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ANTISENSE RNA-MEDIATED INHIBITION OF *GMFAD2-1* ENCODING OMEGA-6-DESATURASE

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SUMMARY

Antisense RNA-mediated inhibition of gene expression is a valuable tool to metabolically engineer the composition of oilseeds to improve their nutritional value and provide the functional properties required for various purposes. To improve the efficiency of *fad 2-1* inhibition and to selectively suppress it in developing seeds of soybean an antisense construct was designed in two stages. In the first stage, the PCR amplified conserved *fad 2-1* gene sequence was cloned in the antisense orientation with respect to seed-specific (vicilin) promoter derived from pCW66 vector. In the second stage, the chimeric cassette was inserted in a plant transforming vector carrying *"bar"* gene and *kanamycin* resistance gene for plant selection. Efficacy of the chimeric construct was checked in the model plant *Arabidopsis thaliana.*

Key words: **Antisense construct, *Arabidopsis*, developing seeds, *fad2-1*, soybean, transformation**

INTRODUCTION

Oils and fats, the glycerol esters of fatty acids play a major role in human nutrition because of their high energy content. Ninety percent of the vegetable oil produced is used for human consumption, predominantly in margarines, shortening, salad oils, and frying oils (Robbelen 1988). The relative composition of saturated and unsaturated fatty acids in seed triacylglycerol is one of the major factor influencing the quality of edible oils. All the major steps in the biosynthetic pathway to triacylglycerols (TAGs) are now known and sequence information for genes encoding most of the enzymes involved is available which facilitates the implementation of the genetic manipulation for improving quality attributes relating both to the nutritional status of oil and its industrial uses. The major challenge in modifying the composition of plant storage oils is to change the degree of fatty acid desaturation. For most of the common food oils including soybean much of interest is centered on reducing the levels of polyunsaturated fatty acids (linoleic and linolenic), contributing to oxidative instability and off flavours, and producing oils rich in monounsaturates (oleic acid).

The most effective way of reducing polyunsaturated fatty acid content in the seed lipids of the soybean is to suppress the expression of *fad 2-1* gene encoding ω 6-desaturase by gene silencing technologies. Post-transcriptional gene silencing (PTGS), a sequence-specific RNA degradation mechanism inherent in eukaryotes has been successfully used to silence gene expression and is induced in plants either through the use of antisense or co-suppression constructs (Kooter and Mol 1993). The major advantage of this approach is the ability to avoid any undesirable effects of global silencing of the target gene by confining the gene suppression to a specific tissue or organ through the use of appropriate tissue-specific promoters to drive the gene-silencing constructs. The down-regulation of expression of *fad 2-1* gene by antisense or sense suppression strategies could lead to the production of high oleic acid soybean oil with

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increased shelf life, stability and improved nutritive value which would obviate the need for any additional chemical hydrogen processing step.

To access the silencing of ω-6 desaturase enzyme in controlling the conversion of oleate to linoleate within the storage lipids, a gene silencing construct was designed and the efficacy of the antisense strategy was, to begin with, tested in *Arabidopsis thaliana* as the 271 bp long coding region of *fad2-1* cloned in the antisense construct is highly conserved in the ω -6-deasturase coding *fad 2* gene of *Arabidopsis thaliana.*

MATERIALS AND METHODS

The molecular biology grade chemicals used in the present study were obtained from Sigma Chemical Company, USA, MBI Fermentas, Amresco, SRL, Gelrose™ LE. Restriction enzymes, ligases, PCR cloning kit, Min-Elute PCR purification Kit, QIA quick gel extraction kit were obtained from Promega, Qiagen, MBI Fermentas, Genei, Bangalore and New England Biolabs. The primers designed for PCR were custom synthesized from Imperial Bio Medics, Bangalore, India.

The plasmid DNA from cDNA clone p137 (Sinha *et al.* 2004) was restricted with *BamH*I and *Hind* III. The released fragment of 1273 bp was eluted and purified using QIA quick gel extraction kit from QIAGEN. The *fad 2-1* specific nucleotide sequence of 1273bp was aligned in Blast search and from the conserved regions, both forward and reverse primers were designed and custom synthesized by Imperial Bio Medics, Bangalore, India, to amplify a 293 bp long PCR product. The primer sequences were as follows:

Forward (FX) : *Xho I* Tm = 67.3°C , 5 '- CCG CTC GAG CGG GGT TGA TGA TGT TAT GGG TT - 3', Reverse (RN) : *Nco* I Tm = 65.0°C 5' - CAT GCC ATG GCA TGA ACC CAT CAT AGG GTC TG - 3'

For PCR amplification the following reagents were added in a 0.2 ml PCR tube: 2.5 µl, 10 x PCR buffer, 2.5 µl, 1 mM dNTP mixture, 1 µl, 100 pmol forward primer, 1 µl, 100 pmol reverse primer, 0.5 µl, 1.25 units Taq DNA polymerase, 5 µl, 50 ng template DNA (1273bp). The final volume was made to 25 µl with sterile double distilled water.

The polymerase chain reaction was carried out in a thermal cycler (Minicycler™, MJ Research) following a programme of initial denaturation at 94°C for 3 min. followed by 36 cycles of denaturation at 94°C for 30 seconds, annealing at 63°C for 1 min and extension at 72°C for 1 min and a final extension at 72°C for 10 min after the completion of the cycles. The gel eluted amplicon was restricted with *Xho* I and *Nco* I and was finally eluted in 10 µl elution buffer.

The pCW66 vector DNA (8225 bp) isolated from its overnight grown culture in LB containing ampicillin (100µg/µl) was restricted with *Xho* I (X) (5294) and *Nco* I (N) (2935). The larger DNA fragment (5866 bp) containing vicilin promoter and terminator was eluted in 10 µl of elution buffer (10 mM Tris-Cl, pH 8.5).The purified restricted (X/N) fragment of amplified DNA (293bp) was ligated to the linearized (X/N) DNA fragment (5866bp) of pCW66 vector. Ligation mixture was incubated at 22°C for 2.30 h in PTC 100™ Peltier Thermal Cycler before transformation into competent cells of *E.coli* strain DH5α (Ausubel *et al.* 1999). The transformants screened on LA plates containing ampicilin (100µg/µl) were subjected to digestion with *Xho* I and *Nco* I to check for the presence of 293 bp amplicon. The chimeric fragment (3477bp) containing *fad2-1* sequence behind vicillin promoter in antisense orientation was then released by restriction of recombinant clone pVconAS with *Eco*RI.

The *EcoR* I fragment (3477bp) containing the expression cassette was ligated to *Eco*RI restricted fragment (8437bp) of the binary vector - pAKVS (12570 bp). The correct insertion and orientation was checked at each stage by restriction enzyme analysis. The ligated chimera (11914bp) was transformed into *E. coli* (DH5α) and the transformants selected on kanamycin plates were confirmed by colony PCR with sequence specific primers. The positive clone pAKVconAS was transformed into *Agrobacterium tumifaciens* strain GV3101 using freeze thaw method. The presence of the chimeric expression cassette was confirmed by colony PCR with bar specific primers BarF- 5' GAACGACGCCCGGCCGACAT 3', BarR- 5' GTCCAGCTGCCAGAAACCCAC 3'. *Arabidopsis thaliana* ecotype *Columbia* was used for all

transformation experiments. Surface sterilized *Arabidopsis* seeds were suspended in 0.1% agarose solution and spread evenly on petri plates containing solid MS medium. The plates were kept for 48hrs at 4^0C to synchronize germination and then incubated in the culture room till the seedlings established themselves at 22-24^{0}C at a photoperiod of 16hrs light and 8hrs darkness. The floral dip transformation system developed by Clough and Bent (1998) was successfully standardized under laboratory conditions and used for *in planta* transformation of *Arabidopsis thaliana* ecotype *Columbia*.

At the beginning of the flowering stage, about 70 wild type *Arabidopsis* plants (T_0) were infiltered with *Agrobacterium* strain containing the antisense construct. Their seeds (T_1) were harvested in five weeks after inoculation. The primary transformants (T_1 plants) from T_1 seeds were selected on MS plates containing 6mgl^{-1} Basta (phosphinothricin) as selective agent. The Basta resistant T_1 plantlets were transferred to soilrite mix and grown to maturity. The genomic DNA from T_1 plants was used for PCR with *bar* specific primers and PCR positive plants were further raised for another generation. Southern hybridization was performed on T_2 plants with *bar* specific probe to confirm the integration. Total lipids were extracted from the T_2 seeds, leaves and roots of T_1 plants by the method of Kartha and Sethi (1957) and the methyl esters of the fatty acids were prepared by trans-esterification (Morrison and Smith 1964) and resolved in a Gas Chromatograph (model 5500, Nucon Engineers, Delhi, India).

RESULTS AND DISCUSSION

Many methods of post-transcriptional gene silencing (PTGS) have been developed that enable the expression of these genes to be precisely down regulated during oil synthesis in the developing seeds, without affecting their expression in other parts of the plant. PTGS has been invoked to modify seed oil fatty acid composition by seed specific expression of a DNA sequence that is complementary to the whole or part of the appropriate target fatty acid biosynthesis gene.

Fad2-1 gene, specifically expressed in seeds (Heppard *et al.* 1996, Tiwari *et al.* 2004) plays a major role in controlling the conversion of oleic acid to linoleic acid. In the present study an antisense construct was designed with a seed specific promoter and terminator sequences derived from pCW66 vector to drive the transcription of the antisense expression cassette. Kusaba (2004) suggested that seed specific expression of dsRNA nullifies the silencing of homologous gene in other tissues where down regulation is not desired because the absence of plasmodesmata between the seed and its surrounding tissues might affect the efficiency of the spread of the silencing signal. Seed specific promoters thus offer a convenient alternative to classical breeding methods or chemical mutagenesis for generating plants with modified fatty acid profile in seeds. For designing the antisense chimera, in the first stage, a conserved sequence, of about 271 bp (from 283-554 bp) of *fad 2-1* gene from soybean sharing 86% identity with *fad 2* of *Arabidopsis* (as the efficacy of the construct was to be checked first in the model plant *Arabidopsis thaliana*) was PCR amplified from p137 cDNA and cloned in pDRIVE vector. The *fad 2-1* fragment (293bp) was amplified using forward and reverse primers (FX, RN) linked to *Xho* I / *Nco* I sites on their 5' ends respectively for the purpose of directional cloning, Fig. 1.

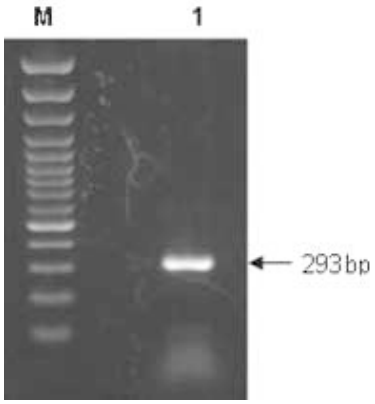

Fig. 1. 293 bp conserved domain amplified from p137 cDNA. L: 100 bp ladder, 1: Amplicon

The plasmid vector pCW66 was restricted with *Xho* I and *Nco* I and the larger fragment (5866 bp) containing 2.8 kb vicilin gene promoter and 0.3 kb vicilin terminator was selected for preparation of the chimeric fragment by directionally cloning the *fad 2-1* amplicon between them at the *Xho* I/*Nco* I sites. The resulting recombinant

plasmid pVconAS (6159 bp) had the 293 bp *fad 2-1* fragment inserted in the antisense orientation relative to the seed-specific vicilin promoter fragment. Restriction analysis confirmed the presence of insert, Fig 2 a,b.

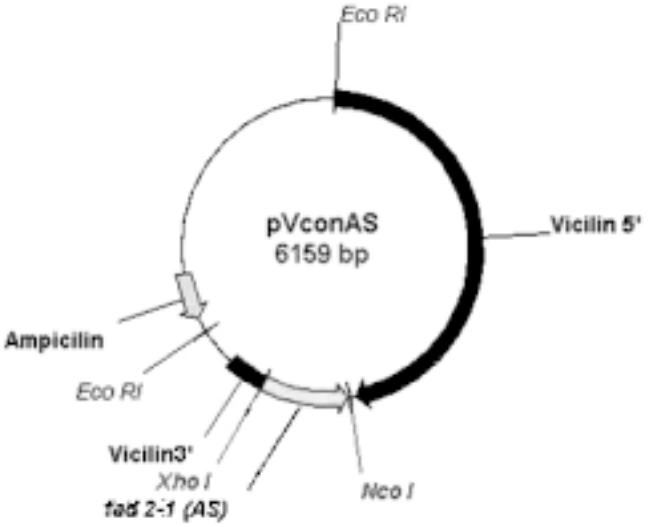

Fig. 2a. Circular map of chimeric gene construct pVconAS (6159bp) representing *fad 2-1* amplicon in antisense orientation relative to vicillin promoter

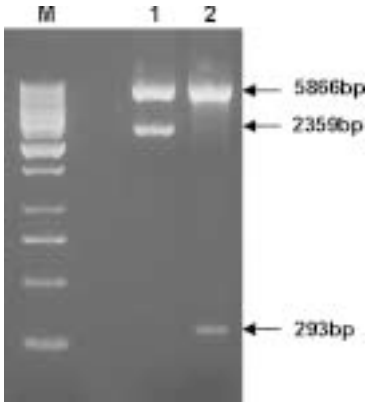

Fig. 2b. Comparative restriction analysis of vector pCW66 (8225 bp) and clone pVconAS (6159 bp) with *Xho* I / *Nco*I on 1.0% agarose gel . M : 1 kb DNA ladder Lane 1 : pCW66 DNA restricted with X/N Lane 2 : pVconAS DNA restricted with X/N

The larger fragment (8437bp) of *Eco*R1 digested binary vector (pAKVS) carrying the kanamycin resistance gene and plant selectable marker "bar" gene was used to clone the 3477kb chimeric cassette released from the recombinant clone pVconAS, to generate an antisense construct pAKVconAS of 11910 bp, Fig 3a. The construct was transformed in *E. coli* (DH5α) and the orientation confirmed by restriction enzyme analysis, Fig 3b. The *"bar"* gene was incorporated in the antisense construct to be used as a screenable, selectable marker linked to the expressed gene cassette to make the identification of the transformed progeny efficient and

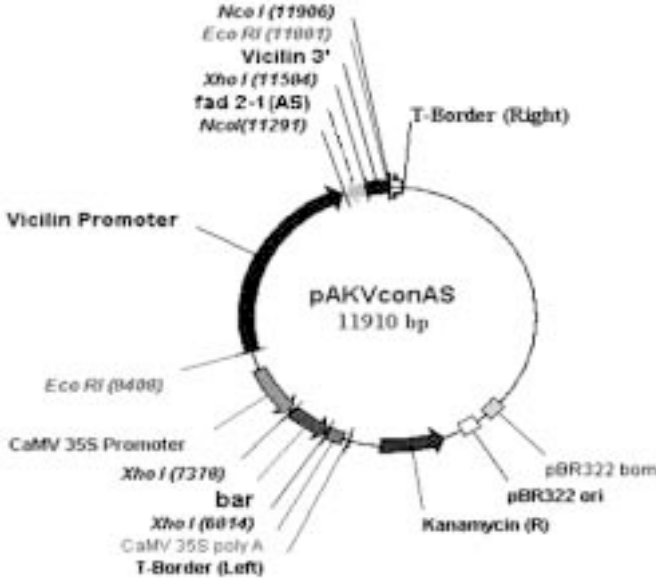

Fig. 3a. A circular map of recombinant clone pAKVconAS (11910 bp) carrying chimeric gene construct along with plant selection marker *"bar"* gene and Kan® gene

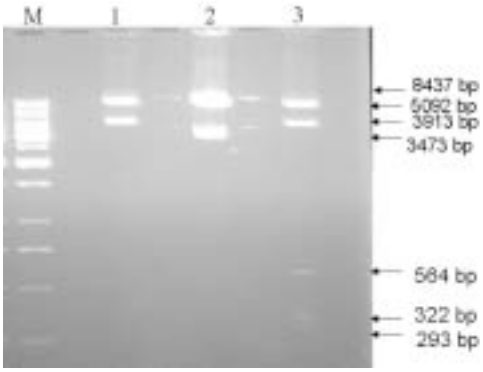

Fig. 3b. Comparative restriction analysis of pAKVS(12570 bp) and pAKVconAS (11910 bp) restricted with *Eco* RI and pAKVconAS with *Xho* I / *Nco* I on 1.0% agarose gel. M: 1kb DNA ladder, Lane 1: *Eco*R I restricted pAKVS, Lane2: *Eco*R I restricted pAKVconAS, Lane3: pAKVconAS restricted with *Xho* I/*Nco* I

fast. The "*bar"* gene confers resistance to the herbicide BASTA and the reliability of the herbicide screening for identification of "*bar*" gene containing plants can be confirmed by genomic Southern hybridization of the randomly selected segregating seedlings. Also, the spraying of herbicide (BASTA) on young soybean seedlings is very dependable and an economic method that can identify "*bar"* containing plants.

Introduction of the antisense construct (pAKVconAS) carrying the truncated *fad 2-1* gene in antisense orientation into soybean genome can be expected to exhibit significantly reduced levels of microsomal ω-6 desaturase, restricted only to seeds. Since only a part of *fad 2-1* gene is present, it will not code for an active enzyme. In transgenic rapeseed, an increased oleic acid content, upto 83%, has been achieved by antisense suppression of *fad 2-1* gene. Further crossing the line with a mutant which accumulates 78% oleic acid resulted in rapeseed line whose oleate level was 88% in TAG (Hitz *et al.* 1994, Topfer and Martini 1995). High oleate transgenics were developed from soybean transformed with *fad 2-1* transcript construct containing the coding region of the target gene in the sense orientation under the control of a seed-specific promoter (Kinney 1994). Similar results i.e. about 79% C18:1 as compared to 22% oleic acid in controls were obtained with antisense expression of w-6 desaturase in soybean (Kinney 1996). These findings clearly indicated the possibility of increasing the oleic acid content to ~85% by seed-specific antisense inhibition of the D-12/ω-6 desaturase. Stearoyl-acyl carrier protein (stearoyl-ACP) desaturase catalyses the first desaturation step in seed oil biosynthesis, converting stearoyl-ACP to oleoyl-ACP. Knutzon *et al.* (1992) developed a seed-specific antisense gene construct of *B. rapa* stearoyl-ACP desaturase to reduce the enzyme activity of stearoyl-ACP desaturase in developing rapeseed embryos during storage lipid biosynthesis. The resulting transgenic plants showed dramatically increased stearate levels in the seeds.

Cartea *et al*. (1998) constructed chimeric genes using seed-specific promoter *(AT 252)* and the coding sequences of *Arabidopsis* D-12 and rapeseed D-15 desaturases in two orientations in order to define the most efficient way to specifically modify the fatty acid composition of transgenic *Arabidopsis thaliana* seeds. The sense strategy led mainly to an over expression of desaturase activity and in some cases to its inhibition, presumably by co-suppression or sense-suppression of the endogenous genes, while the antisense strategy gave a graded range of activity. Over expression was more efficient when the homologous sequence from rapeseed was used in sense construct to transform *Arabidopsis* and on the contrary, under expression both by antisense and sense (co-suppression) strategies were more efficiently achieved on using the *Arabidopsis* sequence.

Sivaraman *et al.* (2004) developed high oleic and low linoleic acid transgenics in a zero erucic acid *Brassica juncea L*. (Indian mustard) line by antisense suppression of the *fad 2* gene. The constructs containing truncated versions of *fad 2* gene of *B. rapa* driven by a truncated napin (seed-specific) promoter were designed for obtaining the desired suppression.

The antisense construct – pAKVconAS was mobilized into *Agrobacterium* strain GV3101 using freeze thaw method and the transformation was confirmed by PCR using *bar* specific primers Fig 4. Since the antisense construct designed in the present study contains a *fad 2-1* fragment bearing homology to *fad 2* of *Arabidopsis*, it was first introduced into this model plant for selection of transformants carrying silencing transgene at 6 mg l^{-1} Basta, Fig 5 a,b.

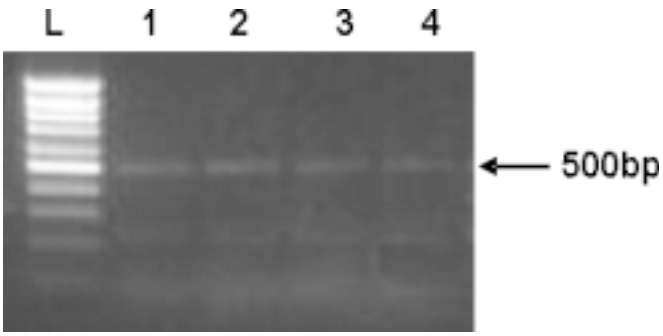

Fig. 4. Colony PCR of *Agrobacterium* containing pAKVS$_{Con}$AS with *bar* specific primers. L: 100bp ladder, Lanes 1- 4: positive colonies showing a 500 bp band

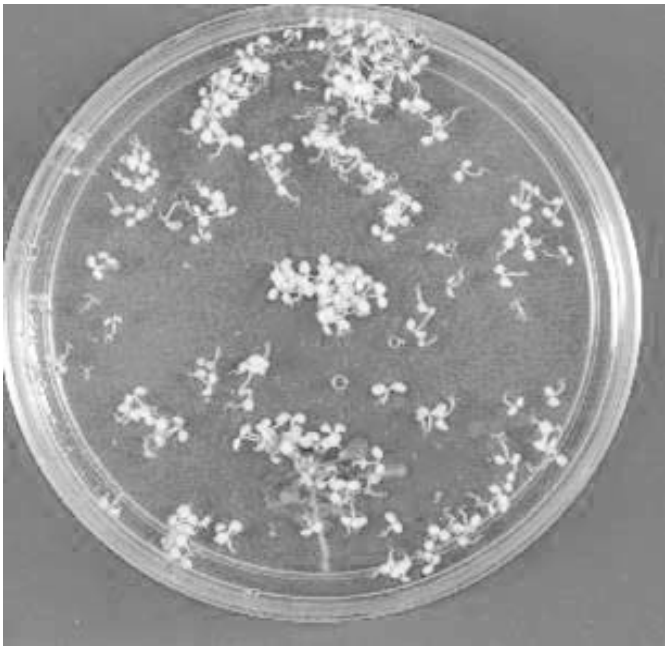

Fig. 5a. Selection of different transformants carrying silencing transgene at 6mg l^{-1} Basta

Fig. 5b. Primary transformants (T1 plants) harbouring gene silencing construct grown in soilrite mix

PCR of genomic DNA of T_1 plants using *bar* specific primers resulted in an amplicon of the expected size (500bp) thereby confirming the integration of trans gene into host genome Fig 6. PCR positive plants (CONAS 8, 34, 37, 52 and 61) were raised for another generation and Southern hybridization was performed on *Eco*R1 digested genomic DNA of the positive transformants (T_2) with radiolabelled *bar* gene as probe.

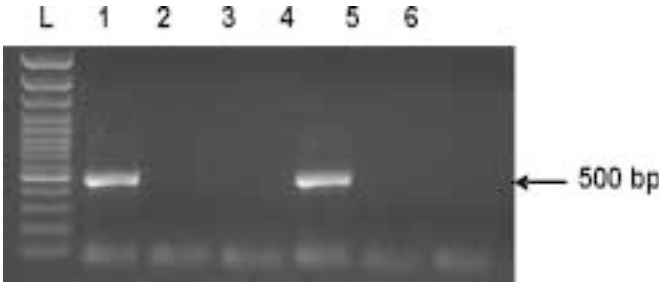

Fig. 6. PCR analysis of T1 lines harboring pAKVconAS fragment with *bar* specific primers. Lane L: 100 bp ladder, Lane 1: pAKVS positive control, Lane: 2-5, various T1 lines, Lane 6: negative control. The arrow indicates the 500 bp amplicon

Southern analysis confirmed the integration of transgene into *Arabidopsis* genome Fig 7. Uniquely sized fragments identified in the blots verified independent transformation events in each of the individual transgenic lines. Bands of different sizes represented different transgenic insertions. Single transgene insertions were observed in CONAS 8 and multiple insertions in CONAS 34, 37, 52 and 61. Also, strongly hybridizing bands in almost all lines, suggested tandem, possibly inverted repeat insertions of transgenes at some loci. Similar type of transgene integrations were also observed in different such experiments (Liu *et al.* 2002, Stoutjesdijk *et al.* 2002). Fatty acid analysis on leaves and roots did not reveal any difference from wild type plants (data not shown).

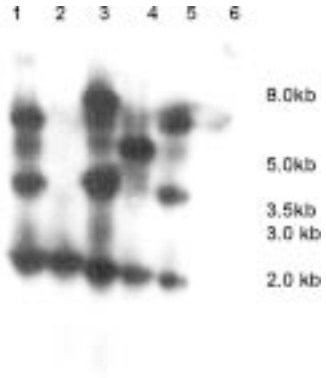

Fig. 7. Southern hybridization of *Eco*RI digested genomic DNA of T2 transgenic plants with radiolabelled *bar* gene fragment) Lane 1: CONAS 34, Lane 2: CONAS 8, Lane 3: CONAS 37, Lane 4: CONAS 52, Lane 5: CONAS 61, Lane 6: Untransformed *Columbia.* The migration of DNA marker is shown on the right

Table 1. Fatty acid composition (%) of total seed lipids from selected T_2 transformants and untransformed (wild-type) plants.

Plant	Fatty acids (%)					
	Palmitic (16:0)	**Stearic (18:0)**	**Oleic (18:1)**	**Linoleic (18:2)**	**Linolenic (18:3)**	**Others**
Wild type	8.6	3.2	14.4	31.9	22.2	19.7
Con AS8	9.3	3.7	14.1	30.5	21.3	21.1
ConAS34	8.1	3.7	14.9	32.1	20.9	20.3
ConAS37	8.8	3.6	28.2	19.5	20.1	19.8
ConAS52	7.9	3.3	15.3	33.1	21.1	19.3
ConAS61	9.2	3.5	16.4	29.7	21.4	19.8

However, in order to evaluate the efficiency of the construct used in the present study, the total fatty acid composition of seed lipids was determined in mature seeds of *Basta* resistant T_2 plants. The seeds of only CONAS 37 exhibited changes in the oil composition compared to wild type (Table 1). The oleic acid showed an increase from 14.4 to 28.2 % in the transgenic accompanied by concomitant decrease of linoleic acid content i.e, 19.5% in the transformants from 31.9% in wild type. Fatty acid analysis on leaves and roots did not reveal any difference from wild type plants (data not shown). The alteration in the fatty acid content in the transgenic seeds would be possible by inhibition of the ù-6 desaturase enzyme via antisense RNA expression using the desaturase gene in conjunction with seed specific promoter. The 86% identity between the transgenic and the endogenous sequence could explain the inhibition of enzyme activity resulting in the relative increase in the substrate i.e, oleic acid content accompanied by the simultaneous decrease in the product i.e, linoleic acid. The under expression strategy using homologous antisense gene was in this case effective to some extent in modifying the fatty acid profile in an individual transgenic line. Result implies the need to obtain a large number of transgenic events to determine the possible range of lines containing the target fatty acid profile. *Arabidopsis* being fast and easy to generate serves a good model to test alterations in the expression of the fatty acid genes in the seeds. Further, germination and progeny development of the transformants with altered fatty acid profile is necessary to check the stability or variability of the expression of silencing of the homologous gene.

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