Transgenic Res (2018) 27:253–263
https://doi.org/10.1007/s11248-018-0070-5

ORIGINAL PAPER

Biofortification of safflower: an oil seed crop engineered for ALA-targeting better sustainability and plant based omega-3 fatty acids

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Received: 14 June 2016 / Accepted: 5 April 2018 / Published online: 11 May 2018

Abstract Alpha-linolenic acid (ALA) deficiency and a skewed n6:n3 fatty acid ratio in the diet is a major explanation for the prevalence of cardiovascular diseases and inflammatory/autoimmune diseases. There is mounting evidence of the health benefits associated with omega-3 long chain polyunsaturated fatty acids (LC PUFA's). Although present in abundance in fish, a number of factors limit our consumption of fish based omega-3 PUFA's. To name a few, overexploitation of wild fish stocks has reduced their sustainability due to increased demand of aquaculture for fish oil and meal; the pollution of marine food webs has raised concerns over the ingestion of toxic substances such as heavy metals and dioxins; vegetarians do not consider fish-based sources for supplemental nutrition. Thus alternative sources are being sought and one approach to the sustainable supply of LC-PUFAs is the metabolic engineering of transgenic plants with the capacity to synthesize n3 LC-PUFAs. The present investigation was carried out with the goal of developing transgenic safflower capable of producing pharmaceutically important alpha-linolenic acid (ALA, C18:3, n3). This crop was selected as the seeds accumulate ~ 78% of the total fatty acids as linoleic acid (LA, C18:2, n6), the immediate precursor of ALA. In the present work, ALA production was achieved successfully in safflower seeds by transforming safflower hypocotyls with Arabidopsis specific delta 15 desaturase (FAD3) driven by truncated seed specific promoter. Transgenic safflower fortified with ALA is not only potentially valuable nutritional superior novel oil but also has reduced ratio of LA to ALA which is required for good health.

Keywords Alpha linolenic acid · Omega-3 fatty acids · Biofortification

Introduction

Oilseed crops such as canola, corn, olive, peanut, safflower, soybean and sunflower are grown primarily for the oil contained in the seeds. However the ratio of accumulation of saturated fatty acids, monounsaturated fatty acids, omega 6 (n6) fatty acids (linoleic acid-LA, 18:2) and omega 3 (n3) fatty acids (alpha-linolenic acid-ALA, 18:3) varies from crop to crop (Kostik et al. 2013). Presence of n6 fatty acids in the diet reduces body fat, lowers the risk of heart disease by reducing the LDL cholesterol, improves brain

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11248-018-0070-5) contains supplementary material, which is available to authorized users.

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function and lowers the risk of osteoporosis in women indicating their importance. Similarly n3 fatty acids regulate cholesterol synthesis, transportation and eicosanoid synthesis (Simopoulas 1991; Kankaanpaa et al. 1999). Dietary consumption of n3PUFAs is known to mitigate conditions such as cardiovascular disease, obesity, metabolic syndrome, diabetes, stroke, rheumatoid arthritis, asthma, inflammatory bowel disease, ulcerative colitis, some cancers and mental decline (Calder 2004; Nugent 2004; Williams and Burdge 2006; Poudyal et al. 2011). However WHO recommends that n6 and n3 fatty acids should be taken in proper ratio of 2–3:1 as skewed ratio increases the incidence of chronic diseases (Stark et al. 2008).

Omega-3 desaturases catalyze the desaturation of n6 fatty acids into n3 fatty acids and belong to the class of methyl-end desaturases. Fatty acid biosynthesis pathway suggests that n3 desaturase called delta 15 desaturase (FAD3) catalyzes the conversion of LA into ALA (first n3 fatty acid of the pathway). ALA acts as the precursor of most important very long chain n-3 fatty acids such as eicosapentanoic acid (EPA, 20:5) and docosahexanoic acid (DHA, 22:6). Although humans are capable of converting ALA into EPA and DHA, they cannot convert LA into ALA due to the lack of FAD3 (Yadav et al. 1993). Thus to consume required amount of n3 fatty acids, ALA must be consumed in diet. Though the oil seed crops such as soybean, mustard etc. are preferred in market due to the nutritional value attributed to the presence of n3 fatty acid called ALA, alternate are being sought due to increase in consumption and requirement (16.34 Mt in 2016–2017 and 20.36 Mt in 2020–2021, respectively). One approach to the sustainable supply of these fatty acids is the metabolic engineering of transgenic plants with the capacity to synthesize n3 fatty acids (Domergue et al. 2005). In this regard choice of a crop with sufficient accumulation of immediate precursor-LA looks promising. One of such crops is safflower, an oil seed crop that occupies seventh place in the area dedicated to oil and accumulates as much as 78% of the total fatty acids as LA in the seeds.

In the present work, an attempt was done to induce ALA biosynthesis in safflower seeds by over expression of Arabidopsis specific delta 15 desaturase (FAD3) driven by truncated seed specific promoter. The gene was selected based on the previous reports wherein ALA accumulation in Arabidopsis plants was reduced to 1–2% in fad3 mutant lines (James and Dooner 1990; Lemieux et al. 1990) or ectopic expression of the gene lead to increase in ALA contents in soybean seeds (Damude et al. 2006; Eckert et al. 2006) confirming the capacity of the gene for catalyzing the desired reaction. To our knowledge this is the first report of fortification of safflower seeds with ALA, an oil seed crop recalcitrant to transformation.

Materials and methods

Plant material and growth conditions

Seeds of safflower (*Carthamus tinctorius* L.) genotype A1 were procured from University of Agriculture Science, Dharwad. For sterilization, seeds were soaked in 0.1% tween 20 (Qualigens Fine Chemicals, Mumbai, India) for 5 min followed by incubation in 2% bavistin (BASF India Ltd, Mumbai) for 30 min, and subsequently rinsed with water. Further sterilization was carried out with 0.1% $HgCl_2$ for 10 min. Traces of sterilant were removed by 5–6 washes with sterile autoclaved water. To eradicate Alternaria contamination, seeds were treated with hot water at 45 °C for 30 min and soaked in water for 2 days to soften the hard seed coat. Mercuric chloride being toxic was handed over to authorized pollution control board disposal agencies as per special waste regulations.

Seeds of *Arabidopsis thaliana* ecotype col-0 were surface sterilized with 0.2% $HgCl_2$ for 2 min followed by several washes with distilled water. The seeds were germinated in soil and plants were maintained in the plant growth chamber (Sanyo, MLR-351H) for 2 months in 16 h light and 8 h dark periods (temperature, 25 ± 1 °C; light intensity, 60–70 μE m^{-2} s^{-1}; RH, 60–70%). Leaves were used for RNA isolation as described by Ghawana et al. (2011).

Seeds of *Glycine max* JSS335 were purchased from University of Agriculture Science, Dharwad. Seeds were germinated in plastic pots in green house (temperature, 25 ± 5 °C; light intensity, 150–200 μE m^{-2} s^{-1}; RH, 60–70% and plants were maintained till seed set stage. Genomic DNA was isolated from seeds by CTAB method as described by Doyle and Doyle (1987).

Molecular characterization of transgenic plants

Cloning of Arabidopsis specific delta 15 desaturase (FAD3)

Total RNA was isolated from Arabidopsis leaves and complementary DNA (cDNA) was synthesized using M-MuLV RT-PCR kit (Genei^{TM}) after digesting with 2 U DNase I. Primer pair, forward: 5′-ATAAGCT TATGGCGAGCTCGGTTTTATC-3′ with restriction site *Hind* III and reverse: 5′-ATGTCGAC TCATGTTCTTTGTCCATTGAG-3′ with restriction site *Sal* I was designed from the Arabidopsis FAD3 sequence available in the GenBank database vide accession number AY078043. Polymerase chain reaction (PCR) was performed and primer pair with cDNA yielded an amplicon of 1.3 Kb involving cycling conditions of initial denaturation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 30 s, 50 °C for 30 s, 72 °C for 1.5 min; cloned in pGEM®-T Easy vector (Promega, USA) and confirmed by sequencing.

Cloning of seed specific promoter β-conglycinin (βCG) from Glycine max

Genomic DNA was isolated from *G. max*. Primer pair forward 5′-ATCCCGGGGCAATCACACACAGT GGACCC-3′ with restriction site *Sma* I and reverse 5′-ATAAGCTTTTCTCGATGATGAAAACCTGGA CC-3′ with restriction site *Hind* III was designed from the sequence information available in NCBI database (Accession No. FN807059.1). Polymerase chain reaction (PCR) was performed and primer pair with DNA yielded an amplicon of 597 bp involving cycling conditions of initial denaturation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 30 s, 54 °C for 30 s, 72 °C for 1.0 min and cloned in Genei T-vector. Sequencing and BLAST X analysis of the cloned gene showed homology with the β-conglycinin (βCG) promoter from *G. max*.

Vector construction

Plant expression vector pCAMBIA2300 was chosen (Hajdukiewicz et al. 1994) to make the gene cassette containing FAD3 under the control of seed specific promoter βCG. βCG was moved from Genei T vector to pCAMBIA2300 plant expression vector by *Sma*I and *Hind*III double digestion. Open reading frame of FAD3 was moved to pCAMBIA2300 vector bone downstream to βCG by *Hind*III and *Sal*I double digestion T-DNA cassette harboring both the genes was transformed in *E. coli* DH5α cells. T-DNA cassette containing both the genes was confirmed by restriction digestion and transformed in Agrobacterium strain EHA105 (Fig. S1–S2a, Electronic Supplementary Material).

Preparation of inoculum

To prepare the inoculums, Agrobacterium colony harboring pCAMBIA2300-βCG-FAD3 was inoculated in LB broth at 28 °C/120 rpm with kanamycin (50 μg ml^{-1}) and rifampicin (10 μg ml^{-1}) as selection pressure. Overnight grown Agrobacterium culture was reinoculated in 50 ml LB broth at 28 °C/120 rpm with kanamycin (50 μg ml^{-1}) and rifampicin (10 μg ml^{-1}) as selection pressure till OD600 reached 1.5. The Agrobacterium culture was centrifuged at 2500 rpm for 5 min. Finally the culture was resuspended to OD600 ~ 0.5 in MS medium containing 0.02% silwet L-77 (surfactant) and acetosyringone (100 μg ml^{-1}). This culture of Agrobacterium harboring pCAMBIA2300-βCG-FAD3 gene cassette was used for transformation of safflower hypocotyls. Agrobacterium culture containing pCAMBIA2300 without gene cassette constituted the control and was prepared exactly as described above.

Optimization of transformation using hypocotyls as explants

The sterilized seeds were germinated in MS medium for 10 days in tissue culture room in 16 h light and 8 h dark periods (temperature, 25 ± 1 °C; light intensity, 70–80 μE m^{-2} s^{-1}; RH, 60–70%). Germinating shoots were cut into 1–3 mm and used as hypocotyls (Fig. S2b, Electronic Supplementary Material). Sonication Assisted Agrobacterium Mediated Transformation (SAAT) is known to improve the transformation efficiency (Trick and Finer 1997; Finer and Larkin 2008); hence to transform hypocotyls with the gene of interest, explants were pricked with sterile needle followed by SAAT. For SAAT treatment, the explants were immersed in 1.5 ml microfuge tubes containing 1 ml of the Agrobacterium suspension. The tubes were placed in floating pad at the centre of a bath sonicator and sonicated for 30 s followed by incubation in Agrosuspension at 28 °C/120 rpm for 30 min. After incubation, the explants were removed from the microfuge tubes, placed on sterile filter paper to

blot off the excess bacteria and then transferred to co-cultivation medium [MS containing 3% sucrose + acetosyringone (200 μM) + BAP (4.44 μM) + cystein (412.67 μM) + ascorbic acid (85.16 μM)] in dark at 24 °C for 3 days. To prevent overgrowth of Agrobacterium during co-cultivation period, the co-cultivation medium was overlaid with double layer of autoclaved filter paper and explants were kept on the filter papers. Following co-cultivation, the infected hypocotyls were washed 3–4 times with autoclaved distilled water followed by cefotaxime (250 μg ml^{-1}) treatment for 1 h. The explants were transferred to callus induction medium [(MS + 2,4-D (4 mg l^{-1}) + TDZ (6 mg l^{-1})] with kanamycin (50 μg ml^{-1}) as selection pressure for 30 days (Fig. S2c, Electronic Supplementary Material). At this stage the calli were transferred to shoot induction medium (SIM) [(MS + cefotaxime (250 μg ml^{-1})- + BAP (4.44 μM) + kanamycin (100 μg ml^{-1})] (Fig. S2d, Electronic Supplementary Material). Shoot bud formation was observed in few calli and the emerging shoots were cut and sub cultured three times in shoot elongation medium (SEM) [(MS + cefotaxime (250 μg ml^{-1}) + BAP (4.44 μM) + kanamycin (150 μg ml^{-1})]. The elongated shoots were transferred to root induction medium (RIM) [MS + cefotaxime (250 μg ml^{-1}) + IBA (10 μM)] without kanamycin selection pressure. After 30 days of incubation in rooting medium, the rooted plants were transferred to small pots containing sterile vermiculite (Fig. S2e, Electronic Supplementary Material) and were covered with polythene bags for 4–5 days to maintain high humidity (70%). After 5 days, the tips of polythene bags were cut to enable gas exchange. The polythene bags were removed after 1 week and plantlets were allowed to grow for 3–4 days. The acclimatized plants were later transferred to soil and maintained in green house (temperature, 25 ± 5 °C; light intensity, 150–200 μE m^{-2} s^{-1}; RH, 60–70%) till seed set stage (Fig S2f-g, Electronic Supplementary Material). Hypocotyls transformed with vector alone without gene cassette (pCAMBIA2300 in EHA105 strain) constituted the control.

DNA isolation and PCR to screen the transformed plants

DNA was isolated from the putative transformed plants kept for seed set in green house (temperature, 25 ± 5 °C; light intensity, 150–200 μE m^{-2} s^{-1}; RH, 60–70%). Leaf tissue (10 mg) was taken and DNA was isolated by the method described by Doyle and Doyle (1987). To avoid contamination of plasmid DNA that could occur due to the presence of Agrobacterium in the infected plants, isolated DNA was fractioned on 0.8% agarose gel. The DNA bands were cut and eluted product was used for amplification to check the presence of transgene. True transformants were further confirmed by reverse-transcription PCR (RT-PCR) wherein RNA was isolated from putative transformed leaf tissues and cDNA was synthesized. Amplification of cDNA was done using FAD3 specific primer pair involving cycling conditions of initial denaturation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 30 s, 50 °C for 30 s, 72 °C for 1.5 min and the amplified fragments were electrophoresed on a 1.2% agarose gel. Samples giving amplification of 1.3 Kb indicated the integration of gene into the plant genome and considered true transformed plants (Fig. S3, Electronic Supplementary Material). Complementary DNA (cDNA) of plants transformed with vector without gene cassette (control) did not give any amplification with FAD3 primer pair. Seeds (T1) were collected from the self pollinated plants which were either transformed with the pCAMBIA2300 gene cassette containing βCG-FAD3 or the pCAMBIA2300 vector without gene cassette (control).

Study of stable inheritance of the transgene

Studies involving stable integration of the gene and its inheritance to next generation were taken up by identifying the true T1 transgenic plants. To achieve this, T1 seeds were collected from all the independent T0 transgenic events and all the seeds of each independent transgenic event were germinated separately in the presence of kanamycin (100 μg ml^{-1}). The plants (T1) surviving kanamycin selection were checked for the presence of transgene by PCR involving FAD3 primer pair. PCR positive plants were bagged at the time of flowering to ensure self pollination. Seeds (T2) were collected from each independent event and pooled together to perform oil profile analysis. Oil profile analysis was done both from homozygous and hemizygous lines as we could not differentiate the homozygous and hemizygous lines due to low seed set in the transformed plants.

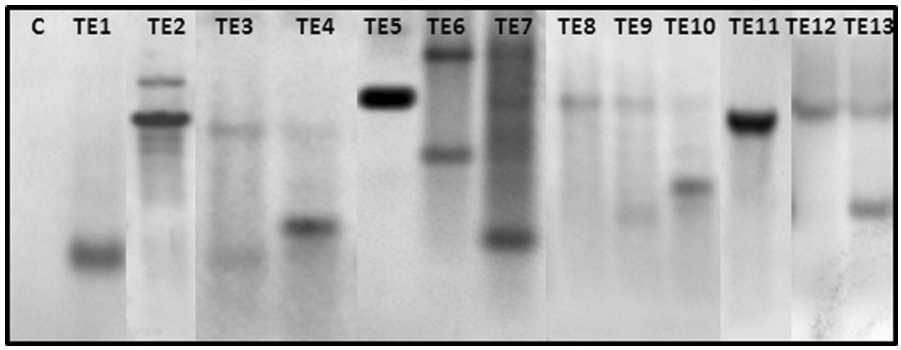

Fig. 1 Southern blot of thirteen independent transformed lines. Genomic DNA was isolated from T1 plants. C is the control plant transformed with empty vector and TE1 to TE13 indicate thirteen independent transgenic events (TE)

Southern blot of T1 plants

Thirteen independent transgenic T1 plants which were confirmed for the presence of transgene by PCR and a T1 plants transformed with vector alone without gene cassette (control) were selected for Southern blot analysis. Leaf samples were harvested; genomic DNA was isolated by CTAB method and digested with *EcoR*1. The digested samples were run on 0.8% agarose gel and blotted on to nylon N + membrane by standard protocol. FAD3 gene was used as probe and labeled with biotin using Biotin DecaLabel DNA Labelling Kit (Thermo Scientific, #K0652). Prehybridization, hybridization and washing of the blotted membrane containing digested DNA were done using standard protocols and detection was carried out using Biotin Chromogenic Detection Kit (Thermo Scientific, #K0661) (Fig. 1).

Tissue specific expression of delta 15 desaturase (FAD3) under control of beta-conglycinin (βCG)

Tissue specific expression of FAD3 was carried out in tissues and developing seeds of T0 plants. Tissues of leaf (L), flower (F), flower bud (FB) and seeds of different developing stages—SS1, SS2 and SS3 [SS1 is the seed stage when seed formation just started (10 days post pollination); SS2 is the seed stage when the seed was developed but not reached to its size (25 days post pollination); SS3 is the seed stage taken when the seeds were fully developed but they were immature without any seed coat (40 days post pollination)] were taken. Control seeds were harvested from the plants transformed with empty vector and raised through tissue culture. RNA was isolated from above samples and cDNA was synthesized (Genei^{TM}) after treating RNA with DNase1. Since FAD3 was under control of seed specific promoter βCG, the gene was used as a handy marker to study the seed specific gene expression. FAD3 specific primer pair, forward: 5′-ATGGCGAGCTCGGTTTTATC-3′ and reverse: 5′-TCATGTTCTTTGTCCATTGAG-3′ yielded an amplicon of 1.3 Kb within exponential phase involving cycling conditions of initial denaturation at 94 °C for 3 min, followed by 28 cycles at 94 °C for 30 s, 50 °C for 30 s, 72 °C for 1.5 min. Actin was used as internal control for expression studies. Gels were documented and integrated density values (IDV) were calculated to determine changes in gene expression (Fig. 2).

Oil profile analysis by GC–MS

Seeds (T2) of each independent transgenic event were pooled together and used for oil profile analysis. Seeds collected from plants transformed with vector alone without gene cassette were taken as control. The oil was extracted by Soxhlet method of extraction (1879). Seeds (∼ 100 mg) were ground to fine powder and incubated overnight with 10 ml HPLC grade hexane. The extract was centrifuged and supernatant was evaporated to dryness. The concentrate was resuspended in 1 ml of 5% H_2SO_4 in methanol and incubated at 80 °C for 2–3 h. To this 1 ml of 1% NaCl was added followed by addition of 500 µl HPLC grade hexane. Upper phase containing oil in hexane phase was taken in separate vial and estimation of fatty acids was carried out using Shimadzu GC/MS–QP2010S. The separation of fatty acids in safflower was achieved using mid-polar DB-23 capillary column (length 30 m, i.d 0.25 mm, film thickness 0.25 µm) using helium as carrier gas at 1 ml min^{-1} column flow with linear pressure mode. Sample (1 µl) was injected through injector maintained at 250 °C with 1:10 spilt.

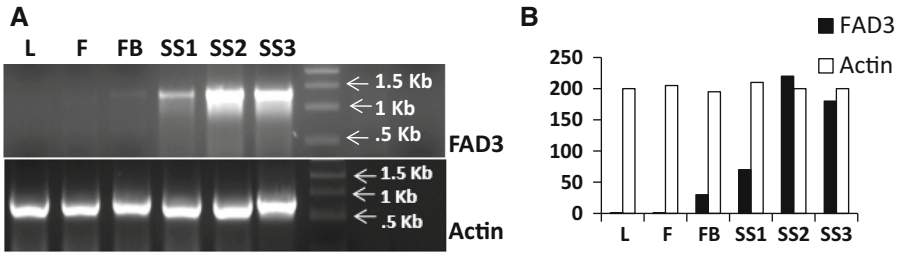

Fig. 2 Semi-quantitative expression of FAD3 under control of seed specific promoter beta conglycinin. Bar diagram on the right shows IDV values of the amplified bands measured by using the Alpha DigiDoc system. Actin was used as an internal control. The amplification of each gene was studied within exponential phase and amplified products were electrophoresed in 1.2% agarose gel. L, leaf sample; F, flower sample; FB, flower bud; SS1, SS2 and SS3 are three different developmental stages of seed; SS1 is the seed stage when seed formation just started, SS2 is the seed stage when the seed was developed but not reached to its size, SS3 is the stage taken when the seeds were fully developed but they were immature without any seed coat

Initial oven temperature was kept at 130 °C for 1 min, then ramped to 170 °C at the rate of 6.5 °C min^{-1}, again ramped to 215 °C at the rate of 2.75 °C min^{-1} and held for 5 min and finally ramped to 230 °C at the rate of 40 °C min^{-1} to elute all fatty acids with baseline separation. The mass detector was kept at 250 °C during the analysis and all mass spectra were recorded in the scan mode at 70 eV (40–500 mz^{-1}). The compounds eluting from the column were identified by comparing their mass pattern with the NIST 05 mass library data base, also the compounds were identified by comparing their retention time with that of reference fatty acid standards. The area percent purity of the each fatty acid was calculated by using their peak area response. All mass spectra were recorded in the scan mode at 70 eV (40–500 mz^{-1}). The samples were injected twice to ensure the repetition of results.

Results

Agrobacterium mediated transformation in Safflower

The gene AtFAD3 is functionally characterized previously and known to catalyze the conversion of linoleic acid into alpha linolenic acid (Yadav et al. 1993). Hence this gene was selected in present work and to drive the expression of gene in seeds, it was cloned downstream to truncated seed specific promoter βCG in plant expression vector 2300, transformed in EHA105 strain of Agrobacterium and used for transformation experiments (Fig. S1, Electronic Supplementary Material). Safflower is an oil seed crop recalcitrant to transformation. Shoot vitrification and poor rooting of in vitro regenerated shoots are the major constraints on tissue cultures of safflower. A method of transformation using hypocotyls as explants was developed (Fig. S2b-e). Sonication treatment of explants for 30 s created microwounds that possibly led to increased accessibility of Agrobacterium to deeper layers of explants and hence sorted out the issue of recalcitrance in safflower. Use of double layer of autoclaved filter papers during co-cultivation period prevented the overgrowth of Agrobacterium thereby increasing the net survival rate of the explants.

In preliminary studies, the infected hypocotyls were cultured on different concentrations of 2,4D and TDZ. Amongst the different media combinations tested, MS containing 4 mg 2,4D and 6 mg TDZ per liter of medium produced highest frequency (58.3%) of calli. The green calli surviving kanamycin selections (50 μg ml^{-1}) were transferred to SIM after 30 days. Though different BAP concentrations were tried, optimal concentration was found to be 4.44 μM wherein 27.4% of the calli (16 nos out of 58.3) responded to shoot emergence. Shoots were elongated to 5–6 cm after three subcultures in shoot elongation medium and elongation frequency was found to be 69.3% (21 shoots obtained from 16 calli out of which an average of 14.7% elongated). Adventitious root formation was found in 6 out of the 14.7 elongated shoots giving a rooting frequency of 43.5%. While maintaining the safflower plants in tissue culture conditions, shoot vitrification was controlled by addition of iota-carrageenan (1.5 g l^{-1}) and increasing the percentage of agar in the medium to 0.9% as described

by Belide et al. (2011). Rooted plants were transferred to green house after acclimatization and maintained till seed set stage. On an average 3.7% of the transformed plants could survive till seed set stage giving a transformation efficiency of 3.7%. Since out of 6 acclimatized plants, an average of 3.7 plants survived in green house till seed set stage, the plant performance in green house was found to be 61.7%. The plants did not exhibit any morphological changes in terms of growth, flowering time and seed set as compared to control plants (data not shown). The regeneration of plants till seed set stage was completed in 12–14 weeks.

Analysis of seed specific promoter β-conglycinin (βCG) from *G. max* and tissue specific expression of delta 15 desaturase (FAD3) in transgenic plants

Sequence analysis of βCG revealed presence of RY elements, E-box, DPBF binding site and SEF1 binding sites indicating the promoter to be seed specific. Seed specificity of βCG was further confirmed by studying the tissue specific expression of FAD3 in transgenic plants. FAD3 did not exhibit (negligible) any expression in leaf (L) and flower (F) sample (Fig. 2). A marginal expression was detected in flower bud (FB). As compared to FB, the expression was higher by 57% in SS1. It further increased by 86.4 and 83.3% in SS2 and SS3, respectively when compared with the expression in FB (Fig. 2). The expression was higher by 68.2 and 61.1% in SS2 and SS3, respectively as compared to SS1. Thus the seed specific expression of the gene was confirmed wherein the expression was observed only in seeds and not in the vegetative tissue.

Southern analysis of T1 plants

Stable integration of the transgene into the plant genome was studied by Southern analysis of thirteen independent T1 transgenic lines whose cDNA showed amplification with FAD3 specific primer pair. The FAD3 probe hybridized to the blotted and digested genomic DNA of transgenic plants and not with the digested DNA of plants transformed with vector alone without gene cassette (control). The transgenic lines showed 1–3 copies of gene by Southern analysis (Fig. 1).

Accumulation of ALA in transgenic safflower seed

Stable integration of the transgene was further confirmed by FAD3 mediated product accumulation in T2 generation. Seeds (T2) of transgenic lines (T1) that were confirmed for the presence of transgene by PCR as well as Southern blot were utilized for oil profile analysis. GC analysis of the seed oil profile showed the presence of fatty acids such as palmitic acid (HA; 16:0), stearic acid (SA; 18:0), oleic acid (OA; 18:1), linoleic acid (LA; 18:2) and alpha linolenic acid (ALA; 18:3). However percent accumulation of different fatty acids varied in samples studied. The transformed seeds could accumulate 0.48, 0.96, 1.59, 0.92, 1.66, 6.50, 1.48, 2.11, 1.66, 0.99, 2.86, 0.58 and 0.94% ALA, respectively of the total fatty acid contents in different independent transformed events which corresponded to 1.34, 2.69, 4.45, 2.58, 4.65, 18.2, 4.14, 5.91, 4.65, 2.77, 8.01, 1.62 and 2.63 mg ALA g^{-1} dwt of the seeds, respectively in different independent events (Table 1). No ALA accumulation was observed in the seeds collected from plant transformed with empty vector (Table 1). Interestingly, transformed line accumulating less ALA had more level of LA whereas the transformed line with high level of ALA showed less accumulation of LA that gave clear evidence that LA is being utilized as substrate for ALA production. Other fatty acids such as palmitic acid, stearic acid and oleic acid (HA, SA, OA) showed minor variations in different samples, but total percent area of fatty acids (HA + SA + OA + LA + ALA) was always hundred.

Discussion

Omega 3 fatty acid deficiency is one of the most prevalent nutritional problems in the world. Alpha-linolenic acid (ALA), an essential n3 polyunsaturated fatty acid is known to reduce the risk of coronary heart disease (CHD) through a variety of biologic mechanisms, including platelet function, inflammation, endothelial cell function, arterial compliance and arrhythmia (Mozaffarian 2005). It acts as the precursor for the biosynthesis of VLC-PUFAs of immense importance. Being essential, our body is not able to synthesis it due to the lack of FAD3 enzyme. However, ones taken in the diet, it can be converted

Table 1 Fatty acid analysis by GC showing variations in fatty acid compositions and accumulation of alpha linolenic acid (ALA) in transgenic seeds

S. no.	Transformation events	Hexadecanoic acid (HA %)	Octadecanoic acid (SA %)	9-Octadecenoic acid (OA %)	9,12-Octadecadienoic acid (LA %)	9,12,15-Octatrienoic acid (ALA %)	ALA mg g^{-1} dry wt. of seed
1.	Control	6.78	2.54	15.20	75.48	…	…
2.	TE1	3.21	8.43	21.34	63.75	0.48	1.34
3.	TE2	2.92	0.87	7.20	87.89	0.96	2.69
4.	TE3	1.82	0.81	4.93	89.87	1.59	4.45
5.	TE4	15.03	4.83	12.28	66.94	0.92	2.58
6.	TE5	18.99	6.82	13.32	59.21	1.66	4.65
7.	TE6	24.55	10.12	7.41	51.42	6.50	18.2
8.	TE7	25.36	10.11	14.75	47.24	1.48	4.14
9.	TE8	5.38	2.02	11.33	78.86	2.11	5.91
10.	TE9	5.35	1.55	7.77	83.50	1.66	4.65
11.	TE10	4.82	1.01	7.59	85.37	0.99	2.77
12.	TE11	5.31	1.81	8.62	81.19	2.86	8.01
13.	TE12	13.74	7.11	12.82	65.75	0.58	1.62
14.	TE13	17.72	10.71	16.24	54.39	0.94	2.63

13 independent FAD3 transformed events showed different levels of ALA accumulation in their T2 seeds. No ALA accumulation was observed in plant transformed with empty vector (control). ALA accumulation in mg g^{-1} dry wt. basis was calculated by multiplying the peak area with 2.8 considering that safflower seeds accumulate 28% oil in their seeds

to VLC-PUFAs due to the presence of desaturases required for the conversion. Although present in abundance in marine food, a number of factors limit our consumption of fish based n3 LC PUFA's. These are (1) reduced sustainability of fish stocks due to over exploitation; (2) concern over the ingestion of toxic substances such as heavy metals, polychlorinated biphenyls (PCBs) and dioxins due to pollution of marine food webs; (3) vegetarians and vegans do not consider fish-based sources for supplemental nutrition. Therefore, alternative sources of these n3 LC PUFA's are clearly desirable, and the concept of obtaining them from higher plants in commercial and sustainable quantities is particularly attractive. Although oil seed crops such as soybean and canola accumulate ALA in their seeds, alternates are being sought due to increased global requirements. To meet the demand, metabolic engineering of transgenic plants with the capacity to synthesize these fatty acids in agronomically viable oil-seed species looks promising.

In this regard, we selected safflower, an oil seed crop to produce n3 LC PUFA—alpha linolenic acid (ALA, C18:3, n3) which is not present in safflower. In safflower, oil accumulation occurs between 10 and 35 days after flowering with high content of LA ($\sim$ 78% of the total fatty acids), but ALA formation is not there (Weselake et al. 1993). ALA synthesis in seeds is mainly catalyzed by FAD3 enzyme (Vrinten et al. 2005). It is reported that over expression of AtFAD3 in soybean and rice led to accumulation of 8.57 and 10.06 mg g^{-1} ALA, respectively which was 23.8- and 27.9-fold higher than that of non-transformants (Liu et al. 2012). Hence in present work Arabidopsis specific FAD3 was isolated from *A. thaliana* ecotype col-0 and cloned in pCAMBIA2300 plant expression vector downstream of truncated βCG seed specific promoter from *G. max* and used in safflower transformations. Seed specific expression was studied in three different developmental stages of transformed seeds—SS1, SS2 and SS3 which corresponded to the seeds collected after 10, 25 and 40 days post pollination, respectively. However it was observed that all the putative T0 transformed plants did not set the seeds and those where seed set was observed was limited to 3–4 seeds/pod. All the seeds of each independent transgenic event were maintained separately. The transformed seeds (T2) could accumulate 1.34 to 18.2 mg ALA g^{-1} dwt of seeds in

independent events (Table 1). Seeds collected from plant transformed with empty vector did not show any ALA accumulation, reflecting the functionality of FAD3. Also it was observed that transgenic line accumulating more ALA had reduced level of LA as compared to transgenic line accumulating less ALA. There was no detrimental change in plant morphology, growth and development (data not shown).

It is predictable that storage protein genes are good source of strong seed specific promoters. One such example of seed specific promoter is α subunit of βCG from soybean used in present study. It is reported that motifs conferring seed-specific expression reside in the proximal region of the promoter, often within 0.5 kb upstream of the transcriptional start (Chamberland et al. 1992; Chandrasekharan et al. 2003; Lindstrom et al. 1990) and are identified through deletion analysis (Thomas 1993; Van der Geest and Hall 1996). DNA sequence analysis of 2S albumin promoter of *Vitis vinifera* L. indicated the presence of seed-specific regulatory motifs within 0.6 Kb regions upstream to transcription start site. GFP expression was found only in cotyledons and not in any of the vegetative tissue indicating the promoter to be seed specific (Li and Gray 2005). Similarly, 1 kb of 5′ flanking partial sequence of LeB4 promoter of *Vicia fabalegumin* mediated high level of seed specific expression in transgenic tobacco indicating that full length promoter is not required for seed specific expression (Baumlein et al. 1991). The RY motif has long been known to be conserved and proven to be functional in terms of seed specificity in soybean (Fujiwara and Beachy 1994; Lelievre et al. 1992). Yoshino et al. (2001, 2006) reported that RY elements of α subunit of βCG regulate the seed specific gene regulation.

Based on these reports, 597 bp of 3′ UTR of the βCG promoter (truncated) was used in the present study. The promoter induced the FAD3 expression specifically in seeds only as evident from tissue specific expression studies (Fig. 2). Although the oil profile analysis was done in T2 progeny, we could not differentiate the homozygous lines from the hemizygous lines due to low seed set in transgenic plants. However analysis of seeds in T2 generation gave an evidence of transgene stability and stable integrity of the gene, thus opening a way of commercialization of transgenic safflower to meet the omega 3 fatty acid demand by transformation of single gene.

However potential limitation of transgenic approach in this crop is that safflower has many wild weedy relatives which intercross to produce fertile offsprings. Gene transfer through interspecific hybridization may introduce traits into commercial crop, creating the potential for invasive hybrid populations (Hauser et al. 1998a, b; Lexer et al. 2003). This leads to concern about transgene escape from cultivated *C. tinctorius* and potential for commercial safflower to cross with its weedy relatives and become feral. Potential factors affecting domestication or weediness of safflower are seed weight, seed colour, presence of pappus, number of seeds produced, time at rosette stage, spininess, time of flowering, time of flowering and shattering versus non-shattering heads. However for hybridization and introgression, the plants should be sympatric in distribution and flower at same time. Analysis reported by Mayerhofer et al. (2011) suggests that outcrossing is not easy under normal conditions and even if it happens and fertile offsprings are produced, hybrids are unlikely to provide any selective advantage and trangene gets deleted in hybrids due to negative selection mechanism against foreign DNA in the species (Mayerhofer et al. 2011). Thus transfer of T-DNA construct from commercial safflower should not have any visible effect on hybrids.

Conclusion

Safflower fortified with ALA and improved n6 to n3 ratio will be nutritionally superior crop with better health benefits. Present work is an attempt done in search of alternative plant based sources of omega-3-fatty acids.

Acknowledgements Authors are thankful to Department of Biotechnology [DBT; Ref No. (BT/PR4077/AGR/2/832/2011)], India, for providing financial assistance. Arti Rani is thankful to the department of science and technology (DST) for providing fellowship under women scientist [WOS (A); Ref No. SR/WOS-A/LS-580/2013 dated 1.8.2014].

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