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Long-Term Anoxia Tolerance[1]

Multi-Level Regulation of Gene Expression in the Amphibious Plant *Acorus calamus* L.

Marcel Bucher and Cris Kuhlemeier*

Institute of Plant Physiology, University of Berne, Altenbergrain 21, CH-3013 Berne, Switzerland

***Acorus calamus* is a monocotyledonous wetland plant that can withstand extremely long periods of anoxia. We have investigated the expression of genes coding for pyruvate decarboxylase (*Pdc*), alcohol dehydrogenase (*Adh*), and fructose-1,6-bisphosphate aldolase (*Ald*) during periods of anoxia ranging from 2 h to 2 months. Upon anoxic incubation, *Pdc* mRNA levels peak at 6 h, followed by *Adh* and *Ald*, which peak at 12 and 72 h, respectively. Subsequently, the mRNA levels of all three genes decline within days to low levels. In contrast, alcohol dehydrogenase (ADH) protein levels increase steadily for at least a week and then remain constant. Native gel electrophoresis demonstrates the presence of two sets of ADH isozymes, one present constitutively, the other enhanced during anoxia. Translation initiation factor 4A protein levels, used as a control, remain constant during 2 months of anoxia. The results suggest that *A. calamus* has developed a complex anaerobic response consisting of differential regulation of transcription, translation, and posttranslational processes.**

Gas diffusion from the atmosphere to the soil is drastically limited during flooding. As a consequence, the respiratory activity of roots and microorganisms rapidly depletes the bulk soil solution of oxygen and anoxic conditions are created in the root environment (Drew and Lynch, 1980). Such conditions are harmful to dryland species and can lead to irreversible damage. The ability to survive oxygen deprivation varies greatly among species. Potato tubers and maize roots, for example, do not survive more than 3 d under anoxia (Sachs et al., 1980; Sieber and Brändle, 1991). Rice seeds and seeds of barnyard grass (*Echinochloa crus-galli*) have the capacity to germinate under flooded or anoxic conditions (Opik, 1973; Kennedy et al., 1980). Seedlings of both species survive at least 4 d under these conditions (Cobb and Kennedy, 1987).

Acorus calamus (Fig. 1) is a monocotyledonous amphibious plant. An important anatomical feature of this plant is the rhizome, a stem morphosis comparable to a potato tuber, which, together with the adventitious roots, remains submerged at all times. In summer, high metabolic activity and photosynthesis in the above-water leaves lead to starch accumulation in the rhizome. Oxygen is supplied predominantly by transport through the shoot (Steinmann and Brändle, 1984; Studer and Brändle, 1984). In fall, the aerial leaves senesce and newly formed leaves remain small and submerged. The entire plant lives submerged during winter and, similar to other marsh plants, is in a resting state with reduced metabolic activity (McKee and Mendelssohn, 1984; Steinmann and Brändle, 1984). It has been reported that submergent macrophytes, which do not die back in winter, show photosynthetic activity under ice and limited light conditions coupled with low water temperatures (Boylen and Sheldon, 1976). This may also be true for *A. calamus*.

In *Schoenoplectus lacustris*, a marsh plant comparable to *A. calamus* with respect to its morphology and life cycle, starch content in the rhizome slightly increases during winter, although total amylase and starch phosphorylase activity are high during this period (Steinmann and Brändle, 1984). This also suggests that there may be photosynthetic activity in *A. calamus* during winter. In spring, active metabolism resumes and allows rapid growth of the young leaves into the air. Thus, despite prolonged oxygen deprivation during winter, *A. calamus* can embark on vigorous growth with the advent of spring (our unpublished observation).

Anoxia tolerance in plants is related to flooding tolerance (Fagerstedt and Crawford, 1987) and involves the concerted action of a number of morphological and metabolic adaptations (Hook, 1984). *A. calamus*, like other flood-tolerant species such as rice, *S. lacustris* L. Palla, *Spartina alterniflora* Loisel, *Glyceria maxima*, *Iris pseudacorus*, and others, has a well-differentiated aerenchyma, which enables internal aeration of flooded parts (Armstrong, 1979) as well as elimination of potentially toxic end products (Monk et al., 1984; Studer and Brändle, 1987). The rhizome stores large amounts of carbohydrate, allowing energy metabolism to continue during prolonged periods of flooding (Steinmann and Brändle, 1984). In plants, glycolysis and fermentation are the main pathways through which carbohydrates are degraded and ATP and NAD^+ are produced (for reviews, see Jackson and Drew, 1984; Walker et al., 1987; Brändle, 1990; Drew and Stolzy, 1991). Ethanolic fermentation is thought to be an advantage during anoxia because ethanol metabolism, unlike that of lactate, does not result in severe cytoplasmic acidosis

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* Corresponding author; fax 41–31–332–20–59.

Abbreviations: ADH, alcohol dehydrogenase; *Adh*, gene for ADH; AEC, adenylate energy charge; ALD, cytoplasmic fructose-1,6-bisphosphate aldolase; *Ald*, gene for ALD; eIF-4A and eIF-5A, eukaryotic translation initiation factor 4A and 5A, respectively; PDC, pyruvate decarboxylase; *Pdc*, gene for PDC.

ligation with *Eco*RI linkers, the cDNA was inserted into the *Eco*RI site of the expression vector λZAP II (Stratagene, La Jolla, CA). Packaging into λ phage was carried out with Gigapack II Gold Packaging Extract (Stratagene). After amplification, plaques were screened with the cDNA clone pZM54 (coding for maize aldolase, kindly provided by W.J. Peacock), resulting in the partial cDNA clone pACAld (coding for *A. calamus* ALD). One hundred thirty-four base pairs of pACAld were sequenced and showed 79% homology at the DNA level to a region between +917 and +1050 in the coding region of pZM54. Library screening with the cDNA clone pZM84 (coding for maize ADH, kindly provided by V. Walbot) gave the partial cDNA clone pACAdh (coding for *A. calamus* ADH). The pACAdh DNA sequence showed 71% homology to the maize *Adh1* region located between +13 and +139 in the coding region (Gerlach et al., 1982). The clones were used for northern blot hybridization.

Oligonucleotide primers based on the maize PDC cDNA sequence (Kelley, 1989), 5′-CGCCATGGAT(CT)TCCAC-(CT)TCGATGGT(GA)TA-3′ and 5′-CCAAGCTTA(CT)G-CCGCCGA(CT)GGGTA(CT)GC-3′, were used to amplify *A. calamus* first-strand cDNA obtained after reverse transcription of poly(A)$^+$ RNA from 24-h anoxically treated leaf tissue. Four *Pdc* fragments were cloned into the *Hin*dII/*Hin*dIII site of pBluescript SK−. Double-stranded templates were sequenced using dideoxy sequencing with T7 polymerase (Pharmacia). Sequencing of the four *Pdc* clones, named pACPdc1, pACPdc5, pACPdc8, and pACPdc12, indicated 100% identity at the nucleotide level between pACPdc1 and pACPdc5 and 100% identity between pACPdc8 and pACPdc12, but only 82% identity between the two groups. This suggests that PDC is encoded by at least two genes. Alternatively, since *A. calamus* is a wild species, allelic complexity in the triploid genome may be possible. pACPdc1 nucleotide sequence showed 87% homology to the coding region from +3084 to +3183 in the maize *Pdc* clone (Kelley, 1989).

Northern Blot Analysis

Total RNA was quantified both spectrophotometrically and visually by the staining of stripped northern blots in 0.02% methylene blue dye in 0.3 M sodium acetate, pH 5.5. Excess dye was gently washed away with water. Equal loads of total RNA were separated on 1.2% agarose glyoxal gels after glyoxylation (Hull, 1985). This is based on the assumption that the amount of rRNA, which makes about 98% of total RNA, is approximately unchanged during incubation (Gallagher, 1982; Dennis et al., 1985; Kuhlemeier et al., 1987). Consistency of total RNA loading was tested by staining for ribosomal RNA bands as described above. Northern blotting and hybridization procedures were carried out under standard conditions (Sambrook et al., 1989). Blots were hybridized with appropriately digested fragments of the plasmids below. pZM84 (maize ADH) and pZMPK437pstkpn (coding for maize PDC and kindly provided by P. Kelley) were randomly labeled DNA probes. The final wash was at 50°C. pZM54 (encoding maize ALD) and pACAld (encoding *A. calamus* ALD) were also randomly labeled. The final wash was at 55°C. pACAdh and pACPdc were used for antisense RNA synthesis. The last wash was at 65°C. Transcript size was determined by comparison of the signals with a glyoxylated BRL 1-kb DNA ladder. After exposure, the blots were stripped by putting them into 0.1% SDS at 98°C for a few minutes, and, subsequently, they were allowed to cool to room temperature. The same blots were used to hybridize to each of the three probes coding for ALD, PDC, and ADH.

Densitometric scanning of appropriately exposed northern blots was performed with a CD 60 densitometer (Digitana, Horgen, Switzerland).

Protein Extraction

Homogenization of tissue frozen in liquid nitrogen was performed exactly as described for RNA extraction using the micro-dismembrator. Proteins were extracted in 0.1 M Tris-HCl, pH 7.5, 0.1% 2-mercaptoethanol, 0.2% PVP, and 5% polyvinylpolypyrrolidone by vortexing and centrifugation in a microcentrifuge at 13,000*g* for 20 min at 4°C. Total protein concentration was measured according to Bradford (1976). The samples, containing final concentrations of 0.125 M Tris-HCl, pH 6.8, 12.5% glycerol, 0.02% bromphenol blue, and 0.025% 2-mercaptoethanol were either used immediately for separation by PAGE or frozen in liquid nitrogen and kept at −80°C.

Western Blot Analysis

Ten micrograms of total soluble protein was separated either in a 12.5% SDS-PAGE gel or in a native gel (Sachs et al., 1980) and transferred to nitrocellulose (Schleicher and Schuell, BA85) on a semi-dry blotter (Millipore). Western analysis was performed as described (Harlow and Lane, 1988) with a 1:1000 dilution of either a rabbit anti-barley ADH antibody (kindly provided by A. Good) or a rabbit anti-tobacco eIF-4A antibody (kindly provided by G.W. Owttrim) and horseradish peroxidase conjugates. Polypeptide size was determined by comparison with Amersham Rainbow mol wt markers run on each gel.

Native Gel ADH Activity Staining

Ten micrograms of total soluble protein was separated by native gel electrophoresis as described (Sachs et al., 1980). ADH activity staining was performed according to Constabel et al. (1990). Pyrazole (0.1 M) acted as a specific ADH inhibitor of all of the isozymes detected. Densitometric scanning showed that the intensity of the bands was in a linear range from 5 to 30 μg of total soluble protein.

In Situ ADH Activity Staining

Rhizomes were surface sterilized in 2% sodium hypochlorite for 2 min and rinsed thoroughly in tap water for 5 min. Rhizome slices of 100 μm were cut with a vibratome (Series 1000, Sectioning System, TPI, St. Louis, MO) and incubated in 0.1 M Tris-HCl, pH 8.7, 80 μL/mL ethanol (95%) for 5 min at room temperature. The staining was carried out in staining solution as described for native gel activity staining for 5 min at room temperature. The staining reaction was stopped in 100% methanol.

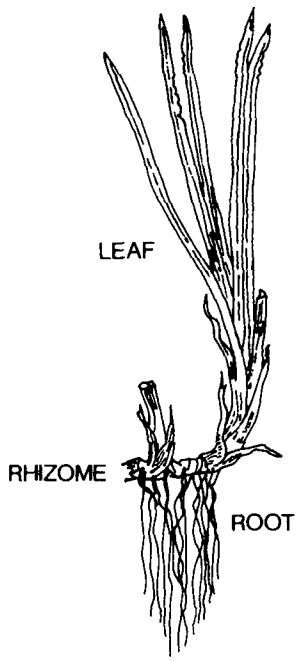

Figure 1. *A. calamus* L. The figure has been adapted from Bursche (1980) and is 1/15 natural size. The rhizome is positioned at the interface between soil and water, and the adventitious roots remain in the soil. New leaves, formed in autumn, remain completely submerged during winter and emerge in early spring.

(Davies, 1980; Hochachka and Mommsen, 1983; Roberts et al., 1984). The AEC (the fraction of the total adenylate pool that is enriched in high-energy phosphate bonds) correlates with the level of residual metabolic activity during limited ATP regeneration (Saglio et al., 1980). In *A. calamus*, AEC values remain high during 48 h of anoxia, in contrast to a dramatic drop observed in potato tubers (Sieber and Brändle, 1991).

During anoxia, the synthesis of various stress proteins is rapidly induced in plants. These so-called anaerobic polypeptides, which have been studied intensively in maize, are involved in fermentation or Glc-P metabolism. They include ALD (Kelley and Freeling, 1984; Kelley and Tolan, 1986), PDC (Wignarajah and Greenway, 1976; Laszlo and St. Lawrence, 1983; Kelley, 1989), and ADH (Sachs and Freeling, 1978; Ferl et al., 1979; Dennis et al., 1984, 1985).

We are interested in *A. calamus* as an example of a long-term anoxia-tolerant plant. In this paper, we report on the molecular-biological characterization of the anaerobic response in *A. calamus*. We have selected three genes that are known from maize and other well-studied systems to code for key components of the anaerobic response and describe their expression under both short-term and long-term oxygen deprivation.

MATERIALS AND METHODS

Plant Material

Acorus calamus L. plants (Fig. 1) were harvested from the Moossee, a lake in the vicinity of Berne, transferred to the greenhouse of the Botanical Institute, and kept partially flooded. *A. calamus* is a nonfertile triploid plant that reproduces vegetatively (Wulff, 1940; Dykyjova, 1980). Therefore, the plant material can be considered genetically homogenous. For incubation, the young rhizome was separated from the old part at a length of 5 to 7 cm and the terminal young leaves were cut at 5 to 7 cm from the leaf base still attached to the rhizome; old leaves were removed and adventitious roots on the young rhizome were trimmed to a length of about 3 cm. Plant material was washed thoroughly with water, surface sterilized in 2% sodium hypochlorite for 5 min, and rinsed well with tap water for 10 min. For all incubations, the plants were placed in groups of three into plastic beakers in 100 mL of sterile water with 50 mg/L of chloramphenicol, without flooding the rhizome to facilitate gaseous exchange, and were incubated in a humidified atmosphere.

Anaerobic Incubation

For anoxic incubation, we used an anaerobic workbench (Anaerobic System, Forma Scientific, Marietta, OH) with N_2 containing 5% CO_2 and 10% H_2 where any traces of O_2 were removed by palladium catalysts. Anoxic conditions were tested with methylene blue anaerobic indicator strips. For 2% and 20% O_2 treatment, we flushed chambers with a humidified gas mixture of N_2 containing 5% CO_2 and either 2 or 20% O_2 at an RH of 80%. Short-term treatment was at 25°C for 24 h in the dark to avoid photosynthetic O_2 production. Long-term treatment was at 25°C in the anaerobic workbench in complete darkness for periods indicated in the figures. Under these conditions, internal O_2 is rapidly depleted from the tissue (Steinmann and Brändle, 1984; Studer and Brändle, 1984). Control plants were flushed with air in the dark. Viability tests of anoxically treated *A. calamus* plants included reexposure in air until shoot and root growth had been established and in vivo staining of rhizome slices by the nitroblue tetrazolium method (Constabel et al., 1990).

RNA Extraction

After anaerobic treatment, the plant material was rinsed briefly in ice water. The organs were frozen in liquid nitrogen and either stored at −80°C or used directly for RNA extraction. Approximately 1 g fresh weight of frozen tissue was homogenized in a Teflon vessel containing a tungsten carbide bowl by vigorous mechanical shaking for 60 s using a microdismembrator (Bender and Hobein, Zürich, Switzerland). RNA was extracted with hot phenol according to Verwoerd et al. (1989). Yields were 464 ± 205, 348 ± 67, and 101 ± 43 μg of total RNA per g of leaf, rhizome, and root tissue, respectively.

cDNA Cloning

Poly(A)$^+$ RNA from *A. calamus* leaves treated anoxically for 24 h was isolated by chromatography of total RNA on oligo(dT)-cellulose (Maniatis et al., 1982). Double-stranded cDNAs were prepared with a cDNA synthesis kit (Pharmacia LKB) according to the manufacturer's instructions. Following

RESULTS

Isolation of *A. calamus* cDNAs Coding for Anaerobic Polypeptides

We chose the genes for *Ald* as a general glycolysis marker and *Pdc* and *Adh* as markers for ethanolic fermentation. We initially had difficulties in grinding the hard rhizome and root tissue with mortar and pestle. Finally, the application of a micro-dismembrator in combination with the hot phenol extraction method of Verwoerd et al. (1989) (see "Materials and Methods") gave satisfactory and approximately equal yields of leaf and rhizome total RNA on a gram fresh weight basis. Yields in roots were generally lower. A cDNA library was generated from poly(A)$^+$ RNA isolated from *A. calamus* leaf tissue kept in anoxia for 24 h. The library consisted of 2 $\times$ 10^5 independent clones with an average insert size of 500 bp. Heterologous screening with maize *Ald* and *Adh* cDNA clones resulted in the isolation of two *Ald* clones and one *Adh* clone. Four *Pdc* clones were obtained using the polymerase chain reaction. Sequence identities are described in "Materials and Methods."

Short-Term Anoxia Response

To investigate how *A. calamus* responds to short-term oxygen limitation at the level of gene expression, we analyzed the mRNA transcript levels of *Ald*, *Adh*, and *Pdc* by northern blots using equal amounts of total RNA extracted from leaf, rhizome, and root. Whereas in many experimental systems anaerobic conditions are created by submergence and gassing of the solution with nitrogen or argon to deplete molecular oxygen, we have carried out the incubations by exposing nonimmersed entire plants to defined gas mixtures. Incubation in the so-called anaerobic workbench included degassing of the organs in a vacuum chamber for a short time prior to anoxic incubation. This ensures that acclimation processes to anoxia (Hole et al., 1992) remain limited, since the desired oxygen concentration is quickly established in the tissue.

All three organs responded to hypoxic (2% O_2) and anoxic (0% O_2) conditions during incubation in the dark for 24 h by increasing the transcript levels of the three genes (Fig. 2). In leaves and rhizomes, transcript levels were higher at 0% than at 2%. In roots, however, the results were variable. In the experiment shown here, *Adh* mRNA levels did not increase, whereas in a replicate experiment there was a 3.5-fold increase (data not shown).

In Figure 2, normoxically treated plants, which were prepared exactly as the anoxically incubated plants, did not show elevated RNA levels of the three genes described. Thus, we are convinced that we can exclude wounding stress as a cause of induction, and that we are indeed studying anoxia response in our system. Moreover, it has been reported that during hypoxia, wounding-inducible mRNAs do not accumulate, whereas they do under aerobic conditions (Butler et al., 1990). Therefore, we think that it is unlikely that wounding and anoxia act synergistically on gene expression.

Aldolase is an enzyme of the main stem of the glycolytic pathway, and it appears surprising that *Ald* mRNA is hardly detectable under normoxic conditions. Hake et al. (1985) also reported on very low aerobic levels of *Ald* mRNA in maize

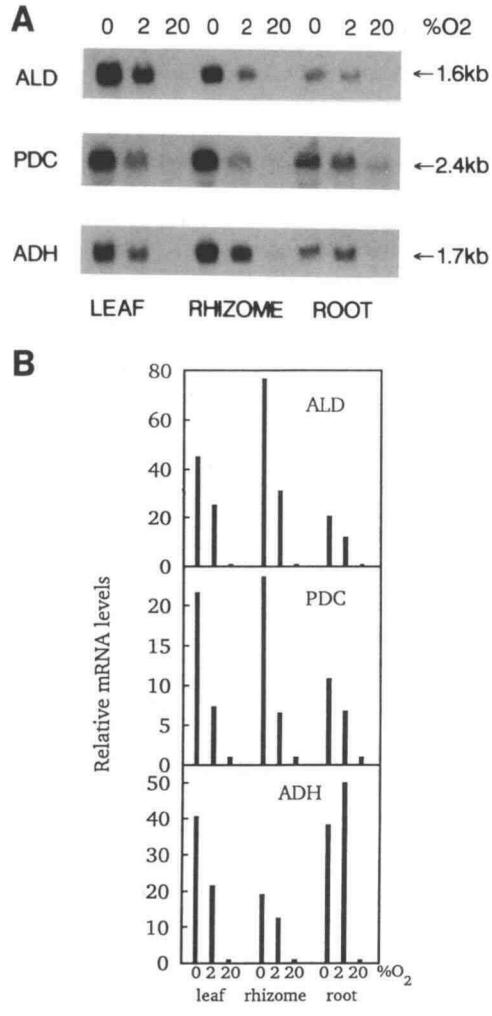

Figure 2. *Ald*, *Pdc*, and *Adh* transcript levels during oxygen deprivation. A, *Ald*, *Pdc*, and *Adh* transcript levels in *A. calamus* were detected by northern blot analysis (10 μg of total RNA per lane). Plants were incubated for 24 h in the dark at 0, 2, or 20% oxygen as indicated at the top of the figure. The RNA on the membrane was hybridized with ^{32}P-labeled DNA probe from pACAld and riboprobe from pACPdc and pACAdh. B, Quantitation of the northern blot analysis was done by scanning autoradiograms with a Digitana model CD 60 densitometer. The mRNA levels for each of the three genes from normoxically grown plants at 20% O_2 were arbitrarily set at 1.0.

primary roots, which were visible only after overexposure of the blots. Thus, under the standard experimental conditions of short-term O_2 deprivation, the expression of all three genes was strongly enhanced in each *A. calamus* organ.

Long-Term Anoxia Response

In most published work, the time of anoxic incubation was limited and rarely exceeded 72 h, except for studies of rice and barnyard grass seedlings (*Echinochloa*), where anoxic incubation was for up to 4 to 10 d, respectively (Cobb and Kennedy, 1987; Kennedy et al., 1990). We investigated gene expression in *A. calamus* leaves and rhizomes exposed to

anoxia for periods ranging from 2 h to 2 months. Anaerobic treatment had little visible effect on *A. calamus* leaves and rhizomes. The leaves grew slightly during the first few days and, although kept in the dark, the green parts of the plant were not etiolated after 2 months of anoxia. In contrast to leaves and rhizomes, roots did suffer under long-term anoxia and were visibly damaged after 96 h of anoxia. *A. calamus* resumed growth of leaves and rhizomes immediately after transfer back to the air; in addition, new leaves and new roots formed.

Figure 3 shows the relative transcript levels of *Ald*, *Pdc*, and *Adh* over 2 months of anoxia both as northern blots (Fig. 3A) and as densitometric scans (Fig. 3B). The time course of induction was different for each of the individual mRNAs, i.e. the three genes reached maximum levels at different time points after the onset of anoxia. *Pdc*, *Adh*, and *Ald* transcript levels peaked at 6, 12, and 72 h, respectively, based on scanning densitometry (Fig. 3B). Transcript levels decreased gradually after this point, although they were still detectable after 2 months. For each of the three genes, the time course of induction was comparable in leaves and in the rhizomes. Thus, the three genes display different induction kinetics, but the anoxia response of each gene is similar in rhizomes and leaves.

Comparison of ADH Protein and Transcript Levels during Long-Term Anoxia

The steep decline in ADH mRNA levels during anoxia was puzzling, since the plants did not appear stressed, even after 2 months of anoxia. Therefore, we investigated whether the ADH protein levels in *A. calamus* reflected the mRNA profiles during long-term anoxia. By western blot analysis, we detected two approximately 46-kD immunologically cross-reactive bands in *A. calamus* leaf and rhizome using a barley anti-ADH antibody (Fig. 4A). It is interesting that the protein gradually accumulated during at least 7 d of anoxia and then remained constant. Considerable amounts of ADH protein could be observed under normoxia and the relative increase during anoxia was only 3- to 5-fold. This was in contrast to the much stronger increase in ADH transcript levels within 12 h and their subsequent decline (Fig. 3), and indicated marked differences between *Adh* transcript levels and the corresponding levels of ADH protein. Leaves and rhizomes appeared to respond to oxygen deprivation in a similar fashion with respect to ADH protein levels.

To demonstrate that the rise in ADH protein levels is specific, we also investigated the levels of two "housekeeping" proteins, eIF-4A (Fig. 4B) (NeIF-4A in Owttrim et al., 1991) and eIF-5A (NeIF-5A in Chamot and Kuhlemeier, 1992) (data not shown) via western analysis using antibodies against the tobacco proteins. The levels of these two proteins remained constant throughout the experiment and, therefore, we assume that the translational machinery remains intact during anoxic treatment in *A. calamus*. This was supported by the immediate resumption of plant growth during postanoxic incubation in the air.

ADH Isozyme Pattern under Anoxia

The high levels of ADH protein under normoxia (Fig. 4A) and the moderate increases in ADH protein levels upon

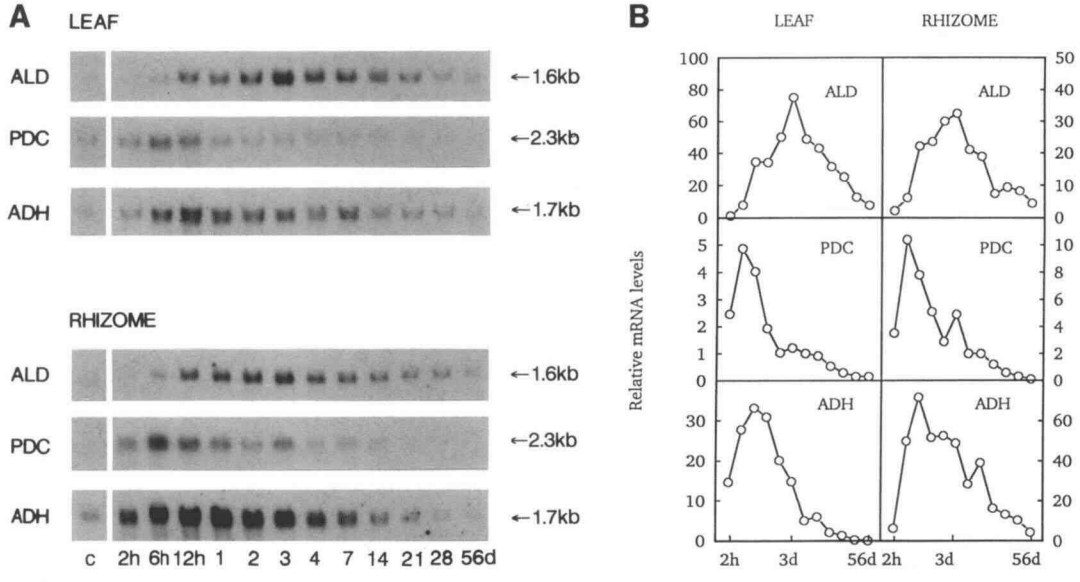

Figure 3. Divergent profiles of mRNA accumulation during 2 months of anoxia in *A. calamus*. A, Transcript levels of *Ald*, *Pdc*, and *Adh* in leaf and rhizome tissue were detected by northern blot analysis (10 μg of total RNA per lane). Plants were incubated in the anaerobic workbench in the dark for the indicated periods. The RNA on the membrane was hybridized with ^{32}P-labeled DNA probe from pACAld and antisense RNA probe from pACPdc and pACAdh. Control (C) plants were incubated in air for 28 d. B, Quantitation of the northern blot analysis was done by scanning autoradiograms with a Digitana model CD 60 densitometer. The mRNA levels for each of the three genes from air-grown control plants were arbitrarily set at 1.0.

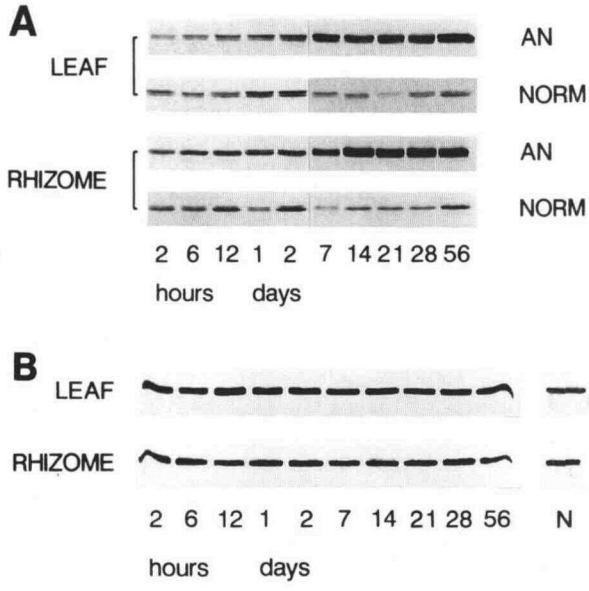

Figure 4. ADH protein accumulates during 2 months of anoxia in *A. calamus*. A, Total protein (10 μg) of anoxically (AN) or normoxically (NORM) treated leaf and rhizome tissue was separated by SDS-PAGE. ADH protein levels were detected with western blot analysis using an anti-barley ADH antibody. Incubation times were as indicated at the bottom of the figure. B, Total protein (10 μg) of anoxically treated leaf and rhizome tissue was separated by SDS-PAGE. eIF-4A protein levels were detected with western blot analysis using an anti-tobacco eIF-4A antibody. Incubation times were as indicated at the bottom of the figure. Proteins from air-grown plants served as a control (N).

anoxic treatment led us to ask whether the specific synthesis of a distinct set of isozymes could contribute to the ADH accumulation during anoxia. Analysis of ADH isozymes by ADH activity staining in native polyacrylamide gels (Fig. 5) revealed at least seven ADH isozymes present at different levels in normoxic *A. calamus* (three strong bands with intermediate electrophoretic mobility and two weak bands with higher and two weak bands with lower mobility). The four weak bands were only faintly visible in the original gel. Incubating the gel in the presence of pyrazole, a specific ADH inhibitor (Constabel et al., 1990), inhibited band formation in the assay (data not shown). Titration experiments indicated that band intensity was linear with protein concentration in the range used and, thus, the assay is at least semiquantitative (see also Heeb and Gabriel, 1984, for a discussion).

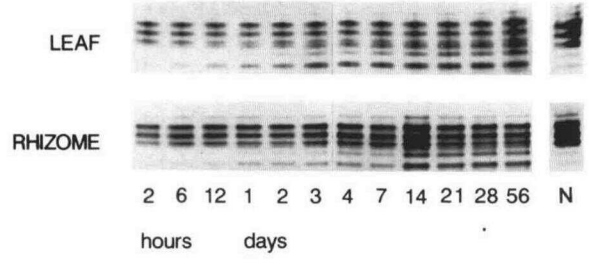

Figure 5. Two groups of ADH isozymes are detected in *A. calamus* upon long-term anoxia by activity staining. Total protein (10 μg) from *A. calamus* leaf and rhizome tissue was separated by native PAGE. ADH isozymes were detected by ADH activity staining (see "Materials and Methods"). Incubation times were as indicated at the bottom of the figure. Proteins from air-grown plants served as a control (N). The seemingly higher activity of rhizome ADH after 14 d of anoxia resulted from 75% excess of total protein accidentally loaded on the gel in this lane only.

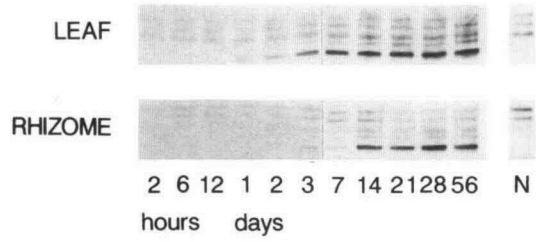

Figure 6. Western analysis shows accumulation of distinct ADH isozymes in *A. calamus* during 2 months of complete anoxia. Total protein (10 μg) from *A. calamus* leaf and rhizome tissue was separated by native PAGE. ADH isozymes were detected by western analysis using an anti-barley ADH antibody. Incubation times were as indicated at the bottom of the figure. Proteins from air-grown plants served as a control (N).

The isozymes detected by activity staining of *A. calamus* ADH during long-term anoxia fell into two groups (Fig. 5). At least five bands (including the two very weak top bands) did not increase upon anoxic incubation. The two ADH bands with the highest electrophoretic mobility, however, were clearly enhanced after onset of anoxia and reached a plateau after 1 to 2 weeks of treatment. There were no organ-specific ADH isozymes detectable in *A. calamus*.

Comparison of ADH Isozyme Activity and Protein Level

The activity staining experiments of Figure 5 indicated the presence of two enhanced ADH isozymes. To confirm that the anaerobically enhanced ADH activity was paralleled by an increase at the protein level, we performed western blotting on the native gels. The barley anti-ADH antibody cross-reacted with all but the faintest protein bands detectable by ADH activity staining (Fig. 6). The western blot closely resembled the pattern obtained by activity staining, except for one quantitative discrepancy. It appears that the isoform with the highest electrophoretic mobility gives a stronger signal in the western blot than in the activity gel in comparison with the other forms (compare Figs. 5 and 6). This will be discussed below.

In situ experiments showed that under both normoxic and anoxic conditions, all cells stained positive for ADH activity (data not shown). Thus, we were unable to demonstrate cell-specific differences in ADH expression.

DISCUSSION

A remarkable feature of the amphibious perennial plant *A. calamus* is its ability to survive extremely long periods of anoxia. We hope that this system will cast new light on the regulation of gene expression in higher plants, which is our

longstanding interest (for review, see Kuhlemeier et al., 1987; Kuhlemeier, 1992).

Expression of the three genes *Pdc*, *Adh*, and *Ald* is not coordinately induced. *Pdc* reacts first and its mRNA levels reach a maximum early in the anaerobic response, whereas *Adh* and *Ald* mRNA levels peak later. This may suggest a primary function for PDC in the onset of the anaerobic response.

In the case of ADH, we were able to compare the mRNA levels with the levels of protein and enzymic activity. The results suggest that *Adh* transcription ceases after 12 h, leading to a gradual decline in mRNA levels. In contrast, ADH protein levels increase gradually for at least 1 week, suggesting that even after the arrest of transcription, translation continues for at least 1 week. This view is supported by the presence of constant levels of eIF-4A protein and by results in other laboratories that strongly indicate that posttranscriptional regulation is involved in the anaerobic response in maize (Sachs et al., 1980; Bailey-Serres and Freeling, 1990; Andrews et al., 1993). Cessation of biosynthetic activities within the first 1 or 2 weeks could improve the energy status of the tissue and enable the plant to keep essential parts of its metabolism on a maintenance level during long-term anoxia.

Our results suggest a third level of regulation of *Adh* gene expression, namely at the level of enzymic activity. Although ADH protein is present at normoxia (Figs. 5 and 6) and thus could allow *A. calamus* to react quickly to flooding, ethanol production is not detected in vivo during normoxia (Studer and Brändle, 1987). Several explanations for this discrepancy are possible. The first is that pyruvate is preferentially entering the tricarboxylic acid cycle via pyruvate dehydrogenase and thus is not available for ethanolic fermentation. Second, PDC activity may be limiting due to unfavorable cytoplasmic pH and thus acetaldehyde, the substrate for ADH, would not be produced. Third, the presence of an ADH inactivator that down-regulates ethanolic fermentation during normoxia has been demonstrated in rice seedlings (Shimomura and Beevers, 1983). Such an inactivator may also operate in *A. calamus*.

Comparison of Figures 5 and 6 indicates that the enhanced form(s) of ADH are more prominent in the western blot than in the activity staining. One explanation for this discrepancy could be that the anti-barley ADH antibody cross-reacts more strongly with the enhanced than with the constitutive forms. If this is true, it would mean that the constitutive ADHs in *A. calamus* are less related to the well-characterized ADHs described so far in the literature. It could also lead to the hypothesis that our *Adh* cDNA hybridizes with the products of inducible *Adh* genes, and that constitutively expressed genes have escaped detection in the northern experiments.

A precedent for this is provided by the maize genes coding for the cytosolic glyceraldehyde-3-P dehydrogenase, *Gpc2* and *Gpc3*, which are differentially regulated during anoxia (Martinez et al., 1989; Russell and Sachs, 1989). A physiological function for the enhanced ADH isozymes has been suggested by Hanson et al. (1984). In barley aleurone layers, the K_m for NADH of the inducible barley ADH isoforms is lower than that of the constitutive forms, similar to the K_m for NADH of lactate dehydrogenase. The authors suggested that an additional response to anoxia was competition by the inducible ADH isozymes with lactate dehydrogenase for the cofactor NADH. This may also be the case for the enhanced *A. calamus* ADH isoforms, which thus could contribute to the control of cellular pH and redox state during long-term anoxia. The constitutively present ADH isozymes in *A. calamus* may lead to preadaptation and may have an early function in the anoxia response.

Our results demonstrate that *A. calamus* has developed a multi-level regulation of glycolytic gene expression at the levels of transcription, translation, and posttranslation to contend with fluctuations in oxygen availability. In future experiments, we intend to characterize further the molecular basis of these responses. In addition, we have initiated a molecular-genetic characterization of the anoxia response of *A. calamus* in its natural habitat.

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