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Mitochondrial Respiration and Hemoglobin Gene Expression in Barley Aleurone Tissue[1]

Xianzhou Nie and Robert D. Hill*

Department of Plant Science, University of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2

Previous studies have shown that plant hemoglobin (Hb) mRNA is expressed in barley (*Hordeum vulgare* L.) aleurone layers during hypoxia. We have examined the effect of a number of respiratory inhibitors on barley aleurone layers to determine the factors that influence Hb gene expression. Respiratory inhibitors that reduce O_2 consumption, such as CO, cyanide, and antimycin A, strongly enhanced Hb mRNA levels. Treatment with the oxidative phosphorylation uncoupler 2,4-dinitrophenol markedly increased O_2 consumption and had a similar positive effect on Hb gene expression. Hb transcript levels were also stimulated by the ATP synthase inhibitor oligomycin. The results suggest that the expression of Hb is not directly influenced by O_2 usage or availability but is influenced by the availability of ATP in the tissue.

Hb exists widely in organisms ranging from prokaryotes to eukaryotes (Appleby et al., 1988; Wittenberg and Wittenberg, 1990). It has been found in a number of plant species (Appleby, 1992), most notably dicots engaged in symbiotic relationships with bacteria. The expression of a plant Hb gene in the seed tissue of a monocot (Taylor et al., 1994) has raised the possibility that the molecule may be more universally present in plants and contribute to plant growth and development in a manner beyond its role in the N_2-fixation process in dicots.

Hb is characterized by its conserved structure, high O_2 affinity, and reversible combination with O_2 in the ferrous state. Its function is normally associated with the facilitation of O_2 diffusion, O_2 storage, and O_2 utilization in organisms. Legume Hb has been well studied in legume symbiosis, where it is believed to act as an O_2 carrier to the symbiosomes of root nodules, supplying O_2 to the bacterial respiratory chain while preserving a low free-O_2 concentration (Appleby, 1992). However, the significance of the presence of Hb in nonnodulating plants has not been resolved. Studies have shown that in nonnodulating dicots Hb occurs mainly in the roots at a concentration of approximately 100 nM, which is probably lower than the concentration of free-dissolved O_2 in the cells (Appleby et al., 1988). This led to the suggestion that Hb in plant roots may serve as an O_2 signal molecule, detecting an O_2 deficit and triggering a shift in the metabolism from an aerobic to an anaerobic pathway.

Hb gene expression is induced in barley (*Hordeum vulgare* L.) aleurone layers as the O_2 concentration in air decreases below 10% (Taylor et al., 1994). Alcohol dehydrogenase (*Adh*) and lactate dehydrogenase (*Ldh*) genes are also induced, but induction becomes apparent at slightly lower O_2 concentrations. In the present study we wished to establish whether Hb gene expression was solely responsive to O_2 or whether other metabolic events within the tissue would trigger its synthesis. We have found that inhibitors that interfere with oxidative phosphorylation induce Hb gene expression and that the availability of ATP may be the determining factor in Hb expression.

MATERIALS AND METHODS

Seeds of barley (*Hordeum vulgare* L. cv Harrington, provided by the Canadian Grain Commission, Winnipeg, Manitoba) were de-embryonated and a small portion of the distal end was cut off. The resulting half-seeds were surface-sterilized for 30 min in 1% (w/v) NaOCl and rinsed thoroughly in distilled water. After 2 d of imbibition at 22°C in darkness, 25 aleurone layers were separated from the starchy endosperm, placed in a sterile 50-mL conical flask containing 1.5 mL of an incubation medium (water or 0.1 M phosphate buffer, pH 7.2), and incubated with slow agitation (65 cycles/min) at room temperature.

Treatment with Respiratory Inhibitors

Unless otherwise stated, freshly prepared barley aleurone layers were incubated in 0.1 M phosphate buffer (pH 7.0) containing various additions. The Cyt *c* oxidase inhibitor KCN was used at a final concentration of 0.8 mM, and the Cyt *c* reductase inhibitor antimycin A (27 mM stock in 2-propanol) was added to give a final concentration of 0.2 mM. Control incubations had equal amounts of 2-propanol without antimycin A. The oxidative phosphorylation uncoupler DNP was used at a concentration ranging from 0 to 1 mM. The phosphorylation inhibitor oligomycin (1 mg/mL stock in 2-propanol) was added to yield a final concentration ranging from 0 to 20 μg/mL. All solutions for this series had an equal amount of 2-propanol.

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* Corresponding author; e-mail rhill@cc.umanitoba.ca; fax 1–204–261–0813.

Abbreviations: DNP, 2,4-dinitrophenol; Hb, hemoglobin.

O_2-Stress Treatment

Isolated barley aleurone layers were incubated under controlled O_2 tensions as described previously (Taylor et al., 1994). For anoxic treatment, each flask was flushed with N_2 for 1.5 min, and the flasks were placed in a 3-L jar, which was in turn purged with N_2 for 1 h and sealed. For treatments longer than 6 h, the jar was reflushed with N_2 for 30 min every 6 h thereafter. In treatments of various O_2 and CO concentrations, aleurone flasks were placed in 1-L jars, which were sealed and purged for 20 min with various O_2/N_2 or $CO/O_2/N_2$ mixtures to vary the O_2 or CO/O_2 partial pressure. The gas mixtures were prepared using a gas mixing system (MKS-232, MKS Instruments, Andover, MA). The jars were re-purged with the same gas mixture for 20 min every 4 h. Samples were harvested at appropriate times.

Northern Blotting

Total RNA was isolated from 50 aleurone layers as described previously (Taylor et al., 1994). Yield was determined by UV spectrophotometric absorbance at a wavelength of 260 nm. Ten micrograms of total RNA was denatured in formaldehyde and formamide, which was electrophoresed on a 1.25% agarose gel containing 2.2 M formaldehyde and a trace amount of ethidium bromide. The RNA was blot-transferred to membranes (Hybond N^+, Amersham) in 20× SSC for 10 to 15 h. Blots were air-dried for 1 h before UV cross-linking on a transilluminator for 3 min. Probes (cDNA inserts isolated from the vectors) were labeled with [α-^{32}P]dCTP (Sambrook et al., 1989). The Hb probe was barley Hb cDNA (Taylor et al., 1994), the *Adh* 1 probe was a gift from A. Good (University of Alberta), and the *Ldh* probe was a gift from A.D. Hanson (University of Florida). An rDNA probe was used for normalization of RNA loading. Prehybridization and hybridization were carried out for 2 and 14 to 16 h, respectively, at 65°C in a hybridization buffer containing 6× SSC, 5× Denhardt's solution, 1% SDS, and 100 μg/mL salmon-sperm DNA. The membranes were washed at 65°C for 30 min in 2× SSC and 0.5% SDS, for 20 min in 2× SSC and 0.1% SDS, and for 5 to 10 min in 0.2× SSC and 0.1% SDS at 65°C. The membranes were exposed to film (XAR-5, Kodak) at −75°C. After exposure, the developed films were scanned with an image system (Imagex, developed by L. Lamari, University of Manitoba, Canada).

O_2 Uptake Measurement

O_2 uptake was measured polarographically with an O_2 electrode (Rank Brothers, Cambridge, UK) for 5 min. The typical incubation medium contained 1.5 mL of 0.1 M phosphate buffer (pH 7.0), seven aleurone layers, and the various chemicals tested.

RESULTS

Effects of CO on Hb, *Adh*, and *Ldh* Gene Expression

The effects of CO on the transcript levels of Hb, as well as on *Adh* and *Ldh*, two key enzymes involved in anaerobic metabolism, are shown in Figure 1. All three genes experienced significant increases in expression under hypoxia (1% O_2). Hb transcripts increased 24-fold under hypoxia, whereas treatment of the tissue with CO under normoxic (20% O_2) conditions gave about an 18-fold stimulation of the Hb transcripts. CO exposure under hypoxic conditions resulted in a 40-fold increase in the Hb mRNA. Hypoxia increased *Adh* and *Ldh* gene expression 35- and 78-fold, respectively, relative to normal atmospheric conditions. The *Adh* and *Ldh* transcript levels were less affected than Hb by CO under normal atmospheric conditions. The effects of CO at 20% O_2 on the *Adh* and *Ldh* gene expression were similar to its effect on Hb expression, increasing 11- and 15-fold after treatment. CO treatment under hypoxic conditions gave a 36-fold increase in *Adh* and a 71-fold increase in *Ldh* mRNA relative to a normoxic control, which was not significantly different from hypoxic treatment alone.

Effects of Cyanide and Antimycin A on Respiration and Hb Gene Expression

CO has the potential to bind to either or both Hb and Cyt *c* oxidase. It is not possible to predict the relative binding affinity of CO for the two proteins without a measure of the relative affinity of barley Hb for O_2 and CO. As a consequence, it becomes difficult to interpret the effect of CO on Hb gene expression. To determine whether interference with mitochondrial electron transport affected Hb gene expression, aleurone layers were incubated in the presence of either KCN or antimycin A under both aerobic and anaerobic conditions (Fig. 2). KCN (0.8 mM) dramatically increased the level of the Hb transcript under normal at-

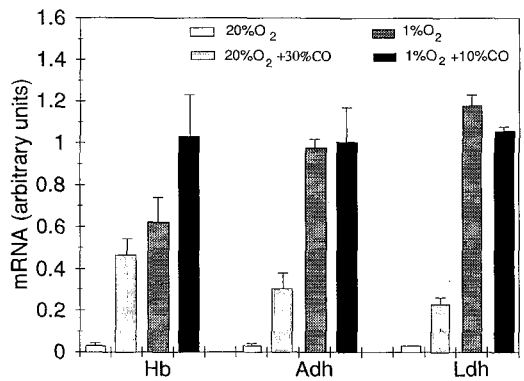

Figure 1. Effects of CO on Hb, *Ldh*, and *Adh* transcription. Freshly prepared barley aleurone layers were incubated in 1.5 mL of the incubation medium containing 10 μg/mL chloramphenicol and 10 mM $CaCl_2$ and then treated with various gas mixtures for 24 h as described in "Materials and Methods." Northern analysis was performed by probing the membrane with the Hb probe first, and then the membrane was stripped of probe by boiling in 0.1% SDS for 5 min. The membrane was reprobed with *Ldh*, then with *Adh*, and finally with the rDNA probe. Signals from each membrane were quantified by scanning. mRNA signals were standardized to their corresponding 26S rRNA signals. Data represent the means and SE values of two replicates per treatment, with each assay performed in duplicate. The data shown in Figures 2 to 5 were obtained similarly.

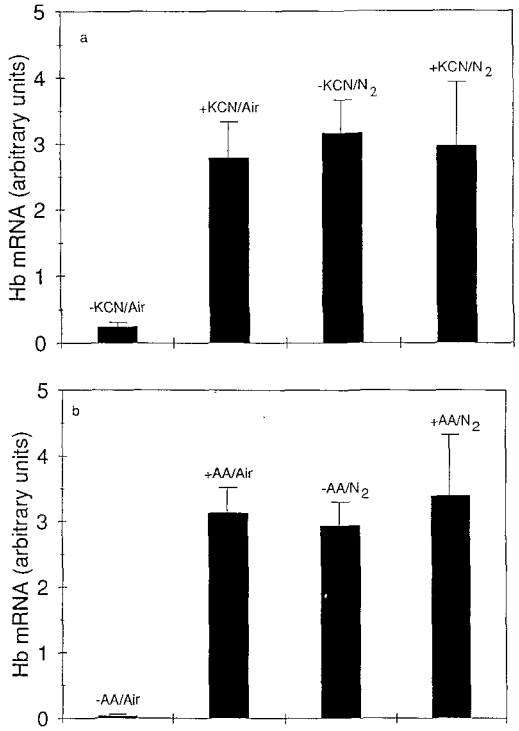

Figure 2. Effects of KCN and antimycin A on Hb transcription. a, KCN effect. Barley aleurone layers were preincubated in different solutions for 1 h. The layers were then set under N_2 or air for 6 h in the same solutions. Northern analysis was carried out. The blot was probed with the Hb probe first, stripped of probe, and reprobed with the rDNA probe. Signals from two separate experiments of two blots each were quantified and standardized to their corresponding 26S rRNA signals. b, Antimycin A (AA) effect. The treatment and quantification procedures were the same as described for a.

mospheric conditions (air atmosphere). These treatments were as effective as anoxia (Fig. 2a) in stimulating gene expression. However, KCN had no additional inhibitory or stimulatory effect on anoxia-induced Hb transcript accumulation. Respiratory studies showed that KCN (0.8 mM) inhibited 25% of the total O_2 uptake whether the respiration was measured 15 min or 6 h after the KCN treatment (Table I).

Antimycin A specifically interferes with the mitochondrial electron transport chain between Cyt *b* and Cyt *c* (Douce, 1985). Respiratory studies demonstrated that antimycin A inhibited 40% of the O_2 uptake of barley aleurone layers measured 15 min or 6 h after treatment (Table I). Under aerated conditions the level of Hb mRNA increased dramatically in aleurone layers treated with antimycin A (Fig. 2b), and under anoxic conditions (N_2 atmosphere) antimycin A had no further effect on the Hb transcript levels.

Effects of DNP on Respiratory Rate and Hb Gene Expression

Inhibition of mitochondrial electron transport may induce Hb gene expression through several different routes. To gain further insight into how this was occurring, other mitochondrial inhibitors were tested. DNP depletes the chemiosmotic potential developed across mitochondrial membranes during electron transport, resulting in an increased electron transport without concomitant ATP production (Douce, 1985). Respiratory experiments indicated that 0.1 mM DNP stimulated O_2 uptake in barley aleurone layers by approximately 80 and 40%, measured 15 min and 6 h after treatment, respectively (Table I). Northern analysis (Fig. 3) showed that under normal air conditions Hb transcript accumulation did not change appreciably when aleurone layers were treated with 10 μM DNP. However, Hb transcripts increased significantly as the concentration of DNP in the incubation medium was increased from 10 to 100 μM. A concentration of 1 mM DNP gave approximately the same response as 100 μM DNP. Under anaerobic conditions 10 μM DNP induced a greater Hb gene response than with DNP or anoxia alone. Concentrations of 100 μM DNP or higher, however, appeared to be less effective.

Effects of Oligomycin on Hb Gene Expression

Whereas DNP inhibits ATP synthesis by depletion of the chemiosmotic potential, oligomycin inhibits DNP by blocking phosphorylation of ADP, which is driven by the chemiosmotic gradient (Douce, 1985). The O_2 uptake of barley

Table I. *The effects of respiratory inhibitors on barley aleurone tissue respiration*

Seven barley aleurone layers were set for an O_2-consumption measurement at 25°C. The data shown are the means of ± SE of two separate measurements.

Treatment	5 min of Preincubation		6 h of Preincubation	
	nmol min^{-1} per layer	*%*	*nmol min^{-1} per layer*	*%*
Control[a]	3.69 ± 0.11	100	2.95 ± 0.08	100
2-Propanol[b]	3.81 ± 0.09	103.2	2.83 ± 0.06	95.9
Antimycin A[c]	2.33 ± 0.03	63.1	2.09 ± 0.03	70.9
KCN[d]	2.83 ± 0.06	76.7	2.09 ± 0.03	70.9
DNP[e]	6.88 ± 0.20	186.5	4.00 ± 0.12	135.6

[a] Control, 0.1 M phosphate buffer, pH 7.0. [b] 2-Propanol, Same amount of 2-propanol as indicated in the antimycin solution in the 0.1 M phosphate buffer, pH 7.0. [c] Antimycin A, 0.2 mM antimycin A, diluted from a 27 mM stock solution in 2-propanol, in 0.1 M phosphate buffer, pH 7.0. [d] KCN, 0.4 mM in 0.1 M phosphate buffer, pH 7.0. [e] DNP, 0.1 mM in 0.1 M phosphate buffer, pH 7.0.

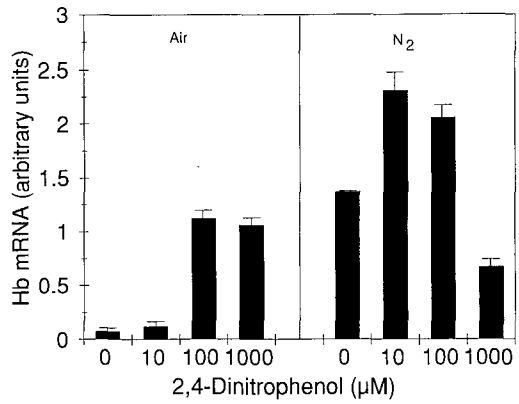

Figure 3. Effects of DNP on Hb transcription. Barley aleurone layers were preincubated in the different DNP concentrations shown for 1 h. The layers were then set under N_2 or air for another 6 h in the same solutions. Northern analysis was performed and the data were analyzed as described in Figure 1.

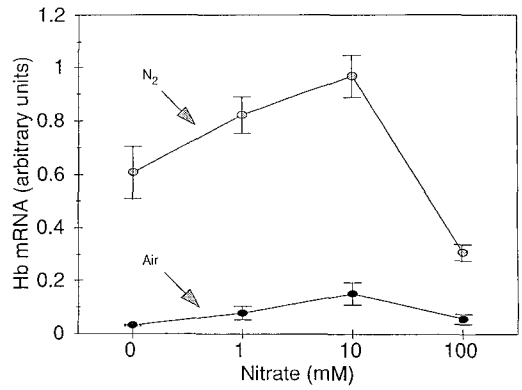

Figure 5. Effects of nitrate on Hb gene expression. Barley aleurone layers were preincubated in 1.5 mL of an incubation medium containing 10 μg/mL chloramphenicol and various concentrations of KNO_3 for 1 h. The layers were then set under N_2 or air for another 24 h in the same solutions. Northern analysis was carried out and the results are presented as indicated in Figure 1.

aleurone layers was not significantly affected by oligomycin in a range from 0 to 20 μg/mL (data not shown). However, northern analysis (Fig. 4) indicated that the Hb transcript levels increased as the concentration of oligomycin increased from 0 to 5 μg/mL under normal atmospheric conditions. At 5 μg/mL the level of the Hb transcripts was approximately the same as that obtained under anaerobic conditions in the absence of oligomycin. There was no further significant increase in the Hb gene expression as the oligomycin concentration was increased to 20 μg/mL.

Effects of Nitrate on Hb Gene Expression

Whereas nitrate has no specific effect on mitochondrial respiration in plants, there is a considerable requirement for reducing equivalents in the conversion of nitrate to ammonia (Kaiser and Huber, 1994). Furthermore, nitrate reductase is induced under O_2 deficiency in roots. The effect of nitrate on the barley aleurone Hb gene expression was determined (Fig. 5). Hb transcript levels increased slightly when the concentration of nitrate increased from 0 to 10 mM under aerobic conditions and then declined as nitrate was increased to 100 mM. Under anaerobic conditions there was a pronounced increase in Hb gene expression in the presence of 1 and 10 mM nitrate, but the addition of 100 mM nitrate inhibited Hb gene expression induced by the anaerobic conditions.

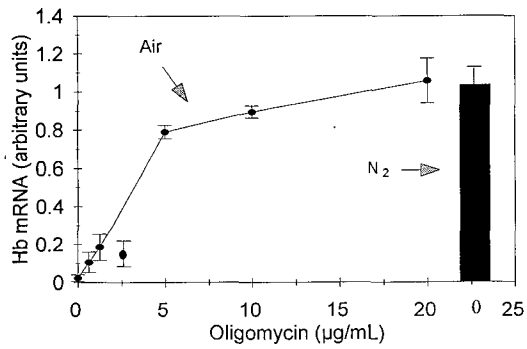

Figure 4. Effects of oligomycin on Hb transcription. Barley aleurone layers were preincubated in the different concentrations shown for 1 h. The layers were then set under N_2 or air for another 6 h in the same solutions. Northern analysis was carried out and presented as indicated in Figure 1.

DISCUSSION

Previous studies have shown that Hb gene expression is up-regulated by low-O_2 stress in bacteria (Wakabayashi et al., 1986), as well as in maize and barley roots and in aleurone layers (Taylor et al., 1994). The changes in gene expression in the presence of CO (Fig. 1) suggest that there may be some differential effect of CO on Hb expression relative to the anaerobic response genes *Adh* and *Ldh*. CO is less effective in promoting *Adh* and *Ldh* gene expression than Hb gene expression under normoxic conditions. Whereas Hb gene expression is further enhanced during hypoxia by CO, there is no significant change in *Adh* and *Ldh* gene expression. This may be attributable to the fact that CO binds tightly to most plant Hbs (Gibson et al., 1989), whereas estimates in leaf tissue suggest that Cyt *c* oxidase may be insensitive to CO at ratios of CO:O_2 below 40:1 (v/v) (Naik et al., 1992).

The results with other respiratory inhibitors indicate that interference with mitochondrial ATP synthesis strongly induces Hb gene expression. Cyanide and antimycin A, inhibitors of mitochondrial electron transport, increased the level of Hb transcripts (Fig. 2). The results with antimycin A indicate that the effect is not due to interference with O_2 binding to a heme structure. DNP, which stimulates electron transport (Table I; Douce, 1985) in the absence of ATP synthesis, also increased Hb expression. The induced Hb expression was, therefore, not the result of inhibited electron transport or the accumulation of reducing equivalents in the tissue. Conditions under

which there was a 37% reduction (antimycin) and an 86% stimulation of O_2 uptake (DNP) induced Hb gene expression, making it unlikely that mitochondrial O_2 availability is directly involved in the induction process. Oligomycin, which specifically inhibits oxidative phosphorylation in mitochondria by inhibiting ATP synthase through binding to the F_o subunit and interfering with H^+ transport through F_o (Voet and Voet, 1990), also increased expression (Fig. 4). This observation, in conjunction with the DNP results, indicates that ATP is a critical component in Hb gene expression.

The induction of the Hb gene under low O_2 tension shares some similarities with the responses of alcohol dehydrogenase and lactate dehydrogenase involved during anaerobic metabolism (Taylor et al., 1994). A close relationship has been shown between energy charge and the rate of ethanol plus lactate production (Saglio et al., 1980). Seeds storing lipids as the main carbon reserve have been observed to have 20 to 50 times the ethanol production during anaerobiosis as those containing starch (Al-Ani et al., 1982). The energy charge of the starchy seeds after several hours of anoxia was greater than 0.6, whereas the lipid-containing seeds maintained energy charges of less than 0.35. Maize roots, exposed to a period of hypoxia prior to experiencing anoxic conditions, remain viable for longer periods (Johnson et al., 1989). This has been attributed to the induction of *Adh*, providing ATP synthesis through anaerobic fermentation. Increased Hb levels, triggered by a transient decline in energy charge, may serve to assist in the adaptation to hypoxia.

There are several possible roles for Hb under hypoxic conditions: (a) it may serve as an O_2 carrier, like myoglobin (Wittenberg and Wittenberg, 1990), to allow mitochondrial respiration to continue during early anoxia; (b) it may act as an electron-transfer protein, as has been suggested for a flavohemoglobin from *Escherichia coli* (Cooper et al., 1994) and the *Vitreoscilla* Hb (Chen et al., 1994; Kallio et al., 1994); and (c) it may function as a sensor to regulate expression of other genes, as has been shown for the heme protein FixL of *Rhizobium meliloti* (Gilles-Gonzalez and Gonzalez, 1993; Gilles-Gonzalez et al., 1994).

Nitrate reduction is an energy-consuming process in which eight electrons are required to reduce a nitrate ion to an ammonium ion (Salisbury and Ross, 1985). The enzyme is induced by nitrate in barley aleurone layers (Ferrari and Varner, 1969). Enzymic activity in root tissue increases rapidly upon depletion of O_2, when ATP levels are low and AMP levels are high (Glaab and Kaiser, 1993). The prevailing evidence supports regulation via phosphorylation (inactivation), dephosphorylation (activation) mechanisms (Kaiser and Huber, 1994). The lack of effect of nitrate on Hb gene expression in air and the approximately 40% increase under anaerobic conditions (Fig. 5) may be due to a combination of increased nitrate reductase synthesis and regulation of nitrate reductase activity. Under aerobic conditions nitrate reductase was synthesized because of the presence of nitrate, but its activity was low as a result of higher ATP levels. With anaerobiosis, nitrate reductase activity was stimulated, resulting in competition for reducing equivalents with *Adh* and *Ldh*. Consequently, ATP levels declined further because of a lack of fermentative ATP synthesis and, therefore, further enhanced Hb transcription. The decline of Hb gene expression at high nitrate concentrations may be due to cytotoxic accumulation of nitrite (Kaiser and Huber, 1994).

In summary, the results strengthen the earlier conclusion (Taylor et al., 1994) that Hb gene expression occurs as a result of O_2 depletion in barley aleurone layers and that the response shares similarities with the anaerobic response for *Adh* and *Ldh*. We have established that O_2 is not directly involved in the regulation of Hb gene expression, since the gene can be induced in the presence of O_2. Furthermore, our results provide evidence that ATP, or some consequence of ATP action, is involved in Hb gene regulation.

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