First Internship Protocol

Quantitative,Transcriptome and Expression Analysis of *Arabidopsis thaliana* and Barley (*Hordeum vulgare* L.) to Analyze their Drought-tolerance Capacity and Identify the Expression Patterns of the Drought-stress Induced Gene ALDH7B4

Presented to

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Abbreviations

WT	Wild Type
MA	Martin
HS	*Hordeum spontaneum*
MO	Moroc
EDTA	Ethylenediaminetetraacetic acid),
BHT	Butylated hydroxytoluene
TBA^1	Thiobarbituric acid
TBA^2	Tert-Butyl Alcohol
TCA	Trichloroacetic acid
MDA	Malondialdehyde
SDS	Sodium dodecyl-sulfate
ALDH	Aldehyde Dehydrogenase
RWC	Relative Water Content
CD	Control Deterioration
OE	Over Expression
KO	Knockout
LPO	Lipid Peroxidation
RES	Reactive electrophile species

1. Introduction

1.1. Drought Resistance in Plants

The mechnisms by which plants acclimate to enviornmental stresses has been gaining more attention by the the plant science community in recent years. Consequently, and due to the advent of more severe and extreme climate and weather patterns, understanding the physiological and molecular background of such tolerance has become a task of utmost importance for plant breeder, insofar that developing this knowldge will help them breed cultivars which are able to withstand the continuously changing environmental conditions and produce higher crop yield (Janiak et al., 2018).

Nonetheless, one of the most potent and deadly environmental stresses affecting a plethora of the world's crop plants is the drought stress. And by taking into consideration global climate change, the frequency of severe drought conditions is expected to increase (Dai, 2012). Kneebone et al. (1992) defines drought in meteorological terms as a conditon caused by a sufficiently long period of dry weather which instigates plant injuries.

Interestingly, due to the huge diversity of plant species across regions that experience extreme dry conditions, plants have evolved a wide range of morphological, physiological, and biochemical adaptive mechnaisms to endure and acclimate to drought stress (Bohnert et al., 1995). Henceforth, scientists have been conduting for the past two decades numerous studies on the morpholigcal traits as well as the underlying biochemical, physiological, and molceular regulatory mechanisms of drought stress, to unravel its eluding cellular and subcellular processes in plants (Reddy et al., 2004).

The four primary mechanisms by which plants acclimate to drought involve drought avoidance (DA), drought tolerance (DT), drought escape (DE), and drought recovery (DR) (Lawlor, 2103). However, the two major mechanisms that govern the processes confering plant tolerance are DA and DT (Yue et al., 2006). Morgan (1984) defines drought toelrnace in plants as their ability to endure and survive low tissue water content through the onset of adaptive traits including the maintenance of cell turgor and increasing protoplasmic resistance.

In contrast, Levitt (1980) defined DA as the ability of plants to maintain a relatively high tissue water content even though the water content of the soil has been reduced. DA is

achieved by various mechanisms that differ between water spenders and water savers. For instance, water spenders maintain high tissue water content by conserving their uptake of water through increased rooting and hydraulic conductances. On the other hand, water savers utilize the already uptaken water effictevely and reduce water loss by reducing transpiration and transpiration area (Morgan, 1984).

It is noteworthy to mention that drought resistance mechanisms which provide plants with the ability to merely survive longer leads to subsitence yield, which is significantly lower than the yield desired from crop plants. This observation can be attributed to the fact that crop plants are grown under optimal conditions for high agricultural production and are exposed to random and short-term drought stress, pressing them to continue to grow normally and yield crops despite being exposed to stressful enviornemnts (Basu et. al., 2016). Thereforfre, comprehending the various mechanisms by which plants respond duly to water stress is the primary challenge to enhancing crop drought tolerance (Deikman et al., 2012).

1.2. Determination of Drought Resistance in Plants

The determination of drought resistance has been proven to be more difficult than that of pointing out other stress resistances (Levitt, 1980). Nevertheless, specific trait or several combined indicators (e.g., DA-related indicators, DT-related indicators, and integrative indicators) have been employed by researchers to asses drought resistance (Luo, 2010).

On one hand, DA-associated indicators encompass moisture maintenance, water uptake, and water use efficiency. On the other thand, physiological parameters pertained to osmotic adjustment (e.g., osmotic potential, proline content, soluble sugar content, and ABA content) as well as parameters related to the alleviation of drought damage (e.g., the acitivities of protective enzymes and chlorophyll content) can be categorized under the DT-associated indicators. However, integrative indicatos governing complex traits related to biomass and economic yield (e.g., fresh weight, dry weight, survival rate, stay-green cpability, seed-setting rate, sppikelet fertility, and grain weight) are experimental methods utilized to assess drought resistance in crop plants. Although these integrative traits are meaningful in breeding plants with enhanced drought resistance, they rarely used to reveal the underlying physiological and molecular mechanisms of drought resistance (Fang and Xiong, 2015).

1.3. ALDH7B4 Gene Description

The Aldehyde Dehydrogenases (ALDH) protein superfamily exhibit a wide variety of roles in plants, which have been studied thoroughly in the model plant *Arabidopsis thaliana*. For instance, ALDHs are involved in ALDHs are involved in the endogenous and exogenous aldehyde metabolism such as carnitine biosynthesis, glycolysis/gluconeogenesis, and amino-acid metabolism (Yang et al. 2011), in which either NAD or NADP is used as a cofactor (Bartels and Sunkar 2005) to irreversibly oxidize a wide range of reactive aldehydes to their corresponding carboxylic acids (Perozich et al., 1999). Moreover, one group of important intermediates in many catabolic and biosynthetic pathways are compounds with aldehydic functional groups. For example, the maintenance of cellular redox balance relies on the ALDH-mediated generation of NADH/NADPH. Also, seed development and maturation requires the involvement of several ALDH enzymes (Shin et al. 2009).

In the light of the aformentioned information, several ALDH genes which are induced under saline and water deficit conditions have been identified in a wide variety of plant species (Chen and Murata, 2002). Nonetheless, ALDH genes may also respond to water logging, exposure to heavy metals, heat, cold, and ultraviolet radiation stressors (Chugh et al. 2011). Indicating that the upregulation of ALDH genes are a common target for the activation of stress response pathways in plants (Bartels and Sunkar 2005) and may play an essential role during osmotic stress adaptation (Chen and Murata, 2002). As such, scientists have a vested economic interest in utilizing ALDH expression to improve the growth and quality of crops cultivated under harsh conditions such as poor soil quality or drought (Bartels and Sunkar 2005).

172 ALDH genes which belong to more than 20 protein families have been identified in eukaryotes. These genes may encode for either substrate specific-enzymes or ALDHs that shows a highly variable substrate-specificity (Sophos and Vasiliou, 2003). The plant ALDH superfamily contains 13 distinct families: ALDH2, ALDH3, ALDH5, ALDH6, ALDH7, ALDH10, ALDH11, ALDH12, ALDH18, ALDH21, ALDH22, ALDH23 and ALDH24 (Broker et al., 2013). The gene of interest, ALDH7B4, belongs to a family 7 of the ALDH superfamily. In addition, members of the ALDH7 protein family are also known as δ1-

piperideine-6-carboxylate dehydrogenases, α-aminoadipic semialdehyde dehydrogenases or antiquitins, reflecting the antique nature of their evolutionary history (Lee et al. 1994).

The availability of the fully sequenced genome of the most studied and analyzed plant, *Arabidopsis thaliana*, porvides an important tool to study the organization and molecular relationships of ALDH genes in detail (Perozich et al., 1999). ALDH7B4 is a poorly studied gene in both *Arabidopsis thailiana* and barley. A previous experiment conducted on *Arabidopsis thaliana* analyzed the function of the first few hundred nucleotides upstream of the translation start codon by isolating the core promoter of ALDH7B4. The promoter was found to play an essential role in wound responses as well as salt and dehydration stress.

The analysis of cis-acting elements involved in stress responsiveness was performed revealing two conserved ACGT-containing motifs proximal to the translation start codon that are essential for the responsiveness to osmotic stress in leaves and in seeds. Nevertheless, it has been found that the integrity of the upstream ACGT motif and a dehydration-responsive element/C-repeat—low temperature-responsive element is of extreme importance for the maintenance of ALDH7B4 expression in seeds and induction by salt, dehydration and ABA in leaves (Missihoun et al., 2014).

Previous studies have coroborrated the involvement of ALDHs in growth, development, and stress adaptation. ALDH7B1, a member of the ALDH7 family, was the first plant ALDH7 plant protein to be discovered. ALDH7B1 of the garden pea (*Pisum sativum*) was shown to be expressed upon dehydration, low temperature, heat shock and ABA (Guerrero et al., 1990). In canola (*Brassica napus*), the upregulation of ALDH7 gene was reported under osmotic stress (Stroeher et al., 1995). Also, the upregulation of the expression of ALDH7B4 in transgenic tobacco (*Nicotiana tabacum*) plants further confirmed the importance of the ALDH7B4 protein in plant stress responses (Raza, 2010).

There is a tremendous amount of diversity in plant genome size, structural organization, and chromosome number, allowing scientists to use genomic and phylogentic data to study the distribution, the evolution, and expression patterns of ALDH genes. And there are many reasons which have impelled us to study the expression of ALDH7B4 in barley. Currently, there are no reports concerning the relationship between the ALDH7B4 gene in barley and drought stress. Therefore, the primary aim of this study is to analyse changes in the

expression patterns of the ALDH7B4 gene in barley. These changes may be helpful for the development of seed varieities that can withstand drought-stres in countries were drought is a prevalent phenomenon.

1.4. Aims of the Study

The study aims to analyze and characterize the expression patterns of the drought-stress induced gene ALDH7B4. The first section of the study was divided into two parts and was conducted on seven barley varieties. Four wild type barley varities named 111, 18780, 4565, and 906 were used to monitor the expression of the gene during the development of the plant. The three stages of dvelopment were monitored: the vegatative stage represented by the tillers, the stage of undeveloped seeds represented by seeds inside kernels, and the generative stage represented by the fully developed seeds. The second part was conducted on three different barely varieties called MA, Mo, and HS. Nevertheless, the propensity of the aforementioned varieties to withstand drought has been verified by conducting a test to reveal their respective relative water content. In addition, to verify the results obtained from the relative water content test on the molecular level, lipid peroxidation assay was performed to measure the level of peroxidation products, which reflects the propensity of the plants to withstand drought-stress.

The different expression patterns of ALDH7B4 gene between non-stressed control and stressed samples were recorded and analyzed. Moreover, the second section of the experiment was performed on the seven *Arabidopsis thaliana* varieties. ALDH7B4 is highly expressed in the reproductive organs of *Arabidopsis thaliana*, hence the different expression patterns in seeds during seed development were characterized before and after exposure to high moisture stress. To achieve the latter, two germination tests were made, one at the onset of the experiment before applying moisture stress to the sample, and a second one after exposing the seeds to high moisture conditions via the application of the control deterioration test. One experiment employing the quick and dirty DNA extraction method was done by me, however, the subsequent steps pertained to genotyping and genotyping PCR were done by the PhD student Abdel Aziz Nasr.

The two primary species in this manuscript were chosen because they allow for a relatively comprehensive look at how ALDH7B4 expressioon may vary between distant species. The central purpose of this manuscript is to expand upon and also consolidate the expression of

ALDH7B4 in *Arabidopsis thailian* and barley in an effort to highlight the role of ALDH7B4 in drought stress.

All the materials and methods utilized to perform the experiments as well as all the results have been reported in the following sections. Appendices have been attached at the end of this report to present the detailed recorded data which couldn't be presented in the body of this internship report.

2. Materials and Methods

2.1. Plant Material

Seven *Arabidopsis thaliana* varieties: wild type (WT), dog1, ALDH7B4 knockout (KO), ALDH7B4 overexpressed (OE), ALDH3F1 KO, ALDH3F1 OE, and ALDH3I1 KO, were chosen for conducting several expirments including the germination test, the control deterioration test, and the quick and dirty DNA analysis.

Three barley varieties were chosen (Martin, MA - *Hordeum spontaneum*, HS - Moroc, MO) for the measurement of the relative water content, analyzing the differences in the expression pattern of the gene ALDH7B4 between control and stressed samples. It is noteworthy to mention that some of these varieties were selected due to their exquisite performance under drought stress. The variety Martin is cultivated in North Africa and it has evolved to tolerate drought stress. Similarly, the variety HS is a pure line of *Hordeum vulgare* and has been chosen for its well adaptation to drought stress. However, Moroc is not well-adapted to drought stress and is considered to be sensitive to it (Ceccarelli et al., 2004).

Four barley (*Hordeum vulgare* L.) varieties (906, Morex - 111, viborg - 4654, Golden promis – 18780, Martin) were selected for the isolation of RNA from seeds, seeds inside kernels, and tillers as well as analyzing the different expression patterns of the gene ALDH7B4 in seeds.

2.2. Germination Test

The germination of four replicates of each plant variety was assessed using the warm standard germination method which is composed of two consecutive steps. In the first step, filter papers are placed on 24 plates (155mm in diameter). Using a marker, each plate was divided into 8 sections, the filter papers were imbibed with distilled water, and the seeds were dispersed equally over them. The plates were then covered and incubated in the cold and dark

room at 4°C for 3 days to promote the germination process and initiate stratification. In the second step, after three days, the plates were transferred to the short-day room for 7 days (8h, 16°C day; 16 h, 16°C night).

The germination test was conducted on seed samples for the following varieities: WT, dog1, 7B4 KO, 7B4 OE, 3F1 KO, 3F1 OE, and 3I1 KO. The numbers of germinated and non germinated seeds were recorded and the percent of germination was evaluated.

The percent of germination was assessed after 7 days of incubation in the short-day room using the following equation:

$$\text{Percent of Germination} = \frac{\text{number of germinated seeds}}{\text{total number of tested seeds}} \times 100$$

2.3. Relative Water Content

For conducting this experiment, three varieties of barley (MA, MO, and HS) have been grown on soil in pots in a greenhouse. Nonetheless, these varieties were separated into two batches with the first batch grown on soil irrigated with water whereas the second batch was grown with soil containing polyethylene glycol to mimic the effect of drought on barley plants in the greenhouse.

From each variety, 20 barely plants were grown (i.e., 20 for MA, 20 for MO, and 20 for HS) in such a way that 10 replicates from each variety represented the control (i.e., 10 for MA C, 10 for MO C, and 10 for HS C) and the other 10 replicates of the same variety represented the stressed plants (i.e., 10 for MA S, 10 for MO S, and 10 for HS S). Therefore, in total, 30 plants were grown on soil irrigated with water while 30 plants were grown on soil irrigated with polyethylene glycol. The plants were then transferred to the short day greenhouse growing chamber (8h, 20°C day; 16 h, 16°C night).

For the first few weeks of growth, all plants in both treatments have been watered equally everyday to make sure that there is enough water available for them to grow. Once the plants have grown a second leaf, half of the tested plant (drought stress treatment) were watered with 50 mL of -1 mPA polyethylene glycol for the next 10 days to initiate the drought stress condition. Whereas the other half (control treatment) was irrigated with water for the same

period of time as the drought stress tratment. The second leaves of each plant were obtained and weighed individually to evaluate their relative water content.

The first step in calculating the relative water content of the leaves requires measuring the fresh weight of these leaves. Hence, 7cm of the second leaf of each plant was cut and weighed on a balance.

The second step is to measure the turgor weight of the leaves. This requires the transfer of leaves into 50 mL falcon tubes and immersing the leaves with distilled water and incubating them at 4°C overnight under dark conditions. On the next day, the leaves were removed from the dark room, removed from the falcon tubes one by one, gently dried with paper towels to remove excess water from their surface, and weighed on a balance.

The third step is to measure the dry weight of the leaves. The leaves were dried in an oven at 80°C for 3 days. Then their weight was measured on a balance.

The relative water content (RWC) was evaluated after drought treatment (10 days after irrigating the stressed plants with 50 mL of -1 mPA polyethylene glycol) using the following equation:

$$\mathrm{RWC} = \frac{\text{fresh weight} - \text{dry weight}}{\text{turgid weight} - \text{dry weight}} \times 100$$ (Pieczynski et al. 2013)

2.4. Control Deterioration Test (CD Test)

The CD test was conducted on seed samples obtained from the following seven varieities: wild type (WT), dog1, ALDH7B4 knockout (KO), ALDH7B4 overexpressed (OE), ALDH3F1 KO, ALDH3F1 OE, and ALDH3I1 KO. From each variety, 8 replicates were made. On a 99 well plate, seeds from each variety were transferred individually to the first 8 wells of the plate. The 99 well plate was then placed in a large plastic container which contains two chambers. The first chamber was reserved for the 99 well plate while the second chamber was reserved for the supersaturated NaCl solution. For preparing the supersaturated NaCl solution, 89.75 g of NaCl was dissolved in 250 mL of distilled water.

Every three days, the following batch of seed replicates was transferred to the 8 wells adjacent to the previously transferred seed replicates. The aforementioned process was repeated for all

the replicates over a period of 24 days. Once all the seed replicates have been transferred to the 99 well plate, the germination test can be commenced.

The CD test was performed on all seven seed varieties and their replicates which have been incubated at a temperature of 37°C and 75% relative humidity, until the last group of seed replicates was tranfered to the 99 well plate.

The 99 well plate was eventually transferred to the cold room for 3 days to stimulate the germination process. Afterwards, the germination test describd in section 2.2 was conducted.

2.5. RNA extraction using Trizol Reagent Method

With the help of a pistol, mortar, and liquid nitrogen, a sufficient amount of plant material was finely grinded and subsequently transferred into 50 ml tubes. Upon the successful grinding, 50 to 100 ml of the grinded plant material (obtained from the following varieties: 111, 1870, 906, 4565, MA C, MA S, MO C, MO S, HS C, and HS S) was transferred into 2 ml Eppendorf and the remaining plant material was stored at -80°C. Next, 1 ml of Trizol Reagent was added to each sample and the resulting mixtures were briefly vortexed. To ensure that the nucleoprotein complexes were completely dissociated, the samples were incubated at room temperature for five minutes. Under the laminar flow hood, 0.2 mL of cholorform was added to each sample and after gently inverting the Eppendorf tubes to homogenize the mixture, the samples were incubated at room temperature for 3 minutes.

The resulting mixtures were then centrifuged (12.000 rpm, 15 minutes, 4°C), separating the mixtures into three phases. The lower phase contained the red-phenol chloroform whereas the upper phase contained the colorless aqueous with the interphase separating the lower and upper phases. The upper aqueous phase which contains the RNA was transferred to new 2 mL Eppendorf tubes. The obtained RNA samples were stored at -80°C.

2.6. cDNA Synthesis Procedure using ReverseAid Reverse Transcriptase

2.6.1. DNase treatment

The DNase treatment procedure is used to treat the total RNA acquired from the plant material (obtained from the following varieties: 111, 1870, 906, 4565, MA C, MA S, MO C,

MO S, HS C, and HS S) were to make sure that there are no traces of DNA contaminating the samples.

After adding 1µl RNase-free DNase I enzyme to 4 µl of total RNA, the samples were incubated at 37°C for 10 minutes. Afterwards, the reaction was stopped by adding 1 µl of 25 mM EDTA and re-incubating the samples at 65°C for 10 min. The resulting samples were separated into two tubes of equal volumes (5 µl of mixture) for positive and negative controls.

2.6.2. cDNA Synthesis

1 µl oligo-dt primer and 0.5 µl MiliQ were added to all positive and negative control samples, gently mixed, and incubated at 65°C for 5 min.

For the treatment of the positive's samples, 2 µl of 5x first strand buffer, 1 µl of 10 mM dNTP Mix, and 0.5 µl of Reverse Transcriptase enzyme were added, making a total volume of 10 µl.

However, for the treatment of the negative control's samples, 2 µl of 5x first strand buffer, 1 µl of 10 mM dNTP Mix, and 0.5 µl of MiliQ Water were added, making a total volume of 10 µl.

Table 2.1. cDNA synthesis components added to the pretreated DNase free cDNA first strand

Positive Treatment		Negative Treatment	
5x First Strand Buffer	2 µl	5x First Strand Buffer	2 µl
10mM dNTP Mix	1 µl	10mM dNTP Mix	1 µl
Reverse Transcriptase Enzyme	0.5 µl	MiliQ Water	0.5 µl
Total Volume	10 µl	Total Volume	10 µl

The tubes were gently mixed and incubated at 42°C for 60 min. The reaction was terminated with the incubation of the samples at 72°C for 5 min. Eventually, the cDNA product was diluted in 40 µl MiliQ with a total volume of 50 µl.

2.7. Quick and Dirty DNA Protocol for PCR Analysis

To commence with the quick and dirty DNA preparation for *Arabidopsis Thaliana* samples, the DNA extraction buffer was prepared by mixing 250 mM NaCl, 25mM EDTA (ethylenediaminetetraacetic acid), 200mM Tris/HCL with pH 7.5, and 0.5% (w/v) SDS (sodium dodecyl-sulfate).

From each plant sample, one leaf was cut and transferred to a 1.5 mL Eppendorf tube. After adding 500 µl of the extraction buffer to each tube, the plant material was homogenized thoroughly using a plastic pestle for 15 seconds, vortexed efficiently, and incubated for 5 min at room temperature. The tubes were then centrifuged at maximum speed for 5 min to pellet debris, and 300 µl of the supernatant were transferred individually to new 1.5 mL Eppendorf tubes. Afterwards, to harvest the nucleic acids, the newly transferred tubes were centrifuged at full speed for 5 minutes. Subsequently, the supernatant was discarded carefully, the nucleic acid pellet was washed with 100 µl 70% ethanol, and the tubes were left to air dry for at least 10 minutes. Finally, the nucleic acid pellet was dissolved in 50 µl distilled water.

2.8. Reference and Gene-of-Interest PCRs

Regular PCR was executed on the previously extracted cDNA samples. The expression of both control and under-stress cDNA samples was compared to a reference gene that is invariably expressed in both the control and stressed plants. Henceforth, forward and reverse ADP primers were used to determine the level of expression of the reference gene. The PCR was repeated multiple times to ensure that the level of expression of every pair of samples (stressed and control) on gel was equilibrated by adjusting the volume of cDNA in every round of PCR. To determine the volume of water that needs to be added, the original water volume (14.3 µl) was subtracted from the volume of cDNA. The optimum volume of cDNA was recorded.

Table 2.2. The components of Regular PCR using ADP primers

Reference Gene PCR	
Distilled water	14.3 µl
10x Buffer	2 µl
ADP Forward Primer	0.4 µl
ADP Reverse Primer	0.4 µl
dNTPs	0.4 µl
Taq Polymerase	0.5 µl
Total Volume	18 µl

Next, the level of ALDH7B4 was determined by using the forward and reverse mlfo primers. The same volumes as those recorded previously from the reference gene primers were used in the gene-of-interest PCR analysis. However, if there were some aberrations in between the stressed and control of each pair of samples, the levels of expression cDNA were equilibrated by changing the volume of cDNA in the following rounds of PCR.

Table 2.3. The components of Gene-of-Interest PCR using mlfo primers

Gene-of-Interest PCR	
Distilled water	14.3 µl
10x Buffer	2 µl
mlfc Forward Primer	0.4 µl
mlfc Reverse Primer	0.4 µl
dNTPs	0.4 µl
Taq Polymerase	0.5 µl
Total Volume	18 µl

2.9. Lipid Peroxidation Assay Method

The thiobarbituric acid (TBA[1]) test was used to measure the level of peroxidation products in the following barely varieties: MA C, MA S, MO C, MO S, HS C, and HS S. The TBA[1] test depends on determining the amount of malondialdehyde (MDA), which is the end product of the lipid peroxidation process (Hodges et al., 1999).

Before starting the experiment, the reagents were prepared. First, 0.1% (w/v) trichloroacetic acid (TCA) was prepared by mixing 0.1 g TCA with 100 mL of distilled water. Second, the 20% (w/v) TCA was prepared by mixing 20 g of TCA with 100 mL of distilled water. Third, 0.65% tert-Butyl Alcohol (TBA[2]) was prepared by mixing 0.65 g of TBA[2] with 100 mL of distilled water. Fourth, Reagent Solution I (RS I) was prepared by adding the previously prepared 20% (w/v) TCA to 0.01% Butylated hydroxytoluene (BHT). Fifth, Reagent Solution II (RS II) was prepared by mixing RS I with 0.65% TBA[2].

The obtained plant tissue was grinded, transferred to 1.5 mL Eppendorf tubes, and homogenized with 1 mL pre-chilled 0.1% (w/v) trichloroacetic acid (TCA) solution. Next, the mixtures were centrifuged (13,000 rpm, 5 minutes, 4°C). Then, 500 µl of the supernatant was transferred to a new Eppendorf tubes and homogenized with one volume of the Reagent Solution II in 15 mL Falcon tubes. After vigorously mixing the samples, they were transferred

to a water bath and boiled at 95°C for 25 min. the reaction was ceased by placing the samples on ice and centrifuging them (5,000 rpm, 5 minutes, 4°C). using a spectrophotometer, the absorbance was measured at three different wavelengths, 440 nm (sugar absorbance), 532 nm (maximum absorbance of pinkish-red chromagen product of the reaction of MDA with TBA), and 600 nm (turbidity). 500 µl of 0.1% (w/v) TCA was used as a reference solution.

The MDA content (nmol ml^{-1}) of the samples was evaluated using the following formula:

$$\text{MDA equivalents (nmol ml\^} - 1) = \frac{[A - B]}{157{,}000} \times 1{,}000{,}000$$

Where A = [(Abs 532_{RSII} – Abs 600_{RSII})] and B = [(Abs 440_{RSII} – Abs 600_{RSII}) × 0.0571]

Furthermore, the MDA content of the fresh weight (nmol ml^{-1} FW) was evaluated using the following formula:

$$\text{MDA equivalents FW} = \frac{\text{MDA equivalents (nmol ml}^{-1}) \times \text{total volme of the extracts (mL)}}{\text{g FW or number of seedlings}}$$

2.10. Isolation of RNA from Seeds Containing High Levels of Starch

The protocol of Wang et al. (2011) was used to extract RNA from seeds containing high levels of starch. Before starting the experiment, the extraction buffer and 20% SDS solution were prepared. Firstly, 3 mL of the extraction buffer was prepared by mixing 200 µl 1.5 M Tris-HCL (pH 9.0) with 60 µl 2% ß-mercaptoethanol and 460 µl MiliQ water. Secondly, 50 mL of the 20% SDS solution was prepared by thoroughly dissolving 10 g SDS in 50 mL distilled water. Subsequently, by using liquid nitrogen in mortar and pestle, the immature seeds were grinded into fine powder. Around 200 mg of grinded seeds were transferred to a 1.5 mL Eppendorf tube and 400 µl of the extraction buffer was added. The samples were mixed thoroughly with a vortexer and incubated at room temperature for 15 min.

After adding 20 µl of 20% SDS into the suspension, the samples were inverted gently five times and incubated at room temperature for 5 min. The mixture was then centrifuged (12,000 rpm, 10 minutes, 4°C). 200 mL of the aqueous phase was transferred to a 1.5 mL Eppendorf tube and two volumes of Trizol (400 mL) were added. The samples were mixed thoroughly using a vortexer and incubated at room temperature for 10 min. Then, 1/5 volume of cholorform (120 µl) was added to the Trizol and mixed thoroughly with a vortexer then

centrifuged (12,000 rpm, 10 minutes, 4°C). 400 µl of the aqueous phase was transferred (avoiding the interphase) to a new 1.5 mL Eppendorf tube, mixed with an equal volume of isopropanol, inverted several times, and precipitated at -20°C for 20 min. Later, the samples were centrifuged (12,000 rpm, 10 minutes, 4°C). The pellet was resuspended gently in 400 µl MiliQ water after discarding the supernatant. After adding 200 µl of citrate saturated phenol (pH 4.3) and 200 µl of chloroform, the mixture was mixed thoroughly using a vortexer. Later, the samples were centrifuged (12,000 rpm, 10 minutes, 4°C). Into a new 1.5 mL Eppendorf tubes, the aqueous phase (200 µl) was carefully transferred while avoiding the interphase, 200 µl of cholorform was added, and the samples were mixed thoroughly with a vortexer. Later, the samples were centrifuged (12,000 rpm, 10 minutes, 4°C). 200 µl of the aqueous phase was transferred to new 1.5 mL Eppendorf tubes. Two volumes of ice-cooled ethanol (400 µl) and 1/10 volume of 3 M sodium acetate (pH 4.8, 20 µl) were added and mixed thoroughly by inversion.

The samples were precipitated at -80°C for 30 min. Later, the samples were centrifuged (12,000 rpm, 20 minutes, 4°C). using a pipette, the supernatant was removed from each sample and 500 µl of ice-cooled ethanol was added, and the tubes were tapped gently to make sure that the pellet has been freed into the ethanol. The samples were centrifuged (12,000 rpm, 5 minutes, 4°C). After discarding the supernatant, the pellet was allowed to air-dry for 10 min at room temperature. 20 µl of MiliQ water was used to dissolve the pellet. Finally, the samples were stored at -80°C for further use.

3. Results and Discussion

3.1. Germination Test

The germination test has been conducted on the seven Arabidopsis thaliana varieties WT, dog1, 7B4 KO, 7B4 OE, 3F1 KO, 3F1 OE, and 3I1 KO to determine the percent of germination of the seeds. Since 4 replicates from each variety was used to conduct the test, data (the number of germinated seeds and the total number of seeds) for each replicate have been evaluated independently (refer to appendices number 1, 2, 3, 4, 5, 6, and 7). Thereafter, the average of the percent of germination for each variety was evaluated.

The final data was presented in table 3.1. Moreover, in figure 3.1., the aforementioned data was represented graphically in a plot chart. The following varieties: WT, dog1, ALDH7B4

OE, ALDH3F1 KO, and ALDH3I1 KO exhibited a relatively high percent of germination above 51 %. Whereas the remaining two varities: ALDH7B4 KO and ALDH3F1 OE revealed a relatively low percent of germination below 31%.

It is typical of the WT to show a relatively high percent of germination (76.88 %). On the other hand, since the dog1 mutant had a 67.1 % average of percent of germination, which is lower than wild type‘s (76.88 %), it can be said that Dog1 gene may play a role in enhancing the germination of the seeds.

Moreover, the over-expression of ALDH7B4 resulted in a 79.2 % average of percent of germination which is high than wild type’s (76.88 %). This shows that over-expressing the gene can increase the number of germinating seeds.

Nonetheless, the knock out of ALDH3F1 exhibited an average of percent of germination of 79.02 %, which is higher than wild type’s (76.88 %), indicating that knocking out this gene won’t affect the rate of germinating seeds. Whereas, the knock out of ALDH3I1 showed a moderate average percent of germination (51.6 %), which is lower than wild type’s type’s (76.88 %). Hence, knocking out ALDH3I1 can dramatically decrease the number of germinating seeds.

Finally, the remaining two varities: ALDH7B4 KO and ALDH3F1 OE revealed a relatively low percent of germination of 30.73 % and 13.5 % respectively, revealling that knocking out ALDH7B4 and over-expressing ALDH3F1 genes results in lower than average percent of germination.

Table 3.1. The average of percent of germination of seven Arabidopsis thaliana varieties WT, dog1, 7B4 KO, 7B4 OE, 3F1 KO, 3F1 OE, and 3I1 KO

Variety	Average of Percent of Germination
WT	76.88911516
dog1	67.89212403
7B4 KO	30.73273912
7B4 OE	79.28410572
3F1 KO	79.02832859
3F1 OE	13.59809231
3I1 KO	51.65274025

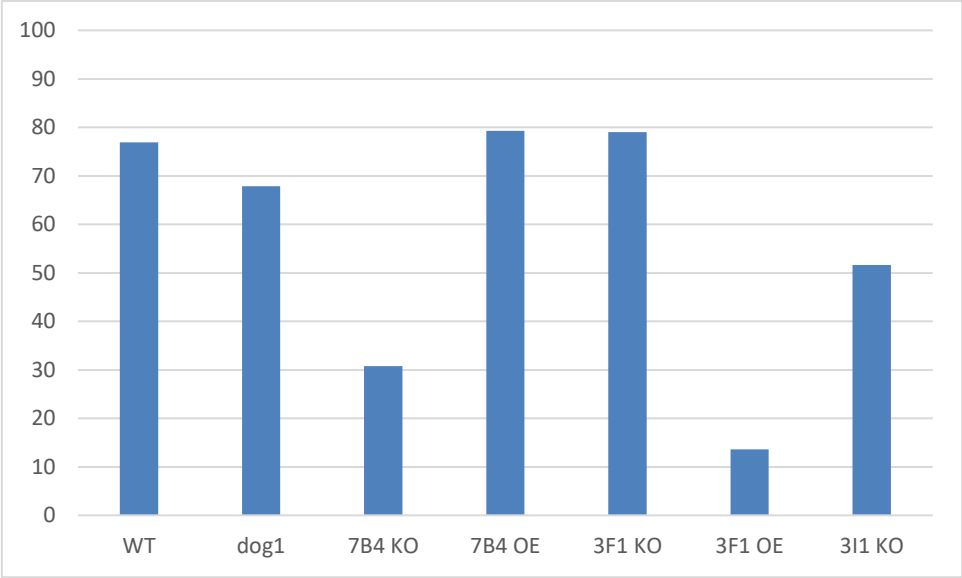

Figure 3.1. Plot Chart of the average of percent of germination of the seven Arabidopsis thaliana varieties WT, dog1, 7B4 KO, 7B4 OE, 3F1 KO, 3F1 OE, and 3I1 KO

3.2. Relative Water Content

The relative water content was determined for the three barley varieties HS, MA, and MO. Each barley variety was divided into two categories, control and stressed. In each category, 10 replicates from the same variety was used to conduct the test. The data which includes the fresh weight, dry weight, and turgor weights for each of the replicates have been evaluated independently (refer to appendices number 8, 9, and 10). Upon obtaining the results, the relative water content for each variety was evaluated, and the average RWC was calculated. The final data is presented in table 3.2. In figure 3.2., the aforementioned data is represented graphically in a plot chart.

The tested drought-tolerant HS and MA barley varities showed consistent results. For instance, the control replicates of HS and MA varieities exhibited a relativley high RWC exceeding 90 %. Likewise, the stressed samples of HS and MA varieities exhibited a RWC ranging between 82-88 %, which is relatively lower than the RWC of the conrol replicates.

However, MO, even though it is a drought-sensitivie variety, which should be drought-sensitive, performed exceptionally well as control (92.38 %) and under stress (85.79 %) exhibiting high RWC. This shows that two new replications of this experiment must be conducted to re-evaluate whether the MO variety is actually drought sensitive or not.

Table 3.2. The RWC of the control and stressed replicates of HS, MA, and MO barley varieties

Variety	Category	RWC Average
HS	Control	95.96030897
HS	Stressed	88.29003871
MA	Control	96.48940598
MA	Stressed	82.3451268
MO	Control	92.38410538
MO	Stressed	85.79401649

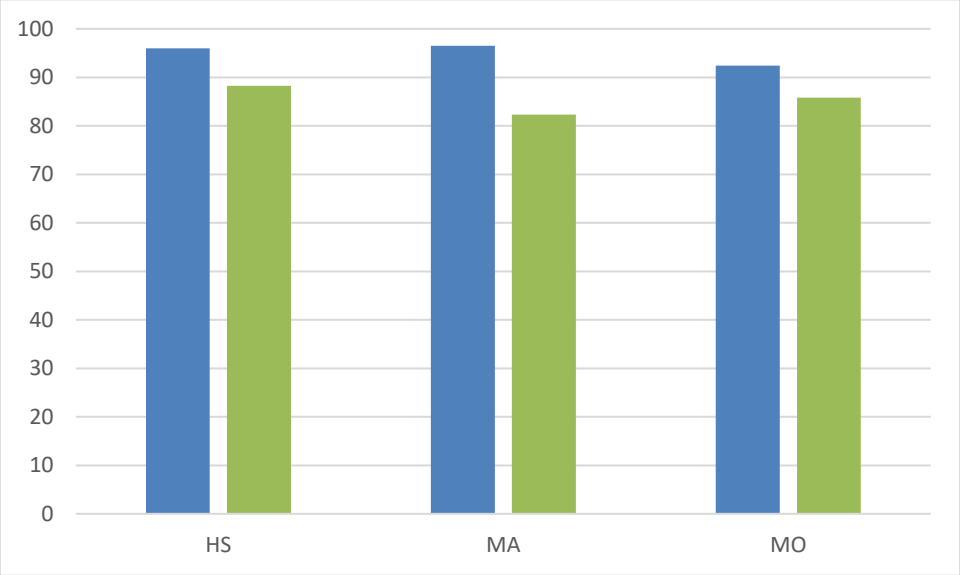

Figure 3.2. Plot chart of the RWC of the control and stressed replicates of HS, MA, and MO barley varieties

3.3. Control Deterioration (CD) Test

The control deterioration test is a vigor test (Powell and Matthews, 2005) designed to study the longevity of seeds, how would seeds germinate when exposed to high moisture stress conditions, and to predict field emergence potential of seed lots of many crops such as such as onion and carrot (Matthews, 1980). The CD test has been chosen for this experiment because it has been propsed to be a more a suitable vigor test for small seeds (Powell and Matthews, 2005).

In this study, the CD test was conducted to evaluate and predict field emergence in the seeds of the following *Arabidopsis thaliana* varieties: WT, dog1, ALDH7B4 KO, ALDH7B4 OE, ALDH3F1 KO, ALDH3F1 OE, and ALDH3I1 KO. As discussed in the methods and

materials section, at the end of the 21st day, the seeds were dispersed over plates, left to germinate for 7 days, and counted. Since 3 replicates from each variety was used to conduct the test, data (the number of germinated seeds and the total number of seeds) for each replicate have been evaluated independently.

The final data presented in table 3.3 shows the percent of germination of the seeds and the average perccent of germination. The detailed data describing each plate have been added to the appendices section (refer to appenices 11, 12, 13, 14, 15, 16, and 17). In figure 3.2., the aforementioned data is represented graphically in a plot chart. After analyzing table 3.3, it has been determined that the percent of germination flucutated erratically throughout the 21 days. The seed emergence percentage ranged between 88 and 0%. Therefore, all of the tested varities didn't show any consistent results.

What was expected, is to see the percentage of germination decrease gradually as the number of days of incubation under stress increase. In other words, there should be inversely proportional relationship between the number of days and percent of germination. However, the results were far from being consistent.

For instance, in the WT variety, the percent of germination increases from 56.74 % in day 21 to 78.47 % in day 18, which follows aforementioned inversely proportional rule. However, the percent of germination decrease from 78.47 % in day 18 to 43 % in day 15, showing a directly proportional relationship between the number of days of incubation and the percent of germination. This kind of fluctuations has been observed in all of the tested samples. Therefore, this experiment is ought to be conducted again, hoping that new and better results can be obtained.

Table 3.3. The percent of germination of seeds of the following varieties WT, dog1, 7B4 KO, 7B4 OE, 3F1 KO, 3F1 OE, and 3I1 KO, which were exposed to high moisture stress.

	Variety						
Days	**WT**	**dog1**	**7B4 KO**	**7B4 OE**	**3F1 KO**	**3F1 OE**	**3I1 KO**
21	56.7442	6.78733	57.2034	63.9216	44.8	46.4286	70.7317
18	78.4722	9.48276	55.9567	68.2741	50.3247	56.6845	76.03
15	43.0556	74.4493	54.8736	75.7576	87.234	54.5064	36.5741
12	78.4983	59.3625	11.6402	63.0372	81.1798	50.1247	0.64935
9	86.2745	78.1646	20.2765	80.7273	82.699	45.2663	0
6	88.1773	67.2862	47.7987	82.1561	74.477	68.0912	1.86567
3	78.9157	69.2547	26.4706	84.7403	94.0594	54.8263	23.3333
0	75	87.963	16.5179	49.789	67.6923	44.3114	38.5301
Average	73.1422	56.5938	36.3422	71.0504	72.8083	52.5299	30.9643

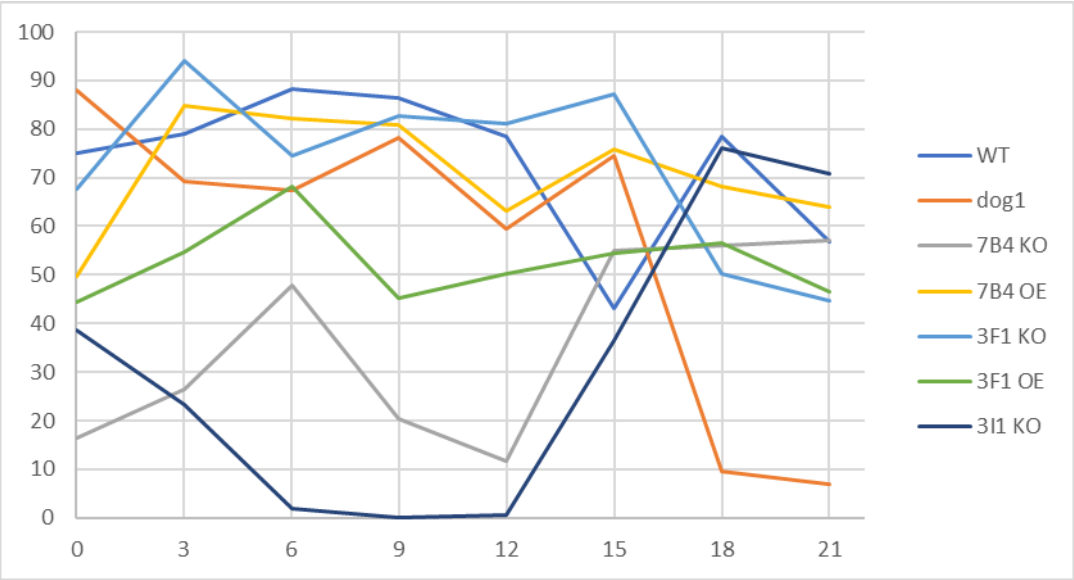

Figure 3.3. Plot chart of the percent of germination of seeds of the following varieties WT, dog1, 7B4 KO, 7B4 OE, 3F1 KO, 3F1 OE, and 3I1 KO, which were exposed to high moisture stress.

However, figure 3.4 shows how a typical graph of a CD test is ought to look like. The graph was obtained from my current supervisor and PhD student Abdel Aziz. In this figure the relationship between the percent of germination and number of days is truly inversely proportional. As the number of days of incubation under stress increase, the percent of germination of seeds is expected to intensely decrease.

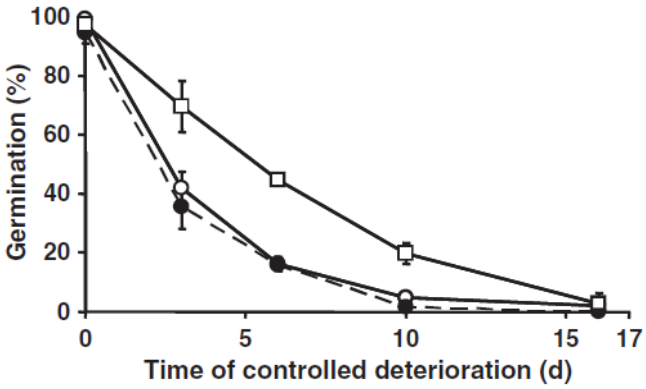

Figure 3.4. Plot chart of a typical scenario where the relationship between the percent of germination and number of days is inversely proportional.

3.4. Reference and Gene-of-Interest PCRs

3.4.1. RNA Extraction

The samples obtained from RNA extraction were run on gel (with ethidium bromide added) at 110 V. Then, the gels were visualized on a UV transilluminator. The results of RNA extraction are shown in figures 3.5, 3.6, and 3.7. All of the extracted RNA samples shown in figures 3.5 and 3.6 displayed coherent results and showed two bands with the upper band representing the 28S rRNA and the lower band representing the 18S rRNA. However, not all of the RNA shown in figure 3.7 showed desirable results. Only three (4654 of sample 1, 111 of sample 2, and 18780 of sample 2) out of six tested samples exhibited desirable results as shown in figure 3.7. The samples which showed prominent results, were used to synthesize cDNA and test the expression of ALDH7B4.

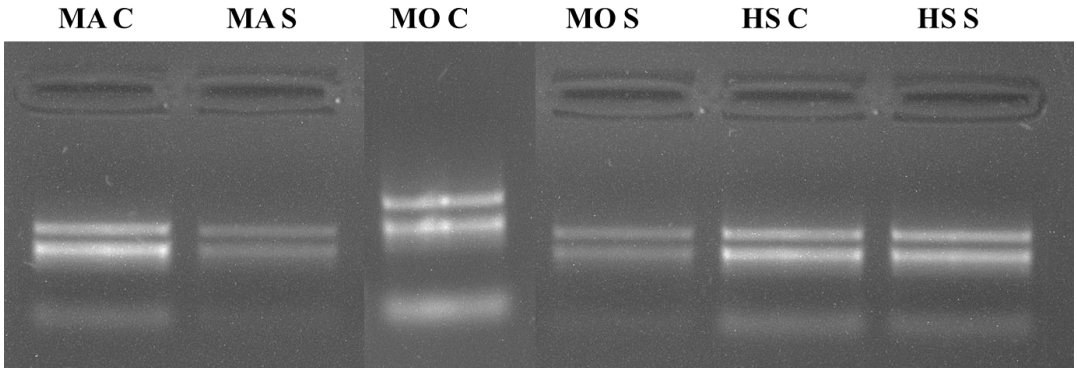

Figure 3.5. RNA extraction result for MA, MO, and HS of *Hordeum* using urea the Trizol Reagent method.

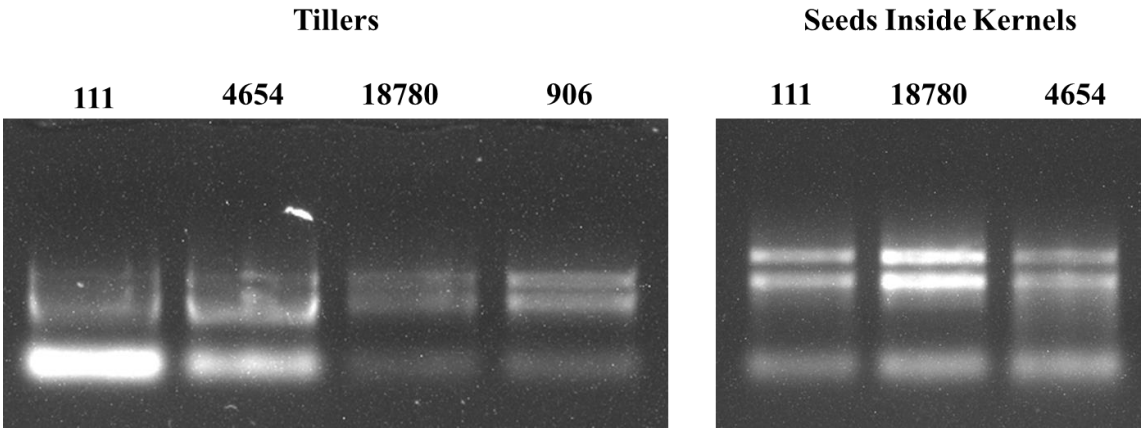

Figure 3.6. RNA extraction result for tillers (111, 4654, 18780, and 906) and seeds inside kernels (111, 4654, and 18780) of *Hordeum* using urea the Trizol Reagent method.

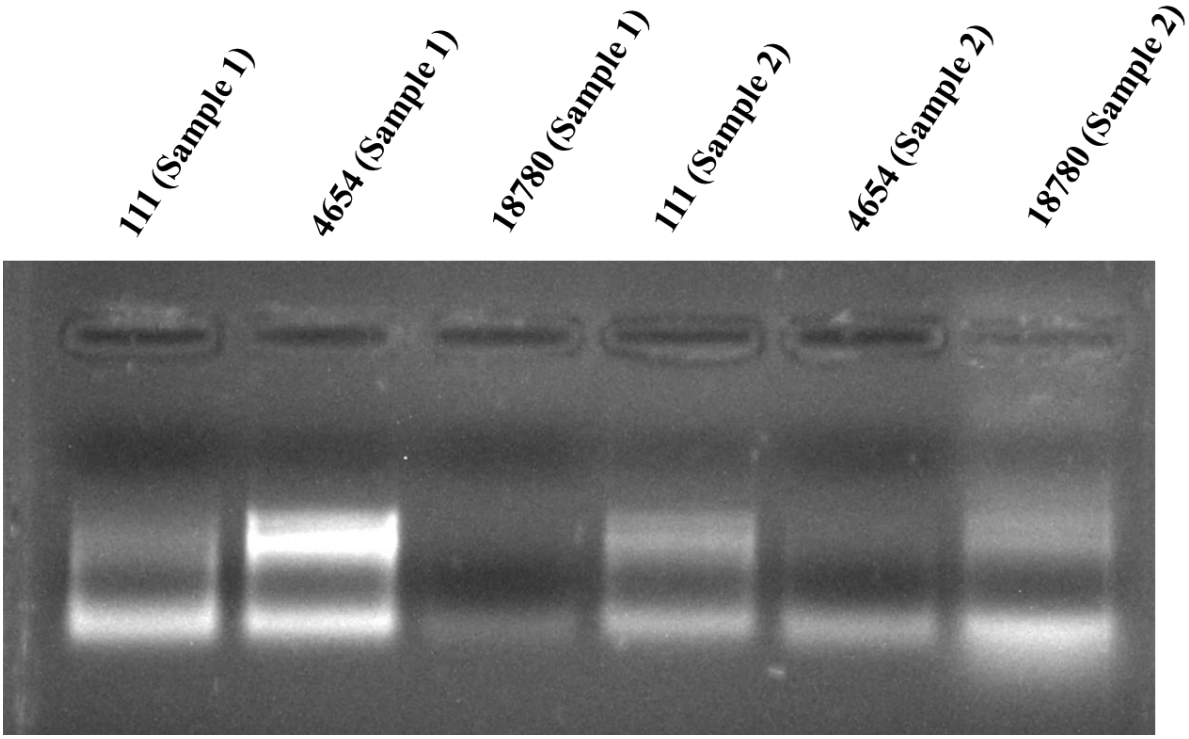

Figure 3.7. RNA extraction result for seeds (111, 4654, 18780, and 906) of *Hordeum* which contains high levels of starch using the protocol of Wang et al. (2011).

3.4.2. cDNA Synthesis

3.4.2.1. cDNA Synthesis of Barley Varieties Using ADP Primers

The run samples of RNA were used to synthesize cDNA samples with ADP primers of the reference gene as discussed earlier in section 2.6.2. In the following section, the results of RNA extraction are shown in figures 3.8, 3.9, 3.10, 3.11, 3.12, and 3.13. All of the synthesized cDNA samples displayed good results and showed one band with the same intensity. The same intensity of the bands reflects the equal expression of the ADP gene in all of the tested samples. There was equal expression between stressed and control samples for MA, MO, and HS and between tillers and seeds inside kernels for 111, 18780, and 4654. The samples which showed prominent results, were then used to synthesize cDNA by using the gene-of-interest's (ALDH7B4 gene) primers (mlfo primers).

It is noteworthy to mention that barley variety number 906 was not analyzed because what could only be collected from the plant was the tillers. Since no seeds and seeds inside kernels were collected, it is difficult to compare the expression patterns since some of data is not available. Therefore, 906 was excluded from my work and the experiments were commenced.

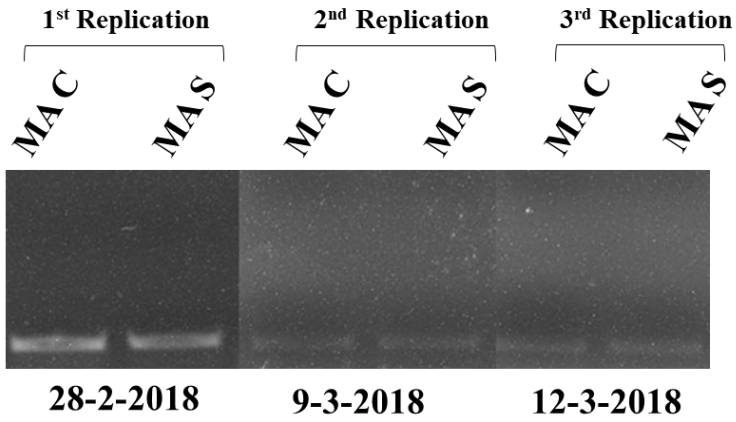

Figure 3.8. cDNA synthesis using ADP primers for control and stressed samples of MA barley variety

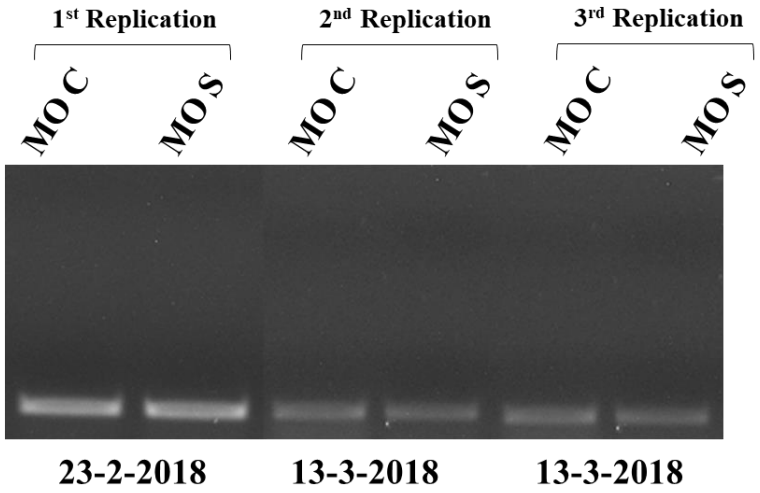

Figure 3.9. cDNA synthesis using ADP primers for control and stressed samples of MO barley variety

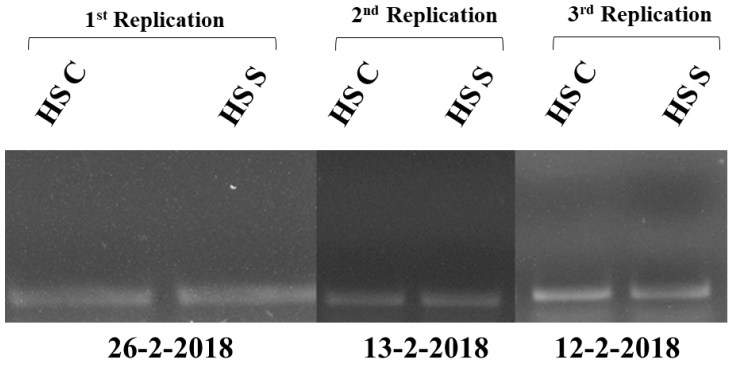

Figure 3.10. cDNA synthesis using ADP primers for control & stressed samples of HS barley variety

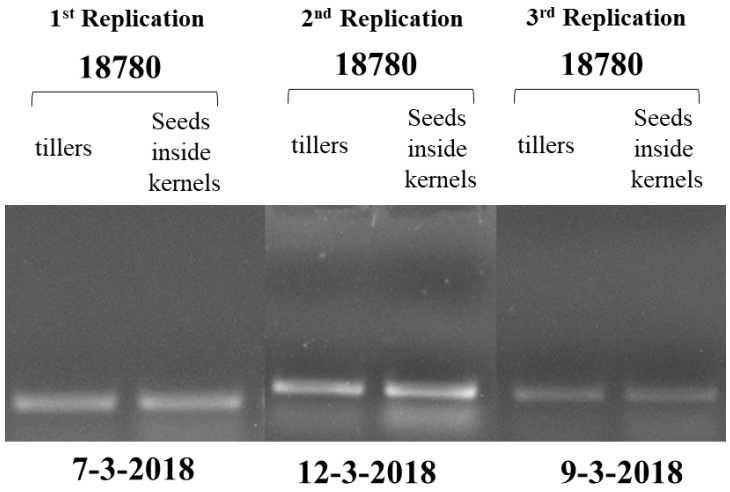

Figure 3.11. cDNA synthesis using ADP primers for tillers and seeds inside kernels samples of 18780 barley variety.

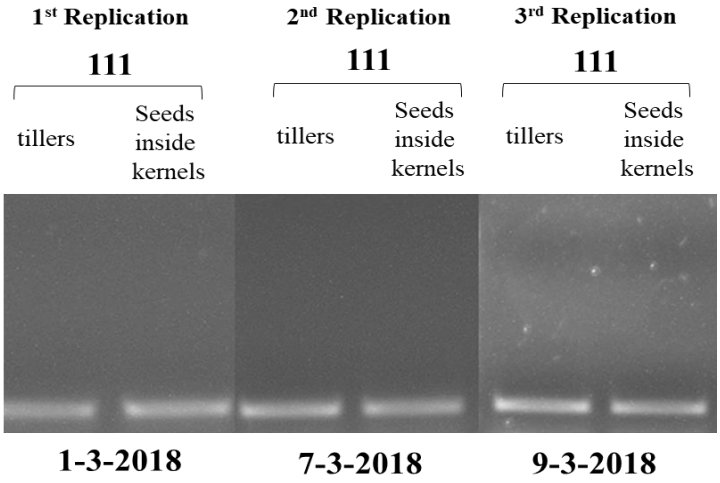

Figure 3.12. cDNA synthesis using ADP primers for tillers and seeds inside kernels samples of 111 barley variety.

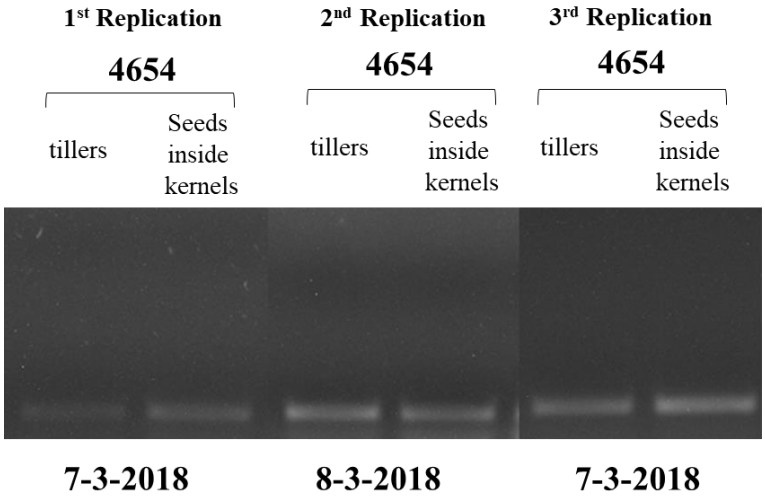

Figure 3.13. cDNA synthesis using ADP primers for tillers and seeds inside kernels samples of 4654 barley variety.

3.4.2.2. cDNA Synthesis of Barley Varieties Using mlfo Primers

After adjusting the samples using ADP primers, the previously run RNA samples were used to synthesize cDNA samples with mlfo primers of the gene-of-interest ALDH7B4. The results of RNA extraction are shown in figures 3.14 and 3.15. All of the synthesized cDNA samples showed acceptable results that reflects the divergence in the expression of ALDH7B4

between our control and stressed MA, MO, and HS barley varieties, as well as between tillers and seeds inside kernels in 111, 1878, and 4654 barley varieties.

In figure 3.14, the stressed samples of MA and HS varieties showed higher expression of ALDH7B4 as compared to control samples. This shows that ALDH7B4 plays an important role in moderating barley drought-tolerance. However, the control and stressed samples of MO showed similar expression levels of ALDH7B4. This indicates that the expression level does not vary whether plant is under stress or not. However, since MO variety is drought-sensitive, then what would be expected is to observe a relatively low expression level in the stressed sample, which is not the case here.

In addition, as shown in figure 3.15, the expression levels of ALDH7B4 was higher in seeds inside kernels than in tillers. Since the seeds inside kernels are in the generative stage and dehydrated, plants produce more of ALDH7B4 to increase their tolerance to long periods of drought stress, before they come in contact with water source and germinate.

Regarding sample number 18780, the reason why only one replication has been described is due to lack of good results in the second and third replication. To confirm the result of the first replication, two more replications should be made.

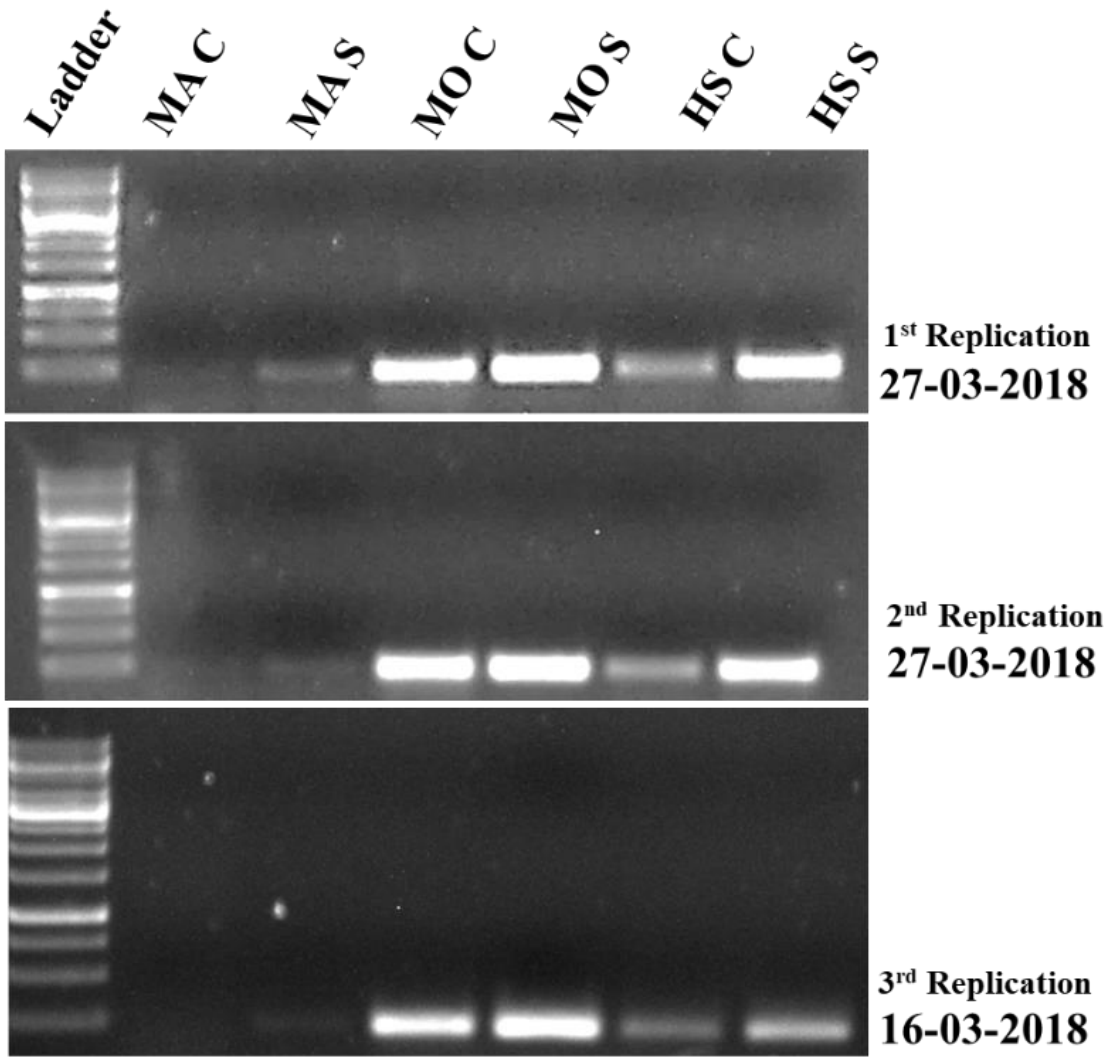

Figure 3.14. cDNA synthesis using mlfo primers for control and stressed MA, MO, and HS barley varieties.

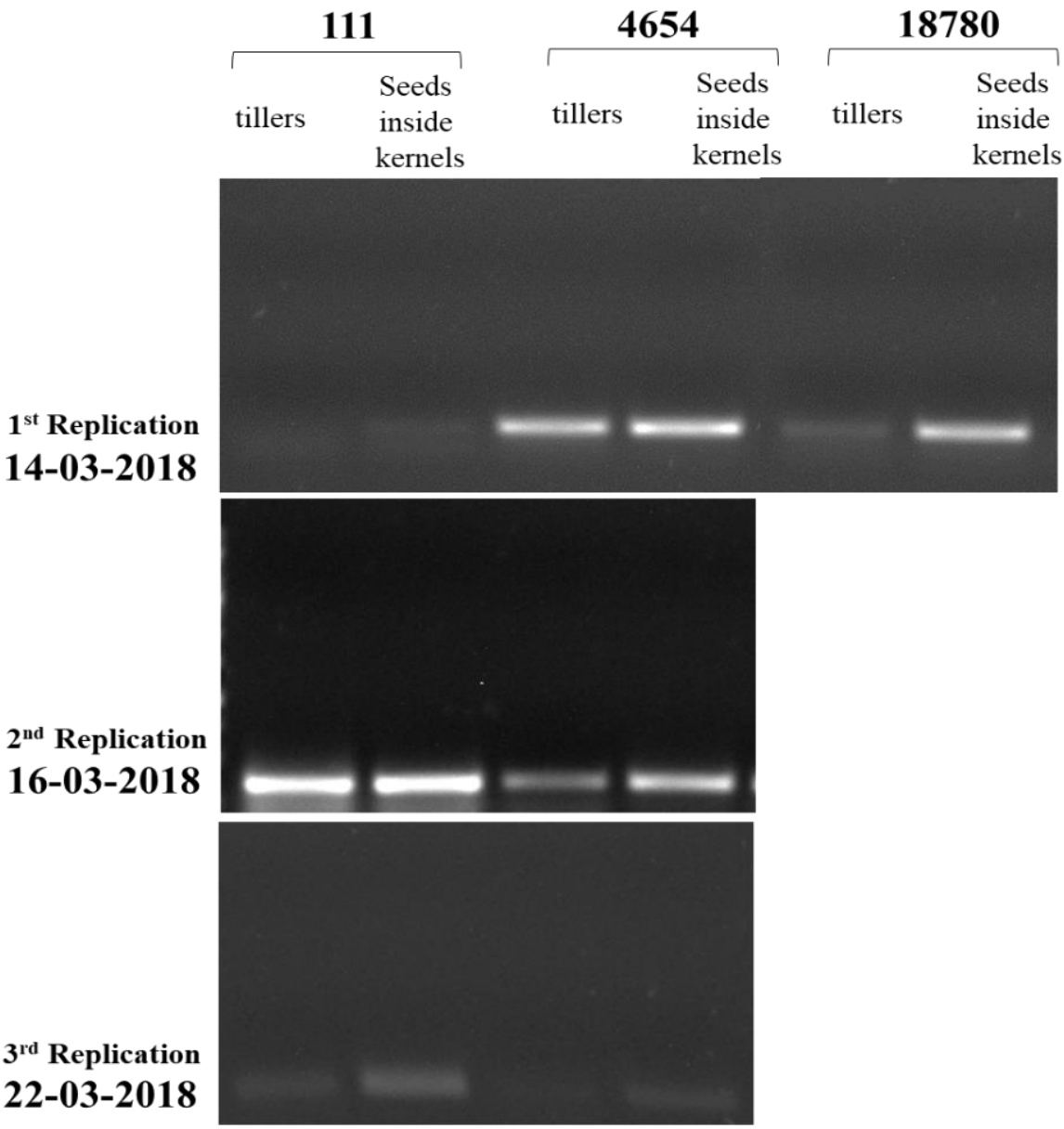

Figure 3.15. cDNA synthesis using mlfo primers for tillers and seeds inside kernels of 111, 18780, and 4654 barley varieties.

3.5. Lipid Peroxidation Assay

Alcoholic fermentation is an extremely important mechanism which allows plans to survive under anaerobic stress. One of the intermediates of alcoholic fermentation which is called acetaldehyde can be reduced by by alcohol dehydrogenase and can be oxidized by ALDHs (Nakazono et al., 2000). Furthermore, ALDHs can act as aldehyde scavengers under conditions which induce oxidative stress. As such, ALDHs are involved in lipid peroxidation

(LPO) where they metabolize reactive aldehydes produced as a consequence of the oxidative degradation of lipid membranes. A large number of LPO-derived aldehydes including 4-hydroxynoneal (4-HNE), 4-oxononenal (4-ONE), and malondialdehyde (MDA) are potent electrophiles. LPO-derived aldehydes can readily form adducts with nucleic acids and proteins. Adduction of nucleic acids and proteins can result in mutations and hinder their function as well as lead to significant fluctuations in homeostasis.

ALDH proteins either confer abiotic stress tolerance or alleviate the stress injuries related to lipid peroxidation derived-byproducts. In this experiment, the main focus was on MDA. As such, a large group of lipid peroxidation products including MDA contain an electrophilic (electron-accepting) α,β-unsaturated carbonyl group that reacts with electron-donor (nucleophilic) atoms common to many biological molecules that are called reactive electrophile species (RES). Thus, it has been proposed for MDA that its reactivity depends on the intra-cellular pH which is a parameter that can change under stress conditions.

The accumulation of aldehydes in cells is achieved when plants undergo excessive lipid peroxidation under stress conditions. The stress-inducible ALDH encoding genes are then activated, initiating the detoxification of aldehydes. Subsequently, the cytosolic pH decreases, leading to the protonation of MDA, making it become potentially reactive (Farmer and Davoine 2007). And in spite of their potential toxicity, MDA generated during oxidative stress were proved to function as powerful gene activator (Sattler et al., 2006; Farmer and Davoine, 2007; Mueller et al., 2008).

However, Missihoun (2010) suggested that a threshold of both intra-cellular MDA contents and lipid peroxidation-derived aldehydes, is necessary to trigger the expression of the target genes. He also hypothesized that the induction of the promoter of ALDH7B4 by aldehydes requires intermediate factors that are probably synthesized de novo.

Furthermore, an increase in the proclivity of plants to tolerate oxidative stress resulting from drought stress is accompanied by a reduction of MDA derived from the lipid peroxidation (Huang et al., 2008). Notwithstanding, it has been argued that under normal physiological conditions with neutral cytosolic pH, MDA remains a latent RES (Missihoun, 2010).

The table below conveys the recorded information pertained to the lipid peroxidation assay. Specimens were measured at different wavelengths (440 nm, 532 nm, and 600nm), and the MDA equivalent (nmol.ml^{-1}) was evaluated.

As mentioned earlier, the lower the MDA the higher is the tendency of the plant to tolerate oxidative and drought stresses. Therefore, MA 3S (0.752), MO 2S (0.677), and HS 1S (0.795) showed a relatively high MDA levels exceeding the average whereas MA 3C (0.0.323), MO 2C (0.4555), and HS 1C (0.309) showed a relatively low MDA levels falling behind the average (refer to table 3.4). To understand whether the MDA equivalent results are in accord with our previous results, the percent change has been calculated. The percent change shows how much the MDA equivalent increased between the control and stressed samples. The higher the value the more drought tolerant the plant is. For MA and MO varieties, the percent change was extremely high (132 % and 156 % respectively), indicating that these two species are drought tolerant. Whereas in MO variety, the percent change was low (48.5 %), which shows that MO is drought-sensitive. However, this contradicts with our previous results obtained from RWC which showed that MO is a drought-tolerant variety. This can be due to two main reasons. First, there might have been a mix up between the MA and MO samples while conducting the RWC experiment. Or, the previous research which showed that MO is a drought-sensitive variety might have been wrong. Be that as it may, the RWC and lipid peroxidation assay experiments are ought to be repeated with newly bought seeds to reveal the tru nature of drought-tolerance of the MO variety. Nevertheless, it can be certainly said that according to the current MDA equivalent results, MO is a drought-sensitive variety.

Table 3.4. MDA equivalent (nmol.ml^{-1}) measured at the following wavelengths (440 nm, 532 nm, and 600nm) for MA 3C, MA 3S, MO 2C, MO 2S, HS 1C, and HS 1S.

	Wavelength			MDA equivalent Calculation			
	440 nm	532 nm	600 nm	A	B	MDA equivalent (nmol ml-1)	Percent Change
Blank	0	0	0	0	0	0	0
MA 3C	0.068	0.067	0.013	0.054	0.003141	0.32394586	
MA3S	0.102	0.141	0.018	0.123	0.004796	0.752889172	132.4120371
MO 2C	0.112	0.094	0.017	0.077	0.005425	0.455894904	
MO2S	0.188	0.184	0.071	0.113	0.006681	0.677192994	48.5414702
HS 1C	0.146	0.106	0.052	0.054	0.005367	0.309761783	
HS 1S	0.245	0.237	0.104	0.133	0.008051	0.795852866	156.924162

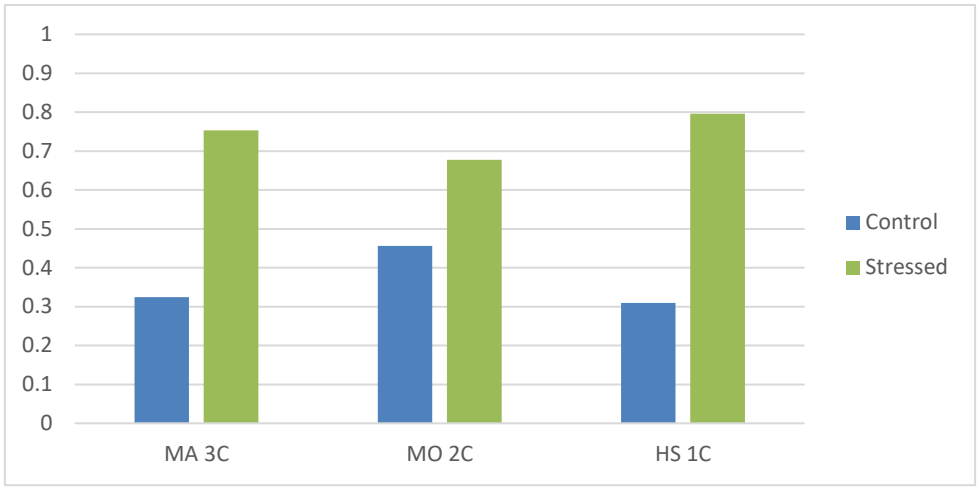

Figure 3.16. Plot chart of MDA equivalent (nmol.ml^{-1}) measured at the following wavelengths (440 nm, 532 nm, & 600nm) for MA 3C, MA 3S, MO 2C, MO 2S, HS 1C, & HS 1S.

4. Conclusion

In this internship, ALDH7B4 gene has been scrupulously studied to determine its role in conferring tolerance to drought and oxidative stresses in barley and *Arabidopsis thaliana*. In summation, running primers on synthesized cDNA targeting the gene-of-interest, analysis of RWC, and the lipid peroxidation analysis all showed that ALDH7B4 is a big player in aiding the studied species in tolerating drought conditions. Although MO variety showed some divergence from the aforementioned conclusion, more replications of the same experiments may prove otherwise. Additionally, the role of other members of the ALDH superfamily (e.g., ALDH3F1 and ALDH3I1 KO) have been explored. Nonetheless, the lack of consistent data in the results of the control deterioration test averted us from giving a defining conclusion regarding the roles of the members of the ALDH superfamily. Utilizing some techniques to control and moderate the constitutive expression of ALDH7B4 gene while under stress may help crop plants acclimate to drought, resulting in increased crop yields and maintaining economic stability in countries where agriculture is the backbone of the economy.

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Appendices

1. The percent of germination of the WT *Arabidopsis thaliana* variety

WT		Plate 1	Plate 2	Plate 3	Plate 4	Sum	Percent of Germination
Section 1	Germinated	46	41	56	0	143	
	Total	58	57	74	0	189	75.66137566
Section 2	Germinated	49	46	48	39	182	
	Total	51	54	59	54	218	83.48623853
Section 3	Germinated	37	35	101	73	246	
	Total	89	58	115	87	349	70.48710602
Section 4	Germinated	102	34	38	91	265	
	Total	111	53	106	103	373	71.04557641
Section 5	Germinated	75	25	95	82	277	
	Total	107	69	130	91	397	69.77329975
Section 6	Germinated	66	29	93	72	260	
	Total	96	53	108	83	340	76.47058824
Section 7	Germinated	55	64	21	49	189	
	Total	72	70	38	63	243	77.77777778
Section 8	Germinated	39	56	96	73	264	
	Total	41	67	104	80	292	90.4109589
						Average	76.88911516

2. The percent of germination of the dog1 *Arabidopsis thaliana* variety

dog1		Plate 1	Plate 2	Plate 3	Plate 4	Sum	Percent of Germination
Section 1	Germinated	48	41	53	64	206	
	Total	57	49	64	75	245	84.08163265
Section 2	Germinated	33	27	37	29	126	
	Total	48	74	49	62	233	54.07725322
Section 3	Germinated	5	3	12	0	20	
	Total	71	66	49	86	272	7.352941176
Section 4	Germinated	55	44	58	41	198	
	Total	63	80	72	95	310	63.87096774
Section 5	Germinated	39	81	70	1	191	
	Total	49	83	79	58	269	71.00371747
Section 6	Germinated	57	36	57	72	222	
	Total	72	65	83	76	296	75
Section 7	Germinated	55	72	97	140	364	
	Total	66	76	105	142	389	93.57326478
Section 8	Germinated	100	92	57	123	372	
	Total	104	107	59	125	395	94.17721519
						Average	67.89212403

3. The percent of germination of the 7B4 KO *Arabidopsis thaliana* variety

7B4 KO		Plate 1	Plate 2	Plate 3	Plate 4	Sum	Percent of Germination
Section 1	Germinated	1	4	0	2	7	
	Total	66	44	42	71	223	3.139013453
Section 2	Germinated	0	44	2	31	77	
	Total	67	62	38	18	185	41.62162162
Section 3	Germinated	61	43	7	71	182	
	Total	85	59	43	81	268	67.91044776
Section 4	Germinated	54	52	23	18	147	
	Total	73	58	39	65	235	62.55319149
Section 5	Germinated	5	22	6	10	43	
	Total	64	49	48	58	219	19.6347032
Section 6	Germinated	3	4	1	5	13	
	Total	56	42	42	28	168	7.738095238
Section 7	Germinated	49	22	11	12	94	
	Total	63	46	54	56	219	42.92237443
Section 8	Germinated	1	0	0	0	1	
	Total	90	76	54	72	292	0.342465753
						Average	30.73273912

4. The percent of germination of the 7B4 OE *Arabidopsis thaliana* variety

7B4 OE		Plate 1	Plate 2	Plate 3	Plate 4	Sum	Percent of Germination
Section 1	Germinated	49	71	56	30	206	
	Total	56	79	76	46	257	80.15564202
Section 2	Germinated	65	44	69	41	219	
	Total	103	71	75	55	304	72.03947368
Section 3	Germinated	34	33	43	47	157	
	Total	54	67	61	63	245	64.08163265
Section 4	Germinated	30	75	69	55	229	
	Total	35	89	78	62	264	86.74242424
Section 5	Germinated	44	40	74	36	194	
	Total	54	53	86	45	238	81.51260504
Section 6	Germinated	49	82	46	67	244	
	Total	63	87	57	79	286	85.31468531
Section 7	Germinated	63	52	67	55	237	
	Total	77	61	82	61	281	84.34163701
Section 8	Germinated	55	29	61	44	189	
	Total	66	37	73	60	236	80.08474576
						Average	79.28410572

5. The percent of germination of the 3F1 KO *Arabidopsis thaliana* variety

3F1 KO		Plate 1	Plate 2	Plate 3	Plate 4	Sum	Percent of Germination
Section 1	Germinated	29	36	38	29	132	
	Total	32	40	45	39	156	84.61538462
Section 2	Germinated	35	31	32	37	135	
	Total	37	36	35	48	156	86.53846154
Section 3	Germinated	0	46	40	36	122	
	Total	45	57	45	46	193	63.21243523
Section 4	Germinated	64	49	44	69	226	
	Total	65	53	51	72	241	93.77593361
Section 5	Germinated	60	54	52	47	213	
	Total	70	59	64	61	254	83.85826772
Section 6	Germinated	50	41	37	44	172	
	Total	54	48	58	55	215	80
Section 7	Germinated	84	57	39	42	222	
	Total	87	65	163	52	367	60.49046322
Section 8	Germinated	52	49	44	36	181	
	Total	56	61	63	47	227	79.73568282
						Average	79.02832859

6. The percent of germination of the 3F1 OE *Arabidopsis thaliana* variety

3F1 OE		Plate 1	Plate 2	Plate 3	Plate 4	Sum	Percent of Germination
Section 1	Germinated	1	29	1	0	31	
	Total	57	53	72	99	281	11.03202847
Section 2	Germinated	1	31	0	0	32	
	Total	82	37	73	43	235	13.61702128
Section 3	Germinated	6	45	0	0	51	
	Total	113	63	97	85	358	14.24581006
Section 4	Germinated	2	26	0	0	28	
	Total	37	35	49	30	151	18.54304636
Section 5	Germinated	1	63	0	0	64	
	Total	50	88	55	79	272	23.52941176
Section 6	Germinated	1	4	0	0	5	
	Total	43	32	44	33	152	3.289473684
Section 7	Germinated	5	20	0	0	25	
	Total	92	56	53	110	311	8.038585209
Section 8	Germinated	0	31	0	0	31	
	Total	42	33	59	54	188	16.4893617
						Average	13.59809231

7. The percent of germination of the 3I1 KO *Arabidopsis thaliana* variety

3I1 KO		Plate 1	Plate 2	Plate 3	Plate 4	Sum	Percent of Germination
Section 1	Germinated	48	26	34	37	145	
	Total	52	35	70	46	203	71.42857143
Section 2	Germinated	55	33	46	46	180	
	Total	71	43	60	69	243	74.07407407
Section 3	Germinated	35	9	24	26	94	
	Total	63	38	64	77	242	38.84297521
Section 4	Germinated	20	15	26	20	81	
	Total	56	39	60	48	203	39.90147783
Section 5	Germinated	18	20	21	15	74	
	Total	43	38	64	44	189	39.15343915
Section 6	Germinated	42	27	35	32	136	
	Total	73	46	59	50	228	59.64912281
Section 7	Germinated	45	21	23	37	126	
	Total	72	46	43	63	224	56.25
Section 8	Germinated	30	14	13	39	96	
	Total	90	37	83	73	283	33.92226148
						Average	51.65274025

8. The fresh weights, turgor weights, dry weights, and Relative Water Content of control and stressed replicates of the HS barley variety

Variety	Category	Replicate	Fresh Weight	Turgor Weight	Dry Weight	RWC
HS	Control	1	0.0476	0.0491	0.0044	96.6443
HS	Control	2	0.0708	0.0749	0.0067	93.9883
HS	Control	3	0.0968	0.0996	0.0074	96.9631
HS	Control	4	0.0773	0.0842	0.0079	90.9567
HS	Control	5	0.112	0.1161	0.0087	96.1825
HS	Control	6	0.0903	0.094	0.0091	95.6419
HS	Control	7	0.0774	0.0796	0.0072	96.9613
HS	Control	8	0.0715	0.0721	0.0037	99.1228
HS	Control	9	0.1226	0.1262	0.0088	96.9336
HS	Control	10	0.1311	0.1359	0.0093	96.2085
Average			0.08974	0.09317	0.00732	95.9603
HS	Stressed	1	0.0795	0.0899	0.008	87.3016
HS	Stressed	2	0.0782	0.0922	0.0094	83.0918
HS	Stressed	3	0.0551	0.0622	0.0061	87.344
HS	Stressed	4	0.0831	0.0901	0.0079	91.4842
HS	Stressed	5	0.1279	0.1401	0.01	90.6226
HS	Stressed	6	0.0744	0.0799	0.0076	92.3928
HS	Stressed	7	0.0943	0.1021	0.0104	91.494
HS	Stressed	8	0.0602	0.0672	0.0071	88.3527
HS	Stressed	9	0.1076	0.1256	0.0093	84.5228
HS	Stressed	10	0.09	0.1025	0.0113	86.2939
Average			0.08503	0.09518	0.00871	88.29

9. The fresh weights, turgor weights, dry weights, and Relative Water Content of control and stressed replicates of the MA barley variety

Variety	Category	Replicate	Fresh Weight	Turgor Weight	Dry Weight	RWC
MA	Control	1	0.0428	0.0444	0.0043	96.01
MA	Control	2	0.0627	0.0658	0.0073	94.7009
MA	Control	3	0.908	0.923	0.0096	98.3578
MA	Control	4	0.1003	0.1068	0.0104	93.2573
MA	Control	5	0.0886	0.0903	0.0086	97.9192
MA	Control	6	0.0889	0.0902	0.0105	98.3689
MA	Control	7	0.1063	0.1093	0.0097	96.988
MA	Control	8	0.0722	0.0761	0.0066	94.3885
MA	Control	9	0.0632	0.0646	0.0066	97.5862
MA	Control	10	0.0727	0.0745	0.0074	97.3174
Average			0.16057	0.1645	0.0081	96.4894
MA	Stressed	1	0.0773	0.0846	0.0071	90.5806
MA	Stressed	2	0.0823	0.11	0.0086	72.6824
MA	Stressed	3	0.1277	0.1426	0.0138	88.4317
MA	Stressed	4	0.0561	0.0711	0.0067	76.7081
MA	Stressed	5	0.0803	0.0959	0.0086	82.1306
MA	Stressed	6	0.0859	0.092	0.0074	92.7896
MA	Stressed	7	0.0707	0.0811	0.0077	85.8311
MA	Stressed	8	0.0873	0.0977	0.0077	88.4444
MA	Stressed	9	0.0414	0.074	0.0068	51.4881
MA	Stressed	10	0.093	0.0981	0.0076	94.3646
Average			0.0802	0.09471	0.0082	82.3451

10. The fresh weights, turgor weights, dry weights, and Relative Water Content of control and stressed replicates of the MO barley variety

Variety	Category	Replicate	Fresh Weight	Turgor Weight	Dry Weight	RWC
MO	Control	1	0.0607	0.0713	0.0066	83.6167
MO	Control	2	0.093	0.1072	0.0107	85.285
MO	Control	3	0.087	0.0901	0.008	96.2241
MO	Control	4	0.0603	0.0649	0.0055	92.2559
MO	Control	5	0.0581	0.0626	0.0058	92.0775
MO	Control	6	0.0484	0.0497	0.0054	97.0655
MO	Control	7	0.0595	0.0633	0.0055	93.4256
MO	Control	8	0.0475	0.0493	0.0037	96.0526
MO	Control	9	0.0591	0.0626	0.0062	93.7943
MO	Control	10	0.0342	0.0361	0.0042	94.0439
Average			0.06078	0.06571	0.00616	92.3841
MO	Stressed	1	0.0955	0.1075	0.0076	87.988
MO	Stressed	2	0.0695	0.0769	0.0072	89.3831
MO	Stressed	3	0.0641	0.0775	0.0053	81.4404
MO	Stressed	4	0.0552	0.0652	0.0052	83.3333
MO	Stressed	5	0.0355	0.0444	0.0033	78.3455
MO	Stressed	6	0.066	0.0766	0.0084	84.4575
MO	Stressed	7	0.0747	0.0864	0.0068	85.3015
MO	Stressed	8	0.0754	0.0816	0.0068	91.7112
MO	Stressed	9	0.0851	0.0941	0.0088	89.449
MO	Stressed	10	0.0695	0.0794	0.0059	86.5306
Average			0.06905	0.07896	0.00653	85.794

11. The percent of germination of the WT *Arabidopsis thaliana* variety in the CD test evaluated at 21, 18, 15, 12, 9, 6, 3, and 0 days after exposure to high moisture stress

Days	WT		Plate 1	Plate 2	Plate 3	Sum	Percent of Germination
21	Section 1	Germinated	43	38	41	122	
		Total	67	77	71	215	56.74418605
18	Section 2	Germinated	61	76	89	226	
		Total	73	94	121	288	78.47222222
15	Section 3	Germinated	25	15	22	62	
		Total	55	46	43	144	43.05555556
12	Section 4	Germinated	72	86	72	230	
		Total	100	96	97	293	78.49829352
9	Section 5	Germinated	100	118	46	264	
		Total	106	122	78	306	86.2745098
6	Section 6	Germinated	52	39	88	179	
		Total	61	52	90	203	88.1773399
3	Section 7	Germinated	66	82	114	262	
		Total	86	103	143	332	78.91566265
0	Section 8	Germinated	40	105	125	270	
		Total	119	108	133	360	75
						Average	73.14222121

12. The percent of germination of the dog1 *Arabidopsis thaliana* variety in the CD test evaluated at 21, 18, 15, 12, 9, 6, 3, and 0 days after exposure to high moisture stress

Days	dog1		Plate 1	Plate 2	Plate 3	Sum	Percent of Germination
21	Section 1	Germinated	7	5	3	15	
		Total	64	84	73	221	6.787330317
18	Section 2	Germinated	10	9	3	22	
		Total	80	87	65	232	9.482758621
15	Section 3	Germinated	66	29	74	169	
		Total	80	45	102	227	74.44933921
12	Section 4	Germinated	72	53	24	149	
		Total	88	82	81	251	59.3625498
9	Section 5	Germinated	81	100	66	247	
		Total	94	112	110	316	78.16455696
6	Section 6	Germinated	87	23	71	181	
		Total	103	65	101	269	67.28624535
3	Section 7	Germinated	101	42	80	223	
		Total	113	99	110	322	69.25465839
0	Section 8	Germinated	130	139	111	380	
		Total	133	152	147	432	87.96296296
						Average	56.5938002

13. The percent of germination of the 7B4 KO *Arabidopsis thaliana* variety in the CD test evaluated at 21, 18, 15, 12, 9, 6, 3, and 0 days after exposure to high moisture stress

Days	7B4 KO		Plate 1	Plate 2	Plate 3	Sum	Percent of Germination
21	Section 1	Germinated	46	44	45	135	
		Total	65	89	82	236	57.20338983
18	Section 2	Germinated	8	78	69	155	
		Total	102	95	80	277	55.9566787
15	Section 3	Germinated	3	83	66	152	
		Total	92	97	88	277	54.87364621
12	Section 4	Germinated	5	10	7	22	
		Total	101	49	39	189	11.64021164
9	Section 5	Germinated	0	27	17	44	
		Total	85	81	51	217	20.2764977
6	Section 6	Germinated	0	41	35	76	
		Total	57	51	51	159	47.79874214
3	Section 7	Germinated	0	26	73	99	
		Total	139	140	95	374	26.47058824
0	Section 8	Germinated	0	3	34	37	
		Total	78	92	54	224	16.51785714
						Average	36.34220145

14. The percent of germination of the 7B4 OE *Arabidopsis thaliana* variety in the CD test evaluated at 21, 18, 15, 12, 9, 6, 3, and 0 days after exposure to high moisture stress

Days	7B4 OE		Plate 1	Plate 2	Plate 3	Sum	Percent of Germination
21	Section 1	Germinated	72	42	49	163	
		Total	84	99	72	255	63.92156863
18	Section 2	Germinated	125	74	70	269	
		Total	162	124	108	394	68.27411168
15	Section 3	Germinated	91	42	67	200	
		Total	103	60	101	264	75.75757576
12	Section 4	Germinated	43	91	86	220	
		Total	119	120	110	349	63.03724928
9	Section 5	Germinated	123	55	44	222	
		Total	136	73	66	275	80.72727273
6	Section 6	Germinated	72	81	68	221	
		Total	81	100	88	269	82.15613383
3	Section 7	Germinated	62	118	81	261	
		Total	78	135	95	308	84.74025974
0	Section 8	Germinated	34	26	58	118	
		Total	73	71	93	237	49.78902954
						Average	71.05040015

15. The percent of germination of the 3F1 KO *Arabidopsis thaliana* variety in the CD test evaluated at 21, 18, 15, 12, 9, 6, 3, and 0 days after exposure to high moisture stress

Days	3F1 KO		Plate 1	Plate 2	Plate 3	Sum	Percent of Germination
21	Section 1	Germinated	58	61	49	168	
		Total	118	160	97	375	44.8
18	Section 2	Germinated	41	92	22	155	
		Total	78	103	127	308	50.32467532
15	Section 3	Germinated	98	90	58	246	
		Total	102	93	87	282	87.23404255
12	Section 4	Germinated	75	136	78	289	
		Total	85	149	122	356	81.17977528
9	Section 5	Germinated	73	104	62	239	
		Total	77	109	103	289	82.69896194
6	Section 6	Germinated	88	80	10	178	
		Total	92	83	64	239	74.47698745
3	Section 7	Germinated	113	71	6	190	
		Total	118	74	10	202	94.05940594
0	Section 8	Germinated	89	129	2	220	
		Total	99	148	78	325	67.69230769
						Average	72.80826952

16. The percent of germination of the 3F1 OE *Arabidopsis thaliana* variety in the CD test evaluated at 21, 18, 15, 12, 9, 6, 3, and 0 days after exposure to high moisture stress

Days	3F1 OE		Plate 1	Plate 2	Plate 3	Sum	Percent of Germination
21	Section 1	Germinated	44	11	36	91	
		Total	78	34	84	196	46.42857143
18	Section 2	Germinated	0	113	99	212	
		Total	109	147	118	374	56.68449198
15	Section 3	Germinated	0	71	56	127	
		Total	68	93	72	233	54.50643777
12	Section 4	Germinated	2	104	95	201	
		Total	151	134	116	401	50.12468828
9	Section 5	Germinated	5	39	109	153	
		Total	156	63	119	338	45.26627219
6	Section 6	Germinated	14	94	131	239	
		Total	109	103	139	351	68.09116809
3	Section 7	Germinated	0	90	52	142	
		Total	81	97	81	259	54.82625483
0	Section 8	Germinated	2	38	34	74	
		Total	71	48	48	167	44.31137725
						Average	52.52990773

17. The percent of germination of the 3I1 KO *Arabidopsis thaliana* variety in the CD test evaluated at 21, 18, 15, 12, 9, 6, 3, and 0 days after exposure to high moisture stress

Days	3I1 KO		Plate 1	Plate 2	Plate 3	Sum	Percent of Germination
21	Section 1	Germinated	37	90	47	174	
		Total	76	110	60	246	70.73170732
18	Section 2	Germinated	122	39	42	203	
		Total	133	49	85	267	76.02996255
15	Section 3	Germinated	52	18	9	79	
		Total	86	73	57	216	36.57407407
12	Section 4	Germinated	0	2	0	2	
		Total	87	103	118	308	0.649350649
9	Section 5	Germinated	0	0	0	0	
		Total	75	138	80	293	0
6	Section 6	Germinated	1	1	3	5	
		Total	120	73	75	268	1.865671642
3	Section 7	Germinated	0	0	70	70	
		Total	66	134	100	300	23.33333333
0	Section 8	Germinated	27	0	146	173	
		Total	81	172	196	449	38.53006682
						Average	30.9642708