

# Nucleotide Levels Do Not Critically Determine Survival of Maize Root Tips Acclimated to a Low-Oxygen Environment<sup>1</sup>

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We tested the hypothesis that ATP levels and energy charge determine the resistance of maize (*Zea mays*) root tips to anoxia. We focused on root tips of whole maize seedlings that had been acclimated to low O<sub>2</sub> by exposure to an atmosphere of 3% (v/v) O<sub>2</sub> in N<sub>2</sub>. Acclimated anoxic root tips characteristically have higher ATP levels and energy charge and survive longer under anoxia than nonacclimated tips. We poisoned intact, acclimated root tips with either fluoride or mannose, causing decreases in ATP and energy charge to values similar to or, in most cases, below those found in nonacclimated anoxic tips. With the exception of the highest fluoride concentration used, the poisoned, acclimated tips remained much more tolerant of anoxia than nonacclimated root tips. We conclude that high ATP and energy charge are not components critical for the survival of acclimated root tips during anoxia. The reduced nucleotide status in poisoned, acclimated root tips had little effect on cytoplasmic pH regulation during anoxia. This result indicates that in anoxic, acclimated root tips either cytoplasmic pH regulation is not dominated by ATP-dependent processes or these processes can continue *in vivo* largely independently of any changes in ATP levels in the physiological range. The role of glycolytic flux in survival under anoxia is discussed.

Evaluation of the relative importance of biochemical parameters as determinants of plant performance under environmental stresses, such as low O<sub>2</sub>, is complicated by the fact that stresses disturb whole networks of biochemical processes (reviewed by Kennedy et al., 1992; Perata and Alpi, 1993; Ricard et al., 1994). Many observed differences between stressed and unstressed, or tolerant and intolerant, plants may well be manifestations of this general disturbance rather than revelations of fundamental processes responsible for the behavior of a particular plant in different environments.

The primary biochemical effect of low O<sub>2</sub> on plant cells is an inhibition of respiratory ATP synthesis, leading to a switch to a lower overall rate of ATP synthesis via fermentation (Beever, 1961). Given the central role of ATP in cell function (Stryer, 1988), the "energy crisis" in plant cells evoked by low O<sub>2</sub> would appear to be the direct cause of the eventual loss of function. The resistance of maize root tips to anoxia can be enhanced by pretreatment of intact

seedlings with hypoxia (2–4% O<sub>2</sub>) (Saglio et al., 1988; Johnson et al., 1989). Associated with this acclimation response is the ability to maintain a high glycolytic rate during long periods of anoxia (Saglio et al., 1988; Hole et al., 1992; Xia and Saglio, 1992) and higher ATP levels and energy charge (Saglio et al., 1988; Johnson et al., 1994). One straightforward interpretation of this phenomenon is that the primary acclimation response leading to improved tolerance of anoxia is the enhanced energy metabolism, which relieves the shortage of ATP and facilitates cell function. Moreover, reported differences between acclimated and nonacclimated root tips involving processes such as intracellular pH regulation (Xia and Roberts, 1994) could be viewed as simple consequences of changes in energy metabolism rather than as a primary manifestation of the acclimation response, since pH regulation involves ATP-dependent reactions (Kurkdjian and Guern, 1989).

Here we report a direct test of the primacy of nucleotide supply in the acclimation response of maize root tips. We studied root tips of intact seedlings. The metabolic flux to ethanol and the amount of ATP in acclimated root tips were reduced using the metabolic inhibitors Man and fluoride. The consequences of these treatments with respect to survival and cytoplasmic pH regulation were monitored. These measurements allow us to report the dependence of survival and cytoplasmic pH regulation on nucleotide levels and the rate of ATP synthesis in acclimated roots.

## MATERIALS AND METHODS

Maize seeds (*Zea mays* L., inbred B73, a kind gift from Pioneer Hi-Bred International, Johnston, IA) were germinated and grown until the seedlings had primary roots approximately 10 cm long as described previously (Roberts and Testa, 1988). Groups of 25 seedlings were then placed in a glass funnel, and the stem was inserted in a polypropylene tube (Poly-Prep column, Bio-Rad) so that the primary roots could be immersed in 15 mL of basal medium [0.7 mM KNO<sub>3</sub>, 0.65 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 0.25 mM KH<sub>2</sub>PO<sub>4</sub>, 0.75 mM NH<sub>4</sub>NO<sub>3</sub>, 0.16 mM MgSO<sub>4</sub>, 50 μM NaCl, 6 μM H<sub>3</sub>BO<sub>3</sub>, 2.25 μM MnSO<sub>4</sub>, 1 μM ZnSO<sub>4</sub>, 25 nM CuSO<sub>4</sub>, 10 nM Na<sub>2</sub>MoO<sub>4</sub>, 5 mM Mes, adjusted to pH 6.2 with KOH], leaving the kernel and shoot above the liquid. The top of

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Abbreviations: HPT, hypoxically pretreated or hypoxic pretreatment; NHPT, not hypoxically pretreated or no hypoxic pretreatment; NTP, nucleoside triphosphate.

the funnel was sealed with two layers of Parafilm and the medium was flushed with the appropriate gas (100% O<sub>2</sub> for NHPT, 3% [v/v] O<sub>2</sub> in N<sub>2</sub> for HPT; in both cases without pre-equilibration) through the bottom of the tube. The pretreatment usually lasted 6 h, and the bubbled medium was renewed once at 3 h unless otherwise stated.

For Man treatments, seedlings in the basal medium were first flushed with 3% O<sub>2</sub> for 3 h, then this medium was replaced with fresh basal medium supplemented with 50 mM Man and 1 mM Pi (instead of the original 0.25 mM), and the hypoxic treatment was continued for another 3 h. The higher Pi concentration was applied during Man treatment to partially compensate for the depletion of intracellular Pi due to accumulation of Man phosphates (Herold and Lewis, 1977; Loughman et al., 1989a, 1989b). Man was not present during anoxia. In treatments using fluoride, fresh basal medium containing 1 or 2 mM NaF was supplied 45 min before anoxia began; the inhibitor was present during subsequent anoxia.

<sup>31</sup>P-NMR spectra were obtained at 202 MHz, using the sample tube and perfusion arrangement described by Xia and Roberts (1994), a dedicated 10-mm probe, and acquisition conditions used by Talbott et al. (1988). Seedlings were transferred to the NMR sample tube just prior to anoxia, after the various pretreatments were administered as described above. A volume of 180 mL of basal medium, without KH<sub>2</sub>PO<sub>4</sub> and MnSO<sub>4</sub>, was used as the perfusing solution. Cytoplasmic pH was measured from the chemical shift of the cytoplasmic Pi resonance, as described by Roberts (1986a). NTP levels were estimated by <sup>31</sup>P-NMR as described by Roberts (1986b).

Viability of root tips of intact seedlings under anoxia was assessed by measuring their ability to elongate when returned to air. Seedlings were subjected to pretreatments and anoxia as in the NMR experiments. After various periods of anoxia, roots of individual seedlings were placed in glass tubes each lined with a strip of filter paper wetted with 0.1 mM CaSO<sub>4</sub> (Roberts and Testa, 1988); these seedlings were then transferred to a humidified tank (exposed to laboratory fluorescent lights at 23 ± 1.5°C), and the elongation after 24 h in air was measured.

For enzymatic determination of nucleotides, batches of 12 intact seedlings were pretreated and subjected to anoxia as for NMR and viability measurements. At the specified time of anoxia, seedlings were quickly frozen in liquid N<sub>2</sub> (the contact with air during transfer lasted less than 3 s, which has been verified to induce no modifications of ATP levels in the tissues). The frozen root tips (5 mm) were then excised for nucleotide extraction and assay as described previously (Saglio and Pradet, 1980). Ethanol production by excised root tips was assayed enzymatically according to the method of Bernt and Gutmann (1974).

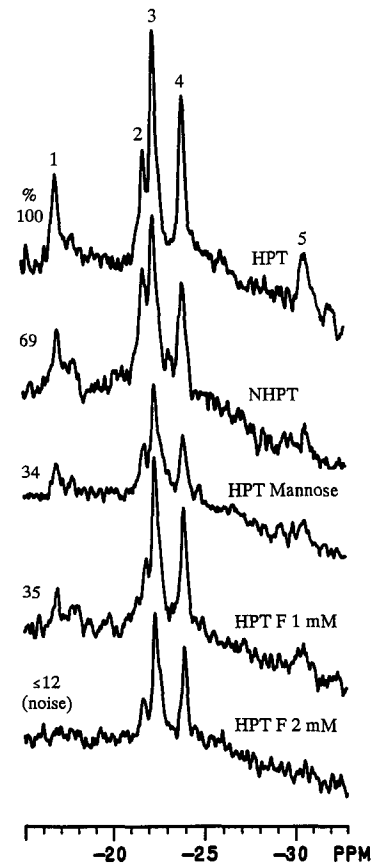
## RESULTS

### Poisoned HPT Root Tips with Lower ATP and Energy Charge than NHPT Tips Remain Tolerant of Anoxia

Acclimated (HPT) root tips of whole maize seedlings were poisoned with either fluoride or Man. Fluoride inhib-

its energy metabolism during anoxia, reflected by reduced flux from Glc to ethanol and lower ATP level and energy charge (Saglio et al., 1983); however, energy metabolism is not disturbed under normoxia (Saglio et al., 1983) or during HPT (data not shown). Man inhibits glycolysis and depletes cytoplasmic phosphate and nucleotide pools (Herold and Lewis, 1977; Loughman et al., 1989a, 1989b). We observed that early in anoxia levels of NTP in poisoned HPT root tips were lower than in NHPT root tips, measured *in vivo* by <sup>31</sup>P-NMR (Fig. 1).

Root tip extracts were analyzed enzymatically for a more complete picture of adenine nucleotide levels and ratios



**Figure 1.** <sup>31</sup>P-NMR partial spectra of nucleotides in intact root tips during anoxia. After 6 h of pretreatment under 3% (HPT) or 100% (NHPT) O<sub>2</sub>, seedlings were transferred to the NMR tube, rinsed briefly with water, and placed in the NMR spectrometer. Sodium fluoride at a final concentration of 1 mM (HPT F 1 mM) or 2 mM (HPT F 2 mM) was added to the perfusing medium 45 min before the onset of anoxia. Man (50 mM in base medium with 1 mM Pi) was applied to the whole seedlings during the last 3 h of hypoxic pretreatment and then removed by a brief washing immediately before anoxic incubation (HPT Man). Spectra shown were obtained over 3 h, from 15 to 195 min of anoxia, and are the summation of data from three separate experiments for each treatment. Peak assignments: 1, γ-NTP; 2, α-NTP; 3 and 4, UDP-Glc; 5, β-NTP. Differences in peak areas are proportional to differences in the amounts of the indicated nucleotides. The relative amount of NTP, expressed on the left of each spectrum as a percentage of the amount in HPT root tips, was obtained from the area of peak 1.

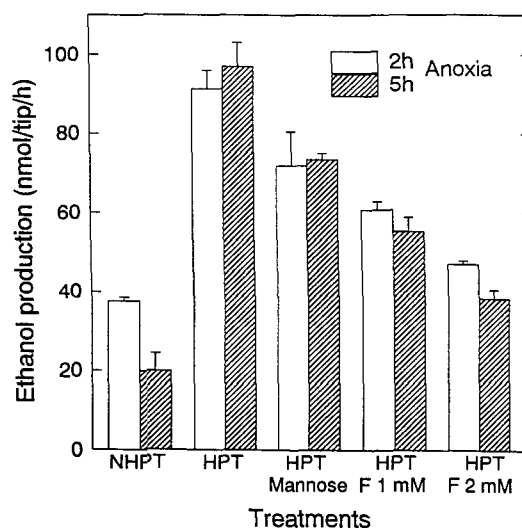
(Table I). The poisons reduced ATP levels, energy charge, and total adenylate pool size in HPT root tips to values similar to, or, in most cases, below those found in NHPT root tips.

Fermentation to ethanol is the principal pathway for ATP production in plants during anoxia (Ricard et al., 1994) and, for a given anoxic tissue, there is a quantitative relationship between energy charge and the rate of glycolysis (Saglio et al., 1980). Therefore, we measured the rate of ethanol production by root tips to infer rates of ATP synthesis during our various treatments. The inhibitor treatments all caused reductions in ethanol production in HPT root tips, although in most cases the flux to ethanol was higher than in NHPT root tips (Fig. 2), in contrast to the effects on nucleotide levels and ratios noted above (Table I). This point is addressed further in "Discussion."

Tolerance of anoxia was tested by following the growth of the intact primary root in normoxia for 24 h, following different times of anoxic treatment. Failure to grow is interpreted to reflect root tip death. Most NHPT root tips died after approximately 8 h of anoxia, as did HPT roots poisoned with 2 mM fluoride (Fig. 3). However, the latter root tips still grew almost twice as fast as NHPT root tips after 4 h of anoxia (Fig. 3), despite having NTP or ATP levels and energy charge far below values in NHPT root tips (Fig. 1; Table I). Remarkably, HPT root tips treated with either 1 mM fluoride or 50 mM Man survived as well as nonpoisoned HPT tips (Fig. 3).

#### Poisoned HPT Root Tips Regulate Cytoplasmic pH Better Than NHPT Tips

We monitored cytoplasmic pH in root tips for two reasons. First, cytoplasmic acidosis is associated with intolerance of low  $O_2$  (Roberts et al., 1984; Kennedy et al., 1992; Perata and Alpi, 1993; Ricard et al., 1994). Second, ATP-dependent processes, such as transmembrane proton pumping, may be important in the regulation of cytoplasmic pH (reviewed by Kurkdjian and Guern, 1989). Hence,



**Figure 2.** Effect of fluoride and Man on ethanol fermentation by anoxic maize root tips. After the various pretreatments and either 2 or 5 h of anoxia (see "Materials and Methods"), 5-mm root tips (fresh weight approximately 4 mg each) were excised and immediately reincubated in a sealed syringe containing 4 mL of base medium. Anoxia was obtained by an intensive 10-min flushing of the medium before and after the addition of root tips. After 50 min of further anoxia, ethanol in the medium was assayed enzymatically. Data points are means  $\pm$  SE of four to eight samples. HPT F 1 mM, Sodium fluoride at a final concentration of 1 mM was added; HPT F 2 mM, sodium fluoride at a final concentration of 2 mM was added.

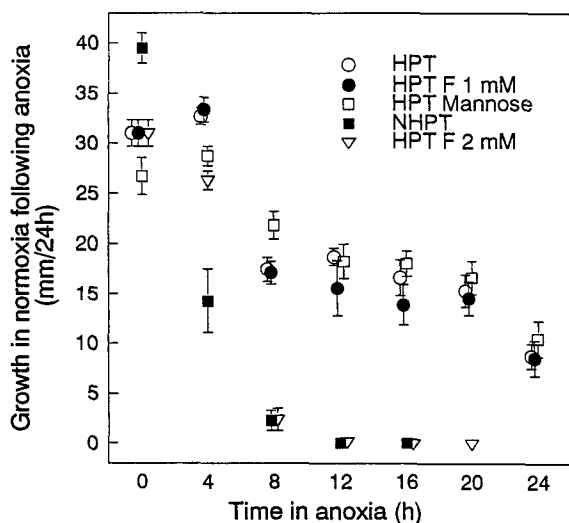
it is reasonable to imagine that maintenance of adequate levels of ATP, presumably higher than in NHPT root tips, which exhibit poor intracellular pH regulation (Xia and Roberts, 1994), might be needed to adequately fuel  $H^+$ -ATPases and permit survival under anoxia. The  $^{31}P$ -NMR measurements of cytoplasmic pH shown in Figure 4 were made on the same samples giving rise to Figure 1. We have previously noted the heterogeneity in cytoplasmic pH of NHPT root tips during anoxia (Xia and Roberts, 1994),

**Table I.** Effects of HPT and metabolic poisons on energy charge, ATP, and total adenylates in 5-mm maize root tips

Data are the averages  $\pm$  SE or range of two to six tests of three independent sets of experiment. Root tip fresh weight is approximately 4 mg each.

Nucleotides	Conditions prior to Sampling	NHPT (n = 6)	HPT			
			Control (n = 6)	Man (n = 4)	1 mM NaF (n = 2)	2 mM NaF (n = 2)
ATP (pmol/tip)	Air	1607 $\pm$ 190	1597 $\pm$ 123	308 $\pm$ 34	1445 $\pm$ 197	1398 $\pm$ 100
	2 h anoxia	494 $\pm$ 14	678 $\pm$ 89	270 $\pm$ 24	324 $\pm$ 61	86 $\pm$ 7
	4-8 h anoxia <sup>a</sup>	373 $\pm$ 30	739 $\pm$ 96	337 $\pm$ 13	259 $\pm$ 16	79 $\pm$ 23
ATP + ADP + AMP (pmol/tip)	Air	2028 $\pm$ 168	2008 $\pm$ 116	695 $\pm$ 52	1968 $\pm$ 242	1857 $\pm$ 130
	2 h anoxia	1437 $\pm$ 41	1343 $\pm$ 104	790 $\pm$ 50	1378 $\pm$ 190	1183 $\pm$ 14
	4-8 h anoxia <sup>a</sup>	1290 $\pm$ 67	1402 $\pm$ 122	862 $\pm$ 27	1124 $\pm$ 40	1087 $\pm$ 9
Energy charge ([ATP + 0.5ADP]/ [ATP + ADP + AMP])	Air	0.87 $\pm$ .02	0.88 $\pm$ .02	0.59 $\pm$ .01	0.84 $\pm$ .02	0.85 $\pm$ .00
	2 h anoxia	0.52 $\pm$ .01	0.67 $\pm$ .02	0.52 $\pm$ .01	0.43 $\pm$ .02	0.18 $\pm$ .01
	4-8 h anoxia <sup>a</sup>	0.46 $\pm$ .04	0.69 $\pm$ .02	0.56 $\pm$ .02	0.43 $\pm$ .02	0.16 $\pm$ .01

<sup>a</sup> Data combined from samples harvested after 4 h (n = 2) and 6 h (n = 2) for NHPT, HPT control, and HPT-Man treatments and 8 h (n = 2) for NaF treatments.



**Figure 3.** Effects of hypoxic pretreatment and metabolic poisons on root tip growth after different periods of anoxia. Experiments were performed in parallel to, and under the same conditions as, experiments using NMR spectroscopy (see "Materials and Methods"). Each point is the mean  $\pm$  SE from 10 to 45 individual roots, including the dead ones, which have a value of zero. HPT F 1 mM, sodium fluoride at a final concentration of 1 mM was added; HPT F 2 mM, sodium fluoride at a final concentration of 2 mM was added.

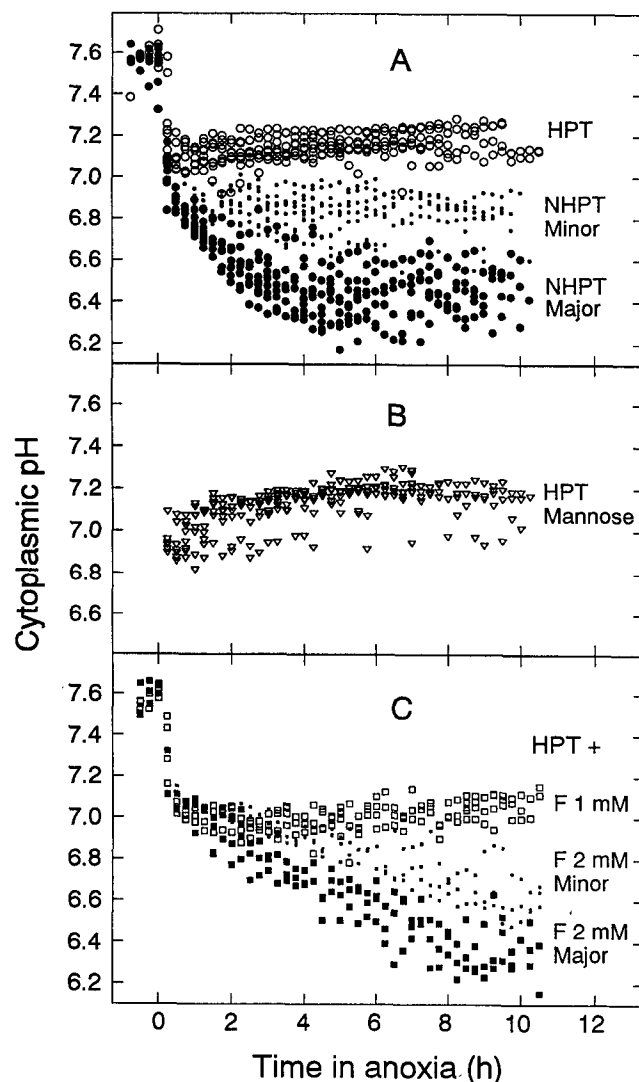
inferred from the existence of two distinct cytoplasmic Pi signals in  $^{31}\text{P}$ -NMR spectra of NHPT roots (Fig. 5, top). Therefore, the data in Figure 4 include information concerning subpopulations of root tip cells differing in cytoplasmic pH and show that the dying root tips exhibit both increased heterogeneity in cytoplasmic pH and increased cytoplasmic acidosis. For example, in NHPT root tips, the cytoplasmic pH in most cells was less than 6.6 after 3 h of anoxia, but a minor proportion of cells maintained their cytoplasmic pH just below neutrality (Fig. 4A), long after the root tips lost the capacity to survive anoxia (Fig. 3). Two such major and minor populations differing in cytoplasmic pH were seen in HPT root tips exposed to 2 mM, but not 1 mM, fluoride (Fig. 4C). The less severe cytoplasmic acidosis early in anoxia in 2 mM fluoride-HPT root tips, relative to NHPT roots (Fig. 4, C and A, respectively), correlates with their respective growth rates when returned to air after 4 h of anoxia (Fig. 3). HPT root tips, in the presence or absence of 1 mM fluoride or 50 mM Man, exhibited excellent cytoplasmic pH regulation near neutrality for at least 10 h of anoxia (Fig. 4), which correlates with their improved survival (Fig. 3).

## DISCUSSION

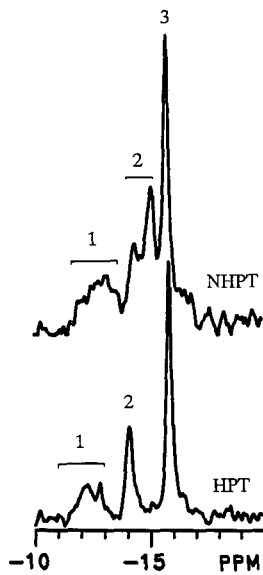
Acclimation to a low  $\text{O}_2$  environment induces a number of metabolic and energetic modifications, somehow linked to modifications of gene expression (Ricard et al., 1994). The main metabolic features identified to date in acclimated root tips during anoxia are (a) higher levels of ATP and energy charge values, which suggest a more efficient energy metabolism (Saglio et al., 1988; Johnson et al., 1994); (b) a sustained and high glycolytic rate (Saglio et al., 1988;

Waters et al., 1991; Hole et al., 1992; Xia and Saglio, 1992); and (c) control of cytoplasmic pH near neutrality (Xia and Roberts, 1994). These observations raise the question of whether any or all of these metabolic modifications are of critical importance in determining survival of acclimated root tips under anoxia.

The use of the poisons Man and fluoride allowed us to dissociate nucleotide levels from cytoplasmic pH regulation and survival of acclimated, intact root tips during anoxia. For HPT root tips during anoxia, both survival (Fig. 3) and cytoplasmic pH regulation (Fig. 4) were unaffected



**Figure 4.** Effects of hypoxic pretreatment and metabolic poisons on cytoplasmic pH during anoxia in intact root tips. Treatments are identical with those described in the legend to Figure 1, except that in some NHPT experiments the seedlings were not preincubated in 100%  $\text{O}_2$  (this had no effect on viability). Data shown were obtained from  $^{31}\text{P}$ -NMR spectra collected successively every 15 min, from three to eight experiments for each treatment. The labels "Major" and "Minor" represent the major and the minor components, respectively, of pH values calculated from the broadened and split cytoplasmic Pi resonance evident in spectra of NHPT roots and HPT roots treated with 2 mM fluoride.



**Figure 5.**  $^{31}\text{P}$ -NMR partial spectra of monophosphate esters and Pi pools in intact root tips during anoxia. After 6 h of pretreatment under 3% (HPT) or 100% (NHPT)  $\text{O}_2$ , seedlings were transferred to the NMR tube, rinsed briefly with water, and placed in the NMR spectrometer. Spectra shown are from single experiments and were obtained over 15 min, starting 4.5 h after the onset of anoxia. Peak assignments: 1, monophosphate esters, such as sugar phosphates; 2, cytoplasmic Pi; 3, vacuolar Pi. More negative Pi chemical shifts correspond to more acidic pH values.

by 1 mM fluoride or 50 mM Man, despite a more than 50% decrease of ATP (Table I; Fig. 1); therefore, we conclude that survival and cytoplasmic pH regulation in HPT root tips are not critically dependent on cellular ATP content. Even HPT root tips treated with 2 mM fluoride, which contained only trace amounts of ATP after 2 h anoxia (Table I), were able to regulate cytoplasmic pH early in anoxia much more effectively than NHPT root tips (Fig. 4) and resumed growth at an almost unaffected rate when returned to air after 4 h of anoxia (Fig. 3). Furthermore, anoxic NHPT root tips neither regulated cytoplasmic pH nor survived very long, despite having higher ATP and energy charge than poisoned HPT root tips, indicating that the poor performance of NHPT root tips under anoxia was not simply due to low ATP levels. NHPT root tip cells died without depleting ATP below levels found in the viable, poisoned HPT tissues (Table I). This observation suggests that acclimation results in modifications in ATP-utilizing enzymes, which increase their avidity for ATP, by increases in some combination of the amounts, catalytic turnover numbers, and affinity for ATP, of these enzymes.

The experiments with fluoride and Man also shed some light on the role played by glycolytic flux to ethanol in survival and cytoplasmic pH regulation during anoxia. The results in Figure 2 suggest a critical threshold of approximately 50 nmol of ethanol per tip per h below which survival is compromised. This apparent threshold is approximately 50% of the flux to ethanol in acclimated controls, indicating that fermentation in nonpoisoned HPT root tips is not a factor limiting their survival under anoxia.

This critical threshold for glycolytic flux under anoxia presumably reflects the minimum rate of supply of ATP necessary to sustain cell function. The maintenance of a critical, relatively high glycolytic flux is consistent with the notion that an increase in the activities of a number of enzymes involved in sugar metabolism may play a role in the acclimation response (reviewed by Ricard et al., 1994). How glycolytic flux is regulated in HPT and NHPT root tips and what is responsible for the decline in glycolytic flux in NHPT root tips early in anoxia (Hole et al., 1992; Xia and Saglio, 1992) (Fig. 2) remain to be elucidated.

The metabolic poisons fluoride and Man reduced ATP levels and energy charge in HPT root tips proportionately much more than glycolytic flux, when compared with values in NHPT root tips (compare Table I with Fig. 2), which indicates that the rate of ATP synthesis is of greater importance than the level of ATP in determining survival during anoxia. These observations also lead to the conclusion that the relationship between energy charge and the rate of glycolysis, which reflects the balance between pathways of ATP regeneration and consumption (reviewed by Raymond et al., 1987), has been altered during the acclimation process. Modification of ATP-utilizing enzymes and glycolytic metabolism, noted above, are two general ways in which the relationships between nucleotide fluxes and nucleotide concentrations, or ratios, could be altered.

The close correlation between survival and cytoplasmic pH, in which loss of viability during anoxia is associated with increased acidosis (compare Figs. 3 and 4), provides additional evidence that cytoplasmic acidosis is a determinant of plant cell tolerance to low  $\text{O}_2$  (Roberts et al., 1984; Kennedy et al., 1992; Ricard et al., 1994; Xia and Roberts, 1994). Our finding that cytoplasmic pH regulation in anoxic, acclimated root tips is unaffected when the rate of ATP synthesis and the level of ATP are significantly reduced invites consideration of the role of ATP-dependent processes in intracellular pH regulation *in vivo*. It has long been thought that plasma membrane and tonoplast  $\text{H}^+$ -ATPases play an important role in intracellular pH regulation in plants (reviewed by Sze, 1984; Kurkdjian and Guern, 1989; Serrano, 1989). As discussed by Saint-Ges et al. (1991), it is reasonable to expect that the activities of these  $\text{H}^+$ -ATPases would be strongly affected by changes in intracellular ATP levels, given the low affinity of these enzymes for MgATP generally found *in vitro*, relative to intracellular nucleotide levels.

However, contrary to this expectation, the results in Table I and Figure 4 indicate that either the  $\text{H}^+$ -ATPases have a much higher affinity for ATP *in vivo* or these proton pumps play a minor role in regulation of cytoplasmic pH in cells under low  $\text{O}_2$ , or both. The work of Mathieu et al. (1986) would indicate that the second possibility is likely the case, such that other mechanisms of pH regulation, including membrane antiports and metabolic proton consumption, play a major role in the disposal of intracellular acid. An effective lactate (or lactic acid) excretion mechanism (Xia and Saglio, 1992; Rivoal and Hanson, 1993; Xia and Roberts, 1994) may play a crucial role in cytoplasmic pH regulation in anoxic, acclimated root tips. Whether any

of these mechanisms are directly or indirectly ATP dependent remains to be determined. We should also point out that the relative importance of ATP-dependent reactions in intracellular pH regulation can vary with environmental conditions. For example, Gout et al. (1992) concluded that, when plant cells are exposed to increasingly acidic environments, the plasma membrane ATPase consumes more and more ATP to extrude protons that leak into the cells. Their results lead to the testable expectation that poisoned HPT root tips would be less tolerant of anoxia than non-poisoned HPT roots at lower extracellular pHs than used in the experiments reported here.

Metabolic reactions driven by the hydrolysis of PPI (reviewed by Taiz, 1986) are of potential relevance to a discussion about ATP-independent metabolic processes involved in survival under anoxia. The observation in anoxic rice of an induction of the glycolytic enzyme PPI:Fru-6-P 1-phosphotransferase (Mertens et al., 1990) and the tonoplast proton-pumping PPIase (Carystinos et al., 1993) leads to the question: what role do these enzymes play in energy metabolism and pH regulation in anoxic plant tissues? We offer three reasons why PPI-dependent enzymes are unlikely to be critical in driving metabolism in anoxic maize root tips. First is the fact that net PPI synthesis is a byproduct of nucleotide-dependent reactions, primarily associated with biosynthesis (Taiz, 1986). The rate of biosynthetic processes such as cell wall and protein synthesis is greatly reduced under anoxia, and therefore the net rate of PPI production must be small under anoxia and much smaller than the rate of ATP synthesis. This point also draws attention to the fact that PPI-dependent reactions are coupled to NTP metabolism rather than being metabolically NTP independent. Second, the cytoplasmic PPI-dependent reactions *in vivo* appear to be near thermodynamic equilibrium and therefore may do little or no work (Roberts, 1990). Third, in nonacclimated maize root tips the activity of PPI:Fru-6-P 1-phosphotransferase is already approximately 50-fold higher than that needed to account for the *in vivo* glycolytic flux, and hypoxic acclimation induces only a small increase of this activity (P.H. Saglio, unpublished data). These last two points suggest that the PPI-dependent enzymes may be analogous to alcohol dehydrogenase in maize root tips: activity of alcohol dehydrogenase in normal root tips does not limit the capacity for energy production, and increases in the activity of this enzyme are not responsible for improved tolerance of anoxia (Roberts et al., 1989; Johnson et al., 1994). These suppositions await direct tests of their validity and clarification of the role of PPI-dependent enzymes in plant cell metabolism.

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