Characterisation of selected *Arabidopsis* aldehyde dehydrogenase genes: role in plant stress physiology and regulation of gene expression

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DECLARATION

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

Bonn, November 2010 **----------**

Tagnon D. MISSIHOUN

DEDICATION

To

My wife:	**Fabienne TOSSOU-MISSIHOUN and our kids Floriane S. Jennifer and Sègnon Anges-Anis**
My parents:	**Lucrèce KOTOMALE and Dadjo MISSIHOUN**
My sister and brothers:	**Mariette, Marius, Ricardo, Renaud, Ulrich**
And my dearest aunts and uncles:	**Hoho, Rebecca, Cyriaque, Dominique, Alphonsine**

CONTENTS

ABBREVIATIONS

4 –MUG	4 –Methylumbelliferyl glucuronide
A	Adenin
ABA	Abscisic acid
ABAL	4-aminobutyraldehyde
ABRE	ABA responsive element
ALDH	Aldehyde dehydrogenase
AMADH	Aminoaldehyde dehydrogenase
Amp	Ampicillin
APAL	3-aminopropionaldehyde
APS	Ammonium persulfate
bp	Nucleotide base pair
BSA	Bovine serum albumin
β-ME	β-mercaptoethanol
bZIP	Basic leucine zipper
C	Cytosine
CaMV	Cauliflower mosaic virus
CAO	Copper Amine Oxidase
cDNA	Complementary DNA
CRT	C-repeat
D	Dalton
DAB	3,3'-diaminobenzidine
dATP	Desoxy-adenosin-triphosphate
dCTP	Desoxy-cytidin-triphosphate
dCTP	Desoxy-cytidin-triphosphate
dGTP	Desoxy-guanosin-triphosphate
DMF	N,N-Dimethylformamid
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
DRE	Dehydration responsive element
DTT	Dithiothreitol
dTTP	Desoxy-thymidin-triphosphate
DW	Dry weight
EDTA	Ethylenediaminetetraacetate
fwt	Fresh weight
fwd	Forward
g	gram

g	Acceleration
G	Guanine
GB	Glycine betaine
gDW	gramme dry weight
GFP	Green Fluorescent Protein
GST	Gluthation-S-transferase
GUS	*E. Coli* β-glucuronidase gene (*uidA*)
h	Hour
HEPES	4-(2-Hydroxyethyl)-1-piperazinethansulfonic acid
His	Histidine
HNE	4-hydroxy-trans-2-nonenal
IgG	Class G immunoglobulin
IPTG	Isopropyl- β -D-thiogalactopyranoside
Kan	Kanamycin sulfate
kb	Kilobase
kDa	Kilodalton
LB	Luria and Bertani medium
LEA	Late Embryogenesis Abundant
M	Molar, mole(s) per liter
mA	Milliamperes
MCS	Multiple cloning site
MDA	malondialdehyde
MES	2-(N-morpholino)ethanesulfonic acid
min	Minute
ml	Milliliter
MOPS	3-(N-morpholino) propanesulfonic acid
MPa	Mega Pascal
mRNA	messenger RNA
MS	Murashige and Skoog (1962)
MW	Molecular weight
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
nm	Nanometers
nt	Nucleotide
OD	Optical density
Oligo (dT)	Oligodeoxythymidylic acid
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PAO	Polyamine oxidase
PBS	Phosphate Buffer Saline

PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
PIPES	Piperazine-N,N,-bis (2-ethanesulfonic acid)
PMSF	Phenylmethanesulphonylfluoride
PUFA	Poly-unsaturated fatty acid
PVP	Polyvinylpyrrolidone
QTL	Quantitative Loci Trait
rev	Reverse
Rif	Rifampicin
RNA	Ribonucleic acid
RNase	Ribonuclease
RES	Reactive Electrophile Species
ROS	Reactive Oxygen Species
rpm	Rounds per minute
RT	Room temperature
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SAP	Shrimp Alkaline Phosphatase
SDS	Sodium dodecyl sulfate
SSC	Saline sodium citrate buffer
ssDNA	Single-stranded DNA
TA	Annealing temperature
TAE	Tris-Acetate-EDTA
Taq	*Thermophilus aquaticus*
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TE	Tris (10mM)-EDTA (1 mM)
TEMED	N,N,N',N'-tetramethylethylenediamine
TM	Melting temperature
Tris	Tris-(hydroxymethyl)-aminomethane
Triton X-100	Poly(ethylenglycolether)n-octylphenol
U	Unit
UV	Ultraviolet
V	Volts
v/v	Volume/volume
w/v	Weight/volume
X	Times
X-Gluc	5-bromo-4-chloro-3-indolyl-β-D-glucuronide

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SUMMARY

The importance of aldehyde dehydrogenase (ALDH) proteins in plant stress responses was investigated in this study by functionally analysing transgenic *Arabidospsis thaliana* ALDH knock-out and over-expressing plants. From the nine ALDH gene families present in *Arabidopsis*, four gene members of the families 10, 3 and 7 have been analysed in this work.

Both ALDH10A8 (AT1G74920) and ALDH10A9 (AT3G48170) belong to the family 10 of the superfamily of ALDH proteins and, based on sequence similarity, they putatively code for betaine aldehyde dehydrogenases (BADHs), enzymes that catalyse the last step of glycine betaine biosynthesis. But, *Arabidopsis* is known not to be able to produce glycine betaine. The function of these two genes was therefore investigated. ALDH10A8 was found to be localized in leucoplasts whereas ALDH10A9 is targeted to peroxisomes. The *ALDH10A8* and *ALDH10A9* transcripts were detected in the plant and were slightly induced by stress treatments. Plants lacking *ALDH10A8* transcripts were found to be drought and salt sensitive, indicating that *ALDH10A8* may be involved in other pathways than the biosynthesis of glycine betaine in *Arabidopsis*. Using betaine aldehyde, 4-aminobutyraldehyde (ABAL) and 3-aminopropionaldehyde (APAL) as substrates, the recombinant ALDH10A9 protein showed both betaine aldehyde and aminoaldehyde dehydrogenase activities, although the affinity to the substrates was low compared to data from the literature. No enzymatic data was obtained for ALDH10A8 as it was not possible to purify sufficient amounts of the enzyme in its active form. Considering the high amino acid sequence similarity between ALDH10A8 and ALDH10A9, I propose that ALDH10A8 may be also active *in vivo* and likely both proteins function as aminoaldehyde dehydrogenases by detoxifying cells from metabolism-derived cytotoxic aminoaldehydes.

The *Arabidopsis ALDH3H1* (*AT1G44170*) gene belongs to the family 3 of the ALDH superfamily. Previous findings showed that *ALDH3H1* transcripts mostly accumulate in roots of 4 week-old plants upon ABA, dehydration and NaCl treatments. Here, the expression analysis was extended to the protein level and in adult plants. Together with the previous observations it is found that the up-regulation of ALDH3H1 protein by salt stress mainly occurs in leaves of plants older than 4 weeks. To understand the function of *ALDH3H1* in the stress response of *Arabidopsis*, transgenic plants over-expressing the ALDH3H1 protein were generated and analysed. It appeared that the constitutive expression of ALDH3H1 did not confer stress tolerance to the transgenic plants. However, the results indicate that the

ALDH3H1 protein can help the plant to cope with stress injuries by alleviating damages from lipid peroxidation.

Besides, the results from this study gives for the first time the experimental evidence that the *ALDH3H1* short transcript variant (*AT1G44170.3* (*T3*)) is expressed in *Arabidopsis*. It is nearly absent or expressed at a very low level in the wild type but accumulates in the *3h1-A* mutant, which carries a T-DNA insertion in the first exon of the *ALDH3H1* locus. The expression of the transcript *T3* is shown to be directed by an alternative promoter comprised within the first intron of this gene. *T3* and other *ALDH3H1* transcript variants (*AT1G44170.1* (*T1*) and *AT1G44170.2* (*T2*)) are found to be differentially expressed in roots and shoots. Sub-cellular localisation experiments indicated that the protein T3 is targeted to the cytosol but its presence could be revealed neither in the *3h1-A* mutant nor the wild type by using ALDH3H1 antibodies. Comparative analysis of the wild type and different T-DNA insertion mutants showed that the transcript *T3* does not functionally compensate the lack of *T1* and *T2* under salt stress. The possible origin and functions of the transcript *T3* are discussed.

It is hypothesized that aldehydes may function as signal molecules and trigger aldehyde dehydrogenase gene expression. To test this hypothesis, transgenic plants expressing the β-glucuronidase (*GUS*) reporter gene driven by the *ALDH7B4* (*AT1G54100*) gene promoter were generated (7B4-GUS). The *ALDH7B4* promoter was found to be constitutively active in naturally desiccation-tolerant organs like seeds and pollen. In addition, both pentanal and trans-2-hexenal activated the promoter. The comparison of the GUS activities revealed that dehydration and NaCl induce the promoter stronger than trans-2-hexenal. To further understand the mechanism of the promoter activation by aldehydes the enzymatic activity of the GUS protein in plant extracts was compared to the accumulation pattern of malondialdehyde (MDA). Except for the methyl viologen treatment, no correlation was found between the GUS activity and the plant MDA content for the other treatments. Moreover, the *in silico* analysis of the *ALDH7B4* promoter region revealed the presence of several stress-related *cis*-elements including one putative dehydration-responsive element/C-repeat – low temperature-responsive element (DRE/CRT-box) and three ACGT-boxes. The functional analysis of these elements suggested that the two proximal ACGT2 and ACGT3 boxes are the most influential ACGT-boxes involved in the salt response of the promoter. To identify factors involved in the aldehyde-induced expression of ALDH genes, a genome-wide mutagenesis approach has been chosen. Seeds from a homozygous transgenic 7B4-GUS plant were treated by the mutagen ethyl methanesulphonate (EMS). A second generation seed population has been generated.

1. INTRODUCTION

1.1 Climate changes and environmental stress

In a world, where population growth exceeds food supply, one of the big challenges in the coming decades is the development of a sustainable agriculture for producing enough food for all people on earth. Such a challenge becomes more and more problematic to be achieved taking into account the progressive and alarming climate changes (IPCC 2001). In a recent publication, The World Bank (2007) has identified climate change as an important risk factor for food production and development and has indicated that the effects are already being felt in Sub-Saharan Africa, where crop production has fallen. Actually, global scarcity of water resources is increasing in addition to environmental pollution, soils and water salinity. These changes strengthen environmental stresses to which plants are subjected everyday and will become soon more severe with desertification that is covering more and more the world's terrestrial area. Abiotic stressors are the primary cause of crop loss worldwide, reducing yields for most major crop plants and thereby leading to fatal economical effects on agriculture. Among the abiotic factors that limit plant productivity, drought and salinity are widespread in many regions and prevent plants from expressing their full genetic potential. Agricultural practices such as irrigation (in areas with low water availability), traditional plant breeding and QTL-based (Quantitative Loci Trait) crop selection have been used to develop new cultivars and to promote a sustainable agriculture. However, these methods are showing their limits as they are time consuming and costly. Modern plant biotechnology aiming to cope with environmental stress effects on crops need to be implemented in addition.

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1.2 Plant stress and mechanisms of tolerance

In natural conditions plants are exposed to a variety of different environmental cues. The variation in environmental conditions are sources of stress ranging from water deficit, drought, salinity, high temperature, freezing, flooding, strong light (abiotic stress), to those induced by soil or air borne pathogens such as fungi, viruses and bacteria (biotic stress). Both biotic and abiotic stresses reduce productivity, delay growth and development and in extreme cases cause the death of the plant. To assure their own integrity plants have developed various mechanisms to cope with stresses (Ingram and Bartels 1996). Studies in stress physiology have attempted to elucidate the biochemical and molecular strategies developed by plants under various stress conditions (Chaves et al. 2003; Flexas et al. 2004). The most common objective of these investigations has been to identify and characterize genes expressed in

plants under stress and thereby discover mechanisms developed by plants to withstand adverse conditions. For example, a small group of angiosperms, termed resurrection plants, has been studied for about 40 years (Gaff 1971; 1987). These plants can still survive after losing more than 90% of their cellular water and they become fully turgid 24 hours after rehydration (Bartels et al. 1990). The study of such naturally desiccation-tolerant species has revealed important aspects of desiccation tolerance (Ingram and Bartels 1996). Several genes have been isolated as responsive to water or salt stress; most of them coding for stress-protective proteins (e.g. LEAs), transcription factors, detoxifying enzymes, enzymes involved in the biosynthesis of compatible solutes and proteins involved in signalling and regulatory pathways (Bartels and Sunkar 2005). Yet, responses to abiotic stress are genetically complex and multigenic. Drought, salt and cold stress responses are interconnected and may produce similar cellular damages. Low temperature may induce mechanical constraints, changes in enzyme activity and decreased osmotic potential (Xiong et al. 2002). Similarly, high salt stress disrupts both osmotic and ionic homeostasis at the cellular and whole plant level. Important changes in ion and water homeostasis could lead to growth arrest and death. As a consequence of such interconnections in cold, drought and salt stress effects, it appears difficult to associate a specific locus or genetic marker with a tolerance trait. However, significant progress has been made so far in elucidating signalling pathways related to environmental stress and identifying gene products involved in the acquisition of stress tolerance.

1.3 Gene products related to abiotic stress

Stress inducible genes can be classified into two groups (Seki et al. 2004): (i) gene products including transcription factors, protein kinases, phosphatases and enzymes involved in phosphoinositide metabolism. These gene products regulate the expression of the other genes in the signalling pathways. They constitute the group of early stress responsive genes as they are rapidly and transiently induced and activate downstream response genes; (ii) gene products that directly protect against stress: these are the molecules that function by protecting cells from damages. They include the enzymes responsible for the synthesis of various compatible solutes, LEA-like proteins, antifreeze proteins, chaperones and detoxification enzymes.

1.3.1 Regulatory pathways of stress-related gene expression in plants

The signal transduction pathways in plants under environmental stresses have been divided into three major types (Xiong et al. 2002) : (i) osmotic/oxidative stress signalling that makes use of mitogen activated protein kinase (MAPK) modules; (ii) Ca^{2+}-dependent signalling that leads to activation of LEA and LEA-like genes such as dehydration responsive elements (DRE)/cold responsive or sensitive transcription factors (CRT) class of genes, and (iii) Ca^{2+}-dependent salt overly sensitive (SOS) signalling that results in ion homeostasis.

1.3.1.1 Osmotic/oxidative stress signalling

Salt and drought stress induce the formation of Reactive Oxygen Species (ROS) such as superoxide, hydrogen peroxide, and hydroxyl radicals, causing extensive cellular damage and inhibition of photosynthesis. This phenomenon is termed oxidative stress. ROS are potentially damaging agents contributing to stress injury in plants. But, they can also act as signal molecules inducing ROS scavengers and other protective mechanisms. Osmotic/oxidative stress is initially perceived by sensors that initiate a cascade of intracellular signals leading to the activation of a set of genes which in many cases correspond to transcription factors encoding genes. Sensors could be the receptor-like kinases (RLKs) found in both animals and plants. They perceive the signal through the extracellular domain that binds a ligand and transmit this signal by their intracellular domain through a kinase activity. In the case of histidine kinase, where the extracellular sensor domain perceives a signal, the cytoplasmic histidine residue is auto-phosphorylated and the phosphoryl moiety is then passed to an aspartate receiver in a response regulator, which may constitute part of the sensor protein or a separate protein. The sensors may be coupled with a downstream mitogen-activated protein kinase (MAPK) cascade (Agrawal et al. 2003) or directly phosphorylate specific targets to initiate cellular responses. The MAPK cascades involved three kinases that are sequentially activated by an upstream kinase. The MAP kinase kinase kinase (MAPKKK), upon activation, phosphorylates a MAP kinase kinase (MAPKK) on serine and threonine residues. This MAPKK in turn phosphorylates a particular MAP kinase (MAPK) on conserved tyrosine and threonine residues. The activated MAPK can then either migrate to the nucleus to activate the target transcription factor(s) directly, or activate additional signal components to regulate gene expression or cytoskeleton-associated proteins or enzyme activities (Rodriguez et al. 2005). The MAP cascades mediate osmotic homeostasis and/or detoxification responses. Secondary signals like hormones and second messengers like inositol phosphates and reactive

oxygen species (ROS) can initiate another cascade of signalling events, which can differ from the primary signalling in time and space (Xiong et al. 2002).

1.3.1.2 Ca^{2+}-dependent signalling

Drought, salt and cold stress have been shown to cause transient Ca^{2+} influx from either apoplastic spaces or internal stores to the cell cytoplasm (Gelli and Blumwald 1997). The concentration of intracellular Ca^{2+} is carefully tuned and specific Ca^{2+} oscillations have been implicated in various physiological processes. Releases of stored Ca^{2+} are controlled by ligands that interact with some ligand-sensitive Ca^{2+} channels. These ligands act as second messengers. Calcium-dependent protein kinases (CDPKs) represent an important group of Ca^{2+} influx sensors in plants mediating stress responses. CDPKs are serine/threonine protein kinases with a C-terminal calmodulin-like domain with up to 4 EF hand motifs that can directly bind Ca^{2+}. CDPKs are activated by abiotic stress and are believed to orchestrate the activation of transcription factors which induce gene expression of LEA and LEA-like proteins. LEA-like genes include the dehydration-responsive element (DRE)/C-repeat (CRT) class of stress-responsive genes.

Plant responses to ionic stress caused by high salinity include restricting salt intake, increased extrusion-compartmentalization and controlled long-distance transport to aerial parts. In *Arabidopsis*, the SOS (Salt Overly Sensitive) pathway is a major signalling pathway required for ion homeostasis under salt stress. The SOS pathway consists of three proteins, SOS1, SOS2, and SOS3. SOS1 is a plasma membrane Na^+/H^+ antiporter (Shi et al. 2000). SOS2 is a member of the SNF1-related protein kinase 3 family (SnRK3) (Liu et al. 2000; Hrabak et al. 2003). SOS3 is a myristoylated calcium-binding protein (Liu and Zhu 1998; Ishitani et al. 2000). An early detectable response to sodium stress is the rise in cytosolic free calcium concentration (Knight 2000). Transient increases in cytosolic Ca^{2+} under salt stress are sensed by SOS3 that forms a complex with SOS2, activating the substrate phosphorylation activity of SOS2. The formation of the complex SOS3-SOS2 is calcium-dependent (Halfter et al. 2000; Liu et al. 2000) and recruits SOS2 to the plasma membrane. The SOS3–SOS2 protein kinase complex phosphorylates SOS1 to stimulate its Na^+/H^+ antiport activity (Qiu et al. 2002; Quintero et al. 2002).

1.3.2 ABA signalling

1.3.2.1 ABA metabolism

Abscisic acid (ABA) is a phytohormone that regulates several aspects of plant development including seed development, desiccation tolerance of seeds and seed dormancy. It also plays a crucial role in the plant response to abiotic (drought, salinity, cold, and hypoxia) and to some extent to biotic stress. Abiotic stress causes an increase in ABA biosynthesis, which is then rapidly metabolized following the removal of the stress. Although the upregulation of ABA biosynthesis in response to osmotic stress is a well-known fact, the signalling pathway by which ABA biosynthetic genes are up-regulated remains to be clarified. A Ca^{2+}-dependent signalling pathway was proposed to regulate the expression of ABA biosynthetic genes such as ZEP (zeaxanthin epoxidase), NCED (9-cis-epoxycarotenoid dioxygenase), AAO (ABA-aldehyde oxidase), and MoCo sulphurase (molybdenum cofactor sulphurase) (Xiong et al. 2002). Biochemical studies suggested that the rate-limiting step is the reaction catalyzed by NCED (Koornneef et al. 1998). As for the ABA catabolism, a cytochrome P450 CYP707A family member was recently identified as ABA 8'-hydroxylase, an enzyme that degrades ABA during seed imbibition and dehydration stress (Kushiro et al. 2004; Saito et al. 2004). CYP707As are strongly induced by exogenous ABA treatment, dehydration, and rehydration.

1.3.2.2 ABA perception

Despite the progess made in ABA metabolism and signal transduction, mechanisms of ABA perception and signal transduction at the early stages were poorly understood until recently. Several proteins including GCR2 (a hypothetical G protein-coupled receptor), GT1 and GT2 (two membrane proteins with homology with G protein-coupled receptors) and CHLH/GUN5 (Mg-chelatase subunit H/GENOMES UNCOUPLED 5) have been proposed as putative ABA receptors (McCourt and Creelman 2008; Klingler et al. 2010). But, the most probable and convincing candidates for ABA receptor have been so far the PYR/PYL/RCAR (PYrabactin Resistance/PYrabactin Resistance-Like/Regulatory Component of Abscisic acid Receptor) proteins (Ma et al. 2009; Park et al. 2009). The PYR/PYL/RCAR receptor family is homologous to the Bet v 1-fold and START (StAR-related lipid transfer) domain proteins and has been independenly shown by different research groups to bind ABA. PYR/PYL/RCAR proteins are proposed to bind ABA and transduce the message through a signalling module involving Protein Phosphatase 2Cs (PP2Cs) and SNF1-related protein kinase 2s (SnRK2s) (Klingler et al. 2010, Hubbard et al. 2010). In this model, the PYR/RCARs act as ABA receptors, the PP2Cs act as negative regulators of the pathway, and SnRK2s act as positive

regulators of downstream signalling (Ma et al. 2009; Park et al. 2009). In the absence of ABA, PP2Cs inhibit SnRK2 protein kinase activity through removal of activating phosphates. ABA is bound by intracellular PYR/PYL dimers, which dissociate to form ABA–receptor–PP2C complexes. Complex formation therefore inhibits the activity of the PP2C in an ABA-dependent manner, allowing activation of SnRK2s. Several SnRK2 targets have been identified both at the plasma membrane and in the nucleus, resulting in control of ion channels, secondary messenger production, and gene expression (Klingler et al. 2010, Hubbard et al. 2010).

1.3.2.3 ABA signal transduction

Two reviews on the current state of knowledge on osmotic and cold stress signalling pathways have been recently published (Shinozaki and Yamaguchi-Shinozaki 2007; Nakashima et al. 2009). The role of ABA in drought and salt stress encompasses two major aspects: water balance and cellular dehydration tolerance. Whereas the role in water balance is mainly through guard cell regulation, the latter role is related to the induction of genes that encode dehydration tolerance proteins in nearly all cells. Stress-responsive genes have been proposed to be regulated by both ABA-dependent and ABA-independent signalling pathways (Shinozaki and Yamaguchi-Shinozaki 2007). Two major *cis*-acting elements, ABRE and DRE/CRT, are found to mediate the ABA-dependent and the ABA-independent gene expression, respectively. The comparison of the promoter region of several ABA-inducible genes has allowed to isolate a conserved sequence, PyACGTGGC, termed as ABA responsive element (ABRE). Several environmentally induced genes contain a similar conserved *cis*-acting element named as the G-box (CACGTGGC) (Menkens et al. 1995). A single copy of ABRE was not sufficient for ABA-responsive transcription. ABRE and coupling elements such as CE1 and CE3 are necessary for the ABA-induced gene transcription. In some cases, an adjacent copy of ABRE or DRE/CRT was found to function as a coupling element. Most of the known coupling elements are similar to ABREs and contain an A/GCGT motif (Hobo et al. 1999). ABREs are bound by the ABRE-binding proteins (AREB) or ABRE-binding factors (ABFs). The activation of the AREB/ABF proteins has been shown to require an ABA-dependent phosphorylation. Recently, several type-2 SNF1-related protein kinases (SnRK2-type) were reported as ABA-activated protein kinases, and were shown to mediate the regulation of stomatal aperture and to function upstream of ABA-responsive expression (Mustilli et al. 2002; Yoshida et al. 2002). These kinases might phosphorylate and activate the AREB/ABF-type proteins (Johnson et al. 2002). The phosphorylation/dephosphorylation-

regulated events appear to play important roles in ABA signalling. Target genes of AREB/ABF-type transcription factors are comprised of LEA genes and ABA- and dehydration stress-inducible regulatory genes, including linker histone H1 and AAA ATPase (Fujita et al. 2005).

ABRE-like motifs are not involved in the ABA regulation of some stress-inducible genes such as *RD22* but interact with some other transcription factors. As example, the induction of the dehydration inducible *RD22* is mediated by ABA and requires protein biosynthesis for its ABA-dependent expression. A MYC transcription factor, AtMYC2 (*RD22BP1*), and a MYB transcription factor, AtMYB2, respectively bind MYC and MYB recognition sites in the *RD22* promoter and cooperatively activate the expression upon dehydration stress (Abe et al. 1997; Abe et al. 2003). The MYC and MYB transcription factors are synthesized upon ABA accumulation, indicating their role in later stages of stress responses. *Arabidopsis RD26* encodes a NAC protein and is induced not only by ABA but also by dehydration, high salinity and jasmonic acid (JA). Functional analysis of the RD26-overexpressing plants indicated that a *cis*-acting element, the NAC recognition site, might function in ABA-dependent gene expression under stress conditions (Fujita et al. 2004). The homeodomain-containing transcription factor ATHB6 functions as a negative regulator downstream of ABI1 in the ABA signal transduction pathway, suggesting that a homeodomain-binding site is a negative *cis*-acting element in ABA-dependent gene expression. Altogether, ABRE is the most important *cis*-acting element; but several other types of *c*is-acting elements also function in ABA-responsive gene expression (Himmelbach et al. 2002).

The dehydration-responsive element (DRE) contains the core sequence A/GCCGAC and is involved in the regulation of gene expression in response to drought, high salinity, and cold stresses in *Arabidopsis* (Yamaguchi-Shinozaki and Shinozaki 1994). Similar *cis*-acting elements, named C-repeat (CRT) and low temperature-responsive element (LTRE), both containing the DRE core motif, are present in cold-inducible genes (Baker et al. 1994; Jiang et al. 1996). DRE/CRT *cis*-acting elements are specifically bound by the dehydration-responsive element binding protein 1 (DREB1)/C-repeat binding factor (CBF) and DREB2 transcription factors in ABA-independent gene expression. These transcription factors contain APETALA2 (AP2)/ethylene-responsive element binding factor (ERF) motif that is specific to plants and functions as a DNA-binding domain. Six DREB1/CBF genes and eight DREB2 genes were found in the *Arabidopsis* genome (Sakuma et al. 2002). These include DREB1A, DREB1B, and DREB1C, which are major transcription factors required for cold-inducible gene expression and DREB2A and DREB2B genes that are the major transcription factors required

for high salinity- and drought-inducible gene expression (Liu et al. 1998; Nakashima et al. 2000). DREB/CBF regulons were found to target multiple genes coding for transcription factors, phospholipase C, RNA-binding proteins, sugar transport proteins, desaturase, carbohydrate metabolism-related proteins, LEA proteins, KIN (cold-inducible) proteins, osmoprotectant biosynthesis proteins, and protease inhibitors (Seki et al. 2001; Fowler and Thomashow 2002; Maruyama et al. 2004). Most of these target genes were shown to function in response to stress. The comparative analysis of the DREB1A and DREB2A downstream target genes indicated that DREB1A has specifically a high affinity to A/GCCGACNT sequences, whereas DREB2A preferentially binds ACCGAC motifs (Sakuma et al. 2006a, b). DREB2 regulons function in both osmotic and heat-shock stress responses.

It has also been reported that some drought-inducible genes do not respond to either cold or ABA treatment, suggesting the existence of another ABA-independent pathway regulating the dehydration stress response. Indeed, in addition to the two major pathways, an ABA-dependent pathway directed by the AREB/ABF regulons and ABA-independent directed by the DREB/CBF regulons, other regulons, including the NAC and MYB/MYC regulons, are involved in abiotic stress-responsive gene expression (Yamaguchi-Shinozaki and Shinozaki 2006; Shinozaki and Yamaguchi-Shinozaki 2007; Nakashima et al. 2009).

1.3.3 Stress inducible proteins and other compounds

One of the mechanisms evolved by plants to cope with the detrimental effects of abiotic stresses is to synthesize specific proteins and compounds that protect the photosynthetic system and other vital macromolecules in different cell compartments. The protective molecules include mainly the LEA (Late Embryogenesis Abundant) and LEA-like proteins (Schneider et al. 1993; Rodrigo et al. 2004), small heat shock proteins (Allamillo et al. 1995) as well as compatible solutes and detoxifying enzymes.

1.3.3.1 LEA proteins

LEA proteins encompass a large group of proteins that are inducible by ABA in immature embryos and accumulate in mature embryos during desiccation. Most of them are rich in hydrophilic amino acids and are water soluble. Based on sequence similarities and biochemical properties they can be divided into five subgroups (Bernacchia and Furini 2004). Group 1 LEA proteins are characterised by a 20-amino-acid-motif (Litts et al. 1987) while proteins in group 2 share a conserved serine and lysine-rich motif and remain soluble after boiling (Bartels and Salamini 2001). Group 3 LEA proteins are characterised by a motif of 11

amino acids, which is predicted to form an α-helix probably involved in structural interactions (Dure et al. 1989). Group 4 and 5 proteins have less conserved sequences and have been suggested to protect membranes and to bind water respectively (Ingram and Bartels 1996). LEA genes are expressed at high levels in the cytoplasm or in chloroplasts upon dehydration and/or ABA treatment in vegetative or callus tissues of *Craterostigma plantagieum* (Ingram and Bartels 1996). Results from *in vitro* studies carried out with LEA or LEA-like proteins (Hara et al. 2001; Bravo et al. 2003) and analysis of transgenic plants over-expressing LEA genes (Zhang et al. 2000; Hara et al. 2003) support the hypothesis that these proteins may function as cellular protectants.

1.3.3.2 Compatible solutes

Compatible solutes also termed by osmoprotectants are small molecules that accumulate in the cell at molar concentrations without any toxic effect and stabilize proteins and cell membranes against denaturing effects of stress (Yamaguchi-Shinozaki et al. 2002). The compounds that fall into this group are amino acids (proline), quaternary ammonium compounds (glycine betaine), polyols and sugars (mannitol, D-ononitol, trehalose, sucrose, fructan) (Nuccio et al. 1999). They are not uniformly synthesized within the plants. They naturally accumulate in some plant species and help plants to cope with stress conditions. Regarding their protective role, several research studies have focused on both biosynthetic and degradation pathways to identify the genes implicated in their metabolism. For most of them, this goal has been achieved and the genes have been used to engineer the metabolism in plant species that do not naturally accumulate the molecules (Rathisanapathi 2000).

1.3.3.2.1 Mannitol, D-ononitol and sorbitol

Mannitol is a major photosynthetic product in many algae and some higher plants and enhances tolerance to water deficit stress primarily through osmotic adjustment (Loescher et al. 1992). *Arabidopsis thaliana* and tobacco do not accumulate mannitol. The introduction of a mannitol dehydrogenase (*mt1D*) gene into tobacco chloroplasts led to oxidative stress tolerance (Shen et al. 1997a). Similarly, overexpression of the *mt1D* gene from *Escherichia coli* in *A. thaliana* plant has conferred tolerance to salt stress compared to the wild type (Thomas et al. 1995). It is suggested that mannitol may scavenge $OH^{\cdot}$ radicals, as shown in tobacco where mannitol protects thioredoxin, ferredoxin, and glutathione and thiol-regulated enzyme phosphoribulokinase from the effects of $OH^{\cdot}$ (Shen et al. 1997b).

Similar findings were reported from D-ononitol and sorbitol. Tobacco transgenic plants transformed with *imt1* gene coding for myo-inositol-o-methyltransferase enzyme involved in the biosynthesis of D-ononitol were more drought tolerant than wild-type plants (Sheveleva et al. 1997). When apple cDNA encoding sorbitol-6-phosphate dehydrogenase was used to transform the plant species *Diospyros kaki*, the photosynthetic activity of the transgenic plants accumulating sorbitol under salt stress was higher than that of the wild type, suggesting that sorbitol may have contributed to the acquired tolerance (Gao et al. 2001).

1.3.3.2.2 Trehalose

Trehalose (α-D-glucopyranosyl-1,1-α-D-glucopyranoside) is a non-reducing disaccharide that is present in many organisms (bacteria, fungi, invertebrates and a few plant species) and functions as reserve carbohydrate and stress protectant, stabilising biological structures under abiotic stress conditions (Goddijn and al. 1999). The *A. thaliana* genome contains 11 genes coding for trehalose phosphate synthase (TPS) and 10 genes for trehalose-6-phosphate-phosphatase (Leyman et al. 2001), although no significant levels of trehalose accumulate in *A. thaliana*. Transgenic *Arabidopsis* plants which accumulated low amounts of trehalose-6-phosphate displayed a drought tolerant phenotype without any visible morphological alterations, except for delayed flowering (Avonce et al. 2004). Likewise, transgenic *Arabidopsis* plants expressing a yeast chimaeric gene coding for the TPS and the carboxy-terminal region of the TPP did not show any morphological alterations and were tolerant to drought, salinity, freezing and heat (Miranda et al. 2007). Recently alfalfa plants transformed with this bifunctional TPS-TPP enzyme showed improved tolerance to multiple abiotic stresses (Suárez et al. 2009). Trehalose was also shown to regulate the carbon metabolism and photosynthesis as well as the development of the embryo and flowering. Useful information about trehalose signalling and the role in plant stress physiology have recently been rewieved by Iturriaga et al. (2009).

1.3.3.2.3 Sucrose

The accumulation of sucrose is usually observed in seeds of many species and especially in fully hydrated tissues of desiccation-tolerant plants (Leprince et al. 1993). In the case of *C. plantagineum*, Bianchi et al. (1991) have observed that an unusual eight-carbon sugar 2-octulose accounts for approximately 90% of total sugars in fully hydrated leaves. But this sugar is rapidly converted into sucrose (representing about 40% of the dry weight (DW)), as soon as dehydration takes place. The reverse process is observed during rehydration. It is

thought that 2-octulose may be the predominant photosynthetic storage sugar that accumulates in leaves during the day and is partially metabolized at night. To date, little is known about the mechanism of this conversion. Nevertheless, studies on sucrose metabolizing enzymes sucrose synthase and sucrose phosphate synthase in *C. plantagineum* revealed that they are differentially expressed along with an up-regulation of glyceraldehyde dehydrogenase (Bartels and Salamini 2001). Also, other plant species have been shown to accumulate sucrose upon dehydration, even though at different levels (Oliver and Bewley 1997). Based on results from *in vitro* experiments where sugars protect biomolecules from denaturing during dehydration (Crowe et al. 1992), it is proposed that sucrose may form glasses like a solid liquid and prevent crystallization or partially replace water molecules in hydration shells, thus preventing the fusion of polar head groups in structural lipids (Bartels et al. 2006).

1.3.3.2.4 Fructans

Fructans are polymers of fructose and are used in many plant species as carbohydrate reserves (Vijn and Smeekens 1999). They accumulate in vacuoles and are thought to be involved in stress tolerance (Vereyken et al. 2003). Indeed, tobacco and sugar beet plants that were transformed with the bacterial fructan synthase gene showed enhanced tolerance to drought stress conditions (Pilon-Smits et al. 1995; Pilon-Smits et al. 1999). But, fructans accumulated at low levels in the transformed plants so that it is suggested that they may either act as regulators or signalling molecules influencing plant metabolism, or as scavengers of ROS (Shen et al. 1997a).

1.3.3.2.5 Proline

Free proline accumulation is observed in several plant species when subjected to environmental stresses (Delauney et al. 1993). It is proposed that proline acts as an osmolyte for osmotic adjustment, stabilizes sub-cellular structures such as membranes and proteins and scavenges ROS. Regulation of proline biosynthesis, degradation and transport in higher plants have been well documented (Kavi-Kishor et al. 2005).

The involvement of proline in the response to water deficit has been demonstrated in tobacco (Roosens et al. 2002). Moreover it has been shown that the gene coding for Δ1-pyrroline-5-carboxylate synthetase (P5CS), involved in the biosynthesis of proline from glutamate, is induced in *A. thaliana* under cold, osmotic stress and ABA application (Kreps et al. 2002). Yet, the accumulation of proline in the cell is also under the control of both transport and catabolism. Indeed, proline is catabolised by proline dehydrogenase (PDH) (Nakashima et al.

1998) and it has been shown that PDH is repressed under abiotic stress and induced by proline and hypo-osmolarity (Nakashima et al. 1998). Moreover, antisense transgenic *Arabidopsis* plants carrying AtProDH cDNA encoding proline dehydrogenase accumulated proline at higher levels than wild-type plants. The transgenic plants were more tolerant to freezing and high salinity stress (Nanjo et al. 1999). Expression of proline metabolizing enzymes and resulting proline levels have been reported for other transgenic plants (De Ronde et al. 2004; Molinari et al. 2004; Su and Wu 2004; De Ronde et al. 2001), seedlings (Phutela et al. 2003; Nayyar 2003; Zhu et al. 2003) and for plants exposed to shock treatments (Igarashi et al. 1997).

1.3.3.2.6 Glycine betaine

Glycine betaine (GB) is a quaternary ammonium compound that occurs naturally in a wide variety of animals, microorganisms and plants. But some species such as *A. thaliana*, tobacco, rice do not accumulate GB (Rhodes and Hanson 1993). As an amphoteric compound, GB can interact with both hydrophilic and hydrophobic domains of macromolecules such as enzymes and protein complexes. *In vitro* experiments have shown that GB stabilizes the structures and activities of proteins and maintains the integrity of membranes against the damaging effects of high salt, heat, cold and freezing (Gorham 1995).

To date, it is known that GB is synthesized via two pathways from two distinct substrates: choline and glycine, respectively. In the two-enzyme pathway, GB is synthesized in a two-step oxidation of choline via the toxic intermediate betaine aldehyde. In higher plants, the reactions are catalysed by choline monooxygenase (CMO) and betaine aldehyde dehydrogenase (BADH). The biosynthesis of GB is stress inducible and its accumulation *in vivo* varies among the species from undetectable levels in non-accumulating plants to up to 400 μmol $(gDW)^{-1}$ in natural accumulators under stress conditions (Rhodes and Hanson 1993). The glycine pathway has been discovered and identified in only two extremely halophilic micro-organisms, *Ectothiorhodospira halochloris* and *Actinopolyspora halophilia* (Nyyssölä et al. 2000). This involves three successive N-methylations, which are catalysed by two S-adenosylmethionine-dependent methyltransferases, GSMT (glycine sarcosine methyltransferase) and SDMT (sarcosine dimethylglycine methyltransferase). The characterisation of these enzymes has allowed engineering non-accumulating plants such as tobacco and *A. thaliana* to synthesize GB. This conferred tolerance to high salt and high temperature, cold and freezing to these species (Holmström et al. 2000; Gao et al. 2000; Alia et al. 1998; Sakamoto et al. 2000).

1.3.3.3 Small RNAs

Regulation of gene expression has been thought until recently to involve only transcription factors and DNA binding sequences in the relevant genes. But recently, small RNA molecules have been discovered to act as important regulatory molecules in both animal and plant organisms. Regulation by short-RNAs can result in both transcriptional and post-transcriptional suppression of gene expression. In plants short-RNAs are grouped into micro-RNAs (miRNAs) and three classes of endogeneous small-interfering RNAs (siRNAs): *trans*-acting siRNA (ta-siRNA), heterochromatic siRNA (hc-siRNA) and natural-antisense RNA (nat-siRNA). Further details on the short-RNAs have been recently published (Phillips et al. 2007). Hypotheses indicating an implication of miRNAs in plant responses to abiotic stress were first provided by Jones-Rhoades and Bartel (2004) who identified novel *Arabidopsis* miRNAs, which were predicted to target superoxide dismutase, laccases and ATP sulphurylases. Moreover, it has been demonstrated that various miRNAs are up or down-regulated by ABA, cold or dehydration (Sunkar and Zhu 2004). Further information concerning stress related small RNAs are summarized in reviews by Phillips et al. (2007), Sunkar et al. (2007) and Jones-Rhoades et al. (2006). Many questions related to the regulation of these small RNAs with respect to their processing and their stability are still open.

1.3.3.4 Reactive Oxygen Species (ROS)

Water deficit under high light leads to an active production of singlet oxygen ($^1O_2$) and superoxide radical anion ($O_2^{\cdot-}$) through the transfer of excitation energy from the photosynthetic systems PSII and PSI to molecular oxygen. This happens when the excess of energy cannot be dissipated via photosynthetic pathways and leads to free radicals generating processes and formation of ROS (Seel et al. 1992a, b; Smirnoff 1993). ROS subsequently interact with diverse macromolecules in the cell leading to cell death in extreme cases (Mundree et al. 2002). To cope with the detrimental effects of ROS, plant cells have evolved various detoxifying systems, both enzymatic and non enzymatic. Besides the negative effects of ROS in the cell, these reactive species have been shown to act as signal molecules. They have been shown to mediate the systemic activation of gene expression in response to pathogen attack (Alvarez et al. 1998), wounding (Orozco-Cardenas and Ryan 1999), high light (Mullineaux and Karpinski 2002) and stomatal closure (Pei et al. 2000). Moreover singlet oxygen, superoxide anion and hydrogen peroxide (H_2O_2) were shown to induce the transcription of specific sets of genes in plant cells (Gadjev et al. 2006). ROS signalling systems also interact with other pathways, like phosphorylation cascades by kinases or Ca^{2+}

signalling. ROS signalling is part of the regulatory network that connects the cell to its environment (Mittler 2002). As consequence of the dual role of ROS, plant cells require at least two different mechanisms to regulate their intracellular ROS concentration: the scavenging of the excess of reactive species (in stress conditions) and the modulation of ROS to low levels for signalling purposes. Many current studies related to ROS have been focused on this issue attempting to elucidate mechanisms responsible of such a balance. Navrot et al. (2007) have recently published a review on the current knowledge on ROS production and scavenging pathways.

1.3.3.5 Aldehydes and the peroxidation of membrane lipids

Aldehydes are often volatile molecules that are generated *in vivo* through the normal cell metabolism. Aldehyde molecules are potentially toxic due to their extreme reactivity with the nucleophilic compounds (nucleic acids, proteins, membrane lipids) present in different cell compartments (Skibbe et al. 2002). Several aldehyde molecules have been reported to be harmful to cells at various levels. These are acetaldehyde, glyceraldehyde-3-phosphate, p-nitrobenzaldehyde, glycoladehyde, phenylacetaldehyde, malondialdehyde (MDA), succinic semialdehyde, propionaldehyde and 4-hydroxy-trans-2-nonenal (4-HNE or HNE) (Ting and Crabbe 1983; Trivic and Leskovac 1994; Hu et al. 2002).

Besides the metabolism, aldehydes are also produced at high levels from the non-enzymatic lipid peroxidation under stress conditions (Bartels 2001; Barclay et al. 1994). In the lipid peroxidation, ROS such as superoxide anion radical and hydrogen peroxide that can directly oxidize lipids are converted to the highly reactive hydroxyl radical ($OH^{\cdot}$) *in vivo* through the Fenton and Haber-Weiss reactions. This radical readily attacks membrane lipids and initiates a radical chain reaction with the poly-unsaturated fatty acids (PUFAs), mainly linoleic and linolenic acids. The PUFAs easily undergo free-radical-catalyzed oxidation to yield racemic peroxy fatty-acid radicals. These radicals may remove a hydrogen atom from adjacent PUFAs in an autocatalytic process, thereby propagating the radical chain reaction and leading to the accumulation of hydroperoxides, predominately in membranes but also in the cytosol. PUFA hydroperoxides and peroxy radicals that have more than two double bonds can be further oxidized, and can undergo intramolecular radical chain reactions to yield unstable bicyclic endoperoxy hydroperoxides similar to prostaglandins in animal cells. These compounds are named phytoprostanes in plants. G1-phytoprostanes deriving from linolenate peroxy radicals are believed to be formed *in situ* in membrane lipids (Mueller 2004). Further spontaneous rearrangements of esterified G1-phytoprostanes leads to the generation of various

phytoprostanes and aldehydes (4-hydroxy-2-nonenal, hexanal, (E) 2-hexenal), of which malondialdehyde (MDA) is one of the end-products (Mueller 2004). Malondialdehyde has been detected in healthy *Arabidopsis* leaves at concentrations in the range of 4–5 nmol/g fresh weight and malondialdehyde has been widely used as a marker of free-radical-catalyzed lipid peroxidation (Weber et al. 2004). Many of the lipid peroxidation products including MDA contain an electrophilic (electron-accepting) α,β-unsaturated carbonyl group that can react with electron-donor (nucleophilic) atoms common to many biological molecules. As such, these molecules are termed reactive electrophile species (RES). As for MDA, it has been proposed that its reactivity depends on the intra-cellular pH, a parameter that can change in stress conditions (Farmer and Davoine 2007). These authors argued that under normal physiological conditions with neutral cytosolic pH, MDA remains a latent RES. Stress conditions cause the decrease of the cytosolic pH, thus leading to the protonation of MDA that becomes potentially reactive with two tautomeric forms of the molecule, one of which being the highly diffusible dialdehyde form. It is believed that MDA and other RES deriving from the non-enzymatic peroxidation of lipids are continuously generated in healthy plant tissues with a high turnover (Farmer and Davoine 2007). Despite their potential toxicity, those aldehydes and oxidized lipids generated during oxidative stress were proved to function as powerful gene activator (Sattler et al. 2006; Farmer and Davoine 2007; Mueller et al. 2008), indicating that a fine-tuning of the intra-cellular concentration of these compounds is very crucial for the cell viability. Findings on the generation, biological activities and mode of action of RES are recently reviewed by Mueller and Berger (2009).

1.3.3.6 Aldehyde dehydrogenases (ALDHs) as ROS-detoxifying enzymes

Abiotic and biotic stresses lead to oxidative stress due to the generation of ROS: hydrogen peroxide, hydroxyl radical and superoxide radical anion. Decreasing ROS levels and reducing their damaging effects appear to represent an important stress-tolerant trait. Indeed, an *Arabidopsis* mutant *pst1*, which exhibits increased salt stress tolerance, was found to have an increased capacity to scavenge ROS (Tsugane et al. 1999). ROS are eliminated in the cells through both enzymatic and non-enzymatic pathways. Non-enzymatic detoxification involves compounds such as ascorbic acid, gluthathione, thioredoxin and carotenoids, while the second pathway requires activities of enzymes like superoxide dismutase, catalase, gluthathione peroxidase and other peroxidases. But, recently it has been proposed that enzymatic scavenging of ROS may also involve proteins of the superfamily of aldehyde dehydrogenases (ALDHs) (Wenzel et al. 2008). Indeed, Ohsawa et al. (2003) showed that the deficiency in

ALDH2 increases susceptibility to oxidative stress in animal cells. Similarly, it was found that transgenic *Arabidopsis* plants over-expressing ALDH proteins are drought and salt stress tolerant compared to the wild type (Sunkar et al. 2003; Kotchoni et al. 2006). Unlike the wild type, these transgenic plants could survive on media supplemented with relatively high amounts of hydrogen peroxide. The ROS content of the transgenic plants measured or visualised in detached leaves was significantly lower than in control plants. Such results highlight the importance of ALDHs in both the cell metabolism and stress physiology and ask for deeper analyses.

1.3.3.7 Aldehyde dehydrogenase genes

Aldehydes could be detoxified either by the reduction of their carbonyl group to alcohol or by oxidation to the corresponding carboxylic acid (Perozich et al. 1999). The latter reaction is catalysed in plants by aldehyde dehydrogenase, $NAD(P)^+$ dependent enzymes (ALDH, EC 1.2.1.3), representing a large protein superfamily, of which members are widely distributed in all organisms including human and plant genomes (Yoshida et al. 1998; Vasiliou et al. 1999; Sophos and Vasiliou 2003). The ALDH superfamily is classified on the basis of their substrate specificity. With respect to this specificity, some ALDHs are known as non-specific ALDHs and as such react with a wide range of aliphatic and aromatic substrates. These include the tetrameric class 1 and 2 ALDHs (cytosolic and mitochondrial) and dimeric class-3 ALDHs (Yoshida et al. 1998). Substrate-specific ALDHs include all the semialdehyde dehydrogenases (SemiALDHs) and betaine aldehyde dehydrogenase (BADH). Although ALDH proteins have been well characterised in humans and their roles have been established as general detoxifying enzymes of biogenic and xenobiotic aldehydes, much remains to be researched on plant aldehyde dehydrogenases with respect to their regulation and function in the plant stress physiology.

The *Arabidopsis* genome contains 14 ALDH genes encoding members of nine ALDH protein families, which comprise eight known families and one novel family (ALDH22) that is so far found only in plants (Kirch et al. 2004). In 1996, Cui et al. isolated the first ALDH gene (*Rf2a*) from maize which encodes a mitochondrial class-2 ALDH. Its activity promotes male gametogenesis and probably participates in anther development (Liu et al. 2001; Liu and Schnable 2002). Other ALDH encoding genes have been identified from plants under stress conditions. For example, an ABA and dehydration inducible aldehyde dehydrogenase gene with homology to class-3 ALDHs has been isolated from the resurrection plant *C. plantagineum* (Kirch et al. 2001). The genes *ALDH3I1* and *ALDH3H1* belonging to class-3

ALDHs were shown to be transcriptionally up-regulated upon dehydration, high salinity or ABA in *Arabidopsis* (Kirch et al. 2001). In addition, the *ALDH7B4* gene that codes for a turgor-responsive ALDH and belongs to family 7 ALDH proteins, showed a strong induction by osmotic stress and ABA (Kirch et al. 2005). Over-expression of ALDH3I1 or ALDH7B4 in *A. thaliana* has conferred osmotic and oxidative stress tolerance to transgenic plants (Sunkar et al. 2003; Kotchoni et al. 2006). A summary of ALDH encoding genes isolated so far from plant species was presented by Kirch et al. (2004). The cellular functions of most of these identified genes remain to be established.

1.3.3.8 Betaine aldehyde dehydrogenases

One of the best studied ALDH coding genes in plant species are betaine aldehyde dehydrogenases (BADH). They have been shown to be expressed in various plants under abiotic stress prior to the accumulation of glycine betaine (Chen and Murata 2002). *A. thaliana* does not accumulate GB, although its genome encodes two members of the ALDH10 protein family, ALDH10A8 and ALDH10A9, which code for BADH proteins based on sequence similarities (Sakamoto and Murata 2002; Kirch et al. 2004). It has been suggested that the non-accumulation of glycine betaine is due to the lack of a functional choline monooxygenase (Nuccio et al. 1998). Previous studies have shown that the *Arabidopsis* choline monooxygenase-like protein (accession nos. NM_119135 and CAB43664) could not complement *Escherichia coli* strains lacking the choline dehydrogenase gene (Hibino et al. 2002). It was therefore suggested that the physiological role of the *Arabidopsis* CMO-like protein might be different from choline oxidation (Hibino et al. 2002). Yet, the functions of the *Arabidopsis ALDH10A8* (*At1g74920*) and *ALDH10A9* (*At3g48170*) genes are so far unclear. A number of recent studies have shown that some BADHs or BADH homologous proteins possess affinity for a range of aminoaldehyde substrates (Trossat et al. 1997; Sebela et al. 2000; Fitzgerald et al. 2009 and references therein). It has been suggested that plant BADH enzymes that possess an AMADH activity are part of the polyamine catabolism and are involved in stress responses through GABA synthesis (Bouchereau et al. 1999; Cona et al. 2006; Petrivalsky et al 2007). Consequently, Fitzgerald et al. (2009) proposed that plant enzymes classified as BADHs belong to two subfamilies: i) the true BADHs comprising proteins with activity and high specificity for betaine aldehyde as substrate and ii) the high BADH homology-aminoaldehyde dehydrogenases (HBH–AMADHs) grouping BADHs that show broader affinity for a range of aminoaldehydes. Whether *Arabidopsis* BADHs possess AMADH activity is so far unknown.

1.3.3.9 Aminoaldehyde dehydrogenases and the polyamine metabolism

Aminoaldehydes can be generated through oxidation of polyamines by copper amine oxidases (CAOs, EC 1.4.3.6) and FAD-containing polyamine oxidases (PAOs, EC 1.5.3.11) (Bouchereau et al. 1999). The oxidative cleavage of the polyamines spermidine and spermine by plant PAOs results in the formation of 4-aminobutyraldehyde (ABAL) and 4-(3-aminopropylamino) butyraldehyde, respectively, with the concomitant formation of 1,3-diaminopropane and H_2O_2 (Sebela et al. 2000). Further oxidation of 1,3-diaminopropane generates 3-aminopropionaldehyde (APAL), a direct precursor of β-alanine, which can be, in turn, trimethylated to yield the osmoprotectant β-alanine betaine (Duhazé et al. 2002; Cona et al. 2006). Aminoaldehydes are further metabolized by NAD-dependent aminoaldehyde dehydrogenases (AMADHs, EC 1.2.1.19). For instance, ABAL is oxidized to 4-aminobutyric acid (GABA), which is a zwitterionic molecule that exists in an unbound form, and can adopt several conformations in solution, including a cyclic structure that is similar to proline. At physiological pH values, GABA is highly soluble in water (Shelp et al. 1999). The role of GABA in salt tolerance has been related to osmotic regulation, detoxification of reactive oxygen radicals, conversion of putrescine to proline and intracellular signal transduction (Smirnoff and Cumbes 1989; Kinnersley and Turano 2000; Bouché and Fromm 2004). Increased CAO and PAO activities have been reported in oat seedlings (Smith 1985), and a response to salt stress has been found to be associated with an increase of diamine propane in tomato leaf explants (Aziz et al. 1998). Similarly, heat stress led to increased levels of PAO and induction of arginine decarboxylase (a polyamine biosynthetic enzyme; EC 4.1.1.19). A concomitant increase of diamine oxidase activity and GABA formation from ABAL was also reported in soybean (Xing et al. 2007). Hence, it has been suggested that plant BADH enzymes that possess an AMADH activity are part of the polyamine catabolism and are involved in stress responses through GABA synthesis (Bouchereau et al. 1999; Cona et al. 2006; Petrivalsky et al 2007).

1.4 Objectives of the study

The objective of this study was to generate and characterize transgenic *A. thaliana* plants over-expressing selected ALDH genes then compare the ALDH over-expressors with their T-DNA insertion mutant counterparts, so as to understand the function of these genes in the development and stress physiology of plants. Moreover, it was planned to identify regulatory factors involved in aldehyde-induced expression of ALDH genes and their regulation patterns, since some preliminary experiments have indicated a putative feed-back *ALDH* expression

taking place when the plants are exposed to aldehydes. More specifically, the work has been divided into the following tasks:

1. Generate *Arabidopsis* transgenic plants over-expressing the ALDH3H1 protein and investigate the physiological characteristics under stress conditions.
2. Perform a functional analysis of *ALDH3H1* T-DNA insertion mutants and compare their physiological and developmental characteristics with those of constitutive ALDH3H1-expressing plants. This should elucidate the functional properties of the ALDH3H1 in the plant response to abiotic stress.
3. Select and characterize *ALDH10A8* and *ALDH10A9* knock-out plants in order to understand the role of these genes in stress physiology and development of *A. thaliana*.
4. Generate and characterize *A. thaliana* transgenic plants expressing the β-glucuronidase reporter gene under the control of the *ALDH7B4* gene promoter.
5. Investigate the responsiveness of the *ALDH7B4* gene promoter to selected aldehyde molecules using the *ALDH7B4-GUS* lines.
6. Perform EMS-based mutagenesis on transgenic plants carrying the *ALDH7B4 promoter::GUS* fusion constructs and generate a mutant population that will be screened to identify mutants affected in the *ALDH* gene activation by aldehyde molecules and other stressors.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant materials

Arabidopsis thaliana ecotype Col-0 was used as wild-type in this work. All transgenic plants were established in this ecotype. The *Arabidopsis* T-DNA insertion mutants used in this work are listed in the **Table 1**.

Table 1 List of the *Arabidopsis* T-DNA insertion mutants used in this study

Lines (ID)	Target gene	Collections	References
SALK_079892	ALDH10A8	SALK	Alonso et al. 2003
KO8-1 (175D06)	ALDH10A8	GABI-Kat	Rosso et al. 2003
KO8-2 (SK24056)	ALDH10A8	SASKATOON	Robinson et al. 2009
KO9-1 (SAIL_502_G10)	ALDH10A9	SAIL	Sessions et al. 2002
KO9-2 (SALK_066181)	ALDH10A9	SALK	Alonso et al. 2003
3h1-A (SAIL_832_A05)	ALDH3H1	SAIL	Sessions et al. 2002
3h1-B (KO69)	ALDH3H1	-	Rios et al. 2002
3h1-C (SAIL_828_D05)	ALDH3H1	SAIL_828_D05	Sessions et al. 2002

2.1.2 Chemicals

Chemicals used in this work were from the following companies: Amersham Buchler-Braunschweig, Boehringer-Manheim, Merck-Darmstadt, Pharmacia-Freiburg, Quiagen-Hilden, Sigma-Deisenhofen, Stratagene-Heidelberg, Biomol-Hamburg, Serva-Heidelberg, Carl Roth-Karlsruhe, Germany.

2.1.3 DNAs, vectors and bacteria

2.1.3.1 cDNAs

cDNAs were initially provided in the pBluescript II SK+/- vector (Stratagene, La Jolla, USA). This vector contains the β-lactamase gene that confers the resistance to ampicillin. The clone pda06974 (RAFL09-93-B10, RIKEN Institute; Seki et al. 2002) containing the full-length ALDH3H1 cDNA (Accession: AY072122) was kindly provided to me by Dr Kirch Hans-Hubert. The clones pda07810 (RAFL07-07-L09, RIKEN Institute) and pda01165 (RAFL05-07-N03, RIKEN Institute) containing respectively the ALDH10A8 (Accession: AY093071) and ALDH10A9 (Accession: AF370333) full-length coding sequences were kindly provided by Jessica Schmitz (2007).

2.1.3.2 Vectors

The plasmid vectors used are listed below. Molecular details of the vectors are provided in the appendix. All vectors used in this work are kept as plasmids at -20°C (Department of Molecular Physiology, Institute of Molecular Physiology and Biotechnology of Plants – IMBIO – University of Bonn). The bacteria are stored in glycerol cultures at -80ºC.

2.1.3.2.1 pJET1.2

This vector is from Fermentas (St. Leon-Rot, Germany) and was used to clone PCR products as described by the manufacturer.

2.1.3.2.2 pBT10-GUS

This vector (Sprenger-Haussels and Weisshaar 2000) contains the coding sequence of the reporter gene β-glucuronidase (GUS/*uidA*). This vector was used to generate the ALDH promoter-GUS fusion constructs. The vector contains the β-lactamase gene conferring the resistance to ampicillin.

2.1.3.2.3 pRTL2-GUS vector

This vector (Carrington et al. 1991) contains a dual 35S promoter, the tobacco etch virus (TEV) leader sequence (lacking the first 12 nucleotides from pTL-7SN), the *GUS* coding sequence and the 35S poly(A) signal. It was used to isolate the 35S promoter fragment that was then cloned in the pBT10-GUS. It contains the β-lactamase gene and can be selected by ampicillin.

2.1.3.2.4 pGJ280

The Green Fluorescent Protein (GFP) expression vector *pGJ280* was constructed by Dr G. Jach (Max-Planck-Institute, Cologne, Germany) and contains in the following order the CaMV35S promoter with a duplicated transcriptional enhancer, the tobacco etch virus translational enhancer, the GFP coding sequence (Tsien 1998) and the CaMV35S polyadenylation site (Reichel et al. 1996).

2.1.3.2.5 pET28a

This is an expression vector used for His-tagged protein over-expression (Novagen, Darmstadt, Germany).

2.1.3.2.6 pBIN19 and pROK2

The plasmid pROK2 (Baulcombe et al. 1986) is a binary vector derivative of pBIN19 (Bevan 1984; Frisch et al. 1995). pROK2 was used to generate ALDH3H1 over-expressing plants. pBIN19 was used to transform *Agrobacterium tumefaciens* with the ALDH- or 35S-GUS constructs. pBIN19 and pROK2 contain the *NPTII* gene coding for the enzyme neomycin phosphotransferase that confers the kanamycin resistance to *A. tumefaciens* cells and plants.

2.1.3.2.7 pPG-Tkan

The vector *pPG-Tkan* was provided by Jessica Schmitz (2007) and contains an engineered *Bam*HI site that allows fusing a target signal sequence in frame downstream to the GFP coding sequence. This vector was used to generate C-terminal GFP fusion constructs.

2.1.3.3 Bacteria

- *Escherichia coli* DH10B (Lorrow and Jessee 1990) Genotype: F-mcrA Ä(mrr-hsdRMS-mcrBC) 80d lacZÄM15 ÄlacX 74 endA1 recA1 deoR Ä(ara, leu)7697 araD139 galU galK nupG rpsL ë. This cell was used as host strain for cloning.
- *Escherichia coli* BL21 (Pharmacia, Freiburg) Genotype: F^- ompT hsdSB (rB^-mB^-) gal dcm (DE3). This bacteria was used to express the recombinant ALDH10A8 and ALDH10A9 proteins.
- *Agrobacterium tumefaciens* GV3101/pmP90RK (Koncz and Shell 1986). This strain was used for transient and stable transformations of wild-type *A. thaliana* (ecotype Col-0) plants.

2.1.4 Enzymes and DNA-marker

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

2.1.5 Software, programs and online tools

- Vector NTI Advance™ 10 (Informax Inc, 2006, North Bethesda, MD, USA).
- ImageQuant Version 5.2 software.
- SPSS for windows v.11.0.1.
- Spectral Imaging EZ-C1 Goldversion 3.20.
- Microsoft Office package 2003.
- ChloroP 1.1 (http://www.cbs.dtu.dk/services/ChloroP).
- SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP).
- TargetP 1.1 (http://www.cbs.dtu.dk/services/TargetP).
- PLACE Web Signal Scan (http://www.dna.affrc.go.jp/PLACE/signalscan.html).
- PlantCare (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

2.1.6 Machines and other devices

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

2.1.7 Membranes

DNA- and protein-blots were performed on the nitrocellulose membrane Protran BA-85 (0.45 µm; Whatman, Maidstone, UK). RNA-blots were done on the nylon membrane Hybond™ (Amersham Biosciences, Buckinghamshire, UK).

2.1.8 Kits

DNA fragments were isolated from agarose gels with the QIAEX II Gel Extraction Kit (Qiagen, Hilden, Germany) and NucleoSpin® Extract II (Macherey–Nagel, Düren, Germany). Midi- and Maxi-preps of plasmid DNA were done using the NucleoBond® Xtra Maxi Kit (Macherey–Nagel; Düren, Germany). PCR products were cloned using the CloneJET™ PCR Cloning Kit, (Fermentas; St. Leon-Rot, Germany). The RT–PCR was performed using the totalscript–OLS® Kit (OLS; Hamburg, Germany) and the RevertAid™ H Minus First Strand cDNA Synthesis Kit, (Fermentas; Burlington, CDA). The HexaLabel™ DNA Labeling Kit from MBI Fermentas (St. Leon-Rot, Germany) was used for ^{32}P-labeling of DNA probes. Site-directed mutations were generated with the QuikChange® II Site-Directed Mutagenesis Kit (Stratagene; Heidelberg, Germany).

2.1.9 Media, buffers and solutions

All media, buffers and solutions, if not otherwise stated, were autoclaved for 20 min at 120ºC at 1.5 bars.

2.1.9.1 Media

MS-medium (per litre):	4.6 g MS-salt; 20 g sucrose; 1 ml vitamin solution (see the composition on the next page); adjust pH to 5.8 with 1M NaOH; 8 g select-agar for solid medium.
LB-medium (per litre):	10 g peptone; 10 g NaCl; 5 g yeast extract; adjust pH to 7.5; 15 g bacto-agar for solid medium.
YEB (per litre):	5 g sucrose; 5 g of meat extract; 5 g peptone; 1 g yeast extract; 2 mM $MgSO_4$ (0.493 g $MgSO_4$); adjust pH at 7.0; 15 g bacto-agar for solid medium.

SOC (per litre):	2% (w/v) trypton; 0.5% (w/v) selected yeast extract; 10 mM NaCl; 10 mM $MgSO_4$; 10 mM $MgCl_2$.

2.1.9.2 Buffers and solutions

Vitamin solution (Plant growth medium):	2 mg/ml glycine; 0.5 mg/ml Niacin (Nicotine acid); 0.5 mg/ml pyridoxin-HCl; 0.1 mg/ml thiamin-HCl. Use 1:1000 dilution of the autoclaved solution; store at 4°C.
50X TAE (Tris-Acetate-EDTA) buffer:	2 M Tris base; 100 mM EDTA, pH 8.0; adjust pH with glacial acetic acid.
RNase A:	10 mg/ml RNase A in milli-Q sterile water; store in aliquots at -20°C for further use.
Denaturing buffer:	1.5 M NaCl; 0.5 M NaOH; store at room temperature.
Neutralizing buffer:	1 M Tris; 1.5 M NaCl, pH 8.0; adjust with concentrated HCl; store at room temperature.
10X DNA loading buffer (10 ml):	25 mg Bromophenol blue; 25 mg Xylencyanol; 0.2 ml 50X TAE; 3 ml glycerine; 6.8 ml sterile distilled water.
10X MEN:	200 mM 3-(N-morpholino) propanesulfonic acid (MOPS); 80 mM sodium acetate: dissolve in water and adjust the pH to 7.0. Then add 0.5 M EDTA pH 8.0 to a final concentration of 10 mM; filter sterilise and store at room temperature. Protect against direct light exposure.
20X SSC:	3 M NaCl; 0.3 M Sodium citrate; store at room temperature.

1X TE buffer:	10 mM Tris-HCl; 1mM EDTA, pH 8.0; store at room temperature.
RNA-, DNA-blot washing buffer:	0.1% (w/v) SDS; 2X SSC; store at room temperature.
100X Denhardt's:	2% (w/v) BSA (fraction V); 2% (w/v) Ficoll-400; 2% (w/v) PVP 360,000. Store in aliquots at -20°C.
Ampicillin (stock solution):	100 mg/ml in water; filter sterilize and store at -20°C; working solution: 1:1000 dilution.
Kanamycin (stock solution):	50 mg/ml in water; filter sterilize and store at -20°C; working solution: 1:1000 dilution.
Rifampicin (stock solution):	50 mg/ml in DMSO; store at -20°C; working solution: 1:500 dilution.
IPTG (stock solution):	100 mM in water; filter sterilize and store at -20°C; working solution: variable concentration.
X-Gal:	40 mg/ml in N, N-dimethylformamide (DMF); protect from light; store at -20°C.

2.2 Methods

2.2.1 Growth conditions

2.2.1.1 Seed culture and plant growth

Plants were grown under approximately 120-150 µE m^{-2} s^{-1} light at 22°C with a day/night cycle of 8/16h if not otherwise stated. For flowering 4-5 week-old plants were moved to a growth chamber with a 16/8h photoperiod. For sterile seed culturing, seeds were surface sterilized in 70% (v/v) ethanol for 2 min then in 7% (v/v) NaOCl (Carl Roth; Karlsruhe, Germany) + 0.1% (w/v) SDS for 10 min, rinsed four times in sterile distilled water and sown on MS-agar plates (Murashige and Skoog 1962). Transgenic ALDH3H1over-expressor seeds were selected on solid media containing 50 µg/ml kanamycin. For soil-based experiments 14 day-old seedlings were transferred into soil-pots and then subjected to various abiotic

stressors. Plant materials were collected and used either immediately or frozen in liquid nitrogen and stored at -70°C.

2.2.1.2 Growth of microorganisms

All *E. coli* strains were cultured at 37°C either in liquid LB medium at 200-300 rpm or on solid LB-agar medium. *Agrobacterium tumefaciens* strains were grown at 28°C in liquid YEB medium at 250 rpm or on solid YEB-agar medium. The cultures were supplemented with appropriate selection markers if required.

2.2.2 Primers

All primers were synthesized from Sigma-Genosys (Steinheim, Germany) and dissolved in sterile water to 100 µM end-concentration. The primer sequences listed in this section are oriented from 5' end to 3' end. The enzyme restriction sites were highlighted in grey. The mutated nucleotides are underlined.

Table 2 List of the primers

Name	Sequence	Restriction site
Generation and analysis of the 35S-ALDH3H1 expressors		
35S-fwd	CAG GTC CCC AGA TTA GCC TT	-
35S-rev	TCC CCC GTG TTC TCT CCA AA	-
CaMV35S-sense (sequencing)	CCC ACT ATC CTT CGC AAG ACC	-
pROK-NPTII-fwd	TGG ATT GCA CGC AGG TTC TC	-
pROK-NPTII-rev	TGG GCG AAG AAC TCC AGC AT	-
Aldh4-RT-fwd (P4)	CGT TTC GCC GGA CTA TAT CTT GAC G	-
Ath-Aldh1c-anti (P5)	TCA ACC AAC TAA GTC CAT GTT TGA	-
Generation and analysis of GUS-fusion constructs		
Aldh7B4 prom 5'	TCC CAC TAC TGA ATT GAC CTT CA	-
Aldh7B4 prom 3'	CTC TGC GCA AGA ATT CAC CCC A	*Eco*RI
5'-pBT10-GUS-fwd	AAT ACG CAA ACC GCC TCT	-
pBIN-HindIII (H_0)	AGC TAT GAC CAT GAT TAC GCC AAG	-
pBIN-EcoRI	CGA TTA AGT TGG GTA ACG CCA GG	-
GUS-Start	GGT TGG GGT TTC TAC AGG ACG	-
GUS-sense	CGT CCT GTA GAA ACC CCA ACC	-
GUS-rev	GAT AGT CTG CCA GTT CAG TTC G	-
INT-Xba-fwd	GCGACGTCTAGAGTTAGGATTCTTTTTTCTCTC	*Xba*I
INT-Xba-rev (I_0)	CTGAGCTCTAGATAAATGAGGAAAGGTCAGTG	*Xba*I
Mutagenesis primers		
7B4_ABRE1_fwd	ACG AAA GCA TAG GAC A**TT** TGA CAC ATG TGA TG	-
7B4_ABRE1_rev	CAT CAC ATG TGT CA**A** **A**TG TCC TAT GCT TTC GT	-
7B4_DRE_fwd	GAT CGT GGC AAT **AAA** **T**AT GAT AAC AAC AAC TCC	-
7B4_DRE_rev	GGA GTT GTT GTT ATC AT**A** **TTT** ATT GCC ACG ATC	-
7B4_AB_fwd	TTA CAA AAG A**TT** TAC A**TT** TCT CTC TCT CTC ACT C	-

7B4_ABRE1_rev	GAG TGA GAG AGA GAG A**AA** TGT A**AA** TCT TTT GTA A	-
His-Tag protein fusion constructs		
AY093071_BamHI_Fwd	ACGGCGAATGGAGAGGATCCATCTT	*Bam*HI
AY093071_SacI_rev	GAGATTTGTACGAGCTCCAGGGAT	*Sac*I
AF370333_NdeI_Fwd	GAGAGAGAGAGAGCATATGGCGAT	*Nde*I
AF370333_EcoRI_rev	CTCAGAGCTTGGAATTCGGTTTGT	*Eco*RI
Analysis of T-DNA insertion mutants and RT-PCRs		
Gene-specific primers		
Ath-ALDH1a-sense (P1)	AGA AGG TTT TTG GAT CGG CGG A	-
Ath-ALDH2-sense	ATC GGC GGA AGC GAG TAA TTT GGT G	-
Ath-ALDH2-anti	TAT GGC GGA TAC CTG ACG GCT GAA TC	-
putExon2-ALDH3H1_fwd (P2)	ATG TTT TAC CAA CAG AGA GTA C	-
Exon3-ALDH3H1_fwd (P3)	CAG CTA AAG AAC TGG ATG GCT C	-
Aldh9-fwd (A3)	ACA ATG GCG ATT CCG ATG CCT ACT C	-
Aldh9-rev (A4)	GTT TCA GGA CCG AAC TAG ACA GAC TGA	-
AY093071_RT_fwd (A1)	GATCTTGCATGGTGGTTCCCGA	-
AY093071_ RT_rev (A2)	AAGCACAAAGATTTGAACAGACAGC	-
10A8_RT2-rev	CCT TCT TCC ATG GGG TCT GA	-
10A8_mid_seq	TTG TCG AGA AGT TGG TCT TCC TCC	-
Aldh8-fwd (B3)	TCA CCA CCT CTA GTA GCA GAG AGA G	-
Aldh8-rev (B4)	GTC TCA GAG CTT GGA AGG AGG TTT G	-
KO10A9_fwd (B1)	ATT GTT CCG TGG AAA TGG GGT GA	-
KO10A9_rev (B2)	TTG GGA CTT TCC GTT TTG GT	-
10A9_mid_seq	TGG TTT AGG AAC TGA AGC AGG TGC	-
AF370333_RT_fwd	TGTTCTTTGTGGAGGAGTTCGTC	-
AF370333_RT_rev	GAAGGGTCTCTTGCTTTATTGGT	-
Ath_Actin2_fwd mitte	GGA ATC CAC GAG ACA ACC TAT AAC	-
Ath_Actin2_rev	GAA ACA TTT TCT GTG AAC GAT TCC T	-
At1g74910_RT_fwd	CGT GTT TCC AGC TTC GAA GCT CTT C	-
At1g74910_RT_rev	GAC GCG GGA CCA TCT CCC GAT AGA	-
T-DNA specific primers		
FISH1 (T1)	CTG GGA ATG GCG AAA TCA AGG CAT C	-
pROK-FISH2 (T2)	CAG TCA TAG CCG AAT AGC CTC TCC A	-
pROK-Lba3 (T3)	ACC CAA CTT AAT CGC CTT GCA GCA C	-
Gabi_10A8_fwd	CTG TGA CTA CAG TCA GCC GTG	-
Gabi_10A8_rev (G2)	GTT TCC GAG ATG GTG ATT GC	-
Sail_10A9_fwd	TAG AGT CGA CCT GCA GGC AT	-
Sail_10A9_rev (S2)	CTG GCA AGT GTA GCG GTC AC	-
pSKTAIL-L3 (L3) (Robinson et al. 2009)	ATA CGA CGG ATC GTA ATT TGT CG	-
LB3 (S3) (Sessions et al. 2002)	TAG CAT CTG AAT TTC ATA ACC AAT CTC GAT ACA C	-
Generation and analysis of GFP-fusion constructs		
pGJ280_fwd	ACG AAT CTC AAG CAA TCA AGC A	-
pGJ280_rev	TGT GCC CAT TAA CAT CAC CA	-
p35S-pROK2	CAC TGA CGT AAG GGA TGA CGC	-
5mtALDH3H1-GFP	TTT TGA ATT CTA ATG TTT TAC CAA CAG AGA GTA CTA GTA TCT CTA CTG AGA AAC TC	*Eco*RI
3mtALDH3H1-GFP	CTG CCG CCA TGG TGA CAC GTC CGA	*Nco*I
10A8_Nco_fwd	AGA TTG GGC CAT GGC ACC TGG AGC TGT TCG TGC	*Nco*I
AY093071_rev_NcoI_ Nterm	CTC TTA AAA CTC TCC ATG GGA AG	*Nco*I
10A8-Bam_Fwd	ATA AGG ATC CAT GGC GAT TCC GAT GCC TAC TC	*Bam*HI

pPG-Tkan_fwd	AAC CAC TAC CTG AGC ACC CA	-
pPG-Tkan_rev	ACG AAA GCT CTG CAG CCA AC	-
Other primers		
pJET_for	CGA CTC ACT ATA GGG AGA GCG GC	-
pJET_rev	AAG AAC ATC GAT TTT CCA TGG CAG	-
T7 promoter_pET28a	TAA TAC GAC TCA CTA TAG GG	-
T7 terminator_pET28a	GCT AGT TAT TGC TCA GCG G	-

2.2.3 Extraction of nucleic acids

2.2.3.1 Extraction of genomic DNA from *A. thaliana*

Arabidopsis tissues (50 – 200 mg) were ground to a fine powder under liquid nitrogen in 2 ml Eppendorf tubes with metal beads. The plant material was then homogenized in 300 µl 2X lysis buffer (0.6 M NaCl, 0.1 M Tris-HCl, pH 8.0, 40 mM EDTA, pH 8.0, 4% Sarcosyl, 1% SDS), 300 µl 2 M urea and 30 µl of equilibrated phenol. After addition of one volume (600 µl) phenol-chloroform-isoamyl alcohol (25:24:1) the suspension was thoroughly mixed and centrifuged for 10 min, 14000 rpm at room temperature. DNA was precipitated from the supernatant with 0.7 volume of isopropanol and pelleted by centrifugation for 15 minutes at 14000 rpm, 4°C. The DNA pellet was washed twice with 70% ethanol, air-dried and dissolved in 25 µl of the re-suspension buffer (10 mM Tris-HCl, pH 8.0 containing 20-40 µg/ml RNAse A). Samples were briefly incubated at 37°C for 5 min to degrade contaminating RNAs. Optionally, RNase was removed by phenol-chloroform extraction and the DNA re-precipitated with absolute ethanol. One-tenth dilution of the samples was used for checking the quality on a 1% agarose gel and to estimate the amount of DNAs with a spectrophotometer. OD 260 and 280 nm were read for every sample. Samples were stored at -20°C.

2.2.3.2 Plasmid DNA mini-prep (Birnboim and Doly 1979; Sambrook et al. 1989)

The plasmid mini-prep from *E. coli* cells was done according to Sambrook et al. (1989). For plasmid preparations from *A. tumefaciens*, clones were inoculated in 2 ml YEB media containing appropriate selection markers and allowed to grow (28ºC, 250 rpm) for about 21 h. The culture was centrifuged at 6000 rpm, RT for 5 min. The bacteria pellet was resuspended in 400 µl solution I and incubated for 10 min at RT without shaking. The suspension was next carefully mixed with 800 µl Solution II and further incubated for 10 min at RT. To obtain high quality plasmid DNA mini-prep, 120 µl of solution IIa and 600 µl of 3 M sodium acetate pH 5.2 were added to the suspension and carefully mixed to avoid shearing the DNA. The mixture was incubated at -20ºC for 15 min then centrifuged at

14000 rpm for 10 min at 4 ºC. The supernatant, which contains the plasmid DNA, was carefully collected and divided into 3 aliquots of 650 µl. To each aliquot, 2 volume of cold absolute ethanol was added and incubated at -80ºC for 15 min. The aliquots were centrifuged (14000 rpm, 10 min at 4ºC); the pellet was resuspended in 500 µl 0.3 M sodium acetate pH 7.0, and 1 ml absolute ethanol. The suspension was incubated at -80ºC for 15 min then centrifuged (14000 rpm, 10 min, 4ºC). The DNA pellet was then washed twice with 70% (v/v) ethanol and air-dried at RT. The dried pellet was dissolved in 50 µl 10 mM Tris-HCl pH 8 containing 20 µg/ml of RNase A and incubated at RT for 15 min. 3 µl of the plasmid DNA were run on a 0.8% agarose gel to check the purity of the DNA.

Solution I: 50 mM Glucose; 10 mM EDTA; 25 mM Tris, pH 8.0; 4 mg/ml Lysozyme (freshly prepared).
Solution II: 0.2 M NaOH; 1% (w/v) SDS (always prepared freshly).
Solution IIa: 2 volume of solution II + 1 volume of Phenol.

2.2.3.3 Purification and precipitation of DNA

To purify a DNA sample from protein residues and other contaminants, the sample was brought to 100-200 µl with sterile distilled water. One volume of phenol/chloroform/isoamyl alcohol (25/24/1) was then mixed with the suspension. After centrifugation at 14000 rpm and room temperature for 5 min the supernatant (upper aqueous phase) was removed and mixed with 0.1 volume 3 M sodium acetate pH 5.2 and 2 volumes of absolute ethanol then incubated at –20°C for 20 minutes. The mixture was centrifuged (14000 rpm, 20 min, 4 °C); the pellet was washed in 70% (v/v) ethanol, air-dried and dissolved in 20 µl sterile Tris-Cl 10 mM, pH 8.0. The quantity of DNA was assayed spectrophotometrically at OD 260 and 280 nm and the samples were stored at -20°C.

2.2.3.4 Extraction of DNA fragments from agarose gels

After enzymatic digestion or PCR amplification of plasmid DNA constructs, the DNA fragment was isolated from agarose gels using the QIAEX II Qiagen extraction Kit or the NucleoSpin® Extract II Kit. The extraction and purification were done after excising the bands from the agarose gel according to the instructions of the kit manufacturer.

2.2.3.5 Extraction of total RNAs from *A. thaliana*

Total RNAs were isolated from about 100 mg *Arabidopsis* seedlings or leaves. The plant tissues were ground to a fine powder under liquid nitrogen with metal beads and resuspended in 500 µl of the extraction buffer (6 M urea, 3 M LiCl, 10 mM Tris-HCl, pH 8.0 and 20 mM EDTA, pH 8.0). Then, one volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added. The suspension was thoroughly mixed and centrifuged for 5 min at 14000 rpm, 4°C. The aqueous phase was successively re-extracted with one volume of phenol-chloroform-isoamyl alcohol and chloroform-isoamyl alcohol (24:1). RNAs were precipitated from the supernatant by adding 0.1 volume of 3 M Na-acetate, pH 5.2 and 0.7 volume of isopropanol. The mixture was kept on ice for 5 min. After centrifugation at 14000 rpm and 4°C for 10 min, the RNA pellet was washed twice with 70% (v/v) ethanol, dried and dissolved in 20-30 µl sterile distilled water.

2.2.4 Qualitative and quantitative estimation of concentrations of macromolecules

2.2.4.1 Qualitative and quantitative estimation of DNA and RNA

The DNA samples were qualitatively monitored in 1% (w/v) agarose gel electrophoresis using 1 kb ladder as a reference. The concentration of the nucleic acids was assayed with spectrophotometer at ODs 260 and 280 nm. A value of OD_{260} = 1 approximately corresponds to 50 µg/µl for a DNA solution or 40 µg/µl for a RNA solution. For a pure DNA sample, the value of OD_{260}/OD_{280} is found between 1.8 and 2. A value of OD_{260}/OD_{280} below 1.8 usually indicates a contamination of DNA preparation with proteins or phenol compounds.

2.2.4.2 Quantitative estimation of protein extracts

Protein concentrations were determined using a Bio-Rad protein assay Kit according to 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. For protein-blot analyses where the Laemmli buffer (1970) 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).

2.2.5 Cloning of DNA fragments

2.2.5.1 Polymerase chain reaction (PCR)

DNA fragments were amplified from various plasmid and genomic sources. A standard PCR reaction in a total volume of 50 µl was prepared as followed:

30-35 µl	H_2O (sterile double distilled)
5.0 µl	10X PCR-buffer
1.5 µl	50 mM $MgCl_2$
2.0 µl	Forward-primer (10 pmol/µl)
2.0 µl	Reverse-primer (10 pmol/ µl)
1.0 µl	10 mM dNTPs
1.0 µl	plasmid DNA (5 ng/µl) or genomic DNA (50-100 ng/ µl) or a bacterial colony
0.5 µl	Taq-polymerase

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

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

TA = annealing temperature = TM ± 4 °C

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

2.2.5.2 Restriction endonuclease treatments

DNA digestion was carried out by restriction endonucleases according to the following criteria: the reaction buffer (10X) was 1/10 of the end volume and 5 U of restriction enzymes was used per 1 µg of DNA to digest. A double digestion was possible per reaction only when

both restriction enzymes can be active in the same buffer; otherwise the digestions were performed sequentially.

2.2.5.3 Dephosphorylation

Linear plasmid vectors were dephosphorylated at their 5' end with shrimp alkaline phosphatase (SAP; Boehringer/Roche, Mannheim, Germany) in order to avoid self-ligation of their cohesive or compatible ends. The reaction was made in 10 µl reaction comprising 1 µl of 10X SAP buffer, 1.0 µl (1 unit) SAP, and adequate amount of the plasmid vector. The mixture was brought to 10 µl with sterile distilled water. The reaction was incubated for 10 min at 37°C followed by inactivation of SAP at 65°C for 15 min.

2.2.5.4 Ligation

To generate recombinant plasmid DNA constructs, the insert-DNA was ligated to a linear vector in a ligation reaction. The ligation was performed in 20 µl reaction volume containing 1X ligase buffer, x µl digested and purified plasmid DNA vector, 1.0 µl T4 DNA ligase (MBI-Fermentas; St. Leon-Rot, Germany or Invitrogen/GibcoBRL; Karlsruhe, Germany), and y µl insert-DNA. The mixture was brought to 20 µl with sterile H_2O and incubated at 16°C for 20 h or according to the ligase manufacturer. For an optimal ligation reaction the molar amount of plasmid vector should be the third of the insert-DNA in the reaction.

2.2.5.5 Transformation

2.2.5.5.1 Calcium-competent *E. coli*

A bacterial culture (100 ml) was allowed to grow (37°C, 250 rpm) till OD_{600} = 0.5, cooled down on ice for 5 min and centrifuged (5 min, 5000 rpm, 4 °C). The pellet was suspended in 1 ml pre-chilled 0.1 M $CaCl_2$ and centrifuged once again as above. The pellet was resuspended in 9.0 ml pre-chilled 0.1 M $CaCl_2$ and centrifuged as above. The pellet was finally resuspended in 1 ml pre-chilled 0.1 M $CaCl_2$ + 15% (v/v) glycerol and stored at -70°C in aliquots of 100 µl of competent cells.

2.2.5.5.2 Transformation of calcium-competent *E. coli*

One µl plasmid DNA (5-10 ng/µl) or 1-5 µl of the ligation product was added to one aliquot of calcium-competent cells (100 µl) and carefully mixed. The mixture was incubated on ice for 1 h and heat-shocked in a water bath at 42 °C for 45 seconds. Cells were diluted with 650 µl LB medium and incubated under agitation (250 rpm) at 37°C for 1 h. Aliquots (100-

200 µl) of the cell suspension were then spread on selective agar-plates and incubated at 37ºC overnight.

2.2.5.5.3 Preparation of electrocompetent *E. coli*

E. coli bacteria were inoculated in 10 ml LB medium and pre-cultured overnight (37°C, 250 rpm). On the following day, the pre-culture was used to start a fresh culture in 200 ml LB medium till OD_{600} = 0.6. The culture was then cooled down on ice for 30 min and centrifuged (5 min, 5000 rpm, 4°C). The cell pellet was washed in 50 ml pre-chilled sterile H_2O, centrifuged as above, re-washed in 25 ml of H_2O and centrifuged again. The pellet was further washed twice in 25 ml 10% (v/v) glycerol and resuspended in 10 ml cold GYT-medium. After centrifugation, the cell pellet was resuspended in 2 ml GYT. Aliquots (50 µl) of competent cells were shock-frozen in liquid nitrogen and stored at -70°C.

GYT: 10% (v/v) glycerol, 0.125% (w/v) selected yeast extract, 0.25% (w/v) trypton.

2.2.5.5.4 Preparation of electrocompetent *A. tumefaciens*

A. tumefaciens was inoculated in 3 ml YEB-Rif medium and pre-cultured for overnight (28°C, 250 rpm). Cells were pelleted, diluted into fresh YEB-Rif (50 ml) and further cultured till OD_{600} = 0.5. The cell culture was cooled down on ice for 30 min and centrifuged (5000 rpm, 4 °C) for 5 min. The pellet was resuspended in cold sterile H_2O and centrifuged as above. The cells were further resuspended in the following solutions with centrifugations (5000 rpm, 10 min, 4 °C) between the suspensions, as follows:

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
1 ml	10% (v/v) glycerol

Aliquots (40 µl) of the last suspension of electrocompetent cells were made, shock-frozen in liquid nitrogen and stored at -70°C.

2.2.5.5.5 Transformation via electroporation (Tung and Chow 1995)

Aliquots of electro-competent cells were thawed on ice before transformation. 1 µl DNA of the ligation product or plasmid DNA (approximately 5-10 ng/µl) was added to the electro-competent cells and carefully mixed in a pre-chilled Electro–cuvette (Bio-Rad, Germany). The DNA was brought into the cells by electroporation after a single pulse of 3 to 5 sec (GenePulser II, Bio-Rad). Cells were immediately diluted in 1 ml YEB-mediun or 800 µl SOC medium and cultured for another 1 h at 37ºC (for *E. coli*) or 2-3h at 28ºC (for *A. tumefaciens*) under agitation at 250 rpm. 100 µl aliquots of the cell culture were spread on selective media and incubated at appropriate temperatures (**Table 3**).

Table 3 Electroporation parameters of *E. coli* and *A. tumefaciens* cells

Transformation parameters	***E. coli***	***A. tumefaciens***
DNA	Ligated vector or plasmid	Only plasmid
Electro–cuvette	1 mm	2 mm
Capacity	25 µF	25 µF
Power	1.6 kV	2.5 kV
Resistance	200 Ω	400 Ω
Incubation Medium	SOC	YEB
Incubation temperature	37ºC	28ºC
Incubation time for selecting clones	12-16 h	48-72 h

2.2.5.5.6 Biolistic transformation of *Arabidopsis* leaves

The preparation of the microcarriers and DNA coating were performed according to Sanford et al. (1993) with a few modifications. Gold particles (1.6 µm diameter) were used as microcarriers for the bombardment and prepared as follows: 30 mg gold particles were weighted into a 1.5 ml microfuge tube and vigorously vortexed in 1 ml of 70% ethanol (v/v) on a platform vortexer for 3-5 min. The particles were further allowed to soak in the ethanol solution for 15 min and spun down (10,000 rpm) for 10 seconds in a microfuge. The supernatant was discarded and the gold particles were washed three times as follows: vigorous vortexing for 1 min in 1 ml sterile water, 1 min pause to let the particles to settle, recovery of particles by briefly spinning in a microfuge and removal of the liquid. After the third wash, 500 µl sterile 50% (v/v) glycerol were added to the particles to bring their concentration to 60 mg/ml, assuming no loss during the preparation. Prepared gold particles were stored at 4°C in 50 µl aliquots for up to one month without decrease in the transformation efficiency. Fifty microlitres of microcarriers were used for the coating procedure: 15 µl plasmid-DNA

(1 µg/µl), 50 µl of 2.5 M $CaCl_2$ and 20 µl of 100 mM freshly prepared spermidine (Sigma S0266, Munich, Germany) were in this order added to the gold suspension while vortexing for 3 min at maximum speed. The suspension was briefly centrifuged as above and the liquid discarded. The particles were washed with 140 µl 70% (v/v) ethanol, spun down, re-washed with 140 µl 100% ethanol and mixed, after centrifugation, with 30 µl 100% ethanol. The particles were gently resuspended by tapping the side of the tube several times then by vortexing at low speed for 2-3 seconds. Fifteen microlitres of the gold suspension were used for each bombardment.

The bombardment procedure followed the PDS-1000/He manufacturer's instruction (Bio-Rad, Munich, Germany). Briefly, a plastic macro-carrier disk containing 15 µl of DNA-coated gold particle (micro-carrier) suspension was placed into the macro-carrier holder along with a stopping metal grid. The system macrocarrier-microcarrier-stopping grid was placed into the launch assembly unit, as described by the manufacturer. Well-expanded *Arabidopsis* leaves were arranged on a plate of solid MS medium and placed at 5-10 cm below the stopping screen. Vacuum was then applied to increase the gas pressure within the bombardment chamber. The release of the pressure led to the burst of the rupture disk and allowed the macro-carrier to eject at high celerity the DNA-coated gold particles into the leaves. The particles were accelerated with a helium pressure of 1150 pounds per square inch (psi) under a vacuum of 27 mm Hg (3.6 MPa). After bombardment the leaves were incubated in water overnight and analysed under a confocal laser microscope within 48 h.

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

Ten to twelve day-old *Arabidopsis* seedlings were transiently transformed by the FAST (Fast *Agrobacterium*-mediated Seedling Transformation) technique based on the co-cultivation of *Agrobacterium* cells (GV3101::pMP90) harbouring 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. 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. 30 to 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% 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 in the case of *ALDH7B4* promoter study, as this gene is induced under prolonged dark conditions. After the co-cultivation period, the medium was replaced with the surface sterilization solution (0.05% sodium hypochlorite) and incubated for 10 min, washed three times with H_2O to remove epiphytic bacteria. Seedlings were finally incubated in 0.5X MS, 500 µg/mL carbenicillin to inactivate remaining *Agrobacterium* cells prior applying the stressors.

2.2.5.5.8 *A. tumefaciens*-mediated stable transformation of *Arabidopsis* plants

Arabidopsis transgenic plants were generated via *A. tumefaciens*-mediated transformation of wild-type (Col-0) plants according to Clough and Bent (1998). The *Agrobacterium* clone carrying the transgene was cultured (28ºC, 250 rpm) in 250 ml YEB/kanamycin/rifampicin (50 µg/mL kanamycin and 50 µg/mL rifampicin) until OD_{600} = 0.7 – 0.8. The cell suspension was added with 0.05% (v/v) of the surfactant Silwet L-77 and collected in a 500 ml beaker. Flowering plants with young inflorescences and un-opened flowers were carefully inverted and immersed in the infiltration medium with gentle rotation for 20 sec. Care was taken to immerge all the inflorescences in the solution. Dipped plants were thereafter returned to trays and covered with plastic bags. A few holes were made on the bags for ventilation. Three days after infiltration the plastic bags were removed and the plants were supported with wooden sticks and grown until they set the first generation of seeds (T1).

2.2.6 Screening methods

2.2.6.1 Blue-white screening of bacterial colonies

This screening method was used to visually identify positive transformants. It was performed after transformation with plasmids carrying the *LACZ* reporter gene. The *LACZ* gene codes for the enzyme β-galactosidase and can be induced by isopropyl thio-β-D-galactoside (IPTG). The β-galactosidase enzyme metabolizes the substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) to a blue product visible in bacteria colonies. The insertion of the transgene within the *LACZ* gene sequence hampers the synthesis of a functional β-galactosidase, making the recombinant clones to appear white in the presence of X-Gal and

IPTG, while untransformed clones become blue. LB-agar plates were first spread with 40 µl of X-Gal (40 mg/ml in N, N-dimethylformamide; DMF) and 40 µl of 100 mM IPTG before the cell plating.

2.2.6.2 Screening for transformed bacterial clones

Bacteria colonies were transferred onto fresh plates and assigned with different numbers, which were considered throughout the screening process. The colonies were either used as DNA source to amplify DNA inserts via PCR amplification (Colony-PCR) or used for individual plasmid DNA mini-prep. The extracted recombinant plasmid DNA was then analysed by restriction enzymes in order to confirm the presence of the insert-DNA and its correct orientation, if required. All plasmid DNA constructs were thereafter sequenced. Optionally, recombinant DNAs were further confirmed by DNA gel blot analysis using specific radioactive probes to detect the fragment of interest.

2.2.6.3 Screening for transgenic *Arabidopsis* seeds

After transformation, the first generation of dried seeds (T1 seeds) was collected. The seeds were surface-sterilized and sown on MS-agar plates containing 50 µg/ml kanamycin. Seeds were let germinate and grow for 15 days. Transgenic T1 seedlings resistant to kanamycin were easily detectable and appeared with green cotyledons and leaves. Instead, non-transgenic seedlings become yellow and died. Transgenic seedlings (T1 lines) were transferred into soil-pots and grown for the next generation of seeds (T2 seeds). Independent parents homozygous for the kanamycin resistance were identified among the T2 plants after scoring the kanamycin resistance rate of the T3 progeny.

2.2.6.4 Preparation of bacterial glycerol stocks

A bacterial colony of interest was picked from a selection plate and grown overnight at 37°C with shaking (220 rpm) in 2 ml LB medium with the appropriate antibiotic. On the day after, 0.5 ml of the bacterial culture was thoroughly mixed to 0.5 ml of autoclaved 100% (v/v) glycerin solution in an Eppendorf tube. The suspension was immediately frozen in liquid nitrogen and stored at -80°C.

2.2.7 Reverse transcriptase (RT)-PCR analysis

For RT-PCR analysis 2-4 µg of total RNAs were treated with 10 U RNase-free DNase I (Roche; Mannheim, Germany) in 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 Totals*cript*-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.

2.2.8 Electrophoresis and blotting methods

2.2.8.1 Agarose gel electrophoresis

The size and the quality of DNA and RNA extracted from plants or bacteria were analysed by 0.8-1.2% (w/v) agarose gel electrophoresis. DNA or RNA was loaded on the gel and separated by electrophoresis (small-size gel chamber: 65-70 mA, 45-60 min; mid-size gel chamber: 100-120 mA, 45-60 min) in 1X TAE buffer using a 1 kb DNA ladder as reference, if required. DNA fragments were visualized under UV light using ethidium bromide staining.

Agarose gel:	0.8-1.2% (w/v) agarose in 1X TAE buffer.
Ethidium bromide solution:	1 mg/l ethidium bromide in 1X TAE buffer.

2.2.8.2 DNA-blot analysis: (Sambrook et al. 1989)

Genomic DNAs were digested with appropriate restriction enzymes and size-fractionated on agarose gel electrophoresis. The gel was successively soaked in 0.25 M HCl for 15 min, alkaline denaturing buffer for 30 min and in neutralising buffer for 30 min with gentle shaking. The gel was blotted overnight on the nitrocellulose Protran BA-85 membrane (0.45 µm; Whatman, Maidstone, UK) using 10X or 20X SSC solution. The membrane was pre-hybridised at 65ºC for 3 h and hybridised overnight to a ^{32}P-labeled probe (see below) at 65ºC in the Southern hybridization buffer (Sambrook et al. 1989). The membrane was subsequently washed (2-3 x 20 min) in the washing buffer (2X SSC, 0.1% (w/v) SDS) and exposed to a Storage Phosphor Imager Screen (Amersham Biosciences; Buckinghamshire, England) for 1 to 6 days. The Phosphor Screen was scanned on Typhoon Scanner 9200 (Amersham Biosciences; Piscataway, NJ) and the picture was documented.

DNA hybridisation buffer (100 mL): 15 ml 4 M NaCl, 10 ml 0.1 M PIPES pH 6.8, 200 µl 0.5 M EDTA pH 8.5, 1 ml 10% (w/v) SDS, 10 ml 100X Denhardt's solution, 63.7 ml distilled H_2O, 100 µl hering sperm or ssDNA (single-stranded DNA).

2.2.8.3 RNA blot analysis

20-30 µg total RNAs were used in the RNA-blot assays. For 100 mL of 1% gel, 1 g of agarose powder was first boiled in 62 ml water, cooled down to 60°C and mixed with 20 mL 10X MEN and 18 mL deionised formaldehyde (37% p. A.). The gel was immediately poured and allowed to solidify at room temperature under a fume hood. RNA samples were diluted with one volume of the RNA-blot loading buffer, heated at 70°C for 5 minutes and immediately loaded onto the gel. The electrophoresis was performed in the RNA running buffer at 100 mA till the blue marker has migrated at least 8 cm from the top of the gel. The gel was directly blotted onto a nylon membrane (Hybond™-N, Amersham Biosciences; Buckinghamshire, UK) overnight using 10X or 20X SSC as Northern-transfer buffer according to Sambrook et al (1989) and Bartels et al (1990). Then, the membrane was allowed to dry shortly between Whatman papers and exposed to the UV-lamp for 3 minutes for cross-linking RNAs to the membrane. Optionally, the membrane was further baked at 80°C for 30 minutes, stained with methylene blue (see below) or directly kept within Whatman papers at room temperature in a cool and dry place. The membrane was pre-hybridized for 3 h at 42 ºC in a shaking water bath and hybridized overnight to a specific radioactive probe in the RNA hybridization buffer (50% (v/v) formamide, 5X SSC, 10 mM PIPES pH 6.8, 0.1% (w/v) SDS, 1X Denhardt's, 100 µl denatured hering sperm or ssDNA). Equal loading of the RNA samples was monitored by hybridizing the same membrane with an actin probe. The membrane was washed (2 x 20 min at 42 ºC and 1 x 20 min at 65 ºC) in RNA-, DNA-blot washing buffer (0.1% (w/v); SDS, 2X SSC).

RNA-blot loading buffer (1 ml): 50 µl 10X MEN, 175 µl 37% (p. A.) deionized formaldehyde, 500 µl formamide, 20 µl 10% (w/v) bromophenol blue and 255 µl 100% glycerin.

RNA running buffer: dissolve 100 ml 10X MEN in 820 ml sterile distilled H_2O; add 80 ml 37% (v/v) deionized formaldehyde to make one litre solution.

2.2.8.4 Staining of the RNA-blot membrane with Methylene Blue

To check the efficiency of the transfer, the RNA-blot membrane was stained with a methylene blue solution (0.04% Methylene Blue in 0.5 M sodium acetate, pH 5.2). The membrane was immersed in the solution for 5-10 minutes at room temperature with gentle shaking. The methylene blue solution was removed and the membrane washed with distilled water until appearance of clear blue-stained RNA bands. A photograph of the membrane was taken. The membrane was either immediately used for pre-hybridization or stored within Whatman papers. The stain could be completely removed from the membrane by washing with 0.1-1% sodium dodecyl sulphate (SDS) or with a pre-hybridization solution containing SDS.

2.2.8.5 Synthesis of α^{32}P-DNA hybridisation probes (Feinberg and Vogelstein 1983)

A cDNA fragment or a PCR fragment from the gene of interest was used to synthesize the probe. The PCR-fragment to use as probe was purified using the NucleoSpin® Extract II Kit. The Hexalabel™-labeling Kit (MBI Fermentas; St. Leon-Rot, Germany) was used for labelling the probe. Briefly, 10 µl of 10X hexanucleotides buffer were added to 100 ng DNA probe and H_2O to a final volume of 40 µl. The probe was denatured by heating for 5 min at 95ºC and immediately cooled down on ice. The reaction was mixed with 3 µl Mix C (dNTPs without dCTP), 2 µl α^{32}P-dCTP (10 µCi/µl) and 1 µl Klenow fragment. After incubation at 37ºC for 10 min, 4 µl dNTP-Mix was added to reaction followed by incubation at 37ºC for 5 min. The reaction was stopped by adding 50 µl of 1X TE pH 8.0. The labelled probe was separated from the non-incorporated nucleotides using a 1 ml Sephadex G-50 column pre-equilibrated with 1X TE buffer. Ten fractions of 100 µl eluates were collected. A Geiger counter was used to identify the eluates with high specific radioactivity. The hot eluates were pooled and the DNA probe was denaturated for 5 min at 95ºC, quickly cooled on ice and immediately used as radioactive probe for hybridisation.

2.2.8.6 Semi-quantitative analyses of the gene expression from the RNA blots

The intensity of the signals on the RNA-blots was quantified using ImageQuant Version 5.2 software. The signal intensity value for each sample and for a specific gene was divided by that of the *ACTIN-2* gene for the same sample. The resulting ratios for each sample were plotted versus the time in Microsoft Excel.

2.2.8.7 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 of Laemmli buffer (Laemmli 1970). 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 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-Cl pH 6.8; 10% glycerin; 2% SDS (w/v); 0.1% bromophenol blue and 0.7 M (≈ 5%) β-mercaptoehanol. Add freshly DTT at 0.1 M final concentration to the needed volume of the buffer just before use.

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

Recombinant proteins were extracted from *E. coli* BL21 (DE3) clones for enzymatic tests. Growing bacteria (OD_{600} = 0.4-0.5) were induced with 1 mM IPTG and further cultured at 22-26°C for 3 h in the dark. 1 ml-culture sample was taken just before and every hour after addition of IPTG then centrifuged at 8000 g, 4°C for 10 min. The supernatants were discarded and the stored at -20°C. The bacteria pellets were resuspended in 200 µl pre-chilled 1X PBS, 5 mM DTT and 1% Triton-X100. The suspensions were sonicated on ice (Branson Sonifier; 6 x 20 seconds) for complete lysis and centrifuged for 10 min at 12000 g, 4°C. 50 µl of the supernatant (soluble proteins) were diluted with one volume 2X Laemmli buffer (1970) while the pellets or "inclusion bodies" were dissolved in one volume 1X Laemmli buffer (1970). These samples were heated at 95°C for 10 min and immediately analysed by SDS-PAGE or stored at -20°C.

2.2.8.9 Extraction and purification of the recombinant ALDH proteins by His-tag affinity-chromatography

Soluble recombinant ALDH proteins were purified by metal ion chromatography on His-tag binding columns under native conditions. The bacteria pellet from 100 ml culture previously treated with IPTG was resuspended in 5 ml extraction buffer (50 mM HEPES-NaOH pH 7.4, 300 mM NaCl, 10% v/v glycerol, 0.1% v/v Triton X-100, pH 8.0; 1.5 mM β-mercapto-ethanol added freshly) + 1 mg/ml lysozyme and 5 mM imidazole, incubated for 30 min on ice and sonicated for complete lysis. The homogenate was centrifuged for 30 min at 12000 g, 4°C and the supernatant filtered through a 0.45 µ membrane. The column was first washed with

3 volumes H_2O, 5 volumes 50 mM $NiSO_4$ and 3 volumes extraction buffer. The filtered supernatant was loaded onto the column and allowed to drain freely by gravity. The column was washed with 10 volumes buffer A and 8 volumes buffer B. The protein was eluted with the buffer C in 250 µl fractions and stabilised by adding 1 mM PMSF, 0.5 mM NAD, 6 mM DTT and glycerol to 50% (v/v). The purity of the protein fractions was verified by SDS-PAGE analysis and the quantity of eluted proteins was estimated by the Bradford assay. Aliquots of the non-purified supernatant and of the flow-through were analysed along with the protein fractions. The column was regenerated with 100 mM EDTA, pH 8.0; 500 mM NaCl; 20 mM Tris-HCl, pH 8.0. All buffers and solutions used for the assay were filter-sterilized (0.45µ).

Buffer A: extraction buffer + 5 mM imidazole
Buffer B: extraction buffer + 20 mM imidazole
Buffer C: extraction buffer + 250 mM imidazole

2.2.8.10 Aldehyde dehydrogenase activity of the recombinant ALDH protein

Affinity-purified ALDH proteins were used for the enzymatic tests. Enzymatic activities were assayed with the spectrophotometer at 340 nm by monitoring the conversion of NAD^+ to NADH at room temperature in 0.5 ml reaction. The reaction contained 100 mM sodium pyrophosphate pH 8.0, 1.5 mM NAD (Roche, Mannheim, Germany) and variable amounts of betaine aldehyde, 4-aminobutyraldehyde and 3-aminopropionaldehyde. Betaine aldehyde chloride was purchased from Sigma-Aldrich (Steinheim, Germany) and directly used in the enzymatic assay. 4-aminobutyraldehyde was prepared from its diethyl acetal (Sigma-Aldrich, Steinheim, Germany) and 3-aminopropionaldehyde from 1-Amino-3, 3-diethoxypropane (Alfa Aesar®, Karlsruhe, Germany). The acetals were hydrolysed at 100°C in 0.1 M HCl in a plugged test tube for 10 min. The reaction was initiated by adding the affinity-purified protein. To determine the kinetic parameters the extinction coeffcient ε for NAD(P)H = 6.22 mM^{-1} cm^{-1} was used. Kinetic constants (Km and Vmax) were calculated assuming Michaelis-Menten-type performance of the enzyme and estimated from Hanes plots (Rudolph and Fromm 1979).

2.2.8.11 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-PAGE was performed according to Laemmli (1970). The gel was made 4% (w/v) acrylamide stacking gel and 12% (w/v) acrylamide separating gel as described below. Protein

samples were boiled for 5 min at 95°C before loading onto the gel (10 cm x 10 cm). The gels were run with 1X SDS-protein running buffer for about 2 h at 10-15 mA in the stacking gel and 20-25 mA in the separating gel. The protein ladder (Fermentas; Burlington, CDA) used contains: β-galactosidase (*E. coli*; 116.0 kDa), Bovine serum albumin (bovine plasma; 66.2 kDa), Ovalbumin (chicken egg white; 45.0 kDa), Lactate dehydrogenase (porcine muscle; 35.0 kDa), Restriction endonuclease *Bsp98I* (*E. coli*; 25.0 kDa), β -lactoglobulin (bovine milk; 18.4 kDa) and Lysozyme (chicken egg white; 14.4).

Table 4 Composition of the SDS-PAGE gel

Stock solution	4% Starking gel (**3 mL**)	12% Separating gel **(7.5 mL**)
Sterile H_2O	2.16 ml	1.92 ml
30%(v/v) Acrylamide	0.50 ml	2.4 ml
1 M Tris-Cl pH 6.8	0.38 ml	-
1.5 M Tris-Cl pH 8.8	-	1.56 ml
10% (w/v) SDS	30 µl	60 µl
10% (w/v) APS	30 µl	60 µl
TEMED	3 µl	2.4 µl

1X SDS-protein running buffer, pH 8.2: 25 mM Tris; 192 mM Glycine; 0.1% SDS. Do not adjust the pH!

2.2.8.12 Coomassie blue staining of SDS-PAGE

The SDS-PAGE was stained with the PageBlue^{TM} Protein Staining Solution from Fermentas (Vilnius, Lithuania). After electrophoresis the gel was placed in a tank and washed three times with distilled water for 10 minutes. Then, 20 ml of the PageBlue^{TM} Protein Staining Solution was added to the gel with gentle agitation for 1 hour or overnight. The staining solution was then removed and the gel rinsed 1-3 times 5 min with 100 ml of distilled water till the gel background has become clear. The staining solution was used up to three times without any decrease of the staining efficiency. Alternatively, the gel was stained with Coomassie blue G-250 according to Zehr et al (1989). The gel was gently submerged in Coomassie staining solution and kept shaking overnight. The gel was then fixed for 1-2 hours, washed 3 times 10 min with water and incubated in the staining solution on a shaker overnight. The gel was distained after several washes with distilled water.

Fixing solution:	50% (v/v) methanol; 10% (v/v) acetic acid.
Coomassie 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 part Staining stock solution + 1 part methanol

2.2.8.13 Ponceau-Red Staining

The Ponceau-Red staining was performed on the Western-blot membrane after protein blotting and before the immuno-detection assay. 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) and gently shaked for 5-10 min. The staining solution was removed and the membrane distained with H_20. The membrane was scanned and the positions of the standard proteins (in the protein size marker) were marked with a pencil.

2.2.8.14 Protein-blot analysis

To specifically detect the protein of interest, the crude protein extract was subjected to SDS–PAGE and electro-blotted at 100 V for 1 h from the gel onto a nitrocellulose Protran BA-85 membrane (Whatman) using a pre-chilled protein-blot transfer buffer (PBTB) (Towbin et al. 1979). The membrane was stained with the Ponceau-red solution (see above) and blocked for 1h at room temperature or overnight at 4°C in the blocking solution. The subsequent immuno-detection assay was performed by incubating the membrane at room temperature for 1h with the gene specific antibody diluted in the blocking solution. The degree of the dilution was empirically determined for each protein in the range from 1:1000 to 1:5000 (v/v) depending on the antibody used. The membrane was washed with TBST as follows: once briefly, once for 15 min and three times for 5min. The membrane was then incubated for 45 min at room temperature with 5000-fold diluted anti-rabbit IgG coupled to horseradish peroxidase (secondary antibody), washed again several times as described above. The presence of the target protein was revealed by using the ECL Plus Western Blotting detection Kit (Amersham, Braunschweig, Germany). The complex antigen-antibody is detected on the membrane by chemiluminescence under a CCD camera (Intelligent Dark Box II, Fujifilm Corporation).

10X TBS, pH 7.5:	200 mM Tris-HCl (24.2 g/L); 1.5 M NaCl (87.6 g/L); add H_2O to 1 L after adjusting the pH with fuming HCl.
TBST solution:	1X TBS + 0.1% (v/v) Tween-20.
Protein-blot transfer buffer (PBTB):	25 mM Tris, 192 mM glycine, 20% (v/v) methanol. Do not adjust the pH!
Blocking solution:	4% (w/v) not-fat dry-milk powder dissolved in TBST solution.

2.2.8.15 Purification of ALDH-specific IgG antibodies from a crude antiserum

In cases where the antiserum did not allow the specific detection of the protein of interest, the antibodies were further affinity-purified as mono-specific antibodies. Briefly, the recombinant form of the protein was first immobilized onto a nitrocellulose membrane after SDS-PAGE. The areas of the membrane containing the protein were revealed by Ponceau staining and were then cut into pieces of 1.5 cm length. These membrane strips were incubated for 1 h at RT in a blocking solution (50 ml of 1X TBS and 4% (w/v) not-fat dry-milk powder). Then, 0.5 ml (or more – depending on the titer) of the crude serum was added to the blocking solution and further incubated with the membrane strips as above. The blocking solution was removed and the strips washed once with 1X TBS, 0.2% Tween-20 for 20 min and 2 x 20 min with 1X TBS. After discarding the last wash, the membrane strips were soaked in "X" volume of 0.2 M glycine-HCl, pH 2.2 for 1 min with gentle vortexing to elute the protein-specific IgGs. The glycine solution was saved in a fresh vial. The membrane strips were neutralized with 1/6 volume "X" of 1 M Tris-Cl, pH 8.8 for 10-20 seconds. The 1/6 vol "X" of 1M Tris-HCl containing residual IgGs from the strips was used to neutralize the glycine solution as well. This mixture contains the protein mono-specific IgGs and was directly added to 50 ml blocking solution and used up to 5 times as primary antibody for immuno-detection in protein-blot assays. The membrane strips were stored in 1X TBS, 0.2% sodium azide at 4°C.

2.2.9 Stress experiments with bacterial cells

For stress experiments, transformed *E. coli* BL21 cells were pre-cultured in 10 ml LB medium containing 50 mg/l kanamycin for 12 h at 37°C. A fresh culture was initiated from the pre-culture. The expression of the recombinant protein was induced by adding IPTG to a

final concentration of 0.1 mM to bacterial cultures at log phase (OD_{600} = 0.3). The bacteria were further incubated at 21°C for 1 h then H_2O_2 and NaCl were added as stressors to a final concentration of 1 mM and 500 mM, respectively. Cell density was measured as OD_{600} at different time-points. The relative cell growth was calculated as the ratio of the OD_{600} in the presence of IPTG + (NaCl or H_2O_2) to the OD_{600} in the presence of only IPTG.

2.2.10 Plant stress treatments

2.2.10.1 Stress treatment of seedlings

For *in vitro* experiments, seeds were sown on MS-agar medium. A germination test was performed as described by Jung et al. (2008) with a few modifications. Approximately 50 seeds were placed on MS-medium containing 2% (w/v) sucrose and different concentrations of NaCl or mannitol. To break the dormancy, seeds were incubated at 4°C for 3 d in the dark prior to be placed in the growth chamber. Six days later the percentage of germinated seeds related to the total number of plated seeds was calculated. Seeds were counted as germinated when the roots and/or the 2 green cotyledons became visible. Next, seedlings were collected and weighed 14 days after germination. Accumulated fresh weight was compared with respect to the genotypes and the treatments. For gene expression analysis, seedlings were removed from the plates and air-dried or incubated in NaCl solutions for the indicated time-periods. Seedling samples were collected, frozen in liquid nitrogen and kept at -70ºC or immediately used for various analyses.

2.2.10.2 Stress treatment of soil-grown plants

Besides the *in vitro* stress experiments, the performance of soil-grown plants was also investigated under stress conditions. Fifteen day-old seedlings were transferred into soil and allowed to grow for two more weeks in short-day conditions before applying stressors.

2.2.10.3 Drought stress treatment

Drought stress was imposed to adult plants by withholding watering for 10-14 days. Leaf samples were collected for phenotypic and biochemical analyses. For gene expression analyses, dehydration experiments were performed with seedlings or detached leaves placed on filter paper and air-dried at room temperature for various time-periods.

2.2.10.4 Salt and Paraquat® stress treatments

To analyse the salt tolerance in adult plants the soil-grown plants were watered every two days with water or water containing different concentrations of NaCl (0-300 mM) for 10-14 days. For Paraquat® treatments, plants were sprayed with 10 µM methyl viologen (Paraquat®). Phenotypic traits were recorded and biochemical analyses (MDA, proline and H_2O_2 contents) were performed on leaves and/or root samples. For gene response studies, salinity and Paraquat® treatments were carried out by incubating up-rooted plants or detached leaves in NaCl solutions (0-250 mM) or a freshly prepared Paraquat® solution for the indicated time-periods.

2.2.11 Biochemical analyses and microscopy

2.2.11.1 Determination of chlorophyll content (Arnon 1949)

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

$C\ (mg\ FW^{-1}) = 0.002 \times (20.2 \times OD_{645} + 8.02 \times OD_{663}) /$ g *FW* where C expresses the total chlorophyll content (chlorophyll A + chlorophyll B).

2.2.11.2 Lipid peroxidation assay

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; Kotchoni et al. 2006). 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 4ºC. 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% 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,

product of the reaction of MDA with TBA) and 600 nm (turbidity). 0.1% (w/v) TCA was used as reference solution. The MDA contents were estimated by the formula:

MDA equivalents (nmol ml^{-1}) = [(A–B)/157 000] x 10^6 where A = [(Abs 532_{RSII} – Abs 600_{RSII})] and B = [(Abs 440_{RSII} – Abs 600_{RSII}) x 0.0571].

MDA equivalents (nmol g^{-1} FW) *= MDA equivalents (nmol ml^{-1}) x total volume of the extracts (ml) /* g *FW or number of seedlings*.

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

2.2.11.3 H_2O_2 measurement

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

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

2.2.11.4 Proline determination

Free proline was determined according to the method of Bates et al. (1973). Approximately 100 mg plant material was ground in liquid N_2 with metal beads and homogenized in 2 ml of 3% (m/v) sulphosalicylic acid. The mixture was centrifuged at 4000 rpm for 5 min. 1 ml of ninhydrin acid and 1 ml of glacial acetic acid were successively added to 1 ml of the supernatant or standard L-proline solution (1, 5, 10, 25 and 50 µM). The mixture was boiled for 60 min and extracted with 2 ml of toluene. Free proline was quantified with a spectrophotometer from the upper organic phase at 520 nm by using a standard curve obtained from various concentrations of L-proline. The following formula was used:

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

2.2.11.5 GUS staining of *Arabidopsis* leaves

To study the expression pattern of *ALDH* gene promoters, transgenic seedlings or adult plant leaves were stained for the β-glucuronidase (GUS) reporter gene expression. The GUS enzyme catalyses the cleavage of the colourless substrate X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) to an intermediate product that undergoes a dimerization leading to an insoluble blue precipitate known as dichloro-dibromo-indigo (ClBr-indigo). The ability of ClBr-indigo to precipitate was used to trace the *in situ* expression of the *GUS* gene driven by the ALDH promoter.

2.2.11.6 GUS-Assay with X-Gluc as substrate (Jefferson et al. 1987)

The *in-situ* detection of the GUS activity was performed according to Jefferson et al. (1987). Plant tissues were incubated in the GUS-staining buffer at 37ºC overnight (14-16h). The tissues were distained in 80% (v/v) ethanol solution at 80ºC where they lose the chlorophyll then kept in 10% (v/v) glycerol. Photographs of the tissues were taken under a dissecting microscope (Nikon SMZ-800; Düsseldorf, Germany).

GUS-staining buffer: 0.5 mg/ml X-Gluc (*NB*: first dilute the X-Gluc in DMF: 100 µl DMF per 10 mg X-Gluc); 50 mM NaH_2PO_4 buffer pH 7.2; 0.1% (v/v) Triton X-100; 8 mM β-mercaptoethanol freshly added.

2.2.11.7 Fluorometric detection of the GUS activity

The fluorometric assay of the GUS activity was carried out from ground plant tissues according to the method of Jefferson et al (1987) with minor modifications. In this assay, the fluorogenic substrate 4-methylumbelliferyl-glucuronid (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, 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 (r*eaction 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 were 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)*].

2.2.11.8 *In situ* visualization of lipid peroxidation-derived aldehydes

Aldehydes that are generated from the lipid peroxidation were stained and visualized *in situ* using Schiff's reagent as described by Yin et al. (2010). Detached leaves that were previously treated with stressors were soaked in the Schiff's reagent (Merck; Darmstadt, Germany) for 20 min, rinsed with a freshly prepared sulfite solution (0.5% [w/v] $K_2S_2O_5$ in 0.05 M HCl) and kept in the sulfite solution. Then leaves were immediately and observed under a dissecting binocular microscope (Düsseldorf, Germany).

2.2.11.9 Microscopic observation of the GFP activity in bombarded leaves

The expression of the GFP in leaves was examined within 48 h after the particle bombardment. Single leaves were mounted in distilled water between two micro-cover glass slides of 0.13 to 0.17 mm thickness (VWR International, Darmstadt, Germany). The leaf was analysed using an inverted confocal laser microscope (Nikon Eclipse TE2000-U/D-Eclipse C1, Nikon; Düsseldorf, Germany) with excitation light at 488 nm and emission at 515 nm. Chloroplast auto-fluorescence was observed with excitation at 543 nm and emission at 570 nm. Images were processed with EZ-C1 version 3.20 (Nikon, Düsseldorf, Germany).

3. RESULTS

3.1 Betaine aldehyde dehydrogenase genes from *Arabidopsis* with different subcellular localizations affect stress responses

Arabidopsis thaliana belongs to the plants that do not naturally accumulate glycine betaine (GB), although its genome contains two genes, *ALDH10A8* and *ALDH10A9,* that code for betaine aldehyde dehydrogenases (BADHs). BADHs were initially known to catalyse the last step of the biosynthesis of GB in plants. But, they can also in some cases oxidize metabolism-derived aminoldehydes to their corresponding aminoacids. This study was undertaken to understand the functional properties of the two *Arabidopsis* BADH coding genes.

3.1.1 Comparison of BADH gene sequences

Both ALDH10A8 (At1g74920) and ALDH10A9 (At3g48170) belong to the family 10 of the superfamily of ALDH proteins. ALDH10A8 and ALDH10A9 mRNAs respectively contain a protein coding sequence of 1506 and 1512 bp. Sequence comparison using the Vector NTI program indicated that both mRNAs share 68.8% of nucleotide identity. At protein level, 89.5% of amino acid sequence homology and 79.0% of amino acid residue identity were found between ALDH10A8 and ALDH10A9. The ALDH10A8 and ALDH10A9 proteins respectively contain 501 and 503 amino acid residues. The last three amino acids of ALDH10A9 constitute the SKL signal that predicts a localization of the protein in peroxisomes (Reumann 2004). Unlike ALDH10A9, ALDH10A8 does not contain any obvious targeting signal. So far, nothing is known on the expression pattern of these genes, nor their functions. These questions were addressed in this study starting from the expression analysis at the transcription level.

3.1.2 Expression patterns of *ALDH10A8* and *ALDH10A9* genes

ALDH10A8 and ALDH10A9 transcript accumulation was analysed in *A. thaliana* (Col-0) wild-type plants. Four week-old plants were removed from the soil and incubated in water supplemented with 100 µM ABA, 200 mM NaCl or 10µM methyl viologen for 72 h. Alternatively, they were kept in water at 4°C for the chilling stress. RNA-blot analyses indicated that both the *ALDH10A8* and *ALDH10A9* transcripts did not significantly increase upon these treatments (**Fig. 1a**). The slight changes in the gene expressions were brought out by the semi-quantitative analysis of signals on the RNA-blots. Based on the expression

pattern of the cold and dehydration responsive gene *RD29A*, it appeared that highest gene expressions started after 8 h of the treatments. With regards to this, the semi-quantitative analysis of the RNA-blot signals at the time-point 8h revealed that *ALDH10A8* and *ALDH10A9* are inducible by ABA, and to a lesser extent by NaCl, chilling and methyl viologen (**Fig. 1b**). As for the dehydration treatment, both genes showed increasing expression over a 24 h period, with ALDH10A8 having all the time the higher level (**Fig. 1c**). These results suggest that the *ALDH10A8* and *ALDH10A9* genes are stress-responsive.

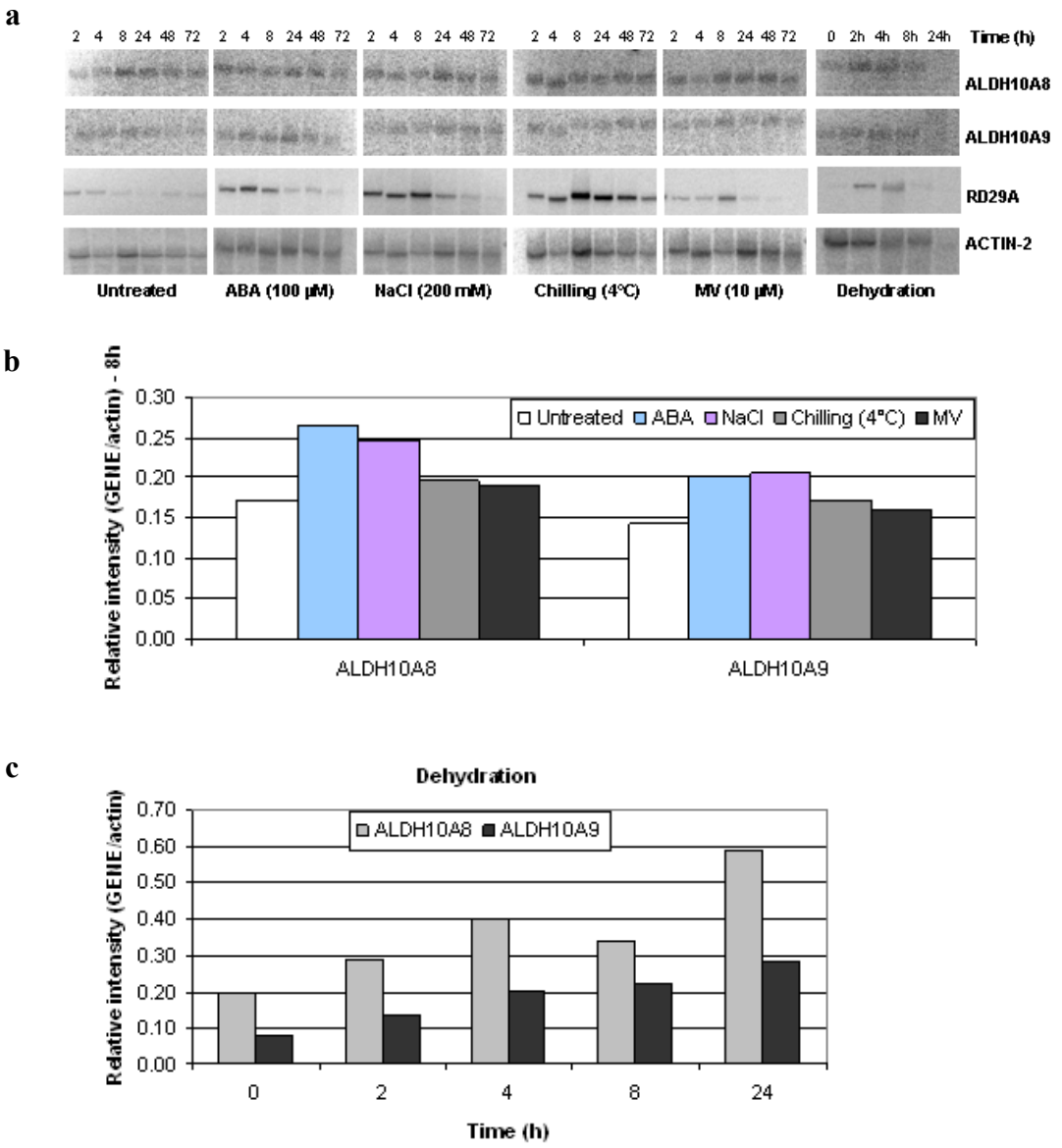


Fig. 1 Expression of the *ALDH10A8* and *ALDH10A9* transcripts in 4 week-old plants under various stress conditions. (**a**) RNA blots: Total RNAs were used for the blots. ^{32}P-labelled *ALDH10A8*, *ALDH10A9*, *Ath-ACTIN2* (An et al. 1996) and *Ath-RD29A* (Yamaguchi-Shinozaki and Shinozaki 1993) cDNA fragments were used as probes. The *Ath-ACTIN2* probe was used to monitor loading of RNAs whereas the *Ath-RD29A* probe allowed monitoring the efficiency of the stress treatments. (**b**,**c**) Semi-quantitative analysis of the RNA-blot signals: The intensity of the signal of each sample was related to that of the *ACTIN-2* gene at the same time-point, as described in Material and methods. Shown are the comparative analyses of *ALDH10A8* and *ALDH10A9* expression under ABA, NaCl, chilling (4°C) and methyl viologen (MV) after 8 h (**b**) and under dehydration (**c**).

3.1.3 Sub-cellular localization of ALDH10A8 and ALDH10A9 proteins

The protein sequence of ALDH10A8 does not contain an obvious sub-cellular localization signal. To identify the sub-cellular localization of this protein, different GFP fusion constructs were generated and analysed. **Table 5** lists all GFP fusion constructs that were analysed.

Table 5 Overview of the aldehyde dehydrogenase-GFP constructs

Construct	Hybrid gene[a]	Position of GFP in the hybrid gene
ALDH10A8-140	1–420 bp 5' of ALDH10A8 fused to GFP	C-terminal
ALDH10A8-73	202–420 bp 5' of ALDH10A8 fused to GFP	C-terminal
ALDH10A8-501	Full-length CDS (1–1503 bp) of ALDH10A8 fused to GFP	N-terminal
ALDH10A9-180	970–1509 bp of 5' ALDH10A9 fused to GFP	N-terminal

[a]Nucleotides are counted from the ATG of the full-length coding sequence of ALDH genes

Two GFP fusion constructs were made in *pGJ280* for the ALDH10A8 cDNA: i) the first 420 nucleotides (amino acids +1 to +140) were fused in frame to the GFP via engineered *Nco*I sites to generate the ALDH10A8-140–GFP expression construct (Schmitz 2007) ii) the cDNA fragment coding for the amino acids +68 to +140 was fused to GFP to yield the construct ALDH10A8-73-GFP (this work), which lacks the first 67 amino acids, as compared to ALDH10A8-140. In addition, the full-length protein coding sequence was sub-cloned downstream of the GFP in the *pPG-Tkan* vector via engineered *Bam*HI sites to yield the GFP-ALDH10A8-501 construct (this work). ALDH10A8-140 and ALDH10A8-73 are N-terminal ALDH-GFP fusion constructs whereas in ALDH10A8-501 the GFP is N-terminal and the ALDH is downstream of the GFP. The expression cassette in ALDH10A8-140 was next subcloned into the *Hin*dIII site of *pBIN19* for stable transformation *in planta* (Schmitz 2007). The *pPG-Tkan* vector was also used for the construction of the ALDH10A9-GFP fusion (Schmitz 2007). It was shown that transgenic plants expressing the ALDH10A8-140 construct show green fluorescing organelles that appear often pairwise and possess cytoplasmic strands (Schmitz 2007; **Fig. 2a-c**). Similar observations were made from *Arabidopsis* leaves transiently transformed by particle bombardement with the same construct (Schmitz 2007). These organelles are smaller than the surrounding chloroplasts, suggesting a localization in leucoplasts (**Fig. 2a-c**).

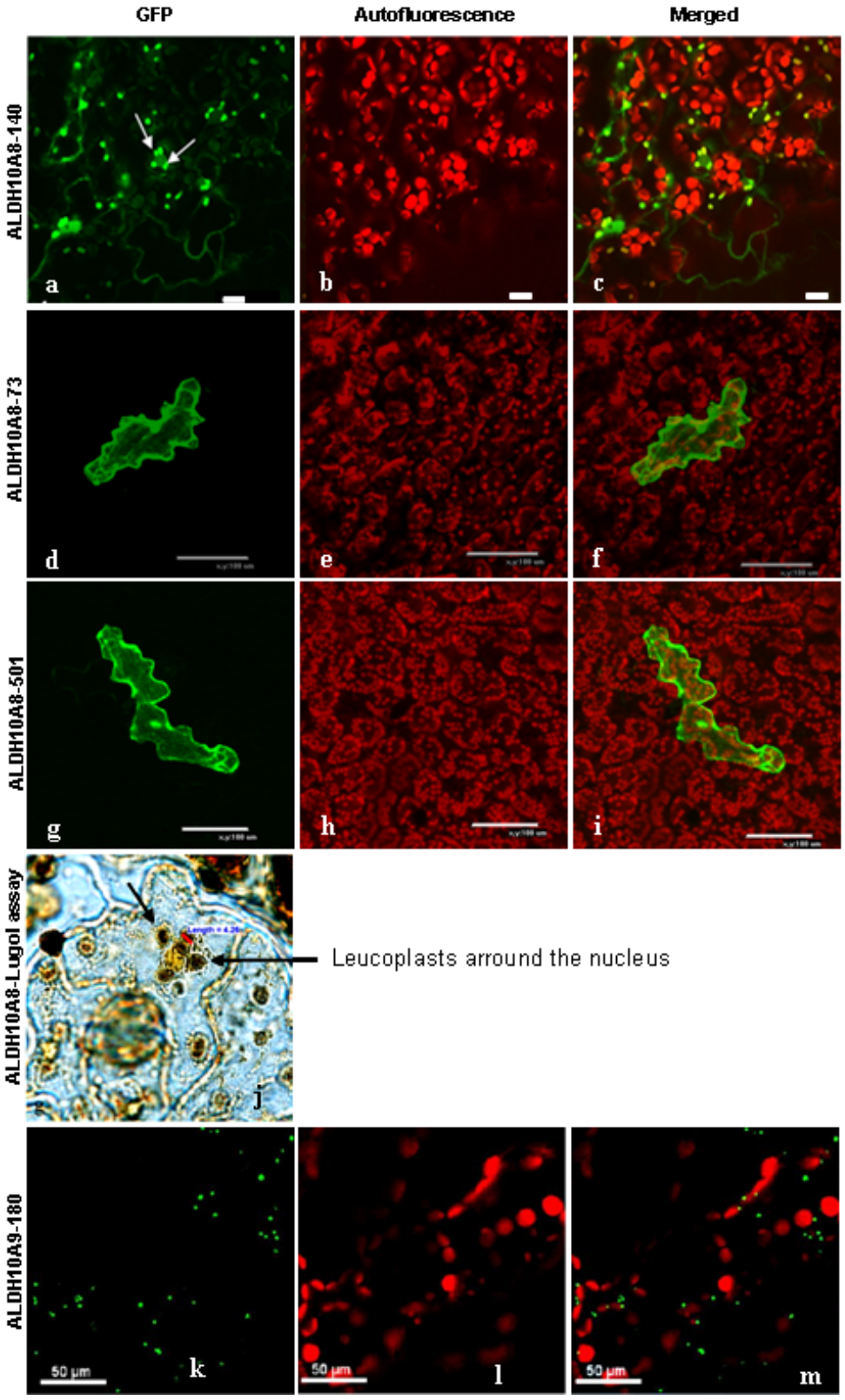


Fig. 2 Sub-cellular localization of ALDH10A8– and ALDH10A9–GFP fusion proteins. ALDH10A8– and ALDH10A9–GFP fusion constructs (Table 5) were expressed in *A. thaliana* plants. The names of the different constructs are indicated on the left hand side. Leaves were viewed under the fluorescence microscope with appropriate filters. (**a–c**) The sub-cellular localization of the ALDH10A8 (N-terminal 140 amino acids)–GFP fusion protein (scale bar: 10 µm). *Please see the rest of the legend on the next page.*

Fig. 2 Sub-cellular localization of ALDH10A8– and ALDH10A9–GFP fusion proteins. (**d–f**) The sub-cellular localization of the ALDH10A8 (N-terminal amino acids: +68 to +140)–GFP fusion protein (scale bar: 100 µM). (**g–i**) The sub-cellular localization of GFP–ALDH10A8 (full length protein; amino acids: +1 to +501) fusion protein (scale bar: 100 µm). (**j**): photographs of epidermis cells of wild-type *Arabidopsis* leaves treated with Lugol reagent (red scale bar: 4 µm); leucoplasts appear brownish and are localized around the nucleus. (**k–m**) The sub-cellular localization of ALDH10A9 (C-terminal amino acids: +324 to +503)–GFP fusion protein (scale bar: 50 µm). (**a**, **d, g, k**) GFP fluorescence alone; (**b, e, h, l**): chlorophyll auto-fluorescence; (**c**, **f, i, m**): merged chlorophyll and GFP fluorescence together. Arrows in (a) indicate fluorescent leucoplasts in cells of transgenic plants, arrows in (j) point to leucoplasts in cells of wild-type plants. Photographs of ALDH10A8-140 and ALDH10A9-180 are from stably transformed plant leaves whereas ALDH10A-73 and ALDH10A8-501 pictures are from transiently transformed leaves.

To support this finding, *Arabidopsis* rosette leaves of wild-type plants were incubated with Lugol reagent for 5-10 min at 37°C. Microscopic observations of epidermis cells from Lugol-treated leaves under bright field showed starch-containing leucoplasts with an intense brown colour coinciding with the fluorescent signal surrounding the nucleus (Schmitz 2007; **Fig. 2j**). Complementary experiments were carried out in this work with two additional constructs, ALDH10A8-73 and ALDH10A8-501. When the first 67 N-terminal amino acids are deleted from ALDH10A8-140 (ALDH10A8-73), the green fluorescence was not observed anymore in the leucoplasts, but in the cytoplasm (**Fig. 2d-f**). Likewise, the GFP signal appeared in the cytoplasm for the ALDH10A8-501 construct, where the original N-terminal part of the gene was placed in the middle of the construct by engineering the GFP protein in front of the ALDH10A8 coding sequence (**Table 5**; **Fig. 2g-i**). These observations confirm the presence of a leucoplast targeting signal within the 67 the N-terminal amino acids of the ALDH10A8 protein. The ALDH10A9 protein is predicted to be localized to peroxisomes as it has the peroxisome targeting-signal SKL (Reumann 2004). Experimental evidence was reported previously (Schmitz 2007; Klug 2008; **Fig. 2k-m**).

3.1.4 Isolation of homozygous ALDH10A8 T-DNA insertion mutants

To investigate the possible function of *ALDH10A8* and *ALDH10A9* genes T-DNA insertion mutants, *SALK_079892*, *KO8-1*, *KO8-2*, *KO9* and *SALK_066181*, were obtained for the *ALDH10A8* and *ALDH10A9* genes. The line *SALK_079892* contains a T-DNA insertion within the 9th intron of *ALDH10A8* and this was confirmed by PCR using the primer pairs A3-T2 and T3-A4. The gene is still expressed in isolated homozygous plants. Therefore, this line was not further analysed. Sequence analysis revealed that the *KO8-1* mutant harbours a T-DNA insertion in the 3'UTR (3'untranslated region; 10 nucleotides after the stop codon TAA in the 15th exon), whereas the *KO9* mutants contained a T-DNA insertion 162 nucleotides upstream of the ATG translation start codon (**Fig. 3**).

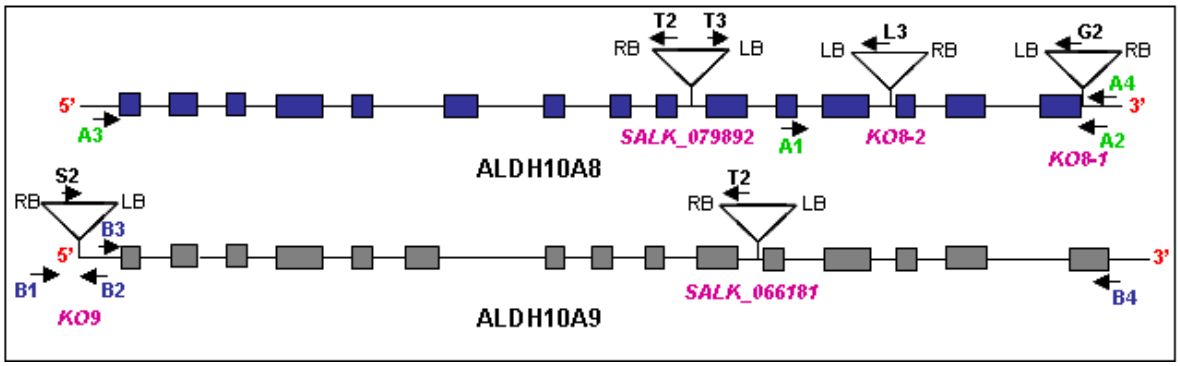


Fig. 3 Schematic representation of the *ALDH10A8* and *ALDH10A9* genes with the location of T-DNA insertion lines. Intron-exon structure of *ALDH10A8* and *ALDH10A9* genes and locations of the T-DNA fragments. 5'UTR, introns and 3'UTR are represented as horizontal lines and exons are depicted as filled boxes according to the organisation of the genes on the TAIR website (http://www.arabidopsis.org). Names of mutants are shown in pink under the insertion site. Arrows represent the primers and their locations in the genes – **A1**: AY093071_RT_fwd; **A2**: AY093071_RT_rev; **A3**: Aldh9-fwd; **A4**: Aldh9-rev; **G2**: Gabi_10A8_rev; **L3**: pSKTAIL-L3; **T3**: pROK-Lba3; **T2:** pROK-FISH2; **B1**: KO10A9_fwd; **B2**: KO10A9_rev; **B3**: Aldh8-fwd; **B4**: Aldh8-rev; **S2**: Sail_10A9_rev. See Materials and methods section for the primer sequences. **LB**: T-DNA left border; **RB**: T-DNA right border.

The presence of the T-DNA was confirmed by PCR with the following primer pairs: A1-G2 for the line *KO8-1* and S2-B2 for *KO9*. Homozygous mutant plants were isolated (**Fig. 4a**) and analysed for the expression of both ALDH genes by RT-PCR. The *ALDH10A8* transcript is absent in *KO8-1* plants as compared to the wild type (**Fig. 4b**). But it was found that the T-DNA insertion in that line also disrupted the expression of the adjacent gene *At1g74910* (**Fig. 4c**). Therefore, the line *KO8-2*, an allelic T-DNA insertion mutant of *KO8-1* was obtained from the Saskatoon *Arabidopsis* T-DNA collection and investigated. The presence of the T-DNA at the indicated position was confirmed as above using the primer pairs A1-L3 and A3-L3 (**Fig. 3**).This mutant represents a single knock-out of the *ALDH10A8* gene (**Fig. 4d**).

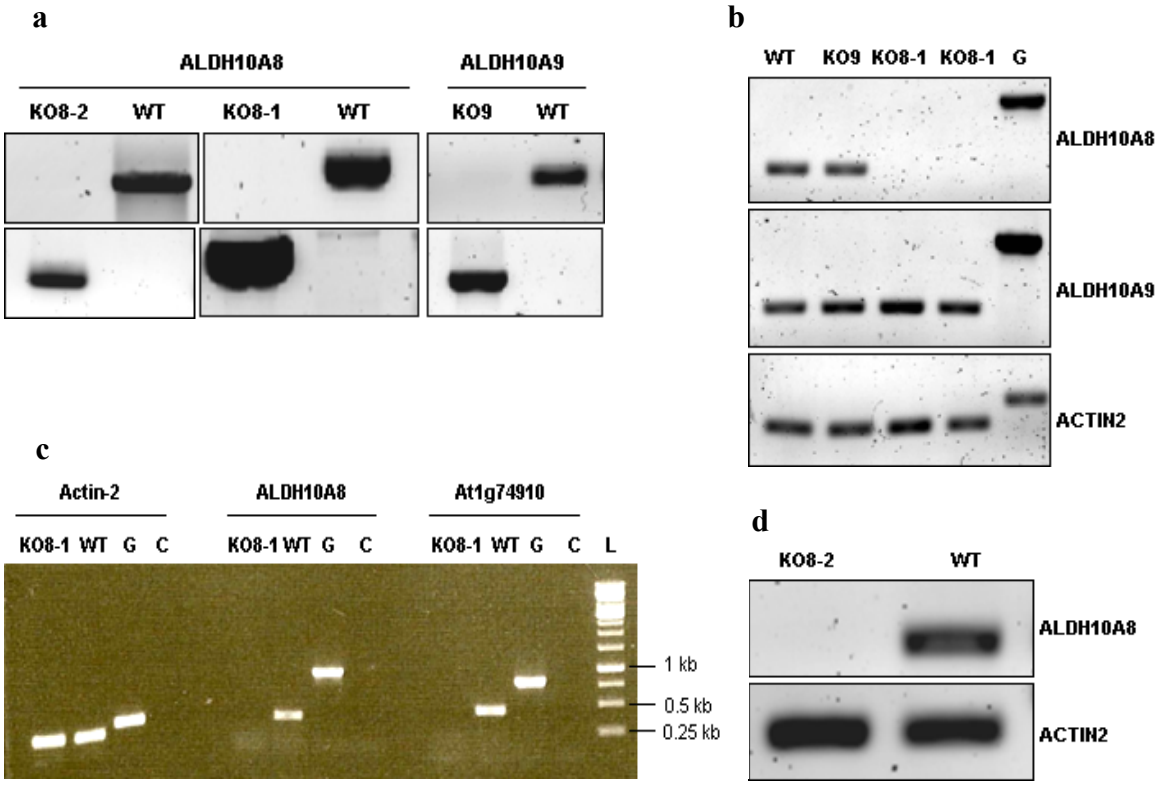


Fig. 4 Molecular characterisation of *ALDH10A8* and *ALDH10A9* T-DNA insertion lines. (**a**) Genotyping of T-DNA insertion lines. Genomic DNA isolated from both wild-type (Col-0) and mutants were used to amplify the *ALDH10A8* (upper middle and left) and *ALDH10A9* (upper right) genes. Similarly, gene/T-DNA junctions were amplified for both genes as described in Materials and methods; **lower middle and left**: *ALDH10A8*; **lower right**: *ALDH10A9*. (**b**) RT-PCR analyses of *ALDH10A8* and *ALDH10A9* transcripts in wild-type (Col-0), *KO8-1* and *KO9*. First-strand cDNAs were synthesized and amplified by PCR from wild-type (Col-0) and homozygous mutants (Col-0 background) total RNA samples. (**c**) RT-PCR analyses of *At1g74910* transcripts in *KO8-1*. *ACTIN-2* and *ALDH10A8* RT-PCRs are shown for comparison. (**d**) RT-PCR analyses of ALDH10A8 transcripts in *KO8-2*. **WT**: wild-type; **KO9**: *ALDH10A9* mutant; **KO8-1** and **KO8-2**: *ALDH10A8* mutants; **G**: wild-type genomic DNA was used as control. **C**: Negative control for PCR performed with water instead of DNA. **L**: 1 kb DNA ladder.

The analysis of the segregation ratio of the herbicide glufosinate ammonium resistance in *KO8-2* seedlings was performed as described by Robinson et al. (2009). It revealed that the line contains a single T-DNA insertion. As for the *KO9* line, the ALDH10A9 transcripts are still present in the plants, indicating that the expression is not affected by the T-DNA insertion. Another T-DNA insertion line (*SALK_066181*) with the T-DNA fragment inserted in the 10th intron of *ALDH10A9* was also screened. The primer pairs B3-B4 and B3-T2 were used for PCR to identified homozygous plants. But again the gene was still expressed as checked by RT-PCR (data not shown). No other knock-out line is available for *ALDH10A9*. Therefore subsequent experiments were only performed with *KO8-2* plants.

3.1.5 Functional analysis of the ALDH10A8 T-DNA insertion mutant *KO8-2*

The susceptibility of *ALDH10A8* deficient plants to abiotic stress was investigated in seedlings and adult plants. Seeds were first germinated and grown *in vitro* on MS-medium supplemented with 100 mM NaCl or 200 mM mannitol. As shown in **Fig. 5** *KO8-2* seedlings showed more chlorosis together with high accumulation of anthocyanins on salt or mannitol containing medium.

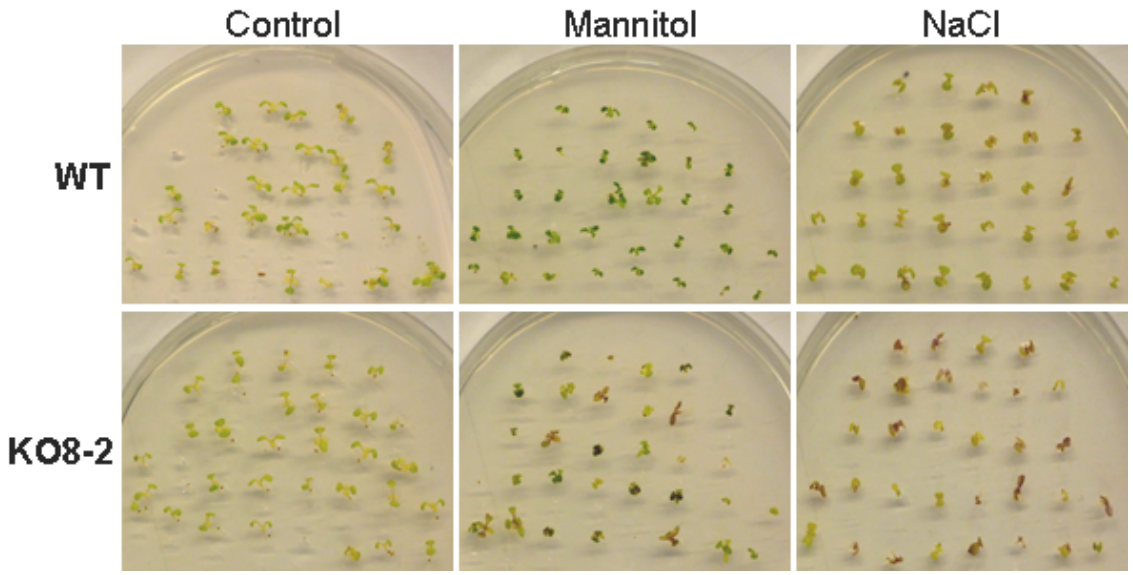


Fig. 5 Photographs of WT and *KO8-2* seedlings on MS-plates 14 days after germination. *A. thaliana* wild-type and transgenic seeds were surface-sterilised and sown on MS-agar plates supplemented either with either 100 mM NaCl or 200 mM mannitol.

Under stress conditions plant membrane lipids are oxidised, which often leads to the accumulation of the toxic compound malondialdehyde (MDA) (Sunkar et al. 2003; Kotchoni et al. 2006). Consistent with the visual observations, there was no difference in MDA contents of WT and *KO8* seedlings under control conditions. However, *KO8-2* plants accumulated more MDA than WT when grown on MS medium supplemented with 100 mM NaCl (**Fig. 6a**). Likewise, the MDA content was higher in mutants than the wild-type when seedlings were grown on MS agar supplemented with 200 mM mannitol (**Fig. 6b**).

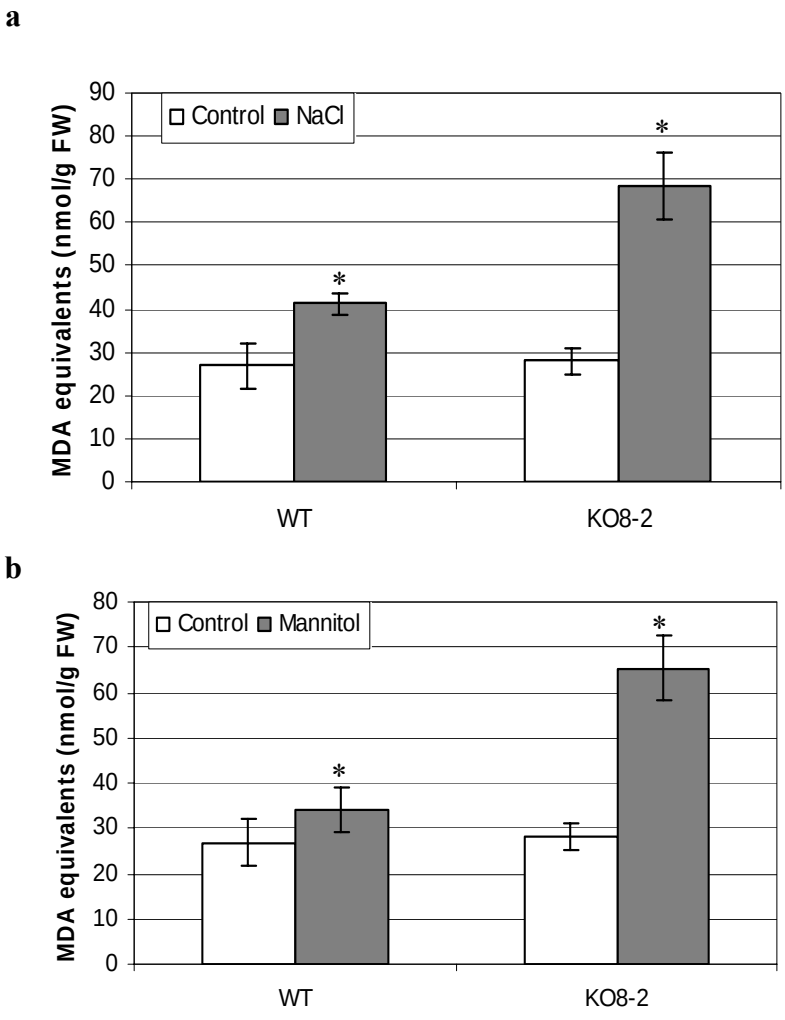


Fig. 6 MDA contents in unstressed and stressed WT and *KO8-2* seedlings. (**a**): 100 mM NaCl; (**b**): 200 mM mannitol. Data are means ± SE from three biological replicates; Differences between WT and *KO8-2* are shown with (*) when significant; $P < 0.05$, Student *t*-test.

To analyse the salt and drought response in adult plants, seedlings were transferred to soil and allowed to grow in pots for two weeks. The pots were watered every two days with water or with water containing 300 mM NaCl for 10 days. Alternatively, drought stress was imposed by withdrawing water for 10 days. As shown in **Fig. 7a** and **Fig. 7b**, adult *KO8-2* plants accumulated higher MDA levels than WT upon salt and drought stress. The MDA contents were similar in regularly watered WT and *KO8* mutants.

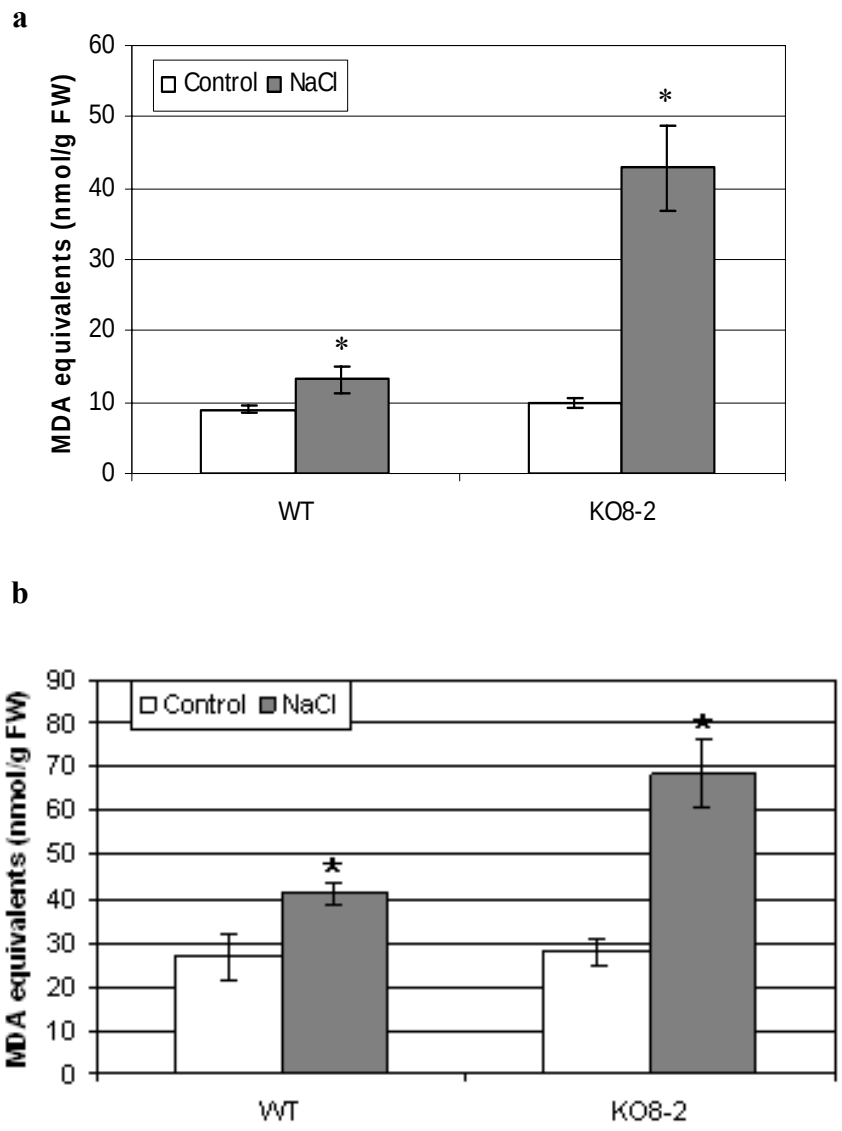


Fig. 7 MDA contents in adult WT and *KO8-2* plants. (**a**): 300 mM NaCl; (**b**): 10 day-drought stress. Data are means ± SE from three biological replicates; Differences between WT and *KO8-2* are shown with (*) when significant; $P \leq 0.05$, Student *t*-test.

Like glycine betaine, proline is a compatible solute that accumulates in plants under stress conditions. Free proline content was quantified from the mutants and the WT under control and stress conditions. No difference was observed in the free proline content of the WT and *KO8* plants (**Fig. 8**).

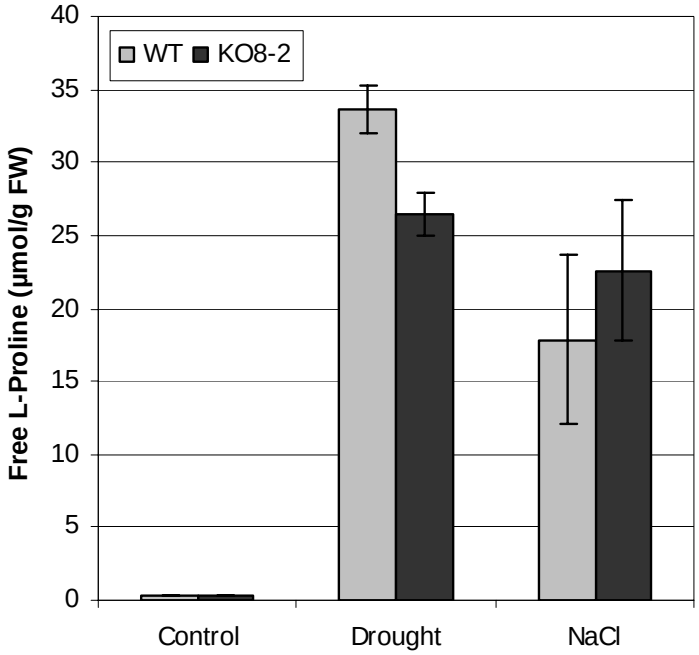


Fig. 8 Free proline content in adult WT and *KO8-2* plants. Drought and 300 mM NaCl were used as stressors for 10 days. Data are means ± SE from three biological replicates.

Altogether, the results show that the disruption of the *ALDH10A8* gene expression has negatively affected the sensitivity of plants to salt and dehydration stresses.

3.1.6 Enzymatic properties of the recombinant ALDH10A9 protein

To examine the function of the *Arabidopsis* BADH coding genes the coding region of *ALDH10A8* and *ALDH10A9* cDNAs were cloned in the pET-28a (+) expression vector (Novagen, Novagen Inc., Madison, WI, USA). An ALDH10A8 cDNA fragment was generated by PCR from the clone pda07810 (RAFL07-07-L09) (RIKEN Institute) with the primers AY093071_BamHI_Fwd and AY093071_SacI_rev. Then, the *Bam*HI/*Sac*I cDNA fragment (+56 to +1618; 478 aa) was subcloned into the *Bam*HI/*Sac*I sites of the pET28a- expression vector yielding a fusion protein of 526 aa with both N-terminal and C-terminal His-tag (6 x His). For ALDH10A9 a cDNA fragment was amplified from the clone pda01165 (RAFL05-07-N03) (RIKEN Institute) with the primers AF370333_NdeI_Fwd and

AF370333_EcoRI_rev. The resulting *Nde*I/*Eco*RI fragment (+1 to +1553; 498 aa) was subcloned into the *Nde*I/*Eco*RI sites of the pET28a-expression vector, also yielding a fusion protein of 538 aa with both N-terminal and C-terminal His-tags (6 x His). The DNA sequences of the pET-ALDH10A8 (pET-10A8) and pET-ALDH10A9 (pET-10A9) plasmids were verified and separately introduced into the *E. coli* strain BL21 (DE3). The expression of the recombinant proteins was induced at 22°C by adding IPTG (isopropyl-β-D-thiogalactopyranoside) to a final concentration of 1 mM. The proteins were thereafter purified from the soluble fraction using a His-tag affinity column. As shown in **Fig. 9**, only small amounts of the recombinant ALDH10A8 were purified under native conditions, but sufficient amounts of purified ALDH10A9 protein were obtained and used for enzymatic activity assays.

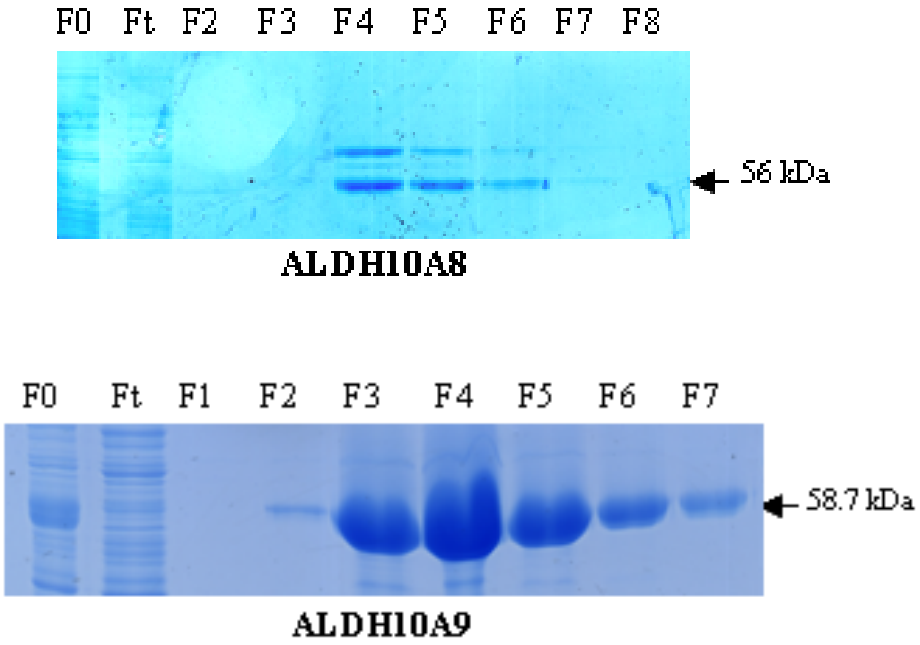


Fig. 9 Purification of the ALDH10A8 and ALDH10A9 recombinant proteins by His-tag affinity chromatography under native conditions. **F0**: total soluble fraction; **Ft**: Flow-through fraction; **F1-F8**: Eluted fractions (250 µl). Five microliters from each fraction were analysed by SDS-PAGE.

The ALDH10A9 recombinant protein converted betaine aldehyde, 4-aminobutyraldehyde (ABAL) and 3-aminopropylaldehyde (APAL) to glycine betaine, GABA and 3-aminopropanoic acid, respectively. With NAD as cofactor the enzyme displayed a Km of 1.1 mM for betaine aldehyde, 6.81 mM for ABAL and 3.7 mM for APAL (**Table 6**).

Table 6 Kinetic parameters of the recombinant ALDH10A9 protein

Substrates	Km [mM]	Vmax [$mM.min^{-1}$]	Vmax/Km [min^{-1}]
Betaine aldehyde	1.1	6×10^{-4}	6.0×10^{-4}
4-Aminobutyraldehyde (ABAL)	6.81	3.1×10^{-3}	4.5×10^{-4}
3-Aminopropionaldehyde (APAL)	3.7	1.0×10^{-3}	2.8×10^{-4}

Enzyme activities were determined using the affinity-purified ALDH10A9 protein from *E. coli* extracts. Substrates were used at a concentration range of 0.5-12 mM. Values are means of at least two independent experiments.

Like previously identified BADHs (Livingstone et al. 2003; Bradbury et al. 2008; Fujiwara et al. 2008), the ALDH10A9 exhibited both betaine aldehyde and aminoaldehyde dehydrogenase activities and could be actively expressed in *E. coli*. The over-expression of either ALDH10A9 or ALDH10A8 in *E. coli* did not render the bacteria more tolerant to sodium chloride or H_2O_2 in comparison to cells transformed with the vector only (**Fig. 10**).

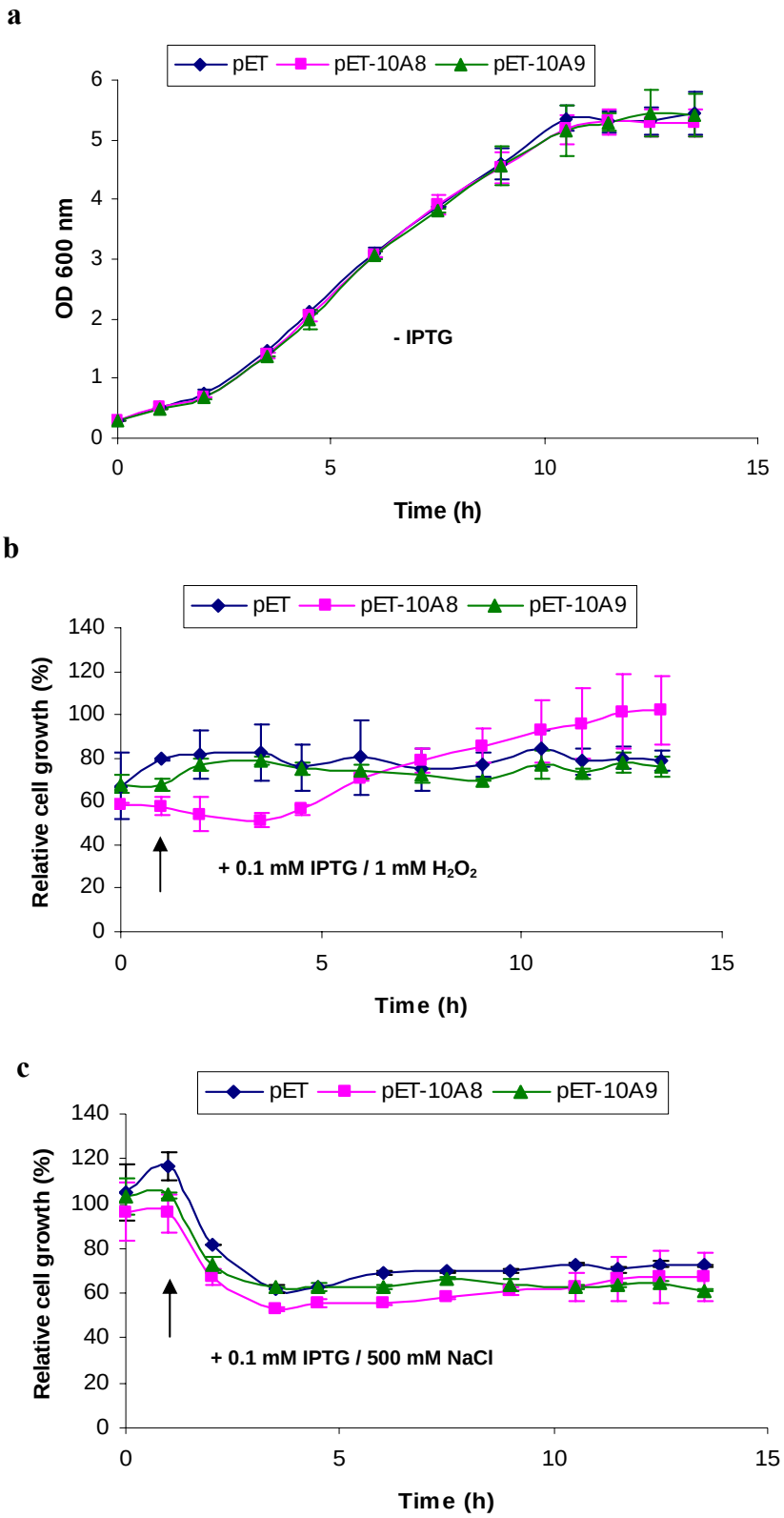


Fig. 10 Expression of ALDH10A8 and ALDH10A9 in *E. coli* BL21 cells. Cells harbouring empty pET28 (pET), pET-10A8 or pET-10A9 vectors were cultured in liquid LB medium supplemented with 1 mM H_2O_2 or 500 mM NaCl. Neither H_2O_2 (**b**) nor NaCl (**c**) were added to the control cultures (**a**). The expression of the recombinant protein was induced by adding IPTG to 0.1 mM final concentration. Cell density was determined at various time-points as absorbance at 600 nm. Arrows indicate the time-point when H_2O_2 or NaCl were added.

3.2 Molecular and functional characterization of the aldehyde dehydrogenase gene *ALDH3H1*

The family 3 of *Arabidopsis* aldehyde dehydrogenase (ALDH) proteins contains three members: ALDH3F1, ALDH3H1 and ALDH3I1. From these members, only *ALDH3I1* has been so far functionally characterized (Sunkar *et* al. 2003; Kotchoni et al. 2006). Here, the expression of the *ALDH3H1* gene has been studied and ALDH3H1 over-expressors and T-DNA insertion mutants have been characterized under various stress conditions.

3.2.1 *ALDH3H1* gene description

The *Arabidopsis ALDH3H1* gene codes for a protein of 484 amino acid residues. ALDH3H1 has a predicted size of 53.2 kDA and an isoelectric point of 8.45, which was calculated by using the Vector NTI program. Phylogenetic analyses revealed that *Arabidopsis* ALDH family 3 proteins are separated from the other plant ALDHs and BADHs (Kirch et al. 2004). ALDH from *C. plantagineum*, ALDH3H1 and ALDH3I1 were found closely related to ALDH proteins from bacteria and animal species (Kirch et al. 2001). The ALDH3H1 protein shares globally 77.0% amino acid homology and 33.6% amino acid identity with ALDH3F1 and ALDH3I1. When compared pairwise, ALDH3H1 was found more similar to ALDH3I1 (66.5% amino acid homology; 53.8% amino acid identity) than ALDH3F1 (60.7% amino acid homology; 46.3% amino acid identity). Previous gene expression studies at transcription level found that from the three members of the ALDH family 3 genes in *Arabidopsis*, only ALDH3I1 and ALDH3H1 are stress-responsive (Sunkar et al. 2003; Kirch et al. 2005). Compared to *ALDH3I1*, *ALDH3H1* transcripts mostly accumulated in roots of 4 week-old plants upon ABA, dehydration and NaCl treatments (Kirch et al. 2005). *ALDH3I1* was more significantly induced both in roots and leaves upon these treatments than *ALDH3H1*. Whether these differences in the gene expression pattern account for any functional importance is so far unknown. Here, the expression pattern of *ALDH3H1* was examined at protein level in leaves of plant older than 4 weeks and the protein was functionally characterized.

3.2.2 Age-dependent accumulation of the ALDH3H1 protein

The expression of the *ALDH3H1* gene was investigated at the protein level by protein-blot followed by immuno-detection using a purified antibody raised against the ALDH3H1 protein. The crude antibodies were produced by BioGenes (Berlin, Germany) from affinity-

purified recombinant ALDH3H1 protein made by Dr. Andreas Ditzer. *Arabidopsis* wild-type plants at different ages were removed from soil and kept in water or 200 mM NaCl for 3 days. Analyses of the crude protein extracts from detached-leaves revealed that ALDH3H1 accumulates in response to salt stress (**Fig. 11**).

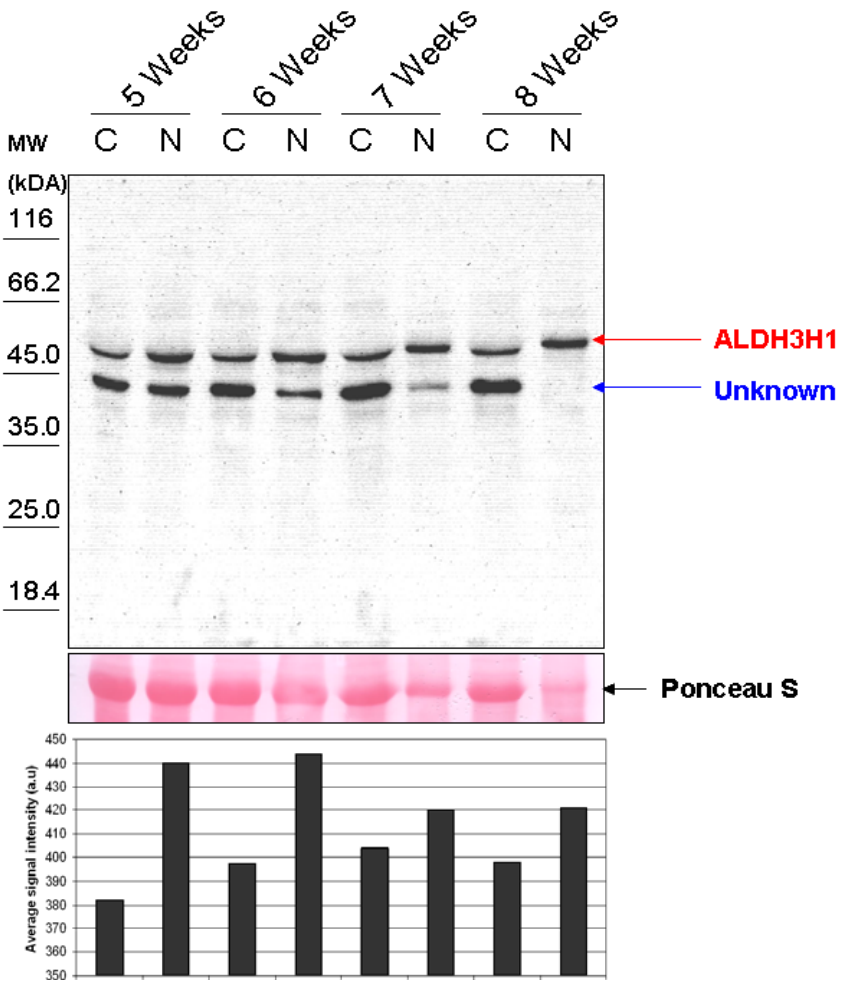


Fig. 11 Age-dependent accumulation of the ALDH3H1 protein. The salt-induced expression of the ALDH3H1 protein (53.2 kDa) in *A. thaliana* (Col-0) leaves was monitored at different stages of development. Up-rooted plants were treated in hydroponic cultures with either water (**C**) or 200 mM NaCl (**N**) for 3 days. **Upper level**: protein-blot; **lower level**: Densitometric analysis of the ALDH3H1 protein signal intensity determined as the average of the pixel intensities in each lane by using ImageQuant v5.2.

Besides, it was found that the antibody cross-reacts with an unknown protein (**Fig. 11**). This unknown protein appeared to be down-regulated in plants older than 6 weeks upon NaCl treatment. Together with the previous observations made on 4 week-old plantlets (Kirch et al. 2005) the present data indicate that the up-regulation of ALDH3H1 protein by salt stress mainly occurs in leaves of adult plants older than 4 weeks after germination.

3.2.3 Generation and molecular characterisation of transgenic plants over-expressing the ALDH3H1 protein

To further understand the role of the ALDH3H1 protein in response to abiotic stress transgenic ALDH3H1 over-expressing plants were generated. A *Bam*HI cDNA (Accession: AY072122) fragment (1730 bp) containing the full-length ALDH3H1 coding region was isolated from the plasmid pda06974 (RIKEN Institute) and sub-cloned into the *Bam*HI site of the binary pROK2 vector (**Fig. 12**). The binary pROK2 vector is a derivative of pBIN19 used for *A. tumefaciens* transformation. The presence and the orientation of the insert were checked by colony-based PCRs using primers specific to the ALDH3H1 cDNA (Aldh4-RT-fwd and Ath.Aldh1c_anti) and by enzymatic digestions of plasmid DNA from kanamycin resistant clones. After sequencing, two clones, SS8 and SA7 having the cDNA fragment inserted in sense and antisense orientation respectively were used to transform *A. tumefaciens* GV3101 strains.

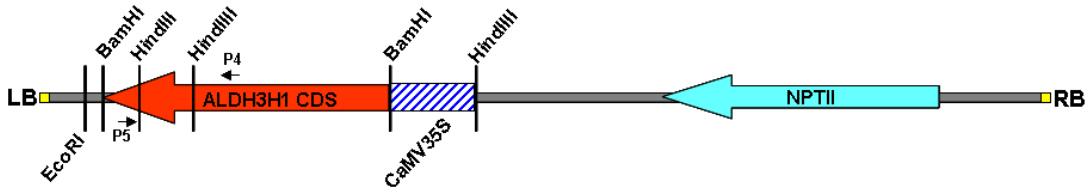


Fig. 12 Schematic diagram of the T-DNA region with the *ALDH3H1* coding sequence. It shows the *35Sprom::ALDH3H1* fusion construct and the gene coding for the kanamycin resistance (NPTII). The small black arrows **P4** and **P5** roughly indicate the location of the primers Aldh4-RT-fwd and Ath.Aldh1c_anti, respectively. **LB**: Left border; **RB**: Right border

Transformants were selected on YEB medium supplemented with kanamycin (50 mg/L) and rifampicin (100 mg/L) and further checked by DNA-blot (**Fig. 13**).

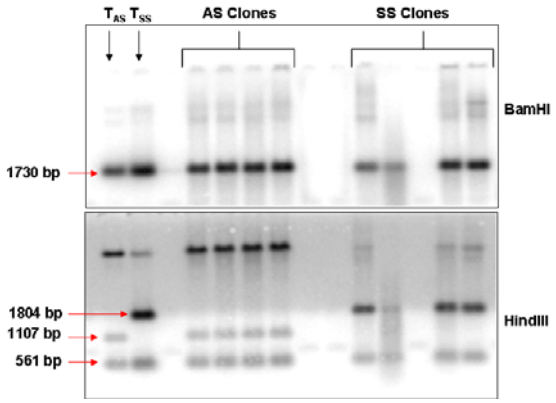


Fig. 13 DNA-blot analysis of recombinant plasmid DNAs isolated from *A. tumefaciens* cells. AS: antisense orientation; **SS**: sense orientation; $\mathbf{T_{AS}}$ and $\mathbf{T_{SS}}$: positive plasmid controls. *Bam*HI digestion was performed to release the cDNA insert whereas *Hin*dIII digestion allowed differentiating insert orientation. The B*am*HI cDNA fragment (1730 bp) containing the ALDH3H1 coding region was used as probe.

An SS8-derived *A. tumefaciens* clone harbouring the CaMV35S::*ALDH3H1* fusion construct was used to transform *Arabidopsis* wild-type plant (ecotype Col-0) using the floral dip method (Clough and Bent 1998). T1 seeds from the dipped plants were screened on MS-agar plates containing kanamycin (50 mg/L) (**Fig. 14**). Kanamycin-resistant seedlings were transferred to soil pots after two weeks and further screened by PCR for the presence of the transgene. Two primers specific to the CaMV35S promoter (35S-fwd and 35S-rev) were used in this assay. The positive transgenic plants derived from the PCR were further analysed by DNA-blot using a CaMV35S promoter fragment as probe to discriminate wild-type and transgenic plants. Results indicated that several plants contained at least one T-DNA fragment inserted into the genome (**Fig. 15**).

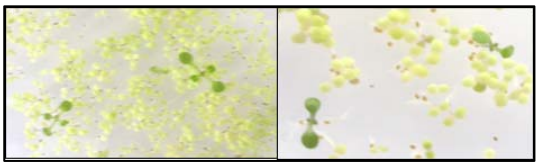


Fig. 14 Selection of putative ALDH3H1 over-expressors. The selection was performed on MS-agar plates containing kanamycin.

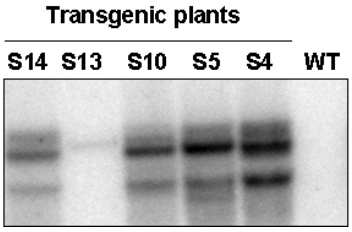


Fig. 15 DNA-blot analyses of putative ALDH3H1 over-expressors. 12 µg genomic DNA (from T2 plants) was digested by *Eco*RI. A CaMV35S promoter fragment was used as probe.

Seed segregation analyses were further performed to identify plants harbouring a single or double functional T-DNA fragment (**Table 7**).

Table 7 Segregation of the kanamycin resistance and deduced number of inserted T-DNA fragments in selected putative ALDH3H1 over-expressors

Lines	Sown seeds	Germinated seeds	Kanamycin Resistant (Kan R)	Germination rate	Kan R rate	T-DNA fragments
S4	56	55	52	0.98	0.94	≥ 2
S5	50	47	45	0.94	0.95	≥ 2
S10	50	50	49	1	0.98	≥ 2
S13	51	48	36	0.94	0.75	1

Segregating T2 plants (second generation after dipped plants) from six (S2, S4, S5, S10, S13, S14) independent transgenic lines were checked for the over-expression of the ALDH3H1 gene at both transcription and protein levels. All lines except S2 were found to constitutively express the gene at high level compared to the wild type. Results on S13 and S10 transgenic plants are shown in **Fig. 16**.

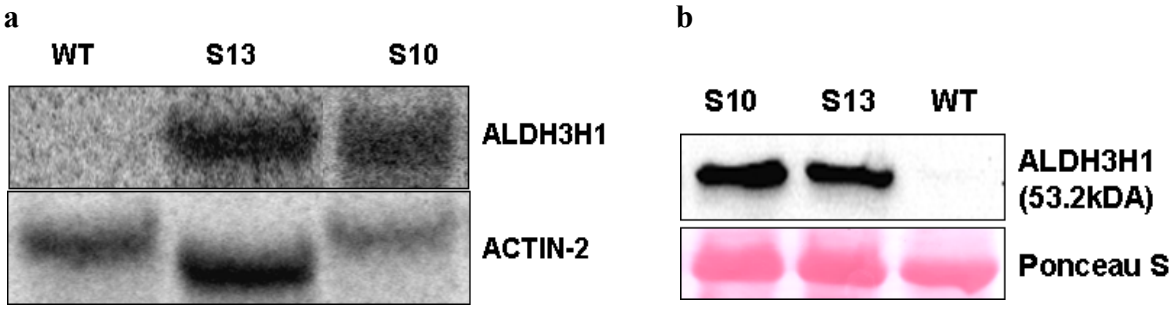


Fig. 16 RNA and protein-blot analyses with the putative ALDH3H1 over-expressors. (**a**) Accumulation of *ALDH3H1* transcripts in ALDH3H1 over-expressors. The RNA blot was performed with 20 µg total RNAs from T2 plants. A *Bam*HI cDNA fragment comprising the ALDH3H1 coding sequence was used as probe. (**b**) Accumulation of the ALDH3H1 protein in ALDH3H1 over-expressors. Fifteen micrograms crude protein extracts from T2 plants were analysed by protein-blot. Antibodies raised against the ALDH3H1 protein were used for the immuno-detection assay. **WT**: Wild type. **S**: Independent ALDH3H1 over-expressing lines.

Homozygous transgenic plants, as determined by the kanamycin resistance throughout the segregation, were isolated. They were subjected along with the wild-type plant to various stress treatments so as to investigate the role of the ALDH3H1 protein in response to abiotic stress. Here are shown the data on the transgenic lines S10 and S13.

3.2.4 Functional characterization of ALDH3H1 over-expressors exposed to various abiotic stressors

RNA and protein-blot analyses have revealed an organ and age-dependent expression pattern of the *ALDH3H1* gene upon drought, ABA or NaCl treatment (Kirch et al. 2005; **Fig. 11**). To understand the function of *ALDH3H1* in the stress response of *Arabidopsis*, transgenic plants over-expressing this protein were compared to the wild type under various abiotic stress conditions. In addition, KO69, a T-DNA insertion mutant for *ALDH3H1* was included in the experiments. This line, hereafter renamed *3h1-B*, carries a T-DNA fragment in the first intron of the *ALDH3H1* gene. This mutant was found to express significantly reduced levels of *ALDH3H1* transcripts as compared to the wild type (Ditzer, unpublished data). Homozygous plants were screened by two different sets of PCR. One involved the use of the *ALDH3H1*

gene specific primers P1 and P5 (**Fig. 17**; panel "Genomic") and the second the combination of P1 with a T-DNA left border primer FISH1 (**Fig. 17**; panel T-DNA).

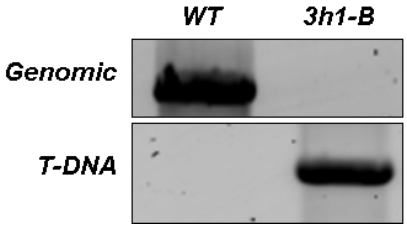


Fig. 17 Screening of the homozygous T-DNA insertion mutant *3h1-B*.

The homozygous status was confirmed by the same PCRs on the segregating plants from a single homozygous parent. The stress experiments were performed on both young and adult wild-type and transgenic plants.

3.2.4.1 *In vitro*-based stress experiments

Wild-type and transgenic plants were sown on MS-agar medium containing different concentrations of NaCl. The germination rate was scored and the effect of NaCl on the growth was measured as fresh weight accumulation. As shown in **Fig. 18** and **19**, wild-type and transgenic plants were similarly affected in their germination rate and seedling growth.

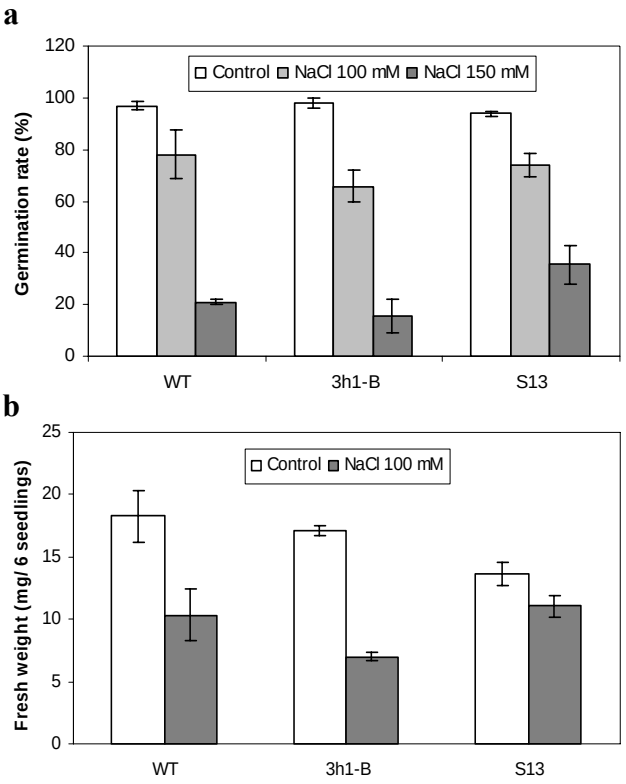


Fig. 18 Germination rate and growth assays. (**a**) The percentage of germinated seeds for each genotype on salt. (**b**) The growth of seedlings on salt assayed as fresh weight accumulation.

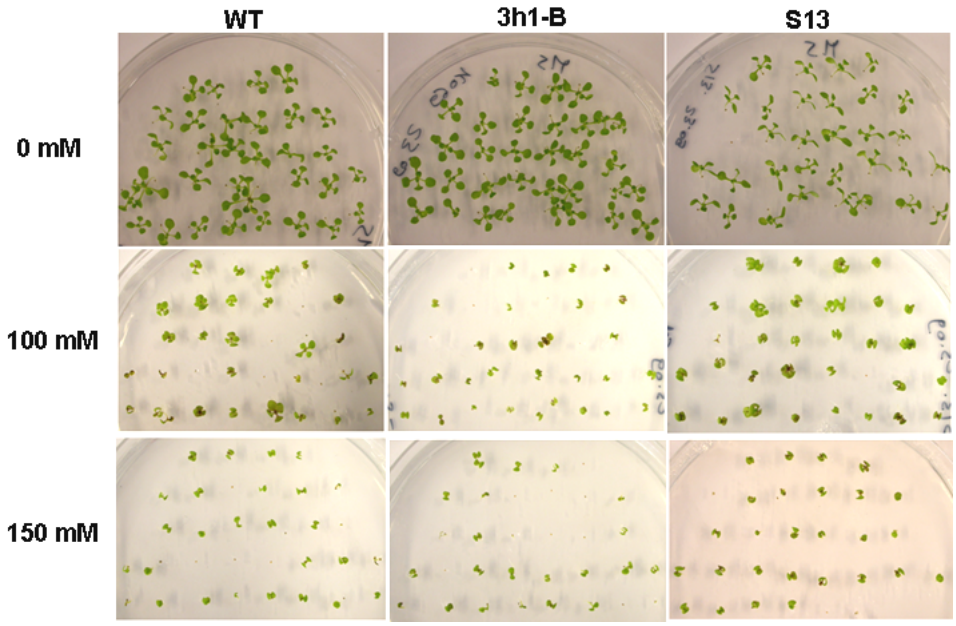


Fig. 19 Photograph of wild-type and transgenic seedlings grown on salt. *A. thaliana* wild-type and transgenic seeds were surface-sterilised and plated on MS-agar plates supplemented with different concentrations of NaCl. Pictures were taken 14 days after germination.

Likewise, all three genotypes have similarly reduced chlorophyll levels on 100 mM NaCl (**Fig. 20**). However, the ALDH3H1 over-expressor S13 accumulated less MDA than the wild type and most significantly less than the knock-down mutant *3h1-B* (**Fig. 20**).

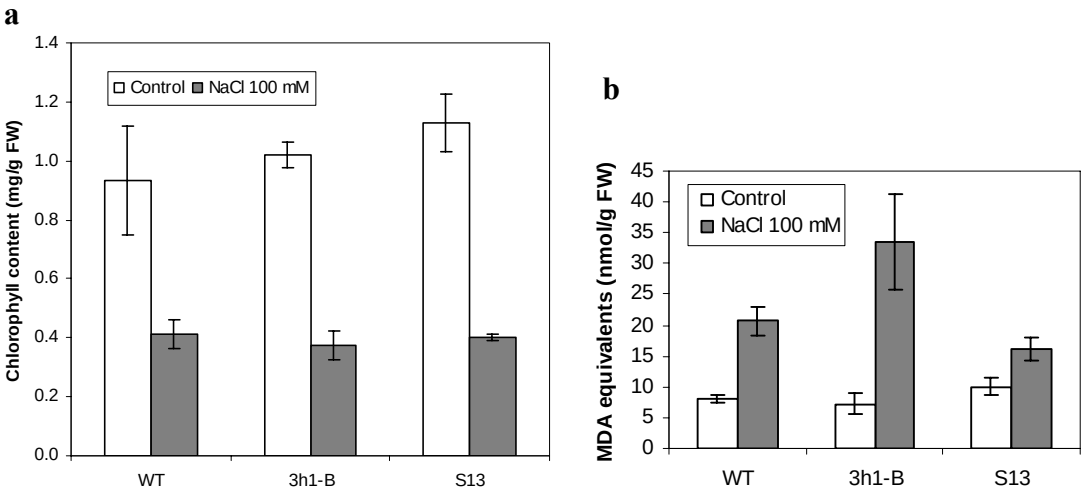


Fig. 20 Chlorophyll and malondialdehyde contents. (**a**) Chlorophyll content. (**b**) Accumulation of malondialdehyde (MDA) on salt.

These observations suggested that without conferring a general abiotic stress tolerance to the plant the over-expression of ALDH3H1 protein alleviated salt stress injuries in seedlings. With regard to the germination rate and the gain of biomass on salt, *3h1-B* plants are as sensitive as the wild type under salt stress; indicating a leaking effect of the mutation. The soil-based salt stress experiments were therefore focused on the wild-type and the ALDH3H1 over-expressors.

3.2.4.2 Soil-based stress experiments

Two week-old wild-type and over-expressing plants (S10 and S13) growing on MS-agar were transferred to soil and allowed to grow for 3 weeks. The salt stress was imposed by watering plants every two days with water containing 0 – 300 mM Nacl. Leaf samples were taken after 7 and 14 days of treatments and used to quantify the MDA and the free L-proline contents. No difference was seen in the MDA and proline contents between wild type and the transgenics after 14 days of salt stress (**Fig. 21**). The accumulation of proline correlated with the severity and the duration of the stress.

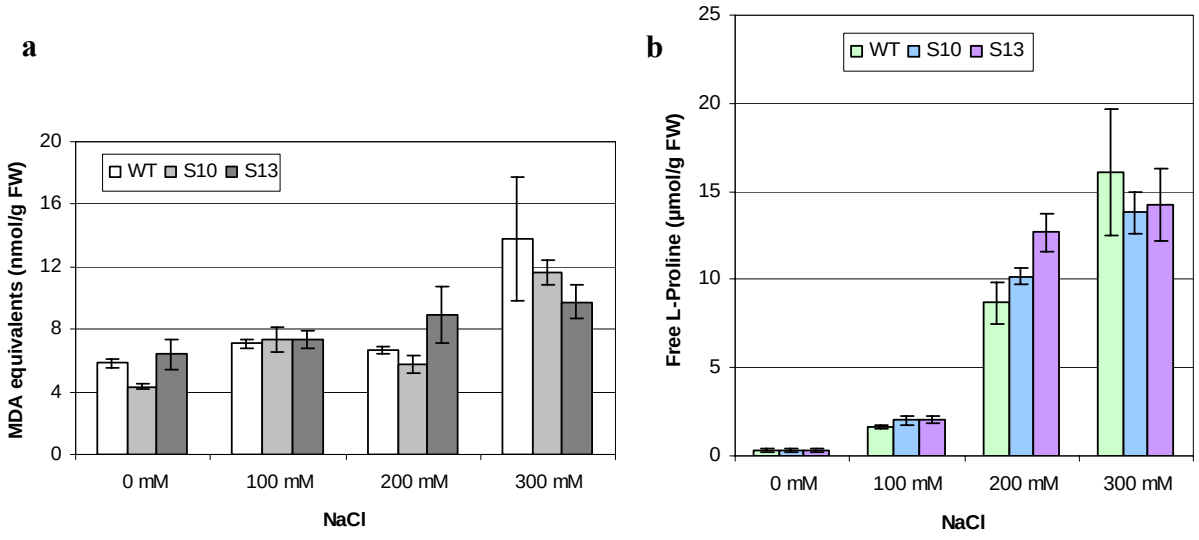


Fig. 21 MDA and free proline contents upon salt stress on soil. (**a**) MDA content; (**b**) Free proline content.

When the plants were subjected to drought stress for 14 days, the ALDH3H1 over-expressors accumulated less MDA and proline than the wild type and the *3H1-B* knock-down mutant (**Fig. 22a,b**). But, this did not improve the fitness of the over-expressors as they all dried out like the wild type. No difference was observed between the wild type and these transgenic plants with regard to the H_2O_2 accumulation (**Fig. 22c**).

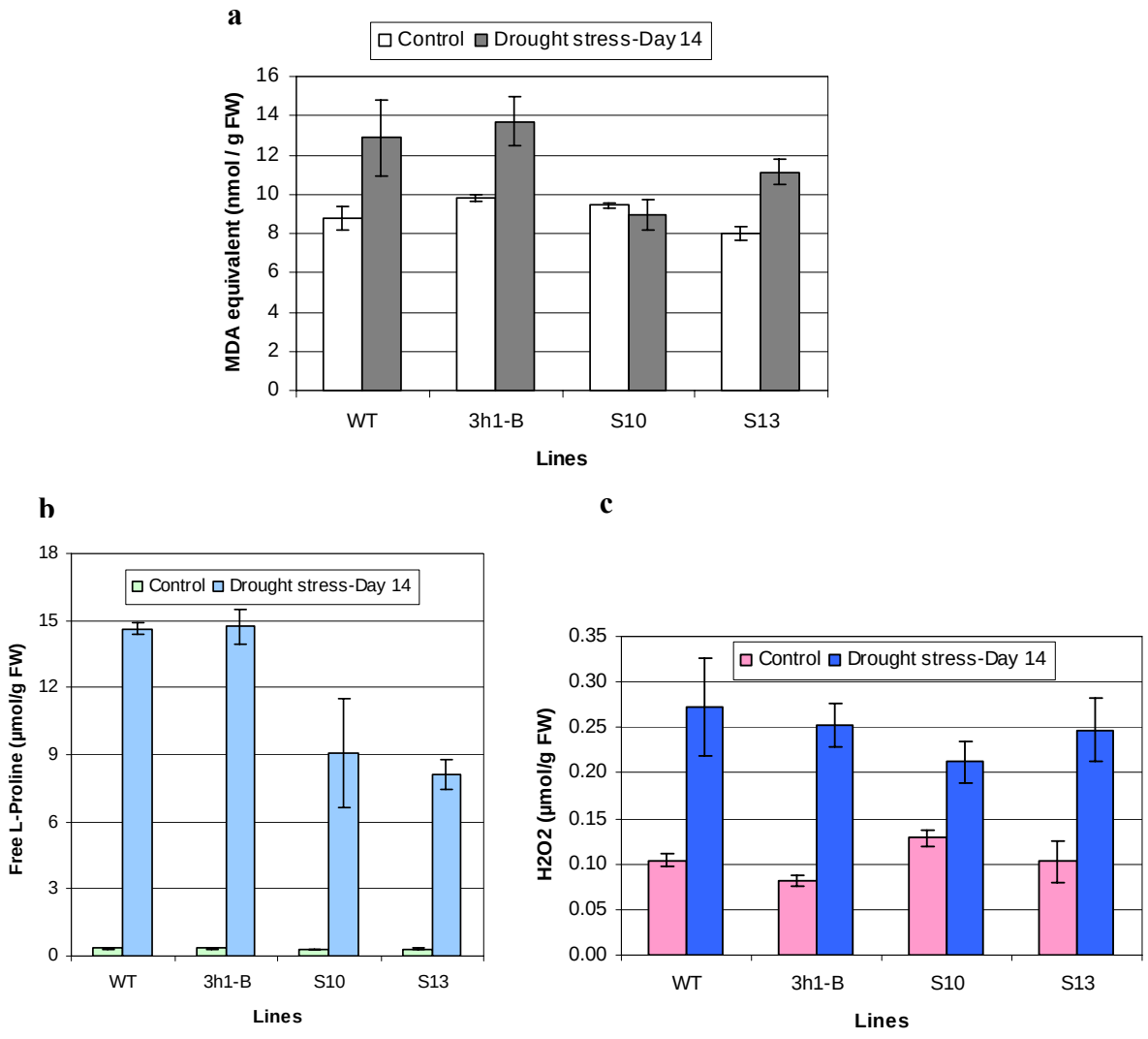


Fig. 22 Accumulation of MDA (a), free proline (b) and H_2O_2 (c) in wild type and transgenic plants upon drought on soil.

The plants were further challenged with methyl viologen (Paraquat®). The methyl viologen is a toxic herbicide which provokes a strong oxidative burst in the plant. Under high light exposure, it causes a rapid depletion of the photosynthetic apparatus leading to necrosis and

plant death. Again, wild-type and ALDH3H1 over-expressing plants showed similar MDA increase after 7 days of methyl viologen treatment (**Fig. 23**).

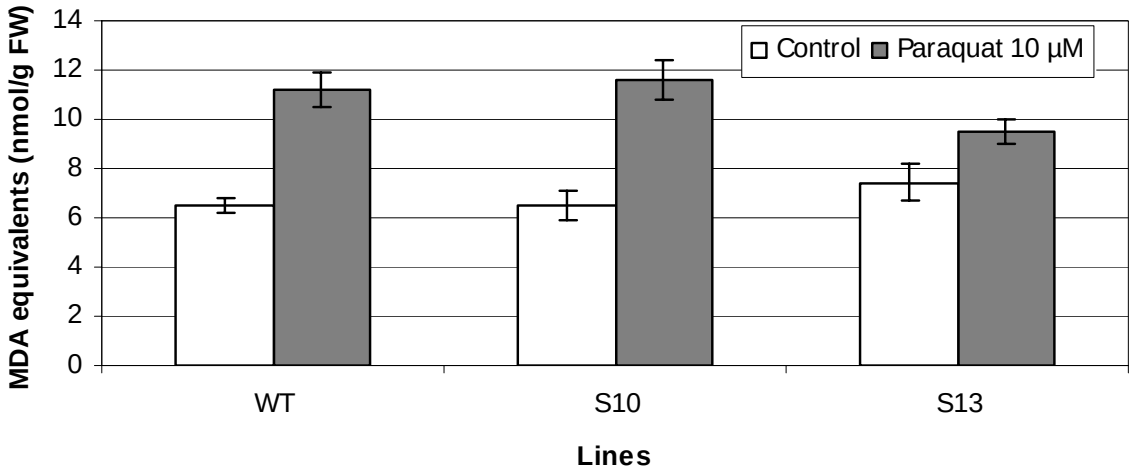


Fig. 23 Accumulation of MDA in the wild type (WT) and ALDH3H1 over-expressors (S10 and S13) upon Paraquat® treatment. Five week soil-growing plants were sprayed with 10µM Paraquat® (methyl viologen) every 2 days for one week. Control plants were watered with tap water. Leaf samples were taken and assayed for their MDA content.

In summary, the over-expression of the ALDH3H1 protein did not confer abiotic stress tolerance to *Arabidopsis*. However, the results indicated a role of the ALDH3H1 protein in alleviating the stress injuries related to lipid peroxidation derived-byproducts. Due to the leaky phenotype of the *3H1-B* mutant, additional T-DNA insertion mutants of the *ALDH3H1* gene were investigated.

3.2.5 Identification and characterisation of further T-DNA insertion mutants of the *ALDH3H1* gene

Two further *ALDH3H1* T-DNA insertion mutants were identified from the SAIL collection (Syngenta Arabidopsis Insertion Library; McElver et al. 2001; Sessions et al. 2002) and the seeds were obtained from the European Arabidopsis Stock Centre (NASC). The line SAIL_832_A05, hereafter named *3h1-A*, carries a T-DNA insertion in the first exon whereas the line SAIL_828_D05, hereafter named *3h1-C*, has the insertion in the seventh exon, according to the predicted protein coding gene model *AT1G44170.1* on the Arabidopsis Information Resource (TAIR) website (http://gbrowse.arabidopsis.org/cgi-

bin/gbrowse/arabidopsis/?name=AT1G44170.1). The locations of the T-DNA insertions for each mutant and those of the primers used in the molecular analyses are depicted in **Fig. 24**.

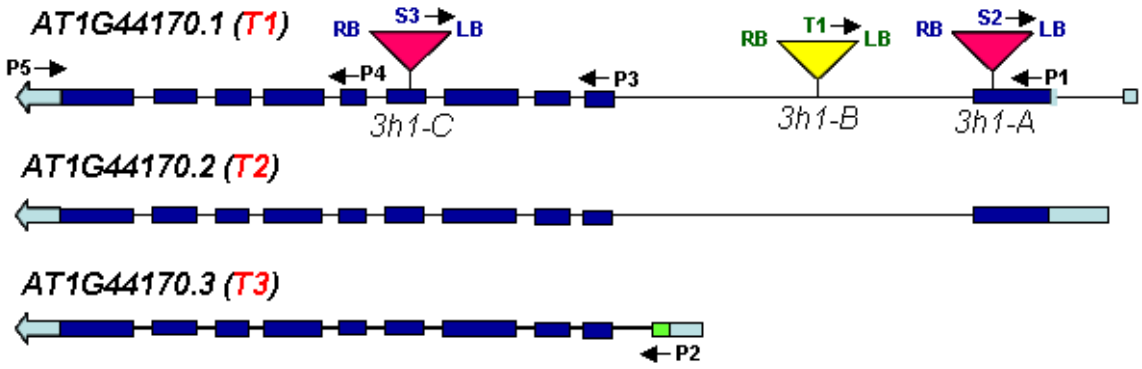


Fig. 24 Schematic representation of the protein coding gene models from the *ALDH3H1* locus including the T-DNA insertion sites for each mutant. ***Small black arrows***: orientation of primers and their approximate position within the gene or the T-DNA. ***Light blue colored arrows***: 3'-UTR. ***Light blue colored rectangles***: 5'-UTR. ***Dark blue-colored rectangles***: exons. ***Green-colored rectangle:*** exon2'. ***Black line between exons***: introns. **LB** and **RB**: left and right borders of the T-DNA fragment. **T1**: primer FISH1. **S2**: primer Sail-10A9-rev. **S3**: primer LB3. **P1-P4**: Exon-specific forward primers. **P5**: Reverse primer used with P1-P4. ***T1-T3***: Transcript variants associated to the gene models.

3.2.6 Molecular characterisation of the *3h1-A* and *3h1-C* mutants

Soil-grown *3h1-A* and *3h1-C* plants were screened by PCR to isolate homozygous mutants. A similar approach as described above for the *3H1-B* mutant was used. The T-DNA specific primers S2 (Sail-10A9-rev) and S3 (LB3; Sessions et al. 2002) were used with the primer P1 to confirm the presence of T-DNA in *3h1-A* and *3h1-C*, respectively (**Fig. 25**).

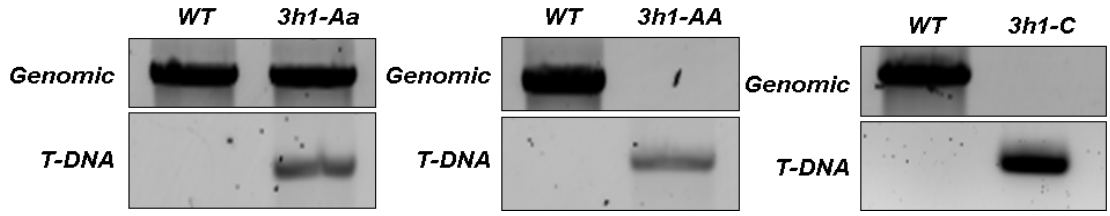


Fig. 25 Screening of the homozygous T-DNA insertion mutant *3h1-A* and *3h1-C*. *3h1-Aa* and *3h1-AA* are respectively *3h1-A* heterozygous and homozygous for the T-DNA insertion.

Next, isolated homozygous *3h1-A* and *3h1-C* mutants were analysed by RT-PCR to check for the expression of gene. First-strand cDNAs were synthesized from total leaf RNAs and used as template for the subsequent PCR reactions. The gene-specific primers P1 and P5 were used to specifically amplify the full-length *ALDH3H1* transcript (*AT1G44170.1*). In parallel, the *Actin-2* gene was amplified to monitor the quality and the amount of the first-strand cDNA

template. Results showed that the *3h1-A* plants did not accumulate the full-length *ALDH3H1* transcript, as compared to the wild type. Instead, they expressed another transcript variant that could be detected when the primer P1 was replaced in the PCR by the primer P4 that is located downstream to the insertion site (**Fig. 26**). The origin of this novel *ALDH3H1* transcript was further investigated.

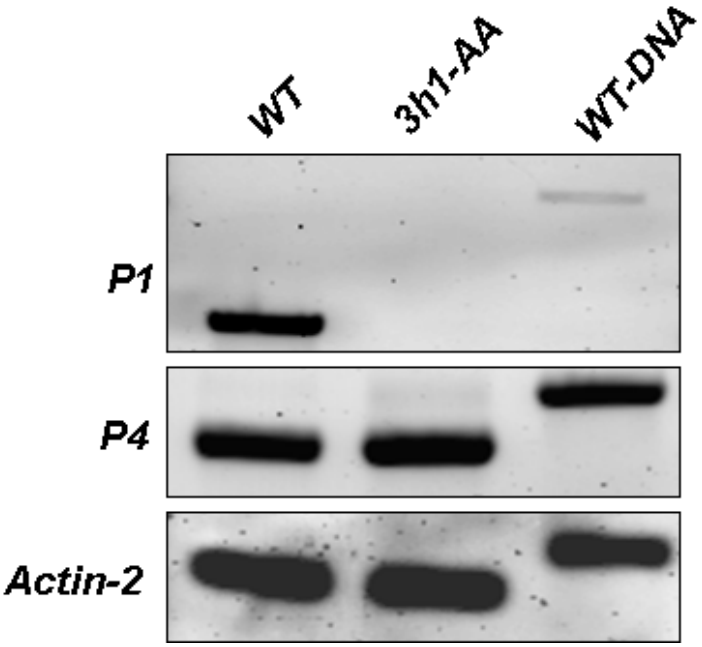


Fig. 26 Analysis of the *ALDH3H1* transcripts in homozygous *3h1-AA* mutants. P1 and **P4** are the forward primers used in combination with the reverse primer P5 for the RT-PCR. The use of **P1** allows to specifically amplify the full-length *ALDH3H1* transcript (*AT1G44170.1*) whereas **P4** cannot discriminate between any other existing *ALDH3H1* transcripts. See **Fig. 24** for the primer locations. **WT**: wild type. WT genomic DNA was used as control.

As depicted in **Fig. 24**, three protein coding gene models (*AT1G44170.1*, *AT1G44170.2* and *AT1G44170.3*) are predicted to derive from the *ALDH3H1* gene locus (*AT1G44170*) (TAIR, http://gbrowse.arabidopsis.org/cgi-bin/gbrowse/arabidopsis/?name=AT1G44170.1).
AT1G44170.1 (T1) and *AT1G44170.2* (T2) primary transcripts have equal numbers of introns and exons with the same organization and sequence. However, they differ in their 5'UTR sequence. In contrast, the *AT1G44170.3* (T3) transcript model differs from the others in its 5'-end as it lacks the first exon completely. Instead, it has a cryptic exon (*exon2'*) of 24 base pairs at the 5'-end, which is embedded in the first intron of the two previous models (**Fig. 24**). Here, *T1* and *T2* were studied as a single variant (*T1*[*T2*]), since transcripts *T1* and *T2* were not distinguished by the primers. The short *ALDH3H1* transcript detected in the *3h1-A* line has never been experimentally identified and characterized before. Based on the predictions, it

was hypothesized that this transcript could correspond to the transcript variant *T3*. Two additional primers were designed to investigate this hypothesis by PCR. One primer (P2) was made of the 24 nucleotides of the *exon2'* and the second (P3) was located in the *exon2*, as shown in **Fig 24**. The primer P2 is to specifically amplify the transcript *T3* whereas the primers P3 and P4 will not discriminate the *ALDH3H1* transcript isoforms. Consistent with the hypothesis, *T3* could be amplified from the homozygous *3h1-A* mutant using the primers P2 and P5. But, it was neither detected in the wild type nor in the *3h1-B* and *3h1-C* lines. As expected, both *3h1-B* and *3h1-C* mutants did not accumulate any of the *ALDH3H1* transcripts (**Fig. 27**).

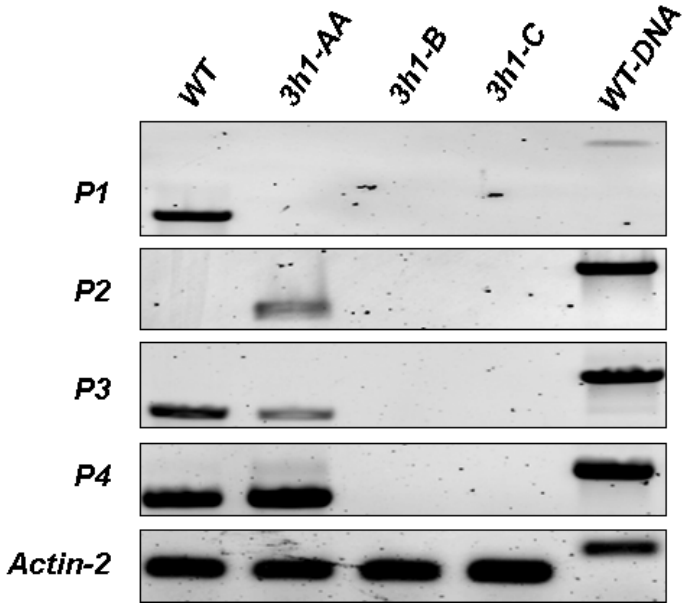


Fig. 27 Comparative analysis of the *ALDH3H1* transcripts in homozygous *3h1-AA, 3h1-B* and *3h1-C* mutants. P1, **P2**, **P3** and **P4** are the forward primers used in combination with the reverse primer **P5** for the RT-PCR. **P1** allows to specifically amplify T1[T2] transcript variants whereas P2 is specific to the isoform T3. **P3** and **P4** cannot discriminate between any of the three *ALDH3H1* transcript isoforms. See **Fig. 24** for the primer locations. **WT**: wild type. WT genomic DNA was used as control.

Based on these observations, the accumulation of the ALDH3H1 protein was next analysed in these mutants. Equal amounts of crude protein extracts from the wild type, the homozygous *3h1-A*, *3h1-B*, *3h1-C* insertion mutants and the over-expressing lines S13 and S10 were analysed by protein-blot followed by immuno-detection using affinity-purified ALDH3H1-specific antibodies. In comparison with the wild-type and the over-expressors, none of the T-DNA insertion mutants were found to express the functional 53.2 kDa ALDH3H1 protein (**Fig. 28**). Moreover, the 46.2 kDa peptide that would derive from *T3* was not observed in the

lanes containing 3h1-A protein samples. Due to these observations, the nature of the transcript *T3* and its expression were further investigated in the *3h1-A* line.

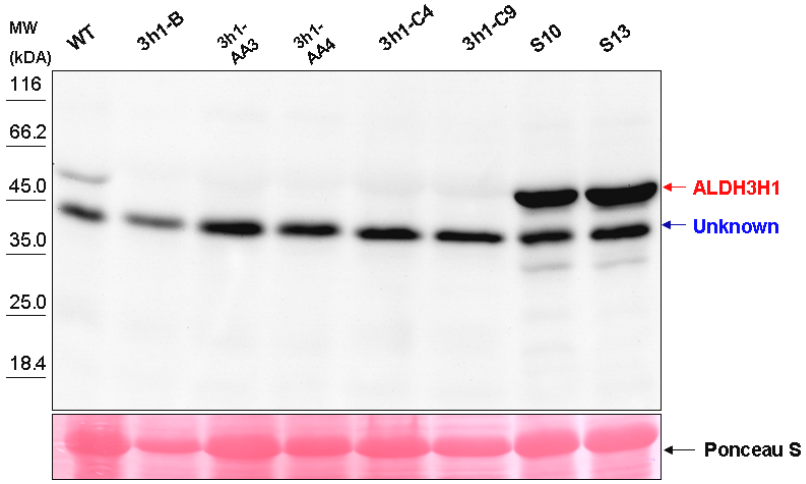


Fig. 28 Comparative analysis of the accumulation of the ALDH3H1 protein in the wild type, the ALDH3H1 over-expressors and T-DNA insertion mutants.

Homozygous and heterozygous *3h1-A* plants were initially compared by RT-PCR. As shown in **Fig. 29**, the heterozygous plants (*3h1-Aa*), in contrast to the homozygous (*3h1-AA*), expressed both the full-length and the short transcripts. This indicates that the insertion of the T-DNA has triggered the expression of the transcript *T3*, since it was not detected in the wild type. Consistent with this, the PCR products from *3h1-A* samples were nearly half of the homozygous-derived PCR products (**Fig. 29**).

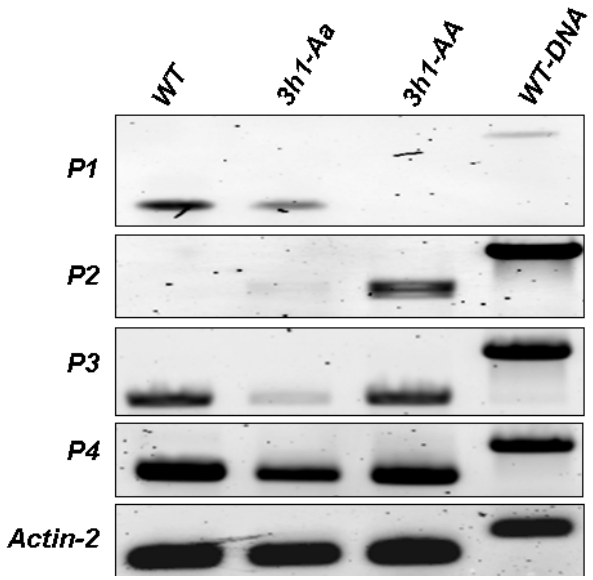


Fig. 29 Comparative analysis of the accumulation of *ALDH3H1* transcripts in wild type, homozygous (*3h1-AA*) and heterozygous (*3h1-Aa*) *3h1-A* mutants. P1, **P2**, **P3** and **P4** are the forward primers used in combination with the reverse primer **P5** for the RT-PCR. **P1** allows to specifically amplify T1[T2] transcript variants whereas P2 is specific to the isoform T3. **P3** and **P4** cannot discriminate between any of the three *ALDH3H1* transcript isoforms.

This actually reflects the mono-allelic origin of the transcripts in heterozygous plants compared to the homozygous plants, in which the T-DNA is simultaneously present (*3h1-AA*) or absent (WT) on the chromosomes doubling the expression of the transcripts.

3.2.7 The origin of the transcript *T3* in the *3h1-A* line

The analysis of the T-DNA insertion mutant *3h1-A* has revealed that this line exclusively expresses a short transcript variant of the *ALDH3H1* gene. The findings indicate that this short transcript derives from the gene model *AT1G44170.3*. But, the intriguing question that remains is to clarify how the transcript T3 is generated. Has the transcript *T3* derived from an alternative splicing of the full-length *ALDH3H1* primary transcripts (*T1* and *T2*) or expressed from an alternative promoter? The first hypothesis is unlikely. The analysis of the genomic DNA in this locus revealed that the mRNA splicing acceptor site does not exist at the junction between the intron sequence and the *exon2'* (**Fig. 30**). Instead of the consensus sequence [GT…AG] at the intron-exon junction of plant protein coding genes the intronic region upstream to the *exon2'* is terminated by an [TA], as highlighted in **Fig. 30**. The existence of an alternative promoter directing the expression of the transcript *T3* has become more plausible and was therefore investigated.

Fig. 30 Partial *AT1G44170.1* gene model sequence including the first intron. Exons and introns are in capital and small letters, respectively. The *exon2'* is colored in green. *Cis*-elements are bold-underlined and the mRNA splicing donor and acceptor sites are bold-colored in red and blue, respectively.

3.2.7.1 Generation and analysis of the *3h1-intron::GUS* construct

To demonstrate that the intronic region can direct the expression of the transcript *T3* a region of the *ALDH3H1* gene locus, +214 to +1457 starting from the ATG codon in the gene model *AT1G44170.1* was amplified from wild-type *Arabidopsis* (Col-0) genomic DNA using the primers INT-Xba-fwd and INT-Xba-rev. These primers were designed with an *Xba*I restriction site. The expected 1244 bp-PCR product was eluted from the agarose gel and then digested by *Xba*I. The resulting *Xba*I-DNA fragment was exchanged with the *ALDH7B4* gene promoter fragment after *Xba*I digestion of 7gB, a pBin19-derived plasmid. The 7gB plasmid harbors the fusion construct *ALDH7B4-promoter::GUS::nospolyA*. The resulting *ALDH3H1-intron::GUS::nospolyA* fusion construct (**Fig. 31a**) was subcloned into *E. coli* DH10B cells. The kanamycin-resistant transformants were screened both by PCR using the INT-Xba-rev and pBIN-HindIII primer pair and sequenced to check for the presence of the *ALDH3H1* gene promoter and its correct orientation. One positive clone was used to transform *A. tumefaciens* competent cells. Resulting *A. tumefaciens* transformants were further screened by PCR as described above and one positive clone was subsequently used.

3.2.7.2 Functional analysis of the *3h1-intron::GUS* construct *in planta*

Functional assays of the fusion construct were performed through the FAST method that allows a transient transformation of *Arabidopsis* seedlings (Li et al. 2009). In comparison with the other plant transient transformation variants, this technique is fast, simple and suitable for construct screening before stable transformation. As shown in **Fig. 31b**, the transiently transformed seedlings expressed the β-glucuronidase (GUS) reporter protein in roots and in cotyledons without any stress treatment.

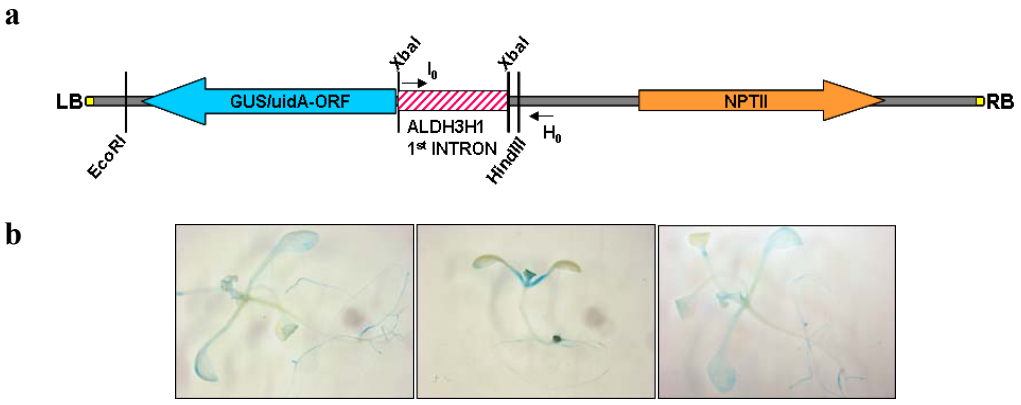


Fig. 31 Generation and functional analysis of the *3h1-intron::GUS* construct. (**a**) Schematic diagram of the engineered T-DNA region. Arrows $\mathbf{I_0}$ and $\mathbf{H_0}$ indicate the approximate location of the primers INT-Xba-rev and pBIN-HindIII, respectively. (**b**) Photographs of transiently transformed *Arabidopsis* seedlings with the *3h1-intron::GUS* fusion construct.

This indicates that the construct is functional and that the intronic fragment is able to direct the transcription of any downstream protein coding DNA fragment. The intronic region +214 to +1457 (starting from the ATG codon in the gene model *AT1G44170.1*) acts as an alternative promoter to drive the expression of the transcript *T3*.

3.2.8 Stress-responsive expression of the transcript *T3*

In silico analysis of the *ALDH3H1* locus by PLACE Web Signal Scan (Prestridge 1991; Higo et al. 1999) revealed the presence of several *cis*-elements between positions +214 and +1457 starting from the ATG codon of the *T1* transcript. As highlighted in **Fig. 30**, two dehydration-responsive element/C-repeat – low temperature-responsive elements (DRE/CRT), one ACGT-box (ABRE) were found. Using the PlantCare tool for *in silico* analysis of promoter sequences (Lescot, 2002) the closest predicted TATA-box and the corresponding transcription start signal (TSS) were respectively found at 133 bp and 103 bp upstream of the ATG of *exon2'*. To test whether these *cis*-elements are functional, the accumulation pattern of the transcript *T3* was investigated in the *3h1-A* plants after dehydration or NaCl treatment. Fifteen day-old homozygous *3h1-A* seedlings were removed from MS-agar plates and either dehydrated on filter paper at room temperature for 1 hour, or incubated in 200 mM NaCl at room temperature for 4 h. The roots and the shoots were separately harvested and immediately frozen in liquid nitrogen. For adult plants, heterozygous *3h1-Aa* plants were used to simultaneously monitor the transcripts *T1*[*T2*] and *T3*. Six week-old heterozygous *3h1-Aa* plants were removed from soil and incubated in water or 250 mM NaCl for 6 h. Leaf and root samples were harversted and used for transcript analyses. The primers P1 and P2 (forward) were used in combination with the primer P5 (reverse) in the RT-PCRs to amplify the transcripts *T1*[*T2*] and *T3* respectively. The pair P4 and P5 amplifies both *T1*[*T2*] and *T3*. As shown in **Fig. 32**, *T3* slightly accumulated in seedling roots upon NaCl and dehydration treatments. But, it was not up-regulated in the shoot. In adult plants, *T3* was up-regulated by salt treatment in both roots and leave. In contrast, *T1*[*T2*] did not increase in roots in response to salt (**Fig. 32**). These observations indicate that the alternative promoter is stress responsive like the upstream *ALDH3H1* gene promoter but both are differentially regulated by salt.

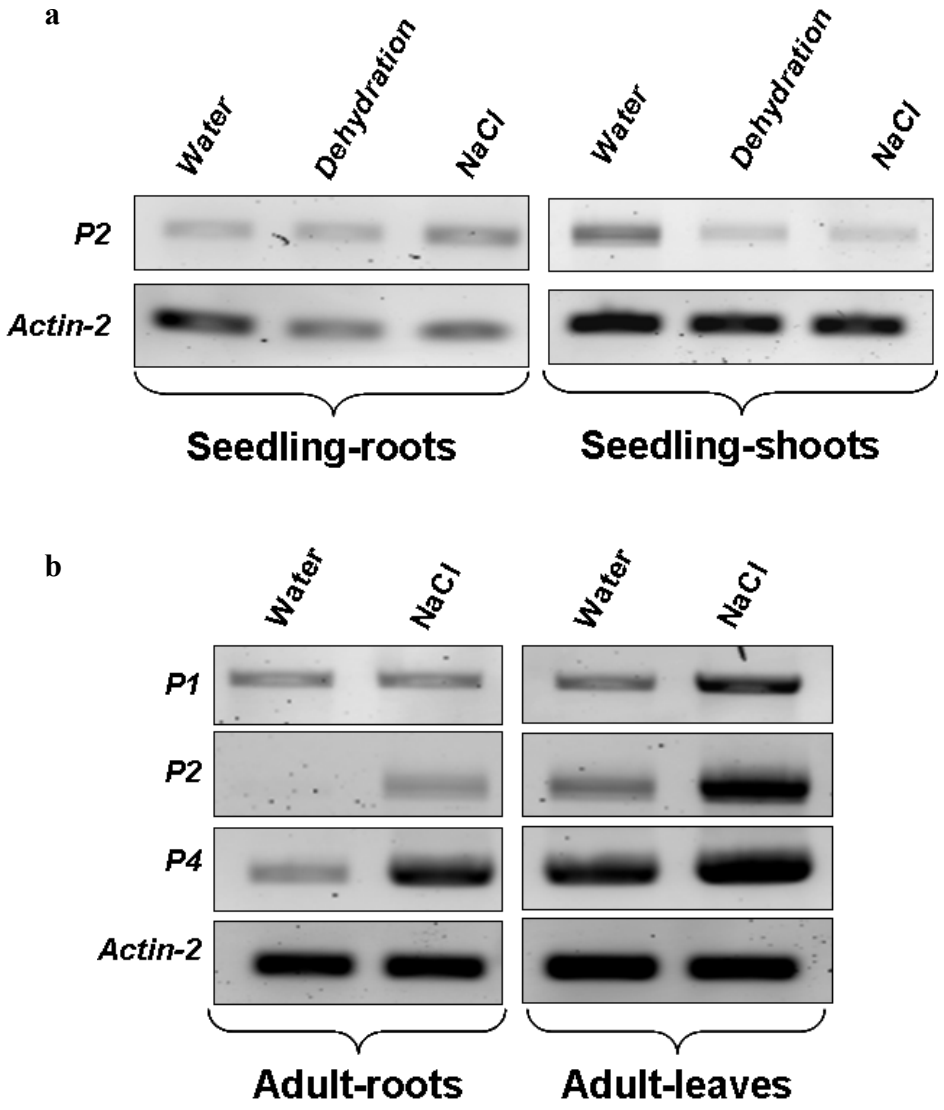


Fig. 32 Expression patterns of the *ALDH3H1 T1*[*T2*] and *T3* transcripts under stress conditions: In seedlings (**a**); in adult plants (**b**). **P1**, **P2**, **P3** and **P4** are the forward primers used in combination with the reverse primer **P5** for the RT-PCR. **P1** allows to specifically amplify T1[T2] transcript variants whereas P2 is specific to the isoform T3. **P4** cannot discriminate between any of the three *ALDH3H1* transcript isoforms. See **Fig. 24** for the primer locations.

3.2.9 Comparative analysis of the *ALDH3H1* T-DNA insertion mutants in response to stress

The phenotype of *3h1-A* and *3h1-C* mutants was further investigated under salt in comparison to the wild type. Seeds from the different genotypes were germinated and grown on MS-agar plates for 6 days. Seedlings were then transferred to fresh MS-agar plates containing 100 mM NaCl. The inhibition of the root growth was assayed for all genotypes after 3 days. As shown

in **Fig. 33** homozygous *3h1-A* and *3h1-C* mutants were affected in their root growth to the same extent; but stronger than the wild type and the heterozygous *3h1-Aa*. There was no phenotypic difference between the heterozygous *3h1-Aa* and the wild type. These observations suggest that the transcript *T3* does not functionally compensate the lack of *T1*[*T2*] under salt stress. However these observations indicate that the ALDH3H1 protein does contribute to cope with stress injuries, as judged by the significant reduction of the root growth in the true *ALDH3H1* knock-out *3h1-C* line compared to the wild type.

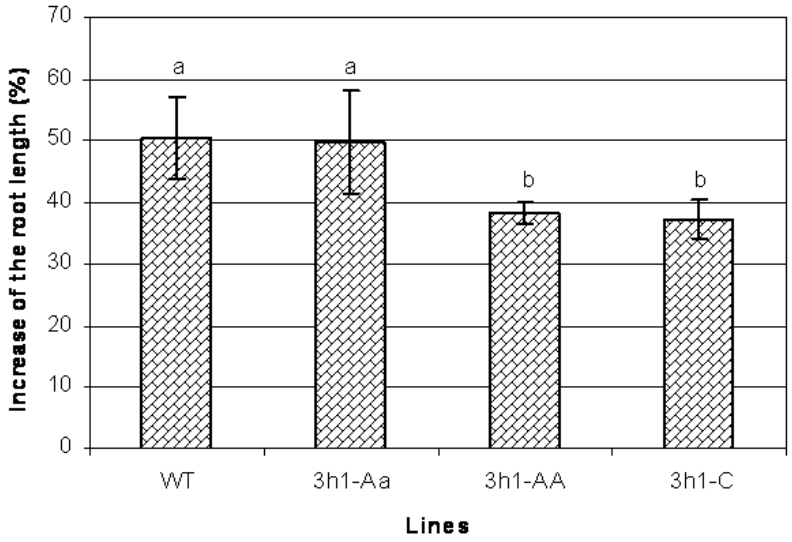


Fig. 33 Testing of the root growth in *ALDH3H1* mutants. The root growth was measured as increase in root length on MS-agar medium supplemented with 100 mM NaCl. The growth on medium without salt was taken as reference for calculating the percentage of inhibition. Series with different letters (a, b) are significantly different (Student *t*-test; *P* value < 0.05).

3.2.10 Sub-cellular localization of the putative protein derived from the transcript *T3*

Although no protein corresponding to the transcript *T3* could be detected by the ALDH3H1 antibodies in protein-blot analyses, it is still plausible that this protein, with an N-terminal end different from that of the full-length ALDH3H1, is produced at a very low level in the cells. Therefore, the putative sub-cellular localization was investigated. The *in silico* analysis of the T3 protein coding sequence in TargetP predicts the corresponding protein to localize in mitochondria. To experimentally validate this prediction, an ALDH3H1 cDNA fragment was amplified by PCR from the plasmid pda06974 (RIKEN Institute) using the primer pair: 5mtALDH3H1-GFP and 3mtALDH3H1-GFP. The primer 5mtALDH3H1-GFP contains the *exon2'* followed by a few nucleotides of the *exon2* (+1593 to +1612 from the *AT1G44170.1* (*T1*) ATG) at the 3'-end. In addition, the 5mtALDH3H1-GFP and 3mtALDH3H1-GFP

primers contain an *Eco*RI and an *Nco*I site respectively to facilitate cloning. The amplified cDNA fragment was purified and sub-cloned in frame and upstream of the Green Fluorescent Protein (GFP) into the *Eco*RI-*Nco*I sites of the pGJ280 vector. Transformants were screened by PCR and sequenced. A single positive clone was used to transiently transform wild-type *Arabidopsis* leaves by particle bombardment. Microscopic observations revealed that the GFP fusion protein specifically accumulated in the cytoplasm (**Fig. 34**). This indicates that, in contrast to the prediction, the putative protein derived from the transcript *T3* localizes in the cytosol, as previously observed for the full-length ALDH3H1 protein (Ditzer, unpublished results).

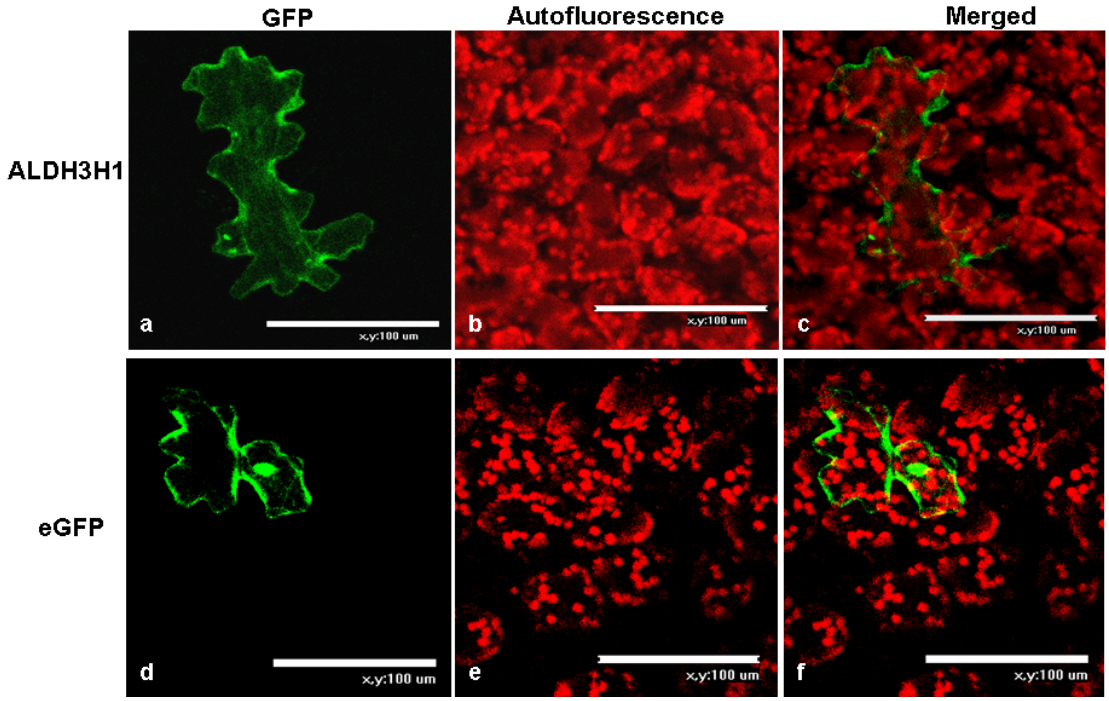


Fig. 34 Sub-cellular localization of the putative AT1G44170.3 (T3) protein. The AT1G44170.3–GFP fusion construct was transiently expressed in *A. thaliana* leaves. Leaves were observed under the fluorescence microscope with appropriate filters. (**a**, **d**) GFP fluorescence alone; (**b, e**): chlorophyll auto-fluorescence; (**c**, **f**): merged chlorophyll and GFP fluorescence together. Scale bars: 100 µm.

In summary, the results from this study give for the first time the experimental evidence that the *ALDH3H1* short transcript variant (*AT1G44170.3* [*T3*]) is expressed in *Arabidopsis*. It is not detected in the wild-type plant but found to be expressed in the *3h1-A* mutant, which carries a T-DNA insertion in the first exon of the *ALDH3H1* locus. The expression of the transcript *T3* is shown to be directed by an alternative promoter comprised within the first intron of this gene. *AT1G44170.1* (*T1*) and *AT1G44170.2* (*T2*) -*T1*[*T2*]- and *AT1G44170.3* transcripts are differentially expressed. *T1*[*T2*] is up-regulated by salt in leaves of adult plants

but not in roots. In contrast, *T3* is up-regulated by salt in both roots and leaves of adult plants. Like *T1*[*T2*], *T3* is induced by salinity in seedling roots but not in shoots. However, the protein T3 could not be detected by the ALDH3H1 antibodies. Functional analyses of the *ALDH3H1* locus through the use of T-DNA insertion mutants and transgenic over-expressing plants indicated that the ALDH3H1 protein can contribute to cope with stress injuries by alleviating damages from lipid peroxidation. However, the constitutive expression of this protein did not confer stress tolerance to the transgenic plants when compared to wild-type plants.

3.3 Responsiveness of the aldehyde dehydrogenase gene *ALDH7B4* to aldehydes

It is well known that plants undergo excessive lipid peroxidation under stress conditions, which at the end leads to the accumulation of aldehydes in cells. Functional analyses of some aldehyde dehydrogenase encoding genes suggested that the detoxification of aldehydes would be performed by the stress-inducible aldehyde dehydrogenases. But the upstream signaling events and factors involved in this response remain to be elucidated. In this chapter this question was addressed by investigating the response of the aldehyde dehydrogenase gene *ALDH7B4* (*AT1G54100*) to aldehydes; further genetic tools were established to study the regulation.

3.3.1 Generation of *ALDH7B4*-promoter::*GUS* expressing plants

To study whether and how aldehyde molecules can affect expression of aldehyde dehydrogenase genes transgenic plants expressing the β-glucuronidase (*GUS*) reporter gene driven by the *ALDH7B4* gene promoter were generated (7B4-GUS). The *ALDH7B4* promoter was amplified by PCR from *A. thaliana* Col-0 genomic DNA using the primer pair Aldh7B4-prom-5’ and Aldh7B4-prom-3’. The PCR product was digested with *Eco*RI and purified from an agarose gel. The resulting 0.64 kb *Eco*RI promoter fragment was purified and subcloned into the pBT10-GUS vector to generate the clones *7gt*. Next, one positive *7gt* clone was digested with *Bam*HI and *Bgl*II to isolate the *ALDH7B4-promoter::GUS::nos_terminator* cassette that was then subcloned into the unique *Bam*HI site of the binary vector pBIN19 to yield the clones *7gB*. A single *7gB* clone was used to transform *A. tumefaciens* cells.

Transformed colonies were selected on YEB + rifampicin (100 mg/L) + kanamycin (50 mg/L) plates and further checked by both enzymatic digestion and PCR. A fragment of 0.4 kb from the GUS coding region was targeted by PCR with the primers GUS-sense and GUS-antisense (**Fig. 35**).

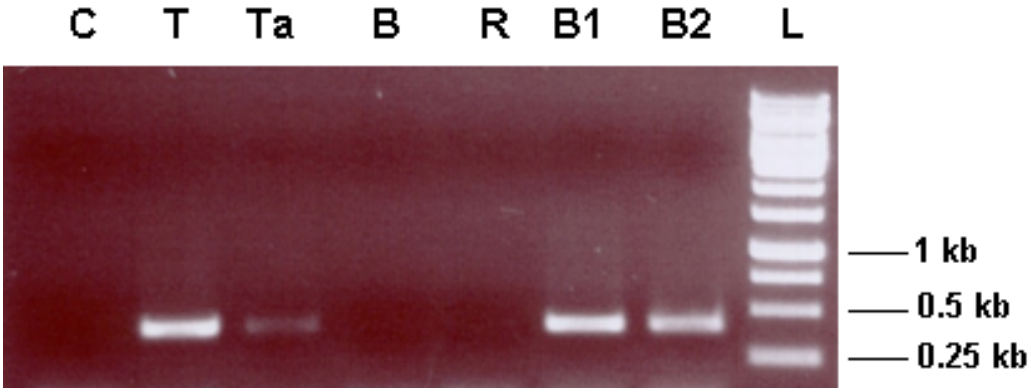


Fig. 35 Screening of the recombinant 7B4-GUS clones by partial amplification of the *GUS* gene. **T**: pBT10-GUS; **Ta**: plasmid 7gB; **B**: vector pBin19; **R**: plasmid pROK2; **B1** and **B2**: plasmid DNA from transformed *A. tumefaciens*. **T** and **Ta** are used as positive controls. **B** and **R** are negative controls as they do not contain any GUS coding sequence. **C**: Negative control for PCR performed with water instead of DNA. **L**: 1 kb DNA ladder.

Similarly, a fusion construct of *CaMV35S-promoter::GUS-nos_terminato*r was made for the control experiments. A *Hin*dIII–*Eco*RI fragment with CaMV35S (dual enhancer) was isolated from pRTL2-GUS vector and subcloned into pBT10-GUS vector. The resulting recombinant plasmids were named *pTS*. Then, the *CaMV35S_promoter::GUS-nos_terminato*r gene cassette was isolated from a *pTS* clone after *Hin*dIII–*Bgl*II digestion and subcloned in the *Hin*dIII and *Bam*HI sites of pBIN19 plant transformation vector to yield *pBS* plasmids. Recombinant *pBS* clones were selected as described above and transferred into *A. tumefaciens*. One recombinant *Agrobacterium* clone was used to transform wild-type *A. thaliana* Col-0 plants to generate 35S-GUS transgenic plants.

3.3.2 Molecular characterization and segregation analysis of the 7B4-GUS and 35S-GUS lines

Independent 7B4-GUS and 35S-GUS transgenic plants were first selected on kanamycin and next checked by genomic DNA-based PCR using the primers pBIN-HindIII and GUS-start. Plants carrying the 7B4-GUS or 35S-GUS constructs were respectively renamed B-lines and R-lines. These independent lines were screened by DNA-blot analyses to check the number of

T-DNA fragments that were integrated in each plant. It was found that the majority of kanamycin-resistant lines harbored at least one T-DNA fragment (**Fig. 36a**). Two independent lines, B8 and B10, showed a single T-DNA insertion (**Fig. 36b**). The segregation of the kanamycin resistance was examined in the T2 progeny of these selected transgenic plants. Consistent with the DNA-blot analyses B8 and B10 lines have a single functional T-DNA insertion making them as good candidates for the gene expression analyses. The DNA-blot analysis revealed the presence of three T-DNA fragments in the line B19. But, seeds from this plant segregated as single locus with the kanamycin resistance, suggesting a linkage between the T-DNA fragments. Alternatively, one or two T-DNA fragments might not be functional. Although many other independent lines were also examined, only results from B8, B10 and B19 are reported in the next paragraphs. No phenotypic difference was observed between these lines and the wild type with respect to the germination rate, growth, flowering time and seed yield.

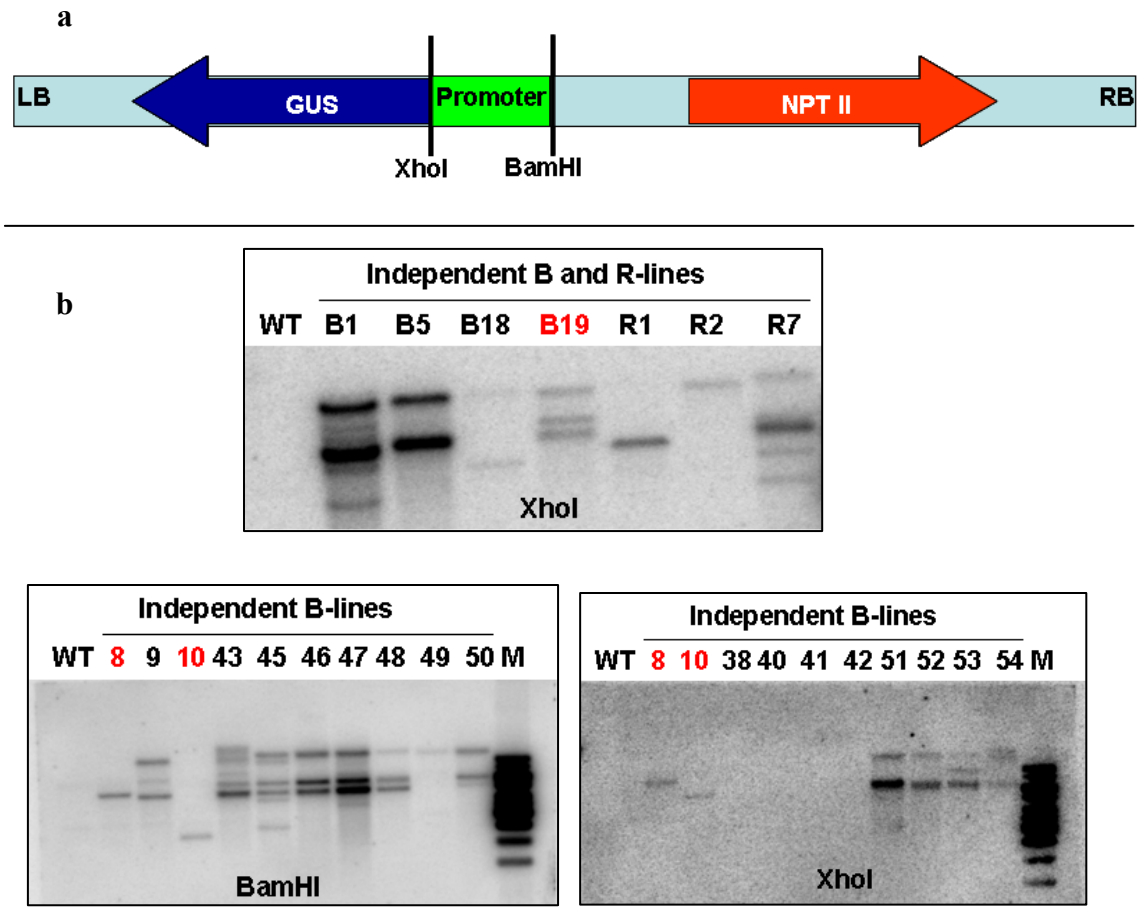


Fig. 36 Analysis of independent transgenic lines expressing the *ALDH7B4-GUS* gene cassette. (**a**): Schematic representation of the T-DNA region. (**b**): Photographs of 3 DNA-blot membranes probed with ^{32}P-labelled NPTII DNA fragment. Twelve micrograms genomic DNA (from T2 or T3 plants) were digested by *Xho*I or *BamHI*. **WT**: Wild type; **M**: DNA size marker.

3.3.3 Activity of the *ALDH7B4* promoter in reproductive organs and seeds

The induction pattern of the *ALDH7B4* gene by various abiotic stressors was previously examined and published (Kirch et al. 2005). These analyses were mainly focused in leaves and roots of young plants and it was found that the expression of the gene is maintained at a relatively low level in the plant under non-stress conditions. Here, the analyses of the *ALDH7B4* expression were extended to reproductive organs and seeds. Homozygous plants were assayed without any previous stress treatments. Floral buds, opened flowers, siliques of different sizes and seeds were harvested and were either immediately stained or frozen in liquid nitrogen. The activity of the promoter was either assayed by visual observation after staining tissues with the GUS-staining buffer or monitored measuring the GUS protein activity. It was found that the *ALDH7B4* promoter is strongly induced during maturation of the siliques and in mature seeds (**Fig. 37**).

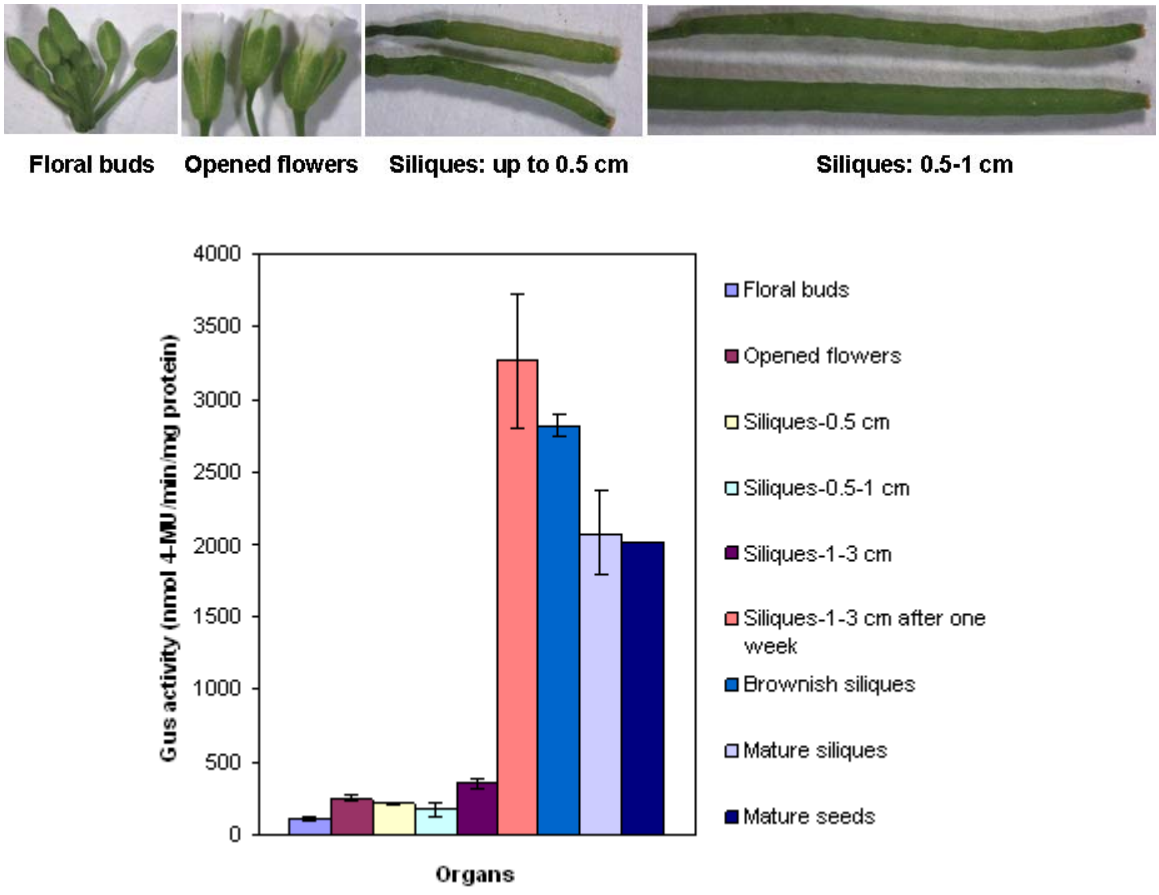


Fig. 37 Quantitative assessment of the activity of the *ALDH7B4* promoter in different organs of *Arabidopsis*. Upper panel: Photographs of the organs analysed. **Lower panel**: The GUS activities in the analysed organs. The ALDH7B4 protein accumulation occurs during the maturation of the silliques and remains at a high level in mature seeds.

Pistil and stamens showed intense blue staining reflecting the activity of the promoter in these organs (**Fig. 38**).

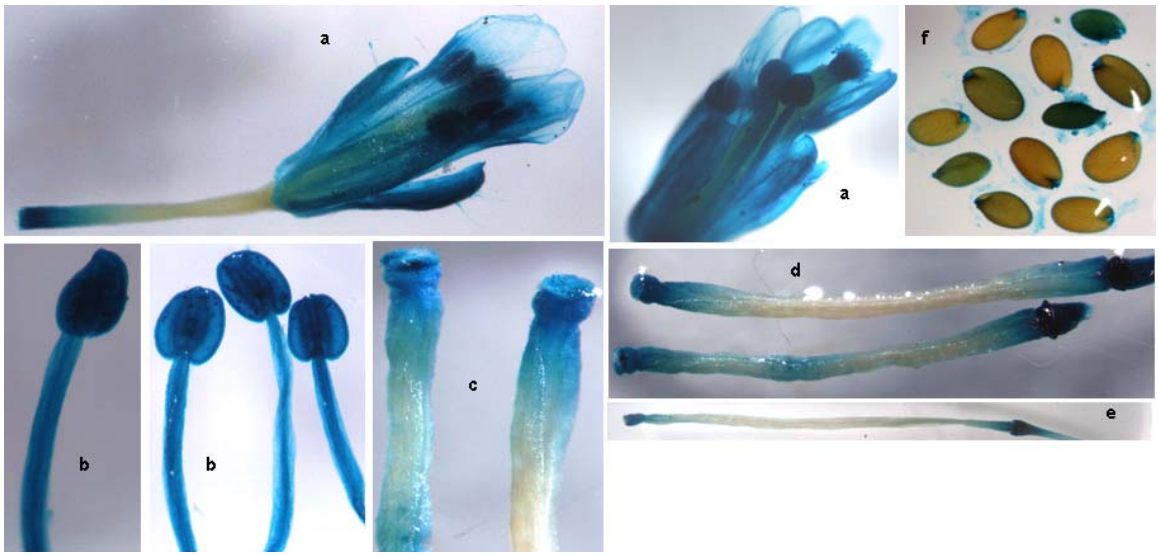


Fig. 38 ***In situ*** **detection of the activity of the** ***ALDH7B4*** **promoter in different organs of** ***Arabidopsis*****.** **a**: flower; **b**: stamens; **c**: pistil; **d**: silliques of 0.5-1 cm; **e**: silliques of 1-1.5 cm; **f**: mature seeds. Plant materials were directly incubated in the GUS-staining buffer after being harversted.

The constitutive activity of the *ALDH7B4* promoter in seeds was further investigated. Crude protein extracts from both wild-type and transgenic seeds were analysed by protein-blot using diluted (1:2500) antibodies raised against the ALDH7B4 protein. The crude antibodies were produced by BioGenes (Berlin, Germany) from affinity-purified recombinant ALDH3H1 protein made by Dr. Andreas Ditzer. As shown in **Fig. 39**, the ALDH7B4 protein accumulated to higher levels in wild-type and transgenic seeds than in leaves.

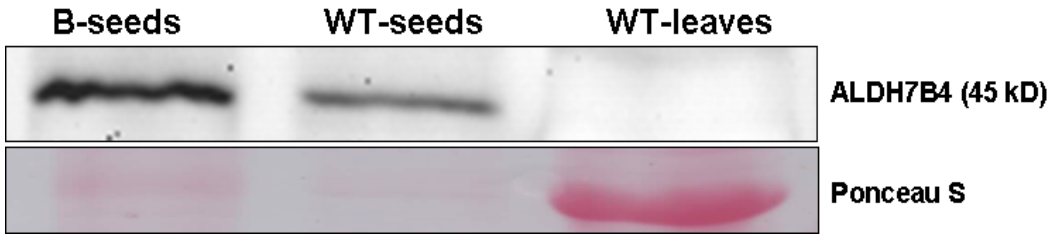


Fig. 39 Immunodetection of the ALDH7B4 protein (54 kDa) by protein-blot analysis of total proteins from wild-type and transgenic ***Arabidopsis*** **leaves or dry seeds. B:** 7B4-GUS line; **WT**: Wild type.

This suggests that the accumulation of ALDH7B4 in the different organs involves *trans*-acting factors shared by both the wild-type and transgenic plants and that this accumulation does not result from the insertion of the transgene. It was argued that the *ALDH7B4* gene is constitutively active in naturally desiccation-tolerant organs like seeds and pollen and thus follows the expression pattern of many LEA (late embryogenesis abundant) genes. Accumulated ALDH7B4 protein would be of great importance during the germination and the growth of seedlings, when the plant metabolism is particularly active and plants are more sensitive to environmental stresses. With regards to this the accumulation of the GUS protein was examined in 14 day-old seedlings. As shown in **Fig. 40**, the activity of the promoter is still intense in untreated seedlings and no visual difference could be seen between the untreated and aldehyde- or NaCl-treated plantlets. However, the background expression of the promoter was low in older seedlings and particularly in adult plants (**Fig. 40**).

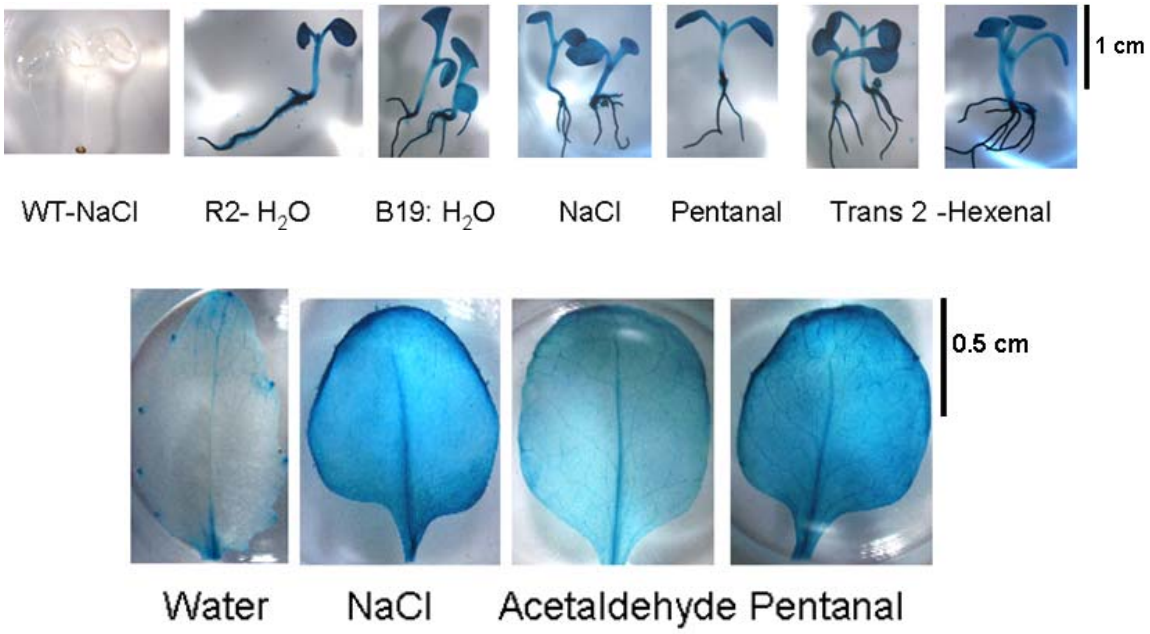


Fig. 40 Activity of the *ALDH7B4* promoter in *Arabidopsis* seedlings and adult plant tissues. Upper panel: 14 day-old plantlets were incubated either in water, 200 mM NaCl or 5 µmol of aldehydes. **WT**: wild type; **R2**: 35S-GUS line; **B19**: 7B4-GUS line. **Lower panel**: Detached leaves from 4 week-old soil-grown 7B4-GUS plants were treated with either water, 200 mM NaCl or 5 µmol of aldehydes.

3.3.4 Activity of the transgenic 7B4-GUS lines in response to aldehyde and abiotic stress treatments

Four week-old B8 and B10 plants grown on soil were used for the experiments. Single leaves were detached from the plants and incubated with water, 10 mM H_2O_2, 100 µM $CuSO_4$, 5 µmol pentanal or trans-2-hexenal, 300 mM NaCl for 24 and 48 h. For the dehydration stress

leaves were slowly dried at room temperature. The *in situ* detection of the GUS activity revealed that these lines expressed a very low level of GUS protein under control condition (water) (**Fig. 41a**). It was found that H_2O_2 and pentanal weakly activated the promoter whereas NaCl and dehydration treatments strongly induced its activity. $CuSO_4$ and trans-2-hexenal moderately activated the promoter in both lines. The promoter activity was also quantitatively assessed. Consistent with the histochemical observations, NaCl treatment led to 8-fold higher GUS expression than trans-2-hexenal, in comparison to the control (water); and the dehydration led to 6-fold higher GUS expression than NaCl (**Fig. 41b**). The promoter was found more inducible by trans-2-hexenal than pentanal.

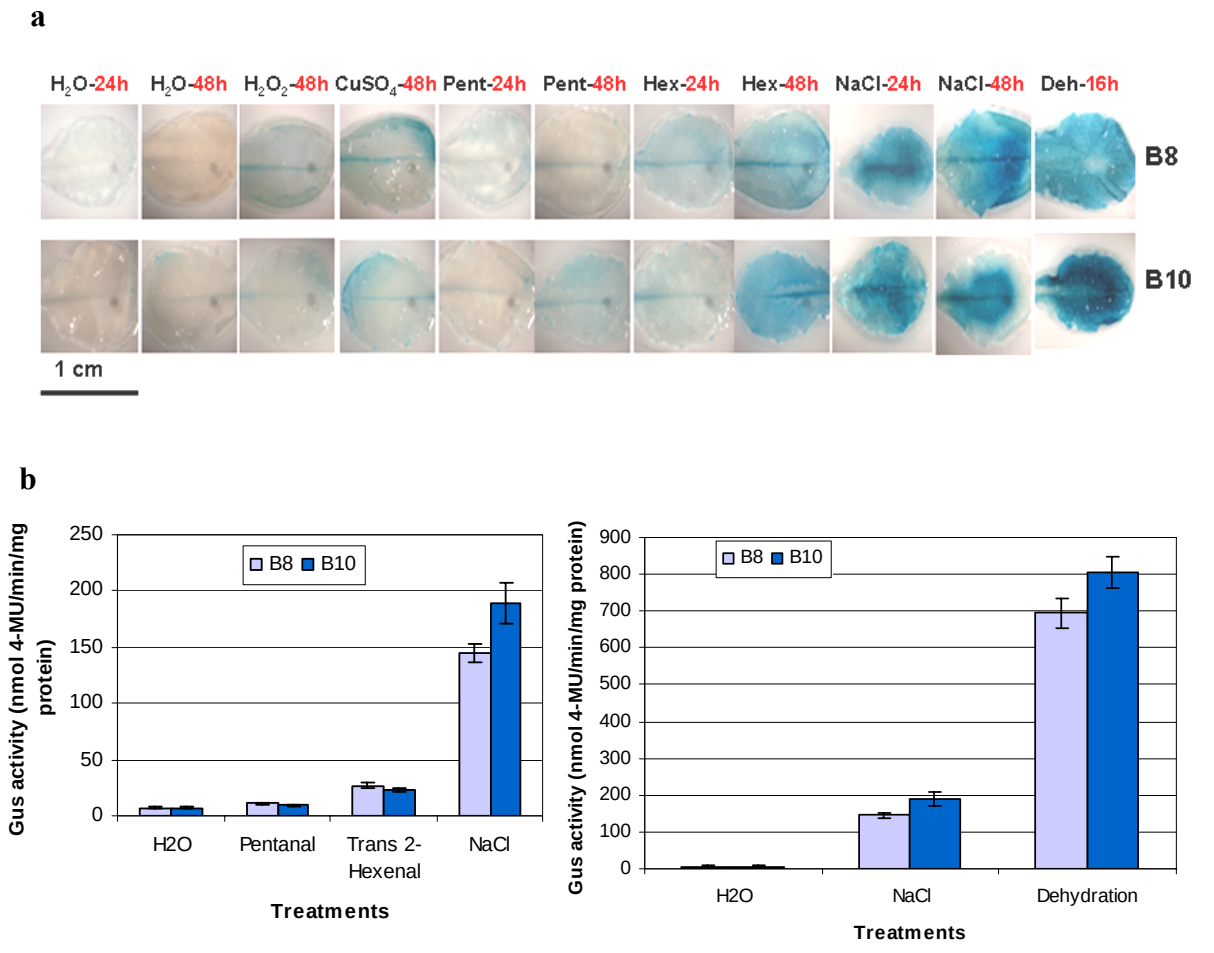


Fig. 41 Activity of the *ALDH7B4* promoter upon abiotic stress treatments. (**a**) *In situ* detection of the activity of *ALDH7B4* promoter in leaves from 4 week-old plants. **Pent**: pentanal; **Hex**: trans-2-hexenal; **Deh**: dehydration. (**b**) Measurement of the *ALDH7B4* promoter-driven GUS activity in transgenic plant tissues in response to different treatments.

The response of the endogenous *ALDH7B4* promoter to the treatments was monitored in parallel by protein-blot analyses using ALDH7B4 antiserum. As shown in **Fig. 42**, similar observations were made as with the GUS reporter protein. The 16 h-dehydration treatment led to the highest expression levels of the endogenous *ALDH7B4* gene, followed by salt (48 h) and trans-2-hexenal (48 h). It therefore appears that the activity of the *ALDH7B4* promoter in the selected transgenic lines (in particular B8 and B10) reflects that of the endogenous gene. The results also demonstrated the responsiveness of the *ALDH7B4* promoter to aldehydes, in particular to trans-2-hexenal.

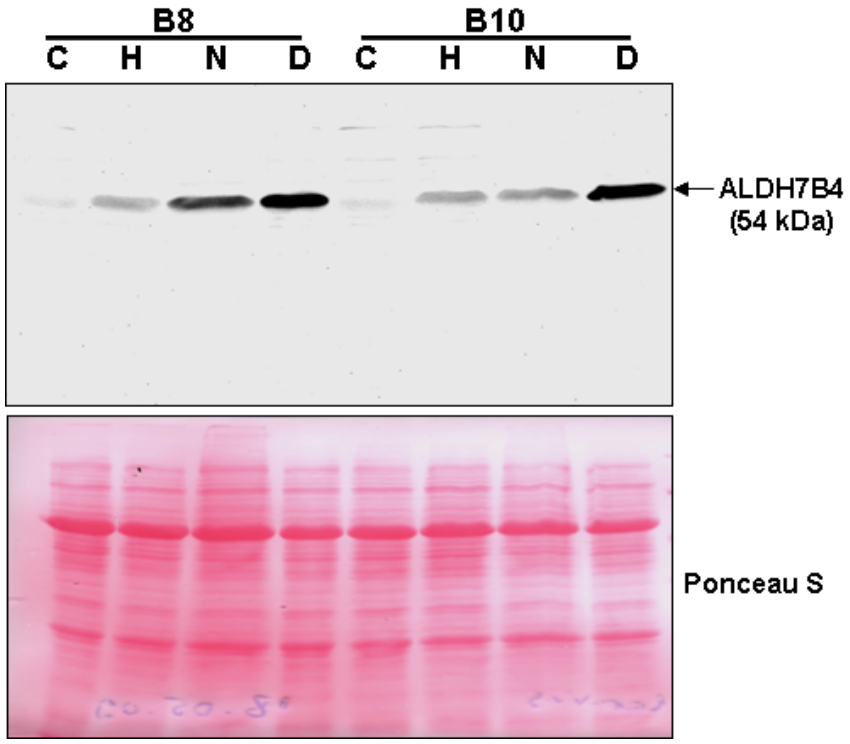


Fig. 42 Protein-blot analysis of the endogenous ALDH7B4 protein accumulation. Leaves from 4 week-old 7B4-GUS plants were either treated by water (**C**), 5 µmol trans-2-hexenal (**H**), 300 mM NaCl (**N**) for 48 h or dehydrated (**D**) for 16 h. ALDH7B4 antibodies were used for the immunodetection.

Like MDA, trans-2-hexenal possesses a double carbon bond conjugated with the carbonyl group, which is a characteristic feature of the compounds referenced as Michael acceptors. Increasing evidence has suggested that these Michael acceptors are capable of triggering the expression of some stress-responsive genes. Therefore, the aldehyde- and stress-induced activation of the *ALDH7B4* promoter was further monitored along with the MDA accumulation in transgenic and wild-type plants.

3.3.5 Comparison of the *ALDH7B4* promoter activation and the MDA accumulation

To further understand the activation of the *ALDH7B4* promoter by aldehydes the enzymatic activity of the GUS protein in plant extracts was compared to the MDA accumulation pattern in leaves of 4 week-old transgenic plants. Leaves were detached from soil-grown plants and incubated in water (as control), 300 mM NaCl, 30 mM H_2O_2, 5 µmol trans-2-hexenal or 50 µM Paraquat® for 8 h. Then, the leaves were divided into pools and used for the *in situ* detection of reductive aldehydes, the quantification of the GUS activity and the determination of the MDA content. As shown in **Fig. 43** no GUS activity above the background was detected in leaves treated with NaCl, H_2O_2 and trans-2-hexenal after 8 h of stress treatments. In contrast, the Paraquat® treatment led to a significant increase of the GUS activity. Accordingly, high amounts of MDA and aldehydes were found in the Paraquat®-treated leaves. Unlike the GUS activity, MDA significantly accumulated in NaCl-, H_2O_2- and trans-2-hexenal-treated leaves after 8 h, as compared to the controls (**Fig. 43**).

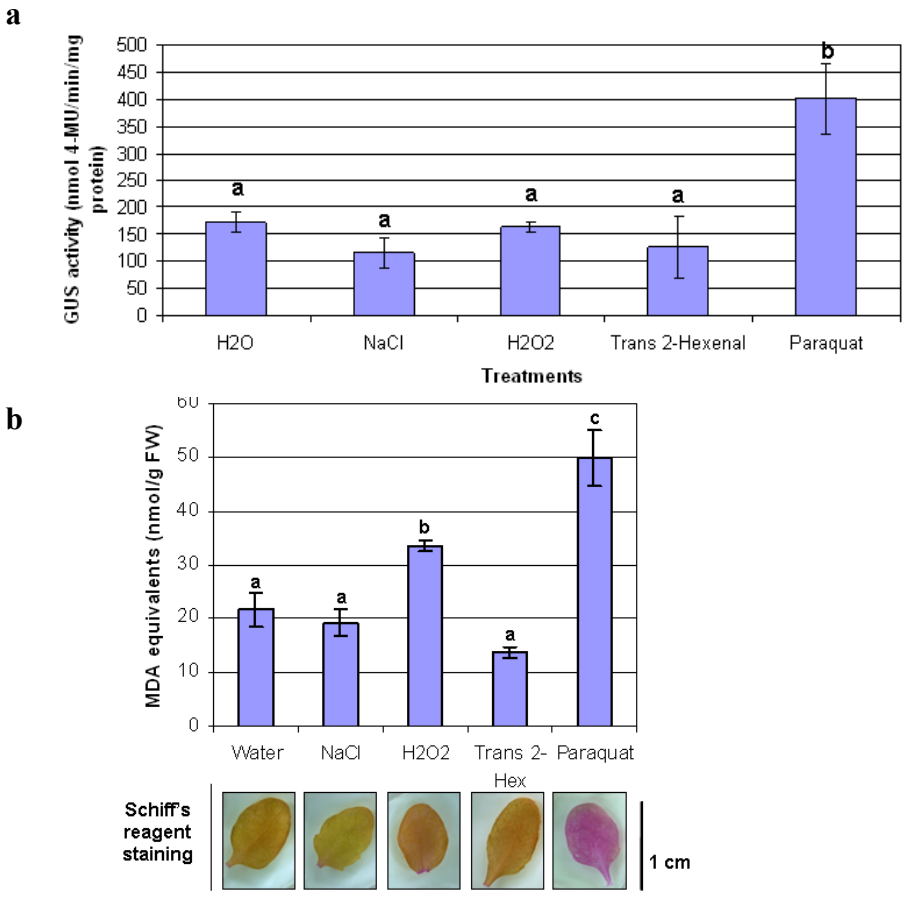


Fig. 43 Comparison of the *ALDH7B4* promoter activation and the MDA accumulation. (**a**) Activity of the *ALDH7B4* promoter measured as the GUS activity from the ALDH7B4-GUS plant extracts. (**b**) **Upper panel**: MDA contents measured in leaf samples from the ALDH7B4-GUS plants used for the GUS activity measurement; **lower panel**: *In situ* detection of reductive aldehydes molecules in treated ALDH7B4-GUS leaf samples by the use of the Schiff's reagent. The series with different letters are significantly different (Student *t* test, $P \leq 0.05$).

Except for the Paraquat® treatment, no correlation was found between the GUS activity and the MDA content for the other treatments. It is therefore difficult to infer a direct relationship between the MDA accumulation and the activation of *ALDH7B4* promoter. Nevertheless, these results support the idea that a threshold of intra-cellular MDA contents, and in general of lipid peroxidation-derived aldehydes, is required to trigger the expression of the target genes. This threshold was probably reached after 8 h of Paraquat® treatment.

3.3.6 Analysis of the *ALDH7B4* gene promoter sequence and effects of the mutation of the DRE and ACGT-boxes

Previous findings along with the results described above suggested that the *ALDH7B4* gene is not only activated by aldehydes but also other abiotic stressors such as dehydration and salt. Consistent with these observations, the *in silico* analysis of the *ALDH7B4* promoter region using PLACE Web Signal Scan (Prestridge 1991; Higo et al. 1999) revealed the presence of numerous stress-related *cis*-elements including one putative dehydration-responsive element/C-repeat – low temperature-responsive element (DRE/CRT-box) (RYCGAC; R=A/G, Y=C/T) and three ACGT-boxes (**Table 8**). The DRE/CRT- and ACGT-boxes respectively form the core of G-boxes and ABA-responsive *cis*-elements (ABRE). Two MYB1 recognition sequences (WAACCA; W=A/T), three MYC recognition sequences (CANNTG; N=A/T/G/C) and one "Elicitor Responsive Element" known as W-box (TTGACC) were also found.

Table 8 List of some *cis*-acting regulatory elements present in the *ALDH7B4* promoter

***Cis*-elements**	**Position (strand)**	**Sequence**	**Description**	**References**
MYB1AT	-615 (+) -478 (+)	WAACCA	MYB recognition site found in the promoters of the dehydration-responsive gene *rd22* and many other genes in *Arabidopsis*; W=A/T.	Abe et al. 2003
MYCCONSENSUSAT	-557 (+) -322 (-) -315 (+)	CANNTG	MYC recognition site found in the promoters of the dehydration-responsive gene *rd22* and many other genes in *Arabidopsis*; Binding site of ATMYC2 (previously known as rd22BP1); N=A/T/G/C; MYC recognition sequence in CBF3 promoter; Binding site of ICE1 (inducer of CBF expression 1) that regulates the transcription of CBF/DREB1 genes in the cold in *Arabidopsis*.	Abe et al. 2003; Chinnusamy et al. 2004.
CBFHV	-385 (-)	RYCGAC	Binding site of barley (*Hodeum vulgare*) CBF1, and also of barley CBF2; CBF = C-repeat (CRT) binding factors; CBFs are also known as dehydration-responsive element (DRE) binding proteins (DREBs); R=A/G; Y=C/T.	Svensson et al. 2006.
ELRECOREPCRP1	-348 (+)	TTGACC	ELRE (Elicitor Responsive Element) core of parsley (*Petroselinum crispum*) PRL genes; consensus sequence of elements W1 and W2 of parsley PRL-1 and PRL-2 promoters; Boxes W1 and W2 are the binding site of WRKY1 and WRKY2, respectively.	Rushton et al. 1996; Eulgem et al. 2000.
ABRELATERD1	-322 (-)	ACGTG	ACGT sequence required for etiolation-induced expression of erd1 early responsive to dehydration) in *Arabidopsis*.	Simpson et al. 2003.
MYBCORE	-213 (+)	CNGTTR	Binding site for all animal MYB and at least two plant MYB proteins ATMYB1 and ATMYB2, both isolated from *Arabidopsis*; ATMYB2 is involved in regulation of genes that are responsive to water stress in *Arabidopsis*.	Urao et al. 1993.
ACGTATERD1	-180 (+) -174 (+)	ACGT	Same as for ABRELATERD1.	See above.

To study interactions between these stress-related *cis*-elements present in the *ALDH7B4* promoter, point mutations were introduced in the ACGT-boxes and the unique putative DRE/CRT-box by site-directed mutagenesis on the *7gt* plasmid that harbors the *ALDH7B4_promoter::GUS-nos_terminator* cassette (**Fig. 44**). Four constructs with different mutations were generated in which the ACGT-boxes were mutated in ATTT whereas the core sequence ATCGAC of the single DRE/CRT-box was substituted to ATATTT. As shown in **Fig. 44** the constructs pA and pD respectively lack the ACGT1 and DRE boxes in comparison to the parental construct *7gt*. ACGT1 and DRE boxes were both mutated in the pAD construct and the boxes ACGT2 and ACGT3 are simultaneously mutated in pAB. These gene expression cassettes, *mutALDH7B4_promoter::GUS-nos_terminator*, were isolated and subcloned into the pBIN19 as described above for the *7gt* clone. Recombinant *Agrobacterium* cells expressing either of these constructs were used to transiently transform *Arabidopsis* seedlings by the FAST method.

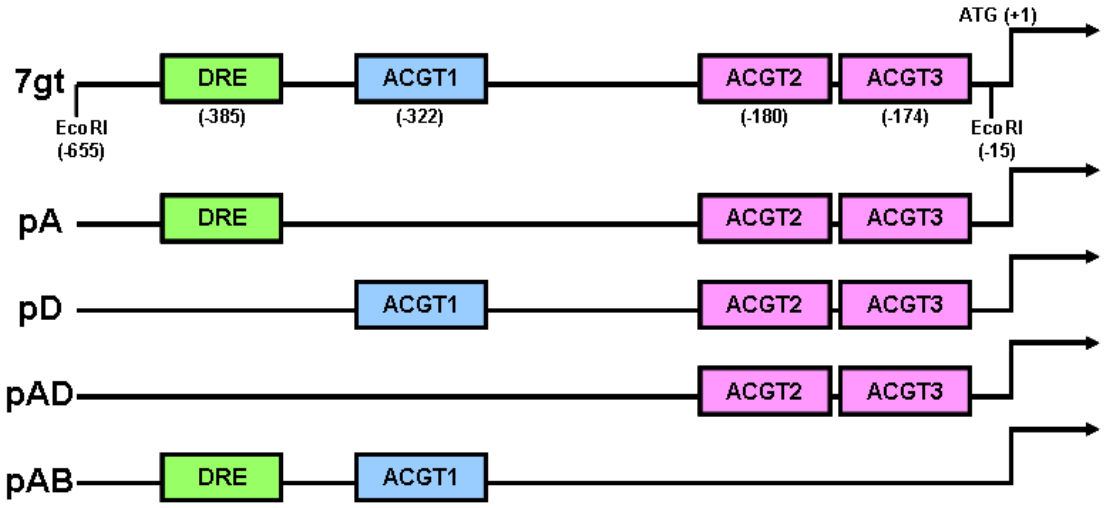


Fig. 44 Schematic representation of the different plasmid constructs with intact (7gt) or mutated (pA, pD, pAD, pAB) DRE and ACGT-boxes within the *ALDH7B4* promoter. The locations of the *cis*-elements within the gene promoter are put in brackets.

The activities of the mutated promoters were compared to those of the non-mutated parental promoter to deduct the effect of the mutations. The simultaneous deletion of the ACGT2 and ACGT3 boxes (pAB) almost abolished the induction of the promoter upon NaCl treatment for 16 h (**Fig. 45**). But, when both ACGT1 and DRE boxes are mutated with ACGT2 and ACGT3 remaining intact (pAD), only one-third of the promoter activity was lost. Single mutation of either of the DRE and ACGT1 boxes also led to the loss of one-third of the promoter activity. The observations indicate the complex interactions which exist between these *cis*-elements in the salt responsiveness of the *ALDH7B4* promoter. They also suggest that the two proximal ACGT2 and ACGT3 boxes are the most influential ACGT-boxes involved in the salt response of the promoter.

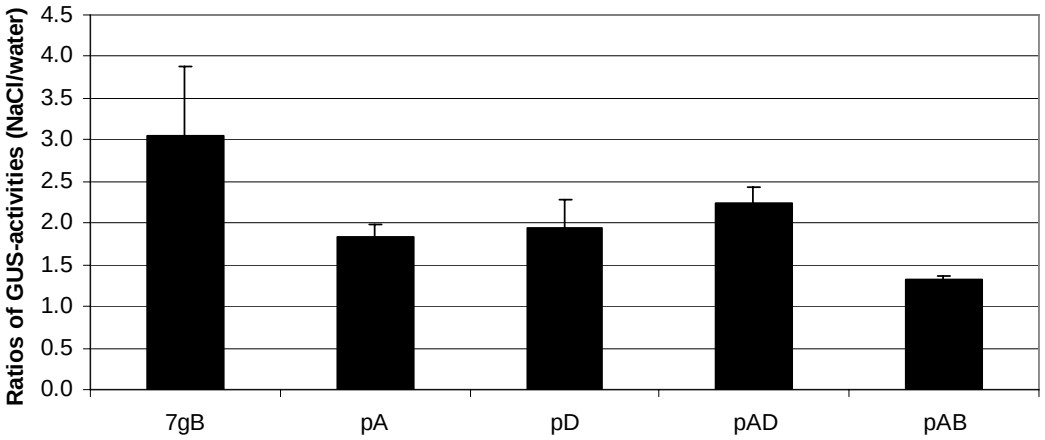


Fig. 45 Effects of the mutation of the DRE and ACGT-boxes within the *ALDH7B4* promoter. Residual GUS activities driven by the mutated promoter in **pA**, **pD**, **pAD** and **pAB** constructs were compared to the activity of the intact promoter in the **7gB** construct. For that, the GUS activities of NaCl-treated samples were divided by the activities of water-treated samples for each construct. The magnitude of the induction is given by this ratio and reported here. The promoter induction by NaCl is weaker in **pAB** than in other constructs. The mutated ACGT2 and ACGT3 boxes in **pAB** may be relevant to the salt response of the *ALDH7B4* promoter.

3.3.7 Production and strategy of screening of the EMS-derived mutant population

The main goal of this project is to identify factors involved in the aldehyde-induced expression of ALDH genes and to elucidate the regulation pattern. A genome-wide mutagenesis approach has been chosen to identify genetic factors involved in this regulation. Seeds from a homozygous transgenic B8 plant containing the ALDH7B4-GUS transgene were treated by the mutagen ethyl methanesulphonate (EMS) to introduce random point mutations in the genome. The EMS-treated seeds were sown on soil to yield the M1 plant population. M2 seeds from M1 plants were harvested in bulks of 15 plants, with a total of

45 bulks. Attempts to screen the M2 plants by aldehydes for any alteration in the GUS expression in comparison to the non-mutated transgenic lines have proved challenging and difficult to undertake. A direct exposure of plants to aldehydes did not work out as expected. In fact, the aldehydes are volatile and spraying them on the plants did not lead to a reproducible effect. An alternative strategy of treatment is the exposure of the plant to a specific concentration of the aldehyde in a tightly closed container. This method has so far been using with individual plants to study the promoter activity in different stress conditions. Yet, the drawback is that many plants cannot be simultaneously treated in a single container. This renders the method inappropriate for a large scale screening. Additionally, the induction of the *ALDH7B4* promoter by aldehydes was not found strong enough to allow a visible clear-cut decision. Taken these together, an indirect approach of screening needs to be tested and used. It may be worth screening the M2 seeds first by NaCl. Once putative mutants with altered GUS expression have been identified, a second round of screening will be performed using aldehydes. This should allow identifying mutants impaired in aldehyde-mediated gene responses. Reasons for proposing such a screening method come from the observations made throughout this study and are further discussed below.

In summary, the responsiveness of the *ALDH7B4* promoter to aldehydes has been examined in this chapter. Using transgenic plants expressing the *ALDH7B4-promoter::GUS* fusion construct it was shown that both pentanal and trans-2-hexenal activated the promoter. However, the induction by trans-2-hexenal was stronger than that by pentanal. Consistent with the previous observations on the *ALDH7B4* transcript and protein analyses, the results confirmed that the promoter is inducible by sodium chloride, copper sulphate, hydrogen peroxide and dehydration, but at different amplitudes. The comparison of the GUS activities revealed that dehydration and NaCl induce the promoter stronger than trans-2-hexenal. Moreover, the *ALDH7B4* promoter was found constitutively active in naturally desiccation-tolerant organs like seeds and pollen thus following the expression pattern of many LEA (late embryogenesis abundant) genes. The difficulties for exclusively using the aldehyde-driven GUS activity as the sole criterion to screen a large mutant population to detect plants defective in the aldehyde-induced expression of ALDH genes were also presented. An alternative screening method is proposed.

4. DISCUSSION

Since completion of the sequencing of the *Arabidopsis* genome (The Arabidopsis Genome Initiative [AGI] 2000), it has been adopted as a model plant of choice for biological and biotechnology research. Its advantages include a small genome size, short life cycle, small stature, prolific seed production. These features in addition to its easiness of transformation have allowed the development of a plethora of genomic resources ranging from DNA stocks (genomic and full-length cDNAs) to seed stocks (natural accessions or ecotypes, near saturation insertion mutant collections, mapping lines, etc.). The importance of using knowledge gained from research on *Arabidopsis* should facilitate the understanding of biological phenomena in crops and other plant species. *Arabidopsis* has been important for studying stress signal transduction and molecular mechanisms leading to stress adaptation or tolerance. The use of forward genetics and the development of reverse genetic tools have been essential to determine the function of numerous genes involved in plant growth, development and stress tolerance acquisition. To date, significant advances have been made but much still remains to be done for numerous gene families, where functional redundancy among closely related genes often obscures their phenotypes. The aldehyde dehydrogenase gene family is such an example and the molecular and functional characterization of some gene members have been undertaken in this work. Namely, two gene members, *ALDH10A8* and *ALDH10A9*, from the ALDH protein family 10 were investigated with respect to their biochemical properties and functions in *Arabidopsis* development and stress response. *ALDH3H1*, a gene member of the protein family 3 has been analysed through the use of both T-DNA insertion mutants and over-expressing lines. Although it is established that aldehyde dehydrogenases primarily oxidize aliphatic and aromatic aldehyde molecules to their corresponding carboxylic acids, their regulation pattern and what links them to plant stress responses is mainly not understood. This aspect has been addressed with transgenic reporter lines expressing the *GUS* gene driven by the stress responsive promoter of *ALDH7B4* (Kirch et al. 2005). The *ALDH7B4* gene is described as a "tugor responsive" gene and codes for a protein of the family 7 of aldehyde dehydrogenases (Kirch et al. 2004; Kotchoni et al. 2006).

4.1 Functional analysis of putative betaine dehydrogenase genes from *Arabidopsis*

Many higher plants accumulate glycine betaine as a compatible solute under stress conditions (Fitzgerald et al. 2009). The biosynthesis of glycine betaine involves two enzymatic reactions

catalysed by choline monooxygenase and betaine aldehyde dehydrogenase. Like rice, *A. thaliana* belongs to those higher plants, which do not accumulate glycine betaine. This has been attributed to the absence of a functional choline monooxygenase that oxidizes choline to betaine aldehyde (Hibino et al. 2002). However, two genes, *ALDH10A8* and *ALDH10A9*, were assigned to code for betaine aldehyde dehydrogenase in the *Arabidopsis* genome (Kirch et al. 2004). Using a loss-of-function mutant, the biological function of the *ALDH10A8* gene has been investigated. The biochemical properties of ALDH10A9 have also been analysed.

4.1.1 *Arabidopsis* BADH coding genes are stress inducible

The analyses revealed that *ALDH10A8* and *ALDH10A9* transcripts are detectable in the plant and are slightly induced by stress treatments. Similar expression patterns have been described for these genes at the GENEVESTIGATOR V3 (http://www.genevestigator.com; Zimmermann et al. 2004) database, where weak inductions of these genes were observed upon various stress treatments. This indicates that *ALDH10A8* and *ALDH10A9* may have a role in maintaining cellular homeostasis under stress conditions. Transcript analyses from some other plant species have however shown a constitutive expression of the BADH coding genes. A BADH homologue in *Avicennia marina* did not respond to salt in leaf or root tissues (Hibino et al. 2001). Similarly, BADH from *Amaranthus tricolor* was not stimulated by salt, drought, H_2O_2, Cu^{2+}, Hg^{+} and high temperature, except for methyl viologen (Bhuiyan et al. 2007). In some plants the *BADH* genes are differentially expressed. For example in rice *BADH2* is constitutively expressed in mature leaf tissue grown under salt stress conditions (Chen et al. 2008; Fitzgerald et al. 2008), whereas *BADH1* is inducible by salt (Nakamura et al. 1997), although rice, like *Arabidopsis*, does not accumulate glycine betaine. The fact that BADH homologues do not respond to stress treatments in the same way in all plant species suggests that they may have different roles, which allow adaptation to a specific stressing compound or environmental conditions. It was observed that young and old plants lacking *ALDH10A8* transcripts were drought and salt sensitive. This indicates that *ALDH10A8* might be involved in other pathways than the biosynthesis of glycine betaine in *Arabidopsis*.

4.1.2 *Arabidopsis* BADHs are probably aminoaldehyde detoxifying enzymes

Previous findings suggested that some BADH enzymes could oxidize aminoaldehyde molecules. Trossat et al. (1997) demonstrated that sugar beet (*Beta vulgaris*) BADH oxidized 3-dimethylsulfoniopropionaldehyde, APAL and ABAL. Similarly, BADH homologues from oat, rice and barley were shown to oxidize a variety of aminoaldehydes including ABAL and

APAL, which were converted to GABA and 3-aminopropionic acid, respectively (Livingstone et al. 2003; Bradbury et al. 2008; Fujiwara et al. 2008; Fitzgerald et al. 2009). Also, pea (*Pisum sativum*, cv Lantra) aminoaldehyde dehydrogenase was shown to oxidise APAL and ABAL (Petrivalsky et al. 2007). Likewise, the ALDH10A9 protein possesses both betaine aldehyde and aminoaldehyde dehydrogenase activities and could oxidize betaine aldehyde, ABAL and APAL *in vitro*, as shown in this work. However, the affinity to the substrates was low compared to data from the literature (**Table 6**). No enzymatic data was obtained for ALDH10A8 as it was not possible to purify sufficient amounts of the enzyme in its active form. But, the salt and drought sensitive phenotype developed by the *ALDH10A8* knock-out mutant suggests that this enzyme is active in the plant. It is possible that ALDH10A9 and likely ALDH10A8 function as aminoaldehyde dehydrogenases and would therefore be involved in the polyamine catabolism through the oxidation of aminoaldehydes resulting from the activity of copper amine oxydase (CAO) and polyamine oxydase (PAO) (Cona et al. 2006). Supporting findings of this hypothesis are our data confirming the peroxisomal localisation of ALDH10A9 and those reporting the peroxisomal targeting of *Arabidopsis* CAO (*At2g42490*, accession no. NM_129810) and PAOs (AtPAO3: *At3g59050*, accession no. AY143905; AtPAO4: At1g65840, accession no. AF364953) (Moschou et al. 2008a; Kamada-Nobusada et al. 2008; Eubel et al. 2008; Reuman et al. 2009). AtPAO3 is shown to catalyze the back-conversion/oxidation of spermine to spermidine and spermidine to putrescine whereas AtPAO4 only oxidizes spermine to spermidine (Kamada-Nobusada et al. 2008; Moschou et al. 2008a). Although any concomitant aminoaldehyde production was not shown for these substrates, it is still plausible that the oxidation of other substrates not tested in these studies will lead to the production of aminoaldehydes, as it is the case in animal cells. The plant polyamine content is shown to increase upon salt, water, cold stress (Bouchereau et al. 1999; Maiale et al. 2004; Yang et al. 2007; Cuevas et al. 2008). Recently, Moschou et al. (2008a, 2008b) showed that AtPAO3 and AtPAO4 accumulated in *Arabidopsis* seedlings upon ABA treatment or mechanical damage and they subsequently suggested that the polyamine catabolism in the apoplast is a key factor that exerts a specific role in abiotic stress responses. Similarly, Petrivalsky et al. (2007) reported that pea AMADH activity increased during wound healing of mechanically injured etiolated seedlings and this was spatially correlated with lignification, a physiological process that involves both CAO and PAO activities. Simultaneous increase of diamine oxydase activity and GABA formation from ABAL was also reported in soybean (Xing et al. 2007). Based on these observations, it is plausible that *Arabidopsis* AMADHs exert their biological effects by detoxifying cells from

metabolism-derived aminoaldehydes, which are cytotoxic (Morgan et al. 1987; Yu et al. 2003). In that way, they would generate GABA that can be directed to the GABA-shunt pathway or accumulate as compatible solute. Indeed, Petrivalsky et al. (2007) found that high GABA accumulation occurred in roots of soybean [*Glycine max* (L.) Merr.] grown on salt; and about 39% of the total GABA pool was derived from polyamine degradation. Smirnoff and Cumbes (1989) reported that GABA possesses *in vitro* hydroxyl-radical-scavenging activity, exceeding that of proline and glycine betaine at the same concentrations (16 mM).

4.1.3 The *ALDH10A8* knock-out mutant is stress sensitive

In the light of what is discussed above the inactivation of *ALDH10A8* might lead to an increase of intracellular levels of toxic aminoaldehydes in *KO8-2* plants. Indeed, our results indicate that the inactivation of *ALDH10A8* rendered *Arabidopsis* plants more sensitive to drought and NaCl. Likewise, Niu et al. (2008) recently reported that transgenic rice RNAi-lines with inhibited *BADH2* expression had a decreased salt stress tolerance, as measured by shoot and root length, weight and root number. Similar observations have also been published on fragrant rice varieties which lack the functional BADH2 enzyme (Fritzgerald et al. 2010). According to the authors, these lines showed greater than 99% inhibition of mature seed production if exposed to 22mM NaCl solution. These findings along with our observations support the idea that BADH coding genes play a role as yet to be clarified in GB non-accumulating plants under drought and salt stress conditions. The enzymatic activities of ALDH10A9 on betaine aldehyde, APAL and ABAL suggest that *Arabidopsis* BADHs may serve as detoxification enzymes controlling the level of aminoaldehydes under stress conditions and during metabolism. The results also confirm that the non-accumulation of glycine betaine in *Arabidopsis* is not due to the absence of a functional BADH protein, but is related to the lack of a functional CMO enzyme as previously reported by Hibino et al. (2002).

4.2 Molecular and functional analyses of the *ALDH3H1* gene locus

4.2.1 What can one learn from over-expressing the ALDH3H1 protein?

One way to identify the biological function of a plant gene is to analyse transgenic lines that are either knock-out mutants for the gene or that over-express the gene at high levels. Compared to the knock-out mutants the over-expressing plants are especially interesting when dealing with gene families with functional redundancy. Both types of transgenic plants have

been used in this study to understand the role of the *ALDH3H1* gene in the development and the stress physiology of *A. thaliana*. From the family 3 of ALDHs only ALDH3I1 has been functionally characterized (Sunkar et al. 2003; Kotchoni et al. 2006). The first evidence of the ALDH involvement in *Arabidopsis* stress response was provided by Sunkar et al. (2003). It was shown that the gene is inducible by NaCl, heavy metals (Cu^{2+} and Cd^{2+}) and chemicals that induce oxidative stress (Paraquat® and H_2O_2). Later, Kirch et al. (2005) reported on the expression analysis of several ALDH genes including *ALDH3H1*. It was shown that *ALDH3H1*, like *ALDH3I1*, is stress inducible and mainly up-regulated by NaCl in the roots of young plants at the transcriptional level. Here, the expression analysis has been extended to the protein level and in adult plants. As explained in the results, the up-regulation of the ALDH3H1 protein by salt stress mainly occurs in leaves of plants older than 4 weeks after germination. In comparison with the previous findings, it appears that ALDH3H1 and ALDH3I1 are differentially expressed in the plants. Sub-cellular localization experiments have revealed that ALDH3H1 is targeted to the cytosol whereas ALDH3I1 contains a plastid signal that directs it to chloroplasts. This implies that these proteins although from the same family could be functionally different. Indeed, transgenic plants constitutively expressing the full-length coding sequence of *ALDH3H1* under the control of the 35S promoter did not develop a strong stress tolerance phenotype in contrast to the ALDH3I1 over-expressors. *Arabidopsis* ALDH3I1 over-expressors showed improved tolerance to NaCl, heavy metals, methyl viologen and H_2O_2 (Sunkar et al. 2003). Consistent with these observations, the ALDH3I1 knock-out mutants were more sensitive to dehydration and salt than the wild type (Kotchoni et al. 2006). These phenotypes were explained by the detoxification of aldehydes, given that the level of thiobarbituric acid reactive substances was decreased by the over-expression of ALDH3I1 and increased in the case of enzyme deficiency. Similar observations were made with ALDH over-expressing lines in other species. Transgenic tobacco (*Nicotiana tabacum*) and *Arabidopsis* plants constitutively expressing the soybean gene GmTP55 that encodes a dehydrogenase motif containing protein of the ALDH7 family have been shown to be tolerant to salinity during germination and to water deficit during plant growth (Rodrigues et al. 2006). These transgenic plants also exhibited enhanced tolerance to oxidative stress with a lower concentration of lipid peroxidation-derived reactive aldehydes. Likewise, it has been reported that transgenic tobacco plants over-expressing the *ALDH22A1* gene from maize (*Zea mays*) showed increased stress tolerance accompanied by a reduction of MDA derived from the lipid peroxidation (Huang et al. 2008). By contrast, the ALDH3H1 over-expressors did not significantly perform better than wild-type plants under stress. The ALDH3H1 over-

expressors showed a slightly improved germination rate and growth compared to the wild type and the knock-down mutant *3h1-B*. Similar observations were made with soil-grown plants. Nevertheless, it is worth to notice the overall lower level of MDA that was found in ALDH3H1 over-expressing plants compared to the wild type and the knock-down mutant *3h1-B*. This tendency is consistent with the findings from the above-cited literature as for that the detoxification of reactive aldehydes derived from the cellular lipid peroxidation is predominantly performed by *ALDH* gene members. As compared to the results from ALDH3I1, the constitutive expression of the ALDH3H1 protein did not confer abiotic stress tolerance to *Arabidopsis*. But this protein may take part to the detoxification of lipid peroxidation-derived aldehydes, contributing therefore to the performance of the plant under stress.

Further, the ALDH3H1 over-expressing plants accumulated less proline than the wild type and *3h1-B* under drought; its accumulation was correlated with the severity and the duration of the stress. Proline is an amino acid that many higher plants accumulate in response to drought, salinity and various environmental stresses (Kavi Kishore et al. 2005). Proline is believed to function as an osmolyte for osmotic adjustment and to stabilize sub-cellular structures such as membranes and proteins and to scavenge ROS. The accumulation of proline in many plant species has been correlated with stress tolerance, and its concentration has been shown to be higher in stress-tolerant than in stress-sensitive plants (Petrusa and Winicov 1997; Nanjo et al. 1999; Nayyar and Walia 2003). But, this correlation is not universal and the toxic effect of proline at high concentrations has also been reported (Lutts et al. 1999; Nanjo et al. 2003). Such contradictory findings could result from the differences in the mechanisms that underlie proline accumulation and degradation or the signaling mechanisms that lead to proline accumulation. But, it may also depend on the actual function of proline in stressed plants. In fact, the proline content was increased, as expected, in wild-type and ALDH3H1 over-expressing plants upon stress, although there was no visible difference in their phenotype. The level of proline after 7 days of salt and drought stress was significantly lower than that found after 14 days, irrespectively of the genotype (data not shown). This suggests that proline accumulation can be at some point understood as a signal to reflect the physiological status of plants. The intracellular concentration of proline could be a biochemical signature reflecting the severity of the stress endured by the plant. With regards to this, one could interpret the lower levels of proline in ALDH3H1 over-expressing plants under drought as a sign that they suffered less from the dehydration than did the wild type and the knock-down mutant *3h1-B*.

Although *ALDH3H1* expression is stress responsive, the study of the over-expressing plants suggests that its function in the detoxification of lipid peroxidation-derived reactive aldehydes is less important than for *ALDH3I1*. In the light of the observations made with transgenic seedlings (**Fig. 18-20**) the ALDH3H1 protein might be more functional in seedlings than adult plants. Since the gene was found to be up-regulated by salt in seedling roots exclusively, it is worth deepening the investigations in this organ. It is plausible that the ALDH3H1 protein, instead of having a primordial role in the stress tolerance acquisition, is involved in the maintenance of the root architecture and the integrity of root tissues under stress conditions. Alternatively, this protein may have another so far unknown biological function, as it has been the case for some other *ALDH* gene members. For instance, it has been shown that *Rf2a*, a maize gene coding for a mitochondrial family-2 ALDH, is a maize nuclear restorer gene (Liu et al. 2001). The protein Rf2a is involved in the anther development and plays a critical role in producing functional male gametes (Liu et al. 2001; Liu and Schnable 2002); although, the molecular mechanisms associated with the restorer function of the *Rf2a* gene remain to be clarified. Similarly, the *Arabidopsis* ALDH2C4 was shown to oxidize sinapaldehyde and coniferaldehyde and thereby is involved in the production of ferulic acid and sinapic acid during lignin biosynthesis (Nair et al. 2004). Recently, Wei et al. (2009) described the ALDH2B4 as the main player among the ALDH2 protein family members involved in the "pyruvate dehydrogenase bypass" pathway in *Arabidopsis* pollen. Indeed, "pyruvate dehydrogenase bypass" pathway plant family 2 ALDHs oxidize acetaldehyde generated via ethanol fermentation, producing acetate for acetyl-CoA biosynthesis via acetyl-CoA synthetase (ACS), as it is the case in yeast. An additional example of the role that ALDH could play in the plant development was provided by Shin et al. (2009). These authors reported that a rice ALDH7 gene (*OsALDH7*) plays an important role in maintaining seed viability by detoxifying the aldehydes generated from lipid peroxidation. Based on these observations, it will be worth to further investigate both ALDH3H1 over-expressors and knock-out mutants in order to uncover any other features related to the function of ALDH3H1.

4.2.2 *ALDH3H1* locus contains an alternative promoter directing the expression of an alternative first exon (AFE) transcript

Since residual *ALDH3H1* transcripts were detected in the *3h1-B* mutant (Ditzer A. unpublished data) it was considered that the absence of a stress phenotype in this line could be due to the leakage of the mutation. I therefore sought to characterize additional T-DNA

insertion mutants. The comparative analysis of *ALDH3H1* transcripts in the mutants *3h1-A* and *3h1-C* allowed to identify the presence of a short transcript (*T3*) different in its 5'end sequence from the longer isoforms *T1* and *T2*. As explained in the results, *T3* originates from the use of an alternative first exon (AFE). AFEs can be produced by alternative promoters, alternative splicing of gene transcripts or through the combination of both mechanisms. Many eukaryotes use AFEs to generate several transcripts from a single gene (Kornblihtt 2005; Chen et al. 2007). Typically, an AFE is defined as the first exon of one splice variant of a gene, but either located downstream of a corresponding AFE of other variants generated by the same gene, or absent from other variants altogether (Luzi et al. 2000; Kimura et al. 2006). Reports on AFEs are mainly based on mammalian genomes, especially mouse and human. But recently a genome-scale analysis has been performed on rice and *Arabidopsis* (Chen et al. 2007). It was found that 5.9 and 5% respectively of the total expressed genes in rice and *Arabidopsis* contain AFEs; and about 58% of AFE-containing gene structures derive from alternative promoters. Consistent with these findings, our observations indicated the existence of a second gene promoter within the 1st intron of the *ALDH3H1* locus, which drives the expression of the short AFE-transcript *T3*. The size of the 1st intron is about 1.2 kb and represents the distance between the upstream exon and the 5'-end of the cryptic *exon2'*. Accordingly, Chen et al. (2007) found that the average distance between the start sites of alternative first exons was about 1.6 kb in *Arabidopsis*. The analysis of other *Arabidopsis ALDH* gene sequences did not reveal any similar intron size, indicating that this feature is unique for *ALDH3H1*. As discussed hereafter the necessity of such an alternative promoter and the function of the deriving transcripts *T3* are not fully understood. Chen et al (2007), however, observed that genes involved in enzymatic reactions and cellular processes were significantly enriched in AFEs in rice and *Arabidopsis*, indicating that the complex transcription mediated by AFEs might be important for the plant cell adaptation to dynamic internal and external environmental changes.

4.2.3 Influence of the use of AFE-transcripts on the protein sub-cellular localization

It is well documented that some AFE-transcripts contain different ATGs, which result in protein isoforms with different N-termini. As consequence, these protein isoforms can be targeted to different cellular compartments or have different functions (Wang et al. 2002; Kitagawa et al. 2005). Unlikely, the ALDH3H1 protein from *T3* was found to be localized in the cytosol (**Fig. 34**). More frequently, AFEs lead to different transcripts that merely differ in their 5'-untranslated region (5'-UTR). The shared downstream exons contain the same

translation start codons (ATGs) and therefore produce identical proteins (Luzi et al. 2000). As shown on the TAIR website and reported here in **Fig. 24** three protein coding gene models could derive from the *ALDH3H1* locus. *T1* and *T2* only differ in their 5'UTR region and would therefore code for a unique protein isoform targeted to the cytosol, as previously shown (Ditzer A. unpublished data). Although the difference between *T1* and *T2* transcript isoforms was not experimentally analysed in this study, it is plausible that they also represent AFE-transcripts. Indeed, Tanaka et al. (2009), in a genome wide comparative study of transcription start sites (TSS) mapping, found that every locus had 1.96 and 2.08 TSS clusters in rice and *Arabidopsis*, respectively. The means of the distances between TSSs were shown 149 bp in rice and 184 bp in *Arabidopsis*, suggesting that *T1* and *T2* could be generated from the use of alternative TSSs located in the upstream promoter.

4.2.4 What can one understand from the differential expression of *ALDH3H1* transcript isoforms?

The analyses revealed that the transcript *T3* has derived from the use of an alternative promoter located within the 1.2 kb-first intron of the *ALDH3H1* locus. Several eukaryotic genes have been found with multiple promoters. Each promoter determines a specific TSS with a first exon, and accordingly, generates a different transcript. The generation of alternative transcripts by the use of multiple promoters was seen as an additional way of generating regulatory diversity and provides a mechanism to synthesize functionally related proteins acting together to mediate a specific biological response (Morello et al. 2002; Parsley and Hibberd 2006; Qi et al. 2007). The expression pattern of the transcript *T3* substantially differs from that of *T1* and *T2* in roots of adult plants, which indicates that the two promoter regions are differentially activated. It is well documented that alternative promoters are often responsible for tissue- or developmental stage-specific gene expression. Besides the different examples described in human genes, Koo et al. (2009) recently showed that the rice MAP Kinase gene *OsBWMK1* generates, by the use of alternative promoters, transcript variants that show distinct expression patterns in response to environmental stresses. They found that one promoter was constitutively active in most tissues at various developmental stages in rice and *Arabidopsis*, whereas the activity of the second promoter was lower and, spatially and temporally restricted. Similarly, Chen et al. (2007) identified a number of putative AFE-containing gene clusters in *Arabidopsis*, which exhibit tissue- and/or development-specific transcription. The diversification of TSSs and transcripts from a single locus were shown to contribute to variations of gene expression patterns and regulation in rice and *Arabidopsis*

(Tanaka et al. 2009). Whether these variations are either gene-specific or intrinsic to *cis*- and *trans*-elements involved in gene expression, is not yet fully elucidated and requires further investigations. Nevertheless, findings suggest they could evolve from the discrepancy in nucleotide features around upstream and downstream TSSs (Tanaka et al. 2009). According to this hypothesis, the most upstream TSS would have retained canonical *cis*-elements, whereas downstream TSSs contained atypical nucleotide features, resulting in the development of novel gene expression patterns.

Despite the fact that *T3* transcripts could be easily detected in *3h1-A* mutants, no corresponding protein was found in those plants. A possible explanation of this feature is that the transcripts *T3* are less stable and undergo a posttranscriptional degradation that prevents their translation into protein. Another more plausible reason could be that the translation efficiency of *T3* transcripts is very poor. Indeed, some of the GC-rich leader exons generated by alternative promoters were shown to be poorly translated (Kozak 1991). Also, the presence of one or more upstream start codon AUGs in the 5'UTR region of AFEs were found to inhibit cap-dependent translation (Kozak 1991). Three ATGs were found between the TATA-Box of the *ALDH3H1* alternative promoter and the functional ATG of the cryptic *exon2'*. This indicates that the presence of these upstream ATGs is not random, and the ATGs could attenuate the translation of *T3* transcripts.

Another more relevant question that arose is the absence of the transcript *T3* in the wild type. One cannot rule out the possibility that the transcript is produced in the wild type at a very low level. Alternative promoters that were 100-fold different in their strength have been documented for mammalian α-amylase genes (Schibler et al. 1983). It is nevertheless intriguing that *T3* transcripts could be detected in heterozygous *3h1-A* mutants along with the other variants. It is possible that the *trans*-acting factors required for the expression of *T3*, although present in the wild type, do not have access to the promoter. It can be hypothesized that the affinity of the *trans*-acting factors to the upstream promoter is much stronger than that to the downstream alternative promoter; making thereby the upstream promoter control the recruitment of the transcription factors and the gene expression. In that case the dominance of the upstream promoter would have been suppressed by the T-DNA in *3h1-A*. This is what apparently happened. The insertion of the T-DNA in the exon close to the upstream promoter (**Fig. 24**) could have affected its integrity or disrupted its native conformation or some important *cis*-elements. Interestingly, *T3* was not detected in *3h1-B* mutants, which have a T-DNA insertion within the first intron at a position distant to the upstream promoter. Nevertheless, the situation in *3h1-B* could be explained by the fact that the T-DNA has

actually disrupted the alternative promoter within the intron. The hypothesis of the dominance of the upstream promoter over the alternative downstream promoter could be further investigated by comparing, for example, the levels of the *GUS* gene activity driven by the alternative promoter in transgenic plants generated in wild type and *3h1-A* mutant background.

4.2.5 Is the *T3* transcript variant relevant for the plant viability?

The comparative analysis between the *3h1-A* mutants, the wild type and the *3h1-C* mutants indicated that *3h1-A* plants were as similarly affected as *3h1-C* mutants in their root growth under NaCl stress. This suggests that the transcript *T3* did not functionally compensate for the lack of *T1* and *T2* transcripts. This perhaps explains why the expression of *T3* is suppressed or inhibited in wild-type plants. However, the possibility cannot be discarded that the transcripts function under certain conditions that were not explored in this study. Nevertheless, it is interesting to understand why the nature has donated this gene an alternative promoter and why the activity of that promoter is maintained low in the wild type. From the general point of view, the alternative promoters provide additional flexibility in the control of expression and function of a gene. But this is not in agreement with the absence of transcripts *T3* in the wild type as observed. The sequence comparison of the 1.2 kb-intron sequence to the genomic sequence of various plant species did not show any significant homologues. Moreover, it has appeared that *ALDH3H1* is the single ALDH coding gene with an alternative promoter identified so far in *Arabidopsis*. The generation of the alternative promoter may be an isolated event that has occurred during evolution. Landry et al. (2003) proposed several events that can explain the origin of alternative promoters. First, it may be through progressive mutations, which eventually create over time a set of new functional motifs that can recruit the transcriptional machinery and that are in a favourable genomic position to serve as a promoter for the downstream gene. Alternatively, it can result from a recombination event duplicating a promoter region and subsequent mutations would affect the strength and tissue specificities of the promoters, or even the sequence depending on the age of the duplication and selective pressures. Other possibilities are the insertion of a transposable element or genomic rearrangements in the vicinity of the gene, creating thereby a novel promoter. Considering that the occurrence of a novel promoter would be accompanied by the generation of new TSSs, downstream promoters or TSSs might produce a truncated protein whose function is deteriorated or lost. In contrast, the generation of upstream mutations would affect downstream canonical transcriptional signals and gene function. Therefore, upstream

mutations would have been negatively selected evolutionary. Regardless the mechanisms that evolve the alternative promoter, such modifications would only persist if they can withstand selective pressure and become beneficial or neutral to the host. Thus, it is more plausible that a slow and gradual accumulation of mutations that result in a novel transcription unit would be the most effective evolutionary path (Landry et al. 2003). Understanding how the regulation of the novel promoter has evolved is another task that requires study of the AFE-containing gene clusters in other species. An insight on this aspect has been brought in a study that compared AFE-containing gene pairs from rice and *Arabidopsis* (Chen et al. 2007). It was respectively found that only 1.4 and 2.9% of all AFE-containing gene clusters in rice and *Arabidopsis* were classified as orthologous groups. By investigating which kinds of gene categories were likely to use AFEs the authors did not find any bias. They therefore concluded that evolutionary conservation of AFEs exists in functional categories instead of individual genes in plant genomes. Mutations leading to novel promoters or TSSs might have occurred to adapt the gene expression to changes over the time. Altogether, the alternative promoter in *ALDH3H1* gene would have independently occurred late after *ALDH* gene diversification in *Arabidopsis*.

4.3 Study of the *ALDH7B4* gene promoter

4.3.1 The *Arabidopsis* antiquitin-like protein ALDH7B4 is a good candidate to investigate aldehyde dehydrogenase gene regulation

ALDH7B4 belongs to the family 7 of plant ALDH proteins that are also named antiquitins. These proteins were given the name “antiquitin” to reflect their antique nature (Lee et al. 1994). The amino acid sequence identity among family 7 members is about 60-70%, making them besides histone H2A proteins one of the most evolutionarily conserved eukaryotic proteins (Lee et al. 1994; Fong et al. 2006). The high degree of sequence similarity between species is probably indicating an essential conserved role within the cell. Plant ALDH7B proteins are believed to function in the regulation of turgor pressure or more generally in plant stress responses. In this regard, the garden pea (*Pisum sativum*) turgor protein 26g (now referred to as ALDH7B1 according to the official nomenclature) was found to be induced by dehydration, low temperature, heat shock and ABA (Guerrero et al. 1990). A similar increase of the gene transcript under osmotic stress was reported in canola (*Brassica napus*) (Stroeher et al. 1995). In *Arabidopsis*, the ALDH7B4 protein showed a strong induction by osmotic stress and ABA (Kirch et al. 2005). Over-expression of ALDH7B4 in *A. thaliana* has

conferred osmotic and oxidative stress tolerance to transgenic plants (Kotchoni et al. 2006). Similarly, transgenic tobacco (*Nicotiana tabacum*) and *Arabidospsis* plants ectopically expressing the soybean (*Glycine max*) antiquitin-like *ALDH7* gene showed overall reduced MDA levels associated with improved drought and high salinity tolerance in addition to decreased sensitivity to hydrogen peroxide and methyl viologen (Rodrigues et al. 2006). Seedlings of a rice T-DNA insertion mutant lacking the ALDH7 protein showed increased sensitivity to cold, heat, salinity, dehydration and methyl viologen (Shin et al. 2009).

Reports on the physiological function of human antiquitin started in 2006 when it was discovered that mutations of antiquitin are responsible for pyridoxine-dependent seizures (Mills et al. 2006; Plecko et al. 2007). Human antiquitin catalyzes the oxidation of α-aminoadipic semialdehyde (α-AASA) in the lysine catabolic pathway (Mills et al. 2006). Mutations in the human antiquitin gene led to a deficiency of the enzymatic activity and the accumulation of α-AASA and its equilibrium product Δ^1-piperidine-6-carboxylate. The latter will undergo Knoevenagel condensation with pyridoxal-5-phosphate, leading to a deficiency of Vitamin B6 required for neurotransmission processes. More recently, the mammal ALDH7A1, a homolog of pea ALDH7B1, was shown for the first time to protect against hyperosmotic stress by generating osmolytes and metabolizing toxic aldehydes (Brocker et al. 2010). Together with their evolutionarily conserved features it appears that ALDH7 proteins play a pivotal role in cells in osmotic homeostasis.

Although ALDH7B4 is classified as aldehyde dehydrogenase, its physiological substrates are still elusive. 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). Enzymatic activities of the recombinant rice ALDH7 protein were reported using MDA, acetaldehyde or glyceraldehyde as substrates (Shin et al. 2009). Similarly, purified recombinant mammalian ALDH7A1 efficiently metabolized a number of aldehyde substrates, including betaine aldehyde, lipid peroxidation-derived aldehydes, and the intermediate lysine degradation product, α-aminoadipic semialdehyde (Brocker et al. 2010). This indicates that ALDH7 proteins can oxidize a wide range of aldehyde substrates and this may predominantly account for their function in stress tolerance.

As reported, *ALDH7B* genes are rapidly induced upon stress, indicating that a relatively short or rapid signalling pathway may govern their expression. The induction of *ALDH7B4* in *Arabidopsis* ABA-deficient and ABA-insensitive mutants was impaired after dehydration, but the expression was nearly unaltered upon NaCl treatment, indicating a divergence of the

signalling pathways (Kirch et al. 2005). It was proposed that the dehydration stress response may be ABA-dependent, whereas salt stress-induced expression is regulated in an ABA-independent manner. In contrast to *ALDH3I1* (another stress responsive *ALDH* gene), of which the salt and dehydration responses were found to be mainly ABA-dependent, the *ALDH7B4* gene would offer the unique advantage of studying *ALDH* gene regulation in both ABA-dependent and -independent situations. Moreover, ALDH7B4 could be detected in both roots and aerial parts of the plant while this feature was absent in all three members of family 3 (ALDH3I1, ALDH3H1, ALDH3F1) and the ALDH22A1 (Kirch et al. 2005) in *Arabidopsis*. All this makes *ALDH7B4* a good candidate for studying the regulation of *ALDH* genes under stress conditions. Transgenic reporter lines which expressed the GUS reporter protein under the control of the *ALDH7B4* promoter were generated. These plants were used to investigate how plants sense the increase in lipid peroxidation-derived aldehydes and which signalling mechanism(s) govern(s) the expression of ALDH proteins in the presence of aldehydes.

4.3.2 Induction patterns of the *ALDH7B4* gene promoter

Transgenic 7B4-GUS lines were generated to study the regulation of *ALDH* genes under stress and by aldehyde molecules. The *ALDH7B4* promoter was found to be constitutively active in mature seeds. The accumulation of the GUS reporter protein started during the silique elongation and reached a peak in mature siliques. The levels of accumulated GUS activity remained high in mature seeds but lower than the levels in mature siliques. The observations were confirmed by protein-blot analysis of wild-type seed protein extracts (**Fig. 39**). The results are consistent with data on the *ALDH7B4* gene in the GENEVESTIGATOR V3 database (http://www.genevestigator.com; Zimmermann et al. 2004). Such an accumulation pattern of antiquitins in seeds has been reported for the rice ALDH7 protein. Seeds from a T-DNA insertion mutant lacking the functional ALDH7 enzyme showed increased sensitivity to aging and accumulated more MDA than the wild-type (Shin et al. 2009). Moreover, seeds of this knock-out mutant accumulated melanoidin as a consequence of the lack of functional ALDH7 protein. Melanoidin is described as a product of a Maillard reaction, where a carbonyl group (for example from aldehydes) is non-enzymatically combined to an amine group (from amino-acid residues in proteins) (Adams et al. 2005; Papetti et al. 2006; Niquet and Tessier 2007). As suggested by Shin et al. (2009) the accumulation of ALDH7 protein during seed maturation and storage may be required for the detoxification of aldehydes, which could affect seed viability. Consistent with this, the

ALDH7B4 gene promoter is also activated during seed desiccation. This suggests that plant antiquitins may play a pivotal role in seed maturation and viability similar to LEA genes.
When the transgenic 7B4-GUS lines were treated with different stressors including aldehydes, it was found that both the chromosomal and transgenic *ALDH7B4* gene promoters were equally activated. These observations are consistent with the previously reported analyses of the *ALDH7B4* gene at both mRNA and protein levels (Kirch et al. 2005; Kotchoni et al. 2006). This shows that the expression of the *GUS* reporter gene reflects the chromosomal *ALDH7B4* gene expression. Therefore, these reporter lines could be confidently used to study the activation pattern of the chromosomal gene under different stress conditions or in the presence of diverse effectors. In addition, these observations suggest that the aldehyde-mediated *ALDH7B4* promoter activation involves some *trans*-acting factors that can specifically recognize and activate the promoter irrespective of its position in the genome.

4.3.3 Biological activities of α,β-unsaturated aldehydes and related oxilipins

Numerous examples of the bioactivity of aldehyde molecules have been reported. Trans-2-hexenal was shown to inhibit root elongation in *Arabidopsis* seedlings. It was proposed that trans-2-hexenal exerted its action through the synthesis and accumulation of GABA (Mirabella et al. 2008). Tocopherol deficiency in the vitamin E2 mutant *vte2-1* of *Arabidopsis* led to enhanced lipid peroxidation and accumulation of MDA as well as stable phytoprostanes shortly after germination. This correlated with the up-regulation of defense-related genes (Sattler et al. 2006). Fujita and Hossain (2003) reported the induction of pumpkin (*Cucurbita maxima* Duch.) glutathione S-transferase (GST) genes by crotonaldehyde and trans-2-hexenal at both mRNA and protein levels. Likewise, many abiotic stress responsive genes were shown to be induced by MDA (Weber et al. 2004). GST1 mRNA was strongly expressed in *vte2-1* mutant seedlings and in response to direct MDA application (Weber et al. 2004; Sattler et al. 2006). Other GST coding genes have been shown to be induced by MDA or oxilipins derived from non-enzymatic lipid peroxidation (Mène-Safrané et al. 2007; Mueller et al. 2008). Dueckershoff et al. (2008) showed that cyclopentenone-oxylipins spontaneously react with several proteins and glutathione *in vitro* and *in vivo*. The conjugation with glutathione could be reversibly catalysed by GSTs and the binding of glutathione to α,β-unsaturated lipid peroxidation products was proposed as a mechanism of detoxification (Farmer and Davoine 2007).
An important feature of the aldehydes with biological activity is the presence of an α,β-unsaturated carbonyl group. It was shown that electrophilic aldehydes containing an

α,β-unsaturated carbonyl were more efficient in gene activation than their aliphatic counterparts (Weber et al. 2004). Consistent with this, trans-2-hexenal induced the *ALDH7B4* promoter stronger than pentanal (**Fig. 41**) and other short-chain aliphatic aldehydes (data not shown).

In 2004, Weber et al. demonstrated that MDA could up-regulate the expression of an array of genes, most of which are related to abiotic stress. The authors postulated that the concentration and the localisation of unbound MDA would be key factors directing its biological activities *in vivo*. About 75% of total MDA content in expanded leaves was shown to originate from trienoic fatty acids while the source of the second pool is so far unknown (Weber et al. 2004). Using triple fatty acid desaturase (*fad3-2fad7-2fad8*) mutants deficient in the accumulation of the trienoic fatty acid, it was found that a basal MDA level is kept in plant tissues in physiological conditions. The biological activity of MDA is mostly derived from changes in the free MDA pool that is dynamic and increases upon stress (Farmer and Davoine 2007). Treating *Arabidopsis* leaves with Diquat (a structural analogue of methyl viologen that causes oxidative burst) Weber et al. 2004 observed 2.7-fold increase of the free MDA content. Similar to these observations, methyl viologen led to a significant accumulation of MDA associated with high *ALDH7B4* promoter activity, as shown here. This underlines the correlation between oxidative stress, MDA accumulation and gene expression. However, the comparison of the promoter activity and MDA accumulation has demonstrated that this correlation is not always true for some stressors, as shown in **Fig 43.** This suggests that other factors besides MDA may be required for *ALDH7B4* promoter activation. The results presented here do support the idea that a threshold of intra-cellular MDA contents, and in general of lipid peroxidation-derived aldehydes, is required to trigger the expression of the target genes. Additional transcription factors may be necessary for a full induction of the target promoters.

4.3.4 How could bioactive aldehydes function as signal compounds?

It is unlikely that MDA or other lipid peroxidation-derived aldehydes directly interact with the target gene promoters in the nucleus. A more plausible mechanism would involve other effectors located in the cytosol or the chloroplasts, where these molecules are predominantly generated under stress conditions. Any significant response over the background expression could not be detected within the first 4 h of treatment, indicating that the induction of the *ALDH7B4* promoter by aldehydes requires intermediate factors that are probably synthesized *de novo*. The fact that the promoter activity increased with the incubation time did support

this idea (**Fig. 41a**). As explained in the previous paragraph, the signalling event is probably triggered as aldehydes accumulate to a certain level that is sufficient to promote their interactions with the target factors. Reactive electrophile species (RES) including MDA and other α,β-unsaturated aldehydes possess a thiol-reactivity that allows them to covalently modify proteins *in vivo* (Mueller and Berger 2009). Such a covalent interaction is proposed to be a determinant for their signalling function. Recent data indicated that MDA and other oxilipin-mediated signalling could first lead to the induction of genes coding for transcription factors, which in turn will trigger the expression of further target genes in a latter phase of the response. A DREB2A encoding gene (*At5g05410*) transcript was found highly up-regulated upon 4 h of MDA treatment (Weber et al. 2004). A parallel between high MDA or phytoprostanes levels and significant up-regulation of a DREB-like AP2 transcription factor (*At2g38340*) was reported in *vte2* mutants (Sattler et al. 2006). The implication of the TGA transcription factors TGA2, TGA5 and TGA6 in cyclopentenone oxylipins-mediated gene expression has also been described (Mueller et al. 2008). Thirty percent of gene induction by 12-oxo-phytodienoic acid (OPDA) and 60% of gene induction by A1-phytoprostanes were absent in the *tga2,5,6* triple knock-out mutant. As MDA and some other alkenals are generated along with these phytoprostanes, the implication of these TGA transcription factors in MDA signalling cannot be excluded. Based on the irreversible feature of RES bounds with thiol residues in proteins, the most obvious signalling pathway is the covalent modification of *trans*-acting factors. This may lead to the direct activation of the transcription factor or its release from a partner that in the absence of signal prevents its access to the promoter. The genetic screening of mutants with altered *ALDH7B4* promoter activation may lead to uncover *trans*-acting factors implicated in α,β-unsatured aldehyde signalling.

4.3.4.1.1 Functional analysis of the *cis*-acting elements in the *ALDH7B4* promoter

Our results from mutated *ALDH7B4* promoter constructs indicated that only the most downstream ACGT-containing motifs (ACGT2 and ACGT3 boxes) are relevant for the salt stress response (**Fig. 44**). By contrast, Kirch et al. (2005) showed that the salt responsiveness of the *ALDH7B4* gene is more likely to occur in ABA-independent manner. This discrepancy may be indicating that these ACGT-containing boxes are not real ABREs. Mehrotra and Mehrotra (2010) recently described that two copies of ACGT element separated by 5 nucleotides imparted salicylic acid induction to a basal promoter whereas ABA-responsiveness was obtained when the distance between both ACGT elements were at least 25 nucleotides. Many plant *cis*-acting elements actually contain the core ACGT-sequence and

the flanking sequence is the key factor determining the function (Guiltinan et al. 1990; Salinas et al. 1992; Busk et al. 1997). ACGT-containing sequences that do not function as ABREs even in ABA inducible promoters were also reported (Kao et al. 1996; Busk et al. 1997). Reports on identical *cis*-acting sequences but with different functions have been documented as well (Busk and Pagès 1998); who suggested that the ABRE is a subset of ACGT-containing elements that is defined by the function rather than by the flanking sequence. Further analyses of the different *ALDH7B4* promoter constructs with respect to ABA treatment are required to uncover the nature and the function of the ACGT2 and ACGT3 boxes. By now, it is also impossible to decide which *cis*-elements in the *ALDH7B4* promoter are involved in the response to aldehyde treatments. The functional analysis of the mutated promoter constructs should be extended to aldehyde molecules to better understand the mechanism of the promoter activation. It is important that these analyses are performed on stably transformed *Arabidopsis* plants. In fact, the co-cultivation method used in this study has some substantial drawbacks as for the important number of replications required for getting meaningful results. The method may not be appropriate for studying gene stress response, as the procedure for the co-cultivation can itself be a stress to the plantlets, compromising the interpretation of the results. Seedlings also fail sometime to resist for 24 h stress application after co-cultivation and die by losing their chlorophyll before the experiments is terminated.

4.3.5 Generation of the EMS-mutagenized seed population and screening strategy

In order to identify signalling proteins involved in the aldehyde-mediated activation of the *ALDH7B4* promoter a forward genetic approach was undertaken in this study. Homozygous seeds from 7B4-GUS reporter plants were treated with EMS and cultivated on soil until the seeds from the next generation (M2) were obtained. The approach is based on the unique feature of EMS to introduce random point mutations throughout the genome. This results in nonsense or mis-sense mutations in the gene promoter or coding regions that can alter gene expression. As such, any mutation in a *trans*-acting factor gene involved in the activation of *ALDH7B4* promoter could result in the alteration of the *GUS* gene expression. Such an alteration can be seen through the absence, down-regulation or up-regulation of the GUS enzymatic activity. Plants with such mutations could be identified from screening the M2 mutant population and further characterized. This approach has been widely used in the past and led to the identification of various protein functions or gene expression pathways (Jackson et al. 1995; Oono et al. 1998; Heiber et al. 2007). Although the use of an EMS-based

mutagenesis approach generally allows finding a mutation in any given gene by screening fewer than 5,000 *Arabidopsis* plants (Feldman et al. 1994), it is clear that the success and the efficiency of this approach are mainly dependent on the screening procedure used. Here, it has appeared that a direct exposure of plants to aldehydes did not work out as expected. In fact, the aldehydes are volatile enough and spraying them on the plants did not lead to a reproducible effect. But, this could be overcome by exposing the whole plant or organs to a specific concentration of aldehyde in a tightly closed container, as described by Weber et al. (2004). This method was used with individual plants to study the promoter activity in different stress conditions. Yet, many plants could not be simultaneously treated in a single container, rendering the method inadequate for a large scale screening. Additionally, the activity of the promoter did not appear to be strong enough induced by aldehydes, as compared to dehydration or salt. As consequence, it was not possible to make a clear-cut decision. An indirect approach of screening may prove useful to overcome the problem. In the light of the results obtained from this work it may consist of screening the M2 plants first by methyl viologen. Once putative mutants with altered GUS expression have been identified, a second round of screening will be performed on those mutant lines using aldehydes. This should allow identifying mutants impaired in aldehyde-mediated gene responses.

4.4 Conclusions and future perspectives

The data presented here contribute to the understanding of the function and regulation of aldehyde dehydrogenases in *Arabidopsis*. Broadening the previous findings, this work provides important data for the so far unknown function of members of family 10, 3 and 7 of ALDH proteins in *Arabidopsis*. Similar to rice, the data showed that *Arabidopsis* BADH homologous genes are involved in the plant stress response but in a way that is still elusive. Additional work is required to shed light on that feature. Functional analyses of BADH over-expressing lines would be a good starting point for investigations. On the other hand, data also pointed out to the aminoaldehyde dehydrogenase activity of ALDH10A8 and ALDH10A9 and their probable contribution to the intracellular GABA content. Considering that the major source of GABA is believed to derive from the decarboxylation of glutamate, the importance of the aminoaldehyde dehydrogenase route could be pharmacologically accessed with inhibitors of glutamate decarboxylase. Moreover, genetic and phenotypic studies can be carried out in association with mutants defective in glutamate decarboxylase activity to better understand the interaction between these metabolic pathways. If the

aminoaldehyde dehydrogenase-mediated GABA synthesis is of any importance, growth of plants on polyamine-enriched media should enhance the polyamine catabolism and stimulate GABA formation. Combined use of the available mutant tools for both the polyamine catabolism and glutamate decarboxylase pathways in association with ALDH10A8/A9 knock-out and over-expressing lines represent a range of research opportunities to consider in order to further clarify functions of *Arabidopsis* BADH coding genes.

Another point addressed in this work is the functional characterization of the *ALDH3H1* gene. The results indicate that unlike ALDH3I1, ALDH3H1 does not significantly contribute to the plant resistance to abiotic stress. It is probable that ALDH3H1 functions in the normal plant metabolism with minor influences under adverse conditions. Another intriguing feature of the *ALDH3H1* gene uncovered in this study is the existence of an alternative promoter within the upstream intron, which directs the expression of an alternative first exon transcript. Although, the data indicated the absence of this transcript in the wild type, it is more likely that this novel transcript variant is expressed at a very low level. A nested RT-PCR approach should help to solve this issue. On the other hand, further experiments are required to deepen the functional relevance of the *ALDH3H1* transcript variants. It would be worth to screen organ development of the different *ALDH3H1* mutant lines with different transcript variants that could not be seen through the experimental approach used in this study.

Finally, the results in agreement with previous reports confirmed the bioactivity of α,β-unsaturated aldehydes in gene induction. The screening of the generated M2 mutant population from the EMS-treated 7B4-GUS seeds will lead to the identification of interesting mutants affected in the *ALDH7B4* promoter response to aldehydes. As discussed above, the use of methyl viologen as a first alternative to a direct application of aldehyde molecules could be more time-saving and efficient for the screening procedure. Considering that the regulation of the promoter by aldehydes involves some *trans*-acting factors, another approach would be to establish a yeast one-hybrid system to isolate direct interacting proteins of the *ALDH7B4* promoter upon treatment of aldehydes or analogue effectors.

5. APPENDICES

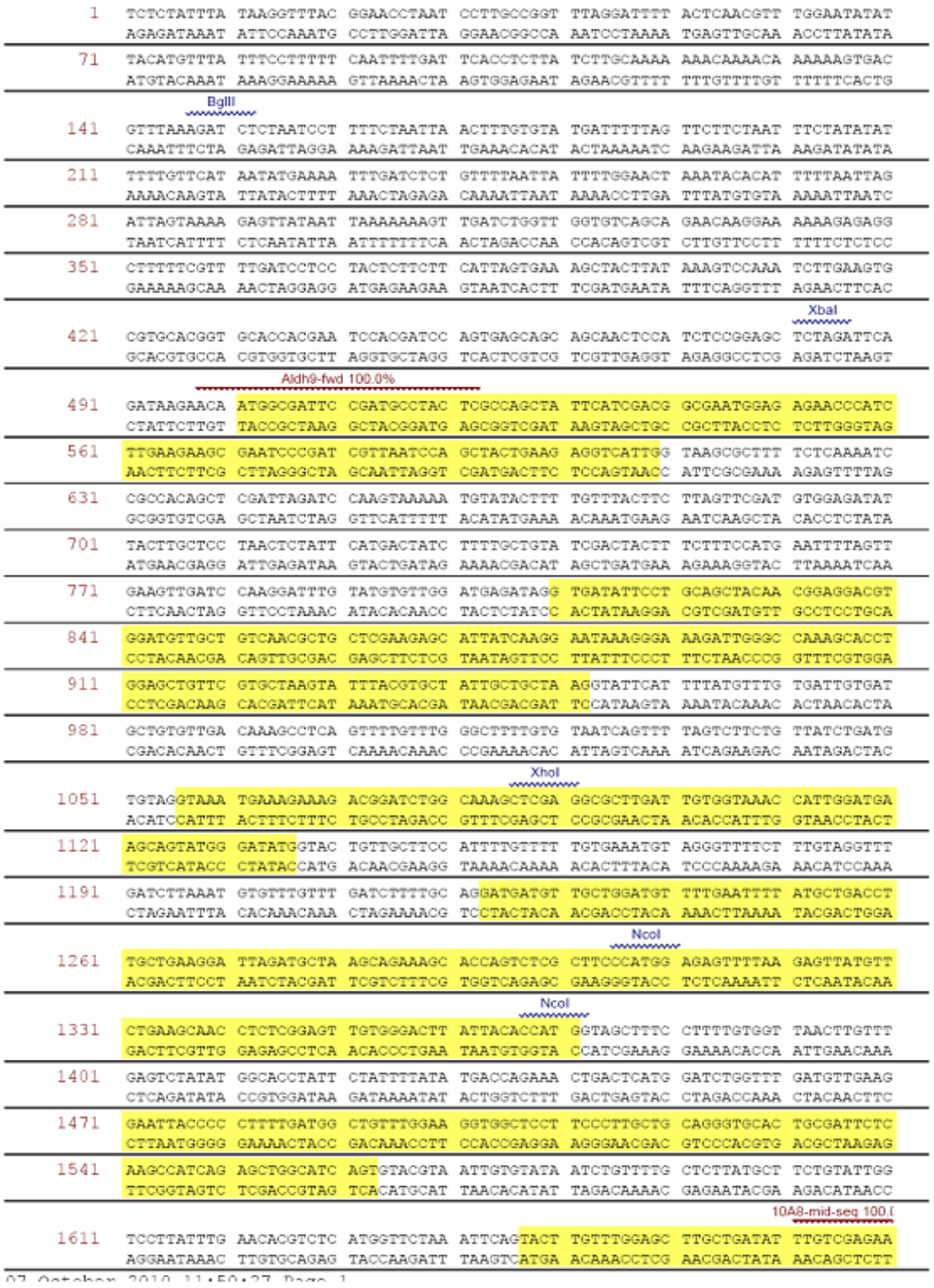
5.1 Accession numbers of the *ALDH* genes

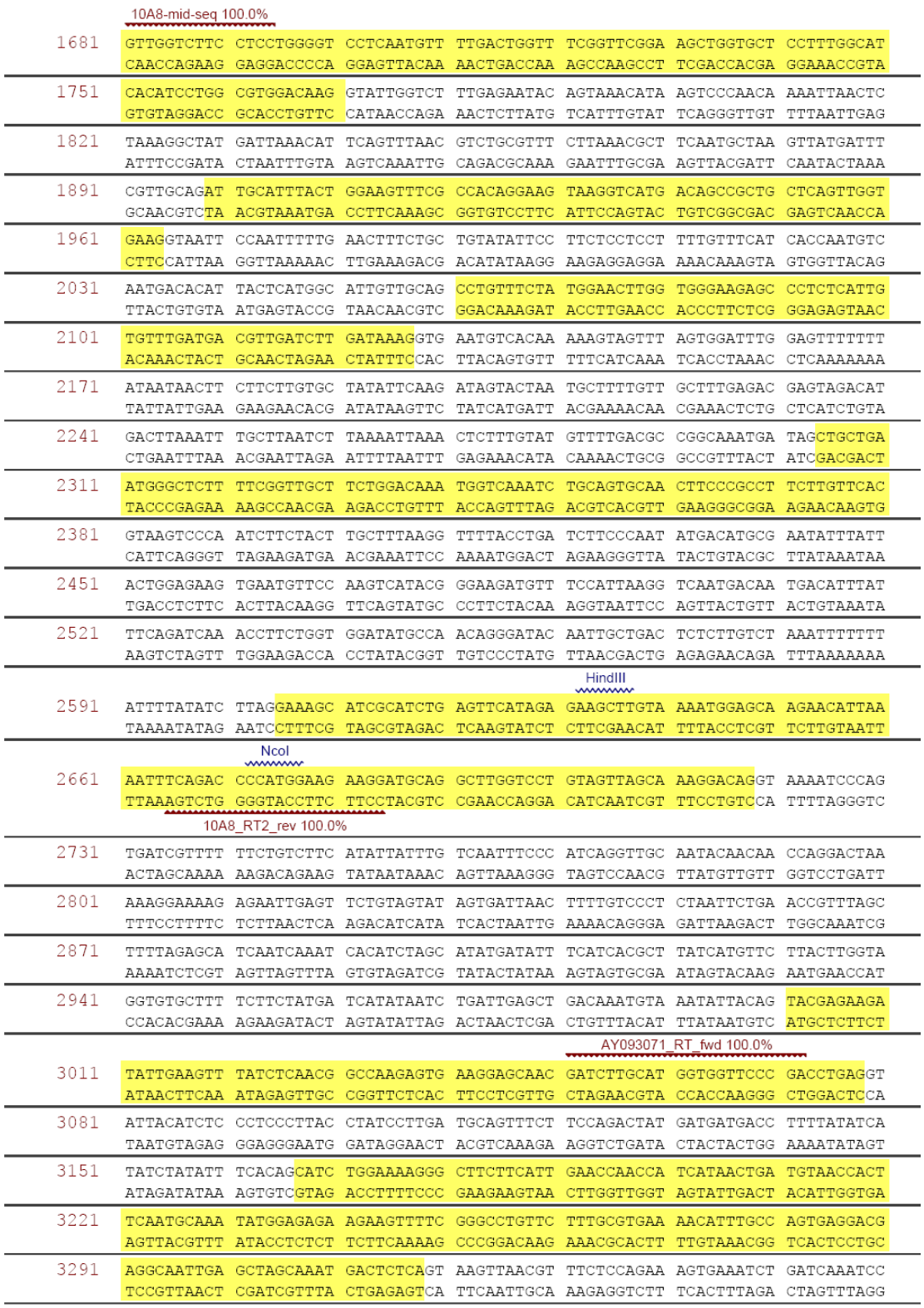
Genes	TAIR Gene name	NCBI GenBank accession	References
ALDH10A8	AT1G74920	AY093071	Kirch et al. 2004
ALDH10A9	AT3G48170	AF370333	Kirch et al. 2004
ALDH3H1	AT1G44170	AY072122	Kirch et al. 2004
ALDH7B4	AT1G54100	AJ584645	Kirch et al. 2004
ACTIN2	AT3G18780	AF428330	An et al. 1996

5.2 Gene sequences

The sequences of the ALDH genes characterized in this work are shown here using the Vector NTI™ Suite program. The exons are shaded in yellow. Regions between exons are introns. Primers are indicated by their name and their locations are showed with a red line. Restriction sites of commonly used restriction enzymes are indicated as well. The *exon2'* of *ALDH3H1* is shaded in turquoise.

5.2.1 *ALDH10A8* gene sequence

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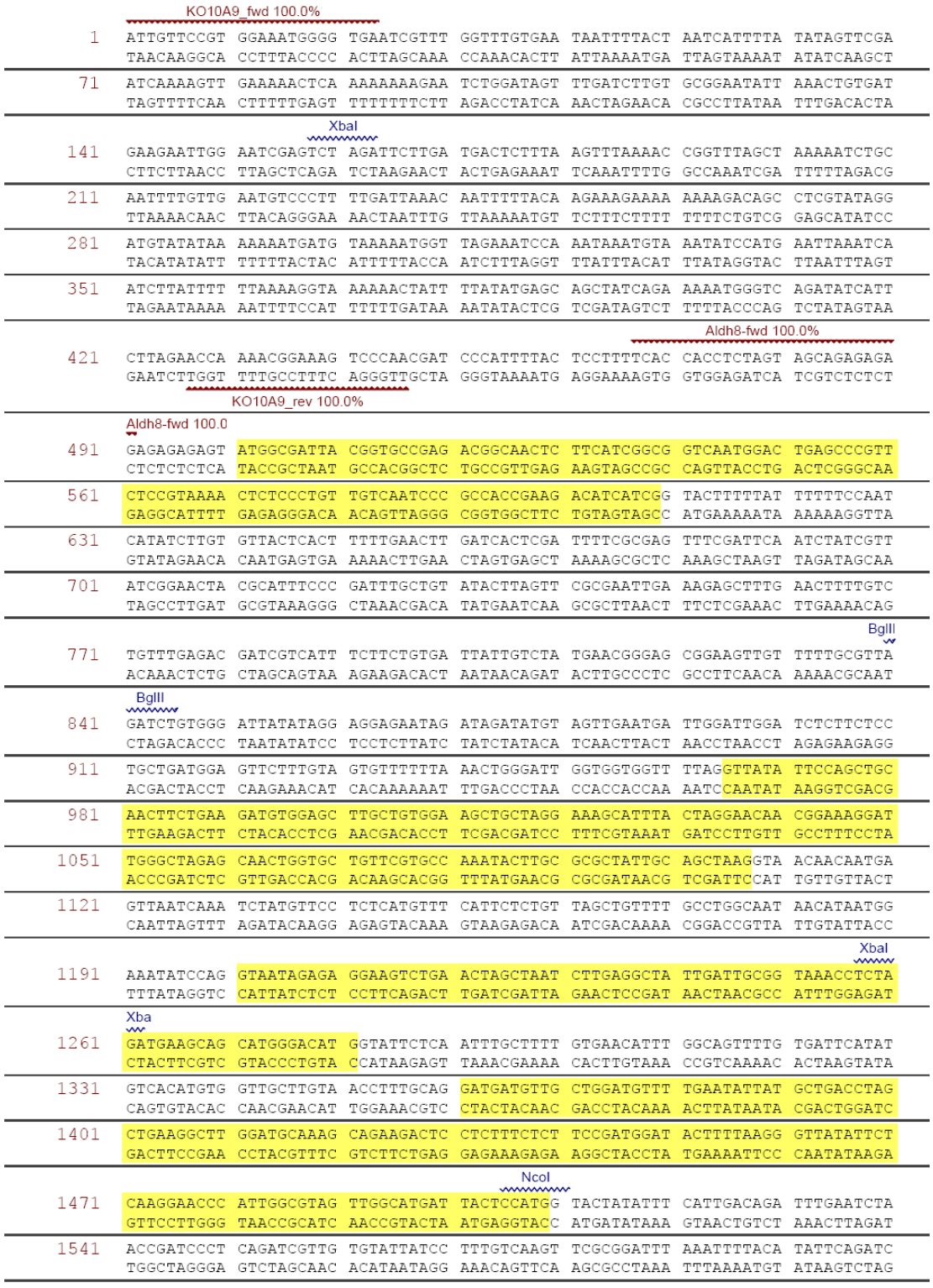

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      GGACTCCATG TAATATAACT GATTAGAAGA TACATACACG GTTTATGATA GATCTCGGGC ATCGTGAATG

3361  AATATTTTAT TTATGTTCTG CAGCATTTAA AGAAGGGATA CTTTGTTGAA CCTGCGATTG TTTCAAATGT
      TTATAAAATA AATACAAGAC GTCGTAAATT TCTTCCCTAT GAAACAACTT GGACGCTAAC AAAGTTTACA
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3431  GACTACTTCA ATGGAAATCT GGAGAGAGGA AGTCTTTGGT CCTGCTCTGT GTGTGAAAAC ATTCTCCACC
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3501  GAGGATGAGG CAATTCAGCT TGCAAATGAC TCTCAGTGAG TGTAGATTTC TCTTCTTATC TTGAAGTAGA
      CTCCTACTCC GTTAAGTCGA ACGTTTACTG AGAGTCACTC ACATCTAAAG AGAAGAATAG AACTTCATCT

3571  ACTCCACTGA TGACGTGTTA GATCATATGC CTGACTTTAT TTGTATTTGC AGATATGGAT TAGCAGGCGC
      TGAGGTGACT ACTGCACAAT CTAGTATACG GACTGAAATA AACATAAACG TCTATACCTA ATCGTCCGCG

3641  AGTATTATCA AATGATCTGG AGCGGTGTGA TCGTGTTAGT AAGGTGTGTA TCTAACTTTT ACTATCAACA
      TCATAATAGT TTACTAGACC TCGCCACACT AGCACAATCA TTCCACACAT AGATTGAAAA TGATAGTTGT

3711  ATAACAATCA ATAACCTCAT GATTGAACTT AATCTCTAAA GTTTTATGAA TCGTAATCAT TGTGTGTTTC
      TATTGTTAGT TATTGGAGTA CTAACTTGAA TTAGAGATTT CAAAATACTT AGCATTAGTA ACACACAAAG

                                                                        NcoI
3781  AGGCTTTCCA GGCGGGAATA GTGTGGGTCA ACTGCTCTCA GCCATGCTTC TGCCAAGCTC CATGGGGTGG
      TCCGAAAGGT CCGCCCTTAT CACACCCAGT TGACGAGAGT CGGTACGAAG ACGGTTCGAG GTACCCCACC

3851  AACCAAACGC AGTGGATTTG GCCGTGAATT GGGAGAATGG TTAGTCTCTT TCGTTAGCTA TAAGTATTAA
      TTGGTTTGCG TCACCTAAAC CGGCACTTAA CCCTCTTACC AATCAGAGAA AGCAATCGAT ATTCATAATT

3921  GATTATCAAA CCATTAGAAA AAGACACTCT CCAACTAGAT GGAATTGTGT CCATAGTAAC TTAAACCGTA
      CTAATAGTTT GGTAATCTTT TTCTGTGAGA GGTTGATCTA CCTTAACACA GGTATCATTG AATTTGGCAT

3991  ATAGATTACC AACAAAATTT GAATTGAAAC ATATTTTCTT CGTCGCAGGG GACTTGAGAA CTACTTGAGT
      TATCTAATGG TTGTTTTAAA CTTAACTTTG TATAAAAGAA GCAGCGTCCC CTGAACTCTT GATGAACTCA

                                  NcoI
4061  GTGAAGCAGG TGACTCAGTA TATCTCTGAT GAACCATGGG GATGGTACAA ACCTCCTTCC AAGCTCTGAG
      CACTTCGTCC ACTGAGTCAT ATAGAGACTA CTTGGTACCC CTACCATGTT TGGAGGAAGG TTCGAGACTC
                                                 Aldh8-rev 100.0%

4131  ACAAACACTA GTTCCTGATA TTTCCTTAAA ACATGTACTC ATGAGAATAT ACCAATAAAG CAAGAGACCC
      TGTTTGTGAT CAAGGACTAT AAAGGAATTT TGTACATGAG TACTCTTATA TGGTTATTTC GTTCTCTGGG
      Aldh8-rev 100.0                                    AF370333_RT_rev 100.0%

4201  TTCTGTAAGA GAAATGGTAT TGCTACTTTA AATTCTTGAA AAAAATGAAA AATAAGATGA GATGATATTT
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4271  CTTTTTGTTT TTCTCAAAAC AACATGCCAT ACAAAAGTAG CAAAAAACTG GCGAAAGATG AATGCTCTTA
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4341  TATGTAACAT ACAAAAGTTA CAGCCTACAG TAAACTTCAG AAAGCAAAGA GGGTATGCGA TCTGTGAATT
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4411  AATGTCGGAT TCTTGCAGTT ACAATTGTGT GGAGAAACTA AACCACATTG ATCAAAAGAA ATGACTCTTG
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4481  AAGCTCTCTA CACGAATGAT GATTGATGTT CACAGGTAAG AGAAGAAGAA GAAGAAGAAG AAGAAGAAGA
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                          BglII
4551  AGAAGAAGAA GAAAAAGAAG ATCTAACGGT GTTGTGATGT ATTAGGTAAT GGATTCTCCT GGGTGACTTC
      TCTTCTTCTT CTTTTTCTTC TAGATTGCCA CAACACTACA TAATCCATTA CCTAAGAGGA CCCACTGAAG

4621  GCTGAACCA
      CGACTTGGT
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5.2.3 *ALDH3H1* gene sequence

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1    TTTATGATTT TTTAATATGT ATGTACAAAC TTTAAATTAC AACTAATATG AAACTGAAGA AATATATAGA
     AAATACTAAA AAATTATACA TACATGTTTG AAATTTAATG TTGATTATAC TTTGACTTCT TTATATATCT

71   AATAAAATAT TTTATTCTTA CTTTGTTGTA ATCAAATTAT TTGTTTTGTT AAAAATTATC AAAAAAAAAA
     TTATTTTATA AAATAAGAAT GAAACAACAT TAGTTTAATA AACAAAACAA TTTTTAATAG TTTTTTTTTT

141  TCTTTCTTGA TATTCTCTTT TTGACGTTTC TTCATCTCTA TAAATATTGT AACGCATGCT TTTTTTTATT
     AGAAAGAACT ATAAGAGAAA AACTGCAAAG AAGTAGAGAT ATTTATAACA TTGCGTACGA AAAAAAATAA

211  ATCAATTTCG AGGAATAAAA GTAAGAAACA TTGCGACAAA AAAAAAAAAA GTAAGAAACA TTTTTTTTCT
     TAGTTAAAGC TCCTTATTTT CATTCTTTGT AACGCTGTTT TTTTTTTTTT CATTCTTTGT AAAAAAAAGA

281  TCTTTTTATG TGTTTTTAAA GAAACTTATC ACTTTTTATT TGGCACGAGC AACGTCAATA TCTACGAAAA
     AGAAAAATAC ACAAAAATTT CTTTGAATAG TGAAAAATAA ACCGTGCTCG TTGCAGTTAT AGATGCTTTT

                    EcoRI                    BamHI
351  GAATATTTAT TTTCTTGAAT TCAAGAAAAA TTCTCGGGAT CCGAACTCAA TATTTGTTCT CTTCTCTTCT
     CTTATAAATA AAAGAACTTA AGTTCTTTTT AAGAGCCCTA GGCTTGAGTT ATAAACAAGA GAAGAGAAGA

421  CTCTCTCTTT GCCCGTGATT ACTGATTACT GTGTTAATTA TTTTTATTCT GCGTACGTTA CGTAAGAGAG
     GAGAGAGAAA CGGGCACTAA TGACTAATGA CACAATTAAT AAAAATAAGA CGCATGCAAT GCATTCTCTC

                                                  Ath-ALDH2-sense 100.0%
                                 Ath-ALDH1a_sense 100.0%
491  ATATTGAGGA ATGGCTGCGA AGAAGGTTTT TGGATCGGCG GAAGCGAGTA ATTTGGTGAC GGAGCTTCGT
     TATAACTCCT TACCGACGCT TCTTCCAAAA ACCTAGCCGC CTTCGCTCAT TAAACCACTG CCTCGAAGCA

561  CGGAGTTTTG ATGATGGTGT GACACGTGGT TATGAATGGA GAGTGACTCA GCTTAAGAAA CTGATGATTA
     GCCTCAAAAC TACTACCACA CTGTGCACCA ATACTTACCT CTCACTGAGT CGAATTCTTT GACTACTAAT

631  TTTGTGATAA TCATGAGCCT GAGATCGTCG CGGCTCTTCG CGATGATCTT GGTAAGCCTG AGCTTGAATC
     AAACACTATT AGTACTCGGA CTCTAGCAGC GCCGAGAAGC GCTACTAGAA CCATTCGGAC TCGAACTTAG

701  TTCTGTTTAT GAGGTTAGGA TTCTTTTTTC TCTCTTTATG ATCTCGCTTT CCCTCAGATT GTTTTTTTTA
     AAGACAAATA CTCCAATCCT AAGAAAAAAG AGAGAAATAC TAGAGCGAAA GGGAGTCTAA CAAAAAAAAT

771  AAAGAGTTTT TTAGGATTGA TTGTGATTGG TCAATTCTTA ATTTGAATCT TTGAATTTTA ATTGAAGATT
     TTTCTCAAAA AATCCTAACT AACACTAACC AGTTAAGAAT TAAACTTAGA AACTTAAAAT TAACTTCTAA

841  CAGTTTCTTG TTATGGTTTA ATCTGTTTGT GTAGTGGCAT TGTGGTATAT AGGATAGATG AAAAGTGAAG
     GTCAAAGAAC AATACCAAAT TAGACAAACA CATCACCGTA ACACCATATA TCCTATCTAC TTTTCACTTC

911  CTCCAAGTTC GATTTTACGG TGTATGATTA GAGTTATGTG GTTATTCATA GAAACTTTTT AGCCTTTTTA
     GAGGTTCAAG CTAAAATGCC ACATACTAAT CTCAATACAC CAATAAGTAT CTTTGAAAAA TCGGAAAAAT

981  CCCGAAATTA GACTCAATCT TTTTAGTCGA AGTAGATTTA AGTTCATAGT TCGGATGTTT TTTTATAGTG
     GGGCTTTAAT CTGAGTTAGA AAAATCAGCT TCATCTAAAT TCAAGTATCA AGCCTACAAA AAAATATCAC

1051 GTAGACTTGC AGAAAACATC CGAGCTATGA ATTTATATCG CTTTTATGAT TAGTTAGAAG TTTTGGAGAA
     CATCTGAACG TCTTTTGTAG GCTCGATACT TAAATATAGC GAAAATACTA ATCAATCTTC AAAACCTCTT

1121 ATTTGATATC CAAGTAATAA AGTATTTGAG TTTATTGTAA TTTTAAGAAG CATGATTGCT TCCGTGATTA
     TAAACTATAG GTTCATTATT TCATAAACTC AAATAACATT AAAATTCTTC GTACTAACGA AGGCACTAAT

1191 CTCAGAAGTT TTGAAAAAAT CCGATTATCA AGTAATAATA CATTTGAGCT ACGAACTTAT AGTAAATTAA
     GAGTCTTCAA AACTTTTTTA GGCTAATAGT TCATTATTAT GTAAACTCGA TGCTTGAATA TCATTTAATT

1261 AAAAAAAAAA CTTGATCACT TCTCTAACTA CTTAGAAATT TGGAGAAATT CCGAATATTT GAGTAATAAA
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1331 ACATCCGAAC TATGAATTTA TAGGAAGTTC AAATAACATG ATCACTTCCA TGATTACTTA AAAGTTTTAG
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1401 AGAAATCCAG ATATTCGATG CCAATCTATC ATTTGAAAAC CAATTATTGG TATGGTTATG AGTCAGAATC
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1471 AGATGTCGCT ACTAATATTT GCGGCTGATA GAATTTGCTT AGTGATGATA TTTCTTTAAT TAGTGGCTGT
     TCTACAGCGA TGATTATAAA CGCCGACTAT CTTAAACGAA TCACTACTAT AAAGAAATTA ATCACCGACA

1541 TTAACACATA AGCCAAACCT CTATAATTGG ATTAACATAT CCCCCTACTT GGTCGGTCTC ATTATTGTCT
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1611 ATGACCTTAG TTGCTACGTA AATGATGTAG TTTGTCGACA CATGTAACCA ACACTGTCTT CAATGCTAAC
     TACTGGAATC AACGATGCAT TTACTACATC AAACAGCTGT GTACATTGGT TGTGACAGAA GTTACGATTG

1681 TTTTTCACAA GTTAATACTA GTATCCATCA GCATAGAATG CAGGTAACTT TATGTTATGT AGGAACCAAT
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1751 ACTATAAAAA TGTTGGGTCC TATATTGAAA GCAAATATAC ATTAAATTTT TCCTTCTCCT ACTGTTAAAC
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1821 TACCATATAA CATTAAATTT CATCACCAAC CTTGTGGGGA TTCCCACGTT TTTCATCACT TGTGTTCCTT
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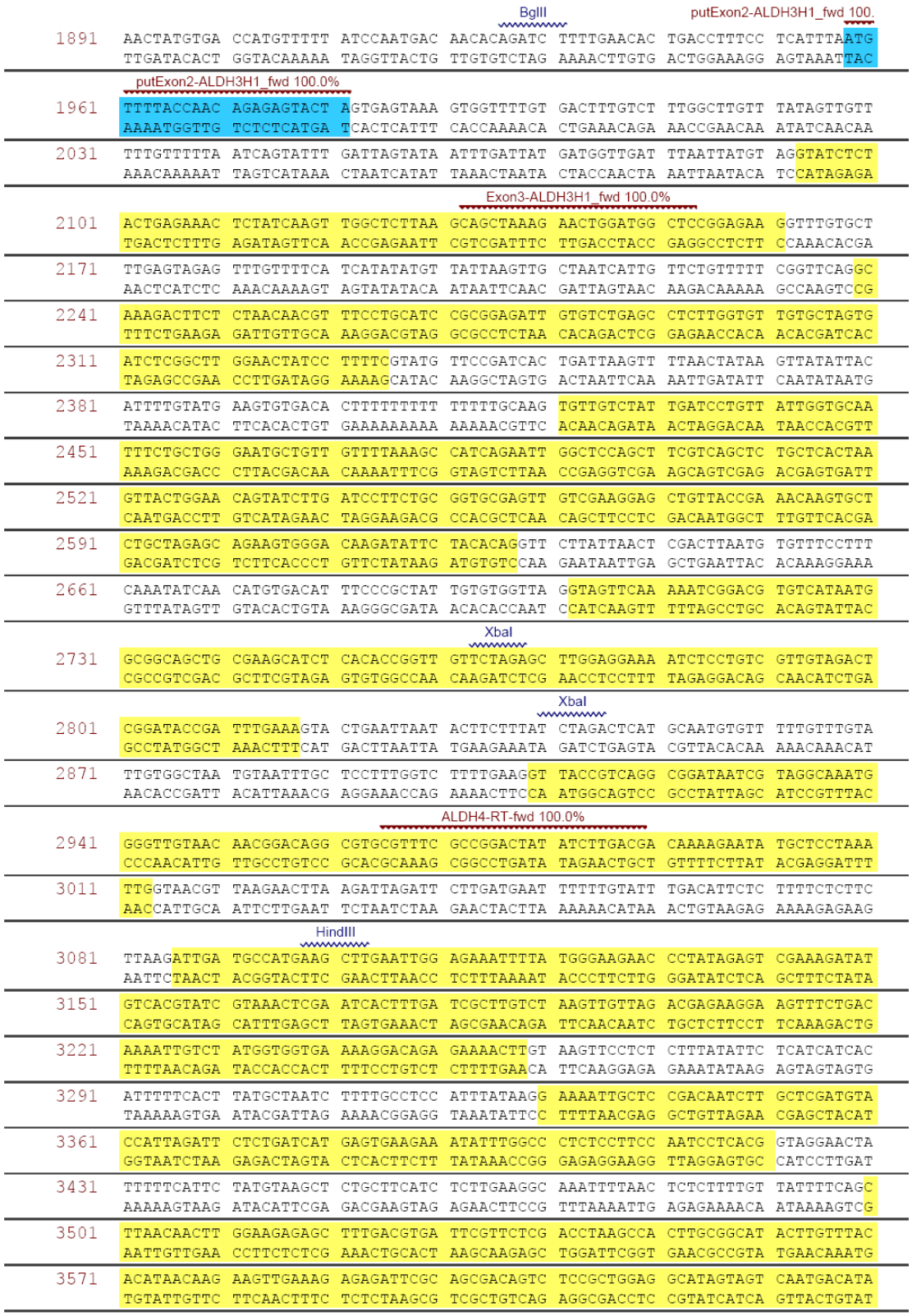

BglII
putExon2-ALDH3H1_fwd 100.
putExon2-ALDH3H1_fwd 100.0%
Exon3-ALDH3H1_fwd 100.0%
XbaI
XbaI
ALDH4-RT-fwd 100.0%
HindIII

```
3641  GCTGTTCATG TAAGAAAGAC ACTAGCTAAA ATACATTGCA CTAGTAGCAT TTTATCATTA CACAAAAGCG
      CGACAAGTAC ATTCTTTCTG TGATCGATTT TATGTAACGT GATCATCGTA AAATAGTAAT GTGTTTTCGC

3711  TTATCATCAT CCGATATTTT GCAGCTTGCA CTTCACACAT TGCCATTCGG AGGAGTTGGT GAAAGTGGAA
      AATAGTAGTA GGCTATAAAA CGTCGAACGT GAAGTGTGTA ACGGTAAGCC TCCTCAACCA CTTTCACCTT

                NcoI
3781  TGGGTGCTTA CCATGGTAAA TTCTCATTTG ATGCTTTTAG TCACAAGAAG GCGGTTCTCT ACAGAAGCCT
      ACCCACGAAT GGTACCATTT AAGAGTAAAC TACGAAAATC AGTGTTCTTC CGCCAAGAGA TGTCTTCGGA

                                        XhoI        HindIII
3851  TTTCGGTGAT TCAGCCGTCA GGTATCCGCC ATACTCGAGA GGAAAGCTTA GATTGTTAAA AGCCCTTGTC
      AAAGCCACTA AGTCGGCAGT CCATAGGCGG TATGAGCTCT CCTTTCGAAT CTAACAATTT TCGGGAACAG
                Ath-ALDH2-anti 100.0%

3921  GACAGCAATA TATTCGATTT ATTCAAAGTC CTTCTCGGTT TAGCTTAAAC GGTAAAAAGA CCGAGGACAC
      CTGTCGTTAT ATAAGCTAAA TAAGTTTCAG GAAGAGCCAA ATCGAATTTG CCATTTTTCT GGCTCCTGTG

3991  TTCCCTTTGT ACCTTATTTA CTTGTTTTAT TTTTCAAACA TGGACTTAGT TGGTTGATAT GTTTTGGTTT
      AAGGGAAACA TGGAATAAAT GAACAAAATA AAAAGTTTGT ACCTGAATCA ACCAACTATA CAAAACCAAA
                                        Ath-ALDH1c_anti 100.0%

4061  GGTGTTGATT CTATGAATAT TGAAGTTGAT AAATAAAAGA TTTTCGGTTG ATTATGGAAA CCGGTTTCTT
      CCACAACTAA GATACTTATA ACTTCAACTA TTTATTTTCT AAAAGCCAAC TAATACCTTT GGCCAAAGAA

4131  CATTCCAACT TGGTTGTTTA CAAAACAAAG AAGACACAAA AGAGTAAGAA TCTCTTTGAT TTTTTTATC
      GTAAGGTTGA ACCAACAAAT GTTTTGTTTC TTCTGTGTTT TCTCATTCTT AGAGAAACTA AAAAAAATAG

4201  TAAACCATGT TGTTAAAGCT CCCCTATAAT GGGAAAGAAA ATCTGTAGCA ACAAA
      ATTTGGTACA ACAATTTCGA GGGGATATTA CCCTTTCTTT TAGACATCGT TGTTT
```

5.2.4 *ALDH7B4* gene promoter sequence

The promoter sequence is shown here with some putative *cis*-elements. The nucleotide pair T-A in bold red was mutated in C-G so as to introduce an *Eco*RI site with the primer ALDH7B4 prom 3'. The resulting *Eco*RI/*Eco*RI region was used as the promoter. Region in black bold indicates the intergenic region between ALDH7B4 (AT1G54100) and the 5'-adjacent gene.

Red-shaded: MYB recognition site (WAACCA; W=A/T)

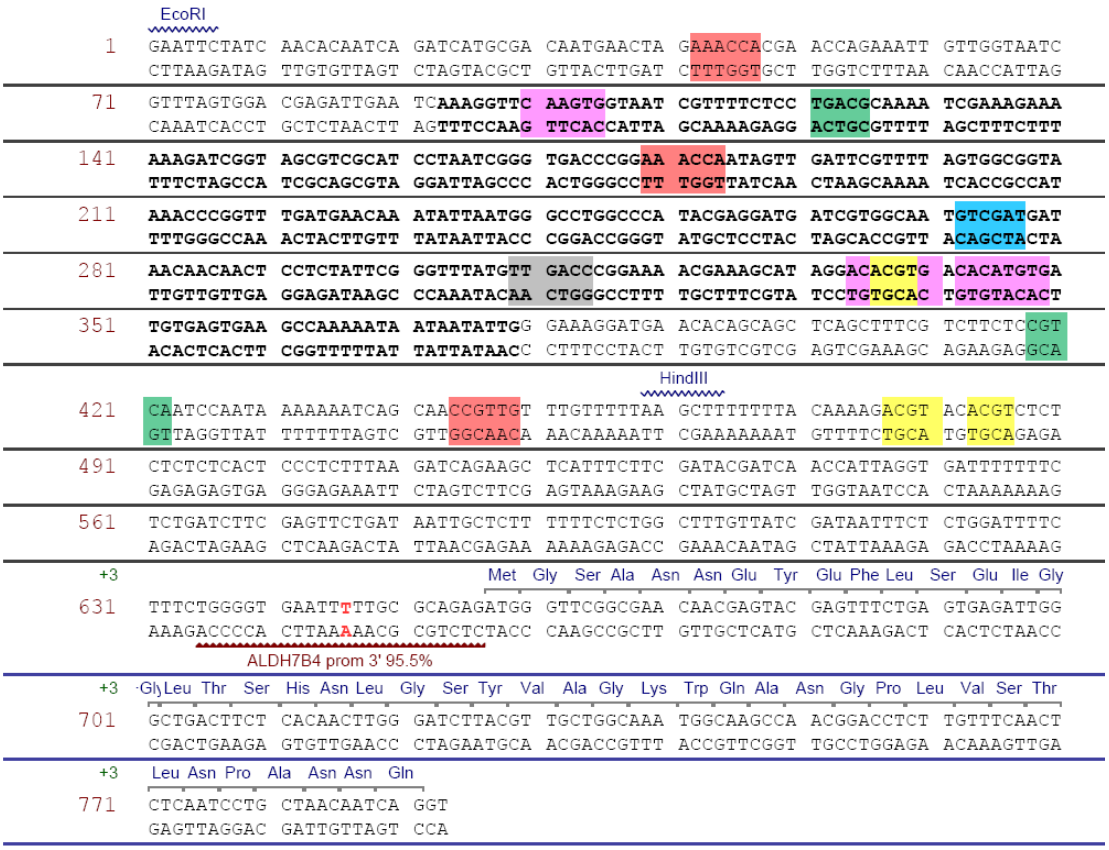
Pink-shaded: MYC recognition site (CANNTG; N=A/T/G/C)

Teal-shade: putative ASF-1 binding site

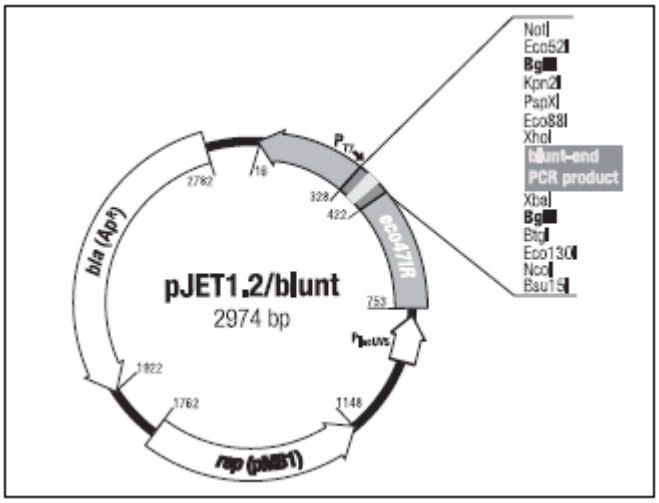
Turquoise-shaded: Putative DRE (RYCGAC; R=A/G; Y=C/T)

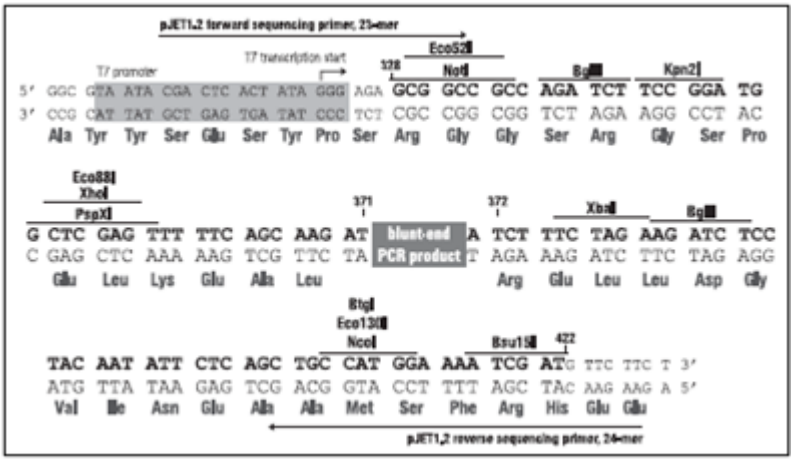
Gray-shaded: Elicitor Responsive Element (Core of W1 and W2 boxes)

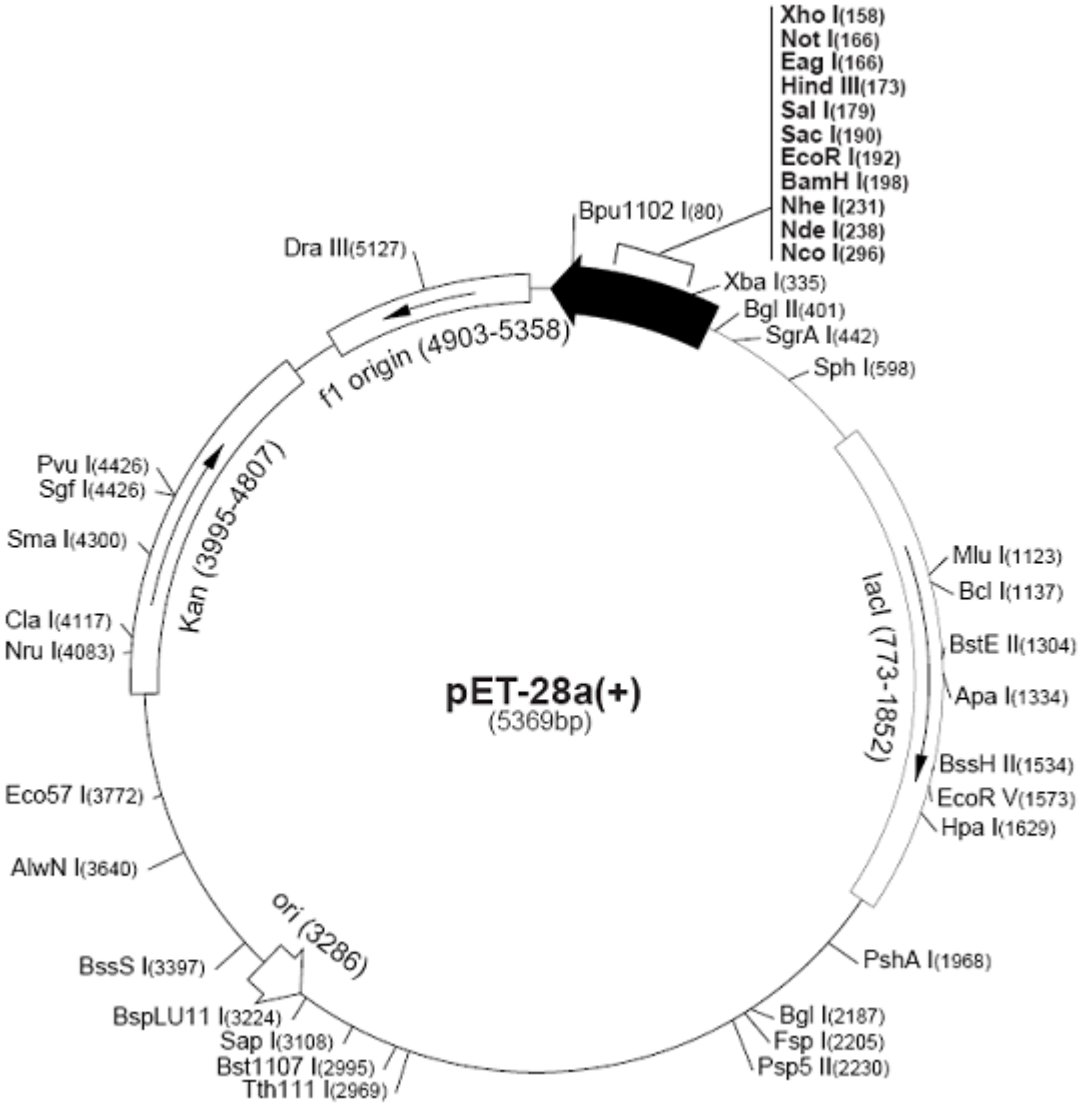
Yellow-shaded: ACGT-Box

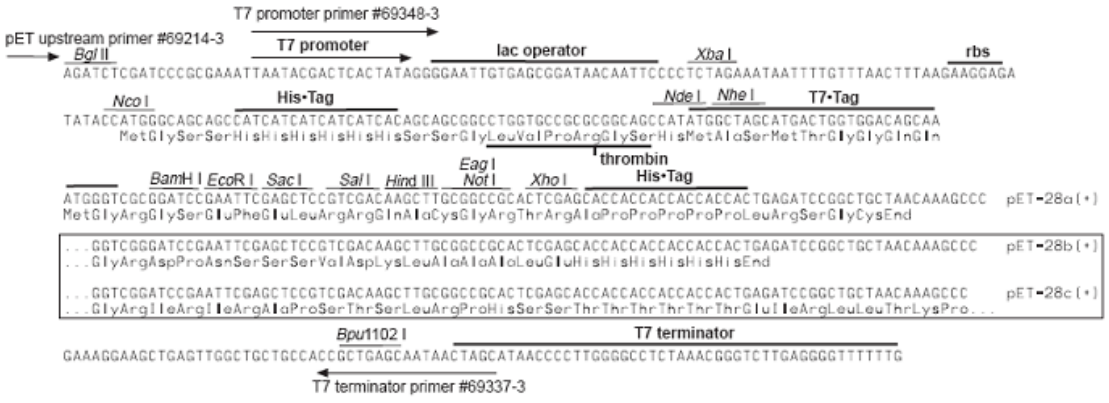


5.3 Vector maps

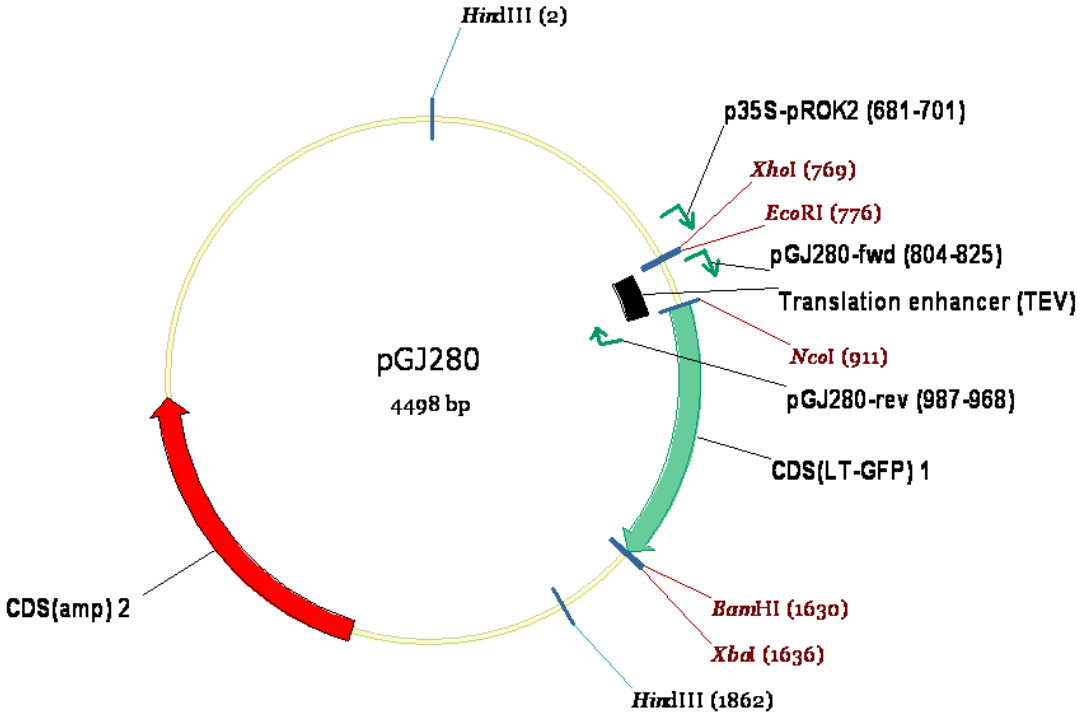








pET-28a-c(+) cloning/expression region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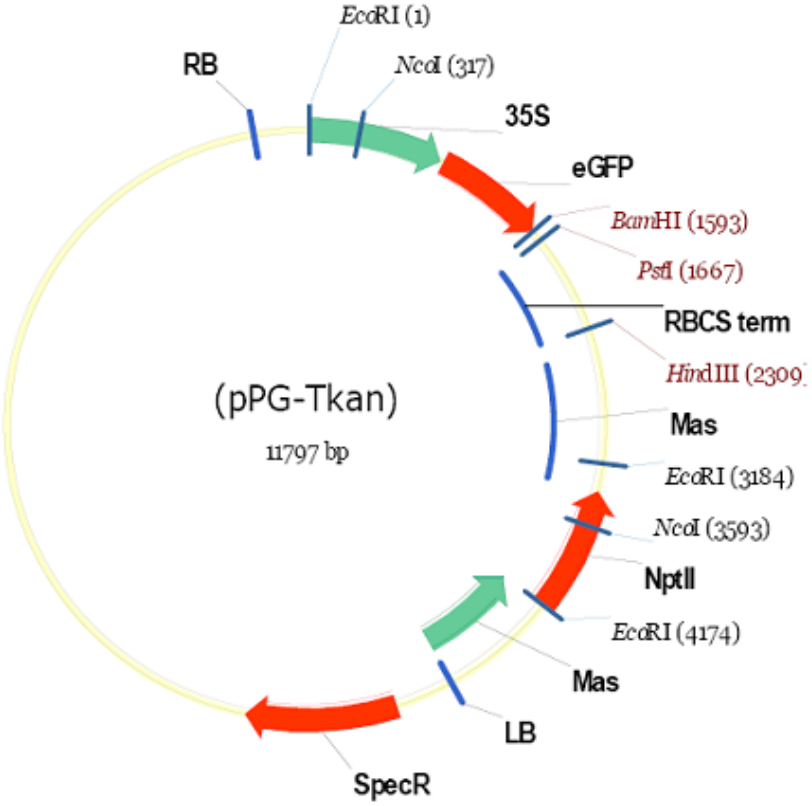
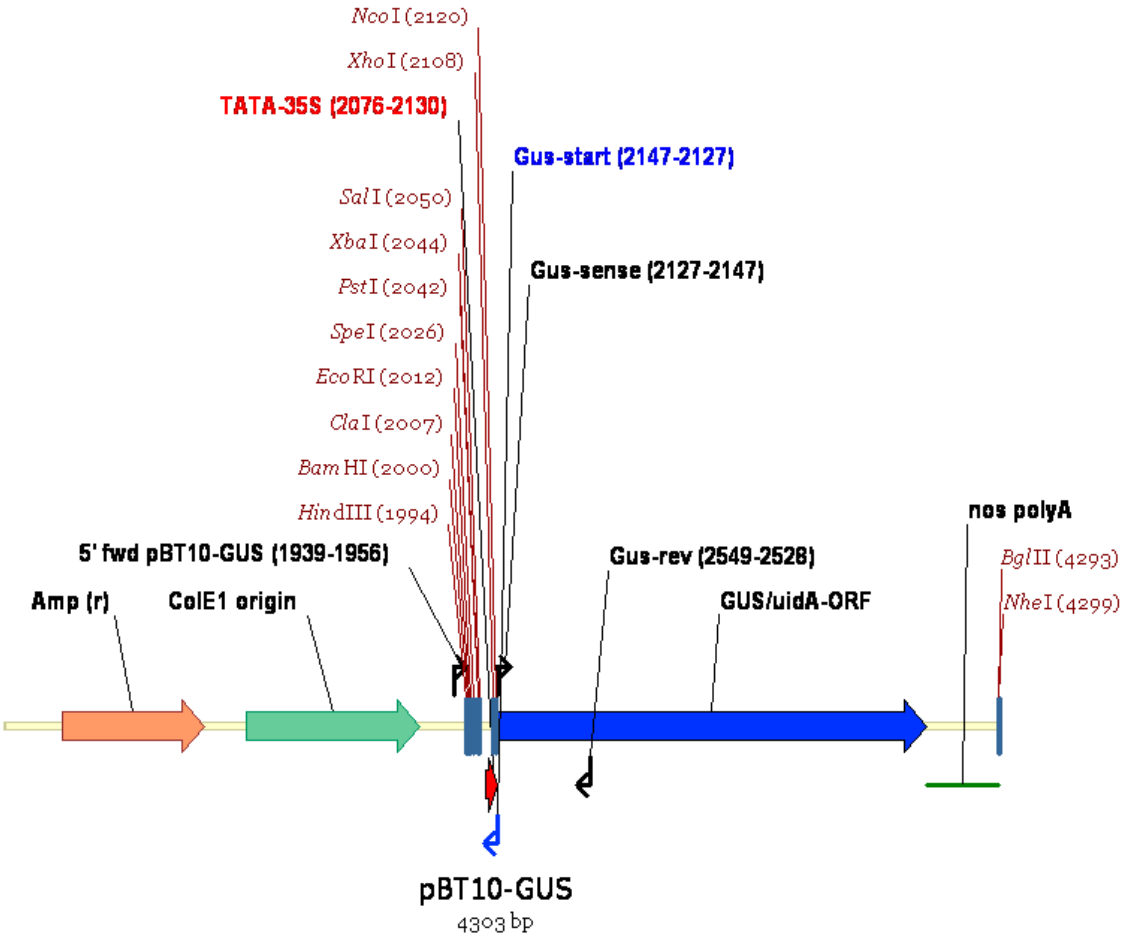
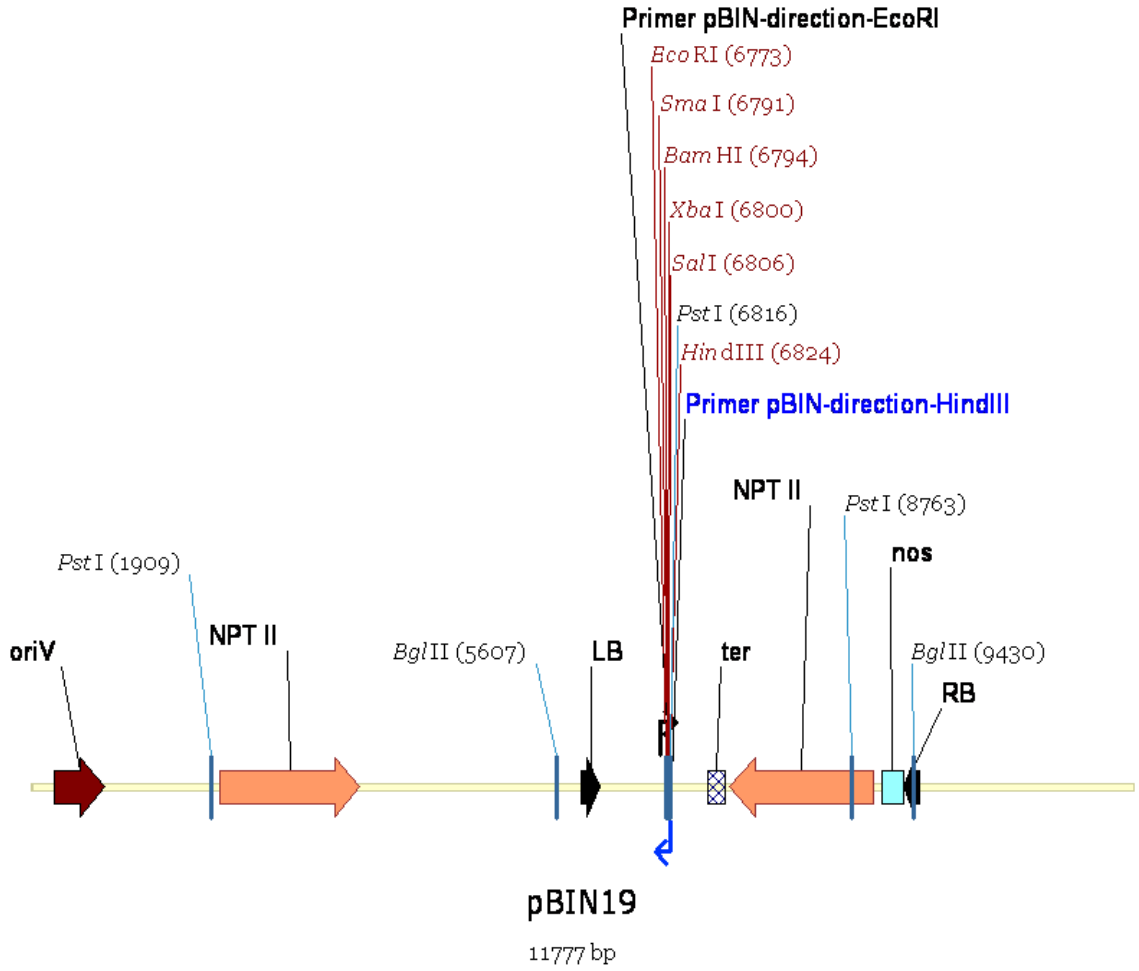
p35S-pROK2 (681-701)
XhoI (769)
EcoRI (776)
pGJ280-fwd (804-825)
Translation enhancer (TEV)
NcoI (911)
pGJ280-rev (987-968)
pGJ280
4498 bp
CDS(LT-GFP) 1
CDS(amp) 2
BamHI (1630)
XbaI (1636)
HindIII (1862)


EcoRI (1)
RB
NcoI (317)
35S
eGFP
BamHI (1593)
PstI (1667)
RBCS term
HindIII (2309)
(pPG-Tkan)
11797 bp
Mas
EcoRI (3184)
NcoI (3593)
NptII
EcoRI (4174)
Mas
LB
SpecR


NcoI (2120)
XhoI (2108)
TATA-35S (2076-2130)
Gus-start (2147-2127)
SalI (2050)
XbaI (2044)
Gus-sense (2127-2147)
PstI (2042)
SpeI (2026)
EcoRI (2012)
ClaI (2007)
BamHI (2000)
HindIII (1994)
nos polyA
5' fwd pBT10-GUS (1939-1956)
Gus-rev (2549-2528)
BglII (4293)
Amp (r)
ColE1 origin
GUS/uidA-ORF
NheI (4299)
pBT10-GUS
4303 bp


Primer pBIN-direction-EcoRI
EcoRI (6773)
SmaI (6791)
BamHI (6794)
XbaI (6800)
SalI (6806)
PstI (6816)
HindIII (6824)
Primer pBIN-direction-HindIII
NPT II
PstI (8763)
nos
PstI (1909)
oriV
NPT II
BglII (5607)
LB
ter
BglII (9430)
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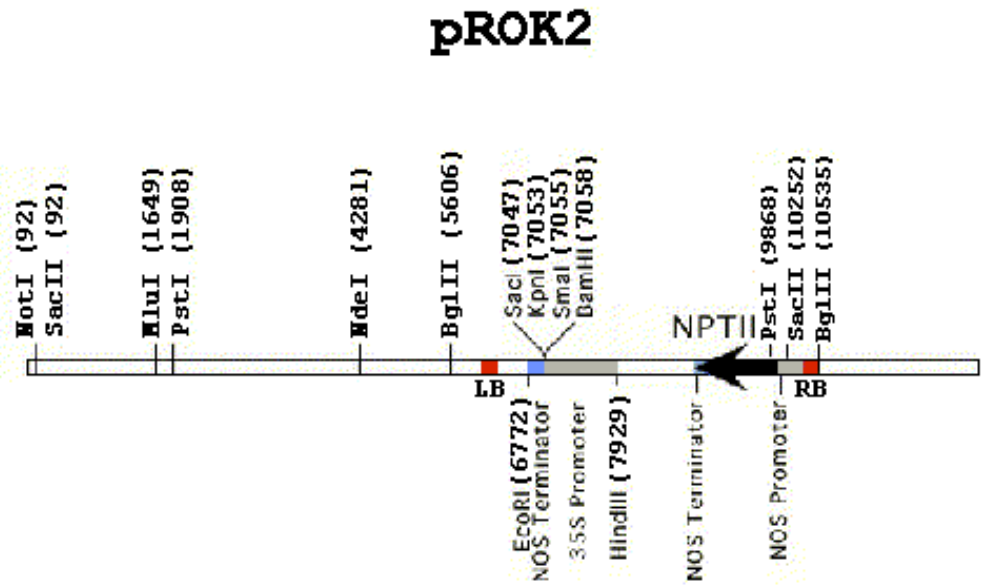
pROK2
NotI (92)
SacII (92)
MluI (1649)
PstI (1908)
NdeI (4281)
BglII (5606)
SacI (7047)
KpnI (7053)
SmaI (7055)
BamHI (7058)
NPTII
PstI (9868)
SacII (10252)
BglII (10535)
LB
RB
EcoRI (6772)
NOS Terminator
35S Promoter
HindIII (7929)
NOS Terminator
NOS Promoter

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8. MEETING AND CONFERENCES ATTENDED WITH POSTER PRESENTATIONS

- 7th Tri-National Arabidopsis Meeting, September 15-18th 2010, Salzburg, Austria.
- The 3rd International Conference (InterDrought) on Integrated Approaches to Improve Crop Production Under Drought Prone Environments, October 11-16th 2009, Shanghai, China.
- 6th Tri-National Arabidopsis Meeting, September 16-19th 2009, Cologne, Germany.
- 5th Tri-National Arabidopsis Meeting, September 10-13th 2008, Zürich, Switzerland.

9. LIST OF PUBLICATIONS

Publications from the PhD thesis:

Missihoun DT, Schmitz J, Klug R, Kirch H-H, Bartels D (2011) Betaine aldehyde dehydrogenase genes from *Arabidopsis* with different sub-cellular localization affect stress responses. Planta 233:369–382.

Other publications:

1. Ahoyo AT, Baba-Moussa L, Anago AE, Avogbe P, Missihoun TD, Loko F, Prévost G, Sanni A, Dramane K (2007) Incidence of infections dues to *Escherichia coli* strains producing extended spectrum betalactamase, in the Zou/Collines Hospital Centre (CHDZ/C) in Benin. Médecine et Maladies Infectieuses 37:746–752.

2. Baba-Moussa L, Akele-Apko MT, Adjobimey T, Missihoun TD, Sanni A (2006) What Do The Inflammatory Cervical Smears Hide? A Comparison Study Of Cytopathology And Pcr In Cotonou. Revue Africaine de Pathologie 5(1):21–26.

3. T. Missihoun, T. Adjobimey, I. Edaye, S. Abley, G. Gbotosho, C. Happi, A. Oduola, A. Sanni, 2005. *Plasmodium falciparum* genetic diversity in children with severe and acute uncomplicated malaria during the raining season in Benin Republic. In: *Abstracts from the 4th MIM Pan-African Malaria Conference, Yaoundé 13-18 November 2005*. Acta Tropica, Suppl. 95S**,** ISSN 0001-706X, S281.