Transcriptomic Responses of Barley (*Hordeum vulgare* L.) to Drought and Salinity

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Abstract Drought and salinity are the main factors limiting plant growth and productivity. With the effects of global warming, severe drought episodes are expected to be widespread, which will certainly lead to decrease in crop production. Therefore, understanding plants' response to drought and salinity stresses is more urgent than ever to reveal molecular mechanisms behind the natural tolerance which, then, can be used in the generation of stress-tolerant crop species. Barley stands out as the most salinity and drought-tolerant crop in *Poaceae* family with its wide range of wild genotypes. Due to its higher tolerance to abiotic and biotic stresses among other crops, it was studied to understand the mechanisms behind the natural tolerance via generation of various genetic resources and databases created by extensive sequence data, microarray studies, next-generation sequencing (NGS), and genetic maps. Large-scale transcriptomic analyses in barley showed that ROS-scavenging enzymes, transcription factors, LEA group proteins, and enzymes coding for osmoprotectants are the prominent groups of genes differentially expressed under salinity and drought stresses. Quantitative real-time PCR was efficiently used to measure transcript levels of stress-related genes under high salt or limited water conditions,

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allowing the prediction of functional characteristics of these genes according to their expression patterns. Small-scale expression studies also revealed the importance of cell and tissue type expression and mode of the stress treatment. However, although there are numerous candidate barley genes that can be used to develop transgenic crops with higher tolerance to salinity and drought, there are only limited isolation and cloning studies with these genes. We highly recommend more detailed studies on this naturally tolerant crop to be able to generate more drought or salt tolerance species via genetic transformation.

Keywords Barley • Transcriptomics • Drought • Salinity • Transgenics

1 Introduction

Environmental drought and soil salinity in arid and semi-arid regions are the major problems for agricultural productivity and threat for plant biodiversity (Godfree 2012; Trenberth et al. 2014). In addition to local drought, model-based analyses of soil moisture, drought indices, and precipitation-minus-evaporation studies are showing an increased risk of global drought in the twenty-first century (Dai 2013). Agricultural drought is a period with dry soils resulting from insufficient precipitation, intense but less frequent rainfalls, high evaporation or agricultural practices preventing water infiltration in the soil (Bot and Benites 2005; Dai 2011). Salinity also negatively affects the quality of soil and limits plant growth and production. Saline soils are formed by the basins with limited or no access to the rivers due to diverse soil types or unsuitable irrigation, poor drainage, and high evaporation. Soil salinity can be in different types as "irrigation-induced" and "transient" dry-land salinity (Läuchli and Grattan 2007). It is estimated that approximately 7 % of total land area and 20 % of the irrigated agriculture land is affected by salinity (Rozema and Flowers 2008; Agarwal et al. 2013).

Climatic changes all around the world with decreasing regional annual rainfall and increasing temperatures, in addition to obligation of agriculture in saline drylands, emphasize the importance of development of stress-tolerant cultivars, especially for important crop species (Cattivelli et al. 2008; Gosal et al. 2009; Mir et al. 2012). Tolerance to abiotic stresses such as drought and salinity is possessed by rare plant species including halophytes and xerophytes. Barley is known to have a significant potential tolerance to both drought and salinity stresses as described by physiological, morphological, and genomics tools; and, therefore, selected as an excellent model organism for stress response studies (Knüpffer et al. 2003; Nevo and Chen 2010; Mian et al. 2011; Roy et al. 2014). Other commonly used model organisms such as *Arabidopsis* and rice cannot complete their life cycle when exposed to 100 mM NaCl, while barley keeps its shoot growth at even higher concentrations of NaCl (200–300 mM) for at least 3 weeks (Munns and Tester

2008). Barley also has wild relatives such as *Hordeum marinum* which can grow in salty marshes and seasides, showing high salinity resistance of an unknown mechanism.

The transcriptome means the complete set of all transcripts in a cell and their quantity, for a physiological circumstance or specific developmental stage (Wang et al. 2009). Today it is known that understanding the transcriptome is important for interpreting the functional elements of the genome (Jain 2011). The key aims of the transcriptomics are: (1) to catalogue all transcripts, including mRNAs, non-coding RNAs, and small RNAs; (2) to determine the transcriptional structure of genes; and (3) to quantify the changing expression levels of each transcript during development and under different conditions (Wang et al. 2009).

In this chapter, we will provide the present knowledge on barley's response to salinity and drought stress as characterized by microarray, RNA sequencing (RNA-Seq) and more specifically quantitative real-time PCR. As many crop plants are subjected to combined stresses of drought and saline conditions in the field, pathways and gene networks overlapping at the molecular level in these two stress factors will also be discussed for a more realistic and useful approach.

2 Barley (*Hordeum vulgare* L.) as a Model

Among the cereal species, barley (*Hordeum vulgare* L.) is one of the oldest in the world and was first cultivated in Neolithic times in Fertile Crescent (Zohary and Hopf 1993; Badr et al. 2000; Morrell and Clegg 2007), from where it spread to the other parts of the world including South Africa, Europe, Near East, North Asia, China, and Japan. Barley is virtually found worldwide since it has a high adaptation to broad range of environments including steppes, savannas, mountains, as well as temperate zones in subtropics and subarctics (Nevo 1992; Hayes et al. 2003; Ullrich 2011). Based on the center of diversity concept of Vavilov, Anatolia (present-day Turkey) is one of the thirty-six agro-ecological groups where cultivated two- and six-rowed barley distributes along with the dry central and wet coastal regions (Knüpffer et al. 2003).

Three gene pools within the *H. vulgare* can be defined as "wild barley," adapted to diverse environments (mostly semi-arid); "landraces," adapted to marginal low-input agricultural regions; and "elite cultivars," grown in high-yielding agricultural regions. Wild progenitor of the barley, *Hordeum vulgare* spp. *spontaneum* C. Koch, which is also known as *Hordeum spontaneum*, has distribution in Fertile Crescent and Irano-Turanian as its primary and Mediterranean and Central Asia as secondary habitats (Zohary and Hopf 1993; Badr et al. 2000). As an example, distribution of wild barley covers from Çanakkale, North of Turkey alongside with the western coastal region to Southeastern Turkey. The comparison of the summer rainfall measurements in Çanakkale (13.4 kg/m^2) and Ceylanpınar (1.76 kg/m^2) shows the extreme climatic differences throughout the area. In addition, agricultural fields of

barley and wheat in Southeastern regions were frequently occupied by *H. spontaneum* during severe drought seasons (A. Karagöz, personal communication). It is well known that genetic diversity of cultivated barley was greatly reduced by domestication in elite cultivars (Morrell and Clegg 2007; Kilian et al. 2010) and different allele frequencies were occurred by the adaptation to different eco-geographical environments in three kinds of gene pools (Badr et al. 2000; Morrell et al. 2014). The observations summarized above imply the importance of wild barley populations for studying abiotic stress tolerance mechanisms.

Full genome sequence information is significant for understanding genome structure, evolutionary relatedness and variation, and thus development of modern strategies for crop breeding programs. Barley is diploid ($2n = 2x = 14$) and has seven chromosome pairs, designated as 1H to 7H with approximately 5.1 Gbp in size (von Bothmer 1992; Dolezel et al. 1998). An oligonucleotide array containing 350,000 high-quality ESTs and an SNP-genotyping platform were previously made available for barley (Close et al. 2009, 2004). High-throughput sequencing of barley genome was initiated on 2009 and resulted in the development of a 4.98 Gbp physical map with expressed barley genes in "Morex" cultivar (Wicker et al. 2009; Steuernagel et al. 2009; Mayer et al. 2011; The International Barley Genome Sequencing Consortium 2012). Results indicated that 84 % of barley genome comprises of mobile elements and repetitive DNA. Based on the homology comparisons with the genomes of close relatives (*Sorghum*, rice, *Brachypodium*) and *Arabidopsis*, barley has 26,159 genes of which 75 % have a multi-exon structure. These results proved the high abundance of alternative splicing in barley. Extensive SNP variation and transcriptionally active regions which are homolog to rice and *Brachypodium* have also been identified in the barley genome (The International Barley Genome Sequencing Consortium 2012).

In addition to a wide range of genomic studies proving large genomic diversity, there are other factors making barley a preferred model plant for abiotic stress research. For example, a high resistance to drought, salinity, and fungal diseases were proven in barley (Knüpffer et al. 2003; Bonman et al. 2005). Barley is also more alkaline tolerant than other cereal species (van Gool and Vernon 2006), and salt tolerance of barley cultivars is higher than bread wheat and other cultivated *Triticeae* (Garthwaite et al. 2005). However, as will be summarized in the following sections, barley was mostly studied on its tolerance to salt stress, and more studies are still needed to understand the mechanisms behind this natural abiotic and biotic tolerance.

3 Large-Scale Transcriptomics for Drought

Plants change their metabolism to reduce adverse effects of cellular dehydration caused by drought through well-conserved molecular and biochemical changes in cellular level. The complex network of metabolic changes can simply be divided into two: (1) changes in single function genes and enzymes; such as accumulation

of osmolytes, radical oxygen scavenging proteins and enzymes, ion transporters, channel proteins, and enzymes involved in lipid biosynthesis; and (2) regulatory proteins including transcription factors, protein kinases/phosphatases and proteinases responsible from the reprogramming of the metabolism in response to dehydration (Cominelli et al. 2013). A diverse family of transcriptional factors (DREB/CBF, ABF, AP2/ERF, bZIP, NAC, MYB, MYC, HD-ZIP, bHLH, NF-Y, EAR, and WRKY) is responsible for changes in gene expression under drought conditions and almost all of them belong to large family of proteins involved in regulation of several plant functions (Bhargava and Sawant 2013; Cominelli et al. 2013; Osakabe et al. 2014).

Transcriptional profiling through microarray technology made a breakthrough on understanding molecular responses of plants to abiotic stress conditions in gene expression level (Kilian et al. 2012). The very first microarray study on drought stress response of barley cv. Tokak plants on transcriptome level was performed by Ozturk et al. (2002) using an in-house printed cDNA array with 1,463 DNA elements from 6 to 10 h shock-drought-stressed leaf and root cDNA libraries constructed by the authors. The shock-drought treatment used in this study was simply done by removing 3-week-old barley plants from pots and leaving them on bench under growth conditions for 6 h (RWC 70 %) and 10 h (RWC 64 %). Hybridization was carried out with Cy3 and Cy5-dUTP labeled drought stressed leaf and root RNA with their appropriate controls. The majority of the drought stress cDNA libraries belonged to no hit (9.5 %) and unclassified protein (27.5 %) categories due to limited protein and gene information available on databanks at that time. Nevertheless, Ozturk et al. (2002) in their study was able to show the differential up-regulation of several stress-responsive genes in leaf and root tissues, including several jasmonate-induced proteins (jasmonate biosynthesis), metallothionein-like proteins, dehydrins (cellular protection), late embryogenesis abundant proteins (cellular protection), Δ 1-pyrroline-5-carboxylate synthetase (proline metabolism), wheat aluminum-induced proteins, several protein phosphatases (signal transduction), actin binding protein (cellular protection), auxin-induced protein, cytochrome P450 homologs, and cell death suppressor proteins. Down-regulated genes mostly belonged to photosynthesis metabolism such as ribulose-biphosphate carboxylase activase, rubisco small-chain precursor, and chlorophyll a/b-binding proteins. As conclusion, the authors cautioned the researchers that shock-stress treatment was effective to clone large number of drought stress-responsive genes but not comparable to field situation; therefore might not be effective to understand time-dependent changes in transcriptome in response to slow-developing drought. Indeed, Talame et al. (2007) hybridized a cDNA array containing 1,654 DNA elements from Ozturk et al. (2002) and RNA from control leaves and roots with 7 days (RWC 91 %) and 11 days (RWC 81 %) slow drought-treated barley Er/Apm variety from ICARDA (well adapted to dry environment) leaves and showed that a lower number of differentially regulated transcripts were obtained by gradual stress compared to shock-like stress treatment performed by Ozturk et al. (2002). Talame et al. (2007) also indicated that although only about 10 % of the transcripts shared similar changes in these two approaches, a considerable number of transcripts showed similar

regulation in both cases, and even concluded that a shock-like treatment can be an effective way of identifying and characterizing alleles involved in adaptive response to dehydration. Indeed, Diab et al. (2004) used cDNA libraries constructed by Ozturk et al. (2002) for QTL analysis in a population of 167 F_8 recombinant inbred lines and identified two candidate genes and ten differentially expressed sequences that were associated with QTLs in drought-tolerant traits.

Another study with cDNA microarray consisting more than 300 DNA elements derived from cold, dehydration, salinity, high light, and copper-treated barley cv. Nure leaves was published on 2004 (Atienza et al. 2004). The authors performed a shock-like dehydration treatment; however, they spotted only 20 genes from 5 h (RWC 89 %) to 10 h (RWC 80 %) drought-treated leaf samples and, therefore, revealed limited information on changes in barley transcriptome in response to dehydration.

On 2004, Affymetrix 22K Barley1 GeneChip Array made available to research (Close et al. 2004). The array contained 21,439 probes derived from 350,000 ESTs from 84 cDNA libraries and 1,145 barley gene sequences from National Center for Biotechnology Information database (NCBI, http://www.ncbi.nlm.nih.gov). Although barley Affymetrix array is available for a while, there is surprisingly low number of publications using this effective platform to identify transcriptomic changes in response to drought. Guo et al. (2009) used 22K Affymetrix Barley 1 microarray to compare transcriptomes of two drought-tolerant barley genotypes (cv. Martin and *Hordeum spontaneum* 41-1) and one drought-sensitive genotype (cv. Moroc9-75) in response to drought during reproductive stage (AWC 70 % in control and 10 % in drought; hybridizations with total RNA from leaf tissues collected at 0, 1, 3, and 5 days on 10 % AWC). The main aim of the study was to identify drought stress tolerance-related genes, and the authors stated identification of 17 genes specifically induced in drought-tolerant genotypes that can be related with tolerance through controlling stomatal closure via carbon metabolism (NADP malic enzyme and pyruvate dehydrogenase), glycine-betaine synthesis (C-4 sterol methyl oxidase), reactive oxygen scavenging (aldehyde dehydrogenase, ascorbate-dependent oxidoreductase), and membrane and protein stabilization (heat shock proteins and dehydrin). In addition, calcium-dependent protein kinase (signal transduction), membrane steroid binding protein (signal transduction), G2 pea dark accumulated protein (anti-senescence), and glutathione S-transferase (detoxification)-related genes were reported to be constitutively expressed in drought-tolerant genotypes, and the authors indicated the possibility of the direct role of these proteins in genotypic differences in drought tolerance. It is important to note that in their studies, Guo et al. (2009) observed differential regulation in a total of 263 genes (collective from all genotypes) which stands for just over 1 % of whole array; whereas Ozturk et al. (2002) in their study reported change in gene expression about 50 % of all cDNA array probes; which indicates that only a certain percentage of plant genome is transcriptionally regulated in response to drought and probes on the microarray are an important point to consider in a study aiming to detect complete transcriptomic changes in response to stress conditions.

The second comprehensive use of Affymetrix 22K Barley1 GeneChip Array was with barley cultivar Morex, where the authors investigated gene expression in the spike organs (lemma, palea, awn, and seed) exposed to drought treatment for 4 days during the grain-filling stage (RWC dropped from 85 to 60 % in lemma, palea, and awn; and from 89 to 81 % in seed) (Abebe et al. 2010). There was almost no change in transcript abundance in seed tissue; whereas the comparison suggested better drought tolerance in lemma and palea than awn. Among the stress defense-related genes, the expression of NADPH oxidase, ribosome inactivating proteins, chitinases, protease inhibitors, and amylases induced upon dehydration. The accumulation of transcripts belonging to late embryogenesis related proteins (LEA) in the lemma, palea, and awn was an indicative of protective roles of LEA proteins during dehydration via retention of water, sequestration of ions, and stabilization of proteins and chaperons of protein folding.

As indicated before, although effective, the information that can be gathered from microarray studies is limited to the extent of DNA elements on the array. Use of new technologies, mainly next-generation sequencing (NGS) via RNA-Seq, on the other hand, makes studies on transcriptome level more feasible and creates larger datasets compared to microarray approaches. There is only one publication using this new approach for the identification of drought stress-responsive transcripts in barley (Bedada et al. 2014). In this study, Bedada et al. (2014) used two wild barley ecotypes (B1K2, desert and B1K30, Mediterranean) and compared transcriptomes of 5 days of drought-stressed leaf tissues (SWC 80 % in control and 30 % in drought stress). Normalized cDNA libraries were sequenced by 454 platform and the authors identified over 800 unique transcripts from each ecotype. The majority of these unique transcripts were homologs of transcription factors (bZIP, bHLH, MYB), heat shock proteins, aquaporins and ERD, LEA, and ABC transporter proteins. As conclusion, Bedada et al. (2014) pointed out the genomic differentiation between the desert and Mediterranean wild barley ecotypes.

4 Large-Scale Transcriptomics for Salinity

Analyzing the functions of stress-inducible genes is important to understand the molecular mechanism of stress tolerance and to improve stress tolerance of crops by genetic manipulation (Seki et al. 2009). During the last decade, DNA microarrays have been the technology of choice for large-scale studies of salt regulated gene expression levels in model plants and crops, such as *Arabidopsis* (Richards et al. 2012), rice (Walia et al. 2005), barley (Walia et al. 2006; Guo et al. 2009), wheat (Kumar et al. 2014), maize (Allardyce et al. 2013), and tobacco (Edwards et al. 2010). Many genes that respond to salinity stress at the transcription level in barley were identified using microarray technology (Ozturk et al. 2002; Ueda et al. 2004; Walia et al. 2006; Gao et al. 2013) (Table 1).

First microarray analysis of barley transcripts under salinity stress was reported by Ozturk et al. (2002). Responses to salinity were investigated by microarray

Table 1 Summary of large-scale transcriptomic analyses for salt stress response in barley

Transcriptome analysis method	Cultivar and stress treatment	No. of up-regulated/ down-regulated genes in shoot tissue	No. of up-regulated/ down-regulated genes in root tissue	Reference
cDNA microarray (1463 DNA elements)	cv. Tokak; 150 mM NaCl; 24 h	29/27	10/9	Ozturk et al. (2002)
cDNA microarray (460 salt-responsive genes)	cv. Haruna-nijyo; 200 mM NaCl; 24 h	62/30	49/14	Ueda et al. (2004)
cDNA micro array (22K barley chip 1)	cv. Morex; 100 mM NaCl and 10 mM $CaCl_2$; 3, 8, and 24 h	339/311	N/A	Walia et al. (2006)
cDNA micro array (22K barley chip 1)	cv. Hua 30 (salt sensitive) cv. Hua 11 (salt tolerant); 300 mM NaCl, 30 mM $CaCl_2$; 6 h	1090/763	864/609	Gao et al. (2013)
mRNA-Seq (Illumina)	cv. Hindmarsh;150 mM NaCl; 12 h	48/62	N/A	Ziemann et al. (2013)

N/A not available

hybridization of 1463 DNA elements derived from cDNA libraries of 6 and 10 h drought-stressed plants. Salt stress was carried out on barley cv. Tokak plants in hydroponic tanks containing one-third strength Hoagland's solution (Hoagland and Arnon 1950) supplied with 150 mM NaCl for 24 h. Equal or greater than 2.5-fold changes in expression of genes were considered significant. There were 37 and 36 transcripts that showed increased or decreased expression level in salt-stressed leaf and root tissues, respectively. A limited number of the overlapping genes were found among up- or down-regulated transcripts in root and leaf tissues such as 60S acidic ribosomal protein P0 and ubiquitins (10 and 11). Metallothionein-like protein type 2 (*MT2*) was the most significantly up-regulated transcript (36-fold) in leaf tissues; however, its expression was not changed in root tissues. While the genes encode allene oxide synthase, basic proline-rich protein (PRB1L) precursor, lipid transfer protein cw 18, glutathione S-transferase (auxin induced), early responsive to dehydration 1 (ERD1), and Δ 1-pyrroline-5-carboxylate synthetase (P5CS) were induced in leaf tissues of barley, fructose-bisphosphate aldolase, elongation factor 1-alpha, ubiquitin 4 and 10, and germin-like protein were the highly induced transcripts in root tissues. The number of the down-regulated transcripts in salt-stressed leaf was threefold higher than the transcripts of root. Auxin-induced protein, late embryogenesis abundant protein LEA14-A, aminopeptidase N were the significantly down-regulated transcripts in leaf tissues.

Ueda et al. (2004) also performed a customized cDNA microarray study using 460 salt-responsive genes obtained by a previous differential display analysis (Ueda et al. 2002). The 15-day-old seedlings of barley cv. Haruna-nijyo were treated with 200 mM NaCl in half-strength Hoagland's solution. Salt-stressed plants were harvested after 1 and 24 h of salt stress. In barley roots, a total of 49 genes were found to be induced during the initial phase under salt stress. After 1 h salt stress, 13 genes showed up-regulation. These genes encode signaling elements such as receptor-like protein and serine/threonine protein kinase as well as stress tolerance genes like plasma membrane protein 3 (PMP3). After 24 h, the genes involved in the biosynthesis and transport of amino acids and genes of the cytochrome P450 family were up-regulated (16-fold) in barley roots. A total of 14 genes were found to be repressed under salt stress in barley roots. The transcript levels of water channel protein 2, phospholipase C, and salt-responsive protein (SalT) were down-regulated within 1 h, while the mRNA abundance of water channel protein 1 and DNA-binding protein CCA1 showed significant down-regulation only by 24 h under stress. The number of up-regulated genes was less in leaves than in roots. Twenty-six of these 460 genes showed increased level of expression. The calcium-dependent protein kinase, phosphatidylinositol-4-phosphate-5-kinase, pleiotropic drug resistance 5-like ATP binding cassette transporter, and *SET1* involved in signal transduction were up-regulated in barley leaves within 1 h under salt stress. After 24 h, stress tolerance genes encoding P5CS, PMP3, aldehyde dehydrogenase, betaine aldehyde dehydrogenase were highly induced in barley leaves. Besides, expression level of the genes encoding enzymes involved in the biosynthesis of amino acids such as methionine synthase and asparagine synthetase, and lipoxygenase related to jasmonic acid biosynthesis were increased significantly. Out of 460 genes, 21 genes showed down-regulation in barley leaves. The genes encoding phosphoenolpyruvate carboxylase and omega-3 fatty acid desaturase were repressed significantly at 24 h time point during salt stress. The authors indicated that the barley root cells responded to salt stress signals earlier than leaf cells based on the observation that larger numbers of the differentially expressed genes existed in roots than in the leaf cells after 1-h salt stress. While expression of PMP3 gene was induced in root cells at 1 h, expression of it was not changed in leaf cells. Although the expression of cytochrome P450 family was highly up-regulated (16-fold) in barley roots after 24 h, they were suppressed in leaf cells. There were also common genes differentially expressed in root and leaf cells after 24 h salt stress including up-regulation of P5CS and aldehyde dehydrogenase, and down-regulation of water channel 2.

Walia et al. (2006) reported the first use of the Affymetrix 22K Barley1 GeneChip Array in identification of transcripts under salt stress. A gradual salt stress was applied to 14-day-old barley cv. Morex plants. The NaCl concentrations were elevated to 100 mM by increments of 25 mM NaCl per day. The plants were harvested at 3, 8, and 27 h after reaching a final concentration of 100 mM NaCl. In the study, three time points based on the physiological status of plants in response to addition of salt were chosen to investigate the reducing growth rate stage (3 h), growth rate recovery stage (8 h), and ion-specific responses (27 h). A significant difference in Na^+ level was found between root (639 ± 124 mmol/kg) and shoot samples

(897 ± 41 mmol/kg). There were 261 probe sets corresponding to 339 unigenes and 234 probe sets (311 unigene) that showed up-regulation and down-regulation at three different time points (3, 8 and 27 h), respectively. A fold change of 1.5 was considered as an indication of significant modulation in gene expression. While less than 10 % of the up-regulated probe sets (25) were overlapped between three distinct time points (3, 8, 27 h), this ratio was less than 6 % among down-regulated probe sets (total number of down-regulated probes were 14). The relatively higher number of induced and repressed genes was observed at time points of 27 and 8 h, respectively. The nodulin MtN3 family protein, P5CS, C-4 sterol methyl oxidase, arginine/serine-rich protein were the common up-regulated probe sets at all three time points. Expression level of the photosystem II 10 kDa polypeptide was increased at all time points under salt stress and reached maximum at 27 h. Peroxidase was the highest down-regulated transcript among the repressed genes. Some of the genes involved in jasmonic acid biosynthesis and jasmonic acid-responsive genes including phospholipase, lipoxygenase, and allene oxide synthase were up-regulated especially at the 3 h time point under salt stress. However, 12-oxophytodienoate reductase, involved in the biosynthesis or metabolism of oxylipin signaling molecules, was significantly down-regulated at the 8 h time point. Walia et al. (2006) also found a number of known jasmonic acid-responsive genes with altered expression under salt stress. These were O-methyltransferase, jasmonic acid-induced proteins, glutathione S-transferase, selenium binding protein, and hordothionins. The expression level of some genes known to respond to other abiotic stress conditions such as osmotic stress and cold were found to be increased under salt stress. Well-known dehydration-responsive genes, *Dhn5* and *RD22* were induced at 3 and 27 h time points. Walia et al. (2006) showed that several well-known sodium transporters and anti-porters such as *HvNHX1* were differentially expressed (at least 1.5-fold) under salt stress at any time point. The osmoprotectants such as proline and glycine betaine are known to be accumulated in plant cells in response to the salt and drought stress in many plants (Kavi Kishor et al. 1995). The gene encoding P5CS, the key enzyme for proline biosynthesis, was the only gene found to be commonly induced in all three studies (Ozturk et al. 2002; Ueda et al. 2004; Walia et al. 2006). Gao et al. (2013) was the first comparing salt-tolerant barley (cv. Hua11) with a salt-sensitive cultivar (cv. Hua30) to identify salt stress-regulated genes on a large scale using Affymetrix 22K Barley1 GeneChip Array. At 7th day of germination, seedlings were suspended in a half-strength Hoagland solution. Salt stress (300 mM NaCl) was applied to 10-day-old seedlings grown in Hoagland's solution supplemented with 30 mM $CaCl_2$ for 6 h. Salt stress was shown to increase the production of reactive oxygen species (ROS) and cause ROS-associated injury (Miller et al. 2010). Therefore, it was concluded that the activities of ROS-scavenging enzymes like superoxide dismutase (SOD) and peroxidase (POD) play important roles in the protection of barley plants under salt stress. Gao et al. (2013) reported that the SOD and POD activities in cv. Hua11 were higher than cv. Hua30 in root and shoot tissues. A total of 1853 (1090 up-regulated and 793 down-regulated) and 1473 (864 up-regulated and 609 down-regulated) differentially expressed probe sets were found in the shoot and root tissues, respectively,

after 6 h salt treatment. When differently expressed genes were compared between Hua11 and Hua30, it was revealed that 916 (62 %) and 842 (45 %) genes were co-regulated in root and shoot tissues, respectively. Gao et al. (2013) concluded that these genes might be responsible for barley intrinsic tolerance to salt stress. Furthermore, the number of salt-responsive genes identified from shoot tissues (1853) was more than that of root tissues (1473). Number of the up-regulated probe sets in shoot tissues of tolerant cultivar (cv. Hua11) was higher than sensitive cultivar (cv. Hua30) with 906 and 685 probe sets, respectively. On the contrary, there were higher number of down-regulated probe sets in root and shoot tissues of cv. Hua30 than cv. Hua11. The number of the significantly changed probe sets related to signal transduction in salt-tolerant genotype was higher than the sensitive genotype in root and shoot. Although 14 receptor-like kinases and 3 mitogen-activated protein kinases were induced in the shoot tissue, expression level of these was not changed in root tissues. In the study, a set of hormone-related genes including jasmonic acid and gibberellins, ethylene, and cytokinin were identified in root and shoot tissues under salt stress. Among them, three lipoxygenases were up-regulated in shoot of cv. Hua11 after salt treatment. In addition, one cytokinin dehydrogenase transcript was significantly up-regulated in root tissues of salt-tolerant genotype. In the salt-tolerant genotype, 30 genes encoding transcription factors (ZIM, WRKY, MYB, CBF, NAC, bHLH) were characterized in response to salt stress in shoot, in addition to 6 genes in root tissue. Most of these genes were up-regulated except 4 from bZIP and AP2 families. Among these genes, especially one C-repeat binding factor and one bHLH transcription factor showed a special expression pattern with respect to the other transcription factors. They were both induced in the shoot tissues of salt-tolerant genotype and down-regulated in the shoot tissues of salt-sensitive genotype. Organic solutes, known as compatible solutes, including amino acids, sugars, and other low molecular weight metabolites, protect the plants from biotic and abiotic stress with osmotic adjustment, protection of membrane integrity, and stabilization of enzymes or proteins (Ashraf and Foolad 2007). After 6 h salt treatment, 15 genes in shoot, and 11 genes in root associated with compatible solutes and secondary metabolites were found to be differentially regulated. All of the genes encoding the compatible solutes such as one proline-degraded enzyme and four sugar-related genes were induced in shoot and root tissues. One of the terpenoid-related genes was down-regulated in roots.

Next-generation sequencing (NGS) technologies have become faster, more accurate, and less expensive in recent years, leading to their widespread application in diverse fields (Hawkins et al. 2010). This technology has been applied mainly in human genetics and medicine, and is now emerging as the most popular high-throughput sequencing technique in plants (Ziemann et al. 2013; Xu et al. 2012; Camilios-Neto et al. 2014). Ziemann et al. (2013) used RNA-seq technology to analyze barley genes involved in salt stress. Two-week-old barley (cv. Hindmarsh) seedlings were treated with 150 mM NaCl in Hoagland's solution (Hoagland and Arnon 1950) for 12 h. Plant leaves were harvested and analyzed by RNA-Seq. The total number of RNA sequence reads, generated in the RNA-Seq experiment from sequencing of salt-stressed and control plants were 26.7 million and 23.7 million,

respectively. The 16.4 million (63 %) reads of salt-stressed cDNAs were uniquely aligned using burrows-wheeler aligner (BWA) (Li and Durbin 2009) according to barley Unigene database. Differential gene expression between the control and salt-stressed samples was analyzed using DESeq software (Anders and Huber 2010). When controlling the false discovery rate (FDR) at 5 %, 110 genes were found differentially expressed under acute salt stress. While 48 of them were significantly up-regulated, 62 of them were significantly down-regulated. Late embryogenesis abundant protein was strongly up-regulated (over 32-fold) under salinity stress. Similarly Ueda et al. (2004) and Walia et al. (2006) were reported that this gene confers salinity tolerance in barley. Other significantly induced genes were cellulose synthase-like protein, lipoxygenase 2.1, protein phosphatase 2C, calcium/calmodulin-dependent protein kinase, as well as those encoding membrane bound proteins such as a peptide transporter, two plasma membrane ATPases and a novel wall-associated receptor kinase. Down-regulated transcripts include those in the jumonji, pumilio RNA binding, and MYB transcription factor classes, and also several transcripts of unknown function.

5 Small-Scale Expression Analyses of Stress-Responsive Genes

Tolerance of barley to drought stress was found to be related with several major classes of genes involved in signaling and protection in the plant cells. In addition to large-scale transcriptome analyses, there are also reports revealing the expressional change in this group of genes in response to abiotic stress conditions. Barley cultivars with contrasting phenotypes for their tolerance to salinity and drought were occasionally used to investigate transcriptional profiles of specific genes involving in stress tolerance. In this part of the chapter, we will try to emphasize the ones that are mostly related to drought and salt stress response and/or tolerance.

The most important group of genes activating by abiotic stresses are transcription factors (TFs) that modulate the expression of stress-related genes. The APETALA 2/ethylene-responsive element binding factors (AP2/ERF) is a large family of TFs in plants and regulates gene expression during abiotic stress responses (Mizoi et al. 2012). In barley, an AP2/ERF gene, dehydration-responsive factor 1 (*HvDRF1*), was shown to be involved in the regulation of stress-responsive genes by an abscisic acid (ABA)-mediated pathway (Xue and Loveridge 2004). Binding motif of *HvDRF1* was identified as T(T/A)ACCGCCTT. Increase in *HvDRF1* by drought, salinity, and ABA treatment was shown to activate *HVA1* gene in barley (Xue and Loveridge 2004). *HVA1* (*Hordeum vulgare* aleurone 1) is a well-known group 3 LEA protein which was previously characterized in aleurone layers, and identified as a responsive gene to drought, salinity, heat, and cold stresses (Hong et al. 1992). Another TF, namely *Hordeum vulgare* dehydration-responsive element binding protein 1 (*HvDREB1*) was characterized as an A-2 group member of the DREB family (Xu et al. 2009). Transient strong induction of this gene was observed

upon high saline concentrations (2 % salt as 60 % NaCl and 40 % Na_2SO_4) and 200 μM ABA treatment suggested a significant role of *HvDREB1* on plant stress response; however, genes associated with *HvDREB1* are still remain to be explored (Xu et al. 2009).

WRKY transcription factors in barley were identified and classified as subgroups 1–3, having the conserved promoter motif of TTGACCT, so-called W-box (Mangelsen et al. 2008). Among this family, *HvWRKY38* was reported to be expressed continuously by freezing and drought stress. *HvWRKY38* was shown to carry W-box motif and induced by dehydration after 30 min of treatment, but slightly reduced in prolonged durations of drought. Drought-response induction of this gene was shown to be ABA-independent (Rushton et al. 2010; Mare et al. 2004). In a comparative study of Tibetan hulless barley, *HvvWRKY2, HvvWRKY5, HvvWRKY19,* and *HvvWRKY46* genes were cloned from a tolerant genotype and their enhanced expression relative to a sensitive wild genotype were demonstrated under artificial drought stress (PEG-6000) (Li et al. 2014). *HvvWRKY2* was also induced by salt stress (250 mM NaCl) proving that this gene is involved in salinity tolerance in barley (Li et al. 2014).

A prominent group of genes that induced by drought stress are dehydrins (*Dhns*) in plants. *Dhn* gene family is well-characterized in barley with 13 members (Choi et al. 1999; Choi and Close 2000; Rodriguez et al. 2005). They are hydrophilic and intrinsically disordered proteins, serve as cryoprotectants in the cell during stressful conditions by yet unknown mechanisms. The interactions of dehydrins with membrane phospholipids, metal ions, water, and other unknown ligands were found to be associated with their cryoprotective functions in the plant cell (Hincha and Thalhammer 2012; Graether and Boddington 2014). Changes in dehydrin gene expression in response to drought were extensively studied in barley. Ten of the barley *Dhn* gene family (*Dhn1-11*) was shown to be up-regulated by dehydration and ABA treatment while *Dhn5* and *Dhn8* were also shown to be induced by cold stress (5 °C) (Choi et al. 1999). The expression of *Dhn13* was significantly increased upon drought, freezing, and chilling stress treatments in cv. Morex (Rodriguez et al. 2005). Similarly, after 8 h of drought stress in Tibetan hull-less barley, *Dhn13* transcript accumulations were observed, but significantly higher in drought-tolerant genotypes (TR1 and TR2) than in sensitive genotypes (TS1 and TS2) (Qian et al. 2008). Tommasini et al. (2008) proved up-regulation of the group of dehydrins including *Dhn1, Dhn2, Dhn3, Dhn4, Dhn7, Dhn9,* and *Dhn10* by drought stress and depending on the developmental stages of coleoptile, embryo, mesocotyl, and seminal roots of barley. In the same study, *Dhn6, Dhn11,* and *Dhn12* were found to be the genes that were not up-regulated upon drought or low temperature, but only expressed in barley embryo. Surprisingly, in wild barley (*H. spontaneum*), *Dhn6* showed differential expression between tolerant and sensitive genotypes and increased transcript accumulation after 12 and 24 h of dehydration (Suprunova et al. 2004). Recently, expression of *Dhn3* and *Dhn9* in flag leaves of a drought-tolerant barley (cv. Yousef) has found to be correlated with physiological traits such as chlorophyll content, osmotic adjustment, stomatal conductance, biomass, and grain yield (Karami et al. 2013). Presence or absence of *cis*-acting regulatory elements in

5′ flanking regions of dehydrins were found to be correlated with the expression patterns and most of the members of dehydrin family were shown to contain abscisic acid-responsive elements (ABRE) and dehydration-responsive elements (DRE) in their promoter regions (Choi et al. 1999; Maruyama et al. 2012). Among the *Dhn* genes, only *Dhn4, Dhn5, Dhn8,* and *Dhn10* are known to be induced by salinity stress in barley (Walia et al. 2005; Du et al. 2011).

Maintenance of ionic and oxidative homeostasis under high saline conditions is an important indication of salinity tolerance in plants (Munns and Tester 2008; Bose et al. 2014; Adem et al. 2014). There is a consensus on the idea that cellular K^+ to Na^+ ratio is a key determinant of salinity tolerance in *Arabidopsis* and monocots (Chen et al. 2007; Hauser and Horie 2010; Shabala et al. 2010; Qiu et al. 2011; Wu et al. 2013). In this context, different genotypes of barley having varying tolerance levels to salinity were studied to characterize the role of ion homeostasis components of tolerance (Chen et al. 2005; Boscari et al. 2009; Qiu et al. 2011; Roslyakova et al. 2011; Adem et al. 2014). Tolerant genotypes of barley were shown to have better control of membrane voltage (a more negative membrane), better ability to pump Na^+ from cytosol to outside, and high antioxidant capacity (Chen et al. 2007; Witzel et al. 2009). Comparative studies with three different barley cultivars showed that the most salt-tolerant genotype accumulated less Na^+ and maintained a stable K^+ concentration in the root during the salinity treatment (Adem et al. 2014). Similarly, wild *Hordeum* species were proven to have better Na^+ and Cl^- exclusion and maintained higher K^+ in the leaf compared to *H. vulgare* L. (Garthwaite et al. 2005). For example, in wild barley, K^+/Na^+ ratio were 5.2 in the leaf in response to 150 mol/m^3 salts, while it was 0.8 in *H. vulgare*. Maintenance of low Na^+ or limiting the Na^+ uptake from roots can be provided by non-selective cation channels (NSCC), Na^+/H^+ antiporters, and tonoplast-located Na^+/H^+ antiporters (Chen et al. 2007). There are several studies conducted for genetic characterization of antiporters and channels in barley. Genes coding K^+ channels, namely *HvAKT1*, *HvAKT2,* and *HvKCO1* were cloned and showed to be differentially expressed in the leaves and roots (Boscari et al. 2009). *HvAKT1* was dominantly expressed in roots whereas *HvAKT2* was expressed in leaves about 20 times higher than in roots. A high affinity *HvHAK4* K^+ transporter is expressed only in the growing leaf tissue. Salinity and K^+ treatments did not affect the expressional profile of the tested genes in a significant manner (Boscari et al. 2009). The "High-Affinity K^+ Transporter" (HKT) gene family was found to be related to salinity tolerance by regulating Na^+ transport within the plant (Huang et al. 2008; Hauser and Horie 2010; Mian et al. 2011; Benito et al. 2014). Studies with HKT family genes particularly showed their involvement in Na^+ transport by mediating Na^+ exclusion from xylem vessels in order to protect shoots from high amounts of Na^+ (Hauser and Horie 2010; Roy et al. 2014). A gene from subfamily 2, *HvHKT2;1* (also known as *HvHKT1*) showed high homology to wheat *TaHKT2;1* and its expression was dominant in roots and then in leaf blades and sheaths under normal conditions (Haro et al. 2005; Huang et al. 2008; Mian et al. 2011). Transcript levels of *HvHKT2;1* increased by 50 mM NaCl and in the absence of K^+ ions, suggest that its expression is regulated by K+ in the growth environment (Haro et al. 2005; Mian et al. 2011). Differential expressions of *HvHKT1* and

HvHKT2 genes were also demonstrated in the roots of Tibetan wild barley genotypes. *HvHKT1* was induced by salinity (150 mM NaCl) in the roots of plants nearly 50 times higher than controls, while the expression of *HvHKT2* was reduced by exposure to salinity as fivefold after 6 h (Qiu et al. 2011). Polymorphisms within the genes also explained the higher tolerance of wild accessions than cultivated barley as proven by SNP-associated studies (Qiu et al. 2011). More data is still required on expression profiles of *HKT* genes in barley in terms of cell types, growth stages, and stress conditions.

NHXs are Na^+/H^+ exchangers located on tonoplast and endosomes in addition to plasma membrane, and involved in ion and pH regulation in plant cells (Rodríguez-Rosales et al. 2009; Bassil et al. 2012). In barley, four *NHX* genes were identified for coding NHX isoforms, namely *NHX1-4*, while there are six homologous genes (*AtNHX1-6*) in *Arabidopsis* (Eckardt and Berkowitz 2011; Roslyakova et al. 2011). Overexpression of *AtNHX1* in *Arabidopsis* plants was resulted in enhanced tolerance to salinity and sustained growth under saline conditions (Apse et al. 2003). Besides ionic homeostasis, intracellular NHX antiporters are associated with significant indirect cellular functions including osmotic adjustment, vesicular traffic, cell volume regulation, and stress response. Roslyakova et al. (2011) reported that content of NHX1-3 isoforms on tonoplast increased by salinity stress (150 mM NaCl) in both leaf and root tissues. Expressions of *NHX1* and *NHX3* increased in early hours of stress while the expression of *HvNHX2* was not changed. *NHX1* expression was shown to be the same in the roots of both tolerant (cv. Elo) and sensitive cultivar (cv. Belogorskii). The comparison indicated early induction of *NHX3* expression in tolerant cultivar. As a result, authors concluded that there is a direct correlation between enhanced *NHX1* and *NHX3* expression and their protein content. In contrast, Adem et al. (2014) in their study showed that *NHX1* expression was increased twofold in a sensitive barley cultivar (cv. Naso Nijo) under salinity compared to control while there was no significant difference in other two tolerant cultivars (cvs. Numar and Golden Promise).

There are also studies reporting the expressional change of one gene in response to salinity. For example, *HvSRG6* (Stress-responsive gene 6) is characterized by differential display (DDRT-PCR) studies, which was mapped in chromosome 7H, within a region that previously was linked to osmotic adaptation in barley (Malatrasi et al. 2002). Accumulation of *HvSGR6* mRNA was shown to be relatively higher in drought-tolerant barley (*H. distichon*) cultivars upon dehydration conditions (Rapacz et al. 2010; Wojcik-Jagla et al. 2012). Upon drought, expression of *HvSRG6* was highly influenced by ABA accumulation, negatively correlated with PSII photochemical activity, and reduced by light, emphasizing the interactions between these factors and water deficit (Malatrasi et al. 2002; Rapacz et al. 2010; Wojcik-Jagla et al. 2012). In contrast, *HvSGR6* expression was not found significantly related to ABA treatment in some barley genotypes (No.62-Unitan, No.3-Keystone, No.53 GK Rezi, No. 73a Compana, No.8 Hazen) (Cseri et al. 2011).

Regulations against oxidative stress and osmotic capacity were reported as important factors for higher salinity tolerance of barley compared to other cereals (Witzel et al. 2009; Roy et al. 2014; Adem et al. 2014). However, information on

transcript levels of genes associated with oxidative and osmotic pathways is still limited for different experimental models. Adem et al. (2014) recently reported that transcriptional changes of antioxidant genes in one specific time point were not informative for evaluating salinity tolerance in contrasting genotypes. Comparison of dehydration treatments also indicated that barley responds differently upon rapid- or slow-developing water stress with respect to antioxidant mechanisms (Talame et al. 2007). Similarly, a study comparing the changes in antioxidant mechanism genes between contrasting barley cultivars (tolerant cv. Martı and sensitive cv. Erginel90) subjected to shock-like and slow-developing dehydration demonstrated a rapid induction of a 2-Cys peroxiredoxin gene, *HvBAS1,* and metallothionein-like protein type 2 (*HvMT2)* in both cultivars upon shock-like dehydration while their expression was relatively low in slow-developing water stress (Fig. 1) (Gürel et al. 2016).

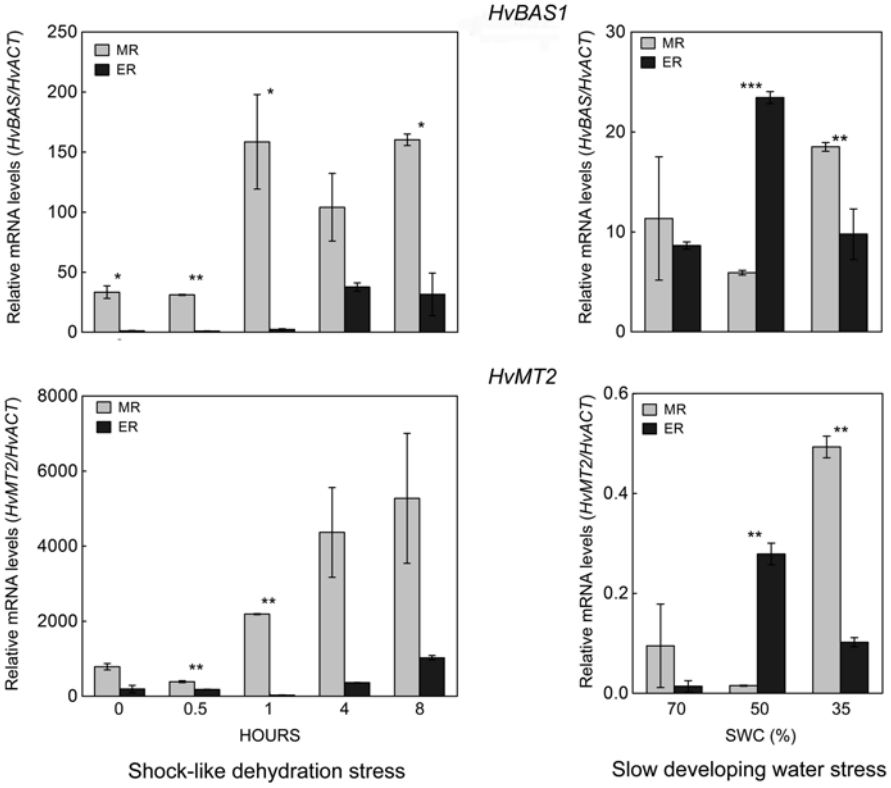

Fig. 1 Relative transcription levels of water stress-responsive genes, *HvBAS1* and *HvMT2* in barley cultivars cv. Martı (MR) and cv. Erginel90 (ER) under shock-like dehydration and slow-developing water stress. Transcript levels were measured by qRT-PCR and normalized to *HvACT*. Asterisks indicate statistical significance at * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$ of the differences between cultivars according to *t*-test

Although there are a number of studies on expressional changes of stress-responsive or tolerance-related genes, the functions of the majority of the genes proven to have altered expression upon stress treatments still remain unknown and there are probably more genes to be discovered.

6 Combined Effects of Drought and Salinity on Transcriptome

Drought and salinity stress combination in nature is the main factor for yield loss in agriculture (Ahmed et al. 2013a). Cellular osmotic stress causing abiotic stress (drought, salinity, heat, cold) response pathways have common components (Hu et al. 2010; Dolferus 2014). The effect of low water and high salt in cellular level especially is quite similar, since both cause reduction in water potential and reprogramming of the metabolism to reduce the adverse effects of dehydration due to low water potential (Ahmed et al. 2013a). Most of the studies on transcriptomal changes, on the other hand, were mainly on the effect of drought or salt stress alone. The combination of stresses is obviously more detrimental to plant and, therefore, the understanding of abiotic stress on plant metabolism should be more focused on the different combinations of these stresses.

The literature on combined effects of abiotic stresses on barley, especially drought and salinity, is quite limited and mainly based on the physiological comparison of dehydration on barley genotypes rather than the changes in gene expression profile (Ahmed et al. 2013b). One outstanding study was published by Ozturk et al. (2002). They used the in-house printed cDNA array consisting 6 and 10 h shock-drought stressed leaf and root DNA elements for hybridization with 24 h 150 mM NaCl stressed 3-week-old barley plants (cv. Tokak). Only 5 % of the 1463 unique transcripts were responsive to salt stress; induction in, for example, metallothionein-like protein type 2, heat shock protein DnaJ homolog Pfj4, allene oxide synthase, glutathione S-transferase, ubiquitin; and reduction in auxin-induced protein, wheat aluminum-induced protein wali5, late embryogenesis abundant protein LEA14-A, and sucrose synthase. There were only a number of transcripts showing co-regulation in response to both drought and salt stresses; allene oxide synthase 1, metallothionein-like protein, ERD1 protein precursor, Δ 1-pyrroline-5-carboxylate synthetase and germin-like protein up-regulated in response to both stresses, and α-amylase, lipid transfer protein cw18, ABC transporter, vacuolar processing enzyme precursor, and an unknown protein from rice were down-regulated.

It is clear that there should be more studies on dose- and time-dependent responses of barley genotypes to drought, salt, and drought and salt combined stress treatments to understand the differential changes on metabolism depending on the these factors (Dolferus et al. 2011; De Mezer et al. 2014). Such studies are also required to create crop interactome maps which will enable researches to analyze

association between phenotypic variation and environmental stress tolerance (Skirycz and Inze 2010; Mochida et al. 2011; Shelden and Roessner 2013; Shanker et al. 2014).

7 Transgenic Approaches for Drought and Salinity Tolerance

It is expected that the human population will reach nine billion by 2050 and the increase in food production should be at least 70 % to provide the demand (Godfray et al. 2010). The crops comprise about 80 % of human food, while the cereals supply the half of the global food production (Langridge and Fleury 2011). In order to meet the growing demand, crop productivity should be increased to 44 million tones (Mt) per year; however, annual increase in global food production was calculated to be around 32 Mt on 2010 and, with changing global climate, there will be an increasing gap between food production and consumption (Tester and Langridge 2010). The main yield loss in agricultural production occurs due to abiotic stresses and in order to meet the growing demand for food, the harmful effects of stress factors on crop yield should be minimized. The best approach is the development of crop varieties with enhanced tolerance to environmental stress conditions either by conventional breeding, or molecular breeding and genetic engineering, or combination of both. There are a number of studies using the identified stress-responsive genes in improvement of barley genotypes to drought and salt stresses using genetic engineering (Table 2).

Transcription factors (TFs) control the expression of many genes, and overexpression of TF genes can help plants to better tolerate abiotic stress (Kasuga et al. 1999; Cominelli et al. 2013). Therefore, transcription factors have always been attractive targets for conferring abiotic stress tolerance to plants. A promising example is the family of drought-responsive element binding proteins (DREBs)/C-repeat binding factors (CBFs). The transgenic overexpression of *HvCBF4* in rice resulted in an increase in tolerance to drought, high salinity and low temperature stresses without growth retardation (Oh et al. 2007). *HsDREB1A* transcription factor isolated from wild barley was cloned to downstream of stress-inducible *HVA1s* promoter and introduced into the apomictic bahiagrass (*Paspalum notatum* Flugge) cultivar cv. Argentine by particle bombardment (James et al. 2008). Overexpression of this gene in bahiagrass plants improved survival and biomass under severe dehydration and salt stress in contrast to control plants. In another study, transformation of *Arabidopsis* with barley *HvDREB1* gene enhanced salinity and drought tolerance (Xu et al. 2009). Overexpression of *HvDREB1* driven by 35S promoter of cauliflower mosaic virus (CaMV) led to the accumulation of transcripts corresponding to *RD29A* in the transgenic *Arabidopsis* plants in addition to its own mRNA. The transgenic plants displayed less severe inhibition of root growth than non-transgenic plants during 100 mM NaCl treatment. Two DREB/CBFs (*TaDREB2* and *TaDREB3*)

Table 2 Summary of transformation studies to improve salinity and drought tolerance using genes encoded by barley and other plant species

Transgene	Origin of the gene	Transformed plant	Performance of transgenic plant	Reference
HvCBF4	Barley	Rice	Drought, salinity, and cold tolerance	Oh et al. (2007)
HsDREB1A	*H. spontaneum*	Bahiagrass plants	Salinity and drought tolerance	James et al. (2008)
HvDREB1	Barley	*Arabidopsis*	Salinity tolerance	Xu et al. (2009)
TaDREB2 and TaDREB3	Wheat	Barley	Drought and frost tolerance	Morran et al. (2011)
HvRAF	Barley	*Arabidopsis*	Salinity and pathogen tolerance	Jung et al. (2007)
HvSNAC1	Barley	Barley	Drought tolerance	Al Abdallat et al. (2014)
AtCIPK16	*Arabidopsis*	Barley	Salinity tolerance	Roy et al. (2013)
HvHVA1	Barley	Rice	Drought tolerance	Babu et al. (2004)
HvHVA1	Barley	Mulberry	Salinity, drought and cold tolerance	Checker et al. (2012)
HvAPX	Barley	*Arabidopsis*	Salinity tolerance	Xu et al. (2008)
HvHKT2;1	Barley	Barley	Salinity tolerance	Mian et al. (2011)
AtAVP1	*Arabidopsis*	Barley	Salinity tolerance	Schilling et al. (2014)

from wheat were used to modulate the stress tolerance in barley and wheat (Morran et al. 2011). Transgenic barley plants constitutively expressing *TaDREB2* and *TaDREB3* genes showed enhanced survival under severe drought conditions but growth retardation and late flowering with lowered grain yield were observed in these transgenic plants (Morran et al. 2011). Alternatively, the growth retardation was eliminated by using an inducible promoter of a maize gene responsive to abscisic acid (*ZmRAB17*).

A barley *HvRAF* (*Hordeum vulgare* root abundant factor) gene encoding an ethylene response factor-type transcription factor was cloned from young seedlings of barley and characterized for its functional importance in biotic and abiotic stress responses (Jung et al. 2007). This gene was also transferred to *Arabidopsis* by floral-dip method to monitor expression patterns of several stress-responsive genes such as *Arabidopsis* putative fungal protein (*PDF1.2*), a serine/threonine protein kinase (*KIN2*), and thaumatin-like protein (*PR5*) (Jung et al. 2007). Overexpression of the full-length *HvRAF* gene in *Arabidopsis* plants led to improved resistance to bacterial soil-borne pathogen *Ralstonia solanacearum* strain GMI1000 and enhanced salinity tolerance. Recently, a gene from *NAC* TF family, namely *HvSNAC1* was

also isolated from drought-stressed barley plants and used for transgenic approach (Al Abdallat et al. 2014). The gene was introduced to barley (cv. Golden Promise) plants via *Agrobacterium tumefaciens* under the control of maize ubiquitin (*Ubi*) promoter (Al Abdallat et al. 2014). Transgenic plants constitutively expressed *HvSNAC1* showed higher drought tolerance with improved productivity and grain yield relative to control plants.

Calcium (Ca^{2+}) serves as a ubiquitous second messenger and regarded as one of the most important molecule involved in plant signaling networks (Yu et al. 2014). Ca^{2+} signaling process is assumed to be one of the earliest events in salt signaling, and may play an essential role in the ion homeostasis leading to salt tolerance in plants (Zhu 2003; Reddy and Reddy 2004). Calcineurin B-like-interacting protein kinases (CIPKs) have important functions in the Ca^{2+} regulated signaling pathways for controlling plant responses to abiotic stresses and regulation of ion homeostasis (Weinl and Kudla 2009; Kudla et al. 2010). Transgenic barley plants overexpressing *AtCIPK16* were shown to have increased salinity tolerance, which was expressed as 20–45 % higher maintenance in biomass under exposure to long-term strong salinity stress (30 days, 300 mM NaCl) compared with control plants (Roy et al. 2013).

Another approach for developing transgenic plants with higher tolerance to salinity and drought stress is transferring single genes that encode functional and structural proteins such as LEA proteins, molecular chaperones, ion transporters, proteins involved in lipid biosynthesis, detoxification enzymes, and key enzymes for osmolyte biosynthesis (Cominelli et al. 2013). Overexpression of the barley *HvHVA1* gene (group 3 LEA protein) under the constitutive control of rice *Act1* promoter led to a significant accumulation of the HVA1 protein in the roots and leaves of transgenic rice plants (Babu et al. 2004). After transformation, the leaf relative water content (RWC) of transgenic plants was higher than control plants. In addition, the transgenic plants showed less reduction in plant growth under drought stress (Babu et al. 2004). The *HvHVA1* gene under a constitutive promoter (*Act1*) was also introduced to mulberry plants and overexpression of the gene conferred tolerance to salt and cold stresses as well as drought in transgenic plants with retardation on plant growth and productivity (Checker et al. 2012). In order to eliminate these effects, authors used another recombinant vector (*pBI121:rd29A:Hva1*), which was constructed under the control of the stress-inducible *Arabidopsis rd29A* promoter. The *rd29A*-regulated transgene expression was also conferred enhanced tolerance against drought, salt, and cold stress. The performance of transgenic mulberry plants against these multi-stress factors was evaluated in the field conditions. *Rd29A:Hva1* plants showed better performance in field conditions upon mild stress than *Act1:Hva1* plants, which performed better in severe stress conditions.

A number of transgenic improvements for abiotic stress tolerance have been achieved by overexpression of detoxifying genes such as glutathione peroxidase (GPX), ascorbate peroxidase (APX), and superoxide dismutase (SOD) (Roxas et al. 1997; Wang et al. 2005; Xu et al. 2008). For example, the transgenic *Arabidopsis* plants overexpressing barley *HvAPX1* gene under the control of the CaMV 35S promoter were shown to enhance salt tolerance compared to wild-type plants (Xu et al. 2008). However, Na^+, K^+, Ca^{2+}, and Mg^{2+} contents of transgenic plants were

not found to be significantly different from wild-type plants. In addition, H_2O_2 content and lipid peroxidation level (determined by measuring the amount of malondialdehyde) upon salt stress were lower in the transgenic plants than in the wild type. As a result, it was concluded that improvement in response to salt stress was achieved by reduction in oxidative stress injury instead of maintenance of cellular ion homeostasis (Xu et al. 2008).

Transporter proteins are important candidates in genetic engineering for enhancing salt and dehydration tolerance in plants. Transgenic barley plants overexpressing a HKT transporter *HvHKT2;1* showed higher growth rates than non-transgenic plants by approximately 25–30 % in response to 50 mM NaCl and 100 mM NCl treatments supplemented with 2 mM K^+ (Mian et al. 2011).

Finally, successful transformations were performed in transgenic plants expressing genes for enzymes involved in proton (H^+) pumps that generate energy for tonoplast transport of solutes into vacuoles. Previously, *Arabidopsis* proton pumping pyrophosphatases (H^+-PPase; *AVP1*) were transferred to different plant species to confer salinity tolerances as reviewed by Agarwal et al. (2013). Very recently, the improvement of salinity tolerance of transgenic barley (cv. Golden Promise) by overexpression of *AtAVP1* was confirmed in saline field trials in addition to assessments in greenhouse conditions (Schilling et al. 2014). The transgenic barley plants were generated via *Agrobacterium*-mediated transformation using a construct including *AtAVP1* under the control of the CaMV 35S promoter (Schilling et al. 2014). In the low-salinity area where the soil electrical conductivity (SEC) was 161 ± 11 µS/cm, the transgenic barley plants had a significantly higher (17–33 %) shoot biomass and higher (23–34 %) grain yield per plant compared to non-transgenic plants and in the high-salinity area (SEC = 1231 ± 155 µS/cm), transgenic barley had higher shoot biomass (30–42 %) and showed improved survival compared to non-transgenic plants. Furthermore, in the high-salinity area, the grain yield per plant of the transgenic barley was significantly higher (79–87 %) than that of non-transgenic plants with the higher number of heads and grains.

In conclusion, several dehydration and salinity stress-related genes have been isolated and characterized in barley and transferred to different plant species to test their efficiency in tolerance mechanism. Equally, genes from wheat and *Arabidopsis* were also used to obtain transgenic barley plants (Table 2). In most cases, the performance of the transgenic plants was evaluated under greenhouse or controlled laboratory conditions. Recently, generated transgenic lines are more frequently being tested under natural field conditions to observe phenotypic responses to single or combination of stress factors. For instance, long-term field testing of transgenic barley for higher drought tolerance is known to be carried out in Australia (Mrízová et al. 2014).

8 Conclusion and Future Perspective

A better understanding of the genes in model plants with natural tolerance to water deficiency and salinity is becoming a priority due to more frequently experienced drought episodes in certain areas and global climatic changes. With its natural tolerance to several abiotic and biotic stress factors in addition to its small genome size, barley was proposed to be an excellent model system for abiotic stress research in cereals.

Transcriptomics which allows deep functional characterization of stress-inducible or stress-associated genes in plants is an important step in omics technologies. The studies using different plant systems and different stress conditions indicated that cellular signalling and metabolic mechanisms like osmotic adjustment, transcriptional regulation, antioxidant system, and single genes such as antiporters, LEA proteins, and ABA-inducible proteins are activated by water deficit and salinity stresses and contribute to survival of plants in stressful conditions. Transcriptomics was also effectively used in barley. A large amount of data was obtained by high-throughput transcriptomic technologies (microarrays and RNA-seq) and used in the identification of differentially regulated genes upon water deficit and salinity in barley (Ozturk et al. 2002; Ueda et al. 2004; Walia et al. 2006; Gao et al. 2013; Ziemann et al. 2013). New methods are still needed for interpretation of such large-scale data, for its incorporation to prior biological knowledge, and for the establishment of the relationships among variables (genes and metabolites) more precisely (Reshetova et al. 2014). Small-scale expression studies, on the other hand, focus on investigating more detailed analyses of gene inductions and supply useful data, particularly in comparison to wild-type and contrasting genotypes differing in their tolerance to drought or salinity.

Breeding is still the most popular approach to generate abiotic stress-tolerant crops. Selection of breeding lines by physiological comparison in response to drought and salt in the field conditions is still not easy and time-consuming, although there are new developments such as high-throughput phenotyping with global positioning systems, meteorological devices and so-called phenomobiles, phenotowers, and thermal imaging sensors (Honsdorf et al. 2013; Araus and Cairns 2014; Chen et al. 2014). Therefore, studies on changes in gene expression patterns in response to drought and salt stress conditions are extremely important not only to understand the basis of tolerance but also to generate molecular markers for the selection of tolerant genotypes in laboratory (Forster et al. 2000). Transgenic approach is an attractive option to develop salt and drought-tolerant crops for near future. In this context, transcription factors (e.g. *HsDREB1A, HvSNAC1*) and a few single genes (*HvHVA1*, *AtAVP1* etc.) cloned from barley and *Arabidopsis were* demonstrated to be efficient to generate plants with increased drought and salt tolerance.

With the large transcriptomic data and resources, barley is being exploited to identify genetic determinants that underlie its high tolerance to abiotic stresses. Wild progenitor of cultivated barley *H. spontaneum* is known to adapt to diverse environments including deserts and cold regions like Tibet (Nevo and Chen 2010;

Zhao et al. 2010), and therefore, is a promising genetic resource for abiotic stress tolerance improvement. Current research indicates the role of epigenetic mechanisms for abiotic stress tolerance and long-term adaptation to stressful conditions. It is now known that in addition to the known genetic determinants, epigenetic mechanisms including the dynamic changes in chromatin and synthesis of small RNAs also contribute to the regulation of gene expression during the stress responses (Mirouze and Paszkowski 2011). There are only two studies with barley in this scope. Papaefthimiou and Tsaftaris (2012) identified a putative jumonji-like histone demethylase, namely *HvPKDM7-1* in barley and showed its up-regulation under drought. Another barley gene coding a putative HvDME protein related to cytosine methylation was highly induced by dehydration stress (Kapazoglou et al. 2013). By having a larger genome than *Arabidopsis* and rice, transcriptomic information from barley may lead to complete resolution of molecular and biochemical networks related to stress response, and may also provide new approaches for the generation of transgenic crops with higher abiotic stress tolerance.

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