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ORIGINAL PAPER

Agrobacterium-mediated transformation of *Brassica juncea* with a cyanobacterial (*Synechocystis* PCC6803) delta-6 desaturase gene leads to production of gamma-linolenic acid

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Abstract Genetic manipulation of the oil-yielding crop plants for better oil quality through biotechnological methods is an important aspect of crop improvement. Due to the inherent absence of the Δ^6-desaturase (d6D) function, *Brassica juncea*, an oil-yielding 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. Cyanobacterial d6D introduces carbon–carbon double bond onto linoleic acid (C18:2) and α-linolenic acid (C18:3) by desaturation processes for production of GLA and octadecatetraenoic acid (OTA) respectively. In the present investigation, d6D coding sequence from *Synechocystis* sp. PCC6803 was cloned by polymerase chain reaction and introduced into *B. juncea* through *Agrobacterium*-mediated transformation technique. Both cytosolic as well as seed-specific expression of d6D were attempted. The transformed plants show production of GLA and OTA in contrast to their absence in the untransformed control plants adducing evidence for introgression and functional expression of the cyanobacterial d6D gene in *B. juncea*.

Keywords Δ^6-Desaturase · γ-Linolenic acid · C18 fatty acid · Genetic manipulation · Transgenic *Brassica* plant

Abbreviations

ALA	α-linolenic acid
BAP	6-benzylaminopurine
CaMV	Cauliflower mosaic virus
d6D	Δ^6-desaturase
2,4,-D	2,4,-dichlorophenoxyacetic acid
FAMEs	fatty acid methyl esters
GLA	γ-linolenic acid
GLC	gas liquid chromatography
GUS	β-glucuronidase
hptII	hygromycin phosphotransferase gene
MS	Murashige and Skoog
nos	nopaline synthase gene
OTA	octadecatetraenoic acid
PCR	polymerase chain reaction
SD	standard deviation
TAG	triacyl glycerol
TLC	thin layer chromatography

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Introduction

Increasing population worldwide resulted in significant increase in oilseed consumption from 40 Mt in 1964 to 347 Mt by 2004 Asian countries being the major contributors to this increasing trend thereby increasing its demand (Agriculture and Agri-food Canada Bi-weekly bulletin 2004). In India alone, consumption rate is increasing at the rate of 6% per annum and per capita availability of oil and fats is 10 kg against world average of 17 kg. While the chances of cultivable area expansion is limited (Hegde 2003), genetic engineering of the crop plant to produce qualitatively improved tailor-made oilseed for edible and industrial purpose may be one of the approaches to meet the increasing demand as opposed to conventional breeding practices (Kinney 1994; Agnihotri and Kaushik 1998, 2000; Cardoza and Stewart 2004). For the qualitative improvement of oil seed crop, most of the genetic modification attempts were mainly directed to *Brassica napus* among the oilseed rape, soybean and sunflower (Purdy 1986; Knutzon et al. 1992; Kinney 1994; Singh et al. 2000; Cardoza and Stewart 2004). At the same time less emphasis has been given to Indian mustard, *Brassica juncea*, one of the major oil producing crop plants of South Asian countries (Mackey 2002; Hegde 2003). Among all oleiferous brassicas cultivated in India, *B. juncea* occupies the largest acreage (Kaushik and Agnihotri 2003). In addition, it is becoming a better choice as oilseed crop of India over other *Brassica* species due to its heat and drought tolerance (Burton 2003; Gupta et al. 2004) as well as for phytoremediation project as it effectively can accumulate heavy metals (Clemente et al. 2005).

Genetic transformation of oilseed *Brassica* has been mostly directed towards traits like male sterility (Denis et al. 1993), fungal pathogen tolerance (Grison et al. 1996), alteration of self-incompatibility phenotype (Stahl et al. 1998; Shiba et al. 2000), low chlorophyll seed variety (Tsang et al. 2003) etc. 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 (Singh et al. 2005). In this respect only a few reports are available so far on *Brassica* with altered fatty acid composition for obtaining qualitatively improved seed oil (Verwoert et al. 1995; Stoutjesdijk et al. 2000). Therefore, it was felt necessary to focus on genetic modification of the fatty acid composition of *B. juncea*, in order to make it more acceptable as nutritionally better food, feed and for industrial utilization.

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 being an active precursor of arachidonic acid (C20:4), which participates in the synthesis of prostaglandin in human being (Napier et al. 1999a). 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 atherosclerotic heart disease (Barre 2001). Therefore, production of oilseed 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 pathway is present in the cyanobacteria (e.g. *Synechocystis*, *Spirulina*) and in a few higher

plants (e.g. *Borago officinale*, *Oenothera*) 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 low chain poly-unsaturated fatty acid, 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 *B. juncea* 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 workers to explore the possibilities of altering the C18 desaturation pathway of plants for production of GLA (Knutzon et al. 1999; Sayanova et al. 1999a; 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 *Lycopersicon esculentum* with borage d6D (Cook et al. 2002), *B. juncea* with *Pythium irregulare* d6D (Hong et al. 2002) and *Oenothera* sp. with borage d6D (Gyves et al. 2004).

However, expression of a cyanobacterial d6D in any oil seed crop such as *B. juncea* has not been reported so far. In this present investigation, an attempt has been made to explore the possibilities of use of a cyanobacterial (*Synechocystis* sp. PCC6803) d6D gene in *B. juncea* to modify the C18 desaturation pathway towards GLA production for obtaining nutritionally superior *Brassica* oil. In addition, accumulation of considerable amount of GLA in seed oil greatly depends on the shuttling of modified acyl group between acyl-phosphatidyl choline and acyl-CoA pool by the action of lysophosphatidyl choline acyl-transferase. Endogenous acyl transferase might not be capable of performing this shuttling effectively (Singh et al. 2005). Our investigation is also aimed to provide a d6D transformed oilseed plant, which can be a good starting material for genetic manipulation with acyl transferase for effective accumulation of GLA in seed oil.

Materials and methods

Strains and growth condition

Synechocystis sp. PCC 6803 (obtained from the National Facility for Marine Cyanobacteria, Bharathidashan University, Tiruchirapalli, India) was grown photoautotrophically in BG11 media (Rippka et al. 1979) at 25°C with mild shaking (40 rpm, Certomat Orbital Shaker) under 12 h light/dark cycle maintained at approx. 3,000 lux. Bacterial cultures of *E. coli* DH5 α was stored as 15% (v/v) glycerol stocks at –80°C and maintained on Luria–Bertani (LB) plates containing 1.4% (w/v) agar. Cells harboring recombinant plasmids were grown and maintained on LB media supplemented either with 100 μg ml^{-1} ampicillin sodium salt (potency ≥845 μg mg^{-1}, stock solution used 100 mg ml^{-1}, Sigma, St. Louis, MO, USA) or with 50 μg ml^{-1} kanamycin sulphate (potency ≥750 μg mg^{-1} stock solution used 50 mg ml^{-1}, Sigma, St. Louis, MO, USA) as required. Growth condition was set at 37°C with constant shaking (180 rpm).

Isolation and cloning of *Synechocystis* d6D (Syn-d6D)

Genomic DNA of *Synechocystis* PCC6803 was isolated from 21-day-old culture following the methods of Majur et al. (1980) and was used as template DNA to isolate the *Syn*-d6D coding sequence (ORF sll0262) from *Synechocystis*. Two oligonucleotide primers were synthesized based on the reported *Syn*-d6D coding sequence available at National Centre for Biotechnology Information (NCBI) database (GenBank Accession no. L11421) as follows: forward primer 5′-TCTAGAGGATCCATGCTAACAGCGGAA-AGA-3′; reverse primer: 5′-CTCGAGGGTAC CGAGCTCTCACGATGCTTT-3′. To facilitate construction of plant expression cassette, flanking *Bam*HI site at 5′ end and *Kpn*I site at 3′ end were added to the respective primers for the *Syn*-d6D coding sequence. A 25 μl PCR reaction mix contained 2.5 μl 10× High Fidelity *Taq* buffer (GIBCO BRL), 0.75 μl of 50 mM $MgSO_4$, 2.5 μl of 2.5 μM each of forward and reverse primers, 0.5 μl of 10 mM dNTPs, 100 ng of template DNA

and 2.5 units of High Fidelity Platinum *Taq* DNA polymerase (GIBCO BRL). Thirty-two cycles of amplification were carried out at 94°C for 1.5 min, 55°C for 1.5 min and 72°C for 2 min with a 10 min final extension at 72°C. The PCR amplified product was first analyzed and then purified using QIAquick Gel Extraction Kit (QIAGEN), digested with *Bam*HI and *Kpn*I and cloned in pBluescriptSK$^+$ cloning vector (Stratagene) to generate p*Sy*DB. The nucleotide sequence of the PCR product was determined and verified with the reported sequence.

Construction of plant expression cassette for *Agrobacterium*-mediated plant transformation

The plasmid p*Sy*DB was digested with *Bam*HI and *Kpn*I to release the ~1.1 kb fragment containing *Syn*-d6D open reading frame. The gel purified fragment was directionally cloned into a pCAMBIA 1301 based binary vector (a gift from Prof. Akhilesh Tyagi, University of Delhi, South Campus, New Delhi, India) which contains GUS as a reporter gene (under the control of CaMV 35S promoter and nopaline synthase terminator), hptII gene as plant selection marker (under the control of CaMV 35S promoter and 35S polyA terminator). Regulatory elements that include a CaMV 35S promoter for constitutive expression of d6D gene in most plant tissues and a nopaline synthase (*nos*) termination sequence was cloned in between *Hin*dIII–*Bam*HI and *Sac*I–*Eco*RI sites of pCAMBIA 1301 respectively. To generate chimeric plant expression vector pBSA1 containing 35S-d6D gene cassette (also called 35S-d6D), the d6D coding sequence from p*Sy*DB clone was inserted into above-mentioned pCAMBIA 1301 between *Bam*HI and *Kpn*I sites.

For seed-specific expression of d6D gene, napin promoter (obtained as a gift from Dr. I.B. Maity, Kentucky University, Lexington, USA) was used to generate the pNidBLA plant expression vector containing napin-d6D gene cassette (also called napin-d6D). The napin promoter and *Syn*-d6D coding sequence was inserted in the pCAMBIA 1301-based plant expression vector (already containing *nos* terminator, hptII and GUS cassette) step by step as follows: *Bam*HI and *Kpn*I fragment containing *Syn*-d6D coding sequence was taken out from pBSA1 and was inserted directionally at *Bam*HI and *Kpn*I site in the pCAMBIA 1301 vector (containing *nos* terminator) and napin promoter was then inserted at *Pst*I and *Sal*I site to obtain the chimeric plant expression vector pNidBLA to drive the seed-specific expression of *Syn*-d6D gene. *Agrobacterium tumefaciens* LBA4404 was mobilized with chimeric plant expression vectors pBSA1 and pNidBLA and were grown overnight in 15 ml AB minimal medium containing rifampicin and kanamycin sulphate (50 μg ml^{-1} each, stock solution used 50 mg ml^{-1} for both, Sigma, St. Louis, MO, USA). The cells were collected by centrifugation and suspended in liquid hormone-free Murashige and Skoog's (MS) medium (Murashige and Skoog 1962). The cell suspension of an O.D. of 0.5–0.6 at 600 nm was then used for plant transformation.

Agrobacterium-mediated transformation of *B. juncea*

Agrobacterium-mediated transformation and in vitro regeneration of *B. juncea* transgenics were performed following the methods of Barfield and Pua (1991). Seeds of *B. juncea* var. B85, a local cultivar of lower Gangetic plains of West Bengal, India (obtained from the Bose Institute experimental farm, Kolkata, India) were surface sterilized and germinated aseptically in plastic screw cap glass jar (height 12.5 cm and inner diameter 6 cm) on 50 ml of half strength hormone free MS medium in dark for 3 days and then transferred to light (16 h light and 8 h dark) for 5 days. Approximately 500 hypocotyl explants (0.5–1.0 cm) from 8-day-old seedlings were cultured for 24–48 h on 30 ml solid hormone free MS medium in 10-mm Borosil glass Petri plate. The explants were placed horizontally on the surface of the media. The pre-cultured explants were incubated with ~20 ml *Agrobacterial* suspension for 10–20 min with occasional gentle shaking in a Borosil glass Petri plate. After infection, the bacterial suspension was removed and explants were transferred and incubated on same pre-culture plate (containing solid MS medium) for co-cultivation keeping the orientation of the

explants as before. After 24 h of co-cultivation, explants were plated (10-mm Borosil glass Petri plate with 30 ml media in each) on shoot induction medium (MS medium supplemented with 0.5–1.0 mg l^{-1} BAP and 0.01–0.05 mg l^{-1} 2,4-D along with 30 μM $AgNO_3$, 250 mg l^{-1} cefotaxime sodium salt, potency 916–964 μg mg^{-1}, obtained from Sigma, St. Louis, MO, USA and 30 mg l^{-1} hygromycin B, stock solution used 5 mg ml^{-1} obtained from Roche Applied Sciences, Indianapolis, IN, USA) and sub-cultured in the same medium at every 10 days interval. Explants showing appearance of green shoot buds were then transferred to plastic screw cap glass jar (dimension was same as mentioned before) containing 50 ml regeneration medium (MS medium supplemented with 0.05–1.0 mg l^{-1} BAP along with 30 μM $AgNO_3$ and 250 mg l^{-1} cefotaxime and 30 mg l^{-1} hygromycin B) for regeneration of multiple shootlets. Elongation and rooting of shootlets was performed in similar glass vessels containing 50 ml hormone free solid MS medium where hygromycin B concentration was reduced to 20 mg l^{-1}. Following rooting, the full-grown plantlets were sub-cultured at monthly intervals in the same medium to maintain the transformed lines and were kept for further analysis. Cultures were maintained throughout at 23 ± 2°C under a 16 h/8 h light–dark cycle with light (140–180 μmol m^{-2} s^{-1}) supplied by cool-white daylight fluorescent lights and 70% relative humidity in Plant Growth Chamber (NK System BIOTRON, LPH 200). Selected healthy positive 35S-d6D and napin-d6D transgenic lines were transferred to soilrite, an artificial soil and nutrient mixture (KEL PERLITE, Bangalore, India) for hardening. After proper hardening, the plantlets were potted in natural soil, kept inside glass house for about 15–20 days and then transferred to natural environmental condition along with control plants for its growth, flowering and seed setting.

Histochemical β-glucuronidase (GUS) assay

Histochemical determination of GUS activity in the plant tissues was performed according to Jefferson (1987). Leaves of untransformed and transformed plants were incubated at 37°C overnight in 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-gluc) solution consisting of 10 mM EDTA, 100 mM sodium phosphate (pH 7.0), 0.5 mM potassium ferrocyanide and 0.1% (w/v) X-gluc and examined visually. β-glucuronidase (GUS) act on its substrate X-gluc and produces soluble colorless primary reaction product, 5-bromo-4-chloro-3-indolyl which oxidized and dimerized due to presence of ferrocyanide in the reaction solution and forms an insoluble, intensely blue final reaction product. Formation of blue coloration is thus indicative of GUS reporter activity in the transformed plant tissue (Vitha et al. 1995).

PCR analysis of transgenic plants

Total DNA was isolated from the leaves of untransformed and transformed plants using CTAB method (Rogers and Bendich 1994). PCR was performed in a total volume of 25 μl containing 200 ng of template DNA and *Syn*-d6D specific primer (same set used for isolation of *Syn*-d6D coding sequence from *Synechocystis*) using identical PCR conditions as described for isolation of the *Syn*-d6D gene. PCR products were verified on 1% agarose gel.

Southern blot analysis

Total genomic DNA was extracted from the leaves of transgenic and non-transgenic control plants for Southern analysis, according to the procedure of Dellaporta et al. (1983) with minor modifications. Twenty μg of DNA was digested overnight with *Pvu*II to confirm the inserted *des* gene in the genome. The digested DNA was separated by electrophoresis on 1% agarose gel. DNA fragments were denatured and transferred to Nylon Membranes, positively charged (Roche) following the manufacturer's instruction. The 1.1-kb PCR amplified *des* gene was labeled with α-[^{32}P]-dCTP using the Rediprime II Random Prime labeling System (Amersham Biosciences) and used as hybridization probe.

Fatty acid analysis

Total lipid was extracted from *Synechocystis* cells, leaves and seeds of untransformed as well as

transformed *Brassica* plants containing the *Syn*-d6D gene following the method of Bligh and Dyer (1959). Fatty acid methyl esters (FAMEs) were prepared and analyzed using a Chemito 1000 gas liquid chromatograph (GLC) equipped with a hydrogen flame ionization detector and BPX-70 megabore capillary column of 30 m length and 0.53 mm inner diameter. Oven temperature for total FAMEs was programmed from 150°C to 240°C at the rate of 8°C/min. Initial and final temperatures were kept constant for 2 min and 10 min, respectively. Injection port and detector temperatures were 250°C and 300°C, respectively. Nitrogen gas was used as carrier with a flow rate of 6.6 ml/min. For unambiguous identification, FAMEs of trienoic acids were separated from others on silver nitrate-coated TLC plates following standard methods (Kates 1986). GLC of the purified FAMEs of the trienoic acids were done as mentioned above. Retention times and cochromatography of standards (obtained from Sigma Chemical Co., St. Louis, MO) were used to identify the fatty acids. Peak identity was based on comigration with GLA and OTA standard in the same chromatographic run for overall separation time of 10–12 min. The average fatty acid composition was determined as the ratio of peak area of each C18 fatty acid normalized to a C17 internal standard. All the data obtained were computed using Winchrom software.

Results and discussion

Cloning of d6D gene from *Synechocystis* PCC6803 and construction of the plant expression vector

An analysis of C18 fatty acid profile showed that the mature seeds of *B. juncea* contain no detectable GLA although presence of ALA was discernible (Fig. 1a). However, in case of *Synechocystis* PCC6803 fatty acid extracts, a distinct peak for GLA was obtained (Fig. 1b). Following this observation, the d6D coding sequence of *Synechocystis* sp. (ORF sll0262) was selected as candidate gene for d6D expression in the target crop *B. juncea.* The *Synechocystis* coding sequence was amplified by PCR as described in the Methods. Absolute sequence similarity was obtained for the PCR amplified product while aligned with the reported gene sequence of ORF sll0262 through pairwise BLAST 2.0 analysis. The PCR amplified product was then used to construct d6D plant expression cassettes.

In diverse plant species, triacylglycerol (TAG) is surrounded by a layer of phospholipids and oleosin protein that forms small discrete intracellular organelle called oil bodies (Tzen et al. 1993). In the oil bodies, TAG act as storage lipids mainly in the seeds. Acylation and hydrolysis of precursor molecules like C18:2, C18:3 in the endoplasmic reticulum (ER) result in the formation of storage

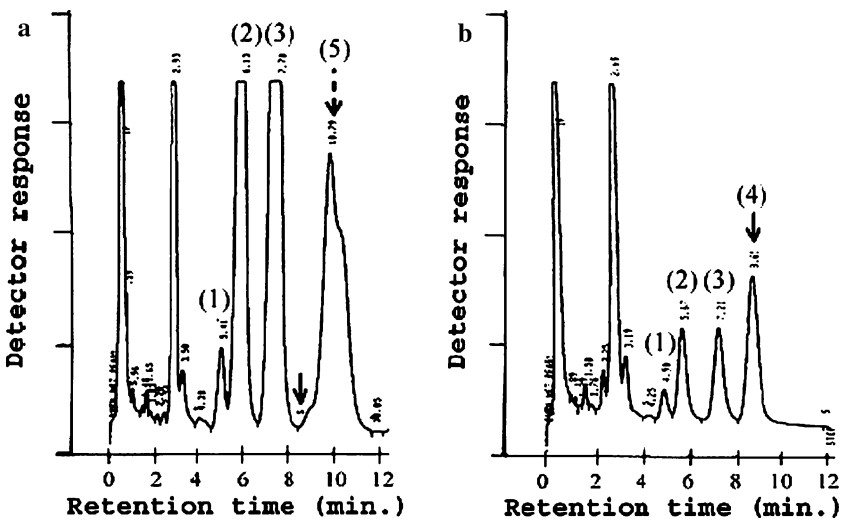

Fig. 1 Gas chromatogram of fatty acid methyl esters extracted from (**a**) mature seeds of untransformed *B. juncea* showing absence of GLA [↓].and presence of ALA [↓] and (**b**) *Synechocystis* sp. PCC 6803 cells showing presence of GLA [↓]. [C18 peaks : (1) 18:0; (2)18:1; (3) 18:2; (4) 18:3 γ; (5) 18:3 α.]

lipid TAG in which GLA is exclusively incorporated (almost 98.5%) at sn-2 position (Hong et al. 2002). The precursor molecules are exported to ER from its cytosolic pool regardless of its site of synthesis (Kinney 1994). Delta-6-desaturation in the cytosol is thought to increase the cytosolic pool of γ-form of C18:3 (GLA) synthesized from the available C18:2 linoleic acid. With this background, *B. juncea*, an oil seed crop plant, was transformed through *Agrobacterium*-mediated transformation technique with two plant expression constructs, one under the control of CaMV-35S promoter and the *nos* terminator element (Fig. 2a) for constitutive expression of d6D in the cytosol and another for its specific expression in the seeds under the control of napin promoter and *nos* terminator (Fig. 2b). *Agrobacterium tumefaciens* strain LBA4404 was used to transfer the T-DNA containing these constructs to *B. juncea*.

Agrobacterium-mediated *B. juncea* transformation and regeneration

Agrobacterium-infected hypocotyl explants showed appearance of green shoot buds within 45–60 days when incubated in shoot induction medium containing hygromycin B, for selection of the transformed explants (Fig. 3a, b). Each of the shoot buds regenerated 4–5 multiple shootlets on culturing in regeneration medium for 30–45 days (Fig. 3c, d). Transfer of individual shootlets in hormone-free solid MS medium (Fig. 3e, f) led to the formation of full grown rooted plantlets within 20–30 days (Fig. 3g, h). All together 500 explants, for each construct, were infected in 5 individual entire experiments (each experiment with total 100 explants divided into 5 replica). A total of 33 putative transgenic lines for 35S-d6D (transformation frequency 6.6%) and 21 putative transgenic napin-d6D lines (transformation frequency 4.2%) were obtained which is close upon the transformation frequency (7.1%) reported by Barfield and Pua (1991) for the hormonal combinations selected in this study. Representative of the best performing lines are documented in Fig. 3. Histochemical GUS assay of the leaves from transformed lines showed appearance of characteristic blue color after incubation in X-gluc solution while leaves of untransformed plants showed no such coloration (Fig. 3i–k) indicating GUS reporter activity in the transformants. Full grown rooted plantlets were then transferred to pot in natural environment (Fig. 3l–n) after proper hardening in soilrite for 10 days.

Genotyping and analysis of gene integration in transformed plants

GUS positive transformed lines were characterized for its genotype by PCR amplification

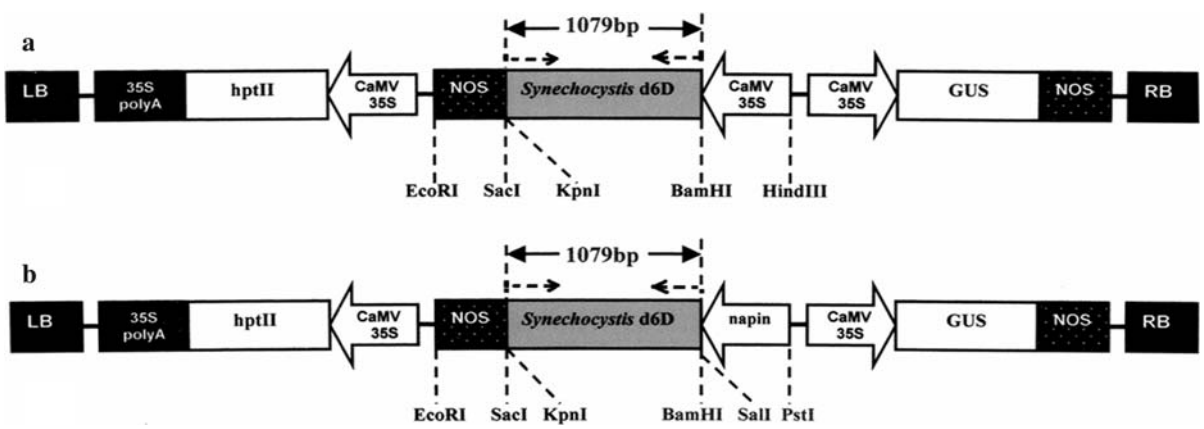

Fig. 2 pCAMBIA 1301-based plant expression construct of chimeric *Synechocystis* Δ^6 desaturase gene: (**a**) under the transcriptional control of CaMV 35S constitutive promoter; (**b**) under the transcriptional control of seed specific *B. napus* napin-promoter. LB: left border and RB: right border. [1,079 bp region of *Synechocystis* d6D is indicated by (⟵ / ⟶) while the location of the primers used for PCR amplification is indicated by (⇢ / ⇠)]

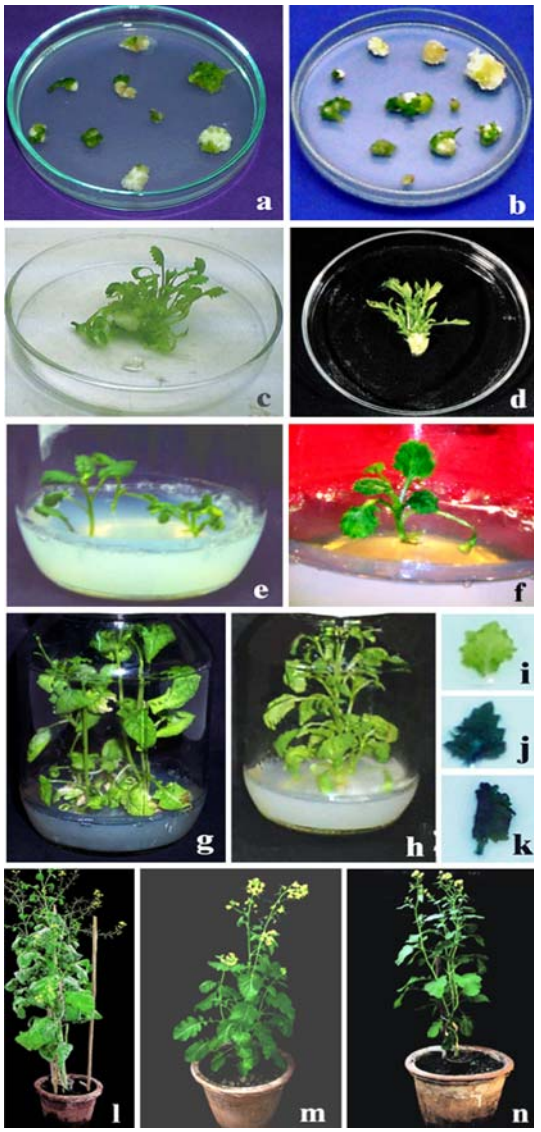

Fig. 3 *Agrobacterium*-mediated transformation of *B. juncea* at different stages of in vitro regeneration, screening of transformed plants and their establishment in natural environmental condition. Appearance of green shoot bud from *Agrobacterium*-infected hypocotyls explants: (**a**) 35S-d6D line, (**b**) napin-d6D line. Regeneration of multiple shootlets: (**c**) 35S-d6D line, (**d**) napin-d6D line. Individual shootlet in hormone-free MS media for its elongation and rooting: (**e**) 35S-d6D line, (**f**) napin-d6D line. Full grown rooted plantlet: (**g**) 35S-d6D line, (**h**) napin-d6D. Histochemical GUS assay of leaf: (**i**) untransformed control plant, (**j**) 35S-d6D line, (**k**) napin-d6D line. Potted plants (at reproductive stages) in natural environmental conditions: (**l**) untransformed control plant, (**m**) 35S-d6D, (**n**) napin-d6D line

of total DNA using *Syn*-d6D specific primers (as indicated in Fig. 2). Only the hygromycin resistant GUS positive lines showed a single amplification product of expected size of ~1.0 kb for *Syn*-d6D whereas no such amplified product was obtained in the untransformed control lines (Fig. 4) indicating the presence of *Synechocystis* d6D gene into the host plant genome.

Introgression of the *Syn*-d6D gene in the T_0 and T_1 generation of the transformed plants were confirmed by Southern blot analysis with *Syn*-d6D specific probe (Fig. 5). For this, total genomic DNA was digested with *Pvu*II and the separated fragments hybridized with the *Syn*-d6D specific probe generating a hybridizable 2.4-kb fragment as confirmed by similarly digested pBSA1 DNA with *Pvu*II used as the positive control in the Southern blot analysis. Hybridization with *Syn*-d6D gene as probe confirmed the introgression of the gene in the plant genome. Stable introgression of the *Syn*-d6D gene was confirmed in T_1 generation as well.

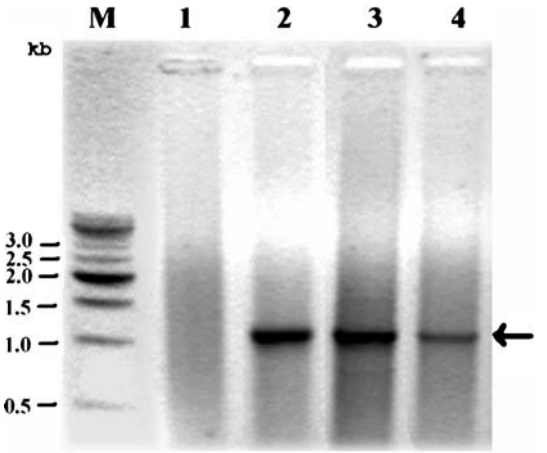

Fig. 4 Genotypic analysis of transformed *B. juncea* lines through PCR amplification with *Syn*-d6D specific primers. M: DNA marker (500-bp ladder), lane 1: untransformed plants as negative control, lane 2: 35S-d6D line, lane 3: napin-d6D line, lane 4: *Synechocystis* d6D as positive control (arrow head indicating the position of amplified product of ≈1079 bp)

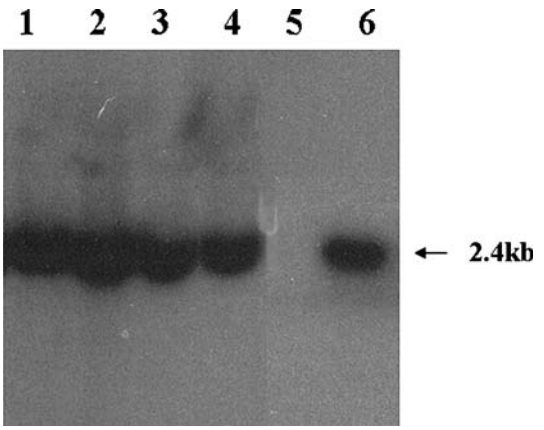

Fig. 5 Southern blot showing the introgression of *Syn*-d6D gene in the primary transgenics and subsequent generations. A total of ~20 μg of plant genomic DNA was digested overnight with *Pvu*II, electrophoresed in 1% TAE-agarose gel, Southern blotted and hybridized with α-[^{32}P]-dCTP-labeled probes of PCR generated *Syn*-d6D gene. Lane 1: napin d6Dline (T_0 generation); Lane 2: napin d6Dline (T_1 generation); Lane 3: 35S-d6D line (T_0 generation); Lane 4: 35S-d6D line (T_1 generation); Lane 5: untransformed plant (negative control); Lane 6: DNA of pBSA1 clone digested with *Pvu*II (positive control)

Fatty acid analysis of untransformed and transformed *B. juncea* plants

FAME analysis of untransformed control seeds showed distinct peak of ALA but lacking GLA (Fig. 6a). In case of 35S-d6D and napin-d6D transformed seeds, in addition to ALA an additional peak for GLA was obtained (Fig. 6b, c). Further, the presence of GLA in transformed seeds was reconfirmed by chromatographic profile of silver nitrate-purified trienoic acid of the same FAME sample. Among C 18:3 fatty acid, only ALA is present in untransformed control but not the GLA (Fig. 6d) while purified C18:3 fatty acids of 35S-d6D and napin-d6D transformed seeds contain both ALA and GLA (Fig. 6e, f). This result indicated that the introgressed d6D gene is expressing functionally in the transformants. Comparatively higher amount of stearic (C18:0), oleic (C18:1) and linolenic (C18:2) acid was obtained in leaf tissues of napin-d6D plants than that of the 35S-d6D plants. However, ALA content of

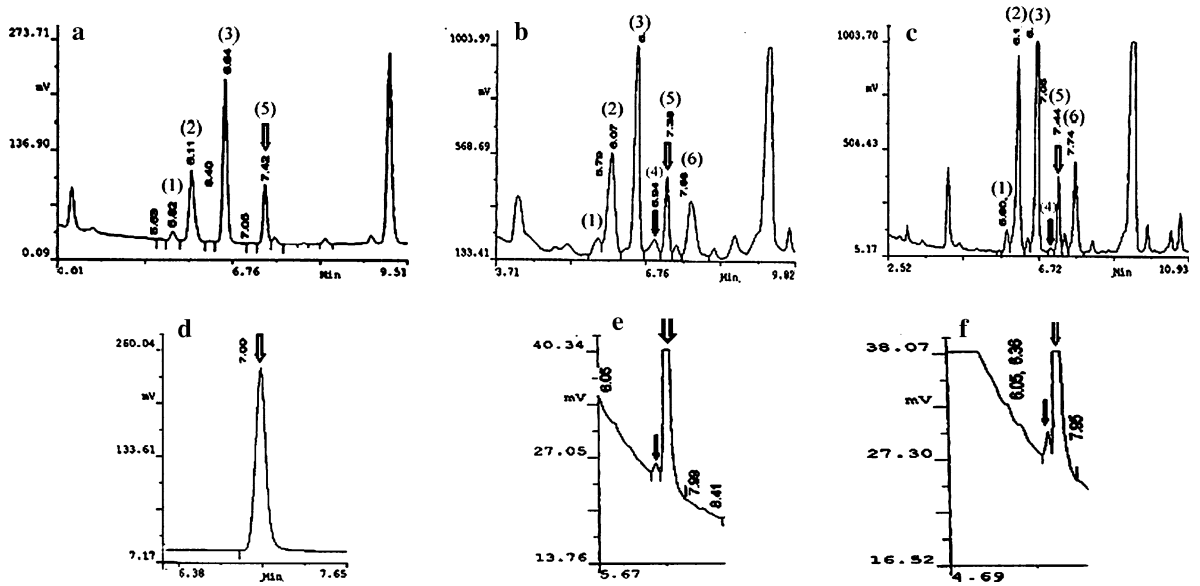

Fig. 6 Gas chromatogram of FAMEs extracted from mature seeds of *B. juncea*; (**a**) untransformed control plant, (**b**) 35S-d6D transformed line, (**c**) napin-d6D transformed line; Gas chromatogram of silver nitrate TLC purified trienoic acid from FAMEs of (**d**) untransformed control plant, (**e**) 35S-d6D transformed line, (**f**) napin-d6D transformed line. (⬇) GLA; (⇩) ALA. 'X' axis = retention time (min.) and 'Y' axis = detector response (mV).); [C18 peaks: (1) 18:0; (2) 18:1; (3) 18:2; (4) 18:3 γ; (5) 18:3 α; (6) 18:4 OTA—octadecatetraenoic acid]

Table 1 C18 fatty acid composition of leaves and seeds of untransformed and transformed *B. juncea* plants

Plant	C18 fatty acid content (%) Mean ± SD; $n = 5$						Ratio 18:3γ/18:3α
	18:0	18:1	18:2	18:3γ	18:3α	18:4 (OTA)	
Untransformed control							
Seed	2.84 ± 1.28	25.85 ± 3.29	34.23 ± 2.89	0.0	18.51 ± 2.35	0.0	0.0
Leaf	9.79 ± 1.08	5.15 ± 1.69	23.01 ± 1.24	0.0	49.01 ± 3.65	0.0	0.0
35S-d6D transformed							
Seed	3.86 ± 0.03	26.46 ± 2.57	39.88 ± 0.90	2.76 ± 0.15	10.76 ± 1.09	15.21 ± 1.74	0.256
Leaf	4.11 ± 1.09	3.06 ± 0.61	10.70 ± 1.94	0.36 ± 0.06	81.73 ± 2.03	0.01 ± 0.001	0.004
Napin-d6D transformed							
Seed	3.27 ± 0.85	33.74 ± 2.52	35.01 ± 3.69	0.44 ± 0.04	8.82 ± 1.25	15.42 ± 1.96	0.049
Leaf	15.48 ± 2.36	5.99 ± 1.06	12.90 ± 2.58	0.0	61.70 ± 4.36	0.0	0.0

Results are taken from the best performing line for each of the transformed categories

N.B.: OTA = Octadecatetraenoic acid

the leaves was more (10.76%) in 35S-d6D plants than that of the napin-d6D (8.82%) (Table 1). When all the positive transformants were considered for their GLA status, the seeds of 35S-d6D line showed the highest of GLA content (2.76%) whereas the C18:3 γ content in the leaves of some of the 35S-d6D lines differed marginally, the values being 0.40 and 0.48 respectively (data not shown). Considering the ratio of C18:3 γ/C18:3 α for all the untransformed and transformed plants, seeds of 35S-d6D line showed the maximum value (0.256) as compared to the rest where the value ranged between zero (for untransformed and for the leaves of napin-d6D line) to 0.049 (seeds of napin-d6D line). It is thus evident that constitutive expression of d6D in the cytosol altered the fatty acid composition with respect to GLA and OTA. Targeted expression of the gene in the seeds showed presence of GLA and OTA in mature seeds but not in leaf tissues. The presence of GLA and OTA in transformed plants indicated that the existing linoleic acid and ALA pool has been shifted towards the synthesis of GLA and OTA respectively by the activity of the introgressed d6D gene.

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 Δ^{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 CaMV 35S promoter showed high level of GLA accumulation in vegetative tissues but in seeds the level was low (Sayanova et al. 1999a; Qiu et al. 2002). But in our case 35S-d6D transformant showed higher level of GLA accumulation in seeds (2.76%) than that of the leaves (0.36%) (Table 1). Moderate production of GLA (3%–9%) in the seed of *B. 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 *M. alpina* Δ^9 desaturase under the control of napin promoter showed high level of GLA production (43%) in seeds of *B. napus* (Kuntzon et al. 1999; Palombo et al. 2000; Liu et al. 2001). Upto 40% of GLA accumulation in the seeds of *B. juncea* was obtained by Hong et al. (2002) upon transformation with *Pythium irregulare* d6D. In contrast, in the present investigation low level of GLA accumulation in seed was obtained when the gene is targeted for seed-specific expression. This may be due to: (1) inefficient movement of GLA from the site of synthesis to be deposited in TAG for high level accumulation in seed oil (Singh et al. 2005), (2) limited access of the appropriate substrate or strong competitive inhibition by the inherent Δ^{15} desaturase or due to biased codon usage in higher plants (Reddy and Thomas 1996). It has further been proposed that the presence of a consensus heme-binding motif, called cytochrome b_5-like domain, at N-terminus of d6D protein, exhibits an essential role in transferring electrons during desaturation process (Mitchell and Martin 1995; Napier et al. 1999b; Sayanova et al. 1999b; Sayanova and Napier 2004). Such domain is absent in the *Synechocystis* protein, unlike other eukaryotic d6D (Laoteng 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, in some of the transformed lines showing cytosolic expression of the gene, we obtained higher level of GLA production as compared to the cyanobacterial d6D expression, previously reported in tobacco by Reddy and Thomas (1996).

In all the earlier reports, the target crops were transformed with eukaryotic d6D. This is the first report on transformation of *Brassica* crop 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 (15.21% and 15.42% in 35S-d6D and napin-d6D transformed plants, respectively, Table 1) with respect to GLA 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 *B. juncea* lines with acyltranferase gene for accumulation of GLA in seeds to a greater extent, may make them nutritionally a more acceptable oilseed crop.

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