RESEARCH ARTICLE

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Oleosin Expression Patterns and Size of Oil Bodies as a Factor in Determining Oil Content in Safflower (*Carthamus Tinctorius* L.) Genotypes

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ABSTRACT

The climate crisis and the Ukraine war have shown the vulnerability of various crop commodities. One of those badly affected is cooking oil, leading to a shortage in several countries. This coupled with the need for healthier cooking oil, increases proportionally with the world population and has resulted in escalated cooking oil prices. Thus, continued evaluation of alternative oil crops that can do well in marginal lands becomes a vital practice to undertake. Safflower is one of the marginalized oil crops with high-quality oil containing essential fatty acids beneficial to human health. Screening safflower genotypes for oil content is critical for its breeding and adoption in non-native areas. Therefore, this study delineates the relationship between oleosin genes and oil bodies in regulating the oil content of safflower seeds. Oleosin genes and oil bodies from the seeds of five safflower genotypes were isolated and quantified using qPCR and fluorescence microscope respectively, and evaluated against the seed oil content. The results showed an inverse relationship where smaller oil bodies were displayed by genotypes with high oil content. A high relative expression of oleosin genes was observed in genotypes with high oil content (Kenya-9819 and Gila). Of the eight Ctoleosin genes that were studied, it was observed that Ctoleosin genes (1, 4, 6, 7, and 8) were highly reliable in characterizing safflower genotypes based on the oil content. Kenya-9819 and Gila genotypes were found to have high oil potential and this was confirmed by a higher accumulation of the oleosin gene. A high correlation coefficient between oleosin, oil content, and oil body was also observed in this study. The findings suggest that selected oleosin genes and oil bodies are important traits to consider when characterizing oil seed crops for oil content.

Keywords: Oil, Oil Bodies, Oleosin, Oilseed Crop, Safflower.

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I. Introduction

Seeds store lipids in the form of small spherical intracellular organelles, called oil bodies, with sizes ranging between 0.5 and 2.0 µm in diameter Leprince *et al*. [1] with Song, Wang, and Rose [2] suggest that some oil body diameters can be 2–3 times larger. The oil body sizes vary with species depending upon the set of surrounding proteins mainly oleosin and the nature of lipids stored [3]. These stored lipids, mainly triacylglycerols (TAGs), provide energy for seed germination and seedling growth [4]. Oil bodies accumulate in maturing seeds, and in seeds with high oil and they fill much of the cytoplasmic space by the start of dormancy Schmidt and Herman [5] and they occupy a larger area in the physiologically mature oilseed which ensures oil body stability [6]. Oleosins which take their names from oil (oleo-) protein (-sin) [7] are the most abundant proteins associated with oil bodies [8] covering about 80 % of the oil body surface [9] and they contain highly conserved amino acid sequences [10]. The role of oleosin proteins is to stabilize the lipid bodies in developing seeds, and mature seeds, and act as the recognition signals for lipase biding in germinating seeds [11], [12]. The formation of oleosin contributes to the buildup of discrete oil bodies in plant tissues thus stabilizing the oil body surface Li and Fan [13] and preventing coalescence of the oil bodies [14]. Oil bodies can be found in other plant tissues such as fruit but only pollen and seeds produce oleosins where the oil bodies are subjected to developmentally regulated desiccation and hydration [5]. Therefore, the expression of oleosin genes is tissue-specific but appears to be universally abundant in seeds that store oil, this is not the case for oil-storing fruits [15], [10].

Previous studies have shown an inverse relationship between the size of oil bodies to the amount of oleosin in maize and most importantly that oil content was highly influenced by oleosin content [16]. Ho *et al*. [17] found that the size of oil bodies in mesocarp tissue of high oil yielding palms were significantly smaller than the oil bodies of low oil yielding palms thereby, confirming the inverse relationship between oil body size and oil yield. A similar study on rice oleosin also confirms that seed oil content is negatively correlated with oil body size and that oleosin participates in the formation of oil bodies and enlarges oil storage capacity [18]. All these studies suggest that the accumulation patterns of oleosins in oil seed crops are an important trait that may be used to screen genotypes for oil content.

Safflower, (*Carthamus tinctorius* L.) is an oil seed crop with high-quality vegetable oil rich in unsaturated fatty acids, mainly oleic acid and linoleic acid. It contains oil content in the range of 20.3 to 35.8 % depending on the genotype [19]. Seed oil content is a very important economic trait for safflower and is considered one of the most important factors affecting the success of safflower adoption in new areas [20], [21]. Safflower oil can be used as a vegetable oil for cooking, margarine production, salad oil, infant formulations, paints, and varnishes [22]–[24]. Oleosin bounded oil bodies have the potential to be incorporated in the food industry, in the preparation of cosmetic products and pharmaceuticals and this makes safflower a perfect candidate for such technology because it has quality oil that is rich in oleic and linoleic acid and low allergic reactions. Oil bodies can be used as an ingredient in dairy like food, beverages, salad dressings, sauces, edible films, coatings and hair, products [25], [6]. Therefore, studies on oil body size and their associated oleosins proteins are of great importance in the establishment of this promising technology. In this study, we isolated and measured the size of oil bodies from mature safflower seeds, extracted and quantified oleosin genes from developing safflower seeds and also extracted and determined the oil content of safflower genotypes. These were done to compare oleosin gene expressions in relation to oil body size and oil content among safflower genotypes. Since studies comparing the relationship between oleosin gene expression, oil body size and oil content of safflower genotypes are limited in the literature. The results of this study may guide in the selection of high oil-yielding safflower genotypes and in breeding for high seed oil content by regulating the levels of oleosin genes surrounding the surface oil bodies.

II. MATERIALS AND METHODS

A. Plant Material

Five safflower genotypes used in this study were, Sina, Gila, Turkey, PI53763, and Kenya- 9819. These genotypes were planted at the Botswana University of Agriculture and Natural Resources (BUAN) Content Farm during the 2020/2021 growing season. This site is located at the latitude of 24° 33’ South and longitude of 25° 54’ East in Sebele, Gaborone in the southern part of Botswana. The plants were grown (for two seasons winter 2021 and summer 2020) until they reached physiological maturity then their seeds were harvested and used for oil content determination and oil bodies isolation. For RNA isolation, the flowers containing immature seeds were harvested 20 days after flowering. The flowers for RNA were carefully cut out and immediately frozen in liquid nitrogen and stored at -80 °C until use. Before RNA extraction, the seeds were separated from the flowers and then ground in liquid nitrogen.

B. Oil Content Determination

Oil from the safflower seeds was quantified using the soxhlet n-hexane extraction method [26]. Firstly about 15 g of safflower seeds were grounded using a pestle and mortar. Then 5 g of crushed safflower seeds were inserted into a soxhlet extractor connected to a round bottom flask containing 150 mL of n-hexane (the solvent). The extraction was conducted as the solvent was heated up to boiling temperature around 70 °C for six hours. After extraction, the solvent was evaporated using a rotary evaporator. The final weight of the oil was determined by weighing and expressed as a percentage of oil content using Equation (1).

$$Oil\ content\ (\%) = \frac{weight\ of\ oil\ extracted}{weight\ of\ seed\ sample}\ x\ 100 \qquad (1)$$

C. Isolation of oil bodies

Isolation and purification of oil bodies were done following the method of Tzen *et al*. [27] with minor modifications. Physiological mature seeds (20 g) were used for oil body isolation whereby, the seeds were homogenized at 4 °C in 50 ml grinding medium (0.6 M sucrose and 10 mM sodium phosphate buffer pH 7.5) with a blender (12 000 rpm) for 90 s. After blending, the homogenate was filtered through two layers of mutton cloth. After filtration, each 200 µl portion of the homogenate was transferred to a 1.5 ml Eppendorf tube, and 200 µl of flotation medium (grinding medium containing 0.4 M sucrose) was layered on top. The tube was centrifuged at 10,000 X g for 20 min and the supernatant was collected and resuspended in 400 µl of detergent washing solution (0.2 M sucrose, 5 mM sodium phosphate buffer pH 7.5, and 0.1 % Tween 20). The resuspension was transferred to two 1.5 ml Eppendorf tubes (200 µl in each), and 200 µl of 10 mM sodium phosphate buffer (pH 7.5) was layered on top, and the tubes were centrifuged at 10,000 X g for 20 min. The supernatant was collected and resuspended in 400 µl of ionic elution buffer (grinding medium additionally containing 2 M NaCl). The resuspension was transferred to two 1.5 ml Eppendorf tubes (200 µl in each), 200 µl of floating medium (grinding medium containing 2 M NaCl and 0.25 M instead of 0.6 M sucrose) was layered on top, and the tubes were centrifuged at 10,000 X g for 20 min. Then, the supernatant was collected and placed in a new 1.5 ml Eppendorf tube then 200 µl of 10 mM sodium phosphate buffer pH 7.5 was layered on top and the tubes were centrifuged at 10,000 X g for 20 min. The supernatant was collected and resuspended in 200 µl of grinding medium mixed with 200 µl of n-hexane and the tube was centrifuged at 10,000 X g for 20 min. The upper hexane layer was removed, then the oil bodies were collected and resuspended in 200 µl of grinding medium. The resuspension was transferred to a new 1.5 ml Eppendorf tube while 200 µl of flotation medium was layered on top, and the tubes were centrifuged. The supernatant was collected and resuspended in a grinding medium and stored at 4 °C till use. This final medium was termed salt-washed oil bodies.

D. Imaging

Identification of oil bodies was done by staining the isolated oil bodies with a Nile red dye as a fluorophore (1 mg of Nile red/ml of acetone) in a ratio of 1:100 v/v Nile red to oil bodies and incubated for an hour at 20 °C. The stained oil bodies appeared as red circles when excited with green light under a fluorescence microscope (Carl Zeiss Scope. A1, Leica Microsystems CMS GmbH, Wetzlar, Germany). Samples were placed on a microscope, covered with a cover slip, then visualized under magnification of 40x, and images were photographed using Axiocam 305 digital camera. A total of six slides were prepared per genotype (3 biological reps plus 3 technical reps). Ten oil bodies were measured per micrograph using the microscope inbuilt software (Zeiss ZEN lite software). Then the average of the ten measured oil bodies was used as the final reading (oil body diameter).

E. RNA Extraction and cDNA Synthesis

Total RNA was isolated from maturing safflower seeds (20 days after flowering) as Lu *et al.* [4] observed that the expression pattern of Ctoleosin genes peaks during 15-25 days post onset of flowering, and decreases thereafter. After collection, the seeds were snap frozen into liquid nitrogen and stored at -80 °C until RNA isolation. Frozen seeds were ground in liquid nitrogen, and total RNA was extracted with the Quick-RNA Kit MiniPrep (Zymo Research Corp, United States of America, Tustin, California) as per the manufacturer's instruction. The quality and quantity of the extracted RNA were checked on both the 0.8% agarose gel and Nanodrop (Thermo Fisher Scientific, USA, Wilmington, DE). Samples with traces of contamination and low concentration of RNA were discarded, and a new extraction was undertaken. Samples with good quality and quantity were subjected to cDNA synthesis. The DNA-free RNA was converted into first-strand complementary DNA (cDNA) using a ReverTra-Ace-α synthesis kit (Toyobo Co., Ltd, Osaka, Japan) with an Oligo (dT) primer (Toyobo Co.LTD, Osaka, Japan). The cDNA synthesis success was checked by amplifying the cDNA using a set of plant reference markers Elongation factor (EF1 and EIF-5A) to check the presence of the amplified band. cDNAs that showed banding were used for quantification of the gene of interest whereas cDNAs that did not show any banding after amplification were redone.

F. Gene Of Interest Selection and Design of Primers

Oleosin genes from various plants' genome databases were used to blast search homologs against the safflower genome in the National Center for Biotechnology Information, U.S. National Library of Medicine (NCBI) website, and were cross-checked with Lu *et al.* [4]. From the identified gene homologs, the sequences were used to design the primers using the Primer3 online software tool. Suitably designed primers for qPCR were assembled and purchased from Inqaba Biotechnical industries (Pty) LTD, South Africa.

G. Quantification of Oleosin Gene Expression

The designed primers (Ctoleosin 1, 2, 3, 4, 5, 6, 7, and 8) were used to quantify the mRNA abundance of oleosin genes in safflower (Table I). The quantification was monitored by Fluorescent, Quantitative Detection System (qPCR instrument) (Bioer, China, Zhejiang) using Light Cycler 480 SYBR Green I Master Kit (Roche, Germany, Mannheim) to amplify 50 ng cDNA using the designed pair of primers. Two reference genes of EF1 and EIF-5A were used as internal standards and their normalized values were used to calculate the relative abundance of respective oleosin mRNAs using the $2^{-\Delta\Delta Ct}$ method. The profiling of mRNA quantification was run with three technical replications.

Relative gene expression (R) was calculated using Equation (2).

$$R = 2^{[\Delta Ct\ sample - \Delta Ct\ control]}$$
$$R = 2^{-\Delta\Delta Ct} \qquad (2)$$

H. Data Analysis

One-way analysis of variance (ANOVA) was performed using Sigmaplot program version 14.0. Treatment means were compared using Fisher's least significant difference (LSD) procedure at a significance level of 5 %. Pearson's correlation coefficient was used to test if there were correlations between oleosins, oil content, and oil bodies.

TABLE I: PRIMERS USED FOR GENE EXPRESSION

Primer name		Sequence
Ctoleosin 1	Ctoleosin 1- F	ATTGATCGCCGTCTTCATCC
	Ctoleosin 1- R	CCGTCACGTACGAGTAGATCCA
Ctoleosin 2	Ctoleosin 2 - F	ATTTTCAGCCCCGTGTTGG
	Ctoleosin 2 - R	CAGAAGAAAACAAACACGGCG
Ctoleosin 3	Ctoleosin 3 - F	TTCAGGAAGAGCCACCAGATCA
	Ctoleosin 3 - R	TGAGCCCTCCGTTTTGCAT
Ctoleosin 4	Ctoleosin 4 - F	ATGGACAACGGCCAACTCAA
	Ctoleosin 4 - R	CCAGTGGAAACGAAAAAGACGA
Ctoleosin 5	Ctoleosin 5 - F	TTCATCCTCTTCAGCCCCATC
	Ctoleosin 5 - R	GCAGTTGACCAGGAACGACAA
Ctoleosin 6	Ctoleosin 6 - F	CAGATACCGTGGACTACGCCA
	Ctoleosin 6 - R	CGTACATGCCCATATCGTGG
Ctoleosin 7	Ctoleosin 7 - F	ATCTTCGGCCCTTTGCTGTT
	Ctoleosin 7- R	AACCCATCCCAACGTAGCAAG
Ctoleosin 8	Ctoleosin 8- F	CCTCATCTTCTTTTCGCCCATC
	Ctoleosin 8 - R	ACCCGAAGACACACAGGAATCC
EF1	EF1 F	TCTGGTGTCACTGCTGAAGG
	EF1 R	TCCTCACCGAAAAGATCCAC
EIF-5A F	EIF-5A F	TGTCCCTCATGTCAACCGTA
	EIF-5A R	GCATCATCAGTTGGGAGCTT

III. RESULTS

Safflower genotypes under study had varying levels of seed oil body diameters (Table II). The oil body diameters ranged between 1.54 to 3.23 µm. Genotype Gila had a significantly smaller oil body diameter of 1.54 ± 0.20 µm while the other genotypes had significantly similar oil bodies with diameters higher than 2 µm. The microscopy imaging showed the presence of large oil bodies among safflower genotypes Kenya-9819, Turkey, Sina, and PI53763 (Fig. 1). The observed oil bodies were spherical in shape (Fig. 1). As for oil content, Gila had a significantly higher oil content of 38.02 ± 0.76 % followed by Kenya-9819 and PI53763 with an oil content of 28.85 ± 0.76 % and 27.85 ± 0.76 % respectively (Table II). The rest of the genotypes had a significantly lower oil content of less than 23 %.

TABLE II: OIL BODY DIAMETER AND OIL CONTENT OF SAFFLOWER SEEDS

Genotype	Oil body diameter (µm)	Oil content (%)
Gila	1.54±0.20b	38.02±0.76a
Kenya-9819	2.83±0.20a	28.85±0.76b
PI53763	3.23±0.20a	27.85±0.76b
Sina	3.20±0.20a	21.83±0.76c
Turkey	2.76±0.20a	20.94±0.76c
f-statistic	11.81***	82.24***

Values followed by dissimilar letters in the same column in treatment are significant at P≤0.05 according to Fischer LSD where n=60 for oil bodies and n=3 for oil content. *: P≤0.05; **: P≤0.01; ***: P≤0.001. ns=not significant. Values in the columns represent the means and SEM.

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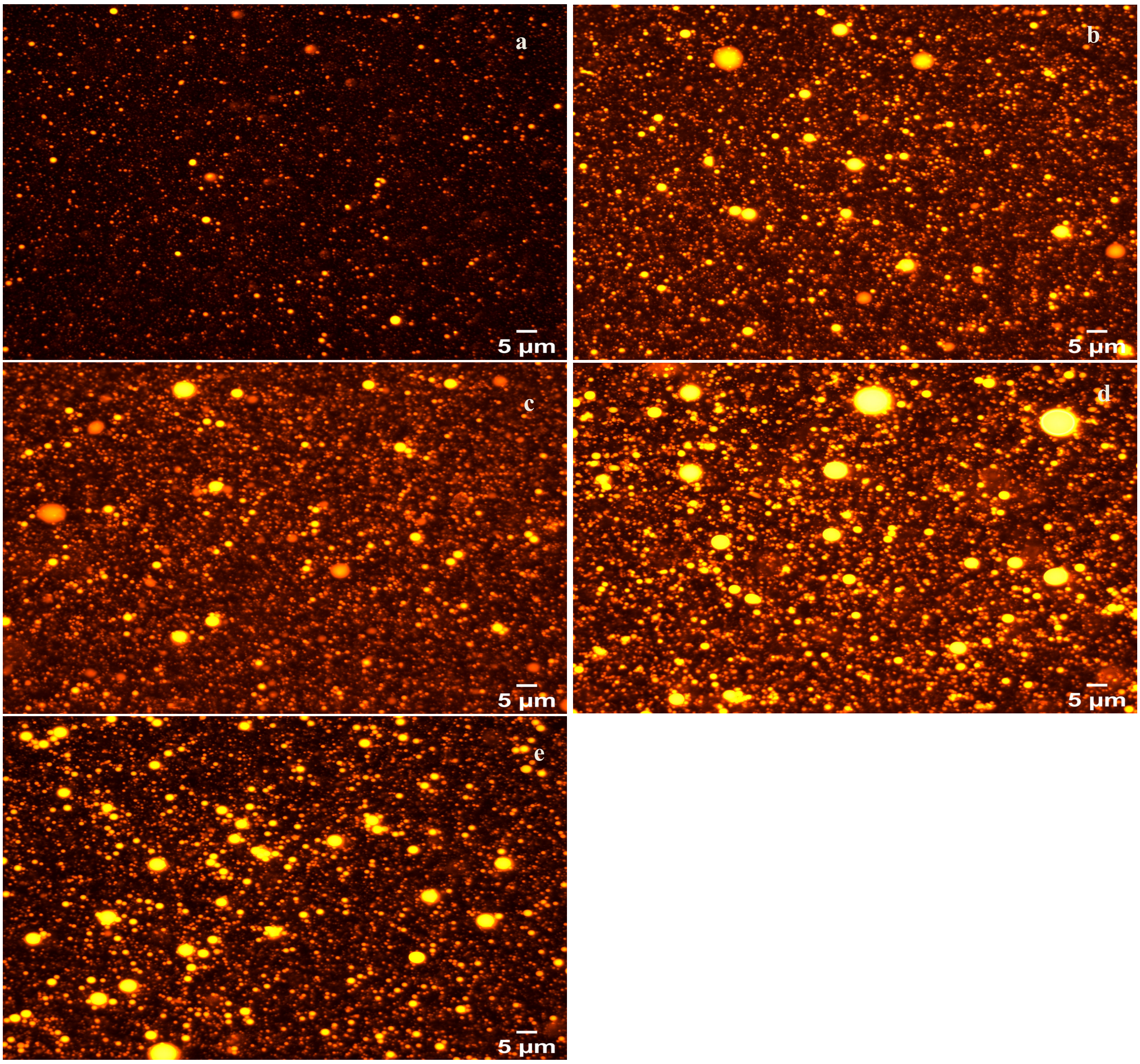

Fig. 1. Microscopic identification of seed oil bodies using fluorescence technique for safflower genotypes a; Gila, b; Kenya, c; PI, d; Sina and e; Turkey.

TABLE III: Pearson correlation coefficient of oil body diameter, oil content, and oleosin

	Oil content	Ctoleosin 1	Ctoleosin 4	Ctoleosin 6	Ctoleosin 7	Ctoleosin 8
Oil body size	-0.53**	-0.24 ns	-0.34 ns	-0.08 ns	0.2 ns	-0.54*
Oil content	1	0.55*	0.45*	0.25 ns	0.21 ns	0.74**

Values for correlation are significant according to Pearson's correlation P≤0.05 *: P≤0.05; **: P≤0.01; ***: P≤0.001. ns=not significant

A total of eight (8) selected genes were used for the relative expression and of the 8 only five (5) gave a satisfactory expression of genes and were used in this study. The relative expression results showed that the oleosin genes were variably expressed in the seeds of safflower genotypes (Fig. 2). Genotypes Kenya-9819 and Gila recorded higher oil content significantly and showed a higher level of gene expression than other genotypes under investigation. Kenya-9819 exhibited the greatest expression level of Ctoleosin genes 1, 6, and 7 while Gila showed the greatest expression level of Ctoleosin genes 1 and 8. All the genotypes showed a significantly similar expression level of Ctoleosin 4. The genotypes which also exhibited lower oil content (Sina and Turkey) showed the lower expression levels of oleosin genes (Fig. 2).

Oil bodies showed a significant negative correlation with oil content and Ctoleosin 8 (Table III). On the other hand, oil content showed a significant positive relationship with Ctoleosin 1, 4, and 8. The results show that 20 % of the studied Ctoleosin genes have a significant correlation with oil body size while 60 % of them have a significant correlation with oil content.

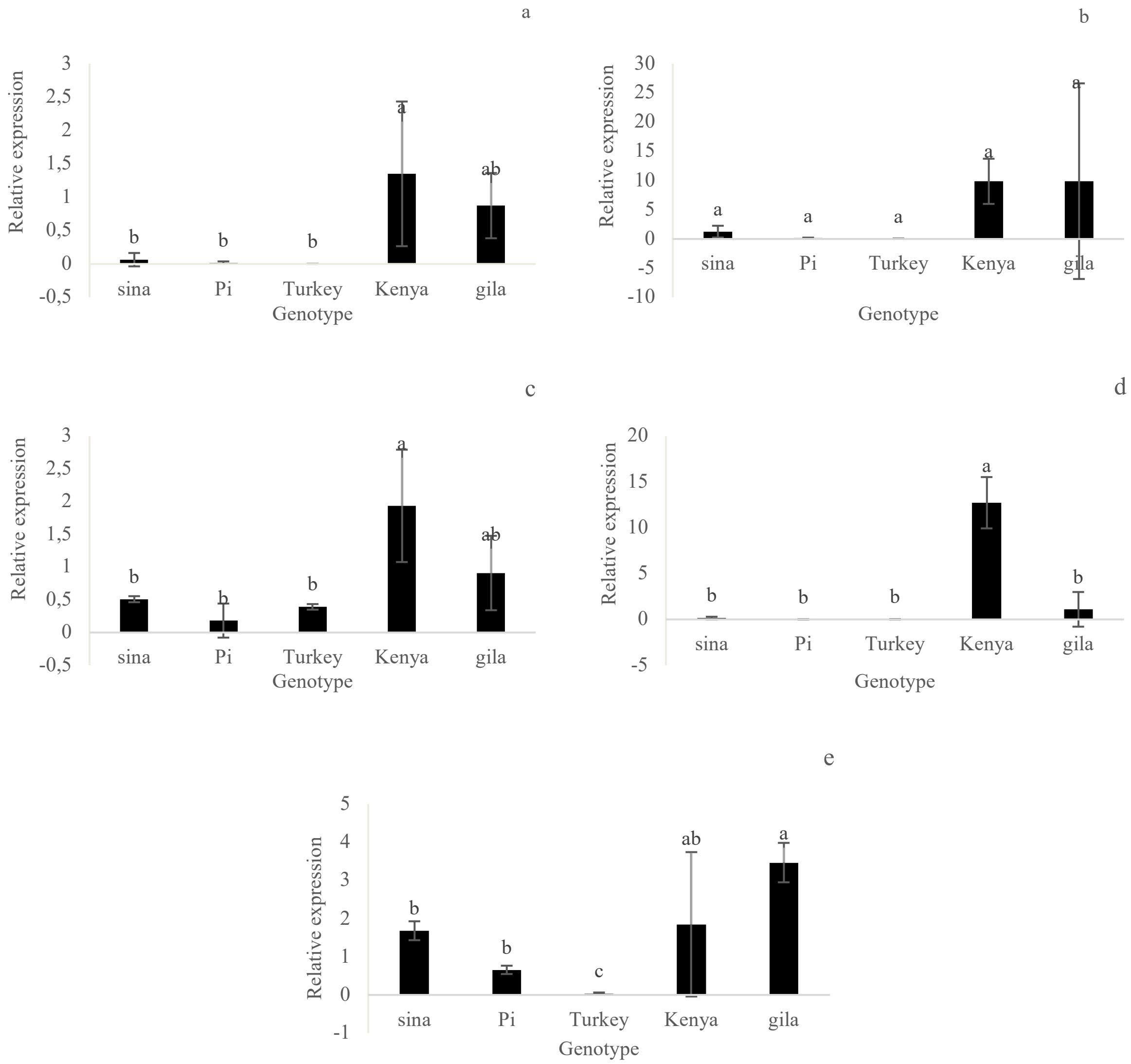

Fig. 2. The relative expression of Ctoleosin 1 (a), 4 (b), 6 (c), 7 (d), and 8 (e) genes in winter planted safflower at 20 days after flowering. Values with dissimilar letters in a treatment indicate significant differences according to Fisher's LSD.

IV. Discussion

The results of the current study on oil content indicated that a larger variation existed in oil content between safflower genotypes. Notably, the oil contents of these genotypes fell in the range of the ones reported in the literature [28], [29]. Oil bodies store seed oil and are vital in understanding oil content in crops [2]. Oil body size has been found to negatively correlate with oil content as smaller oil-bodied crop usually give a better yield of oil. In the current study, an average oil body diameter of safflower seeds ranged between 1.56 and 3.23 µm with an average of 2.7 µm which was higher than those reported in other studies [30], [31]. The variation in oil body diameter could be due to the presence of some overly large oil bodies that were identified in the current study. Further observations by Lan *et al*. [31] showed that oil bodies were nearly monodisperse in size unlike in the present study where a presence of some overly sized oil bodies was observed which led to polydispersity in oil body size. The genotype Gila, which recorded the highest oil content had the smallest oil body diameter which shows that oil body sizes are inversely correlated with oil content as also observed by Mawlong *et al*. [3].

The present results showed a significant variation in the relative expression of different oleosin genes in safflower seeds and this can be attributed to the differences observed in the quantity of oil content recorded from the genotypes studied. The genotypes that had higher oil content (Kenya-9819 and Gila) exhibited the highest oleosin gene content. This then suggests that the oleosin genes can be used to differentiate the safflower genotypes based on the expected oil content. This was further elucidated by a significant positive correlation between the oil content and oleosin gene expression (Table III). Even though safflower oleosin genes have shown to positively correlate with oil content, Ting *et al*. [22] found that seed oleosin genes in maize are expressed independently of the oil contents.

Results of this study have shown that oil body size had a significant inverse correlation with the Ctoleosin genes (Table III). These results are in line with the findings of Siloto *et al*. [14], who reported that a slight reduction in OLEO1 content resulted in larger and homogeneous oil bodies and further reductions led to the production of oil bodies with diverse sizes in Arabidopsis. Conversely, Ting *et al*. [22] found larger oil bodies in high oil-yielding maize and smaller and irregularly shaped oil bodies in low oil-yielding maize. These contradictions could mainly be brought about by the differences in the plant species thus suggesting that expression patterns of oleosins and accumulation of oil bodies are highly influenced by the specie studied.

According to Marin *et al*. [32], the relative amount of oleosins and oil content determine the size of the oil bodies, providing a high surface-to-volume ratio that facilitates lipase access. A strong correlation between oleosins, oil content, and oil bodies was also found in the current study (Table III). This suggests that the amount of oleosin genes present in seed can be used to distinguish between high oil-yielding genotypes and low oil-yielding genotypes.

V. Conclusion

The present work reported the use of oleosin genes in characterizing safflower genotypes based on the oil content, where Kenya-9819 and Gila genotypes were correctly characterized to have high oil potential and this was validated by the smaller oil bodies and higher oil content of the two genotypes. The inverse correlation of the oil bodies and oil content was also identified as an important trait to use when characterizing oil seeds for oil content. A significant correlation between oil content, oil bodies, and oleosin genes obtained from this study suggests that breeding for high oil content in safflower can be achieved by regulating the levels of oleosin genes that are embedding the surface of seed oil bodies and subsequently increasing the oil storage capacity of the seed. Therefore, breeding for higher seed oil yield by targeting the oleosin genes may serve as a novel way of meeting the increasing demands for seed oil.

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Conflict of Interest

We declare that there is no conflict of interest.

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