Development of nutritionally improved cottonseed oils by genetically manipulating seed-specific fatty acid desaturase genes in cotton

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Background

Among the oil seeds, cottonseed is considered to be an important commodity because of its use in food and animal feed throughout the world. Cottonseed contains approximately 23-26% oil and 26-28% protein on a moisture, lint-free seed basis (Cherry, 1984). The current annual world production of cottonseed oil is approximately 4 million metric tons, ranking sixth behind soybean, oil palm, rapeseed, sunflower and groundnut (Oil World Annual, 1998). Global cottonseed production should expand in the future as a result of increased cotton acreage and higher seed yields.

Cottonseed oil is high in saturate

Cottonseed oil contains relatively high level of saturated fatty acid, mainly palmitic acid. It is well established that palmitic acid is a major contributor to the increased levels of total cholesterol in the blood, and especially the low-density lipoprotein (LDL) form of cholesterol which is closely associated with risk of cardiovascular disease. While the stability and high melting point of saturated fatty acid is important for oil's functionality, it is desirable to replace palmitic acid with an alternative saturated fatty acid, i.e. stearic acid, which is proven to be neutral in terms of cardiovascular disease. Since stearic acid has a melting point of about 70°C, the high-stearic oils would be ideal for solid fat applications. For example, it has great potential to substitute for partially hydrogenated plant oils in margarine production and could also be used as a substitute for cocoa butter. Other reported potential industrial uses for stearic acid include production of cosmetics, pharmaceuticals and candles. Unfortunately, there is only a trace amount of stearic acid in the current cottonseed oil.

Hydrogenated cottonseed oil contains *trans* fatty acids

Although polyunsaturated fatty acids (PUFAs) are essential fatty acids, oils containing high levels of polyunsaturates are relatively unstable and prone to oxidation which leads to the shortened shelf life and rancidity following expose to long period of time at high-temperature, such as the process of cooking. Therefore, varying degrees of hydrogenation of the oil are necessary to reduce the level of PUFAs to prolong the shelf life and to achieve the high stability required in deep-frying food service application. Extensive hydrogenation of

cottonseed oil has also been used in margarine production in order to raise the melting point and enable the margarine to solidify. There was a time when hydrogenated vegetable oil was thought to be the best nutritional alternative to saturated animal fats. However, the hydrogenation process results in the formation of undesirable *trans* isomers of unsaturated fatty acids, which are now known to raise the LDL level in a manner similar to palmitic acid.

In contrast, the monounsaturate, oleic acid, has the same LDL-lowering effect as linoleic acid, but is not as susceptible to oxidation as linoleic acid. Oils with increased levels of oleic acid and decreased levels of PUFAs would therefore have the desired properties of cooking oil, without the added expense and potential health hazards of hydrogenation.

Increasing public concern about saturated and *trans* fatty acids

A mandatory labelling requirement for *trans* fatty acids in commercial foods is likely to be implemented in the USA in the year 2002. It requires the *trans* fatty acids be combined and labelled along with the saturated fatty acids. Also, in recognition of the neutral effect of stearic acid on blood cholesterol, Food and Drug Administration (FDA, US) allows the voluntary declaration of stearic acid as a sub-component of saturated fatty acids. Australian and New Zealand Food Authority (ANZFA) is currently considering similar proposals to be implemented here in Australia. Because the cottonseed oil in its current form contains high level of palmitic acid and requires hydrogenation for commercial use, it is reasonable to expect that without genetic improvement, cottonseed oil may loose its future markets to other healthier oilseeds, such as the high-oleic sunflower and soybean. Therefore, it is necessary for the cotton industry to realise the coming changes and react accordingly. Developing new quality types that have the same functionality as the current hydrogenated cottonseed oil, but with greatly reduced content of palmitic and *trans* fatty acids.

Improving cottonseed oil quality

Despite many years of interest in improving cottonseed quality, researches on the quality of cottonseed oil has little impact on the improvement of cotton cultivars. Genetic modification of the fatty acid composition may be accomplished by either mutagenesis or genetic engineering. The induction of genetic variability by mutagenesis has made possible the identification and isolation of mutants with altered fatty acid composition in the seed oils of a number of commercial oilseed crops. The development of novel linseed oil with significantly reduced linolenic acid (Green, 1986) represents a successful example of conventional genetic approaches *via* mutagenesis. However, the desirable mutant types have not been identified in cottonseeds. Recent progress in understanding the genetic and biochemical factors regulating plant lipid composition raises the possibility that this limitation may be alleviated by using cloned genes to create genetically modified plants that produce 'designer' oils, in which the fatty acid composition is precisely tailored to suit particular uses.

Firstly, in order to eventually replace the harmful palmitic acid with its healthier saturated counterpart, stearic acid, our strategy was to raise the level of stearic acid by down-regulating

the stearoyl-ACP Δ9-desaturase which is responsible for the disappearance of stearic acid in cottonseed oil. Secondly, the unstable polyunsaturated fatty acid, linoleic acid which is the dominant fatty acid in cottonseed oil, is synthesized by the microsomal ω−6 desaturase. By genetically silencing the microsomal ω−6 desaturase, we expect to increase the level of its substrate, the relatively stable monounsaturate, oleic acid at the expense of linoleic acid.

Genetic modification of seed quality traits in plants has typically involved in the re-introduction of a copy of the target gene under the control of a seed-specific promoter. The target gene can be arranged in either the sense or antisense orientations relative to the promoter. When a gene is oriented with the translation initiation region immediately following the promoter in a gene construct it is said to be of the 'sense orientation' while the gene in reverse orientation is called 'antisense'. When inserted into a plant genome, both sense and antisense strategies have been demonstrated to down-regulate enzyme activity coded by the endogenous target gene (Heinkoff and Dressen, 1989; van der Krol et al., 1988).

Increasing the stearic acid content of seed oil was one of the first applications of genetic engineering made in the area of plant lipid metabolism. In that case, a cDNA encoding stearoyl-ACP Δ9 desaturase was isolated from *Brassica rapa* and it was expressed in *B. rapa* and *B. napus* in the antisense orientation under the control of seed-specific promoters, and stearic acid was increased up to 40% at the expense of oleic acid (Knutzon 1992). Kinney (1997) reported soybean oil with up to 88% oleic acid content obtained from plants in which an introduced sense copy of the seed specific FAD2 desaturase gene co-suppressed microsomal ω−6 desaturase activity.

However, both sense and antisense strategies are known to have variable and unpredictable effectiveness. This presents a particular problem for cotton since cotton is still relatively difficult to transform, requiring long period of time in tissue culture for regeneration. A novel and highly effective method of producing transgenic, silencing plants recently described by Waterhouse et al. (1998) demonstrated that the inclusion of an inverted repeat within a transgene could substantially increase the frequency of target gene-silenced transgenic plants, potentially making gene-silencing more practicable in cotton.

We have previously described the isolation and characterisation of seed-specific cotton fatty acid desaturase genes, i.e. *ghSAD-1* encoding stearoyl-ACP Δ9-desaturase (Liu et al., 1996) and *ghFAD2-1* encoding microsomal ω−6 desaturase (Liu et al., 1999). Both *ghSAD-1* and *ghFAD2-1* were used in the construction of separate inverted repeat based gene constructs and transformation of cotton cv. Coker315.

Because lipids are essential components of plant cells and are important for plant structure and function, the expression of the fatty acid desaturase silencing constructs must not be modified in organs other than seeds. For this reason, a strong seed-specific promoter derived from the soybean lectin gene was chosen to drive the transcription of the gene constructs.

The individual seeds borne on primary transgenic lines were subjected to preliminary fatty acid analysis. It was found that a number of independent transgenic plants exhibited potentially useful changes in stearic and oleic acids in the seed oil as described below.

High-stearic cottonseed oil

Expression of the stearoyl-ACP Δ9-desaturase gene in the inverted repeat gene construct resulted in the increased level of stearic acid, presumably due to the down-regulation of the corresponding desaturase activity. As summarized in Fig.1, at least half of the total 26 individual transgenic lines transformed with the *ghSAD-1* inverted repeat construct exhibited elevated levels of stearic acid when compared to the wild-type plants. The increase of stearic acid level ranged from minor changes of two or three percent up to much more substantial alterations. The highest stearic acid level was 38%, 15-fold greater than the untransformed control plants. As shown in Fig.3, corresponding to the increase of the stearic acid in the high-stearic line, D9IR-1, the levels of the other three major fatty acids, palmitic, oleic and linoleic acids were all substantially reduced. The reductions of oleic and linoleic acids were expected since they are the products of stearoyl-ACP Δ9-desaturase; but the reduction of palmitic acid is surprising. This is perhaps because of the change of the stearoyl-ACP Δ9-desaturase enzyme activity dramatically altered the components of the flux in fatty acid biosynthesis pathway and therefore affected the effectiveness of other enzymes involved.

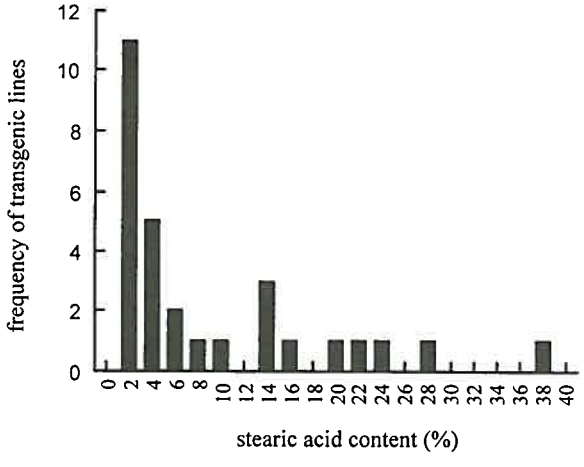

Fig.1 The distribution of stearic acid in transgenic lines transformed with *ghSAD-1* inverted repeat gene construct

High-oleic cottonseed oil

Seventeen out of 29 independent transgenic lines transformed with the microsomal ω−6 desaturase *ghFAD2-1* inverted repeat construct exhibited altered fatty acid composition in the mature seeds of primary transgenic plants (Fig.2). The level of oleic acid increased from about 15% in the untransformed cotton plants up to a range of 26-77%. It is interesting to note that the distribution of oleic acid level among the transgenic lines tend to skew to more extreme phenotype, with oleic acid level around 70%. As indicated by the fatty acid profile of the high-oleic line, D12IR-1 in Fig.3, the increase of oleic acid level is mainly at the expense of linoleic acid. Corresponding to the increase of oleic acid, the linoleic acid level is decreased to as low as 4%. As was the case with the high-stearic lines, the level of palmitic acid was significantly reduced.

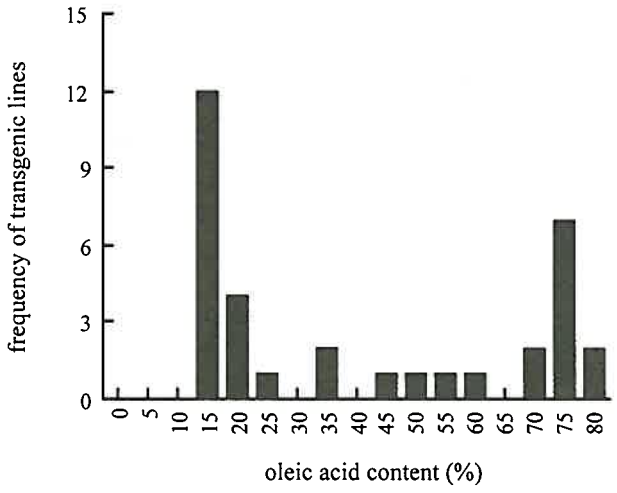

Fig.2 The distribution of oleic acid in transgenic lines transformed with *ghFAD2-1* inverted repeat gene construct

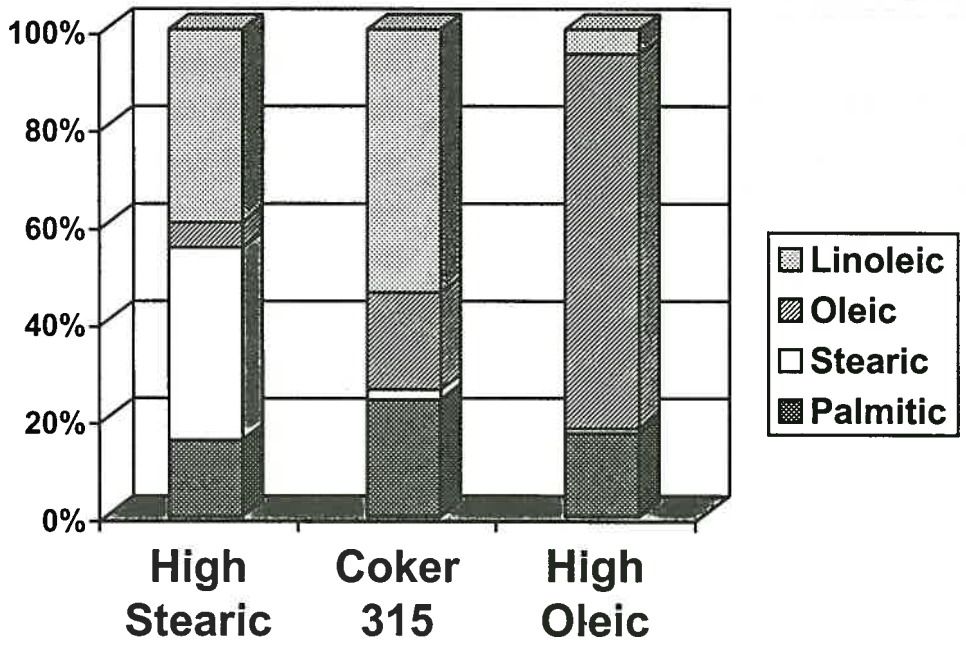

Fig.3 Fatty acid profiles of high-stearic (left), high-oleic (right) lines generated by transformation of inverted repeat desaturase gene constructs. The fatty acid profile of untransformed control, Coker315 is in the middle.

Future prospects

In this current work sponsored by the Australian cotton industry, we have demonstrated that it is possible to achieve healthier and potentially value-added cottonseed oil by altering the expression of fatty acid desaturase genes. While the down-regulation of stearoyl-ACP Δ9-desaturase resulted in accumulation of the healthier stearic and partially replaced the harmful palmitic acid, the silence of the microsomal ω−6 desaturase gene leads to the elevated level of oleic acid at the expense of the unstable polyunsaturated linoleic acid. The inverted repeat mediated gene silencing method offers a highly effective and precise alternative to classical methods of gene inactivation.

The current work shows that cottonseed is quite amenable to genetic modification in the fatty acid profile. For the first time we have demonstrated the ability to increase nutritional value of cottonseed oil without compromising functionality. The high-oleic cottonseed oil is expected to have greater stability than any other seed oil, and have excellent potential for direct use as frying oil in the food service sector without any need for hydrogenation. Likewise the high-stearic cottonseed oil is expected to be suitable for use as hard stock in margarine production without the need for hydrogenation. These materials already offer the

promise of developing cottonseed oil products with greatly improved nutritional appeal to consumers. However, there are further improvements possible to greatly reduce the palmitic acid content of the oil without compromising functionality, something which cannot be done with current cottonseed. Through the present work, we have already achieved a substantial reduction of palmitic acid level in both high-stearic and high-oleic transgenic cotton lines. These lines will provide an excellent platform for the further reduction of palmitic acid without compromising functionality. Secondly, there is a possibility of producing very high value cocoa butter substitute by combining the high-stearic and high-oleic traits into a single line. Such a possibility is currently being explored by carrying out some preliminary intercrossing experiments between a number of selected primary transgenic lines.

Acknowledgment

This work is sponsored by the Australian Cotton R&D Cooperation (CRDC).

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