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Ethanolic fermentation in transgenic tobacco expressing *Zymomonas mobilis* pyruvate decarboxylase

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During oxygen limitation in higher plants, energy metabolism switches from respiration to fermentation. As part of this anaerobic response the expression of genes encoding pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) is strongly induced. In addition there is ample evidence for post-translational regulation. In order to understand this multi-level regulation of the anaerobic response, we provided tobacco with the constitutive capacity of ethanolic fermentation by expressing a PDC gene derived from the obligate anaerobe *Zymomonas mobilis*. The protein accumulated to high levels and was active in an *in vitro* assay. During the first 2–4 h of anoxia, acetaldehyde accumulated to 10- to 35-fold and ethanol to 8- to 20-fold higher levels than in wild-type. Under normoxic conditions no accumulation of acetaldehyde and ethanol could be measured. Instead, the two products may be immediately re-metabolized in tobacco leaf tissue. We show that aerobic fermentation takes place when the respiratory system is inhibited. Although these conditions enhance ethanolic fermentation under normoxia, they fail to increase ADH transcript levels. These results indicate that anaerobic transcription is triggered not by the metabolic consequences of oxygen limitation, but directly through an oxygen-sensing system.

Key words: aerobic fermentation/alcohol dehydrogenase gene expression/anoxia/transgenic tobacco/*Zymomonas mobilis* pyruvate decarboxylase

Introduction

An important aspect of the adaptation to oxygen limitation is the shift from respiration to fermentation. During anoxia, ATP and NAD^+ are generated not in the Krebs cycle and the respiratory chain but via glycolysis and fermentation. In many organisms (including man) lactic acid is a prominent end product of anaerobic metabolism. An unfavorable consequence of lactate accumulation is cytoplasmic acidosis, a decrease in cellular pH which may finally lead to cell death (Roberts *et al.*, 1984a). As an alternative, plants can sustain ethanolic fermentation, which is thought to be advantageous during anoxia since ethanol production, unlike lactate production, does not result in cytoplasmic acidosis (Davies, 1980; Roberts *et al.*, 1984b). Moreover, ethanol can diffuse into the surrounding medium and does not accumulate to toxic levels in the tissue (see for example Rumpho and Kennedy, 1981; Good and Muench, 1993). In several systems studied, high ethanol production and low lactate synthesis correlate with anoxia tolerance (Rumpho and Kennedy, 1981; Rivoal *et al.*, 1989; Sieber and Brändle, 1991; Good and Muench, 1993; Muench *et al.*, 1993).

In ethanolic fermentation, pyruvate is the substrate of pyruvate decarboxylase (PDC), yielding CO_2 and acetaldehyde. Subsequently, acetaldehyde is reduced to ethanol with the concomitant oxidation of NADH to NAD^+ by alcohol dehydrogenase (ADH). Since only two enzymes are involved in this pathway, increasing the flux through the pathway by increasing the amount of one or both of the enzymes by means of genetic engineering appears a feasible strategy. Overexpression of ethanol by genetic engineering has been attempted in yeast with mixed results. Sharma and Tauro (1986) reported a higher ethanol production in yeast mutants overproducing PDC and ADH in stationary phase cells. Schaaff *et al.* (1989) performed a detailed study in yeast in which they overexpressed various glycolytic enzymes either alone or in various combinations, among them either PDC alone or simultaneously with ADH. However, they did not detect an increasing rate of ethanol production in logarithmically growing cultures. The authors suggest that an increase of the flux through glycolysis requires the overproduction of many, if not all, of the glycolytic enzymes.

Roberts *et al.* (1989) examined the role of ADH in the metabolism and survival of hypoxic maize root tips. From a study of isogenic lines differing in ADH activity over a ~200-fold range they concluded that only minimal levels of ADH activity were sufficient to enable ethanol production and confer survival under hypoxic conditions. Therefore, ADH activity in wild-type maize root tips did not limit the capacity for energy production via fermentation, and did not determine viability under extreme hypoxia. This suggests a key regulatory role for the first enzyme, PDC.

There are two advantages of using non-plant genes for overexpression in plant species. First, introducing a homologous gene may lead to down-regulation rather than overexpression of the target gene; this phenomenon is known as co-suppression (Kooter and Mol, 1993). Non-homologous genes are not known to be sensitive to co-suppression. Second, higher levels of an endogenous enzyme may not increase the flux through the system due to metabolic control at the enzyme level. In contrast, non-plant enzymes may be regulated by different mechanisms.

A search through the literature identified PDC from the obligate anaerobe *Zymomonas mobilis* as an attractive candidate for our purposes. PDC is a key enzyme in ethanol formation of *Z.mobilis* and channels 95% of the substrate carbon via acetaldehyde to ethanol (Rogers *et al.*, 1982). The glycolytic pathway in *Zymomonas* differs from eukaryotic species in that *Zymomonas* utilizes the

Entner–Doudoroff pathway (Dawes *et al.*, 1966). Using extracts of *Zymomonas*, Algar and Scopes (1985) suggested that there are no controls on glycolytic metabolism other than the fact that the enzymes are operating at close to maximum capacity. Of the 15 intermediary metabolites tested, none acted as an inhibitor of PDC activity at concentrations of up to 5 mM (Hoppner and Doelle, 1983). The native *Zymomonas* PDC enzyme makes up 4–6% of total soluble protein and has a mol. wt of 200 000 (Bringer-Meyer and Sahm, 1991). The holoenzyme consists of four probably identical subunits. The active enzyme requires two cofactors, thiamine pyrophosphate (TPP) and Mg^{2+}, for its stability. In *Z.mobilis*, PDC does not absolutely require TPP for its catalytic activity. This is in contrast to PDC of germinating maize seeds which was only active in the presence of TPP and Mg^{2+} (Leblová *et al.*, 1989). The *Zymomonas* PDC exhibits a major pH optimum at pH 6.0 and a minor pH optimum at pH 4.3 (Hoppner and Doelle, 1983). Bringer-Meyer and Sahm (1991) compared apparent K_m values for PDC with respect to pyruvate for *Z.mobilis*, *Saccharomyces cerevisiae* and wheat germ PDC. The values were 0.4, 1.1 and 3.6 mM, respectively. Interestingly, *Z.mobilis* PDC has been expressed in *Escherichia coli*, where it had a considerable effect on the amount of fermentation products formed (Bräu and Sahm, 1986). Feeding 25 mM glucose led to ethanol concentrations of up to 41.5 mM while almost no acids were formed. Thus the *Zymomonas* protein accumulates to high levels in at least one foreign organism, it is highly active as a monomer or a homomeric multimer, its activity is not known to be under metabolic control and it has only very limited sequence homology to the plant enzyme (Kelley, 1989).

We present here the overexpression of *Z.mobilis* PDC in transgenic tobacco plants. Our results show that the *Zymomonas* PDC protein accumulates to high levels in tobacco and is active *in vitro* and *in vivo*.

Results

Construction of a chimeric pyruvate decarboxylase gene and introduction into transgenic tobacco

The bacterial PDC gene from *Z.mobilis* was introduced into the plasmid pMOGEN18 (see Materials and methods) via an exact fusion at the ATG of the PDC gene. In this construct the PDC gene is under the control of the 35S promoter, a strong plant promoter originating from the cauliflower mosaic virus (Odell *et al.*, 1985) and the alfalfa mosaic virus translational enhancer which has been shown to increase translation of eukaryotic genes (Jobling and Gehrke, 1987). The 3′ end of the nopaline synthase gene (Depicker *et al.*, 1982) was included to terminate transcription. This chimeric construct encoded a full-length pyruvate decarboxylase protein of 559 amino acids with a calculated mol. wt of 60 000 (Conway *et al.*, 1987).

The chimeric gene construct was cloned as an *Eco*RI–*Sal*I fragment into the corresponding site of the binary vector pMON505 (Figure 1) which was transferred to the *Agrobacterium* strain LBA4404 via triparental mating. Transformation frequencies were low: from six independent experiments eight individual transgenic lines arose. T_0 plants were used to check for successful expression of the chimeric gene. Wild-type plants were used as controls.

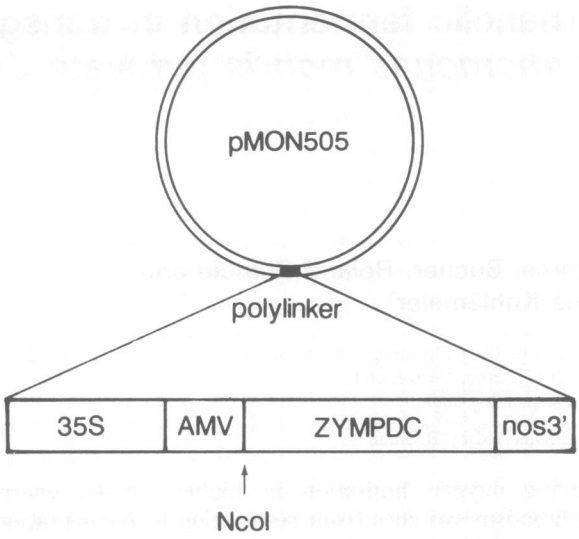

Fig. 1. Structure of the chimeric *Zymomonas* PDC gene construct used for transformation of tobacco plants.

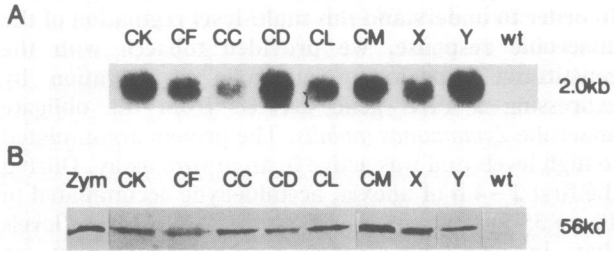

Fig. 2. The *Zymomonas* PDC gene is actively transcribed and translated in leaves of transgenic tobacco. (**A**) *Zymomonas* PDC transcript levels in leaves of eight transgenic T_0 plants were detected by Northern blot analysis (10 μg of total RNA per lane). Leaf samples were taken from normoxic plants and immediately frozen in liquid nitrogen prior to RNA extraction. The different transgenic lines were labelled as indicated on top of the figure. The RNA on the membrane was hybridized with a ^{32}P-labelled DNA probe from *Zymomonas* PDC. Total RNA from wild-type plants served as a control (wt). (**B**) Total protein (30 μg per lane) of leaves of eight transgenic T_0 plants were separated by SDS–PAGE. *Zymomonas* PDC protein levels were detected with Western blot analysis using an anti-*Zymomonas* PDC antibody. Proteins from wild-type plants served as a negative control (wt). 0.5 μg of *Zymomonas* PDC protein purified from transformed *E.coli* (see Materials and methods) served as a positive control (Zym).

Expression of the chimeric construct

In order to demonstrate that the *Zymomonas* PDC gene was actively transcribed, Northern blotting was performed. Total RNA was extracted from transgenic T_0 plants and separated on a glyoxal–agarose gel. After transfer to nylon membrane, the RNA was hybridized to radioactively labelled *Zymomonas* PDC DNA (Figure 2A). For all transgenic lines a signal of ~2 kb was observed. The bands varied in intensity indicating that the transgene was expressed to different levels in individual transgenic lines. While lines 9204-CK, -CD, -CM and -Y had high RNA levels, line 9204-CC showed the lowest RNA concentration. The lines 9204-CF, -CL and -X were intermediate to the other lines mentioned above. In wild-type plants no hybridizing RNA was present, as shown in the control lane.

As a next step we wished to ascertain whether the mRNA was properly translated in the transgenic plants. Therefore,

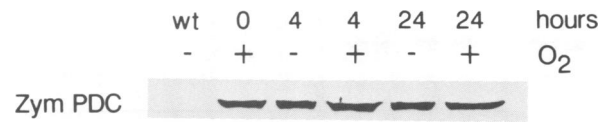

Fig. 3. *Zymomonas* PDC protein levels remain constant during anoxic incubation. Total protein (30 μg per lane) from leaves of transgenic 9204-X F_1 plants were separated by SDS−PAGE. *Zymomonas* PDC protein levels were detected with Western blot analysis using an anti-*Zymomonas* PDC antibody. Proteins from normoxic wild-type plants served as a negative control (wt). Incubation periods were as indicated in the upper line at the top of the figure. Normoxic or anoxic incubation was as indicated on the second line at the top of the figure.

we overexpressed the *Zymomonas* PDC gene in *E.coli* (see Materials and methods). The PDC protein was purified from the inclusion bodies and used to generate anti-*Zymomonas* PDC antibodies in rats. The antibodies were used for Western blotting on total soluble leaf protein of the T_0 plants (Figure 2B). Proteins extracted from normoxically or anoxically incubated leaves of wild-type plants showed no cross-reactive band after immunodetection on the Western blots. This indicated that the antibody was specific for *Z.mobilis* PDC. Figure 2B shows a signal present in total protein extracts from leaf material of transgenic plants, the size of which (56 kDa) correlated with the size of the *Z.mobilis* PDC protein (57 000 ± 4000 kDa) (Bringer-Meyer and Sahm, 1991) as determined by SDS−PAGE. *Zymomonas* PDC protein in lane Zym, purified from *E.coli*, migrated to the same position on the Western blot as the PDC protein in transgenic plants. Whereas PDC RNA levels varied in leaves of different T_0 plants, as shown in Figure 2A, the PDC protein levels were fairly similar in all the primary transformants. Thus, the chimeric *Zymomonas* PDC gene was correctly transcribed and translated and the proteins accumulated to comparable levels in individual transgenic tobacco plants. The *Zymomonas* PDC protein accumulated to ~0.5−1% of total soluble leaf protein, as estimated visually by comparison with known amounts of purified recombinant *Zymomonas* PDC protein on a Western blot.

The *Zymomonas* PDC gene is under the control of the 35S promoter and alfalfa mosaic virus translational enhancer, which are not known to be responsive to oxygen availability. In order to prove that *Zymomonas* PDC protein levels remain constant during anoxia, total protein was extracted from leaves before and after anoxia treatment. Figure 3 represents the levels of *Zymomonas* PDC protein on a Western blot from leaves which were exposed to anoxia for 0, 4 or 24 h. The results show that the protein accumulated to comparable levels under all conditions. Therefore, anoxic incubation had no influence on the levels of *Zymomonas* PDC protein in transgenic tobacco.

PDC activity in vitro

To prove that the overexpressed *Zymomonas* PDC was enzymatically active we established an *in vitro* activity test using yeast PDC as an exogenous control. In the assay, pyruvate was the substrate and PDC activity was coupled to ADH activity which reduces acetaldehyde to ethanol, thus oxidizing NADH to NAD^+. The oxidation of NADH was monitored following the OD_{340nm} on a double-beam spectrophotometer (see Materials and methods). In the

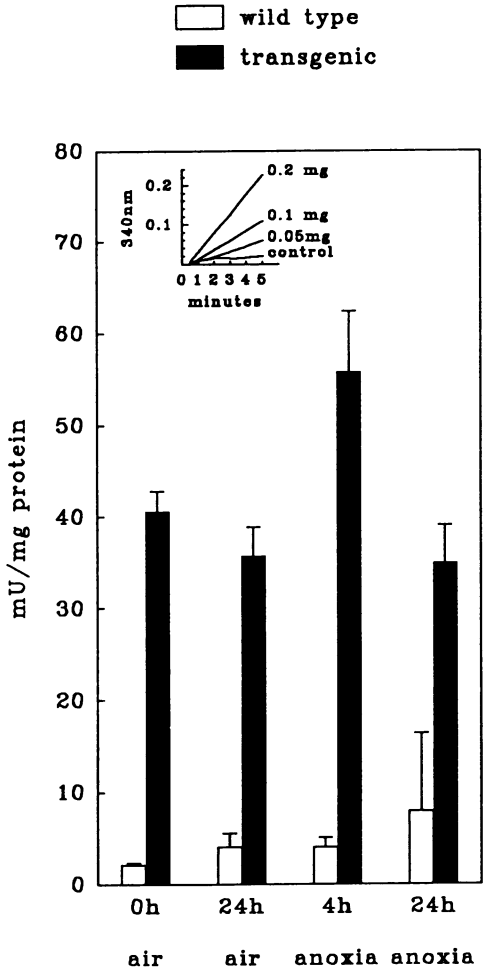

Fig. 4. *In vitro* PDC activity in tobacco leaves. Specific PDC activity in tobacco leaf extracts in mU/mg protein, where 1 U will convert 1.0 μmol of pyruvate to acetaldehyde per minute at pH 6.0 at 25°C. Values represent the average of six individual measurements of split leaves of F_1 plants from the line 9204-X (filled bars) or from wild-type plants (open bars). Incubation conditions were as indicated at the bottom of the figure. **Inset**, absorption differences at 340 nm during NADH oxidation in the PDC activity assay. Leaf material from an F_1 plant of the line 9204-X was extracted according to Materials and methods. Different extract volumes (corresponding amounts of total soluble protein were as indicated at the end of the curves) were assayed for PDC activity for 5 min in a double-beam spectrophotometer. No substrate (pyruvate) was added to the control assay (which contained 0.05 mg of total soluble protein).

experiment shown in Figure 4 (inset) different amounts of extracted leaf material of a 9204-X F_1 plant were tested for PDC activity. This showed that the assay was linear for at least 5 min. It has been reported that 5 mM dithiothreitol (DTT) inhibited the activity of an ADH inhibitor in rice *in vitro* (Shimomura and Beevers, 1983). This was not the case under our assay conditions, since presence of 5 mM DTT did not induce NADH oxidation *in vitro*. Thus, extracts from tobacco leaves presumably did not contain an ADH inhibitor. Pyruvate is also the substrate for lactate dehydrogenase (LDH) and thus, the measured NADH oxidation may reflect in part LDH activity. We tested the proportion of LDH activity according to Laszlo and St Lawrence (1983). From these observations we conclude that also in the wild-type the measured NAD^+ production is due primarily to PDC

activity and not to LDH activity. Figure 4 shows PDC activities as measured in wild-type leaves and leaves from transgenic F_1 plants after anoxic incubation of 0, 4 and 24 h. Under normoxic conditions transgenic plants exhibited ~20 times higher *in vitro* PDC activity in young leaves than wild-type plants. After 4 h of anoxia PDC activity in transgenic plants exceeded wild-type activity 14-fold. After 24 h of anoxia, *in vitro* PDC activity detected in young leaves of transgenic plants was still ~5 times higher than in wild-type plants. This indicated that the *Zymomonas* PDC gene was expressed in transgenic tobacco plants as a functional enzyme which exhibited enzymatic activity *in vitro*. As a consequence, total PDC activity *in vitro* was significantly higher in leaves of transgenic plants than in wild-type plants under both normoxic and anoxic conditions.

Anaerobic fermentation in vivo

To monitor the enzymatic activity of the *Zymomonas* PDC protein *in vivo*, we were interested to detect acetaldehyde (the product of pyruvate decarboxylation) and ethanol (the product of the subsequent reaction catalyzed by ADH). Since acetaldehyde and ethanol are volatile compounds which diffuse out of the tissue, it was possible to measure the accumulation of these two products in the head space via gas chromatography. This enabled us to monitor *in vivo* enzymatic activity of PDC and ADH under anoxic and normoxic conditions in tobacco leaves. Young leaves were split along the midrib and placed into 150 ml gas-tight glass bottles, which were painted black to keep the leaves in complete darkness. One half of the leaf was incubated under normoxic control conditions, while the other half was kept under anoxia. This procedure allowed the comparison of leaf tissue in a similar metabolic state at the onset of the two different treatments. Synthesized acetaldehyde and ethanol were detected by injecting gas samples from the bottles into the gas chromatograph. The results show that in wild-type leaves acetaldehyde and ethanol (Figure 5A and B respectively) were barely detectable after 2 and 4 h of anoxia in the surrounding atmosphere. Thus, there was no appreciable induction of ethanolic fermentation in wild-type plants after short-term anoxia. In contrast, acetaldehyde and ethanol accumulated to higher levels in anoxically incubated leaves of transgenic F_1 plants. Acetaldehyde accumulated to ~10 times and 35 times higher levels per gram of fresh weight than wild-type leaves after 2 and 4 h of anoxia, respectively (Figure 5A). Ethanol levels per gram fresh weight were 8 and 20 times higher in the head space of transgenic leaves than in wild-type leaves after 2 and 4 h of anoxia, respectively (Figure 5B). These results prove that under short-term anoxia the products of ethanolic fermentation accumulated in transgenic plants overexpressing the *Zymomonas* PDC gene, while ethanolic fermentation in wild-type plants was very low. This further suggests that at the onset of anoxia, PDC is a limiting factor and ADH is already available in sufficient quantity. After 24 h of anoxia both wild-type and transgenic leaves exhibited considerable accumulation of acetaldehyde and ethanol in the head space, although standard deviations were high (data not shown). This may well be due to reduced viability. Repression or inhibition of glucose uptake, metabolic rate or changes in the plasma membrane could have occurred. Preliminary results using ultra-red absorbance spectroscopy (URAS)

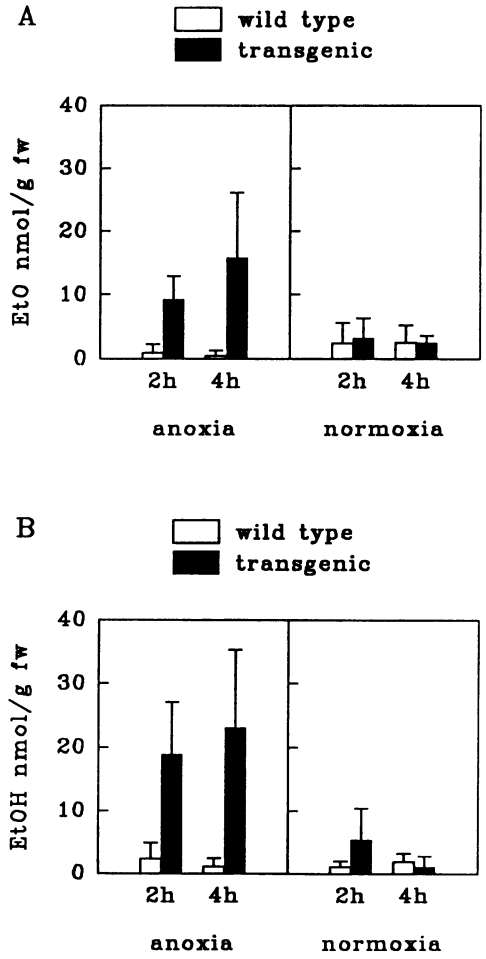

Fig. 5. Head space analysis by gas chromatography. (**A**) Accumulation of acetaldehyde (EtO) in the head space of incubated leaves in nmol/g fresh wt. Split leaves were incubated under either anoxic or normoxic conditions for 2 or 4 h, as indicated at the bottom of the figure. Values represent the average of six individual measurements of leaves of F_1 plants from the line 9204-X or from wild-type plants. (**B**) Accumulation of ethanol (EtOH) in the head space of incubated leaves in nmol/g fresh wt. Acetaldehyde and ethanol were analyzed simultaneously from the same samples.

indicated that CO_2 production was reduced relative to that in normoxic controls. This may indicate that after 24 h the mitochondria are damaged.

Ethanol metabolism under normoxia

Head space analysis of transgenic leaves under normoxic conditions did not reveal significant accumulation of ethanol or acetaldehyde. Thus, although the PDC was highly active *in vitro* (Figure 4) and glucose was infiltrated into the leaves (and thus substrate ought to be available), the end products of ethanolic fermentation did not accumulate. Two possible explanations can be envisaged. The first is that the fermentation pathway is active but the products are remetabolized. The second is that there exists a hierarchy of control and during normoxia carbon preferentially enters the TCA cycle.

We wished to test whether acetaldehyde and ethanol could be metabolized under normoxia via alternative metabolic routes and, therefore, would not accumulate. To do this, ethanol was injected into gas-tight bottles containing split

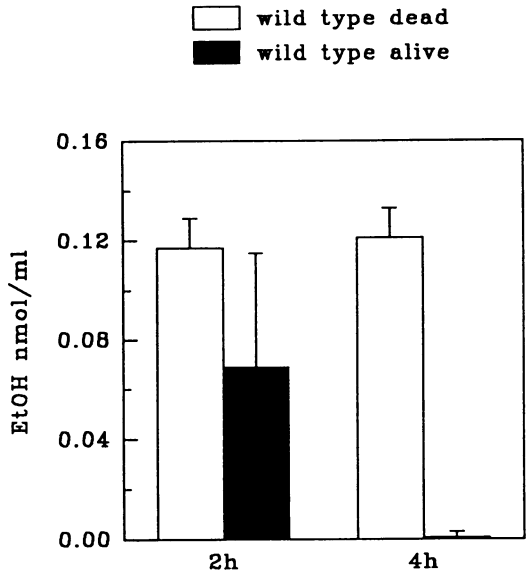

Fig. 6. Ethanol metabolism in wild-type leaves of tobacco. Ethanol (EtOH) was present at 4.6 nmol/ml in 150 ml bottles at the onset of anoxia. Split leaves of wild-type tobacco were incubated under anoxia. After 2 and 4 h gas samples were analyzed for ethanol by gas chromatography. One half of each leaf was killed by boiling for 30 s and served as a control. Values represent the average of three individual measurements.

leaves of wild-type plants under normoxia to a final concentration of 4.6 nmol/ml. One half of the leaves were tested for ethanol metabolism; the other half were killed by immersing in boiling water for 30 s prior to incubation and served as a control. Ethanol rapidly diffused into the dead leaves (Figure 6) to a steady-state concentration of 0.12 nmol/ml in the head space during 4 h of incubation. Thus, the partition ratio of ethanol in the head space and the tissue of tobacco leaves is ~1/40. In living leaves, in contrast to the dead leaves, ethanol concentrations decreased to undetectable levels in the head space. This indicates that ethanol can be metabolized efficiently in tobacco leaves under normoxia.

Aerobic fermentation in transgenic tobacco leaves

Figure 6 shows that ethanol can be metabolized under normoxia. However, these results do not rule out the second explanation, namely that pyruvate preferentially enters the TCA cycle and the respiratory chain. To test for this second possibility we applied two inhibitors. The first, antimycin A (AA), is a specific inhibitor of the respiratory chain which blocks electron transfer between cytochromes *b* and c_1 in the cytochrome pathway (Storey, 1972; Slovacek *et al.*, 1979). The second, (R)-1-aminoethylphosphinate (AEP) is metabolized to acetylphosphinate which acts as a highly specific inhibitor of the pyruvate dehydrogenase complex (PDHC, Laber and Amrhein, 1987; Schönbrunn-Hanebeck *et al.*, 1990). Without the addition of inhibitors, transgenic leaves accumulated very low levels of acetaldehyde or ethanol (Figure 7, right panels, solid bars). Treatment of transgenic leaves with AA or AEP resulted in a significantly enhanced accumulation of acetaldehyde and ethanol (left panels, solid bars). This shows that under conditions where either the respiratory chain or the entrance of pyruvate into the TCA cycle via PDHC is inhibited, aerobic fermentation does take place in transgenic leaves. In contrast to transgenic

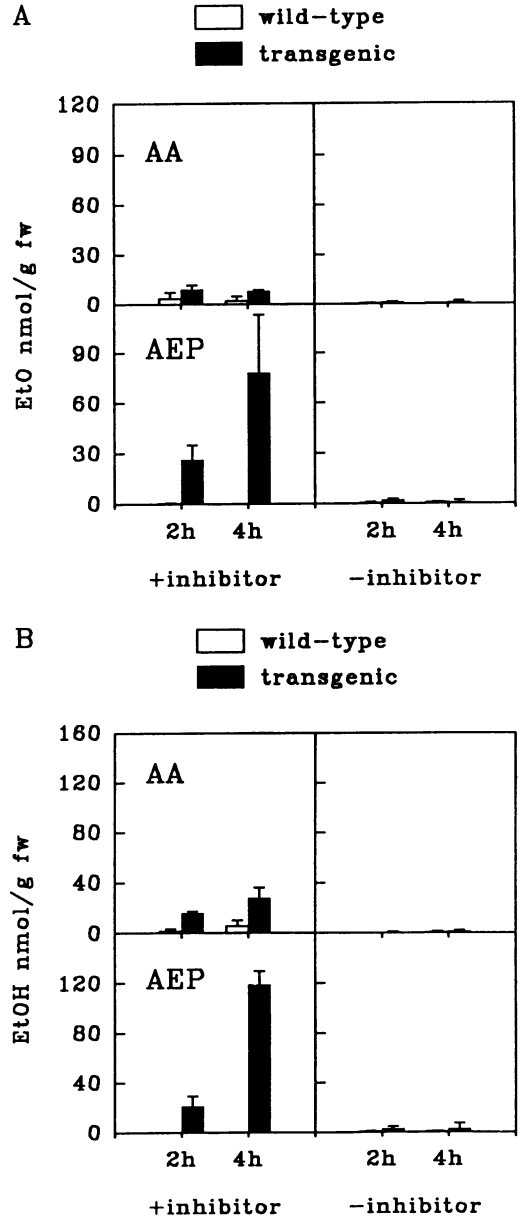

Fig. 7. Head space analysis by gas chromatography. (**A**) Accumulation of acetaldehyde (EtO) in the head space of incubated leaves in nmol/g fresh wt. Split leaves were incubated in the presence of either 2 μM AA or 30 μM AEP for 2 or 4 h, as indicated at the bottom of the figure. Control experiments were performed without inhibitors. Values represent the average of three individual measurements of leaves of F_1 plants from the line 9204-CL or from wild-type plants. (**B**) Accumulation of ethanol (EtOH) in the head space of incubated leaves in nmol/g fresh wt. Acetaldehyde and ethanol were analyzed simultaneously from the same samples.

leaves, inhibitor treatments of wild-type leaves did not result in appreciable accumulation of the two products of ethanolic fermentation (Figure 7, left panels, open bars). Most likely, this is caused by the absence of the PDC enzyme under these conditions.

ADH transcript levels do not increase during aerobic fermentation

In all plant species tested the transcript levels for ADH increase rapidly after the onset of anoxia (Hake *et al.*, 1985). In the previous section we demonstrated that fermentation

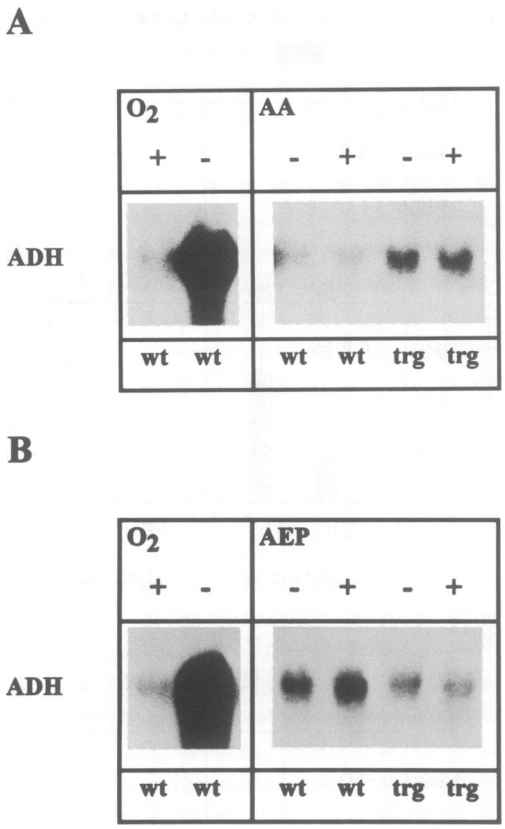

Fig. 8. Inhibitor treatment does not increase ADH transcript levels. (**A**) ADH transcript levels in leaves of wild-type (wt) and transgenic (trg) plants treated in the absence (−) or presence (+) of 2 μM AA for 4 h under normoxia were detected by Northern blot analysis (10 μg of total RNA per lane). Total RNA from wild-type leaves incubated under either normoxia ($+O_2$) or anoxia ($-O_2$) for 2 h served as a control. After incubation leaf samples were taken and immediately frozen in liquid nitrogen prior to RNA extraction. The RNA on the membrane was hybridized with a ^{32}P-labelled DNA probe from a tobacco ADH cDNA. (**B**) ADH transcript levels in leaves of wild-type (wt) or transgenic (trg) plants treated in the absence (−) or presence (+) of 30 μM AEP for 4 h under normoxia. Total RNA from wild-type leaves incubated under either normoxia ($+O_2$) or anoxia ($-O_2$) for 2 h served as a control. In all cases 10 μg of total RNA was loaded per lane.

can take place under normoxia provided that the pathway towards respiration is inhibited and that PDC is present and thus the proper metabolic conditions are created. This raises the interesting question of whether such metabolic conditions can induce the transcription of the ADH gene in the presence of oxygen. To answer this question we isolated an ADH cDNA from tobacco. As can be seen in Figure 8, ADH transcript levels in tobacco are also strongly and rapidly induced by anoxic incubation. However, neither AA nor AEP has any influence on transcript levels of the two genes. Thus, inhibition of the aerobic energy production pathway is sufficient to induce ethanolic fermentation, but it fails to induce anaerobic gene expression.

Discussion

Expression of bacterial genes in higher plants has proven to be a successful strategy and marked effects on plant metabolism and stress tolerance have been achieved in several cases (e.g. Sonnewald, 1992; Wolter *et al.*, 1992; Karchi *et al.*, 1993; Tarczynski *et al.*, 1993; Vernon *et al.*, 1993). We have introduced a gene encoding PDC, a key enzyme in ethanol production in the obligate anaerobe *Z.mobilis*, into tobacco. The introduction of *Zymomonas* PDC into tobacco does not cause any obvious phenotypic differences from controls under standard growth conditions, except possibly a slight growth retardation.

The bacterial PDC is functional in transgenic tobacco, indicating strong evolutionary conservation of the structure and function of PDC from species as distantly related as plants and bacteria. Detectable activity of the transgene product in tobacco indicates that *Zymomonas* PDC is most probably active as a homomer, as proposed by Bringer-Meyer and Sahm (1991).

Zymomonas PDC protein is constitutively present at high levels and active *in vitro* under both normoxic and anoxic conditions in leaves of transgenic tobacco. After 2 and 4 h of anoxia, considerable acetaldehyde and ethanol accumulated in the transgenic plants but not in the wild-type. These results show that PDC is the limiting factor during short-term anoxia and that the concentration of the enzyme is likely to be a key regulator. On the other hand, it appears unnecessary to introduce constitutively expressed ADH, since the acetaldehyde produced by *Zymomonas* PDC can be converted to ethanol by endogenous ADH. Our results are thus in line with those of Roberts *et al.* (1989) who showed that maize mutants with drastically reduced ADH activity still displayed a normal anaerobic reponse.

Even when grown under normoxia, the transgenic plants contained large quantities of *Zymomonas* PDC and sufficient endogenous ADH. These are the only two enzymes uniquely required for ethanolic fermentation. Then why do the transgenic tobacco plants not produce ethanol in the presence of oxygen? The answer to this question is complex. First of all, the experiments shown in Figure 6 demonstrate that tobacco leaves have the capacity to metabolize ethanol aerobically. Thus, ethanol may be produced but immediately converted to acetate by ADH and acetaldehyde dehydrogenase (Cossins, 1978). After the action of an acetyl-coenzyme A synthetase the carbon could enter the TCA cycle. In *S.cerevisiae* this pathway operates after transition from glucose limitation to glucose excess under aerobic conditions (Verduyn *et al.*, 1984). Under such physiological conditions PDH levels are too low to sustain rapid growth and the pathway functions to feed the TCA cycle while bypassing PDH. Evidence for the existence of such a pathway has also been obtained in plants. In aerated leaves of eastern cottonwood, exogenously added labelled ethanol was metabolized to acetaldehyde and then to acetate by alcohol and aldehyde dehydrogenases, and then further processed in general metabolism (MacDonald and Kimmerer, 1993). This pathway may operate in the transgenic plants and could explain why ethanol does not accumulate during normoxia. However, it cannot explain the results of the inhibitor experiments presented in Figure 7. In the case of AA there is no problem: respiration is inhibited and the only possible way to keep energy production going is via fermentation. However, AEP is a very specific inhibitor of PDH (Laber and Amrhein, 1987; Schönbrunn-Hanebeck *et al.*, 1990) and the remobilization pathway, which bypasses PDH, should function. In effect, ethanol production is substantially higher after AEP treatment than after AA treatment. We therefore assume that if the remobilization pathway is functional its capacity must be limited. The lower ethanol production after AA treatment

could easily be explained by partial activity of the TCA cycle with possible accumulation of TCA cycle intermediates such as succinate or malate (Good and Muench, 1993). In the absence of inhibitors, in the air pyruvate preferentially enters the TCA cycle via PDH. Apparent K_ms in the order of 57−250 μM have been reported for various plant PDHs (Randall and Miernyk, 1990). These values are lower than the K_m for *Zymomonas* PDC (0.4 mM; Bringer-Meyer and Sahm, 1991). Thus, under normoxic conditions pyruvate preferentially enters the TCA cycle and small amounts of ethanol may be remetabolized.

One of the best studied aspects of the anaerobic response is the transcriptional induction of the ADH gene. In maize and rice, ADH transcript levels rise within minutes and accumulate to very high levels. This is also the case in tobacco leaves. Under normoxia ADH transcripts are present at low levels (Figure 8A). After 2 h of anoxia a high increase in steady-state transcript levels can be measured. What is the cellular sensor which regulates this transcriptional response? Is it oxygen concentration itself or is it some metabolic consequence of oxygen deficiency? The results shown in Figures 7 and 8 clearly show that metabolic conditions which cause ethanol production do not trigger ADH gene transcription. Thus, it seems likely that the signal for transcriptional activation is either oxygen concentration itself or the concentration of an effector molecule, such as heme, whose synthesis is oxygen-dependent. In yeast, the aerobic induction of *CYC1* and *CYC7* transcription is indeed mediated by heme. In this system heme binds directly to transcription factor HAP1 and thereby activates transcription from the *CYC1* and *CYC7* promoters (Pfeifer *et al.*, 1989). In the case of the anaerobically induced *ANB1* gene, heme can repress transcription in the presence of air (Lowry and Zitomer, 1984; Lowry and Lieber, 1986). In the plant symbiont *Rhizobium meliloti*, the hemoprotein kinase FixL was shown to control the expression of bacterial nitrogen fixation genes in response to low oxygen levels (Lois *et al.*, 1993). Hemoglobin is present not only in leguminous plants, but also in the nitrogen-fixing root nodules of a non-legume, *Parasponia* (Appleby *et al.*, 1983). This raises the possibility that hemoproteins have a general function in plants as oxygen sensors.

Constitutive overexpression of PDC has allowed us to study the physiology of ethanolic fermentation and its relationship to regulation of gene expression. However, our work may also have important applications. Tobacco seedlings are quite tolerant of anoxia, surviving several days of anoxia, and no further improvement was observed in transgenic plants. Introducing *Zymomonas* PDC into flooding-intolerant species such as potato (Sieber and Brändle, 1991) may improve tolerance. In avocado and tomato fruits acetaldehyde is applied commercially to inhibit ripening and cell wall softening (Pesis and Marinansky, 1993). In fruits hypoxic conditions may occur during ripening and acetaldehyde production could be enhanced without using metabolic inhibitors of respiration. Such fruits of transgenic plants are likely to exhibit advantageous properties during storage and flavor development.

Materials and methods

Plasmid construction

The complete *Z.mobilis* PDC gene was present in the plasmid pLOI276 (Conway *et al.*, 1987). Through oligonucleotide-directed mutagenesis an *Nco*I site (CCATGG) was created around the initiator ATG. The sequence from this *Nco*I site to the *Bam*HI site downstream of the stop codon in the polylinker of the plasmid was inserted into plasmid pMOGEN18 (B.Dekker, Mogen, Leiden, The Netherlands, unpublished). This chimeric plasmid contained the constitutive 35S cauliflower mosaic virus promoter (Odell *et al.*, 1985) and the alfalfa mosaic virus translational enhancer (Jobling and Gehrke, 1987) 5′ to an exact fusion to the ATG translation start site of the *Zymomonas* PDC gene and the *nos* 3′ terminator (Depicker *et al.*, 1982) 3′ to the PDC gene. Excision of this construct by *Eco*RI−*Sal*I digestion and cloning between the *Eco*RI and *Sal*I sites of the binary vector pMON505 (Rogers *et al.*, 1987) finally gave rise to plasmid pMON505-9204.

A tobacco ADH cDNA was isolated from a tobacco pollen cDNA library. Identity was verified by partial sequence analysis and comparison with the maize ADH cDNA sequence (Dennis *et al.*, 1984). Details will be published elsewhere.

Tobacco transformation

Vector pMON505-9204 was used to transform leaves of *Nicotiana tabacum* var. Samsun with the *Agrobacterium tumefaciens* strain LBA4404 essentially according to Horsch *et al.* (1985).

Plant material

F_1 plants of primary transformants were tested via Western blotting for the presence of the *Zymomonas* PDC protein. Young leaves of positive transformants and of wild-type *N.tabacum* var. Samsun plants at a similar developmental stage with a length of ~14 cm and a width of ~8 cm were excised and used for further experiments.

RNA extraction and Northern blot analysis

Total RNA was extracted from 200−300 mg of tobacco leaf material on a small scale using the method of Verwoerd *et al.* (1989). The yield was 80 ± 37 μg per gram fresh weight. Total RNA was quantified both spectrophotometrically at 260 nm and visually by the staining of stripped blots in 0.02% methylene blue in 0.3 M sodium acetate, pH 5.5. Excess dye was gently washed away with water. Ten micrograms of total RNA of each sample were loaded onto a 1.2% agarose−glyoxal gel after glyoxylation (Hull, 1985). Northern blotting and hybridization procedures were carried out under standard conditions (Sambrook *et al.*, 1989). Blots were hybridized at 65°C with randomly labelled probes as indicated in the figure legends. The final wash was with 0.1% SSC at 42−48°C. Transcript size was determined by comparison of the signals with a glyoxylated 1 kb DNA ladder (BRL).

Antibody generation

The same 1700 bp *Nco*I−*Bam*HI fragment used for expression in tobacco was cloned into the vector pET-8c (pET-3d) (Studier *et al.*, 1990). This construct was retransformed into the *E.coli* strain pLysS which was cultured in LB medium containing 25 μg/ml chloramphenicol and 100 μg/ml ampicillin until an OD_{660nm} of 0.1−0.2 had been reached. Expression of the heterologous protein was initiated with IPTG at 0.4 mM final concentration for 2 h at 37°C. Inclusion bodies were isolated according to Marston (1987). They were solubilized in 8 M urea and dialyzed overnight at 4°C in 50 mM Tris, 2 mM EDTA at pH 7.5. 3 × 0.1 ml of the purified antigen was injected intramuscularly and 2 × 0.1 ml was injected subcutaneously into rats at a concentration of 1 mg/ml in complete Freund's adjuvant. This procedure was repeated three times every 3 weeks in incomplete Freund's adjuvant to boost antibody production. Two months after the first immunization the rats were bled and the serum was recovered. A 1:1000 dilution of rat serum was used for Western blot analysis.

Protein extraction and Western blot analysis

Protein extraction and Western blotting were performed as described by Bucher and Kuhlemeier (1993). The yield with tobacco leaves was 9.5 ± 2.5 mg per gram fresh weight. Briefly, 10 μg of total soluble protein was separated on a 12.5% SDS−polyacrylamide gel and transferred to nitrocellulose (Schleicher & Schuell). A 1:1000 dilution of rat anti-*Zymomonas* PDC antibody and horseradish peroxidase conjugates were used to detect *Zymomonas* PDC in transgenic tobacco leaf extracts.

Anaerobic incubation

Young tobacco leaves as described above were detached, carefully rinsed in distilled water and split along the midrib with a scalpel. This procedure was performed to guarantee homogenous plant material in comparable physiological states for anaerobic treatment and control treatment. Prior to incubation, split leaves were infiltrated three times at 64 kPa in a solution containing 50 mM glucose and 0.1 mM $CaSO_4$ (Roberts *et al.*, 1984a) for 2.5 min, briefly dried with kitchen paper and transferred into black-painted,

150 ml, air-tight, glass bottles. Anoxic conditions were created as described in Bucher and Kuhlemeier (1993) in an anaerobic work bench for test leaves. Incubation lasted 2, 4 or 24 h.

In vitro PDC activity

Approximately 200–300 mg of leaf tips of incubated split leaves were homogenized in Eppendorf tubes in 500 µl of cold extraction buffer (Laszlo and St Lawrence, 1983) and put on ice. After centrifugation at 13 000 r.p.m. for 10 min in a microfuge at 4°C the supernatant was transferred into a new tube and spun again for 20 min. This supernatant was used to detect PDC activity according to the protocol to detect yeast PDC (EC 4.1.1.1, Sigma P-6810) activity as provided by Sigma. The assay had a total volume of 1.0 ml and contained citrate buffer (0.18 M) at pH 6.0, β-NADH (0.107 mM), ADH (3.3 U/ml) and 33.3 µl extract. The assay was started by the addition of pyruvate at a final concentration of 33 mM. Residual respiratory activity was inhibited by the addition of 0.9 mM KCN. Yeast PDC from Sigma (see above) was used to calibrate the assay conditions. NADH oxidation was measured in 1 ml cuvettes at 340 nm in a double-beam spectrophotometer (Uvicon) for 5 min with six data points. The change in absorbance per minute (ΔA_{340}/min) was calculated from the slope of the regression curve. Protein concentrations were detected according to Bradford (1976). The specific activity of PDC in the extracts was calculated as follows:

$$U/mg = (\Delta A_{340}/min)/[6.22 \times (mg\ protein/ml\ reaction\ mixture)]$$

[1 unit will convert 1.0 µmol of pyruvate to acetaldehyde per minute at pH 6.0 at 25°C.]

Head space analysis of acetaldehyde and ethanol

Split leaves were incubated as described above. After 2, 4 or 24 h, 1 ml samples of head space were taken with a gas-tight syringe (Hamilton) and immediately injected and analyzed by gas chromatography in a Sigma 300 GC (Perkin Elmer) with a flame ionization detector, using a Porapak Q 2 m × 3 mm column (Perkin Elmer). GC conditions were as follows. Column temperature, 100°C; injector temperature, 170°C; detector temperature, 190°C; N_2 carrier gas flow, 30 ml/min. The electrometer signal was processed with an LCI 100 detector (Perkin Elmer). The system was calibrated with ethanol and freshly distilled acetaldehyde as standards. The standard concentrations were in the range of the expected sample signals to ensure linear calculations by the detector.

Inhibitor treatment

Split leaves were infiltrated as described above with the addition of 2 µM antimycin A (AA) (Vanlerberghe and McIntosh, 1992) to the infiltration medium. (R)-1-aminoethylphosphinate (AEP) was added at a concentration of 30 µM. After infiltration, split leaves were transferred into glass bottles as described above under normoxic conditions.

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