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Hypobaric Pressure Effects on Gene Expression, as a Physiological Response of Canola Varieties

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Authors' contributions

This work was carried out in collaboration between both authors. Author BÇ designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author EA managed the analyses of the study and the literature searches. Both authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: The main purpose of our study was to determine the expression levels of genes, which respond to low pressure in canola (*Brassica napus* L.) varieties Californium, Orkan, Jura and Elvis.
Study Design: Canola (*Brassica napus* L.) varieties (Californium, Jura, Orkan and Elvis) placed within specially designed low pressure cabinets were exposed to low pressure (40 Torr ≅ 53 kPa) for durations of 1, 2 or 3 days. Total RNA was isolated from the plants analyzed for genes, *OsNCED, OsABA8, OsZEP* and *TMAC2* by RT-PCR technique. For control, housekeeping gene β-actin was used. As a result *OsABA8ox 1, 2, 3* and *OsNCED* showed increase in expression levels.
Methodology: Canola (*Brassica napus* L.) varieties *Californium, Orkan, Jura* and *Elvis* were provided by the Black Sea Agricultural Research Institute, Samsun – Turkey. Seeds were sown and placed in the plant growth chamber. The 14 days old plants were exposed to low pressure in the low pressure cabinet. RT-PCR reactions were performed in one-tube reaction according to manufacturer's protocol (Access Quick RT-PCR System, Promega, A1701).
Results: The PCR products of *OsNCED, OsZEP, OsABA*, *TMAC2* separated by 2%

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agarose gel electrophoresis were found to be approximately between 200-300bp. The PCR products of *OsABA8* gene (~750bp) in Jura was determined to be increased compared to that of control group. Change of expression of *OsABA8* gene (~750bp) in varieties of *Jura* and *Orkan* were determined and compared to that of control group. *OsABA8* gene region (~300bp) in varieties of both *Californium* and *Elvis*, whose expression of *OsNCED* gene region only (~300bp) in varities of *Jura* and *Orkan* were determined and compared to that of control group.

Conclusion: Any changes have been determined in the expression of *TMAC2* gene which supports the other studies in the literature. As a consequence of this, results obtained from our study have the feature that can give a new direction to other studies. In addition to this, because of there is no yet such a study related to low atmospheric conditions, this study has the characteristics of being the first and fundamental study with this speciality.

Keywords: Hypobaric pressure; Canola; OsNCED, TMAC2, OsABA8, RT-PCR, gene expression; RNA.

1. INTRODUCTION

Canola was cultivated by ancient civilisations in Asia and the Mediterranean. Its use has been recorded as early as 2000 BC in India and has been grown in Europe since the 13 th century, primarily for its use as oil for lamps. Canola was first grown commercially in Canada in 1942 as a lubricant for use in war ships. Canola was first grown commercially in Australia in 1969. Traditionally, *Brassica napus* is unsuitable as a source of food for either humans or animals due to the presence of two naturally occurring toxicants, erucic acid and glucosinolates. However, in the 1970s, very intensive breeding programs in several countries including Australia produced high quality varieties that were significantly lower in these two toxicants. The term ‘canola’ refers to those varieties of *B. napus* that meet specific standards on the levels of erucic acid and glucosinolates. Those cultivars must yield oil low in erucic acid (below 2%) and meal low in glucosinolates (total glucosinolates of 30 μmoles/g toasted oil free meal), and are often referred to as “double low” varieties. Canola is a crop with plants from three to five feet tall that produce pods from which seeds are harvested and crushed to create canola oil and meal. These plants also produce small, yellow flowers, which beautify the environment. Canola seeds contain about 44 percent oil. This large percentage of oil comes in a small package; canola seeds are similar in size to poppy seeds, though brownish-black in color. Although they look similar, canola and rapeseed plants and oils are very different. Canadian scientists used traditional plant breeding in the 1960s to eliminate the undesirable components of rapeseed* and created "canola," a contraction of "Canadian" and "ola." Canola oil is prized for its heart-healthy properties with the least saturated fat of all culinary oils.

Atmospheric conditions of low pressure were tested in the early space programs of United States and the structural and physiologic effects of low pressure was investigated on plants that were grown for nourishing astronauts in 1960s [1]. The results showed that plants can tolerate low atmospheric pressure by giving responses in physiology and development [2-7]. The experiment was combined later with changes in O_2, CO_2 and total gas pressure. Hypoxia by reduced pressure of O_2 caused the most dramatic effect [7,8]. Interest in the exploration of environments beyond Earth’s atmosphere has brought unique challenges to bear on the understanding of the biological systems that will inhabit those environments. Among these challenges are alterations in atmospheric pressure, which are known to have

effects on plant physiology and development [1-7]. Concepts for greenhouses on Mars, on the moon, and in Earth orbit incorporate low atmo-spheric pressures to address engineering and sys-tems limitations [9,10].

Endogenous hormones and environmental factors have a big role in plant development. Abscisic acid (ABA) has important roles in seed development root and stem growth, and also in ripening of embryo, regulation of stoma movement and response to water stress [11]. In ABA synthesis, the key enzyme is the stress responsive 9-cis-epoxycarotenoid dioxygenase (NCED), which transforms 9-cis-neoxanthin and 9-cis violaxanthin to xanthoxin. In watery environment, the amount of ABA decreases but phaseic acid (PA) (which is the oxidized form of ABA) increases. Enzyme ABA-8'-hydroxylase catalyses the oxidation of ABA to PA. This hydroxylase is encoded by 3 genes in rice (*OsABA8ox1*, *2* and *3*). Plants kept submerged in water for a couple hours showed increments in the expression of *OsABA8ox1* gene meanwhile the synthesis of ABA-9-cis-epoxycarotenoid dioxygenase and zeaxanthin epoxidase decreased [12]. Two or more Abscisic acid responsive elements (ABREs) that respond for ABA treatment were found in *Arabidopsis thaliana.* One of those is *TMAC2* (two or more abres-containing gene 2) and it is induced by ABA and NaCl. This gene plays a negative role in ABA and salt stress, besides excessive expression of this gene causes forming short root and late flowering [13]. Characterization of the low pressure response can therefore provide data to guide systems engineering development for bio-regenerative life support, as well as lead to fundamental insights into aspects of desiccation metabolism [14].

To assess directly to molecular events that characterize plant adaptation to low atmospheric pressures, we used hypobaric chamber to determination of gene expression of 1, 2 or 3 days exposure of *B. napus* varieties (Californium, Orkan, Jura and Elvis*)* to 40 Torr.

2. MATERIALS AND METHODS

2.1 Growing Conditions and Plant Nutrition

Canola (*Brassica napus* L.) varieties, Californium, Orkan, Jura and Elvis were provided by the Black Sea Agricultural Research Institute, Samsun – TURKEY. Seeds were planted and placed in the plant growth chamber (Angelantoni Ekochl1500) at 25ºC, 70% humidity and 50-60 miromol^{-2}s^{-1} of cool white fluorescent light 16/8 hours photoperiod.

2.2 Treatment Plants by Low Pressure

The 14 days old plants were exposed to low pressure in the hypobaric pressure cabinet (Nanovak Company) (Fig. 1). First, the whole air in the cabinet was taken out and setup for 40 Torr (≅ 53 kPa). Nitrogen and oxygen levels were adjusted to 98% ($^{v}/_{v}$) $N_2$and 2% ($^{v}/_{v}$) O_2. Plants were kept in plant growth cabinet for 1, 2 and 3 days.

Fig. 1. Hypobaric pressure cabinet

All glassware was sterilized with water including 10% diethylpyrocarbonate (dissolved 10ml diethylpyrocarbonate in 90ml water) at 37ºC for one night followed by autoclaving for two times (20 minutes each) to prevent possible RNase contamination.

2.3 Total RNA Isolation

Total RNA was isolated from canola leaves (60mg) by using Promega, Z3100 kit in both group of plants of the control and the low pressure treated plants following the manufacturer's protocol. Fresh leaves were frozen in liquid nitrogen, pulverized and then lysated in lysis buffer. After that treatment, dilution buffer was added and mixed well, then kept at 70ºC for 3 minutes and centrifuged by 14,000×g for 10 minutes. 95% ($^{v}/_{v}$) ethanol was added into the lysate and mixed well then the mixture was filtered into a collection tube and centrifuged by 13,000×g for 1 minute. After this manual step, RNA isolation was carried on according to total RNA isolation kit (Promega – Z3100) protocol.

2.4 Quantitative RT-PCR

RT-PCR reactions were performed in one-tube reaction according to manufacturer's protocol (Access Quick RT-PCR System, Promega, A1701) by using oligonucleotide primers

(Table 1) initially, a volume of 2 µl total RNA was used for cDNA reaction by using AMV RT enzyme. Then PCR reactions were carried on in the same tube. After an inactivation of AMV RT enzyme at 95ºC for 2 min, 40 amplification cycles were performed as follows: 45 sec at 94℃, 45 sec at 57ºC, and 1 min at 68℃. This was f ollowed by 5 min final extension at 68ºC, and the reaction mix stored at 4ºC.

Table 1. Oligonucleotide primer sequences used for RT-PCR in this study

Primers	Sequences (5'→3')
OsNCED1 F	AGC CTC GGT CTT CCA ATT TT
OsNCED1 R	CAC CCA ACA CAA AAG CTA CG
OsABA8ox1-F	AAGCTGGCAAAACCAACATC
OSABA8ox1-R	CCGTGCTAATACGGAATCCA
OsZEP-F	GGATGCCATTGAGTTTGGTT
OsZEP-R	TGGCTGACTGAAGTCTCTCG
TMAC2-F	TCAGAGAAGACGAGGGCATTTA
TMAC2-R	CATCGTTACATTTTGAAAGTTAGGG
Actin-F	GTTTCCTGGAATTGCTGATCGCAT
Actin-R	CATTATTTCATACAGCAGGCAAGC

RNA and PCR products were separated on agarose gel (1.5% agarose m/v). The electrophoresis was performed at 70V for 40min for total RNAs and for 1hour for PCR products.

3. RESULTS AND DISCUSSION

The PCR products of *OsNCED, OsZEP, OsABA, TMAC2* separated by 2% agarose gel electrophoresis were found to be approximately between 200-300bp (Figs 2 and 3).

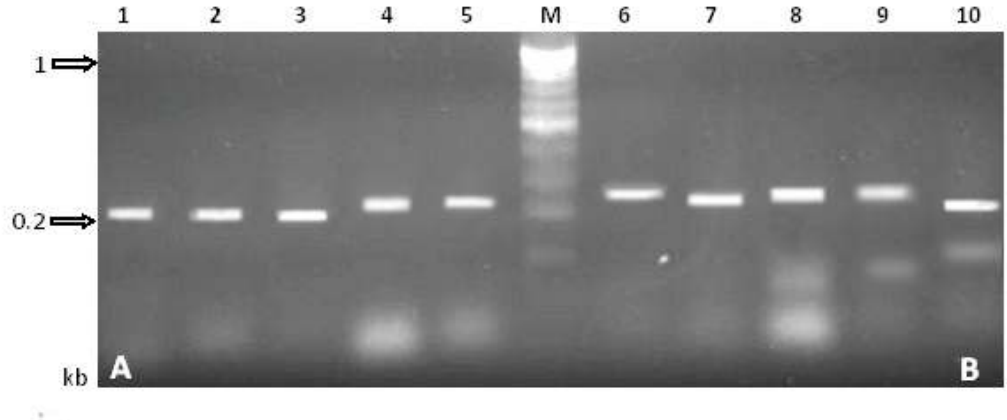

Fig. 2. RT-PCR results at control groups, A) *Californium*, B) *Elvis*, M) Marker (bp), 1,6) *OsNCED*, 2,7) *OsZEP*, 3,8) *OsABA*, 4,9) *TMAC2*, 5,10) Actin *(*housekeeping*)*

The PCR products of *OsABA8* gene (~750bp) in Jura was determined to be increased compared to that of control group (Fig. 4).

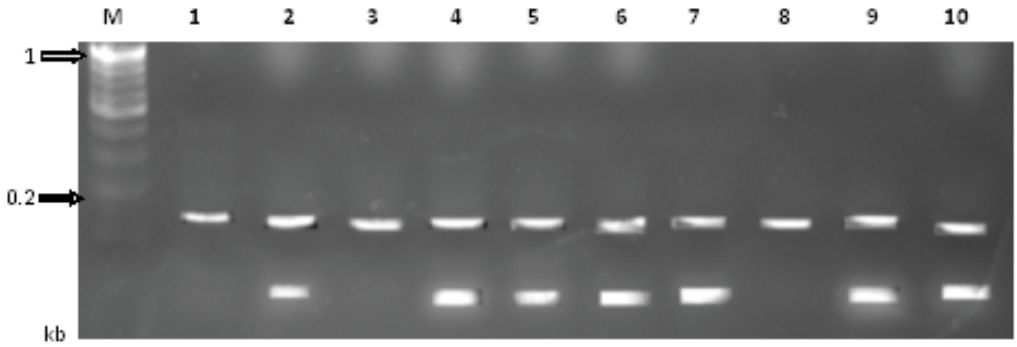

Fig. 3. RT-PCR results at control groups, M) Marker (bp), 1-5) Jura, 6-10) Orkan, 1,6) *OsNCED*, 2,7) *OsZEP*, 3,8) *OsABA*, 4,9) *TMAC2*, 5,10) *Actin (*housekeeping)

RT-PCR was performed by using experimental samples exposed to low pressure for two days and combining with *OsNCED, OsZEP, OsABA8* and *TMAC2* primers. PCR products whose expression of OsABA8 gene (~750bp) in varieties of Jura and Orkan were determined compared to that of control group (Fig. 5).

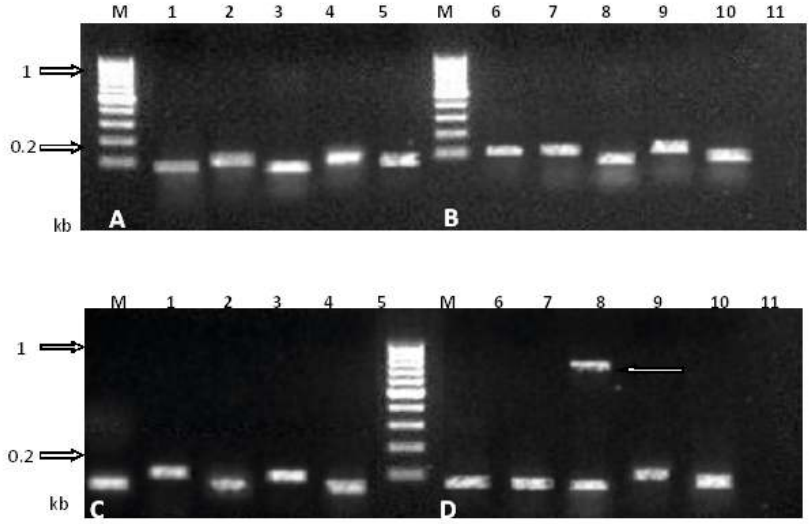

Fig. 4. RT-PCR results of one day treated with hypobaric pressure plants, A) Californium, B) *Orkan*, C) *Elvis* and D) Jura, M) Marker (bp), 1,6) *OsNCED*, 2,7) *OsZEP*, 3,8) *OsABA*, 4,9) *TMAC2*, 5,10) *Actin (*housekeeping) and 11) Negative control

RT-PCR was performed by using experimental samples exposed to low pressure for three days and combining with *OsNCED, OsZEP, OsABA8* and *TMAC2* primers. PCR products whose expression of both *OsABA8* gene region and *OsABA8* gene region (~300bp) in varieties of Californium and Elvis, whose expression of *OsNCED* gene region only (~300bp) invarities of Jura and Orkan were determined compared to that of control group (Fig. 6).

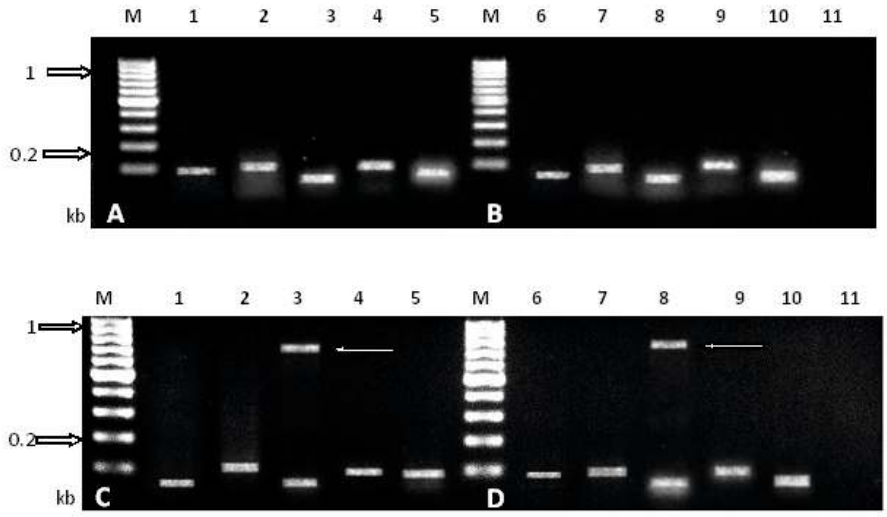

Fig. 5. RT-PCR results of two day treated with hypobaric pressure plants, M) Marker (bp), A) Californium, B) *Orkan*, C) *Elvis* and D) Jura, 1,6) *OsNCED*, 2,7) *OsZEP*, 3,8) *OsABA*, 4,9) *TMAC2*, 5,10) Actin (housekeeping), 11) Negative control

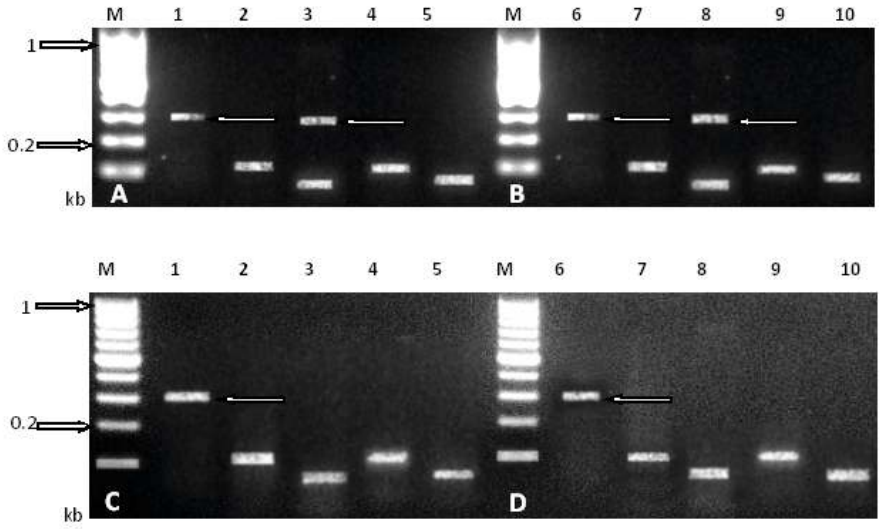

Fig. 6. RT-PCR results of three day treated with hypobaric pressure plants, M) Marker (bp), A) Californium, B) *Orkan*, C) *Elvis* and D) *Jura*, 1,6) *OsNCED*, 2,7) *OsZEP*, 3,8) *OsABA*, 4,9) *TMAC2*, 5,10) Actin (housekeeping)

As a result of this study, when we compared the experimental group with the control group, we didn't determine any changes for *TMAC2* gene region during 1,2 and 3 days period (Figs 4,5 and 6).

In this study, Californium, Elvis, Jura and Orkan varieties of canola (*Brassica napus* L.) plant were exposed to low pressure (40 Torr) along 1, 2 or 3 days. As a result of this, responses that plants gave under low pressure, changes in expression levels of *OsABA8, OsNCED, OsZEP* and *TMAC2* genes were detected. In canola varieties exposed to low pressure along 1, 2 and 3 days, it was determined that expression of *OsABA8* and *OsNCED* genes have been altered.

In study of He et al. [15], under 50kPa and 101kPa pressure for 28 days, it has been demonstrated that lettuce and wheat plants could grow. It has been stated that low pressure promotes plant growth but not changes germination rate. In research done by Paul et al. [16] on *Arabidopsis thaliana*, compared to plants exposed to low and normal pressure, it has been detected that more than 200 genes have made expression in plant exposed to low pressure. It has been stated that these genes are unique and related to water transmission in plant. In addition to this, they have detected that these genes connect with side-lines related to abscisic acid and drought. Guo et al. [17] also have observed changes by planting under low and normal pressure and different partial oxygen pressure in research on wheat; and reported that growth has been encouraged on wheat plant exposed to low pressure.

In study on lettuce by He et al. [18] they have demonstrated effects of growth and ethylene level in plant exposed to low pressure for 10 days. In consequence of this research, it has been detected that ethylene has an impact on carbon-dioxide assimilation (CA), dark phase of respiration (DPR) and growth of lettuce under low and normal pressure; however, there has no notable difference in production of inner ethylene under low and normal pressure. It has been stated that CA is more sensitive to rise of ethylene than DPR.

In our research, expression of *OsABA8, OsNCED, OsZEP* and *TMAC2* genes that are taken place in ABA and ethylene biosynthesis in plants in control and experimental groups were examined by using RT-PCR technique. It has been detected that among plants that were held in 40 Torr pressure for a day, only in Jura variety *OsABA8* gene has been expressed (Fig. 4). Of plants that were exposed to 40 Torr pressure for two days, only in Jura and Orkan varieties *OsABA8* gene has been expressed (Fig. 5).

In addition to this, it has been demonstrated that among plants that were exposed to 40 Torr pressure for merely three days in Californium and Elvis varieties, both *OsABA* gene and *OsNCED* gene have been expressed. Besides, as another difference; of plants in the same group only in Jura and Orkan varieties *OsNCED* gene has been expressed (Fig. 5).

In the study made, compared to canola varieties exposed to low pressure for one and two days, it has been identified that in Jura variety in comparison with others *OsABA8* gene has been expressed in early phase and low pressure stress conditions (Figs. 4 and 5). Compared to canola varieties exposed to low pressure along 1, 2 and 3 days, in all varieties exposed to low pressure along 1, 2 days it has been detected that 750bp-weight member of *OsABA8* gene family has been expressed (Figs. 4 and 5). At the end of third day, differently in all varieties, it is demonstrated that approximately 300bp-weight area of *OsABA8* gene has been expressed (Fig. 6).

The fact that different areas of *OsABA8* gene have been expressed depending on time is considered that this gene takes place in ABA biosynthesis. In research by Paul et al. [16], exposure of plants to an atmosphere of 10kPa compared with the sea-level pressure of 101kPa resulted in the significant differential expression of more than 200 genes between the two treatments. Yang and Choi [19] have determined that ethylene strongly triggers expression of *OsABA8α1* in rice plant under water. On this basis, they have suggested that increase of ethylene concentration in rice plant triggers expression of *OsABA8α1* gene and in consequence of this, casing expansion. Saika et al. [12] have reported that quantity of phosphatidic asit (PA), oxidized form of ABA, increases in amount while ABA decreases. Besides, they have detected that oxidation of ABA catalyses ABA 8'-hydroxylase enzyme and this enzyme is coded three genes (*OsABA8α1*, *2* and *3*) in rice plant. 1-2 hours after rice plant submerged expression levels of *OsNCED* and *OsZEP* genes, responsible for ABA synthesis, decrease. If seeds are treated with ethylene, expression of *OsABAox1* gene is elevated. In submerged rice trunks, it has been observed rapid decrease in ABA amount; some parts of this decrease are directed by ethylene-encouraging *OsABAox1* gene.

In the research of Zeervaart and Creelman [11], it has been demonstrated that abscisic acid plays a great role in regulating responses depending on water stress in leaf, and in drought conditions rise produces firstly in abscisic acid, inner hormone, in leaf. In study by Tan et al. [20] on *Arabidopsis thaliana*, they decided that *OsNCED* gene plays a great role in ABA biosynthesis and ABA synthesis is controlled by *NCED* gene family.

Cooper and Horanic [21] have put forward that ethylene is removes from tissues under low pressure. They have declared that gases are revealed in tissues held under low pressure and these revealed gases prevent from storing internally. They have detected that low pressure doesn't damage to fruits; however, it causes ripening in fruits with ethylene elimination. In the light of findings received in our research, it has been stated that ethylene gas triggers expression of *OsABA8* gene under low pressure in canola varieties. In addition to these findings, it has been detected that different members belonging to *OsABA8* gene family have made expressions depending on time. It has not been come across any

literature about the fact that different members of *OsABA8* gene family have made expression depending on time. With these findings, it can be said that responses revealed in plants exposed to low pressure depending on time are directed through different areas of *OsABA8* gene family.

Saika et al. [12] have reported in their study that *NCED* gene plays a role in ABA biosynthesis as a catalyst; yet, it is not related to ethylene biosynthesis. Within the scope of the study, it has been detected that on first and second day of canola plants exposed to low pressure expression of *NCED* gene hasn't changed; on the other hand, *NCED* gene takes in making expression in all variations on third day. This outcome revealed can be indicator of the fact that *NCED* gene related to ABA biosynthesis in canola plants exposed to low pressure can make expression in later period depending on time.

In other study on *Arabidopsis thaliana* plant by Huang and Wu [13], they identified ABA-control genes (ABREs; ABA-responsive elements). One of these genes, gene 2 (*TMAC2*) including two or more than two ABREs, increases its expression under impact of ABA. This gene makes much more expression in roots than other parts of plants. *TMAC2* gene plays negative role in ABA and salt stress [14,21,22].

In our study, any changes has not been detected in expression of *TMAC2* gene in the way supporting literature researches. Consequently, information from our research is expedient for other new researches to be made. Additionally, this research is the first in its field and with this feature it is a base study because of the fact that any research about low atmosphere conditions like this hasn't been made.

4. CONCLUSION

Characterization of the low pressure response can therefore provide data to guide systems engineering development for bio-regenerative life support, as well as lead to fundamental insights into aspects of desiccation metabolism. Major findings of our study that Californium, Elvis, Jura and Orkan varieties of canola (*Brassica napus* L.) plant were exposed to low pressure (40 Torr ≅ 53kPa) along 1, 2 or 3 days. As a result of this, responses that plants gave under low pressure, changes in expression levels of *OsABA8, OsNCED, OsZEP* and *TMAC2* genes were detected. In canola varieties exposed to low pressure along 1, 2 and 3 days, it was determined that expression of *OsABA8* and *OsNCED* genes have been altered.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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