Physiological, biochemical and molecular responses of different barley varieties to drought and salinity

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Muhammad Tauhid Iqbal

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1. Gutachter: Prof. Dr. Dorothea Bartels

2. Gutachter: PD Dr. Ali Ahmad Naz

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DECLARATION

I hereby declare that the whole PhD thesis is my own work, except where explicitly stated otherwise in the text or in the bibliography.

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Muhammad Tauhid Iqbal

Contents

ABBREVIATIONS

µl	Micro litre
µM	micro Molar
AA	Ascorbic acid
ABA	Abscisic acid
ABA-GE	ABA glucosyl ester
ABF	ABA-responsive element (ABRE)-binding factor
ABRE	ABA-responsive element
ALDH	Aldehyde dehydrogenase
AP2	APETELLA2
APS	Ammonium persulfate
APX	Ascorbate peroxidase
AREB	ABA-Responsive Element Binding protein
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
BALDH	Betaine ALDH
BC	Backcross
bp	Nucleotide base pair
BSA	Bovine Serum Albumin
bZIP	leucine zipper
C	Cytosine
Ca	Calcium
$CaCl_2$	Calcium chloride
Cat	Catalase
Cat	Catalase
CBF	C-repeat Binding Factor
cDNA	Complementary DNA
CFC	Chlorofluorocarbons
CH_4	Methane
cms	cytoplasmic male sterility
CO_2	Carbon dioxide
CRT	C-repeat
Da	Dalton
dCTP	Deoxycytidine triphosphate
ddH_2O	Double distilled water
Dhn	Dehydrin gene
DHN	Dehydrin protein
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphat
DRE	Dehydration-Response Element
DREB	Dehydration-Responsive Element Binding protein
DTT	Dithiothreitol
DW	Dry weight
EDTA	Ethylenediaminetetraacetate
EMS	Ethyl Methane Sulfonate
EPA	Environment protection agency USA
ERF	Ethylene-Responsive Element Binding Factor

FW	Fresh weight
FWD	Forward
g	Gram (weight)
g	Acceleration
Gb	Gigabases
GB	Glycinebetaine
GFP	Green Fluorescent Protein
Glu	Glutamic acid
Gly	Glycine
GPX	Guaiacol peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GST	glutathione-S-transferase
h	Hour
H_2O_2	Hydrogen peroxide
HSP	Heat shock proteins
K^+	Potassium ion
KAc	Potassium acetate
kb	Kilobases
kDa	Kilodalton
Km	Kilometers
KOH	Potassium hydroxide
LB	Lysogeny broth
LEA	Late Embryogenesis Abundant
LiCl	Lithium Chloride
LTRE	Low-temperature-responsive element
Lys	Lysine
M	Molar, mole(s) per litre
mA	milliamperes
MDA	Malondialdehyde
$MgCl_2$	Magnesium chloride
min	Minute
ml	millilitre
mM	milliMolar
MOPS	3-(N-morpholino) propanesulfonic acid
mRNA	messenger RNA
MS	Murashige and Skoog (1962)
MYB	MYeloBlastosis
MYC	MYeloCytomatosis
N_2O	Nitrous oxide
Na^+	Sodium ion
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
O_3	Ozone
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffer Saline
PBTB	Protein-Blot Transfer Buffer
PCR	Polymerase Chain Reaction

Phe	Phenylalanine
PIPES	Piperazine-N,N,-bis (2-ethanesulfonic acid)
POX	Peroxidase
PP2C	Protein Phosphatase 2Cs
Pro	Proline
PUFA	Poly-unsaturated fatty acid
PVP	Polyvinylpyrrolidone
rd29	Responsive to dessication (29)
REV	Reverse
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive Oxygen Species
rpm	Rounds per minute
RT	Room temperature
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
RWC	Relative water contents
SDS	Sodium dodecyl sulfate
Ser	Serine
SOD	Superoxide dismutase
SSC	Saline Sodium Citrate buffer
ssDNA	Single-stranded DNA
ssp	Sub Specie
T_a	Annealing temperature
TAE	Tris-Acetate-EDTA
Taq	*Thermophilus aquaticus*
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TE	Tris (10mM)-EDTA (1 mM)
TEMED	N,N,N',N'-tetramethylethylenediamine
TF	Transcription factor
T_m	Melting temperature
Tris	Tris-(hydroxymethyl)-aminomethane
TW	Turgid weight
U	Units
UV	Ultraviolet
V	Volts
v/v	Volume/volume
w/v	Weight/volume
WLR	Water loss rate
ZFs	Zinc fingers

FIGURES AND TABLES

SUMMARY

Barley *(Hordeum vulgare)* is an important member of grass family *Poaceae*. Among various agricultural crops, barley is considered a model plant due to its important features like short season, tolerance to abiotic stress, large number of varieties and availability of sequenced genome. Ten old barley varieties from different parts of the world; Reisgerste II, Candice, Scarlett, Heilis Frankin, Himalaya USA, Himalaya Nepal, Himalaya Winter, Himalaya Freak, Himalaya Nakt and Himalaya India were selected for studying the drought and salt tolerance mechanisms. All these varieties were grown in hydroponic cultures. Relative water contents (RWC) and water loss rate (WLR) of the plants were measured to have a rough estimation of stress tolerance and water retention rates in plants. The seedlings were subjected to drought stress after two weeks after germination by stopping irrigation. Salt stress was imposed by treating the plants with 200 mM NaCl and 400 mM NaCl solutions for seven days. Although the RWC and WLR in varieties showed that all the barley varieties had a range of oxidative stress tolerance, Himalaya Nakt, Himalaya India and Scarlett were with better water retention capability than others while Himalaya Freak had the least one.

Although the plants were affected by 200 mM salt treatment as well, yet the effect of 400 mM NaCl treatment and drought stress were much more than that of 200 mM NaCl treatment. The amount of total chlorophyll contents estimated from the leaves showed a greater decrease at 400 mM NaCl and drought treatments moreover the decrease was much more in the plants with less water holding capacity, showed the degradation of chlorophyll in the plants.

Proline is an amino acid that contributes in scavenging ROS, hence enhances oxidative stress tolerance in living organisms. Proline content increased in all the varieties on stress treatments. Increase in proline contents in tolerant varieties was more than five times during stress conditions, in less tolerant varieties the observed increase was two times at drought and 400 mM NaCl.

The amounts of MDA and H_2O_2 in the plants show the susceptibility of the plants towards oxidative stress. MDA level in Himalaya Freak was double than that were found in Himalaya Nakt, Himalaya India and Scarlett in drought stress while even more than double 400 mM NaCl. Similarly, amount of H_2O_2 in Himalaya Freak was

almost 1.6 times higher than in tolerant varieties in drought stress while 2.5 times in case of 400 mM NaCl treatment.

Antioxidants are known to inhibit the oxidation of biological molecules thereby protecting the cells against oxidative damage. The activities of different anti-oxidative enzymes like SOD, catalase, peroxidase and glutathione reductase were measured. The activities of SOD and catalase increased in 200 mM NaCl while in 400 mM NaCl and drought stress their activities were at par with control plants in the majority of the varieties. However, in Himalaya Freak it decreased on all the three treatments. The activities of glutathione reductase and peroxidase increased in all varieties on all treatments except for Himalaya Freak, where no significant difference was found at 200 mM NaCl treatment. The activities of anti-oxidative enzymes in the varieties like Scarlett, Himalaya Nakt and Himalaya India were much more than in Himalaya Freak, Reisgerste II and Candice.

In order to correlate the physiological and biochemical changes with molecular changes, the differences in gene expression levels of different dehydrins were analyzed. The reverse-transcriptase polymerase chain reaction (RT-PCR) was performed to analyze the relative expression levels of different dehydrins as indicator of stress tolerance. Among the 13 dehydrins found in barley, Dhn8 and Dhn13 were constitutively expressed in all varieties. Dhn10 and Dhn11 did not express in any variety. The expressions of Dhn1, Dhn6 and Dhn7 correlate with physiological and biochemical data.

To summarize, the physiological, biochemical and molecular analysis of different varieties of Barley at different stress conditions suggests that out of selected 10 varieties, Scarlett, Himalaya Nakt and Himalaya India were found to be most tolerant varieties and Himalaya freak was found to be the most susceptible variety.

1. INTRODUCTION

1.1 Barley- *Hordeum volugare* as experimental plant

Barley is one of the most important cereals. It is also one of the first ever grown among the cultivated grains especially in Eurasia region of world (Zohary and Hopf, 2000). It is also well adapted to the drought, salt and cold stresses.

The barley plant *Hordeum volugare* is classified as

Kingdom: *Plantae* – Plants

Subkingdom: *Tracheobionta* – Vascular plants

Superdivision: *Spermatophyta* – Seed plants

Division: *Magnoliophyta* – Flowering plants

Class: *Liliopsida* – Monocotyledons

Subclass: *Commelinidae*

Order: *Cyperales*

Family: *Poaceae* – Grass family

Genus: *Hordeum* L. – barley

Cereal crops provide about two-thirds of worldwide human calorie intake, both directly and indirectly in the form of meat and milk from animals raised on cereal feed. Barley is among the oldest cereal crop, which ranked fifth in 2014 in terms area of production after wheat, maize, rice and soybean. It is cultivated on approximately 49.4 million hectare (http://faostat.fao.org). Barley is grown primarily for food and malting.

Among cereals, barley is considered as model plant for genetic and physiological studies due to the following features (Saisho and Takeda, 2011);

- It is a true diploid plant with a high rate of self-fertilization i.e. 99%.
- Cross-fertilization is also not difficult.

- Barley has many variants/varieties that are morphologically and physiologically different from others.
- It requires a short season of 2-3 months to complete its life cycle.
- It is cultivated on a variety of environmental conditions.
- It is also considered as tolerant to drought, cold, salinity and alkalinity.
- It contains a large genome 5.1 Gb in size on seven different chromosomes 2n=14 (Barley genomes consortium, 2012)

It is considered that cultivated barley (*Hordeum vulgare ssp. vulgare*) is domesticated from the wild barley (*Hordeum vulgare ssp. spontaneum*) and the near East Fertile Crescent is thought to be the only place where the wild barley was domesticated (Harlan and Zohary, 1966; Nevo, 2006; Zohary et al., 2012) but discovery of *H. vulgare ssp. Spontaneum* on other places like Tibet, Central Asia, Morocco, Libya, Egypt and Ethiopia has raised questions on the only domestication theory (Dai et al., 2012; Molina-Cano et al., 2002). Recent molecular studies proposed Central Asia, 1,500–3,000 Km farther east from the Fertile Crescent (Morrell and Clegg, 2007), and Tibet of China (Dai *et al*., 2012) as additional centers of wild barley domestications, and supported multiple origins of cultivated barley.

1.2 Stress

Stress is an exogenous factor, which has a negative effect on the plants. Usually all the organisms have to face two kinds of stresses: biotic and abiotic. Biotic stresses are consequences of the activities of other organism to the particular organism. However, the abiotic stresses are effects of harsh conditions and environment on that organism. Abiotic stress completely depends on the tolerance level of the particular plant, as the conditions, which are encouraging for one organism, could be unfavorable for the other. Because the higher plants are unable to move, it prompts them to develop some response against these harsh environment, as a result plants attain some special mechanism to manage the situation.

1.2.1 Relationship between global warming and abiotic stress

It has now become a universal truth that the average temperature of globe and rate of rainfall have changed significantly (Fauchereau et al., 2003; Jung et al., 2002). The increase in temperature is due to the increase in the concentration of greenhouse gases like carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O), ozone (O_3) water vapors and chlorofluorocarbons

(CFCs). Since the industrial revolution, the amount of greenhouse gases in the atmosphere has significantly increased. According to the report of environment protection agency USA (EPA 2007), the concentrations of CO_2 and CH_4 have increased by 36% and 148% respectively since 1750, due to the burning of fossil fuels and deforestation.

Global warming is affecting the crop production system in many ways for example, in *Indo-Pak* there are two major crop-growing seasons i.e. summer or *kharif* and winter or *rabi*. The summer rainy season (monsoon) in the *Indo-Pak* provides water to crops of both *Rabi* and *kharif* seasons. As the monsoon occurred in *kharif* and the precipitation at the end of the season provide soil moisture and irrigation for the *rabi* season crop. The global warming has affected the monsoon season all over the world resulting in drought and floods which affected the food grain production (Krishna Kumar et al., 2004; Parthasarathy et al., 1992; Selvaraju, 2003). All the episodes like floods, drought, heat waves, cyclone, and hailstorms cause great problem to the crops.

1.2.2 Drought Stress

Stress is any physiological modification which may change the plant internal equilibrium (Gaspar et al., 2002). Less available water in the soil and continuous loss of water due to transpiration and evaporation is the general cause of drought stress. A different level of drought stress tolerance has been observed in different plants. Drought stress is less loss of water, which causes stomatal closure and limitation of gas exchange while desiccation is the extreme loss of water, which may lead to disturbance of metabolism and cell structure resulting in stopping enzyme-catalyzed reactions (Jaleel et al., 2007; Smirnoff, 1993).

1.2.3 Salt stress

Under high salinity plants suffers from two kinds of stresses, i.e. osmotic stress and ionic stress. Osmotic stress results in reducing or inhibiting the water uptake of plant. While ionic stress causes the accumulation of huge amount of Na^+ which damages the leaves with chlorosis and necrosis (Glenn et al., 1999; Horie et al., 2012; Yeo and Flowers, 1986).

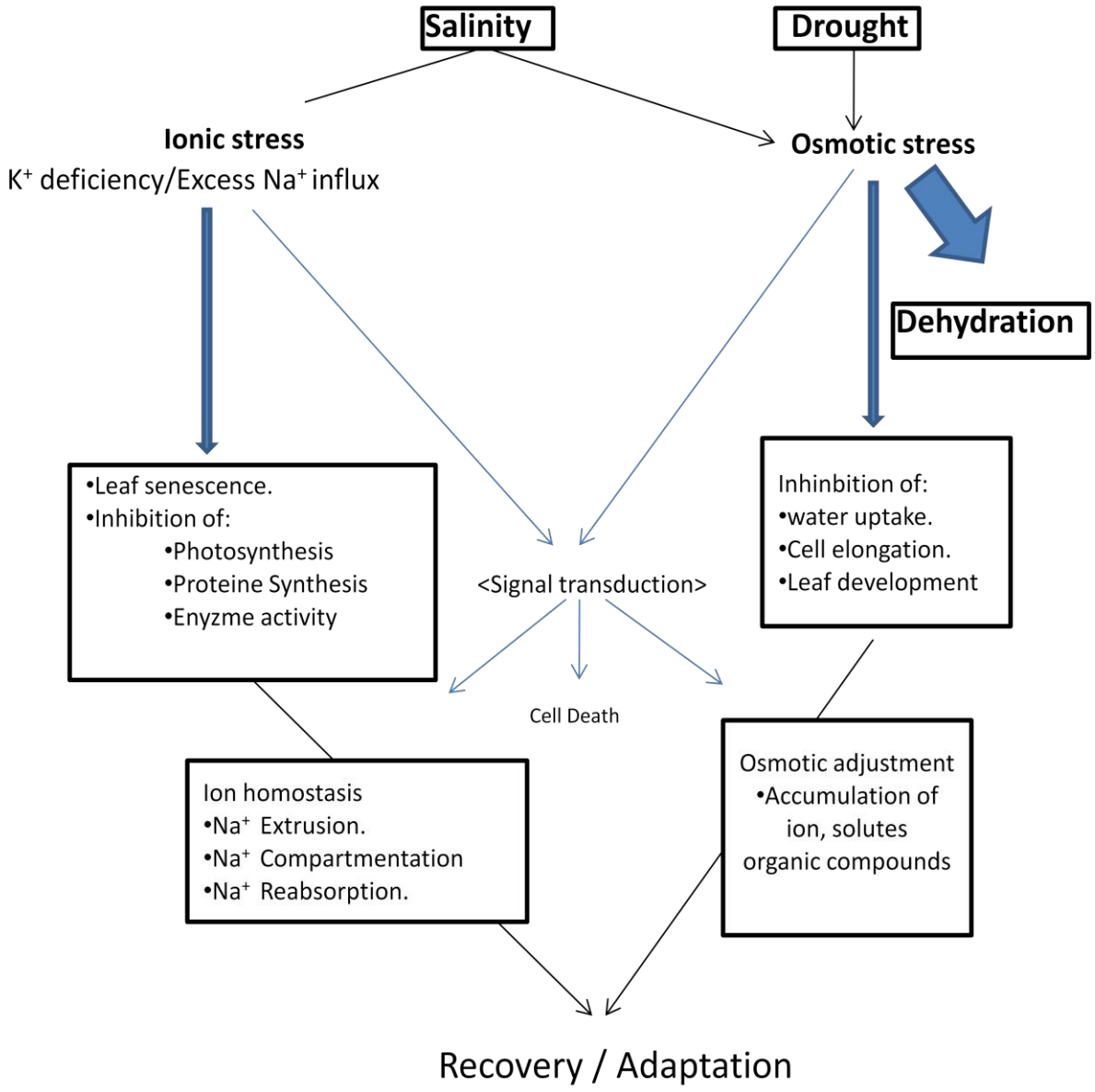

Figure 1.1: Schematic diagram of response of plants on drought and salinity stress. This scheme modified from (Horie et al., 2012).

1.2.4 Effect of Stress on Morphology of Plant

Decrease in leaf area is one of the earliest responses to water deficiency. When the water contents of the plant decrease the volume of the cell is decreased due the shrinkage of the cells. At early stages of plant development drought stress can limit the plant growth has well. Limited water not only affects the area of the leaf but the number of the leaves as well as it decreases growth and number of branches (Taiz and Zeiger, 1998). Water deficiency in soybean decreased the stem length of the plant (Specht et al., 2001). Similarly in potato the plant height decreased in the restricted water environment (Heuer and Nadler, 1995).The citrus plants grown under drought conditions have 25% less height (Wu et al., 2008). The reduction in plant height could be the result of decrease in cell elongation. Under drought conditions disturbance in water flow from the xylem towards surrounding elongating cells causes inhibition in the elongation of the cell (Nonami, 1998). Drought causes impaired mitosis; cell elongation and expansion resulted in reduced growth (Hussain et al., 2008). Water scarcity promotes root growth into the deep moist soil. Inhibition of the leaf elongation causes assimilates to distribute into the root system resulting into the growth enhancement (Taiz and Zeiger, 1998). Under the limited water supply, the root to shoot ratio is higher in comparison with plants having precise water supply as roots are less sensitive to drought than shoots (Wu and Cosgrove, 2000). Under the water deficient environment, a reduction in biomass is observed in majority of the plants like sunflower (Tahir and Mehid, 2001), suger beet (Mohammadian et al., 2005), soybean (Specht et al., 2001), *Poncirus trifoliatae* (Wu et al., 2008) and in barley (Wehner et al., 2015). Shortage of available water affects different pigments in the plants like chlorophyll and carotenoids. A reduction in chlorophyll content was observed in drought stressed cotton (Massacci et al., 2008), sunflower ((Kiani et al., 2008; Manivannan et al., 2007) and chickpea (Mafakheri et al., 2010).

1.2.4.1 Physiological adaptations

As the receptors in the plant cells sense the stress and regulatory mechanisms induce the initiation of cascades of reactions. These stimuli result in the activation of nuclear transcription factors to induce the expression of many genes and proteins (Boudsocq and Laurière, 2005). Disturbance in the water uptake and evaporation equilibrium alters turgor, which results into stomatal closure (Taiz and Zeiger, 1998). The opening and closing of stomata deals with the regulation of transpiration and closed stomata protects water loss from the leaves under stress conditions. Loss of turgor from guard cell resulting in closing the stomata is because of ABA

that enhances K^+ ions efflux from the guard cells. Drought stress causes loss of cell turgor and cell membrane perturbation results into 50% increase in ABA level (Guerrero and Mullet, 1986).

Stomatal closure not only regulates the transpiration but also affects the photosynthesis by limiting the CO_2 supply. Abiotic stresses disturb other photosynthesis components like thylakoid electron transport, the carbon reduction cycle, increased accumulation of carbohydrates, peroxidative destruction of lipids and disturbance of water balance (Allen and Ort, 2001). For example drought stress caused 33% reduction in photosynthesis in maize (Anjum et al., 2011).

1.3 Stress Tolerance

According to Ingram and Bartels (1996), three main techniques have been used to study stress tolerance in plants; a) studying tolerance system in seeds or in the resurrection species as the seeds and resurrection plants have the capability to withstand desiccation; b) by analyzing the mutants from model plants like Arabidopsis; and c) studying tolerance mechanism in crops of agricultural importance. The last approach is more useful as due to the rigorous breeding and invitro selection screened lines with different grades of tolerance are available within single species.

1.3.1 Regulatory Mechanism

Cellular stresses trigger the signaling pathways that control many physiological aspects of the cells. Regulation of gene expression, changes in cell metabolism, protein homeostasis and changes in enzymatic activities are among the major stress responses of organisms. These responses consist of general responses that correspond to many stresses and specific adaptive responses for particular stresses.

1.3.1.1 Regulation of Gene Expression

Regulation of gene expression is among primary stress responses in organisms. Any kind of abiotic stress induces the expression of many genes regulated by complex network (Yamaguchi-Shinozaki and Shinozaki, 2006).

1.3.1.1.1 Transcription Factors

Transcription factors (TFs) are the protein, which control many plant functions like they bind with specific DNA sequence to regulate the transcription of genetic information (Latchman, 1997; Mitsuda and Ohme-Takagi, 2009). Transcription factors do their job alone or by in combination with other proteins making a complex by enhancing or inhibiting recruitment of

polymerase to specific genes (Lee and Young, 2000; Nikolov and Burley, 1997; Roeder, 1996). The cis-elements present in the promoter region of certain genes work together with transcription factors to control the expression of genes to show stress tolerance (Agarwal and Jha, 2010). Some large families of transcription factors are as; basic leucine zipper (bZIP), APETALA 2/ethylene-responsive element binding factor (AP2/ERF), NAM/ATAF1/CUC2 (NAC), WRKY, MYB, Cys2(C2)His2(H2)-type zinc fingers (ZFs), and basic helix-loop-helix (bHLH) and the members of these family are usually characterized according to their features and their role in abiotic stress tolerance (Lindemose et al., 2013).

1.3.1.1.2 ABA responsive Elements (ABRE)

Abscisic acid (ABA) plays a vital role in regulating many of the plant physiological processes including the stimulation of stress related genes (Agarwal and Jha, 2010). ABA is one of the key signals which cause drought and salinity responses and variety of factors like drought, desiccation, excessive water stress, salinity, heat and wounds induce the production of ABA (Farooq et al., 2009; Gómez et al., 1988; Hubbard et al., 2010; Verslues and Bray, 2005). Most of the genes, which are induced by the osmotic stress, are also induced by the exogenous ABA but there are some which are not induced by the external ABA (Chandler and Robertson, 1994; Leung and Giraudat, 1998; Zhu, 2002) suggests that there are ABA dependant and ABA independent pathways to regulate the gene expression. ABA dependant pathways control the genes expression through i) bZIP transcription factors and ABRE cis-regulatory elements (Busk and Pagès, 1998) and ii) MYC and MYB elements and transcription factors (Yamaguchi-Shinozaki and Shinozaki, 1993).

1.3.1.1.3 Dehydration Response Elements (DRE)

Comprehensive molecular studies showed many specific *cis*-regulatory elements are present which control the induction of large number of stress responsive genes under different environmental stresses (Lata and Prasad, 2011). There are several genes, which are induced in the ABA deficient and ABA insensitive mutants, showed that these genes do not require ABA stimulus to express themselves, under stress conditions (Bartels and Sunkar, 2005). A 9bp-conserved sequence (5'-TACCGACAT-3') was recognized as drought responsive element for first time in the promoter region of the drought responsive Arabidopsis gene rd29 (Yamaguchi-Shinozaki and Shinozaki, 1993). After this discovery, it was reported in many studies that drought responsive elements are involved in different abiotic stress responses and is an essential

cis-regulatory element for rd29A induction in the ABA independent response against dehydration in Arabidopsis (Busk and Jensen, 1997; Dubouzet et al., 2003; Kizis, 2002; Liu et al., 1998; Saleh et al., 2005; Yamaguchi-Shinozaki and Shinozaki, 1994).

1.3.1.2 Compatible solute Accumulation

All the plants have developed different mechanism to cope with different environmental stresses (drought, salinity, cold etc) and accumulation of different low molecular organic solutes is one of them (Bohnert et al., 1995). These organic solutes are also called as osmoprotectant, they act as osmolytes to protect the organism from the extreme environmental conditions (Lang, 2007). Osmolytes are non-toxic compounds even at high concentrations. They do not obstruct the regular metabolism and usually accumulate in the cytoplasm under osmotic stress (Chen and Murata, 2002; Flowers et al., 1977; Jones et al., 1977; Yancey, 2005).

There are variety of osmolytes present in the plants, among carbohydrates like glucose, fructose, sucrose, trehalose, raffinose and fructans and among sugar alcohols such as sorbitol, mannitol, glycerol, inositol and methylated inositols. Some of the osmolytes are amino acids like proline, pipecolic acid, some methylated proline-related compounds, like methyl-proline, proline betaine and hydroxyproline betaine, some other betaines, such as glycine betaine, β-alanine betaine, choline *O*-sulphate; and tertiary sulphonium compounds, such as dimethylsulphoniopropionate are also present in plants (Ashraf and Foolad, 2007; Rhodes et al., 2002; Slama et al., 2015).

The osmolytes/osmoprotectants act in maintaining the turgor and as protein and cell structure stabilizer (Yancey et al., 1982)they have capability to change the solvent properties of water (Yancey, 2005). Some osmoprotectants like proline subjects to rapid variations and others like betaines accumulate for longer period of time (Gagneul et al., 2007).

1.3.1.2.1 Sugars and Sugar Alcohols

Under stress conditions non-structural carbohydrates like sucroses, hexoses and sugar-alcohols are accumulated (Bartels and Sunkar, 2005; Briens and Larher, 1982; Yuanyuan et al., 2009). It is also reported that accumulation of sugars in plants correlates with osmotic stress tolerance (Baki et al., 2000; Gilmour et al., 2000; Ramanjulu et al., 1994; Streeter et al., 2001; Taji et al., 2002). The sugars are supposed to guard certain macromolecules and stabilize membrane structure (Bartels and Sunkar, 2005) and they may protect cells by forming glass structure (Black and Pritchard, 2002). Sugars also help to uphold the growth of sink tissues and regulate the

expression of many genes by affecting the sugar sensing system (Hare et al., 1998). Trehalose, which is a non-reducing sugar, soluble in water but chemically it is un-reactive, make it compatible with cellular metabolism even at high concentrations (Slama et al., 2015). However trehalose is present in bacteria, fungi, yeast and nematodes (Bartels and Sunkar, 2005; Fernandez et al., 2010; Lunn et al., 2014). The proposed function of sugar alcohols could be stabilizing macromolecules and promoting scavenging systems for reactive oxygen species (Llanes et al., 2013)

1.3.1.2.2 Glycinebetaine

Glycinebetaine (GB) is extensively found in the majority of plants, animals and microorganisms (Chen and Murata, 2008; Rhodes and Hanson, 1993). The accumulation of glycinebetaine is widely studied in response to drought, salt and extreme temperature conditions (Gorham, 1995) and found that the plants, which accumulate small amounts of glycinebetaine in normal conditions known as natural producers of glycinebetaine, produce a large amount when exposed to abiotic stress (Hussain Wani et al., 2013; Storey et al., 1977). Some crops of economic significance like potato and tomato are unable to produce glycinebetaine neither in normal nor in stressed conditions (McCue and Hanson, 1990).

The accumulation of glycinebetaine differs in different transgenic lines, producing different levels of stress tolerance and the transgenic plants produce only a low level of glycinebetaine, hence producing only limited tolerance (Hayashi et al., 1997; Hussain Wani et al., 2013).

1.3.1.2.3 Proline

Proline is an amino acid and widely distributed osmoprotectant in plants and many other organisms (Delauney and Verma, 1993; McCue and Hanson, 1990). It accumulates in large quantities in response to various environmental stresses (Ali et al., 1999; Kishor et al., 2005) like in drought stress (Hare et al., 1998), in salinity (Hong et al., 2000; Munns, 2005; Rhodes et al., 2002), in low temperature (Naidu et al., 1991), in heavy metals (Bassi and Sharma, 1993; Sharma and Dietz, 2006) and in ultraviolet (UV) radiations etc (Hayat et al., 2012).It accumulates in cytoplasm. Proline is multifunctional amino acid which not only functions as osmolyte for osmotic adjustment but various other functions as to stabilize sub-cellular structure e.g. membranes and proteins, to scavenge free radicals and to buffer cellular redox potential under stress conditions (Kaur and Asthir, 2015; Rodriguez and Redman, 2005). It operates as a

sink of energy to reduce power (Verbruggen et al., 1996) and a source of carbon and nitrogen (Ahmad and Hellebust, 1988; Peng et al., 1996). It also works as protein-compatible hydrotrope (Hayat et al., 2012; Strizhov et al., 1997). It mitigates cytoplasmic acidosis and maintains suitable $NADP^+$/NADPH ratios compatible with metabolism (Hare and Cress, 1997). The concentration of proline is found to be higher in stress tolerant plants as compared to the stress sensitive plants (Fougere et al., 1991; Petrusa and Winicov, 1997). Contrarily there is also a report about antisense ProDH (Proline dehydrogenase) transgenic Arabidopsis plants, where no increase in stress tolerance has been observed even on proline accumulation (Mani et al., 2002).

1.3.1.3 ROS scavenging enzymes

Reactive oxygen species (ROS) mainly consists of peroxides, superoxide, hydroxyl radical, and singlet oxygen (Hayyan et al., 2016) and are very dangerous to DNA, proteins and lipid (Apel and Hirt, 2004; Foyer and Noctor, 2005). Generally, ROS are formed in the metabolism of oxygen, however in stress conditions their quantity increase drastically limiting normal cell functions. In oxidative stress, redox homeostasis is maintained by enzymatic antioxidents like superoxide dismutase (SOD), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), glutathione-S-transferase (GST), and catalase (CAT), and non enzymatic low molecular compounds like ascorbic acid (AA), reduced glutathione (GSH), α-tocopherol, carotenoids, phenolics, flavonoids, and proline (Gill et al., 2011; Gill and Tuteja, 2010; Miller et al., 2010).

1.3.1.4 Protective proteins

Under the majority of abiotic stress situations, some specific stress associated protective proteins accumulate and they are supposed to play a vital role in plants stress response (Bohnert and Sheveleva, 1998; Hoekstra et al., 2001; Ingram and Bartels, 1996). Heat shock proteins, late embryogenesis abundant (LEA)- type proteins are accumulated in huge amount on exposure to drought, salinity and extreme temperature stresses and perform many function in regulating the homeostasis of plants (Hussain et al., 2011).

1.3.1.4.1 Heat Shock Proteins (Hsps)

Heat shock proteins are produced to protect the cell against abiotic stress environment, these were first discovered in response to heat shock (Ritossa, 1962). Further studies revealed that these proteins were found to accumulate in response to cold (Matz et al., 1995), ultraviolet light (Cao et al., 1999), drought (Campalans et al., 2001; Coca et al., 1996; Kawasaki et al., 2000) and

wound stresses (Laplante et al., 1998). Heat shock proteins contain many chaperones, which assist in folding and assembly of proteins during protein synthesis and unfolding and removal of degraded proteins. Heat shock proteins are classified into HSP110, HSP90, HSP70, HSP60, and small HSP based on their molecular weights (Kosová et al., 2015; Xu et al., 2012).

1.3.1.4.2 Late Embryogenesis-Abundant (LEA) Proteins

LEA proteins, is a protein family, which protects the other cellular proteins from aggregation in abiotic stress (Goyal et al., 2005). These proteins were first discovered in cotton seeds at late stages of embryo development (Dure III et al., 1981; Galau et al., 1986; Liang et al., 2013a). In plants, at late stages of seed development, the water contents in the seed decrease with maturation, which induce ABA production. Increase in ABA concludes into the expression LEA genes which ultimately results in the acquisition of stress tolerance (Goldberg et al., 1989; Skriver and Mundy, 1990). Accumulation of LEA proteins and the attainment of desiccation tolerance indicates the correlation between these parameters (Bartels et al., 1988). Generally, in vegetative tissues, LEA proteins only accumulate in osmotic stress situations or on the application of exogenous ABA, and protect the cells (Ingram and Bartels, 1996). Therefore an increased accumulation of LEA protein were found under drought and cold stress (Brini et al., 2007; Crosatti et al., 1995; Houde et al., 1992; Kosová et al., 2008; Vágújfalvi et al., 2000; Vágújfalvi et al., 2003; Vítámvás et al., 2007).

1.3.1.4.2.1 Dehydrins (DHNs)

Dehydrins are a group of intrinsically disordered proteins, which are sub-classified as LEA-II (Bray, 1993) or LEA D-11 (Dure et al., 1989). Like other members of LEA protein family, they also accumulate at late stages of embryogenesis (Allagulova et al., 2003; Goday et al., 1994; Momma et al., 2003). However, in vegetative tissues their accumulation is only possible under cell dehydration conditions like drought, salt or cold etc (Ingram and Bartels, 1996; Ismail et al., 1999; Lisse et al., 1996; Nylander et al., 2001) Accumulation of dehydrin corresponds to the tolerance of the plant towards stress environment (Close, 1997).

The characteristic feature of the dehydrins is lysine rich 15 amino acid long conserved motif (EKK GIM E/DKI KEK LPG) near C terminus. Some other conserved amino acid segments like tyrosine rich segment called Y- segment [(V/T)D(E/Q) YGNP] near N-terminus of dehydrins, S segment (contains 4-10 serine segments) or rarely and less conserved usually rich with polar

amino acid called Φ-segments (Close, 1997). Dehydrins are further classified into Y_nSK_n, Y_nK_n, SK_n, K_n and K_nS depending on the arrangement and number of conserved motives (Campbell and Close, 1997; Close, 1996).

There are many proposed functions of dehydrins. According to Danyluk et al. (1998), dehydrins reduce damages caused by dehydration as they interact membranes within the cells, they may prevent the interaction of membrane bilayers or due to their ability to chelate ions. Dehydrins are also said to perform chaperones like function i.e. stabilization of the membranes, resistance to osmotic pressure and protecting other proteins (Agoston et al., 2011). Some of the functions of dehydrins are related to their particular structure, e.g. YSK_n type dehydrins bind to the lipid vesicles that contain acidic phospholipids and K_nS dehydrins can form bond with metals and are able to scavenge hydroxyl ions (Alsheikh et al., 2003; Asghar et al., 1994), they protect lipid membranes from lipidperoxidation also act as a cryoprotectants. Dehydrin of SK_n and K_n type dehydrins help in attaining cold adaptation (Allagulova et al., 2007; Danyluk et al., 1998; Houde et al., 1995; Zhu et al., 2000). While Y_nSK_m proteins are low molecular weight proteins, which are induced due to drought, stress (Vaseva et al., 2010; Xiao and Nassuth, 2006).

1.3.1.4.2.1.1 Dehydrins in Barley

Until now, thirteen dehydrins protein have been discovered in barley. The architecture and classification of the barley dehydrins are shown in the figure 2 and phylogenetic tree these dehydrins showing how these dehydrin are close to each other is mentioned in figure 3. Majority of the dehydrin protein in barley belong to the Y_nSK_m (8 out of 13).

Dehydrin	Architecture	Class
DHN1	Y S K K	YSK2
DHN2	Y S K K	YSK2
DHN3	Y S K K	YSK2
DHN4	Y S K K	YSK2
DHN5	K K K K K K K K K	K9
DHN6	Y Y S K K K	Y2SK3
DHN7	Y S K K	YSK2
DHN8	S K K K	SK3
DHN9	Y S K K	YSK2
DHN10	S K K K	SK3
DHN11	Y Y S K K	Y2SK2
DHN12	S K K	SK2
DHN13	K S	KS

Figure 1.2: Architecture and classification of barley dehydrins.

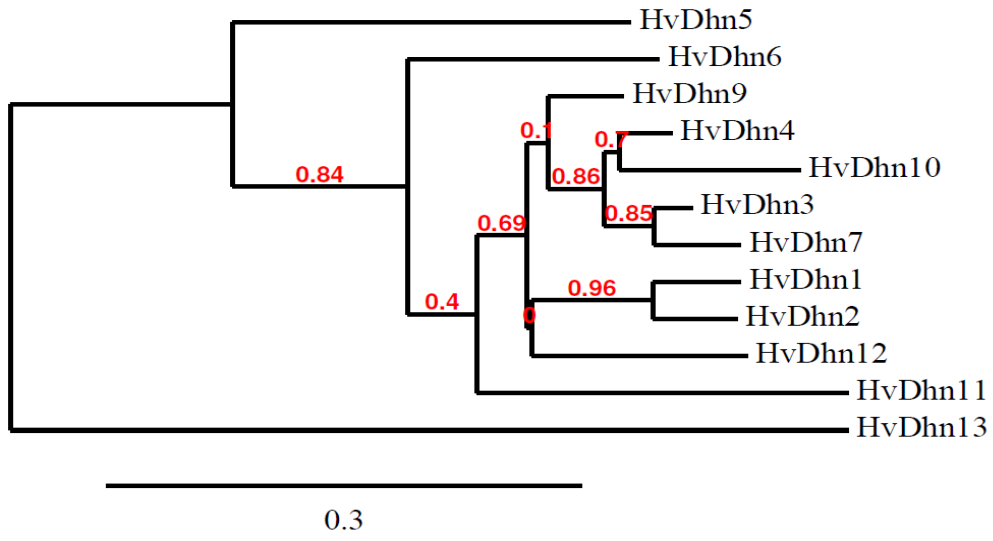

Figure 1.3: Phylogenetic tree of barley dehydrins showing closeness of the each member with other

1.4 Objectives of the studies

The major objective of my study was to evaluate different barley varieties under drought stress and different levels of salinity stress. As barley is tolerant to the abiotic stresses, so keeping in mind all the stresses were given at higher level, to assess the stress tolerance at higher level of stress.

There are many barley varieties with the name Himalaya, but from different regions and climate, so their performance was analyzed, how they differ with each other in tolerating drought and salinity and off course, I added some German varieties as well in this evaluation. Assessment under stress conditions was made on the following parameters.

- Evaluating their physiological behavior under stress.
- Their capability to induce enzymatic antioxidents and non-enzymatic osmolytes as scavenging agents for reactive oxygen species (ROS).
- Molecular evaluation, using expression of dehydrin genes as a marker of stress tolerance.

I was also interested in finding sub-cellular localization of the dehydrin proteins in brley.

1.5 Varieties of Barley

Barley varieties taken for this study originated from different part of the world. Most of them were from German and European origin, however some other from different regions. Majority of them are with the name Himalaya, but their origin was different.

Table 1.1: Name, origin and the growing season of barley varieties

	Name of Variety	**Origin**	**Winter/Spring**
1	CCS140 (Reisgerste II)	Germany	spring
2	Candice	UK	spring
3	Scarlett	Germany	spring
4	Heilis Frankin	Germany	spring
5	Himalaya USA	USA	spring
6	Himalaya Nepal	Nepal	spring
7	Himalaya winter	USA	winter
8	Himalaya Freak	USA	spring
9	Himalaya Nakt	Unknown	spring
10	Himalaya India	India	spring

.


Candice
Reisgerste II
Scarlett
H. Frankin
Him Freak
Him. Winter
Him. USA
Him. Nepal
Him. India

2. Materials and Methods

2.1 Materials

2.1.1 Plant material

Ten different varieties of barley (*Hordeum vulgare* L.) grown in different regions of the world were selected for characterization under control and stress conditions (drought and salt). The cultivars are as given below.

i. Reisgerste II
ii. Candice
iii. Scarlett
iv. Heilis Frankin
v. Himalaya USA
vi. Himalaya Nepal
vii. Himalaya Winter
viii. Himalaya Freak
ix. Himalaya Nakt
x. Himalaya India

2.1.2 Chemicals

Chemicals utilized in this work were ordered from the following companies:

- Amersham Bioscience, Freiburg, Germany
- AppliChem, Darmstadt, Germany
- BIOMOL, Hamburg, Germany
- Clontech, Saint-Germain-en-Laye, France
- Fermentas, St. Leon-Rot, Germany
- FLUKA, Buchs, Switzerland
- Hartmann Analytic GmbH, Stöckheim, Germany
- Hoechst AG, Frankfurt, Germany
- Invitrogen/GibcoBRL, Karlsruhe, Germany
- KMF, Lohmar, Germany
- Merck, Darmstadt, Germany

- Macherey-Nagel, Düren, Germany
- PEQLAB, Erlangen, Germany
- Pharmacia, Freiburg Germany
- Roche, Mannheim, Germany
- Roth, Karlsruhe, Germany
- Sigma-Aldrich, Steinheim, Germany

2.1.3 Kits

For this study, following kits were used:

- NucleoSpin® Extract II (Macherey–Nagel, Düren, Germany)
- RevertAidTM H Minus First Strand cDNA Synthesis Kit, (Fermentas, St. Leon-Rot, Germany)

2.1.4 Enzymes and DNA-marker

Restriction enzymes and their corresponding buffers were from Amersham Pharmacia Biotech (Freiburg, Germany), MBI-Fermentas (St. Leon-Rot, Germany), Roche/Boehringer (Mannheim, Germany), Sigma (Munich, Germany), Invitrogen/GibcoBRL (Karlsruhe, Germany). The DNA marker (1 kb ladder) was from Invitrogen/GibcoBRL (Karlsruhe, Germany).

2.1.5 Microorganisms

2.1.5.1 Escherichia coli DH10B (Lorow and Jessee 1990)

Genotype: F– endA1 recA1 galE15 galK16 nupG rpsL ΔlacX74 Φ80lacZΔM15 araD139 Δ(ara,leu)7697 mcrA Δ(mrr-hsdRMS-mcrBC) λ– . This strain was used as host strain for cloning.

2.1.6 Vector

2.1.6.1 pGJ280

This vector contains following features in following order, a dual CaMV35S promoter followed by a tobacco etch virus translational enhancer, the Green Fluorescent Protein (GFP) coding sequence (Tsien 1998) and the CaMV35S polyadenylation site (Reichel et al. 1996). It also carries a bla gene that confers the ampicillin resistance for selection. This vector was originally

constructed by Dr. G. Jach (Max-Planck-Institute, Cologne, Germany) and was used for protein localization analysis (Willige et al. 2009).

2.1.7 Machines and other devices

- Spectrophotometer SmartSpec 3000, Bio-rad, Hercules, Canada.
- T3-Thermocycler, Biometra, Göttingen, Germany.
- Power supply, Electrophoresis power supply, Gibco BRL, Carlsbad, Canada.
- UV illuminator Intas UV systems series, CONCEPT Intas Pharmaceutical Ltd., Gujarat, India.
- Imaging system Typhoon Scanner 9200 Variable Mode imager, Amersham Biosciences, Piscataway, NJ.
- SDS-PAGE Minigel system, Biometra, Göttingen, Germany.
- Protein blotting cell Criterion blotter, Bio-Rad, Hercules, Canada.
- Chemiluminescence detector Intelligent Dark Box II, FUJIFILM Corporation, Tokyo, Japan.
- Electroporation system GenepulserII Electroporator, Bio-Rad, Hercules, USA
- VersaFluor™ Fluorometer, Bio-Rad, Germany
- Storage Phophor Screen, Amersham Biosciences, Buckinghamshire, England.
- Confocal Laser Scanning Microscope ZE2000 with Laser D-eclipse C1, Nikon, Düsseldorf, Germany.
- Binocular microscope SMZ-800, Nikon, Düsseldorf, Germany.
- Particle Gun Biolistic®, Bio-Rad, Hercules, USA.

2.1.8 Buffers and Solutions

Table 2.1: Buffers and Solutions

Buffers/Solutions	Concentrations	
10X DNA loading buffer (10 ml)	25 mg	Bromophenol blue
	25 mg	Xylencyanol
	200 µl	50X TAE
	3 ml	Glycerol
	6.8 ml	ddH_2O
50X TAE	242 g	Tris base
	57.1 ml	glacial acetic acid
	100 ml	0.5 M EDTA, pH8.0
		add dd H_2O to 1 liter
10X TBE	108 g	Tris base
	55g	Boric acid
	40 ml	EDTA, pH 8.0
		add dd H_2O to 1 liter
1X TE buffer	10 mM	Tris-HCl, pH 8.0
	1 mM	EDTA, pH 8.0
20X SSC	3 M	NaCl
	0.3 M	sodium citrate
		adjust the pH to 7.0 with 1M HCl
10X PCR buffer	670 mM	Tris-HCl pH 8.8
	166 mM	$(NH_4)_2SO_4$
	4.5% (v/v)	Trtion® X-100
	2 mg/ml	Gelatin

	20 mM	$MgCl_2$
RNA extraction buffer	38% (v/v)	Buffer-saturated phenol
	0.8 M	Guanidine thiocyanate
	0.4 M	Ammonium thiocyanate
	0.1 M	Sodium acetate, pH 5.0
	5% (v/v)	glycerol
Glucose/Tris/EDTA (GTE)		
	50 mM	Glucose
	25 mM	Tris-Cl pH 8.0
	10 mM	EDTA
	Autoclave and store at 4 °C	
NaOH/SDS solution	0.2 N	NaOH
	1% (w/v)	SDS
	Prepare immediately before use	
Potassium acetate solution	1.5 M	Tris-HCl, pH 7.4
	1.5 M	NaCl
Laemmli Buffer	62.5 mM	Tris-HCl (pH 6.8)
	10%	glycerol
	2% (w/v)	SDS
	0.1%	Bromophenol blue
	0.7 M	β-mercaptoethanolethanol
	0.1 M	DTT freshly prepared just before use
1X SDS protein running buffer	25 mM	Tris-HCl
	192mM	Glycine
	0.1%	SDS
	pH 8.0 (Do not adjust pH)	

2.1.9 Primers

All the primers were designed using the online web service primer 3 plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and were synthesized from Sigma-Genosys (Steinheim, Germany) or Eurofins (Germany). All the primers were dissolved in TE buffer of a volume mentioned by the manufacturer to a concentration of 100 µM. The working solutions of the primers were prepared by diluting the stock solution to 10 μM. All the primers were stored at -20^{o}C. The primers used in this study are as listed below:

Table 2.2: List of primers.

Nr.	Name	Sequence	Tm.
1	Hv Dhn1 Fwd.	ACAATGGAGTACCAGGGTCA	58
2	Hv Dhn1 Rev.	TCCTTCATCCCCTTCTTCCT	59
3	Hv Dhn3 Fwd.	TCCAGCTCGTCTGAGGATGA	62
4	Hv Dhn3 Rev.	CATGATGCCCTTCTTCTCG	59
5	Hv Dhn4 Fwd.	AGTACCAGGGACAGCAGCAC	60
6	Hv Dhn4 Rev.	GCTGTCCGTAGCCGTAGGT	60
7	Hv Dhn5 Fwd.	CAGCAGACAGGTGGCATCTA	60
8	Hv Dhn5 Rev.	TAGTGCTGTCCAGGCAGCTT	61
9	Hv Dhn6 Fwd.	CTATGGAGGCTCTGGGATTG	60
10	Hv Dhn6 Rev.	ACGTCGTGGGTACCTGTGAT	60
11	Hv Dhn7 Fwd.	GCGTCGATGAGTACGGTAAC	57
12	Hv Dhn7 Rev.	TCCATGATGCCCTTCTTTTC	58
13	Hv Dhn8 Fwd.	AGGGGAAGCTCAAGGAGAAG	60
14	Hv Dhn8 Rev.	CCATGATCTTGCCCAGTAGG	60
15	Hv Dhn9 Fwd.	CACAAGACCCGTGGGATACT	60
16	Hv Dhn9 Rev.	TCTTGTCCATGATCCCCTTC	60
17	Hv Dhn10 Fwd.	AGAAACTTCCGGGAGGTCA	60
18	Hv Dhn10 Rev.	CGTGACCTTGCTGGTTGTAA	60
19	Hv Dhn11 Fwd.	CAGTACGGCAACCCCATC	59
20	Hv Dhn11Rev.	TGATGCCCTTCTTCTTCCTC	60
21	Hv Dhn13 Fwd.	AAGATCGAGGAGAAGCTCCA	59
22	Hv Dhn13 Rev.	TCTCCTTCTTCTCCTTGTGG	57
23	Hv Dhn3 NCO1 Fwd.	TGCACCATGGAGCACGGCCA	66
24	Hv Dhn3 NCO1 Rev.	TTGTCCATGGTGCCCTTCTT	61

2.2 Methods

2.2.1 Growth Conditions

2.2.1.1 Seed Culture and Plant Growth

Seeds of all the ten varieties were put on moist filter paper in Petri-plates at room temperature in the dark. After germination, the germinating plants were transferred to pots with clay pebbles for hydroculture. All the plants were grown under 120-150 μE m^{-2} s^{-1} light with day/night cycle of 16/8 h.

2.2.1.2 Stress Treatment

Until the age of two weeks normal tap water was provided to all the plants with fertilizer WUXAL (WUXAL® Universaldünger). At the age of 15 days, drought stress was induced by stopping water for one week. Salt stress was applied by providing the 200 mM NaCl and 400 mM NaCl solution to the plants while the control plants were still receiving normal tap water.

2.2.2 Morphological Analysis

After one week of stress treatment some plants were analyzed morphologically on the basis of the following parameters.

- Number of leaves per plant
- Shoot length per plant (plant height was taken in centimeter from the beginning of shoot to the longest leaf)
- Root length per plant (Root length was taken in centimeter from the beginning of roots to the longest root)

2.2.3 Plant Material Storage

After one week of stress treatment, the leaves and root samples of the plants were separated and ground in liquid nitrogen using pestle and mortar. All the samples were stored at -80^{o}C in 50 ml tubes for the further experiments.

2.2.4. Water loss rate

The water lose rate (WLR) of a plant determines the tolerance of a plant against drought stress. WLR of plants were calculated according to Suprunova *et al.* (2004). To evaluate the water loss rate, seedlings were grown on wet filter paper in the Petri-plates at room temperature. The weight of the first fully expanded leaf was measured (FW), right after it was cut. Then leaves

were put on filter paper for 24 hours to take (W24) the weight. Total dry weight (DW) was recorded after drying the leaves at 80^{o}C for 24 hours.

WLR(g h^{-1} g^{-1} DW) = (FW - W24)/ (DW× 24)

Where,

WLR = Water loss rate

DW = Dry weight

FW = Fresh weight

W24 = Weight after 24 hours.

2.2.5 Leaf relative water contents of leaves

Leaf relative water contents (RWC) were calculated with the method of Barrs and Weatherley (1962). Leaf relative water contents were measured in control plants and drought treated plants. To provide drought stress ten days old plants were put on a filter paper for 24 hours, while the control plants were watered normally. After 24 hours of treatment fully expanded leaves were excised and fresh weight (FW) was measured immediately. Afterwards the leaves were soaked in the distilled water for 24 hours in darkness at 4^{o}C to record turgid weight (TW). Total dry weight (DW) was then taken after drying them at 80^{o}C for 24 hours. Relative water contents were calculated according to the formula:

$$RWC\ (\%) = [(FW - DW)/(TW - DW)] \times 100$$

In this formula

RWC = Relative water content

FW = Fresh weight

DW = Dry Weight

TW = Turgid Weight

2.2.6 Extraction of Nucleic Acids

2.2.6.1 Extraction of RNA

Total RNA was extracted from leaf and root tissues by the method of Valenzuela-Avendaño et al. (2005). 1.5 ml of the extraction buffer was taken into a 2 ml Eppendorf tubes. Approximately 50 mg of each sample was added to the extraction buffer. After mixing them well by vortexing, the reaction was allowed to stay at room temperature for 10 minutes. To separate the cell debris the sample was centrifuged at 10000 g for 10 minutes at room temperature. The supernatant was transferred into a new tube and 300 µl of chloroform-isoamylalcohol was added to the supernatant. The reaction mixture was shaken vigorously by putting it on a vortex machine at highest speed for 10 seconds. After centrifuging at 10000 g at 4^{o}C for 10 minutes, upper aqueous phase was separated and pipetted out into new Eppendorf tubes. 375 µl of isopropanol and 375 µl of mixture of 0.8 M sodium citrate and 1 M sodium chloride were added. The reaction was incubated at room temperature for 10 minutes. To pellet the RNA the mixture was centrifuged again at 12000 g at 4^{o}C for 10 minutes. The supernatant was eliminated carefully and the pellet was washed with 70% ethanol (v/v) at -20^{o}C and centrifuged at 12000 g at 4^{o}C for 10 minutes. The ethanol was discarded and the pellet was air dried at room temperature and was dissolved into 100 µl of double distilled water.

Extraction Buffer	0.8 M guanidine thiocyanate, 0.4 M ammonium thiocyanate, 0.1 M sodium acetate (pH5.8), 5% glycerol and 38% water-saturated phenol

2.2.6.1.1 Purification of RNA

Extracted RNA was further purified by adding 167 µl of 4 M LiCl,(with final concentration of 2.5 M). The tubes were kept on ice for two hours then the reaction mixture was centrifuged at 14000 g at 4^{o}C for 20 minutes. The supernatant was discarded carefully and the pellet was washed twice with 1ml of 70% ethanol (v/v) at -20^{o}C as described previously. The 70% ethanol was eliminated carefully and the pellet was air dried at room temperature and suspended in 50µl of double distilled water. The quality and quantity were estimated on nanodrop as mentioned previously in the quantification section. The quality was further tested by loading 1 µg of RNA on a 1% agarose gel.

2.2.6.1.2 Removal of genomic DNA contamination from RNA

To eliminate the DNA impurities from the RNA, 1µg of RNA was treated with 1 µl of DNase I, RNase free enzyme (10U/µl, Fermentas, St. Leon-Rot, Germany) in mixture containing 1 µl of 10X reaction buffer and water to make the volume to 10 µl. The reaction was incubated at 37^{o}C for 30 minutes. The reaction was stopped by adding 1µl of 50mM EDTA and incubating at 65^{o}C for 10 minutes.

Reaction buffer 100mM Tris-HCl (pH7.5), 25mM $MgCl_2$ and 1mM $CaCl_2$

2.2.6.2 Plasmid DNA mini-prep from *E.coli* (Birnboim and Doly 1979)

Plasmid DNA was extracted from *E. coli* according to Bimboim and Doly (1979) with minor modifications. A single positive bacterial colony was inoculated in 5 ml LB medium and cultured at 37 °C overnight. Cells from overnight culture were collected in a 2 ml Eppendorf tube by spinning at maximum speed for 30 seconds. After eliminating the supernatant, cells were resuspended in 100 µl GTE solution and let it stood for 5 minutes at room temperature. Then 200 µl NaOH/SDS solution was added, mixed by tapping with fingers and incubated on ice for 5 minutes followed by adding 150 µl potassium acetate solution. Mixed thoroughly by vortexing at maximum speed and incubated on ice for another 5 minutes. The mixture was centrifuged for 3 minutes (13,000 rpm, RT) and supernatant was transferred into a fresh 1.5 ml Eppendorf tube. One volume (450 µl) of phenol/chloroform/isoamyl alcohol (25/24/1) was added and mixed by vortexing for 10 seconds. The upper phase was carefully transferred to a fresh tube after a very short centrifugation (at maximum speed, RT), and mixed with two volumes of 95% ethanol. Materials and Methods 41 The mixture was allowed to stand for 2 minutes at room temperature, then centrifuged for 10 minutes (13,000 rpm, RT) to precipitate the plasmid DNA. The DNA pellet was washed with 70% (v/v) ethanol, air dried and dissolved in TE buffer with 20 µg/ml RNase A. The re-suspended DNA was incubated at 37 °C to remove the RNAs and then stored at -20 °C or directly used for analysis.

Glucose/Tris/EDTA (GTE): 50 mM glucose; 25 mM Tris-Cl, pH 8.0; 10 mM EDTA. Autoclave and store at 4 °C.

NaOH/SDS solution:	0.2 N NaOH; 1% (w/v) SDS. Prepare immediately before use.
Potassium acetate solution:	29.5 ml glacial acetic acid; KOH pellets to pH 4.8; bring to 100 ml with H_2O. Store at room temperature (do not autoclave).

2.2.6.3 Quantification of Nucleic Acid

The quantity and quality of nucleic acid were measured with the help of Biospec-nano Shimadzu Biotech, Japan using 1 µl of nucleic acid. The ratio of values of OD_{260} and OD_{280} (OD_{260}/OD_{280}) was measured that corresponds to the quality of the nucleic acid.

2.2.7 First strand cDNA synthesis

1ul of the 50pmol oligo $(dT)_{18}$ and 1 µl of nuclease free water was added to the DNase I treated RNA sample. The reaction mixture was mixed gently, centrifuged briefly. After incubation at 65ºC for 5 minutes the reaction mixture was placed on ice immediately. The following components were added to the reaction mixture in the following order; 4 µl of 5X reaction buffer, 1 µl of Ribolock ^{TM} RNase inhibitor (20 U/µl), 2 µl of 10 mM dNTP mix and 1 µl RevertAid^{TM} H Minus M-MuLV Reverse Transcriptase (200 U/µl). The tube containing the reaction was mixed gently and centrifuged briefly. Afterwards the reaction was kept at 45ºC for 60 minutes. At the end the reaction was terminated by incubating it at 70ºC for 5 minutes. The prepared cDNA was stored at -80ºC.

2.2.8 Polymerase chain reaction (PCR)

Complimentary DNA (cDNA) fragments were amplified by standard PCR reaction in a total volume of 20 µl as follows:

Final volume (20 µl)	H_2O (sterile double distilled)
2.0 µl	10X PCR-buffer
0.5 µl	50 mM $MgCl_2$
0.5 µl	Forward-primer (10 pmol/µl)

0.5 µl	Reverse-primer (10 pmol/ µl)
0.6 µl	10 mM dNTPs
1.0 µl	cDNA (5 ng/µl)
0.2 µl	Taq-polymerase

The reaction was mixed gently and the PCR was performed in a TRIO-thermoblock (Biometra, Göttingen, Germany). The optimal number of PCR cycles and the annealing temperature was determined empirically for each PCR. A standard PCR programme was as followed:

94°C	5 minutes of denaturing
94°C	30 seconds (30 times) of denaturing
TA	30 seconds (30 times) of primer binding
72°C	45 seconds (30 times) of elongation
72°C	10 minutes for final extension
4°C	for keeping the samples stable until they are collected.

T_A = annealing temperature = $T_M \pm 4$ °C

T_M = melting temperature of the primers. For primers with different TM, the lower one is considered for the calculation of the T

2.2.9 Semi quantitative gene expression level determination

Expressions of the amplified genes through PCR were detected by gel electrophoresis. 1 µl of the DNA loading buffer was added to the PCR product, and was loaded onto a 1% agarose gel. 7 µl of 1 Kb DNA marker was loaded in a separate slot to estimate the size of the product.

2.2.10 DNA extraction from an agarose gel/PCR product purification

To extract and purify the PCR product the NucleoSpin® Extract II Kit was used. The bands of interest were isolated on a UV-light box. For DNA fragments isolated from the gel, 100 mg of agarose gel was dissolved into 200 ml of NTI buffer and was incubated at 50ºC for 10 minutes.

The mixture was vortexed briefly for 2-3 minutes until the pieces of gel completely dissolved. 700 µl of the sample was loaded into NucleoSpin® Gel and PCR Clean-up column. Then the column was centrifuged at 11000 g for 30 seconds. The remaining sample was loaded (if necessary) and the procedure was repeated. For washing, 700 µl of wash buffer (buffer NT3) was added to the columns and centrifuged at 11000 g for 30 seconds. The washing procedure was repeated and the flow through was discarded every time. The column was centrifuged at 11000 g for 2 minutes to remove the NT3 buffer completely and to make the column dry. The column was incubated at 70ºC for 5 minutes to remove ethanol completely. The nucleic acid was eluted by adding 20 µl of buffer NE (elution buffer) to the column which was placed on a new 1.5 ml Eppendorf tube and centrifuged for 11000 g for 1 minute.

2.2.11 Subcellular localization of Protein

2.2.11.1 Preparation of competent *E. coli* (RbCl method)

A single colony was inoculated to 4 ml LB medium and cultured under agitation (200 rpm) at 37 °C overnight. The next day 1 ml pre-culture of cells was inoculated into 100 ml of LB medium and cultured under the same conditions as above until an OD_{600} of 0.35-0.45. The cells were collected in two 50 ml Falcon tubes by centrifuging for 10 minutes (4,000 rpm, 4 °C) and gently resuspended in 15 ml ice-cold TFB I solution without pipetting or vortexing. The suspensions were incubated on ice for 10 minutes and centrifuged as above. Then the cells were resuspended again in 15 ml ice-cold TFB I solution and centrifuged as above. After washing two times with TFB I solution, cells were resuspended in 2 ml ice-cold TFB II solution and aliquots of 50 µl cell suspension were frozen in liquid nitrogen and stored at -80 °C.

TFB I:	30 mM KAc; 100 mM RbCl; 10 mM $CaCl_2.2H_2O$; 50 mM $MnCl_2.4H_2O$; 15% (v/v) Glycerol. Adjust pH to 5.8 using 0.2 M acetic acid and filter sterilize.
TFB II:	10 mM MOPS; 75 mM $CaCl_2.2H_2O$; 10 mM RbCl; 15% Glycerol (v/v). Adjust pH to 6.5 using KOH and filter sterilize.

2.2.11.2 Transformation of competent *E. coli*

One microliter plasmid DNA (10-100 ng/µl) or 1-5 µl of the ligation product was added to one aliquot of competent cells (50 µl) and carefully mixed and then heat-shocked in a water bath at

42 °C for 45 seconds. Cells were diluted with 800 µl LB medium and incubated under agitation (200 rpm) at 37 °C for one hour. Aliquots of 200 µl of the cell suspension were then spread on selective agar-plates and incubated at 37 ºC overnight.

2.2.11.3 Transient expression analysis via particle gun bombardment

Microcarriers and DNA coating were prepared according to the previously described method with some modifications (Sanford et al., 1993)30 mg gold particles (1.6 µm diameter) which were used as microcarriers were weighed into a 1.5 ml Eppendorf tube and washed with 1 ml 100% ethanol with vigorously vortexing for 5 minutes. After sedimentation of the particles, the supernatant was carefully pipetted off and discarded. The gold particles were washed three times as follows: added 1 ml sterile water vortexed for 1 minutes and waited until particles have sedimented again. Took off supernatant and discarded. Repeated the washing step three times and finally dissolved gold particles in 500 µl sterile 50% (v/v) glycerol. Prepared gold particles (60 mg/ml) were stored at 4 °C in 50 µl aliquots for up to one month without decrease in transformation efficiency. One aliquot of the gold particles was used for coating: 25 µg plasmid-DNA, 50 µl of 2.5 M $CaCl_2$ and 20 µl of 100 mM freshly prepared spermidine were in this order added to the gold suspension rapidly while vortexing for 5 minutes at maximum speed. The suspension was briefly centrifuged and the supernatant was discarded. The particles were then washed twice with 140 µl 70% and 100% ethanol, respectively. The covered gold particles were finally suspended in 50 µl 100% ethanol. 25 µl of the gold suspension was used for each bombardment. Bombardment was performed according to the instructions of PDS-1000/He manufacturer. Briefly, a plastic macro-carrier disk with 25 µl of DNA-coated gold particle (micro-carrier) suspension was placed into the macro-carrier holder along with a stopping metal grid. The system macro-carrier and stopping grid was placed into the launch assembly unit as described by the manufacturer. Healthy Arabidopsis leaves or fresh onion epidermises were well arranged in the center of a 1/2 MS solid medium plate and placed at 5-10 cm below the stopping screen. Vacuum was then applied to increase the gas pressure within the bombardment chamber. The release of the pressure led to the burst of the rupture disk and allowed the macro-carrier to eject at high velocity the DNA-coated gold particles into the leaves or onion epidermal cells. The particles were accelerated with a helium pressure of 1150 pounds per square inch (psi) under a

vacuum of 27 mm Hg (3.6 MPa). The leaves or onion epidermis were incubated on 1/2 MS plates for 12-48 h and analyzed under a confocal laser microscope.

2.2.11.4 Subcellular localization of Dehydrin

To study protein localization of barley dehydrin 3 protein (DHN3), the coding sequence of DHN3 was fused to the 5' end of the GFP gene in CaMV35S::GFP vector (pGJ280) (Willige et al., 2009). The barley dehydrin 3 full length sequence was amplified by PCR using HvDhn3NCO1 (F) and HvDhn3NCO1 (R) primer combinations (as mensioned in table of primers) to generate NcoI sites at both ends. The NcoI/NcoI fragments were cloned into the pGJ280 vector to obtain the corresponding translational fusions. Onion cells were transiently transformed via particle bombardment (van den Dries et al., 2011). Protein fluorescence was observed using an inverted confocal laser scanning microscope (Nikon Eclipse TE2000-U/D Eclipse C1, Nikon, Düsseldorf, Germany). The excitation wavelengths were 488 nm for GFP and 543 nm for chloroplast auto-fluorescence and emitted light was detected at 515-530 nm and 570 nm, respectively. Images were captured and processed with EZ-C1 software version 3.20 (Nikon).

2.2.12 Protein analysis

2.2.12.1 Protein extraction from plant tissues

The crude protein was extracted using the method determined by Laemmli (1970). For extraction 50-100 mg ground plant material was homogenized with 150-200µl Laemmli buffer (Laemmli, 1970) by votexing vigorously. The extract was incubated at 95ºC for 5 minutes, and was put on ice to cool it down. The mixture was then centrifuged at 14000 rpm for 5 minutes at room temperature. The supernatant containing total crude protein was collected into a fresh Eppendorf tube and stored at -20ºC. The sample was again boiled for 5 minutes and centrifuged before loading to the gel.

Laemmli Buffer: 62.5 mM Tris-HCl (pH6.8), 10% glycerol, 2% SDS (w/v), 0.1% bromophenol blue and 0.7 M β-mercaptoethanol (approximately 50%), and freshly prepared 0.1 M DTT just before use.

2.2.12.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed by the method described by Laemmli (1970). 4%(w/v) acrylamide stacking gel and 12 % (w/v) acrylamide separating gel was made as mentioned below. Protein samples were boiled for 5 minutes at 95ºC and loaded on to the gel. The electrophoresis was performed in 1X SDS-protein running buffer for about 2 hours at 10-15 mA in stacking gel and 20-25 mA in the separating gel. The components of the protein ladder used (Fermentas; Berlington, CDA) were: β-galactoside (*E.coli;* 116.0kDa), Bovine serum albumin (bovine plasma, 66.2kDa), Ovalbumin (chicken egg white; 45.0 kDa), Lactate dehydrogenase (porcine muscle; 35kDa), Restriction endonuclease *BSP*981(*E. Coli;* 25kDa), β-lactoglobulin (bovine milk; 18.4kDa) and lysozyme (chicken egg white; 14.4 kDa).

1X SDS protein running buffer, pH8.2: 25 mM Tris-HCl, 192mM Glycine and 0.1%SDS. Do not adjust the pH.

Constituents of SDS-PAGE gel.

Table 3.3: Constituents of SDS PAGE gel.

Stock Solution	**4%Stacking gel (3ml)**	**12% Separating gel (7.5 ml)**
ddH_2O	2.16 ml	1.92 ml
30% (v/v) Acrylamide	0.50 ml	2.4 ml
1M Tris-HCl pH6.8	0.38 ml	-
1.5M Tris-HCl pH8.8	-	1.56ml
10% (w/v) SDS	30 µl	60µl
10% (w/v) APS	30 µl	60 µl
TEMED	3 µl	2.4 µl

2.2.12.3 Ponceau red staining

After protein blotting, the membrane was stained using Ponceau-red staining solution to check protein transfer efficiency. The membrane was immersed, protein-side up, in about 50 ml of the staining solution [0.2% (w/v) Ponceau S in 3% (w/v) Trichloroacetic acid (TCA)] and stained for 5-10 minutes with gentle shaking. The staining solution was removed and the membrane was

destained with H2O. The membrane was scanned and the positions of protein markers were marked with a pencil.

2.2.13 Physiological and Biochemical Assays

2.2.13.1 Determination of chlorophyll content

The amount of the chlorophyll in leaf tissues was determined according to Arnon (1949). The leaf tissues (20-60 mg) were ground in Eppendorf tubes with metal beads under liquid nitrogen and homogenized in 2 ml 80% (v/v) aqueous acetone. The suspensions were incubated in the dark at room temperature under shaking for 30 minutes, then centrifuged for 5 min at 10000 rpm at room temperature. The absorption of the extracts was measured at 663 and 645 nm. The chlorophyll content was estimated by the formula:

$$C\ (mg\ FW^{-1}) = 0.002\ x\ (20.2\ x\ OD_{645} + 8.02\ x\ OD_{663}) /\ g\ FW$$

where C expresses the total chlorophyll content= (chlorophyll A + chlorophyll B)

2.2.13.2 Proline determination

Free proline was determined according to the method of Bates et al. (1973). Approximately 100 mg plant material was ground in liquid Nitrogen with metal beads and homogenized in 2 ml of 3% (m/v) sulphosalicylic acid. The mixture was centrifuged at 4000 rpm for 5 minutes. 1 ml of ninhydrin acid and 1 ml of glacial acetic acid were successively added to 1 ml of the supernatant or standard L-proline solution (1, 5, 10, 25 and 50 μM). The mixture was boiled for 60 minutes and extracted with 2 ml of toluene. Free proline was quantified with a spectrophotometer from the upper organic phase at 520 nm by using a standard curve obtained from various proline concentrations, using the following formula:

Free proline content ($\mu mol\ g^{-1}FW$) = (Estimated concentration x volume of extract in L) / g FW.

2.2.13.3 Lipid peroxidation Assay (MDA level)

The level of lipid peroxidation products was determined in the leaf tissues of the barley cultivars using the thiobarbituric acid (TBA) test. In this test the amount of malondialdehyde (MDA) as end product of the lipid peroxidation is calculated (Kotchoni et al., 2006). 50-75 mg of ground leaf material was taken into a 2 ml Eppendorf tube containing 1 ml of chilled 0.1% trichloroacetic acid (TCA) solution (w/v). The mixture was mixed by vortexing and was allowed

to stay at room temperature for 5 minutes, followed by a centrifugation at 13000g for 5 minutes at 4^{o}C. This step was repeated again by re-extracting the pellet with 1 ml of the same solvent. The supernatant was transferred to a fresh tube. 600 µl of the supernatant was added to the same volume of the assay mixture present in 15 ml falcon tube. The sample was mixed thoroughly and incubated at 95^{o}C for 30 minutes. The reaction was stopped by putting the tubes on ice for 30 minutes followed by a centrifugation at 5000 rpm for 5 minutes at 4^{o}C. With the help of spectrophotometer the optical density of the samples were taken at 440 nm, 532 nm and 600 nm. 0.1% (w/v) TCA was the reference solution. The MDA level was calculated by the following formula:

MDA equivalents (nmol/ml) = $[(OD_{532TCA+TBA}-OD_{600TCA+TBA})-(OD_{532TCA}-OD_{600TCA})/157000] x10^{6}$

MDA equivalents (nmol/g fresh weight) = 2 x MDA equivalents (nmol/ml) x Total volume of the extracts (ml) / gram FW

2.2.13.4 H_2O_2 measurement

H_2O_2 was measured according to Velikova et al. (2000). Briefly, 20-60 mg plant material was ground to a fine powder with liquid nitrogen and metal beads in an Eppendorf tube, homogenized in 2 ml of 0.1% (w/v) TCA and incubated for 5 minutes on ice bath. The mixture was centrifuged at 13000 rpm for 10 minutes at 4°C. Then, 0.5 ml of the supernatant was mixed to 0.5 ml of 10 mM potassium phosphate buffer, pH 7.0 and the reaction was started by adding 1 ml 1 M KI. In parallel, 1 ml 1 M KI was mixed with 1 ml of H_2O_2 standards (5, 10, 25, 50 µM) prepared with 10 mM potassium phosphate buffer, pH 7.0. The mixtures were kept in the dark at room temperature for 20 minutes and the absorbance was read at 390 nm using 10 mM potassium phosphate buffer, pH 7.0 as blank. H_2O_2 contents of plant samples were estimated from a standard curve obtained with standards of H_2O_2 by the following formula:

H_2O_2 (µmol g^{-1} FW) = (Estimated concentration x volume of extract in L) /g FW.

2.2.13.5 Activities of Antioxidative enzymes

2.2.13.5.1 Super Oxide Dismutase (SOD) Activity

Activity of superoxide dismutase (SOD) was measured as described by Kakkar et al. (1984). 500 mg of ground leaf material was homogenized with 3.0 ml 50 mM potassium phosphate buffer (pH 6.4) in 15 ml falcon tubes. The whole reaction was centrifuged at 2000g for 10 minutes at room temperature. The 0.2 ml supernatant was mixed into a 2.8 ml assay mixture. The reaction was started by adding of 0.2 ml 780 µM NADH. The mixture was incubated at 30^{o}C for 90 seconds and was stopped by the addition 1.0 ml of glacial acetic acid. The reaction mixture was shaken with n-butanol. The reaction was kept at room temperature for 10 minutes, centrifuged for 1 minute and butanol layer was collected. The color intensity of chromogen in the butanol was calculated at 560nm on spectrophotometer. One unit of enzyme activity is described as the amount of enzyme that gave 50% inhibition of the NBT reduction in one minute. SOD activity is measured in units per milligram of protein. As the reaction was allowed to take place in 90 seconds so the value was divided by the factor 2/3 to calculate the units.

Assay mixture	1.2 ml of 0.025 M sodium pyrophosphate buffer (pH 8.3), 0.1 ml of 186 µM phenazine methosulphate, 0.3 ml of 300 µM Nitroblue tetrazolium and 1 ml of distilled water.

2.2.13.5.2 Determination of Catalase Activity

Catalase Activity was measured by the method of Luck (1965) modified by Sadasivam and Manickam (1992). 200 mg of ground leaf material was dissolved into 1ml of 0.067 M phosphate buffer (pH 7.0). The homogenate was centrifuged at 10000 g for 10 minutes. The 0.1 ml supernatant was immediately added to the experimental cuvette containing 3 ml reaction mixture. The change in optical density (OD) was measured at 240nm in a spectrophotometer. The time took in decreasing the absorbance from 0.45 to 0.4 was recorded. Catalase activity of was determined in terms of units per mg of fresh weight of plants material. The enzyme activity was calculated by the formula:

Units/ml enzyme = (3.45) (df)/ (t) (0.1)

Where:

3.45 = decomposition of 3.45 µmoles of hydrogen peroxide in a 3.0 ml reaction mixture producing a decrease in the A_{240} from 0.45 to 0.40

df = dilution factor

t = minutes required for the A_{240} to decrease from 0.45 to 0.40

0.1 = milliliter of enzyme solution added to the cuvette

Units/mg solid = (units/ml enzyme)/ (mg solid/ml enzyme)

Reaction Mixture: 0.036% (w/w) H_2O_2 in 50 mM phosphate buffer (pH 7.0).

2.2.13.5.3 Peroxidase Activity

Peroxidase activity was measured by the method of Reuveni *et al*. (1992). 200g of ground leaf material was added to a 1 ml of 0.015 M sodium phosphate buffer (pH 6.5). The mixture was centrifuged at 10000 g for 10 minutes. 50µl of the supernatant was added to a 15 ml falcon tube containing the reaction mixture. Increase in optical density (OD) was recorded at 470nm with the help of a spectrophotometer. Peroxidase activity was measured as change in OD per minute per gram of fresh weight of Plant material.

Reaction Mixture 15mM sodium phosphate buffer (pH 6), 1mM H_2O_2 and 0.1mM *o*-methoxyphenol

2.2.13.5.4 Glutathione reductase (GR) Activity

The glutathione reductase activity was recorded according to David and Richard (1983). 200 mg of ground leaf material was dissolved into 1 ml of 0.12 M phosphate buffer (pH 7.2). 100 µl of the supernatant was added to 2 ml Eppendorf tubes containing 1.8 ml reaction mixture. The mixture was allowed to stand for 3 minutes at room temperature and then 100µl of NADPH was added to the reaction. With the help of a spectrophotometer, the absorbance was recorded at 340 nm at intervals of 15seconds for 2 minutes. One unit of GR was calculated as µmol of NADPH oxidized per minute per gram of Fresh weight.

Assay mixture 1ml of 0.12 M potassium buffer (pH 7.2), 0.1 ml of 15 mM EDTA, 0.1 ml of 10 mM sodium azide and 0.1 ml of 6.3 mM oxidized glutathione and 0.5 ml distilled water.

2.2.14 Physico-chemical properties of dehydrins

The molecular mass (MW), isoelectric point (pI), aliphaticindex, instability index and grand average of hydropathy (GRAVY)of the thirteen dehydrin proteins were calculated on the base of amino acid sequences using the ProtParam programme tool accessed online at http://web.expasy.org/cgi-bin/protparam/protparam .

3. RESULTS

In the current study 10 barley varieties, which were adapted to different climatic regions, were used to evaluate their salinity and drought tolerance. Ten different barley varieties: Reisgerste II, Candice, Scarlett, Heilis Frankin, Himalaya USA, Himalaya Nepal, Himalaya Winter, Himalaya Freak, Himalaya Nakt, Himalaya India were grown on artificial clay (see materials and methods). Two weeks after germination the stress treatments such as salt stress and drought stress treatment were given. Salt stress treatments were applied by treating plants with 200 mM NaCl solution or 400 mM NaCl solution. Drought stress was applied by stopping the water supply. All the stresses were applied for seven days, while the control plants were receiving normal water.

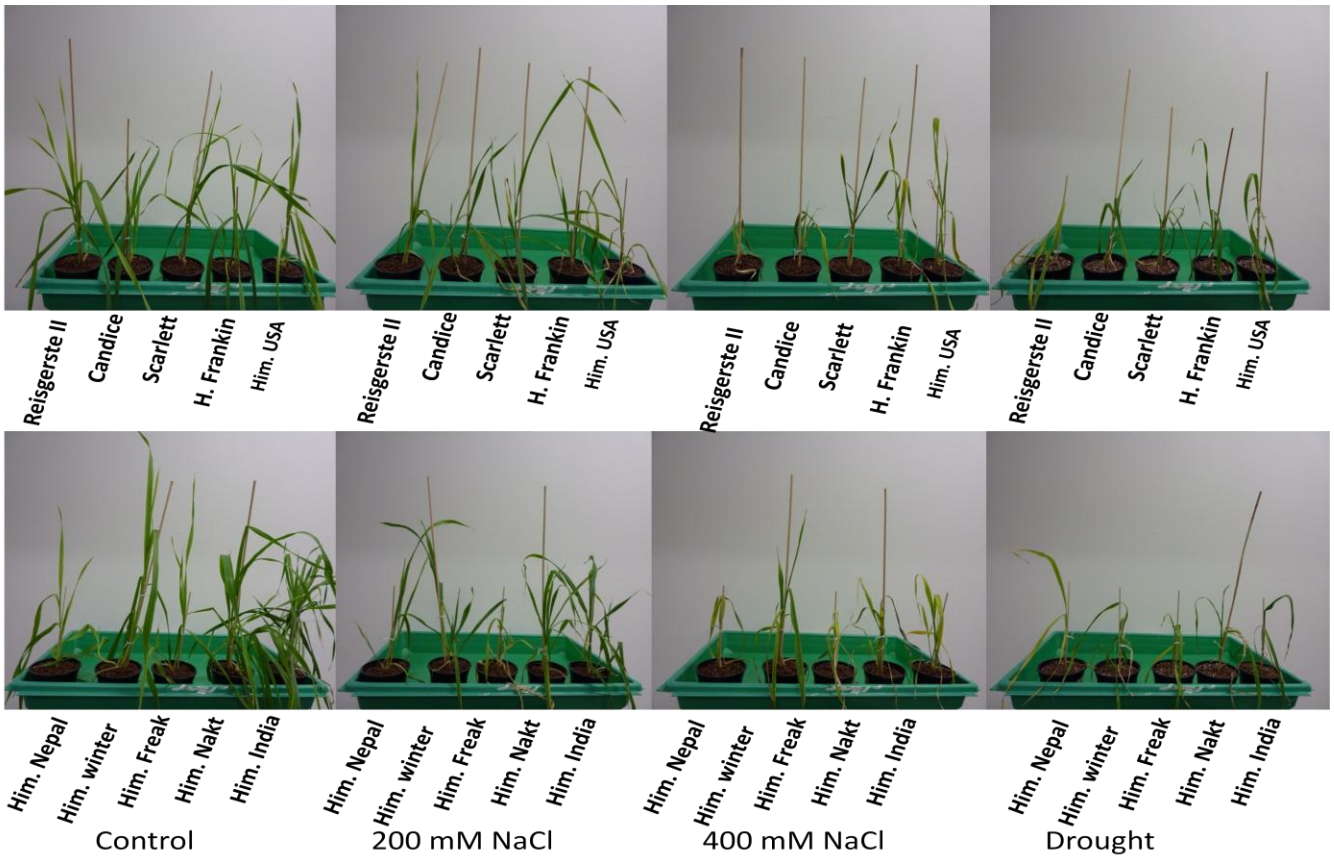

Figure 3.1: Phenotype of ten barley varieties under control, after 200 mM NaCl treatment, after 400 mM NaCl treatment and drought stress.

The phenotypic appearance of the plant showed that all the barley cultivars were not much affected by the 200 mM NaCl treatment, but severely affected by 400 mM NaCl application and drought stress. However, the degree of severity was dependent on the tolerance limit of the plant.

3.1 Growth of the plant

Growth of the plants was measured in terms of total biomass produced, which mainly depends on production of leaves, shoots, and roots of the plant. To evaluate the health (tolerance against stresses) of the all the controlled and stress treated plants, the number of leaves, root lengths and shoot lengths of plants were determined.

3.1.1 Number of leaves

Although all the barley varieties have different numbers of leaves ranging from 8-28 even under control conditions yet all the three stress treatments reduced the number of leaves in all the ten barley varieties under study (Fig. 2).

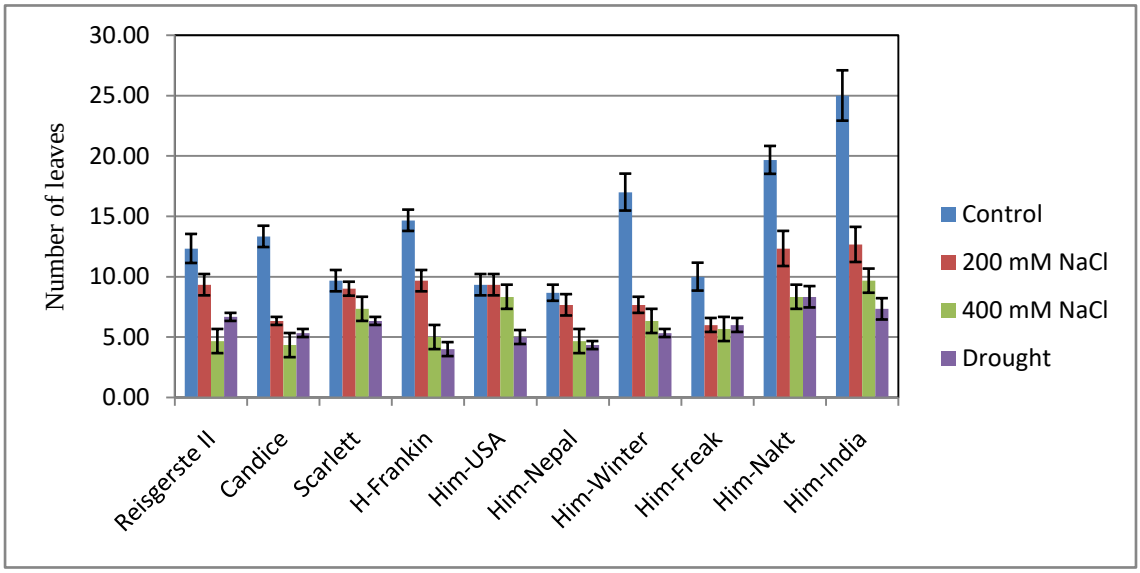

Figure 3.2: Number of leaves of all the studied barley varieties under control, after 200 mM NaCl treatment, 400 mM NaCl treatment and drought stress. Stresses were applied for 7 days to 15 days old plants after germination.

In control conditions, varieties were divided into three categories according to their leaf production after two weeks of germination; varieties with highest number of leaves (20 or more) like Himalaya Nakt and Himalaya India, varieties with medium number of leaves (10-19) like

Reisgerste II, Candice, Heilis Frankin, Himalaya Winter and Himalaya Freak, and with lower number of leaves (less than 10) like Scarlett, Himalaya USA and Himalaya Nepal.

In comparison with control plants there is a significant decrease in number of leaves even at 200 mM NaCl solution treatment in all varieties except in Himalaya USA, Scarlett and Himalaya Nepal where the decreasing tendency was low. Increase in the concentration of salt to 400 mM NaCl resulted in a further decrease of number of leaves. Visually one week of drought stress had a similar impact on the number of leaves as observed for the 400 mM NaCl treatment.

3.1.2 Shoot length

The shoot lengths of the barley plants were measured in centimeters with help of a scale from beginning of roots until the highest leaf.

The barley varieties have different shoot lengths at control conditions ranging from 35-54 cm. At 200 mM NaCl treatment for one week, most of varieties have a similar shoot length as that of the control plants. The barley variety Himalaya Freak performed very bad on all the stresses at 200 mM NaCl.

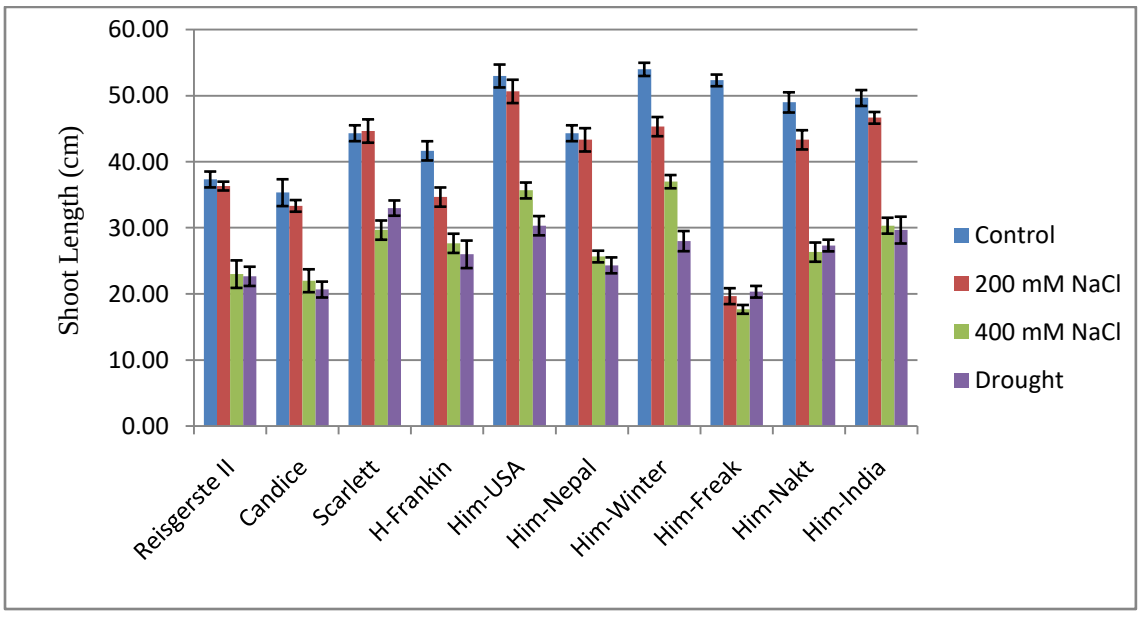

Figure 3.3: Shoot length of all the studied barley varieties under control, after 200 mM NaCl treatment, 400 mM NaCl treatment and drought stress. Stresses were applied for 7 days to 15 days old plants after germination.

Shoot length of all the cultivars decreased tremendously on 400 mM NaCl and drought stress treatment. Himalaya USA and Himalaya winter had longest shoots among all tested varieties at 400 mM NaCl, while Scarlett had longest shoot length at drought stress treatment. The shoot lengths of Reisgerste II and Candice were the lowest on both 400 mM NaCl and drought treatment.

3.1.3 Root length

Root lengths were also measured with scale in centimeters from the point of root emergence until the tip of the longest root.

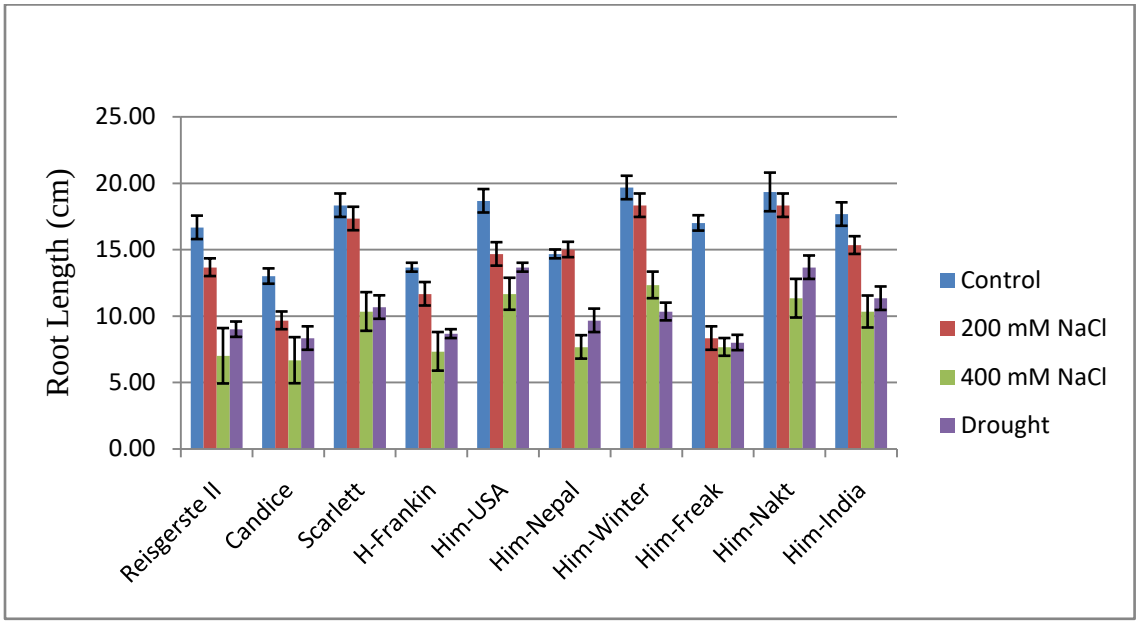

Figure 3.4: Root length of all the studied barley varieties under control, after 200 mM NaCl treatment, 400 mM NaCl treatment and drought stress. Stresses were applied for 7 days to 15 days old plants after germination.

Figure 4 shows that the trend of all the barley varieties in root length was the same as it was observed for shoot lengths. However, the root lengths of most of the cultivars were longer in drought stress than under 400 mM NaCl stress.

3.2 Water Loss Rate (WLR)

Water Loss Rate (WLR) of the all the ten barley varieties were measured to characterize barley varieties on the basis of short term severe drought stress. The variety with the lowest water loss rate was considered to be drought tolerant.

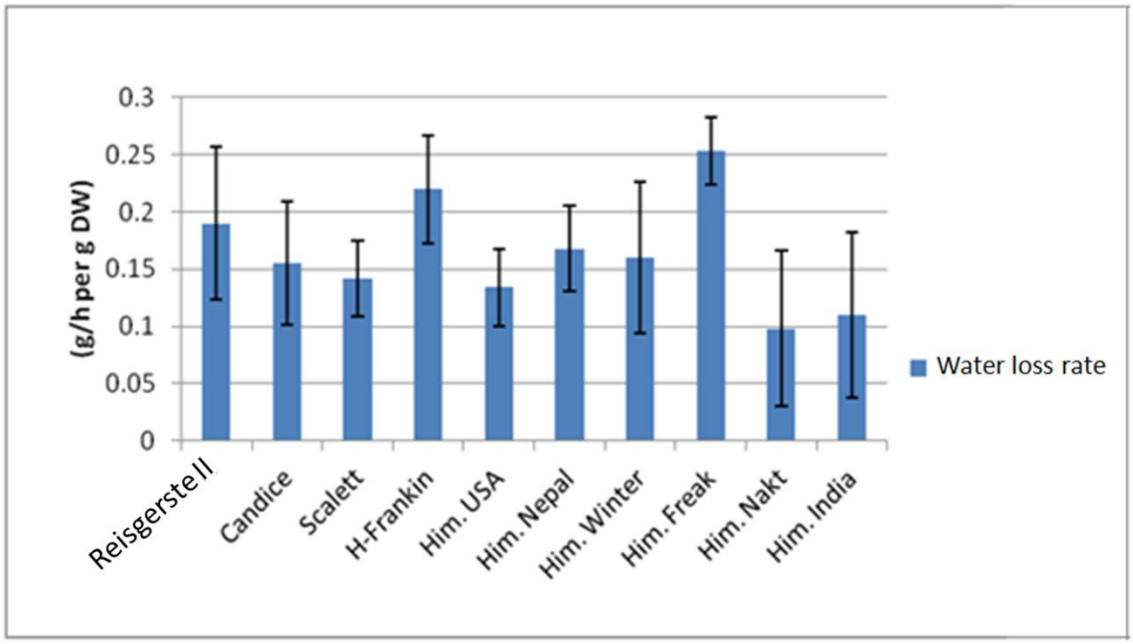

Figure 3.5: Water loss rate (WLR) of all the studied barley varieties.

Himalaya Freak had the highest water loss rate (0.25g/h per g DW) followed by Heilis Frankin (0.22 g/h per g DW) and Reisgerste II (0.18 g/h per g DW) while Himalaya Nakt (0.1 g/h per g DW) and Himalaya India (0.12 g/h per g DW) had the least water loss rate followed by the Himalaya USA (0.14 g/h per g DW) and Scarlett (0.13 g/h per g DW).

3.3 Leaf Relative water content (RWC)

Leaf relative water content is way to measure the water status and the related metabolic activities in the leaf tissues of the plant (Flower & Ludlow 1986). It is the measure of the drought stress tolerance in the plant. The metabolic activities in the plants with higher relative water contents would be more similar with the control plants and would be considered as tolerant plants and vice versa.

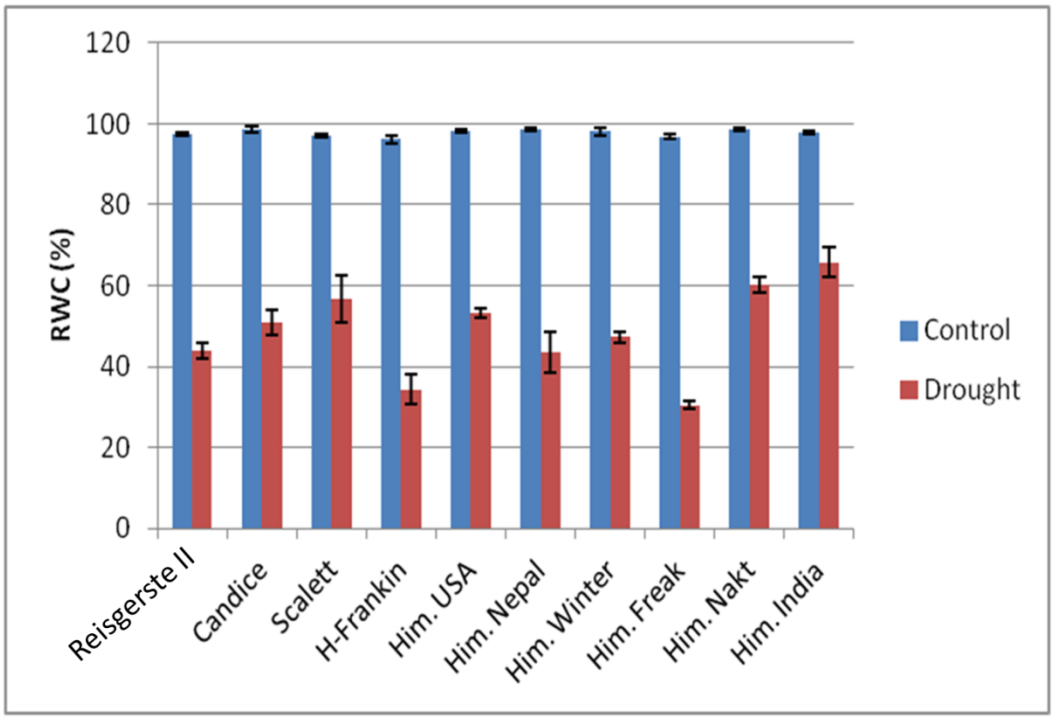

Figure 3.6: Leaf relative water contents of all the studied varieties.

Leaf relative water contents of all the barley varieties decreased on drought application. However, barley variety Himalaya India had the highest RWC (65.62%) followed by Himalaya Nakt (60%) and Scarlett (56.25%). On contrast, Himalaya Freak and Heilis Frankin were most sensitive to drought with RWC of 30% and 33.75% respectively.

3.4 Total chlorophyll content

Chlorophylls are important pigments in the photosynthesis process, the chlorophyll contents are considered as determinants of photosynthesis; the higher the chlorophyll contents the higher would be the rate of photosynthesis. Total chlorophyll contents of all the varieties of barley under study were calculated in all the given stress conditions by the method developed by the Arnon (1949).

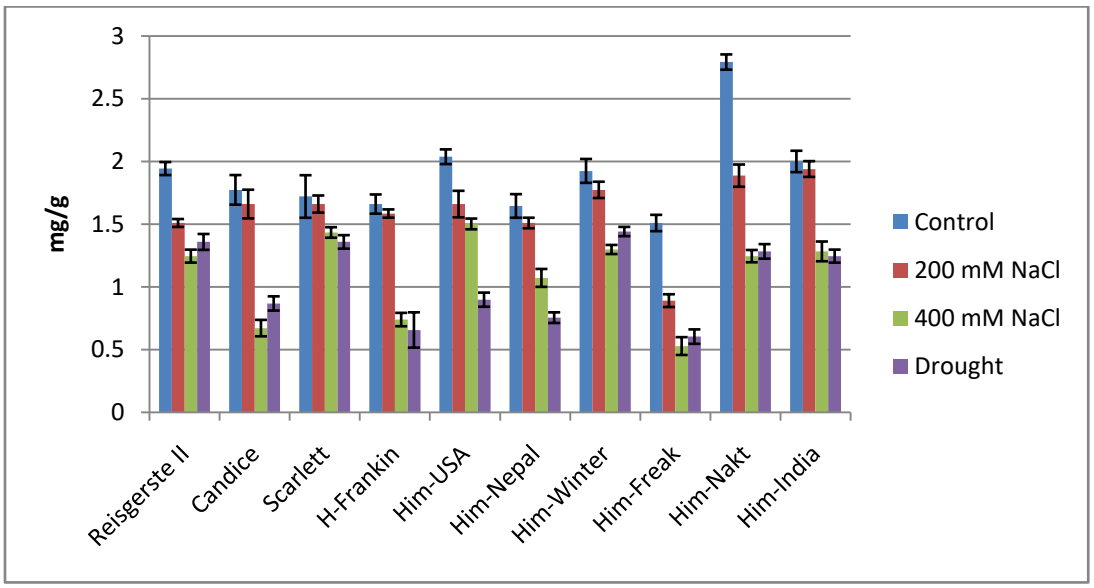

Figure 3.7: Total chlorophyll contents (mg/g of fresh weight) of all the studied barley varieties under control, after 200 mM NaCl treatment, 400 mM NaCl treatment and drought stress.

The control plants of all varieties of barley had almost similar levels of total chlorophyll contents except the Himalaya Nakt (2.89mg/g) with highest and Himalaya Freak (1.51mg/g) with lowest amount of total chlorophyll content among the control plants. Total chlorophyll content in all the varieties decreased in both NaCl and drought treatment and decreased more with increased concentration of NaCl from 200 mM to 400 mM. However at 200 mM NaCl treatment most of the varieties were having similar chlorophyll level as in control plants. Lowest chlorophyll contents at 200 mM NaCl were found in Himalaya Freak (0.89mg/g). At 400 mM NaCl treatment and drought treatment Himalaya Freak had the least amount of chlorophyll (0.63 mg/g and 0.69mg/g respectively), followed by the Candice (0.69 mg/g and 0.87 mg/g respectively) and Reisgerste II (0.87mg/g and 0.98 mg/g respectively).

3.5 Proline Determination Assay

Proline accumulation is also an indicator of stress tolerance. The higher amount of proline in plants, higher would be the tolerance. The free proline contents were measured in control and stress treated plants by the method Bates *et al*. 1973

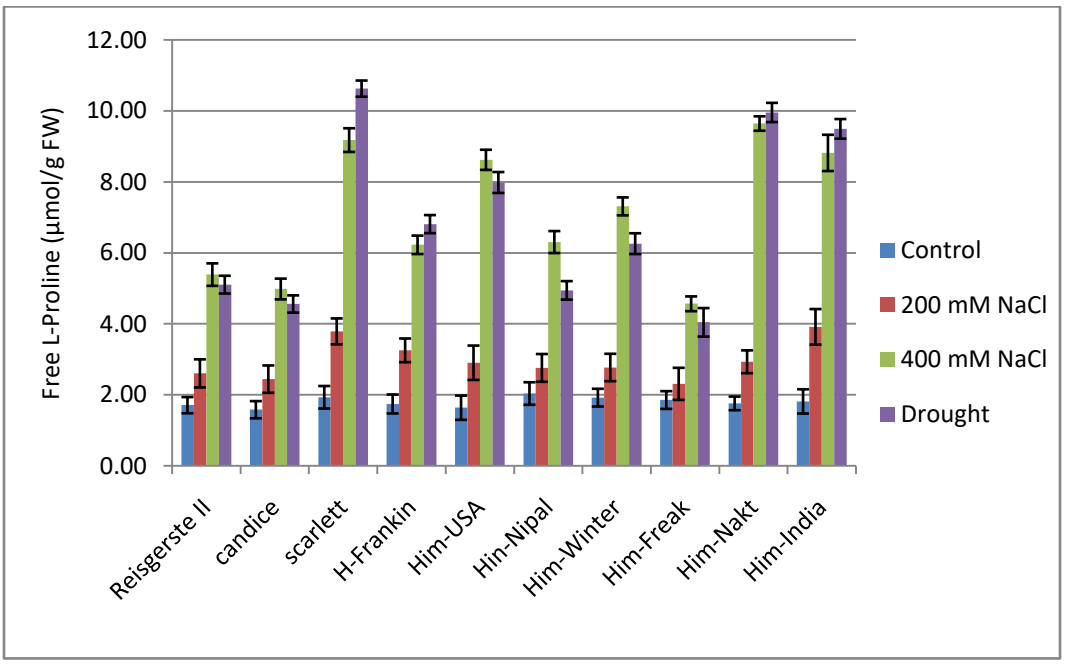

Figure 3.8: Free L-proline content (µmol/g of fresh weight) of all the studied barley varieties under control, after 200 mM NaCl treatment, 400 mM NaCl treatment and drought stress.

All the control plants had the same amount of L-proline. On 200 mM NaCl treatment a small increase was observed in proline contents in all varieties. Highest increase in 200 mM NaCl treatment was found in Himalaya India (1.81 to 3.94 µmol/g) followed by Scarlett (1.93 to 3.79 µmol/g) while the least increase was observed in (1.86 to 2.31 µmol/g). In general, in most varieties no significant difference was found at 200 mM NaCl. However at 400 mM NaCl and drought treatment the proline contents increased almost 2.5 to 5 fold. As in the variety Scarlett, the proline contents were 9.18 and 10.63 µmol/g of fresh weight on 400 mM NaCl and drought treatment respectively followed by Himalaya Nakt (9.64 and 9.96 µmol/g of fresh weight in 400 mM NaCl and drought application respectively) and Himalaya India (8.82 and 9.49 µmol/g of fresh weight in 400 mM NaCl and drought application respectively). On contrast the smallest increase was observed in Himalaya Freak (4.57 and 4.05 µmol/g of fresh weight in 400 mM NaCl and drought application respectively). The other varieties had intermediate proline contents.

3.6 Lipid peroxidation assay

The levels of lipid peroxidation products in the leaves of all salt and drought stressed plants and control plants were determined using the thiobarbituric acid (TBA) test. This test calculates malondialdehyde (MDA) as a final product of lipid the peroxidation process (Hodges et al. 1999; Kotchoni et al. 2006).

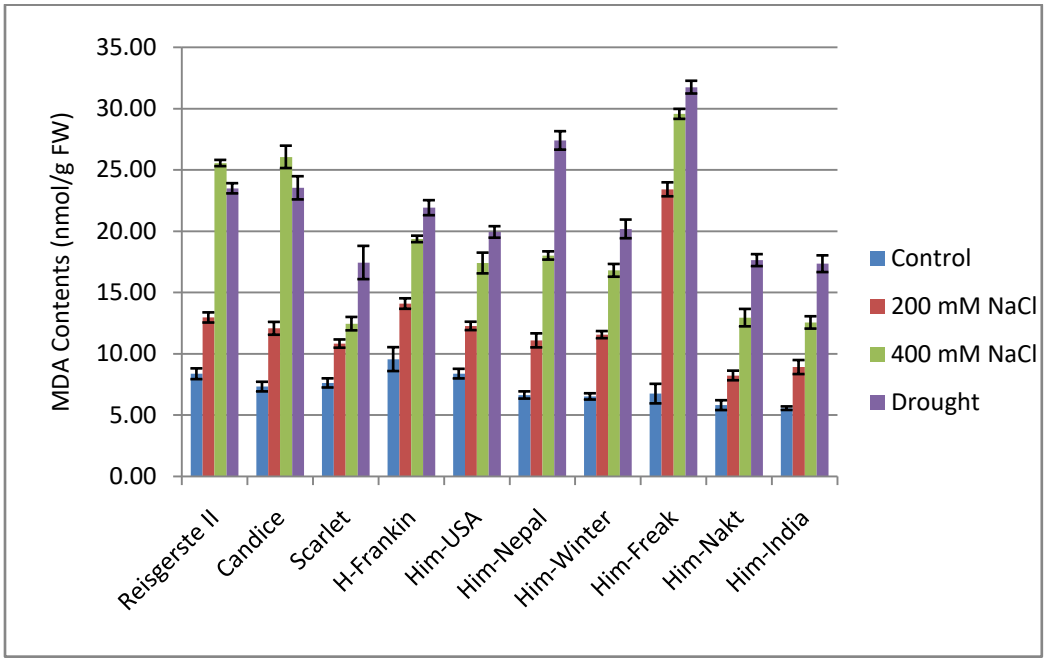

Figure 3.9: Malondialdehyde content (nmol/g of fresh weight) of all the studied barley varieties under control, after 200 mM NaCl treatment, 400 mM NaCl treatment and drought stress.

The MDA levels in the different varieties in different conditions showed that among the control plants all the varieties have almost the same level of MDA. Upon increase of NaCl concentration to 200 mM NaCl the MDA level increased a little bit, maximum increase was observed in Himalaya Freak (6.76 to 23.43 n mol g^{-1} FW). At 200 mM NaCl Himalaya Nakt and Himalaya India had the smallest increase in MDA (5.68 to 8.23 and 5.60 to 8.90 n mol g^{-1}FW respectively). The MDA level in all varieties in all conditions increased with the increase of salt to 400 mM NaCl and drought treatment for one week. In all the varieties except for the Reisgerste II and Candice, MDA accumulation at drought was higher as compared to 400 mM NaCl treatment. Like at 200 mM NaCl, Himalaya Freak had highest MDA even at 400 mM NaCl and drought application (29.97 and 31.75 n mol g^{-1} FW) followed by Himalaya Nepal at drought treatment, Candice and REISGERSTE II at 400 mM NaCl treatment (27.41, 27.43 and 25.16 n mol g^{-1} FW

respectively). The least increase in the accumulation of MDA was observed for the varieties Scarlett, Himalaya Nakt and Himalaya India for all the three stress treatments.

3.7 Hydrogen Peroxide (H_2O_2) Measurement

Hydrogen peroxide is a reactive oxygen species (ROS) and it is an established fact that excess of hydrogen peroxide in the plants leads to oxidative stress (Gill and Tuteja 2010). The amount of hydrogen peroxide that is produced in the leaves in stress conditions was measured according to the method of Velikova *et al*. 2000.

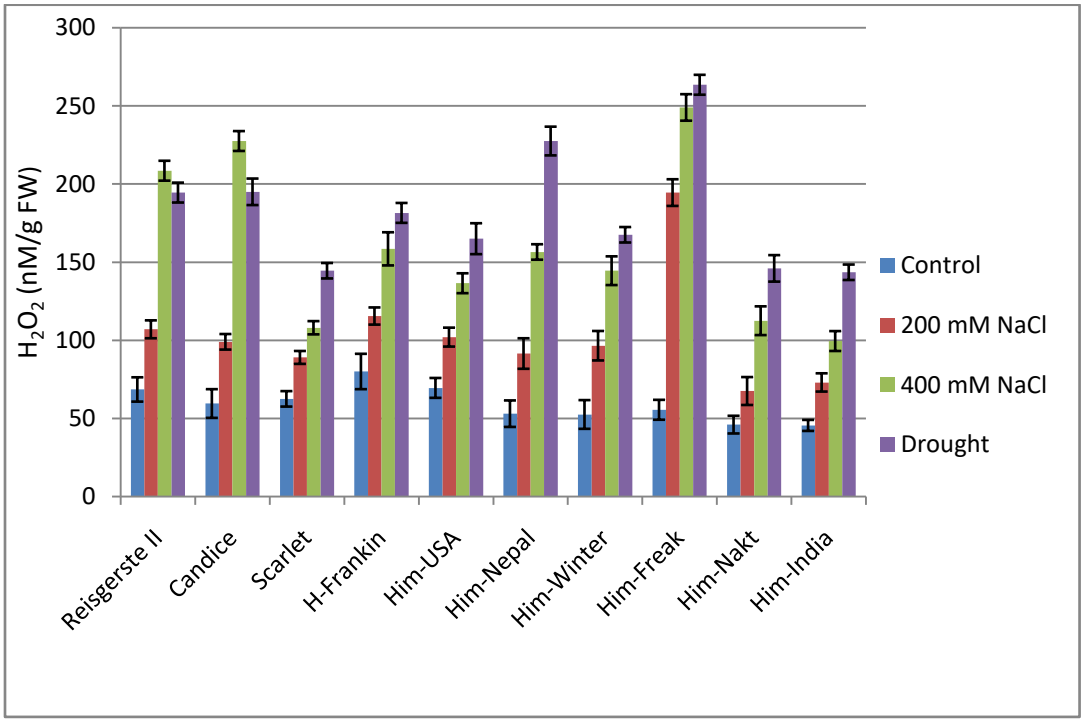

Figure 3.10: Hydrogen peroxide (nmol/g of fresh weight) of all the studied barley varieties under control, after 200 mM NaCl treatment, 400 mM NaCl treatment and drought stress.

Compared to the respective control the amount of hydrogen peroxide (H_2O_2) increased in plants treated with salt and drought stress. Higher amounts of H_2O_2 were found in the plants which were receiving higher concentrations of salt. In the plants treated with 200 mM NaCl the highest amount of H_2O_2 was found in Himalaya Freak (195.24 nmol/g of fresh weight) which was almost 3.5 times higher than in control plants, followed by Heilis Frankin (116.34 nmol/g of fresh weight), the smallest amounts of hydrogen peroxide were seen in Himalaya Nakt and Himalaya India (68.59 and 74.14 nmol/g of fresh weight respectively). In the plants treated with 400 mM

NaCl and in drought treatment, a huge increase was observed ranging from 2 to 5 times. Again highest increase was in Himalaya Freak in both at 400 mM NaCl and drought (249.78 and 264.54 µmol/g of fresh weight respectively) followed by Candice (228.62 and 196.1 nmol/g of fresh weight at 400 mM NaCl and drought treatment respectively), Reisgerste II (209.68 and 195.80 µmol/g of fresh weight at 400 mM NaCl and drought treatment respectively) and Himalaya Nepal at drought (228.33 nmol/g of fresh weight). On contrast the lowest increase was found in Himalaya India (100.59 and 144.53 nmol/g of fresh weight at 400 mM NaCl and drought treatment respectively), Scarlett (108.92 and 145.35 µmol/g of fresh weight at 400 mM NaCl and drought treatment respectively) and Himalaya India (113.51 and 146.97 nmol/g of fresh weight at 400 mM NaCl and drought treatment respectively).

3.8 Anti-oxidative enzymes activities in different barley varieties after drought and salt stress

Reactive oxygen species (ROS) are unavoidable product of respiration in the organism. However ROS production increases in the stress environment. Higher amount ROS can cause damage to the Nucleic acid, proteins and lipids and increase the permeability of the cells (de Carvalho 2008; Gill & Tuteja 2010). An antioxidant is a molecule that inhibits the oxidation of other molecules. The activities of different anti-oxidative enzymes are considered as the determinants of oxidative stress. The amount of activities of anti-oxidative enzymes has positive correlations with abiotic stress tolerance.

3.8.1 Super Oxide Dismutase (SOD) Activity

The superoxide dismutase (SOD) dismutases the oxygen radicals into H_2O_2. Activity of SOD was measured by the method described by the Kakkar *et al.* (1984).

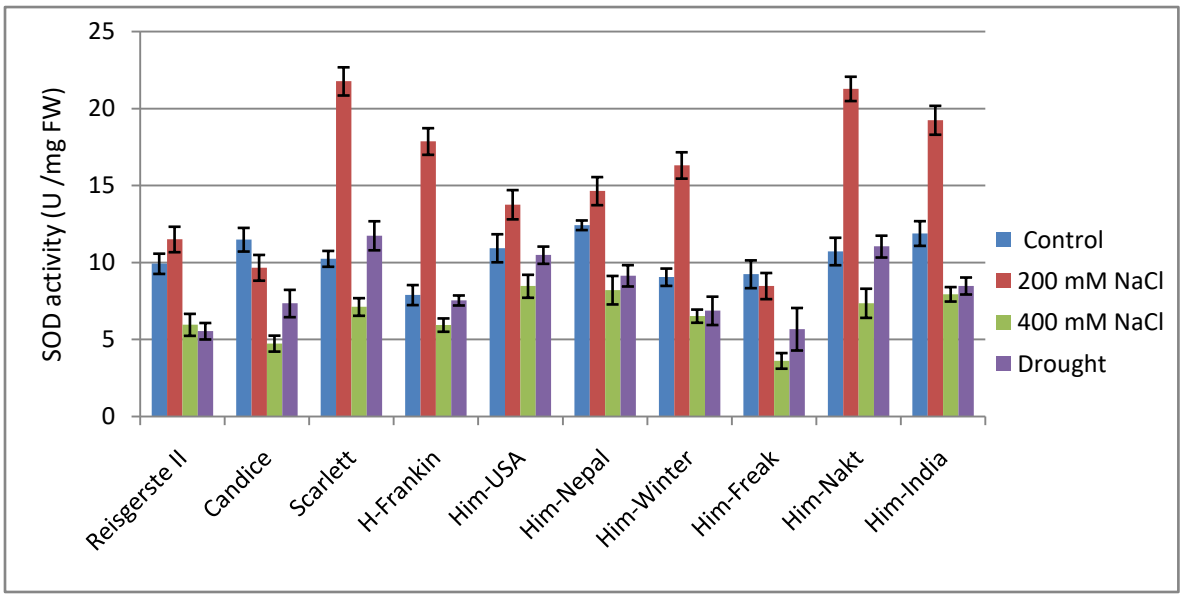

Figure 3.11: Super Oxide Dismutase (SOD) Activity (u/mg of fresh weight) of all the studied barley varieties under control, after 200 mM NaCl treatment, 400 mM NaCl treatment and drought stress.

SOD activity increased in most of the studied barley varieties a lot at the application of 200 mM NaCl. In some of the varieties like Scarlett, Heilis Frankin, Himalaya Nakt and Himalaya India

increase in SOD activity was more than double or almost double than the what it was observed in control plants (10.25 to 21.77 U mg^{-1} of Protein, 7.90 to 17.87 U mg^{-1} of Protein 10.73 to 21.29 U mg^{-1} of Protein and 11.90 19.25 U mg^{-1} of Protein respectively). However in two varieties Candice and Himalaya Freak a minimal decrease was observed in SOD activity of the 200 mM NaCl treated plants. Surprisingly increase in the concentration of NaCl to 400 mM resulted into the huge decrease in SOD activity and in most of the varieties it was even less than which were observed in the control plants. Moreover, the seven days of drought treatment resulted in the decrease of SOD activity in most of the varieties except for the Scarlett where it increased a little (10.25 to 11.76 U mg^{-1} of Protein) and Himalaya Nakt, Himalaya USA and Heilis Frankin where almost SOD activity was observed in comparison with their control plants.

3.8.2 Catalase Activity

Catalase is very important enzyme, which protects the cell from oxidative damage by catalyzing the decomposition of hydrogen peroxide into water and oxygen (Chelikani (2004). Catalase activity was measured by the method of Luck (1974) modified by the Sadasivam and Manikam (1991).

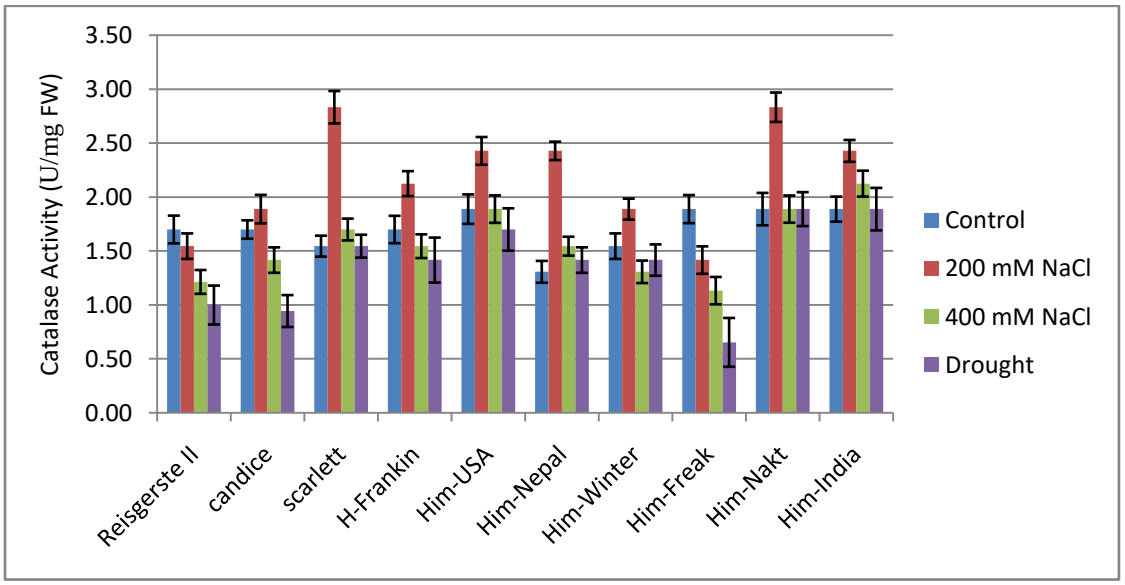

Figure 3.12: Catalase activity (U mg^{-1} min^{-1}) of all the studied barley varieties under control, after 200 mM NaCl treatment, 400 mM NaCl treatment and drought stress.

The graph of catalase activity in barley varieties in different conditions shows that most of the varieties had almost same catalase activity in control plants except for the Himalaya Nepal

having a lowest activity (1.31 U mg^{-1} min^{-1}). With the application of 200 mM NaCl, catalase activity increased in all varieties except in Himalaya Freak (1.9 to 1.38 U mg^{-1} min^{-1}) and Reisgerste II (1.66 to 1.53 U mg^{-1} min^{-1}). In the varieties where it increased, highest increase was observed in Himalaya Nakt (1.89 to 2.94 U mg^{-1} min^{-1}) followed by Scarlett (2.71 U mg^{-1} min^{-1}), Himalaya USA (2.56 U mg^{-1} min^{-1}) and Himalaya India (2.55 U mg^{-1} min^{-1}). Surprisingly with the increase in the concentration of salt to 400 mM NaCl and drought treatment, the catalase activity decreased significantly than which was found at 200 mM NaCl treated plants. In some varieties like Reisgerste II (1.66 to 1.25 and 1.02 U mg^{-1} min^{-1} in 400 mM NaCl and drought treated plants respectively), Candice (1.76 to 1.39 and 0.95 U mg^{-1} Min^{-1} in 400 mM NaCl and drought treated plants respectively), and Himlaya Feak (1.90 to 1.15 and 0.64 U mg^{-1} min^{-1} in 400 mM NaCl and drought treated plants respectively)it decreased significantly from its control plants as well. In Scarlett, Himalaya Nepal and Himalaya India at 400 mM NaCl treatment, higher catalase activity was observed as compared to their control plants. Moreover no significant difference was found in rest of varieties on both 400 mM NaCl and drought treatment.

3.8.3 Glutathione Reductase Activity

Glutathione reductase is an enzyme which converts oxidized Glutathione to the reduced one with the oxidation of NADPH (Halliwell & Gutteridge, 2000). The reduced glutathione is strong reducing agent which protects membranes from peroxidation of ROS. The glutathione reductase activity was calculated according to the method of David and Richard (1983).

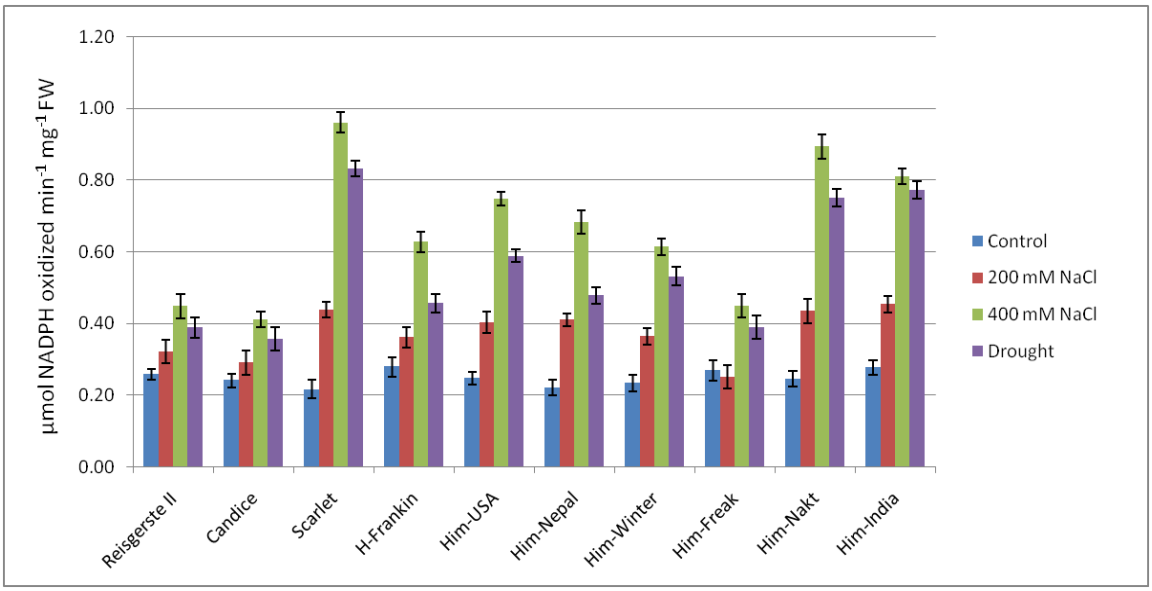

Figure 3.13: Glutathione reductase activity (µmol NADPH oxidized min^{-1} mg^{-1} FW) of all the studied barley varieties under control, after 200 mM NaCl treatment, 400 mM NaCl treatment and drought stress.

Glutathione reductase activity increased under stress condition. At 200 mM NaCl treatment when comparing with their control plants no significant difference was found in varieties Candice (0.24 to 0.29 µmol NADPH oxidized min^{-1} mg^{-1} FW) and Himalaya Freak (0.27 to 0.25 µmol NADPH oxidized min^{-1} mg^{-1} FW). While the remaining varieties had more or less the same glutathione reductase activities, the highest glutathione reductase activity was found in Scarlett both at 400 mM NaCl and drought stress treatment (0.96 and 0.83 µmol NADPH oxidized min^{-1} mg^{-1} FW respectively) followed by Himalaya Nakt (0.89 and 0.75 µmol NADPH oxidized min^{-1} mg^{-1} FW respectively) and Himalaya India (0.81 and 0.77 µmol NADPH oxidized min^{-1} mg^{-1} FW respectively). On the other hand in varieties Reisgerste II (0.45 and 0.39 µmol NADPH oxidized min^{-1} mg^{-1} FW respectively), Candice (0.41 and 0.36 µmol NADPH oxidized min^{-1} mg^{-1} FW respectively) and Himalaya Freak (0.45 and 0.39 µmol NADPH oxidized min^{-1} mg^{-1} FW respectively) were found to have the lowest increase in glutathione reductase activity at 400 mM NaCl and drought treatment.

3.8.4 Peroxidase Acitvity

Peroxidase (POX) is an enzyme, which catalyzes the reduction of H_2O_2. There is a positive correlation between POX activity and the tolerance to the oxidative stress tolerance. POX acitivity in all varieties was measured according the method of Reuveni *et al*. (1992).

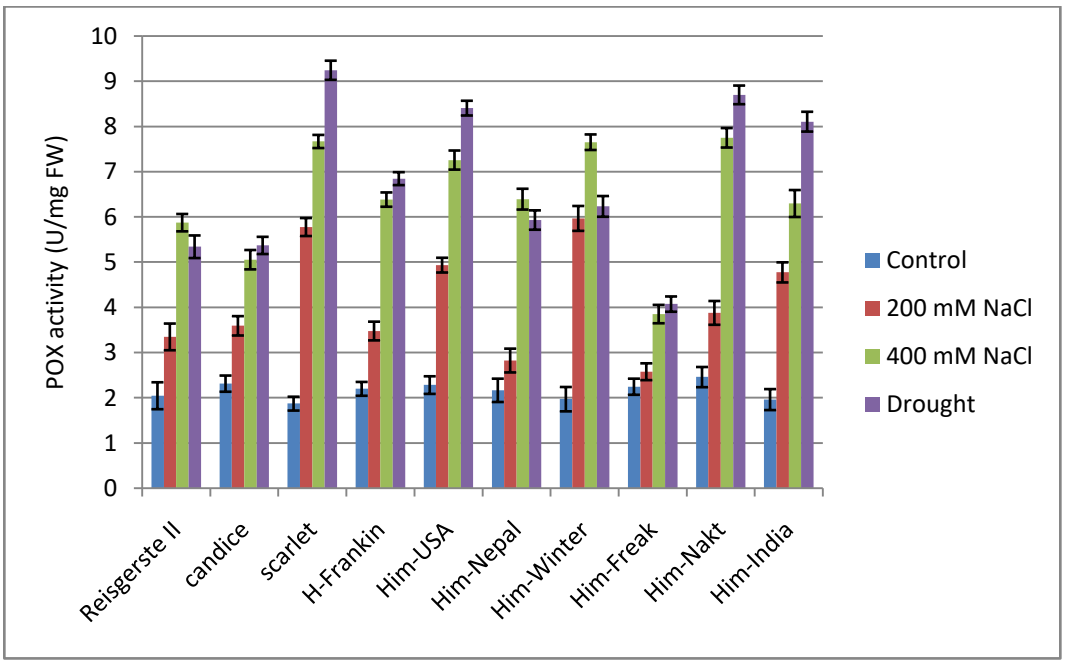

Figure 3.14: Peroxidase activity (U mg^{-1} FW) of all the studied barley varieties under control, after 200 mM NaCl treatment, 400 mM NaCl treatment and drought stress.

The results of peroxidase activity exhibit that there is no significant difference within the control plants of all the varieties. However, with the application of each type of stress POX activity increased except for Himalaya Freak, which had almost similar peroxidase activity as that of control plants (2.25 and 2.58 U mg^{-1} FW at control and 200 mM NaCl respectively), and different varieties showed different peroxidase activities.

3.9 Dehydrins

Dehydrins are group 2 late embryogenesis abundant (LEA) proteins which are known to accumulate in vegetative tissues under dehydration conditions. Accumulation of dehydrins and oxidative stress tolerance are supposed to be positively correlated. Lysine rich 15 amino acid long conserved sequence called K-segment is the characteristic feature of dehydrin. Moreover they have some other conserved sequences like tyrosine rich Y-segment and serine rich S-segment. In Barley 13 dehydrin have been discovered so far. These dehydrins are further classified into different groups according to the presence of Y, S, and K conserved segments as mentioned in the table 1.

3.9.1 Physico-chemical Analysis of different barley dehydrins

Table 3.1: Physico-chemical properties of different barley dehydrins.

Name	Type	Amino acids	MW (KDa)	PI	Instability index	Aliphatic index	GRAVY
Dehydrin 1	YSK_2	139	14.24	8.81	42.17	38.71	-1.077
Dehydrin 2	YSK_2	143	14.42	8.00	35.09	38.39	-1.169
Dehydrin 3	YSK_2	155	15.71	8.07	15.03	31.55	-1.103
Dehydrin 4	YSK_2	247	24.72	8.04	11.10	21.78	-1.024
Dehydrin 5	K_9	575	58.51	6.65	02.58	28.92	-1.161
Dehydrin 6	$Y2SK_3$	502	47.65	8.09	-6.56	31.35	-0.749
Dehydrin 7	YSK_2	181	18.07	9.30	16.14	30.83	-1.001
Dehydrin 8	SK_3	255	27.73	5.21	55.52	62.00	-1.093
Dehydrin 9	YSK_2	146	15.13	9.52	34.94	30.89	-1.151
Dehydrin 10	SK_3	295	29.15	9.67	25.31	34.95	-0.851
Dehydrin 11	Y_2SK_2	232	23.46	6.26	31.31	51.90	-0.738
Dehydrin 12	SK_2	141	14.24	6.59	34.81	42.34	-0.905
Dehydrin 13	KS	106	11.92	6.84	38.23	23.96	-2.223

The molecular weight of barley dehydrins ranges from 11.92 to 58.51 KDa and size from 106 to 575 amino acids. The deydrins are thermostable and hydrophilic. These properties can be judged on the basis of aliphatic index, instability index and grand average of hydropathy index

(GRAVY). The aliphatic index of barley dehydrins which mentions thermostability of protein ranges from 21.78 to 62.00 as in above given table. Instability Index determins the stability of the protein, the protein with instability index value lower than 40 is considered as stable and vice versa. Among the thirteen barley dehydrins, Dhn1 with instability index value (42.17) and Dhn8 with instability index value (55.52) were predicted to be unstable. While the rest of barley dehydrins are stable as they had instability index value less than 40. The positive GRAVY value indicates the hydrophobicity and negative value show hydrophilicity of the protein. The negative GRAVY values of barley dehydrins showed (Table 1) that these dehydrins are hydrophilic in nature.

3.9.2 Barley dehydrin transcript analysis

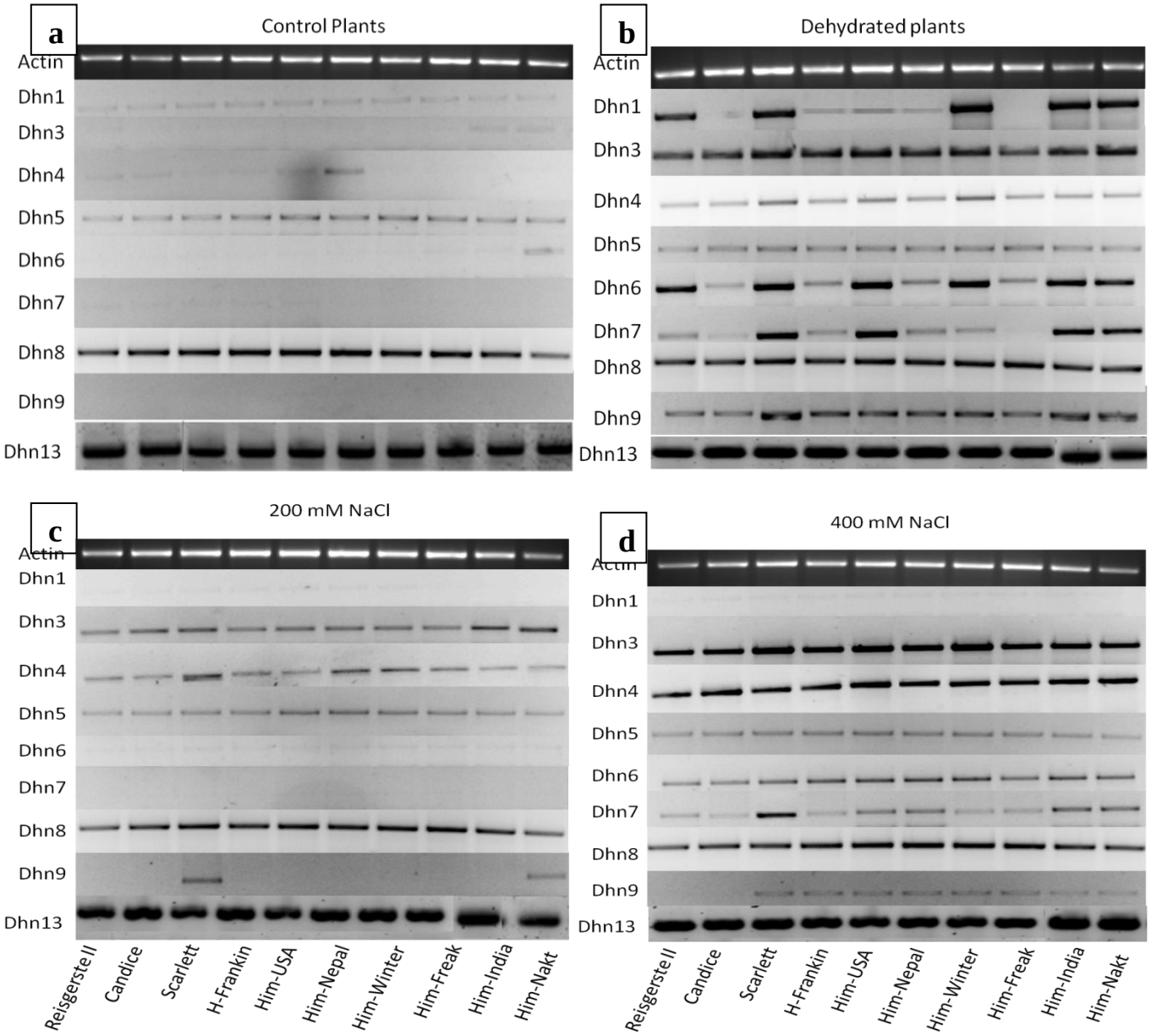

Figure 3.15: Expression analysis of barley dehydrins in control plant (a), drought stressed (b) 200 mM Nacl treated (c) and 400 mM NaCl treated (d). While actin was taken as housekeeping gene.

3.9.2.1 Transcript analysis Dhn1 gene

Dehydrin 1 gene (Dhn1) is one of the thirteen dehydrin genes found in the barley. It belongs to YSK_2 subclass of dehydrin family.

It was not induced in control plants and the plants treated with 200 mM NaCl. However with increase in the concentration of NaCl to 400 mM it was induced at basal level. The application of drought stress resulted in higher level expression in varieties like Reisgerste II, Scarlett, Hmalaya Winter, Himalaya India and Himalaya Nakt and in Himalaya Freak no expression was observed at all, while in rest of the varieties induction of the dhn1 was very low.

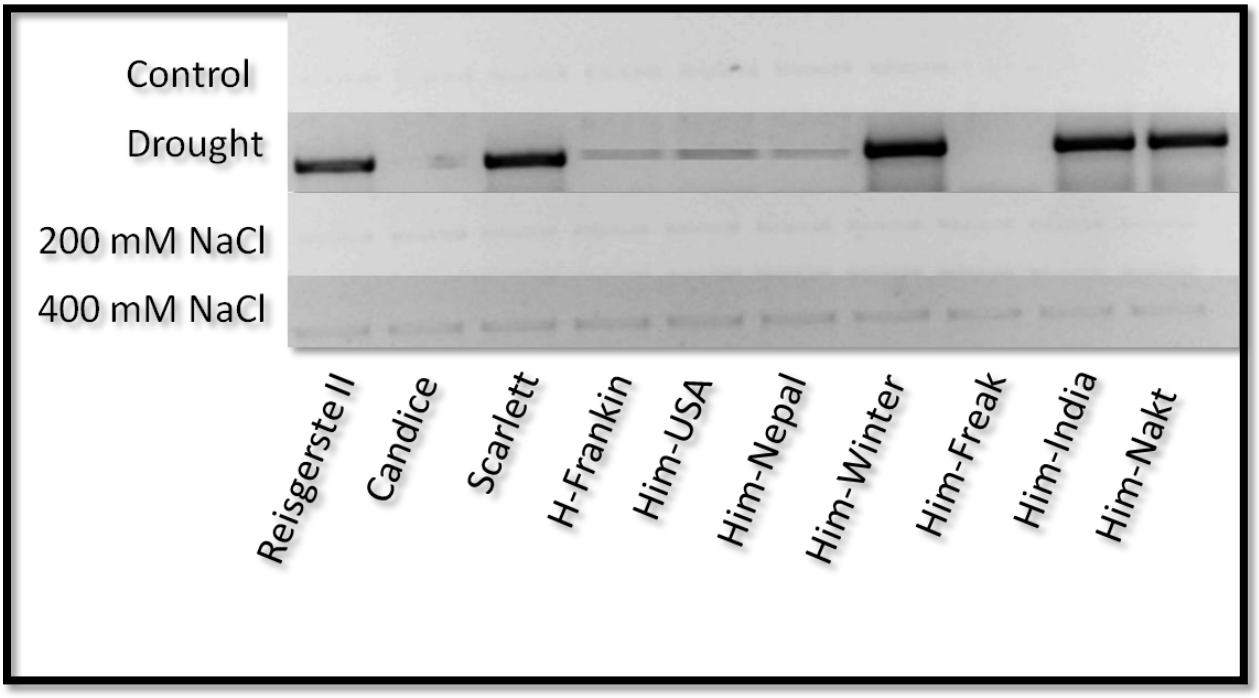

Figure 3.16: Expression analysis Dehydrin 1 (Dhn1) in response to Drought, 200 mM NaCl, and 400 mM NaCl treatment. RNA was extracted from leaves of all the 10 studied barley varieties and RT-PCR was performed as described in the materials and methods.

3.9.2.2 Transcript analysis Dhn2 gene

```
Dhn1        ATGGAGTACCAGGGTCAGCACGGCCACGCCACCGACAAGGTGGAGGAGTACGGCCAGCCC
Dhn2        ATGGAGTACCAGGGACAGACCGGCCGCGCCACCGACAAGGTGGAGGAGTACGGCCAGCCC
            **************:***..*****.**********************************

Dhn1        GTGGCCGGGCACGGCGGTTTCACCGGCGGGCCCACGGGGACGCACGGAGCTGCCGGCGTC
Dhn2        GTGGCCGGCCACGGCGGCGCCACCGGCGGACCCACGGGGACTCACGGCGCCGCCGCCGCC
            ******** ********   *********.********** *****.** **** ** *

Dhn1        GGCGGC---GCGCAGCTCCAGGCGACGAGGGATGGCCACAAGACTGACGGCGTACTTCGA
Dhn2        GCGGGCACGGGGCAGCTCCAGCCGACCAGGGACGACCACAAGACCGACGGTGTCCTGCGC
            *  ***   * ********** **** ***** *.********* ***** **.** **.

Dhn1        CGCTCCGGCAGCTCCAGCTCCAGCTCCTCTGAGGACGACGGCGTGGGCGGGAGGAGGAAG
Dhn2        CGCTCCGGCAGCTCCAGCTCCAGCTCGTCTGAGGACGACGGCGTTGGCGGGAGGAGGAAG
            ************************* ***************** ***************

Dhn1        AAGGGGATGAAGGAGAAGATCAAGGAGAAGCTCCCCGGAGGAGCCCACAAGGACGCCGCA
Dhn2        AAAGGGATGAAGGAGAAGATCAAGGAGAAGCTCCCCGGTGGTGCCCACAAGGACGCCGCC
            **.***********************************:**:*****************.

Dhn1        GGGCAGCAGCAGCAGACGGCGATGGCGGGCGAGTACGCG------GGCACACATGGCACG
Dhn2        GGGCAGCAGCACA---CGCCGGCGGCCGGCGAGTACGCCGGCACCGGCACGCACGGCGCG
            *********** .   ** **. *** ***********       *****.** ***.**

Dhn1        GAGGCCACCGGCGAGAAGAAGGGCGTCATGGACAAGATCAAGGAGAAGCTTCCCGGCGGA
Dhn2        GAGGCCACCGGCGAGAAGAAGGGTGTCATGGACAAGATCAAGGAGAAGCTTCCCGGCGGA
            ********************** ***********************************

Dhn1        CAGCACTGA
Dhn2        CAGCACTGA
            *********
```

Figure 3.17: Alignment of coding sequences of dehydrin 1 with 2.

The alignment data in figure 17 showed that the coding sequence of Dhn1 and Dhn2 are 87% identical. So it was difficult to design gene specific primers which could only amplify Dhn2.

3.9.2.3 Transcript analysis of Dhn3 gene

Dehydrin 3 (Dhn3) is also a YSK_2 dehydrin from barley. In all the studied stress treatments Dhn3 was found to be up-regulated but the degree of expression was different in all treatments and in the varieties as well. In dehydrated plants expression level of Dhn3 in varieties Reisgerste II, Candice, and Himalaya Freak was comparatively less than rest of the varieties. While at 200 mM NaCl treated plants, the induction level was not so strong and was almost same in all the varieties. However the 400 mM NaCl treatment resulted in huge increase in xpression level, strongest bands were found in Scarlett, Himalaya USA and Himalaya Winter.

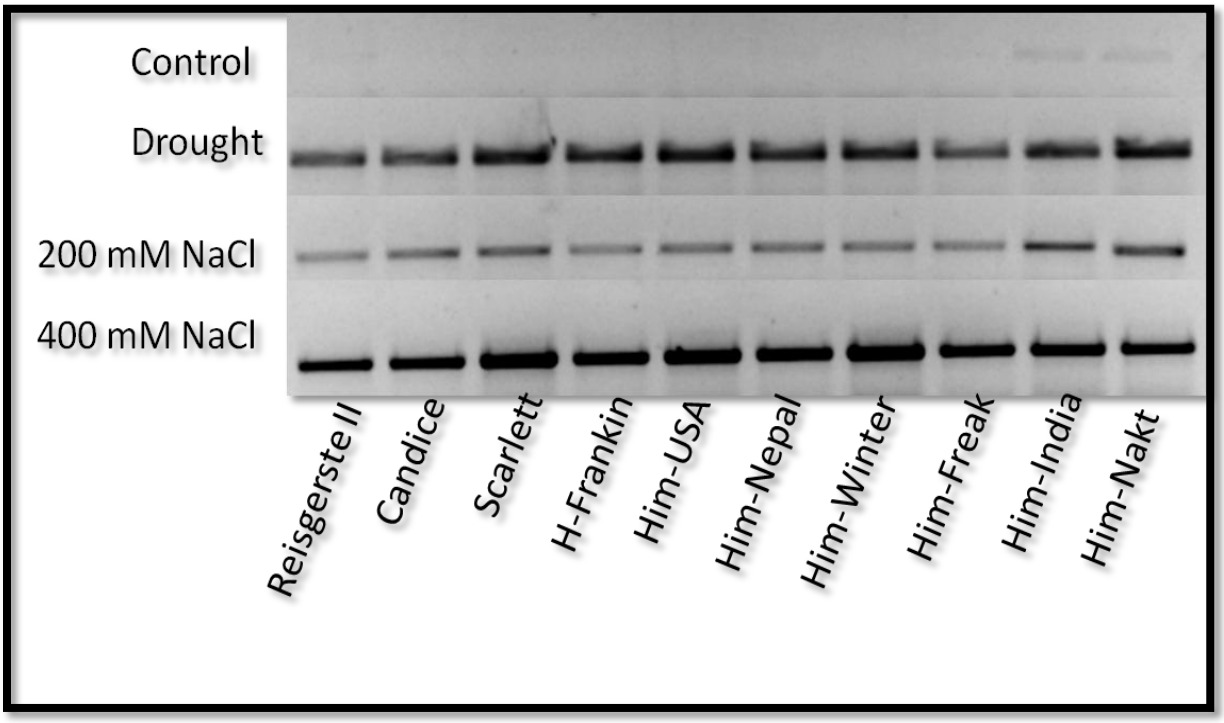

Figure 3.18: Expression analysis Dehydrin 3 (Dhn3) in response to Drought, 200 mM NaCl, and 400 mM NaCl treatment. RNA was extracted from leaves of all the 10 studied barley varieties and RT-PCR was performed as described in the materials and methods.

3.9.2.4 Transcript analysis Dhn4 gene

Barley dehydrin 4 (Dhn4) is also from YSK_2 subclass of dehydrin. Dhn4 was upregulated in all stresses like drought, 200 mM NaCl and 400 mM NaCl in under studied varieties of barley. Among dehydrated plants relatively high expression was observed in Scarlett, Himalaya USA and Himalaya winter. While in 200 mM NaCl treatment, highest induction was in Scarlett, Himalaya Nepal and Himalaya winter. However, at 400 mM NaCl treatment all the varieties had almost same level of expression in all varieties.

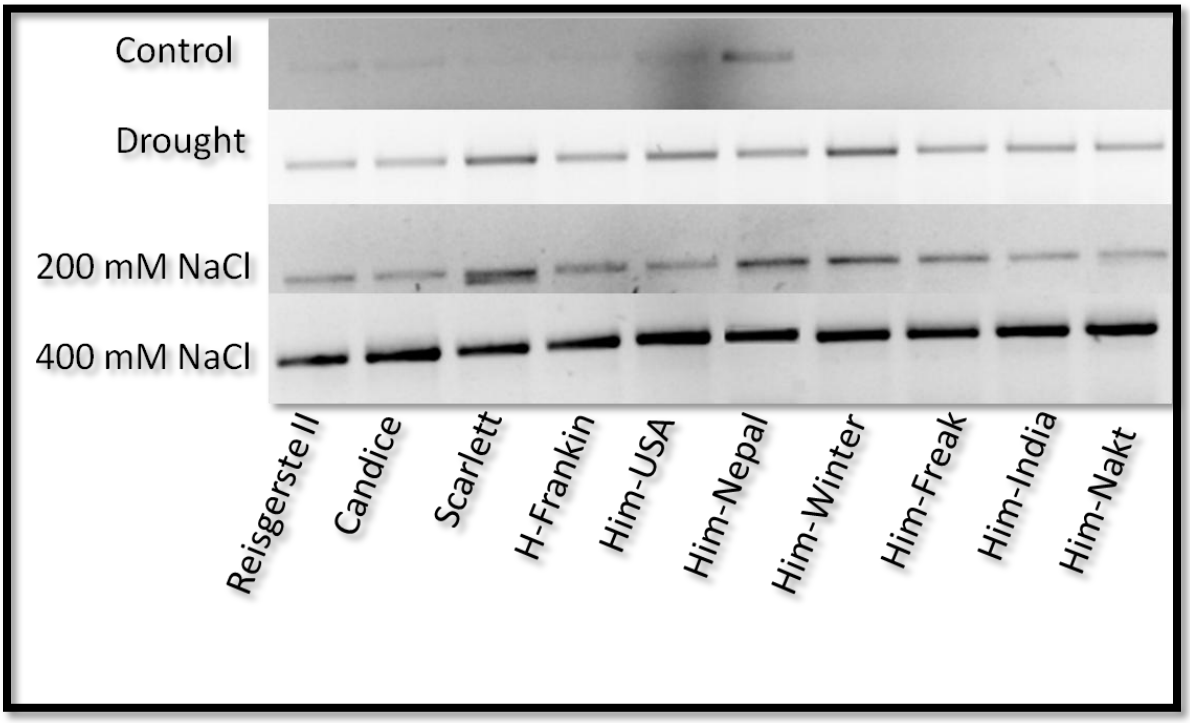

Figure 3.19: Expression analysis Dehydrin 4 (Dhn4) in response to Drought, 200 mM NaCl, and 400 mM NaCl treatment. RNA was extracted from leaves of all the 10 studied barley varieties and RT-PCR was performed as described in the materials and methods.

3.9.2.5 Transcript analysis Dhn5 gene

Dehydrin 5 (Dhn5) gene is the only gene in barley, which belongs K_9 subclass of dehydrin. Upon stress treatments no significant difference was observed in any of the studied varieties. Figure 20 shows a basal level constitutive expression of Dhn5 gene in all stresses in all studied barley varieties.

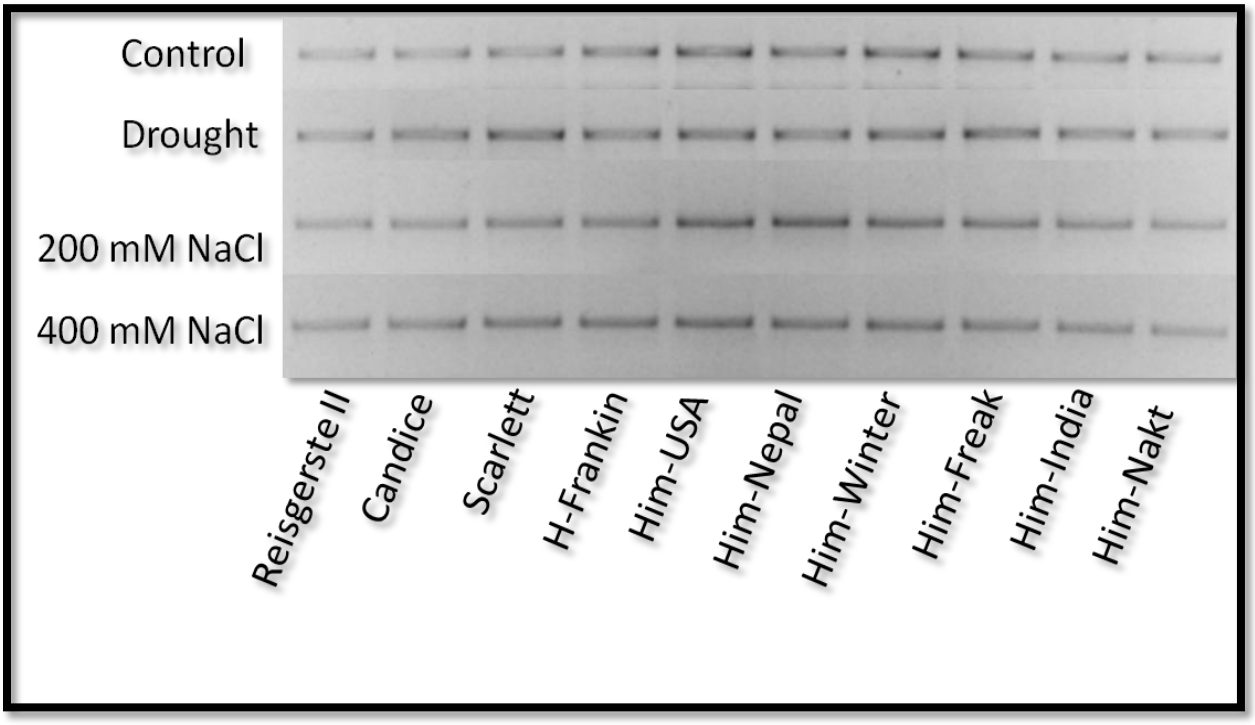

Figure 3.20: Expression analysis Dehydrin 5 (Dhn5) in response to Drought, 200 mM NaCl, and 400 mM NaCl treatment. RNA was extracted from leaves of all the 10 studied barley varieties and RT-PCR was performed as described in the materials and methods.

3.9.2.6 Transcript analysis Dhn6 gene

Dehydrin 6 (Dhn6) belongs to Y_2SK_3 subclass of dehydrin family. The expression of Dhn6 was very strong in some varieties such as Reisgerste II, Scarlett, Himalaya USA, Himalaya Winter, Himalaya India and Himalaya Nakt but in rest of varieties it was expressed only at basal level in drought stress. In case of salt stress no induction was found at 200 mM NaCl treatment, however at increased concentration of NaCl it was expressed in all varieties with varied degree of expression and lowest bands were in Reisgerste II and Candice.

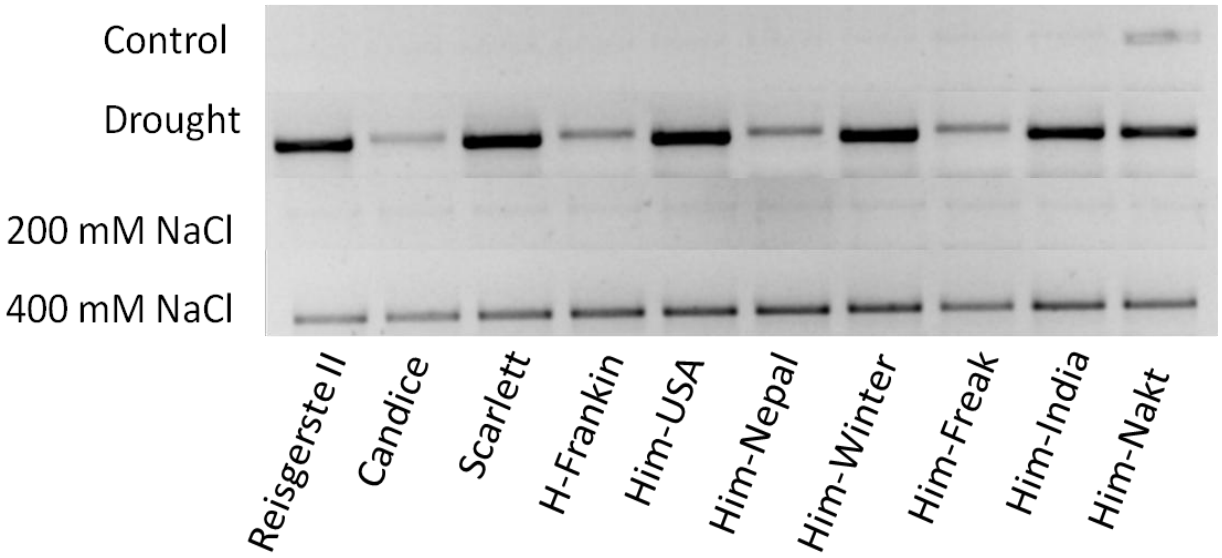

Figure 3.21: Expression analysis Dehydrin 6 (Dhn6) in response to Drought, 200 mM NaCl, and 400 mM NaCl treatment. RNA was extracted from leaves of all the 10 studied barley varieties and RT-PCR was performed as described in the materials and methods.

3.9.2.7 Transcript analysis Dhn7 gene

Dehydrin 7 (Dhn7) is a member of YSK_2 subclass of dehydrin. Dhn7 was not expressed in any of the varieties in case of well watered (control) plants and in plants with 200 mM NaCl treatment. However in dehydrated plants, higher induction was observed in Scarlett, Himalaya USA, Himalaya India, and Himalaya Nakt, however it was not induced in Himalaya Freak and a minimal induction was observed in case of Candice. Dhn7 gene was up-regulated with the application of 400 mM NaCl, and strongest band was observed in Scarlett, remaining varieties had very basal level of expression.

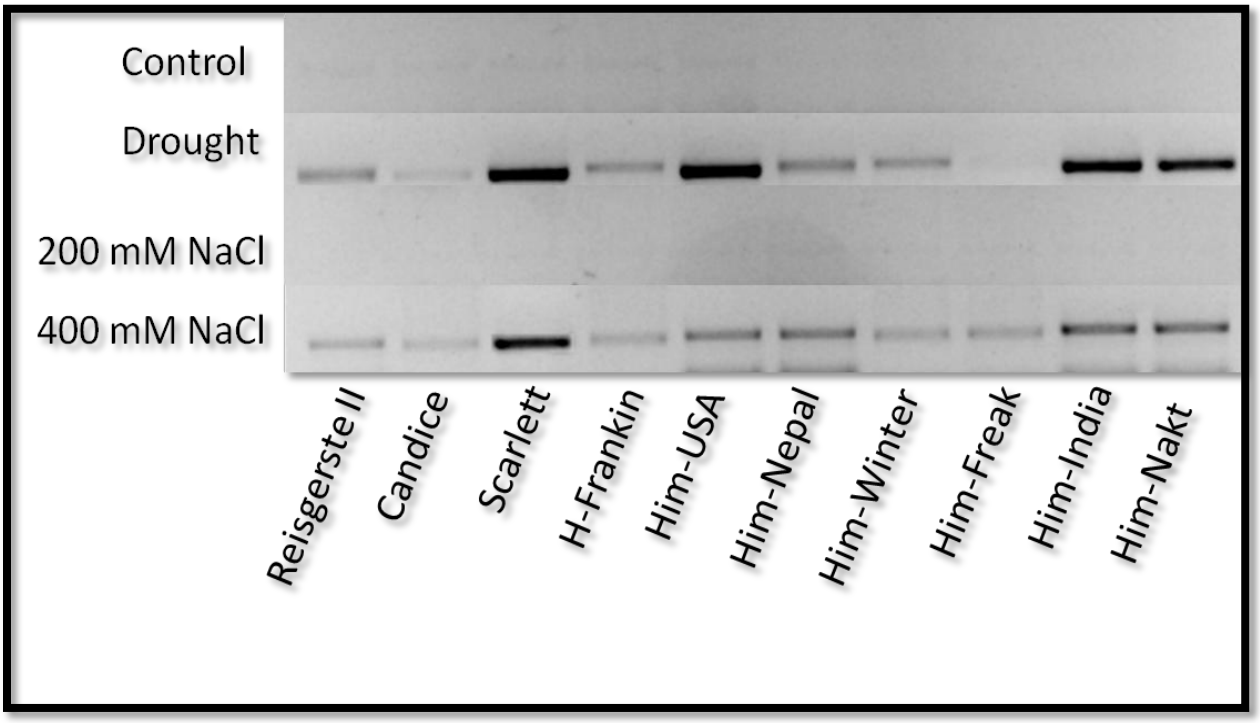

Figure 3.22: Expression analysis Dehydrin 7 (Dhn7) in response to Drought, 200 mM NaCl, and 400 mM NaCl treatment. RNA was extracted from leaves of all the 10 studied barley varieties and RT-PCR was performed as described in the materials and methods.

3.9.2.8 Transcript analysis Dhn8 gene

Dehydrin 8 is a SK_3 type dehydrin in barley. Its expression was very strong constitutive expression as it was induced equally in all the varieties on all the levels of stresses i.e. drought, 200 mM NaCl and 400 mM NaCl.

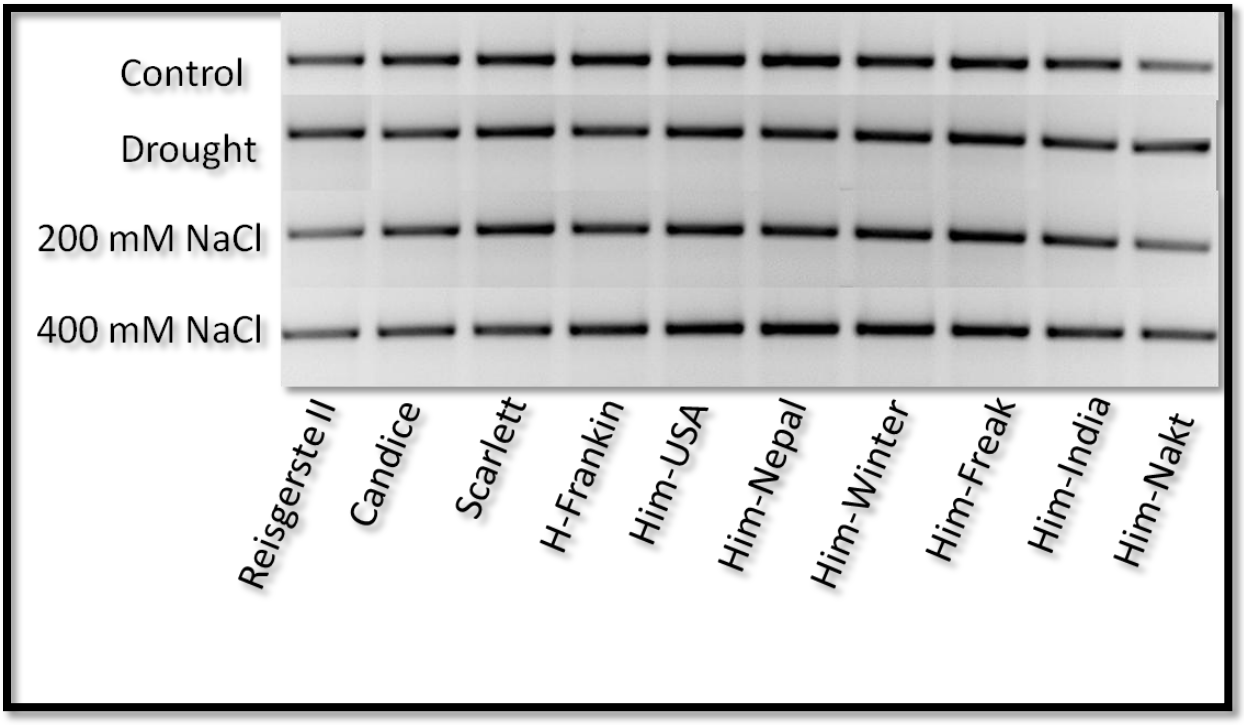

Figure 3.23: Expression analysis Dehydrin 8 (Dhn8) in response to Drought, 200 mM NaCl, and 400 mM NaCl treatment. RNA was extracted from leaves of all the 10 studied barley varieties and RT-PCR was performed as described in the materials and methods.

3.9.2.9 Transcript analysis Dhn9 gene

Dehydrin 9 is a YSK_2 dehydrin. In control plants there is no induction of Dhn9 gene, however it was up-regulated in all varieties in dehydrated plants, strongest induction was found in Scarlett. In plants treated with 200 mM NaCl it was only expressed at Scarlett and Himalaya Nakt, while at 400 mM NaCl application it was not induced only in Reisgerste II and Candice however very basal level expression was observed in rest of varieties.

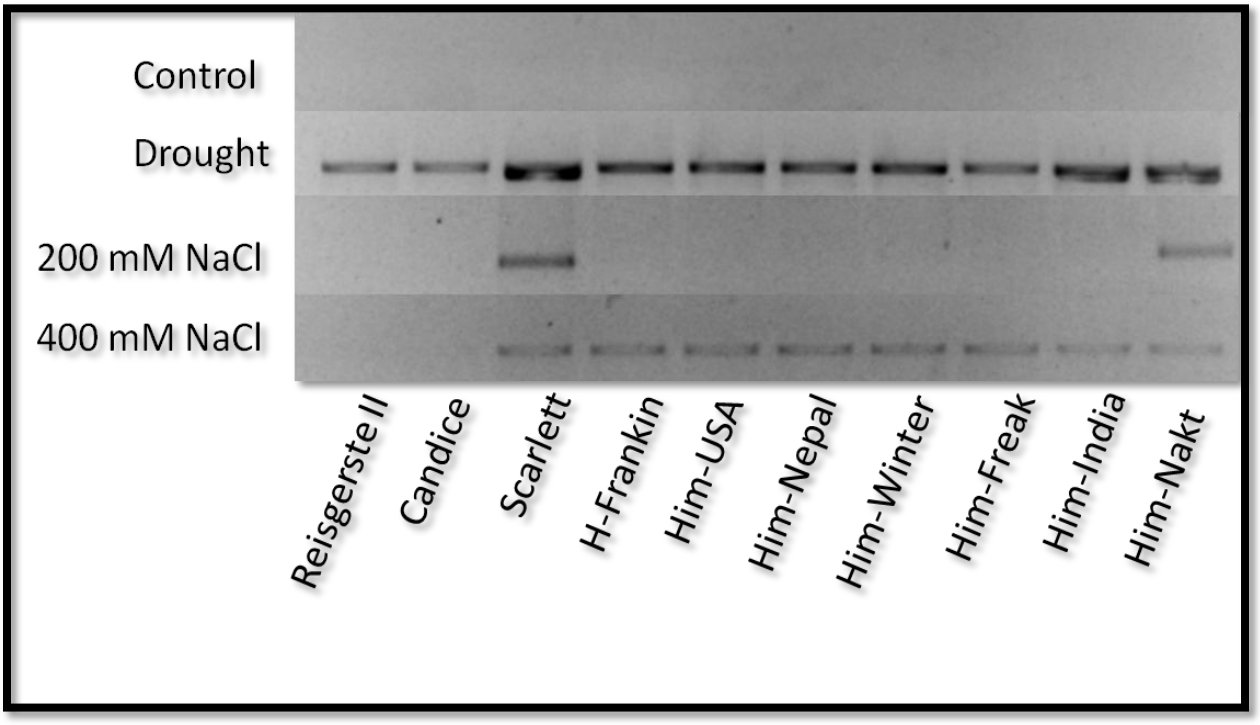

Figure 3.24: Expression analysis Dehydrin 9 (Dhn9 in response to Drought, 200 mM NaCl, and 400 mM NaCl treatment. RNA was extracted from leaves of all the 10 studied barley varieties and RT-PCR was performed as described in the materials and methods.

3.9.2.10 Transcript analysis Dhn10, 11, and 12 gene

Dhn10, 11 and 12 were not observed under control and stressed conditions.

3.9.2.11 Transcript analysis Dhn13 gene

Barley Dehydrin 13 is KS type dehydrin. Its expression was very strong constitutive expression as was in Dhn8 and was induced constitutively in all the varieties at all the three under studied stresses like drought, 200 mM NaCl and 400 mM NaCl.

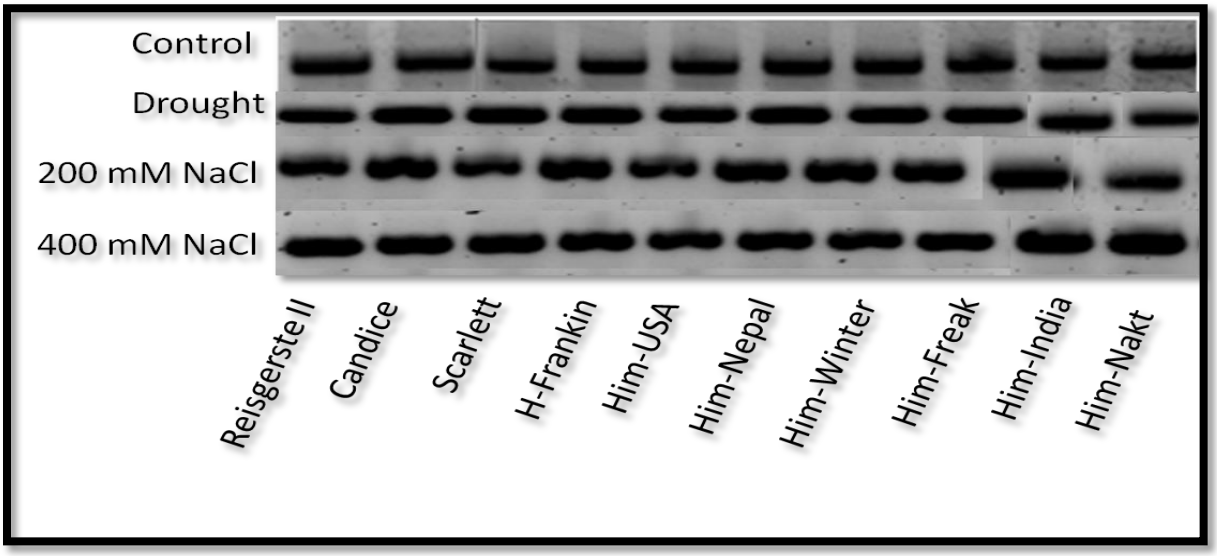

Figure 3.25: Expression analysis Dehydrin 13 (Dhn13) in response to Drought, 200 mM NaCl, and 400 mM NaCl treatment by RT-PCR of RNA extracted from leaves of all the 10 studied varieties.

3.9.3 Immuno Blots Analysis

Western blot analysis was performed to check whether induction of Dhn genes mRNA transcripts correlate with the accumulation of corresponding DHN protein in respective varieties. Dehydrin polyclonal antisera specific to K segment consensus sequence (TGEKKGIMDKIKEKLPGQH) (Close et al.,1993) was used in protein blot analysis. To verify the equal loading of protein, the membranes were stained with Ponceau stain,

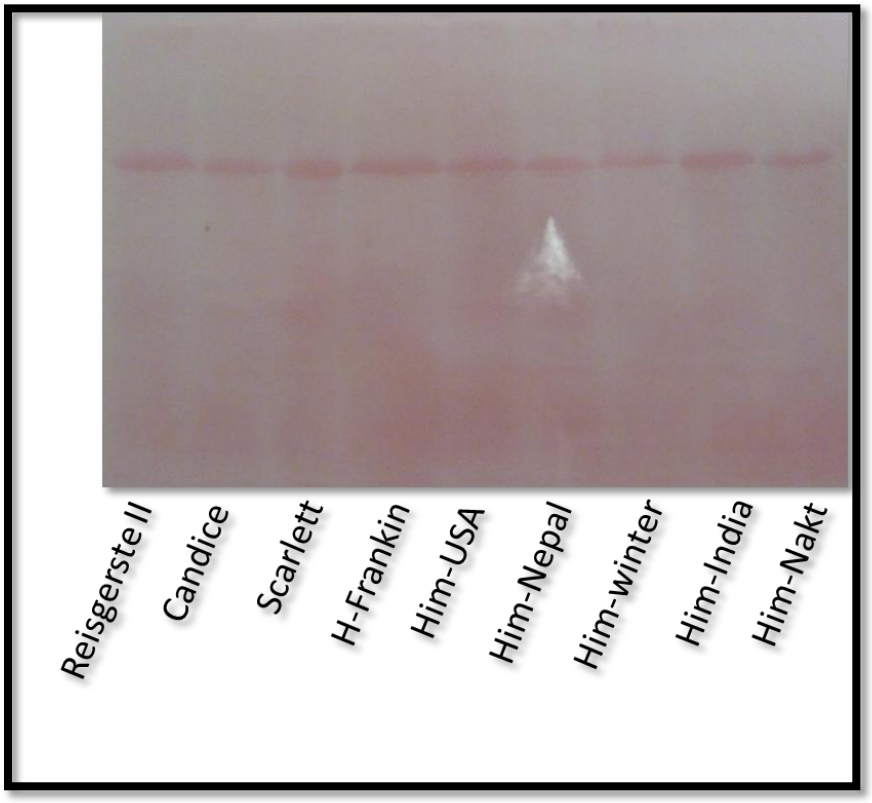

Figure 3.26: Ponceau staining of the membrane to check the equal loading of the protein

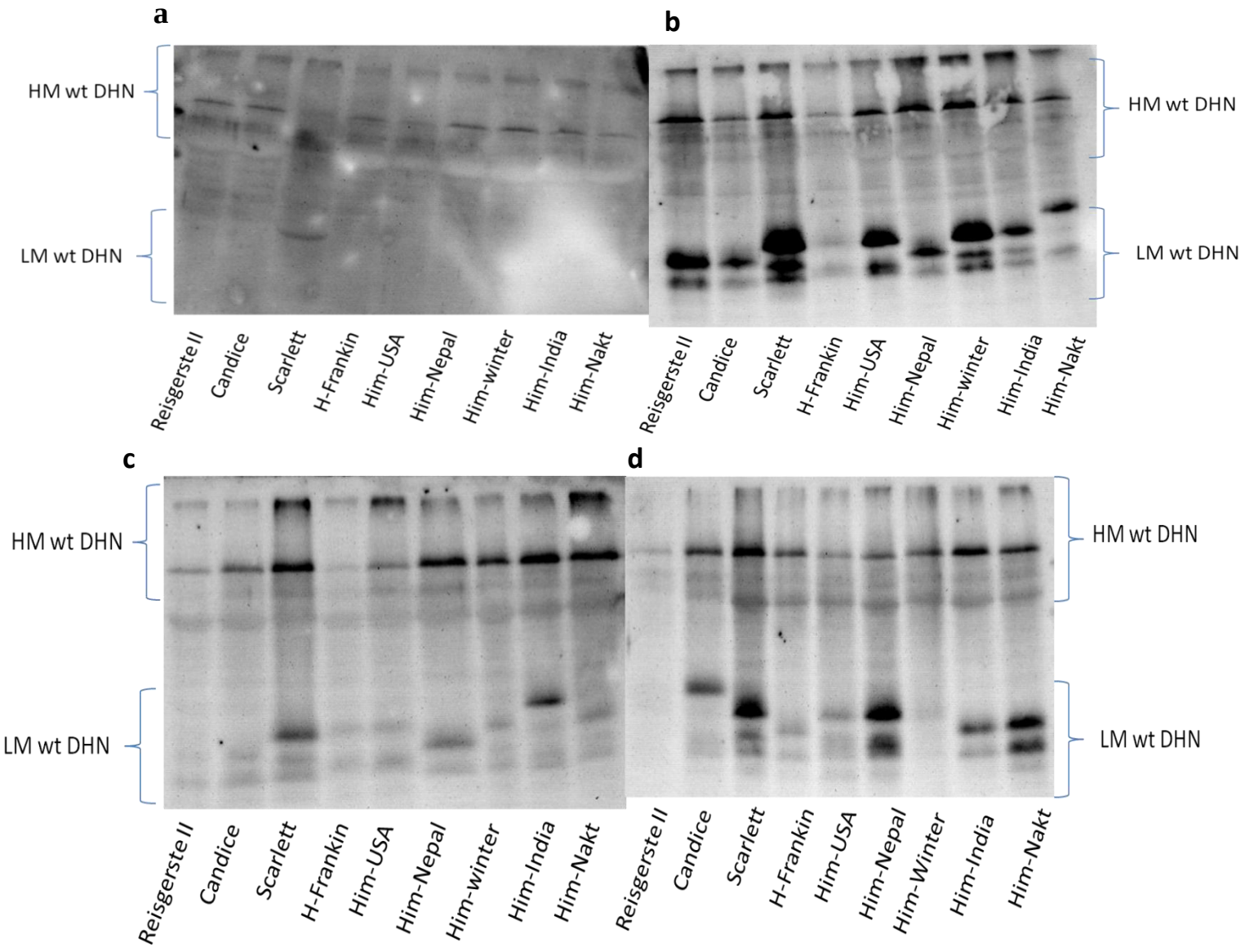

Figure 3.27: Immunoblot analysis of DHN expression in barley varieties under different stress conditions (a) control plants (b) drought treated plants (c) plants at 200 mM NaCl tratment (d) plants treated with 400 mM NaCl.

Immunoblot analysis (Fig. 25) showed that application of stress induced the accumulation of DHN proteins. At 200 mM NaCl (Fig. 25C) application in barley varieties only of low molecular weight dehydrin protein were accumulated. However at 400 mM NaCl treatment the accumulation of low molecular weight dehydrin protein was much higher. Moreover highest accumulation of low molecular weight dehydrin protein was found in dehydrated plants (Fig. 25C). The occurrence of several copies of low molecular weight dehydrin protein superfamily work together.

3.9.4 Sub-cellular Localization of dehydrin proteins

3.9.4.1 Sub-cellular Localization of barley dehydrin3

To determine the subcellular localization of DHN3 from barley, onion epidermal cells were transformed with DHN3 from barley fused with GFP. The distribution of the DHN3 was analyzed through confocal microscope, and it was found to be present in nucleus and cytoplasm, however majorly it was found in nucleus.

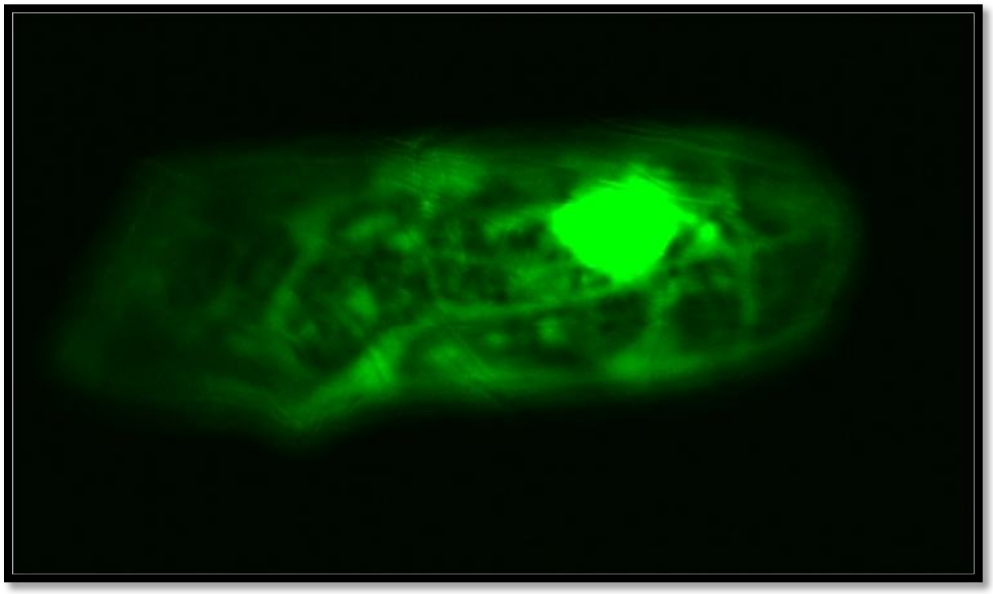

Figure 3.28: Sub-cellular localization DHN3 from barley in onion epidermal cells. Confocal microscopy images of onion cells transformed with DHN3 fused with GFP.

3.9.4.2 Predicted Subcellular Localization of Barley Dehydrins

For the rest of the dehydrin protein subcellular localization were predicted using the following online web service: http://psort.hgc.jp/form.html

Table 3.2: Predicted subcellular localization barley dehydrin using tool from http://psort.hgc.jp/form.html,

Dehydrin	
DHN1	Nucleus Certainty= 0.300 microbody (peroxisome) Certainty= 0.281
DHN2	nucleus Certainty= 0.300 microbody (peroxisome) Certainty= 0.281
DHN3	nucleus --- Certainty= 0.640 microbody (peroxisome) Certainty= 0.300
DHN4	nucleus --- Certainty= 0.640 microbody (peroxisome) Certainty= 0.300
DHN5	microbody (peroxisome) Certainty= 0.64 cytoplasm Certainty= 0.450
DHN6	nucleus --- Certainty= 0.640 microbody (peroxisome) Certainty= 0.300
DHN7	nucleus --- Certainty= 0.640 microbody (peroxisome) Certainty= 0.300
DHN8	nucleus --- Certainty= 0.700 microbody (peroxisome) Certainty= 0.30
DHN9	microbody (peroxisome) Certainty= 0.475 nucleus --- Certainty= 0.300
DHN10	microbody (peroxisome) Certainty= 0.475 nucleus --- Certainty= 0.300
DHN11	nucleus --- Certainty= 0.300 chloroplast stroma --- Certainty= 0.200
DHN12	cytoplasm --- Certainty= 0.450 microbody (peroxisome) --- Certainty= 0.233
DHN13	nucleus --- Certainty= 0.992 chloroplast stroma --- Certainty= 0.200

Table 3.3: Predicted sub-cellular localization of different barley dehydrinin using tool https://wolfpsort.hgc.jp/results/pEY9cf80f1e228e4ce1b2d4ece524a7ef43.html

	Dehydrins	**Type**	**Localization prediction**
1	DHN1	YSK_2	Nucleus/Cytoplasm/Plastids
2	DHN2	YSK_2	Nucleus/Mitochondria/Cytoplasm
3	DHN3	YSK_2	Nucleus/Plastids/Cytoplasm
4	DHN4	YSK_2	Nucleus/Plastids/Cytoplasm
5	DHN5	K_9	Nucleus/Cytoplasm/Mitochondria/Plastids
6	DHN6	YSK_3	Nucleus/Cytoplasm/Peroxisome/Mitochondria
7	DHN7	YSK_2	Nucleus/Cytoplasm/Plastids
8	DHN8	SK_3	Nucleus/Chlorophyll
9	DHN9	YSK_2	Nucleus/Cytoplasm/Plastids
10	DHN10	YSK_3	Nucleus/Cytoplasm/Plastids
11	DHN11	Y_2SK_2	Nucleus/Chlorophyll/Cytoplasm
12	DHN12	YSK_2	Nucleus/Chlorophyll/Mitochondria/Plastids
13	DHN13	KS	Nucleus/Plastids

Predicted sub-cellular localization of different barley dehydrinin using tool https://wolfpsort.hgc.jp/results/pEY9cf80f1e228e4ce1b2d4ece524a7ef43.html, predictions were made on the basis of number of different proteins resembled with query and probability is from high to low.

In-silico analysis of the all the barley dehydrin performed on two different online services showed that all the barley dehydrin had maximum probability to be located in nucleus, with second probability in cytoplasm.

As predicted in in-silico study, the maximum chance of accumulation DHN3 was in the nucleus and same result we found in our studies. In another study by Brini *et al*., 2007 showed that a dehydrin DHN5 in wheat, which is homolog of barley dehydrin DHN4 in barley, was found to present in the nucleus and cytoplasm.

4. DISCUSSION

Barley is a very important cereal which ranked fifth in 2014 on basis of area of production after wheat, maize, rice and soybean (http://faostat.fao.org). Due to its salient features like short life cycle, large number of varieties, capability to grow in different climatic conditions/environment and tolerance to majority of abiotic stress, it is considered as model cereal crop (Saisho and Takeda, 2011). Drought and salinity are the two major produce limiting factors in agriculture (Wang et al., 2003) where salinity alone affects about 800 million hectares of land (Munns, 2005).

Plants have developed complex mechanisms to counter different kinds of stresses. As the agricultural crops have large number of varieties within the specie so, it is also a good approach to study the effect of abiotic stress in the agricultural crops (Ingram and Bartels, 1996). Finding the tolerant plants from the existing varieties can help the breeders in developing the new cultivars with desired traits. Although plants can be screened on the basis of their visible traits like biomass, root/shoot ratio etc, but these traits could be deceiving depending on different environments. Hence, it is essential to evaluate the performance of the plants under stress conditions through molecular, physiological and biochemical methods.

4.1 Growth parameters

In the current study, the stress situations were induced in the ten old barley varieties by treating them with 200 mM NaCl, 400 mM NaCl solution and drought stress. Drought stress was applied by stopping the irrigation of the plants. All these stresses were applied for seven days. Phenotypic observations showed that the health of the plants was negatively correlated with the degree of stress. The 200 mM NaCl treatment affected the barley plants to lesser extent, which could be due to the fact that barley is generally considered as tolerant to abiotic stress. Moreover the behaviour of plants at 400 mM NaCl and drought treatment was more or less same (as the salt stress also causes oxidative stress). In all the treatments reduction occurred in all studied growth parameters like number of leaves, shoot length and root length. This may be due to the shrinkage of the cells due to less availability of water, decrease in cell enlargement, stomatal closure and increase in the reactive oxygen species (ROS) (Daneshmand et al., 2010; Gunes et al., 2007; Meneguzzo et al., 1999; Steduto et al., 2000). Disturbances in Na^+ and Cl^- ion homeostasis could be an additional reason for the decreased biomass in the salt-stressed plants. A big decrease in the numbers of leaves in Himalaya India and Himalaya Nakt was observed

because the growth of these varieties in control treatment was very high, however in the stressed plants they also had higher growth while comparing with corresponding plants of other varieties. Similarly, the decrease in growth was also observed in other plants like in soybean (Specht et al., 2001), potato (Heuer and Nadler, 1995) and in citrus (Wu et al., 2008).

4.2 Water retaining capability

Water loss rate (WLR) is one way to determine the drought tolerance in plants. In WLR assay plant leaves are exposed to severe but short term drought stress and then the amount of water lost by these leaves is calculated. Higher amount of water lost by leaves corresponds to low tolerance and vice versa. The very low water loss rate in Himalaya Nakt and Himalaya India showed the tolerance of these plants to oxidative stress, while on other hand highest water loss was observed in Himalaya Freak. Relative water contents (RWC) in leaves is another way to check plant water retention capability and drought resistance of plant, it depends on how much water plants retain during stress. RWC experiments confirmed the results of WLR experiments.

4.3 Total chlorophyll contents

Chlorophyll is an important green colour pigment in plant leaves which absorbs energy from the light and is necessary for photosynthesis. Severe oxidative stress limits photosynthesis by affecting chlorophyll contents and damaging photosynthesis apparatus (Iturbe-Ormaetxe et al., 1998; Ommen et al., 1999). Chlorophyll contents decreased in all varieties on all stress treatments, however in the majority of varieties at 200 mM NaCl, chlorophyll contents were same as they were in control plants. Although at 400 mM NaCl and drought conditions, chlorophyll contents decreased in all varieties and the minimum amount was found in Himalaya Freak, which was supposed as susceptible to salt and drought conditions in water loss rate and relative water contents experiments. On the other hand tolerant varieties like Scarlett, Himalaya Nakt and Himalya India were having maximum chlorophyll contents. Munné-Bosch and Alegre (1999) correlated this decrease with relative water contents and considered as adaptive feature of plants in water deficiency. Chlorophyll is degraded when reacted with oxygen and salinity enhances the activity of chlorophyllase which degrades the chlorophyll (Rao and Rao, 1981), or inhibitory effects of these ions on other chlorophyll fractions (Ali et al., 2004). These results are in agreement with other studies in some other plants e.g. in wheat (Nyachiro et al., 2001) and chickpea (Mafakheri et al., 2010) in drought and under salinity in rice (Ali et al., 2004). Decrease

in photosynthesis could also be a reason for decreased biomass/growth parameters in our varieties.

4.4 Proline contents

Proline is an important amino acid and a widely distributed osmoprotectant in plants and many other organisms (Delauney and Verma, 1993; McCue and Hanson, 1990). It accumulates in large quantities in response to various environmental stresses (Ali et al., 1999; Kishor et al., 2005) such as in drought stress (Hare et al., 1998), in salinity (Munns, 2005; Rhodes and Hanson, 1993), in low temperature (Naidu et al., 1991) and in heavy metals (Bassi and Sharma, 1993; Sharma and Dietz, 2006). The concentration of proline is found to be higher in stress tolerant plants as compared to the stress sensitive plants (Fougere et al., 1991; Petrusa and Winicov, 1997). In this study, application of any kind of stress resulted in increased accumulation of free proline. However, in the varieties which had higher relative water content like scarlett, Himalaya Nakt and Himalaya India its accumulation was maximum, while in the varieties with lower water contents also had lower increase in free proline contents. In contrast with our results where proline contents increased in all varieties, Binott et al. (2017) while working on Kenyan varieties found that proline contents were increased in the tolerant varieties while decreased in the susceptible except for Karne which was susceptible variety but found to have increased proline contents. It is already established that proline acts as osmoprotectant by maintaining the cell volume and fluid balance (Delauney and Verma, 1993), it also acts as chemical chaperone, metal chelator and ROS scavenging agent (Liang et al., 2013b). Proline also enhances the activities of antioxidative enzymes and Hoque et al. (2007) reported that exogenous application of proline to tobacco suspension cultures exposed to salinity stress resulted in the enhancement of the activities of SOD, catalase, and peroxidase antioxidative enzymes.

4.5 MDA level and Hydrogen peroxide

Lipid peroxidation is generally considered as a marker for stress tolerance in plants as it is a measure of damage to the membrane. The amount of malondialdehyde (MDA), that is produced on the oxidation of polyunsaturated fatty acids, is considered a useful index of general lipid peroxidation. Plants having low MDA level is thought to be tolerant against the respective stress. MDA level decreased in all varieties in both drought and salt stress, and increase in concentration of salt also resulted in increasing the MDA level in all of our varieties, however, this increase was higher in varieties with less water retention capability like Himalaya Freak and

the opposite was observed in the varieties with higher water retaining ability. Many studies in different plant species showed similar results e.g. in Kentucky bluegrass on drought treatment, tolerant varieties had a least MDA level (Xu et al., 2011), in wheat plants exposed to two days of drought treatment, higher accumulation of MDA was observed (Wu et al., 2012). Sairam and Srivastava (2001) found that drought tolerant wheat had lower lipid peroxidation level than susceptible one. Similarly, under salt stress conditions, the levels of MDA were found to be higher in salt sensitive varieties of rice (Demiral and Türkan, 2005), corn (Hamada AbdElgawad et al., 2016; Valentovic et al., 2006), and rapeseed (Farhoudi et al., 2011).

Different metabolic reactions in the plant cells result in the production of reactive oxygen species (ROS). Hydrogen peroxide (H_2O_2), superoxide (O_2^-), hydroxyl ion (OH^-) and nascent oxygen ($^1O_2$) are produced as early response to the oxidative stress (Hossain et al., 2015). The amount of ROS such as H_2O_2 in plant samples can be used as a marker of stress determination in the plants. In this study tolerant varieties like Scarlett, Himalaya India and Himalaya Nakt had much lower H_2O_2 accumulation in their tissue samples so they have lower MDA level than the susceptible variety Himalya Freak. Binott et al. (2017) found that most of the tolerant varieties had lower MDA level in comparison to susceptible Kenyan varieties. Alexieva et al. (2001) who worked on pea and wheat, also found increase in accumulation of H_2O_2 upon exposure to drought and ultra violet radiations. Chakraborty and Pradhan (2012) found that when stress was applied, accumulation of H_2O_2 was higher in drought sensitive varieties of wheat than the tolerant varieties. Sharma et al. (2014) also found similar results in different cultivars of wheat.

4.6 Activity of Antioxidative enzymes

Reactive oxygen species (ROS) is an unavoidable product of metabolic reactions such as respiration and photosynthesis in plants. ROS production increases under stress. Higher amount of ROS can cause damage to the nucleic acids, proteins and lipids and increase the permeability of the cells thereby causing cellular damage (Cruz de Carvalho, 2008; Gill and Tuteja, 2010). Inability of plant to scavenge ROS can result in the death of the plants. Antioxidative enzymes such as superoxide dismutase (SOD) dismutases the oxygen radicals into H_2O_2 and catalase scavenges the H_2O_2, thereby decreasing the ROS levels in plant cells. Higher amounts of antioxidative enzymes produced in the plants on exposure to stress makes plants tolerant to that particular stress. The SOD activity in most of the studied barley varieties increased at 200 mM NaCl application except for the most stress susceptible variety Himalaya Freak. On increase in

concentration of salt to 400 mM NaCl, the SOD activity decreased in most varieties, however the tolerant varieties had higher activity than susceptible varieties. Similarly, in drought stress it also decreased but in some tolerant varieties like Scarlett and Himalaya Nakt it was similar to control plants. Catalase activity at 200 mM NaCl also had similar increase as was found in case of SOD. A higher activity was observed at 400 mM NaCl and drought. The activities of both SOD and catalase were higher in tolerant varieties than in susceptible varieties in both drought and salinity stresses. The decrease in the activities of SOD and Catalase on 400 mM NaCl and drought can be correlated with phenotypic behavior of the varieties. As the damage caused by ROS was much higher in the plants at 400 mM NaCl and drought; it may not let the SOD and catalase to activate properly.

Glutathione reductase (GR) is an enzyme which converts oxidized glutathione to the reduced one with the oxidation of NADPH (Halliwell and Gutteridge, 2015). The reduced glutathione is a strong reducing agent, which protects the membrane from peroxidation caused by ROS. Higher activity of GR corresponds to the higher tolerance in plants. Peroxidase (POX) is an enzyme, which catalyzes the reduction of H_2O_2, hence has a role in scavenging the ROS. The activities of GR and POX were found to be increased in most of the studied barley varieties on all stresses. At 200 mM NaCl treatment, the varieties like Candice and Himalya Freak had similar GR activities as were found in control plants, while in contrast Candice had higher POX activity at 200 mM NaCl. However, with increase in the concentration of NaCl to 400 mM and on drought treatment, all the varieties showed increased GR and POX activities. The increase in the activities of both the enzymes in the tolerant varieties like Scarlett, Himalaya Nakt and Himalaya India were much more than the susceptible varieties like Reisgerste II, Candice and Himalaya Freak.

Another study on the different barley varieties showed that the tolerant varieties had higher SOD and catalase activities than the sensitive varieties (Marok et al., 2013). Chakraborty and Pradhan (2012) while working on wheat varieties found a decrease in catalase and SOD activities on 6 and 9 days of drought stressed plants but an increase in glutathione reducatase and POX activities, while higher activities were observed in the tolerant varieties than in the susceptible varieties. Xu et al. (2011) found the decrease in SOD and Catalase activity in the Kentucky bluegrass plants. However, another researcher Jiang et al. (2010) did not find any change in the activity of SOD and catalase in prairie junegrass under drought. Molina et al. (2002) showed that

the plants adapted to NaCl had higher glutathione reductase activity than the plants which were not adapted to the NaCl. The activities of catalase, POX and GR increased when treated with NaCl in both salt sensitive and salt tolerant cultivars of wheat but SOD activity decreased (Mandhania et al., 2006).

4.8 Dehydrins in barley

Dehydrins are group 2 LEA (Late Embryogenesis Abundant) proteins (Ingram and Bartels, 1996). Like other members of the LEA protein family, dehydrins accumulate at late stages of embryogenesis. As the late stages of embryogenesis mimic drought conditions so, they also exhibit their expression in oxidative stress conditions. Many studies had revealed that dehydrin expression in plants have a positive correlation with the oxidative stress tolerance (Ismail et al., 1999). It was also observed that plants over expressed with dehydrin genes showed greater tolerance upon comparing with wild type plants. In barley, thirteen dehydrins has been discovered belonging to different sub-classes. The varieties selected in this study were from different parts and climates of the world. The purpose this study was to analyze the differential expression of different dehydrin genes during drought, moderate and high levels of salt stress in different barley varieties from different parts of the world. The physico-chemical analysis of different dehydrins showed the hydrophilicity of these barley dehydrins. Hydrophilins are proteins which largely contain charged amino acids, glycine and other small amino acids like alanine, serine, or threonine (Battaglia and Covarrubias, 2013) but usually do not contain tryptophans and cysteines (Garay-Arroyo et al., 2000). Many studies showed that hydrophilins can prevent the inactivation of certain enzymes such as lactate dehydrogenase or malate dehydrogenase under different dehydration levels (Reyes et al., 2008). Inactivation of these enzymes may result into cell death.

Dehydrin classification is based on presence of conserved segments like K segment, Y segment and S segment, depending on their number in the sequence of a particular dehydrin. On the basis of protein sequences given on https://www.ncbi.nlm.nih.gov , DHN1, DHN2, DHN3, DHN4, DHN7 and DHN9 were classified as YSK_2, DHN11 as Y_2SK_2, DHN6 as Y_2SK_3, DHN13 as KS, DHN12 as SK_2, DHN10 and DHN8 as SK_3 and DHN5 was kept under K_9 dehydrins. However, Tommasini et al. (2008) classified DHN6 and DHN10 as YSK3 and DHN12 as YSK_2.

The reverse-transcriptase polymerase chain reaction (RT-PCR) was carried out to check the relative expression levels of different dehydrins in different barley varieties exposed to 200 mM NaCl, 400 mM NaCl and drought conditions. The expression of dehydrins differed in different plants depending on the genotype and type of stress and intensity of stress. Dehydrin 1 (Dhn1) expressed exclusively in drought stress treatment, and strong induction was found only in Himalaya Nakt, Himalaya India, Himalaya Winter, Scarlett and Reisgerste II, however the induction in gene expression was not observed in Himalaya Freak and Candice. Dehydrin 3 (Dhn3) was induced in all stresses and in all varieties, however at 200 mM NaCl the induction was not so strong. Dhn4 was strongly induced only in plants treated with 400 mM NaCl. Dhn5 had a basal level constitutive expression. Dhn6 was also found to be up-regulated at drought treatment and 400 mM NaCl and it was induced strongly in Himalaya Nakt, Himalaya India, Himalaya winter, Himalaya USA, Scarlett, and Candice in dehydrated plants. Dhn7 was expressed strongly in Himalaya India, Himalaya Nakt, Himalaya USA, and Scarlett dehydrated plants and only in Scarlett 400 mM NaCl treated plants, however it did not induce in case of 200 mM NaCl and in Candice and Himalaya Freak in case of drought treatment. Dhn9 was induced in all dehydrated plants but at 400 mM NaCl treatment it was induced on basal level in all except Reisgerste II and Candice, while at 200 mM NaCl a minute expression was found in Scarlett and Himalaya Nakt. Dhn8 and Dhn13 had a constitutive expression in all the studied varieties on all given stress conditions. The dehydrin genes expressed on the basis of variety and severity of the stress. The alignment of the coding sequence of Dhn1 and Dhn2 revealed that the coding sequence of both genes were 87% identical.

Our data on the expression of dehydrin genes showed that induction of Dhn1, Dhn6 and Dhn7 and can be used as markers for drought stress, while Dhn7 can also be used for salt stress in barley varieties. Contrary to our study, de Mezer et al. (2014) suggested that dehydrins like Dhn1, Dhn7 and Dhn9 had higher expression in the varieties with more water loss. Tommasini et al. (2008) reported that Dhn5, Dhn8 and Dhn13 which were constitutively expressed in our study were induced on drought and cold stresses. However, Rodriguez et al. (2005) reported about the constitutive expression of Dhn13 barley which are in agreement with our results. Dhn1 and Dhn9 were only expressed in drought treatment while Dhn3, Dhn4 Dhn6 and Dhn7 were also induced at salt treatment, Binott et al. (2017) while working on the Kenyan varieties also found the similar results. The results of Wang et al. (2014) showed subclasses of wheat dehydrins, KS,

SK_n, K_n and Y_nSK_m were induced on drought treatment however, KS and SK_n subclasses had basal level of expression in control conditions as well.

4.8.1 Sub cellular localization of Dehydrins in barley

Accumulation of dehydrins is specific for different growth parameters, tissues, cells and stresses. Dehydrins may be found in different cell organelles. Post-translational changes like phosphorylation can affect their localization. On the sub cellular level, they can be found in different compartments, such as cytoplasm, nucleus, mitochondria, vacuole and in the plasma membrane. The sub cellular localization of the selected barley dehydrin DHN3 protein from YnSKm group which contain nine members of barley dehydrin out of thirteen was performed. The onion cells were bombarded with GFP (green fluorescent protein) fused with DHN3 protein. The confocal analysis showed the presence of DHN3 protein in the nucleus and cytoplasm. In-silico analysis from two different sources as given in table 3.2 and 3.3 also predicted that the maximum chances of barley DHN3 are to be localized in nucleus. The different studies on the sub cellular localization of dehydrin proteins showed that most of the dehydrins are localized in the nucleus and cytoplasm. Houde et al. (1995) showed the localization of a wheat K_n dehydrin WCS 120 in nucleus and in cytoplasm. While Szabala et al. (2014) found that SK_n dehydrin DHN24 was also localized in the nucleus and cytoplasm. Many of the researchers also showed the presence of many Y_nSK_m type dehydrin to be found in nucleus and cytoplasm e.g. *Avicennia marina* dehydrin AmDHN1 (Mehta et al., 2009) TAS14 from tomato (Godoy et al., 1994), RAB17 from maize (Goday et al., 1994) and RAB21 in rice (Mundy and Chua, 1988). In-silico data in our studies correlate well with above mentioned researches with maximum probability of localization in the nucleus. Another study by Brini et al. (2007) showed that a dehydrin DHN5 in wheat, which is homolog of barley dehydrin DHN4, was found to be present in the nucleus and cytoplasm, which confirms in-silico analysis done in this study with maximum probability of DHN4 in the nucleus.

4.9 Conclusions

Physiological, biochemical and molecular responses of all the varieties in this study showed that drought and salinity caused oxidative stress to plants. All the studied parameters such as growth parameters, water loss rate, RWC, chlorophyll contents, proline contents, MDA levels, H_2O_2 levels, the activities of Antioxidative enzyme such as SOD, catalase, glutathione reductase and peroxidase and the expression levels of dehydrins as molecular markers indicated that different

varieties of barley had different levels of tolerance against drought and salinity. However, three cultivars, Scarlett, Himalaya Nakt and Himalaya India were found to have excellent antioxidative/ ROS scavenging mechanisms which protected the plants from oxidative stresses even at higher levels. Contrarily, Himalaya Freak, Candice and Reisgerste II did not perform well under drought and salinity stresses. So, considering all this physiological, biochemical and molecular data, it can be concluded that Scarlett, Himalaya Nakt and Himalaya India had the highest tolerance, while Candice and Reisgerste II are less tolerant while Himalaya Freak was with least tolerance against drought and salinity among the studied varieties. While other varieties like Heilis Frankin, Himalaya Nepal, Himalaya USA, Himalaya Winter had intermediary tolerance in comparison with other studied varieties. At 200 mM NaCl treatment, Himalaya Freak showed highest susceptibility.

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