MicroRNAs contribute to enhanced salt adaptation of the autopolyploid *Hordeum bulbosum* compared to its diploid ancestor

By

Beibei Liu

A Thesis Submitted to

Saint Mary's University, Halifax, Nova Scotia,

In Partial Fulfillment of the Requirement for

The Degree of Master of Science in Applied Science

July 2017, Halifax, Nova Scotia

Copyright Beibei Liu, 2017

Approved: Dr. Genlou Sun
Professor
Supervisor

Approved: Dr. Doug Strongman
Professor
Committee Examiner

Approved: Dr. Christa Brosseau
Associate Professor
Committee Examiner

Approved: Dr. Gavin Kernaghan
Associate Professor
External Examiner

Date: July 12th, 2017

ABSTRACT

MicroRNAs contribute to enhanced salt adaptation of the autopolyploid *Hordeum*

bulbosum compared to its diploid ancestor

By Beibei Liu

Several studies have shown that autopolyploid species can tolerate abiotic stresses better than their diploid ancestor. However, the underlying molecular mechanism is poorly understood. Whole genome duplication can result in the expansion of miRNA families, and the innovative miRNA-target interaction is vital for adaptive response to various environments. The new microRNAs which were induced by genome duplication, also associated with stress response, and the distinctive microRNA networks in tetraploid and diploid *Hordeum bulbosum* by using high-throughput sequencing. Five miRNAs affected by genome doubling were involved in salt stress response. Of these, miR528b-3p was only detected in the tetraploid plants, and down - regulated in salt stress tetraploid plants relative to that in tetraploid check (CK). Moreover, it was found that miR528b-3p was not only involved in DNA replication and repair but also affected salt stress response. Finally, distinguished microRNAs-targets regulatory networks in both diploid and tetraploid plants were discovered.

July 2017

Dedication

I would like to dedicate this work to my boyfriend Chao Wang who has graduated last year. To wait for me graduating, he stays here for another year with me. There is no doubt that without his support and help in my work, I could not finish this work. I also want to express my special thanks to my loving parents. Although they didn't live with me here, it is their words of encouragement and love that support me to complete my graduate study.

I would also like to thank my family and especially my boyfriend for their love and encouragement whenever I was discouraged, without them this work could not have been completed.

Table of Contents

List of Figures

List of Tables

x

List of Abbreviations

MPV	Middle Parent Value
WGD	Whole Genome Duplication
STG	Signaling transduction genes
RLKs	Receptor-like Kinases
nt	Nucleotide
DCL1	Dicer-like 1

HYL1	Hyponastic Leaves 1
HENA1	Hua Enhancer 1
HST1	Histone deacetylase 1
AGO	Argonaute protein
RISC	RNA-Induced Silencing Complex
TFs	Transcriptional Factors
CK	Control experiment
WL	Water Loss
RWC	Relative Water Content
FW	Fresh Weight
TW	Turgid Weight
DW	Dry Weight
TPM	Transcripts Per Million
CT	Threshold Cycle
qRT-PCR	Quantative real-time PCR

Chapter 1 Introduction

1.1 Preamble

Polyploid organisms contain more than two complete sets of chromosomes, which can be classified as allopolyploid via merging two or more distinct genomes or autopolyploid by multiplying a single genome. Hybrid vigor, like taller stems and larger leaves, is a feature of allopolyploid which has been studied by many researchers. Autopolyploid is more common than once thought, but has received little attention compared with allopolyploid, because autopolyploid is often associated with poorer yield, smaller leaves, *etc.* However, several studies illustrated that autopolyploid organisms have a stronger ability to tolerate environmental stresses compared to its diploid progenitors, including drought, salt and cold stresses. Moreover, it is reported that genome doubling has a great influence on stress tolerance. Nevertheless, the molecular mechanism of how genome doubling may help autopolyploids to tolerate adverse environmental stresses is poorly understood.

MicroRNA are endogenous ~ 21 nucleotides (nt) small RNAs which play an important role in regulating gene expression by targeting mRNA for cleavage or inhibition of translation at the post-transcriptional level. There are two reasons for studying microRNAs in autopolyploid. First of all, microRNAs have been closely associated with stress response, and many stress-related microRNAs have been identified in various plants. Furthermore, it is suggested that genome duplication can retain known or induce new microRNA which could develop new microRNA mechanisms, and the innovative

microRNAs-target interactions are important for adaptive response to various environmental conditions, in addition to maintaining a constant set of microRNAs for basic biological functions. Therefore, it is entirely possible that genome duplication could induce new or maintain known microRNA to help autopolyploid tolerate environmental stress better than its diploid ancestor.

Investigating the microRNA mechanism in autopolyploid species is important in several respects. Firstly, understanding why autopolyploid could tolerate salt stress better than its diploid progenitor could help us better appreciate the significance of the autopolyploid from molecular and evolutionary aspects. Secondly, a better and more comprehensive knowledge of the complex mechanisms involved in the salt stress responses allows for the identification of novel points to engineer the plants, finding a better way to improve stress tolerance. Therefore, this thesis attempts to investigate diploid and tetraploid barley have different ability to tolerate salt stress by analyzing the microRNA sequencing data.

1.2 Objectives of the thesis

The objectives of this thesis are to compare the different ability to tolerate salt stress between diploid and tetraploid barley, and by analyzing the microRNA sequencing data to investigate the reason why autopolyploid and diploid has different ability to tolerate salt stress. Finally, to identify the distinct microRNA regulatory mechanisms between diploid and tetraploid barley under salt stress.

More specifically, this thesis attempts to investigate why diploid and tetraploid *Hordeum bulbosum* have different ability to tolerate salt stress from a microRNAs respect:

- Compare the ability of diploid and tetraploid barley to tolerate salt stress;

- Identify the salt stress-related miRNAs in diploid and tetraploid barley, respectively;

- Discover the differentially expressed microRNAs due to genome duplication;

- Determine the microRNAs which are not only affected by genome duplication, but also involved in salt stress response;

- Identify the distinct microRNAs-target mechanisms in diploid and tetraploid barley under salt stress.

Chapter 2 Literature Review

2.1 Polyploidy

2.1.1 The widespread occurrence of polyploidy in plants

Polyploid species contain more than two complete sets of chromosomes, including allopolyploid via merging two or more distinct genomes and autopolyploid by multiplying a single genome (Fig. 1).[1] Polyploidy is widespread in the plant kingdom, especially in angiosperm; almost 70% of angiosperm are polyploid.[2] Many polyploid species are important crops such as autopolyploid - alfalfa and potato, allopolyploid - wheat, oat, cotton and coffee, etc.[3] It is also reported that the ancestors of most flowering plants are ancient polyploidy which have undergone one or more whole genome duplications.[4] In addition, monocots and dicots are derived from whole-genome duplication and ancestral chromosome fusion events, therefore, almost all modern species are paleopolyploid.[5]
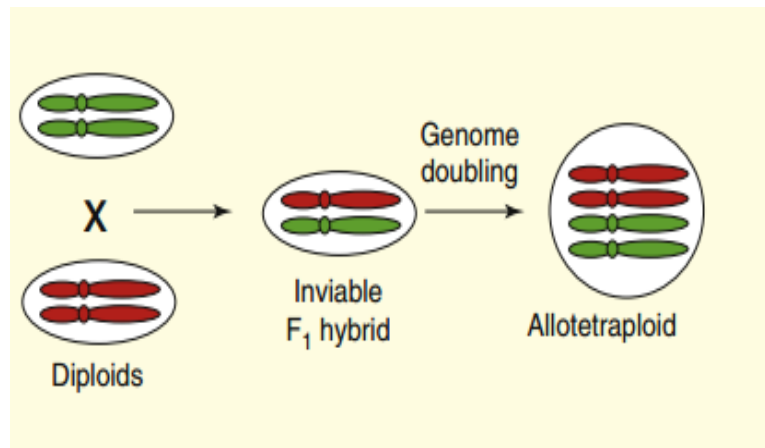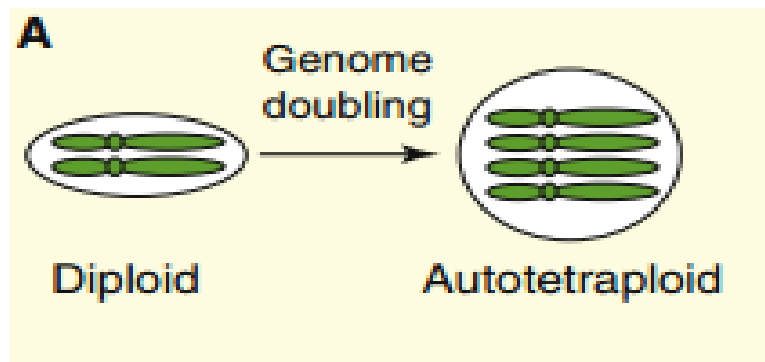
Figure 1. These two graphs illustrate the formation of allopolyploid and autopolyploid (cite from "Chen.K, 2007)[6].

2.1.2 The significance of polyploidy

The prominence of polyploidy indicates that polyploidy have some evolutionary or adaptive advantages over their diploid progenitors which are mainly manifested in phenotypic and physiological aspects.[2] From the phenotypic aspect, it was suggested that polyploidization could cause hybrid vigor compared to the diploid counterparts, such as increased biomass, larger leaves and taller stem, etc.[4] From a physiological perspective, polyploidy plays an important role in the evolution of plants, and is considered as the

determinant of the invasiveness of plants, since genome doubling can alter genetic make-up, physiology and ecology within only one or a few generations which can help polyploidy species survive in strongly fluctuating environments or promote colonization in new environment.[7,8] Many studies have also demonstrated that polyploidy has a stronger ability to tolerate stresses compared to its progenitor, and polyploidy has been regarded as an efficient way to improve environmental stress tolerance in plants.[9] However, the reason why polyploidy have such advantages is poorly known.

2.2 Allopolyploid

2.2.1 The research on hybrid vigor

Hybrid vigor, including larger leaves, taller stem and greater yield, is a typical feature of allopolyploid which has attracted muh attention. Significant effort and time have been devoted towards investigating the molecular mechanism of hybrid vigor. It was suggested that the change of gene expression patterns, involving non-additive gene expression, uniparental gene expression, tissue and developmental stage specific gene expression, underlie the phenotypic and physiological differences between allopolyploid and its diploid ancestor.[7-9] Non-additive gene expression refers to gene expression that does not equal to the middle parent value (MPV) (1 + 1 = 2; alternatively, 0.5 Parent 1 + 0.5 Parent 2 = 1),[10] uniparental gene expression means that only one parental gene is activated or repressed, while the tissue- and developmental- specific gene expression refers to the genes which are expressed in particular tissues or at certain developmental stages.[10-12]

2.2.2 The genome-wide gene expression research in allopolyploid

With the increasing utilization of Next Generation sequencing (NGS) which can help us to detect the gene expression change of a whole genome in one shot, genome-wide gene expression has been studied in many allopolyploids, including *Arabidopsis*, Cotton*, Senecio* and Wheat. Large scale gene expression changes were observed in these allopolyploids compared to their diploid progenitors, including 16% differentially expressed genes in wheat allohexaploid,[13] 30–70% in cotton allotetraploid, 30–60% in *Senecio* allohexaploids,[8] and 5–38% in *Arabidopsis* allotetraploids.[1] It was found that these differentially expressed genes encoding transcripts and regulatory factors may reprogram gene expression networks and induce non-additive phenotypes in allopolyploids, and eventually might partially contribute to the growth vigor in hybrids and allopolyploids compared with those of its diploid progenitors, and these phenotypes might also improve allopolyploid's survival ability.[10] Recent molecular studies have also shown that altered patterns of gene expression might confer upon the polyploidy a broader phenotypic range than that of their progenitors.[11]

2.2.3 The driving force for gene expression change in allopolyploid

The generation of polyploidy is followed by genetic change (chromosomal rearrangements and gene loss) and epigenetic changes (DNA methylation and histone modifications), which could lead to reprograming of gene expression patterns.[10] Genetic changes, mainly including chromosomal rearrangements and gene loss, means the DNA sequence variation induces the phenotypic or physiological differences, while epigenetic changes, mainly involving DNA methylation and histone modifications, refer to the

alteration of phenotype, morphological or molecular without changing the DNA sequence (Fig. 2).[14] Both DNA methylation and histone modifications are very common ways to regulate the gene expression on a transcriptional level. DNA methylation can not only silence the genes, but also silence the transposable element, and histone modification is another way to modify the gene expression. Histone is the primary packing structure of the DNA so the modification of histones can alter the   chromatin state, and thus the transcriptional state of genes, finally affecting gene expression.[14] Thus, in allopolyploid, the genetic and epigenetic changes due to whole-genome duplication could alter the gene expression patterns which might help allopolyploid to develop some non-additive phenotypes compared to its diploid progenitor.
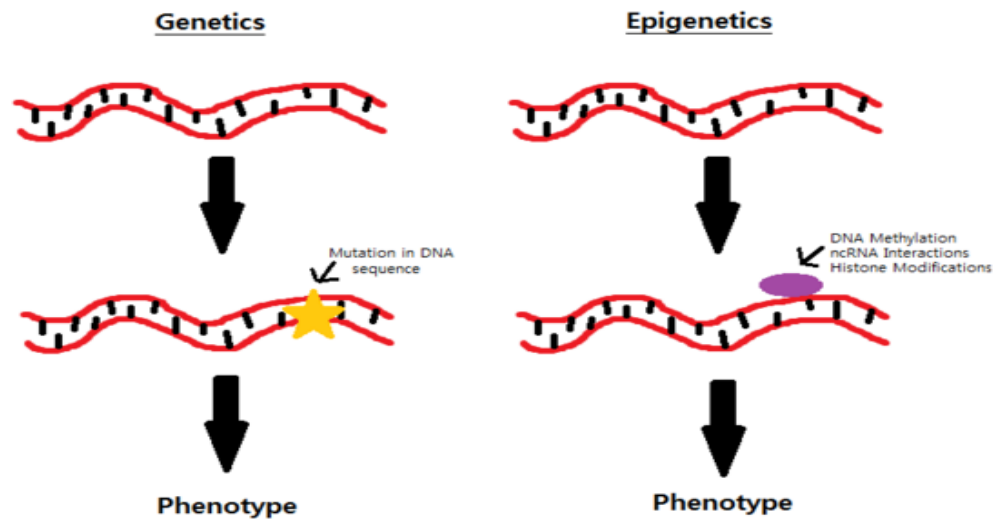
(Google)

Figure 2. This graph displays the genetic and epigenetic mechanisms which can alter the gene expression pattern.

2.2.4 Influence of genome duplication and hybridization on gene expression

Although it is reported that genetic and epigenetic changes could alter gene expression which could induce hybrid vigor in allopolyploid compared to its diploid parents, it is little known about whether the genetic and epigenetic changes are caused by hybridization or polyploidization. Because in allopolyploid, two processes--hybridization and genome duplication are involved (Fig. 1). It was reported that hybridization has a greater influence than genome doubling on gene expression change, in other words, genome duplication has a less effect on gene expression alteration[10,11,15,16]. However, it was identified that autopolyploid also has some differentially expressed genes relative to their diploid progenitors, which was only due to genome duplication rather than hybridization.[16] Currently, the significance of genome doubling *per se* is still unclear, and it is difficult to distinguish the effects of hybridization and genome duplication in allopolyploid. Therefore, a preferred way to understand the role of whole genome duplication is to study autopolyploid whose formation only requires genome doubling.

2.3 Autopolyploid

2.3.1 Effects of genome duplication in autopolyploid

Autopolyploid is more common than once thought, but has received little attention compared with allopolyploid, because autopolyploid is often associated with poorer yield, smaller leaves, etc.[17] Although there is a long-term interest in polyploidy, the significance of genome duplication is unclear. At present, there are two contradicting theories around

the significance of whole genome duplication (WGD). One theory suggests that autopolyploid is an evolutionary dead end because most genome duplication events have occurred near the tips of evolutionary tree of life, that is, polyploidization is often followed by extinction.[18] The second asserts that autopolyploid is a major driving force in evolution, because ancient WGDs occurred in plants and animals resulting in some particularly species-rich groups. [18, 19]

Although the influence of WGD on speciation is controversial, it is widely believed that genome doubling is tightly associated with environmental change,[20] and genome doubling can help plants develop a stronger ability to adapt to various environmental stresses.[17] Two examples can aptly illustrate this point. Firstly, there was a burst of genome duplication at the end of the Cretaceous period corresponding closely with the Cretaceous-Tertiary mass extinction which indicates that there is an association between genome-wide duplication and environmental stress/fluctuation.[21] Similar observations have been made for present-day polyploidy, which are often encountered in unstable and stressful environments such as the overabundance of recently formed polyploidy in the arctic, in particular of high-level polyploidy.[22] The successful range expansion and radiation have been demonstrated in various natural autopolyploid which indicates that genome doubling may represent an evolutionary advantage.[20] Recent studies have illustrated that autopolyploid has a stronger ability to cope with abiotic stresses than its diploid counterparts such as: the tetraploid *Brassica rapa* L,[23] *Citrus*[24] and *Black locust*[3] can deal with salt stress better than their respective diploid progenitors, while the tetraploid *Isatis indigotica* [9] and *Dendranthema nankingense* (Nakai) Tzvel[25] are capable to tolerate the

abiotic stresses better than their respective diploid ancestors. Therefore, genome doubling might play a critical role in the evolution of autopolyploid adapting to adverse environment conditions, which is in accordance with the discovery that genome doubling has a greater influence on stress tolerance than hybridization.[26]

2.3.2 Genome-wide gene expression research in autopolyploid

How whole genome duplication could help autopolyploid tolerate the adverse environmental conditions better than its diploid ancestor is poorly understood. In allopolyploid, large scale gene expression changes due to genetic and epigenetic alterations can account for why allopolyploids develop some phenotypic and physiological differences from its diploid ancestors. Although allopolyploid and autopolyploid share the property of duplicated genomes, the difference in their composition (Fig. 1) will lead to different results during their evolution.[19] Genome-wide gene expression studies have also been carried out in autopolyploid, however, no large-scale gene expression changes were observed. Only 4.3% of all genes showed expression differences between autopolyploid and diploid *Isatis indigotica* [3], and 1% gene expression changes were observed between the diploid and tetraploid rangpur lime (*Citrus limonia*)[27,28] which were consistent with the discovery that genome duplication has little influence on gene expression change. However, there are still some genes which are differentially expressed in autopolyploid compared to its diploid counterpart, and most of the non-additive genes and proteins are related to the stress response.[12] Among the differentially expressed genes, some of them are signaling transduction genes (STG), most STG encode plant receptor-like kinases (RLKs) which

11

play important roles in cell communication with each other and with the environment,[3] and among the six genes that differentially expressed, five are related to in response of the water-deficit stress and expressed higher in tetraploid.[28]

2.3.3 The driving force behind gene expression change in autopolyploid

The mechanism underlying the expression change of stress-related genes in autopolyploid is poorly understood. In allopolyploid, the genetic and epigenetic changes can change the gene expression pattern, but they are pretty rare in autopolyploid.[20] Actually, gene expression can be changed at different levels: transcriptional level and post-transcriptional level. Although many efforts have been devoted to study the effects of transcription factors on gene expression, transcriptional regulation doesn't have the largest influence on gene expression. It is reported that the amount of mRNA does not always have a positive relationship with the final concentration of active gene product (protein) which is the most relevant quantity to the phenotype.[6,12,29] Similar observation was made for re-synthesized *Brassica napus* allotetraploid; only one third differentially expressed genes have non-additive proteins which can be mainly regulated by post-transcriptional modifications.[30] Another research reported that transcriptome difference cannot explain the metabolic changes, there was a discordance between transcriptome and metabolic data which again suggests that post-transcriptional regulation plays an essential role in the gene expression after genome duplication.[17] Therefore, it is probably that post-transcriptional regulation plays an important role in the gene expression.

2.4 MicroRNAs (miRNAs)

2.4.1 The biogenesis of miRNAs in plants

On a post-transcriptional level, small RNAs (siRNAs and miRNAs) have significant influence. MicroRNAs (miRNAs) are endogenous ~ 21 nt small RNAs which play an important role in regulating gene expression by targeting mRNA for cleavage or repression of translation at post-transcriptional level.[31,32] miRNAs are encoded by MIR genes. First, the MIR genes are transcribed by RNA polymerase II (Pol II) and produce the primary miRNAs (pri-miRNAs) with a characteristic hairpin structure, which subsequently processed by DCL1 and assisted by HYPONASTIC LEAVES (HYL1) to form the precursor miRNAs with a partially double-strand stem-loop structure. The precursor miRNAs are processed by DCL proteins to form the miRNA/miRNA* duplex which is then methylated by HUA ENHANCER 1(HENA1) and exported into the cytoplasm by HASTY (HST1). The miRNA is combined with an AGO protein to form the RNA induced silencing complex (RISC) and guides the RISC to bind to target transcripts which can base pair with the miRNA, finally the endonuclease will cut the mRNA near the middle of the miRNA complementary (Fig. 3).[32]

2.4.2 The identification of miRNAs in plants

Since the first microRNA was cloned from *C.elegans*, [33] miRNAs have also been identified in various plants like *Arabidopsis*, [34] wheat (*Triticum aestivum* L.),[35] barley (*Hordeum vulgare* L.),[36] peanut (*Arachis hypogaea* L.),[37] *Gossypium hirsutum*,[38] *Medicago truncatula*,[39] *Citrus trifoliate*,[40] and rice.[41] Although this regulatory mechanism can be

identified in plants, animals even in green algae, there is no miRNA homologue among them.[42]
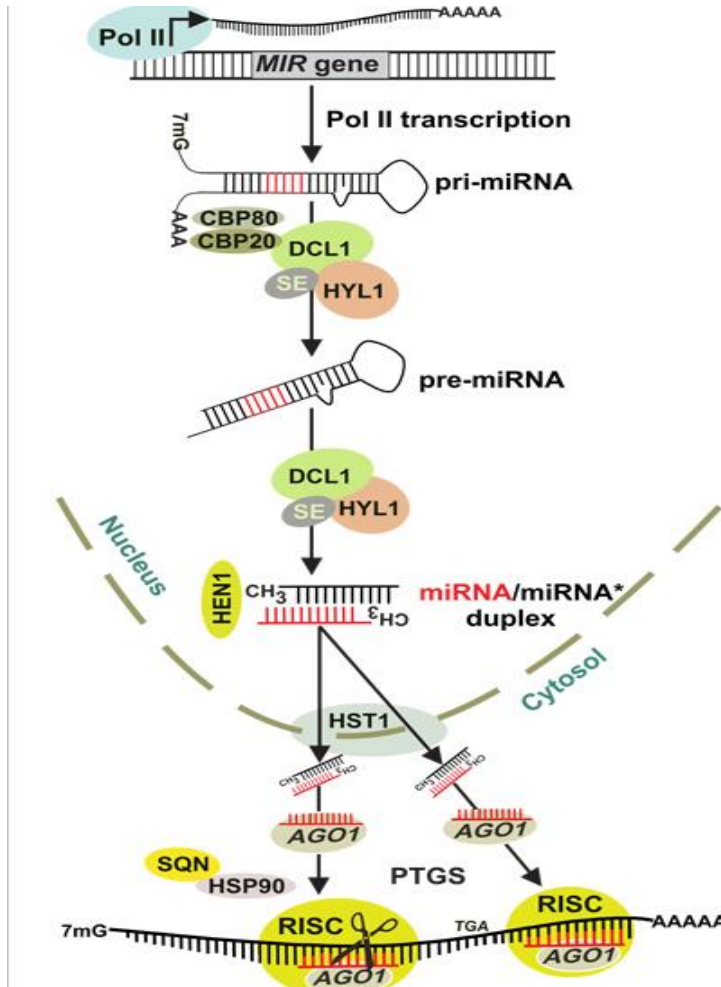
Figure 3. The biogenesis of microRNAs (cite from "Khraiwesha.B, 2012)[32].

2.4.3 The role of miRNAs during genome duplication

Whole-genome duplication is often followed by gene loss, which means genome-wide remove of some but not all redundant genes.[5] Although most of the duplicated genes produced by genome duplication will vanish in subsequent gene loss, some duplicated genes still remain, which are called dosage balance-sensitive duplicates, and gene loss is

14

not a random process and genes which are involved in transcription and signal transduction will be retained, while other genes which are involved in DNA repair and organelle proteins are preferentially lost.[5,43] The retention of dosage balance-sensitive duplicates does not provide an immediate evolutionary advantage, instead, their loss would lead to an immediate disadvantage.[44] In this respect, the maintained regulators might later have facilitated the evolutionary innovations or diversifications, thus creating new phenotype and physiological mechanisms in polyploidy.[22] It has become increasingly clear that the rewiring of the regulatory network following WGD is of major importance.[22]

Whole genome doubling (WGD) is a major mechanism for the expansion of miRNA families which has been identified in many studies. For instance, it has been found that the percentage of microRNAs relative to the total small RNAs increased with the increasing polyploidy.[45] Despite most miRNAs expression are similar to the MPV in hybrids, some significant deviations were observed in allopolyploid [45] which indicates some miRNAs are induced by genome duplication. Similar findings were reported in a study that some miRNAs are just exist in natural allopolyploids but not in interspecies hybrids.[46] In addition, many miRNAs are identified to target transcriptional factors (TFs) [5] which are preferable to be remained during WGD. The co-loss or co-retention of miRNAs and target genes may result in innovative miRNA-target interactions which are important for adaptive response to various environmental conditions, in addition to maintaining to constant set of miRNAs for basic biological functions.[5] Therefore, autopolyploid may not necessarily have a massive change of gene expression pattern, instead, polyploidy can retain more than two alleles per locus during genome doubling and then the duplicate genes

may evolve novel functions, which in theory, would help them better adapt to different stressful environment.[20]

2.4.4 The role of miRNAs in response to biotic and abiotic stresses

miRNAs play a critical role in various physiological and developmental processes including auxin signaling, organ separation, leaf development and reproduction.[32] Recently, it was found that miRNAs are tightly associated with various biotic and abiotic stress responses. Since the first stress-related miRNA was discovered in *Arabidopsis*,[34] many stress-related miRNAs have been discovered in various plants, including the drought induced miRNAs in rice[47] and *Sorghum bicolor* (L.) Moench,[48] the salt-induced miRNAs in maize,[49] the drought- and salt-induced miRNAs in tobacco[50]. By comparing the mRNA and small RNA transcriptome between allohexaploid wheat and its parents, it was found that microRNAs play important role in allopolyploid to adapt to different environmental conditions.[51] Although it is known that genome duplication can induce new miRNAs, and innovative miRNAs—targets are important in polyploids for response to unfavorable environmental conditions, little study has been done to explore the miRNAs that were not only induced by genome duplication but also participated in stress response.

2.5 Soil salinity

2.5.1 The severity of soil salinity

Soil salinity is a major environmental constraint to crop production, affecting an estimated 45 million hectares of irrigated land, and is expected to increase due to global climate changes and as a consequence of many irrigation practices.[52,53] Meanwhile, the fast expansion of industries and exhaustive utilization of fertile soil in many developing

countries have limited the increase of agricultural production and given rise to the demand for reclaiming saline coastal areas or arid lands, where high amounts of soluble salts exist in the soils.[23] Increased soil salt concentrations decrease the ability of a plant to take up water and, once Na^+ and Cl^- are taken up in large amounts by roots, both Na^+ and Cl^- negatively affect growth by impairing metabolic processes and decreasing photosynthetic efficiency.[52] Plants enact mechanisms to alleviate salt stress by reducing water loss while maximizing water uptake. Furthermore, plants minimize the harmful effects of ionic Na^+ stress by exclusion of Na^+ from leaf tissues and by compartmentalization of Na^+, mainly into vacuoles. Despite these tolerance mechanism, salt stress decrease crop yields and is leading to continuing loss of arable land. Such losses are compounded by the additional challenge that agriculture needs to provide enough nutrition for a world population that is rapidly expanding (estimated to reach 9.6 billion by the year 2025).[54]

2.5.2 The development of plant engineering

Many researchers have been focusing on understanding the physiological, genetic and molecular responses to environmental stresses to develop methods and approaches towards improving tolerance and acclimation. Salinity is perceived by sensor system resulting in the activation or suppression of complex regulatory networks which controls the expression of stress related genes to alleviate the detrimental effects and restore cellular homeostasis.[55,56] The classic approach to engineer plants to better tolerate environmental stresses involves strengthening the endogenous system by altering the expression or activity of sensors and signaling/regulatory elements (e.g. kinases, transcription factors), or direct-action genes or effectors (e.g. antioxidant enzymes, heat-shock proteins).[57-59]

Although this strategy has successfully improved the salt toleration of some plants, there are some drawbacks. In the first place, there is complex crosstalk between several regulatory, metabolic and developmental pathways. Thus, while intervening upstream in the signaling network could increase tolerance to different stresses, there is a higher probability of inducing undesired results including growth handicap and developmental alterations. By contrast, overexpression of direct-action genes normally provides enhance performance only against individual sources of stress.[58]   However, plants growing in natural environments are often simultaneously challenged by combination of stresses, for example, drought and heat.

Therefore, a better and more comprehensive knowledge of the complex mechanisms involved in the stress responses allow to identify novel points of intervention, finding a better way to improve stress tolerance. It is reported that manipulations of regulatory systems based on small RNAs including microRNA can achieve a more generalized stress tolerance while keeping a tighter control on the response.[58]

2.6 *Hordeum bulbosum*

Barley was domesticated in the Fertile Crescent about 10,000 years ago. Today barley is the fourth most important cereal crop after wheat, rice and maize.[60] In addition to its agricultural importance, barley is also a widely cultivated crop and one of the most tolerant to salinity among cereal crops, thus it is commonly used as a model plant to study the salinity stress[51,61,62]. *Hordeum bulbosum* L. is a wild barley and has many disease resistant genes that can be transferred into cultivated barley to improve the cultivars. The

chromosome of *H. bulbosum* tends to be eliminated when outcrossing with cultivar barley, which has attracted much attention from crop geneticists and breeders.[51,63,64] Most importantly, *H. bulbosum* includes diploid and natural autotetraploid that adapted to various environmental conditions. Therefore, this thesis attempts to investigate how genome duplication affects miRNAs and finally could help autopolyploid cope with stressful environments by growing the diploid and tetraploid cytotypes of *H. bulbosum* under control and salt stress.

2.7 Hypothesis and Objectives

Whole genome duplication might induce new or maintain known miRNAs to help autopolyploid cope with stress environmental conditions better than its diploid progenitor.

Objectives

- Compare the ability of diploid and tetraploid barley to tolerate salt stress;

- Identify the salt stress-related miRNAs in diploid and tetraploid barley, respectively;

- Discover the differentially expressed microRNAs due to genome duplication;

- Determine the microRNAs which are not only affected by genome duplication, but also involved in salt stress response;

- Identify the distinct microRNAs-target mechanisms in diploid and tetraploid under salt stress.

Chapter 3 Material and Methods

3.1 Physiological Experiment

3.1.1 Salt treatment

Seeds of diploid and tetraploid *H. bulbosum* were provided by the U.S. National Plant Germplasm System. Diploids (14 chromosomes) and tetraploids (28 chromosomes) *H. bulbosum* were identified through chromosome counting. In this study, the diploid (PI 240164) and the tetraploid—(PI 106880) cytotypes were randomly chosen. The seeds of diploid and tetraploid *H. bulbosum* were germinated in a petri dish for one week and then transplanted into soil (PRO-MIX) and grown in the greenhouse (16/8 h day/night and ~20/~16 °C day/night).

Both diploid and tetraploid *H. bulbosum* were divided into control CK groups (diploid CK and tetraploid CK), which were watered with tap water once every two days for two weeks, and the experimental group (diploid and tetraploid salt stress treatment), which were watered with salt (sodium chloride) solution (250 mM) for two weeks (once every two days).[65] Each group has four seedlings. Under salt stress, the ionic and osmotic homeostasis will be disrupted and thus re-establishing the ionic and osmotic balance will be necessary for plants to better cope with salinity stress.[65] From the osmotic point of view, a strong ability to retain water and prevent water loss is critical. Therefore, the water loss (WL) and relative water content (RWC) were measured to determine their abilities to tolerate salt stress between diploid and tetraploid *H. bulbosum*.

3.1.2 Relative water content (RWC)

The first fully expanded leaves were cut to measure the leaf relative water content (RWC).[66] Briefly, the first fully expanded leaves were excised, immediately the fresh weight (FW) was recorded. Then the leaves were immersed in deionized water at 4°C for 24h and the turgid weight (TW) was recorded. Finally, these leaves were dried in a drying oven for 24h at 80 degrees and recorded as DW.[66] The RWC was calculated according to this formula: RWC (%) = [(FW-DW) / (TW-DW)] $*$ 100.[67] To compare the RWC values among different groups, the average RWC values of diploids and tetraploids under CK and salt stress were obtained and boxplot was generated by using RStudio Team (2015) (RStudio: Integrated Development for R. RStudio, Inc., Boston, MA URL http://www.rstudio.com/). Statistical significances between diploid and tetraploid plants under the same treatments were tested using t- test (ns refers to p>0.05, * means p <=0.5, ** means p <=0.01, *** means p<= 0.001)

3.1.3 Water loss (WL)

The second fully expanded leaves were used to measure the water loss (WL).[66] Briefly, the second fully expanded leaves were cut, and the fresh weight (FW) was recorded. Then the leaves were put on the filter paper (WHATMAN) in a petri dish and weighed once each hour for five hours and recorded as F_x: F_1, F_2, F_3, F_4, F_5, respectively. Eventually, the proportions of fresh weight loss were calculated as the percentage of initial fresh weight according to the formula: WL (%) = (FW-F_X)/FW*100. [26,68] The averaged WL values of diploids and tetraploids under salt stress were calculated from the five different measured times and a line graph was generated by using RStudio Team (2015). Statistical

significances between diploids and tetraploids under salt stress were tested using using t-test (ns refers to p>0.05, * means p <=0.5, ** means p <=0.01, *** means p<= 0.001)

3.2 Molecular Experiment

3.2.1 Extraction of RNA

100 mg leaves which were kept in -80℃ refrigerator were used to extract the total RNA using TRIzol reagent according to the TRI Reagent® Protocol. First, the leaves were ground into powders, 1ml TRIzol was added into the tube immediately followed by powder homogenization. Next the homogenized samples were incubated for 5 minutes at room temperature and centrifuged at 12,000*g for 10 minutes at 4℃. After that, the supernatant was extracted to another new tube and 200ul chloroform was added, shaking tube vigorously by hand for 15 seconds and incubating for 2-3 minutes at room temperature. Centrifuging the sample at 12,000*g for 15 minutes at 4℃ and remove the supernatant to a new tube, 500ul isopropanol was added followed by incubating in refrigerator at -20℃ for 24 hours and centrifuging at 12,000*g for 15 minutes at 4℃. Thereafter, the supernatant was discarded, leaving only the pellet which was washed twice with 75% ethanol, followed by air drying for 5-10 minutes. Finally, the RNA pellet was resuspended with 50 ul RNase-free water.

3.2.2 High-throughput sequencing

The concentration of the total RNA was measured using a Nanodrop 2000. Before measuring the concentration of total RNA, 2ul RNase-free water was used to blank the machine, and the results of the blank was stored as a reference. Finally, the total RNA of

four samples (diploid CK, diploid stress, tetraploid CK, tetraploid stress) were sent to the BGI company (BGI Americas Corporation, MA, USA) to perform RNA sequencing using the Hiseq-4000 platform.

3.3 Bioinformatics Analysis

3.3.1 Bioinformatics analysis for conserved miRNA identification

Through high-throughput sequencing performed by BGI Company, raw data was obtained. Before doing any further analysis, quality control is required, that is, getting clean data is very important. Therefore, the impurities of raw data, including low quality sequence, sequence with 5' primer contaminants, sequence without 3' primer, sequence without the insert tag, sequence with poly A, and sequence shorter than 18nt, needed to be removed. [66] The clean data was aligned to the mature miRNAs of all plants in miRBase 21.0, allowing two mismatches by using BLASTN. The highest expression of miRNA for each mature miRNA family was selected and regarded as the temporary miRNA database. The precursors of all the identified miRNAs in the temporary miRNA database will be predicted via Mireap (http://sourceforge.net/projects/mireap/) with the following parameters: minimal miRNA sequence length (18), maximal miRNA sequence length (25), minimal miRNA reference sequence length (20), maximal miRNA reference sequence length (23), maximal copy number of miRNAs on reference (20), maximal free energy (-18kcal/mol), maximal space between miRNA and miRNA* (300), minimal base pairs of miRNA and miRNA* (16), maximal bulge of miRNA and miRNA* (4), maximal asymmetry of miRNA/miRNA* duplex (4), flank sequence length of miRNA precursor

(20). Those which could not form hairpin structure were treated as pseudo-miRNAs and removed. The clean data was aligned to the temporary miRNA database though BLASTN and the expression of each miRNA was generated by summing the count of tags which can align to the temporary miRNA database within two mismatches.

To compare the common and unique miRNAs among different libraries, the venn diagram was generated by using Jveen program.[83] Venn diagrams are commonly used to display list comparison. In biology, they are widely used to show the difference between gene lists from different samples.[69] The expression of each miRNA was normalized to transcripts per million (TPM) according to the following formula: TPM= (actual miRNA count/total count of clean reads) *1,000,000. Based on the normalized expression of each miRNA in the four libraries, boxplot and heat map were generated by using RStudio Team (2015) to compare the expression of miRNAs in the four libraries derived from the 4 treatments. Biology heat maps are typically used in molecular biology to represent the level of expression of many genes across a number of comparable samples such as cells in different states, samples from different patients etc.

3.3.2 Differential expression analysis of miRNAs

To compare the abundance of miRNAs between diploid and tetraploid libraries, the count of each miRNA was normalized to transcripts per million (TPM). TPM=actual miRNA count/total count of clean reads* 1,000,000. Afterwards, the fold change was calculated by using Fold change =log2 (miRNA TPM in tetraploid library/miRNA TPM in diploid library). Positive values mean the miRNAs up-regulated, while the negative values

indicate the miRNAs down-regulated. To identify the polyploidy-specific miRNAs two standards need to be met; (1) normalized count was at least 1TPM in either diploid or tetraploid library (2) log2 ratio of the normalized count in diploid or tetraploid libraries was >1 or < -1. Scatterplots were produced by using RStudio Team (2015) to identify the differentially expressed miRNAs between diploid and tetraploid libraries.

3.3.3 Target gene prediction Gene Ontology (GO) analysis

psRobot, and psRNATarget were used to predict the potential targets of all the miRNAs. For psRobot program, the following parameters were used: for the target prediction- Penalty score threshold: 3.0; Five prime boundary of essential sequence:1; Three prime boundary of essential sequence:31; Maximum number of permitted gaps: 0; Position after which with gaps permitted: 1; For psRNATarget program, we used the following criteria for the target prediction-Maximum expectation: 3.0; Length for complementary scoring (hip size): 20; Target accessibility (UPE): 25.0; Flanking length around the target site for target accessibility analysis: 17; Range of central mismatch leading to translational inhibition: 9-11 nt. All the mutual and unique targets were accepted, [48] and venn diagram was made by using jvenn program.[48] After finding the targets of miRNAs, all the targets were annotated by using Blast2GO and according to the functions of target genes, they were grouped into three ontologies in GO by using RStudio Team (2015): biological process, molecular function, and cellular component.

3.3.4 qRT - PCR validation

26

Total RNA was extracted from four samples (Dip-CK, Dip-stress, Tetra-CK and Tetra-stress) using TRIzol reagent according to the TRI Reagent® Protocol. miRNA -specific stem-loop RT primers were designed according to the published method [70] and used for the transcription of the total RNA extracted from leaves. Reverse transcription was performed by using the QuantiTect Reverse Transcription kit (QIANGEN) following the manufacturer's protocol. Then the cDNA then was used for real- time PCR using specific forward and universal reverse primers (Tale 8). SnoR14 was used as the internal control for the stem-loop qRT-PCR. Quantitative real time PCR was performed using the Green-2-Go-Mastermix (Biobasic) on Applied Biosystems 7000 Sequence Detection System (Life technology, Foster City, CA, USA). Each 20 µl reaction contained 10 µl Green-2-Go-Mastermix, 1 µl (10 µm) forward primer, and 1 µl (10 µm) reverse primer, 7 µl RNase-free water and l µl cDNA template. The PCR profile was 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 10 s. After the reactions were completed, the threshold was manually set (= 0.2) and the threshold cycle (CT) was automatically recorded. (The CT is defined as the fractional cycle number at which the fluorescence signal passes a fixed threshold.) All reactions were performed with three replicates. The relative expression level was calculated by using the Pfaffl method.[49] Relative quantification determines the changes in m RNA levels of a gene across multiple samples and expresses it relative to the levels of an internal control RNA. Pfaffl method refers to the calculations are based on the comparison of the CT values of a gene in different sample, according to the following formula[49]:

$$\text{Relative Expression} = \frac{(\mathbf{Etarget})^{\Delta CTtarget(control-sample)}}{(\mathbf{Ereference})^{\Delta CTreference(control-sample)}}$$

Chapter 4 Results

4.1 Physiological Results

4.1.1 Comparison of RWC between diploid and tetraploid

As shown in Fig. 4a, similar Relative water content (RWC) values were obtained in diploid (93.6%) and tetraploid (92.7%) CK plants and there was no significant difference (p-value 0.59) between the values. Under salt stress, there was a significant difference (p-value 0.036) between the RWC values of diploid and tetraploid, 79.1% and 84.1%, respectively, (Fig. 4a).

4.1.2 Comparison of WL between diploid and tetraploid

For the water loss (WL), the tetraploid had a lower WL value than that for the diploid under salinity stress (Fig. 4b). For diploids under salt stress, the WL values were 9.9%, 17.4%, 23.0%, 29.4% and 35.1%, but in tetraploids, the values were 5.5%, 10.7%, 13.4%, 18.2% and 22.8%. These differences in WL between diploid and tetraploid plants were significant (p-value were: 0.01, 0.005, 0.004, 0.008, 0.01, respectively) (Fig. 4b).
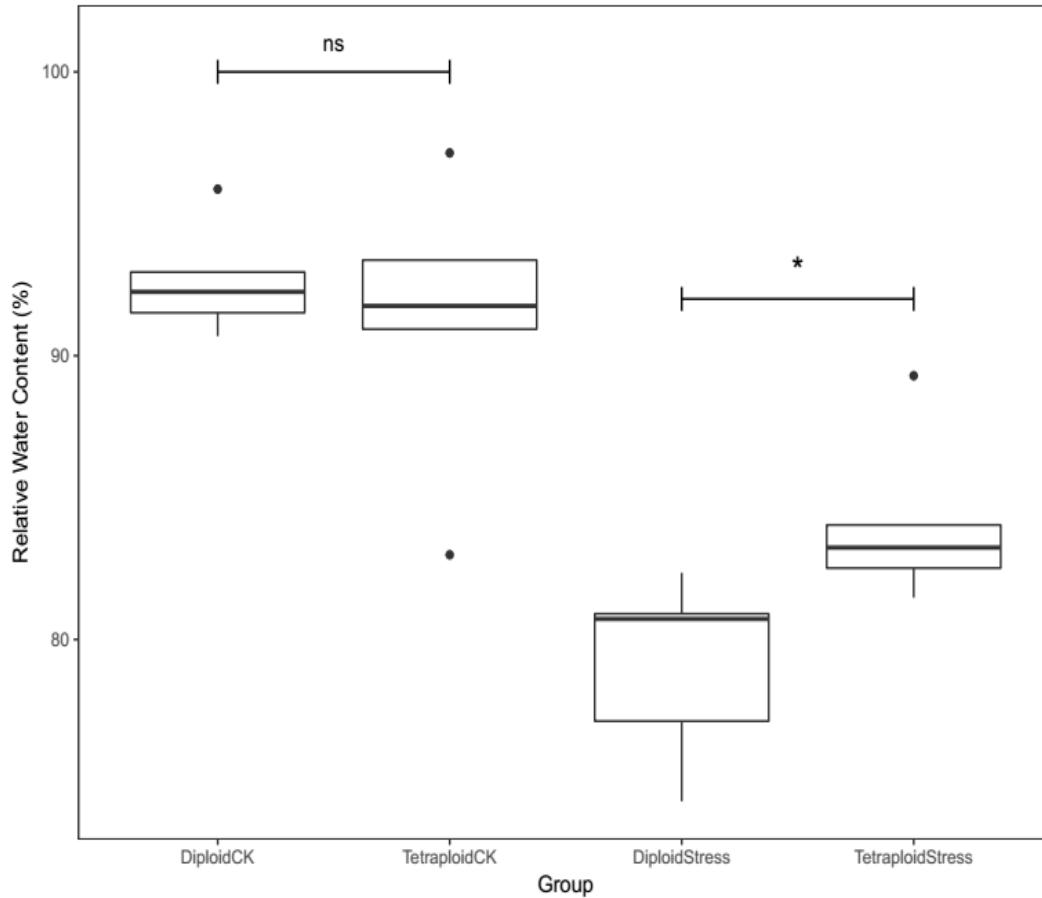
Figure 4a. Analysis of RWC in diploid and tetraploid *H*. bulbosum under control and salt stress. RWC were calculated for both diploid (Dip) and tetraploid (Tetra) under control and salt stress, according to the formula RWC (%) = [(FW-DW)/ (TW-DW)] * 100. Statistical significance by t- test: ns = no significance; * P<0.05.
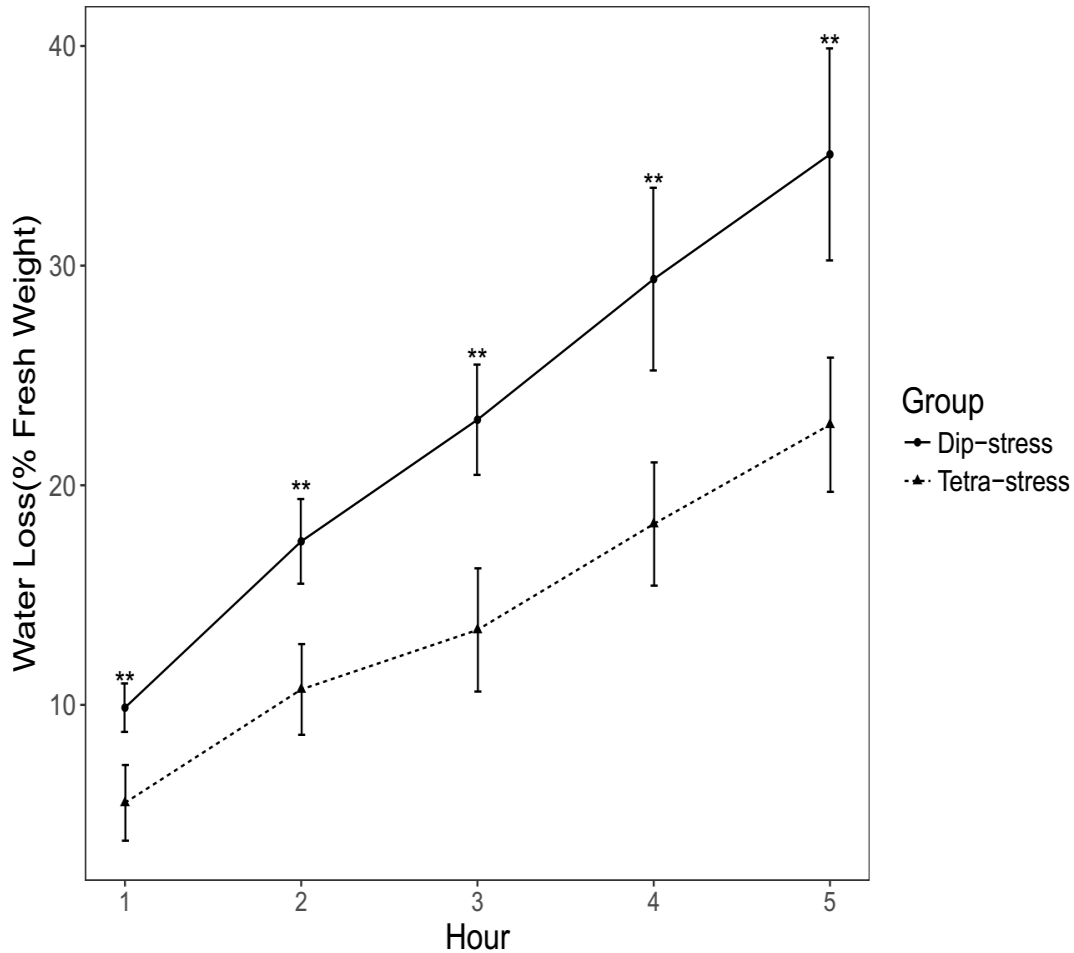
Figure 4b. Analysis of WL in diploid and tetraploid *H. bulbosum* under salt stress.
WL was measured once every one hour for five hours of both diploid and tetraploid under
salt stress (dip-stress and tetra-stress), according to the formula: WL (%) = (FW-
F_X)/FW*100. Statistical significance by Student's test: **$P<0.01$.

4.2 Bioinformatics results

4.2.1 Analysis of the sequencing data

To identify the miRNAs that were induced by genome duplication but also

associated with salt stress, four libraries of small RNAs from diploid and tetraploid *H.*

bulbosum grown under control and salt stress conditions were constructed and sequenced

independently. After filtering and removing the low-quality tags, adaptors, shortages, and adaptor-adaptor ligation, 34296615, 32765430, 34432952 and 34970804 reads from diploid CK, diploid stress, tetraploid CK, and tetraploid stress were obtained. The data of these four libraries were searched against the Rfam database using BLASTN program. The numbers and proportions of different kinds of small RNAs from these four libraries are shown in Table 1-4.

Category	Unique sRNA	Percent (%)	Total sRNA	Percentage (%)
Total	3,703,874	100	34296615	100
miRNA	974	0.03	4180234	12
rRNA	60,920	1.64	3005793	8
snRNA	3,421	0.09	26920	0.1
snoRNA	2,922	0.08	36115	0.1
t RNA	20,225	0.55	2005262	6
Unannotated	36,15412	97.61	25042291	73

Table 1. Distribution of small RNA among different categories in diploid CK

Category	Unique sRNA	Percent (%)	Total sRNA	Percentage (%)
Total	2549221	100	32765430	100
miRNA	987	0.038718	6127916	19
rRNA	46381	1.819419	2150716	7
snRNA	2609	0.102345	37144	0.1
snoRNA	2172	0.085202	41160	0.1
tRNA	11869	0.465593	640943	2
Unannotated	2485203	97.48872	23767551	73

Table 2. Distribution of small RNA among different categories in diploid under salt stress

Table 3. Distribution of small RNA among different categories in tetraploid CK

Category	Unique sRNA	Percent (%)	Total sRNA	Percentage (%)
Total	3082498	100	34970804	100
miRNA	1011	0.03	5150103	15
rRNA	39102	1.27	1899601	5
snRNA	2271	0.08	28981	0.1
snoRNA	1844	0.06	27558	0.1
tRNA	9566	0.31	609392	2
Unannotated	3028704	98.25	27255169	78

Table 4. Distribution of small RNA among different categories in tetraploid under salt stress

Category	Unique sRNA	Percent (%)	Total sRNA	Percentage (%)
Total	1329885	100	34432952	100
miRNA	765	0.06	3609052	10
rRNA	45335	3.41	2961786	9
snRNA	1823	0.14	41605	0.1
snoRNA	1882	0.14	74101	0.2
tRNA	12006	0.90	1537936	4
Unannotated	1268074	95.35	26208472	76

Size profile is an important feature to distinguish miRNA from other small RNAs.

Most mature miRNAs are ~21 nucleotides (nt). The length distribution patterns of these

four libraries were analyzed. The results suggested that the majority of the small RNAs from these four libraries were 24nt and 21nt in size, followed by 22nt and 23nt (Fig. 5). The percentages of miRNAs in tetraploid CK (28.7%) and tetraploid stress (31.5%) were higher than those in diploid CK (25.8%) and diploid stress (21.4%) (Fig. 5).
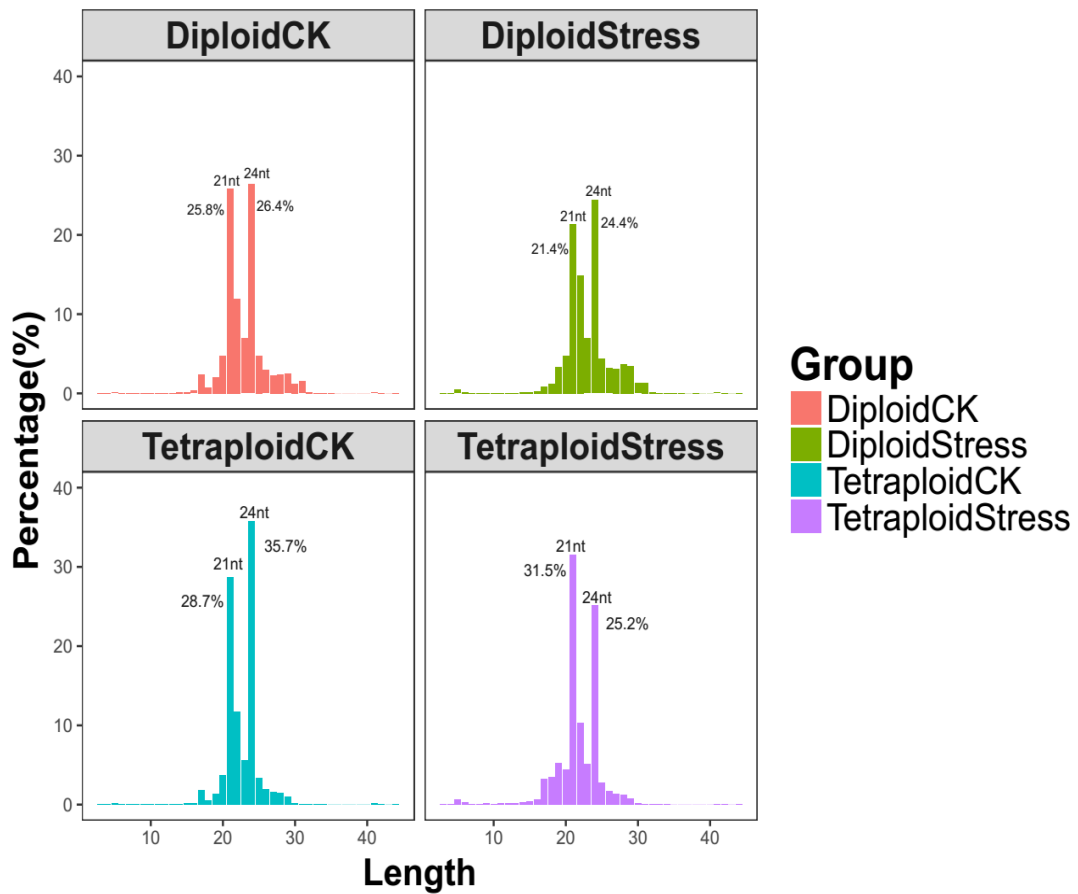
Figure 5. Analysis of length distribution. The length distribution and abundance of small RNAs in diploidCK, diploidStress, tetraploidCK and tetraploidStress libraries.

4.2.2 Identification of conserved miRNAs

37 conserved miRNAs were identified from diploid CK library, 33 conserved miRNAs in the diploids under salt stress conditions, 41 miRNAs from tetraploid CK and 36 conserved miRNAs from tetraploid stress library (Fig. 6). In total, 54 conserved miRNAs were discovered from these four libraries, of which 19 miRNAs were in common in the four libraries. The diploid CK and diploid stress libraries had 27 common miRNAs, tetraploid CK and tetraploid stress had the same 32 common miRNAs, while diploid CK and tetraploid CK libraries had 5 common miRNAs (Fig. 6).

Overall, the miRNAs expression in the diploid plants in the stress treatment were down-regulated compared with those in the diploid CK. However, there was no large-scale change in miRNAs expression patterns between tetraploid plants in stress and tetraploid CK, and between diploid and tetraploid CKs (Figs. 7a and 7b).
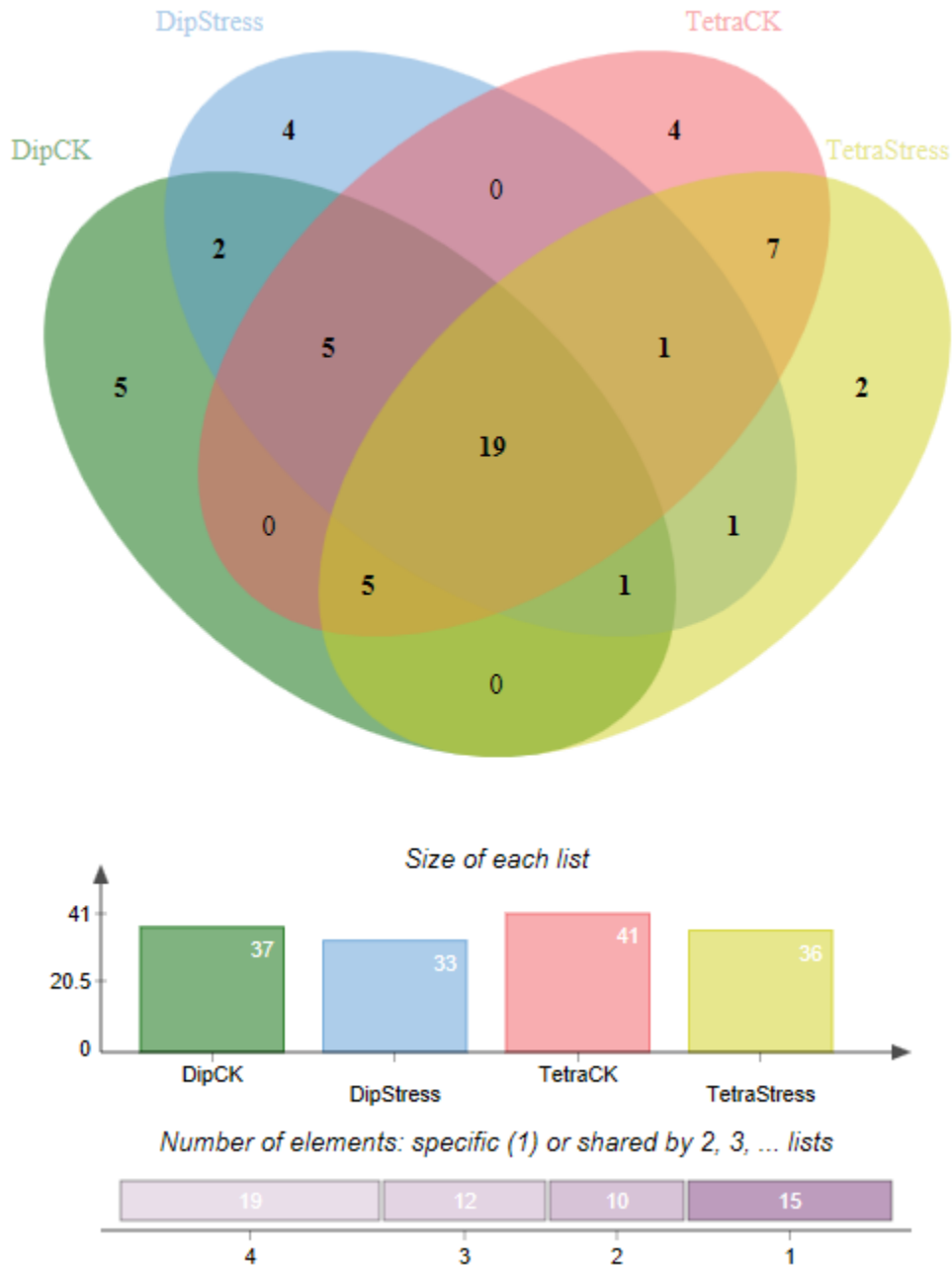
Figure 6. Identification of the conserved miRNAs in different libraries. The Venn diagram illustrates the number of common and unique miRNAs in diploid CK, diploid stress, tetraploid CK and tetraploid stress, the following bar chart showed the total conserved miRNAs in each library (diploid CK, diploid stress, tetraploid CK and tetraploid stress)
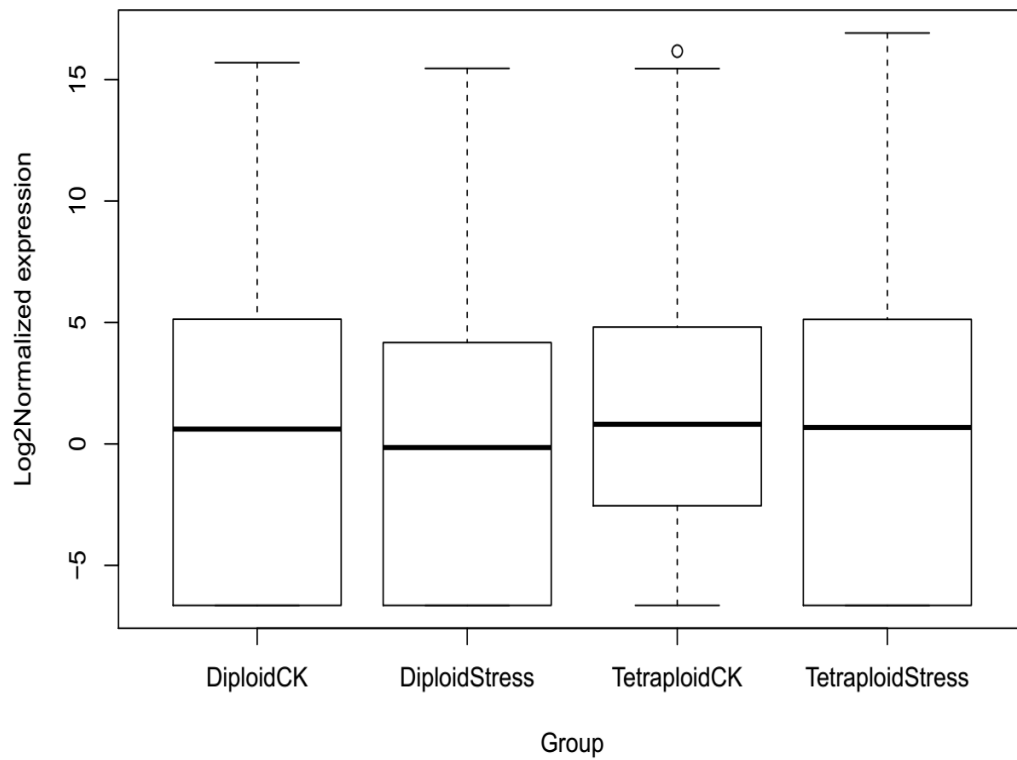
Figure 7a. Overall expression of miRNAs in the four libraries. The boxplot displayed the expression (log2 Normalized expression) of miRNAs in DiploidCK, DiploidStress, TetraploidCK and TetraploidStress libraries (∘ refers to the outlier)
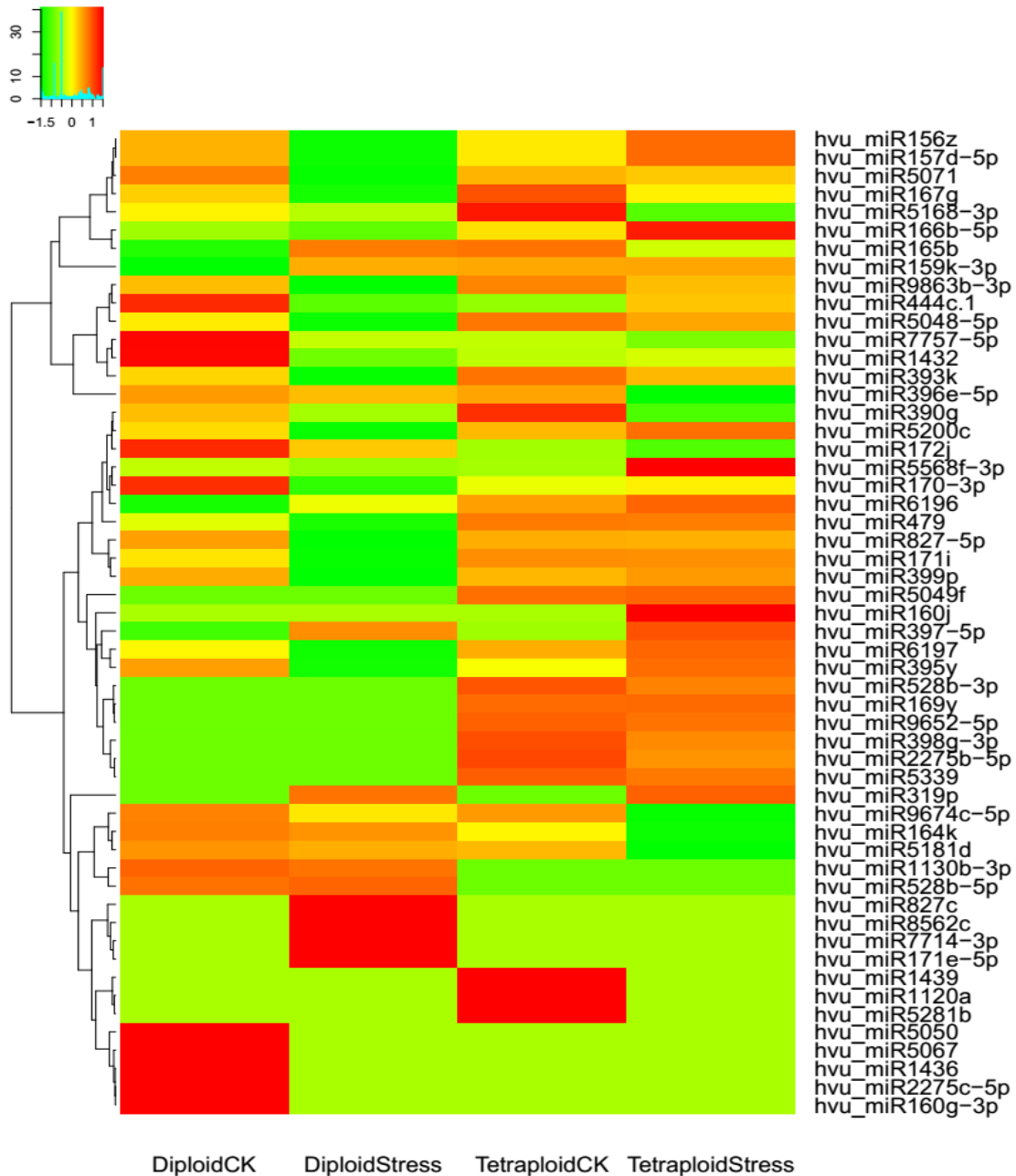
Figure 7b. The overall expression of conserved miRNAs in different libraries. This heatmap showed the expression of each miRNAs in four different libraries – DiploidCK, DiploidStress, TetraploidCK and TetraploidStress, different color means different expression level (green refers to a lower expression, red refers to a higher expression)

4.2.3 miRNAs affected by genome duplication *per se*

To identify the miRNAs which were influenced by genome duplication *per se*, the expression of each miRNA was compared between diploid and tetraploid CKs. The fold change of each miRNA was calculated according to the formula: Fold change = log2 (miRNA TPM in tetraploid CK library/miRNA TPM in the diploid CK library). The results showed that 13 miRNAs were differentially expressed due to the genome duplication *per se*. Of these, 9 miRNAs (miRNA171i, miR479, mir5048-5p, mir6196, miR169y, miR528b-3p, miR159k-3p, miR9652-5p and miR5049f) were over-expressed in tetraploid CK compared to those in diploid CKs, while 4 miRNAs (miR164k, miR1432, miR528b-5p and miR1130b-3p) were down-regulated in tetraploid CK compared with those in the diploid CK (Table 5, Fig. 8a). Among those 9 up-regulated miRNAs, 5 miRNAs (miR169y, miR528b-3p, miR159k-3p, miR9652-5p and miR5049f) were identified in only the tetraploid CK not in diploid CK, while among the 4 down-regulated miRNAs, 2 miRNAs (miR528b-5p and miR1130b-3p) were only identified in diploid control but not in tetraploid control (Table 5).

Table 5. The differentially expressed miRNAsbetween dip- and tetraploid CKs

S. No	MiRBase ID	DC(Dip-control)	TC (Tetra-control)
Down-regulated in TC			
1	Hvu-miR164k		↓
2	Hvu-miR1432		↓
Up-regulated in TC			
1	Hvu-miR171i		↑
2	Hvu-miR479		↑
3	Hvu-miR5048-5p		↑
4	Hvu-miR6196		↑
Detected in DC but not in TC			
1	Hvu-miR528b-5p	D	ND
2	Hvu-miR1130b-3p	D	ND
Not detected in DC but in TC			
1	Hvu-miR169y	ND	D

2	Hvu-miR528b-3p	ND	D
3	Hvu-miR159k-3p	ND	D
4	Hvu-miR9652-5p	ND	D
5	Hvu-miR5049f	ND	D

⬆ means the up-regulated miRNAs in tetraploid control compared to those in diploid control; ⬇ refers to the down-regulated miRNAs in tetraploid control relative to those in diploid control; **D** means the detected miRNAs; **ND** represents the miRNAs which were not detected.
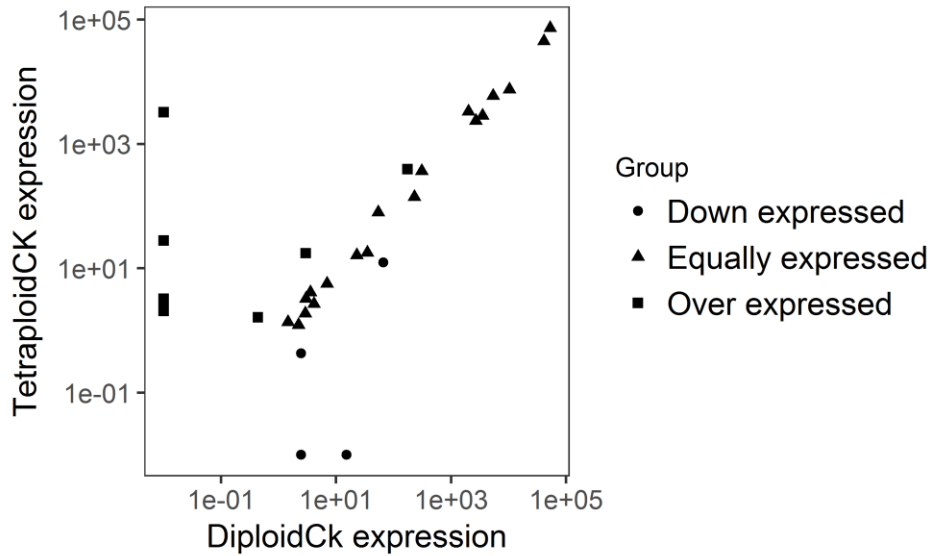
Figure 8a. Identification of differentially expressed miRNAs due to genome duplication. The scatter plot shows the different miRNAs expression pattern between tetraploid-control and diploid-control (square ■ circle● and triangle▲ refer to miRNAs which were up-, down- and equally- regulated in tetraploid-control compared to those in diploid-control).

4.2.4 Salt stress-related miRNAs in diploid and tetraploid plants

To identify salt stress related miRNAs in diploid plants, the fold change of each miRNA between salt stressed diploid and CK plants was calculated according to the formula: fold change = log2 (miRNA TPM in diploid stress library/ miRNA TPM in diploid control library). The result showed that 5 miRNAs (miR319p, miR159-3p, miR6196, miR7714-3p and miR827c) were up-regulated in diploid stressed plants compared to controls, while 16 miRNAs were down-regulated in the diploid stress treatment relative to the diploid controls (Table 6, Fig. 8b). To identify the salt stress related miRNAs in tetraploid plants, the fold change of each miRNA between tetraploid stress treatment and CK was also calculated as for the diploid plants. Only 3 miRNAs (miR396e-

5p, miR9647c-5p and miR528b-3p) were down-regulated and 5 miRNAs (miR319p, miR395y, miR5568f-3p, miR160j and miR6197) were up-regulated in the tetraploid salt stressed plants compared with controls (Table 6, Fig. 8c). By analyzing the differentially expressed miRNAs and the functions of these miRNAs in the diploid stress treatment and tetraploid stress treatment, different miRNAs—targets responses to salt stress in diploid and tetraploid plants were discovered which will be highlighted latter.

Table 6. The differentially expressed miRNAs in diploid and tetraploid stress compared to their respective CKs.

S.NO	MiRBase ID	DS(dip-stress vs dip-CK)	TS(tetra-stress vs tetra-CK
Up regulated under salt in both DS and TS			
1	Hvu-miR319	⬆	⬆
Down regulated under salt in both DS and TS			
1	Hvu-miR396e-5p	⬇	⬇
2	Hvu-miR9647c-5p	⬇	⬇

Up regulated in DS but not change or not detected in TS

1	Hvu-miR159-3p	↑	No change
2	Hvu-miR6196	↑	No change
3	Hvu-miR7714-3p	↑	No change
4	Hvu-miR827c	↑	No detected

Not change or not detected in DS but up regulated in TS

1	Hvu-miR395y	Not detected	↑
2	Hvu-miR5568f-3p	Not changed	↑
3	Hvu-miR160j	Not detected	↑
4	Hvu-miR6197	Not detected	↑

Down regulated under salt in DS but not change in TS

1	Hvu-miR1432	↓	No change
2	Hvu-miR156z	↓	No change
3	Hvu-miR157d-5p	↓	No change
4	Hvu-miR167g	↓	No change
5	Hvu-miR170-3p	↓	No change
6	Hvu-miR393k	↓	No change

7	Hvu-miR171i	↓	No change
8	Hvu-miR399	↓	No change
9	Hvu-miR479	↓	No change
10	Hvu-miR5048-5p	↓	No change
11	Hvu-miR5071	↓	No change
12	Hvu-miR5200c	↓	No change
13	Hvu-miR827-5p	↓	No change
14	Hvu-miR9863-3p	↓	No change

Not change in DS but down regulated in TS

1	Hvu-528b-3p	Not detected	↓

Not detected in DS but expressed in TS

1	Hvu-miR169y	Not detected	No change
2	Hvu-miR5049f	Not detected	No change
3	Hvu-miR9652-5p	Not detected	No change

Detected in DS but not in TS

1	Hvu-miR5181d	No change	Not detected
2	Hvu-miR7757-5p	No change	Not detected

3	Hvu-miR528b-5p	No change	Not detected
4	Hvu-miR164k	No change	Not detected
5	Hvu-miR1130b-3p	No change	Not detected
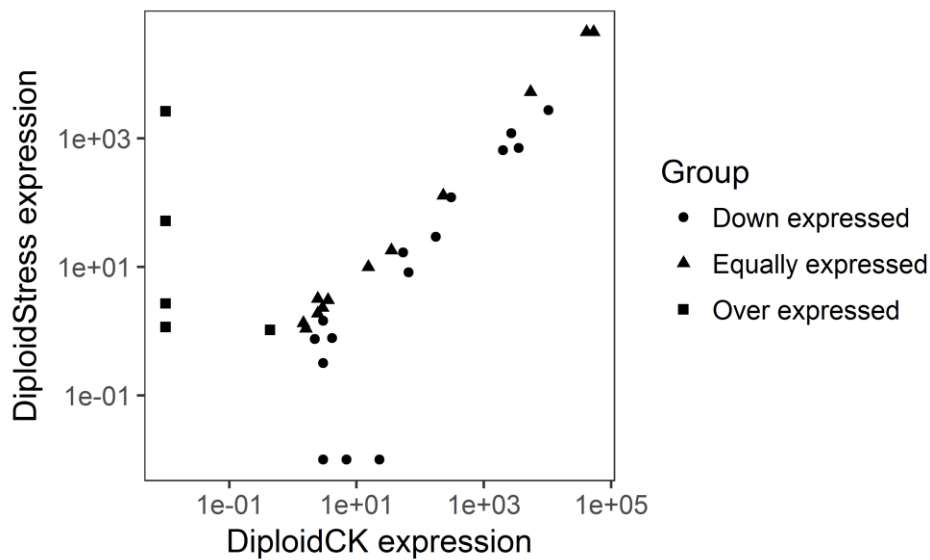
Figure 8b. Identification of differentially expressed miRNAs under salt stress in diploid. This scatter plot shows the different miRNAs expression pattern between dipploid under salt stress and control (square   circle ■triangle   refer to miRNAs which were up- down- and equally- regulated in diploid-stress compared to those in diploid-control).
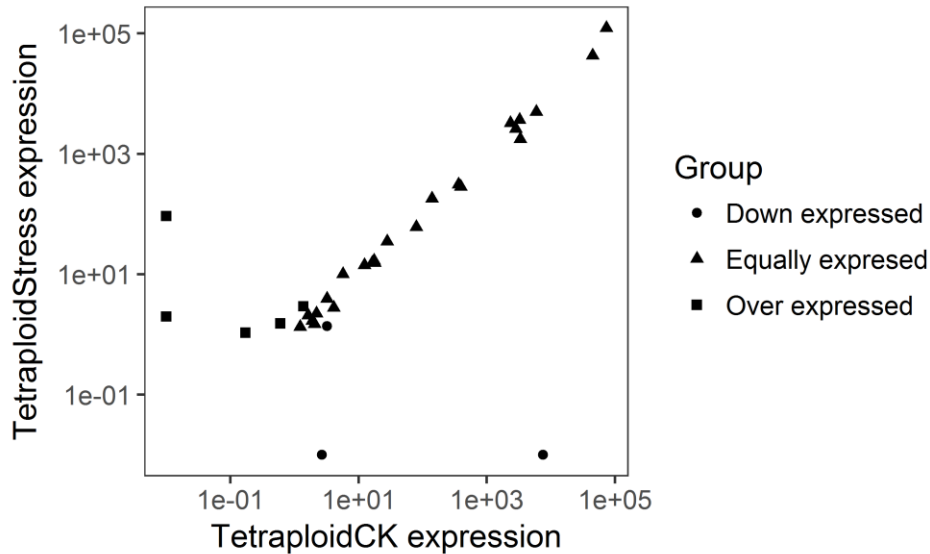
Figure 8c. Identification of differentially expressed miRNAs under salt stress in tetraploid. The scatter plot shows the different miRNAs expression pattern between tetraploid under salt stress and control (square ◼ circle ● triangle ▲ refer to miRNAs which were up- down- and equally- regulated in tetraploid-stress compared to those in tetraploid-control).

4.2.5 Targets of known miRNAs and Gene Ontology (GO)

The target genes of the 54 conserved miRNAs were predicted by using psRNATarget and psRobot. The results suggested that there wre 1251 and 1875 target genes possible based on these analytic tools respectively (Fig. 9a). In total, 2284 targets were predicted for these 54 conserved miRNAs. The functions of the identified targets for miRNAs were annotated (Table 7). These target genes were grouped into three different categories based on their functions: biological process, cell component and molecular function (Fig. 9b).
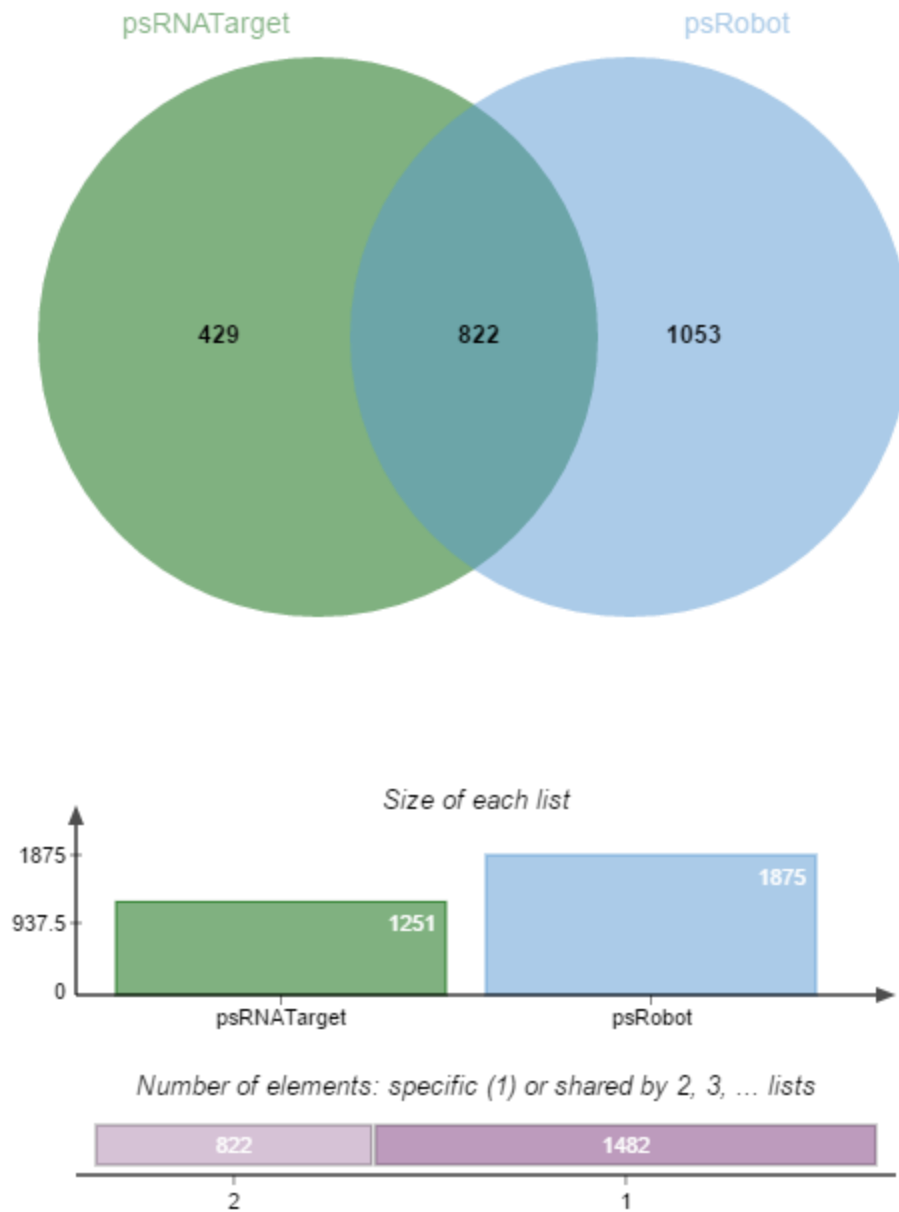
Figure 9a. Prediction of target genes for all the identified miRNAs. This pie chart showed the number of common and unique target genes discovered by two different target-prediction software – psRNATarget and psRobot, while the following bar chart displayed the number of target genes identified by psRNATarget and psRobot each.
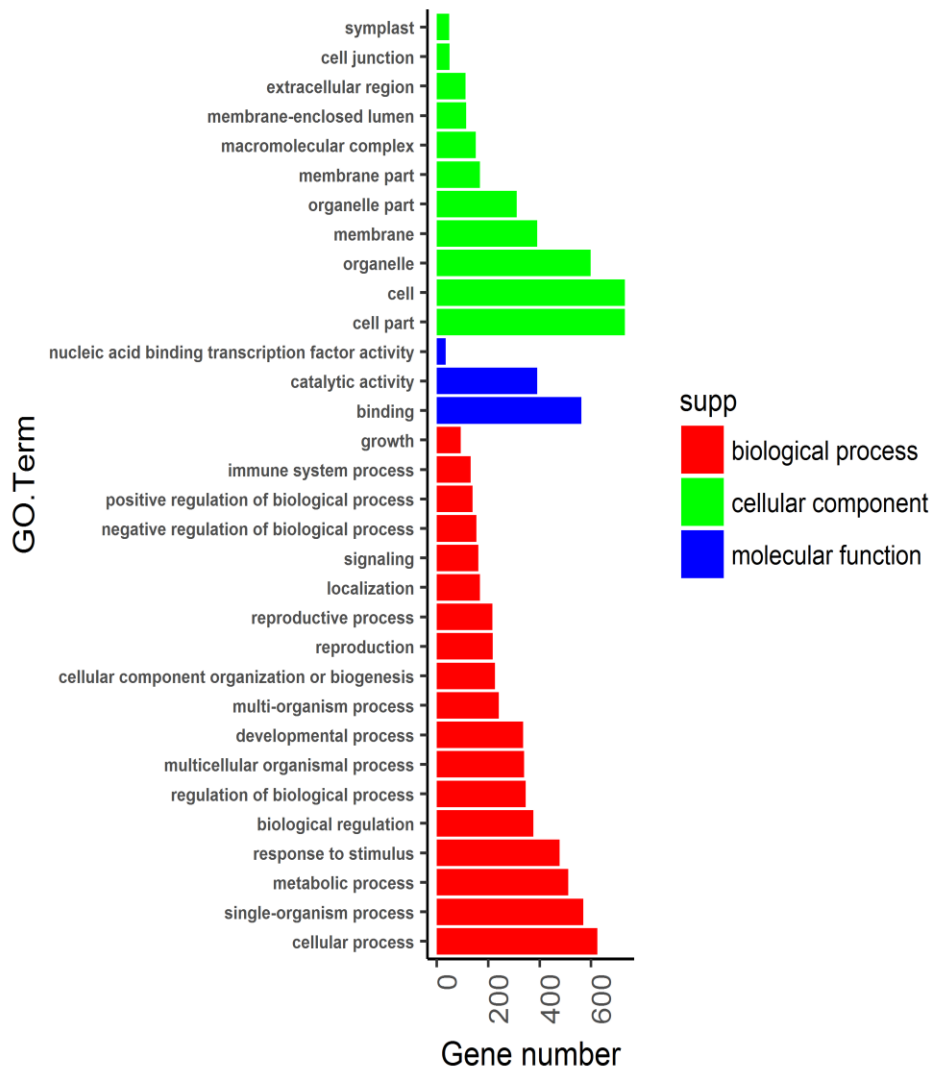
Figure 9b. Gene Ontology (GO). Gene Ontology (GO) analysis of miRNAs target genes identified in diploid and tetraploid under control and salt stress.

4.2.6 miRNAs induced by genome duplication also associated with salt stress response

5 miRNAs (miR171i, miR479, miR5048, mir6196 and miR528b-3p) were affected by genome duplication, and also associated with salt stress. Of these, four miRNAs -- miR171i, miR479, miR5048 and mir6196 were up-regulated in tetraploid CK treatments (Table 5) compared with those in the diploid CK treatment. However, miR171i, miR479 and miR5048-5p were down-regulated in diploid salt stressed plants (Table 6) compared to those in the diploid CK, and miRNA6196 was up-regulated in the diploid stress treatment (Table 6) relative to the CK. In addition, miR528b-3p was only detected in tetraploid control plants, not in diploid controls (Table 5), but was down-regulated in the tetraploid stress treatment compared with that control (Table 6). Target prediction and function annotation indicated that the targets of miRNA171i and miR479 are PNSB (photosynthetic NDH subunit of subcomplex B chloroplastic) (Table 7). The predicted targets of miR528b-3p are ERF2 (Ethylene-responsive transcription factor 2), PLDZ1 (Phospholipase D zeta 1), MZT1B (Mitotic-spindle organizing associated with a ring of gamma-tubulin 1B) and RFA1C (replication A 70 KDa DNA-binding subunit C). As for the other 2 miRNAs, miR5048-5p and miR6196, their functions cannot be precisely predicted.

4.2.7 qRT - PCR validation

To validate the high-throughput sequencing results, 8 miRNAs were randomly selected for qRT-PCR and the results were consistent with the sequencing data (Figs. 10a-c). For example, both the sequencing data and the qRT-PCR results showed that the expression of miRNA164k was down regulated in tetraploid CKs compared to the diploid CKs, while miRNA171i was up regulated in the tetraploid control compared with that in diploid control, miR528b-3p was down regulated in the tetraploid stress treatment relative to the tetraploid control. The specific relative expression level of miR396e-5p in tetraploid stressed plants, miR164k in diploid stress treatment compared to the respective CK, and miRNA171i in tetraploid vs diploid CKs were not completely identical between the qRT-PCR and sequencing data, but expression trends of were similar between qRT-PCR and sequencing data in response to salt stress.
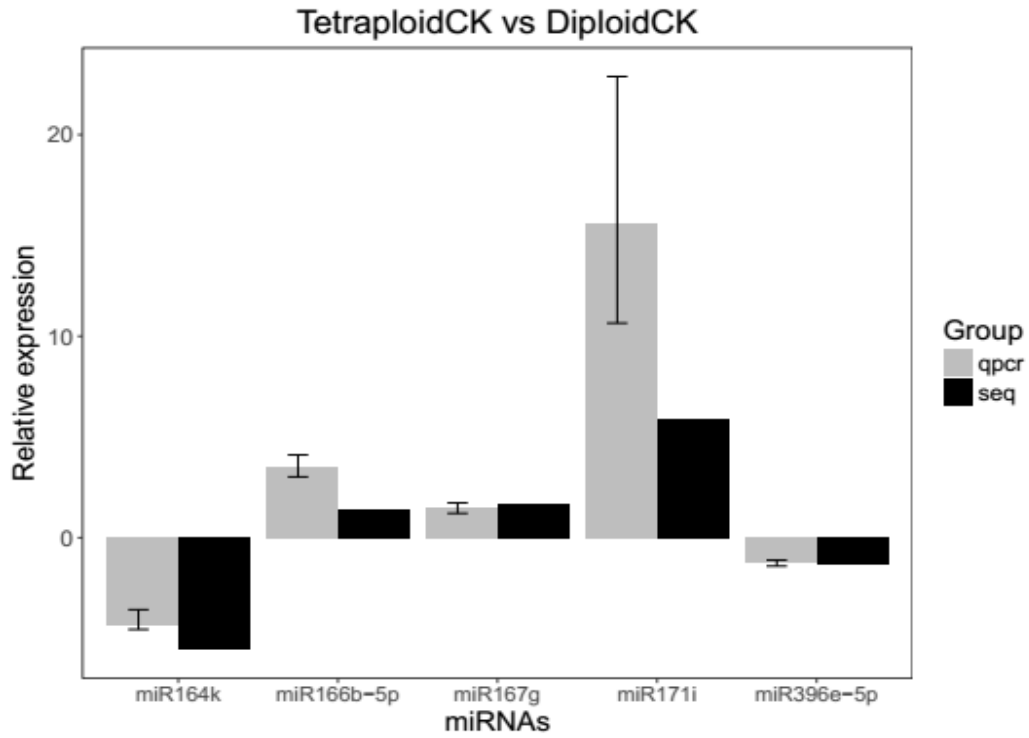
Figure 10a. qRT - PCR validation. This bar plot shows the relative expression of miRNAs in tetraploid CK compared to those in diploid CK by using high throughput sequencing and qRT-PCR, respectively.
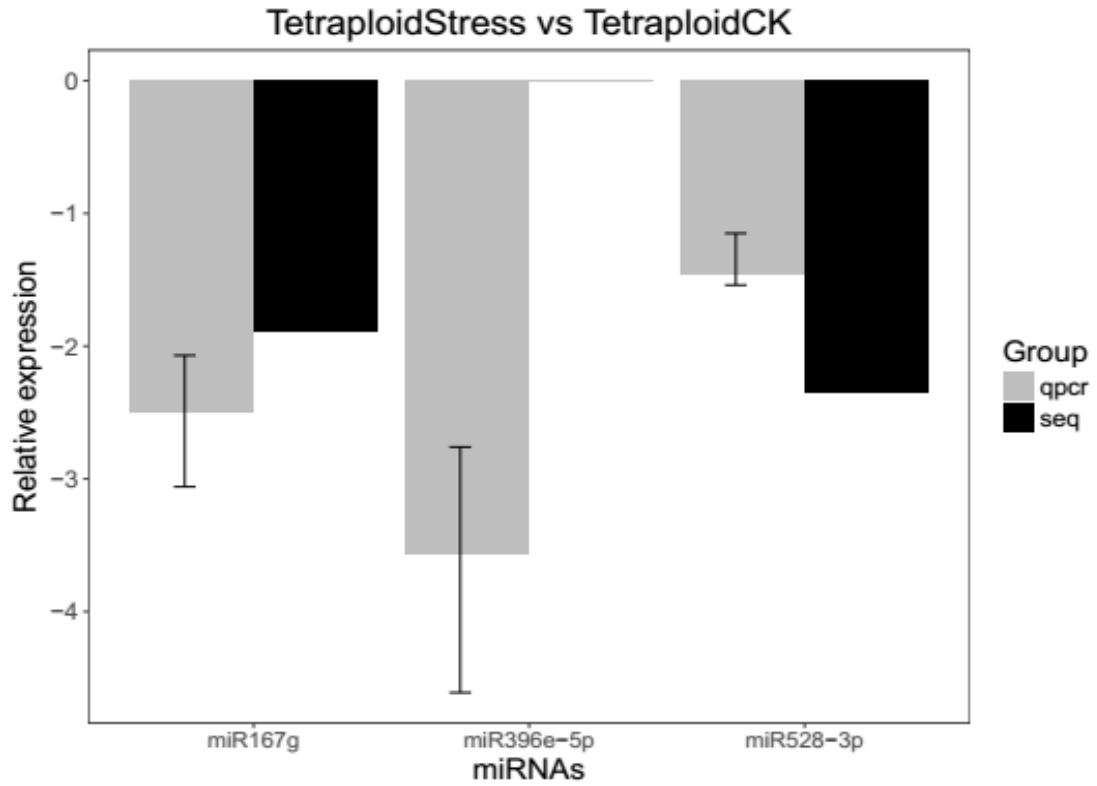
Figure 10b. qRT - PCR validation. This bar plot illustrated the relative expression of miRNAs in tetraploid stress compared to those in tetraploid check (CK) by using high throughput sequencing and qRT-PCR, respectively
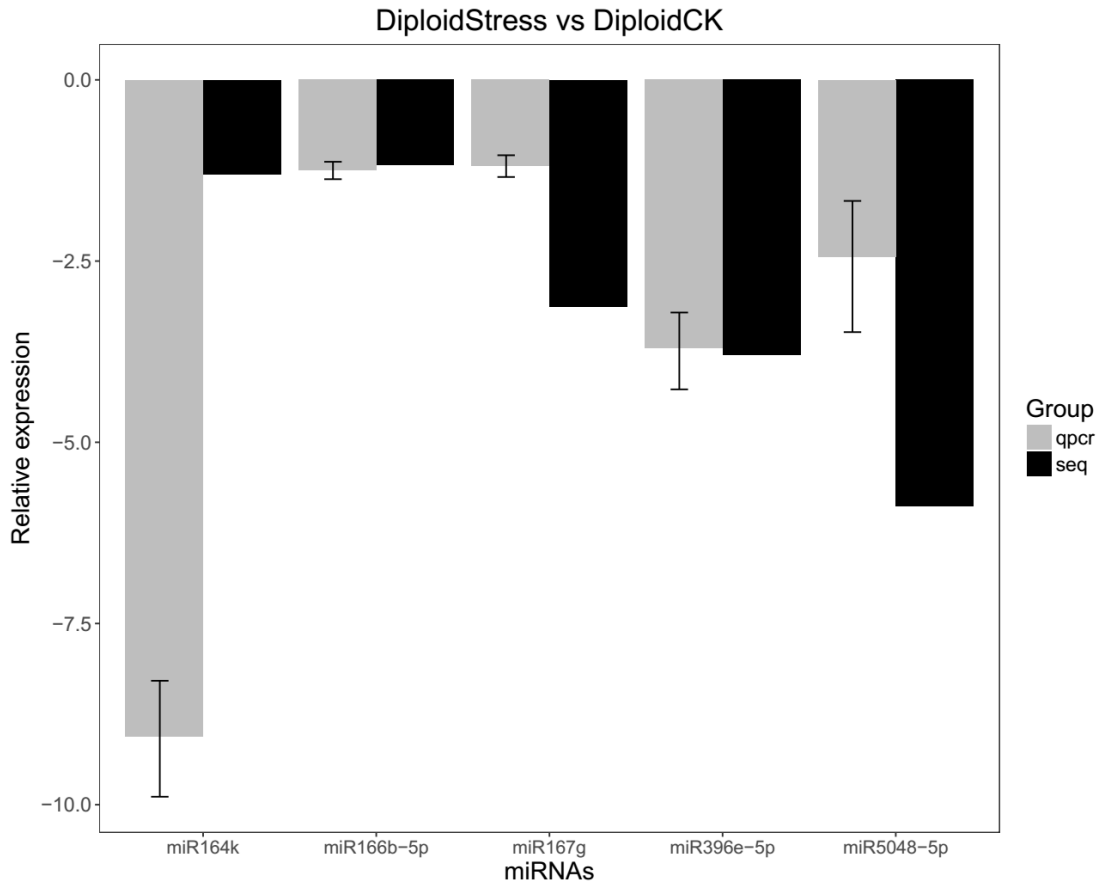
Figure 10c. qRT - PCR validation. This bar plot displayed the relative expression of miRNAs in diploid stress compared to those in diploid CK by using high throughput sequencing and qRT-PCR, respectively.

4.3 The research is other accessions of *Hordeum bulbosum*

4.3.1 Comparison of salt stress tolerance in other accessions of *Hordeum bulbosum*

Another two experiments were carried out to measure the different abilities between diploid and tetraploid in 2016 and 2017, respectively. As shown in Fig. 11a, during 2016, RWC values were obtained in diploid –PI 206565 (93.3%), tetraploid PI 246218 (93.0%) and tetraploid PI 531776 (96.6%) CKs. Under salt stress, the RWC values of diploid PI 206565, tetraploid PI 246218 and tetraploid PI 531776 were 75.8%, 79.7% and 84.8%, respectively, (Fig.11a). For the water loss (WL), both the tetraploid PI 246218 and tetraploid PI 531776 had a lower WL value than that for the diploid PI 206565 under salinity stress (Fig. 11b). For diploid PI 206565 under salt stress, the WL values were 13.5%, 22.1%, 29.9%, 36.9% and 43.0%, but in tetraploid PI 246218, the values were 12.5%, 21.4%, 29.3%, 35.0% and 40.1%, and in tetraploid PI 531776, the values were 11.2%, 18.9%, 24.4%, 29.9% and 35.3% (Fig. 11b).

During 2017, 5 accessions PI 15681, PI 168306, PI 247050, PI 283353, PI 304326, with PI 15681 is a known diploid, were used to determine the different abilities to tolerate salt stress among different accessions. As shown in Fig. 11c, the RWC values were obtained in PI 15681 (99.5%), PI 168306 (96.8%), PI 247050 (93.4%), PI 283353 (95.8%) and PI 304326 (94.3%) CKs. Under salt stress, the RWC values of PI 15681, PI 168306, PI 247050, PI 283353 and PI 304326 were 80.1%, 88.8%, 83.4%, 87.3% and 83.8%, respectively (Fig. 11c).
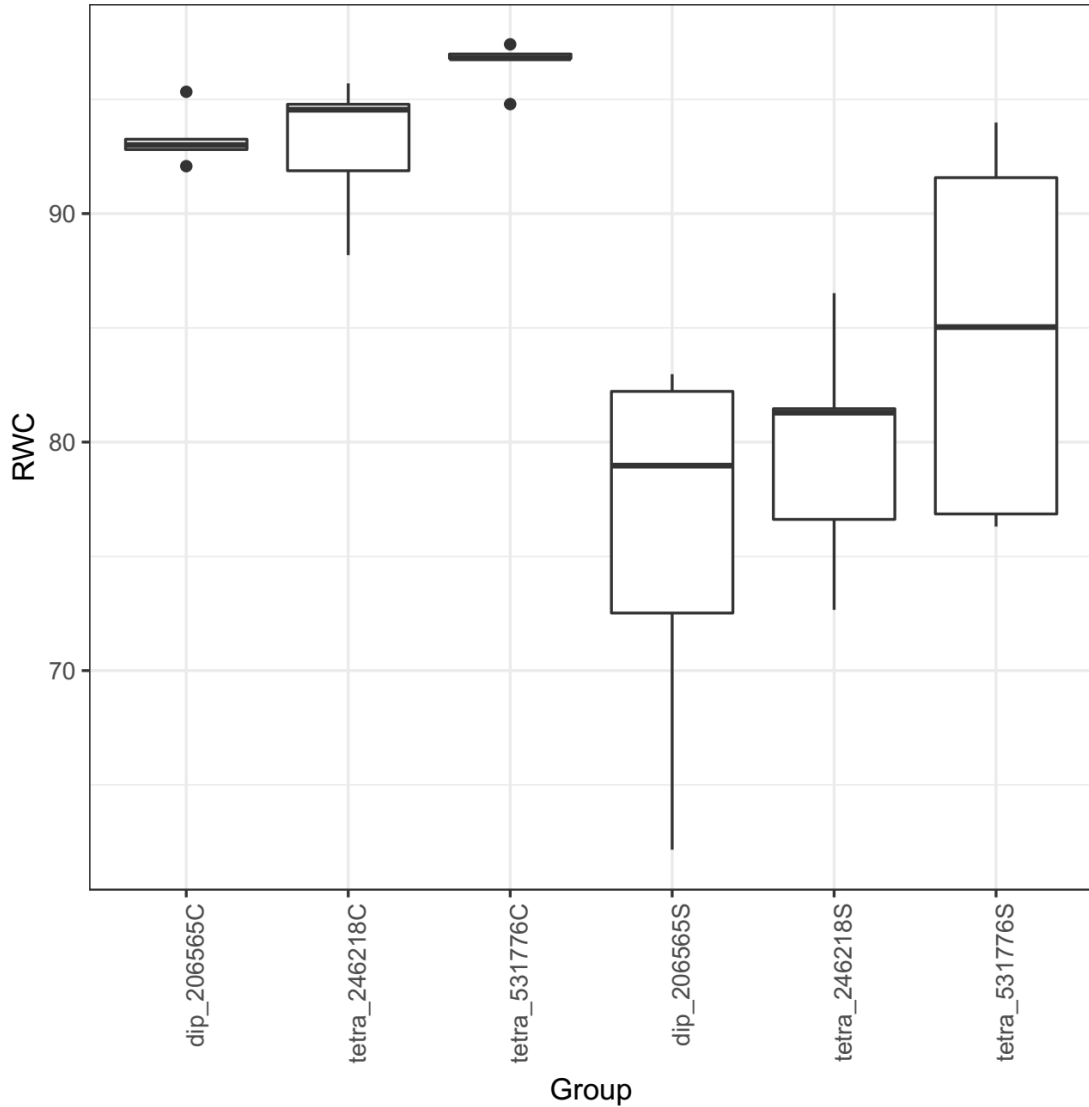
Figure 11a. Analysis of RWC in diploid and tetraploids *H. bulbosum* under control and salt stress in 2016. RWC were calculated for diploid(PI 206565), tetraploid (PI 246218) and tetraploid(PI 531776) under control and salt stress, according to the formula RWC (%) = [(FW-DW)/ (TW-DW)] * 100
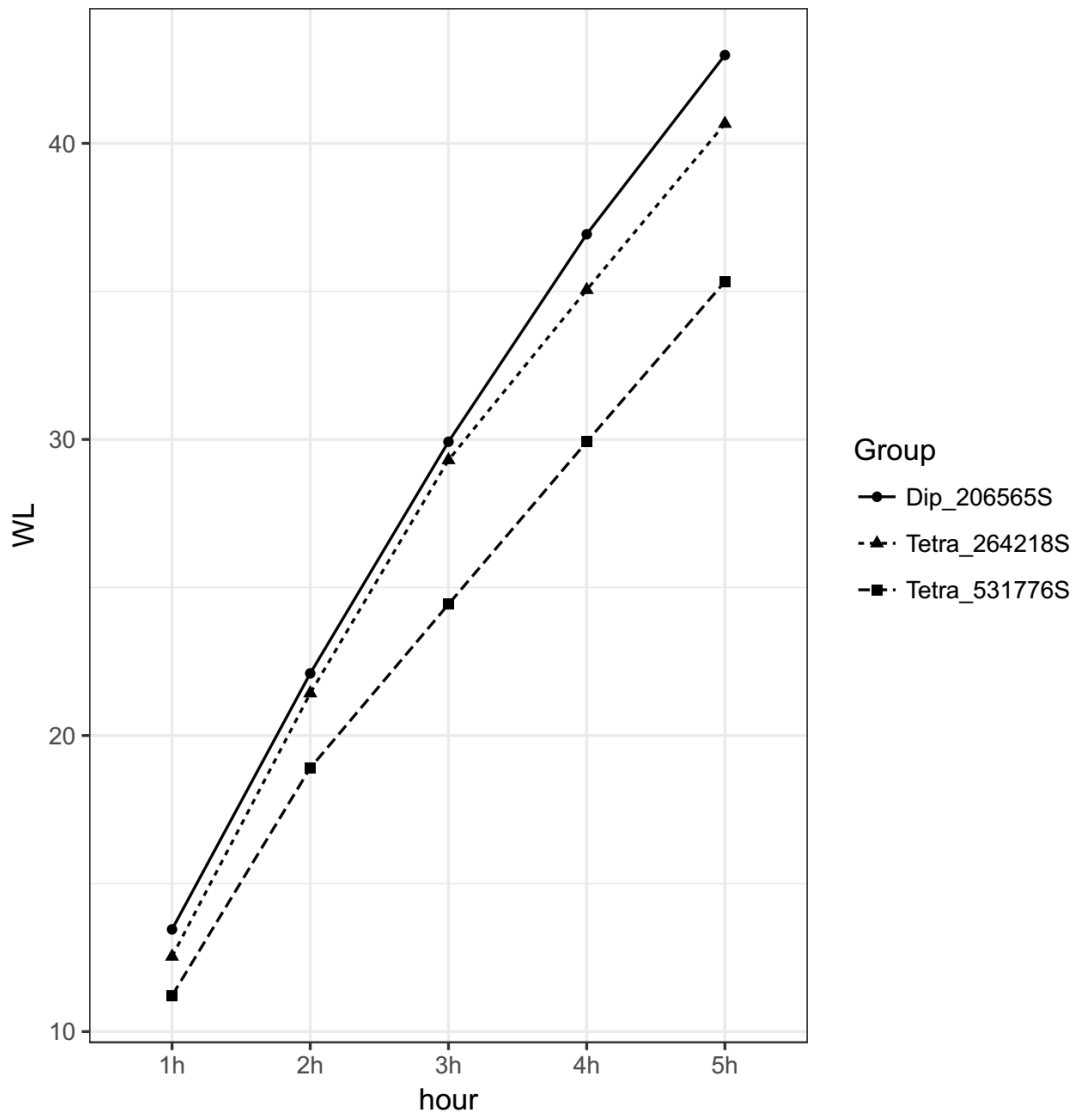
Figure 11b. Analysis of WL in diploid and tetraploids *H. bulbosum* under salt stress in 2016. WL was measured once every one hour for five hours of both diploid and tetraploid under salt stress (dip-stress and tetra-stress), according to the formula: WL (%) = (FW-FX)/FW*100.
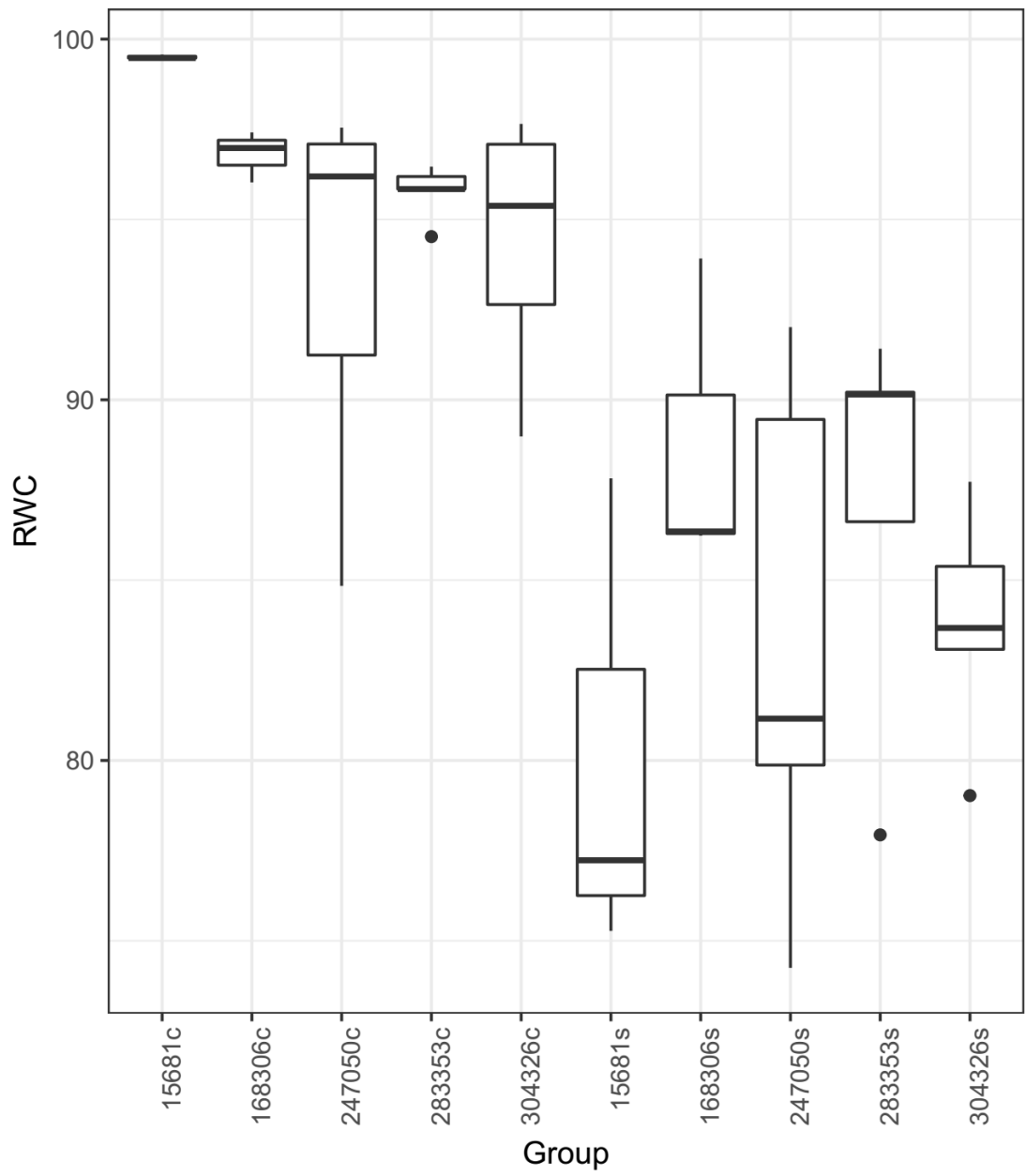
Figure 11c. Analysis of RWC in different accessions of *H. bulbosum* under control and salt stress in 2017. RWC were calculated for both diploid (Dip) and tetraploid (Tetra) under control and salt stress, according to the formula RWC (%) = [(FW-DW)/ (TW-DW)] * 100.

4.3.2 qPCR validation of miRN528b-3p in different accessions of *Hordeum bulbosum*

The relative expression of miR528b-3p under salt stress plants compared to that in the CK plants was detected in PI 15681, PI 168306, PI 246218, PI 247050, PI 283353, PI 304326 and PI 531776 using qPCR. As shown in Fig. 12, the relative expression of miRNA528b-3p in PI 15681, PI 168306, PI 246218, PI 247050, PI 283353, PI 304326 and PI 531776 were 2.4, 1.0,   -1.2, 0.9, 1.1, 1.3 and 1.4 (Fig. 12), with the diploid PI 15681 has the highest relative expression of miRNA528b-3p under salt stress condition compared with that in CK plants.
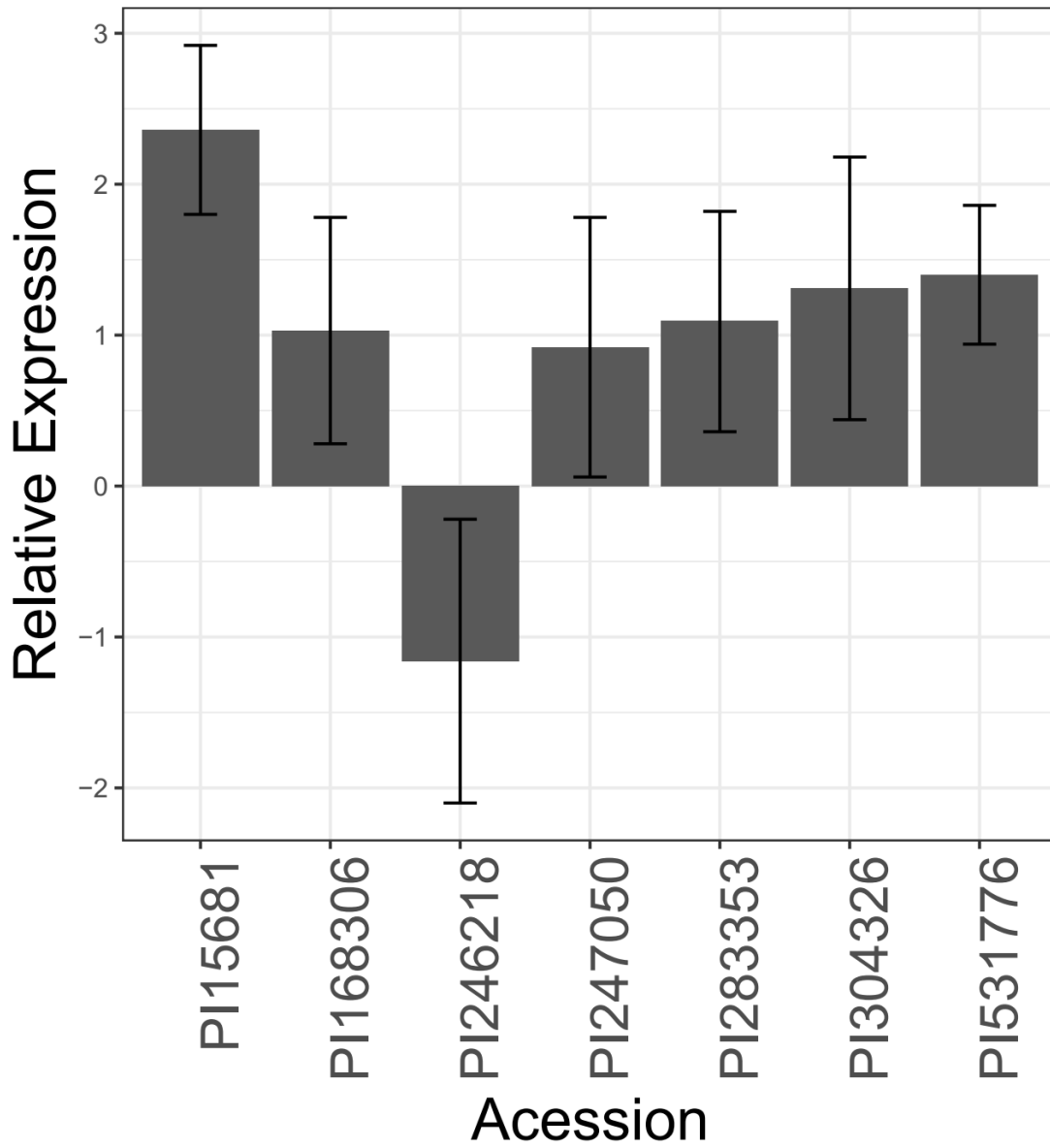
Figure 12. qRT - PCR validation of miR528b-3p. This bar plot illustrated the relative expression of miR528b-3p in tetraploid stress compared to those in tetraploid check (CK) using qRT-PCR.

Chapter 5 Discussion

5.1 Autotetraploid *Hordeum bulbosum* can adapt to salt stress better than diploid plants

In this study, it was found that under salinity stress tetraploids have higher RWC and lower WL values, indicating that tetraploid (plant ID 106880) *H. bulbosum* has a stronger ability to maintain water content and prevent water loss compared with its diploid (Plant ID 240261) counterpart. Therefore, tetraploid *H. bulbosum* can better deal with the salt stress compared to its diploid progenitor, which is consistent with the findings in other plant species.[3, 9, 23-25]. Since only one diploid and one tetraploid accession of *Hordeum bulbosum* were randomly selected two conduct the experiment, it cannot be generalized that all tetraploid plants in the whole *Hordeum bulbosum* species will have a stronger ability to tolerate stress than its diploid ancestor.

5.2 Bioinformatics discussion

5.2.1 Bioinformatics analysis of miRNAs

By analyzing the length distribution of miRNAs in the four libraries (diploid CK, diploid stress, tetraploid CK and tetraploid stress), it was found that the majority of the small RNAs from these four libraries were 24 nt and 21nt in size, followed by 22nt and 23nt (Fig. 5). The length distribution patterns of these four libraries were highly consistent with small RNA sequences in other plants, such as in cultivated barley (*Hordeum vulgare*),[36] peanut (*Arachis hypogaea* L.),[37] *Gossypium hirsutum,*[38] *Medicago truncatula*,

[39] and rice.[41] It was also consistent with the typical size of Dicer-digestion product [36]. It is also found that both tetraploid CK and tetraploid stress libraries have higher percentages of miRNAs (31.5% and 28.7%, respectively) than those in both (salt stressed / CK) diploid libraries (25.8% and 21.4%). This discovery was consistent with the finding that the percentage of microRNAs relative to the total small RNAs increased with the increasing polyploidy, and WGD is a major mechanism for the expansion of miRNA families.[45, 46] 2284 target genes were predicted in total (Fig. 9a) for the 54 conserved miRNAs. As shown in Table 7, most of the targets are transcriptional factors (TFs) such as the predicted targets of mir156z, mir159k-3p, mir164k targeting SPL, GAMYB and NAC, respectively. This is similar to previous findings for maize.[71] Other targets like ARF, ERF, SOD and TCP TFs were also discovered in this study, and were identified to be the salt-induced targets of miRNAs, as reported in previous studies. [71,72]

5.2.2 MiRNAs associated with both genome duplication and stress response

In this study, five miRNAs were not only affected or induced by genome duplication, but were also involved in salt stress response. Among these five miRNAs, four (miR171i, miR479, miR5048-5p and miR6196) were up-regulated in tetraploid CK compared to those in diploid CK, but miR171i, miR479 and miR5048-5p were down-regulated and miR6196 was up-regulated in diploid stress compared to those in the diploid CK. The predicted targets of miRNA171i and miR479 are PNSB (photosynthetic NDH subunit of subcomplex B chloroplastic) (Table 7) which mediates cyclic electron transport and plays an important role in chloro-respiration, and NDH is also required for protection of photosynthetic machinery against oxidative stress. [73] The miR528b-3p was only found

in the tetraploid CK plants and was down-regulated in the stress treatment compared to the CK. The predicted targets of miR528b-3p were ERF2, PLDZ1, MZT1B and RFA1C. PLD was involved in osmotic stress –activated phospholipid signaling, and can generate PA that is a signal molecule to activate the stress-responsive gene expression.[74] ERF2 is involved in phytohormone signal cascades and has been identified as salt-induced targets of miRNAs in previous studies.[71, 72] MZT1B, gamma-tubulin plays an important role in the organization of spindle and participates in the mitotic entry [75] while RFA1C is critical not only for DNA replication but also for efficient DNA repair and recombination. [76] These results may indicate that during genome duplication the new induced miR528b-3p can help to control DNA duplication, repair and cell division, while under salt stress, miR528b-3p was down-regulated which can increase the expression of PLD and ERF to better cope with salt stress.

It is worth noting that miR528b-5p was only detected in diploid CK, while miR528b-3p was only detected in the tetraploid CK (Table 5). The predicted target of miR528b-5p is SOD [71] which was only involved in salt stress response, while the predicted targets of miR528b-3p were not only associated with salt stress but were also involved in DNA replication and repair. Actually, miRNA-5p and miRNA-3p resulted from the same pre-miRNA, and it was proposed that arm switching may contribute to isomiR expression and thus can complicate the regulatory network and may favor keeping the genome compact, because miRNA-5p and miRNA-3p do not target the same gene families [77]. Therefore, the arm switching that occurred in tetraploid may be due to genome duplication. It is reported that the co-retention of miRNAs and target genes may result in innovative

miRNA-target interactions that are critical to an adaptive response to various environmental stimuli in addition to maintaining a constant set of miRNAs for basic biological functions [5]. Thus, in theory, the newly induced miR528b-3p and the over-retained miRNAs (miR171i, miR479, miR5048-5p and miR6196) may develop novel miRNAs—target interaction that can help tetraploid better cope with stressful environmental conditions.

5.2.3 Distinct miRNAs-targets responses to salt stress in diploid and tetraploid plants

In this study, comparison of the functions (Table 7) of all differentially expressed or newly induced miRNAs in diploid and tetraploid salt stressed plants with their respective CKs showed there are distinct miRNAs-targets in response to salt stress in diploids and tetraploids (Figs. 13a-b). The differentially expressed miRNAs in tetraploid stress compared to its CK can decrease energy wastage and activate stress-related genes such as, miR399, which can decrease biosynthesis of starch and amino acids to maintain the supply of energy[78], and miR169, which can target NF-YA, a CCAT-BOX binding transcription factor and regulator of a large number of genes [79]. Although fewer miRNAs are differentially expressed in tetraploid stressed plants than in the diploid stressed plants (Table 7), the differentially expressed and new generated

miRNAs in the salt stressed tetraploids are sufficient to deal with the stress effects, which suggested that some of the differentially expressed miRNAs in diploids under salt stress are redundant. For instance, both miR159-3p and miR319p were predicted to target GAMYB[71], the predicted targets of both miR156z and miR157d-5p were SPL[80], and both

miR399 and miR827-5p regulated the Pi concentration in plants[81]. Theoretically, the more miRNAs were down-regulated, the more energy was consumed to produce protein which will slow the growth [12]. In this case, diploids might need more energy to cope with the salt stress than tetraploids. In turn, diploids have less energy to sustain normal growth compared with tetraploids. All these results indicated that, under salt stress, tetraploids have a more elaborate miRNA—target interaction compared to that in diploids, which can help tetraploids better deal with salt stress and maintain normal growth, and is consistent with the physiological results that tetraploids have a stronger ability to retain water and prevent water loss resulting in better survival under salt stress.

The possible applications of microRNAs in agriculture

Soil salinity is a major constraint on crop productions. Much attention and time have been devoted to study the salt tolerance mechanism and the classic approach which engineers an individual stress related gene can only help plants to tolerate a single stress. However, in natural environment plants face many different stresses at the same time. Many differentially expressed miRNAs have been identified under different stresses and it is also found that one miRNAs can be involved in many different stress responses. In this study, the differentially expressed microRANs due to salt stress in tetraploid and diploid have been identified. For instance, the microRNA-miR528b-3p was not only affected by genome duplication but also was involved in salt stress response. Thus, manipulating the expression of the stress related miRNAs or transfer the stress related miRNAs to plants might help us to improve the stress tolerant ability of plants[25,26].

Chapter 6 Future directions

This work has identified the differentially expressed microRNAs in diploid and tetraploid under salt stress compared with their CK counterparts, as well as a new induced microRNA528b-3p. Therefore, in the future, several studies could be done to further this research as follows:

Using qRT-PCR to detect the expression of the identified salt-stress related microRNAs in this thesis in other accessions of *Hoderum bulbosum*. In this way, it is possible to investigated whether these differentially expressed microRNAs are unique to these two accessions of *Hordeum bulbosum* or a general pattern in the whole species of *Hordeum bulbosum*. Since different accessions of *Hordeum bulbosum* distributed in different part of the world, their growing conditions are significantly different. Therefore, it is possible to analyze the expression of microRNAs associated with the environmental conditions (the temperature, the humidity, and so forth) where these different accessions of *Hordeum bulbosum* were grown.

Using qRT-PCR to detect the expression of miRNA528b in other tetraploid and diploid accessions of Hordeum bulbosum. To see whether all tetraploid accessions of *Hordeum bulbosum* have this microRNA and all diploid counterparts don't have this miRNA. Since miRNA528b-3p was induced by genome duplication, therefore it is worth to investigating the mechanism underlying the induction of new miRNAs.

MiRNA528b-3p is the only miRNA which was induced by genome duplication and also involved in stress response. Even though, the targets of miRNA528b-3p have been predicted by software, it is necessary to devise a molecular experiment to investigate the function of miRNA528b-3p accurately.

Table 7. The function of some miRNAs targets

MiR ID	Predicted targets	Protein annotation	Function	Previous study
miR156z	MLOC_13032	SPL13 Squamosa promoter-binding 13	Flowering time	[79]
	MLOC_37841	SPL16 Squamosa promoter-binding 16		
	MLOC_61297	SPL17 Squamosa promoter-binding 17		
	MLOC_52321	SPL2 Squamosa promoter-binding 2		
	MLOC_11199	SPL3 Squamosa promoter-binding 3		
	MLOC_62426	SPL4 Squamosa promoter-binding 4		
	MLOC_13032	SPL13 Squamosa promoter-binding 13		
	MLOC_37841	SPL16 Squamosa promoter-binding 16		

miR157d-5p		SPL17 Squamosa promoter-binding 17	Flowering time	[80]
	MLOC_61297			
	MLOC_52321	SPL2 Squamosa promoter-binding 2		
	MLOC_11199	SPL3 Squamosa promoter-binding 3		
	MLOC_62426	SPL4 Squamosa promoter-binding 4		
mir159k-3p	MLOC_6041	GAM1 Transcription factor GAMYB	Flowering time and male fertility	[72]
	MLOC_74051	GAM1 Transcription factor GAMYB		
	MLOC_71332	GAM1 Transcription factor GAMYB		
	MLOC_60410	PP139 Pentatricopeptide repeat-containing		
	MLOC_52196	SAP16 Zinc finger AN1 and C2H2 domain-containing stress-associated 16		
mir160j	MLOC_77438	ARFH Auxin response factor 8	Control the formation of root cap	[82]
	MLOC_67174	ARFJ Auxin response factor 10		
	MLOC_56664	ARFM Auxin response factor 13		
	MLOC_69988	ARFR Auxin response factor 18		
	MLOC_64795	ARFV Auxin response factor 22		

mir164k	MLOC_74277	MPK4 Mitogen-activated kinase 4	Necessary for normal embryonic, vegetative and floral development	[83]
	MLOC_53744	NAC22 NAC domain-containing 21		
	MLOC_53745	NAC22 NAC domain-containing 21		
	MLOC_53746	NAC98 NAC domain-containing 98		
mir169y	MLOC_53766	BZIP9 Basic leucine zipper 9	Participates in transcriptions regulation of larger number genes	[47]
	MLOC_53767	HACDA 3-hydroxyacyl-dehydratase		
	MLOC_53768	NFYA3 Nuclear transcription factor Y subunitA-3		
	MLOC_53769	NFYA5 Nuclear transcription factor Y subunitA-5		
	MLOC_53771	NFYA6 Nuclear transcription factor Y subunitA-6		
mir170-3p	MLOC_53777	SCL6 Scarecrow 6	Control the formation of radial organization of the root	[84]
	MLOC_53778	SCL6 Scarecrow 6		
	MLOC_53779	SCL6 Scarecrow 6		

mir171i	MLOC_53781	NADHK Probable NADH kinase	Against oxidative stress	[73]
	MLOC_53782	PNSB5 NAD(P)H dehydrogenase	Mediates cyclic electron	
miR319p	MLOC_53806	GAM1 Transcription factor GAMYB		
	MLOC_53807	GAM1 Transcription factor GAMYB	Flowering and male fertility	[72]
	MLOC_53808	GAM1 Transcription factor GAMYB		
	MLOC_53816	PP139 Pentatricopeptide repeat-containing		
	MLOC_53814	NORK Nodulation receptor kinase		
mir393k	MLOC_9864	TIR1A Transport inhibitor response 1		[85]
	MLOC_56088	TIR1B Transport inhibitor response 1	Regulating auxin response	
	MLOC_51300	CLP1 CLP-SIMILAR PROTEIN 3		
	MLOC_57855	LPE1Leaf permease 1		
mir395y	MLOC_72476	DBR 2-alkenal reductase (NADP(+)-dependent)	Transportation of sulfate and decrease energy wastage	[32,86]
	MLOC_3923	SUT3 Low affinity sulfate transporter 3		

	MLOC_80060	GRF10 Growth-regulating factor 10	Cell proliferation and increase organ size	[73,87]
	MLOC_64055	GRF2 Growth-regulating factor 2		
mir396e-5p	MLOC_67201	GRF6 Growth-regulating factor 6		
	MLOC_4153	RPM1 Disease resistance RPM1		
	MLOC_74620	DOF36 Dof zinc finger PP357		
mir399p	MLOC_52822	Pentatricopeptide repeat-containing	Prevent Pi excess	[87]
	MLOC_53410	UBC24 E2 ubiquitin-conjugating enzyme 24		
	MLOC_70692	ERF2 Ethylene-responsive transcription factor	Salt -induced targets	[71,72]
	MLOC_70374	PLDZ1 Phospholipase D zeta 1	Involved osmotic stress	[74]
	MLOC_56528	MZT1B Mitotic-spindle organizing associated with a ring of gamma-tubulin	Participate in mitotic entry	[75]
mir528b-3p			DNA replication and DNA repair and recombination	[76]
	MLOC_10576	RFA1C Replication factorA 1C		
mir528b-5p	MLOC_57716	HMA5 Probable copper-transporting ATPase 3		

	MLOC_17760	SODCP Superoxide dismutase	Scavenging the ROS	[71,88]
mir827c	MLOC_57566	SPXM2 SPX domain-containing membrane	Control the concentration of Pi in plants	[81]
	MLOC_63586	SPXM2 SPX domain-containing membrane _17046	Control the concentration of Pi in plants	[81]
mir827-5p	MLOC_53112	PP191 Pentatricopeptide repeat-containing		
	MLOC_4112	UBC22 Ubiquitin-conjugating enzyme E2 22	Prevent Pi excess	[81]
mir9863-3p	MLOC_24045	R13L1 Disease resistance RPP13 1	Disease resistant	
	MLOC_60393	R13L2 Disease resistance RPP13 2		
	MLOC_62757	R13L3 Disease resistance RPP13 3		
mir5071	MLOC_1443	RPM1 Disease resistance RPM1	Disease resistant	
	MLOC_31061	RPP13 Disease resistance RPP13		
mir9652-5p	MLOC_4008	C3H19 Zinc finger CCCH domain-containing 19		

			Involved in ABA-independent signaling transduction and activate stress related gene expression	[89,90]
	MLOC_56602	DRIP2 E3 ubiquitin ligase DRIP2		
Mir9674c-5p	MLOC_18343	PPR Mitochondrial Fertility restorer	Control RNA processing and translation in mitochondria and chloroplasts	
	MLOC_43104	PPR Mitochondrial Fertility restorer		
	MLOC_44563	PPR Mitochondrial Fertility restorer		

Table 8. The specific forward and universal reverse primers used for qPCR analysis

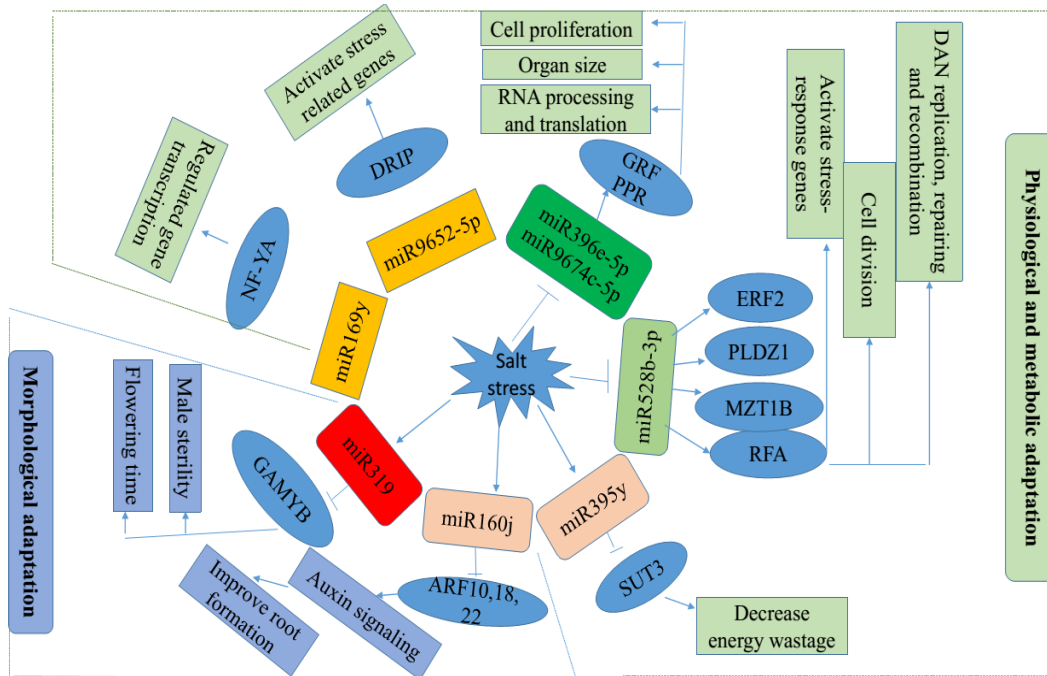| sequence	RT primers (5'->3')	Forward primers (F) (5'->3')	TM	Universal Reverse primer (5'->3')
UUUGGAUUGAAGGGAGCUC	5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC GAG CTC-3'	5'-CGCGCGTTTGGATTGAAGG-3'	60	
UCCACAGGCUUUCUUGAACUG	5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC CAG TTC-3'	5'-CGCGCCTCCACAGGCTTT-3'	60	
UUUGGAUUGAAGGGAGCUCUG	5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC CAG AGC-3'	5'-CGCGCGTTTGGATTGAAGG-3'	60	
UUAGAUGACCAUCAGCAAACA	5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC TGT TTG-3'	5'-CGCGCCTTAGATGACCATC-3'	60	
UGCCUGGCUCCCUGAAUGCCA	5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC TGG CAT-3'	5'-CATGCATGCCTGGCTCCC-3'	60	
UGAAGUGUUUGGGGGAACUC	5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC GAG TTC-3'	5'-CGCGCATGAAGTGTTTGGG-3'	60	
UGACAGAAGAGAGUGAGCAC	5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC GTG CTC-3'	5'-CACGCGTGACAGAAGAGAG-3'	60	5'-CCA GTG CAG GGT CCG AGG-3'
UGAAGCUGCCAGCAUGAUCUGA	5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC TCA GAT-3'	5'-CGCGCATGAAGCTGCCAG-3'	60	
UGAUUGAGCCGUGCCAAUAUC	5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC GAT ATT-3'	5'-CGCGCGTGATTGAGCCGT-3'	60	
UUCCAAAGGGAUCGCAUUGAU	5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC ATC AAT-3'	5'-CGCGCATTCCAAAGGGATC-3'	60	
CCUGUGCCUGCCUCUUCCAUU	5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC AAT GGA-3'	5'-CACTCACCTGTGCCTGCC-3'	60	
UAGCCAAGGAUGACUUGCCUG	5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC CAG GCA-3'	5'-CGCGCGTAGCCAAGGATG-3'	60	
UGGAGAAGCAGGGCACGUGCA	5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC TGC ACG-3'	5'-CGCGCATGGAGAAGCAGG-3'	60	
UGAGCCGAACCAAUAUCACUC	5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC GAG TGA-3'	5'-CGCGCATGAGCCGAACCA-3'	60	
UUUGCAGGUUUUAGGUCUAAGU	5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC ACT TAG-3'	5'-CACGCGTTTGCAGGTTTTAG-3'	60	
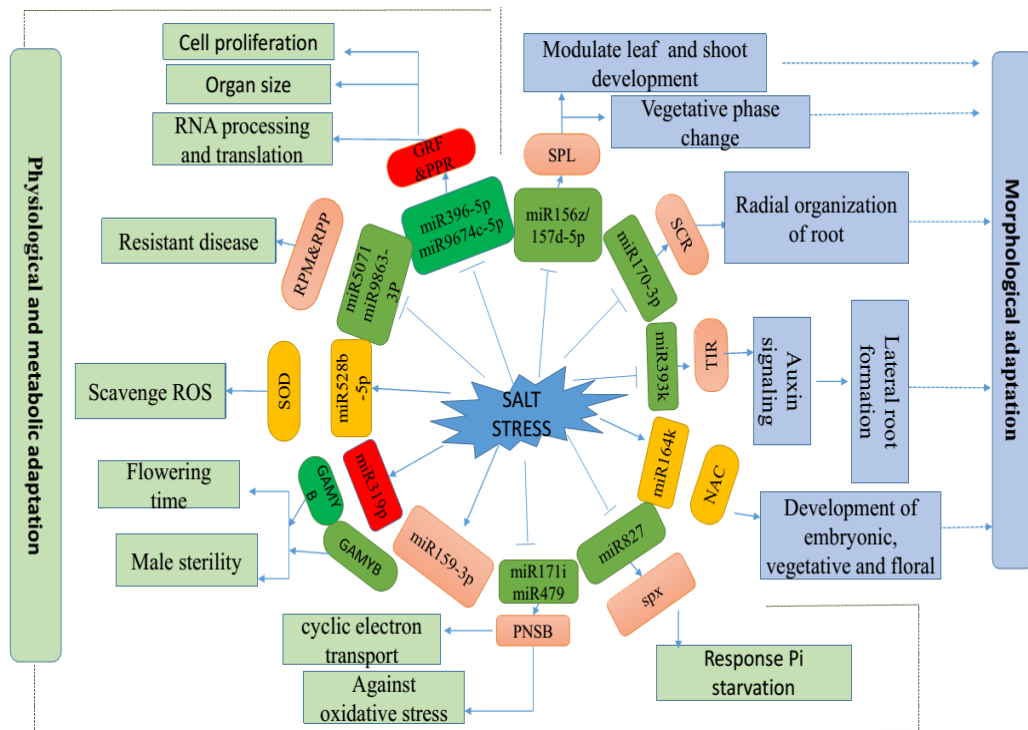
Figure 13a. The miRNAs—targets response to salt stress in diploid *H. bulbosum*. This graph summarizes how the expression of miRNAs are altered under salt stress in diploid.

[green box] means the down-regulated miRNAs both in diploid and tetraploid under salt stress compared with their controls, [dark green box] represents the miRNAs which only were down-regulated in diploid salt stress compared with that in diploid control but not in tetraploid.

[red box] Refers to the miRNAs which were up-regulated both in diploid and tetraploid under salt stress compared to their controls and [orange box] means the miRNAs which were only up-regulated in diploid stress relative to that of diploid control but not in tetraploid. Means induction while refers to inhibition.

Figure 13b. The miRNAs—targets response to salt stress in tetraploid *H. bulbosum*.

means the down-regulated miRNAs both in diploid and tetraploid under salt stress
d with their controls, represents the miRNAs which only were down-regulated
in diploid salt stress compared with that in diploid control but not in tetraploid.

Refers to the miRNAs which were up-regulated both in diploid and tetraploid under salt
stress compared to their controls and ns the miRNAs which were only up-regulated
in diploid stress relative to that of diploid control but not in tetraploid.

Means induction while refers to inhibition.

Chapter 7 References

1. Wang JL, Tian L, Lee HS, et al. Genome wide nonadditive gene regulation in arabidopsis allotetraploids. *Genetics*. 2006; 172:507-517.

2. Stupar RM, Bhaskar PB, Yandell BS, et al. Phenotypic and transcriptomic changes associated with potato autopolyploidization. *Genetics*. 2007; 176:2055-2067.

3. Lu BB, Pan XZ, Zhang L, et al. A genome-wide comparison of genes responsiveto autopolyploidy in *isatis indigotica* using *arabidopsis thaliana* affymetrix genechips. *Plant molecular biology reporter*. 2006; 24:197-204.

4. Fawcett JA, Maere S, Peer YV. Plants with double genomes might have had a better chance to survive the Cretaceous–Tertiary extinction event. *PNAS 106: 5737-5742*. 2009; 106:5737-5742.

5. Abrouk M, Zhang RZ, Murat F, et al. Grass microRNA gene paleohistory unveils new insights into gene dosage balance in subgenome partitioning after whole-genome duplication. *The Plant Cell*. 2012; 24:1776-1792.

6. Chen K, Rajewsky N. The evolution of gene regulation by transcription factors and microRNAs. . *Nature*. 2007; 8:93-103.

7. Beest MT, Le Roux JJ, Richardson DM, et al. The more the better? The role of polyploidy in facilitating plant invasions. . *Annals of Botany*. 2012; 109:1-27.

8. Hegarty MJ, Barker GL, Wilson ID, Abbott RJ, Edwards KJ, Hiscock SJ. Transcriptome shock after interspecific hybridization in senecio is ameliorated by genome duplication. *Current Biology*. 2006; 16:1652-1659.

9. Wang ZM, Wang MY, Liu LK, Meng FJ. Physiological and proteomic responses of diploid and tetraploid black locust (*robinia pseudoacacia* L.) subjected to salt stress. *International Journal of Molecular Sciences*. 2013; 14:2029-2035.

10. Jackson S, Chen ZJ. Genomic and expression plasticity of polyploidy. *Plant Biology*. 2010; 13:153-159.

11. Hegarty M, Hiscock S. Polyploidy: Doubling up for evolutionary success. *Current Biology 17:1669-1674*. 2007; 17:1-3.

12. Ng D, Zhang C, Miller M, Shen Z, Briggs S, Chen ZJ. Proteomic divergence in arabidopsis autopolyploids and allopolyploids and their progenitors. *Heredity*. 2012; 108:419-430.

13. Pumphrey M, Bai J, Laudencia-Chingcuanco D, Anderson O, Gill BS. Nonadditive expression of homoeologous genes is established upon polyploidization in hexaploid wheat. *Genetics*. 2009; 181:1147-1157.

14. Rapp RA, Wendel JF. Epigenetics and plant evolution. *New Phytologist*. 2005; 168:81-91.

15. Parisod C, Holderegger R, Brochmann C. Evolutionary consequences of autopolyploidy. *New Phytologist*. 2010; 186:5-17.

16. Church SA, Spaulding EJ. Gene expression in a wild autopolyploid sunflower series. *Journal of Heredity*. 2009; 100:491-495.

17. Tan FQ, Tu H, Liang WJ, et al. Comparative metabolic and transcriptional analysis of a doubled diploid and its diploid citrus rootstock (C. junos cv. ziyang xiangcheng) suggests its potential value for stress resistance improvement. . *BMC Plant Biology*. 2015; 15:1-14.

18. Peer YV, Maere S, Meyer A. The evolutionary significance of ancient genome duplications. *pespectives*. 2009; 10:1-8.

19. Madlung A. Polyploidy and its effect on evolutionary success: Old questions revisited with new tools. *Heredity*. 2013; 110:99-104.

20. Parisod C, Holderegger R, Brochmann C. Evolutionary consequences of autopolyploidy. *New phytologist*. 2010; 186:5-17.

21. Crawford DJ, Doyle JJ, Soltis DE, Soltis PS, Wendel JF. Contemporary and future studies in plant speciation, morphological/floral evolution and polyploidy: Honouring the scientific contributions of leslie D. gottlieb to plant evolutionary biology. *Philosophical transactions of the royal society*. 2015; 369.

22. Vanneste K, Maere S, Peer YV. Tangled up in two: A burst of genome duplications at the end of the cretaceous and the consequences for plant evolution. (2014) 369:1-13. *Physiological transactions of the royal society B*. 2014; 369:1-13.

23. Meng HB, Jiang SS, Hua SJ, et al. Comparison between a tetraploid turnip and its diploid progenitor (*brassica rapa* L.): The adaptation tosalinity stress. *Agricultural Science in China*. 2011; 10:363-375.

24. Saleh B, Allario T, Dambier D, Ollitrault P, Morillon R. Tetraploid citrus rootstocks are more tolerant to salt stress than diploid. *Competes Rendus Biologies*. 2008; 331:703-710.

25. Liu SY, Chen SM, Chen Y, Guan ZY, Yin DM, Chen FD. In vitro induced tetraploid of *dendranthema nankingense* (nakai) tzvel. Shows an improved level of abiotic stress tolerance. *Scientia horticulturae*. 2011; 127:411-419.

26. del Pozo JC, Ramirez-Parra E. deciphering the molecular bases for drought tolerance in*arabidopsis* autotetraploids. *Plant,Cell and Environment*. 2014; 37:2722-2737.

27. Hegarty MJ, Hiscock SJ. Genomic clues to the evolutionary success of polyploidy plants. *Current Biology*. 2008; 18:435-444.

28. Allario T, Brumos J, Colmenero-Flores JM, et al. Large changes in anatomy and physiology between diploid rangpur lime (*citrus limonia*) and its autotetraploid are not

associated with large changes in leaf gene expression. *Journal of Experimental Botany*. 2011:1-13.

29. Chen ZJ. Genomic and epigenetic insights into the molecular bases of heterosis. *Nature*. 2013; 10:1-12.

30. Marmagne A, Brabant P, Thiellement H, Alix K. Analysis of gene expression in resynthesized brassica napus allotetraploids: Transcriptional changes do not explain differential protein regulation. *New phytologist*. 2010; 186:216-227.

31. Bartel DP. MicroRNAs: Genomics, biogenesis, mechanism and function. *Cell*. 2004; 116:281-297.

32. Khraiwesha B, Zhu JK, Zhu JH. Role of miRNAs and siRNAs in biotic and abiotic stress responses of plants. *Biochimica et Biophysica Acta*. 2012; 1819:137-148.

33. Lee RC, Feinbaum RL, Ambrosts V. The C. *elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell*. 1993; 75:843-854.

34. Liu HH, Tian X, Li YJ, Wu CA, Zheng CC. Microarray-based analysis of stress-regulated microRNAs in arabidopsis thaliana. . *RNA*. 2008; 14:836-843.

35. Yao YY, Guo GG, Ni ZF, et al. Cloning and characterization of microRNAs from wheat (*triticum aestivum* L.). *Genome Biology*. 2007; 8:1-13.

36. Lv SZ, Nie XJ, Wang L, et al. Identification and characterization of microRNAs from barley(*Hordeum vulgare* L.) by high-throughput sequencing. *International Journal of Molecular Sciences*. 2012; 13:2973-2984.

37. Chi XY, Yang QL, Chen XP, et al. Identification and characterization of microRNAs from peanut (*arachis hypogaea* L.) by high-throughput. *PLOS ONE*. 2001; 6:1-10.

38. Ruan MB, Zhao YT, Meng ZH, Wang XJ, Yang WC. Conserved miRNA analysis in *gossypium hirsutum* through small RNA sequencing. *Genomics*. 2009; 94:263-268.

39. Szittya G, Moxon S, Santos DM, et al. High-throughput sequencing of *medicago truncatula* short RNAs identifies eight new miRNA families. *BMC Genomics*. 2008; 9:1-9.

40. Song CN, Wang C, Zhang CQ, et al. Deep sequencing discovery of novel and conserved microRNAs in trifoliate orange (*citrus trifoliata*). *BMC Genomics*. 2010; 11:1-12.

41. Sunkar R, Zhou XF, Zheng Y, Zhang WX, Zhu JK. Identification of novel and candidate miRNAs in rice by high throughput sequencing. *BMC Plant Biology*. 2008;8:1-17.

42. Xie FL, Zhang BH. microRNA evolution and expression analysis in polyploidized cotton genome. *Plant Biological Journal*. 2015;10:1-14.

43. Comai L. The adavantages and disadvantages of being polyploid. *Nature*. 2005;6:836-847.

44. Adams KL, Wendel JF. Polyploidy and genome evolution in plants. *Current Opinion in Plant Biology*. 2005;8:135-141.

45. Eichler MK, Leshkowitz D, Tal L, et al. Wheat hybridization and polyploidization results in deregulation of small RNAs. *Genetics*. 2011;188:263-272.

46. Ha M, Lu J, Tian L, et al. Small RNAs serve as a genetic buffer against genomic shock in *arabidopsis* interspecific hybrids and allopolyploids. *PNAS*. 2009;106:17835-17840.

47. Zhao BT, Liang RQ, Ge LF, et al. Identification of drought-induced microRNAs in rice. . *Biochemical and Biophysical Research Communications*. 2007;354:585-590.

48. Katiyar A, Smita S, Muthusamy SK, Chinnusamy V, Pandey M, Bansal KC. Identification of novel drought-responsive microRNAs and trans-acting siRNAs from *sorghum bicolor* (L.) moench by high-throughput sequencing analysis. *Frontiers in Plant Science*. 2015;6:1-21.

49. Pfaffl, MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research.* 2001, 29; 2002-2007.

50. Ding D, Zhang LF, Wang H, Liu ZJ, Zhang ZX, Zheng YL. Differential expression of miRNAs in response to salt stress in maize roots. *Annals of Botany*. 2009;103:29-38.

51. Frazier TP, Sun G, Burklew CE, Zhan BH. Salt and drought stresses induce the aberrant expression of microRNA genes in tobacco. *Molecular Biotechnology*. 2011;49:159-165.

52. Li AL, Liu DC, Wu J, et al. mRNA and small RNA transcriptomes reveal insights into dynamic homoeolog regulation of allopolyploid heterosis in nascent hexaploid wheat. *Plant Cell*. 2014;26:1878-1900.

53. Stuart JR, Sonia N, Mark T. Salt resistant crop plants. *Current Opinion in Biotechnology*. 2014;26:115-124.

54. Pichu R. Soil processes affecting crop production in salt-affected soils. *Functional Plant Biology*. 2010;37:613-620.

55. Ulrich D, Aaron BS, Tomoaki H, Wei L, Xu GH, and Julian IS. Plant salt-tolerance mechanisms. *Trends in plant science*. 2014;19:371-379.

56. Alessandra P, Giovanni C, Wafa M, Delphine C, Valerie R, Renata DC, Francois L, Raphael M, Patrick O, Bianca EM. Salt-stress induced changes in the leaf proteome of diploid and tetraploid mandarins with contrasting na+ and cl- accumulation behaviour. *Jouranl of plant physiology*. 2013;170:1101-1112.

57. Nobuhiro S, Shai K, Ron M, Gad M. ROS and redox signalling in the response of plants to abiotic stresspce_2. *Plant,Cell and Environment*. 2012;35:259-270.

58. Takashi H & Kazuo S. Research on plant abiotic stress responses in the post-genome era: Past, present and future. *The Plant Jornal*. 2010;61:1041-1052.

59. Julieta V, Anabella FL, Matias DZ. Novel perspectives for the engineering of abiotic stress tolerance in plants. *Current Opinion in Biotechnology*. 2014;26:62-70.

60. Matias D. Zurbriggen , Mohammad-Reza Hajirezaei & Nestor Carrillo. Engineering the future. Development of transgenic plants with enhanced tolerance to adverse environments. *Biotechnology and Genetic Engineering Reviews*. 2010;27:33-56.

61. Raj KP, Rajiv S, Marcos M, Fred AE, Grit H, Benjamin K and Andreas G. Genome-wide association studies for agronomical traits in a worldwide spring barley collection. *BMC Plant Biology*. 2012.

62. Jie X, Snape JW. The resistance of *hordeum bulbosum* and its hybrids with *H.vulgare* to common fungal pathogens. *Euphytica*. 1989;41:273-276.

63. Ortiz L, Gonzale Á, Chueca MC. On the presenge of diploid and tetraploid forms of *hordeum bulbosum* L. east spain. *Aneales Jardfn Botanico de madrid*. 1985;41:1-5.

64. Jabeen Z, Hussain N, Wu DZ, et al. Difference in physiological and biochemical responses to salt stress between tibetan wild and cultivated barleys. *Acta Physiologiae Plantarum*. 2015;37:1-8.

65. Mano Y, Takeda K. Genetic resources of salt tolerance in wild *hordeum* species. *Euphytica*. 1998;103:137-141.

66. Li JB, Luan YS, Liu Z. SpWRKY1 mediates resistance to phytophthora infestans and tolerance to salt and drought stress by modulating reactive oxygen species homeostasis and expression of defense-related genes in tomato. *Plant Cell Tiss Organ Culture*. 2015;123:67-81.

67. Liu H, Yu CY, Li HX, et al. Overexpression of ShDHN, a dehydrin gene from solanum habrochaites enhances tolerance to multiple abiotic stresses in tomato. *Plant Science*. 2015;231:198-211.

68. Suprunova T, Krugman T, Fahima T, et al. Differential expression of dehydrin genes in wild barley, *hordeum spontaneum*, associated with resistance to water deficit. *Plant, Cell and Environment*. 2004;27:1297-1308.

69. Zhu M, Chen GP, Dong TT, et al. SlDEAD31, a putative DEAD-box RNA helicase gene, regulates salt and drought tolerance and stress-related genes in tomato. *PLOS ONE*. 2015; 10:1-20.

70. Philippe B, Jérôme M, Frédéric E, Christophe Dand Christophe K. Jvenn: An interactive Venn diagram viewer. *BMC Bioinformatics*. 2014.

71. Kramer MF. Stem-loop RT-qPCR for miRNAs. *Current protocol molecular biology*. 2011:1-12.

72. Again D, Zhang LF, Wang H, Liu ZJ, Zhang ZX, Zheng YL. Differential expression of miRNAs in response to salt stress in maize roots. . *Annals of Botany*. 2009;103:29-38.

73. Palatnik JF, Wollmann H, Schommer C, et al. Sequence and expression differences underlie functional specialization of Arabidopsis MicroRNAs miR159 and miR319. . *Developmental Cell*. 2007;13:115-125.

74. Ueda M, Kuniyoshi1 T, Yamamoto H, et al. Composition and physiological function of the chloroplast NADH dehydrogenase-like complex in marchantia polymorpha. *The plant journal*. 2012;72:683-693.

75. Zhu JK. Salt and drought stress signal transduction in plants. *Annual Review Plant Biology*. 2002;53:247-273.

76. Zimmerman WC, Sillibourne J, Rosa J, Doxsey SJ. Mitosis-specific anchoring of tubulin complexes by pericentrin controls spindle organization and mitotic entry. *Molecular biology of the cell*. 2004;15:3642-3657.

77. Longhese MP, Plevani P, Lucchini G. Replication factor A is required in vivo for DNA replication, repair, and recombination. *Molecular and cellular biology*. 1994;14:7884-7890.

78. Hu WX, Wang TZ, Yue EK, Zheng S, Xu JH. Flexible microRNA arm selection in rice. *Biochemical and Biophysical Research Communications*. 2014;447:526-530.

79. Zhang ZX, Wei LY, Zou XL, Tao YS, Liu ZJ, Zheng YL. Submergence-responsive microRNAs are potentially involved in the regulation of morphological and metabolic adaptations in maize root cells. *Annals of Botany*. 2008; 102:509-519.

80. Zhao BT, Ge LF, Liang RQ, et al. Members of miR-169 family are induced by high salinity and transiently inhibit the NF-YA transcription factor. *BMC Molecular Biology*. 2009; 10:29.

81. Wang JW, Czech B, Weigel D. miR156-regulated SPL Transcription Factors define an endogenous flowering pathway in arabidopsis thaliana. *Cell*. 2009;138:738-749.

82. Lin S, Santi C, Jobet E, et al. Complex regulation of two target genes encoding SPX-MFS proteins by rice miR827 in response to phosphate starvation. *Plant cell physiology*. 2010;51:2119-2131.

83. Pradhan A, Naik N, Sahoo KK. RNAi mediated drought and salinity stress tolerance in plants. . *American Journal of plant sciences*. 2015;6:1990-2008.

84. Mallory AC, Dugas DV, Bartel DP, Bartel B. MicroRNA regulation of NAC-domain targets is required for proper formation and separation of adjacent embryonic, vegetative, and floral organs. *Current Biology*. 2004;14:1035-1046.

85. Laurenzio LD, Diller JW, Malamy JE, et al. The *SCARECROW* gene regulates an asymmetric cell division that is essential for generating the radial organization of the arabidopsis root. . *Cell*. 1996;86:423-433.

86. Chen ZH, Bao ML, Sun YZ, et al. Regulation of auxin response by miR393-targeted transport inhibitor response protein 1 is involved in normal development in arabidopsis. *Plant molecular biology*. 2011;77:619-629.

87. Kataoka T, Hayashi N, Yamaya T, Takahashi H. Root-to-shoot transport of sulfate in arabidopsis. evidence for the role of SULTR3;5 as a component of low-affinity sulfate transport system in the root Vasculature1. *Plant Physiology*. 2004;136:4198-4204.

88. Huertero CA, Perez RB, Melo DB, et al. Conserved and novel miRNAs in the legume phaseolus vulgaris in response to stress. *Plant Molecular Biology*. 2009;70:385-401.

89. Kim SY, Lim JH, Park MR, et al. Enhanced antioxidant enzymes are associated with reduced hydrogen peroxide in barley roots under saline stress. *Journal of biochemistry and molecular biology*. 2005; 38:218-224.

90. Agarwal PK, Agarwal P, Reddy MK, Sopory SK. Role of DREB transcription factors in abiotic and biotic stress tolerance in plants. *Plant cell Rep*. 2006; 25:1263-1274.

91. Qin F, Sakuma Y, Tran LP, et al. Arabidopsis DREB2A-interacting proteins function as RING E3 ligases and negatively regulate plant drought Stress–Responsive gene expression. *The plant cell*. 2008;20:1693-1707.