



Aldehyde dehydrogenases in *Arabidopsis thaliana*: biochemical requirements, metabolic pathways, and functional analysis

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Aldehyde dehydrogenases (ALDHs) are a family of enzymes which catalyze the oxidation of reactive aldehydes to their corresponding carboxylic acids. Here we summarize molecular genetic and biochemical analyses of selected *Arabidopsis* ALDH genes. Aldehyde molecules are very reactive and are involved in many metabolic processes but when they accumulate in excess they become toxic. Thus activity of aldehyde dehydrogenases is important in regulating the homeostasis of aldehydes. Overexpression of some ALDH genes demonstrated an improved abiotic stress tolerance. Despite the fact that several reports are available describing a role for specific ALDHs, their precise physiological roles are often still unclear. Therefore a number of genetic and biochemical tools have been generated to address the function with an emphasis on stress-related ALDHs. ALDHs exert their functions in different cellular compartments and often in a developmental and tissue specific manner. To investigate substrate specificity, catalytic efficiencies have been determined using a range of substrates varying in carbon chain length and degree of carbon oxidation. Mutational approaches identified amino acid residues critical for coenzyme usage and enzyme activities.

Keywords: aldehyde dehydrogenases, aminoaldehyde dehydrogenases, betaine aldehyde dehydrogenases, coenzyme binding, enzyme activities, stress tolerance

INTRODUCTION

Aldehyde dehydrogenases (ALDHs) represent an evolutionary conserved protein superfamily of NAD(P)⁺-dependent enzymes that metabolize a wide range of endogenous and exogenous aliphatic and aromatic aldehydes, which are toxic, if their steady state level is not strictly regulated. A steadily increasing number of ALDHs has been identified in almost all taxa (Sophos and Vasil-iou, 2003), but a comprehensive structural and functional analysis has so far only been performed on the human ALDHs (Marchitti et al., 2008). Although the completion of a number of genome projects in recent years has led to the identification of ALDH protein superfamilies of some lower and higher plant species, as *Physcomitrella patens*, *Arabidopsis*, maize, and rice (Kirch et al., 2004; Wood and Duff, 2009; Jimenez-Lopez et al., 2010; Kotchoni et al., 2010), their physiological roles are largely unclear. The interest in studying ALDHs is triggered by available data that indicate essential functions of ALDHs in growth, development, and stress adaptation. The maize mitochondrial ALDH2B2 has been identified as the nuclear restorer of cytoplasmic male sterility (Cui et al., 1996; Liu et al., 2001). The rice mitochondrial ALDH family 2 probably is essential for the detoxification of acetaldehyde during re-aeration after submergence (Tsuji et al., 2003), whereas OsALDH7B6 is necessary for seed maturation and maintenance of seed viability by detoxifying aldehydes generated by lipid peroxidation (Shin et al., 2009). Nair et al. (2004) showed that the *reduced epidermal fluorescence1 (ref1)* mutant of *Arabidopsis* is caused by a

mutation in the *ALDH2C4* gene (At3g24503) and that ALDH2C4 plays an important role in the phenyl-propanoid pathway, being involved in ferulic acid and sinapic acid biosynthesis. The *Arabidopsis* ALDH2B4 may play a role in the pyruvate dehydrogenase bypass pathway (Wei et al., 2009).

The activities of aldehyde dehydrogenases seem to be crucial to regulate the imbalanced accumulation of toxic aldehydes in plants exposed to stress conditions. Different plant ALDH genes have been reported to respond to environmental stress conditions such as dehydration, salinity, or excessive light. Overexpression of selected ALDH genes confers enhanced stress tolerance to transgenic plants, which demonstrates the importance of ALDH proteins in stress responses (Deuschle et al., 2001, 2004; Kirch et al., 2001, 2005; Bouché et al., 2003; Sunkar et al., 2003; Kotchoni et al., 2006; Rodrigues et al., 2006).

The *Arabidopsis thaliana* genome encodes 14 genes belonging to nine different aldehyde dehydrogenase families ranging from substrate specific to variable substrate ALDH proteins (Kirch et al., 2004; **Table 1**). In this review we summarize the current knowledge on selected aldehyde dehydrogenases, which have been studied in our laboratory, using genomic, and biochemical tools. The focus has been on the functional analysis of selected members of different *Arabidopsis* ALDH families, implicated in abiotic stress tolerance. A platform encompassing genetic and molecular tools was developed for *Arabidopsis* ALDH genes with a potential role in osmotic stress adaptation and novel ALDHs. Functional

Table 1 | Localization and putative physiological functions of the *Arabidopsis thaliana* ALDH protein superfamily.

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

Data in bold refer to ALDHs that have been analyzed in our own laboratory.

genomics tools have been generated for the stress-related family 3 and family 7 ALDH genes (*ALDH3I1*, *ALDH3H1*, *ALDH3F1*, and *ALDH7B4*), the betaine aldehyde dehydrogenase (*BADH*) homologues *ALDH10A8* and *ALDH10A9*, and the novel plant specific gene *ALDH22A1*.

MOLECULAR, BIOCHEMICAL, AND PHYSIOLOGICAL FEATURES OF *ARABIDOPSIS* ALDEHYDE DEHYDROGENASES SUBCELLULAR LOCALIZATION AND EXPRESSION PATTERNS

Aldehyde dehydrogenases genes were analyzed with regard to expression patterns, subcellular localization, and biochemical properties. Results of these studies are summarized in **Tables 1** and **2**. GFP reporter gene analysis demonstrates that ALDH proteins are targeted to different cellular compartments (**Figure 1**). *ALDH3I1* proteins are targeted to the chloroplasts, while *ALDH3H1*, *ALDH3F1*, *ALDH22A1*, and *ALDH7B4* are found in the cytosol (Kuhns, 2005; Kotchoni et al., 2006; Ditzer, unpublished results). The putative *BADH* proteins, *ALDH10A8* and *ALDH10A9* are targeted to leucoplasts and peroxisomes, respectively (Missihoun et al., 2011), which is in accordance to the localization of *BADH* proteins from other plant species (Weretilnyk and Hanson, 1990; Nakamura et al., 1997, 2001; Shirasawa et al., 2006; Fitzgerald et al., 2009). The differential localization of ALDH enzymes implies a functional specialization and suggests that different compartments may require ALDH proteins with specific biochemical properties. The fact that several ALDHs proteins are conserved and ubiquitously present in plant cells emphasizes their biological importance, although their function is often still unclear.

To elucidate the function of ALDH genes expression was analyzed on the transcript level and by examining transgenic promoter-*GUS Arabidopsis* plants. ALDH genes are expressed

in a tissue specific manner. *GUS* expression in *ALDH3H1* and *ALDH22A1* transgenic plants is much stronger in roots than in leaves. *ALDH3F1::GUS* plants display a very strong expression in the whole plant and in germinating seeds (Kirch et al., 2005; Ditzer, unpublished results). A high *GUS* activity was detected in pistil and stamen, siliques and mature seeds of *ALDH7B4::GUS* transgenic plants (Missihoun, 2011). Plants carrying the *ALDH22A1* promoter-*GUS*-fusion showed constitutive *GUS* expression in all tissues but with variable intensities (**Figure 2A**). *GUS* expression was high in siliques, flowers, roots, and relatively low in leaves under control and stress conditions.

Transcript accumulation analyses demonstrated that five of the analyzed *Arabidopsis* ALDH genes (*ALDH3H1*, *ALDH3I1*, *ALDH7B4*, *ALDH10A8*, and *ALDH10A9*) respond to ABA treatment and abiotic stress conditions such as salt and dehydration, while *ALDH3F1* and *ALDH22A1* are constitutively expressed and appear to be not related to stress (Kirch et al., 2001, 2005; Sunkar et al., 2003; Missihoun et al., 2011).

Stress induces ALDH genes differentially (Kirch et al., 2005). *ALDH3I1*, *ALDH3H1*, *ALDH7B4* promoters are induced in response to abiotic stress conditions (**Table 2**). *ALDH3I1::GUS* plants display a high expression in leaves but not in roots after ABA treatment, dehydration, or NaCl stress, while the situation is *vice versa* for *ALDH3H1* (Kirch et al., 2005). The *ALDH7B4* promoter shows the strongest stress-inducibility, and high *GUS* expression was found in all tissues in response to ABA, dehydration, NaCl, and wounding. In addition, the *ALDH7B4* promoter is also slightly inducible by aldehydes (Höller, 2010; Missihoun, 2011), which may point to a feedback regulation in some metabolic conditions.

To identify possible posttranscriptional regulation steps ALDH protein accumulation was analyzed with specific antibodies. The

Table 2 | Molecular, biochemical, and physiological features of Arabidopsis aldehyde dehydrogenases.

	ALDH3F1	ALDH3H1	ALDH3I1	ALDH7B4	ALDH10A8	ALDH10A9	ALDH22A1
Transcript accumulation	Constitutive expression ^{3,7}	Weakly stress inducible in 4-week-old plantlets, up-regulated in root cultures by ABA, dehydration, NaCl ³	Stress inducible by ABA, dehydration, NaCl, heavy metal, H ₂ O ₂ , and methyl viologen treatment ^{1,2,3}	Strongly stress inducible by ABA, dehydration, and NaCl treatment ³	Weakly stress inducible by ABA, dehydration, NaCl, chilling, and methyl viologen treatment ⁵	Weakly stress inducible by ABA, dehydration, NaCl, chilling, and methyl viologen treatment ⁵	Constitutive expression ^{3,7}
Protein expression	Constitutive expression ⁷	Up-regulated by salt stress mainly in leaves of plants older than 6 weeks ⁷	Stress inducible by ABA, dehydration, NaCl, CuCl ₂ , and H ₂ O ₂ treatment in plants older than 5 weeks ^{4,7}	Strongly stress inducible by ABA, dehydration, NaCl, CuCl ₂ , and H ₂ O ₂ treatment ⁴	Not determined	Not determined	Constitutive expression ⁷
Tissue specificity in transgenic promoter::GUS lines							
Control conditions	Strong expression in whole plant and germinating seeds ⁷	Low to medium expression in seedling leaves and roots, respectively ³	No expression in seedling leaves and roots ³	High expression in pistil and stamen, siliques, and mature seeds ⁷	Not determined	Not determined	High expression in open flowers, carpels, siliques, mature seeds, and roots; low expression in petals, sepals, and leaves ⁷
Stress conditions	Not stress inducible ⁷	High expression in roots but not in leaves after ABA treatment, dehydration, and NaCl stress ³	High expression in leaves but not in roots after ABA treatment, dehydration, and NaCl stress ³	High expression in leaves and roots after dehydration, NaCl stress, and wounding, slightly induced by aldehydes ⁷	Not determined	Not determined	Not stress inducible ⁷
Knock-out mutant phenotype	No different phenotype in comparison to control plants ⁷	Increased MDA levels and inhibition of root growth in comparison to wild-type plants upon salt stress ⁷	Increased sensitivity to dehydration and salt stress and higher MDA levels than wild-type plants ^{2,4}	Increased sensitivity to dehydration and salt stress and higher MDA levels than wild-type plants ⁴	Increased chlorosis and high accumulation of anthocyanin after NaCl and mannitol treatment in seedlings, higher MDA levels than WT in adult plants upon salt, and dehydration stress ⁵	Not determined	No different phenotype in comparison to control plants ⁷

(Continued)

Table 2 | Continued

	ALDH3F1	ALDH3H1	ALDH3I1	ALDH7B4	ALDH10A8	ALDH10A9	ALDH22A1
Overexpressor phenotype	Higher germination rate and reduced accumulation of MDA after NaCl stress in comparison to WT ⁷	No improved stress tolerance, but reduced accumulation of MDA in comparison to WT ⁷	Improved tolerance to dehydration, NaCl, heavy metals, methyl viologen, and H ₂ O ₂ ^{2,4}	Improved tolerance to osmotic (dehydration, KCl, NaCl) and oxidative stress ^{4,7}	Not determined	Not determined	No different phenotype in comparison to control plants ⁷
Enzymatic properties/preferred substrates	Not determined	Medium to long-chain saturated aldehydes (C-6 to C-12) ⁶	Medium to long-chain saturated aldehydes (C-6 to C-12) ⁶	Not determined	Not determined	Betaine aldehyde, 4-aminobutanal, 3-aminopropanal ⁵	Not determined

¹Kirch et al. (2001), ²Sunkar et al. (2003), ³Kirch et al. (2005), ⁴Kotchoni et al. (2006), ⁵Missihoun et al. (2011), ⁶Stiti et al. (2011), ⁷unpublished.

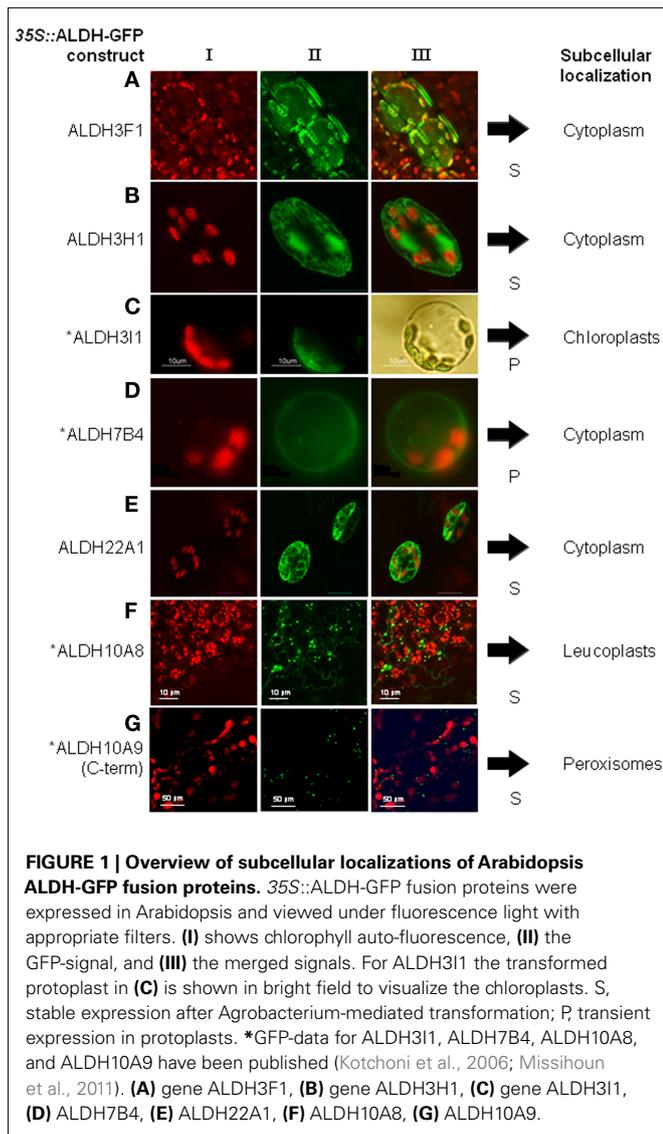


FIGURE 1 | Overview of subcellular localizations of Arabidopsis ALDH-GFP fusion proteins. *35S::ALDH-GFP* fusion proteins were expressed in *Arabidopsis* and viewed under fluorescence light with appropriate filters. (I) shows chlorophyll auto-fluorescence, (II) the GFP-signal, and (III) the merged signals. For ALDH311 the transformed protoplast in (C) is shown in bright field to visualize the chloroplasts. S, stable expression after *Agrobacterium*-mediated transformation; P, transient expression in protoplasts. *GFP-data for ALDH311, ALDH7B4, ALDH10A8, and ALDH10A9 have been published (Kotchoni et al., 2006; Missihoun et al., 2011). (A) gene ALDH3F1, (B) gene ALDH3H1, (C) gene ALDH311, (D) ALDH7B4, (E) ALDH22A1, (F) ALDH10A8, (G) ALDH10A9.

protein blot analyses reveal a developmental-specific expression pattern for two of the analyzed ALDH proteins. The ALDH311 protein is stress inducible in plants older than 5 weeks (approximately 8–10 rosette leaves; Kotchoni et al., 2006; Schmidt, 2006). In contrast, ALDH3H1 accumulates in response to salt stress in 7-week-old (approximately 12–14 rosette leaves) plants and on a lower level in 6-week-old plants (approximately 10–12 rosette leaves; Schmidt, 2006). ALDH3F1 is stress inducible only in young plants at a very low level. ALDH7B4 shows a strong stress response irrespective of age (Kotchoni et al., 2006; Schmidt, 2006). ALDH22A1 proteins accumulate in all tissues but to different amounts. Protein expression is higher in open flowers, carpels, siliques, mature seeds, and roots than in petals/sepals, stamens, and leaves (Figure 2B).

The comparison between transcript expression and protein accumulation as well as the promoter-*GUS* analyses indicate that *ALDH* gene expression is predominantly regulated transcriptionally.

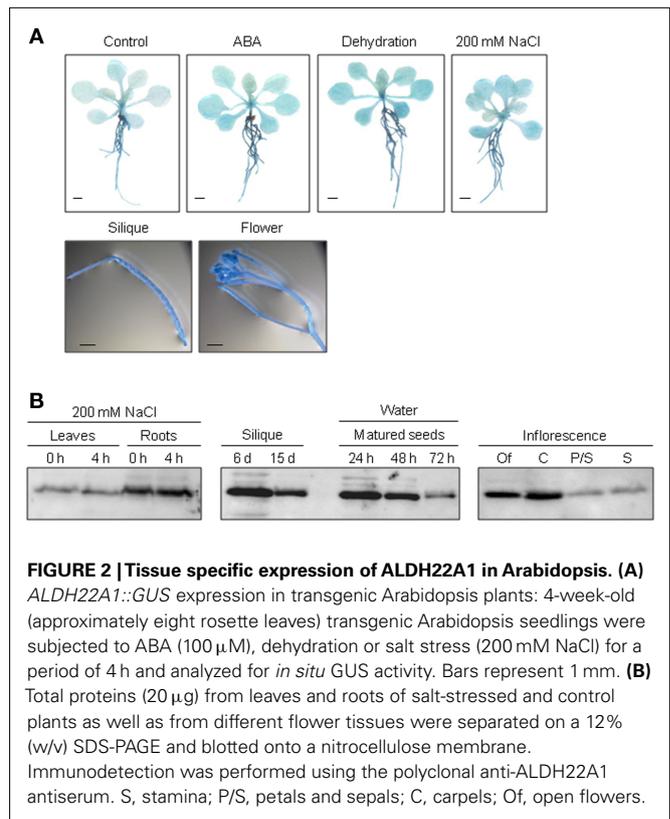


FIGURE 2 | Tissue specific expression of ALDH22A1 in Arabidopsis. (A) *ALDH22A1::GUS* expression in transgenic *Arabidopsis* plants: 4-week-old (approximately eight rosette leaves) transgenic *Arabidopsis* seedlings were subjected to ABA (100 μM), dehydration or salt stress (200 mM NaCl) for a period of 4 h and analyzed for *in situ* GUS activity. Bars represent 1 mm. (B) Total proteins (20 μg) from leaves and roots of salt-stressed and control plants as well as from different flower tissues were separated on a 12% (w/v) SDS-PAGE and blotted onto a nitrocellulose membrane. Immunodetection was performed using the polyclonal anti-ALDH22A1 antiserum. S, stamens; P/S, petals and sepals; C, carpels; Of, open flowers.

FUNCTIONAL SIGNIFICANCE OF ALDHs

The stress inducible ALDH proteins are predicted to be important for the mechanisms of stress adjustment and long-term adaptation. This hypothesis was investigated by analyzing the stress-performance of transgenic plants overexpressing *ALDH* genes and *ALDH*-knock-out lines. Transgenic *Arabidopsis* plants overexpressing *ALDH3F1*, *ALDH311*, or *ALDH7B4* genes are more tolerant to salt (NaCl and/or KCl) dehydration and oxidative stress. They show higher germination rates and reduced accumulation of malondialdehyde (MDA) in comparison to wild-type plants when challenged with salt, drought, or oxidative stress (Sunkar et al., 2003; Kotchoni et al., 2006; Martens, 2009). *ALDH* gene overexpressor phenotypes in transgenic plants are in agreement with physiological and molecular analyses of corresponding *ALDH* T-DNA single and double insertion mutants, which generally reveal an increased sensitivity to dehydration and salt stress and accumulate higher MDA levels than wild-type plants (Figure 3; Sunkar et al., 2003; Kotchoni et al., 2006; Missihoun, 2011; Missihoun et al., 2011). Plants overexpressing *ALDH3H1* do not display improved stress tolerance, although accumulation of MDA under stress conditions was reduced in comparison to wild-type plants (Missihoun, 2011). Thus, this gene may have another function.

The stress tolerant phenotype of transgenic *Arabidopsis* plants overexpressing either *ALDH311* or *ALDH7B4* is further supported by experiments with transgenic tobacco plants (Raza, 2010). Results regarding the protective role of ALDH7B4 are confirmed by

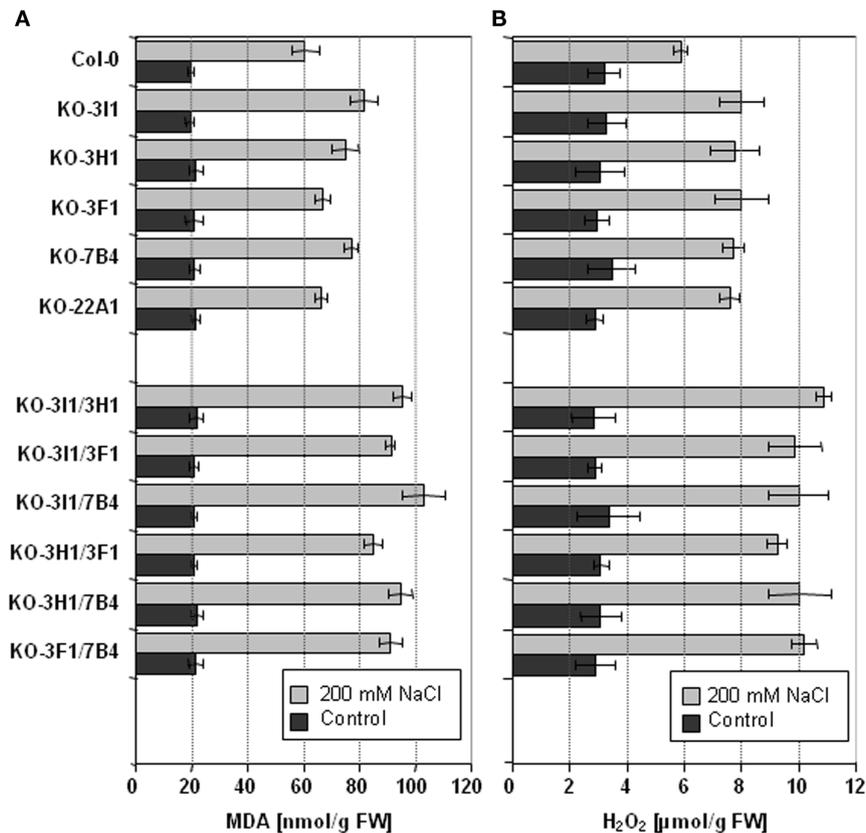


FIGURE 3 | Lipid peroxidation and H₂O₂ accumulation in Arabidopsis ALDH T-DNA knock-out lines. Lipid peroxidation levels (A) and H₂O₂ accumulation (B) of 4-week-old, soil-grown Arabidopsis T-DNA knock-out plants that were irrigated with 200 mM NaCl in tap water every second day for 7 days. Control plants were treated with tap water for the same period.

Lipid peroxidation was determined as the amount of malondialdehyde (MDA) accumulating as an end product of lipid peroxidation (Sunkar et al., 2003). Quantitative H₂O₂ measurements were performed according to Kotchoni et al. (2006). The data represent mean values (\pm SD) of three replicate experiments.

Rodrigues et al. (2006), who report that *Arabidopsis* and tobacco plants ectopically expressing a soybean *ALDH7* gene also show enhanced tolerance to abiotic stress.

ALDH10A8 AND ALDH10A9: BETAINE ALDEHYDE DEHYDROGENASE ORTHOLOGOUS GENES?

Glycine betaine is a protective osmolyte that is synthesized from choline in response to stress by a two-step reaction catalyzed by choline monooxygenase (EC 1.14.15.7) and betaine aldehyde dehydrogenase (BADH; EC 1.2.1.8). *A. thaliana* possesses two BADH orthologs, *ALDH10A8* and *ALDH10A9* (Sakamoto and Murata, 2002; Kirch et al., 2004) but *Arabidopsis*, like rice, belongs to those species that do not accumulate detectable amounts of glycine betaine under stress conditions (Rhodes and Hanson, 1993). The function of the two *Arabidopsis* genes, *ALDH10A8* and *ALDH10A9*, are therefore of particular interest. Xing and Rajashekar (2001) reported the detection of endogenous glycine betaine in *Arabidopsis* using nuclear magnetic resonance (¹H NMR). About 3 μ mol. g⁻¹ dry weight of glycine betaine were found to accumulate in cold acclimated seedlings. The accumulation of glycine betaine was shown to correlate with the freezing tolerance of leaf tissues. No other report indicated endogenous

glycine betaine accumulation in *Arabidopsis*. This confirms the widely accepted observation that *Arabidopsis* may only contain none or hardly measurable amounts of glycine betaine. In comparison naturally glycine betaine accumulating species such as spinach accumulate more than 10 μ mol glycine betaine. g⁻¹ fresh weight in response to salt stress (Di Martino et al., 2003). Interestingly, when *Arabidopsis* plants have been transformed with BADH genes isolated from naturally accumulating plant species, glycine betaine was detected and osmotic stress tolerance has been reported (Fitzgerald et al., 2009).

The absence of a functional choline monooxygenase was proposed to explain the lack of glycine betaine in some plant species (Nuccio et al., 1998). For instance, recombinant protein corresponding to the *Arabidopsis* choline monooxygenase-like gene did not complement *Escherichia coli* strains deficient in choline dehydrogenase (Hibino et al., 2002). The absence of glycine betaine in some plant species could also be due to choline availability (Peel et al., 2010). So far, the reasons for the non-accumulation of glycine betaine in some plant species are still elusive.

Variation seems to exist among betaine accumulating plants with respect to the subcellular localization of glycine betaine and the biosynthetic enzymes (Fitzgerald et al., 2010; Chen and Murata,

2011). Glycine betaine was found to accumulate in spinach chloroplasts and accordingly both, choline monoxygenase and betaine aldehyde dehydrogenase activities were detected in chloroplasts (Robinson and Jones, 1986). In other glycine betaine accumulators such as mangrove and barley no choline monoxygenase and betaine aldehyde dehydrogenase activities were found in chloroplasts (Hibino et al., 2001; Fujiwara et al., 2008). The BADH isoforms in barley, BBD1 and BBD2, are peroxisomal and cytosolic proteins, respectively (Nakamura et al., 2001; Fujiwara et al., 2008). In *Arabidopsis*, the ALDH10A8 and ALDH10A9 proteins are targeted to leucoplasts and peroxisomes, respectively (Missihoun et al., 2011). These observations indicate that glycine betaine may be synthesized in other cellular compartments than chloroplasts or other unknown proteins may be involved in its biosynthesis (Chen and Murata, 2011). Alternatively, BADH genes may have other functions. The latter hypothesis is supported by the recent discovery that the *fgf* mutation associated with the fragrance phenotype in rice resides within the *OsBADH2* gene isoform (Bradbury et al., 2008). This mutation results in a non-functional BADH protein that cannot oxidize 4-aminobutanol to γ -aminobutyric acid (GABA), leading to the accumulation of 2-acetyl-1-pyrroline, responsible for the fragrance. The functional analysis of BADH proteins in naturally non-accumulating glycine betaine species may lead to the discovery of alternative mechanism(s) used by those plants to compensate for the lack of glycine betaine. This was addressed with biochemical and genetic tools that we established for *Arabidopsis*.

We recently reported on the molecular and functional analysis of the *Arabidopsis* ALDH10A8 (*At1g74920*) and ALDH10A9 (*At3g48170*) genes, which are differentially regulated (Missihoun et al., 2011). Generally ALDH10A8 is expressed at higher levels than ALDH10A9. Both genes are responsive to ABA treatment and to a lesser extent to NaCl, dehydration, chilling, and methyl viologen. A T-DNA insertion mutant for the ALDH10A8 gene was found to be sensitive to drought and salinity, indicating that the ALDH10A8 gene product is functional *in planta*, likely in a pathway different from glycine betaine synthesis. Our data point to a possible involvement in the oxidation of aminoaldehydes derived from the degradation of polyamines.

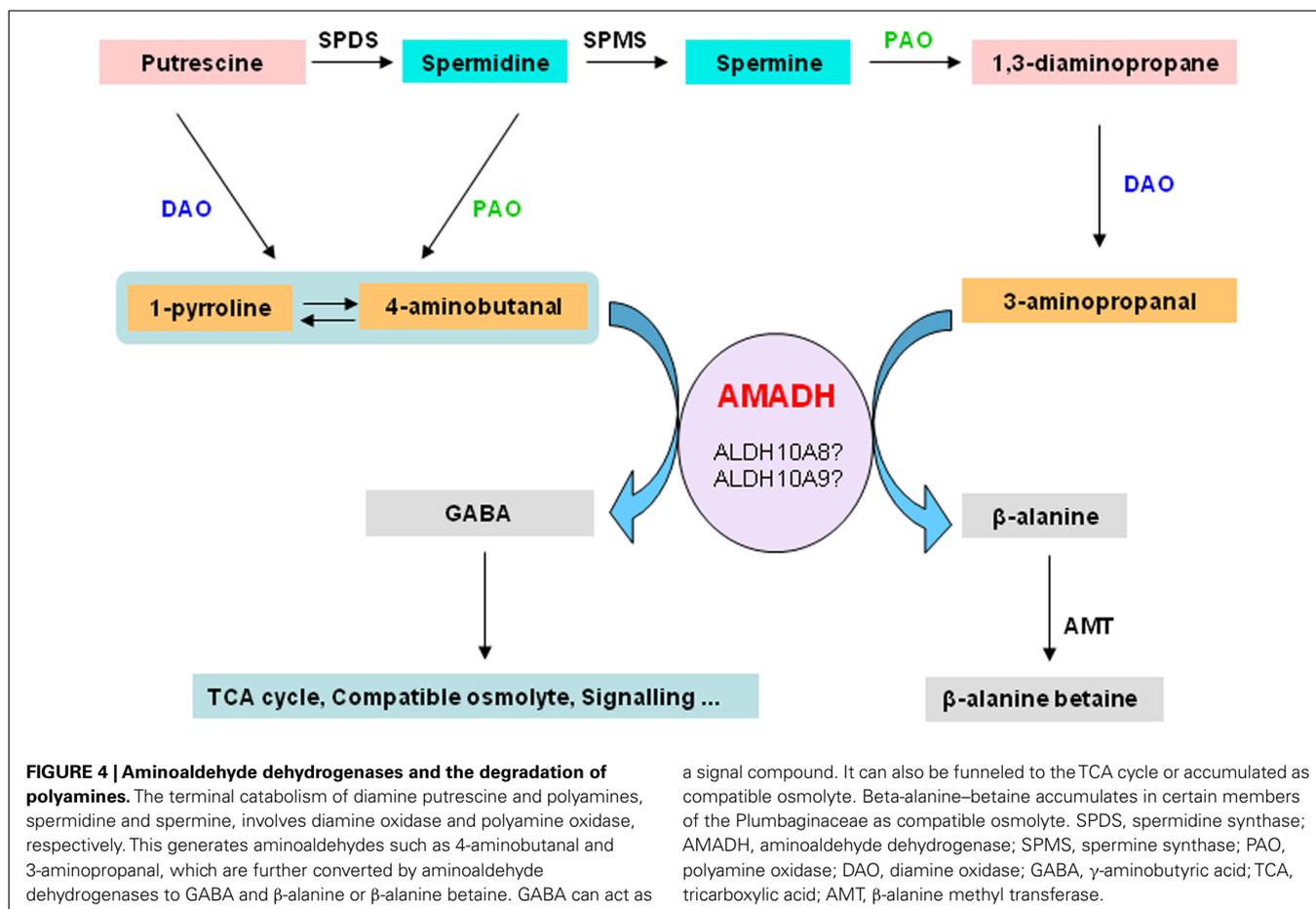
ALDH10A9 recombinant protein is able to metabolize betaine aldehyde as well as the two aminoaldehydes, 4-aminobutanol and 3-aminopropanal, implying that ALDH10A9 might be involved in the polyamine metabolism (Missihoun et al., 2011). As there was no mutant for ALDH10A9 available, this hypothesis was not fully investigated. Only recently, a T-DNA mutant line (SK17639; Robinson et al., 2009) has become available and the analysis of this line should shed some light on the function of this gene. Evidence supporting the involvement of plant aminoaldehyde dehydrogenases (AMADH) in the terminal catabolism of polyamines has been reported. Petrivalsky et al. (2007) found that the activity of pea AMADH increased during wound healing of injured etiolated seedlings, which was spatially correlated with lignification, a physiological process that involves both copper amine oxidase and polyamine oxidase activities. Likewise, simultaneous increase of diamine oxidase activity and the production of GABA from 4-aminobutanol was also reported in soybean (Xing et al., 2007). This resulted in an increase of the GABA contents in roots of

soybean [*Glycine max* (L.) Merr.] grown on salt. The GABA content derived from the activity of AMADH proteins was estimated to be about 39% of the total GABA pool in soybean (Xing et al., 2007). This suggests that the production of GABA from the polyamine catabolism-derived aminoaldehydes under adverse conditions is a general feature shared by both monocots and dicots. We propose that AMADH may link the polyamine metabolism and the tricarboxylic acid cycle via the GABA-shunt (Figure 4). The metabolic AMADH activity can be responsible for the detoxification of toxic aminoaldehydes (Tylichová et al., 2007). Based on sequence similarity, ALDH10A8 and ALDH10A9 represent the best candidates for AMADH in *Arabidopsis*. Whether ALDH10A8 and ALDH10A9 function *in vivo* in this pathway remains to be demonstrated.

As reported by Xing et al. (2007) and illustrated in the Figure 4, GABA can be produced from the oxidation of 4-aminobutanol by AMADH. Several functions have been attributed to GABA in plants, ranging from acting as a compatible solute to a signal molecule (Bouché and Fromm, 2004). Although GABA is believed to derive mainly from the decarboxylation of glutamic acid (Shelp et al., 1999), the question as to how cells control the GABA biosynthesis to match the physiological need is unclear. A complex regulatory mechanism may take place to adjust the GABA levels according to the cellular requirement. The site of GABA synthesis may be an important factor. Thus, GABA produced in the cytosol by decarboxylation of glutamic acid may not play the same role as the one generated by AMADHs in peroxisomes or in plastids. AMADHs also oxidise 3-aminopropanal to beta-alanine (Figure 4). Beta-alanine is a non-proteinic amino acid involved in the biosynthesis of pantothenate, a precursor of coenzyme A. It has also been associated to thermotolerance in transgenic tobacco plants (Fouad and Rathinasabapathi, 2006; Fouad and Altpeter, 2009). Beta-alanine can be trimethylated to beta-alanine–betaine, which accumulates as compatible solutes in certain members of the Plumbaginaceae. It is worth validating the existence of enzymatically active AMADHs in *Arabidopsis* and to establish unequivocally the pivotal role of AMADHs in linking polyamine metabolism to the energy metabolism in plant cells. This will facilitate future investigations of the physiological importance of GABA in plants and help uncover subtle details related to its functions.

BIOCHEMICAL ANALYSIS AND COENZYME AFFINITY STUDIES OF ALDH3H1 AND ALDH3I1

Research on *Arabidopsis* ALDHs was started after it was discovered that Cp-ALDH isolated from the desiccation tolerant plant *Craterostigma plantagineum* is responsive to dehydration and may contribute to detoxify chloroplasts when aldehydes accumulated under stress conditions (Kirch et al., 2001). ALDH3I1 and ALDH3H1 from *Arabidopsis* are the closest homologues to Cp-ALDH (Cp-ALDH shares 81% and 80% amino acid homologies with ALDH3H1 and ALDH3I1, respectively; ALDH3I1 and ALDH3H1 have 79% homology). Cp-ALDH and ALDH3I1 show a similar tissue specific expression, are targeted to the chloroplast, use NADP⁺ as coenzyme in enzymatic reactions and contain a conserved valine in their coenzyme binding site. The cytosolic ALDH3H1 has an isoleucine residue instead of the valine and



only uses NAD^+ as coenzyme. Overexpressing either Cp-ALDH or ALDH3I1 in *Arabidopsis* leads to improved stress tolerance, which was not observed in plants overexpressing ALDH3H1 (Table 2). We addressed the question how the different subcellular localizations and the single amino acid difference determine substrate specificities and coenzyme affinities. These biochemical properties may be important in the observed stress tolerance. Enzymatic activities of recombinant ALDH3H1 and ALDH3I1 proteins were analyzed to identify their substrate requirements. Both display a preference for medium to long-chain saturated aldehydes with dodecanal as most effective substrate (Table 2).

Structures of several ALDHs revealed that they bind the coenzyme in an atypical five stranded open α/β Rossmann fold (Figure 5A; Liu et al., 1997). Different amino acids are important for coenzyme binding and influence coenzyme specificity. Most importantly a lysine residue interacts with the adenine ribose of NAD^+ or the 2'-phosphate of NADP^+ (Perozich et al., 2000). A glutamic acid residue occupies a central position in the coenzyme binding site and coordinates the adenine ribose 2'- and 3'-hydroxyls of the adenine ribose of NAD^+ , while repelling the 2'-phosphate of the ribose of adenosine in NADP^+ (Perozich et al., 2000, Stiti unpublished). Thus, space in the opposite side of the coenzyme binding cleft is required to keep the interacting NADP^+ molecule in an active conformation.

Comparison of the amino acid sequences showed that the NAD^+ -specific ALDH3H1 has an isoleucine instead of a valine in motif 4 of the coenzyme binding cleft. The isoleucine has a large side chain due to an additional methyl group compared to the valine occupying this position in ALDH3I1. A valine residue is highly conserved in this position in all family 3 ALDHs except for ALDH3H1 and ALDH3F1. Therefore it was tested in a site-directed mutagenesis approach whether the unusual isoleucine is the reason for the inability to use NADP^+ . The space occupied by isoleucine may not allow to accommodate the 2'-phosphate of NADP^+ while interaction with NAD^+ is not affected. Thus the isoleucine-200 was substituted by residues with a shorter side chain, valine, or glycine (Stiti et al., 2011). For the generated mutated enzyme ALDH3H1_{Ile200Val}, the cofactor binding cleft is enlarged by 1.4 Å compared to the wild-type enzyme (7.85 Å). The distance from valine-200 to glutamic acid-149 is approximately 9.23 Å as determined by structure modeling. But the width of the cleft is similar to ALDH3I1 (9.22 Å) or the rat ALDH3A1 (9.19 Å; Figure 5B). The latter two enzymes can use either NAD^+ or less effectively NADP^+ . The ALDH3H1_{Ile200Val} mutant is able to use NADP^+ as coenzyme with a $K_m\text{NADP}^+$ in the range comparable to the wild-type ALDH3I1 with hexanal as a substrate ($K_m\text{NADP}^+ = 2300 \mu\text{M}$ for ALDH3H1_{Ile200Val} and $K_m\text{NADP}^+ = 1868 \mu\text{M}$ for ALDH3I1). A minor increase was observed in $K_m\text{NAD}^+$ and no significant

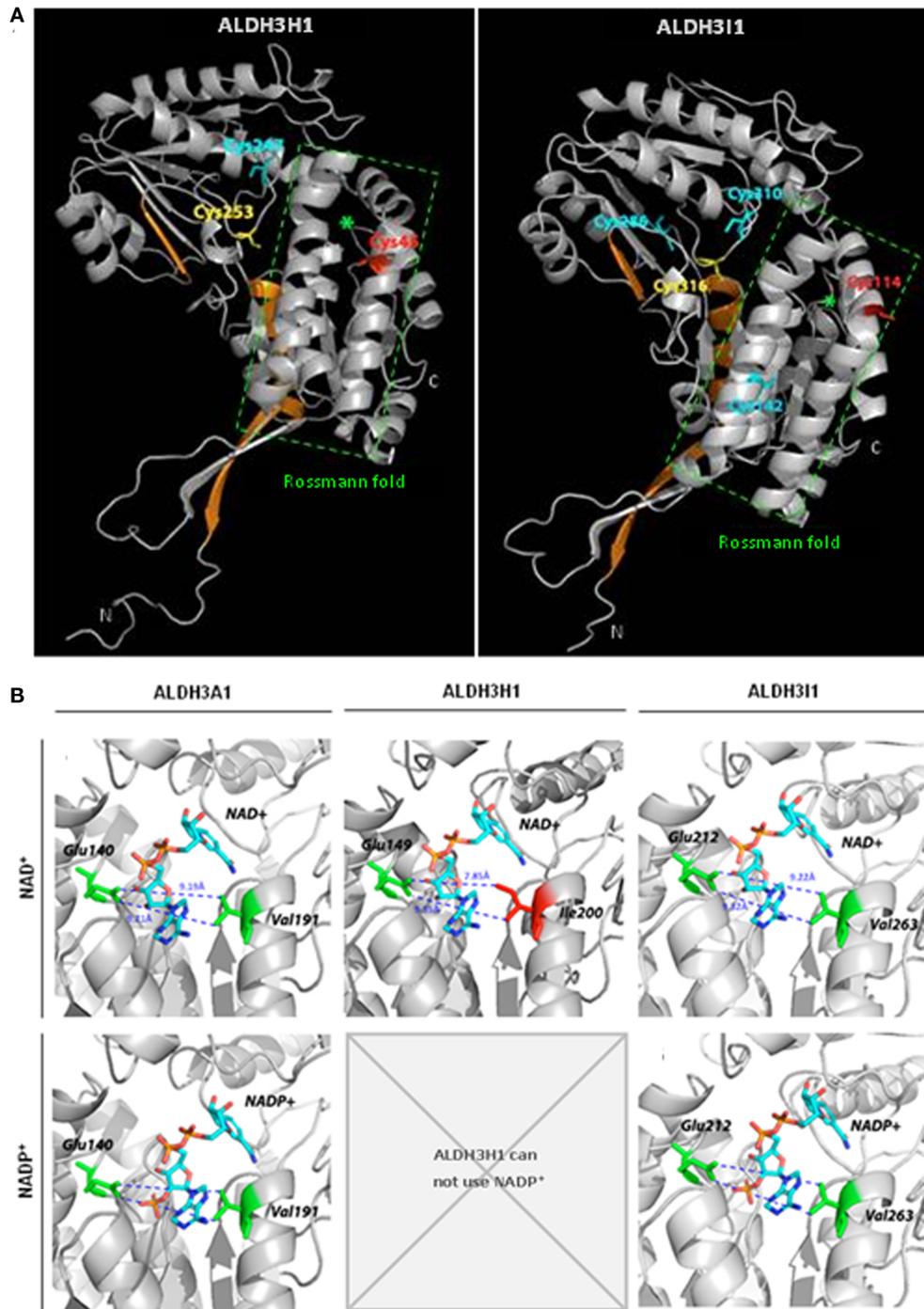


FIGURE 5 | Predicted structures of *Arabidopsis* ALDH3H1 and ALDH3I1 and models of coenzyme binding. (A) Ribbon diagrams of the predicted structures of *Arabidopsis thaliana* ALDH3H1 and ALDH3I1 monomeric subunits. Green asterisks denote the central helices of the coenzyme binding Rossmann fold domain. Catalytic cysteine residues are depicted in yellow, redox-sensitive cysteines in red, other cysteines in cyan (Stiti et al., 2011). Regions highlighted in orange indicate helix α D and sheets β 12 as well as β 13 involved in hydrogen bond-mediated homodimerization in the

functional native homologous ALDH3A1 protein. **(B)** Location of NAD⁺ and NADP⁺ and amino acid residues in the coenzyme binding cleft of the previously reported structure of ALDH3A1 from *Rattus norvegicus* and the predicted structures of ALDH3H1 and ALDH3I1. The predicted structures of ALDH3H1 and ALDH3I1 were built using the crystal structure of *Rattus norvegicus* ALDH3A1 as template. (This figure was redrawn from Stiti et al., 2011, permission to reproduce the figure was obtained from Portland press).

change of V_{max} was observed. Similarly to the wild-type enzyme, ALDH3H1_{Ile200Val} prefers NAD^+ .

Substitution of isoleucine-200 by glycine should enlarge the cleft to reach a width of 11.63 Å, which makes the NAD^+ binding weaker and at the same time increases the affinity for $NADP^+$ ($K_mNAD^+ = 3218\mu M$ vs. $K_mNADP^+ = 1817\mu M$). This demonstrates that the enlargement of the coenzyme binding site resulted in a shift of specificity from NAD^+ to $NADP^+$, which confirms that the distance is critical between the residues occupying the positions 200 and 149 for coenzyme affinity. Substituting isoleucine by glycine provides the space necessary to accommodate $NADP^+$, but the larger space possibly makes the cleft too wide for correct binding of NAD^+ . The isoleucine side chain may force the adenine ribose of NAD^+ to allow interaction with the enzyme surface inside the coenzyme binding pocket. Point mutations in enzymatically critical positions alter coenzyme affinities and may be an evolutionary adaptation to the

requirements of the physiology needed for the function of the ALDH enzymes.

This review demonstrates that despite strict sequence conservations among ALDH enzymes a few changes in gene sequences lead to diversity in enzymatic properties, subcellular localization, expression patterns, and contribution to stress tolerance. A high degree of sequence conservation of ALDH genes between plant species suggests that these alterations must have been positively selected during evolution for the specific role of individual ALDH genes.

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