Physiological and molecular studies of different aldehyde dehydrogenase (*ALDH*) genes in response to high temperature and functional analyses of the *ALDH7B4* promoter in *Arabidopsis thaliana*

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DECLARATION

I hereby declare that the whole Ph.D. thesis is my own work, except where it is explicitly stated marked as citation in the text.

Bonn, 2017

Junyi Zhao

DEDICATION

To

My Parents

Di Yang and Zhao Xuming

CONTENT

LIST OF ABBREVIATIONS

ABA	Abscisic acid
ABRE (ACGT)	ABA-responsive element
ALDH	Aldehyde dehydrogenase
Amp	Ampicillin
APS	Ammonium persulfate
ATAF	*Arabidopsis* transcription activation factor
bp	Base pair
BSA	Bovine serum albumin
BADH	Betaine aldehyde dehydrogenase
β-ME	β-Mercaptoethanol
CaMV	Cauliflower mosaic virus
cDNA	Complementary DNA
CDPK	Calcium-dependent kinase
CRT	C-repeat core motif
CUC	Cup-shaped cotyledon
DMF	N, N-Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
DRE	Dehydration-responsive element
DTT	Dithiothreitol
DW	Dry weight
EMSA	Electrophoretic mobility shift assay
EDTA	Ethylenediamine tetraacetic acid
fwt	Fresh weight
fwd	Forward
GB	Glycine betaine
gDW	Gram dry weight
GFP	Green Fluorescent Protein
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	Kilo Dalton
LB	Luria and Bertani medium
LEA	Late Embryogenesis Abundant
MDA	Malondialdehyde
MOPS	3-(N-morpholino) propanesulfonic acid
mRNA	Messenger RNA
MS	Murashige and Skoog
MW	Molecular weight
NAC	NAM, ATAF1/2, and CUC
NAM	No apical meristem
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
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

Rev	Reverse
Rif	Rifampicin
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
RT-PCR	Reverse transcription-polymerase chain reaction
SDS	Sodium dodecyl sulfate
SSC	Saline sodium citrate buffer
ssDNA	Single-stranded DNA
TAE	Tris-Acetate-EDTA
Taq	*Thermophilus aquaticus*
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TE	Tris (10mM)-EDTA (1 mM)
TEMED	N, N, N', N'-tetramethylethylenediamine
TM	Melting temperature
Tris	Tris-(hydroxymethyl)-aminomethane
Triton X-100	Poly (ethylenglycolether)n-octylphenol
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
YEB	Yeast extract broth
YEPD/YPD	Yeast extract peptone dextrose
YPAD	Adenine supplemented YPD
3-AT	3-Amino-1,2,4-triazole
4-MU	4-Methylumbelliferone
4 –MUG	4-Methylumbelliferyl-β-D-glucuronide

SUMMARY

High temperature, dehydration, salinity, and wounding are major environmental factors limiting crop growth and yield worldwide. The combination of these stresses causes many physiological changes that affect crop yield and quality. In this study, the function of aldehyde dehydrogenase (*ALDH*) genes in response to heat stress alone and in combination with dehydration, salinity, or wounding stress was investigated using selected *ALDH* genes and characterized transgenic *A. thaliana ALDH* double knock-out lines. As heat stress often occurs in combination with other stresses, the purpose of this study was to first investigate the response of selected *ALDH* genes to heat and secondly to a combination of heat and other abiotic stresses. Five selected *ALDH* genes of the families 3, 7 and 10 have been analyzed in this work (*ALDH3H1, ALDH3I1, ALDH7B4, ALDH10A8, and ALDH10A9*). The *ALDH* enzymes produce NADPH and NADH in their enzymatic reactions and also contribute to balancing redox equivalents. Previous studies showed that the expression of selected *ALDH* genes was induced in response to various abiotic stresses, such as dehydration, salinity (NaCl), heavy metals (Cu^{2+} and Cd^{2+}), oxidative stress (H_2O_2) and ABA treatment. Overexpression of *ALDH* genes improved stress tolerance and knock-out lines of *ALDH* genes showed increased sensitivity to stress treatments in *A. thaliana,* suggesting important roles in environmental adaptation. Nevertheless, compared with other stresses, the role of *ALDH* genes in high temperature and stress combinations (heat combined with dehydration, wounding or salinity stress) is unclear.

Expression of selected *ALDH* genes was analyzed on the transcript and protein level at different time points of heat stress and in response to basal or acquired thermotolerance. The experiments were done with seedlings and

mature plants. The results showed that *ALDH* genes, particularly *ALDH7B4*, is strongly induced by heat stress, indicating that *ALDH* genes play a crucial role in protecting plants from high temperature damages. To investigate the involvement of *ALDH* genes in heat stress responses, *ALDH* mutant lines were compared with wild-type plants under heat stress regimes. The comparison of the physiological and biological parameters (survival rates, photosynthesis, lipid peroxidation and chlorophyll content) in T-DNA double mutants of *ALDH* genes and wild-type plants demonstrated that mutant lines are more sensitive to heat stress. Secondly, to understand how the *ALDH* genes are regulated under multiple stresses, expression of selected *ALDH* genes was analyzed in wild-type *A. thaliana* plants under a combination of stress conditions. The physiology of plants was also analyzed including lipid peroxidation and chlorophyll content. A combination of heat stress with dehydration resulted in an increased *ALDH7B4* expression more than in the case of a single stress. Double knock-out lines of *ALDH* genes accumulate more MDA than wild-type under heat-dehydration (H/D) and dehydration-heat (D/H) stress. However, a smaller effect was seen in the chlorophyll content in response to heat combined with dehydration stress treatment. A higher expression of *ALDH7B4* was observed under heat combined with salinity stress than during a single stress. Higher accumulation of MDA in mutant plants than in wild-type plants was detected. When wounding and heat stress was applied in combination, besides *ALDH7B4*, other *ALDH* genes were not induced in response to both heat-wounding (H/W) and wounding-heat (W/H), although the ALDH genes were highly regulated by wounding alone. More MDA accumulates during wounding-heat (W/H) stress than during a single stress. These results suggest that different stress combinations cause different expression patterns of *ALDH* genes. Knock-out of *ALDH* genes results in higher sensitivity to stress

combinations. *ALDH* genes may be involved in the antioxidant defense machinery to eliminate ROS during the heat and combined stress situations.

The study of the *ALDH7B4* promoter in *A. thaliana* showed the functional significance of two *cis*-acting elements, ACGT and DRE/CRT, present in the *ALDH7B4* promoter. This was shown by studying six ALDH *GUS* fusion lines along with lines carrying promoter deletions and base substitutions. The results indicated that DRE/CRT and ACGT1 motifs are vital for the response to heat stress combined with wounding or salt stress. In addition ACGT2 and ACGT3 promoter elements play a crucial role for *ALDH7B4* gene expression and stress responsiveness. Using a yeast one-hybrid screen, several NAC genes, were identified to bind to the *ALDH7B4* promoter including ATAF1 (ANAC002), which is important in plant adaptation to abiotic stress and development. Experiments demonstrated that ATAF1 activates *ALDH7B4* by directly binding to a specific promoter region *in vivo* and *in vitro*. Overexpression of *ATAF1* in *A. thaliana* plants results in elevating the expression of *ALDH7B4* in seeds, seedlings, and mature plants, whereas *ATAF1* T-DNA knock-out plants abolished the expression of *ALDH7B4* in different stages of growth (seeds, seedlings, and mature plants). This part of the study demonstrates that the ATAF1 acts as a DNA-binding transcription factor that activates *ALDH7B4* expression by directly binding to its promoter region.

1. INTRODUCTION

1.1 Climate changes affect global food security

Societies and ecologies are influenced by the multiple interconnect risks and uncertainties between climate changes and food security (Wheeler and Braun 2013). FAO (United Nations Food and Agricultural Organization) predicts that about 2 billion of the global populations of more than 7 billion are food insecure. According to the '2012 Global Hunger Index', the most extreme 'alarming' prevalence of hunger category is in sub-Saharan Africa or South Asia **(Fig. 1)** (von Grebmer et al. 2012; Wheeler and Braun 2013). Since the 1850s, the average global temperature has risen by 0.8°C and by the end of this century, the global mean temperature could be 1.8°C to 4.0°C warmer than at the end of the previous century (Solomon and Manning 2007). **Fig. 2** displays the global impacts of climate change on crop productivity (Bierbaum and Zoellick 2009). Warmer temperature will change the rainfall and enhanced frequency of extreme weather (Trenberth 2005), and increase the risk of drought, reduce photosynthesis rates and the light interception (Tubiello et al. 2007). As a result, the frequency of extreme climatic events and severity of climatic events will have serious consequences for food production and food insecurity (Deryng et al. 2014; IPCC 2014). Heat stress damage of plants is particularly severe during the reproductive period including controls of the rate of plant metabolic processes that ultimately influence the production of biomass, fruits and grains (Teixeira et al. 2013).

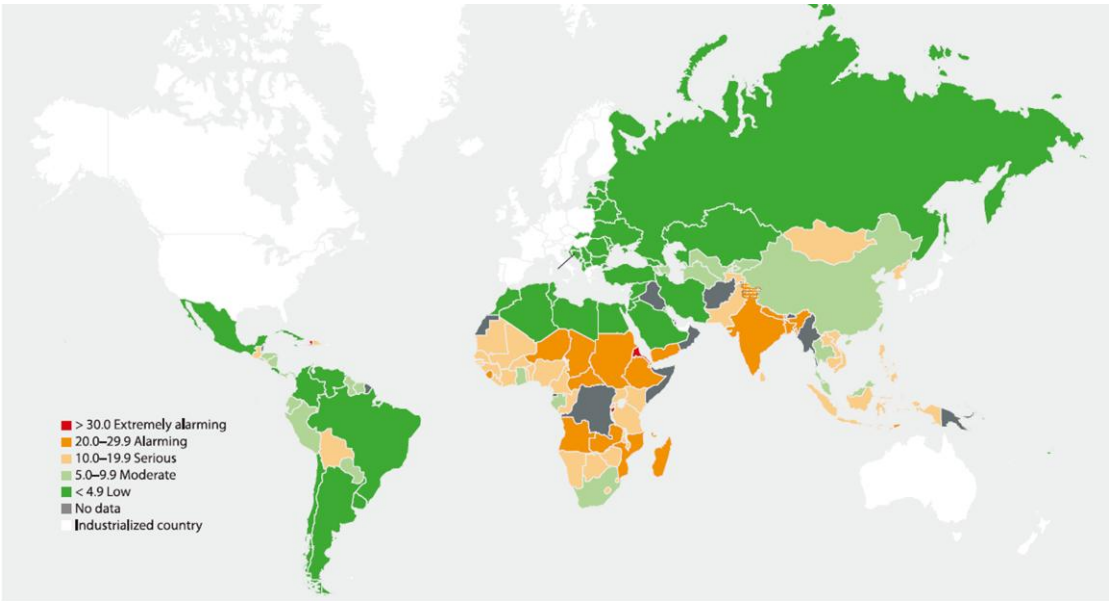


Fig. 1 Global distribution of hunger as quantified by the 2012 Global Hunger Index. 120 countries estimate the proportion of people who are undernourished, the proportion of children under five years old who are under- weight, and the mortality rate of children younger than age five (von Grebmer et al. 2012).

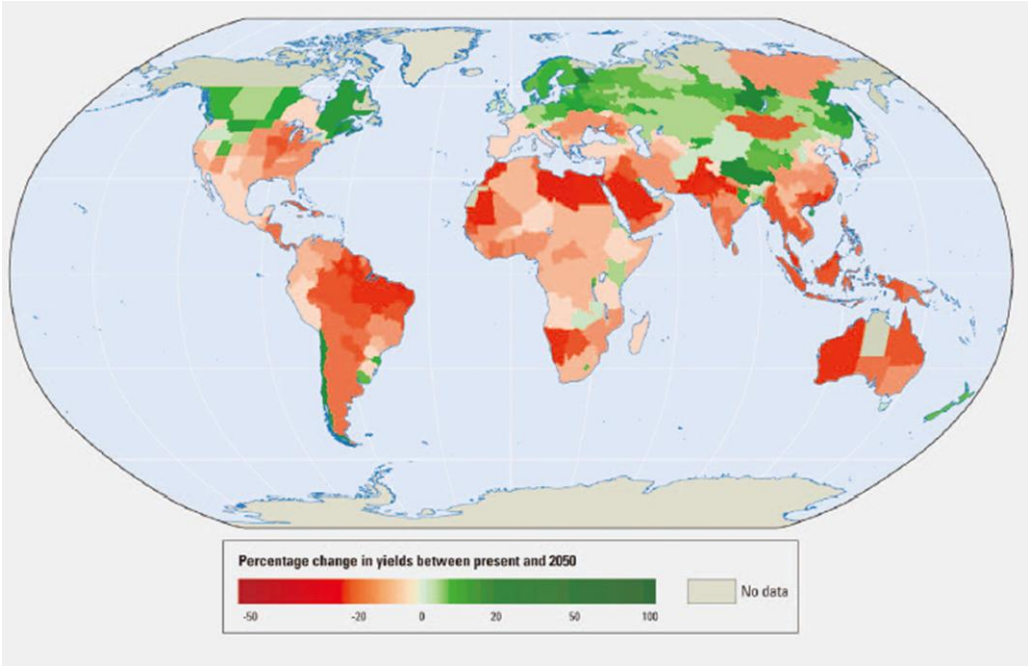


Fig. 2 Global impacts of climate change on crop productivity from simulations published in 2010 (Bierbaum and Zoellick 2009)**.**

1.2 Major effects of high temperature on plants

Temperature above the normal optimum is sensed as heat stress by all living organisms (Kotak and Larkindale 2007). Heat stress is a major environmental stress that limits the growth, metabolism, and productivity in global, causing multifarious, and often adverse effects **(Fig. 3)** (Hasanuzzaman et al., 2013a), including elevated respiration, reduced photosynthetic rates, altered timing of the circadian clock, induction of the protein unfolding, aggregation and degradation, loss of membrane integrity and acceleration of senescence (Prasch and Sonnewald 2015).

Heat stress also differentially alters the internal morphology of plants, including stability of various proteins, membranes, nucleic acids, cytoskeleton structures, and alters the efficiency of enzymatic reactions in the cell, causing a state of metabolic imbalance (Ruelland and Zachowski 2010; Mittler et al. 2012; Hasanuzzaman et al. 2013a). Larkindale et al. revealed that in plants, 5% of the transcriptome is upregulated more than twofold in response to heat stress (Larkindale et al. 2005). On the other hand, heat stress also represses expression of genes involved in cell growth, including histones and DNA polymerases and deregulation of DNA methylation and transposon activation (Pecinka et al. 2010).

Fig. 3 Major effects of high temperature on plants (Hasanuzzaman et al. 2013)**.**

1.2.1 Responses of plants to heat stress

Because plants are sessile organisms, they have to modify their metabolism to avoid damage caused by heat (Mittler et al. 2012). The ability of plants to respond and acclimate to severe heat stress is generally known as basal thermotolerance (Lee and Schöffl 1996; Hong and Vierling 2001). Acquired thermotolerance induced by pre-exposure to elevated but non-lethal temperatures that enable organisms to survive a subsequent severe heat stress that would be lethal in the absence of the preconditioning of the heat treatment (Landry et al. 1989). Acquired thermotolerance enhances basal thermotolerance and heat endurance through a transition to “efficient” cellular performance when reached to acclamatory homeostasis (Bokszczanin and Fragkostefanakis, 2013).

The first observations of damages caused by heat stress are sunburns on leaves, branches and stems, leaf abscission, shoot and root growth inhibition and fruit damage (Prasad et al. 2006; Wahid 2007). Some research showed that decreased relative growth rates under heat stress also causing the reduction in net assimilation rates (Morales et al. 2003). During germination stage of plants, a high temperature decrease the germination percentage and loss of vigor were observed in soybean and tobacco plants (Ren et al. 2009; Dobra et al. 2010). In *A. thaliana*, heat-treated seeds displayed reduced germination percentage, plant emergence, abnormal seedlings, and ultimately results in poor vigor seedlings (Toh et al. 2008). The major consequence of heat stress leads to membrane permeability and can generate excess reactive oxygen species (ROS), which cause oxidative stress (Asada 2006). Oxidative stress negatively affects plant vegetative organs and development of reproductive (Qi, Wang et al. 2011). Moreover, oxidative stress caused by heat stress also increased the membrane peroxidation lipids and accumulation of MDA levels in several crops. In rice and wheat, heat stress reduced the antioxidant enzyme activities that increased MDA contents in leaves (Hurkman et al. 2009; Savicka 2010). Similarly, increased membrane damage and MDA contents were also observed in cotton, sorghum, and soybean (Rahman et al.,2009; Xu et al., 2011). Heat stress results in ROS accumulation at the plasma membrane can also cause membrane depolarization and trigger programmed cell death (Qi et al. 2011). In plants, physiological processes such as photosynthesis and respiration are more sensitive to heat stress (Wahid 2007). Heat stress has a negative influence on chloroplast, carbon metabolism of the stroma and photochemical reactions in thylakoid lamellae (Cheng et al. 2009a). In addition, the photosystem II (PSII) activity and amount of photosynthetic pigments are reduced under heat stress (Suwa et al. 2010). In soybean, rice, tobacco and oak leaves, heat stress

significantly decreased total chlorophyll content, chlorophyll a content, chlorophyll a/b ratio, and Fv/Fm ratio. As a consequence, decreased in sucrose content, sugar content and soluble sugars content were observed (Hurkman, Vensel et al. 2009, Suwa, Hakata et al. 2010, Djanaguiraman, Prasad et al. 2011, Tan and Meng et al. 2011). Reproductive tissues of plants also showed a high sensitivity to heat stress. A significant decrease or abortion in floral buds and flowers and increase sterility were observed under heat shock stress (CAO et al. 2008; McClung and Davis 2010).

1.2.2 Adaptation of plants to heat stress

In order to counteract the harmful effects of heat stress on cellular metabolism, plants respond to heat stress by reprogramming their transcriptome, proteome, metabolome and lipidome (Bita and Gerats 2013). Such changes are intended to establish a new balance of metabolic processes that can enable the organism to function, survive and even reproduce at a higher temperature. Heat stress can change membrane properties and activate a calcium channel, which follows the inward flux of calcium thought to activate signal transduction events and alter in an 'acclimation' process **(Fig. 4)** (Mittler et al. 2012). As shown in **Fig. 4**, that acclimation pathways act as the primary heat sensing mechanism of plants (highlight in yellow). In plants, at least four sensors have been proposed to trigger the heat stress response. Heat-induced changes may occur in protein stability and exposure of hydrophobic residues of proteins that may trigger the unfold protein response (UPR) sensors in the cytosol and the endoplasmic reticulum (ER), histone eviction in the nucleus, accumulation of ROS and alteration in cellular energy levels, and unfolding of RNA species that could act as riboswitches or affect spliceosome and miRNAs function (Mittler et al. 2012).

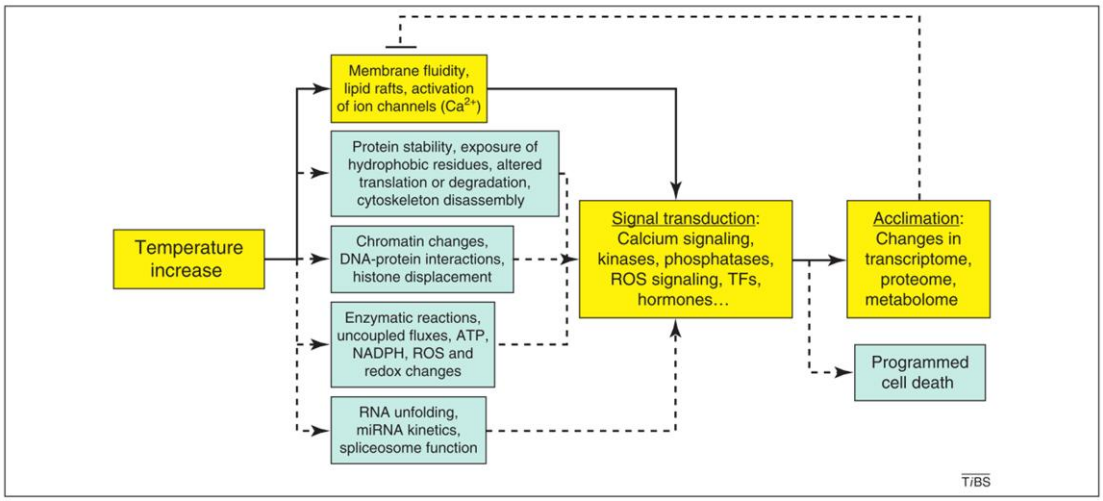


Fig. 4 A schematic model for temperature sensing in plants. Increase in ambient temperature affects many different processes (Mittler et al. 2012).

There are two types of plant adaptation to heat stress, avoidance and tolerance mechanisms **(Fig. 5)**. Avoidance or escape mechanisms involve in turn away from leaf blades from light and orient the leaves parallel to sun rays, transpiration cooling, and alteration of membrane lipid compositions (Hasanuzzaman et al. 2013a). Under heat stress, avoidance mechanisms in plants involved in early maturation and decreased yield (Borras-hidalgo 2005). The tolerance mechanisms are defined as the ability of the plant to grow and produce an economic yield under heat stress (Wahid 2007; Hasanuzzaman et al. 2013a). Plants have evolved various mechanisms for heat stress, including signaling cascades and transcriptional control, ion transporters, expression of stress proteins, antioxidant defense and osmoprotectants (proline, glycine betaine, and trehalose) **(Fig. 5)** (Kocsy et al. 2002; Borras-hidalgo 2005). The accumulation of heat shock proteins (HSPs) under control of heat stress transcription factors (HSFs) is presumed to play an important role in the heat stress response in plants (Kotak and Larkindale 2007). HSPs were strongly induced by heat stress, including HSP100, HSP90, HSP70, HSP60 and small HSP proteins (sHSPs). These HSPs have important roles in renaturing

proteins which denatured by heat stress (Rizhsky et al. 2004; Bokszczanin and Fragkostefanakis 2013; Ohama et al. 2016). Zhong et al. (2013) suggested that in *A. thaliana*, sHSP21 is essential for chloroplasts under heat stress. Besides HSP protection mechanism, other transcripts encode proteins that are involved in calcium signaling, protein phosphorylation, phytohormone signaling, sugar and lipid signaling and metabolism, RNA metabolism, translation, primary, and secondary metabolisms, transcription regulation and responses to different stresses (Nover et al. 2001; Mittler et al. 2012).

In plants, heat stress tolerance is closely related to antioxidative capacity. Former studies reported that heat-acclimated species have lower production of ROS than non-acclimated plants from heat-induced oxidative stress conditions (Xu et al. 2006; Hasanuzzaman et al. 2013a). Tolerant plants overcome the damages of ROS with generating of various enzymatic and non-enzymatic ROS scavenging and detoxification systems (Apel and Hirt 2004; Sharma et al. 2012). The activities of antioxidant enzymes, such as catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD), peroxidase (POX) and glutathione reductase (GR) are heat stress sensitive (Almeselmani et al. 2006; Chakraborty and Pradhan 2011). Ascorbic acid (AsA), Glutathione (GSH), tocopherol and carotene, which involved in antioxidant metabolites also protect plants against oxidative stress (Sairam et al. 2000). GSH levels, the enzymes involved in GSH synthesis and the ratio of GSH/GSSG also increased in wheat (Kocsy et al. 2002). Antioxidant enzymes showed contribute to maintaining the chlorophyll content and reduce membrane damages index during most of the stages in wheat, rice, and maize (Almeselmani et al. 2006; Kumar et al. 2012). Taken together, as temperature increased, so will expression of the antioxidative enzymes. The temperature increased activities are different in tolerant and susceptible varieties (Chakraborty and Pradhan 2011).

Recent studies indicating that not only transcription factors are essential during heat stress, but also epigenetic regulators and small RNAs, has an important role in adaption to heat stress (Ohama et al. 2016). Methylation of histone H3 lysine 4 (H3K4) is involved in the sustained expression of heat stress-inducible genes after heat stress (Zhang et al. 2009b). Moreover, small RNAs, including miR398 and miR156 affect the heat stress response by regulating the activity of various transcription factors (Guan et al. 2013; Stief et al. 2014).

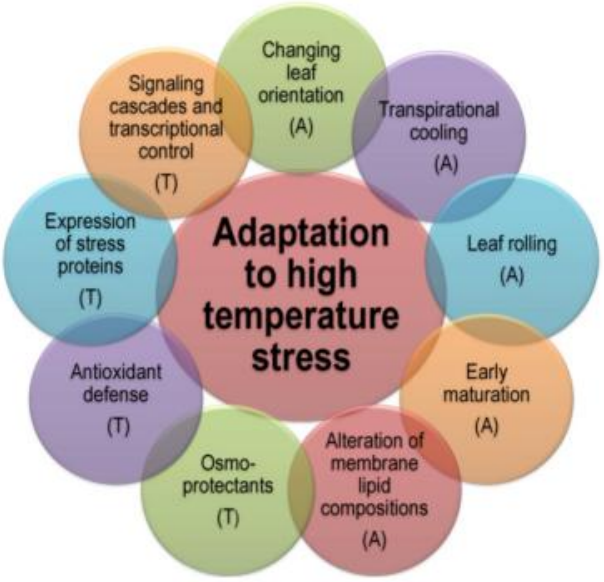


Fig. 5 Different adaptation mechanisms of plants to high temperature. A: Avoidance, T: Tolerance (Hasanuzzaman et al. 2013a)

1.2.3 Heat stress signal transduction

Multiple external and internal signaling pathways are involved in the heat stress response, which controls activation of heat shock proteins (HSPs) and regulates various responses. There are multiple signal transduction molecules involved in stress-responsive gene activation. Some major signal molecules are the Ca^{2+}- dependent protein kinases (CDPKs), mitogen-activated protein kinases (MAPK/MPKs), nitric oxide (NO), sugar, which acts as signaling

molecule and phytohormones (salicylic acid, abscisic acid, gibberellin, jasmonic acid, and brassinolide) (Ahmad et al. 2012). These signaling molecules, HSPs, and transcription factors work together and activate the expression of heat-induced genes, which are involved in detoxifying pathways and activate the essential enzymes and structural proteins to maintain cellular homeostasis (Hasanuzzaman et al. 2013a).

In general, there are four different sensors of cellular responses in plants response to heat stress, including activation of a plasma membrane (PM) channel, a histone sensor in the nucleus and two unfolded protein sensors in the endoplasmic reticulum (ER) and cytosol (Mittler et al. 2012). It was reported that heat is sensed at the plasma membrane and Ca^{2+} channels are opened by modulation of membrane fluidity. This would allow a specific inward flux of extracellular Ca^{2+} ions into the cytoplasm, which can regulate multiple signaling pathways (Saidi et al. 2009a). There are more than forty putative Ca^{2+} channels in *A. thaliana* genome, which probably located in the plasma membrane and may act as the sensors to heat stress (Ward et al. 2009). Moreover, it has been reported that the CaLM3 in is essential for heat stress signaling in *A. thaliana* (Zhang et al. 2009a). As shown in **Fig. 6**, heat stress negatively affects membrane stability and activates a PM Ca^{2+} channel, leading to an inward flux of Ca^{2+}. Ca^{2+} binds the calmodulin CaLM3 and activates various protein kinases, transcriptional regulators of HSP (HSFs, MBF1c, WRKY, and DREB) and NADPH/respiratory burst oxidase protein D (RBOHD), which can trigger multiple mitogen-activated protein kinases (MAPKs) (Sangwan et al. 2002). ROS that derived from RBOHD can lead to membrane depolarization and trigger the ROS/redox signaling, which would further activate downstream pathways through protein including MBF1c, certain HSFs, MAPKs, and SnRKs (Bokszczanin and Fragkostefanakis 2013). In addition, heat-induced changes in membrane stability also trigger lipid

signaling via the enzyme phospholipase D (PLD) and a PIPK (phosphatidylinositol 4, 5-bisphosphate kinase). Molecules involved in lipid signaling like phosphatidic acid (PA), PIP2 (phosphatidylinositol phosphate kinase), and IP3 (D-myo-inositol-1, 4, 5-trisphosphate) accumulate under heat stress (Mishkind et al. 2009). The accumulation of these signaling molecules could in turn cause opening of channels and the triggering of a Ca^{2+} influx (Nover et al. 2001). Another sensor of cellular responses in plants to heat stress is a histone sensor in the nucleus. A research of *ARP6* gene in *A. thaliana* encodes a subunit of the SWR1 complex, which is involved in response to heat stress (Kumar and Wigge 2010).

Besides activation of a plasma membrane (PM) channel and a histone sensor in the nucleus, heat-induced exposure of hydrophobic residues of proteins may also trigger the unfolded protein response (UPR) in the cytosol and the endoplasmic reticulum (ER) (Mittler et al. 2012). Plants need to cope with the negative effects of the accumulation of unfolded proteins after heat stress, the UPR is a signaling pathway activated in cells in response to stress that triggers protein instability in the ER (Bernales et al. 2006). Because the UPR activation required Ca^{2+} signal from the PM, the UPR protective mechanisms are less sensitive as the Ca^{2+} channel and not the primary sensor to heat stress (Saidi et al. 2009b; Mittler et al. 2012). Changes stability in the ER cause a further increase in the level of chaperone transcripts which targeted in the ER and activate of brassinosteroid pathway (Che et al. 2010). There are two UPR signaling pathways in plants, proteolytic processing which involved in bZIP and RNA splicing factor, such as IRE1. Sidrauski et al. (2007), suggested that IRE1 have both serine/threonine protein kinase and endoribonuclease activity. Under heat stress, bZIP members in *A. thaliana* AtbZIP17 and AtbZIP28 respond to UPR (Che et al. 2010). The cytosolic UPR activated by unfolded proteins in the cytosol. In *A. thaliana* which is likely to be linked to the heat

shock promoter element and the specific heat shock proteins, especially HsfA2, which regulated by alternative splicing and nonsense-mediated mRNA decay (NMD) (Sarkar et al. 2009). Recent studies suggested that HsfA1 acts as 'master regulators' involved in the activation of transcriptional networks during heat stress. HsfA1s have been predicted to directly regulate the expression levels of heat stress-responsive transcription factors, such as DREB2A, HsfA2, HsfA7a, HsfBs, and MBF1C. Knockout of *HsfA1* in *A. thaliana* caused the decreased expression of many heat stress-responsive genes and reduced heat stress-sensitive phenotypes (Yoshida et al. 2011; Ohama et al. 2016). In addition to HSFs, overexpression of transcription factors also showed improved heat stress tolerance (Gao and Han 2009; Kim et al. 2011). Díaz *et al*reported that *HOT5* gene, which encoded the type III alcohol dehydrogenase 5 (*ADH5*, *GSNOR*) is required for heat stress acclimation (Díaz et al. 2003). NAC transcription factors, NAC019 and JUB1 are involved in the heat stress response (Wu et al. 2012; Guan et al. 2014).

Changes in metabolic and ROS signaling also contribute to heat stress responses (HSR). It has been suggested that heat stress can cause the accumulation of ROS, which could act as signals to trigger the HSR (Rizhsky et al. 2004). However, under heat stress, the accumulation of ROS is also an active response, which mediated by ROS-producing enzymes and inhibits of the ROS- producing enzyme NADPH oxidase (KÖNIGSHOFER et al. 2008). As described above, ROS that derived from RBOHD can lead to membrane depolarization and trigger the ROS/redox signaling. Thus, RBOHD is required for heat stress signal transduction by direct binding of calcium, which is regulated by protein kinases (Suzuki et al. 2011). An influx of Ca^{2+} mediated by calcium channels at the PM could activate RBOHD and lead to the accumulation of ROS. In addition, ROS accumulation in cells can trigger programmed cell death (PCD) in plants (Mittler et al. 2012).

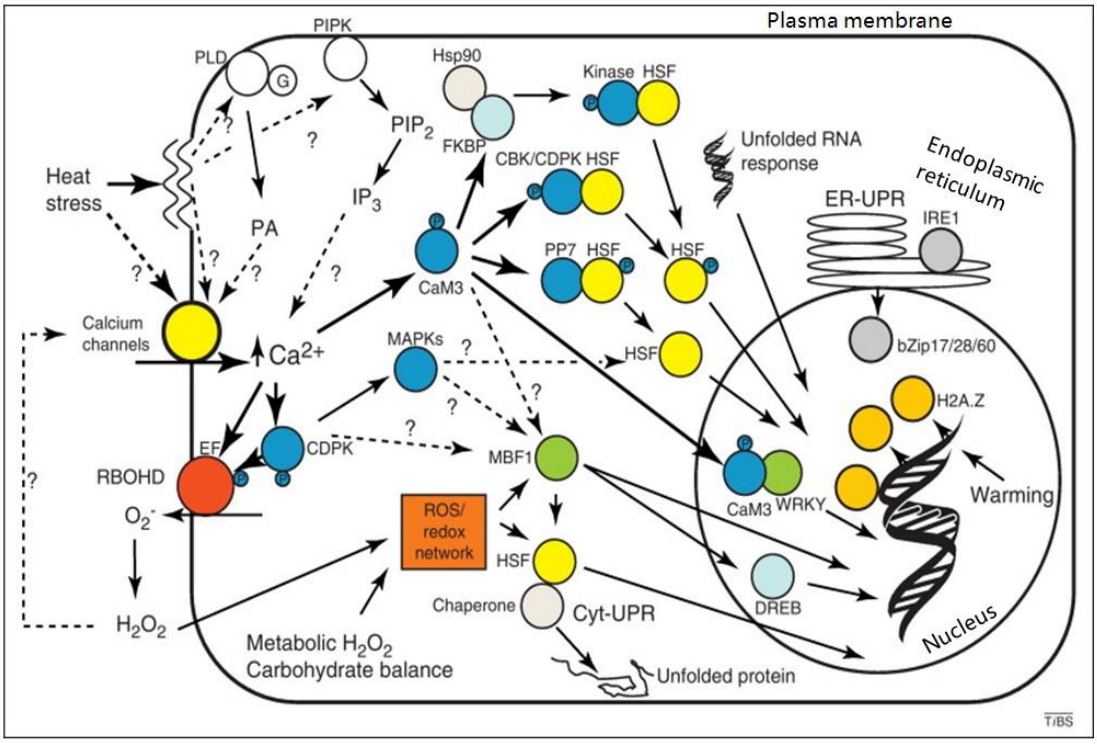


Fig. 6 Signal transduction pathways activated in response to heat stress in plants (Mittler et al. 2012).

1.3 Combination of stresses

It is the simultaneous occurrence of multiple abiotic stresses, rather than one particular stress condition, including heat, drought, salinity and pathogen attack or wounding stress (Mittler 2006; Suzuki et al. 2014). Compared with individual stress, the combination stresses caused more damage to crops (Suzuki et al. 2014). Recent studies revealed that the acclimation of plants to a combination of different environmental stresses is unique and cannot be directly deduced from studying the response of plants to each of the different stresses applied individually (Wang et al. 2004; Suzuki et al. 2016). Specific physiological and molecular responses of plants to stress combinations and single stresses were identified (Barnabás et al. 2008). Transcriptome analysis of *A. thaliana* plants subjected to different abiotic and biotic stresses as single stresses, or in combination, revealed that almost 60% of the transcripts only

expressed under stress combinations (Hasanuzzaman et al. 2013b). The stress requires a new defense or acclimation response. Thus, transgenic plants with enhanced tolerance to biotic or abiotic stress conditions should be also tested for their tolerance to a combination of different stresses (Greco et al. 2012). **Fig. 7** summarizes many of the stress combinations that could have a significant impact on agricultural production (The 'Stress Matrix') (Mittler 2006). When heat stress combined with drought, salinity, ozone, pathogen and UV light, displayed potential negative interactions to plants. Whereas, combined ozone and drought/pathogen/UV light shown potential positive interactions. Because several environmental stimuli can affect plant development in combination, numbers of stress-responsive signal transduction pathways have to be integrated to adapt resource allocation between defense, growth, and reproduction **(Fig. 8)**. The protein kinase networks transduced signals from phytohormone, ROS, Ca^{2+}, lipid, and metabolite to cellular adaptations (Prasch and Sonnewald 2015).

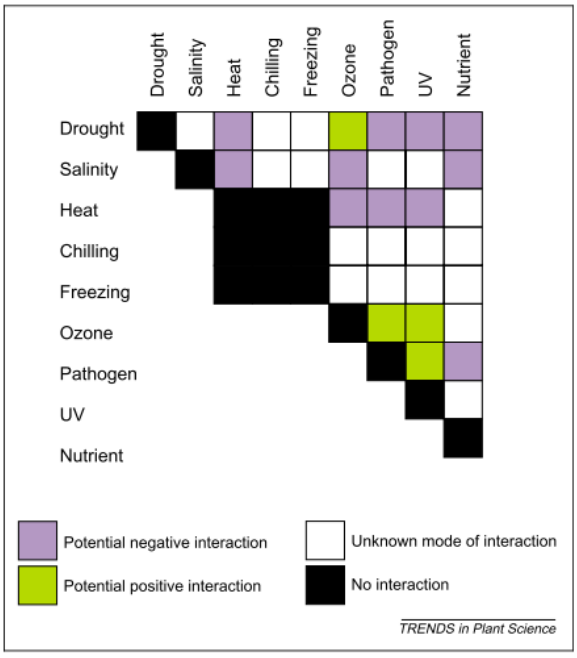


Fig. 7 Agriculturally important stress combinations ('The Stress Matrix') (Mittler 2006)**.**

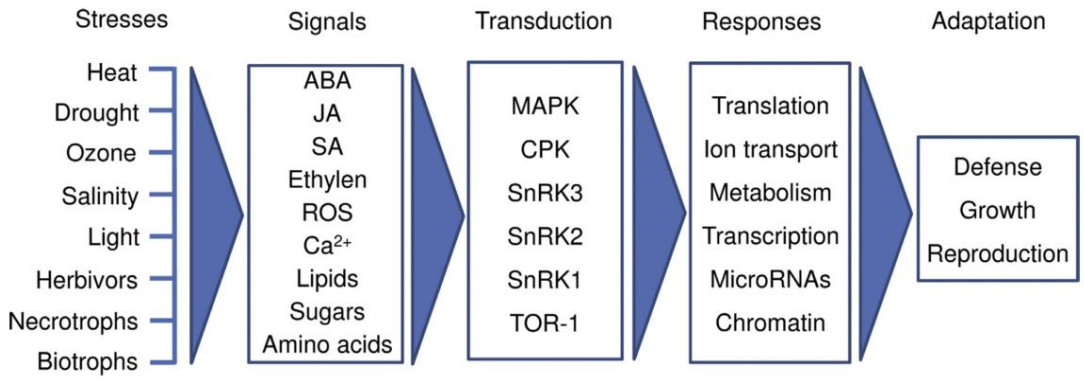


Fig. 8 Crucial events in the signal transduction pathway activated by biotic and abiotic stress factors (Prasch and Sonnewald 2015)**.**

1.3.1 A combination of heat and dehydration stress

High temperature and dehydration stress represent the two most frequent abiotic stress combination occurring in natural environments, which affects plant growth and yield (Savin and Nicolas 1996; Rizhsky et al. 2002a; Rizhsky et al. 2004). Physiological changes of plants subjected to individual stress and a combination of heat and dehydration stress found that stress combination has some unique aspects, including low photosynthesis, closed stomata and high leaf temperature (Rizhsky et al. 2002a). Recently, Suzuki et al. pointed out the combined effects of heat and dehydration were generally additive, suggesting a certain degree of independence between the mechanisms regulating the responses of plants to dehydration or heat stress (Suzuki et al, 2014).Studies on transcriptome profiling of individual and combination revealed almost 770 transcripts that only express in stress combination conditions. Similarly, several unique metabolites accumulate specifically during stress combination (Rizhsky et al. 2004). Antioxidant mechanisms play an important role in the response of plants to a combination of dehydration and heat stress (Suzuki et al. 2014). Koussevitzky et al. reported that cytosolic ascorbate peroxidase 1 (APX1) protein was shown to accumulate during

dehydration, heat stress and their combination, and a knock-out mutant of *APX1* was more sensitive to stress combination than wild-type plants (Koussevitzky et al. 2008).

1.3.2 A combination of heat and salinity stress

The effects of salinity stress could be worse when combined with heat stress because enhanced transpiration could cause increased uptake of Na^+ (Keleş and Öncel 2002). A recent study demonstrated that the detrimental effects on tomato plants caused by salinity stress were partially counteracted by heat stress when stresses were combined (Rivero et al. 2014). Nevertheless, Suzuki pointed out *A. thaliana* plants were more susceptible to the combination of salt and heat stress than to each of the different stresses applied individually (Suzuki et al. 2016). Heat stress can cause changes in membrane fluidity that affect the function of membrane-bound ion transporters. Salinity stress can cause Na^+ toxicity that affects K^+ uptake, and cause the damage of enzymatic activities and inhibition of metabolic pathways (Keleş and Öncel 2002). According to the RNA-seq results of *A. thaliana* in response to a combination of salt and heat stress(Suzuki et al. 2016), the enhanced expression of about 700 genes unique to the stress combination are observed after stress combination. Moreover, many of the transcripts that specifically associated with ABA are upregulated. A recent study on a heat and salt combination of stresses also found responses of heat more dominant than salt stress (Rasmussen et al., 2013). The ROS-related membrane lipid peroxidation in *A. thaliana* plant accumulated under either heat or salt stress (Katsuhara et al. 2005). In addition, chlorophyll content of *A. thaliana* leaf was significantly reduced in response to both heat stress and a combination of salinity and heat stress (Suzuki, 2016).

1.3.3 A combination of heat and wounding stress

Plants attacked by a vast range of pests and pathogens (wounding stress), including fungi, bacteria, viruses, nematodes and herbivorous insects. Wounding stress damages plant tissues also triggers pathways for pathogen invasion (Cheong et al. 2002). It has been reported that high temperature is able to influence metabolism and plant defense responses (Mittler and Blumwald 2010; Mittler et al. 2012). Combined occurrence of abiotic and biotic stress may result in synergistic or antagonistic interactions (Zhu et al. 2010). Many abiotic stress conditions are shown to weaken the defense mechanisms of plants (Mittler and Blumwald 2010; Atkinson and Urwin 2012). In defense responses against biotic and abiotic stress conditions phytohormones such as SA, JA, ethylene, and ABA have been described to act synecdochical and antagonistically, known as signaling crosstalk (Fujita et al. 2006). Under stress treatment conditions, Ca^{2+} and ROS are produced independently of biotic and abiotic stress applications and it is assumed that ABA appears to be centrally positioned between ROS and SA signaling (Kissoudis et al. 2014). The findings of research on the triple stress of heat, drought, and virus revealed that heat is the major stress factor and the combination of abiotic stresses almost abolished the defense response induced by virus treatment. Virus-treated plants showed increased expression of defense genes. However when plants additionally subjected to heat and drought stress, the expression of defense genes were abolished (Prasch and Sonnewald 2013).

1.4 Aldehyde dehydrogenase (*ALDH*) genes in *Arabidopsis thaliana*

Aldehyde dehydrogenase (*ALDH*) genes are a family of enzymes, which catalyze the oxidation of reactive aldehydes to their corresponding carboxylic acids. Many kinds of researches on human-ALDH proteins demonstrated their

function in detoxification pathways of cellular metabolisms (Brocker et al. 2010; Stagos et al. 2010). ALDHs are a family of NAD (P) $^{+}$-dependent enzymes with an oxidative function (Kirch et al. 2001). The *A. thaliana* genome encodes 14 genes, classify into nine aldehyde dehydrogenase families (Kirch et al. 2004) **(Fig. 9)**. In this study, I focused on stress-related family 3 and family 7 *ALDH* genes (*ALDH3I1*, *ALDH3H1*, *ALDH3F1*, and *ALDH7B4*) and two Betaine aldehyde dehydrogenase (*BADH*) homologs *ALDH10A8* and *ALDH10A9*. The *A. thaliana* family 3 has three *ALDH* isoforms: *ALDH3F1*, *ALDH3I1*, and *ALDH3H1*. These three isoforms share more than 60% amino acid identity with *C. plantagineum* family 3. ALDH family 7 proteins are highly conserved among plants and animals. A comparative analysis of amino acid sequences derived from animal and plant species shows more than 60% sequence identity (Kirch et al. 2004).

Protein	Locus	Localization	Putative function or pathway involved	Reference
ALDH2B4	At3g48000	Mitochondria	Pyruvate dehydrogenase bypass pathway	Wei et al. (2009)
ALDH2B3	At1g23800	Mitochondria		
ALDH2C4	At3g24503	Cytosol	Phenyl-propanoid pathway (ferulic acid and sinapic acid biosynthesis)	Nair et al. (2004)
ALDH3F1	**At4g36250**	**Cytosol**	**Variable substrate ALDH, stress-regulated detoxification pathway**	**This review**
ALDH3H1	**At1g44170**	**Cytosol**	**Variable substrate ALDH, stress-regulated detoxification pathway**	**This review**
ALDH3I1	**At4g34240**	**Chloroplasts**	**Variable substrate ALDH, stress-regulated detoxification pathway**	**Sunkar et al. (2003); Kotchoni et al. (2006)**
ALDH5F1	At1g79440	Mitochondria	Succinic semialdehyde dehydrogenase, involved in GABA-shunt pathway, stress-regulated detoxification of ROS intermediates, and in the patterning of *Arabidopsis* leaves along the adaxial–abaxial axis	Bouché et al. (2003); Bouché and Fromm (2004); Toyokura et al. (2011)
ALDH6B2	At2g14170	Mitochondria	Putative methylmalonyl semialdehyde dehydrogenase	
ALDH7B4	**At1g54100**	**Cytosol**	**Turgor-responsive, stress-regulated detoxification pathway**	**Kotchoni et al. (2006)**
ALDH10A8	**At1g74920**	**Leucoplasts**	**Putative stress-regulated AMADH, involved in the oxidation of aminoaldehydes derived from polyamine degradation**	**Missihoun et al. (2011)**
ALDH10A9	**At3g48170**	**Peroxisomes**		
ALDH11A3	At2g24270		Non-phosphorylating GAPDH	
ALDH12A1	At5g62530	Mitochondria	Δ^1-Pyrroline-5-carboxylate dehydrogenase, stress-regulated pathway, essential for proline degradation, and protection from proline toxicity	Deuschle et al. (2001, 2004); Miller et al. (2009)
ALDH22A1	**At3g66658**	**Cytosol**	**Plant specific ALDH**	**Kirch et al. (2005), this review**

Fig. 9 Localization and putative physiological functions of the *Arabidopsis thaliana* ALDH protein superfamily (Stiti et al. 2011).

Previous studies revealed that ALDH enzymes have different subcellular localizations, suggesting ALDH enzymes participate in specific biochemical processes. ALDH3I1 is localized in the chloroplast, while ALDH3F1 and

ALDH3H1 are found in the cytosol (Kirch et al. 2004). Transcript accumulation analyses demonstrated that five of the analyzed *ALDH* genes (*ALDH3H1*, *ALDH3I1*, *ALDH7B4*, *ALDH10A8*, and *ALDH10A9*) expression in response to ABA treatment, salt, dehydration, heavy metals and H_2O_2, while *ALDH3F1* and *ALDH22A1* are constitutively expressed at low level (Kirch et al. 2005; Stiti et al. 2011). Promoter-GUS analysis indicating that *ALDH3I1*, *ALDH3H1*, the *ALDH7B4* promoters are induced in response to abiotic stress conditions. The *ALDH7B4* promoter shows high GUS expression in all tissues in response to ABA, dehydration, NaCl, and wounding (Stiti et al. 2011; Missihoun Ph.D. dissertation 2010). *Cis*- elements of *ALDH7B4* promoter involved in stress responsiveness were also analyzed in our lab. Two conserved ACGT-containing motifs close to the translation start codon were important for the responsiveness to osmotic stress in leaves and in seeds (Missihoun et al. 2014). Early studies also found that plants overexpressing *ALDH3F1*, *ALDH3I1*, or *ALDH7B4* genes are more tolerant to salt, dehydration and oxidative stress (Kotchoni et al. 2006). T-DNA single and double insertion mutants of *ALDH*, display an increased sensitivity to dehydration and salt stress, along with accumulating higher MDA levels than wild-type plants (Sunkar et al. 2003; Kotchoni et al. 2006). These results indicate that *ALDH* genes function as aldehyde-detoxifying enzymes, also as ROS scavengers and lipid peroxidation-inhibiting enzymes (Kirch et al. 2005). So far, the response of *ALDH* genes to high temperature (heat) stress and heat stress combined with other abiotic stresses has not been investigated.

1.5 The NAC transcription factors

It is well known that transcription factors and their targeting *cis*-acting elements act as molecular switches for gene expression, regulating temporal and spatial gene expression (Badis et al. 2009). NAC [No apical meristem (**N**AM), *A.*

thaliana transcription activation factor (**A**TAF) and cup-shaped cotyledon (**C**UC)] family proteins are plant-specific transcription factors (Nuruzzaman et al. 2013). This family represents the largest transcription factors of land plants of more than a hundred members in plant genomes. There are at least 151 and 117 NAC family members in rice and *A. thaliana* (Nuruzzaman et al. 2010), 163 in *Populus trichocarpa*, 163 in *Glycine max L*., 152 in *Nicotiana tabacum* and 48 in *Hordeum vulgare L*. (Rushton et al. 2008; Hu et al. 2010; Le et al. 2011). NACs also reported being present in the moss *Physcomitrella patens* (Shen et al. 2009).

1.5.1 Structure of NAC transcription factors

The NAC transcription factors contain a general structure that consists of a highly conserved NAC domain at the N-terminus and a variable C-terminal region. The N-terminal region includes the DNA binding domain, whereas C-terminal region thought to play an important role in the determination of the target genes (Jensen et al. 2009). The transcriptional regulatory domain in C-terminal is highly variable, which may contribute to the functional diversity in different NAC proteins. The NAC domain has five sub-domains (A, B, C, D and E). Studies suggested that the sub-domain C and D are responsible for DNA binding, while sub-domain B and E are more diverse than others (Ernst et al. 2004; Wang et al. 2011). It has been noted that the structure of NAC domain is unique. Unlike the classical helix-turn-helix motif, the NAC domain contains a twisted β-sheet surrounded by helical elements (Ernst et al. 2004).

1.5.2 Biological functions of NACs

NAC transcription factors play important roles in multiple biological processes such as plant development from shoot meristem to auxin signaling (Olsen et al.

2005; Nakashima et al. 2012), response to stresses and hormone signals. When genes encoding NAC transcription factors are overexpressed in plants, robust phenotypes including salt and drought tolerance have been observed (Tran et al. 2004). Studies indicated that NAC proteins also involved in response to biotic stress (Wu et al. 2009; Xia et al. 2010). Microarray analysis overexpressing of *ANAC072* (*RD26*) plants showed up-regulation of stress-inducible genes and ABA-responsive genes suggesting that *RD26* is involved in regulation of drought-responsive genes in an ABA-dependent pathway (Tran et al. 2004). Expression of NACs regulated by several *cis*-acting elements contained in the promoter region. These *cis*-elements including ABREs, DREs, LTREs (Low-temperature responsive elements), MYB and MYC binding sites, W-Box, jasmonic acid-responsive element and salicylic acid-responsive element (Nakashima et al. 2012).

The transcript level of *ATAF1* increases in response to wounding, H_2O_2, and ABA. Moreover, Yamamizo et al. (2016) revealed that ATAF1 as a positive regulator of leaf senescence and drought tolerance and mutants of ATAF1 show hyposensitivity to ABA. There is controversial evidence regarding the function of *ATAF1* in biotic and abiotic (drought) stress. Overexpression of *ATAF1* showed negative regulation of defense responses against necrotrophic fungal in both Wang and Wu's research, while the results regarding the expression of *PR1* gene were opposite (Wang et al. 2009; Wu et al. 2009). The opposite conclusions also occurred regarding *ATAF1* in response to drought stress conditions. One study pointed out that ATAF1 negatively regulates drought-responsive genes by using *ataf1* mutant lines (Lu et al. 2007; Jensen et al. 2008). However, Wu et al. reported that ATAF1 positively regulates drought-responsive genes via overexpression lines of *ATAF1* (Wu et al. 2009). Moreover, ATAF1 also contribute to ABA synthesis by directly regulating the expression of related enzymes (Jensen et al. 2013).

The NAC transcription factors regulate of their downstream genes by binding to a consensus sequence in their promoter regions. NAC proteins can bind CaMV 35S promoter, CGT (G/A) and CACG as the core-DNA binding motif was identified in the promoter of drought-inducible *ERD1* gene in *A. thaliana* (Tran et al. 2004; Nakashima et al. 2007), called NAC binding sites (NACBS). The NACBS are also present in the promoter of *PR* genes indicating that it responsive to biotic stress (Seo et al. 2010).

1.6 Objectives of the study

This study focused on the expression of aldehyde dehydrogenase (*ALDH*) genes and physiological changes in *Arabidopsis thaliana* under high temperature and a combination of stresses and functional analyses of the *ALDH7B4* promoter. This work was mainly comprised of three sections.

1. Study the response of selected *ALDH* genes to heat and in combination with other abiotic stresses.

a. Expression of selected *ALDH* genes in *A. thaliana* wild-type seedlings and mature plants subjected to heat stress

b. Changes in the physiology (including survival rate, photosynthesis, lipid peroxidation and chlorophyll content) of two T-DNA knockout mutant lines *KO6/62* (knock-out of *ALDH7B4* and *ALDH3F1*) and *KO6/76* (knock-out of *ALDH7B4* and *ALDH3I1*) in comparison with wild-type plants in response to heat stress.

c. Phenotypic changes and expression analysis of selected *ALDH* genes in *A. thaliana* wild-type plants during a combination of stresses (heat stress combined with dehydration, salt or wounding)

d. Changes in the physiology (including analysis of lipid peroxidation and chlorophyll content) of two T-DNA knockout mutant lines *KO6/62* (knock-out of *ALDH7B4* and *ALDH3F1*) and *KO6/76* (knock-out of *ALDH7B4* and *ALDH3I1*) in comparison with wild-type plants during a combination of heat stress with other abiotic stresses.

2. Functional analysis of the importance of putative *cis*-elements in the *ALDH7B4* promoter.

The aim of this part was to focus on two selected *cis*-elements (DRE, ACGT1, ACGT2 and / 3) and other putative *cis*-elements in the promoter of *ALDH7B4* gene in response to heat and stress combinations.

a. The *Arabidopsis thaliana* lines carrying *ALDH7B4* promoter-GUS fusions constructs were examined under heat and stress combinations to analyze the importance of promoter elements in response to stress combinations.

b. Identification of putative transcription factor that regulate*ALDH7B4* using a yeast one-hybrid screening.

3. Functional analysis of the transcription factor ATAF1

a. Investigation of DNA binding of the ATAF protein using DIG-labeled electrophoretic mobility shift assays (EMSAs).

b. Transcriptional activation or repression using transient transformation assays.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant materials and growth conditions

2.1.1.1 Plant materials

Arabidopsis thaliana (ecotype *Col-0*) was used as wild type in this work. All transgenic plants were established in the *A. thaliana* ecotype *Col-0*.

T-DNA knock-out mutant lines *KO6/62* (knockout of *ALDH7B4* and *ALDH3F1*), *KO6/76* (knockout of *ALDH7B4* and *ALDH3I1*) and *A. thaliana* lines carrying *ALDH7B4 promoter-GUS* fusion constructs (B8, AB, AD, P1, P2, and P3) were kindly provided by Dr. Tagnon Missihoun (Missihoun 2010; Missihoun et al. 2014).

Seeds of ATAF1 overexpression lines ATAF1-OX1 and ATAF1-OX2 (Wu et al. 2009), and two *ATAF1* T-DNA insertion mutant lines SALK_64806 and SALK_057618 were obtained from Dr. Qi Xie (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China).

2.1.1.2 Growth conditions

For sterile culturing of seeds, *A. thaliana* seeds were surface sterilized in 70% (v/v) ethanol for 2 min then in 7% (v/v) NaOCl (C. Roth; Karlsruhe, Germany) + 0.1% (w/v) SDS for 10 min, rinsed four times with sterile distilled water and sown on MS-agar plates. Before sowing, the seeds were kept for at least 48 h for vernalization at 4°C. All plants were grown under 120-150 µE m^{-2} s^{-1} light at 22°C with a day/night cycle of 8/16 h. For flowering, four- week-old plants were moved to long-day condition (16/8 h photoperiod) growth chamber. Transgenic plants were selected on MS agar plates containing 50 mg/l kanamycin.

2.1.2 Bacteria and growth conditions

2.1.2.1 Bacteria strains

Escherichia coli DH10B

This strain was used as a host for cloning experiments, recombinant plasmids and subsequent sequencing of the plasmid inserts.

Escherichia coli BL21

This strain was used to express recombinant ATAF1 proteins.

Agrobacterium tumefaciens GV3101/pmP90RK

This strain was used for transformation of wild-type *A. thaliana* (ecotype *Col-0*).

Saccharomyces cerevisiae YM4271

This yeast strain has been used for yeast one-hybrid screening. Uracil, leucine, and histidine were used as markers for selection.

2.1.2.2 Growing conditions of bacteria cultures

The different conditions for bacteria cultures are listed in **Table 1** Solid agar plates were incubated with adequate aerobic conditions in the dark. Liquid cultures were grown on a shaker at 200 rpm. Antibiotics were added to select the transformed bacteria.

Table 1 Growing conditions of different bacteria strains.

	E. coli *DH10B*	*E. coli* *BL21*	*A. tumefaciens* *GV3101*	*S. cerevisiae* *YM4271*
Medium	LB	LB	YEB	YEPD or SD
Temperature	37°C	37°C	28°C	30°C
Incubation	10-18 h	14-18 h	48-72 h	3-6 days
Antibiotics	Amp, Kan	Amp, Kan, Carb	Rif, Kan	SD dropout

2.1.2.3 Glycerol stocks of bacteria strains

For long-term storage, the bacteria strains were stored in 30% (v/v) glycerol at -80°C fridge. A single bacterial colony with plasmids was grown in 3 ml of liquid LB-medium containing the antibiotic at 37°C for 10-18 h. 0.5-1 ml of the culture was mixed vigorously with 1 ml of autoclaved 100% (v/v) glycerol and immediately frozen in liquid nitrogen. The final glycerol concentration is 25%-30% (v/v). Glycerol stocks were stored at -80°C until further use.

2.1.3 Chemicals, enzymes, and kits

2.1.3.1 Chemicals and enzymes

The chemicals, enzymes, associated buffers and markers that were used in this study were obtained from the following companies: Amersham (Braunschweig, Germany), AppliChem GmBH (Darmstadt, Germany), Apolloscientific (Bredsbury, CZ), Bio-budget Techologies GmbH (Krefeld, Germany), Biomol (Hamburg, Germany), Bio-Rad (Munich, Germany),

Boehringer (Mannheim, Germany), Clontech (Heidelberg, Germany), Difco (New York, USA), Duchefa Biochemie (Haarlem, Netherlands), Ferak Laborat GmBH (Berlin West, Germany), Fermentas (St. Leon-Rot, Germany), Invitrogen (Karlsruhe, Germany), Labomedic (Bonn, Germany), Macherey-Nagel (Düren, Germany), Merck (Darmstadt, Germany), New England Biolabs (NEB, Ipswich, E), Pharmacia (Uppsala, Sweden), Promega (Mannheim , U.S.), Roth (Karlsruhe, Germany), Sigma-Aldrich (Munich, Germany), TH. Geyer (Renningen, DE), and Stratagene (Heidelberg, Germany).

2.1.3.2 Kits

Nucleo Spin Extract II (Machery-Nagel; Düren, DE)

The DNA fragment was extracted from the agarose gel by dissolving it in the binding buffer and then added to the silica membrane (SiO_2). The membrane binds to the DNA by adsorption at high concentrations of chaotropic salts. TE buffer was used for elution.

CloneJET™ PCR Cloning Kit, (Fermentas; St. Leon-Rot, Germany)

The kit is designed for the efficient cloning of PCR fragments into the vector pJET1.2/blunt.

RevertAid™ H Minus First Strand cDNA Synthesis Kit, (Fermentas; Burlington, Canada)

The cDNA synthesis for reverse transcription polymerase chain reaction was performed using the RevertAid™ H Minus First Strand cDNA Synthesis Kit, (Fermentas; Burlington, Canada).

DIG Gel shift Kit, 2nd generation, (Roche; Roche Diagnostics Deutschland GmbH, Germany)

DNA-protein interactions were performed by 'gel mobility shift' assays using this kit. Because free DNA and DNA-protein complexes migrate differently during gel electrophoresis, they can be separated and detected on native polyacrylamide or agarose gels.

2.1.4 Media and solutions

All media and related solutions were autoclaved at least 20 min at 121°C and 1.2 bars. Antibiotics were added when media for agar plates were cooled during continuous stirring to about 60°C. After solidification, the plates were packaged and stored at 4°C in the dark.

2.1.4.1 Media

MS medium (Murashige and Skoog): 4.6 g/l MS salt, 20 g/l sucrose, 1 ml/l vitamin stock solution, adjust pH to 5.8 with KOH, 8 g/l agar-select for solid medium. This medium was used for plant germination and growth.

1/2 MS-medium: 2.15 g/l MS-salt mixture, 20 g/l sucrose; 150 mg/l ascorbic acid, 100 mg/l citric acid, 2 ml/l 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 5.8with KOH, 8 g/l agar-select for solid medium.

1/4 MS-medium liquid**:** 1.1 g/l MS-salt mixture, 20 g/l sucrose, 1 ml/l vitamin stock solution, 1% (w/v) sucrose, adjust pH to 6.0 with KOH, 0.005% (v/v) Silwet® L-77 (Crompton Corporation, USA). This medium was used for the FAST assay (see 2.2.4.6).

LB medium: 20 g/l Lennox LB powder (10 g Tryptone, 5 g yeast extract, 5 g NaCl) or 35 g/l Lennox LB Agar powder (10 g Tryptone, 5 g yeast extract, 5 g NaCl, 15 g agar) for solid medium were used for growing *E. coli*.

SOC medium: 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, adjust pH 7.0 with NaOH. After filter steriled, 10 mM $MgSO_4$ and 10 mM $MgCl_2$ were added. This medium was used for transformation of *E. coli*.

YEB medium: 5 g/l sucrose, 5 g/l bacto-peptone, 5 g/l meat extract, 1 g/l bacto-yeast extract, adjust pH to 7.0 with NaOH, 15 g/l agar-select for solid medium. 2 mM $MgSO_4$ were added after autoclaving. This medium was used for cultivation of *A. tumefaciens.*

YPD (YEPD) medium: 20 g/l peptone, 10 g/l yeast extract, pH 6.5, 20 g/l agar-select for solid medium. 50 ml/l of 40% (w/v) glucose were added after autoclaving. This medium was used for growing *S. cerevisiae*.

SD medium: 6.7 g/l Yeast Nitrogen base without amino acids (Difco) adjust pH 5.8 with NaOH, 20 g/l agar-select for solid medium. 50 ml/l 40% (w/v) glucose and 100 ml/l of 10 x Dropout-solution were added after autoclaving. This medium was used as a minimal medium for selection of *S. cerevisiae*.

2.1.4.2 Solutions

Ampicillin stock solution: 100 mg/ml in Milli-Q water, filter sterilized and aliquoted. Storage at -20°C, final working concentration is 100 µg/ml.

Kanamycin stock solution: 50 mg/ml in Milli-Q water, filter sterilized and aliquoted. Storage at -20°C, final working concentration is 50 µg/ml.

Rifampicin stock solution: 50 mg/ml were dissolved in DMSO by vortexing, filter sterilized and aliquoted. Storage at -20°C, final working concentration is 100 µg/ml.

Carbenicillin stock solution: 50 mg/ml in Milli-Q water, filter sterilized and aliquoted. Storage at -20°C, final working concentration is 50 µg/ml.

Vitamin stock solution: 2 mg/ml glycine, 0.5 mg/ml nicotinic acid, 0.5 mg/ml pyridoxine HCl, 0.1 mg/ml thiamine-HCl in Milli-Q water, store at 4°C. 1:1000 dilution was used in the medium.

10X Dropout solution**:** 200 mg/l adenine hemisulfate salt; 200 mg/l arginine HCl; 200 mg/l histidine HCl monohydrate; 300 mg/l isoleucine; 300 mg/l lysine HCl; 1,000 mg/l leucine; 200 mg/l methionine; 500 mg/l phenylalanine; 2,000 mg/l threonine; 200 mg/l tryptophan; 300 mg/l tyrosine; 200 mg/l uracil; 1500 mg/l valine. Different amino acid dropout solutions were prepared without histidine, uracil, and leucine, or a combination of histidine, uracil, and leucine. The solutions were autoclaved and stored at 4°C. This medium was used with SD medium for selection of auxotrophic yeast.

3-AT stock solution: 1 M 3-amino-1, 2, 4-triazole in Milli-Q water. Filter sterilized and stored at 4°C. Using 0 mM to 60 mM of 3-AT in SD dropout medium to select the *HIS3* expression. 3-AT is a competitive inhibitor of *HIS3* protein in histidine synthesis.

1× TE buffer: 10 mM Tris-HCl; 1 mM EDTA; pH 8.0, store at room temperature.

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; adjust pH to 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.

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% (w/v) 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.

2.1.5 Vectors

All the vectors used in this work are kept as plasmids at -20°C or in glycerol stock at -80°C. The vectors used in this study were listed below (molecular details of vectors are shown in the Appendix).

pJET1.2/ blunt

This vector designed for blunt-end cloning and it is already linearized with blunt ends (Thermo Scientific, St. Leon-Rot, Germany). It contains the β-lactamase

gene, ampr, which confers resistance to ampicillin, the *Eco47IR* gene, which allows positive selection of transformants. This gene codes for the Eco47I restriction enzyme, and when the enzyme is expressed, it is toxic to *E. coli*. When the *Eco47IR* gene is disrupted by the insertion of DNA into the cloning site, the gene will no longer be expressed and the transformed cells will grow and divide on selective media.

R4L1pDEST_LacZi (Mitsuda et al. 2010)

The plasmid R4L1pDEST_LacZi is a derivative of the yeast integration and pLacZi vector (Clontech). The *attR4-ccdB/Cmr-attL1* cassette was inserted into the MCS in front of the *lacZ* reporter gene under the control of the iso-1-cytochrome C promoter of yeast. A *Col E1 Ori* and a *bla* gene for ampicillin resistance allow the reproduction of the vector in *E. coli*. The *ccdB* gene encoded protein is toxic for the *E.coli* strains. URA3 as a selection marker for integration of the linearized vector into the yeast genome.

R4L1pDEST_HISi (Mitsuda et al. 2010)

The plasmid R4L1pDEST_HISi is a derivative of the yeast integration and pLacZi vector (Clontech). The *attR4-ccdB/Cmr-attL1* cassette was inserted into the MCS in front of the reporter gene *HIS3* under the control of a minimal promoter of *HIS3*. The *ccdB* gene encoded protein is toxic for the *E.coli* strains. HIS3 or URA3 are as a selection marker for integration of the linearized vector into the yeast genome.

TFpDEST_GAD424 (Mitsuda et al. 2010)

This plasmid is a carrier of *A. thaliana* factors. The library contains 1,498 transcription factors and was used for the yeast one-hybrid screening. cDNAs of selected transcription factors are carried without the stop codon and are introduced via a gateway system in the vector pGAD424 (Clontech match

markers) (Mitsuda et al. 2010). The particular transcription factors are fused to the activation domain of the yeast transcription activatorGAL4. A pMB1 Ori and ampicillin resistance (*bla*) are included in *E. coli* for propagation. The *LEU2* gene is used for selection in yeast. Minimal ADH1 promoter provides a low expression of the fusion protein and an SV40 T-antigen nuclear localization sequence is used to target the protein in yeast.

pBT10-GUS (Sprenger‐Haussels and Weisshaar 2000)

This vector was used to produce promoter-GUS constructs. It contains the *uidA* reporter gene of *β-glucuronidase* (GUS) and the *bla* gene of β-lactamase for ampicillin resistance. Replication of Col E1-jump from *E. coli* provides a high copy number.

pGJ280 (Kotchoni et al. 2006)

This vector contains a double CaMV35S promoter followed by a tobacco etch virus translational enhancer, the Green Fluorescent Protein (GFP) coding sequence, and the CaMV35S polyadenylation site. A *bla* gene confers the ampicillin resistance for selection. This vector was originally constructed by Dr. G. Jach (Max-Planck-Institute, Cologne, Germany).

pBIN19 (Frisch et al. 1995)

pBIN19 is a binary vector used for the transformation of *Agrobacterium tumefaciens* for produce transgenic plants using the floral dip method (Clough and Bent 1998). The plasmid containing the *nptIII* gene for selection of transformed bacteria, as well as a T-DNA region in the MCS.

pET43.1b (Novagen)

This vector is designed for cloning and high-level expression of peptide sequences fused with the 491 amino acid Nus•Tag (Novagen, Darmstadt,

Germany). It carries upstream His-Tag, S-Tag, optional C-terminal HSV-Tag, thrombin and enterokinase cleavage sites, multiple cloning sites in all reading frames and compatible with Origami/Origami B, rosetta-gami, /rosetta-gami B host strains to promote proper folding of expressed proteins.

2.1.6 Primers

The primer sequences **(Table 2)** were synthesized by Eurofins MWG Operon (Ebersberg, Germany) and dissolved in TE buffer to give a stock solution of 100 µM, the final concentration is 100 µM. All primers were designed using the "Oligo 6" and "ApE" software.

Table 2 List of primers

Name	**5'-3' sequence**	**Restriction site**
Expression analysis of *A. thaliana ALDH* genes		
Ath_Actin 2_fwd	GGAATCCACGAGACAACCTATAAC	
Ath_Actin2_rev	GAAACATTTTCTGTGAACGATTCCT	
ALDH7B4RT-Fwd	GAAGCAATAGCCAAAGACACACGC	
ALDH7B4RT-Rev	GATATCTCGATTATCGTAGGCTCC	
ATH-ALDH5 FWD	GAAGCCATGGAAGCTATGAAGGAGAC	
ATH-ALDH5 REV	GTCTCTGTCTCTCACTTTCCCCCTT	
Ath-ALDH2-sense	ATCGGCGGAAGCGAGTAATTTGGTGTAT	

Ath-ALDH2-anti	TATGGCGGATACCTGACGGCTGAATC	
AraAld_Fwd	CTACTGGATGTGCCTGAAGCATC	
AraAld_Rev	CATGAGTCTTTAGAGAACCCAAAG	
AY093071-RT-fwd	GATCTTGCATGGTGGTTCCCGA	
AY093071-RT-rev	AAGCACAAAGATTTGAACAGACAGC	
AF370333-RT-fwd	TGTTCTTTGTGGAGGAGTTCGTC	
AF370333-RT-rev	GAAGGGTCTCTTGCTTTATTGGT	
RD29B FOR	ACCAGAACTATCTCGTCCCAAA	
RD29B REV	CGGAGAGAGGTAGCTTTGTCAT	
HSP70_FOR	GGTGGTGGTACTTTTGATG	
HSP70_REV	TTGTCTTTCAGAAGATCTAT	
Yeast one-hybrid screening		
pHISi_fwd	CGACGGCCAGTGAATTGTA	
pHISi_rev	GACAGAGCAGAAAGCCCTA	
pLacZi_fwd	GTCTGTGCTCCTTCCTTCG	
pLacZi_rev	GTGTGTGTATTTGTGTTTGC	
GAL4AD_fwd	CTATTCGATGATGAAGATACCCC	
GAL4AD_rev	CGTTTTAAAACCTAAGAGTCAC	
Expression of ATAF1 protein		

ATAF1 Fwd for pet43	ACTGGATCCGATGTCAGAATTATTACAGTT	BamHI
ATAF1 Rev for pet43	TAACTCGAGGTAAGGCTTCTGCATGTA	XhoI
S-tag	GAACGCCAGCACATGGAC	
Oligos for electrophoretic mobility shift assay		
ALDH7B4 promoter oligo_Fwd	AAGCATAGGACACGTGACACATGTGATGTGAGTGAAGCC	
ALDH7B4 promoter oligo_Rev	GGCTTCACTCACATCACATGTGTCACGTGTCCTATGCTT	
Transactivation assays		
ATAF1 Fwd-pGJ280	CCCCCATGGCAGAATTATTACAGTT	NcoI
ATAF1 Rev-pGJ280	TTCCCTTCTAGTAAGGCTTCTGCAT	
p35s-pROK2	CACTGACGTAAGGGATGACGC	
pBIN-HindIII	AGCTATGACCATGATTACGCCAAG	
pBIN-EcoRI	CGATTAAGTTGGGTAACGCCAGG	

2.1.7 Database software and online tools

The nucleic acid sequences of plasmids and genes are derived from the NCBI database (National Centre for Biotechnological Information: www.ncbi.nlm.nih.gov) and TAIR (The *Arabidopsis* Information Resource: www.arabidopsis.org).

For editing and analyzing DNA sequences, ApE-A plasmid Editor and VectorNTI (Invitrogen, Carslbad, CA) were used in this study. Oligo 6 (Molecular Biology Insights, Inc. DBA Oligo, Inc.) was used for design and analysis of primers.

For Blasts of nucleic acid and protein sequences the functions BLASTN, BLASTP and BLASTNP were used by NCBI. Alignments were performed using the ClustalW function in mega VectorNTI 4.0.

Putative *cis*-elements of the promoter were searched using PLACE Web Signal Scan (http://www.dna.affrc.go.jp/PLACE/signalscan.html) and Plant Care ((http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Sigma plot Version 12.3 and SPSS for windows v.11.0.1 were used for managing and analysis of statistics data and research.

ImageJ (National Institutes of Health, U.S.) is a Java-based image processing program and is an open source image processing program for multidimensional image data with a focus on scientific imaging. It was used for analysis of root elongation of *A. thaliana* seedlings.

2.1.8 Machines and devices

Spectrophotometer**:** SmartSpec 3000, Bio-rad, Hercules, Canada. Bio Spec-nano Spectrophotometer for Life Science, Japan.Ultrospec 2000 UV/Visible Spectrophotometer, Germany.

PCR machine: T3-Thermocycler, Biometra, Göttingen, Germany

pH meter**:** SCHOTT GLAS, Mainz, Germany

Electroporator**:** Gene Pulser II, Bio-Rad, Hercules; CA; USA

Imaging system**:** Typhoon 9200, Amersham Biosciences, Piscatway; NJ; USA

Chemiluminescence detector: Intelligent Dark Box II FUJIFILM, FUJIFILM Corporation, Tokyo; JPN

Power supply: Electrophoresis power supply, Gibco BRL, Carlsbad, Canada.

Protein blotting cell: Criterion blotter, Bio-rad, Hercules, Canada.

UV illuminator: Intas UV systems series, CONCEPT Intas Pharmaceutical ltd., Gujarat, India.

SDS-PAGE: Minigel system, Biometra, Göttingen, Germany.

Sonicator: Ultrasonic Processor UP200S, Hielscher- Ultrasound Technology, Teltow; Germany.

Fluorometer: VersaFluorTM Fluorometer, Bio-rad, Germany.

Scanner: Image Scanner III, GE Healthcare; Piscataway; NJ; USA.

2.1.9 Membrane

Protein transfer (Western blot) was performed on nitrocellulose membrane Protran BA-85 0.45 µm (Whatmann, Maidstone, UK).

2.1.10 Antibodies

For the immune-detection of proteins the following antibodies were used:

Primary antibodies: Monospecific antibody against ALDH7B4, ALDH3F1, ALDH3H1, and ALDH3I1 were purified from crude serum (BioGenes GmbH, Berlin; Germany)(Kirch et al. 2001b; Kotchoni et al. 2006).

Secondary antibodies: Monoclonal anti-biotin-peroxidase antibody produced in mouse BN-34 (Sigma- Aldrich; St. Louis; MO, USA). Anti-rabbit IgG from donkey directly coupled to horseradish peroxidase (HRP) (Sigma-Aldrich; St. Louis; MO, USA).

2.2 Methods

2.2.1 Stress treatment

Arabidopsis thaliana wild-type (ecotype *Col-0*), two *ALDH* double knock-out mutant lines (*KO6/62* and *KO6/76*) (Missihoun, PhD dissertation 2010), six *ALDH7B4 promoter*-GUS fusions lines (B8, AB, AD, P1, P2 and P3) (Missihoun et al. 2014) and ATAF1 overexpression (OX1 and OX2)/ knock-out (*ataf1* and *ataf2*) lines (Wu et al. 2009) were used for stress treatments.

2.2.1.1 Heat stress treatment

Heat stress for different time points: Plants at different developmental stages were subjected to 45°C heat stress for 1 h, 3 h, 6 h, 12 h, 24 h and 72 h. Ten-day-old seedlings after germination were directly heated in the incubator and allowed to recovery in the growth chamber. Four-week-old soil-grown plants were kept in the short-day conditions to grow before applying heat stress.

Thermotolerance treatment: Basal thermotolerance (Ba) treatments consisted of heating the plant material to 45°C, while acquired thermotolerance (Ac) tests were done by heating the seedlings or plants initially to 38°C for 90 min, then leaving the plants at room temperature for 120 min, before finally heating to 45°C for 2 h. All heat treatments were performed in the dark. Recovery was in a growth chamber at 22°C for 5 days in the light (Larkindale et al. 2005).

Seed thermotolerance test: A seed thermotolerance test was performed after seeds were sown on MS-agar medium and incubated at 4°C for 3 days in the dark to break the dormancy. Seeds were heat-treated at 45°C for 1 h, 3 h, 6 h and 12 h immediately after removal from the cold and then allowed to grow an additional 2 d, 3 d, 4 d, 7 d and 9 d for germination analysis. Untreated control samples were placed at 22°C in the same way as the experimental samples.

Root length assay: plants were grown for 7 days under short day conditions, then the seedlings were exposed to heat treatment and after that returned for 3 days to the growth chamber and then the root lengths were measured.

2.2.1.2 Dehydration, wounding and salt stress treatments

The performance of soil-grown plants was investigated under stress conditions. Four-week-old plants were subjected to different stresses. Plants were wounded by treating the leaf surfaces with abrasive sandpaper and afterward kept for four hours under short day conditions. Dehydration was imposed by withdrawing watering for 6–7 d to reach a relative water content of 75% in the plants. Salt stress treatment was performed by watering soil-grown plants every two days with water containing 300 mM NaCl for 10-14 days; control plants were watered with water only.

2.2.1.3 Treatments using a combination of stresses

A combination of dehydration and heat stress was performed either by subjecting dehydrated plants (typically 6-7 days) to a heat stress treatment (45°C for 6 h) or by exposing plants first to 45°C for 6 h followed by dehydration. A combination of wounding and heat stress was applied by wounding the leaves first followed by 45°C for 6 h and *vice versa*. A

combination of salt and heat treatment was performed by first watering the plants with 300 mM NaCl for 10-14 days followed by exposing plants for 6 h to 45°C and *vice versa*. All experiments were performed in triplicates and repeated at least three times. All tissues collected were divided and used in parallel for molecular and metabolic analyses.

2.2.2 Extraction of nucleic acids

2.2.2.1 Isolation of total RNAs from *A. thaliana*

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% (v/v) 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.

2.2.2.2 Extraction of plasmid DNA from *E. coli*

An overnight culture (2-5 ml) of a single positive bacterial colony were collected and centrifuged for 30 sec at a maximum speed 13200 rpm. The supernatant was removed, the pellet was resuspended in 100 µl of resuspension buffer GTE and allowed to stand for 5 min at room temperature. 200 microliters of lysis buffer (NaOH/SDS) was added to the resuspended cells and mixed gently by tapping with fingers. The mixture was incubated on ice for 5 min. In this step, the solution became viscous and slightly clear. 150 µl of potassium acetate solution was added to the lysate and mixed gently by inversion then centrifuged at a maximum speed 13,200 rpm for 3 min at room temperature. The supernatant was transferred to a fresh 1.5 ml Eppendorf tube. One volume of phenol/chloroform/isoamyl alcohol (25/24/1) was added and mixed by vortexing for 10 sec. The mixture was centrifuged for 3 min (13000 rpm, RT) and 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% (v/v) ethanol. The mixture was centrifuged for 3 min (13000 rpm, RT) and 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% (v/v) ethanol.

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

NaOH/SDS solution: 0.2 M NaOH; 1% (w/v) SDS. Freshly prepared before use.

Potassium acetate solution: 29.5 ml glacial acetic acid; add KOH pellets to pH 4.8; bring to 100 ml with H_2O. Store at room temperature (do not autoclave).

2.2.2.3 Extraction of plasmid DNA from yeast cells

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 (14000 rpm, RT). The top aqueous phase was transferred to a fresh tube and mixed with 2.5 volume of 100% (v/v) ethanol and 1/10 volume of 3 M sodium acetate (pH 5.2). Plasmid DNA was precipitated by centrifuging for 10 min (14000 rpm, RT) and washed with 70% (v/v) ethanol. The plasmid DNA was then resuspended in 20 µl TE buffer and 2 µl was used to transform *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.

2.2.2.4 Genomic DNA extraction from *A. thaliana*

Two to three leaves of *A. thaliana* (50-100 mg) were frozen in liquid nitrogen. Plant material was ground to a fine powder in 2 ml Eppendorf tubes using 3 big (2x5mm) and 2 small (2x3mm) metal beads. The vortex was pre-cooled to support grinding and 2.0 ml Eppendorf tubes were used. Freshly prepared mixture of 375 µl 2x lysis buffer and 375 µl 2M urea solution were added to the plant material and vortexed for a few seconds to homogenize it with plant material. Then 37.5 µl phenol was added and vortexed briefly. The mixture was transferred into new 2.0 ml centrifuge tubes and kept on ice till other samples were undergone the same step. Then 750 µl of phenol: chloroform: isoamyl alcohol (25: 24: 1) mixture was added and centrifuged for 10 minutes at 13000 rpm at room temperature (room temperature). Supernatants were transferred into new tubes and 0.7 volume (500 µl) of isopropanol was mixed by gently inverting the tubes. Centrifugation was done for 30 minutes at 4°C

and 13000 rpm. The supernatant was carefully discarded and pellets were washed with 1 ml 70% (v/v) ethanol (2-3 min centrifugation). The ethanol was carefully discarded and pellets were air-dried for a few minutes. The pellets were dissolved in 300 µl Tris + RNase and kept at 37°C for 1-2 hours. At this step, the pellet was stored at 4°C overnight or addition of equal volume 300µl phenol: chloroform: isoamyl alcohol (25: 24: 1) followed by mixing gently and one more centrifugation for 5 minutes at 13000 rpm were done to eliminate the RNA. The supernatant was transferred to a new 1.5 ml centrifuge tube and the DNA was precipitated (at least 1 hour at -70°C) with 0.1 volume (30 µl) 3 M sodium acetate pH 5.2 and 2.5 volume (750 µl) absolute ethanol. Final centrifugation was performed at 4°C for 20 to 30 minutes with 13000 rpm. Pellets obtained were washed with 70% (v/v) ethanol, dried and finally dissolved in 40 µl Tris and RNase buffer.

2x Lysis Buffer: 0.6 M NaCl, 0.1 M Tris pH 8.0, 40 mM EDTA. 4% (w/v) sarcosyl, 1% (w/v) SDS.

Tris + RNase (1 µl/ml of Tris): RNase Stock 100 mg/ml dilution (in 10 mM Tris pH 8.0) 1:1000.

2.2.2.5 Extraction of DNA fragments 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.

2.2.2.6 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 decrease. 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.

Agarose gel electrophoresis was used as the standard method for testing the quality and quantity of DNA and RNA. DNA and RNA samples were separated on horizontal electrophoresis with a concentration of agarose ranging from 1% to 2% (w/v) of agarose. The gels were prepared in 1x TAE buffer containing 1:1000 (v/v) ethidium bromide (10 mg/ml). Ethidium bromide intercalates with double-stranded DNA and can be visualized by excitation through UV-light (245nm).

2.2.3 Cloning of DNA fragments

2.2.3.1 Polymerase chain reaction (PCR)

The polymerase chain reaction is a standard method in molecular biology used to amplify DNA fragments. The PCR reaction mixture in a total volume of 20 µl was prepared as below:

H_2O (sterile double distilled)	15.6 µl
10x PCR-buffer with $MgCl_2$	2.0 µl
10 mM dNTPs	0.4 µl
Forward-primer (10 pmol/µl)	0.4 µl
Reverse-primer (10 pmol/ µl)	0.4 µl
Taq-polymerase (5 U/µl)	0.2 µl
Template	1.0 µl

Reactions were homogenized and the PCR was performed in a TRIO-thermo block (Biometra, Göttingen, Germany). The optimal number of PCR cycles and the annealing temperature was determined empirically for each PCR. A standard PCR program was as followed:

Initial denaturing	94°C	5 min
Denaturing	94°C	30 sec (30-35 times)
Annealing	Ta	30 sec(30-35 times)
Elongation	72°C	40 sec (30-35 times)
Final extension	72°C	5 min
Storage	4°C	

The annealing temperature (Ta) was set 5°C below the melting temperature of the used primers (Tm). For primers with different Tm values, the lower value is considered for the calculation of the Ta.

2.2.3.2 Restriction endonuclease digestions

DNA digestion was carried out by restriction endonuclease according to the following criteria: 1 U of restriction enzymes was used per 1 µg of DNA with the reaction buffer (10x) 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 10x 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 labeled “A”. 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 30 µl was left in the tube “A”. All tubes were kept on the 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” **(Fig. 10)**. Mix 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), all the samples were loaded on the gel immediately after incubation.

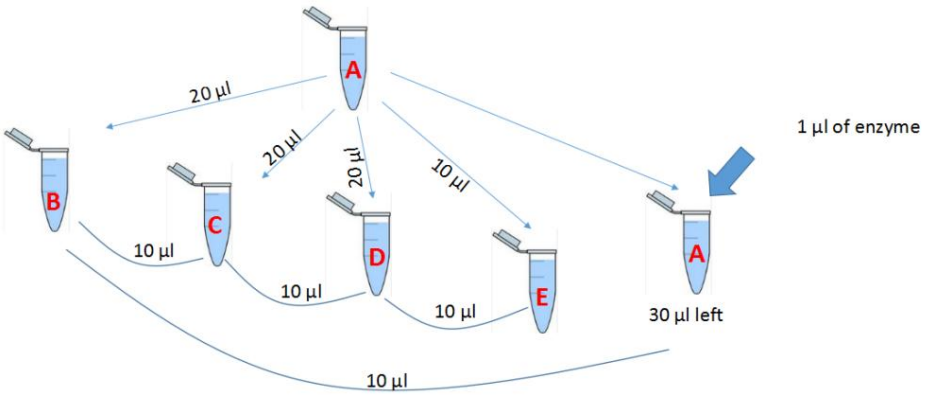


Fig. 10 Scheme of partial digestion with restriction endonuclease

2.2.3.3 DNA dephosphorylation

The linearized plasmid vector was dephosphorylated by antarctic phosphatase (AnP) (New England Biolabs, Beverly, MA; USA) to prevent DNA recircularization during the ligation step. Phosphatase is an enzyme that removes the phosphates from both ends of the linear DNA to prevent self-ligation of the plasmid DNA. The dephosphorylation reaction was comprised of 1 µl of 10x antarctic phosphatase buffer, 1 µl of antarctic phosphatase (5 unit) and an adequate amount of plasmid. The mixture was filled up to 10 µl with sterile water. The reaction was incubated at 37°C for 15 min for 5' extensions and 60 min for 3' extensions. The restriction enzyme was inactivated by heating at 70°C for 5 min.

2.2.3.4 Ligation

The final step in the construction of recombinant plasmids is the insertion of double-stranded DNA into a compatibly digested vector. This reaction is catalyzed by T4 DNA ligase (Roche, Mannheim; Germany). This enzyme enhances the formation of covalent phosphodiester linkages, which permanently join the nucleotides together. The ligation reaction was performed in a 20 µl final volume comprising 2 µl 10x ligase buffer, 1 µl plasmid DNA vector, 1 µl T4 DNA ligase and Y µl of insert DNA. The volume was filled up to 20 µl with sterile water and incubated at 16°C for 20 h. The amount of plasmid DNA in the reaction should be the third of the insert DNA to ensure an efficient ligation. The ligation product was used for bacterial transformation.

2.2.4 Transformation

2.2.4.1 Preparation of chemically competent *E. coli*

A single colony from freshly streaked LB Agar plates with *E. coli* strain *DH10B* was cultured in 5 ml LB for overnight (37°C and 200 rpm). 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 (4000 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.

2.2.4.2 Transformation of chemically competent *E. coli*

1-2 µl plasmid DNA (5 -50 ng/ µl) or ligated plasmid DNA construct was added to one aliquot of calcium-competent cells (100 µl) and carefully mixed. The mixture was incubated on ice for half an hour and then heat shock was given in a water bath at 42°C for 45 sec and cooled again on ice for 2 min. LB medium (800 µl) was added to the transformed cells and further incubated (37°C, 250 rpm) for 1 h for recovery. Before plating, 1:10 and 1:100 dilutions of the transformed cells were made with LB medium. Aliquots (100-200 µl) of the

diluted cells were spread on selective LB agar plates and incubated at 37°C overnight.

2.2.4.3 Preparation of electrocompetent *A. tumefaciens*

A single colony of Agrobacterium (GV3101 or LBA4404) was picked from a freshly streaked plate and pre-cultured in 3 ml YEB+Rif medium and incubated in a shaker (225 to250 rpm) for 14-16 hours at 28°C. The Agrobacterium culture from this pre-culture was poured in fresh 50 ml YEB+Rif medium and incubate similarly for about 8 hours till theOD_{600}=0.5. The bacterial cells were incubated for 30 min on ice. The agrobacterium cells were then passed through a series of centrifugations each time 5000 rpm, 10 min, 4°C and then again resuspended in the subsequent solutions:

25 ml 1 mM Hepes, pH 7.5; 12.5 ml 1 mM Hepes, pH 7.5; 10 ml 10% (v/v) Glycerol, 1 mM Hepes, pH 7.5; 5 ml 10% (v/v) Glycerol, 1 mM Hepes, pH 7.5; 2 ml 10% (v/v) Glycerol. The pellet was finally dissolved in 1 ml 10% (v/v) glycerol. 40 µl aliquots were made and immediately frozen with the help of liquid nitrogen and then stored at -80°C.

2.2.4.4 Transformation of *A. tumefaciens* via electroporation

Competent *A. tumefaciens* cells were thawed on ice. One microliter plasmid DNA (approximately 10-50 ng/µl) was added to the electrocompetent 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 (GenePulser II, Bio-Rad). Cells were immediately diluted in 1 ml YEB-medium and incubated for 2-3 h at 28°C under agitation (250 rpm).

100-200 µl aliquots of the cell culture were spread on selective media and incubated at 28°C for 2-3 days.

2.2.4.5 Transformation of yeast (Gietz and Schiestl 2007)

High-efficiency yeast transformation used the LiAc/SS carrier DNA/PEG method. 1. Inoculate 2-5 ml of liquid YPAD or 10 ml SC and incubate with shaking overnight at 30°C. 2. Count o/n culture and inoculate 50 ml of warm YPAD to a cell density of 5 x 106/ml culture. 3. Incubate the culture at 30°C on a shaker at 200 rpm until it's equivalent to 2 x 107 cells/ml. This will take 3 to 5 hours. This culture will give sufficient cells for 10 transformations. 4. Harvest the culture in a sterile 50 ml centrifuge tube at 3000 x g (5000 rpm) for 5 min. 5. Pour off the medium, resuspend the cells in 25 ml sterile water and centrifuged again. 6. Pour off the water, resuspend the cells in 1.0 ml 100 mM LiAc and transfer the suspension to a 1.5 ml tube. 7. Pellet the cells at top speed for 15 sec and remove the LiAc with a micropipette. 8. Resuspend the cells in a final volume of 500 ml (2 x 109 cells/ml).9. Boil a 1.0 ml sample of SS-DNA for 5 min. and quickly chill in ice water.10. Vortex the cell suspension and pipette 50 µl samples into labeled tubes. Pellet the cells and remove the LiAc with a micropipette. 11. The basic "transformation mix" consists of:

240 µl	50% (w/v) PEG
36 µl	1.0 M LiAc
50 µl	SS-DNA (2.0 mg/ml)
X µl	Plasmid DNA (0.1 - 10 µg)
34-X µl	Sterile ddH_2O

360 µl	Total

12. Vortex each tube vigorously until the cell pellet has been completely mixed. Usually takes about 1 min. 13. Incubate at 30°C for 30 min. 14. Heat shock in a water bath at 42°C for 30 min.15. Centrifuge at 6-8000 rpm for 15 sec and remove the transformation mix with a micropipette. 16. Pipette 1.0 ml of sterile water into each tube and resuspended the pellet by pipetting it up and down gently. 17. Plate from 2 to 200 µl of the transformation mix onto SD-minus plates. If plating less than 200 µl deliver into a pool of not more than a final volume of 200 µl of sterile water on the plate.18. Incubate the SD minus plates for 2 - 4 days to recover transformants.

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

Ten to twelve-day-old *A. thaliana* seedlings were transiently transformed by the FAST (Fast Agrobacterium-mediated Seedling Transformation) technique based on the co-cultivation of Agrobacterium cells (GV3101::pMP90) harboring the transgene in a binary vector with the seedlings in a medium containing the surfactant Silwet® L-77. One day before co-cultivation, a single colony of *A. tumefaciens* was inoculated into 2 ml of YEB medium with appropriate antibiotics (50 µg/ml kanamycin and 50 µg/ml Rifampicin) and cultured at 28°C for 18-24 h. On the day of co-cultivation, the saturated Agrobacterium culture was diluted to OD_{600} = 0.3 in 10 ml of fresh YEB medium without antibiotics. The cells were further grown at 28°C under vigorous agitation until the OD_{600} reading reaches 1.5-2.0. After centrifugation at 6000 g for 6 min, the cell pellet was resuspended in 10 ml of washing solution (10 mM $MgCl_2$). The cell suspension was again pelleted by centrifugation at 6000 g for 5 min and resuspended in 1 ml washing solution as above. About 50 seedlings were carefully transferred from plates into a clean

100 x 20 mm Petri dish filled with 20 ml of co-cultivation medium (1/4 MS, 0.005% [v/v] Silwet® L-77). Agrobacterium cell suspension was added to the co-cultivation medium to a final density of OD_{600} = 0.5 and mixed well by gentle shaking. The Petri dish was wrapped with aluminum foil and incubated in the plant growth chamber for 36-40 h. Plates were kept without aluminum foil. After the co-cultivation period, the medium was replaced with the surface sterilization solution (0.05% [w/v] sodium hypochlorite) and incubated for 10 min, washed three times with H_2O to remove epiphytic bacteria. Seedlings were finally incubated in 0.5 X MS, 500 µg/ml carbenicillin to inactivate remaining *Agrobacterium* cells prior to applying the stressors.

2.2.5 Screening

2.2.5.1 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.

2.2.5.2 Screening for transformed yeast clones

To screen for positive yeast baits, colonies that were present on selective plates (SD-His-Ura for Yeast one-hybrid assay) were picked to perform yeast colony PCR using specific primers to amplify DNA inserts. For a yeast one-hybrid 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.

2.2.6 Reverse transcriptase (RT)-PCR analysis

2-4 µg of total RNAs were treated with 10 U RNase-free DNase I (Roche; Mannheim, Germany) in a 10 µl reaction containing 1x DNase I buffer (20 mM Tris/HCl pH 8.4; 50 mM KCl and 2 mM $MgCl_2$) at 37°C for 5 min. Then, 1 µl of 25 mM EDTA was added and the reaction was heated at 65°C for 15 min to deactivate the DNase I. First-strand cDNA synthesis was performed using the Totalscript-OLS® Kit (OLS, Hamburg, Germany) or the RevertAid™ H Minus First Strand cDNA Synthesis Kit, (Fermentas, Burlington, CDA) using the protocol provided with the kit.

2.2.7 Extraction of proteins

2.2.7.1 Protein extraction from plant tissues (Laemmli 1970)

Crude proteins were extracted from 50-100 mg plant tissues ground under liquid nitrogen and with metal beads by vigorous vortexing. The plant material was then homogenized with 150-200 µl Laemmli buffer. The plant extract was transferred into a fresh tube to recuperate the metal beads. The extract was heated at 95°C for 5 min, cooled down on the ice and centrifuged at room temperature at 14000 rpm for 5 min. The supernatant containing crude total proteins was collected in a fresh tube and stored at -20°C. Samples were heated up at 95°C for 2 min before loading on the gel.

Laemmli buffer (1X): 62.5 mM Tris-HCl pH 6.8; 10% (v/v) glycerol; 2% (w/v) SDS; 0.1% (w/v) bromophenol blue and 0.7 M β-ME. Add freshly DTT at 0.1 M final concentration to the needed volume of the buffer just before use.

2.2.7.2 Quantification of proteins

Protein concentrations were determined using a Bio-Rad protein assay Kit (Bradford 1976). Sample aliquots (5-10 µl) were mixed with 200 µl Bio-Rad protein assay kit and brought to 1000 µl with sterile H_2O. When the Laemmli buffer was used to extract proteins from the plant tissues, 5 µl of the protein sample was first diluted in 100 µl of 100 mM potassium phosphate buffer, pH 6.8. The mixture was incubated at room temperature for 10 minutes to precipitate the SDS salt. The suspension was then centrifuged at high speed and room temperature for 5 min. The supernatant (about 100 µl) was carefully transferred to a fresh tube and mixed with 700 µl sterile distilled H_2O and 200 µl Bradford reagent (Bio-Rad). The suspensions were incubated at room temperature for 2-5 min followed by an OD measurement at 595 nm. The amount of protein was estimated from a standard curve established from defined concentrations of bovine serum albumin (BSA).

2.2.7.3 Extraction and analysis of recombinant ATAF1 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 (14000 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% (v/v) Triton X-100. The suspensions were sonicated on ice (4X20 sec) for complete lysis and centrifuged for 10 min (12,000 g, 4°C). Supernatant (soluble proteins) were diluted with one volume 2X sample buffer while the pellets were suspended in one volume 1X 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_2HPO_4; 0.24 g/l KH_2PO_4.

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

2.2.7.4 Extraction and purification of the recombinant ATAF1 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 x 20 sec). The cell suspension was centrifuged for 30 min (12000 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 ddH_2O, charged with 5-bed volumes 50 mM $NiSO_4$, 3-bed volumes of ddH_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 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-ME (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 pH to 8.0 with NaOH.

2.2.7.5 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-PAGE is a technique used for separation of negatively charged proteins based on their ability to move under an electrical current. SDS is added as a detergent to remove secondary and tertiary protein structures. It maintains the proteins as polypeptide chains. The SDS coats the proteins proportionally to their molecular weight. The gel consists of 4% (w/v) acrylamide stacking gel and 12% (w/v) acrylamide separating gel as described in the table below **(Table 3)**. Samples were mixed with Laemmli buffer and heated at 95°C for 5 min to denature the proteins before loading on the gel. Electrophoresis was performed using 1x SDS-running buffer. The intensity of the current is 10 milliamperes for the stacking gel and 20 milliamperes for the separating gel.

1X SDS-protein running buffer: 25 mM Tris; 192 mM glycine; 0.1% (w/v) SDS. No need to adjust pH levels.

Table 3 Composition of SDS-PAGE

Stock solution	4% Stacking gel	12% Separating gel
1 M Tris-HCl pH 6.8	0.38 ml	--
1.5 M Tris-HCl pH 8.8	--	1.56 ml
10% (w/v) SDS	30 µl	60 µl
10% (w/v) APS	30 µl	60 µl
30% (v/v) Acrylamide	0.5 ml	2.4 ml
H_2O	2.16 ml	1.92 ml
TEMED	3 µl	2.4 µl

2.2.7.6 Coomassie blue staining

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.

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.

2.2.7.7 Ponceau red staining

Ponceau red staining was performed to check the transfer of protein from the gel to the membrane. The membrane was immersed, protein side up, in about 100 ml of the staining solution (0.2% [w/v] Ponceau S in 3% [w/v] TCA) with gentle shaken for 5-10 min. The staining solution was removed and the membrane destained with H_20. The membrane was scanned and the positions of the standard proteins were marked with a pencil.

2.2.7.8 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 1979). After staining, 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 1:1000 to 1:5000 (v/v). The membrane was incubated at room temperature for 1 h and was washed with TBST as follows: 1X rinse, 1X 15 min, and 3X 5 min. The membrane was then incubated for another 45 min at room temperature with a 5000-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).

<u>10X TBS, pH 7.5:</u> 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 37% HCl.

<u>TBST solution:</u> 1X TBS + 0.1% (v/v) Tween-20

Protein-blot transfer buffer (PBTB): 25 mM Tris, 192 mM glycerol, 20% (v/v) methanol. Do not adjust the pH levels.

Blocking solution: 4% (w/v) non-fat dry-milk powder dissolved in TBST solution.

2.2.8 DIG (digoxigenin) labeled electrophoresis gel mobility shift assay (EMSA)

DIG-labeled EMSA was conducted using the DIG Gel Shift Kit (2nd Generation, Roche). The DNA labeling reaction was conducted following the manufacturer's instruction: 39 bp DNA fragments of the *ALDH7B4* promoter were annealed and diluted to 10 ng/µl by ddH_2O, incubated with labeling buffer, $CoCl_2$-solution, DIG-dd UTP solution and terminal transferase at 37°C for 20 min. 0.2 mM EDTA was added to stop the labeling reaction.

Various amounts of purified ATAF1 and DNA fragments were mixed and incubated at 37°C for 20 min in EMSA binding buffer (pH 7.2, 20 mM Tris, 50 mM NaCl, 10 mM EDTA, 4 mM DTT, 5% (v/v) glycerol, 0.5 mg/ml BSA). Following the binding reaction, samples were electrophoresed on 6% non-denaturing polyacrylamide gel in 0.5xTBE buffer. After electrophoresis, free DNA probes or DNA-protein complexes were transferred to positively charged nylon membrane by capillary transfer in 10xSSC buffer. The membrane was incubated with blocking buffer followed by the alkaline phosphatase-conjugated antibody (anti-digoxigenin-AP). The DIG-labeled 39 bp DNA fragments are visualized by an enzyme immunoassay using anti-DIG-AP, Fab-fragments, and the chemiluminescent substrate CSPD. The generated chemiluminescent signals are recorded on the imaging device (Intelligent Dark Box II FUJIFILM, FUJIFILM Corporation, Tokyo; JPN).

2.2.9 Physiological and biochemical assays

2.2.9.1 Lipid peroxidation assay (MDA analysis)

The level of lipid peroxidation products was measured in the plant tissues by using the thiobarbituric acid (TBA) test, which determines the amount of malondialdehyde (MDA) as the end product of the lipid peroxidation process (Hodges et al. 1999). The plant tissues (20-60 mg) were ground in Eppendorf tubes as described above and homogenized in 1 ml pre-chilled 0.1% (w/v) trichloroacetic acid (TCA) solution. The homogenates were centrifuged at 13000 rpm for 5 min at 4°C. When using more starting plant material the pellet was once again re-extracted with 1 ml of the same solvent and the supernatants were collected in a fresh tube and thoroughly mixed. 500-600 µl of the supernatant were added to one volume of the Reagent Solution II (RSII; RSI + 0.65% [w/v] TBA) in a 15 ml-Falcon tube. The samples were vigorously mixed and boiled at 95°C in a water bath for 25 min. The reaction was stopped by placing the tube on ice and the samples were centrifuged at 5000 rpm for 5 min at 4°C. Absorbances were read with a spectrophotometer at 440 nm (sugar absorbance), 532 nm (maximum absorbance of pinkish-red chromagen, a product of the reaction of MDA with TBA) and 600 nm (turbidity). 0.1% (w/v) TCA was used as the reference solution.

The amount of MDA was calculated with the following formula (where FW= fresh weight):

MDA equivalents (nmol/ml) = [(A-B)/157000] x 106

$A = (Abs\ 532_{RSII} - Abs\ 600_{RSII})$

$B = (Abs\ 440_{RSII} - Abs\ 600_{RSII})$ x0.0571

MDA equivalents (nmol/g FW) = MDA equivalents (nmol/ml) x total volume of the extracts (ml) / g FW or number of seedlings.

Reagent Solution I (RSI): 20% (w/v) TCA and 0.01% (w/v) Butylated hydroxytoluene (BHT)

2.2.9.2 Determination of chlorophyll content

Total chlorophyll was extracted in 80% (v/v) aqueous acetone based on the work of MacKinney (1941) and spectrophotometrically quantified according to Arnon (Arnon 1949). For the extraction, 0.2 g plant material was used and suspended in 2 ml extraction buffer and incubated in the dark under shaking at room temperature for 30 min. The suspension was centrifuged (5min, 10000 rpm, RT) and the OD of the supernatants was measured at 663 nm and 645 nm in plastic cuvettes. The chlorophyll content was estimated by the following formula as described:

$C\ (mg\ FW^{-1}) = 20.2 \times OD_{645} + 8.02 \times OD_{663}$

Where C expresses the total chlorophyll content (chlorophyll A + chlorophyll B) in mg/l of extraction solution.

2.2.9.3 Gas exchange and chlorophyll fluorescence

Mature leaves of four-week-old plants were used for measuring photosynthesis with the GFS-3000 gas exchange system (Walz, Effeltrich, Germany). Measurements were performed at 22°C and a relative humidity of 60%.The light-saturated photosynthetic rate was determined at 0-1800 µmol $m^{-2}s^{-1}$with $[CO_2]$ = 350 ppm. To generate a light response curve, photosynthetic measurements were conducted at PPFD intensities of 1800, 1500, 1000, 500, 400, 300, 200,100 and 0. The following parameters were assessed:

(1) Assimilation of yield of CO_2

(2) Effective quantum yield of PSII [ΔF/Fm'= (Fm'- Fs)/Fm']

(3) Non-photochemical quenching coefficient [NPQ = (Fm-Fm')/Fm']

(4) Maximum quantum yield of photosystem II (PSII) [Fv/FM= (Fm-Fo)/Fm]

2.2.9.4 GUS-assay with X-Gluc as substrate (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-b-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°C overnight or for a shorter time period. The tissues were destained by removing chlorophyll in 80% (v/v) ethanol solution at 80°C and then kept at 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 ; 0.1% (v/v) Triton X-100; 8 mM β-mercaptoethanol freshly added.

2.2.9.5 Quantification of GUS activity

The fluorometric assay of the GUS activity was carried out from ground plant tissues. In this assay, the fluorogenic substrate 4-methylumbelliferyl-glucuronide (4-MUG; Sigma, Steinheim, Germany) is cleaved by the enzyme to the fluorescent product 4-methylumbelliferone (4-MU). About 50-100 mg of plant material was ground as described above with metal beads, homogenized in 100-150 µl extraction buffer (50 mM sodium phosphate, pH 7, 10 mM EDTA, 0.1% (v/v) TritonX-100, 0.1% (w/v) Na-lauryl sarcosine) and centrifuged (14000 rpm, 4°C) for 10 min. The protein concentration of the crude extract was determined from 5 µl of each sample by the Bradford assay (Bradford 1976) using a kit (Bio-rad). Then, a 10-15 µl (sample volume) plant extract was mixed with one volume 1 mM 4-MUG and the reaction was incubated at 37°C. A control reaction was made with one volume 4-MUG and one volume extraction buffer without plant extract. Five microlitres (volume per test) were removed periodically from each reaction and diluted in 2 ml (reaction volume) stop buffer (0.2 M sodium carbonate: Na_2CO_3, pH 9.5). 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. Standard solutions of Na_2CO_3, pH 9.5, containing 5, 10, 25, 50 and 100 nM 4-MU were used to generate a standard curve (FI versus pmol 4-MU) and to calculate the slope x (FI/pmol 4-MU). Samples ’FI values was also plotted versus time (min). The slope y (FI/min) was calculated for each sample and for the control reaction. Each y-value was corrected by subtracting the y-value of the control reaction. The specific GUS activity for each sample was expressed as 4-MU pmol/min/mg protein by the following formula:

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

2.2.9.6 Colony lift assay for β-galactosidase activity

A Whatman filter WH10311897 that fits the Petri dishes was prepared and placed onto the yeast colonies grew on an 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 3 mm 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; 100 µl 4% (w/v) X-Gal; 11 µl β-mercaptoethanol.

3. RESULTS

3.1 Study of aldehyde dehydrogenase (*ALDH)* genes under heat stress

3.1.1 Expression analysis of different aldehyde dehydrogenase (*ALDH)* genes from *A. thaliana* under heat stress

3.1.1.1 Phenotype of *A. thaliana* wild-type plants subjected to heat stress

To assess the impact of heat stress on phenotypic changes in plants, we first examined the morphological changes resulting from high temperature stress. Ten-day-old seedlings and four-week-old plants of *A. thaliana* wild-type were analyzed under neutral, high temperature and recovery conditions **(Fig. 11)**. *A. thaliana* plants were treated at two growth stages with different heat stress treatments by keeping plants at 45°C for 0 h, 1 h, 3 h, 6 h, 12 h, 24h or for basal (45°C/120 min, 22°C/3 d) and acquired (38°C/90 min, 22°C/120 min, 45°C/120 min, 22°C/3 d) thermotolerance. Basal (Ba) thermotolerance is the inherent ability to survive exposure to temperatures above the optimal growth temperature. Acquired (Ac) thermotolerance is induced by a short acclimation period at moderately high (but survivable) temperatures or by treatment with other non-lethal stresses prior to heat stress (Larkindale et al., 2005).

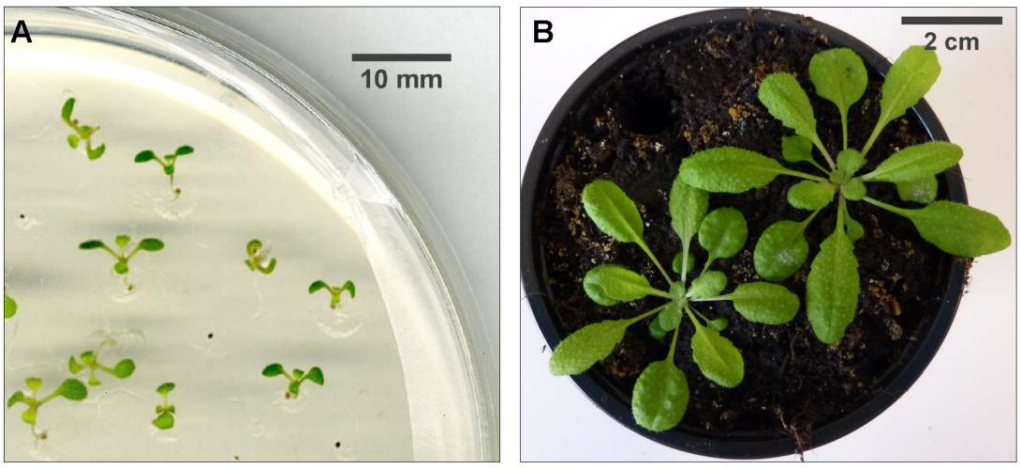


Fig. 11 The phenotype of *A. thaliana* plants.

(A)*A. thaliana* wild-type seedlings grown on MS agar plates in the light for 10 days.

(B)*A. thaliana* wild-type plants grown in soil in 12 cm plastic pots in a growth chamber with short day light conditions for 4-5 weeks.

All seedlings survived 1 h heat treatment. When the treatment was increased to 3 h, the survival rate decreased and during the recovery conditions, most of the seedlings showed morphological changes including brown color and wilting. The margins of the cotyledons showed dark green and yellowish brown color after 6 h at 45°C and after 12 h treatment all seedlings turned yellow, bleached and did not recover **(Fig. 12)**. The seedlings were less affected by the acquired thermotolerance stress programme than by the basal heat stress treatment. Similarly, four-week-old *A. thaliana* wild-type plants were exposed to 45°C from 1 h to 72 h. No visible stress symptoms were observed up to exposure to 12 h heat stress. Exposure for 24 h resulted in upwards curled leaf margins and the entire plants were dried up after recovery **(Fig. 13)**. No viable plants were recovered when they were kept for 36 h or longer at 45°C.

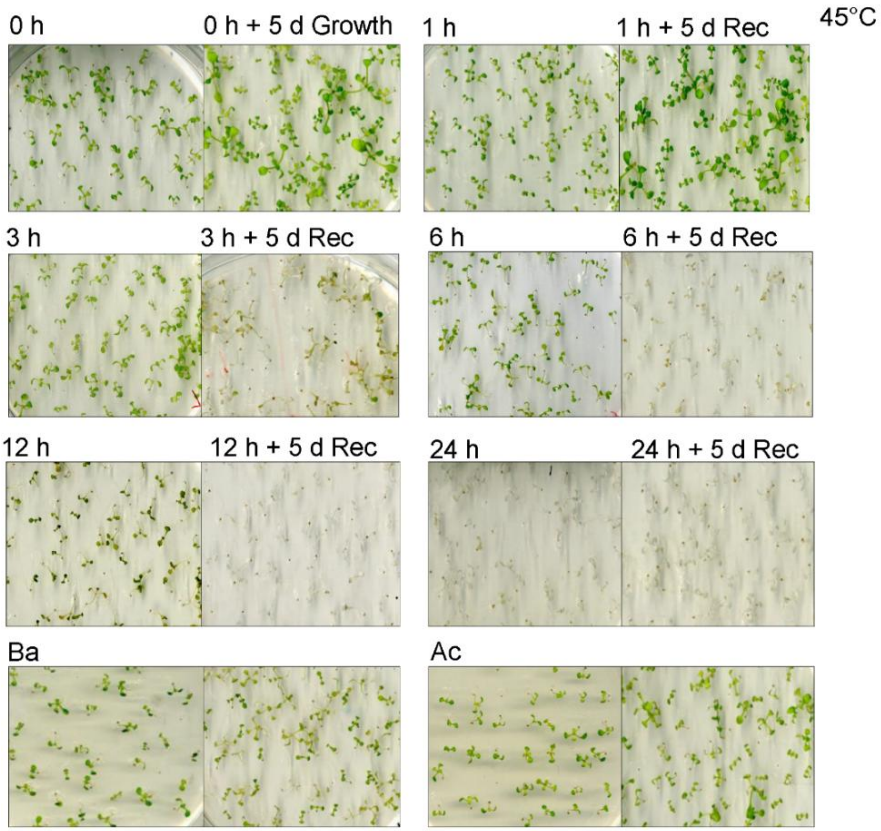


Fig. 12 The phenotype of ten-day-old seedlings subjected to 45°C heat treatment for different time periods. Wild-type seedlings were treated under 45°C at 0 h, 1 h, 3 h, 6 h, 12 h, 24h, basal heat stress and acquired thermotolerance after grown at MS medium for 10 days. Ba: basal heat stress, Ac: acquired thermotolerance, Rec: recovery.

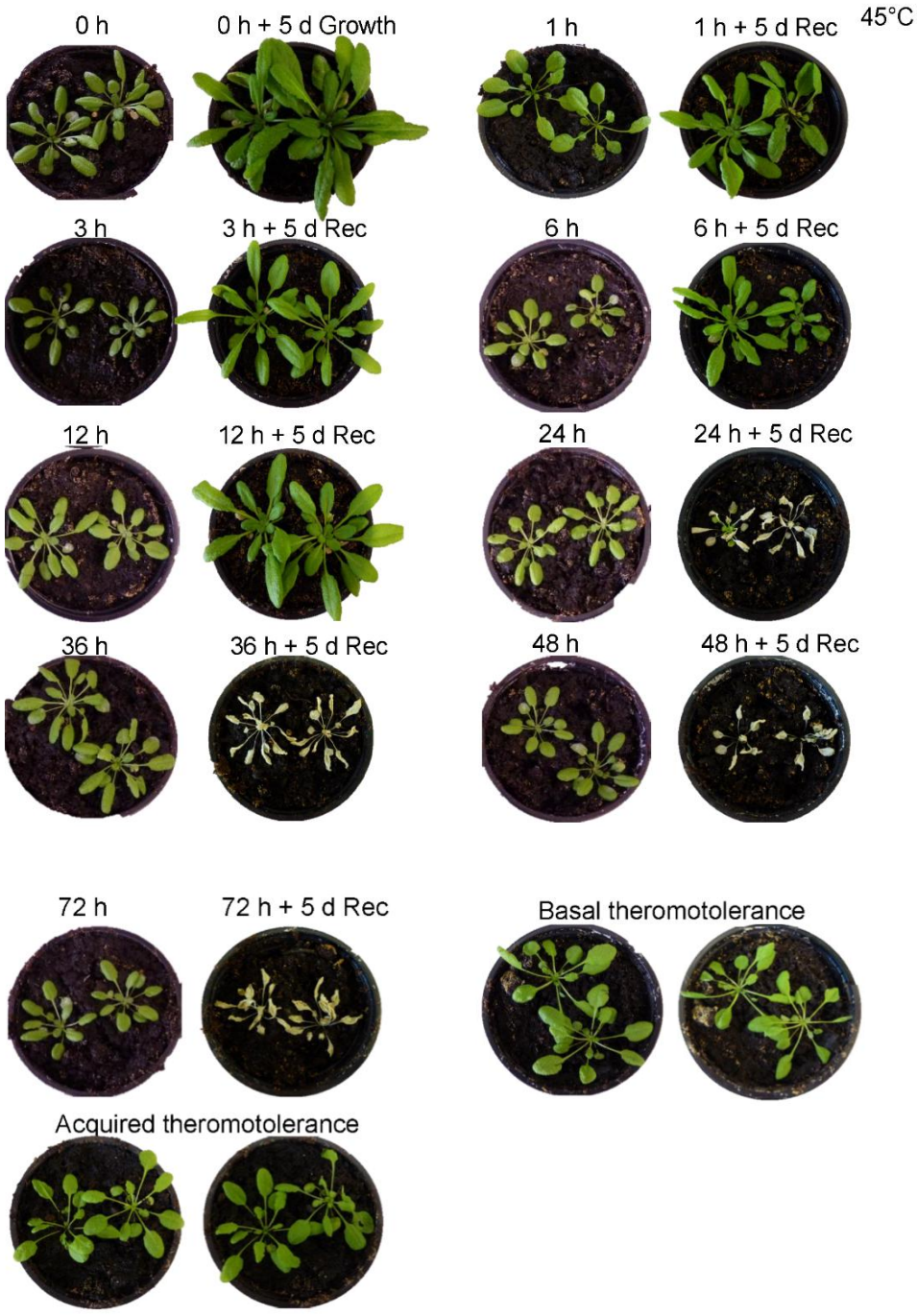


Fig. 13 The phenotype of four-week-old seedlings subjected to 45°C heat treatment for different time periods. Plants were kept at 45°C heat treatment for different time periods (0–24h, basal heat stress or acquired thermotolerance). Ba: basal heat stress, Ac: acquired thermotolerance, Rec: recovery.

3.1.1.2 Expression analyses of selected *ALDH* genes under heat stress treatment

To test whether high temperature influences the expression of *ALDH* genes, six *ALDH* genes (*ALDH7B4, ALDH3I1, ALDH3H1, ALDH3F1, ALDH10A8,* and *ALDH10A9*) were selected. *RD29B* and *HSP70* were used as negative and positive controls respectively. *RD29B* encodes a protein that is induced in response to water deprivation such as desiccation, cold and high-salt but not heat stress(Uno et al. 2000). *HSP70* encodes heat shock proteins and was used as the marker to monitor high temperature stress (Sung et al. 2001). Total RNAs from 6 h and 12 h heat-treated seedlings and 36 h heat-treated four-week-old plants were degraded **(Fig. 14A and 4B)**. For this reason, we selected 0 h, 1 h, 3 h, Ba and Ac stress treatment for ten-day-old seedlings and 0 h, 1 h, 3 h, 6 h, 12 h, 24 h, Ba and Ac (details in material and methods) for four-week-old plants to investigate physiological and biochemical performances.

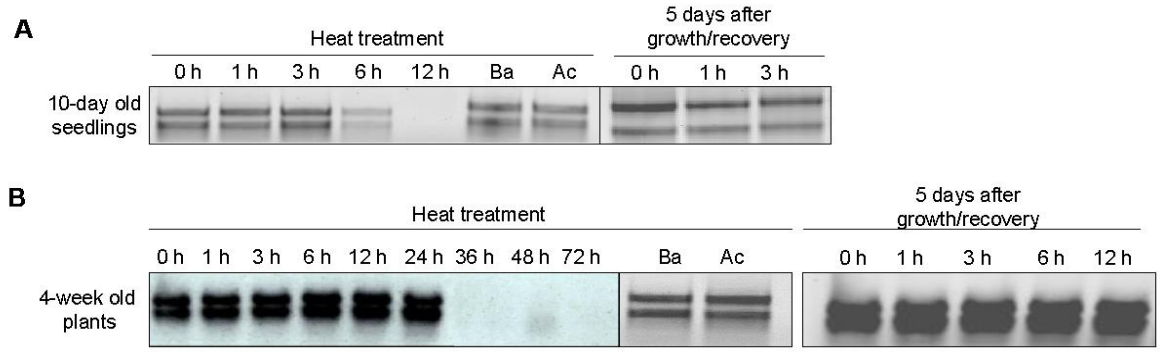


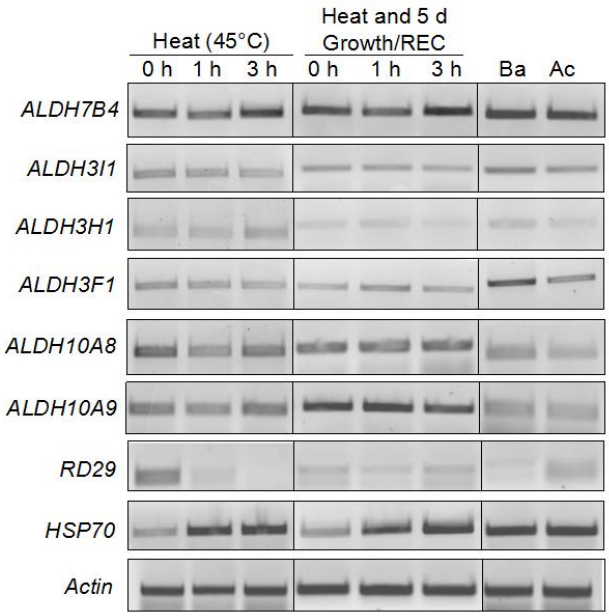
Fig. 14 RNA isolation from ten-day-old seedlings and four-week-old plants exposed to 45°C for the indicated length of time, Ba, Ac and recovery conditions.

(A) RNAs of heat treated Ba, Ac, and recovery in seedlings were isolated. Ba: basal heat stress, Ac: acquired thermotolerance, Rec: recovery.

(B) RNAs of heat treated Ba, Ac, and recovery in leaf tissue of 4-week old plants were isolated.

In 3 h heat-stressed seedlings *ALDH7B4* is slightly increased **(Fig. 15A and B)**. *ALDH3I1*, *ALDH3F1*, *ALDH10A8* and *ALDH10A9* transcripts do not change under heat stress and recovery conditions, while *ALDH3H1* transcript accumulated at a lower level during recovery. The expression of *ALDH7B4* and *ALDH10A9* showed no obvious difference under Ba and Ac thermotolerance. *ALDH3I1*, *ALDH3F1* and *ALDH10A8* transcripts accumulated under Ba stress. HSP70 increased in response to high temperature and *RD29B* was hardly detected in heat stressed samples. Expression analysis of *ALDH7B4* in four-week-old plants showed that the transcripts accumulated in 6 h and 12 h heat-stressed plants and declined thereafter **(Fig. 15 C and D)**. *ALDH3I1* showed up-regulation after 1 h heat stress, while down-regulation after 12 h and 24 h heat stress. Accumulation was slightly increased after 1 h under recovery conditions. *ALDH3H1* and *ALDH3F1* are expressed at low levels in response to high temperature stress whereas higher expression was observed during recovery. Transcripts of *ALDH10A8* and *ALDH10A9* were constitutively expressed at a high level during recovery. *ALDH7B4*, *ALDH10A8,* and *ALDH10A9* transcripts were higher expressed in Ba than Ac.

A



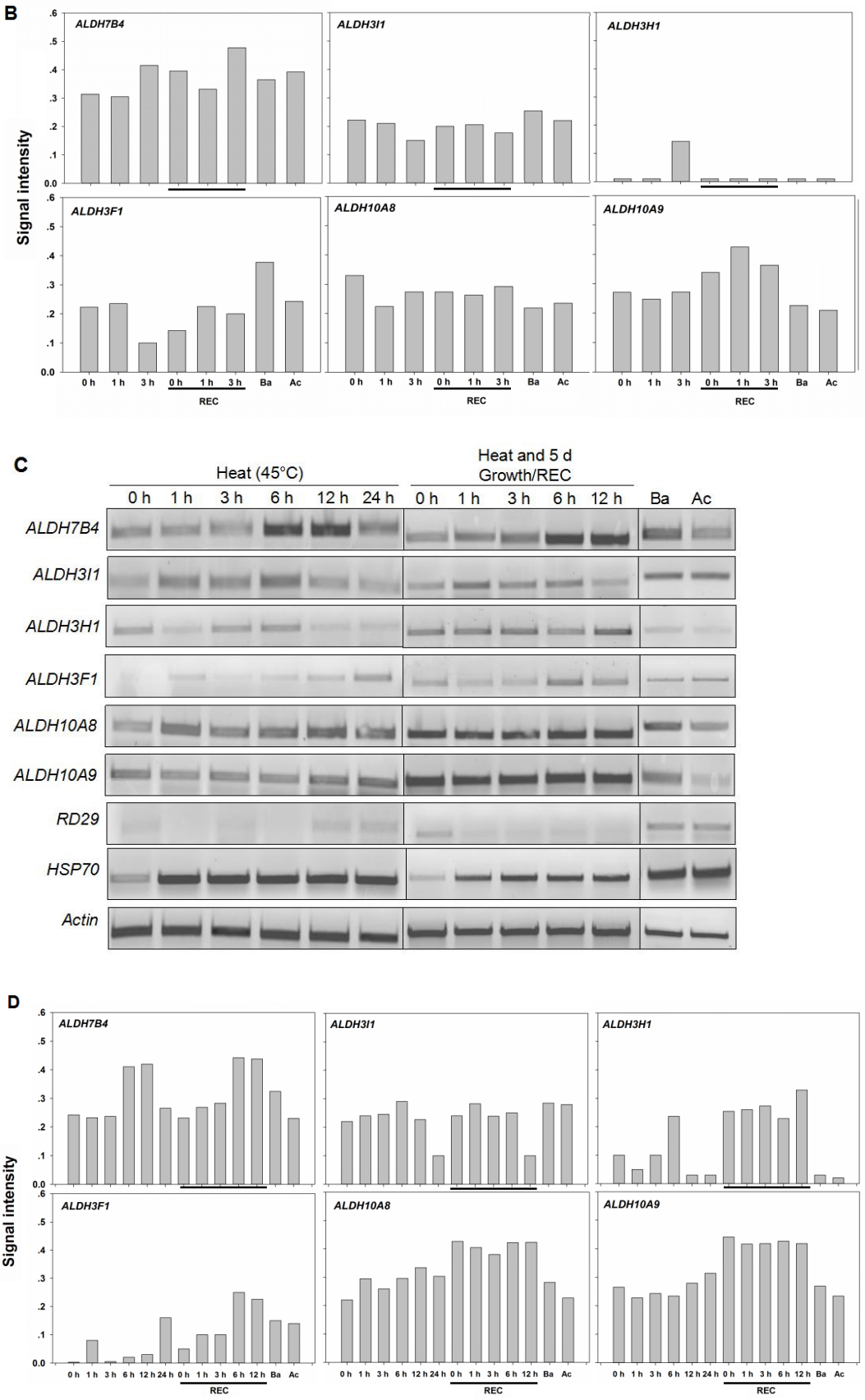

B
ALDH7B4
ALDH3I1
ALDH3H1
ALDH3F1
ALDH10A8
ALDH10A9
Signal intensity
0 h 1 h 3 h 0 h 1 h 3 h Ba Ac
REC
C
Heat (45°C)
Heat and 5 d Growth/REC
0 h 1 h 3 h 6 h 12 h 24 h 0 h 1 h 3 h 6 h 12 h Ba Ac
ALDH7B4
ALDH3I1
ALDH3H1
ALDH3F1
ALDH10A8
ALDH10A9
RD29
HSP70
Actin
D
ALDH7B4
ALDH3I1
ALDH3H1
ALDH3F1
ALDH10A8
ALDH10A9
Signal intensity
0 h 1 h 3 h 6 h 12 h 24 h 0 h 1 h 3 h 6 h 12 h Ba Ac
REC

Fig. 15 Gene expression analysis of selected *ALDH* genes in wild-type plants under heat stress. Expression of *ALDH7B4*, *ALDH3I1*, *ALDH3H1*, *ALDH3F1*, *ALDH10A8* and *ALDH10A9* in *A. thaliana* wild-type were analysis by RT-PCR.

(A) Transcript levels of *ALDH* genes in *A. thaliana* wild-type ten-day-old seedlings under different heat stress treatment, basal heat stress, acquired thermotolerance and recovery conditions, as determined by RT-PCR. RT-PCR specific for *ALDH* genes and control genes mRNA was carried out on total RNA (2 µg) extracted from different time intervals after imposing heat stress. Actin was used as a reference gene to monitor the cDNA quality.

(B) Quantification of *ALDH* genes expression relative to actin transcript levels in ten-day-old seedlings of *A. thaliana* in response to heat stress.

(C) The expression level of *ALDH* genes of wild-type 4-week-old plants under different heat stress times, basal heat stress, acquired thermotolerance and recovery conditions.

(D) Quantification of *ALDH* gene expression relative to actin transcript levels in four-week-old wild-type plants of *A. thaliana* in response to heat stress.

3.1.1.3 Protein blot analysis of different selected ALDHs under heat stress conditions

To determine whether changes in transcript accumulation are reflected at the protein level, ALDH proteins were analyzed using antibodies against ALDH7B4, ALDH3I1, ALDH3H1 and ALDH3F1 proteins (no antibodies are available against ALDH10A8 and ALDH10A9). ALDH7B4 was induced after 3 h and Ba treatment in seedlings **(Fig. 16A)**. Also, ALDH3F1 increased after 3 h of heat stress but not after Ba and Ac treatment. Heat stress caused only a slight increase in the expression level of ALDH3I1 protein after 3 h heat treatment, the same observation was made for ALDH3H1 **(Fig. 16A)**. ALDH protein accumulation was also analyzed in four-week-old plants in response to high temperature. **(Fig. 16B)**. ALDH7B4 and ALDH3I1 were upregulated after exposure to 45°C. A slightly increased expression was observed for ALDH7B4 after 3 h, 6 h and ALDH3I1 after 12 h in recovery conditions. ALDH3H1 and ALDH3F1 showed nearly constant expression in response to heat stress,

whereas a low expression was detected of ALDH3F1 during recovery. The protein blots confirmed the transcript analysis and showed that mainly the *ALDH7B4* gene is expressed in response to high temperature.

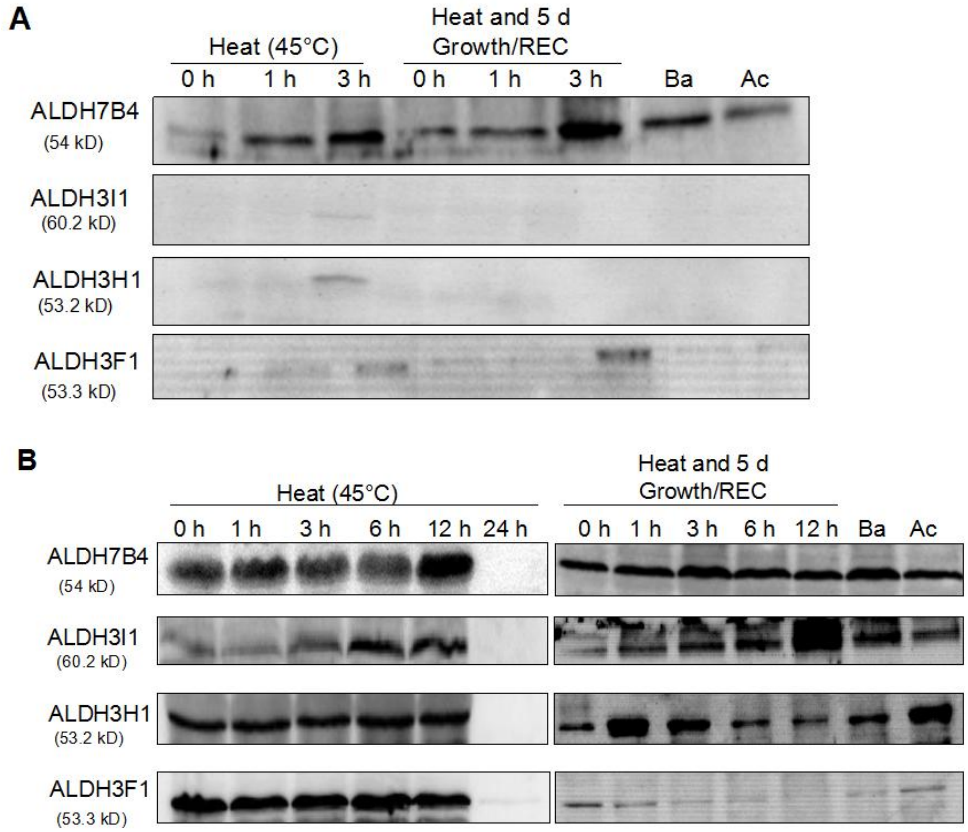


Fig. 16 Protein blot analysis of selected ALDHs under heat stress treatment.

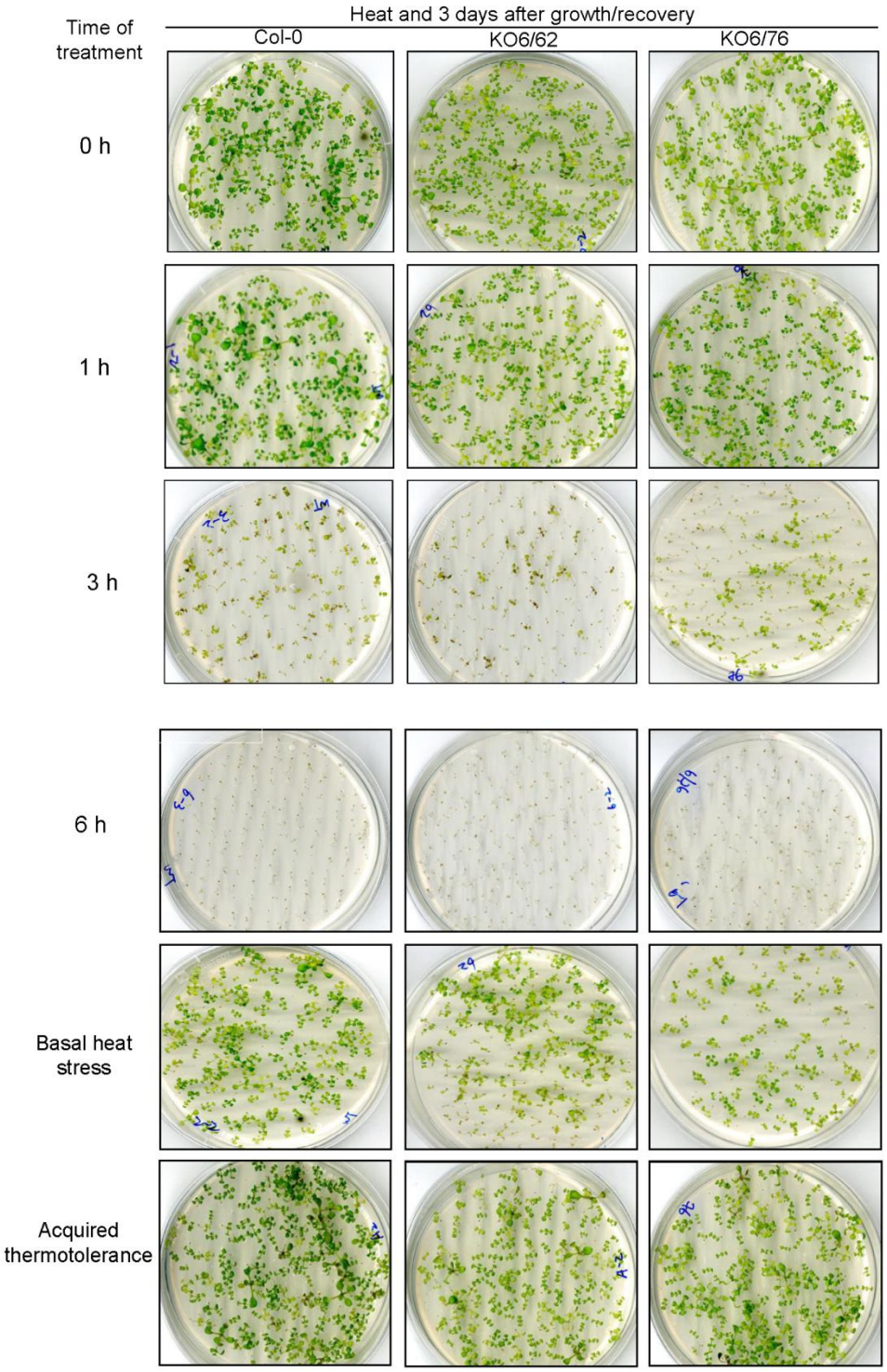
(A) Protein blot analysis of protein extracts from ten-day-old *A. thaliana* wild-type seedlings before heat stress at 0 h, during heat stress (45°C for 1 h, 3 h, Ba, and Ac), and during recovery (5 days after growth/recovery 1 h and 3 h).

(B) Protein blot analysis of protein extracts from four-week-old *A. thaliana* wild-type plants before heat stress as 0 h, during heat stress (45°C for 1 h, 3 h, 6 h, 12 h, 24 h, Ba and Ac), and recovery (5 days after growth/recovery 1 h, 3 h, 6 h and 12 h).

3.1.2 Survival rates of two *ALDH* knock-out lines under heat stress

To investigate whether *ALDH* genes have an effect on heat stress response pathways, two *ALDH* double knock-out mutant lines *KO6/62* (knock-out of

ALDH7B4 and *ALDH3I1*) and *KO6/76* (knock-out of *ALDH7B4* and *ALDH3F1*) were compared with wild-type plants. All of the *ALDH* mutants identified from previous screens were sensitive to abiotic stress than wild-type plants (Kotchoni et al. 2006). Therefore it is hypothesized that double loss-of-function of *ALDH* mutants imposes the more severe effect on plants than single mutants in response to stress. Survival rates were measured after 3 days recovery of ten-day-old seedlings at different times of heat stress. No difference of visual phenotype was found between the mutant lines and wild type under non-stress conditions **(Fig. 17)**. No differences were seen between the mutant and wild-type after 1 h heat stress and Ac treatment. Wild-type plants showed better growth than the mutants after 3 h and Ba, especially, parts of seedlings from *KO6/62* and *KO6/76* were more bleached with chlorosis and stopped growing after 3 h treatment. Wilde type plants have given 50% and 23% higher values of survival rates than mutant lines after 3 h stress. The survival rate from mutant lines was also lower than wild-type in Ba heat-treated (57% and 85%, respectively). These results indicate that loss of *ALDH7B4*, *ALDH3F1*and *ALDH3I1* make the plants more sensitive and less resistant to high temperature stress during early development.


Time of treatment
Heat and 3 days after growth/recovery
Col-0
KO6/62
KO6/76
0 h
1 h
3 h
6 h
Basal heat stress
Acquired thermotolerance

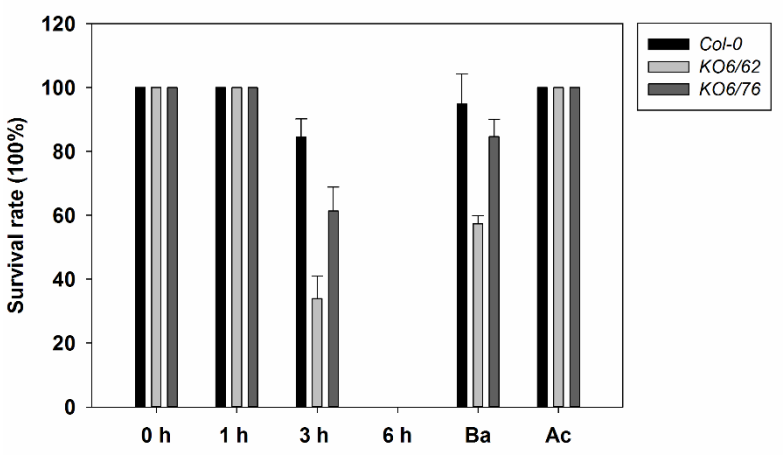


Fig. 17 Survival rates in *ALDH* double knock-out mutant plants. *A. thaliana* wild-type and double mutant (*KO6/62* and *KO6/76*) seedlings exposed to heat stress. Percentage of 10-day-old seedlings scored as surviving after 3 days heat stress at different time points, basal heat stress and acquired thermotolerance, The survival rate (%) and standard errors were calculated based on results from three independent experiments; error bars represent SD. Black bars, wild-type; dark-grey bars, *KO6/62*; light-grey bars, *KO6/76* double mutant.

3.1.3 Analysis of root length of two *ALDH* knock-out lines under heat stress

Seedlings were grown on MS-medium for 7 d, then they were subjected to Ba and Ac heat thermotolerance regimes and subsequently the lengths of the hypocotyls were measured **(Fig. 18A)**. The roots of the mutant seedlings (*KO6/62* and *KO6/76*) were slightly shorter than the roots of wild-type seedlings under non-stress conditions **(Fig. 18B)**. However, the lengths of roots in wild-type seedlings were significantly longer than the roots of the mutant seedlings under Ba and Ac regimes. Seedlings of all genotypes were better adapted in Ac than in Ba treatment to heat stress. This result shows that root growth is severely affected in the *ALDH* mutant seedlings after exposure to high temperature regimes.

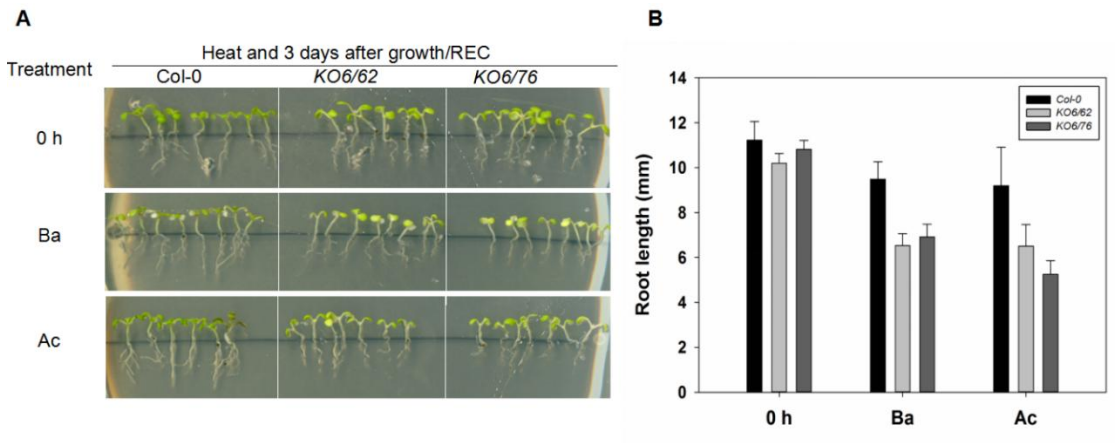


Fig. 18 The response of *KO6/62* and *KO6/76* double mutant lines and wild type plants to basal heat stress and acquired thermotolerance.

(A) Seedling development of *KO6/62* and *KO6/76* double mutant lines and the wild type exposed to basal heat stress and acquired thermotolerance. The photographs were taken 10 days after germination.

(B) Root length of seedlings of *KO6/62* and *KO6/76* double mutant lines and the wild type exposed to basal heat stress and acquired thermotolerance. Root length was calculated as the mean ± SD of 50 seedlings for each line.

3.1.4 Lipid peroxidation assay of two *ALDH* knock-out lines under heat stress

Under stress conditions, plant membrane lipids are oxidized, which often leads to the accumulation of the toxic compound malondialdehyde (MDA). MDA is a product of lipid peroxidation and accumulates when plants are grown under adverse conditions. Heat-shock conditions are known to cause membrane peroxidation (Wahid et al. 2007), and as a consequence, MDA levels could rise in heat-stressed tissues. Ten-day-old seedlings of wild-type, *KO6/76,* and *KO6/62* lines were subjected to Ba and Ac treatment. MDA levels increased upon heat stress, especially under basal heat stress. *KO6/76* and *KO6/62* accumulated more MDA than wild-type **(Fig. 19 Left)**. MDA levels increased linearly with the increase of the exposure time to high temperature (45°C) and four-week-old *KO6/76* and *KO6/62* plants accumulated more MDA level than wild-type plants when exposed to heat stress treatments **(Fig. 19 Right)**. A

sharp increase of MDA is seen in double mutant lines after 6 h heat stress and MDA continued to accumulate up to 24 h heat treatment. MDA levels are lower under Ac treatment than Ba stress regime. A significantly higher MDA content was found in *KO6/76* than in *KO6/62* after 1 h, 12 h, Ba and Ac indicating a role for *ALDH3I1* in heat stress protection. High levels of MDA seem to be correlated with decreased tolerance to high temperature. Overall, high levels of MDA resulted in decreased tolerance to high temperature. The higher concentration of MDA in *ALDH7B4*, *ALDH3F1* and *ALDH3I1* deficient plants than in wild-type plants under heat stress indicates that *ALDH* genes might be involved in cellular mechanisms, retarding the peroxidation of membrane lipids and improving the ability of resistance and adaptation of plants to heat stress.

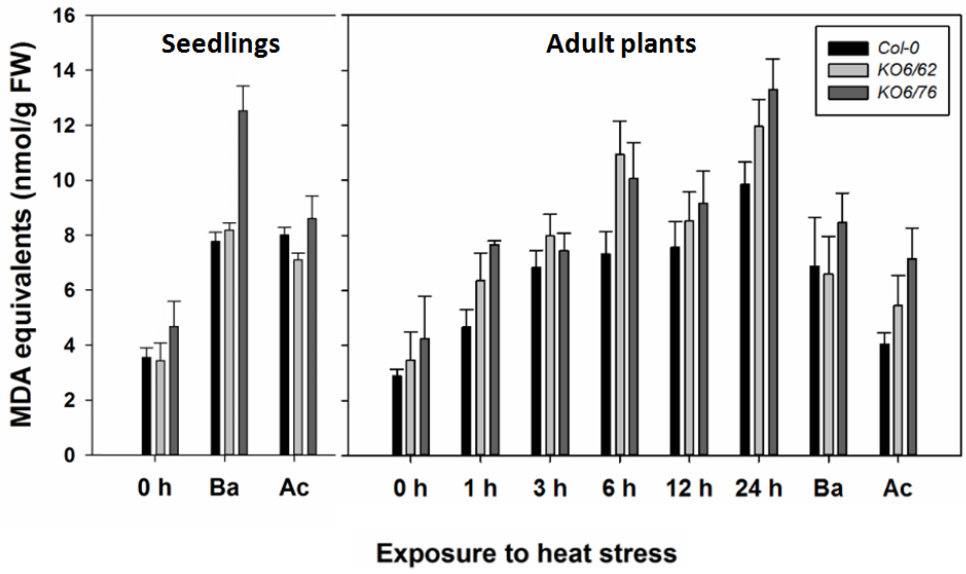


Fig. 19 Lipid peroxidation as measured by MDA levels in wild-type *Arabidopsis* and double mutant plants after heat treatment. Lipid peroxidation in ten-day-old seedlings **(Left)** and four-week-old **(Right)** wild-type and *KO6/62* and *KO6/76* mutant plants after heat stress at different time points (1 h, 3 h, 6 h, 12 h and 24 h exposure to 45°C), basal heat stress (Ba) and acquired thermotolerance (Ac). Data are the mean ±SD of three independent experiments. Black bars: wild-type; dark-grey bars: *KO6/62* mutant; light-grey bars: *KO6/76* mutant.

3.1.5 Chlorophyll content of two *ALDH* knock-out lines under heat stress

ALDH enzymes play important antioxidant roles by producing NAD(P)H, directly absorbing UV radiation and scavenging hydroxyl radicals *via* cysteine and methionine sulfhydryl groups (Singh et al. 2013). Because antioxidants were implicated in protecting the photosynthetic apparatus from oxidative damage, chlorophyll contents were quantified in the *ALDH* mutant lines. There is no significant difference in total chlorophyll content between wild-type and *ALDH* double knock-out seedlings **(Fig. 20)**. Chlorophyll content gradually declined with the length of heat stress in all the mutant and wild type four-week-old leaf discs. Total chlorophyll content was only slightly reduced in*KO6/76* and *KO6/62* as compared to wild-type during treatment **(Fig. 20)**. The results indicate that chlorophyll content of both wild-type and double mutants were constantly reduced with the increasing duration of heat treatment. More importantly, data reveal that photosynthetic parameters of *ALDH* genes deficient plants were slightly more sensitive than wild type plants when subjected to high temperatures.

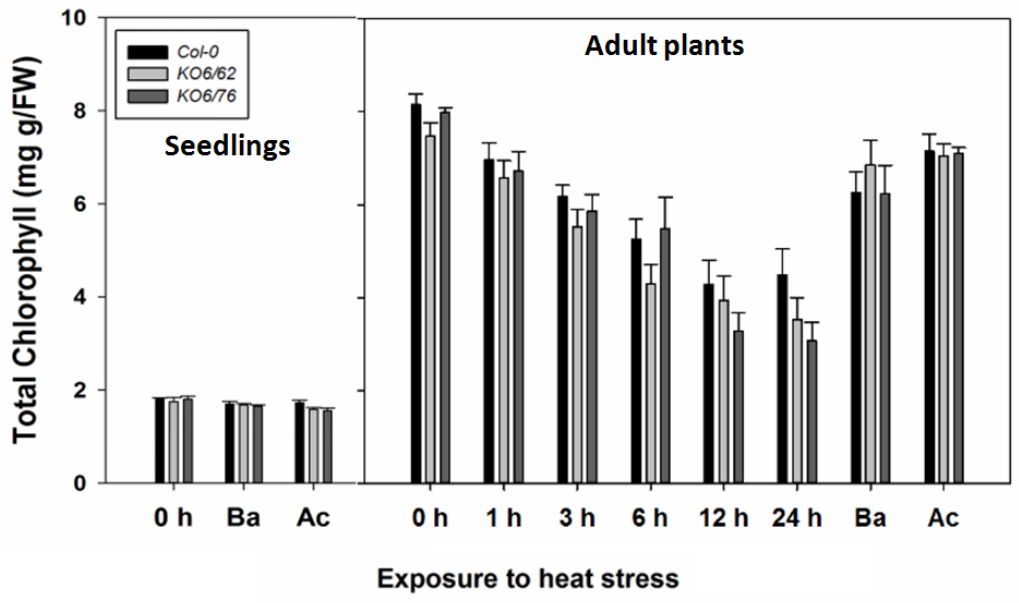


Fig. 20 Chlorophyll contents of *KO6/62* and *KO6/76* lines and the wild type plants under heat stress treatment. Chlorophyll contents of ten-day-old seedlings

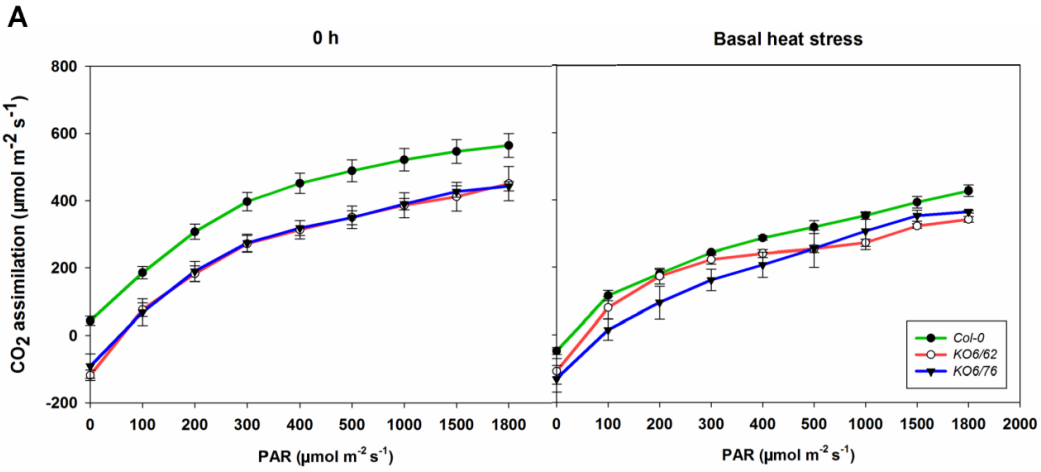
(Left) and leaves of four-week-old **(Right)** wild-type and *KO6/62* and *KO6/76* mutant lines after heat stress treatments at different time points (1 h, 3 h, 6 h, 12 h and 24 h exposure to 45°C), basal heat stress (Ba) and acquired thermotolerance (Ac). Data are the mean ±SD of three independent experiments. Black bars, wild-type; dark-grey bars, *KO6/62*; light-grey bars, *KO6/76* double knock-out mutant.

3.1.6 Analysis of photosynthesis efficiency during heat stress of two *ALDH* knock-out lines

Photosynthesis is an important process that is detrimentally affected by extreme environmental conditions such as high temperature. In order to investigate further functions of *ALDH* genes on improved photosynthesis, photosynthetic efficiency was compared at all light intensities (0-1800 µmol $m^{-2}s^{-1}$) for wild-type, *KO6/62* and *KO6/76* double mutant under normal and Ba conditions. CO_2 assimilation, the efficient quantum yield of PSII, non-photochemical quenching (NPQ), fluorescence and maximum fluorescence ratio (Fv/Fm,) were determined after exposure of leaves to light for 3 minutes at different intensities.

CO_2 assimilation is the ability that plants assimilate carbon from carbon dioxide in the atmosphere to form metabolically active compounds. Results showed that three genotypes under control condition had higher CO_2 assimilation level than under Ba treatment **(Fig. 21A)**. Wild-type plants offered the highest assimilation ability in comparison with *KO6/62* and *KO6/76* indicating that the loss of *ALDH* genes may affect photosynthetic efficiency. The efficient quantum yield of PSII was demonstrated that quantum yield (qE) of PSII, known as the light-adapted test. **Fig. 21B** showed that qE was only slightly reduced in*KO6/62* and *KO6/76* as compared to wild type under non-stress conditions whereas dramatic lower qE were detected in double mutant lines, and the *aldh7b4aldh3i1*double mutant showed the lowest quantum yield of all lines analyzed. In addition, non-photochemical quenching (NPQ) consists of

the rapid dissipation of excess excitation energy as heat. **Fig. 21C** showed that NPQ was only slightly affected in all mutants. Under normal conditions, the mutants exhibited a lower steady state NPQ than did the wild-type plants. Consistently, a lower NPQ activation in *ALDH* defecting lines was observed under Ba heat stress suggesting that the capacity of dissipating excitation energy as the heat was higher in wild-type with respect to the mutants. The maximum photochemical efficiency of PSII (Fv/Fm) stands for plant stress affects photosystem II in a dark-adapted state or optimal/maximal quantum yield of PSII. The significant reduction in Fv/Fm was observed in mutant lines under heat stress **(Fig. 21D)**. These findings fit with previous studies of chlorophyll content, which found that ALDH deficient plants were affected more than wild type when subjected to high temperatures, indicating that ALDHs may be involved in protecting the photosynthetic apparatus from oxidative damage.



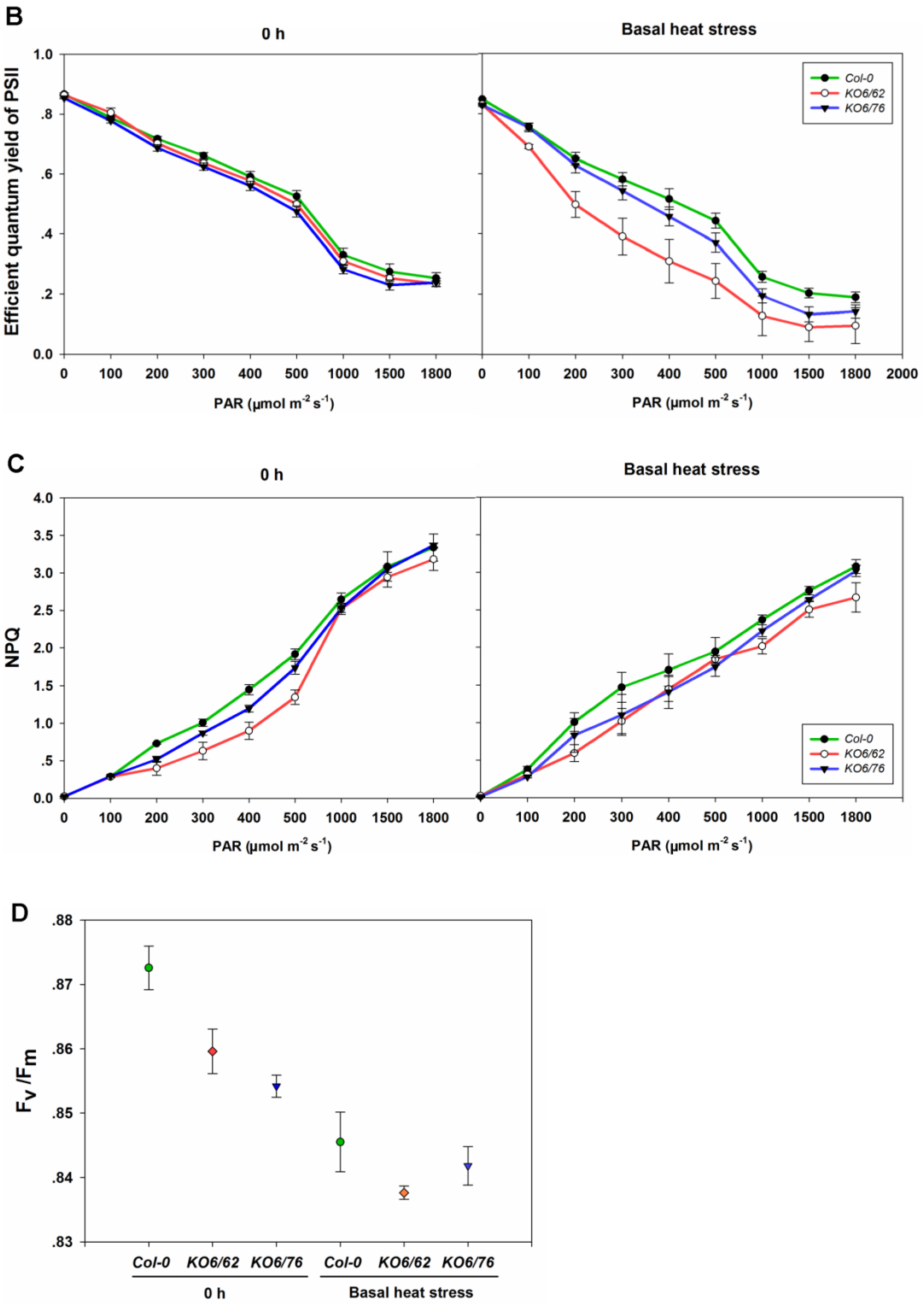


Fig. 21 Analysis of photosynthesis apparatus in wild-type, *KO6/62* and *KO6/76* leaves.

(A) CO_2 assimilation in wild-type, *KO6/62* and *KO6/76* leaves subjected to control and basal heat stress conditions, respectively, under various light intensities.

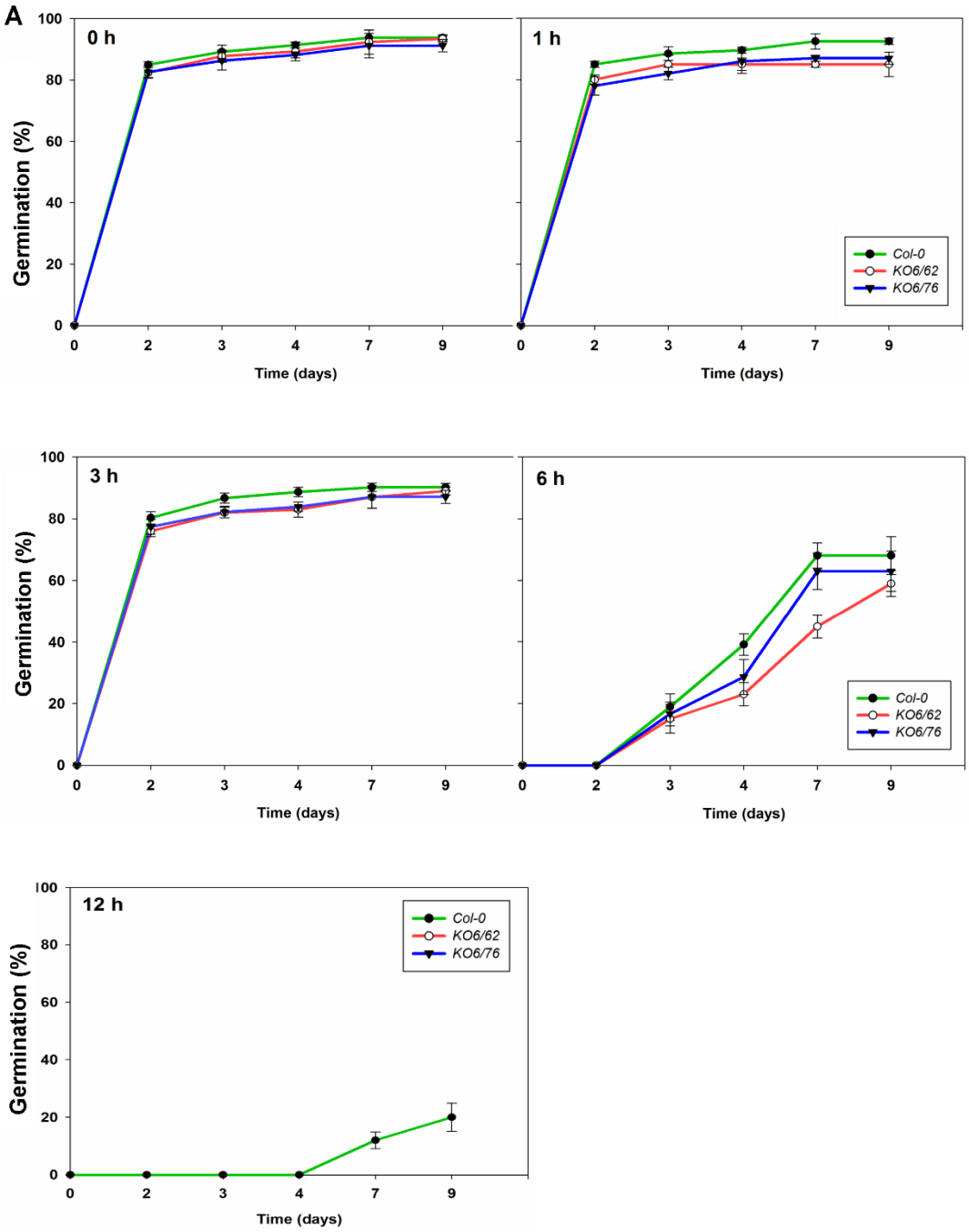
(B) Quantum yield of PSII in wild-type, *KO6/62* and *KO6/76* leaves subjected to control and basal heat stress conditions.

(C) Total leaves of non-photochemical quenching under control and basal heat stress treatment.

(D) Leaves excised from 4-week-old wild-type, *KO6/62* and *KO6/76* were treated under basal heat stress and the kinetics of the Fv/Fm was measured. Values are means ±SD (n=8).

3.1.7 Evaluation of two *ALDH* knockout lines for seed thermotolerance

In our previous report, we have shown *ALDH3F1-GUS and ALDH7B4-GUS* transgenic plants display a very strong expression in the whole plant and in germinating seeds (Kirch et al. 2005; Stiti et al. 2011).Therefore, we investigated if *ALDH* genes were involved in seed thermotolerance. The seeds of the *ALDH* deficient mutants *KO6/62* and *KO6/76* were placed on MS medium to test their ability to germinate directly at 45°C for 0 h, 1 h, 3 h, 6 h, 12 h and recover at growth chamber for 2 d, 4 d, 7 d and 9 d afterward **(Fig. 22A)**. The data presented showed that nearly 100% germination was observed of wild-type, *KO6/62*, and *KO6/76* under normal conditions. The *KO6/62* and *KO6/76* mutants all show a lower germination percentage compared with wild types after 1 h, 3 h, and 6 h. Moreover, none of the double mutant seeds germinated after 12 h heat treatment. Based on germination results, lipid peroxidation in the germinating seedlings was analyzed **(Fig. 22B)**. The seeds of wild-type plants and two double mutant lines were treated as described above. The seedlings were harvested after 10 days of growing on MS medium, the MDA level was measured. The results demonstrated that mutant lines after 3 h of treatment can accumulate more MDA than wild-type, especially after 6 h. The results of this study indicated that lack of *ALDH* genes affects the ability to germinate under high temperatures.


A
0 h
1 h
3 h
6 h
12 h
Germination (%)
Time (days)
Col-0
KO6/62
KO6/76

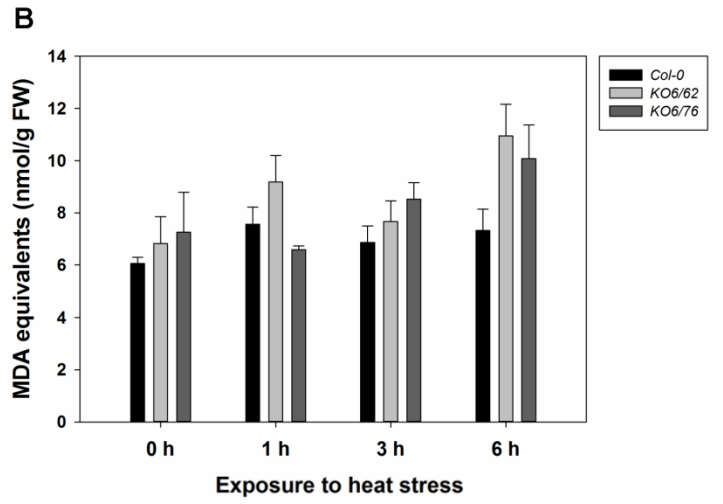


Fig. 22 Germination and seedlings growth of double-mutant (*KO6/62* and *KO6/76)* seeds and wild-type exposed to heat stress.

(A) Seeds were treated for different heat time point and germinated on MS agar plate. The photographs show 9-d-old seedlings. Percentage of seeds germinated was scored after 2, 3, 4, 7 and 9 days by heating to 45°C for 0 h, 1 h, 3 h, 6 h and 12 h.
(B) MDA level in the germinating seedlings derived from the thermotolerance test of wild type, *KO6/62,* and *KO6/76.* Data are the mean ±SD of three independent experiments.

3.2 Study of aldehyde dehydrogenase (*ALDH)* genes in *A. thaliana* under combination of stresses

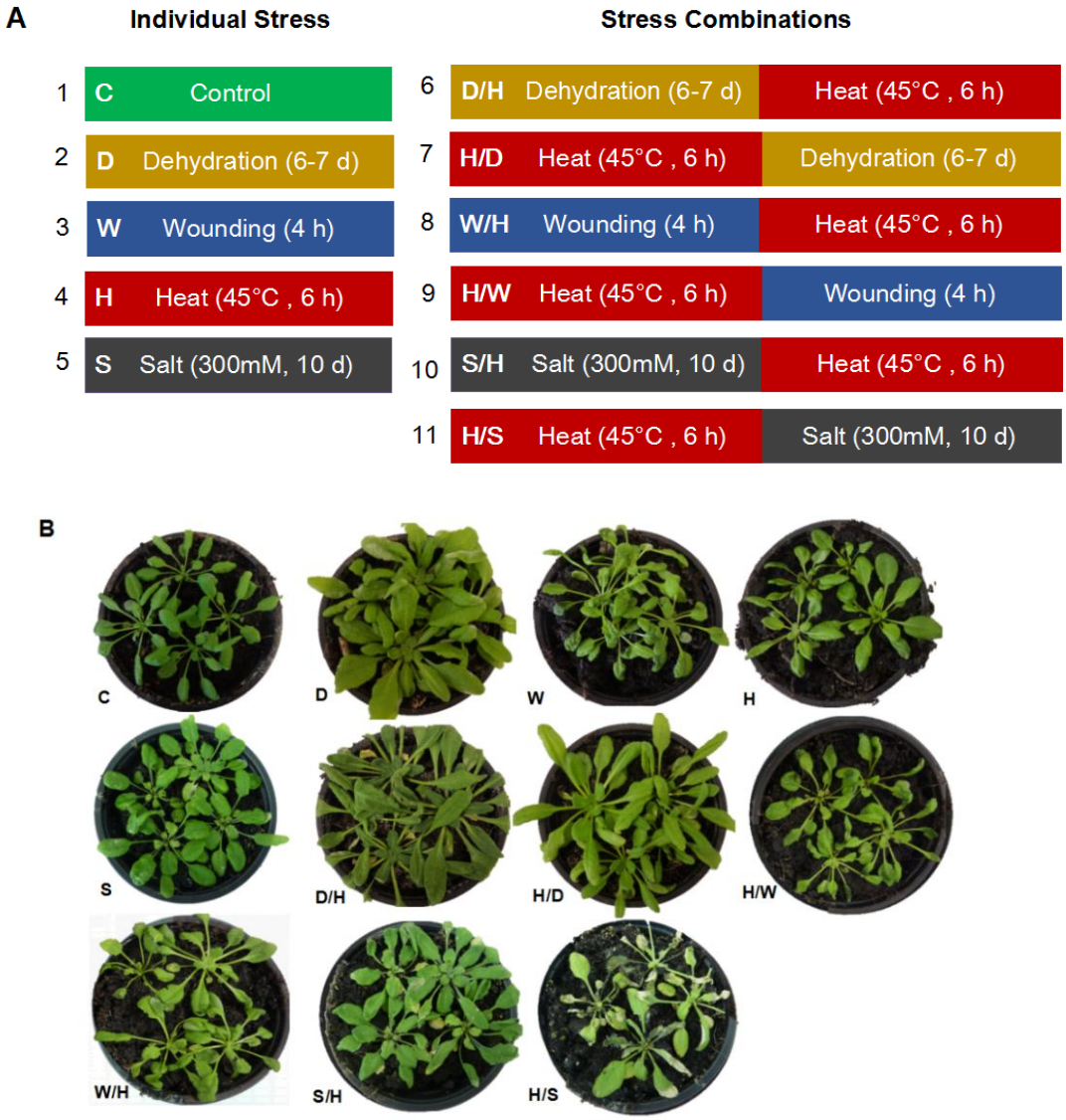
3.2.1 Expression analysis of different aldehyde dehydrogenase (*ALDH)* genes from *Arabidopsis thaliana* under combination of stresses

Individual abiotic stresses are well studied recently, but much less is known about the effect of multiple, co-occurring stress factors, despite the fact that multiple stresses are probably the rule under natural conditions (Holopainen and Gershenzon 2010).Therefore we have asked the question how the different stressors influence each other using *ALDH* genes as an example. First, the stress combination treatments were established and the corresponding physiological changes analyzed. *A. thaliana* wild-type plants were subjected to a combination of stress conditions and the performance was compared with plants subjected to a single stress 5 days after growth at

non-stress conditions (recovery). The single stress treatments and the stress combinations are depicted in **Fig. 23A.** The design of the stress applications attempts to describe the conditions that occur in the field when a relatively prolonged period of dehydration (D) or salinity (S) is combined with a short period of high temperature (H) stress. High temperature stress was also combined with wounding (W) stress. **Fig. 23B** shows that plants after wounding stress (W), dehydration-heat stress (D/H), heat-wounding (H/W) stress heat-salt (H/S) stress and wounding-heat W/H showed signs of injuries, some leaves after D/H and H/S treatment turned yellow and dried up. However, plants subjected to heat-dehydration (H/D) stress showed no severe symptoms which might be due to the fact that the plants recovered from the heat stress during seven days of dehydration at non-stress temperature 22°C.

To analyze the effect of stress combinations on target gene expression *ALDH* gene expression was monitored at the transcript and protein level. The abiotic stress-related *A. thaliana ALDH* genes *ALDH7B4*, *ALDH3H1*, *ALDH3I1*, *ALDH3F1* and two betaine aldehyde dehydrogenases *ALDH10A8* and *ALDH10A9* were selected for these studies **(Fig. 23C)**. RT-PCR analysis indicated that *ALDH7B4* transcripts increased under all single stresses applied and accumulated to even higher levels after D/H, H/D, W/H and H/S treatment. Expression of ALDH3I1 was strongly induced upon wounding and slightly in response to salt stress, but transcripts remained low after a combination of stress treatments. ALDH3H1 accumulated at low levels in all treatments. Similarly, ALDH3F1 was also not much expressed under all stress conditions. ALDH10A8 and ALDH10A9 were constitutively expressed and did not change either in response to single stresses or stress combinations. Protein blots showed that ALDH7B4 protein was induced by all stress conditions, and accumulated abundantly under W, D/H and H/S stress treatment **(Fig. 23D)**. The ALDH3I1 protein was up-regulated in response to W and H treatment and

accumulated weakly after D/H and W/H. The expression of ALDH3H1 protein was induced under W, S, and D/H and H/D, also slightly induced by heat and H/S treatment. The ALDH3F1 protein was constitutively expressed in response to individual stress and expression was only detected in response to D/H and H/S stress combinations **(Fig. 23D)**. Taken transcript and protein expression together, ALDH7B4 and ALDH3H1 expression levels are increased under D/H and H/S stress combination.



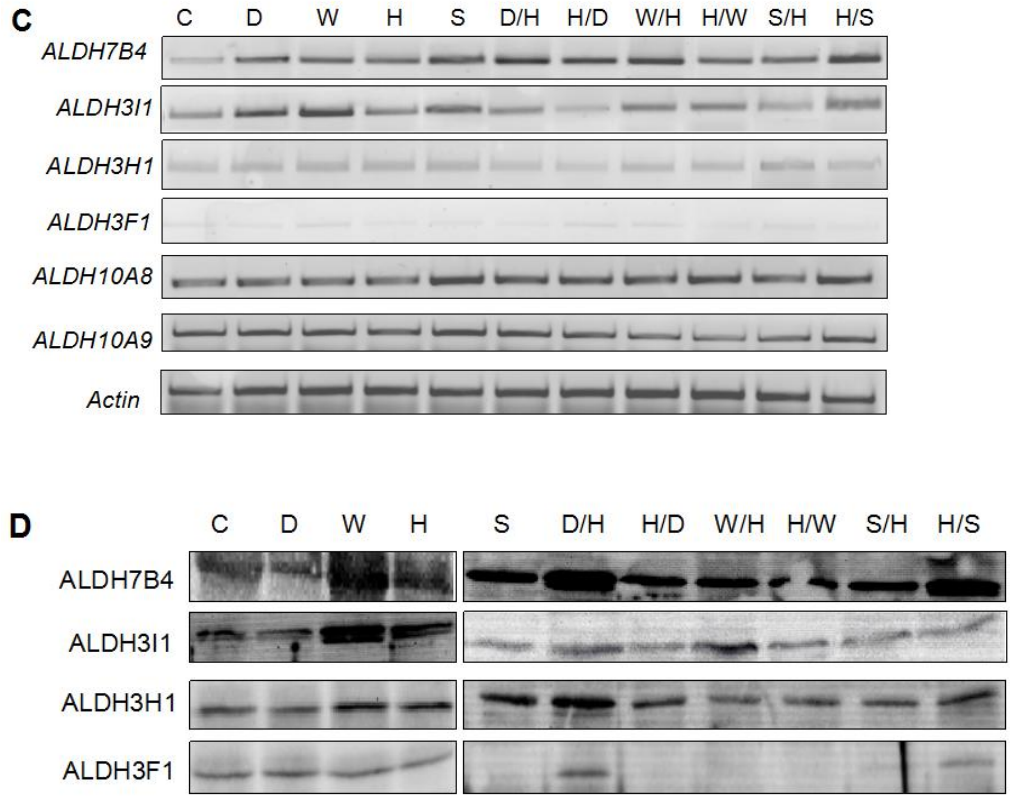


Fig. 23 Effect of stress combinations studied in four-week-old *A. thaliana* plants.

(A) The experimental design of stress applications. *A. thaliana* wild-type plants subjected to different individual (2-5) and combination stresses (6-11). Dehydration stress is imposed by withdrawing water from plants for 6-7 day (2), the corresponding relative water content is about 75%. Wounding was imposed by incubating the plant at short day growth chamber for 4h after pressed the leaves by sandpaper (3). Heat is imposed by keeping the plants at 45^{o}C for 6 h (4), salinity stress is watered the plant every two days with water containing 300 mM NaCl for 10 days (5). A combination of dehydration and heat stress was performed by dehydration-treated plants followed by a heat stress treatment or heat stress comes first and followed by dehydration (6 and 7). A combination of wounding and heat stress was performed by wounding the plants for 4 h followed by heat stress or heat stress before wounding stress (8 and 9). Similarly treatment orders to heat and salt combination stress (10 and 11).

(B) Phenotypic analysis of four-week-old *A. thaliana* plants subjected to a single stress and stress combinations. C: control, D: dehydration, W: wounding, H: heat, S: Salt, D/H: dehydration followed by heat stress, H/D: heat stress followed by dehydration, W/H: wounding stress followed by heat stress, H/W: heat stress followed by wounding, S/H: salt stress followed by heat stress, H/S: heat stress

followed by salt stress.

(C) Transcript accumulation analyzed by RT-PCR using gene specific primers to amplify the transcripts from total RNA (2 µg) extracted after different stress treatments (details see materials and methods).

(D) Protein blot analysis of protein extracts from four-week-old plants subjected to different stress treatments(C).

3.2.2 Analysis of lipid peroxidation of two *ALDH* knock-out lines under a stress combination

To gain more insights into the potential functions of selected *ALDH* genes under a combination of stress, malondialdehyde formation (MDA) in *KO6/62* and *KO6/76* mutant lines in comparison with wild-type plants were studied under treatment. From the above studies, the two *ALDH* double knock-out mutant lines were more sensitive than wild-type under different heat stress conditions. Here, a level of oxidative stress caused by a combination of stress was evaluated by measuring MDA in wild-type and *KO6/62* as well as *KO6/76* mutant lines. An elevation in MDA levels was observed after a single stress and stress combinations. In comparison with other stress conditions, D/H and H/S caused a significant increase in accumulation of MDA content, moreover, mutant lines had significantly higher MDA levels than stressed wild-type plants **(Fig. 24)**. The result corroborates the role for *ALDH* genes in stress protection by lowering lipid peroxidation.

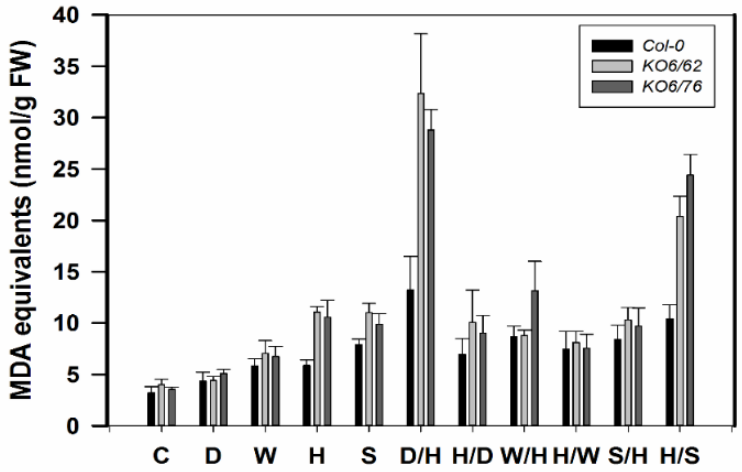


Fig. 24 MDA equivalents of four-week-old plants of *KO6/62* and *KO6/76* double mutant lines and wild type plants subjected to individual and a combination of stresses. Data are the mean ±SD of three independent experiments. C: control, D: dehydration, W: wounding, H: heat, D/H: dehydration followed by heat stress, H/D: heat stress followed by dehydration, W/H: wounding stress followed by heat stress, H/W: heat stress followed by wounding. Black bars, wild-type: dark-grey bars: *KO6/62*; light-grey bars: *KO6/76* mutant.

3.2.3 Analysis of chlorophyll content of two *ALDH* knock-out lines under a stress combination

Chlorophyll content also tested in these three genotypes under individual and combination stress conditions. The results showed that leaves from heat, D/H, W/H, S/H and H/S treatments have less chlorophyll content than other conditions, but wild-type and knock-out mutants have similarly reduced chlorophyll levels **(Fig. 25)**. These results corroborate a role for *ALDH* genes in stress protection by lowering lipid peroxidation. The leaves from heat, D/H, W/H, S/H and H/S treatments have less chlorophyll content under other conditions, but wild-type and knock-out mutants have similarly reduced chlorophyll levels which were also observed for heat stress condition.

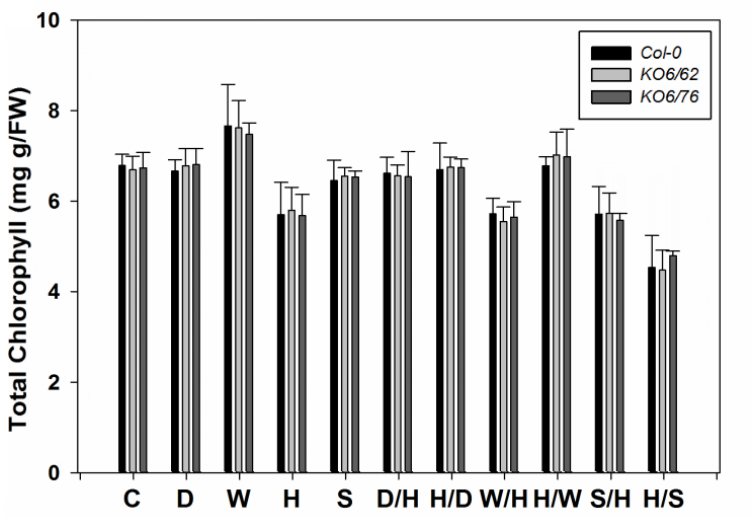


Fig. 25 Chlorophyll levels in response to single stress and a combination of stresses. Chlorophyll levels were determined in four-week-old wild-type and *KO6/62*

as well as *KO6/76* mutant lines. Data are the mean ±SD of three independent experiments. C: control, D: dehydration, W: wounding, H: heat, D/H: dehydration followed by heat stress, H/D: heat stress followed by dehydration, W/H: wounding stress followed by heat stress, H/W: heat stress followed by wounding. Black bars, wild-type: dark-grey bars: *KO6/62*; light-grey bars: *KO6/76* mutant.

3.3 Study of *ALDH7B4* promoter in *A. thaliana*

3.3.1 Functional analysis of the *cis*-elements within *A. thaliana ALDH7B4* promoter in response to stress combinations

3.3.1.1 Information and structures of *A. thaliana ALDH7B4* promoter mutation lines

The research of high temperature and stress combinations on the promoter activities of *ALDH* genes is based on the former studies in our lab (Missihoun et al. 2014).The *ALDH7B4* promoters are induced in response to abiotic stress conditions. High *GUS* reporter gene expression was found in all tissues in response to ABA, dehydration, and NaCl. Moreover, the promoter was found to be responsive to wounding stress, suggesting that ALDH7B4 may also be involved in response to plant pathogens (Missihoun et al. 2014).To investigate more details about the responsiveness of the *ALDH7B4* promoter fragment to heat and a combination of stresses, interactions between stress-related *cis*-elements present in *ALDH7B4* promoter have been studied and five constructs with six different mutations (AB, AD, P1, P2, and P3) were used in this work. The promoter-GUS constructs were generated by Dr. Missihoun in our lab **(Fig. 26)**. *A. thaliana* transgenic lines A, D, AD and AB contain point mutations in the promoter sequence while P1, P2 and P3 line were 5' end deletion lines.P1 (−11 to −474), P2 (−11 to −335), and P3 (−11 to −219), the different promoter constructs are depicted in **Fig. 26**. ACGT2/3 was mutated in the AB construct, AD construct lacks a DRE and ABRE1 MYC box in comparison to the full length of the promoter (B8). The P1 construct is lacking

two MYB boxes and three heat shock sequence elements. An additional DRE box was missing in the P2 construct. The P3 construct only has MYB, ACGT2/3 and heat shock elements left.

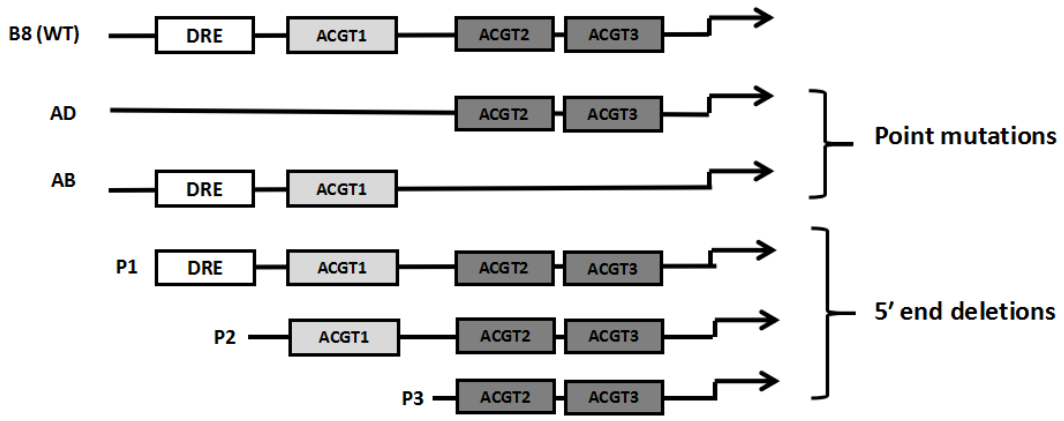
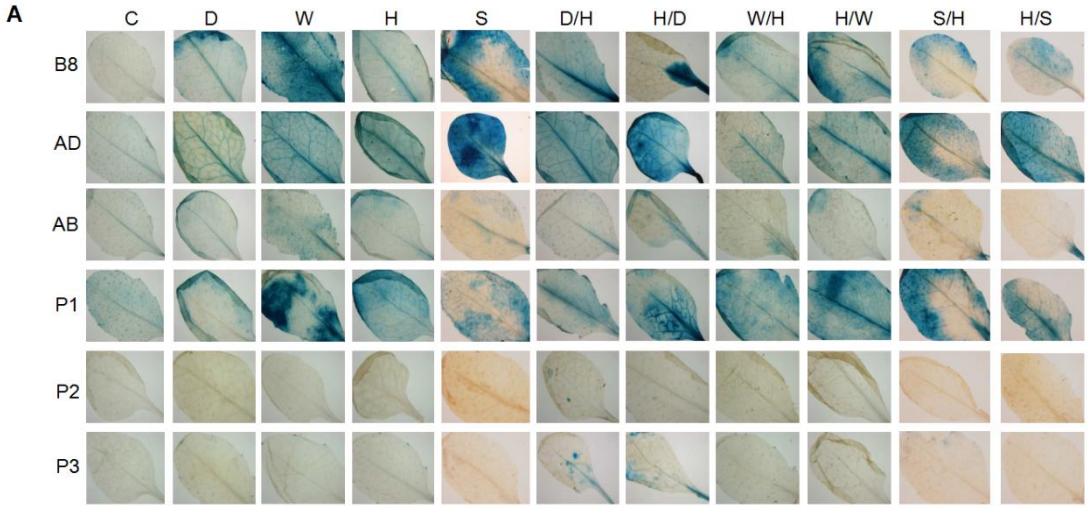


Fig. 26 Schematic representation of*ALDH7B4* promoter fragments fused to GUS (Missihoun et al. 2014)**.** B8 (WT) = "full promoter", 646bp fragments as the *promoter-GUS* reporter construct for expression studies used. AD and AB = point mutations in putative cis elements DRE of ATCGAC to ATATTT; ABRE of ACGT to ATTT; the MYC remains recognizable despite the mutation of ACGT1. Promoter deletion lines P1-P3 were also used as *promoter-GUS* reporter constructs for expression studies.

3.3.1.2 Characterization of *A. thaliana ALDH7B4* promoter deletion GUS expression lines in response to different stresses

To address the functional significance of the individual *cis*-elements present in *ALDH7B4* promoter deletion constructs were analyzed under a combination of stresses. We also evaluated the individual stress effects of dehydration, heat, wounding and salt treatment on promoter-GUS expression. Patterns of accumulation of GUS in promoter deletion lines were firstly investigated under various abiotic stresses in four-week-old leaves using GUS tissue staining **(Fig. 27)**. Low GUS activity was observed under control conditions in all six independent lines. A moderate increase in GUS activity was found when plants

were grown under dehydration stress. Higher accumulation patterns of GUS expression were obtained for B8, AD and P1 lines than the AB line. GUS activity decreased drastically in P2 and P3 lines. Incubation of plants after 4 h wounding stress resulted in a rapid increase in GUS activity whereas the level of GUS in AB, P2 and P3 was severely reduced and showed weak staining. Remarkably, an increase of GUS activity in AD and P1 occurs when plants were kept at 45°C for 6 h. Fluorimetric measurements of GUS activity indicated that short promoter P2 and P3 lines showed nearly no GUS activity, although some potential stress elements are predicted in this promoter. High GUS activity was found in B8 and AD lines under salt treatment. Combined dehydration/heat and salt/heat showed nearly similar levels of GUS expression as single stress. While lower GUS activities in wounding/heat combination stress conditions than individual stress were observed. The strong inductions were observed for AD and P1 line. Likewise, GUS expression in P2 and P3 was lost upon deletion of the ACGT1 and DRE motifs, but a slight increase in GUS activity was observed in P3 after D/H and H/D treatment. Taken together, the results indicate that ABRE/MYC (ACGT2/3) elements, as well as ACGT1 and DRE/CRT in the *ALDH7B4* promoter, are necessary to mediate its expression under individual abiotic stress and stress combinations.



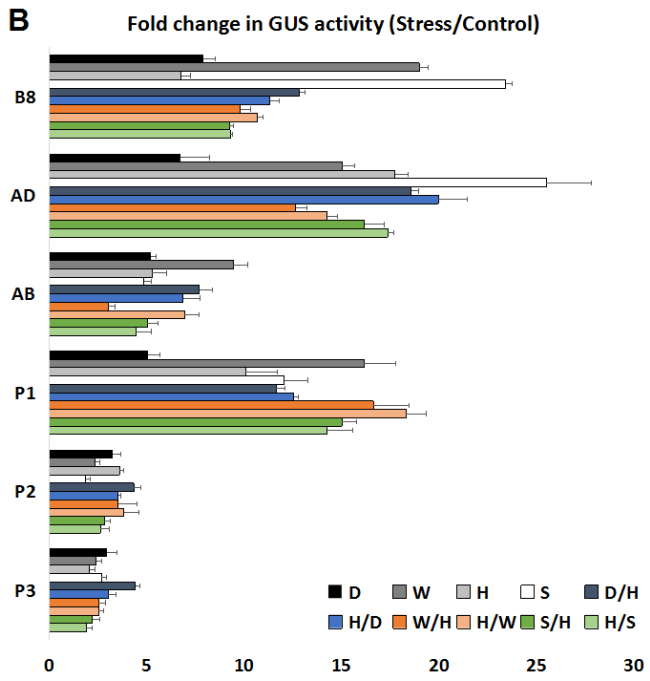


Fig. 27 Activities of the deleted *ALDH7B4* promoters under different stress conditions.

(A) *In situ* detection of the GUS activity. Four-week-old wild-type and independent transgenic *A. thaliana* plants harboring different the *ALDH7B4* promoter fragments fused to the GUS reporter gene were subjected to different stresses. B8, AB, AD, P1, P2 and P3 represent different *ALDH7B4* deletion GUS expression lines (details see **Fig. 26**) C: control, D: dehydration, W: wounding, H: heat, D/H: dehydration followed by heat stress, H/D: heat stress followed by dehydration, W/H: wounding stress followed by heat stress, H/W: heat stress followed by wounding.

(B) The activity of the *ALDH7B4* promoter deletion constructs measured as enzymatic activities of the GUS reporter protein under individual and combination stress conditions. Error bars correspond to the SD of three independent replicates.

3.3.2 Identifying transcription factors interacting with *A. thaliana ALDH7B4* promoter

3.3.2.1 Yeast one-hybrid screening system and associated *cis*-elements in *A. thaliana ALDH7B4* promoter

To focus on possible proteins that bind to putative *cis*-elements in*ALDH7B4* promoter, a yeast one-hybrid screening was performed. In this technique, the interaction between two proteins (bait and prey) is detected via *in vivo* reconstitution of a transcriptional activator that turns on expression of a reporter gene. Detection is based on the interaction of a transcription factor (prey) with a bait DNA sequence upstream of a reporter gene (*HIS3* or *LacZ*). To ensure that DNA binding results in reporter gene activation, cDNA expression libraries are used to produce hybrids between the prey and a strong trans-activating domain. The advantage of cloning transcription factors or other DNA-binding proteins via one-hybrid screenings, compared to biochemical techniques, is that the procedure does not require specific optimization of *in vitro* conditions. An improved system with a library of 1,498 transcript factors (TFS) from *A. thaliana* was used (Mitsuda et al. 2010). The screening system including the transcription factor library and the vectors used are described in **Table 4**.

Table 4 Vectors for the yeast one-hybrid screening

Vector	Fragment
TFpDEST_GAD424	Transcript factors library
R4L1pDEST_LacZi	Reporter gene *lacZ*
R4L1pDEST_HISi	Reporter gene *HIS3*

Transcription factors bind to characteristic recognition sequences *cis*-elements which can trigger the transcription (activators) or block (repressors). In this work, the promoter of *ALDH7B4* from *A. thaliana* was examined. Various *cis*-elements of the promoter involved in stresses have been described here and in former studies (Missihoun 2010). *ALDH7B4* showed tolerance to salt (NaCl and/or KCl), dehydration and oxidative stress. Previous studies have shown that the expression of the *ALDH7B4* gene both by abiotic stress factors such as drought and salt, as well as aldehydes, wounding stress can be induced. Sequence analysis of the promoter sequence of *ALDH7B4* has been done using the databases PLACE Web Signal Scan (www.dna.affrc.go.jp/PLACE/) and RIKEN *Arabidopsis* Genome Encyclopedia and (www.rarge.psc.riken.jp/). A diagram with the position of these elements can be found in **Fig. 28**. The *ALDH7B4* promoter contains "Drought Responsive Element" (DRE or CBF; RYCGAC) motif and three ACGT motifs (ACGT1, ACGT2 and ACGT3), four MYC consensus sequences (E-box), four heat shock sequence elements (AGAAn), two AtMYB1-binding motifs (WAACCA) and two MYB consensus sequences (CNGTTR). DREs are bound by "DRE binding proteins" (DREBs) and "C-repeat binding factors" (CBF), mainly functional in the ABA-independent gene induction by cold and drought stress. ACGT boxes are at the core of "ABA Responsive Elements" (ABREs). Both MYCs and MYBs lead to an ABA-inducible gene expression by cold and drought stress.

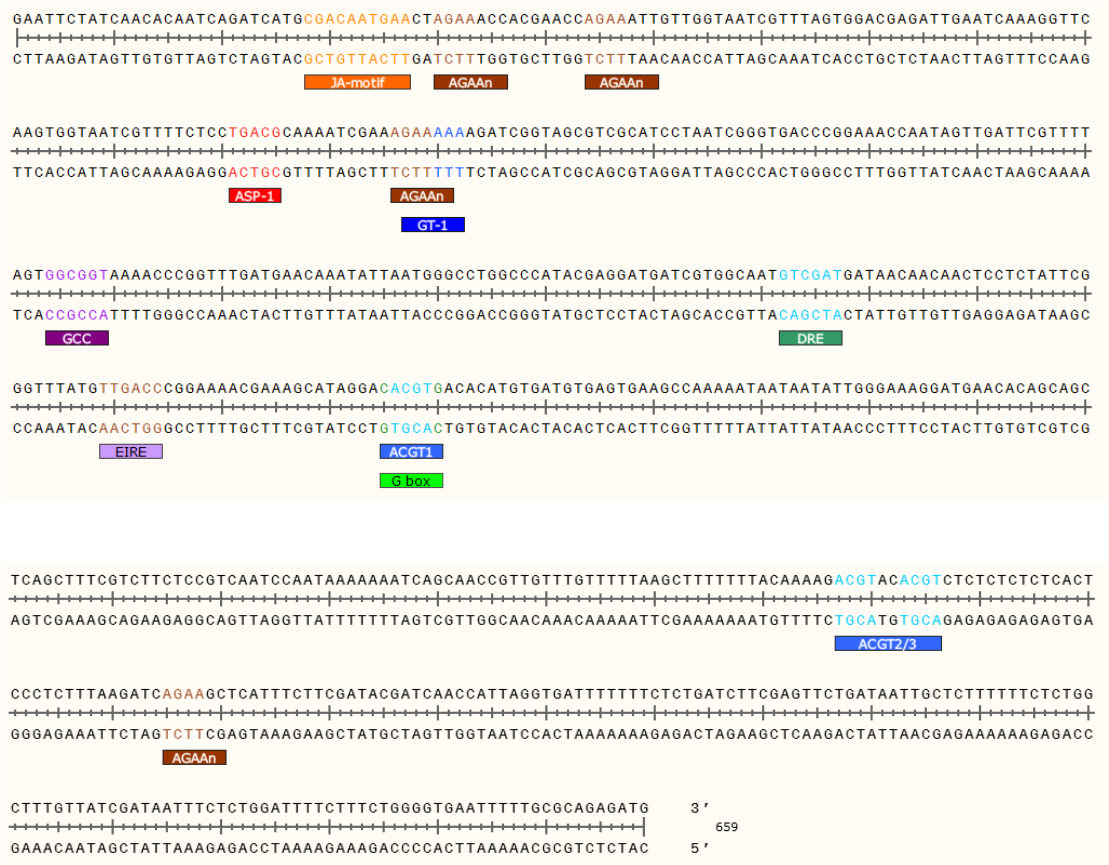


Fig. 28 Full promoter sequence of the *ALDH7B4* gene and localization of stress associated *cis*-elements. The full promoter of *ALDH7B4* with localization of stress associated *cis*-elements. The figure was created by Snap Gene Viewer (659 bp), the nucleic acid sequence of the *ALDH7B4* promoter in *A. thaliana* with color-marked, identified *in silico* of *cis*-element motifs. CGTCAATGAA: JA-motif; AGAAn: heat shock sequence elements; CACGTG: G-box motif; TGACG: ASF-1 binding site; GAAAAA: GT-1 motif; GGCGGT: GCC box; RYCGAC/ACGT: DRE motif/ACGT; TTGACC: EIRE motif.

3.3.2.2 Confirmation of yeast-one-hybrid DNA-bait strains and transformation on a small scale

Two construct strains have been generated and used for the yeast one- hybrid DNA-baits (Schmidt 2011). The target element of *ALDH7B4* promoter fragment was selected from 606 and 607 bp **(Fig. 29).** The plasmids *R4L1pDEST_HISi* and *R4L1pDEST_LacZi* containing the promoter fragment

were retested and amplified using primers pHISi_fwd/pHISi_rev and pLacZi_fwd/pLacZi_rev respectively. The PCR product was transformed into *E. coli* DH10B competent cells and reconfirmed by DNA sequencing. The *R4L1pDEST_HISi-ALDH7B4PH* and *R4L1pDEST_LacZi-ALDH7B4PL* constructs were linearized with the restriction enzymes XhoI and NcoI **(Fig. 30)**.

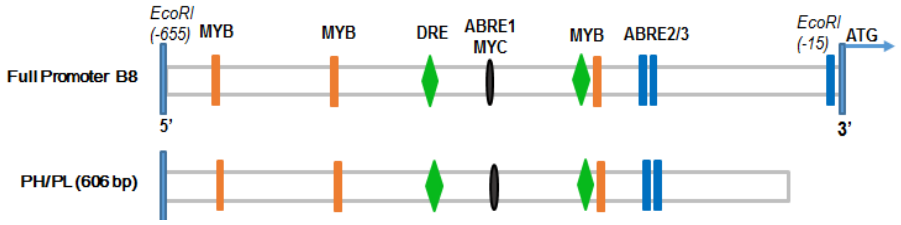


Fig. 29 The schematic representation of *ALDH7B4* full promoter and promoter fragments for yeast one-hybrid screening. B8= full promoter; PH / PL = promoter fragments for *HIS3* and *lacZ* reporter in yeast one-hybrid screening (difference: 3 'end of PL is 1 bp longer than PH).

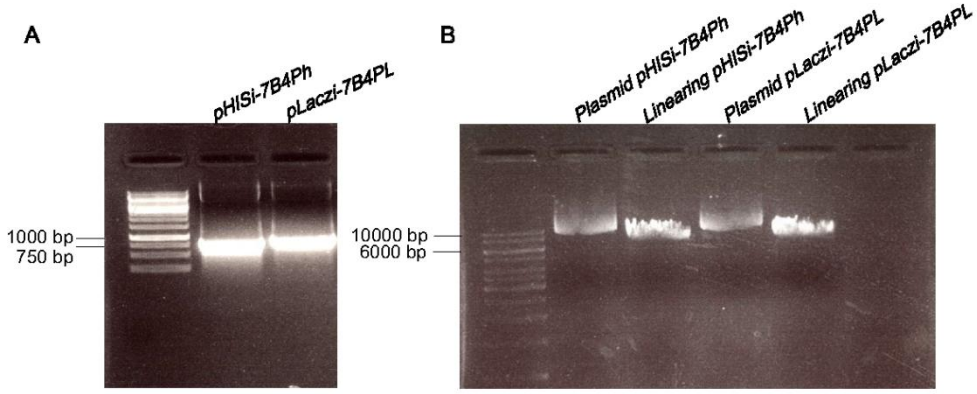


Fig. 30 Two DNA-bait constructs were amplified by PCR and linearized by different restriction enzymes.

(A) The *ALDH7B4* promoter fragment was amplified using primers pHISi_fwd and pHISi_rev for the plasmid of pHISi-7B4PH (606 bp), pLacZi_fwd and pLacZi_rev for the plasmid of pLacZi-7B4PL (607 bp).

(B) Lane 1 and 3 are undigested R4L1pDEST_HISi-7B4Ph and R4L1pDEST_LacZi-7B4PL plasmids respectively. Lane 2 is XhoI digested

R4L1pDEST_HISi-7B4Ph, and lane 4 is NcoI digested R4L1pDEST_LacZi-7B4PL.

After linearization, the constructs were simultaneously transformed into the yeast strain YM4271, the *pHISi-7B4PH* constructs were followed simultaneously and transformed into the yeast strain YM4271 on a small scale and screened on SD-His selection medium**(Fig. 31)**. The second transformation was also performed with pLacZi-7B4PL into YM4271-pHISi-7B4PH screened on SD-His/-Ura medium. The yeast strain was cultured on the SD-His-Ura medium and integration of the constructs was tested by PCR using two pairs of primers pHISi_fwd/pHISi_rev and pLacZi_fwd/pLacZi_rev.

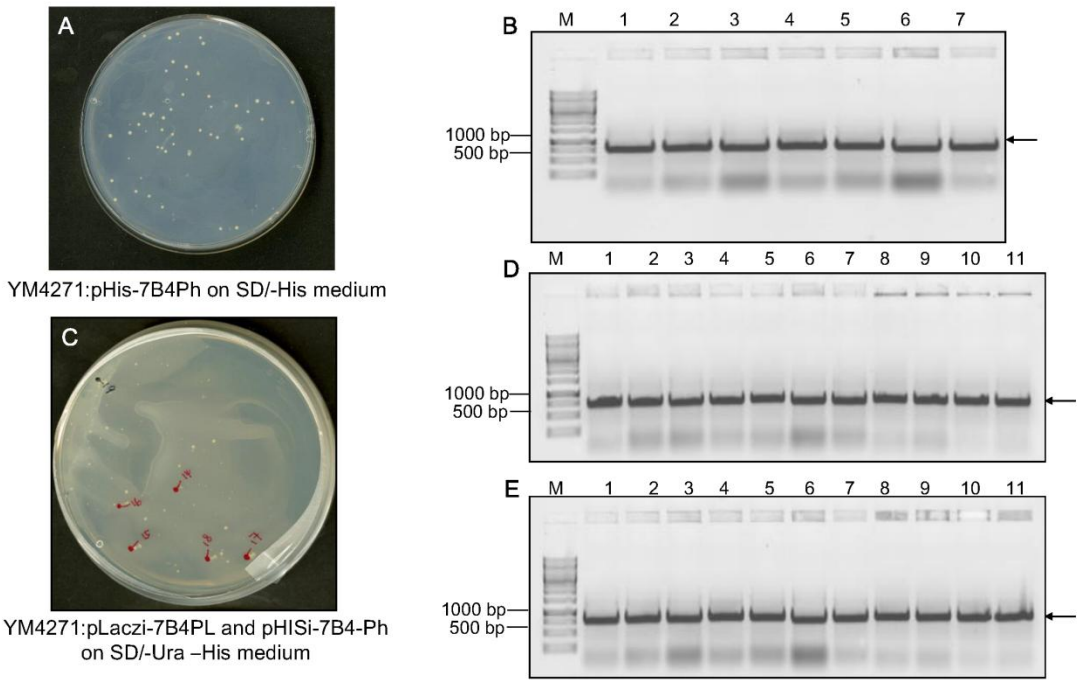


Fig. 31 Transformation of pLaczi-7B4pl and pHis-7B4Ph into yeast YM4271 on a small scale.

(A) Transformation of pHis-7B4ph into YM4271 on a small scale and screened on SD-His selected medium.

(B) Positive clones from SD-His selected medium were confirmed by colony PCR (606 bp).

(C) Transformation of pLaczi-7B4PL into YM4271: pHis-7B4Ph on a small scale and screened on SD-His-Ura selected medium.

(D) Colonies from SD-His-Ura selected medium were confirmed by colony PCR using the primer pHISi_fwd/pHISi_rev (606 bp).

(E) Colonies from SD-His-Ura selected medium were confirmed by colony PCR using the primer pLacZi_fwd/pLacZi_rev (607 bp).

3.3.2.3 Measurements of autoactivation of yeast one-hybrid DNA-bait strains

The autoactivation assay is the method that tests the level of expression of integrated *DNA bait-reporters* lacking an AD-prey clone. Autoactivation strength was measured by two different tests, which measure the expression of two reporter genes (*HIS3*) and *β-galactosidase* on colony lift assays **(Fig. 32)**. *HIS3* gene expression was measured by growing yeast strains on selective media lacking histidine and uracil. The activity of the *HIS3* reporter was quantified by increasing amounts of a competitive inhibitor of HIS3 (3-AT). The concentration of 3-AT was performed from 0 mM to 60 mM. The integrant with the lowest auto-activity concentration of 3-AT that inhibited growth was considered as the activation strength of the gene construct so that transcript factors -DNA interactions can be retested (Doerks, Copley et al. 2002). Fifteen positive yeast colonies were selected that contain the full *ALDH7B4* promoter bait. After incubation at 30 ºC for 5-7 days, the growth of each DNA-bait-reporters strain was scored on the different media **(Fig. 32A)**. Theβ-gal colony lift assays were used to perform the activity test of the *LacZ* reporter gene that grew on the SD/-His-Ura medium at 37 ºC for 0 h-48 h without a competitive inhibitor of HIS3 **(Fig. 32B)**.

According to the activation strength in these two assays for autoactivation, all fifteen strains showed minimal or growth on 15-60 mM concentration of the 3-AT medium. Only strain No. 11 has a strong signal in the β-Gal assay. The

integrated strain, which has the lowest autoactivity for both reporters, was selected to decrease the number of false protein- DNA interactions in the subsequent assays. By comparison of activation strength in both assays strain No. 7 was chosen as the optimally integrated strain, also because it grew well under none competitor conditions and showed no strong signal in the colony lift assays.

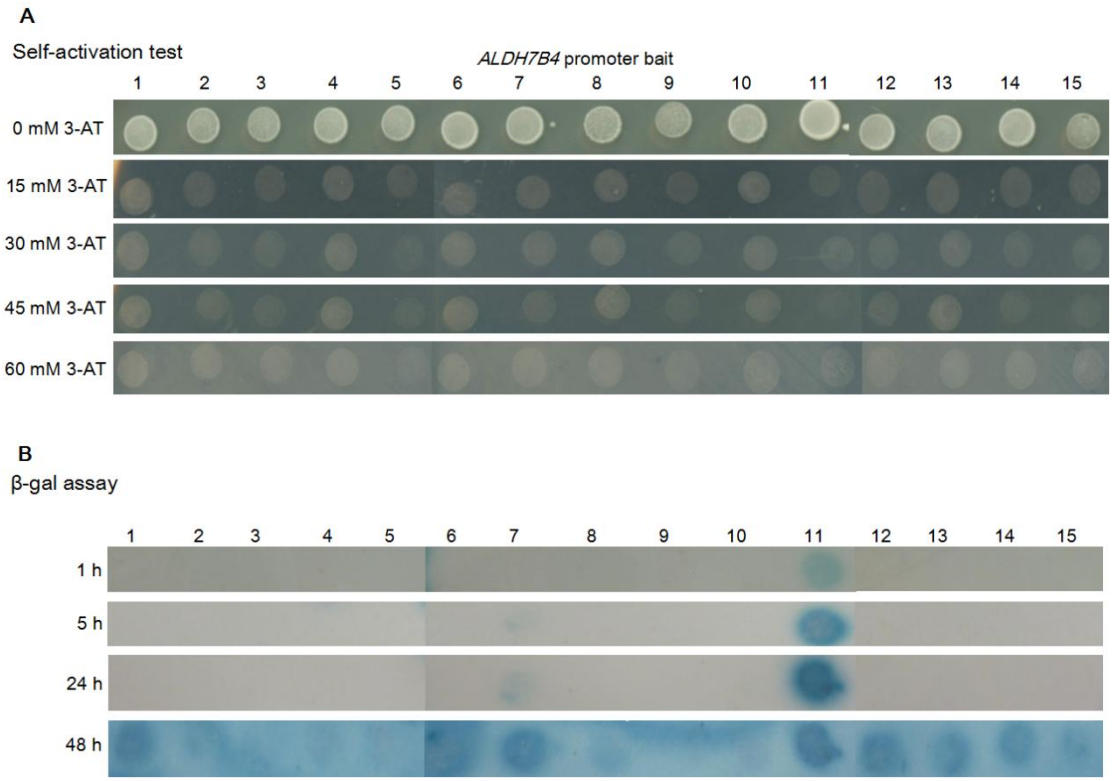


Fig. 32 Activation strength of yeast one-hybrid DNA-bait-reporters constructs.

(A) Autoactivation assay of the *HIS3* reporter gene on SD/-His-Ura media of 3-AT (15 mM-60 mM) after 3 days at 30ºC.

(B) Autoactivation assays of the *LacZ* reporter gene after 1 h, 5 h, 24 h and 48 h incubation at 37ºC.

3.3.2.4 Prey library transformation on a large scale

For the screening of the vector pGAD424 carrying the transcript factors -only prey library of *A. thaliana* transcription factors was used (Mitsuda et al. 2010). This library was introduced into yeast by transformation on a large scale. After the transcriptional autoactivation assay, bait constructs were specially adapted for interaction screens. 'Validated baits No.7' were transformed with 6 µg of the library. 5,000-10,000 independent yeast colonies were subjected to selection on the selected medium after incubating at 30 ºC for 5-7 days. Five hundred of the colonies that grew well on SD/-His-Ura-Leu plates were collected and stored at -80ºC. All the five hundred colonies were then picked and transferred on SD/-His-Ura-Leu+15 mM 3-AT medium and the second round of selection on SD/-His-Ura-Leu+30 mM 3-AT plates to observe their growth conditions before proceeding to colony lift β-galactosidase assays. Each individual colony was identified by a β-Gal colony lift assay and was compared with the results of increasingHIS3inhibitor concentrations **(Fig. 33A)**. The assay of histidine resistance and selection for β-galactosidase activity resulted in 127 positive colonies. The transcript factors in these “double-positive” colonies were PCR-amplified using the primers GAL4AD_For /GAL4AD_Rev and sequenced for identification **(Fig. 33B)**. 68 of these 127 clones were positive with an average size of 1.5 kb.

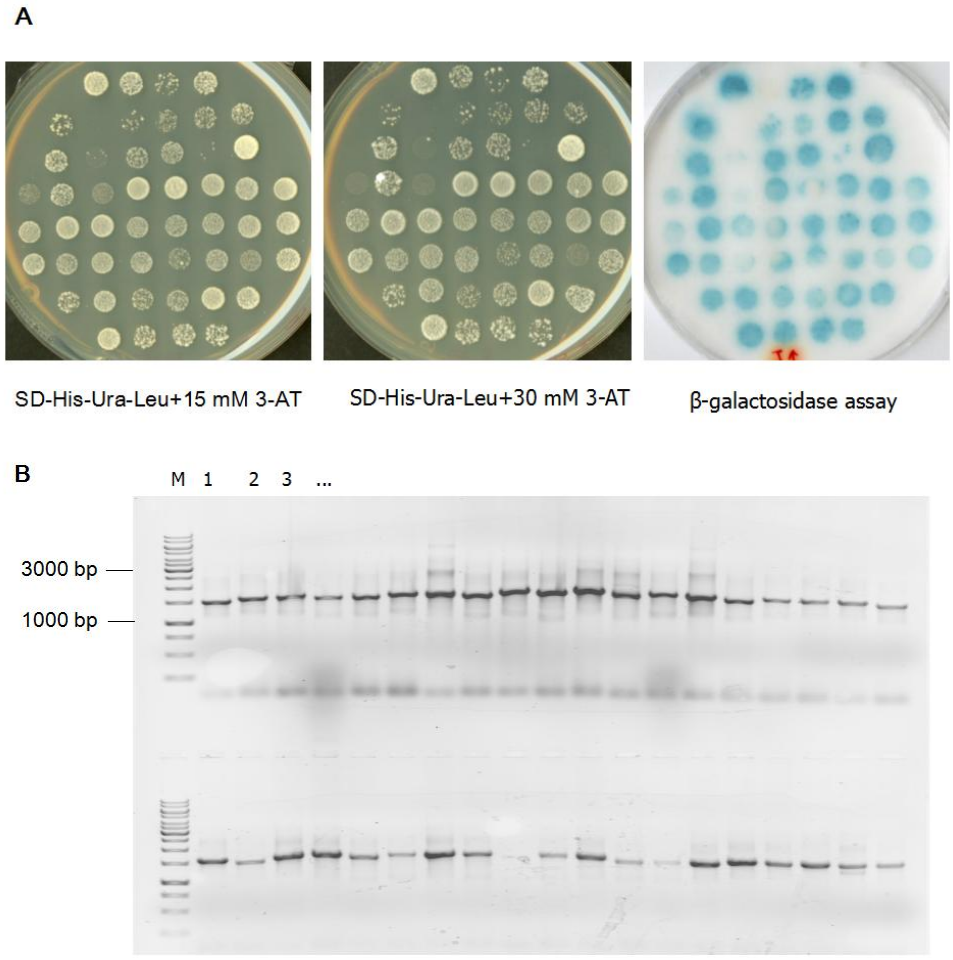


Fig. 33 Yeast one-hybrid library screening.

(A) The growth of yeast strains on SD-His-Ura-Leu+15 mM 3-AT and SD-His-Ura-Leu+30 mM media. The β- Gal assays with these strains after 5 h incubation at 37 ºC.

(B) 127 colonies were confirmed to contain an insert by colony PCR using the primers GAL4AD_For and GAL4AD_Rev. M= marker. 1, 2, 3…= PCR product from individual colonies.

3.3.2.5 Identification of DNA-protein interactors and retesting of interactions by Gap-repair

Based on observations of nutritional selection, the β-galactosidase assays and the result of PCR amplification, sixteen from sixty-eight PCR-amplified

colonies were picked and identified by DNA sequencing **(Table 5)**. The identified transcript factors all belong to NAC superfamily. There were 8 isolates of the NAC domain-containing protein 2 (accession number: AT1G01720) and 4 isolates of the ATAF-like NAC-domain transcription factor 3 (accession number: AT3G15500). Clones encoding the NAC domain-containing protein 102, NAC domain-containing protein 25, NAM-like protein 18 and NAC domain-containing protein 19 were unique isolates. There are reports showing that transcript levels increase in response to wounding and abscisic acid in ANAC002; ANAC102 appears to have a role in mediating response to low oxygen stress (hypoxia) in germinating seedlings and has functions in sequence-specific DNA binding transcription factor activity; ANAC025 involved in multicellular organismal development and regulation of transcription; ANAC018 is homologous to the petunia gene NAM which is required for the development of the shoot; ANAC019 encodes an NAC transcription factor whose expression is induced by drought, high salt, and abscisic acid. It was reported that detailed DNA binding assays of NAC transcription factors (ANAC019, ANAC055, and ANAC072/RD26) determined NARS (NAC recognition sequences) ANNNNNCNNNNNNNACACGCATGT, containing CATGT and harboring CACG as the core DNA-binding site (Tran, Nakashima et al. 2004). CGTG and GTGC are also reported to be the NAC protein binding sites. These sequences motifs are in the *ALDH7B4* promoter **(Table 6).**

Table 5 Isolated transcription factors binding to the *ALDH7B4* promoter.

Isolated TF (by locus No.)	Times isolated	TF family	Description
Full *AtALDH7B4* promoter bait screening			
AT1G01720	8	NAC superfamily	NAC domain-containing protein 2 (ANAC002, ATAF1)
AT3G15500	4	NAC superfamily	ATAF-like NAC-domain transcription factor 3 (NAC055, NAC3)
AT5G63790	1	NAC superfamily	NAC domain-containing protein 102 (NAC102)
AT1G61110	1	NAC superfamily	NAC domain containing protein 25 (NAC025)
AT1G52880	1	NAC superfamily	NAM-like protein 18 (NAC018)
AT1G52890	1	NAC superfamily	NAC domain-containing protein 19 (NAC019)

Table 6 Repetition of recognition sequences of NAC protein binding sites in the *ALDH7B4* promoter.

Putative NAC protein binding sites in the *ALDH7B4* promoter sequence			
Binding sites	CACG	CGTG/A	CATGTG
Repetitions	3	3	1

In order to confirm the interaction of these sixteen interactors with the *ALDH7B4* promoter, Gap-repair assays were performed because it is important to retest each of the interactions identified from the transcript factors-only library screen. Not all the “double-positive” yeast colonies might be the result of prey interacting with the DNA-bait strain, but the autoactivators by bait itself (Walhout and Vidal 2001). The sixteen “double-positive” yeast clones were transferred from a fresh SD/-Ura-His-Leu plates and incubated overnight at 30°C. For each positive candidate, a yeast plasmid miniprep was prepared and transformed into *E. coli DH10B*. The amplified fragments

were re-tested using the primers GAL4AD_For and GAL4AD_Rev **(Fig. 34A)**. All the plasmids were retransformed into the strain *YM4271* carrying the DNA-bait plasmid No. 7 which has the lowest autoactivity for both reporters (details in 3.3.2.3). Plasmids were successfully transformed and after 3-5 days of incubation at 30°C on SD medium containing 30 mM 3AT and lacking histidine, uracil, and leucine, colonies grew on plate medium. These sixteen candidates interact with the DNA-bait and were also confirmed by histidine and the absence of β-galactosidase activity **(Fig. 34B).** All sixteen interactors grew well on 30 mM 3-AT selection medium and the colony lift assay indicated that these sixteen "double-positive" colonies interacted due to the prey protein binding and interaction did not arise by the bait yeast autoactivation.

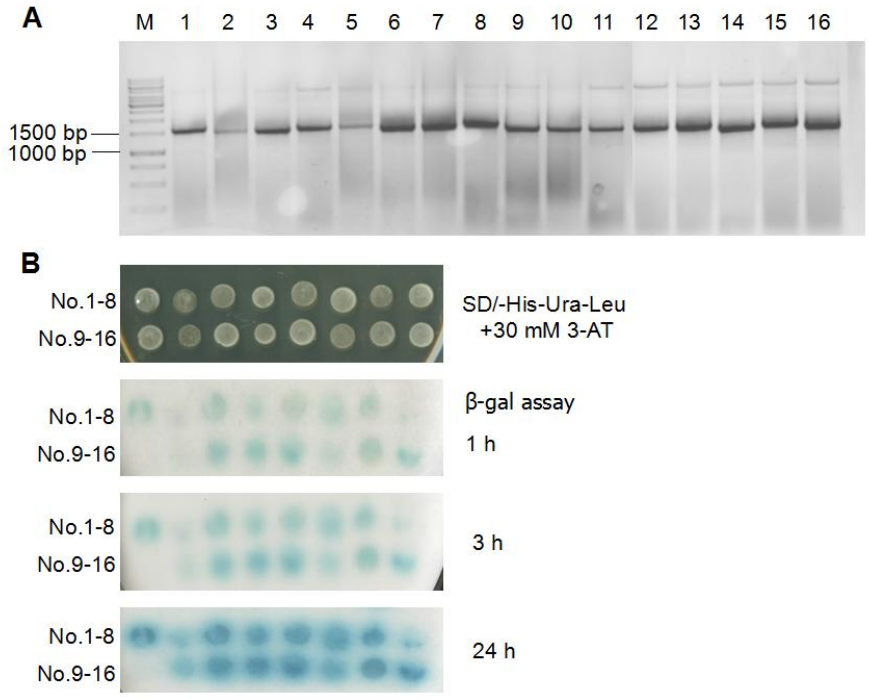


Fig. 34 Re-tested the yeast one-hybrid candidates by Gap-repair assays.

(A) Yeast plasmid miniprep was prepared of sixteen candidates and transformed into *E. coli DH10B*. They were re-tested by amplification using the primers GAL4AD_For and GAL4AD_Rev. M= marker; No.1-16: individual positive candidates.

(B) Confirmed by autoactivation test of *HIS3* and *LacZ* reporter gene. The growth of sixteen candidates which interact with the DNA-bait strain No. 7 on SD-His-Ura-Leu media with 30 mM after 3 d at 30°C to test *HIS3* reporter gene; colony lift assays after incubation for 1 h, 3 h, and 24 h at 30°C to test the *LacZ* reporter gene.

3.4 Study of the yeast one-hybrid transcription factor candidate ATAF1 protein

3.4.1 ATAF1 protein is involved in regulation of *ALDH7B4* and its role in stress tolerance

Based on the results of transcription factors binding assays to the *ALDH7B4* promoter, we noticed that the NAC domain-containing protein 2 (ANAC2/ATAF1) was several times identified independently in the yeast one-hybrid screening. ATAF1 (AT1G01720) is located on chromosome 1 with 3 exons and 2 introns **(Fig. 35)**. It codes for a protein of 289 amino acid residues and has a predicted molecular weight of 32.92 kDa with an isoelectric point of 6.52 **(Fig. 36)**. The NAC domain is subdivided into five subdomains. The overall structure of the NAC domain monomer consists of a very twisted antiparallel beta-sheet, which packs against an N-terminal alpha-helix on one side and one shorter helix on the other side surrounded by a few helical elements. The structure suggests that the NAC domain mediates dimerization through conserved interactions including a salt bridge, and DNA binding through the NAC dimer face rich in positive charges (Puranik et al. 2012). The NAC (NAM/ATAF/CUC) family of proteins is a major group of plant-specific transcription factors involved in plant development, senescence, secondary cell wall formation and stress responses (Olsen et al. 2005; Hu et al. 2006; Nakashima et al. 2012). ATAF1 was one of the first NAC-domain proteins identified in *A. thaliana*. It was reported that expression of the *ATAF1* gene was induced by drought and ABA treatments(Wu et al. 2009). The constructed

ATAF1 also observed using green fluorescent protein (GFP) fusion protein was localized in the nucleus and the C-terminal domain exhibits transcriptional activity in yeast (Lu et al. 2007). Recent studies reveal that ATAF1 integrates ABA- and H_2O_2- dependent signaling with senescence through the direct regulation of key photosynthesis and senescence transcriptional cascades (Garapati et al. 2015a). In *A. thaliana*, the predicted functional partners of ATAF1 protein are shown in **Fig. 37**.

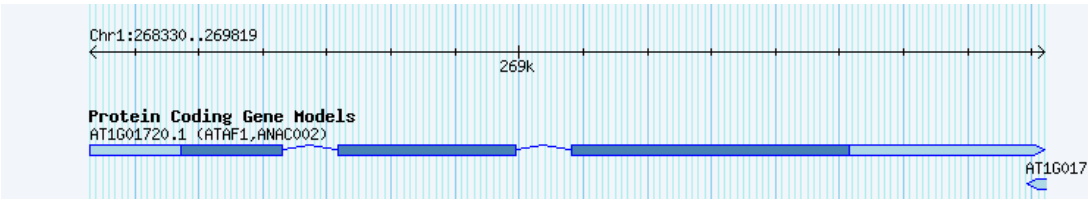


Fig. 35 Coding gene models of *ATAF1* in *A. thaliana* chromosomes. *ATAF1* (AT1G01720) in *A. thaliana* viewed in Gbrowse (https://gbrowse.arabidopsis.org). The *ATAF1* is located on chromosome 1 with three exons (highlighted by three disconnected dark blue bold line) and two introns (two of disconnected thin line).

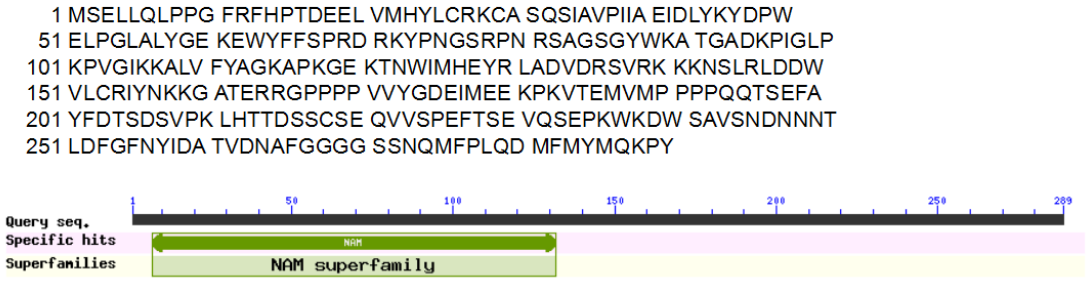


Fig. 36 The protein sequence of ATAF1. ATAF1 has 289 amino acids (top) and a predicted NAM domains (bottom)(accession: PF02365, from +8 to +131) using NCBI protein blast software (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

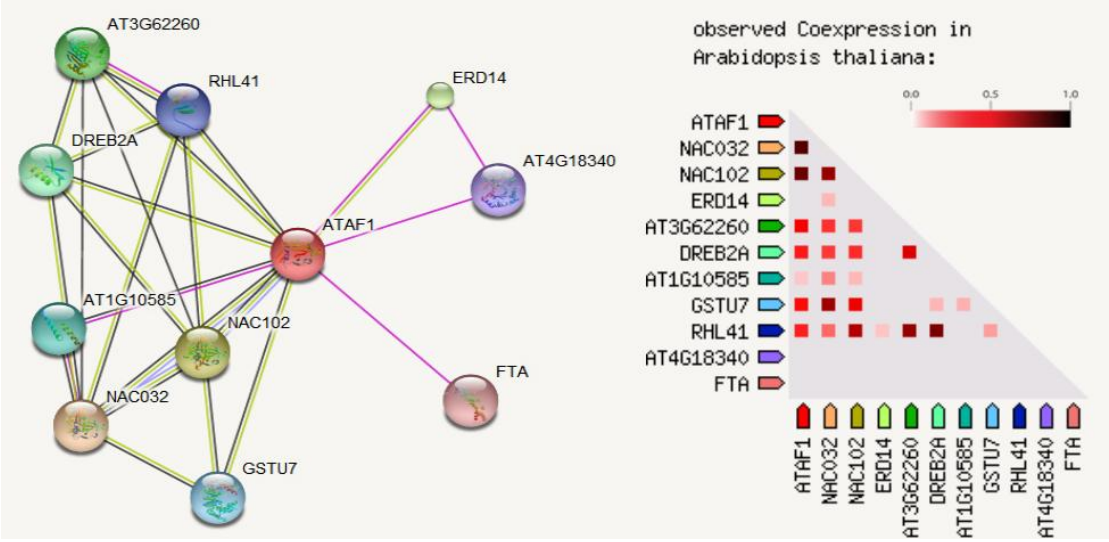


Fig. 37 Predicted functional partners of the ATAF1 protein. The network comes from the string (http://string-db.org), a database of known and predicted protein interactions. Predicted partners are shown (left): NAC domain containing protein 32; AC domain containing protein 102; Dehydrin ERD14 putative protein phosphatase 2C 49; DRE-binding protein 2A; Basic helix-loop-helix domain-containing protein; Glutathione S-transferase tau 7; RESPONSIVE TO HIGH LIGHT 41; Glycosyl hydrolase family 17protein; Farnesyltransferase A. In the triangle-matrices at the right side, the intensity of color indicates the level of confidence that two proteins are functionally associated, given the overall expression data in the organism.

3.4.2 Purification of the recombinant protein ATAF1 to investigate its DNA binding ability

3.4.2.1 ATAF1 protein expression and purification

To further verify the ATAF1 and *ALDH7B4* promoter interactions directly, we used *in vitro* electrophoretic mobility shift assays (EMSA). **Fig. 38** illustrates the flowchart of recombinant protein expressed in *E. coli* BL21 cells. Due to the coding sequence of *ATAF1* has 3 of exons and 2 of introns **(Fig. 35)**, the sequence of the opening reading frame (ORF) (+142 to +1185 bp, 289 aa) of *ATAF1* was amplified from the cDNA of *A. thaliana* wild-type plants with the primers ATAF1_Fwd (BamH1) for pET43 and ATAF1_Rev for pET43 (Xho1). The amplified ORF sequence with restriction enzyme sites was first cloned into

the pJET1.2 cloning vector, retested by colony PCR **(Fig. 39)**. The results showed that the sequence of the pJET1.2 -ATAF1 is in corresponds to the ATAF1 ORF sequence.

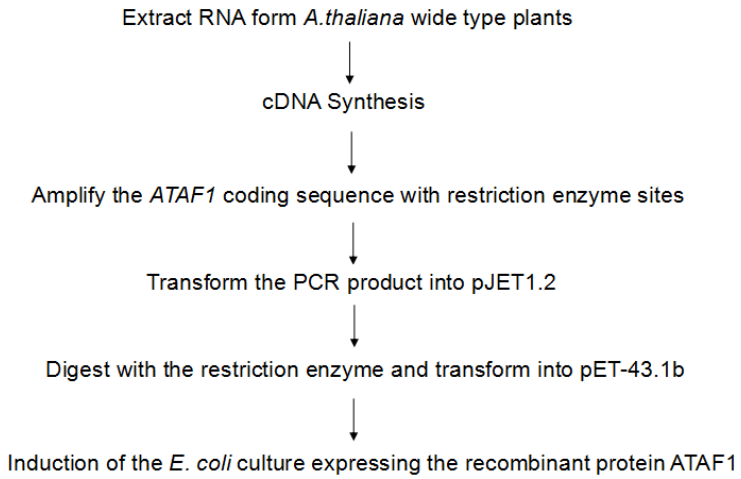


Fig. 38 Flowchart of recombinant protein expressed in *E. coli BL21* cells.

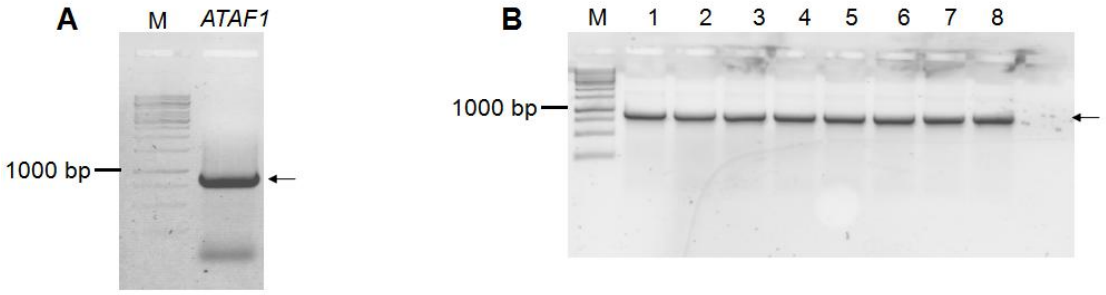


Fig. 39 Amplified the full length of *ATAF1* ORF sequence and re-tested by colony PCR.

(A) The ATAF1 ORF sequence was amplified from cDNA sequence and transform to pJET 1.2 vector.

(B) The 870 bp PCR product was retested by colony PCR using primers ATAF1_Fwd for pet43 and ATAF1_Rev for pet43. M= marker; lane 1 to lane 8=individual clone of pJET1.2-ATAF1 was picked after transformation.

The pJET1.2-ATAF1 was digested with BamH1 and Xho1 and subcloned into the pET43.1b expression vector, which has an N-terminal Nus-Tag fusion partner, and upstream a His-Tag fusion tags with the same restriction enzyme sites to induce the recombinant protein. After restriction enzyme digestion and ligation with an expression vector, pET43.1b- ATAF1 plasmid was confirmed by colony PCR using the primers Colin-DOWN/S-tag and sequencing **(Fig. 40)**, then introduced into *E. coli* strain BL21 for expression. Synthesis of the recombinants ATAF1 protein was induced in LB medium using a final concentration of 1 mM IPTG. The fusion protein was purified by His-tag affinity column under native conditions with 20 mM imidazole. The bacteria were collected by centrifugation and lysed, and then the supernatants and pellets were collected and subjected to SDS-PAGE. The recombinant ATAF1 protein was successfully induced and purified **(Fig. 41)**. The molecular weight of the expression product is 95.7 kDa (ATAF1 protein plus Nus-His Tag), which matches the predicted size. Purified ATAF1 protein was desalted using a PD-10 column and through dialyzed against 14% Glycerin; 15 mM HEPES; 8 mM Tris-HCl, pH 7.5; 120 mM KCl; 0.14 mM EDTA; 7 mM mercaptoethanol and 0.1 mM PMSF.

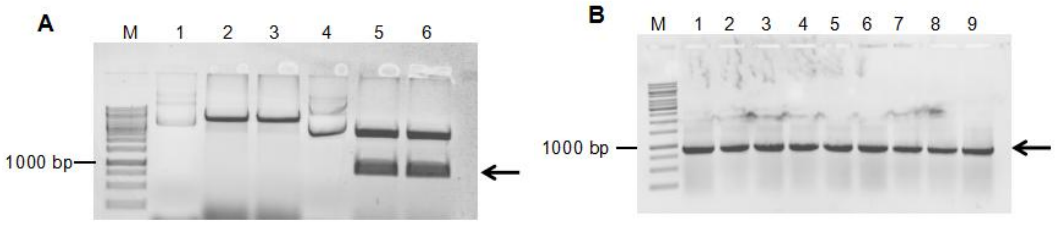


Fig. 40 The pET43.1b and pJET1.2-ATAF1 plasmids digested with restriction enzymes.

(A) Lane 1 and 4 are undigested pET43.1b and pJET1.2-ATAF1 plasmids. Lane 2 and 3 are pET43.1b plasmids (7275 bp), which digested withBamH1/Xhol. Lane 5 and 6 are pJET1.2-ATAF1 plasmids, which digested withBamH1/Xhol. The band indicated by a black arrow at the bottom is ATAF1 ORF sequences (870 bp).

(B) The 870 bp digested product was ligated into the digested pET43.1b plasmid and finally transformed into the *E. coli* strain BL21. Re-tested by colony PCR using primers Colin-DOWN and S-tag. Lane 1 to lane 9=individual clones, clone "pET43.1b -ATAF1" was picked after transformation. Black arrow: positive clones.

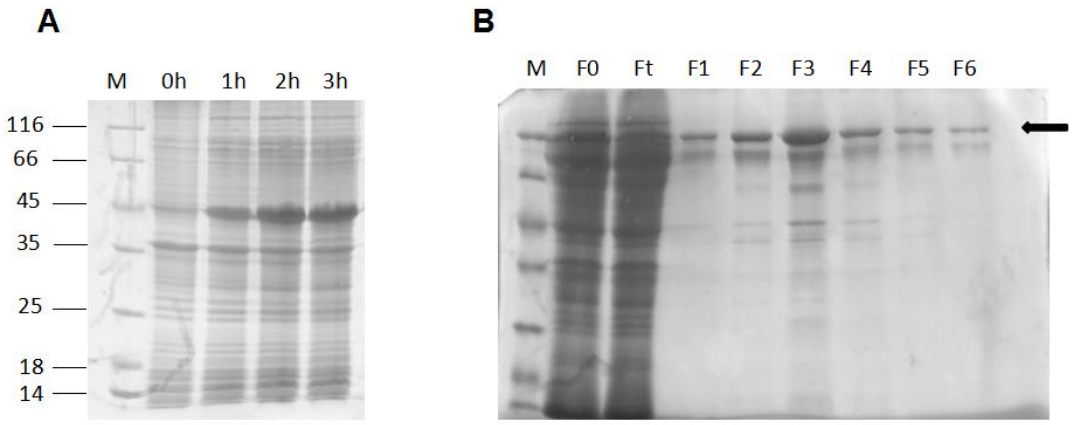


Fig. 41 SDS-PAGE analysis of protein extracts after induction and purification of the ATAF1 recombinant protein.

(A) Induction the recombinant protein with IPTG for 0 h, 1 h, and 3 h. M: Protein molecular weight marker.

(B) The purified ATAF1 recombinant protein. F0: Total soluble fraction; Ft: Flow-through fraction; F1-F6: Eluted fractions. 15 microliters from each fraction were analyzed by SDS-PAGE. Black arrow: purified product (95.7 kDa).

3.4.2.2 Detecting ATAF1 protein–*ALDH7B4* promoter sequence interactions by electrophoretic mobility shift assay (EMSA)

EMSA experiments were performed to determine whether ATAF1 protein could specifically interact with the putative NAC motifs in the sequence of the *ALDH7B4* promoter *in vitro*. In the electrophoretic mobility shift assay, solutions of proteins and nucleic acids are combined and the resulting mixtures are subjected to electrophoresis under native conditions through polyacrylamide or agarose gels. After electrophoresis, distribution of species

containing nucleic acid is determined, usually by autoradiography of ^{32}P-labeled (digoxigenin -11-ddUTP labeled in this study) nucleic acid. In general, protein–nucleic acid complexes migrate more slowly than the corresponding free nucleic acid. Annealed pairs of oligonucleotides designed from the putative NAC protein binding sites (see 3.3.2.5) from *ALDH7B4* promoter were used as probes or competitors in EMSAs. A labeled 39 bp double-stranded oligonucleotide (5'AAGCATAGGACACGTGACACATGTGATGTGAGTGAAGCC-3') containing the consensus CACG, GTGC, and CATGTG motifs are shown in **Fig. 42**.

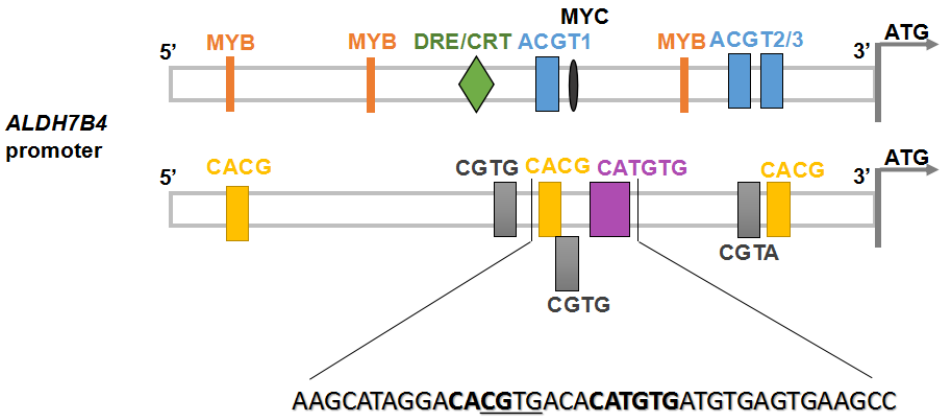


Fig. 42 Oligonucleotides from the *ALDH7B4* promoter for EMSAs. The full length of the *ALDH7B4* promoter has three of CACG motifs, two of CGTG motifs and one of CATGTG motif. A 39 bp of oligonucleotides was used as double-stranded DNA.

The chemiluminescent detection is presented in **Fig. 43**, Specific binding without competition was observed for ATAF1 and the labeled oligonucleotides (lane 2). Cross-competition experiments were carried out using a 50-fold excess of unlabeled oligonucleotides, resulting in weakening of the shifted bands (lane 3). No band was observed in the absence of either protein or oligonucleotides (lane 1). PET43.1b empty vector was used as a negative control (lane 4-6). The result of the EMSAs indicates that the ATAF1

transcription factor can interact with a fragment of the *ALDH7B4* promoter directly.

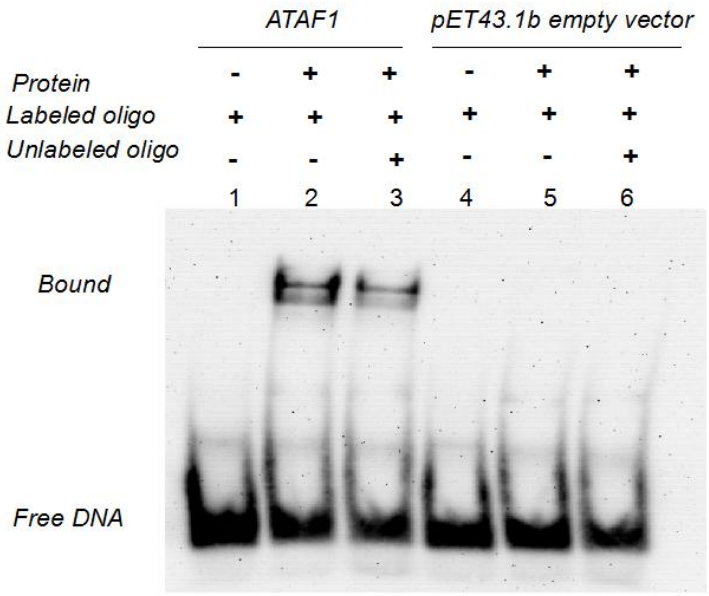


Fig. 43 EMSA analysis of the putative NAC motifs in the *ALDH7B4* promoter. Gel-shift assays were performed using DIG-11-ddUTP-labelled double-stranded probes as described in the materials and methods. Competition experiments were performed with a 50-fold molar excess of unlabeled double-stranded oligonucleotides (lane 3 and lane 6).

3.4.3 ATAF1 functions as a transcriptional activator that regulates the expression of *ALDH7B4*

A previous study had shown that the ATAF1 can specifically bind to 39 bp of oligonucleotides of the *ALDH7B4* promoter containing CACG, CGTG, and CATGTG motif, and therefore I aimed to determine whether the ATAF1 protein is able to mediate ALDH7B4 gene expression *in vivo*. Transactivation assays (FAST assay) were performed using ten-day-old of *A. thaliana* wild-type seedlings. The control reporter construct contains the ALDH7B4 promoter-GUS cassette in the binary vector pBIN19 and a GUS reporter gene. The effector plasmid consisted of double 35S promoter of Cauliflower mosaic

virus (CaMV) fused to cDNA fragments encoding *ATAF1*. Seedlings were cotransfected with “control reporter” and also “effector/ reporter” that contained the *GUS* reporter gene, the full-length of the *ALDH7B4* promoter sequence (control reporter) and a“35S_ATAF1” effector plasmid **(Fig. 44)**.

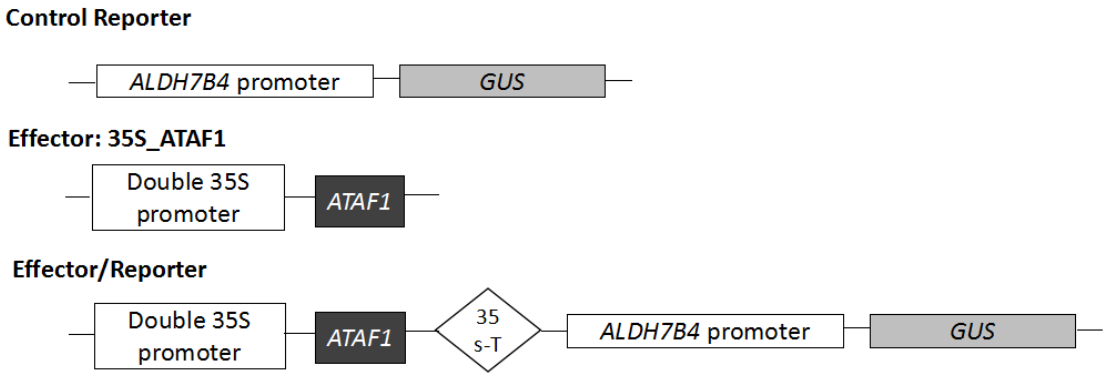


Fig. 44 Schematic illustration of the constructs for the FAST assay. The sequence of the ALDH7B4 promoter with GUS reporter gene as control reporter. cDNA fragments encoding *ATAF1* with double 35S promoter (Effector) were cloned into the plant expression vector pBIN19with the reporter gene plasmids (Effector/Reporter).

The full sequence of ATAF1 ORF (882 bp) construct was first amplified using primers ATAF1 Fwd-pGJ280 and ATAF1 Rev-pGJ280 with the restriction enzymes sites XbaI and NcoI from the cDNA of *A. thaliana* plants into the pJET1.2 cloning vector and re-tested by colony PCR **(Fig. 45AB)**. The pJET1.2-ATAF1 was partially digested with XbaI/NcoI (pJET1.2 has one XbaI restriction site), the fragment of 882 bp band was separated on a 1.5% agarose gel extracted and purified **(Fig. 45CD)**. The ATAF1 fragment with XbaI/NcoI sites was then cloned into the pGJ280 that was digested with the same restriction enzyme, which contains the double CaMV 35S promoter. The CaMV 35S_ATAF1_teminator cassette was subsequently isolated from the pGJ280 vector digested with HindIII site and cloned into the binary vector pBIN19 (35S_ ATAF1) and confirmed by colony PCR **(Fig. 45EF)**. Finally, the

ALDH7B4-promoter cassette was isolated from the pBT10-GUS vector digested with BamHI/BglII and cloned into BamHI digested pBIN19-35S_ATAF1 plasmid.

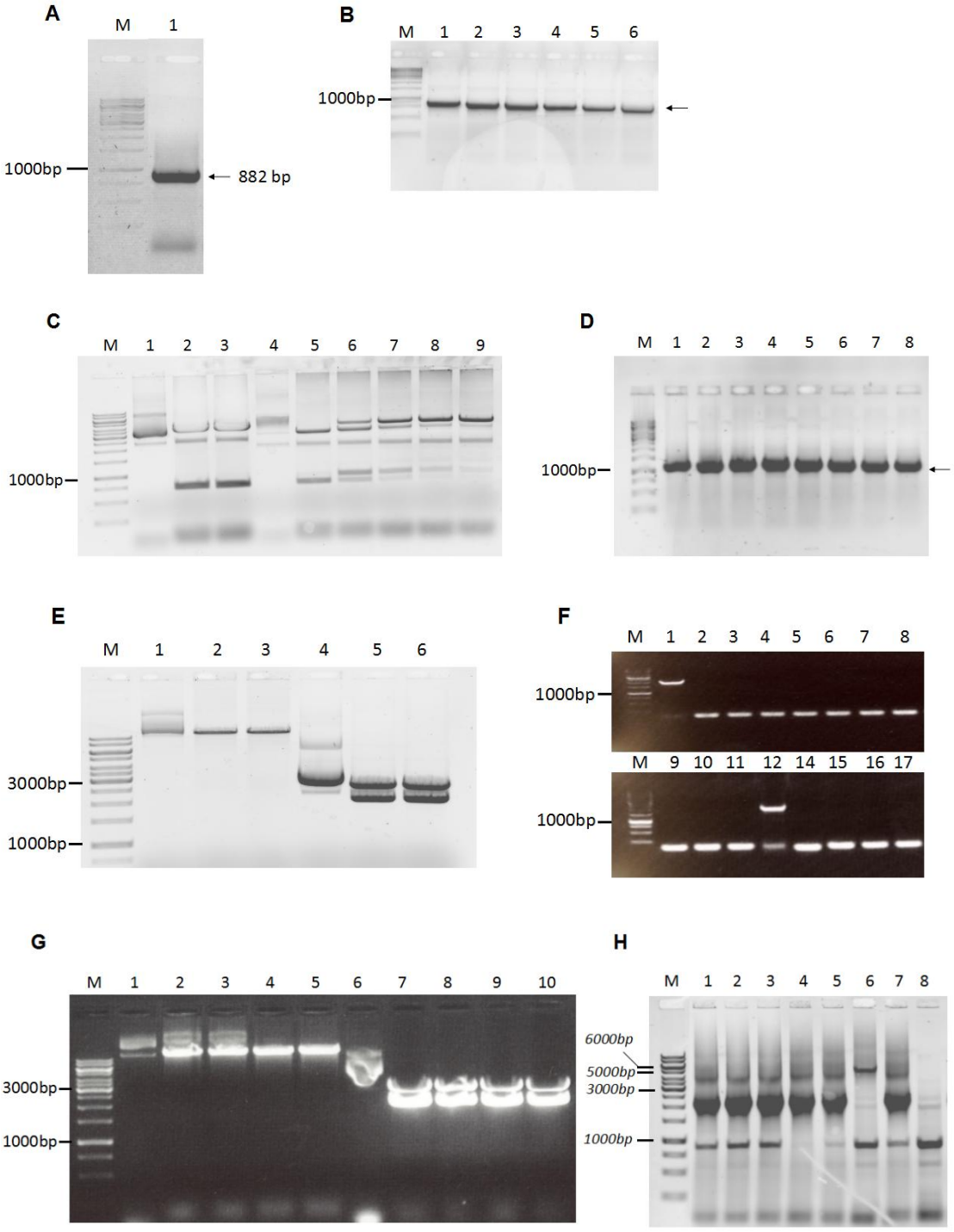


Fig. 45 Generating the constructs for FAST transient expression assays.

(A) Amplified cDNA fragment encoding *ATAF1* (lane 1: 882 bp).

(B) The 882 bp PCR product transformed into pJET1.2 was retest by colony PCR using primers ATAF1 Fwd-pGJ280 and ATAF1 Rev-pGJ280. Lane 1 to lane 6: the individual clone of pJET1.2-ATAF1.

(C) Double digest the vector pGJ280 and partial digested the pJET1.2-ATAF1 with XbaI/NcoI. Lane 1 and lane 4 are undigested pGJ280 and pJET1.2-ATAF1; lane2 and lane3 are double digested pGJ280 (the upper band are extractions from the gel and purified for ligation), lanes 5-9 are partially digested with XbaI/NcoI. The 882 bp fragment was separated by different concentration of enzymes using partial digestion method (described in material and method 2.2.3.2).

(D) Partially digested 882 bp was cloned into double digested pGJ280 vector and confirmed by colony PCR using primers p35S-pROK2-SA and ATAF1 Rev-pGJ280, Lane 1 to lane 8: individual clones of pGJ280-ATAF1. The size of the PCR product is 1110 bp.

(E) Digest the binary vector pBIN19 and pGJ280-ATAF1 construct with HindIII. Lane 1 and lane 4 are undigested pBIN19 and pGJ280-ATAF1, lane 2 and lane 3 are digested pBIN19, lane 5 and lane 6 are digested pGJ280-ATAF1 (the upper band are extraction from the gel and purified for ligation).

(F) Digested pGJ280-ATAF1 with double 35S promoter-ATAF1 cloned into pBIN19, retested by colony PCR using the primers pBIN19_EcoRI and pBIN19_HindIII. Lane 1 to lane 17: the individual clone of pGJ280-ATAF1. Lane 1 and lane 12 are positive clones. The size of the PCR product is 2154 bp.

(G) Construct pBIN19-35S-ATAF1 was digested with BamHI (lane 2-5) and the pBT10-GUS-7B4 promoter construct was digested with BamHI and Bglll (lane 7-10, upper band was isolated from the gel and purified). Lane 1 and lane 6 are undigested pBIN19-35S-ATAF1 and pBT10-GUS-7B4 promoter.

(H) Colony PCR was performed using the primers pBIN19_EcoRI and pBIN19_HindIII. Lane 1 to lane 8: individual clones after transformation. The size of the PCR product is 4845 bp.

Expression of each of the “Control reporter” or “Effector/Reporter” constructs in seedlings significantly transactivated the expression of the GUS reporter gene **(Fig. 46A).** When seedlings were cotransfected with a GUS reporter gene

fused to a full length of the *ALDH7B4* promoter sequence and the ATAF1 effector plasmid, the degree of induction of the GUS reporter gene was significantly higher. Lower GUS activity in "Control reporter" transgenic seedlings was observed and further quantified by measured as GUS enzymatic activities **(Fig. 46B)**. These results demonstrate that ATAF1 indeed functions as transcriptional activator involved in the expression of *ALDH7B4* in *Arabidopsis*.

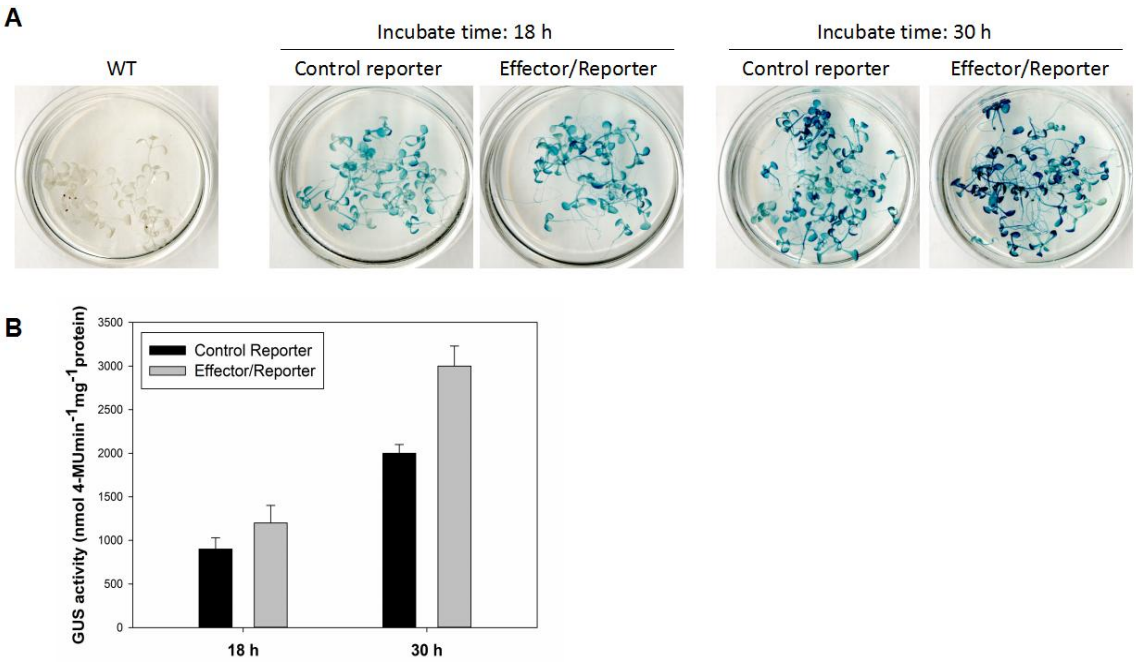


Fig. 46 Transactivation potential of selected ATAF1 transcription factors.

(A) GUS reporter protein expression from "control reporter" construct and "effector/reporter" construct in transformed *A. thaliana* seedling observed by GUS-staining assay, wild-type seedlings as the negative control.

(B) GUS protein accumulation from "control reporter" and "effector/reporter" constructs measured as enzymatic activities of the GUS reporter protein.

To further investigate whether ATAF1 can up-regulate the expression of *ALDH7B4*, transgenic *A. thaliana* plants overexpressing ATAF1 (ATAF1-OX1 and ATAF1-OX2) and T-DNA knock-out mutant lines of ATAF1 were used for RT-PCR and protein blot of *ALDH7B4* expression analysis (*ataf1-1*:

SALK_64806 and *ataf1-2*: SALK_057618 were obtained from Dr. Xie Qi (Wu et al. 2009). To confirm the overexpression and T-DNA mutant lines of *ATAF1*, the seeds of transgenic lines were germinated on selection medium for further analysis **(Fig. 47AB)**. Basta-resistant (overexpression lines) and kanamycin-resistant (T-DNA mutant lines) seedlings were transferred to soil pots after two weeks and further screened by PCR for the presence of the transgene. Two T-DNA insertion lines showed a weak band using LP and RP primer, which are*ATAF1* gene specific primers **(Fig. 47C)**. Transgenic plants were further analyzed by RT-PCR using *ATAF1* specific primers to discriminate the expression in wild-type and transgenic plants. Moreover, transcript accumulation of endogenous *ALDH7B4* was compared between wild-type and ATAF1-overexpression and T-DNA insertion mutant plants **(Fig. 47D)**. Overexpression of ATAF1 caused a statistical increase of the transcript accumulation of the endogenous *ALDH7B4* when compared with wild-type and ATAF1 knock-out plants **(Fig. 48A)**. In addition, our previous studies revealed that the *ALDH7B4* gene is abundantly expressed in embryos of seeds (Kirch et al. 2005; Stiti et al. 2011). Therefore we also investigated whether ATAF1 is involved in up-regulation of *ALDH7B4* during early embryos stages (in seeds and seedlings) using protein blot analysis. Consistent with the regulation pattern of mature plants, the protein level of ALDH7B4 was higher in ATAF1-OX and lower in *ataf1* than in wild-type plants **(Fig. 48B)**. These results suggest that the *ALDH7B4* promoter is activated by ATAF1.

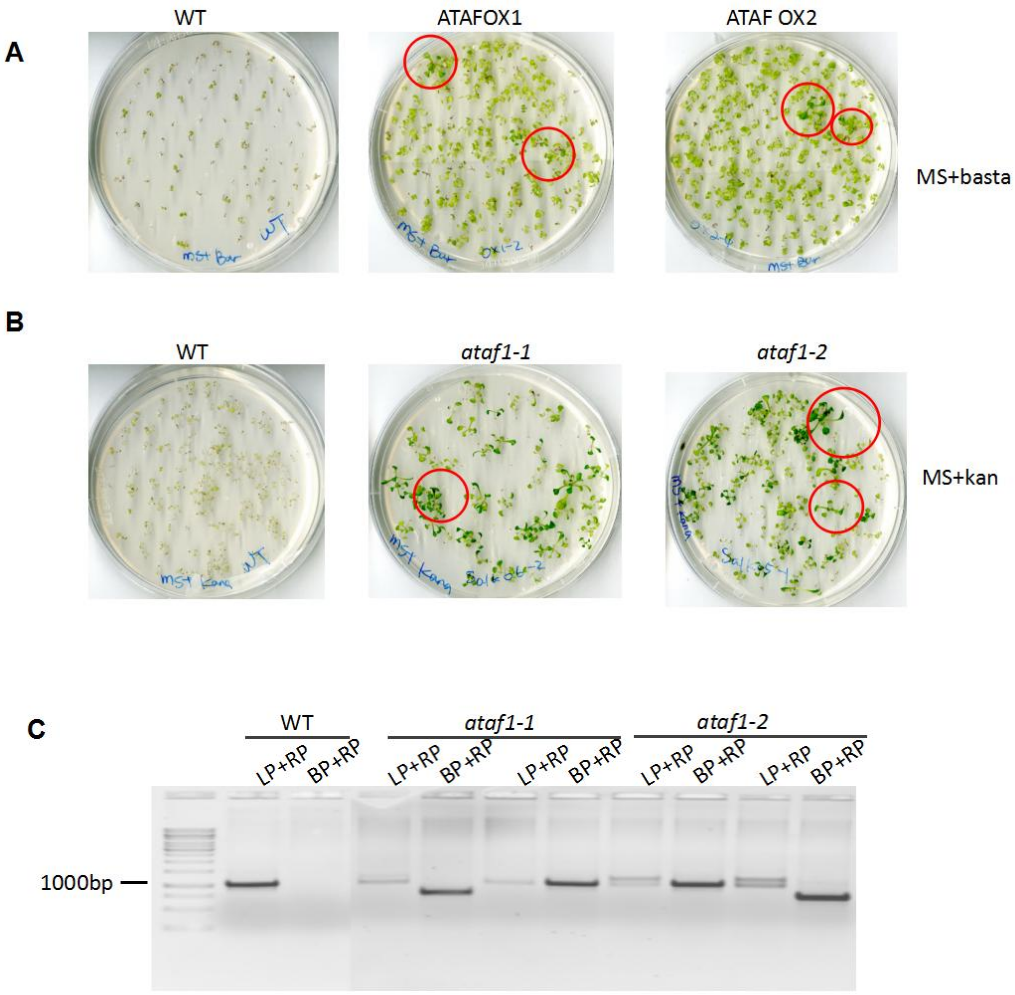


Fig. 47 Confirmation of transgenic lines carrying overexpression constructs of *ATAF1* or deletions of ATAF1 and expression of *ALDH7B4*.

(A) Photograph of wild-type, ATAF1T-OX1 and ATAF1-OX2 lines grown in a growth chamber with short day light conditions on MS medium containing 8 mg/l Basta. Selection of putative ATAF1 over- expressors as circled in red. Pictures were taken 14 days after germination.

(B) Photograph of wild-type, T- DNA insertion line *ataf1-1* and *ataf1-2* MS medium with 50 mg/l kanamycin. Selection of putative ATAF1 T-DNA insertion mutants as circled in red. Pictures were taken 14 days after germination.

(C) Genotyping of T- DNA insertion line *ataf1-1* and *ataf1-2.* Genomic DNA isolated from both wild-type (*Col-0*) and independent mutants. The complete coding sequence of *ATAF1* was amplified using gene specific primers ATAF1_fwd (LP) and ATAF1_rev

(RP); gene/T-DNA junction was amplified using T-DNA primer LBa3 and gene specific primer ATAF1_rev (RP).

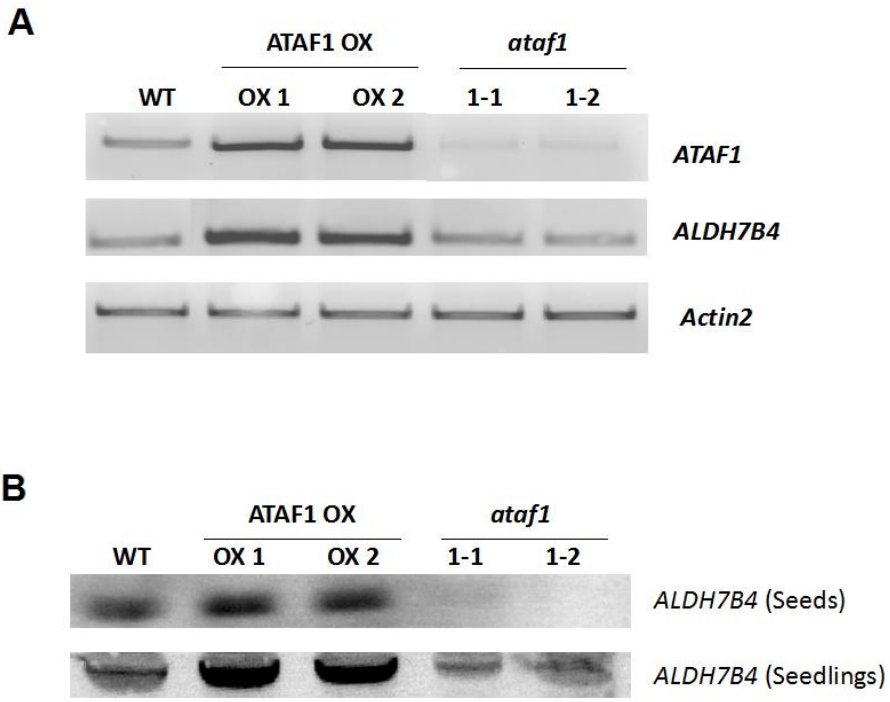


Fig. 48 Endogenous ALDH7B4 expression in wild-type (WT) and independent ATAF1-overexpression (OX1 and OX2) and ataf1 (1-1 and 1-2) *A. thaliana* plants.

(A) Endogenous *ALDH7B4* gene expression in four-week-old wild-type, independent ATAF1 overexpression, and *ataf1 A. thaliana* plants. Reverse transcription PCR analysis was performed with 25 cycles for *actin2*, *ATAF1,* and *ALDH7B4* amplification.

(B) Endogenous ALDH7B4 protein expression in seeds and two-week-old seedlings in wild-type, independent ATAF1-overexpression, and *ataf1 A. thaliana* lines.

4. DISCUSSION

4.1 Expression profiles of the *A. thaliana ALDH* genes in response to heat stress

In this study, we analyzed the function of selected *ALDH* genes in *A. thaliana* plants subjected to heat stress either alone or in combination with dehydration, salinity, and wounding stresses. By disrupting the stability of various proteins, membranes, and cytoskeleton, heat negatively affects several processes in plants including germination, growth, development, and reproduction (Bita and Gerats 2013; Weis and Berry 1988). Our analysis of the expression of selected ALDH genes in response to heat stress indicated that ALDH genes play a crucial role in protecting plants from high temperature damage. The ALDH genes were shown to be involved in the aldehyde-detoxification and ROS-elimination processes. Previous studies showed that the expression of selected *ALDH* genes was induced in response to various abiotic stresses, such as dehydration, salinity (NaCl), heavy metals (Cu^{2+} and Cd^{2+}), oxidative stress (H_2O_2) and ABA treatment (Kirch et al. 2001). Overexpression of *ALDH* genes improved stress tolerance and knock-out of ALDH lines showed increased sensitivity to stress treatment in *A. thaliana* (Sunkar et al. 2003; Kotchoni et al. 2006). In this study, we found that the expression of *ALDH* genes, particularly *ALDH7B4*, was strongly induced by heat stress at two different growth stages **(Fig. 15)**. The ALDH3I1 expression was weakly induced by high temperature in mature plants. Although the expression of ALDH3H1 and ALDH3F1 were weak during heat treatment, their transcripts accumulated during recovery. In Antarctic microalga, an increase in total ALDH activity occurred following heat exposure. They suggest that the ALDH 5 and ALDH 6 isozymes have important roles in heat tolerance (Choi and Lee 2012). Hibino et al. (2001) showed the high stability of the mangrove BADHs

(betaine aldehyde dehydrogenase) at high temperature(Hibino et al. 2001). In our study, two potential BADH-encoding genes in *A. thaliana*, ALDH10A8 and ALDH10A9 are constitutively expressed and accumulated at a high level during recovery **(Fig. 15)** suggesting they also may function as protectants in response to heat stress, although naturally, *A. thaliana* do not synthesize glycine betaine under stress conditions (Quan et al. 2004; Wahid and Close 2007; Missihoun et al. 2015). The increased expression of ALDH genes during heat stress and during recovery suggest that these genes may play important roles in heat stress response in plants.

4.2 Knock-out mutants of *ALDH3* and *ALDH7* are heat sensitive

Heat stress often decreases root growth, the number of roots and root diameter (Khurana et al. 2013). In potato, heat stress causes a dramatic change in assimilating allocation between shoot and roots, reduced starch content and reduced dormancy (Hancock et al. 2014). Tian et al. (2009) reported that in heat-adapted thermal *Agrostis scabra*, the architecture changes and hypocotyls and petioles elongate resembling the morphological responses of high temperature (Tian et al. 2009).To evaluate the importance of *ALDH* genes in the plant tolerance to heat, two mutants of aldehyde dehydrogenase (*KO6/62* and *KO6/76*) seedlings were examined for their survival rate and root growth under heat stress. Similarly to the observation by Tian et al. (2009), the two ALDH mutants showed heat-defective phenotypes, lower survival rates, and slightly shorter root length were observed under different heat stress treatment **(Fig. 17 and 18)**. These findings suggest that ALDH3I1, ALDH3H1, and ALDH7B4 are involved in protecting the plants during the early growth period from heat damage. Like other abiotic stresses, heat stress results in the production of ROS and triggers oxidative stress responses (Potters et al. 2007). ROS causes damage to a wide range of

cellular components such as the photosynthetic apparatus or mitochondrial and chloroplast electron transport chains (Xu et al. 2006). The heat stress increases leaf temperature, reduces the antioxidant enzyme activities, and ultimately increases MDA contents in leaves of rice and wheat plants (Rahman et al. 2009; Savicka 2010). Hence, membrane lipid saturation is considered as an important element in high temperature tolerance. Bouche et al. (2003) revealed T-DNA mutants of *SSADH1* (*ALDH5F1*) in *A. thaliana* are dwarf plants and display an enhanced sensitivity to heat stress which is corrected with a rapid increase in the levels of hydrogen peroxide, suggesting that this gene restricts levels of ROS intermediates in plant defense against environmental stress. In our study, a significantly higher MDA level was found in the *ALDH* mutants than in wild-type plants under heat stress in both seedlings and adult plants **(Fig. 19)**. This result on the lipid peroxidation reveals that ALDH3I1, ALDH3H1, and ALDH7B4 contribute to the detoxification of ROS during heat-induced oxidative stress. This is in agreement with the previous observations on the *ALDH3I1* and the *ALDH7B4* single knock-out mutants subjected to salinity and dehydration stresses (Kotchoni et al. 2006).

Photosynthesis is one of the most heat sensitive physiological processes in plants. Photosystem II (PSII) activity is greatly reduced and even stops under heat stress (Morales et al. 2003), and the ability of the plant to maintain leaf gas exchange and CO_2 assimilation rates under heat stress directly correlates with heat tolerance (Yang et al. 2006). Heat-treated leaves of ALDH mutant lines exhibit lower CO_2 assimilation, reduced efficient quantum yield, lower NPQ activation and a significant reduction in Fv/Fm when compared to *A. thaliana* wild-type plants**(Fig. 21)**. Similar observations were made in soybean, rice, tobacco and oak leaves, where heat stress significantly decreased total chlorophyll content, chlorophyll a content, chlorophyll a/b ratio, and Fv/Fm ratio

(Hurkman et al. 2009; Suwa et al. 2010; Djanaguiraman et al. 2011; Tan et al. 2011). This indicated that the protective role of ALDH3I1, ALDH3H1 and ALDH7B4 is required to maintain membrane fluidity and to support leaf gas exchanges and photosynthesis. The ALDHs may function by reducing lipid peroxidation of chloroplasts and thylakoid membranes under high temperature conditions.

Germination is the plant growth stage mostly affected by heat. In *A. thaliana* seed, reduced germination and plant emergence, abnormal seedlings, poor seedling vigor, reduced radicle and plumule growth of germinated seedlings are major impacts caused by heat stress (Toh et al. 2008). In wheat and tomato, the rate of germination and seedling establishment were strictly prohibited under heat stress (Cheng et al. 2009b). We found that seeds of the ALDH mutants exposed to high temperatures had lower germination rate than the wild-type seeds. In parallel, higher amounts of MDA accumulated in both mutant lines than in the wild-type plants **(Fig. 22)**. Altogether, the results of this study demonstrated that *ALDH* genes contribute to the survival of plants at high temperature.

4.3 Basal versus Acquired Thermotolerance

All the organisms have an inherent ability to survive exposure to temperatures above the optimal growth temperature (basal thermotolerance). Acquired thermotolerance is induced by a short acclimation period at moderately high (but survivable) temperatures to heat stress (Hong and Vierling 2000).In this study, *A. thaliana* wild-type plants showed a less defective phenotype in the acquired (Ac) treatment than in the basal thermotolerance (Ba) treatment when assayed as ten-day-old seedlings **(Fig. 12)** but not at later growth stages **(Fig. 13)**.These results are consistent with observations from Massie et al. (2003)

and Larkindale et al. (2005) indicating pretreatment with moderate temperatures allows plants to acquire thermotolerance to withstand supra-optimal temperatures. Expression analysis of *ALDH* genes displayed increased levels in response to both Ba and Ac in wild-type plants. The expression of *ALDH* genes was lower after Ac than in Ba **(Fig. 15)**. Analysis of survival rates, root length and chlorophyll content in ALDH double mutants showed a stronger decrease in response to Ba than to Ac **(Fig. 17, 18 and 20)**. Higher MDA levels after Ba than Ac was also observed **(Fig. 22)**. These results suggested that the ALDH mutants are more impaired in Ba than in Ac. Previous studies suggested that different pathways could be involved in Ba and Ac (Larkindale and Knight 2002; Larkindale et al. 2005).Larkindale et al. (2005) indicated that oxidative damage is a major component in heat damage under Ba conditions. Analysis of mutants with defects in genes relating to antioxidant metabolism (*vtc1*, *vtc2*, *npq1*, and *cad2*) showed more sensitivity after Ba than after Ac in seven-day-old seedlings, whereas genes related to ABA signaling appears to be more critical for Ac treatment. Previous studies from our lab have indicated that *ALDH* genes play a crucial role in antioxidant systems of plants exposed to abiotic stress conditions (Kotchoni et al. 2006). Therefore, ALDHs act as the antioxidant is more likely to be critical in Ba, indicating that *ALDH* mutants are defective in some pathways that are essential in Ba.

4.4 ALDH7B4 contribute to a combination of dehydration, salt and/or heat stress tolerance

In the field, plants are often exposed to more than one stress parameters. For this reason, there is a need to understand the nature of responses to multiple stresses (Suzuki et al. 2014). The response of plants to a combination of different stresses is difficult to predict the response of plants to each of the different stresses applied individually (Mittler 2006). Photosynthesis was

shown to be sensitive to drought or heat stress (Chaves et al. 2009). Carmo-Silva et al. (2012) and Demirevska et al. (2010) revealed that in cotton and tobacco plants, the photosynthetic rate increased under heat stress and a combination of drought and heat stress, with a more severe impact on the combined stress condition. In our study, we analyzed *ALDH* genes in response to multiple stress situations including heat stress combined with drought, salinity or wounding in *A. thaliana* plants. Heat stress alone had a detrimental effect on *A. thaliana* plants **(Fig. 12 and 13)**, but as shown in **Fig. 23B**, a combination of stress treatments showed a more severe growth defects including yellowed and dried-up of leaf margin than did an individual stress. In *A. thaliana*, during heat stress, plants increase their stomatal conductance in order to cool their leaves by transpiration (Vile et al. 2012). However, if the heat stress occurred simultaneously with drought, plants would not be able to open their stomata and their leaf temperature would increase by 2–5°C (Rizhsky et al. 2002b; Rizhsky et al. 2004). This may explain the effects of the multiple stresses on the growth of the *ALDH* mutants.

To better understand how the *ALDH* genes are regulated under multiple stresses, we have analyzed their expression patterns. We found that heat combined with dehydration resulted in an increased ALDH7B4 expression more than in the case of a single stress **(Fig. 23 C and D)**. Consistently, transcriptome analysis of *A. thaliana* plants subjected to a combination of heat and drought found the transcript of *ALDH7B4* elevated more than fourfold (Rizhsky et al. 2004). Our observation about the expression of the *ALDH7B4* gene was therefore supported by the conclusion that the combined effects of heat and drought were generally additive (Suzuki et al. 2014). In parallel with the gene expression data, we found that the double knock-out mutants accumulate more MDA than wild-type plants under H/D and D/H **(Fig. 24).** However, no significant effect was seen on the chlorophyll content in *A.*

thaliana plants under H/D and D/H compared with individual stresses **(Fig. 25)**. In fact, the antioxidant enzymes play an important role in the response of plants to a combination of drought and heat stress. A null mutant of cytosolic ascorbate peroxidase 1 (APX1) was more sensitive to this stress combination than wild-type plants (Koussevitzky et al. 2008). We noted that the order in which the stresses were applied also matters. We observed that the expression of ALDH genes was higher after D/H than H/D, and consistently, the plants had more severe symptoms in the D/H assay than in the H/D assay (**Fig. 23B**). This suggests that dehydration alone may cause more damage to the plant than may do heat and that the ALDH regulon in the dehydration response overlap but supersedes that of the heat response.

Suzuki et al, (2016) pointed out *A. thaliana* plants were more susceptible to the combination of salt and heat stress than to each of the different stresses applied individually. Based on our transcript and protein analysis, ALDH7B4, ALDH3I1, and ALDH3H1 can be induced by either heat or salinity stress. An increased expression of ALDH7B4 was observed under H/S stress than single stress **(Fig. 23C and D)**. Moreover, H/S can accumulate more ALDH7B4 than S/H. a significant elevation of MDA was detected during H/S. Higher accumulation of MDA in mutant plants than wild-type plants **(Fig. 24 and 25)**. My results are consistent with those by Suzuki (2016). In contrast to drought, salinity and heat, wounding stress imposes a mechanical damage to the plant by enabling thereby a pathogen invasion. We found that the phenotype caused by a combination of heat and wounding stress *A. thaliana* plants was more severe than that observed for either individual stress treatment **(Fig. 23B)**. Abiotic stressors were shown to weaken the defense mechanisms of plants (Mittler and Blumwald 2010; Atkinson and Urwin 2012), which suggests that the regulation of *ALDH* genes by heat stress may overlap with that of drought, salinity, wounding, and pathogens. Indeed, the analysis of the *ALDH7B4*

promoter activation indicated that *ALDH7B4* could be induced by pathogen and herbivore attacks as well as by drought and high salinity (Missihoun et al. 2014). *ALDH7* found induced by the fungus in rice (Wu et al. 2007). When wounding and heat stress was applied in combination, besides ALDH7B4, other ALDHs were not induced in response to both H/W and W/H, although they were highly regulated by wounding alone. Moreover, the ALDH7B4 during W/H shown higher expression than H/W or H but lower than W at the protein level. The ALDH3I1 also showed higher expression after W/H than H/W **(Fig. 23D)**. Similarly, Prasch and Sonnewald (2013) showed that virus-treated plants displayed enhanced expression of defense genes, which was abolished in plants additionally subjected to heat and drought stress. Suzuki et al. (2014) suggested that after stress treatment Ca^{2+} and ROS are produced, independently of biotic and abiotic stress applications and ABA seems to be centrally positioned between ROS and SA signaling. However, most of the interactions between ROS, ABA and SA are antagonistic. Wounded leaves of *A. thaliana* produce reactive oxygen species (ROS) within minutes (Beneloujaephajri et al. 2013). In our study, we found that the plants accumulated more MDA contents during W/H than single stress. The mutants have higher MDA levels than wild-type plants **(Fig. 24)**. Therefore, we proposed that ALDH7B4 may also function as peroxidation-inhibiting enzymes during a combination of heat and wounding stress.

Taken together, these results suggest that different stress combinations cause different expression patterns of ALDHs. ALDH7B4 is strongly induced by D/H, H/S and slightly in W/H, suggesting that different signaling pathways are triggered in plants during these combinations. Knock-out of *ALDH* genes resulting in more MDA contents during the combination of these stresses. These results indicate that ALDHs may involve an antioxidant defense machinery to eliminate ROS during stress combinations, which is the key

pathway of plants to tolerate to stress combinations(Demirevska et al. 2010; Ahmed et al. 2013).

4.5 The *ALDH7B4* promoter activities in response to heat and combined stress conditions

ALDH family 7 members are highly conserved among animals and plants, indicating an essential role within the cell. ALDH7B4 belongs to the family 7 of plant ALDH proteins, which were given the name “antiquitin” to reflect their antique nature (Pauline Lee 1994). Functional studies of ALDH7B4 using knock-out and over-expressing lines indicated that the protein is involved in the detoxification of lipid peroxidation-derived aldehydes including MDA (Kotchoni et al. 2006). Induction of *ALDH7B4* in *A. thaliana* ABA-deficient and ABA-insensitive mutants was impaired after dehydration, but the expression was nearly unaltered upon NaCl treatment, indicating different signaling pathways (Kirch et al. 2005). Based on previous studies, Missihoun et al, (2014) proposed that the *ALDH7B4* gene would offer the unique advantage to study*ALDH* gene regulation in both ABA-dependent and –independent conditions (Missihoun 2010; Missihoun et al. 2014).

It is generally known that stress signaling is regulated by both ABA-dependent and ABA-independent pathways. ABA-responsive elements (ABREs; PyACGTGGC), G-box elements (CACGTGGC) and DRE/CRT (A/GCCGAC) motifs were found to mediate the ABA-dependent and the ABA-independent gene expression, respectively (Kim et al. 2011).Therefore, the transgenic *A. thaliana* plants harboring *ALDH7B4* promoter-GUS lines (mutation of ABREs and DRE/CRT motifs) were generated by Missihoun to study the regulation of *ALDH* genes under heat stress and stress combinations. Missihoun et al. (2014) reported that the *ALDH7B4* promoter was found to be responsive to various individual stresses, including wounding, salt, and dehydration. *Cis*-acting

elements involved in stress responsiveness were analyzed and two conserved ACGT-containing motifs proximal to the translation start codon were found to be essential for the responsiveness to osmotic stress in leaves and in seeds. An upstream ACGT motif and a DRE/CRT element were found to be necessary for *ALDH7B4* expression in seeds and induction by salt, dehydration and ABA in leaves (Missihoun et al. 2014). Measurement of the GUS activities from mutated *ALDH7B4* promoter plants under single stress treatment indicated that an increase of the GUS activity upon dehydration, wounding or salt treatments are in agreement with previously reported observations (Kotchoni et al. 2006). In this study, I found that besides osmotic and wounding stress, heat stress is another main trigger for*ALDH7B4* induction in leaves. A strong decrease of GUS activities was observed for the AB line (mutation of ACGT2 and ACGT3) under both single and all combined stress conditions (D/H, H/D, W/H, H/W, S/H and H/S), while AD line shown was less affected by all stress treatment, including dehydration, wounding, heat, salinity, D/H, H/D, W/H, H/W, S/H and H/S stress. These results were indicating that besides response to dehydration and salinity, *cis*-acting elements ACGT2 and ACGT3 also plays a crucial role in response to wounding, heat and combined stress conditions **(Fig. 27)**. The ACGT1 motif and the DRE/CRT element also found to be important in response to heat and combined stress in leaves. Previous studies revealed that both DRE/CRT and ACGT1 are functioning together with ACGT2 and ACGT3 for induction by salt and dehydration (Missihoun et al. 2014). In this study, analysis of P1, P2 and P3 promoter deletion lines demonstrated that the ACGT1 and the DRE/CRT elements might be vital for*ALDH7B4* induction during heat stress combined with wounding or salt stress, as a lower responsiveness to W/H, H/W, S/H and H/S compared with each individual stress was observed in AD lines **(Fig. 27)**.

4.6 ATAF1 acts as transcriptional activator and regulates the expression of *ALDH7B4*

NAC proteins are plant-specific transcription factors, which contain a highly conserved N-terminal DNA-binding domain (DBD), known as the NAC domain, which is also responsible for the oligomerization into dimeric proteins (Olsen et al. 2005). The C- terminal region of NAC members is more diverse, intrinsically disordered, and functions as a transcription regulatory domain (Jensen et al. 2009). Kleinow et al. (2009) pointed out that the NAC domain consists of five DNA binding sub-domains A-E and suggested sub-domain A plays a major role in dimerization of NAC proteins. The DNA binding domain (DBD) is contained within the sub-domains C and D. The core NAC binding sequences (CGT[G/A]), some NAC genes had NAC binding sequenced in their own promoters, implying an NAC regulatory network through auto-regulatory and cross-regulatory mechanisms (Kim et al. 2014). ATAF1 (ANAC002) belongs to the ATAF sub-family (ATAF1, ATAF2, ANAC032 and ANAC102), and was predicted to be a DNA binding protein with an NAC domain, which is an N-terminal module of 7 -131 amino acids. Transcription regulation of stress responsive NAC (SNAC) transcript factors has been revealed by the presence of several stress-responsive *cis*-acting elements in the promoter regions. DNA binding ability of ATAF1 was tested *in vitro* via an EMSA assay in this study **(Fig. 43)**. The results were confirmed that ATAF1 can directly bind to the core-DNA binding motif in the *ALDH7B4* promoter.

To further characterize the ATAF1 act as transcription activator or repressor to regulate *ALDH7B4* expression, the ATAF1 binding ability was measured using transactivation assays *in vivo*. When the *ALDH7B4::GUS* reporter gene containing the *ALDH7B4* promoter was expressed together with the effector ATAF1, the *ALDH7B4::GUS* expression increased **(Fig. 46)**. *ALDH7B4* transcript abundance was higher in *ATAF1* overexpression lines but lower in

ataf1 knockout mutants compared with the wild-type in seeds, seedlings and mature plants **(Fig. 48)**. Thus, I conclude that ATAF1 positively regulates *ALDH7B4* expression by directly binding to its promoter region. The majority of reports of NAC transcription factors have indicated that NACs play a role in the transcriptional reprogramming associated with plant immune response (Nuruzzaman et al. 2013). In addition, results from global transcriptome profiling, *NAC* genes showed enhanced expression during natural leaf senescence in *A. thaliana*, which including *ATAF1*, *NAC055, NAC018, NAC019* and *NAC102* (Breeze et al. 2011). More recent studies revealed that ANAC092/ORE1 was activated by ATAF1, which also induced ABA-mediated senescence with the direct regulation of ABA-related genes, such as *NCED3* and *ABCG40*, repressed the expression of chloroplast maintenance transcript factors GLK1 by direct binding to the promoters of both genes (Jensen et al. 2013; Garapati et al. 2015). Transcript analysis revealed that RD29-regulated genes are involved in the detoxification of ROS (e.g. glutathione-S-transferases and glutathione redoxins), defense, and senescence (Fujita et al. 2004; Balazadeh et al. 2011). Similarly to RD26 signaling pathways, the expression of ATAF1 is induced by drought, high-salinity, mechanical wounding, ABA, JA, SA and H_2O_2 (Wu et al. 2009). Previous findings showed that ALDH7B4 function as aldehyde-detoxification enzyme and also as efficient ROS scavengers (Kotchoni et al. 2006). As ATAF1 positively regulates *ALDH7B4* expression by directly binding to its promoter region, this is the first evidence that detoxification of ROS may be closely related to ATAF1- mediated stress responses. We also found that overexpression or knockout of ATAF1 at the seeds or seedling stage strongly affected ALDH7B4, indicating that ATAF1 is also required for *ALDH7B4* gene expression in young tissues.

4.7 Conclusions

This study attempted to identify the role of *Arabidopsis thaliana* aldehyde dehydrogenase (*ALDH*) genes in response to high temperature and stress combinations (heat combined with dehydration, wounding or salinity). The data presented here provide information on the *ALDH* genes expression profiles under heat stress and combined stress that mimic field conditions. *ALDH3H1*, *ALDH3I1*, *ALDH7B4* and two BADH-encoding genes *ALDH10A8* and *ALDH10A9* have been analyzed in this work. The results show that *ALDH* genes, particularly *ALDH7B4*, is strongly induced by heat stress in seedlings as well as adult plants. High level of ALDH10A8 and ALDH10A9 was found during recovery, suggesting that these genes function in the plant through the biosynthesis of the osmoprotectant glycine betaine. An increased expression of *ALDH7B4* was observed under heat combined with dehydration, salinity or wounding stress. Measurements of changes in physiological parameters such as survival rates, photosynthesis, lipid peroxidation and chlorophyll content of T-DNA double mutants of *ALDH* genes and wild-type plants demonstrated that mutant lines are more sensitive to heat stress and combined stress conditions. These results indicate that *ALDH* genes not only play a crucial role in protecting plants from high temperature damage but are also involved in the antioxidant defense machinery to eliminate ROS during the heat and combined stress conditions.

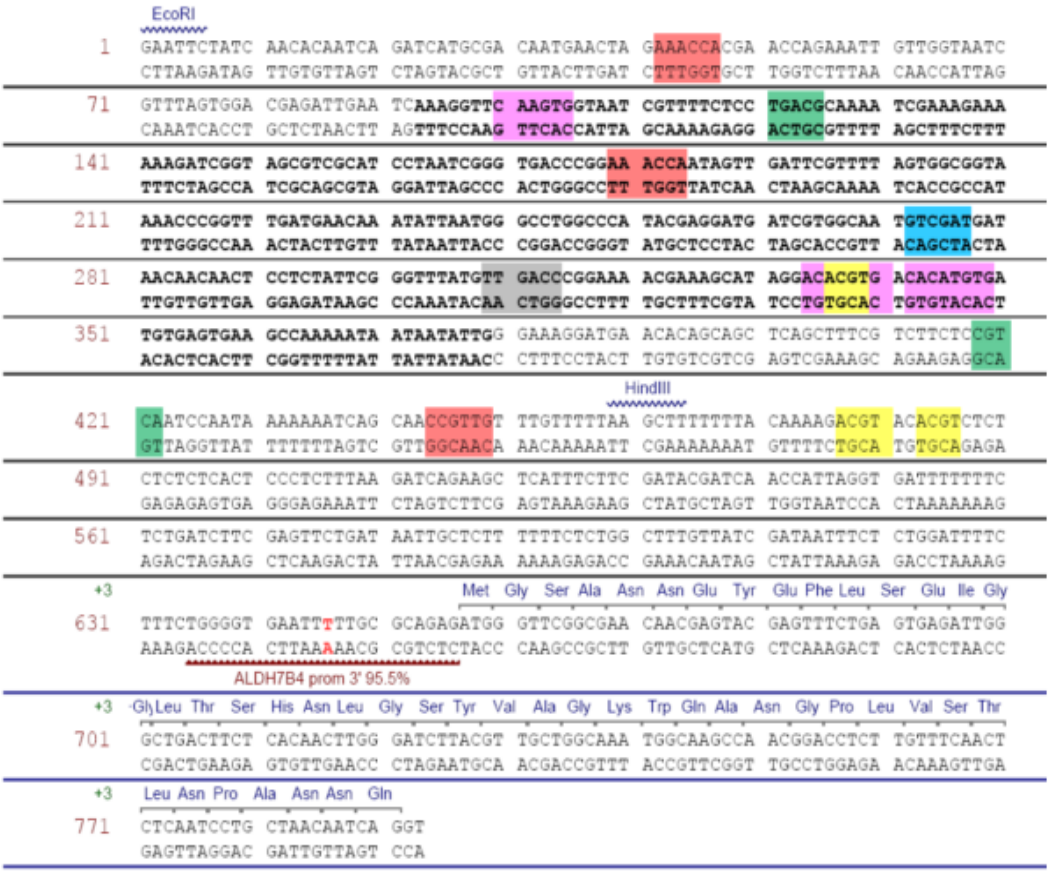
Another point addressed in this work is functional analyses of the *ALDH7B4* promoter in response to heat and stress combinations. First, the functional significance was analyzed of the *cis*-acting elements, ACGT and DRE/CRT, using six GUS fusion lines along with the deletion and base-substitution lines. The results show that besides in response to dehydration and salinity, the *cis*-acting elements ACGT2 and ACGT3 also play a key role in response to wounding, heat and combined stress conditions. The upstream ACGT1 motif

and DRE/CRT element were found to be necessary for *ALDH7B4* under heat and combined stress in leaves. Analysis of P1, P2 and P3 promoter deletion lines demonstrated that ACGT1 and DRE/CRT are vital for heat combined with wounding and salt stress, as a lower responsiveness to W/H, H/W, S/H and H/S compared with each individual stress was observed in AD lines. Putative transcription factors binds to the *ALDH7B4* promoter was identified using a yeast one-hybrid screening. Several NAC transcription factors, including ATAF1 (ANAC002), were isolated. These transcription factors play a critical role in plant adaptation to abiotic stress and development. Experiments utilizing molecular techniques demonstrated that ATAF1 activates *ALDH7B4* by directly binding to a specific promoter region *in vivo* and *in vitro*. Overexpression of ATAF1 in *A. thaliana* results in elevated expression of ALDH7B4 in seeds, seedlings, and mature plants, whereas ATAF1 T-DNA knock-out plants abolished the expression of ALDH7B4 at different growth stages. This study demonstrated ATAF1 as a DNA-binding transcription factor that activates *ALDH7B4* expression by directly binding to its promoter, indicating that detoxification of ROS may be closely linked to ATAF1- mediated stress responses.

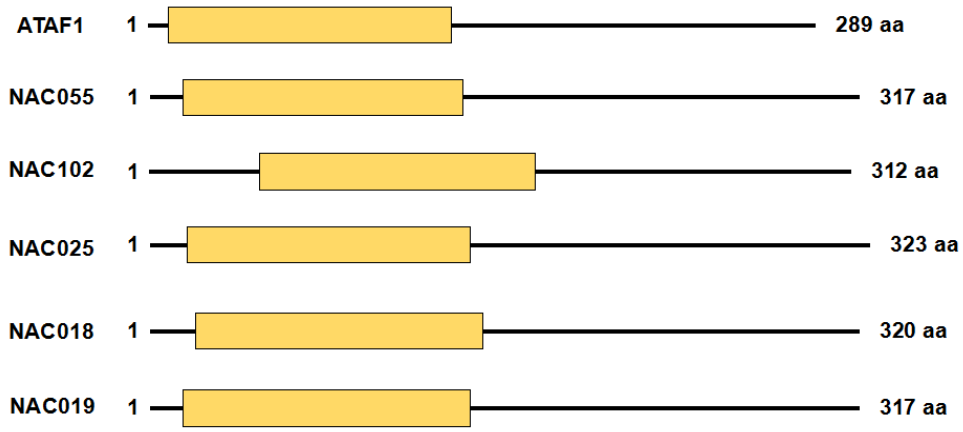
5. APPENDIX

ALDH7B4 gene promoter sequence:

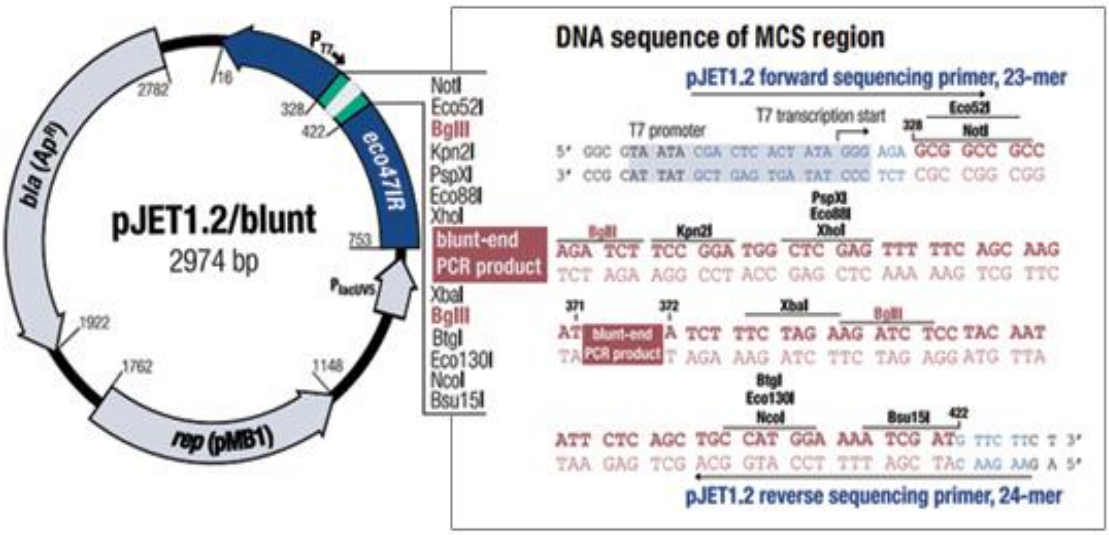
Red: MYB recognition site (WAACCA; W=A/T) Pink: MYC recognition site (CANNTG; N=A/T/G/C) Green: putative ASF-1 binding site Turquoise-shaded: Putative DRE (RYCGAC; R=A/G; Y=C/T) Gray: Elicitor Responsive Element (Core of W1 and W2 boxes) Yellow: ACGT-Box (Missihoun 2010).



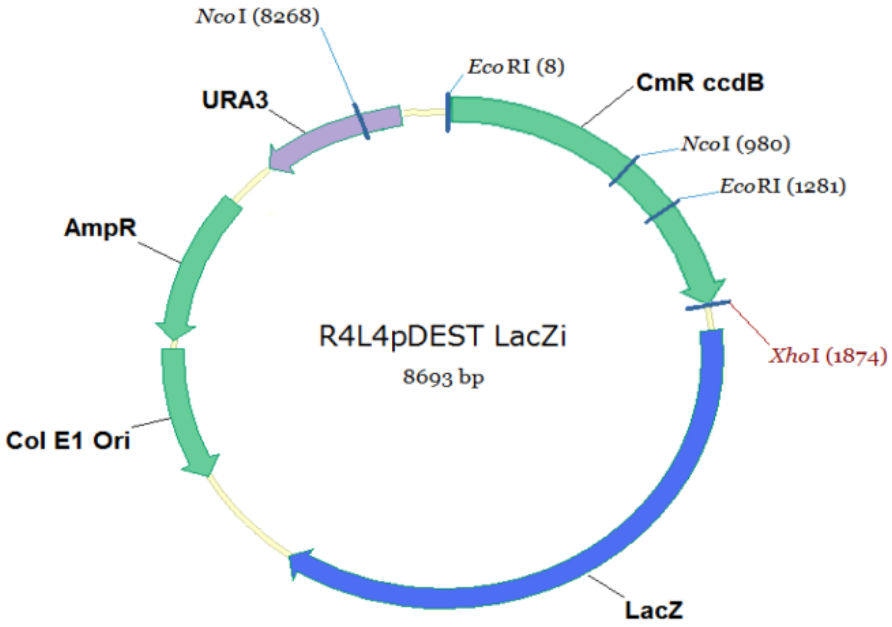
NAC domain view of six isolated transcript factors by yeast one-hybrid:



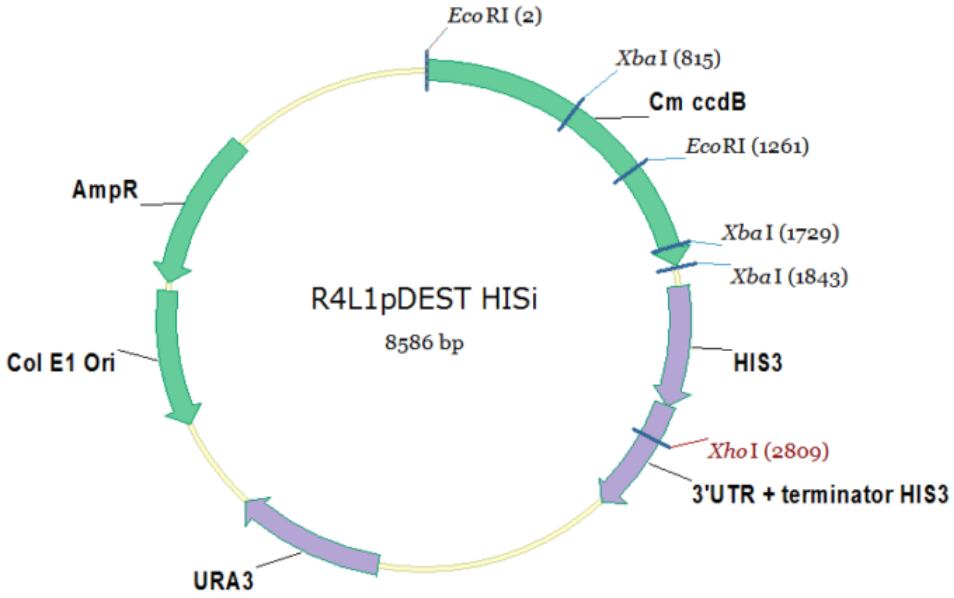
pJET1.2/blunt:



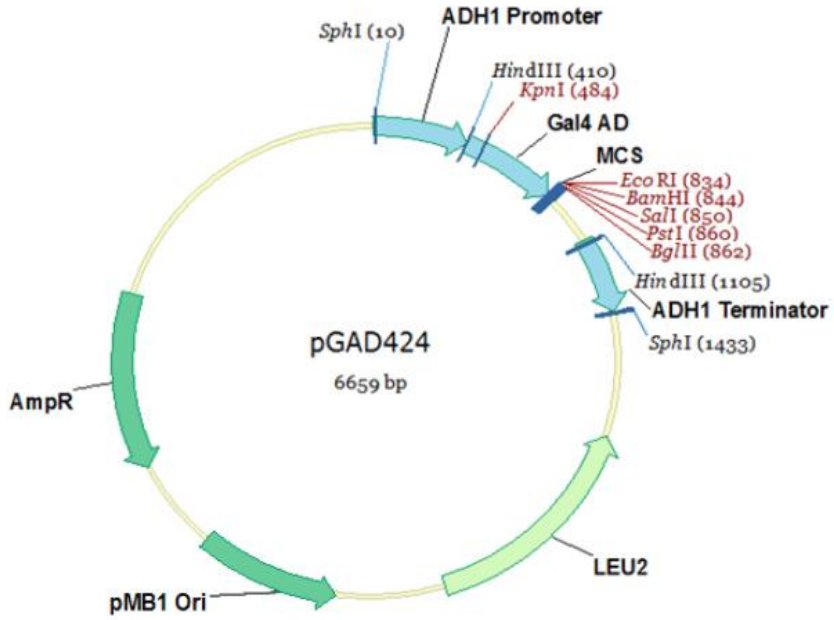
R4L1pDEST-lacZi:



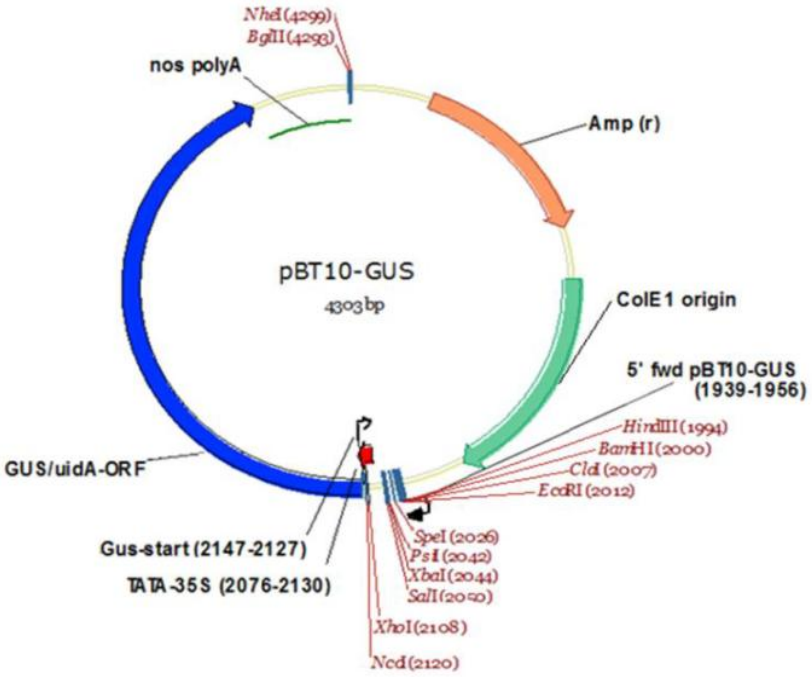
R4L1pDEST-HISi:



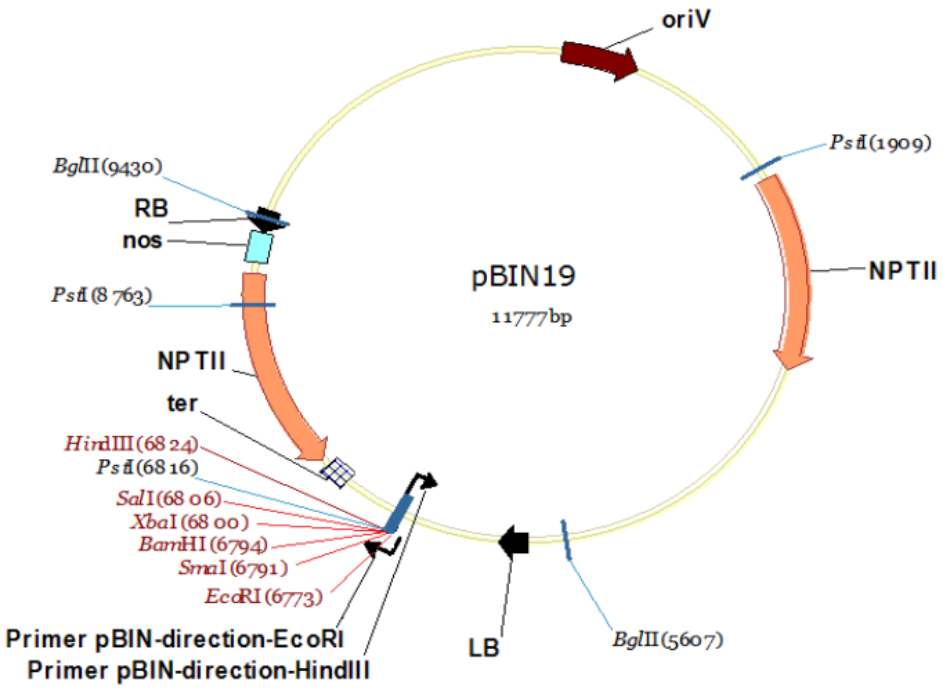
pDEST-GAD424:



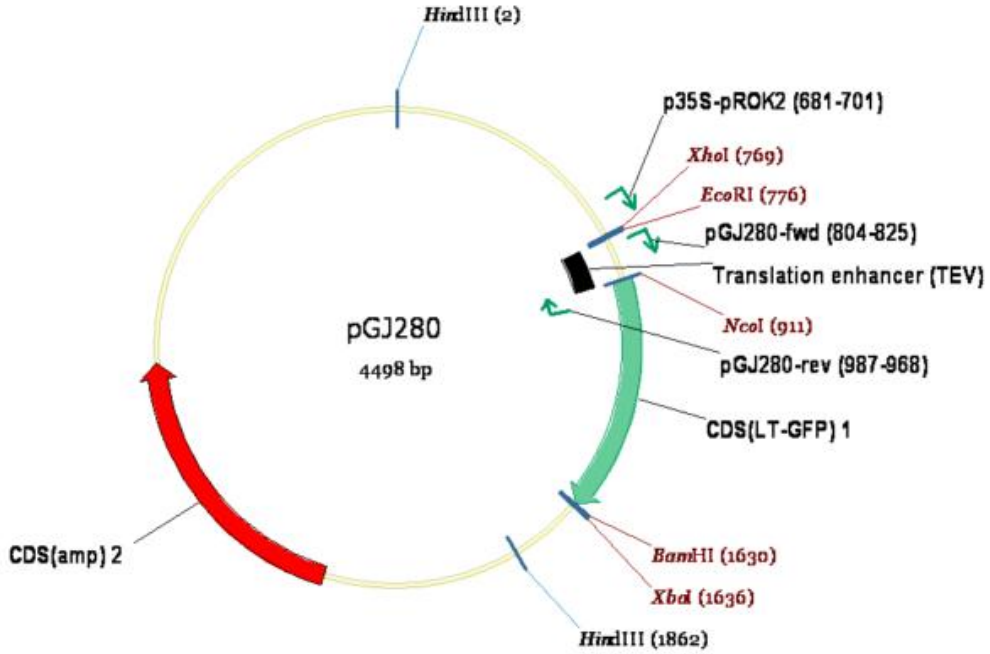
pBT10-GUS:



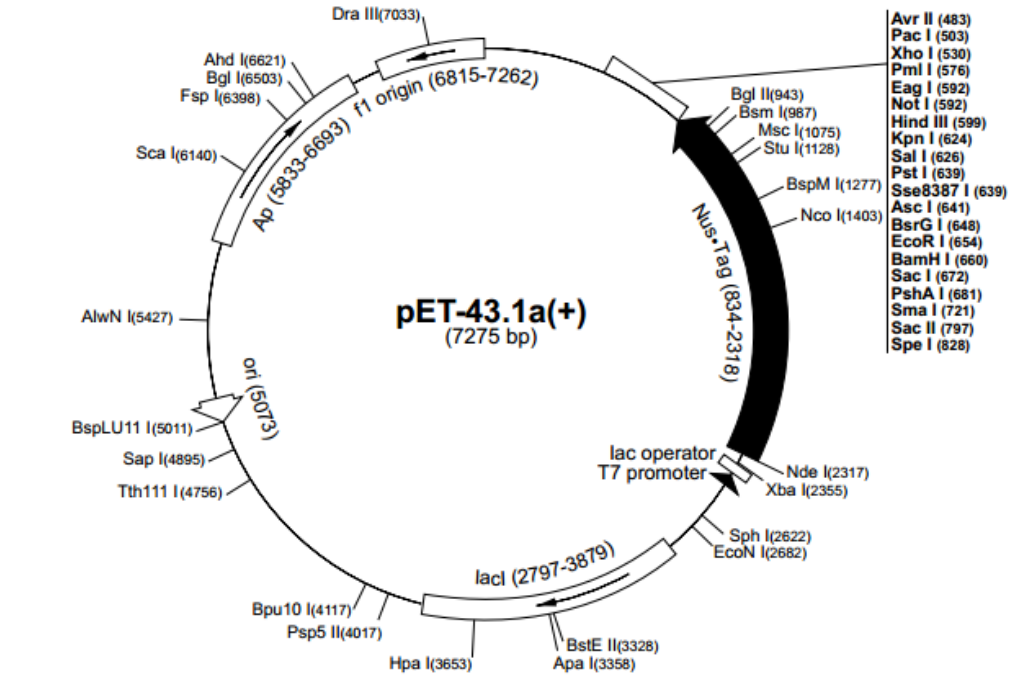
pBIN19:

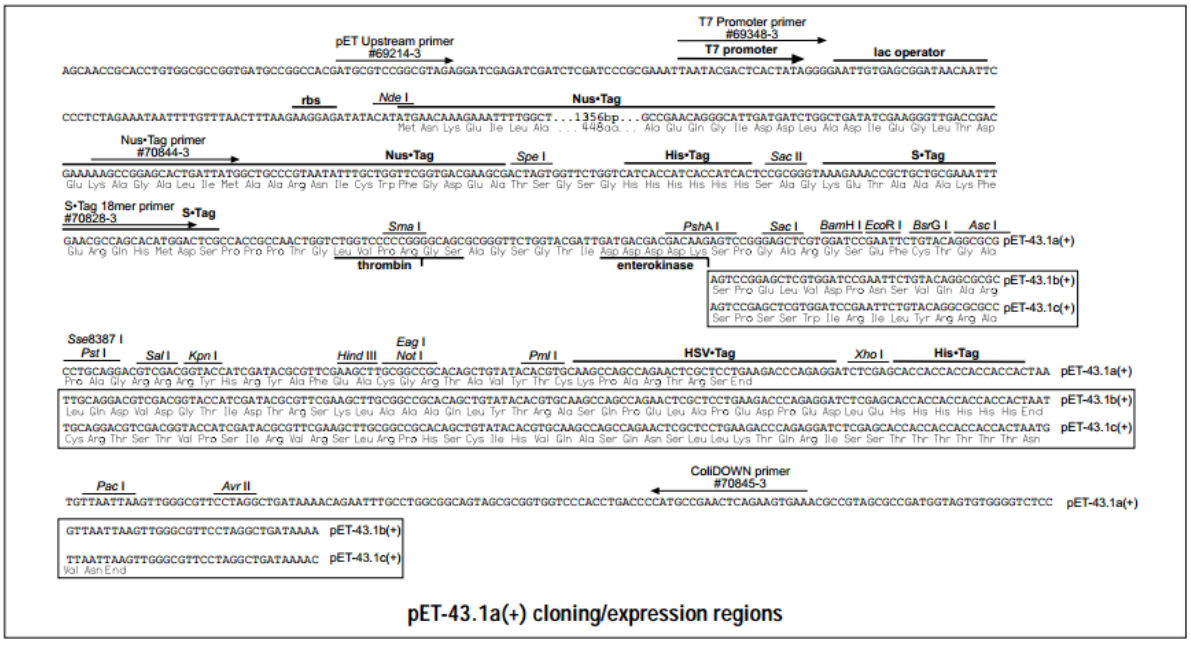


pGJ280:



pET43.1b:





pET-43.1a(+) cloning/expression regions

6. FIGURES AND TABLES

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