Development of nutritionally superior *Brassica napus* and *B. juncea* oils using RNAi-mediated gene silencing

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Abstract

Canola oil is recognized as a superior healthy edible oil. However, in recent years, industry has expressed concern regarding the increasing levels of saturated fatty acids in canola oil. RNAi-mediated post-transcriptional gene silencing technique will be used to silence the palmitoyl-ACP thioesterase gene (FatB) that produces palmitic acid thus reducing the level of this nutritionally undesirable saturated fatty acid in both *B. napus* and *B. juncea* oils. We have isolated the full-length FatB gene sequences from *B. napus* and *B. juncea* using reverse transcriptase PCR (RT-PCR) and rapid amplification of cDNA ends PCR (RACE-PCR). An inverted repeat unit of a conserved region of *B. napus* FatB gene (~740-bp), interrupted by a spliceable intron, was cloned into a binary vector pWVec8-Fp1. A seed-specific promoter (Fp1) previously isolated from *B. napus* was used to control the transcription of the trangene. *Agrobacterium*-mediated transformation of elite breeding lines of *B. napus* and *B. juncea* with FatB silencing construct using hygromycin B as the selectable agent is currently underway. FatB silencing construct has also been transformed into the close relative model plant, *Arabidopsis thaliana* in order to get early evidence regarding the effectiveness of this construct. We have adopted a strategy of transforming *Brassica* plants with a dual silencing construct containing inverted-repeat sequences of FatB and microsomal Δ12 destaurase (Fad2) genes. This is to target the reduction of palmitic acid level and simultaneous elevation of oleic acid level. The development of low-palmitic and high-oleic *B. napus* and *B. juncea* varieties will enable Australian growers to remain competitive in quality-sensitive canola export markets.

Media summary

The development of low-palmitic and high-oleic *B. napus* and *B. juncea* varieties will enable Australian growers to remain competitive in quality-sensitive canola export markets.

Key words

Fatty acids, RNAi, Transgene, Low-palmitic, High-oleic

Introduction

Nutritional benefits associated with the low levels of saturated fatty acids found in *Brassica* oils (~7%) have been a significant factor in the success of canola oil in Canada, Australia and United States. However, in recent years the Canadian industry has expressed concern that the level of saturated fatty acids in canola oil has been increasing as a result of decreased plantings of the canola species, *Brassica rapa*. The adverse effects of saturated fat on blood cholesterol and its implications for cardiovascular disease, is well documented. There are two different classes of serum cholesterols based on its carrier, the high-density lipoproteins (HDL) and the low-density lipoproteins (LDL). HDL is beneficial as it is associated with the removal of cholesterol from the blood stream, while LDL is undesirable as it is responsible for the movement of cholesterol within the bloodstream. Saturated fatty acid, palmitic acid in particular, has the most significant LDL cholesterol raising effect (Zock *et al.* 1994).

Brassica oil contains around 35% polyunsaturated fatty acids. It is normally hydrogenated in order to achieve the stability required for food applications requiring prolonged shelf life and for high temperature frying of foods. However, partial hydrogenation may lead to the formation of *trans*-fatty acids, which can raise LDL in a manner similar to palmitic acid. On the other hand, the monounsaturated oleic acid can

lower LDL-cholesterol and at the same time it can confer high stability required by the food industry without the need for hydrogenation. Therefore the ideal healthy cooking oil, especially those used for industrial food applications, should be rich in oleic acid and low in palmitic acid.

In recent years, post-transcriptional gene silencing (PTGS) techniques have been successfully employed for the production of *B. napus* and *B. juncea* varieties rich in oleic acid (Stoutjesdijk *et al.* 2000) and for high-oleic and high-stearic varieties in cotton (Liu *et al.* 2002). A similar strategy is adopted in this project to lower palmitic acid and raise oleic acid levels. We have chosen FatB encoding palmitoyl-ACP thioesterase as the target gene to reduce palmitic acid. Palmitoyl-ACP thioeserase is the soluble plastid enzyme responsible for the cleaveage of palmitic acid off its acyl carrier protein and is crucial for the final accumulation of palmitic acid in seed oil. There are two classes of thioesterases in plant kingdom, according to their substrate preferences either for unsaturated (FatA) or saturated fatty acids (FatB). In order to raise oleic acid content in this project, the expression of the microsomal Δ12-desaturase (Fad2) gene that converts oleic acid to linoleic acid is being targeted. Furthermore, the simultaneous silencing of both FatB and Fad2 genes using a single transgene construct containing inverted-repeat of the fused FatB and Fad2 gene sequences is also being explored.

Methods

Isolation & characterization of thioesterase genes (FatA & FatB)

Total RNAs were isolated from developing seeds of *B. napus* and *B. juncea*. RT-PCR was performed using a pair of degenerate primers based on conserved FatB sequences. Full-length cDNAs were obtained by extending at both 5' and 3' ends of the DNA fragments using RACE-PCR.

Silencing FatB expression in B. napus and B. juncea lines

An inverted-repeat construct containing 740-bp of the relatively conserved region of the FatB gene was made using pKANNIBAL vector, which separates the two repeats with a spliceable intron. This was followed by subcloning into a binary vector pWVec8-Fp1, which contains a napin seed-specific promoter. Similar strategies were used to make the dual silencing construct containing FatB and FAD2 gene sequences. The two genes were linked as a whole unit prior to the cloning into such a silencing construct. Genetic transformation of elite Australian *B. napus* and *B. juncea* lines via *Agrobacteium* infection is currently being carried out. The fatty acid composition in the initial transformants and subsequent generations will be analysed using gas chromatography.

Results and Discussion

Initially a 1.1 kb fragment of FatB gene from both *B. napus* and *B. juncea* was isolated by RT-PCR using degenerate primers designed from amino acid sequences conserved between *Arabidopsis*, cotton and rice FatB genes. The 1.1 kb FatB sequence information was used to isolate the full-length FatB gene sequences from *B. napus* and *B. juncea*, using RACE-PCR. The isolated DNA sequences from *B. napus* and *B. juncea* share significant sequence homology particularly to the FatB gene of *Arabidopsis* (~90% homology). A phylogenetic tree generated using various amino acid sequences of FatA and FatB thioesterases clearly distinguished the isolated fragments from *B. napus* and *B. juncea* as putative FatB thioesterase genes (Figure 1).

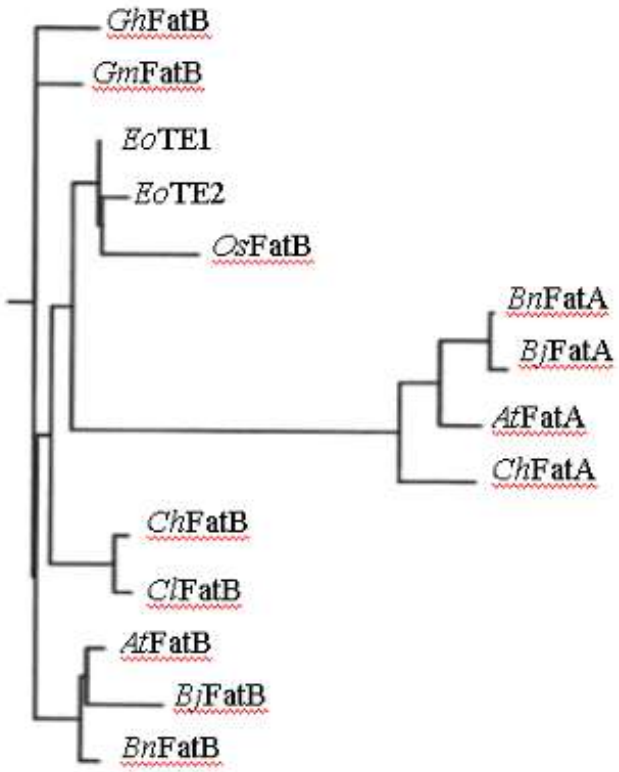

Figure 1. Phylogenetic tree using various amino acid sequences of FatA and FatB thioesterase genes. At – Arabidopsis thaliana; Bj – Brassica juncea; Bn – Brassica napus; Ch – Cuphea hookeriana; Cl – Cuphea lanceolata; Eo – Elaeis oleifera; Gh – Gossypium hirsutum; Gm – Garcinia mangostana; & Os – Oryza sativa

Using a 740 bp nucleotide region of the *B. napus* FatB gene sequence, an inverted-repeat (IR) gene silencing construct has been made. The 740 bp region selected to make the construct shows significant sequence homology (more than 90%) for FatB sequences of *B. napus*, *B. juncea* and *Arabidopsis thaliana* and at the same time this region was less homologous (less than 40%) to FatA sequences of these three species. The *B. napus* FatB gene silencing contruct was PCR-amplified with the appropriate restriction enzymes added to the 5' end of the primers and sequentially cloned into similarly cut pKANNIBAL vector system. Ultimately this construct was cloned into a binary vector pWVec8-Fp1 under the transcriptional control of a napin seed-specific promoter (Figure 2A). Elite Australian *B. napus* and *B. juncea* breeding lines are currently being transformed using *Agrobacterium*-mediated transformation of hypocotyl segments, with hygromycin B as the selectable agent.

A 500 bp fragment of the Fad2 gene sequence has been isolated from *B. napus* using PCR strategies and this fragment has been fused with the 740 bp region of FatB gene. An inverted repeat of this chimeric unit has been made in the pKANNIBAL vector and subsequently cloned into a binary transgene construct (Figure 2B). The FatB-Fad2 dual silencing construct should simultaneously silence both FatB and Fad2 genes. The FatB and FatB-Fad2 silencing construct has also been transformed into *Arabidopsis thaliana* using the Agroinfiltration transformation method. This will allow us to access some early evidence regarding the effectiveness and stability of the current strategies.

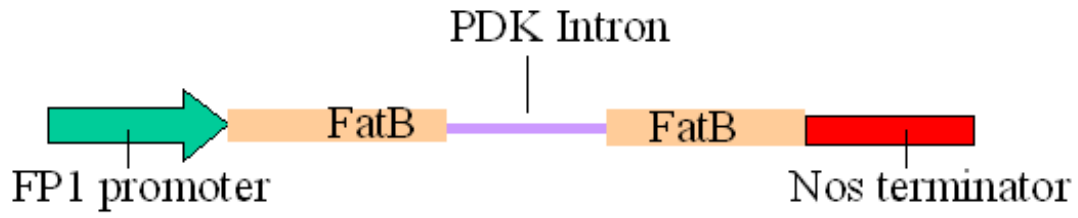

Figure 2A. Inverted-repeat construct to silence FatB expression in *B. napus* and *B. juncea*.

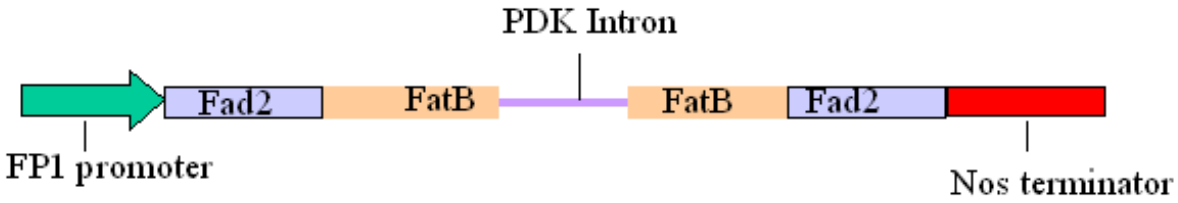

Figure 2B. Inverted-repeat construct to silence FatB and Fad2 expression in *B. napus* and *B. juncea*.

Summary

FatB and FAD2 genes have been isolated using RT-PCR and RACE-PCR from *B. napus* and *B. juncea.* The designed silencing constructs specifically targeting FatB is expected to reduce palmitic acid content in the seed oil. By simultaneously silencing FatB and Fad2 genes the development of low-palmitic and high-oleic *B. napus* and *B. juncea* oils is targeted, without segregation of these two desirable traits in subsequent generations. The development of low-palmitic and high-oleic *B. napus* and *B. juncea* varieties will enable Australian growers to remain competitive with international canola markets.

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