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The ethanolic fermentation pathway supports respiration and lipid biosynthesis in tobacco pollen

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Summary

Rapid pollen tube growth requires a high rate of sugar metabolism to meet energetic and biosynthetic demands. Previous work on pollen sugar metabolism showed that tobacco pollen carry out efficient ethanolic fermentation concomitantly with a high rate of respiration (Bucher *et al.*, 1995). Here we show that the products of fermentation, acetaldehyde and ethanol, are further metabolised in a pathway that bypasses mitochondrial PDH. The enzymes involved in this pathway are pyruvate decarboxylase, aldehyde dehydrogenase and acetyl-CoA synthetase. Radiolabelling experiments show that during tobacco pollen tube growth label of ^{14}C-ethanol is incorporated into CO_2 as well as into lipids and other higher molecular weight compounds. A role for the glyoxylate cycle appears unlikely since activity of malate synthase, a key enzyme of the glyoxylate cycle, could not be detected.

Keywords: fermentation; ethanol; PDH-bypass; ADH; tobacco pollen; sugar metabolism

Introduction

Pollen tubes are the fastest growing plant cells known. This rapid growth puts high demands on energy production (Taylor and Hepler, 1997). Indeed, growing pollen tubes have been shown to respire at least 10 times faster than green leaf tissue (Dickinson, 1965; Tadege and Kuhlemeier, 1997).

Bucher *et al.* (1995) discovered that in germinating tobacco pollen a high respiration rate coincides with a significant level of ethanolic fermentation. Until then, ethanolic fermentation in plants had been observed only in vegetative plant tissues. Under low oxygen conditions and other stresses, such as cold and dehydration, the pathway was induced. It was proposed that under stress conditions, which damage the mitochondrial ATP-generating machinery, the cells resort to ethanolic fermentation to regenerate NAD^+ for the support of glycolytic ATP production (for review see Tadege *et al.*, 1999).

In this process, the regeneration of NAD^+ is coupled to the ADH-catalysed conversion of acetaldehyde to ethanol. Plants mutated in *ADH* grow well, except under hypoxic conditions (Freeling and Bennet, 1985). In contrast to vegetative tissues the flux through the ethanolic fermentation pathway in tobacco pollen is primarily controlled by sugar supply rather than by oxygen availability (Bucher *et al.*, 1995; Tadege and Kuhlemeier, 1997). It was proposed that at low sugar levels pyruvate is converted into acetyl-CoA by pyruvate dehydrogenase (PDH), whereas at high sugar levels pyruvate will be shunted into the ethanolic fermentation pathway, resulting in the production of significant amounts of acetaldehyde and ethanol (Tadege and Kuhlemeier, 1997). This is reminiscent of what is observed in *Saccharomyces cerevisiae*, where ethanol production is known to take place at high sugar levels (Pronk *et al.*, 1996).

The two enzymes responsible for ethanolic fermentation, present in tobacco pollen at very high levels, are pyruvate decarboxylase (PDC), which converts pyruvate into acetaldehyde plus carbon dioxide, and alcohol dehydrogenase (ADH), which converts acetaldehyde into ethanol (Bucher *et al.*, 1995; Tadege and Kuhlemeier, 1997) For full reactions see Figure 1.

Since ethanolic fermentation is so prominent in pollen it might be expected that *ADH* mutants have an impaired fertility. However, *ADH* mutants appear to be perfectly fertile and heterozygotes segregate in the normal Mendelian ratios (Freeling and Bennet, 1985). In *ADH* null

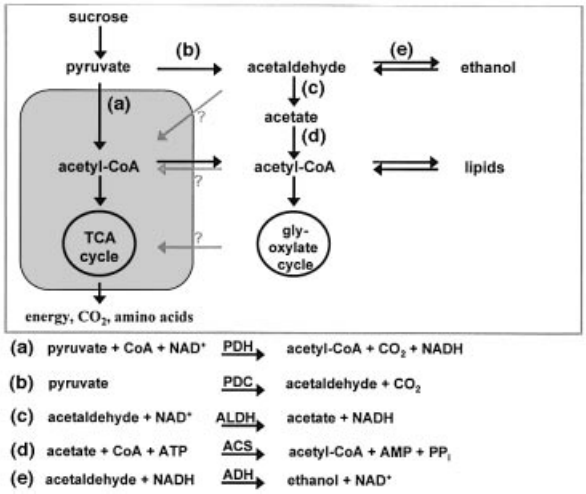

Figure 1. Proposed model for pyruvate utilization in pollen.
Pyruvate is converted into acetyl-CoA by PDH (a) and enters the TCA-cycle. Pyruvate can also be converted into acetyl-CoA by the concerted action of the enzymes PDC (b), ALDH (c) and ACS (d). Acetaldehyde can be converted to ethanol by ADH (e).
The acetyl-CoA produced by the PDH-bypass can enter the TCA-cycle in the form of succinate, which is produced in the glyoxylate cycle, or it can be directed to lipid synthesis. PDH = pyruvate dehydrogenase; PDC = pyruvate decarboxylase; ALDH = aldehyde dehydrogenase; ACS = acetyl-CoA synthetase; ADH = alcohol dehydrogenase.

mutant pollen, ethanol production and regeneration of NAD would not be possible. This suggests that in pollen the main purpose of ethanolic fermentation may not be the support of glycolytic ATP production.

Therefore, we decided to investigate, whether aerobic fermentation in pollen could serve similar functions as in yeast. In this organism both sugars and ethanol can be metabolised. Sugars are converted into pyruvate, which can then be further metabolised to acetyl-CoA through two alternative reaction sequences. The first reaction sequence occurs in the PDH-complex and the second reaction sequence involves the action of the enzymes PDC, aldehyde dehydrogenase (ALDH) and acetyl-CoA synthetase (ACS), which together constitute the so-called PDH-bypass (Pronk *et al.*, 1994). The ethanol produced through ethanolic fermentation is re-metabolised through ADH, ALDH and ACS (de Jong-Gubbels *et al.*, 1997; Wang *et al.*, 1998; Wasungu and Simard, 1982).

The enzymes PDH (Thelen *et al.*, 1999), a pollen specific PDC (PDC2) (Bucher *et al.*, 1995; Tadege and Kuhlemeier, 1997), ADH (Bucher *et al.*, 1995) and ALDH (Op den Camp and Kuhlemeier, 1997) have been detected in significant amounts in tobacco pollen. As in yeast, PDH and the PDH-bypass could function in the conversion of pyruvate into acetyl-CoA in tobacco pollen.

Recent work from Liu *et al.* (2001) suggests that the maize *rf2* gene, which encodes an aldehyde dehydrogenase, plays an important physiological role in anther development. Plants mutated in *Rf2* showed anther development arrest. Arrested anthers were smaller and failed to shed pollen. How exactly Rf2 is involved in anther development is not known. Work from Tadege and Kuhlemeier (1997) however, suggests a role for ALDH as part of a PDH-bypass. They demonstrated that ethanolic fermentation coincides with accumulation of ALDH in pollen late in anther development.

In pollen, the end product of the PDH-bypass, acetyl-CoA, could be available for respiration in the mitochondria and/or for synthesis of higher molecular weight compounds such as lipids and amino acids. In the glyoxylate cycle cytosolic acetyl-CoA could be converted into succinate and malate, molecules that can be transported into the mitochondria. A model describing pyruvate metabolism in tobacco pollen is depicted in Figure 1.

We hypothesize that in growing pollen tubes, the PDH-bypass functions in sugar metabolism, to support energy production and the biosynthesis of higher molecular weight compounds. To test this hypothesis, we used ^{14}C-ethanol to radiolabel growing tobacco pollen tubes. Ethanol can only enter general metabolism through ADH, ALDH and ACS, and thus is diagnostic for the PDH-bypass. Utilization of the PDH-bypass could result in the production of acetyl-CoA, which might be used for respiration, but also for biosynthetic purposes.

Results

Pollen tube growth on culture media designed for the radiolabelling experiments

To detect the accumulation of metabolic end products we supplied trace amounts of ^{14}C-ethanol and ^{14}C-sucrose to growing pollen tubes. Ethanol can readily be converted into acetaldehyde and thus enter the PDH-bypass. In contrast, sucrose can enter both main pathway and PDH-bypass. Since a high level of sucrose stimulates ethanol production (Bucher *et al.*, 1995), it was also desirable to test the effect of a high level of sucrose (100 mM) on the metabolism of ^{14}C-ethanol.

An essential requirement for the radiolabelling experiments was to design pollen culture media that allowed for optimal pollen tube growth. Therefore, growth speed of pollen tubes was analysed in several media (2 mM ethanol; 2 mM sucrose; 2 mM ethanol plus 2 mM sucrose; 2 mM ethanol plus 100 mM sucrose). Growth speed on these media was comparable (65 $\pm$ 8 μm h^{-1}; 67 $\pm$ 10 μm h^{-1}; 68 $\pm$ 9 μm h^{-1}; 74 $\pm$ 7 μm h^{-1}, respectively). For the radiolabelling experiments the media containing 2 mM ethanol plus 2 mM sucrose and 2 mM ethanol plus 100 mM sucrose were used. In these media either ethanol or sucrose was supplied as the ^{14}C labelled compound.

Table 1. Distribution of the label over the pollen fractions and the culture medium after 3 h of labelling, expressed as percentage of total applied radioactivity. Means and standard deviations are based on values from at least three independent experiments

	^{14}C-sucrose + 2 mM ethanol	^{14}C-ethanol + 2 mM sucrose	^{14}C-ethanol + 100 mM sucrose
Fraction	Mean ± SD	Mean ± SD	Mean ± SD
Amino acids	0.07 ± 0.05	0.28 ± 0.05	0.21 ± 0.02
H_2O soluble	2.42 ± 0.36	0.41 ± 0.09	0.32 ± 0.02
CO2	2.78 ± 0.55	4.44 ± 0.91	2.14 ± 0.53
lipids	0.50 ± 0.16	2.27 ± 0.20	2.25 ± 0.21
Culture medium	88.9 ± 3.35	88.1 ± 1.95	88.8 ± 1.98
Total retrieved from 100% input	94.7 ± 2.88	95.5 ± 1.19	94.0 ± 1.49

The PDH-bypass can be used to support energy production and lipid biosynthesis

Pollen were incubated for up to 3 hours in the synthetic media, in the presence of ^{14}C-ethanol or ^{14}C-sucrose. After extraction of the pollen, label from ^{14}C-ethanol and ^{14}C-sucrose could be detected in CO_2, lipids, amino acids and the residual H_2O soluble material (Table 1). Over the time period of 3 hours the increase in incorporation into the fractions was approximately linear (data not shown).

It was observed that incorporation of ^{14}C-ethanol in these compounds also took place at high sucrose levels, i.e. at a high level of ethanolic fermentation (Table 1). Thus, ethanol metabolism occurs under conditions that allow a flux through the PDH-bypass.

Incorporation into amino acids was low on both ^{14}C-ethanol and ^{14}C-sucrose. On ^{14}C-sucrose most label was incorporated into CO_2 and H_2O soluble compounds. On ^{14}C-ethanol however, most label was incorporated into CO_2 and lipids. Assuming that the incorporation from ^{14}C ethanol accurately reflects the flux through the bypass, it can be calculated that the bypass accounts for approximately 20% of the total flux from sucrose. The calculation is based on the assumption that sucrose labelling (5.8%, Table 1) reflects the flux through both main pathway and bypass, while labelling with ethanol (7.4%, Table 1) reflects the flux through the bypass. 0.5 ml of culture medium contains 2 μmol C-atoms in ethanol (1 μmol of ^{14}C-ethanol) whereas 12 μmol C-atoms are present in sucrose (1 μmol ^{14}C-sucrose). This would mean that 5.8% of 12 μmol C-atoms from sucrose and 7.4% of 2 μmol C-atoms from ethanol are metabolised by the pollen.

The assumption is only correct if ^{14}C-ethanol freely exchanges with the internal acetaldehyde pool. Moreover, if the internal acetaldehyde pool is large relative to the externally supplied ^{14}C-ethanol, the flux through the bypass will be underestimated. This may well be the case, especially at high sucrose concentrations (Bucher *et al.*, 1995).

Taken together, the results indicate that ^{14}C-ethanol can be used significantly for energy production as well as for the biosynthesis of lipids.

Label of ^{14}C-ethanol and ^{14}C-sucrose is incorporated into amino acids

Assuming that ^{14}C-sucrose will be used through PDH and the PDH-bypass, whereas ^{14}C-ethanol will only be used through the PDH-bypass, the resulting incorporation patterns in lipids and amino acids may differ for both labelled carbon sources. Therefore we determined the identity of the individual amino acids and lipids.

Amino acid fractions of radiolabelled pollen were purified and separated by HPLC. Incorporation into a number of amino acids could be detected (Figure 2). Differences that can be observed are the high incorporation of label from ^{14}C-sucrose into alanine or threonine (the HPLC method used did not allow discrimination between incorporation into alanine and threonine), whereas glycine is the highest labelled amino acid after ^{14}C-ethanol labelling.

The formation of labelled alanine from ^{14}C-sucrose can easily be explained by direct transamination of the glycolytic intermediate pyruvate. On ^{14}C-ethanol we expect no formation of sugars (Figure 1) and thus, no pyruvate and alanine formation. Indeed, no significant incorporation into ^{14}C-alanine could be detected. Instead, glycine and/or glutamine and serine and/or asparagine were labelled under these conditions. Glycine and serine are synthesized through the photo-respiratory pathway, although there is no evidence that this pathway functions in pollen. In yeast, glycine and serine biosynthesis proceed by two pathways, a 'glycolytic' pathway, using 3-phos-

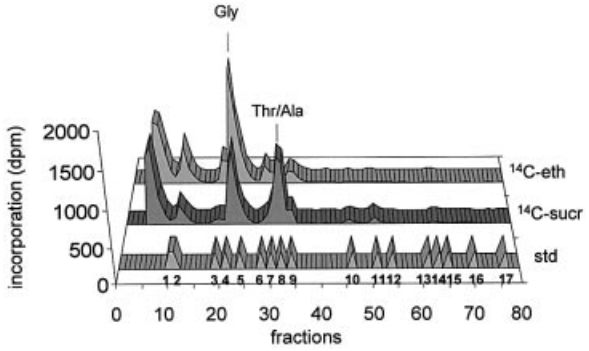

Figure 2. ^{14}C-labelled amino acids separated by HPLC. (back) Amino acids of pollen labelled with ^{14}C-ethanol. (middle) Labelled with ^{14}C-sucrose. (front) Non-radioactive amino acid standard solution (std), containing: (1) Asp, (2) Glu, (3) Ser/Asn, (4) Gly/Gln, (5) His, (6) Arg, (7) Thr, (8) Ala, (9) Pro, (10) Tyr, (11) Val, (12) Met, (13) Cys, (14) Ile, (15) Leu, (16) (Phe), (17) Lys.

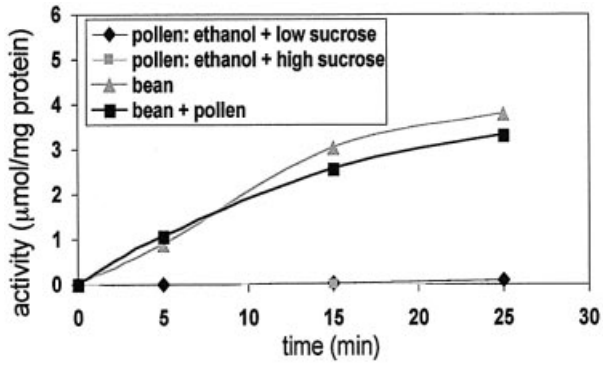

Figure 3. Malate synthase activity from pollen tubes grown on ethanol and sucrose.

phoglycerate, and a 'gluconeogenic' pathway, using glyoxylate (Melcher and Entian, 1992).

Minor amounts of radioactivity were also found in aspartate, glutamate, arginine, threonine and proline.

Taken together, on ^{14}C-ethanol label accumulated only in amino acids from TCA-cycle and possible photorespiratory related pathways, while the shikimate and pyruvate derived amino acids were not labelled.

The absence of malate synthase activity in germinated tobacco pollen indicates that the glyoxylate cycle is not operative.

Conceivably, amino acids could be synthesized from ethanol through the action of the glyoxylate cycle, which products malate and succinate can be transported into mitochondria.

The enzymatic activity of malate synthase in germinated tobacco pollen was measured and found to be undetectable (Figure 3). MS activity in the positive control, cotyledons of germinated common bean (*Phaseolus vulgaris* L.), was significant. To exclude the possibility that some pollen factor in the protein extract was responsible for inactivation of MS, proteins samples of pollen and bean were mixed. The addition of the pollen extract to the bean extract did not significantly decrease MS activity. Thus, we conclude that MS activity in pollen is absent.

The absence of sucrose could induce activity of the enzymes ICL and MS of the glyoxylate cycle (Kudeilka and Theimer, 1983) and could lead to the induction of expression of the corresponding genes (Ismael *et al.*, 1997; McLaughlin and Smith, 1994). As a consequence, pollen tubes that grow exclusively on the two-carbon compound ethanol, may use the glyoxylate cycle to produce anaplerotic substrates for the TCA-cycle (Eastmond and Graham, 2001). MS activity was measured in total protein extracts of pollen cultured on 2 mM ethanol for 4 hours. No enzyme activity could be detected (data not shown). The results suggest that the glyoxylate cycle does not operate in tobacco pollen metabolism during tube growth.

Label of ^{14}C-ethanol and ^{14}C-sucrose was incorporated into the same lipid classes but the distribution over the individual fatty acids differed

To determine which lipid classes were synthesized from ^{14}C-ethanol and ^{14}C-sucrose, the lipid extract was separated on thin layer chromatography (TLC) plates.

One-dimensional separation and autoradiography revealed that label of both ^{14}C-ethanol and ^{14}C-sucrose were incorporated mainly into the polar lipids and triacylglycerols, while minor amounts of label appeared in free fatty acids, sterols and some unidentified components (Figure 4a).

The 2-dimensional separation and autoradiography of polar lipids demonstrate that from both precursors the label was predominantly incorporated into the phospholipids (Figure 4b,c). With ^{14}C-sucrose also the galactolipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) were labelled to a small extent.

Although in general the same lipid classes were synthesized on ^{14}C-ethanol and ^{14}C-sucrose, it is possible that these lipids contain different fatty acids. In order to localize the label in the different building blocks of lipids, alkaline hydrolysis was carried out yielding fractions of fatty acids, non-saponifiable and water-soluble polar moieties.

With both ^{14}C-ethanol and ^{14}C-sucrose as precursors most label appeared in the fatty acids, accounting for about 70% of the total. Minor amounts of radioactivity appeared in the non-saponifiable and water-soluble fractions, as shown in Figure 5a.

The separation of fatty acids according to their unsaturation revealed that saturated as well as mono- and dienoic

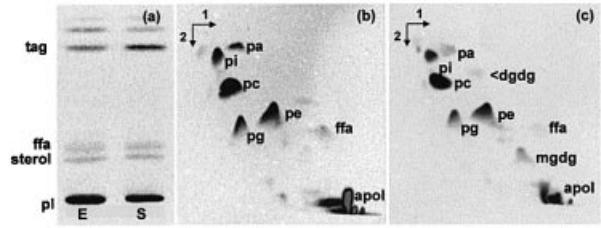

Figure 4. Analysis of labelled polar and apolar lipids.
(a) One-dimensional TLC. E = ^{14}C-ethanol; S = ^{14}C-sucrose.
(b) 2D-TLC of ^{14}C-ethanol-derived lipids. 1 = 1th dimension; 2 = 2nd dimension.
(c) 2D-TLC of ^{14}C-sucrose-derived lipids.
(pa = phosphatidic acid, pc = phosphatidyl choline, pe = phosphatidyl ethanolamine, pg = phosphatidyl glycerol, pi = phosphatidyl inositol, ffa = free fatty acids, apol = apolar lipids, dgdg = digalactosyl diacylglycerol, mgdg = monogalactosyl diacylglycerol, tag = triacylglycerol, pl = polar lipids.)

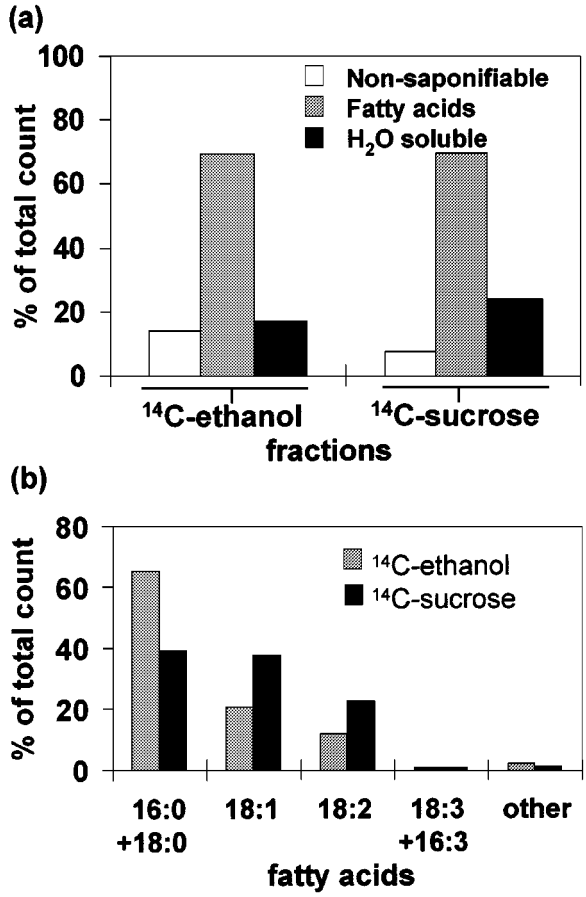

Figure 5. Incorporation of ^{14}C-ethanol and ^{14}C-sucrose into fatty acids, non-saponifiable and water-soluble moieties.
(a) Distribution of the label among the different building blocks.
(b) Distribution of the label among the different fatty acids.
Data presented refer to the mean of duplicate samples.

fatty acids were labelled, whereas almost no label ended up in the trienoic acids. Interestingly, on ^{14}C-sucrose the incorporation into mono- and dienes (at the expense of saturated fatty acids) was higher than on ^{14}C-ethanol (Figure 5b).

The observed quantitative differences in the distribution of label over the fatty acids may have been determined by the pathway(s), through which the flux of ^{14}C-label took place.

Discussion

The results presented in this work show that tobacco pollen can incorporate radiolabelled ethanol into a variety of compounds (Table 1). Based on the data we suggest that pyruvate can be converted to CO_2 both through PDH and through a PDH-bypass, which encompasses the enzymes PDC, ALDH and ACS (Figure 1). Moreover, this pathway appears to support the biosynthesis of amino acids and, importantly, lipids. No glyoxylate cycle activity was present and thus the bypass does not serve a gluconeogenic function.

Labelled compounds derived from ^{14}C-ethanol are used for energy production in the mitochondria.

The high incorporation of label from ^{14}C-ethanol into CO2 and the incorporation of label into amino acids derived from TCA-cycle intermediates, suggests that the PDH-bypass can support respiration by providing compounds to the TCA cycle.

In *Brassica napus* pollen mRNAs of isocitrate lyase (ICL) and malate synthase (MS) were detected at low levels and the glyoxylate cycle is thought to be functional there (Zhang *et al.*, 1994). The data of Figure 3 indicate that malate synthase activity is below the detection level in tobacco pollen, and that the conversion of acetyl-CoA into malate or succinate by the glyoxylate cycle does not take place at a significant rate.

Direct transport of acetyl-CoA into mitochondria might occur. However in plants, the existence of a carnitine/acetylcarnitine shuttle for transport of acetyl-CoA into mitochondria has remained unproved (Roughan *et al.*, 1993). Instead, the PDH-bypass intermediate acetaldehyde could be the compound that enters mitochondria. In plants, specific ALDHs are localized in the mitochondria (Liu *et al.*, 2001; Nakazono *et al.*, 2000) and in the cytoplasm (Li *et al.*, 2000). Therefore, in pollen mitochondria acetaldehyde could be converted into acetate by ALDH.

How acetate could be converted into acetyl-CoA in the mitochondria is not known. Acetyl-CoA synthetase, the enzyme responsible for this conversion is thought to be plastidial (Ke *et al.*, 2000).

Incorporation of label from ^{14}C-ethanol into lipids

The radiolabelled lipids derived from ^{14}C-ethanol, probably became part of the large lipid pool that had been accumulated during pollen development. Most likely, these lipids become integrated into the endoplasmatic reticulum, dictyosomes, secretory vesicles and the rapidly extending plasma membrane of the growing pollen tube (Dorne *et al.*, 1988; Kappler *et al.*, 1986).

The comparison of lipid labelling obtained on ^{14}C-sucrose with that on ^{14}C-ethanol revealed a similar distribution of label over the lipid classes (Figure 4). This shows that at the level of lipid classes, the observed difference in total incorporation (Table 1) is a quantitative rather than a qualitative one. Thus, fatty acids produced from precursors that originated from the main pathway and the PDH-bypass were all directed to the same lipid biosynthesis machinery.

Importance of PDH main pathway, PDH-bypass and ethanol production

Previous work has established that protein levels of PDC and ADH are high in tobacco pollen (Bucher *et al.*, 1995; Tadege and Kuhlemeier, 1997). The flux through PDC and ADH increased at high glucose levels and it was estimated that under such conditions more than half of the glucose was fermented rather than respired (Bucher *et al.*, 1995; Tadege and Kuhlemeier, 1997). In the present experiments we estimate that at least 20% of the metabolized sucrose flows through the PDH-bypass. Thus, flux through PDC is significant.

Work on maize shows that mitochondrial ALDH is encoded by *Rf2*, the nuclear restorer of T-type cytoplasmic male sterility (Liu *et al.*, 2001). In maize, ALDH loss-of-function mutants compromise pollen development, supporting the notion that ALDH plays a role not only as a restorer of fertility but in normal pollen development, as well.

It could even be questioned whether the main pathway through mitochondrial PDH is at all present in pollen. Tobacco pollen germinate normally on the PDH-inhibitor 1-aminoethylphosphinate (AEP), but fail to grow on the ALDH-inhibitor disulfuram (Laber and Amrhein, 1987; Op den Camp and Kuhlemeier, 1997). This suggests that PDH is dispensable in pollen. On the other hand, the E1α subunit of PDH is present at high levels in pollen (Thelen *et al.*, 1999).

To assess the importance of PDH, the PDH-bypass and ethanol fermentation for pollen tube growth, the generation of mutant pollen, which lack activity of ADH, PDH and/or one of the PDH-bypass enzymes, is highly desirable. The generation of mutant pollen and its genetic and biochemical analysis may shed light on the functions of these pathways in pollen metabolism.

Experimental procedures

Pollen tube growth on culture media designed for the radiolabelling experiments

Pollen were collected from *Nicotiana tabacum* Samsun plants and stored at –80°C until further use.

Growth medium for culture of pollen tubes was essentially according to Read *et al.* (1993), except that the medium did not contain PEG, and 2 mM NH_4Cl instead of casein acid-hydrolysate. Media were filter-sterilized with FP30 0.2 μm filter units (Schleicher & Schuell, Riehen, Switzerland) and stored at –20°C until required.

Pollen were cultured for 1 hour in growth medium containing 100 mM sucrose and then transferred to medium containing 2 mM ethanol, 2 mM sucrose, 2 mM ethanol plus 2 mM sucrose or 2 mM ethanol plus 100 mM sucrose.

Pollen tubes were observed and photographed with a SMZ 1500 microscope (Nikon, Küsnacht, Switzerland) coupled to a DKC-5000 digital photo camera (Sony, Schlieren, Switzerland).

Radiolabelling with ^{14}C-ethanol and ^{14}C-sucrose

Radioactive labelling. Samples of 3.0 ± 0.05 mg pollen were incubated in 65 × 18 mm glass bottles with screwcaps (Merck, Dietikon, Switzerland) in 500 μl of culture medium. Pollen were pre-incubated for 1 hour in a medium without radioactive carbon source, to allow for germination. The pre-incubation medium contained 100 mM sucrose as a carbon source. After the pre-incubation the pollen were transferred to culture medium containing 2 mM ethanol plus 2 mM sucrose in which one of the two carbon sources was labelled, or 2 mM ^{14}C-ethanol plus 100 mM sucrose. Pollen samples were always incubated with 1.0 μCi of the labelled carbon source. 1–^{14}C-ethanol was purchased from Hartmann Analytic (Zürich, Switzerland) and uniformly labelled ^{14}C-sucrose was purchased from Anawa (Wangen, Switzerland). The 3.0 mg pollen samples were incubated for 0, 10, 15, 30, 60, 120 or 180 minute periods. $^{14}CO_2$ was trapped in a well containing 250 μl 2 M KOH. At the end of the labelling period pollen samples were quickly collected on 13 mm ∅ cut 595 filter paper (Schleicher & Schuell, Riehen, Switzerland) in SX filter holders (Millipore, Volketswil, Switzerland) and washed with 10 ml non-radioactive medium.

Extraction of the pollen was done essentially according to Bligh and Dyer (1959). Lipids in the chloroform solution were cleaned of water and methanol on a column containing anhydrous Na_2SO_4 (Fluka, Buchs, Switzerland). Amino acids in the water-methanol solution were purified over a column containing Dowex 50 W X 8 (Fluka, Buchs, Switzerland).

Scintillation counting. To determine radioactivity a 100-μl sample of each fraction was added to 2 ml Opti-Fluor scintillation cocktail (Packard, Groningen, The Netherlands) in 6.5 ml snap-twist vials (Semadeni, Bern, Switzerland) and counted with a LS 6500 scintillation counter (Beckmann, Nyon, Switzerland). Incorporation into the total fractions was calculated.

Incorporation for every fraction was calculated as the percentage of the input count, the input count being 1.0 μCi. Averages

and standard deviations were calculated from at least three independent experiments.

Analysis of lipids and amino acids

HPLC of amino acids. Amino acids were purified as described above. Purified amino acids were coupled to phenyl-isothiocyanate (Pierce, Lausanne, Switzerland) and samples containing equal amounts of radioactivity were separated by HPLC on a System Gold solvent module 126 coupled to detector module 166 (Beckmann, Nyon, Switzerland) on a Nova-Pak C_{18} 5μ column (Millipore Waters, Volketswil, Switzerland).

Fractions were collected in 6.5 ml snap-twist vials on an LKB Redifrac automated fraction collector (Pharmacia, Dübendorf, Switzerland) at a rate of 5 fractions per minute. Fractions were counted as described above (scintillation counting). To identify single amino acids on the chromatogram an amino acid standard solution (product no. 20088, Pierce, Lausanne, Switzerland) was separated under identical conditions. The method used did not allow us to fractionate the amino acids alanine and threonine in such a way that we could determine which of these two amino acids was significantly labelled (Figure 2).

One-dimensional thin layer chromatography of apolar lipids. Pollen lipids (50000 dpm) were applied as a streak on a 20 × 10 cm silicagel plate (Merck, Dietikon, Switzerland) using a linomat III automatic streak applicator (Camag, Muttenz, Switzerland). Lipids were separated with petroleum ether-diethylether-acetic acid (70 : 30 : 1, v/v/v). Dried plates were sprayed with primuline and viewed under UV light (366 nm). To identify lipids 30 nmoles of free fatty acid 18 : 3 (ffa), triacylglycerol (TAG) and sterol were used as standards.

Two-dimensional thin layer chromatography of polar lipids. Pollen lipids plus (20000 dpm) plus 1 mg of non-labelled leaf lipids as carrier were spotted on a 20 × 20 cm silicagel plate (Merck, Dietikon, Switzerland). For separation in the first dimension, chloroform-methanol-water (65 : 25 : 4, v/v/v) and for separation in the second dimension chloroform-methanol-isopropylamine-ammonia (65 : 35 : 0.5 : 5, v/v/v/v) were used as solvents (Hofmann and Eichenberger, 1997). Lipid spots were detected under UV light (366 mm) after spraying the plate with 2′,7′-dichlorofluorescein (0.05% in ethanol). For the identification of single components a TLC plate was loaded with 0.8 mg non-radioactive tobacco leaf lipids and sprayed with specific reagents. Phospholipids were stained with molybdenum blue, phosphatidylcholine (pc) was identified by staining with Dragendorff reagent, phosphatidylethanolamine (pe) was identified by spraying with ninhydrin and glycolipids were identified with anthrone reagent (Vogel and Eichenberger, 1990).

A Cs 170–7328 molecular imaging screen (Biorad, Glattbrugg, Switzerland) was exposed to the TLC plate for 1 week. To visualize the radioactive labelled lipids the imager screen was scanned with a Molecular Imager System GS 525 (Biorad, Glattbrugg, Switzerland). Identified radioactive spots were scraped from the plates, the lipids dissolved in 500 μl chloroform, mixed with 4 ml scintillation cocktail and counted as described above.

Lipid hydrolysis. To labelled pollen lipids, 0.4 mg non-radioactive leaf lipid was added. Then, 1 ml of KOH-H_2O-ethanol (1 : 2 : 20, g/v/v) was added and the mixture was kept at 70°C for 30 min After adding 1 ml of H_2O, the alkaline solution was washed 4 times with 2 ml hexane. Pooled hexane portions were washed with 0.5 ml 0.5 M NaOH. After evaporating the solvent the residue contained the non-saponifiable lipids. Alkaline solutions were pooled, acidified with 0.15 ml HCl (32%) and then washed 4 times with 2 ml hexane. Pooled hexane portions containing free fatty acids were washed with 0.5 ml H_2O. After evaporation of the solvent, fatty acids were esterified with diazomethane (Vogel and Eichenberger, 1992). The lipid hydrolysis experiments were performed *in duplo*.

Ag-TLC of fatty acid methyl esters. Samples were spotted on a TLC plate (Merck, Dietikon, Switzerland), which had been soaked in a solution of $AgNO_3$ (10% in acetonitrile) and dried for 2 min in a warm air stream. The plate was developed in toluene-acetonitrile (97 : 3, v/v) and spots were visualized under UV light after spraying with dichlorofluorescein. Rf of saturated, monoene, diene and triene compounds were 0.79, 0.63, 0.51 and 0.34, respectively. The Ag-TLC experiments were performed *in duplo*.

Scintillation counting. To lipid samples or silicagel scraped from (Ag-)TLC plates, 2–6 ml methanol and 5 ml toluene containing 0.7% butyl-PBD were added. A TriCarb 2000CA Liquid Scintillation Analyzer (Packard, Groningen, The Netherlands) was used for counting.

Malate synthase activity

Common beans (*Phaseolus vulgaris* L.) were germinated on humid soil for 5 days in the dark. Pollen were incubated as decribed above for the labelling experiments, with non-labelled carbon sources.

Protein was extracted from germinated pollen and bean cotyledons in 50 mM Tris/Mes pH 7.8, 0.3 M sorbitol, 1% PVP40, 5 mM EGTA, 10% glycerol, 1 mM DTT and 1 mM PMSF. For the *in vitro* assay the complete reaction mixture contained 84.5 mM Tris/HCl pH 8.0, 0.03% Triton X–100, 4 mM $MgCl_2$, 0.1 mM DTNB, 0.1 mM acetyl-CoA, 1.5 mM glyoxylate and 150 μg protein extract. To check the pollen extract for the presence of an inhibitory factor, 150 μg protein extracts of the pollen and the bean cotyledons were mixed (1 : 1). The adsorbance was measured at 412 nm over a period of 25 minutes (Miernyk *et al.*, 1979). Averages and standard deviations of activity of three protein samples were calculated.

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