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EXPRESSION OF *CTFAD2* GENE FOR EARLY SELECTION IN SAFFLOWER OLEIC/LINOLEIC OIL CONTENT

I. Buyuk*, E. Bolukbasi and S. Aras

Department of Biology, Faculty of Science, Ankara University, Ankara-06100, Turkey
*Corresponding Author email: ilker.buyuk@ankara.edu.tr

ABSTRACT

Safflower oil constitutes an important source of oleic acid and linoleic acid which can be used for food and industrial applications. Because of their importance, nowadays, breeders are focused on selection of high-oleic or linoleic safflower breeding lines for particular end use purposes. For this aim, we investigated the expression levels of six *CtFAD2* genes at leaf and root tissues of four Turkish safflower varieties (LINAS, OLAS, Remzibey-05 and Dincer) by qRT-PCR to find out suitable genes for the early selection of safflower lines with desired oleic/linoleic content. qRT-PCR results showed that all the genes exhibited differential expression levels among different safflower varieties. The highest mRNA levels of all *CtFAD2* genes in the leaves were observed in LINAS safflower variety which is a high-linoleic type safflower variety having high yielding and high oil capacity. Among all six *CtFAD2* genes, mRNA levels of *CtFAD2-1*, *CtFAD2-5* and *CtFAD2-10* genes were interestingly quite similar among leaf tissues of all safflower varieties. Results of the current study suggest that, according to their expression levels in leaf tissues, *CtFAD2-10*, *CtFAD2-5* and *CtFAD2-1* genes are appeared as good candidates for early selection of high oleic/high linoleic safflower varieties and can be conveniently used in breeding studies.

Keywords: *CtFAD2*, safflower, qRT-PCR, oleic, linoleic.

INTRODUCTION

Safflower, also known as saffron or American saffron is an annual, drought resistant oil crop which has broad-leaves with barbs or without barbs, flowers with red, orange and white colors, and has an oil content of 30 to 50%. It is admitted to be originated from South Asia, firstly seeded in BC period and disseminated to the world from Egypt (Kolsarici and Eda 2002). Today 25 wild species of safflower is known to spread out worldwide and some of these species can be easily found in natural habitats of different regions of Turkey (Singh and Nimbkar 2006; Babaoglu and Guzel 2015).

At first it was used for medical purposes and due to its dye ingredient found in its flowers, it was also used in the food and textile industry for dyeing purposes (Singh and Nimbkar 2006). Today, especially it is taken advantage of its oil content in its seeds. Safflower, one of the medicinal and aromatic plants, has a very high ratio of total unsaturated fatty acids which also has an importance in terms of human health. While the ratio of total unsaturated fatty acids in the sunflower plant is about 86%, the safflower plant has the ratio of around 90-93% (Mundel 2008; Kartha 2010).

Safflower oil constitutes an important source of monounsaturated and polyunsaturated fatty acid for the human and oleic acid (C18:1Δ9) and linoleic acid (C18:2Δ9,12) are the most important fatty acids. Both oleic and linoleic acid can be used for food and industrial applications (Chen *et al*. 2015). Because of their importance, nowadays breeders are focused on selection of high-oleic or high-linoleic safflower breeding lines for particular end use purposes (Chen *et al*. 2015). For this aim, in the early selection process, developing a method used to identify the oleic/linoleic capacities of safflower varieties has a great importance for the breeders.

FAD genes encodes for desaturase enzymes and in most of the oil crops, these enzymes are known to be responsible for the conversion of oleic acid to linoleic acid. In varieties with high oleic content, these enzymes are blocked especially after the blooming and as a consequence very less linoleic acid is produced (Garces and Macha 1993). After the cloning of *FAD2* gene firstly discovered in *Arabidopsis thaliana*, the orthologous DNA sequences in soybean, cotton, oilseed rape, peanut (ground-nut) and flax have been characterized. Except in *Arabidopsis*, in other plant species, the *FAD2* gene is known to be encoded by small gene families (Cao *et al.* 2013).

In the current study, we hypothesized that the oleic/linoleic composition differences in the different safflower varieties could be detected by expression analysis of the members of *CtFAD2* gene family. Based on this hypothesis, we quantified the expression levels of six *CtFAD2* genes at leaf and root tissues of four Turkish safflower varieties (LINAS, OLAS, Remzibey-05 and Dincer) by qRT-PCR to find out suitable genes for the early selection of safflower lines with desired oleic/linoleic content.

MATERIALS AND METHODS

Plant material: Four different Turkish safflower (*Carthamus tinctorius* L.) varieties (LINAS, OLAS, Remzibey-05 and Dincer) which were obtained from 'The Directorate of Trakya Agricultural Research Institute' and 'The Transitional Zone Agricultural Research Station' were used for the study. High-oleic (C18:1) and high-linoleic (C18:2) safflower varieties were selected for the study. High-linoleic ones were LINAS and Dincer varieties and high-oleic ones were OLAS and Remzibey-05 varieties.

Safflower seeds belong to these four safflower varieties were germinated and grown hydroponically in pots containing 0.2 L of modified 1/10 Hoagland's solution. Four safflower varieties with three biological replicates, each consisting of one pot with five plants were grown in a controlled environmental growth chamber with light of 250 mmol m^{-2} s^{-1} photosynthetic photon flux at 25 ^{o}C, and with 70 % relative humidity.

RNA extraction: Total RNA was extracted from leaf and root tissues of each safflower varieties using the Trizol RNA extraction protocol followed by RNeasy mini kit (Qiagen, Cat no: 74104) to cleanup (Chomczynski and Mackey 1995). The quantity and quality of RNA was determined by NanoDrop Lite Spectrophotometer and also confirmed by gel electrophoresis which contains 1.5% agarose and formaldehyde. RNAs were stored at -80 °C.

First strand cDNA synthesis: A two-step procedure was used for real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Reverse transcription reactions were performed with 2 µg of RNA, 2.5 µM Anchored-oligo(dT)18, 1X Transcriptor High Fidelity Reverse Transcriptase Reaction Buffer, 20 U Protector Rnase Inhibitor, 1 mM deoxynucleotide Mix, 5mM DTT and 10 U Transcriptor High Fidelity Reverse Transcriptase using the High fidelity cDNA Synthesis Kit (Roche). The quantity and quality of cDNA was determined by NanoDrop Lite Spectrophotometer.

qRT-PCR analysis of *CtFAD2* genes: Real-time PCR was performed using Light Cycler ® Nano System (Roche). Gene-specific qRT-PCR primers (Table 1) for six *CtFAD2* genes and actin (ACT) were designed using Primer 3 software (Rozen and Skaletsky 2000) based on the sequence information of safflower genes available in the databank (http://www.ncbi.nlm.nih.gov/). Amplifications of the PCR product were monitored via SYBR Green I dye. The qRT-PCR analysis contained three biological replicates, consisting of three technical replicates.

Statistical methods: The abundance of target gene transcripts was normalized to ACT and set relative to Remzibey-05 variety according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Changes in relative expression levels (REL) of the gene were checked for statistical significance according to one way ANOVA. The results were considered statistically significant if the P-value was <0,05 in the Dunnett test.

RESULTS AND DISCUSSION

Information about fatty acid composition of safflower varieties: Fatty acid composition and oil content data of LINAS, OLAS, Remzibey-05 and Dincer safflower varieties were obtained from The Directorate of Trakya Agricultural Research Institute' and The Transitional Zone Agricultural Research Station and shown in Table 2.

LINAS and OLAS are newly registered safflower varieties which have high total oil content and oil yield according to the records obtained from 'The Directorate of Trakya Agricultural Research Institute' (Babaoglu and Guzel, 2015).

mRNA levels of six *CtFAD2* genes: The highest mRNA levels of all *CtFAD2* genes in the leaves were observed in LINAS safflower variety (Previous name: TRE-ASL09/14 safflower line) which is a high-linoleic type safflower variety having high yielding and high oil capacity which was registered in 2013 by Trakya Agricultural Research Institute, Turkey (Figure 1).

Among all six *CtFAD2* genes, mRNA levels of *CtFAD2-1*, *CtFAD2-5* and *CtFAD2-10* genes were interestingly quite similar among leaf tissues of Remzibey-05, OLAS, LINAS and Dincer safflower varieties. According to the mRNA levels of all these four genes, mRNA levels from high to low were observed in the leaves of LINAS, Dincer, Remzibey-05 and OLAS safflower varieties, respectively (Figure 2). The highest mRNA levels were observed in the leaves of LINAS variety while the lowest levels were observed in OLAS. OLAS safflower variety (Previous name: TRE-ASO12/08 safflower line) which is a high-oleic type safflower variety having high yielding and high oil capacity was registered in 2015 by Trakya Agricultural Research Institute, Turkey.

Comparative analysis of *CtFAD2-1*, *CtFAD2-5* and *CtFAD2-10* mRNA levels between the leaves of LINAS and OLAS safflower varieties revealed that mRNA levels in the leaves of LINAS variety were 15-fold higher for *CtFAD2-10*, 27-fold higher for *CtFAD2-1* and 82-fold higher for *CtFAD2-5* gene compared to the mRNA levels in OLAS variety. The highest mRNA level in the leaves of LINAS variety was observed for *CtFAD2-5* gene.

When the mRNA levels of six *CtFAD2* genes in the roots are evaluated, it is clearly seen that there are some critical differences compared to the mRNA levels obtained from the safflower leaves (Figure 3). Despite of

these differences, the highest mRNA levels of *CtFAD2-1*, *CtFAD2-5* and *CtFAD2-10* genes were again observed in the roots of LINAS safflower variety. Remzibey-05 and Dincer safflower varieties revealed similar mRNA levels in roots in terms of these three *CtFAD2* genes (Figure 4).

To compare mRNA levels of all *CtFAD2* genes between leaf and root tissues, we selected one of the safflower varieties used in the current study. Results of comparison showed that *CtFAD2-8*, *CtFAD2-5*, Ct-*FAD2*-1 and *CtFAD2-10* genes expressed at higher levels in the root tissues of safflower plants than the leaf tissues. Expression of *CtFAD2-7* gene seems higher in the root tissues while mRNA levels of *CtFAD2-11* were seen similar between both tissues of safflower plant (Figure 5).

We here report on the mRNA levels of six *CtFAD2* genes in four different Turkish safflower varieties. OLAS and LINAS are newly registered and named safflower varieties which have better performances than the Dincer and Remzibey-05 varieties according to the 'The Republic of Turkey Ministry of Food, Agriculture and Livestock' reports (Babaoglu and Guzel 2015). These two new safflower varieties (OLAS and LINAS) are thought that they can contribute to the lessening of the oil deficit in Turkey next years. Hence it is important to perform research studies with these new safflower varieties in detail. Seed oil of many Turkish safflower varieties has been analyzed by various researchers Camas *et al.* (2007) and Cosge *et al.* (2007) however, gene expression analysis of these *CtFAD2* genes has not been reported before.

In plants, the common feature of the membranes is the high-level fatty acid (FA) unsaturation that is executed by two types of fatty acid desaturases. Fatty acid desaturase-2 (*FAD2*) is located in the endoplasmic reticulum (ER) and fatty acid desaturase-6 (*FAD6*) is located in plastids (encode two ω-6 desaturases). These desaturases convert oleic acid (18:1) to linoleic acid (18:2) by adding a double bond at the ω-6 position. Fatty acid desaturase-3 (*FAD3*) is located in the ER and fatty acid desaturase-7 (*FAD7*) or fatty acid desaturase-8 (*FAD8*) are located in plastids encode three ω-3 desaturases which are responsible for converting of linoleic acid (18:2) to linolenic acid (18:3) (Zhang *et al.* 2012). *FAD*s have significant diversity in terms of their sequences and expression levels (Warude *et al.* 2006). Recent studies were focused on identifying the *FAD2* genes in many plant species and in the first *FAD2* studies only a single copy of a *FAD2* gene was identified in Arabidopsis and maize (Belo *et al.* 2008). But afterwards multiple copies of the *FAD2* gene were found in some plant species like soybean and safflower up until today. In the study of Cao *et al.* (2013), 11 tissue specific *CtFAD2* genes were identified in a wild type safflower (linoleic) and they analyzed their differential expression profile. In the current study, to establish correlations between six *CtFAD2* gene expression and oleic/linoleic composition, we examined mRNA levels of *CtFAD2* genes using safflower varieties differing in linoleic and oleic content.

Table 1. Sequences, melting temperatures of primers and accession numbers of *CtFAD2* genes used in qRT-PCR

Gene		Sequence (5'-3')	Tm (°C)	Accession no.
***CtFAD2*-1**	Forward	GTGTATGTCTGCCTCCGAGA	60 °C	KC257447.1
	Reverse	GCAAGGTAGTAGAGGACGAAG		
***CtFAD2*-5**	Forward	CAATACGGTAGAGGCCACACAG	60 °C	KC257451
	Reverse	ATCATCTCTTCGGTAGGTTATG		
***CtFAD2*-7**	Forward	CGAATCACACCCACGGGATC	60 °C	KC257453.1
	Reverse	CTAAAGAATTTCCATGGTGTTAC		
***CtFAD2*-8**	Forward	GAGCAACGGAGAGAAGTAACC	60 °C	KC257454
	Reverse	GAGGGATGATAGAAAGAGGTCC		
***CtFAD2*-10**	Forward	CCAACAAACAAACCATCTCTCG	60 °C	KC257456.1
	Reverse	GAGAGACGGTGGAAGTAGGTG		
***CtFAD2*-11**	Forward	CCATTGATCCACCCTTCACCTTA	60 °C	KC257457.1
	Reverse	AAAGACATAGGCAACAACGAGATC		
ACTIN	Forward	TGAGCAAGGAAATCACGGCT	60 °C	AF282624
	Reverse	TCCTCCGATCCAGACACTGT		

The data obtained by the qRT-PCR experiments showed that all the genes which are members of *FAD2* family exhibited differential expression levels among different safflower varieties used in the current work. Especially, mRNA levels from safflower leaves seem more suitable then the ones observed in the roots for distinguishing different safflower varieties in terms of their oleic/linoleic levels. Comparative analysis of *CtFAD2* genes between leaf and root tissues of safflower varieties revealed that the expression of *CtFAD2-10*, *CtFAD2-8*, *CtFAD2-5* and *CtFAD2-1* genes are higher in the roots than the leaves of four Turkish safflower varieties. The data obtained from the study by Cao *et al.* (2013) is consistent with the results of the current study

(Cao *et al*. 2013). They found that the expression of *CtFAD2-5*, *CtFAD2-8* and *CtFAD2-10* genes are higher in root tissues with relatively low levels detected in leaf tissues as it was detected in the current study.

LINAS is a newly registered variety and have better performances than the other Turkish safflower varieties in terms of many parameters such as high seed yield, high oil content, yield stability and also wide adaptation ability in all over the Turkey. As it was expected, all *CtFAD2* genes revealed higher mRNA levels in LINAS variety compared to the OLAS, Remzibey-05 and Dincer varieties (Babaoglu and Guzel, 2015).

It is known that *FAD2* genes are responsible for converting oleic acid to linoleic acid in oil crop plants (Chen *et al*. 2015). Therefore in the current study we aimed to investigate whether these genes can be used for early selection of high oleic or high linoleic safflower varieties. Results of the current study suggest that, according to their expression levels in leaf tissues, *CtFAD2-10*, *CtFAD2-5* and *CtFAD2-1* genes are appeared as good candidates for early selection of high oleic/high linoleic safflower varieties and can be conveniently used in breeding studies.

Table 2. Fatty acid composition and oil contents of safflower varieties used in the current study

Safflower varieties	Oleic/Linoleic acid %	Total oil %	Registration date
LINAS	71.3 Linoleic	39.9	2013
OLAS	70.0 Oleic	39.2	2015
Remzibey-05	37.3 Oleic	29.9-32.1	2005
Dincer	15.6 Oleic	29.1-31.9	1983

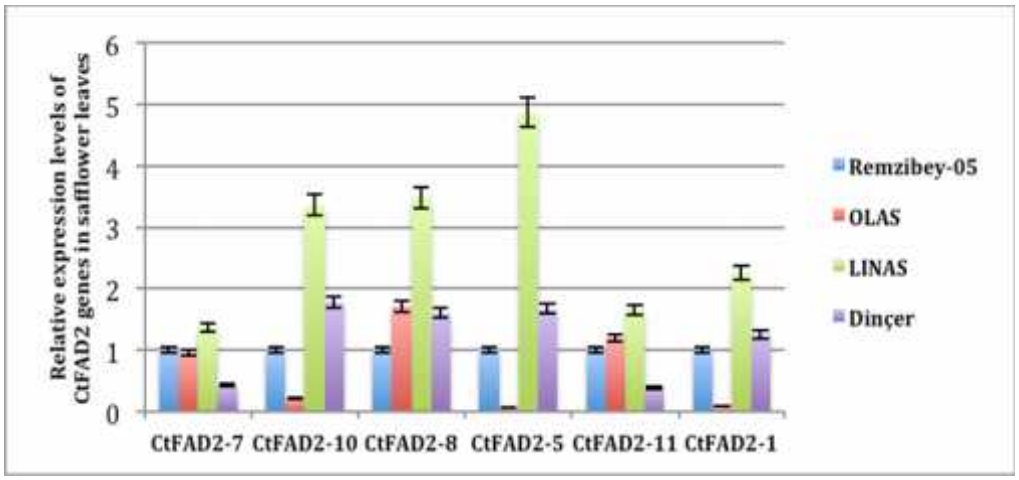

Figure 1. Relative expression levels of six *CtFAD2* genes in the leaves of four safflower varieties (n=3)

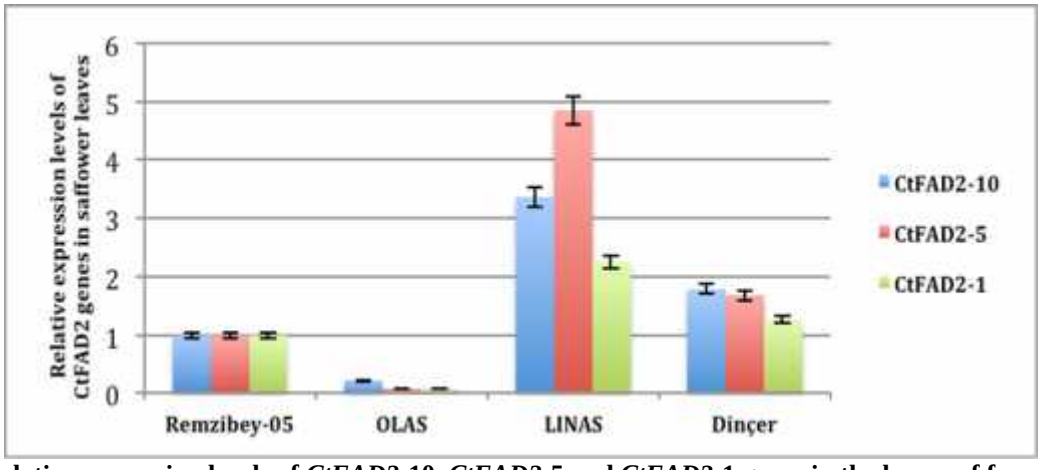

Figure 2. Relative expression levels of *CtFAD2*-10, *CtFAD2*-5 and *CtFAD2*-1 genes in the leaves of four safflower varieties (n=3)

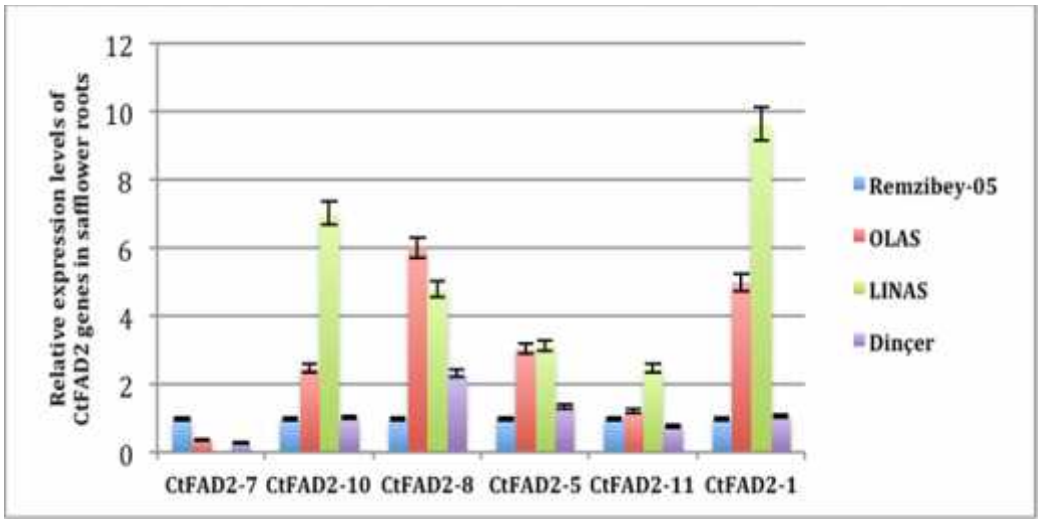

Figure 3. Relative expression levels of six *CtFAD2* genes in the roots of four safflower varieties (n=3)

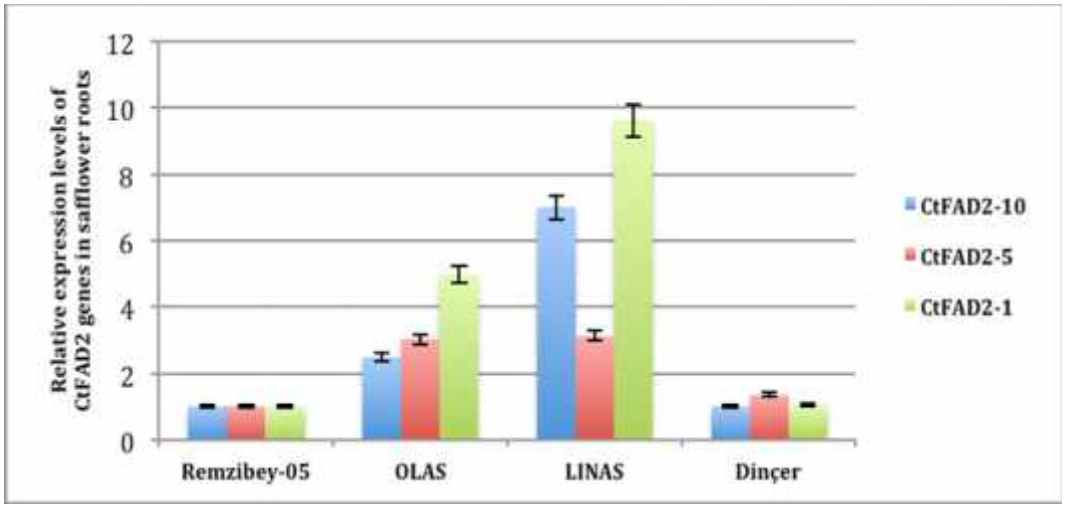

Figure 4. Relative expression levels of *CtFAD2*-10, *CtFAD2*-5 and *CtFAD2*-1 genes in the roots of four safflower varieties (n=3)

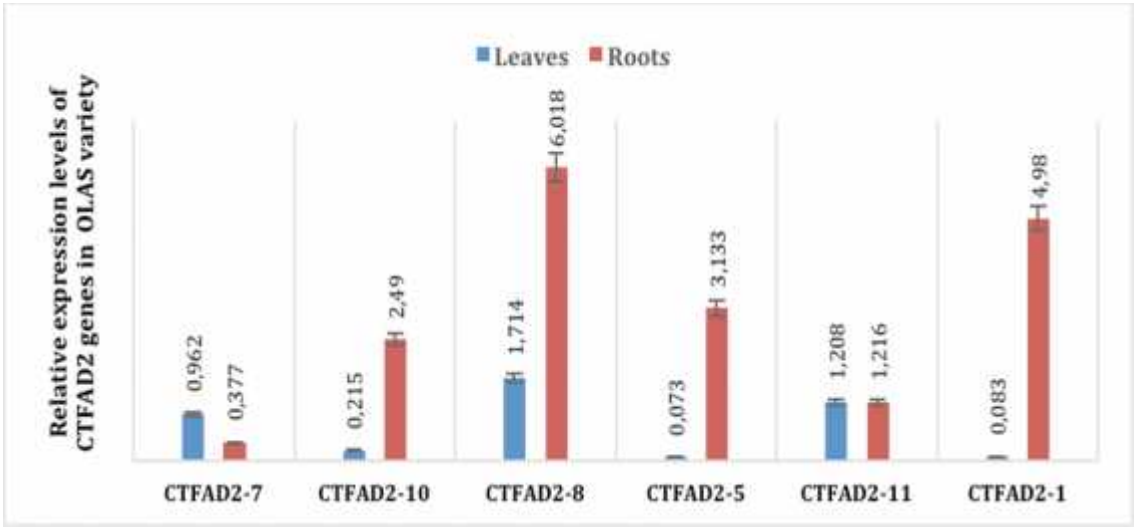

Figure 5. Relative expression levels of six *CtFAD2* genes between leaf and root tissues of OLAS safflower variety

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