Salt stress response single genes expression in diploid and autopolyploid *Hordeum bulbosum*

by

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Abstract

Soil salinity is a worldwide problem, which hinders the growth of crop yields and brings great challenges to the world. To deal with the reduction of agricultural land, research and cultivating the development of salt-tolerant crops are urgently needed. Autotetraploidy has played an important role in plant evolution and plant speciation. An autotetraploid wild grass species (*Hordeum bulbosum*) has advantages in high-salt environments. However, the function of single genes in autopolyploid for salt tolerance is poorly unknown. Physiological activities related to a single functional gene are important for the salt resistance in barley; the release of protective metabolites is promoted by the single function gene. The purpose of this study is to compare the difference in gene expression of single genes between diploid and autotetraploid wild barley. The diploid and autotetraploid wild barley that were used in this study were both incubated with saline and water before comparing their level of gene expression of single genes. The results showed that there is no consistent expression change for all target genes between diploid and autotetraploid, which indicated that polyploidization could result in the gene expression change, either increase or decrease based on each gene. Even if the use of polyploid crops to combat land salinization requires further research, the prospects of salt-tolerant crops are promising.

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Introduction

1.1 Why soil salinization poses a great challenge

Soil salinization changes fertile land into an unproductive one by impairing crop growth and development. There are three ways of soil salinization that damage plant growth: (1) due to an increased concentration of solute in the soil, the osmotic potential of the root cells of the water in the soil decreases, which limits the water uptake of plants (Isayenkov & Maathuis, 2019); (2) the accumulation of soil solute in plants adversely affects the physiological activities of plants, better known as cytotoxicity, including an excessive uptake of sodium (Na^+) and chloride (Cl^-) ions that cause drying of leaves, damage to fruit and impairment of root growth (Nachshon, 2018); and (3) nutritional imbalance in plants caused by competition of sodium and chloride with nutrients like calcium, potassium and nitrates, all of which are essential for plant growth (Munns & Tester, 2008; Tester & Davenport, 2003).

The causes of soil salinity are manifold. Some human activities and global warming exacerbate soil salinization. Inaccurate irrigation habits in agriculture (using highly saline irrigation water), lack of a good drainage system, and over-exploitation caused by groundwater inversion may all lead to salinization (Nachshon, 2018). In addition, global warming caused by human activities increase the frequency and severity of droughts and heatwaves and other extreme weather events. These events accelerate the evaporation of water, therefore with more solutes in the water that are left in the soil (Nachshon, 2018).

Currently, one-fifth of cultivated land worldwide is being stressed by soil salinization, and around one-third of irrigated agricultural land globally are salinized as a consequence of poor agricultural practices (Nachshon, 2018). The affected cultivated land area is still growing, as it is anticipated that around half of the world's agricultural land will be salinized by 2050 (Food and Agricultural Organization of the United Nations, 2009). Moreover, due to the continuous and rapid growth of global population, the existing agricultural cultivation is not sufficient to meet the demands. A huge challenge is that food production worldwide must increase by 60% in order to meet the demands of additional 2.3 billion newborns by 2050 (Food and Agricultural Organization of the United Nations, 2009; Singh, 2015).

Various environmental pressures, droughts, floods, pests, and soil salinity, among others, affect crop production. Soil salinity is one of the greatest environmental stresses that hinder crop productivity worldwide. Since most farmers, particularly those in developing countries, cannot afford the cost of reclaiming saline soils, increasing the salt tolerance of crops is crucial (Munns et al., 2006). Thus, research and cultivation of salt-tolerant crops are highly necessary.

1.2 Advantage of Polyploids

A polyploid is an organism with more than two sets of chromosomes. Polyploidization plays a vital role in plant evolution and speciation. Polyploids are frequently found in extreme environments. They can adapt to many extreme environments, including salty, drought or cold environments. They also manifest better

disease and pest resistance, and present a broader environmental adaptability than their diploid ancestors (Stebbins, 1950; Moore & Purugganan, 2005; Birchler et al., 2010).

The autopolyploid *Hordeum bulbosum* is used in this study. The primary difference between autopolyploid and allopolyploid is that the former is a containment of multiple sets of chromosomes that are derived from a single species, whereas the latter is the containment of multiple sets of chromosomes that are derived from different species. Autopolyploids are formed from genomes of the same species, while allopolyploids are associated with hybridization. To eliminate interferences caused by hybridization, this study uses the autopolyploid *H. bulbosum* for comparison with its diploid ancestor.

1.3 General information of *Hordeum bulbosum*

H. bulbosum originates from the west of Iran, and is distributed worldwide (Hamed & Hojjatolah, 2011). It is prominent as a wild grass species and is a close relative of cultivated barley (*Hordeum vulgare L.*). Presently, barley (*Hordeum vulgare L.*) is a significant food source for humans. There are up to 30 additional wild *Hordeum* species, but only *H. bulbosum* can easily crossbreed with *H. vulgare*, and is the only member of the secondary gene pool (Nair, 2019). As a representative of the barley gene pool, *H. bulbosum* is a valuable source for richening barley gene diversity, especially for improving the resistance and tolerance of barley to the pathogens in its environment (Walther et al., 2000, Ruge et al., 2003). It is a perennial and obligate outbreeder (self-incompatible), and is widely distributed from the Mediterranean and extended to Afghanistan and Tajikistan in Central Asia (von Bothmer et al., 1995). It is caespitose,

and its culms are 50 to 100 cm long with swollen features at the base (Culm-internodes glaucous). The leaf-sheath auricles of *H. bulbosum* are falcate. The ligule has a ciliate membrane. The leaf blades are 10 to 20 cm long and 3 to 7 mm wide with scabrous, glabrous or pilose surfaces. The inflorescence is composed of racemes. Rhachis is fragile at the nodes, with spikelet packing broadside to it. Its internodes are oblong. The spikelets of *H. bulbosum* are well-developed (von Bothmer et al., 1995).

The autopolyploid *H. bulbosum* and the diploid *H. bulbosum* can be found worldwide. In the Western world, the distribution of *H. bulbosum* is mostly as a diploid, while tetraploids predominate in Asia (Devaux, 2003).

1.4 Mechanism of Barley response to salinity

To avoid damage caused by high saline concentration in the soil, plants must have the ability to sense salt stress, transduce stress signals and regulate their metabolism. Plants have developed the capacity to sense both hyperosmotic component and the ionic Na^+ component of the stress. Hyperosmotic stress may be perceived by a mechanically gated Ca^{2+} channel, as plant hyperosmotic sensors are closely associated with Ca^{2+} channels (Zhao et al., 2020). A recent study has presented that glycosyl inositol phosphorylceramide (GIPC) sphingolipids may function as salt stress sensors in plants (Jiang et al., 2019).

Plants overcome salt stress by regulating osmotic pressure. When solute in the soil lowers the external water potential and compromises the plants' ability to take up water,

the plants increase their uptake of inorganic ions, and their growth resumes (Zhao et al., 2020).

Some halophytes have formed special structures. They accumulate excessive Na^+ in their vacuoles, making them adapt to high salinity. For instance, seeds of salt-tolerant barley absorb water and minimize K^+ losses by taking up sodium during germination under salinity stress (Zhang et al., 2010).

1.5 Transcriptional regulation and gene expression

The two major methods for plants to cope with salt stress are facilitating the production of protective metabolites and controlling the expression of downstream genes (Agarwal et al., 2013). Hence, the induction of various genes falls into two broad groups: single function genes and regulatory genes (Mwando et al., 2019). This study focuses on single function genes.

The synthesis of transporters or channel proteins, and the release of protective metabolites such as osmolytes, lipid biosynthesis genes and antioxidative enzymes, are promoted by the single function gene (Mwando et al., 2019). Potassium ion (K^+) is the major cationic inorganic nutrient that is utilized for osmotic regulation, cell growth and enzyme activation in plants. K^+ transport is regulated by single function genes *HvHAK1* and *HvAKT1* (Fulgenzi et al., 2008; Mwando et al., 2019). Some studies showed that the mutations in the barley *HvHAK1* potassium transporter lead to an improved K^+-- nutrition and enhanced resistance to salt stress (Mangano et al., 2008). In barley, the Na^+/H^+ antiporter in vacuolar membranes transports Na^+ from the cytoplasm to the vacuoles, and stimulates osmotic balancing in the barley cell with its environment by accumulating Na^+

in the vacuoles. The vacuolar Na^+/H^+ antiporter is regulated by *HvNHX1* (Fukuda et al., 2004), *HvNHX3* (Roslyakova et al., 2009) and *HvNHX4* (Mwando et al., 2019). The expression of these genes increases salt stress tolerance in the roots and leaves of barley. Sodium ions are transported and compartmentalized in the vacuoles through two types of H^+ pumps (V-ATPase and H+ -ATPase) and vacuolar pyrophosphatase, all regulated by *HVA/68* and *HvHVP1* (Zhu, 2002).

1.6 Goal of this study

Several existing studies demonstrated that autopolyploids have a better capability to tolerate abiotic stress than diploid ancestors; however, the molecular mechanism remains unclear (Sun et al., 2019). A recent study discussed that microRNAs are related to better salt tolerance in autopolyploid (Liu & Sun, 2017). However, the function of single genes in autopolyploid for salt tolerance is vague.

The primary objective of this study is to evaluate if polyploidization results in stress response gene expression change by using autopolyploid and diploid *H. bulbosum* as a case study. The goal is to explore the differences in gene expression between autopolyploid and diploid *H. bulbosum* by comparing their single function genes. The hypothesis is that salt response genes have different expressions in autopolyploid and diploid *H. bulbosum*, and that polyploidization results in gene expression change.

Materials and methods

2.1 Plant growth

The cultivation of the accessions of *Hordeum bulbosum* was conducted by Maggie Scarrow. Four diploid individual and four tetraploid individual *Hordeum bulbosum* (wild barley) were utilized in this study. There were six pots – three plants in each pot – from each accession of *H. bulbosum*. These pots were divided into two groups: three pots comprised the control group, and the other three were the salt treatment group. The plant cultivation duration was 28 days. Day 1-14 was the treatment period; the plants in the salt treatment group were treated once with 300ml 250mM NaCl solution, while the control group was incubated with 300ml H_2O. Day 15-28 was the recovery period; all groups were made to grow in H_2O. The pots were watered every two days continuously for 28 days. The pots were grown inside a greenhouse situated on the campus of Saint Mary's University in Halifax, Canada (latitude 44.636325N, longitude -63.594416W) from May 4th, 2020 to June 1st, 2020. The temperatures were set at 25/18°C (day/night), and were controlled by heating and ventilation. Apart from natural solar radiation, the plants received additional lighting provided by 600W SON-T Green Power lamps (Philips, Belgium). The minimum photosynthetic photon flux density (PPFD) at bench height from the additional lighting was 300 μmol $m^{-2}s^{-1}$. The photoperiod of the said lighting was set as 18/6 hours (day/night).

2.2 RNA isolation

RNA isolation was performed by Maggie Scarrow. The three newly sprouted leaves from each individual (young leaves) were obtained for RNA isolation on June 2nd. 150 mg of leaves from each individual were measured using a high-precision balance scale (Mettler Toledo AE163) and were immediately frozen in liquid nitrogen before grinding using a mortar and pestle. Each sample was lysed by adding 2 ml of TRlzol, 0.4 ml chloroform and centrifuge (10,000 rpm for 5 mins and 10,000 rpm for 15 mins). The top clear layer was then transferred for RNA isolation. RNA was isolated by adding isopropanol (0.5 ml per 1 ml TRlzol) and applying centrifugation at 10,000 rpm for 10 mins. The isolated RNA was washed with 75% EtOH (1 ml per 1 ml TRlzol) then centrifuged at 7,500 rpm for 5 mins. The RNA was resuspended in 50 µl RNase-free water after discarding the supernatant from the RNA wash. The RNA samples were then stored at -80°C immediately after applied a 60°C, 15 mins water bath.

2.3 RNA quality determination

The quality and purity of the RNA template was measured prior to analysis of the gene expression. Electrophoresis to identify the RNA's integrity and overall quality was performed by Maggie Scarrow and Zhilian Cheng. 2 ul of isolated total RNA was used with Nano-drop to determine the concentration and purity. Quality was detected on 1.0% bleach gels (1.5g agarose, 1.22ml bleach and 148.78ml 1x TAE Buffer) (Bio-Basic inc, CA). Good-quality RNA that was not degraded was used in the succeeding steps, while degraded samples were discarded.

2.4 cDNA synthesis and quality determination

Compared to RNA, cDNA has the advantages of storage resistance and more stability. Thus, the RNA was synthesized into cDNA before qPCR. The iscript cDNA synthesis kit (Bio-Rad Inc., US) was used to synthesize cDNA. The reaction protocol followed its manual, and the complete reaction mix was incubated in Bio-Rad T100 Thermal cycler (Bio-Rad Inc., US) using the following protocol setup: (1) priming for 5 min at 25 °C; (2) reverse transcription for 20 min at 46 °C; and, (3) RT inactivation for 1 min at 95 °C.

To confirm the successful synthesis of cDNA, cDNA was electrophoresed in 1.0% agarose gel with 1X TAE, stained with ethidium bromide, and visualized on gel documentation system. Finally, the synthesized cDNA was diluted to 1/75 with nuclease-free water.

2.5 Gene expression analysis

Gene expression of *H. bulbosum* was studied by real-time RT-PCR technique using gene-specific primers by Zhilan Cheng. Gene oligo primers (ordered from Bio Basic Canada Inc, ON, Canada) (Table 1) of K+ transporters (HvHAK1 and HvAKT1), Na+/H+ antiporters (HvNHX1, HvNHX3, and HvNHX4), vacuolar H+ -ATPase (HvHVA/68) and vacuolar H inorganic pyrophosphatase (HvHVP1) were used for cDNA amplification. The cDNA and their corresponding primers were mixed with iTaq

universal SYBR® Green reaction mix (Bio-Rad inc, US) – following manufacture's instruction. The iTaq universal SYBR® Green reaction mix amplificated cDNA with specific primer. The reaction was performed in the CFX96 Touch Deep Well Real-Time PCR Detection System as per the manual of CFX Maestro Software (Bio-Rad inc, US). The protocols set up for qPCR were stage 1, 50°C for 2 mins; stage 2, 95°C for 10 mins; stage 3, 95°C for 15 s; stage 4, 56°C for 30 s; and, stage 5, 60°C for 1 min. There were 40 cycles from stages 3 to 5.

Each sample was repeated thrice in a Bio-Rad 96 PCR plate, sealed with a MicroAmp Optical Adhesive Film Kit (Thermo Fisher Scientific, US). Real-time PCR data were analyzed by comparison to the normalized gene expression (ΔΔCq) level of target gene using the CFX Maestro™ software (Bio Rad, US), while Actin was applied as the control primer.

2.6 Electrophoresis gel verification product

To determine whether the primers bind to a specific sequence as designed, the size of the product can be roughly measured by using 1.0% agarose gel electrophoresis (Bio-Basic inc, CA) with 1X TAE electrophoresis. 25-500bp Low Range DNA Marker A (Bio-Basic inc, CA) was used as size reference to compare with the qPCR product to get a product size range. Then primer blast (National Center for Biotechnology Information, US) was used to calculate the theoretical product size. If the actual product size matches the theoretical product size, then the specific primer is considered to be correctly bound with the designed fragment.

Table 1. List of Primers for gene expression analysis by RT-PCR

Gene name	Gene function	Sense Primer	Anti sense primer	Prouduct size (bp)
HvACTIN	Housekeeping	5'- CGTGTTGGATTCTGGTGATG -3'	5'- AGCCACATATGCGAGCTTCT -3'	208
HvHAK1	K^+ transport	5'- TGGTGATAGGCGATGGAAC-3'	5'- GAGAGACCCATCCACTCTTC -3'	324
HvHKT1	Na^+/K^+ -symport	5'- ACTTTGCCGTGATCCATATC -3'	5'- CTGGATTCTTGATCATGAGC -3'	327
HvHVA/68	H^+ pumps	5'- AGTACAGTCTGCAGGATACTG -3'	5'- GAGTACTTTGAGAGCGCCTG -3'	350
HvHVP1	H^+ pumps	5'- TACCCACTCCTAATCAGCTC -3'	5'- GCTTGTGTAGTACTCTGTAATG -3'	306
HvNHX1	Na^+/H^+ antiporter	5'- TCCAGGTGAAGAAGAAGCAG -3'	5'- GTGGCATCGTTCACAACAC -3'	274
HvNHX2	Na^+/H^+ antiporter	5'- GGCTATCTTCTCAGCAACC -3'	5'- CAAGAACGGTGCTGGTGAG -3'	227
HvNHX3	Na^+/H^+ antiporter	5'- CGATGCGACATCAGTTGTG -3'	5'- CAGGTATAGTGCGACATTAC -3'	312
HvNHX4	Na^+/H^+ antiporter	5'- AGGAGTAATGCAGCAGGAG -3'	5'- GTTTCAGCCAAAGATGATAGC -3'	344
HvAKT1	K^+ transport	5'- GGGTTCGATGTGCAAAGCTC -3'	5'- CTCCCGTATGTTCTCCGCATG -3'	236

Result

3.1 Product analysis

The size of the qPCR products of the target genes were between 200 bp and 350 bp (Figures 1 & 2). The markers were used as the standard, and the qPCR product sizes between 200 and 300 bp were Actin, *HvAKT1*, *HvHVA/68*, *HcNHX1* and *HvNHX2*. The product sizes between 300 and 400 bp were *HvHVP1*, *HvHAK1*, *HvNHX3* and *HvNHX4*. In melt peak diagrams, most of the curves were single peaks, while a few had small peaks when encountering the largest peak (Figures 3& 4).

3.2 Different ploidy on the expression of salt-stressed genes

In highly saline environments, some autopolyploid exhibit better adaptability. To compare and study the differences in adaptation caused by ploidy, this experiment analyzed the expression of specific salt-related genes of *H. bulbosum* in different ploidy. Diploid and autopolyploid under the same treatment were evaluated for different target genes.

Due to variability among individuals, some accessions have very low relative normalized expression (RNE) levels. The RNE levels of genes *HvHAK1* (Figure 5) and *HvHVA/68* in autopolyploid (Figure 7) are higher than that in diploid. For genes *HvHKT1* (Figure 6), *HvNHX1* (Figure 10), *HvNHX2* (Figure 11) and *HvNHX4* (Figure 13), the expression level in autopolyploids and diploid is not obvious difference. The expression

of genes *HvHVP1* (Figure 8), *HvAKT1* (Figure 9) and *HvNHX3* (Figure 12) in diploids was slightly greater than in autopolyploid.

3.3 Treatments on salt related gene expression

To study the adaptability of *H. bulbosum* of the same ploidy in a saline environment, both diploid and autopolyploid were cultivated under salt stress and no salt stress conditions. The subjects under different treatments were then compared.

The expression of the target gene greatly varies. For genes *HvHAK1* (Figure 5), *HvHVA/68* (Figure 7), *HvNHX1* (Figure 10), *HvNHX3* (Figure 12) and *HvNHX4* (Figure 13), the RNE levels of tetraploid individual 2 (treated) and diploid accession # 1 (treated) were significantly higher than those of the remaining individuals. Diploid accession # 2 (treated) also had a high RNE level for target gene *HvAKT1* (Figure 9).

However, for target gene *HvHKT1* (Figure 6), except for diploid individual 1 (untreated) and tetraploid individual 2 (untreated), which manifested slightly higher expressions, the other individuals had relatively average RNE levels. The RNE level of diploid individual 2 (untreated) was the highest, followed by tetraploid individual 2 (untreated) for *HvHVP1* (Figure 8). The RNE levels of genes *HvHKT1* (Figure 6), *HvHVP1* (Figure 8) and *HvAKT1* (Figure 9) in tetraploid individual 2 (treated) were close to zero.

To summarize, two individuals (D1_treated and T2_treated) with saline treatment had significantly higher RNE levels for most salt-related genes (*HvHAK1*, *HvHVA/68*,

HvNHX1, *HvNHX3*, *HvNHX4* and *HvAKT1* for the diploid individual, and *HvNHX2* for the tetraploid individual) than in other individuals. However, in other genes (*HvHKT1* and *HvHVP1*), this trend did not exist.

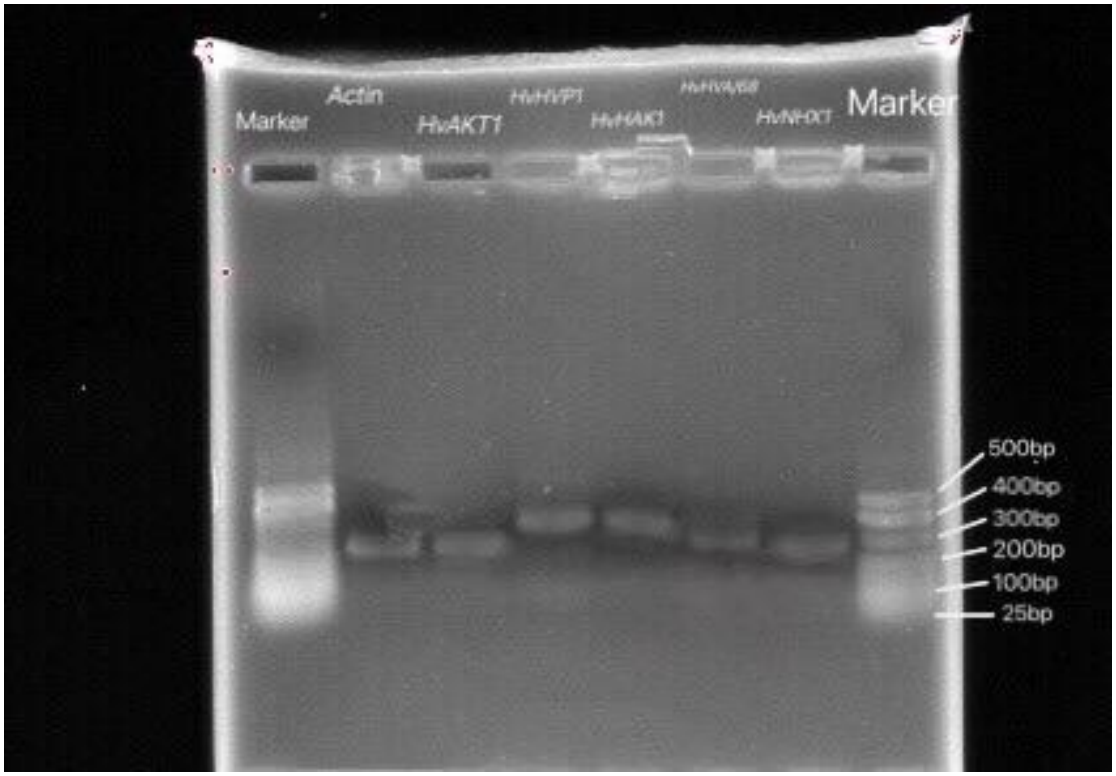

Figure 1. Agarose gel electrophoresis image of qPCR products with target gene specific primer. DNA size marker in column 1 &8, Actin on column 2, *HvAKT1*in column 3, *HvHVP1* in column 4, *HvHAK1* in column 5, *HvHVA/68* n column 6, *HvNHX1* in column 7.

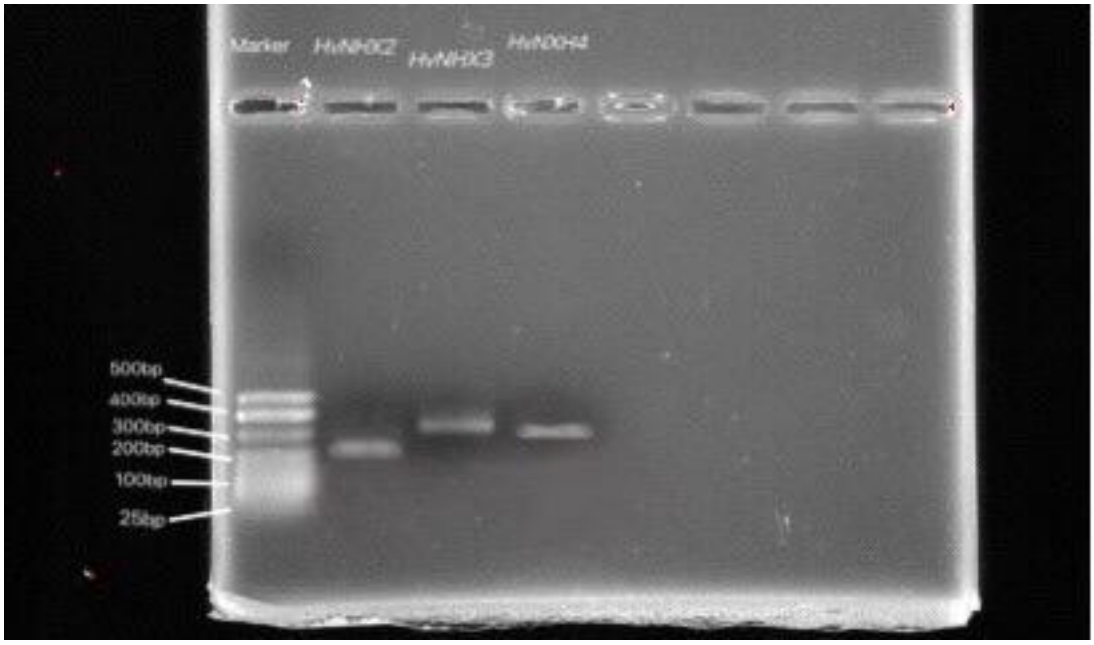

Figure 2. Agarose gel electrophoresis image of qPCR products with target gene specific primer. DNA size marker in column 1, *HvNHX2* in column 2, *HvNHX3* in column 3, *HvNXH4* in column 4.

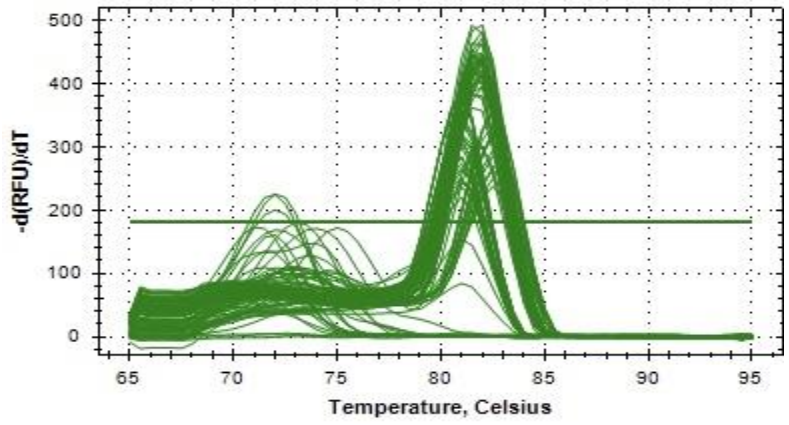

Figure 3. The melt peak of Tm analysis of the negative value of the change in relative fluorescence unit (RFU) over the change in temperature (−dRFU/dT) versus temperature (degrees Celsius) for housekeeping gene (Actin) and target gene *HvAKT1*, *HvHKT1* and *HvHVP1*.

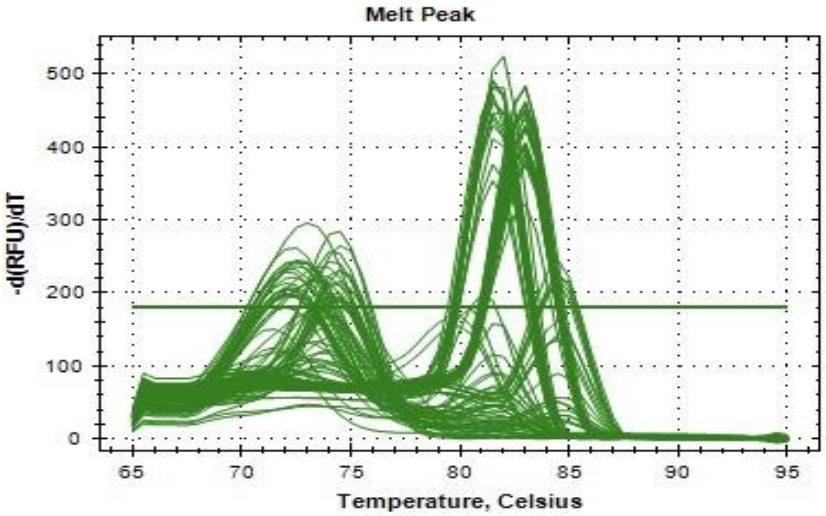

Figure 4. The melt peak of Tm analysis of the negative value of the change in relative fluorescence unit (RFU) over the change in temperature (−dRFU/dT) versus temperature (degrees Celsius) for housekeeping gene (Actin) and target gene *HvHAK1, HvHVA/68* and *HvNHX1*.

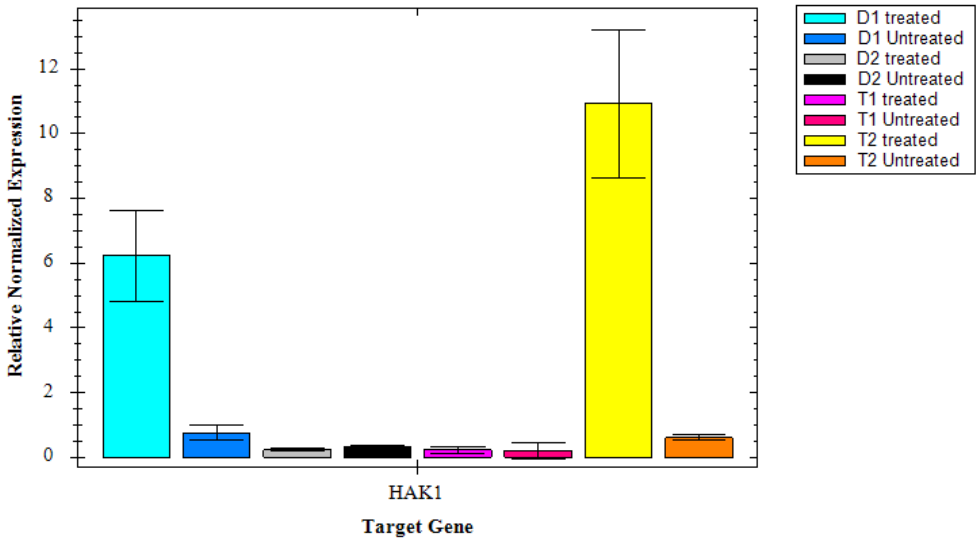

Figure 5. The Bar chart of relative normalized expression of target gene *HvHAK1* for different ploidy (diploid and tetraploid), individual and treatment (Salt-treated and control) with standard error of the mean expression. D1&D2 refer to diploid individual 1 and diploid individual 2, T1&T2 refer to tetraploid individual 1 and tetraploid individual 2. There are three replicates for each sample. Relative normalized expression (ΔΔCq) calculated using Actin as housekeeping gene.

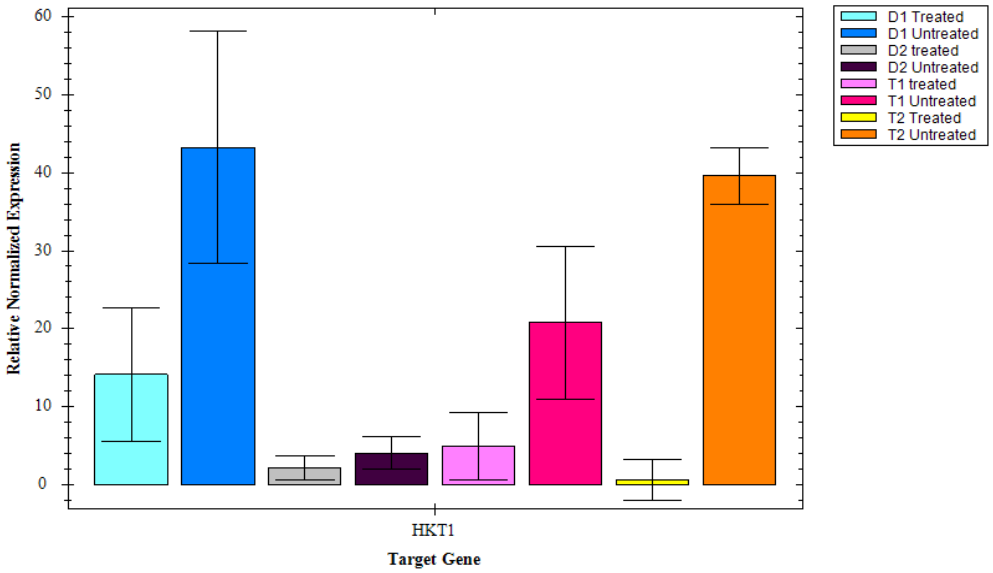

Figure 6. The Bar chart of relative normalized expression of target gene *HvHKT1* for different ploidy (diploid and tetraploid), individual and treatment (Salt-treated and control) with standard error of the mean expression. D1&D2 refer to diploid individual 1 and diploid individual 2, T1&T2 refer to tetraploid individual 1 and tetraploid individual 2. There are three replicates for each sample. Relative normalized expression (ΔΔCq) calculated using Actin as housekeeping gene.

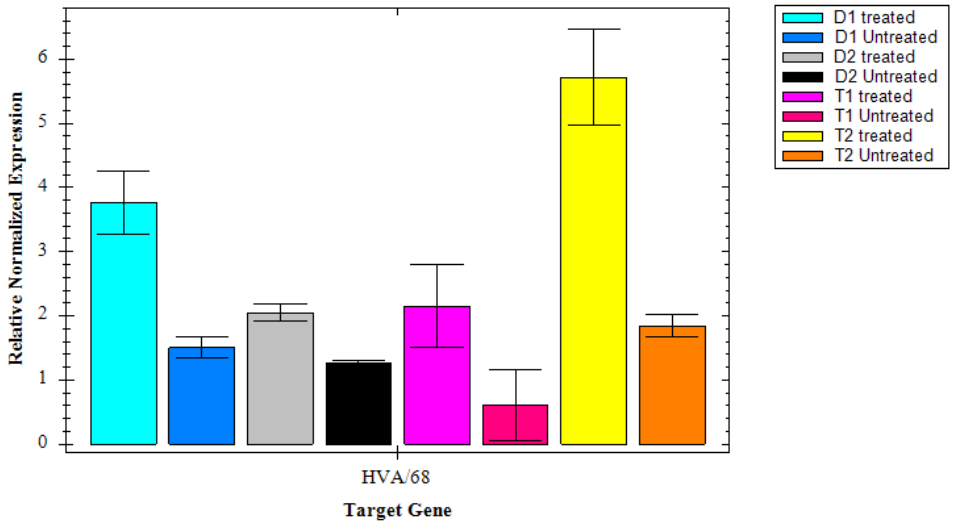

Figure 7. The Bar chart of relative normalized expression of target gene *HvHVA/68* for different ploidy (diploid and tetraploid), individual and treatment (Salt-treated and control) with standard error of the mean expression. D1&D2 refer to diploid individual 1 and diploid individual 2, T1&T2 refer to tetraploid individual 1 and tetraploid individual 2. There are three replicates for each sample. Relative normalized expression (ΔΔCq) calculated using Actin as housekeeping gene.

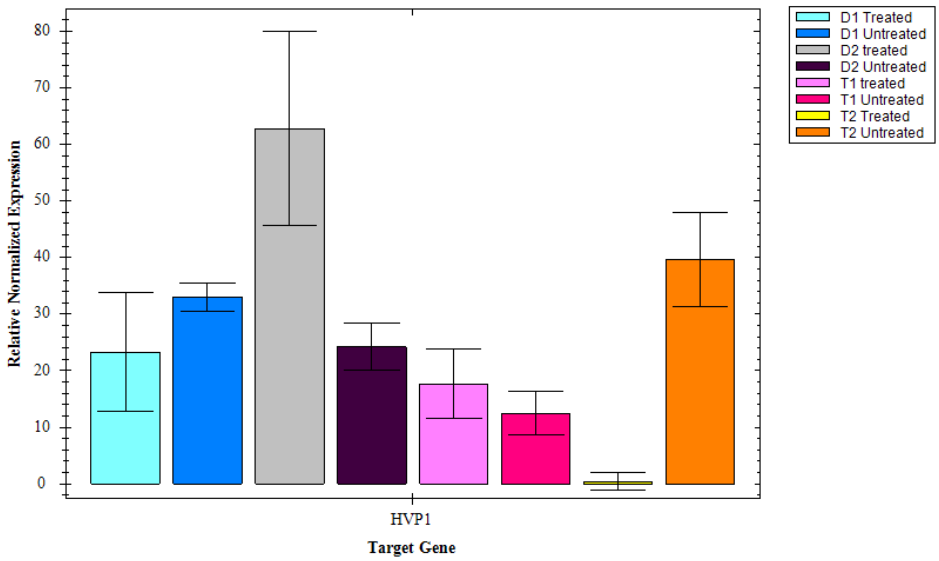

Figure 8. The Bar chart of relative normalized expression of target gene *HvHVP1* for different ploidy (diploid and tetraploid), individual and treatment (Salt-treated and control) with standard error of the mean expression. D1&D2 refer to diploid individual 1 and diploid individual 2, T1&T2 refer to tetraploid individual 1 and tetraploid individual 2. There are three replicates for each sample. Relative normalized expression (ΔΔCq) calculated using Actin as housekeeping gene.

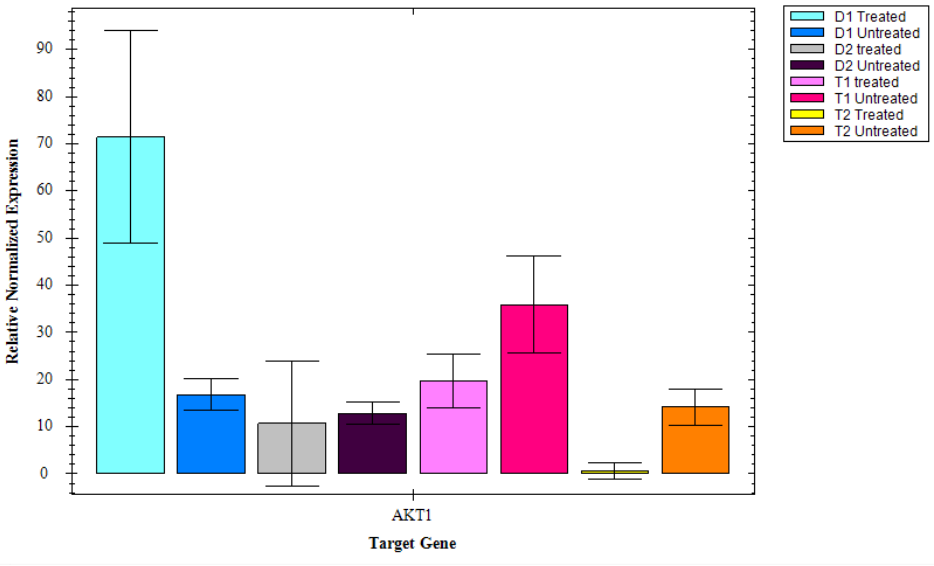

Figure 9. The Bar chart of relative normalized expression of target gene *HvAKT1* for different ploidy (diploid and tetraploid), individual and treatment (Salt-treated and control) with standard error of the mean expression. D1&D2 refer to diploid individual 1 and diploid individual 2, T1&T2 refer to tetraploid individual 1 and tetraploid individual 2. There are three replicates for each sample. Relative normalized expression (ΔΔCq) calculated using Actin as housekeeping gene.

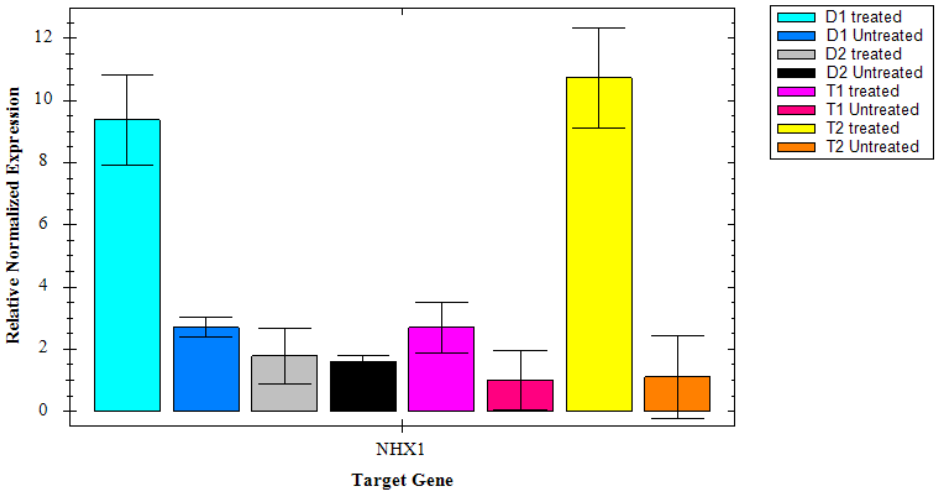

Figure 10. The Bar chart of relative normalized expression of target gene *HvNHX1* for different ploidy (diploid and tetraploid), individual and treatment (Salt-treated and control) with standard error of the mean expression. D1&D2 refer to diploid individual 1 and diploid individual 2, T1&T2 refer to tetraploid individual 1 and tetraploid individual 2. There are three replicates for each sample. Relative normalized expression (ΔΔCq) calculated using Actin as housekeeping gene.

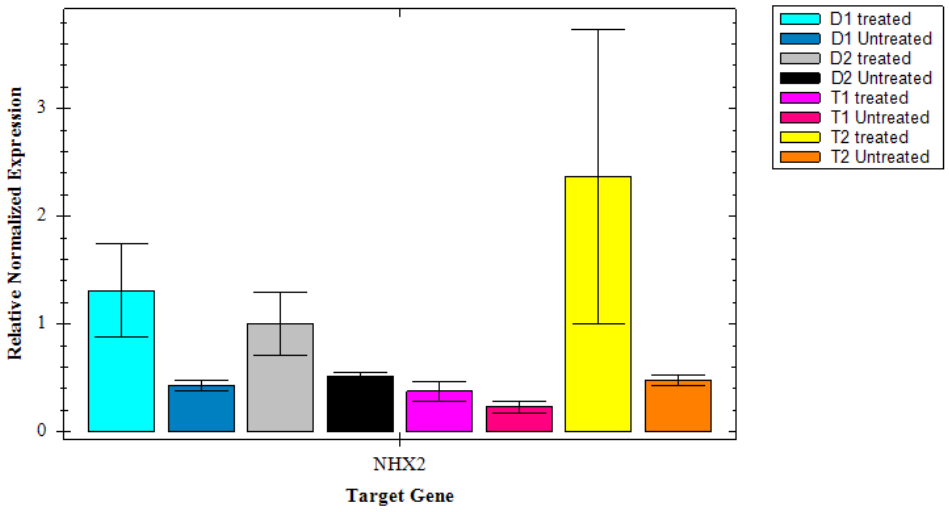

Figure 11. The Bar chart of relative normalized expression of target gene *HvNHX2* for different ploidy (diploid and tetraploid), individual and treatment (Salt-treated and control) with standard error of the mean expression. D1&D2 refer to diploid individual 1 and diploid individual 2, T1&T2 refer to tetraploid individual 1 and tetraploid individual 2. There are three replicates for each sample. Relative normalized expression (ΔΔCq) calculated using Actin as housekeeping gene.

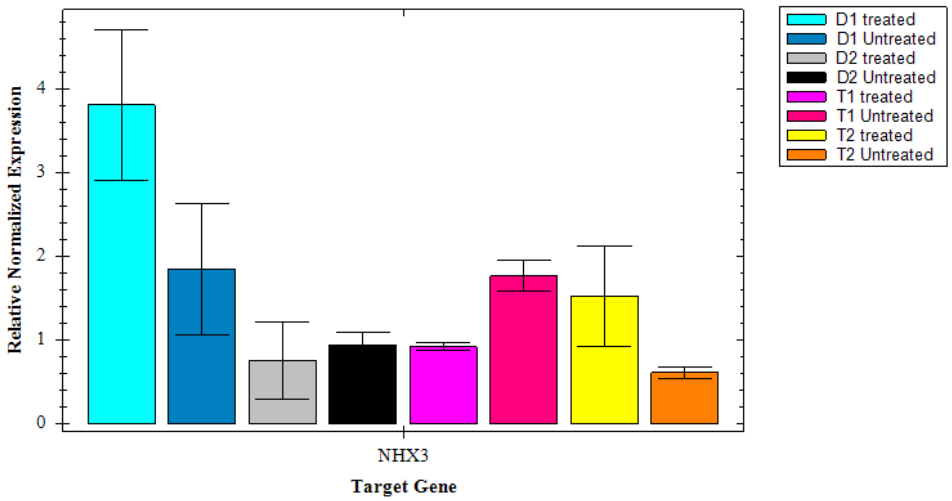

Figure 12. The Bar chart of relative normalized expression of target gene *HvNHX3* for different ploidy (diploid and tetraploid), individual and treatment (Salt-treated and control) with standard error of the mean expression. D1&D2 refer to diploid individual 1 and diploid individual 2, T1&T2 refer to tetraploid individual 1 and tetraploid individual 2. There are three replicates for each sample. Relative normalized expression (ΔΔCq) calculated using Actin as housekeeping gene.

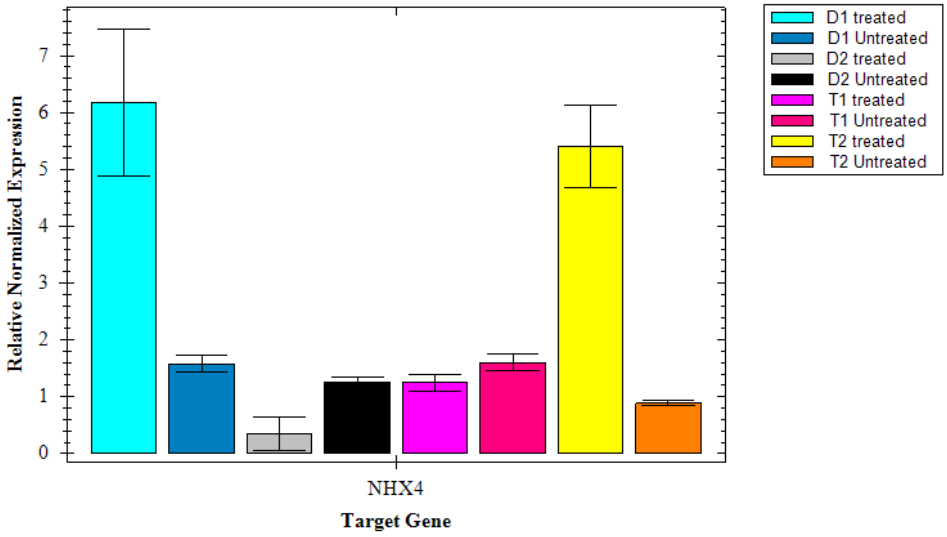

Figure 13. The Bar chart of relative normalized expression of target gene *HvNHX1* for different ploidy (diploid and tetraploid), individual and treatment (Salt-treated and control) with standard error of the mean expression. D1&D2 refer to diploid individual 1 and diploid individual 2, T1&T2 refer to tetraploid individual 1 and tetraploid individual 2. There are three replicates for each sample. Relative normalized expression (ΔΔCq) calculated using Actin as housekeeping gene.

Discussion

4.1 Correct qPCR product

To ensure that the primer would be bound to the designed sequence, the qPCR products were analyzed by agarose gel electrophoresis. The theoretical product sizes as calculated by the primer blast were: Actin, 208 bp; *HvAKT1*, 236 bp; *HvHVP1*, 306 bp; *HvHAK1*, 324 bp; *HvHVA/68*, 250 bp; *HvNHX1*, 274 bp; *HvNHX2*, 227 bp; *HvNHX3*, 312 bp; and *HvNHX4*, 344 bp. It can be deduced from the results that the product sizes fall within the range that matches primer blast analysis.

In melt peak diagrams, most of the curves were single peaks, which proved the appearance of a single product. However, there were a few that had small peaks when encountering the largest peak. Generally, if there are two peaks in the melting curve, it indicates that there are two amplicons, and no single product is produced. However, this is not the case in this experiment. The products were analyzed by electrophoresis gel, and the results showed that the curves generated a single amplicon, validating a single PCR product. A possible reason why small peaks were formed was perhaps due to the high primer concentration, as it might be a non-specific primer binding and primer dimer formation.

4.2 There is no consistent expression change for all target genes between diploid and autotetraploid.

Polyploidization is common among plants. The doubling of the genome caused by polyploidization is a driving force for plant evolution and is the primary way for plants to adapt to their natural environment. When the plant genome is doubled, theoretically all of the genes are doubled. Will these genes remain the same and just multiply by two? In this experiment, the salt resistance-related single gene expressions of diploid and autotetraploid *H. bulbosum* were tested. Autotetraploid *H. bulbosum* had the same chromosomes as diploid *H. bulbosum*. If a single gene related to salt resistance is maintained the same in autotetraploid except for doubling the ancestral chromosome complement, theoretically, the gene expression in autotetraploid should be double that of diploid.

From the experiment outcomes, among the target genes monitored in this experiment, polyploidization did not result to constant changes in the expression of salt-tolerant genes. Except for target genes *HvHAK1* and *HvHVA/68*, the expression in autotetraploid was slightly higher than that in diploid; even those genes did not reach double the RNE of diploid. Among the other target genes, none of the autotetraploid individuals presented higher expression levels than diploid; for target genes *HvHVP1*, *HvAKT1* and *HvNHX3*, the gene expression of autopolyploid was lower than that of diploid. Based on this outcome, it is presumed that polyploidization does not result to constant changes in gene expression.

Why does the duplicate gene caused by gene doubling in autotetraploid not continue to be expressed as in diploid? A possible reason is that, during the evolution of polyploid, some duplicated genes are lost, and others may be retained. A study (Liu et al., 2015) on soybeans revealed the loss and retention of duplicated genes formed by plant polyploidization.

For major genetic changes like polyploidization and whole genome duplications (WGDs), most duplicate gene copies will be lost over time (Lynch & Conery, 2000; Karev et al., 2004), while few duplicates will remain. The repetitive genes that are reserved tend to be certain functional genes (Geiser et al., 2016; Li et al., 2016). In the evolution process, the mechanisms of deletion and retention of duplicate genes caused by polyploidy are still unclear.

4.3 Treated Individuals tend to have higher gene expressions than untreated individuals

Plants are indispensable in the environment. They rely on and influence each other. The impact of environmental changes on plants has always been a hotspot in biological and agricultural research. In the past, the development of salt-tolerant crops did not attain much success, which might be due to the fact that, in addition to genetic factors, the growth of crops was also cumulatively influenced by various environmental stresses (Jamil et al., 2011). With the increase in soil salinization, in order to examine the adaptation mechanisms of the *H. bulbosum* gene under salt stress, both diploid and tetraploid barley were cultivated without salt and with salt conditions.

The gene expression levels of plants were not static. As certain environments put pressure on the individual, related genes may be upregulated to help plants better survive. Under highly saline specific conditions, some cellular responses are mediated by osmotic potential changes in the initial stage of salt stress (Ueda et al., 2004), which can be the cause of increases in the expression of a specific gene. Some existing studies have conducted related experiments. Toranj et al. (2020) studied the gene expression of salinity stress in rose madder (*Rubia tinctorum*). They found that the expression of the vacuolar H^+-pyrophosphatase pump (AVP) and tonoplast Na^+/H^+ antiporters (NHX) were up-regulated under salt stress condition.

Fukuda et al. (2004) tested the expression levels of vacuum H^+-pyrophosphatase in barley, H^+-ATPase subunit A and Na^+/H^+ antiporter-related genes in barley under salt stress and indicated that salt stress increased the transcription level of some related genes. They further mentioned that salt stress led to the increase in the transcription level of *HvNHX1* in barley, with the results of this study further supporting their view. However, they also concluded that the transcript levels of *HvHVP1* was increased under salt stress and coordinated with *HvNHX1* in barley roots as a response to salt and osmotic stresses. Conversely, the present experimental data were opposite to their conclusions, the results indicated that the expression of *HVP1* on tested individuals was relatively low under salt stress (Figure 8). Similar to *HvNHX1*, the expression of *HvNHX3* (Figure 12) during salt stress was increased several-fold in roots and leaves of barley seedlings in previous studies (Roslyakova et al., 2009). In this experiment, only a treated diploid had higher *HvNHX3* expression.

A study (Fulgenzi et al., 2008) suggested a regulatory effect of the ionic environment on the contribution of *AKT1* (Figure 9) and *HAK1* (Figure 1) transporters. They believe that a high-concentration ion environment will increase the expression of *AKT1*, but the effect of ion concentration on the *HAK1* transporter is still unknown. In this study, the expression of *HAK1* tends to increase in a high ion concentration environment, this may suggest that the environmental ion concentration will affect the expression of *HAK1*. From the result, only small part accessories have increased the expression of *AKT1* under high ion concentration. Qiu et al (2011) evaluated salinity tolerance and analysis of the allelic function of *HvHKT1* in Tibetan wild barley. They pointed out that the increased expression of *HvHKT1* contributes to the salt tolerance of barley. The results in the present study are not consistent with their opinion, the salt-treated accessories did not have higher *HvHKT1* expression (Figure 6).

The results presented that the expression of different genes in plants under salt stress was not constant, as there were more salt-treated individuals that showed higher expressions of salt-tolerant genes. It could be implied that different accessions collected from adverse environmental pressures could change the expressions of related genes in plants.

4.4 Limitations of this study

The two main outcomes of this study are as follows: (1) polyploidization changes the expression of salt resistance-related single gene in *H. bulbosum*, and no constant change between autotetraploid and diploid has been found; and, (2) salt-treated individuals tend

to have higher gene expressions than untreated individuals. However, from a statistical perspective, due to the small number of accessions used in this experiment, the deviation of data caused by individual variations resulted to a weak reliability of the experiment.

Similar limitations are reflected in sample type selection. In this experiment, only the new leaves of plants were used; relatively, gene expressions in other parts could be different. Especially for the rhizome of plants, it should be noted that it has the most direct contact with the soil and salt for a plant, and that the expression of salt resistance-related genes in rhizomes may be contrasting from that of leaves.

The selection of the target genes is a limitation of polyploidization analysis. There are more than one kind of gene that are related to salt resistance in barley. This experiment focuses on the analysis of single genes. Related studies (Agarwal et al., 2013) have presented that regulatory genes that control the expression of downstream genes also play a great function in salt resistance of barley. Regulatory genes like *bZIP, WRKY, AP2, NAC, C2H2* zinc finger gene, and *MYC/MYB* (myelocytomatosis/ myeloblastosis) are called transcription factors (TFs) (Mwando et al., 2019). These factors are the most vital regulators of gene expression under salinity stress (Gupta & Huang, 2014). They interact with diverse cis-elements (non-coding DNA that regulates the transcription of neighboring genes) in the promoter regions of various downstream genes and modify their expressions. There are several different types and functions of TFs. For instance, *DREB* is a dehydration-related TF that promotes the gene expression of barley for salt tolerance (Mwando et al., 2019).

4.5 Future research

As discussed, this study has limitations. Expanding the number and types of samples in future research can improve the reliability of the results. In addition, salt stress-related regulated gene expressions may be tested in future studies to evaluate if polyploidization results in regulatory gene expressions change under salt stress, and if such changes correspond well with what has been detected for function genes.

Conclusion

The results indicated that polyploidization can result in gene expression changes, whether an increase or a decrease based on each gene. Duplicate genes caused by polyploidization may either be lost or retained. Individuals treated with salt stress are more likely to have higher or lower expressions of single genes related to salt resistance depending on the function of each gene in the metabolic pathway.

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