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RESEARCH PAPER

Analysis of the role of the pyruvate decarboxylase gene family in *Arabidopsis thaliana* under low-oxygen conditions

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ABSTRACT

Plants under low-oxygen conditions adapt their metabolism by inducing the fermentative pathway, with ethanol as the predominant end product. Activities of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) are required for this pathway. While a single gene encodes ADH in *Arabidopsis*, a family of four genes codes PDC. The availability of microarray data sets enabled the relative importance of the four *PDC* genes under low oxygen to be assessed, and revealed that, contrary to previous published evidence, not only *PDC1* but also *PDC2* plays a role under hypoxic conditions. We observed a high level of expression, both at transcript and protein levels of PDCs, even under aerobic conditions when ADH is almost absent. This suggests that PDC has a role under aerobic conditions, which is not coupled to fermentative metabolism. The expression of both *PDC1* and *PDC2* is strongly up-regulated under low oxygen. *PDC1* is predominantly present in roots, while *PDC2* appears to be leaf-specific. We showed that mutations in both *PDC1* and *PDC2* result in lower tolerance to submergence.

INTRODUCTION

In aerobic organisms, oxygen is a key requirement for efficient energy production. When the supply of oxygen is reduced, plants can experience rapid and profound physiological consequences, including gene expression, cellular metabolism and energy consumption (Gupta *et al.* 2009; Mustroph *et al.* 2010). Plants may undergo oxygen deprivation because of variations in the environment where they live, such as sudden floods or severe rainfall, leading to soil waterlogging and submergence (Bailey-Serres & Chang 2005). Submerged plant organs experience a decline in oxygen concentration and light supply, and a consequent reduction in energy production, which negatively affects plant growth and crop production (Bailey-Serres & Voesenek 2010). However, flooding is not the only cause of hypoxia in plant tissues. In fact, a gradient in oxygen concentration is often found through dense tissues, such as in bulky organs and developing seeds (van Dongen *et al.* 2004). In the case of seeds, the oxygen level can decrease to below 1% under the seed coat (van Dongen *et al.* 2004).

Throughout evolution, plants have acquired different adaptations in order to have a better chance of surviving flood conditions. These stress responses are found at both a cellular level and within whole plants, and lead to metabolic and developmental changes (Bailey-Serres & Chang 2005). When hypoxic conditions occur, plants immediately alter their gene expression and start to synthesise a set of anaerobic polypeptides (ANPs), which are specifically involved in glycolysis and ethanol fermentation (Sachs *et al.* 1980, 1996; Drew 1997; Dennis *et al.* 2000). A drop in cytoplasmic pH leads to the inactivation of lactate dehydrogenase (LDH) and the activation of pyruvate decarboxylase (PDC; Felle 2005). This means that lactate production is only transient and is replaced by ethanol as an end product of fermentative metabolism. The pyruvate, which is generated by glycolysis, is metabolised to ethanol in two steps *via* pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH), with the concomitant re-oxidation and recycling of NADH. Re-oxidation and recycling are essential to allow ATP production through glycolysis to continue in the absence of the mitochondrial re-oxidation of NADH. ADH and PDC have been studied in many plant species that differ in their degree of anoxia tolerance, such as maize, rice, *Rumex palustris* and *Arabidopsis thaliana* (Perata & Alpi 1993; Dolferus *et al.* 1997, 2003; Ellis *et al.* 1999; Visser *et al.* 2003; Loreti *et al.* 2005).

The significant role of both ADH and PDC during anoxic stress has been supported through genetic evidence. ADH is a key player in flooding tolerance: if it is knocked out, there is a reduction in low-oxygen tolerance (Ellis *et al.* 1999; Ismond *et al.* 2003). A number of *adh* mutants have been characterised in different species, such as maize (*Zea mays*; Freeling & Bennett 1985), tobacco (*Nicotiana tabacum*; Rousselin *et al.* 1990) and *Arabidopsis* (Jacobs *et al.* 1988). In *Arabidopsis*, the *adh1* null mutant has lower survival rates in low-oxygen conditions. In rice, an anoxia-tolerant species, coleoptile elongation is reduced in the *reduced adh activity* (*rad*) mutant on submergence. The *rad* null mutant shows a reduction in the ADH

protein because of a point mutation in the *ADH1* gene, and a consequent vulnerability to submergence stress, suggesting that a functional ADH protein is important for submergence tolerance (Matsumura *et al.* 1995, 1998; Saika *et al.* 2006). Furthermore, the maize *Adh1⁻* mutant that is unable to synthesise the alcohol dehydrogenase1 (ADH1) isozyme, is very susceptible to anoxia (Roberts *et al.* 1985; Andrews *et al.* 1994; Johnson *et al.* 1994). ADH overexpression can confer enhanced anoxia tolerance, resulting in hairy roots and improved root growth (Shiao *et al.* 2002), but has no effect on flooding survival (Ismond *et al.* 2003). Overall, this evidence has proved that ADH has an essential role in fermentation and low-oxygen tolerance.

Although the conversion of pyruvate to acetaldehyde, catalysed by PDC, is considered to be rate limiting in ethanol fermentation, a regulatory role for PCD has not yet been fully described. In *Arabidopsis*, four genes encode PDC, and the overexpression of *PDC1* and *PDC2* has been shown to improve survival under low-oxygen conditions (Ismond *et al.* 2003). Only the *pdc1* mutant has been characterised and shows a marked susceptibility to anoxia, indicating the importance of the *pyruvate decarboxylase1* gene under oxygen deprivation (Kürsteiner *et al.* 2003).

Modulation of the transcriptional programme is a plant strategy to overcome unfavourable conditions. Tolerance to anoxia is often mediated through changes in gene expression, leading to the production of proteins that are then involved in this process (Timperio *et al.* 2008). In addition, it has been suggested that plants regulate gene expression in response to developmental stage. Unlike other organisms, plants develop continuously, with the formation of new organs throughout the life cycle (Schmid *et al.* 2005).

We were curious to see how altered environmental conditions, such as anoxia, affect transcriptional responses depending on the morphological stage of the plant. We investigated the expression profile and protein levels of PDC in different *Arabidopsis* organs subjected to anoxia at different developmental stages. Our results demonstrated that both *PDC1* and *PDC2* genes are important in the response to low-oxygen stress, and in addition that PDC1 and PDC2 proteins are differentially expressed in roots and leaves.

MATERIAL AND METHODS

Plant material and growth conditions

Arabidopsis thaliana Col-0, its *pdc1* (*At4g33070*) and *pdc2* (*At5g54960*) T-DNA insertion lines (obtained from the Nottingham Arabidopsis Stock Centre, Nottingham, UK), SALK line N660027 and SAIL line N862662, respectively, were used in this study. Homozygous lines were identified *via* PCR screening of genomic DNA using gene-specific primers together with T-DNA-specific primers (primer LBb1 for the SALK line, and LB1 for the SAIL line; see Table S1). For anoxia experiments, seeds were sown on soil and plants were treated in an anoxia chamber, as described below. The submergence tolerance experiment reported in Fig. 4 was performed using 5-week-old plants (stage 1.14) grown in a gravel medium with a nutrient solution containing 1.25 mM KNO_3, 1.50 mM $Ca(NO_3)_2$, 0.75 mM $MgSO_4$, 0.50 mM KH_2PO_4, 50 μM KCl, 50 μM H_3BO_3, 10 μM $MnSO_4$, 2.0 μM $ZnSO_4$, 1.5 μM $CuSO_4$, 0.075 μM $(NH_4)_6Mo_7O_{24}$ and 72 μM Fe-diethylenetriamine pentaacetate (Gibeaut *et al.* 1997). In all experiments, plants were grown in a growth chamber with a photoperiod of 12-h light/12-h dark (120 μM·photons·m^{-2}·s^{-1}) at 22 °C day/18 °C night. Samples were harvested and immediately frozen in liquid nitrogen. Developmental stages were defined according to Boyes *et al.* (2001).

Anoxia treatments

Anoxia treatments were carried out in the dark. An enclosed anaerobic workstation (Anaerobic System model 1025; Forma Scientific, Marietta, OH, USA) was used to provide an oxygen-free environment for seedling incubation. This chamber uses palladium catalyst wafers and desiccant wafers to maintain strict anaerobiosis at less than 10 mg·ml^{-1} oxygen (according to the manufacturer's specifications). High-purity N_2 was used to initially purge the chamber, and the working anaerobic gas mixture was N_2:H_2 with a ratio of 90:10. Three independent replicated experiments were performed for each experimental condition. Each independent experiment consisted of four replicated samples pooled before RNA extraction.

Submergence tolerance assay

The submergence treatment was performed on all three genotypes. Five-week-old plants were submerged in deionised water for 8 days with photoperiod 12 h/12 h, light/dark. After recovery (3 days), surviving and dead plants were counted and photographed. Tolerance assays were repeated three times using 20 plants per genotype in each replication.

RNA isolation, cDNA synthesis and real-time qRT-PCR analysis

Total RNA was extracted from each sample as previously described (Perata *et al.* 1997), with minor modifications (omission of aurintricarboxylic acid) to make the protocol compatible with the subsequent PCR procedures. Electrophoresis using a 1% agarose gel was performed for all RNA samples to check for RNA integrity, followed by spectrophotometric quantification. Contaminating DNA was removed using a TURBO DNA-free kit (Ambion, www.ambion.com). RNA was then reverse-transcribed using an iScript TM cDNA synthesis kit (BioRad Laboratories, www.bio-rad.com). Expression analysis of *PDC* genes, *PDC1* (*At4g33070*), *PDC2* (*At5g54960*), *PDC3* (*At5g01330*) and *PDC4* (*At5g01320*), was performed by real-time PCR using an ABI Prism 7300 sequence detection system (Applied Biosystems). Quantitative PCR was performed using 40 ng cDNA and iQTM SYBR® Green Supermix (Biorad Laboratories, Hercules, CA), according to the manufacturer's instructions. Expression of *Ubiquitin10* (*At4g05320*) was used as an endogenous control. Relative expression levels were calculated using GeNorm (http://medgen.ugent.be/~jvdesomp/genorm). For a list of primers used and designed by the QuantPrime Tool (http://quantprime.mpimp-golm.mpg.de; Arvidsson *et al.* 2008), see Table S2.

SDS-PAGE and immunoblotting

Samples (about 500 mg) were ground in liquid nitrogen. An extraction buffer, described by Siddique *et al.* (2008), was

added in a 1:2 ratio (plant tissue:buffer). Total protein content was quantified with a BCA Protein Assay (Pierce, Thermo Fisher Inc., Rockford, IL USA). SDS-PAGE was performed on a 10% Criterion polyacrylamide gel (BioRad). Blotting on an Amersham Hybond-P polyvinylidene difluoride membrane was performed with a Trans-Blot Turbo system (Bio-Rad). Immunoblotting was performed using Immun-Star HRP Chemiluminescent Detection Kits (BioRad). Antibodies against PDC were purchased from Agrisera (product code AS10 691; Agrisera, Vännäs, Sweden). A densitometric analysis of the protein signals on the Western blots was performed with the software package UVP VisionWork LS (Ultra-Violet Products, Cambridge, UK). Amido black staining was performed to check equal loading. The blot was stained for 5 min [0.1% amido black (Sigma Aldrich, Milan, Italy), 45% methanol, 10% acetic acid] and then washed in a destaining solution (90% methanol/2% acetic acid/8% water) for 2 min.

Assay of PDC activity

The PDC activity was assayed following Ismond *et al.* (2003) with some modifications. Plants were harvested and frozen in liquid nitrogen, and homogenised in the extraction buffer [50 mM phosphate buffer (pH 7.0), 5 mM $MgCl_2$, 5 mM EDTA, 500 μM thiamine pyrophosphate (TPP), 100 μM phenylmethylsulphonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 5 μM leupeptina)]. The extract was clarified by centrifugation at 15,000 *g* at 4°C for 15 min. The supernatant was directly assayed for enzyme activity. The protein amount in each sample was quantified using the BCA protein assay (Pierce). PDC activity was assayed in 50 mM MES-NaOH (pH 6.0), 5 mM $MgCl_2$, 500 μM TPP, 1 mM DTT, 670 μM NADH, 6 mM sodium pyruvate and 1.16 $U{\cdot}ml^{-1}$ ADH (Sigma Aldrich). The reaction started with pyruvate and was monitored at 340 nm using a spectrophotometer.

RESULTS

PDC GENES ARE DIFFERENTIALLY EXPRESSED UNDER LOW-OXYGEN STRESS

In the absence of oxygen, an increase in PDC activity has been well documented in plant species such as rice, wheat and maize. Among the four rice *PDC* genes isolated, *PDC1*, *PDC2* and *PDC4* are anoxia responsive (Rivoal *et al.* 1997). These three *PDC* genes isolated from maize are hypoxia responsive (Peschke & Sachs 1993). In *Arabidopsis*, four *PDC* genes are present: *PDC1* (*At4g33070*), *PDC2* (*At5g54960*), *PDC3* (*At5g01330*) and *PDC4* (*At5g01320*) (Kürsteiner *et al.* 2003). *PDC1* and *PDC2* are induced by several stresses but never at the level observed under anoxia (Kürsteiner *et al.* 2003). These genes are expressed at all developmental stages (Fig. S1). The Affymetrix ATH1:22K microarrays available in Genevestigator (Hruz *et al.* 2008) were related to the Col-0 ecotype and the HRE1-HRE2 overexpressor, in which the transcript level increases during oxygen depletion (Licausi *et al.* 2010), and in seedlings, roots and shoots subjected to low-oxygen stress conditions, *e.g.* anoxia, hypoxia and submergence (Fig. 1). The expression profile highlighted the induction (in red) of *PDC1* and *PDC2* genes in most of the treatments in the data set. The expression of *PDC3* and *PDC4* genes, on the other hand, was not affected (yellow) by any of

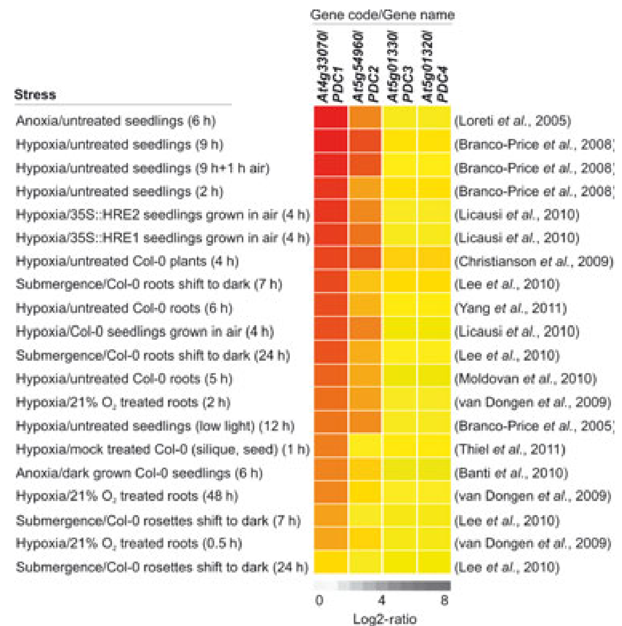

Fig. 1. PDC gene expression in response to different low-oxygen conditions. Anoxia, hypoxia and submergence stresses affect expression of all four PDC genes in the *Arabidopsis* Col-0 ecotype and in several mutants and various tissues. PDC*1* corresponds to the 253416_AT probeset, PDC*2* to 17922_AT, while PDC*3* and *PDC4* are represented by a single probeset in Affymetrix ATH1:22K, namely 251112_S_AT. Data (expressed as Log2) were retrieved using Affymetrix ATH1:22K microarrays from the Genevestigator web tool, and are shown as a heat map.

the low-oxygen treatments (Fig. 1). Taken together, these observations suggest that *PDC1* and *PDC2* genes are involved in low-oxygen responses, whereas *PDC3* and *PDC4* are not affected by hypoxia/anoxia conditions.

Characterisation of *PDC* genes in anoxia-treated plants at different development stages

To examine whether *PDC* genes are differentially expressed during plant development, *Arabidopsis* plants (Col-0) were studied at different growth stages (Boyes *et al.* 2001; Fig. S2). The plate-based platform used stages 1.0, 1.02 and 1.04, whereas all the other stages under investigation were grown in a semi-hydroponic system. Although the data retrieved from Genevestigator showed that *PDC3* and *PDC4* genes were not regulated by low-oxygen stress (Fig. 1), it has not yet been demonstrated whether their expression patterns could be influenced by plant age. We thus used qRT-PCR to study the expression of all four *PDC* genes.

The heat-map showing qRT-PCR analysis at different stages revealed that *PDC1* and *PDC2* were constitutively expressed under aerobic conditions. *PDC3* was expressed in young tissues and in roots even in aerobic conditions, whereas *PDC4* was absent in almost all the stages and organs analysed, except for stage 8.0, where *PDC4* was down-regulated in anoxia treatment (Fig. 2A). Overall, the data indicated that 2 h anoxia did not have a marked influence on *PDC3* and *PDC4* mRNA expression levels, whereas it had a clear inductive effect on *PDC1* and *PDC2* expression (Fig. 2A). To obtain an overview of *PDC* gene regulation under anoxia at all development stages, the

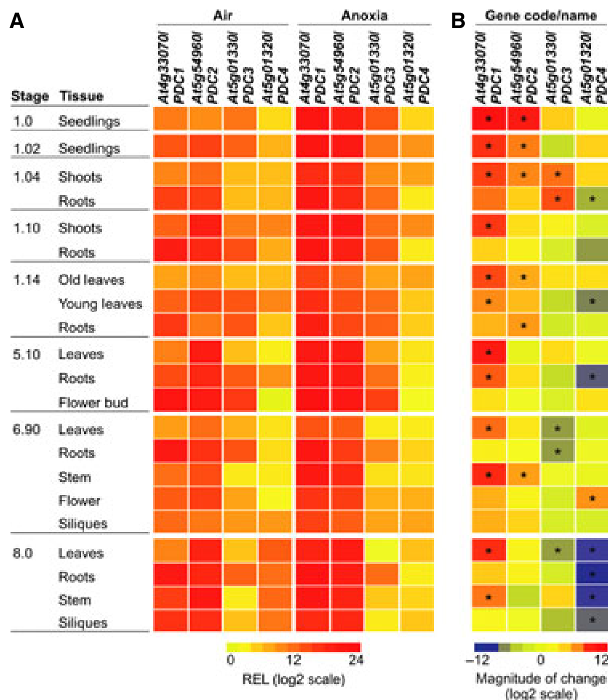

Fig. 2. (A) Effects of 2 h of anoxia on the expression profile of PDC genes of *Arabidopsis* at different growth stages. Classification of growth stages is based on Boyes *et al.* (2001). mRNA levels were determined with RT-qPCR using gene-specific primers (listed in Table S2). Relative expression levels were calculated by setting the expression to 1 for *PDC1* in old leaves at stage 1.14. Data are the mean of three replicates. (B) Responsiveness to anoxia of PDC genes. The expression data in panel A are shown as fold change (log2) calculated as the ratio between the control (air) and anoxia (2 h) for each growth stage and tissue. Statistical significance for comparison 'anoxia *versus* air' is reported (two-way ANOVA with Bonferroni *post-hoc* test $P \leq 0.05$), and values that differ significantly are marked with *.

relative transcript levels shown in Fig. 2A are displayed as a heat-map of a log2 ratio between air and 2 h anoxia (Fig. 2B). *PDC1* was induced by anoxia especially in the leaves and stems; *PDC2* was induced in seedlings and at stage 1.14; whereas *PDC3* seemed to be induced only at stage 1.04 (Fig. 2B); *PDC4* expression appeared to be unaffected or even repressed by low-oxygen conditions (Fig. 2B). The high expression of *PDC1* and *PDC2* under anoxia (Fig. 2A) and the induction of these genes (Fig. 2B) suggest that not only *PDC1* (Kürsteiner *et al.* 2003) but also *PDC2* is involved in the response to anoxia. The protein level of ADH was also monitored in seedlings and plants at stage 1.14 (Fig. S3), revealing that ADH is low in air in most tissues, with the exception of roots. The anoxic treatments induced protein accumulation of ADH very rapidly in seedlings, while induction in leaves was modest and slow (Fig. S3).

Tissue-specific expression of *PDC1* and *PDC2* genes

Both *PDC1* and *PDC2* appear to be predominantly involved in anoxia responses, but it remains unclear whether they are functionally redundant. To assign them a specific role under anoxia, we analysed two knockout mutants, *pdc1* and *pdc2* (Fig. S4). As both genes were significantly induced at stage 1.14 (Fig. 2), we investigated anoxia responses in the mutants at this development stage (Fig. 3). Stage 1.14 represents an early pre-flowering stage, with the rosette formed by old leaves, young leaves and roots (Fig. S2). Col-0, *pdc1* and *pdc2* plants were therefore grown until they reached stage 1.14, and then

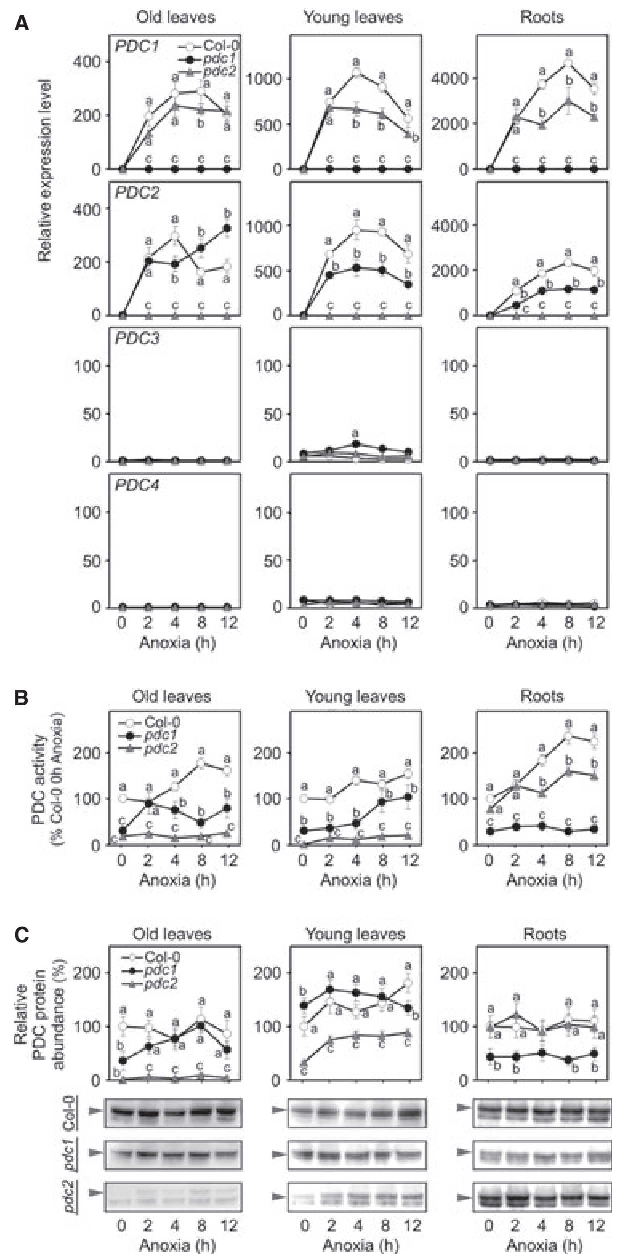

Fig. 3. Effect of anoxia on expression of PDC in wild type (Col-0) and *pdc1* and *pdc2* mutants of *Arabidopsis*. Plants at the 1.14 stage were studied during a time course of anoxia. (A) Analysis of PDC*1*, PDC*2*, PDC*3* and PDC*4* mRNA levels with RT-qPCR using gene-specific primers (listed in Table S2). Relative expression levels were calculated setting the expression to 1 of Col-0 old leaves at time 0. Data are means of three replicates (±SD). (B) PDC enzyme activity. Data are expressed as a percentage of Col-0 at time 0. (C) Immunoblot analysis of PDC wild type (Col-0) and *pdc1* and *pdc2* mutants. Data were normalised to protein quantification of a major protein band stained using amido black (Fig. S6) used as a loading control. The data represent means (±SD) of three independent immunoblots. Differences among genotypes were evaluated with two-ANOVA (Bonferroni *post-hoc* test $P \leq 0.05$).

treated under anoxia for up to 12 h. The responses, both at transcript and protein levels, were monitored in air (0 h), used as a reference, and at 2, 4, 8 and 12 h under anoxia (Fig. 3). Data were expressed using the aerobic level detected in old leaves as a reference in order to compare expression levels in different organs. *PDC1* mRNA was highly induced in Col-0 and *pdc2* genotypes, but absent in the *pdc1* mutant, whereas *PDC2* was induced in Col-0 and *pdc1*, but absent in the *pdc2* mutant (Fig. 3A). Although we checked *PDC3* and *PDC4* transcript levels, these isoforms did not compensate for *PDC* expression when the other isoforms were knocked out.

We were interested in checking PDC enzymatic activity in the different genotypes and tissues. In the *pdc1* mutant, PDC enzyme activity was anoxia-responsive and, in both young and old leaves, it was higher than in the roots at all the time points in the experiment (Fig. 3B). In the *pdc2* mutant, on the other hand, PDC enzyme activity was higher in roots (Fig. 3B). To verify whether that the results obtained for PDC enzyme activity resulted in correspondingly different protein levels, we checked the amount of PDC using immunoblotting analysis (Fig. 3C). In *pdc1,* the protein level was lower in roots compared to that in Col-0 and *pdc2*, whereas in *pdc2* the protein level was high in roots and very low in leaves (Fig. 3C). These results suggest that PDC1 accounts for most of the PDC enzymatic activity in root tissues, whereas PDC2 is the major player for PDC activity in leaves (Fig. 3C). This was confirmed when looking at the presence of PDC in plants at stage 6.90 (Fig. S5).

Both PDC1 and PDC2 contribute to anoxia tolerance in *Arabidopsis*

In *Arabidopsis*, fermentative genes are strongly induced under low-oxygen conditions (Dolferus *et al.* 1997; Loreti *et al.* 2005). To better understand the role of PDC1 and PDC2 in low-oxygen conditions, we performed a tolerance test using Col-0, *pdc1* and *pdc2*. We submerged *Arabidopsis* plants (stage 1.14) for 8 days and examined their survival during recovery. Eighty per cent of wild-type plants survived 8 days of submergence, however survival dropped to 60% and 35% in *pdc1* and *pdc2* genotypes, respectively (Fig. 4), indicating that they are both involved in the metabolic acclimation to low-oxygen conditions.

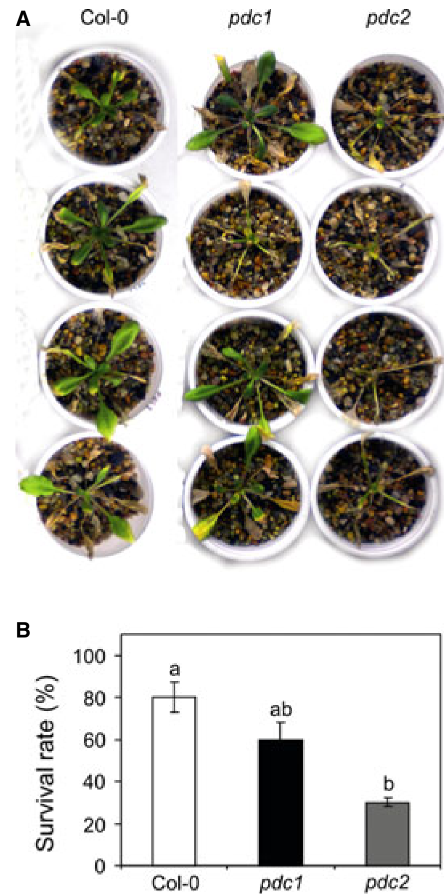

Fig. 4. Effect of submergence on survival in the wild type (Col-0) and the *pdc1* and *pdc2* mutants. (A) Photograph of plants subjected to submergence for 8 days followed by a recovery phase of 3 days. (B) Survival rate of wild-type (Col-0) *Arabidopsis* and the *pdc1* and *pdc2* mutant plants. The number of plants recovering from the submergence treatment is expressed as a percentage (n = 3 experiments, each with 20 plants for each genotype). Values that are significantly different (one-way ANOVA $P \leq 0.05$) from each other are marked with different lowercase letters in the graph.

DISCUSSION

Alcoholic fermentation through the coupled activity of PDC and ADH (Roberts *et al.* 1984) is of great importance for a plant's ability to survive short periods of hypoxia or anoxia (Ricard *et al.* 1994; Drew 1997; Tadege *et al.* 1999). In *Arabidopsis*, a single *ADH* gene is present, which, when knocked out, results in reduced tolerance to low oxygen. PDC, on the other hand, is represented through four different genes, but Kürsteiner *et al.* (2003) identified only one, namely *PDC1*, as being important for anaerobic metabolism. These authors carried out a very comprehensive analysis of the PDC gene family, and concluded that *PDC1* was important under low oxygen, since a *pdc1* mutant is impaired in tolerance to anoxia (Kürsteiner *et al.* 2003). These authors did not investigate *PDC2*, as it was not induced by anoxia in their experiments. We believe that the conclusions of Kürsteiner *et al.* (2003) now need to be revised on the basis of the large collection of microarray data sets obtained for *Arabidopsis* under both hypoxic and anoxic conditions, showing that not only *PDC1* but also *PDC2* is up-regulated when the plant senses low-oxygen conditions (Fig. 1). *PDC3* and *PDC4* mRNAs were not markedly induced by anoxia, suggesting that these two genes are unlikely to play a role during anoxic stress (Figs 1, 2).

Although our results highlight that both *PDC1* and *PDC2* are required for survival under low-oxygen conditions, they do not appear to be functionally redundant. The expression levels of both *PDC1* and *PDC2* were already high under aerobic conditions (Fig. 2A) and were strongly induced by anoxia (Fig. 3). The high level of PDC protein observed in aerobic tissues raises a question regarding its metabolic role. Western blots revealed a comparably high level of ADH protein only in roots (Fig. S3), suggesting that PDC is not involved in ethanol production in aerobic leaves. Tadege *et al.* (1999) proposed that PDC could act as a by-pass for the activity of pyruvate dehydrogenase (PDH). This is interesting, especially because not only PDC but also aldehyde dehydrogenase (ALDH) is constitutively present in aerobic leaves (Kürsteiner *et al.* 2003). Zabalza *et al.*

(2009) showed that pyruvate levels can affect the rate of oxygen consumption, and it is tempting to speculate that the PDC-dependent PDH by-pass prevents cellular build-up of excess pyruvate, which might negatively affect respiration. The expression pattern of *PDC* mRNA does not provide enough clues to rule out functional redundancy, however immunoblot analysis of the *pdc1* and *pdc2* mutants highlighted tissue-specific presence of PDC1 and PDC2. The level of PDC protein in leaves of the *pdc2* mutant was very low, while an almost normal level was detected in *pdc1*.

Conversely, a lower level of PDC was detected in roots of *pdc1* but not in *pdc2* roots (Fig. 3C, Fig. S5). These results suggest that PDC1 plays a dominant role in roots, while PDC2 is leaf-specific. However, we did not observe a consistent difference in intolerance symptoms in either *pdc1* or *pdc2*, which both underwent leaf death when submerged (Fig. 4). This is possibly explained by the negative impact of root death (in *pdc1*) on the rosette leaves and the direct impact of the lack of PDC2 in leaves of the *pdc2* mutant. The role of PDC3 and PDC4 is still unknown, but it is unlikely that they contribute to metabolism in tissues subjected to low-oxygen availability.

Under low-oxygen conditions, both *PDC1* and *PDC2* transcripts are induced. This process is mediated by the oxygen-sensing mechanism described in Licausi *et al.* (2011), based on oxygen-dependent destabilisation of the RAP2.12 transcription factor. Indeed, both *PDC1* and *PDC2* were up-regulated in aerobic plants that express an oxygen-insensitive version of RAP2.12, and down-regulated in hypoxia when RAP2.12 was repressed using an artificial miRNA approach (Licausi *et al.* 2011). The RAP2.12-dependent mechanism is also involved in the up-regulation of ADH (Papdi *et al.* 2008; Licausi *et al.* 2011), which leads to the coordinated expression of genes required to support fermentative metabolism under low-oxygen conditions. The overexpression of both *PDC1* and *PDC2* resulted in higher tolerance to hypoxia, while the over-expression of *ADH* is without obvious consequences (Ismond *et al.* 2003). Ismond *et al.* (2003) attempted to down-regulate PDC1 and PDC2, but without consequences in terms of tolerance. As Ismond *et al.* (2003) stated, real knockouts would be needed to reveal the importance of PDC1 and PDC2 in low-oxygen tolerance, as described in this work.

We conclude that *PDC1* and P*DC2* are both responsive to anoxia and are required for survival under low-oxygen conditions. The role of PDC3 and PDC4 is unclear at present, as is the high level of PDC protein observed in aerobic tissues, although the possible role of PDCs as a PDH bypass (Tadege *et al.* 1999) is worth further investigation.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. List of primers used to detect T-DNA insertions.

Table S2. List of the primers used for gene expression analysis.

Figure S1. Expression pattern of *PDC* genes throughout the development of *Arabidopsis*, based on Genevestigator ® microarray data sets.

Figure S2. Classification of developmental stages (Boyes *et al.* 2001).

Figure S3. Immunoblot analysis of ADH and PDC in seedlings at stage 1.0 and at stage 1.14.

Figure S4. Map of the T-DNA insertions in the *pdc1* and *pdc2* mutants and their phenotype.

Figure S5. Immunoblot analysis of PDC in organs from plants at stage 6.90.

Figure S6. Loading controls for the immunoblot analysis.

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