PROGRESS IN THE DEVELOPMENT OF NUTRITIONALLY IMPROVED AND VALUE-ADDED COTTONSEED OILS

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Introduction

Cottonseed oil is highly polyunsaturated and is routinely hydrogenated to achieve greater stability for cooking applications and functionality for margarine production. The combination of *trans*-fatty acids that result from hydrogenation, and naturally high levels of saturates make hydrogenated cottonseed oil nutritionally undesirable because both components are implicated in rasing blood cholesterol. Increasing consumer attention will be focused on these negative features of hydrogenated cottonseed oil in the future as a result of the likely introduction of compulsory labelling of *trans* and saturated fatty acid content. The recent development of nutritionally-improved forms of competing vegetable oils (e.g. sunflower, rapeseed, soybean) will create a situation of increasing market discrimination away from the current cottonseed oil.

We have previously reported the breakthrough in producing the world's first high-oleic (HO) and high-stearic (HS) cottonseed oils (Liu et al., 2000). As a result of transforming cotton with a hairpin RNA (hpRNA) construct expressing inverted repeat (IR) of cotton microsomal Δ12-desaturase (*ghFAD2-1*) the oleic acid level in cottonseed oil increased from normally 15% up to 78%, mainly at the expense of linoleic acid which is reduced down to as low as 4%. Likewise, the silencing of the cotton Δ9-desaturase (*ghSAD-1*) by a similar approach led to an increase in stearic acid level from normally 2% to as high as 40%. The HO and HS cottonseed oils are expected to be directly usable in current cottonseed oil applications without the need for hydrogenation and thereby avoiding production of nutritionally undesirable *trans* fatty acids. The greatly improved nutritional value of these products should ensure that they readily replace hydrogenated cottonseed oil and imported palm oil in the food industry. Here we report our recent progress in the further development of the homozygous HO and HS transgenic cotton lines and their hybrids. The inheritance of these novel traits has also been studied. We also described a new generation of hpRNA constructs aimed at further improving the effectiveness and specificity of the genetic modification of fatty acid composition, as well as simultaneous modification of multiple traits using a single gene construct.

Inheritance of the HO and HS traits

In the majority of the HO lines, the high-oleic trait which resulted from the silencing of *ghFAD2-1* gene expression is inherited as a completely dominant trait. In the initial segregating populations, both

homozygous and heterozygous seeds contain about 77% oleic acid, indicating the near-maximum suppression of the *ghFAD2-1* gene. Several independently derived transgenic HO lines were bred to homozygosity and the novel fatty acid composition was found to be stably inherited. In contrast, the expression of the high-stearic trait in the HS lines appeared to be more complex with a relatively wide variation among individual seeds. A similar range in stearic acid levels was observed between individual seeds in populations that were homozygous for the *ghSAD-1*-IR transgene, suggesting that the variation is non-genetic.

The peculiar but stable inheritance of HO and HS was also demonstrated in the cross hybridisation progenies. By intercrossing the HS and HO genotypes we have demonstrated that it was possible to simultaneously down-regulate both *ghFAD2-1* and *ghSAD-1*. To our best knowledge, this is also one of the first desmonstrations that the hpRNA induced transcriptional gene silencing (PTGS) in independent genes can be combined without any diminution in the degree of silencing. In such an F_2 population (Fig. 1), all the high-oleic seeds, both homozygous and heterozygous, contained similar fatty acid composition. High-stearic seeds, however, displayed greater variations of stearic acid level. The relatively high variation in fatty acid composition of the seeds containing both *ghFAD2-1*-IR and *ghSAD-1*-IR transgenes is likely due to the high variation in stearic acid level as a result of action of the *ghSAD-1*-IR transgene. Nevertheless, the sum of stearic acid and oleic acid levels was a constant figure of about 77%. This reflects the substrate and product relationship between these two fatty acids. The seed to seed variation of fatty acid composition in these hybrids persisted in subsequent generations where both transgenes were homozygous.

By March 2002, two high-oleic and two high-stearic lines each having a single functional transgene locus have been selected and bred to homozygosity. Approximately 2.5 kg cottonseed from each of the four lines were harvested. Oil extraction was performed on a fraction of the seeds to enable a range of functional tests at Food Science Australia, while the rest of seeds will ultimately be used for a small field trial for seed increase and primary observation of agronomic performances. Under the glasshouse condition, both HO lines appear to have a normal physiological behaviour with no notable phenotypic differences from the wild-type cotton. However, both HS lines have reduced germination rate and there is a correlation between the high stearic acid level and poor germination rate. This phenomenon is consistent with previous findings in high-stearic soybean and rapeseed lines (Rahman et al., 1997). This problem can be alleviated by germinating the seeds at high temperatures or on sucrose-supplemented culture media, however the problem will need to be overcome before HS cottonseed lines can be field tested or grown commercially.

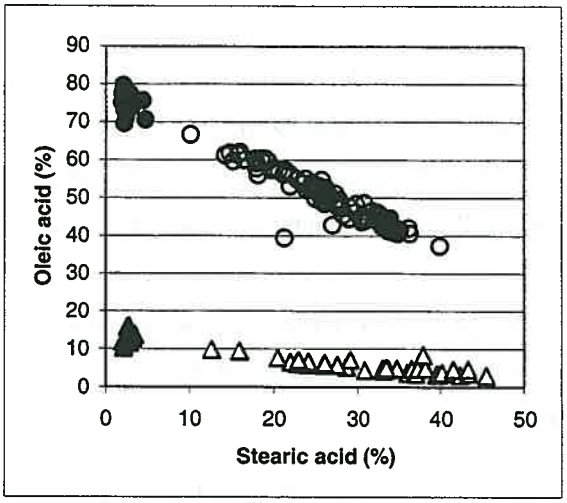

Fig. 1. Stearic and oleic acid accumulation in an F_2 seed population derived from a cross between the HO and HS lines. Seeds containing only the *ghSAD-1*-IR transgene (△), seeds containing only the *ghFAD2-1*-IR transgene (●) and seeds containing both *ghSAD-1*-IR and *ghFAD2-1*-IR transgenes (○) and the seeds containing neither of the two genes (▲).

Genetic manipulation of palmitic acid content

The relatively high level of palmitic acid is a major disadvantage of conventional cottonseed oil in the increasingly health-conscious vegetable oil market. Although its synthesis was not directly targeted by the silencing of either *ghSAD-1* or *ghFAD2-1* desaturase, palmitic acid has been reduced from normally 26% down to 15% in both HS and HO cottonseed oils. Since then, we have placed additional high priority on making further reductions in palmitic acid content to maximize the nutritional improvement and ensure that the new oil keeps pace with similar developments with other competing vegetable oils. It is expected that a substantial reduction of palmitic acid is achievable without compromising the stability of HO or HS cottonseed oil.

Furthermore, we are also interested in producing very a high-value cocoa butter substitute by tailoring the fatty acid composition in cottonseed oil. Cocoa butter is the most highly-valued plant lipid and it commands a large price premium relative to all other vegetable oils. In cocoa butter, the dominant species of triacylglyceride is the so-called POS type which contains equal proportions of palmitic, oleic and stearic acids. It is the POS triglyceride that gives cocoa butter its unique physical property of sharp melting point at body temperature. In the homozygous seeds containing both HS and HO traits, stearic acid level ranged between 20-40% and the oleic acid level varied between 37-57%. The palmitic acid level remained constant at 15%. Consequently, a logical step is to increase the palmitic acid level (from 15% up to 30%).

If, as expected, this increase is at the expense of oleic and linoleic acids then careful selection may lead to a fatty acid profile very close to that of cocoa butter.

There are two enzymes directly responsible for the accumulation of palmitic acid in cottonseed oil, i.e. palmitoyl-ACP thioesterase (FatB) and keto-acyl synthase II (KASII). FatB cleaves the palmitic acid off the palmitoyl-ACP and therefore makes it available for triacylglycerol assembly, whereas the KASII extends palmitoyl-ACP to stearoyl-ACP pulling palmitic acid away for the biosynthesis of downstream fatty acids. Therefore, the further reduction of palmitic acid can perhaps be achieved by either down-regulating the FatB activity or over-expression of KASII. The adoption of the opposite approach, i.e. the over-expressing a FatB gene or moderate down-regulation of KASII activity, can perhaps also lead to production of cocoa butter substitute.

We have isolated a number of candidate genes encoding FatB or KASII enzymes. It appears that both FatB and KASII are encoded by multigene families. Many copies of these genes are responsible for the biosynthesis of membrane lipids which are very important to physiological functions and tolerances to environmental stresses and they must remain active in the vegetative tissues while being silenced in the seeds. Therefore, further studies on the molecular characterisation of these genes are being carried out in order to accurately pinpoint the gene member(s) that are specifically expressed during the seed oil biosynthesis and responsible for the accumulation of palmitic acid in cottonseed oil. This information will enable the specific targeting of the key genes by using gene-specific DNA fragments, such as untranslated regions (UTRs), in the subsequent genetic transformation of cotton.

Increasing the silencing efficiency and simultaneous targeting of multiple genes

A dramatically increased number of high-oleic lines was produced when the transforming of cotton involved *ghFAD2-1*-IR construct containing a spliceable intron (Wesley et al., 2001). We have also established that a gene-specific UTR fragment as short as 98-bp is sufficient to silence the target cotton fatty acid desaturase genes. This made it possible to consider silencing multiple genes simultaneously by a single construct. We have previously demonstrated that the high-oleic and high-stearic traits can be combined by sexual crossing of the relevant transgenic lines. As the transgenes are inserted at different locations they will segregate in subsequent generations making the task of stacking modified traits through crossing a laborious and time-consuming one and effectively limiting the number of genes that can be combined. An alternative strategy is to use a single chimeric transgene to simultaneously suppress multiple genes by fusing partial DNA sequences together (Abbott et al., 2002). We are currently evaluating the effectiveness of this approach to down-regulate the cotton *ghFatB-1*, in combination with *ghSAD-1* and *ghFAD2-1* silencing. Approximately 300-bp cDNA fragments from each of three genes, *ghSAD-1*, *ghFAD2-1* and *ghFatB-1*, were fused together as a whole unit. In the binary construct, this chimeric gene is separated from its inverted repeat by a spliceable intron derived from *ghFAD2-1*. This approach is aimed at further reducing the palmitic acid level in the high-oleic and high-stearic germplasm.

Summary

We have developed a number of independent transgenic cotton lines containing homozygous high-oleic, or high-stearic or both traits. Monitoring the inheritance of the novel HO or HS traits and their general physiological characteristics, such as the germination rate among a number selected transgenic cotton has advanced our understanding of genetic manipulation of fatty acid composition and the limitations. The recent study has also highlighted a number of challenges we are facing, such as overcoming the lower germination rate of high-stearic seeds and the increase of palmitic acid to the desired level for use as cocoa butter substitute. Currently cottonseed oil is a undervalued product and there are a number of improvements to be made besides the initiatives we have already made. It is desirable to remove cyclopropenoid fatty acids and gossypol from cottonseeds while retaining them as they are in other tissues. Some concerted efforts are being made to identify the genes responsible for the production of these compounds. Approaches similar to those we have used in the production of HO and HS cottonseed oils can also be adopted to achieve such goals. In the current study we have developed techniques to silence a target gene with very short gene-specific UTR fragments. This will not only enhance the specificity of gene silencing but also facilitate the silencing of multiple genes simultaneously by using a single chimeric gene construct. It is envisaged that a new cottonseed having multiple improvements, such as the high-oleic, low-palmitic, zero cyclopropeniods and zero-gossypol should be available in the future. Most importantly, as technology advances and communications with the public improve, the considerable health benefits of such a cottonseed oil should eventually be taken up widely as an alternative to chemically hydrogenated vegetable oils.

References

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