The *Pyruvate decarboxylase1* Gene of Arabidopsis Is Required during Anoxia But Not Other Environmental Stresses[w]

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Ethanolic fermentation is classically associated with flooding tolerance when plant cells switch from respiration to anaerobic fermentation. However, recent studies have suggested that fermentation also has important functions in the presence of oxygen, mainly in germinating pollen and during abiotic stress. Pyruvate decarboxylase (PDC), which catalyzes the first step in this pathway, is thought to be the main regulatory enzyme. Here, we characterize the *PDC* gene family in Arabidopsis. PDC is encoded by four closely related genes. By using real-time quantitative polymerase chain reaction, we determined the expression levels of each individual gene in different tissues, under normal growth conditions, and when the plants were subjected to anoxia or other environmental stress conditions. We show that *PDC1* is the only gene induced under oxygen limitation among the *PDC1* gene family and that a *pdc1* null mutant is comprised in anoxia tolerance but not other environmental stresses. We also characterize the expression of the aldehyde dehydrogenase (*ALDH*) gene family. None of the three genes is induced by anoxia but *ALDH2B7* reacts strongly to ABA application and dehydration, suggesting that ALDH may play a role in aerobic detoxification of acetaldehyde. We discuss the possible role of ethanolic fermentation as a robust back-up energy production pathway under adverse conditions when mitochondrial function is disturbed.

The ethanolic fermentation pathway branches off the main glycolytic pathway at pyruvate. In the first step, pyruvate is the substrate of pyruvate decarboxylase (PDC), yielding CO_2 and acetaldehyde. Subsequently, acetaldehyde is reduced to ethanol with the concomitant oxidation of NADH to NAD^+ by alcohol dehydrogenase (ADH). In the present day aerobic atmosphere, ethanolic fermentation is used only by specialized organisms or under particular conditions. In plants, it has been studied because of its relevance in flooding tolerance where plant cells switch from aerobic respiration to anaerobic fermentation (for review, see Drew, 1997).

ADH has been the subject of numerous genetic studies, and *adh* mutants have been reported for a number of species, including maize (*Zea mays*; for review, see Freeling and Bennett, 1985), tobacco (*Nicotiana tabacum*; Rousselin et al., 1990), and Arabidopsis (Jacobs et al., 1988). Maize *adh* null mutants are sensitive to strict anoxia but no obvious phenotype is apparent in acclimated plants (Johnson et al., 1994). Experiments with isogenic maize lines differing in ADH activity over a approximately 200-fold range indicated that ADH activity does not limit the capacity for energy production by ethanolic fermentation unless there is a reduction in activity to less than 1% of wild-type levels (Roberts et al., 1989). These results suggest that ADH is present in large excess and are inconsistent with the idea of ADH as the regulatory enzyme of this pathway.

Overexpression of a bacterial PDC in transgenic tobacco resulted in constitutive high and active protein levels under both normoxic and anoxic conditions (Bucher et al., 1994). The acetaldehyde produced by the bacterial and the endogenous PDC, was converted efficiently to ethanol by the endogenous ADH. This suggests a key regulatory role for the first enzyme of ethanolic fermentation, PDC. Overexpression of Arabidopsis genes for the enzymes ADH and PDC improved the tolerance of Arabidopsis roots to low oxygen conditions (Dennis et al., 2000; Shiao et al., 2002). Loss-of-function mutations in *PDC* would provide a valuable tool to understand the importance of ethanolic fermentation in flooding tolerance, but such mutants have not yet been reported in plants.

A new and exciting aspect of ethanolic fermentation is the suggested involvement in stress signaling and response to environmental stresses other than low oxygen (Tadege et al., 1999). The micro-array expression profiles from stressed plants often reveal increases in *ADH* and/or *PDC* expression (Desikan et al., 2001; Seki et al., 2002). Furthermore, specific analysis of the *ADH* gene from rice (*Oryza sativa*), maize, and Arabidopsis showed *ADH* to be induced by cold (Christie et al., 1991), wounding (Kato-Noguchi, 2001), dehydration (Dolferus et al., 1994), and the phytohormone abscisic acid (ABA; Hwang and Van-Toai, 1991; de Bruxelles et al., 1996), in line with the observation from the micro-array experiments. Preliminary data on expression of the *PDC* genes from

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Arabidopsis during abiotic stresses have been reported (Dolferus et al., 1997). Thus it is conceivable that ethanolic fermentation is part of a general response to environmental stress.

Additional evidence indicating a role in biotic stress is provided by experiments with transgenic potato plants overexpressing bacterial PDC. These plants accumulated acetaldehyde and showed a lesion-mimic phenotype (Tadege et al., 1998). Furthermore, several markers normally associated with plant defense were expressed, and lesion formation was accompanied by a significant resistance to a fungal pathogen. The cell death was developmentally and environmentally regulated and the drastic effect on sugar export suggested a link between carbohydrate metabolism and disease susceptibility.

Although *PDC* and *ADH* gene induction has been demonstrated, ethanol and acetaldehyde production as a result of stress treatment has only been reported for red pine (*Pinus resinosa*) and birch (*Betula* spp.) seedlings exposed to sulfur dioxide, water deficiency, freezing, and ozone (Kimmerer and Kozlowski, 1982). A comprehensive investigation of *PDC* and *ADH* gene induction and the determination of acetaldehyde and ethanol production during stress treatments would provide valuable information on how ethanolic fermentation is involved in the response to abiotic or biotic stress.

One problem during aerobic stress is toxicity of reactive acetaldehyde. One of the pathways for the detoxification of aldehydes to less reactive forms is the oxidation to carboxylic acids by aldehyde dehydrogenase (ALDH). ALDH converts acetaldehyde to acetate concomitantly reducing NAD^+ to NADH. In plants, *ALDH* is strongly expressed in germinating pollen of tobacco (op den Camp and Kuhlemeier, 1997), and it is essential for the restoration of fertility in male sterile maize plants (Cui et al., 1996; Liu et al., 2001). During anoxia, however, results from tobacco leaves indicated that *ALDH* is not induced (op den Camp and Kuhlemeier, 1997). In contrast to these results, *ALDH2B5* is anaerobically induced in rice seedlings (Nakazono et al., 2000). Enhanced activity of ALDH would deplete the NAD^+ pool in the cytosol and consequently block glycolysis. Therefore, speculations about the possible function of *ALDH* during anoxia need to take into account the requirement for NAD^+ regeneration during glycolysis. We therefore investigated whether Arabidopsis *ALDH* is induced during anoxia as compared with other environmental stresses.

In this paper, we present results demonstrating the requirement for ethanolic fermentation during anoxia and its involvement during adaptation to other environmental stresses in Arabidopsis. Expression levels of the relevant mRNA from *PDC*, *ADH*, and *ALDH* were determined quantitatively. The identification of a *pdc1* mutant by T-DNA insertional mutagenesis allowed us to study the function for *PDC1* in Arabidopsis.

RESULTS

Characterization of the *PDC* Gene Family in Arabidopsis

Screening several gene and protein databases for Arabidopsis Columbia (Col-0) sequences with the protein sequence of tobacco PDC2 (Bucher et al., 1995) revealed four open reading frames with high homology to tobacco PDC2. Two open reading frames had very high homology with the GenBank entries *PDC1* (U71121) and *PDC2* (U71122) cloned from the accessions C24 and Landsberg *erecta* (L*er*), respectively. The corresponding open reading frame in the accession Col-0 were annotated in the Arabidopsis Genome sequence as *PDC1*, *PDC2*, *PDC3*, and *PDC4* (Table I). Arabidopsis *PDC1* is located on chromosome 4, *PDC2* is located on chromosome 5, and *PDC3* and *PDC4* genes are positioned at the end of chromosome 5 and are separated by only 1,600 bp. An alignment of the four Arabidopsis PDC and the tobacco PDC2 protein sequences revealed a high overall consensus in all the functional motifs including the catalytic site (Fig. 1). Two anaerobic response elements can be found in the promoter of *PDC1* (Hoeren et al., 1998). No G-Box (ABA-responsive element) could be found in the promoter of *PDC1* in contrast to the Arabidopsis *ADH* gene (Dolferus et al., 1994). No known stress-related elements were identified in the promoter regions of *PDC2*, *PDC3* and *PDC4*.

Expression of PDC in Various Organs Determined by Real-Time Quantitative Reverse Transcriptase (RT)-PCR

Preliminary experiments indicated that under standard growth conditions, each of the four *PDC* genes was expressed at low levels, at or below the limit of detection by RNA gel-blot analysis (data not shown). To analyze the mRNA accumulation of the four different genes coding for PDC, we used real-time quantitative RT-PCR technology (see "Materials and Methods" and supplementary material, which can be

Table I. *Comparison of amino acid identity for PDC proteins from tobacco and Arabidopsis*

Nos. indicate the percentage of amino acid identity done by a global alignment with a Blossum 35 matrix. Gene accession nos.: NtPDC2 (X81855), AtPDC1 (AT4G33070), AtPDC2 (AT5G54960), AtPDC3 (AT5G01330), and AtPDC4 (AT5G01320).

	NtPDC2	AtPDC1	AtPDC2	AtPDC3
NtPDC2	–			
AtPDC1	80.4	–		
AtPDC2	78.9	81.5	–	
AtPDC3	80.6	85.2	77.8	–
AtPDC4	80.6	88.6	80.6	89.8

Figure 1. Partial comparison of the amino acid sequences of PDC proteins from Arabidopsis and orthologs in other species. Partial amino acid sequence alignments of PDC proteins from *Zymomonas mobilis* (ZymPDC; GenBank no. M15368), tobacco (NtPDC2; GenBank no. X81855), and Arabidopsis (AtPDC1, AT4G33070; AtPDC2, AT5G54960; AtPDC3, AT5G01330; and AtPDC4, AT5G01320). Active site is highlighted with asterisk. Alignments were generated using the Multalin software v5.4.1 with the Blossum 62-12-2 matrix (Corpet, 1988). Black boxes indicate high consensus levels (100%); gray boxes indicate low consensus levels (> 50%).

viewed at http://www.plantphysiol.org). RNA was isolated from various organs, and the abundance of the four *PDC* mRNA and the single-*ADH* mRNA in these tissues was quantified based on separate RNA isolations derived from three independent experiments (Fig. 2). *ADH* was expressed in all organs. The highest expression was observed in imbibed seeds (50-fold higher compared with 14-d-old seedlings). The expression profile of *PDC1* closely matched *ADH* expression. Expression of *PDC2*, *PDC3*, and *PDC4* could also be seen in most organs but, in contrast to *ADH* and *PDC1*, expression was not particularly high in imbibed seeds (Fig. 2A). The real-time RT-PCR method can also be used to compare absolute transcript abundances of genes in mRNA preparations (Fig. 2B). *PDC1* transcripts were the dominant *PDC* mRNAs in roots, flowers, siliques, and seeds. In siliques and seeds, more than 96% of all *PDC* transcripts belonged to the *PDC1* class. *PDC3* was abundant in whole seedlings, roots, and shoots, whereas *PDC2* and *PDC4* transcripts contributed overall only minor proportions to the total *PDC* mRNA population.

PDC1 Is Strongly Induced during Anoxia

In maize and Arabidopsis, strong induction of fermentation genes takes place in anaerobic conditions (Dolferus et al., 1985; Bailey-Serres et al., 1988; Conley et al., 1999). RNA gel blotting was performed with specific probes from the 3′-untranslated regions of the four *PDC* genes of Arabidopsis. A strong hybridization signal from *ADH* and *PDC1* appeared after 3 h of anoxia and declined slowly during prolonged anoxia (Fig. 3A). Expression of *PDC2*, *PDC3*, and *PDC4* was not detected, consistent with the weak expression previously observed for these genes (Fig. 2). These results suggest that *PDC1* is the only gene induced in anaerobiosis among the *PDC* gene family in Arabidopsis.

To settle the question of whether *PDC2*, *PDC3*, and *PDC4* are induced during anoxia to levels too low to be detected by classical RNA gel-blot analysis, quantitative RT-PCR technology was used. As a positive internal control we amplified the *ADH* RNA, which is known to be strongly induced by anoxia (Dolferus et al., 1985). As expected, *ADH* showed a approximately 200-fold induction of transcript amounts after a 24-h anoxia treatment of seedlings compared with the non-

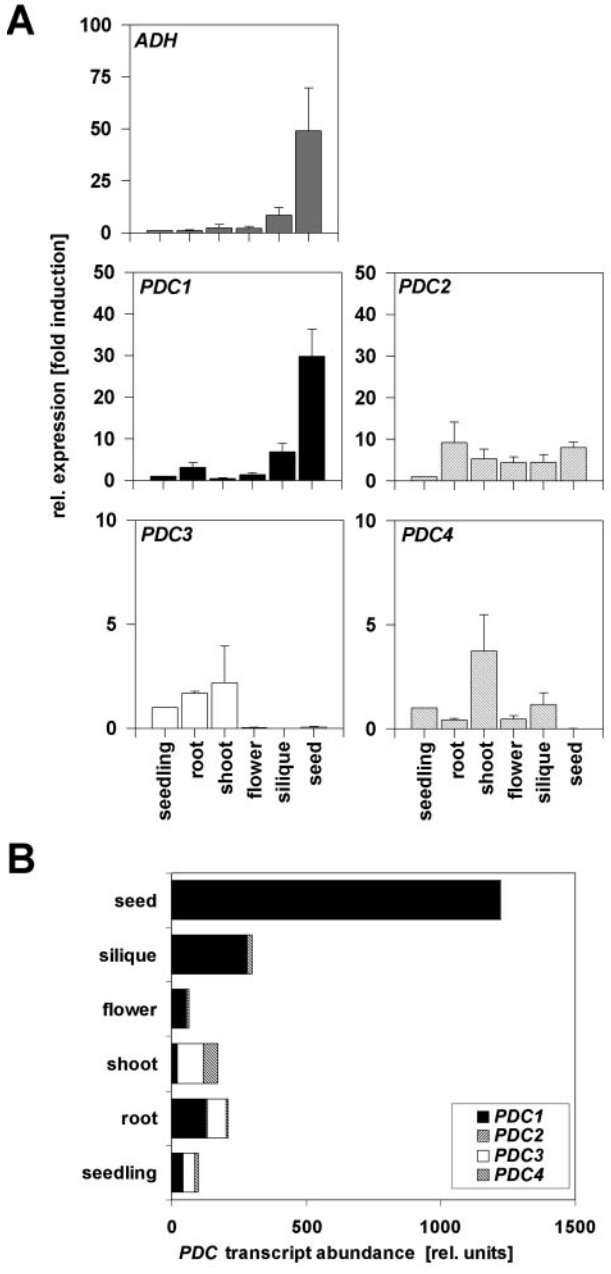

Figure 2. Relative expression level and transcript abundance of fermentation genes in aerobic conditions during development. A, Quantification of mRNA levels was achieved by using a quantitative real-time PCR system (see "Materials and Methods"). *ADH* and *PDC* expression levels were normalized with respect to the internal control *ACT2* and are plotted relative to the expression from whole seedlings. Data bars represent the mean ± SE level of transcripts from three experiments with independent RNA extractions. Note different scales as *y* axis. B, *PDC* transcript abundance in different organs. Total *PDC* mRNA in seedlings = 100. Contribution from individual genes according to A is represented.

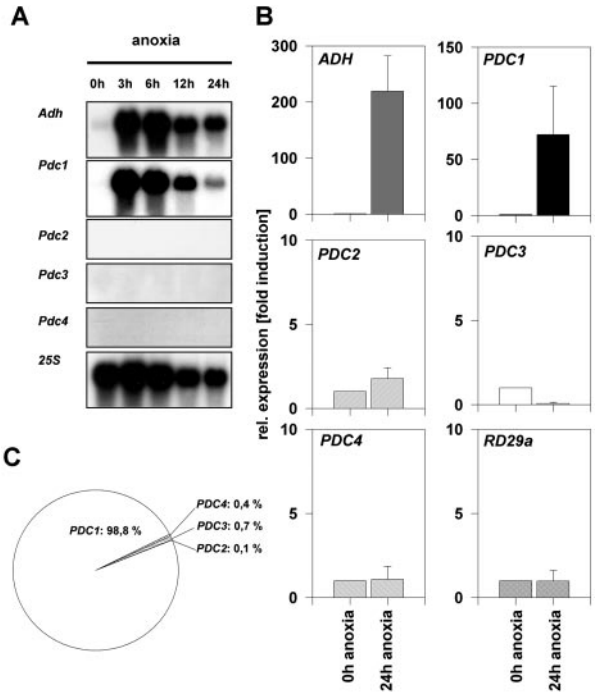

Figure 3. Relative expression level and transcript abundance of fermentation genes during anoxia. A, RNA gel-blot analysis from seedlings treated with different periods of anoxia. Hybridization probes specific to each of the *PDC* genes were used. *25S* hybridization was used as control. B, Quantification of *ADH* and *PDC* transcripts from seedlings submitted to 24-h anoxia using the a quantitative real-time PCR system. Expression of *RD29a* was used as a control. Expression levels were normalized with respect to the internal control *ACT2* and are plotted relative to the expression at 0-h anoxia. Data bars represent the mean ± SE level of transcripts from three experiments with independent RNA extractions. Note different scales as *y* axis. C, *PDC* transcript abundance in anoxia-treated seedlings. Contribution from individual genes according to A is represented.

treated control (Fig. 3B). Expression analysis of the well-characterized stress-gene *RD29a* (Gilmour and Thomashow, 1991; Nordin et al., 1991; Yamaguchi-Shinozaki and Shinozaki, 1994) under an anoxic atmosphere showed this gene not to be induced. As already seen in the RNA gel-blot analysis, *PDC1* was strongly (approximately 70-fold) induced by anoxia. *PDC2*, *PDC3*, and *PDC4* were not induced by anoxia, but low levels of transcripts were detected. The relative transcript abundance is very low for *PDC2* (approximately 1,600 times less), *PDC3* (approximately 130 times less), and *PDC4* (approximately 250 times less) compared with the transcript abundance of *PDC1* during anoxia. The proportion of *PDC1* transcripts in anoxia-treated cells is about 99%. The mRNA abundance of *PDC2*, *PDC3*, and *PDC4* represent under anoxia only about 1% of all *PDC* transcripts (Fig. 3C).

Identification and Molecular Analysis of the *pdc1* Mutant

The expression analysis revealed *PDC1* to be the only strongly expressed gene of the *PDC* gene family during anoxia. *PDC2*, *PDC3*, and *PDC4* are dramatically less abundant than *PDC1* transcripts and we therefore focused further on the *PDC1* gene. To study the role of *PDC1* in Arabidopsis, the T-DNA insertion collection of the Torrey Mesa Research Institute was screened. A multiple sequence alignment with the *PDC1* sequence against the database of the T-DNA-derived flanking sequences was performed, which resulted in an alignment representing a high probability of identity with a particular line in the collection. Using a primer from the T-DNA left border plus a gene-specific primer, a single PCR product was identified in this line, and subsequent sequencing of the fragment revealed an insertion in the third exon of *PDC1*, at position 1,312 bp after the ATG in the coding sequence (Fig. 4A). This mutant line was designated *pdc1*.

At position 1,385 bp in the inserted T-DNA sequence of *pdc1*, a potential stop codon was identified. Furthermore, the thiamine pyrophosphate-binding site (an essential cofactor of the enzyme) is located 85 bp after the insertion site in the wild-type coding sequence. This analysis indicated a complete disruption of the *PDC1* gene. To confirm this, RNA was isolated from Col-0 and L*er* wild type, and *pdc1* (Col-0) and the *adh* mutant (L*er*) were subjected to a 6-h anoxic period (Fig. 4B) and analyzed by RNA gel-blot analysis. *PDC1* gene expression was observed in the two different wild-type accessions and in the *adh* mutant, but *pdc1* mutant plants showed no transcripts at all after 6 h of anaerobic conditions.

To investigate whether other members of the *PDC* gene family might compensate for *PDC1*, real-time RT-PCR analysis was performed. *PDC1* transcripts in *pdc1* mutants were not detectable either in normoxic or in anoxic conditions, confirming the data from the RNA gel-blot analysis (Fig. 4C). None of the other three genes showed increased transcript levels in *pdc1* mutant seedlings treated by anoxia compared with the wild type. Thus, the three *PDC* genes do not compensate for a complete loss of *PDC1* transcripts in *pdc1* mutant plants by induced expression. *ADH* expression is not influenced in the *pdc1* mutant. Conversely, *PDC1* expression is not influenced in the *adh* mutant (Fig. 4B).

The *pdc1* Mutant Is More Susceptible to Anoxia

Development of *pdc1* mutant plants and survival during anoxia were investigated. Under standard growing conditions, no morphological phenotype was apparent (data not shown). To reveal the possible consequences of a null mutation in *PDC1*, attention was focused on the survival of plants under anoxia. Studies on the differential adaptation of shoots and roots of Arabidopsis to low oxygen indicated that ethanol fermentation is essential in roots but not in shoots (Ellis et al., 1999). We therefore analyzed the ability of the root tip of *pdc1* mutants to

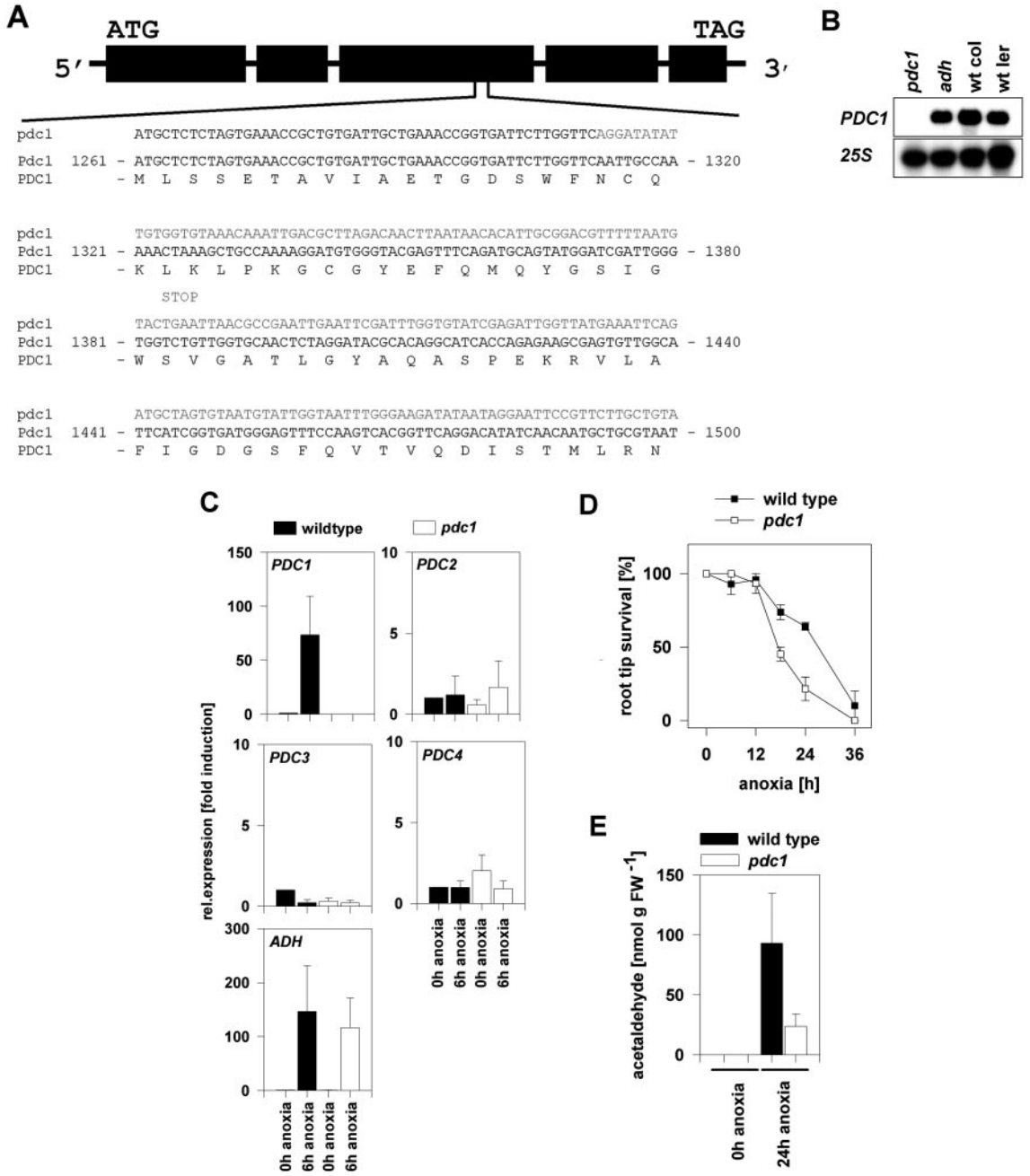

Figure 4. Interference of a T-DNA insertion in PDC1 with gene expression and survival under anoxia. A, Structure of the Arabidopsis *PDC1* gene. DNA sequences of the wild type and the mutant line showing the T-DNA-derived sequences in bold and the wild-type amino acid sequence. A potential stop codon in the reading frame of the T-DNA insertion in *PDC1* is indicated. B, Expression level in *pdc1* and *adh* seedlings treated for 6 h under anoxia. Total RNA (10 μg) from wild-type seedlings of the accessions Col-0 and L*er* and from *pdc1* (Col-0) and *adh* (L*er*) were subjected to RNA gel-blot analysis with a specific probe from the 3′-untranslated region of *PDC1*. *25S* labeling indicated equal RNA loading. C, Quantification of mRNA levels of *PDC* and *ADH* genes in wt and *pdc1* mutant seedlings under normoxia (0-h anoxia) or after 6-h anoxia. Expression levels were normalized with respect to the internal control *ACT2* and are plotted relative to the expression at 0-h anoxia. Data bars represent the mean ± SE level of transcripts from three experiments with independent RNA extractions. D, Survival of *pdc1* and wt seedlings under anoxia. The survival of the existing root tip was scored from seedlings subjected to different periods of anoxia and a 3-d-long recovering time. Black squares represent wild-type seedlings; white squares represent mutant seedlings. Data represent the average of three survival experiments with independent plant material ± SE. E, Production of acetaldehyde in roots submitted to 24-h anoxia measured by gas chromatography. Detached roots were infiltrated with 50 mM Glc and 0.1 mM $CaSO_4$ and were incubated for 24 h in an anoxic bench or in normal atmosphere. Values represent the average of between three and five measurements of different populations of seedlings ± SE.

survive prolonged anoxia (Fig. 4D). After 12 h of anoxia, viability declined in wild-type and *pdc1* root tips. This susceptibility to anoxia was more pronounced in the mutant line, which revealed a significant decrease ($P < 0.025$) of the survival rate of *pdc1* root tips after 18 h relative to wild-type plants. After 24 h, the survival ability of the wild type was more obvious; 64% of wild-type plants still had healthy root tips, whereas *pdc1* root tips survived significantly less well ($P < 0.05$). After 36 h of complete anoxia, *pdc1* roots were all dead, whereas some wild-type roots still survived. To analyze the consequences of the lack of *PDC1*, we determined the product of the PDC enzyme, acetaldehyde, in mutant roots during anoxia. Mutant *pdc1* roots produced considerably less acetaldehyde than the wild type (26%; Fig. 4E), but the decrease was less than the reduction in total *PDC* transcript levels (1% of wild-type mRNA level). No production of acetaldehyde was observed in aerobic conditions. Taken together, the lack of *PDC1* results in a 99% reduction in *PDC* mRNA, in a 74% reduction in acetaldehyde concentration, and in a pronounced susceptibility to anoxia in root tips.

Induction of Ethanolic Fermentation by Environmental Stresses Other Than Anoxia

The induction of expression of the *ADH* gene in several species by environmental conditions such as cold, osmotic stress, or wounding suggested a role for PDC in these conditions. Therefore, we analyzed the behavior of *PDC* transcripts of Arabidopsis in seedlings subjected to a range of abiotic stresses. Seedlings were treated with cold, mannitol, ABA, salinity, wounding, and paraquat (a herbicide that exacerbates O_2^- radical production and is used to study oxidative stress; Penninckx et al., 1998), and gene expression was quantified. The Arabidopsis *ADH* gene is known to be induced by cold, mannitol, and ABA treatment (Dolferus et al., 1994; de Bruxelles et al., 1996). Our analysis confirmed these earlier results and demonstrated *ADH* induction in response to salt stress, wounding, and paraquat treatments (Fig. 5A). The transcript pattern obtained for the known stress-inducible gene *RD29a* was in agreement with the literature (Gilmour and Thomashow, 1991; Nordin et al., 1991; Yamaguchi-Shinozaki and Shinozaki, 1994). *RD29a* was strongly induced by ABA, salinity, cold, and dehydration, whereas wounding and paraquat treatments induced the *RD29a* gene only weakly.

Next, we analyzed the stress-induced expression levels for all four *PDC* genes of Arabidopsis. *PDC1* was induced by all treatments; the strongest induction was observed by ABA application (23-fold compared with the control treatment). Cold, salinity, mannitol, wounding, and paraquat induced *PDC1* to comparable levels (8- to 10-fold). *PDC2* was induced by several stresses compared with the control treatment (Fig. 5A). *PDC3* and *PDC4* mRNA levels were not markedly affected by any treatment. However, the absolute transcript abundance of *PDC2* in the different stress-treated plants was always lower compared with *PDC1* (Fig. 5B). *PDC3* and *PDC4* transcripts are in several treatments more abundant than *PDC1*, although these genes are not markedly induced by these conditions compared with the control treatment (Fig. 5, A and B).

Because both *PDC* and *ADH* genes are inducible under stress conditions, we decided to measure the products of the pathway, acetaldehyde and ethanol, by gas chromatography. We found acetaldehyde production to be stimulated by ABA and paraquat treatment and during salt and wounding stress, but not during cold and mannitol treatments (Fig. 5C). In all cases, the amount of acetaldehyde and ethanol produced was much less than under anoxia.

The expression data show that *PDC1* is strongly inducible under abiotic stress conditions, that *PDC2* contributed only marginally to total *PDC* levels, and that *PDC3* and *PDC4* show no induction. Detection of acetaldehyde and ethanol during stress treatments demonstrated that ethanolic fermentation was occurring during these conditions. To see whether the loss of *PDC1* results in an enhanced susceptibility of the *pdc1* mutant to environmental stress conditions, we determined the germination rate and the root elongation on agar plates containing different concentrations of sodium chloride and mannitol, which represent a salt stress and a dehydration stress, respectively. In both assays, no considerable differences were observed between wild-type and mutant plants in the germinating rate, and only slight effects were observed on root elongation (Fig. 6), indicating that germination under osmotic stress conditions is presumably independent of *PDC1*.

ALDH Transcript Levels Do Not Increase during Anoxia But during Environmental Stress

Acetaldehyde is a potentially toxic molecule that can be detoxified through the action of ALDH. We therefore determined the transcript profiles of each of the three Arabidopsis *ALDH* genes (Skibbe et al., 2002). RNA gel-blot analysis of anoxia-treated seedlings was carried out for each of the three genes *ALDH2B4*, *ALDH2B7*, and *ALDH2C4* using gene-specific probes. The results showed no measurable increases of Arabidopsis *ALDH* transcript levels during anoxia (Fig. 7A).

To reveal whether *ALDH* genes are induced by environmental stresses other than anoxia, we performed RNA gel-blot analysis with RNAs derived from seedlings treated with different stresses. ABA and mannitol treatments strongly induced the transcription of the putative mitochondrial *ALDH2B7* gene (Fig. 7B). Wounding and salinity induced the

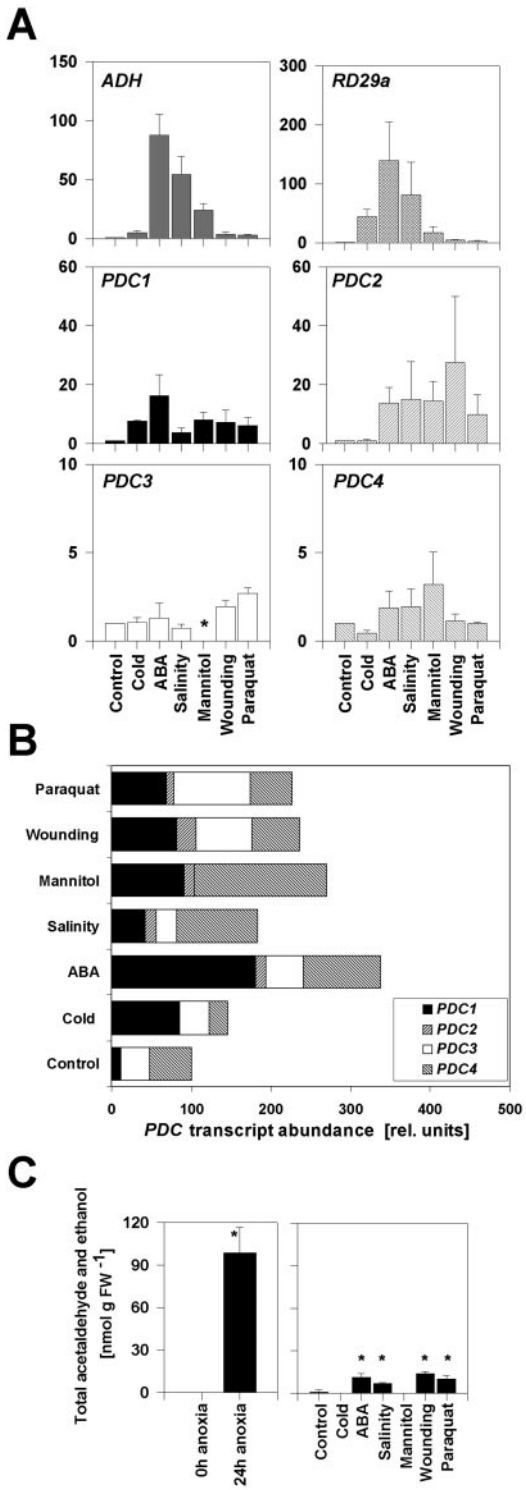

Figure 5. Relative expression levels of fermentation genes under different abiotic stresses and total acetaldehyde and ethanol production by leaves submitted to anoxia or other stress treatments. A, Quantification of mRNA of *ADH*, *PDC*, and *RD29a* transcripts with the a quantitative real-time PCR system from seedlings treated with different abiotic stresses. Expression levels were normalized with respect to the internal control *ACT2* and are plotted relative to the control treatment. Data bars represent the mean ± SE level of transcripts from three experiments with independent RNA extractions. Asterisk indicates no detection of amplification. Note different scales as *y* axis. B, *PDC* transcript abundance in different treatments. Total *PDC* mRNA in control-treated seedlings = 100. Contribution from individual genes according to A is represented. C, Leaves were subjected to the indicated treatments. Measurements were made by gas chromatography. Values represent the mean ±SE of between three and five measurements of different leaves. Asterisks indicate significant differences relative to the control treatment, $P < 0.005$).

gene to a lesser extent, whereas cold and paraquat had no effect on the expression of *ALDH2B7*. These results indicate that there may be a function for *ALDH2B7* during an abiotic stress response. The expression pattern of the putative cytosolic gene *ALDH2C4* and the putative mitochondrial gene *ALDH2B4* was only marginally influenced by stress treatments. The housekeeping gene *ACTIN2* is barely regulated by any of the stresses, validating its use as the reference gene for the quantitative real-time PCR experiments.

DISCUSSION

Dominance of *PDC1* Transcript Abundance during Anoxia

The four *PDC* genes annotated in the Arabidopsis Genome Sequence Bank are highly homologous to each other and to other plant *PDC* genes (Fig. 1; Table I). All functional motifs are conserved, and thus they are likely to encode proteins with bona fide PDC enzymatic activity. This is supported by the reduced accumulation of the product of the enzyme, acetaldehyde, in the *pdc1* null mutant (Fig. 4E). No conserved stress-related cis-elements were present in any of the other *PDC* genes.

The transcript abundance of all *PDC* genes under normal conditions was at the detection limit of classical RNA gel-blot analysis preventing accurate quantitation. Quantitative real-time RT-PCR enabled us to determine transcript levels of each of the *PDC* genes independently. The careful analysis of the mRNA populations with three independent experiments and RNA extraction for the real-time RT-PCR analysis made it possible to obtain reliable expression profiles. We could demonstrate that among the four *PDC* genes only *PDC1* showed appreciable variation in expression between different tissues and high levels of induction by anoxia. Previous sequence inspection reported two anaerobic response elements in the 5′ upstream region of *PDC1* from Arabidopsis (Hoeren et al., 1998). In siliques and seeds, *PDC1* transcripts alone make up more than 96% of the total *PDC* mRNA. Imbibed seeds and developing or ripening fruits are compact tissues, and it is conceivable that they experience hypoxia. Thus, the high *PDC1* expression in anoxic seedlings, seeds, and fruits may reflect activation of a common signaling pathway. However, the regulation of the *PDC* genes during development might depend on a separate signaling

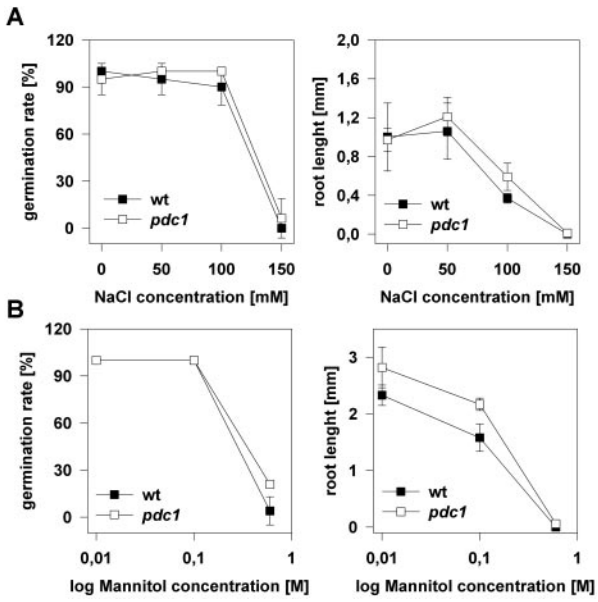

Figure 6. Survival rate of germinating pdc1 and wild-type seeds on plates containing different concentrations of sodium chloride or mannitol. Surface-sterilized *pdc1* and wild-type seeds were placed on 0.85% (w/v) agar plates containing the indicated concentrations of either NaCl or mannitol. After an incubation at 4°C for 3 d to break seed dormancy, plates were transferred to the growth room under long-day conditions (23°C ± 2°C). Percentage of germination was scored after 1 week, and root length of germinated seeds was measured. Data represent the mean ± SE of four replicate experiments. A, Germination on NaCl-containing plates. B, Germination on mannitol-containing plates.

pathway than the one involved in response to anoxia, as demonstrated for the regulation of *ADH* expression by ABA and during anoxia (Dolferus et al., 1994; de Bruxelles et al., 1996).

Under normal conditions *PDC1* transcripts make up about 10% to 40% of the total *PDC* mRNA in seedlings (Figs. 2B and 5B). Under oxygen limitation, *PDC1* transcripts are strongly induced during anoxia and represent in these cells about 99% of the total *PDC* mRNA (Fig. 3C). This dominant status of one *PDC* gene in Arabidopsis is different from tobacco. In tobacco, at least two copies exist for PDC (Bucher et al., 1995), one of which is induced anaerobically in vegetative tissues, whereas the other is constitutively expressed in reproductive organs. This is an additional difference with Arabidopsis, where *PDC1*, *PDC2*, and *PDC4* are expressed in flowers (Fig. 2A).

Mutation of *PDC1* Results in Susceptibility to Anoxia

Root tips of *pdc1* mutant seedlings are less tolerant to oxygen deprivation than the wild type (Fig. 4D), indicating that the reduced *PDC* levels compromise survival. It should be noted that for up to 12 h, there is no significant difference, but prolonged anoxia results in the accelerated death of the root tip in mutant plants. The real-time PCR analysis showed that under anoxia, the total *PDC* mRNA concentration in *pdc1* mutants was reduced to approximately 1% of wild-type levels. No compensation of the loss of *pdc1* expression through induction of the other genes was observed (Fig. 4C). The absence of the *PDC1* transcript in the *pdc1* mutant had no effect on the level of expression of *ADH*, and respectively, the absence of *ADH* transcripts in the *adh* null-mutant did not influence the expression of *PDC1* (Fig. 4, B and C). This indicates, that the regulation of the expression of these two genes is independent of the presence of the other. The reduction of *PDC* mRNA levels is accompanied by a reduced production of acetaldehyde and ethanol. However, there is a quantitative difference between the reduction in mRNA and enzyme product levels, with acetaldehyde accumulating to 26% of wild-type levels (Fig. 4E). We envisage three possibilities for the comparatively high acetaldehyde production in *pdc1* knock-out roots. First, acetaldehyde production may be the result of the activity from the enzymes encoded by *PDC2*, *PDC3*, and *PDC4*. The latter mRNAs may be more efficiently translated, or the proteins be more stable or have a higher specific activity. Second, in the anoxically induced wild type, PDC enzymes may be in large excess, and the flux through the pathway may be limited by other factors than enzyme abundance. Third, acetaldehyde in *pdc1* roots may be the product of a yet unknown PDC-independent pathway. In any case, knock-out of the major gene, *PDC1*, compromises survival under oxygen deprivation.

PDC1 Is Induced by Stress

Increase of *PDC* expression has been reported by cold and mannitol treatments in Arabidopsis (Dolferus et al., 1997; Conley et al., 1999) and by cold treatments in rice and maize (Christie et al., 1991).

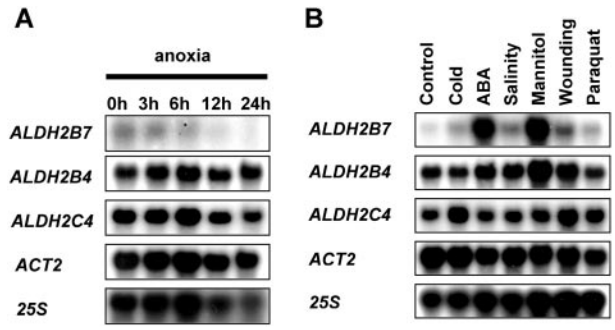

Figure 7. Relative expression level of ALDH genes during anoxia and during other stresses. A, RNA gel-blot analysis from seedlings subjected to different periods of anoxia. Hybridization probes specific to the *ALDH* genes were used. *ACT2* and *25S* hybridization were used as controls. Accession numbers: *ALDH2B7* (AT1G23800), *ALDH2B4* (AT3G48000), and *ALDH2C4* (GenBank no. AF349448). B, RNA gel-blot analysis of *ALDH* transcripts from seedlings treated with different abiotic stresses. *ACT2* and *25S* hybridization were used as controls.

Our results also indicate a strong induction of the *PDC1* gene and an increased production of fermentation metabolites by a broad range of stresses other than anoxia (Fig. 5C). The increase of expression, however, was lower than during anoxia. In addition, we were able to detect the production of fermentation metabolites in stress-treated seedlings, although to a lesser extend than during anoxia. The measured production of fermentation metabolites might be an underestimation, because acetaldehyde and ethanol might have been remetabolized.

Taken together, these results suggest the involvement of ethanolic fermentation in response to abiotic stress, as indicated both at the transcriptional level and by the accumulation of ethanolic fermentation products. Abiotic stress can disrupt respiratory activities and it has been proposed that compensation for impaired mitochondrial function will occur by shifting the metabolism form aerobic to anaerobic fermentation (Levitt, 1980).

However, germination assays with the *pdc1* knockout plants on osmotic pressure producing medium, indicated, that *PDC1* seems not to be essential for survival in these conditions (Fig. 6). Thus, ethanolic fermentation might be a part of abiotic stress adaptation but how much it contributes to stress-tolerance has to be investigated.

Arabidopsis ALDHs: A Function in Detoxification of Products Derived from Reactive Oxygen Species?

None of the three *ALDH* genes from Arabidopsis were induced during anoxia (Fig. 7A). This is in agreement with the situation in tobacco leaves (op den Camp and Kuhlemeier, 1997) and in contrast to the results of Nakazono et al. (2000), who showed increased expression of *ALDH2B5* in submerged rice seedlings, although there was incongruence between the pattern of ALDH2B5 protein and *ALDH2B5* mRNA data. Activity of ALDH during anoxia would deplete the essential NAD^+ pool in any compartment and thus adversely affect anoxia tolerance. Any model postulating the operation of ALDH during anoxia would have to explain how NAD^+ is regenerated under these conditions.

ALDH2B7 expression is strongly induced by cold and mannitol treatments (Fig. 7B). This increase in transcripts suggests a specific function or regulation in stress response. Op den Camp and Kuhlemeier (1997) in tobacco and Skibbe et al. (2002) in Arabidopsis showed that ALDHs convert acetaldehyde to acetate in vitro. In addition, Liu and Schnable (2002) demonstrated through an in-depth analysis a functional specialization of the two maize mitochondrial ALDHs. Similarly, the putative mitochondrial located ALDH2B7 of Arabidopsis might be involved in an ABA-mediated response to dehydration. Removing toxic aldehydes may prevent membrane damage in the mitochondria. This pathway might operate in addition to the cytosolic activity of ADH in detoxification of acetaldehyde. A similar detoxification pathway was also put forward by Møller (2001) to explain how ALDH could prevent pollen abortion in T-cytoplasm maize plants. Interestingly, another Arabidopsis *ALDH* gene, which clusters to the class 3 ALDHs (oxidizing aromatic aldehydes and fatty aldehydes) is also induced in response to dehydration and ABA treatment (Kirch et al., 2001). ALDH activity during stress might occur independent of ethanol fermentation. We envisage therefore a possible role of ethanolic fermentation as a robust energy production pathway in the cytosol in conditions where the mitochondrial ATP machinery is damaged.

MATERIALS AND METHODS

Plant Growth Conditions

Seeds of Arabidopsis, accessions Col-0 or L*er* were surface sterilized and plated on 0.5× Murashige and Skoog medium containing 10% (w/v) Suc, vitamins (1×10^{-4}% myo-inositol, 1×10^{-6}% nicotinic acid, 1×10^{-6}% pyrodoxin-HCl, 1×10^{-5}% thiamine-HCl, and 2×10^{-6} % Gly) and 0.85% (w/v) agar. Plates were incubated at 4°C for 3 d to break seed dormancy and then transferred to the growth room (23°C ±2°C, 16-h-light/8-h-dark cycles) in vertical position for 2 to 3 weeks as described by Chung and Ferl (1999).

Identification of *pdc1*

The *pdc1* mutant was identified in the T-DNA population (accession Col-0) originating at the Torrey Mesa Research Institute (San Diego) by a random sequencing based screen of the T-DNA left border (line no. 688.D02). Genetic analysis by PCR using primers from the left border (5′-TAG CAT CTG AAT TTC ATA ACC AAT CTC GAT ACA C-3′) and a *PDC1*-specific primer (5′-ACA TTC AGA AGA TGC TCT CTA GTG AAA C-3′) identified homozygous mutant lines and enabled the isolation of a fragment covering the insertion site for sequencing. Homozygous *pdc1* mutant plants were further examined. The *adh* mutant in the background L*er* was obtained directly from the Nottingham Arabidopsis Stock Center online catalog (35 N8095; http://nasc.nott.ac.uk/home.html).

Stress Treatments

For anoxic treatments, 2- to 3-week-old plantlets on plates were placed in an anoxia work bench (Forma Scientific, Mariatta, OH) in the dark. Cold and mannitol treatments were performed as previously described by Dolferus et al. (1994) with the following modifications: Before the beginning of the treatments, seedlings were transferred to petri dishes containing 12 mL of liquid 0.5× Murashige and Skoog solution for 24 h (16-h-light/8-h-dark cycle) on a shaker at 70 rpm. For the cold treatment, the plates were then transferred to 11°C in the dark for 24 h on a shaker. For the salt stress, the seedlings were shaken in liquid 0.5× Murashige and Skoog solution containing 300 mM NaCl for 4 h in the light. For the wounding treatments, leaves were wounded three times each with forceps, kept in light and harvested after 4 h. For paraquat (methyl viologen, Sigma-Aldrich, St. Louis) treatment, a 25 μM solution was sprayed and the plant kept in light, and seedlings were harvested after 4 h. For ABA treatment ([+]-cis,trans-ABA, Sigma-Aldrich), the plants were incubated with 10 μM ABA as described by de Bruxelles et al. (1996) in the light.

Acetaldehyde Measurements in the Gas Phase

Roots of hydroponically grown plants or leaves from plants grown on soil were prepared as follows. Material was washed three times with distilled water. Roots or leaves were then infiltrated with a 50 mM Glc and 0.1 mM $CaSO_4$ solution three times for 2.5 min under vacuum. Infiltrated samples were briefly blotted on tissue paper, placed in 10-mL gas-tight

glass-bottles, and sealed after transfer either to the anaerobic working bench and kept in the dark according to Bucher and Kuhlemeier (1993) or to aerobic atmosphere, also kept in the dark. After 24 h, 2.5-mL samples of head spaces were taken with a gas-tight syringe (Hamilton, Bonaduz, Switzerland) and immediately injected and analyzed by gas chromatography as described by Bucher et al. (1994) with the following modifications: column temperature, 180°C; injector temperature, 190°C; and detector temperature, 220°C.

Survival Assay for Anoxia, Dehydration, and Salt Stress

Survival assay for anoxia was done according to Ellis et al. (1999) with the following modifications: Before anoxic conditions, seedlings were transferred for 24 h to plates containing a liquid Murashige and Skoog solution. The medium was removed and substituted with fresh medium, which was gassed for 1 h with N_2. After the incubation in the anoxic bench, seedlings were placed on recovery plates, and the positions of the root tip were scored on the back of the plates. The recovery phase lasted for 3 d where the plates were incubated vertically under diffuse light conditions. Root tip survival was scored as the ability of the main root tip to extend beyond the mark scored after the treatment.

Survival assay for dehydration and salt stress was performed on Murashige and Skoog plates containing the indicated concentrations of either mannitol or NaCl. Seeds of wild type and pdc1 were surface sterilized and placed on the indicated plates. These were then incubated at 4°C for 2 d and then transferred to the growth room (23°C ±2°C, 16-h-light/8-h-dark cycles) in vertical position for 1 week. Germination rate and root length were scored after this period.

RNA Extraction and Northern-Blot Analysis

Total RNA was isolated from seedlings of control or stress treatments or mutants and transferred to nylon membranes as described by Caderas et al. (2000). Hybridizations were carried out under standard conditions with randomly labeled probes (Sambrook et al., 1989). ALDH probes were generated from a cDNA by PCR from using the following primers: ALDH2C4, 5′-GTT ACC GGA GAT CAA ATT CAC CAA G and 5′-CAG TCA AGA TCA AAC CCG AAG TAG C; ALDH2B4, 5′-TCA GCT TCC TCT CCC TTA CTG TTT CG and 5′-ACT TCG TCT CGT TCG CCC TCT TTA TC; and ALDH2B7, 5′-AAG ATA CAG TAA CCT CGC TGC TGC TG and 5′-CCA ATG CCA CTC ATC TTA ACC CTC C. No cross-hybridization could be detected among the probes (data not shown). To determine expression of the housekeeping gene ACTIN2 (EMBL no. ATU41998), blots were hybridized with an ACT2 probe generated by RT-PCR (forward primer, 5′-ATT CAG ATG CCC AGA AGT CTT GTT-3′; reverse primer, 5′-GAA ACA TTT TCT GTG AAC GAT TCC T-3′

Reverse Transcription and PCR Optimization

Total RNA (2 μg per reaction) was DNase I treated. First-strand synthesis of cDNA was performed by using oligo(dT) primer and avian myeloblastosis virus RT. The following primers were used for RT-PCR experiments: ADH forward primer, 5′-AGT TGT GGT TTG TCT ACT GGG TTA G-3′, and reverse primer, 5′-AGA GTC CTC TCA TTC AAG AAA TTC A-3′; PDC1 forward primer, 5′-CTC GTT GAC GCC ATT CAT AAC-3′, and reverse primer, 5′-CCA TGA TAA AGC GTA CAT GGA A-3′; PDC2 forward primer, 5′-TTT GGT AGT GTC TTC ACC GTT C-3′, and reverse primer, 5′-TTC TTG GGA TGG GAT CTC AAC-3′; PDC3 and PDC4 forward primer, 5′-CTG GTC TTG TCG ATG CTA TTC A-3′, and reverse primer, 5′-AAA CTT TGT CAA CAA GGG GTT C-3′; PDC4 reverse primer, 5′-CAC CAT CAA TGG TAA TGG TAC A-3′; RD29a forward primer, 5′-GTG GAG AAG ATC TCT ACC GAG AAG G-3′, and reverse primer, 5′-CAT CAA AGA CGT CAA ACA AAA CAC A-3′; and ACT2 forward primer, 5′-ATT CAG ATG CCC AGA AGT CTT GTT-3′, and reverse primer, 5′-GAA ACA TTT TCT GTG AAC GAT TCC T-3′. Primers were optimized for amplification in a gradient cycler with various annealing temperature from 47.5°C to 63.1°C.

Quantitative Real-Time PCR

For quantitative real-time PCR experiments, the LightCycler system (Roche Diagnostics, Mannheim, Germany) was used. For PCR-reactions, a mastermix of the following reaction components was prepared (the end-concentration is indicated in parentheses): 12 μL of water, 2 μL of MgCl2 (2.5 mM), 1 μL of forward primer (0.5 μM), 1 μL of reverse primer (0.5 μM), and 2 μL of LightCycler (Fast Start DNA Master SYBR Green I, Roche Diagnostics). LightCycler mastermix was filled in the LightCycler glass capillaries, and 2 μL of cDNA was added as PCR template. Capillaries were closed, centrifuged, and placed into the LightCycler rotor. The following LightCycler experimental run protocol was used: denaturation program (95°C for 10 min), amplification and quantification program repeated 45 to 55 times (95°C for 15 s, annealing temperature: 63°C for 15 s for all primer combinations except for PDC3 amplification:58°C, 72°C for 20 s with a single fluorescence measurement), melting curve program (65°C–95°C with heating rate of 0.1°C s^{-1} and a continuous fluorescence measurement), and finally a cooling step at 40°C.

For relative quantification, PCR efficiencies for each gene were determined as follows: Standard curves for each gene was performed using the cDNA with the highest abundance of the gene to cover the range of all template concentrations. For PDC2, an external standard curve was performed on a purified PDC2-PCR fragment. Real-time PCR efficiencies (E) were calculated from the given slopes in the LightCycler software of the standard curves according to the equation: $E = 10\ [-1/\text{slope}]$. PCR efficiencies for the used target and reference genes: ADH, 1.95; ACT2, 1.99; RD29a, 1.90; PDC1, 1.88; PDC2, 1.70; PDC3, 1.57; and PDC4, 1.92 (see also supplementary materials; they can be viewed at www.plantphysiol.org). Crossing points, defined as the point at which the fluorescence rises above the background fluorescence was determined using the "Fit Point Method" in the LightCycler software 3.5.3 (Roche Diagnostics). cDNA abundance of the PDC genes was calculated by crossing point differences between amplification of the different genes after a baseline adjustment. Gene-specific PCR efficiency was used to calculate the induction level. A mathematical model, which was shown recently to hold in experimental environments, was used to quantify the expression of target genes relative to the expression of a reference gene (Pfaffl, 2001). The relative expression ratio of the target genes were calculated based on their efficiencies (E) and the crossing points (CP) deviation of an unknown sample versus a control and expressed in comparison to the reference gene *ACT2* as shown in equation 1.

$$ratio = \frac{(E_{target})^{(CP_{control} - CP_{sample})}}{(E_{reference})^{(CP_{control} - CP_{sample})}} \quad (1)$$

where relative expression [fold induction] means x-fold induction of the expression of gene A relative to the standard gene B compared by a control experiment.

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