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Biofortification of safflower oil with gamma linolenic acid through transgenic approach using delta -6 -desaturase gene from *Borago officinalis*

I.Swarnalatha Devi[1,3] , Ansari N.A.[1] and V. Dinesh kumar[2]
[1]Department of Genetics and Plant Breeding, ANGRAU, Rajendranagar, Hyderabad 500 030, India
[2]Directorate of Oilseeds Research, Rajendranagar, Hyderabad 500 030
[3] sld_66@yahoo.co.in

Abstract
The present investigation has been taken up with the goal of developing transgenic safflower producing gamma linolenic acid (GLA), a pharmaceutically important fatty acid, in the seed oil. With high levels of linoleic acid in the seed oil, safflower forms an ideal target for production of GLA. The strategy envisaged includes developing transgenic safflower expressing the delta-6-desaturase gene from *Borago officinalis* under napin promoter so that the linoleic acid in the seeds of safflower will be desaturated to yield GLA in the seed oil. We report here the development of suitable gene cassettes for achieving this goal. Gene specific primers for delta 6-desaturase synthesized using the sequences available in the database were used for amplifying the gene from genomic DNA isolated from *Borago officinalis*. The obtained amplicon of the expected size (1347 bp) was cloned in T/A cloning vector confirmed by restriction analysis and sequencing. The sequence data was subjected to BLAST analysis, which confirmed the cloning of delta-6-desaturase. To achieve the expression of the delta-6-desaturase gene in the seeds of safflower, seed specific napin promoter was cloned from *Brassica napus* using standard PCR technique. The isolated gene delta-6-desaturase was cloned under this napin promoter for expressing in the seeds of safflower to effect production of GLA in safflower seed oil. This gene cassette was cloned into binary vector pCAMBIA1390 and the confirmed clone was mobilized into *Agrobacterium* strain LBA4404. Putative transgenic safflower shoots have been obtained using this vector and the analysis of these shoots is in progress.

Key words: GLA - Gamma linolenic acid - seed oil – transgenic – safflower – napin - delta 6 desaturase

Introduction

Advances in recombinant DNA technology and plant transformation over the past few years have allowed the introduction of 'novel' traits into plant species. One area of interest in this rapidly expanding field of plant biotechnology is the modification of the lipid profile of oilseeds (Topfer et el., 1995). This is particularly attractive as a target for manipulation because the end products have significant commercial value (as foods, pharmaceuticals or industrial raw material) and because the lipids of oilseeds are synthesized by a well-defined pathway (Shanklin and Cahoon, 1998). Plants, unlike animals produce a large array of different fatty acids and these are usually found in the storage lipid triacylglycerol (TAG) (Stymne and Stobart, 1993) .To date, over 300 different types of fatty acids have been reported in plants, a number of which are of interest as targets for exploitation. One such fatty acid is gamma linolenic acid (GLA; 18: 3 delta 6,9,12), which is used as a general health supplement, and is also registered pharmaceutically used oil against conditions such as eczema and mastalgia (Horrobin, 1990). GLA has been shown to reduce the risks of, or have positive effect on, those disorders that are associated with a low level of this fatty acid (Horrobin, 1990,1992). As a result, there is considerable interest in the large-scale production of GLA in oilseed crops.

Gamma linolenic acid is naturally available in a few oilseed plants such as Borage, *Oenothera,* and Black currant. Gamma linolenic acid is found in human milk and in small amounts in a wide variety of common foods, notably organ meat (Horrobin 1990 Huang, Y S and Ziboh A, 2001). Borage oil is the richest supplemental source (17 to 25% gamma linolenic acid) followed by

black current oil (15 to 20%) and evening primrose oil (7 to 10%). However, these are not conventional crop plants and they yield low levels of oil per unit area basis. Therefore, there is considerable interest in both increasing the gamma linolenic acid in natural sources and /or increasing the productivity of the plant and for the production of gamma linolenic acid in a conventional oil crop, which does not otherwise produce gamma linolenic acid naturally. For such an approach, the ideal crops are those edible oilseed crops, which accumulate higher proportion of linoleic acid viz. Sunflower and Safflower, as linoleic acid is the precursor for GLA production

Safflower is a commercially important agricultural crop. Safflower oil primarily comprises the saturated fatty acids palmitic (c16:0), stearic (c18:0) and unsaturated fatty acids oleic (c18: 1) and linoleic (c18:2). However, Safflower plants naturally produce only negligible amounts of GLA. As such, transgenic Safflower plants with seeds containing higher levels of GLA than that occurring naturally would have great utility. We describe the isolation of genomic DNA clone encoding the delta 6 desaturase from leaves of borage plant (*Borago officinalis L*), using a PCR – based strategy, cloning the same under seed specific promoter napin and transforming safflower and tobacco with the developed gene cassette. The transgenicity of the obtained shoots was established using gene specific PCRs and Southern analysis.

Materials and Methods

Delta-6-desaturase gene was isolated from genomic DNA of Borage seedlings using polymerase chain reaction (PCR). The primers 5' gga tcc atg gct gct caa atc aag aaa tac and 3'gaa ttc tta acc atg agt gtg aag agc (restriction enzyme sites included in the primers have been underlined) were used in the PCR reaction. The primers introduced a *Bam HI* and an *Eco RI* sites at the 5' and 3' end of the ORF for ease in subsequent sub- cloning. The PCR product was verified by sequencing analysis. Seed specific promoter of napin gene (BcNA1) was isolated using genomic DNA obtained from *Brassica napus* seedlings and the same was confirmed by sequencing. Isolated delta-6-desaturase gene was cloned downstream of the confirmed napin promoter. The Δ^6–desaturase expression cassette was cloned into the binary vector pCAMBIA 1390 that resulted in the plant transformation vector pDD1390.

Safflower Transformations

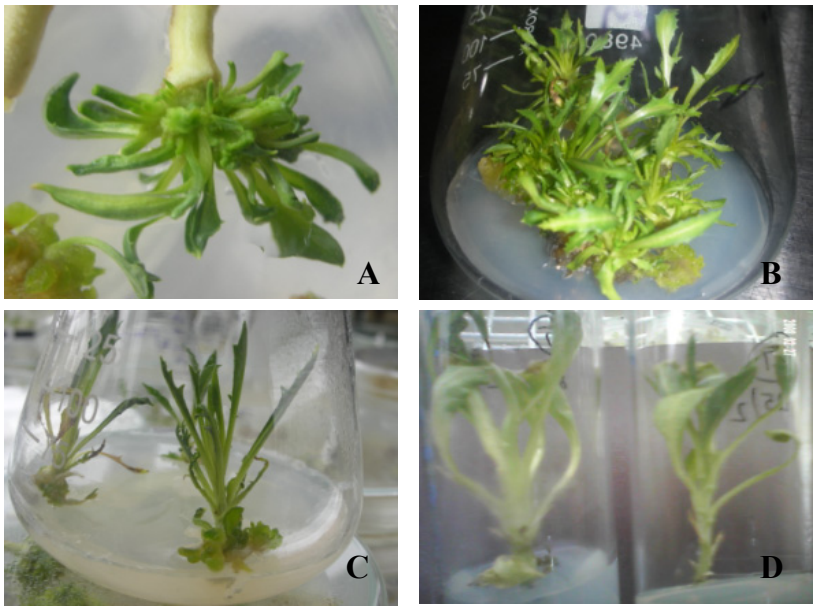
The T-DNA plasmid pDD1390 was mobilized into *A. tumefaciens* strain LBA 4404 through electroporation(Ying et al,1999). Safflower *cv* HUS-305 was transformed with this construct using the protocol standardized in the lab. Seedling derived hypocotyl explants were co-cultivated with the *Agrobacterium* strain and the transformed shoots were selected using Hygromycin as the selection agent. Putative transformed shoots were confirmed for the presence of the introduced gene cassette using PCR.

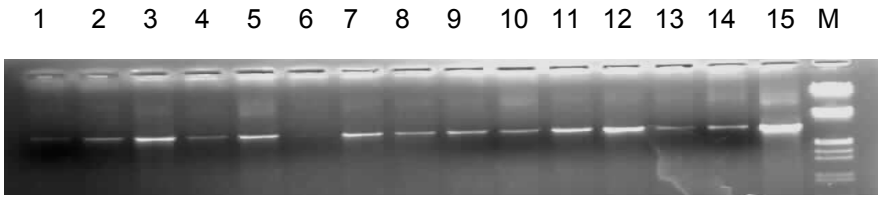
Results

Production of Transgenic Safflower Containing the *Borage officinalis* Δ^6 Desaturase

Gamma linolenic acid and STA are produced via desaturation of linoleic and α-linolenic acids respectively (Sayanova et al., 1997). A genomic DNA clone of the *B. officinalis* Δ^6–desaturase gene was generated via PCR from DNA isolated from young leaves of the herb. The Δ^6–desaturase gene was cloned between the seed specific promoter napin and the 35S poly A sequence. The gene cassette was cloned into binary vector and the confirmed binary vector was mobilized into Agrobacterium.

Safflower transformation experiments were carried out employing the protocol standardized in our lab using hygromycin as selection agent. A total of 155 primary transgenic shoots were developed and they are currently being subjected for rooting and acclimatization. Transgenicity of the shoots was confirmed using gene specific PCRs.(Fig.1)





E. Transgenic safflower clones confirmation through PCR

Lane :1 to 15 transgenic safflower plants (Promoter+gene) entire cassette of 3.0 kb
Lane :16 EcoRI+HindIII double digest marker

Fig 1. Transformation of safflower with the developed construct

A. Initiation of multiple shoots from the hypocotyls explants
B. Proliferation of shoots
C. Elongation of shoots
D. Rooting of the transformed shoots
E. Confirmation of the transgenics through PCR

The same construct was used for transformation of tobacco variety (*Nicotiana xanthii*) as a model crop. Routine leaf disc transformation method was followed. A total of 125 putative transgenic tobacco plants were produced. These plants were analysed for the presence of gene cassette using gene specific PCR (Fig.2).

To further confirm the introduction of gene cassette in to the tobacco genome, genomic DNA was isolated from the leaves of either a GLA –positive transgenic tobacco plant or a control plant that had been subjected to the same tissue culture regime. The DNA samples were analyzed by southern blotting and probed with the delta- 6- desaturase gene sequence from the plasmid DNA of pBDD DNA. This analysis identified tobacco plants carrying single copy insert of the gene cassette. Such plants have been acclimatized and they are being analyzed for the expression of

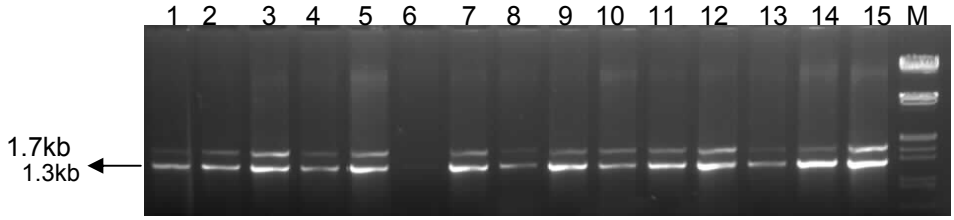


Fig 2 Confirmation of transgenic tobacco plants through PCR

Lane: 1 to 5 and 7 to 15 shows transgenic clones
Lane: 6 untransformed clone
Lane: 16 EcoRI + Hind III Double digest marker

delta-6-desaturase using RT-PCR. The seeds obtained from these plants shall be subjected to fatty acid profiling to study the effect of introduced gene in altering the fatty acid profile.

Discussion and Conclusion

Linoleic and alpha-linolenic fatty acid pools in plants can be converted to GLA and STA, respectively, via the action of a delta 6 desaturase. Sayanova et al (1997) inserted the *Borago officinalis* delta 6 desaturase gene down stream of the napin promoter and the cassette was introduced into tobacco. These produced significant levels of gamma linolenic acid and moderate levels of STA in vegetative tissue; however, relatively low levels of the novel fatty acids were produced in mature seed, < 3.0% GLA and STA (Sayanova et al, 1999). The reason that transgenic tobacco plants with the heterologous seed- expressed promoter did not produce high levels of delta 6 desaturase fatty acids in seeds has not been clearly stated. They have opined (Sayanova et al., 1999 that a single gene insertion might not be enough because of the limitation or inability of the host enzymes to efficiently use unusual fatty acids as the substrate (Ohlrogge and Browse 1998). It is evident that the low level production of delta 6 desaturase fatty acids in tobacco is due to the low level of expression of functional desaturase. However, when the same delta-6-desaturase gene from *Borago officinalis* has been expressed in transgenic soybean and Brassica there has been a significantly high levels of GLA in the seeds of such plants.

In our case we have produced the transgenics in both tobacco and safflower and confirmed that the transgenics carry the introduced gene cassette. Further analysis with the plants will confirm the effect of the introduced genes cassette in altering the fatty acid profile. Already safflower transgenic plants expressing high levels of GLA have been realized and are subjected to field trials in United States (**http://www.arcadiabio.com**). This study indicating that there is no serious problem of over expressing GLA in seeds of safflower, the incorporation of GLA in tryglycerides and also confirm that GLA could be incorporated into seed oil without any deleterious effects on the plants.

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