Gene expression in response to salt stress mediated by genome doubling in *Hordeum bulbosum*

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Abstract

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Soil salinity impacts crop plants globally and can lead to the death of the plant due to incapacitation of cellular processes. To increase survival, plants have evolved many adaptation mechanisms to tolerate salt stress including genome duplication resulting in polyploid plants and molecular modifications like N^6-methyladenosine (m6A) RNA modification. How beneficial the m6A modification is to plant survival when faced with salt stress is still being largely studied. The species of study included the diploid and autotetraploid species of wild-type barley (*Hordeum bulbosum*) which are common in nature, with the tetraploids being known for surviving in high salinity environments. This study aimed to compare differences in gene expression of the diploid and tetraploid treated and control environments using salt response and m6A genes. To analyze salt tolerance, control plants were watered with tap water, and treatment plants were watered with a salt solution. RNA was collected from both control and treatment plants to be used for gene expression analysis. The expected results are that the salt treated plants will have changed gene expression for salt response and m6A genes compared to the control plants.

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1st May, 2023

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1. Introduction

1.1 The negative implications of soil salinity

Soil salinity is identified when there is an accumulation of soluble salts within the soil that is great enough to interfere with plant development (Allbed et al, 2014). Soil salinity is a global issue; in 2016 approximately 932.2 Mha of land was affected by soil salinity, with soil salinity hotspots occurring in regions within Pakistan, China, United States, India, Argentina, Sudan, and numerous countries in Central and Western Asia (Daliakopoulos et al., 2016). Soil salinity can be caused by poor irrigation water (water with high salt levels) or certain agricultural practices (Gabriel et al., 2012). As a result of high sensitivity levels, many important crop plants are negatively affected by soil salinity (Jiang et al., 2013).

1.2 Plant Stress Responses

Plants can be challenged by multiple environmental stressors simultaneously including salinity, drought and extreme temperatures from living in different climate zones on Earth or those caused by global warming (Thoen et al., 2017). It's important to understand the mechanisms underlying plant responses and potential tolerance to stress. Plants react to stress by either stress avoidance through protecting themselves from stress, or stress tolerance by adapting and living under stressful environments (Kosová et al., 2011). The intensity and length of stress response in plants is based on the stress intensity and duration (Kosová et al., 2011).

The stress response in plants can be identified at different stages. The first stage is an alarm phase which is triggered by shock from the non-acclimated plant, followed by an acclimation phase featuring new homeostasis formulation under the stressful environment, a maintenance phase where the stressful environment persists and the new homeostasis is maintained, and an exhaustion phase occurs when plants perish under the stress of a new homeostasis (Kosová et al., 2011).

The activation of the stress response in some plants results in changes in the composition of plant transcriptome, proteome, and metabolome (Kosová et al., 2011). An example of how plants tolerate stress is by altering their transcriptional and posttranscriptional gene regulatory processes (Shoaib et al., 2021). Multiple transcriptions and epigenetic factors such as DNA and RNA methylations will become activated as part of the stress response of transcriptional regulation (Shoaib et al., 2021). Proteins are important for change in transcriptional modification as they regulate transcript and protein level response (Kosová et al., 2011). RNA binding proteins are responsible for regulating the stress response through intron splicing, precursor-RNA processing, RNA turnover, and translational control which is regulated by posttranscriptional stress response (Shoaib et al., 2021).

1.3 Genome Duplication and Polyploidy as Adaptations to Stress

Plants face many great environmental stressors in life that require them to have adaptations to survive; one adaptation that has been prevalent throughout history during stressful and extinction level events is whole genome duplication, resulting in polyploid plants (Liu & Sun, 2017). A polyploid species contains two or more complete sets of chromosomes (Huang & Zhu, 2019). Polyploid can be defined as autopolyploid where the multiple sets of chromosomes come from one species, or allopolyploid where two or more sets of the chromosome come from two or more species (Huang & Zhu, 2019).

Polyploidy in plants has been identified as a key factor that allows them to tolerate abiotic stress due to polyploids having larger cells which allows them to contain more RNA and DNA (Forrester et al., 2020). It is also suggested that for some plants the autopolyploids have a greater capacity at withstanding stress than the diploid ancestor species (Liu & Sun, 2017). This difference is seen in some plants because autopolyploids contain non-additive genes which play

a role in stress response (Liu & Sun, 2017). For some plant species where diploid and tetraploid accessions exist, the tetraploid individual can survive in ecologically extreme niches (Hijmans et al., 2007). Several studies have shown that the tetraploid accessions of turnip, citrus, and black locust have higher salt tolerance than the diploid species when faced with salt stress allowing them to tolerate extremely salinized environments (Esposito et al., 2021).

Genome duplication is an important molecular process that aids in the ability of plants to tolerate environmental stressors. Through neofunctionalization (i.e., the retention of ancestral functions on one copy of the duplicated chromosomes and novel function on the other copy), and subfunctionalization (i.e., both copies of the duplicated chromosomes retain ancestral gene function) duplication events can designate new roles in plant growth and resistance (Zhu et al., 2021). This allows for chromosomal duplication to play a vital role in stress response due to the presence of a larger gene set, which could include an increased number of beneficial stress tolerant genes (Yona et al., 2012). When plants undergo the process of becoming polyploids this involves genome duplication that leads to changes in genetic and epigenetic levels (Esposito et al., 2021). Genome doubling and hybridization cause genome-wide transcriptional and splicing level change (Qin et al., 2021). During the formation of polyploids, the plant will face genomic shock caused by the genome doubling. It has been found that the change in micro-RNAs (MiRNA) caused by genome duplication alleviate some of the stresses (Esposito et al., 2021). MiRNAs also function in various plant growth stages, developmental transition, and stress response of biotic and abiotic conditions (Esposito et al., 2021).

1.4 Plant Responses to Salt Stress

Salt stress is highly prevalent throughout the world and affects many types of crop plants. It has been estimated that approximately 6-10% of soil around the world is affected by salt

concentrations, where one-third of that land is used for agricultural purposes (Murphy, 2003). High salt concentrations are more common in arid countries or in desert environments that have low annual rainfall, as well as in coastal estuaries and saltmarshes where high salt concentrations are due to the movement of saltwater tides and currents (Murphy, 2003).

Plants require salt to grow, but the majority of plants can only tolerate a narrow range of salt concentration before cellular processes become negatively affected (Murphy, 2003). Salt stress in plants occurs when there is a high concentration of salt ions in the soil which decreases the amount of water available in the soil (Murphy, 2003; Medina et al., 2020). High salt concentrations occur for several reasons including low precipitation, increased surface evaporation and poor irrigation practices that lack proper drainage (Dong et al., 2020). High salt concentrations in the soil results in plants facing salt stress due to an uptake of salt ions caused by the reduction of available soil water, which causes disruption of ion homeostasis where the interior of the plant cells become highly toxic (Dong et al., 2020).

In response to salt stress plants will undergo a variety of physiological changes which may include decreased rates of leaf gas exchange (e.g., photosynthesis, stomatal conductance), the formation of reactive oxygen species (ROS), decreased water content in cells, or complications involving protein folding (Medina et al., 2020; Zheng et al., 2021; Dong et al., 2020). An increased uptake of sodium (Na^+) also causes decreased calcium (Ca^{2+}) and potassium (K^+) uptake which results in reduced growth and leaf chlorosis (Medina et al., 2020). Both sodium and chloride ions have a negative effect on plant growth and development when taken up in extremely high concentrations, resulting in a decreased uptake of water and other nutrients (Dong et al., 2020). Due to less water availability in soil the salt stress response in plants is greatly impacted by the combined effect of dehydration and osmotic stress (Medina et al., 2020).

To prevent cell death due to high salt concentrations, plants can deter osmotic stress by accumulating compatible solutes or combat ROS by producing oxygen scavengers (Dong et al., 2020). Compatible solutes function in salt tolerance by drawing more water into the cells of plants (Medina et al., 2020). Oxygen scavengers combat ROS by using enzymes including superoxide dismutase (SOD) and catalases (CAT) to detoxify oxygen free radicals (Vaidanathan et al., 2003).

To maintain ion homeostasis, some species secrete the toxic ions through Na^+ ion transporters that can function under high salt concentrations to transport Na^+ ions out of the cell and decrease cell toxicity (Garriga et al., 2017). Other species maintain homeostasis by compartmentalizing the toxic ions in vacuoles (Dong et al., 2020).

1.5 Role of N^6-methyladenosine (m6A) modification in plant salt response

N^6-Methyladenosine (m^6A) is a type of mRNA methylation that is characterized as an epitranscriptomic modification that includes defined writers (add the methyl group to adenosine), readers (interpret the m6A marks), and erasers (remove the methyl group from adenosine) (Hu et al., 2021). An epitranscriptomic modification refers to the modification of an RNA molecule that does not alter the RNA sequence (Ruocco et al., 2020). The mRNA modification- m6A- occurs when an adenosine molecule gets an additional carbon methyl group added to the nitrogen of the 6^{th} carbon on the adenine group (Figure 1) (Hu et al., 2021). The presence of m6A modifications affect many stages of mRNA expression by affecting splicing, the export from nucleus-to-cytoplasm, the ability of translation, structural rearrangements, and shaping the interaction of RNA-proteins (Ruocco et al., 2020). It has been discovered that m6A modifications are responsible for the mRNA regulation at all stages (Ruocco et al., 2020). This is theorized to be

true due to the presence of high density m6A sites located around stop codons, at 3' untranslated regions (3'UTRs), and present at the beginning of codon coding regions (Ruocco et al., 2020).

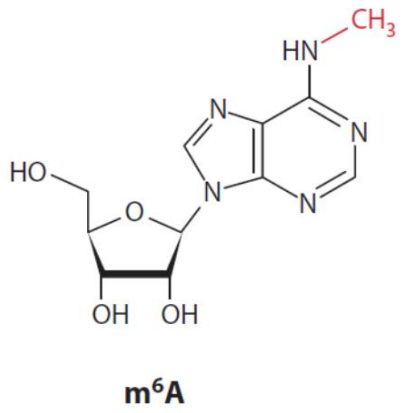

Figure 1. An adenosine molecule that has been transfigured into an m6A modification with the addition of a carbon methyl group highlighted in red. The methyl group (red) binds to the nitrogen group on the 6th carbon of the adenine.

The m6A modification is the most abundant mRNA modification identified in eukaryotes and has been found to influence plants by regulating the salt tolerance response (Zheng et al., 2021). During times of stress the plant will undergo DNA and RNA methylation, including m6A modifications, which is categorized as a transcriptional regulatory process (Hu et al., 2021). A study done by Anderson et al (2018) demonstrated the correlation between m6A methylation and salt stress response in *Arabidopsis thaliana*. The study found that under salt stress trials the transcript abundance of m6A increased compared to trials under the control conditions (Anderson et al., 2018).

Studies have linked the increased expression of m6A regulatory genes to the presence of a larger genome size caused by genome duplication (Zhu et al., 2021). Expansion of m6A regulatory genes is accomplished through whole genome duplications and chromosomal rearrangements which aids to trigger the activation of the m6A genes (Zhu et al., 2021).

1.6 Function of m6A writers, readers, and erasers

The m6A modification is a reversible process as the adenosine base can be turned into an m6A modification and turned back into an adenosine molecule with the addition and removal of a methyl group (Hu et al., 2021; Shoaib et al., 2021; Ruocco et al., 2020). There are three fundamental components that allow the m6A modification to be reversible: the writers, readers, and erasers. The m6A writers are methyltransferases that are responsible for depositing m6A marks and add the methyl group to adenosine (Hu et al., 2021; Shoaib et al., 2021; Ruocco et al., 2020). The name methyltransferase comes from the ability of m6A writers to transfer a methyl group from a different larger group known as S-adenosylmethionine onto the adenosine N^6-amine group (Arribas-Hernández & Brodersen, 2020a). Different writers found in plants include MTA, MTB, FIP37, and HAKA1 (Ruocco et al., 2020). RNA demethylase is commonly referred to as the m6A erasers which are responsible for removing the m6A marks from an adenosine by removing the methyl group (Hu et al., 2021; Ruocco et al., 2020). The m6A erasers include enzymes from the ALKBH family which are enzymes that catalyze oxidative dealkylation of N-methylated nucleotides (Arribas-Hernández & Brodersen, 2020a). RNA-binding proteins play a role by acting as the m6A readers that bind onto a transcript containing m6A and interpret the m6A marks (Hu et al., 2021; Ruocco et al., 2020). The importance of m6A readers in plant stress response was found in a study by Zhu et al (2021), who determined that m6A reader genes were expressed in response to abiotic stress (Zhu et al., 2021). As writers, readers and erasers work together, the results found by Zhu et al (2021) demonstrate how an increase in m6A reader gene expression must indicate an increase in the gene expression of m6A writers and erasers. How genome duplication affects the expression of m6A writer, reader, and eraser components is currently unknown.

1.7 Objectives and Questions

In this thesis, I have characterized the effects of genome duplication in response to salt stress. Three main questions that I addressed were:

1) Does genome doubling change the expression of genes related to salt stress?
2) Does genome doubling change the expression of transcription factors that regulate salt stress related genes?
3) Does genome doubling change the expression of m6A genes in response to salt stress?

1.8 Study Species

Barley is an important crop plant that can be cultivated across the globe and is the fourth most important crop (Wendler et al., 2017). In this paper I focused my research on *Hordeum bulbosum L.*, commonly known as bulbous barley (Hoseinzadeh et al., 2020). *Hordeum bulbosum* is an important species for barley agriculture as it is a valuable source for genetic diversity regarding crop improvement (Wendler et al., 2017). It has been found that compared to other species of barley, *H. bulbosum* is highly resistant to diseases that affect barley plants (Hoseinzadeh et al., 2020). Another important characteristic of *H. bulbosum* is that both the diploid and tetraploid occur naturally (Wendler et al., 2017). Diploid *H. bulbosum* has 2n=14 chromosomes, and tetraploid species have 4n=28 chromosomes (Wendler et al., 2017).

2. Materials and Methods

2.1 Plant Growth and Experimental Design

Six accessions of *Hordeum bulbosum* were grown inside a greenhouse on the Saint Mary's University campus in Halifax, Canada (latitude 44.636325N longitude -63.594416W) from May 4th, 2020, to June 1st, 2020 (Scarrow, 2021). The accessions included three diploids

and three tetraploids. There were three plants per pot, with six pots for each accession. The heating and ventilation were controlled, with temperatures set at 25/18°C (day/night).

The plants were germinated inside from seed in a petri dish with some water for two weeks before treatments began. After two weeks the seeds were transferred to pots. The pots were divided into two groups: three of the pots were designated to receive the salt treatment, and the other three were designated as the control. The salt treatment consisted of watering the plants with 300mL of 250mM NaCl solution prepared using tap water. The control was watered with 300mL of tap water. Treatments were administered to the plants from day 1-14 (after germination period). A recovery period occurred from days 15-28 where both groups were watered with 300mL of tap water every two days.

At 28 days old, the plants were moved outside, and the clones of the original plants were separated using a scalpel to cut the bulbs of the clones growing from the original bulb and placed in a new pot. Both the original and repotted clones received water every third day and were fertilized using Water Soluble All Purpose Plant food (Miracle-Gro) once a week.

2.2 RNA Isolation

RNA isolation was performed by collecting a sample from each plant of three newly sprouted leaves (young leaves). A high-precision balance scale (Mettler Toldeo AE163) was used to weigh 150mg of leaves per individual which was frozen immediately in liquid nitrogen, and a mortar and pestle were used to grind the leaves into powder. The TRIzol Reagent Protocol was used to lyse the samples by adding 2 ml of TRIzol and 0.4ml chloroform and centrifuged (10 000 rpm for 5 minutes). RNA isolation was conducted by transferring the top clear layer to a new centrifuge tube. Isopropanol was added (0.5 ml per 1 ml TRIzol) before being centrifuged at 10 000 rpm for 10 minutes for the RNA to be isolated. A 75% ethanol alcohol (EtOH) was used to

wash the isolated RNA (1 ml EtOH per 1 ml TRIzol) then centrifuged for 5 minutes at 7500 rpm. The supernatant was discarded from the RNA before being resuspended in 50 µl of RNase-free water. The RNA samples were placed in a 15-minute water bath at 60°C and then immediately stored in -80°C (Scarrow, 2021).

2.3 RNA quality determination

Before analysis of gene expression, the RNA template was assessed for quality and purity purposes. The integrity and quality of the RNA was determined by conducting electrophoresis. Concentration and purity of RNA was assessed using 2 µl of isolated total RNA with a Nano-drop (Scarrow, 2021). A 1.0% bleach gel (1.5g agarose, 1.22ml bleach and 148.78 ml 1x TAE Buffer) (Bio-Basic Inc, CA) was used to analyze the quality of RNA. The RNA was determined to be good quality if it showed three clear bands without smear and was then used in the following analysis.

2.4 cDNA synthesis and quality determination

RNA was synthesized into cDNA before real time-qPCR (RT-qPCR) due to cDNA being more stable and being stored for up to 12 months without degradation. The iScript cDNA synthesis kit (Bio-Rad) was used for cDNA synthesis. The iScript kit protocol was followed with the samples containing the reaction mix (100 µl of 5x iScript Reaction mix, 25 µl of iScript Reverse Transcriptase, and 1.5 mL of Nuclease-free water to make 25 reactions) being incubated in Bio-Rad T100 Thermal cycler (Bio-Rad Inc., US) with the following synthesis steps being used: (1) priming at 25°C for 5 minutes, (2) reverse transcription at 46°C for 20 minutes, (3) RT inactivation at 95°C for 1 minute.

The cDNA was diluted to a series of concentrations: ½, 1/10, 1/20, 1/40, 1/50 and 1/75 using nuclease-free water before being used for qPCR analysis. The qPCR test found that 1/75 dilution showed good amplification and was used for the following analyses.

2.5 Gene expression analysis

Analysis of gene expression was done using RT-qPCR with primers synthesized by Bio Basic Canada Inc, ON, Canada (Table 1), which included function genes in response to salt, such as K+ transporters (HvHAK1 and HvAKT1), Na+/H+ antiporters (HvNHX1, HvNX3, and HvNHX4), vacuolar H+ -ATPase (HvHVA/68) and vacuolar H inorganic pyrophosphatase (HvHVP1) (Cheng, 2021), transcriptional factors and m6A function genes. M6A genes that were isolated included m6A writers (MTA, MTB, FIP37, and HAKA1), m6A readers (ECT4, ECT3, and ECT1) and m6A erasers (ALKBH6, ALKBH8, ALKBH9, and ALKBH10). A mixture of cDNA and the associated primer was created with iTaq universal SYBR® Green reaction mix (Bio-Rad inc, US) added in accordance with manufacturer's instructions. A Bio-Rad 96 PCR plate was sealed with a MicroAmp Optical Adhesive Film Kit (Thermo Fisher Scientific, US). Each sample was repeated three times. Amplification of cDNA with the corresponding primer was amplified using the CFX96 Touch Deep Well RT-qPCR Detection System (Bio-Rad inc, US). The RT-qPCR protocol was set as follows: stage 1, 95°C for 10 minutes; stage 2, 95°C for 15 seconds, stage 3, 56°C for 30 seconds, stage 4, 60°C for 1 minute. Stages 2-5 were repeated for a total of 40 cycles.

Actin was used as the control and repeated two times. The data collected from the RT-qPCR was analyzed by comparison to the normalized gene expression (△△Cq) level of the target gene using the CFX Maestro^{TM} software (Bio Rad, US).

Table 1. List of primers used in RT-PCR

Gene name	Primer name	Sequence (5'-3')	Size product (bp)
HvHAK1	HvHAK1F	TGGTGATAGGCGATGGAAC	321
	HvHAK1R	GAGAGACCCATCCACTCTTC	
HvAKT1	HvAKT1F	GGGTTCGATGTGCAAAGCTC	235
	HvAKT1R	CTCCCGTATGTTCTCCGCATG	

HvHKT1	HvHKT1F	ACTTTGCCGTGATCCATATC	326
	HvHKT1R	CTGGATTCTTGATCATGAGC	
HvHVA/68F	HvHVA/68F	AGTACAGTCTGCAGGATACTG	251
	HvHVA/68R	GAGTACTTTGAGAGCGCCTG	
HvHVP1	HvHVP1 F	TACCCACTCCTAATCAGCTC	305
	HvHVP1R	GCTTGTGTAGTACTCTGTAATG	
HvNHX1	HvNHX1 F	TCCAGGTGAAGAAGAAGCAG	273
	HvNHX1R	GTGGCATCGTTCACAACAC	
HvNHX2	HvNHX2F	GGCTATCTTCTCAGCAACC	235
	HvNHX2R	CAAGAACGGTGCTGGTGAG	
HvNHX3	HvNHX3F	CGATGCGACATCAGTTGTG	320
	HvNHX3R	CAGGTATAGTGCGACATTAC	
HvNHX4	HvNHX4F	AGGAGTAATGCAGCAGGAG	343
	HvNHX4R	GTTTCAGCCAAAGATGATAGC	
HvMT2	HvMT2F	TCAGTCGAATCAACACATGGA	266
	HvMT2R	CACGAGGACGGAACTAAAGC	
HvBAS1	HvBAS1F	CGTCACCAAATCGATCTCAAA	141
	HvBAS1R	TCCACACTACGGCCAATACC	
HvLHCB	HvLHCBF	TCTGAGGGTGGTCTCGATTA	99
	HvLHCBR	CAACAAGACCCATGAGAAGG	
HvWRKY12	HvWRKY12F	CTACCGGTGCACACATCAAG	157
	HvWRKY12R	GACCTGCATCTGGGTGAGTA	
HvDRF2	HvDRF2F	TGAGACGATCAAGCAATGGA	195
	HvDRF2R	CGAATTTCAGCAACCCACTT	
HvACTIN	HvACTINF	CGTGTTGGATTCTGGTGATG	208
	HvACTINR	AGCCACATATGCGAGCTTCT	
HvDREB1	HvDREB1F	ATGGAGACCGGGGGTAGC	583
	HvDREB1R	GCAGACTCAAACTCATCCTTGG	
HvZIP1	HvZIP1F	GCGCAAGCAGGCTGAATGTGA	299
	HvZIP1R	TATGCATACTACAACTGCGCCAGCA	
HvNAC005	HvNAC005F	CCATGTGAACAGCAGCGGCAAC	67
	HvNAC005R	CCGACGTTGAGGCTGGTGAATC	
HvNAC027	HvNAC027F	ACGGCTACGTGAACCACGACAC	97
	HvNAC027R	CAAGCTGCCGCTGGATCTCTTC	
APETALA2	APETALA2F	TGATGGACGACGGCAACTTCTG	67
	APETALA2R	ATGTTGTGATGGGCGGTGATCC	
MTA	MTAF	CTTATCCCCTTCGCCGAGAT	170
	MTAR	CTGTCCTCAGCTATGCGTCG	
MTB	MTBF	TCATCGTGCACCAGGAATAACA	125
	MTBR	CTACGCCATCACCAACCCAAA	

FIP37	FIP37F	CCTGCAGGAACCAATCCAGA	113
	FIP37R	GCAGCTTCTCTTTTCTTCGCT	
HAKAI	HAHAIF	GCCTGTGCAAGAAGTGATTCC	173
	HAHAIR	AGGAGATTTGCATGAGCTTCAG	
ECT4	ECT4F	AGCTCAGCCGTTTCTTTGGA	217
	ECT4R	CCACATTAGCACCTGGCAGA	
ECT3	ECT3F	GCTGGATCGAATGGACTGGA	214
	ECT3R	GTTGAGCATACATGCCAGGTG	
ECT1	ECT1F	CCTTCAGGGCAATGGGAAGA	147
	ECT1R	CTCTCCAACAGTTGAAACAGGAG	
ALKBH1	ALKBH1F	ACCGAAAACGGGATCCCAAA	188
	ALKBH1R	GGCGGCTTGTAGAGCTTGTA	
ALKBH2	ALKBH2F	TTTGAGGTACAGCGGCCATC	89
	ALKBH2R	GGGCTTCATGGACCTCTTTC	
ALKBH6	ALKBH6F	TCACACCCCATGGAAAGCTC	189
	ALKBH6R	GGAAGTCTGTATAAGCCTGATCT	
ALKBH8	ALKBH8F	TTGGCGAAAAGGCGAGTTCA	213
	ALKBH8R	GTGTGGAGACAAACCTACACC	
ALKBH9B	ALKBH9F	TGTGTGCCAGACAGTTGCAT	162
	ALKBH9R	TTCTCCAGGACCAGCGATTT	
ALKBH10B	ALKBH10F	ACCTGGACAACCCCATTTCC	92
	ALKBH10R	CTTGTAGTTGCCGTTGCTGTC	
ATAF1	ATAF1F	GTACTACGAGGTGAGGCCGT	251
	ATAF1R	CGAGTCGGACGGATTCTCAG	
Myb	MYB56F	CACCGACAACTCCGTCAAGA	292
	MYB56R	TGCATTAGCTTCCTCCGACG	
PiP1	PiP1F	GAGAGCAAGGGAGAGCGTCAT	229
	PiP1R	GACGTAGAGGAAGAGGAAGGTG	
BGLU12	BGLU12F	CTGAAAACGGCGTCGATGAA	248
	BGLU12R	CTTGGGGTACCGCTTCCTAC	
PP2C	PP2CF	ATCGACTTCAAGCCCAACCT	265
	PP2CR	AGATCACGTCCCAAACCCC	
GI	GIF	CAACTGCTTACAGCCGAGGA	193
	GIR	TCATCCAAGGGTGTTGTCGG	
GSTU 17	GSTU17F	CGACAAGTTTCCTACGGCGA	236
	GSTU17R	CGTCGAGAAGCCTGTGTTCA	
RD22	RD22F	GTCCACGTCAACGTCTCACC	137
	RD22R	GCCATGAAATGGACGCTCATC	
CBL3	CBL3F	GGAGTTTGTTCGGTCCCTCC	254
	CBL3R	CTTTCCATTCGTCGGGGTCT	
CBL2	CBL2F	CGCCAAGGGAAGATTCGAGA	189

	CBL2R	GCCTACTCCGTTTTGGGACA	
SOS1L	SOS1LF	GTAAAGTGGACAAGTCAGAGACTCA	215
	SOS1LR	TCCTGCCACATGAAATCCTCAATA	

2.6 Statistical Analysis of RT-PCR results

To analyze the results of the gene expression from RT-qPCR the mean, standard deviation, and variance of the three trials of each primer were calculated. Four different comparisons were performed: comparison between the diploid control and treated, comparison between the polyploid control and treated, comparison between the control diploid and control tetraploid, and comparison between the treated diploid and treated polyploid species. To determine significance, P-value less than or equal to 0.05 were considered statistically significant using a two way ANOVA (Appendix A, Appendix B). To determine whether genome doubling changed the expression of m6A and salt stress-related genes, I compared expression of genes between diploid and polyploid accessions using the normalized data calculated by subtracting the mean of the housekeeping gene (actin) from the mean of the three repeats for each primer.

3. Results

3.1 qPCR amplification

Gene expression for a total of 44 primers was analyzed using RT-qPCR. Eighteen primers had to be excluded from analysis due to poor gene amplification caused by one or more of the trials failing to amplify which include *HAK1*, *AKT1*, *HVA/68F*, *HVP1*, *NHX1*, *NHX2*, *NHX3*, *NHX4*, *MT2*, *APTALA*, *MTA*, *ALKBH1*, *ALKBH2*, *MYB*, *CBL3*, *ZIP1*, *HAKA1*, *ECT4*. Poor amplification was identified by the amplification graph produced by the RT-qPCR where trials did not start to amplify until cycle 30-40.

3.2 Comparison of diploids and tetraploids for the salt treated plants

The gene amplification data collected from RT-qPCR was normalized by subtracting the mean of the housekeeping gene, *ACTIN*, from the mean of the three trials of the primer. Each primer was tested using cDNA collected from six accessions of plants, three diploid (accession number: 311, 240, 210) and three tetraploid accessions (accession number: 287, 283, 106). Each accession was tested under salty and control environments. The three diploid trials were compared with one another, and the three tetraploid trials were compared with one another to confirm whether each ploidy of plant for each primer demonstrated the same pattern, either all trials of the same ploidy were all positive (upregulated) or all negative (downregulated).

For the salt treated trials six genes showed upregulated expression in the diploid species: *HKT1*, *LHCB*, *NAC005*, *NaC027*, *ALKBH8*, and *SOS1L* (Table 2). Ten genes showed upregulated expression in both the salt treated tetraploid and the salt-treated diploid accessions: *DREB1*, *MTB*, *FIP37*, *ECT3*, *ECT1*, *ALKBH9*, *ATAF*, *GI*, *RD22*, and *CBL2* (Table 2). For the salt treated tetraploid plants there were three genes that showed upregulated expression: *ALKBH6* (Figure 2), *ALKBH10*, and *BGLU12* (Table 2). A couple of the primers for salt treated plants had negative amplification: *BAS1* (diploid and tetraploid), *GSTU17* (diploid). All other primers for the salt treated trials had a mixture of positive and negative values for the normalized data and cannot be used for further comparison in this study: *HKT1* (tetraploid), *DRF2* (diploid and tetraploid), *LHCB* (tetraploid), *WRKY12* (diploid and tetraploid), *NaC005* (tetraploid), *NaC027* (tetraploid), *ALKBH6* (diploid), *ALKBH8* (tetraploid), *ALKBH10* (diploid), *PiP1* (diploid and tetraploid), *BGLU12* (diploid), *PP2C* (diploid and tetraploid), *GSTU17* (tetraploid), *SOS1L* (tetraploid) (Table 2).

3.3 Comparisons of the diploids and tetraploids for the control plants

Comparisons of the gene expression for the control trials of the three diploid species, and the three tetraploid species were conducted to determine whether each ploidy had the same pattern: either all positive values (upregulated) or all negative values (downregulated) (Table 3). The *ATAF* gene showed upregulated expression among the three diploid accessions in the control group (Figure 3). Seven genes including *NaC005, ALKBH6, PP2C, GI, RD22, CBL2,* and *SOS1L* displayed upregulated expression among all three accessions of tetraploid species in control. Eleven genes *DRF2, WRKY12, DREB1, NaC027, MTB, FIP37, ECT3, ECT1, ALKBH9, ALKBH10,* and *PiP1* showed upregulated expression in both diploid and tetraploid accession in control. A couple of primers for the control treated plants had amplification with all negative values: *BAS1* (diploid and tetraploid), and *ALKBH8* (tetraploid). All other primers for the control treated trials had a mixture of positive and negative values for the normalized data and cannot be used for further comparison in this study: *HKT1* (diploid and tetraploid), *LHCB* (diploid and tetraploid), *NaC005* (diploid), *ALKBH6* (diploid), *ALKBH8* (diploid), *ATAF* (tetraploid), *BGLU12* (diploid and tetraploid), *PP2C* (diploid), *GI* (diploid), *GSTU17* (diploid and tetraploid), *RD22* (diploid), *CBL2* (diploid), and *SOS1L* (diploid).

3.4 Comparison of the control and salt treated diploids

The control and treatment plants of the same ploidy were compared by comparing the control diploid to the salt treated diploids. Many of the primers that were analyzed did not have any consistent difference between the diploid control and diploid salt treated plants. Many primers between the control diploid and the salt treated diploid did not have a consistent pattern of one group having a much lower or higher gene expression. The primers with no consistent difference include: *DREB1*, *NaC027*, *MTB*, *FIP37*, and *ATAF*. The expression of *ECT3* (Figure

4) and *ECT1* in the salt treated diploids had a lower gene expression than the control treated diploids (Table 2 & 3).

3.5 Comparison of the control tetraploid and salt treated tetraploids

The control and treatment plants of the same ploidy were compared by comparing the gene expression of the control tetraploids to the salt treated tetraploids. A few of the primers analyzed did not have a consistent difference between the control and salt treated stress, neither the control or the salt treated tetraploid had a higher or lower gene expression. The primers with no consistent difference in normalized gene expression include: *ALKBH10* and *RD22*. The expression of nine genes including *DREB1*, *MTB*, *FIP37*, *ECT3*, *ECT1*, *ALKBH6*, *ALKBH9*, *GI* and *CBL2* (Figure 5) in the salt treated tetraploids had a much lower gene expression than in the control tetraploids (Table 2 & 3).

3.6 Comparison of the control diploid and control tetraploid

Gene expression levels were compared between the control diploid plants and control tetraploid plants. The majority of the primers analyzed did not show any consistent difference, either higher or lower, for normalized gene expression between the control diploid and the control tetraploid. The primers with no consistent difference include: *DRF2*, *WRKY12*, *NaC027*, *FIP37*, *ECT3*, *ECT1*, and *ALKBH10* (Table 3). Three genes, *DREB1* (Figure 6), *ALKBH9*, and *PiP1* demonstrated a higher normalized gene expression for the control tetraploid plants than the control diploid plants. The expression of *MTB* was around the same between the control diploid and control tetraploid (Table 3).

3.7 Comparison of the salt treated diploids and salt treated tetraploids

Comparison of the gene expression between the treated diploid plants and the treated tetraploid plants revealed one primer that showed a consistent pattern of difference between the salt treated diploids and tetraploids. The salt-treated diploid for the primer *CBL2* (Figure 7) had a

higher normalized gene expression compared to the salt-treated tetraploid (Table 2). The rest of the primers that were analyzed showed no consistent difference when comparing the salt treated diploid to the salt treated tetraploid: *DREB1*, *MTB*, *FIP37*, *ECT3*, *ECT1*, *GI*, and *RD22*. A couple of the primers showed similar normalized gene expression for both the salt treated diploid and the salt treated tetraploid: *ALKBH9* and *ATAF* (Table 2).

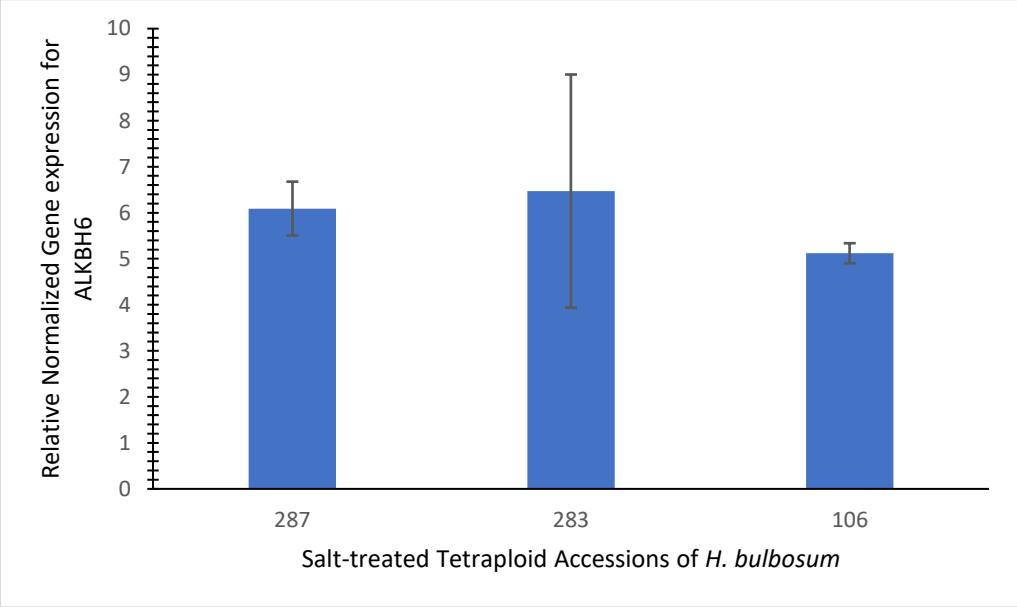

Figure 2. The bar chart of relative normalized gene expression of *HvALKBH6* for the tetraploid accessions of *H. bulbosum* salt-treated group. The bars represent the normalized data of the three replicates of *HvALKBH6* with the variation between the trials represented by the error bars. Relative normalized gene expression (△△Cq) was calculated using Actin as the housekeeping gene.

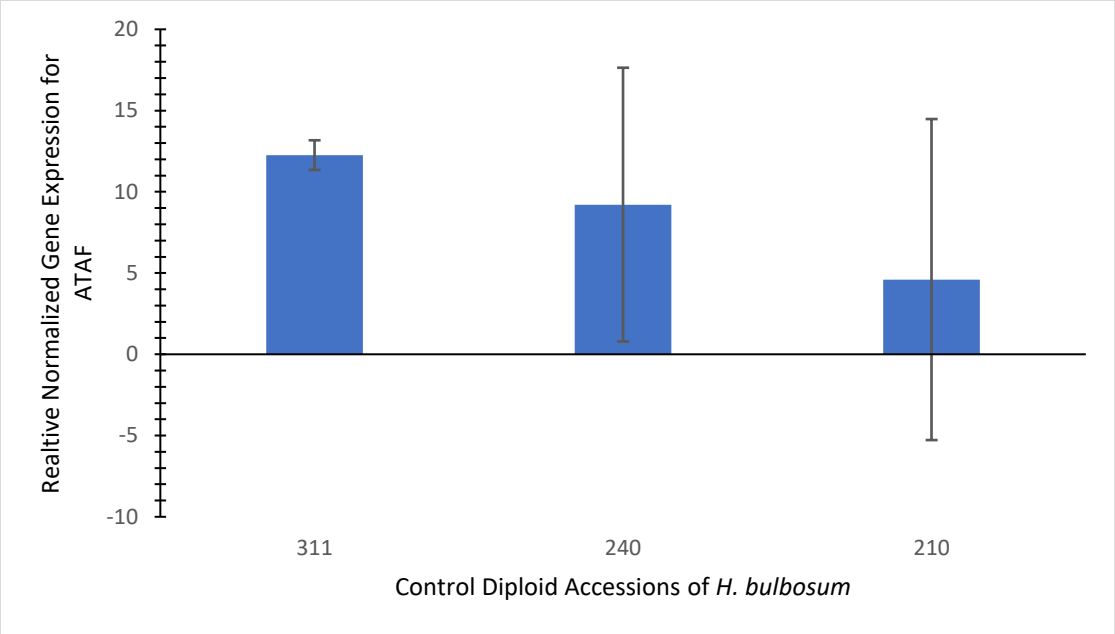

Figure 3. The bar chart of relative normalized gene expression of *HvATAF* for the diploid accessions of the *H. bulbosum* control group. The bars represent the normalized data of the three replicates of *HvATAF* with the variation between the trials represented by the error bars. Relative normalized gene expression (△△Cq) calculated using Actin as the housekeeping gene.

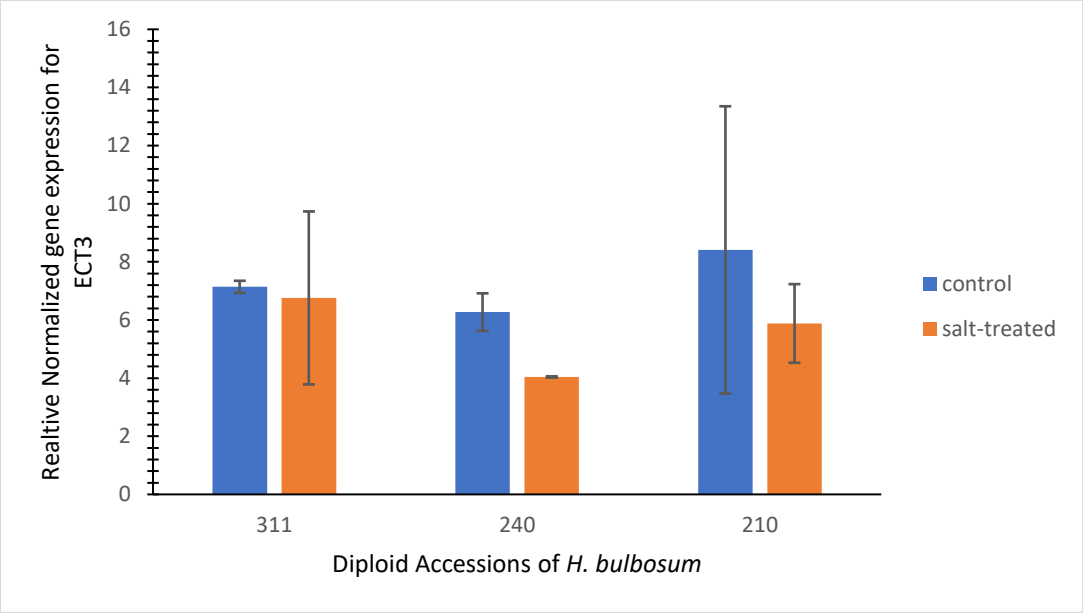

Figure 4. The bar chart of relative normalized gene expression of *HvECT3* for the diploid accessions of *H. bulbosum* for the salt-treated and control groups. The bars represent the normalized data of the three replicates of *HvECT3* with the variation between the trials represented by the error bars. Relative normalized gene expression (△△Cq) calculated using Actin as the housekeeping gene.

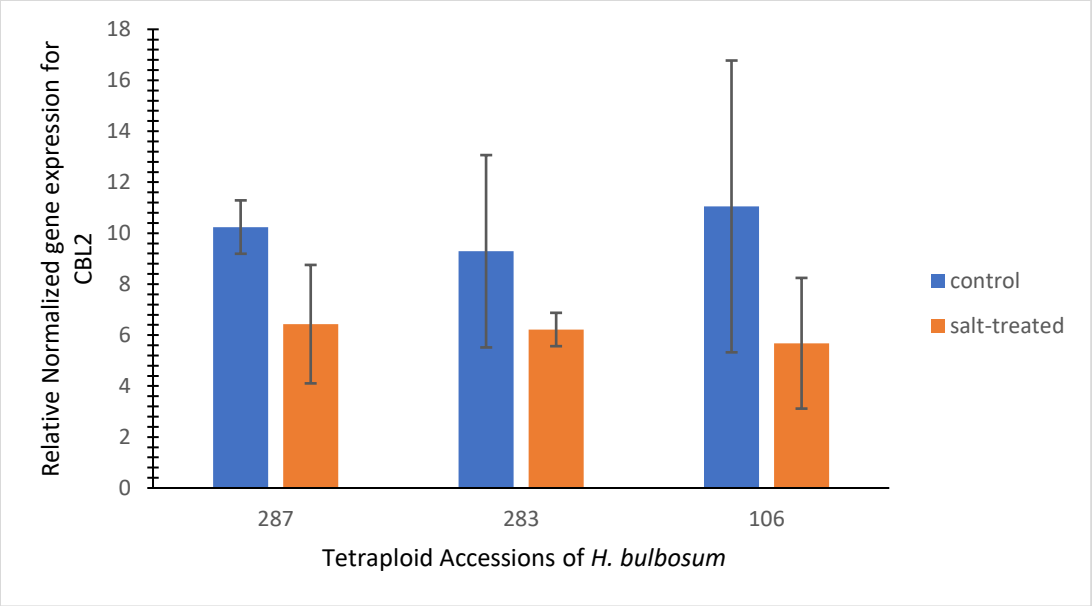

Figure 5. The bar chart of relative normalized gene expression of *HvCBL2* for the tetraploid accessions of the salt-treated and control *H. bulbosum*. The bars represent the normalized data of the three replicates of *HvCBL2* with the variation between the trials represented by the error bars. Relative normalized gene expression (△△Cq) calculated using Actin as the housekeeping gene.

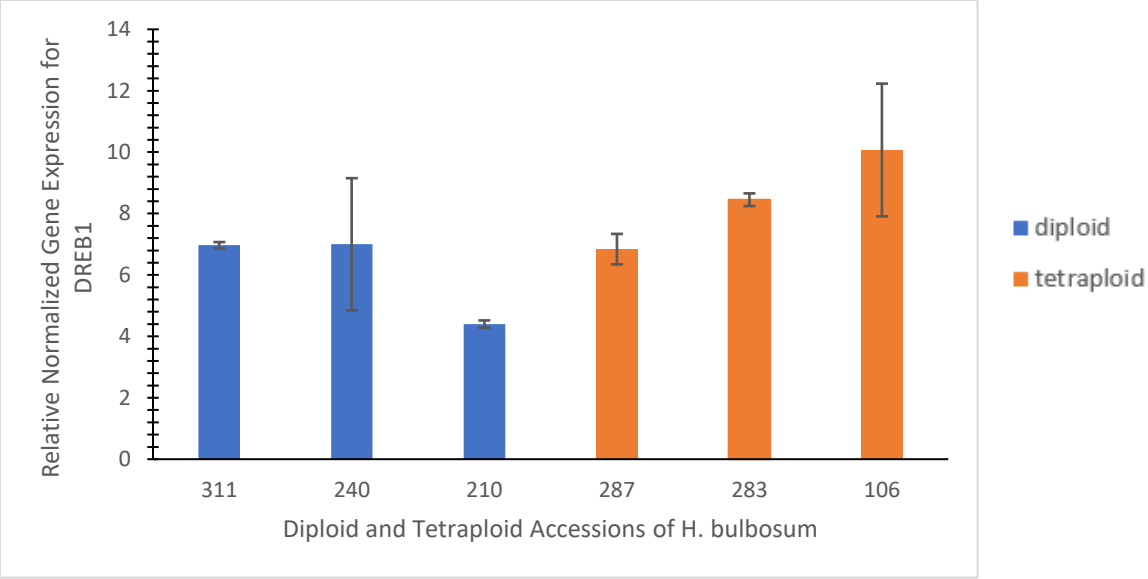

Figure 6. The bar chart of relative normalized gene expression of *HvDREB1* for the diploid and tetraploid accessions of the control grown *H. bulbosum*. The bars represent the normalized data of the three replicates of *HvDREB1* with the variation between the trials represented by the error bars. Relative normalized gene expression (△△Cq) calculated using Actin as the housekeeping gene.

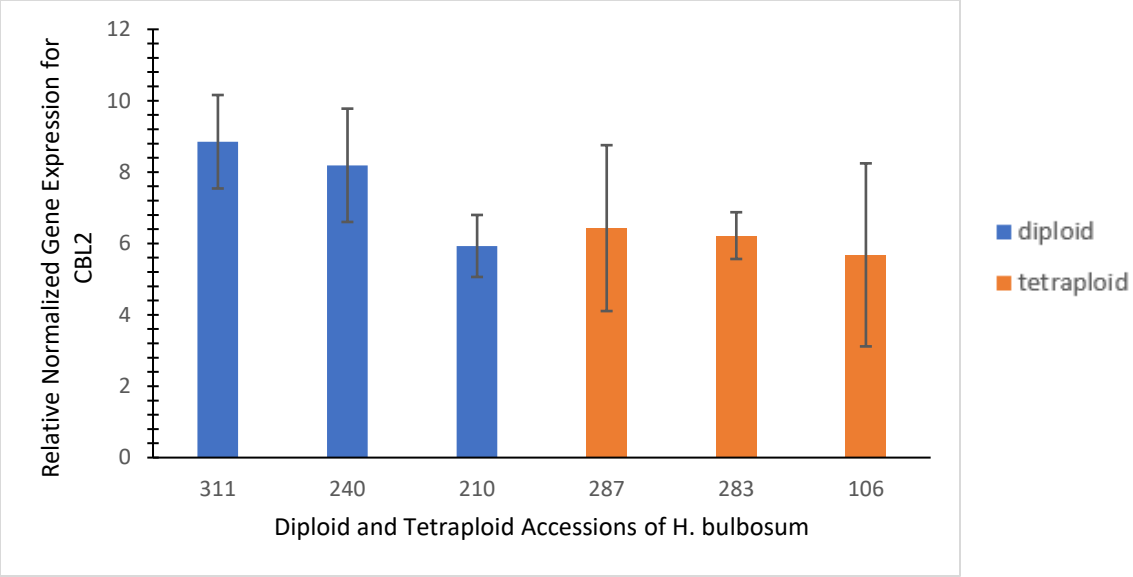

Figure 7. The bar chart of relative normalized gene expression of *HvCBL2* for the tetraploid accessions of the salt-treated *H. bulbosum*. The bars represent the normalized data of the three replicates of *HvCBL2* with the variation between the trials represented by the error bars. Relative normalized gene expression (△△Cq) calculated using Actin as the housekeeping gene.

Table 2. Normalized data for each accession of *H. bulbosum* in the salt treated samples

Primer	Accession	Mean value for each Primer	Mean value for Actin	Normalized data
HKT1	311	37.74	22.67	15.07
	240	35.48	23.28	12.20
	210	35.49	23.47	12.02
	287	24.85	25.74	-0.89
	283	22.56	25.10	-2.54
	106	25.17	24.79	0.38
BAS1	311	23.09	23.47	-0.38
	240	25.11	25.56	-0.45
	210	25.01	25.19	-0.18
	287	26.28	26.72	-0.44
	283	25.80	25.51	0.29

	106	25.30	26.18	-0.88
LHCB	311	26.06	24.04	2.02
	240	27.90	26.23	1.67
	210	27.32	25.89	1.43
	287	24.49	22.85	1.64
	283	22.01	22.05	-0.04
	106	26.27	25.21	1.06
WRKY12	311	12.67	23.70	-11.03
	240	27.73	25.56	2.17
	210	29.00	25.19	3.81
	287	12.35	27.46	-15.11
	283	25.39	25.50	-0.11
	106	34.76	26.21	8.55
DRF2	311	31.42	23.47	7.95
	240	31.57	26.23	5.34
	210	20.80	25.89	-5.09
	287	32.63	26.72	5.91
	283	22.86	25.51	-2.65
	106	32.10	26.18	5.92
DREB1	311	31.42	27.58	3.84
	240	32.40	26.01	6.39
	210	30.85	25.97	4.88
	287	30.15	23.89	6.26
	283	31.03	25.30	5.73
	106	33.51	28.24	5.27
NaC005	311	27.80	27.58	0.22
	240	26.91	26.01	0.90
	210	26.16	25.97	0.19
	287	27.43	23.89	3.54
	283	26.56	25.30	1.26
	106	25.78	28.24	-2.46
NaC027	311	34.44	23.70	10.74
	240	33.93	25.37	8.56
	210	33.71	25.04	8.67
	287	32.94	27.46	5.48
	283	23.99	25.50	-1.51
	106	32.47	26.21	6.26
MTB	311	29.93	25.21	4.72
	240	29.39	24.12	5.27
	210	30.58	24.45	6.13

	287	31.69	25.80	5.89
	283	31.88	25.79	6.09
	106	31.24	25.83	5.41
FIP37	311	30.14	25.21	4.93
	240	30.55	24.12	6.43
	210	29.72	24.45	5.27
	287	31.53	25.80	5.73
	283	31.53	25.79	5.74
	106	30.64	25.83	4.81
ECT3	311	32.48	25.72	6.76
	240	29.49	25.45	4.04
	210	30.60	24.72	5.88
	287	33.06	26.92	6.14
	283	32.49	26.23	6.26
	106	32.10	27.06	5.04
ECT1	311	31.07	25.72	5.35
	240	28.00	25.45	2.55
	210	28.13	24.72	3.41
	287	30.94	26.92	4.02
	283	30.92	26.23	4.69
	106	31.15	27.06	4.09
ALKBH6	311	20.97	24.38	-3.41
	240	30.52	24.10	6.42
	210	29.00	23.71	5.29
	287	32.90	26.81	6.09
	283	32.60	26.13	6.47
	106	32.98	27.86	5.12
ALKBH8	311	33.38	24.38	9.00
	240	31.69	24.10	7.59
	210	31.72	23.71	8.01
	287	11.35	26.81	-15.46
	283	35.43	26.13	9.30
	106	22.81	27.86	-5.05
ALKBH9	311	30.81	24.70	6.11
	240	29.03	23.79	5.24
	210	29.91	23.43	6.48
	287	31.42	26.15	5.27
	283	32.56	26.33	6.23
	106	33.51	27.33	6.18
ALKBH10	311	29.22	24.70	4.52

	240	26.89	23.79	3.10
	210	19.47	23.43	-3.96
	287	30.33	26.15	4.18
	283	33.99	26.33	7.66
	106	31.26	27.33	3.93
ATAF1	311	35.17	25.04	10.13
	240	32.54	26.79	5.75
	210	34.95	24.31	10.64
	287	35.29	27.75	7.54
	283	35.92	25.76	10.16
	106	34.85	26.19	8.66
PiP1	311	22.05	25.04	-2.99
	240	31.19	26.79	4.40
	210	33.05	24.31	8.74
	287	33.92	27.75	6.17
	283	22.62	25.76	-3.14
	106	32.49	26.19	6.30
BGLU12	311	32.22	25.98	6.24
	240	34.49	29.26	5.23
	210	0.00	28.50	-28.50
	287	31.36	27.82	3.54
	283	33.87	26.98	6.89
	106	32.01	27.59	4.42
PP2C	311	31.97	25.98	5.99
	240	34.32	29.26	5.06
	210	12.87	28.50	-15.63
	287	22.84	27.82	-4.98
	283	32.63	26.98	5.65
	106	31.07	27.59	3.48
GI	311	29.51	25.76	3.75
	240	29.71	26.21	3.50
	210	32.25	26.40	5.85
	287	31.16	28.50	2.66
	283	32.80	26.93	5.87
	106	30.75	26.60	4.15
GSTU17	311	25.71	25.76	-0.05
	240	24.83	26.21	-1.38
	210	25.31	26.40	-1.09
	287	39.08	28.50	10.58
	283	13.09	26.93	-13.84

	106	37.70	26.60	11.10
RD22	311	32.60	26.32	6.28
	240	32.54	25.70	6.84
	210	35.29	26.15	9.14
	287	33.14	27.95	5.19
	283	34.66	25.80	8.86
	106	31.97	27.14	4.83
CBL2	311	32.67	23.82	8.85
	240	33.47	25.28	8.19
	210	31.43	25.50	5.93
	287	32.09	25.66	6.43
	283	32.70	26.48	6.22
	106	32.35	26.67	5.68
SOS1L	311	33.62	23.82	9.80
	240	32.23	25.28	6.95
	210	31.20	25.50	5.70
	287	31.27	25.66	5.61
	283	22.29	26.48	-4.19
	106	31.35	26.67	4.68

Table 3. The mean value of the three trials of primer normalized using the mean of actin for each accession of the control treatment *H. bulbosum*.

Primer	Accession	Mean value for each Primer	Mean value for Actin	Normalized data
HKT1	311	11.48	23.38	-11.90
	240	33.89	22.81	11.08
	210	11.63	23.46	-11.83
	287	23.77	24.09	-0.32
	283	23.98	24.86	-0.88

	106	32.59	23.95	8.64
BAS1	311	23.66	24.35	-0.69
	240	23.51	24.14	-0.63
	210	24.35	24.76	-0.41
	287	23.46	24.09	-0.63
	283	24.17	25.00	-0.83
	106	24.07	25.01	-0.94
LHCB	311	23.05	22.52	0.53
	240	22.19	25.37	-3.18
	210	25.06	26.87	-1.81
	287	23.01	22.41	0.60
	283	22.69	22.61	0.08
	106	20.82	22.72	-1.90
WRKY12	311	36.92	25.89	11.03
	240	31.33	24.14	7.19
	210	32.05	24.76	7.29
	287	25.75	24.86	0.89
	283	34.24	24.54	9.70
	106	32.93	25.68	7.25
DRF2	311	31.86	24.35	7.51
	240	34.32	25.37	8.95
	210	32.02	26.87	5.15
	287	32.26	24.09	8.17
	283	34.36	25.00	9.36
	106	30.02	25.01	5.01
DREB1	311	31.49	24.52	6.97
	240	31.40	24.40	7.00
	210	30.19	25.79	4.40
	287	31.34	24.50	6.84
	283	33.52	25.07	8.45
	106	35.10	25.03	10.07
NaC005	311	27.87	24.52	3.35
	240	26.83	24.40	2.43
	210	23.84	25.79	-1.95
	287	27.19	24.50	2.69
	283	26.06	25.07	0.99
	106	26.37	25.03	1.34
NaC027	311	33.06	25.89	7.17
	240	33.71	24.03	9.68
	210	33.74	24.75	8.99

	287	33.16	24.86	8.30
	283	33.83	24.54	9.29
	106	33.66	25.68	7.98
MTB	311	31.77	25.16	6.61
	240	29.37	23.28	6.09
	210	30.43	24.15	6.28
	287	30.85	22.33	8.52
	283	31.64	25.07	6.57
	106	31.22	24.72	6.50
FIP37	311	31.52	25.16	6.36
	240	28.71	23.28	5.43
	210	30.64	24.15	6.49
	287	31.39	22.33	9.06
	283	31.18	25.07	6.11
	106	30.78	24.72	6.06
ECT3	311	33.32	26.18	7.14
	240	30.36	24.09	6.27
	210	32.12	23.71	8.41
	287	31.71	25.33	6.38
	283	33.18	25.91	7.27
	106	32.70	25.58	7.12
ECT1	311	31.51	26.18	5.33
	240	28.09	24.09	4.00
	210	29.79	23.71	6.08
	287	29.94	25.33	4.61
	283	30.65	25.91	4.74
	106	30.64	25.58	5.06
ALKBH6	311	21.49	26.12	-4.63
	240	31.80	23.28	8.52
	210	30.09	23.60	6.49
	287	32.73	25.10	7.63
	283	32.61	25.89	6.72
	106	31.87	25.61	6.26
ALKBH8	311	22.73	26.12	-3.39
	240	31.33	23.28	8.05
	210	32.22	23.60	8.62
	287	11.59	25.10	-13.51
	283	12.06	25.89	-13.83
	106	22.79	25.61	-2.82
ALKBH9	311	31.09	25.69	5.40

	240	28.90	23.24	5.66
	210	30.26	23.83	6.43
	287	30.95	24.30	6.65
	283	32.19	25.38	6.81
	106	31.88	25.20	6.68
ALKBH10	311	30.78	25.69	5.09
	240	28.78	23.24	5.54
	210	30.26	23.83	6.43
	287	30.70	24.30	6.40
	283	31.78	25.38	6.40
	106	31.47	25.20	6.27
ATAF1	311	37.58	25.32	12.26
	240	34.62	25.41	9.21
	210	29.27	24.67	4.60
	287	35.23	25.06	10.17
	283	23.38	25.78	-2.40
	106	0.00	26.04	-26.04
PiP1	311	33.96	25.32	8.64
	240	30.48	25.41	5.07
	210	30.44	24.67	5.77
	287	34.12	25.06	9.06
	283	35.12	25.78	9.34
	106	34.69	26.04	8.65
BGLU12	311	33.85	27.46	6.39
	240	35.63	27.54	8.09
	210	0.00	29.05	-29.05
	287	32.49	26.18	6.31
	283	35.01	27.58	7.43
	106	23.50	27.14	-3.64
PP2C	311	30.32	27.46	2.86
	240	35.85	27.54	8.31
	210	23.72	29.05	-5.33
	287	30.81	26.18	4.63
	283	32.21	27.58	4.63
	106	31.11	27.14	3.97
GI	311	30.26	26.12	4.14
	240	29.75	25.45	4.30
	210	21.81	27.07	33.33
	287	28.31	24.42	3.89
	283	31.91	25.93	5.98

	106	32.17	26.52	5.65
GSTU17	311	38.59	26.12	12.47
	240	36.83	25.45	11.38
	210	25.48	27.07	-1.59
	287	25.36	24.42	0.94
	283	12.80	25.93	-13.13
	106	13.09	26.52	-13.43
RD22	311	22.23	27.48	-5.25
	240	34.44	25.88	8.56
	210	34.76	25.45	9.31
	287	32.68	25.49	7.19
	283	33.55	25.68	7.87
	106	31.06	25.21	5.85
CBL2	311	33.79	24.11	9.68
	240	31.33	24.20	7.13
	210	11.07	24.37	-13.30
	287	33.37	23.13	10.24
	283	34.04	24.75	9.29
	106	36.16	25.11	11.05
SOS1L	311	33.54	24.11	9.43
	240	32.56	24.20	8.36
	210	21.81	24.37	-2.56
	287	30.33	23.13	7.20
	283	34.10	24.75	9.35
	106	33.01	25.11	7.90

4. Discussion

4.1 qPCR amplification

The primers that did not amplify well could be caused by poor primer design, or even low-quality cDNA. The difference in gene expression level for the various primers could be due to the accession of *H. bulbosum*. In this study we used 6 accessions of *H. bulbosum*, each originating from a different country. The different ecological niches each plant normally survives

could be a reason for different expression levels of different genes (Glennon et al., 2014). Each country the *H. bulbosum* accessions originated from would most likely have different environments with each plant requiring different survival needs therefore requiring different genes to survive (Thoen et al., 2017). Most likely the genes we targeted amplified poorly as the different accessions need different genes to survive. Dong et al (2020) noted for their experiment on testing salt tolerance in Gossypium (cotton plants) that the difference in salt stress response could be due to the different accessions of plants. Zhang et al (2014) noted that for physic nut seedlings, the plants had differing levels of salt-tolerant gene expression for the roots and leaves. In this study, cDNA was made from RNA that was collected from only the leaves of *H. bulbosum*. If plant material for both the leaves and the roots had been collected for analysis this would have better represented the gene expression of *H. bulbosum* due to a larger sampling effort and potentially less of a difference between the accessions could have been seen.

4.2 Comparison of diploids and tetraploids for the salt treated plants and control plants

Our main objectives were to see if the genome doubling changes the gene expression of salt-tolerant and m6A genes. Unfortunately, many primers we tested did not have all positive values or all negative values for the same ploidy. This meant many primers had to be excluded from the study as only primers where the ploidy levels had the same pattern of gene expression could be compared to other ploidy levels and treatments.

A lot of the primers that were amplified did have positive values for all three trials. The primers that amplified with positive values are all the upregulated areas of the targeted genes. The primers that amplified with all negative values are the downregulated areas of the targeted genes. This study found a similar finding for the comparison of the salt treated diploids and tetraploids for the control treated diploids and tetraploids. The primers that amplified with all

positive values for the three trials were identified as those that upregulated from the targeted genes. The primers that amplified with all negative values for the three trials were identified as being downregulated from the targeted gene. In the salt treated group the downregulated genes included *BAS1* (diploid and tetraploid), and *GSTU17* (diploid). The downregulated genes in the control group included *BAS1* (diploid and tetraploid), and *ALKBH6* (tetraploid).

4.3 Comparison of the control and salt treated diploids

One objective of this study was to see if genome doubling changes the expression of (1) genes related to salt stress, (2) transcription factors that regulate salt stress related genes and (3) m6A genes in response to salt stress. The results showed there was no consistent difference either higher or lower between the control and salt treated diploids. This is an indication that the targeted m6A and salt tolerant genes do not change gene expression when the diploid *H. bulbosum* activates the salt stress response. It has been commonly found in some plants between the diploid and tetraploid accessions, that the tetraploid would survive in higher salinity conditions compared to the diploid (Liu & Sun, 2017). Dong et al (2020) found when comparing the salt tolerance levels of diploid and tetraploid cotton plants that the tetraploids did not outperform the diploids in moderate and high salinity environments. This indicates that the diploid accessions of some plants are better at withstanding salt stress than the tetraploid accessions.

The gene expression for the primers *ECT3* and *ECT1* was lower in the salt treated diploid plants than the control treated diploid plants. This is an indication that *ECT3* and *ECT1* play an important role in salt tolerant response in *H. bulbosum* as the level of gene expression in the salt treated plants decreases from control treated plants. It has been found that ECT genes (*ECT2*, *ECT3*, *ECT4*) function to stimulate growth and proliferation in organ primordia (Arribas-

Hernández et al., 2020b). When the gene transcript for ECT genes contain m6A it was noted that ECT genes play an important role for m6A pathways in plants (Arribas-Hernández et al., 2020b). It was also seen that when ECT gene transcripts had reduced m6A levels the plants showed delayed development and slower root and stem growth (Arribas-Hernández., 2020b). These findings highlight the importance of m6A not only for stress response but for plant development and growth.

4.4 Comparison of the control tetraploids and salt treated tetraploids

The gene expression for salt treated tetraploids was lower than the gene expression for control treated tetraploids for primers including *DREB1*, *MTB*, *FIP37*, *ECT3*, *ECT1*, *ALKBH6*, *ALKBH9*, *GI* and *CBL2*. These results demonstrated the objective that genome doubling does change gene expression of m6A and salt tolerant genes as the results show that the m6A genes and the salt tolerant genes were targeted by the salt-treated tetraploids. When *H. bulbosum* activates the stress response it is assumed that the plant would want to express as many beneficial genes as it can to increase the chance of survival (Yona et al., 2012). The beneficial genes would be any gene relating to salt tolerance. As the salt treated tetraploids showed a lower gene expression for m6A and salt-tolerant genes it is seen that these genes (*DREB1*, *MTB*, *FIP37*, *ECT3*, *ECT1*, *ALKBH6*, *ALKBH9*, *GI* and *CBL2*) are important for salt tolerance and help the tetraploid plants to survive salt stress. Karan et al (2012) had similar results with rice plants in that the salt treated plants had a lower gene expression for salt-tolerant genes than the control plants. Based on the idea that tetraploids have a higher salt tolerance than diploid accessions, Xie et al (2020) tested wheat plants under salty conditions and found that the higher the ploidy of wheat varieties the better the salt tolerance. These results are an indication that genome duplication is beneficial for salt tolerance as the wheat plants with higher ploidy levels, the plants that underwent genome duplication, can better withstand salt stress. Our results indicate that the

salt-treated tetraploids showed gene expression for a wide variety of salt-tolerant and m6A genes under salty environments.

4.5 Comparison of the control diploids and control tetraploids

There was no consistent difference, either increase or decrease, found between the control diploids and the control tetraploids. As tetraploids are individuals with 2 sets of chromosomes and were historically diploid until extreme environments adapted them to become tetraploid (Liu & Sun, 2017), it was thought that tetraploids would hold more genetic material including DNA and genes as they hold two times the chromosomes of a diploid (Yona et al., 2012). As a result of the tetraploids potentially having twice the amount of cellular material this could mean that tetraploids would have twice the amount of m6A and salt tolerant genes. The findings of this study indicated this is not the case. Many of the primers that were analyzed comparing the control diploids and control tetraploids demonstrated that the control tetraploids did not differ in gene expression for m6A and salt tolerant related genes. The results contradict the findings of Karan et al (2012) where the experiment noted a difference in the expression of methylated transcripts for diploid and tetraploid rice plants under salt stress. This finding by Karan et al (2012) suggested that methylation or genes that code for methylation will have different gene expression levels in plants with different ploidy levels. As m6A is identified as an mRNA methylation, our results do not correspond well with those found by Karan et al (2012).

It has been demonstrated that m6A can be beneficial for a range of plant stress responses including drought tolerance and pathogen response in addition to benefiting early plant development (Anderson, 2018). Due to m6A being important for early plant development it can be assumed that m6A genes are present within plants starting from seeds to fully grown adult plants. Our results of the control plants clearly indicated that the plants show gene expression for

m6A even when the plants have not activated any stress response. This could mean that gene expression does not change as the plants develop, including when the plants experience different stressful environments. In this study, we only collected plant material at a single point during plant development. If we had potentially collected plant samples from *H. bulbosum* as seeds, young seedlings, and fully grown plant we would have been able to use RT-qPCR to identify if m6A and salt tolerant gene expression in *H. bulbosum* stays the same throughout the lifetime of the plant.

4.6 Comparison of the salt treated diploids and salt treated tetraploids

There was no consistent difference, either increased or decreased in gene expression for the majority of the primers when testing to see if genome doubling changed gene expression of m6A and salt-tolerant genes in salt treated diploids and tetraploids. Only *ALKBH9* and *ATAF* demonstrated similar gene expression for the salt treated diploids and salt treated tetraploids. Based on the objectives of this study, it was found that the genome doubling did not change the gene expression for *ALKBH9* and *ATAF* in the salt treated tetraploid plants. The objectives were to determine the effects of genome doubling concerning expression, transcription factors and m6A genes. Based on the results from this study the salt treated tetraploids did not show changed gene expression when *H. bulbosum* was faced with salt stress. The genome doubling did not result in the tetraploids showing increased or decreased expression for m6A genes or transcription factors.

As a crop plant, barley has been identified as the most salt tolerant of cereal crops (Liu & Sun, 2017). Due to barley being highly tolerant to salt stress this could have potentially had an impact on our results. Potentially by increasing the NaCl concentration above 250 mM we would start to see a clearer difference in m6A and salt tolerant gene expression between the diploid and

tetraploid salt treated plants. In the plant species Roma, which has been identified as a more salt-sensitive plant, a large difference in gene expression of m6A was seen between the control and salt treated plants (Zheng et al., 2021). This finding by Zheng et al (2021) furthers the idea that *H. bulbosum* was not tested under high enough salt concentrations to initiate a difference in gene expression between diploid and tetraploid plants due to barley being a highly salt tolerant plant.

4.7 Future Work

This experiment was successful in identifying a difference in gene expression levels in diploid and tetraploid individuals but failed to detect a significant difference in gene expression for different treatment groups. One way to improve this experiment would be to include additional treatment groups of higher or lower NaCl concentrations. Liu & Sun (2017) demonstrated that the salt tolerance response in *H. bulbosum* is activated in NaCl concentrations of 250 mM. It has also been documented that *H. bulbosum* is the most salt tolerant individual of the cereal crops (Yona et al., 2012). By adding an experimental group in which *H. bulbosum* are grown in a salty environment greater than 250 mM, experimenters can confirm the results of Liu & Sun (2017) by testing to see what salt concentration overwhelms the salt stress response resulting in the death of the plant.

Given the ability of m6A to be beneficial for an assortment of stress responses in plants including drought and disease tolerance (Anderson, 2018), it would be interesting to test *H. bulbosum* under different stressful conditions. By exposing *H. bulbosum* to drought, experimenters could see if gene expression changes. As m6A has been shown to help in drought stress response, experimenters can predict that drought tolerant genes like m6A will increase gene expression in *H. bulbosum* exposed to drought related stressful environments.

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Appendices

Appendix A. The ANOVA statistical analysis of the *H. bulbosum* treatment groups (control and salt treated) for the primers used for gene expression. All tests that were significant at P<0.05 (*), significant at P<0.01 (**), significant at P<0.001 (***).

Source	degrees of freedom	sum squared	mean squared	F-value	P-value	Significance
HKT1	1	24.63	24.631	6.85	0.0148	*
BAS1	1	13.57	13.567	14.81	0.000499	***
LHCB	1	74.33	74.33	21.56	4.96E-05	***
WRKY12	1	13.5	13.48	0.999	0.326	
DRF2	1	137.3	137.32	2.331	0.136	
DREB1	1	4.68	4.681	1.734	0.197	
ZIP1	1	13.52	13.519	6.021	0.0198	*
NAC005	1	1.54	1.542	0.726	0.4	
NAC027	1	0.62	0.6246	0.273	0.605	
MTB	1	0.08	0.0803	0.083	0.775	
FIP37	1	0.004	0.0036	0.004	0.95	
HAKA1	1	4.97	4.965	2.806	0.103	
ECT4	1	0.233	0.2327	0.264	0.611	
ECT3	1	2.51	5.512	1.153	0.29	
ECT1	1	0.04	0.0427	0.02	0.887	
ALKBH6	1	0.7	0.7033	0.277	0.603	
ALKBH8	1	0.06	0.06	0.018	0.894	
ALKBH9	1	0.98	0.9768	0.422	0.52	
ALKBH10	1	1.58	1.577	0.395	0.534	
ATAF1	1	1.89	1.887	0.297	0.59	
PiP1	1	0.59	0.592	0.16	0.692	
BGLU12	1	18.46	18.456	8.341	0.00755	**
PP2C	1	3.53	3.534	0.702	0.409	
GI	1	0.71	0.709	0.31	0.581	
GSTU17	1	0.266	0.266	0.255	0.618	
RD22	1	0.04	0.0368	0.018	0.893	
CBL2	1	13.29	13.291	4.998	0.0325	*
SOS1L	1	3.08	3.078	1.496	0.23	
TUBULIN	1	1.04	1.035	0.746	0.394	

Appendix B. The ANOVA statistical analysis of the *H. bulbosum* ploidy levels (diploid and tetraploid) for the primers used for gene expression. All tests that were significant at P<0.05 (*), significant at P<0.01 (**), significant at P<0.001 (***).

Source	degrees of freedom	sum squared	mean squared	F-value	P-value	Significance
HKT1	1	0.34	0.337	0.074	0.788	
BAS1	1	4.77	4.767	4.057	0.0519	
LHCB	1	37.76	37.76	8.348	0.00668	**
WRKY12	1	98.3	98.3	9.394	0.00478	**
DRF2	1	1.3	1.25	0.02	0.889	
DREB1	1	10.24	10.242	4.046	0.0525	
ZIP1	1	0.17	0.1694	0.064	0.802	
NAC005	1	0	0.0001	0	0.994	
NAC027	1	0.44	0.4363	0.19	0.666	
MTB	1	12.41	12.414	20.42	7.16E-15	***
FIP37	1	0.004	0.0036	0.004	0.95	
HAKA1	1	7.43	7.426	4.375	0.044	*
ECT4	1	2.675	2.675	3.307	0.0781	.
ECT3	1	11.85	11.845	6.222	0.0176	*
ECT1	1	14.59	14.592	8.778	0.00553	**
ALKBH6	1	30.18	30.182	18.61	0.000144	***
ALKBH8	1	39.56	39.56	22.78	6.71E-05	***
ALKBH9	1	39	39	32.55	2.08E-06	***
ALKBH10	1	50.46	50.46	20.12	8.32E-05	***
ATAF1	1	12.64	12.64	2.106	0.157	
PiP1	1	36.77	36.77	14.28	0.00065	***
BGLU12	1	4.85	4.854	1.787	0.192	
PP2C	1	29.59	29.595	7.099	0.0123	*
GI	1	3.18	3.176	1.437	0.239	
GSTU17	1	2.313	2.3132	2.423	0.133	
RD22	1	9.02	9.025	5.202	0.0292	*
CBL2	1	6.41	6.411	2.231	0.145	
SOS1L	1	1.81	1.812	0.865	0.359	
TUBULIN	1	10.87	10.869	9.982	0.00338	**