*Plant Molecular Biology* **35:** 343–354, 1997.



# Aerobic fermentation during tobacco pollen development



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

*Key words:* acetaldehyde, alcohol dehydrogenase, cytoplasmic male sterility, fermentation, pollen, pyruvate decarboxylase, tobacco

## Abstract

In vegetative organs of plants, the metabolic switch from respiration to fermentation is dictated by oxygen availability. The two genes dedicated to ethanolic fermentation, pyruvate decarboxylase and alcohol dehydrogenase, are induced by oxygen deprivation and the gene products are active under oxygen stress. In pollen, these two genes are expressed in a stage-specific manner and transcripts accumulate to high levels, irrespective of oxygen availability. We have examined the expression pattern of pyruvate decarboxylase and alcohol dehydrogenase at the protein level in developing pollen and show that the active proteins are localized to the gametophytic tissue and begin to accumulate at microspore mitosis. A flux through the ethanolic fermentation pathway could already be detected very early in pollen development, occurring in all stages from premeiotic buds to mature pollen. This flux was primarily controlled not by oxygen availability, but rather by sugar supply. At a high rate of sugar metabolism, respiration and fermentation took place concurrently in developing and germinating pollen. We propose that aerobic fermentation provides a shunt from pyruvate to acetyl-CoA to accommodate the increased demand for energy and biosynthetic intermediates during pollen development and germination. A possible undesirable side-effect is the potential accumulation of toxic acetaldehyde. Our results support a model for cms-T-type male sterility in maize, in which degeneration of the tapetum is caused by the toxic effects of acetaldehyde on mitochondria weakened by the presence of the URF13 protein.

## Introduction

Ethanolic fermentation in plants is generally thought of as an adaptation to oxygen deprivation [13]. The main purpose of this pathway is the regeneration of NAD to allow glycolytic ATP production in the absence of respiration. Only two enzymes are required for the production of ethanol from the glycolytic intermediate pyruvate: pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH). These two enzymes are present at low levels under normoxic conditions but their synthesis is strongly induced by oxygen deprivation [1, 25, 29, 44].

In baker's yeast (*Saccharomyces cerevisiae*), the regulation of the fermentation pathway is quite different. When yeast is grown in a glucose-limited medium, the direct pathway from pyruvate to acetyl coenzyme A (acetyl-CoA) via the pyruvate dehydrogenase (PDH) complex is fully active and no ethanol is detectable [16, 39]. Upon transition to a glucose-rich medium, however, ethanolic fermentation commences immediately [39, 43, 53]. This phenomenon of aerobic ethanolic fermentation in glucose excess has been attributed to a limited respiratory capacity [39] or a restricted assimilatory capacity of *S. cerevisiae* [53]. It has become quite clear now that at high glucose levels baker's yeast uses two pathways (a direct path and a bypass) for pyruvate conversion under ambient $O_2$ levels. In the direct path, PDH catalyzes the conversion of pyruvate to acetyl-CoA which is further processed in the tricarboxylic acid (TCA) cycle [41, 42]. The so-called PDH bypass is an indirect path whereby pyruvate is first decarboxylated to acetaldehyde, then oxidized to acetate and finally converted to acetyl-CoA

by the sequential actions of pyruvate decarboxylase (PDC), aldehyde dehydrogenase (ALDH) and acetyl coenzyme A synthetase (ACS), respectively [42]. The acetyl-CoA formed in this way can enter the glyoxylate cycle for the generation of biosynthetic intermediates or join the TCA cycle, depending on the energy status of the cell (for review see [56]).

In plants, the fermentation pathway is not known to operate under ambient oxygen tension except in a few instances. For example, root tips produce ethanol under atmospheric oxygen conditions [4, 11]. This has been attributed to the tightly packed cell layers in root meristems and stele, which causes diffusional impedance, and to the high $O_2$ demand of these tissues. The combined action of both diffusional impedance and high $O_2$ demand is held responsible for stelar anoxia in root tips [2, 45, 50]. The existence of such an increasing core of anaerobiosis in onion roots was noted very early by Berry and Norris [5]. Tree trunks also produce some ethanol [11], but the explanation similarly calls for the insufficient diffusion of oxygen into the innermost cellular compartments and the hypoxic nature of the interior of tree trunks [11]. Plants are also known to resort to ethanolic fermentation when they are stressed. Kimmerer and Kozlowski [27] have shown that red pine and paper birch seedlings fumigated with $SO_2$ produce appreciably high levels of acetaldehyde and ethanol. The anoxia-inducible *Adh* gene of *Arabidopsis thaliana* has been shown to be induced by environmental insults such as cold and drought [15]. The physiological significance of PDC and ADH induction and alcoholic fermentation by environmental stress, other than $O_2$ limitation, has not been resolved.

We previously introduced a heterologous PDC gene into tobacco and showed that it was constitutively expressed at high levels. However, even though both enzymes of the fermentation pathway were present, ethanol accumulation could not be measured under aerobic conditions unless respiration was impaired by inhibitors [8]. This indicates that under atmospheric conditions pyruvate preferentially enters the TCA cycle through PDH and that aerobic fermentation is not at all the rule in vegetative tissues.

In the pollen grain, the control of the ethanolic fermentation pathway appears to be very different from vegetative tissues, and in some respects resembles the situation found in baker's yeast. In tobacco pollen, abundant ADH and a pollen specific isoform of PDC (PDC2) have been found [7]. Transcript levels for both genes were not influenced by oxygen concentration. Interestingly, the flux through the ethanolic pathway appeared to be controlled by sugar supply rather than oxygen availability [7]. It is not clear what the function of this pathway in pollen could be. Obviously, pollen must invest energy into synthesizing abundant PDC and ADH at a time of high energy demand, yet ADH null mutants appear to have no phenotype [18]. It is thus difficult to attribute an essential function to fermentation in pollen. Here we study the expression of PDC and ADH and the flux through the pathway throughout pollen development and demonstrate that a significant flux occurs much earlier than we previously thought. The significance of these findings and a possible connection to cytoplasmic male sterility are discussed.

## Materials and methods

### *Growth of plants*

Tobacco plants (*Nicotiana tabacum* cv. Samsun) were grown in the greenhouse, under a 16:8 light/dark cycle and a temperature of 18 to 25 °C.

### *Isolation of microspores and protein extraction*

Anthers of tobacco were disrupted with a Polytron PT 3000 (Kinematica AG) at high speed for about 30 s and microspores from anthers at different stages were isolated [46]. About 200 mg tissue were homogenized in microfuge tubes and proteins were extracted as described [8]. The extract was either used immediately for enzymatic assays or frozen in liquid nitrogen and stored at −80 °C for protein determination and western analysis. For determination of enzymatic activity in whole anthers, proteins were extracted in the same way from the whole anther tissue instead of isolated microspores. All manipulations were performed at 4 °C. Protein concentrations were determined using the BioRad protein assay.

### *Antibody generation and western blotting*

A 1 kb internal fragment of TobPDC1 cDNA was cloned in the *Bam*HI site of pET-8c (pET-3d) vector [49] creating a translational fusion, and introduced into pLysS strain of *Escherichia coli*. The recombinant protein was purified from the inclusion bodies and antibodies were generated in rabbit. Generation of antibody against *Zymomonas* PDC was described before [8]. Since both antibodies detect a single band of the

correct size in crude pollen extracts and react with recombinant TobPDC1, we believe that each specifically recognizes both TobPDC1 and TobPDC2. Ten or thirty micrograms of total soluble proteins were separated on a 12.5% SDS-PAGE and transferred to nitrocellulose (Schleicher & Schuell) as described [9]. A 1:1000 dilution of rat anti-*Zymomonas* PDC antibody, rabbit anti-TobPDC1 antibody or rabbit anti-barley ADH antibody (kindly provided by A. Good) and horseradish peroxidase conjugates were used to detect PDC and ADH proteins.

*PDC and ADH enzymatic assays*

PDC activity was measured as described [8]. Plant lactate dehydrogenase inhibitors ATP and oxamate at final concentrations of 20 mM and 1 mM, respectively, were used to check lactate dehydrogenase activity in the assay [29]. ADH activity was measured with a test kit for yeast ADH (Sigma).

*Ethanol determination in anther tissue*

A 200 mg portion of anthers from thoroughly rinsed buds was either directly frozen in liquid nitrogen for extraction or vacuum infiltrated with 50 mM glucose and 0.1 mM $CaSO_4$ solution [8] for 2 min. Infiltrated samples were briefly blotted on tissue paper and incubated in brown bottles in a 30 °C water bath for 2 h. Incubated samples were transferred to Eppendorf tubes and frozen in liquid nitrogen. Frozen samples were homogenized and deproteinized in 1 ml perchloric acid (6%) according to Sieber and Brändle [48]. The homogenate was spun in a microfuge at 13 000 rpm for 10 min at 4 °C. The supernatant was neutralized with an ice cold 5 M $K_2CO_3$ solution and spun again for 10 min. This supernatant was used for determination of ethanol concentration. Average recoveries by adding known amounts of ethanol to frozen samples were 87%. Ethanol concentrations were measured with a test kit (Boehringer Mannheim).

*Acetaldehyde and ethanol measurements in the gas phase*

*Anthers.* Flower buds were rinsed three times with sterile water and 200 mg anthers were infiltrated with glucose solution as above. The infiltrated samples were briefly blotted on tissue paper and sealed in 33 ml brown bottles under normoxic conditions. For 40% $O_2$ treatment, the bottles were filled with the nutrient solution and sealed with Teflon septa. Two needles were introduced into the septum, one connected to a 40% $O_2$ gas jar and the other open to the air to allow displacement [22]. A 40% level of $O_2$ was monitored until all the solution was removed. Both needles were taken off and all samples were incubated in a 30 °C water bath for 2 h. Anoxia was imposed in an anaerobic work bench as described [8]. Gas samples (1 ml) were removed from the headspace with a Hamilton syringe and injected into a Sigma 300 GC (Perkin Elmer) equipped with flame ionization detector. GC conditions were as described [8].

*Mature pollen.* Freshly collected 25 mg pollen were washed 3 times with diethyl ether in Eppendorf tubes according to Hoekstra and Bruinsma [20] and dried under vacuum. Five drops of glucose solution (see above) were added to the dried pollen on a prewet filter paper and sealed in 33 ml bottles. Air was removed and replaced by 40% $O_2$ through two different needles, as above, but using bottles without solution. Not more than one min elapsed between addition of glucose and bubbling in 40% $O_2$. The bottles were immediately incubated in a 30 °C water bath and 1 ml gas samples were analyzed by the GC as above at 2, 5, 10, 20 and 30 min of incubation.

*Respiration measurements*

Oxygen consumption and carbondioxide production by roots, leaves, and anthers were measured using Gilson differential respirometer. A 200–500 mg portion of tissues was rinsed with distilled water and infiltrated with glucose solution for 4 min under vacuum and incubated in 1 ml glucose solution at 30 °C. Two Warburg vessels were used for each sample, one containing 0.3 ml of 2 M KOH with 3 pieces of filter paper in the central compartment to trap $CO_2$ and the other without KOH. The samples were equilibrated for 10 min and measurements were carried out for 1 h with reading every 15 min. The amount of $O_2$ consumed and $CO_2$ produced was calculated from the gas equation PV = nRT in which both $O_2$ and $CO_2$ were assumed to occupy 22.4 litres per mole at standard temperature and pressure. Respiratory quotient (RQ) is the amount of $CO_2$ produced over $O_2$ consumed. Similar measurements were performed with respiration solution alone or with the medium after taking out the tissues and showed no measurable contamination. At the end of the measurement, the tissues together with the medium were

frozen in liquid nitrogen and extracted with perchloric acid for enzymatic determination of ethanol.

Oxygen uptake was also measured in the presence of inhibitors with a Rank Brothers oxygen electrode at 30 °C. Pollen were washed 3 times with diethyl ether, dried under vacuum, and 12 mg were used per 5 ml respiration solution (25 mM 2-morpholinoethane sufonic acid pH 5.9, 300 mM sucrose, 1 mg/ml casein hydrolysate, 1.6 mM $H_3BO_4$, 1 mM KCl, 1 mM $CaCl_2$, 0.8 mM $MgSO_4$, 0.03 mM $CuSO_4$). Leaves were rinsed with sterile water, cut into small pieces and 100 mg were used per 5 ml medium in complete darkness. Inhibitors were added either at the beginning or after 10 min of incubation at final concentrations of 2 $\mu$M antimycin A (AA) and 1 mM salicylhydroxamic acid (SHAM), both obtained from Sigma and dissolved in DMSO. The same amount of DMSO alone was used for negative controls.

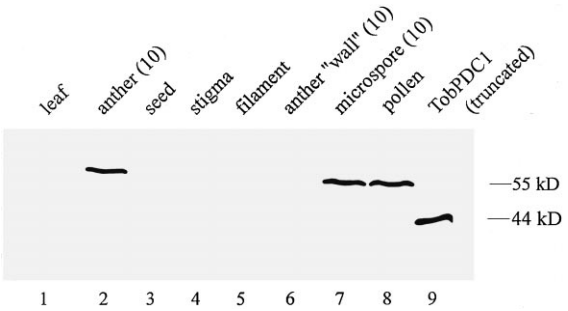


*Figure 1.* Organ-specific expression of PDC protein. Proteins were separated on 12.5% SDS-PAGE and transferred to nitrocellulose filter. A rat polyclonal antibody raised against *Zymomonas mobilis* PDC protein was used to probe the filter. Thirty $\mu$g of total soluble proteins from each indicated organ were loaded in lanes 1–6. The amount of proteins in lanes 7 and 8 were three times less. Anthers at stage 10 (lane 2) were separated into microspores (lane 7) and remaining material (anther wall, lane 6). Lane 9 shows 20 ng of a truncated form of recombinant TobPDC I protein that was expressed and purified from *E. coli*, shown here as a control. Numbers at the bottom indicate lanes.

## Results

### *Developmental regulation of PDC and ADH protein expression in tobacco pollen*

Previous studies have shown that PDC is encoded by at least two genes in tobacco. The PDC1 mRNA is present in vegetative tissues and is induced by oxygen limitation. The PDC2 gene encodes a protein which is very similar to PDC1, but has a 26 amino acid insertion in the C-terminus [7]. The PDC2 mRNA is highly expressed in the pollen grain. It is a typical 'late gene' in that its transcripts begin to accumulate at microspore mitosis (stage 6, according to Koltunow *et al.* [28], where stage 1 stands for meiosis complete, stage 6 for the time of microspore mitosis and stage 12 for dehisced pollen). To examine whether the levels of PDC protein reflect the reported pattern of PDC transcripts, we performed western blots using an antibody raised against *Zymomonas* PDC. This antibody recognized both PDC2 (present at high levels in pollen) and recombinant TobPDC1 (Figure 1, lanes 8 and 9). Of all the other tissues analyzed, a signal could only be detected in anthers (Figure 1, lane 2). When anthers were dissected, PDC-reactive material was found in the isolated microspores, but not in the remaining maternal tissue (Figure 1, lanes 6 and 7). We were never able to detect PDC protein in roots and leaves even after anaerobic induction (data not shown). Similar negative results were obtained with an antibody raised against recombinant TobPDC1 (data not shown). From these

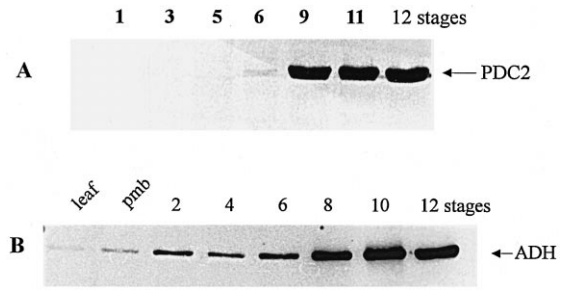


*Figure 2.* Developmental regulation of PDC and ADH proteins. Total soluble proteins were isolated from the microspores of the indicated stages and separated as described in Figure 1. A. PDC protein at different stages of tobacco pollen development. A 30 $\mu$g portion of protein was loaded per lane. B. ADH protein accumulation at various stages of tobacco pollen development. In leaves and premeiotic buds 20 $\mu$g of protein were loaded, in all other lanes 10 $\mu$g. Numbers indicate the stage of the microspores (see text); pmb, premeiotic buds without sepals.

results we conclude that in vegetative tissues, even after induction, PDC1 is present at levels considerably lower than those found in pollen.

Figure 2 shows the accumulation of PDC and ADH protein in isolated microspores during tobacco pollen development. The PDC protein (Figure 2A) could first be detected at about stage 6, reached a peak between stages 7 and 9 and remained more or less stable thereafter. The ADH protein exhibits a similar expression pattern as PDC2, except that in early stages and in leaves low levels of ADH protein could be observed (Figure 2B). The absence of PDC2 protein at the earli-

er stages could be due to its complete absence or to the inability of the antibody to detect very low levels. Based on the results presented in Figure 1, we assume that low levels of PDC protein would escape detection. In rice coleoptiles and maize root tips, the level of PDC has been reported to be 17–65 times lower than that of ADH [36] and therefore PDC levels may indeed be generally low.

### In vitro *PDC and ADH enzymatic activity*

We next examined the *in vitro* enzymatic activity of PDC in the microspores (Figure 3A). In leaves, a very low PDC activity was measured. Because other pyruvate-dependent NADH oxidations might contribute to the measured activity (see Materials and methods), we cannot exclude the possibility that this very low activity does not represent PDC activity. From premeiotic buds to microspores at stage 10, a steady increase in activity could be observed, with a somewhat lower activity in mature pollen (Figure 3A). A comparison at stage 10 between whole anthers (10A) and isolated microspores (10) showed that in the latter at least a two-fold enrichment was obtained. This indicates again that PDC activity is localized in the gametophytic tissue. Similar results were obtained for ADH activity (Figure 3B). Comparison of activity at stage 10 between whole anthers (10A) and isolated microspores (10) also showed more than a two-fold increase in the microspores.

### *Aerobic ethanolic fermentation in tobacco anthers*

The previous experiments established that both PDC and ADH accumulated to very high levels after microspore mitosis, and that before this time point levels were very low or even below the detection limit (for PDC). To determine when metabolic flux through the ethanolic fermentation pathway began, we measured ethanol levels during pollen development. Anthers were either put directly into liquid nitrogen for extraction, or infiltrated with a 50 mM glucose solution for 2 min and incubated for 4 h before extraction. Tissue concentration of ethanol was determined enzymatically. In anthers which were directly frozen, ethanol appeared in the early stages of gametophyte development and declined after microspore mitosis (Figure 4A). In leaves no ethanol could be detected, in agreement with previous measurements. Infiltration with glucose had a dramatic effect in inducing ethanolic fermentation in anthers (Figure 4B, note the difference in scale with Figure 4A), but not in leaves.

### *Effect of oxygen concentration on ethanolic fermentation*

The obvious explanation for the effect of glucose on ethanol production is that sugar supply regulates ethanolic flux, as it does in yeast. However, we wished to examine possible effects of oxygen availability, as this is the major regulator of ethanolic fermentation in leaves and roots. We set up three control experiments. First, we reasoned that if $O_2$ were limiting, incubation in 40% $O_2$ would increase respiration and decrease fermentation. Second, we determined the kinetics of fermentation. If pollen were $O_2$ limited, one would expect that respiration initially occurs at a high rate, and then declines when $O_2$ is depleted with a concomitant rise of fermentation. Third, we decided to directly measure $O_2$ consumption during fermentation.

As the first control experiment, anthers were infiltrated with nutrient solution, incubated with air or 40% $O_2$ for 2 h at 30 °C and gas samples from the head space were analyzed by gas chromatography. The results presented in Figure 5 show the effect of elevated $O_2$ concentration on the levels of end products of fermentation. Acetaldehyde and ethanol production could be detected at all stages of pollen development in both ambient and 40% $O_2$ concentrations (Figure 5A and B). As a negative control, anthers were infiltrated with the buffer without glucose and incubated under normoxic conditions. Both acetaldehyde and ethanol accumulated to much lower levels measured in the absence of glucose (Figure 5C) suggesting once again that aerobic fermentation in anthers is regulated by sugar availability. Incubation of stage 10 anthers under anoxic conditions, on the other hand, resulted in a two-fold increase both in acetaldehyde and ethanol levels (data not shown). This indicates that ambient $O_2$ concentration does not dramatically alter accumulation of acetaldehyde and ethanol.

To quantitate the ethanolic flux with respect to respiration and verify by a different approach that aerobic fermentation is substantial in anthers, we determined the rate of $O_2$ consumption and $CO_2$ production manometrically. In roots and leaves, $O_2$ consumption paralleled $CO_2$ production with a respiratory quotient (RQ) of approximately 1.0 (Figure 6). In anthers, however, higher level of $CO_2$ production could be measured compared with $O_2$ consumption, yielding RQs of 1.5 and 1.2 for stages 2 and 10, respectively

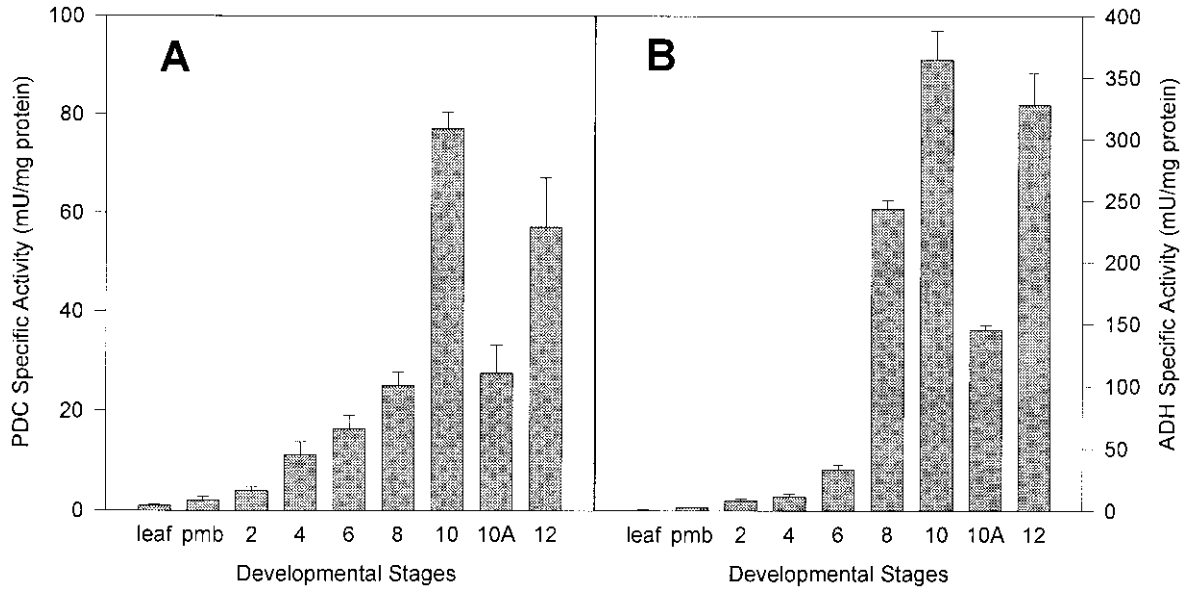


*Figure 3.* *In vitro* PDC and ADH enzymatic activity. A. PDC specific activity in leaf, premeiotic buds (pmb) and isolated microspores at different stages of development. Whole anthers at stage 10 (10 A) were included for comparison. One unit will convert 1.0 $\mu$mol of pyruvate to acetaldehyde per minute at pH 6.0 at 25 °C. B. ADH specific activity in leaf, premeiotic buds (pmb) and microspores at different stages. Anthers at stage 10 (10A) were included for comparison. One unit will convert 1.0 $\mu$mol of ethanol to acetaldehyde per minute at pH 8.8 at 25 °C. Numbers indicate the stages of microspore development. Values represent the mean ± SD of five independent measurements each made in duplicate. pmb, premeiotic buds without sepals; A. anthers.

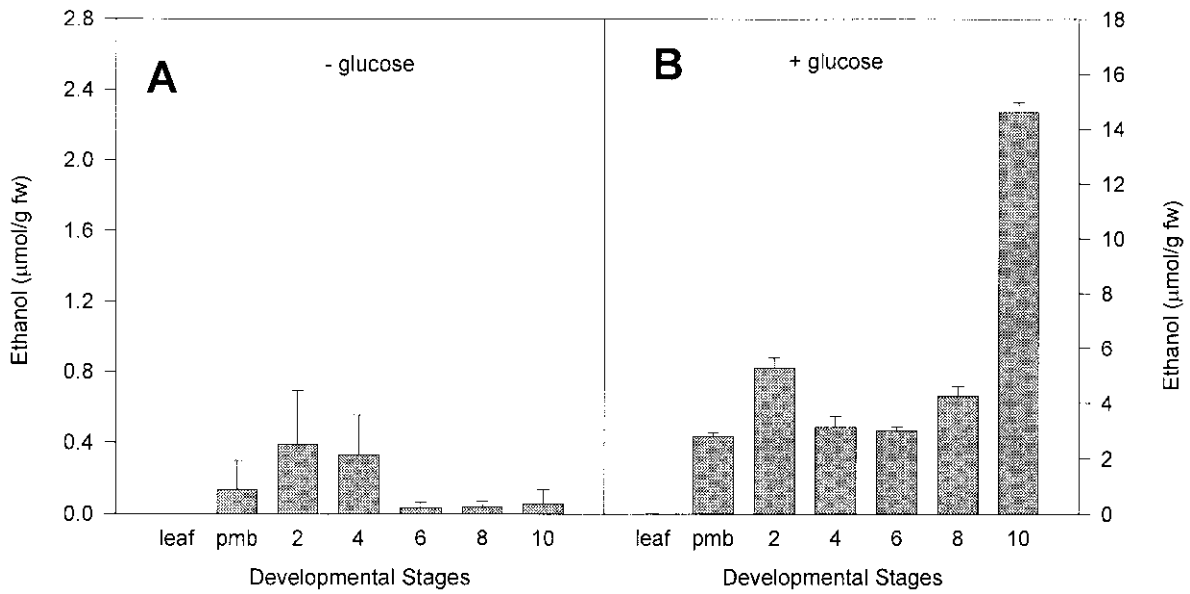


*Figure 4.* Enzymatic determination of ethanol concentration in anther tissues with or without glucose. A. Anthers were removed from the flower at the indicated stages and immediately frozen in liquid nitrogen. Frozen samples were extracted and deproteinized as in materials and methods. B. Anthers were infiltrated with 50 mM glucose and 0.1 mM $CaSO_4$ solution for 2 min under vacuum. Afterwards, anthers were briefly blotted on tissue paper and incubated in brown bottles at 30 °C in a water bath for 4 h. The incubated samples were treated as in A. Premeiotic buds (pmb) are whole buds without sepals. Values are the mean ± SD of three individual measurements each made in duplicate.

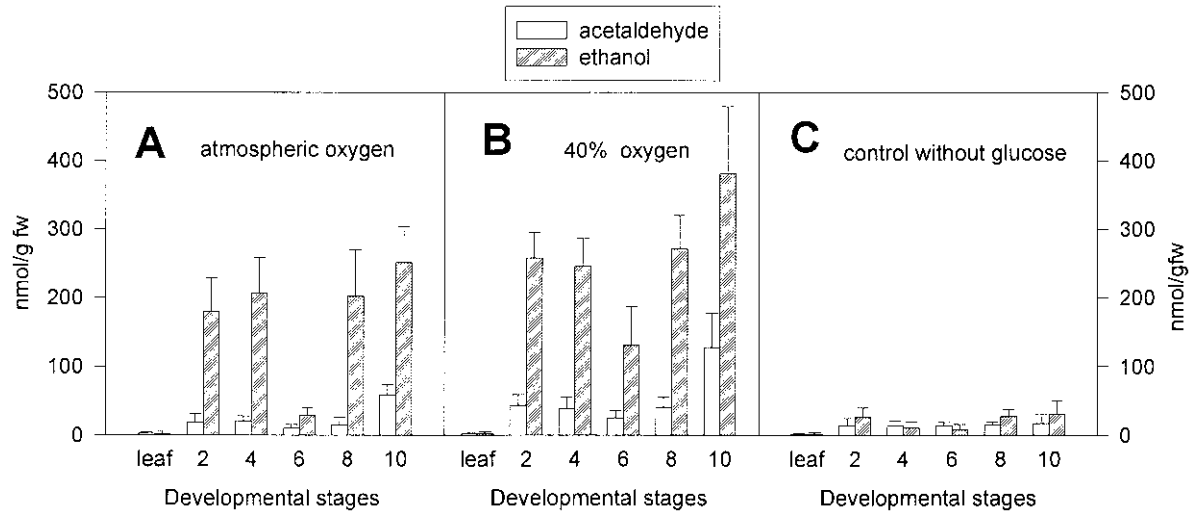


*Figure 5.* Acetaldehyde and ethanol accumulation in the gas phase. Anthers at the indicated stages were infiltrated with a nutrient solution as above and incubated under atmospheric condition (panel A) or under 40% oxygen (panel B) in a 30 °C non-shaking water bath for 2 h. In panel C anthers were incubated as in A, but infiltrated with the buffer alone without glucose. One ml gas samples were removed from the headspace with a gas tight syringe and injected into the GC. Values represent the mean ± SD of six independent measurements.

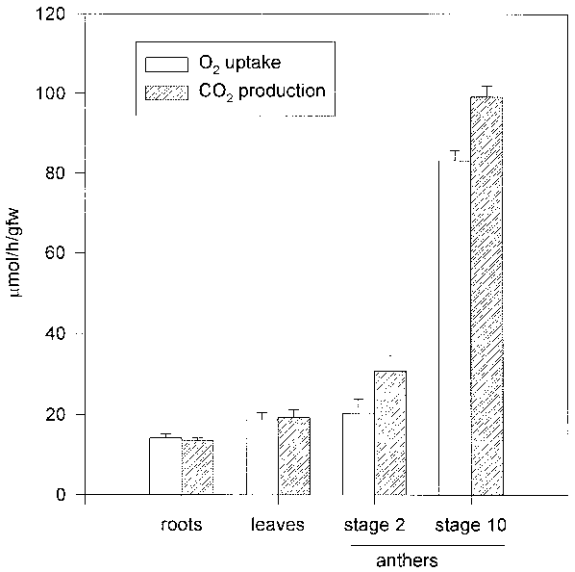


*Figure 6.* Respiratory behaviour in different tissues of tobacco. Either 200 mg anthers or 500 mg roots or leaves were infiltrated with glucose solution and incubated in 1 ml of the infiltration solution at 30 °C. $O_2$ consumption and $CO_2$ production were measured manometrically in a Gilson differential respirometer.

(Figure 6). From Figure 6 it can be calculated that the extra $CO_2$ production at stages 2 and 10 amounts to 11 and 16 $\mu$mol/h per g fresh weight (F.W.), respectively. This matches the amount of ethanol produced (12 and 15 $\mu$mol/h per g f.w., respectively) as determined enzymatically (data not shown). Thus, assuming that only sugars are metabolized, respiration yields 6 mol $CO_2$ per mole of glucose and fermentation gives 2 mol $CO_2$ per mole of glucose, and that other oxidative processes are not differentially activated during pollen development, we can roughly estimate that at least 38 to 60% of the dissimilated carbon comes from fermentation.

As the second control experiment, Figure 7A shows a time course analysis of acetaldehyde and ethanol production under 40% $O_2$ tension in mature pollen. We detected acetaldehyde and ethanol in the head space at the earliest time point technically possible. In the first 2 min of incubation, acetaldehyde and ethanol reached up to 0.6 and 0.5 nmol per mg pollen, respectively (Figure 7A). This massive burst is at least fifty fold higher compared to that measured in leaf tissue of the same cultivar after 4 h of anoxic incubation [8]. In addition, the acetaldehyde and ethanol levels increased steadily and did not show any abrupt increase during the first 30 min of incubation time.

Finally, we measured oxygen consumption in respiring pollen using an oxygen electrode in the presence and absence of respiration inhibitors, AA (inhibitor of the cytochrome pathway) [8, 52] and SHAM (inhibitor of the alternative oxidase pathway) [52]. In the absence of inhibitors, $O_2$ uptake by pollen proceeded linearly for at least 10 min (Figure 7B, inset) and 10 times faster than the rate observed in leaf (Fig-

ure 7B). When AA and SHAM were applied, $O_2$ uptake was found to be blocked by more than 95%. This inhibition was observed both when inhibitors were applied immediately or after 10 min of incubation (Figure 7B, inset). These results demonstrate that pollen respires very rapidly and is not oxygen limited in a nutrient solution for more than 10 min while ethanol and acetaldehyde could steadily accumulate in the headspace without a measurable lag. This demonstrates the coexistence of respiration and fermentation.

## Discussion

Aerobic ethanolic fermentation seems to be a universal phenomenon in pollen and developing microspores. In addition to *Nicotiana tabacum*, we have also measured substantial acetaldehyde and ethanol production in the pollen of *Nicotiana plumbaginifolia* and *Zea mays* (T. Mandel and C. Kuhlemeier, unpublished results). Scott *et al.* [47] reported that 40 mM sucrose is toxic to barley microspores while 0.2 mM sucrose and 40 mM maltose support survival. During incubation in 40 mM sucrose, but not in 0.2 mM sucrose or 40 mM maltose, a high level of ethanol accumulated, in agreement with our results. Although these authors did not directly measure respiration rates, they hypothesized that ethanol accumulation occurs when high respiration causes oxygen depletion. The tight sporopollenin wall of the microspore was also thought to easily impede oxygen diffusion. Our results argue against oxygen depletion as the trigger for ethanol production. Instead, we propose that the flux through the fermentative pathway is primarily triggered by sugar availability. The data in Figure 5 show that doubling of $O_2$ level does not decrease fermentation rate. More importantly, respiration is linear for at least the first 10 min (Figure 7B, inset) while ethanol accumulation begins within minutes, without a measurable lag (Figure 7A). Moreover, we consider impedance of $O_2$ diffusion by the pollen coat as unlikely. Sporopollenin formation is not an early event in pollen development [35], yet we detected ethanolic fermentation at all stages of pollen development including premeiotic buds (Figure 4). Since a multitude of anatomical changes takes place throughout pollen development, it does not seem reasonable to assume that a single cellular compartment is a physical barrier to oxygen diffusion from premeiotic buds to mature pollen. The critical oxygen pressure and the rate of $O_2$ diffusion into the microspores at various stages should be carefully evaluated to address this issue precisely. Taken together, our data suggest that respiration and ethanolic fermentation take place concurrently during tobacco pollen development, and that sugar availability is the prime factor that triggers the latter.

The coexistence of ethanolic fermentation and respiration on glucose medium has been well documented in at least one eucaryotic organism, baker's yeast. In *S. cerevisiae*, the concerted action of PDC, ALDH and ACS provides a functional bypass to the mitochondrial PDH to produce more acetyl-CoA in situations of a high rate of sugar metabolism [42]. Since PDH has a higher affinity for pyruvate than PDC [41, 54], during glucose-limited growth of *S. cerevisiae*, pyruvate preferentially enters the TCA cycle via PDH [54]. In glucose excess media, however, there is no competition for pyruvate and a significant proportion of pyruvate metabolism also occurs via PDC [42]. The demonstration that (1) PDH negative disruption mutants grow in glucose-limited chemostat cultures without ethanol production [42], (2) PDC negative disruption mutants exhibit very slow growth rates in glucose-grown batch cultures [21], and (3) acetyl-CoA synthetase 1 and 2 (ACS1-ACS2) double mutants are not viable on glucose media [51] are some very elegant pieces of evidence that argue for the existence and functionality of the PDH bypass in yeast.

It seems likely that a similar situation exists in developing pollen. Amplification of mitochondrial number by 40-fold in the tapetum and by 20-fold in the sporogenous tissue of maize anthers has been reported [30, 55]. This mitochondrial amplification has been related to an increased energy demand during pollen formation (see [32] for review). Our data on oxygen consumption by respiring pollen shows that pollen respire at least 10 times faster than green leaf tissue (Figure 7B). This high rate of respiration and increased energy demand during pollen development and germination could be associated with active cell division and fast growth. Under such circumstances, the demand for acetyl-CoA and other biosynthetic intermediates must be enormous. In particular, the degeneration and formation of various tissues during pollen development, the formation of unique substances such as sporopollenin, the deposition of waxy materials on the pollen coat, and the synthesis and regulation of about 10 000 anther-specific transcripts [23, 24] must impose unusually high demands for energy and key biosynthetic intermediates. Under such conditions, the PDH to acetyl-CoA direct path may not be sufficient to meet all requirements. This might be the

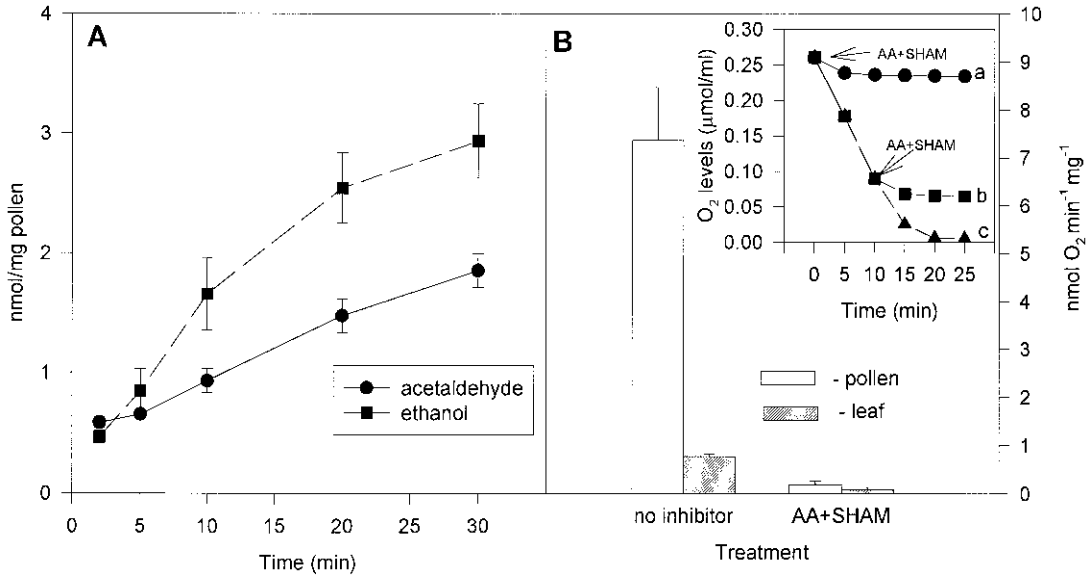


*Figure 7.* Oxygen uptake and time course of acetaldehyde and ethanol production by mature pollen. A. Freshly collected pollen were washed twice with diethyl ether and dried under vacuum. The dried pollen were put on a wet filter paper and immediately sealed in the presence of glucose under 40% oxygen. The bottles were incubated in a non shaking water bath at 30 °C and 1 ml gas samples were analyzed by the gas chromatograph at the indicated time points. B. Washed pollen or rinsed leaf pieces were incubated in 5 ml respiration medium at 30 °C in an oxygen electrode. Inhibitors (AA + SHAM) were added either at the beginning or 10 min after incubation in the respiration medium. The inset shows the traces of three representative measurements. Arrows indicate the time points of inhibitor addition. a, inhibitors added at the beginning; b, inhibitors added after 10 min; c, control without inhibitors.

trigger for the operation of the PDH bypass in pollen, with the observed ethanol production being just a protection against toxic acetaldehyde accumulation. The probable routes of pyruvate utilization are summarized diagrammatically in Figure 8. This model proposes 3 possible fates of the acetyl-CoA that is derived from acetaldehyde via acetate: (1) it can be used for energy production in the TCA cycle; (2) it can be used for lipid biosynthesis in the plastids; and (3) it can be converted to malate and succinate in the glyoxysomes to serve as a biosynthetic intermediate. The partitioning of acetate and acetyl-CoA between the cytoplasmic organelles, however, remains to be uncovered. There is evidence for ethanol metabolism in plants which would involve ALDH and ACS [8, 10, 34]. We have recently cloned *Aldh* from a tobacco pollen cDNA library and show that it is highly expressed in pollen and pistils (R. op den Camp and C. Kuhlemeier, accompanying paper). In that paper, we present additional evidence for the existence of the PDH bypass in pollen. Further characterization of ALDH and ACS, antisense approaches, and metabolite labelling experiments should provide us with a definitive molecular clue about the functionality of the bypass in the male gametophytic tissue.

We would like to draw attention to the toxic effects of acetaldehyde and/or ethanol that are produced in large quantities during pollen development. Acetaldehyde is a very strong cell toxin and is implicated as one of the major causes of tissue injury and cell death imposed by anoxia and post-anoxia [3, 40]. Perata and Alpi [37], while studying ethanol metabolism, pinpointed acetaldehyde as the cause of ethanol-induced injury in carrot cells. Acetaldehyde is thought to exert its toxic side-effects by forming acetaldehyde-protein adducts [6, 38]. Acetaldehyde is volatile and chemically reactive, which makes it difficult to reliably determine its concentration in tissues. However, since with anthers the concentration in the gas phase is at least 10 times higher than with anoxic leaves, we assume that the tissue concentration is also much higher. Acetaldehyde could, therefore, very well be the cause of cell death in the reported experiments with barley microspores [47].

Acetaldehyde could also be the 'hypothetical pollen factor' [17] that causes cytoplasmic male sterility. Cytoplasmic male sterility (cms) is a maternally inherited trait observed in a wide range of species and is characterized by the inability of plants to produce viable pollen [57], for reviews see [19, 31, 32]. In

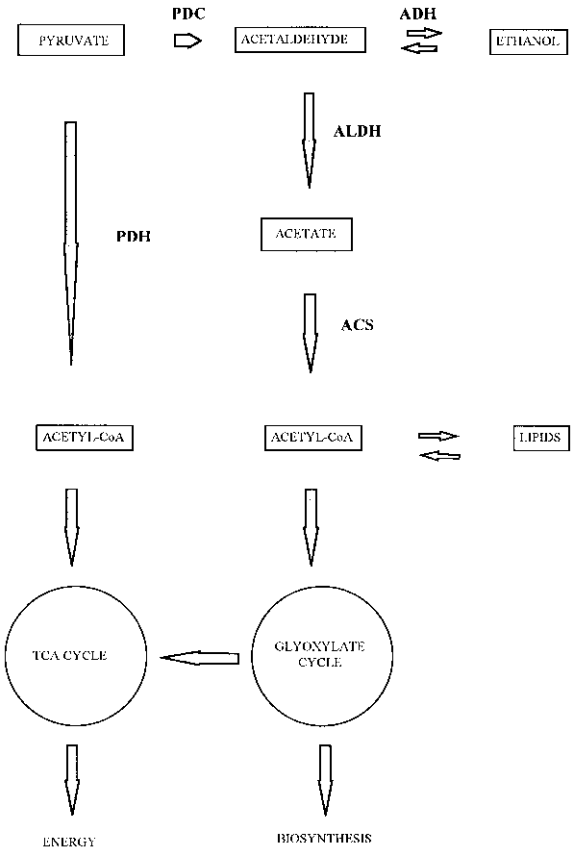


*Figure 8.* Proposed pathways of pyruvate utilization in developing pollen. Acetyl-CoA could be formed either directly from pyruvate in the mitochondria or indirectly through the fermentation pathway. Mitochondrial acetyl-CoA mainly goes to the production of energy in the TCA cycle. The acetyl-CoA derived from acetate may serve a biosynthetic function in the plastids or after being reshuffled in the glyoxysomes may join the TCA cycle to replenish biosynthetic intermediates or generate energy depending on the energy demand of the cell. $\beta$-oxidation of fatty acids could also supply acetyl-CoA to the glyoxylate cycle in situations of glucose scarcity presumably during pollen germination analogous to the usual function of glyoxysomes in the germination of oily seeds.

the best studied system, cms-T maize, the male sterility phenotype is associated with the expression of a unique mitochondrial protein called URF13. URF13 is expressed in almost all tissues, but only anthers were found to be sensitive. For this reason it has been argued that there might be a pollen-specific substance, 'pollen factor', that interacts with URF13 to cause pollen abortion [33]. Attempts to identify this pollen-specific substance have not been successful [32]. Two nuclear genes designated *Rf1* and *Rf2* act jointly to restore male fertility in cms-T maize. *Rf1* has been shown to decrease the abundance of URF13 by about 80% [14, 26]. *Rf2* has recently been cloned and sequenced and turned out to encode a putative aldehyde dehydro-

genase [12]. The data presented here show that acetaldehyde accumulates much earlier than would have been expected based on the 'late' expression pattern of PDC and ADH. Thus, at the developmental stages when degeneration of the tapetum leads to male sterility in cms plants, acetaldehyde is already synthesized. Our data support models in which the combination of toxic acetaldehyde and mitochondria weakened by URF13 causes tissue degeneration specifically during anther development.

## Acknowledgements

We thank all members of our lab for their stimulating discussions and helping hands during the course of this work. We are particularly grateful to Dr A. G. Good for the generous gift of the anti-ADH antibody; R. op den Camp, Drs R. Brändle, A.J. Fleming, M. Bucher, C. Brunold, T. ap Rees and P. S. Schnable for stimulating discussions, critically reading of the manuscript and sharing their unpublished results. M. T. is supported in part by fellowships from the European Human Capital Mobility Programme and The Roche Research Foundation.

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