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ORIGINAL ARTICLE

Xianzhou Nie · Douglas C. Durnin
Abir U. Igamberdiev · Robert D. Hill

Cytosolic calcium is involved in the regulation of barley hemoglobin gene expression

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Abstract Hemoglobin gene expression is upregulated during hypoxia. To determine whether the induction occurs via similar mechanisms that have been proposed for other hypoxically induced proteins, barley (*Hordeum vulgare* L.) aleurone layers were treated with various agents that interfere with known components of signal transduction. Ruthenium red, an organelle calcium channel blocker, inhibited anoxia-induced hemoglobin (Hb) and alcohol dehydrogenase (EC 1.1.1.1) (Adh) gene expression in a dose-dependent manner. The divalent ionophore, A23187, combined with EGTA also dramatically reduced anoxia-induced *Hb* and *Adh* expression. Normal induction of *Hb* by anoxia in EGTA-treated cells was restored by adding exogenous Ca^{2+} but not Mg^{2+}, suggesting that cytosolic calcium is involved in *Hb* and *Adh* regulation. W-7, a calmodulin antagonist, did not affect anaerobically induced *Hb* and *Adh* expression even though it induced *Hb* under aerobiosis. A3, a protein kinase inhibitor, did not significantly affect anaerobically induced *Hb*, but did significantly upregulate the gene under aerobic conditions. The results indicate that calmodulin-independent anaerobic alteration in cytosolic Ca^{2+} and protein dephosphorylation are factors in *Hb* induction.

Keywords Alcohol dehydrogenase · Anaerobiosis · Gene expression · Hemoglobin · *Hordeum vulgare* aleurone · Signal transduction

Abbreviations *Adh*: Alcohol dehydrogenase · *CaM*: Calmodulin · *GA*: Gibberellic acid · *EGTA*: Ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid · *Hb*: Hemoglobin · *IDC*: Iodine dextrin color · *RR*: Ruthenium red

X. Nie · D. C. Durnin · A. U. Igamberdiev · R. D. Hill (✉)
Department of Plant Science, University of Manitoba,
R3T 2N2 MB, Winnipeg, Canada
E-mail: rob_hill@umanitoba.ca
Fax: +1-204-4747528

*Present address:*X. Nie
Agriculture and Agri-Food Canada, 850 Lincoln Rd,
Fredericton, 20280, E3B 4Z7 NB, Canada

Introduction

Plant hemoglobins and hemoglobin genes have been isolated and characterized in several plant species including both monocotyledons and dicotyledons (Taylor et al. 1994; Andersson et al. 1996; Duff et al. 1997; Trevaskis et al. 1997). While some information has been obtained about the physiological function of hemoglobin (Hb) in plants, there is little information about the regulation of their gene expression. Previous results (Taylor et al. 1994) indicate that Hb gene expression is induced within 1–2 h of imposition of hypoxia. This expression occurs at both transcriptional and translational levels (Duff et al. 1998; Guy et al. 2002). In addition to hypoxic stress, the induction can be achieved by incubating the tissue with respiratory inhibitors, suggesting that *Hb* gene expression is associated with ATP levels or some consequence of ATP action (Nie and Hill 1997) as opposed to oxygen levels, per se. The objective of this study was to assess the possible signal transduction routes by which *Hb* gene regulation is accomplished.

Ca^{2+} is involved in many events in plants subjected to either developmental cues or environmental stimuli (Trewavas and Knight 1994). For instance, Ca^{2+} plays a role in phytochrome-mediated processes (Wei and Deng 1996; Shacklock et al. 1992), mechanical stimuli responses (Knight et al. 1992), phytohormone actions (Gilroy 1996; Gilroy and Jones 1992; Allan et al. 1994) and responses to microbial invasion (Knight et al. 1991). Ca^{2+} has also been suggested to function as a physiological transducer of anaerobiosis in plants (Subbaiah et al. 1994a, b, 1998).

Many cellular processes in plants and animals are controlled by the reversible phosphorylation of proteins,

a process which is often mediated by calcium (Hunter 1995). In plant cells, there is accumulating evidence that indicates that protein phosphorylation/dephosphorylation is modulated in response to different external signals including light, gravity, hormones and stress factors (for reference see Smith and Walker 1996). A number of processes that require protein phosphorylation/dephosphorylation have been identified by using protein kinase and/or phosphatase inhibitors (Smith and Walker 1996).

In this study, the effects on *Hb* expression of a number of compounds that interfere at different stages of Ca^{2+}-modulated signal transduction or protein phosphorylation have been examined. *Adh* gene expression has also been monitored as a control, since the expression of this gene relative to Ca^{2+} has been extensively studied (Subbaiah et al. 1994a, b, 1998). The results suggest that Ca^{2+} plays a major role in the signal transduction pathway leading to hemoglobin synthesis in plants.

Materials and methods

Barley aleurone layers

Seeds of barley (*Hordeum vulgare* L. cv Harrington, provided by the Canadian Grain Commission, Winnipeg, Manitoba) were dehusked in 50% H_2SO_4 for 2 h followed with thorough rinsing in tap water. The seeds were air-dried at room temperature and stored at 4°C.

The dehusked seeds were de-embryonated and a small portion of the distal end was removed. The resulting half seeds were surface-sterilized for 30 min in 1% NaOCl and rinsed thoroughly in distilled water. After 2 days of imbibition at 22°C in darkness, the aleurone layers were separated from the starchy endosperm. Twenty-five layers were placed in a sterile 50-ml conical flask containing 1.5-ml incubation solution (see figure legends for details) and incubated with slow agitation (65 cycles/min) at room temperature as described previously (Nie and Hill 1997).

Anoxia treatment

Isolated barley aleurone layers were incubated under controlled environments (N_2 or air) as described previously (Taylor et al. 1994). For anoxia 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. The jar was reflushed with N_2 for 10 min once or twice in a 6-h incubation period. Samples were collected and frozen in liquid nitrogen after the anoxia treatment. Samples that were not used immediately were stored at −75°C.

Northern blot analysis

Fifty aleurone layers were ground in liquid nitrogen, and total RNA was isolated as described previously (Taylor et al. 1994). Total RNA was quantified according to its absorbance at 260 nm. Ten micrograms of total RNA were denatured and electrophoresed on a 1.25% agarose gel, containing 2.2 M formaldehyde and a trace of ethidium bromide (Sambrook et al. 1989), and finally transferred to Hybond N^+ membranes (Amersham) with 0.05 M NaOH for 10–15 h. The membrane was rinsed briefly in 2× SSC buffer and air-dried. The membrane was hybridized with a ^{32}P labeled *Hb* probe at 65°C as described previously (Nie and Hill 1997). After 12–16 h of hybridization, the membrane was washed at 65°C for 30 min in 2× SSC, 0.5% (w/v) SDS, then 20 min in 2× SSC, 0.1% (w/v) SDS and 5–10 min in 0.2× SSC, 0.1% (w/v) SDS at 65°C. The membranes were exposed to Kodak XAR-5 film at −75°C for an appropriate period of time. After being stripped by boiling the filter in 0.1% SDS (w/v) for 5 min, the membrane was reprobed with *Adh* 1 probe and autoradiographed. The membrane was finally stripped and reprobed with 26S ribosomal DNA probe as described above. The resulting signals (northern blot bands on the film) were quantified by scanning the intensity and area of the band using the software, Image J (http://rsb.info.nih.gov/ij/).

Probe preparation

Probes (cDNA inserts isolated from the vectors) were labeled with [α-^{32}P]dCTP according to Sambrook et al. (1989). Hb probe was barley *Hb* cDNA (Taylor et al. 1994). Adh probe was barley *Adh*1 cDNA (a gift from Allen G. Good, University of Alberta, Edmonton, AB, Canada). 26S Ribosomal DNA probe was used as an internal standard to normalize RNA loadings.

Alpha-amylase analysis

Aleurone layers were treated with various Ca^{2+} signal transduction inhibitors using the conditions described under Anoxia treatment. After 6 h under a nitrogen gas atmosphere, they were rinsed with distilled water and incubated for 24 h in air, with gentle shaking, in 1.5 ml of water containing 1 μM GA_3 and 1 mM $CaCl_2$. The aleurone layers were ground in their incubation medium in a Teflon-glass homogenizer at 4°C and, after centrifugation, the supernatant was assayed for α-amylase activity (Briggs 1961). Activity is expressed as iodine dextrin color (IDC) units. One IDC unit is defined as the amount of enzyme required to change the A_{540} of a β-limit dextrin solution from 0.6 to 0.4 in 100 min.

Results

Effect of calcium antagonists and inhibitors on GA-induced α-amylase activity

Treatment with various inhibitors did not result in death of the aleurone cells as, after removal of the inhibitors, the aleurone layers retained the ability to respond to GA with GA-stimulated α-amylase activity (Table 1). All of the compounds that are used to affect *Hb* or *Adh* gene expression have previously been used in aleurone experiments and, at the concentrations used, have not affected aleurone viability although calcium antagonists are known to interfere with GA-regulated processes (Bethke and Jones 1994). In addition, W-7 has been shown to inhibit the pea mitochondrial pyruvate dehyrogenase complex, inhibiting mitochondrial respiration (Miernyk et al. 1989). Some compounds (EGTA, A23187 + EGTA, W-7) showed a stimulation of activity after replenishment of Ca^{2+}.

Effects of ruthenium red (RR) on *Hb* and *Adh* gene expression

Ruthenium red modifies Ca^{2+} release by blocking intercellular Ca^{2+} channels (Trewavas and Knight 1994). From Fig. 1, it can be seen that under aerobic conditions, increased concentrations of RR caused small increases in *Adh* mRNA, with 25 μM RR yielding a significant increase compared to the untreated sample. A slight rise was also observed in *Hb* mRNA. Under anoxic (N_2) conditions, as the concentration of RR was increased, *Hb* transcript levels decreased, becoming significant at concentrations above 25 μM. A similar pattern was found for *Adh* mRNA. Unlike maize roots (Subbaiah et al. 1994b), however, exogenously added Ca^{2+} failed to restore the normal anoxic responses for *Adh* and *Hb* in barley aleurone layers after RR was removed from the incubation medium.

Effects of EGTA and A23187 on *Hb* and *Adh* gene expression

To gain more information about possible involvement of calcium in *Hb* and *Adh* regulation in barley aleurone layers, a divalent ionophore, A23187 (Reed and Lardy 1972), and a metal chelator, EGTA, were employed. Under aerobic conditions, A23187 and A23187 combined with EGTA appeared to increase *Hb* transcript level slightly. Under anaerobic (N_2) conditions, A23187 decreased *Hb* mRNA levels slightly, while EGTA significantly reduced the mRNA levels by two- to threefold (Fig. 2). Using A23187 combined with EGTA, to further deplete Ca^{2+} levels, resulted in additional significantly decreased *Hb* mRNA levels. A similar pattern was found in *Adh* transcripts (Fig. 2).

Table 1 Gibberellic acid-stimulated α-amylase activity in aleurone layers after treatment with inhibitors

Treatment	IDC Units/layer
Medium (– GA)	8.0 ± 0.4
Medium	668 ± 39
Ruthenium red (50 μM)	639 ± 39
A23187 (2 μM)	523 ± 38
EGTA (5 mM)	949 ± 19
A23187 (2 μM) + EGTA (5 mM)	890 ± 36
W-7 (1 mM)	1025 ± 37
A3 (200 μM)	558 ± 66

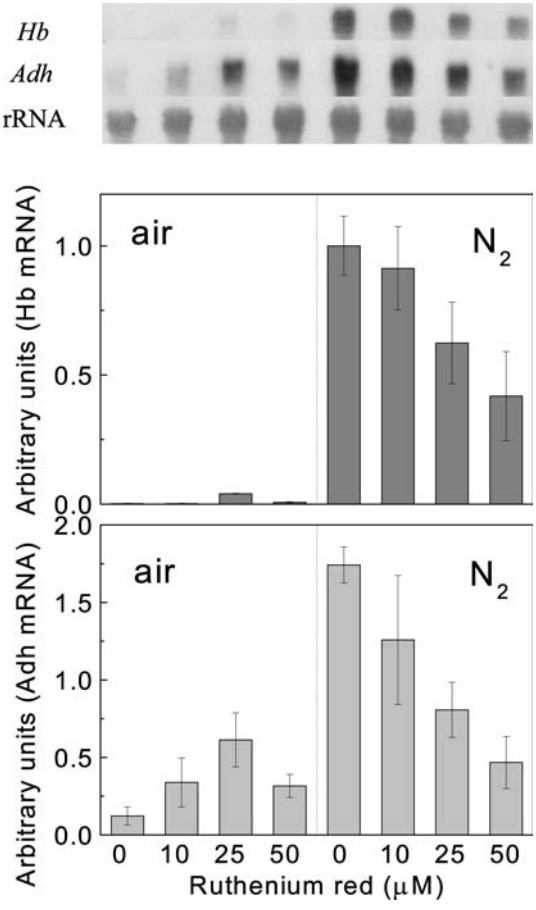

Fig. 1 Effect of ruthenium red on *Hb* and *Adh* expression. Barley aleurone layers were preincubated in sterilized double-distilled water containing various concentrations of ruthenium red from 0 to 50 μM for 1 h. The layers were then placed under anoxia (N_2) or air for 6 h. Total RNA was isolated. Northern blot analysis was carried out as described in the Materials and Methods. The filter was probed with the barley *Hb* probe first. After being stripped by boiling in 0.1% SDS for 5 min, the membrane was reprobed with an *Adh1* probe and finally with a ribosomal DNA probe. Signals from each membrane were quantified by image analysis. Blots shown are representative ones. Messenger RNA signals were normalized to the corresponding 26S ribosomal DNA signals. The data for *Hb* and *Adh* are presented as means of three replicates. *Top panel* Northern blots showing profiles of *Hb*, *Adh* and ribosomal RNA at various ruthenium red concentrations; *middle panel* relative intensity of *Hb* RNA in top panel, using ribosomal RNA intensities as loading controls; *bottom panel* relative intensity of *Adh* RNA in top panel, using ribosomal RNA intensities as loading controls

To determine whether the effect of EGTA and A23187 was due to Ca^{2+} removal, exogenous Ca^{2+} and Mg^{2+} were added back to the barley aleurone layers after removing EGTA and A23187 from the incubation medium. Under aerobic conditions, no significant differences were observed amongst these treatments (Fig. 3). However, under anaerobic conditions, both *Hb* and *Adh* mRNA levels in the tissue were significantly decreased by the treatment of EGTA combined with A23187. Addition of Ca^{2+} largely restored the response of *Hb* and *Adh* expression under anaerobiosis after removing EGTA and A23187; while in the treatment in which exogenous Mg^{2+} was added after removing EGTA and A23187, no significant changes were observed.

Effects of W-7, a calmodulin antagonist, on *Hb* and *Adh* expression

Calmodulin (CaM) is involved in many calcium-mediated processes (Bowler and Chua 1994). W-7, a

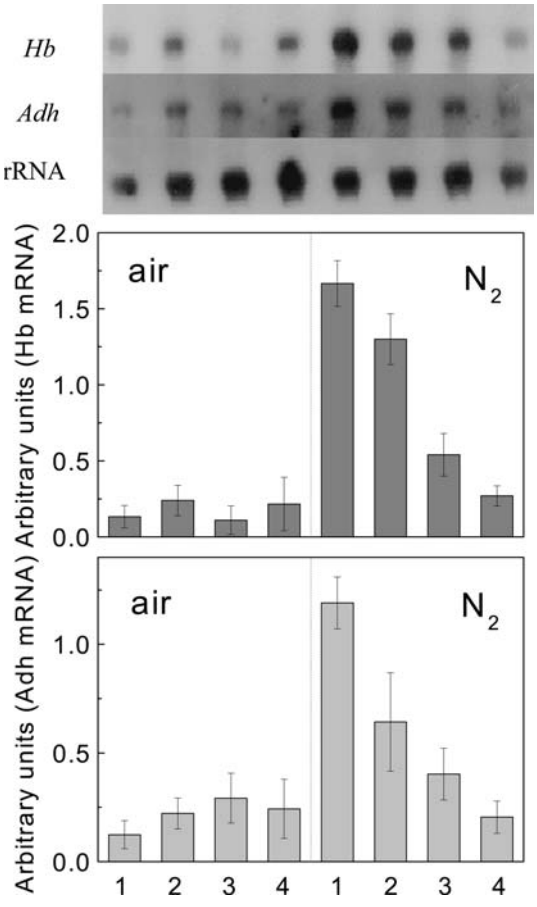

Fig. 2 Effects of EGTA and A23187 on *Hb* and *Adh* expression. Barley aleurone layers were preincubated in sterilized double-distilled water containing various components (EGTA, 5 mM, and/or A23187, 2 μM) for 2 h. The solution was removed and replaced with sterilized double-distilled water followed with further treatment under N_2 or air for 6 h. Total RNA was isolated. Northern blot analysis was carried out subsequently. The blot was probed with the *Hb* probe first, and then stripped and reprobed with the *Adh1*, and finally with the ribosomal DNA probe as described in Fig. 1 and Materials and Methods. Blots shown are representative ones. Signals from the blots were quantified and normalized to the corresponding 26S ribosomal DNA. The data for *Hb* and *Adh* are presented as means ± SE of three replicates. Treatments are *1* H_2O; *2* A23187; *3* EGTA; *4* EGTA + A23187. *Top panel* Northern blots showing profiles of *Hb*, *Adh* and ribosomal RNA at various treatments; *middle panel* relative intensity of *Hb* RNA in top panel, using ribosomal RNA intensities as loading controls; *bottom panel* relative intensity of *Adh* RNA in top panel, using ribosomal RNA intensities as loading controls

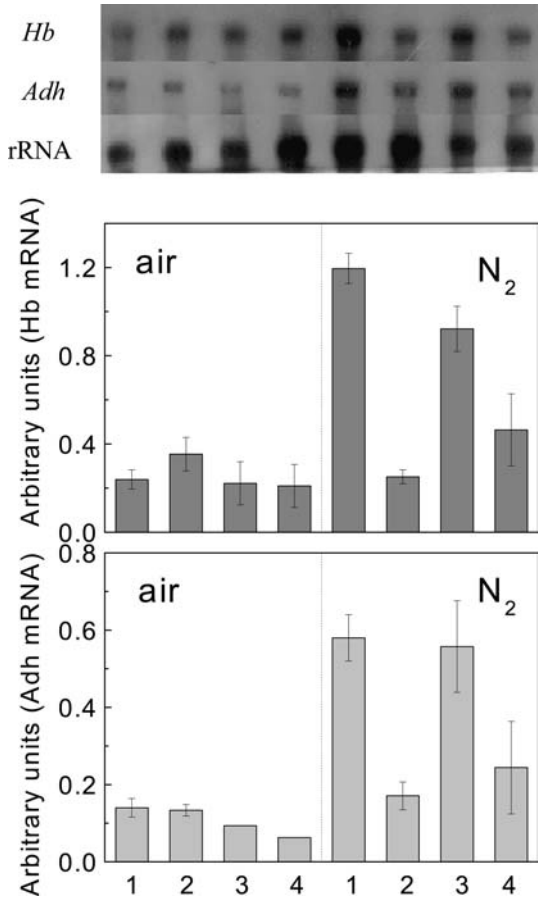

Fig. 3 Effects of Ca^{2+} and Mg^{2+} on *Hb* and *Adh* expression. Barley aleurone layers were preincubated in water or EGTA (5 mM) plus A23187 (2 μM) for 2 h as described in Fig. 2. The layers incubated in EGTA + A23187 were washed carefully with sterilized-double-distilled water several times in order to remove remaining residues of EGTA and A23187. The layers were then incubated with water or $CaCl_2$ (1 mM) or $MgCl_2$ (5 mM) for 1 h. Thereafter, the layers were placed under N_2 or air for another 6 h in the same solutions. Total RNA was isolated, and Northern blot analysis was carried out and presented as described in Fig. 1 and Materials and Methods. Blots shown are representative ones. The data for *Hb* and *Adh* are presented as means ± SE of three replicates. Treatments are *1* H_2O; *2* EGTA + A23187; *3* EGTA + A23187 + Mg^{2+}; *4* EGTA + A23187 + Ca^{2+}. *Top panel* Northern blots showing profiles of *Hb*, *Adh* and ribosomal RNA at various treatments; *middle panel* relative intensity of *Hb* RNA in top panel, using ribosomal RNA intensities as loading controls; *bottom panel* relative intensity of *Adh* RNA in top panel, using ribosomal RNA intensities as loading controls

calmodulin antagonist, was used in this study to investigate the possible involvement of CaM in *Hb* and *Adh* regulation in barley aleurone tissue. Figure 4 shows the effect of different concentrations of W-7 on *Hb* and *Adh* mRNA. Under aerobic conditions, both *Adh* and *Hb* transcripts were enhanced by W-7, with increases for *Hb* transcripts becoming significant at 100 μM, and *Adh* transcripts at 10 μM. Under anaerobic conditions, no significant changes in *Hb* mRNA levels were observed in the treatments in which W-7 concentration was less than 100 μM. *Adh* transcripts, however, increased significantly in the presence of 10 μM W-7. As the concentration of W-7 increased to 1,000 μM, the levels of both transcripts significantly decreased.

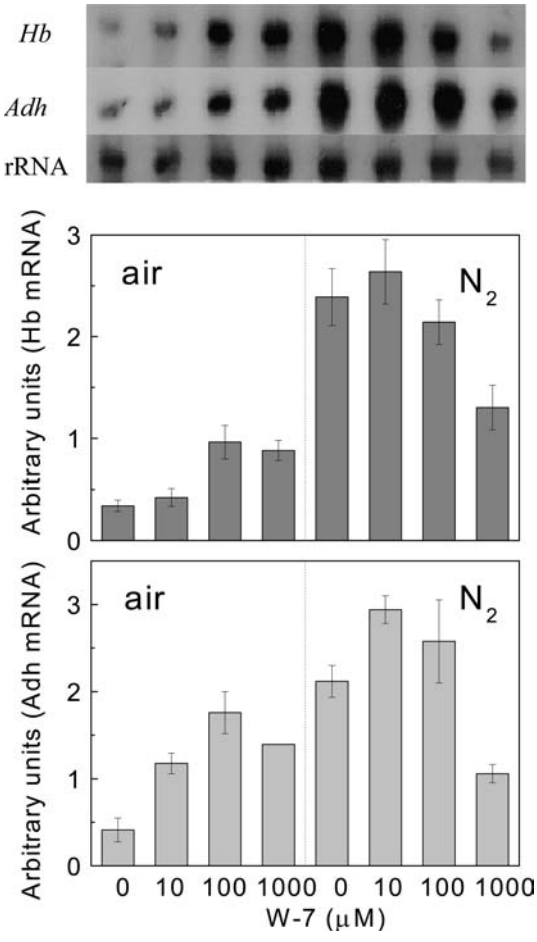

Fig. 4 Effects of W-7, a calmodulin antagonist, on *Hb* and *Adh* expression. Barley aleurone layers were preincubated in different concentrations of W-7 ranging from 0 to 1,000 μM (diluted from the stock of 26.5 mM in water) for 1 h. The layers were then placed under N_2 or air for another 6-h treatment in the same solutions. Total RNA was isolated, and Northern blot analysis was carried out and presented as described in Fig. 1 and Materials and Methods. Blots shown are representative ones. The data shown are means ± SE values of four replicates. *Top panel* Northern blots showing profiles of *Hb*, *Adh* and ribosomal RNA at various W-7 concentrations; *middle panel* relative intensity of *Hb* RNA in top panel, using ribosomal RNA intensities as loading controls; *bottom panel* relative intensity of *Adh* RNA in top panel, using ribosomal RNA intensities as loading controls

Effect of A3, a protein kinase inhibitor, on *Hb* and *Adh* expression

Reversible protein phosphorylation, catalyzed by protein kinases and protein phosphatases, has been demonstrated to play a role in the regulation of many biological processes (Smith and Walker 1996). A3, a protein kinase inhibitor, was applied at various concentrations in barley aleurone layers, and its effects on *Hb* and *Adh* expression were observed. Under aerobic conditions, both *Adh* and *Hb* transcriptions were enhanced by 10 and 100 μM concentrations of A3 (Fig. 5). Under anaerobic conditions, no significant changes were observed in *Hb* mRNA levels at various concentrations of A3 ranging from 0 to 200 μM. However, under the same conditions, as the concentration of A3 increased to 100 μM, *Adh* mRNA levels decreased 50%, and at 200 μM, *Adh* transcript levels decreased three- to fourfold.

Discussion

Cytosolic calcium is involved in many physiological processes ranging from environmental stimuli to developmental cues. Anoxia-induced cytosolic Ca^{2+} fluxes and their impacts on cellular injury have been well studied in both animal (Gasbarrini et al. 1992a, b) and plant systems (Subbaiah et al. 1994a, b, 1998; Subbaiah and Sachs 2003; Kuo et al. 1996; Yang and Poovaiah 2003; Reddy and Reddy 2004). Transcript levels of genes involved in calcium signaling are significantly altered in response to oxygen limitation in concert with the induction of class-1 hemoglobin in *Arabidopsis* (Liu et al. 2005). Although Ca^{2+} is stored mainly in the rough endoplasmic reticulum (Denton et al. 1980) or vacuole (Trewavas and Knight 1994), the hypoxic Ca^{2+} signal is mainly mitochondrial (Subbaiah et al. 1998). Mitochondria release calcium in the cytosol under hypoxic conditions (Logan and Knight 2003; Subbaiah and Sachs 2003). Elevation of the cytosolic Ca^{2+} results in cytochrome *c* release by mitochondria and other events affecting energy metabolism in hypoxic cells (Virolainen et al. 2002).

Maize seedlings, treated with RR, an organellar Ca^{2+} channel blocker, show poor recovery after a brief anoxia treatment (Subbaiah et al. 1994b). This has been attributed to the abolition of an anoxia-induced Ca^{2+} influx ($[Ca^{2+}]i$) in the presence of ruthenium red. Ca^{2+} influx, thus, is considered to be an early signal transducer of oxygen depletion in the environment (Subbaiah et al. 1994a, b). Indeed, maize cells have been shown to respond to anoxia by elevating $[Ca^{2+}]$ within a minute or two (Subbaiah et al. 1994a). The observation that RR decreased levels of anoxia-induced *Hb* and *Adh* transcripts in barley aleurone layers (Fig. 1) is consistent with the results reported for maize seedlings, suggesting that a similar mechanism exists in both tissues in

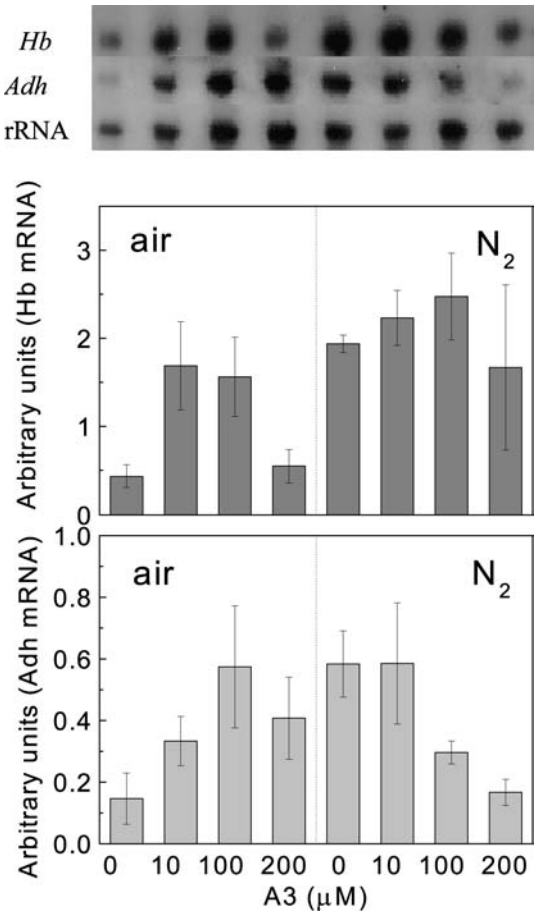

Fig. 5 Effects of A3, a protein kinase inhibitor, on *Hb* and *Adh* expression. Barley aleurone layers were preincubated in various concentrations of A3 ranging from 0 to 200 μM (diluted from a stock of 31.2 mM in dimethyl sulfoxide (DMSO); each solution had an equal amount of DMSO) for 1 h. The layers were then placed under N_2 or air for another 6 h in the same solutions. Total RNA was isolated, and Northern blot analysis was carried out and presented as described in Fig. 1 and Materials and Methods. The data shown are means ± SE of four replicates. *Top panel* Northern blots showing profiles of *Hb*, *Adh* and ribosomal RNA at various A3 concentrations; *middle panel* relative intensity of *Hb* RNA in top panel, using ribosomal RNA intensities as loading controls; *bottom panel* relative intensity of *Adh* RNA in top panel, using ribosomal RNA intensities as loading controls

response to oxygen deprivation. The very minor increase in *Hb* level and more significant increase in *Adh* under normoxic condition as a result of RR treatment may be linked to the blocking of aerobic Ca^{2+} uptake from the cytosol, the opposite process to Ca^{2+} release to the cytosol in hypoxia.

Further evidence of Ca^{2+} involvement in anaerobiosis of barley aleurone cells comes from experiments involving the divalent ionophore, A23187 and the calcium chelator, EGTA. A23187 stimulates a Ca^{2+}-ATPase in the endoplasmic reticulum (ER) of red beet (Giannini et al. 1987) and radish (Rasi-Caldogno et al. 1989), and a Ca^{2+}-ATPase has been described in barley aleurone ER (Bush et al. 1989). The ionophore, used alone, had less effect on both *Hb* and *Adh* transcription than EGTA, but *Adh* transcription was significantly inhibited by the ionophore under anaerobic conditions (Fig. 2). The aleurones were not treated with gibberellic acid (GA) in this experiment, which has been shown to stimulate the ER Ca^{2+}-ATPase (Bush et al. 1989). Furthermore, at low extracellular Ca^{2+} concentrations, A23187 has been shown to have little effect on cytoplasmic Ca^{2+} levels (Felle et al. 1992). Either or both of these factors could explain the response we observed in A23187. As shown in Fig. 2, however, EGTA alone or A23187 combined with EGTA, inhibited anoxia-induced *Hb* and *Adh* transcription. The inhibitory effect of A23187 plus EGTA could be overcome after removing the inhibitors by adding Ca^{2+}, but not Mg^{2+}, to the medium (Fig. 3).

Although Ca-dependent calmodulin (CaM) acts along with Ca^{2+} in many processes such as mechanical stimuli and red/far-red responses in plants (Bowler and Chua 1994), the CaM antagonist, W-7, failed to show an inhibitory effect on anoxia-induced gene transcription except at a concentration of 1,000 μM, the highest concentration tested in this investigation (Fig. 4). The presence of W-7 did, however, result in a small, but significant, increase in both *Hb* and *Adh* expression under aerobic conditions. W-7 has been shown to inhibit the mitochondrial pyruvate dehydrogenase complex (Miernyk et al. 1989), affecting mitochondrial respiration and, since inhibition of mitochondrial respiration has been shown to induce hemoglobin formation (Nie and Hill 1997), it is likely that the W-7-induced *Hb* expression under aerobic conditions is a result of this effect. The inhibition of mitochondrial respiration may affect Hb expression by a proposed mechanism involving Ca^{2+} release to the cytosol (Yang and Poovaiah 2003).

The response of *Hb* and *Adh* expression to A3 is complex. Under anoxic conditions, A3 had no significant effect on *Hb* expression whereas, beyond 10 μM, there was a significant inhibition of *Adh* expression (Fig. 5). Under aerobic conditions, expression of both genes was significantly increased in the presence of 10 μM A3. The *Hb* results can be explained if it is assumed that existence of a protein in a dephosphorylated state is necessary for *Hb* expression. Under aerobic conditions, where cell energy status is normal, inhibition of a protein kinase by A3 prevents phosphorylation of the protein. Under anaerobic conditions, where lack of oxygen results in declining the ability to synthesize ATP, lack of substrate ATP for the protein kinase limits phosphorylation and little additional effect of the protein kinase inhibitor is observed. Alternatively, the protein kinase needed to inhibit *Hb* expression may be repressed under anaerobic conditions. While the change is nonsignificant, the increase in *Hb* expression with increasing A3 under anaerobic conditions would also support the position that a dephosphorylated protein is a component in the signal transduction pathway leading to *Hb* expression. There is a negative effect on gene expression at higher concentrations of A3 in all instances which, assuming a more sensitive protein kinase

involved in *Adh* expression, may explain the *Adh* results at A3 concentrations greater than 10 μM under anoxia.

The results presented in this paper provide a starting point for further work to elucidate the signal transduction pathway for Hb synthesis, particularly in relation to Ca^{2+}. We have established that, unlike in mammalian systems, oxygen per se is not the primary signal inducing Hb expression in plants (Nie and Hill 1997). The present results indicate that compounds that interfere with cytosolic Ca^{2+} within the cell inhibit Hb expression (Figs. 1, 2, 3) and that restoring Ca^{2+} to depleted tissues restores *Hb* induction (Fig. 3). Thus, the initial steps of a hypothetical scheme for a *Hb* signal transduction pathway may be as follows: a decline in oxygen levels within the cells gradually reduces mitochondrial respiration rates, resulting in decreased transmembrane potentials and ATP levels; this, in turn, interrupts Ca^{2+}-ATPases and Ca^{2+}/nH^{+} antiporters that are efflux transporters maintaining cellular Ca^{2+} homeostasis (Bush 1995); the increase in cytosolic Ca^{2+} released from mitochondria and possibly other cell compartments, begins the signal transduction process leading to Hb expression. We have shown that cells overexpressing hemoglobin are able to maintain energy charge and ATP levels (Sowa et al. 1998; Dordas et al. 2003; Igamberdiev et al. 2004), due to participation in NO oxidation via Hb/NO cycle (Igamberdiev and Hill 2004). The presence of hemoglobin could, therefore, re-establish ATP levels, restoring Ca^{2+} homeostasis and switching off further hemoglobin synthesis. This would be in keeping with the observation that aleurone cells and embryos develop maximum expression of hemoglobin in early germination and within 24 h of oxygen deprivation (Nie 1997; Duff et al. 1998; Guy et al. 2002).

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