synechocystis   LAIALAAFSFNVGHDANHNAYSSNPHINRVLGMTYD--FVGLSSFLWRYRHNYLHHTYTN 132
S.platensis     LGFGVSAVGFNISHDGNHGGYSKYQWVNYLSGLTHD--AIGVSSYLWKFRHNVLHHTYTN 133
C.elegans       LALAWQQFGW-LTHEFCHQQPTKNRPLNDTISLFFGNFLQGFSRDWWKDKHN-THHAATN 213
H.sapiens       LATSQAQAGW-LQHDYGHLSVYRKPKWNHLVHKFVIGHLKGASANWWNHRHF-QHHAKPN 225
                :.      .: : *:  *         .           * *   *. .*   **   *

Borago          SLEY-------DPDLQYIPFLVVSSKFFGSLTSHFYEKRLTFDS-LSRFFVSYQHWTFYP 256
Echium          SLDY-------DPDLQYIPFLVVSSKLFSSLTSHFYEKKLTFDS-LSRFFVSHQHWTFYP 256
Mucor           VSGE-------DPDIDTAPILLWDEFAVANFYGSLKDNASGFDRFIAEHILPYQTRYYFF 280
Rhizopus        VHGH-------DPDIDTAPVLLWDEYASAAYYASLDEEPTMISRFLAESVLPHQTRYYFF 271
Mortierella     VHGE-------DPDIDTHPLLTWSEHALEMFSDVPDEE---LTRMWSRFMVLNQTWFYFP 267
Pythium         LHSAKDEGFIGDPDIDTMPLLAWSKEMARKAFESAHGP----------FFIRNQAFLYFP 273
Synechocystis   ILGH-------DVEIHGDGAVRMSPEQEHVGIYR-----------------FQQFYIWG 167
S.platensis     ILGH-------DVEIHGDELVRMSPSMEYRWYHR-----------------YQHWFIWF 168
C.elegans       VIDH-------DGDIDLAPLFAFIPGDLCKYKASFEKA--------ILKIVPYQHLYFTA 258
H.sapiens       IFHK-------DPDVNMLHVFVLGEWQPIEYGKKKLKY----------LPYNHQHEYFFL 268
                          * ::.    .                                  *
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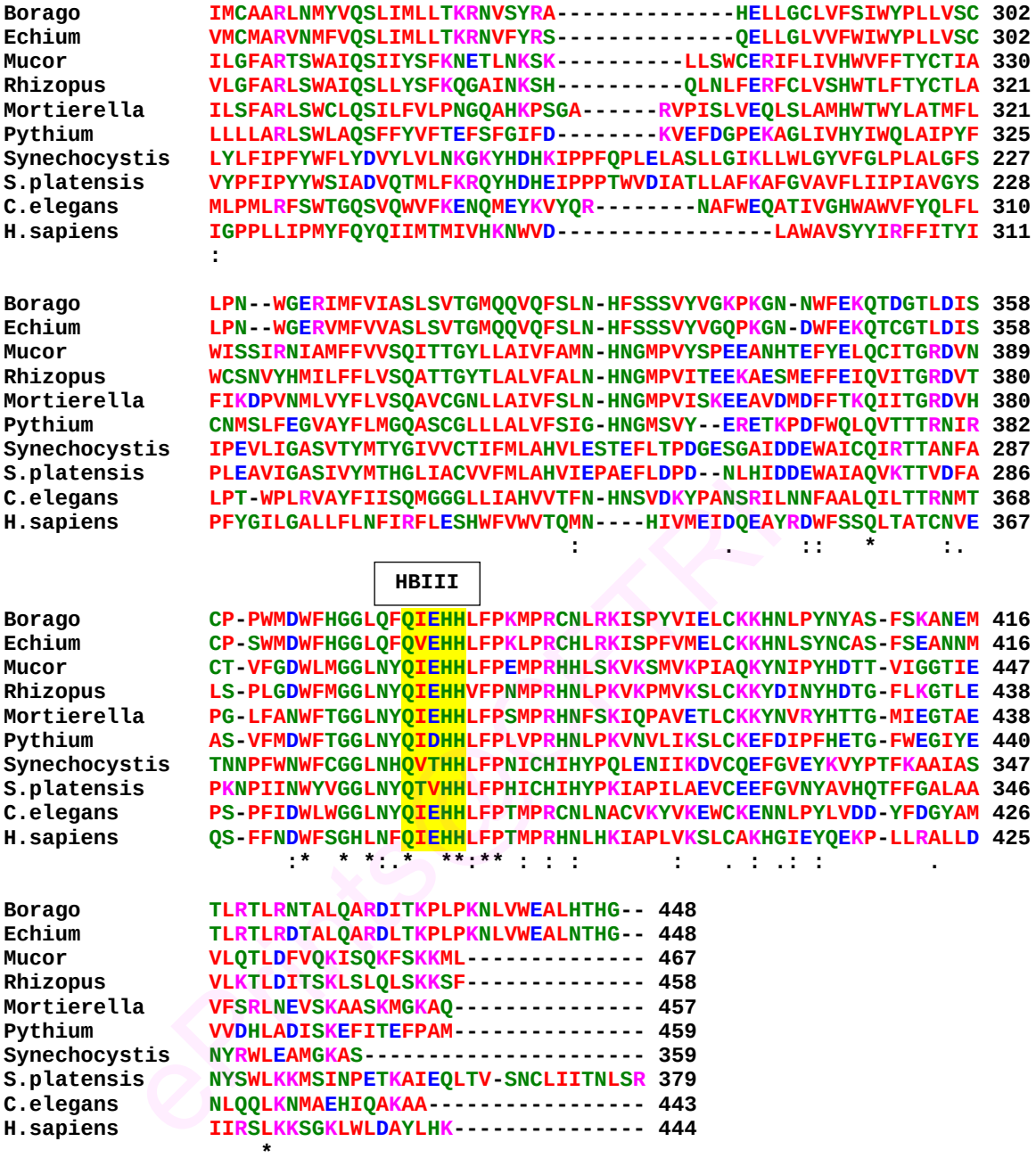

Figure 22. Comparison of the deduced amino acid sequence of delta-6 desaturase from *Spirulina platensis* (FJ752023) with other organisms *viz.*, *Borago officinalis* (BOU79010), *Echium plantagenum* (AY952780), *Mucor circillinoides* (AB052086), *Rhizopus sp.* (NK030037), *Mortierella isabellina* (AF306634), *Pythium irregulare* (AF419296), *Synechocystis sp.* (L11421), *Caenorhabditis elegans* (AF031477) and *Homo sapiens* (AF084559). The signature motifs common to fatty acid desaturases are highlighted and are represented as HBI, HBII and HBIII. The symbol at the bottom of the alignment denotes the degree of conservation ("*****" - identical in all sequences; "**:**" - conserved substitutions and "**.**" semi-conserved substitutions of amino acid residues) observed in each column.

Table 26. Percentage sequence identity of delta-6 desaturase with other organisms

Organism	*S. Platensis*	*Synechocystis*	*Borage*	*Echium*	*C. elegans*	*Mortierella*	*Mucor*	*Pythium*	*H. sapiens*	*Rhizhopus*
S. Platensis	100	50	15	13	15	17	12	15	16	13
Synechocystis		100	13	16	17	13	13	16	13	15
Borage			100	85	24	28	25	28	24	26
Echium				100	23	28	25	27	24	26
C. elegans					100	30	29	29	22	30
Mortierella						100	45	38	22	47
Mucor							100	34	22	57
Pythium								100	26	35
H. sapiens									100	23
Rhizhopus										100

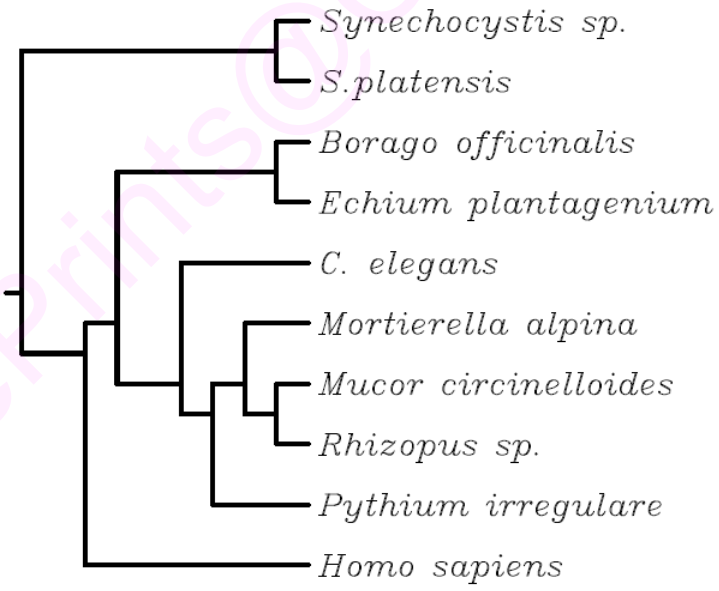

Figure 23. Phylogenetic relationship between delta-6 desaturase of *Spirulina platensis* (FJ752023) with other organisms *viz.*, *Borago officinalis* (BOU79010), *Echium plantagenum* (AY952780), *Mucor circillinoides* (AB052086), *Rhizopus sp.* (NK030037), *Mortierella isabellina* (AF306634), *Pythium irregulare* (AF419296), *Synechocystis sp.* (L11421), *Caenorhabditis elegans* (AF031477) and *Homo sapiens* (AF084559).

3.10.4. Functional analysis of pCAMBIAD6d in transgenic soybean

The D6d expression cassette from the recombinant plant expression vector pRT 100 was sub-cloned into the binary vector pCAMBIA1305.2 using the restriction enzyme *Hind III*. The resultant recombinant binary vector pCAMBIAD6d was mobilized into the *Agrobacterium tumefaciens* strain EHA105 which was used for soybean transformation. In order to produce GLA from linoleic acid in soybean, it is necessary to express the delta-6 desaturase gene (Figure 3, under section 1.3.4.1). To determine whether the *Spirulina* D6d gene could catalyze the synthesis of delta-6 desaturated fatty acids in transgenic plants, the gene was expressed in soybean plants under the control of a strong constitutive CaMV35S promoter and poly A terminator (Figure 24). Both linoleic acid and α-linolenic acid act as a substrate for the delta-6 desaturase gene and results in the formation of GLA and OTA respectively.

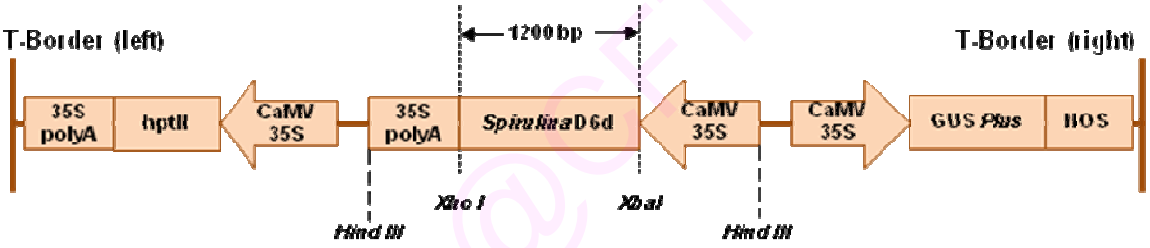

Figure 24. Recombinant pCAMBIAD6d vector showing the region between the T-border and D6d gene insert

3.10.4.1. *Agrobacterium* mediated transformation of soybean

Agrobacterium mediated soybean transformation was carried out as previously described under section 2.6.8 using embryonic axis explants and hygromycin for selection of transformants. The embryonic tips turned green, increased in size and grew normally in the co-cultivation medium for 5 days. The explants when transferred to selection medium showed the appearance of multiple shootlets within 30-45 days. Transfer of individual shootlets to rooting medium resulted in the formation of roots within 4 weeks. A total of 100 explants were infected in 4 individual experiments (each experiment with a total of 25 explants). A total of 4 putative transgenic lines (transformation efficiency of 4.0%) were obtained for the hormonal combinations used in this study. Histochemical GUS assay of the leaves from transformed lines showed the appearance of characteristic blue colour after incubation in X-gluc solution while leaves of untransformed plants showed

no such coloration (Figure 25) indicating GUS reporter gene activity in the transformants. Fully grown rooted plantlets were then transferred to pots in transgenic green house after proper hardening for 10 days. No phenotypic differences were evident among the different transgenic lines. These plants grew and matured at a normal rate, were fully fertile and were morphologically similar to the control plants.

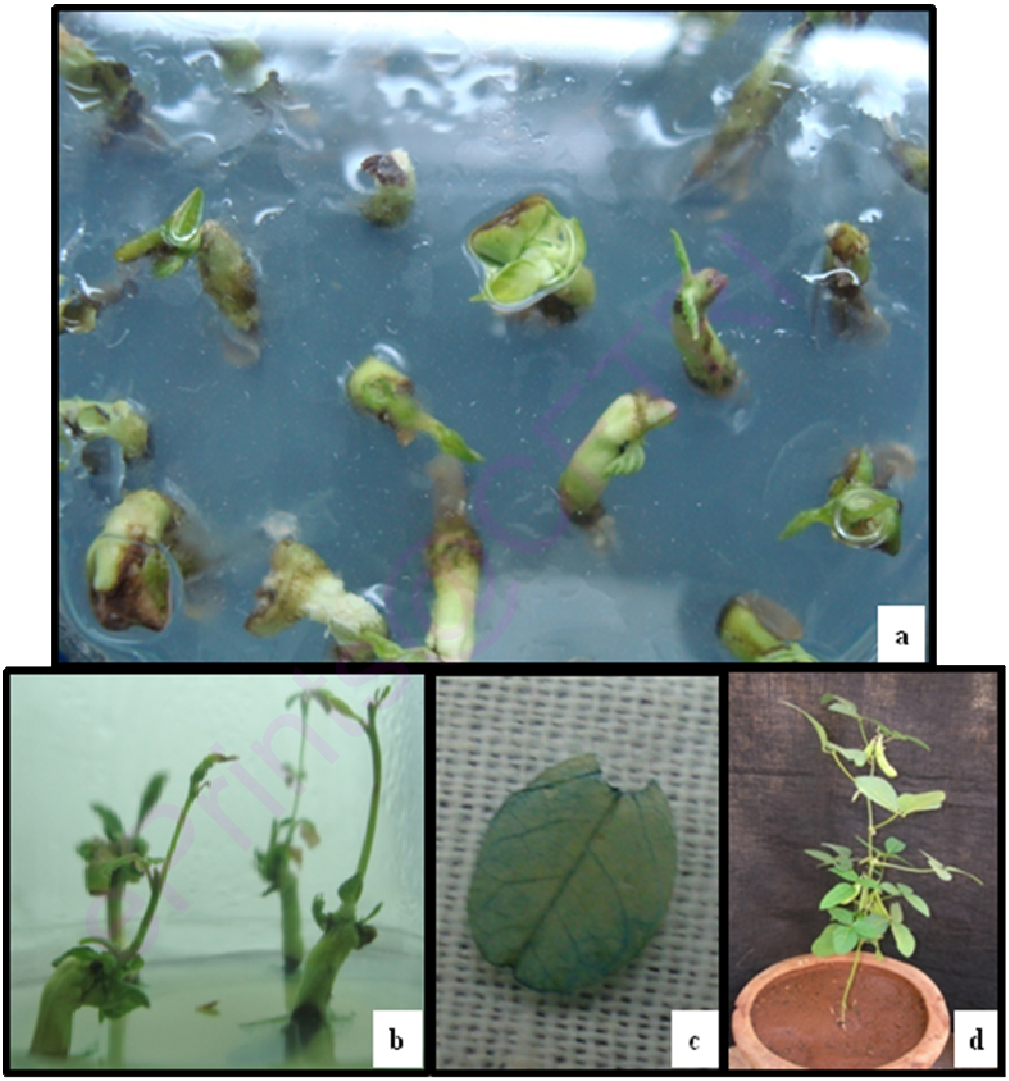

Figure 25. *Agrobacterium* mediated transformation of soybean cv. JS 335 using pCAMBIAD6d plasmid construct; (a) Co-cultivation of embryonic axis explants in co-cultivation media; (b) Regeneration of multiple shootlets from embryonic axis explants in selection media; (c) histochemical GUS assay of leaf from transformed plant; and (d) potted plant of transformed soybean.

3.10.4.2. Genotyping and analysis of gene integration in transformed plants

GUS positive transformed lines were characterized for its genotype by PCR amplification of total genomic DNA using *hptII* gene and *S. platensis* D6d gene specific primers (as indicated in Table 18 under section 2.6.9.1). Only the hygromycin resistant GUS positive lines showed a single amplification product of expected size (~1.2 kb), whereas no such amplified product was obtained in the untransformed control lines (Figure 26) indicating the presence of *S. platensis* D6d gene into the host plant genome.

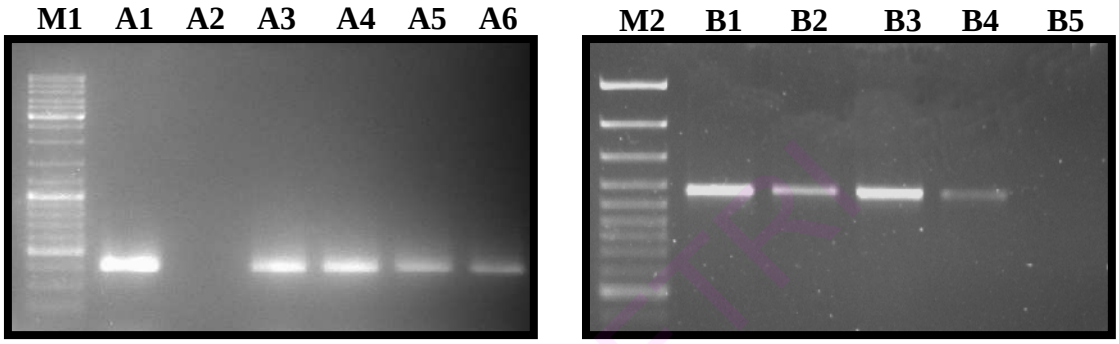

Figure 26. Genotypic analysis of transformed soybean lines through PCR amplification of *hptII* and D6d gene specific primers; A1 to A6 – PCR amplicon (480 bp) of *hptII* gene specific primers (*hptII* F & *hptII* R); A1- positive control (pCAMBIAD6d), A2 - control plant, A3 to A6 - transformed plants, M1 – 10 kb DNA ladder; B1 to B5 – PCR amplicon (1200 kb) of D6d gene specific full length primers ($D6dF_3$ & $D6dR_3$); B1 to B4 - transformed plants, B5 - control plant, M2 – 3 kb DNA ladder.

3.10.4.3. Southern blot analysis

Introgression of the D6d gene in the T_0 generation of the transformed plants were confirmed by Southern blot analysis with *hptII* gene as probe (Figure 27). For this, total genomic DNA was digested with *Xba I* and *Cfr 421*. It is expected that, the restriction enzyme *Xba I* would cut once through the multiple cloning site and the enzyme *Cfr421* would cut outside the T-DNA area. The separated fragments were hybridized with the *hptII* gene specific probe generating a hybridizable 2.6 kb fragment as confirmed by similarly digested pCAMBIAD6d plasmid DNA. Two to three bands of different sizes were obtained (Figure 27) when genomic DNA of transformed plants were digested and probed as above. Hybridization with *hptII* gene as probe confirmed the introgression of the D6d gene in the plant genome.

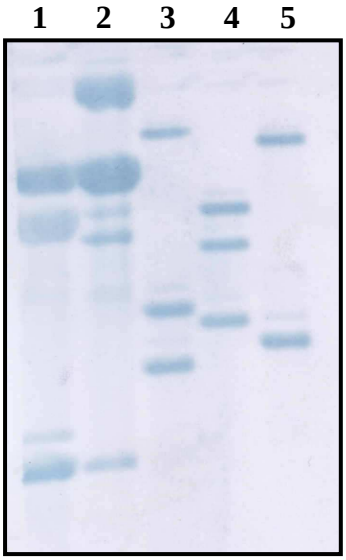

Figure 27. Southern blot showing the introgression of *S. platensis* D6D gene in the genome of transformed soybean plants; Lane 1 - positive control DNA of plasmid pCAMBIA1305.2; Lane 2 - plasmid DNA of pCAMBIAD6d; and lane 3 to 5 - genomic DNA of transformed soybean plants. The DNA was double digested with *Xba I* and *Cfr 421* hybridized with hpt gene probe.

3.10.4.4. Fatty acid analysis

Fatty acid methyl esters (FAMEs) were prepared both from transformed and untransformed soybean seeds and are analyzed by gas liquid chromatography (GC). Representative GC profiles are shown in Figure 28. FAME chromatogram of soybean transformants showed two prominent peaks in addition to ALA (Figure 28c) which are not present in the control plants (Figure 28b). These peaks had retention times identical to the FAME standards of GLA (Figure 28a) and OTA. GC-MS analysis of the FAMEs further confirmed the identity of these peaks. The mass peak *m/z* = 292 and 290 indicated the molecular mass of the methyl derivative of GLA and OTA respectively (Figure 29). The fragmentation pattern was also identical to that of the standards.

The proportions of C18 fatty acids from the total lipid fractions prepared from the seeds of control and transformed soybean plants are given in Table 27. Both the delta-6 desaturated products (*viz*., GLA and OTA) are absent in the control plants, whereas they account for about 3.8% and 6.2% of the total C18 lipids in the transformed plants respectively. The presence of both GLA and OTA indicates that the delta-6 desaturase used both linoleic and α-linolenic acid as substrates and this may be responsible for the

decreased contents of linoleic and α-linolenic acids observed in the transgenic plants. These results suggest that the recombinant vector pCAMBIAD6d encoding the enzyme delta-6 fatty acid desaturase is functionally expressed in the transgenic soybean plants by catalyzing the formation of GLA and OTA, from linoleic and α-linolenic acids respectively.

Table 27. C18 Fatty acid composition of transformed and control seeds of soybean cv. JS 335*

Fatty acids	C18 Fatty acid composition (%)	
	Control	Transformants
Stearic acid (C18:0)	5.8 ± 1.2	5.2 ± 1.6
Oleic acid (C18:1)	22.5 ± 3.1	20.6 ± 2.6
Linoleic acid (C18:2)	55.2 ± 4.1	53.1 ± 3.7
α-linolenic acid (C18:3)	14.8 ± 2.8	9.6 ± 2.1
γ-linolenic acid (C18:3)	0.00	3.8 ± 1.8
OTA (C18:4)	0.00	6.2 ± 2.4

*Values are mean ± SD

The fatty acid composition of transgenic seeds is shown in Table 27. It is clear that the production of delta-6 desaturated fatty acids occurs at the cost of two major fatty acids, linoleic and α-linolenic acid. Proportions of oleic and stearic acids in transgenics are slightly, but not significantly, reduced compared with those of the control. The content of α-linolenic acid in the transgenics was dramatically reduced. In the untransformed plants, α-linolenic acid accounts for more than 15% of the total C18 fatty acids, whereas in transgenics, the level was reduced to 9.7%. As compared with the reduction of α-linolenic acid in transgenics, the decrease in linoleic acid in transgenics is less dramatic but still significant. In the untransformed control plants, linoleic acid accounts for more than 56% of the total C18 fatty acids in seeds, whereas in transgenics, the level was reduced to less than 54%. The results are expected due to the fact that these two fatty acids form the substrate for the delta-6 desaturase enzyme. However, the expression of GLA and OTA is a value addition of significance from the point of view of enhancement of nutritional quality of oil.

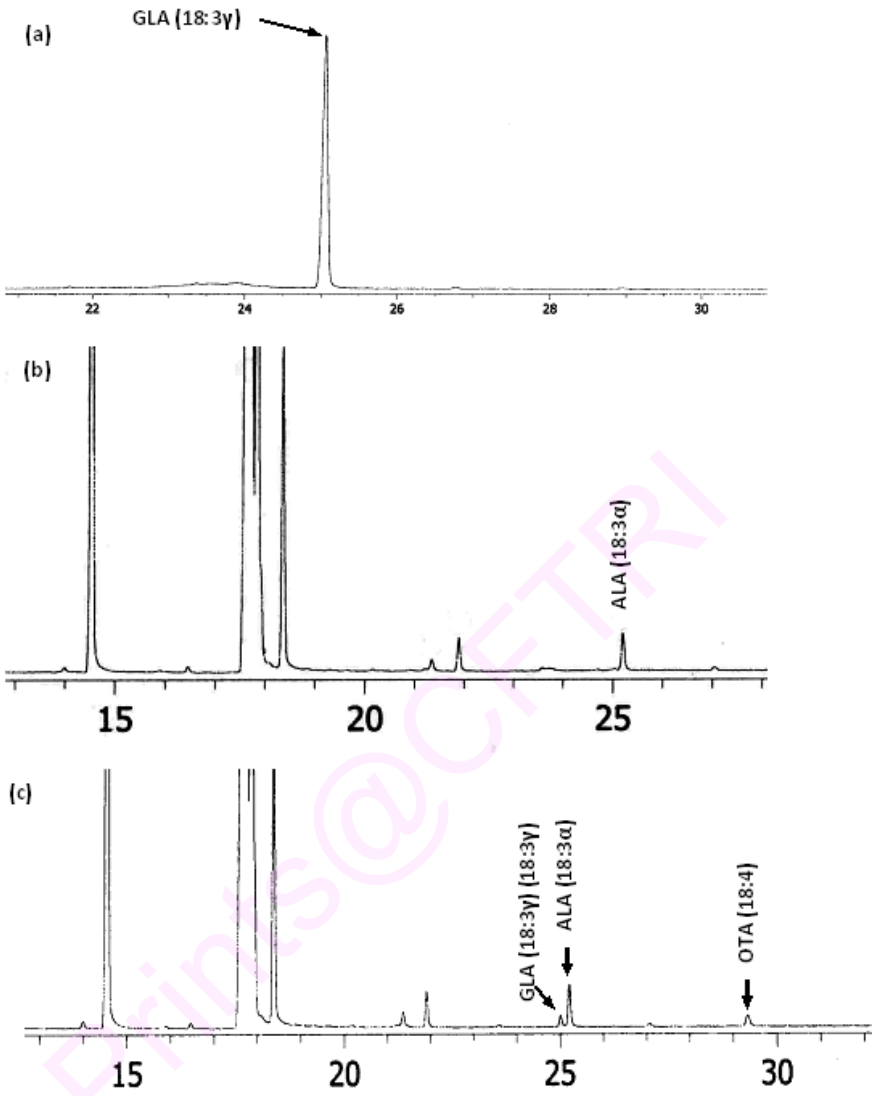

Figure 28. Gas chromatographic analysis of fatty acid methyl ester (FAME) derivatives from mature seeds of soybean cv. JS 335; (a) FAME derivative of GLA standard; (b) untransformed control plant; and (c) transformed plant showing the presence of GLA and OTA.

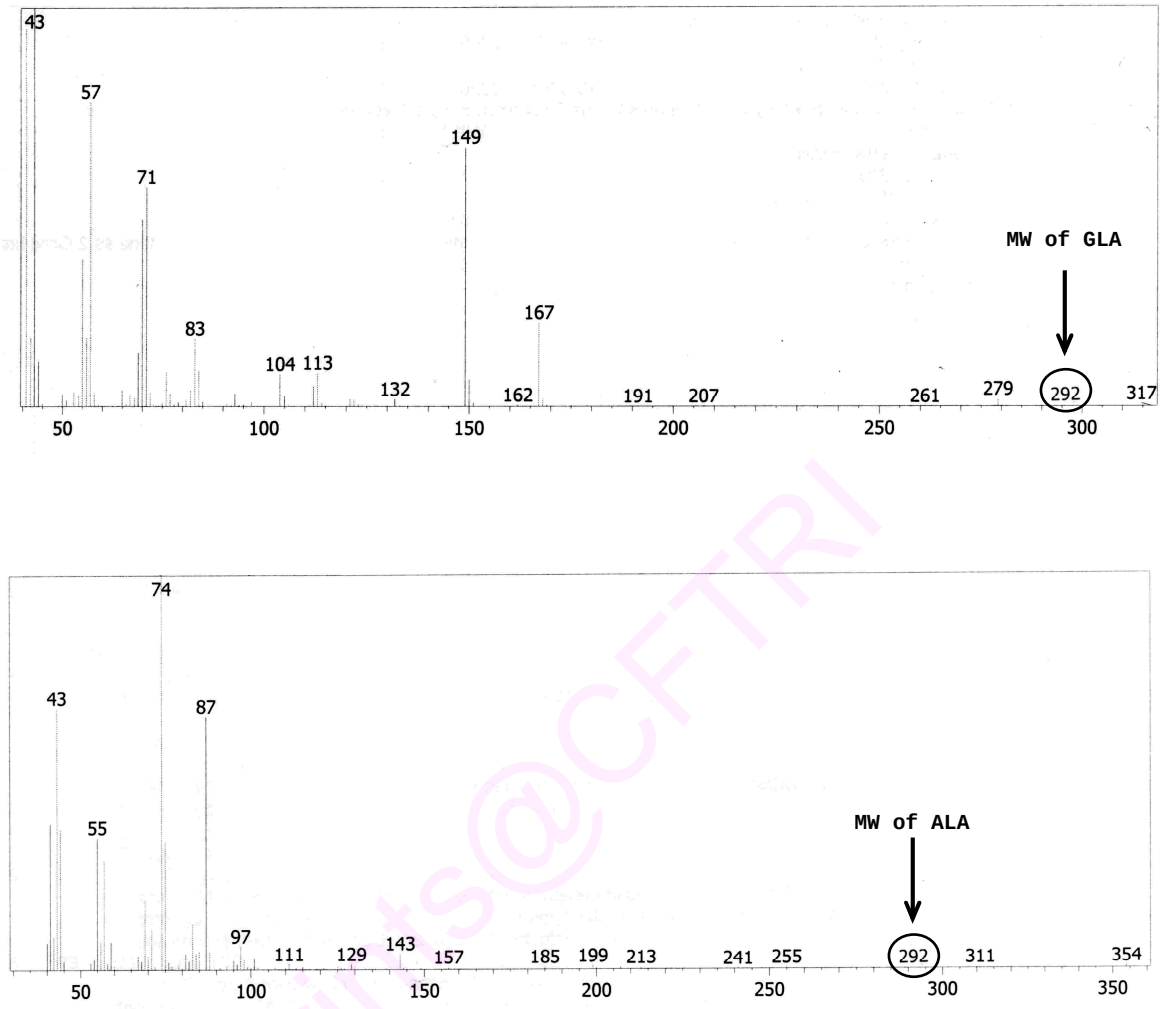

Figure 29. Mass spectrum obtained from the GC–MS analysis of the methyl ester derivatives of the fatty acids extracted from transformed soybean seeds; (a) fragmentation pattern of GLA; and (b) Fragmentation pattern of ALA as analyzed by GC-MS.

3.10.4.5. Isoflavone analysis

A typical HPLC chromatogram of all the 12 isoflavone compounds present in soybean seeds is shown in Figure 30. Genistein, daidzein and glycitein as well as their β-glucosides (genistin, daidzin, glycitin), acetyl-β-glucosides (acetylgenistin, acetyldaidzin, acetylglycitin) and malonyl-β-glucosides (malonylgenistin, malonyldaidzin, malonylglycitin) were successfully separated and identified using the applied HPLC conditions. Isoflavone contents of the transformed and control plants of the soybean cultivar JS 335 as analyzed by HPLC are shown in Table 28.

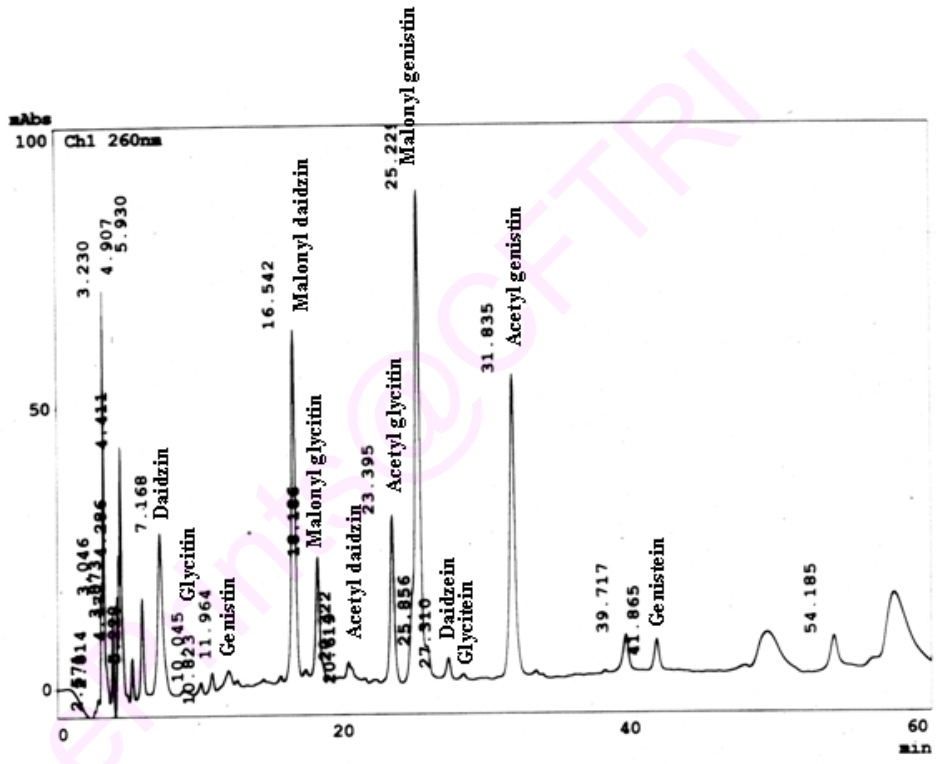

Figure 30. Chromatogram of all 12 isoflavone compounds in soybean seeds of cv. JS 335

Table 28. Isoflavone concentration of control and transformed soybean seeds of cv. JS 335 (dry weight basis)[a]

Isoflavones	Control ($\mu g\ g^{-1}$)	Transformants ($\mu g\ g^{-1}$)
Daidzin	38.6 ± 1.82	50.0 ± 5.59
Malonyl daidzin	212.8 ± 8.78	198.9 ± 9.54
Acetyl daidzin	3.9 ± 1.06	6.1 ± 0.82
Daidzein	15.0 ± 3.02	18.0 ± 0.41
Glycitin	15.7 ± 1.20	24.2 ± 1.50
Malonyl glycitin	23.3 ± 1.95	26.1 ± 1.99
Acetyl glycitin	0.5 ± 0.25	2.6 ± 0.26
Glycitein	nd	nd
Genistin	51.2 ± 2.88	60.7 ± 2.71
Malonyl genistin	382.0 ± 9.70	448.3 ± 5.27
Acetyl genistin	1.1 ± 0.33	5.4 ± 1.47
Genistein	9.3 ± 0.50	7.3 ± 0.51
Total Isoflavones	753.4 ± 3.65	847.4 ± 5.19

[a]Values are mean ± SD; nd-not detected

The total isoflavone content of transformed and untransformed soybean plants were 753.4 and 847.4 $\mu g\ g^{-1}$ respectively. Among the total isoflavones, genistin and its glucosides accounts for a major share of 59 and 62%, followed by daidzein and its glucosides (36 and 32%), and glycitein and its glucosides (5 and 6%) in case of untransformed and transformed plants respectively. Among the glucosides the malonyl glucosides accounts for around 80%, followed by the β-glucosides (15%), aglycones (3%) and acetyl glucosides (1%) of the total isoflavones in both the control and transformed plants. Thus there was no significant variation among the isoflavone compounds between the control and transformed soybean plants. Although the total isoflavone contents varied between the control and transformed plants with the later recording higher isoflavone content of 12% compared to the control, the increase was not statistically significant as analyzed by student t- test at 5% probability level.

3.11. Discussion

Many attempts have been made in the cloning, characterization and manipulation of fatty acid desaturases from various sources over the last few years. Interest in this field can be explained by the ability of fatty acid desaturases to produce a wide range of PUFA of nutritional and pharmaceutical importance (Damude and Kinney, 2008). Here we provide evidence for the identification of the D6d gene product as functional delta-6 desaturases. First, a high similarity of their protein sequences was found with other previously characterized D6d, mainly of *Spirulina maxima* and *Synechocystis sp.* belonging to cyanobacteria. However, as it has been pointed out (Napier et al., 1999), sequence similarity among desaturases should be considered cautiously before assigning a particular function to an unknown protein. For example, a sunflower desaturase, showing a high homology to the *Borago* D6d (BOD6DES), was identified as a Δ^8-desaturase active on sphingolipids rather than on glycerolipids (Sperling et al., 1998). This is further supported by directed mutagenesis experiments in which few amino acid changes in a desaturase resulted in drastic alterations in the substrate specificity regarding acyl chain length and desaturation position (Cahoon et al., 1997). Nevertheless, clustering analysis of desaturases from other sources separates with a high reliability the group integrated by *Spirulina* desaturase from that of the other fatty acid desaturases, thus indicating that the cloned *Spirulina* products are likely to be delta-6 desaturase.

The alignment of delta-6 desaturases revealed three histidine rich motifs conserved among all the fatty acid desaturases, together with two histidine residues adjacent to the third motif, which are conserved among the acyl-lipid desaturases of cyanobacteria. Hongsthong et al., (2004) by mutation studies found that the conserved amino acids among cyanobacterial D6d *viz*., H313, H315, G136 and E140 are critical for desaturase activity and are thus likely to be located on the cytoplasmic phase of the membrane. They also proposed that these residues play a part in forming the active site, while the three histidine motifs possibly plays a role in providing the iron catalytic center. Diaz et al., (2002) reported that the hydropathy plots of several acyl lipid desaturases show the presence of a hydrophobic segment located between the first two histidine clusters. Thus they proposed that this hydrophobic segment might be involved in substrate recognition.

The fatty acid profile in soybean is approximately 13% palmitic, 4% stearic, 18% oleic, 55% linoleic and 10% α-linolenic acids. This is suggestive that a significant substrate pool for the delta-6 desaturase activity is present, making this crop an ideal target for the production of these two novel fatty acids. The results presented here demonstrate that using a cyanobacterial delta-6 desaturase gene, transgenic soybean lines can be obtained that can produce significant levels of these novel fatty acids (Table 27). Nutritional benefits have been attributed to diets supplemented with GLA. The total lipid content of the transformed seeds of the soybean cv. JS 335 was 22%, in which GLA accounts for ~ 3.4% of the total fatty acids. Horrobin (1992) suggested a range of daily intake of 25-50 mg of GLA for nutritional purposes, 100-150 mg for therapeutic use and 500-2000 mg of GLA to provide pharmacological effects. Also the per capita intake of edible oil was estimated to be 20 g per day (WHO, 2009). Thus the GLA content of the obtained transgenic plants is sufficient enough to meet the daily requirement for nutritional purposes. In addition, OTA the precursor to endogenous EPA and DHA acid may also act as an effective omega-3 fatty acid from nutritional point of view

Modulating seed metabolism in a major oil crop such as soybean can serve as a cost effective route for the production of high value molecules such as GLA and OTA. This in turn provides the consumer an additional option to acquire the health benefits from these nutraceuticals without altering their dietary consumption (Knutzon and Knauf, 1998). In the present study however, the level of GLA is not as high as the reported expression in planta of the eukaryotic D6d either alone or in combination with delta-12 desaturase (Palombo et al., 2000; Liu et al., 2001; Hong et al., 2002). Tobacco and flax transformed with borage D6d under the control of CaMV35S promoter showed high level of GLA accumulation in vegetative tissues but in seeds the level was low (Sayanova et al., 1999; Qiu et al., 2002). Moderate production of GLA (3–9%) in the seed of *Brassica juncea* was obtained by Qiu et al., (2002) when borage D6d gene was expressed under the control of napin promoter. Expression of a D6d along with *Mortierella alpina* delta-9 desaturase under the control of napin promoter showed high level of GLA production (43%) in seeds of *Brassica napus* (Liu et al., 2001; Palombo et al., 2000). Upto 40% of GLA accumulation in the seeds of *Brassica juncea* was obtained by Hong et al., (2002) upon transformation with *Pythium irregulare* D6d. It has been proposed that the presence of a consensus heme-binding motif, called cytochrome b5 like domain, at N-terminus of D6d protein, exhibits an essential role in transferring electrons during desaturation process (Napier et al., 1999; Sayanova et al., 1999; Sayanova and Napier, 2004). Such

domain is absent in the *Spirulina platensis* D6d protein, unlike other eukaryotic D6d (Laoten et al., 2000). This may contribute towards the low level of cyanobacterial D6d activity than that of the eukaryotic desaturase in planta. Nonetheless, in the present study, we obtained significant level of GLA production as compared to the *Synecocystis* D6d expression, previously reported in tobacco by Reddy and Thomas (1996).

In the earlier reports, the target crops were transformed with eukaryotic D6d. This is the first report on transformation of soybean with a prokaryotic D6d for the production of GLA that can be considered as an avenue for use of prokaryotic desaturase gene(s) to alter seed oil composition of crop plant. Presence of considerably higher amount of OTA in seeds of transformed lines (6.2%, Table 27) with respect to GLA (3.8%) under the present conditions, indicate that ALA serves as a better substrate for the cyanobacterial D6d rather than linoleic acid. Synthesis of higher amount of OTA in the seeds of transgenic plants, as compared to those reported earlier, opened up the possibilities of these transgenic lines to be considered as value added crop with industrial utilization. Further manipulation of these D6d expressing soybean lines with acyl-transferase gene for accumulation of GLA in seeds to a greater extent, may make them nutritionally a more acceptable oilseed crop.

Intake of soy isoflavones and polyunsaturated fatty acids compliment each other in reducing menopausal disorders, breast cancer, osteoporosis and age related disorders in humans and animals (Bruce et al., 2005; Lucas et al., 2009). Hence isoflavones levels and fatty acid composition of soybean were analyzed in the control and transformed plants. The results indicated that while the fatty acid profile is altered by the formation of GLA and octadecatetraenoic acid, there is no significant difference in the isoflavone composition of transformed and untransformed plants. These results suggest that there is no negative effect of delta-6 desaturase on the isoflavone content and composition of soybean seeds between the control and transformed soybean seeds. The marginal increase in the total isoflavones content of transformed soybean seeds compared with that of the control may be attributed to the stress factor (Sakthivelu et al., 2008) that the transgenic plants is possibly exposed to due to the integration of foreign gene(s).

3.12. Summary

The 1.2 kb coding region of the delta-6 desaturase gene from *Spirulina platensis* was isolated by polymerase chain reaction using gene specific primers and inserted in between the *Xho I* and *Xba I* site of the plant expression vector pRT 100 by directional cloning. The recombinant plasmid was verified by restriction digestion, PCR and gene sequencing. Computational analysis of the deduced amino acids from the cloned gene sequence showed 98% similarity with the targeted protein sequence. Multiple sequence alignment revealed three conserved Histidine motifs among the compared delta-6 desaturase enzymes of cyanobacteria, fungus and higher plants. The expression cassette (delta-6 desaturase gene along with CaMV35S promoter and 35S poly A terminator) was further sub-cloned into the *Hind III* restriction site of the binary vector pCAMBIA 1305.2, to obtain the recombinant binary plasmid pCAMBIAD6d. The resultant recombinant plasmid was subsequently used to transform soybean cv. JS 335 by *Agrobacterium* mediated transformation technique. The transformed plants showed the production of GLA (3.8%) and OTA (6.2%) in contrast to their absence in the untransformed control plants adding evidence for the introgression and functional expression of the cyanobacterial D6d gene in soybean. Moreover there was no deleterious or unintended effect of this genetic transformation (with D6d gene) in the transgenic plants, as noticed for the isoflavones levels, which is important for this crop from bioefficacy point of view. The level of expression of GLA obtained in this study is likely to be sufficient to meet the daily requirements from nutrition point of view.

Summary & Conclusion

SUMMARY OF RESULTS

Research work undertaken for this study was aimed to establish a genetic transformation protocol for soybean and to express the delta-6 desaturase gene for the production of gamma linolenic acid in soybean. The major findings and the future prospects of this entire research work can be summarized as follows,

1. Soybean genotypes (Hardee and JS 335) when subjected to *in vitro* regeneration with different plant hormones viz., TDZ, BAP and GA_3 against the explants hypocotyl and cotyledonary node recorded a maximum frequency of shoot bud tissues for cotyledonary nodes in the media containing 0.2 mg l^{-1} Thidiazuron. Elongation of these shoot buds was best achieved in 0.5 mg l^{-1} GA_3, compared to other combinations. Among the explants cotyledonary nodal explants gave a maximum of 8 shoots/ explant. The shoot buds showed better elongation with maximum shoot length of 5.7 cm on medium containing 0.5 mg l^{-1} GA_3. The regenerated shoots were rooted on MS basal medium with 0.5 mg l^{-1} Indole butyric acid (IBA).

2. Triacontanol at low concentration of 5 µg l^{-1} significantly increased the frequency of shoot buds for both cotyledonary node and shoot tip explants compared to the control. A maximum of 14 shoots/ explant was obtained from the cotyledonary nodal explants on media containing 50 µg l^{-1} GA_3 and 5 µg l^{-1} TRIA with an average shoot length of 10 cm. TRIA also increased the number of roots proportionately upto 10 µg l^{-1}, enhanced root length with a reduced length of internodes and stimulated branching alone and also in combination with other hormones. It was concluded that triacontanol can be successfully used as a growth regulator in both the multiplication and rooting phase of soybean micro propagation.

3. The *Agrobacterium tumefaciens* mediated transformation efficiency of soybean as analyzed by transient GUS expression showed 33.7% for embryonic axis and 17.9% for cotyledonary node explants. Addition of cysteine during co cultivation enhanced the T-DNA delivery into the cells with a notable decrease in enzymatic browning of the explants. Also a resting period of 7 days was found to improve the selection of transgenics by eliminating selection pressure in the early shoot induction phase. It was found that the embryonic axis transformation system has

advantages in terms of easy handling and less time consuming as compared with cotyledonary nodal transformation system and hence can be effectively used for genetic improvement of soybean.

4. The full length gene sequence (1239 kb) of delta-6 desaturase gene was amplified from *Spirulina platensis* by polymerase chain reaction and cloned into the binary vector pCAMBIA1305.2 at the *Hind III* site, under the control of CaMV35S constitutive promoter and 35S poly A terminator to generate the recombinant binary vector pCAMBIAD6d. The cloned delta-6 desaturase gene sequence exhibited 98% similarity with the target protein sequence and showed three histidine rich motifs, which may act as an active site for the enzyme activity.

5. *Agrobacterium* mediated transformation of embryonic axis explants of soybean cv. JS 335 with the recombinant plasmid (pCAMBIAD6d) resulted in a transformation efficiency of 4%. The fatty acid profile of putative transformed plants showed the formation of GLA (3.8%) and octadecatetraenoic acid (6.2%) in contrast to their absence in the control plants adding evidence for the introgression and functional expression of the cyanobacterial delta-6 desaturase gene in soybean. The isoflavone profiles of transformed and control plants did not show any significant difference. The results indicated that while the fatty acid profile is altered by the formation of GLA and octadecatetraenoic acid, there is no unintended effects of the cloned gene, especially on isoflavones levels, which is an important phytochemical constituent of soybean seeds.

In recent years engineering of fatty acid pathway in higher plants for enhancing their seed oil content and composition is gaining importance. The results from the present study suggest that cyanobacterial desaturase gene(s) is useful for producing GLA in oilseed crops. The level of expression of GLA obtained in this study is likely to be sufficient to meet the daily requirements of GLA from nutrition point of view. Further selection and manipulation of these delta-6 desaturase expressing soybean lines for stability and enhanced accumulation of GLA in seeds will result in a nutritionally superior vegetable oil crop.

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Appendix

List of appendices

Appendix A:

Luria and Bertaini (LB) media composition:

Component	Amount (g l^{-1})
Tryptone	10
Yeast extract	5
NaCl	5

Dissolve in dd H_2O and adjust the volume to one litre. Adjust the p^H to 7 with 2 *N* NaOH and autoclave. For solid media, add 15 g l^{-1} agar and autoclave.

Appendix B:

Preparation of 50 TAE buffer:

Dissolve 242 g of Tris-base in 800 ml sterile dd H_2O. Add 57.1 ml glacial acetic acid and 100 ml of 0.5 *M* EDTA of pH 8.0 [Dissolve 93.06 g $Na_2EDTA.2H_2O$ in 300 ml of dd H_2O. Adjust the pH to 8.0 with 2 *N* NaOH and the final volume to 500 ml with dd H_2O]. Adjust the volume to one litre and store at room temperature.

Appendix C:

Zarrouk's media composition:

The following chemicals are weighed separately and mixed in the distilled water.

Chemical compound	Formula	g l^{-1}
Solution A		
Sodium hydrogen carbonate	$NaHCO_3$	16.0
Solution B		
Dipottasium hydrogen phosphate	K_2HPO_4	0.5
Sodium nitrate	$NaNO_3$	2.5
Potassium sulphate	K_2SO_4	1.0
Sodium chloride	NaCl	1.0
Magnesium sulphate	$MgSO_4$	0.2
Calcium chloride	$CaCl_2$	0.04
Ferrous sulphate	$FeSO_4$	0.01
Ethylenediamino tetrachloro acetic acid	EDTA	0.08
Trace elements (A_5 Solution)*		1 ml l^{-1}

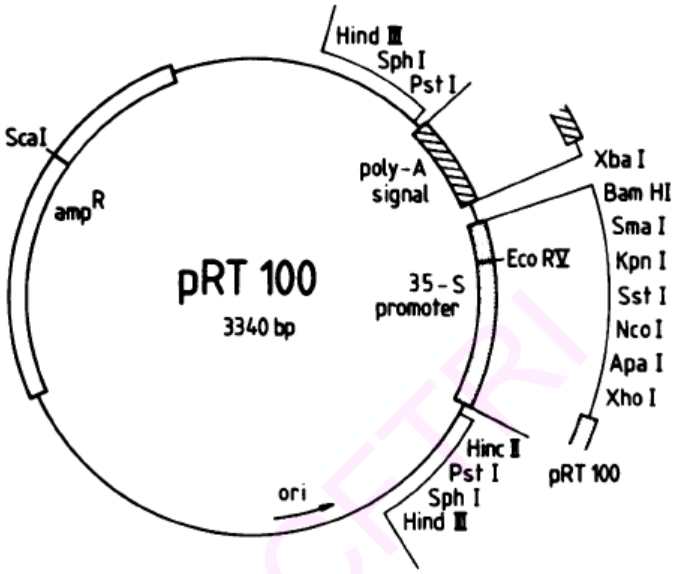
*****A_5 Solution Preparation

Chemical compound	g l^{-1}
Manganese chloride	1.81
Molybdic acid	0.017
Zinc sulphate	0.222
Copper sulphate	0.079
Boric acid	2.86

Autoclave solutions A and B separately in conical flasks. After autoclaving and cooling, they should be mixed well before use. For preparation of slants add agar (2.0%) in solution B, before autoclaving.

Appendix D:

Vector map of pRT 100



The plasmid pRT100 (3340 bp) carrying the 35S promoter (CaMV 35S) and the polyadenylation (poly A) signal of CaMV strain Cabb B-D were constructed in modified polylinkers of pUC18/192.

Appendix E:

Preparation of chemically competent cells: Chemically competent cells of the *E. coli* strain DH5α can be prepared as follows,

1. Streak out a loop of the E. coli strain from the frozen glycerol stock stored at -70 °C onto the surface of an LB plate using a sterile platinum wire loop.
2. Invert the plate and incubate at 37 °C for 12 to 16 h. Bacterial colonies will become visible.
3. Loop out a single colony from the plate and inoculate in 10 ml of LB media. Incubate at 37 °C overnight with shaking at 200 rpm.
4. Add 200 µl of the bacterial culture to 50 ml of fresh LB media and incubate at 37 °C for 12 to 16 h, until the OD_{600} reaches 0.45 to 0.55.

5. Transfer the cells in 1.5 ml eppendorf tubes and keep them in ice for 10 mins. Recover the cells by centrifuging at 4000 g for 10 min at 4 °C
6. Drain off the supernatant and resuspend the cells in 1 ml of ice-cold 0.1M $CaCl_2$ and incubate them in ice for 45 mins.
7. Recover the cells by centrifuging at 4000 g for 10 mins at 4 °C and gently resuspend them in 200 µl of ice cold 0.1M $CaCl_2$ solution.
8. Store the cells in 20% glycerol at -70 °C until use.

Appendix F:

Transformation protocol for chemically competent cells of *E. coli* strain DH5α with recombinant plasmids:

1. Thaw the frozen competent cells and distribute them into 4 eppendorf tubes with 50 µl each.
2. Add the recombinant plasmid (ligated material) 2 µl, 4 µl etc., into the eppendorf tubes and incubate on ice for 30 mins.
3. Heat shock at 42 °C for 2 min and place on ice for 1 min.
4. Add 800 µl of LB liquid media and vigorously shake at 200 rpm at 37 °C for 30 mins.
5. Recover the cells by centrifuging at 4000 g for 5 mins and plate 50 µl of culture per LB plate containing 50 µg ml^{-1} ampicillin. Quickly spread the cells over the entire surface using a flame sterile bent glass rod.
6. Invert the plates and incubate them in a 37 °C incubator for 12 to 16 h until the colonies are visible.

Appendix G:

Transformation of *Agrobacterium tumefaciens* with binary plasmid (Freeze-thawing method):

1. Prepare the competent cells of *Agrobacterium tumefaciens* strain EHA 105 as mentioned in Appendix E.
2. Thaw the competent glycerol stocks of *Agrobacterium tumefaciens* strain EHA 105 strain (100 µl) on ice for 5 min.
3. Add (1µg μl^{-1}) of the recombinant plasmid to the competent cells and mix by gentle tapping.
4. Freeze the mixture in liquid N_2 for 5 min, followed by thawing at 37 °C for 5 min and transfer to ice for 2 min.
5. Add 900 µl of LB media and vigorously shake for 200 rpm for 1 h at 28 °C.
6. Pellet the content and plate 50 µl of the suspension in LB plates with 1 mg l^{-1} rifampicin and 50 mg l^{-1} kanamycin along with X-gal and IPTG for blue white colony screening.
7. Incubate them at 28 °C for 24 h until blue-white colonies are visible.

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References

LIST OF REFERENCES

Abbadi, A., Domergue, F., Bauer, J., Napier, J. A., Welti, R., Zahringer, U., Cirpus, P. and Heinz, E. (2004) Biosynthesis of very-long-chain polyunsaturated fatty acids in transgenic oilseeds: constraints on their accumulation. *Plant Cell,* 16: 2734–2748.

Adam, O. (1989) Linoleic and Linolenic Acids Intake. In: Dietary omega-3 and omega-6 fatty acids: Biological effects and Nutritional Essentiality. Series A: Life Sciences. Vol. 171. C Galli and AP Simopoulos, eds; pp 391-402. New York: Plenum-Press.

Agaba, M., Tocher, D. R., Dickson, C. A., Dick, J. R. and Teale, A. J. (2004) Zebrafish cDNA encoding multifunctional fatty acid elongase involved in production of eicosapentaenoic (20:5n-3) and docosahexaenoic (22:6n-3) acids, *Marine Biotechnol.*, 6: 251–261.

Alonso, L. D. and Maroto, F. G. (2000) Plants as 'chemical factories' for the production of polyunsaturated fatty acids. *Biotechnology Advances,* 18: 481–497.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990) Basic Local Alignment Search Tool. *J. Mol. Biol*., 215: 403-410.

Anthony, M. S. (2000) Soy and Cardiovascular Disease: Cholesterol Lowering and Beyond. *J. Nutr.*, 130: 662S–663S.

Arao, T. and Yamada, M. (1994) Biosynthesis of polyunsaturated fatty acids in the marine diatom *Phaeodactylum tricornutum*. *Phytochemistry*, 35:1177–81.

Arao, T., Sakaki, T. and Yamada, M. (1994) Biosynthesis of polyunsaturated lipids in the diatom, *Phaeodactylum tricornutum*. *Phytochemistry*, 36: 629–35.

Awang, D. V. C. (1990) Borage. *Can. Pharm. J.*, 123: 121-126.

Azam, M. M. E., Singh, P. and Ghanim, A. (1997) Triacontanol and triterpenes from *Tecomella undulata*. In: Mukherjii AK (ed) World Forestry Congress, Anatylya, Turkey 13–22 Oct. 1997 (Vol 3) topic 15.

Bailey, M. A., Boerma, H. R. and Parrott, W. A. (1994) Inheritance of tumor formation in response to *Agrobacterium tumefaciens* in soybean. *Crop Sci.*, 34: 514–519.

Bailey, M. A., Boerma, H.R. and Parrott, W. A. (1993) Genotype-specific optimization of plant regeneration from somatic embryos of soybean. *Plant Sci*., 93: 117–120.

Bajpai, P. K., Bajpai, P. and Ward, O. P. (1991) Optimization of production of docosahexaenoic acid (DHA) by *Thraustochytrium aureum* ATCC 34304. *J. Am. Oil Chem. Soc.,* 68: 509–514.

Bajpai, P. and Bajpai, P. K. (1993) Eicosapentaenoic acid (EPA) production from microorganisms: a review. *J. Biotechnol.,* 30: 161–83.

Balyan, S. S., Pal, S. and Dutt, P. (1994) Triacontanol effect on growth and yield parameters of CKP-25 variety lemongrass w. Ind. *Perfum.,* 38: 60–64.

Bandyopadhyay, S., Ghosh, S., Chaudhuri, S. S. and Bhattacharyya, D. K. (2001) Production of γ-linolenic acid by *Rhizopus nigricans* sssd-8. *J. of Oleo Science,* 50: 641-647.

Barclay, W. R., Meager, K.M. and Abril, J.R. (1994) Heterotrophic production of long-chain omega-3 fatty acids utilizing algae and algae-like microorganisms. *J. Appl. Phycol.,* 6: 123–129.

Barnes, W.M. (1977) Plasmid detection and sizing in single colony lysates. *Science*, 195: 393-394.

Barre, D. E. (2001) Potential of evening primrose, borage, black currant and fungal oils in human health, *Ann. Nutr. Metab.,* 45: 47–57.

Barwale, U. B., Meyer, M. M. and Widholm, J. M. (1986) Screening of *Glycine max* and *Glycine soja* genotypes for multiple shoot formation at the cotyledonary node. *Theor. Appl. Genet.,* 72: 423–428.

Beaudoin, F., Michaelson, L. V., Hey, S. J., Lewis, M. J., Shewry, P. R. and Sayanova, O. (2000) Heterologous reconstitution in yeast of the polyunsaturated fatty acid biosynthetic pathway, *Proc. Natl. Acad. Sci. USA,* 97: 6421–6426.

Belarbi, E. H., Molina, E. and Chisti, Y. (2000) A process for high yield and scaleable recovery of high purity eicosapentaenoic acid esters from microalgae and fish oil. *Enzyme Microbiol. Technol.,* 26: 516–29.

Bhella, R. S. and MacKenzie, S. L. (1995) Nucleotide sequence of a cDNA from *Limnanthes douglasii* L. encoding a delta-15 linoleic acid desaturase. *Plant Physiol.,* 108: 861.

Biernbaum, J. A., Houtz, R. L. and Ries, S. K. (1988) Field studies with crops treated with colloidally dispersed triacontanol. *J. Am. Soc. Hort. Sci.*, 113: 679–684.

Bligh, E. G. and Dyer, W. J. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.*, 37: 911–917.

Bolton, G. W., Nester, E. W. and Gordon, M. P. (1986) Plant phenolic compounds induce expression of the *Agrobacterium tumefaciens* loci needed for virulence. *Science*, 232: 983–985.

Booth, J. R., Cahoon, R. E., Hitz, W. D., Kinney, A. J. and Yadav, N. S. Inventors. March 8, 2006. Nucleotide sequences of a new class of diverged delta-9 stearoyl-ACP desaturase genes. *European Patent Publication*, EP1311659.

Bowen, B. A. (1993) Markers for plant gene transfer. In: Kung S, Wu R (eds) Transgenic plants: engineering and utilization, vol 1. Academic Press, San Diego, pp 89–123.

Broun, P., Gettner, S. and Somerville, C. (1999) Genetic engineering of plant lipids. *Ann. Rev. Nutr.*, 19: 197-216.

Brown, P. (1994) Baby formula needs essential extras. *New Sci.*, November: 1952.

Browse, J. and Somerville, C. (1991) Glycerolipid synthesis: biochemistry and regulation. *Ann. Rev. Plant Physiol. Plant Mol. Biol.*, 42: 467–506.

Bruce, A., Watkins, B. A., Reinwald, S., Li, Y. and Seifert, M. F. (2005) Protective actions of soy isoflavones and n-3 PUFAs on bone mass in ovariectomized rats. *J. of Nutr. Biochem.*, 16: 479-488.

Budziszewski, G. J., Croft, K. P. C. and Hildebrand, D. F. (1996) Uses of biotechnology in modifying plant lipids. *Lipids*, 31: 557–69.

Buhr, T., Sato, S., Ebrahim, F., Xing, A., Zhou, Y., Mathiesen, M., Schweiger, B., Kinney, A. J., Staswick, P. and Clemente, T. (2002) Ribozyme termination of RNA transcripts down-regulate seed fatty acid genes in transgenic soybean. Pl*ant J.*, 30: 155–163.

Byrne, M. C., McDonnell, R. E., Wright, M. S. and Carnes, M. G. (1987) Strain and cultivar specificity in the *Agrobacterium*-soybean interaction. *Plant Cell Tissue Organ Cult.*, 8: 3–15.

Cahoon, E. B., Lindqvist, Y., Schneider, G., and Shanklin, J. (1997) Redesign of Soluble Fatty Acid Desaturases from Plants for Altered Substrate Specificity and Double Bond Position. *Proc. Natl. Acad. Sci., USA*, 94: 4871–4877.

Cardoza, V. and Stewart, C. N. (2004) *Brassica* biotechnology: progress in cellular and molecular biology. *In Vitro Cell. Dev. Biol. Plant*, 40: 542-551.

Carlson, J. B. and Lersten, N. R. (1987) Reproductive morphology. In: Soybeans: Improvement, Production, and Uses; Second Edition. Ed. J. R. Wilcox. pp. 95-134. American Society of Agronomy. Madison, Wisconsin.

Carmen, S. J. M., Ballester, A. and Vieitez, A. M. (2001) Effect of thidiazuron on multiple shoot induction and plant regeneration from cotyledonary nodes of chestnut. *J. Hort. Sci. Biotechnol.*, 76: 588–595.

Carroll, K. K. and Kurowska, E. M. (1995) Soy Consumption and Cholesterol Reduction: Review of Animal and Human Studies, *J. Nutr.*, 125: 594S–597S.

Cassagne, C., Lessire, R., Bessoule, J. J., Moreau, P., Creach, A., Schneider, F. and Sturbois, B. (1994) Biosynthesis of very long chain fatty acids in higher plants. *Prog. Lipid Res.*, 33: 55–69.

Certik, M., Balteszova, L. and Sajbidor, J. (1997) Lipid formation and gamma linolenic acid production by mucorales fungi grown on sunflower oil. *Lett. Appl. Microbiol.*, 25: 101-105.

Chapkin, R. S. (1992) Reappraisal of the Essential Fatty Acids, In: Fatty Acids and Their Health Implications, edited by C.K. Chow, Marcel Dekker, New York, Chapter 18, pp.429–435.

Chen, R., Matsui, K., Ogaw, M., Oe, M., Ochiai, M., Kawashima, H., Sakuradani, E., Shimizu, S., Ishimoto, M., Hayashi, M., Murooka, Y. and Tanaka, Y. (2006) Expression of Δ^6, Δ^5 desaturase and GLELO elongase genes from *Mortierella alpina* for production of arachidonic acid in soybean [*Glycine max* (L.) Merrill] seeds. *Plant Sci*., 170: 399–406.

Chibnall, A. C., Williams, E. F., Latner, A. L. and Piper, S. H. (1993) The isolation of *n*-triacontanol from lucerne wax. *Biochem. J.*, 27: 1885–1888.

Cho, H. J., Farrand, S. K., Noel, G. R. and Widholm, J. M. (2000) High-efficiency induction of soybean hairy roots and propagation of the soybean cyst nematode. *Planta,* 210: 195–204.

Christianson, M. L., Warnick, D. A. and Carlson, P. S. (1983) A morphogenetically competent soybean suspension culture. *Science,* 222: 632-634.

Christou , P., Mc Cabe, D. E., and Swain, W. F. (1988) Stable transformation of soybean callus by DNA-coated gold particles. *Plant physiology,* 87: 671-674.

Christou, P., Mc Cabe, D. E., Martinell, B. J. and Swain, W. F. (1990) Soybean genetic engineering - commercial production of transgenic plants. *Trends in Biotechnology,* 8: 145-151.

Chung, C. H., Kim, J. L., Lee, Y. C. and Choi, Y. L. (1999) Cloning and characterization of a seed-specific ω-3 fatty acid desaturase cDNA from *Perilla frutescens*. *Plant Cell Physiol*., 40: 114–8.

Conti, E., Stredansky, M., Stredansky, S. and Zanetti, F. (2001) Gamma linolenic acid production by solid state culture of mucorales strain on cereals. *Biores. Technol.,* 76: 283-286.

Cook, D., Grierson, D., Jones, C., Wallace, A., West, G. and Tucker, G. (2002) Modification of fatty acid composition in tomato (*Lycopersicon esculentum*) by expression of a borage delta-6 desaturase. *Mol. Biotechnol.,* 21: 123–128.

Coupland, K., Coupland, D. and Packer, C. (1996) New sources of stearidonic acid and the moderation of inflammation by plant oils containing stearidonate. ISSFAL 1996.

Crosby, D. G. and Vlitos, A. J. (1959) Growth substances from Maryland Mammoth Tabasco: long chain alcohols. *Contrib. Boyce Thompson Inst.,* 20: 283.

Damude, H. G. and Kinney, A. J. (2008) Enhancing Plant Seed Oils for Human Nutrition. *Plant Physiology*, 147: 962–968.

Damude, H. G., Zhang, H., Farrall, L., Ripp, K. G., Tomb, J. F., Hollerbach, D. and Yadav, N. S. (2006) Identification of bifunctional Δ^{12}/ω-3 fatty acid desaturases for improving the ratio of ω-3 to ω-6 fatty acids in microbes and plants. *Proc. Natl. Acad. Sci. USA,* 103: 9446–9451.

Dan, Y. and Reichert, N. A. (1998) Organogenic regeneration of soybean from hypocotyl explants. *In Vitro Cell Dev. Biol.,* 34P: 14–21.

Das, B., Goswami, L., Ray, S., Ghosh, S., Bhattacharyya, S., Das, S. and Majumder, A. L. (2006) *Agrobacterium*-mediated transformation of *Brassica juncea* with a cyanobacterial (*Synechocystis* PCC6803) delta-6 desaturase gene leads to production of gamma-linolenic acid. *Plant Cell Tiss. Organ Cult.,* 86: 219–231.

Das, T., Huang, Y. S. and Mukerji, P. (2000a) Delta-6 desaturase and GLA biosynthesis: a biotechnology perspective, In: Huang Y. S. and V.A. Ziboh (Eds.), Gamma-Linolenic Acid: Recent advances in Biotechnology and Clinical Applications, AOCS Press, Champaign, IL, pp. 6–23.

Das, T., Thurmond, J. M., Bobik, E., Leonard, A. E., Parker-Barnes, J. M., Huang, Y. S. and Mukerji, P. (2000b) Polyunsaturated fatty acid-specific elongation enzyme. *Biochem. Soc. Trans.*, 28: 658–660.

Das, T., Thurmond, J. M., Leonard, A. E., Parker-Barnes, J. M., Bobik, E., Chuang, L. T., Huang, Y. S. and Mukerji, P. (2002) In: Huang, Y. S. and Ziboh, V. A. (Eds.), γ-Linolenic Acid—Recent Advances in Biotechnology and Clinical Applications, AOCS Press, Champaign, IL, pp. 40–44.

Delzer, B. W., Somers, D. A. and Orf, J. H. (1990) *Agrobacterium tumefaciens* susceptibility and plant regeneration of 10 soybean genotypes in maturity groups. *Crop Sci.,* 30: 320–322.

Devi, M. K. A., Gondi, M., Sakthivelu, G., Giridhar, P., Rajasekaran, T. and Ravishankar, G.A. (2009) Functional attributes of soybean seeds and products with reference to isoflavone content and antioxidant activity. *Food Chemistry,* 114: 771-776.

Di, R., Purcell, V., Collins, G. B. and Ghabrial, S. A. (1996) Production of transgenic soybean lines expressing the bean pod mottle virus coat protein precursor gene. *Plant Cell Rep.,* 15: 746–750.

Diaz, A. R., Mansilla, M. C., Vila, A. J. and Mendoza, D. (2002) Membrane topology of the acyl-lipid desaturase from *Bacillus subtilis*. *Biol. Chem.,* 277: 48099–48106.

Dixon, R. A. (1993) Isoflavonoids: Biochemistry, Molecular biology and Biological functions, In: *Comprehensive Natural Products Chemistry*. Elsevier, Amsterdam, pp 773–823.

Dodds, J. H. and Roberts, L. W. (1982) In: Experiments in Plant Tissue Culture. Cambridge University Press, Cambridge, London, New York.

Domergue, F., Abbadi, A., Ott, C., Zank, T. K., Zahringer, U. and Heinz, E. (2003) Acyl carriers used as substrates by the desaturases and elongases involved in very long-chain polyunsaturated fatty acids biosynthesis reconstituted in yeast. *J. Biol. Chem.*, 278: 35115–35126.

Domergue, F., Lerchl, J., Zahringer, U. and Heinz, E. (2002) Cloning and functional characterization of *Phaeodactylum tricornutum* front end desaturases involved in eicosapentaenoic acid biosynthesis. *Eur. J. Biochem.*, 269: 4105–4113.

Donaldson, P. A. and Simmonds, D. H. (2000) Susceptibility to *Agrobacterium tumefaciens* and cotyledonary node transformation in short season soybean. *Plant Cell Rep.*, 19: 478–484.

Droste, A., Pasquali, G. and Bodanese-Zanettini, M, H. (2002) Transgenic fertile plants of soybean [*Glycine max* (L.) Merrill] obtained from bombarded embryogenic tissue. *Euphytica*, 127: 367–376.

Droste, A., Pasquali, G. and Bodanese-Zanettini, M. H. (2000) Integrated bombardment and *Agrobacterium* transformation system: an alternative method for soybean transformation. *Plant Mol. Biol. Rep.*, 18: 51–59.

Dye, F., Berthelot, K., Griffon, B., Delay, D. and Delmotte, F. M. (1997) Alkylsyringamides, new inducers of *Agrobacterium tumefaciens* virulence genes. *Biochimie*, 79: 3–6.

Dyerberg, J. (1986) Linolenate derived polyunsaturated fatty acids and prevention of atherosclerosis. *Nutr. Rev.*, 44: 125–34.

Eckert, H., LaVallee, B., Schweiger, B. J., Kinney, A. J., Cahoon, E. B. and Clemente, T. (2006) Co-expression of the borage Δ^6 desaturase and the *Arabidopsis* Δ^{15} desaturase results in high accumulation of stearidonic acid in the seeds of transgenic soybean. *Planta*, 224: 1050–1057.

Falco, S. C., Guida, T., Locke, M., Mauvais, J., Sanders, C., Ward, R. T. and Webber, P. (1995) Transgenic Canola and soybean seeds with increased lysine. *BioTechnology*, 13: 577–582.

Falcone, D. L., Gibson, S., Lemieux, B. and Somerville, C. (1994) Identification of a gene that complements an *Arabidopsis* mutant deficient in chloroplast ω-6 desaturase activity. *Plant Physiol.,* 106: 1453–9.

Finer, J. F. and McMullen, D. (1991) Transformation of soybean *via* particle bombardment of embyrogenic suspension culture tissue. *In vitro Cell Dev. Biol. Plant.* 27: 175–182.

Finer, J. J. and Nagasawa, A. (1988) Development of an embryogenic suspension culture of soybean (*Glycine max* Merrill.). *Plant Cell Tissue Org. Cult.,* 15: 125–136.

Finer, K. R. and Finer, J. J. (2000) Use of *Agrobacterium* expressing green fluorescent protein to evaluate colonization of sonication assisted *Agrobacterium*-mediated transformation of treated soybean cotyledons. *Lett. Appl. Microbiol.,* 30: 406–410.

Fisk, H. J. and Dandekar, A. M. (1993) The introduction and expression of transenes in plant. *Sci. Hortc.*, 55: 5-36.

Frame, B., Shou, H., Chikwamba, R., Zhang, Z., Xiang, C., Fonger, T., Pegg, S. E., Li, B., Nettleton, D., Pei, D. and Wang, K. (2002) *Agrobacterium* mediated transformation of maize embryos using a standard binary vector system. *Plant Physiol.*, 129: 13–22.

Franklin, G., Carpenter, L., Davis, E., Reddy, C. S., Al-Abed, D., Abou Alaiwi, W., Parani, M., Smith, B., Goldman S. L. and Sairam, R. V. (2004) Factors influencing regeneration of soybean from mature and immature cotyledons. *Plant Growth Regulation*, 43: 73–79.

Fratini, R. and Ruiz, M. L. (2003) A rooting procedure for lentil (*Lens culinaris* Medik.) and other hypogeous legumes (pea, chickpea and *Lathurus*) based on explant polarity. *Plant Cell Rep*., 21: 726–732.

Friedt, W. and Luhs, W. (1998) Recent developments and perspectives of industrial rapeseed breeding. *Fett/Lipid,* 100: 219–26.

Fukuchi, M. M., Savin, K., Cornish, E., Tanaka, Y., Ashikari, T., Kusumi, T. and Murata, N. (1995) Senescence-induced expression of a homologue of Δ^9-desaturase in rose petals. *Plant Mol. Biol.,* 29: 627–35.

Gamborg, O. L., Miller, R. A. and Ojima, K. (1968) Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res.*, 50: 151–158.

George, E. F. (1993) The derivation, preparation, and use of plant tissue culture media. *In*: *Plant propagation by tissue culture (Part I)*. 2nd edition. Exegetics Limited, England. pp: 344-419.

Gibson, S., Arondel, V., Iba, K. and Somerville, C. (1994) Cloning of a temperature regulated gene encoding a chloroplast ω-3 desaturase from *Arabidopsis thaliana*. *Plant Physiol*., 106: 1615–21.

Gill, I. and Valivety, R. (1997) Polyunsaturated fatty acids, part 1: occurrence, biological activities and applications. *Trends Biotechnol*., 15: 401–9.

Gill, R. and Saxena, P. K. (1992) Direct somatic embryogenesis and regeneration of plants from seedling explants of peanut (*Arachis hypogaea*): promotive role of thidiazuron. *Can. J. Bot.*, 70: 1186-1192.

Giridhar, P., Indu, E. P., Ravishankar, G. A. and Chandrasekar, A. (2004) Influence of triacontanol on somatic embryogenesis in *Coffea arabica* and *Coffea canephora. In Vitro Cell. Dev. Biol. Planta.*, 40: 200–203.

Girke, T., Schmidt, H., Zähringer, U., Reski, R. and Heinz, E. (1998) Identification of a novel delta6-acyl-group desaturase by targeted gene disruption in *Physcomitrella patens*. *Plant J*., 15: 39–48.

GLA monograph. (2004) Gamma-linolenic acid (GLA) monograph. *Alternative medicine review*, 9: 70-78.

Goffman, F. D. and Galletti, S. (2001) Gamma-linolenic acid and tocopherol contents in the seed oil of 47 accessions from several *Ribes* species. *J. Agric. Food Chem.*, 49: 349–354.

Gunstone, F.D. (1998) Movements towards tailor-made fats. *Prog. Lipid Res.*, 37: 277–305.

Gunstone, F. D. (1992) Gamma linolenic acid-occurrence and physical and chemical properties. *Prog. Lipid Res.*, 31: 145–61.

Gurr, M. I. and Harwood, J. L. (1991) Lipid Biochemistry. London: Chapman & Hall, 1991.

Gyves, E. M., Sparks, C. A., Sayanova, O., Lazzeri, P., Napier, J. A. and Jones, H. D. (2004) Genetic manipulation of γ-linolenic acid (GLA) synthesis in a commercial variety of evening primrose (*Oenothera sp.*). *Plant Biotechnol*., 2: 351.

Hadi, M. Z., McMullen, M. D. and Finer, J. J. (1996) Transformation of 12 different plasmids into soybean *via* particle bombardment. *Plant Cell Rep*., 15: 500–505.

Hamada, T., Kodama, H., Nishimura, M. and Iba, K. (1994) Cloning of a cDNA encoding tobacco omega-3 fatty acid desaturase. *Gene*, 147: 293–4.

Hangarter, R., Ries, S. K. and Carlson, P. (1978) Effect of triacontanol on plant cell cultures *in vitro. Plant Physiol*., 61: 855–857.

Hansen, G., Das, A. and Chilton, M. D. (1994) Constitutive expression of the virulence genes improves the efficiency of plant transformation by *Agrobacterium*. *Proc. Natl. Acad. Sci. USA*, 91: 7603–7607.

Hao, Y. L., Mei, X. H., Zhao, F., Yin, S., Li, R. H. and Luo Y. B. (2008) Expression of *Mucor circinelloides* gene for Δ^6 Desaturase results in the accumulation of high levels of γ-Linolenic acid in transgenic tobacco. *Russian Journal of Plant Physiology*, 55(1): 77–81.

Harwood, J. L. (1988) Fatty acid metabolism. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 39: 101–88.

Harwood, J. L. (1996) Recent advances in the biosynthesis of plant fatty acids. *Biochim. Biophys. Acta*, 1301: 7–56.

Heiduschka, A., and Luft, K. D. (1919) Fette Oel der Samen der Nachtkerze (*Oenothera biennis*) und uber eine neune linolensaure. *Arch. Pharmazie*, 257: 33-69.

Heinz, E., Zank, T., Zaehringer, U., Lerchl, J. and Renz, A. (2001) Patent: WO 0159128-A 16-Aug-2001.

Helgeson, J. P. (1979) Tissue and cell suspension culture. In: *Nicotiana*: procedures for experimental use. R.D. Durbin (ed.). U.S. Dept. Agric., Technical bulletin 1586: 52-59.

Henz, B. M., Jablonska, S., Van-de-Kerkhof, P. C., Stingl, G., Blaszczyk, M., Vandervalk, P. G., Veenhuizen, R., Muggli, R. and Raederstorff, D. (1999)

Double-blind, multicentre analysis of the efficacy of borage oil in patients with atopic eczema. *Br. J. Dermatol.*, 140: 685–688.

Heppard, E. P., Kinney, A. J., Stecca, K. L. and Miao, G. H. (1996) Developmental and growth temperature regulation of two different microsomal omega-6 desaturase genes in soybeans. *Plant Physiol.*, 110: 311–9.

Hiei, Y., Ohta, S., Komari, T. and Kumashiro, T. (1994) Efficient transformation of mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *Plant J.*, 6: 271-282.

Hinchee, M. A. W., Connor-Ward, D. V., Newell, C. A., McDonnell, R. E., Sato, S. J., Gasser, C. S., Fischhoff, D. A., Re, D. B., Fraley, R. T. and Horsch, R. B. (1988) Production of transgenic soybean plants using *Agrobacterium*-mediated DNA transfer. *Bio/Technology*, 6: 915–922.

Hiruta, O., Kamisaka, Y., Yokochi, T., Futamura, T., Takebe, H. and Satoh, A. (1996) Gamma linolenic acid production by a low temperature resistant mutant of *Mortierella ramanniana*. *J. fermen. Bioeng.*, 82: 119-123.

Hong, H., Dalta, N., Reed, D. W., Covello, P. S., MacKenzie, S. L. and Qiu, X. (2002) High-level production of γ-linolenic acid in *Brassica juncea* using a Δ^6-desaturase from *Pythium irregulare*. *Plant Physiol*., 129: 354–362.

Hongsthong, A., Deshnium, P., Paithoonrangsarid, K., Cheevadhanarak, S., Tanticharoen, M. (2003) Differential responses of the three acyl-lipid desaturases to immediate temperature reduction occurred in two lipid membranes of *Spirulina platensis* strain C1. *J. Biosci. Bioeng.*, 96: 519–524.

Hongsthong, A., Subudhi, S., Sirijuntarat, M. and Cheevadhanarak, S. (2004) Mutation study of conserved amino acid residues of *Spirulina* Δ^6-acyl-lipid desaturase showing involvement of histidine 313 in the regioselectivity of the enzyme. *Appl. Microbiol. Biotechnol.*, 66: 74–84.

Horiguchi, G., Kawakami, N., Kusumi, K., Kodama, H. and Iba, K. (1998) Developmental regulation of genes from microsome and plastid omega-3 fatty acid desaturases in wheat (*Triticum aestivum* L.). *Plant Cell Physiol*., 39: 540–4.

Horrobin, D. F. (1992) Nutritional and medical importance of gamma-linolenic acid. *Prog. Lipid Res.*, 31: 163–94.

Horsch, R. B., Fry, J. E., Hoffman, N. L., Eicholtz, D., Rogers, S. G. and Frankley, R. T. (1985) A simple and general method for transferring genes into plants. *Science,* 227: 1229-1231.

Hu, C. Y. and Wang, L. (1999) *In planta* soybean transformation technologies developed in China: procedure, confirmation and field performance. *In Vitro Cell Dev. Biol. Plant.,* 35: 417–420.

Huang, Y. S. and Ziboh, V. A. (2000) Gamma linolenic acid: An Introduction, In: Y.-S. Huang, V.A. Ziboh (Eds.), Gamma-Linolenic Acid: Recent advances in Biotechnology and Clinical Applications, AOCS Press, Champaign, IL, pp. 6–23.

Huang, Y. S., Chaudhary, S., Thurmond, J. M., Bobik, E. G., Yuan, L., Chan, G. M., Kirchner, S. J., Mukerji, P. and Knutzon, D. S. (1999) Cloning of Δ^{12}- and Δ^{6}-desaturases from *Mortierella alpina* and recombinant production of gamma-linolenic acid in *Saccharomyces cerevisiae*. *Lipids,* 34: 649–59.

Huang, Y. S., Mukerji, P., Das, T. and Knutzon, D. S. (2001) Transgenic Production of Long-Chain Polyunsaturated Fatty Acids. *Hamazaki T, Okuyama H (eds): Fatty Acids and Lipids - New Findings.World Rev Nutr Diet. Basel, Karger*, vol 88, pp 243-24.

Huang, Y. S., Pereira, S. L. and Leonard, A. E. (2004) Strategic Enzymes for transgenic biosynthesis of long-chain polyunsaturated fatty acids. *Biochimie,* 86: 793–798.

Huetteman, C. A. and Preece, J. E. (1993) Thidiazuron: a potent cytokinin for woody plant tissue culture. *Plant Cell Tissue Org. Cult.* 33: 105–119.

Hulanicka, D., Erwin, J. and Block, K. (1964) Lipid metabolism of *Euglena gracilis, J. Biol. Chem.*, 239: 2778–2787.

Hymowitz, T. (1970) On domestication of soybean, *Economic Botany*, 24: 408–421.

Hymowitz, T. and Harlan, J. R. (1983) Introduction of soybeans to North America by Samuel Bowen in 1765. *Economic Botany*, 37: 371–379.

Hymowitz, T. and Newell, C. A. (1980) Taxonomy, speciation, domestication, dissemination, germplasm resources and variation in the genus *Glycine.* In "Advances in Legume Science" pp. 251-264 (eds. R. J. Summerfield and Bunting, A. H.) Kew: Royal Botanic Gardens.

Hymowitz, T. and Singh, R. J. (1987) Taxonomy and Speciation. In "Soybeans: Improvement, Production and Uses" 2nd ed. *Agronomy Monograph*, 16: 23-48.

Iacono, J. M. and Dougherty, R. M. (1993) Effects of polyunsaturated fats on blood pressure. *Annu Rev. Nutr*., 13: 243–60.

Innis, S. M. (1991) Essential fatty acids in growth and development. *Prog. Lipid Res.*, 30: 39–103.

Ishida, Y., Saito, H., Ohta, S., Hiei, Y., Komari, T. and Kumashiro, T. (1996) High efficiency transformation of maize (Zea mays L.) mediated by *Agrobacterium tumefaciens. Nat. Biotechnol.*, 14: 745-750.

Jareonkitmongkol, S., Sakuradani, E. and Shimizu, S. A. (1993) A novel D5-desaturasedefective mutant of *Mortierella alpina* 1S-4 and its dihomo-γ-linolenic acid productivity. *Appl. Environ. Microbiol.*, 59: 4300–4304.

Jefferson, R. A., Kavanagh, T. A. and Bevan, M. W. (1987) GUS fusions: Beta – glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO. J.*, 6: 3901-3907.

Kaneda, Y., Tabei, Y., Nishimura, S., Harada, K., Akihama, T. and Kitamura, K. (1997) Combination of thidiazuron and basal media with low salt concentrations increases the frequency of shoot organogenesis in soybeans [*Glycine max* (L.) Merr.]. *Plant Cell Reports.* 17: 8–12.

Kendrick, A. and Ratledge, C. (1992) Lipids of selected molds grown for production of ω-3 and ω-6 polyunsaturated fatty acids. *Lipids*, 27: 15–20.

Kennedy, M. J., Reader, S. L. and Davies, R. J. (1993) Fatty acid production characteristics of fungi with particular emphasis on gamma linolenic acid production. *Biotech. Bioeng.*, 42: 625-634.

Kinney, A. J. (1994) Genetic modification of the storage lipids of plants. *Curr. Opinion Biotechnol.*, 5: 144–51.

Kinney, A. J. and Knowlton, S. (1998) Designer oils: the high oleic soybean. In: S. Roller, S. Harlander, eds, Genetic Modification in the Food Industry. Blackie, London

Kinney, A. J., Cahoon, E. B., Damude, H. G., Hitz, W. D., Kolar, C. W. and Liu, Z. B. (2004) Enhancing Plant Seed Oils for Human Nutrition August 10, 2004. Production of very long chain polyunsaturated fatty acids in oilseed plants. International Patent Publication WO2004071467

Kita, Y., Nishizawa, K., Takahashi, M., Kitayama, M. and Ishimoto, M. (2007) Genetic improvement of the somatic embryogenesis and regeneration in soybean and transformation of the improved breeding lines. *Plant Cell Rep.,* 26: 439-447.

Klein, T. M., Wolf, E. D., Wu, R. and Sanford, J. C. (1987) High velocity microprojectiles for delivering nucleic acids into living cells. *Nature,* 327: 70-73.

Knauf, V. C., Shewmaker, C., Flider, F. J., Emlay, D. and Ray, E. (2006) November 30, 2006. Safflower with elevated gamma-linolenic acid. International Patent Publication. WO2006127789.

Knight, S. L. and Mitchell, C. A. (1987) Stimulating productivity of hydroponic lettuce in controlled environments with triacontanol. *Horti. Sci.,* 22: 1307–1309.

Knutzon, D. S. and Knauf, V. (1998) Manipulating seed oils for polyunsaturated fatty acid content. p. 287–304. *In* J. Harwood (ed.) Plant lipid biosynthesis: Fundamentals and agricultural applications. Cambridge Univ. Press, Cambridge.

Knutzon, D. S., Thurmond, J. M., Huang, Y. S., Chaudhary, S., Bobik, E. G., Chan, G. M., Kirchner, S. J. and Mukerji, P. (1998) Identification of Δ^5-desaturase from *Mortierella alpina* by heterologous expression in bakers' yeast and canola. *J. Biol. Chem.,* 273: 29360–29366.

Ko, T., Lee, S., Krasnyanski, S. and Korban, S. S. (2003) Two critical factors are required for efficient transformation of multiple soybean cultivars: *Agrobacterium* strain and orientation of immature cotyledonary explant. *Theor. Appl. Genet.,* 107: 439–447.

Komatsuda, T. and Ohyama, K. (1988) Genotypes of high competence for somatic embryogenesis and plant regeneration in soybean *Glycine max*. *Plant Cell Rep.,* 75: 695–700.

Komatsuda, T., Lee, W. and Oka, S. (1992) Maturation and germination of somatic embryos as affected by sucrose and plant growth regulators in soybeans, *Glycine*

gracilis Skvortz and *Glycine max* (L.) Merr. *Plant Cell Tissue Org. Cult.*, 28: 103–113.

Kridl, J. C., inventor. April 2, 2002. Methods for increasing stearate content in soybean oil. U.S. Patent US6365802

Kulandaivelu, K. (1996) Ameliorating effect of triacontanol on salt stressed *Erythrina variegata* Lam. Seedlings. Changes in growth, biomass, pigments and solute accumulation. *Biol. Plant.,* 38: 133–136.

Kyte, J. and Doolittle, R. (1982) A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.,* 157: 105-132.

Lackey, J. A. (1981) Phaseoleae. In: Advances in legume systematics. Royal Botanic Gardens. Kew.

Laoten, K., Mannontarat, R., Tanticharoen, M. and Cheevadhanarak, S. (2000) Δ^6-desaturase of *Mucor rouxii* with high similarity to plant Δ^6-desaturase and its heterologus expression in *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Commun.,* 279: 17-22.

Lassner, M. (1997) Transgenic oilseed crops: a transition from basic research to product development. *Lipid Technol*., 1: 5–9.

Lazzeri, P. A., Hildebrand, D. F. and Collins, G. B. (1985) A procedure for plant regeneration from immature cotyledon tissue of soybean. *Plant Mol. Biol. Rep.,* 3: 160–167.

Lazzeri, P. A., Hildebrand, D. F. and Collins, G. B. (1987) Soybean somatic embryogenesis: Effects of hormones and culture manipulations. *Plant Cell Tissue Org. Cult.,* 10: 197–208.

Lees, A. M. and Korn, E. D. (1966) Metabolism of unsaturated fatty acids in protozoa, *Biochemistry*, 5: 1475–1481.

Leonard, A. E., Bobik, E. G., Dorado, J., Kroeger, P. E. Chuang, L. T., Thurmond, J. M. et al., (2000a) Cloning of a human cDNA encoding a novel enzyme involved in the elongation of long-chain polyunsaturated fatty acids. *Biochem. J.*, 350: 765–770.

Leonard, A. E., Kelder, B., Bobik, E. G., Chuang, L. T., Parker B. J. M., Thurmond, J. M., Kroeger, P. E., Kopchick, J. J., Huang, Y. S. and Mukerji, P. (2000b) cDNA cloning and characterization of human Δ^5-desaturase involved in the biosynthesis of arachidonic acid. *Biochem. J.*, 347: 719–724.

Leonard, A. E., Pereira, S. L. and Huang, Y. S. (2004) Elongation of long-chain fatty acids. *Prog. Lipid Res.*, 43: 36–54.

Lersten, N. R. and Carlson, J. B. (1987) Vegetative morphology. In: Soybeans: Improvement, Production, and Uses; Second Edition. Ed. J. R. Wilcox. pp. 49-94. American Society of Agronomy. Madison, Wisconsin.

Lichtenstein, A. H., Matthan, N. R., Jalbert, S. M., Resteghini, N. A., Schaefer, E. J. and Ausman, L. M. (2006) Novel soybean oils with different fatty acid profiles alter cardiovascular disease risk factors in moderately hyperlipidemic subjects. *Am. J. Clin. Nutr.*, 84: 497–504.

Liu, H., Yang, C. and Wei, Z. (2004) Efficient *Agrobacterium tumefaciens* mediated transformation of soybeans using an embryonic tip regeneration system. *Planta*, 219: 1042–1049.

Liu, J. W., Huang, Y. S., DeMicheles, S., Bergana, M., Mobike, E. H. C., Chuang, L. T., Mukherjee, P. and Knutzon, D. (2001) Evaluation of seed oil from a canola plant genetically transformed to produce high level of γ-linolenic acid. In: Huang Y-S, ZibohA(eds) γ-Linolenic acid: recent advances in biotechnology and clinical applications. AOCS Press, Champaign IL, pp 61–71.

Liu, K. Ph.D. (2004), Soybeans as Functional Foods and Ingredients, AOCS Press.

Liu, S. J., Wei, Z. M. and Huang, J. Q. (2008) The effect of co-cultivation and selection parameters on *Agrobacterium*-mediated transformation of Chinese soybean varieties. *Plant Cell Rep.*, 27: 489–498.

Los, D. A. and Murata, N. (1998) Structure and Expression of Fatty Acid Desaturases. *Biochim. Biophys. Acta*, 1394: 3-15.

Lucas, M. R. D., Asselin, G., Merette, C., Poulin, M. J. and Dodin, S. (2009) Effects of ethyl-eicosapentaenoic acid omega-3 fatty acid supplementation on hot flashes and quality of life among middle-aged women: a double-blind, placebo-controlled, randomized clinical trial. *Menopause*. 16: 357-366.

Ma, F. W., Wang, J. C. and Rong, W. (1990) Effects of plant growth regulators on *in vitro* propagation of apple cultivar Fuji. *J. Fruit Sci.*, 7: 201–206.

Mahadevappa. M., Murthy, R. A. K. and Biradar, B. B. (1989) Effect of triacontanol on rice seedling weight and grain yield. *Int. Rice Res. News Lett.*, 14: 26.

Malik, K. A. and Saxena, P. K. (1992) Regeneration in *Phaseolus vulgaris* L.: High frequency induction of direct shoot formation in intact seedlings by N^6-benzylaminopurine and thidiazuron. *Planta:* 186: 384–389.

Mamatha, S. S., Ravi, R. and Venkateswaran, G. (2008) Medium optimization of gamma linolenic acid production in *Mucor rouxii* CFR-G15 using RSM. *Food Bioprocess Technol*., 1: 405–409.

Mann, M. L., Skirvin, R. M. and Chu, M. C. (1982) Effects of autoclaving on the pH of tissue culture media. *Hort. Science*, 17: 534.

Marotoa, F. G., Jose, A., Cardenasa, G., Rodriguez, R. J., Vilches, F. M., Adamc, A. C., Polainac, J. and Alonso, D. L. (2002) Cloning and Molecular Characterization of the Δ^6-Desaturase from Two *Echium* Plant Species: Production of GLA by Heterologous Expression in Yeast and Tobacco. *Lipids*, 37: 417-426.

Mauro, A. O., Pfeiffer, T. W. and Collins, G. B. (1995) Inheritance of soybean susceptibility to *Agrobacterium tumefaciens* and its relationship to transformation. *Crop Sci*., 35: 1152–1156.

McCabe, D. E., Swain, W. F., Martinell, B. J. and Christou, P. (1988) Stable transformation of soybean (*Glycine max*) by particle acceleration. *Bio/Technology*, 6: 923–926.

Meesapyodsuk, D., Reed, D. W., Savile, C. K., Buist, P. H., Ambrose, S. J. and Covello, P. S. (2000) Characterization of the regiochemistry and cryptoregiochemistry of a *Caenorhabditis elegans* fatty acid desaturase (FAT-1) expressed in *Saccharomyces cerevisiae. Biochemistry*, 39: 11948–11954.

Messina, M., Messina, V. and Setchell, K. D. R. (1994) The Simple Soybean and Your Health, Avery Publishing Group, Garden City Park, New York.

Meurer, C. A., Dinkins, R. D, and Collins, G. B. (1998) Factors affecting soybean cotyledonary node transformation. *Plant Cell Re*p., 18: 180–186.

Meyer, A., Cirpus, P., Ott, C., Schlecker, R., Zahringer, U. and Heinz, E. (2003) Biosynthesis of docosahexaenoic acid in *Euglena gracilis*: biochemical and molecular evidence for the involvement of a delta 4-fatty acyl group desaturase. *Biochemistry,* 42: 9779–9788.

Meyer, A., Kirsch, H., Domergue, F., Abbadi, A., Sperling, P., Bauer, J., Cirpus, P., Zank, T. K., Moreau, H., Roscoe, T. J., Zahringer, U. and Heinz, E. (2004) Novel fatty acid elongases and their use for the reconstitution of docosahexaenoic acid biosynthesis. *J. Lipid Res.,* 45: 1899–1909.

Michaelson, L. V., Lauzarus, C. M., Griffiths, G., Napier, J. A., Stobart, A. K. (1998) Isolation of a Δ^5-fatty acid desaturase gene from *Mortierella alpina*. *J. Biol. Chem.,* 273: 19055–19059.

Millar, A. A., Wrischer, M. and Kunst, L. (1998) Accumulation of very-long-chain fatty acids in membrane glycerolipids is associated with dramatic alterations in plant morphology. *Plant Cell,* 11: 1889–902.

Miquel, M. F. and Browse, J. (1994) High-oleate oilseeds fail to develop at low temperature. *Plant Physiol.,* 106: 421–7.

Miwa, T. K., Mikolajczak, K. L., Earle, F. R. and Wolff, I. A. (1960) Gas-chromatographic characterization of fatty acids. Identification constants for mono- and dicarboxylic methyl esters. *Anal. Chem.*, 32: 1739-1742.

Mohamed, S. V., Sung, J. M., Jeng, T. L. and Wang, C. S. (2006) Organogenesis of *Phaseolus angularis* L.: high efficiency of adventitious shoot regeneration from etiolated seedlings in the presence of N6-benzylaminopurine and thidiazuron. *Plant Cell Tiss. Organ Cult.,* 86: 187–199.

Mok, M. C., Mok, D. W. S., Turner, J. E., Armstrong, D. J., Shudo, K., Isogai, Y. and Okamoto, T. (1982) Cytokinin activity of N-phenyl-N'-1,2,3,-thiadiazol-5ylurea (thidiazuron). *Phytochemistry*, 21: 1509–1511.

Monsanto, (2008) Monsanto and the Solae Company to collaborate on soybased omega-3 portfolio. Monsanto Company. www.monsanto.com/ features/omega.asp

Muhid, F., Nawi, W., Kader, A. J., Yusoff, W. M. and Hamid, A. A. (2008) Effects of metal ion concentrations on lipid and gamma linolenic acid production by *Cunninghamella sp*. 2A1. *On Line Jl .of Biological Sci.,* 8: 62-67.

Mukerji, P., Huang, Y. S., Das, T., Thurmond, J. M., Leonard, A. E. and Pereira, S. L. (2002) Protein and cDNA sequences of delta-4 desaturases isolated from fungi and therapeutical uses thereof. International Patent Publication WO 2002090493.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.,* 15: 473–497.

Murata, N. and Wada, H. (1995) Acyl-lipid desaturases and their importance in the tolerance and acclimatization to cold of cyanobacteria. *Biochem. J.,* 308: 1–8.

Murphy, D. J. (1993) Structure, function and biogenesis of storage lipids bodies and oleosins in plants. *Prog. Lipid Res.,* 32: 247–80.

Murthy, B. N. S., Murch, S. J. and Saxena, P. K. (1995) TDZ-induced somatic embryogenesis in geranium (*Pelargonium x hortorum* Bailey cv. Ringo rose) cotyledonary cultures. *Plant Cell Rep.,* 15: 423–426.

Mustady, L., Los, D. A., Gombos, Z. and Murata, N. (1996) Immunocytochemical localization of acyl-lipid desaturases in cyanobacterial cells: evidence that both thylakoid membranes and cytoplasmic membranes are the sites of lipid desaturation. *Proc. Natl. Acad. Sci. USA,* 93: 10524-10527.

Napier, J. A., Michaelson, L. V. and Stobart, A. K. (1999) Plant desaturases: harvesting the fat of the land. *Curr. Opin. Plant Biol.,* 2: 123–127.

Nunberg, A. N., Li, Z., Bogue, M. A., Vivekananda, J., Reddy, A. S. and Thomas, T. L. (1994) Developmental and hormonal regulation of sunflower helianthinin genes: proximal promoter sequences confer regionalized seed expression. *Plant Cell,* 6: 473–486.

Obregon, E. G. A., Prieto, S. D. L., De la Riva, G. A., Perez, M., Selman, H. G. and Vazquez, P. R. I. (1999) *Agrobacterium* mediated Japonica rice transformation: a procedure assisted by an antinecrotic treatment. *Plant Cell Tissue Organ Cult.,* 59: 159–168.

Ohlrogge, J. B. and Jaworski, J. G. (1997) Regulation of fatty acid biosynthesis. *Ann. Rev. Plant Physiol. Plant Mol. Biol.,* 48: 109–136.

Okayasu, T., Nagao, M., Ishibashi, T. and Imai, Y. (1981) Purification and partial characterization of linoleoyl-CoA desaturase from rat liver microsomes. *Arch. Biochem. Biophys.,* 206: 21-28.

Okuley, J., Lightner, J., Feldmann, K., Yadav, N., Lark, E. and Browse, J. (1994) *Arabidopsis FAD2* gene encodes the enzyme that is essential for polyunsaturated lipid synthesis. *Plant Cell*, 6: 147–158.

Olhoft, P. M. and Somers, D. A. (2001) L-Cysteine increases *Agrobacterium* mediated T-DNA delivery into soybean cotyledonary node cells. *Plant Cell Rep.,* 20: 706–711.

Olhoft, P. M., Flagel, L. E., Donovan, C. M. and Somers, D. A. (2003) Efficient soybean transformation using hygromycin B selection in the cotyledonary node method. *Planta,* 216: 723–735.

Owens, L. D. and Cress, D. E. (1985) Genotypic variability of soybean response to *Agrobacterium* strains harboring Ti or Ri plasmids. *Plant Physiol.*, 77: 87–94.

Padley, F. B., Gunstone, F. D. and Harwood, J. L. (1994) Occurrence and characteristics of oils and fats, In: F.D. Gunstone, J.L. Harwood, F.B. Padley (Eds.), The Lipid Handbook, second ed, Chapman and Hall, London, 1994, pp. 47–223.

Page, R. D. M. (1996) TREEVIEW: An Application to Display Phylogenetic Trees on Personal Computers. *Comput. Appl. Biosci.*, 12: 357–358.

Palanichelvam, K., Oger, P., Clough, S. J., Cha, C., Bent, A. F. and Farrand, S. K. (2000) A second T-region of the soybean-supervirulent chrysopine- type Ti plasmid pTiChry5, and construction of a fully disarmed *vir* helper plasmid. *Mol. Plant Microbe. Interact.,* 13: 1081–1091.

Palombo, J. D., DeMichele, S. J., Liu, J. W. and Huang, Y. S. (2000) Comparison of growth and fatty acid metabolism in rat fed diets containing equal levels of α-linolenic acid from high α-linolenic acid canola or borage oil. *Lipids*, 35: 975–981.

Parent, C. A., Lagarde, M., Venton, D. L. and LeBreton, G. C. (1992) Selective modulation of the human platelet thromboxane A_2/ prostaglandine H_2 receptor by eicosapentaenoic and docosahexaenoic acids in intact platelets and solubilized platelet membranes. *J. Biol. Chem.,* 267: 6541–6547.

Parker, B. J. B., Das, T., Bobik, E., Leonard, A. E., Thurmond, J. M., Chuang, L. T., Huang, Y. S. and Mukerji, P. (2000) Identification and characterization of an

enzyme involved in the elongation of ω-6 and ω-3 polyunsaturated fatty acids. *Proc. Natl. Acad. Sci. USA*, 97: 8284-8289.

Parrott, W. A., Dryden, G., Vogt, S., Hildebrand, D. F., Collins, G. B. and Williams, E. G. (1988) Optimization of somatic embryogenesis and embryo germination in soybean. *In Vitro Cell Dev. Biol.*, 24: 817–820.

Parrott, W. A., Hoffman, L. M., Hildebrand, D. F., Williams, E. G. and Collins, G. B. (1989) Recovery of primary transformants of soybean. *Plant Cell Rep*., 7: 615–617.

Passey, A. J., Barrett, K. J. and James, D. J. (2003) Adventitious shoot regeneration from seven commercial strawberry cultivars (*Fragaria* x *Ananassa* Duch.) using a range of explant types. *Plant Cell Rep*., 21: 397–401.

Paz, M. M., Martinez, J. C., Kalvig, A. B., Fonger, T. M. and Wang, K. (2006) Improved cotyledonary node method using an alternative explant derived from mature seed for efficient *Agrobacterium* mediated soybean transformation. *Plant Cell Rep.*, 25: 206–213.

Paz, M. M., Shou, H., Guo, Z., Zhang, Z., Banerjee, A. K. and Wang, K. (2004) Assessment of conditions affecting *Agrobacterium* mediated soybean transformation using the cotyledonary node explant. *Euphytica*, 136: 167–179.

Peck, M. D. (1994) Interactions of lipids with immune function II-experimental and clinical studies of lipids and immunity. *J. Nutr. Biochem.*, 5: 514–521.

Pereira, S. L., Huang, Y. S., Bobik, E. G., Kinney, A. J., Stecca, K. L. and Mukerji, P. (2003) A novel omega-3(ω-3) fatty acid desaturase involved in the biosynthesis of eicosapentaenoic acid. *Biochem. J.*, 378: 665–671.

Pereira, S. L., Leonard, A. E., Huang, Y. S., Chuang, L. T. and Mukerji, P. (2004) Identification of two novel microalgal enzymes involved in the conversion of the ω-3 fatty acid eicosapentaenoic acid (EPA) to docosahexaenoic acid (DHA). *Biochem. J.*, 384: 357–366.

Poehlman, J. M., and Sleper, D. A. (1995). *Breeding field crops* (4th ed.). Ames, IA: Iowa State University Press.

Poirier, Y., Ventre, G. and Nawrath, C. (2000) High-frequency linkage of co-expressing T-DNA in transgenic *Arabidopsis thaliana* transformed by vacuum infiltration of *Agrobacterium tumefaciens*. *Theor. Appl. Genet.*, 100: 487–493.

Preez, J. C., Immelman, M., Kock, J. L. F. and Kilian, S. G. (1997) The effect of acetic acid concentration on the growth and production of gamma-linolenic acid by *Mucor circinelloides* CBS 203.28 in fed-batch culture. *World J. of Micro. Biotech.*, 13: 81-87.

Pueppke, J. L. (1996) The genetics and biochemical basis for nodulation of legumes by rhizobia. *Crit. Rev. Biotechnol.*, 16: 1–51.

Qi, B., Fraser, T., Mulford, S., Dobson, G., Sayanova, O., Butler, J. et al., (2004) Production of very long chain polyunsaturated omega-3 and omega-6 fatty acids in plants. *Nat. Biotechnol.*, 22: 739–745.

Qiu, X., Hong, H. and MacKenzie, S. L. (2001) Identification of a Δ^4 fatty acid desaturase from *Thraustochytrium sp*. involved in the biosynthesis of docosahexanoic acid by heterologous expression in *Saccharomyces cerevisiae* and *Brassica juncea*. *J. Biol. Chem.*, 276: 31561–31566.

Qiu, X., Hong, H. P., Datla, N., MacKenzie, S. L., Tayler, C. D. and Thomas, L. T. (2002) Expression of borage Δ^6-desaturase in *Saccharomyces cerevisiae* and oilseed crops. *Can. J. Bot.*, 80: 42–49.

Ranch, J. P., Oglesby, L. and Zielinski, A. C. (1985) Plant regeneration from embryo derived tissue cultures of soybeans. *In Vitro Cell Dev. Biol.*, 21: 653–658.

Reddy, A. S. and Thomas, T. L. (1996) Expression of a cyanobacterial Δ^6-desaturase gene results in γ-linolenic acid production in transgenic plants. *Nat. Biotechnol.*, 14: 639–642.

Reddy, A. S., Nuccio, M. L., Gross, L. M. and Thomas, T. L. (1993) Isolation of a Δ^6-desaturase gene from the cyanobacterium *Synechocystis* sp. strain PCC 6803 by gain of function expression in *Anabaena sp*. strain PCC 7120. *Plant Mol. Biol.*, 27: 293–300.

Reddy, B. O., Giridhar, P. and Ravishankar, G. A. (2002) The effect of triacontanol on micropropagation of *Capsicum frutescens* and *Decalepis hamiltonii*. *Plant Cell Tiss. Org. Cult.*, 71: 253–258.

Reichert, N. A., Young, M. M. and Woods, A. L. (2003) Adventitious organogenic regeneration from soybean genotypes representing nine maturity groups. *Plant Cell Tissue and Organ Culture*, 75: 273–277.

Ries, S. and Houtz, R. (1983) Triacontanol as a plant growth regulator. *Hort. Sci.*, 18: 654–662.

Ries, S. K. and Wert, V. (1988) Rapid elicitation of second messengers by nanomolar doses of triacontanol and octacosanol. *Planta*, 173: 79–87.

Riley, J. P. (1949) The seed fat of *Oenothera biennis* L. *J. Chem. Soc.*, 2728-2731.

Rozen, S. and Skaletsky, H. J. (2000) Primer3 on the www for general users and for biologist programmers. In: Bioinformatics methods and protocols, Methods in molecular biology (Vol. 132). (S. Misener and Krawetz, S. A. ed). Humana Press, Totowa, New Jersy, pp: 365-386.

Sairam, R. V., Franklin, G., Hassel, R., Smith, B., Meeker, K., Kashikar, N., Parani, M., Alabed, D., Ismail, S., Berry, K. and Goldman, S. L. (2003) A study on the effect of genotypes, plant growth regulators and sugars in promoting plant regeneration via organogenesis from soybean cotyledonary nodal callus. *Plant Cell Tissue and Organ Culture*, 75: 79–85.

Saitou, N., and Nei, M. (1987) The Neighbor-Joining Method: A New Method for Reconstructing Phylogenetic Trees. *Mol. Biol. Evol.*, 4: 406–425.

Sakamoto, T., Wada, H., Nishida, I., Ohmori, M. and Murata, N. (1994) Delta-9 acyl-lipid desaturases of cyanobacteria. *J. Biol. Chem.*, 269: 25576–80.

Sakthivelu, G., Akitha Devi, M. K., Giridhar, P., Rajasekaran, T., Ravishankar, G.A., Nikolova, M. T., Angelov, G. B., Todorova, R. M. and Kosturkova, G. (2008) Isoflavone composition, phenol content and antioxidant activity of Soybean seeds from India and Bulgaria. *J. of Agri. and Food Chem.*, 56 (6): 2090-2095.

Sakthivelu, G., Giridhar, P., Sarada, R. and Ravishankar, G. A. (2009) *Arthrospira (Spirulina) platensis* (Sp-6) delta-6 desaturase (D6d) gene, complete cds, GenBank: FJ752023.1, http://www.ncbi.nlm.nih.gov/nuccore/224813785

Sambrook, J. and Russell, D. W. (2001) *Molecular cloning. A laboratory manual* 3/e (*Vol. I to III*). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Santarem, E. R. and Finer, J. J. (1999) Transformation of soybean [*Glycine max (L.) Merill*] using proliferative embryogenic tissue maintenance on semi solid medium. *In Vitro Cell Dev. Boil. - Plant*, 35: 451-455.

Santarem, E. R., Trick, H. N., Essig, J. S. and Finer, J. J. (1998) Sonication assisted *Agrobacterium*-mediated transformation of soybean immature cotyledons: optimization of transient expression. *Plant Cell Rep*., 17: 752–759.

Sarmiento, C., Ross, J. H. E., Herman, E. and Murphy, D. J. (1997) Expression and sub-cellular targeting of a soybean oleosin in transgenic rapeseed. Implications for the mechanism of oil body formation in seeds. *Plant J*., 11: 783–796.

Sato, S., Xing, A., Ye, X., Schweiger, B., Kinney, A. J., Graef, G. and Clemente, T. E. (2004) Production of γ-linolenic acid and stearidonic acid in seeds of marker free transgenic soybean. *Crop Sci*., 44: 646–652.

Sayanova, O. and Napier, J. A. (2004) Eicosapentaenoic acid: biosynthetic routes and the potential for synthesis in transgenic plants. *Phytochemistry*, 65: 147–158.

Sayanova, O. V., Beaudoin, F., Michaelson, L. V., Shewry, P. R. and Napier, J. A. (2003) Identification of *Primula* fatty acid Δ^6-desaturases with n-3 substrate preferences. *FEBS Lett.*, 542: 100–104.

Sayanova, O., Davies, G. M., Smith, M. A, Griffiths, G., Stobart, A. K., Shewry, P. R. and Napier, J. A. (1999) Accumulation of Δ^6-unsaturated fatty acids in transgenic tobacco plants expressing a Δ^6-desaturase from *Borago officinalis*. *J. Exp. Bot.*, 50: 1647–1652.

Sayanova, O., Smith, M. A., Lapinskas, P., Stobart, A. K., Dobson, G., Christie, W. W., Shewry, P. R. and Napier, J. A. (1997) Expression of a borage desaturase cDNA containing an N-terminal cytochrome b5 domain results in the accumulation of high levels of Δ^6-desaturated fatty acids in transgenic tobacco. *Proc. Natl. Acad. Sci. USA*, 94: 4211–16.

Schmidt, H., Dresselhaus, T., Buck, F. and Heinz, E. (1994) Purification and PCR-based cDNA cloning of a plastidial ω-6 desaturase. *Plant Mol. Biol.*, 26: 631–42.

Schmidt, H., Sperling, P. and Heinz, E. (1993) Biochemistry and molecular biology of membrane and storage lipids of plants. American Society for Plant Physiologists, Rockville.

Shan, Z., Raemakers, K., Tzitzikas, E. N., Ma, Z., Visser, R. G. F. (2005) Development of a highly efficient, repetitive system of organogenesis in soybean [*Glycine max* (L.) Merr]. *Plant Cell Rep.*, 24: 507–512.

Shanklin, J. and Cahoon, E. B. (1998) Desaturation and related modifications of fatty acids. *Ann. Rev. Plant Physiol. Plant Mol. Biol.*, 49: 611-641.

Shetty, K., Asano, Y. and Oosawa, K. (1992) Stimulation of *in vitro* shoot organogenesis in *Glycine max* (Merrill.) by allantoin and amides. *Plant Sci.*, 81: 245–251.

Simonopoulos, A. P. (1989) Executive Summary. In: Dietary omega-3 and omega-6 fatty acids: Biological effects and Nutritional Essentiality. Series A: Life Sciences. Vol 171. Galli, C. and Simopoulos, A. P. eds; pp 391-402. New York: Plenum-Press, 1989.

Simonopoulos, A. P. (1991) Omega-3 fatty acids in health and disease and in growth and development. *Am. J. Clin. Nutr.*, 54: 438–463.

Singh, G. and Chandra, R. K. (1988) Biochemical and cellular effects of fish and fish oils. *Prog. Food Nutr.*, 12: 371–419.

Singh, R. J. and Hymowitz, T. (1999) Soybean genetic resources and crop improvement. *Genome*, 42: 605-616.

Singh, S. P., Zhou, X. R., Liu, Q., Stymne, S. and Green, A. G. (2005) Metabolic engineering of new fatty acids in plants. *Curr. Opin. Plant Biol.*, 8: 197–203.

Slabas, A. R. and Fawcett, T. (1992) The biochemistry and molecular biology of plant lipid biosynthesis. *Plant Mol. Biol.*, 19: 169–91.

Somen. C. K. and Seetalakshmi, K. K. (1991) Growth response of seedlings of *Acacia*, *Alianthus* and *Casuaria* to triacontanol (Vipul). *Indian J. For.*, 14: 46–50.

Somers, D. A., Samac, D. A. and Olhoft, P. M. (2003) Recent advances in legume transformation. *Plant Physiol.*, 131: 892–899.

Somerville, C. and Browse, J. (1991) Plant lipids: metabolism, mutants, and membranes. *Science*, 252: 80–87.

Soyatech. (2004) Inc., *Soya & Oil Bluebook,* Bar Harbor, Maine. http://www.soyatech.com/index.php

Sperling, P., Schmidt, H. and Heinz, E. (1995) A cytochrome b5-containing fusion protein similar to plant acyl lipid desaturases. *Eur. J. Biochem.*, 232: 798–805.

Sperling, P., Zähringer, U., and Heinz, E. (1998) A Sphingolipid Desaturase from Higher Plants. Identification of a new cytochrome b5 fusion protein. *J. Biol. Chem.*, 273: 28590–28596.

Spychalla, J. P., Kinney, A. J. and Browse, J. (1997) Identification of an animal omega-3 fatty acid desaturase by heterologous expression in *Arabidopsis. Proc. Natl. Acad. Sci. USA*, 94: 1142–1147.

Srivastava, N. K. and Sharma, S. (1990) Effect of triacontanol on photosynthesis, alkaloid content and growth in opium poppy (*Papaver somniferum* L.). *Plant Growth Regul.*, 9: 65–71.

Stoutemyer, V. (1981) Triacontanol: A promising new growth promoting substance. *Fuchsia. Fan.,* 41: 9–10.

Stoutemyer, V. and Cook, R. (1987) Update on Triacontanol. *The Orchid Advocate*, 13: 86–87.

Streekstra, H. (1997) On the safety of *Mortierella alpina* for the production of food ingredients, such as arachidonic acid. *J. Biotechnol.*, 56: 153–165.

Tantos, A., Meszaros, A. and Kissimon, J. (1999) The effect of triacontanol on micropropagation of balm, *Melissa officinalis* L. *Plant Cell Rep.,* 19: 88–91.

Thomas, J. C. and Katterman, F. R. (1986) Cytokinin activity induced by thidiazuron. *Plant Physiol.,* 81: 681–683.

Thompson, J. D., Higgins, D. G. and Gibson, T.C. (1994) CLUSTALW: Improving the Sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucl. Acids Res.,* 22: 4673–4680.

Tocher, D. R., Leaver, M. J. and Hodgson, P. A. (1998) Recent advances in the biochemistry and molecular biology of fatty acyl desaturases. *Prog. Lipid Res.*, 37: 73–117.

Tomlin, E. S., Branch, S. R., Chamberlain, D., Gabe, H., Wright, M. S. and Stewart, C. N. J. (2002) Screening of soybean, *Glycine max* (L.) Merrill, lines for somatic embryo induction and maturation capability from immature cotyledons. *In Vitro Cell Dev. Biol.*, 38: 543–548.

Tonon, T., Harvey, D., Larson, T. R. and Graham, I. R. (2003) Identification of a very long chain polyunsaturated fatty acid delta4-desaturase from the microalga *Pavlova lutheri*. *FEBS Lett*., 553(3): 440–444.

Traitler, H., Wille, H. J. and Studer, A. (1988) Fractionation of blackcurrant seed oil. *J. Am. Oil Chem. Soc.*, 65: 755-760.

Traitler, H., Winter, H., Richeli, U. and Ingenbleck, Y. (1984) Characterization of γ-linolenic acid in *Ribes* seed. *Lipids*, 19: 923-928.

Trautwein, E. A. (2001) ω-3 Fatty acids-physiological and technical aspects for their role in food. *Eur. J. Lipid Sci. Technol.*, 103: 45–55.

Trick, H. N. and Finer, J. J. (1998) Sonication-assisted *Agrobacterium*-mediated transformation of soybean [*Glycine max* (L.) Merrill] embryogenic suspension culture tissue. *Plant Cell Rep*., 17: 482–488.

Tzitzikas, E. N., Bergervoet, M., Raemakers, K., Vincken, J. P., Lammeren, A. and Visser, R. G. F. (2004) Regeneration of Pea (*Pisum sativum* L.) by a cyclic organogenic system. *Plant Cell Rep*., 23: 453–461.

Ullmann, A., Jacob, F. and Monod, J. (1967) Characterization by *in vitro* complementation of a peptide corresponding to an operator-proximal segment of the beta-galactosidase structural gene of *Escherichia coli*. *J. Mol. Biol*., 24: 339-343.

Ulsamer, A. G., Smith, F. R. and Korn, E. D. (1969) Lipids of *Acanthamoeba castellanii*. Composition and effects of phagocytosis on incorporation of radioactive precursors. *J. Cell. Biol.*, 43: 105–114.

USDA nutrient database, 2008. http://www.nal.usda.gov/fnic/foodcomp/search/

Van, N. J. P., Zimmerman, R. H. and Fordham, I. (1986) Thidiazuron stimulation of apple shoot proliferation *in vitro*. *Hort. Science*, 21: 516–518.

Vincze, T., Posfai, J. and Roberts, R. J. (2003) NEBcutter: a program to cleave DNA with restriction enzymes. *Nucleic Acids Res.*, 31: 3688-3691.

Von W.K.P., J.G. Olsen, K. Arnvig, S. Larsen, In: J.L. Harwood, P.J. Quinn (Eds.), Recent Advances in the Biochemistry of Plant Lipids, Portland Press, 2001, pp. 601–607.

Wada, H., Gombos, Z. and Murata, N. (1990) Enhancement of chilling tolerance of a cyanobacterium by genetic manipulation of fatty acid desaturation. *Nature,* 347: 200–3.

Wallis, J. G. and Browse, J. (1999) The Δ^8-desaturase of *Euglena gracilis*: an alternate pathway for synthesis of 20-carbon polyunsaturated fatty acids. *Arch. Biochem. Biophys.,* 365: 307–316.

Wang, C.Y. and Wixon, R. (1999) Phytochemicals in Soybeans and Their Potential Health Benefits. *INFORM,* 10(4): 315–321.

Wang, H. J. and Murphy, P. A. (1994) Isoflavone content in commercial soybean foods. *J. Agric. Food Chem.,* 42: 1666–1673.

Watts, J. L. and Browse, J. (1999) Isolation and characterization of a Δ^5-fatty acid desaturase from *Caenorhabditis elegans. Arch. Biochem. Biophys.*, 362: 175–182.

WHO (2009) Global and regional food consumption patterns and trends. http://www.who.int/nutrition/topics/3_foodconsumption/en/index3.html

WHO/FAO. (1977) Dietary Fats and Oils in Human Nutrition. Report of an Expert Consultation. Rome: FAO.

Willis, A. L. (1981) Nutritional and pharmacological factors in eicosanoid biology. *Nutr. Rev.*, 39: 289-301.

Wingmore, S. J., Ron, J. A., Falconer, J. S., Plaster, C. E., Tisdale, M. J., Carter, D. C. and Faron, K. C. H. (1996) The effect of PUFA on the progress of cachexia in patients with pancreatic cancer. *Nutrition,* 12: 27–30.

Wordragen, M. F. and Dons, H. J. M. (1992) *Agrobacterium tumefaciens* mediated transformation of recalcitrant crops. *Plant Mol. Biol. Rep.,* 10: 12-36.

Wright, M. S., Koehler, S. M., Hinchee, M. A. and Carnes, M. G. (1986) Plant regeneration by organogenesis in *Glycine max. Plant Cell Rep.,* 5: 150–154.

Wright, M. S., Ward, D. V., Hinchee, M. A., Carnes, M. G. and Kaufman, R. J. (1987a) Regeneration of soybean (*Glycine max* L. Merr.) from cultured primary leaf tissue. *Plant Cell Rep*., 6: 83–89.

Wright, M. S., Williams, M. H., Pierson, P. E. and Carnes, M. G. (1987b) Initiation and propagation of *Glycine max* L. Merr.; Plants from tissue cultured epicotyls. *Plant Cell Tissue Org. Cult.,* 8: 83–90.

Wroblewski, T., Tomczak, A. and Michelmore, R. (2005) Optimization of *Agrobacterium* mediated transient assays of gene expression in lettuce, tomato and *Arabidopsis*. *Plant Biotechnol, J.*, 3: 259–273.

www.sopa.org

www.soystats.com

Xian, M., Nie, J., Meng, Q., Liu, J., Bi, Y. and Zhen, K. (2001) Production of gamma linolenic acid by *Mortierella isabellina* grown on hexadecanol. *Lett. Appl. Microbiol.*, 33: 367-370.

Yadav, N. S., Wierzbicki, A., Aegerter, M., Caster, C. S., Perez-Grau, L., Kinney, A. J., Hitz, W. D., Russell, J., Schweiger, B., Stecca, K. L., Allen, S. M., Blackwell, M., Reiter, R. S., Carlson, T. J., Russell, S. H., Feldmann, K. A., Pierce, J. and Brown, J. (1993) Cloning of higher plant omega-3 fatty acid desaturases. *Plant Physiol.*, 103: 467–76.

Yan, B., Reddy, M. S. S., Collins, G. B. and Dinkins, R. D. (2000) *Agrobacterium tumefaciens* mediated transformation of soybean [*Glycine max* (L.) Merrill] using immature zygotic cotyledon explants. *Plant Cell Rep*., 19: 1090–1097.

Yip, W. K. and Yang, S. F. (1986) Effect of thidiazuron, a cytokinin active urea derivative, in cytokinin dependent ethylene production systems. *Plant Physiol.*, 80: 515–519.

Yun, C. H. and Kim, H. I. (1986) Differences in callus formation from different explant sources and effect of plant growth regulators in plant regeneration in rice (*Oryza sativa L*). *Res. Rep. Rur. Dev. Admin.*, 1: 113–116.

Zank, K. U., Zahringer, J. L. and Heinz, E. (2000) Cloning and functional expression of the first plant fatty acid elongase specific for delta(6)- polyunsaturated fatty acids, *Biochem. Soc. Trans.*, 28: 654–658.

Zarrouk, C., (1996) Contribution a l'etude d'une Cyanophycee. Influence de divers facteurs physiques et chimiques sur la croissance et la photosynthese de Spirulina maxima (Setch et Garner) Geitler. Ph.D. Thesis Univ. Paris, France.

Zeng, P., Vadnais, D. A., Zhang, Z. and Polacco, J. C. (2004) Refined glufosinate selection in *Agrobacterium*-mediated transformation of soybean [*Glycine max* (L.) Merrill]. *Plant Cell Rep.*, 22: 478–482.

Zhang, Z. Y., Xiang, A. Q., Staswick, Q., et al (1999) The use of glufosinate as a selective agent in *Agrobacterium*-mediated transformation of soybean. *Plant Cell Tissue Org. Cult.*, 56: 37–46.

Zhao, Z., Gu, W., Cai, T., Tagliani, L., Hondred, D., Bond, D., Schroeder, S., Rudert, M. and Pierce, D. (2001) High throughput genetic transformation mediated by *Agrobacterium tumefaciens* in maize. *Mol. Breed.*, 8: 323–333.

Zhou, J., Ma, H., Guo, F. and Luo, X. (1994) Effect of thidiazuron on somatic embryogenesis of '*Cayratia japonica'*. *Plant Cell Tissue Org. Cult.*, 36: 73–79.

Zolfaghari, R., Cifelli, C. J., Banta, M. D. and Ross, A. C. (2001) Fatty acid delta(5)-desaturase mRNA is regulated by dietary vitamin A and exogenous retinoic acid in liver of adult rats. *Arch. Biochem. Biophys.*, 391: 8–15.

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Publications

LIST OF PUBLICATIONS

1. **Sakthivelu, G.,** Akitha Devi, M. K., Giridhar, P., Rajasekaran, T., Ravishankar, G.A., Nikolova, M. T., Angelov, G. B., Todorova, R. M. and Kosturkova, G. Isoflavone composition, phenol content and antioxidant activity of Soybean seeds from India and Bulgaria. *Journal of Agricultural and Food Chemistry*, 2008, 56 (6): 2090-2095.

2. **Sakthivelu G.,** Akitha Devi M.K., Giridhar P., Rajasekaran T., Ravishankar G.A., Nedev T. and Kosturkova G. Drought induced alterations in growth, osmotic potential and *in vitro* shoot multiplication of soybean cultivars. *General and Applied Plant Physiology*, 2008, Special issue 34(1-2), 103-112.

3. Kosturkova, G., Todorova, R., **Sakthivelu, G.,** Akitha Devi, M. K., Giridhar, P., Rajasekaran, T. and Ravishankar, G. A. Response of Bulgarian and Indian soybean genotypes to drought and water deficiency in field and laboratory conditions. *General and Applied Plant Physiology*, 2008, Special issue 34(3-4), 239-250.

4. Akitha Devi, M. K., Mahendranath Gondi, **Sakthivelu, G.**, Giridhar, P., Rajasekaran, T. and Ravishankar, G.A. Functional attributes of soybean seeds and products with reference to isoflavone content and antioxidant activity. *Food Chemistry*, 2009, 114(3): 771-776.

5. Nedev T., Todorova R., Kosturkova G., Akitha Devi M.K., **Sakthivelu G.,** Giridhar P., Rajasekaran T., Ravishankar G.A. Variation in *in vitro* morphogenic response to growth regulators in soybean genotypes from India and Bulgaria. *Bioautomation*, 2007, 8: 193-200.