Plant and Soil **178:** 75–82, 1996.
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Glycolytic gene expression in amphibious *Acorus calamus* L. under natural conditions

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Received 3 February 1995. Accepted in revised form 4 August 1995

Key words: cold acclimation, ecophysiology, fermentation, flooding, gene expression

Abstract

Acorus calamus L is an amphibious plant, which is exposed to periods of flooding and consequently hypoxic conditions as a part of its natural life cycle. Previous experiments under laboratory conditions have shown that the plant can survive for two months in the complete absence of oxygen, and that during this period the expression of genes encoding the glycolytic enzymes fructose-1,6-bisphosphate aldolase (ALD), pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) is induced in leaves and rhizomes (Bucher and Kuhlemeier, 1993). Here we studied the expression of ALD and ADH through two years in the natural habitat of *A. calamus*. Under natural conditions roots and rhizomes were always submerged but newly grown leaves emerged in spring; in autumn the leaves senesced and the whole plant was submerged again. High *Ald* and *Adh* mRNA levels in leaf and rhizome were found only in winter when the leaves were entirely submerged. Upon leaf emergence in spring the mRNA levels rapidly declined. Under controlled experimental conditions expression of *Ald* and *Adh* was not induced by low temperature. The combination of laboratory and field experiments supports the hypothesis that oxygen deprivation rather than low temperature is a major regulator of glycolytic gene expression in *A. calamus*. The possible role of other environmental factors is also discussed.

Abbreviations: ADH – alcohol dehydrogenase, *Adh* – gene encoding ADH, ALD – cytoplasmic fructose-1,6-bisphosphate aldolase, *Ald* – gene encoding ALD, PDC – pyruvate decarboxylase, *Pdc* – gene encoding PDC.

Introduction

Flooding limits the gaseous diffusion of oxygen into the plant by several orders of magnitude and results in decreased internal oxygen concentrations (Armstrong et al., 1994; Drew, 1990; Stünzi and Kende, 1989). In most higher plants such oxygen deprivation causes severe damage and even death. Marsh plants, however, are frequently exposed to flooding as a part of their normal life cycle, and therefore must have developed mechanisms to efficiently deal with oxygen deprivation.

The object of this study, *Acorus calamus* L, is a monocotyledonous plant which grows at the shore of the Moossee, a lake in the vicinity of Berne. *A. calamus* lives completely submerged from late fall to early spring and thus experiences hypoxic or even anoxic conditions during a large part of its life cycle (for review see Brändle, 1990). Upon emergence of the leaves in spring, air can be transported via the aerenchyma to the lower parts of the plant (Armstrong et al., 1994). The *A. calamus* rhizome is positioned at the water-soil interphase and as a consequence is totally submerged throughout its lifetime. It is dependent on leaf emergence for sufficient aeration during the growth phase. Being the overwintering organ, the rhizome generates new shoots in late autumn which stay small during winter and grow rapidly in the following spring. Young rhizomes are generated in summer and

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serve as starch storage organs during winter. Starch accumulates in old rhizomes of *A. calamus* from June on, and is mobilized in the next spring (Haldemann and Brändle, 1986).

Low oxygen levels impair respiratory activity and consequently energy production. This leads to major adaptations in the plant. Among them is transcriptional induction of glycolytic and fermentative gene expression (for review see Sachs, 1991). In previous studies we studied glycolytic gene expression in *A. calamus* under defined laboratory conditions (Bucher and Kuhlemeier, 1993). We showed that upon anoxic incubation transcript levels of the three glycolytic genes *Pdc*, *Adh* and *Ald* peaked at different time points. Both in leaves and rhizomes *Pdc* mRNA levels reached a maximum at 6 h, followed by *Adh* and *Ald*, which peaked at 12 and 72 h, respectively. Thus, the three genes displayed different induction kinetics, but the anoxia response of each gene was similar in rhizomes and leaves.

It is obvious that the life cycle of *A. calamus* in its natural habitat is far more complex than the defined laboratory conditions under which we and others have so far studied the regulation of gene expression in response to flooding. Light and temperature are important environmental factors, which vary continuously. Under aerobic conditions light has been shown to upregulate the expression of glycolytic genes, such as glyceraldehyde-3-phosphate dehydrogenase in tobacco and maize (Russell and Sachs, 1989; Shih and Goodman, 1988) or the sucrose synthase gene Ss2 in wheat (Maraña et al., 1990). During hypoxia *A. calamus Ald*, *Pdc* and *Adh* levels were lower in the light than in complete darkness (Bucher, 1992). Low temperature increased *Adh* rRNA levels in maize (Christie et al., 1991) and Ss1 mRNA levels in wheat (Maraña et al., 1990).

In this paper we studied the expression of *Ald* and *Adh* genes in *A. calamus* under natural conditions, where a concerted action of multiple environmental factors may influence gene expression.

Materials and methods

Plant material

A. calamus plants were harvested from the Moossee, a lake in the vicinity of Berne (47° 1.5′ N; 7° 28.4′ E; 521 m above sea level). *A. calamus* is a non-fertile triploid plant that reproduces vegetatively (Dykyjova, 1980; Wulff, 1940). Carolus Clusius imported one rhizome to Vienna in 1574, which is thought to be the ancestor of all *A. calamus* plants at the Moossee (Steck, 1893). The population in the Moossee was introduced in the 1860s by the physician Dr med Rudolf König (von Büren, 1942). Therefore, the documentary evidence suggests that the plant material used in this study is genetically homogeneous.

Three plants were harvested weekly or biweekly from the Moossee around 2:30 p.m. Immediately after harvest 5 to 6 cm of the young rhizome tissue was separated from the older tissue, and the terminal young leaves were cut at a distance of 3 to 4 cm from the leafbase; old leaves were removed and adventitious roots were cut away from the rhizome. Plant material was thoroughly washed in distilled ice-cold water, sliced, frozen in liquid nitrogen, transferred to the laboratory, and stored at −80 °C.

For the controlled environment experiments, *A. calamus* plants were taken from the greenhouse and treated as described (Bucher and Kuhlemeier, 1993; Bucher et al., 1994). Incubation at 4 °C was in a cold room, in the absence of light.

Temperature and oxygen measurements

The oxygen concentration and the water temperature were recorded at the site of plant harvest with an Oximeter OXI 92 and an oxygen electrode EO 92 (WTW, Weilheim, Germany). For this purpose, 500 mL of water from just above the rhizome was collected in a sealable glass bottle. Oxygen concentration and water temperature were recorded immediately after sampling. Values are the mean of two measurements. Single measurements varied less than 1% or 1 °C.

Northern blot analysis

Frozen plant material was homogenized by microdismembration as described previously (Bucher and Kuhlemeier, 1993). RNA was extracted with hot phenol according to Verwoerd et al. (1989). Yields were 464 ± 205 and 348 ± 67 μg of total RNA per g of leaf and rhizome tissue, respectively (n=20). No significant seasonal differences in the total RNA content per gram fresh weight were observed. Therefore, we assume that the amount of rRNA, which is around 98% of the total RNA, remains approximately constant during the season. This is general practice (e.g. Gallagher

and Ellis, 1982; Kuhlemeier et al., 1987), also in the field of flooding research (Dennis et al., 1985).

Total RNA was quantified both spectrophotometrically, and visually by staining of stripped northern blots in 0.02% (w/v) methylene blue dye in 0.3 *M* sodium acetate, pH 5.5. Excess dye was gently washed away with water. The rhizome samples from May 2, 12, 29, Dec 26, 1992 and Jan 29, 1993 yielded degraded RNA, and the results from these samples were omitted from the analysis. Ten μg of total RNA were separated on 1.2% agarose glyoxal gels after glyoxylation (Hull, 1985). Consistency of total RNA loading was tested by staining for ribosomal RNA bands as described above. Northern blotting and hybridization procedures were carried out as described previously (Bucher and Kuhlemeier, 1993). The same blots were used to hybridize with appropriately digested fragments of the plasmids below. pAC*Ald* (encoding *A. calamus* ALD) was randomly labeled. The final wash was at 55 °C. pACAdh (encoding *A. calamus* ADH) was used for antisense RNA synthesis. The last wash was at 65 °C. Despite considerable effort we were unable to obtain satisfactory northern blots with the *Pdc* probe, which we used successfully with RNA isolated from laboratory grown *A. calamus* plants (Bucher and Kuhlemeier, 1993).

Hybridizing probe was visualized and quantitated by autoradiographic and storage phosphor imaging techniques. Screen-enhanced autoradiography was performed by exposing over night to Fuji X-ray film NIF RX at −80 °C using Kyokko high plus amplifying screens. The films were developed in LX24 X-ray film developer (Kodak).

Storage phosphor imaging of blots was performed at room temperature using imaging screens (Bio-Rad) previously exposed for 15 min to a Bio-Rad eraser to erase any residual signal. After exposure the screens were immediately scanned using the Scanner GS-250 Molecular Imager (Bio-Rad) and the signal areas were viewed on a PC monitor and quantitated using image analysis software (Phosphate Analyst Software, Version 1.0, Bio-Rad Laboratories, California, USA). The signals of the RNA samples from 11 June 1992 were arbitrarily set as 1 for the calculation of relative expression levels in leaf and rhizome. At this time point leaves were vigorously growing and were fully aerated.

Results and discussion

Developmental and environmental changes during the year

A. calamus plants were harvested from the Moossee, from 25 January 1991 to 29 January 1993. The life cycle of *A. calamus* is depicted in Figure 1 which shows leaf growth in relation to the water level from May 1992 to January 1993. Leaf growth began at the end of the winter 1992, and in the middle of May 1992 the leaves reached the water surface. After emergence, continuous rapid growth led to leaf lengths of up to 148 cm. In the summer new rhizomes were formed. From July to the end of September leaf length of newly formed leaves gradually declined to values of around 100 cm. During the growth phase from spring until autumn the leaves remained above the water level. From the end of July 1992 the aerial leaves started senescing and they had died by the end of September. By that time the plants had developed short overwintering leaves and were completely submerged. Throughout the year the rhizomes, which lie at the soil-water interface as well as the roots remained flooded. A similar development was also observed in the season of January 1991 to winter 1992. Thus, one main characteristic in the life cycle of *A. calamus* is the occurrence of periods of only partial submergence with flooded rhizomes and roots, but emerged leaves in spring and summer, followed by periods of complete submergence during late autumn and winter.

An important difference between this study and previous laboratory experiments is the fluctuation in water temperature under natural conditions. Figure 2 shows the water temperature around the rhizomes at the time-points of harvest of the plants. In February 1991 a layer of ice and snow up to 12 cm covered the lake and the temperature of the water below sank to 1 °C. From March to the middle of August the temperature gradually increased to 27 °C and later decreased to 17 °C in early October and to 6 °C in late December. In 1992 water temperature fell to 3 °C on February 7 and ice covered the lake from January 9 to February 2. Water temperature reached 28 °C on August 7. During winter 1993 no ice covered the lake and the water temperature was not recorded to fall below 4.9 °C. Thus, periods of leaf emergence coincided with water temperatures between 17 and 27 °C. During complete submergence the water temperature surrounding the rhizome ranged from 1 °C to about 17 °C. Oxygen concentration in the water close to the soil-water interphase varied from 6.5

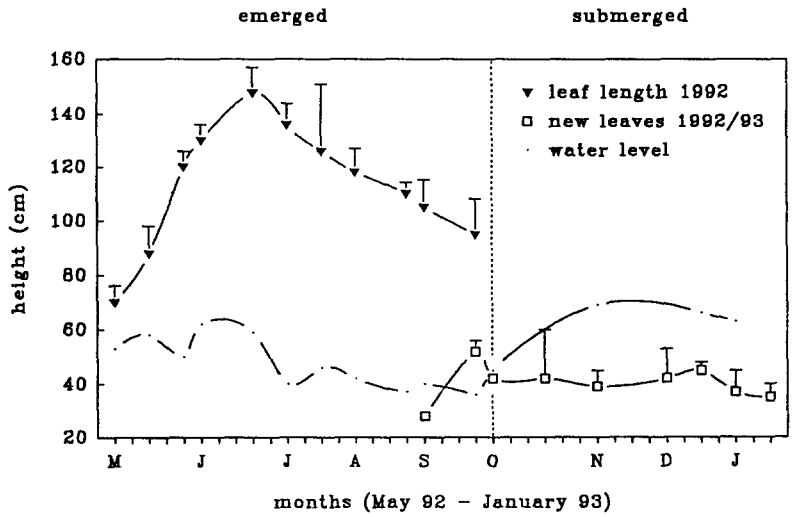

Fig. 1. Length of leaves from May 1992 until January 1993 and water table above the rhizomes of *A. calamus*. Length of leaves from May to September 1992 (diamonds) and length of mainly submerged overwintering leaves from September 1992 to January 1993 (open squares). Dots represent single measurements of height of water. Values, with standard deviation, represent the average of terminal leaf lengths of three individual plants.

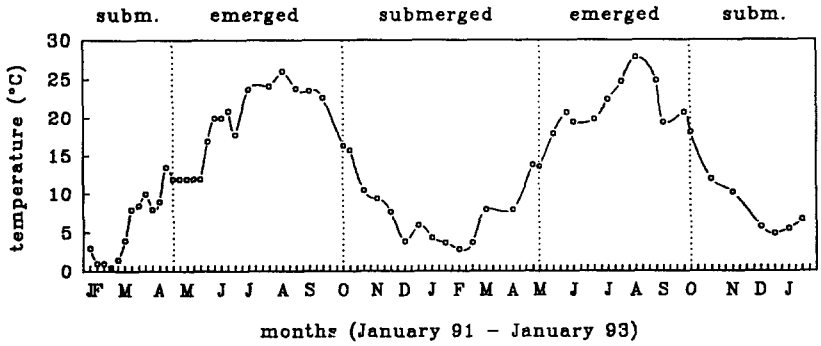

Fig. 2. Temperature fluctuations in the Moossee above the rhizomes in the natural habitat from 25 January 1991 to 29 January 1993. At each day of harvest the water temperature was measured above the rhizomes at 2:30 p.m.

to 17 mg L^{-1} that is generally above 80% saturation. Thus the water surrounding the rhizome was well aerated throughout the year, such that some diffusion of oxygen from the water into the leaves and the rhizome would be possible. The water level recorded over the rhizomes varied from 30 to 69 cm from 1991 to 1993, except for a few times after heavy rainfall in Summer 1991, when water levels temporarily rose to about 80 cm.

Seasonal variations in gene expression

A. calamus plants were collected from the Moossee at weekly or biweekly intervals, and total RNA was isolated. Northern blots were hybridized with probes for *Ald* and *Adh*. Figure 3 shows a representative part of the data obtained by exposing the hybridized blots to X-ray film. It can be seen that good quality mRNA, was present in the samples. A quantitative evaluation of all the data is presented in Figure 4. During the course of two years both *Ald* and *Adh* transcripts accumulated to high levels in winter. They remained high throughout spring until leaf emergence in April 1991 or May 1992, respectively, and then were low during summer. In both October 1991 and 1992, *Ald* and *Adh* transcript levels increased to high levels at the time that the aerial leaves died off. Thus, in both organs high mRNA levels of *Ald* and *Adh* coincided fairly well with periods of complete submergence (Fig. 3 and 4). In previous experiments under controlled conditions we showed that oxygen

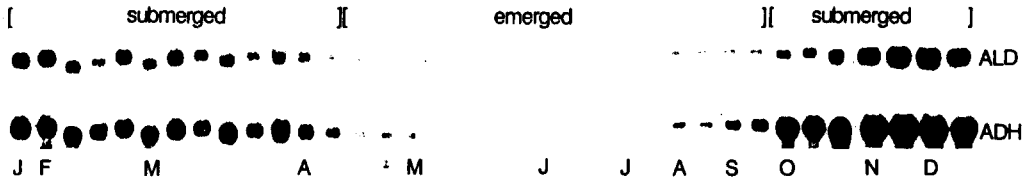

Fig. 3. Profiles of mRNA accumulation during the season in rhizome and terminal leaves of *A. calamus* under natural conditions. Transcript levels of *Ald* and *Adh* in rhizome tissue were detected by northern blot analysis (10 μg of total RNA per lane). The period when the leaves have risen above the water surface is designated (emerged).

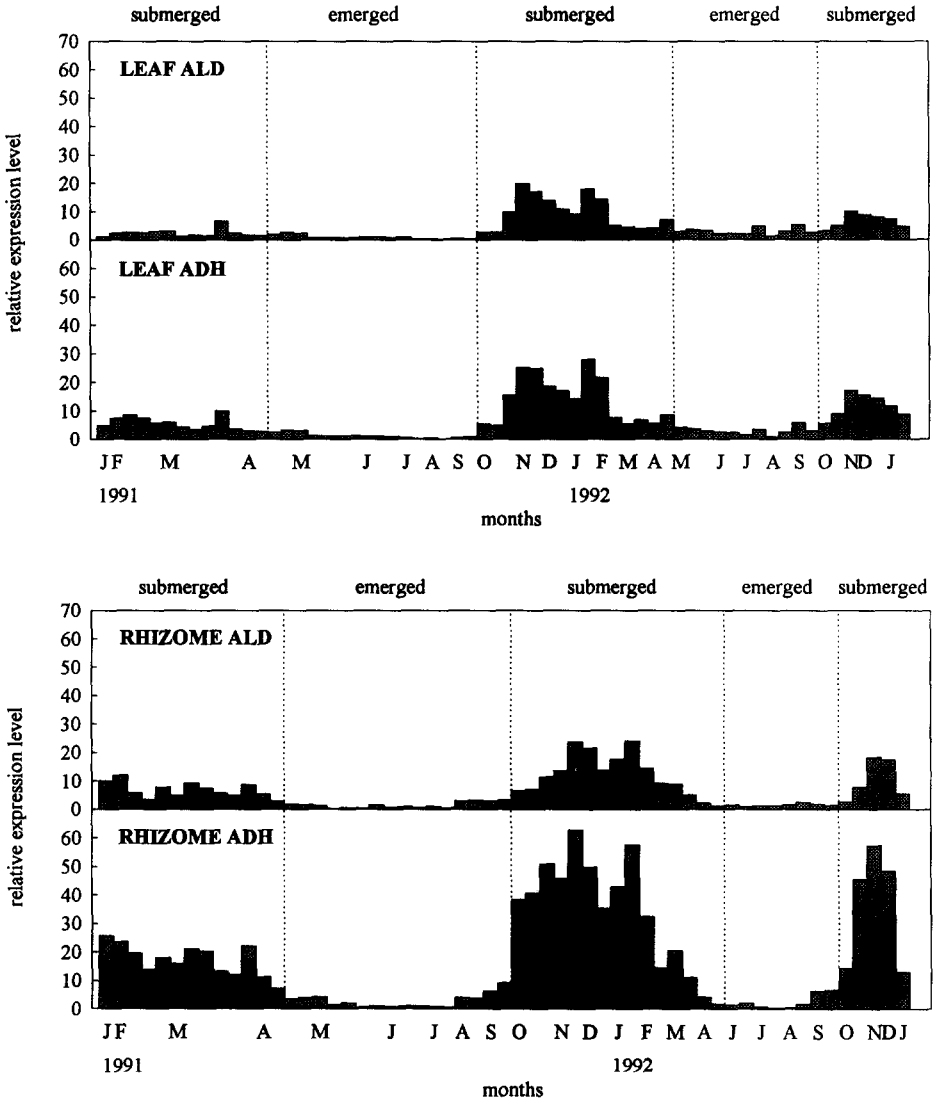

Fig. 4. Profiles of mRNA accumulation in rhizome and terminal leaves of *A. calamus* during two years under natural conditions. Transcript levels of *Ald* and *Adh* in leaf tissue and in rhizome tissue were detected by northern blot analysis (10 μg of total RNA per lane). Five rhizome samples in which the RNA was degraded were omitted from the analysis. The period when the leaves have risen above the water surface is designated (emerged). The mRNA levels from 11 June 1992 on the northern blots were arbitrarily set as 1.

deprivation could trigger a major induction of *Ald* and *Adh* gene expression (Bucher and Kuhlemeier, 1993). Thus the laboratory experiments support the hypothesis that in the field oxygen deprivation induces gene expression as well. It may be noted here that in contrast to the controlled experiments, in the field the rhizomes remained permanently flooded. Thus the fluctuations in gene expression in this organ imply that the rhizome responds to the oxygen status of the leaves.

Alternative environmental cues for Ald *and* Adh *induction*

Although a correlation between high transcript levels and submergence is readily apparent, some discrepancies must be pointed out. First, transcript levels rose initially during submergence, but their decline started before the leaves emerge. For instance, in leaves, transcript levels in March were already down to the June level (Fig. 4). A similar phenomenon was also observed when rhizomes were exposed to long term anoxia under controlled conditions: transcripts rose rapidly during the first hours of anoxia, but then declined gradually, such that after two months of anoxia mRNA levels were almost back to the levels found in the control plants grown under ambient atmosphere (Bucher and Kuhlemeier, 1993). Thus, it appears that both in laboratory and field experiments transcript levels are under complex control, probably involving both transcriptional and post-transcriptional mechanisms.

Second, especially in rhizomes a small but possibly significant rise in transcript levels could be observed in fall before the plants were fully submerged (Fig. 4). This might reflect the onset of leaf senescence and an accompanying breakdown of communication between the leaf and the rhizome. Alternatively, this elevation in transcript levels might be caused by the decrease in temperature during the fall. To some extent high transcript levels coincide with low temperatures, although the correlation is not perfect. For instance, lower temperatures in 1991 are accompanied by lower transcript levels than in 1992 (Figs. 3 and 4). Cold induced gene expression has been observed in many systems (Guy, 1990; Kurkela et al., 1988). In fact there is a single report in the literature demonstrating cold induction of the maize *Adh1* (Christie et al., 1991). In order to obtain some insight into this issue, we incubated *A. calamus* plants in the laboratory at 4 °C and ambient oxygen concentration (Fig. 5). Whereas control plants incubated under anoxia showed strong induction of *Ald*

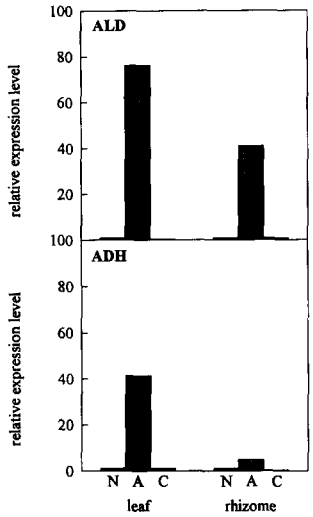

Fig. 5. *Ald* and *Adh* are not induced by cold treatment. *A. calamus* plants were incubated for 24 h at room temperature and normoxic (N), at room temperature in an anaerobic workbench (A), and under normoxia at 4 °C (C). Ten μg total RNA was isolated, and analyzed by northern blotting. The mRNA levels from the normoxic, room temperature samples were arbitrarily set to 1. The data represent the average of two experiments.

and *Adh* expression, both in leaves and rhizomes, cold treatment under normoxic conditions did not enhance expression. Obviously, it cannot be ruled out that under modified conditions an effect of low temperature might become apparent. However, these results are a first indication that temperature is not a major regulator of *Ald* and *Adh* gene expression under natural conditions, as well. It certainly remains possible that cold acts in concert with other environmental factors, such as flooding or shading.

We have not systematically measured the effects of light intensity and day length on *Ald* and *Adh* expression. Laboratory experiments showed that during hypoxia, mRNA levels for *Ald*, *Pdc* and *Adh* were higher in complete darkness than during light incubation (Bucher, 1992). Especially when ice covered the lake, light intensity may have been very low, and it is conceivable that near darkness further enhanced gene expression in winter.

Although the rhizome is permanently flooded throughout the year, *Ald* and *Adh* mRNA levels fluctuate in a pattern similar to that demonstrated in leaves. Additionally, the quantitation of the results revealed that changes in relative expression levels of both genes were similar in each organ (rhizome or leaf). This suggests that *Ald* and *Adh* gene regulation is similar in the two organs and strongly indicates that the rhizome

is dependent on leaf emergence for sufficient aeration and respiratory activity. This is supported by studies on internal oxygen concentrations in rhizomes of *A. calamus* (Studer and Brändle, 1984), where it was shown that the oxygen concentration is mainly determined by air ventilation through the leaf aerenchyma and not by oxygen diffusion from the water into the rhizome.

Haldemann and Brändle (1986) measured a peak of ADH activity and ethanol accumulation in the rhizome at about the onset of growth, that is before leaf emergence. In contrast, *Ald* and *Adh* mRNA levels peaked in winter, and were declining during the period of vigorous spring growth (Fig. 4). This discrepancy between increasing enzymatic activity and declining transcript levels could mean that ADH protein was present in sufficient amounts at the onset of leaf growth. This is in accordance with our previous laboratory experiments (Bucher and Kuhlemeier, 1993) and suggests strongly that ADH activity is not exclusively transcriptionally regulated, but that post-transcriptional regulation may play an important role in the natural habitat.

In conclusion, our results show that in the natural habitat, rhizomes and leaves of the amphibious plant *Acorus calamus* L. coordinately regulate the expression of genes involved in glycolytic and fermentative energy metabolism. The combination of laboratory and field experiments indicates that oxygen deprivation is a major regulator for induction of gene expression. Up till now plant molecular biologists have mostly limited themselves to studying individual environmental cues under controlled laboratory conditions, and avoided the exceedingly complex responses of plants to their natural environment. Here, we have used the methods of molecular biology to study a problem in ecophysiology.

Acknowledgements

We thank Dr Michel Weber for his cooperation in the field, Therese Mandel for her help with Figure 4 and Dr Andrew Fleming for critical reading of the manuscript. We also wish to thank Daniela Gilgen for her computer work on Figure 4. This work was supported in part by the Swiss National Science Foundation grant No 31-26324.89.

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Section editor: F J de Bruijn