Comparative studies of selected stress responsive *DREB* and *ALDH* genes in *Arabidopsis thaliana*, *Eutrema salsugineum* and *Hordeum vulgare*

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Comparative studies of selected stress responsive *DREB* and *ALDH* genes in *Arabidopsis thaliana*, *Eutrema salsugineum* and *Hordeum vulgare*

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DECLARATION

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

Bonn, February 2015 **Quancan Hou**

谨以此论文献给我的父母和我终将逝去的青春

CONTENTS

ABBREVIATIONS

3-AT	3-Amino-1,2,4-triazole
4-MU	4-Methylumbelliferone
4 –MUG	4-Methylumbelliferyl-β-D-glucuronide
A	Adenine
ABA	Abscisic acid
ABRE	ABA responsive element
ALDH	Aldehyde dehydrogenase
AMADH	Aminoaldehyde dehydrogenase
amiRNA	Artificial mircoRNA
Amp	Ampicillin
APS	Ammonium persulfate
bHLH	Basic helix-loop-helix
BLAST	Basic local alignment search tool
β-ME	β-Mercaptoethanol
bp	Nucleotide base pair
BSA	Bovine serum albumin
bZIP	Basic leucine zipper
C	Cytosine
CaMV	Cauliflower mosaic virus
CBF	C-repeat binding factor
cDNA	Complementary DNA
CRT	C-repeat
d	Day
D	Dalton
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DMF	N,N-Dimethylformamid
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
DRE	Dehydration responsive element
DREB	Dehydration-responsive element binding
DTT	Dithiothreitol
dTTP	Deoxythymidine triphosphate
DW	Dry weight
EDTA	Ethylenediaminetetraacetate
fwt	Fresh weight
fwd	Forward
g	Gram
g	Acceleration
G	Guanine
GB	Glycine betaine
gDW	Gram dry weight
GFP	Green Fluorescent Protein
GST	Gluthation-S-transferase
GUS	E. coli β-glucuronidase gene (uidA)
h	Hour
HEPES	4-(2-Hydroxyethyl)-1-piperazinethansulfonic acid

His	Histidine
IgG	Class G immunoglobulin
IPTG	Isopropyl-β-D-thiogalactopyranoside
Kan	Kanamycin sulfate
kb	Kilobase
kDa	Kilodalton
LB	Luria and Bertani medium
LEA	Late embryogenesis abundant
Leu	Leucine
M	Molar, mole(s) per liter
mA	Milliampere
MCS	Multiple cloning site
MDA	Malondialdehyde
min	Minute
ml	Milliliter
MOPS	3-(N-morpholino) propanesulfonic acid
MPa	Mega Pascal
mRNA	Messenger RNA
MS	Murashige and Skoog (1962)
MW	Molecular weight
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
nm	Nanometers
nt	Nucleotide
OD	Optical density
Oligo (dT)	Oligodeoxythymidylic acid
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PIPES	Piperazine-N,N,-bis (2-ethanesulfonic acid)
PMSF	Phenylmethanesulphonyl fluoride
PUFA	Poly-unsaturated fatty acid
PVP	Polyvinylpyrrolidone
QTL	Quantitative loci trait
Rev	Reverse
Rif	Rifampicin
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
rpm	Rounds per minute
RT	Room temperature
RT-PCR	Reverse transcription-polymerase chain reaction
SAP	Shrimp alkaline phosphatase
SD	Synthetic defined media
SDS	Sodium dodecyl sulfate
sec	Second
SSC	Saline sodium citrate buffer
ssDNA	Single-stranded DNA
TA	Annealing temperature
TAE	Tris-acetate-EDTA
Taq	Thermophilus aquaticus

TBA	Thiobarbituric acid
TBE	Tris-borate-EDTA
TCA	Trichloroacetic acid
TE	Tris (10mM)-EDTA (1 mM)
TEMED	N,N,N',N'-tetramethylethylenediamine
TF	Transcription factor
TM	Melting temperature
Tris	Tris-(hydroxymethyl)-aminomethane
Triton X-100	Poly(ethylenglycolether)n-octylphenol
Trp	Tryptophan
U	Unit
Ura	Uracil
UV	Ultraviolet
V	Volts
v/v	Volume/volume
w/v	Weight/volume
WT	Wild type
X	Times
X-Gal	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside
X-Gluc	5-bromo-4-chloro-3-indolyl-β-D-glucuronide
Y1H	Yeast one hybrid
Y2H	Yeast two hybrid
YEB	Yeast extract broth
YEPD/YPD	Yeast extract peptone dextrose
YPAD	Adenine supplemented YPD

SUMMARY

Drought and salinity are the most severe abiotic stresses limiting agricultural production worldwide. Plant adaptive responses to these stresses involve stress signal perception, signal transduction to cytoplasm and nucleus, and gene expression to produce regulatory or protective proteins. CBF/DREBs are important transcription factors regulating the expression of a set of stress-associated downstream genes. Aldehyde dehydrogenases (ALDH) are considered detoxification enzymes to eliminate toxic aldehydes that accumulate under stress conditions. In this study, expression profiles of five barley *CBF/DREB* genes were investigated under a simulated slow progression of drought stress similar to field conditions. Aldehyde dehydrogenases were studied in the glycophyte *A. thaliana* and the *Eutrema* halophytes in a comparative manner with a focus on the mechanisms regulating the expression of the *ALDH7B4* gene under osmotic stress. The transcription factors regulating the promoter of *EsALDH7B4* were identified and the identified transcription factor bHLH146 was molecularly characterized.

Studies on barley *CBF/DREB* genes showed that the barley genome is rich in CBF/DREB1 subfamily genes but contains relatively few DREB2 subfamily genes. Two DREB2 subfamily genes *HvDREB1* and *HvDRF1.3* were constitutively expressed under both laboratory and complex field conditions, suggesting that they function as housekeeping genes. In contrast, expression of three other analyzed CBF/DREB1 subfamily genes did not show a clear pattern under the given conditions especially *HvCBF1*. Results from this study suggest that the knowledge obtained from laboratory conditions is not always identical to the data obtained in the complex field conditions.

In addition to the early responsive regulatory proteins under stress conditions, this study also focused on ALDHs which is one type of important late responsive protective proteins. By searching public databases, 16 and 17 *ALDH* genes were genome-wide identified from halophyte models *E. parvulum* and *E. salsugineum*, respectively. Phylogenetic analysis of ALDH protein sequences indicated that *Eutrema* ALDHs are closely related to those of *A. thaliana*, and members within these species possess nearly identical exon-intron structures. Gene expression analysis under different salt stress conditions showed that most of the *ALDH* genes have similar expression profiles in *A. thaliana* and *E. salsugineum* except for *ALDH3H1*,

ALDH7B4, and *ALDH10A8*. Transcripts of *ALDH3H1* and *ALDH7B4* increased in response to NaCl at higher salt levels in *E. salsugineum* than in *A. thaliana*, whereas *ALDH10A8* showed a different expression pattern under high salt in *E. salsugineum* and in *A. thaliana*. *EsALDH7B4* promoter deletion analysis revealed that a conserved G-box motif is important for the gene expression while a specific "TC" rich motif in the *EsALDH7B4* promoter represses gene expression in transgenic *A. thaliana* plants. This study also demonstrated that the genetic background plays an important role in the regulation of gene expression as the *EsALDH7B4* promoter showed a lower activity in transgenic *E. salsugineum* than in transgenic *A. thaliana* plants. Yeast one-hybrid screening identified the putative transcription factors that can regulate *EsALDH7B4*. Consistent with the results from promoter deletion assays, many members from bZIP and bHLH families that interact with the G-box motif were identified. A transcription factor MYB_like had been predicted as a transcriptional repressor and was identified using the "TC" rich motif as a bait.

This study also characterized the unknown *A. thaliana* transcription factor bHLH146. EMSA and DNA footprinting assays showed that bHLH146 did not directly bind to the *EsALDH7B4* promoter fragment and therefore has no direct G-box binding ability. Transient expression of bHLH146-GFP fusion protein in *A. thaliana* leaves and onion epidermis showed that the bHLH146 protein localized mainly in the nucleus. Spatiotemporal expression patterns of bHLH146 were investigated by placing a GUS reporter gene downstream of its promoter. GUS activity was detected in various organs reflecting that bHLH146 is expressed in almost all organs in *A. thaliana*. Expression of *bHLH146* was down-regulated by salt stress, which is opposite to the expression pattern of *ALDH7B4*. Further analysis suggests that bHLH146 may act as a transcriptional repressor. Yeast two-hybrid screening revealed that bHLH146 interacts with other G-box binding bHLH proteins bHLH49, bHLH69, and bHLH76. These results support the speculation that bHLH146 regulates G-box containing gene expression by forming heterodimers and thus inhibiting the DNA binding of the partners. In addition, several GTPase related proteins were also identified as interactors, which implies that bHLH146 is involved in many regulatory processes. *bHLH146* gene silencing lines by artificial microRNA and overexpression lines were generated and analyzed. Seedlings of bHLH146 overexpression lines showed a short hypocotyl and had a large open apical hook angle in the dark, which suggests it might also be involved in photomorphogenesis. This is supported by the observation

that bHLH146 overexpression lines exhibited some abnormal ectopic petal, carpel, or stamen structures. Progeny of bHLH146 T-DNA insertion heterozygous lines exhibit a non-Mendelian segregation and only heterozygous plants were obtained. An explanation for these phenotypes observed in bHLH146 overexpression and T-DNA insertion lines could be that bHLH146 interacts with GASA4 and TPTC proteins. No phenotypic difference was observed between *bHLH146* gene silencing lines, *bHLH146* and *At2g18969* double silencing lines and the wild-type plants.

1. INTRODUCTION

1.1 Global drought and salinity stress and food crisis

In the last 100 years, the global atmospheric temperature has increased by approximately 0.75 ˚C. However, the rate of global warming has accelerated over the last 25 years, at a speed of over 0.18 ˚C per decade (WHO 2009). It is predicted that global atmospheric temperature will rise by approximately 4 ˚C by 2080, consistent with a doubling of atmospheric CO_2 concentration (Turral *et al.* 2011). Climate change will significantly affect agriculture by increasing water demand and by reducing water availability in areas where irrigation is most needed, thereby aggravating drought stress that is already a worldwide problem (**Fig. 1**). By 2050, around four billion people (about 40% of the projected global population of 9.4 billion) from 54 countries will face the problem of water stress or scarcity. (Gardner-Outlaw and Engelman 1997; UNFPA 1997). According to the FAO Land and Plant Nutrition Management Service, over 6% of the world's land is affected by either salinity or sodicity (**Table 1**). Due to the continuous irrigation, a significant proportion of cultivated land is salt-affected. Out of the current 230 million ha of irrigated land, 45 million ha are salt-affected soils (19.5 percent) and of the almost 1,500 million ha of dry land agriculture, 32 million are salt-affected to varying degrees (2.1 percent). It is estimated that drought and salinity together affect more than 10% of arable land, causing average

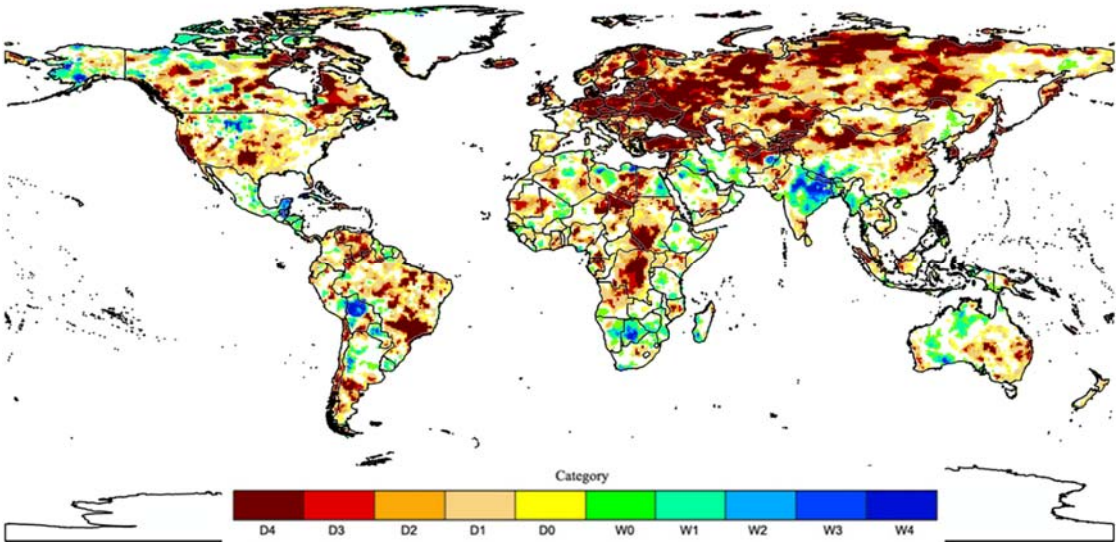


Fig. 1 Overview of global drought occurrence. Global drought information obtained from Global Integrated Drought Monitoring and Prediction System (GIDMaPS http://drought.eng.uci.edu/) (March. 2014). Color bar representation D0: abnormally dry; D1: moderate drought; D2: severe drought; D3: extreme drought; D4: exceptional drought; W0: abnormally wet; W1: moderate wetness; W2: severe wetness; W3: extreme wetness; W4: exceptional wetness.

yield losses of more than 50% of major crops worldwide (Boyer 1982). On the other hand, the global grain production needs to be doubled by the year 2050 to meet the ever-growing demands of the population (Cassman 1999; Tilman *et al.* 2002).

Table 1 Regional distribution of salt-affected soils, in million hectares

Regions	Total area	Saline soils		Sodic soils	
	Mha	Mha	%	Mha	%
Africa	1,899	39	2	34	1.8
Asia, the Pacific and Australia	3,107	195	6.3	249	8
Europe	2,011	7	0.3	73	3.6
Latin America	2,039	61	3	51	2.5
Near East	1,802	92	5.1	14	0.8
North America	1,924	5	0.2	15	0.8
Total	12,781	397	3.10%	434	3.40%

Source: http://www.plantstress.com/

Water availability, food security and how to cope with environmental stress to develop sustainable agriculture in the context of global climate change are the urgent issues that human beings have to consider (Brown and Funk 2008). Although traditional plant breeding methods have long been implemented and have generated some crop varieties with improved stress tolerance, they are time and labor consuming (Flowers 2004). Classical breeding may not cope with the food crisis that would occur. To accelerate the crop breeding process, direct introduction of genes by genetic engineering is more attractive and serves as a quick solution for improving stress tolerance (Dunwell 2000). Modern plant biotechnological approaches are mature to be applied to generate abiotic stress-tolerant crops. However, understanding the mechanisms of plant tolerance to abiotic stress is a fundamental step and still needs a long way to go. With the development of high-throughput sequencing technologies, more and more genomes of plant species have been sequenced and many more sequencing projects are carried out. The availability of genome sequences allows plant biologists to compare abiotic stress sensitive plants and tolerant plants and identify abiotic stress-related genes and important cellular pathways. Dissection of the tolerance nature of the extremophilic plants will help to unravel the molecular basis of plant stress tolerance.

1.2 Molecular mechanisms of drought and salt tolerance in plants

As sessile organisms, plants are exposed to many types of environmental stresses such as drought, cold, salinity, high temperature and others. Among the various abiotic stresses, water stress caused by drought and salt is the most prevalent abiotic stress that challenges plants. Both drought and salt stress largely affect plant physiology and metabolism and result in numerous changes. Physiological changes including leaf wilting or abscission, reduction in leaf area, stimulation of root growth, changes in relative water content (RWC), *etc.* occur in plants thereby affecting the plant metabolism. At the cellular level, drought and salt stress causes osmotic stress and removal of water out of the cytoplasm thereby leading to cellular dehydration. These stresses also cause the accumulation of reactive oxygen species (ROS) in the cells, which then in turn cause oxidative damage and affect cellular structures and metabolism negatively. ROS disrupt cellular homeostasis by reacting with lipids, proteins, pigments, and nucleic acids resulting in lipid peroxidation (LP), membrane damage, and the inactivation of enzymes, thus affecting cell viability (Bartels and Sunkar 2005; Lata and Prasad 2011).

Although most of the changes have serious consequences of stress injury, plants have evolved sophisticated mechanisms to adapt to drought and salt stress. Except for the ionic component in salt stress, responses to drought and salt stresses are largely identical. These similarities include metabolic processes such as a decrease in photosynthesis and increase in the levels of stress-related plant hormones like abscisic acid (ABA) and jasmonic acid (JA). High intracellular concentrations of sodium and chloride ions are an additional problem of salinity stress. According to Zhu (2002), the adaptive responses can be generally grouped into three control aspects: 1) homeostasis which is mainly relevant to salt stress, namely reestablishment of cellular homeostasis under stress conditions; 2) stress damage control or detoxification to repair stress damages; 3) growth control through coordinate cell division and expansion to levels suitable for the particular physiological conditions. Molecular and cellular responses to drought and salt stress include stress signal perception, signal transduction, gene expression and finally metabolic changes leading to stress tolerance (Xiong *et al.* 2002; Bartels and Sunkar 2005; Agarwal *et al.* 2006; Lata and Prasad 2011).

1.2.1 Drought and salt signal perception

Although many different sensors are expected to sense the multiple stress signals, no plant molecule has unambiguously been identified as an osmosensor so far. However, there are reports to show that drought, salt, and cold stress induce transient Ca^{2+} influx into the cellular cytoplasm (Knight 1999). Therefore, it was speculated that channels responsible for this Ca^{2+} influx might represent one type of sensor for these stress signals (Xiong *et al.* 2002). A putative plant osmosensor osca1 was isolated recently using forward genetic screens. *osca1* reduced hyperosmolality-induced Ca^{2+} increase, displays impaired osmotic Ca^{2+} signaling in guard cells and root cells, and attenuated water transpiration regulation and root growth in response to osmotic stress (Yuan *et al.* 2014). *Arabidopsis* histidine kinase1 (AtHK1) has been proposed as a plant osmosensor some time ago because it can complement the osmosensitivity of yeast osmosensor mutants *SLN1* (Urao *et al.* 1999; Osakabe *et al.* 2013). Further study showed that AtHK1 not only is involved in the water stress response during early vegetative stages of plant growth but also plays a unique role in the regulation of desiccation processes during seed formation (Wohlbach *et al.* 2008). The tobacco membrane-located receptor-like protein NtC7 was also suggested as a putative osmosensor (Bartels and Sunkar 2005). Its membrane location was confirmed in onion epidermis cells by transiently expressing a NtC7-green fluorescent protein (GFP) fusion protein. Its transcripts were found to accumulate rapidly and transiently within 1 h upon wounding, salt and osmotic stress (Tamura *et al.* 2003). Also, the activity of the plant histidine kinase cytokinin response 1 (Cre1) is regulated by changes in turgor pressure in a similar manner to yeast's SLN1, which suggests it as a putative candidate for sensing osmotic stress in plants (Reiser *et al.* 2003). In salinity stress, the plasma membrane Na^+/H^+ antiporter SOS1 (SALT OVERLY SENSITIVE1) is a candidate for sensing Na^+ (Zhu 2002, 2003). The transport activity of SOS1 is essential for Na^+ efflux from *Arabidopsis* cells and overexpression *SOS1* improves salt tolerance in *A. thaliana* (Shi *et al.* 2003). The suggested role as a Na^+ sensor is based on its structure. The SOS1 protein has 10-12 transmembrane domains and a long tail that is predicted to reside in the cytoplasm (Shi *et al.* 2000).

1.2.2 Cellular signal transduction upon sensing stress stimuli

After sensing the external stress stimuli, plant responses are activated by initiating different signaling cascades. In contrast to signal perception, various components of the signal transduction have been identified. The signal transduction pathways comprise a network of

protein-protein reactions and signaling molecules (ROS, Ca^{2+}, phospholipid-derived molecules, salicylic acid, nitric oxide *etc.*). The best-studied pathways are MAPKinase, phosphatases and phospholipid signaling pathways.

1.2.2.1 MAPKinase and phosphatases pathways

Reversible protein modification is an important mechanism by which organisms regulate cellular processes in response to environmental cues. Protein phosphorylation is the best-studied protein modification although other protein modifications like ubiquitination and sumoylation have become prominent. The mitogen-activated protein kinase (MAPK) cascades are common signaling modules in eukaryotic cells including plants. A general feature of MAPK cascades is their composition of three functionally linked protein kinases. The upstream MAPKK kinase (MAPKKK) activates MAPK kinase (MAPKK) through phosphorylation of conserved threonine and/or serine residues. Activated MAPKK further activates MAP kinase (MAPK) through phosphorylation of conserved threonine and tyrosine residues (Bartels and Sunkar 2005). The activation of the cytoplasmic MAPK can translocate to other sites in the cytoplasm to phosphorylate specific enzymes or cytoskeletal components (Robinson and Cobb 1997). More commonly, activation of the cytoplasmic MAPK module often induces translocation of the MAPK into the nucleus where the kinase can activate genes through phosphorylation of transcription factors (Tyerman *et al.* 2002). In *Arabidopsis*, at least 20 MAPK, 10 MAPKK and 60 MAPKKK genes have been identified based on sequence similarities (Riechmann 2000). Transcript levels for a number of these protein kinases increase to osmotic and other stress treatments (Mizoguchi *et al.* 2000).

MAPK pathways can mediate signaling of an extracellular stimulus and bring about specific responses. It is extrapolated from the studies on mammals that transient and low level MAPK activation may contribute to stress tolerance in plants, whereas prolonged and high level activation may be detrimental to the organism (Bartels and Sunkar 2005). Phosphatases provide modulation and reversibility of the phosphor regulatory mechanism. Therefore, phosphatases counteract the negative effects caused by high activation of MAPKs. There are two major groups of phosphatases, with different substrate specificities: phosphoprotein (serine/threonine) phosphatases (PPases) and phosphotyrosine (protein tyrosine phosphatases or PTPases). PPases are further classified into four groups (PP1, PP2A, PP2B, and PP2C) based on their biochemical and pharmacological properties

(Cohen 1989). The PTPases form three subgroups: receptor-like PTPases, intracellular PTPases, and dual specific PTPases. In yeast, expression of genes encoding PTPases is often up-regulated by the MAPK pathway, forming a negative feedback loop for MAPK regulation (Wurgler-Murphy *et al.* 1997). In *Arabidopsis*, both AtPTP1and AtMKP4 respond to salt stress (Xu *et al.* 1998). AtPTP1 dephosphorylates AtMPK4 resulting in a complete loss of enzyme activity indicating that the negative feedback loop for MAPK regulation also exists in plants (Huang *et al.* 2000).

1.2.2.2 Phospholipid signaling

Phospholipids not only have important structural roles, but they also mediate osmotic stress signals in plants. It is hypothesized that phospholipids are cleaved by phospholipases, which generate a multitude of phospholipid-derived signal molecules. Phospholipases are typically grouped into four major classes based on their site of lipid hydrolysis: phospholipase C (PLC), phospholipase D (PLD), and phospholipase A1 and A2 (PLA1 and PLA2) (Wang 2002). PLC cleaves the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2) to produce the soluble secondary messengers inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). PLD catalyzes the hydrolysis of membrane phospholipids, generating phosphatidic acid (PA) and free head groups. PLA2 cleaves phospholipids at the sn-2 position and results in lysophospholipids and free fatty acids. IP3, DAG and PA are the major phospholipid-derived signaling molecules under osmotic stress. Phospholipid signaling may be regulated through G-proteins and maybe tightly linked with calcium. In *Arabidopsis*, PLC1 is induced by salt and drought (Hirayama *et al.* 1995). The activation of PLC leads to the synthesis of IP3 and DAG. Studies have shown that IP3 levels also increase rapidly in response to hyperosmotic stress as well as exogenous ABA (DeWald *et al.* 2001; Takahashi *et al.* 2001; Xiong *et al.* 2001b). IP3 releases Ca^{2+} from internal stores while DAG may be rapidly phosphorylated to PA or activates a protein kinase C. In guard cells, the induced Ca^{2+} increase in the cytoplasm by IP3 triggers stomatal closure (Sanders *et al.* 1999). Plant PLDs, PLDα, PLDβ, PLDγ and PLDδ contain a Ca^{2+} binding domain which is not found in PLDs from other organisms (Wang 2002). It is speculated that this feature indicates a direct regulation of PLD by calcium (Bartels and Sunkar 2005). Dehydration stress rapidly activates PLD activity in *Arabidopsis* and in the desiccation tolerant plant *Craterostigma plantagineum* (Frank *et al.* 2000; Katagiri *et al.* 2001). However, drought stress-induced PLD activity was found higher in the drought-sensitive

cowpea cultivar than in the drought-resistant cultivar (Maarouf *et al.* 1999). Consistent with this finding, blocking PLD activity resulted in reduced stress injury and improved freezing tolerance (Zhu 2002). These results imply that PLD activation reflects membrane damage during stress injuries. Therefore, it was suggested that the PLD product, PA, has involved a signaling role to alleviate stress injury (Zhu 2002). However, the targets of PA in plants are still unknown. It was speculated that PIP kinase, PDK (phosphoinositide dependent kinase), MAPK pathway, K^+ channel are possible targets (Munnik and Meijer 2001; Bartels and Sunkar 2005).

1.2.2.3 ABA and osmotic stress signaling

The phytohormone abscisic acid (ABA) serves as a central endogenous messenger in the abiotic stress response. This is illustrated by ABA-deficient mutants as these mutants perform poorly under both drought and salt stress and even die if the stress persists (Xiong *et al.* 2001a). ABA plays a major role in water balance mainly through guard cell regulation, whereas the role in cellular dehydration tolerance is through induction of genes that encode proteins involved in conferring dehydration tolerance.

A major breakthrough has been achieved in understanding the mechanism of ABA in plant intracellular signaling. Several important ABA receptors PYR1/PYLs/RCARs (pyrabactin resistance 1/Pyr-likes/regulatory component of ABA receptors; subsequently referred to as PYRs) were discovered (Ma *et al.* 2009; Sang-Youl Park *et al.* 2009). Detailed review articles have been published emphasizing the molecular basis of the core regulatory network in ABA response (Umezawa *et al.* 2010; Qin *et al.* 2011). According to the model by Qin *et al.* (2011), in the absence of ABA, ABA signaling negative regulators two type 2C protein phosphatases (PP2Cs)/ABA insensitive 1 and 2 (ABI1 and ABI2) dephosphorylate the sucrose non fermenting1-related protein kinase 2 (SnRK2) and keep them inactive. When the nuclei accumulate ABA, PYR/PCAR receptors bind ABA and interact with PP2Cs to inhibit their phosphatase activity, subsequently causes the SnRK2 kinases to become active. Activated SnRK2 further phosphorylates AREB/ABF transcription factors that directly bind ABREs (ABA-responsive elements) in the promoters of ABA-responsive genes, which leads to gene expression. In addition, the ABA receptors are also involved in ion channel control in guard cells. ABA can induce stomata closure to decreases transpiration to reduce water loss, which in turn leads to lower photosynthesis rates. Once ABA is bound by PYR/RCAR in guard cells, PP2C activity is inhibited, which

activates SnRK2E/OST1 (open stomata 1). Activated OST1 phosphorylates the potassium channel KTA1, and reduces its K^+ uptake activity (Sato *et al.* 2009). On the other hand, OST1 phosphorylates the ion channel SLAC1 (slow anion channel-associated 1) to promote Cl^- efflux (Negi *et al.* 2008). Calcium-dependent protein kinases 21 and 23 (CPK21 and CPK23) also interact with SLAC1 and phosphorylates SLAC1 (Geiger *et al.* 2010), which depends on Ca^{2+} signaling. Consequently, turgor and ionic changes of guard cells determine stomata closure.

1.2.3 Gene expression changes during drought and salt stress

Among the drought- and salt- responsive genes, a small number of genes are induced very quickly (within minutes) and often transiently. These genes are designated as "early-response genes" and are typically transcription factors. In contrast, the "delayed-response genes" comprise the majority of the stress-responsive genes. They are activated more slowly (within hours), and their expression is often sustained. Induction of "early-response genes" does often not require *de novo* protein synthesis because all signaling components are already present. The upstream transcription factors for "early-response genes" are constitutively expressed and are regulated by stress at the posttranslational level such as phosphorylation changes (Zhu 2002). Expression of "delayed-response genes" is usually regulated by the "early-response genes". Therefore, using "early-response genes" for genetic engineering is a powerful way to generate stress-tolerant plants as their overexpression can lead to the up-regulation many downstream "delayed-response genes" genes (Kasuga *et al.* 1999).

Transcriptome analysis using microarray or RNA sequencing technologies revealed the genes that are induced by stress could be categorized into two groups (Bohnert *et al.* 2001; Fowler and Thomashow 2002; Seki *et al.* 2002). One group usually comprises "early-response genes" coding for regulatory proteins, including transcription factors (bZIP, MYB, NAC and DREB, *etc.*), protein kinases (MAP kinase, CDP kinase, and transcription-regulation protein kinase, *etc.*) and proteinases (phosphoesterases and phospholipase C, *etc.*) involved in signal transduction and gene regulation. The other group consists of functional proteins such as enzymes for osmolyte biosynthesis (betaine, proline and sugars, *etc.*) to balance compatible solutes; detoxification enzymes (superoxide dismutase, aldehyde dehydrogenase, catalase, *etc.*) to remove toxic products which are generated under abiotic stress; membrane proteins (water channel proteins and membrane transporters) that

maintain water movement through membranes; and proteins for the protection of macromolecules (LEA protein, chaperons and mRNA binding protein, *etc.*). Plant tolerance to abiotic stress can also be improved by introduction of genes encoding these functional proteins (Sunkar *et al.* 2003).

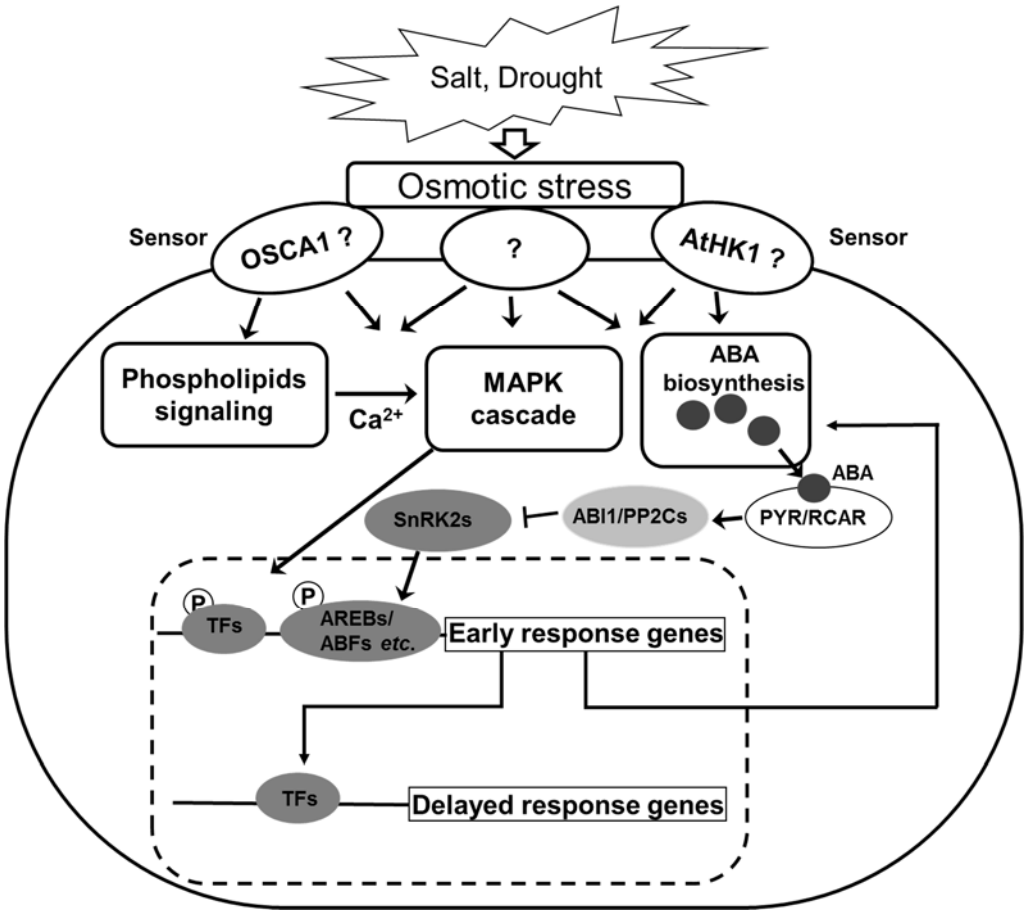


Fig. 2 Model showing regulation of drought and salt stress responsive genes. Model integrating cellular stress sensing, signal transduction cascades (activation of phospholipid signaling and MAPK cascade, and ABA signaling cascade), and transcription cascade leading to the expression of responsive genes. (This model was modified from Zhu (2002) and Qin *et al.* 2011).

Fig. 2 shows a general model of drought and salt stresses induced responsive gene expression based on the model by Zhu (2002) and Qin *et al.* (2011). This model integrating stress sensing, activation of MAP kinase cascades, phospholipid signaling cascade, ABA signaling cascade and transcription cascade leading to the expression of delayed-response genes.

1.3 Role of transcription factors in abiotic stress tolerance in plants

Transcription factors (TFs) are regulatory proteins that interact with other transcriptional regulators, including chromatin remodeling/modifying proteins, to employ or obstruct RNA polymerases to the DNA template (Udvardi *et al.* 2007). Up-regulated expression of various abiotic stress-related genes by interaction between transcription factors and *cis*-elements present in the promoter region is the main way of plants to tolerate abiotic stress (**Fig. 3**). In the *A. thaliana* genome, more than 5% of the genes (~ 1700) encode transcription factors (Riechmann and Ratcliffe 2000). Many transcription factors have been identified that are important in regulating plant responses to different stresses, including basic-domain leucine-zipper (bZIP) (Uno *et al.* 2000), NAC (NAM, ATAF1,2, CUC2) (Olsen *et al.* 2005), WRKY (Eulgem *et al.* 2000), AP2/EREBP (APETLA2/ethylene responsive element binding protein) (Mizoi *et al.* 2012). Although most of the stress-inducible genes are also induced by ABA (Shinozaki and Yamaguchi-Shinozaki 2000), there are many stress responsive genes that are induced independent of ABA as illustrated by ABA mutants. For example, stress-induced *rd29A* expression was detected in *aba* (ABA-deficient) or *abi* (ABA-insensitive) *Arabidopsis* mutants (Yamaguchi-Shinozaki and Shinozaki 1993), suggesting the existence of ABA-independent signal transduction cascades besides the ABA-dependent pathway (Yoshida *et al.* 2014). Among the transcription factors, bZIP/ABRE and MYC/MYB are ABA-dependent while NAC and

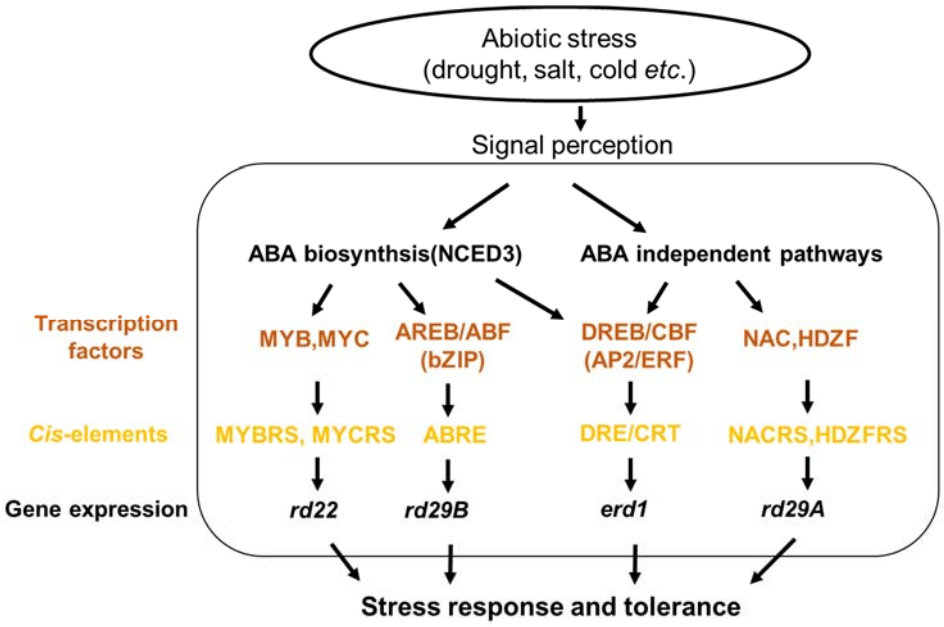


Fig. 3 Schematic representation of cellular signal transduction pathways from stress signal perception to gene expression. Expression of stress responsive genes via interactions between *cis*-elements and transcription factors. (This figure was modified from Agarwal *et al.* 2006).

DREBs are ABA-independent (Lata and Prasad 2011). There are both ABA-dependent and -independent TFs in the AP2/EREBP family (**Fig. 3**). The ability of transcription factors in acting as master regulators has been regarded as a powerful tool for genetic engineering as their overexpression can lead to the up-regulation of a whole array of genes under their control approach to modify complex traits in crop plants (Agarwal *et al.* 2006).

1.3.1 The CBF/DREB transcription factors

The dehydration responsive element binding proteins (DREBs) or C-repeat binding factors (CBFs) are transcription factors that belong to the APETALA 2/ethylene-responsive element binding factor (AP2/ERF) family, which is unique to plants. All the members of the AP2/ERF superfamily contain at least one conserved 58-59 amino acid domain (AP2/ERF domain) that binds to the GCC box and the C-repeat CRT/dehydration responsive element (DRE) involved in the expression of cold and dehydration responsive genes (Gu *et al.* 2000). Based on the number of copies of AP2/ERF domains and their sequence similarity, the AP2/ERF proteins have been subdivided into five subfamilies, namely AP2, RAV, DREB, ERF and others (Sakuma *et al.* 2002). The AP2 subfamily contains two AP2/ERF domains; the RAV subfamily contains one AP2/ERF domain and one additional B3 DNA-binding domain while members of other three subfamilies contain only a single AP2/ERF domain.

The first isolated cDNAs encoding DRE binding proteins, CBF1 (CRT binding factor1), DREB1A, and DREB2A were identified through yeast one-hybrid screening from *A. thaliana* (Stockinger *et al.* 1997; Liu *et al.* 1998). Since then, many stress-inducible DREBs have been isolated from numerous plants, including dicots such as oilseed rape (*Brassica napus*) and tomato (*Solanum lycopersicum*) (Jaglo *et al.* 2001), monocots such as rice (*Oryza sativa*) (Dubouzet *et al.* 2003) and barley (*Hordeum vulgare*) (Xue and Loveridge 2004), and moss (*Physcomitrella patens*) (Liu *et al.* 2007). The DREBs from different species are conserved in the binding domain. In AtDREB2A, OsDREB2A and AtDREB1A proteins, the 14th valine and 19thglutamic acid are conserved in the ERF/AP2 domain. In OsDREB1-type proteins, valine is conserved at both the 14th and 19thpositions except for OsDREB1C, where a glutamic acid is located at the 19th position. Other DREB1-type proteins in monocots (barley, wheat, and rye) also have a conserved valine in the 19th position. The conserved nature of the DREB2-type protein suggests that these proteins have similar binding specificity in different plants. These proteins specifically bind to the DRE

sequence (TACCGACAT) and activate the expression of genes containing a DRE motif. The DREB proteins were first identified to bind the promoter of the drought-responsive gene *rd29A* of *Arabidopsis* (Yamaguchi-Shinozaki and Shinozaki 1994). Binding analysis showed that both AtDREB1A/CBF3 and AtDREB2A specifically bind to six nucleotides (A/GCCGAC) of DRE (Sakuma *et al.* 2002). AtDREB1A prefers to bind A/GCCGACNT while AtDREB2A prefers ACCGAC (Maruyama *et al.* 2004). Competitive DNA binding assays also demonstrated that AtDREB1A binds to ACCGAC and GCCGAC with the same efficiency while OsDREB1A prefers GCCGAC compared to ACCGAC (Dubouzet *et al.* 2003).

CBF/DREBs play an important role in abiotic and biotic stress tolerance as they can induce a set of abiotic stress-related genes and confer stress tolerance to plants. CBF/DREB1 and DREB2 are two main subgroups of the DREB subfamily, involved in low temperature and dehydration triggered signal transduction pathways, respectively. *Arabidopsis* contains six *CBF/DREB1* and eight *DREB2* genes. The expression of *AtDREB1* is induced by cold, but not by dehydration or high salt stress (Liu *et al.* 1998; Shinwari *et al.* 1998). A similar result was reported for *CBF* genes which showed high expression in response to low temperature treatment and maximum expression was detected 1 h after exposure to 4 °C (Medina *et al.* 1999). The expression of *AtDREB2A* and its homolog *AtDREB2B* were induced by dehydration and high salt stress, but not by cold stress (Liu *et al.* 1998; Nakashima *et al.* 2000). *CBF/DREB* genes have been overexpressed in different plant species. All the results are similar and showed that plants exhibit strong tolerance to drought, high salinity or freezing stress. For example, transgenic *Arabidopsis plants* overexpressing *DREB1A/CBF3* or *DREB1B/CBF1* showed pronounced tolerance to drought, freezing and high salinity (Jaglo-Ottosen *et al.* 1998; Liu *et al.* 1998; Kasuga *et al.* 1999). Overexpression of the *Arabidopsis DREB1/CBF* genes in transgenic tobacco or *Brassica napus* induced expression of downstream genes and improved the freezing tolerance of transgenic plants (Jaglo *et al.* 2001; Kasuga *et al.* 2004). AtDREB2A contains a negative regulatory domain and deletion of this domain makes AtDREB2A constitutively active. Overexpression of this active form resulted in growth retardation of transgenic *Arabidopsis* and up-regulation of many stress-inducible downstream genes and improved tolerance to drought stress but with minimal tolerance to freezing stress (Sakuma *et al.* 2006). Overexpression of *AtDREB2C* also induce the expression of many heat stress-inducible genes improving thermal tolerance of transgenic *Arabidopsis* plants (Lim *et al.* 2007). Ectopic expression of rice *OsDREB2B*

or maize *ZmDREB2A* in *Arabidopsis* exhibited improved drought and heat stress tolerance but the plants exhibited a dwarf phenotype under non-stress conditions (Qin *et al.* 2007; Matsukura *et al.* 2010). All of these observations suggest that DREB1 and DREB2 regulons can be used to improve the drought, salinity, and freezing stress tolerance of important crops by genetic engineering. To avoid the drawbacks caused by constitutive expression, a stress-activated promoter needs to be used to drive the expression of the CBF/*DREB* genes (Kasuga *et al.* 1999).

1.3.2 The bHLH transcription factors

The basic/helix-loop-helix (bHLH) proteins are a superfamily of transcription factors that are widely distributed in all three eukaryotic kingdoms. The bHLH family is defined by the bHLH signature domain (~60 amino acids) which comprises two functionally distinct regions: the basic region and the HLH (helix-loop-helix) region. The basic region that located at the N-terminal end of the domain is involved in DNA binding and consists of ~18 hydrophilic and basic amino acids. The HLH region at the C-terminal end constitutes mainly hydrophobic residues that form two amphipathic α-helices separated by a loop structure of variable sequence and length. Based on the number of basic residues in the basic region, the bHLH proteins are divided into two major categories: DNA binding bHLHs and non-DNA binding bHLHs. More than 120 bHLH proteins are predicted to bind DNA as they have an average of six basic residues in the basic region. These DNA binding bHLHs are subdivided further into E-box (CANNTG) binders and the non-E-box binders based on the presence or absence of the two specific residues in the basic region: 13^{th} glutamic acid and 16^{th} arginine (Toledo-Ortiz *et al.* 2003). The E-box binding bHLHs can be categorized further into subgroups based on the type of E-box recognized. There are three residues in the basic region of the bHLH proteins: histidine/lysine, glutamic acid, and arginine at positions 9, 13, and 17 which constitute the best-understood G-box (CACGTG) recognition motif (Shimizu *et al.* 1997). The rest of the bHLHs with E-box binding capacity lacking the conserved residues to bind a G-box were defined as non-G-box binders. Around ten bHLH proteins in *Arabidopsis* have five to eight basic residues in their basic region but lack the sequence specificity for an E-box. The proteins that have DNA binding ability, but do not recognize an E-box are defined as non-E-box binding proteins. At least 27 AtbHLHs are predicted not to bind DNA as they only have an average of 3.8 basic residues in their basic region (Toledo-Ortiz *et al.* 2003). It is speculated that these non-DNA binding bHLHs

act as negative regulators of E-box binding bHLHs through the formation of heterodimers (Toledo-Ortiz *et al.* 2003; Hao *et al.* 2012).

The number of characterized bHLHs has increased in recent years, revealing that bHLH proteins are involved in many regulatory processes in plants, animals and yeast (Atchley and Fitch 1997; Robinson and Lopes 2000; Stevens *et al.* 2008). In plants, bHLH proteins have been reported to function in a myriad of regulatory processes including shoot branching (Komatsu *et al.* 2001), root (Ohashi-Ito and Bergmann 2007), trichome (Payne *et al.* 2000), stomata (Kanaoka *et al.* 2008), pollen (Ko *et al.* 2014), flower and fruit development (Rajani and Sundaresan 2001; Szécsi *et al.* 2006; Gremski *et al.* 2007), symbiotic ammonium transport (Kaiser *et al.* 1998), hormone signaling (Friedrichsen *et al.* 2002; Lee *et al.* 2006), light signaling (Ni *et al.* 1998; Huq and Quail 2002; Roig-Villanova *et al.* 2007; Leivar *et al.* 2008), and under abiotic and biotic stress responses (Smolen *et al.* 2002; Chinnusamy *et al.* 2003; Song *et al.* 2013). The bHLHs form one of the largest transcription factor families in plants and many novel atypical bHLHs have been identified recently (Carretero-Paulet *et al.* 2010). The biological and molecular functions of the novel and uncharacterized bHLHs still have to be investigated.

1.4 Aldehyde dehydrogenase (ALDH) superfamily in plants

Environmental stress like drought and high salinity induce the rapid generation of reactive oxygen species (ROS), which subsequently cause excessive accumulation of aldehydes in plant cells. Aldehydes are also intermediates in a range of metabolic pathways, but excessive amounts of aldehydes interfere with the metabolism and can be toxic to the cells (Jakobyz and Ziegler 1990; Lindahl 1992; Bartels 2001). Aldehyde dehydrogenase (ALDH) enzymes contribute to aldehyde homeostasis and are considered to be “aldehyde scavengers” to eliminate toxic aldehydes (Sunkar *et al.* 2003; Rodrigues *et al.* 2006). The ALDH superfamily comprises a group of $NAD(P)^+$-dependent enzymes that metabolize a wide range of endogenous and exogenous aliphatic and aromatic aldehyde molecules by oxidation to their corresponding carboxylic acids (Lindahl 1992; Yoshida *et al.* 1998). In addition to acting as aldehyde scavengers, ALDHs are involved in a broad range of metabolic functions including participating in intermediary metabolism such as amino acid and retinoic acid metabolism or generating osmoprotectants, such as glycine betaine (Ishitani *et al.* 1995). Aldehyde dehydrogenase enzymes produce NADPH and NADH in their enzymatic reactions and thus may contribute to balancing redox equivalents.

Table 2 Number of ALDH family members identified in representative species

		ALDH family																									
Taxonomy	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	Total No.	References
Herbaceous dicotyledon	*A. thaliana*	-	3	3	-	1	1	1	-	-	2	1	1	-	-	-	-	-	2	-	-	-	1	-	-	16	Kirch *et al.* (2004)
Herbaceous dicotyledon	*E. parvulum*	-	3	3	-	1	1	1	-	-	2	1	1	-	-	-	-	-	2	-	-	-	1	-	-	16	Hou and Bartels (2014)
Herbaceous dicotyledon	*E. salsugineum*	-	3	4	-	1	1	1	-	-	2	1	1	-	-	-	-	-	2	-	-	-	1	-	-	17	Hou and Bartels (2014)
Herbaceous dicotyledon	*G. max*	-	5	1	-	-	-	4	-	-	6	2	-	-	-	-	-	-	-	-	-	-	-	-	-	18	Kotchoni *et al.* (2012)
Herbaceous monocotyledon	*O. sativa*	-	5	5	-	1	1	1	-	-	2	1	1	-	-	-	-	-	2	-	-	-	1	-	-	20	Gao and Han (2009)
Herbaceous monocotyledon	*Z. mays*	-	6	5	-	2	1	1	-	-	3	1	1	-	-	-	-	-	2	-	-	-	1	-	-	23	Jimenez-Lopez *et al.* (2010); Zhou *et al.* (2012)
Herbaceous monocotyledon	*S. bicolor*	-	5	4	-	1	1	1	-	-	2	1	1	-	-	-	-	-	2	-	-	-	1	-	-	19	Sophos and Vasiliou (2003); Brocker *et al.* (2013)
Woody plant	*V. vinifera*	-	5	4	-	3	3	2	-	-	2	2	1	-	-	-	-	-	2	-	-	-	1	-	-	25	Zhang *et al.* (2012)
Woody plant	*M. domestica*	-	13	7	-	2	2	2	-	-	2	3	2	-	-	-	-	-	4	-	-	-	2	-	-	39	Li *et al.* (2013)
Woody plant	*P. trichocarpa*	-	4	6	-	1	4	2	-	-	2	3	1	-	-	-	-	-	2	-	-	-	1	-	-	26	Brocker *et al.* (2013)
Fern	*S. moellendorffii*	-	6	2	-	1	1	1	-	-	1	6	1	-	-	-	-	-	1	-	-	1	1	2	-	24	Brocker *et al.* (2013)
Moss	*P. patens*	-	2	5	-	2	1	1	-	-	1	5	1	-	-	-	-	-	1	-	-	1	-	1	-	21	Wood and Duff (2009)
Algae	*O. tauri*	-	-	1	-	1	-	-	-	-	1	1	1	-	-	-	-	-	-	-	-	-	1	-	-	6	Wood and Duff (2009)
Algae	*C. reinhardtii*	-	1	-	-	1	1	-	-	-	1	1	1	-	-	-	-	-	1	-	-	-	1	-	1	9	Wood and Duff (2009)
Algae	*V. carteri*	-	1	-	-	-	1	-	-	-	1	1	1	-	-	-	-	-	1	-	-	-	1	-	-	7	Brocker *et al.* (2013)
Mammal	*H. sapiens*	6	1	4	1	1	1	1	1	1	-	-	-	-	-	1	-	-	1	-	-	-	-	-	-	19	Yoshida *et al.* (1998); Jackson *et al.* (2011)
Mammal	*M. musculus*	7	1	5	1	1	1	1	1	1	-	-	-	-	-	1	-	-	1	-	-	-	-	-	-	21	Jackson *et al.* (2011)

"-" no member of the ALDH gene family was identified in the corresponding species.

Aldehyde dehydrogenases are found throughout all taxa and have been classified into 24 distinct families based on protein sequence identities. These families are numbered according to the criteria from the ALDH Gene Nomenclature Committee (AGNC) (Vasiliou *et al.* 1999). The plant ALDH superfamily contains 14 distinct families: ALDH2, ALDH3, ALDH5, ALDH6, ALDH7, ALDH10, ALDH11, ALDH12, ALDH18, ALDH19, ALDH21, ALDH22, ALDH23 and ALDH24. The families ALDH10, ALDH12, ALDH19, ALDH21, ALDH22, ALDH23 and ALDH24 are specific to plants, whereas the remaining families have mammalian orthologues (**Table 2**). There are a few *ALDH* genes identified in algae species, 7 *ALDH* genes in the colonial algae *Volvox carteri* (Brocker *et al.* 2013), 6 and 9 *ALDH* genes in the unicellular algae *Ostreococcus tauri* and *Chlamydomonas reinhardti*i, respectively (Wood and Duff 2009). The *ALDH* gene numbers increased in the moss *Physcomitrella patens* which contains 21 members including all plant ALDH gene families except for ALDH22 (Wood and Duff 2009). *P. patens* has gained two novel gene families, ALDH21 and ALDH23, and displays an increase of genes in the ALDH3 and ALDH11 gene families. The expansion of the *ALDH* genes in bryophytes such as *P. patens* may be related to the transition from aquatic to amphibious life. Structural and developmental complexity increases and additional protection may be needed against environmental stresses encountered during the transition (Cronk 2001). When plants completed their life cycles on land, many genes associated with aquatic life were lost and genes required for adaptation to terrestrial stressors were expanded. Gene loss or expansion also occurred in the ALDH superfamily. Green plants have retained 9 ALDH family members from lower plants encompassing ALDH2, ALDH3, ALDH5, ALDH6, ALDH10, ALDH11, ALDH12, ALDH18 and ALDH22. Although the ALDH7 genes are widely present in plants and animals and are highly conserved throughout evolution, they are not reported in the algae (Wood and Duff 2009; Brocker *et al.* 2013). The ALDH21, ALDH23 and ALDH24 protein families are present in *C. reinhardtii* or *P. patens* but have been lost in many vascular plants. So far, only in tomato a single gene of the ALDH19 family has been identified and encodes a γ-glutamyl phosphate reductase involved in proline biosynthesis (García-Ríos *et al.* 1997). Other *ALDH19* genes have not been reported in higher plants.

Most of the studied plant *ALDH* genes are expressed in response to high salinity, dehydration, heat, water logging, oxidative stress or heavy metals (Sunkar *et al.* 2003; Kirch *et al.* 2005; Gao and Han 2009), suggesting important roles in environmental

adaptation. Several studies have demonstrated that ectopic overexpression of *ALDH* genes enhances plant tolerance to abiotic stress (Sunkar *et al.* 2003; Kotchoni *et al.* 2006; Rodrigues *et al.* 2006). Besides the ALDH superfamily in the genetic model plant *Arabidopsis thaliana* (Kirch *et al.* 2004), ALDH gene families from several plant species have been reviewed (Brocker *et al.* 2013); these include the algae *C. reinhardtii* and *O. tauri*, the moss *P. patens* (Wood and Duff 2009), the vascular plants rice (Gao and Han 2009), maize (Jimenez-Lopez *et al.* 2010; Zhou *et al.* 2012), soybean (Kotchoni *et al.* 2012), grape (Zhang *et al.* 2012) and apple (Li *et al.* 2013). No reports are available for halophytic plants.

1.5 Barley as a model crop plant and *Eutrema* as new model plants for stress research

1.5.1 Barley as a model crop plant

Barley (*Hordeum vulgare L.*), a member of the grass family *Poaceae*, is a major cereal grain. It was one of the first cultivated grains and is now grown widely (Salamini *et al.* 2002). According to a ranking of cereal crops in the world from Food and Agriculture Organization (FAO), barley was the fourth both in terms of quantity produced (144.8 million tons) and in area of cultivation (49.8 million hectares) in the year 2013 (http://faostat3.fao.org/). Barley is widely adapted to diverse environmental conditions and is more tolerant to cold, drought, alkalinity and salinity than its close relative wheat (Nevo *et al.* 2012). As a result, barley remains a major food source in some regions like Tibet of China. Barley is widely used as animal fodder, as a source of fermentable material for beer and certain distilled beverages, and as a component of various health foods. It is used in soups and stews, and in barley bread of various cultures. Barley grain is particularly high in soluble dietary fiber, which significantly reduces the risk of serious human diseases including type II diabetes, cardiovascular disease and colorectal cancers that affect many people worldwide (Collins *et al.* 2010).

Barley has a big genome of around 5.1 gigabases distributed over seven pairs of nuclear chromosomes, one mitochondrial and one chloroplastic chromosome. Barley has traditionally been considered as a model for plant genetic research because of several essential features. These features include that it is diploid with a high degree of inbreeding; has a low chromosome number (2n = 14) with large size; the ease of cross-breeding and cultivation in a wide range of climatic conditions (Saisho and Takeda 2011). In addition,

the completion of the barley genome sequencing project in 2012 provides a platform to advance gene discovery, and genome-assisted crop improvement of cultivated barley (Mayer *et al.* 2012).

1.5.2 *Eutrema* as new model plants for stress research

Arabidopsis thaliana is an excellent model to understand basic developmental and physiological processes in plants as well as for understanding mechanisms of the rapid evolutionary process associated with genome duplication and polyploidization. The use of *A. thaliana* for research could be traced to the late 1800s (Meyerowitz 2001). It offers important advantages for basic research in genetics and molecular biology: small genome size, short life cycle, self-fertile, prolific seed production and easily transformable. However, it also has some disadvantages. Besides the limitations on developmental study as it does not produce fruit and it is a dicot, it is also a stress-sensitive species, so there are also limitations on exploration of stress adaptive and dependent responses in this plant.

Eutrema parvulum or *Schrenkiella parvula* (formerly known as *Thellungiella parvula*) and *Eutrema salsugineum* (formerly known as *Thellungiella salsuginea* or *Thellungiella halophila*) belong to the Brassicaceae family and are close relatives of *A. thaliana*. Older studies on taxonomic diversity, phylogeny and geographic distribution of the *Eutrema* species have been neglected and caused some confusion over the species' names in some publications. The names *Eutrema parvulum* and *Eutrema salsugineum* which are currently used in the National Center for Biotechnology Information (NCBI) were used in this study despite that German and Al-Shehbaz (2010) suggested *Schrenkiella parvula* as a new name for *Eutrema parvulum* (German and Al-Shehbaz 2010).

E. parvulum and *E. salsugineum* are halophytes and tolerate high salt concentrations (Inan *et al.* 2004; Orsini *et al.* 2010). They are excellent models for revealing the mechanisms on abiotic stress tolerance because they have a short life cycle, are self-fertile, have a small genome, good seed production and are genetically transformable. Because of these characters, *Eutrema* species were recommended as halophyte model plants a decade ago (Zhu 2001). The availability of the genome sequences allows comparative analyses between these species, which have a close phylogenetic relationship, but with extremely divergent adaptations. The *E. salsugineum* and *E. parvulum* genome is approximately 50% and 15% larger than that of *A. thaliana*, respectively. The higher content of transposable

elements in *E. salsugenium* is the main reason for its genome expansion besides tandem duplications of single copy genes (Wu *et al.* 2012). Comparative genomic analysis showed that *A. thaliana* and *E. salsugineum* share 95.2% and 93.7% of all their gene families, respectively (Wu *et al.* 2012).

1.6 Objectives of the study

The current study has been performed in the crop plant barley and the salt tolerant plant *E. salsugineum* as well as in the genetic model plant *A. thaliana*. The studies were focused on drought- and salt- stress induced gene expression. The work is divided into three main sections.

1. Analysis of CBF/DREB gene expression in field-grown barley

To date, our knowledge on CBF/DREB transcription factors in plant drought responses is mainly derived from the model plant *A. thaliana*. Although there are reports about CBF/DREB transcription factors in response to drought stress in crops, most of the experiments were performed using a short period of a dehydration shock under controlled laboratory conditions. Therefore, the objective of this section was to analyze the phylogenetic relationships of CBF/DREB transcription factors between barley and the well-characterized rice and *A. thaliana* plants; investigate the transcriptional changes of barley CBF/DREB genes to long periods of drought stress under a simulated slow drought stress that occurs in field conditions. The work was divided into the following tasks:

(1) Isolate barley CBF/DREBs from *in silico* data and perform an alignment with CBF/DREBs from rice and *A. thaliana*.
(2) Construct a phylogenetic tree to analyze the evolutionary relationship of CBF/DREB transcription factors among barley, rice and *A. thaliana*.
(3) Expression analysis of five selected barley *CBF/DREB* genes (*HvCBF1*, *HvCBF2*, *HvCBF4*, *HvDREB1*, and *HvDRF1.3*) in eight barley varieties under both controlled laboratory conditions and simulated field drought stress conditions.

2. Comparative analysis of the *ALDH7B4* gene in *A. thaliana* and *E. salsugineum*

The second objective of this study was to study the regulation of expression of the stress inducible *ALDH7B4* gene in the salt sensitive glycophyte *A. thaliana* and in the salt tolerant

halophyte *E. salsugineum* in response to salt stress to differentiate between a stress sensitive and stress tolerant plant in two closely related species. To achieve this goal, the *ALDH7B4* promoter activity of *A. thaliana* and *E. salsugineum* was analyzed in different genetic backgrounds. To identify the regulatory *cis*-elements in the *EsALDH7B4* promoter as well as interacting transcription factors. This section has the following objectives:

(1) Using database searches, a genome-wide identification was carried out of the *ALDH* superfamily genes from *E. parvulum* and *E. salsugineum* halophytes.
(2) Studies of gene organization, copy number, phylogenetic and expression analyses of *ALDH* genes from *A. thaliana*, *E. parvulum* and *E. salsugineum*.
(3) Promoter analysis of *AtALDH7B4* and *EsALDH7B4* by using *AtALDH7B4::GUS* and *EsALDH7B4::GUS* transgenic lines.
(4) Perform *EsALDH7B4* promoter deletion assays to identify the *cis*-elements important in regulation of *EsALDH7B4* expression.
(5) Using yeast one-hybrid assays to identify transcription factors that can regulate the *EsALDH7B4* expression.

3. Molecular and functional characterization of the identified unknown transcription factor bHLH146

This section describes the characterization of the unknown transcription factor bHLH146 that was identified from the yeast one-hybrid screen (second section). The characterization of the bHLH146 included:

(1) DNA binding ability investigation using EMSA and DNase I footprinting.
(2) Subcellular localization assays.
(3) Spatial and temporal expression analysis
(4) Transcriptional activation or repression investigation.
(5) Yeast two-hybrid screening to identify interacting proteins.
(6) Generate and characterize overexpression and artificial microRNA transgenic lines.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant materials

Arabidopsis thaliana ecotype Col-0, *Eutrema salsugineum* ecotype Shandong and eight German barley spring cultivars (*Hordeum vulgare*) out of the so-called barley "core set" were utilized in this study. Seeds for wild type *A. thaliana* and two T-DNA insertion mutants SALK_060203 and SAIL_536_E01 that target to bHLH146 (*At4g30180*) were obtained from the Nottingham Arabidopsis Stock Centre (NASC), UK. Seeds for *E. salsugineum* were originally obtained from Dr. R. Bressan (Purdue University, USA). Four old barley cultivars, *Ack Bavaria*, *Apex*, *Perun* and *Sissy* were ordered from Dr. Benjamin Kilian (IPK Gatersleben) and four modern cultivars *Beatrix*, *Djamila*, *Streif* and *Ursa* were obtained from Dr. Jens Léon (INRES, University of Bonn). All transgenic plants were established in the *A. thaliana* ecotype Col-0 or in the *E. salsugineum* ecotype Shandong. *AtALDH7B4::GUS* and *EsALDH7B4::GUS* transgenic *A. thaliana* plants were established earlier (Tagnon Missihoun 2010, Magdalena Gruca 2012) and seeds were kindly provided to me.

2.1.2 Chemicals

Chemicals used in this work were from the following companies: Amersham Bioscience (Freiburg, Germany); AppliChem (Darmstadt, Germany); Becton Dicknson and Company (Sparks, USA); BIOMOL (Hamburg, Germany); Clontech (Saint-Germain-en-Laye, France); Duchefa Biochemie bv (Haarlem, Netherland); Fermentas (St. Leon-Rot, Germany); Grüssing (Filsum, Germany); Heirler-Cenovis (Radolfzell, Germany); Macherey-Nagel (Düren, Germany); Merck (Darmstadt, Germany); PEQLAB (Erlangen, Germany); Roth (Karlsruhe, Germany); Sigma-Aldrich (Steinheim, Germany); Spiess-Urania Chemicals (Hamburg, Germany); Th. Geyer (Renningen, Germany); Tokyo Chemical Industry Co., Ltd (Tokyo, Japan); VWR International S.A.S (Langenfeld, Germany).

2.1.3 Enzymes and DNA-marker

Restriction enzymes, T4 DNA ligase, alkaline phosphatase, Phusion Green High-Fidelity DNA polymerase and their corresponding buffers were from MBI Fermentas (St. Leon-Rot, Germany), Taq DNA Polymerase was from Ampliqon (Ulm, Germany), DNA Ruler 1 kb ladder # SM0311 was from MBI Fermentas, (St. Leon-Rot, Germany).

2.1.4 Kits

DNA fragments were isolated from agarose gels with the NucleoSpin® Extract II (Macherey–Nagel, Düren, Germany). PCR products were cloned using the CloneJET™ PCR Cloning Kit, (Fermentas; St. Leon-Rot, Germany). The RT–PCR was performed using the RevertAid™ H Minus First Strand cDNA Synthesis Kit, (Fermentas; Burlington, Canada). The HexaLabel™ DNA Labeling Kit from MBI Fermentas (St. Leon-Rot, Germany) was used for ^{32}P-labeling of DNA probes.

2.1.5 Membranes and Whatman papers

Protein-blots were performed using nitrocellulose membranes Protran BA-85 (0.45 µm; Whatman, Maidstone, UK). DNA- and RNA-blots were done on nylon membranes Hybond™ (Amersham Biosciences, Buckinghamshire, UK). Whatman paper filter WH10311897 (Schleicher & Schuell, Dassel, Germany) and Whatman 3MM Chr paper (GE Healthcare, Buckinghamshire, UK) were used for yeast colony colorimetric assay and blotting assays.

2.1.6 Media, buffers and solutions

All media, buffers and solutions, if not otherwise stated, were autoclaved for 20 min at 121 ºC at 1.5 bars.

2.1.6.1 Media

MS-medium (1L): 4.6 g MS-salt mixture; 20 g sucrose; 1 ml vitamin solution (see “2.1.6.2”); adjust pH to 5.8 with 1 M NaOH; 8 g bacto-agar for solid medium.

½ MS-medium (1L):	2,15 g MS-salt mixture; 20 g sucrose; 150 mg ascorbic acid; 100 mg citric acid; 2 ml vitamin solution (0.5 g/l ascorbic acid, 0.5 g/l Niacin, 2.5 g/l pyridoxine-HCl, 50 g/l myo-Inositol); adjust pH to 5.8 ; 8 g bacto-agar for solid medium.
LB-medium (1L):	Stir to suspend 20 g Lennox LB powder (10 g Tryptone, 5 g yeast extract, 5 g NaCl) or 35 g Lennox LB Agar powder (10 g Tryptone, 5 g yeast extract, 5 g NaCl, 15 g agar) for solid medium in 1 liter water.
YEB-medium (1L):	5 g sucrose; 5 g of meat extract; 5 g peptone; 1 g yeast extract; 2 mM $MgSO_4$ (0.493 g $MgSO_4$); adjust pH to 7.0; 15 g bacto-agar for solid medium.
YPD(YEPD)-medium (1L):	10 g yeast extract; 20 g peptone; 20 g glucose; 20 g bacto-agar for solid medium.
SD medium (1L):	6.7 g Yeast nitrogen base without amino acids; 850 ml H_2O; 20 g bacto-agar for solid medium; adjust pH to 5.8. Add 50 ml sterile 40% glucose and 100 ml of the appropriate sterile 10× Dropout Solution after autoclaving.

2.1.6.2 Buffers and solutions

Vitamin solution (Plant growth medium):	2 mg/ml glycine; 0.5 mg/ml Niacin (Nicotine acid); 0.5 mg/ml pyridoxine-HCl; 0.1 mg/ml thiamine-HCl. Use 1:1,000 dilution of the autoclaved solution; store at 4 °C.

10× Dropout Solution (1L):	200 mg adenine hemisulfate salt; 200 mg arginine HCl; 200 mg histidine HCl monohydrate; 300 mg isoleucine; 300 mg lysine HCl; 1,000 mg leucine; 200 mg methionine; 500 mg phenylalanine; 2,000 mg threonine; 200 mg tryptophan; 300 mg tyrosine; 200 mg uracil; 1500 mg valine. Autoclave and store at 4 ºC.
50× TAE buffer:	2 M Tris base; 100 mM EDTA; pH 8.0, adjust pH with glacial acetic acid.
10× TBE buffer:	890 mM Tris base; 890 mM boric acid; 20 mM EDTA; pH 8.0.
10× DNA loading buffer (10 ml):	25 mg Bromophenol blue; 25 mg Xylencyanol; 0.2 ml 50× TAE; 3 ml glycerol; 6.8 ml sterile distilled water.
20× SSC:	3 M NaCl; 0.3 M sodium citrate; store at room temperature.
1× TE buffer:	10 mM Tris-HCl; 1 mM EDTA; pH 8.0, store at room temperature.
RNA-, DNA-blot washing buffer:	0.1% (w/v) SDS; 2× SSC; store at room temperature.
100× Denhardt's:	2% (w/v) BSA (fraction V); 2% (w/v) Ficoll-400; 2% (w/v) PVP 360,000. Store in aliquots at -20 °C.
Z-buffer:	60 mM Na_2HPO_4; 40 mM $NaH_2PO_4.2H_2O$; 10 mM KCl; 1 mM $MgSO_4.7H_2O$; adjust pH to 7.0 with 10 N NaOH.

50 mM phosphate buffer (pH 7.0):	Mix of 21.1 ml 0.2 M NaH_2PO_4, 28.9 ml 0.2 M Na_2HPO_4 and 150 ml H_2O.
4% (w/v) X-Gal:	40 mg/ml in N, N-dimethylformamide (DMF); protect from light; store at -20 °C.
10% (w/v) X-Gluc:	100 mg/ml in N, N-dimethylformamide (DMF); prepare freshly or store at -20 °C.
GUS staining solution:	1 ml 10% X-Gluc diluted in 200 ml 50 mM phosphate buffer; 0.1% (v/v) Triton X-100; 8 mM β-mercaptoethanol freshly added.
RNase A (stock solution):	10 mg/ml RNase A in milli-Q sterile water; store in aliquots at -20 °C.
IPTG (stock solution):	100 mM IPTG in water; filter sterilize and store at -20 °C; working solution: variable concentration.
Ampicillin (stock solution):	100 mg/ml in water; filter sterilize and store at -20 °C; working solution: 1:1,000 dilution.
Kanamycin (stock solution):	50 mg/ml in water; filter sterilize and store at -20 °C; working solution: 1:1,000 dilution.
Rifampicin (stock solution):	50 mg/ml in methanol; add ~5 drops 10 N NaOH per milliliter to facilitate dissolving. Alternatively dissolve in DMSO; store at -20 °C; working solution: 1:500 dilution.

2.1.7 Vectors, microorganisms and cDNA libraries

2.1.7.1 Vectors

The plasmid vectors and microorganisms used in this work are listed below. Molecular details of the vectors are shown in the appendix. All the vectors used in this work are kept as plasmids at -20 °C or in glycerol stock as bacteria strains at -80 °C. All the microorganisms including bacteria and yeasts are stored in glycerol at -80 °C (Department of Molecular Physiology, Institute of Molecular Physiology and Biotechnology of Plants (IMBIO), University of Bonn).

2.1.7.1.1 pJET1.2/blunt

This vector is a linearized cloning vector from Thermo Scientific (St. Leon-Rot, Germany). It contains a lethal gene which is disrupted by ligation of a DNA insert into the cloning site. Therefore, only cells with recombinant plasmids are able to grow. It contains a *bla* gene that confers resistance to ampicillin. This vector was used to clone PCR products according to the descriptions of the manufacturer.

2.1.7.1.2 pBT10-GUS (Sprenger-Haussels and Weisshaar 2000)

This vector contains the coding sequence of the reporter gene β-glucuronidase (GUS/*uidA*). It was used to generate promoter-GUS fusion constructs. The vector contains a *β-lactamase* gene that confers the ampicillin resistance for selection.

2.1.7.1.3 pBIN19 (Bevan 1984; Frisch *et al.* 1995)

The binary vector pBIN19 was used to transform *Agrobacterium tumefaciens* for generating transgenic plants. pBIN19 contains the *NPTII* gene coding for the enzyme neomycin phosphotransferase that confers kanamycin resistance to *A. tumefaciens* cells and plants.

2.1.7.1.4 pGJ280

This vector contains following features in 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). Since it also contains a dual CaMV35S promoter and a translational enhancer, the vector was also used to make overexpression constructs in this study.

2.1.7.1.5 pET28a

This vector carries an N-terminal His-Tag/thrombin/T7-Tag configuration plus an optional C-terminal His-Tag sequence and a Kan^R gene that codes for kanamycin resistance. It was used for His-tagged protein overexpression (Novagen, Darmstadt, Germany).

2.1.7.1.6 pRS300

This vector is a backbone for expressing plant artificial miRNAs, it contains the miR319a precursor which was cloned via the SmaI site in pBSK and an Amp^R gene that confers ampicillin resistance. This vector was obtained from Dr. Detlef Weigel (Max Planck Institute for Developmental Biology, Tübingen, Germany).

2.1.7.1.7 R4L1pDEST_LacZi (Mitsuda *et al.* 2010)

This plasmid is a derivative of the yeast integration and reporter vector pLacZi (Clontech/TAKARA) into which an unconventional gateway cassette *attR4-ccdB/Cm^r-attL1* has been introduced for cloning (Mitsuda *et al.* 2010). The cassette was inserted into the *SmaI* site in front of the reporter gene *LacZ* under the control of the *iso-1-cytochrome c* promoter from yeast.

The lethal *ccdB* gene serves for positive selection during cloning with BP and LR clonase. A *bla* gene for ampicillin resistance and a *Col E1 ori* allows the propagation of the vector in *E. coli*. *URA3* is used as a selectable marker for the integration of the linearized vector into the yeast genome.

2.1.7.1.8 R4L1pDEST_HISi (Mitsuda *et al.* 2010)

This plasmid is a derivative of the yeast integration and reporter vector pHISi (Clontech/TAKARA) into which an unconventional gateway cassette *attR4-ccdB/Cm^r-attL1*

has been introduced for cloning (Mitsuda *et al.* 2010). The cassette was inserted into the *SmaI-SacII* site in front of the reporter gene *HIS3* under the control of a minimal promoter of *HIS3*. The lethal *ccdB* gene serves for positive selection during cloning with BP and LR clonase. A *bla* gene for ampicillin resistance and a *Col E1 ori* allows the propagation of the vector in *E. coli*. *HIS3* or *URA3* are used as selectable markers for the integration of the linearized vector into the yeast genome.

2.1.7.1.9 pDEST_GAD424 (Mitsuda *et al.* 2010)

This plasmid is a derivative of the vector pGAD424 (Clontech/TAKARA) into which a conventional gateway cassette *attR1-ccdB/Cm^r-attR2*, followed by three in-frame stop codons was inserted via the SmaI site (Mitsuda *et al.* 2010). This plasmid is the carrier of a library of 1,498 transcription factors, which was used for the yeast one-hybrid screening. cDNA of selected transcription factors without a stop codon was introduced through a Gateway system into the vector. Each TF gene was thereby fused to the activation domain of the yeast GAL4 transcription activator. It contains *pMB1 ori* and ampicillin resistance (*bla*) for propagation in *E. coli*. Gene *LEU2* serves for selection in yeast. A minimum *ADH1* promoter provides a low expression of the fusion protein and the *SV40 T-anti* gene nuclear localization sequence is used for targeting the protein into the yeast nucleus (Chien *et al.* 1991).

2.1.7.1.10 pAS2-1 (Harper *et al.* 1993)

This plasmid was used to construct the yeast two-hybrid protein-bait. It generates a fusion of the GAL4DNA-BD and a protein of interest that cloned into the MCS in the correct reading frame. pAS2-1 carries the wild-type yeast *CYHS2* gene, which confers sensitivity to cycloheximide in transformed cells. pAS2-1 is a shuttle vector that replicates autonomously in both *E.coli* and *S. cerevisiae* and carries the *bla* gene, which confers ampicillin resistance in *E.coli*. It also contains the *TRP1* nutritional gene that allows yeast auxotrophs to grow on limiting synthetic media.

2.1.7.1.11 pACT2 (Li *et al.* 1994)

This plasmid is used for establishing library for yeast two-hybrid screening. It generates a fusion of the GAL4-AD, a HA epitope tag, and a protein of interest (or protein encoded by a

cDNA in a fusion library) cloned into the MCS in the correct reading frame. pACT2, which is derived from pACT (1), contains a unique EcoR I site in the MCS. The hybrid protein is expressed at high levels in yeast host cells from the constitutive ADH1 promoter; transcription is terminated at the ADH1 transcription termination signal. The protein is targeted to the yeast nucleus by the nuclear localization sequence from the SV40 T-antigen which has been cloned into the 5' end of the GAL4 AD sequence. pACT2 is a shuttle vector that replicates autonomously in both *E. coli* and *S. cerevisiae* and carries the *bla* gene which confers ampicillin resistance in *E. coli*. pACT2 also contains the *LEU2* nutritional gene that allows yeast auxotrophs to grow on limiting synthetic media.

2.1.7.2 Microorganisms

2.1.7.2.1 *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.

2.1.7.2.2 *Escherichia coli* BL21 (Pharmacia, Freiburg)

Genotype: F^- ompT gal dcm lon $hsdS_B(r_B^- m_B^-)$ λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]).

This strain was used to express recombinant proteins.

2.1.7.2.3 *Escherichia coli* DB3.1 (Invitrogen)

Genotype: F^- gyrA462 endA1 glnV44 Δ(sr1-recA) mcrB mrr hsdS20(r_B^-, m_B^-) ara14 galK2 lacY1 proA2 rpsL20(Sm^r) xyl5 Δleu mtl1.

E. coli DB3.1 contains a specific mutation in the DNA gyrase (*gyrA462*) that makes it resistant to the lethal *ccdB* gene product. It was used as a host strain for propagation of the two plasmids R4L1pDEST_lacZi and R4L1pDEST_HISi.

2.1.7.2.4 *Agrobacterium tumefaciens* GV3101/pmP90RK (Koncz and Schell 1986)

Genotype: C58C1 pMK90RK, Rifr, Gmr, Kmr.

This strain was used for transformation of wild-type *A. thaliana* (ecotype Col-0) and wild-type *E. salsugineum* (ecotype Shandong) plants.

2.1.7.2.5 *Saccharomyces cerevisiae* YM4271 (Liu *et al.* 1993)

Genotype: MATa, ura3-52, his3-200, ade2-101, lys2-801, leu2-3, 112, trp1-901, Tyr1-501, gal4D, gal8D, ade5 :: hisG.

This yeast strain has been used for yeast one-hybrid screening. It contains mutations in different genes, such as *ura3*, *his3*, and *leu2*. Because of these mutations, the strain loses the ability to synthesize the corresponding enzymes or amino acids. Thus, uracil, leucine and histidine can be used as selection markers.

2.1.7.2.6 *Saccharomyces cerevisiae* Y190 (Durfee *et al.* 1993)

Genotype: MATa, gal4-542, gal80-538, his3-200, trp1-901, ade2-101, ura3-52, leu2-3,112, URA3:: GAL-LacZ, Lys2::GAL1-HIS3,cyh^r)

This yeast strain has been used for yeast two-hybrid screening. It contains mutations in different genes, such as *try3*, *his3*, and *leu2*. Because of these mutations, the strain loses the ability to synthesize the corresponding enzymes or amino acids. Thus, tryptophan, leucine, and histidine can be used as selection markers.

2.1.7.3 cDNA libraries

The cDNA library that was used for yeast two-hybrid analysis was kindly provided by Dr. Csaba Koncz (Max plank institute for Plant Breeding Research, Cologne, Germany). This library was an oligo (dT) primed cDNA library prepared in the plasmid pACT2 using mRNA from an *A. thaliana* cell suspension. The other cDNA library that was used for yeast one-hybrid analysis was obtained from Dr. Nobutaka Mitsuda (National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Japan). This library is composed only of 1,498 TFs from *A. thaliana* and is cloned in the plasmid pDEST_GAD424 by gateway cloning.

2.1.8 Software, programs and online tools

- Vector NTI Advance Version 11.5.1.
- ImageQuant Version 5.2 software.
- SigmaPlot Version 12.3.
- Mega for windows Version 5.0.
- Spectral Imaging EZ-C1 Goldversion 3.20.
- Microsoft Office package 2013.
- Primer3web (http://primer3.ut.ee/)
- DNA baser (http://www.dnabaser.com/).
- Phytozome (http://phytozome.jgi.doe.gov/).
- PLACE Web Signal Scan (http://www.dna.affrc.go.jp/PLACE/signalscan.html).
- TRANSFAC® Professional Suite from BIOBASE (http://www.biobase-international.com).
- Spidey in NCBI (http://www.ncbi.nlm.nih.gov/spidey/).
- fancyGENE (http://bio.ieo.eu/fancygene).
- PlantCare (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).
- Sequence alignment (http://multalin.toulouse.inra.fr/multalin/).

2.1.9 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.
- Conductivity Meter Set, Qcond 2400, Darmstadt, Germany.
- UV illuminator Intas UV systems series, CONCEPT Intas Pharmaceutical ltd., Gujarat, India.
- Imaging system Typhoon Scanner 9200 Variable Mode imager, Amersham Biosciences, Freiburg, Germany.
- 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.
- Olympus FV1000 confocal laser scanning microscope with the FV10-ASW1.7 software, Olympus, Hamburg, Germany.
- Binocular microscope SMZ-800, Nikon, Düsseldorf, Germany.
- Particle Gun Biolistic®, Bio-Rad, Hercules, USA.

2.2 Methods

2.2.1 Growth conditions and treatments

2.2.1.1 Seed culture and plant growth

If not otherwise stated, *A. thaliana* and *E. salsugineum* plants were grown under approximately 120-150 µE m^{-2} s^{-1} light with a day (22 °C) /night (18 °C) cycle of 8/16 h and 16/8 h, respectively. For flowering 4~5-week-old *A. thaliana* were moved to a growth chamber with a 16/8 h photoperiod. For MS medium culturing, seeds were surface sterilized in 70% (v/v) ethanol for 2 min then in 7% (v/v) NaClO + 0.1% (w/v) SDS for 10 min, rinsed four times in sterile distilled water and sown on MS-agar plates (Murashige and Skoog 1962). Transgenic *A. thaliana* seeds were selected on solid media containing 50 µg/ml kanamycin whereas transgenic *E. salsugineum* seeds were selected on solid media containing 25 µg/ml kanamycin. For soil-based experiments, 14-day-old seedlings were transferred into soil-pots and then subjected to various abiotic stressors. Plant materials were collected and used either immediately or frozen in liquid nitrogen and stored at -80 °C.

2.2.1.2 Stress treatment of seedlings

Two-week-old *A. thaliana* seedlings were removed from the MS-agar medium plates and put on Whatman paper, air-dried at room temperature for 30 min for dehydration stress or incubated in 200 mM NaCl solutions for 2 h for salt stress. Seedling samples were collected; they were either used immediately or frozen in liquid nitrogen and kept at -80 ºC.

2.2.1.3 Stress treatment of soil-grown plants

The performance of soil-grown plants was investigated under stress conditions. Six-week-old plants were subjected to different stresses. Salt stress was given using different concentrations of NaCl (0-600 mM) for several days. Drought stress was applied by withholding watering for 10-14 days. Dehydration stress was given by taking the whole plant out of the soil and placing it on Whatman paper and air-dried at room temperature for various time-periods. Wound stress was given by cutting leaves with scissors or by treating the leaf surface area with abrasive sandpaper; well watered plants served as control. Leaf samples were collected, frozen in liquid nitrogen and stored at -80 °C for further analyses.

2.2.1.4 Drought stress under field conditions

Eight genotypes of spring barley, *Ack Bavaria*, *Apex*, *Perun*, *Sissy*, *Beatrix*, *Djamila*, *Streif* and *Ursa* were used in this study. The plants were grown in 22 × 22 cm plastic pots containing 11.5 L of Terrasoil® (a mixture of top soil, silica sand, milled lava and peat dust, Terrasoil®, Cordel & Sohn, Salm, Germany) in a plastic tunnel which enables natural growth behavior under water controlled conditions. The pots were arranged in a spilt plot design with four replications. Seeds were sown on April 4th, 2011 and April 2nd, 2012. A computer mediated drip irrigation facility was used for water supplication three times per day (6:15 am, 12:15 pm and 6:15 pm) to keep the volumetric water content (VWC) at 40% in each pot. The drought stress treatment started 30 days after sowing (DAS). To simulate the slow drought stress occurred in the field, water content in the pots was decreased to the permanent wilting point (15% VWC) within 21 days and let it stay at 15% for another seven days. Subsequently the pots under drought condition were re-watered after 28 days of the treatment to gain approximately 40% VWC within a few hours. During the whole process, the well-watered pots that were continuously kept at 40% VWC served as controls.

2.2.1.5 High temperature stress under laboratory conditions

Four genotypes of spring barley *Ack.Baravia, Beatrix, Djiamila* and *Streif* were used for high temperature stress experiments. Seeds were germinated and plants were grown in plastic pots containing potting soil under 120-150 µE $m^{-2}s^{-1}$ light at 22 °C with a day/night cycle of 16/8 h.

10-day-old plants were transferred into the other chamber under 120-150 µE $m^{-2}s^{-1}$ light at 28 °C with a day/night cycle of 16/8 h for the high temperature stress for two weeks while plants kept in the first chamber served as controls. Leaf tissues were collected one and two weeks after starting the stress treatment. Samples were frozen in liquid nitrogen and stored at -80 °C.

2.2.1.6 Growth of microorganisms

E. coli strains were cultured at 37 °C either on solid LB-agar or in liquid LB medium at 200-250 rpm. *A. tumefaciens* strains were grown at 28 °C on solid YEB-agar medium or in liquid YEB medium at 250 rpm. The cultures were supplemented with appropriate antibiotics for selection if required. *S. cerevisiae* strains were grown at 30 °C on solid YEPD-agar/SD-agar or in liquid YEPD-medium/SD-medium at 250 rpm, amino acid drop out SD media were used as selection markers, if required.

2.2.2 Extraction of nucleic acids

2.2.2.1 Extraction of genomic DNA from plants (CTAB) (Rogers and Bendich 1985)

This method was used where high quality DNA was required. 50-200 mg powdered plant tissue material was homogenized in 500 µl 2× CTAB-buffer (pre-warmed at 65 °C) and incubated at 65 °C in a water bath for 5-30 min. After incubation, 400 µl chloroform/isoamyl alcohol (24/1) was added, mixed thoroughly by vortexing and centrifuged for 5 min (14,000 rpm, RT). The upper aqueous phase was transferred to a fresh Eppendorf tube and mixed with 0.1 volume of pre-warmed (at 65 °C) 10× CTAB-buffer and 400 µl chloroform/isoamyl alcohol (24/1), then centrifuged as above. The upper aqueous phase was transferred to another fresh Eppendorf tube and one volume CTAB-precipitation buffer was added, mixed and centrifuged for 10 min (14,000 rpm, RT) to precipitate the DNA. The precipitated DNA pellet was air-dried and resuspended in 300 µl high salt TE buffer and then 600 µl of 100% ethanol was added, mixed thoroughly by inverting the tubes several times and DNA was reprecipitated by incubating the tube at -20 °C for at least 15 min. The DNA pellet was collected by centrifugation for 15 min (14,000 rpm, 4 °C) and washed with 70% ethanol, air-dried and dissolved in TE buffer plus 20 µg/ml RNase A.

2× CTAB-buffer: 100 mM Tris-HCl, pH 8.0; 2% (w/v) CTAB; 20 mM EDTA, pH 8.0; 1.4 M NaCl; 1% (w/v) polyvinylpyrrolidone (PVP-40,000).

10× CTAB-buffer: 700 mM NaCl; 10% (w/v) CTAB.

Precipitation buffer: 50 mM Tris, pH 8.0; 10 mM EDTA, pH 8.0; 1% (w/v) CTAB.

High salt TE buffer: 10 mM Tris, pH 8.0; 1 mM EDTA, pH 8.0; 1 M NaCl.

2.2.2.2 Fast genomic DNA extraction (Urea method)

This method was used for rapid DNA extraction from large numbers of putative transgenic plants; the DNA was used for PCR to confirm the presence of the transgene. One or two leaves from 4-week old plants were frozen in a 2 ml Eppendorf tube under liquid nitrogen and ground with metal beads. The ground material was homogenized in 300 µl 2× lysis buffer and 300 µl 2 M urea. Then one volume (600 µl) of phenol/chloroform/isoamyl alcohol (25/24/1) was added and mixed thoroughly. The suspension was centrifuged for 10 min (14,000 rpm, RT) and the supernatant was transferred to a fresh tube. 0.7 volume of ice-cold isopropanol was added to the supernatant, mixed well and allowed to stand for 5-10 min on ice. DNA was precipitated by centrifuging for 15-20 min (14,000 rpm, 4 °C). The DNA pellet was washed with 1 ml ethanol, air-dried and dissolved in TE buffer plus 20 µg/ml RNase A.

2× lysis buffer: 0.6 M NaCl; 0.1 M Tris-HCl, pH 8.0; 40 mM EDTA, pH 8.0; 4% (w/v) Sacrosyl; 1% (w/v) SDS.

2.2.2.3 Purification and precipitation of DNA

To purify a DNA sample from protein residues and other contaminants, the sample was brought to 100-200 µl with sterile distilled water. Add one volume of phenol, vortex at slow speed for 10 sec and spin briefly at room temperature. The supernatant was transferred into a fresh 1.5 ml tube; add equal volume of chloroform, vertex and spin as above. After transferring the supernatant into another fresh 1.5 ml tube, 2 volumes of ice-cold 95% ethanol, 0.1 volume 3 M sodium acetate (pH 4.5) and 20-40 µg glycogen were added and vortex as above. The mixture was centrifuged for 20 min (14,000 rpm, 4 °C) and the pellet was washed with 70% (v/v) ethanol, air-dried and dissolved in 20 µl sterile TE buffer.

2.2.2.4 Total plant RNA extraction (Valenzuela-Avendaño *et al.* 2005)

Total plant RNAs were extracted according to Valenzuela-Avendaño *et al.* (2005) with minor modifications. Plant tissue was ground to a fine powder with liquid nitrogen using an RNase-free mortar and pestle. 50-100 mg powder was homogenized in 1.5 ml of extraction buffer (freshly prepared from stock solutions) and incubated for 10 min at room temperature. After incubation, the mixture was centrifuged for 10 min (10,000 *g*, RT) and the supernatant was transferred to a fresh tube. 300 µl of chloroform/isoamyl alcohol (24/1) was added to the supernatant and mixed thoroughly by vortexing for 10 sec. The suspension was centrifuged for 10 min (10,000 *g*, 4 °C) and the clear upper aqueous phase was transferred into another fresh tube. 375 µl of isopropanol and 375 µl of 0.8 M sodium citrate/1 M sodium chloride were added, mixed thoroughly and the sample was allowed to stand at room temperature for 10 min. Then the sample was centrifuged for 10 min (12,000 *g*, 4 °C) and the pellet was washed with of pre-cooled (-20 °C) 70% ethanol, air-dried and dissolved in distilled milli-Q H_2O.

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.

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

Plasmid DNA was extracted from *E. coli* according to Birnboim 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 sec. After eliminating the supernatant, cells were resuspended in 100 µl GTE solution and let it stood for 5 min at room temperature. Then 200 µl NaOH/SDS solution was added, mixed by tapping with fingers and incubated on ice for 5 min followed by adding 150 µl potassium acetate solution. Mixed thoroughly by vortexing at maximum speed and incubated on ice for another 5 min. The mixture was centrifuged for 3 min (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 sec. 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.

The mixture was allowed to stand for 2 min at room temperature, then centrifuged for 10 min (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 resuspended 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).

2.2.2.6 Plasmid DNA mini-prep from yeast

Yeast cells were collected into a 1.5 ml Eppendorf tube either from liquid culture or scraped from colonies grown on solid medium and resuspended in 200 µl lysis buffer. Then 200 µl phenol/chloroform (25/24) was added together with 0.3 g of acid-washed glass beads (425-600 µm). The suspension was vortexed vigorously for 5-10 min to break the cell wall and centrifuged for 5 min (14,000 rpm, RT). The top aqueous phase was transferred to a fresh tube and mixed with 2.5 volume of 100% ethanol and 1/10 volume of 3 M NaAc (pH 5.2). Plasmid DNA was precipitated by centrifuging for 10 min (14,000 rpm, RT) and washed with 70% ethanol. The plasmid DNA was then resuspended in 20 µl TE buffer and 2 µl was used for transforming *E. coli*.

Lysis buffer:	2% (v/v) Triton X-100; 1% (w/v) SDS; 100 mM NaCl; 10 mM Tris-HCl pH 8.0; 1 mM EDTA.

2.2.2.7 DNA fragments extraction from agarose gels

DNA fragments of PCR products or from enzymatic digestions of plasmid DNA constructs were isolated from agarose gels using the NucleoSpin® Extract II Kit. The extraction and purification were done after excising the bands from the agarose gel following the instructions of the kit manufacturer.

2.2.3 Qualitative and quantitative estimation of nuclei acids and proteins

2.2.3.1 Qualitative and quantitative estimation of DNA and RNA

The DNA and RNA samples were qualitatively monitored by electrophoresis on a 1% (w/v) agarose gel using the 1 kb ladder as a reference. The concentration of the nucleic acids was measured with a spectrophotometer at ODs 260 and 280 nm. A value of OD_{260}=1 approximately corresponds to 50 µg/ml for a dsDNA or 40 µg/ml for RNA. The reading at 280 nm determines the amount of protein in a sample. Pure preparations of DNA and RNA have an OD_{260}/OD_{280} ratio of 1.8 and 2.0, respectively. If there is contamination with proteins or phenol, the OD_{260}/OD_{280} ratio will be less. Strong absorbance around 230 nm can indicate that organic compounds or chaotropic salts are present in the purified nucleic acids. Generally, the lower the ratio of OD_{260}/OD_{230} the higher the amount of salt that is present. As a guideline, the OD_{260}/OD_{230} ratio should be greater than 1.5.

2.2.3.2 Quantification of protein concentrations of protein extracts

A Bio-Rad protein assay Kit was used for determining protein concentrations (Bradford 1976). Protein samples (5-10 µl) were mixed with 200 µl Bradford reagent from the Bio-Rad protein assay kit and brought to 1,000 µl with sterile H_2O. For samples where the Laemmli buffer (Laemmli 1970) was used to extract proteins from plant tissues, the SDS salt was precipitated using potassium phosphate buffer. Five microliter of the protein sample was diluted in 100 µl of 100 mM potassium phosphate buffer. The mixture was incubated at room temperature for 10 minutes, then centrifuged at room temperature with a high speed for 5 min. 100 µl supernatant was carefully transferred into a fresh tube and mixed with 200 µl Bradford reagent (Bio-Rad) and 700 µl sterile distilled H_2O. After 5-10 min incubation at room temperature, the OD of the mixtures was measured at 595 nm. The amount of protein was calculated from a standard curve established from defined concentrations of bovine serum albumin (BSA).

2.2.4 Primer design and annealing of oligonucleotides

2.2.4.1 Primers

All primers were designed using the online tool "Primer3web" (http://primer3.ut.ee/) and the quality was rechecked using online tool "OligoEvaluator" (http://www.oligoevaluator.com/). After quality evaluation, the primers were synthesized by Sigma-Genosys (Steinheim, Germany) or Eurofins MWG Operon (Ebersberg, Germany) and dissolved in TE buffer to a final concentration of 100 µM. The primer sequences are listed in **Table 3** and are oriented from 5'end to 3'end. The restriction enzyme sites are shaded in grey.

Table 3 List of primers

Name	Sequence	Restriction site
Expression analysis of barley *DREB* genes		
HvActin_For	CCCAGCATTGTAGGAAGGC	
HvActin_Rev	CCTCGGTGCGACACGGAGC	
HvEF1α_For	CGTTGCTGTGAAGGATCTGA	
HvEF1α_Rev	GCAAAGGTCTCCACAACCAT	
HvCBF1_For	AACAGTGAGGGCTTCTCGAC	
HvCBF1_Rev	GTCCATACCATCGTCCATCC	
HvCBF2_For	AGGAGCAAGACTACATGACG	
HvCBF2_Rev	CAAGCTCGCGTAGTAGGACC	
HvCBF4_For	GACCAAGTTCCACGAGACG	
HvCBF4_Rev	GTTCTCTGGCCTCGCTCTT	
HvDRF1.3_For	AGCCTGGAAGGAAAAAGCGACCTC	
HvDRF1.3_Rev	ATCCTGCACAGGGAAGTTGG	
HvDREB1_For	TGGTCCAGATTCGGTTGCT	
HvDREB1_Rev	CATCGCCCTCTTGGTACTCT	
HvHSP17_For	ATGTCGATCGTGAGGCGTAG	
HvHSP17_Rev	GCCACTTGTCGTTCTTGTCC	
HvHSP70_For	TGCTATTGCCTACGGTTTGG	
HvHSP70_Rev	TCAAGATGCTTCCCTGCACT	
HvALDH7B4_For	TCAAATGGTTCAGCAACAGG	
HvALDH7B4_Rev	GGCATAAAGGACAGGACCAA	
Expression analysis of *ALDH* genes in *A. thaliana* and *E. salsugineum*		
AtActin_For	GGAATCCACGAGACAACCTATAAC	
AtActin_Rev	GAAACATTTTCTGTGAACGATTCCT	
TsActin2_For	GGAATCCACGAGACGACCTATAAC	
TsActin2_Rev	GAAGCATTTTCTGTGAACAATCCCT	

At/TsALDH3H1_For	CAGCTGCGAAGCATCTCACA
At/TsALDH3H1_Rev	GGCTTAGGTCGAGAACGAATCA
At/TsALDH3I1_For	GATGCAGGAAGAGATATTTGGAC
AtALDH3I1_Rev	CATGAGTCTTTAGAGAACCCAAAG
TsALDH3I1_Rev	CATGAGTCTTGAGAGAAGCCAA
At/TsALDH7B4_For	GAAGCAATAGCCAAAGACACACGC
At/TsALDH7B4_Rev	GATATCTCGATTATCGTAGGCTCC
At/TsALDH10A8_For	GTTGCTTCTGGACAAATGGTC
At/TsALDH10A8_Rev	TTAATCCAGACAATCCCAGCTT
At/TsALDH10A9_For	TGGACAAGCTGGTAAAGTGGAC
At/TsALDH10A9_Rev	ATCCCCATGGTTCATCAGAGAT
Ts2C4_fwd	CCAAAAATGGTTGATGGGAC
EsALDH2C4_fwd	GAGAGAGAGATGGAGAACGGT
EsALDH2C4_rev2	GGTTCTTTTACTTGAATTTCATC
Ts2C5_For	TCGTCGGTCTATGTTCGGATA
EsALDH2C4_rev1	TTTTTACATCCAAGGGGAATTGT
Ts2C6_For	GGAGATAGATTATGGATAAAGTTTGG
EsALDH2C4_rev3	CAAGATTAATGAACGAATAAATTAATG
Ts2C6_fwd2	CGTGTCGTAGAAGAGGTGGC
Ts2C6_rev2	TCCGAACATAGACCGACGAG
Ts2C6_fwd3	GGGCAAAGATTATCAACAAATTC
Ts3F2_For	AATCAGCCATGGAAGCTATGA
Ts3F2_Rev	GAAGTCTCTCGCTTACCATTTG
Ts3f2_rev2	TTCACGACTGAATTACGACTAT
TsALDH7B4iso_For	CCTCGGAAGTTACGTTGGTG
TsALDH7B4iso_Rev	GTCGCACAAACAAGCACAAC
Plasmid primers	
pJET1.2_For	CGACTCACTATAGGGAGAGCGGC
pJET1.2_Rev	AAGAACATCGATTTTCCATGGCAG
GUS-sense	CGTCCTGTAGAAACCCCAACC
GUS-rev	GATAGTCTGCCAGTTCAGTTCG
5'-pBT10-GUS-fw	AATACGCAAACCGCCTCT
GUS-Start	GGTTGGGGTTTCTACAGGACG
pBIN-HindIII	AGCTATGACCATGATTACGCCAAG
pBIN-EcoRI	CGATTAAGTTGGGTAACGCCAGG
pHISi_For	CGACGGCCAGTGAATTGTA
pHISi_Rev	GACAGAGCAGAAAGCCCTA
pLacZi_For	GTCTGTGCTCCTTCCTTCG
pLacZi_Rev	GTGTGTGTATTTGTGTTTGC
GAL4AD_For	CTATTCGATGATGAAGATACCCC
GAL4AD_Rev	CGTTTTAAAACCTAAGAGTCAC

pAS2_1fwd	TCATCGGAAGAGAGTAG	
pAS2_1rev	CTGAGAAAGCAACCTGAC	
pACT2_fwd	TAAAAGAAGGCAAAACGATGT	
pACT2_Gal4AD	GCGTTTGGAATCACTACAGG	
p35s-pROK2	CACTGACGTAAGGGATGACGC	
pGJ280_rev	TGTGCCCATTAACATCACCA	
T7 promoter	TAATACGACTCACTATAGGG	
T7 teminator	GCTAGTTATTGCTCAGCGG	
pBSKamiR-A	CTGCAAGGCGATTAAGTTGGGTAAC	
pBSKamiR-B	GCGGATAACAATTTCACACAGGAAACAG	
pROK-Lba3	ACCCAACTTAATCGCCTTGCAGCAC	
pDAP101-LB3 (S3)	TAGCATCTGAATTTCATAACCAATCTCGATACAC	
Primers for generating different constructs		
T.h.ALDH7B4prom1 Fwd	CACTAGAATTCCACCATGTATTACATATAC	EcoRI
T.h.ALDH7B4prom1 Rev	TCTCTTCTAGAAATTCACCCAACA	Xba I
ThA7pro-3de	ATGACTAGTGTGCTTGACCCTGCCTA	SpeI
ThA7pro-5de	ATTACTAGTTGTCGCCATTGTTGCCA	SpeI
TsA7pro2	GGTGAATTCCGTTTTATTGCACAT	EcoRI
TsA7pro3	GTGGCAACAATGGCGACGAATTC	EcoRI
TsA7pro6	CGAACTAGTCGCTGCTACTTCCT	SpeI
TsA7pro8	AAAGAATTCATAGGACACGTGGCAA	EcoRI
TsA7pro_SalI	GGATTAGGCAGGGTCGACCAC	Sal I
TsA7pro_XbaI	AGGATCTAGATTAGGCAGGGTCAA	Xba I
At4g30180rev_SalI	AAAGTCGACGTCAAGTACTATCTTGA	Sal I
At4g30180for_NcoI	TCTCCCATGGAGAGGCAAAT	Nco I
At4g30180rev_NcoI	AAACCATGGTACTATCTTGAACAATG	Nco I
At4g30180ProFwd2	GTAGAATTCAGTTTCAAAGATGTTT	EcoRI
At4g30180ProRev	CAATCTAGAAACTATTTTTGCTGC	XbaI
Primers for specific gene expression or amplifying specific fragments		
HvALDH7B4prom Fwd	CATGAGCCAGCAAGAGCGTGA	
HvALDH7B4prom Rev	TCGGCGAGGAACTGGTGCT	
Es7B4pfwd	TGGCGACAACTTCTCCTC	
Es7B4prev	ATTCACCCAACAAAAAATCAA	
At4g30180_for	TCTCATATGGAGAGGCAAATC	Nde I
At4g30180_rev	GTCAAGTACTATCTTGAACAATGG	
bHLH146_For	TGGAGAGGCAAATCATAAACAGG	
bHLH146_Rev	TCCGCCTTCTTTATCGGGTCT	
At2g18969For	AACCAACAAACAAAGTTTAGAACA	
At2g18969Rev	TCTTCGTCGCTGTCTTGGT	
Primers for making atifical microRNAs		
S-I-miR-s	gaTCTGTTTATGATTTGCCCCTCctctctttgtattcca	

S-II- miR-a	agGAGGGGCAAATCATAAACAGAtcaaagagaatcaatga	
S-III-miR*s	agGAAGGGCAAATCAAAAACAGTtcacaggtcgtgatatg	
S-IV-miR*a	gaACTGTTTTTGATTTGCCCTTCctacatatatattccta	
B-I-miR-s	gaTATGTTTCACGGTTTGTTCTCctctctttttgtattcca	
B-II-miR-a	agGAGAACAAACCGTGAAACATAtcaaagagaatcaatga	
B-III-miR*s	agGAAAACAAACCGTCAAACATTtcacaggtcgtgatatg	
B-IV-miR*a	gaAATGTTTGACGGTTTGTTTTCctacatatatattccta	
Oligo annealing primers		
TCEcoRI	**AATT**CTCCTCCCTTCCCCTCCCTTCCCCTCCCTTCCCCT	EcoRI ends
TCXbaI	**CTAG**AGGGGAAGGGAGGGGAAGGGAGGGGAAGGGAGGAG	Xbal ends
TCSalI	**TCGA**AGGGGAAGGGAGGGGAAGGGAGGGGAAGGGAGGAG	SalI ends

2.2.4.2 Annealing of oligonucleotides

Oligonucleotides were resuspended in annealing buffer to a stock concentration of 100 µM, 10 µl of the top and bottom strand oligonucleotides were diluted in 80 µl annealing buffer in a 1.5 ml tube (10 µM for each oligo after dilution). The tubes were then incubated in a heating block or water bath at 95 °C for 10 min, then heating was switched off and the tubes were allowed to cool down slowly to room temperature by leaving the tubes in the heating block or water bath (takes ~1h). If sticky ends were designed to be produced upon annealing, the annealed oligonucleotides can directly go to ligation with an approximate molar ratio of 3:1 (insert to vector).

Annealing buffer: 10 mM Tris, pH 7.5; 1 mM EDTA; 50 mM NaCl.

2.2.5 DNA fragments cloning

The process of DNA fragment cloning involves DNA fragment preparation, digestion, dephosphorylation (depends), ligation and transformation into host cells. Methods of each process used in this study are described in this section except for transformation as it is described in "2.2.6".

2.2.5.1 Polymerase chain reaction (PCR)

DNA fragments were amplified from various plasmid and genomic DNA sources. A standard PCR reaction was performed in a total volume of 20 µl as follows:

15.6 µl H_2O (sterile double distilled)
2.0 µl 10× PCR-buffer with $MgCl_2$
0.4 µl 10 mM dNTPs
0.4 µl Forward-primer (10 pmol/µl)
0.4 µl Reverse-primer (10 pmol/ µl)
0.2 µl Taq-polymerase (5 U/µl)
1.0 µl plasmid DNA (5-100 ng/µl) or genomic DNA (50-300 ng/µl) or a bacterial or yeast colony
The mixtures were homogenized and the PCR reaction was performed in a TRIO-thermo block (Biometra, Göttingen, Germany). Annealing temperature and cycle numbers were determined empirically for each PCR. A standard PCR program was as follows:

1. 95 °C 3 min for initial denaturation (for yeast colony PCR or GC high fragments, this step was extended to 6 min)
2. 95 °C 30 sec for cycling denaturation
3. 50-65 °C 30 sec for primer annealing (the annealing temperature is about 3–5 °C below the Tm of the primers used)
4. 72 °C for extension (1 min/kb), go to step 2 for 21-35 times.
5. 72 °C 5-10 min for final extension
6. 4 °C for holding the samples until they were collected.

2.2.5.2 Agarose gel electrophoresis

PCR products or DNA and RNA samples from plants or bacteria were analyzed in an agarose gel (0.8-1.5% (w/v)). DNA or RNA was loaded on the gel and separated by electrophoresis (small-size gel chamber: 65-90 mA, 20-60 min; mid-size gel chamber: 100-140 mA, 30-60 min) in 1× TAE or 1× TBE buffer using a 1 kb DNA ladder as reference when required. DNA fragments were visualized under UV light using ethidium bromide staining and results were recorded on a Typhoon Scanner 9200 (Amersham Biosciences, Freiburg, Germany).

Agarose gel: 0.8-1.5% (w/v) agarose in 1× TAE buffer.
Ethidium bromide solution: 1 mg/l ethidium bromide in 1× TAE buffer.

2.2.5.3 Restriction endonuclease digestions

DNA digestion was carried out by restriction endonucleases according to the following criteria: 1 U of restriction enzymes was used per 1 µg of DNA with the reaction buffer (10×) was 1/10 or 1/5 of the end volume. A double digestion was performed only when both restriction enzymes are active in the same buffer; otherwise the digestions were performed sequentially. In some cases partial digestion was performed according to the following procedure: using 10× restriction enzyme buffer and sterile distilled H_2O to dilute the plasmid or PCR product to a final volume of 100 µl and the tube was labelled "A" and placed on ice. Aliquots of 20 µl were removed from the tube "A" to 3 tubes labeled "B", "C", "D" and 10 µl in a tube labeled "E" while 30µl was left in the tube "A". All tubes were kept on ice. Add 1 µl of restriction enzyme only to the tube labeled "A". Mix well and transfer 10 µl from tube "A" to tube "B". Transfer 10 µl of tube "B" to tube "C", then 10 µl of tube "C" to tube "D", and finally 10 µl of tube "D" to tube "E". Mixed well every time and then all tubes were incubated at the recommended temperature for the restriction enzyme between 15 min to 1h (different restriction enzymes have different efficiency in digestion, usually incubate 30 min), all the samples were loaded on the gel immediately after incubation.

2.2.5.4 Dephosphorylation

Linear plasmid vectors with compatible sequences in two cohesive ends were dephosphorylated at their 5'end with alkaline phosphatase to avoid self-ligation. The reaction was made in a 10 µl reaction volume comprising of 1 µl of 10× SAP buffer, 1.0 µl (1 unit) alkaline phosphatase, and adequate amount of the plasmid vector DNA. The mixture was adjusted to 10 µl with sterile distilled water. The reaction was incubated for 10 min at 37 °C followed by inactivation of alkaline phosphatase at 65 °C for 15 min.

2.2.5.5 Ligation

To generate recombinant plasmid DNA constructs, the insert-DNA was ligated to a linearized vector in a ligation reaction. The ligation was performed in a volume of 10 µl containing 1× ligase buffer, x µl digested, purified plasmid DNA vector, 1 unit T4 DNA ligase, and y µl insert-DNA. The mixture was adjusted to 10 µl with sterile H_2O and incubated at 22 °C for 10 min

to 1 h. For an optimal ligation reaction, the molar ratio of the insert-DNA and plasmid vector should be 3:1-5:1.

2.2.6 Transformation methods

2.2.6.1 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 min (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 min 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.

2.2.6.2 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 sec. Cells were diluted with 800 µl LB medium and incubated under agitation (200 rpm) at 37 °C for 1 h. Aliquots of 200 µl of the cell suspension were then spread on selective agar-plates and incubated at 37 ºC overnight.

2.2.6.3 Preparation of electrocompetent *A. tumefaciens*

Several *A. tumefaciens* colonies were inoculated in 3 ml liquid YEB medium supplemented with rifampicin and pre-cultured at 28 °C under agitation (250 rpm) overnight. Cells from

overnight culture were pelleted and diluted into 50 ml fresh YEB medium with rifampicin and further cultured until OD_{600} to 0.5. The cell culture was incubated on ice for 30 min and centrifuged for 5 min (5,000 rpm, 4 °C). The supernatant was discarded and the pellet was resuspended in 25 ml cold sterile H_2O and centrifuged as above. The cells were then washed with the following solutions in the described order with centrifugation for 10 minutes (5,000 rpm, 4 °C) between each washing step.

1. 25 ml 1 mM Hepes pH 7.5
2. 12.5 ml 1 mM Hepes pH 7.5
3. 10 ml 10% (v/v) glycerol, 1 mM Hepes pH 7.5
4. 5 ml 10% (v/v) glycerol, 1 mM Hepes pH 7.5
5. 2 ml 10% (v/v) glycerol
6. 1 ml 10% (v/v) glycerol

Aliquots of 40 µl of the last cell suspension were shock-frozen in liquid nitrogen and stored at -80 °C.

2.2.6.4 Transformation of *A. tumefaciens* via electroporation (Tung and Chow 1995)

Competent *A. tumefaciens* cells were thawed on ice. One microliter plasmid DNA (approximately 10-50 ng/µl) was added to the electro-competent cells, mixed briefly and transferred to a pre-chilled 2 mm Electroporation–cuvette (Bio-Rad, Germany). The DNA was brought into the cells by electroporation after a single pulse of 3 to 5 sec with the following parameters: 25 µF Capacity, 2.5 kV power, 400 Ω resistance (GenePulser II, Bio-Rad). Cells were immediately diluted in 1 ml YEB-medium and incubated for 2-3 h at 28 ºC under agitation (250 rpm). 100-200 µl aliquots of the cell culture were spread on selective media and incubated at 28 ºC for 2-3 d.

2.2.6.5 Transformation of yeast (Gietz and Schiestl 2007)

High-efficiency yeast transformation was done according to Gietz and Schiestl (2007), the specific procedure was as follows:

1) Two to four yeast colonies were inoculated into 25 ml 2× YPAD medium in a 500 ml flask

and incubated at 30 ºC under agitation (250 rpm) until the OD_{600} reached 1.0 (1×10^7 cells/ml, it took 16-20 h).

2) Yeast cells (2.5×10^8 cells) were collected in a 50 ml tube from the culture by centrifuging for 3 min (3,000 *g*, RT).
3) The supernatant was discarded and the cells were resuspended in 50 ml of pre-warmed (30 ºC) 2× YPAD (SD-Try selective medium was used for Y2H in case plasmid loss). The cell suspension was transferred into a fresh sterile flask and incubated as in step 1 until the OD_{600} to 2.0 (2×10^7 cells/ml).
4) The cells were harvested in a 50 ml tube by centrifuging as above and washed with 25 ml sterile H_2O. For a small-scale transformation (when making a bait), the procedure of step 5 was followed and for a big scale transformation (10× single transformation (library screening)), the procedure of step 6 was followed.
5) For a small-scale transformation, the yeast cells were resuspended in 1 ml of sterile water and transferred to a sterile 1.5 ml tube. Cells were pelleted by centrifuging for 30 sec (maximum speed at RT) and resuspended in a final volume of 1 ml with sterile water. 100 µl of the cell suspension was transferred to different 1.5 ml tubes and pelleted by centrifuging for 30 sec (maximum speed at RT). 326 µl of transformation mix solution and 34 µl plasmid DNAs (single plasmid or multiple plasmids) were added to each tube containing the cell pellets. The cells were resuspended in the transformation mix solution by vortexing vigorously.
6) For a big scale transformation, the yeast cells were washed once more with 25 ml sterile water and centrifuged for 3 min (3,000 *g*, RT). After completely removing the supernatant, 3.26 ml of transformation mix and 340 µl of library preparation corresponding to 6 µg of Y1H TF library or 100 µg Y2H cDNA library were added to the cell pellet. Cells were then resuspended by vortexing vigorously.
7) Prepared yeast transformation suspension tubes were placed in a water bath at 42 ºC and incubated for 40 min, tubes were inverted to mix the suspension every 5 minutes.
8) After incubation, cells were collected by centrifuging for 30 sec (at maximum speed, RT) and cells were resuspended in 1 ml of sterile water for a small scale transformation while cells were collected by centrifuging for 2 min (1,000 *g*, RT) and resuspended gently in 10 ml of sterile water for a big scale transformation.

9) For Y2H or Y1H small scale transformations, 200 µl of resuspended cells were spread on each selective medium plate (92 × 16 mm [Ø × height]) and 10 µl of yeast suspension diluted in 90 µl sterile water was spread on a control plate. For a big scale Y2H transformation, 10 µl of yeast suspension was diluted in 90 µl sterile water and spread on SD-Trp and SD-Trp-Leu medium plates (92 × 16 mm [Ø × height]) to calculate the transformation efficiency. The remaining suspension (500 µl on each plate) was spread on SD-Trp-Leu-His + 3-AT medium plates (150 × 20 mm [Ø × height]). For a big scale Y1H transformation, 10 µl of yeast suspension was diluted in 90 µl sterile water and spread on SD-His-Ura and SD-His-Ura-Leu medium plates (92 × 16 mm [Ø × height]) to calculate the transformation efficiency. The remaining suspension (500 µl on each plate) was spread on SD-His-Ura-Leu + 3-AT medium plates (150 × 20 mm [Ø × height]). All plates were incubated at 30 ºC for 3-7 days.

Transformation mix:	2.4 ml 50% PEG; 360 µl 1 M LiAc; 500 µl SS-DNA (2 mg/ml
(10× single transformation)	boiled and cooled down on ice).

2.2.6.6 Transient expression analysis via particle gun bombardment

Microcarriers and DNA coating were prepared according to the reference 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 min. After sedimentation of the particles, the supernatant was carefully pipetted off and discarded. The gold particles were washed three times as follows: add 1 ml sterile water vortex for 1 min and wait until particles have sedimented again. Take off supernatant and discard. Repeat the washing step three times and finally dissolve 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 min 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 were used for each bombardment.

Bombardment was performed according to the instruction 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.

2.2.6.7 *A. tumefaciens*-mediated transient transformation of *Arabidopsis* seedlings: FAST assay (Li *et al.* 2009)

Ten-day-old *Arabidopsis* seedlings were transiently transformed by co-cultivation with *A. tumefaciens* cells. The day before co-cultivation, a single *A. tumefaciens* colony was inoculated into 2 ml LB medium with appropriate antibiotics (50 µg/ml kanamycin and 50 µg/ml rifampicin). After growth at 28 °C for 18–24 h, saturated culture was diluted the next day into 10 ml fresh YEB medium to OD_{600} = 0.3 and was grown until the OD_{600} reached more than 1.5. Bacteria cells were harvested through centrifugation at 6,000 *g* for 5 min and washed once with 10 ml washing solution (10 mM $MgCl_2$). After centrifuging at 6,000 *g* for another 5 min, the pellet of bacteria cells was resuspended in 1 ml washing solution. In a clean Petri dish (92 × 16 mm [Ø × height]), 30–50 10-day-old *Arabidopsis* seedlings were soaked with 20 ml co-cultivation medium containing 1/4 MS, 1% sucrose, 0.005% (v/v) Silwet L-77 and *A. tumefaciens* cells at final density of OD_{600} = 0.5. Co-cultivation was carried out in the dark by wrapping the Petri dish with aluminum foil. Plates were kept at the same temperature as seedlings were grown for 36–40 h. After the co-cultivation period, the medium was replaced with surface sterilization solution (0.05% v/v sodium hypochlorite) and incubated for 10 min,

washed three times with H_2O to remove epiphytic bacteria. Seedlings were finally incubated in 1/2 MS, 500 µg/ml carbenicillin to inactivate remaining *Agrobacterium* cells prior to applying the stressors or analysis.

2.2.6.8 *A. tumefaciens*-mediated stable transformation of *A. thaliana* or *E. salsugineum* plants (Clough and Bent 1998)

An *Agrobacterium* colony carrying the transgene was cultured at 28 ºC (250 rpm) in 250 ml YEB medium with appropriate antibiotics (50 µg/ml kanamycin and 50 µg/ml rifampicin) until OD_{600} reached 0.7-0.8. The cell suspension was added with 0.05% (v/v) of the surfactant Silwet L-77 and transferred into a 500 ml beaker. Flowering plants with young inflorescences and unopened flowers (siliques were removed before the first dipping) were carefully inverted and immersed in the infiltration medium with gentle rotation for 30 sec. Dipped plants were thereafter returned to trays and covered with plastic bags. A few small holes were made in the bags for ventilation. The plastic bags were removed one day after infiltration. To increase the transformation efficiency, one week after the dipping the plants were again treated as above. For generating transgenic *E. salsugineum*, 5-7 times of dipping was required. After dipping, the plants were grown until the first-generation seeds (T1) were harvested.

2.2.7 Screening methods

2.2.7.1 Screening for transformed bacterial clones

Bacteria colonies that can grow on fresh plates containing appropriate antibiotics were assigned with different numbers to amplify DNA inserts via PCR amplification (colony-PCR). PCR products from different bacteria colonies that showed the correct size were taken as positive clones. Alternatively, recombinant plasmid DNA from individual colonies was extracted and digested with restriction enzymes to confirm the presence of the inserted DNA. All plasmid DNA constructs were confirmed by DNA sequencing before further analysis.

2.2.7.2 Screening for transformed yeast clones

To screen for positive yeast baits, colonies that were present on selective plates (SD-His-Ura for Y1H and SD-Try for Y2H) were picked to perform yeast colony PCR using specific primers

to amplify DNA inserts. For Y1H library screening, big yeast colonies present on selective plates SD-His-Ura-Leu + 15 mM 3-AT plates were resuspended in 50 µl sterile water and then 5 µl were inoculated onto the same fresh plates and as well as onto media supplemented with higher concentrations of 3-AT (SD-His-Ura-Leu + 30 mM 3-AT). Colonies which grew well on high concentrations of 3-AT plates and rapidly generated a high amount of the blue compound as compared with the bait in β-galactosidase assays were taken as positive clones. Beta-galactosidase assays were first performed for all plates for the Y2H library screening, because one white colony in a β-galactosidase assay in an autoactivation test was selected as the bait. Colonies that became blue were resuspended in 50 µl sterile water and 5 µl were spread onto SD-Try-Leu-His + 25 mM 3-AT plates and SD-Try-Leu-His + 50 mM 3-AT plates. Colonies which also grew well on 50 mM 3-AT plates were taken as positive clones.

2.2.7.3 Screening for transgenic *A. thaliana* and *E. salsugineum* seeds

After transformation, the first generation of dried seeds (T1 seeds) were collected. The seeds were surface-sterilized and sown on MS-agar plates containing 50 µg/ml and 25 µg/ml kanamycin for *A. thaliana* and *E. salsugineum* seeds, respectively. After growing for two or three weeks, transgenic T1 seedlings resistant to kanamycin were distinguished from non-transgenic seedlings as they showed green cotyledons and true leaves. Instead, non-transgenic seedlings became yellow and died. Transgenic seedlings (T1 lines) were transferred to soil-pots and covered with a translucent plastic cover for several days. When the seedlings started to generate new leaves, the cover was removed, and the next-generation seeds (T2 seeds) were collected.

2.2.8 Preparation of bacterial and yeast glycerol stocks

A bacterial colony of interest was picked from a selection plate and grown overnight at 37 °C with shaking (200-250 rpm) in 2 ml LB medium for *E. coli* and YEB medium for *A. tumefaciens* with appropriate antibiotics. On the day after, 500 µl of the bacterial culture was thoroughly mixed with 500 µl of autoclaved 100% (v/v) glycerol solution in a sterilized tube. The suspension was immediately frozen in liquid nitrogen and stored at -80 °C.

Yeast of interest was cultured in SD medium with the appropriate selection at 30 °C with shaking at 250 rpm. 500 µl of the yeast culture was mixed with 125 µl of autoclaved 100% (v/v) glycerol in a sterilized tube and directly stored at -80 °C.

2.2.9 Reverse transcriptase (RT)-PCR analysis

For RT-PCR analysis 2-4 µg of total RNAs were treated with 10 U RNase-free DNase I in 10 µl reaction containing 1× DNase I buffer (20 mM Tris/HCl pH 8.4; 50 mM KCl and 2 mM $MgCl_2$) at 37 °C for 30 min. Then, 1 µl of 25 mM EDTA was added and the reaction was heated at 65 °C for 10 min to deactivate the DNase I. First-strand cDNA synthesis was performed using the RevertAid^{TM} H Minus First Strand cDNA Synthesis Kit, (Fermentas, Burlington, CA) according to the protocol provided with the kit.

2.2.10 Blotting methods

2.2.10.1 DNA-blot analysis

Genomic DNAs (5-25 µg) were completely digested in a 200-µl volume with appropriate restriction enzymes. The digested DNAs were precipitated using 1/10 volume of NaAc, 2.5 volume of ethanol and 40 µg glycogen. The pellets were washed with 70% ethanol, air dried, dissolved in 18 µl TE buffer and size-fractionated on a 0.8% agarose gel till the loading dye migrated to 3/4 of the gel (electrophoresis was run in 0.5× TBE buffer at 20 V overnight). The gel was then stained with ethidium bromide and was scanned using Typhoon Scanner together with a ruler. After recording the digestion results, the gel was successively soaked in 0.25 N HCl (15 min), denaturation buffer (2× 20 min) and neutralization buffer (2× 20 min) with gentle shaking. After these treatments, the gel was blotted on Hybond-N^+ nylon membrane overnight using 20× SSC solution. The membrane was incubated at 80 °C for 1 h or exposed on a UV table for 3 min for cross-linking DNA fragments to the membrane. The membrane was stored between Whatman papers or hybridized immediately. The membrane was pre-hybridized at 65 ºC for 3 h and hybridized overnight in the presence of a ^{32}P-labeled probe at 65 ºC in hybridization buffer. The membrane was subsequently washed (2-3 times 20 min) in the washing buffer (2× SSC, 0.1% (w/v) SDS) and exposed to a Storage Phosphor Imager Screen (Amersham Biosciences, Buckinghamshire, England) for 1 to 6 days. The Phosphor

Screen was scanned on a Typhoon Scanner 9200 (Amersham Biosciences, Freiburg, Germany) and the result was documented.

Denaturing buffer: 1.5 M NaCl; 0.5 M NaOH. Store at room temperature.

Neutralizing buffer: 1 M Tris; 1.5 M NaCl, adjust to pH 8.0 with concentrated HCl, store at room temperature.

Hybridization buffer: 5× SSC; 5× Denhardt's solution; 1% (w/v) SDS; 100 µg/ml single-stranded DNA. Prepare immediately before use.

2.2.10.2 RNA-blot analysis

15-30 µg total RNAs were mixed with one volume of the RNA-blot loading buffer, after heating the mixture at 70 °C for 5 minutes, the samples were immediately loaded onto the gel. (For making 100 ml of 1% gel, 1 g agarose was boiled in 62 ml water, mixed with 20 ml 10× MEN and 18 ml deionised formaldehyde (37%) after the agarose cooled down to 60 °C). The electrophoresis was run in the RNA running buffer at 100 mA until the blue marker migrated at least 8 cm. The gel was directly blotted onto the Hybond-N^+ nylon membrane overnight using 20× SSC as transfer buffer (Bartels *et al.* 1990). After blotting overnight, the membrane was shortly dried and exposed to a UV lamp for 3 min to fix the RNA on the membrane. Alternatively, the membrane was incubated at 80 °C for 30 min and stained with methylene blue to monitor the transfer efficiency and the equal loading of the RNA samples. Pre-hybridization was done by incubating the membrane in the RNA hybridization buffer at 42 ºC in a shaking water bath for 3 h and subsequently hybridized overnight to a radioactively labelled probe. The membrane was washed (2-3 times 20 min) in washing buffer (2× SSC, 0.1% (w/v) SDS) and exposed to a Storage Phosphor Imager Screen for 1 to 3 days. The Phosphor Screen was scanned on Typhoon Scanner 9200 and the result was documented.

RNA-blot loading buffer (1 ml): 50 µl 10× MEN; 175 µl 37% deionized formaldehyde; 500 µl formamide; 20 µl 10% (w/v) bromophenol blue and 255 µl 100% glycerol.

10× MEN: 200 mM 3-(N-morpholino) propanesulfonic acid (MOPS); 80 mM sodium acetate: dissolve in water and adjust the pH to 7.0.

	Then add 0.5 M EDTA (pH 8.0) to a final concentration of 10 mM. Store at room temperature, protect against light exposure.
RNA running buffer (1L):	100 ml 10× MEN; 80 ml 37% (v/v) deionized formaldehyde; 820 ml sterile distilled H_2O.
RNA hybridization buffer:	50% (v/v) formamide; 5× SSC; 10 mM PIPES pH 6.8; 0.1% (w/v) SDS; 1× Denhardt's; 50 µg/ml denatured herring sperm or ssDNA. Prepare immediately before use.

2.2.10.3 Staining of the RNA-blot membrane with methylene blue

To check the transfer efficiency and the equal loading of the RNA samples, the RNA-blot membrane was stained with a methylene blue solution (0.04% methylene blue in 0.5 M sodium acetate, pH 5.2). The RNA-blot membrane was immersed in the methylene blue solution for 5-10 minutes at room temperature with gentle shaking. The methylene blue solution was collected and the membrane was washed with distilled water until clear blue-stained RNA bands were visible on the membrane. The membrane was either immediately used for pre-hybridization or sandwiched between Whatman papers for storage.

2.2.10.4 Synthesis of α^{32}P-labelled DNA hybridization probes (Feinberg and Vogelstein 1983)

A DNA fragment from the gene of interest was amplified and purified using the NucleoSpin® Extract II Kit and was used for synthesizing the probe. The radiolabelled DNA probes were prepared following the manufacturer's recommendations of the HexaLabel™ DNA Labeling Kit (Fermentas, St. Leon-Rot, Germany) with minor modifications. 100 ng purified probe was mixed with 10 µl of 10× hexanucleotides buffer and H_2O to a final volume of 40 µl. The mixture was heated at 95 ºC for 5 min for denaturation and immediately cooled down on ice. Spun down briefly, 3 µl Mix C (dNTPs without dCTP), 2 µl α^{32}P-dCTP (10 µCi/µl) and 1 µl Klenow fragment were added and incubated at 37 ºC for 5 min. Then 4 µl dNTP mix was added and incubated for another 5 min at 37 ºC. After incubation, the reaction was stopped by adding 1 µl of 0.5 M EDTA (pH 8.0). The labelled probe was purified using NucleoSpin® Extract II Kit and denatured again by incubating at 95 ºC for 5 min, cooled on ice and added to the hybridization buffer.

2.2.10.5 Protein extraction from plant tissues (Laemmli 1970)

Total soluble plant proteins were extracted using "Laemmli" sample buffer. 50-100 mg powdered plant material was homogenized with 150-200 µl of Laemmli buffer by thorough vortexing in a 1.5 ml Eppendorf tube. The extract was boiled for 5 min in a water bath or heating block and centrifuged for 5 min (13,000 rpm, RT) to separate insoluble material. The supernatant containing crude total proteins was collected in a fresh tube and stored at -20 °C. Samples were heated at 95 °C for 5 min before loading on the gel.

Laemmli buffer (1×): 62.5 mM Tris-Cl pH 6.8; 10% (v/v) glycerol; 2% SDS (w/v); 0.1% (w/v) bromophenol blue and 0.7 M (≈ 5%) β-mercaptoehanol (add freshly just before use).

2.2.10.6 Extraction and analysis of recombinant proteins from *E. coli* cells

Recombinant proteins were extracted from *E. coli* BL21 (DE3) clones to check the induction efficiency and to see whether they were secreted or in "inclusion bodies". The recombinant proteins were induced by adding 1 mM IPTG when the bacteria OD_{600} up to ~0.6 and further cultured at 22-26 °C for 3 h in the dark. 1 ml-culture sample was collected before and every hour after IPTG added, then centrifuged for 2 min (14,000 rpm, 4 °C). The supernatants were discarded and the pellets were stored at -20 °C. The bacteria pellets were resuspended in 200 µl ice-cold PBS, 5 mM DTT and 1% Triton X-100. The suspensions were sonicated on ice (sonicator Dr. Hielscher; 4× 20 sec) for complete lysis and centrifuged for 10 min (12,000 *g*, 4 °C). 50 µl of the supernatant (soluble proteins) were diluted with one volume 2× sample buffer while the pellets were suspended in one volume 1× sample buffer. These samples were heated at 95 °C for 10 min and analyzed by SDS-PAGE or stored at -20 °C.

PBS: 8 g/l NaCl; 0.2 g/l KCl; 1.44 g/l Na_2HPO4; 0.24 g/l KH_2PO4.

2× sample buffer: 4% SDS; 20% (v/v) glycerol; 120 mM Tris, pH 6.8; 0.01% Bromphenol blue, 0.2 M DTT (added freshly before use).

2.2.10.7 Extraction and purification of recombinant proteins by His-tag affinity-chromatography

Soluble His-tagged recombinant proteins were purified by metal ion chromatography on His-tag binding columns under native conditions. The bacterial pellets from 100 ml IPTG-treated culture was resuspended in 5 ml buffer A plus 1 mg/ml lysozyme, incubated on ice for 30 min and sonicated until the cell suspension became translucent (6× 20 sec). The cell suspension was centrifuged for 30 min (12,000 *g*, 4 °C) and the supernatant was filtered through a 0.45 µm membrane. Before loading the supernatant onto the column, the column was washed with 3-bed volumes dH_2O, charged with 5-bed volumes 50 mM $NiSO_4$, 3-bed volumes of dH_2O to remove the free $NiSO_4$ and equilibrated with 3-bed volumes of buffer A. The filtered supernatant was then loaded onto the column and allowed to drain freely by gravity. The column was washed with 10-bed volumes buffer A and 8-bed volumes buffer B. The protein was eluted with the buffer C in 500 µl fractions. The purity of the protein fractions was verified using SDS-PAGE analysis and the quantity was estimated using the Bradford assay. Aliquots of the non-purified supernatant and of the flow-through were analyzed along with the protein fractions. The column was regenerated with strip buffer. All buffers and solutions used for the assay were prepared with autoclaved H_2O.

Buffer A: 50 mM NaH_2PO_4, pH 7.4; 300 mM NaCl; 5 mM imidazole; 10% (v/v) glycerol; 0.1% (v/v) Triton X-100; 1 mM β-mercaptoethanol (add freshly). Adjust to pH 8.0 with NaOH.

Buffer B: 50 mM NaH_2PO_4, pH 7.4; 300 mM NaCl; 30 mM imidazole; 10% (v/v) glycerol; 0.1% (v/v) Triton X-100; 1 mM β-mercaptoethanol (add freshly). Adjust to pH 8.0 with NaOH.

Buffer C: 50 mM NaH_2PO_4, pH 7.4; 300 mM NaCl; 250 mM imidazole; 10% (v/v) glycerol; 0.1% (v/v) Triton X-100; 1 mM β-mercaptoethanol (add freshly). Adjust to pH 8.0 with NaOH.

Strip buffer: 100 mM EDTA, pH 8.0; 500 mM NaCl; 20 mM Tris-HCl, pH 7.9.

2.2.10.8 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-PAGE was performed according to the discontinuous gel system of Laemmli (Laemmli 1970). In this gel system, proteins are concentrated in the 4% (w/v) acrylamide stacking gel before they are resolved in the 12% (w/v) acrylamide separation gel. Protein samples were boiled at 95 °C for 5 min before loading onto the gel (10 cm × 10 cm). The gels

were run with 1× SDS-protein running buffer for about 2 h at 10-20 mA in the stacking gel and 20-40 mA in the separating gel. The protein markers used contain β-galactosidase (E. coli; 116.0 kDa), bovine serum albumin (bovine plasma; 66.2 kDa), ovalbumin (chicken egg white; 45.0 kDa), lactate dehydrogenase (porcine muscle; 35.0 kDa), restriction endonuclease Bsp98I (*E. coli*; 25.0 kDa), β -lactoglobulin (bovine milk; 18.4 kDa) and lysozyme (chicken egg white; 14.4).

4% stacking gel (2 ml): 1.44 ml sterile H_2O; 0.27 ml 30% (v/v) acrylamide; 0.25 ml 1 M Tris pH 6.8; 20 µl 10% (w/v) SDS; 20 µl 10% (w/v) APS; 2 µl TEMED.

12% separating gel (6 ml): 1.92 ml sterile H_2O; 2.4 ml 30% (v/v) acrylamide; 1.56 ml 1.5 M Tris pH 8.8; 60 µl 10% (w/v) SDS; 60 µl 10% (w/v) APS; 2.4 µl TEMED.

1× protein running buffer: 25 mM Tris; 192 mM glycine; 0.1% (w/v) SDS. Do not adjust the pH.

2.2.10.9 Coomassie blue staining of SDS-PAGE

The SDS-PAGE was stained with Coomassie blue G-250 according to (Zehr *et al.* 1989). After electrophoresis, the gel was submerged in fixation solution for 1-2 hours with gentle shaking. The fixation solution was discarded and the gel was washed with water (3 times 10 min) and then incubated in the Coomassie staining solution on a shaker overnight. The gel was destained after several washes with distilled water and the result was documented by a camera or a scanner.

Fixing solution: 50% (v/v) methanol; 10% (v/v) acetic acid.

Staining stock solution: 100 g/l ammonium sulfate; 1% (v/v) phosphoric acid; 0.1% (w/v) Coomassie blue G-250.

Coomassie staining solution: 4 volumes staining stock solution + 1 volume methanol.

2.2.10.10 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 min with gentle shaking. The staining solution was removed and the membrane

was destained with H_2O. The membrane was scanned and the positions of protein markers were marked with a pencil.

2.2.10.11 Protein-blot analysis

Protein-blot analysis was performed to detect the protein of interest. Separated proteins from SDS-PAGE gels were transferred onto a nitrocellulose Protran BA-85 membrane (Whatman) using a pre-chilled transfer buffer in an electro-blotting system at 70 V for 1-2 h (Towbin *et al.* 1979). After staining with the Ponceau-red solution (see above), the membrane was blocked for 1 h at room temperature or overnight at 4 °C in the blocking solution. The blocking solution was replaced by fresh blocking solution with the protein specific antibody diluted in the range from 1:1,000 to 1:5,000 (v/v). The membrane was incubated at room temperature for 1 h and was washed with TBST as follows: 1× rinse, 1× 15 min and 3× 5 min. The membrane was then incubated for another 45 min at room temperature with 5,000-fold diluted secondary antibody (anti-rabbit IgG coupled to horseradish peroxidase) and was washed again with TBST as described above. Binding of antibodies was revealed using an ECL Plus Western blotting detection kit (Amersham, Braunschweig, Germany), the chemiluminescence signal was detected under a CCD camera (Intelligent Dark Box II, Fujifilm Corporation).

Transfer buffer:	25 mM Tris; 192 mM glycine; 20% (v/v) methanol. It is not necessary to adjust the pH.
10× TBS (pH 7.5):	200 mM Tris-HCl (24.2 g/l); 1.5 M NaCl (87.6 g/l); add H_2O to 1 L after adjusting the pH with HCl.
TBST solution:	1× TBS + 0.1% (v/v) Tween-20.
Blocking solution:	4% (w/v) non-fat dry-milk powder dissolved in TBST.

2.2.11 Electrolyte leakage test

The electrolyte leakage test was performed as a measure for plant tissue damage. Background of the ion-leakage test is that cellular membranes of dead tissues will be disrupted and ions will leak into the medium. Thus, the conductivity of the medium will increase. To perform the test, three leaves were punched out from the analyzed plants at the base of the petiole and were transferred as quickly as possible into a test tube containing 3 ml of distilled water. The leaves

were incubated at room temperature for 30 to 60 minutes. Conductivity of the solution was measured in each tube. The test tubes were thoroughly capped and then incubated at 80 °C for 30 min in a water bath to obtain the total ions. After the test tubes were cooled down to room temperature, the conductivity in each tube was re-measured. Relative electrolyte leakage was calculated by dividing the initial conductivity reading by the reading after the heat treatment.

2.2.12 *In situ* detection and quantification of GUS (β-glucuronidase) activity

2.2.12.1 *In situ* detection by GUS staining (Jefferson *et al.* 1987)

To study the expression pattern of a specific gene, the promoter of the gene was fused to the β-glucuronidase (GUS) reporter gene. The GUS enzyme hydrolyses the colorless substrate X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) to an intermediate product that undergoes a dimerization leading to an insoluble blue dye known as dichloro-dibromo-indigo (ClBr-indigo). ClBr-indigo can be easily detected in plant cells and is a very sensitive staining reaction for the detection. Therefore, the spatial and temporal expression of the gene of interest can be traced by detecting the GUS activity in different organs and at different developmental stages of transgenic plants. GUS activity can be either visualized as *in situ* staining or determined fluorometrically as a quantitative measurement. For *in situ* staining analysis, plant tissues were incubated in the GUS-staining buffer at 37 ºC overnight or for a shorter time period. The tissues were destained by removing chlorophyll in 80% (v/v) ethanol solution at 80 ºC and then kept in 10% (v/v) glycerol. Photographs of the tissues were taken by a camera or under a dissecting microscope (Nikon SMZ-800; Düsseldorf, Germany).

GUS-staining buffer: 0.5 mg/ml X-Gluc; 50 mM phosphate buffer, pH 7.0 (see "2.1.6.2"); 0.1% (v/v) Triton X-100; 8 mM β-mercaptoethanol freshly added (optional).

2.2.12.2 Quantification of GUS activity

The GUS activity was quantified by a fluorometric assay according to the method of Jefferson *et al.* (1987) with some modifications. The principle of the assay is that the fluorogenic substrate 4-methylumbelliferyl-glucuronid is cleaved by the GUS enzyme to the fluorescent product 4-methylumbelliferone (4-MU) and the fluorescence of 4-MU can be measured with

excitation at 365 nm and emission at 455 nm on a spectrofluorimeter (Bio-Rad). Proteins were extracted from 50-100 mg ground plant material with 100-200 µl extraction buffer and centrifuged for 10 min (14,000 rpm, 4 °C). The protein concentration of the crude extract was determined from 5 µl of the extract by the Bradford assay. The reaction was started when 15 µl (sample volume) plant extract was mixed with 15 µl 2 mM 4-MUG (dissolved in extraction buffer) at 37 °C and incubated until the reaction was completed. A control reaction was made with one volume extraction buffer and one volume 2 mM 4-MUG. 5 µl (volume per test) reaction mix were removed every 10 min (2×) after each reaction started and diluted in 2 ml stop buffer (0.2 M Na_2CO_3) separately. The fluorescence intensity (FI) of the samples was read in a fluorometer (Bio-Rad) using filters with excitation at 365 nm and emission at 455 nm. Each sample's FI value versus time (FI/min) was calculated by subtracting the FI value versus time (min) of the control reaction. Standard solutions of Na_2CO_3, containing 5, 10, 25, 50 and 100 nM 4-MU were used to generate a standard curve FI versus pmol 4-MU (FI/pmol 4-MU). The specific GUS activity was expressed as 4-MU pmol/min/mg protein by the formula below for each sample.

GUS activity of extract (pmol 4-MU/min/mg protein) = [(FI/min) / (FI/pmol 4-MU)] × [reaction volume (ml) / sample volume (ml)] × [1 / volume per test (ml)] × [1 / extract concentration (mg protein/ml)].

Extraction buffer: 50 mM sodium phosphate, pH 7.0 (see "2.1.6.2"); 10 mM EDTA; 0.1% (v/v) Triton X-100; 0.1% (w/v) Na-lauryl sarcosine; 10 mM β-mercaptoethanol freshly added.

2.2.13 Colony colorimetric assay for β-galactosidase activity

A Whatman filter WH10311897 that fits the petri dishes was prepared and placed onto the yeast colonies grown on a SD or YPD plate. The Whatman filter was lifted from the YPD or SD plate carefully using a forceps to make sure every yeast colony was transferred onto the Whatman filter. Place the filter yeast side up in a liquid nitrogen bath for 10 sec. The frozen filter was placed with the yeast facing upwards onto two layers of Whatman 3MM Chr paper which had been soaked completely in a reaction solution in a new petri dish. Air bubbles

between the Whatman filter and the Whatman papers were removed quickly while the Whatman filter thaws. The plate was then incubated at 37 °C. Blue coloring was regularly checked over a maximum period of a 24 h. Pictures were taken to document the amount of blue compound generated by each yeast lysate.

Reaction solution (6.11 ml): 6 ml Z-buffer (see "2.1.6.2"); 100 µl 4% (w/v) X-Gal; 11 µl β-mercaptoethanol. Prepare freshly before use.

2.2.14 Microscopic observation of the GFP activity in bombarded leaves and FM4-64 stained cells

The expression of the GFP in bombarded leaves or onion epidermis was examined 12 h after the particle bombardment. Single leaves or onion epidermis were placed in distilled water between two micro-cover glass slides of 0.13 to 0.17 mm thickness (VWR International, Darmstadt, Germany). The GFP signal was scanned under an inverted confocal laser microscope (Nikon Eclipse TE2000-U/D-Eclipse C1, Nikon; Düsseldorf, Germany) with excitation light at 488 nm and emission at 515 nm. Chloroplast auto-fluorescence was observed with excitation at 543 nm and emission at 570 nm. Images were captured with EZ-C1 version 3.20 (Nikon, Düsseldorf, Germany).

The hypocotyl cells of *A. thaliana* seedlings were visualized using confocal microscopy. Five-day-old *A. thaliana* seedlings grown on MS media under dark conditions were placed at 4 ºC for 5 minutes and stained with 2.5 µg/ml FM4-64 for 10 minutes at 4 ºC. Fluorescence microscopy was performed using an Olympus FV1000 confocal laser-scanning microscope (Olympus, Hamburg, Germany). The red fluorescent dye FM4-64 was excited by the 488 nm laser and emission was filtered between 650 and 750 nm. Images were edited using the Olympus Fluoview Vers. 2.0b software.

3. RESULTS

3.1 Study on *DREB/CBF* genes in Barley

3.1.1 Phylogenetic analysis of barley *DREB/CBF* genes

DREB transcription factors are specific to plants and occur ubiquitously. The DREB transcription factors contain a highly conserved AP2/ERF DNA-binding domain of around 58~61 amino acids (Lata *et al.* 2011; Lata and Prasad 2011). The N-terminal amino acid region is mainly composed of a nuclear localization signal (NLS) and a conserved Ser/Thr-rich region adjacent to the AP2/ERF DNA binding domain. The Ser/Thr-rich region is thought to be responsible for phosphorylation of DREB proteins (Liu *et al.* 1998). The C-terminal region is predicted to be functional in the trans-activation activity (Stockinger *et al.* 1997). The 14th valine and the 19th glutamic acid residues in the binding domain are crucial for the binding of DREBs to the DRE core sequences (Liu *et al.* 1998). Alignment of AP2 domains in the DREB subfamily proteins isolated from *Arabidopsis*, rice and barley showed that the 14th valine is completely conserved while the 19th glutamic acid is substituted by valine in some DREB1 subfamily members in the monocots rice and barley (**Fig. 4**). Besides these two residues, many amino acid residues like the 6th, 8th and 18th arginine, the 13th, 27th tryptophan, 29th glycine, 38th alanine, 42th aspartic acid, 56th asparagine (highlighted in yellow in **Fig. 4**) are conserved in the analyzed members.

To further clarify the phylogenetic relationships of barley DREBs with other species, a phylogenetic analysis was carried out based on the similarities of the AP2 domains in the DREB subfamily proteins isolated from these three species using the Neighbor-Joining method of MEGA 5.0 software (**Fig. 5**). The phylogenetic analysis showed that DREB1 and DREB2 subfamily members are separated on the tree across the three species except for barley CBF7 and DREB1. This indicates that DREB1 and DREB2 are arisen by gene duplication before speciation and differentially functionalized to regulate downstream genes that are involved in different pathways. It was also observed that AP2 domains could differentiate monocots from dicots particularly in the DREB1 subfamily.

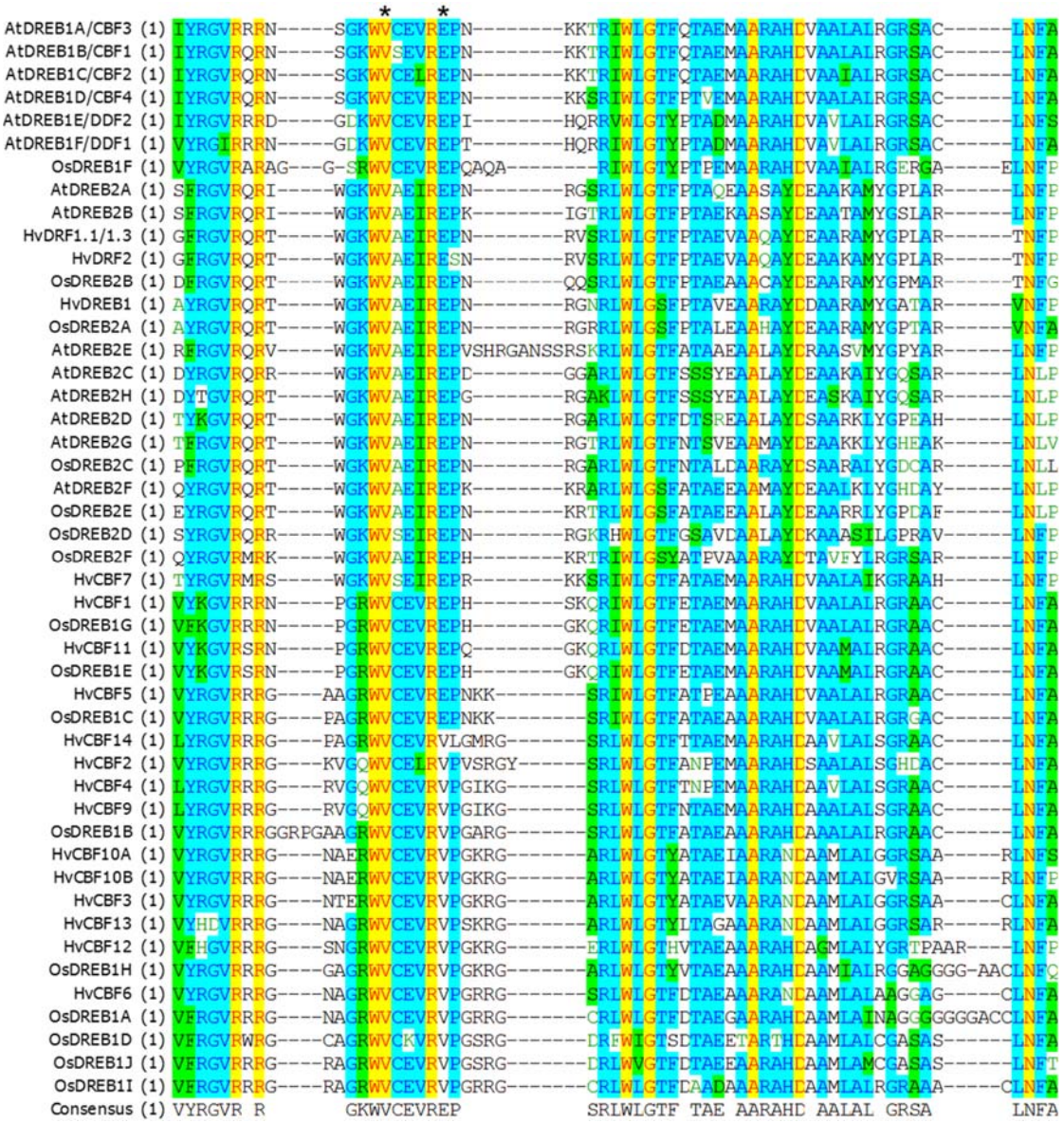


Fig. 4 Amino acid alignments of ERF/AP2 domains of DREB proteins from *A. thaliana*, barley and rice. AtDREB1A (BAA33791), AtDREB1B (BAA33792), AtDREB1C (BAA33793), AtDREB1D(Q9FJ93), AtDREB1E (Q9SGJ6), AtDREB1F (Q9LN86), AtDREB2A (BAA36705), AtDREB2B(BAA36706), AtDREB2C (Q8LFR2), AtDREB2D(Q9LQZ2), AtDREB2E (O80917), AtDREB2F (Q9SVX5), AtDREB2G (P61827), AtDREB2H (Q9SIZ0), OsDREB1A (A2Z389), OsDREB1B (Q8GUW4), OsDREB1C (A2Y8S6), OsDREB1D (Q6REU5), OsDREB1E (A2XWL6), OsDREB1F (A2WZI4), OsDREB1G (A2X899), OsDREB1H (A2Z388), OsDREB1I (Q0J3Y6),OsDREB1J (A2YXQ7), OsDREB2A (A2WL19), OsDREB2B(Q5W6R4), OsDREB2C (Q84ZA1), OsDREB2D(Q65WX1), OsDREB2E (Q10R18), OsDREB2F (BAF15993), HvDRF1.1 (AAO38209), HvDRF1.3 (AAO38211), HvDRF2 (AAO27884), HvCBF1 (AAX23686), HvCBF2 (AAM13419), HvCBF3 (AAX23694), HvCBF4 (AAX23695), HvCBF5 (AAX28952), HvCBF6 (AAX28953), HvCBF7 (AAX28954), HvCBF9 (AAX28955), HvCBF10A (AAX23713),HvCBF10B (AAX28956), HvCBF11 (AAX28957), HvCBF12 (ABA01491), HvCBF13 (ABA01492), HvCBF14 (ABA01493), HvDREB1 (AAY25517). The asterisks indicate the conserved crucial valine and glutamic acid residues in the DREB proteins. Identical, highly conservative and conservative amino acid residues are highlighted in yellow, light blue and green respectively.

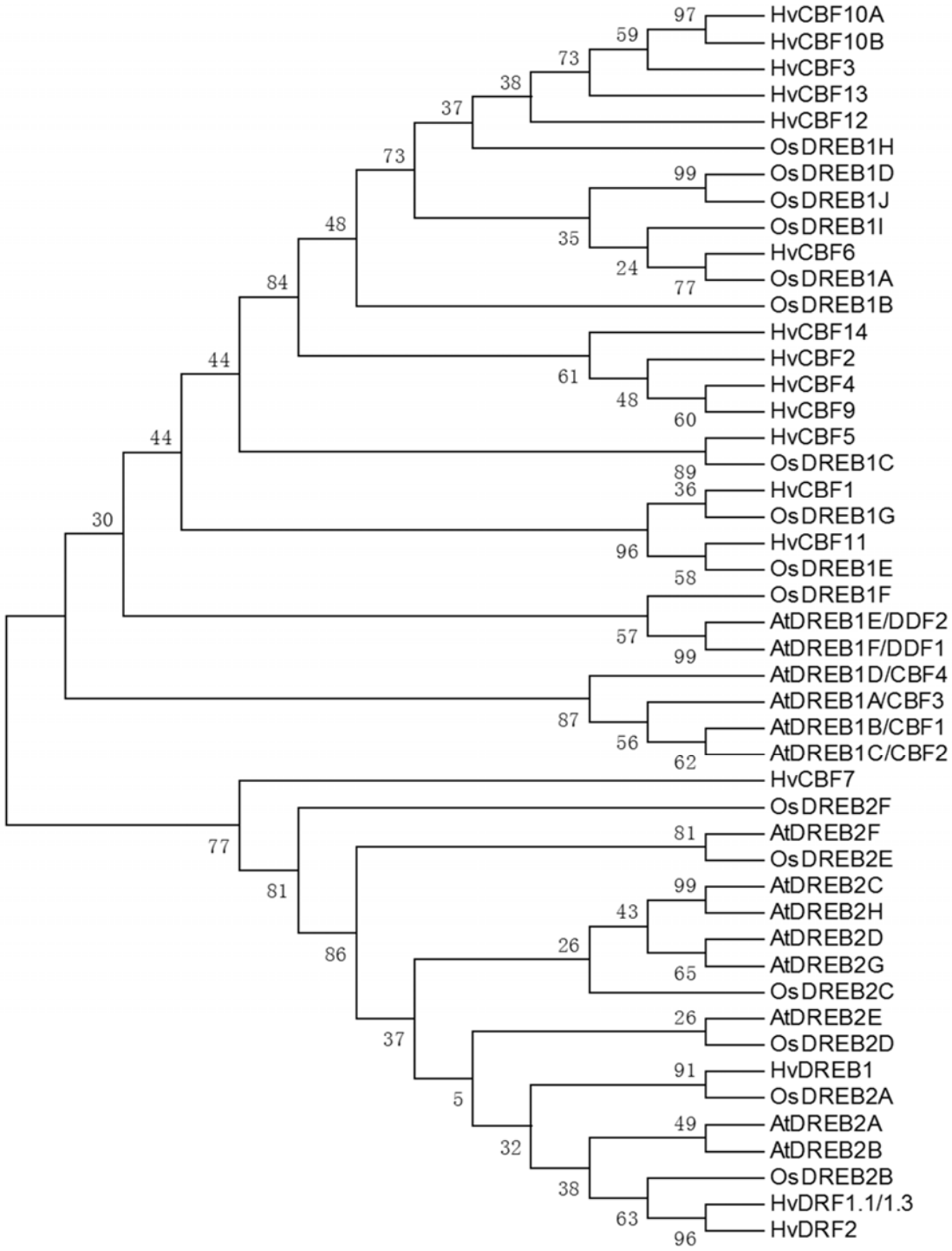


Fig. 5 Phylogenetic analysis of *A. thaliana*, barley and rice DREB proteins. ERF/AP2 domain amino acid sequences of each DREB protein from *A. thaliana*, barley and rice (same members to **Fig. 3**) were aligned using the ClustalW program of MEGA 5.0 software. The unrooted Neighbor-Joining (NJ) tree was constructed with MEGA 5.0 software based on the alignments. Bootstrap values from 500 replicates are indicated at each branch.

3.1.2 Expression analyses of selected *DREB/CBF* genes in different barley varieties

3.1.2.1 Expression profiles of five *DREB/CBF* genes under field drought stress conditions in different barley varieties

Expression of five *DREB/CBF* genes from eight spring barley genotypes was investigated using field simulated experimental condition. Growth conditions and drought stress treatment are described in "2.2.1.4" (**Fig. 6**). The objective of this study was to simulate slow development of drought stress that occurs in real field conditions. Therefore, the drought stress was given gradually using particular water supplying facilities. The gene expression was analyzed from leaf material harvested weekly during the treatments. The same experiment was repeated two times in the year 2011 and the year 2012.

Fig. 6 Barley growth conditions of the field experiment. Plants were grown in 22 × 22 cm plastic pots containing 11.5 L of Terrasoil® in a plastic greenhouse tunnel. This allows to have natural growth condition under water-controlled conditions. Water was supplied with a computer mediated drip irrigation system to control the VWC (volumetric water content) in each pot.

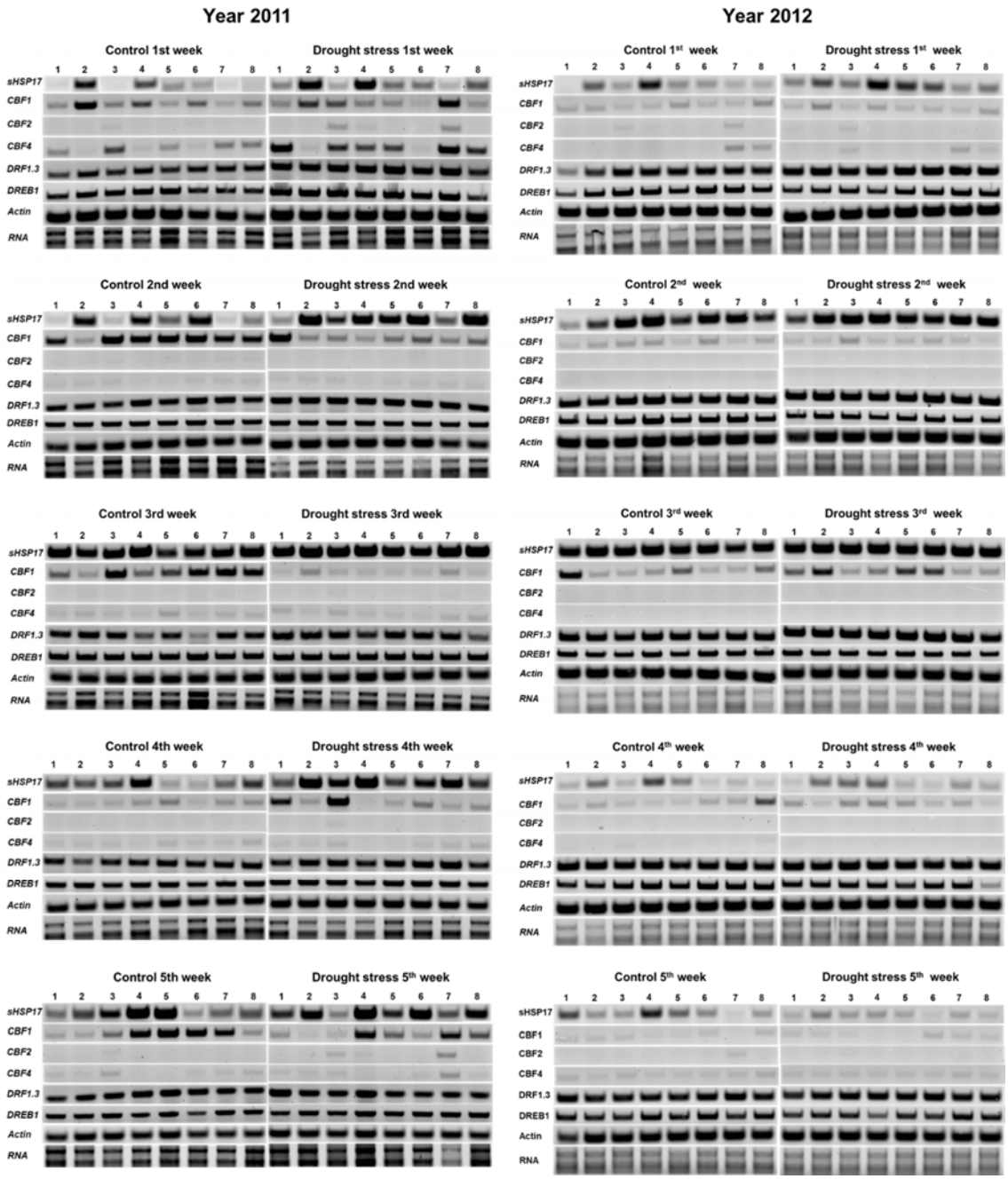


Fig. 7 Gene expression analysis of five *DREB* genes in eight different barley varieties under progressive drought stress. Expression of *CBF1, CBF2, CBF4, DRF1.3, DREB1* in leaf tissues of barley plants grown under well-watered and progressive drought stress in a plastic tunnel in the years of 2011 and 2012. The control plants were watered regularly, while water was progressively withheld for drought-treated plants until the water content in the pots declined to the permanent wilting point (15% VWC) within 21 days (the 1st week to the 3rd week). Then the stressed plants were grown at permanent wilting point for another seven days (the 4th week). After the drought stress treatments, the pots were re-watered to 40% VWC within a few hours and the plants were allowed to grow after re-watering for one week (the 5th week) before samples were harvested. Gene expression was analyzed for each week, *actin* was used as a reference gene to show the cDNA quality. 1, 2, 3, 4, 5, 6, 7, 8 represent different varieties *Ack.Baravia, Apex, Beatrix, Djiamila, Perun, Sissy, Streif* and *Ursa*, respectively.

The expression patterns of five barley *DREB/CBF* genes were analyzed during the two years (2011, 2012). The results are shown in **Fig. 7**. Expression of *HvDREB1*, *HvDRF1.3* and *HvCBF2* showed a clear pattern, *HvDREB1* and *HvDRF1.3* were constitutively expressed while nearly no transcript of *HvCBF2* was detected in all cultivars under the given control and drought conditions. In contrast, expression of *HvCBF1* and *HvCBF4* was complicated. The transcript *HvCBF4* was only detected in the first week of the year 2011, and it showed a drought stress-inducible pattern especially in the cultivars *Ack.Baravia* and *Streif*. However, accumulation of the *HvCBF4* transcript was not detected during the next weeks and even in the first week of the year 2012 when the plants had the same developmental age as the first week of the year 2011. It was assumed that other environmental factors caused the discrepancy between the two years. Expression of *HvCBF1* was more complex, as it did not show a pattern under the given treatments. To better understand the expression profile of *HvCBF1*, the dynamic expression profiles of *HvCBF1* from different varieties under the given conditions were analyzed and are shown in **Fig. 8**.

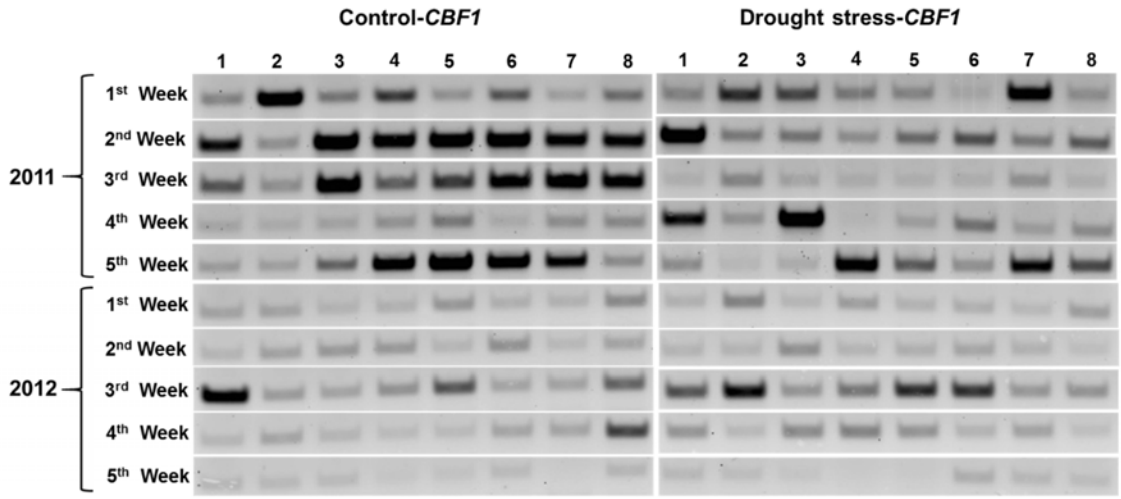


Fig. 8 Dynamic expression profiles of *CBF1* under control and drought stress conditions. Conditions and treatments were the same as in **Fig. 7**. 1, 2, 3, 4, 5, 6, 7, 8 represent different varieties *Ack. Baravia, Apex, Beatrix, Djiamila, Perun, Sissy, Streif and Ursa*, respectively.

Fig. 8 shows that expression of *CBF1* is upregulated in response to drought in some varieties like *Streif* in the first week and *Ack.Baravia* and *Beatrix* in the fourth week of the year 2011 while it is down-regulated in other varieties like in the second and the third week of the year 2011. The result was also not repeatable in the two years. In general, the transcript of *CBF1*

accumulated more in all varieties in the year 2011 than in the year 2012. This is probably due to a combination of other environmental factors. From the data of the year 2011, expression of *CBF1* is probably related to the developmental stage since its expression kept changing under well-watered conditions. In *Apex*, it is expressed in the first week in control conditions while other varieties started to express *CBF1* in the second week. Expression in all varieties was decreased during the fourth week of the experiment. However, it is expressed again in the varieties *Dajamila*, *Perun*, *Sissy* and *Streif* after rehydration in the fifth week.

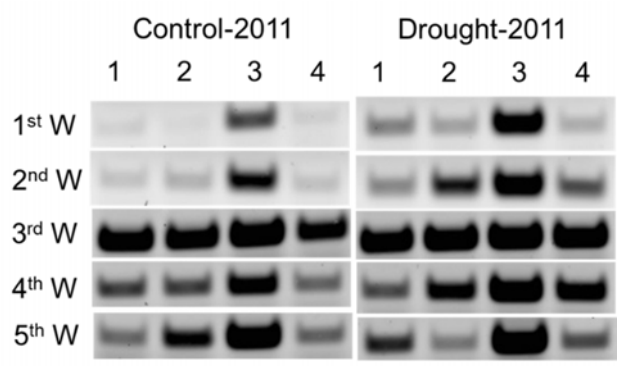


Fig. 9 Expression patterns of *sHSP17* under control and drought stress conditions. Conditions and treatments were the same as in **Fig. 7**. 1,2,3,4, represent expression patterns of different varieties *Ack.Baravia, Beatrix, Djiamila* and *Streif*, respectively.

Since environmental factors were not under control in the field conditions except for water supply, *sHSP17* that encodes a heat shock protein was taken as a marker to monitor the temperature changes. *sHSP17* was not only inducible by high temperature, but also inducible by drought stress as upregulation was clear under drought stress in the four selected varieties after the 1st week, the 2nd week and the 4th week **(Fig. 9)**. *sHSP17* was highly expressed in all varieties in the third week of 2011 and the second and the third week of 2012 (**Fig. 7** and **Fig. 9**). The average temperatures of these three weeks were indeed higher than during other weeks of the experiment according to the weather record (**Fig. 10**). The maximal temperatures during the time of the experiment were recorded in these three weeks (**Fig. 10**). Whether the temperature was a factor that can influence the expression of theses *DREB* genes was addressed, and high-temperature experiments were performed under laboratory conditions.

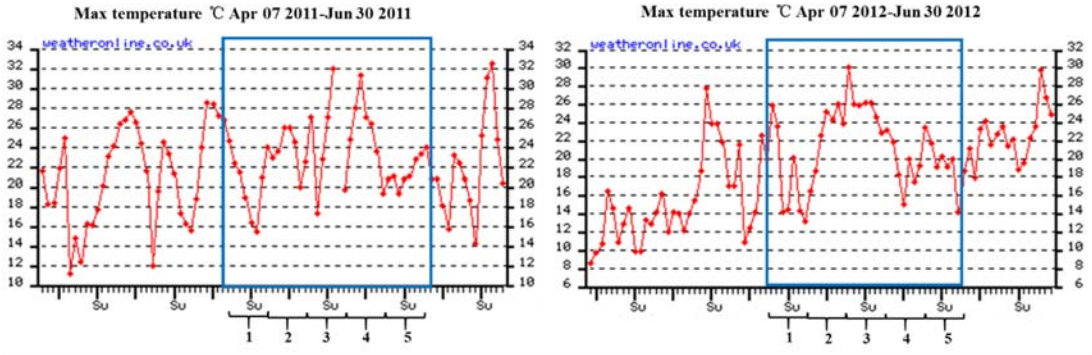


Fig. 10 Maximal temperatures during the field experiment. Maximal temperatures from April 07th to June 30th in 2011 and 2012 in Bonn, Germany were retrieved from http://www.weatheronline.co.uk/. 1,2,3,4 and 5 indicate the five sampling weeks of the experiment.

3.1.2.2 Expression profiles of selected *DREB/CBF* genes under high temperature stress conditions in different barley varieties

To test whether temperature, the most possible environmental factor that might be a co-factor of drought stress, influences the expression of *DREB* genes, four varieties *Ack.Baravia, Beatrix, Djiamila and Streif* were selected, and a high temperature experiment was performed under controlled laboratory conditions as described in "2.2.1.5". Here two genes *HvsHSP17* and *HvHSP70* which encode two heat shock proteins were used as markers to monitor high temperature stress. As expected, *HvsHSP17* was strongly upregulated by high temperature after the barley plants were grown one week under 28 °C. High temperature-induced expression of *HvHSP70* only showed a very slight upregulation after two weeks of high temperature stress (**Fig. 11**). Although transcripts of *HvDRF1.3* and *HvDREB1* did not accumulate as much as they did in the drought stress experiment, they showed constitutively expression in all varieties both under control and high temperature conditions. No significant differential expression of *HvCBF4* was detected between control and high temperature stress although an upregulation was observed in some varieties under drought stress conditions during the 1st week of the year 2011. Only varieties *Ack.Bavaria* and *Djamila* showed a slight upregulation by high temperature stress in the 2nd week. *HvCBF1* and *HvCBF2* showed a slight down-regulation in response to high temperature stress. Under control conditions, a lower amount of transcripts of *HvCBF2* was detected in the 2nd week than in the 1st week. Combining the expression profile in the drought stress experiment, it was assumed that the expression of

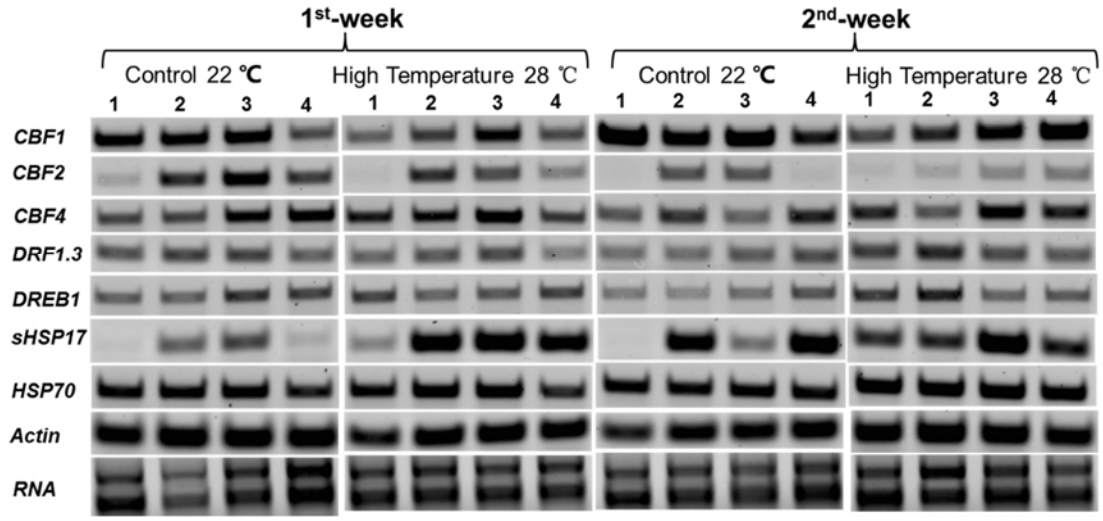


Fig. 11 Gene expression analysis of selected *DREB* genes in different barley varieties under high temperature stress. Expression of *CBF1, CBF2, CBF4, DRF1.3* and *DREB1* in leaf tissues of barley plants grown in a laboratorial chamber. Seeds were germinated and plants were grown in plastic pots containing potting soil under 120-150 μE $m^{-2}s^{-1}$ light at 22 °C with a day/night cycle of 16/8h. 10-day old plants were transferred into the other chamber with the same conditions except for the temperature was 28 °C as the high temperature stress over two weeks while plants kept in the original chamber served as control. Gene expression was detected one and two weeks after starting the stress treatment. *Actin* was used as a reference gene to monitor the cDNA quality. 1,2,3,4 represent the varieties *Ack.Baravia, Beatrix, Djiamila* and *Streif*, respectively.

this gene kept decreasing as the plant develops from a young seedling to a mature plant. Under control conditions, *HvCBF1* was highly expressed in the varieties *Ack.Baravia, Beatrix, Djiamila* while a weaker expression was shown in *Streif*. However, expression intensities of all the four varieties became stronger in the 2nd week than in the 1st week under control conditions. Interestingly, while expression of the former three varieties was repressed by high temperature both in the 1st week and the 2nd week, high temperature stress induced expression of *HvCBF1* was observed in the variety *Streif* in the 2nd week. A similar expression pattern was also recorded in the field drought stress experiment, drought stress repressed the *HvCBF1* expression in most of the 8 varieties but not in *Ack.Baravia*, *Beatrix* and *Streif* at some time points. The *HvCBF1* expression pattern is difficult to summarize since different varieties showed different expression profiles under the same conditions, even in the same genotype the expression intensity of some varieties showed dynamic changes under non-stress conditions. In conclusion, genotype, developmental stage, drought stress and high temperature stress are putative factors that influence *HvCBF1* expression.

3.2 Study on aldehyde dehydrogenase (*ALDH*) genes in *A. thaliana* and *E. salsugineum*

3.2.1 Comparative study of the aldehyde dehydrogenase (*ALDH*) gene superfamily in the glycophyte *A. thaliana* and *Eutrema* halophytes

The role of ALDH proteins in plant abiotic stress tolerance has been reported in the literature and the *ALDH* gene superfamily has been reviewed from unicellular algae to flowering plants. However, the ALDH superfamily in halophytes has not been investigated. A comparative study of the ALDH superfamily in the stress sensitive glycophytes and stress tolerant *Eutrema* halophytes should provide a better understanding of the evolution of the ALDH superfamily in plants and the role of ALDH in stress tolerance.

3.2.1.1 Database searches and annotation of *Eutrema ALDH* genes

The protein databases of *Eutrema parvulum* were searched in Thellungiella home with BLASTP (http://thellungiella.org/) and of *Eutrema salsugineum* in Phytozome with TBALSTN (http://www.phytozome.net/). The following ALDH protein sequences were used as queries for the searches: 16 *A. thaliana* ALDHs (ALDH2B4, ALDH2B7, ALDH2C4, ALDH3H1, ALDH3I1, ALDH3F1, ALDH5F1, ALDH6B2, ALDH7B4, ALDH10A8, ALDH10A9, ALDH11A3, ALDH12A1, ALDH18B1, ALDH18B2 and ALDH22A1), three *Selaginella moellindorffii* ALDHs (ALDH21A1, ALDH23B1 and ALDH23B2), one S*olanum lycopersicum* ALDH (ALDH19) and one *Chlamydomonas reinhardtii* ALDH (ALDH24A1). In addition, the same queries were used to search against the *E. salsugineum* protein database obtained from (http://omicslab.genetics.ac.cn) with BLASTP by using the prfectBLAST2.0 program (Santiago-Sotelo and Ramirez-Prado 2012). All sequences with an E-value < $1e^{-6}$ were selected for manual inspection. The Pfam domain PF00171 (ALDH family), PF00070 (ALDH cysteine active site) and PF00687 (ALDH glutamic acid active site) searches (http:// http://pfam.xfam.org/) were performed to confirm the candidate sequences as ALDH proteins. The confirmed *Eutrema* ALDH protein sequences were annotated using the criteria established by the ALDH Gene Nomenclature Committee (AGNC) (Vasiliou *et al.* 1999).

Table 4 *ALDH* genes in *A. thaliana*, *E. parvulum* and *E. salsugineum*

A. thaliana (119.67MB[a])				*E. parvulum (137.07MB[a])*				*E. salsugineum (231.89MB[a])*				
Gene name	**Accession No.**	**Locus**	**Length (aa)[b]**	**Gene name**	**Accession No.**	**Locus**	**Length (aa)[b]**	**Gene name**	**Accession No.**	**Locus**	**Phytozome ID**	**Length (aa)[b]**
AtALDH2B4	NP_190383.1	AT3G48000	538	*EpALDH2B4*	AFAN01000020.1	Tp5g13980	538	*EsALDH2B4*	XM_006404245.1	Ts5g15070	Thhalv10010271m	538
AtALDH2B7	NP_564204.1	AT1G23800	534	*EpALDH2B7*	AFAN01000001.1	Tp1g21180	540	*EsALDH2B7*	XM_006415991.1	Ts1g21300	Thhalv10007303m	541
AtALDH2C4	NP_566749.1	AT3G24503	501	*EpALDH2C4*	AFAN01000010.1	Tp3g22290	502	*EsALDH2C4*	XM_006418675.1	Ts3g24340	Thhalv10002492m	501
AtALDH3H1	NP_175081.1	AT1G44170	484	*EpALDH3H1*	AFAN01000005.1	Tp1g33460	482	*EsALDH3H1*	XM_006393677.1	Ts1g33100	Thhalv10011438m	483
AtALDH3I1	NP_567962.1	AT4G34240	550	*EpALDH3I1*	AFAN01000039.1	Tp7g32020	548	*EsALDH3I1*	XM_006412189.1	Ts7g33420	Thhalv10024844m	547
AtALDH3F1	NP_195348.2	AT4G36250	484	*EpALDH3F1*	AFAN01000040.1	Tp7g33990	484	*EsALDH3F1*	XM_006411952.1	Ts7g35460	Thhalv10025045m	485
								EsALDH3F2	XM_006409133.1	Ts3g33290	Thhalv10022980m	502
AtALDH5F1	NP_178062.1	AT1G79440	528	*EpALDH5F1*	AFAN01000027.1	Tp5g34490	527	*EsALDH5F1*	XM_006389827.1	Ts5g36850	Thhalv10018394m	527
AtALDH6B2	NP_179032.1	AT2G14170	607	*EpALDH6B2*	AFAN01000013.1	Tp3g27540	537	*EsALDH6B2*	XM_006409602.1	Ts3g28400	Thhalv10022637m	538
AtALDH7B4	NP_175812.1	AT1G54100	508	*EpALDH7B4*	AFAN01000005.1	Tp1g40550	508	*EsALDH7B4*	XM_006392654.1	Ts1g42160	Thhalv10011405m	508
								PseudoEsALDH7B4	AHIU01008199.1	Ts4g13390	scaffold_10: 4569302..4570132	
AtALDH10A8	NP_001185399.1	AT1G74920	496	*EpALDH10A8*	AFAN01000027.1	Tp5g30060	501	*EsALDH10A8*	XM_006390308.1	Ts5g32510	Thhalv10018437m	502
								PseudoEsALDH10A8	AHIU01013287.1	Ts7g03810	scaffold_14: 4330373..4330927	
AtALDH10A9	NP_190400.1	AT3G48170	503	*EpALDH10A9*	AFAN01000020.1	Tp5g13870	503	*EsALDH10A9*	XM_006404229.1	Ts5g14910	Thhalv10010305m	504
AtALDH11A3	NP_001189589.1	AT2G24270	496	*EpALDH11A3*	AFAN01000016.1	Tp4g03260	503	*EsALDH11A3*	XM_006404869.1	Ts4g04290	Thhalv10000267m	503[c]
AtALDH12A1	NP_568955.1	AT5G62530	556	*EpALDH12A1*	AFAN01000008.1	Tp2g25700	556	*EsALDH12A1*	XM_006394321.1	Ts2g30990	Thhalv10003935m	557
AtALDH18B1	NP_181510.1	AT2G39800	717	*EpALDH18B1*	AFAN01000019.1	Tp4g22080	717	*EsALDH18B1*	XM_006411149.1	Ts4g25140	Thhalv10016322m	716
AtALDH18B2	NP_191120.2	AT3G55610	726	*EpALDH18B2*	AFAN01000020.1	Tp5g06860	726	*EsALDH18B2*	XM_006403379.1	Ts5g07350	Thhalv10010150m	733
AtALDH22A1	NP_974242.1	AT3G66658	596	*EpALDH22A1*	AFAN01000009.1	Tp3g05630	596	*EsALDH22A1*	XM_006407856.1	Ts3g05680	Thhalv10020345m	596

a, genome size in mega base; b, length of deduced amino acid sequence; c, length of amino acid deduced from two data sets.

Here, 16 *ALDH* genes were identified from *E. parvulum* using the available genome sequences (**Tables 2, 4**). In the case of *E. salsugineum*, the same ecotype (ecotype: Shandong) had been sequenced twice by two different research groups Wu *et al.*, 2012 and Yang *et al.* 2013. When *ALDH* genes were identified from the two genome datasets, different results were obtained. Seventeen putative ALDH genes were identified from the dataset of Yang *et al.* (2013) and 19 putative *ALDH* genes were identified from the dataset of Wu *et al*. (2012). The datasets differ in three putative orthologous of *ALDH2C4* which are designated as *Ts3g24340*, *Ts5g11880* and *Ts5g11870* in the dataset of Wu *et al*. (2012) and only one ortholog designated as *Thhalv10002492m* was found in the dataset of Yang *et al.* (2013) in Phytozome. The discrepancy is most likely due to an assembly error. The question was experimentally addressed by amplifying the *ALDH2C4* gene from genomic DNA and cDNA of *E. salsugineum*. Only one amplicon was obtained from genomic DNA, but three different fragments were amplified from cDNA. These results demonstrate that there is one orthologous of *ALDH2C4* in the *E. salsugineum* genome, which generates three different transcripts most likely by

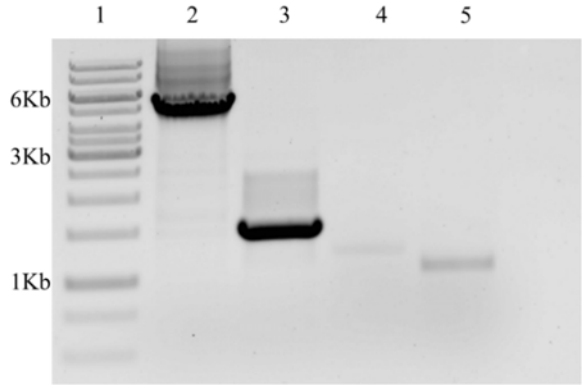


Fig. 12 Amplification of *EsALDH2C4* from genomic DNA and cDNA using different primer combinations. Lane 1: molecular-weight size marker, lane 2: *EsALDH2C4* amplified from genomic DNA using the following primer combinations EsALDH2C4_fwd/EsALDH2C4_rev1, lanes 3, 4, 5 are different EsALDH2C4 transcripts amplified from 6-week old *E. salsugineum* leaf cDNA using primer combinations EsALDH2C4_fwd/EsALDH2C4_rev1, EsALDH2C4_fwd/ EsALDH2C4_rev2 and EsALDH2C4_fwd/ EsALDH2C4_rev3, respectively.

alternative splicing. The existence of the transcripts was verified in RT-PCR reactions (**Fig. 12**). All transcripts are constitutively expressed, and the expression does not change under different salt conditions. The *EsALDH2C4.1* transcript is abundantly expressed and transcripts *EsALDH2C4.2* and *EsALDH2C4.3* are only weakly expressed (**Figs. 12, 15A**). Although

Ts3g24340 and *Thhalv10002492m* which represent the *EsALDH2C4* gene have the same DNA sequence in the two datasets, the protein sequences are not identical, because different open reading frames (ORF) were predicted from the two datasets. Our PCR analysis suggests that *Thhalv10002492m* is the correct prediction as the corresponding transcript was detected from the cDNA whereas a fragment corresponding to *Ts3g24340* could not be amplified. Similar ORF prediction discrepancies were observed for the orthologous of *ALDH11A3* and *ALDH12A1* in *E. salsugineum* that are designated as *Ts4g04290*, *Ts2g30990* and *Thhalv10000267m*, *Thhalv10003935m* in the two datasets, respectively.

In addition, two fragments were identified that resemble part of ALDH7B4 and ALDH10A8 but with stop codons designated as *Ts4g13390* and *Ts7g03810* in the genome dataset of Wu *et al*. (2012) respectively, but no annotation exists in the genome dataset of Yang *et al.* (2013). It was possible to amplify two cDNA fragments using *Ts4g13390* specific primers. DNA sequencing analysis showed that the two transcripts were derived by alternative splicing, but no correct open reading frames exist. The results do not support the prediction of Wu *et al.* (2012), and the two fragments with homologies to *ALDH7B4* and *ALDH10A8* are considered to be pseudogenes.

Using the ALDH nomenclature criteria (Vasiliou *et al.* 1999), the ALDH proteins from the two *Eutrema* species fall into ten families based on their sequence identities. In both *Eutrema* species, six families are represented by a single gene (families 5, 6, 7, 11, 12 and 18), whereas the remaining four families contain multiple members (families 2, 3, 10 and 18). To classify the *ALDH* genes, a formal name was given for each *ALDH* gene of the two *Eutrema* species following the suggested nomenclature system (**Table 4**). According to the nomenclature criteria, sequences that share 60% identity or more should be grouped into a subfamily (Vasiliou *et al.* 1999), therefore, the paralogue of *EsALDH3F1* was designated as *EsALDH3F2*.

3.2.1.2 Evolutionary relationships of *ALDH* genes between *Arabidopsis* and *Eutrema*

As organisms evolve, the genetic materials accumulate mutations over time causing phenotypic changes and speciation. Therefore, molecular phylogenetics is an informative way to reconstruct the evolutionary history. To explore the function and evolutionary process of *Eutrema ALDH* genes, an unrooted phylogenetic tree was constructed from the alignment of full-length protein sequences with *Arabidopsis*. The two pseudogenes were excluded from the phylogenetic tree construction as they do not encode complete proteins. All of the EpALDHs, EsALDHs and AtALDHs fall into ten families with well-supported bootstrap values (**Fig. 13A**). The topology is similar to those trees that were constructed with ALDH proteins from other plant species. Families 2, 10 and 5 cluster together, families 3 and 22 are connected by a node with a high bootstrap value, indicating a close evolutionary relationship among these families. Family 18 was excluded by several other studies because the genes encode bifunctional proteins which contain a glutamate 5-kinase signature (PS00902) in the N-terminal part and a gamma-glutamyl phosphate reductase signature (PS01223) in the C-terminal part instead of a common aldehyde dehydrogenases glutamic acid active site (PS00687) or cysteine active site signature (PS00070). Therefore, the family 18 has a distant phylogenetic relationship with other ALDH families. The new family 3 member EsALDH3F2 generates a new branch on the tree and is separated from 3F1 with a high bootstrap value, indicating that this new gene already diverged from its homolog *ALDH3F1*.

Exon-intron structural divergence plays a pivotal role in the evolution of multiple gene families. To obtain further insight into the evolutionary history of the *Eutrema* ALDH superfamily, a comparison was made of the full-length cDNA sequences with the corresponding genomic DNA sequences. As mentioned above, the two available genome datasets predict different open reading frames (ORF) for EsALDH11A3. The exon-intron analysis showed that neither dataset predicts an exon-intron structure similar to the ORF of AtALDH11A3 and EpALDH11A3. In the dataset of Wu *et al*. (2012) the last exon is lost and the ORF in the dataset of Yang et al. (2013) has lost the first four exons. Thus, the exon-intron structure of *EsALDH11A3* shown in **Fig. 13** is the combination of the two predicted ORFs.

Two more *E. salsugineum ALDH* genes *EsALDH2C4* and *EsALDH12A1* show discrepancies with regards to the predicted ORFs between the two genome datasets. Here we used the predictions from the dataset of Yang *et al.* (2013) as this gives an exon-intron structure

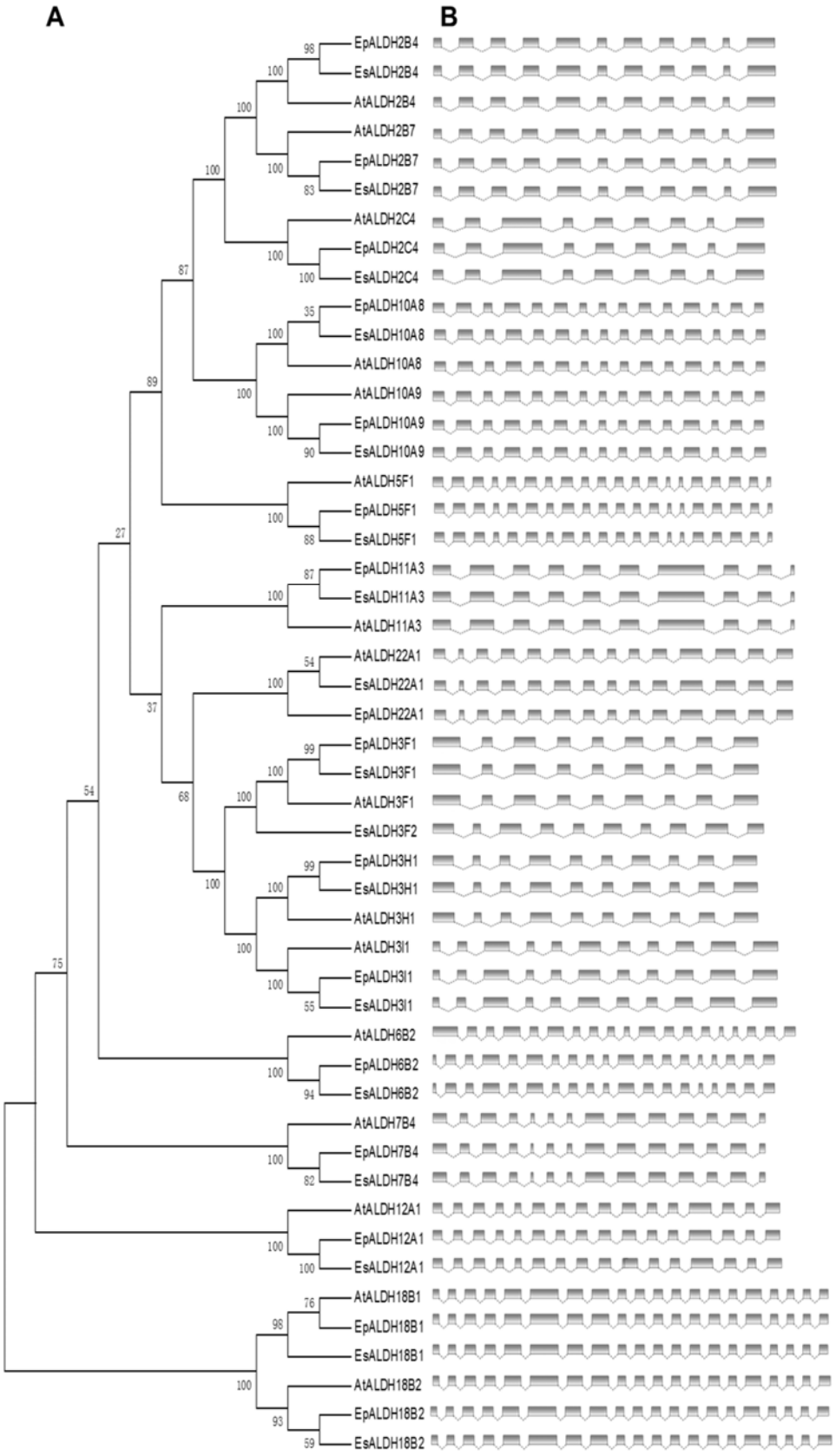


Fig. 13 Phylogenetic analysis and exon-intron structures of *A. thaliana, E. parvulum* and *E. salsugineum ALDH* genes. (**A**) Multiple alignments of ALDH protein sequences from *E. parvulum*, *E. salsugineum* and *A. thaliana* were performed using the ClustalW program of MEGA 5.0 software. The unrooted Neighbour-Joining (NJ) tree was constructed with MEGA 5.0 software based on the alignments. Bootstrap values from 500 replicates are indicated at each branch. (**B**) Exon-intron structures of *ALDH* genes from *A. thaliana*, *E. parvulum* and *E. salsugineum*. Exons are represented by grey boxes and are drawn to scale by using FancyGene (http://bio.ieo.eu/fancygene/). Line angles connecting exon boxes represent introns and are not drawn to scale.

comparable to *A. thaliana* and *E. parvulum*. *ALDH* genes from the three species have the same exon numbers with nearly identical exon lengths (**Fig. 13B**), except for the first exon of *Arabidopsis AtALDH6B2* which is longer than in the two *Eutrema* species (**Fig. 14**). Genes within the same subfamily like subfamily ALDH2B, ALDH10A and ALDH18B show more than 60% sequence identity and have the same exon-intron structure in all three species (**Fig. 13B**). The high degree of sequence identity and similar exon-intron structures of *ALDH* genes within each family suggests that gene duplications most likely occurred before *A. thaliana* and *Eutrema* were separated. Contrary to the ALDH superfamily in other analysed species (Gao and Han 2009; Zhang *et al.* 2012; Zhou *et al.* 2012), there is no intron loss in *Eutrema*, indicating a close evolutionary relationship between *Arabidopsis* and *Eutrema*. However, different intron lengths were observed in almost all families. Longer introns were found in several *E. salsugineum ALDH* genes, especially for *EsALDH2C4* (**Fig. 14**). This finding is

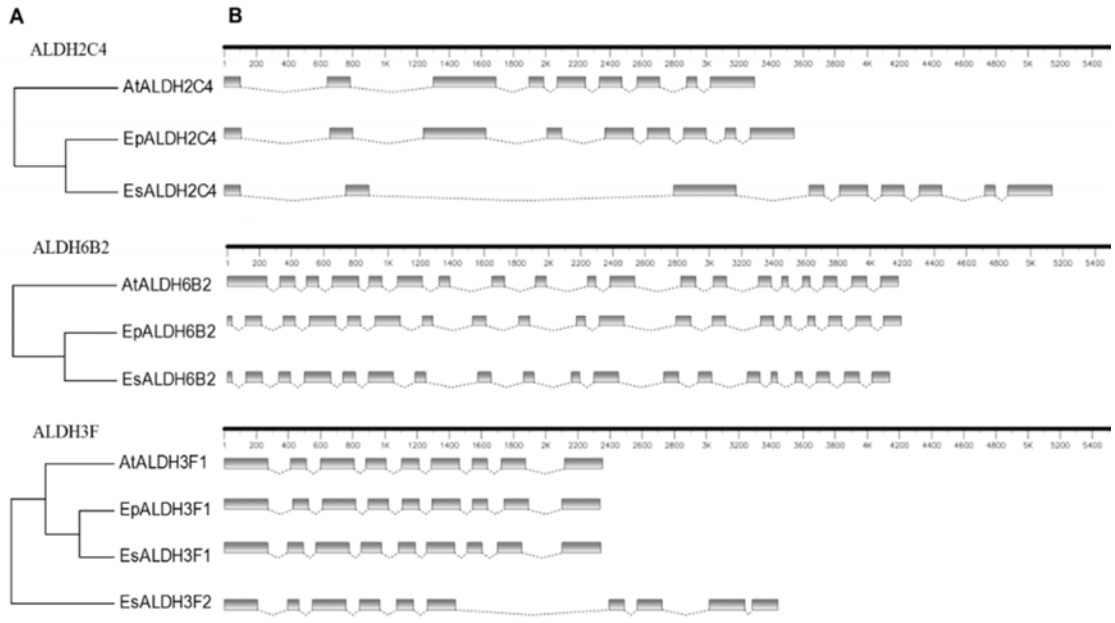


Fig. 14 Phylogenetic relationship and exon-intron structures of three selected *A. thaliana, E. parvulum* and *E. salsugineum ALDH* genes. The genes *ALDH2C4, ALDH6B2* and *ALDH3F* were selected for this analysis. (**A**) The phylogenetic relationship was constructed using MEGA 5.0. (**B**) Exons are represented by grey boxes and line angles connecting two boxes represent introns. The scale bars at the top are scaled by number of nucleotides. Exons and introns are drawn to scale by using FancyGene (http://bio.ieo.eu/fancygene/).

consistent with reports that *E. salsugineum* and *A. thaliana* genes have similar average exon lengths whereas the average intron length is about 30% larger in *E. salsugineum* than in *A.*

thaliana (Wu *et al.* 2012). The new gene *EsALDH3F2* has one more exon than its paralogue *EsALDH3F1* and the orthologue *AtALDH3F1* and *EpALDH3F1*, suggesting that *EsALDH3F2* diverged from *EsALDH3F1*, which is consistent with the phylogenetic analysis (**Fig. 13**). The divergence in gene structure is mainly due to differences in intron sequences while the ALDH protein sequences are highly conserved in these three species indicating that the enzymes kept their roles during evolution.

3.2.1.3 Expression profiles of stress-related *ALDH* genes from *A. thaliana* and *E. salsugineum* under salt stress conditions

Expression of the abiotic stress-related genes *ALDH3H1*, *ALDH3I1*, *ALDH7B4* and two betaine aldehyde dehydrogenases *ALDH10A8* and *ALDH10A9* have been investigated in *Arabidopsis* (Kirch *et al.* 2005; Missihoun *et al.* 2010). To get information on these *ALDH* genes in halophytes, the expression was examined in response to salt stress in both *A. thaliana* and *E. salsugineum* after two and five days of salt treatments (**Fig. 15A**) using RT-PCR with

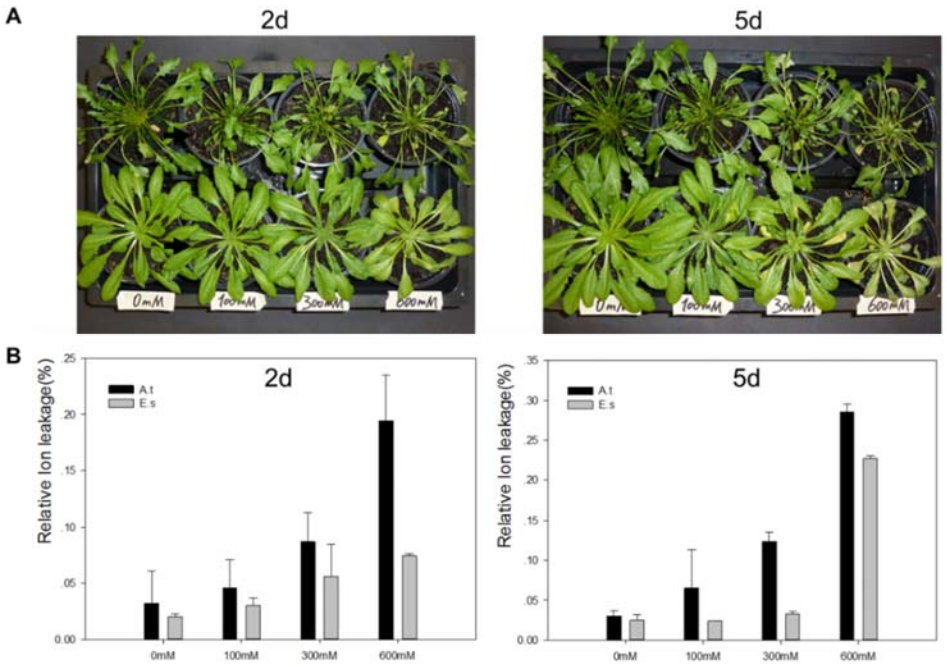


Fig. 15 Performance and ion leakage of *A. thaliana* and *E. salsugineum* under different salt stress conditions. (**A**) 6-week-old *A. thaliana* and *E. salsugineum* plants were treated with 100 mM, 300 mM or 600 mM NaCl while well watered plants serve as control. Photographs were taken 2 days and 5 days after NaCl stress application. The upper and the bottom plants were *E. salsugineum* and *A. thaliana*, respectively. (**B**) The effect of salt stress on ion leakage (% of total electrolyte leakage) of leaf samples from salt-treated *A. thaliana* and *E. salsugineum* as shown in (**A**).

a variable number of amplification cycles. Compared to *A. thaliana*, the halophyte *E. salsugineum* had a lower relative ion leakage during the whole stress process (**Fig. 16**). Since the ion leakage reflects the extent of membrane damage, the result confirms that the halophyte *E. salsugineum* was more salt tolerant than *A. thaliana*.

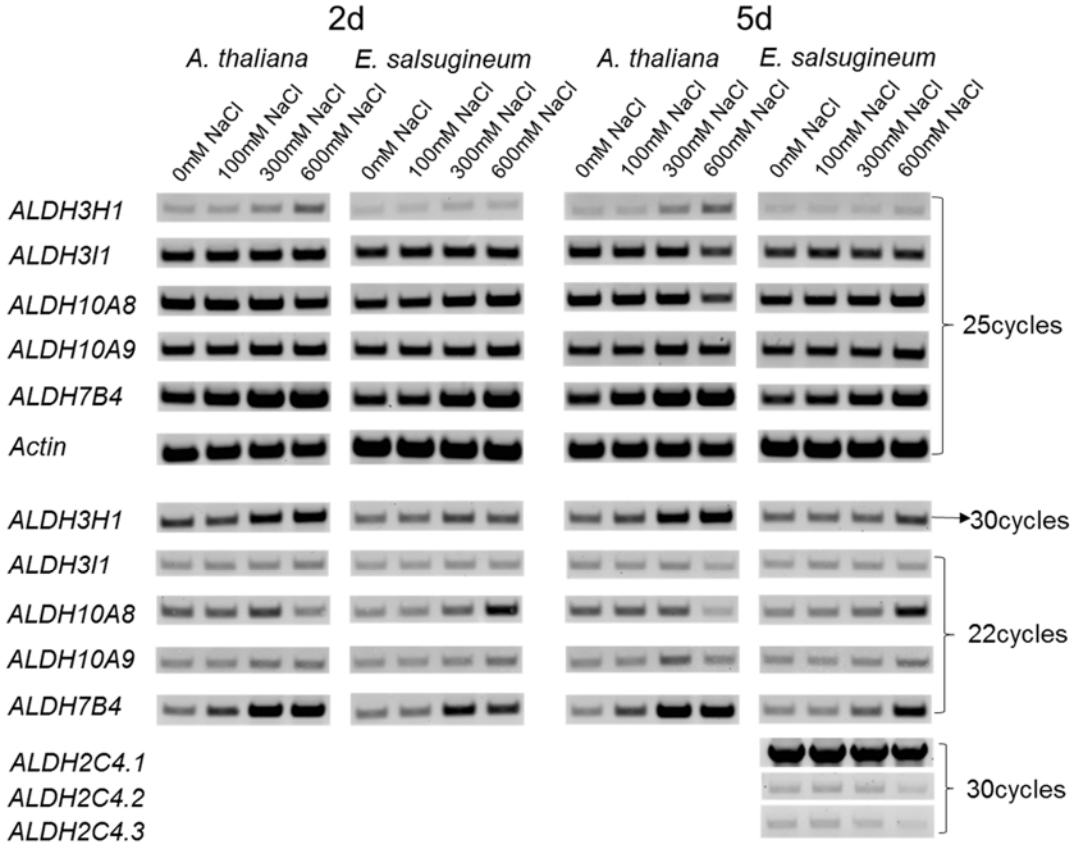


Fig. 16 Expression patterns of stress-associated *ALDH* genes from *A. thaliana* and *E. salsugineum* under variable salt stress. Leaf samples were collected from the salt treated plants as shown in **Fig. 15** (**A**). Expression patterns of stress-associated *ALDH3H1, ALDH3I1, ALDH7B4, ALDH10A8* and *ALDH10A9* genes from *A. thaliana* and *E. salsugineum* were determined by reverse transcription PCR analysis. Transcript abundances were determined using 22, 25 or 30 cycles for amplification. *Actin* was used as reference gene to monitor the cDNA quality. Three different *EsALDH2C4* transcripts were detected in samples of 5 day-treated *E. salsugineum* leaf samples after 30 cycles.

First, expression was recorded by using 25 cycles and then the cycle numbers were decreased from 25 to 22 for highly expressed transcripts and increased from 25 to 30 cycles for weakly expressed transcripts (**Fig. 16**). Under the stress conditions used here *ALDH10A9* is constitutively expressed in both *A. thaliana* and *E. salsugineum*, although it was reported to be weakly induced by different abiotic stressors (Missihoun *et al.* 2010). *ALDH10A8* shows a

differential accumulation in *A. thaliana* and *E. salsugineum*. It was constitutively expressed under low salt stress conditions in *A. thaliana* and down-regulated in high salinity conditions while it was upregulated in 600 mM NaCl in *E. salsugineum*.

Under the salt treatments used here *ALDH3I1* transcripts were constitutively expressed in *Arabidopsis* and *Eutrema*; this result is different to other reports (Kirch *et al.* 2005; Kotchoni *et al.* 2006). *ALDH3H1* shows the lowest expression of all examined transcripts and it is induced at high salt concentrations in *Arabidopsis* and *Eutrema* with a slightly lower level in *Eutrema* (**Fig. 16**). *ALDH7B4* accumulates in response to salt similarly as *ALDH3H1,* but it is more abundant than *ALDH3H1*. Expression of *ALDH7B4* is induced by salt in *Arabidopsis* and *Eutrema*, but the induction is delayed and at a lower level in *Eutrema* (**Fig. 16**).

3.2.2 Analysis of the regulation of the aldehyde dehydrogenase *7B4* (*ALDH7B4*) gene in the glycophyte *A. thaliana* and the halophyte *E. salsugineum*

ALDH7B4 plays an important role in abiotic stress adaptation and tolerance. Expression analysis showed that *ALDH7B4* is only highly expressed in *E. salsugenium* when aldehydes accumulate under high salt conditions. This suggests that a regulatory mechanism allows adaptation to high salt in *E. salsugineum*. Here, the stress inducible gene *ALDH7B4* was taken as an example to compare the regulatory mechanisms between the glycophyte *A. thaliana* and the halophyte *E. salsugineum*. The research should contribute to answer the questions: whether halophytes use regulatory mechanisms which are different from glycophytes and whether the difference accounts for variations in tolerance or sensitivity.

3.2.2.1 Alignment of promoter sequences and genomic organization of *ALDH7B4* in *A. thaliana* and *E. salsugineum*

The turgor responsive member ALDH7B4 is highly conserved throughout evolution in higher plants. Although salt-induced *ALDH7B4* expression was observed in both *A. thaliana* and *E. salsugineum*, it only occurs under high salt conditions in the halophyte *E. salsugineum* (**Fig. 16**). The different expression patterns between the two species were further confirmed by RNA blot analysis (**Fig. 17**).

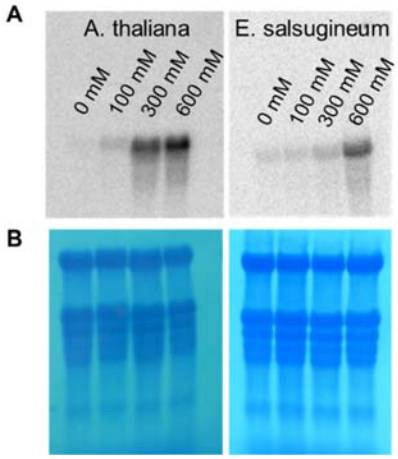


Fig. 17 Expression of the *ALDH7B4* transcripts in 6-week-old *A. thaliana* and *E. salsugineum* under salt stress conditions. (**A**) RNA blots: 20 µg of total RNAs from materials shown in **Fig. 15** were used for the blots. ^{32}P-labelled *ALDH7B4* cDNA fragments were used as probes. (**B**) Methylene blue-stained membrane to monitor the equal loading of RNAs and the blotting efficiency.

The gene organization and putative promoter regions were examined to identify elements that regulate transcript expression. Similar spatial organizations of the *ALDH7B4* gene were found in the genome of the two species. A gene encoding a cation calcium exchanger 4 protein (designated as AT1G54115 and Thhalv10012396m in *A. thaliana* and *E. salsugineum*, respectively) is present in the opposite orientation upstream of the *ALDH7B4* gene in both species. A gene encoding a membrane fusion protein (*AT1G54110*) was found between the cation calcium exchanger 4 gene and the *ALDH7* gene in *A. thaliana* but not in *E. salsugineum* (**Fig. 18A**). Due to this insertion, the *ALDH7B4* gene in *A. thaliana* has a very short putative promoter as the intergenic region between *ALDH7B4* and its upstream gene is short.

The *ALDH7B4* coding sequence is highly conserved among the Brassicaceae family (Missihoun *et al.* 2014), to investigate whether the sequence conservation extends to the non-transcribed promoter region, nucleotide sequences of the 0.65 kb of *AtALDH7B4* and 0.75 kb of *EsALDH7B4* region upstream of the ATG translation start codon were compared (**Fig. 18B**). The alignment showed that the promoter region is much less conserved compared with the coding region, although some conserved blocks were found. Noticeable is a region in *E. salsugineum* which is rich in T and C and which is not present in *A. thaliana*.

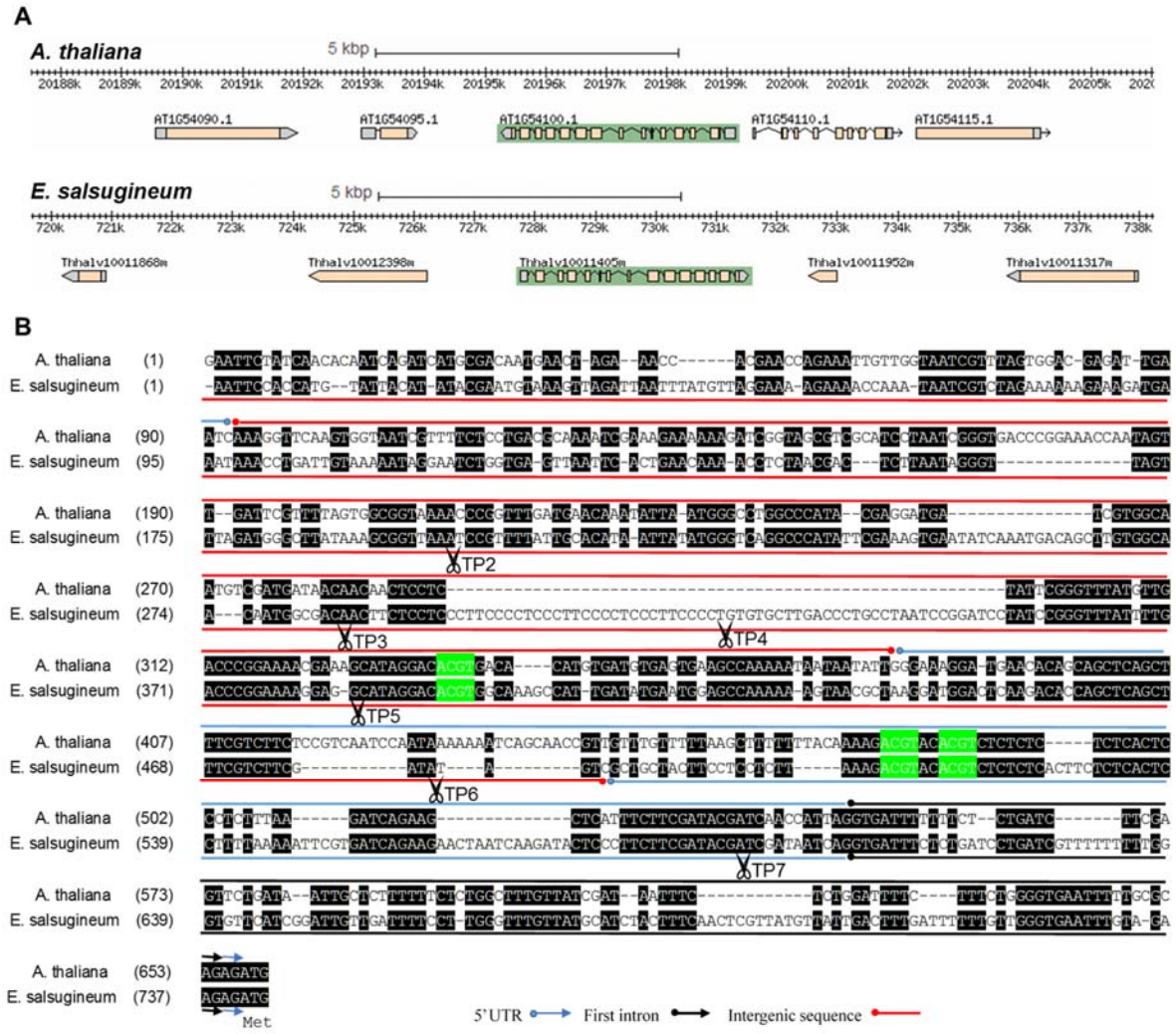


Fig. 18 Promoter sequence alignment and genomic organization of *ALDH7B4* in *A. thaliana* and *E. salsugineum*. (**A**) Genomic organization of *ALDH7B4* and neighboring genes in *A. thaliana* and *E. salsugineum* viewed in Gbrowse environment (http://phytozome.jgi.doe.gov/). The *ALDH7B4* genes are highlighted in dark green background in the middle along with two 5' and 3' adjacent genes. Arrows indicate 5' and 3' orientation of the genes. (**B**) Nucleotide sequence alignment of the putative promoter regions of *ALDH7B4* orthologs in *A. thalina* and *E. salsugineum*. The sequences were retrieved from the Phytozome v10 database (http://phytozome.jgi.doe.gov/) and aligned using the Align X tool of the Vector NTI Advance® 11 software. Three conserved ACGT-containing motif were highlighted in green, scissors indicate promoter deletion positions as described in "3.2.2.5.1".

3.2.2.2 Generation and molecular characterization of plants expressing *AtALDH7B4*-promoter::*GUS*, *EsALDH7B4*-promoter::*GUS* and *HvALDH7B4*-promoter::*GUS*

To investigate whether and how environmental stress can affect the expression of *ALDH7B4* in a glycophyte and a halophyte, transgenic plants were generated expressing the β-glucuronidase (GUS) reporter gene driven by the *EsALDH7B4* gene promoter. The

EsALDH7B4 promoter was amplified by PCR from *E. salsugineum* (Shandong) genomic DNA using the primers T.h.ALDH7B4prom1 Fwd and T.h.ALDH7B4prom1 Rev. The PCR product was digested with EcoRI and then partially digested with XbaI and purified from an agarose gel. The resulting 731 bp EcoRI/XbaI promoter fragment was purified and cloned into the pBT10-GUS vector to generate the clone TP0. Next, plasmid from a TP0 clone was digested with EcoRI and BglII to isolate the *EsALDH7B4-promoter::GUS::nos_terminator* cassette that was then subcloned into the EcoRI/BamHI double digested binary vector pBIN19. A positive clone was used to transform *A. tumefaciens* cells.

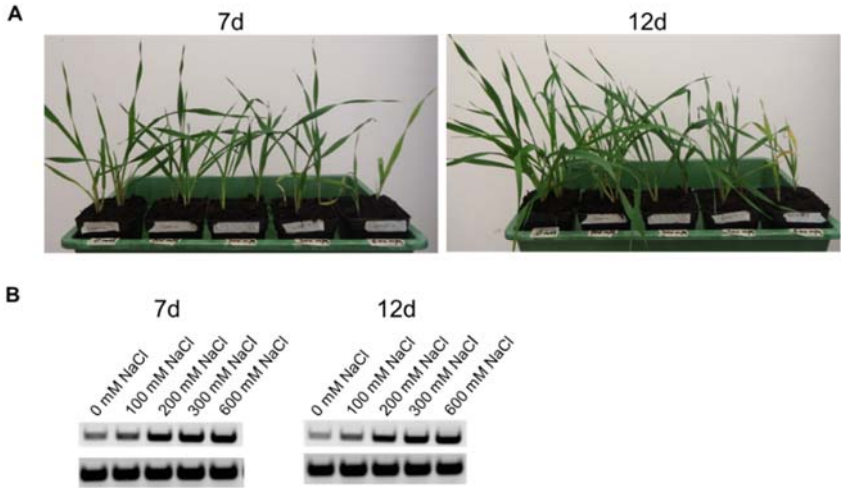


Fig. 19 Expression of the *ALDH7B4* transcripts in barley under salt stress conditions. (**A**) 10 day-old barley plants (variety: Beatrix) were treated with 100 mM, 200 mM, 300 mM or 600 mM NaCl while well watered plants serve as control. Photographs were taken 7 days and 12 days after NaCl stress application. (**B**) Leaf tissues were harvested and transcript (*AK356265.1*) abundances were determined 7 days and 12 days after NaCl stress application. *EF1α* was used as a reference gene to monitor the cDNA quality.

In addition, to investigate how *ALDH7B4* is regulated in monocots, expression of *HvALDH7B4* in barley was also investigated under salt stress conditions. The result showed the *HvALDH7B4* was also salt inducible (**Fig. 19**). Therefore, a fusion construct of barley *HvALDH7B4*-promoter::*GUS-nos_terminator* was also made for comparing the monocot *ALDH7B4* promoter activity with the promoters from dicots. The *HvALDH7B4* promoter was amplified by PCR from barley (cultivar Beatrix) genomic DNA using the primers HvALDH7B4prom Fwd and HvALDH7B4prom Rev. The PCR product was digested with NcoI and purified from an agarose gel. The resulting 1094 bp promoter fragment was purified and cloned into the pBT10-GUS vector to generate the clones harboring *HvALDH7B4-promoter::GUS*. Next, the

HvALDH7B4-promoter::GUS::nos_terminator cassette was sub-cloned into the binary vector pBIN19 and transformed into *A. tumefaciens* cells as described above.

Transformed *A. tumefaciens* cells were selected on YEB medium contains rifampicin (100 mg/l) and kanamycin (50 mg/l) and confirmed by PCR. A recombinant *Agrobacterium* clone harboring *HvALDH7B4-promoter::GUS::nos_terminator* cassette was used to transform wild-type *A. thaliana* (ecotype Col-0) plants. To understand the role of genetic background on gene expression, recombinant *Agrobacterium* clones harboring *AtALDH7B4-promoter::GUS::nos_terminator* (Missihoun 2010) and *EsALDH7B4-promoter::GUS:: nos_ terminator* cassettes were transformed into both wild-type *A. thaliana* (ecotype Col-0) and *E. salsugineum* (ecotype Shandong) plants.

The floral dip method was used to generate both transgenic *A. thaliana* and *E. salsugineum* with some modifications as described in "2.2.6.8". T1 seeds from the dipped plants were screened as described in "2.2.7.3". Kanamycin-resistant *A. thaliana* and *E. salsugineum* seedlings that showed green cotyledons and true leaves (**Fig. 20**) were transferred to soil pots after two weeks and three weeks, respectively. The insertion of the GUS constructs was further confirmed by PCR using primers pBIN-EcoRI and GUS-Start.

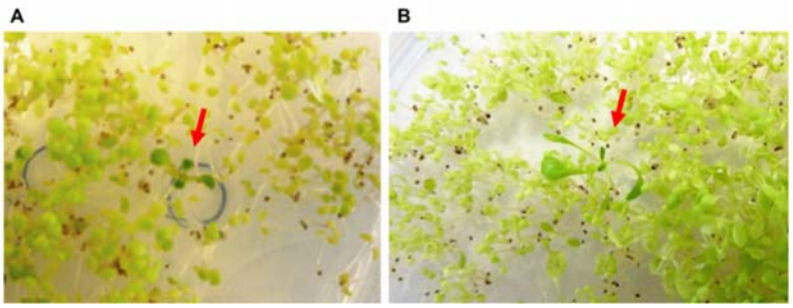


Fig. 20 Selection of transgenic *A. thaliana* and *E. salsugineum* plants. The selection was performed on MS-agar plates containing 50 mg/l kanamycin for *A. thaliana* selection (**A**) and 25 mg/l kanamycin for *E. salsugineum* selection (**B**). Red arrows indicate the putative transgenic plants.

Five positive transgenic *A. thaliana* plants harboring the *EsALDH7B4-promoter::GUS:: nos_terminator* cassette were further analyzed by DNA-blot using a *GUS* gene fragment to check the number of T-DNA fragments that were integrated in each plant. Results indicated that all five kanamycin-resistant lines harbored more than one T-DNA fragment (**Fig. 21**). No phenotypic differences were observed for these lines and the wild type with regard to germination rate, growth, flowering time and seed yield. The E7-2, E7-4, E7-5 lines harboring

three or four T-DNA insertions were used for the next experiments.

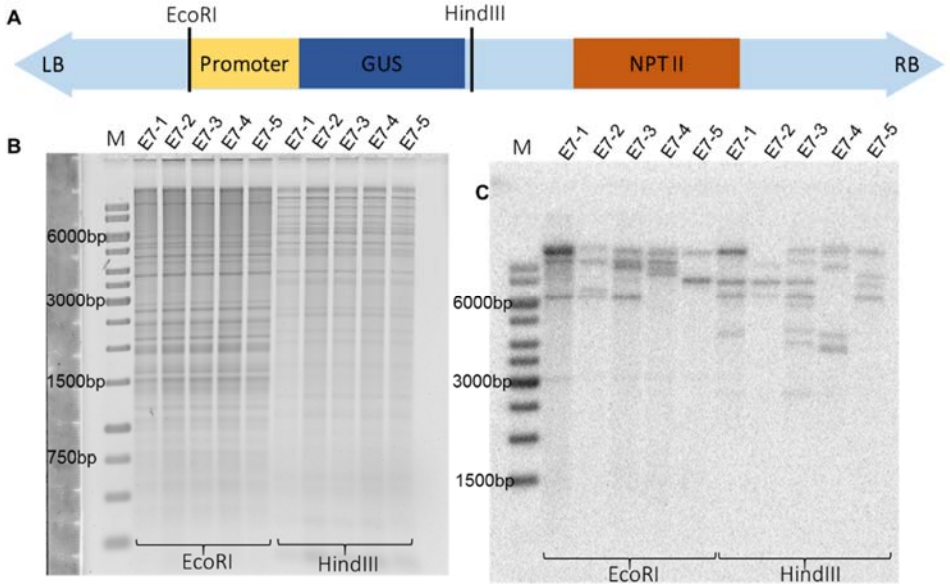


Fig. 21 Analysis of five independent transgenic *A. thaliana* lines expressing the *EsALDH7B4-GUS* gene cassette. (**A**): Schematic representation of the T-DNA region. (**B**): Fifteen micrograms genomic DNA (from T3 plants) were digested with EcoRI or HindIII and separated on a 0.8% agarose gel. (**C**): Photographs of DNA-blot membranes probed with a ^{32}P-labelled GUS fragment. M: DNA size marker. E7-1, E7-2, E7-3, E7-4 and E7-5 represent independent transgenic *A. thaliana* lines.

3.2.2.3 Activity of the *EsALDH7B4* and *HvALDH7B4* promoters in different organs

The spatial expression of the *AtALDH7B4* gene was previously examined (Missihoun *et al.* 2014). Here, expression of the *EsALDH7B4* was examined in leaves, reproductive organs and seeds in both transgenic *A. thaliana* and *E. salsugineum* plants. Homozygous plants grown under non-stress conditions were analyzed. Rosette leaves, cauline leaves, floral clusters, open flowers, green siliques, mature siliques and seeds were harvested and either immediately stained or frozen in liquid nitrogen for further analysis. The activity of the promoter *EsALDH7B4* was firstly analyzed visually after staining tissues with the GUS-staining buffer (**Fig. 22**).Activity of *EsALDH7B4* promoter was detected in all examined organs in transgenic *A. thaliana* while weaker activity was detected in leaves of transgenic *E. salsugineum* (**Fig. 22**).

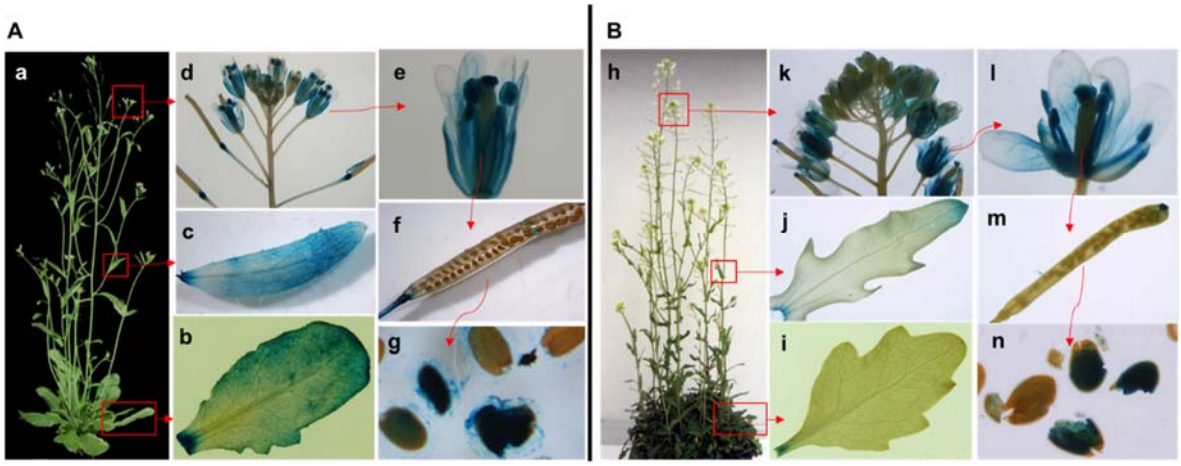


Fig. 22 *In situ* detection of the activity of the *EsALDH7B4* promoter in different organs of transgenic *A. thaliana* (A) and *E. salsugineum* (B) plants. Rosette leaves (**b, i**), cauline leaves (**c, j**), floral clusters (**d, k**), open flowers (**e, l**), siliques (**f, m**) and manually broken seeds (**g, n**) were incubated in the GUS-staining buffer after being harvested. **a**, **h** indicate morphological structure models of flowering *A. thaliana* and *E. salsugineum* plants, respectively.

However, the blue staining was also observed in reproductive organs in transgenic *E. salsugineum,* and the GUS enzymatic activity was then measured in theses organs. Results revealed that the *EsALDH7B4* promoter was strongly induced during maturation of the siliques in both transgenic *A. thaliana* and transgenic *E. salsugineum* and in mature seeds (**Fig. 23**), which is similar to the activity of the *AtALDH7B4* promoter in transgenic *A. thaliana* (Missihoun *et al.* 2014). In contrast, nearly no GUS activity was detected for the *HvALDH7B4* promoter in transgenic *A. thaliana* in all of the analyzed organs (data not shown).

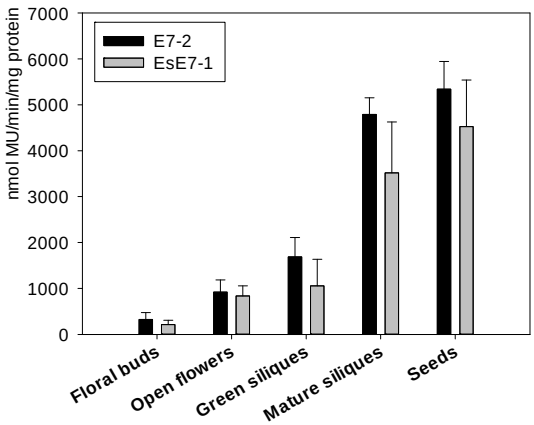


Fig. 23 Quantitative measurements of the activity of the *EsALDH7B4* promoter in reproductive organs and seeds of transgenic *A. thaliana* and *E. salsugineum*. E7-2 and EsE7-1 represent transgenic *A. thaliana* and *E. salsugineum* plants harboring the same *EsALDH7B4::GUS* cassette, respectively.

3.2.2.4 Comparative analysis of *ALDH7B4* promoter activities of *A. thaliana* and *E. salsugineum* in response to different stress factors

To investigate whether *AtALDH7B4* and *EsALDH7B4* promoters have similar regulatory patterns, the promoter activities in response to different stress factors were compared. Six-week-old independent transgenic *A. thaliana* plants harboring three copies of *AtALDH7B4::GUS* or *EsALDH7B4::GUS* cassettes in each line were subjected to different stresses. Salt stress was given by watering with 250 mM NaCl for 10 days; drought stress was given by withholding watering for 14 days; dehydration was applied by removing plants from pots and drying them for 16 h; wound stress was executed by cutting leaves with scissors or by treating the leaf surface with abrasive sandpaper; well watered plants served as control. Materials were collected after these treatments and were either immediately stained or frozen in liquid nitrogen for further analysis. Results from *in situ* detection and enzymatic quantitative assay showed both *AtALDH7B4* and *EsALDH7B4* are salt, drought and wound responsive and have similar abilities to drive *GUS* expression under salt and drought stress conditions (**Fig. 24A**). Consistent with these observations, the *in silico* analysis of the *EsALDH7B4* promoter region using MATCH program of TRANSFAC® Professional Suite subscribed from BIOBASE (http://www.biobase-international.com) revealed that in theory 35 different transcription factors could bind to the 731bp *EsALDH7B4* promoter region. Among these predicted members, there are some stress associated transcription factors including ABA-responsive *cis*-elements binding protein (ABF), G-box binding protein (GPB), heat shock transcription factor (HSFA2), MYB domain protein 15 (Myb-15) (**Table 5**).

Table 5 Predicted transcription factor binding sites in the 731 bp promoter sequences of *EsALDH7B4*

TF name	Position (strand)	Sequence
BPC1	91 (+)	AGAAAg
ASR-1	231 (-)	TGGGT
GT-1	253 (+)	GTGAAta
PLT1	282 (-)	aatgGCGACa
PBF	383 (+)	aAAAGG
PBF	383 (-)	aAAAGG
ABF1	389 (+)	aggcataggACACGtggcaaagcca
ABF1	389 (+)	aggcataggACACGtggcaaagcc
ABF	394 (+)	taggacACGTGgcaaag

GBP	396 (+)	ggaCACGTggca
GBF	396 (-)	ggacACGTGgca
CPRF-1	397 (-)	gacACGTGgc
Opaque-2	397 (-)	gacACGTGgc
CPRF-3	397 (-)	gacACGTGgc
TAF-1	397 (+)	gacACGTGgc
HBP-1a	397 (+)	gacACGTGgc
CPRF-1	397 (+)	gaCACGTggc
CG1	397 (+)	gaCACGTggca
CPRF-3	397 (+)	gaCACGTggc
HBP-1a	397 (-)	gaCACGTggc
OCSBF-1	399 (+)	CACGT
GBF1	399 (+)	cACGTGgca
OCSBF-1	400 (-)	ACGTG
ABI5	400 (+)	ACGTGg
PLT1	422 (-)	aatgGAGCCa
HSFA2	429 (+)	CCAAAa
TGA1A	513 (-)	aGACGTa
OCSBF-1	520 (+)	CACGT
PBF	544 (-)	CCTTTt
PBF	544 (+)	CCTTTt
HSFA2	639 (-)	tTTTGG
ASR-1	642 (-)	TGGGT
ASR-1	671 (-)	TGGGT
Myb-15	676 (-)	tTGTTA
ASR-1	727 (-)	TGGGT

The *EsALDH7B4* promoter showed a stronger ability to drive *GUS* expression than the *AtALDH7B4* promoter under non-stress and wound stress conditions in transgenic *A. thaliana* (**Fig. 24B**). This was not consistent with the low expression of the endogenous *EsALDH7B4* gene, which can be either caused by the different genetic background of *A. thaliana* and *E. salsugineum* or it could be due to the promoter fragment analyzed which is not the full promoter of the endogenous *EsALDH7B4* gene. Therefore, it is necessary to examine the activity of the *EsALDH7B4::GUS* construct also in transgenic *E. salsugineum* to find out whether the genetic background has some influence on the promoter strength. Interestingly, while long-term drought stress highly induced the activities of both *AtALDH7B4* and *EsALDH7B4* promoters, short-time dehydration stress only slightly induced their activities in transgenic *A. thaliana* plants. This suggests plants use different regulatory mechanisms in response to long-term drought stress and short-term dehydration stress.

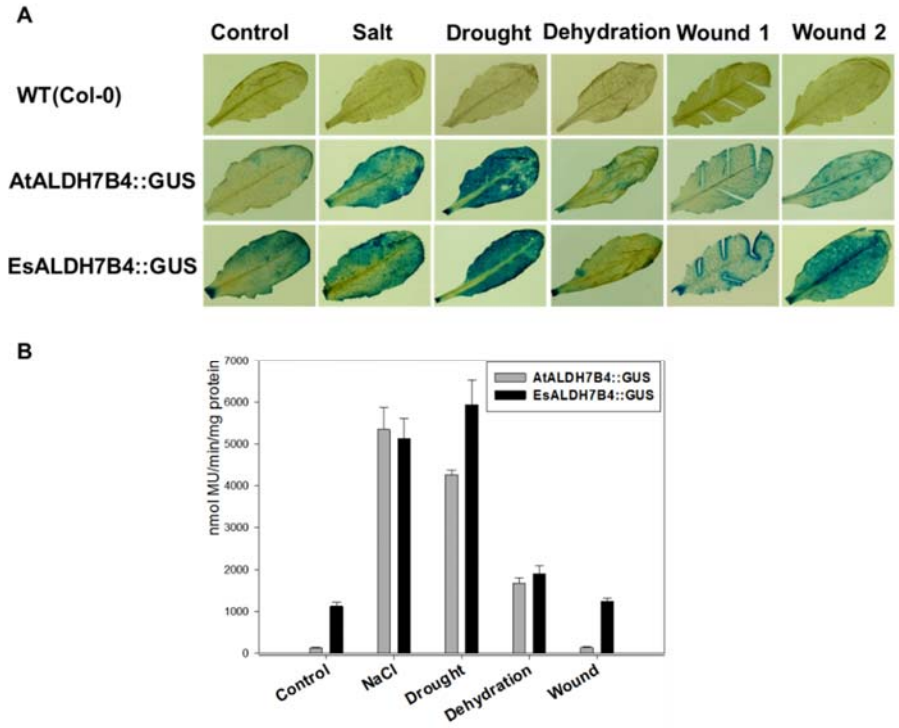


Fig. 24 Activities of the *AtALDH7B4* and *EsALDH7B4* promoters under different stress conditions. 6-week-old wild-type (Col-0) *A. thaliana* and independent transgenic *A. thaliana* plants harboring *AtALDH7B4::GUS* and *EsALDH7B4::GUS* cassettes were subjected to different stresses. Salt stress was given using 250 mM NaCl for 10 days; drought stress was given by withholding watering for 14 days; dehydration was applied by removing plants from pots and drying them for 16 h; wound stress was given by cutting leaves with scissors or by treating the leaf surface area with abrasive sandpaper; well watered plants served as control. (**A**) *In situ* detection of the GUS activity. (**B**) Salt-, drought-, dehydration-, and wounding-induced activities of *A. thaliana* and *E. salsugineum ALDH7B4* promoters measured as enzymatic activities of the GUS reporter protein.

3.2.2.5 Functional analysis of the *cis*-elements within the *EsALDH7B4* promoter in response to different stress factors

3.2.2.5.1 Generation of *EsALDH7B4* promoter deletion lines

To experimentally identify regulatory regions involved in the transcriptional control of the gene expression, promoter deletion analysis was performed. Taking TP0 plasmid that harbors the *EsALDH7B4_promoter::GUS-nos_terminator* cassette as template, *EsALDH7B4* promoter fragments of different lengths were amplified using GUS-Start primer and primers with introduced restriction enzyme sites. Fragments were amplified using the primer GUS-Start combined with primers TsA7pro2 (EcoRI), TsA7pro3 (EcoRI), ThA7pro-3de (SpeI), TsA7pro8 (EcoRI) and TsA7pro6 (SpeI) were digested with XhoI and the corresponding

restriction enzymes (EcoRI, EcoRI, SpeI, EcoRI and SpeI, respectively) and then cloned into the pBT10-GUS vector resulting in TP2, TP3, TP4, TP5, TP6 plasmids, respectively (**Fig. 25**). The TP7 plasmid (**Fig. 25**) was generated directly by digesting TP0 plasmid with ClaI and XbaI and ligated to the pBT10-GUS vector digested with the same enzyme.

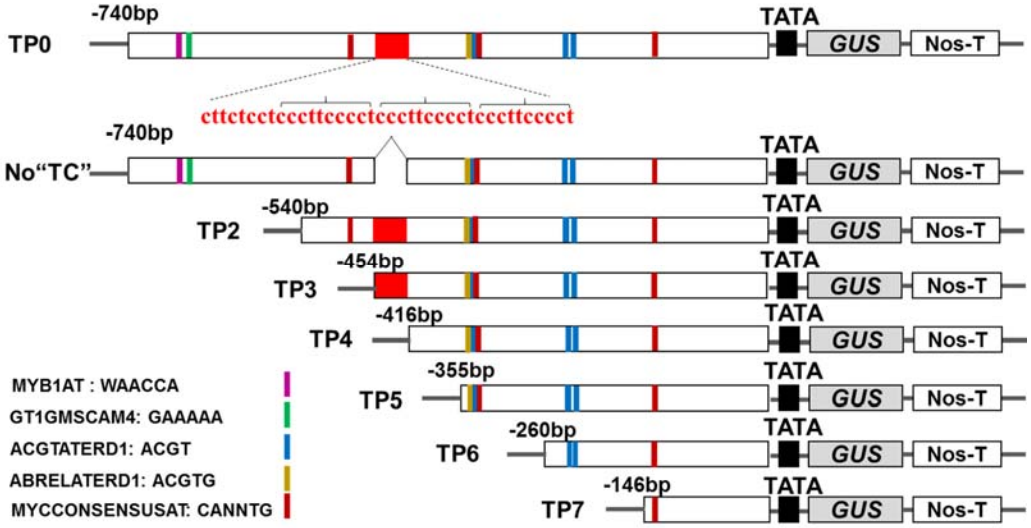


Fig. 25 Distribution of putative *cis*-acting regulatory elements within the promoter of *EsALDH7B4* and schematic representation of the mutated versions of the *EsALDH7B4* promoter region. TP0 denotes the 0.75 kb wild-type promoter fragment. No"TC" denotes the "TC" region deleted TP0. TP2, TP3, TP4, TP5, TP6, and TP7 derived from sequential 5' end deletions of the wild-type promoter. The deletion positions are shown in **Fig. 18B**.

The alignment of the *EsALDH7B4* and *AtALDH7B4* promoters showed a 38 bp "TC" rich motif does not exist in *A. thaliana* but is present in *E. salsugineum*. To analyze the relevance of the "TC" motif, the 38 bp "TC" motif was deleted by substituting a single nucleotide adenine "A". To delete the "TC" motif from the *EsALDH7B4* promoter, fragments upstream and downstream of the "TC" motif were amplified from the TP0 plasmid using primer combinations 5'-pBT10-GUS-fw/ThA7pro-5de and GUS-Start/ThA7pro-3de. These fragments were digested with restriction enzymes EcoRI/SpeI and SpeI/NcoI, respectively. The digested upstream "TC" fragment was first cloned into the EcoRI/SpeI digested pBT10-GUS vector resulting in plasmid 5LpBT10-GUS. The 5LpBT10-GUS plasmid was further digested with SpeI/NcoI and ligated with the SpeI/NcoI digested downstream "TC" fragment and finally resulted in the plasmid No"TC". The schematic representation of all mutated versions of the *EsALDH7B4* promoter is shown in **Fig. 25**.

The *EsALDH7B4_promoter::GUS-nos_terminator* cassette from the plasmids described above were isolated using restriction enzymes EcoRI and BglII for plasmids No"TC", TP2, TP3, TP4, TP5 and TP6 and HindIII and BglII for plasmid TP7. The isolated cassettes were then sub-cloned into the EcoRI/BamHI or HindIII/BamHI digested binary vector pBIN19 to yield the corresponding *E. coli* DH10B clones. Plasmids from positive *E. coli* DH10B clones were transformed into *A. tumefaciens* cells separately. Recombinant *Agrobacterium* clones were used to transform wild-type *A. thaliana* (ecotype Col-0) and wild-type *E. salsugineum* (ecotype Shandong) plants to generate transgenic plants harboring promoter GUS fusion constructs.

3.2.2.5.2 Characterization of *EsALDH7B4* promoter deletion *GUS* expression lines in response to different stress factors

Due to the difficulty in transforming *E. salsugineum*, four transgenic *E. salsugineum* plants were firstly obtained and analyzed (two harboring TP0, one harboring No "TC" and one harboring TP6). The activities of the deleted promoters were compared with the TP0 promoter in transgenic *A. thaliana* lines to analyze the effect of the deletions. In addition, the barley promoter GUS fusion transgenic lines were also analyzed to compare the monocot

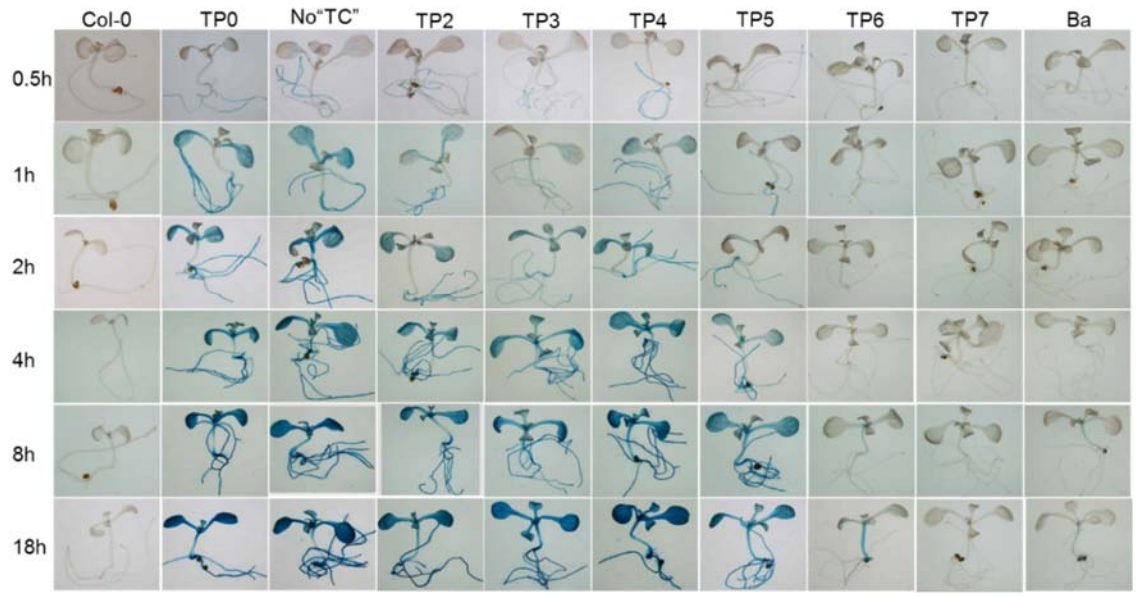


Fig. 26 Activities of the deleted *EsALDH7B4* promoters in 2-week-old *A. thaliana* seedlings. 2-week-old transgenic *A. thaliana* seedlings grown under control conditions were taken from MS medium and then incubated in GUS staining buffer. Photos were taken at different time points to compare the GUS activity in different lines. TP0, No"TC", TP2, TP3, TP4, TP5, TP6 and TP7 represent different *EsALDH7B4* deletion GUS expression lines (details see **Fig. 24**). Ba represents the *HvALDH7B4* promoter GUS expression line.

HvALDH7B4 promoter activity. Three independent T2 generation transgenic plants were analyzed for each promoter GUS deletion line of the transgenic *A. thaliana* plants. GUS activity was firstly detected in 2-week old seedlings using *in situ* GUS staining. There was no significant difference in TP0, No"TC", TP2, TP3 and TP4 except for TP4 that showed a slightly stronger GUS activity than TP3. Lower GUS activity was observed for TP5. GUS activity was nearly abolished in TP6 and no GUS activity was detected in TP7 (**Fig. 26**). Only very slight GUS activity was detected in *HvALDH7B4::GUS* fusion lines in 2-week-old seedlings (**Fig. 26**).

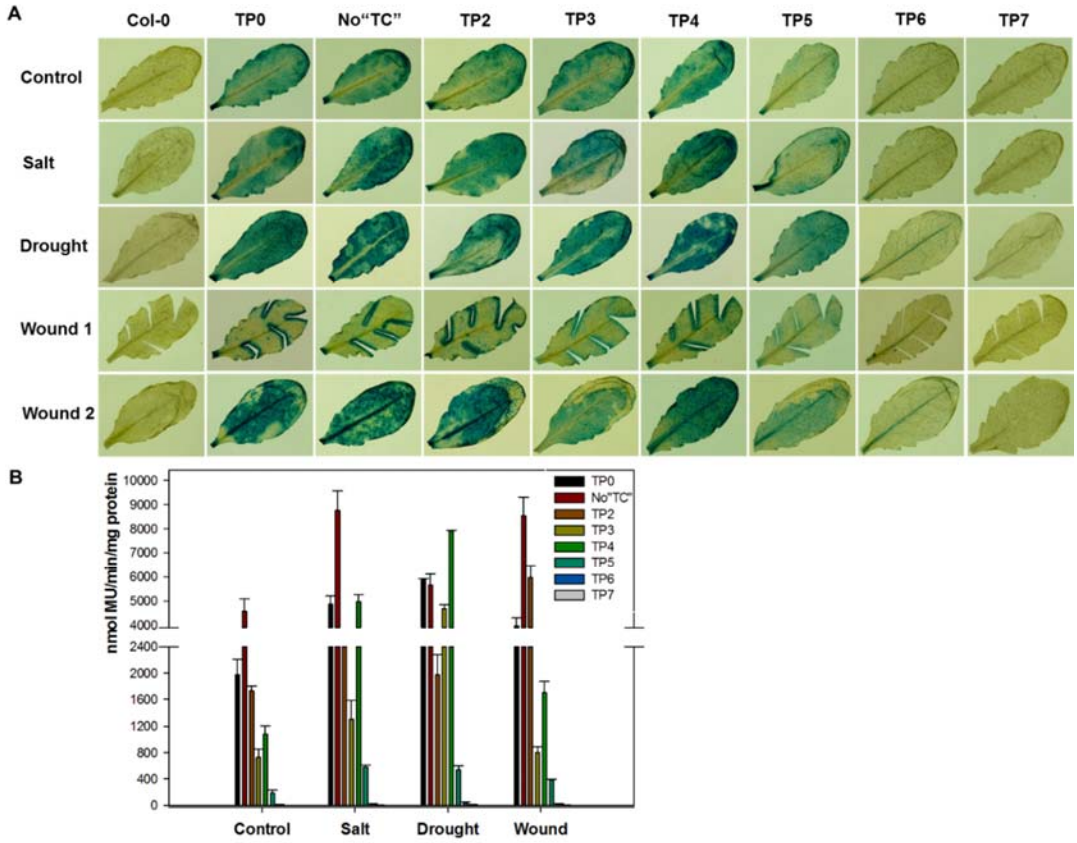


Fig. 27 Activities of the deleted *EsALDH7B4* promoters under different stress conditions. 6-week-old wild-type (Col-0) and independent transgenic *A. thaliana* plants harboring different *EsALDH7B4* promoter fragments fused to the GUS reporter gene were subjected to different stresses. Salt stress was given using 250 mM NaCl for 10 days; drought stress was given by withholding watering for 14 days; wound stress was given by cutting leaves with scissors or by treating the leaf surface with abrasive sandpaper; well watered plants served as control. TP0, No"TC", TP2, TP3, TP4, TP5, TP6 and TP7 represent different *EsALDH7B4* deletion GUS expression lines (details see **Fig. 24**) (**A**) *In situ* detection of the GUS activity. (**B**) Activity of *EsALDH7B4* promoter deletion constructs measured as enzymatic activities of the GUS reporter protein under non-stress, salt, drought, and wound stress conditions.

The deleted promoter activities in response to different stress factors were then compared in 6-week-old transgenic *A. thaliana* plants. Stress treatments were made as described in "3.2.2.4". Consistent with the result of 2-week-old seedlings, GUS activity was detected in the deletion lines TP2 to TP5. Only slight GUS activity was detected in TP6 and no GUS activity was detected in TP7 lines. Higher GUS activity was detected in the TP5 lines in response to stress treatments (**Fig. 27A, B**). This indicates that the region which was deleted from TP5 plays a crucial role for gene expression and stress responsiveness. The TP3 lines showed a weaker GUS activity than TP4, which is particularly obvious after wounding stress (**Fig. 27A**). Quantitative GUS assays confirmed that the TP3 lines have lower GUS activity than TP4 lines in all treatments (**Fig. 27B**). The No"TC" lines also showed significant lower GUS activity than TP0 lines except for the drought stress. The decreased GUS activity in lines with the deleted "TC" motif was observed in all the treatments by comparing the TP0 and no "TC" lines, and TP3 and TP4 lines.

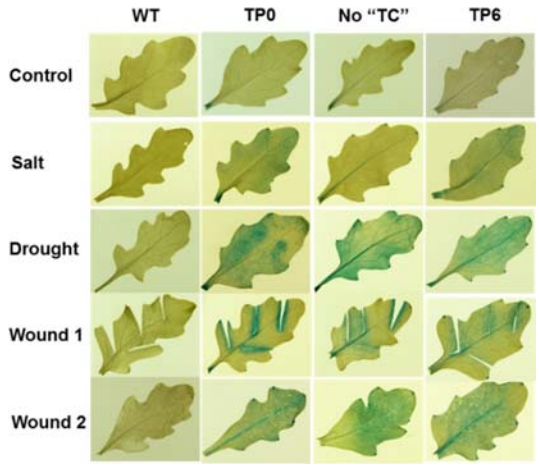


Fig. 28 *In situ* detection of GUS activity of the deleted *EsALDH7B4* promoters in transgenic *E. salsugineum* under different stress conditions. 6-week-old wild-type (Shandong) and transgenic *E. salsugineum* plants harboring different *EsALDH7B4* promoter fragments fused with the GUS reporter gene were subjected to different stresses. Salt stress was given using 250 mM NaCl for 10 days; drought stress was given by withholding watering for 14 days; wound stress was given by cutting leaves with scissors or by treating the leaf surface with abrasive sandpaper; well watered plants served as control. TP0, No"TC" and TP6 represent different *EsALDH7B4* deletion GUS expression lines (details see **Fig. 24**), WT represents the wild-type *E. salsugineum* plants.

The same stress treatments had also been applied to the transgenic *E. salsugienum* lines. In contrast to the results from transgenic *A. thaliana* lines, the TP6 promoter still shows a high activity in transgenic *E. salsugineum*. However, only one TP6 transgenic *E. salsugienum* line

was analyzed (**Fig. 28**). The "TC" motif which showed a repressor effect in transgenic *A. thaliana* did not show this effect in *E. salsugineum* because TP0 and No"TC" lines did not show a difference in the GUS staining. The *EsALDH7B4* promoter activity as reflected by GUS expression in all the treatments especially under non-stress conditions was lower in transgenic *E. salsugineum* than in transgenic *A. thaliana*.

3.2.2.6 Identifying transcription factors interact with *EsALDH7B4* promoter

Yeast one-hybrid (Y1H) assay is a "gene-centered" (DNA-to-protein) genetic method to identify DNA-protein interactions between regulatory DNA sequences and TFs. Briefly, the DNA-bait is separately cloned upstream of two reporter genes (*HIS3* and *LacZ*), and both constructs are integrated into the yeast genome. Reporter gene expression will be activated through the activation domain (AD) of the yeast transcription factor GAL4 in the presence of the fused prey protein which physically interacts with the DNA-bait. Since the Y1H system uses the prey fusions with the activation domain, both transcriptional activators and repressors can be identified as long as the prey can interact with the DNA-bait. Here the TP0 *EsALDH7B4* promoter was used as DNA-bait with the aim to identify TFs that are involved in regulating *EsALDH7B4* expression. In addition, the 38 bp "TC" motif was also used as DNA-bait to identify transcription factors that only interact with this "TC" motif.

3.2.2.6.1 Generating yeast one-hybrid DNA-bait strains

The plasmids R4L1pDEST_HISi and R4L1pDEST_LacZi were used to generate yeast one-hybrid DNA-baits (Mitsuda *et al.* 2010). Because both plasmids contain a lethal gene Cm^R *ccdB* that targets DNA gyrase, only an *E. coli* strain with a specific mutation in the gyrase, such as DB3.1, can be used to propagate *ccdB*. Therefore, the R4L1pDEST_HISi and R4L1pDEST_LacZi plasmids were propagated and isolated from 5 ml overnight *E.coli* DB3.1 culture, and digested with EcoRI/XbaI and EcoRI/SalI, respectively (**Fig. 29A**).

The *EsALDH7B4* promoter fragment was amplified using primers 5'-pBT10-GUS-fw and GUS-Start from the TP0 plasmid containing the *EsALDH7B4*-promoter::GUS::nos_terminator cassette, then digested with the restriction enzymes EcoRI/SalI and EcoRI/XbaI, respectively. For the EcoRI/XbaI digest, the fragment was first completely digested with EcoRI and then partially digested with XbaI (there is a XbaI restriction site within the *EsALDH7B4* promoter

fragment). This partial digest resulted in four bands (835bp, 760bp, 731bp, 656bp) (**Fig. 29B**). After separation of these bands on a 1.5% agarose gel, the 731bp band was extracted and purified. The 731bp and 737bp fragments resulted from the EcoRI/XbaI and EcoRI/SalI digested PCR product were cloned into the R4L1pDEST_HISi and R4L1pDEST_LacZi plasmids, respectively and transformed into *E. coli* DH10B. Recombinant plasmids containing the constructs were isolated from positive *E. coli* DH10B clones and confirmed by DNA sequencing. The R4L1pDEST_HISi and R4L1pDEST_LacZi constructs were then linearized with the restriction enzymes XhoI and NcoI, respectively. After linearization, the constructs were simultaneously transformed into the yeast strain YM4271 and screened on SD-His-Ura medium.

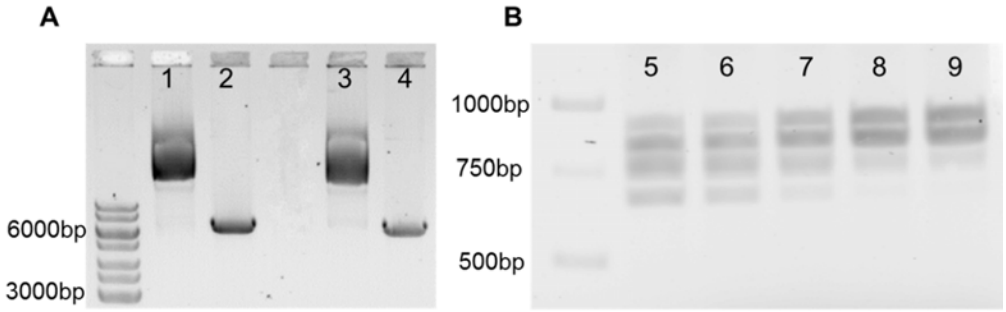


Fig. 29 Restriction enzyme digestion of the plasmids and partial digestion of the *EsALDH7B4* promoter fragment. (**A**) Lane 1 and 3 are undigested R4L1pDEST_HISi and R4L1pDEST_LacZi plasmids respectively. Lane 2 is EcoRI/Xbal digested R4L1pDEST_HISi plasmid (6809bp), and lane 4 is EcoRI/SalI digested R4L1pDEST_LacZi plasmid (6833bp). (**B**) The *EsALDH7B4* promoter was amplified from the TP0 plasmid using primers GUS-Start and 5'-pBT10-GUS-fw. The 908 bp PCR product was first completely digested with EcoRI, the resulting 835 bp fragment was further partially digested as described in "2.2.5.3" with progressively lower concentrations of XbaI and shown on a 1.5% agarose gel (lanes 5, 6, 7, 8, 9). The four bands which resulted from the XbaI partial digest are 835 bp, 760 bp, 731 bp and 656 bp, respectively.

For constructing the "TC" motif bait, the oligo annealing method was used. The oligonucleotide TCEcoRI was annealed with TCXbaI and TCSalI oligonucleotides separately. Since EcoRI, XbaI and SalI sticky ends were designed in the oligonucleotides, the annealed double-stranded oligonucleotides were directly ligated with the EcoRI/XbaI digested R4L1pDEST_HISi and EcoRI/SalI digested R4L1pDEST_LacZi plasmids. The clones with the positive constructs were isolated and confirmed by DNA sequencing. Linearization and yeast transformation were performed as described above.

3.2.2.6.2 Testing autoactivation of yeast one-hybrid DNA-bait strains

Autoactivity is the expression of the integrated DNA-bait::reporters in the absence of an AD-prey clone. The reason for autoactivation is likely due to the fact that an endogenous yeast activator binds to the DNA-bait. Different integrated strains from the same transformation can show varying levels of autoactivity because the integrated strains are harboring different numbers of DNA-bait::report cassettes. It is important to select and use the integrated strain which has the lowest autoactivity for both reporters to decrease the number of false protein-DNA interactions in the subsequent assays. The clones were tested for autoactivation in the following way. The integration of the constructs was tested by PCR using two pairs of primers pHISi_For/pHISi_Rev and pLacZi_For/pLacZi_Rev. Ten positive yeast colonies that are integrated with the full *EsALDH7B4* promoter and seven positive colonies that integrated with the "TC" motif bait were picked, and each colony was resuspended in 60 µl sterile H_2O. Five microliter of each colony mixture was then pipetted to SD-His-Ura media containing 0 mM, 15 mM, 30 mM and 45 mM 3-AT to test for autoactivation of the reporter *HIS3*. After incubation at 30 ºC for 5-7 days, the growth status of each integrated strain was recorded on the different media visually (**Fig. 30A**). The yeast that grew on the SD-His-Ura medium without competitor 3-AT was then used to perform colorimetric assays to test the autoactivation of the *LacZ* reporter gene (**Fig. 30B**).

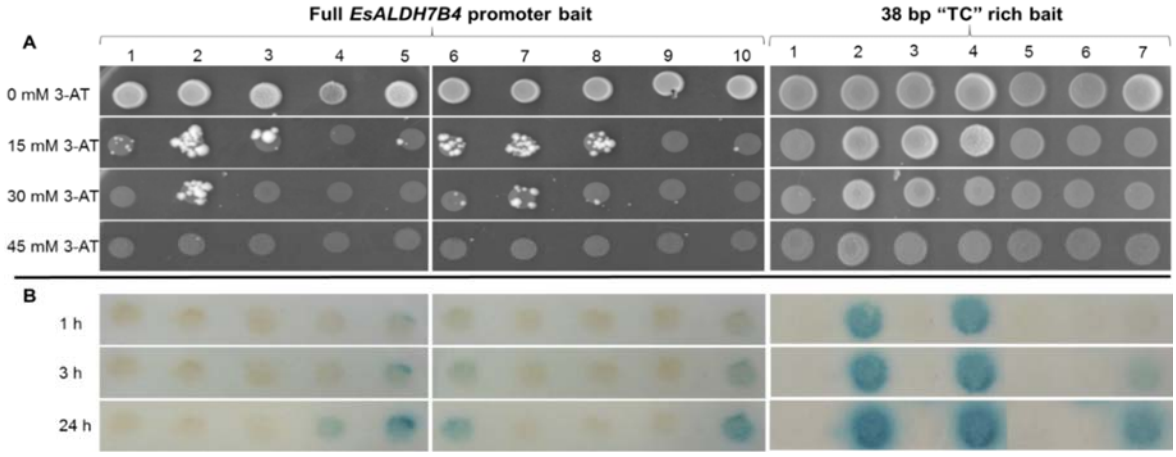


Fig. 30 Yeast one-hybrid autoactivation test readout. (**A**) Autoactivation test of the *HIS3* reporter gene. (Top panel) Growth of different integrated strains on SD-His-Ura media after 3 d at 30 ºC. (Three panels below) Growth after 7 d on SD-His-Ura media containing progressively higher concentrations of 3-AT(15 mM, 30 mM and 45 mM). (**B**) Autoactivation test of the *LacZ* reporter gene. The results of β-galactosidase assays with these strains, after 1h, 3h and 24h incubation at 37 ºC. Arabic numbers represent different integrated strains.

From the result of the autoactivation test of the full *EsALDH7B4* promoter bait, the integrated strains 1, 2, 3, 6, 7 and 8 were continued to grow on with 15 mM 3-AT medium while strains 4, 5, 9 and 10 showed minimal or no growth (**Fig. 30**). If no blue stain was observed in the colorimetric assay of β-galactosidase activity, this might indicate a problem either with the reporter construct or with the integration, and such strains were not selected. Therefore, combining the autoactivation test result of the two reporter genes, strain 5 was chosen as the optimal integrated strain, also because it grew very well under none competitor (0 mM 3-AT) conditions. Similarly, the clone 7 was chosen as the optimal integrated strain for the "TC" motif bait (**Fig. 30**).

3.2.2.6.3 Prey library transformation and identification of DNA-protein interactors

The optimal *EsALDH7B4* promoter integrated strain 5 and the "TC" motif integrated strain 7 were transformed with 6 µg of TF-only library (Mitsuda *et al.* 2010) and screened on SD-His-Ura-Leu+15 mM 3-AT media. After 5 days incubation at 30 ºC, more than 10,000 colonies appeared on the screening medium plates. The large colonies were replicated on fresh SD-His-Ura-Leu+15mM 3-AT medium and medium with a higher concentration of 3-AT (SD-His-Ura-Leu+30mM 3-AT plates) to further confirm their growth status. A colony-lift β-galactosidase assay was performed to validate the selection.

From their growth status and the β-galactosidase assay (**Fig. 31**), it is seen that the colonies which rapidly generate high amounts of the blue compound in the colorimetric assay also grew better on SD-His-Ura-Leu+15 mM 3-AT media even on a higher concentration of 3-AT medium (30 mM). These colonies activated both reporter genes indicating that GAL4AD fused preys (TFs) interacting with the DNA-baits and activating the reporter gene expression through the GAL4AD. The TFs in these "double-positive" colonies were amplified by yeast colony PCR using primers GAL4AD_For and GAL4AD_Rev and subsequently identified by DNA sequencing.

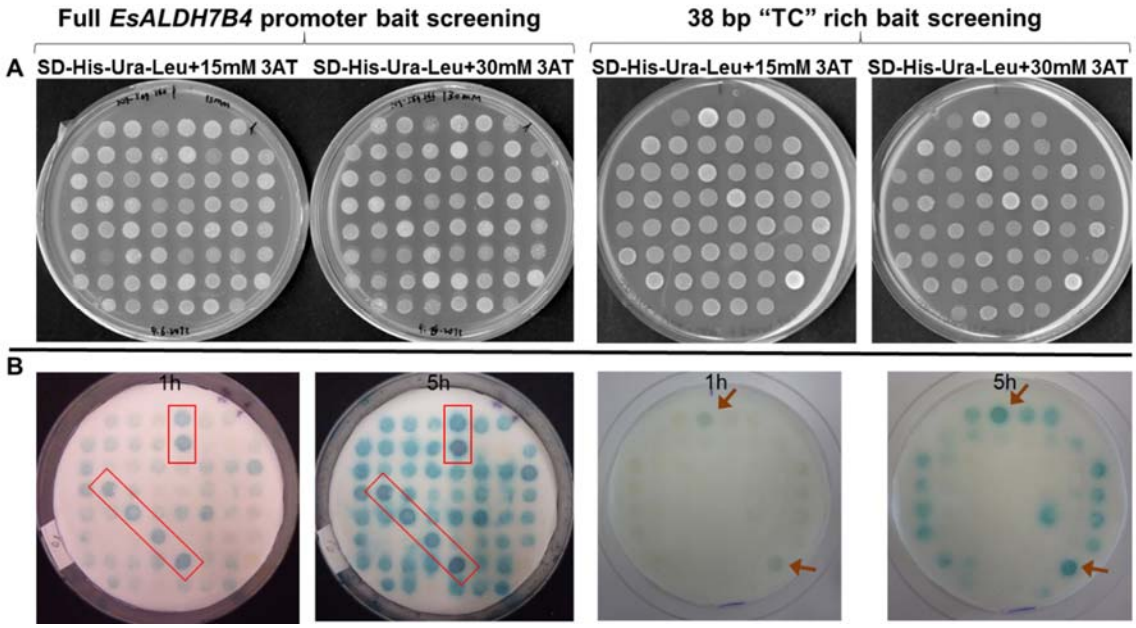


Fig. 31 Yeast one-hybrid library screening readout. (**A**) The growth of yeast strains that may contain bait-prey combinations on SD-His-Ura-Leu+15 mM 3-AT and SD-His-Ura-Leu+30 mM media after 3 d at 30 ºC. (**B**) The results of aβ-galactosidase assay with these strains, after 1 h and after 5 h incubation at 37 ºC. The red box or arrows indicate samples of putative positive clones harboring protein-DNA interactions.

Table 6 Isolated TFs binding to the *EsALDH7B4* promoter and the 38 bp "TC" element

Isolated TF (by locus No.)	Times isolated	TF family	Description	*Cis*-binding motif
Full EsALDH7B4 promoter bait screening				
AT4G36730	4	bZIP-G	G-box binding factor 1	CCACGTGG
AT3G62420	3	bZIP-S	basic region/leucine zipper 53	ACTCAT
AT5G11260	2	bZIP-H	transcription factor HY5	ACACGTGG
AT2G46270	1	bZIP-G	G-box binding factor 3	CACGTG
AT3G49760	1	bZIP-S	basic leucine-zipper 5	ACGT
AT1G03040	1	bHLH	transcription factor bHLH7	CANNTG
AT5G65320	1	bHLH	transcription factor bHLH99	CANNTG
AT3G19860	1	bHLH	transcription factor bHLH121	CANNTG
AT5G09460	1	bHLH	transcription factor bHLH143	CANNTG
AT4G30180	1	bHLH	uncharacterized bHLH146	?
AT3G23240	1	ERF/AP2	ERF1B	GCC
AT1G53170	1	ERF/AP2	ERF8	GCC
AT1G28360	1	ERF/AP2	ERF12	GCC
AT1G22190	1	ERF/AP2	ERF058	GCC
AT1G64380	1	ERF/AP2	ERF061	GCC
AT1G22985	1	ERF/AP2	ERF069	GCC
AT1G72360	1	ERF/AP2	ERF073	GCC
AT1G12890	1	ERF/AP2	ERF088	GCC
AT3G23230	1	ERF/AP2	ERF098	GCC

AT4G24540	1	MADS	MADS-box protein AGL24	CC[A/T]$_6$GG
AT1G01530	1	MADS	protein agamous-like 28	CC[A/T]$_6$GG
AT5G62165	1	MADS	protein agamous-like 42	CC[A/T]$_6$GG
AT3G30260	1	MADS	protein agamous-like 79	CC[A/T]$_6$GG
AT2G15660	1	MADS	protein agamous-like 95	CC[A/T]$_6$GG
AT5G23090	1	NF-YB13	nuclear factor Y, subunit B13	CCAAT
AT1G08970	1	NF-YC9	nuclear factor Y, subunit C9	CCAAT
AT5G43250	1	NF-YC13	nuclear factor Y, subunit C13	CCAAT
AT3G09230	1	MYB	myb domain protein 1	
AT5G40330	1	MYB	transcription factor MYB23	
AT3G58630	1	Myb-like	DNA binding	
AT1G02040	1	C2H2ZnF	C2H2-type zinc finger protein	
AT5G48890	1	C2H2ZnF	protein LATE FLOWERING	
AT1G14687	1	ZF-HD	homeobox protein 32	
AT3G06740	1	GATA	GATA transcription factor 15	WGATAR
AT3G12130	1	C3H	inc finger CCCH domain	
AT1G47655	1	DOF	Dof zinc finger protein DOF1.6	
AT5G14000	1	NAC	NAC domain protein 84	
AT2G21900	1	WRKY	WRKY transcription factor 59	
AT1G04550	1	AUX/IAA	transcriptional regulator	
AT1G14510	1	Alfin1-like	protein alfin-like 7	
AT5G57180	1	Orphan	chloroplast import apparatus 2	
AT1G32030	1	DUF	uncharacterized protein	
AT5G02470	1	E2F/DP	protein DPA	
AT2G01930	1	BBR-BPC	basic pentacysteine1	
AT2G04890	1	GRAS	scarecrow-like protein 21	
AT3G61790	1		E3 ubiquitin-protein ligase SINAT3	
38 bp "TC" rich bait screening				
AT5G56840	1	Myb_like	myb-like transcription factor	
AT5G24520	1		TRANSPARENT TESTA GLABRA 1	

W = T or A; R = G or A; N=A or T or C or G DUF=Domain of unknown function

For the *EsALDH7B4* promoter screening, 52 PCR fragments amplified from independent positive colonies were sequenced, and 46 transcription factors were identified. Several TFs were isolated more than once (**Table 6**). The identified TFs mainly belong to bZIP, bHLH, ERF/AP2, MADS, NF-Y and MYB gene families. The consensus *cis*-elements that can interact with bZIP, bHLH and ERF/AP families are present in the *EsALDH7B4* promoter bait. Although many members from NF-Y and MADS-box families were identified, the exact *cis*-elements which can interact with TFs from these families could not be found in the *EsALDH7B4* promoter fragment used here. The reason could be that these transcription factors have DNA binding sites different from those reported in the literature. There are reports showing that DNA

binding sites for a given transcription factor can vary in the affinity of the transcription factor for the different binding sites (Wittkopp 2010). However, it also possible that the isolated clones are false positive yeast clones. It is assumed that the *cis*-element CACGTG in the *EsALDH7B4* promoter plays a key role for the *EsALDH7B4* expression because when it was deleted the GUS activity was nearly completely lost in the GUS fusion *A. thaliana* lines TP6 (**Figs. 26, 27**). Since this *cis*-element is the consensus sequence of both bZIP and bHLH family TFs, the bZIP and bHLH family transcription factors may be critical for *EsALDH7B4* expression. For the "TC" motif screening, only two putative positive yeast clones were obtained and after DNA sequencing, they were identified as MYB_like transcription factor (AT5G56840) and TRANSPARENT TESTA GLABRA 1 binding factor. It is predicted that the MYB_like transcription factor is a transcriptional repressor (Ikeda and Ohme-Takagi 2009), which is consistent with the promoter deletion result for the 38 bp "TC" rich motif has a negative role in *EsALDH7B4* expression (**Fig. 27B**). However, the interaction between the identified MYB_like transcription factor and the "TC" rich DNA sequence needs to be further analyzed.

3.3 Characterization of the bHLH146

3.3.1 Genome organization of bHLH146 and synteny with its paralog in *A. thaliana*

Among the identified transcription factors from the yeast one-hybrid screening, an uncharacterized bHLH (basic/helix-loop-helix) family transcription factor bHLH146 (At4g30180) attracted our attention as it was predicted to lack the basic region and lost its DNA binding ability. It codes for a protein of 158 amino acid residues and has a predicted molecular weight of 17.8 kDa with an isoelectric point of 9.53. The nuclear localization signal (NLS) "RKKR" was predicted in the N-terminal (Brameier *et al.* 2007). Sequences that form helix structures are predicted in the C-terminal region (**Fig. 32**). Amino acids (~15) in front of the helix region of bHLH proteins comprise the basic region which determines the DNA binding activity of the protein. However, the sequence of the basic region of this protein has a very low similarity with other bHLH family proteins and lacks the Glu-13/Arg-17 which is necessary for binding to the E-box (5'-CANNTG-3'), thus it was predicted as a non-DNA binding HLH protein (Toledo-Ortiz 2003).

```
        10         20         30         40         50         60
MERQIINRKK RVFSLEPNKN PSAVFTRKYT SHLVPALKKL NMNKNSSKQT VKHEVDMALA

        70         80         90        100        110        120
LSAQEFAWSR FLLQKLSSSS NPTTTTSSSS DGIRILERPD KEGGNEEGGI EERLRELKKL

       130        140        150
LPGGEEMNVE EMLSEIGNYI KCLELQTIAL KSIVQDST
```

Fig. 32 Protein sequence analysis of bHLH146. Sequences highlighted in red, dark yellow and yellow are the predicted nuclear localization signal (http://www.sbc.su.se/~maccallr/nucpred/), the helix structure domain (https://www.predictprotein.org/) and the basic region, respectively.

Sequence comparison revealed that bHLH146 is a paralog of At2g18969. At2g18969 was excluded from the bHLH family in early reviews (Bailey *et al.* 2003; Heim 2003; Toledo-Ortiz 2003) but identified as a novel bHLH protein recently (Carretero-Paulet *et al.* 2010). It encodes a protein with a length of 175 amino acids and its locus in the *A. thaliana* genome shows a synteny with the locus of *bHLH146* (**Fig. 33**). Here, the focus is mainly on the transcription

factor bHLH146. Its DNA binding characteristics, sub-cellular localization, expression profiles, protein-protein interaction have been studied. *bHLH146* overexpression, artificial mircoRNA silencing lines and T-DNA knockout lines were obtained and analyzed.

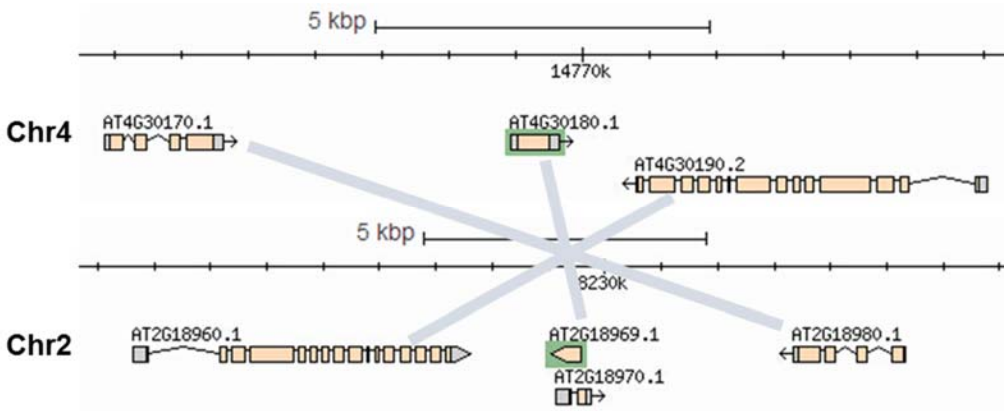


Fig. 33 Genome organization of *bHLH146* and synteny with its paralog on *A. thaliana* chromosomes. Genomic organization of *bHLH146* (*At4g30180*) and its paralog *At2g18969* in *A. thaliana* viewed in Gbrowse environment (http://phytozome.jgi.doe.gov/). The *bHLH146* and *At2g18969* are highlighted by a dark green background in the middle along with upstream and downstream adjacent genes. Arrows indicate 5' and 3' orientation of the genes, paralogs in different chromosomes are linked by grey lines.

3.3.2 Purification of the recombinant protein bHLH146 to investigate its DNA binding ability

To confirm whether the transcription factor bHLH146 can interact directly with DNA fragment, its DNA binding ability was tested *in vitro* by EMSA (Electrophoretic Mobility Shift Assay) and DNase I footprinting assay. The recombinant His-bHLH146 protein was produced in *E. coli* and was purified using a His-tag affinity column. The coding sequence was amplified from *A. thaliana* genomic DNA with the primers At4g30180_for and At4g30180rev_SalI since the gene was encoded by a single exon (**Fig. 33**). The amplified coding sequence (+1 to +474; 158 aa) was subcloned into the pET28a (+) expression vector via NdeI/SalI sites yielding a fusion protein of 178 aa with N-terminal His-tag (6 × His). The DNA sequence of the pET28-bHLH146 plasmid was verified by DNA sequencing and introduced into the *E. coli* strain BL21 (DE3). The expression of the recombinant protein was induced at 22 °C by adding IPTG (isopropyl-β-D-thiogalactopyranoside) to a final concentration of 1 mM. The proteins were purified from the soluble fraction using a His-tag affinity column. As shown in **Fig. 34**, the recombinant bHLH146 protein was induced (**Fig. 34A**) and was purified under native

conditions (**Fig. 34B**). Sufficient amounts of purified bHLH146 protein were obtained and dialyzed against the solution 5 mM Tris, pH 8.0; 10 mM NaCl; 0.1mM EDTA; 1 mM DTT and 0.5mM PMSF.

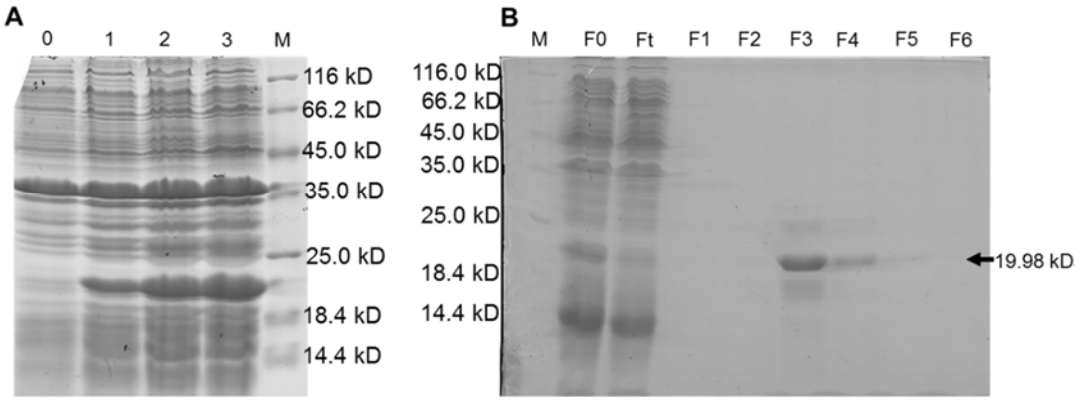


Fig. 34 Induction and purification of the bHLH146 recombinant protein. (**A**) The overexpression of the protein was induced by incubating *E. coli* cultures with IPTG for 3 hours. **M**: Standard protein marker. **0, 1, 2, 3**: proteins of 1 ml samples were analyzed after induction for 0, 1, 2, 3 hours on SDS-PAGE. (**B**) The bHLH146 recombinant protein was purified by His-tag affinity chromatography under native conditions. **F0**: Total soluble fraction; **Ft**: Flow-through fraction; **F1-F6**: Eluted fractions (500 µl). Ten microliters from each fraction were analyzed by SDS-PAGE.

The dialyzed protein was freeze-dried and used for EMSA and DNase I footprinting analyses as well as to generate antibodies. EMSA and DNase I footprinting assays were performed by Shanghai Biotechnology Corporation (http://www.shbiotech.org/). The results of the EMSA assay showed that the bHLH146 did not bind the 452 bp *EsALDH7B4* promoter (amplified with primers Es7B4pfwd and Es7B4prev) that contains the E-Box *in vitro* (**Fig. 35A**). No

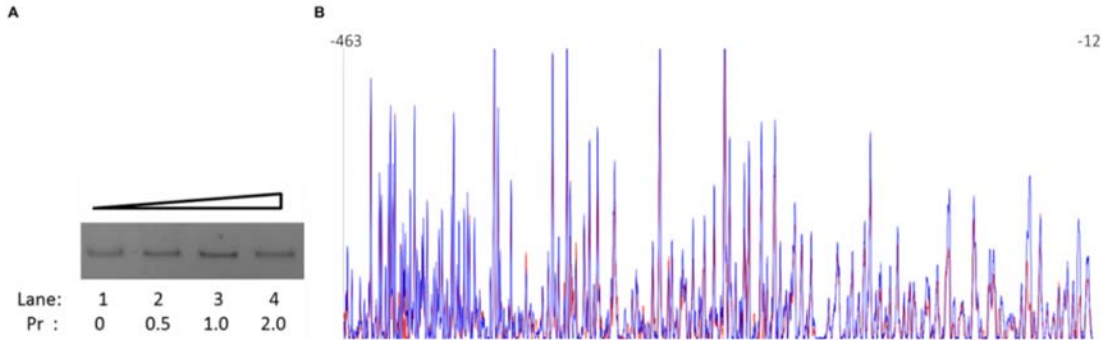


Fig. 35 Results of EMSA and DNase I footprinting assay. (**A**) Electromobility shift assay (EMSA) of *EsALDH7B4* promoter (452 bp) incubated with increasing amounts of His6-bHLH146 (Pr) shown from lanes 1 to 4. (**B**) DNase I footprinting experiment. The sense strand sequence was analyzed. The numbers above refer to nucleotide positions relative to the translation start site (+1) of the *EsALDH7B4* gene. Electropherograms show the combined protection patterns after digestion with DNase I following incubation with His6-bHLH146. The y axis shows the relative fluorescence intensity on an arbitrary scale.

protected region was identified from the DNase I footprinting experiment (**Fig. 35B**). These results indicate that the bHLH146 transcription factor may not interact with DNA directly as it was predicted.

3.3.3 Sub-cellular localization of bHLH146

bHLH146 was reported to be an HLH member. To get experimental evidence for the sub-cellular localization of this protein, GFP fusion constructs were generated and analyzed. The full-length bHLH146 coding sequence was amplified from genomic DNA using the primers At4g30180for_NcoI and At4g30180rev_NcoI. The fragment was then cloned into the pGJ280 vector and fused in frame to the GFP via the NcoI site to generate the bHLH146-GFP expression construct. The construct was transformed into *E. coli* DH10B. Plasmids extracted from *E.coli* were transiently transformed by particle bombardment into *Arabidopsis* leaves and onion epidermis cells. The green fluorescence was clearly observed in both the nucleus and cytoplasm from transformed *Arabidopsis* leaf cells and onion epidermis cells. High intensity of green fluorescence was observed in the nuclei, which indicates bHLH146 is mainly localized in nucleus (**Fig. 36**).

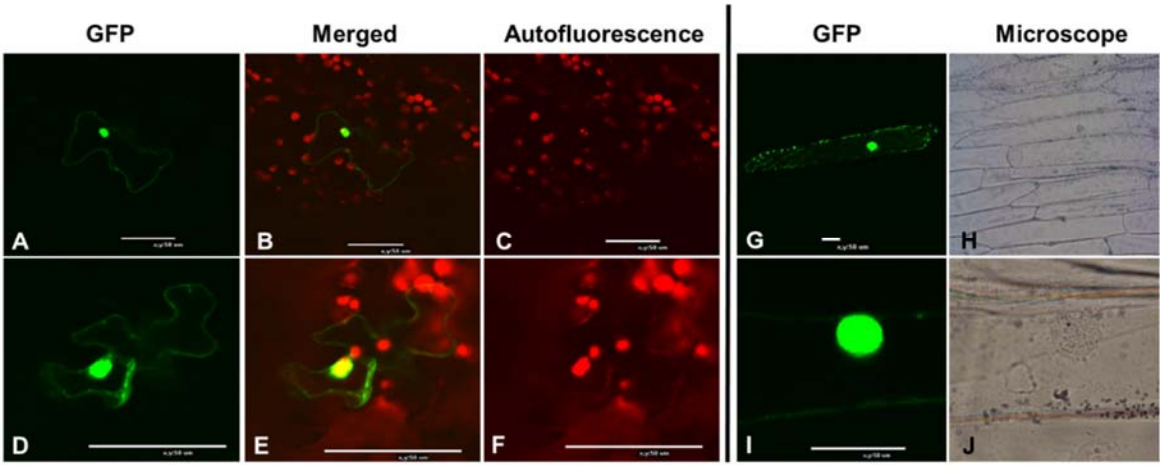


Fig. 36 Sub-cellular localization of bHLH146-GFP fusion proteins. The bHLH146-GFP fusion construct was expressed in leaves of *A. thaliana* (**A**, **B**, **C**, **D**, **E**, **F**) and onion epidermal cells (**G**, **H**, **I**, **J**). Two transformed cells of each in leaves and onion epidermis were viewed under the fluorescence microscope with appropriate filters (scale bar: 50 µm) or normal light microscope. (**A**, **B**, **G**, **H**) GFP fluorescence alone, (**B**, **E**) Merged chlorophyll and GFP fluorescence, (**C**, **F**) chlorophyll auto-fluorescence and (**H**, **J**) corresponding transformed epidermal cells viewed under the normal light microscope.

3.3.4 Identifying proteins that interact with bHLH146 using a yeast two-hybrid system

Proteins that interact with each other are expected to participate in the same cellular processes (Oliver 2000). On this basis, the finding that an unknown protein binds to a protein of known function will provide a clue in which cellular pathways the unknown protein participates. This information will often contribute to understanding the role of the protein in a complex pathway (Coates and Hall 2003). Yeast two-hybrid is a method for protein-protein analysis, which is based on the fact that TFs are composed of separate domains, a DNA binding domain (DNA-BD) and one or more activation domains (AD). BD binds to specific DNA sequences referred to as upstream activation site (UAS) in yeast while AD directly interact with the RNA polymerase II complex to transcribe the gene downstream of the UAS (Keegan *et al.* 1986). An interaction between a bait protein (fused to the GAL4-BD) and a prey protein (fused to the AD) generates a novel transcriptional activator with binding affinity to a GAL4-responsive UAS. This factor then activates reporter genes that have upstream GAL4-responsive elements in their promoters and makes the protein-protein interaction phenotypically detectable. In this study, the GAL4 system and the yeast strain Y190 were used in which two reporter genes (*HIS3* and *LacZ*) are integrated under the control of a GAL4-responsive UAS.

3.3.4.1 Generating yeast two-hybrid protein-bait strains and autoactivation test

The coding sequence of bHLH146 was amplified from *A. thaliana* Col-0 genomic DNA using the primers At4g30180for_NcoI and At4g30180rev_SalI. The PCR product was digested with the restriction enzymes NcoI and SalI and then cloned into the NcoI and SalI digested pAS2-1 vector to generate the bHLH146-GAL4-BD fusion construct. The construct was confirmed by DNA sequencing and subsequently transformed into the yeast strain Y190. Six positive colonies were selected from SD-Try medium and confirmed by PCR using the primers pAS2_1fwd and pAS2_1rev. Then they were tested for autoactivation. Each colony was resuspended in 60 µl sterile H_2O and 5µl of the mixture was pipetted to SD-Try-His media with 0 mM, 15 mM, 25 mM, 50 mM, 75 mM and 100 mM 3-AT to test autoactivation of the *HIS3* reporter gene.

Autoactivation test of *HIS3* reporter showed that growth of all the selected six positive yeast clones was inhibited by 50 mM 3-AT (**Fig. 37**). Autoactivation of the *LacZ* reporter was also

tested by a colorimetric β-galactosidase assay. The result shows that none of the six yeast clones can accumulate any blue compound within 24 hours. Finally, the positive clone number 6 was chosen as the optimal bait because it grew well under the non-3-AT conditions and its growth can completely inhibited by 25 mM 3-AT.

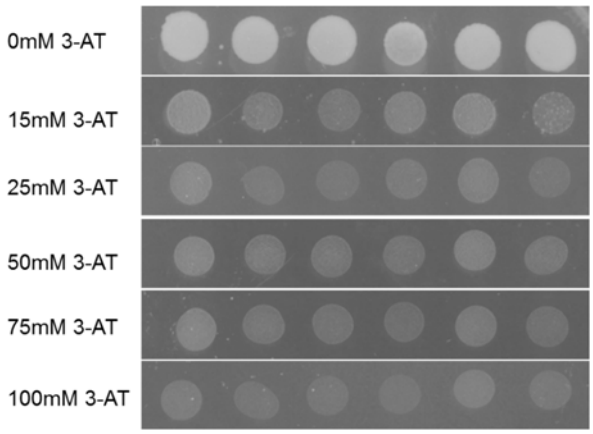


Fig. 37 bLH146 yeast two-hybrid autoactivation test readout. (Top panel) The growth of six positive strains grown on SD-Try media after 3 d of incubation at 30 ºC. The five panels below show the growth status after 5 d on SD-Try-His media containing progressively higher concentrations of 3-AT (15 mM, 25 mM, 50 mM, 75 mM and 100 mM).

3.3.4.2 Prey library transformation and identification of protein-protein interactors

The optimal bait strain 6 was transformed with 150 µg oligo (dT)-primed cDNA library prepared in the plasmid pACT2 using mRNA from an *A. thaliana* cell suspension (Németh *et al.* 1998). The transformed cell suspension was spread on SD medium lacking leucine, tryptophan and histidine plus 25 mM 3-AT for screening. After 5 days of incubation at 30 ºC, more than 1 million colonies were obtained. To rapidly identify colonies that contain interacting preys, colorimetric β-galactosidase assays were performed directly with these colonies (**Fig. 38**).

The colonies that generated blue stain in β-galactosidase assays were picked and resuspended in 60 µl sterile H_2O. Five microliter of each colony suspension was replicated on different SD media to further confirm that they contain the interacting preys from the *HIS3* reporter. In total, 28 positive yeast colonies were obtained (**Fig. 39**).

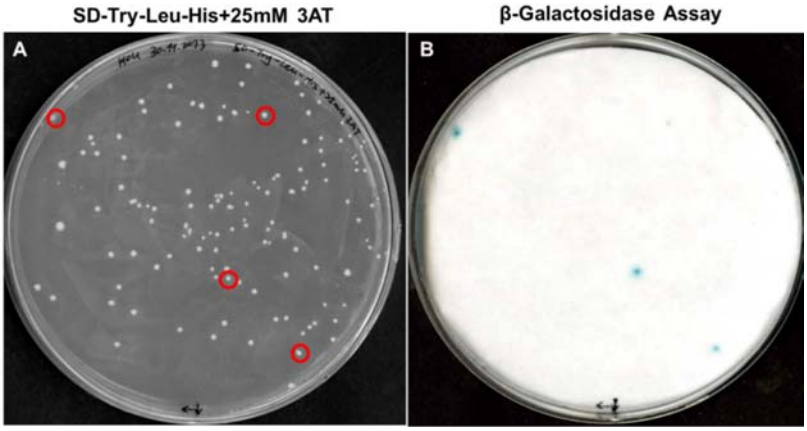


Fig. 38 Yeast two-hybrid library screening readout. (**A**) Example of the growth of the library transformed yeast bait colonies grown on SD-Try-Leu-His + 25 mM 3-AT media after 5 d incubation at 30 ºC. (**B**) The results of a β-galactosidase assay with these colonies after 12 h of incubation at 37 ºC. Colonies that accumulated blue stain were identified based on their positions on the corresponding growth media plates (marked with red circles shown in (**A**)).

The prey proteins in these 28 positive yeast colonies screened for *HIS3* and *LacZ* reporter activities were amplified by yeast colony PCR using the primers pACT2_fwd and pACT2_Gal4AD. However, no band was amplified from four yeast colonies, and two bands were amplified from one of the positive yeast colonies (**Fig. 40**). The PCR products of 23 positive yeast colonies were directly sequenced after purification. The identified prey proteins are listed in **Table 7**.

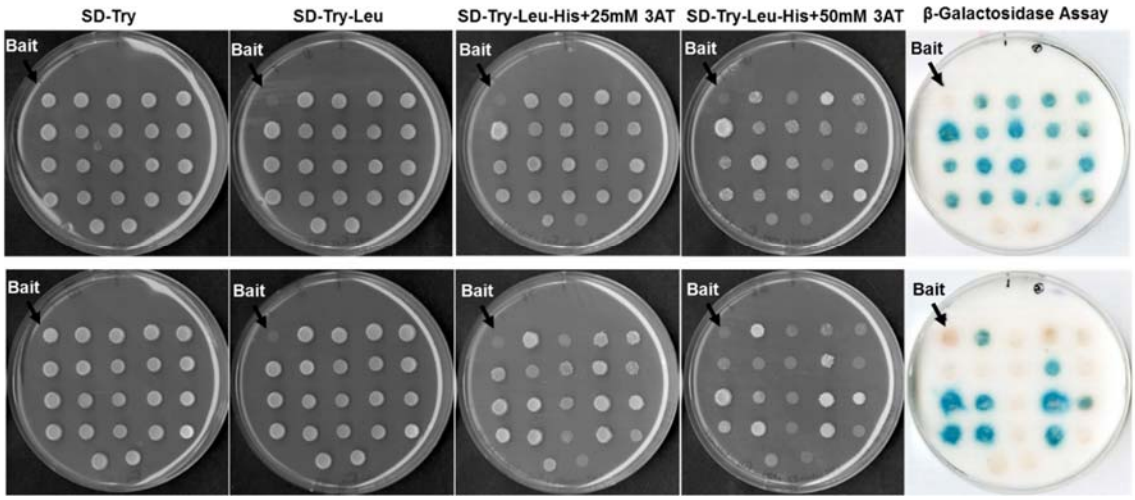


Fig. 39 bHLH146 yeast two-hybrid library screening readout. Colonies that accumulated blue compound were replicated on different SD media. (Photos in black background) The growth of yeast colonies that contain putative interacting preys on different SD media after 5 d incubation at 30 ºC. (Photos in white background) Results of β-galactosidase assays with these colonies after 12 h incubation at 37 ºC.

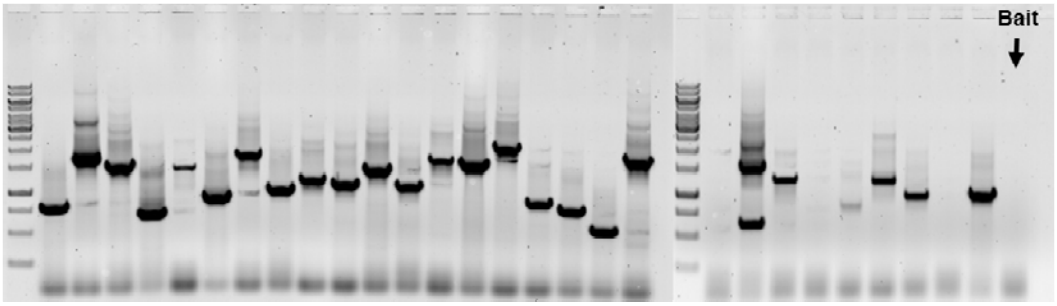


Fig. 40 PCR products of positive yeast colonies in bHLH146 yeast two-hybrid screening. Coding sequence of the prey proteins from the positive yeast colonies in bHLH146 Y2H screening were amplified by yeast colony PCR. The last lane is the negative control using the bait colony as template.

Table 7 Isolated proteins that interact with bHLH146 as identified by yeast two hybrid assays

Isolated protein	Description
AT1G68920	transcription factor bHLH49
AT4G30980	transcription factor bHLH69
AT1G26260	transcription factor bHLH76
AT1G26260	transcription factor bHLH76
AT1G04480	60S ribosomal protein L23
AT1G04270	40S ribosomal protein S15-1
AT2G17265	homoserine kinase (3-hydroxy-3-methylglutaryl-coenzyme A reductase 2)
AT5G56760	serine acetyltransferase 1
AT3G63220	F-box/kelch-repeat protein SKIP30
AT5G20020	GTP-binding nuclear protein Ran-2
AT3G16640	translationally-controlled tumor protein-like protein
AT5G12860	dicarboxylate transporter 1
AT4G12880	early nodulin-like protein 19
AT5G06860	polygalacturonase inhibitor 1
AT1G62380	1-aminocyclopropane-1-carboxylate oxidase 2
AT2G44100	guanosine nucleotide diphosphate dissociation inhibitor 1
AT5G15230	gibberellin-regulated protein 4 (GASA4)
AT1G51020	uncharacterized protein
AT5G06590	uncharacterized protein
AT5G39570	uncharacterized protein
AT5G47060	uncharacterized protein
AT3G01430	uncharacterized protein
AT5G14410	uncharacterized protein

3.3.5 Expression profiles of *bHLH146*

Spatial and temporal expression patterns of a gene suggest its role in plant development. The spatiotemporal expression patterns of *bHLH146* were identified by placing a *GUS* reporter gene downstream of its promoter. The promoter fragment of *bHLH146* was amplified using the primers At4g30180ProFwd2 and At4g30180ProRev and digested with restriction enzymes EcoRI and XbaI. The amplified 972 bp promoter fragment (-974 to -3 relative to translation start site) was cloned into the EcoRI/XbaI digested pBT10-GUS vector, and the construct was propagated in *E. coli* DH10B. A plasmid from one positive clone was digested with EcoRI and BglII to isolate the *bHLH146-promoter::GUS::nos_terminator* cassette, which was then sub-cloned into the EcoRI/BamHI digested binary vector pBIN19. A single positive clone was used to transform *A. tumefaciens* cells and further transformed into wild-type *A. thaliana* (ecotype Col-0) plants. GUS activity was observed in varies organs from three independent transgenic plants (**Fig. 41**). The bHLH146-promoter GUS activity was also compared with the gene expression data from Genevestigator. The data from 53 samples in Genevestigator showed that the expression of bHLH146 is at a low level and decreases as the plant develops from a young

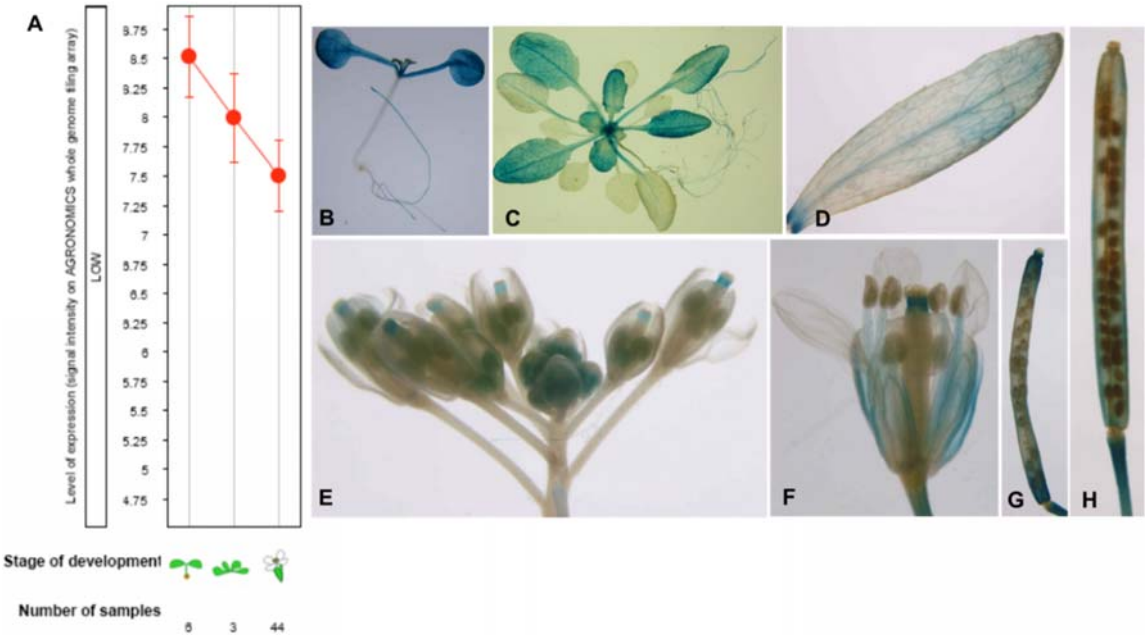


Fig. 41 Spatiotemporal expression of *bHLH146*. (**A**) *bHLH146* expression at different developmental stages. Microarray data were obtained from Genevestigator (https://www.genevestigator.com/gv/), (**B–H**) *In vivo* analysis of the *bHLH146* expression pattern using *bHLH146*::GUS reporter lines. GUS staining in (**B**) seedling, (**C**) rosette leaves, (**D**) cauline leaf, (**E**) floral cluster, (**F**) opened flower, (**G**) young silique, (**H**) mature silique.

seedling to the adult plant at the flowering stage. It is expressed at a low level during the whole develop process. This is in agreement with the GUS reporter gene observations. The young seedlings showed the strongest blue staining followed by vegetative leaves, whereas in reproductive organs the blue staining was only observed in styles reflecting the activity of the gene in this organ (**Fig. 41**). Interestingly, GUS staining showed an inhomogenous pattern in the roots of 2-week old seedlings and leaves of the vegetative stage.

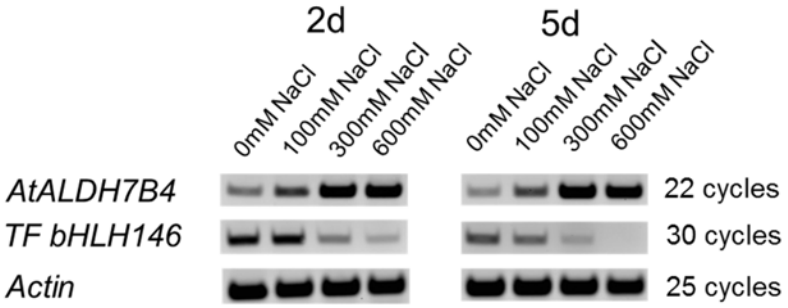


Fig. 42 Expression patterns of *ALDH7B4* and *bHLH146* under salt stress conditions. The plant material was the same as used for PCR analysis shown in **Fig. 16.** Plants were treated for two days (2d) or five days (5d) with different concentrations of NaCl (0 mM, 100 mM, 300 mM and 600 mM). Transcript abundance of *ALDH7B4* and *bHLH146* were determined using 22 and 30 cycles for amplification, respectively. *Actin* was used as reference gene to monitor the cDNA quality.

The gene response to salt stress was investigated using the same cDNA which was used in "3.2.1.3". Compared with the highly expressed *ALDH7B4* which shows clear expression profiles using 22 cycles, *bHLH146* was expressed at a low level after 30 cycles of amplification (**Fig. 42**). Although the expression of bHLH146 was low, expression was down-regulated under salt stress conditions. This suggests that the gene may be responsive to osmotic stress.

3.3.6 bHLH146 act as a transcription repressor

Expression of *bHLH146* was down-regulated by salt stress while *ALDH7B4* expression is upregulated under salt stress. Although bHLH146 did not bind to the promoter of *EsALDH7B4*, the yeast two-hybrid screening revealed that it can interact with some other bHLH transcription factors like bHLH49 and bHLH76 which are CIB (CRY2-interacting bHLH) proteins. There is evidence that CIB proteins can bind to an E-box (5'-CANNTG-3') (Liu *et al.* 2008; Liu *et al.* 2013). These studies showed the CIB proteins bind to an E-box with highest binding affinity with G-box (5'-CACGTG-3'). They bind to DNA in the form of homodimers or heterodimers, and both of the members in dimerization should have the DNA-binding ability. When these G-

box binding bHLH proteins dimerized with non-DNA binding bHLH proteins like bHLH146, these heterodimers are non-DNA binding complex. Therefore, it was investigated whether bHLH146 negatively regulates *ALDH7B4* expression in an indirect manner using ALDH7B4 promoter GUS reporter constructs.

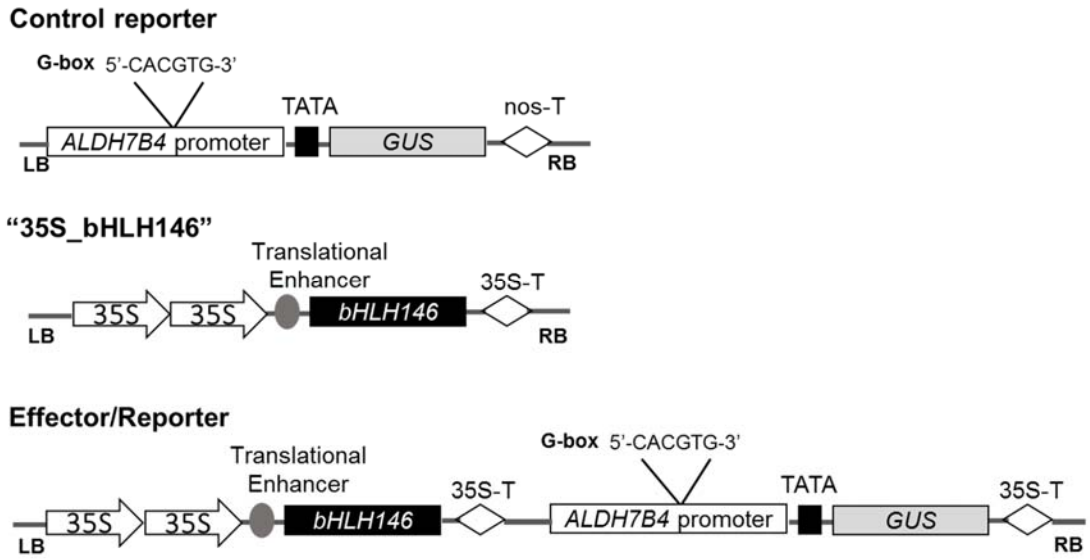


Fig. 43 The schematic diagram of the constructs used in the FAST transient expression assay. *ALDH7B4* promoter (white rectangle) and DNA sequence of the G-box, the double 35S promoter (white arrow), TATA box (black square), translational enhancer (grey ellipse), GUS reporter gene (grey rectangle), bHLH146 effector gene (black rectangle), terminator (white rhombus), and T-DNA [left border (LB) and right border (RB)] are indicated.

The construct that contains the *ALDH7B4-promoter::GUS::nos_terminator* cassette in the binary vector pBIN19 was used as the control GUS reporter (**Fig. 43**). To construct a *bHLH146* over-expressing effector, the coding sequence of bHLH146 was amplified from genomic DNA using the primers At4g30180for_NcoI and At4g30180_rev. The PCR product was cloned into the pJET1.2 vector, and the plasmid was digested with restriction enzymes NcoI and BglII. The resulting fragment was then subcloned into NcoI and BamHI digested pGJ280 vector. The *CaMV 35S_bHLH146_teminator* cassette was subsequently isolated from pGJ280 vector via the HindIII site and finally cloned into the binary vector pBIN19. This resulted in the construct "35S_ bHLH146". The construct "35S_ bHLH146" was used to generate *bHLH146* over-expressing transgenic plants (see next section). Here the FAST method (Li *et al.* 2009) was used for transient transformation of *Arabidopsis* seedlings, therefore the reporter and the effector was made in one construct to exclude the influence of unequal transformation

efficiency. For this, the *ALDH7B4-promoter::GUS::nos_terminator* cassette was isolated from the pBT10-GUS vector (Missihoun *et al.* 2014) with BamHI and BglII and subcloned into the BamHI site of the construct "35S_bHLH146" which resulted in the final effector and reporter construct "ALDH7B4::GUS_35S_ bHLH146" (**Fig. 43**).

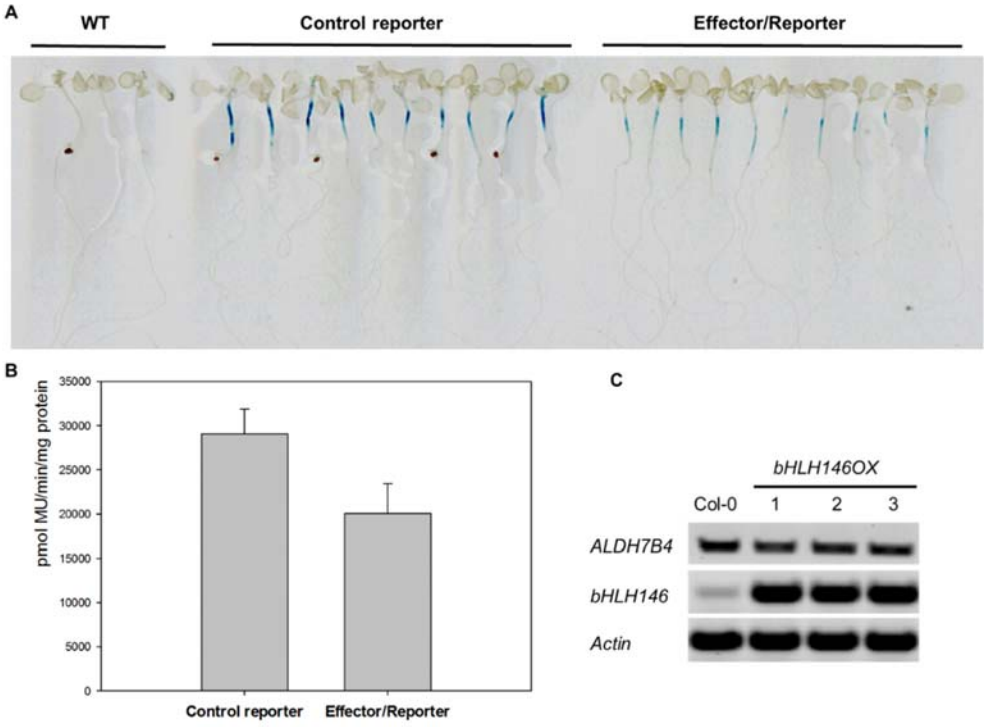


Fig. 44 bHLH146 represses *ALDH7B4* promoter activity. The control reporter construct and effector/reporter constructs (details see **Fig. 43**) were transformed into 2-week old *A. thaliana* using FAST method. (**A**) GUS reporter expression from control reporter construct and effector/reporter construct in transformed *A. thaliana* seedlings shown by GUS-staining, wild type (WT) seedlings as negative control. (**B**) GUS protein accumulation from control reporter and effector/reporter constructs measured as enzymatic activities of the GUS reporter protein. Error bars indicate S.E of three technical repeats. (**C**) Endogenous *ALDH7B4* expression in 6-week-old wild type (Col-0) and independent bHLH146-overexpression *A. thaliana* plants. Reverse transcription PCR analysis was performed with 28 cycles for *actin* and *bHLH146* and 25 cycles for *ALDH7B4* amplification.

The constructs of "Control reporter" and "Effector/Reporter" were transformed into two-week old wild-type seedlings separately as described in "2.2.6.7". A weaker blue staining in "Effector/Reporter" transgenic seedlings than in "Control reporter" transgenic seedlings was observed (**Fig. 44A**). The GUS activities in "Control reporter" and "Effector/Reporter" transgenic seedlings were further quantified by measuring the GUS enzymatic activities. Lower GUS activity in "Effector/Reporter" transgenic seedlings than in "Control reporter"

transgenic seedlings was further confirmed (**Fig. 44B**). In addition, transcript accumulation of endogenous *ALDH7B4* was compared between wild type and bHLH146-overexpression plants. Consistent with the GUS-reporter transient transactivation assay, the transcription level of *ALDH7B4* was slightly lower in bHLH146-overexpression plants than in wild-type plants (**Fig. 44C**). These results suggest that *bHLH146* is involved in repressing *ALDH7B4* expression, which is most likely through the G-box *cis*-element.

3.3.7 Generation of *bHLH146* over-expressing lines and *bHLH146* silencing and double *bHLH146* and *At2g18969* silenced transgenic plants

To further investigate the role of the transcription factor bHLH146, the T-DNA Express database was searched for T-DNA insertion mutants. Two T-DNA insertion lines SALK_060203 and SAIL_536_E01 were obtained from NASC (The European Arabidopsis Stock Centre). However, for unknown reasons, genotyping showed that only heterozygous plants for the SALK_060203 line were present on selection medium even after growing three generations (**Fig. 45**).

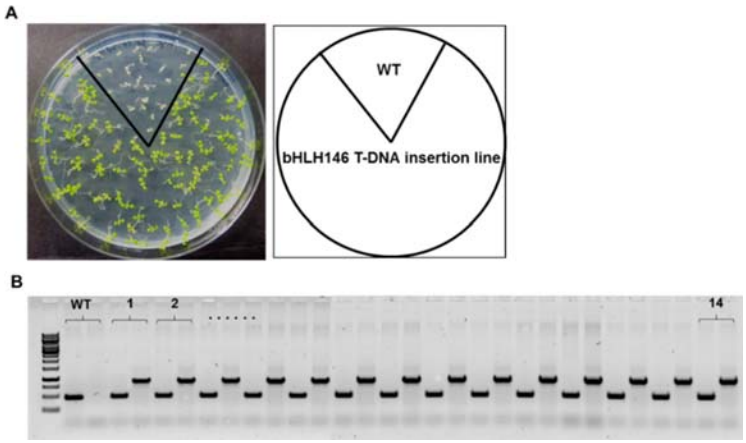


Fig. 45 Progeny of bHLH146 T-DNA insertion lines exhibit a non-Mendelian segregation (**A**) Growth of wild type and bHLH146 T-DNA insertion line SALK_060203 on MS medium with 50 mg/l kanamycin under the short-day condition. Photograph was taken when the seedlings were two-week old. (**B**) Genotyping of T-DNA insertion line SALK_060203. Genomic DNA isolated from both wild type (Col-0) and independent mutants as shown in (**A**). Complete CDS of *bHLH146* was amplified using gene specific primers At4g30180_for and At4g30180_rev (lower bands); gene/T-DNA junction was amplified using T-DNA primer LBa3 and gene specific primer At4g30180_rev (upper bands). Arabic numerals represent independent mutant plants.

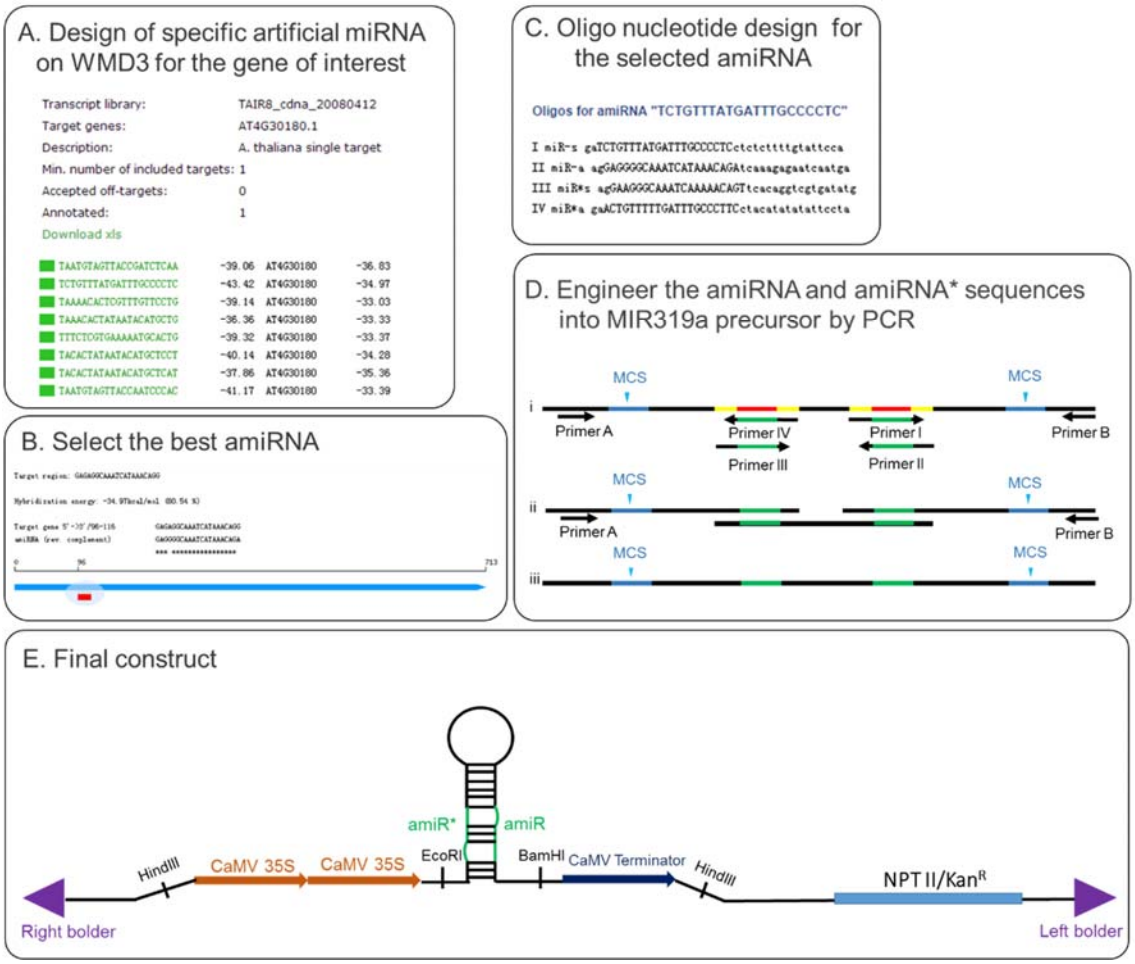


Fig. 46 Work flow of artificial microRNA cloning. (**A**) Specific artificial microRNAs that target the gene of interest were designed on WMD3 website (http://wmd3.weigelworld.org). (**B**) Select the best microRNA from the given candidates according to the selection criteria on WMD3. (**C**) Primer design for the selected microRNA on WMD3, choose the vector "RS300 21mer (MIR319a Arabidopsis thaliana)". (**D**) Engineering of the amiRNA and amiRNA* sequences into the *Arabidopsis* endogenous MIR319a precursor by PCR. (i) The original miRNA and miRNA* sequences of pRS300 (red) will be replaced by the artificial miRNA sequences (green) during the first PCRs. Sequences in RS300 complementary to the primers are indicated in yellow and the multiple cloning sites in blue. (ii)The three PCRs on pRS300 as template (A+IV, II+III, I+B) result in 3 DNA fragments. (iii) Fusion PCR on the 3 PCR products from (ii) with primers A+B results in one DNA fragment for subsequent cloning. (**E**)The final construct contains the cassette of CaMV35s_engineered MIRNA precursor_CaMV terminator in the binary vector pBIN19.

Unfortunately, after examining the T-DNA junction sequence in the SAIL_536_E01 line it was found that the T-DNA junction sequence is targeted to two different genes. Therefore, these two T-DNA insertion lines were not used. Instead, artificial microRNAs "TCTGTTTATGATTTGCCCCTC" targets bHLH146 and "TATGTTTCACGGTTTGTTCTC" targets both *bHLH146* and its paralog *AT2G18969* were designed from the Web MicroRNA

Designer (http://wmd3.weigelworld.org/) to reduce the level of the *bHLH146* transcript and *bHLH146* as well as *AT2G18969* transcripts. Primers for each microRNA were designed from Web MicroRNA Designer. amiRNA and amiRNA* sequences were then engineered into the endogenous MIR319a precursor using plasmid pRS300 as a template. After DNA sequence confirmation, the engineered amiRNA precursors were digested from pRS300 using EcoRI/BamHI and cloned into EcoRI/BamHI digested pGJ280 plasmid and amiRNAs were placed behind the CaMV 35S promoter. The 35S promoter::amiRNA cassettes were then isolated using HindIII from pGJ280 and cloned into the binary vector pBIN19 via the HindIII site. The whole procedure is shown in **Fig. 46**. Binary plasmids containing amiRNAs

Fig. 47 Representative wild type and bHLH146 overexpressing plants grown under normal conditions. (**A**) Seedling, (**D**) Leaves, (**F**) Floral buds and (**H**) flowers of wild-type plants; (**C**) Seedling, (**E**) Leaves, (**G**) Floral buds and (**I**) Flowers of bHLH146 over-expressing plants. (**B**) Rosettes of wild type (left) and bHLH146 overexpressing plants (right); (**J**, **K**) Siliques of wild type (left) and bHLH146 overexpressing plants (right).

precursors were transformed into wild-type *A. thaliana* Col-0 plants as described in "2.2.6.8". *bHLH146* overexpression lines were also generated using the construct "35S_ bHLH146" to compare the phenotype or performance under different treatments. Stable transformation of *A. thaliana* Col-0 plants was done as described in "2.2.6.8". The first generation of *bHLH146* overexpression lines and *bHLH146* silencing, *bHLH146* and *At2g18969* double silencing transgenic plants have been obtained. No obvious phenotype was observed for the silenced lines in the first generation of transgenic plants grown under non-stress conditions. Interestingly, lines overexpressing bHLH146 showed phenotypes different from wild-type plants. These phenotypes include curly leaves, dwarf, dark green leaves, strange flowers and short siliques (**Fig. 47**). This was observed in independent *bHLH146* overexpression lines.

3.3.8 *bHLH146* is involved in photomorphogenesis

CIB proteins interact with CRY proteins which are photolyase-like blue-light receptors that mediate light responses. The result of the yeast-two hybrid screening showed that bHLH146 interacts with CIB proteins, which implies bHLH146 might also be involved in light-mediated processes. To verify this speculation, seeds from the first generation of the transgenic plants were germinated on MS medium and grown in the dark. After 5 days, bHLH146 overexpressing plants displayed a short hypocotyl and an open apical hook (**Fig. 48A**). However, no significant phenotype difference was observed between wild-type plants and bHLH146 single silencing and bHLH146 and At2g18969 double silencing transgenic plants (**Fig. 48A**). This might be because the silencing effect is not sufficient, as the accumulation of the bHLH146 transcript is still detectable in these silencing lines (**Fig. 48B**). Examination under a confocal microscope showed that the hypocotyl cell length of bHLH146 overexpressing plants was much shorter than that of wild-type plants (**Fig. 48C**), indicating that the short hypocotyl phenotype is at least partially due to reduced cell elongation. Varying degrees of dwarfisms were observed in independent bHLH146 overexpressing lines (**Fig. 48D**). Gene expression analysis showed that the severity of the dwarf phenotypes is correlated with *bHLH146* accumulation levels, a higher level of *bHLH146* expression leads to more severe dwarfism of the plants (**Fig. 48D, E**). Further analysis will be done on these overexpression and silencing transgenic plants.

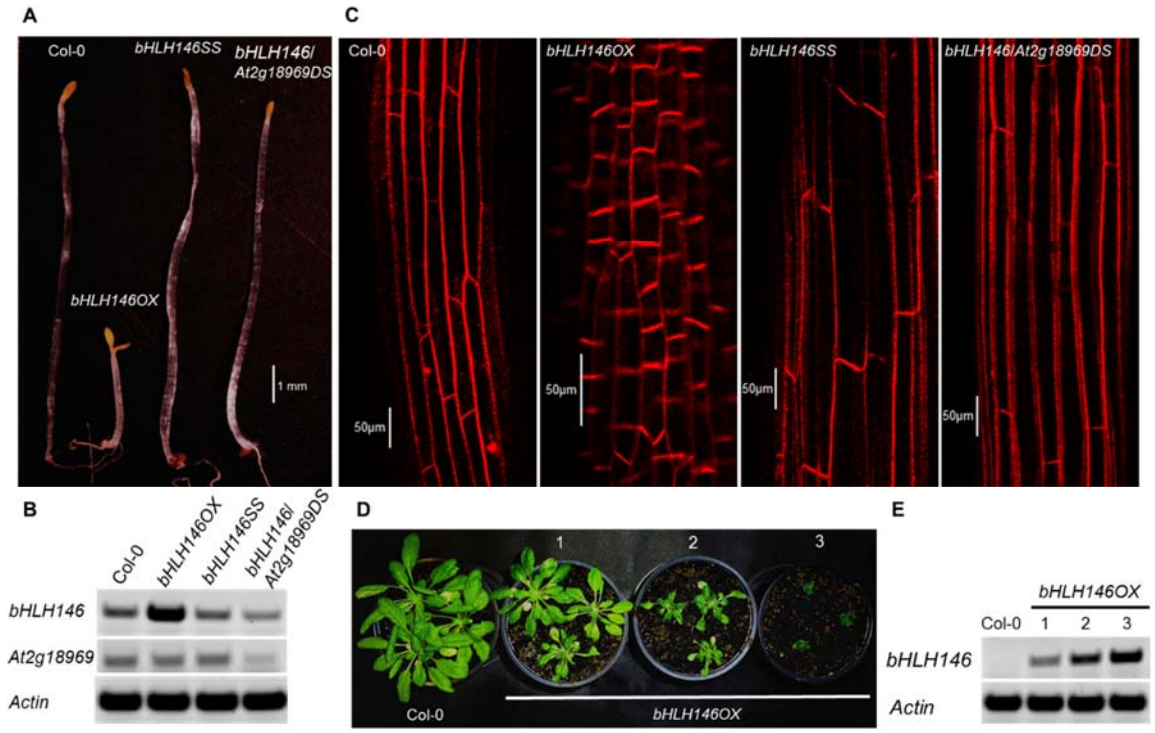


Fig. 48 bHLH146 inhibits cell elongation and is involved in photomorphogenesis. (**A**) bHLH146-overexpressing transgenic plants (*bHLH146OX*) showed a short hypocotyl and opened apical hooks in the dark. Artificial mircoRNA mediated *bHLH146* single silencing (*bHLH146SS*) and *bHLH146* and *At2g18969* double silencing (*bHLH146/At2g18969DS*) transgenic plants did not show significant phenotype differences compared to wild-type (Col-0) plants. Seedlings were grown in the dark for 5 d. Scale bar= 1 mm. (**B**) transcript accumulation of *bHLH146* and *At2g18969* in the plants are shown in (A), reverse transcription PCR was performed with 30 cycles of amplification. (**C**) Hypocotyl cells of *bHLH146OX* are shorter than those of wild-type plants. Seedlings shown in (A) were stained with FM4-64 and examined using a confocal microscope. Scale bar= 50 µm. (**D**) bHLH146-overexpressing transgenic plants (bHLH146OX) show dwarfism. Plants were grown for 6 weeks under short-day conditions. (**E**) *bHLH146* transcript levels in the bHLH146OX plants are shown in (D), reverse transcription PCR analysis was performed with 21 cycles for *bHLH146* amplification.

4. DISCUSSION

Drought and salinity are the most severe abiotic stresses limiting agricultural production. In the past twenty years, *A. thaliana* has been widely used as a genetic model plant to unravel the molecular basis of stress tolerance. Using this model plant, much knowledge has been obtained about how plants perceive, respond and adapt to various stress signals. Many genes and gene products that involve responses to drought and high-salinity stresses have been identified. *A. thaliana* is an excellent model to understand fundamental physiological, biochemical and molecular processes of flowering plants, but it is a stress-sensitive species. Therefore, it is difficult to explore the mechanisms of abiotic stress tolerance which have evolved in some other plant species, the so-called extremophiles. *Eutrema* species as close relatives of *Arabidopsis* have been suggested as a model for molecular research into plant stress tolerance (Bressan *et al.* 2001; Zhu 2001; Amtmann *et al.* 2005). The sequencing of the *Eutrema* genomes provide an invaluable resource for expanding potential comparisons at the biochemical, physiological and molecular level, exploiting orphan genes that are specific for these halophytes and comparing the alterations in transcription and *cis*-element structures of promoters (Dassanayake *et al.* 2011; Wu *et al.* 2012; Yang *et al.* 2013). The importance of using knowledge gained from research on model plants should facilitate the understanding of biological phenomena in crops and other plant species. However, using a model plant such as *Arabidopsis* or *Eutrema* cannot cover all the principles which are present in crops. As most of the crops are monocots, some of the mechanisms of tolerance to abiotic stresses may be different between monocots and dicots. So transferring knowledge from dicot model plants to the major crops is often not possible (Tester and Bacic 2005). With the development of high-throughput sequencing technologies, many other plant genomes besides model plants have been sequenced including many crops. The sequencing of the barley (*Hordeum vulgare L.*) genome not only provides an essential reference for genetic research and breeding on one of the world's first domesticated crops, but also allows to test the molecular biology knowledge gained from the plant model *A. thaliana* (Mayer *et al.* 2012).

4.1 Study of barley *CBF/DREB1* genes

4.1.1 Barley contains a large number of *CBF/DREB1* subgroup members but only a few members in the *DREB2* subgroup

The barley CBF/DREB genes share a similar structure both *inter se* and with CBF/DREBs of other species. The alignment of the AP2 DNA binding domain showed that barley, rice and *A. thaliana* share a highly conserved AP2 binding domain. Phylogenetic analysis showed that DREB1 and DREB2 subfamily members were clearly separated on the tree across the three species except for HvCBF7 and HvDREB1, which suggests that DREB1 and DREB2 subgroups were present prior to speciation. Reports demonstrate that these two subgroup genes are induced by different environmental factors and thus probably are involved in different pathways. In *Arabidopsis*, expression of the *AtDREB1* gene is induced by cold, but not by dehydration or high salinity (Liu *et al.* 1998; Shinwari *et al.* 1998). Similarly, *CBF/DREB1* genes also showed high expression in response to low-temperature treatment and the maximum transcript accumulation was detected one hour after exposure to 4 °C (Medina *et al.* 1999). The expression of *AtDREB2A* and *AtDREB2B* was induced by dehydration and high salt stress, but not by cold stress (Liu *et al.* 1998; Nakashima *et al.* 2000).

Although barley contains a large number of *CBF/DREB1* subgroup members, only a few members were identified in the *DREB2* subgroup. While *Arabidopsis* and rice contain eight and six members in the DREB2 subfamily, respectively, only three members (HvDREB1, HvDRF1 and HvDRF2) have been reported in barley. However, *HvDRF1* could generate three transcripts *HvDRF1.1*, *HvDRF1.2* and *HvDRF1.3* by alternative splicing. Cereals such as rice and barley have more members in the *CBF/DREB1* subfamily than *Arabidopsis* which contains six *CBF/DREB1* genes (Haake *et al.* 2002; Sakuma *et al.* 2002). This suggests that the expansion of the *CBF/DREB1* subgroup genes in rice and barley might occur after monocots and dicots split. Furthermore, the *CBF/DREB1* subgroup members expanded more in barley and wheat (Badawi *et al.* 2007) than in rice. The copy numbers variations of CBF/DREB1 among barley, wheat and rice could be due to their domestication patterns. While barley and wheat are tolerant to low-temperature rice is sensitive to low-temperature. The copy number difference among these plants supports the *CBF/DREB1* subgroup as a primary regulator of cold-stress responses.

4.1.2 Expression patterns of barley *CBF/DREB* genes are complex

High-throughput screening techniques such as microarray or RNA-Seq analysis have been performed to monitor gene expression changes in barley in response to drought and drought-related stresses. (Ozturk *et al.* 2002; Ueda *et al.* 2004; Talame *et al.* 2006; Walia *et al.* 2006; Guo *et al.* 2009). Most of these studies were conducted with a short period of dehydration shock (Ozturk *et al.* 2002; Ueda *et al.* 2004; Walia *et al.* 2006), only two of these reports simulated slow drought stress (Talame *et al.* 2006; Guo *et al.* 2009). Therefore, transcriptional changes in response to longer periods of water stress may not have been identified, even though they may be crucial to understand adaptation under field conditions (Talame *et al.* 2006). CBF/DREBs are considered as the major regulators of abiotic stress responses. Investigation of expression changes of CBF/DREBs under the simulated slow development of drought stress similar to field conditions can reflect expression profiles under real field conditions. This allows a comparison between controlled laboratory conditions and field conditions and may provide further insight into understanding drought tolerance of barley plants under realistic agricultural production conditions.

In this study, the *HvCBF4* transcript was only detected in the first week of the stress treatment of the year 2011 while the *HvCBF1* transcript was detected in all of the cultivars in the stress treatment of the year 2011 with an indistinct pattern (**Fig. 7**). So far, *HvCBF1* and *HvCBF4* expression has been reported only in response to cold-stress (Xue 2002; Oh *et al.* 2007). Contrary to the report of Xue (2003) in which *HvCBF2* was constitutively expressed in barley leaves, no *HvCBF2* transcript was detected in all cultivars in this study. *HvDREB1* and *HvDRF1.3* genes which are members of the *DREB2* subfamily, were shown to be up-regulated during drought stress (Xue and Loveridge 2004; Xu *et al.* 2009) whereas in the current study a constitutive expression pattern was observed for both the genes in field conditions as well as during high temperature stress conditions. These differences could be explained by different plant materials and different research designs. While four-week-old barley seedlings were used for the stress treatments in this study, ten-day-old barley seedlings were used in the literature. However, when ten-day-old barley seedlings were used for high temperature experiment, the *HvCBF2* transcript was detected in all the four cultivars under control conditions, which is consistent with the results obtained by (Xue 2003). This indicates that besides the cold induction, expression of *HvCBF2* probably also depends on the age and developmental state.

The other reason to explain the discrepancy could be that the treatments were different, and in fact, the treatments that applied here were different from those reported in the literature. Here we simulated drought stress conditions that occur in the field in a plastic greenhouse tunnel, in which the growth conditions vary according to external climate conditions, whereas in other experiments barley seedlings were air-dried on filter paper for a short period, which is a dehydration shock. The investigation of gene expression should be examined under controlled laboratory conditions to eliminate other environmental factors. However, it is important to note that plants face multiple abiotic stress factors in reality, which can interact and influence plant response at the physiological and molecular level. Expression profiles expected from literature are not found under field condition. Therefore, these experiments suggest that gene expression needs definitely to be tested for each new conditions and species.

4.2 Aldehyde dehydrogenase (*ALDH*) in *A. thaliana*, *Eutrema* halophytes and barley

4.2.1 Genomic organization, copy number and expression profiles of *ALDH* genes are mainly conserved in *A. thaliana* and *Eutrema* halophytes

In this study, 16 and 17 genes encoding members of 10 ALDH families were identified from *E. parvulum* and *E. salsugineum*, respectively (**Table 4**). *E. parvulum* has the same number of *ALDH* genes as *A. thaliana* while one more paralog designated as *EsALDH3F2* was identified in *E. salsugineum*. The *EsALDH3F2* gene is probably arisen by gene duplication after *E. salsugineum*, *E. parvulum* and *A. thaliana* diverged. Additionally, two pseudogenes resembling ALDH7B4 and ALDH10A8 were identified in *E. salsugineum*. According to Wu *et al.* (2012), these pseudogenes are located on different chromosomes as their corresponding paralogs. It is possible that they resulted from ectopic recombination of repetitive elements such as transposable elements as the genome of *E. salsugineum* contains a high number of repetitive sequences. Families 2, 5 and 10 are clustered together which suggests that these families have probably derived from a common ancestor. ALDH18 does not contain the conserved ALDH active site and thus it is the most distantly related family, which agrees with observations in other plant species.

Except for the ALDH family 3 in *E. salsugineum*, copy numbers are identical in *A. thaliana* and the two *Eutrema* species. This is in contrast to *HKT1* (sodium-potassium co-transporter)

genes related to the maintenance of ion equilibrium. These gene families are expanded in *Eutrema* with three *HKT1* genes in *E. salsugenium* and two *HKT1* genes in *E. parvulum* while only one copy is present in *A. thaliana* (Dassanayake *et al.* 2011; Ali *et al.* 2012; Wu *et al.* 2012). However, another comparative study on copy numbers in families among all the functional categories including transcription factors and SOS-like genes found that *A. thaliana* and *E. salsugenium* contain equal or near-equal numbers, and in most cases, *A. thaliana* has more copies than *E. salsugineum* (Yang *et al.* 2013). ALDH7 family genes are considered to be ancient genomic DNA sequences as they are broadly present in plants and animals and are highly conserved throughout evolution. Unlike soybean and woody plants *V. vinifera* and *P. trichocarpa* which contain multiple members of family 7, there is only one member in each of the two *Eutrema* species as in many other plants. It is not clear why this family has been lost from both marine unicellular algae species *O. tauri* and freshwater unicellular algae species *C. reinhardtii* and multicellular species *V. carteri* (Wood and Duff 2009; Brocker *et al.* 2013). It suggests that this family of proteins may play a fundamental role in land organisms, but it is not essential for aquatic algae.

High-throughput transcript analysis revealed that a number of universally stress-responsive genes are constitutively expressed in *Eutrema* (Inan *et al.* 2004), therefore expression of stress-associated *ALDH* genes was compared under different salinity stress conditions in *A. thaliana* and *E. salsugineum*. This was not observed for the selected *ALDH* genes. Most *ALDH* genes have a similar expression pattern in *A. thaliana* and *E. salsugineum* indicating that most *ALDH* genes are probably not functionally connected with the extreme salinity tolerance of *E. salsugineum*. However, the two salt responsive transcripts *ALDH3H1* and *ALDH7B4* increased in response to higher NaCl levels in *E. salsugineum* than in *A. thaliana* indicating that higher NaCl levels are required in *E. salsugineum* than in *A. thaliana* to trigger defense reactions. A differential expression profile was only observed for *ALDH10A8* which is down-regulated in *A. thaliana* under 600 mM NaCl but is upregulated in *E. salsugineum*. This differential regulation is probably due to differences in the promoter structures in the two species, but the functional promoter elements need to be identified experimentally. Comparison of the *ALDH7* promoter between *A. thaliana* and *E. salsugineum* showed a longer putative promoter region in *E. salsugineum* than in *A. thaliana* with several conserved blocks of functional promoter motifs in both species (Missihoun *et al.* 2014). Alterations in the sequences and *cis*-element

structures of promoters for other orthologous genes have also been found in these two species (Wu *et al.* 2012). Studies carried out so far indicated that genes involved in response to salinity are almost identical in *Arabidopsis* and *Eutrema* halophytes (Zhu 2000). This implies that the halophytic characteristics of *E. salsugenium* may be due to specific regulatory mechanisms rather than to increased copy numbers of genes or epigenetic effects on translational regulation. The analyses of the *ALDH* genes in *Arabidopsis* and *Eutrema* are in agreement with Zhu (2001) who hypothesized that halophytes generally use similar salt tolerance effectors and regulatory pathways as glycophytes and that subtle differences in the regulatory network account for variations in tolerance or sensitivity (Zhu 2001). Nevertheless there are some potential orphan genes in the genome of the *Eutrema* halophytes which also may contribute to tolerance mechanisms.

4.2.2 The *ALDH7B4* gene is a good candidate to compare gene regulatory mechanisms between the glycophyte *A. thaliana* and the halophyte *E. salsugineum*

Based on a survey of the distribution of halophytes in angiosperms, halophytes were found in 117 families and 34 orders (Saslis-Lagoudakis *et al.* 2014). Many of the families with a high proportion of halophytes are found in the orders Alismatales, Caryophyllales, Malphigiales, Fagales and Zygophyllales. Twenty-one out of 3710 species are halophytes in the Brassicaceae family to which *A. thaliana* and *E. salsugineum* belong. The distribution of halophytes among taxonomic groups shows that halophytes are found in at least a quarter of the angiosperm families, and most salt tolerant species have close non-salt tolerant relatives. Proteins from salt tolerant species have homology with non-salt tolerant relatives and have similar functions. This implies that halophytes may use different regulatory mechanisms to produce functionally similar homologous proteins that confer salt tolerance.

ALDH7B4 belongs to the ALDH7 family which is also known as antiquitins and is highly conserved throughout evolution. The amino acid sequence identity among members of the family 7 of ALDH proteins is about 60-80%, making them to one of the most evolutionarily conserved eukaryotic proteins (Lee *et al.* 1994; Fong *et al.* 2006; Wu *et al.* 2007). The high degree of sequence similarity among species indicates an essential conserved role. Studies have shown that expression of plant *ALDH7B4* is turgor-responsive (Kirch *et al.* 2004). The garden pea (*Pisum sativum*) ALDH7B1 (previously was known as turgor protein 26g) was found to

be induced by dehydration (Guerrero *et al.* 1990). The homolog btg-26 from *Brassica napus* was reported to be induced by dehydration, high salinity, low temperature, heat shock and ABA (Stroeher *et al.* 1995). In *Arabidopsis*, the ALDH7B4 protein showed a strong induction by osmotic stress and ABA (Kirch *et al.* 2005). Transgenic *Arabidopsis* plants overexpressing *ALDH7B4* showed osmotic and oxidative stress tolerance (Kotchoni *et al.* 2006). Similarly, reduced MDA levels and decreased sensitivity to hydrogen peroxide and methyl viologen were observed in tobacco and *Arabidopsis* ectopically expressing the soybean (*Glycine max*) *ALDH7* gene (Rodrigues *et al.* 2006), whereas rice *ALDH7* T-DNA insertion mutants showed increased sensitivity to dehydration, salinity, cold, heat and methyl viologen (Shin *et al.* 2009). Taken together, these studies suggest that ALDH7B4 plays an important role in abiotic stress adaptation and tolerance. Although salt-induced *ALDH7B4* expression was observed in both *A. thaliana* and *E. salsugineum*, it only occurred under high salt conditions in the halophyte *E. salsugineum* (**Figs. 16, 17**). *ALDH7B4* is highly expressed in *E. salsugenium* only when it is necessary under high salt conditions when aldehydes accumulate. This suggests that a regulatory mechanism allows adaptation to high salt in *E. salsugineum*. Therefore, *ALDH7B4* was chosen to compare regulatory mechanisms between glycophyte *A. thaliana* and halophyte *E. salsugineum*.

4.2.3 The genetic background plays an important role in the regulation of gene expression

ALDH7B4 was shown salt inducible in both *A. thaliana* and *E. salsugineum* as well as in barley. However, while the endogenous *HvALDH7B4* transcript was inducible under salt stress conditions, GUS activity was not detected in the *A. thaliana* harboring the *HvALDH7B4::GUS* cassette under variable conditions including salt stress. This can most likely be explained by the different genetic backgrounds. The lower efficiency of monocot promoters in dicots has already been reported (Schledzewski and Mendel 1994; Shimamoto 1994). Promoters derived from monocot species often fail to exhibit a regulated pattern of expression in transgenic dicots, although the corresponding transcripts are highly regulated in monocots (Shimamoto 1994). For example, maize polyubiquitin1 promoter and the *Emu* promoter were very weakly expressed in tobacco, and no activity of the rice actin1 promoter was detected in tobacco (Schledzewski and Mendel 1994). The weak activities of the strong monocot promoters in the dicot plants could be due to the presence of an intron. Studies on the processing of monocot

and dicot pre-mRNAs in tobacco allowed Keith and Chua (1986) to conclude that differences may exist in sequences required for RNA processing between monocot and dicot plants. Monocot introns are spliced at lower rates in dicots than in monocots (Keith and Chua 1986). Similarly, Goodall and Filipowicz (1991) indicated that monocots differ from dicots in their mechanism of intron recognition. They showed that monocot splicing seems to be more "permissive" than dicot splicing since the monocot maize was shown to be able to recognize and splice many introns that were spliced poorly or not at all in tobacco (Goodall and Filipowicz 1991). The importance of the first intron of the Gos-2 gene was also demonstrated by Assem *et al.* (2002) who showed that the maize Gos-2 promoter is highly efficient in increasing the transcription level in maize in the presence of an intron, while the transcription rate decreased in the presence of an intron between the promoter and the reporter gene in tomato (Assem *et al.* 2002). Here, the 1.1 kb nucleotide sequence upstream of the translation start codon of the *HvALDH7B4* gene was analyzed, and the presence of an intron is highly possible within this fragment.

In this study, one of the objectives was to transform the different *EsALDH7B4::GUS* cassettes into both *A. thaliana* and *E. salsugineum* and to compare how the same promoters work in different genetic backgrounds. However, due to difficulties in transformation and obtaining *E. salsugineum* transformants, only four transgenic *E. salsugineum* plants have been obtained, two harboring TP0, one harboring No“TC” Tp0 and one harboring TP6. Since *A. thaliana* and *E. salsugineum* belong to the same family and are close relatives, a similar GUS activity from the same promoter-GUS cassette was expected in the different transgenic genetic backgrounds. Compared to transgenic *A. thaliana* lines, TP0 and No“TC” TP0 showed lower GUS activity in the transgenic *E. salsugineum* plants under the given treatments (**Fig. 28**). These results are consistent with the gene expression patterns of the endogenous *EsALDH7B4* that has a higher threshold in responding to salt stress, which indicates that the 731 bp *EsALDH7B4* promoter fragment has retained the activation pattern of the chromosomal *EsALDH7B4* gene. However, the similar GUS activity of *EsALDH7B4* and *AtALDH7B4* promoter in transgenic *A. thaliana* indicates that *EsALDH7B4* promoter lowered its threshold to respond to salt and drought stress as the saying “when in Rome, do as Romans do”. The genetic background somehow likes the so-called “Rome” and the inner physiological environment in the specific plants likes the “Rome unique culture” decides or domesticates the gene expression (what Romans do), no

matter whether the gene is endogenous or exogenous. In addition, GUS activity of the short promoter TP6 still can be detected in transgenic *E. salsugineum*, whereas nearly no GUS activity was shown in three independent transgenic *A. thaliana* plants harboring the same TP6::GUS cassette. The different expression patterns of endogenous *ALDH7B4*, *ALDH3H1* and *ALDH10A8* present in *A. thaliana* and *E. salsugineum* could be explained either from different *cis*-elements or due to different genetic backgrounds. Using the GUS reporter, the same *EsALDH7B4::GUS* cassette showed different performance in *A. thaliana* and *E. salsugineum*. This suggests that the genetic background may be important in regulating gene expression as the effect from *cis*-elements was excluded.

4.2.4 Proposed pathways regulating *EsALDH7B4* expression

Although the intergenic region between *ALDH7B4* and the up-stream gene is longer in *E. salsugineum* than in *A. thaliana* (**Fig. 18**), conserved nucleotide blocks are present within the 0.6 kb region upstream of the translation start codon in five selected Brassicaceae species including *A. thaliana* and *E. salsugineum* (Missihoun *et al.* 2014). The endogenous *EsALDH7B4* gene is responsive to high salinity stress. The promoter-GUS reporter gene analysis in *A. thaliana* showed similar response patterns to stress factors for *AtALDH7B4* and the *EsALDH7B4* promoters thus suggesting that the two promoters located within the 0.6 kb regions share key *cis*-elements conferring similar stress responsiveness. The *AtALDH7B4* promoter has been well studied by promoter deletion and point mutations. Results revealed that three ACGT-containing *cis*-elements are important for the gene expression. The motifs ACGT2 and ACGT3 near to the translational start codon are relevant for salt and dehydration stress responses (Missihoun *et al.* 2014). Consistent with the *EsALDH7B4* promoter deletion analysis, GUS activity was not detected in TP7 deletion lines in which all the three ACGT motifs were deleted. The ACGT1 motif distal to the translation start codon is the core sequence of the G-box (CACGTG) in both *AtALDH7B4* and *EsALDH7B4* promoters. Results from both promoter deletion analysis and yeast one-hybrid screening showed that this motif plays a crucial role in regulating expression of *EsALDH7B4*. GUS activity was nearly abolished when the G-box was deleted in TP6 lines in transgenic *A. thaliana* (**Figs. 26, 27**) and some identified transcription factors are from bZIP, bHLH families that bind to the G-box, thereby indicating that several transcription factors regulate the *EsALDH7B4* expression through the G-box. The

ACGT-containing motifs are conserved in the analyzed *AtALDH7B4* and *EsALDH7B4* promoter regions, but one DRE/CRT *cis*-element in the *AtALDH7B4* promoter that proved to be essential for activation by ABA is not present in the *EsALDH7B4* promoter. In contrast, the *EsALDH7B4* promoter contains a "TC" rich motif (mainly composed of three "CCCTTCCCC" repeats) that does not exist in the *AtALDH7B4* promoter. Analysis showed that the "TC" motif played a negative role in the expression of *EsALDH7B4* when tested in transgenic *A. thaliana*. However, the repression effect was not observed in transgenic *E. salsugineum* by comparing TP0 and No "TC" transgenic lines (**Fig. 28**). These differences either might be due to the different genetic backgrounds or could also be the result of not having enough different transgenic *E. salsugineum* lines available. Yeast one-hybrid screening using the "TC" motif as bait identified a MYB_like transcription factor which has been reported as a transcriptional repressor (Ikeda and Ohme-Takagi 2009). There is no information available about the DNA binding motif which is recognized by the MYB_like transcription factor, but the role of repressor supports the result from the *EsALDH7B4*promoter deletion analysis in transgenic *A. thaliana* plants. The interaction between the MYB_like transcription factor and the "TC" motif could be another reason for the very low expression of endogenous *EsALDH7B4* under low salt conditions. It is suggested that the MYB_like transcription factor is either modified or degraded under high salt conditions, thereby releasing the repression of *EsALDH7B4* expression.

The results from previous and present studies indicate that *EsALDH7B4* like *AtALDH7B4* codes for versatile osmotic stress-responsive proteins involved in both biotic and abiotic stress responses. We propose a model, which combines our previous results on *AtALDH7B4* (Kirch *et al.* 2005; Missihoun *et al.* 2014) and the data from this study with the currently available literature to summarize our understanding of *EsALDH7B4* regulation in response to osmotic stress in vegetative tissues and seeds (**Fig. 49**). In this model, *EsALDH7B4* as well as *AtALDH7B4* is induced by osmotic stress generated externally (wounding, dehydration, salt) and from endogenous processes (seed desiccation). Similar to *AtALDH7B4*, the expression of *EsALDH7B4* in response to wounding, dehydration or salt is likely to be mediated by PLD- and ABA-signaling components. The dehydration response may additionally be through lipid signaling (Kirch *et al.* 2005). As in leaves, the expression of *EsALDH7B4* in seeds appears to involve both PLD- and ABA-signaling pathways. At the promoter level, the ACGT-containing

motifs are required for the induction by salt, dehydration and wounding. The ACGT1 motif which is the core sequence of the G-box in the promoter is particularly important for both *AtALDH7B4* and *EsALDH7B4* expression. The "TC" motif that is specifically present in the *EsALDH7B4* promoter might be responsible for activating gene expression under high salt conditions in *E. salsugineum*. This makes *EsALDH7B4* highly expressed in *E. salsugenium* only when it is necessary. It indicates that gene regulation allows adaptation to high salt in *E. salsugineum*. bZIP, bHLH,and ERF/AP2 transcription factors and the MYB_like transcription factor proteins interact with the G-box, GCC-motifs, and the "TC" rich motif both in leaves and seeds to regulate gene expression.

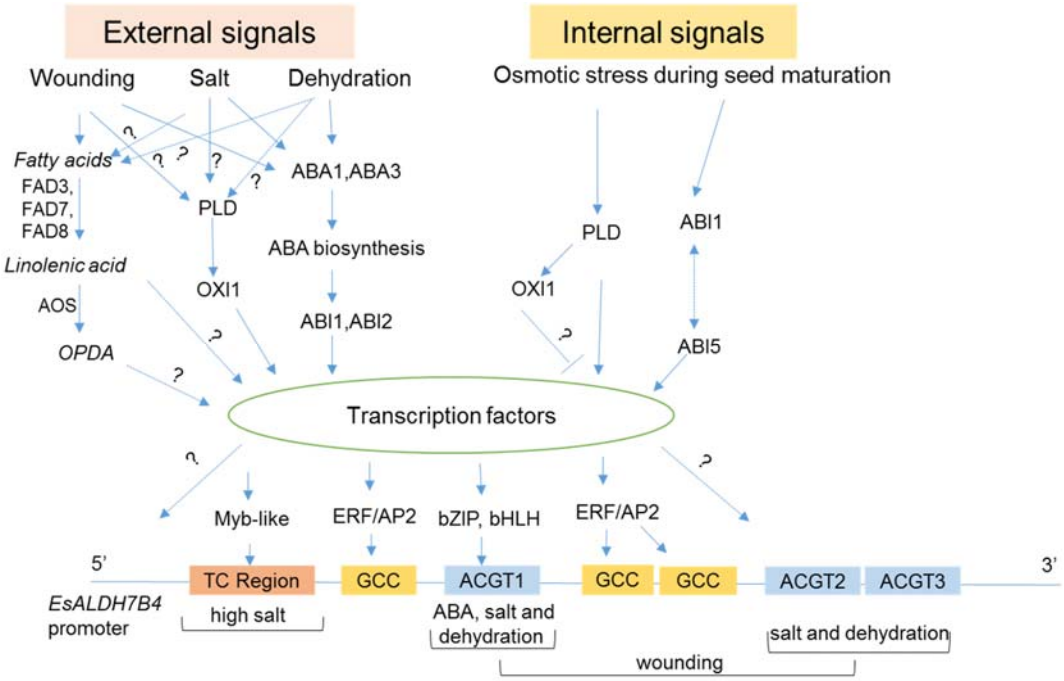


Fig. 49 Proposed regulatory pathways for osmotic stress-induced *EsALDH7B4* expression in leaves upon external stimuli and in seeds. In this model, *EsALDH7B4* is induced by osmotic stress generated externally (wounding, dehydration, salt) or from endogenous processes (seed desiccation). The expression of *EsALDH7B4* in response to wounding, dehydration, salt and in seeds is mainly mediated by PLD- and ABA-signaling components. bZIP, bHLH, ERF/AP2 family transcription factors especially the two identified G-box binding proteins and the MYB_like transcription factor are the main *trans*- regulators. At the promoter level, the ACGT 1, ACGT 2 and ACGT 3 motifs are required for the induction by salt and dehydration. Continued arrows indicate steps or routes for which evidence has been provided either from previous work, this study, or from the literature. Dotted arrows with a question mark indicate hypothetical routes or steps, which require experimental validation. The discontinued line with a question mark indicates a probable inhibition. Compounds are in italics. Proteins are in capital letters and regular font. OPDA 12-oxo-phytodienoic acid, ABA abscisic acid, FAD fatty acid desaturase, ABA1, ABA3 ABA biosynthesis genes, ABI1, ABI2, ABI5 ABA insensitive/ABA-signaling genes, PLD phospholipase D, OXI1 oxidative signal-inducible 1, AOS allene oxide synthase. This model is based on the previous model established by Missihoun *et al*. (2014).

4.2.5 *Pros* and *cons* of *E. salsugineum* being a model

As discussed above, *Arabidopsis* has limitations on exploration of stress adaptive and dependent responses in this plant. To explore the putative genes and gene products that involve responses to varied abiotic stress conditions and to understand the evolution of plant adaptation to extreme environments, scientists tried to find good stress-tolerant plant models. Given the long history of *Arabidopsis* as a model system, plants that are useful for stress physiological studies at the genomic level are those which are closely related to it. *E. salsugineum* is a close relative of *Arabidopsis* and belongs to the same Brassicaceae family. Dating analysis showed that the *E. salsugineum* and *Arabidopsis* diverged around 43.2 million years ago (Yang *et al.* 2013). Its close relationship to *Arabidopsis* and its growth at saline, cold, freezing, and resource-poor habitats makes it a good model for studying plant adaptation to extreme environments. *E. salsugineum* resembles *Arabidopsis* in many features including self-fertility, small genome, good seed production and genetically transformable by using *Agrobacterium tumefaciens*. In addition, the genome sequence of *E. salsugineum* has become available which facilitates gene discovery and comparative studies (Wu *et al.* 2012; Yang *et al.* 2013).

Besides the advantages, *E. salsugineum* also has some disadvantages as an experimental system. Despite its ability to grow in extreme conditions, its slow growth rate is reflected in nearly every developmental stage including germination and flowering. Compared with *Arabidopsis*, the wild-type *E. salsugineum* needs a three-week period of vernalization to flower, which makes its lifecycle longer than *Arabidopsis*. Although it can be genetically transformed using the floral dip method, the ratio of positive seeds is lower than for *Arabidopsis*. The reason for the difficulty is probably due to the slow growth rate of *E. salsugineum* as the floral bud did not open during the optimal period for *Agrobacterium* infection. In contrast, unopened floral buds of *Arabidopsis* can open within 24 hours after dipping. Thus *Agrobacterium* could still infect the opened floral *Arabidopsis* bud efficiently. In addition, *E. salsugineum* seedlings grown on MS medium were vulnerable especially to kanamycin in the medium. During the screening process, different kanamycin concentrations (20, 25, and 50 μg/ml) were tested. It was found that 25 μg/ml of kanamycin was optimal as the lower concentration (20 μg/ml) did not suppress the growth of the wild-type seedlings and the higher concentration (50 μg/ml) killed the positive seedlings. During the screening procedure, the light intensity is a critical factor, as high light intensity (approximately 13,000 Lux) would kill the putative positive

seedlings grown on MS medium in the presence of 25 μg/ml kanamycin. Therefore, low light intensity (approximately 800 Lux) is required for a successful screening process. Care also has to be taken when the putative positive seedlings are transferred from MS medium to soil, and a translucent plastic cover is absolute necessary for covering the transferred seedlings, otherwise the seedlings will rapidly dry and die after being transferred to soil. Despite these disadvantages, *E. salsugineum* is a good choice for a stress-tolerant model as salt tolerance in angiosperms is rare, with only 0.25% of angiosperm species able to complete their life cycles in saline conditions (Flowers *et al.* 2010).

4.3 Characterization of the unknown transcription factor bHLH146

4.3.1 bHLH146 acts as a transcriptional repressor

Transcription factors generally contain one or more DNA-binding domains, a trans-activating domain and an optional signal sensing domain. However, bHLH146 was predicted to be a non-DNA binding protein as it lacks the conserved amino acids Glu-13 and Arg-17 that are necessary for binding to the E-box in the basic region (Toledo-Ortiz *et al.* 2003). Its DNA binding ability was further tested *in vitro* using EMSA and DNA footprinting assays (**Fig. 35**). The result was consistent with the previous prediction that bHLH146 cannot directly bind to the *EsALDH7B4* promoter fragment which contains a G-box motif. The result does not rule out that bHLH146 is a non-DNA binding protein, but at least it is a non-G-box binding protein according to our results.

Localization analysis showed that this protein is mainly targeted to the nucleus (**Fig. 36**) which suggests that the protein might regulate gene expression by forming a complex with other proteins. It has been widely shown that transcription factors from bHLH (Hao *et al.* 2012; Liu *et al.* 2013), bZIP (Lee 1992), WRKY (Chi *et al.* 2013), NR, MADS-box, HD-ZI families (Amoutzias *et al.* 2008) usually interact with other transcription factors mainly forming dimers within the same family members. When the *AtALDH7B4::GUS* reporter gene containing the 0.6 kb *AtALDH7B4* promoter was expressed together with the effector bHLH146, the *AtALDH7B4::GUS* expression was decreased (**Fig. 44**). These results suggest that bHLH146 acts as a transcriptional repressor most likely by forming heterodimers with other G-box binding activators perhaps by blocking the G-box binding site. The animal non-DNA binding proteins ID-HLHs have been shown to act as negative regulators of MyoD by inhibiting the

sequence-specific DNA-binding activities of MyoD and E47 (Fairman *et al.* 1993). Similar interactions have also been reported in plants. PAR1 (HLH protein) inhibits PIF4 (bHLH protein) mediated transcriptional activation by forming heterodimers, and thus blocking PIF4 DNA binding (Hao *et al.* 2012). The results from the yeast two-hybrid screening support this assumption. Three bHLH proteins (bHLH49, bHLH69, bHLH76) were identified. bHLH76 and bHLH49 are both from the subfamily 18 and are also known as CIB5 (CRY2-interacting bHLH 5) and CIL1 (CIB1 LIKE PROTEIN 1) that are involved in floral initiation by interacting with CRY2 (cryptochrome 2) (Liu *et al.* 2013). bHLH69 belons to the subfamily 17 and was reported to be involved in the modulation of circadian rhythm (Hanano *et al.* 2008) and root hair development (Karas *et al.* 2009). bHLH69 is one of the three *Arabidopsis* homologs of the *Lotus japonicus* ROOTHAIRLESS1 (LjRHL1) gene and thus also known as LRL2. All of the three bHLH proteins were predicted to bind to an E-box (Toledo-Ortiz 2003). It was shown that CIB5 and CIL1 have the highest binding affinity to a G-box both *in vitro* and *in vivo* (Liu *et al.* 2008; Liu *et al.* 2013).

Two genes encoding enzymes involved in protein modification (i.e., homoserine kinase and serine acetyltransferase) were also identified from the yeast two-hybrid screening. Although homoserine kinase has the highest activity with the substrate homoserine, L-threonine is a competitive inhibitor of the substrate L-homoserine and both L-threonine and L-homoserine bind to the same site of the non-allosteric *E. coli* homoserine kinase (Burr *et al.* 1976). A homoserine kinase isozyme has also been reported in *Pseudomonas aeruginosa* with *in vivo* phosphoserine phosphatase activity (Patte *et al.* 1999; Singh *et al.* 2004). Phosphorylation and acetylation are key posttranslational modifications that may affect properties of a transcription factor critical for its biological functions (Soutoglou *et al.* 2000; Whitmarsh and Davis* 2000). As the bHLH146 protein contains several serine and threonine residues (**Fig. 32**), it may be possible that the bHLH146 will be activated or deactivated through phosphorylation or acetylation under specific physiological conditions. In addition, bHLH146 also interacts with the F-box/kelch-repeat protein SKIP30 which is a component of SCF(ASK-cullin-F-box) E3 ubiquitin ligase complexes (Risseeuw *et al.* 2003). The interaction between bHLH146 and SKIP30 suggests that ubiquitin can target bHLH146 under special conditions, which leads to degradation of bHLH146. A GTP-binding nuclear protein Ran-2 was also shown to interact with bHLH146. The GTP-binding nuclear protein Ran-2 have been reported involve in

nucleocytoplasmic transport and required for the import of protein into the nucleus and also for RNA export (Moore and Blobel 1993). This may be the pathway how bHLH146 is transported to the nucleus. The nuclear localization of bHLH146 has been experimentally confirmed in this study (**Fig. 36**). Thus, bHLH146 could be imported into the nucleus with the help of a GTP-binding nuclear protein Ran-2 by recognizing the NLS of bHLH146 (**Fig. 32**). Based on these assumptions, a model is proposed to show how bHLH146 functions and regulates gene expression (**Fig. 50**).

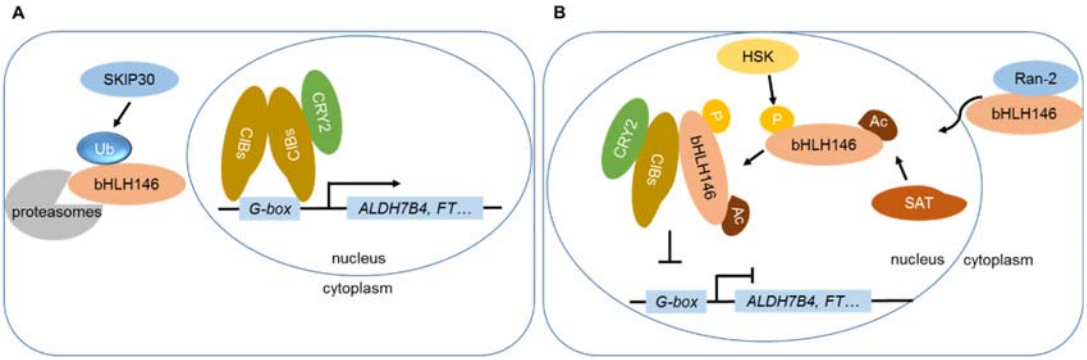


Fig. 50 A hypothetical model to show how bHLH146 regulates transcription. (**A**) Once bHLH146 is targeted by SKIP30, SKIP30 mediates ubiquitination thereby leading to the degradation of bHLH146. Under these circumstances, the CRY2 linked CIB proteins can form homodimers or heterodimers with CIB proteins in the nuclei and regulate downstream gene expression. (**B**) bHLH146 can be transported into nuclei with the help of Ran-2, it can then be activated by phosphorylation or acetylation with HSK or SAT, respectively. The native or activated bHLH146 forms non-G-box binding heterodimers with the CRY2 linked CIB proteins, thus preventing CIBs from binding to DNA. Thus, inhibits CIB-mediated transcriptional activation. (The rectangle and ellipses represent plant cells and nuclei, respectively).

In this model, bHLH146 acts as a repressor of gene expression by forming heterodimers with G-box binding bHLH proteins. Once the bHLH146 protein is targeted by SKIP30, SKIP30 will introduce E3 ubiquitin to bHLH146 which leads to degradation of bHLH146. Therefore, G-box binding bHLH proteins (here use CIBs as they were experimentally confirmed to interact with bHLH146) can dimerize with G-box binding bHLH proteins without competition from bHLH146. Thus, these G-box binding bHLH proteins can activate downstream gene expression (**Fig. 50A**). However, when bHLH146 is released from SKIP30 and transported into nuclei with the help of RAN-2, it will form heterodimers with G-box binding bHLH proteins. These heterodimers form a non-G-box binding complex as bHLH146 does not have a G-box binding ability. This will inhibit G-box binding bHLH protein-mediated transcriptional activation (**Fig. 50B**). In this model, it is suggested that posttranslational

modifications will activate bHLH146 interaction with G-box binding bHLH proteins. However, posttranslational modifications on bHLH146 still need to be experimentally demonstrated.

4.3.2 Biological pathways which might involve bHLH146

Yeast two-hybrid screening provided some evidence in which pathways bHLH146 may participate. Among the identified interactors, several members (GTP-binding nuclear protein Ran-2, Guanosine nucleotide diphosphate dissociation inhibitor 1 and translationally-controlled tumor protein-like protein) are associated with GTPase. As GTPases act as molecular switches for a vast number of cellular processes in all eukaryotes (Vernoud *et al.* 2003), interaction with these GTPase modulators indicates that bHLH146 might be involved in a broad range of cellular processes. The GTP-binding nuclear protein Ran-2 is a small GTPase which cycles between GDP- and GTP-bound states. This GTP binding and hydrolysis is linked with transport into or out of the nucleus (Moore and Blobel 1993; Vernoud *et al.* 2003). Therefore, it is assumed that Ran-2 plays a role in nucleocytoplasmic transport of bHLH146. Guanosine nucleotide diphosphate dissociation inhibitor 1 (GDI 1) negatively regulates members of the Rab GTPase family antagonizing the activity of the GDP/GTP exchange factors (GEF). GDIs form a stable complex with GDP-bound Rab proteins (Žárský *et al.* 1997). They thereby prevent binding of Rab GTPases to membranes and promote the dissociation of GDP-bound Rab GTPases from membranes. The translationally controlled tumor protein (TCTP) is an important component of the TOR (target of rapamycin) signaling pathway, the major regulator of cell growth in animals and fungi (Berkowitz *et al.* 2008). TCTP is a modulator of GTPase activity although opposite effects have also been reported as the guanine nucleotide-free chaperone (Thaw *et al.* 2001) or guanosine nucleotide diphosphate dissociation inhibitor of the translation elongation factor eEF1A (Cans *et al.* 2003). Progeny of heterozygous transgenic *Arabidopsis* plants harboring a T-DNA insertion in *TCTP* gene showed a non-Mendelian 1:1 segregation, from which only wild-type and heterozygous plants were obtained. The reason for the non-Mendelian segregation is because the *tctp* knockout impedes the growth of the pollen tube, thereby reducing its competitiveness against wild-type pollen (Berkowitz *et al.* 2008). In this study, progeny of *bHLH146* T-DNA heterozygous plants also showed a non-Mendelian segregation in which only heterozygous plants were obtained. It will be very interesting to investigate whether these abnormal segregation patterns are due to

the interaction of bHLH146 and TCTP because bHLH146 interacts with TCTP *in vivo* (**Table 7**).

Besides these GTPase associated proteins, several other proteins were identified as interactors with bHLH146 (**Table 7**). Two ribosomal proteins, the 60S ribosomal protein L23 and the 40S ribosomal protein S15-1, were also identified. This indicates that bHLH146 has an affinity to ribosomal proteins. In response to biotic stress conditions, plants accumulate polygalacturonase-inhibiting proteins to reduce the activity of fungal polygalacturonases that hydrolyze the homogalacturonan of plant cell-wall pectin. Disease-induced polygalacturonase inhibitor proteins (PGIPs) are major defense proteins which play an important role in resistance to infection by pathogens. Expression analysis indicated that the transcription level of polygalacturonase inhibitor 1 (PGIP1) was up-regulated in response to infection with *Stemphylium solani* that caused hypersensitive cell death (Di *et al.* 2012). Antisense expression of the *AtPGIP1* gene reduced PGIP protein accumulation and thereby increasing the susceptibility towards *Botrytis cinerea* infection (Ferrari *et al.* 2006). These results indicated that PGIP contributes to basal resistance to this pathogen and that PGIP plays a role in *Arabidopsis* innate immunity. bHLH146 can interact with PGIP1 suggesting its involvement in plant defense pathways together with PGIP1. Plant genes whose expression is induced in legumes by *Rhizobium* bacteria upon nodulation were initially referred to as nodulins. Nodulin-like proteins were also found in non-nodulating plant species (Denancé *et al.* 2014). Several nodulin-like proteins were recently shown to be transporters of various solutes (Chen *et al.* 2010; Ladwig *et al.* 2012). Early nodulin-like (ENODL) proteins are related to phytocyanins (blue copper proteins that bind a single copper atom and function as electron transporters) but lack amino acid residues required for copper binding. AtENODL1, 13-15 and 17 were identified in a phosphoproteomic analysis of plasma membranes treated with elicitors of plant immunity (Benschop *et al.* 2007) while AtENODL9 was accumulated in mature sieve elements which participate in phloem loading/unloading and solute transport (Khan *et al.* 2007). Therefore, AtENODL9 was proposed to play a role in carbohydrate transport (Denancé *et al.* 2014). Not much information is available about AtENODL19, which was shown to be an interactor of bHLH146 from this study. Also, the dicarboxylate transporter 1 (DIT1) interacts with bHLH146. DIT1 is considered to be an essential component in photorespiratory nitrogen recycling (Renné *et al.* 2003). A recent study demonstrated that it also functions as an

oxaloacetate/malate transporter exporting reducing equivalents to the cytosol to prevent photo-inhibition (the malate valve) in *Arabidopsis*. Transport of dicarboxylates across the chloroplast envelope plays an important role in transferring carbon skeletons to the nitrogen assimilation pathway and exporting reducing equivalents to the cytosol to prevent photo-inhibition (the malate valve). Although bHLH146 was shown to interact with DIT1, it needs to be further investigated whether bHLH146 is involved in photo-inhibition. Further, two hormone-related proteins were also identified as bHLH146 interactors. 1-aminocyclopropane-1-carboxylate oxidase 2 (ACO2) is involved in ethylene biosynthesis and gibberellic acid-stimulated *Arabidopsis* (GASA), which plays a role in gibberellin (GA) response. The *Arabidopsis* loss-of-function *aco2* mutant is impaired in 1-aminocyclopropane-1-carboxylic acid (ACC)-mediated reversion of the ABA-induced inhibition of seed germination (Linkies *et al.* 2009). Overexpression of GASA4 suppressed ROS accumulation in plants, and the transgenic seeds were partially resistant to the NO donor sodium nitroprusside (SNP) (Rubinovich and Weiss 2010). Phenotypes of GASA4 overexpression lines occasionally exhibited meristem identity changes in which the plants underwent the transition to flowering and produced floral meristems, followed by a reversion to normal indeterminate inflorescence development. Flowers of GASA4 overexpression lines exhibited mosaic floral organs, most frequently were ectopic carpel or stamen structures (Roxrud *et al.* 2007). Interestingly, similar phenotypes were also observed in bHLH146 overexpression lines (**Fig. 47**). As bHLH146 interacts with GASA4, it is possible that these phenotypes result from the pathway that is regulated by bHLH146-GASA4 complex. It is worth to notice that six of the proteins interact with bHLH146 are uncharacterized proteins. This indicates that bHLH146 may also be involved in yet unknown pathways and needs further investigation.

4.4 Conclusions and future perspectives

Abiotic stress conditions alter gene expression profiles of various genes involved in various metabolic pathways. The data presented here provides information of barley *DREB* gene expression profiles under drought stress similar to stress that occurs in field conditions. The work on aldehyde dehydrogenases in this study broaden the previous findings on the osmotic stress-responsive aldehyde dehydrogenases *7B4* gene expression regulation in both glycophyte *A. thaliana* and halophyte *E. salsugineum*. This work also provides important data for the yet

uncharacterized *A. thaliana* transcription factor bHLH146 and these data will contribute towards understanding its role in multiple processes.

Copy number variations, phylogenetic relationships of *CBF/DREB* genes between *A. thaliana*, rice and barley indicated that *CBF/DREB* genes are diverged between monocots and dicots. The barley genome is rich in CBF/DREB1 subfamily genes but contains relatively few DREB2 subfamily genes. Expression analysis revealed that two DREB2 subfamily genes *HvDREB1* and *HvDRF1.3* were constitutively expressed under laboratory and field conditions, indicating that these DREB2 genes play a role as housekeeping genes in barley. In contrast, expression of three other analyzed CBF/DREB1 subfamily genes did not show a clear pattern under the given conditions. The results of this study demonstrate that the field stress conditions are more complex when compared to laboratory conditions. Therefore, the knowledge of gene expression obtained from laboratory conditions cannot always betransferred to complex field conditions under which agricultural production takes place.

Although the involvement of *ALDH* gene families in detoxification of aldehydes during stress conditions and their significance during different abiotic stress adaptation and tolerance has been reviewed in many plants, until now no reports are available on *ALDH* gene regulation in salt stress tolerant halophytes. The availability of the genome sequences of both the halophytes *E. parvulum* and *E. salsugineum* which are close relatives of *A. thaliana* helped in identifying the *ALDH* genes and studying their regulation. Sixteen and 17 *ALDH* genes were identified by genome-wide analysis from the halophyte models *E. parvulum* and *E. salsugineum*, respectively. Genomic organization, copy number, sub-cellular localization and expression profiles of *ALDH* genes are mainly conserved between *A. thaliana*, *E. parvulum* and *E. salsugineum*. Except for the expression of *ALDH7B4*, *ALDH3H1*, and *ALDH10A8,* no major differences were observed which may contribute to salinity tolerance in *Eutrema* halophytes. Transcripts of *ALDH3H1* and *ALDH7B4* increased in response to NaCl at higher salt levels in *E. salsugineum* than in *A. thaliana*, whereas *ALDH10A8* showed a different expression pattern under high salt in *A. thaliana* and *E. salsugineum*. This indicates that the regulation of transcription may be better adapted to high salt in *E. salsugineum* than in *A. thaliana*. Then the focus was on the gene *ALDH7B4,* which showed osmotic responsive expression in both *A. thaliana* and *E. salsugineum*. *EsALDH7B4* promoter deletion analysis in transgenic *A. thaliana* plants revealed a conserved G-box motif which is important while a specific “TC” rich motif

in the *EsALDH7B4* promoter represses the gene expression in transgenic *A. thaliana*. Yeast one-hybrid screening identified more than 40 putative transcription factors that interact with the *EsALDH7B4* promoter. Consistent with promoter deletion results, many members from bZIP, bHLH families interacting with the G-box motif were identified. The interaction between the "TC" rich motif and the identified MYB_like transcription factor from yeast one-hybrid screening needs to be further confirmed using independent, biochemical methods.

There has been no report yet on bHLH146. In this study, we have been trying to characterize bHLH146 from two aspects: molecular characterization including expression profiles, DNA binding ability and cellular localization; and functional characterization using "gain of function" and "loss of function" mutants. In addition, a yeast two-hybrid screening was performed with the aim to get a clue in which cellular pathways bHLH146 may participate. Results from these studies so far showed bHLH146 does not bind to a G-box but is mainly localized in the nucleus and acts as a transcriptional repressor. bHLH146 can interact with other G-box binding bHLH proteins such as bHLH49, bHLH69 and bHLH76, which supports the hypothesis that bHLH146 regulates downstream gene expression in an indirect manner. Since bHLH49 and bHLH76 are CIB proteins and regulate FT (FLOWERING LOCUS T) transcription and floral initiation. Therefore, it is assumed that bHLH146 might also be involved in the same pathway together with these well-studied CIB proteins. In addition, several GTPase related proteins were identified as interactors, which implies that bHLH146 is involved in many regulatory processes. Seedlings of bHLH146 overexpression lines showed a short hypocotyl and had a large open apical hook angle in the dark, which suggests it might be involved in photomorphogenesis. bHLH146 overexpression lines also occasionally exhibited abnormal ectopic petal, carpel or stamen structures while progeny of bHLH146 T-DNA insertion lines are heterozygous plants and exhibit a non-Mendelian segregation, and only heterozygous plants were obtained. An explanation for these phenotypes could be that bHLH146 interacts with GASA4 and TPTC proteins. It is necessary to cross the bHLH146 T-DNA insertion lines and wild type plants to identify the reason for the non-Mendelian segregation. Further analysis of bHLH146 overexpression lines and silencing lines by artificial microRNA should provide a better understanding of the functions of bHLH146.

5. APPENDICES

5.1 Accession numbers of the analyzed genes

Gene names	NCBI GenBank accession	Other database accession
AtALDH3H1	NM_179439.3	TAIR: AT1G44170
AtALDH3I1	NM_119588.5	TAIR: AT4G34240
AtALDH7B4	NM_179476.2	TAIR: AT1G54100
AtALDH10A8	NM_001198470.1	TAIR: AT1G74920
AtALDH10A9	NM_114686.3	TAIR: AT3G48170
AtbHLH146	BT010967.1	TAIR: AT4G30180
At2g18969	NM_001124873.1	TAIR: AT2G18969
AtActin2	U41998.1	TAIR: AT3G18780
EsALDH3H1	XM_006393677.1	Phytozome: Thhalv10011438m
EsALDH3I1	XM_006412189.1	Phytozome: Thhalv10024844m
EsALDH7B4	XM_006392657.1	Phytozome: Thhalv10011684m
EsALDH10A8	XM_006390308.1	Phytozome: Thhalv10018437m
EsALDH10A9	XM_006404229.1	Phytozome: Thhalv10010305m
EsActin	XM_006406501.1	Phytozome: Thhalv10020906m
HvCBF1	AY785836.1	
HvCBF2	AY785840.1	
HvCBF4	AY785848.1	
HvDREB1	KJ699390.1	
HvDRF1.3	AF521303.1	
HvHSP17	Y07844.1	
HvHSP70	L32165.1	
HvALDH7B4	AK356265.1	
HvActin	U21907.1	
HvEF1α	Z50789.1	

5.2 Gene sequences

The sequences of the analyzed genes in this work are shown here using the Vector NTI software.

5.2.1 *EsALDH7B4* gene promoter sequence

The promoter sequence of *EsALDH7B4* is shown here with some putative cis-elements. The nucleotides in red were mutagenized to introduce restriction enzyme sites EcoRI and XbaI with the primers T.hALDH7B4prom1 Fwd and T.hALDH7B4prom1 Rev, respectively. The resulting EcoRI/XbaI region was used as the TP0 promoter.

Green-shaded: “TC” rich motif

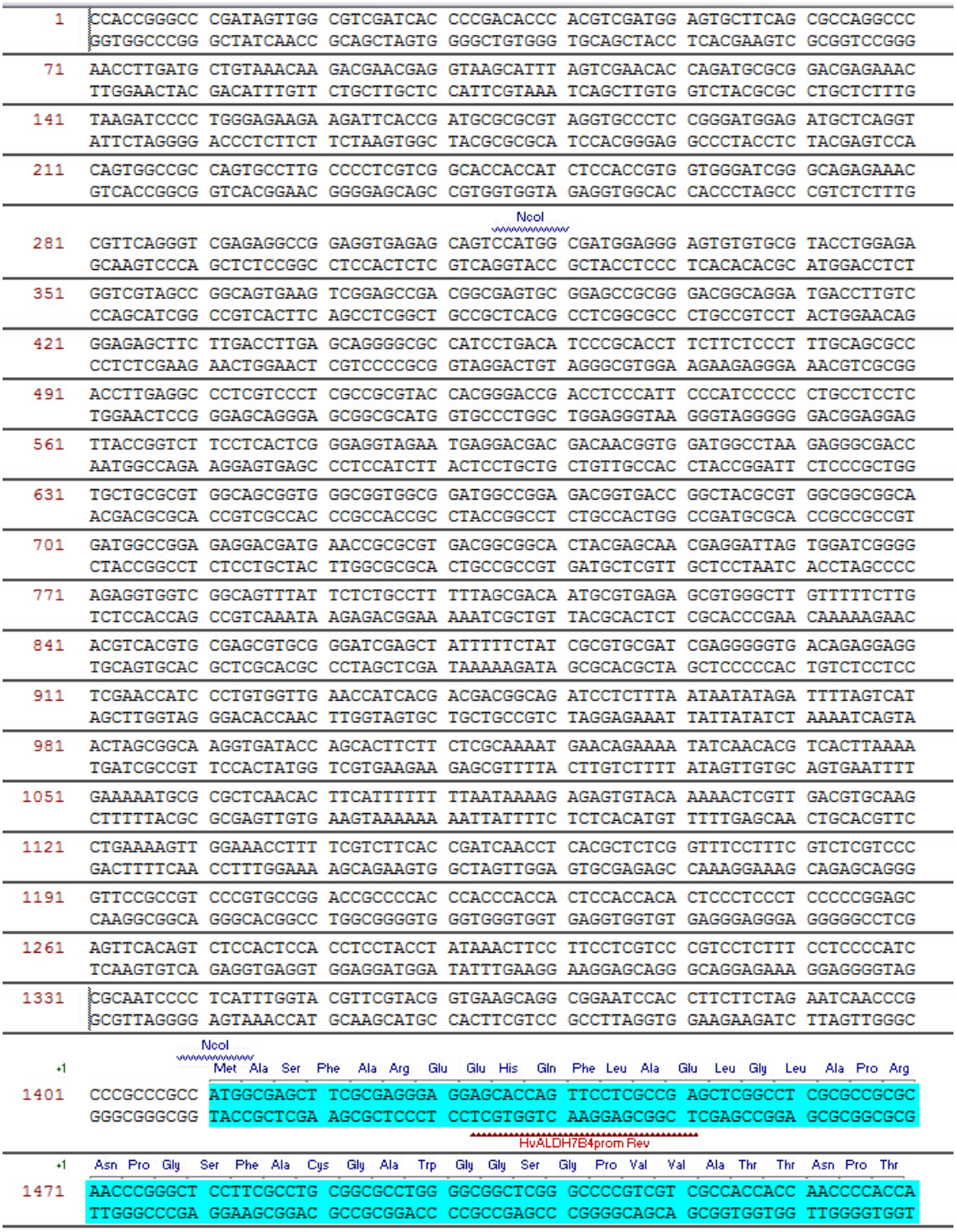
Turquoise-shaded: G-box

Pink-shaded: ACGT-Box

```
                                  T.h.ALDH7B4prom1 Fwd
   1  TCTTTAGGGA TTCACTATAA TTCCACCATG TATTACATAT ACGAATGTAA AGTTAGATTA ATTTATGTTA
      AGAAATCCCT AAGTGATATT AAGGTGGTAC ATAATGTATA TGCTTACATT TCAATCTAAT TAAATACAAT
                                        XbaI
  71  GGAAAAGAAA ACCAAATAAT CGTCTAGAAA AAAGAAAGAT GAAATAAACC TGATTGTAAA AATAGGAATC
      CCTTTTCTTT TGGTTTATTA GCAGATCTTT TTTCTTTCTA CTTTATTTGG ACTAACATTT TTATCCTTAG
 141  TGGTGAGTTA ATTCACTGAA CAAAACCTCT AACGACTCTT AATAGGGTTA GTTTAGATGG GCTTATAAAG
      ACCACTCAAT TAAGTGACTT GTTTTGGAGA TTGCTGAGAA TTATCCCAAT CAAATCTACC CGAATATTTC
                TsA7pro2
 211  CGGTTAAATC CGTTTTATTG CACATAATTA TATGGGTCAG GCCCATATTC GAAAGTGAAT ATCAAATGAC
      GCCAATTTAG GCAAAATAAC GTGTATTAAT ATACCCAGTC CGGGTATAAG CTTTCACTTA TAGTTTACTG
                TsA7pro3                                                ThA7pro-3de
 281  AGCTTGTGGC AACAATGGCG ACAACTTCTC CTCCCTTCCC CTCCCTTCCC CTCCCTTCCC CTGTGTGCTT
      TCGAACACCG TTGTTACCGC TGTTGAAGAG GAGGGAAGGG GAGGGAAGGG GAGGGAAGGG GACACACGAA
                      ThA7pro-5de
 351  GACCCTGCCT AATCCGGATC CTATCCGGGT TTATTTTGAC CCGGAAAAGG AGGCATAGGA CACGTGGCAA
      CTGGGACGGA TTAGGCCTAG GATAGGCCCA AATAAAACTG GGCCTTTTCC TCCGTATCCT GTGCACCGTT
 421  AGCCATTGAT ATGAATGGAG CCAAAAAAGT AACGCTAAGG ATGGACTCAA GACACCAGCT CAGCTTTCGT
      TCGGTAACTA TACTTACCTC GGTTTTTTCA TTGCGATTCC TACCTGAGTT CTGTGGTCGA GTCGAAAGCA
                TsA7pro6
 491  CTTCGATATA GTCGCTGCTA CTTCCTCCTC TTAAAGACGT ACACGTCTCT CTCACTTCTC TCACTCCTTT
      GAAGCTATAT CAGCGACGAT GAAGGAGGAG AATTTCTGCA TGTGCAGAGA GAGTGAAGAG AGTGAGGAAA
 561  TAAAAATTCG TGATCAGAAG AACTAATCAA GATACTCCCT TCTTCGATAC GATCGATAAT CAGGTGATTT
      ATTTTTAAGC ACTAGTCTTC TTGATTAGTT CTATGAGGGA AGAAGCTATG CTAGCTATTA GTCCACTAAA
 631  CTCTGATCCT GATCGTTTTT TTTTGGGTGT TCATCGGATT GTTGATTTTC CTTGGGTTTG TTATGCATCT
      GAGACTAGGA CTAGCAAAAA AAAACCCACA AGTAGCCTAA CAACTAAAAG GAACCCAAAC AATACGTAGA
 701  ACTTTCAACT CGTTATGTTA TTGACTTTGA TTTTTTGTTG GGTGAATTTG TAGAAGAGAT GGGTTCTGCG
      TGAAAGTTGA GCAATACAAT AACTGAAACT AAAAAACAAC CCACTTAAAC ATCTTCTCTA CCCAAGACGC
                                                 T.h.ALDH7B4prom1 Rev
 771  AACAAAGAGT ACGAGTTTCT GAGTGAGATT GGGTTGAGTT CTCACAACCT CGGAAGTTAC GTTGGTGGCA
      TTGTTTCTCA TGCTCAAAGA CTCACTCTAA CCCAACTCAA GAGTGTTGGA GCCTTCAATG CAACCACCGT
```

5.2.2 *HvALDH7B4* gene promoter sequence

The putative *HvALDH7B4* promoter sequence was retrieved from MIPS barley genome database (ftp://ftpmips.helmholtz-muenchen.de/plants/barley/public_data/). The first exon is shaded in turquoise. Position of the reverse primer is marked with a red line. The sequence between the NcoI restriction enzyme sites was used in this study.



5.2.3 *bHLH146* genomic sequence

The genomic sequence of *bHLH146* shown here covers the complete coding sequence and the analyzed promoter sequence in this study. The sequence was retrieved from the Phytozome v9.1 database (www.phytozome.net). The nucleotides in red were mutagenized to introduce restriction enzyme sites with different primers for multiple purposes. Positions of the primers were marked with read lines.

```
   1  TGACAAATAA AACGTATTAA TAACCCAATT ACTGACCACA TGCATCTTAA CTTTATGTTA ATATGCGTAG
      ACTGTTTATT TTGCATAATT ATTGGGTTAA TGACTGGTGT ACGTAGAATT GAAATACAAT TATACGCATC

                                                          At4g30180ProFwd2
  71  TAAAGAAACT TGTATCTACG TCATATTAGT TACAGTGGCA GTAAAATTCA GTTTCAAAGA TGTTTGTCTT
      ATTTCTTTGA ACATAGATGC AGTATAATCA ATGTCACCGT CATTTTAAGT CAAAGTTTCT ACAAACAGAA
 141  GATATGTTGT ACAGATCACC AATTGAACTA CCACTAATAA TAACTAAATT TCTGTTTAGC AAGTTACCAT
      CTATACAACA TGTCTAGTGG TTAACTTGAT GGTGATTATT ATTGATTTAA AGACAAATCG TTCAATGGTA
 211  CTGTATCCAA ATATCGCAAA AGTAGTGGGT AGTGAAACCC AAATTCATCA CACATGCAGC ATAAAAAAAA
      GACATAGGTT TATAGCGTTT TCATCACCCA TCACTTTGGG TTTAAGTAGT GTGTACGTCG TATTTTTTTT
 281  AGAAGGTCAA ATGGTATATA TAATATGAGG GAGATCTATA TTAAGATAAT ATGGGTAGAA ATGACATAGA
      TCTTCCAGTT TACCATATAT ATTATACTCC CTCTAGATAT AATTCTATTA TACCCATCTT TACTGTATCT
 351  TTGATCAAAC TAATTAGGAG ATTGGTCCAA GGACACATAC ACATGCATAC ATGTATTATG TATATACAAA
      AACTAGTTTG ATTAATCCTC TAACCAGGTT CCTGTGTATG TGTACGTATG TACATAATAC ATATATGTTT
 421  GTGTGTGAGT GAGTGAGTGA GAGAGAGAGA GGTTAAGAGG AAGGAACATG TGAAAGCATT AAGAGAAGGT
      CACACACTCA CTCACTCACT CTCTCTCTCT CCAATTCTCC TTCCTTGTAC ACTTTCGTAA TTCTCTTCCA
 491  GGGGTTTTGT ATTGATGTCT TGTTCTTGGA ATGGAGCCAC ATGGTAAAAG GGTCTCTCAA AGCACCTCTT
      CCCCAAAACA TAACTACAGA ACAAGAACCT TACCTCGGTG TACCATTTTC CCAGAGAGTT TCGTGGAGAA
 561  TAGGGGTAGT TGTGATGCAG CCAAATTAGA GCTCTTGCAT GTGACTCCAT GTGTTTTTTC TTCCCTCACC
      ATCCCCATCA ACACTACGTC GGTTTAATCT CGAGAACGTA CACTGAGGTA CACAAAAAAG AAGGGAGTGG
 631  CATTATTATT CCCAAATTCT TCTTTTCTTA TTTTTCTCTC TTTATTTATG TATATATATA TATATATATA
      GTAATAATAA GGGTTTAAGA AGAAAAGAAT AAAAAGAGAG AAATAAATAC ATATATATAT ATATATATAT
 701  TATATATACA CACTCTCTTT TTGTTTTCGT GTATTTTCTA AAAATAAATG TATTATACAA AAAAACATAT
      ATATATATGT GTGAGAGAAA AACAAAAGCA CATAAAAGAT TTTTATTTAC ATAATATGTT TTTTTGTATA
 771  ATTAGTTGAT GATATTCAAA TTCCGTTGTT TTTAAAATAT TTGTCTAAAA AAGAAAACTA CTAACTACGA
      TAATCAACTA CTATAAGTTT AAGGCAACAA AAATTTTATA AACAGATTTT TTCTTTTGAT GATTGATGCT
 841  CTTTCAAATA GTCTTGTGTT TTGTACGTGA ACGAAAAATA ATAACACTCC CAAACATATT ACCTCATATA
      GAAAGTTTAT CAGAACACAA AACATGCACT TGCTTTTTAT TATTGTGAGG GTTTGTATAA TGGAGTATAT
 911  TGTATACAAA GAAAATTAAG TTCTACAACT TTACTTTATA CATCATCAAC GCTTGTTATA TACAAACAAA
      ACATATGTTT CTTTTAATTC AAGATGTTGA AATGAAATAT GTAGTAGTTG CGAACAATAT ATGTTTGTTT

 981  CGTAGGACAC CGCTCTTCTC TTTCTTTGTC TCTCTCATAT TCTAAGTTTG CTATATATGT AACTTTCTCC
      GCATCCTGTG GCGAGAAGAG AAAGAAACAG AGAGAGTATA AGATTCAAAC GATATATACA TTGAAAGAGG

                                                          bHLH146_For
                                                  At4g30180for_NcoI
                                                  At4g30180_for
 +3                                                       Met  Glu  Arg  Gln  Ile  Ile  Asn  Arg  Lys  Lys  Arg
1051  CTTGTAGACT TTCATTTGCA GCAAAAATAG TTTCTCATAT GGAGAGGCAA ATCATAAACA GGAAGAAACG
      GAACATCTGA AAGTAAACGT CGTTTTTATC AAAGAGTATA CCTCTCCGTT TAGTATTTGT CCTTCTTTGC
                                  At4g30180ProRev
 +3  Arg Val  Phe  Ser  Leu  Glu  Pro  Asn  Lys  Asn  Pro  Ser  Ala  Val  Phe  Thr  Arg  Lys  Tyr  Thr  Ser  His  Leu  Val
1121  AGTGTTTTCT CTTGAACCAA ACAAGAACCC TAGTGCAGTT TTCACGAGAA AATACACAAG CCACTTGGTT
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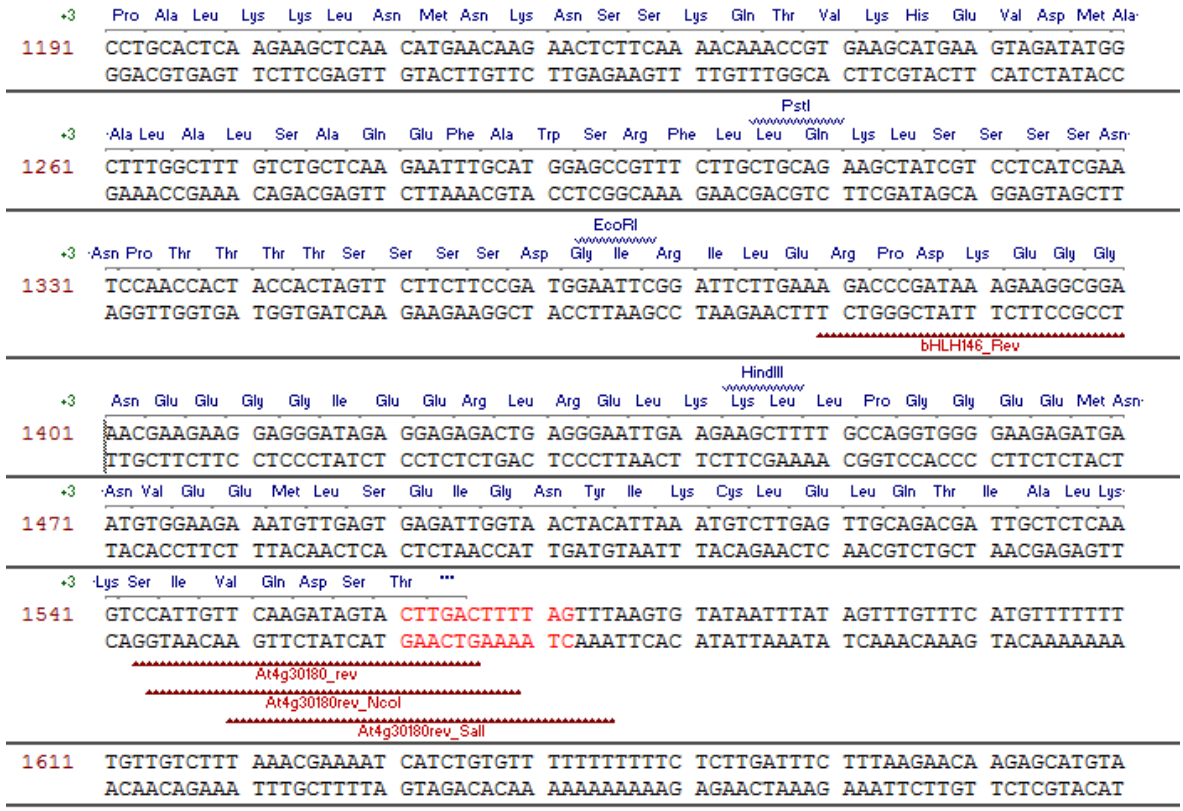
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GGACGTGAGT TCTTCGAGTT GTACTTGTTC TTGAGAAGTT TTGTTTGGCA CTTCGTACTT CATCTATACC
PstI
+3 Ala Leu Ala Leu Ser Ala Gln Glu Phe Ala Trp Ser Arg Phe Leu Leu Gln Lys Leu Ser Ser Ser Ser Asn
1261 CTTTGGCTTT GTCTGCTCAA GAATTTGCAT GGAGCCGTTT CTTGCTGCAG AAGCTATCGT CCTCATCGAA
GAAACCGAAA CAGACGAGTT CTTAAACGTA CCTCGGCAAA GAACGACGTC TTCGATAGCA GGAGTAGCTT
EcoRI
+3 Asn Pro Thr Thr Thr Thr Ser Ser Ser Ser Asp Gly Ile Arg Ile Leu Glu Arg Pro Asp Lys Glu Gly Gly
1331 TCCAACCACT ACCACTAGTT CTTCTTCCGA TGGAATTCGG ATTCTTGAAA GACCCGATAA AGAAGGCGGA
AGGTTGGTGA TGGTGATCAA GAAGAAGGCT ACCTTAAGCC TAAGAACTTT CTGGGCTATT TCTTCCGCCT
bHLH146_Rev
HindIII
+3 Asn Glu Glu Gly Gly Ile Glu Glu Arg Leu Arg Glu Leu Lys Lys Leu Leu Pro Gly Gly Glu Glu Met Asn
1401 AACGAAGAAG GAGGGATAGA GGAGAGACTG AGGGAATTGA AGAAGCTTTT GCCAGGTGGG GAAGAGATGA
TTGCTTCTTC CTCCCTATCT CCTCTCTGAC TCCCTTAACT TCTTCGAAAA CGGTCCACCC CTTCTCTACT
+3 Asn Val Glu Glu Met Leu Ser Glu Ile Gly Asn Tyr Ile Lys Cys Leu Glu Leu Gln Thr Ile Ala Leu Lys
1471 ATGTGGAAGA AATGTTGAGT GAGATTGGTA ACTACATTAA ATGTCTTGAG TTGCAGACGA TTGCTCTCAA
TACACCTTCT TTACAACTCA CTCTAACCAT TGATGTAATT TACAGAACTC AACGTCTGCT AACGAGAGTT
+3 Lys Ser Ile Val Gln Asp Ser Thr ***
1541 GTCCATTGTT CAAGATAGTA CTTGACTTTT AGTTTAAGTG TATAATTTAT AGTTTGTTTC ATGTTTTTTT
CAGGTAACAA GTTCTATCAT GAACTGAAAA TCAAATTCAC ATATTAAATA TCAAACAAAG TACAAAAAAA
At4g30180_rev
At4g30180rev_NcoI
At4g30180rev_SalI
1611 TGTTGTCTTT AAACGAAAAT CATCTGTGTT TTTTTTTTTC TCTTGATTTC TTTAAGAACA AGAGCATGTA
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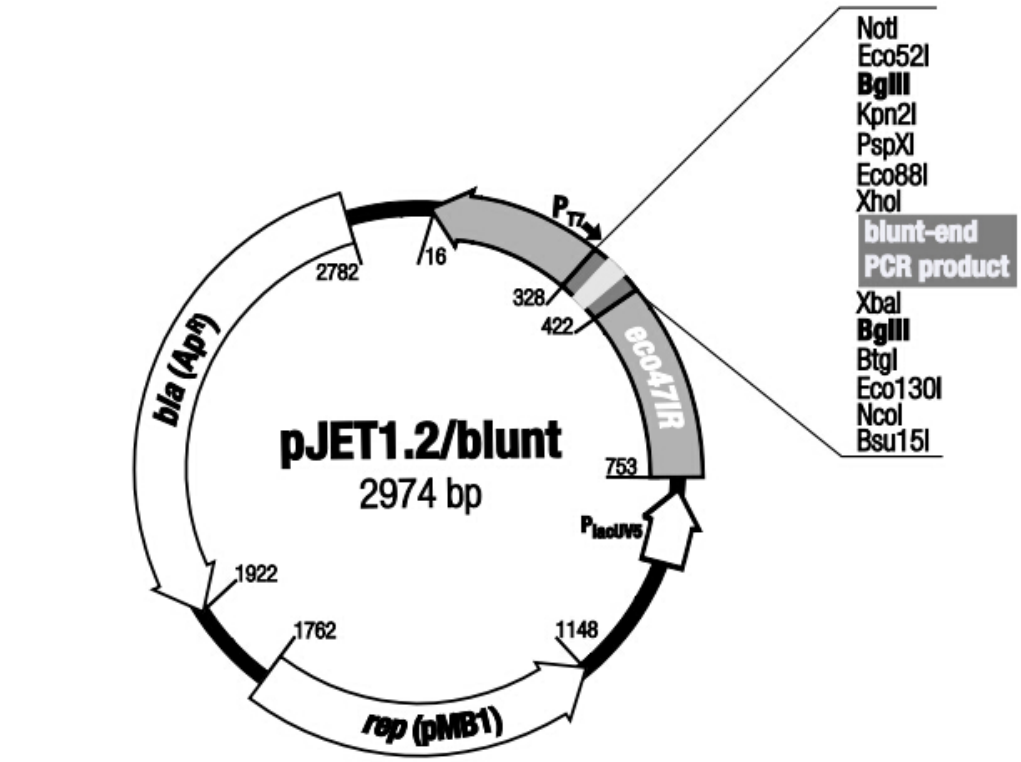
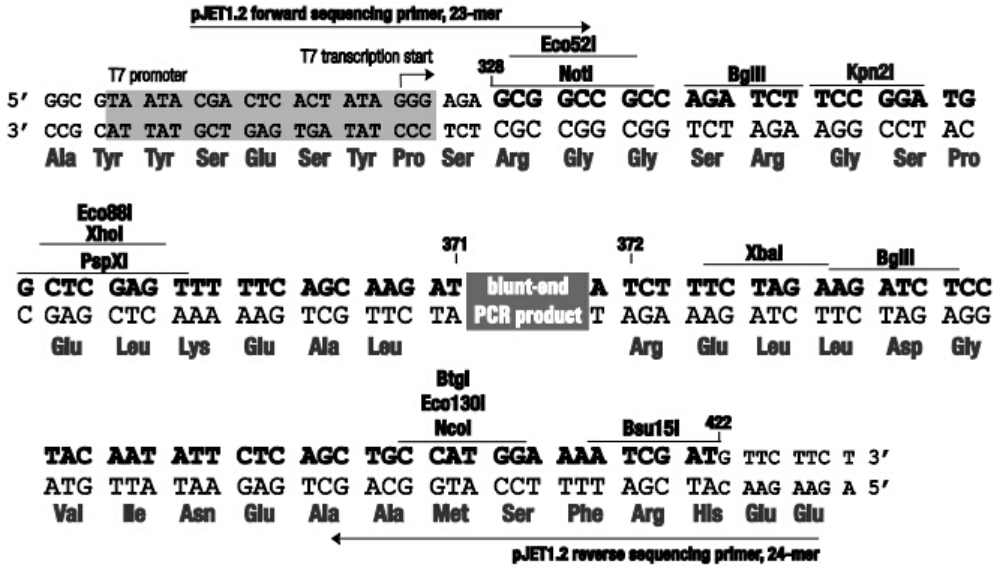
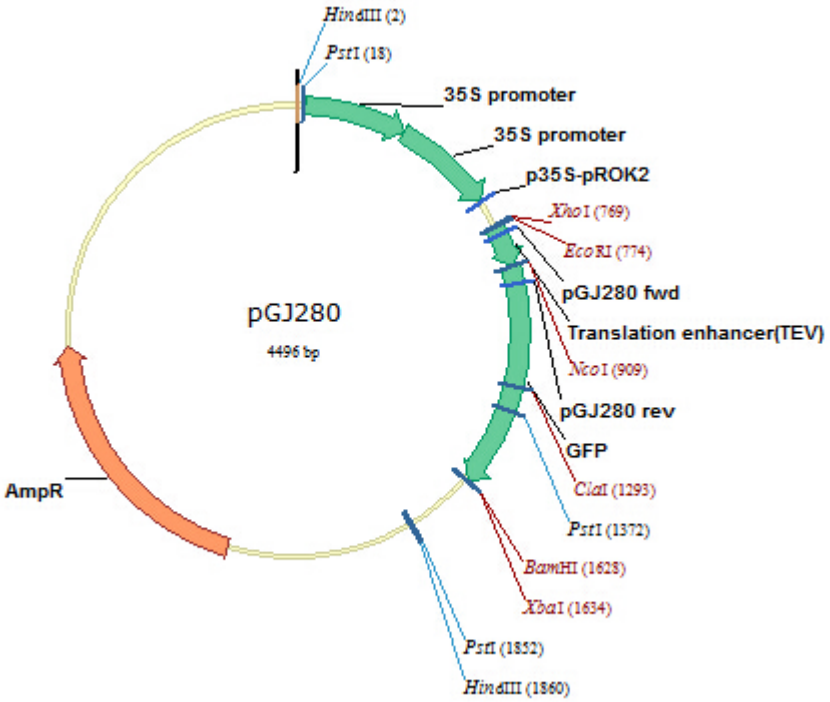
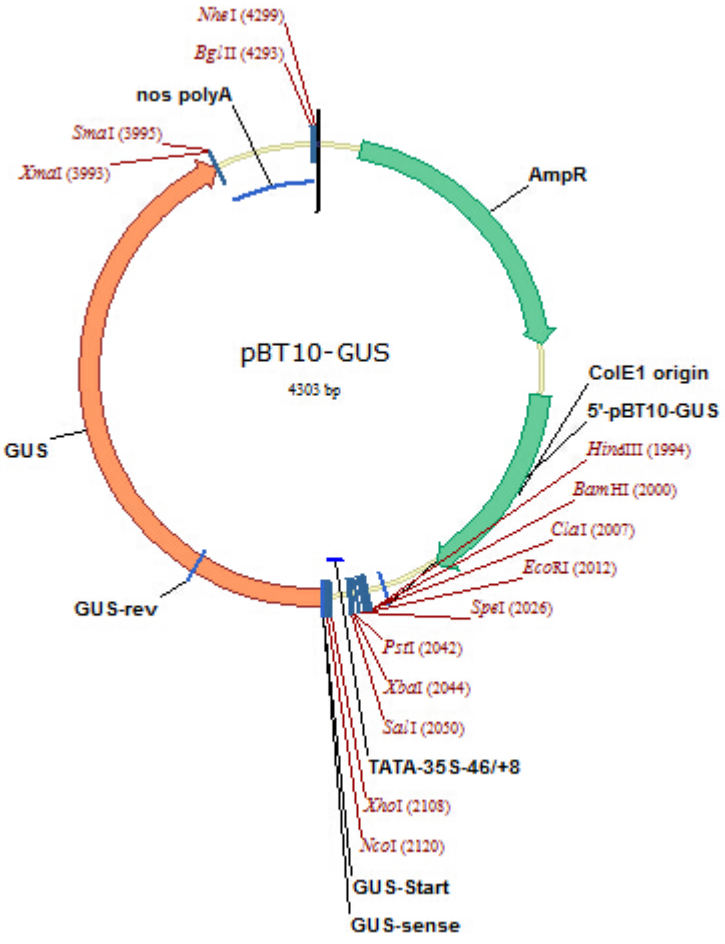
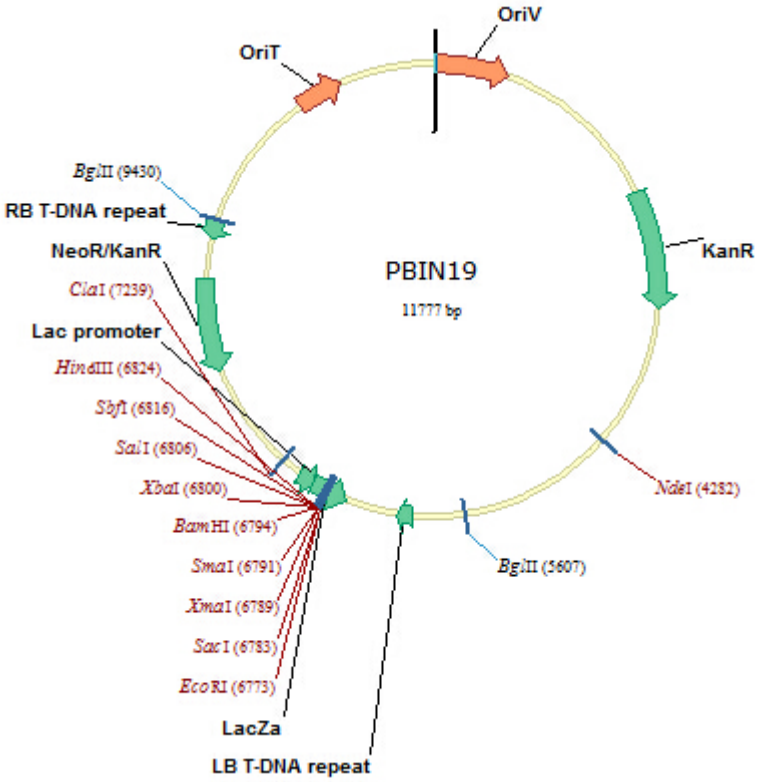
5.3 Vector maps

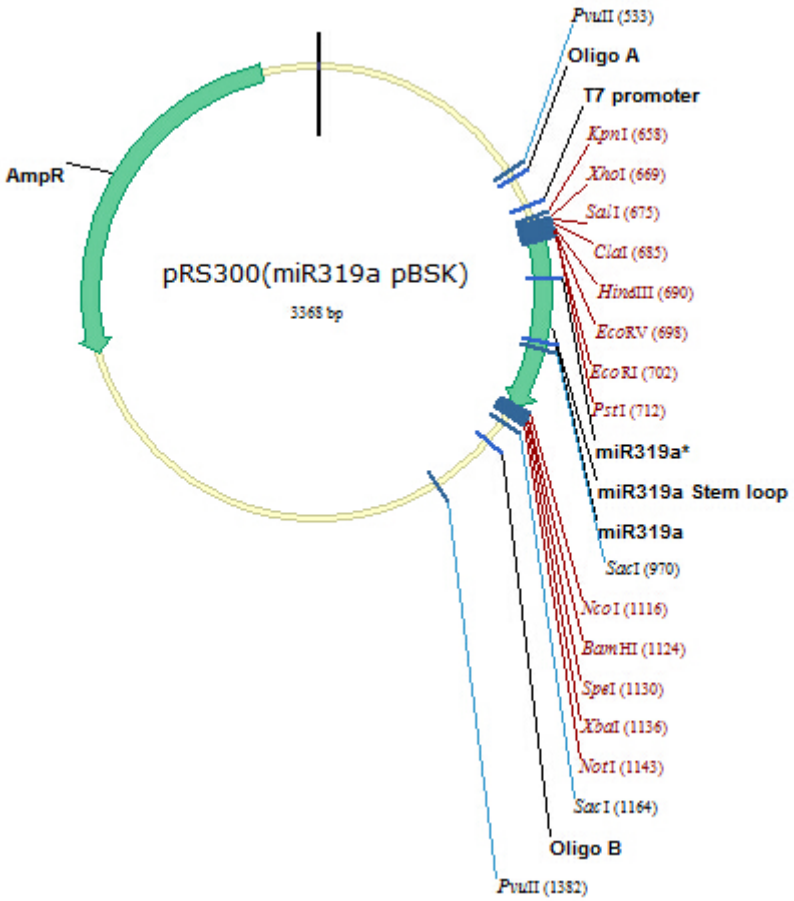


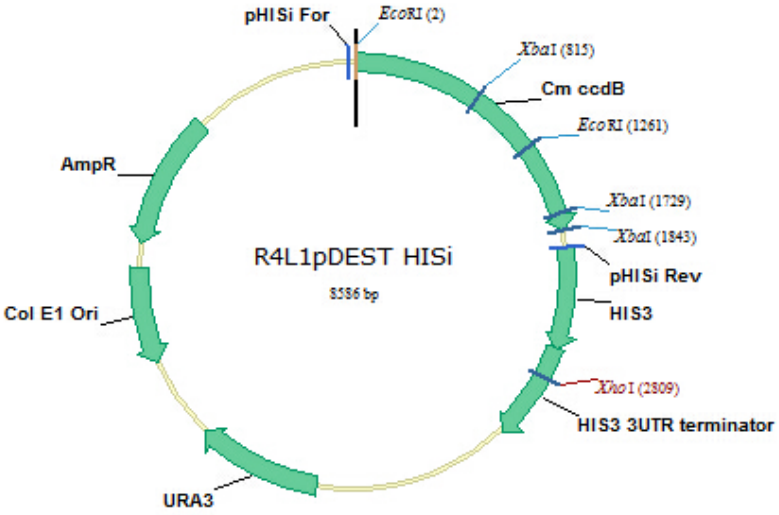


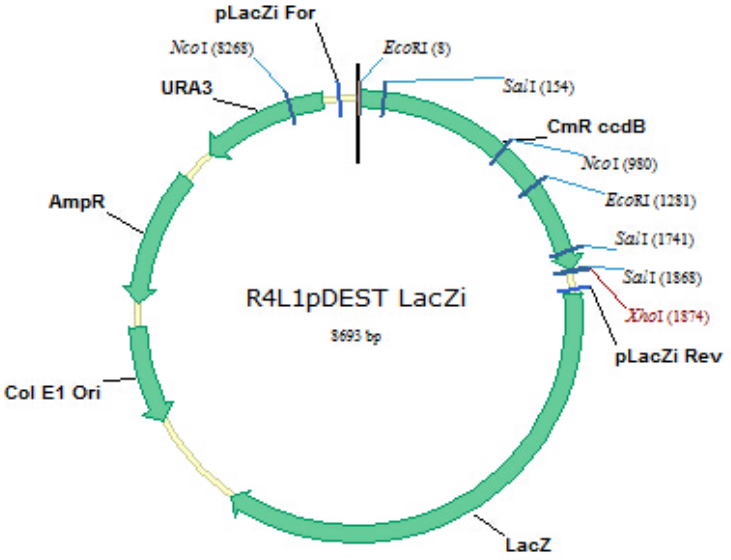

HindIII (2)
PstI (18)
35S promoter
35S promoter
p35S-pROK2
XhoI (769)
EcoRI (774)
pGJ280 fwd
Translation enhancer(TEV)
NcoI (909)
pGJ280 rev
GFP
ClaI (1293)
PstI (1372)
BamHI (1628)
XbaI (1634)
PstI (1852)
HindIII (1860)
pGJ280
4496 bp
AmpR

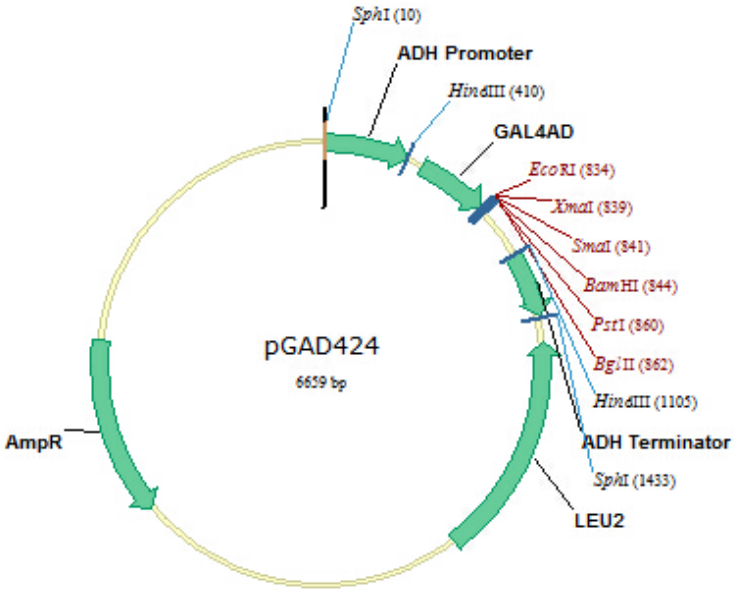

NheI (4299)
Bgl II (4293)
nos polyA
SmaI (3995)
XmaI (3993)
AmpR
pBT10-GUS
4303 bp
ColE1 origin
5'-pBT10-GUS
HindIII (1994)
BamHI (2000)
ClaI (2007)
EcoRI (2012)
SpeI (2026)
PstI (2042)
XbaI (2044)
SalI (2050)
TATA-35S-46/+8
XhoI (2108)
NcoI (2120)
GUS-Start
GUS-sense
GUS
GUS-rev


OriV
OriT
BglII (9430)
RB T-DNA repeat
NeoR/KanR
ClaI (7239)
Lac promoter
HindIII (6824)
SbfI (6816)
SalI (6806)
XbaI (6800)
BamHI (6794)
SmaI (6791)
XmaI (6789)
SacI (6783)
EcoRI (6773)
LacZa
LB T-DNA repeat
PBIN19
11777 bp
KanR
NdeI (4282)
BglII (5607)


PvuII (533)
Oligo A
T7 promoter
KpnI (658)
XhoI (669)
SalI (675)
ClaI (685)
HindIII (690)
EcoRV (698)
EcoRI (702)
PstI (712)
miR319a*
miR319a Stem loop
miR319a
SacI (970)
NcoI (1116)
BamHI (1124)
SpeI (1130)
XbaI (1136)
NotI (1143)
SacI (1164)
Oligo B
PvuII (1382)
AmpR
pRS300(miR319a pBSK)
3368 bp


pHISi For
EcoRI (2)
XbaI (815)
Cm ccdB
EcoRI (1261)
XbaI (1729)
XbaI (1843)
pHISi Rev
HIS3
XhoI (2309)
HIS3 3UTR terminator
URA3
Col E1 Ori
AmpR
R4L1pDEST HISi
8586 bp


pLacZi For
NcoI (8268)
EcoRI (8)
SalI (154)
URA3
CmR ccdB
NcoI (980)
EcoRI (1281)
SalI (1741)
SalI (1868)
XhoI (1874)
pLacZi Rev
AmpR
Col E1 Ori
LacZ
R4L1pDEST LacZi
8693 bp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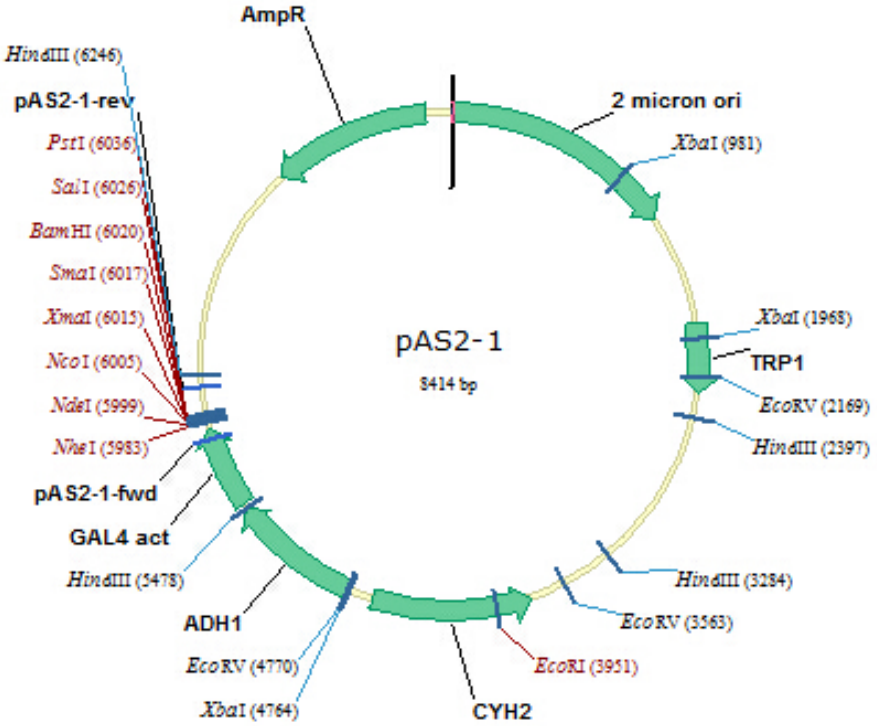
ADH Promoter
HindIII (410)
GAL4AD
EcoRI (834)
XmaI (839)
SmaI (841)
BamHI (844)
PstI (860)
BglII (862)
HindIII (1105)
ADH Terminator
SphI (1433)
LEU2
AmpR
pGAD424
6659 bp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HindIII (6246)
pAS2-1-rev
PstI (6036)
SalI (6026)
BamHI (6020)
SmaI (6017)
XmaI (6015)
NcoI (6005)
NdeI (5999)
NheI (5983)
pAS2-1-fwd
GAL4 act
HindIII (5478)
ADH1
EcoRV (4770)
XbaI (4764)
CYH2
EcoRI (3951)
EcoRV (3563)
HindIII (3284)
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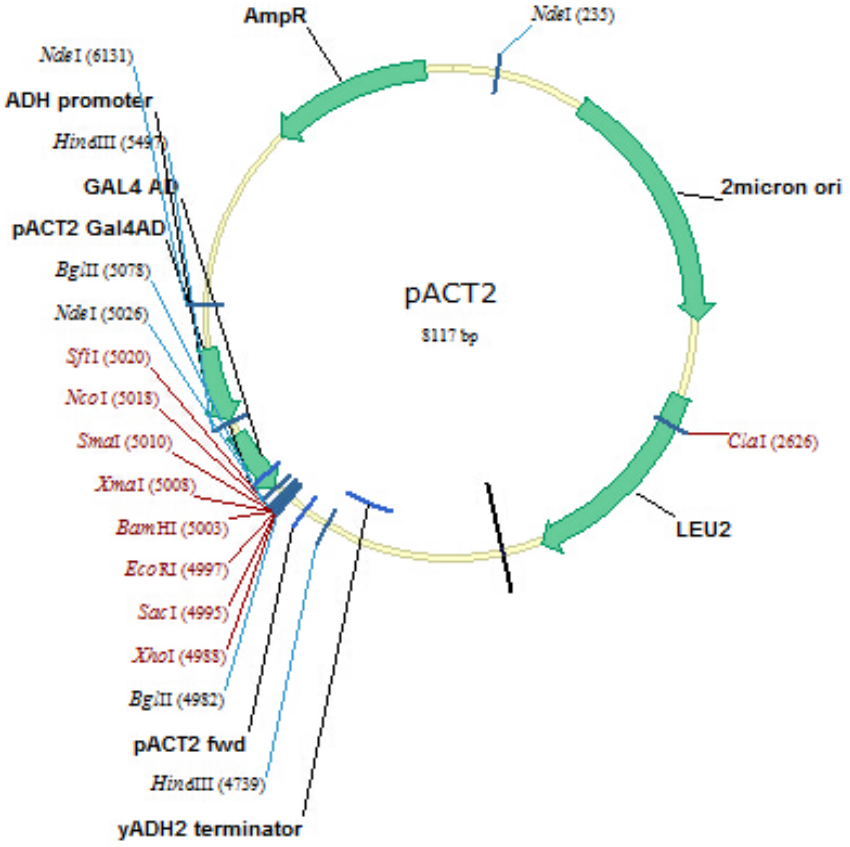
EcoRV (2169)
TRP1
XbaI (1968)
XbaI (981)
2 micron ori
pAS2-1
8414 bp


AmpR
NdeI (235)
NdeI (6131)
ADH promoter
HindIII (549?)
GAL4 AD
pACT2 Gal4AD
BglII (5078)
NdeI (5026)
SfiI (5020)
NcoI (5018)
SmaI (5010)
XmaI (5008)
BamHI (5003)
EcoRI (4997)
SacI (4995)
XhoI (4988)
BglII (4982)
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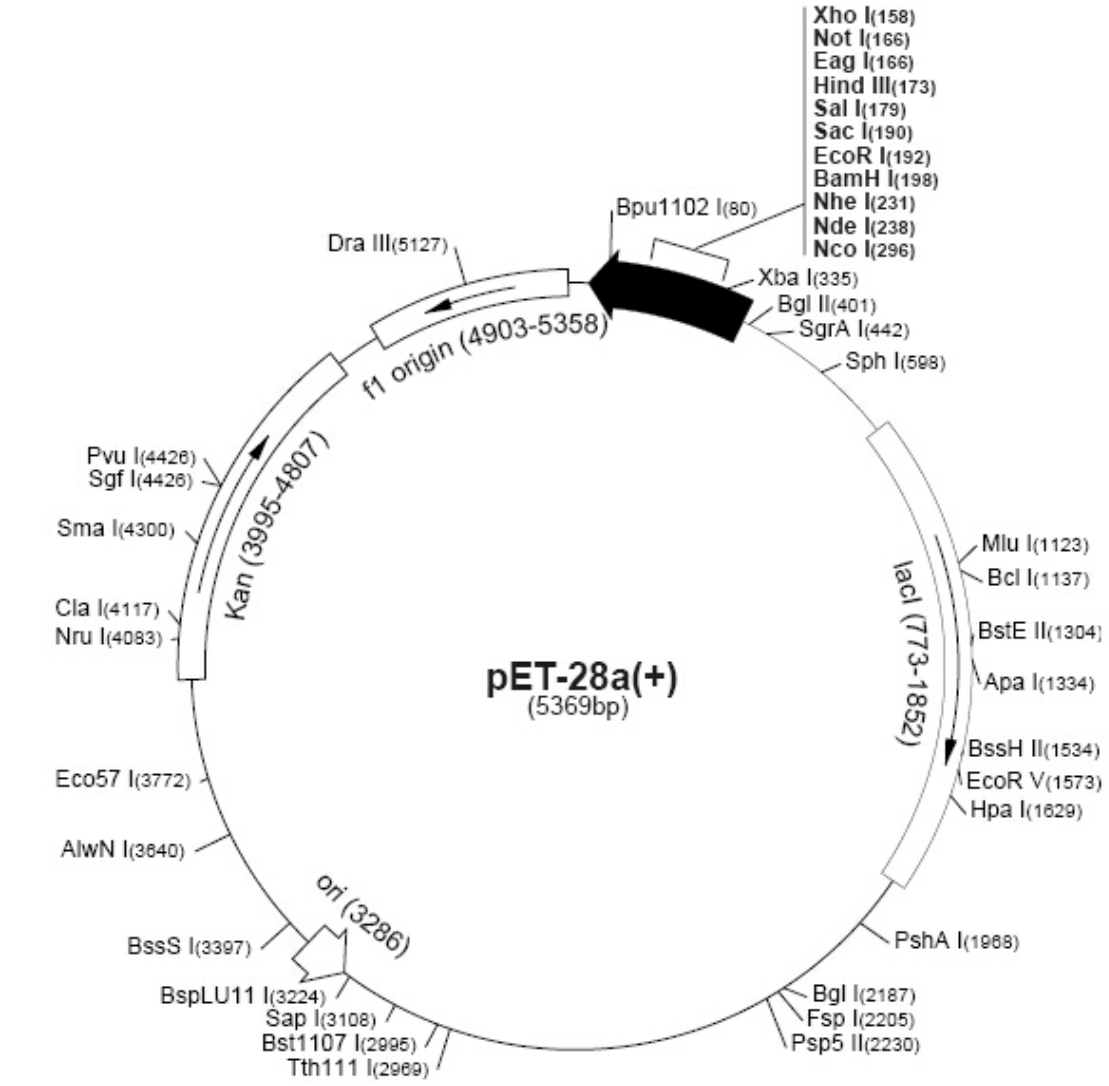
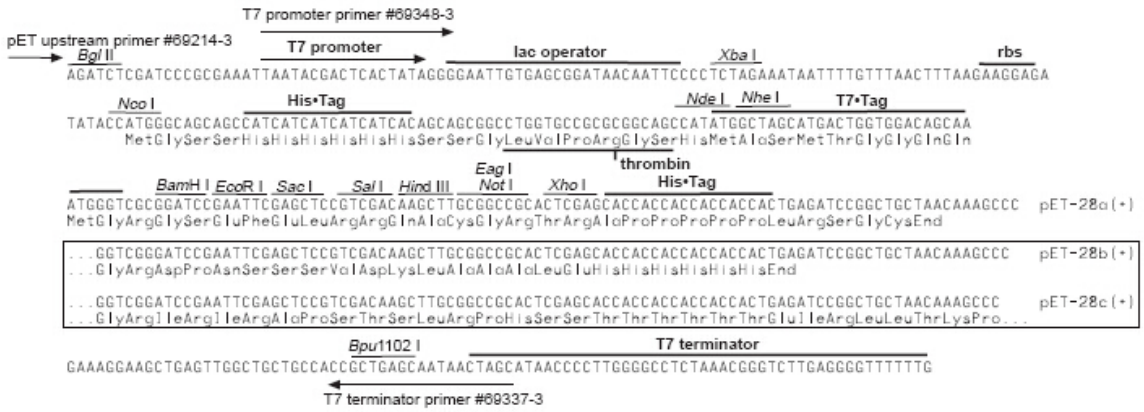
HindIII (4739)
yADH2 terminator
2micron ori
ClaI (2626)
LEU2
pACT2
8117 bp





pET-28a-c(+) cloning/expression region

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7. FIGURES AND TABLES

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9. CONFERENCES WITH POSTER PRESENTATIONS

- EU COST WG2 “Halophyte Biochemistry and Physiology” Training School, July 3rd-10th, 2011, Hannover, Germany.
- COST Action FA0901 “Putting Halophyte to Work- From Genes to Ecosystems” - Final Meeting April 9th-10th 2014, Coimbra, Portugal.

10. PUBLICATIONS

1. Missihoun TD, Hou Q, Mertens D & Bartels D. **(2014).** Sequence and functional analyses of the aldehyde dehydrogenase 7B4 gene promoter in Arabidopsis thaliana and selected Brassicaceae: regulation patterns in response to wounding and osmotic stress. *Planta*, **239** (6), 1281-1298.

2. Hou Q. & Bartels D. **(2015)** Comparative study of the aldehyde dehydrogenase (ALDH) gene superfamily in the glycophyte Arabidopsis thaliana and Eutrema halophytes**.** *Annals of Botany*, **115**, 465-479.