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Aerobic fermentation in tobacco pollen

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Abstract

We characterized the genes coding for the two dedicated enzymes of ethanolic fermentation, alcohol dehydrogenase (ADH) and pyruvate decarboxylase (PDC), and show that they are functional in pollen. Two PDC-encoding genes were isolated, which displayed reciprocal regulation: PDC1 was anaerobically induced in leaves, whereas PDC2 mRNA was absent in leaves, but constitutively present in pollen. A flux through the ethanolic fermentation pathway could be measured in pollen under all tested environmental and developmental conditions. Surprisingly, the major factor influencing the rate of ethanol production was not oxygen availability, but the composition of the incubation medium. Under optimal conditions for pollen tube growth, approximately two-thirds of the carbon consumed was fermented, and ethanol accumulated into the surrounding medium to a concentration exceeding 100 mM.

Introduction

Alcohol dehydrogenase (ADH) is one of the most important models in plant genetics. Over the past thirty years numerous publications have dealt with the isolation and characterization of various ADH mutants and with the structure and regulation of the ADH-encoding genes [14, 15, 20, 31, 36, 39; for reviews of older literature, see 18, 21]. Detailed studies have shown that ADH gene expression is under strict developmental and environmental control. Under normal growth conditions, for example, the ADH gene is expressed at low levels in most tissues, but when the plant is deprived of oxygen, ADH mRNA accumulates to high levels [18, 21]. There is ample evidence to indicate that the capacity of ethanolic fermentation is a key factor in flooding tolerance [10, 42, 43].

In plants grown under normal oxygen tension ADH mRNA levels are low in most tissues, one of the exceptions being pollen. During the later stages of pollen development, after microspore mitosis, ADH becomes one of the major proteins [47]. The function of ADH during pollen development has not been investigated. Could it function in ethanolic fermentation, similar to its role

The nucleotide sequence data reported will appear in the GenBank and EMBL Nucleotide Sequence Databases under the accession numbers X81853 (TobADH1), X81854 (TobPDC1) and X81855 (TobPDC2).

during oxygen limitation in other tissues? ADH catalyzes a reversible reaction, and it also accepts other primary alcohols besides ethanol [3]. If ADH is used to synthesize ethanol, the activity of pyruvate decarboxylase (PDC), the first enzyme in the pathway, is also required. This enzyme is known to be induced in maize roots, but the presence of PDC in pollen has not been investigated [27, 40].

In recent work, we manipulated the ethanolic fermentation pathway in the leaves of tobacco plants by expressing a PDC gene derived from the obligate anaerobe *Zymomonas mobilis* [6]. In the transgenic plants ethanol production was approximately ten times higher under anoxic incubation. This work led to two conclusions that are relevant here. First, in tobacco leaves PDC synthesis is the rate-limiting step in ethanol production, whereas the ADH enzyme is present in sufficient quantities. Second, pyruvate preferentially enters the respiratory pathway and ethanol only accumulates when respiration is inhibited, either by anoxic incubation or by applying specific respiratory inhibitors. It has been well documented for many species that pollen begin to respire at a high rate immediately after rehydration [12, 22, 24]. Thus, if the regulatory mechanisms found in leaves operate in pollen, it seems unlikely that ethanolic fermentation will occur in pollen. On the other hand, in yeast the co-existence of respiration and fermentation is well known, the flux through the ethanolic pathway being primarily regulated not by oxygen availability but by carbon source (for review see [19]).

Here we report on the isolation of ADH and PDC genes from tobacco, and we show that gene products are used in tobacco pollen to synthesize ethanol at a considerable rate, even in an oxygenic atmosphere.

Materials and methods

Plant growth conditions

Pollen was harvested from greenhouse-grown *Nicotiana tabacum* plants using a 35 μm mesh connected to a vacuum cleaner. Pollen was either used directly or frozen at -80 °C. Only pollen with an *in vitro* germination rate of at least 80% was used. Before experiments, dry pollen was washed three times with diethyl ether, dried under vacuum and subsequently preimbided (hydrated) for 2 h in a humid chamber at 37 °C. Germination medium consisted of 18.75 mM 2-(*N*-morpholinoethane) sulphonic acid-KOH pH 5.9, 0.29 M sucrose, 1.6 mM H_3BO_3, 1 mM KCl, 1 mM $CaCl_2$, 0.8 mM $MgSO_4$, 0.3 μM $CuSO_4$, 0.1% casamino acids. Anaerobic incubations were carried out in an anaerobic workbench as described [7].

Molecular-genetic techniques

A cDNA library from pollen poly(A)$^+$ RNA [5] was screened at low stringency (5 × SSC, 48 °C [37]) with the insert of plasmid pZM793, which contains the maize ADH1 cDNA [11]. Two positive plaques were identified. The first one, designated TobADH1, was completely sequenced. TobADH2 was sequenced from the termini and turned out to be almost identical to TobADH1.

A tobacco PDC encoding fragment was isolated by reverserse transcriptase-coupled polymerase chain reaction (RT-PCR). Tobacco leaves were subjected to anoxia for 24 h and total RNA was isolated [53]. Six μg was reverse transcribed with AMV reverse transcriptase in a 15 μl reaction volume. One μl of the product was amplified with degenerate primers [7], corresponding to nucleotides 325 to 344 and 1598 to 1576 of the maize PDC cDNA sequence [27]. After 30 cycles of 1 min/94 °C, 1 min/50 °C and 3 min/72 °C, a band of the expected size of 1400 bp was electroeluted and cloned into the plasmid vector Bluescript KS(−). The resulting plasmid was designated tobPDC1. When the same procedure was applied to total or poly(A)$^+$ RNA from mature pollen, no PCR products were obtained. The TobPDC1 fragment was randomly labelled with ^{32}P-dCTP and used to probe the pollen cDNA library. One positive clone was obtained, which was named TobPDC2.

Northern blotting and DNA sequence analysis were performed according to standard methods. The following probes were used: for TobPDC1 the entire 1400 bp insert obtained by PCR; for TobPDC2, a fragment derived of the 3′ end of the cDNA (nt 1318–2098); for TobADH1, the entire insert of 1600 bp; for translation initiation factor eIF-4A, the entire 1370 bp insert of NeIF-4A10 [38]. The signals on the northern blots of Fig. 5 were quantified using a BioRad Model GS250 phosphorimager.

Sequence analysis was done with the University of Wisconsin GCG programs FETCH, FASTA, LINEUP and PRETTY.

Measurement of fermentation enzymes and products

PDC enzymatic activity in whole extracts was measured spectrophotometrically by coupling to ADH-catalyzed NADH oxidation [6].

For detection of acetaldehyde and ethanol by gas chromatography, 10 mg aliquots of preimbibed pollen were placed in 13 ml Lumac vials. For normoxic treatment, the vials were tightly capped with rubber gaskets and incubated for 2 h and 4 h in a 30 °C water bath without shaking. Aliquots of 1 ml were withdrawn with a gas-tight syringe and analyzed in a gas chromatograph as described before [6]. For anaerobic treatment the vials were placed in an anaerobic work bench and incubated for 2 h and 4 h at 30 °C.

O_2 consumption and CO_2 production were determined in a Gilson differential respirometer. Twenty mg of preimbibed pollen were suspended in 1 ml H_2O or germination medium in 19 ml Warburg vessels. After 10 min equilibration time, the vessels were closed and the gauges set to zero. Measurements were made at 10 min intervals. O_2 consumption was determined by trapping CO_2 with 0.3 ml 2 M KOH in the central compartment. During equilibrium and measurement the vessels were shaken at 88 rpm. The sum of O_2 consumption and CO_2 production was measured without KOH. The respiratory quotient RQ is the volume of CO_2 produced divided by the volume of O_2 consumed.

Ethanol concentrations in the liquid medium were determined enzymatically using a kit from Boehringer Mannheim (cat. no. 176 290).

Results

ADH gene structure and expression

A cDNA library prepared from poly(A)$^+$ RNA of mature tobacco pollen was screened at low stringency with a fragment of the maize ADH1 gene. One of the resulting cDNA clones, TobADH1, was highly similar to ADH sequences from other higher plants. Northern blot analysis was performed to determine the organ specificity of ADH gene expression in tobacco. As can be seen from Fig. 1A, in all organs tested the ADH

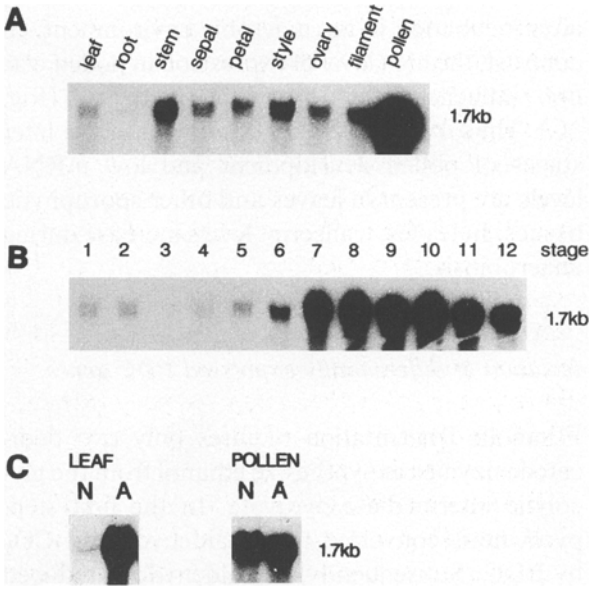

Fig. 1. Analysis of ADH mRNA expression. Ten μg of total RNA, isolated from each of the indicated organs, was separated on 1.2% glyoxal gels and blotted onto nylon membranes. The final washes were in 0.1× SSC at room temperature. A. Expression in various organs. B. Time course of expression during anther development. Stages were according to Koltunow *et al.* [29]. RNA prepared from stages 1–11 was from total anther tissue, that is, including sporophytic tissue. Stage 12 was dehisced pollen. C. Detached leaves (left panel) or pollen (right panel) were incubated for 4 h either under normoxic (N) or anoxic (A) conditions.

gene is expressed at low levels, except for pollen, in which a very high signal was detected.

Total RNA was isolated from anthers at different stages of development. Stage 0 coincides with meiosis, stage 6 with microspore mitosis, stage 12 is mature pollen [29]. In stages 1–11 RNA was isolated from total anthers, i.e., including sporophytic tissues that do not express ADH (and presumably PDC), whereas stage 12 RNA was prepared from pure pollen. Equal amounts of total RNA were analyzed on northern blots. Very low levels of ADH mRNA were found up to stage 6, then at microspore mitosis there was a strong increase, with a peak at stage 10, shortly before anthesis. At stage 12 the level declined, despite the fact that the RNA was isolated from pure pollen and not from whole anthers (Fig. 1B). In leaves grown under normal atmospheric conditions ADH was expressed at very low levels, but mRNA levels increased drastically after incubation in an anaerobic environment. In contrast, the high level of expression in pollen was not influenced by oxygen availability (Fig. 1C). Thus, ADH is highly expressed in the later stages of pollen development and low mRNA levels are present in leaves and other sporophytic tissues. In leaves, transcript levels increase during anaerobiosis.

Isolation of differentially expressed PDC genes

Ethanolic fermentation requires only two dedicated enzymes to synthesize ethanol from the glycolytic intermediate pyruvate. In the first step, pyruvate is converted to acetaldehyde and CO_2 by PDC. Subsequently, acetaldehyde is reduced to ethanol with concomitant reoxidation of NADH by ADH. If ADH functions in the reducing direction to synthesize ethanol, PDC ought to be present as well. Therefore, we decided to determine whether PDC is expressed in pollen.

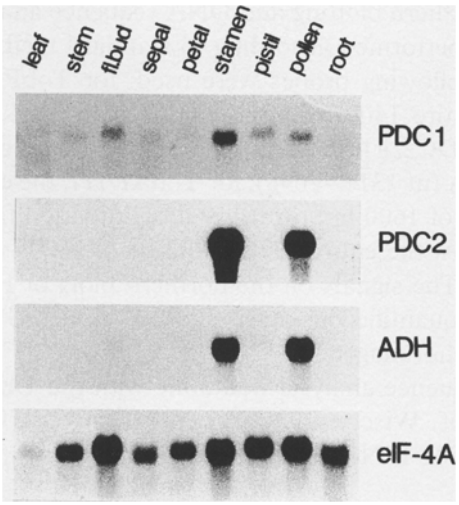

Fig. 2. Organ-specific expression of PDC genes. Northern blotting conditions were as in Fig. 1. Fl. bud, floral buds.

Screening of the pollen cDNA library with a maize cDNA probe did not yield hybridizing plaques. Similarly, primers based on conserved regions of the maize PDC sequence, which had previously enabled us to clone a cDNA from the marsh plant, *Acorus calamus* [7], failed to amplify first-strand cDNA from pollen poly(A)$^+$ RNA (results not shown). When the same primers were used on first strand cDNA derived from anoxically treated tobacco leaves, a fragment of the expected size of 1400 bp was readily obtained. This fragment was cloned and sequenced. The sequence is likely to encode a fragment of a functional PDC as it is closely related to the maize PDC (80% amino acid identity). Identity with the yeast and *Zymomonas* enzymes was 33% and 39%, respectively. This cDNA was designated TobPDC1, and was used as a probe to detect PDC transcripts in northern blot experiments (Fig. 2). PDC1 expression was found at low levels in all organs, including pollen. As a constitutive control we used a fragment of the gene coding for translation initiation factor eIF-4A. This gene was pre-

Fig. 3. Amino acid sequence comparison of PDC proteins. Amino acid sequences are from TobPDC1 (EMBL accession number X81854), TobPDC2 (X81855), maize PDC (M17555), yeast PDC (X04675, with corrections [25, 52]), and *Zymomonas mobilis* PDC (M15393). Dots indicate gaps. The underlined amino acids, T388, T390, G413, I415, D444, S446 and N471 of the yeast enzyme, are among the ones that contact the thiamine and Mg^{2+} cofactors, as inferred from the crystal structure [16].

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  TobPDC2                                MDGSVAKG TSCIQDSQSS SVIANTDATL  28
 MaizePDC      METLLAGNPA NGVAKPTCNG VGALPVANSH AIIATPAAAA ATLAPAGATL  50
 YeastPDC                                                    MSEITL   6
  ZymoPDC                                                      MSYT   4
Consensus

  TobPDC2       GRHLARRLVE IGIQDVFSVP GDFNLTLLDH LIAEPRLKNI GCCNELNAGY  78
 MaizePDC       GRHLARRLVQ IGASDVFAVP GDFNLTLLDY LIAEPGLTLV GCCNELNAGY 100
 YeastPDC       GKYLFERLKQ VNVNTVFGLP GDFNLSLLDK IYEVEGMRWA GNANELNAAY  56
  ZymoPDC       VGTYLAALVQ IGLKHHFAVA GDYNLVLLDN LLLNKNMEQV YCCNELNCGF  54
Consensus             L         F    GD NL LLD                NELN

  TobPDC1       AADGYARARG VGACVVTFTV GGLSVLNAIA GAYSENLPLI CIVGGPNSND
  TobPDC2       AADGYARARG VGACVVTFTV GGLSVLNAIA GAYSENLPVI CIVGGPNTND 128
 MaizePDC       AADGYARSRG VGACAVTFTV GGLSVLNAIA GAYSENLPVV CIVGGPNSND 150
 YeastPDC       AADGYARIKG MSCIITTFGV GELSALNGIA GSYAEHVGVL HVVGVPSISS 106
  ZymoPDC       SAEGYARAKA DAAAVVTYSV GALSAFDAIG GAYAENLPVI LISGAPNNND 104
Consensus        A GYAR          T  V G LS    I  G Y E         G P

  TobPDC1       YGTNRILHHT IGLQDFSQEP RCFQTVTCYR AVVNNLEDAH ELIDTAVSTA
  TobPDC2       YGTNRILHHT IGLPDFSQEL RCFQTVTCYQ AVVNNLDDAH EQIDRAISTA 178
 MaizePDC       YGTNRILHHT IGLPDFSQEL RCFQTITCYQ AIINNLDDAH EQIDTAIATA 200
 YeastPDC       QAKQLLLHHT LGNGDFTVFH RMSANISETT AMITDIATAP AEIDRCIRTT 156
  ZymoPDC       HAAGHVLHHA LGKTDYHYQL EMAKNITAAA EAIYTPEEAP AKIDHVIKTA 154
Consensus            LHH   G  D                            A    ID    T

  TobPDC1       LKESKPVYIS IGCNLPGIPH PTFSREPVPF ALSPRLSNMM GLEAAVEAAA
  TobPDC2       LKESKPVYIS ISCNLPAIPH PTFSRDPIPF SLSPRLSNKR GLEAAVDAAV 228
 MaizePDC       LRESKPVYIS VSCNLAGLSH PTFSRDPVPM FISPRLSNKA NLEYAVEAAA 250
 YeastPDC       YVTQRPVYLG LPANLVDLNV PAKLLQTPID MSLKPNDAES EKEVIDTILA 206
  ZymoPDC       LREKKPVYLE IACNIASMPC AAPGPASALF NDEASDEASL NAAVEETLKF 204
Consensus            PVY      N

  TobPDC1       EFLNKAVKPV LVGGPKMRVA KASDAFVELS DACGYAVAVM PSAKGLFPEH
  TobPDC2       TFLSKAVKPV MIGGPKLRVA KACDAFVELA DSSGYAMAVM LQPKGLVAEQ 278
 MaizePDC       DFLNKAVKPV MVGGPKIRVA KAREAFAAVA DASGYPFAVM PAAKGLVPEH 300
 YeastPDC       LVKDAKNPVI LADACCSRHD VKAETKKLID LTQF.PAFVT PMGKGSISEQ 255
  ZymoPDC       IANRDKVAVL VGSKLRAAGA EEAAVKFADA LG...GAVAT MAAAKSFFQK 251
Consensus

  TobPDC1       HSHFIGTYWG AVSTAFCAEI VESADAYLFA GPIFNDYSSV GYSLLLKKEK
  TobPDC2       HPHFIGTYWG AVGTSYCAEI VESADAYLFA GPIFNDYSSV GYSLLIKKEK 328
 MaizePDC       HPRFIGTYWG AVSTTFCAEI VESADAYLFA GPIFNDYSSV GYSLLLKREK 350
 YeastPDC       HPRYGGVYVG TLSKPEVKEA VESADLILSV GALLSDFNTG SFSYSYKTKN 305
  ZymoPDC       KTALHRYLMG EVSYPGVEKT MKEADAVIAL APVFNDYSTT GWTDIPDPKK 301
Consensus             G                 AD          D

  TobPDC1       AIIVQPDRVT IGNGPAFGCV LMRDFLAALA KRLKHNPTAF ENYHRIYVPE
  TobPDC2       SIIVQPDRVV IGNGPAFGCV LMKDFLSELA KKIKKNETAY ENYRRIFVPE 378
 MaizePDC       AVIVQPDRMV VGDGPAFGCI LMPEFLRALA KRLRRNTTAY DNYRRIFVPD 400
 YeastPDC       IVEFHSDHMK IRNATFPGVQ MKFVLQKLLT NIADAAKGYK PVAVPARTPA 355
  ZymoPDC       LVLAEPRSVV VNGVRFPSVH LKDYLTRLAQ KVSKKTGALD FFKSLNAGEL 351
Consensus

  TobPDC1       GHPLKCEPKE ALRVNVLFQH IQNMLSGDSV VIAETGDSWF NCQKLKLPKG
  TobPDC2       GTPLKSEPNE PLRVNVLFQH IQKMLSDETA VIAETGDSWF NCQKLKLPEG 428
 MaizePDC       REPPNGKPNE PLRVNVLFKH IKGMLSGDSA VVAETGDSWF NCQKLRLPEG 450
 YeastPDC       NAA..VPAST PLKQEWMWNQ LGNFLQEGDV VIAETGTSAF GINQTTFPNN 403
  ZymoPDC       KKAAPADPSA PLVNAEIARQ VEALLTPNTT VIAETGDSWF NAQRMKLPNG 401
Consensus                    L             L      V AETG_S F        P

  TobPDC1       CG........ .......... ........YE FQMQYGSIGW SVGATLGYAQ
  TobPDC2       CGYVTNNSLS AWYPFYLQTL EEKSSCCRYE FQMQYGSIGW SVGATLGYAQ 478
 MaizePDC       CG........ .......... ........YE FQMQYGSIGW SVGATLGYAQ 474
 YeastPDC       TY........ .......... ........GI SQVLWGSIGF TTGATLGAAF 427
  ZymoPDC       AR........ .......... ........VE YEMQWGHIGW SVPAAFGYAV 425
Consensus                                            G IG     A  G A

  TobPDC1       A....APEKR VIACIGDGSF QVTAQDISTM LRCGQRTIIF LINNGGY
  TobPDC2       S....VPKKR VISCIGDGSF QVTAQDVSTM IRCEQKNIIF LINNGGYTIE 524
 MaizePDC       A....AKDKR VIACIGDGSF QVTAQDVSTM LRCGQKSIIF LINNGGYTIE 520
 YeastPDC       AAEEIDPKKR VILFIGDGSL QLTVQEISTM IRWGLKPYLF VLNNDGYTIE 477
  ZymoPDC       G....APERR NILMVGDGSF QLTAQEVAQM VRLKLPVIIF LINNYGYTIE 471
Consensus              R  I   GDGS  Q T Q    M  R        F   NN GYTIE

  TobPDC2       VEIHD..GPY NVIKNWNYTG LVDAIHNGEG NCWTMKVRTE EELTEAIATA 572
 MaizePDC       VEIHD..GPY NVIKNWDYTG LVNAIHNSEG NCWTMKVRTE EQLKEAIAMA 568
 YeastPDC       KLIHGPKAQY NEIQGWDHLS LLPTF..GAK DYETHRVATT GEWDKLTQDK 525
  ZymoPDC       VMIHD..GPY NNIKNWDYAG LMEVFNGNGG YDSGAGKGLK AKTGGELAEA 519
Consensus         IH     Y N I  W     L

  TobPDC2       TGEKKDCLCF IEVIVHKDDT SKELLEWGSR VCSANGRPPN PQ*         614
 MaizePDC       TGDKKDCLCF IEVIVHKDDT SKELLEWGSR VSAANSRPPN PQ*         610
 YeastPDC       SFNDNSKIRM IEVMLPVFDA PQNLVEQAKL TAATNAKQ*              563
  ZymoPDC       IKVALANTDG PTLIECFIGR EDCTEELVKW GKRVAARQQP *           559
Consensus                                    E
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viously shown to be expressed in all plant organs, mRNA levels being highest in actively growing cells [37, 38].

The observation that the PDC1 probe hybridized to pollen mRNA was somewhat surprising as neither by screening the cDNA library with the maize PDC probe, nor by PCR-based methods had we been able to detect PDC gene expression in this organ (see above). These results could be explained by assuming that there exists a second PDC gene whose transcript does not hybridize to the maize cDNA or to the PCR primers used. The signal obtained in pollen would then be the result of cross-hybridization with the PDC1 gene. To test this, we rescreened the pollen library with the PDC1 probe and indeed obtained a full-length cDNA related to PDC1. This clone was designated TobPDC2. Over the area in which TobPDC1 and TobPDC2 can be compared they display 75% identity at the nucleotide and 83% identity at the amino acid level. TobPDC2 displays 79% identity to maize PDC, 35% to yeast PDC and 41% to *Zymomonas* PDC. Most strikingly, TobPDC2 contains a 26 amino acid insertion in the C-terminal part of the protein, which is present in no other PDCs (Fig. 3).

TobPDC2 is highly expressed in anthers and pollen, but no expression could be observed in sporophytic tissues (Fig. 2). In Fig. 4 the expression of the PDC genes during development of the anther is presented. In the top panel the PDC1 fragment was used as a probe. When the stringency of the post-hybridization washes was increased compared to the conditions employed in Fig. 2, the signal was reduced to a very low level. Thus, we assume that the PDC1 gene is not or hardly expressed during anther development. The 3′ part of the PDC2 cDNA hybridized to the anther RNAs in a pattern very similar to that seen for the ADH gene (compare Fig. 1B). As a control, we used again the gene encoding translation initiation factor eIF-4A. This gene is expressed at all stages of anther development with expression rising at microspore mitosis (between stages 6 and 7), in agreement with the general increase in transcriptional activity which occurs at this time [34, 51].

The experiments described above clearly show that in tobacco plants grown under standard conditions the two genes are expressed in different patterns. However, also under oxygen limitation they behave differently (Fig. 5). The PDC1 gene is not or only very lowly expressed in pollen, but in leaves mRNA levels increase about 20-fold upon anoxic induction. In contrast, the PDC2 gene is expressed in pollen, but is not expressed in leaves, irrespective of whether oxygen is present or not. ADH is expressed in pollen and in anoxically induced leaves, as observed before in tobacco and other plants. The translation factor

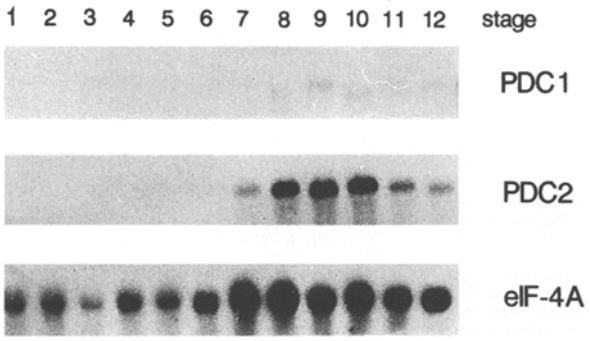

Fig. 4. Time course of expression of PDC genes during anther development. Stages were according to Koltunow *et al.* [29]. Northern blotting conditions were as in Fig. 1, except that the final wash was at 65 °C.

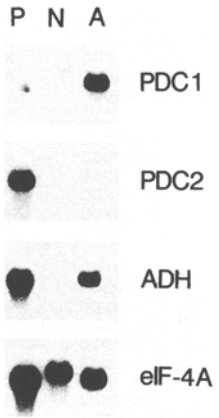

Fig. 5. Differential expression of PDC and ADH genes. Total RNA was isolated from dry pollen (P), from detached leaves incubated in air for 2 h (N), or for 2 h under anoxia (A). Northern blotting conditions were as in Fig. 2, except that the final wash was at 65 °C.

eIF-4A probe serves again as a control. These results show that the two PDC genes are expressed reciprocally: PDC1 is expressed in diploid plant tissues in response to oxygen limitation, PDC2 is constitutively expressed only in the male gametophyte.

Measurement of in vitro *PDC activity*

Several reports claim that gene expression during pollen development is under post-transcriptional control (for review, see [34, 51]). Therefore, we wished to determine whether PDC enzymatic activity was present in pollen. Pollen was collected from greenhouse-grown plants and total protein was extracted directly from dry pollen or from pollen imbibed under various conditions. PDC activity was measured using the same conditions as previously established for measuring *Zymomonas mobilis* PDC expressed in transgenic tobacco [6]. Activities of approximately 150 mU/mg protein were measured in dry pollen, which is much higher than the values obtained with pistils or with anoxically treated leaves (Fig. 6A). Oxygen availability had no influence on the measured activity, neither had the composition of the incubation medium (Fig. 6B and 6C). Therefore, pollen contains large amounts of PDC enzymatic activity, and this *in vitro* activity is not influenced by the environmental conditions under which the pollen are incubated.

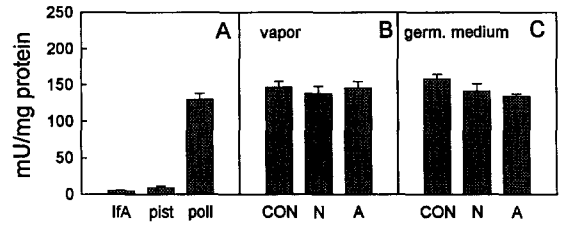

Fig. 6. PDC enzymatic activity. A. Activity was measured in leaves subjected to anoxia for 4 h (lfA), pistils (pist), and mature dry pollen (poll). B. Pollen preimbibed (CON), pollen preimbibed and subsequently for 4 h exposed to humid air (N) or anoxia (A). C. As B, except that incubation was in 0.1 ml germination medium on a piece of filter paper. Values represent the average of three individual measurements.

Activity of PDC and ADH in vivo

The previous experiments document that the enzymes of ethanolic fermentation are present in pollen at high concentration and lead to the most important question of whether there is a flux through the pathway in the normal course of pollen development. To test this hypothesis, pollen were hydrated in humid air and then transferred to closed containers, either in normal air or in an anaerobic environment. After 2 and 4 h, samples were withdrawn from the headspace and acetaldehyde and ethanol concentrations were determined by gas chromatography (Fig. 7). Both under normoxic and anoxic conditions acetaldehyde and ethanol were produced. Interestingly, accumulation of the end-product, ethanol, was only about twofold higher in the absence of oxygen.

From the results of Fig. 7 the quantitative importance of fermentation relative to respiration cannot be reliably calculated. For this, one would need to determine the exact volume of the imbibed pollen and the partitioning of acetaldehyde and ethanol between the gas phase and the living tissue, neither of which are known. In order to determine the quantitative importance of fermentation, respiration and ethanol production were measured in aqueous media. Incubation was ei-

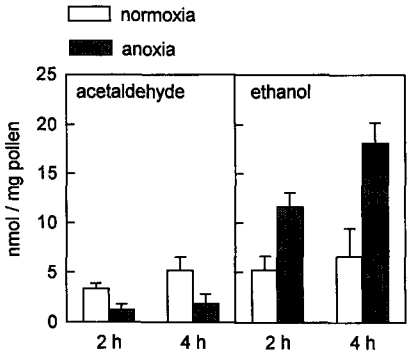

Fig. 7. Acetaldehyde and ethanol production in the presence and absence of oxygen. Pollen were preimbibed and transferred to closed containers, held at 30 °C, either in air, or in anoxic atmosphere. After 2 h and 4 h, 1 ml samples were withdrawn for analysis of acetaldehyde, and ethanol by gas chromatography. Values are the average of five individual measurements.

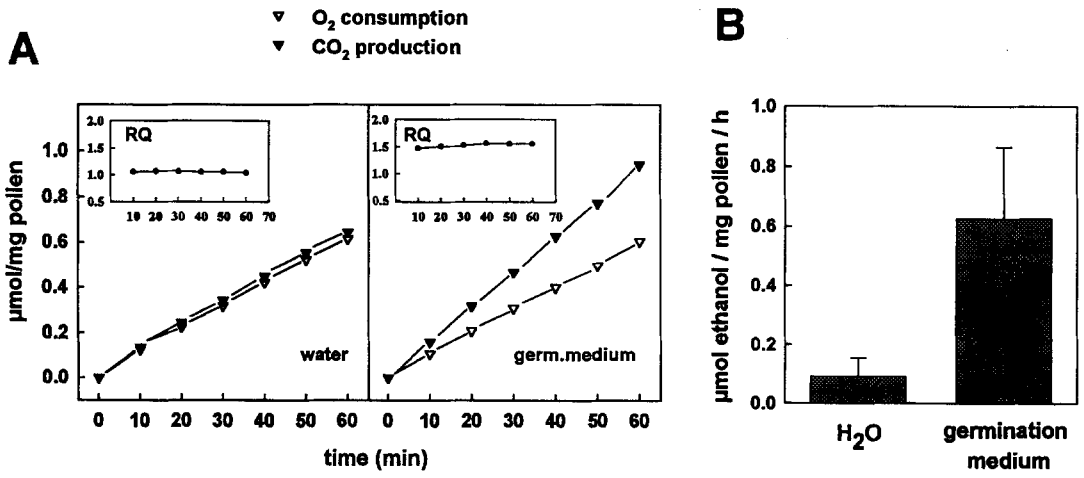

Fig. 8. Respiration and ethanol accumulation in pollen. A. O_2 consumption and CO_2 production were measured in a Warburg apparatus, as detailed in Materials and methods. B. After 1 h the vessels were opened and ethanol concentration in the medium was determined enzymatically. Each point is the average of at least 12 independent measurements.

ther in pure water or in a synthetic medium which is thought to mimic the chemical environment found on the stigma. Pollen germination in pure water was less than 5%, whereas in the germination medium it was at least 80%. Oxygen consumption and carbon dioxide production were measured manometrically in a respirometer. Incubation in H_2O gave rise to an O_2 consumption rate of 0.58 µmol per hour per mg pollen (Fig. 8A). CO_2 production was only marginally higher, yielding an RQ of 1.06 (RQ=CO_2 produced over O_2 consumed). Assuming that only sugars are being metabolized, that no other oxidative processes take place, and that the measurements are free of systematic errors, it can be calculated that 20% of the carbon is fermented. However, none of the above-mentioned assumptions need to be entirely correct, and therefore, ethanol production could in reality be negligible. In order to resolve this issue, ethanol in the medium was measured enzymatically. As can be seen from Fig. 8B, ethanol accumulated at a rate of 0.07 ± 0.05 µmol per hour per mg pollen, within the range of the calculated value.

In germinating pollen, the situation was very much different. While O_2 was consumed at a rate comparable to that found with hydrated, non-germinating pollen, CO_2 production was much higher, leading the RQ values between 1.5 and 1.6 (Fig. 8A). Ethanol accumulated at a rate of 0.6 µmol per hour per mg pollen (Fig. 8B). This means that in germinating pollen about two-thirds of the dissimilated carbon enters the ethanolic fermentation pathway.

Under conditions that favor pollen tube growth, ethanolic fermentation apparently is of major importance. We wondered to what levels ethanol would accumulate in the medium and at what level ethanol would become toxic. Various

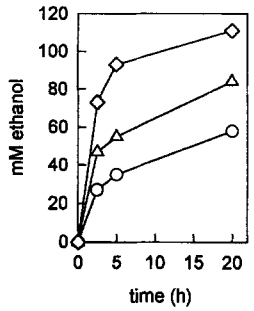

Fig. 9. Ethanol accumulation. Various quantities of pollen were weighed, hydrated, and suspended in 1 ml of germination medium. At the indicated intervals 100 µl samples were withdrawn and ethanol concentration was measured in the supernatant. Cultures were shaken at 90 rpm. ○, 19.2 mg pollen; △, 54.7 mg pollen; ◇, 99.7 mg pollen. One representative experiment is shown.

amounts of preimbibed pollen were incubated in a fixed volume of germination medium under normal atmospheric conditions and ethanol concentrations were determined at different time points. A maximum concentration exceeding 100 mM was reached after 20 h (Fig. 9). The value of 100 mM is approximately the concentration at which exogenously added ethanol becomes inhibitory for pollen tube growth (data not shown).

Discussion

The results described here demonstrate that ADH in pollen is active in the reducing direction, and, just as during oxygen deprivation in sporophytic tissues, it functions in the production of ethanol. However, the regulation of ethanolic fermentation in gametophyte and sporophyte is different. At the level of gene regulation, PDC is encoded by at least two distinct genes: PDC1 is induced during anoxia in leaves, whereas PDC2 is constitutively expressed in pollen only. Recently, the structure and function of yeast and *Zymomonas* PDC were analyzed by X-ray crystallography and site-directed mutagenesis [2, 8, 13, 16]. Inspection of the structure and comparison with the deduced amino acid sequences (Fig. 3) indicate that most of the residues that interact with the thiamine and Mg^{2+} cofactors are conserved between the PDCs from tobacco, maize, yeast and *Zymomonas*. The most striking feature of PDC2 is the presence of a 26 amino acid insertion relative to all other PDCs. This insertion is located between two highly conserved domains which are thought to be critical for catalytic activity. It will be interesting to determine how this extra sequence influences the properties of the enzyme.

Pollen were incubated under various environmental conditions, ranging from a normal atmosphere to complete absence of oxygen, and from pure water to a medium rich in growth supporting substances. It is not known exactly what environment the pollen encounters after landing on the stigma. The cells of the transmitting tract, which the pollen traverse on their way to the ovary, form a compact tissue without internal air spaces [26], and low oxygen tensions in stigmas have indeed been measured [33, 49]. Stigma exudates are chemically complex and their composition may vary considerably between species [28, 30]. *In vitro* media for pollen tube growth need to be optimized for each species [41, 50]. The medium used here promotes high rates of pollen tube germination and growth, and therefore, it may reasonably be assumed that it contains the essential components of the stigma exudate. Thus, it seems very likely that ethanol will also be produced *in vivo*, probably at a substantial rate. What is surprising is that ethanol accumulates to such high levels *in vitro* (Fig. 9). It could well be that *in vivo* the synthesized ethanol is remetabolized by the pistil through the action of ADH, aldehyde dehydrogenase and acetyl-coA synthetase, as has been proposed for other tissues [6, 9]. Anoxia only moderately stimulated ethanol production (Fig. 7), suggesting that oxygen availability is not a major inducer of fermentation. In contrast, incubation in germination medium led to an approximately ten-fold increase in ethanol production, compared to incubation in water (Fig. 8). In both water and germination medium the diffusion of oxygen to the pollen can be assumed to be constant, suggesting that not oxygen, but the availability of carbohydrates, or other components of the germination medium, and thereby the capacity to generate pollen tubes, had a major influence on the rate of ethanolic fermentation. This is very different from what we found in tobacco leaves [6]. In those experiments we also infiltrated a carbohydrate (glucose), but a flux through the fermentation pathway could only be measured when respiration was inhibited. It could be that during pollen tube growth respiration operating at a maximal rate is insufficient to fulfill the requirement for energy, and hence fermentation is used as an accessory energy-generating pathway. Such aerobic ethanolic fermentation may also be of importance in other fast growing tissues. Lateral buds and root tips display high RQ values in the growth zone, but not in the mature tissues [4, 45, 46]. Growing meristematic regions may resort to fermentation, not because

oxygen diffusion from the environment is impaired [1], but because energy demand exceeds the respiratory capacity.

The ADH null genotype is normally transmitted to the offspring [18, 44, 55], and consequently, in mixed pollinations ADH null pollen cannot have a serious competitive handicap relative to wild-type pollen. Here we have shown that ethanolic fermentation is of major importance under *in vitro* conditions which mimic the natural conditions found when pollen penetrate the pistil. How can it be that the absence of a major energy-generating pathway has no effect on competitiveness? One explanation is that fermentation is a redundant pathway and that its elimination is insufficient to compromise competitiveness under standard environmental conditions. This hypothesis is supported by the results of knock-out experiments in transgenic mice: in several cases the inactivation of important genes did not result in any measurable phenotype, and the simplest hypothesis is that the encoded proteins are dispensable (for a thoughtful and stimulating discussion, see [17]). On the other hand, male gametogenesis is characterized by very rapid growth, and consequently enormous demands are placed on energy supply [32, 54]. Moreover, the rate of pollen tube growth is thought to be an important parameter for reproductive success [35, 48]. It is hard to understand why a plant would invest energy in synthesizing a superfluous protein during a critical stage of its life cycle. Our results show that ethanolic fermentation is a major pathway during pollen tube formation. Careful experiments will be necessary to address its function *in vivo*.

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References

1. Armstrong W, Gaynard TJ: The critical oxygen pressures for respiration in intact plants. Physiol Plant 37: 200–206 (1976).
2. Baburina I, Gao Y, Hu Z, Jordan F, Hohmann S, Furey W: Substrate activation of brewers' yeast pyruvate decarboxylase is abolished by mutation of cysteine 221 to serine. Biochemistry 33: 5630–5635 (1994).
3. Barman TE: Enzyme Handbook, vol 1, p. 499. Springer-Verlag, Berlin (1969).
4. Berry LJ, Norris Jr WE: Studies on onion root respiration. I. Velocity of oxygen consumption in different segments of root at different temperatures as a function of partial pressure of oxygen. Biochem Biophys Acta 3: 593–606 (1949).
5. Brander KA, Kuhlemeier C: A pollen specific DEAD box protein related to translation initiation factor eIF-4A from tobacco. Plant Mol Biol, in press (1995).
6. Bucher M, Brändle R, Kuhlemeier C: Ethanolic fermentation in transgenic tobacco expressing *Zymomonas mobilis* pyruvate decarboxylase. EMBO J 13: 2755–2763 (1994).
7. Bucher M, Kuhlemeier C: Long term anoxia tolerance: multilevel regulation of gene expression in the amphibious plant *Acorus calamus* L. Plant Physiol 103: 441–448 (1993).
8. Candy JM, Duggleby RG: Investigation of the cofactor-binding site of *Zymomonas mobilis* pyruvate decarboxylase by site-directed mutagenesis. Biochem J 300: 7–13 (1994).
9. Cossins EA: Ethanol metabolism in plants. In: Hook DD, Crawford RRM (eds) Plant Life in Anaerobic Environments, pp. 169–202. Ann Arbor Scientific Publishers, Ann Arbor, MI (1978).
10. Davies DD, Grego S, Kenworthy P: The control of the production of lactate and ethanol by higher plants. Planta 118: 297–310 (1974).
11. Dennis ES, Gerlach WL, Pryor AJ, Bennetzen JL, Ingles A, Llewellyn D, Sachs MM, Ferl RJ, Peacock WJ: Molecular analysis of the alcohol dehydrogenase (*Adh1*) gene of maize. Nucl Acids Res 12: 3983–4000 (1984).
12. Dickinson DB: Germination of lily pollen: respiration and tube growth. Science 150: 1818–1819 (1965).
13. Diefenbach RJ, Candy JM, Mattick JS, Duggleby RG: Effects of substitution of aspartate-440 and tryptophan-487 in the thiamin diphosphate binding region of pyruvate decarboxylase from *Zymomonas mobilis*. FEBS Lett 296: 95–98 (1992).
14. Dolferus R, Jacobs M, Peacock WJ, Dennis ES: Differential interactions of promoter elements in stress responses of the *Arabidopsis Adh* gene. Plant Physiol 105: 1075–1087 (1994).
15. Dolferus R, Van den Bossche D, Jacobs M: Sequence analysis of two null mutant alleles of the single *Arabidopsis Adh* locus. Mol Gen Genet 224: 297–302 (1990).

16. Dyda F, Furey W, Swaminathan S, Sax M, Farrenkopf B, Jordan F: Catalytic centers in the thiamin diphosphate dependent enzyme pyruvate decarboxylase at 2.4 Å resolution. Biochemistry 32: 6165–6170 (1993).
17. Erickson HP: Gene knock-outs of *c-src*, transforming growth factor β1, and tenascin suggest superfluous, nonfunctional expression of proteins. J Cell Biol 120: 1079–1081 (1993).
18. Freeling M, Bennett CB: Maize *Adh1*. Ann Rev Genet 19: 297–323 (1985).
19. Gancedo C, Serrano R: Energy yielding metabolism. In: Rose AH, Harrison JS (eds) The Yeasts, vol. 3, pp. 205–259. Academic Press, London (1989).
20. Gaut BS, Clegg MT: Molecular evolution of the *Adh1* locus in the genus *Zea*. Proc Natl Acad Sci USA 90: 5095–5099 (1993).
21. Gerlach WL, Sachs MM, Llewellyn D, Finnegan EJ, Dennis ES: Maize alcohol dehydrogenase: a molecular perspective. In: Blonstein AD, King PJ (eds) A Genetic Approach to Plant Biochemistry, pp. 73–100. Springer-Verlag, Wien (1986).
22. Hoekstra FA: Mitochondrial development and activity of binucleate and trinucleate pollen during germination *in vitro*. Planta 145: 25–36 (1979).
23. Hoekstra FA, Bruinsma J: Respiration and vitality of binucleate and trinucleate pollen. Physiol Plant 34: 221–225 (1975).
24. Hoekstra FA, Bruinsma J: Control of respiration of binucleate and trinucleate pollen under humid conditions. Physiol Plant 48: 71–77 (1980).
25. Hohmann S: Structure and expression of yeast pyruvate decarboxylase structural genes. In: Bisswanger H, Ullrich J (eds) Biochemistry and Physiology of Thiamin Diphosphate Enzymes, pp. 106–114. VCH, Weinheim, Germany (1991).
26. Jansen MAK, Sessa G, Malkin S, Fluhr R: PEPC-mediated carbon fixation in transmitting tract cells reflects style-pollen tube interactions. Plant J 2: 507–515 (1992).
27. Kelley PM: Maize pyruvate decarboxylase mRNA is induced anaerobically. Plant Mol Biol 13: 213–222 (1989).
28. Knox RB: Pollen-pistil interactions. In: Linskens HF, Heslop-Harrison J (eds) Encyclopedia of Plant Physiology, New Series, vol. 17, pp. 508–608. Springer Verlag, Berlin (1984).
29. Koltunow AM, Truettner J, Cox KH, Wallroth M, Goldberg RB: Different temporal and spatial gene expression patterns occur during anther development. Plant Cell 2: 1201–1224 (1990).
30. Konarska RN, Linskens HF: Physiology and biochemistry of the stigmatic fluid of *Petunia hybrida*. Planta 71: 372–387 (1966).
31. Kyozuka J, Olive M, Peacock WJ, Dennis ES, Shimamoto K: Promoter elements required for developmental expression of the maize *Adh1* gene in transgenic rice. Plant Cell 6: 799–810 (1994).
32. Levings III CS: Thoughts on cytoplasmic male sterility. Plant Cell 5: 1285–1290 (1993).
33. Linskens HF, Schrauwen J: Measurement of oxygen tension changes in the style during pollen tube growth. Planta 71: 98–106 (1966).
34. Mascarenhas JP: Gene activity during pollen development. Ann Rev Plant Physiol Plant Mol Biol 41: 317–338 (1990).
35. Mulcahy DL: The rise of the angiosperms: a genecological factor. Science 206: 20–23 (1975).
36. Olive M, Peacock WJ, Dennis ES: The anaerobic response element contains two GC-rich sequences essential for binding a nuclear protein and hypoxic activation of the maize *Adh1* promoter. Nucl Acids Res 19: 7053–7060 (1991).
37. Owttrim GW, Hofmann S, Kuhlemeier C: Divergent genes for translation initiation factor eIF-4A are coordinately expressed in tobacco. Nucl Acids Res 19: 5491–5496 (1991).
38. Owttrim GW, Mandel T, Trachsel H, Thomas AAM, Kuhlemeier C: Characterization of the tobacco eIF-4A gene family. Plant Mol Biol, 26: 1747–1757 (1994).
39. Paul AL, Ferl RJ: *in vivo* foorptinting reveals unique *cis*-elements and different modes of hypoxic induction in maize *Adh1* and *Adh2*. Plant Cell 3: 159–168 (1991).
40. Peschke VM, Sachs M: Multiple pyruvate decarboxylase genes in maize are induced by hypoxia. Mol Gen Genet 240: 206–212 (1993).
41. Read SM, Clarke AE, Bacic A: Requirements for division of the generative nucleus in cultured pollen tubes of *Nicotiana*. Protoplasma 174: 101–115 (1993).
42. Roberts JKM, Callis J, Wemmer D, Walbot V, Jardetzki O: Mechanism of cytoplasmic pH regulation in hypoxic maize root tips and its role in survival under hypoxia. Proc Natl Acad Sci USA 81: 3379–3383 (1984).
43. Roberts JKM, Chang K, Webster C, Callis J, Walbot V: Dependence of ethanolic fermentation, cytoplasmic pH regulation, and viability on the activity of alcohol dehydrogenase in hypoxic maize root tips. Plant Physiol 89: 1275–1278 (1989).
44. Rousselin P, Lepingle A, Faure J-D, Bitoun R, Caboche M: Ethanol-resistant mutants of *Nicotiana plumbaginifolia* are deficient in the expression of pollen and seed alcohol dehydrogenase activity. Mol Gen Genet 222: 409–415 (1990).
45. Ruhland W, Ramshorn K: Aerobe Gärung in aktiven pflanzlichen Meristemen. Planta 28: 471–514 (1938).
46. Ruhland W, Ullrich H: Aerobe Gärung in wachsenden Pflanzengeweben. Ber Sächs Akad Wiss Math Phys 88: 11–20 (1936).
47. Schwartz D: Genetic control of alcohol dehydrogenase. A competition model for regulation of gene action. Genetics 67: 411–425 (1971).
48. Snow AA, Spira TP: Pollen vigour and the potential for sexual selection in plants. Nature 352: 796–797 (1991).

49. Stanley RG, Linskens HF: Oxygen tension as a control mechanism in pollen tube rupture. Science 157: 833–834 (1967).
50. Tupy J, Rihova L: Changes and growth effect of pH in pollen tube culture. J Plant Physiol 115: 1–10 (1984).
51. Twell D: The diversity and regulation of gene expression in the pathway of male gametophyte development, in press (1995).
52. Ullrich J, Kellermann J, Schmidt W: Homeotetrameric pyruvate decarboxylase (PDC; EC 4.1.1.1.) from ABYS1 yeast: C- and N-terminals and some kinetic properties. In: Bisswanger H, Ullrich J (eds) Biochemistry and Physiology of Thiamin Diphosphate Enzymes, pp. 99–105. VCH, Weinheim, Germany (1991).
53. Verwoerd TC, Dekker BM, Hoekema A: A small-scale procedure for the rapid isolation of plant RNAs. Nucl Acids Res 17: 2362 (1989).
54. Warmke HE, Lee S-LJ: Pollen abortion in T cytoplasmic male-sterile corn (*Zea mays*): a suggested mechanism. Science 200: 561–563 (1978).
55. Wisman E, Ramanna MS, Zabel P: Isolation of two independent allyl alcohol resistant *Adh-1* null mutants in tomato following selection of pollen and seeds. Plant Science 95: 79–88 (1993).