Development of High Oleic Acid *Brassica juncea* by Antisense Technology

I. Shivaraman, P.K.Burma, V.Gupta, N. Arumugam, A. Mukhopadhyay, Y. S. Sodhi, A. K. Pradhan and D. Pental*

Department of Genetics and Centre for Genetic Manipulation of Crop Plants

University of Delhi South Campus, Benito Juarez Road, New Delhi-110021, India

ABSTRACT

Transgenics in zero erucic acid *B. juncea* were developed with two different antisense constructs to fad2 gene of *B. rapa*, one (pASfad2.1) encoding antisense RNA corresponding to cDNA from 315 to 1251 bp and the other (pASfad2.2) encompassing the 5' region of RNA from 1 to 1251 bp under the control of seed specific napin promoter. Single copy transgenics developed by pASfad2.2 construct were found to be more efficient in suppression of C18:2 level than the transgenics developed by pASfad2.1construct. On the basis of the C18:2 level of the seed, the BC1 and T1 transgenics developed by pASfad2.2 construct were found to segregate in 1:1 and 1:2:1 ratio, respectively. T2 seeds homozygous for antisense gene (As/As) derived from two independent transgenics had mean C18:1content more than 74% with as high as 82% in some seeds. These homozygous lines are being tested for stability of different fatty acids and oil content.

Key Words: Antisense RNA, *Brassica juncea*, *fad2*, linoleic acid, linolenic acid, oleic acid.

INTRODUCTION

Brassica juncea is a major oilseed crop of north India and is grown in more than six million hectares of land mostly under rainfed conditions during the winter season. All the commercial varieties of *B. juncea* grown in India are very high in erucic acid (C22:1) content (approximately 50%) in their seed-oil. We developed a '0' erucic line of *B. juncea* (VH486) by pedigree breeding from a cross between an illadapted '0' erucic line (Heera) and the most widely cultivated Indian cultivar Varuna (unpublished). The line VH486 in comparison to Varuna contains much higher levels of oleic (C18:1), linoleic (C18:2) and linolenic acid (C18:3) (Table 1). Although the C18:2 and C18:3 (PUFA) fractions form an essential component of human nutrition, the increased number of double bonds in the chemical structures of the PUFAs makes them highly susceptible to oxidation. Therefore, oils high in linoleic acid and linolenic acid deteriorate rapidly upon exposure to air, especially at high temperatures, making the oil unhealthy for human consumption (Röbbelen and Nistch 1975).

Improvement of the nutritional value of the seed oil through genetic engineering is a major thrust area of research in plant biotechnology (Przybylski and Mag 2002). Many of the genes involved in fatty acid biosynthetic pathway have been characterized and isolated. The microsomal Δ12 desaturase gene coding for the enzyme fatty acid desaturase 2 (FAD2) is primarily responsible for more than 90% of the PUFA in non-photosynthetic tissues including seeds (Miquel and Browse 1992). There have been a number of reports wherein the introduction of seed-specific antisense and sense *fad2* constructs have led to a reduction in the PUFA levels in oilseed crops (Töpfer et al. 1995). We have used antisense RNA methodology for the suppression of *fad*2 expression in order to develop low PUFA lines in the '0' erucic line (VH486) of *B. juncea*. We report the development

and characterization of transgenic lines with high oleic acid fraction (around 74%) and lower levels of polyunsaturated fractions as compared to VH486 using the antisense constructs.

MATERIALS AND METHODS

From the reported partial *fad2* cDNA sequence from *B. rapa* cv. Agena, a full length *fad2* gene sequence of 1465bp was assembled by cloning of 5' and 3' – region of *fad2* cDNA sequence by rapid amplification of cDNA ends (RACE) and sequencing. Two different *Agrobacterium* constructs, pASfad2.1 containing a 937bp fragment (from nucleotide 315 to 1251)and pASfad2.2 containing a 1251bp fragment (from +1 to 1251) of the *fad2* cDNA in antisense orientation and driven by the promoter of the napin gene (*napA*) were developed. *Agrobacterium* mediated transformations were carried out using the hypocotyl explants of *B. juncea* '0' erucic line VH486, following the protocol described by Mehra et al. (2000). Putative transgenics were transferred to the field and grown under containment. Transgenic plants were selfed and/ or backcrossed to VH486, to obtain T1 and BC1 seeds respectively. Single copy transgenics were identified by southern hybridization. Fatty acid analysis of seeds was conducted by Gas chromatography (GC). The cumulative effects of *fad2* activity during seed fatty acid synthesis were determined as ratio of the total fatty acid products of desaturation to the total amount of fatty acid substrate available called, oleic desaturation ratio (ODR).

RESULTS AND DISCUSSION

Analysis of C18:2 levels from BC1 (backcrossed to control parent) seeds of single copy transgenics indicated that the antisense construct, pASfad2.2, encoding the 5' region of the fad2 transcript was more efficient in bringing about suppression of C18:2 as compared to the construct, pASfad2.1. Genetic analysis of individual BC1and T1 (selfed seeds of T_0 transgenics) seeds of pASfad2.2 containing lines, exhibited 1:1 and 1:2:1 segregation respectively for C18:2 levels. On the basis of the above segregation, the seeds were classified as putative homozygote (As/As) containing <12%, putative heterozygote (As/-) containing greater than 12% but less than 20% and putative null homozygote (-/-) containing greater than 20% (comparable to the control) of C18:2 in the seeds. It was also observed that putative homozygotes (As/As) showed better suppression of C18:2 level as compared to putative heterozygotes.

T1 seeds of two transgenic lines (3.18 and 3.53) were germinated and half seed method was used for GC analysis. On the basis of the GC analysis, three seedlings each belonging to putative As/As and As/- categories were grown to maturity to obtain T2 seeds. Fatty acid analysis of single seed indicated that all the seeds from putative As/As plants showed 18:2 level less than 12% while putative heterozygotes (As/-) showed segregation into three categories i.e. C18:2>20%, 20%>C18:2>12% and C18:2<12% (data not shown). The mean C18:1 content of T2 seeds developed from putative homozygotes (As/As) plants was more than 74% with as high as 82% in some seeds (Table 1). The antisense technology has been effectively used to reduce the level of C18:2 fraction in '0' erucic acid lines.

Transgenics developed in this study would allow the development of high oleic acid (C18:1) varieties with sufficient amounts of C18:2 and C18:3 in a ratio of 1:1 (Table 1). Edible oils with high percentage of oleic acid (C18:1) have been reported to be beneficial in terms of their property of improving stability of the oil and their ability of lowering the low density lipoproteins in blood (Chang and Huang 1998). Amongst edible oils, olive oil has the highest fraction of oleic acid (around 77.9%). Olive oil has around 6.33% linoleic acid and only traces of linolenic acid. The two homozygous transgenic lines developed in this study have 9% linolenic acid besides having 74% oleic acid and 8-9% linoleic acid in their seed-oil. Extensive nutritional studies carried out in India have shown that the presence of linolenic acid in mustard oil is highly beneficial for the vegetarians and low income sections of the society (Ghafoorunissa 1998). Despite the reduction in the PUFA fraction, the substantial levels of C18:3 in seed-oils of our transgenics might therefore be very

useful for proper nutritional balance among the vegetarians and low income sections of India, who do not get linolenic acid from other food sources.

Table 1**.** Fatty acid analysis of seeds (a minimum of 20 seeds were tested for each line) of *Brassica juncea* lines Varuna, Heera, VH486 and two transgenic lines 3.18 and 3.53 (developed in this study) containing antisense *fad2* sequence in homozygous condition.

Cultivar	**%SFA***		**%MUFA***			**%PUFA***	
	C16:0	C18:0	C18:1	C20:1	C22:1	C18:2	C18:3
Varuna	2.4±0.4	0.8±0.1	12.8±1.4	6.3±0.5	46.8±2.2	15.1±1.2	12.9±1.3
Heera	5.9±0.6	1.3±0.2	39.5±3.7	0.6±0.1	-	42.3±3.0	10.1±1.6
VH486	4.1±0.4	1.1±0.1	53.4±3.6	1.1±0.1	-	23.9±2.1	15.5±1.8
Transgenic line 3.18	2.2±2.1	1.2±0.1	74.8±4.1	1.8±0.4	-	9.5±1.6	9.3±1.3
Transgenic line 3.53	4.5±0.2	1.6±0.2	74.3±2.9	1.7±0.1	-	8.2±1.4	9.1±1.5

* SFA – Saturated fatty acid, MUFA – Monounsaturated fatty acid, PUFA – Polyunsaturated fatty acids

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REFERENCES

Chang N.W. and Huang P.C. 1998. Effects of the ratio of polyunsaturated and monounsaturated fatty acids on rat plasma and liver lipid concentration. Lipids. 33: 481-487.

Ghafoorunissa. 1998. Requirements of dietary fats to meet nutritional needs and prevent the risk of atherosclerosis – An Indian perspective. Indian J. of Med. Res. 108: 191-202.

Mehra S., Pareek A., Bandyopadhyay P., Sharma P., Burma P.K. and Pental D. 2000. Development of transgenics in Indian oilseed mustard (*Brassica juncea*) resistant to herbicide phosphinothricin. Curr. Sci. 78: 1358-1364.

Miquel M. and Browse J. 1992. *Arabidopsis* mutants deficient in polyunsaturated fatty acid synthesis. Biochemical and genetic characterization of a plant oleoyl-phosphatidylcholine desaturase. J. Biol. Chem. 267: 1502-1509.

Przybylski R. and Mag T. 2002. Canola/rapeseed oil. In: Gunstone F.D. (ed.), Vegetable oils in food technology: Composition, properties and uses, Blackwell Publishing, CRC Press, USA/Canada, pp. 98-127.

Röbbelen G. and Nistch A. 1975. Genetical and physiological investigations on mutants for polyenoic fatty acids in rapeseed (*Brassica napus*, L). I. Selection and description of new mutants, Z. Pflanzenzüchtg. 75: 93-105.

Töpfer R., Martini N. and Schell J. 1995. Modification of plant lipid synthesis. Science. 268: 681-685.