Molecular and physiological characterization of transgenic *Arabidopsis* plants expressing different aldehyde dehydrogenase (*ALDH*) genes.

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Bonn, Oktober 2004 Oloni Simeon Kotchoni

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ABBREVIATIONS

ABA	Abscisic acid
ALDH	Aldehyde dehydrogenase
Amp	Ampicillin
APS	Ammonium persulfate
A-th	*Arabidopsis thaliana*
BSA	Bovine serum albumin
Cp	*Craterostigma plantagineum*
CTAB	Cetyltrimethylammoniumbromide
Da	Dalton
DAB	3,3'-diaminobenzidine
DATP	Desoxy-adenosin-triphosphate
DCTP	Desoxy-cytidin-triphosphate
DGTP	Desoxy-guanosin-triphosphate
DMF	Dimethylformamid
dNTPs	Desoxy-nucleootide triphosphate
dTTP	Desoxy-thymidin-triphosphate
EDTA	Ethylenediaminetetraacetate
GST	Gluthation-S-transferase
GUS	E. coli â-glucuronidase gene
HEPES	4-(2-Hydroxyethyl)-1-piperazinethansulfonic acid
HNE	4-hydroxy-trans-2-nonenal
IPTG	Isopropyl-â-D-thiogalactopyranoside
Kana	Kanamycin
MCS	Multiple cloning site
MOPS	3-(N-morpholino) propanesulfonic acid
4 –MUG	4 –Methylumbelliferyl glucuronide
NADH	Nicotinamide adenine dinucleotide

NADPH	Nicotinamide adenine dinucleotide phosphate
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PIPES	Piperazine-N,N,-bis (2-ethanesulfonic acid)
PVP	Polyvinylpyrrolidon
Rif	Rifampicin
RNase	Ribonuclease
RT	Room temperature
SDS	Sodium dodecyl sulfate
SSC	Saline sodium citrate buffer
TAE	Tris-acetate-EDTA
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Tris-(hydroxymethyl)-aminomethane
Triton X-100	Poly(ethylenglycolether)$_n$-octylphenol
X-Gluc	5-bromo-4-chloro-3-indolyl-â-D-glucuronide

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SUMMARY

Various reactive molecules such as aldehydes and their intermediates accumulate in plants exposed to environmental stress conditions. These molecules are highly toxic and can cause peroxidation of cellular lipids, protein and nucleic acid modifications. Due to the potential cytotoxicity of these molecules in living cells, various aldehyde dehydrogenase (ALDH) proteins are involved in maintaining a careful balance of cellular accumulation of the toxic aldehyde molecules by converting them into their non-toxic corresponding carboxylic acids. To investigate the biological role of plant-ALDHs and their involvement in abiotic stress tolerance mechanisms, several transgenic *Arabidopsis* plants containing different *Arabidopsis*- and *Craterostigma-ALDH*-cDNA constructs have been generated and characterized under various abiotic stress conditions. Cellular and tissue specific localization of *ALDH* gene expression *via GUS* reporter gene fusion showed that *ALDH3I1*, *ALDH3H1* and *Cp-ALDH* are stress inducible genes. The experiments also revealed that *ALDH3I1* and *Cp-ALDH* expression is leaf specific, while the stress-inducible expression of *ALDH3H1* is restricted to roots. Immunological experiments showed that ALDH3I1 protein accumulations were triggered by ABA, paraquat (methyl viologen, a chemical that induces oxidative stress), and H_2O_2 treatment, indicating that the signal transduction leading to *ALDH* gene expression is responsive to ABA and reactive oxygen species (H_2O_2). The overexpression of *ALDH* genes controls in return the excessive accumulation of ROS, which occurs as a result of environmental stress. This confers thereby an enhanced tolerance to stress. Molecular and biochemical characterizations of selected transgenic plants exposed to stress treatments revealed that transgenic plants overexpressiong the *ALDH* genes showed significant tolerance to a

wide range of abiotic stress conditions especially dehydration, salt stress (NaCl, KCl), heavy metal toxicity (copper and cadmium) and low temperature exposure in comparison to the wild-type plants. The loss of *ALDH* gene functions or a repression of endogenous *ALDH* gene expression in kock-out and antisense transgenic plants respectively correlates with sensitivity to various abiotic stress treatments. The overexpression of *ALDH* genes was found to significantly reduce the level of lipid peroxidation, and the amounts of reactive oxygen species (H_2O_2, O_2^-) in plants exposed to dehydration and salt stress conditions. These findings suggest that aldehyde dehydrogenase genes play a crucial role in aldehyde detoxification and antioxidant systems of plants exposed to abiotic stress conditions. Understanding the regulatory mechanisms of *ALDH* gene expressions in plants could prove to be a promising way to generate transgenic plants that can cope with multiple abiotic and even biotic stress conditions.

1. INTRODUCTION

The adaptation of plants to environmental stress has been widely studied in a number of plant species (Holmstrom et al 2000, Zhang et al 2003, Zhu et al 2003). Major research efforts have been focused on the isolation of stress-inducible genes as a means to understand the molecular and physiological events underlying the adaptation process in plants exposed to stress (Ingram and Bartels 1996, Shinozaki and Yamaguchi-Shinozaki 2000, Ramanjulu and Bartels 2002, Seki et al 2003, Shinozaki and Dennis 2003, Kirch et al 2004). Availability of water is one of the most important and determinant factors for geographical distribution and plant productivity (Bartels 2001a). Living organisms are exposed to different kinds of stressors, which include pathogen attacks, air pollution, drought, salt stress, temperature, light intensity, and nutritional limitation. Since plants have limited mechanisms to avoid stress, they require flexible means of adaptation to constantly changing environmental conditions (Arora et al 2002). The response and adaptation of plants to these stresses is however very complex and highly variable (Ingram and Bartels 1996). This includes generally the expression of specific sets of genes, structural changes of membranes, changes in metabolic processes, production of secondary metabolites, repression of some active genes and expression of various stress-regulatory factors (Ramanjulu and Bartels 2002, Shinozaki et al 2003, He and Gan 2004). In extreme environmental constraints, plants undergo cellular damages despite all adaptive machineries used to cope with the stress conditions. Long term external stimuli lead generally to the production of reactive oxygen species (ROS) such as hydroxyl radicals, singlet oxygen, superoxides and hydrogen peroxide (Bartels 2001b, Kotchoni and Bartels 2003), and various other by-products, which include reactive aldehydes

molecules (Kirch et al 2001, Kotchoni and Bartels 2003). These molecules are highly toxic and can easily attack cellular macromolecules such as nucleic acids, proteins, carbohydrates and phospholipids (Skibbe et al 2002, Sunkar et al 2003). Therefore regulating the accumulation of ROS and removing the production of aldehydes and their intermediates in plants exposed to environmental stress is essential for cell viability.

Aldehyde dehydrogenases (ALDH, EC 1.2.1.3) have been reported as a group of enzymes that play a crucial role in stress relevant detoxification of aldehydes produced as result of lipid peroxidations, and these enzymes are widely characterized in humans and animals (Lindahl 1992, Yoshida et al 1998). In contrast, ALDH characterizations in plants have been relatively limited. Here, physiological and molecular studies were carried out to characterize plant-ALDHs in response to abiotic stress and to gain insight into processes by which their activities limit cellular damage caused by toxic aldehydes. This work reveals that manipulating *ALDH* genes in plants could be a promising way to generate transgenic plants that can cope with a wide range of environmental stressors.

1.1. Water deficit and salt stress: two abiotic stresses with common detrimental effects in plants

Water deficit and salt stress represent one of the most complex physiological phenomena that limit plant growth and productivity (Bartels 2001b) by imposing osmotic stress (alteration of osmotic potential) to plant tissues. The most detrimental effect of high amounts of salt in the soil is the disruption of water uptake by the plants. Generally, plants take up water from soil through roots in the presence of an osmotic potential slope between soil and plant. Osmotic stress causes an alteration in extracellular solute

concentrations and a subsequent flux of water from the plant cells (Bohnert et al 1995). The loss of water from the cells causes a decrease in turgor and an increase in concentrations of intracellular solutes, which subsequently put a strain on membranes and macromolecules. Increasing salt concentrations in soil therefore leads to water deficit and associated detrimental effects in plants (accumulation of several toxic solutes such as chlorine, aldehyde molecules, excess sodium ions, and deactivation of enzymes) as a result of a decline of the osmotic potential difference between plant extra- and intracellular compartments (Ramanjulu and Bartels 2002). In addition, severe water deficit negatively affects photosynthetic reactions (Kaiser 1979). In such conditions, the chloroplasts are generally exposed to excess excitation energy leading to the production of toxic substances such as superoxides, peroxides generally known as reactive oxygen species (ROS) and toxic aldehyde molecules, which damage membranes, enzymes and macromolecules (Kaiser 1979, Sunkar et al 2003).

Presently around 18 % of global farmland suffers of water deficit, and farming in those lands is possible only under irrigation systems (Somerville and Briscoe 2001). Up to 40 % of the global food supply is produced from the irrigated farmlands (Somerville and Briscoe 2001). However, irrigated lands in arid regions are susceptible to salinisation. Naturally occurring salinisation is mainly a consequence of capillary water level elevation and subsequent evaporation of saline groundwater (salt remains in the soil). Salinisation phenomenon therefore results from irrigation water practice, which generally contains higher salt concentrations in comparison with the rain-water (Somerville and Briscoe 2001). A progressive accumulation of water-soluble salt, especially sodium chloride (NaCl), sodium carbonate (Na_2CO_3) and calcium chloride ($CaCl_2$) from arid

irrigated lands leads irreversibly to saline soils. In addition to these, there are several other adverse downstream effects associated with salt and drought stress. They lead to an increased pH-level of the soil, and crop plants generally fail to grow under high pH growth conditions. Furthermore, salinity causes degradation of the soil structure, leading to soil surface pudding and therefore negatively affecting soil-gas exchange (Somerville and Briscoe 2001). It is clear that arable lands may be irreversibly lost through the detrimental effects of salt and drought stress if care is not taken (Bartels 2001a).

1.2. Responses of plants to environmental stress

Plants live in an environment in which they must acclimatize in order to ascertain their viability and perpetuation. Environmental stress is perceived as an external factor imposing detrimental growth conditions to plants during their life cycle (Kim at al 2003). For plants, environmental stress is grouped into two stress categories; biotic stress (pathogen attack) and abiotic stress. The latter includes water deficit, heavy metal pollution, chilling and freezing, heat stress, UV irradiation, salinity, oxygen deficiency and nutrient deficiency (Yamaguchi and Kamiya 2001). Abiotic stress is often difficult to tackle, because the different developmental stages of the plant (seed germination, seedling development, seed maturation and senescence) are differentially affected by the stress conditions (Ingram and Bartels 1996, Kermode 1997). For instance, drought or desiccation tolerance is part of the normal developmental cycle in higher plants especially during seed maturation and seed dormancy (Ingram and Bartels 1996, Koornneef et al 2002, He and Gan 2004). Under normal growth conditions, most of the up-regulated genes during drought stress are induced towards the final stage of seed maturation i.e.

when the embryo reaches its lowest water content, and during seed dormancy. This stage of plant development enables the embryo to survive adverse environmental conditions (Baskin and Baskin 1998, Ramanjulu and Bartels 2002). Seed dormancy is believed to be an evolutionary survival strategy because the end of the reproductive growth of parental plants is often the beginning of an extended period of unfavourable environmental conditions (Baskin and Baskin 1998, He and Gan 2004). However, water deficit for instance is perhaps one of the most prevalent causes of crop yield loss because of the strong link between transpiration and photosynthesis.

Plants must however maintain their cellular water status in a normal homeostasis in order to survive adverse conditions. As already mentioned, plants use various morphological and physiological strategies to cope with the stress conditions. Morphological approaches of plant responses to abiotic stress (water deficit) include: development of deep root systems, stomatal closure, reduction of surface proportionally to the entire volume of the plant by dropping-off of leaves and retention of water molecules in specific water tissues especially in succulent plants (Ingram and Bartels 1996, Holmstrom et al 2000). The physiological strategies of plant adaptation to stress include: ion exclusion, ion transport and cell wall modification by an increased flexibility, osmotic adjustments and osmoprotection (Ingram and Bartels 1996, Pastori and Foyer 2002, Yamaguchi-Shinozaki et al 2002). Osmotic adjustment is a physiologically efficient mechanism by which plants produce osmoprotectants, therefore protecting cells by turgor maintenance of roots and shoots in response to water deficit as well as salt stress (Yamaguchi-Shinozaki et al 2002). Osmoprotectants such as proline, glycine betaine and sugars (mannitol, fructans, saccharose) are well documented and known to help plants to overcome the effects of

water deficit and salt stress (Bartels 2001b). In addition, plants accumulate specific proteins such as LEA proteins (late embryogenesis abundant proteins) and chaperones (heat-stress induced proteins) in response to abiotic stress (Ingram and Bartels 1996). LEA proteins and chaperones have been reported to be involved in protecting macromolecules like enzymes, lipids and mRNA (Ingram and Bartels 1996, Yamaguchi-Shinozaki et al 2002). LEA proteins accumulate mainly in embryos during seed desiccation and in response to water stress. The correlation between LEA gene expression and/or LEA protein accumulation and stress tolerance in a number of plant species provided evidence for the role of the LEA proteins in stress tolerance mechanisms (Ingram and Bartels 1996). Transgenic rice ectopically expressing a barley-HVA1 gene (LEA gene) shows a significantly increased tolerance to water deficit and salinity (Xu et al 1996). The expression of HVA1 gene caused a delayed development of damage symptoms in the transgenic rice when compared to the wild-type rice under stress conditions (Xu et al 1996).

On the other hand, abiotic stress leads to the production of reactive oxygen species (ROS), which cause extensive damage to cells and inhibit photosynthetic reactions (Kaiser 1979). The damaging phenomenon referred to as oxidative damage is the consequence of oxidative stress, the stress resulting from almost all abiotic stresses (Ramanjulu and Bartels 2002). Stressed plants increase the production of specific antioxidants, which are needed for the reduction of active oxygen species. These enzymes include ascorbate peroxidases, glutathione peroxidases and gluthatione reductase (Assada 1992, Mittler 2002). Recently, aldehyde dehydrogenases have been reported to display a probable antioxidative ability in *Arabidopsis thaliana* (Sunkar et al 2003). Molecular

studies have revealed that protein phosphorylation and dephosphorylation via kinases and phosphorylases are important signal transduction mechanisms used by plants to sense the external stimuli and to programme an adequate response based on extremely fine and highly coordinated regulatory system to express stress-related gene and/or repress several other set of genes whose functions disrupt the stress adaptation mechanism (Ingram and Bartels 1996, Kovtun et al 2000). Endogenous ABA levels have been reported to increase as a result of environmental stress and are therefore thought to be involved in signal transduction mediating the up regulation of several abiotic stress-inducible genes (Ramanjulu and Bartels 2002, He and Gan 2004). A genome-wide survey of gene expression in *Arabidopsis thaliana* revealed that about 1354 genes whose expression was either enhanced or suppressed after ABA treatment (Hoth et al 2002, He and Gan 2004) were related to abiotic stress tolerance mechanisms.

Here, particular attention has been focused on physiological and molecular analyses of *ALDH* gene expression and their corresponding proteins. Although the expression of several genes in many plant species responding to abiotic stress has been studied, little is known about the aldehyde dehydrogenase genes and their potential role in plants exposed to abiotic stress. To carry out such investigation, *Arabidopsis thaliana* has been selected as model taking advantage of the potent molecular and genetic tools available from this species. Its short life cycle (approximately 40 to 50 days) makes it possible to conveniently carry out several experimental trials within a short time period. This plant species is therefore an appropriate candidate for thorough experimental analyses to establish research findings, before they are extended to crop plants.

1.3. Aldehyde dehydrogenases: Importance in cellular metabolism

Aldehydes are long-lived molecules that can be generated from various endogenous sources (metabolism of amino acids, carbohydrates, vitamins and lipids) and exogenous sources such as abiotic and biotic stress (Sophos and Vasiliou 2003, Sunkar et al. 2003). Acetaldehyde, glyceraldehydes 3-phosphate, p-nitobenzaldehyde, glycolaldehyde, phenylacetaldehyde, malondialdehyde (MDA), succinic semialdehydes, propionaldehyde, 4-hydroxy-trans-2-nonenal (4-HNE or HNE) are the most frequently recorded molecules that are highly reactive and harmful to cells (Ting and Crabbe 1983, Trivic and Leskovac 1994). They cause genotoxicity i.e. chromosomal aberrations and DNA adducts (Comporti 1998), protein inhibition and biophysical changes of lipid membranes as illustrated in Figure 1 below showing the interaction of 4-HNE (aldehyde) with cellular molecules (Hu et al 2002). The generation of aldehydes either during normal cell metabolism or under external stimuli must be regulated in order to avoid cell developmental arrest. Aldehydes are generally oxidized into their corresponding non-toxic carboxylic acids by aldehyde dehydrogenases (ALDH, EC 1.2.1.3). A vast literature exists on human-ALDHs proving their function in detoxification pathways of cellular metabolisms (Yoshida et al 1998, Kikonyogo et al 1999, Ohsawa et al 2003). ALDHs are a family of $NAD(P)^+$-dependent enzymes with a common oxidative function (Kirch et al 2001). However, the subtle differences in their structure and arrangements of subunits allow them to be grouped into subfamilies (Vasiliou et al 1999, Sophos and Vasiliou 2003, for details see Nomenclature and classification of ALDHs).

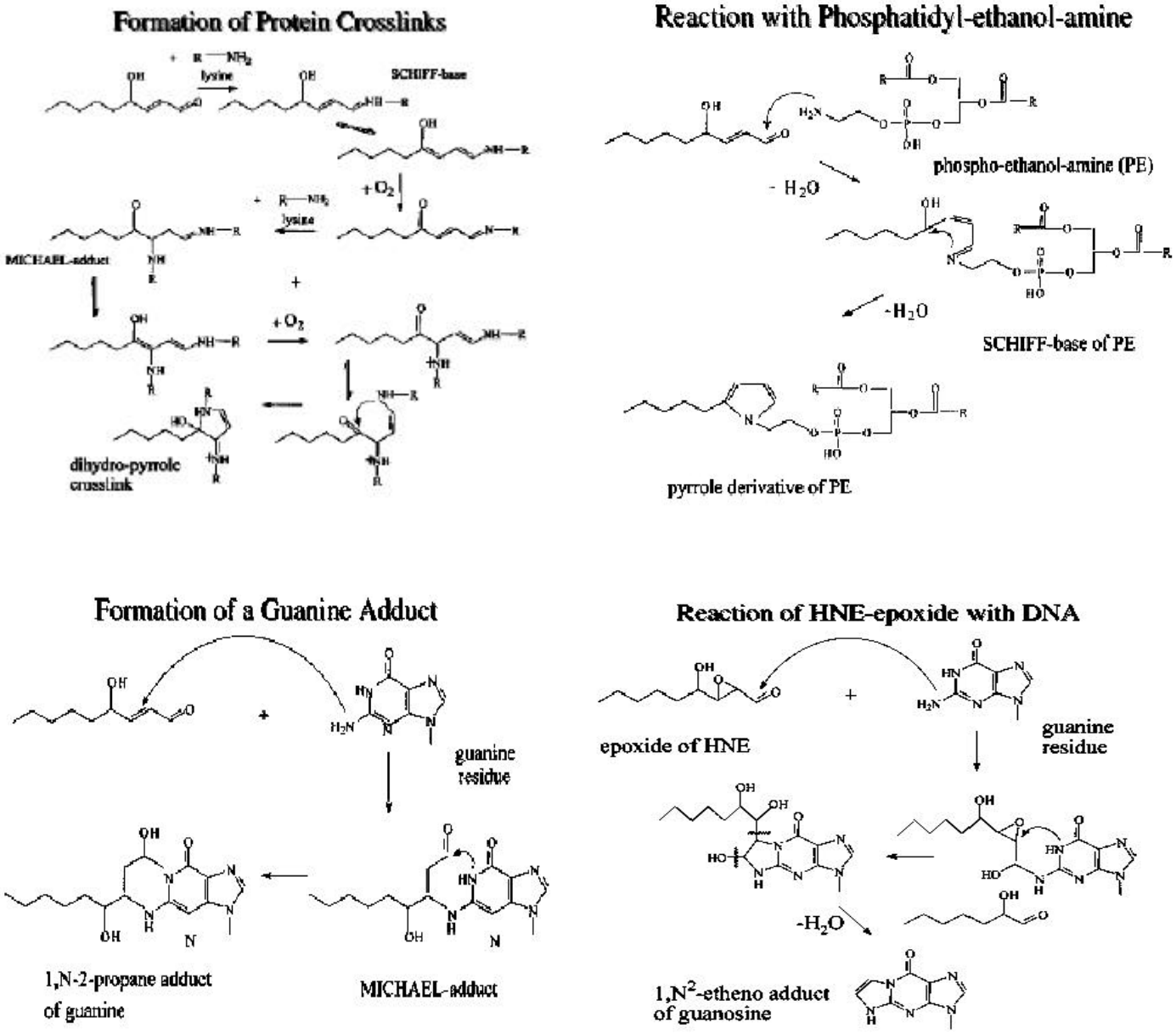


Figure 1: Interaction of reactive aldehydes (HNE) with macromolecules (protein, lipids and DNA) causing alteration of their structure and active sites.
Ref. Chen et al 1998, Guichardant et al 1998, Karlhuber et al 1997, Subramaniam et al 1997, Wacker et al 2001.

ALDHs are widely expressed in tissues and subcellular compartments and are important in cell defence against exogenous and endogenous aldehydes such as those derived from

lipid peroxidation (Lindahl and Petersen 1991, Vasiliou et al 1996). Cytosolic class 3 ALDH enzymes are reported to oxidize medium (6 to 9)-chain-length saturated and unsaturated aldehydes but not short chain aliphatic aldehydes, such as malondialdehyde and 4 –hydroxyalkenals (Lindahl and Petersen 1991), while class 2 *ALDHs* are largely expressed in cytosol and transported into the mitochondrial matrix space where they are mainly responsible for the oxidation of acetaldehyde generated during *in vivo* oxidation of ethanol coupled with energy production through the use of acetyl-CoA in the TCA cycle (op den Camp and Kuhlemeier 1997, Canuto et al 2001, Tsuji et al 2003). Apart from their aldehyde detoxification ability, ALDHs also contribute to the production of acetyl-CoA (metabolism of ethanol-derived acetaldehyde). During low oxygen conditions, pyruvate is directly converted into acetaldehyde by pyruvate decarboxylase (PDC). Acetaldehyde (a toxic molecule) is converted into acetate (a non-toxic molecule) by aldehyde dehydrogenase. Acetate is thereafter transformed into acetyl-CoA by acetyl-CoA synthetase (ACS) and supplied as a substrate for the TCA cycle (energy biosynthesis) (op den Camp and Kuhlemeier 1997, Tsuji et al 2003). Another possible role of ALDHs is to supply NAD(P)H for respiration. The supply of NAD(P)H by class 2 ALDHs during the oxidation of ethanol into acetyl-CoA represents a unique alternative way for cellular economy during biosynthetic reactions (Liorente and de Castro 1977). The oxidation of aldehydes into corresponding carboxylic acids by ALDHs concomitantly converts NAD^+ into NADH in the mitochondria (Kirch et al 2001). This irreversible oxidative reaction contributes to a rapid recovery of respiration and ATP synthesis. Aldehyde dehydrogenases are therefore considered crucial for cellular metabolism due to their multifunctional properties (Vasiliou et al 1996, Kirch et al 2001).

Canuto et al (1996) demonstrated that enhancement of lipid peroxidation by cell enrichment with arachidonic acid and treatment with pro-oxidants inhibit the effect of class 3 ALDH due to a probable decrease of class 3 *ALDH* gene transcripts. When such cell treatment resulted in the complete inhibition of the class 3 ALDH protein synthesis, cell death followed. However, class 3 ALDHs oxidize relatively small amounts of saturated and unsaturated aldehyde molecules. A large number of the other unsaturated, saturated and hydroxylated aldehydes generated during peroxidation of cellular lipids are therefore oxidised by different ranges of other ALDHs that are equally important in cellular metabolism. In 1996, the first gene encoding a plant mitochondrial ALDH, the restorer gene of fertility 2 gene (rf2), was identified in maize (Cui et al 1996). Subsequently, two other *ALDH* genes (*Aldh2a* and *Aldh2b*) were identified in tobacco; *Aldh2a* transcript and Aldh2a protein were found to be present at high levels in floral tissues, especially stamens, pistils and pollen (op den Camp and Kuhlemeier 1997) indicating the importance of ALDH activity not only in the detoxification process but also in general metabolism of cells. In addition, the biosynthesis and accumulation of glycine betaine has been reported in various plant species in response to salinity and drought (Kishitani et al 2000, Nakamura et al 2001). Higher plants synthesize glycine betaine via a two-step oxidation of choline (Nakamura et al. 2001). In the last step of glycine betaine biosynthesis, betaine aldehyde is catalysed into glycine betaine by betaine aldehyde dehydrogenase (BADH). Glycine betaine acts as non-toxic osmolyte in the cytoplasm and probably plays its osmoprotective role against detrimental effects of drought and salt stress by turgor maintenance during osmotic tress (Holmstrom et al 2000, Nakamura et al 2001, Zhu et al 2003).

1.4. Aldehyde dehydrogenases: Nomenclature and classification

Presently more than 500 independent *ALDH* genes with detected ALDH protein or protein-like activity have been identified (Sophos and Vasiliou 2003). A nomenclature for ALDH proteins has been established by the *ALDH* Gene Nomenclature Committee (www.uchsc.edu/sp/sp/alcdbase/aldhcov.html). The nomenclature is based on relatively simple criteria (Vasiliou et al 1999); in which the *ALDH* genes are grouped in families and each family is represented by the root symbol (*ALDH*) followed by a number (family number), a capital letter indicating the sub-family, which is followed by a number identifying the individual gene as illustrated in Table 1 (for details see Kirch et al 2004). The ALDH superfamily is furthermore categorized on the basis of their substrate specificity. Based on this, some ALDHs are known as non-specific ALDHs. Non-specific ALDHs react with a wide range of substrates (aliphatic and/or aromatic aldehydes) and include the tetrameric class 1 and 2 ALDHs (cytosolic and mitochondrial) and dimeric class 3 ALDHs (Yoshida et al 1998). Some other ALDHs are known as substrate specific ALDHs and these include all the semialdehyde dehydrogenases (SemiALDHs), betaine aldehyde dehydrogenase (BADH), while others are grouped as *ALDH*-like genes as described in table 1, which summarizes some plant-*ALDH* genes and their putative functions.

1.5. Plant-*ALDH* gene expression and its relationship to abiotic stress

Plants respond to various stressors by expressing specific sets of genes. Accumulation of several *ALDH* gene transcripts have been reported in various plant species exposed to

abiotic stress. However, many of these genes code for proteins with unknown functions in the mechanisms of plant adaptation to abiotic stress. Recently, *ALDH3H1* (*Ath-ALDH4*) and *ALDH3I1* (*Ath-ALDH3*) encoding two novel aldehyde dehydrogenases belonging to non-specific class 3 ALDHs have been reported from *A. thaliana* (Kirch et al 2001) and overexpression of *ALDH3I1* in transgenic plants improves tolerance to multiple environmental stresses (Sunkar et al 2003). Table 1 shows *ALDH* genes whose transcripts were detected under various abiotic stressors in plants. The BADH genes are the most widely characterized (Table 1) probably due to their ability to code for a substrate specific ALDH protein (BADH protein) whose activity produces glycine betaine a potential osmoprotectant allowing normal metabolic functions to continue in cells under osmotic stress (Weretilnyk and Hanson 1990, Holmstrom et al 2000, Zhu et al 2003). These findings have drawn attention on the glycine betaine synthesis pathway and their corresponding genes to produce transgenic plants resistant to osmotic stress. Nonetheless, our current knowledge of several other ALDH protein activities to trigger abiotic stress tolerance in plants is still limited.

Table 1: Plant-*ALDH* gene families with functions likely to be related to environmental stress tolerance.

Classification	ALDH nomenclature	Putative function (former name)	Source	Subscellular localization
Nonspecific ALDHs	ALDH2B3	NaD^+-Aldehyde dehydrogenase (ALDH2b)	*A. thaliana*	Mitochondria
	ALDH2B4	Aldehyde dehydrogenase (ALDH2a)	*A. thaliana*	Mitochondria
	ALDH2C4	Aldehyde dehydrogenase (ALDH1a)	*A. thaliana*	Cytosol
	ALDH3F1	Aldehyde dehydrogenase (ALDH5)	*A. thaliana*	Nd
	ALDH3H1	Aldehyde dehydrogenase (ALDH4)	*A. thaliana*	Chloroplast
	ALDH3I1	Aldehyde dehydrogenase (ALDH3)	*A. thaliana*	Chloroplast
	ALDH7B4	Turgor-ALDH (ALDH6)	*A. thaliana*	Nd
	ALDH22A1	Putative Aldehyde dehydrogenase (ALDH7)	*A. thaliana*	Secretory pathway
Substrate specific ALDH	ALDH5F1	Succinate semiALDH (SSALDH1)	*A. thaliana*	Mitochondria
	ALDH6B2	Methylmalonate semiALDH(MMSALDH)	*A. thaliana*	Mitochondria
	ALDH10A1	Betaine ALDH (SBADH)	*Sorghum bicolor*	Nd
	ALDH10A2	Betaine ALDH (BADH)	*Beta vulgaris*	Nd
	ALDH10A3	Betaine ALDH (BADH)	*Atriplex hortensis*	Nd
	ALDH10A4	Betaine ALDH (BADH)	*Amaranthus hypochondriacus*	Nd
	ALDH10A6	Betaine ALDH (BADH)	*Hordeum vulgare*	Nd
	ALDH10A7	Betaine ALDH (BADH)	*Spinacia oleracea*	Nd
	ALDH10A8	Putative Betaine ALDH (putBADH)	*A. thaliana*	Nd
	ALDH10A9	Betaine ALDH (BADH)	*A. thaliana*	Mitochondria
	ALDH11A3	Glyceraldehyde 3-P dehydrogenase (GAPDH)	*A. thaliana*	Nd
	ALDH11	Glyceraldehyde 3-P dehydrogenase (GrapC-Crat)	*C. plantagineum*	Cytosol
ALDH-like proteins	ALDH7B1	Turgor-ALDH-like protein (PSCC26G)	*Pisum sativum*	Nd
	ALDH7B3	Turgor-ALDH-like protein (BNBTG26)	*Brassica napus*	Nd
	ALDH12A1	Delta1-pyrroline5-carboxylate dehydrogenase (P5CDH)	*A. thaliana*	Mitochondria
	ALDH18B1	Delta1-pyrroline5-carboxylate synthase (P5cS-1)	*Medicago savita*	Nd
	ALDH18B1	Delta1-pyrroline5-carboxylate synthase (P5cS)	*Oryza sativa*	Nd
	ALDH18B1	Delta1-pyrroline5-carboxylate synthase (Pro2)	*Solanum esculentum*	Nd

Ref: Vasiliou et al (1999), Skibbe et al (2002), Kirch et al (2001), Sunkar et al (2003), Kotchoni and Bartels (2003), Sophos and Vasiliou (2003), Kirch et al 2004, Nd = not determined.

1.6. Reactive oxygen species and ROS-scavenging function of ALDHs

Molecular oxygen is produced during photosynthesis in plant cells and is directly used during photorespiration. Both reactions (production and the use of oxygen) have positive and negative effects on plant metabolism (Arora et al 2002). Under adverse growth

conditions, molecular oxygen undergoes sequential electron reduction (Izumi and Schroeder 2004) leading to the production of superoxide anion, hydrogen peroxide, hydroxyl radical and hydroperoxyl radical generally known as reactive oxygen species (ROS). Plants exposed to stress show a high level of photo-inhibition followed by development of chlorosis (Wise and Naylor 1987). ROS are immediately produced upon exposure of plants to environmental stress and mediate subsequent molecular peroxidations, which are perceived as oxidative stress in cells (Wise and Naylor 1987). ROS generally inactivate enzymes and damage important cellular components. Their effects include the induction of lipid peroxidation, fatty acid de-esterification and membrane breakdown (Goel and Sheoran 2003). It is clear that the capacity and activity of the antioxidative defence system are crucial in limiting the oxidative damage caused by the excess production of ROS. Oxidative stress is essentially a regulatory process, which is dependent on the equilibrium between the ROS generation and the antioxidative capacities under given stress conditions (Bartels 2001b). When the antioxidative systems are at the upper level of the equilibrium the plant becomes tolerant to the stress, otherwise the plant will be damaged and viability is stopped. Therefore, under stress conditions plants activate the efficient ROS-scavenging systems that protect them from cellular damage. The ROS-scavengers include superoxide dismutase (SOD), which catalyses the disproportionation of superoxides into molecular oxygen and H_2O_2 (Scandalios 1993), ascorbate peroxidase responsible for the removal of H_2O_2, and glutathione reductase that is responsible for the protection of thiol groups on enzymes (Goel and Sheoran 2003).

The effects of abiotic stress in plants are coupled with the generation of toxic by-products such aldehyde molecules that are addressed in this work. One of the most important

pathways of the aldehyde metabolism is their oxidation into carboxylic acids. This reaction leads to the production of NAD(P)H, which represents a potential donor of electrons during the reduction of hydrogen peroxide and the protection of enzymatic thiol groups by ascorbate peroxidase and glutathione reductase respectively (Arora et al 2002). The oxidation of the carbonyl group is considered as a general detoxification process and is very crucial in avoiding molecular attack. Therefore, the bonus of NAD(P)H production during ALDH activity indirectly reduces the accumulation of ROS in the cell and promotes thereby the ALDHs as one of the potential ROS-scavenging enzymes. Ohsawa et al (2003) demonstrated that deficiency in mitochondrial ALDH2 increases vulnerability to oxidative stress in animal cells. Their findings suggest that aldehyde dehydrogenases are involved in the antioxidative defence system and their deficiency enhances oxidative stress. Therefore ALDHs enhance the efficiency of reducing oxidative damage in cells undergoing environmental stress by detoxifying the cytotoxic products of lipid peroxidation and reducing the accumulation of ROS. Further molecular and biochemical analyses on plant ALDHs are worthy to be accomplished to fully understand the functions of these genes responses to environmental stress.

1.7. Objectives of the study

The generation of transgenic plants offers a unique way by which one can study the role of a transgene or the subsequent effect of the loss of specific genes in the host plants. In order to understand the biological functions of plant ALDHs in conferring tolerance to multiple abiotic stresses, this study was planned with the following objectives:

1. To carry out the expression analysis of aldehyde dehydrogenase (*ALDH*) genes in response to various abiotic stressors in order to establish the relationship between these genes and the responses of plants to environmental stress.
2. To produce specific ALDH antibodies by immunising rabbits with recombinant GST-ALDH fusion proteins to raise ALDH-antisera against plant ALDHs in order to investigate patterns of ALDH protein accumulation.
3. To analyse several independent transgenic plants with different transgenes such as: constitutive *ALDH*-expressing plants, stress-inducible *ALDH* expressing lines, antisense *ALDH*-suppressive lines and *ALDH* knock-out plants.
4. To characterize the physiological and molecular downstream effects of *ALDH* overexpression, reduction or repression of *ALDH* transcript accumulation and the loss of ALDH functions in selected transgenic plants responding to abiotic stress.
5. To carry out comparative biochemical studies such as lipid peroxidation, reactive oxygen species accumulation, chlorophyll content, biomass accumulation with transgenic and wild-type plants to establish the functions of *ALDH* genes in plants with respect to abiotic stress.
6. To identify the expression of *ALDH* genes in plant cells by using the *GUS* reporter gene fusion as tool for gene expression analyses.

2. MATERIALS AND METHODS

2.1. Plant materials

Arabidopsis thaliana ecotypes C-24 and Col-0 and were used in this work. Transgenic plants overexpressing *ALDH3I1* and *Cp-ALDH* cDNAs under control of the CaMV35S-promoter were established earlier (Kelbert 2000, Heuft 2000) and seeds (T3) were kindly provided to me. All plants were grown (see growth conditions in section 2.7) and subjected to various abiotic stresses (see plant stress treatments in section 2.13). Untreated and stress treated plant samples were collected and used either immediately for analyses or frozen in liquid nitrogen and stored at –70 °C for further use.

2.2. 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, Roth-Karlsruhe, Germany.

2.3. DNAs, vectors and bacteria

The plasmid vectors and bacteria used in this work are listed below. Molecular details of the vectors are provided in the appendix. All the 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 were stored in glycerol cultures at -80 ºC, and were available for direct use.

cDNAs for *ALDH3I1* and *ALDH7B4* used in this study were provided by Kirch H-H. C2-cDNA and C2-promoter was kindly provided by Ditzer A. Isolation of ALDH3I1, ALDH3H1 and Cp-ALDH promoter sequences and construction of promoter-GUS fusion was done earlier by Sunkar R. and ALDHpromoter-GUS fusions in pBIN19 were kindly provided to me.

2.3.1. pBluescript II SK +/-

This vector (Stratagene, La Jolla, USA) was used as cloning vector for the *Arabidopsis-ALDH* (*ALDH3I1*, *ALDH3H1*)- and the *Craterostigma-ALDH* (*Cp-ALDH*)-cDNA constructs. The vector contains a gene sequence encoding for â-lactamase and can therefore be selected by ampicillin.

2.3.2. pBT10gus vector

This vector (Sprenger-Haussels and Weisshaar 2000) contains the *GUS* gene used as reporter gene for the constructs and the molecular analysis of the *ALDH* promoter-*GUS* fusions. The vector contains the â-lactamase gene and can be selected by ampicillin. *ALDH3I1* promoter-*GUS*, *ALDH3H1* promoter-*GUS* and *Cp-ALDH* promoter-*GUS* were subsequently isolated from pBT10gus vector and inserted into pBIN19 vector for *Agrobacterium tumefaciens* transformation (see section 2.11.6.5).

2.3.3. pBIN19 and pROK2

pROK2 vector (Baulcombe et al 1986) is a binary vector derivative of pBIN19 (Bevan 1984, Frisch et al 1995). PROK2 was used to clone the *35S-ALDH7B4* cDNA construct

into *Agrobacterium tumefaciens*, which was then used for plant transformation. pBIN19 and pROK2 contain the *NPTII* gene encoding for kanamycin resistance as plant selectable marker.

2.3.4. pGEX 5x1

This vector was used for the expression of the GST-ALDH3I1 fusion protein (Amersham Pharmacia Biotech, Freiburg) used as antigene the raise ALDH3I1 antibodies from immunized rabbits. The *ALDH3I1* cDNA (1.4 kb) was isolated from EcoRI digestion of pBluescript-*ALDH3I1* cDNA recombinant plasmid and fused to the N terminal (EcoRI) site of GST (for details see production of antibody in section 2.9.8) and induced by adding IPTG.

2.3.5. pcC C2 vector (Ditzer et al 2001)

This vector contains the *Craterostigma plantagineum* C2 cDNA (Ditzer 1999, Ditzer 2003). The promoter of the C2 gene (760 bp) was isolated after EcoRI digestion of 1.0 kb PCR amplification fragment of C2 promoter from pcC C2 recombinant vector and fused to *ALDH3I1* cDNA in pBIN19 in order to obtain a *C2-ALDH3I1* cDNA construct (for detail see forward and reverse primer used for PCR amplification of C2 promoter (1.0 kb) in section 2.8).

2.3.6. *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⁻

2.3.7. *Escherichia coli* BL21 (Pharmacia, Freiburg)

Genotype: F- ompT hsdSB ($r_B^- m_B^-$) gal dcm (DE3).

This bacterium strain was used for the expression of GST-ALDH protein fusion.

2.3.8. *Agrobacterium tumefaciens* LBA 4404

This bacteria Sm^r, (Rif^r), vir-region (Ooms et al 1982, Hoekema et al 1983) was used in the infectious process of plant *Agrobacterium tumefaciens*-mediated transformation.

2.4. Enzymes and markers

Restriction enzymes and their corresponding buffers were from Amersham Pharmacia Biotech (Freiburg), MBI-Fermentas (St. Leon-Rot), Roche/Boehringer (Mannheim), Sigma (Muenchen), Invitrogen/GibcoBRL (Karlsruhe). The DNA marker (1 kb ladder) was from Invitrogen/GibcoBRL (Karlsruhe) and the protein standards such as Phosphorylase (97 Kda), Albumin bovine (66 Kda), Albumin egg (45 Kda), Carbonic anhydrase (29 Kda), á-Lactalbumin (14 Kda) were from Sigma (Muenchen).

2.5. Software programmes

Adobe Photoshop 6.0

Vector NTI™ suite 5.5 (Informax Inc, 1999, North Bethesda, MD, USA)

Corel Photo Paint 8

2.6. Media, buffers and solutions

All media, buffers and solutions used were sterilized either by filter sterilization or by autoclaving for 20 min at 120 ºC at 1.5 bars. Seedlings were grown on MS (Duchefa)-agar plates as described by Murashige and Skoog (1962).

2.6.1. Media

MS-medium (per litre):	4.6 g MS-salts, 20 g sucrose, 1 ml vitamins, adjust pH to 5.8 with 1M NaOH, and 8 g selected agar (optional).
LB-medium (per litre):	10 g peptone, 10 g NaCl, 5 g yeast extract, adjust pH to 7.5, and 15 g selected agar (optional) for agar plate cultures.
YEB (per litre):	5 g saccharose, 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, and 15 g Select agar (optional) for agar plate cultures only.
SOC:	2% (w/v) trypton, 0.5% (w/v) selected yeast extract, 10 mM NaCl, 10 mM $MgSO_4$, 10 mM $MgCl_2$

2.6.2. Buffers and solutions

10 x TAE buffer:	0.4 M Tris-acetate, 20 mM EDTA, pH 8,0.
RNase A + T_1:	1mg/ml RNase A, 10000 U/ml RNase T_1, 10 min heating, and cooling at room temperature, and stored at -20°C for further use.
Denaturing buffer:	1.5 M NaCl, 0.5 M NaOH without adjusting the pH and stored at room temperature.

Neutralizing buffer:	1 M Tris, 1.5 M NaCl, pH 8.0 was adjusted with concentrated HCl, stored at room temperature.
10 x blue gel loading buffer:	25 mg Bromophenol blue, 25 mg Xylencyanol, 1 ml 10 x TAE (as above), 3 ml glycerol, and 6 ml sterile distilled water (sd H_2O).
20 x SSC:	3 M NaCl, 0.3 M Sodium citrate, stored at room temperature.
1 x TE buffer:	10 mM Tris-HCl, 1mM EDTA, pH 8.0 and stored at room temperature.
Washing buffer:	0.1 % (w/v) SDS, 2 x SSC. Stored at room temperature.
Vitamin solution:	2 mg/l glycine, 0.5 mg/l Niacin (Nicotine acid), 0.5 mg/l pyridoxin-HCl, 0.1 mg/l thiamine-HCl. Use 1:1000 dilution of the solution.

2.7. Growth conditions

2.7.1. Germination of seeds

For seed germination, seeds were surface sterilized in 70 % (v/v) ethanol for 2 min and then in 7 % (v/v) NaOCl, 0.1 % (w/v) SDS for 30 min, rinsed three times in sterile distilled water and sown on MS-agar plates. All transgenic seeds were selected on MS-agar plates containing 50 mg/l kanamycin (end concentration). Both wild-type and transgenic seeds were grown under approximately 7000-8000 lux light at 22 °C with a day/night cycle of 16/8h. Seven to 12 day-old seedlings were transferred into soil trays of

3:1 soil: vermiculite, or into hydroponic cultures and then subjected to various abiotic stressors.

2.7.2. Growth of microorganisms

All different strains of *E. coli* were incubated and cultured either in liquid LB medium at shaking with 300 rpm or in solid LB-agar medium at 37 °C, while the Agrobacteria were incubated in liquid YEB medium at 250 rpm or in solid YEB-agar medium at 28 °C. The microorganism cultures were incubated with appropriate selection markers.

2.8. Primers

2.8.1. Primers (5’ 3’) for *GST-ALDH3I1* construct

Identity	Sequence	Restriction enzyme
Ara5Eco (fwd)	GAA**GGAATTC**GCTGTGGTAAAGGAGCAAGCATC	EcoRI
Ara3EcoI (rev)	GAA**GGAATTC**TCATGAGTCTTTAGAGAACCCAAAG	EcoRI

Ara5Eco and Ara3EcoI primers were designed for the amplification of 1.4 kb *ALDH3I1* cDNA fragment subcloned into EcoRI site of pGEX 5.1 expression vector.

2.8.2. Primers (5’ 3’) for Cp -C2 promoter cDNA

C2-PromEcoRI (fwd) EcoRI	GATCTAAACTC**GAATTC**ACACCTG	
C2-Ext (rev)	CTTCTGATGTCCTCGCATCG	None

C2-PromEcoRI and C2-Ext primers were designed to amplify 1.0 kb C2 promoter cDNA fragment from pcC C2 plasmid (Ditzer 2003).

C2-Prom SphI	TTCTATTCTT**GCATGC**GTGG	SphI

C2-Prom SphI was designed for the sequencing of C2-prom from pBin-*C2-ALDH3I1* plasmid.

2.8.3. Primers (5’ 3’) for *35S-ALDH7B4* construct

ALDH7B4 (fwd)	AGATGGGTTCGGCGAACAAC	None
ALDH7B4 (rev2)	TTAACGAGTAAATCTCTGAAA	None

ALDH7B4 (fwd) and *ALDH7B4* (rev) primers were designed for amplification of 1.5 Kb *ALDH7B4* cDNA fragment from pROK2-*ALDH7B4* construct.

P35S-pROK2	CACTGACGTAAGGGATGACGC	None

This primer was used for the sequencing of *ALDH7B4* fragment from 5’ end of 35S-promoter site of pROK2 plasmid.

ALDH7B4-zum promotor	CGTAATCCTCTAGAGAAGCTTC	None

ALDH7B4-zum promotor was designed for sequencing the *ALDH7B4* fragment from 3’ end towards 35S-promotor of the pROK2 plasmid.

2.9. Extraction of nucleic acids

2.9.1. Extraction of RNA from *Arabidopsis thaliana*

Plant material (250 mg) from stress-treated and untreated seedlings was frozen in liquid nitrogen and ground to a fine powder. The total RNA was extracted according to Bartels et al (1990). The plant material was homogenised in 500 µl of 80 ºC pre-warmed RNA extraction buffer:phenol (1:1 i.e. 250 µl:250 µl) and then resuspended in 250 µl chloroform:isoamylalcohol (24:1) for 30 sec followed by centrifugation (14000 rpm) at room temperature for 5 min. The supernatant was mixed with one volume of 4 M LiCl and kept overnight on ice or at 4 ºC. The mixture was then centrifuged (14000 rpm, 4 ºC, 20 min) and the pellet dissolved in 250 µl d H_2O, to which 0.1 volume of 3 M sodium acetate pH 5.2 and 2 volume of 100 % ethanol were added and kept at –70 ºC for 2 h. The RNA extract was finally recovered in the pellet after centrifugation (14000 rpm, 4 ºC, 10

min), washed twice with 70 % (v/v) ethanol, air-dried and resuspended in 25 µl RNase-free water and stored at –70 ºC. The concentration of the extract was quantified spectrophometrically at 260 nm. The quality of the extraction was ascertained by measuring the OD at 260/280 nm and confirmed by agarose gel fractionation (1 % [w/v] agarose) followed by ethidium bromide staining, visualized under UV light.

RNA extraction buffer: 100 mM LiCl, 100 mM Tris, 10 mM EDTA, 1% (w/v) SDS, pH 8.0, add one volume of phenol solution (immediately) before pre-warming the mixture.

2.9.2. Extraction of genomic DNA from *Arabidopsis thaliana* (Doyle and Doyle 1989)

Plant material (500 mg) was ground in liquid nitrogen and dissolved in 7.5 ml of pre-warmed (60 ºC) DNA extraction buffer and further incubated at 60 ºC (in water bath) for 30 min. The mixture was resuspended in one volume chloroform/isoamylalcohol (24/1) and centrifuged at 20 ºC for 10 min at 1600 x g. The supernatant was mixed with 2/3 volume (5 ml) isopropanol and incubated at room temperature for 2 h. The mixture was centrifuged as above and the pellet resuspended in 15 ml 76 % (v/v) ethanol, 10 mM ammonium acetate and centrifuged again (10 min, 1600xg, RT). The supernatant was carefully discarded; the pellet air-dried and then resuspended in 1 ml 10 mM Tris-HCL pH 8.0. To this mixture 10 µl RNase A+T_1 (1 µg/ µl) was added and incubated at 37 ºC for 30 min. 2 ml 10 mM Tris-HCl pH 8.0; 1 ml 7.5 M ammonium acetate pH 7.7 and 10 ml 100 % ethanol were then added to the mixture and incubated at –20 ºC for 2 h. The mixture was centrifuged (10 min, 14000 rpm, 4 ºC) and the pellet (genomic DNA)

washed twice in 70 % (v/v) ethanol, air-dried and resuspended in 100 µl 10 mM Tris-HCl pH 8.0 and stored at –20 ºC.

DNA extraction buffer: 3.5 % (w/v) CTAB, 100 mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0, 1.4 M NaCl, 0.2 % (v/v) â-mercaptoethanol which should be added *in-situ* (before DNA extraction).

2.9.3. Plasmid DNA (mini-prep) (Birnboim and Doly 1979)

For plasmid mini-prep, transformed *Agrobacterium tumefaciens* or *E. coli* clones were inoculated in 2 ml YEB or LB media containing appropriate selection markers respectively and allowed to grow (28 ºC, 250 rpm) for about 21 h for *A. tumefaciens* and 16 h at 37 ºC and 300 rpm for *E. coli*. The bacteria were centrifuged (6000 rpm, 5 min, RT) and the pellet resuspended in 400 µl solution I and further incubated for another 10 min at RT without shaking. Solution II (800 µl) was added to the suspension, carefully mixed and further incubated for 10 min at RT. In order to obtain a 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 breaking of the DNA. The mixture was incubated at –20 ºC for 15 min, and then centrifuged at 14000 rpm for 10 min at 4 ºC. The supernatant, which contains the plasmid DNA was carefully taken and 3 aliquots of 650 µl of it were made. To each aliquot, 2 volume of cold 100 % ethanol was added and incubated at –80 ºC for 15 min. The aliquot was centrifuged (14000 rpm, 10 min at 4 ºC) and the pellet was resuspended in 500 µl 0.3 M sodium acetate pH 7.0, and then in 1ml 100 % ethanol. The suspension was incubated at –80 ºC for 15 min. The plasmid

DNA was recovered in the pellet after centrifugation (14000 rpm, 10 min, 4 ºC). The plasmid DNA was washed twice in 70 % (v/v) ethanol and air-dried at RT. The dried pellet was dissolved in 50 µl 10 mM Tris-HCl pH 8. Finally 3 µl of RNase A+T_1 (Sigma) was added to the solution and incubated at RT for 15 min. A test gel (0.8 % agarose gel) of 9 µl of the plasmid prep was made to monitor the purity of the extraction.

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

2.9.4. Precipitation and purification of DNA

To the DNA aliquot solution as prepared in sections 2.9.2 and 2.9.3, x volume of distilled water was added (to make 100 µl end volume), which is suspended into 100 µl of phenol/chloroform/isoamyl alcohol (25/24/1) and vortexed if necessary. The suspension was centrifuged (14000 rpm, 20 min, RT) and the supernatant was mixed with 0.1 volume 3 M sodium acetate pH 5.2, 2.5 volume of absolute ethanol and incubated at –20 °C for 2 h or overnight. The mixture was thereafter centrifuged (14000 rpm, 20 min, 4 °C) and the pellet washed in 70 % (v/v) ethanol, air-dried and resuspended in 20 µl sterile water. The OD was measured at 260 nm to determine the DNA concentration and the DNA solution stored at –20 °C.

2.9.5. Extraction of DNA fragments from agarose gels

After restriction enzyme digestion or PCR amplification of plasmid DNA constructs, DNA bands or plasmid inserts were isolated from agarose gels using QIAEX II Qiagen extraction kit. The extraction and purification was done after excising the bands from the agarose gel followed by the purification according to the instructions of the manufacturer (Qiagen).

2.10. Protein extraction

2.10.1. Protein extraction from *Arabidopsis* plant material

Plant material (500 mg) was frozen in liquid nitrogen, ground and directly homogenized in 500 µl Laemmli buffer (Laemmli 1970). The samples were briefly vortexed and incubated at 95 °C for 5 min. Samples were then spun down (14000 rpm) for 5min at RT. Supernatants containing the crude protein extracts were transferred into new eppendorf tubes and stored at –20 °C. For protein analysis, samples were incubated at 95 °C for 2 min before loading on an SDS-PAGE gel.

Laemmli Buffer: 62.5 mM 0.5 M Tris-HCl pH 6.8, 10 % (v/v) glycerol, 2 % (w/v) SDS, 5 % (v/v) â-mercaptoethanol, 0.1 % (w/v) bromophenol blue (the buffer was stored at 4 °C)

2.10.2. Protein extraction from bacteria

150-200 ml LB culture of bacteria (300 rpm, 37 °C) was grown overnight with selected antibiotics. A 1:10 dilution was made into fresh LB medium and further incubated (300

rpm, 37 °C, 3 h) in presence or absence of protein inducer (IPTG: 0.1-1.0 mM). The bacteria cultures were centrifuged (5000 rpm, 5 min, RT) and the supernatant used to check for soluble protein, while the bacteria pellets were used to isolate inclusion bodies. For the inclusion body protein preparation, the pellet was resuspended in 100 µl of MTPBS (0.15 M NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4), 1 % Triton x 100 and sonificate (5 x 20 sec) on ice with one min interval followed by centrifugation (14.000 rpm, 10 min, 4 °C) in order to separate the soluble protein. The pellet, which contains the inclusion body protein was resuspended into 200-500 µl protein extraction buffer (Laemmli buffer) and incubated at 95 °C for 5 min, cooled in ice and used for protein analyses or stored at –20 °C.

2.10.3. Production of antibody

In order to produce a specific antibody against the plant proteins, PCR amplification of *ALDH3I1* cDNA fragment (1.4 kb) was amplified from pBluescript-*ALDH3I1* cDNA recombinant plasmid (for details see primers designed for the amplification of the 1.4 kb *ALDH3I1* cDNA in section 2.8) and fused to the N terminal GST protein at EcoRI site of a pGEX 5.1 vector. The recombinant plansmid was sequenced and checked for correct orientation. This construct was used to transform *E. coli* BL21 via electroporation. The fusion protein (GST-cDNA) was induced by IPTG, purified from inclusion bodies and used as antigen to induce the production of specific antibody through immunization of rabbits. A total amount of 150 mg of purified protein was provided for the immunization of the rabbits.

The immunization of the rabbit was carried out by BioGenes mbH, Berlin. The antigen was injected into the rabbit in order to raise the specific ALDH3I1antisera. Preimmune serum (1.5 ml) was collected from the rabbits prior to the immunization. The first boost and the second boost were carried out at weekly intervals and the third boost/antiserum was collected two weeks after the second boosting i.e. a total period of one month for the rabbit to produce the antiserum. 20-50 ml antiserum was collected per immunized rabbit, which was used at 1:500 dilutions as antiserum to analyse the plant protein expression pattern under abiotic stress.

2.11. Qualitative and quantitative estimation of concentrations of macromolecules

2.11.1 Qualitative and quantitative estimation of DNA and RNA

Extracted nucleic acids (DNA and RNA) were qualitatively monitored in 1 % (w/v) agarose gel electrophoresis using 1 kb ladder. The concentration of the nucleic acids was spectrophotometrically determined at OD of 260 and 280 nm. A value of OD_{260} = 1 corresponded to 50 µg/µl DNA solution while OD_{260} = 1 corresponded to 40 µg/µl RNA solution. For a pure DNA extraction, the value of OD_{260}/OD_{280} must be between 1.8 and 2. A value of OD_{260}/OD280 below 1.8 means a contamination of DNA preparation with proteins or phenolic compounds. For the purity of RNA extraction, OD_{260}/OD_{280} value must be higher than 2. A value of OD_{260}/OD_{280} below 2 indicates a contamination of RNA extraction with proteins or phenolic compounds.

2.11.2. Quantitative estimation of protein extracts

The estimation of protein concentration was carried out using a BioRad protein assay kit according to Bradford (1976). Protein aliquots (100 µl) were mixed with 200 µl BioRad protein assay kit and brought to 1000 µl with sterile H_2O (700 µl). The suspensions were incubated at room temperature for 5 min followed by an OD measurement at 595 nm. The quantification of the protein aliquots was calculated by calibrating the measurement of each sample with the OD (595 nm) of a definite concentration (10 µg/µl) of standard proteins (generally BSA).

2.12. Cloning of DNA fragments

2.12.1. Primers designed for cloning

For PCR amplification, DNA sequencing and various plasmid DNA constructs, specific primers were designed with the following criteria:

The GC content of a primer must be approximately 50%, and the melting temperature (T_M) should be according to Faust rules $T_M = 4 (G+C) + 2 (A + T)$ where G, C, A, T represent the DNA bases of the primer sequence. T_M should be approximately between 60 to 65 °C. Based on the fact that GC are complemented (linked) with three hydrogen bonds, the primers preferentially ended with at least one dGTP or dCTP. The primers were designed to avoid self-complementation forming thereby a secondary structure. Forward and reverse primers of each PCR reaction were designed to have approximately the same T_M.

The primers were designed to contain at least at their 5'-end a minimum of 2 bp after BamHI site, 1 bp after EcoRI, 3 bp after Hind III, 4 bp after Sal I, 4 bp after SphI, 4 bp

after Pst I, and 2 bp after Xba I in order to ensure a possible and subsequent digestion of the amplified DNA fragments by the above mentioned enzymes.

2.12.2. Polymerase chain reaction (PCR)

DNA fragments were amplified from various plasmid DNA constructs or genomic DNA as described below:

For each PCR reaction a total volume of 50 µl solution was prepared as followed:

30-35 µl sd H_2O

5.0 µl 10 x PCR-buffer (GibcoBRL/Invitrogen)

1.5 µl 50 mM MgCl2 (GibcoBRL/Invitrogen)

2.0 µl Fwd-primer (10 pmol/µl)

2.0 µl Rev-primer (10 pmol/ µl)

1.0 µl 10 mM dNTPs

1.2 µl plasmid DNA (5 ng/ µl) or PCR product (5 ng/ µl) or bacterial clones (tooth picks), or 1 to 5 µl of genomic DNA solution

1.5 µl 1:10 diluted Taq-polymerase (Pluthero 1993) into Taq-buffer

Taq-buffer: 50 mM Tris, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 50 % (v/v) glycerol, pH 7.9, filter sterilized and stored at 4 °C.

Each PCR aliquot (50 µl) was mixed. PCR reactions were performed in a TRIO-thermoblock (Biometra, Göttingen). The optimal number of PCR cycles and the

annealing temperature was determined empirically per PCR reaction. A standard PCR-programme was as followed:

94 °C	3 min of denaturing
94 °C	30 sec 3(6 times) of denaturing
TA	30 sec (36 times) of primer binding
72 °C	30 sec (36 times) of DNA -synthesis
72 °C	5 min at the end of the reaction
4 °C	indicating the end of PCR running programme

T_A = annealing temperature = T_M – 4 °C

T_M = melting temperature

2.12.3. 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 be digested. A double digestion was possible per reaction only when both restriction enzymes used the same buffer otherwise the digestions were performed chronologically.

2.12.4. Dephosphorylation

Digested DNA fragments were dephosphorylized at their 5’ end with shrimp alkaline phosphatase (SAP, Boehringer/Roche, Mannheim) in order to avoid religation or recycling of cohesive-ends of plasmid DNA during DNA recombination.

The reaction was made in 9 µl (end volume) comprising of 0.9 µl phosphorylation buffer (10 x), 1.0 µl (1 unit) shrimp alkaline phosphatase, and appropriate µg plasmid DNA. The mixture was brought to 9 µl with sterile distilled water. The solution was incubated for 10 min at 37 °C and followed by inactivation of SAP at 65 °C for 15 min. A dephosphorylation of blunt-ended DNA fragments was carried out at 37 °C for 60 min followed by inactivation of SAP by incubating the mixture at 65 ºC for 15 min..

2.12.5. Ligation

For plasmid DNA constructs, different DNA inserts were ligated in various independent DNA-recombination experiments. The ligation reaction was brought to 10 µl (end volume), which comprises 1.0 µl ligase buffer (10 x), x µl digested plasmid DNA vector (dephosphorylated or not), 1.0 µl T4-ligase (Roche), and y µl DNA insert. The mixture was brought to 10 µl with sterile H_2O and incubated at 16 °C for 20 h. For a good ligation reaction the amount of plasmid vector must represent the third of the DNA insert in the mixture.

2.12.6. Transformation

2.12.6.1. Calcium-competent *E. coli*

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

2.12.6.2. Transformation of Calcium-competent *E. coli*

One ìl plasmid DNA (5 -50 ng/ µl) or ligated plasmid DNA construct was brought to 100 µl with cold 0.1 M $CaCl_2$ and added to one aliquot of calcium-competent cells (100 µl) and carefully mixed. The mixture was incubated in ice for 1 h and immediately heat shocked in a water bath at 42 °C for 120 sec. LB medium (650 µl) was added to the transformed cells and further incubated (37 °C, 250 rpm) for 1 h. Before plating, 1:10 and 1:100 dilutions of the transformed cells were made with LB medium. Aliquots (100-200 µl) of the diluted cells were spread on selective plates and incubated at 37 ºC overnight.

2.12.6.3. Electrocompetent *E. coli*

E. coli bacteria were inoculated in 10 ml LB medium, incubated (37 °C, 12 h, 250 rpm) and resuspended into 200 ml new LB medium and further incubated till OD_{600} = 0.6. The culture was cooled on ice for 30 min and centrifuged (5 min, 5000 rpm, 4 °C). The pellet was washed firstly in 50 ml cold sd H_2O, centrifuged as above and secondly washed in 25

ml sd H_2O and centrifuged as well. The pellet was further washed twice in 25 ml 10 % (v/v) glycerol and resuspended in 10 ml cold GYT-medium. The cell suspension was centrifuged and the pellet resuspended in 2 ml GYT. Aliquots (50 µl) of the cell suspension 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

2.12.6.4. Electrocompetent *Agrobacterium tumefaciens*

A. tumefaciens was inoculated in 3 ml YEB_{Rif} medium, incubated (28 °C, 16 h, 250 rpm) and resuspended into fresh YEB_{Rif} (50 ml) and further incubated till OD_{600} = 0.5. The cell culture was cooled in 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 pellet was resuspended in the following solutions with centrifugations (5000 rpm, 10 min, 4 °C) between the suspensions.

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 were made, shock frozen in liquid nitrogen and stored at –70 °C as electrocompetent *A. tumefaciens*.

2.12.6.5. Transformation via electroporation (Tung and Chow 1995)

Aliquots of electrocompetent cells were cooled in ice before transformation. About 1 to 2 µl DNA of a ligated vector or specific plasmid DNA (approximately 5-10 ng/µl) was added to the competent cells and carefully mixed in a precooled electrocompetent cuvette, which undergoes 3 to 5 sec electroporation (GenePulser II, BIO-RAD) for the transformation. 1 ml YEB-mediun or 800 µl SOC was added to the transformed cells and further incubated in a glass tube for another 1 h at 37 ºC (for *E. coli* transformation) or 3 h at 28 ºC (for *A. tumefaciens* transformation) shaking at 250 rpm. 100 µl aliquots of the cells were finally plated out overnight with the selected antibiotics at appropriate temperatures as indicated below.

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

2.12.6.6. *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis* plants

Wild-type plants were transformed via *Agrobacterium tumefaciens*-mediated transformation with the appropriate *ALDH* cDNA construct under the control of either the 35S-CaMV promoter or a stress-inducible promoter using a modified method of Clough

and Bent (1998). The wild type seedlings were allowed to grow to the stage of inflorescence. The *Agrobacterium* clone carrying the transgene were incubated (28 ºC, 250 rpm) in 250 ml YEB/Kanamycin/Rifampycin till OD_{600} = 0.9 and centrifuged (5000 rpm, 10 min, 4 ºC) in a 250 ml centrifuged bottles. The pellets were resuspended in a minimum volume of 400 ml infiltration medium (0.01% (v/v) Silwet 77 in YEB) and further incubated using a stirring magnet till OD_{600} = 0.8. The infiltration medium was prepared in a 500 ml beaker to enable the wild-type seedlings to be immersed in the solution for transformation. Slowly the pots containing the wild-type *Arabidopsis* plants were carefully inverted and immersed in the infiltration medium while stirring for 1 min. Care was taken to submerge all the earlier inflorescence of the seedlings in the solution. The seedlings were thereafter placed back in the trays and sealed in plastic bags. Few holes were made in the bags for aeration. Three days after infiltration the plastic bags were removed and the seedlings were supported together with a stick and allowed to grow till the first generation of seeds (T_1).

2.12.7. Screening methods

2.12.7.1. Screening for positive transformed clones

Using a replica plating technique, cell clones were transferred into new plates and assigned appropriate numbers, which were considered throughout the screening process. The clones were either used as DNA source to amplify DNA inserts via PCR amplification or used for individual plasmid mini prep from which appropriate DNA digestions were carried out using specific restriction enzymes in order to check the DNA fragments and confirm the correct insertion. In addition, all transformed cells were

further confirmed by DNA gel blot analysis using specific radioactive probes (see section 2.11.4.) to detect the DNA bands.

2.12.7.2. Screening for transgenic *Arabidopsis* seeds

After transformation, the first generation of seeds (T_1) was collected, surface sterilized and sown on MS-agar plates containing appropriate selection marker (50 mg/l kanamycin final concentration). After 15-days of growth, transgenic seeds were able to germinate and produce green leaves in the presence of the marker while the non-transgenic seeds were not able to grow with true green leaves. The positive seedlings (transgenic lines) were transferred into soil trays and allow to grow for the next generation of seeds (T_2).

2. 13. Electrophoresis and blotting methods

2.13.1. Agarose gel electrophoresis

In order to ascertain the quality and/or the specificity of DNA digestions, all nucleic acid molecules (genomic DNA, RNA) extracted from plants and all plasmid DNAs extracted from bacteria were checked in 0.8-1.2 % (w/v) agarose gel electrophoresis. For a quantitative electrophoresis, approximately 1-1.5 µg of DNA solution (1 µg/µl) was loaded in the gel and electrophoretically separated (65-70 mA, 45-60 min) in 1 x TAE buffer using a 1 kb ladder (GigcoBRL/Introgen) as reference marker. The detection of DNA fragments was carried out under UV light using ethidium bromide staining.

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

2.13.2. RNA blot analysis

RNA aliquots (30 µg) were separated in formaldehyde-agarose (1.2 % w/v) gels using RNA running buffer and then transferred overnight into Hybond-nylon membrane (Amersham) by means of capillary adsorption blot via Northern-transfer buffer (20 x SSC) according to Sambrook et al (1989) and Bartels et al (1990). The membrane was pre-hybridised (3 h, 42 ºC) in a shaking water bath and hybridised overnight with a specific probe (see below: section 2.11.4.) in the RNA hybridisation buffer (50 % (v/v) formamide, 5 x SSC, 10 mM PIPES pH 6.8, 0.1 % (w/v) SDS, 1 x Denhardt's, 100 µl denatured hering spermDNA (ssDNA: Biomol). Equal amounts of RNA aliquots in the gels were controlled by hybridising the same membrane with an actin probe or with a ribosomal probe pTA71 (Gerlach and Bedbrock 1979). The membrane was thereafter washed (2 x 20 min at 42 ºC and 1 x 20 min at 65 ºC) in blot -washing buffer (0.1 % (w/v) SDS, 2 x SSC). The membrane was then exposed to X-ray film (X-OMAT, Kodak) and stored at –70 ºC for a specific period and developed.

12 % (w/v) RNA blot-agarose gel:	1.8 g agarose gel, 30 ml 5 x Mops, 93 ml sd H_2O, 27 ml 37 % (v/v) formaldehyde.
5 x MOPS:	41.7 g/l MOPS, 4,1 g/l Na-acetate, 10 ml/l 0.5 M EDTA pH 7.0
RNA blue marker:	100 µl 5 x MOPS, 175 µl 37 % (v/v) formaldehyde, 500 µl formamide, 0.2 µl 10 % (w/v) bromophenol blue.

RNA running buffer:	200 ml/l 5 x MOPS, 80 ml/l 37 % (v/v) formaldehyde, add sd H_2O to make one litre solution.
100 x Denhardt's:	2% (w/v) BSA (fraction V), 2 % (w/v) ficoll 400, 2 % (w/v) PVP 360,000.

2.13.3. DNA blot analysis

Genomic DNA was digested with appropriate restriction enzymes and size-fractionated in an agarose gel, (section 2.11.1). The gel was incubated for 30 min in alkaline denaturing buffer and 30 min in neutralising buffer in a shaking water bath and blotted overnight on a Protran BA 85-membrane (Schleicher and Schuell, Dassel) using 20 x SSC blotting buffer. The membrane was pre-hybridised (65 ºC) for a minimum period of 3 h, and hybridised overnight with the ^{32}P-labeled probe (see below: section 2.11.4.) at 65 ºC in Southern hybridisation buffer (Sambrook et al 1989). The membrane was subsequently washed (3 x 20 min) in washing buffer (2 x SSC, 0.1 % (w/v) SDS) and thereafter exposed to X-ray film and kept at –70 ºC for film development.

DNA hybridisation buffer:	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 100 x Denhardt's, 63.7 ml sd H_2O, 100 µl ssDNA.

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

An appropriate size of a cDNA sequence was PCR-amplified using specific forward and reverse primers. The fragments were purified using QIAEX-kit (Qiagen) purification and used as probes for DNA and RNA blot analysis as described by Bartels et al (1990). Hexalabel^{TM}-labeling kit (MBI Fermentas, Hannover) was used for the probe labelling. 10 µl of hexanucleotides were added to 1-2 µl cDNA and H_2O was added to a final volume of 40 µl. The probe was denatured by heating for 5 min at 95 ºC and immediately cooled in ice. 3 µl Mix C (dNTPs without dCTP) was added and then 2 µl ^{32}P-dCTP and 1 µl Klenow fragment were added, carefully mixed and incubated for 10 min at 37 ºC. Finally 4 µl dNTP-Mix was added and incubated for another 5 min at 37 ºC. The reaction was stopped by adding 50 µl of 1x TE pH 8.0. The labelled probe was separated from the non-incorporated nucleotides through a 1 ml Sephadex G-50 column pre-equilibrated with 1 x TE buffer. Ten fractions of 100 µl were eluted (100 µl of 1 x TE per tube). The Geiger counter was finally used to measure the eluates in order to identify the synthesized DNA probe. The tubes, which showed the first peak of labelling were pooled, incubated for 5 min at 95 ºC, cooled immediately on ice and used as probe for the hybridisation.

2.13.5. SDS polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-PAGE was performed according to Laemmli (1970). The gel was made of 4 % (w/v) acrylamide stacking gel and 12 % (w/v) acrylamide separating gel as described below. Protein samples were boiled for 5 min and cooled on ice before loading onto the gel (10 cm x 10 cm) and run with 1 x SDS-protein running buffer at 20 mA for 2 h. The protein standards (protein markers, Sigma) used were: Phosphorylase (97 Kda), Albumin

bovine (66 Kda), Albumin egg (45 Kda), Carbonic anhydrase (29 Kda), á-Lactalbumin (14 Kda).

Stock solution	4 % Starking gel	12 % Separating gel
30 % (v/v) Acrylamide*	0.65 ml	4 ml
0.5 M Tris-Cl pH 6.8	1.25 ml	-
1.5 M Tris-Cl pH 8.8	-	2.5 ml
10 % (w/v) SDS	50 µl	100 µl
Sterile H_2O	3.05 ml	3.4 ml
10 % (w/v) APS	25 µ l	50 µl
TEMED	5 µl	5 µl
Total volume	5.30 ml	10 ml

* Purchase from BIO-RAD

5 x SDS-protein running buffer: 15 g Tris, 72 g glycerol, 5 g SDS, pH 8.2 per litre final volume.

2.13.6. Protein staining methods

2.13.6.1. Coomassie blue staining of SDS-PAGE

The SDS-PAGE was stained with Coomassie blue R-250 according to Zehr et al (1989) in order to visualize the proteins in the gel. The gel was gently submerged in staining solution and kept shaking and then distained (3 h) at room temperature with distaining solution.

Staining solution:	0.1 % (w/v) Coomassie blue R-250, 50 % (v/v) methanol, 7 % (v/v) glacial acetic acid.
Distaining solution:	50 % (v/v) methanol, 7 % (v/v) glacial acetic acid.

2.13.6.2. Ponceau staining

Protein detection on the membrane was performed by ponceau staining (0.2 % (v/v) ponceau S in 3 % (w/v) TCA) before carrying out the antibody detection of specific protein synthesis.

2.13.7. Protein blot analysis

After SDS-PAGE, separated protein samples were transferred from the gel onto a Protran nitrocellulose BA 85-membrane (Schleicher and Schuell, Dassel) using a protein blot transfer buffer (PBTB) as described by Towbin et al (1979). In order to detect the accumulation of specific plant proteins under stress conditions, the membrane was probed with specific antiserum in a milk (3 % w/v)-TTBS buffer and anti-rabbit IgG horse-radish peroxidase-linked antibodies (1:10000) (Sigma) were used as secondary antibodies. Binding of antibodies was detected using an ECL Plus Western blotting detection kit (ECL-Amersham Pharmacia biotech.).

PBTB	25 mM Tris-HCl pH 7.5, 19.2 mM glycine, 20 % (v/v) methanol
TTBS buffer	20 mM Tris-HCl pH 7.5, 137 mM NaCl, 0.1 % (v/v) Tween 20

2.14. Plant stress treatments

2.14.1. Stress treatment of seedlings

For salt stress experiments, plant seeds were allowed to germinate and were grown in solid (MS-plates, soil) and liquid media containing different concentrations of NaCl or KCl.

For H_2O_2 treatments, seeds were placed on filter paper pre-wetted with water containing different concentrations of H_2O_2. Plates were kept for 4 days at 22 ºC in the presence of 2500 lux white light. The H_2O_2 solution was changed daily to avoid conversion of H_2O_2 into H_2O. The seedlings were collected, frozen in liquid nitrogen and used for various analyses or kept at –70 ºC for later use.

2.14.2. Stress treatment of plants transferred into soil

For the stress treatment of adult seedlings, 15 day-old seedlings grown on MS-agar plates were transferred into pots or trays containing soil/vermiculite (3:1) and allowed to grow for a further week at 22 ºC for acclimation before applying stress treatments.

2.14.2.1. Dehydration stress treatment

Dehydration stress was imposed to adult seedlings by withholding watering for a maximum period of two weeks for phenotypic and biochemical analyses. For molecular analysis, dehydration experiments were performed with plants placed on filter paper and air-dried at room temperature for various time points.

2.14.2.2. Salt stress treatments

Salinity stress was carried out by irrigating the seedlings with NaCl or KCl solutions (0, 100, 200, 300, 400 mM) every two days for a period of 2 weeks. Parallel experiments were performed in hydroponic cultures containing different concentrations of salts. Phenotypic traits and biochemical characterizations (chlorophyll contents, fresh weight accumulation, MDA accumulation, ROS generation) of the seedlings were recorded after an appropriate time of exposure to stress (generally one to two weeks).

2.14.2.3. ABA treatments

The seedlings grown in soil were removed and placed in water containing 100 µM ABA (cis/trans isomers) and incubated in a growth chamber for various time points. The plant materials were frozen in liquid nitrogen and used for subsequent molecular analyses.

2.14.2.4. Cu and paraquat and H_2O_2 treatments

The Cu treatments were performed with seedlings put in water containing 200 µM $CuCl_2$ for different time points. Likewise, the seedlings were put in 10 µM paraquat (methyl viologen) or 5 mM H_2O_2 solutions in independent experiments respectively for various indicated time points. Methyl viologen (MV) inhibits the photosysthem I (PSI), therefore leading to the accumulation of reactive oxygen species in chloroplasts (Sunkar et al 2003). The plant material was frozen in liquid nitrogen and used for different physiological and molecular analyses.

2.15. Determination of chlorophyll content

Chlorophyll was extracted in 80% (v/v) aqueous acetone based on the work of MacKinney (1941) and spectrophotometrically quantified according to Arnon (1949). For the extraction, 0.2 g plant materials were used and suspended in 2 ml extraction buffer and incubated in the dark under shaking at room temperature for 30 min. The suspension was centrifuged (5min, 10000 rpm, RT) and the OD of the supernatants was measured at 663 nm and 645 nm. The chlorophyll content was estimated by the following formula as described by Arnon (1949): $C= 20.2 \times OD_{645} + 8.02 \times OD_{663}$ where C expresses the total chlorophyll content (chlorophyll A + chlorophyll B) in mg/l of extraction solution.

2.16. Lipid peroxidation assay

The level of lipid peroxidation was measured in the plant cells according to the thiobarbituric acid (TBA) test, which determines the malondialdehyde (MDA) as the end product of the lipid peroxidation reaction (Heath and Packer 1968, Loreto and Velikova 2001). Plant materials (0.2 g) were homogenized in 5 ml 0.1 % (w/v) trichloroacetic acid (TCA) solution on ice. The homogenates were centrifuged at 10,000 x g for 5 min at 4 ºC and the supernatants were collected in clean test tubes. 1 ml of 20 % (w/v) TCA containing 0.5 % (w/v) TBA was added to a 0.5 ml aliquot of the supernatant. The mixture was kept in boiling water for 30 min and immediately cooled on ice. After centrifugation at 10,000 x g for 10 min, the OD of the supernatant was taken at 532 nm and 600 nm. The absorbance at 600 nm was subtracted from the absorbance at 532 nm, and the MDA concentration was calculated using its extinction coefficient 155 mM^{-1} cm^{-1}

(Heath and Packer 1968). No significant readings were obtained without addition of the reactive TBA.

2. 17. *In vivo* detection of H_2O_2 by the DAB-uptake method and H_2O_2 measurements

For the localization of H_2O_2 generation as result of abiotic stress, leaf materials were cut from stress treated and unstressed plants and placed in 1mg/ml 3,3'-diaminobenzidine (DAB)-HCl, pH 3.8 (Sigma, a low pH is necessary in order to solubilize DAB) and adjust the pH to 7.5 after solubilization. Samples were incubated in a growth chamber for 8 h and cleared by boiling the leaves in 80 % (v/v) ethanol for 2 h and imbedded in 10 % (v/v) glycerol. The accumulation of H_2O_2 was observed as brownish stains in the leaves (Thordal-Christensen 1997).

For H_2O_2 measurement a modified method of Rao et al (2000) was used. Briefly, 200 mg of plant material was treated with the DAB up-take method as described above, followed by chlorophyll clearing by boiling the sample in 96 % (v/v) ethanol (20 min) and immediately homogenized in 1 ml of 0.2 M $HClO_4$ in a precooled pestle and mortar. The mixtures were incubated in ice for 5 min and centrifuge (10,000 g, 10 min, 4 °C). The optical density (OD_{450}) of the supernatants was measured as described by Tiedemann (1997) and the H_2O_2 concentrations were obtained via standard solutions of 0.2 M $HClO_4$, containing 5, 10, 25, 50 µMol H_2O_2 (Sigma), which were used to calibrate the data at the same OD (450 nm) during each assay run.

2.18. Assay for production of superoxide anions in plants exposed to stress conditions

The detection of the superoxide anion (O_2^-) was based on its ability to reduce nitroblue tetrazolium (NBT). Stress treated and untreated plant materials were immerged in 3 ml of 0.05 % (w/v) NBT, 10 mM sodium azide (NaN3) in potassium phosphate buffer pH 7.5 and incubated in room temperature for 1 h to allow a maximum reduction of NBT by the generation of O_2^- produced by the plants. The solution was then heated at 85 ºC for 15 min and immediately cooled on ice. The OD_{580} of the solution was then measured. The amounts of O_2^- generated in plants were expressed as increased absorbant unit (AU) per hour of reaction and per mg of seedling fresh weight (AU/h/mg Fwt) as described by Doke (1983).

Potassium phosphate buffer: 8.6 ml of A + 94.4 ml of B

A= 9.08 g/l KH_2PO_4, B= 1.88 g/l K_2HPO_4

2. 19. Detection of cell viability (Widholm 1972)

To assess cell viability, stress treated and untreated leaves from adult seedlings were imbedded in fluorescein diacetate staining solution. Fluorescein diacetate dye is absorbed only by viable cells and constitutes therefore a specific method to detect the levels of cell survival under stress conditions. Plant samples were submerged in a mixture of phenol, lactic acid, glycerol, distilled water (1:1:1:1) containing 0.01% (w/v) fluorescein diacetate from a stock solution of 0.1% (w/v) fluorescein diacetate in acetone stored at –

20 °C. The fluorescein diacetate stock solution should not be older than one week; otherwise a new stock solution must be prepared before use.

2.20. *GUS* staining of *Arabidopsis* plants

In order to identify the expression pattern of *ALDH* genes in the plants, young (13 day-old) and adult (4 week-old) transgenic plants carrying *GUS-ALDH* constructs were treated with NaCl (200 mM), dehydrated or treated with ABA for a period of 4 h in petridishes, while the control plants were treated with distilled water for the same period or directly used without treatment to analyse the expression and localization pattern of *GUS* activity. The *GUS* enzyme catalyses the cleavage of X-Gluc (a colourless substrate), which undergoes a dimerization leading to a final insoluble blue precipitate known as dichloro-dibromoindigo (ClBr-indigo). The ability of ClBr-indigo to immediately precipitate upon formation was used to trace the location site of *GUS* activity under the control of the *ALDH* promoters, allowing thereby an analysis of tissue specific localization of gene expressions.

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

Treated and untreated *Arabidopsis* plants were incubated overnight at 37 ºC in *GUS*-staining solution (a minimum volume of the staining solution was used). The seedlings were thereafter incubated in two changes of 80 % (v/v) ethanol solution at 80 ºC to distain the chlorophyll. The seedlings were finally submerged in 10% (v/v) glycerol and a photograph of the seedlings was taken to show the expression pattern of the *ALDH* genes in the plants.

GUS-staining solution: 3 mM X-Gluc (0.075 g/50 ml) 50 mM NaH2PO4 buffer pH 7.2 (always freshly prepared) 0.1% (v/v) Triton x100 (0.5 ml of 10% (v/v) Triton stock solution) 8 mM â-ME (28 µl/50 ml).

The staining solution was made in 50 ml falcon tubes and care was taken to minimize the use of excessive staining solution.

2.20 .2. Fluorometric *GUS* activity assay

The fluorometric *GUS* activity was carried out from homogenized wild-type and transgenic seedlings after the indicated period of ABA treatment (100 µM), NaCl (200 mM) or dehydration treatment according to a modified method of Jefferson et al (1987). 100 mg of plant material was homogenized in 100 µl extraction buffer (50 mM sodium phosphate, pH 7, 10 mM EDTA, 0.1 % (v/v) Triton x 100, 0.1 (v/v) Na-lauryl sarcosine) and centrifuged (14000 x g, 4 C) for 10 min. 10 µl aliquots of the supernatant were incubated in 50 µl of 1mM 4-methylumbelliferyl-glucuronid (4-MUG, Sigma) at 37 C. The mixtures were removed periodically and added to 1 ml stop buffer (0.2 M Na-carbonate: Na_2CO_3, pH 9.5) and then used to perform the assay. Standard solutions of Na_2CO_3, pH 9.5, containing 5, 10, 25, 50 nmol 4-methylumbelliferone (4-MU) were used to calibrate the data during each running assay. Using a RF-1501 spectrofluorophotometer (Shimadzu) with an excitation at 365 nm and a measuring emission at 455 nm, the specific *GUS* activity was expressed in 4-MU pmol/min/mg protein extract. Protein concentrations were determined using the Bradford-protein assay

in a Biorad kit (Bradford 1976). The data recorded represent mean values of triplicate experiments.

3. RESULTS

3.1. Involvement of *ALDH* genes in stress tolerance mechanism

Before investigating the role of aldehyde dehydrogenase in stress tolerance processes, *ALDHs* were first analysed for their involvement in plant responses to abiotic stress. In these experiments, wild-type plants were exposed to various abiotic stressors and used to analyse the *ALDH* mRNA accumulation profile and the corresponding protein accumulation pattern in stressed and unstressed plants. A direct involvement of these genes in stress-response mechanisms is expected to result in an up-regulation of these genes and the accumulation of their corresponding proteins under stress conditions.

3.1.1. Induction of *ALDH* genes under various abiotic stressors

The transcript accumulation of *ALDH* genes was investigated in wild-type *Arabidopsis* plants under dehydration and different concentrations of NaCl treatment. RNA blot analyses showed a progressive accumulation of *ALDH3I1* transcripts from 4 h of dehydration treatments (Figure 2 b). Under NaCl treatment, a significant increased accumulation of *ALDH3I1* was observed from 2 h of salt (250 mM NaCl) treatment (Sunkar et al 2003) as shown in Figure 2 c. An increasing accumulation of *ALDH3I1* mRNA transcript was observed in WT plants from 200 mM of NaCl treatment after 24 h of different concentrations of NaCl exposure (0 to 400 mM) as shown in Figure 2 a. For further molecular studies, a concentration of 200 mM NaCl was selected for salt stress because higher concentrations of salt stress damaged the leaf materials for subsequent biochemical analyses such as histochemical localization of *GUS* activity in the plants exposed to stress conditions.

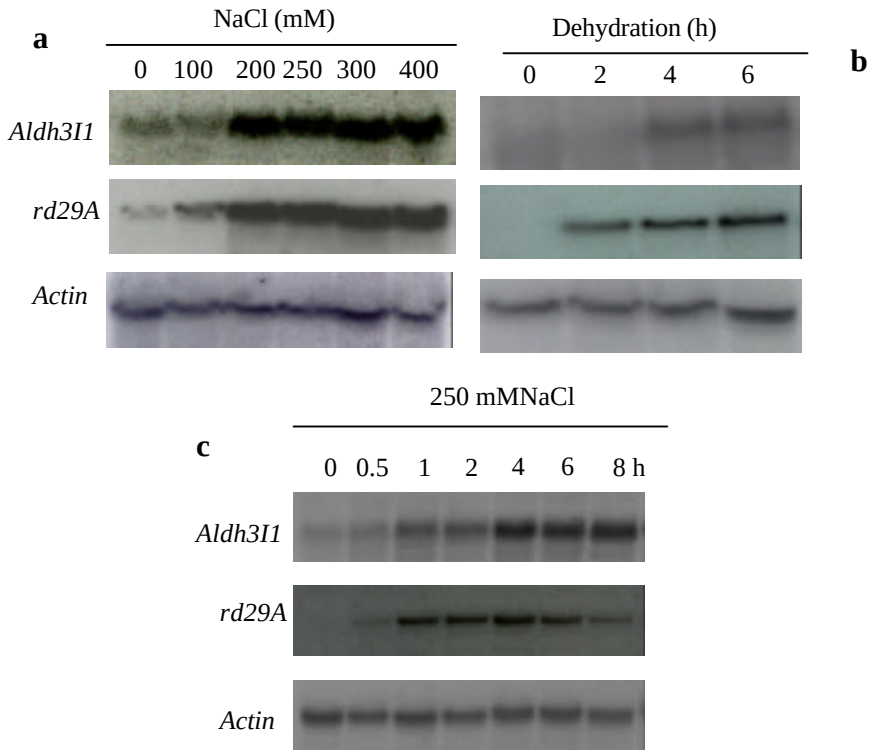


Figure 2: Induction of the *ALHD3I1* gene in wild-type plants exposed to dehydration and salt stress
rd29A = stress-responsive gene in Arabidopsis plants, used to monitor the osmotic stress status of the plants (Yamaguchi-Shinozaki and Shinozaki 1993), Actin = actin probe was used to confirm the equal loading of total RNA samples in the gel. *ALDH3I1*-cDNA (870 bp) was used as probe to detect the *ALDH3I1* transcript accumulation in RNA blot analyses. The temporal accumulation pattern of *ALDH3I1* transcripts (c) in response to NaCl (250 mM) was adopted from Sunkar et al (2003), with permission.

3.1.2. ALDH protein accumulation pattern under stress conditions

3.1.2.1. Production of ALDH3I1 antibody

For ALDH protein detection in plants, a specific ALDH antibody was raised and used in different protein blot analyses to gain insight into the kinetic expression of the protein under stress conditions. In order to raise the specific ALDH3I1 antibody, the coding region of the *ALDH3I1* gene (1.5 kb) was amplified and ligated (at EcoRI site) to the N terminal GST of pGEX 5.1 vector as illustrated in Figure 3. The construct was used to

transform *E. coli* BL21 and grown at 37 °C for 3 h in the presence or absence of IPTG used as protein inducer. SDS-PAGE analysis of *E. coli* BL21 protein followed by Coomassie blue staining confirmed the production of ALDH3I1 protein band (54 kDa) from the crude protein extract analysis (Figure 4 a). The band was excised, purified and used as antigene to raise the production of antibody during immunization of rabbits. The antibody raised recognizes the ALDH3I1 protein band (54 KDa) in a protein blot analysis experiment of *E. coli* BL21-protein extract under IPTG induction (Figure 4 c). No band was detected with the pre-immune ALDH3I1 serum (Figure 4 b). The immunoreaction was therefore specific because the antibody could uniquely compete with the ALDH3I1 protein used for the immunization (Figure 4 c). Under IPTG induction, the different protein sizes; GST (about 30 KDa), *ALDH3I1* insert (54 KDa), and GST-*ALDH3I1* fusion (84 KDa) were clearly identified as a result of partial digestion of the recombinant protein construct during the incubation of *E. coli* BL12 (Figure 4 c).

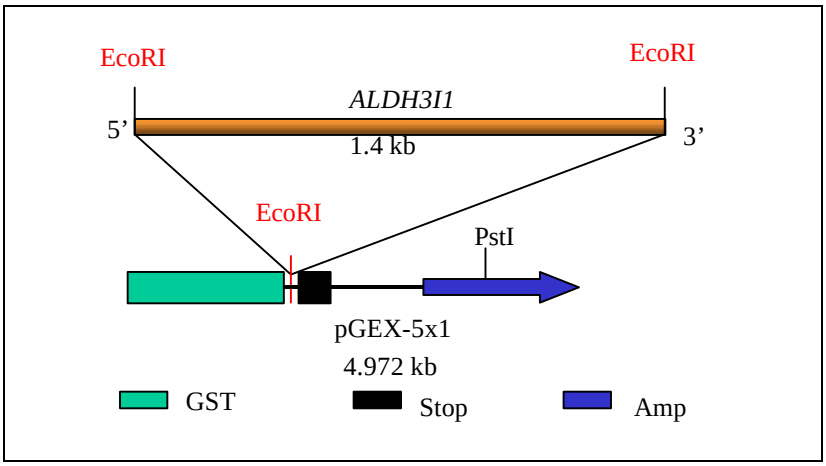


Figure 3: *GST-ALDH3I1* cDNA fusion construct in pGEX-5x1 vector
The coding sequence of *ALDH3I1* was amplified and ligated at the EcoRI site of the N terminal GST site of pGEX-5x1 and cloned in *E. coli* BL21.

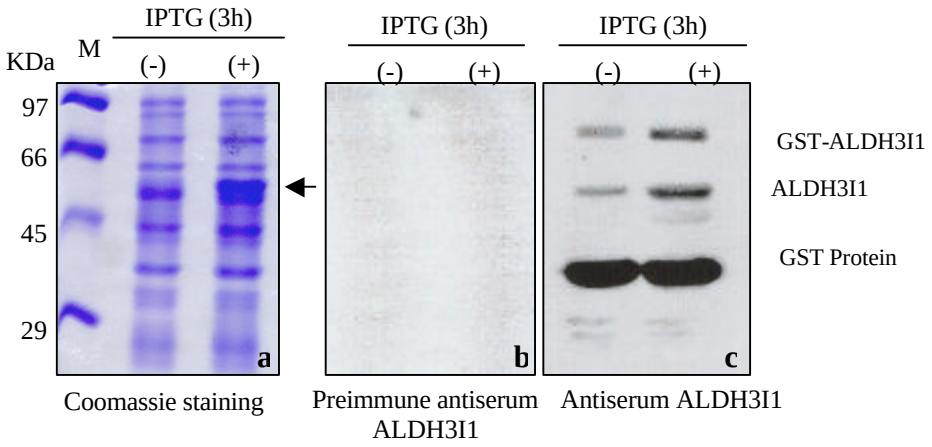


Figure 4: Production of the ALDH3I1 antibody and detection of induced ALDH3I1 protein in *E. coli* BL21

ALDH3I1 protein was excised from the SDS-PAGE gel (see arrow), purified and used to raise specific ALDH3I1 antibody in rabbits. Preimmune antiserum shows no background (b) in protein blot analysis of BL21-protein extract, while the ALDH3I1 antibody recognised the expected protein bands after IPTG induction (c). The arrow indicates the induction of ALDH3I1 protein (54 kDa) in SDS-PAGE staining with Coomassie blue. 50 ìg total protein was loaded per line in the g els.

3.1.2.2. Kinetic analysis of ALDH3I1 protein accumulation pattern

Wild-type Arabidopsis plants were subjected to various abiotic stresses (dehydration, salt stress, hydrogen peroxide, cold stress, paraquat, copper, and ABA treatment) over 72 h of stress exposure and used to investigate ALDH3I1 protein accumulation pattern via protein blot analyses (Figure 5). ALDH3I1 protein accumulated in response to all stress treatments but was differently induced by the stress conditions. ALDH3I1 protein was strongly expressed during dehydration, salt stress and heavy metal (copper) treatment, but weakly expressed in response to cold treatment (Figure 5 a). In order to gain insight into the regulatory mechanism of the protein induction pattern, several reagents such as ABA, ROS (hydrogen peroxide), and paraquat were used to trigger the expression of the protein.

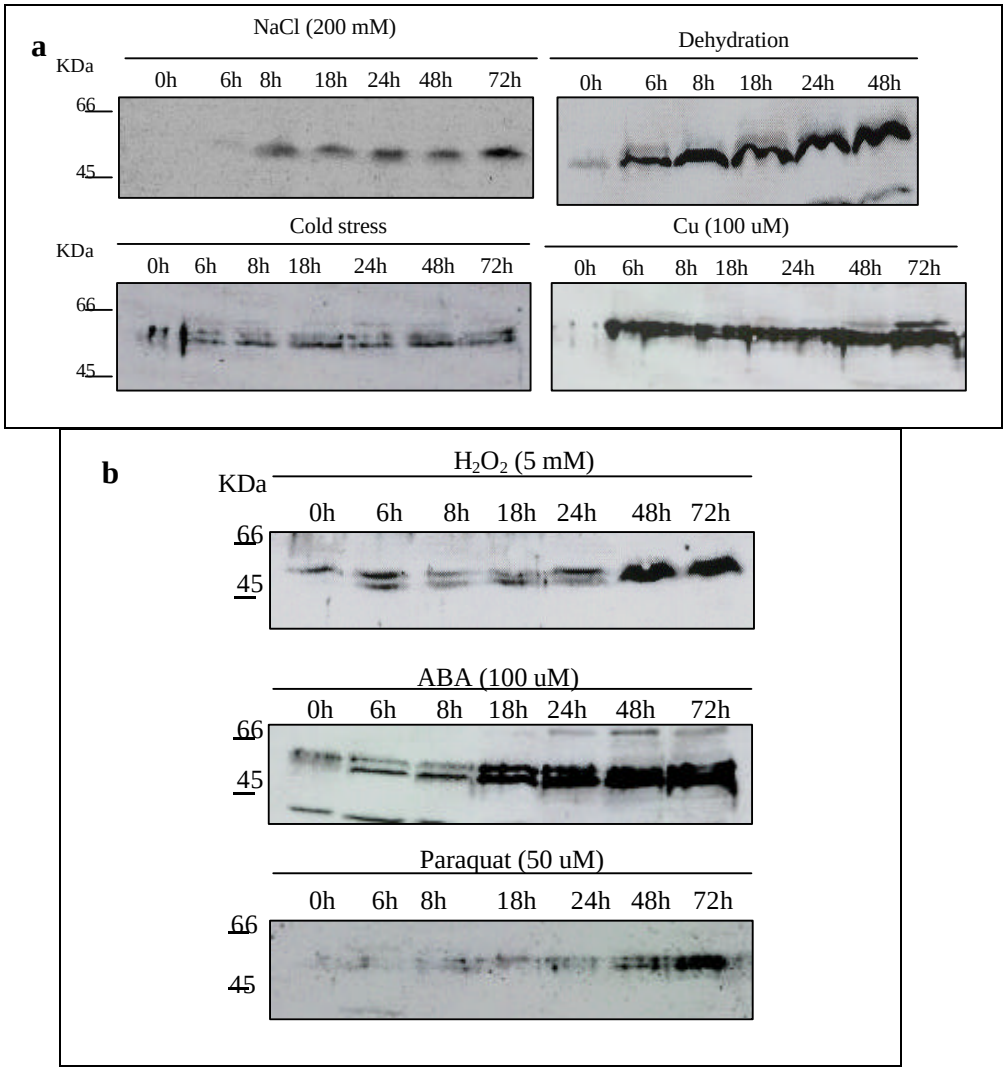


Figure 5: Kinetic analysis of the ALDH3I1 protein accumulation under various abiotic stressors
Wild-type plants were exposed to different abiotic stressors for indicated time period and used to analyse ALDH3I1 protein expression (a). ABA, H_2O_2 and paraquat applications mediate the accumulation of ALDH3I1 protein (b). The cold stress was imposed to the plants by incubating them in cold room (4 °C) for indicated time period.

Figure 5 b revealed the accumulation of two strong protein bands under ABA, hydrogen peroxide and paraquat (methyl viologen) treatment (a chemical that induces oxidative stress). The detection of the second band could be interpreted by a probable degradation of the ALDH3I1 protein, which occurred during the protein extraction from the plant or as the precursor of the active accumulating ALDH3I1 protein, which could still be

recognized by the antibody. The protein detection mediated by ABA and hydrogen peroxide was similar to the protein accumulation pattern observed under dehydration and salt stress. These results suggest that the ALDH3I1 protein expression pathway is probably cross-linked to the ABA biosynthesis pathway and the accumulation of ROS (H_2O_2) in plants exposed to environmental stress.

3.2. Molecular characterization of transgenic plants

The analysis of *ALDH*-transcript and ALDH-protein accumulation has revealed that the *ALDH3I1* gene is responding to a range of abiotic stress conditions. It was then planned to investigate the biological role of *ALDH* genes in plant responses to those stresses. Several independent transgenic Arabidopsis plants carrying different constructs of *ALDH* cDNA sequences both in sense and antisense orientation were thus used in molecular and physiological studies to gain insight into the role of *ALDH* genes in stress tolerance mechanisms.

3.2.1. Characterization of different plasmid DNA constructs

For the generation of transgenic plants, the full length of different *ALDH*-cDNA sequences encoding *ALDH3I1* and *Cp-ALDH* were fused to the constitutive CaMV-35S gene promoter in the pBIN19 vector in sense or antisense orientation (Kelbert 2000, Heuft 2000) as shown in Figure 6. These constructs were stably transferred into wild-type Arabidopsis thaliana via *Agrobacterium tumefaciens*-mediated transformation (Kelbert 2000, Heuft 2000). In addition, a stress-inducible promoter (C2-promoter) isolated from a resurrection plant *Craterostigma plantagineum* (Ditzer 2003) was alternatively used to

replace the constitutive CaMV-35S promoter and fused to *ALDH3I1*-cDNA (sense orientation) in pBIN19 (Figure 7), in order to generate stress-inducible *ALDH3I1* transgenic plants. In addition, *ALDH7B4*-cDNA was cloned from pBluescript (pBs) and ligated to the constitutive CaMV-35S promoter at the SmaI site of the pROK2 vector as shown in Figure 8. The binary pROK2 vector is a derivative of pBIN19 used for *Agrobacterium tumefaciens* transformation.

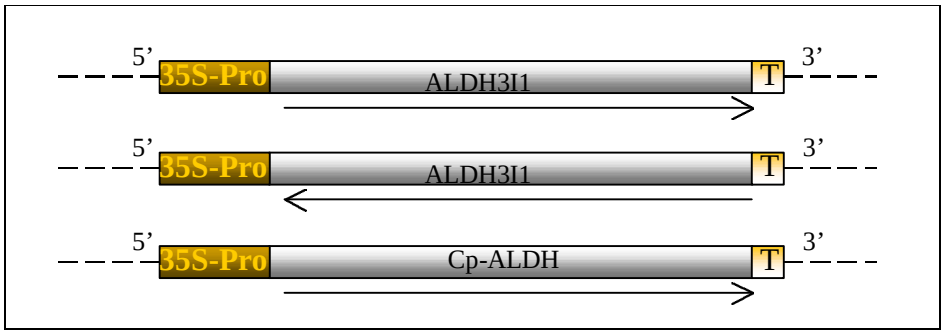


Figure 6: *ALDH*-cDNA constructs in pBIN19 for *Agrobacterium tumefaciens* transformation
35S-Pro: Constitutive CaMV-35S gene promoter; T: stop codon (terminator)
ALDH3I1 →: *Arabidopsis ALDH3I1* gene inserted in sense orientation
ALDH3I1 ←: *Arabidopsis ALDH3I1* gene inserted in antisense orientation
Cp-ALDH →: *Craterostigma ALDH* gene inserted in sense orientation
(Ref. Kelbert 2000, Heuft 2000)

All constructs were checked for correct orientation by specific genomic DNA digestion, DNA blot analysis (Figure 7, 8) and confirmed by DNA sequencing. The constructs were used to stably transform wild-type Arabidopsis plants via *Agrobacterium tumefaciens*-mediated transformation. The insertion of transgenes in the host plants was checked by PCR amplification analyses and DNA blot analyses in T2 progeny plants. Subsequent molecular and physiological analyses were carried out on transgenic plants from independent lines of the T2 or T3 generations.

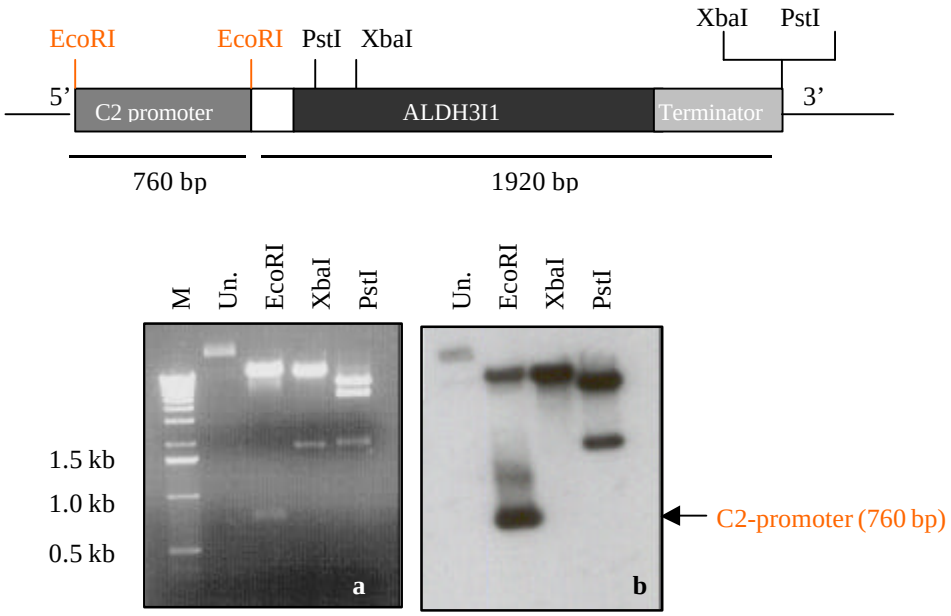


Figure 7: *C2 promoter-ALDH3I1* cDNA construct in pBIN19
The recombinant pBIN plasmid was digested with the indicated restriction enzymes. The photograph (a) shows the Et.br. staining of the agarose gel, and (b) shows the autoradiograph of the membrane after probing the blot with the ^{32}P-labeled 760 bp EcoRI-EcoRI C2 promoter fragment. M = marker, Un. = Undigested recombinant pBIN vector.

Table 2 summarizes the names of the transgenic plants used in this work and the origin of the transgenes in all transgenic plant populations generated. The letters **S**-**A3** in the name indicates a transgenic plant carrying a ***35S***-*ALDH****3I1*** cDNA sense orientation. The initial **AS**-**A3** indicates a transgenic plant carrying a ***35S***-*ALDH****3I1*** cDNA construct in **antisense** orientation. **C**-**A3** indicates a transgenic plant carrying a ***C2 promoter***-*ALDH****3I1*** cDNA construct in sense orientation. **CP** indicates a transgenic plant carrying a ***35S-CpALDH*** cDNA construct in sense orientation. **S**-**A7** indicates a transgenic plant carrying a ***35S***-*ALDH****7B4*** cDNA construct in sense orientation. The initial **knock** indicates ***knock-out T-DNA insertions*** in the ***ALDH3I1*** coding sequence. The letter **P** in the name indicates **the independent transgenic plant** (Table 2).

Table 2: Nomenclature and molecular characterizations of the transgenic plants

Number of transgenic plants analysed	Name of transgenic plants*	cDNA constructs or T-DNA insertion mutants	Origin of transgene
6	**S-A3P1-6**	*335S-ALDH3I1* cDNAsense	*Arabidopsis thaliana*
12	**AS-A3P1-12**	*35S-ALDH3I1* cDNA antisense	*Arabidopsis thaliana*
3	**C-A3P1-3**	*C2 promoter-ALDH3I1* cDNA	C2 promoter is from *C. plantagineum* and ALDH3I1 from *A. thaliana*
4	**Knock1-4**	T-DNA (KONCZ 16843) *ALDH3I1*	
3	**S-A7P1-3**	*35S-ALDH7B4* cDNA sense	*Arabidopsis thaliana*
12	**CP1-12**	*35S-CpALDH* cDNA sense	*Craterostigma plantagineum*

* S-A3 = sense *35S-ALDH3I1* cDNA construct; AS-A3 = antisense *35S-ALDH3I1* cDNA construct; C-A3 = sense *C2 promoter-ALDH3* cDNA construct; Knock = T-DNA knock-out insertion of *ALDH3I1*; S-A7 = sense *35S-ALDH7B4* cDNA construct; CP = sense *35S-CpALDH* cDNA construct; P = independent transgenic plant.

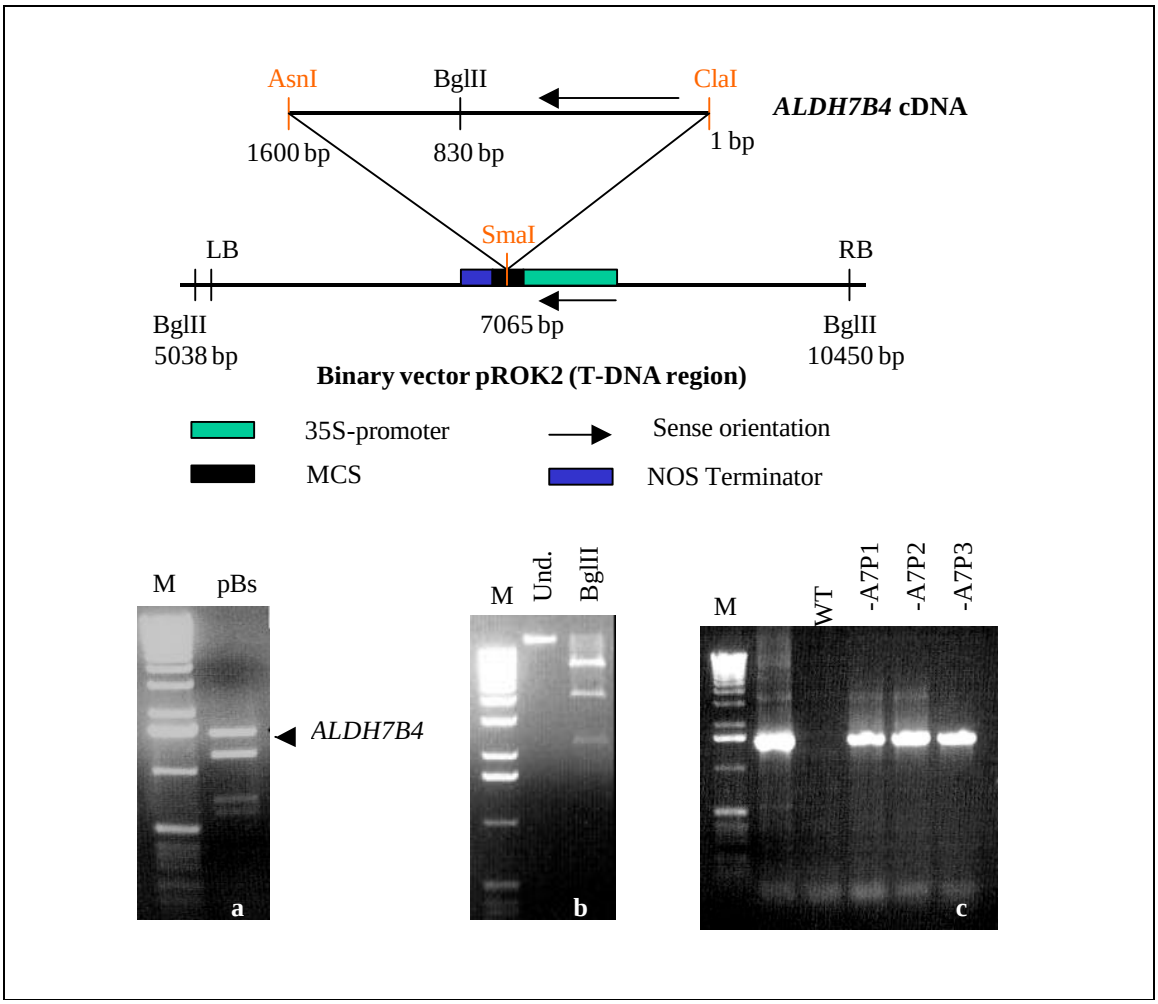


Figure 8: *35S-ALDH7B4* construct in pROK2 binary vector and detection of transgene in the transgenic plants.
ALDH7B4-cDNA was cloned from pBluescript (pBs) vector (digested with ClaI, SnI, SspI) as shown in the gel (a), and inserted at the SmaI site (MCS) of pROK2 and checked for correct orientation by DNA sequencing and BglII digestion of the recombinant pROK plasmid (gel b). *ALDH7B4* transgenes were checked and confirmed in transgenic plants by PCR amplification of the insert in the plant genomic DNA (gel c). Und. = Undigested recombinant pROK2 vector.

CaMV-35S gene is a constitutive promoter leading to a constitutive expression of the inserted gene, while the C2-promoter induces the expression of the inserted gene only under stress conditions. The antisense orientation of the inserted gene under the control of the constitutive promoter leads to the expression of the complementary strand (RNA_{as}) of the endogenous *ALDH3I1* gene. It is expected that the heterologous cDNA is sufficient

for mRNA suppression. This strategy aimed to complement the endogenous *ALDH3I1*-DNA strand (RNA_s: the coding sequence of *ALDH3I1* gene) in the plants and to repress the protein formation. The result should lead to a double RNA_s-RNA_{as} strand status, which is not translatable by the ribosomal machineries into ALDH protein (Baier and Dietz 1999). To produce high-suppression intensities, the *ALDH3I1* cDNA was fused to the constitutive CaMV-35S promoter (Holtorf et al 1995). The antisense *ALDH3I1* transgenic plants were therefore expected to show a suppressive status of *ALDH3I1* gene expression.

3.2.2. Characterization of T-DNA insertion mutants of *ALDH3I1* knock-out transgenic plants

The expression of the *ALDH3I1* gene was knock-out by T-DNA insertions in the coding region of the *ALDH3I1* gene in order to study the subsequent effects of loss of ALDH function in plants exposed to abiotic stress. Four independent homozygous knock-out lines (knock#1, 2, 3, 4) carrying a T-DNA (KONCZ 16843) insertion in the second intron (position 27470 bp) of the *ALDH3I1* gene (Szabados and Koncz 2003, Schlingensiepen 2003, Kirch and Koncz, unpublished) were investigated under stress conditions. The number 16843 indicates the number of the independent transgenic plant with the knockout T-DNA insertion for the *ALDH3I1* out of the whole T-DNA insertion collection (Szabados and Koncz 2003). Figure 9 shows the genomic structure of the *ALDH3I1* gene and the position of the T-DNA insertions. PCR analysis of the T-DNA insertion in this line (16843) revealed that the T-DNA was inserted as two copies in inverse orientation with the RB of each T-DNA copy at the centre and their LB at the periphery as indicated

by the arrows in the Figure 9 (Schlingensiepen 2003). The number of introns and exons in *ALDH3I1* gene were deduced from the sequence alignment of the *ALDH3I1* cDNA and the genomic sequence of the *ALDH3I1* gene using the Vector NTI™ Suite programme. The progeny of the knock-out mutants were checked and confirmed for homozygocity by RT-PCR analysis (Ditzer, unpublished). These T-DNA insertions interrupt the coding sequence of the gene leading therefore to a non-active ALDH3I1 protein. ALDH3I1 protein accumulation was completely absent in those knock-out mutants even under salt stress treatment as shown by the results of protein blot analysis (Figure 18). These knock-out mutants were therefore regarded as good negative candidates in comparison with the transgenic plants overexpressing *ALDH* genes for studying the role of *ALDH* genes.

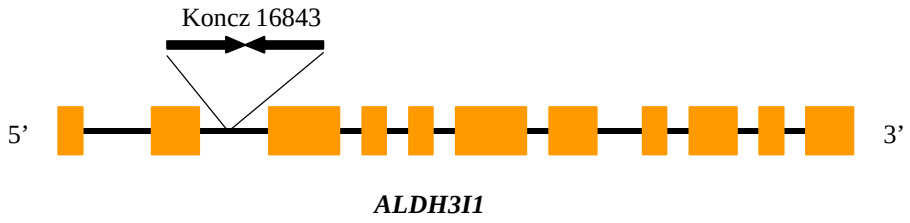


Figure 9: *ALDH3I1* gene structure and the location of the T-DNA insertion.
The orange boxes represent the exons, while the black lines represent the introns in the *ALDH3I1* gene sequence.

3.2.3. DNA blot analysis of transgenic plants

In order to investigate the number of *ALDH* transgene copies in the transgenic plants, genomic DNAs were digested with EcoRI restriction enzyme in independent experiments and probed with ^{32}P-labelled *NPTII* (the gene encoding for kanamycin resistance) or

probed with other specific probes (C2 promoter-probe) designed to detect the transgene copies. Figure 10 shows the number of bands in independent transgenic lines corresponding to the number of transgene copies stably incorporated into the transgenic plants.

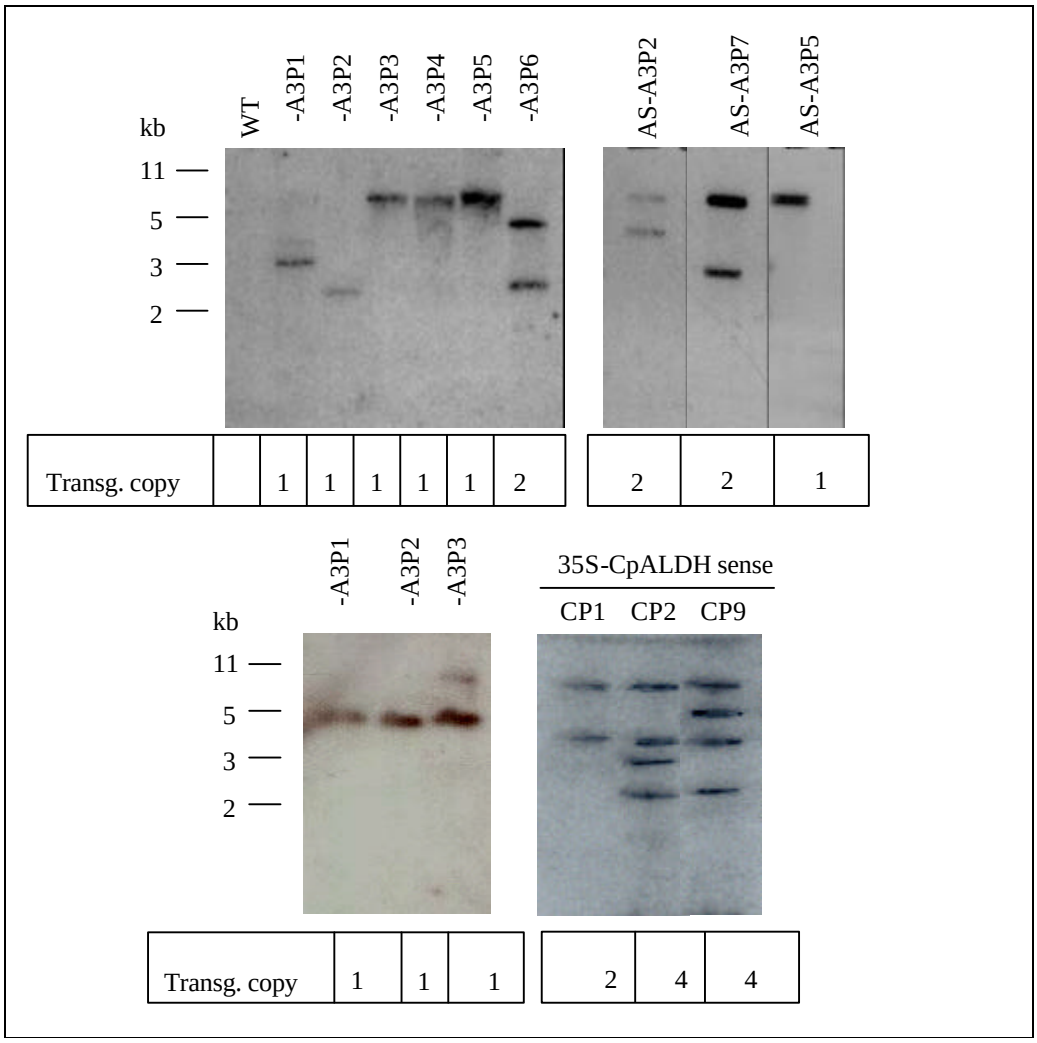


Figure 10: DNA blot analysis of transgenic *Arabidopsis* plants
Genomic DNA samples were digested with EcoRI and probed with the ^{32}P-labeled *NPTII* probe (for *35S-ALDH3I1* sense/antisense, *35S-CpALDH* sense). The ^{32}P-labeled C2 promoter (580 bp) probe was used for the *C2-ALDH3I1* sense transgenic plants. The sizes of molecular mass markers are indicated at the margin. Transg. copy = number of transgene copies stably integrated into the host genomic DNA.

3.2.4. *ALDH* promoter-*GUS* reporter gene constructs

In order to investigate the *ALDH* gene expression pattern in plant responses to abiotic stress, the *GUS* reporter system was used. Various *ALDH* promoters were isolated by designing specific forward and reverse primers and fused to the *GUS* reporter gene (Table 3). The promoters were cloned into the pCR cloning vector (pCR2.1), sequenced from both ends and subsequently fused to the reporter gene (*GUS*) in the pBT10 vector as described in Table 3. These constructs were inserted into the pBIN19 vector or directly used from the pBT10 vector to transform Arabidopsis plants via *Agrobacterium tumefasciens*-mediated transformation.

Table 3: Primers designed to construct the *ALDH promoter-GUS* reporter genes.

PCR amplification of the *ALDH* promoter	Designed Primers for *ALDH* promoter amplification	Insertion sites in pB-10*GUS* plasmid
ALDH3I1 prom. (0.9 kb)	Fwd.: 5'TGAAGATCGGTGTGGCAGATTCCA3' Rev.:5' ACTTCGTCAT**GAATTC**GGTTCAG 3'	HindIII/EcoRI
ALDH3H1 prom. (1.52 kb)	Fwd.: 5'TGCATCACACAATGACAACTTTACTC3' Rev.: 5'TCCTCAATATCTCTCTTACGTAACG3'	XhoI/SpeI
Cp-ALDH prom (0.9 kb)	Fwd.: 5'CAGAATAGTAGGC**AAGCTT**TC3' Rev.: 5'ACGC**GTCGAC**TTCCTTTTATTCTTTTG3'	HindIII/SalI

The promoter sequence analysis of *ALDH3I1*, *ALDH3H1* and *CpALDH* genes revealed some putative cis-elements at various locations in the promoters. A comparative analysis of putative cis-elements in these promoters is shown in Table 4. The promoter of the *ALDH3H1* gene, which is constitutively expressed (Figure 11, 12, Kirch et al 2001) lacks DRE like core motif, while the *ALDH3I1*-promoter and the *Cp-ALDH* promoter contain a DRE like core motif (Table 4) (For detail, see promoter sequences in appendices: section

5). DRE like core motifs (drought responsive elements) are motif elements mainly responsible for the upregulation of genes under drought and salt stress, while ACGT-like ABRE motifs are mainly responsible for the upregulation of genes under ABA treatment (Ingram and Bartels 1996).

Table 4: Putative *cis*-elements present in *ALDH31, ALDH3H1* and *Cp-ALDH promoters*.

DRE like core motif	G Box like	ACGT-like ABRE motif	TATA Box
CCGAC	CACGTG	ACGT	TATA
(-147 bp)	(-549 bp)	(-115 bp)	(-47 bp)
		(-200 bp)	
		(-740 bp)	
none	(-1050)	(-170 bp)	(-48 bp)
	(-1070)	(-880 bp)	
		(-990 bp)	
		(-994 bp)	
(-147)	none	(-247)	(-95)

Orange boxes indicate the motif elements with their position in *ALDH3I1 promoter* sequence. The grey boxes indicate the motif elements with their position in *ALDH3H1 promoter* sequence. The black boxes indicate the motif elements and their position in the Cp-*ALDH promoter* sequence.

3.2.4.1. The *GUS* reporter gene system to study *ALDH* gene expression in plants

The bacterial â-glucuronidase gene (uidA or gusA) commonly referred to as *GUS* gene was used as reporter gene to study the expression pattern of plant-*ALDH* genes under salt stress, dehydration and ABA treatment. The promoter region of *ALDH* genes including

the ATG start codon of the *ALDH* structural gene sequences were fused to the *GUS* gene and transformed into Arabidopsis plants via *Agrobactrim tumefaciens*-mediated transformation. The histochemical localization of *GUS* activity in transgenic plants exposed to stress was carried out to gain insight into the regulatory mechanisms of *ALDH* gene expression. Transgenic plants screened under dehydration (4h) and 200 mM NaCl treatment (4h) indicated that the *ALDH3I1* gene (from *Arabidopsis thaliana*) and the Cp-*ALDH* gene (from *Craterostigma plantagineum*) were highly expressed mainly in leaves, while the *ALDH3H1* gene (from *Arabidopsis thaliana*) was mainly expressed in roots (Figure 11). Microscopic observations of stressed leaves and roots revealed that *GUS* activity under the control of *ALDH3I1* promoter is located in chloroplasts probably as a result of artificial diffusion of the blue precipitate (indigo) into chloroplasts (Figure 12).

In addition, *GUS* activity was quantitatively assayed using a fluorometric substrate 4-methylumbelliferone (4-MU) in order to estimate the expression level under stress conditions. Table 5 presents the levels of *GUS* activity in leaves and roots of independent seedlings exposed to different abiotic stresses. The *GUS* activity induced by *ALDH3I1* promoter is located in leaves and not in roots. This activity is induced 20-60 times higher in leaves as in roots and also 20-60 times higher in stress leaves than unstressed leaves (Table 5). In contrast, induction of *GUS* activity by *ALDH3H1* promoter shows a constitutive low expression in leaves and induced 10-20 times higher in roots under stress conditions (Table 5). In untreated plants, *GUS* activity is induced by *ALDH3H1* promoter 6-10 times higher in roots than in leaves (Table 5). In all growth conditions *GUS* activity was insignificant in wild-type plants (Table 5).

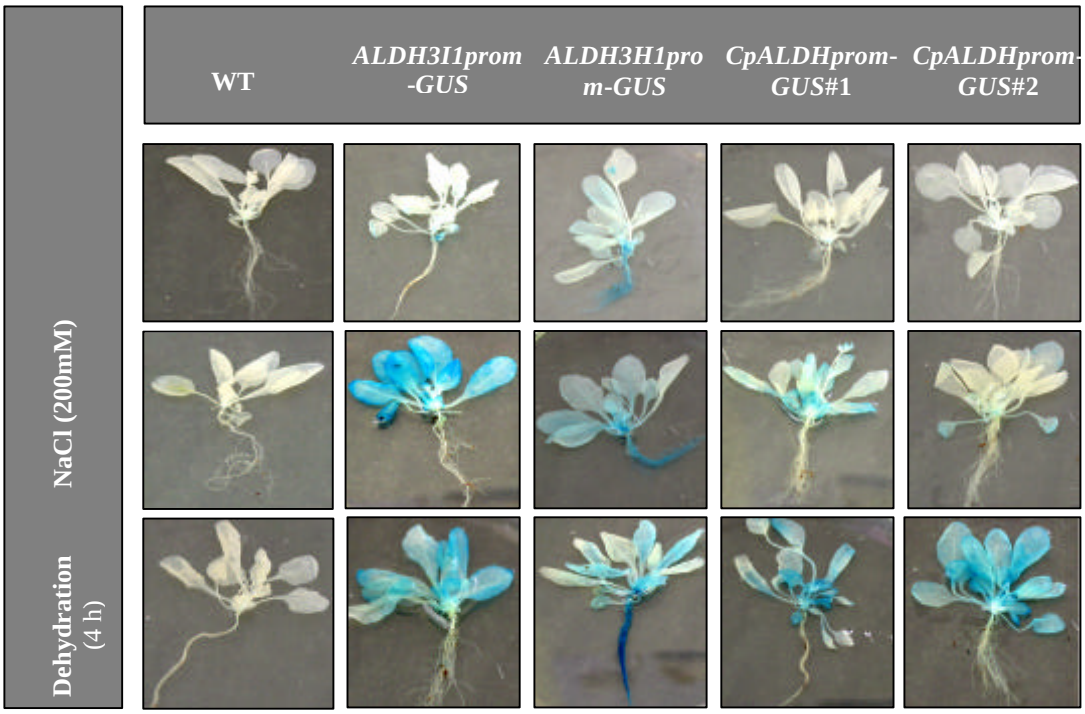


Figure 11: Histochemical localization of *GUS* expression in transgenic plants under salt stress and dehydration.
Four week-old seedlings grown on soil were subjected to the indicated stress conditions for 4h to avoid damage of the leaf materials during *GUS*-staining.

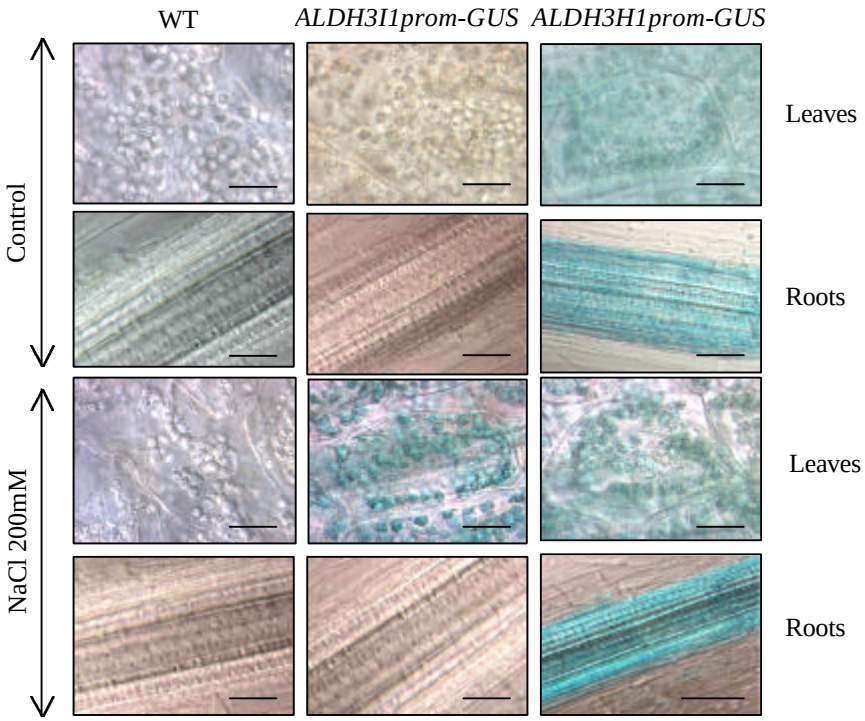


Figure 12: Microscopic analysis of *ALDH-GUS* gene expressions in plants exposed to salt stress treatments

Seedlings were subjected to salt stress conditions. Leaf and root materials were prepared from the treated and untreated plant samples and observed under microscope. Bars = 40 µm

Table 5: *GUS* activity in wild-type and transgenic plants exposed to dehydration, NaCl and ABA treatments

Plant materials	Stress conditions	Specific *GUS* activity (4-MU pmol/min/mg protein)					
		ALDH3I1prom-GUS			*ALDH3H1prom-GUS*		
		0 h	4 h	10 h	0 h	4 h	10 h
Leaves	ABA (100 ìM)	192 ± 13	3843 ± 30	6428 ± 35	277 ± 18	373 ± 26	390 ± 25
	NaCl (200 mM)	192 ± 13	4427 ± 47	7725 ± 41	277 ± 18	382 ± 29	393 ± 33
	Dehydration	192 ± 13	4265 ± 32	6260 ± 32	277 ± 18	352 ± 30	352 ± 27
Roots	ABA (100 ì M)	189 ± 16	199 ± 21	186 ± 13	778 ± 20	4007 ± 33	5742 ± 42
	NaCl (200 mM)	189 ± 16	197 ± 16	198 ± 18	778 ± 20	4095 ± 47	8040 ± 37
	Dehydration	189 ± 16	193 ± 19	193 ± 22	778 ± 20	4440 ± 60	7271 ± 44

Each value represents the average *GUS* activity (± SD) of triplicate experiments. *GUS* activity in WT plants was insignificant (94 ± 15 pmol 4-MU/min/mg protein) under all conditions tested and is not included in the Table.

Although ALDH3I1, ALDH3H1 and Cp-ALDH protein sequence analysis revealed 70 to 80% homology to each other (Kirch et al 2001, Figure 13), their gene expression patterns in plants exposed to stress are diverse. The differences observed in the putative cis-elements of the *ALDH*-promoter sequences (Table 4) may be partially responsible for the differences of *ALDH* gene expression patterns in the stressed plants. The presence of the DRE like core motifs in *ALDH3I1* and *Cp-ALDH* promoters indicates the stress inducible status of those genes (*ALDH3I1*, *Cp-ALDH*), and probably the reason why *ALDH3I1* and *Cp-ALDH* transcripts significantly increased under drought and salt stress treatment (Kirch et al 2001). The presence of ACGT-like ABRE motifs (ABA responsive elements) in all the promoters is probably the reason why these genes (*ALDH3I1*, *ALDH3H1*, *Cp-ALDH*) respond to exogenous application of ABA (Kirch et al 2001, Schlingensiepen 2003). However, *ALDH3H1* is also a stress-inducible gene and its expression is restricted to roots (Figure 12). These results suggest that putative *cis*-element analysis of the promoters is not sufficient to reveal detailed information regarding their stress inducible expression status in plants response to environmental stress.

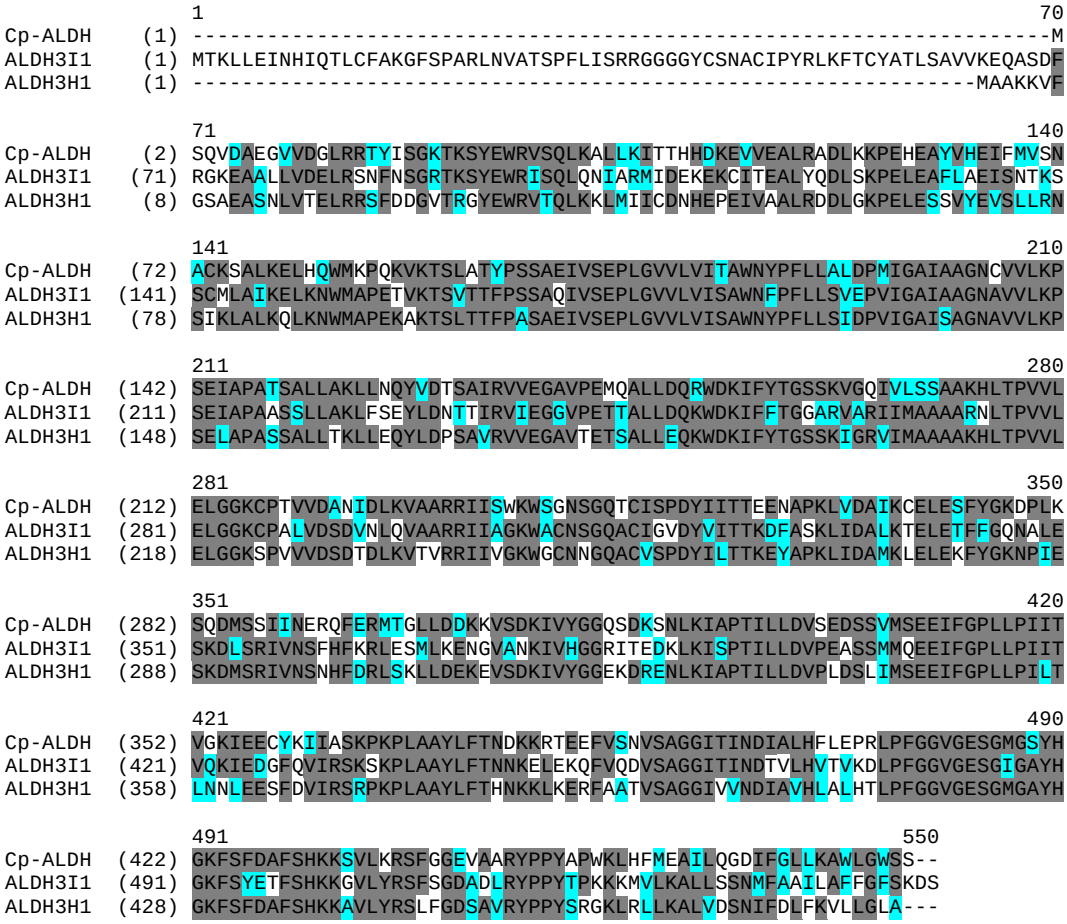


Figure 13: Amino acid sequence homology between ALDH3I1, ALDH3H1 and Cp-ALDH proteins. In the alignment, identical amino acids are shaded in grey and conserved sequence motifs are shaded in blue.

3.2.5. Expression analysis of *ALDH* genes in transgenic plants

To establish the relationship between *ALDH* genes and abiotic stress conditions, the expression of *ALDH* genes was analysed in the wild-type and transgenic plants exposed to various external stimuli. RNA blot analyses showed that the *ALDH3I1* gene was constitutively expressed in *35S-ALDH3I1* sense-transgenic plants (S-A3P1-6 except S-A3P5) (Figure 14). Likewise, *35S-CpALDH* sense transgenic plants (CP1 CP9) and *35S-*

ALDH7B4 sense transgenic plants (S-A7P1, S-A7P2, S-A7P3) constitutively expressed *Cp-ALDH* and *ALDH7B4* transcript respectively (Figure 14 and 16). However, the *35S-ALDH3I1* antisense transgenic plants showed a suppressive expression of the same gene as expected (Figure 14). These results indicate that antisense expression of *ALDH3I1* gene was able to repress the expression of endogenous *ALDH3I1* gene by a RNA-RNA complementation.

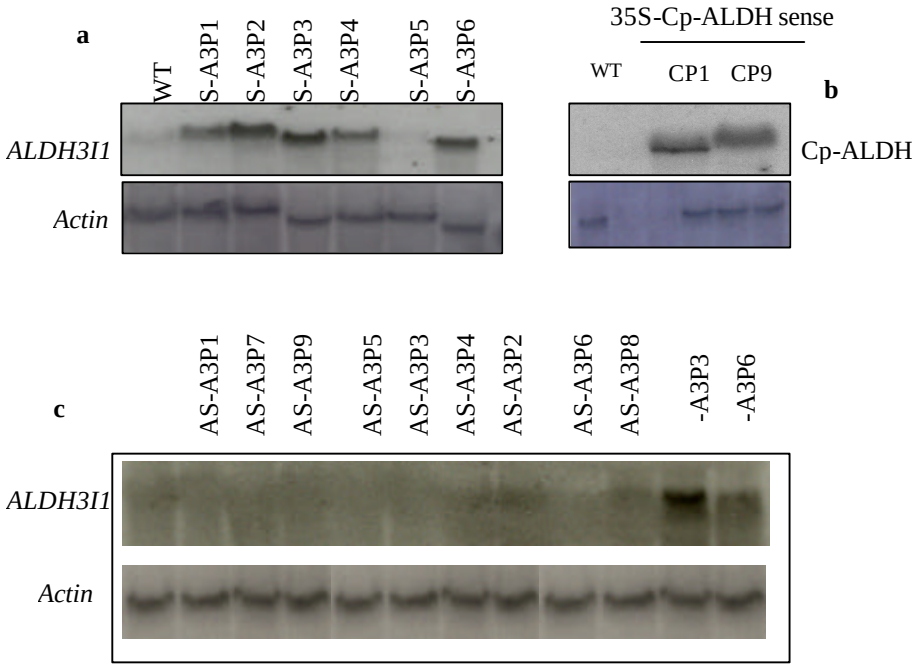


Figure 14: Expression of *ALDH* genes in non-stressed transgenic plants
(a): *ALDH3I1* transcript accumulation pattern in WT and transgenic plants (S-A3P1-6) carrying 35S-*ALDH3I1* cDNA sense construct, (b): *Cp-ALDH* transcript accumulation pattern in WT and transgenic plants (CP1, CP9) carrying the *35S-Cp-ALDH* cDNA sense construct, (c): *ALDH3I1* transcript accumulation parttern in the WT and transgenic antisense plants (AS-A3P1-9) and in transgenic sense plants (S-A3P3, S-A3P6). The actin transcript profile accumulation was used to monitor the equal loading of RNA in the gel.

ALDH3I1 and *ALDH7B4* genes were overexpressed under salt stress (NaCl 100 mM) in transgenic S-A3P- and S-A7P-transgenic plants, while transgenic antisense plants still exhibit a repressed expression of the *ALDH3I1* gene even under NaCl (200 mM)

treatment (Figure 15, 16). These results demonstrate that the transgenic plants really presented the expected phenotype at the transcriptional (production of *ALDH*-mRNAs) level (Figure 14, 15, 16). On the other hand, transgenic plants expressing *ALDH3I1* gene under the control of the C2 promoter showed a similar expression profile as wild-type plants under control conditions (non-stressed plants), but these plants showed an increasing accumulation of the *ALDH3I1* transcript and the corresponding protein under stress conditions as (Figure 17, 18). The upregulation of the *ALDH3I1* gene obtained in C-A3P-lines under salt stress conditions confirmed the expected expression pattern of those transgenic plants at the transcriptional (Figure 17) and translational level (Figure 18).

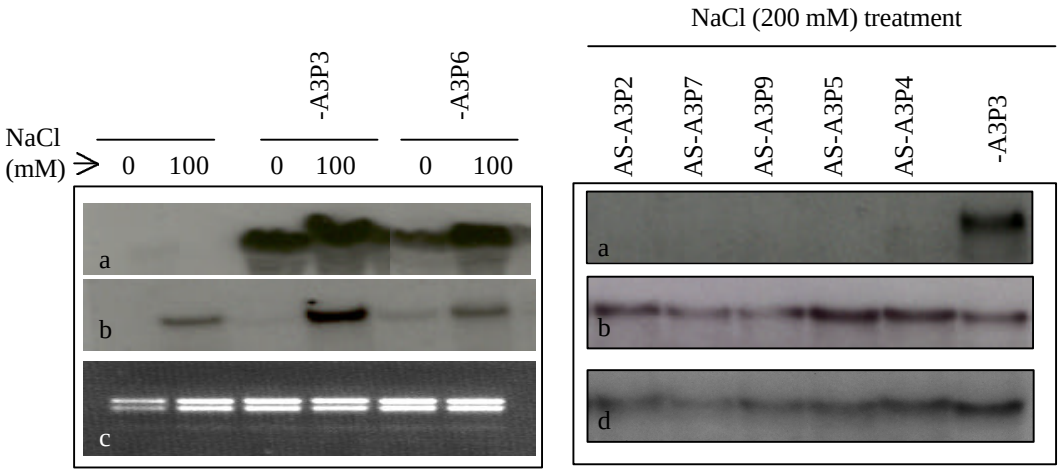


Figure 15: Expression profile of the *ALDH3I1* gene in transgenic sense and antisense lines under salt stress.
(a): *ALDH3I1* transcript expression, (b): rd29A transcript profile expression, (c): rRNA under ethidium bromide staining, (d): Actin transcript profile expression. Salt stress (NaCl) was applied to four week-old seedlings in soil for a period of 72 h.

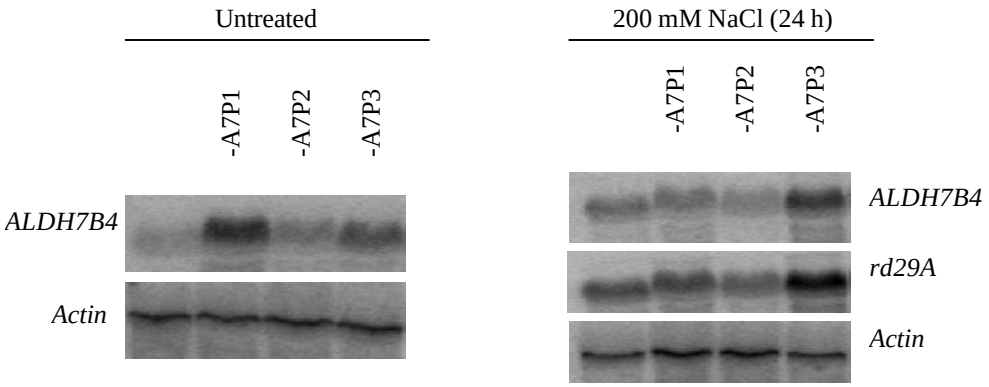


Figure 16: *ALDH7B4* transcript accumulation in wild-type and transgenic plants under stressed and non-stressed conditions
The *rd29A* probe was used to check the osmotic stress status in *Arabidopsis* plants, while the actin probe was used to check the equal amounts of RNA loaded in the gel.

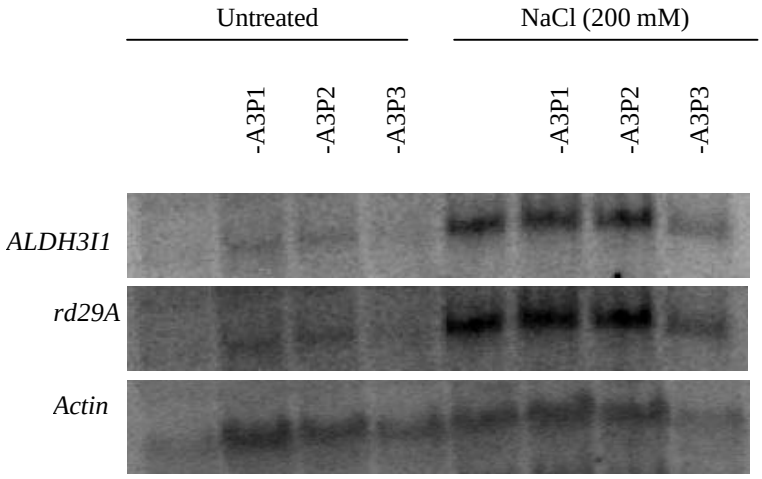


Figure 17: *ALDH3I1* transcript accumulation in wild-type and C-A3P-transgenic plants under stressed and non-stressed conditions.
The *rd29A* probe was used to check the osmotic stress status in *Arabidopsis* plants, while the actin probe was used to check the equal amounts of RNA loaded.

3.2.6. Analysis of ALDH-protein accumulation in transgenic plants

The expression of the *ALDH3I1* gene was investigated at the protein level in WT, transgenic *ALDH3I1* overexpressing plants (S-A3P1-6), transgenic stress-inducible *ALDH3I1* expressing plants (C-A3P1-3), and in *ALDH3I1* knock-out mutants (Knock1-

4). The plants were treated with salt stress (NaCl 200 mM) for 24 h and used for protein analysis. The results showed no ALDH3I1 protein accumulation in the knock-out mutants under control and NaCl (200 mM) treatment, while transgenic plants overexpressing the *ALDH3I1* gene (S-A3P1-6) showed a constitutive ALDH3I1 protein accumulation (Figure 18). On the other hand, transgenic plants expressing the *ALDH3I1* gene under the control of the stress inducible C2-promoter (C-A3P1-3) showed an increasing ALDH3I1 protein accumulation only under stress conditions. Their protein accumulation profile was similar to the wild type under control conditions (untreated plants) (Figure 18). These results indicate that all the transgenic plants showed the expected expression pattern at the protein level respectively.

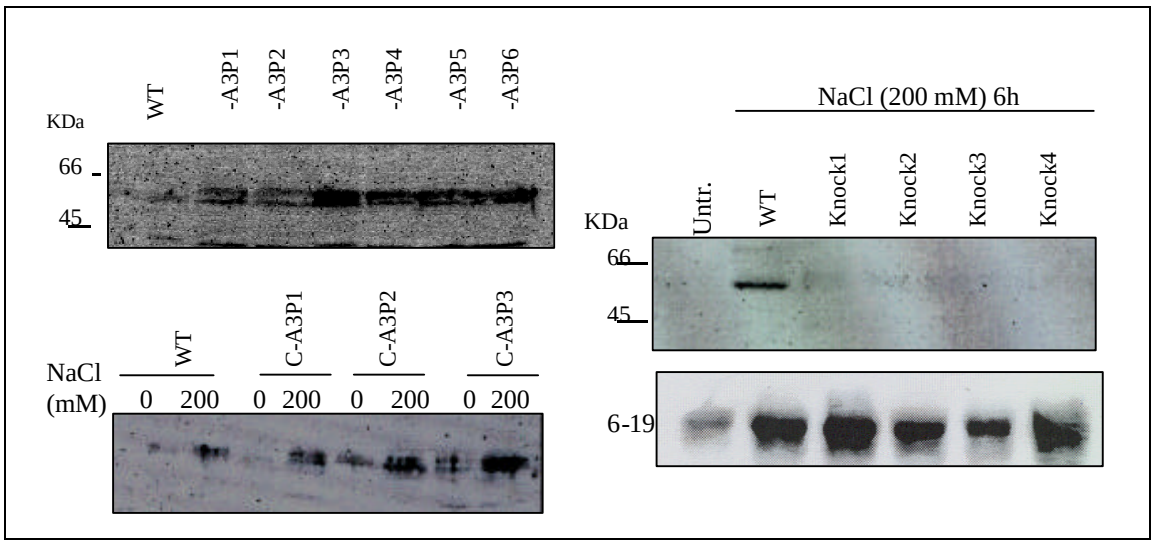


Figure 18: Constitutive and stress inducible accumulation of ALDH3I1 protein in transgenic *Arabidopsis* plants and loss of the protein in knockout mutants
Salt stress (NaCl 200 mM) was applied to adult seedlings (4 weeks old) in soil for a period of 72 h. Untr. = Untreated wild type seedlings. Expression of the 6-19 protein was investigated to confirm the salt stress status of the plants. Antibodies against 6-19 polypeptide from *C. plantagineum* also recognise *Arabidopsis thaliana* homologues (Ingram and Bartels 1996). 6.-19 cDNA encodes for a D11-LEA protein related polypeptide, which is upregulated under osmotic stress and ABA treatment (Piatkowski et al 1990).

3.3. Physiological and biochemical characterization of wild-type and transgenic plants exposed to various stressors

3.3.1. Plant responses to salt stress exposure

The response of plants to salt stress was checked in different media (MS-agar, soil and hydroponic cultures) containing different concentrations of NaCl and KCl. Figure 19 shows the seed germination and the seedling development of independent plants in MS-NaCl media. No growth differences were observed in wild-type and all transgenic plants in the absence of stress (control conditions), but significant growth differences appeared after two weeks of seedling development under stress (NaCl) conditions. All tested transgenic seeds overexpressing the *ALDH* genes grew faster than the wild type despite stress exposure, and developed their first true leaves faster than the wild-type plants (Figure 19 a, c). However, the antisense transgenic seeds and the knock-out mutants showed retarded growth under salt stress and generally failed to germinate at 150 mM NaCl (Figure 19 a, b). Table 6 summarizes the biochemical analyses (chlorophyll content, fresh weight accumulation, MDA accumulation) in the seedlings exposed to the stress. In antisense and knock-out mutants the MDA and Chlorophyll content were not determined at 100 and 150 mM NaCl because those seedlings failed to grow with true leaf development (Figure19, Table 6) and therefore sample collection for biochemical assay was not possible in those transgenic lines under such stress conditions. This observation suggests that antisense and knock-out mutant plants are more sensitive to salt stress than the wild-type and transgenic plants overexpressing *ALDH* genes. Under salt treatments, the level of lipid peroxidation expressed as result of MDA measurements was higher in WT plants than in the transgenic plants overexpressing *ALDH* genes (Table 6).

Inversely the chlorophyll contents and fresh weight accumulation in transgenic plants overexpressing *ALDH* genes were higher than in WT (Table 6), indicating that transgenic S-A3P-lines, S-A7P-lines and C-A3P-lines showed a reduced level of oxidative stress and were able to cope with the stress conditions better than the wild-type, antisense and *ALDH3I1* knock-out mutant seedlings (Figure 19, Table 6).

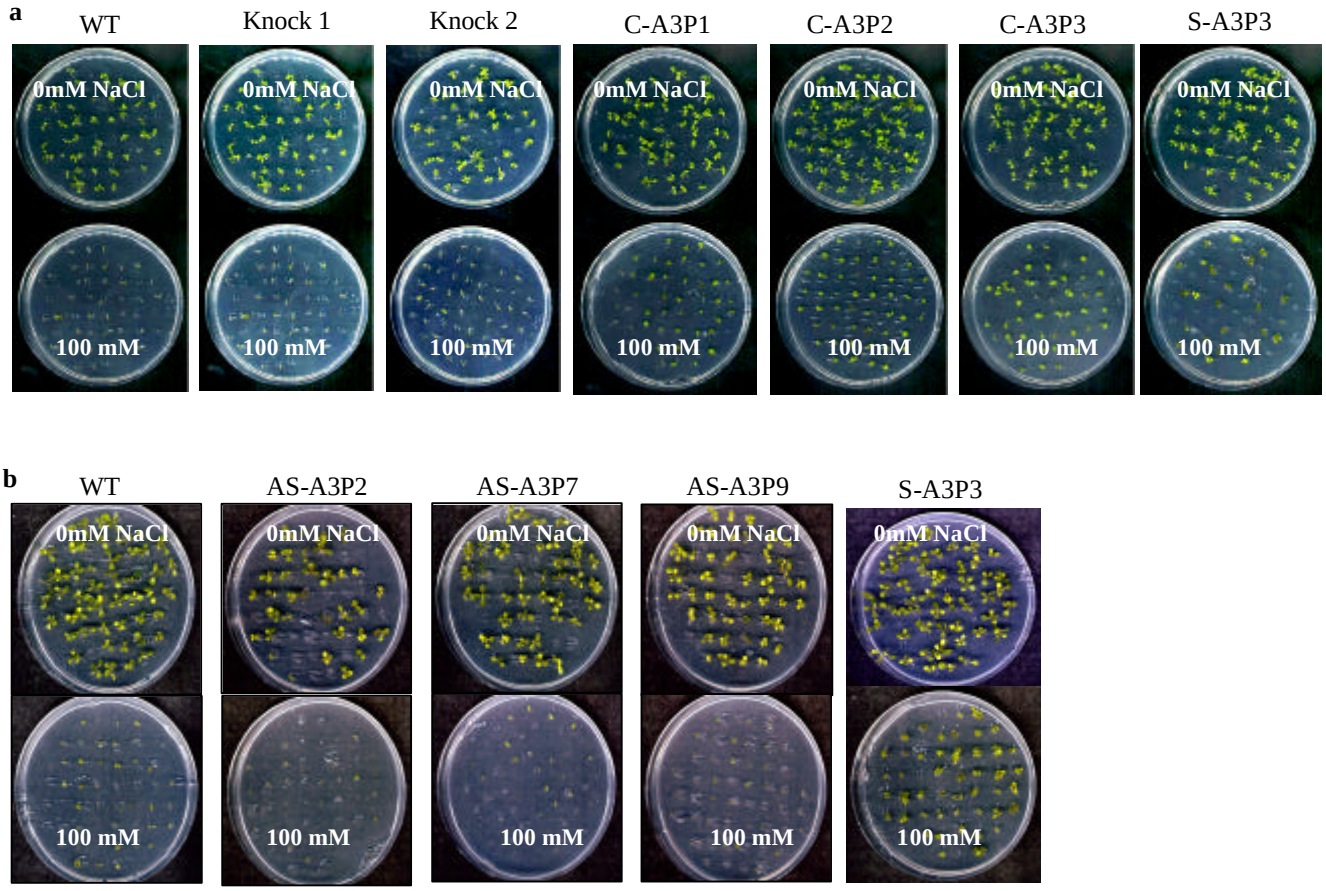


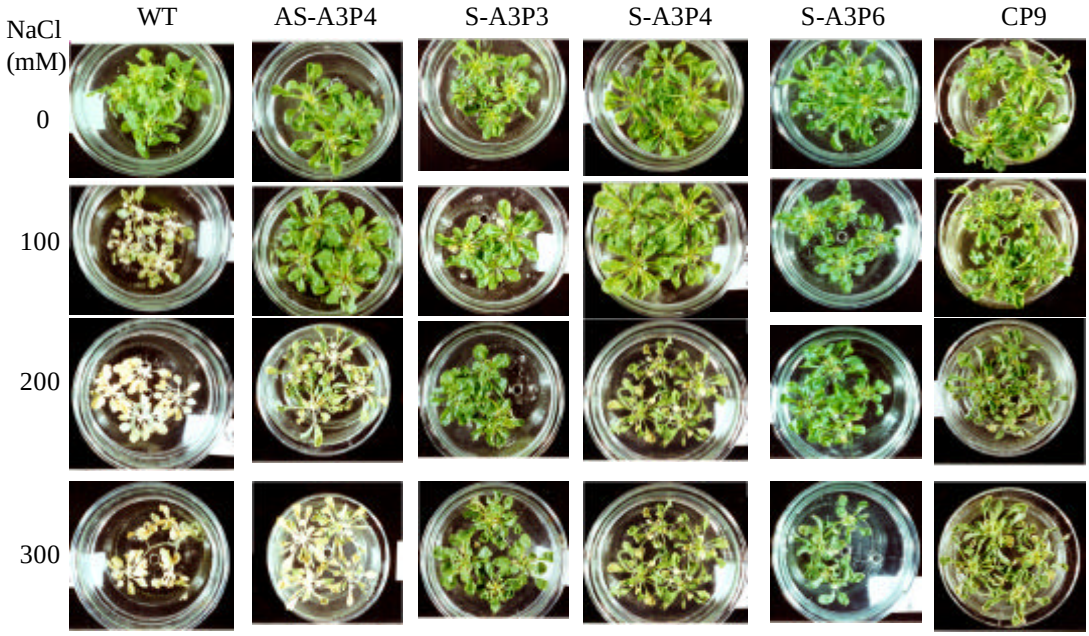
Figure 19: seed germination and early seedling development of wild-type and transgenic plants exposed to salt stress.
(a): Phenotype of 15 day-old wild-type and selected *35S-ALDH3I1* sense (AP3) and *C2prom-ALDH3I1* sense transgenic lines (C-A3P1, C-A3P2, C-A3P3) and *ALDH3I1* knock-out mutants (knock 1, knock 2) growing in MS-NaCl (0-100 mM), (b): 15 day-old wild-type, *35S-ALDH3* antisense (AS-A3P2, AS-A3P7, AS-A3P9) and one of the *35S-ALDH3* sense (AP3) transgenic lines growing in MS-NaCl (0-100 mM), (c): 20 day-old wild-type and *35S-Ath-ALDH7B4* sense (S-A7P1, S-A7P2, S-A7P3) transgenic lines growing in MS-NaCl (0-150 mM).

Table 6: Comparative studies of biochemical analysis of seedling development under salt stress

DNA constructs	Seedlings	MS-NaCl (mM) media											
		Control (0 mM NaCl)				100 mM NaCl				150 mM NaCl			
		F.W	TL	Chl	MDA	F.W	TL	Chl	MDA	F.W	TL	Chl	MDA
	WT	19±2	+++	9±2	6±2	8±2	+ - -	4±2	16±2	ND	- - -	ND	ND
35S-ALDH3I1 sense	S-A3P3	22±4	+++	10±2	5±1	**18±1**	+++	**9±2**	**7±1**	**4±1**	++ -	ND	ND
	S-A3P6	27±2	+++	11±4	5±2	**15±2**	+++	**8±1**	**8±2**	**ND**	+ - -	ND	ND
35S-CpALDH sense	CP9	23±5	+++	9±3	6±1	**14±3**	+++	**8±2**	**7±2**	**2±0**	+ - -	ND	ND
35S-ALDH7B4 sense	S-A7P1	26±5	+++	10±2	7±1	**22±4**	+++	**9±2**	**7±0**	**8±1**	+++	**6±1**	**11±4**
	S-A7P2	27±3	+++	15±3	6±2	**25±3**	+++	**9±3**	**7±3**	**5±0**	+++	**5±0**	**12±4**
	S-A7P3	29±4	+++	12±2	5±1	**20±5**	+++	**8±1**	**6±1**	**7±2**	+++	**8±2**	**14±2**
C2-ALDH3I1 sense	C-A3P1	27±6	+++	12±2	6±1	**15±3**	++ -	**7±1**	**7±2**	ND	ND	ND	ND
	C-A3P2	20±2	+++	11±3	5±2	**16±3**	+++	**8±2**	**6±2**	ND	ND	ND	ND
	C-A3P3	24±3	+++	13±2	6±2	**19±2**	+++	**8±1**	**8±3**	ND	ND	ND	ND
35S-ALDH3I1 antisense	AS-A3P2	25±2	+++	9±4	6±2	ND	- - -	ND	ND	ND	- - -	ND	ND
	AS-A3P7	19±3	+++	10±2	8±1	ND	- - -	ND	ND	ND	- - -	ND	ND
	AS-A3P9	27±5	+++	12±4	7±1	ND	- - -	ND	ND	ND	- - -	ND	ND
ALDH3I1 knockout mutants	Knock 1	26±2	+++	11±2	7±2	ND	- - -	ND	ND	ND	ND	ND	ND
	Knock 2	20±4	+++	15±5	8±2	ND	- - -	ND	ND	ND	ND	ND	ND

Seedlings exposed to salt stress were used for biochemical analyses after 14 days of development in MS-NaCl conditions. F.W = fresh weight accumulation (mg/20 seedlings), Chl = chlorophyll content (mg/20 seedlings) MDA = malondialdehyde accumulation (nmol/mg F.W), TL = true leaf development, (+++) = 100 % true leaf development, (++-) = 75 % true leaf development, (+--) = 25 % true leaf development, (---) = no record of true leaf development, ND = data not determined. The data represent the mean values ± SD of three replicate experiments. The recorded boldfaced values showed significant stress tolerance improvements when compared to those of the wild type under similar stress conditions.

a



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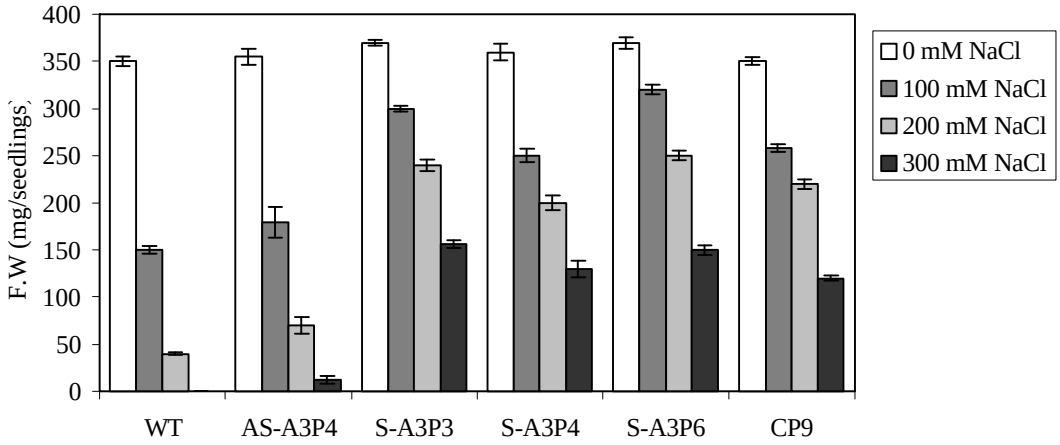


Figure 20: Responses of plants to NaCl treatments in hydroponic cultures
Three weeks old plants were transferred to hydroponic cultures containing indicated salt concentrations and allowed to grow for another two weeks. The photograph (a) represents the phenotype of the plants after one week of salt stress exposure. The average mean values of fresh weight accumulation ± SD of three replicate experiments were recorded (b). F.W = fresh weight accumulation of the plants.

Salt stress (NaCl) was also applied to mature plants (3 week-old) transferred either into soil/vermiculite (3:1) or into hydroponic cultures and allowed to grow for another two

weeks under salt stress (NaCl) treatments. Figure 20 a shows the phenotype of the plants exposed to salt stress in hydroponic cultures and Figure 20 b presents the fresh weight accumulation of the plants exposed to salt stress. No significant differences were observed in untreated plants. However, the wild type was found more sensitive to salt treatment than the transgenic lines. The wild type developed signs of wilting earlier than the transgenic plants overexpressing *ALDH* genes (Figure 20 a). The transgenic plants (S-A3P3, S-A3P4, S-A3P6, CP9) even remained green after 10 days of salt stress, while the wild-type and the antisense transgenic leaves (WT, AS-A3P4) withered away at the same time of stress exposure (Figure 20 a).

Figure 21 shows the phenotypic traits of selected transgenic lines in soil experiments under different concentrations of NaCl and KCl treatments. The results showed significant salt stress tolerance improvement in transgenic plants overexpressing *ALDH* genes (S-A3P3, S-A7P1, S-A7P2, S-A7P3, C-A3P1, C-A3P2) under NaCl and KCl treatments. No significant stress tolerance differences were observed between S-A3P/S-A7P-transgenic plants carrying *35S-ALDH* cDNA constructs and the C-A3P-transgenic plants carrying the *C2-ALDH* cDNA constructs (Figure 21). Figure 22 shows the lipid peroxidation assays in those plants exposed to various concentrations of salt (NaCl) treatments in soil experiments. It was obvious that transgenic plants overexpressing *ALDH3I1*, and *ALDH7B4* genes showed an improved stress tolerance in comparison with the wild-type and the *ALDH3I1* knock-out plants. The plants presented similar stress tolerant phenotypes to that of hydroponic-NaCl cultures. The levels of lipid peroxidation recorded in these plants are similar to that of MS-agar and hydroponic culture experiments i.e. higher lipid peroxidation in WT and knock-out mutants and lower lipid

peroxidation in transgenic plants overexpressing *ALDH* genes under stress conditions. These results show that the observed phenotypes under stress conditions were not limited to specific developmental stages of the plants.

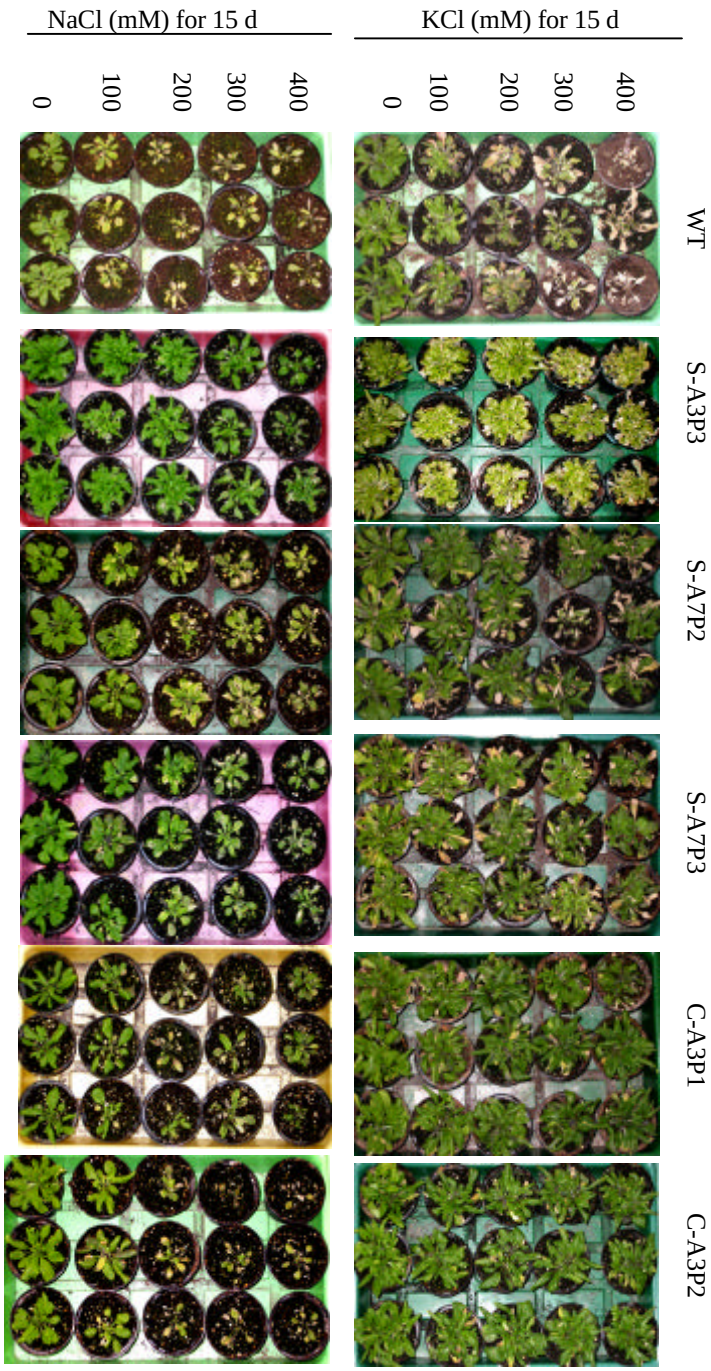


Figure 21: Comparative phenotypic traits of wild-type and transgenic plants exposed to different concentrations of NaCl and KCl treatments

Plants transferred to soil -pots were allowed to acclimate for one week and watered with the indicated salt concentrations for a specific stress exposure period. The photograph represents an overview of three replicate experiments showing stress toler ance improvement of transgenic plants C -A3P1, S -A3P3, S -A7P2 and S -A7P3 in comparison with the wildtype plants

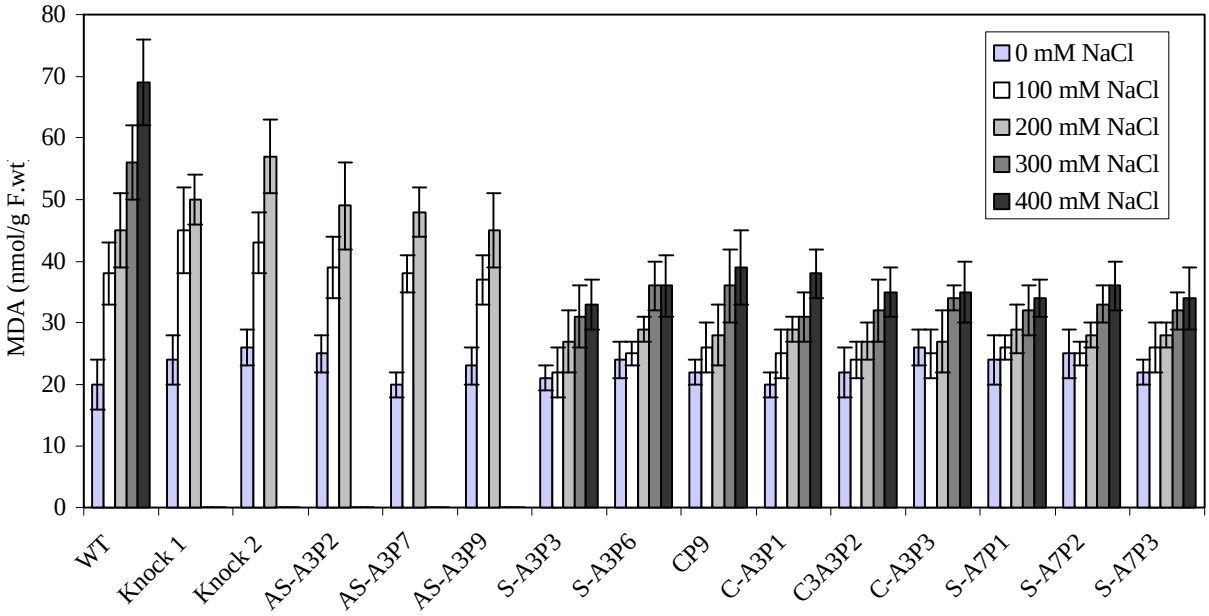


Figure 22: Lipid peroxidation values expressed as MDA content in plants exposed to 7 days of salt (NaCl) stress treatments.
The MDA content in knock 1, knock 2, AS-A3P2, 7 and 9 was not determined at 300 and 400 mM NaCl treatment because the plant material at those stress conditions was not enough to carried out the assay.
Data represent the mean values ± SD of three independent experiments. ND = not determined.

3.3.2. Responses of plants to dehydration stress

Seed germination and early seedling development was monitored in MS-agar under water deficit conferred by different concentrations of polyethylene glycol (PEG 8000, Sigma). PEG was added into pre-cooled autoclaved MS-agar media because changes occur in PEG chemical properties when autoclaved. PEG was used to lower the water content in the agar plates. Seeds were germinated and allowed to grow in MS-agar containing PEG concentrations ranging from 0 to 20 % (w/v) PEG. MS-agar media failed to solidify at PEG concentrations higher than 20 %. Figure 23 shows the levels of lipid peroxidation

and the fresh weight accumulation of seedlings after 15 days of water deficit exposure. First investigations showed that concentrations between 0 and 5 % (w/v) PEG revealed no growth differences between the wild-type and the transgenic plants. The experiments were therefore carried out with 10 and 20 % (w/v) PEG. Significant growth differences were observed after one-week of seedling growth. Transgenic plants overexpressing *ALDH* genes grew better and showed less signs of lipid peroxidation than the wild-type plants under water deficit as confirmed by their fresh weight accumulation and their level of MDA production (Figure 23). The wild-type seeds failed to germinate at 20 % (w/v) PEG, while the transgenic lines showed approximately 25 % of seed germination with true leaf development at water deficit conditions conferred by 20 % (w/v) PEG.

To test the responses of plants to drought stress, 12 to 14 day-old seedlings grown in MS-agar plates were transferred into trays containing soil/vermiculite (3:1) and allowed to acclimate for another 7 days before applying the drought stress. The soil was allowed to dry by withholding the watering for 15 days. The wild-type plants showed more wilting symptoms after one week of dehydration than the transgenic *ALDH* overexpressing plants (S-A3P3, S-A7P1-3, C-A3P1-3) (Figure 24).

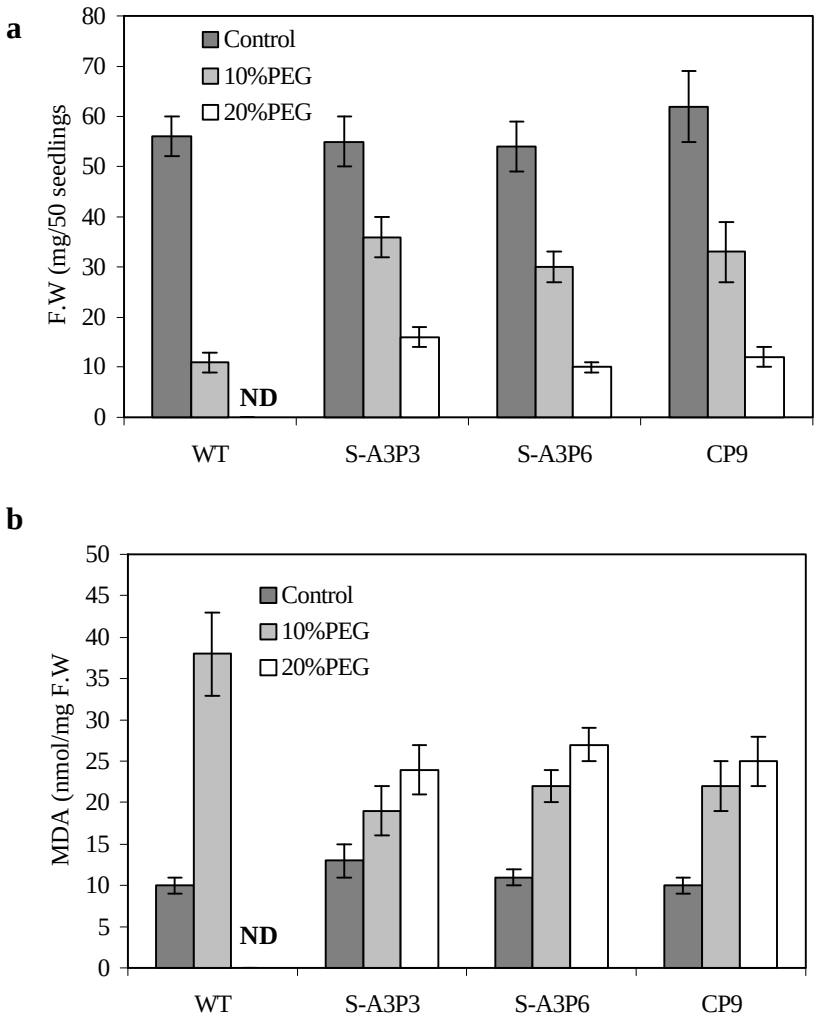


Figure 23: Fresh weight accumulation (a) and lipid peroxidation (b) in wild-type and transgenic plants exposed to water deficit conferred by addition of PEG 8000 into MS-media.
F.W = fresh weight accumulation (mg/50 seedlings), MDA = malondialdehyde accumulation (nmol/mg F.W), ND = data not determined because WT seeds failed to germinate at 20 % (w/v) PEG. The data represent the mean values ± SD of three replicate experiments.

On the other hand, transgenic plants with stress-inducible *ALDH3I1* phenotype (C-A3P-lines) were more tolerant to dehydration stress than the transgenic plants constitutively expressing *ALDH* genes (S-A3P3, S-A7P1, S-A7P2, S-A7P3) (Figure 24). After 10 days of dehydration, C-A3P1 and C-A3P2 plants were still green showing phenotype of enhanced dehydration tolerance compared to wild-type and the transgenic plants (S-A3P3, S-A7P1, S-A7P2, S-A7P3) constitutively expressing *ALDH* genes. Figure 25 shows the level of lipid perodixation in transgenic plants constitutively expressing *ALDH3I1*, *Cp-ALDH*, *ALDH7B4* and the stress inductive *ALDH3I1* gene expression (*C2-ALDH3I1*) under dehydration treatment. The results showed that MDA production in the wild-type plants was two times higher than in the transgenic plants overexpressing *ALDH* genes under drought stress. The levels of MDA content were elevated both in wild-type and in all the transgenic plants, but the degree of lipid peroxidation was significantly lower in transgenic plants overexpressing *ALDH* genes than in stressed wild-type plants (Figure 25). Transgenic C-A3P-lines showed even lower amount of MDA accumulation than the transgenic plants constitutively expressing *ALDH3I1* gene (S-A3P lines, CP lines, S-A7P lines) under dehydration treatment (Figure 25). The *ALDH3I1* knock-out mutant plants (Knock 1 and 2) showed more sensitivity to dehydration than the wild type as illustrated in Figure 26. The phenotypes of knock-out mutants under dehydration correlated with the expected results and confirmed thereby the involvement of *ALDH* genes in stress tolerance mechanisms.

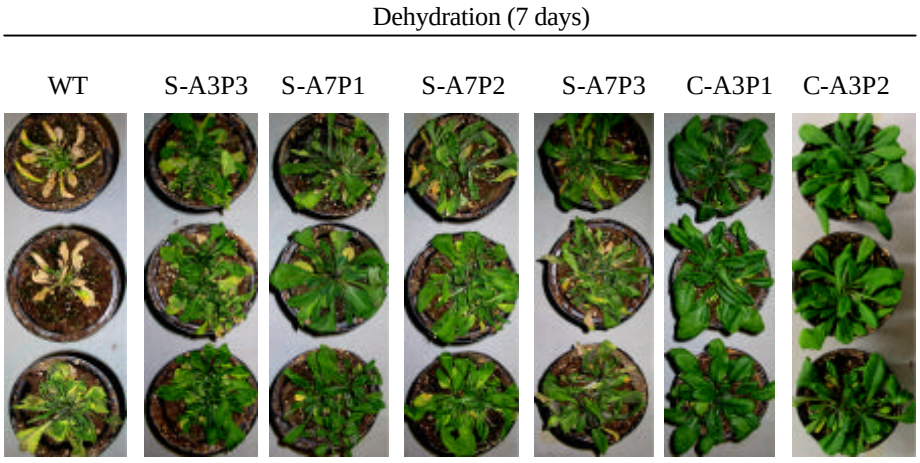


Figure 24: Drought tolerance in transgenic plants overexpressing *ALDH3I1* and *ALDH7B4* genes
The plants growing in pots were exposed to dehydration stress for 15 days. Photograph shows the phenotypes of independent plants after 7 days of dehydration exposure. Each line is presented in triplicate.

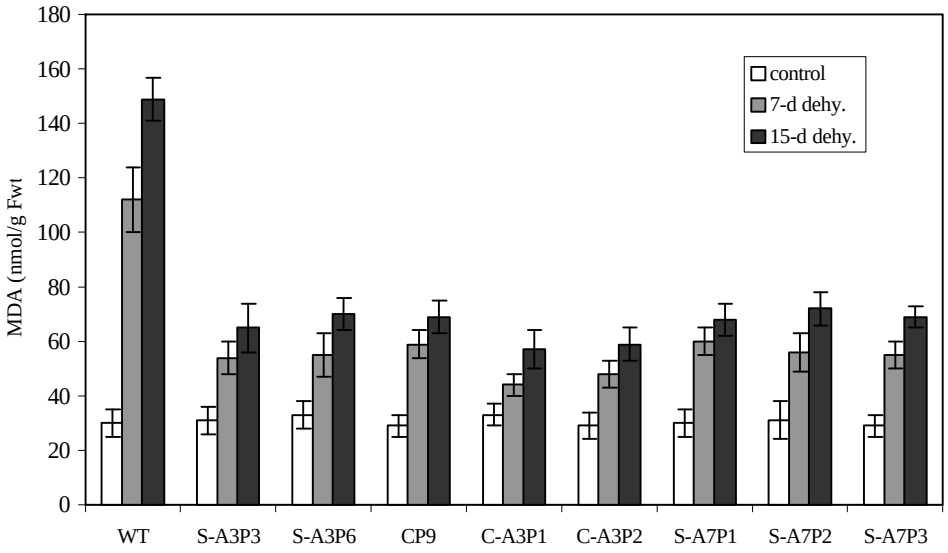


Figure 25: Estimation of lipid peroxidation level expressed as the amounts of MDA contents in plants exposed to dehydration treatments.
Seedlings transferred to soil-trays were allowed to acclimate for one weeks and dehydration was imposed for indicated periods by withholding to water the plants. The data represent the mean values ± SD of three replicate experiments. Dehy. = dehydration treatment.

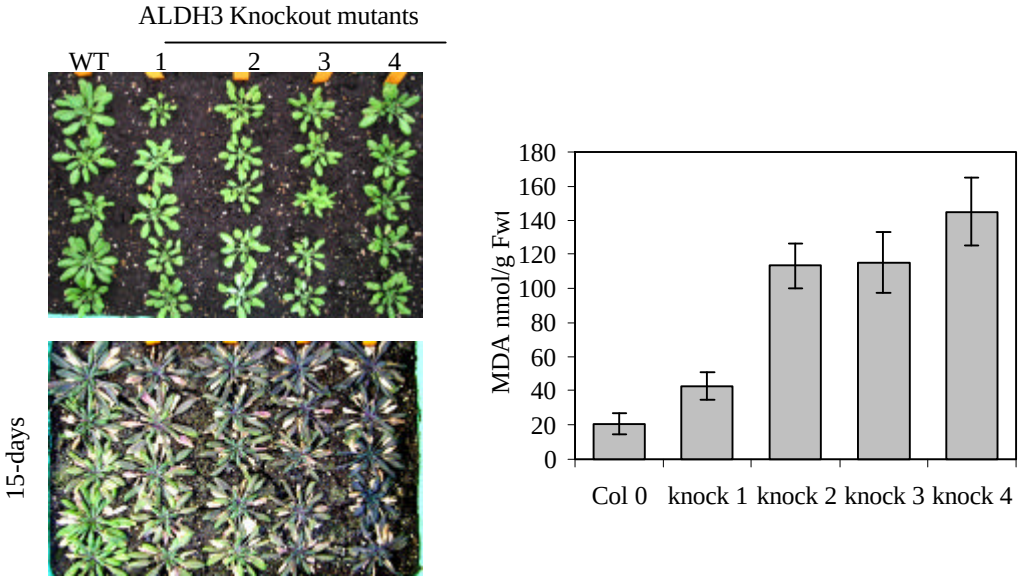


Figure 26: Sensitivity of *ALDH3I1* knock-out mutant plants to dehydration.
Dehydration was imposed to the plants for a period of 15 days and samples were collected for lipid peroxidation assays. Data represent mean values ± SD of three replicate experiments. The photograph shows stress sensitivity of the mutant plants in comparison with the wild-type plants.

3.3.3. Responses of plant to hydrogen peroxide exposure

In order to evaluate the role of *ALDH* genes to confer tolerance to oxidative stress, wild-type and independent transgenic plants were exposed to hydrogen peroxide, a causal agent of oxidative stress in plants. The tolerance of plants to H_2O_2 was analysed during seed germination and early stages of seedling development. Seeds were germinated in liquid media containing different concentrations of H_2O_2. Figure 27 shows the rates of seed germination after 4 days of growth. 5 mM H_2O_2 inhibits wild-type and antisense transgenic seed germination, while the transgenic seeds overexpressing *ALDH* genes (S-A3P3, S-A3P6) were able to germinate and produce true leaves under the same conditions (Figure 27).

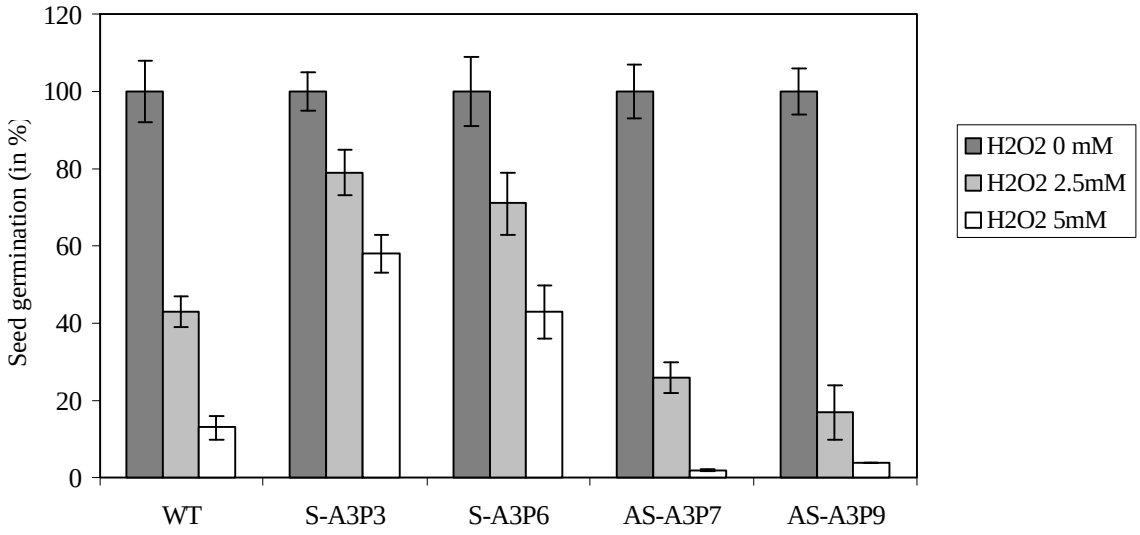


Figure 27: Effect of hydrogen peroxide during early stage of seed germination.
Seeds were allowed to germinate in indicated concentrations of hydrogen peroxide for 4 days and the germination rate was recorded. Data represent mean values ± SD of three replicate experiments.

3.3.4. ROS scavenging effects of ALDHs and oxidative stress tolerance in transgenic plants

The role of ALDHs as ROS scavengers was first investigated by measuring the accumulation of endogenous superoxide ion and hydrogen peroxide in WT, S-A3P3 and S-A3P6 plants under various abiotic stresses. As shown in Figure 28 the production of superoxide ion in the wild-type plants was found to be about four times higher than that of the transgenic lines under 24 h of dehydration treatment (Figure 28 a). Since hydrogen peroxide is very reactive, its accumulation in the plants was assayed after 4 h of plant exposure to salt stress (200 mM NaCl) and dehydration. Figure 28 b shows that higher amounts of hydrogen peroxide were produced in wild-type seedlings than in transgenic plants (S-A3P3, S-A3P6) under stress conditions. These findings promote the role of ALDHs as ROS scavengers and may explain the oxidative stress tolerance in transgenic S-A3P3 and S-A3P6 plants. Tolerance of the transgenic plants to this stress has been

further checked by an assay for the level of MDA production in the plants. The results showed a reduced accumulation of MDA in comparison with the wild type under dehydration and salt stress (Figure 25).

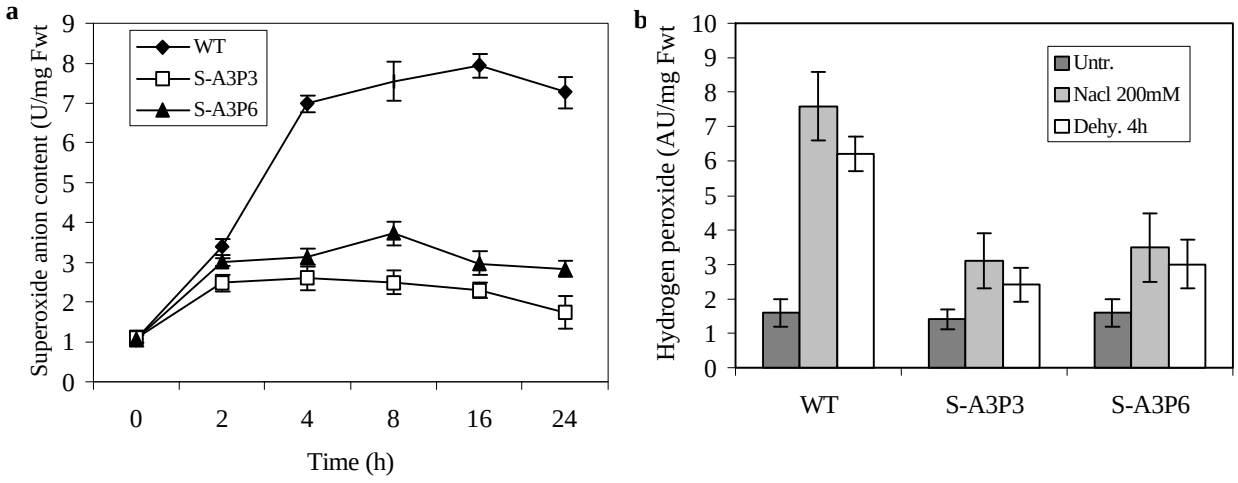


Figure 28: Reduced accumulation of superoxide anion and hydrogen peroxide in transgenic plants constitutively expressing *ALDH3I1* gene under abiotic stress.
AU = mean absorbance multiplied by 1000 without any further conversions (Tiedemann 1997) ± SD of three replicate experiments. (a) = superoxide anion content in seedlings exposed to dehydration over 24 h period. (b) = Hydrogen peroxide accumulation in seedlings exposed to salt stress and dehydration for 4 h. Untr. = Untreated seedlings used as control.

The accumulation of H_2O_2 was further monitored by an *in vivo* assay in transgenic plants overexpressing *ALDH3I1* (S-A3P3), *ALDH7B4* gene (S-A7P1, 2, 3), and in transgenic C-A3P-plants expressing the *ALDH3I1* gene under the control of the C2-promoter. Figure 29 shows the *in vivo* detection of H_2O_2 in some selected plants and Figure 30 shows the level of H_2O_2 accumulation in the plant tissues exposed to stress conditions. Salt stress induces an increased accumulation of H_2O_2 in the wild-type as well as in the transgenic lines (S-A3Ps, S-A7Ps, C-A3Ps), but the rate of accumulation was much lower in transgenic *ALDH* overexpressing plants (S-A3Ps, S-A7Ps, C-A3Ps) than in the wild-type

plants (Figure 29, 30). These results confirmed the potential of ALDH protein activities to reduce the H_2O_2 accumulation in plants exposed to abiotic stress. The reduction of H_2O_2 accumulation in those plants correlated with improved stress tolerance in comparison with the wild-type and the knockout mutant plants (Figure 19, 20, 21).

The level of cell viability was further analysed in plants exposed to salt stress conditions. Figure 31 shows the *in vivo* detection of viable cells via fluorescein diacetate staining under fluorescence microscope observation. Fluorescein diacetate is a specific staining compound, absorbed only by vital cells, and constitutes therefore a good detection method to localize intact and vital cells *in vivo* (Widholm 1972). The results show that transgenic plants overexpressing *ALDH* genes present more viable cells than the wild-type plants under stress conditions (Figure 31), probably because of the reduced levels of MDA and ROS accumulation in those transgenic plants. This protective status of transgenic plant cells probably is due to the higher level of ALDH protein activity in comparison with the wild-type plants as shown by the protein blot analysis under stress conditions (Figure 5).

It is evident from the above biochemical analyses that transgenic plants overexpressing *Arabidopsis ALDH* genes are more tolerant to dehydration and salt stress than the wild-type plants due to the overexpression of *ALDH* genes, subsequently reducing the level of cellular and molecular damages caused by accumulation of reactive aldehydes and ROS in plant cells. The overexpression of *Arabidopsis ALDH* genes therefore provides a protective status to cells under stress conditions.

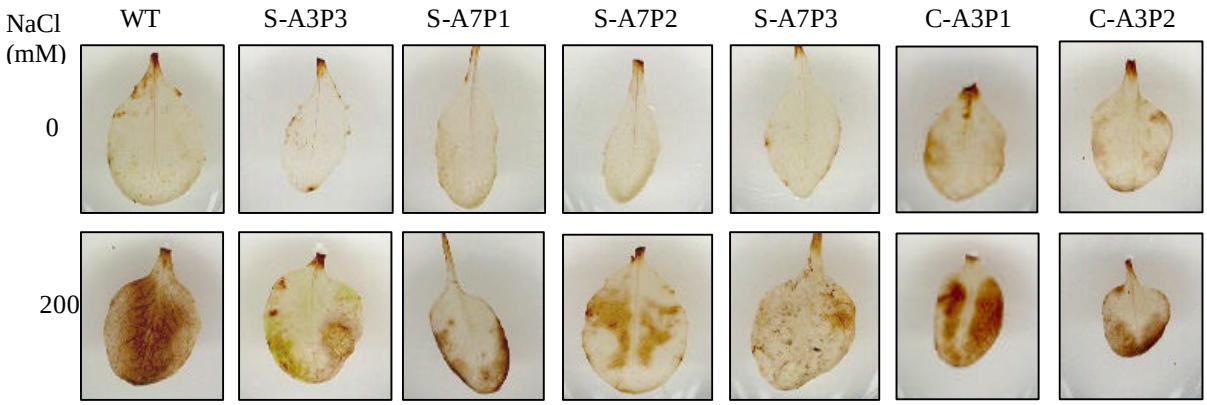


Figure 29: *In vivo* detection of H_2O_2 accumulation in plants exposed to salt stress via DAB method
Wild-type and transgenic plants subjected to different concentrations of NaCl treatment and used to assess the in vivo accumulation of H_2O_2 after one week of salt treatments. The level of H_2O_2 accumulation correlates with the intensity of brownish of the leaves according to DAB method.

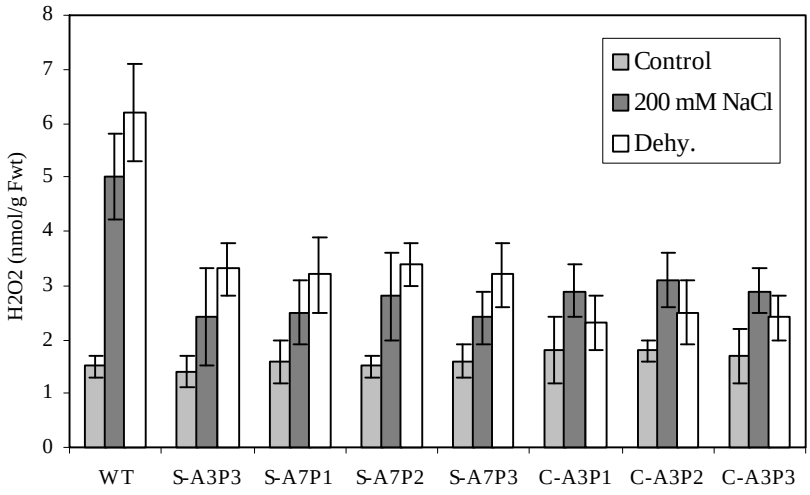


Figure 30: Quantitative estimation of ROS (H_2O_2) generation in plants exposed to dehydration and salt stress.
Seedlings were exposed to various stress conditions for 24 h and then used to check the amounts of ROS (H_2O_2) generated in the plant cells. Data represent mean values ± SD of three replicate experiments. Dehy.= Dehydration treatment.

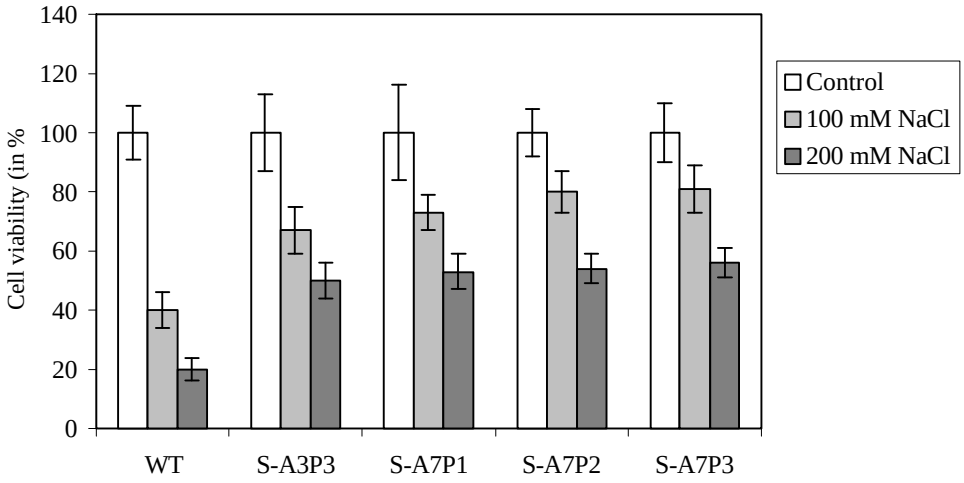


Figure 31: Microscopic detection of viable plant cells under salt stress
Leaf materials were collected from one week-stressed and unstressed plants to assess the amounts of viable plants cells via fluorescein diacetate vital staining solution. The viable cells were fluorescent and able to be counted under microscope observation. The data were estimated in percentage of viable cells (± SD) of three replicate experiments.

4. DISCUSSION

Controlling gene expression is one of the key-regulatory mechanisms used by living cells to accomplish specific biological functions under a given growth condition. In the last decade, increasing amounts of stress-inducible genes have been characterized in various plant species (Kishor et al 1995, Ingram and Bartels 1996, Ramanjulu and Bartels 2002, Shinozaki and Dennis 2003). Some stress-inducible aldehyde dehydrogenase genes have been also characterized in plants (Cui et al 1996, op den Camp and Kuhlemeier 1997, Deuschle et al 2001, Kirch et al 2001, Liu et al 2001, Liu and Schnable 2002, Sunkar et al 2003, Bouché et al 2003, Tsuji et al 2003, Kirch et al 2004). Nowadays, the advance in molecular technique has provided new ways to analyse the transcriptomes of plants involved in environmental stress tolerance mechanisms (Scheideler et al 2002, Shimono et al 2003). This technology has greatly contributed to our current understanding of molecular and physiological mechanisms of stress tolerance in plants. Whereas the role of some proteins as regulatory factors or as biosynthetic enzymes in development and adaptation of plants to environmental stress are known, the involvement and role of aldehyde dehydrogenase proteins in plants to confer environmental stress tolerance is just emerging (Kirch et al 2001, Kirch et al 2004). Apart from the well-studied substrate specific BADH gene, which is involved in the synthesis of the osmoprotectant glycine betaine in plants responding to osmotic stress (Weretilnyk and Hanson 1990, Zhu et al 2003), the biological role of most stress-inducible aldehyde dehydrogenase genes in relation to abiotic stress tolerance is unknown. It is only in 2004 that Kirch et al (2004), based on the release of the complete genome sequences of *Arabidopsis thaliana* examined for the first time the phylogenetic and molecular relationship of all *ALDH*

genes in *Arabidopsis* plants and the functional features of the corresponding ALDH protein families. Their contribution provided a revised and unified nomenclature for plant-*ALDH* genes (Kirch et al 2004), which was adopted by the *ALDH* Gene Nomenclature Committee (AGNC) (Vasiliou et al 1999). The revised *ALDH* gene nomenclature according to Kirch et al 2004 has been used here for the sake of conformity. The study undertaken here contributes uniquely to our knowledge of plant ALDH functions with regard to their involvement to confer tolerance under various stress conditions. The data presented here demonstrate that ALDHs display a crucial antioxidative role coupled with aldehyde detoxification in mechanisms of plant responses to abiotic stress.

4.1. Regulation of *Arabidopsis ALDH* gene expressions in response to abiotic stress

The increased accumulation of *Arabidopsis-ALDH* mRNAs in plants exposed to dehydration and salt stress (Figure 2, Kirch et al 2001, Sunkar et al 2003) suggested that some *Arabidopsis-ALDH* genes are involved in the phenomenon of plant adaptation to abiotic stress. The analysis of ALDH3I1 (formerly known Ath-ALDH3), ALDH3H1 (formerly known as Ath-ALDH4) and Cp-ALDH protein sequence relationship showed a high homology (70 %) to each other (Kirch et al 2001). However, recent studies revealed that several *Arabidopsis-ALDH* genes are differentially expressed in plant tissues exposed to stress conditions (Schlingensiepen 2003). This indicates that the highly homologous *Arabidopsis-ALDH* genes are probably controlled by diverse regulatory mechanisms. In this study as well as in previous studies (Kirch et al 2001, Sunkar et al 2003, Schlingensiepen 2003), *ALDH* transcript accumulations and their corresponding protein

accumulations were analysed under exogenous applications of plant hormone ABA, hydrogen peroxide and paraquat in order to study the regulatory pattern of *ALDH* genes in *Arabidopsis* plants under specific stress elicitors. *ALDH3I1*, *ALDH3H1*, *ALDH3F1*, *ALDH7B4* genes were differentially induced in shoots and roots by ABA (Schlingensiepen 2003). Likewise, the *Craterostigma-ALDH* (*Cp-ALDH*) gene was upregulated in the whole plants exposed to exogenous treatment of ABA (Kirch et al 2001). RT-PCR analysis revealed a rapid accumulation of the *ALDH3I1* transcript in plants exposed to 1 h of hydrogen peroxide treatment (Sunkar et al 2003). This data suggested that *ALDH3I1*, *ALDH3H1* and *Cp-ALDH* genes might probably be induced by an accumulation of endogenous ABA and *ALDH3I1* by an endogenous accumulation of ROS (H_2O_2) under stress conditions or that there might be a cross-link expression pathway between the *ALDH* transcript accumulation and ROS/ABA biosynthesis. This hypothesis was confirmed here by the ALDH-protein accumulation analyses after ABA, H_2O_2 and paraquat treatment (Figure 5). The Cp-ALDH protein displayed an increasing accumulation from 6 h to 72 h of ABA treatment (Kirch et al 2001), while the ALDH3I1 protein was found to progressively accumulate from 2 h to 72 h of ABA, paraquat, and H_2O_2 treatments (Figure 5). These findings point out the potential of ABA and ROS to trigger the synthesis of ALDH-proteins.

Recently, direct genetic evidence showed that activation of other enzymes such as membrane bound NAD(P)H oxidases in root hair growth is under the control of ABA-ROS signalling transduction pathway (Foreman et al 2003). The expression of NAD(P)H oxidases was triggered by accumulation of ABA and ROS, and the transcript accumulation of these genes correlated not only with root hair elongation but also with

stomatal closure (Foreman et al 2003). Such kind of signal transduction modulated by endogenous accumulation of ABA and ROS might also be the probable transduction cascade pathway leading to the upregulation of stress-inducible *ALDH* genes in *Arabidopsis thaliana*. Microarray analyses of transcript accumulation under stress conditions (Scheideler et al 2002, Shimono et al 2003, Seki et al 2002) revealed that ABA is involved in the signal transduction process of environmental stress tolerance in various plant species. Accumulation of endogenous ABA leads to an upregulation of a specific set of stress inducible genes via MAPK cascade pathway (Kovtun et al 2000, Xiong and Yang 2003). ROS accumulation is beneficial to some extent to the adaptation mechanisms of plants to abiotic stress (Allen and Tresini 2000, Dat et al 2000). Increasing literature suggests that ROS probably act as one of the earliest factors that induce the expression of defence-related genes such as GST, encoding glutathione S-transferase, and PAL, encoding Phenylalanin-ammonia lyase (Desikan et al 1998, Grant et al 2000). Lopez-Huertas et al (2000) asserted that H_2O_2 induces the expression of genes required for peroxisome biogenesis. Peroxisomes are organelles of direct importance for antioxidant defence. ROS is also believed to play a pivotal role in the phenomenon of cross-tolerance, in which exposure to one stress can induce tolerance to other stresses (Bowler and Fluhr 2000).

A previous report on *Arabidopsis-ALDH* gene expression revealed that *ALDH3H1* is constitutively and weakly expressed in wild-type plantlets (Kirch et al 2001), This work revealed that *ALDH3H1* is significantly expressed in roots under ABA, NaCl and dehydration treatments (Figure 11, 12, Table 5), confirming that the expression of the *ALDH* gene superfamily responds to various regulatory pathways. These results suggest

that *ALDH3H1* gene may be involved in several cellular metabolic pathways including stress in plants. Not all stress-inducible genes require an accumulation of endogenous ABA and/or ROS to trigger their expression under stress conditions (Ramajulu and Bartels 2002). However, both ABA/ROS-dependent and –independent regulatory systems of gene expression operate in highly defined functional connections to confer tolerance under a given stress condition (Ramanjulu and Bartels 2002). Based on *ALDH* transcript and ALDH protein accumulation analyses under various elicitors, the scheme below (Figure 32) illustrates our current knowledge of signal cascade transduction leading to the upregulation of the stress-inducible *ALDH* gene families in *Arabidopsis thaliana* exposed to various abiotic stresses. The scheme indicates the signal transduction pathways leading to the upregulation of plant-*ALDH* gene expressions under stress conditions.

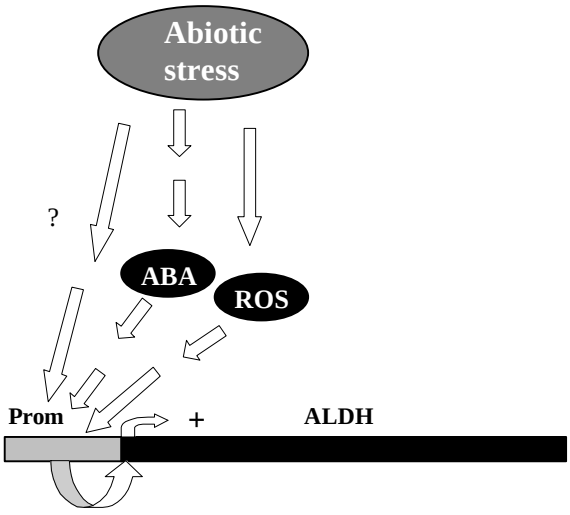


Figure 32: A schematic representation of the regulatory pathway of stress-inducible *ALDH* genes in *Arabidopsis thaliana*.
Arrows indicate the signal transduction cascade leading to the expression of the gene under stress exposure. As a result of the signal transduction, transcription factors bind to the promoter at specific stress responsive sequence elements and trigger the upregulation of the gene (+).

4.1.1. Tissue-specific localisation of ALDH proteins

To study tissue specificity of ALDH genes, analyses were carried out in transgenic plants expressing the GUS reporter gene under the control of different *ALDH* promoters. The results indicated that *ALDH3I1*-GUS and *CpALDH*-GUS were mainly expressed in leaf tissues, while *ALDH3H1*-GUS was concentrated in roots under dehydration, salt stress and ABA treatments. The GUS detection was also confirmed by quantitative analysis of specific GUS activity under stress condition. The results revealed significant increase levels of GUS activity in leaves and particularly in chloroplasts in transgenic plants transformed with *ALDH3I1* promoter-GUS construct under stress conditions (Figure 11, 12, Table 5). The GUS fusion system has been used in various studies of plant gene expression, especially for promoter analysis, for dissecting gene families and for protein targeting studies (Schmitz et al 1990). Previous studies indicated that Cp-ALDH is located in plastids (Kirch et al 2001). The amino acid sequence analysis of Cp-ALDH could not however provide evidence for a specific chloroplast targeting sequence (Kirch et al 2001). The sequence analysis has also predicted the ALDH3I1 protein to be localised in chloroplasts, because it has an appropriate targeting sequence (Kirch et al 2001). However, results in this work could not support a chloroplast localisation of ALDH3I1 protein activity because the *ALDH3I1*-GUS construct contains the *ALDH3I1* promoter without a downstream fragment of the *ALDH3I1* structural gene, which may have appropriate chloroplast-targeting sequences. It can be hypothesized that the chloroplast localisation of the GUS activity could be due to an artificial diffusion of the blue precipitates (ClBr-indigo). Therefore, the data shown are not sufficient to provide

detailed information about the organellar localisation of ALDH3I1, ALDH3H1 and Cp-ALDH proteins. The expression of *ALDH3I1* and *Cp-ALDH* gene is leaf specific under stress conditions, while *ALDH3H1* is constitutively and weakly expressed in the whole plant but highly concentrated in roots under stress treatments. These genes (*ALDH3I1, ALDH3H1, Cp-ALDH*) however belong to the same family 3 *ALDH* genes but display a diverse tissue specific expression pattern under stress condition. This diverse expression pattern points to a crucial ALDH protein function specificity respectively in a highly coordinative physiological mechanism to confer abiotic stress tolerance in plants. It would be interesting to gain detailed information about their functional specificity to enhance abiotic stress tolerance in higher plants.

4.2. Phenotypic analysis of transgenic *Arabidopsis* plants under stress conditions

4.2.1. Transformation of *Arabidopsis thaliana* with *ALDH* cDNA constructs and molecular characterization of the transgenic plants.

Transgenic techniques have become a powerful tool to address analysis of gene functions in plants, especially in identifying physiological roles of novel proteins (Aoyama and Chua 1997, Bartels 2001b, Shinozaki et al 2003). These techniques often include transfer of stress-inducible genes to improve tolerance to a specific stress condition (Holmberg and Buelow 1998). In this work a set of transgenic plants overexpressing *ALDH3I1*, *ALDH7B4* and *Cp-ALDH* genes were used (for detail see Table 2) in order to study the physiological role of ALDH in plants, particularly under abiotic stress conditions. In addition several independent transgenic lines with repressed and knock-out *ALDH* gene expressions were studied together with the wild type in order to gain insight into the

extent of *ALDH* gene involvement in processes of stress tolerance mechanisms in plants. *ALDH3I1* and *Cp-ALDH* genes belong to the class 3 stress-inducible ALDH gene family (Kirch et al 2001), while *ALDH7B4* is a stress-inducible or turgor *ALDH* gene in pea (Guerrero et al 1990, Wood and Krayesky 2002) responding to a range of abiotic stresses including dehydration, low temperature, heat shock and high concentrations of ABA (Wood and Krayesky 2002, Kirch et al 2004). The main objective of this work was to generate and characterize transgenic plants overexpressing stress-inducible *ALDH3I1*, *Cp-ALDH*, and *ALDH7B4* genes in order to improve osmotic and oxidative stress tolerance in higher plants. Generally, a comparative study including knock-out mutant plants and transgenic plants overexpressing a specific gene is an ideal strategy to gain information on the functional involvement of a gene of interest in cellular metabolism. Downstream effects of reduced or loss of function in antisense or knock-out mutant plants compared with the transgenic plants overexpressing the same gene are adequate parameters to evaluate the extent of the gene involvement in stress tolerance mechanisms. For instance, the loss of function in the NAD(P)H oxidase gene (atrbohC) by T-DNA insertion has demonstrated its involvement in root hair growth (Foreman et al 2003, Kwak et al 2003). Wong et al (2004) used the same down-regulation approach to demonstrate that metallothionin is a crucial reactive oxygen scavenger in rice. To test the hypothesis that ALDH protein activities display a protective function in plant responses to abiotic stress, *Arabidopsis* mutants with reduced *ALDH* transcript/protein accumulation (antisense and knock-out transgenic plants) were subjected to various abiotic stresses together with the wild-type and the *ALDH* overexpressing transgenic *Arabidopsis* plants.

4.2.2. Transgenic plants overexpressing *ALDH* genes

Several stress-inducible genes encoding for key enzymes such as osmolyte biosynthesis, and detoxification enzymes have been overexpressed in transgenic plants and this has conferred a stress tolerant phenotype. Attempts to improve osmotic stress tolerance in plants include the use of genes encoding enzymes for biosynthesis of various osmoprotectants such as *E. coli* mannitol 1-phosphate dehydrogenase for mannitol synthesis (Tarczynski and Bohnert 1993), delta-1-pyrroline-5-carboxylate synthetase for proline synthesis (Kishor et al 1995, Igarashi et al 1997) and betaine aldehyde dehydrogenase for glycine betaine (Ishitani et al 1995, Takabe et al 1998, Zhu et al 2003). In those transgenic approaches, only a single gene for a protective protein or enzyme was overexpressed under the control of the constitutive CaMV 35S promoter in the transgenic plants. Here, *ALDH3I1* and *ALDH7B4* genes were successfully overexpressed in independent transgenic *Arabidopsis* plants using the same transgenic approaches. The transgenic plants (S-A3Ps, S-A7Ps, C-A3Ps) showed an increased expression of the ectopic *ALDH3I1* and *ALDH7B4* genes (Figure 15,16). The overexpression of *ALDH3I1*, *ALDH7B4* and *Cp-ALDH* genes in transgenic plants (S-A3Ps, S-A7Ps, C-A3Ps and CPs) confers enhanced tolerance to dehydration and salt stress as shown by phenotypic analyses in plants exposed to different concentrations of NaCl in MS-media as well as in soil-experiments (Figure 19, 20, 21). These results suggest that overexpression of *ALDH* genes could confer stress tolerance at any developmental stage of the plants. The stress tolerance was furthermore confirmed at the seed germination stage, where transgenic plants displayed approximately 40 % of seed

germination with proper true leaf development in salty-soil (200 mM NaCl), while the wild-type seeds failed to germinate under such conditions. The improved stress tolerance in these plants could be explained by a higher activity of ALDH proteins as a result of increased expression level of the ectopic genes in comparison with the wild type. The level of toxic aldehyde by-products, which accumulated in plant tissues under stress conditions, was highly reduced in the transgenic plants (S-A3Ps, S-A7Ps, C-A3Ps, CPs) in comparison with that of the wild-type plants as a result of reduced level of MDA accumulation in those transgenic lines (Table 6). Several other research observations also point to a role of plant-aldehyde dehydrogenases in osmotic stress tolerance. Velasco et al (1994) have reported about the molecular characterization of ALDH11 protein family (GapC-Crat), a cytosolic GAPDH from the resurrection plant *Craterostigma plantagineum*. The mRNA and enzymatic activity of GAPDHc was significantly increased in response to dehydration and exogenous application of ABA. From a proteomic study of the *Arabidopsis* seeds, a cytosolic GAPDH peptide was identified to be associated with the desiccation process of seeds, indicating the importance of these enzymes for desiccation tolerance (Gallardo et al 2001). In addition, characterization of cDNAs encoding the GAPDH from a desert halophyte *Atriplex nummularia* L. was shown to play a crucial role in osmotic stress tolerance (Nui et al 1994). Wood et al (1999) used expressed sequence tags (EST) analysis to discover several genes including *ALDH* genes that are likely to be involved in vegetative desiccation tolerance in the moss *Tortula ruralis*. In addition Chen et al (2002) characterized several cDNAs at the transcriptional level including *ALDH7B6* confirming thereby the findings of Wood et al (1999). Here, overexpression of the *ALDH3I1* and *ALDH7B4* genes was clearly proven to

confer tolerance to dehydration and salt stress in transgenic plants. Understanding the processes by which plant-ALDH activities limit the cellular damage caused by toxic aldehydes may represent a critical protective strategy for plants to survive osmotic and oxidative stress.

4.2.3. Induced repression of endogenous *ALDH*-transcript accumulation in antisense transgenic plants

One of the most crucial metabolic alterations in plant response to abiotic stress is the silencing of a specific set of genes (Ingram and Bartels 1996) whose functions render the plant vulnerable to the ongoing stress condition. The silencing of protein functions could be achieved by antisense expression of the gene or by inactivating the messenger RNAs (mRNAs) by short RNAs referred to as RNA interference (RNAi) (Jorgensen 1990, Romano and Macino 1992). Antisense- and RNAi-directed silencing or knock-down of gene expression has been used successfully in various research applications for rapid and reliable analysis of gene functions in living cells (Dykxhoorn et al 2003). In order to initiate target mRNA silencing, it is first necessary for an introduced gene and the homologous endogenous target gene to specifically interact. Plausibly, the recognition/initiation phase of RNA silencing is promoted by the presence of homologous RNA transcripts alone (perhaps above a certain threshold level), or alternatively, by the expression of aberrant and/or incorrectly processed RNA transcripts (Baulcombe 1996) that operate through a double strand RNA (dsRNA) intermediate, inducing the formation of siRNAs. Both small sense- and small antisense-RNA molecules have been detected during early stages of RNA silencing in various organisms.

An RNA silencing-like mechanism was first described in plants following attempts to overexpress gene constructs encoding key enzymes in the anthocyanin biosynthesis pathway, in transgenic petunia (Napoli et al 1990, van der Krol et al 1990). Contrary to their expectation, the pigmentation in the flowers and the endogenous gene mRNA transcript levels of transformed plants were not enhanced (Napoli et al 1990, van der Krol et al 1990).

The functional analysis of *Arabidopsis-ALDH* genes was here investigated by exploring the antisense-*ALDH3I1* transcript accumulation, which induced the repression of endogenous *ALDH3I1* gene through the control of the constitutive CaMV 35S promoter in transgenic *Arabidopsis* plants. Since the overexpression of the gene conferred tolerance to drought and salt stress, it was then hypothesised that antisense repression of the endogenous gene in transgenic plants could result into stress sensitivity. Efficiency of antisense repression of the *ALDH3I1* gene was tested by RNA blot analysis (Figure 14, 15). Transgenic antisense lines AS-A3P1, AS-A3P2, 3, 5, 7 and AS-A3P9 showed a complete repression of the *ALDH3I1* gene, while AS-A3P4 showed a 60 - 75 % reduction level of the endogenous *ALDH3I1* transcript (Figure 14). The levels of lipid peroxidation and ROS generation in antisense transgenic plants under stress conditions were relatively higher than in the wild-type and in transgenic plants overexpressing *ALDH3I1* gene. These results confirmed that *ALDH* genes are involved in mechanisms of abiotic stress tolerance in plants and their repression in antisense transgenic lines induced vulnerability to several abiotic stresses. This molecular approach has been used successfully in functional characterization of genes in various plant species exposed to stress conditions. The antisense expression of the prl1 gene in *Arabidopsis* plants resulted in root

elongation arrest (Nemeth et al 1998). These results point to the involvement of the prl1 gene in processes of root elongation in plants. In addition Oervar and Ellis (1997) demonstrated that expression of an antisense construct, comprising about 45 % of the 3' coding region of tobacco catalytic ascorbate peroxidase (APX), could reduce significantly both the endogenous APX mRNA levels and the APX catalytic activity in those plants. Their findings also showed that transgenic plants with reduced endogenous APX mRNA and APX catalytic activity displayed a significantly higher level of ozone injury following very high ozone exposure, indicating that cytosolic APX is an important factor in oxidative stress management in tobacco plants following ozone exposure. Likewise, the results presented here showed that antisense repression of *ALDH3I1* resulted not only in sensitivity to dehydration and salt stress but also to vulnerability to lipid peroxidation and oxidative stress. In the presence of 2.5 mM H_2O_2 the antisense *ALDH3I1* transgenic seeds completely failed to germinate, confirming their vulnerability to oxidative stress. This study is the first successful antisense suppression of *ALDH3I1* gene that proves the involvement of *ALDH* gene in abiotic stress tolerance in plants.

4.2.4. *ALDH* knock-out in transgenic T-DNA insertion mutant plants

Another powerful approach of functional characterization of proteins is the screening of mutagenized populations by techniques that create protein inactivations using T-DNA insertions. In the last decade, increasing amounts of transgenic plants with specific protein inactivations have been generated in various studies by *Agrobacterium tumefaciens*-mediated T-DNA insertions (Østergaard and Yanofsky 2004). This approach was successfully used by Finkelstein (1994) to identify two novel *Arabidopsis* loci

(ABA-insensitive 4 and 5: ABI4, ABI5) involved in ABA sensitivity at seed germination, dormancy and stomatal regulation. Recently Nair et al (2004) have used this approach (T-DNA insertion) to demonstrate that REDUCED EPIDERMAL FLUORESCENCE 1 (REF1) gene encodes an aldehyde dehydrogenase that is involved in ferulic and sinapic acid biosynthesis in *Arabidopsis thaliana*. REF1 is needed for the accumulation of cell wall-bound ferulic acid in higher plants and mutant plants with defective REF1 activity accumulate reduced amount of ferulic and sinapic acid in the plant cell walls (Nair et al 2004). REF1 has been reported to have a useful application in crop improvement because of its role in cross-linking cell wall-bound polysaccharides to lignin (Grabber et al 2000, Grabber et al 2002). *Agrobacterium tumefaciens*-mediated *ALDH3I1* T-DNA insertion was used here to identified *ALDH3I1* knock-out transgenic plants in order to study downstream effects of ALDH3I1 protein function under various abiotic stress conditions. The knock-out mutants displayed a higher level of sensitivity to dehydration and salt stress and showed signs of wilting five days earlier than the wild-type and transgenic *ALDH3I1* overexpressing lines. The protein blot analysis showed a complete loss of the ALDH3I1 protein accumulation in the selected knock-out mutant plants. Phenotypic sensitivity of the knock-out transgenic plants to the stress treatments could be attributed to the loss of ALH3I1 protein function. The level of lipid peroxidation is higher in the knock-out mutants than in the wild-type and the transgenic plants overexpressing *ALDH* genes. Bouché et al (2003) have used T-DNA insertion mutagenesis to elucidate the potential of mitochondrial succinic-semialdehyde dehydrogenase (SSADH) of GABA shunt in protecting *Arabidopsis* plants against excessive accumulation of ROS under abiotic stress. Under 3 weeks of light exposure, ssadh-mutant plants accumulate higher

amounts of ROS in their leaves when compared to the wild-type plants (Bouché et al 2003). These data confirm the role of *ALDH* genes in plant protection against the effects of lipid peroxidation and generation of ROS. It is therefore hypothesized that reduced function of *ALDH* genes could lead to susceptibility to various abiotic stress conditions. The loss of ALDH function could irreversibly weaken the oxidative defence systems of plants and render them vulnerable to various environmental stresses. This results are in agreement with the findings of Bouché et al (2003) about a mitochondrial succinic-semialdehyde dehydrogenase (SSADH) of GABA shunt, which is requested to protect *Arabidopsis* plants against excessive accumulation of ROS.

4.3. Protective effects of *ALDHs* against lipid peroxidation

To check the level of cell damage in the plant tissues under stress conditions, the accumulation of MDA as result of polyunsaturated fatty acid oxidations was determined. Lower lipid peroxidation occurred in transgenic plants (S-A3Ps, CPs, S-A7Ps, C-A3Ps) overexpressing the *ALDH* genes than in the wild-type plants under all different stress conditions tested. Aldehyde molecules derived from lipid peroxidation are highly reactive and stable (Sunkar et al 2003). They can easily diffuse and attack proteins and nucleic acids far away from their production site. It is believed that the enhanced detoxification of aldehydes and their intermediates in transgenic plants overexpressing *ALDH* genes reduce the level of lipid peroxidation, which might also improve the photosynthetic reactions in those plants. Several other genes were reported to reduce lipid peroxidation in plants under environmental stress (Oberschall et al 2000). Overexpression of a novel

aldose/aldehyde reductase protects transgenic plants from lipid peroxidation under chemical and drought stress conditions (Oberschall et al 2000).

The overexpression of *ALDH3I1* and *ALDH7B4* genes in transgenic plants was proven to significantly reduce the level of lipid peroxidation under drought and salt stress (Figure 22, 23, 25). These results suggest that the higher ALDH protein activities detected in those plants leads to a scavenging effect of toxic by-products such as aldehyde molecules and their intermediates accumulating as a result of stress conditions. The transgenic plants with a reduced or a loss of ALDH function (transgenic *ALDH3I1* antisense lines and *ALDH3I1* knock-out mutants) were more sensitive to dehydration and salt stress than the wild-type and the overexpressing transgenic lines (Figure 19, Table 6). The relatively high level of lipid peroxidation recorded in the antisense and knock-out mutant plants indicated a higher accumulation of aldehyde by-products as a result of the loss of aldehyde dehydrogenase activity in comparison with the wild-type and the transgenic plants overexpressing ALDH genes. These results confirm the protective role of ALDH proteins against lipid peroxidation. Trans-4-hydroxy-2-nonenal (4-HNE) is one of the major aldehyde molecules produced during lipid peroxidative reactions. 4-HNE generally results from radical-initiated degradation of polyunsaturated fatty acids such as linoleic and arachidonic acids, two relatively abundant fatty acids in cells (Hu et al 2002). In addition, the aldehyde by-products can be further metabolised to an epoxide form that can interact with DNA to form exocyclic etheno-guanine, -adenine, and –cytosine adducts (Chung et al 1996). On the basis of reduced lipid peroxidation and ROS levels detected in transgenic plants overexpressing ALDH3I1, ALDH7B4, Cp-ALDH proteins, probably certain ALDH proteins of families 3 and family 7 can be regarded as highly efficient

enzymes in detoxifying aldehyde by-products and in the protection of macromolecules such as DNA and other proteins.

4.4. ROS generation and antioxidative effects of ALDHs

ROS are products of the normal cellular metabolism that can cause oxidative stress/damage to living tissues when produced in excess. Under stress conditions, the production of ROS is usually exacerbated, which subsequently leads to the disruption of electron transport systems (Kovtun et al 2000, Arora et al 2002). In such conditions, organelles with highly oxidizing metabolic activity or with sustained electron flows such as chloroplasts, and mitochondria are functionally disrupted by the production of excessive ROS (Wise and Naylor 1987, Goel and Sheoran 2003). Within the photosynthetic apparatus, photosystem II (PSII) is mostly affected by drought, particularly within the oxygen-evolving complex and the reaction centres (Toivonen and Vidaver 1988). Protection against oxidative stress is complex and includes both enzymatic and non-enzymatic components (Bowler et al 1992). The key enzymatic systems in cell defence against oxidative damage are superoxide dismutases (Baum and Scandalios 1979, Bowler et al 1992, Tertivanidis et al 2004), glutathione reductase and glutathione oxidase (Price et al 1994). The physiological and molecular correlations between elevated aldehyde dehydrogenase gene expressions in the presence of H_2O_2 and stress tolerance suggest that aldehyde dehydrogenase genes also might enhance the stress-defence potential of plants against oxidative damage. The transgenic plants (S-A3Ps, C-A3Ps, and S-A7Ps) expressing increasing amounts of *ALDH* genes were highly tolerant to abiotic stress and accumulated reduced amounts of ROS. Bouché et al (2003)

has shown that a mitochondrial succinic-semialdehyde dehydrogenase belonging to family 5 *Arabidopsis ALDH* (*ALDH5F1*) is required to restrict levels of reactive oxygen intermediates in plants. Their report reveals that the mitochondrial succinic-semialdehyde dehydrogenase is essential for normal plant growth, at least in part by suppressing the accumulation of H_2O_2 generated under light and heat stresses. The potential of mitochondrial succinic-semialdehyde dehydrogenase to restrict the ROS accumulation is explained by its ability to supply NADH and succinate under conditions that inhibit the tricarboxylic acid (TCA) cycle and impair respiration (Bouché et al 2003). In addition, a mitochodrial Ä^1-pyrroline-5-carboxylate dehydrogenase in *Arabidopsis thaliana* was revealed to protect against proline toxicity (Deuschle et al 2001). Proline toxicity in plant lead to production of ROS in plant (Madeo et al 1999), supporting the hypothesis that mitochodrial Ä^1-pyrroline-5-carboxylate dehydrogenase in *Arabidopsis thaliana* could protect plants against oxidative stress (Deuschle et al 2001).

The direct involvement of ALDH function in antioxidative processes is confirmed by the high accumulation of ALDH3I1 protein under exogenous application of H_2O_2 in wild-type plants (Figure 5). In addition, the qualitative and quantitative estimation of H_2O_2 content in leaves of plants exposed to salt stress (Figure 29, 30) clearly point to a direct function of ALDH enzymes as ROS (H_2O_2)-scavengers. Canuto et al (1996) demonstrated in animal cells that enhanced lipid peroxidation by cell enrichment with arachidonic acid and treatment using pro-oxidants inhibits the effect of class 3 ALDH due to a probable decrease of class 3 *ALDH* gene transcripts, and when such cell treatment resulted in the complete inhibition of the class 3 ALDH protein synthesis, cell death followed. Moreover, deficiency in a mitochondrial aldehyde dehydrogenase has

been reported to increase vulnerability to oxidative stress in PC12 cells (Ohsawa et al 2003). Probably a similar antioxidative role of ALDHs may be effective in plants. Transgenic plants overexpressing *ALDH* genes were able to display a higher ALDH protein activity leading to an efficient detoxification process of toxic aldehyde molecules generated during stress conditions. It is therefore hypothesized that ALDH proteins not only have a role in detoxification of aldehyde molecules but also participate directly to ROS-scavenging and antioxidative processes in responses of plants to environmental stress.

To study the ability of ALDH protein activity to protect cellular structures, plant cell viability in both wild-type and transgenic lines were investigated under stress conditions. Various rapid staining strategies such as protoplasmic streaming or cyclosis, plasmolysis and fluorescein diacetate (Wildholm 1972) have been used to detect viable plant cells in different experiments. In 1970, fluorescein diacetate was successfully used to detect viable pollen in plants (Heslop-Harrison and Heslop-Harrison 1970). Fluorescein diacetate was used here to detect cell viability in plants exposed to stress conditions. Transgenic plants overexpressing *ALDH* genes (S-A3P3, S-A7P1, 2, 3) showed a higher amount of fluorescent cells under 100 and 200 mM NaCl treatments (Figure 31). Wild-type plants showed no fluorescence under 200 mM NaCl treatment, indicating that a higher activity of ALDH proteins in those transgenic plants leads to an efficient detoxification of aldehyde molecules, subsequent reduction of excessive ROS production and thus higher viability. This clearly explains the better protective status of plant tissues against oxidative damage in the transgenic plants and the higher amount of cell viability observed in those plants when compared to the wild-type plants under the same stress

conditions (Figure 31). The above results demonstrate that aldehyde dehydrogenases display a broad functional spectrum that includes toxic aldehyde detoxification, inhibition of lipid peroxidation, ROS-scavenging effects, and antioxidative properties in order to maintain plant tissue integrity under abiotic stress conditions. This could be the reason why transgenic plants expressing higher levels of ALDH proteins are more resistant to salt and drought stress than wild-type plants (Sunkar et al 2003). The findings discussed here contribute to our understanding about the role of plant-ALDH proteins as ROS-scavengers and antioxidative enzymes to confer tolerance to abiotic stress. It is therefore suggested that results from thorough investigations of regulatory mechanisms of *ALDH* gene expression in plants could provide an excellent way to obtain transgenic plants that can cope with various environmental stresses.

4.5. Concluding remarks

From the above results, it is clear that understanding the molecular basis of aldehyde dehydrogenase action and the interaction of ALDHs with other stress-inducible proteins in plants could provide a broad basis of generating transgenic plants that cope with multiple stresses. Results of this work showed that some members of the ALDH protein superfamily (ALDH3I1, ALDH7B4) and Cp-ALDH are involved in antioxidative defence systems in plants exposed to abiotic stress. The perturbation of *ALDH3I1* gene expression resulting in the loss of the corresponding protein functions correlates with vulnerability to oxidative stress. This work demonstrates that the overexpression or the silencing or knock-out expression of these genes greatly alters the process of abiotic stress adaptation in *Arabidopsis thaliana*. Overexpression of aldehyde dehydrogenase

genes may be crucial for plants to cope with environmental stresses such as drought and salt stress. It will be interesting to further investigate the overexpression of members of aldehyde dehydrogenase gene superfamily in various agronomically valuable crops with the aim of improving crop tolerance to multiple environmental stressors. Studies carried out in *Arabidopsis thaliana* have made a major contribution to the current understanding of *ALDH* involvement in molecular and biochemical basis of abiotic stress tolerance in plants (Busch and Fromm 1999, Deuschle et al 2001, Liu et al 2001, Kirch et al 2001, Bouché et al 2003, Sunkar et al 2003, Kirch et al 2004). The results presented here notably demonstrate that combinatory approaches of RNA silencing via antisense-RNA expression, T-DNA knock-out insertion and the overexpression of the same gene provides appropriate data to gain understanding of the biological function of the gene. Therefore, this work represents a valuable contribution in understanding the function of some members of aldehyde dehydrogenase gene superfamily and their potential to confer osmotic and oxidative stress tolerance in higher plants. It is believed that generating transgenic plants with double overexpression of member of class 3 and class 7 *ALDH* genes such as *ALDH3I1* and *ALDH7B4* could be a promising way to increase abiotic stress tolerance enhancement in higher plants.

5. APPENDICES

5.1. Accession numbers of the ALDH genes

ALDH3I1 gene:

GenBank accession number: AJ30691 (Kirch et al 2001, Kirch et al 2004)

ALDH3H1 gene:

GenBank accession number: AY072122 (Kirch et al 2004)

ALDH7B4 gene:

GenBank accession number: AJ584645 (Kirch et al 2004)

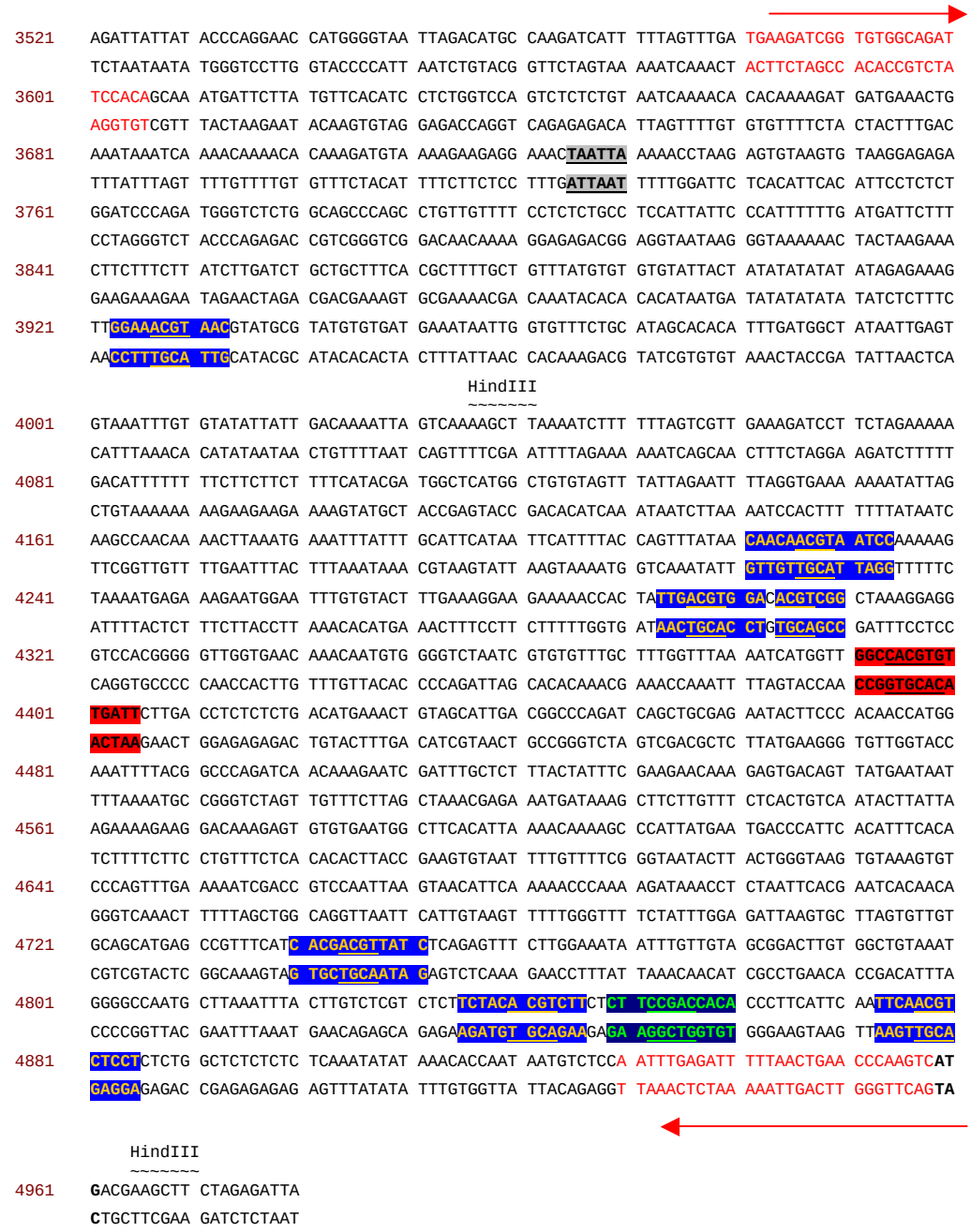
Cp-ALDH gene:

GenBank accession number: Cp-ALDH AJ306960 (Kirch et al 2001).

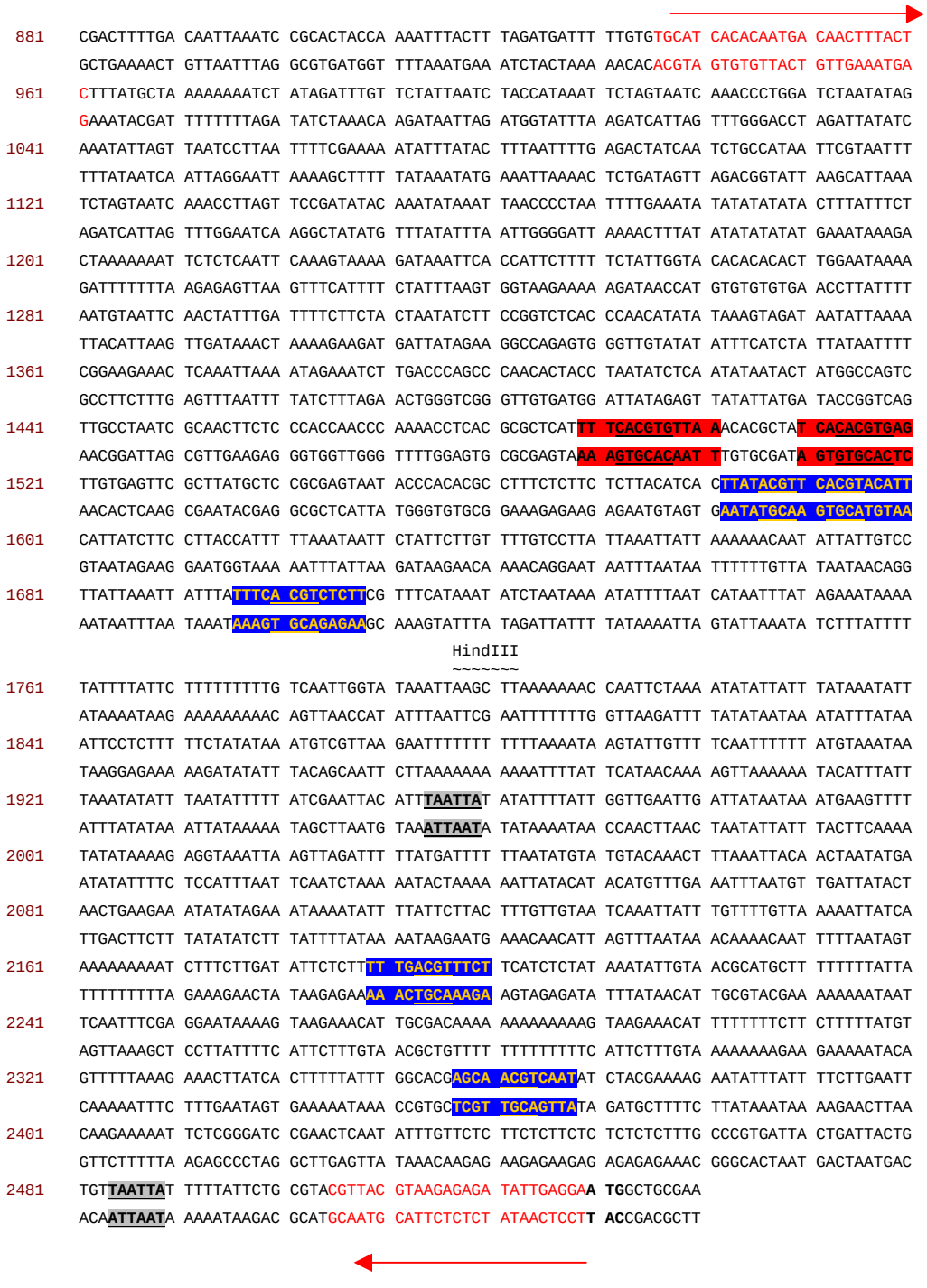
5.2. Promoter sequences of the ALDH genes

The promoter sequences of the ALDH genes are here shown using the Vector NTI™ Suite programme. The TATA boxes are shaded in grey. The G box like is shade in red. The DRE like core motif is in green and shaded in blue. The ACGT-like ABRE motif is in yellow and shaded in blue. The forward and reverse primers are shown in red arrows and the ATG start codon is boldfaced in black.

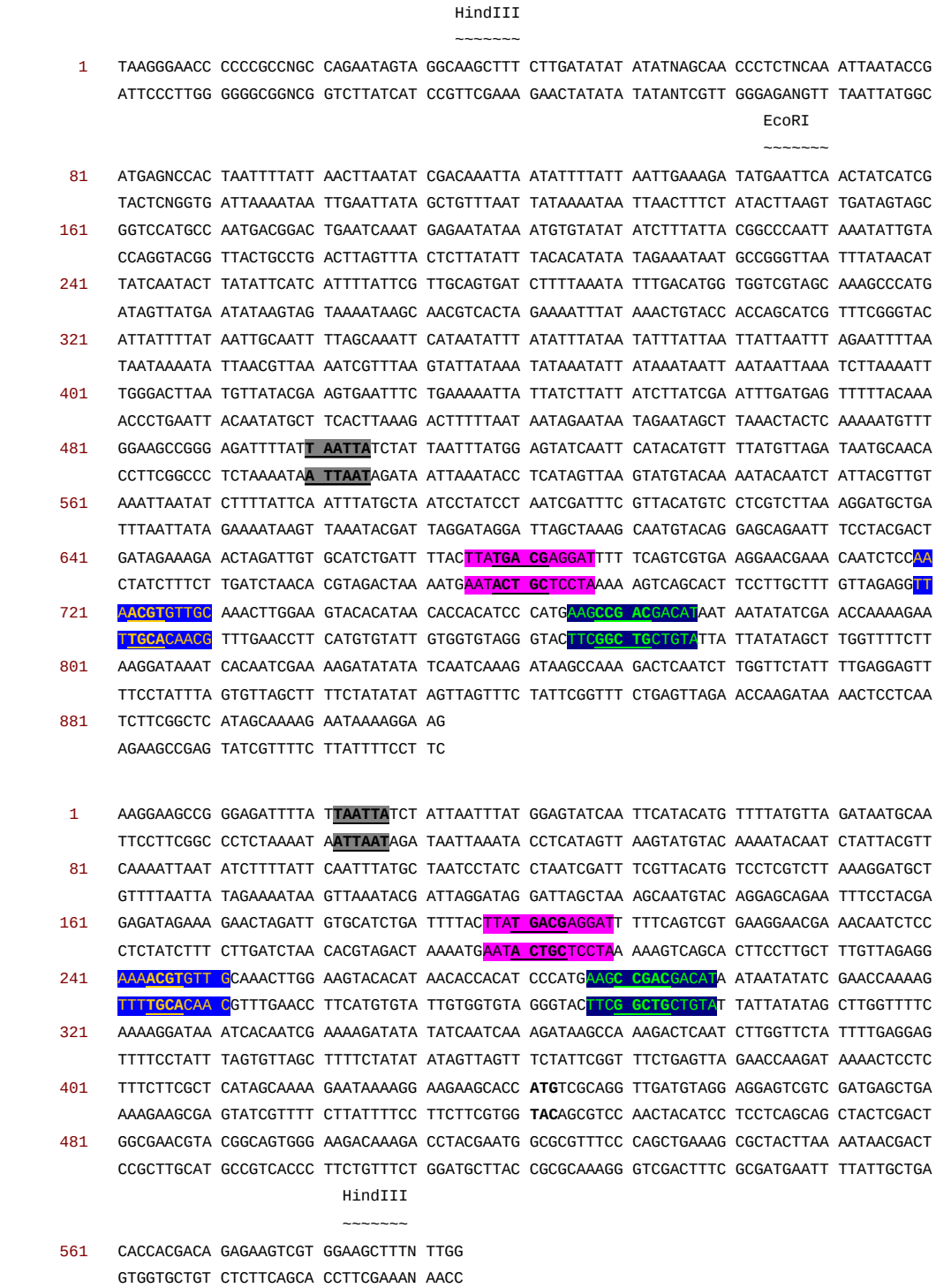
ALDH3I1 Promoter



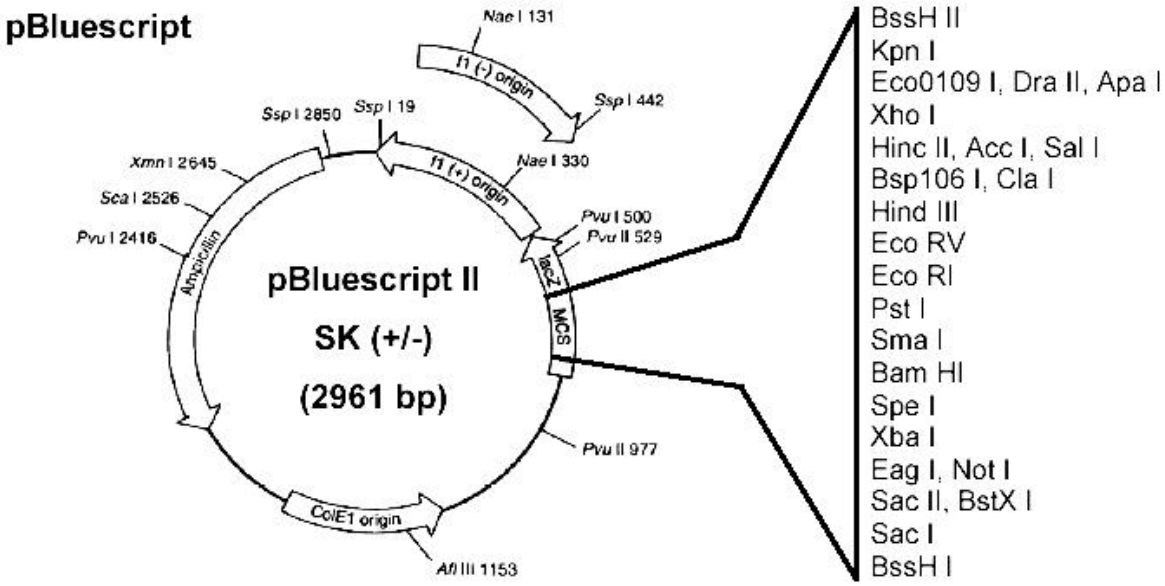
ALDH3H1 Promoter

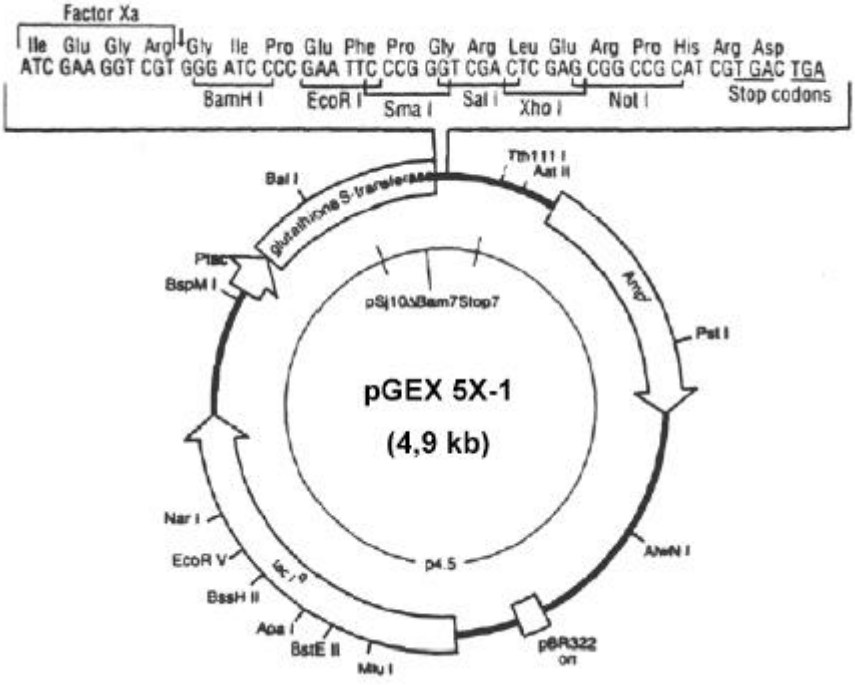


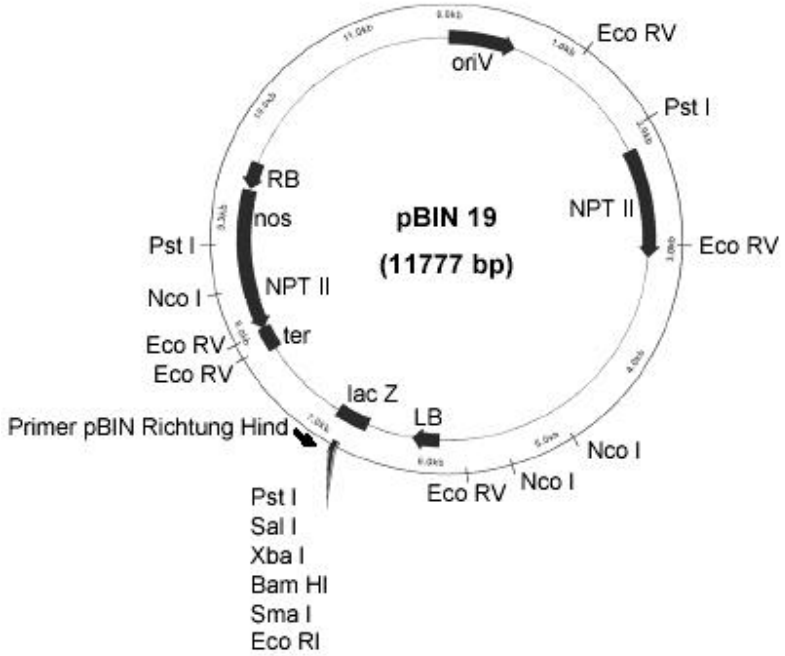
Cp-ALDH promoter

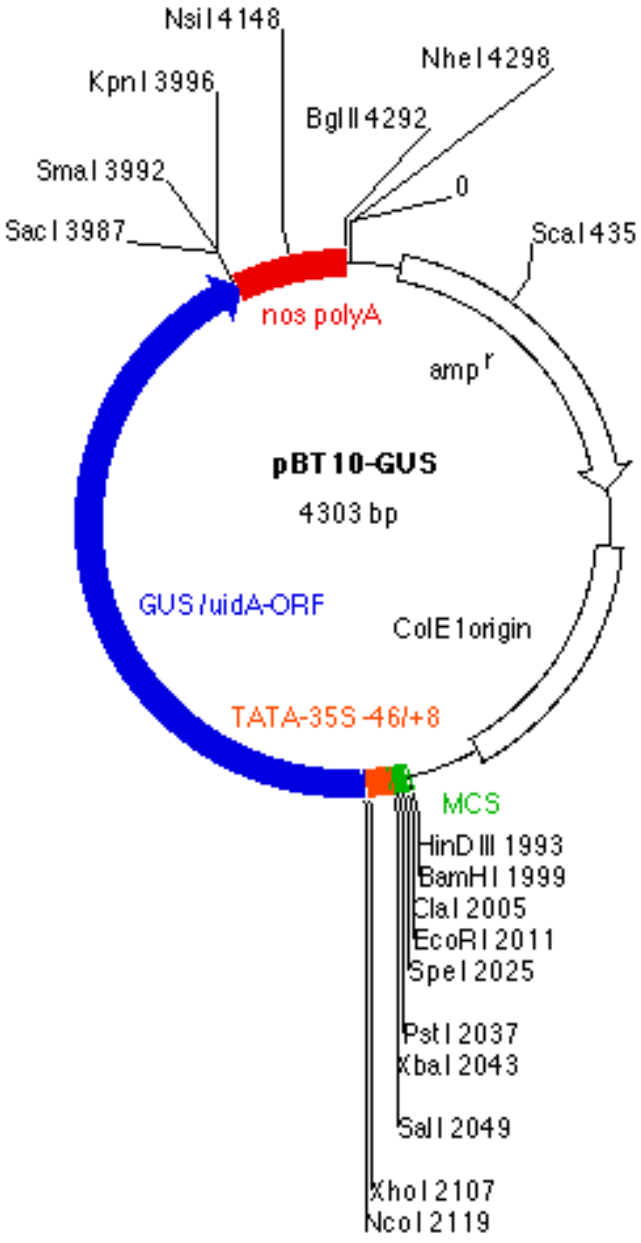


5.3. Map of the vectors with restriction enzyme positions




Factor Xa
Ile Glu Gly Arg Gly Ile Pro Glu Phe Pro Gly Arg Leu Glu Arg Pro His Arg Asp
ATC GAA GGT CGT GGG ATC CCC GAA TTC CCG GGT CGA CTC GAG CGG CCG CAT CGT GAC TGA
BamH I
EcoR I
Sma I
Sal I
Xho I
Not I
Stop codons
Bal I
Ptac
BspM I
Nar I
EcoR V
BssH II
Apa I
BstE II
Mlu I
Pst I
AlwN I
pGEX 5X-1
(4,9 kb)
p4.5
pBR322 ori


oriV
Eco RV
Pst I
RB
nos
NPT II
Eco RV
pBIN 19
(11777 bp)
Pst I
Nco I
NPT II
Eco RV
ter
Eco RV
lac Z
LB
Primer pBIN Richtung Hind
Nco I
Eco RV
Nco I
Pst I
Sal I
Xba I
Bam HI
Sma I
Eco RI


NsiI 4148
NheI 4298
KpnI 3996
BglII 4292
SmaI 3992
0
SacI 3987
ScaI 435
nos polyA
amp r
pBT10-GUS
4303 bp
GUS/uidA-ORF
ColE1origin
TATA-35S -46/+8
MCS
HinD III 1993
BamHI 1999
ClaI 2005
EcoRI 2011
SpeI 2025
PstI 2037
XbaI 2043
SalI 2049
XhoI 2107
NcoI 2119

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