Genetic Improvement of Oil Quality in Sesame (*Sesamum indicum* L.): Assembling Tools

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Abstract

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Sesame (*Sesamum indicum* L.) seed is source of high quality edible oil for which the market niche can be expanded by developing cultivars with altered fatty acid composition through conventional breeding or genetic transformation. There is need to know the natural diversity in oil quality to facilitate breeding, while reliable regeneration and transformation protocols need to be developed for genetic engineering. The seed oil diversity in thirty sesame accessions from East Africa over three years, and the regeneration and floral transformation potential of selected accessions were studied. The effectiveness of a cloned sesame oleate desaturase was tested in *Arabidopsis thaliana*.

Significant variation was observed among the accessions for the oil content and the quantity of individual fatty acids in each of the three years of field evaluation. There was positive correlation between oil content and stearic and oleic acid levels. Shoot regeneration was achieved from cotyledon explants, whereas hypocotyl explants only gave callus and roots. Some accessions were more amenable to regeneration than others. A high rate of sesame transformation using *Agrobacterium tumefaciens* carrying a binary vector containing the neomycin phosphotransferase (NPT) II gene for kanamycin resistance and the enhanced green fluorescent protein (EGFP) reporter gene was achieved through floral dipping, pollen infiltration and suspension drop. Pollen infiltration and suspension drop produced higher transformation frequencies than floral dip. *Agrobacterium* strains EHA 105 and GV3101 gave higher transformation than GV2260. The best sesame cultivars for transformation were Mtwara-2 and McBlack. A Δ12 oleate desaturase cDNA isolated from immature seeds had its function confirmed by its complementation of the *fad2-2* mutant phenotype of *A. thaliana*. The cDNA was also expressed in transgenic *A. thaliana* lines that synthesize epoxy, hydroxy and acetylenic fatty acids, and shown to influence the accumulation of linoleic and the unusual fatty acids.

The major contribution of this study is the development of novel regeneration and transformation techniques for sesame, which open new avenues for the genetic improvement of the crop.

Keywords: Sesamum indicum, oil quality, fatty acids, regeneration, genetic transformation, *Agrobacterium*, Δ12 oleate desaturase.

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The race is not to the swift, or the battle to the strong
(Ecclesiastes 9: 11, Holy Bible)

To my family
With gratitude for your unfailing love
And support through the years

Commit to the Lord whatever you do and your plans will succeed.
(Proverbs 16:3, Holy Bible)

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Appendix

Papers I-IV

This thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Were, B. A., Onkware, A. O., Gudu, S., Welander, M. & Carlsson, A. S. 2006. Seed oil content and fatty acid composition in East African sesame (*Sesamum indicum* L.) accessions evaluated over three years. *Field crops research* (In press).

II. Were, B. A., Gudu, S., Onkware, A. O., Carlsson, A. S & Welander, M. 2006. *In vitro* regeneration of sesame (*Sesamum indicum* L.) from seedling cotyledon and hypocotyl explants. *Plant cell tissue and organ culture* (In press).

III. Were, B. A., Carlsson, A. S., Welander, M., Onkware, A. O. and Gudu, S. 2006. Floral transformation of sesame (*Sesamum indicum* L.) using *Agrobacterium tumefaciens*. *Plant cell reports* (Submitted).

IV. Were, B. A., Onkware, A. O., Gudu, S., Welander, M. & Carlsson, A. S. 2006. Expression of a *Sesamum indicum* Δ12 desaturase alters levels of unusual fatty acids in seed oil of transgenic *Arabidopsis thaliana* (Manuscript).

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Introduction

Botany and Ecology

Sesame (*S. indicum* L. Syn *S. orientale* L., Pedaliaceae) is an erect herbaceous annual crop that grows to a height of 0.4 m to 2 m tall. The plants are often highly branched, but some varieties are relatively unbranched. Stems are square with grooves. Leaves are hairy on both sides with variable shape (ovate to lanceolate) and size, and may be opposite or alternate. Bell-shaped, pale purple to white flowers begin to develop at leaf axils within 6-8 weeks after planting. A single flower is produced at each leaf axil starting from the lower axils and the plant continues blooming until the uppermost flowers on the stem are open (Day, 2000). Multiple flowering is common in varieties with opposite leaves (Oplinger *et al*., 1990). Sesame is predominantly self-pollinated, although cross pollination by insects is common (Pathirana, 1994). The fruit is an oblong capsule, 1 to 3 inches long, containing 50 to 100 or more seeds. The seeds are oval and may be white, yellow red, brown or black in colour. The seeds mature 4 to 6 weeks after fertilization. Sesame grows indeterminately, producing new leaves, flowers and capsules at the same time (Fig 1) as long as the weather conditions permit. The growth cycle is completed within 70 to 180 days depending on the variety and growth conditions.

Sesame is well adapted to high growth temperatures of up to 40^{o}C and drought. However, it requires adequate moisture at sowing and early growth and at least 300 to 400 mm of rainfall per season for reasonable yields. Rainfall late in the season prolongs growth and increases seed loss due to shattering. The crop can grow on a range of soil but performs best on well-drained, fertile medium textured soils with neutral pH. It has a very low tolerance to salinity and is highly susceptible to water logging.

Origin and distribution

Sesame is one of the oldest cultivated crops known to man. It was a highly prized oilseed in the ancient world because of its resistance to drought, the ease to extract oil from seeds and the high stability of the oil (Langham & Wiemers, 2002). Archeological remains of sesame dating back to 5,500 BC have been found in the Harappa valley in the Indian subcontinent (Bedigian & Harlan, 1986). The origin of the crop has been a major subject of discussion, with proposals for an African or Indian domestication. Based on various lines of evidence including cytogenetics, biochemical composition, nuclear DNA marker comparisons and cultural history to name a few, Bedigian (2003, 2004) has concluded that this species was first domesticated on the Indian subcontinent. From there, it spread to Africa, the Mediterranean, and the Far East, and into the Americas following trade routes. Today sesame is widely grown in China, Japan, Korea, Turkey, India, USA, South America and parts of Africa as an oil seed.

Fig 1 Sesame plants in bloom, with capsules, open flowers and immature buds on the same plant.

Sesame production and utilisation as an oil crop

India and China are the world's largest producers of sesame, followed by Myanmar, Sudan, Uganda, Nigeria, Pakistan, Ethiopia and Bangladesh (FAOSTAT data, 2005). In 2004, the total world production was about 3.26 million tons grown on 6.67 million hectares, of which 26.4% was produced in Africa. Sesame currently ranks sixth in the world production of edible oil seeds (Table 1) and twelfth for vegetable oil produced (Table 2).

Table 1 World production of major oil seeds in 2004

Oil crops	Production (Million tons)
Soybeans	204,266,176
Seed Cotton	71,981,922
Rapeseed	46,255,508
Groundnuts in Shell	35,723,285
Sunflower Seed	26,108,358
Sesame Seed	3,257,448
Linseed	1,902,688
Castor Beans	1,282,807
Safflower Seed	604,157
Mustard Seed	703,738

Source: http://faostat.fao.org/

FAOSTAT data, 2005. Last updated July 14 2005

Table 2 World production of vegetable oil in 2002

Oil type	Production (million tons)
Oil of Soya Beans	30,059,155
Oil of Palm	25,457,205
Oil of Rape and mustard seed	12,445,419
Oil of Sunflower Seed	8,129,760
Oil of Groundnuts	5,061,279
Oil of Cotton Seed	3,775,093
Oil of Coconuts	3,162,393
Oil of Palm Kernels	3,163,362
Oil of Olive	2,546,306
Oil of Maize	1,984,614
Oil of Rice Bran	1,077,353
Oil of Sesame Seed	755,578
Oil of other crops	3,324,959

Source: http://faostat.fao.org/

FAOSTAT data, 2005. Last updated August 27 2005

The low ranking of sesame on the vegetable oil market may be attributed to several factors including low seed yield, difficulty to mechanise harvesting and strong competition from more cheaply produced vegetable oils such as palm and coconut oil. In addition, there has been limited breeding effort, largely due to the fact that it is mainly produced in resource-poor developing countries that cannot sustain long-term improvement programs for the crop. The establishment of sesame as a major crop has been extremely slow considering its long history. The use of modern plant breeding knowledge and new technologies could benefit research aimed at improving the crop (Ram *et al*., 1990).

Uses of sesame

Sesame is grown primarily for its nutritious seed that is rich in linoleic acid, protein, and calcium as well as vitamin E, and small quantities of vitamins A, B1 and B2. Nearly 70% of the world's sesame seed is processed into oil and meal while the remainder is channelled to food and confectionery industries (Morris, 2002). The oil is mainly used in cooking and salad, and for making margarine. It is also used in cosmetics preparations, pharmaceutical products, paints, soaps and insecticides (Ashri, 1989; 1998). The meal left after oil extraction contains 35-50% protein and makes a rich feed for poultry and livestock.

Sesame seed is used on bread, buns, cookies, health snacks and as an additive to breakfast cereal mixes. The seeds may be eaten whole either raw or roasted and

salted, or mixed with lemon and honey (Chalbe), but are often ground into paste (Tahini), which may also be sweetened with sugar (Halva). Sesame seed is used as an ingredient in many recipes, added whole or pounded. In Africa, the paste is used as a spread and in preparing soups and sauces.

Oil content and Fatty acid composition in sesame seeds

Sesame has a relatively superior oil quantity as well as quality in comparison to many major oil crops. The oil content ranges from 34.4% to 59.8% but is mostly about 50% of seed weight (Ashri, 1989, 1998). Values of up to 63.2% have been reported in some varieties (Baydar, Turgut & Turgut, 1999; Uzun, Ülger & Çağirgan, 2002). Both genetic and environmental factors influence the oil content in sesame. Late maturing cultivars are reported to have higher oil content than early ones (Yermanos *et al*., 1972). Uzun, Ülger & Çağirgan. (2002) observed that indeterminate cultivars accumulated more oil than determinate ones. Variation also occurs between capsules at different positions on the same plant, such that seeds from the basal capsules on the main stem contain more oil than those located towards the apex and on side branches (Mosjidis & Yermanos, 1985; Muthuswamy & Thangavelu, 1993). Black seeded cultivars often have lower oil content than brown or white seeded ones, indicating a possible linkage between oil content and seed coat colour. Kamal-Eldin & Appelqvist (1994a) have attributed the low oil content in black seeded sesame to a high amount of crude fibre in the seed coats. Black seed coats are usually thicker than lighter coloured ones.

The sesame genus has limited variability in the seed fatty acid proportions (Kamal-Eldin *et al*., 1992). The seed fatty acid composition varies considerably among the different cultivars of sesame worldwide (Yermanos *et al*., 1972; Brar, 1982; Baydar, Turgut & Turgut, 1999). The oil contains four major fatty acids namely, palmitic, stearic, oleic and linoleic acids, along with small quantities of vaccenic, linolenic, arachidic, behenic and eicosenoic acids (Weiss, 1983; Kamal-Eldin *et al*., 1992; Ashri, 1998; Were, Lee & Stymne, 2001). Oleic and linoleic acids occur in nearly equal amounts, constituting about 85% of the total fatty acids.

Cultivars with exceptionally high (> 60%) oleic or linoleic acid are rare (Baydar, Turgut & Turgut, 1999). Uzun, Ülger & Çağirgan. (2002) found differences in stearic, oleic and linoleic acids between determinate and indeterminate cultivars. Determinate cultivars generally have higher stearic and oleic acids, and lower linoleic acid compared to indeterminate ones. Capsule position on the plant also affects the relative quantities of the fatty acids; palmitic, stearic and oleic acids tend to increase up the stem while linoleic acid decreases (Brar, 1977). The fatty acid composition is strongly influenced by environmental factors. Linoleic acid content has been reported to increase under cool growing conditions (Uzun, Ülger & Çağirgan, 2002).

Dietary and health benefits of sesame oil

The fatty acid composition is a major determinant of edible oil quality. Oils having high oleic acid content, in combination with low quantities of saturated and polyunsaturated fatty acids (PUFAs) are commercially and nutritionally desirable. Saturated fatty acids are associated with high risk of heart disease. Whereas PUFAs are known to be beneficial for human health, high PUFA quantity in edible oil is undesirable as they are readily oxidized yielding products that are potentially harmful to human health, and which give off-flavours and odours to foods. Sesame oil has a low level of saturated fatty acids (< 15%) and approximately equal quantities of mono- and polyunsaturated fatty acids. The oil is nutritionally valuable as a source of linoleic acid which is essential to man.

Despite having a high content of linoleic acid, sesame oil is unusually stable to oxidation compared to other vegetable oils with a similar fatty acid composition. This feature is attributed to antioxidant activities of sesamol and sesaminol together with tocopherols present in the oil (Kamal-Eldin & Appelqvist, 1994b). A combination of the high stability and a nutritionally acceptable fatty acid composition contributes significantly to the excellent oil quality, making it a high-value edible oil.

Recent studies have shown that sesame oil is beneficial in lowering cholesterol levels and hypertension (Sankar *et al*., 2004; Frank, 2005), and reducing the incidence of certain cancers (Hibasami *et al*., 2000; Miyahara *et al*., 2001). These health enhancing effects of sesame oil are explained by the low level of saturated fatty acids as well as the activity of antioxidants mainly sesamin. Sesamin is known to enhance the availability and functioning of vitamin E (tocopherol). An elevated concentration of γ-tocopherol in the blood is associated with reduced risk of heart disease and some cancers e.g. of the upper gut. Thus, sesame oil could be beneficial for enhancing health by improving the vitamin E levels in the body (Frank, 2005).

Biosynthesis of fatty acids and triacylglycerol in oil seeds

De novo fatty acid synthesis

The mechanisms of fatty acid biosynthesis and oil formation in plant cells have been extensively reviewed (Ohlrogge & Browse, 1995; Slabas, Simon & Brown, 2001; Voelker & Kinney, 2001). Briefly, there are two main sites for glycerolipid synthesis in plants namely, plastids and endoplasmic reticulum. Saturated fatty acids up to 18-carbon and oleic acid are synthesised in plastids, starting with acetyl-coenzyme A (CoA) as the precursor (Fig 2). In the first step of plant fatty acid synthesis malonyl-CoA is formed by the carboxylation of acetyl-CoA, in an adenosine triphosphate (ATP)-dependent reaction catalysed by the enzyme acetyl-CoA carboxylase (ACCase). Malonyl-CoA:ACP transacylase then converts the malonyl-CoA to malonyl-acyl carrier protein (ACP), which is used in condensation reactions involving several enzymes of the fatty acid synthase (FAS) II complex. These enzymes subsequently add two carbon units derived from malonyl-ACP to

the growing acyl chain. Malonyl-ACP is first condensed to acetyl-CoA by β-ketoacyl-ACP synthase (KAS) III to produce a four-carbon acyl-ACP. The next seven cycles of condensation catalysed by KAS I yield palmitoyl-ACP, a C16 acyl thioester, which is then elongated by KAS II to stearoyl-ACP the final product of the FAS complex. Most of the stearoyl-ACP is converted to oleoyl-ACP by Δ9-stearoyl-ACP desaturase through unsaturation at the C-9/ C-10 position. Due to the high activity of this enzyme nearly all the stearic acid synthesised in plant cells is converted to oleic acid.

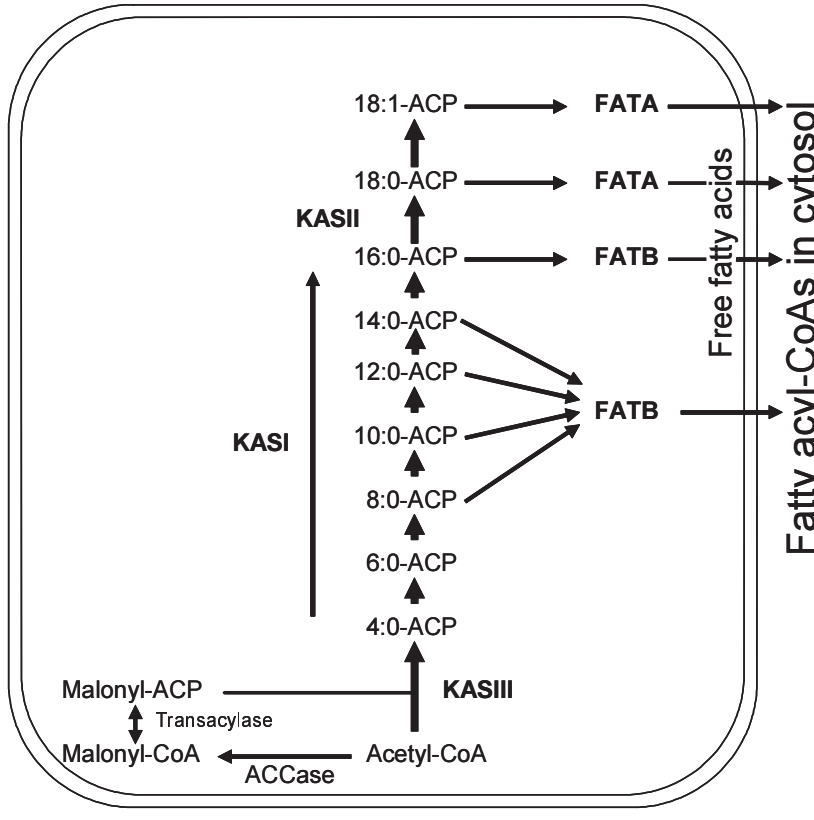

Fig 2 Basic pathway for fatty acid biosynthesis in plastids

Following synthesis in the plastid, the fatty acyl-ACP esters (mainly palmitoyl, stearoyl and oleoyl-ACPs) are either used directly for lipid synthesis in the plastid or hydrolysed by fatty acyl-ACP thioesterases (FATA and FATB) to free fatty acids and exported to the cytosol. In the cytosol, the fatty acids are esterified to coenzyme A and used as substrates for lipid formation in the endoplasmic reticulum (ER). The relative activities of KAS II and palmitoyl-ACP thioesterase determine the proportions of palmitic and stearic acids. Plants with high KAS II activity produce a large amount of 18-carbon fatty acids. In some species like coconut, *Cuphea* and oil palm (kernel) the main products of FAS activity are short

to medium-chain fatty acids (C8-C14) because of premature termination of acyl chain elongation by the specific FATB enzymes.

Extraplastidial elongation of C18 fatty acids

Some plants accumulate very long chain fatty acids (VLCFAs) and their derivatives like waxes. One such fatty acid is erucic acid (22:1Δ13) that is abundant in cruciferous plants. VLCFAs are formed when stearic and oleic acids exported into the cytoplasm are elongated in the acyl-CoA pool (Fig 3) by an ER-associated fatty acid elongase (FAE) system with similar activity as the FAS. Four enzymes namely an elongase, two reductases and a dehydrase constitute the FAE system. The elongase, 3-ketoacyl-CoA synthase (KCS) is the key component for synthesis of VLCFAs as demonstrated by complementation studies in low erucic acid rape seed, LEAR (Lassner, Lardizabal & Metz, 1996). The KCS catalyses the condensation of fatty acyl chains with malonyl-CoA thereby increasing the chain length by two carbon units with every reaction cycle.

Oleic acid desaturation and similar modifications outside the plastid

The proportion of oleate-ACP used for plastidial lipid synthesis or hydrolysis by a thioesterase to free oleic acid determines the fate of the acid for modification within or outside the plastid. The oleate exported from the plastid is either modified further to produce other fatty acids or gets incorporated into glycerolipids (Fig 3). In the ER, oleic acid may be modified through desaturation, hydroxylation, epoxygenation or acetylenation while esterified to phospholipids, preferably phosphatidylcholine (PC). Most commonly, oleate (in form of oleate-PC) is sequentially converted to linoleate and α-linolenate by membrane-bound Δ12 and Δ15 desaturases encoded by the fatty acid desaturase (FAD) genes, FAD2 (Okuley *et al*., 1994) and FAD3 (Yadav *et al*., 1993). The desaturation occurs on fatty acyl chains at the sn-2 position of PC (Stymne & Appelqvist, 1978).

The desaturase-introduced double bonds in linoleic and linolenic acids are separated from each other by a methylene group. Fatty acids with other functional groups or non-methylene-interrupted double bonds result from the modification of oleic and linoleic acids by desaturase-like enzymes encoded by genes believed to have evolved from the FAD2. These enzymes have high sequence homology to the FAD2, with the catalytic function likely to be defined by difference in only a few amino acids (Broun *et al*., 1998a).

The recently characterized fatty acid hydroxylases from *Ricinus communis* (Loo *et al*., 1995) and *Lequerella fendleri* (Broun, Boddulpalli & Sommerville, 1998b) catalyze the introduction of a hydroxyl group instead of a double bond at the Δ12 position of oleic acid producing ricinoleic acid (12-OH-18:$1^{\Delta 9}$). Besides ricinoleic acid other hydroxylated fatty acids like densipolic and lesquerolic acids are also accumulated in these plants. Δ12-acetylenases and -epoxygenases now known to catalyse the formation of fatty acids with triple bonds and epoxy groups have also been discovered (Lee *et al*., 1998). Another type of divergent FAD2 enzymes, the conjugases, introduce conjugated double bonds instead of the commonly formed methylene-interrupted cis double bonds (Cahoon *et al*., 1999). These have been

isolated from a few plant species, e.g. *Impatiens balsamica*, and *Calendula officinalis*. α-parinaric acid (18:4$^{\Delta 9\text{-cis, } 11\text{-trans, } 13\text{-trans, } 15\text{-cis}}$) and calendic acid (18:3 $^{\Delta 8\text{-trans, } 10\text{-trans, } 12\text{-cis}}$) are examples of conjugated fatty acids. Unlike the Δ12-hydroxylases, the FAD2-like acetylenases, epoxygenases and conjugases utilise linoleic acid as a substrate for acyl chain modification.

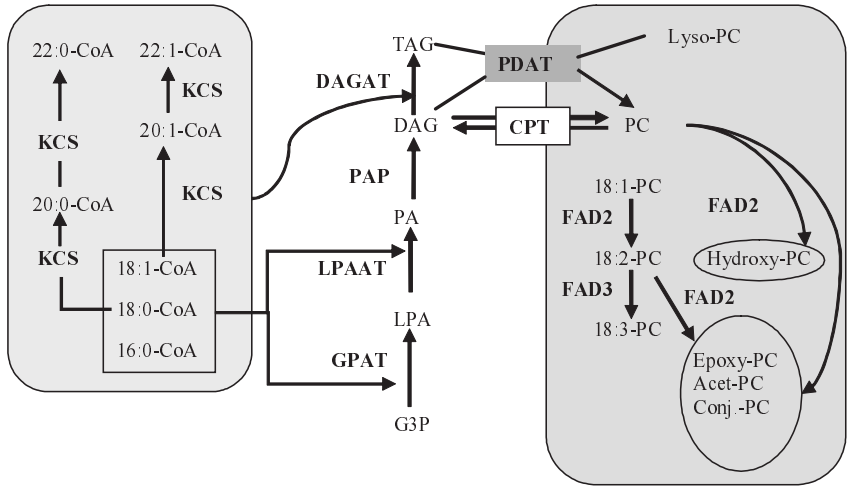

Fig 3: Pathways for modification of fatty acyl residues in the endoplasmic reticulum. Pale grey box to the left shows the elongation steps; darker grey box to the right shows desaturase and desaturase-like reactions.

A feature common to all the divergent FAD2 enzymes characterized at present is that their expression in transgenic plants leads to accumulation of oleic acid, which has been attributed to competition with the endogenous FAD2 (Broun, Boddulpalli & Sommerville, 1998b; Cahoon *et al*., 1999; Thomæus, Carlsson & Stymne, 2001). Whereas many of the divergent FAD2 enzymes modify the Δ12 position of C18-fatty acid substrates, some have also been identified that can modify an existing Δ9 double bond (Cahoon *et al*., 2001). The addition of new functional groups to acyl chains gives rise to a vast range of fatty acid species with varied properties and functions that could be used in industrial products like paints, lubricants, nylon and cosmetics.

Triacylglycerol assembly

Triacylglycerol (TAG) biosynthesis in the endoplasmic reticulum proceeds through three consecutive steps of glycerol-3-phosphate (G3P) acylation (Ohlrogge & Browse, 1995) (Fig 3). The G3P is provided by the G3P dehydrogenase reduction of dihydroxyacetone phosphate. In the first step, an acyl moiety, preferably saturated, from acyl-CoA is introduced at the sn-1 position of glycerol-3-phosphate in a reaction catalysed by a glycerol-3-phosphate acyltransferase (GPAT), generating lysophosphatidic acid (LPA). In most oil seeds the sn-2 position of lysophosphatidic acid is filled by an unsaturated fatty acid producing a

diacylglycerol (DAG)-phosphate, also called phosphatidic acid (PA). The enzyme responsible for the second acyl transfer is lysophosphatidic acid acyltransferase (LPAAT). Phosphatidic acid phosphatase (PAP) then removes the phosphate group from phosphatidic acid, releasing DAG. The DAG either undergoes a final acylation by DAG acyltransferase (DAGAT) to form TAG or it can be used in reversible PC-DAG interconversion to enter membrane lipid synthesis. The PC-DAG interconversion by a choline phosphotransferase (CPT) provides a means by which the fatty acids modified on PC enter into TAG formation. An additional enzyme involved in TAG synthesis, phospholipid diacylglycerol transferase (PDAT) was recently characterized (Dahlqvist *et al*., 2000). PDAT forms TAG (and lyso-PC) by transferring a fatty acid residue from the sn-2 position of PC to a DAG. The TAG formed through these reactions is deposited in oil bodies for storage as an energy reserve.

Modification of fatty acid composition in plant storage oils: problems, progress and prospects

In field crops such as oilseed rape, peanuts, sunflower and sesame, oil constitutes about 45% of the seed weight. Most of the oil is used for human consumption, mainly in shortenings, margarine, salad oils and frying oils. Only a small proportion of about 10% finds use in manufacturing industries as lubricants and in the production of detergents, coatings, and paints.

Vegetable oils may sometimes lack the properties best suited for their intended use. For instance, they could have undesirable nutritional attributes such as high proportion of saturated fatty acids in comparison to the more acceptable unsaturated forms, or have melting behaviour that contributes to poor quality of spreads. Such deficient oils would need to be modified to attain the desired properties. Modification of lipid properties is conventionally done by chemical processes namely, partial hydrogenation, fractionation or interesterification (Bhattacharyya, Bhattacharyya & De, 2000; Timms, 2005). These processing methods, however, are expensive and sometimes yield undesirable products in the edible oils. For example, during the hydrogenation of highly unsaturated oils for making margarine and shortenings trans fatty acids, known to confer health risks in man, are formed (Mozaffarian *et al*., 2004).

Development of crop varieties producing oils with quality appropriate for specific market needs presents a better alternative to chemical modification of vegetable oils and a means to circumvent the short-comings associated with the technology. One way to achieve this is by domesticating wild plants that accumulate oil with characteristics of interest. However, the long time scale (of over 20 years) needed to adapt them to cultivation and the requirement for re-modelling of agricultural machinery and processing equipment present a major limitation to development of novel oil crops. Efforts to domesticate and develop certain species for non-edible oil production have not been rewarding as in the case

of evening primrose that would be a good source of γ-linoleic acid for the pharmaceutical industry (Lapinskas, 1993).

In a variety of cultivated oil crops, the fatty acid composition has been modified by means of conventional breeding methods to meet various consumer demands. Using sexual hybridization as well as induced mutagenesis, new varieties of oil crops have been generated which have diverse composition of fatty acids. Examples include the breeding and establishment of LEAR for edible oil, and the development of high-oleic varieties of soybean, sunflower, brassica oilseeds and peanut (Burton *et al*., 2004). Though successful, conventional breeding relies on the naturally occurring variation within a species or genus and is therefore limited to cross compatible taxa. Some of the variation breeders have used is due to spontaneous random mutations affecting fatty acid synthesis e.g. in LEAR although they are very rare. Induced mutagenesis has been used to create additional diversity in seed fatty acid composition, as was done when developing high linoleic acid linseed (Linola) from a high linolenic acid variety (Green, 1986). However, induced mutagenesis is disadvantageous as it lacks precision, generating many plants with defects and for this reason entails extensive screening of lines to eliminate the bulk of abnormal ones. Undesirable traits such as late flowering, reduced vigour and low seed yield are obtained alongside the phenotype of interest in mutant lines. This method is therefore unreliable for creating variants in which only one locus influencing synthesis of a specific fatty acid is disrupted.

Current research effort is directed towards creating plant oils having diverse fatty acid composition by genetic engineering of the established oil crops. This approach is superior to those previously used owing to its precision and applicability across taxa. By using molecular techniques, it is possible to modify specifically the seed oil quality while keeping the rest of the genetic background of the plant constant. Using techniques such as antisense repression, co-suppression and inverted repeat silencing, transgenic oil crops having novel fatty acid profiles have been generated (Cartea *et al*., 1998; Stoutjesdijk *et al*., 2002). Examples include high-stearate rape (Knutzon *et al*., 1992), high-laurate rape (Voelker *et al*., 1992), and high-oleate cotton (Liu, Singh & Green, 2002) among others.

The major edible oils (listed in Table 2) contain predominantly unsaturated 18 carbon fatty acids and palmitic acid. Key targets for genetic modification of these oils both for edible and industrial uses have been identified (Murphy, 1999). One goal for modification of these oils for edible use is to increase the amount of palmitic and stearic acids in order to minimise the need of hydrogenation in the production of dietary fats. Another important target is to increase stability of oil, achieved by reducing the levels of unsaturated fatty acids especially linolenic acid. However, linoleic and linolenic acids are essential to man and need to be kept at acceptably high levels in dietary fats.

In the case of plant industrial oil, there is a wide range of fatty acids of interest including many from wild species that remain a target for commercial production in transgenic crops. Examples of such fatty acids include lauric, petroselinic, ricinoleic, vernolic and γ-linolenic acids. There has been some success with a few

of these in oilseed rape and soybean but there remains need to increase the quantity of the specific fatty acids for cost effective use of the modified crops. With a better understanding of the biosynthetic pathway for uncommon fatty acids it will be possible to achieve this in the major oil crops.

Prospects and challenges to modifying seed oil composition in sesame

Molecular aspects

A detailed knowledge of the metabolic pathways involved in the biosynthesis of fatty acids is a prerequisite for genetic engineering of the seed fatty acid composition. Although the pathway for sesame is not documented, the fatty acid profile suggests synthesis via the known route common to most major oil crops. Various genes encoding enzymes involved in fatty acid synthesis have been isolated from the species and characterized. Yukawa *et al*. (1996) isolated two copies of the Δ9 stearoyl-ACP desaturase expressed in seeds. Recently, a gene encoding a microsomal Δ12 oleoyl-PC desaturase was simultaneously cloned by two research groups and characterized in sesame (Jin *et al*., 2001) and arabidopsis (present work, Paper IV). Elsewhere, a Δ15 linoleoyl-PC desaturase cDNA has been isolated (GenBank Accession E12718) although reports on their characterization are lacking. The expression pattern of the cloned genes is well-understood. Additional genes that would be useful in modifying the fatty acid composition in sesame oil are the KAS II and palmitoyl-ACP thioesterase. But to the best of my knowledge, no KAS, acyl-ACP thioesterases or elongases from sesame have been characterized to date.

Table 3 shows a summary of target enzymatic steps that could be modulated by genetic engineering to alter the seed fatty acid profile leading to accumulation of novel oils in sesame. Using the various modification strategies (last column in Table 3), it is possible to specifically vary the proportions of different fatty acids i.e. saturated or monounsaturated to polyunsaturated acids and consequently create new oils that would be suited for other uses besides the common use of sesame oil for frying and salad dressing. Considering that conventional sesame oil is beneficial to human health, it seems appropriate that further improvement of quality should focus on producing oils with new dietary, cosmetic, pharmaceutical and nutraceutical uses that would embrace the known advantageous properties of the oil. To be able to increase palmitic acid content using molecular tools, it may be necessary to clone KAS II and palmitoyl-ACP thioesterase genes from the species. Alternatively, one could utilize clones already isolated from other species like arabidopsis and sunflower.

An important requirement for genetic modification of oil composition is the availability of a strongly expressed seed-specific promoter. Besides, the promoter should display correct temporal expression of the introduced genes since the synthesis of various storage products is developmentally regulated. In sesame, fatty acid synthesis begins early (9 days after fertilisation) during seed development (Chung *et al*., 1995), and therefore, a late-expressing promoter would

be unsuitable. Promoters to the seed expressed Δ9 and Δ12-desaturase genes have been cloned and their expression pattern characterized (Yukawa *et al*., 1996; Jung *et al*., 2004). These promoters are strong and turned on at the onset of lipid synthesis, making them ideal candidates for future use in the engineering of sesame oil composition.

Table 3 Possible targets for genetic modification of sesame oil.

Target oil	Advantage/use	Approach
High-palmitate	Reduced need for hydrogenation; margarine; shortenings; soaps; lubricating grease	Down-regulate β-ketoacyl synthase II activity; Over-express palmitoyl-ACP Thioesterase
High-stearate	Reduced need for hydrogenation; margarine or cocoa butter substitute; cosmetics; pharmaceuticals	Down-regulate stearoyl-ACP desaturase
High-oleate	Increased oil stability; better frying oil; pharmaceuticals; soaps; cosmetics	Down-regulate oleoyl-ACP desaturase.
High-linoleate or linolenate	Improved nutritional value; salad oil; coatings; cosmetics; paints	Over-express Δ12 and Δ15 desaturases

Gene transfer methods

A suitable procedure for transferring genes of interest into a plant is needed to achieve the goal of modifying oil composition. Methods that have been successfully used to transform plants include particle bombardment, PEG-transfection of protoplasts and *Agrobacterium*-mediated transformation (Hansen & Wright, 1999). Each system has its advantages and limitations, necessitating continuous improvement of the systems and development of new ones. The efficiency of a given delivery system is dependent on the species to be transformed. *Agrobacterium*-mediated transformation is the most commonly used method for dicotyledonous species. Transformation protocols have been developed for most of the major oil crops including oilseed rape (Damgaard, Jensen & Rasmussen, 1997; Khan *et al*., 2003), soybean (Ko, Nelson & Korban, 2004), sunflower (Hewezi *et al*., 2002), cotton (Leelavathi *et al*., 2004) and peanut (Egnin, Mora & Prakash, 1998) among others, all involving the use of *A. tumefaciens*.

Agrobacterium-mediated transformation has a number of advantages over direct transformation methods. A low copy number of the transgene is transferred, potentially leading to fewer problems with transgene silencing and instability (Hansen, Shillito & Chilton, 1997). Using this method, large DNA fragments can be successfully transferred at a time (Hamilton, 1997). In addition, single cells are transformed and whole plants regenerated, minimizing the possibility of forming

mosaic plants that are more frequent when direct transformation methods are used (Enríquez-Obregón *et al*., 1998).

In addition, there are many different strains of *Agrobacterium* to select from that have varying ability to infect and transform plants. Their degree of virulence is determined by the chromosomal background and the tumour-inducing plasmids they contain (Hellens, Mullineaux & Klee, 2000). Strains with the C58 chromosomal background including GV2260 (octopine), GV3101 (nopaline), EHA 101 (nopaline) and EHA105 (succinamopine) among others are highly virulent, making them effective for the transformation of even the recalcitrant species. Therefore, one has a variety of strains from which to select the most suitable for transformation of a given plant species.

A major drawback to the application of *Agrobacterium*-mediated transformation is that it usually requires the regeneration of whole fertile plants from tissue culture. This has proved difficult to achieve for many important crop species, unfortunately, including sesame. Thus, the *Agrobacterium* system will not function with plants that cannot be regenerated from culture or tolerate wounding. In addition, tissue culture occasionally produces unwanted somaclonal variants, necessitating intensive screening of transgenic lines for phenotypes only resulting from expression of the transgene (Hansen & Wright, 1999). Tissue culture-dependent transformation is laborious, expensive and requires great expertise.

Species with difficulties to regenerate may be transformed using *in planta* methods, which avoid the tissue culture step and associated problems. *In planta* transformation involves the delivery of transgenes into intact plants either as naked DNA or by means of *Agrobacterium*. A number of approaches to in planta transformation have been used in a variety of plant species. These include inoculation of seeds with *Agrobacterium* (Feldmann & Marks, 1987) followed by selection of progeny, vacuum infiltration of seedlings or flowering plants (Trieu *et al*., 2000), floral dip (Clough & Bent, 1998) or spraying flowers with *Agrobacterium* (Chung, Chen & Pan, 2000) and sometimes by applying DNA to styles of recently pollinated flowers (Shou, Palmer & Wang, 2002). Of all these methods, floral infiltration has been the most successfully used in several species, and shows promise for broader application to diverse crops. The simplicity of the method makes it a very attractive alternative to tissue culture based methods. With the now increasing understanding of germ-line transformation, it seems possible to develop robust *in planta* transformation protocols for other plant species besides arabidopsis.

In vitro regeneration and transformation of sesame

A number of traits such as low grain yield, sensitivity to certain environmental stresses, and insufficient diversity in oil composition need genetic improvement. Some of the desirable traits e.g. disease and pest resistance are present in wild relatives but cannot be introgressed into the crop because of interspecific hybridization barriers. Establishment of an efficient method of gene transfer, which circumvents taxonomic barriers, would speed up the development of new varieties by facilitating the use of both conventional breeding and biotechnology.

A variety of techniques for cell, tissue and organ culture including embryo rescue, micropropagation and regeneration that would be useful in sesame improvement, have been investigated (Ashri, 1998). Although much effort is put into developing tissue culture systems for the crop, it has proved to be very problematic to regenerate. Regeneration through somatic embryogenesis has been achieved from seedling-derived callus (Mary & Jayabalan, 1997; Xu, Jia & Hu, 1997) and zygotic embryos (Ram *et al*., 1990) but with low efficiency in callus cultures. There are various reports on adventitious shoot regeneration through a callus phase from hypocotyl and cotyledon explants (Rao & Vaidyanath, 1997; Taşkin & Turgut, 1997; Younghee, 2001), with frequencies ranging between 4.5% and 25.8%. In some cases shoot formation was not achieved (Batra, Dhingra & Gogna, 1991; Ganesh *et al*., 1993). Therefore, efficient *in vitro* regeneration is yet to be established for the crop.

The availability of an efficient regeneration protocol is a key requirement for developing a transformation method for any given plant species. The prevailing problem of recalcitrance to regeneration presents a major obstacle to the application of genetic transformation on this crop. So far, the use of genetic engineering for the improvement of sesame has been limited by lack of a transformation system. Taşkin, Ercan & Turgut. (1999) have demonstrated that sesame is susceptible to transformation by *A. tumefaciens* but failed to regenerate transgenic plants from callus. Transformation by *A. rhizogenes* has been used for the production of novel compounds in sesame roots (Jin *et al*., 2005). So far there are no reports on regeneration of transgenic plants from transformed sesame roots. The pollen tube pathway of transformation which has been reported to be irreproducible (Shou, Palmer & Wang, 2002), was recently used to produce transgenic disease resistant sesame lines transformed with genes obtained from wild *Sesamum* species (Gao *et al*., 2004).

The present study

Background and justification

Most of the vegetable oil used in East African countries is imported. During the mid-1980s surplus quantities of inexpensive palm oil entered the East African market, leading to a drastic reduction in the production of oil crops including sesame. Vegetable oil imports, however cheap they may be, increase the foreign exchange debits to the perpetually poor economies of these countries. In Kenya, about 380,000 t of edible oil are consumed annually with only about 10% produced locally (FAOSTAT Data, 2005). To make up for the deficit in its domestic supply, the country spends over $ 145 million on vegetable oil importation every year. East African countries would benefit through reviving oil crop production to meet their needs. Sesame, reputed to be one of the most important oil crops in Kenya and Tanzania (Chimbi & Kafiriti, 1989), Sudan (Ahmed, 1989), Ethiopia (Belayneh, Weyessa & Urage, 1989), and Egypt (Ibrahim, 1989) is one candidate for improvement. To promote its production in Eastern Africa, a regional research sub-network with a focus on sesame has been established (Omran, 1989).

FAO Experts Consultations (Anon, 1985, Ashri, 1989) in the past have recommended oil quality improvement as one of the key breeding goals that would contribute to increased production of sesame since this would lead to better market competitiveness for the crop. Breeding for high grain yield has also been proposed. Most of the breeding work in Eastern Africa has focussed on increasing seed yield (Omran, 1989; Ayiecho & Nyabundi, 1997), while the case of oil quality remains unresolved.

The aim of this project was to develop conventional and biotechnological tools that would be used in sesame improvement programs towards diversifying the fatty acid composition of the seed oil. Such work would help expand the market niche for sesame oil, thereby contributing to increased cultivation of the crop and reduce the added expenditure on edible oil imports in East Africa. In addition, a better market competitiveness would translate to having a sure source of income for sesame farmers, thereby improving their livelihood. From a long-term perspective the present work, through development of regeneration and transformation methods that are currently lacking, creates a platform for the future improvement of other traits in sesame using biotechnology.

There is insufficient variability in the fatty acid composition of sesame oil. This project will identify cultivars with high oil content and a composition different from the rest that will later be developed further by genetic modification for the production of novel oils. Genetic transformation of sesame with certain genes involved in fatty acid synthesis will provide a means to effect changes in oil composition. Several of these genes have been cloned from sesame (reviewed herein), and many other useful ones from various plant species. For example, the acyl-ACP thioesterases (Hawkins & Kridl, 1998) cloned from mangosteen (*Garcinia mangostana*) and used to transform canola resulting in a dramatic

increase in stearic acid from the normal 2% in wild type plants to a maximum of 22% in transgenic lines. Once the required tools for such manipulation, which include the genes and promoters, and a suitable method for gene transfer are in place similar results will also be achievable in sesame.

Objectives

The long-term objective of the research is to change the fatty acid composition of seed oil in order to create new uses for sesame oil, and thus improve its market competitiveness. The immediate objectives of this research were to:

1. Assess variability in the oil content and fatty acid composition of East African sesame as a means of identifying useful cultivars for incorporation into breeding programs;

2. Develop an *in vitro* regeneration protocol for East African cultivars of sesame that could be used for transformation;

3. Develop methods for gene transfer to sesame using *A. tumefaciens*;

4. Clone and characterise the seed specific microsomal Δ12 oleoyl-PC desaturase gene(s) from sesame as a prerequisite to modifying the oleic/linoleic acid proportion in the seed oil.

Materials and Methods

Plant material

The sesame accessions used in this work were collected from Western and Coastal provinces of Kenya, and Uganda and Tanzania in the years 1999 and 2000. The collection is maintained at the Department of Botany, Moi University in Kenya. Materials for fatty acid analysis were field-grown while those used for floral transformation were raised under controlled temperature, relative humidity and lighting in a climate chamber at the Department of crop Science, SLU. *Arabidopsis thaliana* plants of the Columbia-O ecotype used in part of the study were also grown under controlled conditions.

Oil content and fatty acid analysis

Field evaluation of the sesame germplasm was initiated in the year 2002 in Western Kenya and repeated yearly up to 2004. Seeds harvested from the field experiments were analysed by gas liquid chromatography to determine the oil content and fatty acid composition, and results for the various accessions compared over the three-year period (Paper I).

In vitro regeneration

Sesame regeneration experiments are described in detail in paper II. Four separate experiments were carried out to optimize conditions for regeneration of sesame from callus. Initially, the working hormone concentration range was established for cotyledon explants using ½MS basal medium with 3% (w/v) sucrose. Thereafter, various combinations of modified Murashige & Skoog (1962) basal medium with N6 or ½MS macronutrients and plant growth hormones were tested for regeneration from cotyledon and hypocotyls explants. After identifying the better basal medium, the most promising hormone combinations were further tested to select the optimum. The optimized conditions were used for comparison of eight cultivars for their potential to regenerate shoots.

Effect of sucrose concentration on shoot regeneration

In one of the regeneration experiments conducted, the effect of sucrose concentration on callus induction and shoot formation from cotyledon explants was examined. The culture medium comprised MS basal medium containing N6 macronutrients with 20 µM of cytokinin (20 µM TDZ or 10 µM TDZ + 10 µM BA) and 2 µM IAA in combination with 1, 2, 3, 4 or 5 % (w/v) sucrose. Explants were prepared and cultured as described in paper II.

Anatomical studies on tissue cultured material

Cultures at different stages of development were sampled for anatomical study to ascertain the origin of the regenerating shoots. The cultures were fixed in 4% paraformaldehyde in phosphate buffer saline (pH 7.2) for 2 hours. The material was dehydrated in an ethanol-xylene series and thereafter embedded in histological wax. Sections of 7 µ thickness were made using a rotary microtome fitted with a razor blade. The sections were mounted onto slides dewaxed and rehydrated in a xylene-ethanol series, then stained with 0.1% Toluidine Blue. The slides were viewed under a light microscope and photographed.

Floral transformation

The potential use of floral transformation on sesame was investigated using the NPT II marker and EGFP reporter genes. The binary vector carrying the genes was transformed into three strains of *A. tumefaciens*. Transformation was achieved by floral dip (Paper III), pollen infiltration or one of two approaches to the *Agrobacterium* suspension drop method described in detail below.

In the floral dip transformation method the influence of flower bud age, surfactant concentration and bacterial strain were investigated. In each of the methods tested, seeds harvested from inoculated sesame plants were screened for transformants on selection medium containing a previously determined amount of kanamycin (75 mg/L). Transformation was confirmed by the polymerase chain reaction (PCR) and microscopic visualization of EGFP expression. Transformants were analysed up to the second generation to ascertain stable transmission of the introduced genes.

Alternative methods of floral transformation, to floral dip were also tested. Three cultivars of sesame namely, Mtwara-2, McBlack and McWhite were used in the floral transformation experiments involving three alternative approaches to inoculation of mature flowers. The effect of *Agrobacterium* strain on floral transformation was tested using the three strains, EHA 105, GV2260 and GV3101. Ten millilitre cultures of the bacteria were grown in LB (Sambrook, Fritsch & Maniatis, 1989) for one day, then the LB replaced with GYPC medium (Tjokrokusumo, Heinrich & Wylie, 2000) and the cultures grown for a second day. Bacterial culture medium contained antibiotics as in the floral dip study (Paper III). One millilitre batches of the culture were centrifuged at 14,000 revolutions per minute for 3 minutes to pellet the *Agrobacterium*. For pollen infiltration, the *Agrobacterium* pellet was resuspended in 1 ml of the pollen germination medium of Pfahler, Pereira & Barnet. (1997) containing about 50 mg of fresh pollen and vacuum infiltrated immediately for 20 minutes at -80 Pa. Pressure was released slowly and the suspension centrifuged as before. The pellet was then applied to stigmas of flowers that had been emasculated before. In the other two inoculation methods, the bacterial pellet was washed once in the floral dip medium (Paper III) instead of the pollen germination medium. The bacterial pellet was then applied to stigmas of emasculated flowers before pollination with freshly collected, non-infiltrated pollen. Alternatively, the pellet was applied to stigmas of flowers that had self-pollinated early in the day of inoculation (pollination before inoculation).

Cloning and characterisation of FAD2

PCR-based cloning was used to isolate a full-length cDNA encoding Δ12 oleate desaturase from developing sesame seeds (Paper IV). Reverse-transcriptase PCR was performed using gene-specific primers. A plant expression cassette bearing the cDNA was constructed and transformed into *A. tumefaciens* strain GV3101 for transformation of various *A. thaliana* plant lines. The seeds of transformants was analysed by gas liquid chromatography to evaluate the effect of the introduced gene from sesame on the fatty acid composition of transgenic *A. thaliana*, and in that way confirm its function in fatty acid synthesis.

Summary of results and discussion

Oil content and Fatty acid composition

Genetic variability for oil content and fatty acid composition in sesame is well documented (Yermanos *et al*., 1972; Ashri, 1998; Baydar, Turgut & Turgut, 1999). However, whereas the oil content varies widely (34-63%) among cultivars, there is less diversity for the fatty acid composition even at the genus level. It would be useful to have unique cultivars with either high oleic or linoleic acid, but this are very rare. In fact, mutants for high oleic acid have not been reported. Germplasm screening has occasionally unveiled such naturally occurring mutations in other oil crops (Burton *et al*., 2004; Rojas-Barros *et al*., 2004). Although many diverse cultivars of sesame are grown in East Africa, information on their oil content and

composition is limited. Stable oil-related phenotypes are valuable both in commercial production and variety development. Therefore, this study was conducted to identify accessions having inherently high oil content and unique fatty acid profiles that could be considered for incorporation into sesame breeding programs in the region (Paper I).

Initially a method for simultaneous extraction of oil from intact seeds and methylation of the fatty acids was standardised using two accessions representing black and white seeded types. Soaking seed samples overnight in methylating reagent followed by heating at 90^{o}C for 80 or 100 minutes gave comparable oil content in both accessions, with a difference of about 4% between the two time points. Methylation for 80 minutes was considered complete and therefore adopted for subsequent analyses.

The thirty accessions of sesame differed in oil content in all years registering mean values between 28.7% (Stewa in 2004) and 51% (Ug6 in 2003). Year to year variation was prevalent within accessions but a few including Morogoro, Majengo, Ug7 and Ug4 had relatively consistent oil content. The oil content was highest in 2003 for most accessions. These results have revealed that there is sufficient diversity for oil content in sesame from East Africa. Although superior accessions with oil content above 55% were not encountered, they would probably be found if a larger collection is screened. The accessions which exhibited low variability across the years together with those showing potential for high oil yield will be valuable in the development of improved cultivars for the trait.

The accessions had a similar fatty acid pattern but differed in the quantity of individual fatty acids. Linoleic acid was the main fatty acid with range values of 42.9-54%, followed by oleic (31.6-42%), palmitic (7.2-9.7%) and stearic (3.8-5.6%) acids. Minor components included linolenic (0.5%), arachidic (0.5%), eicosenoic (0.2) and traces of palmitoleic, behenic and erucic acids. These results are similar to those already documented (Kamal-Eldin *et al*., 1992; Ashri, 1998). Comparison of the accessions based on the variability in oleic and linoleic acids over the years showed that Ahero304, Indian, Busia, Bumala and Lungalunga had a stable composition. In contrast, Tan 3, Ug9 and Majengo varied a lot in the oleic and linoleic acid content. Several accessions had relatively high linoleic acid (> 50%). These accessions, which were relatively dissimilar from the rest, could be useful for breeding cultivars with improved oil quality upon further evaluation and selection. However, the present material lacks variation that would be useful for breeding high oleic sesame and therefore there remains need to introduce such variation.

Oil content correlated positively with stearic and oleic acids and negatively with palmitic and linoleic acids. Similar relationships have been reported in other oil crops (Holland, Frey & Hammond, 2001; Möllers & Schierholt, 2002). These findings indicate that it may be possible to select for high oil content concurrently with high stearic and oleic acids. The utility of these relationships in breeding should however be established through further investigation.

Correlations were also observed among the fatty acids. Palmitic and linoleic acids were negatively correlated with both stearic and oleic acids. Stearic and oleic acids were positively correlated to each other while oleic and linoleic acids had a negative correlation. Flagella *et al*. (2002) found similar relationships among the fatty acids in sunflower. These results reflect the link between the different fatty acids in their synthetic pathway (Ohlrogge & Browse, 1995).

In this study, several genotypes of sesame namely, Ug4, Majengo, Morogoro, Ug7, Ahero304, Indian, Busia, Bumala, Lungalunga, Tan 3 and Ug9, have been identified as having potential for use in breeding for altered fatty acid composition and high oil content in sesame. However, the accessions will need to be evaluated further for environmental effects on seed composition before they can be used in breeding programs.

In vitro regeneration

Sesame is difficult to regenerate through tissue culture. However, there is some progress in developing procedures for regeneration of adventitious shoots (Rao & Vaidyanath, 1997; Taşkin & Turgut, 1997; Younghee, 2001) but efficient systems are still lacking. The occurrence of strong genotypic differences in regeneration capacity limits the use of already established methods to only a few cultivars. *In vitro* regeneration of sesame cultivars from Kenya and East Africa as a whole is not documented. A reliable regeneration protocol is required for the development of tissue culture-dependent transformation of the crop. In this study some factors that influence adventitious shoot regeneration were investigated (Paper II). These included macronutrients, explant type, plant growth regulators, sucrose concentration and genotype. The study aimed at optimising conditions for shoot regeneration from selected sesame genotypes.

Results showed significant interaction between macronutrients, explant type and hormone combinations for shoot and root regeneration. In the presence of hormones, modified MS medium containing N6 macronutrients gave better shoot formation and reduced the tendency to regenerate roots compared to medium with ½MS macronutrients. Variation in regeneration response to different culture medium has been previously demonstrated in sesame (Rao & Vaidyanath, 1997). The main difference between N6 and ½MS is the nitrate to ammonium ratio (4:1 and 2:1 respectively). High nitrate to ammonium ratio could promote *in vitro* shoot formation (Welander, 1988; Ramage & Williams, 2002) and might account for the difference in regeneration response observed when N6 and ½MS were compared.

Cotyledon explants produced shoots from callus originating from epidermal and sub-epidermal cell layers at the basal part of the explants (Fig. 4). Hypocotyl explants did not regenerate any shoots but formed callus and roots. Shoot regeneration has been achieved at very low frequency from both cotyledons and hypocotyls before (Rao & Vaidyanath, 1997; Younghee, 2001). The reason for this disparity is unknown, but it could be as a result of differences in culture conditions as well as the genotypes used.

The growth hormones thidiazuron (TDZ) and indole-3-acetic acid (IAA) were superior to benzyladenine (BA) and naphthalene acetic acid (NAA), respectively for shoot regeneration. Only roots were formed on regeneration medium containing BA and NAA. Replacing NAA with IAA resulted in shoot regeneration on medium with BA and improved the response in treatment with TDZ. The best hormone combination was 20 µM TDZ and 2.5 µM IAA. TDZ is reportedly more effective than BA for regeneration in many herbaceous plants (Casanova *et al*., 2004; Winkelmann, Kaviani & Serek, 2005) but has not been used for sesame regeneration before. According to our results, TDZ is more suitable for regeneration of this crop compared to BA which is commonly used.

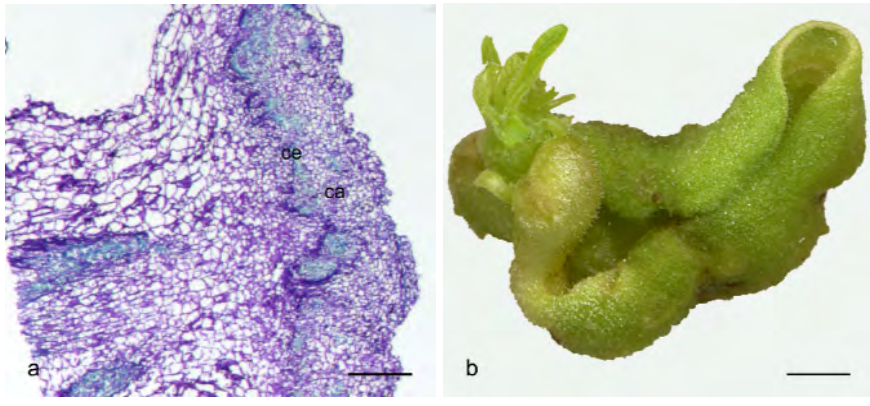

Fig 4 Regeneration of shoots cotyledon base. (a) Longitudinal section through cotyledon explant at three weeks of culture, showing callus (ca) formation from the cut edge (ce); bar = 100 µm. (b) Shoots at the proximal end after 6 weeks of culture on regeneration medium; bar = 3 mm.

Sucrose at 2-3% (w/v) gave the highest number of shoots produced per responding explant and reduced the incidence of vitrified shoots. Similar results have been obtained for micropropagation of sesame (Gangopadhyay, Poddar & Gupta, 1998). Sucrose is a carbon source but also plays a regulatory role on morphogenesis (George, 1993).

Ex-El was the best cultivar, producing 4.4 shoots per regenerated explant at a regeneration frequency of 63%. The lowest regeneration frequency (39%) and number of shoots produced per explant (1.8) were recorded for Siaya and McWhite, respectively. Cultivar differences have also been reported in past studies on sesame (Rao & Vaidyanath, 1997).

In this study we have established a regeneration protocol for sesame. We show that efficient shoot regeneration in sesame can be achieved using a combination of N6 macronutrients, cotyledon explants, TDZ, IAA and suitable genotypes. Since the regeneration proceeds through callus phase, this method will be useful in developing a tissue culture-based transformation system for the crop.

Genetic transformation

There is need to develop an efficient system for gene delivery into sesame. The initial idea was to develop a tissue culture-dependent transformation protocol for our material. However, our early results on regeneration did not look promising as the frequencies achieved were extremely low. Besides, tissue culture-dependent transformation procedures are usually time-consuming, expensive and require a lot of expertise. Because of these we endeavoured to use floral transformation which is relatively versatile to work with although it is not presently applicable to many species. A number of approaches to floral transformation have been successfully used in other crops. Among them are floral dip (Curtis & Nam, 2001), *Agrobacterium* suspension drop (Tjokrokusumo, Heinrich & Wylie, 2000) and pollen infiltration (Tjokrokusumo, Heinrich & Wylie, 2000; Li *et al*., 2004), which were tested for sesame transformation in the present work. Various factors that could influence transformation were investigated for the different methods.

A pre-determined kanamycin concentration of 75 mg/L was used to select transformants *in vitro*. Transformation was confirmed using the polymerase chain reaction (PCR) and visualization of green fluorescent protein (GFP) expression in kanamycin resistant plants (Fig. 5). For the floral dip method (paper III), transformants were obtained at a frequency of 3.4% when 0.05% (v/v) of the surfactant Silwet L-77 was added to the dipping medium. A lower concentration of 0.02% (v/v) was ineffective for transformation, yielding no transgenic plants. Similar results have been obtained for radish where 0.05% Silwet L-77 was optimal for transformation (Curtis & Nam, 2001). We also found that flower bud age significantly influenced transformation. The best stage for transformation by floral dipping was when the buds were about 3-4 weeks from anthesis.

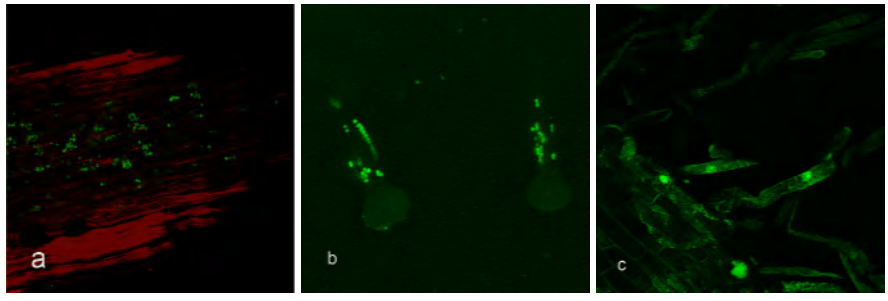

Fig 5 Green fluorescence protein expression in various organs of transformed sesame. (a) Stem (b) Leaf trichomes (c) Root hairs

Transformation frequencies as high as 13.9%, could be achieved in immature buds within that age window. Immature buds have also been shown to be the best target for floral dip transformation in various species, although the ideal timing varies among species. In addition, our results also revealed that buds dipped about two weeks from anthesis led to low seed set. It is possible that the detergent used could have affected reproductive development in these buds, leading to low seed recovery. Similar observations have been reported for *Arabidopsis lasiocarpa*

(Tague, 2001). Detergents are known to induce male sterility in some plants (Singh & Chauhan, 2003), which could have been the case in the present study. The two strains of *A. tumefaciens* used for floral dip here both gave rise to transformants. However, strain EHA 105 was more effective than GV2260. Out of the 74 transformants identified from the screened seeds, 51 were from plants dipped with EHA 105 and 23 from dipping with GV2260. The two strains differ in virulence, with EHA 105 being supervirulent and therefore better than GV2260 in infection and gene transfer.

In the comparison of the three alternative methods of inoculating mature flowers for transformation (Table 4), the best transformation rate (mean 49.8%) was obtained when *Agrobacterium* suspension was applied to the stigma before pollination. However, when the *Agrobacterium* suspension was applied to the stigma after self-pollination the transformation frequency was very low (mean 4.6%). Pollen, infiltrated with *Agrobacterium* and applied to the stigma gave a mean transformation frequency of 14.2%. These results contrast with the study by Tjokrokusumo, Heinrich & Wylie. (2000), who obtained similar transformation frequencies, when they compared inoculation before pollination and pollen infiltration transformation. Our findings might suggest that the procedure used for pollen infiltration in the present study would require some optimization to achieve better results. Most likely the concentration of the various components of the pollen germination medium needs to be tested to determine the optimal medium composition. The medium used here gives up to 37% germination (Pfahler, Pereira & Barnet, 1997). The low germination frequency could have contributed to reduced efficiency of the pollen infiltration procedure for transformation. *Agrobacterium* strains EHA 105 and GV3101 gave similar frequencies of transgenic plants (23% and 25.4%) while GV2260 had the lowest (6.7%), indicative of differences in strain virulence. Genotypic differences for transformation were evident among the three cultivars tested. The best cultivar was McBlack (27.3%) while McWhite (4.5%) was the least susceptible to transformation by inoculation of mature flowers. Genotypic differences for transformation using *Agrobacterium* are commonly encountered (Haliloglu & Baenziger, 2003; Dabauza & Pena, 2003), and are attributed to the inherent variation in resistance to infection by *Agrobacterium*. Highly resistant genotypes give rise to fewer transformants than susceptible ones.

This work has for the first time achieved delivery of foreign DNA into sesame by *A. tumefaciens* using four different approaches to floral transformation. Overall transformation frequencies ranging between 3.4% and 50% were obtained depending on the method used. Pollination after inoculation of mature flowers yielded the highest number of transformants, and could be used when a large number of transformants is required. This approach is however disadvantageous as it is labour-intensive and therefore unsuitable for ordinary transformation work, which can be more conveniently accomplished using the floral dip method.

Table 4 Transformation of sesame by pollen infiltration and *Agrobacterium* suspension drop methods

Treatment combination			No. of seeds selected on kanamycin	No. of kanamycin resistant seeds	% Kanamycin resistant seeds
Inoculation Method	Strain	Cultivar			
A	EHA 105	McBlack	149	93	62.4
A	GV3101	Mtwara-2	228	137	60.1
A	GV2260	McWhite	89	2	2.2
A Summary			466	232	49.8
B	EHA 105	McWhite	293	16	5.5
B	GV3101	McBlack	235	4	1.7
B	GV2260	Mtwara-2	790	41	5.2
B Summary			1318	61	4.6
C	EHA 105	Mtwara-2	67	8	11.9
C	GV3101	McWhite	108	4	3.7
C	GV2260	McBlack	37	18	48.6
C Summary			212	30	14.2

A: Inoculated before pollination; B: Inoculated after pollination; C: Pollen infiltration

Molecular cloning and characterisation of a seed-specific FAD2 from sesame

The microsomal Δ12 oleoyl-PC desaturase enzyme, which is responsible for the conversion of oleic acid ($18{:}1\Delta^{9\ cis}$) to α-linoleic acid ($18{:}2^{\Delta 9,\ 12\ cis}$) in oilseeds is encoded by the FAD2 locus (Okuley *et al*., 1994). Jin *et al*. (2001) cloned a cDNA from sesame and characterized it by sequence comparison to Δ12-oleate desaturases but did not present definitive proof of its function. In the recent past, some enzymes have been identified and shown to possess close structural and functional affinity to the FAD2 protein (Lee *et al*., 1998; Broun *et al*., 1998a; Cahoon *et al*., 1999). For this reason, it was deemed necessary to confirm the identity of the cDNA isolated from sesame (paper IV by complementation studies in the *A. thaliana fad2-2* mutant (Lemieux, Somerville & Browse, 1990) deficient in ER-desaturation of oleic acid. This work was also intended to provide proof that the gene was functional and could be used for genetic modification of the fatty acid composition of sesame in future.

Based on the present knowledge about the FAD2-like enzymes oleic acid modification at the Δ12 position should yield five kinds of fatty acids namely, hydroxylated, epoxidated, acetylenic, conjugated and trans fatty acids (Jaworski & Cahoon, 2003). These unusual fatty acids have potential use as an alternative

source of industrial raw materials for commercial production of polymers, coatings and lubricants currently made from petroleum, a non-renewable resource. Therefore, there has been a growing interest to produce novel oils containing high amounts of these fatty acids in commercially important crops. However, transgenic oil crops developed to date do not accumulate unusual fatty acids to economically viable levels for use in industry. Studies on the transgenic production of the unusual fatty acids by FAD2-like enzymes suggest that substrate availability might partly be the reason for the low quantities (Singh *et al*., 2001). We therefore tested this view by expressing the sesame FAD2 cDNA in transgenic *A. thaliana* lines producing hyxdroxy, epoxy and acetylenic fatty acids (paper IV).

Expression of the cDNA from sesame gene in seeds of the *fad2-2* mutant completely restored the accumulation of linoleic acid. Wildtype Columbia-O plants expressing the sesame gene produced up to 5% more linoleic acid compared to untransformed controls. The results confirm that the cDNA cloned from sesame encodes a functional FAD2 enzyme. The cDNA will therefore be useful in engineering oil quality in sesame.

Co-expression of the desaturase with a FAD2-like hydroxylase, epoxygenase or acetylenase yielded contrasting results. In lines expressing both the desaturase and hydroxylase, linoleic acid levels increased at the expense of hydroxylated fatty acids whose quantity diminished greatly. Oleic acid decreased three-fold in these plants in comparison to those expressing the hydroxylase alone. This result was expected since oleic acid is the substrate for Δ12-hydroxylation. Here we have confirmed that reduced substrate availability contributes to the low yield of hydroxy fatty acids in transgenic *A. thaliana*. Transgenic epoxy lines expressing the sesame desaturase produced a higher amount of unusual fatty acids and linoleic acid than observed in lines without the additional desaturase. This finding conforms to the report by Singh *et al*. (2001) who achieved higher levels of epoxy fatty acids co-expressing Δ12-epoxygenase and desaturase from *Crepis palaestina*. Our results demonstrate that a functional Δ12-desaturase regardless of origin could help raise the yield of epoxy fatty acids in transgenic plants. However, in spite of the increase the levels of epoxy fatty acids are still too low compared to those accumulated in wild the species, implying that other contributing factors need to be established. In the case of the acetylenase, co-expression with the desaturase resulted in an increase in linoleic acid accompanied by an unexpected decrease in crepenynic acid. This has not been reported before, and implies that for the acetylenase linoleic acid substrate availability is not a key factor limiting crepenynic acid production. Taken together, results obtained from co-expression studies show that substrate availability alone cannot explain the low yield of unusual fatty acids in transgenic plants. Thomæus, Carlsson & Stymne. (2001) found that transgenic plants expressing FAD2-like genes retained relatively high amounts of the modified fatty acids in polar lipids, an indication that there may be some deficiency in the mechanism of their channelling into TAGs leading to low quantities in seed oils. To increase the yield of these fatty acids, it might be necessary to co-transform the FAD2-like genes with an acyltransferase like PDAT having specificity for the transfer of acyl groups from the sn-2 position of PC to

TAG, as it has been suggested before (Stahl *et al*., 2004). It remains to be determined whether this approach will solve the problem.

This study has demonstrated that a seed-specific sesame cDNA with close similarity to Δ12 oleate desaturases encodes a functional enzyme. The study also confirmed that substrate availability only partly contributes to the low yield of unusual fatty acids in transgenic plants.

Conclusions and future considerations

To a large extent the present work has achieved its goal of putting together the tools necessary for improvement of oil quality in sesame. The work has shown that significant diversity exists for oil content and fatty acid composition in the sesame germplasm evaluated. Sesame accessions with potential use as parental lines in future breeding for improvement of these traits have been identified. An improved method for the regeneration of sesame from seedling cotyledons has been developed, which upon further optimization will be useful for establishing an *in vitro* transformation system for the crop. In the meantime, floral transformation procedures described herein for the first time in sesame can be used. These procedures yield transformants at high frequency and will be instrumental in the improvement of the crop through genetic engineering. For engineering the fatty acid composition, a seed specific FAD2 gene has been isolated from sesame and confirmed to be functional. In the future, this gene could be silenced to increase the oleic acid content or over-expressed to raise the level of linoleic acid in seeds sesame. With increased production of linoleic acid in the seeds, an additional unsaturation step by an introduced Δ6 or Δ15 desaturase transgene should lead to the accumulation of linolenic acids which are lacking in sesame oil. From the study on FAD2-like enzymes, it has become clear that while substrate availability might contribute to low yield of unusual fatty acids in transgenic plants, the problem is more complex than it appears.

The main focus for the future should be to proceed with utilizing the available resources for genetic improvement of sesame oil. Improvement through conventional methods should be pursued alongside genetic engineering although this might require identifying more sources of variation for significant alteration of the fatty acid composition. With regard to development of regeneration and transformation procedures, it may be necessary to improve the methods described in the present work. In the case of *in vitro* regeneration screening diverse cultivars to identify better responding ones should suffice to achieve high frequency regeneration. However, the regeneration protocol in its present form could be used to initiate transformation experiments and further optimization attempted. Floral transformation by pollen infiltration and *Agrobacterium* suspension drop methods need further investigation of the parameters tested in this work using more systematic analysis that would unveil interactions among the various factors. In this way it will be possible to spell out the optimal working conditions for these methods. Further, with the pollen infiltration method, it will be interesting to know

if adjustments to the composition of the pollen germination medium could improve the efficiency of the method.

There are many other traits of interest such as resistance to diseases and pests that need to be introduced into sesame. The use of gene technology will facilitate the development of sesame lines having such desirable characteristics.

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