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Patterns of mitochondrial gene expression in rapeseed leaves (*Brassica napus* L.) at early growth stage in response to drought stress

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ABSTRACT
Drought stress adversely affects a plant's growth and productivity. Wide ranges of molecular disorders could be caused by the production of reactive oxygen radicals. Plant cells have developed potential systems to prevent such damage by scavenging and reducing the reactive oxygen species (ROS). In this study, both the genotypes of oilseed rape-tolerant and sensitive to drought-were exposed to polyethylene glycol (PEG)-induced osmotic stress at various intervals to screen the expression of mitochondrial genes that are involved in oxidizing excessive NAD(P)H without producing adenosine triphosphate (ATP). Results showed that the maximum number of *alternative oxidase* 1a (*AOX*1a) gene expression occurred in Hyola308 after 12 hours of water stress. Meanwhile, no change was observed in other sampling times. However, in SLM046, the gene expression had gradually been increased during the stress and the maximum expression was observed after 24 hours of stress. The expression of *uncoupler* (*UCP*) gene, in SLM046, was increased during the water stress and its maximum expression was observed at eight and 24 hours after the stress. However, the maximum *UCP* expression in Hyola308 occurred around the 12-hour mark after the stress as an *AOX* gene expression. Moreover, the expression of *external NADPH dehydrogenase* (*exNDH*) was increased at the early hours of the stress in Hyola308 while the same was done during the final hours of stress in SLM046. Our results showed that high activity of the mitochondrial genes, alone or together, could also be an important factor in drought tolerance in oilseed rape crop by detoxifying the harmful effects of the ROS.

Keywords: Canola, drought stress, mRNA quantification, oxidative stress, quantitative real-time PCR.

الگوهای بیان ژن‌های میتوکندریایی در برگ *Brassica napus* L. در پاسخ به تنش خشکی

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چکیده
خشکی رشد گیاه و تولید محصول را به طور نامطلوبی تحت تأثیر قرار می‌دهد و طیف وسیعی از اختلالات مولکولی در این خصوص ناشی از تولید رادیکال‌های فعال اکسیژن است. سلول‌های گیاهی برای مقابله با این اثرات مخرب از یکسری مکانیسم‌های دفاعی برخوردارند که با جمع آوری انواع اکسیژن فعال و احیای آنها به آب، از آسیب به مولکول‌های زیستی پیشگیری می‌نمایند. در پژوهش حاضر به منظور ارزیابی بیان ژن‌هایی که در میتوکندری می‌توانند برای جلوگیری از احیای بیش از حد زنجیر تنفسی، NAD(P)H اضافی را بدون تولید ATP اکسید کنند، از دو ژنوتیپ متحمل و حساس کلزا استفاده شد. نتایج نشان داد بیشترین افزایش بیان ژن آلترناتیواکسیداز برگ رقم Hyola308 (حساس) ۱۲ ساعت پس از اعمال تنش خشکی بود و در زمانهای دیگر تغییرات چندانی در بیان آنها مشاهده نشد ولی در رقم SLM046 (مقاوم) با افزایش زمان تنش، بیان ژن به تدریج افزایش یافته و در ۲٤ ساعت دارای بیشترین میزان بیان بود. در مورد ژن آنکوپلر میزان بیان در SLM046 با افزایش زمان تنش خشکی روند صعودی داشت و در ۸ و ۲٤ ساعت دارای بیشترین میزان بیان بود ولی میزان بیان ژن UCP در Hyola308 همانند بیان ژن AOX در ۱۲ ساعت پس از اعمال تنش دارای بیشترین مقدار بود. در Hyola308 میزان بیان ژن NADPH دهیدروژناز اکسترنال در آغاز اعمال تنش افزایش یافت ولی در SLM046 در ساعت پایانی افزایش تصاعدی بیان ژن موردنظر دیده شد. به نظر می‌رسد افزایش فعالیت این ژن‌ها به تنهایی یا با هم‌دیگر می‌تواند عامل مهمی در بالا بردن میزان تحمل گیاه کلزا به تنش خشکی از طریق خنثی سازی تاثیرات مضر انواع گونه فعال اکسیژن باشد.

واژه‌های کلیدی: کلزا، تنش خشکی، PCR در زمان واقعی، تنش اکسیداتیو.

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Introduction

Oilseed rape (*Brassica napus* L.) is one of the most important industrial crops that are mainly grown to make edible oil (Kholdebarin, 2004). Abiotic stresses, such as drought and salinity, conduct a series of changes in the plant that lead to biochemical, molecular, morphological, and physiological processes, and result in declining plant growth and development (Wang *et al*., 2003). The changes caused by various stressful conditions occur due to a secondary stress (usually osmotic or oxidative) that perturbs the structural and functional stability of membrane proteins and disrupts the cellular homeostasis (Shinozaki and Yamaguchi-Shinozaki, 2000; Zhu, 2001). Plant mitochondria could prevent the excessive reduction of the respiratory chain through NAD(P)H oxidation without producing ATP (Heldt and Piechulla, 2010). The factors that cause NAD(P)H oxidation without ATP production possess three active energy-dissipating systems-the plant uncoupling protein (PUCP) (Pastore *et al*., 2000), the ATP-sensitive plant mitochondrial potassium channel (PmitoKATP) (Pastore *et al*., 1999), and the alternative oxidase (AOX) (Pastore *et al*., 2001) with the ability to control the ROS production (Pastore *et al*., 2007), as well as the *rotenone-insensitive external NAD(P)H dehydrogenases* (*exNDH*) and a highly active malate/oxaloacetate (MAL/OAA) shuttle, both causing cytosolic NAD(P)H oxidation (Pastore *et al*., 2003).

The ROS production can be decreased through an alternative channeling of electrons in the electron transport chain (ETC) by a group of enzymes called AOX. Activating the alternative oxidase can reduce the production of hydrogen peroxide (Edreva, 2005). Previous studies have shown the reduction effects of AOX on the ROS production under different stresses. It is coupled with the ubiquinol pool and catalyzes the four-electron reduction of molecular oxygen to water (Li *et al*., 2013). An increase in AOX or the capacity of response to water deficit has been observed in wheat leaves (Bartoli *et al*., 2005; Vassileva *et al*., 2009), but no increase in the gene could be found in soybean leaves (Ribas-Carbo *et al*., 2005). It was also noted that the drought decreases leaf AOX transcript in *Medicago* (Filippou *et al*., 2011).

The UCPs-as integral mitochondrial membrane proteins-catalyze a proton conductance across the membrane, squandering the mitochondrial proton gradient (Krauss *et al*., 2005). Despite the heat generation, the UCP is not believed to be present in plants for thermogenesis purposes (Sweetlove *et al*., 2006). The activity of the PUMPs is increased by hydroxynonenal or the ROS, and hampered by purine nucleotide (Smith *et al*., 2004). Transgenic plants with high levels of AtPUMP1 show a high tolerance level to oxidative stress (Brandalise *et al*., 2003). The UCP may also be able to decide when the ROS levels become increase (Rhoads *et al*., 2006).

Alternative NAD(P)H dehydrogenases (NDH) were located in the inner membrane of the plant mitochondria internally, oxidizing matrix NAD(P)H, and externally, oxidizing cytosolic NAD(P)H (Liu *et al*., 2008). In a potato, two *NDH* genes-*St-nda1* and *St-ndb1*-were identified as the encoding internal and external enzymes respectively (Rasmusson and Agius, 2001). Arabidopsis contains seven *NDH* genes, four of which belong to the *ndb* gene family (Michalecka *et al*., 2003). An expression of the potato *ndb1* gene, a homolog of fungal and bacterial type-II

NDH, was introduced into tobacco (Michalecka *et al*., 2004). In comparison with the wild type, transgenic lines with high transcript and protein levels for *St-NDB1* had up to three-fold enhanced activity of *external NDH* (*ex NDH*) in the isolated mitochondria.

In this study, both tolerant and susceptible genotypes of canola were subjected to drought stress for screening the expression of the mitochondrial genes involved in the stress. The identification of the mitochondrial alternative factors, involved in drought stress, could be useful in conferring tolerance to the drought-susceptible species via gene manipulation.

Materials and Methods

Plant material

Two canola (*Brasica napus* L.) cultivars, including Hyola308 (susceptible to drought) and SLM046 (tolerant to drought), were prepared from the Seed and Plant Improvement Institute (SPII), Karaj, Iran.

Plant growth and stress treatment

The seeds were sterilized with 2.5% sodium hypochlorite for 10 minutes and then washed repeatedly with distilled water. The sterilized seeds were placed on wet paper in a filter and stored at 25°C in the dark until germination. The germinating seeds were transferred into small pots and were grown hydroponically for six days (a fountain pump with gravitational flow producing a mean flow rate of 6550 Lux, the average temperature was at 23±2 °C, and the photoperiodic lighting with a light to dark ratio of 16:8 h). The plants were irrigated with a half-strength Hoagland's nutrient solution (Hoagland and Arnon, 1950), which was changed every 48 hours until the plant developed five to six expanded leaves. In this study, two canola genotypes were grown in the half-strength Hoagland solution and two conditions were considered for the plants in the 4–5 leaves stage. Besides, 10% osmotic potential (-0.15 MPa) was imposed by PEG 6000 under normal conditions.

The amount of PEG_{6000} for each osmotic potential was calculated in accordance with the Michel and Kaufmann (Michel and Kaufmann, 1973) method and the sampling was done at 4, 8, 12, and 24-hour post treatment (hpt). The samples were immediately frozen in liquid nitrogen and then placed in the freezer at –80°C before processing.

RNA extraction, cDNA synthesis, and Quantitative real-time PCR:

The RNA extraction was performed in accordance with the protocol of TM RNX-plus kit (CINAGENE). The DNase-I (Fermentas) treatment was applied to remove the residual DNA. The cDNA synthesis was performed in accordance with the instructions mentioned in the Fermentas kit. The RNA quality and quantity of the extracted samples were controlled using agarose gel electrophoresis and spectrophotometry respectively.

The actin was used as an internal reference gene and also to calculate the relative expression levels of specific genes. Sequences of the specific gene primers are listed in Table 1. Candidate genes involved in the stress tolerance were identified by searching databases such as NCBI (http://www.ncbi.nlm.nih.gov/) and EBI (https://www.ebi.ac.uk). Moreover, the TCOFEE software was used for the sequence alignment (http://tcoffee.crg.cat/apps/tcoffee/do:mcoffee) and specific primer pairs were designed using the primer3 (http://primer3.ut.ee) programs.

The size of the PCR product varied from 157 to 196 base pairs and the melting point ranged from 52.1°C to

56.8°C, according to the (G+C) percentage and length of the bands.

Table 1. Primer pairs used for relative expression analyses of AOX1a, UCP and ex *NDH* and *Actin* (internal control) genes in response to water stress in *Brassica napus* L.

Gene	Primer sequence	Melting Tem (◦C)	PCR production length	NCBI accession number
Actin-F *Actin*-R	5-TCCCGAGTATTGTTGGTCGT-3 5-TCCATGTCATCCCAGTTGCT-3	54	157	AF111812
*AOX*1a mit-F *AOX*1a mit -R	5´-GCGGTTGGATCTGGACTACT-3´ 5´-TAGCGATTCCTTTCCCTCCC -3´	56.8	171	JX110773
UCP mit -F *UCP* mit -R	5´-GCTCTGTGGACTGGTCTTGG -3´ 5´-TAACCACGTCAACAGGGGAA-3´	52.1	196	XM_013847803
ex NDH mit - F *ex NDH* mit - R	5´- GAAGAAGAAGGTGGTGCTGC-3´ 5´- GAGCTTCAACAGTGCCACAA-3´	54	158	XM_ 013806534

The PCR amplification was performed using the CFX Manager™ Software (Bio-Rad), in accordance with the manufacturer's instructions. The qRT-PCR conditions were as follows: initial denaturation at 95°C for 3 minutes then at 95°C for 10 seconds, annealing between 52.1°C to 56.8°C (depending on the primer used) (Table 1) for 20 seconds, an extension at 72°C for 15 seconds, and the final extension at 72°C for seven minutes.

Each sample was analyzed in triplicate and the average of Ct values was used for further quantification. In order to reveal the absence of contamination or primer dimers, a non-template control (NTC) reaction was performed with each primer pair. The $2^{-\Delta\Delta CT}$ method was used for the quantitative analysis (Livak and Schmittgen, 2001).

Results and Discussion

The results showed that the maximum AOX1a expression in the leaves of Hyola308 was observed around 12 hours after the drought stress, whereas its expression was significantly low at 4, 8 and 24 hours after the stress (Fig. 1). The AOX1a expression has gradually been increased in the SLM046 leaves during the drought stress. It reached its maximum level around 24 hours after the stress.

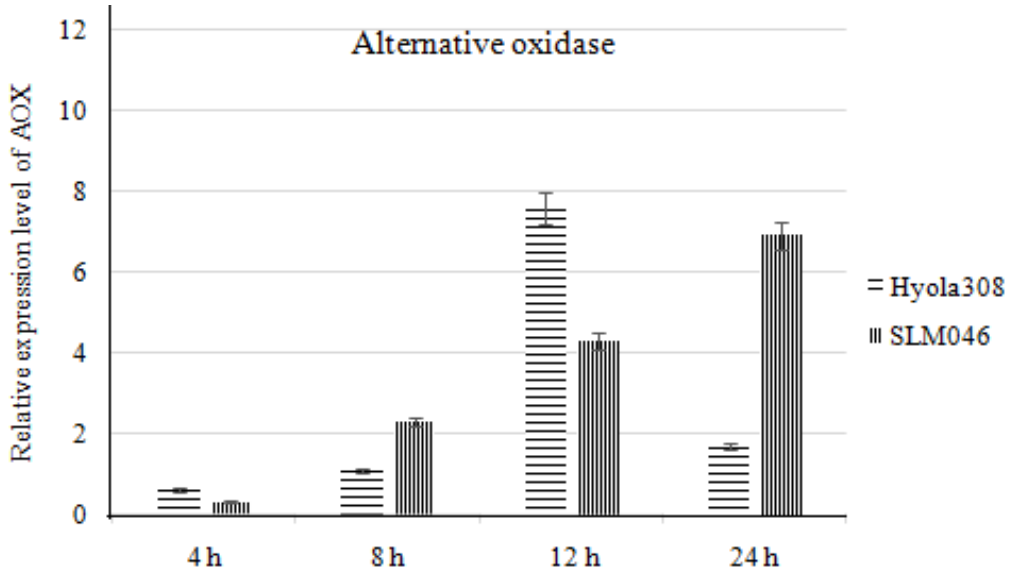

Figure. 1 Expression pattern of *AOX*1a gene using Real-time PCR under drought stress in seedling SLM046 and Hyola308.

The mtETC is the main source of ROS generation under drought stress. AOX1a might also oxidize the excess reductants to provide a dissipation mechanism for preventing damage to the photosynthesis process (Fu *et al*., 2012). Previous researchers have also shown that the absence of AOX1 gene decreases the growth ability or tolerance under stress. In addition, other records have suggested that the over-expression of AOX1 could increase stress tolerability by producing less ROS (Vanlerberghe *et al.*, 2009).

The AOX1 gene with the enhanced stable expression in the tolerant genotype (SLM046) could be considered as being responsible to stress conditions. Therefore, considering the gradual increase of *AOX*1a expression in the SLM046 genotype, an alternative oxidase pathway could be considered one of the oxidative stress resistance pathways in plants. Although the increased AOX1a expression was observed in Hyola308 at 12 hpt, the expression decreased at 24 hpt. The results showed that with increasing stress duration, the AOX1a expression in the tolerant SLM046 genotype was higher than that in the sensitive genotype. Activation of this gene in the resistant genotype could prevent the ROS formation in the electron transport chain (Edreva, 2005).

As a result, the damage caused by the oxidative stress would decrease and the AOX1a expression would directly couple with the oxidation of ubiquinol by reducing O_2 to H_2O.

The expression of the UCP gene was observed in the SLM046 genotype at 8 and 24 hpt in a biphasic manner. The expression in other time points was not significant; hence, there is no need to mention them (Fig. 2). In Hyola308, the maximum level of the UCP expression was observed at 12 hpt.

The activity of the UCP gene was enhanced using mitochondrial preparations obtained from wheat seedlings subjected to middle and high salt stress. Therefore, the UCP gene involves in the ROS detoxification (Pastore *et al*., 2007). Salinity and drought stress tolerance was investigated in the transgenic tobacco plants that over-express an *AtUCP1* from *Arabidopsis thaliana*. The seeds of AtUCP1 transgenic lines were germinated sooner and the mature plants showed a better response to the salt and drought stresses, compared to the wild-type plants. In addition, the transgenic plants showed a low accumulation of hydrogen peroxide in the stressed leaves compared to the wild-type plants (Begcy *et al.*, 2011). Several studies showed that the UCP plays an important role in the preservation of mitochondrial function under stressful and normal conditions (Vercesi *et al.*, 1995) and this protection was related to lower oxidative stress (Begcy *et al.*, 2011).

As compared to the susceptible canola genotype, the UCP and AOX1a genes showed a higher expression after a long time of exposure to water stress in the drought tolerant genotype. According to the role of AOX1a and UCP in the scavenging mitochondrial ROS, this is the responsibility of both the genes, or either of them. Either of these genes could solely reduce the ROS amount in the absence of the other (Rhoads *et al*., 2006).

The maximum expression of the *external alternative NAD(P)H-ubiquinone oxidoreductase B2 (External NADPH-dehydrogenases, ex NDH*) gene was observed in the susceptible Hyola308 leaves at the mark of 4 hours and then the gene expression significantly decreased at 8, 12 and 24 hpt (the lowest expression level) (Fig. 3). Expression of this gene in the drought resistant plant leaves, SLM046, increased in the last hours, presenting the maximum level of expression at 24 hpt.

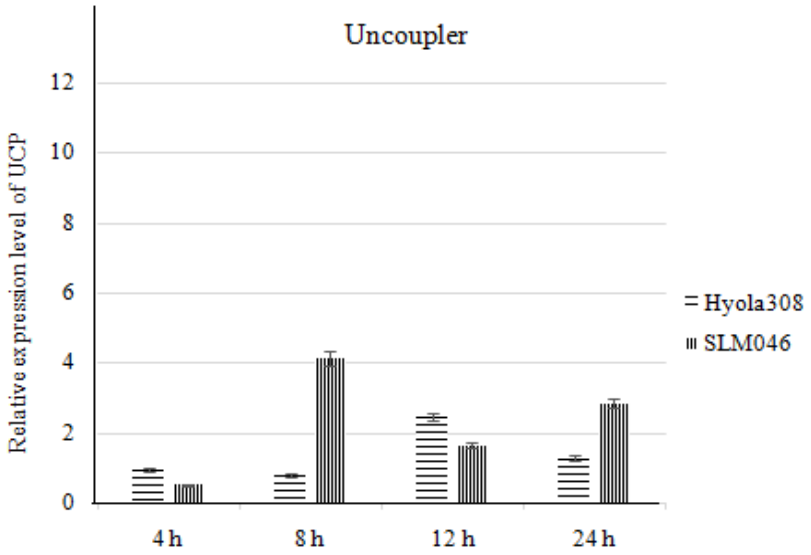

Figure. 2 Expression pattern of *UCP* gene using Real-time PCR under drought stress in seedling SLM046 and Hyola308.

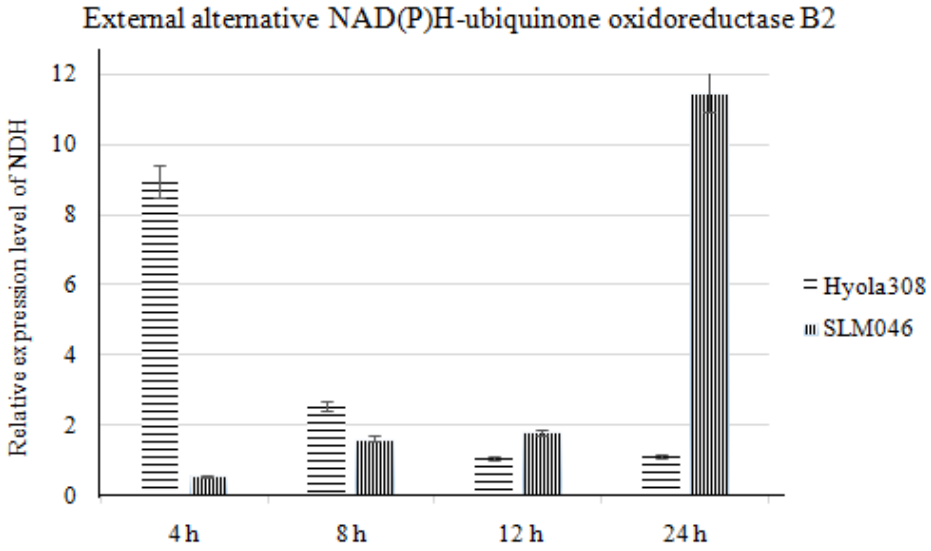

Figure. 3 Expression pattern of *exNDH* gene using Real-time PCR under drought stress in seedling SLM046 and Hyola308.

Peroxidase and *NDH*, as key important antioxidative enzymes, have been reported in olive plants under salinity conditions (Valderrama *et al.*, 2006). Also, it was observed that the capacity of matrix NAD(P)H oxidation through the rotenone-insensitive pathway has considerably decreased in the *Arabidopsis* mutant plant line (Atndi1) (Moore *et al.*, 2003).

After decreasing photosynthesis under drought stress, the products of light reactions such as NAD(P)H and ATP would not be used in the following reactions (Calvin cycle), and the production of ROS would be increased (Türkan *et al.*, 2005).

In the tolerant genotype, SLM046, the plants presenting the higher ex-NDH gene expression in the final hours of the stress reduced the higher amount of NAD(P)H. The results also showed that the NDH gene expression in the SLM046 genotype during the final

hours of the stress was far better than in the Hyola308 genotype. Thus, it can be concluded that SLM046 was able to decrease the oxidative stress, produced by the reactive oxygen, and by reducing the amount of NAD(P)H, prevent the damage caused by the drought, and also lead to decrease in oxidative stress produced by the reactive oxygen. The result showed that the susceptible genotype, Hyola308, could not maintain an increased *ex NDH* expression. Therefore, NAD(P)H would accumulate in the cell, and consequently, the ROS would also accumulate in the cell, which will lead to cell death (Mittler *et al.*, 2004).

Appropriate modulation of redox equivalents is known to significantly curtail the photoinhibitory damage (Shabnam *et al.*, 2015). Normally, the rate of light-driven $NADP^+$ reduction is balanced with the simultaneous oxidation of NADPH through carbon assimilation (i.e. the Calvin–Benson cycle) and other assimilatory pathways (Taniguchi and Miyake, 2012; Kramer *et al*., 2004). However, whenever the rate of light-induced generation of NADPH and reduced ferredoxin exceed their rate of oxidation, reductants accumulate in the thylakoid membranes and the stroma region. This results in cellular redox imbalance that favors an increase in the one-electron reduction of molecular oxygen, leading to the generation of the ROS (Schmitt *et al*., 2014). The ROS, in turn, causes photoinhibition by either damaging the components of the photosynthetic machinery (Photosystem II or PS-II) or inactivating the PS-II repair mechanisms through the suppression of protein synthesis (Nishiyama *et al.*, 2011). Thus, the cellular capacity to modulate the $NAD(P)H/NAD(P)^+$ ratio is critical not only for the redox control of the metabolism but also to restrain the oxidative stress (Shabnam *et al.*, 2015).

One of the major differences is the highly complex mETC (mitochondrial electron-transport chain), which, in particular, contains a system of alternative pathways—type-II NAD(P)H dehydrogenases and AOXs (alternative oxidases)—that allow a larger flexibility in the oxidation of NAD(P)H in plants (Rasmusson and Wallström, 2010). Plant type-II NAD(P)H dehydrogenases and AOXs are energy bypasses around the large multiprotein complexes of oxidative phosphorylation. Type-II NAD(P)H dehydrogenases reduce ubiquinone, and thus, circumvent respiratory complex-I, whereas the AOX genotype bypasses complexes III and IV of the cytochrome pathway by directly oxidizing ubiquinol (Rasmusson *et al*., 2004; Vanlerberghe and McIntosh, 1997; Fernie *et al*., 2004; Rasmusson *et al*., 2008). Respiration through the proton-pumping complexes I, III, and IV creates a proton gradient that is used by the ATP synthase for the production of ATP. Type-II NAD(P)H dehydrogenases and AOXs do not pump protons. Therefore, the activity of these proteins leads to a lower ATP production, and thus, decreases the respiratory energy conservation. In comparison, the changes in the activity of the AOX alone should simultaneously affect both the matrix and cytosolic pools of NADH and NADPH. Changes in the uncoupling protein activity should likewise modify the rate of oxidation for several mETC substrates due to an increase in ubiquinol oxidation in the presence of a lower electrochemical proton gradient across the inner mitochondrial membrane. However, in combination, an increase in the enzymatic capacity of an NAD(P)H dehydrogenase can direct an increased rate in AOX or the uncoupling protein to make the mETC preferentially utilize a particular reductant source, and that, too, in an

elevated flux (Rasmusson and Wallström, 2010).

Conclusion

Plant growth was reduced after the drought stress through changes in photosynthesis and leaf water condition. On the other hand, cells prevent the oxidative stress through the changes in gene expression. Considering the importance of *AOX*1a, *UCP*, and *exNDH* genes in reducing the oxidative stress caused by the drought stress, the decreased and increased expressions of these genes would lead to an increase or decrease in the levels of oxidative stress. The *AOX*1a gene reduced ubiquinone through oxidization and reduced molecular oxygen to water. Moreover, the *UCP* gene inhibited the ATP production in the mitochondria by dissipating membrane potential and uncoupling oxidation from phosphorylation. Also, *ex-NDH* gene reduced the amount of NAD(P)H by oxidizing NAD(P)H without producing ATP. The results showed that the expression of these studied genes in the tolerant genotype, SLM046, in comparison with the susceptible genotypes, Hyola308, increased more significantly after the drought stress, especially at the latest hours of the treatment. Therefore, a high expression of these genes, alone or together, could be considered an important factor behind the enhanced drought stress tolerance and the manipulation of these genes could be used to increase drought tolerance in canola plants.

This was the first attempt made to investigate some mitochondrial alternative factors of *Brassica napus* L. leaves in response to water stress. The information generated here could be useful for germplasm enhancement.

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