Enhanced Low Oxygen Survival in Arabidopsis through Increased Metabolic Flux in the Fermentative Pathway[1]

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We manipulated the enzyme activity levels of the alcohol fermentation pathway, pyruvate decarboxylase (PDC), and alcohol dehydrogenase (ADH) in Arabidopsis using sense and antisense overexpression of the corresponding genes (*PDC1*, *PDC2*, and *ADH1*). Transgenic plants were analyzed for levels of fermentation and evaluated for changes in hypoxic survival. Overexpression of either Arabidopsis *PDC1* or *PDC2* resulted in improved plant survival. In contrast, overexpression of Arabidopsis *ADH1* had no effect on flooding survival. These results support the role of PDC as the control step in ethanol fermentation. Although *ADH1* null mutants had decreased hypoxic survival, attempts to reduce the level of PDC activity enough to see an effect on plant survival met with limited success. The combination of flooding survival data and metabolite analysis allows identification of critical metabolic flux points. This information can be used to design transgenic strategies to improve hypoxic tolerance in plants.

Plants are constantly challenged by environmental stresses that reduce crop yield (e.g. salinity, low temperature, drought, and flooding). Soils with excess water account for 15.7% of the arable land in the United States, and flooding accounted for 16.4% of the crop insurance claims, making it the second highest cause of crop loss in the United States (Boyer, 1982). Traditionally, plant breeders have selected directly for increased stress tolerance, however, selection for flooding tolerance has been performed only in rice (*Oryza sativa*), where a genetic mapping approach was used to identify major and minor genes involved in submergence tolerance (Sripongpangkul et al., 2000; Xu et al., 2000). Transgenic plants with increased tolerance to drought, salinity, low temperatures, or a combination of these stresses (Nelson et al., 1998; Huang et al., 2000) were obtained by introducing genes for the overaccumulation of benign compounds (e.g. Gly betaine, mannitol, and Pro) that are believed to act as osmolytes. Other groups have increased stress tolerance in Arabidopsis by overexpressing transcription factors (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999; Yamaguchi-Shinozaki and Shinozaki, 2001) or signal transduction components (Piao et al., 2001; Tamminen et al., 2001).

There is substantial variation among terrestrial crop species in their ability to tolerate waterlogging conditions. Even rice, which is adapted to life in a flooded environment, incurs damage if the shoots are completely submerged for periods of time (Drew, 1997; Quimio et al., 2000). Genetic variation in flooding tolerance has been found in rice, maize (*Zea mays*), barley (*Hordeum vulgare*), and Arabidopsis. In some cases, this variation results from specific mutants (Harberd and Edwards, 1982; Dolferus et al., 1985; Lemke-Keyes and Sachs, 1989; Ricard et al., 1998). Several studies have focused on the effect of overexpression of genes on flooding tolerance. Zhang et al. (2000) overexpressed a gene involved in cytokinin biosynthesis and demonstrated improved flooding tolerance in Arabidopsis. Grichko and Glick (2001) overexpressed a bacterial ACC deaminase under the control of three different promoters and were able to produce transgenic tomato (*Lycopersicon esculentum*) plants that had increased flooding tolerance.

Because higher plants are sessile and obligate aerobes, they have evolved a number of mechanisms to survive the hypoxic conditions that are generated by flooding. These mechanisms include morphological adaptations, such as formation of adventitious roots (Lorbiecke and Sauter, 1999; Mergemann and Sauter, 2000) and aerenchyma, and a number of complex metabolic adaptations (for review, see Drew, 1997; Liao and Lin, 2001). Studies on maize root tips have led to the conclusion that several factors are involved in improved hypoxic tolerance, all of which are associated with the fermentation pathway. These include better energy status due to higher rates of glycolysis and ethanol fermentation, limitations of lactate accumulation, and better regulation of cytoplasmic pH (Roberts et al., 1984a, 1984b, 1985; Ricard

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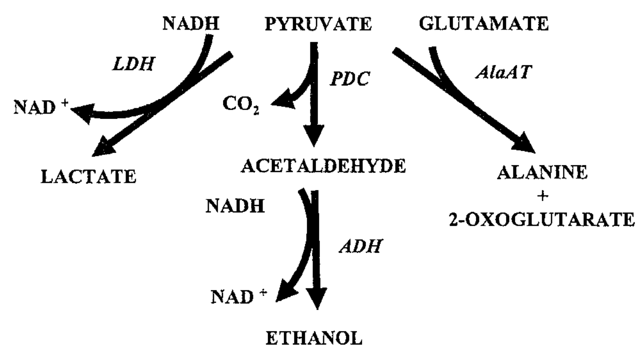

Figure 1. Under low oxygen stress conditions, plants activate three main fermentation pathways that use pyruvate as starting substrate.

et al., 1998). The de novo synthesis of the fermentative enzymes (Fig. 1; pyruvate decarboxylase [PDC]; alcohol dehydrogenase [ADH]; lactate dehydrogenase [LDH]; and Ala aminotransferase [AlaAT]) and other anaerobic polypeptides occurs upon exposure to low oxygen conditions within the plant root (Sachs et al., 1980). The fermentative products of pyruvate include the end products lactate, ethanol, and Ala, which are produced by three separate pathways (Fig. 1).

This study focuses on the manipulation of the ethanol fermentation pathway. PDC (EC 4.1.1.1) catalyzes the first step, which is responsible for the irreversible conversion of pyruvate to acetaldehyde. ADH (EC 1.1.1.1) then converts acetaldehyde to ethanol, with the concomitant regeneration of NAD^+ (Fig. 1). The regeneration of NAD^+ from NADH is thought to be the most important function of the alcohol fermentation pathway under low oxygen stress conditions, because this function is impaired by the inactivation of oxidative phosphorylation. This function is vital, because in the absence of NAD^+, glycolysis ceases (Kennedy et al., 1992; Robertson et al., 1994). Although the importance of the fermentation pathway in hypoxia survival is known, the switch from respiration to fermentation is not completely understood. A large amount of research has been conducted on the role of ADH, however, to date, no one has overexpressed ADH to determine whether increased ADH activity would improve flooding tolerance. Less research has been done on PDC, but several groups have overexpressed this gene in different plants (Tadege et al., 1998; Quimio et al., 2000), and its importance as a critical control point is becoming apparent. In Arabidopsis, four genes encode PDC activity. *PDC1* is the main anaerobically induced gene and is expressed primarily in the roots, whereas *PDC2* is constitutively expressed at low levels in both roots and leaves (R. Dolferus, unpublished data). Only one *ADH* gene (*ADH1*) has been identified in Arabidopsis (Dolferus and Jacobs, 1984). The anaerobic induction and promoter function of this gene has been well documented (Dolferus et al., 1994; Dennis et al., 2000).

In this paper, we report the effect of overexpressing the enzymes of ethanol fermentation, PDC and ADH, in Arabidopsis. The combination of enzyme activity data and metabolite data from all enzymes involved in the hypoxic response allows us to critically examine carbon flow along the glycolytic and ethanol fermentation pathway. In conjunction with the survival assay, we provide a comprehensive look at overexpression of ADH and PDC and their role in hypoxic tolerance. We show that overexpression of either *PDC1* or *PDC2* improved plant survival under low oxygen conditions. This confirms that PDC is the metabolic control point in the alcohol fermentation pathway. *ADH1* overexpression had no effect on flooding tolerance, but normal levels of *ADH1* expression were shown to be critical to plant survival under low oxygen conditions.

RESULTS

Generation and Assay of Transgenic Lines for Enzyme Activity

Transgenic Arabidopsis lines containing the *PDC1*, *PDC2*, and *ADH1* sense and antisense constructs (under the control of the 35S promoter) were screened in the T2 generation by northern-blot hybridizations (data not shown). The line with the highest mRNA expression levels was selected. For the antisense constructs, we selected those lines with the most significant reduction in mRNA levels. We then used enzyme activity assays to confirm the mRNA data and to select the best overexpressing/antisense lines. Homozygous T3 lines were selected from these transgenic lines, and the levels of mRNA and enzyme activity were again confirmed in this generation. The lines overexpressing *PDC1*, *PDC2*, or *ADH1* were assigned the prefix "Ox" (Overexpressing). Lines showing a reduced enzyme activity were generated using antisense constructs for *PDC1* and *PDC2* and were assigned the prefix "Ue" (Under-expressing). In the case of *ADH1*, an *adh1*$^-$ null mutant was selected in C24 background using allyl alcohol selection (Jacobs et al., 1988). Under normal growth conditions, no obvious phenotypic differences were observed between any of the over- and under-expressing transgenic plants, or the *adh1*$^-$ mutant compared with C24 control plants.

Enzyme activities were determined for PDC (Fig. 2, A and B), ADH (Fig. 2, C and D), LDH (Fig. 2, E and F), and AlaAT (data not shown), under aerobic and hypoxic conditions in the selected lines. In roots and shoots, the levels of PDC activity were six to ten times higher for Ox*PDC1* and Ox*PDC2*, respectively, under either aerobic or hypoxic conditions. The least expressing *PDC* lines, Ue*PDC1* and Ue*PDC2*, were originally selected because they had mRNA levels reduced to 30% to 50% of wild-type levels under aerobic and hypoxic conditions (data not shown). However, in terms of activity (Fig. 2, A and B), these lines had only marginally lower PDC activity compared with the wild type under both control and hypoxic conditions.

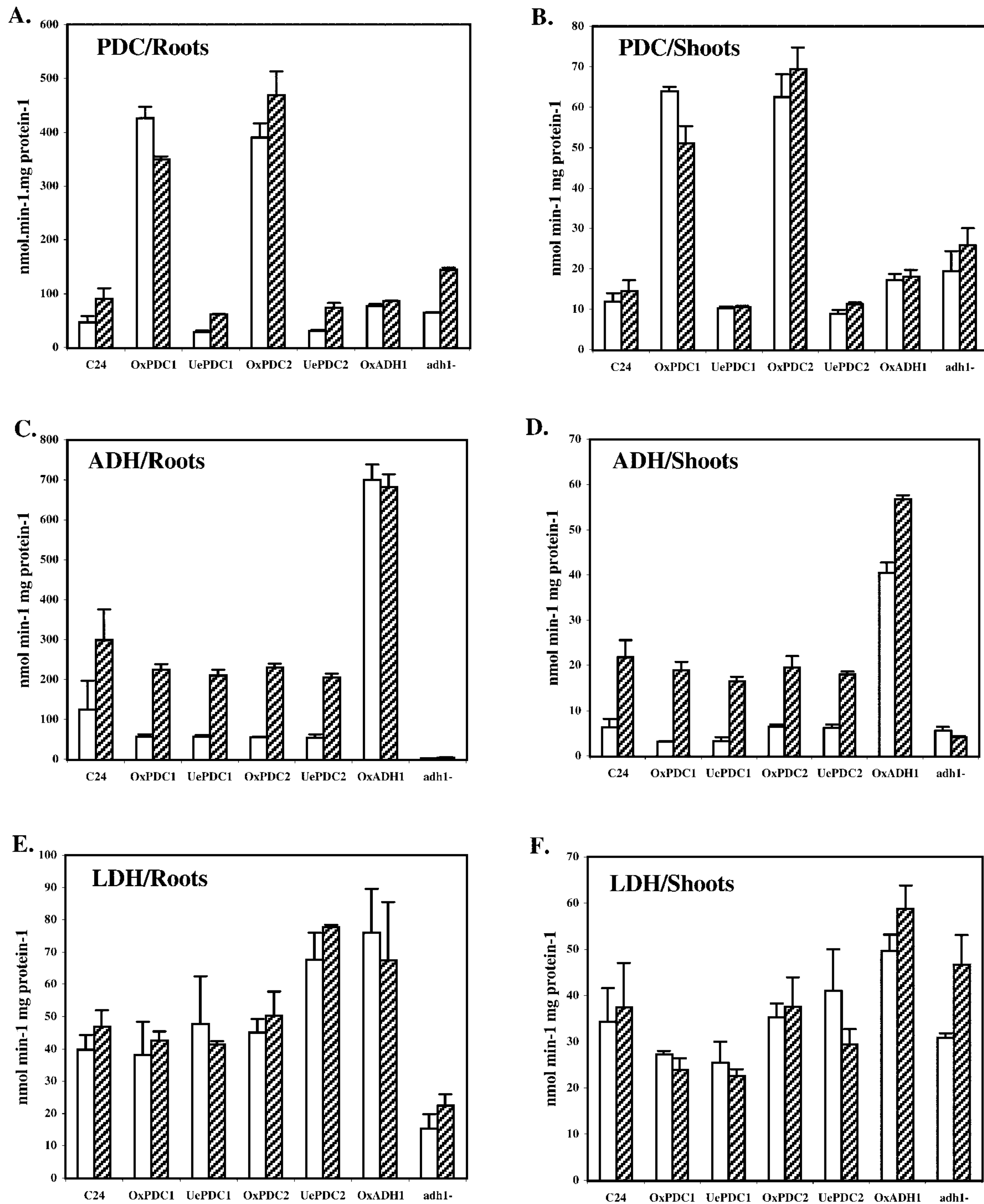

Figure 2. Activities of PDC, ADH, and LDH in roots and shoots of wild-type, transgenic, and null mutant Arabidopsis plants. Plants were grown for 4 weeks and then treated under aerobic (white column) or hypoxic (hatched column) conditions for 24 h as described (see "Materials and Methods"). Results shown are the mean of three replicates with SEs from one representative experiment. A, PDC activity in roots. B, PDC activity in shoots. C, ADH activity in roots. D, ADH activity in shoots. E, LDH activity in roots. F, LDH activity in shoots.

The Ox*ADH1* line showed a 2- to 3-fold increase in ADH activity under hypoxic conditions in both roots and shoots compared with wild-type roots and shoots, whereas activities were increased 7- to 8-fold under aerobic conditions (Fig. 2, C and D). The ethyl methanesulfonate-induced *adh1*$^{-}$ mutant had negligible ADH activity compared with the C24 wild type (Fig. 2, C and D). In this *adh1* null mutant, Glu99 and

His101 were mutated to Asp and Lys, respectively, in a highly conserved area of the ADH enzyme (Dolferus et al., 1990).

The over- or under-expression of *PDC1* or *PDC2* had no effect on the levels of ADH activity in either roots or shoots (Fig. 2, C and D), nor was the total PDC activity in roots and shoots significantly affected by overexpression of *ADH1* (Fig. 2, A and B). The Ue*PDC2* and Ox*ADH1* lines displayed increased levels of LDH activity in the roots, but in shoots, only Ox*ADH1* showed slightly increased LDH activity levels (Fig. 2, E and F). In the $adh1^-$ line, LDH levels were decreased in roots but not in shoots (Fig. 2, E and F). No significant changes of LDH activity were observed by overexpressing the *PDC1* or *PDC2* genes. No differences in AlaAT activity were detected between any of the transgenic or mutant genotypes and the C24 genotype (data not shown).

Manipulation of PDC Levels

To examine the effect of the introduced *PDC* transgenes, metabolites for the key compounds in the fermentative pathway were analyzed in roots, shoots, and the medium surrounding the roots under hypoxic conditions (Figs. 3 and 4). For acetaldehyde, only the liquid Murashige and Skoog medium was measured, because previous experience has indicated that levels in the medium accurately reflect intracellular levels, because acetaldehyde passes easily through intracellular membranes into the medium.

Ox*PDC2*, Ox*PDC1*, and Ue*PDC2* lines had decreased levels of pyruvate in roots and shoots under hypoxic conditions (Fig. 3, A and B). The decrease in pyruvate levels was associated with a 2- or 4-fold increase of acetaldehyde in the medium for Ox*PDC1* and Ox*PDC2* (Fig. 3C). Both lines also showed a corresponding increase in ethanol concentration in the medium (1.6 times; Fig. 3D). Ox*PDC1* was the only line that showed significantly higher ethanol levels in roots (1.8 times) and shoots (Fig. 3, D and E).

The effect of *PDC* overexpression on the metabolites of the other fermentation pathways that are derived from pyruvate was also measured (Fig. 4). Decreased levels of both lactate and Ala were found in the roots, shoots, and Murashige and Skoog medium for Ox*PDC1*. These metabolites remained unchanged in Ox*PDC2* (Fig. 4, A–F). No significant differences were observed in Glu or 2-oxoglutarate levels for Ox*PDC2*, but Ox*PDC1* had significantly lower levels of Glu in the medium (data not shown). These data show that overexpression of the Arabidopsis PDC1 and PDC2 enzymes, although overexpressed to similar total activity levels (Fig. 2), had different effects on metabolite accumulation, suggesting that they may function differently.

If overexpression of PDC leads to increased carbon flow through the ethanol pathway, then repression of PDC could act to decrease carbon flow and reduce ethanol levels. But the decrease in PDC activity that we observed in the Ue*PDC* lines was not nearly as dramatic as the increase in activity of the overexpressing lines (Fig. 2). Residual PDC activity in the Ue*PDC* lines could still account for a significant flow of carbon through the alcohol fermentation pathway. The levels of pyruvate, acetaldehyde, and ethanol production of Ue*PDC1* and Ue*PDC2* were not significantly different from wild type (Fig. 3, A–F), but Ue*PDC1* and Ue*PDC2* both showed increased levels of lactate production in roots but not in leaves (Fig. 4, A and B). This suggests that under hypoxic conditions even a minor reduction in PDC activity may be sufficient to increase the amount of carbon flow from pyruvate to lactate. Only small differences were observed in lactate and Ala (Fig. 4) and in Glu and 2-oxoglutarate levels (data not shown).

Manipulation of ADH Activity Levels

Pyruvate levels were not affected in roots, but they decreased in shoots of the Ox*ADH1* and $adh1^-$ lines (Fig. 3, A and B). Acetaldehyde levels were reduced to 0.1% of wild-type levels in roots in Ox*ADH1*, whereas the $adh1^-$ null line had a 9-fold increase in acetaldehyde production in the medium (Fig. 3C). Although there were no significant changes in tissue ethanol levels in roots or shoots of the Ox*ADH1* line (Fig. 3, E and F), a strong reduction in ethanol production (80%) was observed in the roots of the $adh1^-$ mutant; no change was found in the shoots (Fig. 3, D and E). No significant changes were found in lactate or Ala levels in roots, shoots, or the medium for Ox*ADH1* transgenic lines, whereas the $adh1^-$ mutant showed a 3- to 4-fold increase in lactate levels in the medium and in roots (Fig. 4, A–C).

PDC Overexpression Enhances Low Oxygen Stress Survival

Survival assays were conducted on each genotype to assess the contribution of the transgene to whole-plant survival under low oxygen conditions. We used two experimental conditions that were previously shown to discriminate between wild-type and $adh1^-$ null mutants (Ellis et al., 1999): a 24-h nonhypoxic pretreatment (24-h NHPT), in which the plants were subjected to 0.1% (v/v) O_2 for 24 h, and a 48-h hypoxic pretreatment (48-h HPT), in which the plants received a 24-h pretreatment of 5% (v/v) O_2 before receiving 48 h of 0.1% (v/v) O_2. Plants were then allowed to recover on vertical plates for 2 weeks, as illustrated in Figure 5. Survival, based on the ability to recover from the treatments, was quantified by measuring root tip growth, root weight, and shoot weight (Table I). For each transgenic line, we carried out a minimum of two survival assays with three repeats for each experiment.

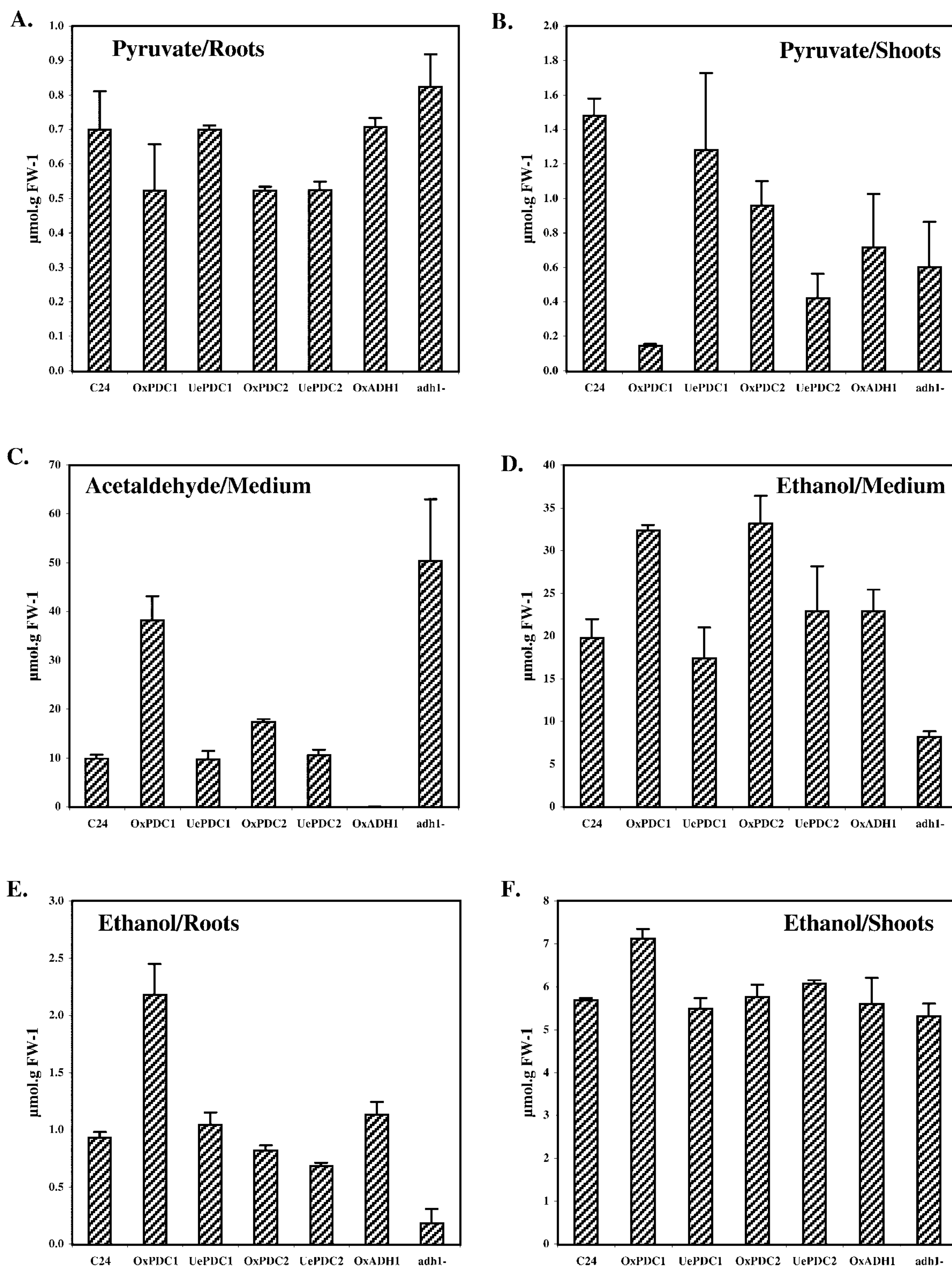

Figure 3. Effect of PDC and ADH manipulation on metabolic products of ethanol fermentation under hypoxic conditions. Plants were grown for 4 weeks and then hypoxically induced for 24 h. The levels of pyruvate, acetaldehyde, and ethanol were then determined (see "Materials and Methods"). Results shown are the mean of three replicates with SEs from one representative experiment. Metabolite levels are presented for pyruvate in roots (A) and shoots (B), acetaldehyde content in the Murashige and Skoog growth medium (C), and ethanol in the medium (D), roots (E), and shoots (F). Values have been normalized against C24 wild type.

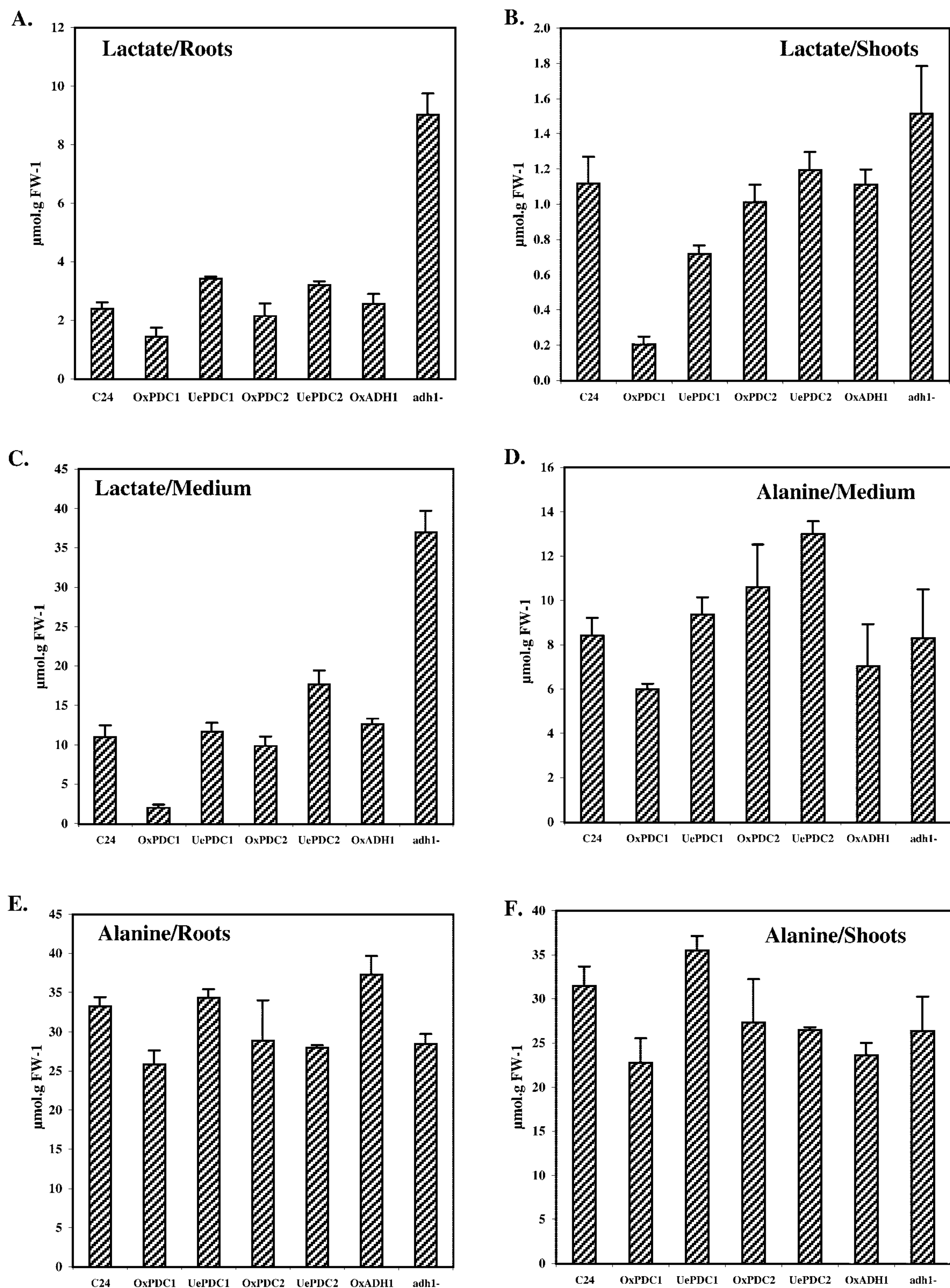

Figure 4. Effect of PDC and ADH manipulation on other fermentative products under hypoxic conditions. Plants were grown for 4 weeks and hypoxically induced for 24 h, and then the levels of lactate and Ala were determined (see "Materials and Methods"). Results shown are the mean of three replicates with SEs from one representative experiment. Metabolite levels are presented for lactate in roots (A), shoots (B), and Murashige and Skoog growth medium (C); and Ala in the medium (D), roots (E), and shoots (F). Values have been normalized against the C24 wild type.

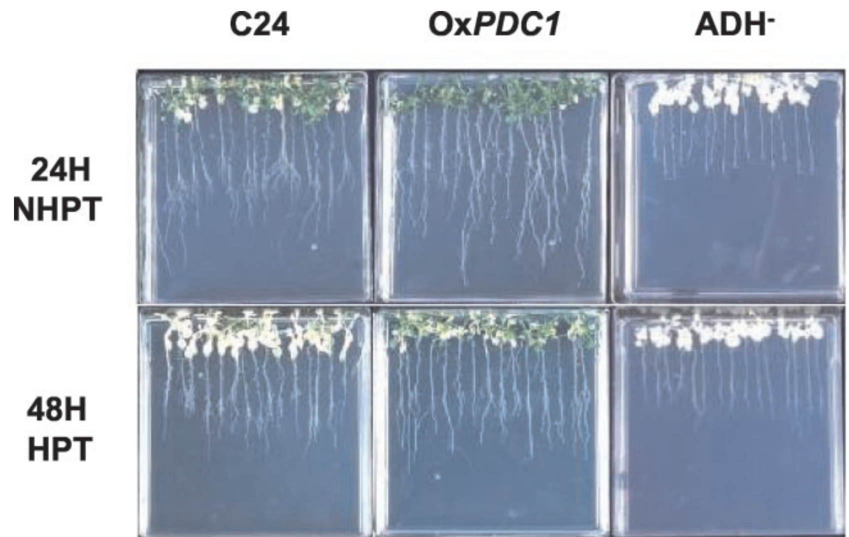

Figure 5. Survival assay results of Arabidopsis seedlings subjected to a low oxygen treatment (0.1% [v/v] O_2) with or without hypoxic (5% [v/v] O_2) pretreatment. Seedlings were exposed to two treatments: low oxygen (0.1% [v/v] O_2) conditions for 24 h without HPT (24H NHPT) or low oxygen for 48 h with a 24-h 5% (v/v) O_2 HPT (48H HPT). After these treatments, seedlings were transferred to vertical plates for a 2-week recovery period under normal growth chamber conditions. A representative plate for each environment was selected. Column 1, wild type (C24); column 2, PDC overexpressor (Ox*PDC1*); and column 3, *adh1*$^-$ mutant. The treatments are in row 1, 24-h NHPT; in row 2, 48-h NHPT; and in row 3, 48-h HPT.

Both the Ox*PDC1* and Ox*PDC2* lines performed better than the control line in the 24-h NHPT and 48-h HPT treatments. These data suggest that PDC enzyme activity levels are a limiting factor for survival of moderately severe oxygen conditions. We did not see any significant differences between C24 and the two Ue*PDC* lines that we tested. The Ox*ADH1* line did not perform better than the C24 wild-type line, but as was previously shown (Ellis et al., 1999), survival of the *adh1*$^-$ line was reduced compared with the C24 wild type (Fig. 5). In the recovery period after the 24-h NHPT treatment, the *adh1*$^-$ line showed a reduction in root tip growth, whereas none of the other lines showed any significant change (Table I). When HPT was used before low oxygen stress (48-h HPT), both PDC overexpressing lines showed improved root tip growth, whereas the *adh1*$^-$ line still performed more poorly than the C24 wild type (Table I).

The data for the root and shoot weight measurements were less consistent than those for root tip growth (Table I), which could partially be due to the variation in stringency of the stress treatment between the two independent experiments. Ox*PDC1* showed increased root formation in the 24-h NHPT and the 48-h HPT treatments. In contrast, root development of the Ox*PDC2* line was higher in the 48-h HPT treatment, but in one of the 24-h NHPT treatments, results were significantly worse than for C24. No significant changes were found for the Ue*PDC1*,

Table I. *Effect of PDC and ADH manipulation on survival and growth of roots and shoots of wild-type, transgenic, and null mutant Arabidopsis plants presented as a percentage of C24 control*

Seedlings were exposed to two treatments: low oxygen (0.1% [w/v] O_2) conditions for 24 h without HPT (24H NHPT) or low oxygen conditions for 48 h with a 24-h 5% (w/v) O_2 HPT (48H HPT). After these treatments, seedlings were transferred to vertical plates for a 2-week recovery period under normal growth chamber conditions. Root tip growth and root and shoot weight were quantified for six lines used in this study. The performance of each of the lines is presented as an averaged percentage compared to the C24 wild type. The results of two independent experiments are presented; each experiment consists of three repeats, and statistical analysis results are presented. *t* test confidence levels are indicated as follows: †, $P < 0.05$; ‡, $P < 0.01$.

Line	Root Tip Growth				Root Weight				Shoot Weight			
	24H NHPT	*t* Test	48H HPT	*t* Test	24H NHPT	*t* Test	48H HPT	*t* Test	24H NHPT	*t* Test	48H HPT	*t* Test
	mm				*mg*							
Ox*PDC1*	128.9	0.059	157.2	0.022†	113.1	0.026†	212.3	0.008‡	139.9	0.052	108.7	0.635
	137.2	0.137	202.3	0.029†	151.2	0.019†	156.4	0.357	105.2	0.131	179.4	0.036†
Ue*PDC1*	88.6	0.726	96.6	0.853	79.7	0.106	105.8	0.744	59.7	0.112	128.1	0.231
	66.9	0.200	102.1	0.818	75.9	0.118	110.5	0.531	99.3	0.985	97.1	0.809
Ox*PDC2*	109.0	0.151	157.9	0.042†	46.8	0.017†	132.2	0.012†	113.6	0.031†	176.5	0.052
	109.0	0.432	136.3	0.000‡	107.7	0.546	134.8	0.000‡	183.9	0.001‡	112.1	0.489
Ue*PDC2*	113.5	0.520	108.4	0.655	73.8	0.026†	210.0	0.021†	62.4	0.019†	127.6	0.299
	120.0	0.324	108.4	0.228	111.2	0.005‡	113.4	0.008‡	112.8	0.399	101.4	0.361
Ox*ADH1*	118.4	0.604	48.7	0.096	73.3	0.165	91.1	0.475	70.8	0.109	77.4	0.070
	65.0	0.084	48.7	0.096	89.5	0.719	81.7	0.545	79.2	0.436	113.1	0.720
ADH1$^-$	19.7	0.017†	0.0	0.002‡	120.0	0.581	26.0	0.008‡	64.9	0.025†	121.3	0.455
	60.5	0.014†	46.7	0.007‡	109.8	0.475	91.5	0.032†	176.7	0.020†	36.6	0.000‡

Ue*PDC2*, and Ox*ADH1* lines, whereas the *adh1*$^-$ showed decreased root formation in the 48-h HPT treatment. For the shoot weight measurements, only the Ox*PDC2* line showed significantly higher shoot weights in the 24-h NHPT treatment. The data for the 24-h NHPT (Ox*PDC1*) and 48-h HPT (Ox*PDC1* and Ox*PDC2*) treatments showed increased shoot weights but were not significant due to variation within each experiment.

DISCUSSION

In the absence of oxidative phosphorylation, the glycolytic conversion of Glc to pyruvate is the only means to generate limited amounts of ATP under anaerobic conditions. Plants then switch to fermentation pathways to regenerate NAD^+ (Roberts et al., 1984a, 1984b). Of the three main fermentation pathways in plants, only two—alcohol and lactic acid fermentation—regenerate NAD^+ (Fig. 1). Ala fermentation does not produce NAD^+, but Ala accumulation under low oxygen conditions is thought to play a role in nitrogen assimilation (Good and Muench, 1993).

The three fermentation pathways are relatively simple, enabling us to start a metabolic engineering approach in Arabidopsis. The three final metabolites of these pathways are also easily measured, and because they have a common starting point, pyruvate, we can monitor changes in metabolites and study the impact on carbon flow in the branches of the fermentative pathway. We established a survival assay to study the influence of metabolic changes on low oxygen stress survival (Ellis et al., 1999).

We have successfully developed plants with modified levels of PDC and ADH activities. Six different lines were analyzed for over- and under-expression of these enzymes using enzymatic, metabolic, and survival data. This allows us to study their role in ethanol fermentation and their impact on the other branches in the fermentation pathway. Overexpression of any of these genes using the cauliflower mosaic virus 35S promoter resulted in high levels of constitutive enzyme activity that was insensitive to hypoxia (Fig. 2). Overexpression of either *PDC1* or *PDC2* enhanced plant tolerance to low oxygen conditions (Fig. 5; Table I). Similarly, Quimio et al. (2000) found that enhancement of PDC levels in rice corresponded with an increase in submergence tolerance. Our results indicate that increased PDC activity results in increased carbon flow through the ethanol pathway, as reflected by an increase in both acetaldehyde and ethanol pool sizes (Fig. 3). This ultimately results in increased ATP and NAD^+ production. However, overexpression of a bacterial PDC gene in transgenic tobacco (*Nicotiana tabacum*) did not result in improved hypoxic tolerance (Tadege et al., 1998). There may be several reasons for the differences between our study and theirs. First, different plant species have different tolerances to flooding, consequently what is observed in rice may vary from what is observed Arabidopsis or tobacco. Second, our levels of PDC overexpression were significantly higher than those observed by Tadege et al. (1998). Third, the use of a bacterial gene may be inappropriate in this instance, because the enzyme may be regulated in a different fashion. This may also explain the their observation that in roots, the ethanol fermentation pathway is substrate limited. Tadege et al. (1998) suggests that in roots, oxygen levels play an important role in controlling flux through the ethanolic pathway, rather than PDC, and they suggest different regulatory mechanisms in roots, shoots, and pollen. Therefore, although the role of PDC as a control point in ethanol fermentation has been recognized, overexpression of PDC and its contribution to plant survival seems to depend on the species being studied, on the tissue of interest, and possibly on the origin of the transgene used in the experiments.

The PDC overexpressors produce much higher levels of acetaldehyde (Fig. 3), as has been observed in PDC-overexpressing tobacco plants (Bucher et al., 1994). Acetaldehyde is a highly reactive chemical effecting cellular damage by forming acetaldehyde-protein adducts (Perata et al., 1992; Braun et al., 1995). It has been suggested that acetaldehyde accumulation in *adh1* mutants is the major cause of cellular damage during anoxic and postanoxic conditions (Jackson and Armstrong, 1999). Despite this, the Ox*PDC1* and Ox*PDC2* performed better than C24 in the survival assay (Fig. 5). This could mean that endogenous ADH levels in Arabidopsis are sufficient for removing harmful levels of acetaldehyde or that acetaldehyde leaks out into the medium efficiently enough to prevent internal injury. Maize mutants with reduced levels of ADH were still able to withstand hypoxia, suggesting that ADH in normal plants is in excess (Roberts et al., 1989).

The Ue*PDC1* and Ue*PDC2* lines displayed no visible changes in low oxygen tolerance relative to the wild type. These transformants also did not show significant differences in pyruvate, acetaldehyde, and ethanol production, although there are small differences at the level of lactate and Ala (Table I; Figs. 3 and 4). Given our current knowledge about gene silencing (Waterhouse et al., 2001b), it is unlikely that the *PDC2* antisense could silence expression of the other *PDC* genes in Arabidopsis, but *PDC1* antisense expression could silence the closely related *PDC3* and *PDC4* genes. Approaches to reduce PDC levels include more effective antisense constructs or the use of T-DNA or transposon-tagged Arabidopsis mutants.

The *adh1*$^-$ mutant had minimal ADH activity, resulting in a dramatic increase in acetaldehyde in the medium surrounding the roots (Fig. 3). The mutant also had poor tolerance to low oxygen conditions

(Fig. 5). These results confirm earlier studies showing that at least low to normal levels of ADH activity are necessary for short-term, waterlogging tolerance (Johnson et al., 1994; Ellis et al., 1999). Overexpression of *ADH1* did not affect carbon flow and ethanol levels did not change, suggesting that wild-type Arabidopsis ADH levels are already in excess in anoxic or hypoxic roots and that ADH levels above a threshold do not correlate with the ability to survive anoxia. Overexpression of ADH therefore does not improve hypoxic survival rates (Fig. 5). High levels of ADH activity may be of greater importance when plants are returned to aerobic conditions (Thomson and Greenway, 1991; Drew, 1997). Accumulated ethanol in roots can be remetabolized to acetaldehyde and then converted to acetate and acetyl-CoA with the enzymes acetaldehyde dehydrogenase (EC 1.2.1.4/EC 1.2.1.5) and acetyl-CoA synthase (EC 6.2.1.1). This results in a reduction of carbon loss in the recovery period after anoxia (Thomson and Greenway, 1991).

PDC activity occurs at a critical branch point between aerobic and anaerobic metabolism (Roberts et al., 1989; Waters et al., 1991a, 1991b), and PDC activity is closely related to the in vivo rates of fermentation (Bouny and Saglio, 1996). In yeast, pyruvate flows preferentially into the tricarboxylic acid cycle because the pyruvate dehydrogenase complex has a much higher affinity for pyruvate. However, in conditions of sugar over-supply, pyruvate dehydrogenase is bypassed, and much of the pyruvate is channeled through PDC (Pronk et al., 1996). In plants, the acidification of the cytoplasm during anoxia may be responsible for this metabolic switch. According to the Davies-Roberts hypothesis, cytoplasmic pH drops during the transition from normoxia (pH 7.3–7.4) to anoxia (pH 6.8) in maize root tips (Davies et al., 1974; Davies, 1980; Roberts et al., 1984a, 1984b, 1985; Gancedo and Serrano, 1989). Below pH 7.0, LDH is inhibited (Rivoal and Hanson, 1994), and the activity of PDC increases dramatically, due to its acidic pH optimum (Morrell et al., 1990; Fox et al., 1994). What is not known is whether the different PDC isoforms have different pH optima. Our data show that overexpression of the *PDC1* gene—but not *PDC2*—leads to a reduction in metabolites of the other fermentation pathways (lactate and Ala; Fig. 4). This could indicate that the K_m of *PDC1* for pyruvate would favor entry of the substrate into the ethanol fermentation pathway.

Although our lines overexpressing the anaerobically inducible (*PDC1*) and constitutively expressed (*PDC2*) gene had similar PDC activity levels and low oxygen survival properties, our results indicate that PDC1 and PDC2 may have different enzymatic properties. Overexpression of the *PDC1* gene, but not *PDC2*, leads to a reduction in metabolites of the other fermentation pathways (lactate and Ala; Fig. 4). Biochemical characterizations have demonstrated that, like yeast and some bacterial PDC enzymes, plant PDC has nonlinear kinetics and is activated by its substrate pyruvate (Mücke et al., 1996; Dietrich and König, 1997). The PDC2 enzyme, which is constitutively expressed at low levels in roots, is quite different from the anaerobically induced enzyme (R. Dolferus, unpublished data) and may have different kinetic properties. This could result in very different in vivo kinetics within the cell and corresponding changes in the levels of different metabolites.

The experiments described in this paper were carried out with plants grown in Murashige and Skoog medium containing 3% (w/v) Suc. Therefore our data showing that PDC overexpression increases metabolic flux through the alcohol fermentation pathway and improves low oxygen stress survival may only be true when sugar supply is adequate. Under sugar-limiting conditions (e.g. in the field), a faster depletion of carbohydrate stores may lead to decreased survival. An exogenous supply of Glc has been shown to ameliorate the effects of anoxia (Xia and Saglio, 1990; Waters et al., 1991a, 1991b; Hole et al., 1992; Xia et al., 1995; Ricard et al., 1998; Tadege et al., 1998). Thus, the flow of carbon through to ethanol may only be effective when high levels of sugars are available.

In conclusion, our data support the idea that PDC is a critical flux control point in hypoxic metabolism and that PDC activity levels can determine plant survival under low oxygen conditions. PDC activity controls carbon flow through the ethanol fermentation pathway and affects flow through the lactate and Ala pathways. On the basis of this study, improved flooding survival may be achieved by overexpressing a plant *PDC1* or *PDC2* gene. Whether overexpression of both PDC and ADH activity would further improve low oxygen stress survival remains to be established. Additional work is also required to determine first, whether continual overexpression has significant negative effects on productivity and second, whether the results of the survival assay can vary, depending on the conditions of evaluation.

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Stress Treatment

Arabidopsis ecotype C24 was used in all our experiments. Seeds were germinated, and seedlings were grown as outlined by Ellis et al. (1999) on Murashige and Skoog medium containing 3% (w/v) Suc and 0.5% (w/v) agar. After 3 weeks growth, plants were transferred to liquid Murashige and Skoog medium and placed on a rotary shaker for 7 d to promote root growth. These plants were then used for all further tests. Hypoxic treatment of plants was carried out for 24 h in a 5% (v/v) O_2/95% (v/v) N_2 environment in the dark as described previously (Howard et al., 1987), and then tissue was harvested for enzyme, metabolite, and northern analyses.

Development of Transgenic Plants

Standard cloning and recombinant DNA methods were used throughout (Maniatis et al., 1982). Sense and antisense constructs expressing *PDC1* and

PDC2 (GenBank accession nos. U71121 and U71122, respectively; R. Dolferus, unpublished data), and a sense construct expressing *ADH1* were developed as previously described (Dennis et al., 2000). The cauliflower mosaic virus 35S promoter was used to drive overexpression of all three genes. Constructs were introduced into plants using a modified root transformation protocol (Dolferus et al., 1994). The ethyl methanesulfonate *adh1*$^{-}$ mutant was isolated using allyl alcohol selection as described previously (Jacobs et al., 1988). Sequencing of the *adh1* allele was performed using synthetic oligonucleotides created to identify mutations within the *ADH1* gene. The University of Wisconsin GCG program was used for sequence analysis.

RNA Extractions and Northern-Blot Analysis

RNA extractions, blot hybridizations using RNA probes, and filter washings were performed as described previously (Dolferus et al., 1994). Signals were detected by laying filters upon phosphor imager screens (Molecular Dynamics, Sunnyvale, CA) and then quantified. To normalize for sample loading differences, signal strengths were divided by their respective ubiquitin signals (Dolferus et al., 1994).

Enzyme and Metabolite Assays

Each overexpressing line was assessed alongside its matching underexpressing line and the wild-type C24 line, in both aerobic and hypoxic environments (Howard et al., 1987). Three plates with 50 plants of each plant line were used in each environment. Roots and shoots were collected separately along with 1 mL of liquid medium from each plate and then quick frozen in liquid nitrogen and stored at −70°C for further processing. Enzymes were extracted with a 3:1 ratio (milliliters per gram fresh weight) of extraction buffer to tissue. The extraction buffer for ADH, LDH, and AlaAT was made fresh each day (5 mM EDTA, 1 mM dithiothreitol, 10 mM Cys, 0.1 mM phenylmethylsulfonyl fluoride, 5 μM leupeptin, and 0.1 M Tris-HCl, pH 7.7). Acid-washed sand and polyvinylpolypyrrolidone (20%–50% of tissue weight) was added during tissue grinding. ADH and AlaAT activity were measured spectrophotometrically using a SpectraMax^{+} ELISA plate reader (Good and Crosby, 1989a, 1989b). LDH activity was measured as an increase in A_{490} using the protocol of Korzeniewski and Callewaert (1983). The PDC extraction buffer and assay (Laszlo and Saint Lawrence, 1983) was performed with the addition of proteinase inhibitors (Cys, leupeptin, and phenylmethylsulfonyl fluoride) to the PDC extraction buffer in the same final concentrations as in the ADH extraction buffer.

Gas chromatography (GC) was used to determine acetaldehyde and ethanol concentrations in the liquid medium. Roots of 3-week-old plants were harvested, transferred into 10-mL syringes containing 10 mL of growing medium gassed with 100% argon to exclude N_2. The syringes were outfitted with 18-gauge needles containing glass wool at the air lock to prevent roots from clogging the needle. After the plunger had been inserted into the syringe, a 1-mL argon gas bubble was pulled into the syringe's chamber, and the needle tip was fitted with a rubber stopper to prevent gas exchange or leakage. The syringes were left on a rotary shaker at room temperature. Within a 24-h period, 1-mL samples were taken at regular intervals and stored at 4°C. Fresh weights of the roots were taken at the experiment's conclusion. Measurement of acetaldehyde and ethanol in the medium was performed using a Varian 3700 GC, fitted with a Varian 800 series auto-sampler and flame ionization detector. The stainless steel column (2-m × 2.16-mm i.d.) was packed with Poropak QS. The GC set-up is as follows: injector, 170°C; detector, 250°C; column programmed between 110°C to 130°C; and N_2 carrier gas flow, 30 mL min^{-1}. Calibration was done using samples spiked with known amounts of acetaldehyde and ethanol. All other metabolites were assayed as described previously. In brief, roots were ground in sand and perchloric acid in a 3:1 (w/v) ratio as described by Good and Muench (1993). Shoots were ground in a Corning homogenizer using a methanol-chloroform-water mixture and then separated as described by Lerma et al. (1988). Pyruvate, ethanol, lactate, Glu, 2-oxoglutarate, and Ala levels were measured spectrophotometrically as described by Good and Muench (1993), but adapted for a 96-well ELIZA plate reader (SpectraMax^{+}). All assays were carried out at room temperature.

Survival Assay

The survival assay protocol of Ellis et al. (1999) was used to assess the flooding tolerance of each transgenic line. The only modifications included gassing the medium for 15 min instead of the original 5 min, and plants were transferred to plates containing 0.8% (w/v) agar instead of 1.0% (w/v) agar for the recovery period. The experimental and the wild-type plants were put into one of two different oxygen treatments: 24 h at 0.1% (v/v) O_2 without hypoxic pretreatment (24-h NHPT) and 48 h at 0.1% (v/v) O_2 after a 24-h HPT at 5% (v/v) O_2 (48-h HPT). Shoot and root tip survival, shoot and root weights, and growth of the primary root tip were recorded at the end of the recovery period.

Data Analysis

Two separate survival assay experiments were completed for each transgenic or mutant line. The data for each measurement for the two experiments were analyzed using a two-tailed Student's *t* test. For each experiment the measured means between the transgenic/mutant and wild-type plants were used for the *t* test. Differences were considered significant providing that differences from both experiments had $P < 0.05$. Presented values in the table indicate significant differences where $0.01 \leq P < 0.05$ and where $P < 0.01$. Data for all enzyme and metabolite experiments are based on a minimum of two separate experiments. Each experiment consisted of three replicate plates per treatment with a C24 control included in each hypoxic chamber as a control. Student's *t* test (two-tailed) was used to assess whether there was a significant difference between the means of the wild-type and transgenic/mutant plants.

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