Plant ALDH10 Family

IDENTIFYING CRITICAL RESIDUES FOR SUBSTRATE SPECIFICITY AND TRAPPING A THIOHEMIACETAL INTERMEDIATE*

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Background: Plant aminoaldehyde dehydrogenases (AMADHs) detoxify ω-aminoaldehydes from several metabolic pathways.

Results: Two of five new AMADHs exhibit unusual kinetic properties. A thiohemiacetal intermediate was trapped in a crystal structure.

Conclusion: Five critical residues can modulate substrate specificity, and a new substrate was identified.

Significance: The present findings allow sequence-based predictions of AMADH substrate specificity linked with the production of individual osmoprotectants in plants.

Plant ALDH10 family members are aminoaldehyde dehydrogenases (AMADHs), which oxidize ω-aminoaldehydes to the corresponding acids. They have been linked to polyamine catabolism, osmoprotection, secondary metabolism (fragrance), and carnitine biosynthesis. Plants commonly contain two AMADH isoenzymes. We previously studied the substrate specificity of two AMADH isoforms from peas (PsAMADHs). Here, two isoenzymes from tomato (Solanum lycopersicum), SIAMADHs, and three AMADHs from maize (Zea mays), ZmAMADHs, were kinetically investigated to obtain further clues to the catalytic mechanism and the substrate specificity. We also solved the high resolution crystal structures of SIAMADH1 and ZmAAMADH1a because these enzymes stand out from the others regarding their activity. From the structural and kinetic analysis, we can state that five residues at positions 163, 288, 289, 444, and 454 (PsAMADHs numbering) can, directly or not, significantly modulate AMADH substrate specificity. In the SIAMADH1 structure, a PEG aldehyde derived from the precipitant forms a thiohemiacetal intermediate, never observed so far. Its absence in the SIAMADH1-E260A structure suggests that Glu-260 can activate the catalytic cysteine as a nucleophile. We show that the five AMADHs studied here are capable of oxidizing 3-dimethylsulfoniopropanaldehyde to the cryo- and osmoprotectant 3-dimethylsulfoniopropanionate. For the first time, we also show that 3-acetamidopropionaldehyde, the third aminoaldehyde besides 3-aminoaldehyde and 4-aminobutyraldehyde, is generally oxidized by AMADHs, meaning that these enzymes are unique in metabolizing and detoxifying aldehyde products of polyamine degradation to nontoxic amino acids. Finally, gene expression profiles in maize indicate that AMADHs might be important for controlling ω-aminoaldehyde levels during early stages of the seed development.

In plants, polyamines like spermine, spermidine, and putrescine are involved in various developmental processes and stress responses (1). Polyamines are degraded by copper diamine oxidases (EC 1.4.3.22, formerly 1.4.3.6) and FAD-containing polyamine oxidases (PAOs; EC 1.5.3.14 and 1.5.3.17), leading to the production of reactive and cytotoxic ω-aminoaldehydes, such as 3-aminoaldehyde (APAL) or 4-aminobutyraldehyde (ABAL; in equilibrium with Δ1-pyrroline) plus hydrogen peroxide (2, 3). The oxidation of ω-aminoaldehydes by aminoaldehyde dehydrogenase (AMADH) ends up with the formation of the nontoxic metabolites β-alanine and γ-aminobutyric acid (GABA), respectively (4, 5).

Studying the physiological aspects of plant AMADHs (EC 1.2.1.19) has become attractive for economic reasons. It has been shown that an AMADH gene mutation leads to the acetylation of free ABAL (or its cyclic form Δ1-pyrroline) and accumulation of 2-acetyl-Δ1-pyrroline, a potent flavor component

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The atomic coordinates and structure factors (codes 4I9B, 4I8Q, and 4I8P) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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5 The abbreviations used are: PAO, polyamine oxidase; ABAL, 4-aminobutyraldehyde; ACAPAL, 3-acetamidopropionaldehyde; ALDH, aldehyde dehydrogenase; NALDH, human ALDH; AMADH, aminoaldehyde dehydrogenase; APAL, 3-aminoaldehyde; BMS, 3-dimethylsulfoniopropanionate; DMSPAL, 3-dimethylsulfoniopropanaldehyde; GABA, 4-quinidinobutyraldehyde; PCAL, pyridine carboxaldehyde; TMABAL, N,N,N-trimethyl-4-aminobutyraldehyde; GB, glycine betaine; NR, nicotinamide riboside; qPCR, quantitative PCR; GABA, γ-aminobutyric acid.
conferring a fragrance to several rice varieties like jasmine and basmati or to soybean (6, 7). Plant AMADHs have also been studied for their role in stress responses and in the production of the osmoprotectants glycine betaine (GB) and dimethylsulfoxoniopropionate (DMSP) by the oxidation of betaine aldehyde (BAL) and 3-dimethylsulfoxoniopropanaldehyde (DMSPAL), respectively (8, 9, 10).

AMADHs are classified in different aldehyde dehydrogenase (ALDH) families because of the established criteria stating that ALDH sequences have to be 40% identical to create a family. Because plant AMADHs display between 35 and 39% sequence identity with bacterial, fungal, fish, or mammalian AMADHs, they have recently been classified in the separate family ALDH10 (11). Several enzymes of the ALDH9 family covering mammalian AMADHs (12) and of the ALDH10 family from various species have been independently shown to oxidize a wide range of aminoaldehydes and other aldehydes (Fig. 1). Therefore, they have been also called 4-aminobutyraldehyde dehydrogenases (EC 1.2.1.19), 4-trimethylaminobutyraldehyde dehydrogenase (EC 1.2.1.47), 4-guanidinobutyraldehyde dehydrogenase (EC 1.2.1.54), and BADH (EC 1.2.1.8).

Plant AMADHs share around 70–80% sequence identity. One complete AMADH sequence is usually known for many plant species. However, ongoing genome analyses have gradually shown the presence of two genes coding for two homologous proteins of at least 80% sequence identity. Interestingly, most plant AMADHs carry the C-terminal peroxisomal targeting signal S(A)KL and are found in peroxisomes (13, 14). We have previously identified two pea AMADHs (Pisum sativum; PsAMADH1 and PsAMADH2) and analyzed their crystal structures (4). Plant AMADHs are dimeric and possess a 14-Å-long substrate channel in each monomer. There are three strictly conserved residues essential for the catalysis, Asn-162, Cys-294, and Glu-260, which lie in PWNYP, GQI(V)CSATSR, and ELGGKSP consensus motifs. The three catalytic residues (Asn, Cys, and Glu) lie at the substrate channel bottom and together form the active site. The catalytic mechanism follows the well described sequential binding model valid for the ALDH superfamily (15, 16). Aminoaldehyde substrates undergo a nucleophilic attack by the catalytic cysteine, leading to a thioester formation (i.e., a covalent intermediate) and the subsequent hydride transfer to NADH. The conserved glutamate residue functions as a general base activating a water molecule. Such a molecule performs a nucleophilic attack at the thioester acyl-sulfur bond, resulting in the release of the amino acid.

Using site-directed mutagenesis performed on PsAMADH2 (17), we showed that two totally conserved aspartate residues located at the entrance of the substrate channel (Asp-110 and -113) are essential for high affinity binding of ω-aminoaldehydes (APAL is the best substrate). We also noticed that the activity on several aminoaldehyde substrates is affected by Trp-288. In order to predict the substrate specificity differences in AMADH pairs from a single species and between species and because only a few plant AMADH pairs were reported and studied (4, 6, 18), we cloned and biochemically characterized five ALDH10 members (19) (Table 1): two tomato (Solanum lycopersicum) isoenzymes SlAMADH1 and SlAMADH2 and three maize (Zea mays) isoenzymes ZmAMADH1a, ZmAMADH1b, and ZmAMADH2. Both tomato AMADHs share 80% sequence identity. ZmAMADH1a and -1b differ in 13 amino acids only (97% sequence identity). ZmAMADH2 and both ZmAMADH1a/1b are 75% sequence identical.

Based on kinetic results, we decided to co-crystallize SIAMADH1 and ZmAMADH1a with NADH. The dimeric structures were determined at 1.90 and 1.95 Å resolution, respectively. Although both soaking and co-crystallization with substrates were attempted and despite using the active-site...
TABLE 1

| Source | Common name | Systematic name | GenBank™ accession |
|--------|-------------|----------------|-------------------|
| Z. mays | ZmAMADH1a | ALDH10A8 | JQ184593 |
| Z. mays | ZmAMADH1b | ALDH10A9 | JN635700 |
| Z. mays | ZmAMADH2 | ALDH10A5 | JQ184594 |
| S. lycopersicum | SLIMADH1 | ALDH10A12 | AY796114 |
| S. lycopersicum | SLIMADH2 | ALDH10A13 | FJ228482 |

base mutant SIAMADH1-E260A for this purpose, obtaining AMADH in such a complex was unsuccessful. However, ethylene glycol and polyethylene glycol (PEG) aldehyde molecules from the crystallization solution were found to mimic a substrate bound at the substrate binding site in ZmAMADH1a and SIAMADH1 structures, respectively. Moreover, we obtained the first crystal structure of a thiohemiacetal intermediate in AMADH family; a PEG aldehyde bound covalently to the catalytic cysteine forms an intermediate in one subunit of the SIAMADH1 structure in which only ADP moiety of NAD$^+$ is ordered. The whole NAD$^+$ is ordered in ZmAMADH1a and SIAMADH1-E260A structures and adopts the common conformation observed in ALDH structures. Combining structural analysis and kinetics, we can state that five residues at positions 163, 288, 289, 444, and 454 (pS AMDH numbering) can significantly modulate AMADH substrate specificity. Trp-288 and Tyr-163 are essential for the high affinity to $\alpha$-aminoaldehyde substrates. The absence of this Trp in addition to the presence of a Thr (instead of Cys in other enzymes) in SIAMADH1 leads to a broader substrate specificity due to an enlargement of its substrate channel. We can predict that AMADHs containing Ile-444 have only a negligible activity with BAL. Conversely, those containing an Asn at position 289 may display BAL activity. For the first time, we identify a new and common substrate for plant AMADHs.

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Purification of AMADHs from Tomato and Maize—The total RNA from apical meristems and leaves of 7-day-old tomato seedlings (S. lycopersicum cv. Amateur) and 5-day-old maize seedlings (Z. mays cv. Sachara) was extracted using the Midi spin column kit (Macherey-Nagel, Düren, Germany), and the cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen). SIAMADH1 and -2 and ZmAMADH1a, -1b, and -2 cDNA sequences were originally obtained in this work and submitted to the EMBL/GenBank™ database. The SIAMADH1 ORF (1515 bp; EMBL/GenBank™ accession number AJ976114) was amplified using a primer pair with a BamHI (5'–CATGGGATCCGCTGTTTGGCCAGCGAGAAG-3') and Xhol site (5'-CGTCTCGAGAAGGTTTGGTTTCCG-3') and a downstream primer with the KpnI site (5'-GCGGCCGCTTATACCCGCTGACG-3') and a downstream primer with the KpnI site (5'-AGATGGAATGCTTACGCTTACCTGTTTTGAG-3') were used. All three maize genes were cloned with primer pairs containing EcoRI and Xhol sites. ZmAMADH1a ORF (1518 bp; GQ184593) was amplified using the following primers: 5'-ATTGAATTC-TGCCCTGCGCAGCGAGTTCCGGG-3' and 5'-CTTCGAGCTGTTGCAACATTAACGACGACGTTCGAGTTTCCAGTTTACGC-3'. ZmAMADH1b ORF (1518 bp; JN635700) was amplified using the primer pair 5'-ATTGAATTC-TGCCCTGCGCAGCGAGTTCCGGG-3' and 5'-CTTCGAGCTGTTGCAACATTAACGACGACGTTCGAGTTTCCAGTTTACGC-3' and ZmAMADH2 ORF (1521 bp; GQ184594) was cloned using the primer pair 5'-ATAGAATTCGCGCCGCGCCGACGACGTTCGAGTTTCCAGTTTACGC-3' and 5'-ATACTCGAGTCACTCAGTTAAGATGCGAGTGCTGTT-3'.

The amplified sequences were inserted into a pCDFDuet vector (Novagen, La Jolla, CA), sequenced, and transformed into T7 express Escherichia coli cells for expression as N-terminal His$_6$ tag proteins. Enzymes were produced and purified on a column filled with Co(II)-charged IDA-Sepharose 6B (Sigma-Aldrich) as described previously (20). The complete purification from a 600-ml culture yielded about 20 mg of recombinant SIAMADHs. Specific activity values measured with 1 mM APAL as a substrate were 3.1 units mg$^{-1}$ for SIAMADH1, 3.6 units mg$^{-1}$ for SIAMADH2, 2.6 units mg$^{-1}$ for ZmAMADH1a, 1.9 units mg$^{-1}$ for ZmAMADH1b, 4.1 units mg$^{-1}$ for ZmAMADH2.

For crystallization, the plasmid was transformed into E. coli BL21 pLYS S (Novagen). The cells were grown in 2YT medium at 37 °C to an A$_{600}$ = 0.6, the temperature was reduced to 20 °C, and expression was induced with 0.5 mM isopropyl 1-thio-$\beta$-galactopyranoside overnight. The cells were suspended in buffer A (50 mM Tris-HCl buffer, pH 8, 20 mM imidazole, and 500 mM NaCl) and disrupted by sonication. The lysate was cleared by centrifugation at 20,000 × g at 4 °C for 30 min. The supernatant was filtered through a 0.22-$\mu$m membrane (Millipore) and loaded on a 5-ml nickel-nitriloacetic acid-agarose column (GE Healthcare). After washing with 10% buffer B (50 mM Tris-HCl, pH 8, 300 mM imidazole, and 500 mM NaCl), elution was performed with 100% buffer B. Fractions containing AMADH were pooled, and the buffer exchange was performed on a HiLoad 26/60 Superdex 200 prep grade (GE Healthcare), using 50 mM Tris-HCl, pH 8, 150 mM NaCl. Fractions were analyzed by SDS-PAGE, and those containing AMADH were pooled, concentrated using Vivaspin-10kDa (Sartorius) at 14 mg/ml, and stored at –20 °C. The identity of the purified proteins was confirmed by peptide mass fingerprinting on a Microflex LRF20 MALDI-TOF mass spectrometer (Bruker Daltonik, Bremen, Germany) after SDS-PAGE and in-gel digestion (21).

Substrates, Activity Assays, and pH Optimum Determination—Elementary aliphatic aldehydes, pyridine carboxaldehydes, and N,N-dimethyl-4-aminobutyraldehyde, APAL, and ABAL diethylacetals were purchased from Sigma-Aldrich. DMSPAL and diethylacetals of 4-amino-2-hydroxybutyraldehyde, diethylacetals of 4-amino-2-hydroxybutyraldehyde, 4-guanidinobutyraldehyde (GBAL), 4-guanidino-2-hydroxybutyraldehyde, and 3-guanidinopropionaldehyde were all synthetic preparations (9, 22).

Diethyl acetals of 4-guanidino-2-hydroxybutyraldehyde, 4-guanidinobutyraldehyde, and 3-guanidinopropionaldehyde were all synthetic preparations (9, 22). Diethyl acetal iodosides of N,N,N-trimethyl-3-aminopropanoic acid, 4-hydroxybutyraldehyde, and 4-hydroxybutyraldehyde were all synthetic preparations (9, 22). Diethyl acetal iodosides of N,N,N-trimethyl-3-aminopropanoic acid, 4-hydroxybutyraldehyde, and 4-hydroxybutyraldehyde were all synthetic preparations (9, 22). Diethyl acetal iodosides of N,N,N-trimethyl-3-aminopropanoic acid, 4-hydroxybutyraldehyde, and 4-hydroxybutyraldehyde were all synthetic preparations (9, 22).
Plant AMADHs share a similar pH optimum. The pH optima of ZmAMADH1a, ZmAMADH2, and SIAMADH2 fall within the pH range of 9.4–9.8 (i.e., similarly to that of pea or rice AMADHs) (4, 6). The pH optima of SIAMADH1 and ZmAMADH1b are slightly more basic (pH ~10.2).

**Crystallization and Data Collection—**Crystallization conditions using commercial kits (Classics, PEGs II, and JCSG I suites from Qiagen) were screened in sitting-drop vapor diffusion experiments using a Cartesian nanodrop robot (Genomic Solutions) on IMAGIF (CNRS, Gif sur Yvette, France). SIAMADH1 with 5 mM NAD⁺ was crystallized over reservoirs containing 23% PEG 1000, 0.1 M HEPES, pH 7.5. ZmAMADH1a with 5 mM NAD⁺ was crystallized over reservoirs containing 16% PEG 4000, 0.1 M HEPES, pH 7.5, and 10% isopropyl alcohol. The E260A mutant of SIAMADH1 with 5 mM NAD⁺ was crystallized over reservoirs containing 15% PEG 1500, 0.1 M HEPES, pH 7.5, and 10% glycerol. Crystals were transferred to a cryoprotectant solution (mother liquor supplemented with 25% PEG 4000 for SIAMADH1 or 20% glycerol for the E260A mutant and ZmAMADH1a) and flash-frozen in liquid nitrogen. Diffraction data were collected at 100 K on the PROXIMA 1 beamline (SOLEIL Synchrotron, Saint-Aubin, France). Diffraction intensities were integrated with the program XDS (26). Data collection and processing statistics are given in Table 2.

**Molecular Replacement, Refinement, and Final Model—**The structures of SIAMADH1 and of ZmAMADH1a were solved by molecular replacement with Phaser (27) using the dimer structures of PsAMADH2 (Protein Data Bank code 3IWJ) as a search model. The structure of the SIAMADH1-E260A mutant was also solved with Phaser using a monomer of the WT structure.
as a search model. Model refinement was performed with BUSTER-TNT (28). Electron density maps were evaluated using COOT (29) for manual refinement. In the SlAMADH1 structure, the maps revealed clear density for the ADP moiety of the NAD⁺ coenzyme. In contrast, the side chain of the active-site base Glu-260 is disordered. For this reason, we set the occupancy of each atom forming the carboxylate at 0. In the ZmAMADH1a and SlAMADH1-E260A structures, the electron density of the whole NAD⁺ molecule was observed. Glu-262 (equivalent to Glu-260) is well defined in the maize structure. Refinement details of the three structures are shown in Table 2. Molecular graphics images were generated using PyMOL (Schoedinger, LLC, New York).

qPCR Analysis—The total RNA from 10-day-old and 2 month-old tomato plants (cv. Amateur) for reverse transcription was isolated using the RNAqueous kit and Plant RNA Isolation Aid solutions from Ambion (Austin, TX). Isolated RNA was treated twice by the TURBO DNase-free kit (Ambion) to remove all traces of genomic DNA contamination and to minimize bias in qPCR data. First-strand cDNA was synthesized by RevertAid H Minus Moloney murine leukemia virus reverse transcriptase and oligo(dt) primers (Fermentas, Vilnius, Lithuania). Diluted cDNA samples were used as templates in real-time PCRs containing TaqMan Gene Expression Master Mix (Applied Biosystems), both primers at 300 nM concentrations, and 250 nM TaqMan MGB probe. For the SlAMADH1 gene, the primer pair 5′-TGAGATGTATCAGGAAAATGG-3′ plus 5′-CCAATGGTTCAGCGAAGATCTAC-TCT-3′ and a probe (5′-AATTCTGACAACTTAAACGG-MGB-3′) were used. For the SlAMADH2 gene, the following primer pair and probe were used: 5′-GTAGTGTTTTGGACGTGACCTT-3′, 5′-TCTCGCGAGCTACATAGCT-3′, and 5′-FAM-AATGGAGTCTCGAGAACT-3′. For the ZmAMADH1a gene, the following accession codes: 4I9B for SlAMADH1 in complex with NAD⁺ (P. sativum) (24); 4I8P for ZmAMADH1a in complex with NAD⁺ (Z. mays) (25); DIQ and 2 (26, 27); 4I98 for SlAMADH1 (28); 4I98 for ZmAMADH1a (29); 4I9B for SlAMADH1 (30); and 4I8P for ZmAMADH1a (31).

RESULTS AND DISCUSSION

Crystal Structures of SlAMADH1, ZmAMADH1a, and ZmAMADH1-E260A—Because SlAMADH1 and ZmAMADH1a differ in terms of activity from the other new studied AMADHs from tomato (S. lycopersicum) and maize (Z. mays) as well as those from peas (see “Substrate Catalysis and Specificity”), their crystal structures were solved at high resolution (Table 2). We also crystallized SIAMADH1-E260A mutant to trap an intermediate or a bound substrate, but these attempts were unsuccessful. However, we have been able to trap an intermediate in the SIAMADH1 structure, thanks to the PEG solution used as precipitant in the crystallization condition.

The asymmetric unit of SIAMADH1 and ZmAMADH1a crystals contains a dimer, which is the active form in solution in accordance with results from gel permeation chromatography (molecular mass estimates between 106 and 112 kDa). The asymmetric unit of SIAMADH1-E260A is composed of one molecule, which forms a dimer by the crystallographic symmetry. The dimers of tomato, maize, and pea (P. sativum) AMADHs are very similar, sharing the same oligomerization interface, folding, and topology. A major difference is that...
**SIAMADH1** is shorter by three residues in the loop from residue 63 to 70 when compared with ZmAMADH1a and PsAMADHs.

All monomers are identical, with root mean square deviations for 494 Cα atoms between 0.6 and 0.8 Å. They adopt the characteristic ALDH fold, consisting of an NAD(P)+ coenzyme binding domain (residues 1–131, 152–261, and 456–480), a catalytic domain (residues 262–455), and an oligomerization domain (132–151 and 481 at the C terminus). They all contain a monovalent cation present in a conserved intrasubunit cavity close to the coenzyme binding site. The cation cavity in SIAMADH1 (and also in ZmAMADH1a) is formed by the three equivalent carbonyl groups of Ile-31 (Val-30), Asp-99 (Asp-101), and Leu-189 (Leu-191) and the carboxyl group of Asp-99 (Asp-101). The nature of the protein ligands and the distances of around 2.4–2.6 Å are typical for a bound sodium ion (32). This ion probably allows maintenance of the protein structural integrity and/or stabilizes the position of a loop involved in NAD+ binding (15).

The oligomerization domains connect both monomers by protruding over the surface of the neighboring subunits around substrate channel entrances, whereas the NAD+ binding sites reside on the opposite side of the dimer. The catalytic cysteine lies between the NAD+ and the substrate binding sites. All enzymes were co-crystallized with NAD+. Whereas the whole well defined NAD+ molecule adopts an extended conformation typical for oxidized NAD+ (15) in ZmAMADH1a and SIAMADH1-E260A structures, the nicotinamide riboside (NiR) moiety is disordered in the SIAMADH1 structure, as frequently observed in structures of NAD(P)+-dependent ALDHs, including PsAMADH2 (4, 33, 34). With the bound coenzyme fully observed in electron density maps, the catalytic cysteine points toward the substrate channel (attacking conformation), allowing the nicotinamide ring to interact with the active-site base Glu. In contrast, when the NiR moiety is mobile, the catalytic cysteine adopts the resting conformation by pointing toward the NAD+ cavity and prevents the NiR moiety from being in the vicinity of the active-site base glutamate. In this state, mixed conformations of NAD+ and NADH coenzyme are observed.

**Coenzyme Binding Site**—The coenzyme binding site is highly conserved in the plant AMADH family. The adenine moiety of NAD+ inserted in a hydrophobic pocket flanked by two helices (αD and αE) makes no polar contact with the enzyme, so that the free amino group is exposed to the solvent. The O2B and O3B oxygen atoms of the ribose and both α- and β-phosphate oxygen atoms establish identical hydrogen bonds with the enzyme as described for PsAMADH2 (4). All five AMADHs

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**TABLE 3**

| Ligand    | K_m (μM) | k_cat (s^-1) | k_cat/K_m | K_i (μM) | β    |
|-----------|----------|--------------|-----------|----------|------|
| NAD⁺      | 72.7     | 3.7          | 19.6      | 19.6     | 0.4  |
| TMABAL    | 17.1     | 0.2          | 85.4      | 85.4     | 0.3  |
| GBAL      | 85.3     | 4.5          | 5.3       | 5.3      | 0.9  |
| APAL      | 41.5     | 6.0          | 85.4      | 85.4     | 1.0  |
| ABA      | 57.100   | 2.2          | 1.9       | 1.9      | 0.7  |
| BAL       | 2054.7   | 4.6          | 102       | 102      | 0.7  |
| 3-PCAL    | 316.7    | 6.9          | 103       | 103      | 0.8  |
| 4-PCAL    | 50.4     | 2.8          | 104       | 104      | 0.7  |

**TABLE 4**

| Ligand    | K_m (μM) | k_cat (s^-1) | k_cat/K_m | K_i (μM) | β    |
|-----------|----------|--------------|-----------|----------|------|
| NAD⁺      | 91.9     | 5.8          | 86.2      | 86.2     | 0.8  |
| TMABAL    | 6.0      | 1.2          | 1.2       | 1.2      | 0.8  |
| GBAL      | 5.8      | 1.6          | 3.5       | 3.5      | 0.7  |
| APAL      | 9.2      | 11.8         | 9.2       | 9.2      | 1.0  |
| ABA      | 28.4     | 12.0         | 12.0      | 12.0     | 1.0  |
| BAL       | 14.6     | 0.6          | 0.6       | 0.6      | 0.7  |
| 3-PCAL    | 24.9     | 0.7          | 0.7       | 0.7      | 0.7  |
| 4-PCAL    | 24.9     | 0.7          | 0.7       | 0.7      | 0.7  |
display similar reaction rates and affinity for NAD$^+$ ($K_m$ values are in the range of 10$^{-5}$ µM; Tables 3 and 4). These enzymes are NAD$^+$-specific because they possess a conserved glutamate (Glu-188 in PsAMADH2 numbering), preventing the binding of the 2' phosphate group of NADP$^+$, as described previously (4). Thus, their activity with NADP$^+$ is very low (Fig. 2).

A screening test using NAD$^+$ analogs with substitutions at the nicotinamide ring in the presence of APAL as a substrate shows that these compounds are less efficient as coenzymes (Fig. 2). 3-Acetylpyridine-NAD$^+$ is the best electron acceptor and leads to ~45% activity for ZmAMADH1a and ZmAMADH1b (as well as for both PsAMADHs) and ~75% activity for both SIAMADHs and ZmAMADH2 compared with that with NAD$^+$. Thio-NAD$^+$ drastically decreases AMADH activity (between 4.5 and 25%), especially that of ZmAMADH2 and PsAMADH1. 3-Pyridinealdehyde-NAD$^+$ is generally not accepted as a coenzyme for our studied AMADHs. To our knowledge, there is so far only one detailed analysis of the coenzyme binding site using various NAD$^+$ analogs performed on human ALDH1 (ALDH1A1), ALDH2, and AMADH (ALDH9A1) (35). The human ALDHs (hALDHs) significantly differ in their activity with nicotinamide analogs, and these differences are rather difficult to interpret in terms of interactions with the conserved catalytic residues. For example, thio-NAD$^+$, which is inactive with hALDH2 (reduced $V_{max}$ and unaffected $K_m$), is a good coenzyme of hAMADH and a weak coenzyme for hALDH1. Similarly, 3-pyridinealdehyde-NAD$^+$, which is a coenzyme for hALDH1 and hALDH2, is inactive for hAMADH as well as for plant AMADHs. Indeed, 3-pyridinealdehyde-NAD$^+$ acts as a strong competitive inhibitor of hAMADH and might form a thiohemiacetal linkage with the catalytic cysteine, preventing its reduction (36).

**Substrate Catalysis and Specificity—**A large screening study performed using 1 mM substrates, such as natural $\omega$-aminoaldehydes, including APAL, ABAL, GBAL, TMABAL, and BAL (Fig. 1), proves that all five plant AMADHs from tomato and maize most readily oxidize propionaldehyde and butyraldehyde natural and synthetic aminoaldehydes (Fig. 3). The highest reaction rates are obtained with APAL for all maize enzymes and SIAMADH2. Only SIAMADH1 displays the highest activity for ABAL. Methyl and guanidine derivatives of APAL and ABAL are also well oxidized, with 10–60% rates on average (relative to that of the best substrate), whereas hydroxylated derivatives are oxidized more slowly. Taken together, the three ZmAMADHs and SIAMADH2 resemble both PsAMADHs with respect to their substrate preference. Unexpectedly, SIAMADH1 exhibits much broader substrate specificity than SIAMADH2 and the other studied AMADHs. This enzyme oxidizes much better not only the guanidine derivatives 3-guanidinopropionaldehyde and GBAL but also 3- and 4-pyridine carboxaldehydes (3- and 4-PCAL; ~40% of the rate with ABAL) and several $n$-alkyl aldehydes like valeraldehyde and capronaldehyde (~15% of the rate with ABAL). One more unexpected result is that ZmAMADH1a shows a high activity with BAL, comparable with ABAL oxidation (~16% compared with APAL). To shed light on these surprising results, we focused on SIAMADH1 and ZmAMADH1a for a crystallographic study.

SIAMADH1 structure reveals well-defined electron density maps in the substrate binding site of both subunits despite the absence of a co-crystallized or soaked substrate. The only compound considered as a potential ligand comes from the PEG solution used as a precipitant for crystallization. A di(hydroxyethyl)ether molecule was straightforward modeled. A continuous electron density between the SG atom of the catalytic cysteine Cys-295 and the C1 atom of the di(hydroxyethyl)ether molecule was straightforward modeled. A continuous electron density between the SG atom of the catalytic cysteine Cys-295 and the C1 atom of the di(hydroxyethyl)ether molecule suggests a 1.7-Å-long covalent bond in subunit A (Fig. 4A). The density shape at C1 is not flat and satisfies an $sp^3$ hybridization expected for a thiohemiacetal intermediate. The oxygen atom of the hydroxyl group at C1 is hydrogen-bonded to the Cys-295 amide nitrogen (2.9 Å) and to the ND2 atom of Asn-162 (2.8 Å), two groups formerly postulated to be involved in the formation of the oxyanion hole and to stabilize the thiohemiacetal and thioacyl intermediates (37) (Fig. 4B). So far, the only reported intermediate in the ALDH family corresponding to the E268A glyceraldehyde-3-phosphate dehydrogenase mutant (38) is a thioester intermediate due to the
flat density observed at the C1 atom, corresponding to a $sp^2$ hybridization.

The existence of the covalent bond and the disordered Glu-260 side chain strongly suggests that the enzyme is turning over in the crystal with a highly mobile Glu-260. Kinetic measurements with PEG solutions (PEG 1000 and 1500) used for crystallization confirm the presence of aldehydes and show an activity of about a 1% rate compared with that with capronaldehyde (data not shown). The presence of an aldehyde in PEG solution was also mentioned in an hALDH2 crystallographic study, where we believe that the soaked crotonaldehyde substrate was arbitrarily taken as 100%. The measurements were performed with 1 mM substrates in 0.15M Tris-HCl buffer, pH 9.0, containing 1 mM NAD$^+$.

The following substrates were tested: APAL, N,N,N-trimethyl-3-aminopropionaldehyde (TMAPAL), 3-guanidinopropionaldehyde (GPAI), ABAL, N,N-dimethyl-4-aminobutyaldehyde (DMABAL), TMAPAL, 4-amino-2-hydroxybutyraldehyde (AHBAL), GBAL, 4-guanidino-2-hydroxybutyraldehyde (GHBAL), BAL, acetaldehyde (C2), propionaldehyde (C3), butyraldehyde (C4), valeraldehyde (C5), capronaldehyde (C6), enanthaldehyde (C7), and pyridine carboxaldehydes (PCAL). Specific activity values with 1 mM APAL were 3.1 units mg$^{-1}$ for SIAMADH1, 3.6 units mg$^{-1}$ for SIAMADH2, 2.6 units mg$^{-1}$ for ZmAMADH1a, 1.9 units mg$^{-1}$ for ZmAMADH1b, and 4.1 units mg$^{-1}$ for ZmAMADH2. Error bars, S.D.

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The presence of either intermediate or product/substrate in the active site does not induce conformational changes. The finding of a thiohemiacetal intermediate in only one subunit (subunit A) and the presence of either a substrate or carboxylate product in the other subunit would imply that in the context of dimer and the ALDH10 family, the hydride transfer (from NAD$^+$ to NADH followed by thioester formation) occurs in each subunit in turn. Otherwise, the covalent bond (between the catalytic cysteine Cys-295 in attacking conformation and a substrate molecule) would be randomly observed in both subunits. At the moment, we cannot conclude whether this property would be unique for the ALDH10 family or also apply for other ALDHs. Indeed, in the only other known case of the Streptococcus E268A-glyceraldehyde-3-phosphate dehydrogenase mutant (38), a thioester intermediate in all four subunits of the tetramer was observed due to a very slow deacylation rate allowing the accumulation of the intermediate in each subunit.

FIGURE 3. Substrate specificity of ALDH10 isoenzymes from tomato (top) and maize (bottom). The activity of each AMADH isoenzyme with the best substrate was arbitrarily taken as 100%. The measurements were performed with 1 mM substrates in 0.15m Tris-HCl buffer, pH 9.0, containing 1 mM NAD$^+$. The extended conformations typical for oxidized NAD$^+$ in the SIAMADH1-E260A structure proves that no turnover occurs in the crystal, although PEG was used as a precipitant. We verified that this mutant is inactive with the aldehyde in PEG solutions and capronaldehyde, at pH 7.0 and in the same buffer used in the crystallization solution, explaining the unsuccessful attempts at obtaining intermediate or bound substrates by soaking methods. At this pH, the catalytic cysteine ($pK_a \sim 8.0$) (39) should carry a thiol group and needs to be deprotonated to the nucleophilic thiolate to function properly. Because an intermediate was trapped in the WT enzyme, the acylation reaction is slow enough to observe the intermediate accumulation in the crystal (i.e. the nucleophilic activation is faster). Therefore, the absence of an intermediate formation in the mutant structure suggests that Glu-260 was required to activate the catalytic cysteine in the WT structure. The same function has been shown for the equivalent glutamate in hALDH2 (40, 41). However, under physiological conditions occurring in plant peroxisomes (a basic pH), we believe that the role of this glutamate is rather for proton abstraction from the hydrolytic water.

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Role of Trp-288 and Tyr-163 in Catalysis of Aliphatic and Aromatic Aminoaldehydes—A comparison of the substrate channel residues in tomato, maize, and pea AMADHs shows that almost all residues are evolutionarily highly conserved (Table 5). Noticeably, two residues at positions 288 and 453 (PsAMADH numbering) are less preserved. Trp-288 could be substituted by Ala, Phe, or Ser, and Cys-453 could be substituted by Ser or Thr.

The five studied AMADHs exhibit the highest $k_{cat}$ value with APAL (Tables 3 and 4). However, based on the $k_{cat}/K_m$ ratio, APAL is the best substrate only for three of them (SIAMADH2, ZmAMADH1a, and ZmAMADH1b) as well as for PsAMADH2 (4), which all possess Trp-288 (Trp-289 in SlAMADH numbering, Trp-290 or -291 in ZmAMADHs). SIAMADH1 and ZmAMADH2, which contain an Ala at this position, prefer TMABAL as the best substrate, accented by the increased $K_m$ values for APAL and ABAL. PsAMADH1 containing a phenylalanine also prefers TMABAL (4). Saturation curves for the five enzymes show that a strong excess substrate inhibition appears at relatively low substrate concentrations (Fig. 5, A–E). The inhibition appears at higher substrate concentrations for SlAMADH1 and ZmAMADH2 lacking Trp-288. This is in line with the behavior of the PsAMADH2-W288A mutant showing $K_m$ values for APAL and ABAL 2–3-fold higher than those for the WT enzyme (17). The preference of TMABAL for Trp-288-absent AMADHs appears either directly due to the increase in the substrate channel diameter and the loss of possible π-electron stacking interaction with the electrophilic protonated amino group of the substrate or indirectly by affecting the position of the Tyr-163 side chain (see “BADH Activity of ZmAMADH1a and SIAMADH1; Role of Cys-446 and Asn-290”). Similar kinetic results have been reported for two rice (OsAMADHs) and barley (HvAMADHs) isoenzymes (6, 18). OsAMADH2 and HvAMADH2, which contain the Trp-288 residue, display significantly lower $K_m$ values for APAL or ABAL than OsAMADH1 and HvAMADH1 lacking this tryptophan.

The five studied AMADHs oxidize 3- and 4-PCAL, representing heterocyclic aminoaldehydes with a tertiary nitrogen atom (Fig. 3). Interestingly, the product of 3-PCAL oxidation, named nicotinic acid, is methylated to the alkaloid trigonelline in plants, which acts among others as an osmoprotectant. AMADHs lacking the Trp-288 residue, such as SlAMADH1, ZmAMADH2, and PsAMADH1, should be more efficient, as demonstrated by the PsAMADH2-W288A mutant showing a 4 times higher rate with 3-PCAL than the WT enzyme (17). However, the activity of ZmAMADH2 and PsAMADH1 is not as high as that of SIAMADH1, which displays similar $K_m$ values for 3- and 4-PCAL as for the best aminoaldehyde substrates (Tables 3 and 4). Therefore, it becomes clear that such a change in SIAMADH1 kinetics does not only result from the Trp-288 absence.

### FIGURE 4. SIAMADH1 substrate channel with a bound intermediate. A, a close-up view of the active site in subunit A showing a continuous electron density from the $F_o - F_c$ omit map contoured at $3\sigma$ between the catalytic Cys-295 and the PEG aldehyde, which forms a thiohemiacetal intermediate. B, a covalent bond is formed between the SG atom of Cys-295 and the C1 atom of the PEG aldehyde. Hydrogen bonds between the intermediate and enzyme residues (Cys-295 and Asn-162) are shown as dashed lines. The angle geometry of this intermediate is shown. C, a close-up view of the active site in subunit B showing a discontinuous electron density map between the catalytic cysteine and the PEG aldehyde. Residues are labeled.
between SlAMADH1 and known plant AMADH structures reveals a displacement of four residues (from Thr-454 to Asp-457), which open the channel cavity in SlAMADH1. Thr-454, the most affected residue, is shifted by 1.8 Å away from the active site, allowing its side chain to make a hydrogen bond with the conserved Gln-485 NE1 atom from the oligomerization domain of the other subunit (Fig. 6B). Thr-454 is thus directly involved in the dimer interface interaction. The position of the Thr-454 side chain leads to a large and less polar channel interior, resulting in high rates of oxidation of aliphatic and aromatic aldehydes by SlAMADH1 compared with SlAMADH2 and both PsAMADHs (Figs. 3 and 5, D and E). Indeed, in PsAMADH1/2, the side chains of Ser-453/Cys-453 make the channel interior smaller by pointing inward and polar for substrate interaction (Fig. 6C). Therefore, the combination of the two residues, Ala-289 and Thr-454, distinguishes SlAMADH1 for its broad substrate specificity. The role of Thr-454 is emphasized by the PsAMADH2-C453A mutant, which does not display any significant differences from the WT enzyme (17).

BADH Activity of ZmAMADH1a and SlAMADH1; Role of Cys-446 and Asn-290—BAL, formed by choline monooxygenase (42) in plants, is oxidized by AMADH/BADH to the osmoprotectant GB. Under stress conditions, high levels of GB have been found in leaves of diverse families of dicots and monocots, including barley, wheat, and rye (43).
vars synthesize considerably lower levels of GB, and naturally occurring GB-deficient inbred lines have also been identified (44). Moreover, certain maize varieties with tomato and pea do not accumulate GB due to the loss of a functional CMO (choline monooxygenase) gene (45–47), correlated with the very low activity of ZmAMADH1b, ZmAMADH2, SlAMADH2, and PsAMADHs with BAL (below 1.5% compared with that with APAL; Fig. 3) (4). Interestingly, ZmAMADH1a exhibits a good activity with BAL (**K**m/4 = 14 μM, **k**cat = 0.6 s⁻¹). SIAMADH1 oxidizes BAL significantly only at high concentrations, as indicated by the corresponding **K**m value (Tables 3 and 4).

Based on AMADH structures, four strictly conserved residues, Tyr-163, Trp-170, Trp-288, and Trp-459 (Tyr-165, Trp-172, Trp-289, and Trp-461 in ZmAMADH1a), seem to be arranged suitably for cation–π interactions with the trimethylammonium group of BAL, which becomes sandwiched between Tyr-163 and Trp-459 upon binding. A structural comparison of the four plant AMADH structures (ZmAMADH1a, SlAMADH1, and PsAMADH1/2) reveals a difference between the position of Trp-461 in ZmAMADH1a and those of the equivalent tryptophans in the other three structures. Indeed, its side chain is shifted by 1.6 Å (corresponding to a rotation of 23° along the Cβ-Cγ axis) away from Tyr-163, leading to an enlargement of the substrate channel favorable for binding of the bulky trimethylammonium group (Fig. 6D). Ile-444 in PsAMADHs (Ile-445 in SIAMADH1) prevents Trp-459 from adopting the observed position in ZmAMADH1a due to a steric hindrance. Thus, the equivalent cysteine (Cys-446) in ZmAMADH1a, located in the second sphere of the substrate interaction, is responsible for the high activity with BAL, as shown in spinach AMADH/BADH (SoBADH; Protein Data Bank code 4A0M) (48). Notably, the concerned tryptophan and cysteine residues adopt the same position in ZmAMADH1a and SoBADH structures. HvAMADH2, which also contains a cysteine, exhibits a good BAL activity (18). Moreover, AMADHs from members of the Amaranthaceae family (amaranth, spinach, and sugarbeet), from several monocots like barley or wheat, and from several grasses like sorghum or velvetgrass, known for their high BADH activity, possess such a cysteine (or an alanine) (49, 50, 10, 51). The low or negligible BADH activity of ZmAMADH1b, ZmAMADH2, SIAMADH2, and PsAMADHs correlates with the presence of Ile-444 (Table 5). However, despite the presence of Ile-445 in SIAMADH1, its activity with BAL resembles that of the mutant Y163A from PsAMADH2 (17). Here the structural comparison shows that Tyr-163, in SIAMADH1, has no hydrogen bond involving its hydroxyl group (conversely, there is a hydrogen bond with the Thr-289 side chain in PsAMADH, which is

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**FIGURE 6.** Substrate channels of SIAMADH1, ZmAMADH1a, and PsAMADH2. A, a comparison between the substrate channel volumes of SIAMADH1 (in red) and PsAMADH2 (in blue mesh; Protein Data Bank code 3WJ). The enlarged volume of SIAMADH1 comes from the absence of Trp-109 and Trp-288 and a displacement at position 453; B and C, a transversal volume section of substrate channel of SIAMADH1 (in red) and PsAMADH2 (in blue), both with a docked ABAL ligand. These images show the enlarged middle section of the substrate channel of SIAMADH1, which occurs due to the presence of Ala-289 and Thr-454 residues compared with the presence of Trp-288 and Cys-453 in PsAMADH2. Thr-454 is hydrogen-bonded to Gln-485 from the other subunit and thus is involved in the dimer interface. Amino acid residues are shown as sticks and labeled. The total surface of the cavities was calculated using Hollow with 0.5-Å grid spacing and a 1.4-Å interior probe. The ABAL molecule (in black) is shown for illustration. It was docked into the active site using AutoDock, and its carbonyl oxygen atom establishes a hydrogen bond to the catalytic cysteine Cys-295/Cys-294, whereas the amino group is hydrogen-bonded to Tyr-163/Tyr-163; D and E, a substrate channel structural comparison of ZmAMADH1a (in purple) and SIAMADH1 (in orange) with PsAMADH2 (in blue, used as the reference for residue numbering shown in parentheses). Cys-446 in ZmAMADH1a allows a conserved Trp to move away from Tyr-165, thus opening the channel for a bulkier BAL molecule. Asn-290 in SIAMADH1 could have a similar role on the opposite side, allowing Tyr-163 to move toward Asn-290, thus opening the channel. Residues labeled in red are not totally conserved in plant AMADHs.
ALDH10 Family from Tomato and Maize

replaced by Asn-290 in SIAMADH1). Asn-290 does not interact with Tyr-163, which could move toward Asn-290 and away from the channel substrate. The flexibility of Tyr-163 in SIAMADH1 would allow bulkier BAL to pass to the catalytic cysteine. Thus, Asn-290 in SIAMADH1, located in the second sphere of substrate interaction, might have a similar role like that of Cys-446 in ZmAMADH1a, in terms of the channel widening, acting on the opposite side on the substrate channel. Another interesting different movement of Tyr-163 is observed in SIAMADH1 and PsAMADH2 structures compared with the two others. Its hydroxyl group can move 1.1 Å (corresponding to a rotation of 9° along the Cα-Cβ axis) toward Ala-289 and the equivalent Phe-288 residue, respectively, which is only possible due to the absence of Trp-288 at this position (Fig. 6F). Consequently, the nature of the residue at position 288 will have a significant effect on the interaction between the Tyr-163 side chain and the substrate.

Interestingly, human ALDH9 (ALDH9A1, also called E3 isoenzyme) has been shown to prefer TMABAL (Km = 1.4 μM) over ABAL (Km = 8–14 μM) and BAL (Km = 120 μM) (23). The low Km value for ABAL indicates that this enzyme is involved in GABA formation (reviewed in Ref. 53). So far, APAL and GBAL have not been tested with this enzyme. The other well studied ALDH9 from cod liver also shows a high preference for TMABAL. Fish ALDH9 from cod liver (Protein Data Bank codes 1A45 and 1BPW) (54) shows a higher kcat/Km ratio for BAL over aliphatic and aromatic aldehydes, and this is why the enzyme is called BADH (55). To our knowledge, other ω-aminoaldehydes have not yet been tested, but the active site preserves aromatic residues responsible for AMADH activity, typical for the ALDH10 family, namely Tyr-167 (Tyr-163 in ALDH10), Trp-174 (Trp-170), and Phe-466 (Trp-459). The variable Trp-288 in ALDH10 is replaced by Leu-291.

**ACAPAL and DMSPAL Dehydrogenase Activities**—Besides the two natural compounds APAL and ABAL, known to be among the best substrates of all plant AMADHs studied so far, we show for the first time that ACAPAL may also be generally oxidized (Fig. 5F). Therefore, these enzymes are unique in metabolizing and detoxifying characteristic aldehyde products of polyamine degradation to nontoxic acids. SIAMADH1 shows 85% activity with ACAPAL compared with that with APAL, whereas SIAMADH2 displays only 30% activity. For ZmAMADHs, the activity is between 43 and 55%, and for PsAMADHs, it is between 28 and 35%. With respect to ACAPAL oxidation by AMADH isoenzymes in a single species, the activity of the Trp-288-absent AMADH isoenzyme is higher. APAL mainly originates from the "polyamine backconversion pathway" via the oxidation of spermine (to spermidine) and spermidine (to putrescine) on the exo-side of N4-nitrogen by plant PAOs (56, 57). Likewise, the oxidation of N1-acetylsperrmine and N1-acetylspermidine ends up with ACAPAL. APAL or ACAPAL is oxidized by AMADH to β-alanine and 3-acetamidopropionate, respectively. β-Alanine can subsequently be methylated to the osmoprotectant β-alanine betaine (58). It is notable that three of five Arabidopsis PAOs are localized in peroxisomes (57, 59, 60). Another part of polyamine catabolism is localized outside peroxisomes because the existence of apoplastic and symplastic PAOs has also been reported (56, 57). These PAOs also oxidize polyamines on the endo-side of N4-nitrogen, producing 1,3-propanediamine (from spermine) and ABAL (from spermidine). Because plant PAOs seem to have no activity or negligible activity with putrescine (57, 59), ABAL apparently originates from putrescine oxidation by plant copper diamine oxidases. It is then oxidized by AMADHs to GABA.

DMSPAL accumulates in many marine algae and a few higher plant species, including the Asteraceae and Poaceae families (9, 61). So far, DMSPAL has been shown to be oxidized by sugar beet and amaranth AMADHs/BADHs (10). Here we show that all studied AMADHs are capable of oxidizing it to the cryo- and osmoprotectant DMSP sharing similar properties with betaines (8, 61) (Fig. 5F). Indeed, SIAMADH1 and SIAMADH2 oxidize this compound at a 36 and 10% rate, respectively, compared with that with their best substrate. Likewise, ZmAMADHs display rates between 8 and 16% compared with those with APAL and both PsAMADHs at a 22% rate. The universal ability of AMADHs to convert DMSPAL seems related to its stable positive charge at the dimethylsulfonio group and its structural similarity with APAL, which is protonated at the primary amino group under physiological conditions. As well as ω-aminoaldehydes, DMSPAL is trapped into a nucleophilic cage formed by the carboxylic and aromatic residues at the active site of AMADH.

**AMADH Gene Expression and Subcellular Localization**—qPCR analysis shows that the expression of both tomato AMADH genes is relatively different. In contrast to the SIAMADH1 gene, the SIAMADH2 gene is highly and ubiquitously expressed in all analyzed tissues (Fig. 7). Whereas SIAMADH2 is well expressed in roots at 10 days after germination and at 6 weeks, SIAMADH1 is not expressed at all and expressed at a low extent (by 2 orders of magnitude), respectively. Both genes are expressed in cotyledons at 10 days after germination and later in flower organs (petals, carpel, and stamens) and senescent leaves. These data indicate that SIAMADH2 is playing a major role in all tomato organs in detoxifying toxic ω-aminoaldehydes. The observed high activity of both SIAMADHs with APAL and ABAL is in a good agreement with previous data obtained from in vivo experiments on tomatoes, where the formation of β-alanine and GABA was observed as a result of polyamine degradation (62).

Because of the incomplete knowledge of tomato genome, it was impossible to localize the tomato genes, whereas those for maize could be mapped. The paralogs ZmAMADH1a and ZmAMADH1b are located on chromosomes 4 and 1, respectively. The gene of ZmAMADH2 is located on chromosome 10. The three maize genes are expressed differently (Fig. 7). A major difference concerns the ZmAMADH2 gene, which is markedly less expressed than the others. Transcript abundance of ZmAMADH2 is higher in silks at 0 and 5 days after pollination and in the kernel at 5 days after pollination. ZmAMADH1a (coding for the AMADH isoform with higher BADH activity) is highly expressed in young and mature leaves as well as in roots of younger plants. Transcript abundance of ZmAMADH1b is weaker in leaves but higher in roots compared with that of ZmAMADH1a. ZmAMADH1b is also highly expressed in silks at 0 days after pollination and in the kernel at 5 days after pol-
lination. Both ZmAMADH1 genes are well expressed in tassels. These data indicate that AMADHs might be important for controlling ω-aminoaldehyde levels during early stages of the seed development.

Recently, several studies have shown that AMADH2 gene mutation, naturally occurring in several rice varieties, is responsible for their fragrance (6, 63, 64). AMADH2 was found to be transcribed constitutively in all tissues tested except for roots. The fragrance appears due to high abundance and subsequent acetylation of free ABAL and accumulation of 2-acetyl-1-pyrroline. Traditional aromatic cultivars often have undesirable properties, including poor crop yield, susceptibility to pests and diseases, and strong shedding. Although their causes have not been analyzed so far, based on our results, these will be a combination of accumulation of toxic aminoaldehydes and lower content of zwitterionic products (γ-alanine betaine, GABA, and others) having osmo-, cryo-, and heat-protecting properties.

Most plant AMADHs, including rice AMADHs and HvAMADH1, carry the SKL C-terminal peroxisomal targeting signal (PTS-1) (14, 65). All of our studied AMADHs except for SlAMADH1 contain PTS-1. Indeed, SIAMADH1 ends with a tripeptide SKN, which is not a peroxisomal targeting signal, making SIAMADH1 probably localized in the cytosol. The loss of the peroxisomal signal in one of the two AMADH isoforms has been observed in many plant species. For example, in Arabidopsis, one isoform (with SKL signal) is localized in peroxisomes, whereas the other (KSPN motif at the C terminus) is targeted to leucoplasts (66). Similarly, in barley, one isoform was demonstrated to be localized in peroxisomes, whereas the second appears to reside in the cytosol (65). The exact subcellular localization of SIAMADH1 remains unclear.

The phylogenetic analysis of numerous AMADH/BADH sequences from monocots and dicots shows that there are two homologous sequences per species among flowering plants and an additional one for maize. Thus, we believe this number can be higher in species with genome duplications. Among monocots, including maize, rice, and barley, two orthologous AMADH subgroups can be identified (Fig. 8). One subgroup contains a cluster of the Triticeae (Poaceae family) predictable as wide substrate-specific AMADHs (due to the presence of Phe-288 and Thr-454 in PsAMADH numbering; Fig. 8, highlighted in yellow). Moreover, the presence of Asn-289 implies that Tyr-163 is mobile, affecting the substrate binding. A similar cluster is found in dicots from the Solanaceae family. The majority of members in the other monocot subgroup display significant BADH activity due to the presence of Ala-444 or Cys-444 residue favoring BAL binding (48). They also possess the Trp-288 residue. Interestingly, higher BAL activity correlates in part with nonperoxisomal targeting. For example, HvAMADH2, wild rye AMADH2, and wheat AMADH1 miss the SKL targeting signal and carry a C-terminal KAPAN motif instead. In the dicots, such a clustering only exists among closely related species. Here again, sequences of AMADHs with BADH activity from the Amaranthaceae, Acanthaceae, and Salicaceae families carry the KSP C-terminal signal as well as Ala-444 and Trp-288. Such a variable targeting indicates that AMADH genes might be differently responsive to, for example, stress conditions in various plants. The differential subcellular localization of AMADHs may arise from the interconnection of

FIGURE 7. Expression profiles of two SIAMADH and three ZmAMADH genes in various organs as evaluated by qPCR. Transcript abundance is expressed as gene copy number in 1 ng of total RNA amplified by qPCR, normalized to EF1α, and recalculated as primer pair efficiency. Tomato samples, including roots and cotyledons, were collected at 10 days after germination (DAG); shoot apices (stem plus leaves), stems below flowers (including sepals), stems below fruits, petals, reproductive organs, green fruit, roots, and senescent leaves were collected from 6-week-old plants grown on a soil/peat mixture (2:1, v/v). Maize samples were collected from 3-month-old plants unless indicated otherwise. Tassels, silks, and kernels were collected at several days before, during, and after pollination (DAP). Error bars, S.D.
several metabolic pathways. Several PAOs are targeted to peroxisomes, where they generate APAL and ACAPAL from polyamines, which are detoxified by peroxisomal AMADHs. Other PAOs as well as copper diamine oxidases are localized in the cytoplasm. Copper diamine oxidases produce ABAL from putrescine, which is oxidized to GABA. In species accumulating GB, AMADHs are often found in chloroplasts (as well as choline monooxygenase), where they convert BAL to GB.

CONCLUSIONS

In this work, we continued studies on substrate specificity of plant AMADHs. After previous analysis of two PsAMADHs, we now cloned five more genes: two from tomato and three from maize. We produced and purified the corresponding enzymes (SlAMADH1, SlAMADH2, ZmAMADH1a, ZmAMADH1b, and ZmAMADH2) to characterize them kinetically. Because two of them stand out from the others in terms of substrate specificity, SIAMADH1 having the broadest range of substrates and ZmAMADH1a having high BADH activity, we determined their crystal structures. From structural comparisons, we observe that SIAMADH1 is distinguished by its large and less polar substrate channel due to residues at positions 289 and 454 (288 and 453 in PsAMADHs), which are responsible for this large specificity. We can also predict that AMADHs containing Ile-444 (PsAMADH numbering) have a negligible BADH activity, and those containing Asn-289 may display some BADH activity. The wide substrate specificity of SlAMADH1 allowed us to trap a thiohemiacetal intermediate in the structure, never observed before, which, in addition to the SlAMADH1-E260A structure, suggests a bifunctional role of the active-site base glutamate. In addition to its known role for the activation of the hydrolitic water, it can activate the protonated catalytic cysteine.

FIGURE 8. Phylogenetic analysis of the plant ALDH10 family (AMADHs). The unrooted phylogenetic consensus tree shows two distinct groups of AMADHs, one from dicots (solid line) and the other from monocots (dashed line). PsAMADH numbering is used to point out crucial residues modulating substrate specificity. AMADHs exhibiting a significant BADH activity are highlighted in green. AMADHs predictable as broad substrate specific enzymes are highlighted in yellow due to the presence of Thr-454. The presence of Asn-289 implies that Tyr-163 is mobile, affecting the substrate binding. The enzymes from maize (ZmAMADHs) and tomato (SIAMADHs) analyzed in this work are shown in boldface type, as are the previously characterized reference enzymes from peas (PsAMADHs) (4), rice (OsAMADHs) (6), and barley (HvAMADHs) (18). The internal labels give bootstrap frequencies for each clade. See “Experimental Procedures” for the corresponding GenBank™ accession numbers.
Based on results of the entire study, we can now state that a tryptophan (Trp-288 in PsAMADHs), which is found in more than three-quarters of AMADH sequences, together with the conserved cluster of Tyr-163, Trp-170, and Trp-459, is responsible for the high affinity to ω-aminaldehydes with APAL as the best substrate. AMADHs devoid of this tryptophan prefer TMABAL either directly due to the increase in the substrate channel diameter (and consequent loss of possible π-electron stacking interaction with the electrophilic protonated amino group of the substrate) or indirectly by affecting the position of the Tyr-163 side chain involved in substrate interaction. For the first time, we identified ACAPAL as a possible common substrate for all plant AMADHs. We also showed that the enzymes are capable of oxidizing DMSPAL to form the cryo- and osmo-protectant DMSP.

It becomes now obvious that AMADHs, depending on the plant species, are capable of oxidizing a wide range of naturally occurring ω-aminaldehydes, resulting in the production of various amino acids that may directly or after a further conversion function as osmoprotectants. Thus, in plants, AMADHs control levels of ω-aminaldehydes, which are produced under normal and stress conditions. Current transgenic studies on the overexpression of AMADH/BADH in plants conferring increased abiotic stress tolerance are aimed especially at GB production. Related engineering of GB synthesis in species with nonfunctional choline monooxygenase is also targeted rather narrowly. In the context of the present work, the wide substrate specificity of plant AMADHs indicates that there is a possibility of altered production of β-alanine betaine, GABA, or γ-butyro-betaine, which is worthy of consideration for the development and analysis of stress-tolerant transgenic crops. The latter compound may also be involved in the biosynthetic pathway of l-carnitine in plants, which shares similar features with pathways in mammals and fungi. In addition to its role in the mitochondrial transport of fatty acids, l-carnitine was also shown to confer tolerance to abiotic stress (52, 67).

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REFERENCES

1. Bouchereau, A., Aziz, A., Larher, F., and Martin-Tanguy, J. (1999) Polymamines and environmental challenges. Recent development. Plant Sci. 140, 103–125
2. Šebela, M., Frébort, I., Petivalsky, M., and Peč, P. (2002) Copper/tota quinine-containing amino oxidasenes. Recent research developments. In Studies in Natural Products Chemistry, Vol. 26, Bioactive Natural Products, Part G (Atta-ur-Rahman, ed) pp. 1259–1299, Elsevier, Amsterdam
3. Li, W., Yuan, X. M., Ivanova, S., Tracey, K. J., Eaton, J. W., and Brunk, U. T. (2003) 3-Aminopropanal, formed during cerebral ischaemia, is a potent lysosomotropic neurotoxin. Biochem. J. 371, 429–436
4. Tylichová, M., Kopečný, D., Moréřa, S., Briozzo, P., Lenobel, R., Snégaroff, J., and Šebela, M. (2010) Structural and functional characterization of plant aminodehyde dehydrogenase from Pisum sativum with a broad specificity for natural and synthetic aminodehydes. J. Mol. Biol. 396, 870–882
5. Bouché, N., and Fromm, H. (2004) GABA in plants. Just a metabolite? Trends Plant Sci. 9, 110–115
6. Bradic, L. M., Gillies, S. A., Brushett, D. J., Waters, D. L., and Henry, R. J. (2008) Inactivation of an aminodehydrogenase is responsible for fragrance in rice. Plant Mol. Biol. 68, 439–449
7. Ariket, S., Yoshihashi, T., Wanchana, S., Uyen, T. T., Huang, N. T., Wong-pornchait, S., and Vanavichit, A. (2011) Deficiency in the amino aldehyde dehydrogenase encoded by GmAMADH2, the homologue of rice Os2AP, enhances 2-acetyl-1-pyridine biosynthesis in soybeans (Glycine max L.). Plant Biotechnol. J. 9, 75–87
8. Rhodes, D., and Hanson, A. D. (1993) Quaternary ammonium and tertiary sulfonium compounds in higher plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 44, 357–384
9. James, F., Paquet, L., Sparace, S. A., Gage, D. A., and Hanson, A. D. (1995) Evidence implicating dimethylsulfoxoniopropionaldehyde as an intermediate in dimethylsulfoxoniopropionate biosynthesis. Plant Physiol. 108, 1439–1448
10. Kirch, H. H., Bartels, D., Wei, Y., Schnable, P. S., and Wood, A. J. (2004) The ALDH gene superfamily of Arabidopsis. Trends Plant Sci. 9, 371–377
11. Sophos, N. A., Vasiliiou, V. (2003) Aldehyde dehydrogenase gene superfamily. The 2002 update. Chem. Biol. Interact. 143, 5–22
12. Nakamura, T., Yokota, S., Muramoto, Y., Tsutsui, K., Oguri, Y., Fukui, K., and Takabe, T. (1997) Expression of a betaine aldehyde dehydrogenase gene in rice, a glycinebetaine nonaccumulator, and possible localization of its protein in peroxisomes. Plant J. 11, 1115–1120
13. Kopecny, D., Tylichova, M., Santen, P., Briozzo, P., Ferrero, J., Moreira, S., Joly, N., and Thomas, H. (2011) Coregulation of an aminoaldehyde dehydrogenase in rice, a glycinebetaine nonaccumulator, and possible localization of its protein in peroxisomes. FEBS J. 278, 3130–3139
14. Fujiwara, T., Hori, K., Ozaki, K., Yokota, Y., Mitsuya, S., Ichiyamag, T., Hattori, T., and Takabe, T. (2008) Enzymatic characterization of peroxi- somal and cytosolic betaine aldehyde dehydrogenases in barley. Physiol. Plant 134, 22–30
15. Brotcher, C., Vasiliiou, M., Carpenter, S., Carpenter, C., Zhang, Y., Wang, X., Kotchoni, S. O., Wood, A. J., Kirch, H. H., Kopečný, D., Nebert, D. W., and Vasiliiou, V. (2013) Aldehyde dehydrogenase (ALDH) superfamily in plants. Gene nomenclature and comparative genomics. Planta 237, 189–210
16. Tylichová, M., Briozzo, P., Kopečný, D., Ferrero, J., Moréřa, S., Joly, N., Snégaroff, J., and Šebela, M. (2008) Purification, crystallization and preliminary crystallographic study of a recombinant plant aldehyde dehydrogenase from Pisum sativum. Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun. 64, 88–90
17. Šebela, M., Štovsová, T., Havlis, J., Wielsh, N., Thomas, H., Zdráhal, Z., and Shevchenko, A. (2006) Thermostable trypsin conjugates for high-throughput proteomics. Synthesis and performance evaluation. Proteomics 6, 2959–2963
18. Šebela, M., Brauner, F., Radová, A., Jacobsen, S., Havlis, J., Galuszka, P., and Pec, P. (2000) Characterisation of a homogeneous plant aminode-
hyde dehydrogenase. *Biochim. Biophys. Acta.* **1480**, 329–341
23. Vaz, F. M., Fouchier, S. W., Olfman, R., Sommer, M., and Wanders, R. J. (2000) Molecular and biochemical characterization of rat γ-trimethylamino-nobutylaldehyde dehydrogenase and evidence for the involvement of human aldehyde dehydrogenase 9 in carnitine biosynthesis. *J. Biol. Chem.* **275**, 7390–7394
24. Wood, P. L., Khan, M. A., and Moskal, J. R. (2007) The concept of “aldehyde load” in neurodegenerative mechanisms. Cytotoxicity of the polyamine degradation products hydrogen peroxide, acrolein, 3-aminopropanal, 3-acetamido-propanal and 4-aminobutanal in a retinal ganglion cell line. *Brain Res.* **1145**, 150–156
25. Holt, A., Degenhardt, O. S., Berry, P. D., Kapty, J. S., Mithani, S., Smith, D. J., and Di Paolo, M. L. (2007) The effects of buffer cations on interactions between mammalian copper-containing amine oxidases and their substrates. *J. Neural Transm.* **114**, 733–741
26. Kabasch, W. (2010) XDS. *Acta Crystallogr. D Biol. Crystallogr.* **66**, 125–132
27. McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C., and Read, R. J. (2007) Phaser crystallographic software. *J. Appl. Crystallogr.* **40**, 658–674
28. Blanc, E., Roversi, P., Vonrhein, C., Flensburg, C., Lesa, S. M., and Brucigne, G. (2004) Refinement of severely incomplete structures with maximum likelihood in BUSTER-TNT. *Acta Crystallogr. D Biol. Crystallogr.* **60**, 2210–2221
29. Emsley, P., Cowtan, K. (2004) Coot. Model-building tools for molecular graphics. *Acta Crystallogr. D Biol. Crystallogr.* **60**, 2126–2132
30. Edgar, R. C. (2004) MUSCLE. Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **32**, 1792–1797
31. Guindon, S., and Gascuel, O. (2003) A simple, fast and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst. Biol.* **52**, 696–704
32. Zheng, H., Chruszcz, M., Lasota, P., Lebioda, L., and Minor, W. (2008) Data mining of metal ion environments present in protein structures. *J. Inorg. Biochem.* **102**, 1765–1776
33. Lamb, A. L., and Newcomer, M. E. (1999) The structure of retinal dehydrogenase type II at 2.7 Å resolution. Implications for retinal specificity. *Biochemistry* **38**, 6003–6011
34. Hill, E. J., Chou, T. H., Shih, M. C., and Park, J. H. (1975) Covalent binding of 3-pyridinealdehyde nicotinamide adenine dinucleotide and substrates to glyceraldehyde 3-phosphate dehydrogenase. *J. Biol. Chem.* **250**, 1734–1740
35. Steinmetz, C. G., Xie, P., Weinier, H., and Hurley, T. D. (1997) Structure of mitochondrial aldehyde dehydrogenase. The genetic component of ethanol aversion. *Structure* **5**, 701–711
36. D’Ambrosio, K., Paolot, A., Talfournier, F., Didierjean, C., Benedetti, E., Aubry, A., Branlant, G., and Corbier, C. (2006) The first crystal structure of a thioacylenzyme intermediate in the ALDH family. New coenzyme conformation and relevance to catalysis. *Biochemistry* **45**, 2978–2986
37. Brauwer, F., Sebela, M., Négrőff, J., Pé, P., and Meunier, J. C. (2003) Pea seedling aldehyde dehydrogenase. Primary structure and active site residues. *Plant Physiol. Biochem.* **41**, 1–10
38. Wang, X., and Weiner, H. (1995) Involvement of glutamate 268 in the active site of human liver mitochondrial (class 2) aldehyde dehydrogenase as probed by site-directed mutagenesis. *Biochemistry* **34**, 237–243
39. Mann, C. J., and Weiner, H. (1999) Differences in the roles of conserved glutamic acid residues in the active site of human class 3 and class 2 aldehyde dehydrogenases. *Protein Sci.* **8**, 1922–1929
40. Brouquise, R., Weigel, P., Rhodes, D., Yocum, C. F., and Hanson, A. D. (1989) Evidence for a ferredoxin-dependent chloride monooxygenase from spinach chloroplast stroma. *Plant Physiol.* **90**, 322–329
41. Ishitani, M., Arakawa, K., Mizzuno, K., Kishitani, S., and Takabe, T. (1993) Betaine aldehyde dehydrogenase in the Gramineae. Levels in leaves of both betaine-accumulating and non-accumulating cereal plants. *Plant Cell Physiol.* **34**, 493–495
42. Brunk, D. G., Rich, P. J., and Rhodes, D. (1989) Genotypic variation for glycinebetaine among public inbreds of maize. *Plant Physiol.* **95**, 1122–1125
43. Lerma, C., Rich, P. J., Ju, G. C., Yang, W.-J., Hanson, A. D., and Rhodes, D. (1991) Betaine deficiency in maize. Complementation tests and metabolic basis. *Plant Physiol.* **95**, 1113–1119
44. Mákelä, P., Jokinen, K., Kontturi, M., Peltonen-Sainio, P., Pfeu, E., and Somersalo, S. (1998) Folar application of glycinebetaine, a novel product from sugar beet, as an approach to increase tomato yield. *Ind. Crop. Prod.* **7**, 139–148
45. Charlot, A. J., Donarski, J. A., Harrison, M., Jones, S. A., Godward, J., Oehlschläger, S., Arques, J. L., Ambrose, M., Chinoy, C., Mullineaux, P. M., and Domoney, C. (2008) Responses of the pea (*Pisum sativum* L.) leaf metabolome to drought stress assessed by nuclear magnetic resonance spectroscopy. *Metabolomics* **4**, 312–327
46. Díaz-Sánchez, Á. G., González-Segura, L., Mújica-Jiménez, C., Ruíñó-Píñera, E., Montiel, C., Martínez-Castilla, L. P., Muñoz-Clares, R. A. (2012) Acetaldehyde residues critical for the specificity for betaine aldehyde of the plant ALDH10 isoenzyme involved in the synthesis of glycine betaine. *Plant Physiol.* **158**, 1570–1582
47. Weigel, P., Weretilnyk, E. A., and Hanson, A. D. (1986) Betaine aldehyde oxidation by spinach chloroplasts. *Plant Physiol.* **82**, 753–759
48. Hígino, T., Meng, Y. L., Kawamitsu, Y., Uehara, N., Matsuda, N., Tanaka, Y., Ishikawa, H., Baba, S., Takabe, T., Wada, K., Ishii, T., and Takabe, T. (2001) Molecular cloning and functional characterization of two kinds of betaine-aldehyde dehydrogenase in betaine-accumulating mangrove *Avicennia marina* (Forsk.) Vihr. *Plant Mol. Biol.* **45**, 353–363
49. Valenzuela-Soto, E. M., and Muñoz-Clares, R. A. (1994) Purification and properties of betaine aldehyde dehydrogenase extracted from detached leaves of *Amaranthus hypochondriacus* L. subjected to water deficit. *J. Plant Physiol.* **143**, 145–152
50. Marchitti, S. A., Brocker, C., Stagos, D., and Vasiliou, V. (2008) Non-P450 aldehyde oxidizing enzymes: the aldehyde dehydrogenase superfamily. *Expert Opin. Drug Metab. Toxicol.* **4**, 697–720
51. Johansson, K., El-Ahmad, M., Ramaswamy, S., Hjelmqvist, L., Jörnvall, H., and Eklund, H. (1998) Structure of betaine aldehyde dehydrogenase at 2.1 Å resolution. *Protein Sci.* **7**, 2106–2117
52. Hjelmqvist, L., Norin, A., El-Ahmad, M., Griffiths, W., and Jörnvall, H. (2003) Distinct but parallel evolutionary patterns between alcohol and aldehyde dehydrogenase. Addition of fish/human betaine aldehyde dehydrogenase. *Cell Mol. Life Sci.* **60**, 109–114
53. Marchitti, S. A., Brocker, C., Stagos, D., and Vasiliou, V. (2008) Non-P450 aldehyde oxidizing enzymes: the aldehyde dehydrogenase superfamily. *Expert Opin. Drug Metab. Toxicol.* **4**, 697–720
54. Tavladoraki, P., Rossi, M. N., Saccuti, G., Perez-Amador, M. A., Pollicelli, F., Angelini, R., and Federico, R. (2006) Heterologous expression and biochemical characterization of a polyamine oxidase from Arabidopsis involved in polyamine back conversion. *Plant Physiol.* **141**, 1519–1532
55. Takahashi, Y., Cong, R., Sagor, G. H., Nitsu, M., Berberich, T., and Ku- sano, T. (2010) Characterization of five polyamine oxidase isoforms from sugarbeet, as an approach to increase tomato yield. *Plant Cell Physiol.* **50**, 2126–2132
56. Kamada-Nobusada, T., Hayashi, M., Fukazawa, M., Sakakibara, H., and Nishimura, M. (2008) A putative peroxisomal polyamine oxidase, AtPAO4, is involved in polyamine catabolism in Arabidopsis *thaliana.*
61. Paquet, L., Rathinasabapathi, B., Saini, H., Zamir, L., Gage, D. A., Huang, Z.-H., and Hanson, A. D. (1994) Accumulation of the compatible solute 3-dimethylsulfoniopropionate in sugarcane and its relatives, but not other gramineous crops. *Aust. J. Plant Physiol.* **21**, 37–48

62. Rastogi, R., and Davies, P. J. (1990) Polyamine metabolism in ripening tomato fruit. I. Identification of metabolites of putrescine and spermidine. *Plant Physiol.* **94**, 1449–1455

63. Chen, S., Yang, Y., Shi, W., Ji, Q., He, F., Zhang, Z., Cheng, Z., Liu, X., and Xu, M. (2008) Badh2, encoding betaine aldehyde dehydrogenase, inhibits the biosynthesis of 2-acetyl-1-pyrroline, a major component in rice fragrance. *Plant Cell* **20**, 1850–1861

64. Niu, X., Tang, W., Huang, W., Ren, G., Wang, Q., Luo, D., Xiao, Y., Yang, S., Wang, F., Lu, B. R., Gao, F., Lu, T., and Liu, Y. (2008) RNAi-directed down regulation of OsBADH2 results in aroma (2-acetyl-1-pyrroline) production in rice (*Oryza sativa* L.). *BMC Plant Biol.* **8**, 100

65. Nakamura, T., Nomura, M., Mori, H., Jagendorf, A. T., Ueda, A., and Takabe, T. (2001) An isozyme of betaine aldehyde dehydrogenase in barley. *Plant Cell Physiol.* **42**, 1088–1092

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

67. Charrier, A., Rippa, S., Yu, A., Nguyen, P. J., Renou, J. P., and Perrin, Y. (2012) The effect of carnitine on *Arabidopsis* development and recovery in salt stress conditions. *Planta* **235**, 123–135

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**ALDH10 Family from Tomato and Maize**