Expression analysis of *HvSAP* genes encoding Stress-Associated Proteins in response to salinity and drought stresses in barley (*Hordeum vulgare* L.)

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Declaration:

I certify that this thesis does not contain material which has been accepted for the award of any degree or diploma; and to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text of this thesis.

Results presented within this thesis were used in the preparation of a manuscript submitted to BMC plant biology, (IF) impact factor = 3.670, on 30 of October 2019 which is awaiting publication: (1) SUPP-D-19-00258. Baidyussen A., Aldammas M., Kurishbayev A., Myrzabaeva M., Zhubatkanov A., Sereda G., Porkhun R., Sereda S., Jatayev S., Langridge P., Schramm C., Jenkins C., Soole K., Shavrukov Y. Identification, gene expression and genetic polymorphism of Zinc finger A20/AN1 stress-associated genes, HvSAP, in salt stressed barley from Kazakhstan.

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Abbreviations:

AUK	Auksinyai
%	Percent
ANOVA	Analysis of variance
AST	Astana
Bb	Base pairs
cDNA	Complementary
DW	Dry weight
F	Forward
FW	Fresh weight
GP	Golden Promise
GRA	Granal
H_2O	Water
Hv	Hordeum vulgare L. (barley)
Kb	Kilobase Pair
mL/µL	Millilitres/Microlitres
mM	Milli-Molar
MW	Molecular Weight
NaCl	Sodium chloride
NAT	Natali
^oC	Celsius
PCR	Polymerase chain reaction
PR	Pamyaty Raisi
qPCR	Quantitative Polymerase Chain Reaction
R	Reverse
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse transcription quantitative real-time polymerase chain reaction
SAP	Stress association protein
TFs	Transcription factor
TNF	Tumor necrosis factor
TZ	Tzelinniy Golozerniy
TZE	Tzelinniy2005
ZF	Zinc finger

Abstract

Abiotic stresses, such as drought and salt, affect the growth and productivity of crop plants and are serious threats to agriculture. Members of the Stress Associated Protein (SAP) family contain A20, AN1, or both A20/AN1 zinc finger domains at the N- or C-terminus and have been shown to be strongly stress-responsive in many plants, however little is known about this gene family in barley. In this study, 17 *HvSAP* genes were identified with strong homology to known *OsSAP* genes in rice. Five novel genes, *HvSAP5*, *HvSAP6*, *HvSAP11*, *HvSAP12* and *HvSAP15*, were described and functionally characterized in this study. The regulation of gene expression is a key factor in plant adaptation to stress. The aim of this study was to identify all *HvSAP* genes in barley (*Hordeum vulgare* L.), and to analyse the expression of selected genes in response to salinity, drought and to combined salt and drought stress in barley plants. The five studied *HvSAP* genes showed a great diversity in response to abiotic stresses in barley cultivars from Kazakhstan. Under salt stress, *HvSAP5*, *HvSAP6*, *HvSAP12* and *HvSAP15* genes were highly expressed in leaves of all studied barley cultivars. The *HvSAP11* gene was mostly non-responsive to salinity. In contrast, exposure to drought caused significant up-regulation of *HvSAP6* and *HvSAP11* genes in all studied barley cultivars, while expression of the remaining genes, *HvSAP5*, *HvSAP12* and *HvSAP15*, were genotype-dependent. The combination of both salinity and drought stress did not show a simple additive effect, or sum of responses to the two stresses, but rather quite different responses. Only the *HvSAP6* gene was highly expressed in all barley accessions and across the three categories of stress applied. Most, but not all, genotypes had significant up-regulation of *HvSAP5*, *HvSAP11* and *HvSAP15* gene expression in response to combined stress, while the expression of *HvSAP12* was so variable that it was not possible to make any conclusions about the response of this gene,

Chapter 1. Literature review

1.1. Introduction and background to the study

Like all other kinds of living organisms, plants experience various environmental stress factors that affect their growth and survival (Park et al., 2018). Abiotic stress refers to any environmental condition that affects the ability of plants to develop, grow, and produce grain, fruit and vegetables below optimal levels (Rahnama et al.2010; Quados, 2011).

Modern agriculture faces the challenge of sustainably feeding an ever-increasing population using limited arable land (FAO, 2017). This requires consistently high agricultural yields. So, it is of paramount importance to overcome abiotic stresses, which cause an estimated 50% loss in crop yields worldwide (Boyer, 1982; Bray, *et al*., 2000; Dixit, 2011). Environmental stress can also reduce about 75% of plant yield potential (Mousavi, *et al*., 2016).

Deforestation and global warming have resulted in rainfall reduction, and an increase in average temperature (Spracklen, *et al*., 2012). This results in an increase in the duration, intensity and frequency of heat stress and drought. Drought, which is characterized by the absence of sufficient soil moisture, occurs during periods of reduced rainfall and water scarcity (Jaleel, *et al*., 2009). Soil moisture is important to counteract the water loss due to transpiration, and to prevent negative water balance, desiccation, shrivelling, withering of plants, and a consequent reduction in photosynthetic output and agricultural yield (Boyer, 1982; Bray, *et al*., 2000; Taiz and Zeiger, 2002). Drought is not only the major yield-limiting stress to crops, but also limits their distribution (Cruz de Carvalho, 2008).

1.2. Abiotic stress in plants

The challenge of modern agriculture to sustainably feed an ever-increasing population, despite limited arable land, requires unfailingly high productivity and output. Abiotic stresses such as drought and salinity, which are widespread global problems, are some of the biggest reasons for reduction of plant yield potential (Atkinson and Urwin, 2012).

Drought and salinity are two major abiotic stresses for plants, in many parts of the world (Atkinson and Urwin, 2012). The increase of the average temperature, due to global warming, leads to increased transpirational water loss, but deforestation has resulted in reduction in rainfall, in most places (Spracklen et al., 2012). This increases the hardness and frequency of droughts, thus negatively impacting photosynthetic output and agricultural yield (Boyer, et al., 1982; Bray, et al., 2000) (Figure 1).

1.3. Salinity

Salinity is the increased amount of water-soluble salts in the soil. Salinity has been documented to affect more than 800 million hectares of agricultural land, which corresponds to 6% of the total global land area (Munns and Tester, 2008; Rengasamy, 2010). (FAO, 2008; Rengasamy, 2010). The presence of salts in water reduces water potential. As water moves from a region of higher water potential (less salts) to a region of lower water potential (more salts), salinity hinders osmotic uptake of water by plants. Thus, more water has to be supplied to overcome salinity, to meet the same water requirement of the plant. Incorrect use of irrigation is a common cause of increased soil salinity in the world (Epstein et al., 1980). When combined with drought, the severity of the problem is accentuated.

Plant responses to water scarcity and salinity at the molecular level include the accumulation of osmolytes and expression of stress-related genes, while responses at the cellular level include closure of stomata, repression of cell growth, reduction of photosynthesis, and activation of photorespiration (Dixit, 2011). As stress is realised, responses are carried out by signal transduction at the molecular level to alter the activities of stress-inducible transcription factors (TFs) in order to tide over unfavourable conditions.

Increased evaporation of water from soil increases salinity. Similarly, an insufficient supply of water for drainage (which leaches away excess salts) due to water scarcity, also increases the salts in soil. This surplus of ions, especially those of sodium and chloride, causes soil salinity (Munns and Tester, 2008).

1.3.1. Salt stress in plants

Salt stress can change the physiological, biochemical and morphological status of the barley (Banzai et al., 2002; Benvades, 2000). A high NaCl concentration creates a major decrease in growth, affecting leaf area, fresh and dry weight of leaves and roots (Ghoulam et al., 2002).

Excess salt, particularly Na^+ and Cl^-, affect the functioning of cell membranes (Shavrukov et al., 2013). So, ions are sequestered in the vacuole by activation of the Salt Overly Sensitive (SOS) pathway (Yang et al., 2009). Other changes at the molecular level include the accumulation of osmolytes, and the expression of stress-related genes. The expression of these genes leads to stress tolerance and enables the plant to overcome the unfavourable condition.

Excess salinity can reduce the CO_2 intake of plants, limiting diffusion and impairing the process of photosynthesis. The secondary outcome of excess salinity is oxidative stress, which results from multiple stresses and causes damage to the photosynthetic machinery and other physiological processes within the plant (Negrao et al., 2016).

1.4. Drought

Drought is defined as a situation with low water availability for plants as compared with the required amount for its sustainable growth (Mueen et al., 2013). Drought hinders the growth, quality of seed and yield of crops. The effects of drought have been aggravated as a result of increased food demands for enormously growing population and limiting water resources. Occurrence and distribution of rainfall, moisture storing capacity of soil and evaporative demands are important factors that aggravate the severity of drought (Riaz et al., 2013). Climate change has increased severity of drought and high temperature resulting in yield reduction of many cereal crops such as barley, wheat and maize (Lobell and Field, 2007). Water scarcity after germination and seedling establishment can severely affect crop yield production. Water deficit results in loss of plant turgor which inhibits cell elongation and reduces photo assimilation and the availability of metabolites required for cell division.

Obstructed cell elongation and impaired mitosis leads to reduced growth, including root growth, which further limits the extent to which the plant can access scarce soil water resources. Water deficit also results in stomatal closure to minimize the water loss, which lessens Rubisco activity and in turn leads to reduced photosynthesis (Farooq et al., 2009).

1.4. 1. Drought tolerance in plants

The ability of plants to live, grow and reproduce despite a restricted supply of water or to face periodic changes in water shortage is drought tolerance (Turner, 1979). Tolerance can be achieved either by drought avoidance or dehydration tolerance (Kramer and Boyer, 1995). Different factors affect the plant response to drought stress: plant genotype, duration and severity of stress (Chaves et al., 2003), and the pattern of gene expression (Denby and Gehring, 2005), respiratory activities (Ribas-Carbo et al., 2005) and photosynthetic activity (Flaxes et al, 2004). Drought adversely affects the various stages in the barley plant's life cycle, from seed germination to plant maturity (Aspinall et al., 1964). The effects of drought on barley crop yields depends on the duration and severity of stress (Anjum et al., 2011). Barley plants can be especially susceptible during very sensitive and important stages of development, such as spike formation, anthesis and the initial stages of grain development and grain filling (Aspinall, 1965). Pollination is affected by abortion of the embryonic sac and dehydration of stigma and pollen under stress conditions. Drought can decrease the numbers of grains and spikes per plant and ultimately decrease the yield (Mamnouie et al., 2010).

1.5. *SAP* genes

Stress-associated proteins (SAPs) are a class of TFs containing the A20/AN1 zinc-finger domains that play important roles in stress response in both plants and animals (Vij and Tyagi, 2008). These include coordination of signal transduction such as in the Tumor necrosis factor-α induced human umbilical vein endothelial cells, known as NF-κB and TNF-α pathways (Dixit et al., 1990; Dixit and Dhankher, 2011). The *SAP* genes in plants are known to be induced in response to multiple abiotic environmental stresses, such as drought, heavy metals, cold, wounding, and flooding (Dixit and Dhankher, 2011).

Image removed due to copyright restriction.

Figure 1: Overview of salt- and drought-stress responses in plants. Salt and drought affect cell physiology and metabolism and, as consequence, reduce plant growth. Stress signaling is perceived by the cell, which elicits stress-signaling pathways that involve transcriptional remodeling, metabolic changes and altered hormonal activity. Bacterial activity may affect the latter. A positive stress response leads to plant tolerance to the stress, while a negative response leads to growth inhibition (Forni et al., 2017).

SAPs are transcription factors that occupy the central hub in the plant stress response, controlling tolerance to multiple environmental stress factors, and their overexpression greatly improves stress tolerance (Hilbricht et al., 2002). The function of these *SAPs* appears to be conserved in different plant species: overexpression of rice *SAPs* has been shown to improve to cold and salt stress tolerance in tobacco (Mukhopadhyay et al., 2004). *SAP* genes of *Arabidopsis*, rice, peach, alyssum, barrel clover, tomato, banana, apple and the resurrection plant species, *Selaginella lepidophylla*, have all been studied to some extent (Gimeno-Gilles et al., 2011, Solanke et al., 2009, Dansana et al., 2014). However,, barley *SAP* genes (*HvSAP*) are wholly uncharacterized. As barley exhibits superior stress tolerance, it is postulated that the expression of *HvSAP* can be associated with better tolerance to abiotic stresses and higher yield, even in suboptimal growth conditions compared to other plant species.

Since *SAP* genes share some sequence similarity among plant species, *HvSAP* have been tentatively identified using computational prediction (Giri et al., 2013). Genes encoding *SAPs* are upregulated during stress. The present study exploits this property to experimentally determine the *HvSAP* genes relevant to salinity and drought, by growing barley seedlings under those conditions, and identifying which of the tentatively identified *HvSAP* genes is upregulated in response to stresses using quantitative PCR. Experimental verification is necessary for their characterization to provide high yield even in marginal and non-cultivable lands.

The *SAP*-like genes, containing the A20/AN1 domains, induced by stress or ABA, were also discovered in rice, and overexpression was found to confer tolerance to multiple abiotic environmental stresses such as drought, heavy metals, cold, wounding, and flooding (Mukhopadhyay, et al., 2004; Dixit and Dhankher, 2011). Interestingly, the function of these

SAP genes appears to be conserved in different plants because, the overexpression of SAPs from rice have been shown to improve salt stress tolerance in tobacco (Mukhopadhyay, et al., 2004; Kanneganti and Gupta, 2008).

Following these studies, a genomic computational search of all putative A20 domain- or AN1 domain-containing proteins revealed that both domains were present together (Vij and Tyagi, 2006). These sequences were used to generate a Hidden Markov Model to predict *SAP* gene family members in rice and *Arabidopsis* genomes. The prediction provided 18 proteins in rice and 14 in *Arabidopsis*, of which rice *SAP* genes were experimentally verified using quantitative PCR (Vij and Tyagi, 2006). Similar studies were also used to identify, validate, isolate and characterize the A20/AN1 domain-containing *SAP* gene family in tomato, cotton and apple (Solanke et al., 2009, Dong et al., 2018).

Further studies of *SAP1* in rice showed that it mediated positive water-stress tolerance by modulating endogenous stress-related genes, while *SAP7* was a negative regulator (Dansana et al., 2014, Sharma et al., 2015). Interaction studies of rice SAP1 protein have revealed that it interacts with itself, as well as with a close homolog, SAP11, and other proteins such as a receptor-like cytoplasmic kinase, an aminotransferase and a pathogenesis-related protein (Giri, et al., 2011; Tyagi, et al., 2014; Kothari, et al., 2016). Rice SAP1 and SAP11 were shown to increase basal resistance to pathogens (Tyagi, et al., 2014; Kothari, et al., 2016). Transcriptomic studies were also carried out for *SAP1* and *SAP16* in rice, which showed *SAP1* was a positive regulator of water-stress tolerance, and *SAP16* was a negative regulator of photosynthesis during drought (Dansana et al., 2014, Wang et al., 2016).

Studies of *SAP* genes in *Arabidopsis* have revealed that SAP5 and SAP9 proteins were localized in the nucleus, and both function in the proteasome pathway and are induced by stress (Kang et al., 2011, Kim et al., 2015). The SAP12 protein was found to undergo redox-dependent regulation by a change in quaternary structure at 4°C and under salt stress (Ströher et al., 2009).

Studies have also characterized one *SAP* gene each from banana *MusaSAP1*, barrel clover, and the halophyte alyssum *LmSAP* (Sreedharan, et al., 2012; Gimeno-Gilles, et al., 2011; Charrier, et al., 2012; 2013; Saad, et al., 2018). The latter two *SAP* genes, *AtSAP10* and *AtSAP13*, were also expressed in transgenic tobacco where they were found to confer abiotic tolerance to heavy metals (Charrier, et al., 2012; 2013; Saad, et al., 2018). A *SAP* was also characterized from peach and was found to confer water retention during drought stress in transgenic plum (Lloret, et al., 2017). However, barley *SAP* genes (*HvSAP*) remain uncharacterized in the literature.

1.6. Barley biology and human use

Barley (*Hordeum vulgare* L.), is a globally-grown major cereal, ranking fourth, only behind wheat, rice and maize (FAO, 2017). It is one of the grains first used for cultivation nearly 10,000 years ago (Zohary and Hopf, 2000). Barley is used for human consumption in health foods, as well as for animal fodder, and for industrial production of distilled beverages (Simon, 1963). Barley is highly adaptable, and is naturally tolerant to salinity, drought and fungal infections (Gürel et al., 2016). Indeed, barley is considered the most salt-tolerant cereal, and was found to complete its life-cycle using only a limited amount water, even with high salt concentrations (Munns et al., 2006). This makes barley an attractive model for stress biology research (Gürel et al., 2016). Barley grains contain, 65–68% starch, dietary fibres 11-

34% 10–17% protein, 4–9% β-glucan, 2–3% free lipids and 1.5–2.5% minerals (Baik and Ullrich, 2008). The global harvested area produces nearly 141 million metric tons per year of barley (FAO, 2017).

Aim:

The aim of this study to assess if the conserved functions of *HvSAP* are involved in tolerance to salinity and drought in barley. Since little is known about *HvSAP* genes, the specific aims were to characterize *HvSAPs* in barley plants and to analyse the expression of *HvSAP* target genes in barley in response to salinity, drought and both stresses together. A further aim was to assess whether the expression profiles of *HvSAP* in leaves is associated with tolerance to salinity and drought in barley plants, by recording gene expression in a range of different cultivars and testing for associations.

Objective:

- To identify *SAP* gene family members in barley and to characterize the genes for salinity tolerance in barley cultivars;
- To determine *SAP* expression level under salinity, drought and combined stress in barley plants;
- To study a possible role of the *SAP* gene family.

Hypotheses:

- *HvSAP* gene expression is associated with barley plant tolerance to abiotic stresses, such as salinity and drought;
- Barley varieties show differing expression profiles of *HvSAP* genes that correlate to their stress tolerance.

Chapter 2. Material and Methods

In this study, eight Kazakh barley cultivars: Natali (NAT), Auksinyai (AUK), Granal (GRA), Astana 2000 (AST), Tzelinniy2005 (TZE), Tzelinniy Golozerniy (TG), Pamity Raisy (PR) and Golden Promise (GP) were used, chosen due to their differing tolerance to drought and salinity, with the lattermost strain being the reference genotype.

Of the Kazakh barley cultivars selected, AST is a standard barley cultivar in Kazakhstan; GRA, a parent of the segregating population; PR, an elite barley feed cultivar in Kazakhstan; and TG, a hull-less or 'naked-seed' cultivar developed for the production of animal feed. Due to its susceptibility to drought and salinity, TG was used as the reference 'sensitive' genotype. All plants were grown under a natural day/night light cycle in a greenhouse facility from March to May 2019 at Flinders University.

2.1. Seed germination

Barley seeds were germinated on paper-towels soaked in Milli-Q water for 10 days, then seedlings were transferred to the greenhouse. Plants were grown in both Hydroponics and 6-inch diameter pots containing BioGro soil (Plant Research Centre, Waite Campus, University of Adelaide). Both hydroponic tubs and pots were placed in the greenhouse during growth.

In hydroponics (Figure 2), young plants were grown in two boxes (tubs) containing 12 L of growth solution: one box was used as a control and another box used for salt treatment. To further study the effect of salinity on 14-day-old barley seedlings, the growth solution was changed after three weeks to conduct NaCl experiment. In order to perform the salinity experiment, a single fully developed leaf was harvested from individual plants at 0, 3, 7 and 14 days after the start of NaCl application in hydroponics.

For soil culture (Figure 3), seedlings (10 day-old) were transplanted into 16 pots containing 1.2 kg of soil to study the effect of drought, salinity and the two stresses combined (salinity and drought), with appropriate controls. In pots, the youngest fully developed leaf was used for *HvSAP* gene expression analyses under drought or salt stress by harvesting at 0, 5, and 15 days after the application of the stress. Three biological replicates were used in each studied cultivar, in each treatment and control. Leaf samples were frozen immediately in liquid nitrogen and then kept at -80^{o}C for subsequent RNA extraction.

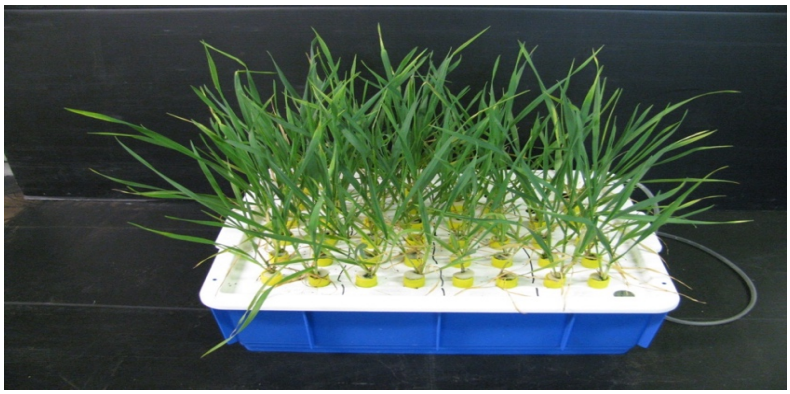

Figure 2: An overview of hydroponics systems

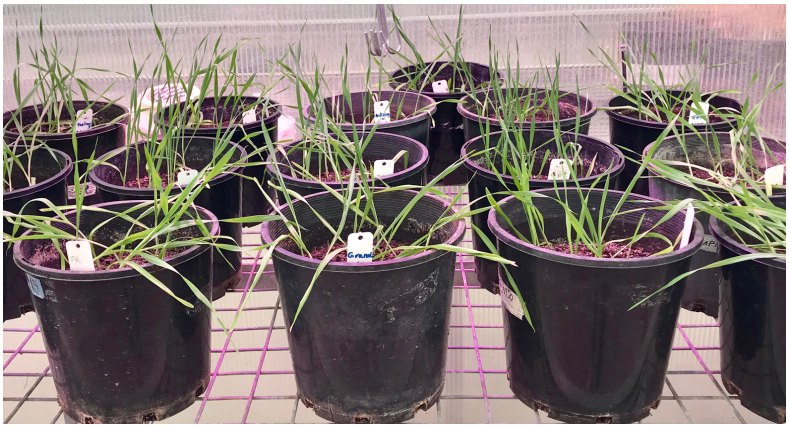

Figure 3: Plants of barley cultivars from Kazakhstan grown in a soil experiment for drought and combined stresses (salt and drought) in the greenhouse of Flinders University.

2.2. Salt stress treatment in hydroponics and collecting of leaf samples

Five-day-old seedlings were transferred into a hydroponics set-up using the described method (Atkinson and Urwin, 2012, Shavrukov et al., 2013) with the following modifications: Two

tubs, each with a 12 L capacity, were covered with lids drilled with 1 cm diameter holes. A foam piece was gently wrapped around the middle of the transferred seedling for support, enabling plants to be secured in the holes with their roots placed in the Growth solution. Constant aeration of the media was provided by aquarium pumps. Further details, including the composition of the Growth solution and an image of the hydroponics set-up with growing plants are presented in Shavrukov et al., (2013).

The hydroponic solution pH remained in the near neutral range (pH=6.5-7.0) throughout the experiment, as confirmed by regular monitoring with a pH-meter (Activon, Model 20, Adelab Scientific, Australia) with no further adjustment required. The Growth solution was topped up daily and replaced completely every 10 days. When plants reached three-weeks old, NaCl was added to one box (designated as salt stressed), by adding 25 mM NaCl increments twice daily, for three days, to reach a final concentration of 150 mM NaCl. Incremental additions are applied to avoid sudden salt shock (Shavrukov, 2013). Supplementary $CaCl_2$ was added to maintain constant calcium activity as in the initial Growth solution (0.98 mM Ca^{2+}). The tub with Control plants were grown identically without the addition of NaCl and $CaCl_2$.

All leaves were collected from three individual plants and placed into 10 ml plastic tubes, making three independent biological replicates for each genotype, treatment and collection time-points (Days 0, 3, 7 and 14 after initiation of salt application). Tubes with leaf samples were immediately frozen in liquid nitrogen and kept at -80°C until RNA extraction.

2.3. Drought stress treatment experiment

Five-day-old seedlings were transplanted into 8 pots (10 plants of each cultivar per pot) were placed in the greenhouse. Each cultivar was grown in the two groups of pots - control and

treatment. After three weeks of growth with watering twice a week, water was withdrawn from the pots designated for drought-treated plants. Leaves were collected at different time points. Pots with soil were weighed daily during watering to maintain a constant level of soil moisture until the start of the drought experiment. At Days 5, 10, and 15, leaves were collected from drought stressed plants. Leaves of Control plants were sampled in Day 0 and 15.

Similar to the experiment with salinity, all leaves from three individual plants under drought stress and Controls were sampled into 10 ml plastic tubes, making three independent biological replicates for each individual genotype. As before, tubes with leaf samples were immediately transferred to liquid nitrogen and kept at -80°C until RNA extraction.

2.4. The combined (salinity and drought) stress treatment experiment

One-month old barley plants of the same size were divided into two groups: Control and combined stress experiment (salt and drought). The stressed plants were treated first with drought stress by withdrawing watering for a week with subsequent salinity treatment. Similar to the methods described above for the salt stress experiment, 150 mM NaCl was added with increments of 25 mM NaCl, twice a day over three days to avoid sudden salt shock. Control plants were grown identically without the addition of NaCl or calcium or withdrawn watering. Control leaves were collected at Days 0 and 15. Leaves were sampled exactly the same as described above for salinity and drought with time-points at Day 5, 10, and 15 after start of salt application. Tubes with leaf samples were immediately transferred in liquid nitrogen and kept at -80°C for further RNA extraction.

2.5. Biomass measurements of shoot and root fresh weight and dry weight

For biomass measurements, fresh weight (FW) was recorded for shoots and roots at 0 and 15 days. Plant material was then dried at 65°C for 72h for dry weight measurement (DW). Biomass data were used to score and estimate tolerance to salinity, drought and both stresses (salt and drought) together compared to controls.

Five replicates of the control and treated plants (five plants of each) were harvested and both shoot and root of each plant was collected separately for estimation of shoot and root fresh / dry weight. The plant fresh weight was measured immediately after the plants were harvested. The shoot dry weights were measured following incubation of the plant shoot at 65°C for 72h.

2.6. Molecular analysis

RNA extraction was performed as per the protocol described in the Section below. cDNA was synthesised using a ProtoScript II Reverse Transcriptase kit (NEB) and qRT–PCR carried out using a KAPA SYBR FAST Universal Kit (Kapa Biosystems, USA) following the manufacturer's protocol. Two reference genes: ADP-ribosylation factor 1-like protein (*HvADP*) and glycolytic glyceraldehyde-3-phosphate dehydrogenase (*HvGAP*) were used for normalisation of gene expression.

2.6.1. RNA isolation

Total RNA was extracted from the leaf tissue of all samples using TRIzol -like reagent as described earlier (Shavrukov et al. 2013). The RNA isolation procedure began by powdering leaf tissue in liquid nitrogen and adding 1 ml of TRIzol to the ground tissue. The samples were then incubated at room temperature for 5 minutes and 200 ul of chloroform was added

to the supernatant. This allowed the mixture to separate into a lower phenol-chloroform phase, an interphase and a colourless upper aqueous phase. The tubes were then vortexed well and the mixed solution was incubated at room temperature for about 10 min. The tubes were then centrifuged in a refrigerated bench centrifuge at maximum speed, 12,000 rpm for 20 minutes at 4°C. The upper aqueous phase was transferred into a new microtube and 500 ul of cool isopropanol was added to the solution and mixed, followed by incubation at room temperature for 20 minutes. The tubes were then centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was discarded, and the resulting RNA pellet was washed with 75 % ethanol, and the tubes were again centrifuged at maximum speed, 12,000 rpm for 5 minutes at 4°C. Finally, the pellet was air-dried for about 20 min before 25 ul of nuclease-free water was added. RNA solutions were stored at -80°C, and RNA concentration was measured by Nanodrop spectrophotometer at 260 nm. RNA quality was checked on a 1.5% agarose gel.

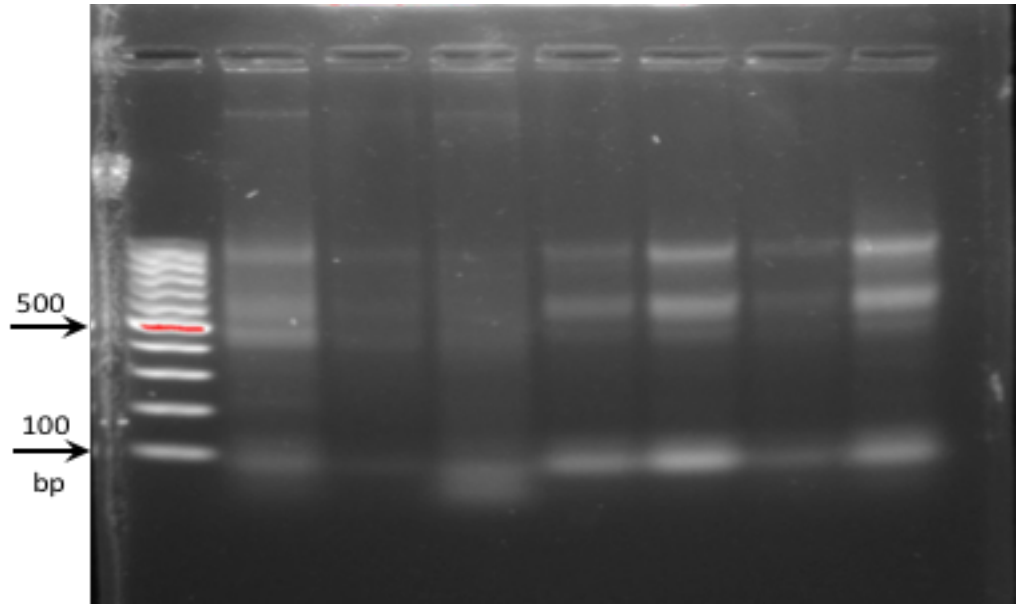

Figure 4: RNA quality check by electrophoresis of 1 µl of each RNA sample on 1.5% agarose gel. M indicates the 100 bp Plus DNA Ladder. Numbers indicate the different cultivars at various time points.

2.6.2. cDNA synthesis

To set up the cDNA synthesis reactions, 2 µg of RNA sample was used for each reaction. RNA samples were diluted with water to make a final volume of 9 µl. In a sterile microfuge tube, 2 µl of 50 µM Oligo $d(T)_{20}$ and 1 µl of 10 mM dNTP were added to make up the final volume of the 12 µl reaction. The reaction tubes were mixed, heated for 5 min at 65°C and then placed on ice for 1 min. One µl of DNase was added and tubes were incubated for 15 min at room temperature 22°C. The following components were then added: 4 µl of ProtoScript11 Reaction buffer, 2 µl of 0.1M DTT, 0.3 µl of Murine RNase Inhibitor, 0.25 µl of ProtoScript11 RT, and sterile water to the final volume. The final reactions of 20 µl were incubated in a PCR cycler at 42°C for 1 hour. The final reaction after dilution was used to perform qPCR to record expression of the target genes. The cDNA samples were stored at -20°C until the next step.

50 µM Oligo $d(T)_{20}$	2.0 ul
10 mM dNTP	1.0 µl
DNase	1.0 µl
5 × ProtoScriptII Reaction Buffer	4.0 µl
0.1M DTT	2.0 µl
Murine RNase Inhibitor	0.3 µl
ProtoScriptII RT	0.25 µl
H_2O	0.45 µl
Total volume	11µl

2.6.3. Semi-quantitative RT-PCR

- 1 µl of the M-MLVRT was added.
- The mixture was incubated at 42°C for 60 minutes. To inactivate the enzyme, the reaction was then incubated at 70 °C for 10 min.

The cycling conditions for RT-PCR were as follows:

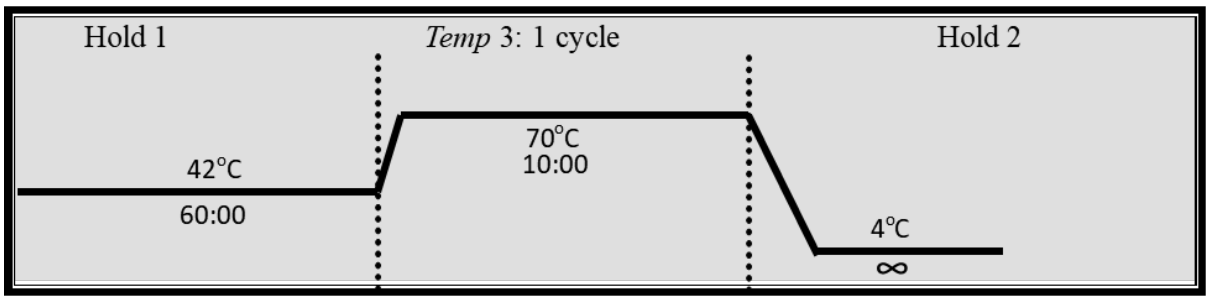

Figure 5. RT-PCR thermal cycling conditions for cDNA

2.6.4. Quantitative PCR (qPCR)

For qPCR, 10 µl reactions were used, which comprised 4 µl cDNA, 5 µl qPCR KAPA qPCR Master Mix, KAPA, and 1 µl of gene specific primers (Table 1). These reactions were carried out in 96-well optical reaction plates.

Table 1: Summary of primer sequences used in this study

Entry	Name of gene/primer	Amplicon (bp)	F / R	Sequence (5' - 3')
1	HvSAP5-qPCR	136	F	CCTCCTCTTTTGACAGCATCGTC
			R	GGACCAGCGATGTCAGCAGG
2	HvSAP6-qPCR	165	F	GTGGCAGAGATGAAGGATGAAGC
			R	GTGCATCGAGCAGAAGGTGTCT
3	HvSAP11-qPCR	163	F	CGTTCCCGCTCTTCGACAAGC
			R	AACCCCGTCAGGCCCACG
4	HvSAP12-qPCR	139	F	CAAAGCCGCCCAGCAACCGA
			R	GCCTTCTTGTAGTCGAATGAGCAT
5	HvSAP15-qPCR	160	F	CATTGTCGTCTGTGCTGTTCGTT
			R	TCCTCCCGACCCACAGTTTTATAA

Transcript abundance in response to salinity and drought stresses was measured by qRT-PCR using KAPA SYBR FAST Universal Kit (Kapa Biosystems, USA) using the manufacturer's

protocol. As indicated in Section 2.6, two reference genes were used for normalisation of gene expression: ADP-ribosylation factor 1-like protein (*HvADP*) and Glycolytic glyceraldehyde-3-phosphate dehydrogenase (*HvGAP*).

Table 2. Composition of Master-mix for qPCR

	1 Master-mix
2 x KAPA	5
Primer mix (3 uM)	1
cDNA	4
Total volume	10

The thermal cycling conditions were as follows:

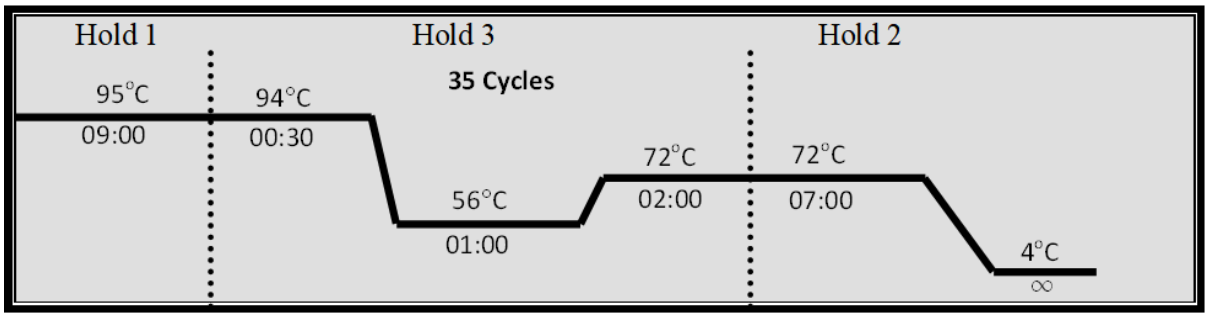

Figure 5: RT-PCR thermal cycling conditions for cDNA.

2.7. Statistical analysis

Standard Excel software was used to calculate and analyse means and standard error using ANOVA. In order to estimate the probabilities for significance, Student's *t*-test was used. A correlation analysis was performed using Tests of Between-Subjects Effects (IBM SPSS, Statistics Desktop 25.0.0.0).

Chapter 3. Results

3.1. The effect of salinity on barley biomass (Hydroponics experiment)

In this study, eight barley cultivars were analysed to see the effect of 150 mM NaCl on growth and leaf morphology relative to controls. Four cultivars, AUK, GP, NAT and TZE, did not show any significant effect following salt application. Whereas the shoot dry weights of four cultivars, AST, GRA, PR and TG, were significantly decreased after salt treatment as compared to the Controls (Figure7). Based on the salt tolerance experiment with eight cultivars, four of them were selected: AST, GRA, PR and TG, all of which were found to be significantly different compared to Controls after salinity application. Conversely, the dry weight of roots of the four selected cultivars after salt application did not show significant differences among the barley accessions (Figure 7).

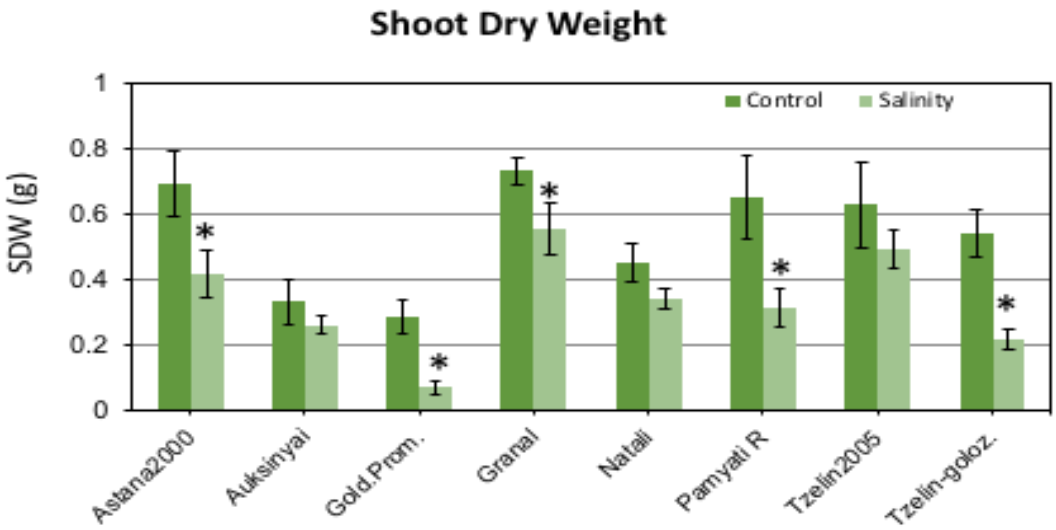

Figure 7. Biomass production in eight barley cultivars at Day 14: Astana 2000 (AST), Auksinyai (AUK), Golden Promise (GP, Reference genotype sensitive to the stresses), Granal (GRA), Natali (NAT), Pamity Raisi (PR), Tzelinniy 2005 (TZE), and Tzelinniy Golozerniy (TG). After plants reached three-weeks old, NaCl (150 mM) was added in increments and plants grew for a further 14 days. The error bars represent means ± standard deviation of six replicates. Asterisks (*) indicate significant ($p<0.05$) differences between genotypes.

The phenotypes of AST and GRA plants in the salinity stress experiment were found to be more tolerant; showing no apparent affects from salt application and appearing visually to be healthier as compared to PR and TG. By contrast, the leaves of PR and TG plants were more affected and were smaller after salt treatment compared to Controls (Figure 8).

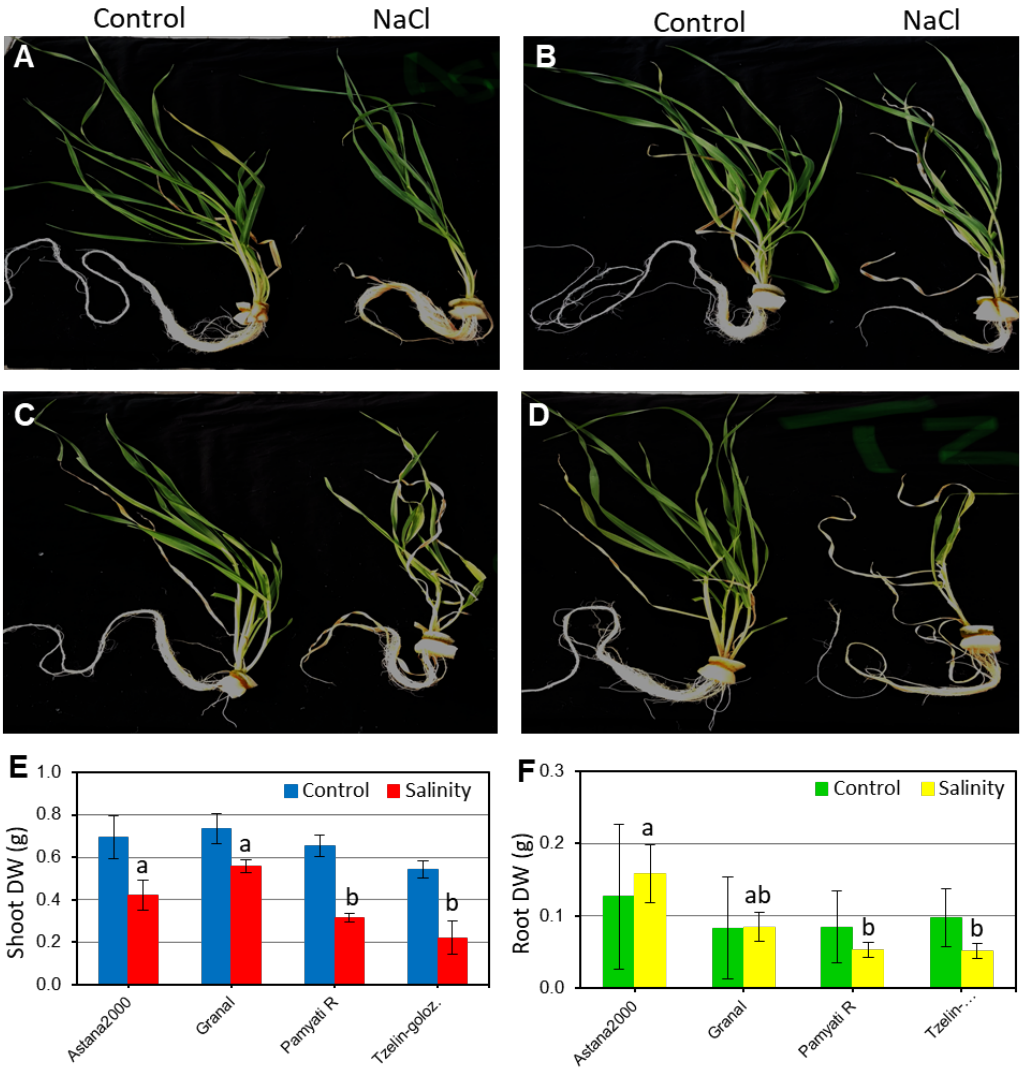

Figure 6: Hydroponic phenotypes of for four barely cultivars with control and salt treatment at Day 14. (A) Astana, (B) Granal, (C) Pamyati Raisi, (D) Tzelinniy Golozerniy. (E) dry weight of shoots of four cultivars with and without salt treatment. (F) dry weight of roots of four cultivars with and without salt treatment. Experiments were performed in triplicate, n=6, each. Different letters indicate significant ($p<0.05$) differences between genotypes, analysed by ANOVA.

In the salt tolerance experiment, the relative dry weight (DW) of whole plants was calculated using a Relative dry weight formula [(DW_{NaCl} / DW_{cont}) × 100], in which the total dry weight of salt-stressed plants was subtracted from the total dry weight of Control plants (without NaCl). The data show that the Relative dry weight of AST, GRA, PR and TG were 70%, 79%, 50%, and 45%, respectfully. The DW of GRA (79%) and AST (70%) were significantly higher compared to the DW of other plants such as PR (50%) and TG (45%) as shown in Figure 9. Therefore, AST and GRA lost much less water compared to PR and TG.

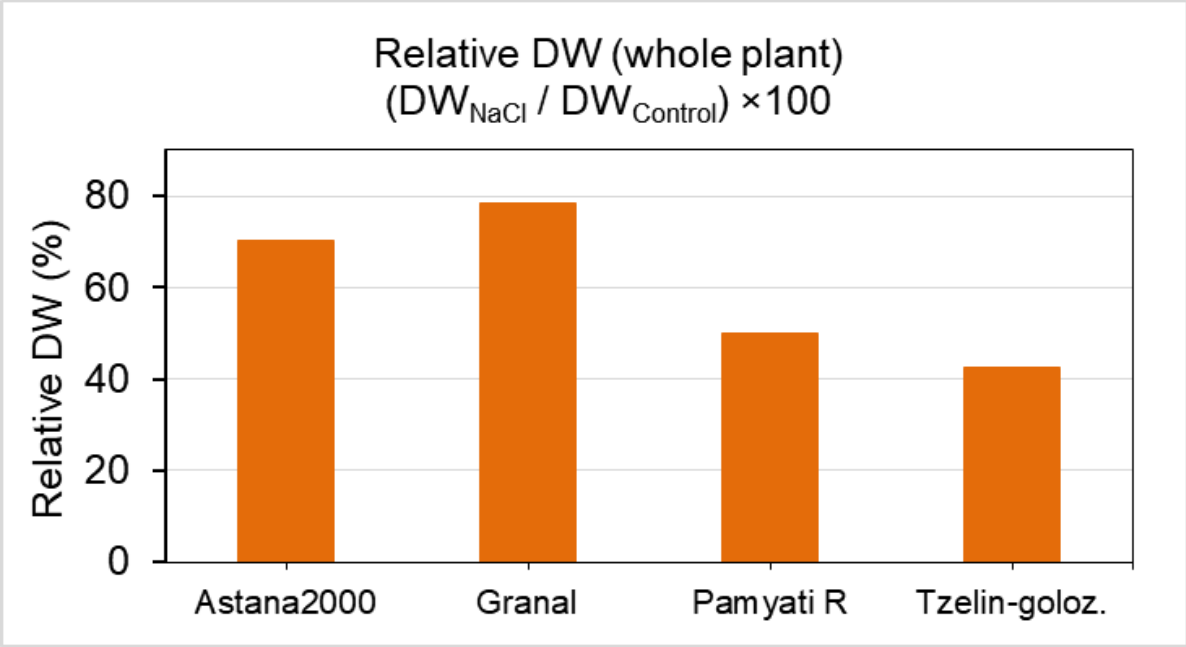

Figure 9. Relative dry weight of whole plants (both shoots and roots) of four barley cultivars: Astana (AST), Granal (GRA), Pamyati Raisi (PR) and Tzelinniy golozerniy (TG) after salt application over a period of 3 days.

3.2. Analysis of *HvSAP* gene expression using semi-quantitative RT-PCR

Semi-quantitative RT-PCR was used to observe the expression levels of the genes. The analysis of 17 identified *HvSAP* genes using bulked cDNA samples (a mixture of 1 µl of each cDNA sample from control, drought and salt treatments) revealed five highly expressed genes including *HvSAP5*, *HvSAP6*, *HvSAP11*, *HvSAP12* and *HvSAP15* (Figure 10). Two genes, *HvSAP1* and *HvSAP16*, showed less intense, but still clear expression levels. The remaining *HvSAP* genes showed no or very poor expression, including multiple bands for the *HvSAP9a* gene. Thus, five highly expressed genes were selected for further study.

Image removed due to copyright restriction.

Figure 10. Agarose gel of semi-quantitative RT-PCR targeting 17 *HvSAP* genes (indicated at the top) using cDNA from plants of barley cultivars grown under drought and salt stress, compared to Controls (Con). M, 100 bp DNA ladder (Bioline, Australia).

3.3. Expression analysis of five *HvSAP* genes in response to salt stress treatment

Almost all *HvSAP* genes examined had moderately high or very high expression after 3 days and especially after 7-days exposure to salt stress (Figure 11). The greatest increase in transcript levels were found for *HvSAP5* (7-fold), *HvSAP6* (12-fold), *HvSAP12* (6-fold), and *HvSAP15* (11-fold). In this study, three cultivars out of four showed similar expression levels of the genes *HvSAP6* and *HvSAP12* at day 3 and day 7. Whereas, in all four studied barley genotypes, the elevated expression of *HvSAP5* and *HvSAP15* transcripts was observed on day 7 (Figures 11A and 11E). However, in non-stressed, control plants, the expression level of the four genes varied from very low (0.35 Relative expression units in *HvSAP15*) to extremely low in *HvSAP5*, *HvSAP6* and *HvSAP12* (Figure 11A, 11B and 11D). Interestingly, in this study we found that the expression level of the *HvSAP11* gene was significantly higher in Controls compared to the other four *HvSAP* genes' expressions (Figure 11F). Moreover, in all four study cultivars, the initial level of *HvSAP11* expression varied between 0.5 and 1.7 Relative expression units, which was significantly higher than the other four *HvSAP* genes (Figure 11F). Under salt stress, the expression of *HvSAP11* was found to be significantly higher (2.5-fold) at Day 7 in GRA, while it was down-regulated during all periods of the experiment at day 3 to day 14 in TG (Figure 11C).

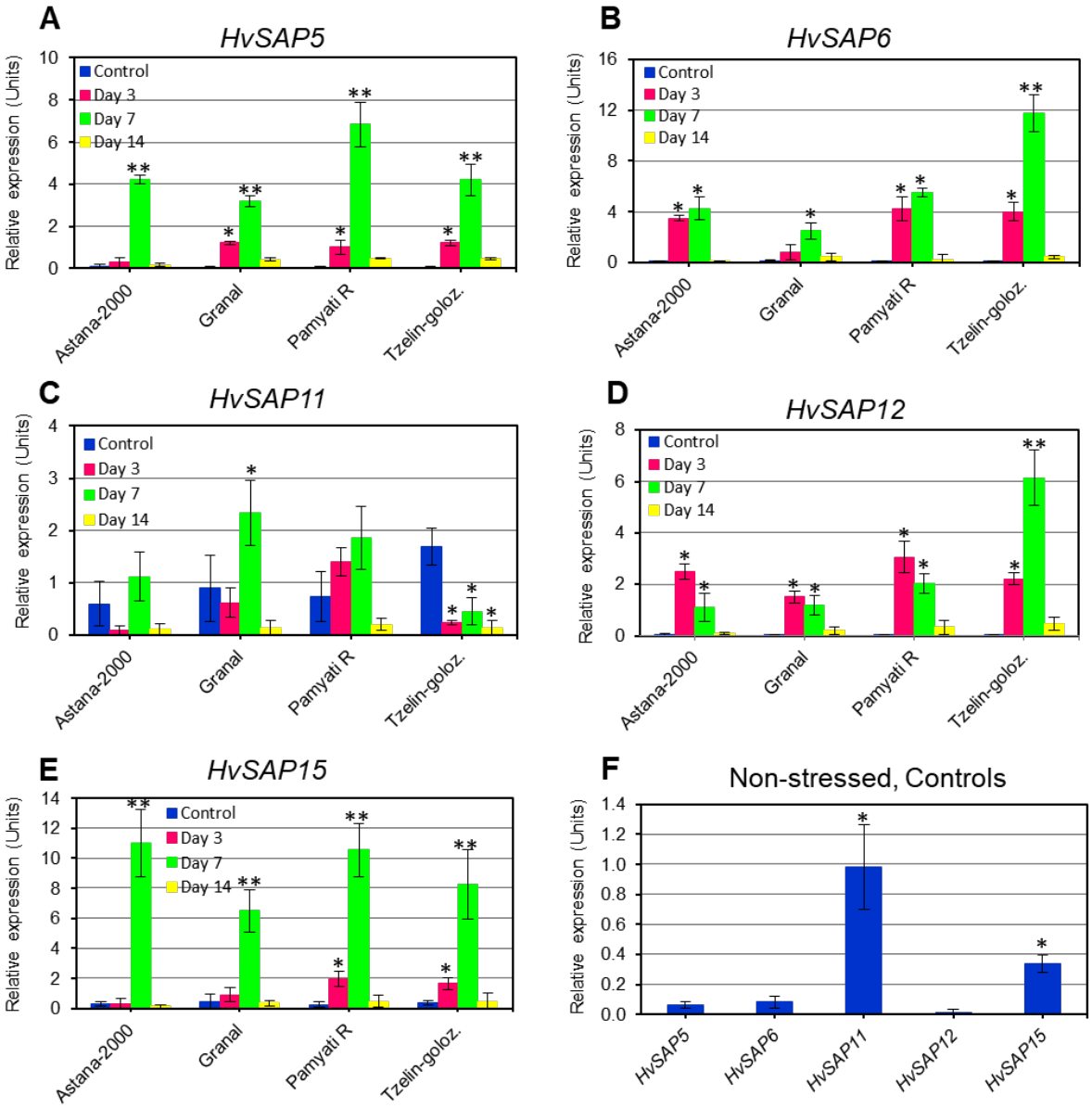

Figure 11. Relative expression levels (fold change) of five *HvSAP* genes: (**A**) *HvSAP5*; (**B**) *HvSAP6*; (**C**) *HvSAP11*; (**D**) *HvSAP12*; (**E**) *HvSAP15*, in leaves of four barley cultivars grown in hydroponics with 150 mM NaCl for 0, 3, 7 and 14 days. (**F**) Averaged expression of the five selected genes in Control plants of four studied barley accessions for comparison. Expression data were normalised using the averages of two reference genes, *HvADP* and *HvGAPDH*, and presented as the average ± SE of three biological and two technical replicates for each genotype and treatment. Significant differences are indicated by asterisks, compared to Controls within each experiment, and for each barley accession (**A-E**) and among studied genes (**F**): * P>0.95; ** P>0.99, calculated using ANOVA.

3.4. The effect of drought tolerance on barley biomass, soil experiment

In this experiment, eight barley cultivars: AST, AUK, GP, GRA, NAT, PR, TZ and TG, were used to determine the effect of drought after 10 days of growth. The four cultivars (AUK, GP, NAT and TZE) did not show any significant effect after drought stress, with biomass similar to the Controls (Figure 12). However, Fresh weights of shoots (SFW) in four cultivars, AST, GRA, PR and TG, were significantly decreased after exposure to drought, relative to the Controls. After 15 days, pots were re-watered, and plants were allowed to recover. SFW of the four barley cultivars were found to be significantly different compared to Controls. Thus, these four barely cultivars (AUK, GP, NAT and TZE), were selected for further study (Figure 12).

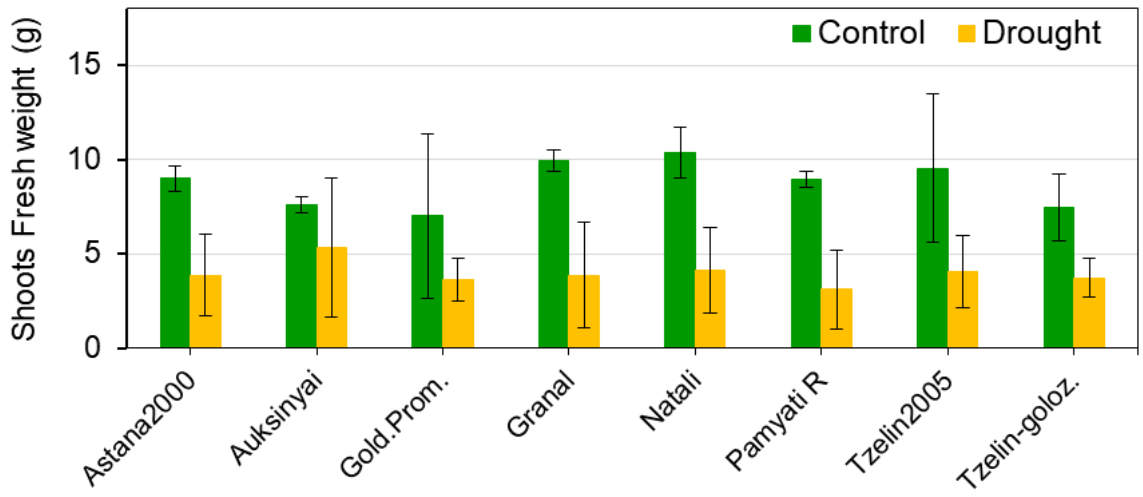

Figure 12. Biomass of eight barley cultivars: Astana 2000 (AST), Auksinyai (AUK), Granal (GRA), Natali (NAT), Pamity R (PR), Tzelinniy2005 (TZE), and Tzelinniy Golozerniy (TG), and a reference genotype sensitive to the stresses, Golden Promise (GP). The figure shows the effect of drought stress on Shoot fresh weight (SFW) of 15-day old seedlings. The error bars represent ± standard deviation of five biological replicates.

In the soil experiment, the four barley cultivars showed significant differences between Control and treated plants in their morphology after 15 days. Moreover, Control plants appeared healthier than the treated plant which lost more leaves after 15 days in the recovery period. The Figure below shows the fresh weight of both shoots and roots of plants after 15-day water recovery (Figure 13E, 13F).

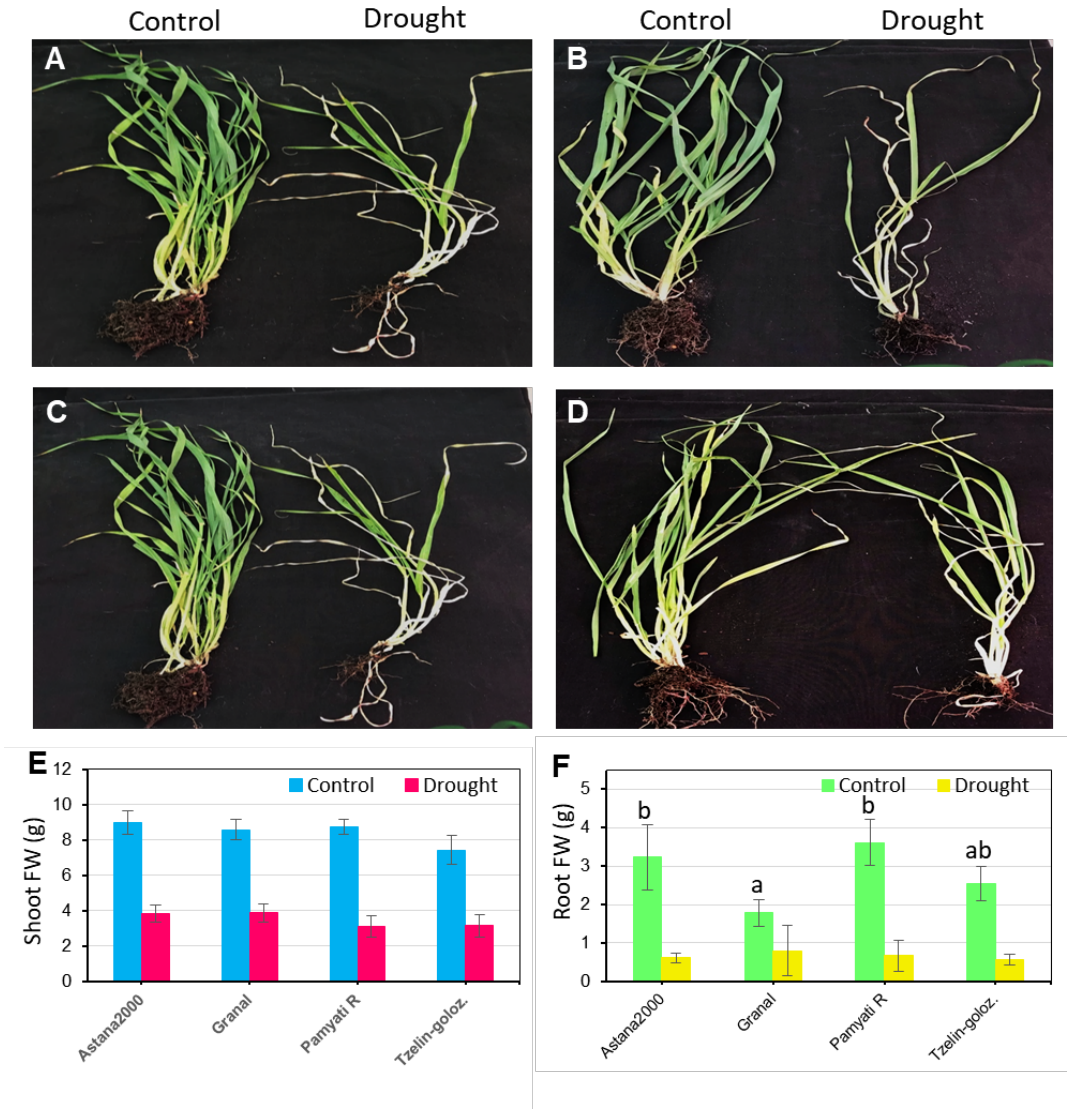

Figure 13. Phenotypes and Fresh weight (FW) biomass of four cultivars in Controls and drought treatment. (A) Astana, AST; (B) Granal, GRA; (C) Pamyati Raisi, PR; (D) Tzelinniy Golozerniy. TG; (E) FW of shoots in four cultivars in Controls and under drought stress; (F) FW of roots in four cultivars in Controls and under drought. Experiments were performed in triplicate, n=6 in each experiment. Different letters indicate significant ($p<0.05$) differences between analysed genotypes by ANOVA.

3.5. Expression analysis for four barley cultivars of five *HvSAP* genes in response to drought stress treatments

Expression level varied significantly among *HvSAP5, HvSAP6, HvSAP11, HvSAP12*, and *HvSAP15* (Figure 14). Expression of *HvSAP5* in AST and GRA was found to be similar at Days 5 and 10, and after rewatering at Day15, compared to Controls (Figure 14A). However, the expression level of *HvSAP5* in PR and TG was found to be significantly higher only after rewatering at Day 15 relative to the Controls (Figure 14A). The *HvSAP6* gene showed a higher expression level at Day 10 in all four studied barley genotypes. However, after rewatering of plants at Day 15, up-regulation of *HvSAP6* expression continued in AST but it was down-regulated in the three other barley accessions, GRA, PR and TG, compared to Controls (Figure 14B). The *HvSAP11* gene was highly expressed at Day 10 and after rewatering at Day 15 in AST, GRA and TG (Figure 14C). Barley cv. PR recorded a significant expression level of *HvSAP11* at Day 10 compared to Controls (Figure 14C). A significantly higher expression of *HvSAP12* was shown at Day 10 in GRA (Figure 14D). In contrast, the other three cultivars, AST, PR and TG, showed no significant differences across all time periods (Figure 14D). Moreover, the expression level of *HvSAP15* was found to be high at Days 5 and 10 in PR compared to the rest of cultivars: AST, GRA and TG, which did not show any significant difference in any time-points (Figure 14E). However, in four genes, *HvSAP6, HvSAP11, HvSAP12* and *HvSAP15*, the expression levels in Control plants (Under non-drought condition), were very low, from 0.01-fold to 0.04-fold in *HvSAP12* and in *HvSAP15*, respectively, compared to expression levels of the Reference genes (Figure 14F). Three genes, *HvSAP5*, *HvSAP6* and *HvSAP11,* had higher expression levels in Controls compared to *HvSAP12* and *HvSAP15*, but were still much smaller, at 0.15-0.45 expression units, compared to the Reference genes used for normalisation (Figure 14F).

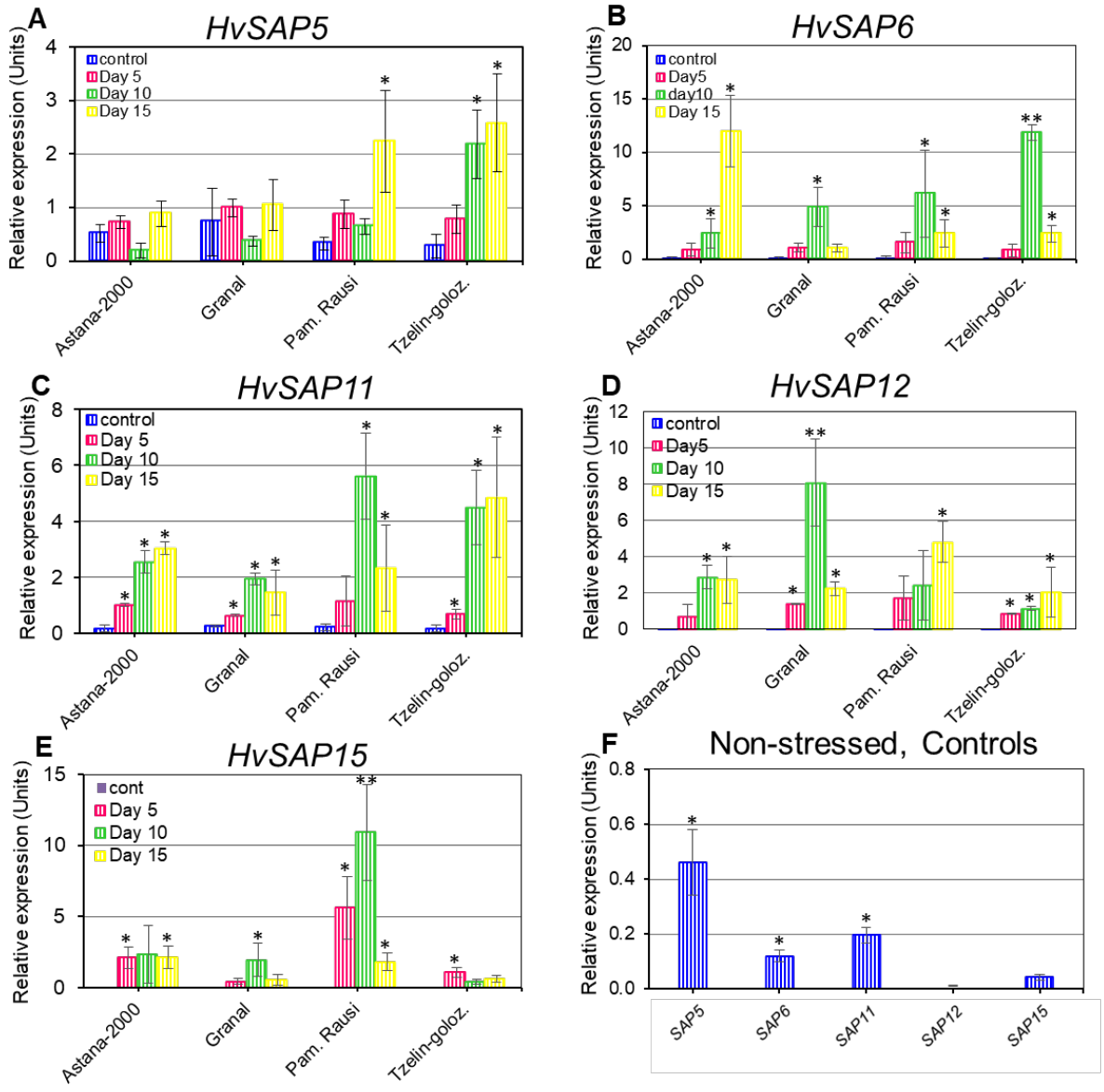

Figure 14. Relative expression levels (fold change) of five *HvSAP* genes: (**A**) *HvSAP5*; (**B**) *HvSAP6*; (**C**) *HvSAP11*; (**D**) *HvSAP12*; (**E**) *HvSAP15*, in leaves of four barley cultivars grown in soil for 0, 5, 10 and 15 days. (**F**) Averaged expression of the five selected genes in Control plants of four studied barley accessions for comparison. Expression data were normalised using the average of two reference genes, *HvADP* and *HvGAPDH*, and presented as the average ± SE of three biological and two technical replicates for each genotype and treatment. Significant differences are indicated by asterisks compared to Controls within each experiment, and for each barley accession (**A-E**) and among studied genes (**F**): * P>0.95; ** P>0.99, calculated using ANOVA.

3.6. The effect of combined salinity and drought stress on barley biomass in soil

Fresh weights of shoots (SFW) in all eight studied barley cultivars, AST, AUK, GP, GRA, NAT, PR, TZE and TG, were significantly reduced in plants exposed to increasing levels of salinity and water deficit stress (combined salt and drought) relative to Controls after 15 days. However, no significant differences were found, either in Controls or in stressed plants, among the eight studied barley accessions (Figure 15). Similar to the methods described above for the salt stress experiment, a further 150 mM NaCl was added in increments of 25 mM NaCl, twice a day over three days, to avoid sudden salt shock. Control plants were grown identically without addition of NaCl or calcium, or withdrawing of water.

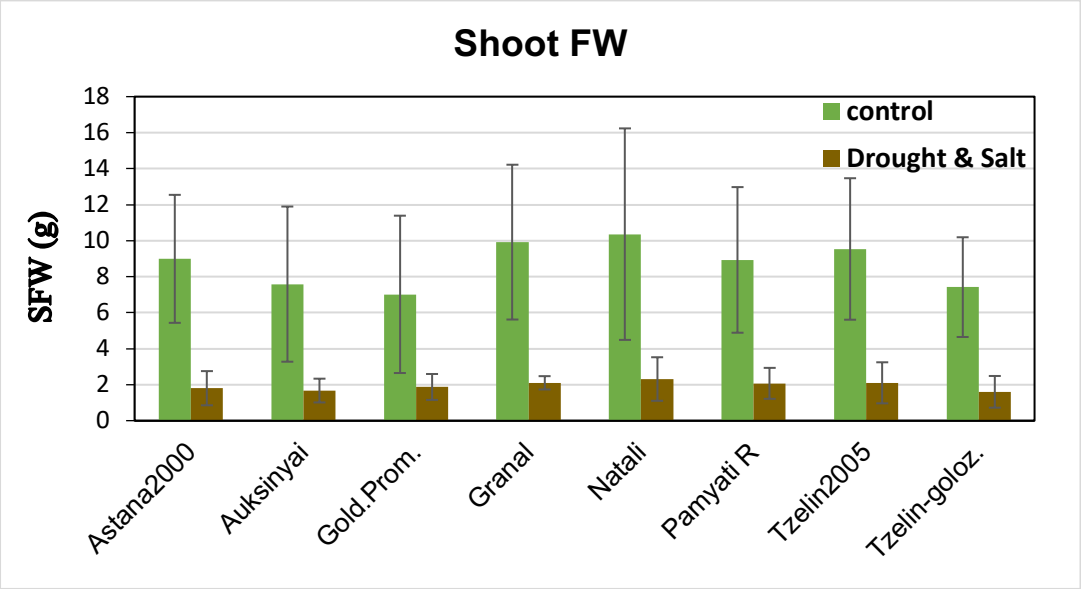

Figure 15. Shoot fresh weight (SFW) of eight barley cultivars in Controls and under combination drought and salinity stresses. Experiment was performed in triplicate, n=6 in each replicate. 150 mM NaCl was added in increments of 25mM over three days. Control plants were grown identically without addition of NaCl, calcium or withdrawing of water.

3.7. Expression analysis of five *HvSAP* genes in four barley cultivars in response to combined salinity and drought stress treatments

Most *HvSAP* genes had a high level of expression on both Days 10 and 15 after exposure to combined salt and drought stresses. For *HvSAP5*, the transcript started to increase from an early stage at Day 5 and continued at a high level during all time-points until Day 15 in three barley cultivars, with the exception of TG. The greatest expression level of *HvSAP5* was found for PR (0.5-fold) on Day 10, but this was still lower than the reference genes (Figure 16A). *HvSAP6* showed a larger, gradual increase to reach a much higher expression level. A similar level between Days 5-15 was found in AST and TZ, showing down-regulation on Day 15, but expression remained unchanged in GRA and PR (Figure 16B). Three cultivars, GRA, PR, and TZ showed a significantly high level of *HvSAP11* on Day 10 compared to Controls (Figure 16C). The *HvSAP12* gene had quite variable expression, as evident in the large error bars, so no conclusion can be made about the relevance of this gene (Figure 16D). Significant up-regulation of *HvSAP15* expression was found on Days 10 and 15 in PR and TZ, but error bars were quite large in some samples (Figure 16E). In Control (non-stressed) plants, the level of expression ranged from very high expression (0.7- and 0.38-fold) in *HvSAP5* and *HvSAP11*, respectively, to an extremely low level of about 0.07-fold in three other *HvSAP* genes (Figure 16E).

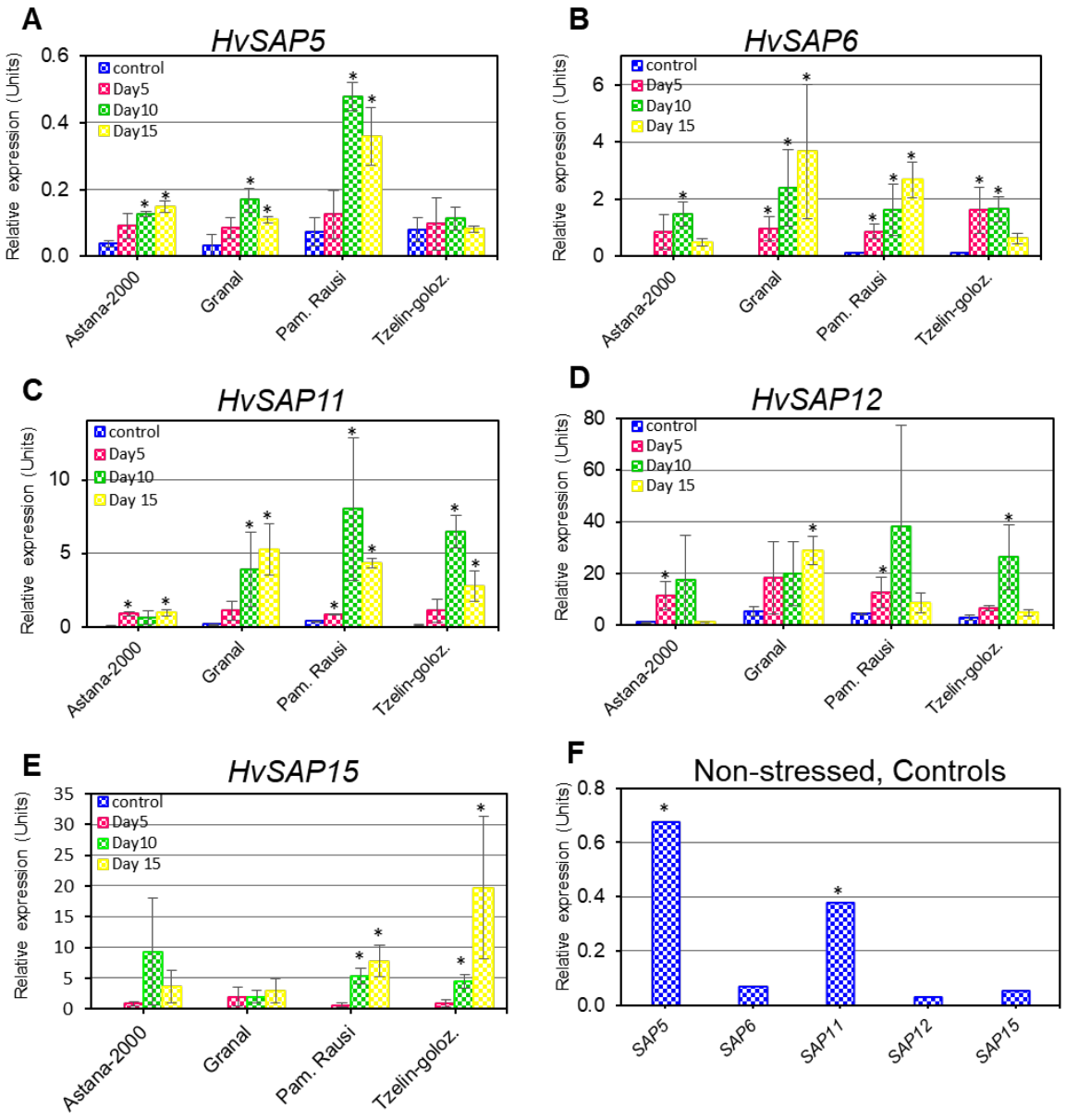

Figure 16. Relative expression levels (fold change) of five *HvSAP* genes: (**A**) *HvSAP5*; (**B**) *HvSAP6*; (**C**) *HvSAP11*; (**D**) *HvSAP12*; (**E**) *HvSAP15*, in leaves of four barley cultivars grown in soil for 0, 5, 10 and 15 days. (**F**) Averaged expression of the five genes in Control plants of four studied barley accessions for comparison. Expression data were normalised using the averages of two reference genes, *HvADP* and *HvGAPDH*, and presented as the average ± SE of three biological and two technical replicates for each genotype and treatment. Significant differences are indicated by asterisks compared to Controls within each experiment, and for each barley accession (**A-E**) and among studied genes (**F**): * P>0.95; ** P>0.99, calculated using ANOVA.

Chapter 4. Discussion

In the current study, *HvSAP* genes were studied for the first time, in barley plants under control (non-stressed) and NaCl treatment in hydroponic experiments; as well as in combined salinity and drought stress experiments in pots with soil. The SAP genes in barley were identified and described earlier in our manuscript.

There is very little published information about *SAP* gene expression in plants grown under favourable conditions in different plant species. *SlSAP* genes in tomato show a much greater resemblance to *Arabidopsi*s than to those of monocot plant species. However, it is noteworthy to compare the results shown in tomato with our findings on *HvSAP* barley transcripts. Very strong *SlSAP1* and *SlSAP10* expressions were found in tomato seedling, 9-days-old without any stress (Solanke et al., 2009). These genes are are closely related to rice *OsSAP1*, *OsSAP11* and *OsSAP15*, which perfectly match our results for *HvSAP11* and *HvSAP15*, which show the highest levels of transcription in Controls - non-stressed barley plants (Figure 13). Three other barley genes found in our study, *HvSAP5, HvSAP6* and *HvSAP12*, showed much lower levels of gene expression in plants grown under favourable conditions. Therefore, our results are similar to those in tomato, where *SlSAP4*, *SlSAP5* and *SlSAP9* have been clustered in the same clade of the phylogenetic tree (Solanke et al., 2009).

In our study, five out of 17 identified *HvSAP* genes showed high expression profiles under salt stress, while other *HvSAP* genes had no or very low transcript levels. For comparison, all five orthologous genes in rice were up-regulated in response to salinity stress (Vij and Tyagi, 2006). The highest level of 3.5-fold increased expression was reported in *OsSAP5* and *OsSAP12*, followed by *OsSAP6* and *OsSAP11* (3-fold), and *OsSAP15* (1.5-fold). In general, our results for the five *HvSAP* genes show a similarity to those results published in rice, with

the difference being that *HvSAP15* compared to *OsSAP15* transcripts showed very high (6-11-fold) rather than relatively low (1.5-fold) expression in barley and rice, respectively.

In addition, more conflicting observations were made in the comparison between other *OsSAP* and *HvSAP* genes. Three genes, *OsSAP7*, *OsSAP10* and *OsSAP14*, showed 5.5-, 9- and 10-fold up-regulation in rice seedling under salt stress (Vij and Tyagi, 2006), while orthologous barley genes (*HvSAP7*, *HvSAP10* and *HvSAP14*) in our study did not show any amplification with bulked cDNA in semi-quantitative RT-PCR, and as a result were excluded from further study. There are several possible reasons for such notable differences in orthologous gene expression. Firstly, four barley cultivars were used in our study, which is much more representative compared to a single rice accession published earlier (Vij and Tyagi, 2006). Rice seedlings were grown for 7 days in trays with cotton bases soaked with water prior to NaCl application, while barley plants in our experiment were three weeks-old and grown in hydroponics with Growth solution. The rice seedlings were exposed to 200 mM NaCl for a very short time (6 h), while leaves from barley plants in our experiments were sampled after 3, 7 and 14 days, as a long-term salinity treatment with 150 mM NaCl.

However, from our point of view, the major important difference between the experiments with rice (Vij and Tyagi, 2006) and our experiments with barley is methodological, which can dramatically change the interpretation of the results. Although it was not explicitly written, we assume that rice seedlings were simply transferred from the wet tray into a beaker with 200 mM NaCl and exposed for 6 h (Vij and Tyagi, 2006). Such a sudden transfer (in one step) of plants from non-stressed conditions into solution with 200 mM NaCl would likely cause ‘osmotic shock’ and cell plasmolysis as protoplasts detach from the cell wall, particularly in roots. Therefore, the gene expression reported (including *SAP* genes) were

possibly the result of ‘salt shock’ rather than ‘salt stress’ as occurs with gradual (in several steps) NaCl application, as described earlier (Shavrukov, 2012). In our experiments with barley plants, high transcript levels of five genes (*HvSAP5*, *HvSAP6*, *HvSAP11*, *HvSAP12* and *HvSAP15*) were found after gradual elevation of NaCl concentration in Growth solution, and these genes indeed were responsive to salt stress. In contrast, the three reported genes in rice, *OsSAP7*, *OsSAP10* and *OsSAP14* (Vij and Tyagi, 2006), were likely responsive to the strong osmotic/salt shock rather than salt stress and should be viewed with greater caution regarding the method of NaCl application.

A similar situation arises for the comparison of *HvSAP* gene expression in barley with *SlSAP* genes in 9-day-old tomato seedlings transferred into media with 200 mM NaCl for 1 and 8 hrs (Solanke et al., 2009). All five *HvSAP* genes identified as responsive to gradual salt stress have similarity with the corresponding genes in tomato, while a group of *SlSAP6*, *SlSAP12* and *SlSAP13* genes are likely expressed in response to salt shock.

In the barley drought study, we report a functional characterization of the *HvSAP* genes, where all *HvSAP* were differentially regulated by drought. Drought stress caused dramatic increases in transcript levels in shoots, reaching as high as 12-fold in *HvSAP6*, 11-fold in *HvSAP15* and 8-fold in *HvSAP12* on Day 10 (Figure 14B, E and C). In addition, on Day15 the highest level of expression reached 12-fold in *HvSAP6*. Most high expression in barley *HvSAP* were found at Day 10 under drought stress, similar to the previous study that showed that most *MdSAP* genes were induced within 2-8 days of treatment in apple (Saad et al., 2010, Dong et al., 2018).

From our results, the performance of *HvSAP11* under drought stress presented strong levels of tolerance to drought stress when barley plants were grown under drought for 10 to 15 days. Similarly, two *SAP* genes from other plant species, *OsiSAP8* from rice (Kanneganti et al, 2008) and *AlSAP* from *Aeluropus littoralis* (Ben Saad et al, 2010), have been shown to provide strong tolerance to drought stress (Saad et al., 2010).

Other results from the drought stress study showed that *HvSAP15* was strongly up-regulated on Day 10, which is similar to what was reported for *AtSAP15* in *Arabidopsis* plants, leading to enhanced drought tolerance (Dong et al., 2018).

In the combined stress study, we analysed the expression of the *HvSAP* genes under drought and salt stress. Whereas the expression of *HvSAP5*, *HvSAP-11* and *HvSAP-15* were significantly induced, the expression level for *HvSAP12* was strongly down-regulated. Therefore, multiple stress likely causes damage to growing plants in different ways, even if the plants are tolerant to a particular type of environmental stress. For example, *Jatropha curcas*, a highly heat-tolerant species, suffers more from the combination of salinity and heat stress than from either of these stresses alone (Quinn et al., 2015).

The analysis of functional effects of combined salt and drought stresses to barley plants show that all studied *HvSAP* genes were differentially regulated in response to multiple abiotic stresses. Also, it seems the functions of these genes are not well defined. Moreover, it was reported earlier that most of the *SAP* genes isolated from cotton were induced by one or more abiotic stresses as shown by qRT-PCR analysis (Guo et al., 2009). Not all *SAP* genes have shown to be induced by multiple stress, however some members of the rice *SAP* family were shown to be induced for salt and drought (Vij and Tyagi, 2006).

In our study, some *HvSAP* genes were up-regulated at the early stage on Day 5 after the beginning of the combined stress, and some of them continued to be highly expressed until the late time-point on Day 15. A previous study of different genes (*AtCDPK1, AtCDPK2*, and *OsCDPK7*) encoding a calcium-dependent protein kinase, showed a high and rapid induction early in response to drought and salt stress (Saijo et al., 2001). Furthermore, the *MusaSAP1* gene in banana has been shown to record a transcript level that remained up-regulated even after several days of stress treatment (Sreedharan et al., 2012).

Similar findings have been described for the overexpression of *AtSAP5* in cotton and *Arabidopsis*. The overexpression of *AtSAP13* and *MusaSAP1* in *Arabidopsis* and banana leads to greater drought and salt tolerances. *PsSAP1* gene expression was slightly but significantly affected by abiotic stresses such as cold, heat, drought and salinity, in the flower buds of peach (Dong et al., 2018).

Chapter 5. Conclusion and future work

In conclusion, novel information was presented in this study about the selected *HvSAP* gene family members in response to abiotic stresses. Also, this present study has characterized a zinc-finger protein gene from barley and unraveled a determinant of abiotic stress tolerance that may be used for study in other crop plant species. The expression of barley *HvSAP* genes could be induced by multiple abiotic stresses, including salt and drought.

This study provides a comprehensive analysis of *HvSAP* genes in leaves and may aid in future efforts to identify the functions of A20/AN1-type proteins and the responses of barley under a variety of abiotic stresses. All the above results may help to further understand the mechanisms of barley tolerance to stress conditions and provide candidate genes for breeding of stress tolerant crops. *SAP* genes have been identified as important regulators in plant tolerance to various stresses. *HvSAP* genes, and *HvSAP12* particular, play an important role in the tolerance of barley plants to salinity and drought, and are associated with higher grain yield in field trials. Thus, these results showed that *HvSAP11* and *HvSAP15* are potentially useful candidate genes for tolerance to salt and drought abiotic stress in barley plants.

The above result may help us to further understand the mechanisms of barley defence to stress condition and provide candidate genes for breeding of stress resistant crops. Further characterisation of the *HvSAP* gene will provide a useful genetic resource for enhancing abiotic stress tolerance in plant. In future work, the analysis may be extended to study more barley accessions from other countries, for example Saudi Arabia, where the environmental conditions of the Middle East are also characterised by soil salinity and drought. Furthermore, barley is also considered an essential crop in Saudi Arabia.

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