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Application of marker-assisted selection for *ahFAD2A* and *ahFAD2B* genes governing the high-oleic acid trait in South African groundnut cultivars (*Arachis hypogaea* L.)

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One of the major shortcomings in cultivated groundnut in South Africa is the short shelf-life of the product due to rancidity of the oil rich seeds. Polyunsaturated fatty acids are more susceptible to oxidation than mono-unsaturated residues. Thus, it would be preferable to adjust the oleic acid: linoleic acid ratio to a more favourable one. The high-oleic acid trait in groundnut was reported to be dependent on two homeologous oleoyl-PC desaturase genes, *ahFAD2A* and *ahFAD2B*. Breeding of new cultivars with this characteristic can be time-consuming and expensive when doing fatty acid analysis in every generation for selection of the progeny with the highest oleic acid: linoleic acid ratio. Marker-assisted selection was applied to the local groundnut breeding program with the utilization of real-time polymerase chain reaction (PCR). The inheritance of the high oleic trait was followed in the 4th backcross progeny and revealed that all combinations of the two genes were found, except the ol_2ol_2 homologous mutant. The highest oleic acid percentage was found in progeny with all four mutant alleles ($ol_1ol_1ol_2ol_2$).

Key words: High-oleic, real-time PCR, oleoyl PC desaturase, marker-assisted selection.

INTRODUCTION

Two genomes, A and B, (*2n = 4x*, AABB) are involved in the genetic make-up of the cultivated allotetraploid groundnut (*Arachis hypogaea* L.). Groundnut is better known worldwide as peanut and to a lesser extent as earthnut, monkey nut and goobers. Groundnut is not indigenous to South Africa as it originated from between Southern Bolivia and Northern Argentina in South America where it was discovered in 1502. From there, traders spread the crop worldwide (Pattee and Stalker, 1995; Seijo et al., 2007; CGIAR, 2010, 2011). Groundnut is among the most widely produced legume crops worldwide (Weiss, 2000; CGIAR, 2010). Groundnuts are used as food (raw, roasted or boiled), animal feed (pods, seeds and plant material) and for industrial raw material. Groundnut products include flour, oil, peanut butter, confectionary and paste. Optimal kernel quality, such as that present in high-oleic acid kernels, is thus, essential for good nutritional value and shelf life of products. High-oleic governing genes dramatically increase the oleic/linoleic acid ratio (O/L) and therefore shelf life of groundnut (Hinds et al., 1992; Damude and Kinney, 2008; Braddock et al., 2006; Henry, 2009; CGIAR, 2010; Pretorius et al., 2010).

Groundnut represents an important source of protein (20 to 36%), oil (45 to 50%) – containing essential fatty acids, carbohydrates (±20%), fibre, folacin, phosphorus,

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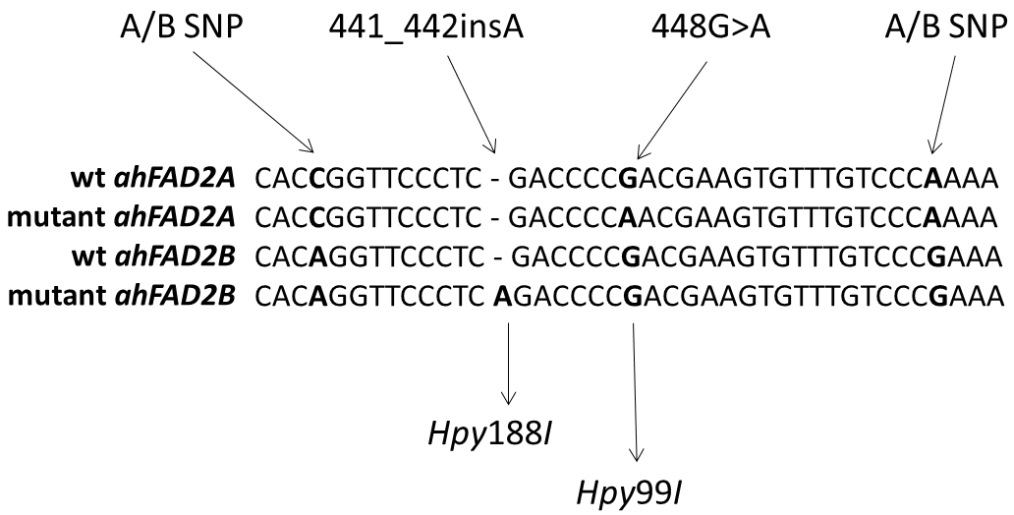

Figure 1. Genomic structure of the recessive mutants in two homeologous oleoyl-PC desaturase genes *FAD2A* and *FAD2B* caused by two SNPs (Chu et al., 2009).

magnesium, zinc, iron, potassium, calcium, vitamins riboflavin, thiamine, niacine and three forms of fat-soluble tocopherols (α, γ and δ).

Tocopherols are the most important lipid-soluble antioxidants discovered by Herbert Evans in 1922 (Shute, 1957; Packer et al., 2001; Mazhar and Basha, 2003; Munné-Bosch, 2005). Two fatty acids namely a mono-unsaturated oleic acid (C18:1) (MUFA) and a poly-unsaturated linoleic acid (C18:2) (PUFA) play major roles in the rancidity rate of groundnut products.

A natural and induced mutation (caused by two SNPs) 'silenced' two homeologous oleoyl-PC desaturase recessive genes *ahFAD2A* and *ahFAD2B*; so the high O/L ratio are controlled by 2 loci OL_1 and OL_2 and are responsible for the high-oleic acid trait (Figure 1). Patel et al. (2004) reported on an insertion of the same miniature inverted-repeat transposable element (MITE) in the *ahFAD2B* gene in another chemically induced mutant (Mycogen-Flavo) and the previously characterized M2-225 mutant.

Products made from kernels with a high percentage linoleic acid and a low percentage oleic acid shows quick oxidation resulting in rancidity, off-flavors and a shorter shelf life for these products. Inexpensive oils, containing high percentages of saturated fatty acids may improve the calcium and iron intakes but they increase saturated fatty acid intake, one of the major risks for developing cancers, obesity, cardiovascular and inflammatory diseases (Pala et al., 2001; Elmadfa and Kornsteiner, 2009; Galli and Calder, 2009; Gerber, 2009; Melanson et al., 2009; Sanders, 2009; Skeaff and Miller, 2009; Vassiliou et al., 2009; FAO, 2010; Vorster and Kruger, 2007). Molecular studies are of utmost importance as it enhances the pace at which high- and intermediate genotypes can be identified in the offspring in conventional breeding programmes (Chu et al., 2007, 2009; Singkham et al., 2010).

The aim of this study was to determine the pattern of heritability of the *FAD2* alleles in South African groundnut backcross populations of commercialized cultivars using molecular methodology.

MATERIALS AND METHODS

Plant material

A backcross (BC) program for transferring the high-oleic acid genes *ahFAD2A* and *ahFAD2B* from high-oleic parents (UF-85, Guat and Atete) to low-oleic commercially produced South African cultivars Akwa, Kwarts and Harts, as well as to a breeding line with resistance to the peanut pod nematode (caused by *Ditylencus africanus*) and a breeding line (ICGV90087 selection) with resistance to early leaf spot caused by *Cercospora arachidicola* Hori., late leaf spot caused by *Cercosporidium personatum* Berk. and Curt. (classified by Jenkins in 1938), web blotch caused by *Phoma arachidicola* (Chock.) Taber, Petit and Philly (classified by Woronichin in 1924) and rust caused by *Puccinia arachidis* Spegazinni (classified by Spegazzini in 1884) (Pretorius, 2006) was done (2008 to 2011).

Fatty acid analysis

For the fatty acid analysis of the genotypes involved in the BC, oil was extracted from 10 g ground kernels, dissolved in hexane and centrifuged to separate the meal from the oil. The oil was converted to fatty acid methyl esters (FAME) according to AOCS Ce- 2-66 and analysed using gas chromatography (Gertz et al., 2000; Anyasor et al., 2009). The oil was weighed (0.02 to 0.03 g in triplicate) and 1 ml of 0.5 M NaOH in methanol was added. The tubes were heated for 10 min at 85°C and then cooled to room temperature. 1 ml of 14% boron trifluoride was added, the tubes were vortexed and returned to the water bath for 10 min. After cooling to room temperature, 1 ml water and 1 ml hexane were added and the tubes vortexed. The

phases were separated and the organic (top) layer, containing the FAMEs was dried under nitrogen gas and analysed with a gas chromatograph. The gas chromatograph was equipped with a split/splitless capillary injector and flame ionisation detector (FID). The gas chromatograph utilised a DB-23 capillary column (90 m × 250 µm × 0.25 µm). Nitrogen was used as carrier gas. The detector and injector temperature was 300°C. The column programme was: 145°C for 5 min, then to 216°C at 5°C/min and held at this temperature for 5 min, then to 240°C at 3.3°C/min and held for 10 min. The profile of fatty acids of all the parents involved in the backcrosses was done using the polymeric triglyceride exclusion HPLC method and measured by a nuclear resonance analyser (Oilseed Board, 1996; ARC-Irene Analytical Services, 2003; PPECB, 2000-2007).

Homogenized samples (100 g) from germplasm entries and all potential high-oleic acid progenies were tested for oleic content by using an Atago Palette digital handheld refractometer (PR-301 alpha) to identify high-, intermediate and low-oleic genotypes. This unit allows the user to input the coefficient into the formula: (concentration = Brix × coefficient) to display the concentrations of the samples. Rancidity (RANCIMAT) tests (to compare oxidative heat stability of groundnut oil at frying temperatures) and oleic- and linoleic acid (O/L) ratios were done (Gertz et al., 2000). The selected genotypes were then planted in the greenhouse to multiply.

DNA analysis

The parental as well as the BC's genotypes were tested for the high-oleic molecular markers. Young leaves were collected from single plants, freeze-dried and DNA was extracted using a modified cetyltrimethylammoniumbromide (CTAB) method (Singsit et al., 1997). A multiplex Real-Time PCR assay developed by Barkley et al. (2009, 2011) was used to detect wild type and mutant alleles of the *FAD2A* mutation G448A (Barkley et al., 2011). The sequences of forward and reverse primers were 5'-GCC GCC ACC ACT CCA ACA-3' and 5'-GTT ATA CCA TGA TAC CTT TGA TTT TGG TTT TG-3', respectively. Two TaqMan probes targeted the wild type allele (VIC) and mutant allele (6FAM), with 5' reporter fluorophores, 3' minor groove binders (MGB) and 3' non-fluorescent quenchers (NFQ), namely 5'-VIC CCT CGA CCG C**G**A CG MGBNFQ-3' (Ol_1) and 5'-6FAM CCT CGA CCG C**A**A CG MGBNFQ-3' (ol_1), as synthesised by Applied Biosystems (London, UK). PCR reactions were carried out in 25 µl containing 0.4 ng/µl genomic DNA, 1 x SensiMix II Probe (Bioline, Celtic Molecular Diagnostics, South Africa), 0.16 µM of forward and reverse primers, 0.4 µM VIC probe and 0.3 µM 6FAM probe. The reaction conditions consisted of 1 cycle of 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and 65°C for 1 min, with a final cycle of 60°C for 30 s. All Real-Time PCR was carried out in a BioRad CFX96 cycler.

The *FAD2B* mutation was detected by the same forward primer as for *FAD2A* and reverse primer 5'-TGG TTT CGG GAC AAA CAC TTC-3' (Barkley et al., 2009). Two TaqMan probes were synthesised as aforementioned with the sequences 5'-VIC ACA GGT TCC CTC GAC MGBNFQ-3' and 5'-6FAM ACA GGT TCC CTC **A**GA C MGBNFQ-3'. The PCR reaction was carried out as aforementioned. Each PCR reaction included duplicate non-template controls as well as positive (UF85) and negative (Akwa) controls.

Statistical analysis

The Real-Time PCR data were subjected to an allele discriminant analysis using the CFX Manager™ software from Bio-Rad. Unknown samples were assigned to homozygote or heterozygote groups using the RFU of positive and negative control samples.

RESULTS AND DISCUSSION

Real-Time PCR was used for detection of *FAD2* alleles on both the A and B genome and to test the heredity in BC4 progenies according to Barkley et al. (2009, 2011). Mono- and heterozygotes for both alleles belonging to the two genes could be distinguished in the allele discriminant analysis (Figure 2). Parents of the backcross progenies as well as 500 individual BC4 plants were subjected to Real-Time PCR. UF85 was used as positive control and was homozygous for the double mutant $ol_1ol_1ol_2ol_2$, whereas, Akwa (negative control) was homozygous for the wildtype $OL_1OL_1OL_2OL_2$. Results show that both SA Juweel and ARC-Oleic2 were homozygous for the mutant alleles, $ol_1ol_1ol_2ol_2$. Both these high oleic acid cultivars displayed an increase in fluorescence with the 6FAM-A probe for the *FAD2A* mutant allele (Figure 2a), as well as with the 6FAMinsA for the *FAD2B* mutant allele (Figure 2b). The low oleic acid kultivar, Akwa, displayed strong fluorescence with both the VIC-probes for the wild type alleles of *FAD2A* and *FAD2B* (Figure 2e and f). Heterozygous progeny displayed high fluorescence for both alleles for these genes (Figure 2e and f). A distinctive grouping of samples could be identified with the allele discriminant analysis (Figure 2g and h) for both genes. Progeny containing allele 1 or allele 2 of the *FAD2A* gene could be clearly distinguished from heterozygous samples displaying equal fluorescence for both alleles. The same tendency was observed for progeny carrying the *FAD2B* mutant and/or wild type allele. The technique is easily applicable to large numbers of samples and very quick to perform.

The parents as well as the end products of the breeding program, the cultivars SA Juweel and ARC-Oleic2, were subjected to gas chromatography to determine the fatty acid composition and verify the results (Table 1). Using a constructed scale of low-oleic (0 to 49% oleic acid); intermediate-oleic (50 to 69%) and high-oleic (> 70%), the backcrosses were grouped into seven possible allele-groups (Figure 3) according to the amount of mutant alleles present as identified with Real-Time PCR.

It is interesting to observe that with only one mutant allele, ol_1 or ol_2 present (heterozygote), the percentage low to intermediate offspring was almost the same, but with two mutant alleles from *FAD2A*, ol_1ol_1, a much higher percentage low-oleic offspring than intermediate-oleic offspring occurred. Inheritance of both mutant alleles, $ol_1ol_1ol_2ol_2$, led to the highest number of offspring with high oleic acid. Only single plants were found with intermediate oleic acid, with no plants having low oleic acid content. Individual BC4 plants that were homozygous for the mutant alleles of both SNPs were selected for use in the breeding program and further advanced to develop the two cultivars, SA Juweel and ARC-Oleic2. It appeared that the MITE described by Patel et al. (2004) was not present in any of the parents involved in the backcrosses (results not shown).

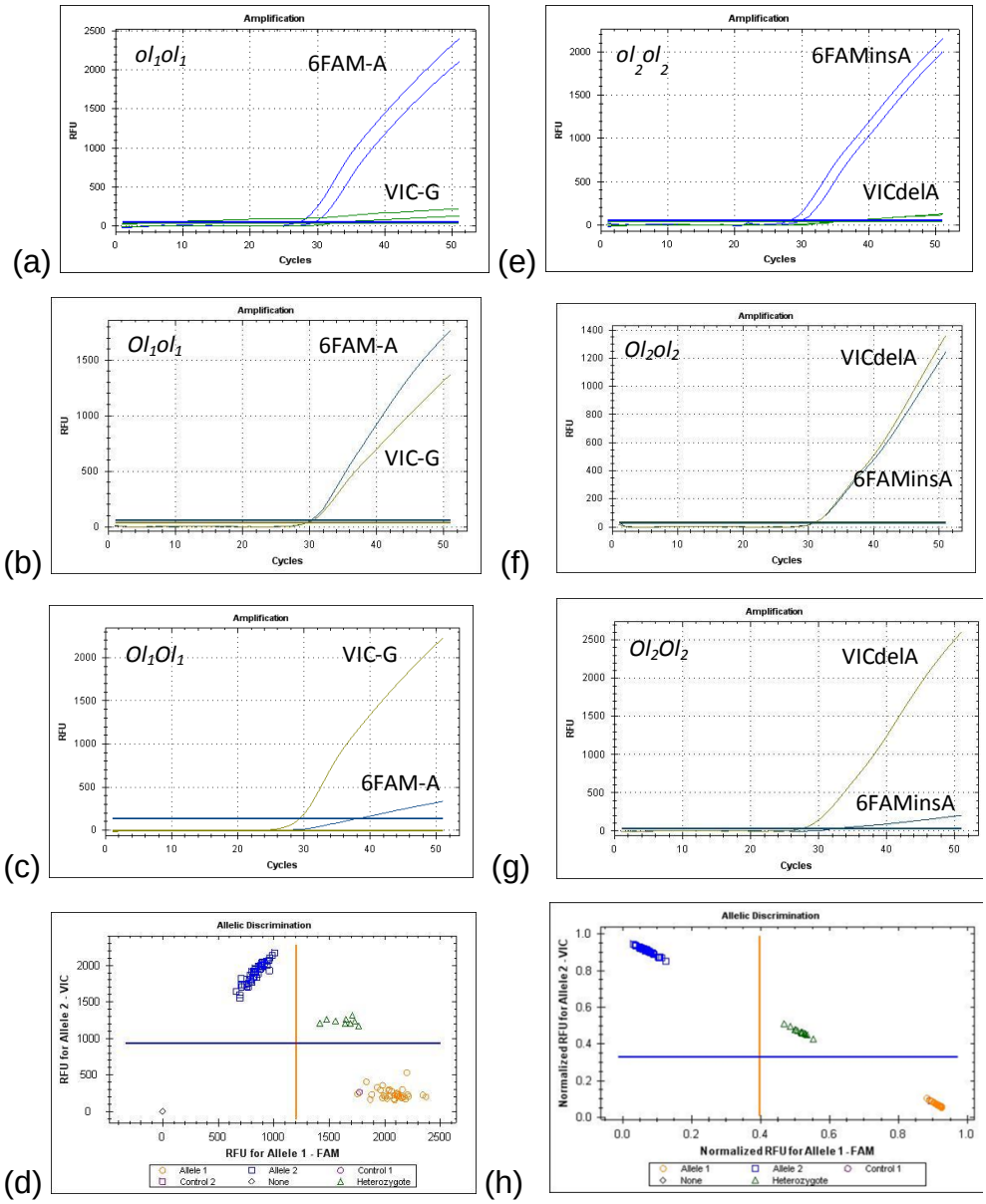

Figure 2 Real-Time PCR profiles of (a) and (e) SA Juweel and ARC-Oleic2; (b) and (f) heterozygote; (c) and (g) Akwa with TaqMan probes for *ahFAD2A* and *ahFAD2B* respectively. (d) Allele discrimination test *ahFAD2A* and (h) *ahFAD2B* of backcross individuals.

Predicting shelf life of a product is easily linkable, percentage wise to Rancimat values. Rancidity (Rancimat) tests are used to compare oxidative heat stability of groundnut oil at frying temperatures (Gertz et al., 2000: 546-551; PPECB, 2003-2007). If the Rancimat value of Akwa and Kwarts is 3.53 h and the shelf life of products hypothetically equals 100%, then products made from SA Juweel with a Rancimat value of 20.3 h will have a shelf life percentage of 575%. So SA Juweel has potentially a ±5 times longer shelf life than Akwa and Kwarts (PPECB 2000-2007). Where only one or two of the alleles were inherited by the offspring, they had low to intermediate values of oleic acid. Plants with two ol_2 alleles and one ol_1 allele had higher oleic acid values than plants with two ol_1 alleles and one ol_2 allele (Figure 3). There must be other factors such as the linoleic acid content; besides these two genes influencing the offspring's oleic percentage. The O/L ratio will in turn influence the rate that products will become rancid. Almost 100% of the offspring with the double mutant allele ($ol_1ol_1ol_2ol_2$) had a high-oleic acid content. Again, there must be other factors present influencing the expression of the $ol_1ol_1ol_2ol_2$ as some offspring showed intermediate-oleic percentages. Wherever heterozygotes of the allele

Table 1. Genotypes of parentage involved in the development of commercial cultivars.

Cultivar	*FAD2A*		*FAD2B*		% Oleic	O/L
Akwa	Ol_1Ol_1	Wildtype	Ol_2Ol_2	Wildtype	40.73	1.09
Harts	Ol_1Ol_1	Wildtype	Ol_2Ol_2	Wildtype	35.10	0.81
Kwarts	Ol_1Ol_1	Wildtype	Ol_2Ol_2	Wildtype	39.31	1.11
Namark	Ol_1Ol_1	Wildtype	Ol_2Ol_2	Wildtype	41.08	1.01
Natal Common	Ol_1Ol_1	Wildtype	Ol_2Ol_2	Wildtype	43.01	1.10
ARC-Opal	Ol_1Ol_1	Wildtype	Ol_2Ol_2	Wildtype	39.07	1.02
Sellie	Ol_1Ol_1	Wildtype	Ol_2Ol_2	Wildtype	40.18	1.01
Atete	Ol_1ol_1	heterozygote	Ol_2Ol_2	Wildtype	54.01	2.15
Guat	Ol_1ol_1	heterozygote	Ol_2Ol_2	Wildtype	53.65	2.09
Tufa	ol_1ol_1	mutant	Ol_2Ol_2	Wildtype	54.56	2.21
UF85-1241	ol_1ol_1	mutant	ol_2ol_2	mutant	77.5	16.94
SA Juweel	ol_1ol_1	mutant	ol_2ol_2	mutant	78.76	12.22
ARC-Oleic2	ol_1ol_1	mutant	ol_2ol_2	mutant	78.8	19.96

*O/L: Oleic/linoleic ratio. *Low-oleic acid content (0 to 49%); *Intermediate-oleic acid content (50 to 69%); *High-oleic acid content (>70%).

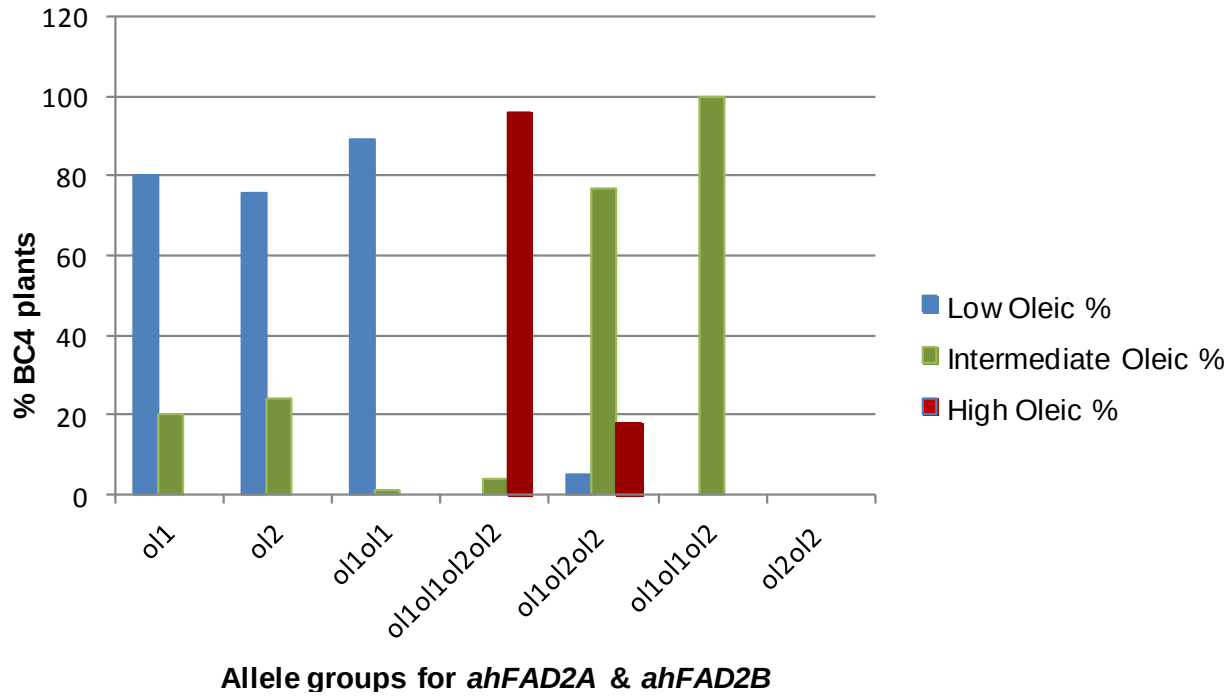

Figure 3. Frequency of allele groups transferred to backcross individuals as identified by Real-Time PCR. Bars indicate % of plants within the specific allele group.

were present, the offspring showed segre-gating oleic percentages; although, the $ol_1ol_1ol_2OL_2$ heterozygote offspring was 100% intermediate-oleic types. It was also interesting that not one of the BC4 offspring was homozygous for the ol_2, mutant alleles. This is in accordance with the study of Chen et al. (2010) who also noted that the genotype $OL_1OL_1ol_2ol_2$ has not actually been detected from natural populations and thus far has only been found in segregating progenies from controlled crosses.

Other crops planted in South Africa include pumpkin, dry bean, soybean, canola, olive, sunflower and maize. The fatty acid analysis for these crops in comparison with groundnut is summarized in Figure 4 (Norden et al., 1987;

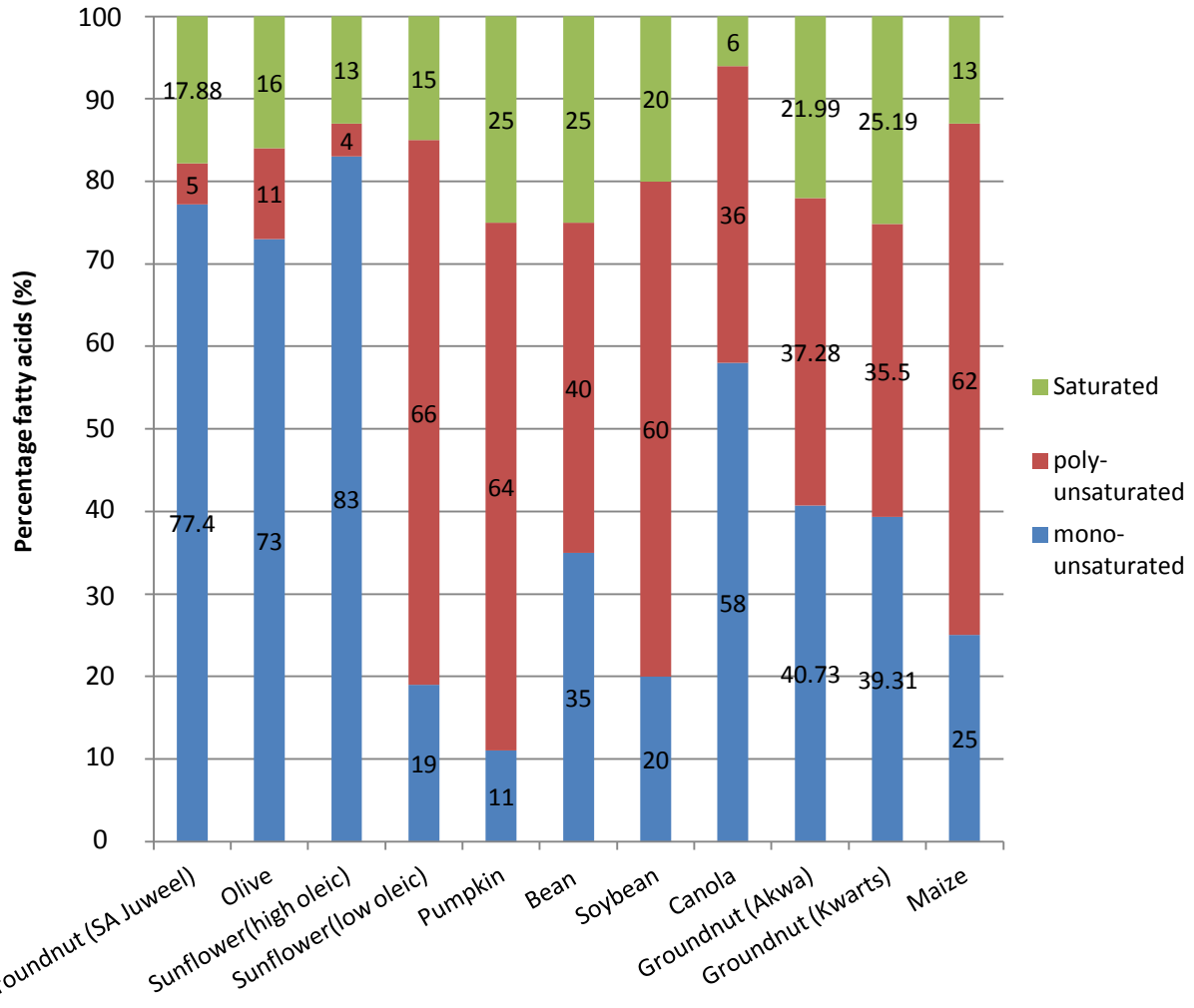

Figure 4. Comparison of fatty acid percentages in the oil (per 100 g sample) of different crops.

Gertz et al., 2000; Özcan and Seven, 2003; Sanders et al., 2003; Anon, 2005; Stevenson et al., 2007; Farooqui, 2010; Shin et al., 2010). It is clear that SA Juweel compares very well with olive and high oleic sunflower, which places it in a very competitive position considering health purposes and shelf life. Rancidity is most prevalent in oils with high levels of polyunsaturated fats (Moore and Knauft, 1989); thus, with the low percentage of linoleic acid present; this cultivar will provide long-term oxidative stability and be a valuable commodity for human consumption.

Conclusion

The Real-Time PCR assays as developed by Barkley et al. (2009, 2011) were successfully applied in the local groundnut breeding program. Molecular marker-assisted breeding programs make it possible to follow the inheritance of the oleic acid trait and provide a huge benefit for the selection of superior cultivars as less time and thus less finance is needed. Instead of low-cost energy-dense food containing a high percentage saturated fatty acids, rural and urban people can plant the easily cultivated groundnut (use a small space, so it can even be planted in house gardens), containing a high percentage mono- and poly-unsaturated fatty acids. A diet high in MUFA's and PUFA's can combat obesity, resulting in the occurrence of a smaller percentage of cardiovascular diseases.

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