

Changes in 2S albumin gene expression in *Moringa oleifera* under drought stress and expected allergenic reactivity in silico analysis

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Abstract An experiment was conducted to explore the effect of water deficit on 2S albumin gene expression in *Moringa oleifera* with a predication of its allergenic reactivity. Drought was applied to 20-day-old plants for 1 month by limiting the irrigation water to half the control level. Soil water content and plant relative water content of drought treatment were about 50 and 70% of control values, respectively at the end of the stress period. Additionally, in relation to the control plants, drought reduced plant height (− 34%), leaf number (− 16%), total plant biomass (− 60%), shoot dry mass (− 37%) and root dry mass (− 21%). Conversely, root/shoot ratio was enhanced due to this stress. Quantitative polymerase chain reaction (qPCR) results revealed that drought enormously reduced 2S albumin gene expression in leaves and roots. Under control conditions, the relative

albumin gene expression in roots was about half that of the leaves. Paratope and epitope prediction showed that albumin 31 which is a member of 2S albumin family, has eight cysteine residues and 25% of solvent-exposed regions. This makes albumin 31 a proposed target for food allergy investigations. Our interesting finding was that drought decreased 2S albumin expression in this species. This possibly will lower the allergenic reactivity and could enhance the feed value of *M. oleifera* for human health.

Keywords Albumin · Antigen · Drought · Epitope · Gene expression · *Moringaceae* · Paratope

1 Introduction

Moringa oleifera Lam. (Moringaceae) is a rapid growing and widely distributed tree in tropical and subtropical areas. It is known as horse radish or drumstick tree and has multiple usages with nutritional, medicinal and economic values (Anwar et al. 2006). Nearly every part of *Moringa* can be used for food, medication and industrial purposes (Busani et al. 2011). Recently, *M. oleifera* has been cultivated in some countries, and the aqueous extract of its leaves has been commercially explored as a dietary supplement (Hagiwara et al. 2016). Crude protein content of its dry leaves is about 30% and its value appeared to

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depend on environmental conditions (Busani et al. 2011; Amira et al. 2015).

Drought is considered one of the most constraints in agriculture. This disturbance has many detrimental impacts on agricultural plants such as inhibiting growth and photosynthesis, changing the ionic balance and plant morphology, and decreasing photosynthetic pigments (Chen et al. 2011). Proteins are fundamental compounds for all functions in plant cells. The changes in protein content in response to water deficit are not regular. Protein content in plant leaves may be decreased, unchanged or increased due to drought (Bakalova et al. 2008). These changes in protein levels are mainly due to the alteration in gene expression and are an essential part of plant adaptation to environmental disturbances (Merewitz et al. 2011).

Albumins are proteins which are soluble in water and coagulable by heat and are considered a major storage protein fraction (Shewry and Pandya 1999). 2S albumins are sulfur-rich proteins, primary sources of carbon and nitrogen, representing about 20–60% of total soluble proteins and are involved in plant defense against biotic and abiotic stresses (Maria and Bârsan 2001; Costa et al. 2015). Recently, Mo-CBP3 protein, which is a member of the albumin family, was identified from *M. oleifera* seeds and exhibited an inhibition of germination and mycelial growth of phytopathogenic fungi (Freire et al. 2015). However, this protein may induce an allergic response in human (Moreno and Clemente 2008). In this respect, Pinto et al. (2015) isolated and purified Mo-CBP3 protein from *M. oleifera* mature seeds and found that this protein has high sequence homology with allergens. Salinity was found to increase or decrease albumin content in plant tissues, depending on severity of stress and stage of plant development (Duca and Bârsan 2001), but the effect of drought on albumin level is rarely studied. This knowledge about albumin prompted us to investigate its expression level under water deficit conditions. Furthermore, it was also interesting to hypothesize a link between the effect of drought on albumin expression level and its possible role in allergy.

Immunoglobulins (known as antibodies) are protein substances found in vertebrates and produced in response to a wide range of foreign objects (called antigens) such as microorganisms, cancer cells and specific types of food. In human, there are five immunoglobulin classes found in the blood:

immunoglobulin A (IgA), immunoglobulin D (IgD), immunoglobulin E (IgE), immunoglobulin G (IgG) and immunoglobulin M (IgM) (Solomon and Weiss 1995). The 2S albumins have been reported as a main group of plant food allergens. They have the ability to interact with IgE antibody in human causing allergy (Lehmann et al. 2006; Moreno and Clemente 2008).

Although the impacts of drought on the physiology, biochemistry, gene expression and yield of economic plants have been widely documented (Leprince and Buitink 2010; Öztürk et al. 2016), its effect on albumin gene expression in *M. oleifera* green leaves does not appear in the literature. Additionally, the impact of water deficit on the crop feed value for human health has been rarely studied. Our hypothesis is that drought decreases 2S albumin gene expression in *M. oleifera* plants and consequently decreases the plant allergenic reactivity when used in human or animal feeding. Therefore, this study was undertaken to investigate the effect of water deficit during the vegetative growth stage on plant growth and 2S albumin expression in *M. oleifera* leaves. Furthermore, the allergy effect of 2S albumin was predicted in silico analysis.

2 Materials and methods

2.1 Seeds source

Seeds of *M. oleifera* Lam were obtained from the Agriculture Research Centre, Cairo, Egypt.

2.2 Growth conditions

Seeds were surface sterilized with 0.25% sodium hypochlorite solution for 5 min and washed thoroughly with distilled water. Then the seeds were soaked in distilled water for 12 h and sown in 50 plastic pots (25 cm in width × 30 cm in height) filled with 6 kg soil (2 clay: 1 sand) using 5 seeds/pot. The pots were kept in a greenhouse, whereas the plants were subjected to natural day/night conditions (minimum/maximum air temperature and relative humidity were 27/33 °C and 59/67%, respectively, at mid-day, during the experimental period). The pots were irrigated to field capacity (FC) when soil water content had decreased to 60% FC. Twenty days after sowing, the plants were reduced to one seedling per pot and

each pot received 1 g NPK (nitrogen, phosphorus and potassium) fertilizer (19–19–19+TE) (N:19% P₂O₅:19% K₂O:19% EDTA-Zn:0.05% B:0.15% MgO:0.2% EDTA-Fe:0.02% EDTA-Mn:0.02%). After that, the pots were arranged in control or drought treatments with 25 replicates for each treatment. Control plants were conducted continuously as before thinning. Drought was applied for 1 month by withdrawing water until 50% FC. At the end of the drought period, the plants were harvested and samples were taken for growth, relative water content (RWC) and 2S albumin gene expression analyses. In addition, soil samples were collected for monitoring soil moisture content.

2.3 Soil water content

Soil water content (SWC) was estimated by obtaining the wet mass of a soil sample (W1), oven drying the soil sample for 48 h at 105 °C and weighing the dry soil sample (W2). The SWC was expressed as a percentage of the mass of dry soil.

2.4 Growth analysis

Shoots and roots of each treatment were collected and weighed separately to obtain the fresh masses and oven dried at 80 °C for 48 h to have the dry masses (g).

2.5 Relative Water Content (RWC)

Leaf discs from each treatment were obtained by a sharp cork borer and weighed to obtain their fresh mass (FM). Then these discs were floated on distilled water at 20 °C in dim light for 5 h. After the 5-h hydration period, the turgid leaf discs were rapidly blotted dry and weighed to obtain the turgid mass (TM). The discs were then oven dried at 80 °C for 24 h and weighed to obtain the dry mass (DM). Leaf RWC was calculated by the following equation: $RWC (\%) = [(FM - DM)/(TM - DM)] \times 100$.

2.6 2S albumin gene expression procedures

Procedures for evaluating the 2S albumin (Mo-CBP3-1) gene (GenBank: AHG99682.1) expression were carried out at Biotechnology institute, Suez Canal University, Egypt. Plant samples (fourth leaves from

shoot apex and roots) were frozen by snap freezing in liquid nitrogen and kept at – 80 °C. After that, three main steps were carried out as follows: RNA extraction using a total RNA purification kit (Jena Bioscience) was performed based on the manufacturer's instructions. The samples were treated with DNase in order to remove possible DNA contamination. Quality and quantity check of RNA were done with a 1% agarose gel and spectrophotometry (nanodrop 2000c, Thermo Scientific, USA). The second step was cDNA synthesis using a Script cDNA synthesis kit (Jena Bioscience). The extracted RNA was used for synthesizing the first cDNA in the presence of forward and reverse primers (ATGGCAAAGCTCACTCTCCT and GCGCTGTTGGGTCTGAAATT, respectively). In addition, two actin gene primers (CTGGACTC TGGTGATGGTGT and ATTTCCCGCTCTGCAGT AGT) GenBank: JQ081230.1) were used for amplifying the gene sequence as an internal control. Finally, quantitation of DNA was achieved by Quantitative polymerase chain reaction (qPCR) applied Biosystems 7500 and qPCR Green Master kit (Jena Bioscience). After Initial denaturation of the gene sequences at 95 °C for 2 min, three different temperatures were applied to the mixture for 40 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 20 s and elongation at 72 °C for 30 s. The genes amplification was repeated three times and the mean value was taken for the three reads. The relative gene expression results were analyzed using a $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

2.7 Antibody–antigen interaction

Four albumin protein sequences (in *Moringa oleifera*) were submitted to two prediction servers in order to determine the possible protein active sites involved in food allergy. These servers are ProInflam (Gupta et al. 2016) and AllerHunter (Muh et al. 2009). Antibody i-Patch server (Krawczyk et al. 2013) was used to predict paratopes (antigen-binding sites). EpiPred server was used to predict epitopes (antibody-binding sites) (Krawczyk et al. 2014). The five immunoglobulins (IgA, IgD, IgE, IgG and IgM with their PDB IDs: 1ow0, 1zvo, 5g64, 5jii and 2rcj respectfully were uploaded to the servers as antibodies. It was known that *M. oleifera* contains four isoforms of 2S albumins (Mo-CBP3) (Freire et al. 2015). Mo-CBP3-1 (GenBank: AHG99682.1) was selected for in silico

Table 1 Effect of drought on soil water content, growth parameters and RWC of *Moringa oleifera* plants

Treatments	Parameters							
	Soil water content (%)	Plant height (cm)	Leaf number	Total plant fwt (g)	Shoot dwt (g)	Root dwt (g)	Root/Shoot ratio	RWC (%)
Control	26.3 ± 0.98	79.3 ± 2.2	16.0 ± 0.36	58.0 ± 1.53	7.3 ± 0.18	2.3 ± 0.04	0.31 ± 0.03	90.7 ± 3.2
Drought	14.2* ± 0.43	59.0* ± 1.4	13.3* ± 0.42	24.3* ± 0.80	3.1* ± 0.07	1.8* ± 0.05	0.59* ± 0.02	65.8* ± 1.8

Values are mean ± SE (n = 3–6). An asterisk (*) indicates a significant difference at $P < 0.05$ based on *T* test

experiments because its structure was determined and the PDB file was available. In this study, Mo-CBP3-1 was given the name albumin 31 and was uploaded as an antigen (PDB: 5dom). The amino acid sequences of the other three isoforms (GenBank: AHG99680.1, AHG99681.1 and AHG99683.1) were submitted to SWISS-MODEL server to search for templates and build models. The server selected 5dom structure as the best template with > 70% identity. For this reason, there was no need to build structure models of the other isoforms.

2.7.1 Preparation of PDB files for protein–protein docking

The pdb files opened with WordPad and the HETATM section was removed. Chimera software was used to prepare pdb files for docking (Pettersen et al. 2004). Next, the prepared pdb files were energy minimized to make protein at its minimal energy state (stable state). Energy minimization was running within the Chimera software (minimize structures tool) with parameters: 1000 steps of steepest descent, step size 0.002 nm, followed by 10 steps of conjugate gradient minimization (step size 0.002 nm) with no atoms fixed, hydrogen atoms included and charged residues assigned. The two Fab regions (Fragment, Antigen Binding) H and L chains of IgE protein were used for docking to albumin 31.

2.7.2 Protein–protein docking and analysis

Several complex solutions were generated using HEX 8.0.0 software (Ritchie and Venkatraman 2010) with default parameters (Correlation type was Shape + Electro, Number of Solutions was 100). Protein–protein binding affinity was predicted by PRODIGY server (Vangone and Bonvin 2015). Docked solution with the highest binding affinity

was selected. Protein–protein interaction analysis was performed using P I C: Protein Interactions Calculator (Tina et al. 2007) and PDBsum (Laskowski et al. 2005).

2.8 Statistical analysis

Data of the drought experiment were presented as means of 3–8 replicates ± SE. The significant difference between means at $P < 0.05$ was evaluated by *T* test using SPSS 18 package.

3 Results

3.1 Changes in soil moisture and plant relative water content

The applied drought decreased soil water content (as appeared in Table 1). At the end of the drought period, soil water content of water deficit treatment was about 50% of that shown by the control soil. This shortage in soil water content adversely affected leaf RWC. RWC of *M. oleifera* leaves was reduced in response to drought to 70% of the control plants.

3.2 Changes in growth parameters

Drought reduced all evaluated growth parameters in comparison with the control (as shown in Table 1 and Fig. 1). Drought reduced plant height (– 34%), number of leaves (– 16%), total plant fresh mass (– 60%), shoot dry mass (– 37%) and root dry mass (– 21%). Conversely, root/shoot ratio was enhanced due to water deficit. Drought increased the plant root/shoot ratio by 90%. Plant fresh mass and shoot dry mass appeared to be the most affected parameter by drought, whereas the number of leaves was the least affected one.



Fig. 1 Effect of drought on the growth of *Moringa oleifera* (left, control; right drought)

3.3 Changes in 2S albumin gene expression

In control plants, the expression of 2S albumin was almost 50% higher in their leaves than roots. Drought reduced 2S albumin gene expression in both leaves and roots (as shown in Fig. 2). However, under drought, 2S albumin expression was the same between leaves and roots.

3.4 Paratope and epitope prediction

The first step to investigate the role of albumin 31 in food allergy in human was to predict the possible amino acids that are required for binding with immunoglobulins. The prediction was performed by ProInflam server by which several binding sites were proposed (data not shown). In addition, AllerHunter server revealed that all the four albumin proteins in *M. oleifera* were predicted as a potential cross-reactive allergen. The second step was the use of Antibody i-Patch and EpiPred servers for predicting the paratopes and epitopes. Every chain in five antibodies (as shown in Table 2) were analyzed individually. The results showed that albumin 31 was able to contact with IgD (all chains), IgE (all chains except A and B) and IgM (all chains except U). The prediction results also revealed that there was no antibody–antigen contact between IgA, IgG and albumin 31.

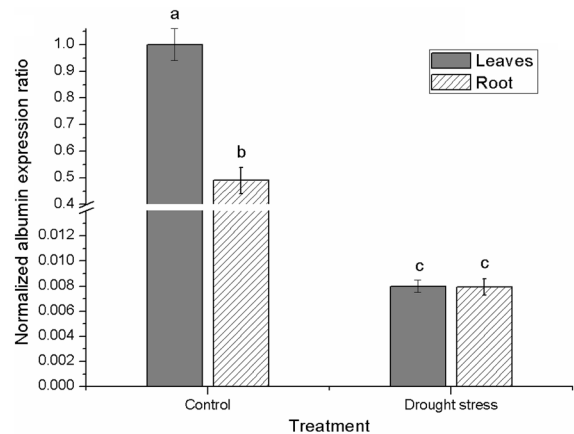


Fig. 2 Changes in relative albumin gene expression in *Moringa oleifera* leaves and roots in response to drought. Values are mean $\pm$ SE. For control and droughted plants, different letters represent significant differences ($P < 0.05$) between leaves and roots

The final step was protein–protein docking using HEX 8.0.0 software to study IgE–albumin complex, which is the most reported and studied food allergic interaction. Figure 3 shows the docked IgE–Albumin31 complex. The best docked complex was selected based on its free energy change (ΔG) which was $-16.9 \text{ kcal mol}^{-1}$ and its dissociation constant (Kd) which was $3.7 \times 10^{-13} \text{ M}$. Critical amino acids of albumin31 involved in the formation of hydrogen bonds were ARG143, ARG154 and ARG158. Albumin 31 would be able to interact with IgE chain L forming more contacts compared to chain H. Table 3 shows the statistics for all the interface interactions between H and L chains of IgE and albumin 31 (A). The supplementary material provides more details about the protein complex, including the atoms of each pair of residues involved in the interaction and the contact types. This docked complex structure can be a possible therapeutic target for designing an anti-food allergic drug.

4 Discussion

Water shortage especially in arid and semi-arid areas exposes the economic plants to many detrimental effects (Younis et al. 2000; Ibrahim and Aldesuquy 2003; Öztürk et al. 2016). In our study, the adverse

Table 2 Paratope and epitope sites

Antibody	Chain	Paratopes	Epitopes
IgA (PDB ID: 1ow0)	No contact	No contact	No contact
IgD (PDB ID: 1zvo)	A (and B)	31,32,34,35,36,37,38,86,87,89	57,61,112,113,116,117,120,121,123,124,125,
	C (and D)	31,33,34,35,36,40,53,56,59,99,104	126,127,130,131,132,133,134,135,136,138,139, 140,142,143,153,154,156,157,158
IgE (PDB ID: 5g64)	H (and I)	100,101,102,103,104,105,106,107,108,109	57,61,112,113,116,117,120,121,123,124,125,
	L (and M)	31,32,33,34,36,54,56,93,95,104	126,127,130,131,132,133,134,135,136,138,139, 140,142,143,153,154,156,157,158
IgG (PDB ID: 5jii)	No contact	No contact	No contact
IgM (PDB ID: 2rcj)	A (and 9A)	34,35,36,37,58,60,97,106,107,108	57,61,112,113,116,117,120,121,123,124,125,
	C (and 9B)	34,35,36,48,84,85,87,89,90,97	126,127,130,131,132,133,134,135,136,138,139, 140,142,143,153,154,156,157,158

9A = chains B,E,F,I,J,M,N,Q,R

9B = chains D,G,H,K,L,O,P,S,T

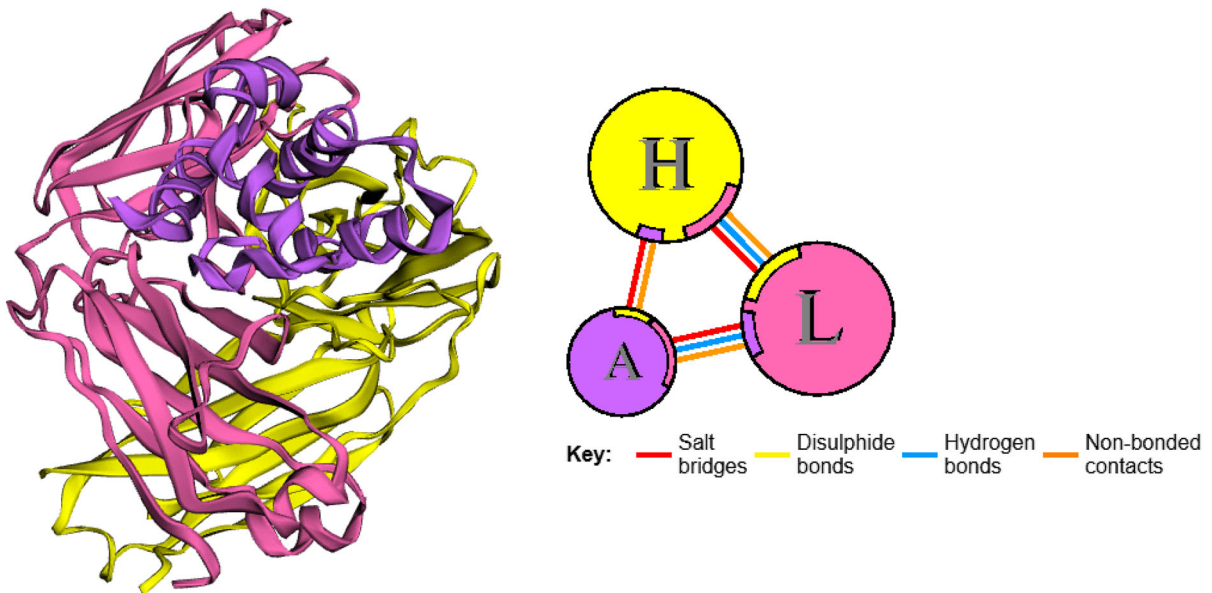


Fig. 3 Left, Docked IgE-Albumin31 complex. Yellow is H chain, Pink is L chain of IgE and purple is albumin 31. Right, Schematic diagram of interactions between protein chains. Interacting chains are joined by colored lines, each representing a different type of interaction, as per the key above.

The area of each circle is proportional to the surface area of the corresponding protein chain. The extent of the interface region on each chain is represented by a colored wedge whose color corresponds to the color of the other chain and whose size represents the interface surface area. (Color figure online)

effects of one-month drought on both shoot and root growth of young *M. oleifera* plants are expected results and were compatible with the observed reduction in soil moisture and RWC of leaves. These results are in agreement with the findings of Sapeta et al.

(2013) who exposed *Jatropha curcas* to a 28-day progressive drought and observed reduced shoot mass, leaf emergence, leaf area and number of leaves. The negative impact of water deficit on the plant growth could be attributed to the decrease in primary

Table 3 Statistics for the interfaces between H and L chains of IgE and albumin 31 (A)

Chains	No. of interface residues	Interface area (Å ²)	No. of salt bridges	No. of disulphide bonds	No. of hydrogen bonds	No. of non-bonded contacts
A–H	11:13	680:656	1	–	–	74
A–L	23:28	1295:1147	1	–	8	543
H–L	32:38	1851:1872	1	–	10	258

metabolic processes such as photosynthesis (Des Marais et al. 2012). The reduction of *M. oleifera* growth was reflected by the fresh mass rather than the dry mass of the plant parts, although fresh mass must be handled with care in plant physiology. The increased root/shoot ratio at the end of the drought period indicated that biomass partitioning in *M. oleifera* was changed to enhance root growth and this may be considered drought avoidance mechanism (Ibrahim 2013).

2S albumins protein is a major soluble protein in plant tissues, rich in Sulphur amino acids and is involved in plant defense against both biotic and abiotic stresses (Shewry and Pandya 1999; Costa et al. 2015). Analysis of protein profile in *M. oleifera* leaves revealed that albumin proteins constituted about 10% of the total soluble proteins (Teixeira et al. 2014). The obtained qPCR results showed that 2S albumin expression was considerably reduced in the leaves and roots of *M. oleifera* in response to drought stress. This reduction in gene expression revealed the inhibition of albumins biosynthesis and this is compatible with the observed reduction in plant growth and biomass. This reduction in albumin as storage proteins could be a regulatory mechanism to enhance the plant tolerance to drought and it could be under control of some transcriptional factors. These transcriptional factors may repress 2S albumin gene expression and induce some other genes which are involved directly in protection of cellular membranes and proteins under drought, such as LEA (late embryogenesis abundant) proteins genes (Wang et al. 2003; Kudo et al. 2017).

Our finding that 2S albumin gene expression in the leaves was higher than that in the roots of irrigated plants revealed tissue-specific regulation for this gene at the transcriptional and/or posttranscriptional level. This paper may be the first report on changes in albumin gene expression in *M. oleifera* leaves and roots. However, our results are distinct from those by Chen et al. (2004) who could not detect the 2S

albumin gene expression in leaves of castor Plant (*Ricinus communis* L), but detected it in developing seeds.

In the present study, it was predicted that albumin 31 has a possible role in food allergy in human. 2S Albumins are considered as main food allergens for two reasons. First, their structure is very stable comprising of a high number of cysteine residues allowing them to survive in the harsh conditions in the gut. The other reason is that 2S Albumins consist of many flexible and solvent-exposed regions forming suitable binding sites for immunoglobulins (Moreno and Clemente 2008). Albumin 31 contains similar structural features mentioned above. It has 8 cysteine residues and 25% of solvent-exposed regions. This makes albumin 31 a proposed target for food allergy investigations. To the best of our knowledge, this is the first in silico study, which links albumins in *M. oleifera* with immunoglobulins and food allergy. However, a study by Hagiwara et al. (2016) suggested that an aqueous extract of *M. oleifera* leaves contains compounds that can prevent the release of histamine and act as an anti-allergic compound. On the other hand, a western blotting of the *Moringa* seed extract indicated the presence of IgE proteins in the patient's serum. They also reported that *M. oleifera* seeds powder induces asthma (Poussel et al. 2015).

5 Conclusion

The decrease in *M. oleifera* growth due to drought was associated with a reduction in the relative 2S albumin gene expression in plant leaves and roots. In silico analysis indicated that albumin 31, which is a member of 2S albumin family, was an allergic agent. Therefore, the applied water deficit had some positive effect by decreasing the possible food allergy. To the best of our knowledge, this is the first report about albumin gene expression in *M. oleifera* leaves and roots and its

expected allergenic effect. Future work are needed to disclose the effect of drought on albumin gene expression in *M. oleifera* seeds.

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