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Dependence of Ethanolic Fermentation, Cytoplasmic pH Regulation, and Viability on the Activity of Alcohol Dehydrogenase in Hypoxic Maize Root Tips[1]

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ABSTRACT

We examined the role of alcohol dehydrogenase (ADH) in the metabolism and survival of hypoxic maize (*Zea mays* L.) root tips. The dependence of the rate of ethanolic fermentation, cytoplasmic pH, and viability on the activity of ADH in maize root tips during extreme hypoxia was determined. Maize lines with ADH activities differing over about a 200-fold range were studied. Effects of genetic background were controlled by comparing pairs of F4 progeny of crosses between mutant (low ADH activity) and reference inbred lines. The capacity of hypoxic root tips to perform ethanolic fermentation exhibited a dependence on ADH activity only at activities found in *Adh1* nulls. The ability of maize root tips to withstand prolonged and extreme hypoxia was likewise independent of ADH activity, except at the lowest activities. Root tips that exhibited lower tolerance of hypoxia had more acidic cytoplasm during extreme hypoxia. We conclude that the activity of ADH in normal maize root tips does not limit the capacity for energy production via fermentation, and does not determine viability under extreme hypoxia. The significance of the induction of ADH activity in plants by hypoxia is discussed.

Fermentation of sugars to ethanol is the primary mechanism of energy production in hypoxic maize roots (1, 8). Ethanolic fermentation permits continuous production of ATP without cytoplasmic acidosis, in contrast to lactic fermentation (14). Reduction of acetaldehyde to ethanol is catalyzed by alcohol dehydrogenase (ADH[3]), which in maize is encoded by two unlinked loci, *Adh1* and *Adh2*. Two observations in particular have provided indirect evidence that the activity of ADH may play a critical role in the metabolism, and so survival, of plants under hypoxia. First, tissues of maize having very low ADH activity (as a result of either mutations in *Adh* genes or developmental control of *Adh* expression) are less tolerant of hypoxia than tissues exhibiting ADH activities typical of wild-type kernels or root tips (4, 12, 13, 15–17). For example, in a comparison of maize lines differing in ADH activity, Chen *et al.* (4) found that kernels containing 5% of wild-type ADH activity were unable to germinate when submerged under air-saturated water (hypoxia), while kernels containing 13% of normal ADH levels were as capable as wild type in tolerating this hypoxic stress. Second, ADH activity increases in maize roots (and other tissues) during hypoxia, because the expression of *Adh* genes and translation of their messages are selectively enhanced (see ref. 2, for review). Such behavior can be viewed as an adaptive response—selected for during evolution—resulting in improved energy metabolism and survival in hypoxic plant tissues. However, use of these correlative observations to assign a critical role for ADH in the metabolism and survival of normal plant tissues is equivocal. With the exception of studies using mutants, comparisons of tissues differing in ADH activity—for example, leaves *versus* roots, or hypoxically pretreated *versus* aerobic organs—are confused by a multitude of other, uncontrolled differences. And the demonstration, using mutants, that very low ADH activity results in both decreased ethanolic fermentation and tolerance of hypoxia, does not speak to the issue of whether ADH activity in normal plants is in excess, or limits metabolism and tolerance of hypoxia.

In this paper we describe the effect of ADH activity on the metabolism and survival of hypoxic maize root tips. Maize lines exhibiting a wide range of ADH activities were examined. Comparisons were made between four *Adh1* mutants and reference *Adh1* alleles, using F4 populations to randomize genetic background. We show that the metabolism and viability of hypoxic maize root tips becomes limited by ADH activity only at values approaching those found in *Adh1* nulls.

MATERIALS AND METHODS

Generation of Maize Lines Differing in ADH Activity

Four different pairs of F4 populations of maize (*Zea mays* L.) were derived from four crosses in which one parent was a mutant line that exhibited either low or no ADH1 activity, and the other parent was a reference inbred line with an ADH1 activity of a different electrophoretic mobility: *Adh1-S908* (low specific activity of ADH1) × *Adh1-FunkF*; *Adh1-Fm335* (low level expression of *Adh1*) × *Adh1-U725*; *Adh1-Cm* (low specific activity of ADH1) × *Adh1-1F*; *Adh1-F460* (no ADH1 activity) × *Adh1-1S*. For a review of these *Adh1* alleles, see Freeling and Bennett (6). Seed for these crosses

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[3] Abbreviation: ADH, alcohol dehydrogenase (EC 1.1.1.1).

was generously provided by Professors M. Freeling (University of California, Berkeley) and D. Schwartz (Indiana University). F2 seed from these four crosses were planted in the field, and the individuals were screened for ADH activity by sampling prop roots, performing native PAGE, and staining for ADH activity (5, 18). Plants identified as homozygous at the *Adh1* locus were selfed. The resultant homozygous F3 ears with the same *Adh1* allele were pooled and either selfed or sib-crossed the next summer, to produce sufficient seed for analysis (about 1200 g per experiment). In addition, homozygous F3 individuals from the cross *Adh1-F460* × *Adh1-1S* were reciprocally crossed to produce a ninth population that had only one functional *Adh1* gene; this population was examined in a three-way comparison with the F4 segregated progeny of the two parent lines. Production of the lines as described above randomizes between the populations in each set (pairs or triad) the genetic background, including the ADH activity from *Adh2* expresssion. Thus, differences in ADH activity between the two F4 populations of each pair were solely the result of differences in the expression at the *Adh1* locus, and loci tightly linked to this gene.

Glycolytic Flux to Ethanol, Cytoplasmic pH, and Viability

These parameters were measured in perfused 2 mm maize root tips as described previously (15) with three major exceptions. First, stainless steel or glass tubing was used between the reservoir of nitrogen-saturated 50 mM Glc and the samples to eliminate diffusion of oxygen into the perfusate through plastic tubing. Second, experiments were performed at 25°C. These conditions constituted somewhat more severe conditions than employed previously, hence root tips died earlier under hypoxia. (In this study, as in previous studies [14, 15], oxygen tension is reduced in the hypoxic treatment such that ATP production from oxidative phosphorylation is less than the rate of substrate level phosphorylation; our hypoxic conditions can therefore be described as extreme hypoxia.) Third, cytoplasmic pH was estimated from the chemical shift of cytoplasmic Pi in ^{31}P NMR spectra obtained with a General Electric GN500 spectrometer, operating at 202 MHz. The ability of root tips to survive hypoxia was measured by scoring for recovery and growth following a given period of hypoxia. Samples of 20 root tips were removed from hypoxia after various periods of time, weighed, placed on filter paper wetted with 50 mM Glc (containing the antibiotics gentamycin and amphotericin B at 50 and 2.5 mg/L, respectively), and weighed again after 48 h. Root tip samples exhibiting no growth were classified as dead; samples that grew were considered alive. Survival time under hypoxia was determined as the time, following the onset of hypoxia, when root tips lost their ability to recover and grow.

Enzyme Assays

ADH and pyruvate decarboxylase assays were performed according to Shimomura and Beevers (19) and Kimmerer (10), respectively. Enzyme activities are given in milliunits (nmol substrate produced or consumed per min) per mg protein. Under the conditions used in these experiments, induction of ADH activity was not observed (data not shown). Protein was assayed according to Bradford (3), using bovine serum albumin as the standard.

RESULTS

We measured the rate of ethanolic fermentation, survival, and cytoplasmic pH in root tips differing in ADH activity. Our experiments consisted of comparisons of three pairs and one triad of maize lines. Within each comparison, genetic background was randomized so the lines differed only in ADH activity. Thus, any differences in the metabolism of the populations of each set could be unambiguously ascribed to differences in ADH activity. While each pair possessed the same genetic background (to *Adh1*, and loci tightly linked to *Adh1*), the different pairs did not. Hence, any metabolic differences between the different paired lines could result from many factors in addition to ADH activity. With the exception of lines exhibiting the very lowest ADH activities, however, we found no such metabolic differences between lines in our preliminary experiments. Consequently, we present results of a study of the nine maize lines together.

Root tips of maize lines differing in ADH activity over about a 200-fold range exhibited similar capacities for fermentation to ethanol, except when ADH activity was reduced to about 10 milliunits per mg protein or lower (Fig. 1A). The fluxes to ethanol given in Figure 1A were those at about 1 h following the onset of extreme hypoxia, when fermentation rates are highest (15). Thereafter, glycolytic flux decreases continuously (15), presumably because of exhaustion of fermentable substrate in the cell cytoplasm. The different root tip samples also contained similar activities of pyruvate decarboxylase, which catalyzes the first reaction unique to ethanolic fermentation (Fig. 1B; mean activity 42, SD ± 21, nmol/min/mg protein).

We have previously shown that maize root tips must contain sufficient ADH to prevent prolonged lactic fermentation, which leads to cytoplasmic acidosis and premature death under hypoxia (14, 15). In this study we found that both cytoplasmic pH regulation (Fig. 1C) and the ability of root tips to survive prolonged hypoxia (Fig. 1D) were largely independent of ADH activity. Only at ADH activities found in *Adh1* nulls (about 10 milliunits ADH/mg protein) was cytoplasmic pH and viability lower than in wild type.

DISCUSSION

Our observation that the flux to ethanol is insensitive to changes in ADH activity near the values normally seen in maize root tips is similar to findings with other metabolic pathways. Decreases in the amount of any one enzyme, even a supposed 'key regulatory' enzyme, by 50% have little impact on fluxes along metabolic pathways *in vivo* (see ref. 20 for review). It appears that control of flux along metabolic pathways *in vivo* is normally distributed among several enzymes, such that the activity of no single enzyme is 'rate limiting' in wild-type cells. Significant changes in flux *in vivo* have been found when the activity of a specific enzyme falls to 10 to 20% of wild type (see, for example, refs. 7 and 9); only then does that one enzyme significantly limit flux. Our results show that only when ADH activity is below about 20 milli-

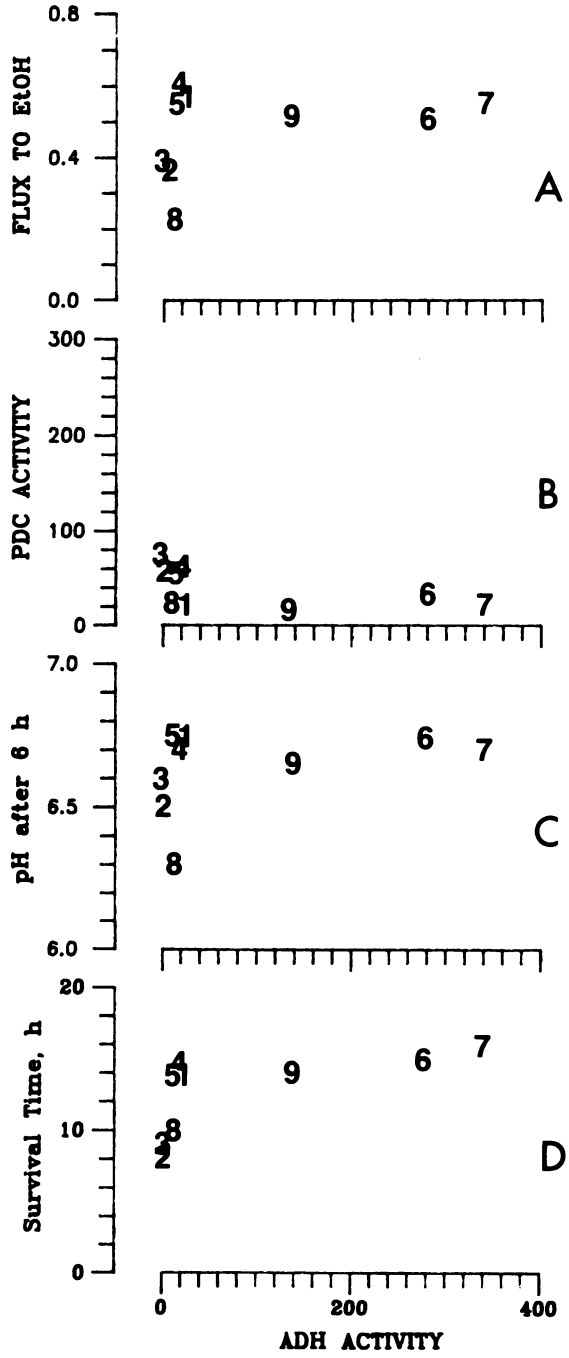

Figure 1. Dependence of metabolism and viability in hypoxic maize root tips on ADH activity in nine maize lines. ADH was assayed in extracts of root tips harvested prior to the start of hypoxia; units, nmol NADH produced/min/mg protein. Data points in each graph are represented with numbers, which specify the line from which the *Adh1* alleles were derived: 1, *Adh1-U725*; 2, *Adh1-Fm335*; 3, *Adh1-S908*; 4, *Adh1-FunkF*; 5, *Adh1-Cm*; 6, *Adh1-1F*; 7, *Adh1-1S*; 8, *Adh1-F460*; 9, one each from *Adh1-1S* and *Adh1-F460*. A, Maximum rate of ethanol production (about 1 h after the onset of hypoxia); units, μmol/min/g fresh weight. B, Pyruvate decarboxylase activity in root tip extracts; units, nmol NADH consumed/min/mg protein. C, Cytoplasmic pH after 6 h of hypoxia. D, Time of root tip death under hypoxia.

units (nmol NADH produced/min) per mg protein, are maize root tips limited both in their capacity to ferment sugars to ethanol, and their ability to survive prolonged and severe hypoxia. Thus, while ADH activity does limit metabolism and survival in mutant maize root tips which have the very lowest activities, in the 'normal' maize root tips, represented in this study by four different reference lines (described in "Materials and Methods"), ADH was not limiting (Fig. 1). A similar result was obtained by Middleton and Kacser (11) in a study of the dependence of ethanol consumption by fruit flies (*Drosophila melanogaster*) on ADH activity; only *Adh* nulls showed a significantly reduced capacity to metabolize ethanol and survive exposure to 15% ethanol.

Further experiments are required to define the dependence of metabolism in hypoxic root tips on ADH activities at levels below approximately 20 milliunits/mg protein. This question could be unambiguously examined by comparisons of F3 or F4 progeny of crosses between the lines expressing low ADH activity. Such comparisons would eliminate differences in genetic background which could affect metabolism under hypoxia; such contributions may account for differences in the behavior of the maize lines having very low ADH activity, apparent in Figure 1.

From these results we infer, first, that any increase in ADH activity in maize root tips above approximately 20 milliunits per mg protein induced by hypoxia (2) cannot, at least alone, serve to improve the energy metabolism of this tissue during oxygen deprivation. Second, the increase in tolerance of maize root tips to severe hypoxia induced by a pretreatment under mild hypoxia (16) cannot, at least alone, be due to increases in ADH activity above approximately 20 milliunits per mg protein; one or more of the many other factors that change during a period of mild hypoxia must be responsible for the acclimation. A role for the induction of ADH activity in improving energy metabolism and tolerance of hypoxia in organs containing very low ADH activity (such as maize leaves) is perhaps more probable, but remains to be established. In tissues containing very low ADH activity, it is likely that factors other than ADH may be able to mitigate the consequences of a reduced capacity for ethanolic fermentation. Finally, there is the question of whether the induction of select enzymes under hypoxia is a reaction to the stress *per se*, or to a sensed shortage of those proteins. It is possible that the signals and mechanisms responsible for the selective synthesis and translation of *Adh* mRNAs observed during hypoxic induction operate independently of the amount of ADH in cells, and so without reference to whether or not ADH activity is limiting cell function.

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