The roles of aldehyde dehydrogenases (ALDHs) in acetyl-CoA biosynthesis and root elongation in Arabidopsis

by

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CHAPTER 1. GENERAL INTRODUCTION

Introduction

Aldehydes are usually deleterious to biological systems and can be oxidized by aldehyde dehydrogenases (ALDHs, EC 1.2.1) into carboxylic acids. Over 550 ALDH genes have been identified across virtually all species, and those from eukaryotes were classified into more than 20 families (Sophos and Vasiliou, 2003). Family 2 ALDHs are mitochondrial or cytosolic homotetrameric enzymes. Their physiological functions remain to be addressed. Studies from maize and rice (Liu and Schnable, 2002; Nakazono et al., 2000; Tsuji et al., 2003) suggested a role of Family 2 ALDHs during ethanolic fermentation, which is catalyzed by pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) and generates acetaldehyde and ethanol. The detoxification of acetaldehyde by ALDH produces acetate, which can serve as the substrate for acetyl-CoA synthetase (ACS) to synthesize acetyl-CoA.

In yeast, the PDC-ALDH-ACS pathway is termed the pyruvate dehydrogenase (PDH) bypass (Boubekeur et al., 2001). Studies conducted in the Kuhlemeier lab (Mellema et al., 2002; op den Camp and Kuhlemeier, 1997; Tadege and Kuhlemeier, 1997) demonstrated that ethanol could be used for lipid production in tobacco pollen and thus suggested, but did not prove, the presence of PDC-ALDH-ACS pathway for generating acetyl-CoA to be used in *de novo* fatty acid synthesis in plastids. Genetic evidence is needed to establish the presence of the PDH bypass in plants. This study uses a reverse genetics approach to compare the incorporation rates of ^{14}C-ethanol into fatty acids in *aldh* mutants versus their wild type controls. It thereby provides the first

direct evidence for the presence the PDH bypass in plants by showing lower incorporation rates in *aldh* mutants as compared to wild type.

Additionally, by demonstrating that the *aldh2B4;aldh2B7* double mutant grew shorter roots than wild type, this study expands our understanding of the physiological functions of Family 2 ALDHs in plants.

Dissertation organization

This dissertation contains a manuscript prepared for journal publication (Chapter 2) and an Appendix. They were all written by Yanling Wei with the advice of Dr. Schnable's.

Chapter 2 describes ^{14}C-ethanol feeding experiments that provide direct genetic evidence for the presence of PDH bypass in plants, and a novel finding that mitochondrial Family 2 ALDHs play a role in root elongation. The ^{14}C-ethanol feeding experiments were conducted in Dr. David Oliver's lab under his supervision and with Dr. Ming Lin's invaluable help. Most of the other experimental work was performed by Yanling Wei. The process of screening for T-DNA insertion lines from the University of Wisconsin was, however, already well underway before Yanling Wei joined the project.

The Appendix serves as a summary of results screening T-DNA insertion lines for the PDC genes in Arabidopsis.

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CHAPTER 2. THE ROLES OF ALDEHYDE DEHYDROGENASES (ALDHS) IN ACETYL-COA BIOSYNTHESIS AND ROOT ELONGATION IN ARABIDOPSIS

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Abstract

Aldehyde dehydrogenases (ALDHs, EC 1.2.1) oxidize aldehydes into carboxylic acids, and thus are important in regulating the level of toxic aldehydes. ALDHs from eukaryotes were classified into more than 20 families. In mammals Family 2 ALDHs detoxify acetaldehyde and a similar function has been suggested in plants. Specifically, it has been hypothesized that plant Family 2 ALDHs oxidize acetaldehyde generated via ethanolic fermentation and produce acetate for acetyl-CoA biosynthesis through acetyl-CoA synthetase (ACS) in plastids, similar to the yeast pathway termed the "pyruvate dehydrogenase (PDH) bypass". *Arabidopsis. thaliana* contains three Family 2 ALDHs, two of which are mitochondrial and the other cytosolic. To test for the presence of the PDH bypass in plants, plants homozygous for T-DNA insertion alleles of the three encoding genes were fed with ^{14}C-ethanol along with wild type controls. The comparisons between the mutant and wild type in their incorporation rates of ^{14}C-ethanol into fatty acids provided direct evidence for the presence of PDH bypass in plants, which may only involve one of the mitochondrial Family 2 ALDHs rather than the other two. Although none of the single, double or triple mutants exhibited novel phenotypes when grown on soil, seedlings of the double mitochondrial *aldh* mutants grew shorter roots than wild type on MS media, suggesting a role for mitochondrial Family 2 ALDHs during root elongation.

Introduction

Aldehydes vary in length and in characteristics of their alkyl chains and are all usually deleterious to biological systems due to their chemical reactivity. Aldehyde dehydrogenases (ALDHs, EC 1.2.1) oxidize aldehydes into carboxylic acids, using NAD^+ or $NADP^+$ as a co-factor. As such ALDHs play an important role in detoxifying aldehydes that are generated endogenously or introduced from the environment. ALDHs are very diverse in that some only use either NAD^+ or $NADP^+$ as the co-factor, while others can use both, some oxidize only a limited number of aldehydes, while others have broader substrate spectra, and ALDHs exist in various subcellular compartments, including the cytosol, mitochondria, plastids and microsomes.

Over 550 ALDH genes have been identified across virtually all species, and those from eukaryotes were classified into more than 20 families (Sophos and Vasiliou, 2003). Family 2 ALDHs are mitochondrial or cytosolic homotetrameric enzymes. A human mitochondrial Family 2 ALDH, ALDH2, has been well studied and it detoxifies acetaldehyde generated via alcohol intake (Li et al., 2004). Family 2 ALDHs in plants have gained attention since the cloning of *rf2a* gene, a nuclear restorer gene for cytoplasm male sterility in maize, which encodes a mitochondrial Family 2 ALDH, RF2A (Cui et al., 1996). Although the molecular mechanisms associated with the restorer function of *rf2a* gene remains to be resolved, substantial studies on plant Family 2 ALDHs, particularly in maize, have provided clues as to the physiological functions of Family 2 ALDHs in plants.

Consistent with the physiological function of human Family 2 ALDH in detoxifying acetaldehyde, Liu and Schnable (2002) demonstrated that acetaldehyde is one

of the best substrates *in vitro* for RF2A, based on the ratio of K_{cat} to K_m. In addition, one of the mitochondrial Family 2 ALDHs in rice may be responsible for efficient detoxification of acetaldehyde during re-aeration after submergence of rice plants (Nakazono et al., 2000; Tsuji et al., 2003). These studies all suggest a role of Family 2 ALDHs during ethanolic fermentation, which is catalyzed by pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) and generates acetaldehyde and ethanol. The detoxification of acetaldehyde by ALDH produces acetate. In yeast and mammals, it has been established that acetyl-CoA synthetase (ACS) can utilize acetate to synthesize acetyl-CoA, both in the mitochondria (Klein and Jahnke, 1979; Yamashita et al., 2002) and in the cytosol (Akamatsu et al., 2000; Loikkanen et al., 2002).

The *Arabidopsis. thaliana* ACS is targeted to the plastid and is encoded by a single gene (Behal et al., 2002; Ke et al., 2000; Lin et al., 2003). In plastids acetyl-CoA is utilized for *de novo* fatty acid biosynthesis. Although the acetyl-CoA pool generated by ACS from acetate seems redundant for fatty acid biosynthesis, ACS is hypothesized to play a specialized role in certain cells and tissues (Behal et al., 2002; Ke et al., 2000). Besides, the redundancy of acetyl-CoA pool from ACS observed above might be due to the low concentration of acetate, probably 0.05 mM (less than one third the *Km* of this enzyme) (Bao et al., 2000; Behal et al., 2002). Indeed, the feeding of radio-labeled acetate indicates that isolated plastids can use exogenous acetate for fatty acid synthesis (Kang and Rawsthorne, 1994). To understand how and when plant ACS contributes to the acetyl-CoA pool in plastids, the study of acetate biosynthesis would be the key, which is readily diffusible across membranes. One potential pathway would be through

ALDH utilizing acetaldehyde generated via ethanolic fermentation by PDC from pyruvate or by ADH from ethanol.

In yeast, the PDC-ALDH-ACS pathway is termed the pyruvate dehydrogenase (PDH) bypass and used to generate acetyl-CoA involving both cytosolic and mitochondrial ALDHs (Boubekeur et al., 2001), predominantly when PDH is mutated (Pronk et al., 1994). In plants, studies conducted in the Kuhlemeier lab have demonstrated that high expression levels of PDC (Tadege and Kuhlemeier, 1997) and ALDH (op den Camp and Kuhlemeier, 1997) coincide with high rates of ethanolic fermentation in tobacco pollen (Tadege and Kuhlemeier, 1997). They supplied growing pollen tubes with ^{14}C-ethanol and found part of the label was incorporated into fatty acids, indicating that ethanol can be used for fatty acid biosynthesis (Mellema et al., 2002), presumably via the ADH-ALDH-ACS pathway. In order to provide direct evidence for the presence of PDH bypass in plants, this study utilizes a reverse genetics approach in Arabidopsis to prove the involvement of the ALDHs during the flux from ethanol into fatty acids.

There are three Family 2 ALDHs in Arabidopsis (Kirch et al., 2004), one of which, ALDH2C4, is localized to the cytosol. One physiological function of ALDH2C4 is the production of ferulic acid and sinapic acid during lignin biosynthesis (Nair et al., 2004). The other two ALDHs, ALDH2B4 (Millar et al., 2001; Heazlewood et al., 2004) and ALDH2B7 (Skibbe et al., 2002), are targeted to mitochondria. Their physiological functions have not yet been described.

In addition to demonstrating the presence of the PDH bypass in plants, this study expands our understanding of the physiological functions of Family 2 ALDHs in plants.

Results

Identification of null mutants of the three Family 2 ALDH genes

aldh2B4-1 and *aldh2B7-1* T-DNA knockout lines were identified from the Arabidopsis Knockout Facility at the University of Wisconsin. *aldh2C4-1, aldh2C4-2,* and *aldh2B4-2* T-DNA knockout lines were identified from the Salk Institute T-DNA insertion library database.

PCR screening of plants from each line with a T-DNA left border primer coupled with a gene specific primer, either upstream or downstream of the insertion, revealed the structures and insertion sites of the causative T-DNA insertions (Table 1).

All of the above lines are null in that they do not accumulate transcripts detectable by RT- PCR applied to RNA isolated from whole plants, while wild type control give detectable transcripts (Figure 1).

Direct evidence for the presence of PDH bypass for acetyl-CoA biosynthesis

An ideal experiment to directly test for the presence in plants of the PDH bypass (the PDC-ALDH-ACS pathway) would be to compare the differences between wild type and mutants of involved genes in the incorporation of ^{14}C-pyruvate or ^{14}C-acetaldehyde into acetyl-CoA. However, because pyruvate is unstable and acetaldehyde is toxic, ^{14}C-ethanol was used instead, which should be predominantly, if not all, oxidized to acetaldehyde via ADH. Because there are not established means to detect acetyl-CoA, flux through the PDH bypass was measured by determining the incorporation of ^{14}C-ethanol in extracted saponifiable lipids, which should consist primarily of fatty acids (Behal et al., 2002).

^{14}C-ethanol was fed to two to three tissues: whole seedlings, seedling leaves, and inflorescences. The incorporation rates were compared between single or double *aldh* mutants and their wild type siblings. No differences were detected in two examined tissues (whole seedlings or seedling leaves) between the *aldh2C4* homozygous mutants from either allele (*aldh2C4-1* and *aldh2C4-2*) and their wild type siblings. We, therefore, conclude that the cytosolic Family 2 ALDH is either not involved in the ADH-ALDH-ACS pathway, or can be compensated for by the mitochondrial paralog(s).

Comparisons were also made on all of the three tissues among *aldh2B4* single mutant (*aldh2B4;ALDH2B7*), *aldh2B7* single mutant (*ALDH2B4;aldh2B7*), *aldh2B4;aldh2B7* double mutant and their wild type siblings (*ALDH2B4;ALDH2B7*), that are in a WS uniform genetic background (Methods).

Comparisons on whole seedlings and inflorescences (Figure 2) showed that the *aldh2B4* single mutant and the *aldh2B4;aldh2B7* double mutant both exhibited lower incorporation rates of ^{14}C-ethanol into fatty acids than their wild type controls. In contrast the rate of incorporation for the *aldh2B7* single mutant did not differ from wild type controls. No differences in rate of incorporation were observed in seedling leaves among any examined genotypes (data not shown).

Similar results were obtained when comparing Columbia wild type plants to the single and double mutant plants that had a mixed Columbia and WS background (Methods) (data not shown).

Expression analyses of Family 2 ALDH genes

To understand the expression patterns of the three Family 2 ALDH genes, quantitative Real-Time PCR (qRT-PCR) was conducted on RNA extracted from different

tissues from adult wild-type WS plants. These analyses indicate that *ALDH2B4* and *ALDH2C4* have similar expression patterns, i.e. they are both constitutively expressed in roots, rosette leaves, stems, cauline leaves, flowers and green siliques, with varying levels across tissues; *ALDH2B7* has a different expression pattern, i.e., it is predominantly expressed in flower buds compared to other tissues (Figure 3). qRT-PCR from whole wild-type plants with three to four primer pairs for each gene showed that in both Columbia and WS ecotypes, *ALDH2B4* has a much higher expression level than *ALDH2C4* and *ALDH2B7,* with *ALDH2B7* the lowest (Figure 4).

Mitochondrial Family 2 ALDH double mutant grow shorter roots than wild type

There are no obvious phenotypes associated with any of the single, double or triple ALDH mutants when grown on soil. When planted on MS media, however, the double homozygous mutant (*aldh2B4;aldh2B7*) seedlings produced shorter roots than their wild type siblings (*ALDH2B4;ALDH2B7*). This comparison was conducted with F2:F3 plants that are in WS uniform background. Although not all families exhibited such deviations from the expected ratios, only a single double homozygous mutant was obtained after screening 144 F2 plants. Consequently, this comparison was made between progeny of the single F2 double homozygous mutant versus progeny of five of its wild type F2 siblings.

Each of the five wild type populations was paired with the double mutant population. Each pair was planted side by side on a same plate, with five plates as replicates for each comparison. Root length was measured for each individual six-day-old seedling and compared between the two genotypes. Statistical analyses showed that each wild type population had longer roots than the double mutant (Table 3).

Similar experiments were performed to compare all of the four genotypes, *aldh2B4* single mutant (*aldh2B4;ALDH2B7*), *aldh2B7* single mutant (*ALDH2B4;aldh2B7*), *aldh2B4;aldh2B7* double mutant and their wild type siblings (*ALDH2B4;ALDH2B7*) from a F2 segregation population obtained by selfing a F1 plant heterozygous for both genes. Three populations all gave a least squares mean order of wild type or *aldh2B7* single mutant, *aldh2B4* single mutant, and double mutant (long to short). The comparison between the double mutant and each of the other three genotypes was statistically significant but that among the other three genotypes was not. Therefore, we do not have evidence to distinguish the performance of the two single mutants.

Similar results were obtained when comparing *aldh2B4;aldh2B7* double mutant plants in a mixed Columbia and WS genetic backgrounds to wild-type Columbia plants (data not shown).

Discussion

The presence of PDH bypass for acetyl-CoA biosynthesis in Arabidopsis

The PDC-ALDH-ACS pathway is termed the PDH bypass in yeast (Boubekeur et al., 2001). Studies conducted in the Kuhlemeier lab (Mellema et al., 2002; op den Camp and Kuhlemeier, 1997; Tadege and Kuhlemeier, 1997) demonstrated that ethanol could be used for fatty acid biosynthesis in tobacco pollen and thus suggested, but did not prove, the presence of PDC-ALDH-ACS pathway for generating acetyl-CoA to be used in *de novo* fatty acid synthesis. Genetic evidence is needed to establish the presence of the PDH bypass in plants. This study uses a reverse genetics approach to compare the incorporation rates of ^{14}C-ethanol into fatty acids in *aldh* mutants versus their wild type

controls. It thereby provides the first direct evidence for the presence the PDH bypass in plants by showing lower incorporation rates in the *aldh2B4* single mutant and *aldh2B4;aldh2B7* double mutant as compared to wild type.

The *aldh2C4* and *aldh2B7* single mutants did not show differences relative to wild type. Because the ALDH2C4 and ALDH2B7 can both oxidize acetaldehyde, at least *in vitro* (Skibbe et al., 2002), this result suggests either the independence of ALDH2C4 and ALDH2B7 from the PDH bypass or that they are involved in the PDH bypass but their absence can be compensated for by the presence of ALDH2B4. This would be consistent with their much lower expression levels relative to *ALDH2B4* (Figure 3 and 4).

Our study established the presence of the PDH bypass in whole seedlings and inflorescences of plants at least under our experimental conditions. This suggests that the PDH bypass is not specifically present in pollen (Mellema et al., 2002) and may be functional in other tissues. This is supported by our qRT-PCR results, which show constitutive expression of the involved gene *ALDH2B4*. The reverse transcriptase PCR assay from the Kuhlemeier lab (op den Camp and Kuhlemeier, 1997), which was qualitative, also detected mitochondrial Family 2 ALDH expression in various tissues of tobacco. However, their quantitative Northern blot analyses gave different results than ours. While we are consistent in showing the high ALDH expression levels in stems, theirs did not detect expression of either *TobAldh2A* or *TobAldh2B* in leaf, while ours showed high *ALDH2B4* expression levels in both rosette leaves and cauline leaves comparable to stems. Nevertheless, the expression patterns of *TobAldh2A* and *TobAldh2B* look more similar to that of *ALDH2B7*, which is predominantly expressed in

flower buds that contain developing pollen. Despite the comparisons made above, due to the lack of full length sequence of *TobAldh2B* and thus lack of phylogenetic relationships among these genes, plus potential differences in sensitivity between the two different techniques, it is hard to make a conclusion yet.

Physiological functions of mitochondrial Family 2 ALDHs in Arabidopsis

Although there are no obvious phenotypes associated with any of the single, double or triple Family 2 ALDH mutants when grown on soil, *aldh2B4;aldh2B7* double mutant seedlings grown on MS media produce shorter roots than do similarly treated wild type siblings. This phenotype was observed on double mutants from either of two different *aldh2B4* mutant alleles in combination with a single *aldh2B7* mutant allele. It was also observed in two genetic backgrounds: a uniform WS genetic background and a mixed Col / WS background. Because we did not get evidence of single gene effect when comparing the single mutant versus the wild type, we conclude that both of the mitochondrial Family 2 ALDHs are involved in root elongation.

Because root elongation requires fatty acids for the synthesis of cell membranes, we first hypothesized that the *aldh2B4;aldh2B7* double mutant grew shorter roots because of the disruption of the PDH bypass caused by the absence of mitochondrial Family 2 ALDHs. To test this hypothesis ^{14}C-ethanol feeding studies were conducted on roots, but in preliminary experiments differences in incorporation rates were not observed among genotypes (data not shown). One possible explanation for this result is that the elongation zone is too small a portion of the whole roots to impact incorporation rates. However, the inconsistency between the ^{14}C-ethanol feeding and the root length comparison experiments, i.e. the previous suggests *ALDH2B4* single gene effect while

the latter does not have evidence for that, suggests a possibility that the role of mitochondrial Family 2 ALDHs in root elongation is not due to their involvement in the PDH bypass. This is supported by the usually broad substrate spectra of Family 2 ALDHs (Liu and Schnable, 2002; Skibbe et al., 2002). Consequently, the mitochondrial Family 2 ALDHs may contribute to root elongation via some other pathway than the PDH bypass.

Materials and Methods

Growth of plants

Wild type and T-DNA insertion lines of Arabidopsis seeds were planted in soil or on standard MS media (Murashige and Skoog, 1962) with addition of 1% sucrose after surface-sterilization with 50% bleach plus 0.01% triton X-100. After planting, they were allowed to imbibe for 2-4 days at 4°C before transfer to 24 hour light conditions in a growth chamber at 22°C under a light intensity of 110 ± 5 μmol m^{-2} S^{-1}.

Identification and Genotyping of T-DNA knockout lines

aldh2B4-1 (CSJ2971) and *aldh2B7-1* (CSJ989) T-DNA knockout lines were identified in association with the Arabidopsis Knockout Facility at the University of Wisconsin following their standard procedures (http//:www.biotech.wisc.edu/arabidopsis). *aldh2C4-1* (SALK_027911), *aldh2C4-2* (SALK_024974), and *aldh2B4-2* (SALK_078568) T-DNA knockout lines were identified in the Salk Institute T-DNA insertion library database (http://signal.salk.edu/cgi-bin/tdnaexpress) by BLAST searches.

The T-DNA left border primers JL202 (5'-CATTTTATAATAACGCTGCGGACATCTAC -3') and LBa1 (5'-

TGGTTCACGTAGTGGGCCATCG -3') were used in combination with gene specific primers to genotype lines from the University of Wisconsin and from the SALK Institute, respectively (Table 1). LBa1 was used instead of LBb1 (see SALK website) because under our PCR conditions LBb1 can self amplify a band of ~450 bp from Columbia and WS wild type plants. (This has already been included in the FAQs on SALK website. Seems that many people have the same issue. Should we still include that in the manuscript?)

Generation of double and triple mutants

To obtain double mutants, crosses were made between plants homozygous for different single mutants to generate a plant that was heterozygous for both. Progeny from this plant were then genotyped. The *aldh2B4;aldh2B7* double mutant in the uniform WS genetic background carried the *aldh2B4-1* and *aldh2B7-1* alleles, and that in a mixed Columbia and WS background carried the *aldh2B4-2* and *aldh2B7-1* alleles. The two types of triple mutants carried the *aldh2B4-1, aldh2B7-1* and one of the two *aldh2C4* alleles.

Polymerase Chain Reaction (PCR)

Each PCR reaction included 0.2 mM dNTP, 2.0 mM $MgCl_2$, 0.5 µM of each primer, and *Taq* polymerase in a total volume of 20 µl. PCR reactions were conducted for 32 cycles, with each cycle conducted at 94° for 30 sec, followed by the appropriate annealing temperature for 45 sec, and then extended at 72° for 1 min.

^{14}C feeding and isolation of fatty acids

An intact seedling removed from the soil with roots cleaned using a paper towel, one seedling leaf, or one apical portion of the inflorescence, was weighed, and placed into a 1.5 ml eppendorf tube containing 100 µl of carrier (20 mM ethanol) plus 1 µCi of ^{14}C-ethanol (Sigma product # 312975). Tubes were incubated in a growth chamber with the lid open. After four hours plant tissues were dried with a paper towel and placed into 1 ml Hexane:Isopropanol (3:2) solution for short-term storage if needed. Fatty acids were isolated using the protocol of Behal et al. (2002).

Tissue collection, RNA isolation and Reverse-Transcriptase PCR

Tissues were collected from one month old adult plants growing on soil in the growth chamber as described above, harvested in the following order: green siliques, open flowers, flower buds, cauline leaves, stems, rosette leaves and roots (washed by water and dried with paper towel). After harvest, samples were immediately submerged in liquid nitrogen and stored at –80°C until RNA isolation was performed.

Tissues were ground with a mortar and pestle in liquid nitrogen, and RNA was isolated with a modified "acid guanidinium thiocyanate-phenol-chloroform extraction" method (Chomczynski and Sacchi, 1987; Puissant and Houdebine, 1990) as described in Huang et al. (2005), except for a slightly different recipe for Trizol (38% phenol equilibrated pH 4.3, 1M guanidine thiocyanate, 1M ammonium thiocyanate, 0.1M sodium acetate pH5, 5% glycerol).

First strand cDNA was synthesized with poly dT primer using SuperScript II RNase H Reverse Transcriptase (Cat. No. 18064-014, Invitrogen, CA).

Two microliters of the first strand cDNA were used for PCR to test the transcription of the T-DNA insertion alleles. All primer pairs flank at least one intron.

The following pair of primers was used for *aldh2C4-1* and *aldh2C4-2*: 5'-AACTTCTCCACAACCTTATCGTAT -3' (forward) and 5'-ACGGAGCCACGACGGTGAAGTTAC -3' (reverse).

The following pair of primers was used for *aldh2B4-1* and *aldh2B4-2*: 5'-CTACTGGATGTGCCTGAAGCATC -3' (forward) and 5'-CATGAGTCTTTAGAGAACCCAAAG -3' (reverse).

For *aldh2B7-1*, the primer sequences are 5'- AGTACCAATGCTTGCTAGGG -3' (forward) and 5'- AGCTTGTAATGTGGCTCCAG -3' (reverse).

The primer sequences used for the positive control actin2 (Accession number U37281) are the same as used by Sunkar et al. (2003).

Quantitative Real-Time PCR

Procedures similar to those described in Swanson-Wagner et al. (2006) were used. The criteria for designing primers (Table 2) using Primer 3 (Rozen and Skaletsky, 2000) were as follows: Tm, 58°C to 61°C, no difference >2°C between the primers in a pair; primer length, 19–24 bp; GC content, 45–55%; amplicon length, 100–200 bp. Only primers yielding a single product in conventional PCR and qRT-PCR were used. qRT-PCR was conducted by using an Mx4000 multiplex quantitative PCR system (Stratagene). A human gene (GenBank accession no. AA418251) was spiked into each reaction as an external reference for data normalization.

qRT-PCR data were initially analyzed by using MX4000 analysis software. Ct values for each target gene and reference gene were calculated by using baseline-

corrected, ROX-normalized parameters. Three technical replicates were included in each plate, and the average Ct value for each gene of interest was normalized within a plate to the human reference gene by computing 2^ΔCt (reference – target) (Livak and Schmittgen, 2001) to indicate the relative amount of expression level compared to the reference gene. This was normalized again by the amount difference of starting RNA. The 2^ΔCt (reference – target) values from three biological replicates were used to calculate standard errors.

Root length measurement and statistical analysis

Photographs were taken of the plants grown on MS media and Image J software (http://rsb.info.nih.gov/ij/) was used to measure root lengths.

The PROC GLM procedure of the SAS statistical software package was used to compare root length among genotypes. Plate was considered a random factor.

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Figure Legends

Figure 1. Mutants used in this study do not accumulate detectable levels of ALDH transcripts. RNA samples extracted from whole plants were subjected to RT-PCR using primers specific for the indicated genes (Methods). m/m and +/+ designate RNA samples from plants homozygous for the mutant and wild type alleles, respectively, of the indicated genes. The *actin2* gene serves as a positive control for RNA quality.

Figure 2. The incorporation rate of ^{14}C-ethanol into fatty acids in whole seedlings (A) and inflorescences (B). Reported values are based on the averages of four biological replicates. Each replicate consisted of the indicated tissue from an individual plant.

Figure 3. Expression patterns of Family 2 ALDH genes in Arabidopsis across different tissues. Three biological replicates were included in the experiment for each tissue, each replicate from an individual plant.

Figure 4. Transcript levels of the three Family 2 ALDH genes in Arabidopsis whole adult plants of Columbia and WS wild type. The standard errors indicated by the error bars were calculated by taking three or four primer pairs as replicates for each gene, four for *ALDH2C4*, four for *ALDH2B4* and 3 for *ALDH2B7* (Table 2).

Table 1 Gene specific primers used along with T-DNA left border primer[1] for genotyping by PCR

	T-DNA location	Gene Specific Primers		Insertion feature[2]
		Forward	Reverse	
aldh2C4-1 (SALK_027911)	Exon 3	5'-GCACAACTACTCA TTTTTTTCT-3'	5'-TTGCGGCTGCGGC TTGCATTATCT-3'	↔
aldh2C4-2 (SALK_024974)	Exon 5	5'-TTGATGCGGTTGA CGGTGGAAAAT-3'	5'-AACTTCTCCACAA CCTTATCGTAT-3'	←
aldh2B4-1 (CSJ2971)	Intron 7	5'-GTTGGTCCTGCTC TTGCTTGTGGTAA-3'	5'-TCGTTCGCCCTCT TTATCACCTCATC-3'	→
aldh2B4-2 (SALK_078568)	Intron 1	5'-ATTCAAAGTACGG CAACACAAACCAAG AG-3'	5'-TTACCACAAGCAA GAGCAGGACCAAC-3'	↔
aldh2B7-1 (CSJ989)	Exon 7	5'-TTGAGACTTGGGA TAATGGGAAACCT-3'	5'-AAGAAAACTGTG ACGGTAATAATCGG-3'	←

To determine the insertion sites, the PCR products were sequenced. As a consequence of the imprecision of T-DNA integration (Mayerhofer et al., 1991) only exon / intron positions rather than exact insert sites are provided. [1] JL202 was used for lines from the University of Wisconsin and LBa1 for those from the SALK institute. [2] Two arrows facing against each other indicates that PCR was positive from the T-DNA left border primer in combination with both of the forward and reverse gene-specific primers. A single left facing arrow indicates that PCR was positive only when the T-DNA left border primer was paired with the forward primer. A single right facing arrow indicates that PCR was positive only when the T-DNA left border primer was paired with the reverse primer.

Table 2 Gene specific primers used for qRT-PCR

	Forward	Reverse
ALDH2C4	5’-GATCAACACGGTTTCGAGGT-3’	5’-GCATAACGACGGATTTGGTT-3’
	5’-GATCAACACGGTTTCGAGGT-3’	5’-ACATCCAAGGGGAATTGTGA-3’
	5’-GAACCAATTGGAGTGGTTGG-3’	5’-GTTGAGCACACCATCAGGAAT-3’
	5’-GAACCAATTGGAGTGGTTGG-3’	5’-CCGCTTCTTTTGAGAGATGG-3’
ALDH2B4	5’-CTTTTTCAGCTTCCTCTCCC-3’	5’-TGATGAGGAGCTGTGTGTGAG-3’
	5’-TGGACAGATCATACCGTGGA-3’	5’-GCATAGAAAGCCGTGAGAGG-3’
	5’-TGGACAGATCATACCGTGGA-3’	5’-AGACCCGCTTCAAGGAAAAG-3’
	5’-AACAGGGTTTCAAGGGCTTT-3’	5’-GTGACGACTGCCTTGATCTG-3’
ALDH2B7	5’-ACCAGCTTTAGCTTGCGGTA-3’	5’-TAGCCCCAAATCCAGAAACT-3’
	5’-CGCTCTTTCATGTCCTCCTC-3’	5’-CAACGAATCTTCCACCGATT-3’
	5’-GGTACGGTTTAGCTGCTGGA-3’	5’-CCCTCCAAATGGAATTGATG-3’

Table 3 Root length comparisons between the progeny of an *aldh2B4;aldh2B7* double mutant and that of five of its wild type siblings

Genotype pair number	*ALDH2B4; ALDH2B7*	*aldh2B4; aldh2B7*	p value
1	1.4 ± 0.1	0.9 ± 0.1	< 0.0001
2	1.1 ± 0.1	0.7 ± 0.1	0.0002
3	1.5 ± 0.1	1.0 ± 0.1	< 0.0001
4	1.3 ± 0.1	0.9 ± 0.1	0.0002
5	1.4 ± 0.1	1.3 ± 0.1	0.0168

Each of the five wild type populations was paired with the double mutant population. Each pair was planted side by side on a same plate, with five plates as replicates for each comparison. Root length (cm) was obtained for 4-6 six-day-old seedlings for each genotype on each plate. An average was calculated out of them as a plate mean and the average of the five plate means are provided as least squares means for each genotype in each pair.

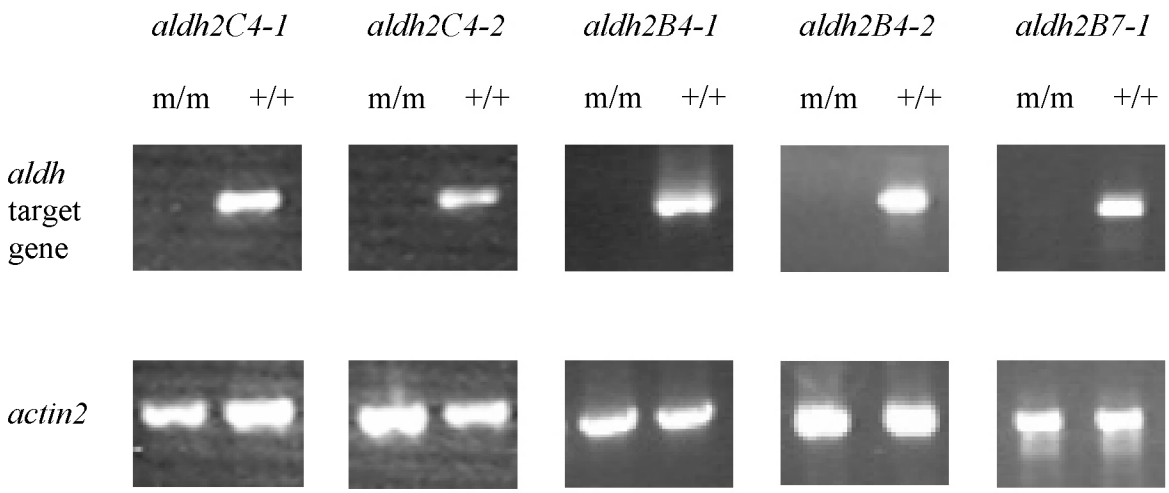

Figure 1 Wei et al.

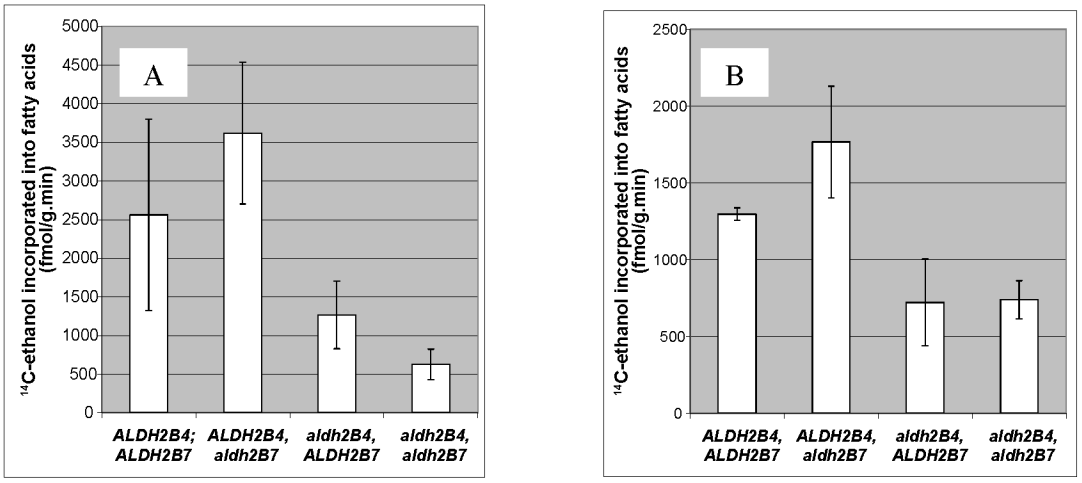

Figure 2 Wei et al

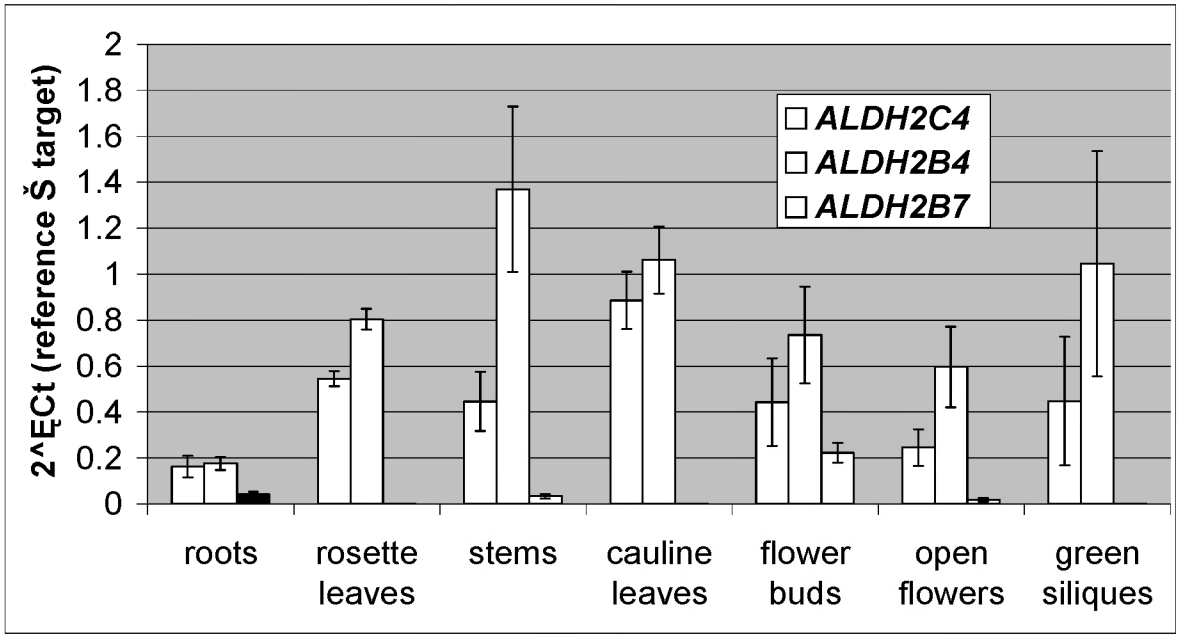

Figure 3 Wei et al.

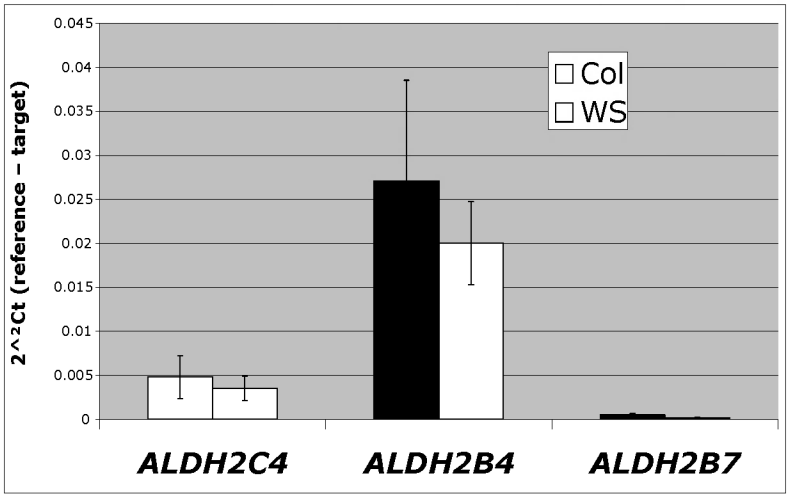

Figure 4 Wei et al.

Supplementary materials

Despite the differences in root length difference between *aldh2a, aldh2b* double mutant and their wild type siblings on MS media, there were no physiological defects associated with the double mutant either growing on MS media or soil. From an evolutionary point of view, the *ALDH* genes themselves must be important under special conditions. To determine the conditions under which *ALDH* genes are of particular importance, the *aldh2a, aldh2b* double mutant and their wild type siblings were subject to various stress treatments. However, we did not find any stress condition under which that the double mutant is different from the wild type control. For the purpose of possible follow up on this, treatment methods are provided as follow.

4×10^{-5} M Rose Bengal (for oxidative stress treatment), 10^{-5} M ABA, 20 mM acetaldehyde (after autoclaving) or 0.5% ethanol (after autoclaving) were added to MS media for various treatments. For cold treatment, plants were placed at 4°C in the dark for 24 hours. For heat treatment, plants were placed put at 40°C in the dark for 12 hours. For hypoxia treatments, plates were filled with water covering plants and incubated in a sterile hood for 6 hours covered with foil to prevent photosynthesis.

APPENDIX. THE MUTANT SCREENINGS FOR PYRUVATE DECARBOXYLASE (PDC) GENES IN ARABIDOPSIS

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The study in this thesis showed direct evidence for the presence of an acetyl-CoA biosynthesis pathway in plants, sequentially characterized by pyruvate decarboxylase (PDC), aldehyde dehydrogenase (ALDH), and acetyl-CoA synthetase (ACS). However, the physiological significance of this pathway remains to be further addressed, in order for which we tried to obtain Arabidopsis mutants in *PDC* genes also. There are four *PDC* genes in Arabidopsis, *PDC1*, *PDC2*, *PDC3* and *PDC4*. Although no thorough characterization has been applied on the mutants, this Appendix serves as a summary of the current progress on the mutant screenings.

pdc1-CSJ4016 and *pdc2-CSJ3667* T-DNA knockout lines were identified in association with the Arabidopsis Knockout Facility at the University of Wisconsin following their standard procedures (http//:www.biotech.wisc.edu/arabidopsis). *pdc1-SALK_018840, pdc1-SALK_090204, pdc2-SALK_053097, pdc2-SALK_066678, pdc3-SALK_087974*, and *pdc3-SAIL_151_G05* T-DNA knockout lines were identified in the Salk Institute T-DNA insertion library database (http://signal.salk.edu/cgi-bin/tdnaexpress) by BLAST searches.

For genotyping the above lines, T-DNA left border primer JL202 (5'-CATTTTATAATAACGCTGCGGACATCTAC -3') was used in pair with gene specific primers for genotyping those lines from University of Wisconsin, LBa1 (5'-TGGTTCACGTAGTGGGCCATCG -3') for genotyping those from SALK institute, and LB1 (5'- TGGTTCACGTAGTGGGCCATCG -3') for the line from Syngenta Arabidopsis Insertion Library (SAIL), *pdc3-SAIL_151_G05* (Table 1).

Reverse-Transcriptase PCR applied on RNA isolated from whole plants revealed that *pdc1-CSJ4016, pdc1-SALK_018840, pdc1-SALK_090204* and *pdc2-CSJ3667* did not give detectable transcripts (Figure 1), while *pdc2-SALK_053097* gave wild type size transcripts. Other lines have not been determined yet.

Table 1 Gene specific primers used along with T-DNA left border primer[1] for genotyping

	T-DNA location	Forward Primer flanking T-DNA insertion	Reverse Primer flanking T-DNA insertion	Insertion feature[2]	Transcription characterization[3]
pdc1-CSJ4016	1 bp down-stream of 3' UTR	5'-GATTAAGA ACTGGAACT ACACTGGTC-3'	5'-CGTTCACT GTCAAAATC TCTCCTGTTT AA-3'	↔	Null
pdc1-SALK_018840	Exon 2	5'-TCCTTCCT CCGCTATCA CCATCAACT-3'	5'-TTCACACT TCAATGGCT TACCTTCAG-3'	↔	Null
pdc1-SALK_090204	Exon 2	5'-TCCTTCCT CCGCTATCA CCATCAACT-3'	5'-TTCACACT TCAATGGCT TACCTTCAG-3'	↔	Null
pdc1-SALK_125892	Exon 3	Same as *pdc1-SALK_018840*		→	ND
pdc1-SALK_128445	Intron 2	Same as *pdc1-SALK_018840*		↔	ND
pdc2-SALK_053097	~250 bp upstream of 5' UTR	5'-AATCTATA GTCAAATCC AAATCGTAA-3'	5'-AGGAACG GAGAAGACA TCGGTGACG C-3'	→	WT
pdc2-SALK_066678	~40 bp upstream of 5' UTR	Same as *pdc2-SALK_053097*		↔	ND
pdc2-CSJ3667	Exon	5'-TTTAGTCG TCATCCTGTT CCGTTCAT-3'	5'-AGTCCAG CATTTTCCTT CTCCATTGT-3'	↔	Null
pdc3-SALK_087974	Exon 3	5'-CGACAAG GCGATAGCA ACAGC-3'	5'-CCCACAT CCTTTAGGC AGCTT-3'	↔	ND
pdc3-SAIL_151_G05	~300 bp upstream of 5' UTR	5'-AAACGGA AAAACCCTA ATCGCCTAA A-3'	5'-CCCACATC CTTTAGGCA GCT T-3'	↔	ND

[1] JL202 was used for lines from the University of Wisconsin and LBa1 for those from the SALK institute.

[2] Two arrows facing against each other indicates that PCR was positive from the T-DNA left border primer using both of the forward and reverse gene-specific primers. A single left facing arrow indicates that PCR was positive only when the T-DNA left border primer was paired with the forward primer. A single right facing arrow indicates that PCR was positive only when the T-DNA left border primer was paired with the reverse primer.

[3] "Null" and "WT" indicate that homozygous mutant plants did not give detectable transcripts or gave wild type size transcripts, respectively, by Reverse-Transcriptase PCR. "ND" stands for "Not Determined".

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