The Plant Journal (1998) **14**(3), 327–335

Anoxia tolerance in tobacco roots: effect of overexpression of pyruvate decarboxylase

Million Tadege, Roland Brändle and Cris Kuhlemeier
Institute of Plant Physiology, University of Berne, Altenbergrain 21, CH-3013 Berne, Switzerland

Summary

Plant survival during flooding relies on ethanolic fermentation for energy production. The available literature indicates that the first enzyme of the ethanolic fermentation pathway, pyruvate decarboxylase (PDC), is expressed at very low levels and is likely to be rate-limiting during oxygen deprivation. The authors expressed high levels of bacterial PDC in tobacco to study the modulation of PDC activity *in vivo*, and assess its impact on the physiology of ethanolic fermentation and survival under oxygen stress. In contrast to leaves, wild-type normoxic roots contained considerable PDC activity, and overexpression of the bacterial PDC caused only a moderate increase in acetaldehyde and ethanol production under anoxia compared to wild-type roots. No significant lactate production could be measured at any time, making it unlikely that lactate-induced acidification (LDH/PDC pH-stat) triggers the onset of ethanol synthesis. Instead, the authors favour a model in which the flux through the pathway is regulated by substrate availability. The increased ethanolic flux in the transgenics compared to the wild-type did not enhance anoxia tolerance. On the contrary, rapid utilisation of carbohydrate reserves enhanced premature cell death in the transgenics while replenishment of carbohydrates improved survival under anoxia.

Introduction

Plants switch from respiration to fermentation when oxidative phosphorylation is curtailed by oxygen limitation, as is often the case during flooding in the natural environment. This switch involves the selective synthesis of a set of polypeptides (Sachs *et al.*, 1980) including the enzymes required for ethanolic and lactic acid fermentation. However, our understanding of the exact molecular nature of the switch is far from complete. According to the widely accepted Davies–Roberts pH-stat hypothesis, the plant's primary response to oxygen limitation is characterised by a burst of lactate production which lowers the cytoplasmic pH. This leads to the activation of PDC and the inhibition of LDH (Davies *et al.*, 1974), thereby causing a shift from lactic acid to ethanolic fermentation. Essentially, lactate production is transitory and plants rely on ethanolic fermentation for metabolic energy during prolonged periods of anoxia. When lactate accumulation is not stopped, cytoplasmic acidification is assumed to cause cell death and thereby flooding intolerance (Roberts *et al.*, 1984a, 1984b). Although these experiments do not warrant generalisation, the hypothesis has quite often been extended to other systems as well (Rivoal and Hanson, 1994). In fact, high ethanol and low lactate production appear to correlate well with anoxia tolerance in a large number of species (Good and Muench, 1993; Pfister-Sieber and Brändle, 1994; Rivoal *et al.*, 1989).

The Davies–Roberts hypothesis appears to be inconsistent with some of the metabolic changes observed under oxygen stress. In shoots of wheat seedlings (Menegus *et al.*, 1989) and maize root tips (Saint-Ges *et al.*, 1991), cytoplasmic acidification and lactate accumulation did not have the same time course. In rice seedling shoots, anoxia imposition led to alkalinization of the cell sap rather than acidification (Menegus *et al.*, 1989). In barley roots (Hoffman *et al.*, 1986) and potato tubers (Pfister-Sieber and Brändle, 1994), induction of LDH activity and sustained production of lactate and ethanol were evident during prolonged periods of anoxia. Even in the highly flooding tolerant, halophytic *Limonium* species, lactate production was maintained throughout the anoxic period (Rivoal and Hanson, 1993). Based on these and other reports, it seems necessary to critically examine the role of transient lactate production as the inducer of ethanolic fermentation.

We are interested in the regulation of ethanolic fermentation and its function in flooding tolerance. We chose to approach the problem from a genetic point of view using molecular tools to alter one character at a time in an otherwise genetically uniform background. Ethanolic fermentation requires only two dedicated enzymes, PDC and ADH. Most studies until now have focused on ADH, the second enzyme in the pathway. Biochemical studies indicate that ADH is very active even at very low concentrations, and is never limiting except in *Adh1* null alleles (Lemke-Keyes and Sachs, 1989; Roberts *et al.*, 1989). The level of PDC is lower than that of ADH by a factor of 17–65 (Morrell and Greenway, 1989) and its activity is very close to the rate of ethanolic fermentation *in vivo* (Morrell *et al.*, 1990; for a recent review see Drew, 1997). Therefore, PDC activity is considered to be a key regulator of ethanolic fermentation

Received 27 August 1997; revised 3 February 1998; accepted 10 February 1998.
*For correspondence (fax +41 31332 2059; e-mail cris.kuhlemeier@pfp.unibe.ch).

under conditions of oxygen limitation (Morrell *et al.*, 1990; Waters *et al.*, 1991).

Since the concentration of PDC in the cell is thought to be a major element in the control of ethanolic flux, we introduced a bacterial PDC into tobacco and studied the effect of this single gene addition on the regulation of ethanolic fermentation (Bucher *et al.*, 1994). In wild-type tobacco leaves, PDC levels were barely detectable, whereas in the transgenics PDC activity increased to 20-fold of the wild-type levels. In the absence of O_2, or when respiration or entry into the TCA cycle was blocked with inhibitors, a large increase in ethanol and acetaldehyde production was measured in the transgenics compared to the wild-type (Bucher *et al.*, 1994). Thus, in oxygen-limited leaves ethanolic flux is determined by the concentration of PDC.

Here, we study the effect of bacterial PDC overexpression in tobacco roots, and compare the results with those obtained in leaves and pollen. We propose a unified model for the regulation of ethanolic flux in those three organs. Moreover, we assess the effect of PDC overexpression on viability under anoxia.

Results

Expression and activity of Zymomonas *PDC in tobacco roots*

Previously, we overexpressed PDC from the obligate anaerobic bacterium *Zymomonas mobilis* under the control of the 35S promoter and measured the effect on sugar metabolism in tobacco leaves (Bucher *et al.*, 1994). Two reasons prompted us to investigate the effect of PDC overexpression in roots. First, roots are the physiological relevant organs which are immediately exposed to oxygen deprivation during flooding. Second, the use of roots enables us to measure the energy status of the tissue without the complication of photosynthesis.

Western blot analysis indicated that the transgene was correctly expressed in the roots of the F1 progeny of the tobacco, and that the protein accumulated to a similar level in all the transformants (Figure 1a). Roots incubated for 4 and 12 h in the presence (+) or absence (–) of O_2 showed similar levels of protein accumulation (Figure 1b). This is to be expected because the activity of the 35S promoter is not known to be influenced by oxygen availability. For further analysis we selected one of the transformants (line X).

Next, we measured PDC enzymatic activity in wild-type and transgenic roots. Under normoxic conditions the *in vitro* PDC enzymatic activity of transgenic roots showed a twofold increase compared to the wild-type (Figure 2a). This is a much lower increase than in leaves, where we found a 20-fold increase (Bucher *et al.*, 1994). This difference between leaves and roots is caused not by a

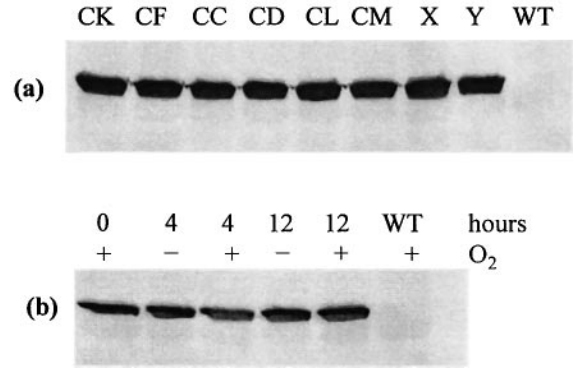

Figure 1. *Zymomonas* PDC protein expression in tobacco roots.
(a) Total protein (40 μg per lane) of eight independent transgenic F_1 roots were separated by SDS-PAGE. *Zymomonas* protein levels were detected using anti-*Zymomonas* PDC antibody. Proteins from the wild-type tobacco roots (WT) were used as a negative control. The transgenic lines are indicated by letters on top of each lane.
(b) Transgenic roots (line X) were subjected to anoxic incubation for 4 h or 12 h as indicated by numbers above the figure. (+) indicates the presence and (–) indicates the absence of oxygen during the incubation period. Total soluble proteins (20 μg per lane) were analysed as in (a). Proteins from normoxic wild-type roots (WT) served as a negative control. Representative samples of two experiments are shown.

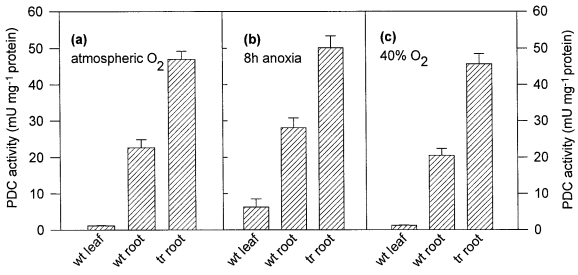

Figure 2. PDC *in vitro* enzymatic activity at different oxygen levels.
Specific PDC activity in transgenic (tr) and wild-type (wt) tobacco. One unit (U) will convert 1.0 μmol of pyruvate to acetaldehyde per minute at pH 6.0 at 25°C.
(a) Plants were grown at ambient oxygen tension.
(b) Plants were grown as in (a), but then transferred to anoxia for 8 h before protein extraction.
(c) Plants were grown in a 40% O_2 environment.

low activity of the transgene in roots, but by a high basal level of PDC activity in wild-type roots compared to leaves (Figure 2a). When detached roots were subjected to anoxia for 8 h, PDC activity in the wild-type increased by only 25%, whereas in the transgenics no significant increase was measured. Anoxic leaves showed a measurable increase in PDC activity, but even the induced level was far below the root level (Figure 2b). There could be two explanations for the high PDC activity in normoxic roots. Either a high basal level exists which is not influenced by oxygen availability, or the culture regime might temporarily or partially cause hypoxia. To discriminate between these possibilities, we cultivated the seedlings suspended in a box where they were misted with nutrients and 40% O_2, thus avoiding any possible hypoxia. Under such conditions, the *in vitro* PDC activity of both the wild-type and the transgenic roots

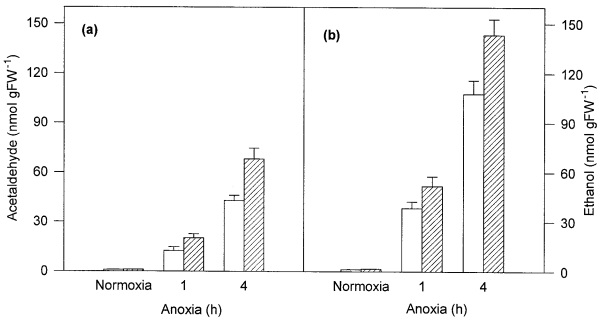

Figure 3. Head space analysis of acetaldehyde and ethanol under anoxia by gas chromatography.
Excised roots were divided into two halves and each half was infiltrated with glucose and briefly blotted on tissue paper. One half was incubated under anoxia for 1 h or 4 h as indicated and the other half served as a control (normoxia). Acetaldehyde (a) and ethanol (b) concentrations were determined in the same sample by gas chromatography. Open bars, wild-type; hatched bars, transgenic.

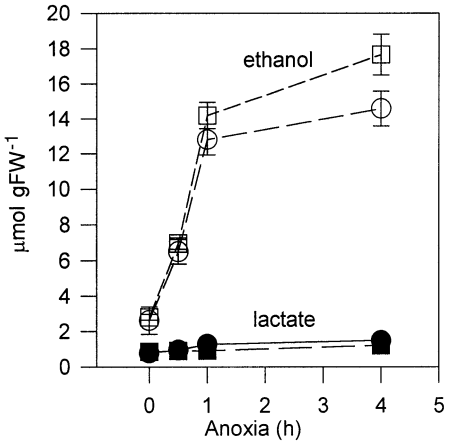

Figure 4. Lactate and ethanol detection in the root tissue under anoxia.
Transgenic and wild-type excised roots were infiltrated with glucose and incubated from 0 to 4 h under anoxia. Lactate and ethanol concentrations in a neutralised extract were determined enzymatically. Circles, wild-type; squares, transgenic.

remained invariably high (Figure 2c). Therefore, the high basal activities in panel (a) were not caused by oxygen limitation. These results demonstrate one major difference between tobacco leaves and roots; namely a constitutively present endogenous PDC enzymatic activity in the latter but not in the former.

Acetaldehyde, ethanol and lactate measurement

To determine if the PDC enzyme is also active *in vivo*, we measured the products of fermentative metabolism in the gas phase, as well as in the tissue. When excised root apices were infiltrated with glucose and incubated for 4 h under normoxic conditions, ethanol and acetaldehyde production in the gas phase by both the transgenic and wild-type roots was below the detection limit of the gas chromatograph. Anoxic incubations, on the other hand, led to the accumulation of acetaldehyde and ethanol within 1 hour (Figure 3a and b). Wild-type roots showed an appreciable ethanolic flux under anoxia, but the transgenics produced consistently higher (40% and 33%, respectively) levels of acetaldehyde and ethanol. Thus, the difference between the wild-type and transgenic roots was not as pronounced as the eight- to 35-fold seen in leaves. This can readily be explained by the high levels of PDC in wild-type roots but not in wild-type leaves (see also Bucher *et al.*, 1994). Whereas gas chromatography provides a fast and reliable way of measuring the accumulation of both acetaldehyde and ethanol, it is difficult to quantitatively relate concentrations measured in the gas phase to tissue concentrations (see Experimental procedures). Moreover, the technique is limited to volatile compounds and thus excludes lactate. Therefore, we measured ethanol and lactate concentrations in the tissue plus medium by enzymatic methods. These experiments revealed that even under normoxic conditions tobacco roots contain a small but significant amount of ethanol in their tissue. When anoxia was imposed onto the system, ethanol rose within 30 min. The transgenics produced slightly higher levels of ethanol at 1 h and 4 h of anoxia (Figure 4). Lactate production, however, was barely detectable under normoxic conditions, and remained very low during at least the first 4 h of anoxia. Similar results were obtained for alanine (data not shown). This clearly shows that ethanol synthesis can be rapidly induced without an increase in lactate production.

This leaves us with a question – if not a lactate-induced pH decrease, what then controls the carbon flux to ethanol? Why does PDC not operate at a high rate under normoxia as it does under anoxia? Ethanolic flux could be directly regulated by an oxygen-sensing system, as is the case for induction of ADH gene expression (Bucher *et al.*, 1994), or alternatively flux might be controlled indirectly by some metabolic consequence of oxygen deprivation. To resolve this issue, we used inhibitors to inhibit respiration in the presence of oxygen. Antimycin A (AA) is a specific inhibitor of the cytochrome oxidase pathway, and salicylhydroxamic acid (SHAM) is an inhibitor of the alternative oxidase pathway (Vanlerberghe and McIntosh, 1997). When AA and SHAM are applied together, electron transport through the respiratory chain is impaired and the effects resemble those of anoxia although oxygen is available. When roots were treated with a combination of AA and SHAM, acetaldehyde and ethanol accumulated to high levels. This treatment caused about a 35% increase in acetaldehyde and a 25% increase in ethanol levels in the transgenic roots compared to the wild-type (Figure 5 roots). In contrast, the increase in leaves was about 22-fold for acetaldehyde and sevenfold for ethanol in the transgenics relative to the wild-type (Figure 5 leaves).

The largest difference between transgenic and wild-type

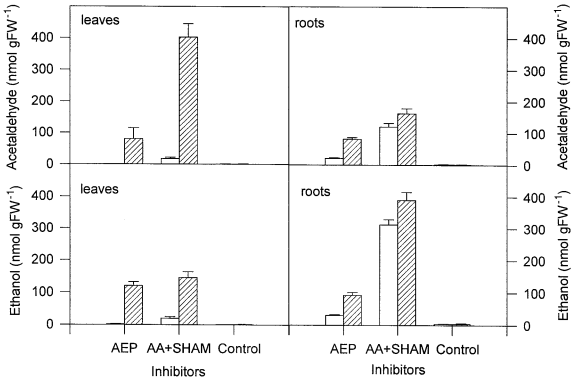

Figure 5. Head space analysis of acetaldehyde and ethanol in the presence of respiration inhibitors.
Roots or leaves were divided into two halves and one half was infiltrated with glucose in the presence of either AEP or AA and SHAM for 5 min. The other half was infiltrated with only glucose and used as a control. Both the controls and inhibitor treated samples were incubated for 4 h at 25°C and 1 ml gas samples from the head space were analysed by gas chromatography. Open bars, wild-type; hatched bars, transgenic.

roots was observed when the entry of pyruvate to the tricarboxylic acid cycle was inhibited by (R)-1-aminoethylphosphinate (AEP). AEP is quickly metabolised to acetylphosphinate which is a very specific and potent inhibitor of the pyruvate dehydrogenase complex (Laber and Amrhein, 1987). This inhibition of the pyruvate dehydrogenase complex (PDH) by AEP caused a fourfold and threefold increase in acetaldehyde and ethanol levels, respectively, in the transgenics compared to the wild-type (Figure 5 roots). In transgenic leaves, a high level of ethanolic flux could also be measured during AEP treatment, but not in the wild-type leaves (Figure 5 leaves). The absence of ethanolic fermentation in wild-type leaves can be explained by the absence of PDC activity in leaves but not in roots (Figure 2a). These inhibitor experiments point to the important fact that ethanolic fermentation in roots can be induced in the presence of oxygen, and thus that fermentation is not regulated by oxygen concentration directly, but more likely indirectly by the metabolic consequences of oxygen deprivation.

Viability under stress

The above experiments demonstrate that the transgenic roots have a somewhat higher capacity for producing ethanol than the wild-type under conditions of oxygen limitation or inhibitor treatment. Since ethanolic fermentation is the major route of energy production under anoxia in tobacco, we wished to test whether this enhanced capacity of ethanol production improves survival in the absence of oxygen. Under greenhouse conditions, the transgenic plants looked normal and showed no obvious phenotype except that they were slightly retarded in height about 5–8 weeks after germination. After this time period the differences became indistinct and the transgenics flowered at about the same time as the wild-type. When 5-week-old whole plants were subjected to anoxia in anaerobic work bench, differences in survival could not be detected. Both the transgenic and wild-type plants survived up to 48 h of anoxia as evaluated by their ability to resume shoot growth when re-exposed to air. After 60 h of anoxic incubation more than 85% of the plants died, and none survived after 72 h of treatment, but there was no difference between the transgenic and the wild-type (data not shown). Similar data were obtained with germinating seeds and young seedlings.

Further investigation of the energy status of the roots under anoxia revealed a difference between the wild-type and the transgenics. Table 1 shows the energy budget of wild-type and transgenic roots under anoxia. Adenylate energy charge (AEC) is a term quite often used to assess the relative proportion of ATP, ADP and AMP in a given system and is expressed as the ratio (ATP + 0.5 ADP)/(ATP + ADP + AMP). Under normoxic conditions, the AEC values were about 0.8, both for the transgenic and the wild-type. During the first 4 h of anoxic incubation, the AEC declined drastically at a similar rate for the transgenic and wild-type (Table 1). After 4 h, in the wild-type roots the reduction of the AEC continued at a lower rate and at about 12 h the AEC stabilised around 0.38. On the other hand, the transgenics increased their AEC values after 4 h of anoxia. This result could be interpreted as indicating that overexpression of bacterial PDC improves the energy status of the root. However, a drastic reduction in the total adenylate nucleotide (ADN) levels was measured during the course of anoxia. This reduction was even more pronounced in the transgenics than in wild-type roots (Table 1). Thus, the total concentration of high energy adenylates available was lowered, and the increase in AEC alone is unlikely to signify increased anoxia tolerance. In order to assess damage to the membranes, we measured the conductivity of the roots after increasing periods of anoxia. Indeed, leakage of ions from the roots increased with anoxic incubation time, the transgenics showing more damage than the wild-type (Figure 6). Under normoxic conditions, the transgenics and the wild-type behaved in the same way to leakage of electrolytes.

Finally, we evaluated the ability of excised, anoxically treated root apices to re-establish normoxic ATP values after re-exposure to air. Several reports have shown that upon re-exposure to air, plant roots regain their normoxic ATP levels in less than 10 min provided that they are not damaged; anoxic maize root tips in which roots showing 10% or lower ATP recovery were classified as dead (Bouny and Saglio, 1996; Xia and Saglio, 1992). This approach can be used to quantitatively estimate cell death during anoxia.

When roots were infiltrated with glucose, blotted on tissue paper and incubated under anoxia without further

Table 1. Adenylate nucleotide levels under anoxia in transgenic and wild-type tobacco roots

Anoxia (h)	Roots	ATP (nmol gFW^{-1})	ADP (nmol gFW^{-1})	AMP (nmol gFW^{-1})	AEC
0	wild-type	60.17 ± 4.35	22.45 ± 2.17	4.88 ± 0.35	0.81
	transgenic	55.95 ± 4.67	20.21 ± 1.98	5.25 ± 0.33	0.81
4	wild-type	7.65 ± 0.57	8.52 ± 0.81	8.99 ± 0.79	0.47
	transgenic	5.74 ± 0.39	5.19 ± 0.60	6.46 ± 0.62	0.48
12	wild-type	4.21 ± 0.25	6.51 ± 0.29	8.44 ± 0.39	0.39
	transgenic	2.61 ± 0.13	1.86 ± 0.15	2.45 ± 0.21	0.51
24	wild-type	2.00 ± 0.28	2.73 ± 0.23	3.99 ± 0.33	0.38
	transgenic	1.66 ± 0.22	1.82 ± 0.25	1.70 ± 0.19	0.50

Values represent the mean ± SE of three experiments each made in triplicate.

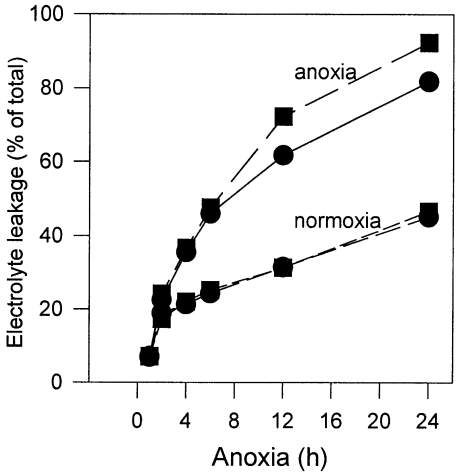

Figure 6. Electrolyte leakage of transgenic and wild-type tobacco roots under anoxia.
Roots were incubated in distilled water under anoxia and the conductivity of the medium was measured at different time points. For controls, roots were incubated under normoxia. Values are the mean of three experiments each made in triplicate. Circles, wild-type; squares, transgenic.

addition of glucose, a steep decrease in the ability to re-establish normoxic ATP levels was observed between 8 and 12 h. Both the transgenic and wild-type roots survived 4 and 8 h of anoxia, but at 12 h only 12.5% of the ATP was recovered from the transgenics compared to the 20.3% in the wild-type (Figure 7a). This shows that, between 8 and 12 h of anoxia, the transgenic roots became more sensitive to the stress than the wild-type. After 12 h of anoxia, both the transgenic and wild-type roots had died, estimated from less than 10% recovery of normoxic ATP concentration.

The decreased viability of the transgenics might be caused by acetaldehyde toxicity. However, there is another explanation. From the data in Figure 4 it can be calculated that during anoxia, root tips produced approximately 12 μmoles gFW^{-1} h^{-1} of ethanol, which would correspond to 6 μmole gFW^{-1} h^{-1} of glucose metabolised. In contrast, during normoxia the same root tips respired at a rate of 14 μmole O_2 gFW^{-1} h^{-1} (data not shown), which would correspond to 2.3 μmole gFW^{-1} h^{-1} glucose metabolised. Thus, there is a considerable Pasteur effect and, in fact, the transgenic root tips may die prematurely because they deplete their carbohydrate reserves more rapidly than the wild-type. Therefore, we repeated the above experiment, but with infiltration of glucose every 6 h. Under these conditions excised wild-type and transgenic tobacco roots survived for more than 48 h of anoxia. Between 48 and 72 h of anoxia, the ATP recovery dropped below 10% and remained low thereafter indicating irreversible damage and death (Figure 7a). At all time points, no significant differences were observed between the transgenic and wild-type. This is in agreement with the survival of the intact seedlings under anoxia as nutrients may not be limiting yet during the first 48 h. Measurement of ethanol production rate during repeated glucose feeding (Figure 7b) shows that at 6 and 12 h of anoxia, the transgenics produced more ethanol than the wild-type. After 24 h, ethanol production was drastically reduced and no differences were observed between transgenic and wild-type. Thus, it appears likely that the reduced viability of the transgenics was caused by enhanced membrane leakage and reduced availability of substrates.

Discussion

The present work completes our studies on the regulation of ethanolic fermentation in tobacco. We constitutively expressed high levels of bacterial PDC and studied ethanol production in three different organs: leaves (Bucher *et al.*, 1994), pollen (Bucher *et al.*, 1995; Op den Camp and Kuhlemeier, 1997; Tadege and Kuhlemeier, 1997) and roots (this study). The combined results show that ethanolic fermentation is regulated very differently in the three organs.

(i) In wild-type leaves, very little PDC enzyme is present under normoxia, and consequently leaves produce no measurable ethanol in the presence of oxygen (Bucher *et al.*, 1994). In the absence of oxygen, PDC synthesis is induced and ethanol is produced. Constitutive expression of bacterial PDC enhances ethanol production under

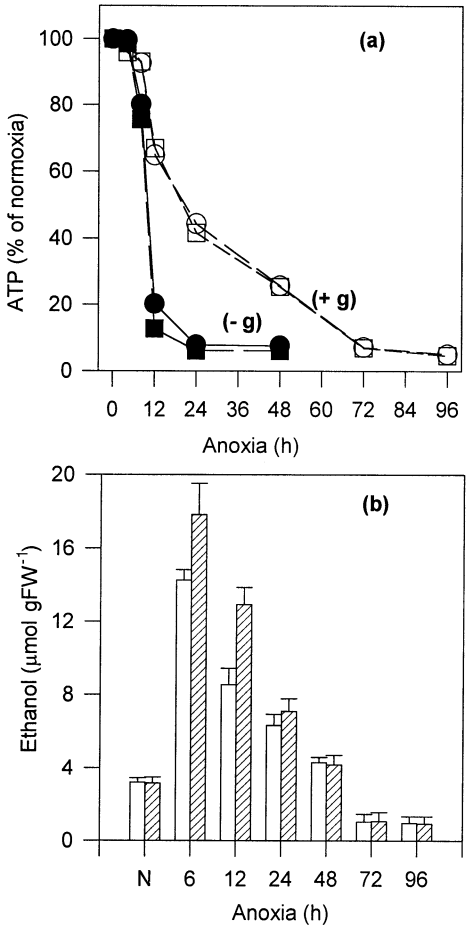

Figure 7. Viability assessment of tobacco roots under anoxia and rate of ethanol production in the medium.
(a) Roots were infiltrated with glucose solution at the beginning of the experiment, blotted on tissue paper and incubated for various periods under anoxia at 25°C (–g) or roots were infiltrated and incubated under anoxia in glucose medium. Every 6 h, the old medium was withdrawn and replaced by fresh deoxygenated glucose solution (+g). At the end of each incubation period, the roots were re-exposed to air for 30 min before extraction and the total ATP recovered was measured. Those which showed 10% or lower ATP recovery were classified as dead. Values are the mean of at least four experiments each made in triplicate. Circles, wild-type; squares, transgenic; (–g), glucose infiltration only once; (+g), repeated glucose infiltration.
(b) Rate of ethanol production during repeated glucose feeding. Roots were infiltrated with fresh deoxygenated glucose solution every 6 h as in (+g) and samples were taken from the medium at the indicated time points. Values are the mean ± SE of four experiments. Open bars, wild-type; hatched bars, transgenic; N, normoxia.

anoxia, indicating that the size of the flux is determined by the amount of PDC. However, the high levels of transgenic PDC do not lead to ethanol production under normoxia, and therefore ethanolic flux is not only dependent on PDC levels, but also directly or indirectly controlled by oxygen availability.

(ii) Pollen contains high levels of PDC, similar to transgenic leaves. Surprisingly, in contrast to transgenic leaves, pollen actively ferments in the presence of oxygen. Thus, ethanolic flux is not controlled by oxygen availability. Instead, we demonstrated that in pollen the controlling factor is substrate availability (Bucher *et al.*, 1995; Tadege and Kuhlemeier, 1997).

(iii) In roots, PDC is present at high levels under normoxia, and when oxygen is withdrawn, enzyme levels rise only marginally. This is very similar to the situation in pollen. Yet, in contrast to pollen, ethanol production is low under normoxia, and high during oxygen limitation. Thus, as in transgenic leaves, flux is controlled primarily by oxygen availability.

In summary, in tobacco leaves, flux is controlled by PDC concentration and oxygen levels; in roots it is controlled by oxygen levels; and in pollen by substrate levels. Although it is obviously possible that regulation in these three organs proceeds by distinct and independent mechanisms, we believe that all the observations can be explained within the framework of a single theory, which is based on the different K_m's of PDH and PDC for pyruvate. Pyruvate can enter two pathways, either the TCA-cycle via PDH, or ethanolic fermentation catalysed by PDC. Intracellular concentrations for pyruvate of the order of 0.1 mM have been measured in parsnip and carrot roots, with an increase being observed after anoxic treatment (Davies *et al.*, 1974; Faiz-ur-Rahman *et al.*, 1974). This is close to the K_m's for pyruvate of 57 and 79 μM for mitochondrial PDHs from pea leaves and etiolated maize shoots, respectively (Randall and Miernyk, 1990). In contrast, the K_m's for pyruvate for rice, pea and maize PDC are in the order of 0.25–1 mM (Lee and Langston-Unkefer, 1985; Mücke *et al.*, 1995; Rivoal *et al.*, 1990). Moreover, pyruvate was shown to bind co-operatively to the rice enzyme (Rivoal *et al.*, 1990), leading the authors to emphasize the importance of intracellular pyruvate concentration for the activation of PDC. Based on the accumulated evidence we propose that ethanolic flux is regulated by a PDH/PDC-stat. Under normoxic conditions pyruvate will be preferentially metabolised by PDH, and ethanolic fermentation will only occur when available pyruvate cannot be utilised by PDH. This can be the case in leaves and roots when entry into the TCA cycle and respiratory chain is blocked, as happens during anoxia or after application of respiratory inhibitors. Alternatively, pyruvate will be shunted towards PDC when the respiratory pathway is working at maximal capacity, as is the case in pollen.

The Davies–Roberts theory postulates a central role for lactate fermentation in the initial phase of the anaerobic response. This theory requires (i) that lactate accumulates transiently before the onset of ethanolic fermentation; (ii) that PDC activity is limiting flux; and (iii) that PDC activity is regulated by pH. We could not measure any significant lactate accumulation in tobacco roots in the early stages of oxygen deprivation, thus it seems unlikely that a LDH/PDC pH-stat regulates ethanolic flux in tobacco. Other

evidence indicates that a drop in pH may be caused by proton leakage from the acidic vacuoles, and that such a pH shift could conceivably activate PDC (Roberts *et al.*, 1984a). However, PDC activity *in vivo* and the flux through the ethanolic pathway may not be under the strict control of pH at all. In tobacco roots and transgenic leaves there is a high concentration of PDC, and yet flux is very low in the presence of oxygen. This would require a very sharp pH optimum, as indeed has been shown in pea seed crude extracts (Davies *et al.*, 1974). However, *Z. mobilis* PDC has a very broad pH range with activities between pH 7.5 and pH 6.0 varying less than 20% (Neale *et al.*, 1987). Thus, if flux were regulated through the pH dependence of PDC, transgenic tobacco should produce ethanol during normoxia. This is not the case and, therefore, other mechanisms must be invoked to explain the absence of flux during normoxia in the transgenics. As stated above, we propose that the intracellular pyruvate concentration determines the activity of PDC *in vivo* and thereby ethanolic flux.

Anoxia and inhibitor treatments are not the only factors that trigger ethanolic flux. For example, water deficit, exposure to cold, exposure to ozone, and fumigation with SO_2 (Kimmerer and Kozlowski, 1982) have been shown to cause aerobic production of ethanol and acetaldehyde. We hypothesise that such treatments impair mitochondrial function, and thereby shunt carbon flux away from TCA cycle and respiratory chain towards PDC. It seems plausible that plant cells fall back on inefficient but robust ethanolic fermentation as an immediate alternative for ATP production whenever the much more complicated and delicate mitochondrial pathway is compromised.

Plants can survive anoxia far longer than most other higher eukaryotes, and this difference is generally attributed to the capacity of plants to carry out ethanolic fermentation. Marsh plants, for instance, produce vast amounts of ethanol. But will an increase in ethanolic flux in a plant improve its flooding tolerance? Maize *Adh1*-null mutants survive anoxia for less time than do wild-type (Roberts *et al.*, 1989), which clearly shows that ethanolic fermentation is required for survival. However, those experiments did not address the question of whether enhanced fermentation would cause enhanced survival, because an increase of ADH activity above a low threshold level did not increase flux, presumably because PDC was limiting. In more recent experiments, tolerance was routinely enhanced by hypoxic pretreatments (Andrews *et al.*, 1994; Bouny and Saglio, 1996; Johnson *et al.*, 1994; Xia and Roberts, 1994; Xia and Saglio, 1992). However, such pretreatments are likely to lead to complex physiological changes in the plants (see Drew, 1997 for review) and thus it is hard to pinpoint the cause of the enhanced tolerance. In our experiments we increased ethanolic flux during anoxia by the addition of a single gene, and thus we could compare isogenic lines under identical growth conditions. Our experiments show that an increase in ethanol production does not lead to enhanced anoxia tolerance (Figure 7). On the contrary, the transgenic plants died slightly earlier. Survival could be prolonged by repeated infiltration with glucose. The data in Figures 6 and 7 suggest that the decreased tolerance of the transgenic roots is caused by enhanced membrane leakage (due to elevated levels of ethanol and acetaldehyde) and by the depletion or reduced transport (see Saglio, 1985) of carbohydrate reserves. This is in agreement with the observation that in rice the ability to degrade α-amylase is one of the major factors that contribute to anoxia tolerance (Perata *et al.*, 1992). Similarly, in hypoxically acclimated maize root tips, improved hexokinase activities correlate with improved survival under anoxia in the presence of exogeneous sugar (Bouny and Saglio, 1996). Thus, in most crop plants improvement of flooding tolerance will require modification of multiple characters.

Experimental procedures

Growth of plants

The wild-type and transgenic tobacco plants (*Nicotiana tabacum cv* Samsun) were grown in a growth room under a 16 h:8 h light:dark cycle and a temperature of 18–25°C. After germination in soil, 3-week-old seedlings were transferred to pots which were filled with inert hydroculture clay beads (Luwasa, Switzerland) and nutrients were supplied as liquid. For 40% O_2 treatment, the roots of the seedlings were washed with water and the clean roots were suspended in a plastic box. The lid of the box was made with holes in such a way that each hole supports the growth of one plant in which the roots were allowed to grow inside the box while the shoots were exposed to the atmosphere. The box was connected to a 40% O_2 jar and to a nutrient solution at its lower side. Roots were grown for 2–3 weeks in this way while being flashed with 40% oxygen and misted with nutrient solution.

Protein extraction and analysis

Protein extraction, Western blotting, and PDC enzymatic assays were as described previously (Bucher *et al.*, 1994).

Inhibitor treatment, acetaldehyde and ethanol measurement in the gas phase

The roots or leaves of 5- to 7-week-old plants were prepared as follows. Roots were washed several times with distilled water and 4 cm of the root apex was excised. The excised roots were blotted on tissue paper, divided into two halves and weighed. Leaves were rinsed with water and the fourh leaf, as counted from the top, was split into two halves along the midrib. Each half was blotted on a tissue paper and weighed. For both roots and leaves, each half (0.5 g) was infiltrated with 50 mM glucose and 0.1 mM $CaSO_4$ solution for 5 min under vacuum in the presence or absence of inhibitors. One half was always used for the anoxia or inhibitor treatment and the other half used as a control. Inhibitors were added to the infiltration solution at concentrations of 30 μM AEP,

2 μM AA, and 1 mM SHAM. AA and SHAM were obtained from Sigma, AEP was a generous gift of N. Amrhein (ETH, Zurich). Anoxia was imposed in an anaerobic work bench (Forma Scientific Mariatta, Ohio, USA) according to Bucher *et al.* (1994). Infiltrated samples were briefly blotted on tissue paper, sealed in 33 ml Lumac brown bottles and incubated at 25°C for the specified periods. At the end of the incubation time, 1 ml gas samples were withdrawn from the head space and analyzed by gas chromatography as described (Bucher *et al.*, 1994). It is important to note that gas chromatography measures only what is in the gas phase and does not indicate the absolute tissue concentrations. Acetaldehyde tends to be more in the gas phase than in the aqueous phase compared to ethanol, but we do not know the partition coefficients of each, and thus the gas phase values should be considered as relative measurements. Values are the mean ± SE of at least five experiments unless otherwise stated.

Determination of adenylate nucleotide levels

Six to 7-week-old roots were washed several times with distilled water and 0.5 g of approximately 4 cm root apices were infiltrated with the above glucose solution in the anaerobic work bench three times for 2.5 min. Samples were briefly blotted on tissue paper and incubated under anoxia at 25°C, or transferred back to air for normoxic controls. At the end of each incubation period, tissues were killed with liquid nitrogen while still under anoxia or air. For the ATP recovery measurements of Figure 7, tissues were either treated as above or incubated in 2 ml glucose medium, and every 6 h the medium was withdrawn and fresh deoxygenated glucose was added. At the end of the incubation time, samples were exposed to air for 30 min before freezing in liquid nitrogen according to Bouny and Saglio (1996). Frozen samples were ground to fine powder in liquid nitrogen with precooled pestle and mortar, and extracted with 6% perchloric acid according to Sieber and Brändle (1991). ATP was measured using the luciferin/luciferase system (Lumac/3M , Schaasberg, The Netherlands) according to Sieber and Brändle (1991) using commercial ATP (Sigma) as internal standard. ADP and AMP were converted stepwise to ATP and their concentration was determined quantitatively. For ethanol and lactate determination in the tissue (Figure 4), tissues were incubated in 2 ml glucose solution for the specified period and frozen together with the medium for extraction. Concentrations in the neutralised extract were measured enzymatically using a kit from Boehringer (Mannheim, Germany).

Determination of ethanol production rate in the medium

The rate of ethanol production during repeated glucose feeding under anoxia was determined as follows. Root apices were washed several times with distilled water and 0.5 g samples were infiltrated with 2 ml of the glucose solution and incubated at 25°C under anoxia or normoxia for 6 h. At the end of 6 h, samples were taken from the medium of both anoxic and normoxic incubations. All the medium from the anoxic samples was withdrawn and replaced by a fresh deoxygenated glucose solution. The medium was exchanged in this way every 6 h and incubation continued for 96 h, taking samples at the indicated time points. Thus, each time point in Figure 7(b) represents the amount of ethanol produced in the medium during the last 6 h.

Measurement of membrane leakage

Approximately 4 cm root apices were rinsed three times with distilled water and cut into 1 cm pieces. These were put into a beaker containing 50 ml distilled water and the conductance of the water was measured immediately and recorded as time zero. The samples were then transferred to anoxia and conductance was measured at intervals up to 24 h. After 24 h of anoxia, samples were put at –20°C for 24 h and then heated to 70°C for 10 min. Conductance was measured after cooling the samples down to room temperature. At this point all the membranes and cellular compartments were assumed to be disrupted and leakage was considered to be 100%. All the other measurements were expressed as a percentage of this value according to Knowles and Knowles (1989). For controls, conductance was measured in the same way but roots were incubated under normoxia.

Acknowledgements

We thank Dr I. Dupuis for comments on the manuscript. M.T. was supported in part by the Roche Research Foundation. This work was partly funded by grant 31–42020–94 from the Swiss National Science Foundation to R.B.

References

Andrews, D.L., Drew, M.C., Johnson, J.R. and Cobb, B.G. (1994) The response of maize seedlings of different ages to hypoxic and anoxic stress. Changes in induction of *Adh1* mRNA, ADH activity, and survival of anoxia. *Plant Physiol.* **105**, 53–60.

Bouny, J.M. and Saglio, P.H. (1996) Glycolytic flux and hexokinase activities in anoxic maize root tips acclimated by hypoxic pretreatment. *Plant Physiol.* **111**, 187–194.

Bucher, M., Brander, K.A., Sbicego, S., Mandel. T. and Kuhlemeier, C. (1995) Aerobic fermentation in tobacco pollen. *Plant Mol. Biol.* **28**, 739–750.

Bucher, M., Brändle, R. and Kuhlemeier, C. (1994) Ethanolic fermentation in transgenic tobacco expressing *Zymomonas mobilis* pyruvate decarboxylase. *EMBO J.* **13**, 2755–2763.

Davies, D.D., Grego, S. and Kenworthy, P. (1974) The control of the production of lactate and ethanol by higher plants. *Planta*, **118**, 297–310.

Drew, M.C. (1997) Oxygen deficiency and root metabolism: Injury and acclimation under hypoxia and anoxia. Annu. Rev. Plant Physiol. *Plant Mol. Biol.* **48**, 223–250.

Faiz-ur-Rahman, A.T.M., Trewavas, A.J. and Davies, D.D. (1974) The Paster effect in carrot root tissue. *Planta (Berl.)*, **118**, 195–210.

Good, A.G. and Muench, D.G. (1993) Long-term anaerobic metabolism in root tissue. Metabolic products of pyruvate metabolism. *Plant Physiol.* **101**, 1163–1168.

Hoffman, N.E. and Bent, A.F.and.Hanson, A.D. (1986) Induction of lactate dehydrogenase by oxygen deficit in barley root tissue. *Plant Physiol.* **82**, 658–663.

Johnson, J.R., Cobb, B.G. and Drew, M.C. (1994) Hypoxic induction of anoxia tolerance in roots of *Adh1* null *Zea mays* L. *Plant Physiol.* **105**, 61–67.

Kimmerer, T.W. and Kozlowski, T.T. (1982) Ethylene, ethane, acetaldehyde and ethanol production by plants under stress. *Plant Physiol.* **69**, 840–847.

Knowles, N.R. and Knowles, L.O. (1989) Correlations between electrolyte leakage and degree of saturation of polar lipids from aged potato (*Solanum tuberosum*) tuber tissue. *Ann. Bot.* **63**, 331–338.

Laber, B. and Amrhein, N. (1987) Metabolism of 1-amino-ethylphosphinate generates acetylphosphinate, a potent inhibitor of pyruvate dehydrogenase. *Biochem. J.* **248**, 351–358.

Lee, T.C. and Langston-Unkefer, P.J. (1985) Pyruvate decarboxylase from *Zea mays* L. I. Purification and partial characterisation from mature kernels and anaerobically treated roots. *Plant Physiol.* **79**, 242–247.

Lemke-Keyes, C.A. and Sachs, M.M. (1989) Anaerobic tolerant null: a mutant that allows *Adh1* nulls to survive anaerobic treatment. *J. Heredity*, **80**, 316–319.

Menegus, F., Catteruzza, L., Chersi, A. and Fronza, G. (1989) Differences in the anaerobic lactate-succinate production and in the changes of cell sap pH for plants with high and low resistance to anoxia. *Plant Physiol.* **90**, 29–32.

Morrell, S. and Greenway, H. (1989) Evidence does not support ethylene as a cue for synthesis of alcohol dehydrogenase and pyruvate decarboxylase during exposure to hypoxia. *Aust. J. Plant Physiol.* **16**, 469–475.

Morrell, S., Greenway, H. and Davies, D.D. (1990) Regulation of pyruvate decarboxylase *in vitro* and *in vivo. J. Exp. Bot.* **41**, 131–139.

Mücke, U., König, S. and Hübner, G. (1995) Purification and characterisation of pyruvate decarboxylase from pea seeds (*Pisum sativum cv.* Miko). *Biol. Chem.* **376**, 111–117.

Neale, A.D., Scopes, R.K., Wettenhall, R.E.H. and Hoogenraad, N.J. (1987) Pyruvate decarboxylase of *Zymomonas mobilis*: isolation, properties, and genetic expression in *Escherichia coli. J. Bacteriol.* **169**, 1024–1028.

Op den Camp, R. and Kuhlemeier, C. (1997) Aldehyde dehydrogenase from tobacco pollen. *Plant Mol. Biol.* **35**, 355–364.

Perata, P., Pozueta-Romero, J., Akazawa, T. and Yamaguchi, J. (1992) Effect of anoxia on starch breakdown in rice and wheat seeds. *Planta*, **188**, 611–618.

Pfister-Sieber, M. and Brändle, R. (1994) Aspects of plant behaviour under anoxia and postanoxia. *Proc. Royal Soc. Edinburgh*, **102B**, 313–324.

Randall, D.D. and Miernyk, J.A. (1990) The mitochondrial pyruvate dehydrogenase complex. In *Methods in Plant Biochemistry, Enzymes of Primary Metabolism* Volume 3 (Lea P.J., ed.). London: Academic Press, pp. 175–192.

Rivoal, J. and Hanson, A.D. (1993) Evidence for a large and sustained glycolytic flux to lactate in anoxic roots of some members of the halophytic genus *Limonium. Plant Physiol.* **101**, 553–560.

Rivoal, J. and Hanson, A.D. (1994) Metabolic control. of anaerobic glycolysis: overexpression of lactate dehydrogenase in transgenic tomato roots supports the Davies-Roberts hypothesis and points to a critical role for lactate secretion. *Plant Physiol.* **106**, 1179–1185.

Rivoal, J., Ricard, B. and Pradet, A. (1989) Glycolytic and fermentative enzyme induction during anaerobiosis in rice seedlings. *Plant Physiol. Biochem.* **27**, 43–52.

Rivoal, J., Ricard, B. and Pradet, A. (1990) Purification and partial characterisation of pyruvate decarboxylase from *Oryza sativa. Eur. J. Biochem.* **194**, 791–797.

Roberts, J.K.M., Callis, J., Jardetzky, O., Walbot, V. and Freeling, M. (1984a) Cytoplasmic acidosis as a determinant of flooding intolerance in plants. *Proc. Natl Acad. Sci. USA*, **81**, 6029–6033.

Roberts, J.K.M., Callis, J., Wemmer, D., Walbot, V. and Jardetzky, O. (1984b) 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.

Roberts, J.K.M., Chang, K., Webster, C., Callis, J. and Walbot, V. (1989) 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.

Sachs, M.M., Freeling, M. and Okimoto, R. (1980) The anaerobic proteins of maize. *Cell*, **20**, 761–767.

Saglio, P.H. (1985) Effect of path or sink anoxia on sugar translocation in roots of maize seedlings. *Plant Physiol.* **77**, 285–290.

Saint-Ges, V., Roby, C., Bligny, R., Pradet, A. and Douce, R. (1991) Kinetic studies of the variations of cytoplasmic pH, nucleotide triphosphates (^{31}P NMR) and lactate during normoxic and anoxic transitions in maize root tips. *Eur. J. Biochem.* **200**, 477–482.

Sieber, M. and Brändle, R. (1991) Energy metabolism in rhizomes of *Acorus calamus* (L.) with regard to their anoxia tolerance. *Bot. Acta*, **104**, 279–282.

Tadege, M. and Kuhlemeier, C. (1997) Aerobic fermentation during tobacco pollen development. *Plant Mol. Biol.* **35**, 343–354.

Vanlerberghe, G.C. and McIntosh, L. (1997) Alternative Oxidase: From gene to function. Annu. Rev. Plant Physiol. *Plant Mol. Biol.* **48**, 703–734.

Waters, I., Morrell, S., Greenway, H. and Colmer, T.D. (1991) Effect of anoxia on wheat seedlings II. Influence of O_2 supply prior to anoxia on tolerance to anoxia, alcoholic fermentation, and sugar levels. *J. Exp. Bot.* **42**, 1437–1447.

Xia, J.-H. and Roberts, J.K.M. (1994) Improved cytoplasmic pH regulation, increased lactate efflux, and reduced cytoplasmic lactate levels are biochemical traits expressed in the root tips of whole maize seedlings acclimated to a low oxygen environment. *Plant Physiol.* **105**, 651–657.

Xia, J.-H. and Saglio, P.H. (1992) Lactic acid efflux as a mechanism of hypoxic acclimation of maize root tips to anoxia. *Plant Physiol.* **100**, 40–46.