Plant Molecular Biology **35:** 355–365, 1997.

Aldehyde dehydrogenase in tobacco pollen

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Received 23 January 1997; accepted in revised form 2 June 1997

Key words: aldehyde dehydrogenase, cytoplasmic male sterility, fermentation, glyoxylate cycle, pollen, tobacco

Abstract

Acetaldehyde is one of the intermediate products of ethanolic fermentation, which can be reduced to ethanol by alcohol dehydrogenase (ADH). Alternatively, acetaldehyde can be oxidized to acetate by aldehyde dehydrogenase (ALDH) and subsequently converted to acetyl-CoA by acetyl-CoA synthetase (ACS). To study the expression of ALDHs in plants we isolated and characterized a cDNA coding for a putative mitochondrial ALDH (*TobAldh2A*) in *Nicotiana tabacum*. TobALDH2A shows 54–60% identity at the amino acid level with other ALDHs and shows 76% identity with maize Rf2, a gene involved in restoration of male fertility in cms-T maize. *TobAldh2A* transcripts and protein were present at high levels in the male and female reproductive tissues. Expression in vegetative tissues was much lower and no induction by anaerobic incubation was observed. This suggests that TobALDH expression is not part of the anaerobic response, but may have another function. The use of specific inhibitors of ALDH and the pyruvate dehydrogenase (PDH) complex indicates that ALDH activity is important for pollen tube growth, and thus may have a function in biosynthesis or energy production.

Introduction

Aldehydes are highly reactive and long-lived molecules that may have a variety of effects on biological systems. Aldehydes cause their effects by reacting with cellular nucleophiles, including proteins and nucleic acids. They can be generated from a virtually unlimited number of endogenous (metabolism of amino acids, carbohydrates, vitamins and lipids) and exogenous (alcohol, smog and smoke) sources. Among the most effective pathways for aldehyde metabolism is their oxidation to carboxylic acids by aldehyde dehydrogenases (ALDH, EC 1.2.1.3). ALDHs are a family of NAD(P)-dependent enzymes with common structural and functional features that catalyze the oxidation of a broad spectrum of aliphatic and aromatic aldehydes. A vast literature exists on human ALDHS, which function in the detoxification pathway of dietary ethanol [16]. Subtle differences in levels and properties of ALDH are thought to be major determinants of susceptibility to ethanol-related diseases. Classes 1 and 3 contain both constitutively expressed and inducible cytosolic forms, whereas class 2 consists of constitutive mitochondrial enzymes [16].

In plants, four ALDHs have been reported. Three are betaine-aldehyde dehydrogenases (BADH, EC 1.2.1.8), cloned from spinach, sugar beet and barley [12, 19, 32]. Betaine, formed by the oxidation of betaine aldehyde by BADH, accumulates in response to salt stress or water deficit. Betaine acts as a nontoxic or protective cytoplasmic osmolyte, allowing normal metabolic function to continue [32]. The fourth putative ALDH is encoded by *Rf2*, a nuclear restorer gene of Texas cytoplasmic male sterility (cms-T) in maize. Cms-T is a maternally inherited trait that causes male sterility as a consequence of a degeneration of the tapetal layer of anthers. The sterility effect of cms-T is mediated by the mitochondrial gene *T-urf13*, encoding URF13, a polypeptide of 13 kDa that resides in the inner mitochondrial membrane. The selective degeneration of the tapetum is paradoxical because URF13

The nucleotide sequence data reported will appear in the GenBank and EMBL Nucleotide Sequence Databases under the accession number Y09876.

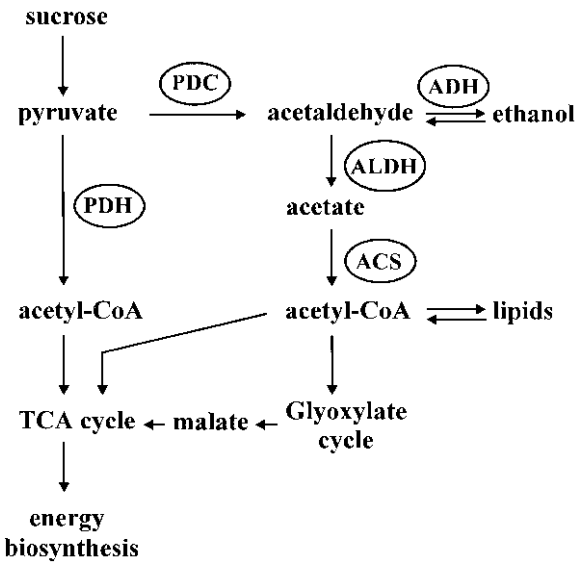

Figure 1. Proposed model for pyruvate utilization in pollen. Pyruvate can be directly converted to acetyl-CoA by PDH and enter the TCA cycle, or it can be converted to acetaldehyde by PDC. Acetaldehyde can be reduced to ethanol, or by the action of ALDH, it can be converted to acetate. This acetate can be used by ACS to produce additional acetyl-CoA. This acetyl-CoA can enter the TCA-cycle, be used in lipid biosynthesis or be used by the glyoxysomes to synthesize malate, which in turn can supplement the TCA cycle and enable biosynthetic reactions.

is expressed in many maize tissues. Tissue-specific degeneration could be explained if tissues differ in their requirements for mitochondrial function. Alternatively, it is possible that a tapetum-specific compound exists that is a prerequisite for URF13-induced toxicity [15]. The effects of URF13 can be reversed by the nuclear genes *Rf1* and *Rf2*. Rf1 reduces URF13 expression by nearly 80%, Rf2 does not affect URF13 expression. The sequence homology between Rf2 and ALDHs leads to the attractive hypothesis that acetaldehyde produced by the pollen interacts with URF13 to cause male sterility and that this defect can be alleviated by ALDH mediated detoxification of acetaldehyde [5].

During oxygen limitation in higher plants, energy metabolism switches from respiration to fermentation. In ethanolic fermentation, pyruvate is the substrate of pyruvate decarboxylase (PDC), yielding CO_2 and acetaldehyde. Subsequently, acetaldehyde is reduced to ethanol with the concomitant oxidation of NADH to NAD^+ by alcohol dehydrogenase (ADH). Ethanolic fermentation in leaves and roots is an adaptation to oxygen limitation, and acetaldehyde and ethanol only accumulate when respiration is inhibited. We previously showed that both genes are expressed at high levels in pollen, even under aerobic conditions [3]. Under optimal conditions for pollen tube growth, more than half of the carbon consumed is fermented, and ethanol accumulates in the surrounding medium to a concentration exceeding 100 mM. In pollen, the flux through the pathway is not regulated by oxygen limitation, but by carbohydrate levels. In the accompaning paper an additional function for the presence of PDC and ADH in pollen is proposed [29]. Acetaldehyde produced by PDC could be converted into acetyl-CoA by ALDH and acetyl-CoA synthetase (see Figure 1). The acetyl-CoA formed could be used by the glyoxylate cycle for biosynthesis of malate or for direct lipid biosynthesis. To investigate the validity of this model we isolated a cDNA encoding an aldehyde dehydrogenase from tobacco. We show that tobacco *Aldh* is not induced under anoxic conditions, but that it is highly expressed in pollen and pistil. A possible function of ALDH in pollen metabolic pathways is discussed.

Materials and methods

Plant growth conditions

Plants of *Nicotiana tabacum* cv. Samsun were grown in the greenhouse, under an 16:8 light/dark cycle and temperature of at least 18 °C. Mature pollen were harvested using a 35 μm mesh filter connected to a vacuum cleaner. Pollen was germinated in 25 mM MES-KOH pH 5.9, 0.3 M sucrose, 3.0 mM $Ca(NO_3)_2$, 1.6 mM H_3BO_3, 1.0 mM KNO_3, 0.8 mM $MgSO_4$, 30 μM $CuSO_4$ and 0.1% (w/v) caseine hydrolysate [30]. 1-aminoethylphosphinate (AEP), a specific inhibitor of PDH [26], was added to a final concentration of 90 μM. Disulfiram (dissolved in DMSO), an inhibitor of ALDH [13], was added to a final concentration of 30 μM.

Seeds were sterilized by washing for 2 min in 70% ethanol, 15 min in 1.3% sodium hypochlorite followed by three washes of sterile water. The seeds were germinated on MS medium (0.4%, Serva) pH 6.0, containing 1% sucrose and 0.7% bactoagar (Difco). AEP was added in a final concentration of 30 μM, disulfiram was added in a final concentration of 30 μM. Anaerobic incubations were performed as described in Bucher *et al.* [2].

Library screening and cDNA sequencing

Two cDNA libraries from pollen and leaf poly(A)$^+$ RNA [1] were used to screen at low stringency (5× SSC, 48 °C) for an *Aldh*. A total of 3×10^5 plaques from each library were screened. The insert of EST clone 51D7T7 from Arabidopsis Biological Resource Center at Ohio State [20] was used as a probe. One positive clone from the pollen library was identified and sequenced by the dideoxy chain termination method. Fragments derived from *Bal*31 exonuclease treatment of the positive clone were subcloned and internal primers were used for sequencing. Analysis of the DNA sequence and predicted amino acid sequence were performed using the GCG Sequence Analysis Software Package, version 8.0. The 5′/3′-RACE Kit from Boehringer Mannheim was used to isolate the 5′ end of the transcript, using three nested primers: 5′-AGGCCATGGTCCTTCGTC-3′ (408–391), 5′-GTTCCACATTGACAGCTGG-3′ (235–217) and 5′-GCTTATCCACAATGATC-3′ (145–129).

Southern blot analysis

High-molecular-weight total genomic DNA was isolated from young leaves of *N. tabacum* as described by [28]. The DNA (10 μg) was digested with *Eco*RI, *Hin*dIII or *Xba*I, electrophorezed in 0.7% agarose gels and transferred to Nytran-N (Schleicher & Schuell). Blotting and hybridization procedures were carried out under standard conditions [25]. Blots were hybridized at 65 °C with randomly labelled probes made from *TobAldh2A* and the final wash was in 0.1× SSC, 0.1% SDS at 65 °C.

Northern blot analysis

RNA was extracted from pollen and various tissues from *N. tabacum* essentially as described by Schrauwen *et al.* [27]. Total RNA was quantified both spectrophotometrically at 260 nm and visually by the staining of blots in 0.02% methylene blue in 0.3 M sodium acetate, pH 5.5. Excess dye was washed away with water. A 10 μg portion of total RNA of each sample was loaded onto a 1.0% agarose-glyoxal gel after glyoxylation. Northern blotting and hybridization procedures were carried out under standard conditions [25]. Blots were hybridized at 65 °C with randomly labelled probes from *TobAldh2A*, *TobAdh1* and *NeIF-4A10* and the final wash was in 0.1× SSC, 0.1% SDS at 65 °C.

Antibody generation and western blotting

A 1192 bp fragment of *TobALDH2A* (880–2071), containing the conserved regions from ALDHs,was cloned into the *Nde*I-*Bam*HI sites from pET-14B (Novagen), introducing a His-Tag sequence at the N-terminus of the ca. 36 kDa fragment. The translational fusion was introduced into the pLysS strain of *Escherichia coli*. The recombinant protein was purified from the inclusion bodies and purified on a His-Tag binding column according to the pET System Manual (Novagen), using solutions containing 6 M urea. The purified proteins were separated through a 12.5% SDS-PAGE gel and electroeluted. The antibodies were generated in rabbit as described before [2].

Total soluble proteins were isolated by grinding in liquid nitrogen and extracting in 100 mM Tris-Cl pH 8.0, 1 mM EDTA, 10% glycerol, 0.1% Triton X-100, 0.1% 2-mercaptoethanol, 0.2% PVP and 1% PVPP. A 30 μg portion of total protein was separated on a 10% SDS-PAGE gel and transferred to nitrocellulose (Schleicher & Schuell). A 1:1000 dilution of rabbit anti-TobALDH or rabbit anti-eIF4A antibody [21] and horseradish peroxidase conjugates were used to detect ALDH and eIF-4A proteins.

Expression of TobALDH2A *in* E. coli *and measurement of* in vitro *enzymatic activity*

A primer(5′-CTTCTAGACATATGTCAAGAGGTTTG ATCATTGTGG-3′, 104–139) was used to introduce a *Nde*I site in the 5′ region of the *TobAldh2A* cDNA by PCR. This introduces an ATG start site at nt 113 and changes Lys-21 (the last amino acid of the putative mitochondrial targeting sequence) into a methionine. The obtained fragment was cloned into the *Nde*I-*Bam*HI sites from pET-3A (Novagen). The translational fusion was introduced into the pLysS strain of *E. coli*. An overnight culture was diluted 10 times and induced with 0.4 mM IPTG. The bacteria were grown for three hours at 30 °C and harvested by centrifugation (5 min, $5000 \times g$). After washing in 100 mM HEPES-NaOH pH 7.4, the bacteria were sonicated in 100 mM HEPES-NaOH pH 7.4, 1 mM EDTA, 10% glycerol and 0.1% Triton X-100, centrifuged (5 min, $15\ 000 \times g$) and the clarified supernatant was used for activity tests. As a negative control, a translational fusion of eIF-4A2 in pET-3A was used. Aldehyde dehydrogenase activity was determined spectrophotometrically at 340 nm by the conversion of NAD into NADH in a buffer containing 100 mM sodium pyro-

phosphate pH 9.5, 1.3 mM NAD, 100 μM substrate and 100 μg total protein extracts [7, 35].

Reverse transcriptase-PCR

The reverse transcriptase and subsequent PCR reactions were performed as described by Fleming *et al.* [8]. The primers P1 (5′-GCTCTAGACTCGTGTGTTT ACCTCTCGTC-3′) spanning 62–82 (plus an additional 5′ *Xba*I restriction site) and P4 (5′-CGGATCCGACGTACAACCATTGGTAC-3′) spanning 566–541 (creating a *Bam*HI site by introducing 3 mismatches at the 3′ end) were used to amplify and subclone regions in the 5′ ends of *TobALDH2A* and *2B*, and subsequently sequenced. To show the presence of both transcripts in a total RNA extraction, a combination of primers P1 with P2 and P1 with P3 was used (see Figure 3A). P2 5′-GCTTATCCACAATGATC-3′ is *TobALDH2A* specific (145–129) and P3 5′-GTTCCACATTGACAGCTGG-3′ primes on both *TobALDH2A* and *2B* (235–217 on *TobALDH2A*).

Results

Isolation of a cDNA encoding an ALDH

Two cDNA libraries derived from poly(A)$^+$ RNA from mature pollen grains and leaves of *Nicotiana tabacum* were screened at low stringency with an *Arabidopsis* EST clone encoding a putative ALDH. No positive clones were found in the leaf library. From the pollen library, the clone with the strongest signal was further analyzed. This clone, termed *TobALDH2A*, was 2010 bp in length but at the 5′ end of the cDNA no in frame ATG was found and there was an open reading frame from the third nucleotide on. Using the 5′-RACE technique with three nested primers, we isolated an additional 61 bases with an in frame ATG at position 54. The predicted molecular mass of the encoded protein is 59.3 kDa and the protein contains a putative mitochondrial targeting sequence (amino acids 1–21) [9]. In agreement with the classification of ALDHs in which mitochondrial ALDHs are called class 2, we designated our clone *TobALDH2A*. The deduced amino acid sequence shows high identity throughout the sequence with ALDHs from different organisms. All the conserved amino acids characteristic for ALDHs are present in TobALDH 2A (Figure 2A): the catalytic site Val-Thr-Leu-Glu-Leu-Gly-Gly-Lys

A)

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TobALDH2A  AAKSNLKPVT LELGGKSPFI VCEDADIDTA VEQAHFALFF NQGQCCCAGS
Maize Rf2  AAKSNLKTVT LELGGKSPFI IMDDADVDHA VELAHFALFF NQGQCCCAGS
   Chicken AGKTNLKRVT LELGGKSPNI IFADADLDEA AEFAHIGLFY HQGQCCIAGS
     Human AGSSNLKRVT LELGGKSPNI IMSDADMDWA VEQAHFALFF NQGQCCCAGS
Aspergillus AAKSNLKKVT LELGGKSPNI VFDDADIDNA ISWANFGIFF NHGQCCCAGS
      BALD AAQ.LVKPVT LELGGKSPIV VFEDVDIDKV VEWTIFGCFW TNGQICSATS
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B)

	TobALDH	Rf2	Chicken	Human	Aspergillus
Maize Rf2	77				
Chicken	56	56			
Human	60	61	67		
Aspergillus	54	53	58	58	
Spinach BADH	42	41	41	43	41

Figure 2. Comparison of the amino acid sequences of ALDHs from different sources. The deduced amino acid sequence of the *TobAldh2A* was aligned with corresponding ALDH sequences from maize cms-T restorer gene *Rf2* [5], chicken [10], human mitochondrial [11], *Aspergillus* [22] and spinach betaine-aldehyde dehydrogenase [32]. A. Amino acid alignment showing the main conserved regions (grey background) of ALDHS. B. Amino acid identities (in %) between the different ALDH.

(amino acid position 306–313 in TobALDH2A) in which the glutamate is the most important amino acid [31], the Cys at position 343 as the active site [7] and the histidine residue at position 276 necessary for the correct folding of the native ALDH [35]. Ser-115 [24] and Glu-528 [6], both with a proposed function in NAD$^+$ binding, are not conserved in the ALDHs from tobacco, maize and *Aspergillus*. TobALDH 2A has the highest identity (77%) with maize Rf2 (Figure 2B).

Analysis of the number of TobAldh *genes*

RT-PCR reactions were performed using total RNA from pollen. The resulting cDNA fragments were cloned and sequenced. Out of 12 different reactions, two distinct cDNA fragments were isolated: the first matched the isolated *TobAldh2A* and a second one represented a new putative mitochondrial targeted gene which we designated *TobAldh2B* (Figure 3A). Within the 237 bp sequenced, *TobAldh2A* and *2B* differed in 29 nucleotides, including a gap of 18 nucleotides in *TobAldh2B*.

To determine whether both transcripts are present in various organs of the plant, RT-PCR was performed using two sets of primers. One set of primers (primer 1 and primer 2) specifically amplified *TobAldh2A* (Figure 3B) and a second set of primers (primer 1 and primer 3) amplified both *TobAldh2A* and *TobAldh2B*

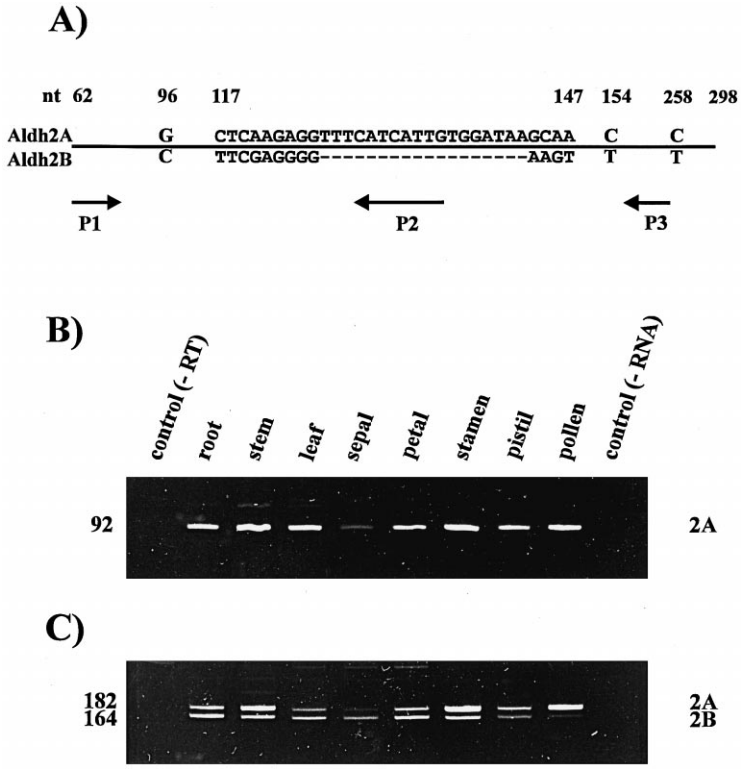

Figure 3. RT-PCR analysis reveals the presence of two *TobAldh* transcripts. A. Schematic drawing of the differences in nucleotide sequence between *TobAldh2A* and *TobAldh2B* (the number and differences in nucleotides (nt) are indicated). The A of the initiator ATG is nt 54. The arrows indicate the primers (see Materials and methods) used for the PCR reaction. B. and C. RT-PCR products using the primers P1 and P2 or P1 and P3, respectively. The sizes of the bands correspond to the predicted 84 bp and 162/180 bp, respectively. The DNA was separated on a 10% acrylamide-TBE gel.

(Figure 3C). As can be seen from Figure 3B and 3C, bands with the predicted sizes could be detected in all organs tested. We must point out that, because of the exponential nature of PCR, these results have to be interpreted qualitatively and are not indicators of the relative expression levels in the various organs (see below). Southern blot analysis of genomic DNA was performed at high stringency to determine the number of genes coding for *Aldh* (Figure 4). *Eco*RI cuts at the 3′ end, *Hin*dIII cuts in the middle and *Xba*I does not cut *TobAldh2A* cDNA. Analysis of the Southern blot reveals the presence of two to three *Aldh* genes, which is consistent with the presence of two different transcripts in tobacco.

Aldh *gene expression is not coordinated with* Adh *expression*

The previous experiments (Figure 3) demonstrated that tobacco contains at least two expressed *Aldh* genes, which is to be expected in alloploid tobacco. However, to determine quantitatively the level of *Aldh* gene expression northern blot analysis was performed. Northern blots were prepared with equal amounts of total RNA isolated from different tobacco organs and hybridized with a randomly labelled probe from *TobAldh2A*. Because the homology between *TobAldh2A* and *TobAldh2B* is very high we expect this probe to hybridize with both transcripts. In addition, an *Adh* cDNA probe was used as a control for the presence of transcripts encoding enzymes involved in ethanolic fermentation [3], and a housekeeping gene encoding the eucaryotic translation initiation factor 4A10 (NeIF-4A10) as a constitutive control. The *Aldh* genes are highly expressed in stamen, pistil and pollen (Figure 5). The signal in the stamens can probably be assigned to the presence of pollen in this organ. *Aldh* is expressed at a lower level in stem tissue. Comparison of *Aldh* and *Adh* shows a similar pattern of expression for both genes, except for the high expression of *Aldh* in pistil

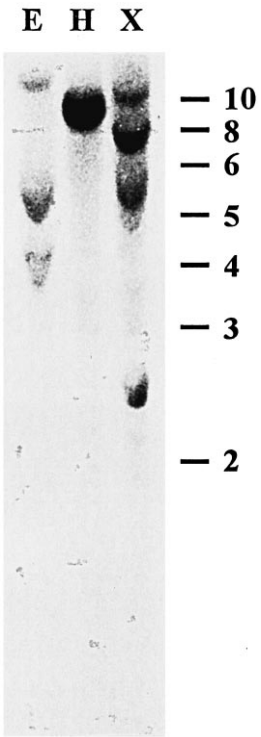

Figure 4. Genomic Southern blot analysis of *N. tabacum*. About 10 μg of genomic DNA was digested with *Eco*RI (E), *Hin*dIII (H) or *Xba*I (X) and size fractionated by electrophoresis through an 0.7% (w/v) agarose gel. The blot was hybridized with the cDNA insert of *TobAldh2A*. Migration positions of a 1 kb DNA ladder (Gibco-BRL) are indicated.

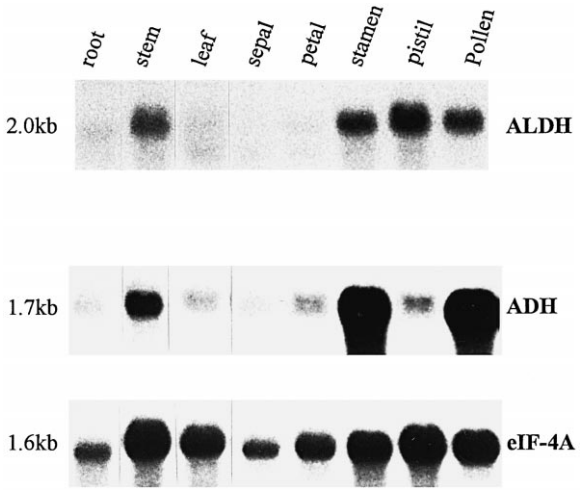

Figure 5. Northern blot analysis of *TobAldh* gene expression in various tissues. Total *N. tabacum* RNA (10 μg), isolated from the indicated organs, was separated on 1% (w/v) glyoxal gels. The blot was probed with the complete cDNA inserts from *TobAldh2A*, *TobAdh1* or *NeIF-4A10*. The approximate sizes of the hybridizing bands are shown in kb to the left.

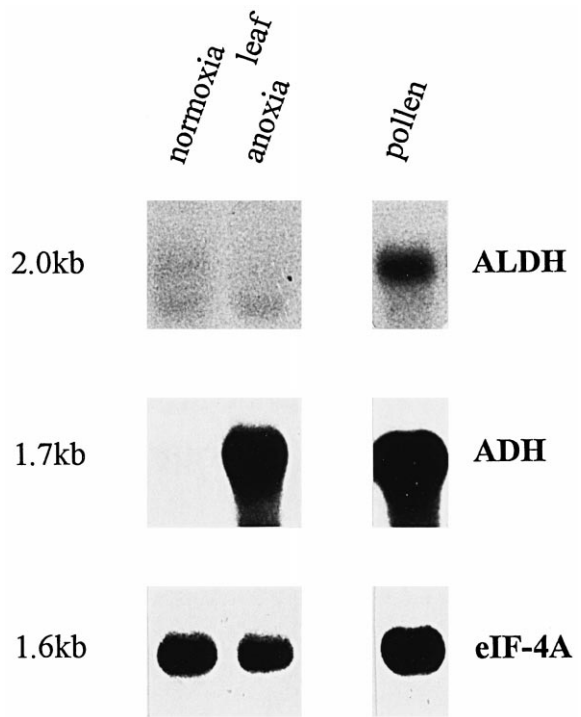

Figure 6. *TobAldh2A* is highly expressed in mature pollen and is not induced under anoxia in leaves. Ten micrograms of total RNA from leaves incubated under normoxic or anoxic conditions and from pollen was separated on 1% (w/v) glyoxal gels. The blot was probed with the complete cDNA insert from *TobAldh2A*, *TobAdh* or *NeIF-4A10*. The approximate sizes of the hybridizing bands are shown in kb to the left.

tissue. The quantitative differences of the expression of NeIF-4A10 in various tissues are not due to unequal loading, but are likely to reflect the relative transcriptional activity of this class of housekeeping genes (for discussion see [18 and 21]).

In leaves grown both under normal atmospheric conditions and in an anaerobic environment, *TobAldh2A* was expressed at a very low level, but mRNA levels were high in pollen (Figure 6), in contrast with *Adh* which is also highly expressed in pollen, but whose expression is drastically increased in leaves during anaerobic incubation. Thus, *TobAldh2A* is not coordinately expressed with the other genes involved in ethanolic fermentation. *TobAldh2A* is highly expressed in pollen, as are *Pdc* and *Adh*, but, unlike *Pdc* and *Adh*, *TobAldh2A* transcript levels are high in pistil, and not increased during anaerobiosis in leaf tissue.

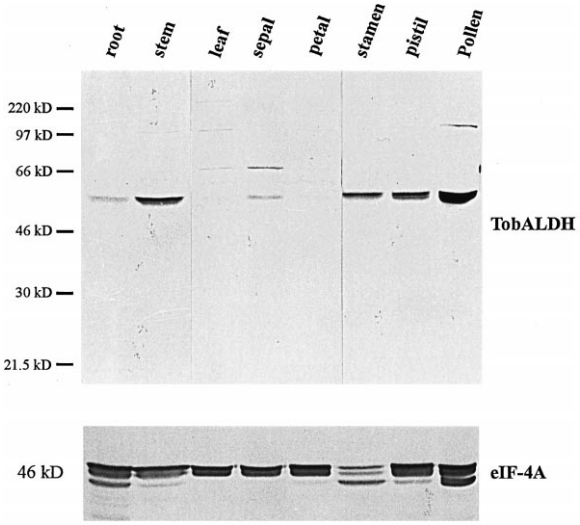

Figure 7. Western blot from various tissues from tobacco. Protein from *N. tabacum* (30 μg), isolated from the indicated organs, was separated on SDS-PAGE gel (12.5%). The blot was probed with an antibody raised against TobALDH2A. As a control, a duplicate blot was probed with an antibody raised against eIF-4A2.

Analysis of TobALDH *proteins*

Mature pollen are rich in proteins, lipids and messenger RNAs which are stored to be used during pollen germination and pollen tube growth [14]. In particular, mRNAs of the late group may not be used immediately but be stored and translated only upon hydration and pollen germination. In order to investigate the TobALDH protein level, we raised a polyclonal antibody in rabbit against a fusion protein of TobALDH2A overexpressed in *E. coli* (see Material and methods). The antiserum was tested and showed a strong cross-reaction with the original antigen (the TobALDH2A fusion protein). A weaker cross-reaction was seen with commercial ALDH from yeast (Boehringer) and no signal was seen with BSA (data not shown).

This antibody was used to detect ALDH protein in the different organs from tobacco via Western blotting. The TobALDH antibody reacted with two proteins in the range of 56 to 57 kDa in stem, stamen, pistil and pollen samples (Figure 7). A weaker signal was seen with root and petal tissue. These results largely confirm the data on mRNA transcript levels obtained by northern blot analysis. An antibody against translation initiation factor eIF-4A [21] was used as a control.

In vitro *enzymatic activity of TobALDH2A*

It was not possible to follow the enzymatic activity of ALDH in pollen extracts by measuring the change in absorbance at 340 nm via the formation of NADH with acetaldehyde or propionaldehyde as a substrate, since the presence of other NAD-linked dehydrogenases interfered with the determination of ALDH activity. Therefore, we overexpressed the mature TobALDH2A in *E. Coli*. Total protein extracts were used in an *in vitro* assay using several substrates. TobALDH2A has an activity for acetaldehyde of 74.5 ± 13.4 nmol/min per mg total protein extract (Figure 8). The enzyme was also highly efficient with propionaldehyde as a substrate, but had hardly any activity for DL-glyceraldehyde or betaine-aldehyde (65.9 ± 12.7, 4.8 ± 4.5 and 3.2 ± 3.0 nmol/min per mg total protein extract, respectively). The addition of 30 μM disulfiram, a potent inhibitor of ALDHs [13], blocked the aldehyde dehydrogenase activity almost completely. Protein extracts from *E. coli* overexpressing eIF-4A showed hardly any activity with all the substrates tested (1.6 ± 2.3 nmol/min per mg total protein extracts using acetaldehyde as substrate). These results show that TobALDH2A is able to use acetaldehyde as a substrate, and has characteristics similar to the well-studied human liver acetaldehyde dehydrogenases.

Inhibitor treatment of PDH and ALDH

The hypothesis we would like to test is that ALDH is part of an indirect metabolic pathway for the synthesis of acetyl-CoA, bypassing the direct route via PDH (see Figure 1). In order to test the relative importance of the direct and indirect pathways we used inhibitors of ALDH and PDH. The first compound, (R)-aminoethylphosphinate (AEP) (kindly provided by Prof. N. Amrhein), is metabolized to acetylphosphinate which acts as a highly specific inhibitor of PDH [26]. Addition of 30 μM AEP to germinating seeds resulted in the death of the seedlings (Figure 9). On the other hand, addition of up to 90 μM AEP to germinating pollen had no effect on the germination frequency or on the growth of the pollen tube.

The second compound we used was disulfiram, an inhibitor of ALDH [13]. Seedlings grown on 30 μM disulfiram formed fewer roots and showed a somewhat retarded growth (compared to wild-type), but were perfectly viable. These relatively mild effects of disulfiram on seedlings can be explained by the possible func-

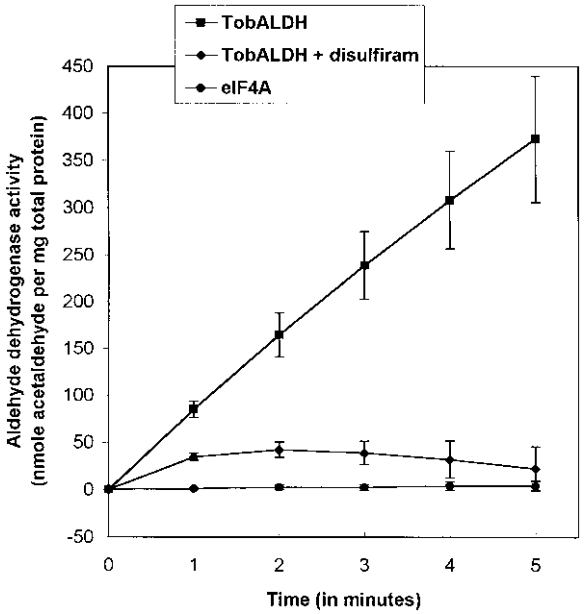

Figure 8. TobALDH2A overexpressed in *E. coli* has an acetaldehyde dehydrogenase activity. Overexpressed TobALDH2A is able to use acetaldehyde as a substrate in an *in vitro* assay. The addition of 30 μM disulfiram blocks the aldehyde dehydrogenase activity. *E. coli* overexpressing eIF-4A2 was used as a negative control. Values are means $\pm$ SD of at least three separate preparations.

tion of ALDH in general detoxification of aldehydes. However, when 30 μM disulfiram was added to pollen, it prevented pollen from growing. Thus, whereas inhibition of PDH had a drastic effect on seedlings, but not on pollen tube germination and growth, inhibition of ALDH primarily affected pollen. These experiments clearly indicate that major differences in metabolism between seedlings and pollen exist, and that ALDH is likely to have an important function in pollen. We realize that inhibitor experiments should be interpreted with caution and in particular disulfiram may not be entirely specific for ALDHs. However, the drastic differences in the effects of the inhibitors on leaves and pollen encourage us to initiate more definitive experiments involving specific antisense inhibition of TobALDH.

Discussion

The protein encoded by *TobAldh2A* is most related to mammalian ALDHs (60%) involved in ethanol detoxification [16], and to maize Rf2 (77%), a putative ALDH involved in restoration of male fertility [5]. Similarity with plant betaine aldehyde dehydrogenases is considerably lower (42%). *In vitro* enzymatic activity measurements with recombinant TobALDH2A show that the enzyme has a high activity for acetaldehyde (Figure 8). Thus, the data suggest that TobALDH2A is a mitochondrial acetaldehyde dehydrogenase. Ethanol metabolism by leaves has been reported for several plants, suggesting that an ADH/ALDH/ACS pathway operates in this organ [17]. However, in tobacco leaves the capacity of such a pathway was very limited [2]. The low expression of *TobAldh2A* in leaves (Figure 5), its failure to be induced by anoxia (Figure 6) and the inability of leaves to survive when PDH activity was inhibited (Figure 9), similarly indicate that this pathway is not important for normal metabolism in leaves. The primary function of ALDH in leaves could be the detoxification of occasional aldehydes.

The situation in pollen is very different. In the male gametophyte, *TobAldh2A* is highly expressed, and inhibition of ALDH prevents pollen growth, whereas pollen germinate and grow in the presence of the PDH inhibitor AEP (Figure 9). These results indicate that the PDH bypass is functional and important in pollen. Preliminary results indicate that *Acs* is highly expressed in pollen (R.G.L. op den Camp, M. Bäuerlein, B. Müller-Röber, C. Kuhlemeier, unpublished results), and thus, all the enzymes of the bypass are present. The acetyl-CoA generated could be used for energy production by the TCA cycle (see Figure 1). For a more complete discussion of the other possible pathways see the accompanying paper [29]. Pollen produce acetaldehyde and ethanol, even under aerobic conditions [3] and the expression of ALDH could protect the pollen against the hazardous effects of acetaldehyde. The high levels of ALDH in pistils (Figures 5 and 7) might be needed to metabolize acetaldehyde and/or ethanol diffusing from the growing pollen tube into the pistil. The protein most related to TobALDH2A is the protein encoded by the recently isolated maize *Rf2* gene [5]. *Rf2* has been well characterized genetically as a nuclear gene which restores fertility of plants containing cms-T cytoplasm. If the hypothesis that *Rf2* restores fertility by detoxifying acetaldehyde is correct, it should be possible to induce male sterility by antisense expression of *Aldh* in URF13 transformed tobacco [4].

The scenario outlined above suggests a function for ALDH in detoxification of acetaldehyde, coupled with energy production through the use of acetyl-CoA in the TCA cycle. However, in analogy to recently developed yeast models, a biosynthetic function may also be considered. In yeast, acetyl-CoA formed

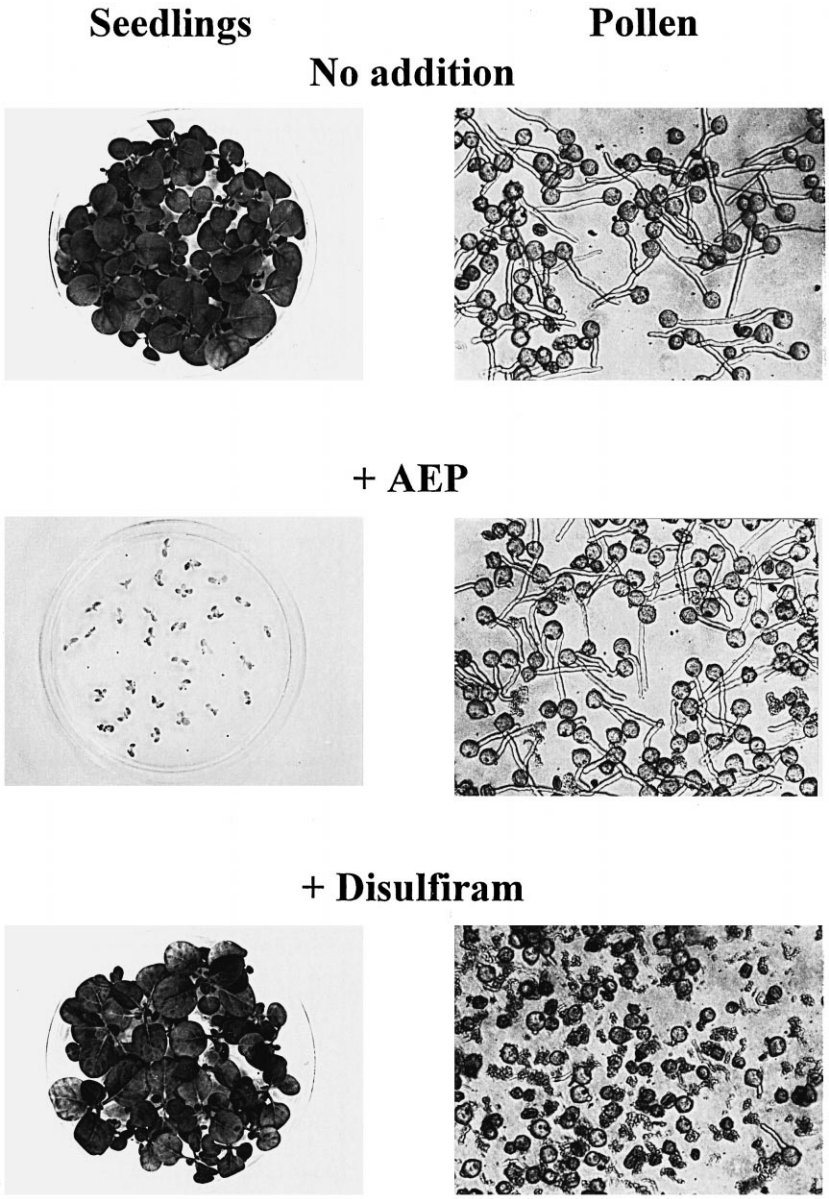

Figure 9. Effect of PDH and ALDH inhibitor treatment on seedling growth and pollen tube germination and growth. Seeds were germinated on a medium containing 30 μM AEP or 30 μM disulfiram and photographed after 40 days. Pollen were germinated in a medium containing 90 μM of AEP or 30 μM disulfiram. After 2 h pollen were photographed.

through PDC/ALDH/ACS is thought to be converted to malate in the glyoxylate cycle. Malate fed into the TCA cycle is used for biosynthetic purposes, and only when the flux of acetaldehyde to acetate through ALDH and ACS is saturated, ethanol formation commences [23]. A similar pathway might operate in pollen and the conversion of acetaldehyde to alcohol by ADH could be a safety valve protecting the pollen against excess acetaldehyde. This could explain why pollen which lack ADH activity (ADH null mutants) [33] have no disadvantage to the wild type pollen when growing through the pistil. It has been shown that the transcripts encoding the enzymes specific for the glyoxylate cycle (isocitrate lyase and malate synthase) are present in pollen from *Brassica napus* [34], and thus it is conceivable that a glyoxylate cycle exists in tobacco pollen.

Our results show that TobALDH is highly expressed in pollen and pistils and may be involved in a functional PDH bypass. In the near future we are planning to test these hypotheses by feeding germinating pollen with radioactive substrates to determine the actual flow of metabolizes, and by genetically manipulating ALDH levels.

Acknowledgements

We would like to thank Million Tadege, Drs Andrew Fleming and Isabelle Dupuis for critically reading the manuscript and Dr Stefano Canevascini for stimulating discussions. This work was financially supported by the Swiss National Science Foundation.

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