Haemoglobin expression in germinating barley

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Abstract

Polyclonal antibodies to purified recombinant barley haemoglobin (Hb) have been raised in rabbits and used to investigate its expression in monocotyledonous plants. Very little or no Hb expression was observed in dry barley seeds but germination resulted in the expression of Hb which peaked at 2–3 days after imbibition. Hb expression was also observed in maize, wheat, wild oat and *Echinochloa crus-galli* seeds during germination. Dissection of tissues from the barley seedlings showed that most of the haemoglobin was expressed in the root and seed coat (aleurone layer), with very little in the coleoptile. Imbibition of half-seeds or excised embryos resulted in the expression of haemoglobin. ATP measurements of barley embryos showed that ATP levels quickly increase after imbibition. α-Amylase activity was also determined in embryos to correlate Hb expression with a well-characterized germination response. The results demonstrate that Hb expression is a normal consequence of germination.

Keywords: Haemoglobin, germination, barley, embryos, aleurones

Introduction

Plant haemoglobins (Hb) have been known to exist in the root nodules of legumes for almost 60 years (Kubo, 1939; Keilin and Wang, 1945). Over the years, haemoglobins have been positively identified in three non-leguminous dicotyledonous plants; *Parasponia andersonii*, *Trema tomentosa* and *Casuarina glauca* (Appleby *et al*., 1983; Bogusz *et al*., 1988; Christensen *et al*., 1991). Recently, researchers in our laboratory isolated a Hb cDNA from barley and demonstrated that the gene was expressed in seed and root tissues under anaerobic conditions (Taylor *et al*., 1994), providing further evidence to support the contention that plant Hbs have a common origin (Landsmann *et al*., 1986). Since Hb has now been demonstrated to occur in two of the major divisions of the plant kingdom, it is likely that an Hb gene is present in the genome of all higher plants (Brown *et al*., 1984; Bogusz *et al*., 1988; Appleby, 1992; Taylor *et al*., 1994; Andersson *et al*., 1996; Hardison, 1996).

Hb is induced by events, such as low oxygen tension, that affect the level of ATP in tissue (Nie and Hill, 1997). Recently, barley haemoglobin has been expressed in *E. coli* (Duff *et al*., 1997). The recombinant barley haemoglobin is a dimer with unique ligand binding characteristics. Oxygen binding to the recombinant Hb is extremely tight suggesting that it does not function in facilitated diffusion.

This paper describes the expression of haemoglobin protein during barley germination to gain insight into the role of the protein in germination processes. We have raised antibodies to the protein to identify and quantify its expression *in vivo*. We have also measured ATP and α-amylase activity under the same conditions. Our results demonstrate that haemoglobin is expressed during normal seed germination.

Materials and methods

Chemicals and plant material

All biochemicals were from Sigma Chemical Co. or Gibco-BRL. Purified recombinant barley haemoglobin (Hb) was obtained as previously described (Duff *et al*., 1997). Barley half-seeds were made by removing the embryo end of the dry seed. Dry barley embryos were obtained according to Johnston and Stern (1957).

Germination and treatments

Seeds (from barley, maize, rice, wild oat and *E. crus-galli*) were germinated in the dark at 22°C in wetted, rolled absorbent paper. The appropriate tissue was removed and frozen in liquid nitrogen until used. Embryo-less seeds and isolated embryos were either frozen in liquid N_2 immediately (dry control) or incubated on Petri plates.

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Abbreviations: Hb=haemoglobin; MOPS= 3-[*N*-Morpholino] propanesulfonic acid; PAGE=polyacrylamide gel electrophoresis; PBS= phosphate-buffered saline.

Haemoglobin protein extraction

The frozen tissue was ground in a mortar and pestle with a 1:1 ratio (w/v) of extraction buffer (Duff *et al*., 1997). The ground homogenate was then centrifuged for 10 min at 27 000 *g* to remove debris. The clarified crude extract was then immediately frozen in liquid N_2.

α-Amylase extraction and assay

α-Amylase was extracted by grinding embryos in a glass Duall tissue grinder (Kontes) with 200 mM sodium acetate, pH 5.5, containing 1 mM $CaCl_2$ and centrifuging at 17 000 *g*. α-Amylase activity was measured in the supernatant (Briggs, 1961). One IDC unit is defined as the amount of enzyme necessary to convert 1 mmol of substrate per minute at 25°C.

ATP extraction and assay

ATP was extracted and assayed according to a standard procedure (Lowry and Passonneau, 1972).

Hb antibody production

Purified Hb samples were dialysed against phosphate-buffered saline (PBS) (50 ml potassium phosphate, pH 7, 150 mM NaCl) overnight and then mixed 1:1 with TiterMax (CytRx Co., Georgia). For the primary injection, 300 mg of Hb in PBS mixed with TiterMax adjuvant was injected into each of three New Zealand white rabbits. Subsequent injections did not include TiterMax. Booster injections of 100 mg per rabbit were performed 4, 9, 12 and 14 weeks after the initial injection. Antibody titre was monitored by dot blots from small samples of serum (1–5 ml) collected 7 days after each injection. The final serum was collected by heart puncture 12 days after the final injection. The blood was allowed to stand for 15–30 minutes at room temperature and then centrifuged at 5000 *g* for 10 minutes to collect the serum.

Immunotitration of Hb antibodies

Immunotitration of Hb was performed by immuno-precipitation of purified recombinant Hb by crude anti-Hb immunoserum according to a previous method (Lin *et al*., 1989). The amount of Hb was determined by A_{412} measurements (Duff *et al*., 1997).

Affinity chromatography of Hb antibodies

The Hb affinity column was prepared according to the manufacturer's instructions (Bio-Rad Laboratories Bulletin 1085). One mg of Phenyl Superose-purified Hb (Duff *et al*., 1997) was desalted against coupling buffer (0.1 M MOPS, pH 7.5 containing 0.3 M NaCl) and then coupled to 1 ml Affi-gel 15 by gentle agitation overnight at 4°C. The coupled gel was poured into a 1-ml column (column diameter 1 cm). The excess liquid was drained from the column and assayed for protein and Hb. The column was then washed for at least 10 bed volumes with 100 mM Tris, pH 8.0 containing 0.04% sodium azide. For affinity purification of Hb antibodies 10 ml of immune serum was applied to the Hb affinity column (5 ml/h, 4°C), which had been extensively washed with PBS, containing 0.02% (w/v) sodium azide. Following washing with at least 10 bed volumes of PBS the antibody was eluted with 100 mM citrate buffer, pH 2.8 at 1 ml/min. The eluted antibody was then dialysed against Affi-gel coupling buffer.

SDS-PAGE and protein immunoblotting

SDS-PAGE was performed using a Bio-Rad Mini-Protean II apparatus according to an established protocol (Laemmli, 1970). Proteins were electroblotted onto pure nitrocellulose membrane in an LKB 2005 Transfor apparatus according to an established protocol (Bio-Rad Laboratories: Protein Blotting: A guide to transfer and detection). Immunoreactive proteins on the nitrocellulose membranes were detected according to an established method (Bio-Rad Laboratories: Protein Blotting: A guide to transfer and detection) using an empirically determined dilution of primary antibody and the goat anti-rabbit alkaline phosphatase conjugate secondary antibody system.

Protein determination

Protein amounts were determined using the Bio-Rad protein assay reagent (Bradford, 1976) with bovine γ-globulin as a protein standard.

Calculation of Hb concentration in plant tissue

Hb concentration from plant tissue was calculated by protein immunoblots in which plant Hb and a standard curve of known concentrations of recombinant Hb were compared by densitometric scanning using a Sony CDD/RGB colour video camera and ImageX for DOS.

Results

The immunotitration of Hb antibodies is shown in Figure 1. I_{50} for the immunoremoval (precipitation) of Hb was 25 600 µl serum/mg protein. In terms of I_{50} the titre of the Hb antibodies compared well with the titre of antibodies raised against other soluble plant proteins

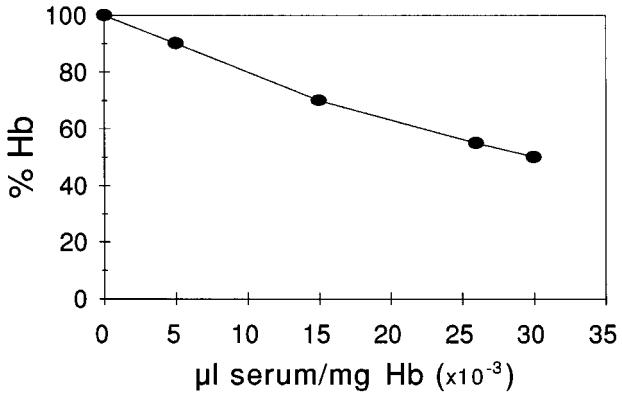

Figure 1. Effect of the addition of rabbit anti-recombinant Hb crude immune serum on the amount of soluble recombinant Hb. Immunoprecipitation was direct and did not involve the use of protein A.

(e.g. Plaxton, 1989; Duff *et al*., 1991; Duff *et al*., 1997) and was high enough to effect reasonable immunotitration of purified recombinant protein and detect the recombinant Hb on protein immunoblots (Fig. 2). The immunoprecipitation was not extended beyond 50% immunoremoval as the spectrophotometric assay at 412 nm was not sufficiently sensitive to detect the Hb remaining in solution. Protein immunoblots showing the immunoreactive proteins from an *E. coli* crude extract probed with affinity-purified Hb antibodies are shown in Figure 2. A single major band at about 18.5 kDa, the molecular weight of recombinant Hb, can be observed on the blot probed with the affinity-purified antibody.

The amount of Hb in dry barley seeds was below the limit of detection for these antibodies. During germination, Hb contents increased up to about 2 or 3 days after the start of imbibition, declining slightly during the remaining time examined (Fig. 3). The concentration of Hb at 3 days was 50 mg/g fresh weight or 0.07% of the total soluble protein. The highest contents of Hb (in proportion to total protein) were observed at day 3 in the root (0.3% total soluble protein), followed by the seed coat (0.1% total soluble protein), and the coleoptile (barely detectable) (Fig. 4). Wheat, wild oat, maize and *Echinochloa crus-galli* (but not rice) also expressed Hb during germination (Table 1). The highest amounts were observed in maize seeds where the calculated value was 0.041% total soluble protein at 7 days' imbibition. Recombinant barley Hb was used as a standard protein. The amounts shown are, therefore, expressed relative to barley Hb. Accurate levels of Hb could not be obtained as the level of cross-reactivity of the barley antibody between species is not known. Indeed, this may be the reason for the lack of detection of Hb in rice.

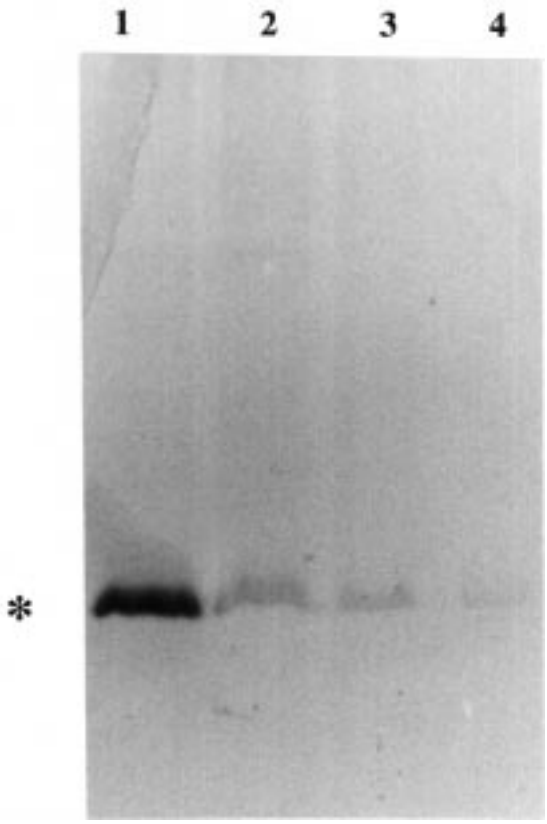

Figure 2. Protein immunoblot showing the immunoreactive proteins from an *E. coli* crude extract to affinity purified Hb antibody. Lane 1, 100 mg protein; lane 2, 30 mg protein; lane 3, 15 mg protein; lane 4, 5 mg protein. The affinity purified antibodies were diluted 1:100. The asterisk marks the 18.5-kDa recombinant Hb band.

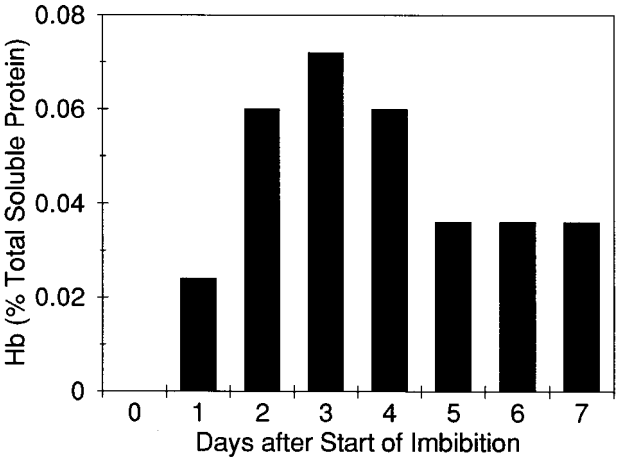

Figure 3. Amounts of Hb in crude extracts made from germinating barley seeds. Amounts were calculated from protein immunoblots as explained in Materials and methods and expressed in terms of total soluble protein.

Hb and ATP levels were determined in mature excised embryos and half-seeds (Fig. 5). Hb could not be detected in either dry excised embryos or dry half-seeds, but was detectable in embryos after 4 h of imbibition and half-seeds after 24 h (Fig. 5A). Amounts of ATP increased as the embryo was imbibed reaching a maximum at 1 h, after which no further increases were observed up to 6 h (Fig. 5B).
α-Amylase synthesis was determined in embryos (Fig. 6). Activity of the enzyme increased markedly after 24 h of imbibition.

Discussion

Antibodies have been produced in rabbit to recombinant barley Hb protein and have been affinity purified (Figs 1, 2). We have previously shown that Hb expression can occur as a result of anoxic stress (Taylor *et al*., 1994) and is linked to ATP availability (Nie and Hill, 1997). Oxygen availability is often considered a factor restricting the rate of germination (Bewley and Black, 1994) and so the presence of Hb in germinating seeds was investigated.

Hb was expressed in whole seeds (Table 1, Figs 3, 4), embryo-less half-seeds (Fig. 5) and excised embryos (Fig. 5) during germination. The fact that haemoglobin was expressed in both embryo-less half-seeds and excised embryos indicates that the gene is independently responsive to signals in both tissues and suggests that both the aleurone layer and the embryo may experience oxygen deficiencies during the imbibition process. In the excised embryo, haemoglobin was induced between 4 and 6 h after imbibition (Fig. 5). Since germination and the early stages of seedling growth are known to be periods of high metabolic demand (Bewley and Black, 1994), this data is consistent with the proposed concept that a demand on energy charge or ATP requirement is primarily responsible for Hb induction (Nie and Hill, 1997; Sowa *et al*., 1998). Major changes in ATP content of the embryos did occur within 1 hour after imbibition (Fig. 5) consistent with previous reports (Osborne, 1983). Protein hydration, protein synthesis and nucleotide synthesis are among the first events of germination. These early events, which consume large amounts of ATP, may well be a factor in the observed induction of Hb synthesis at 4–6 h after imbibition. However, induction occurs well before the major increase in α-amylase secretion (Fig. 6), a period of high metabolic demand, and so the relationship between Hb synthesis and energy availability needs further clarification.

In half-seeds, there is an apparent induction of Hb during imbibition, without the use of gibberellic acid to stimulate the synthesis of hydrolytic enzymes (Fig. 5A). Nie and Hill (1997) did not show appreciable amounts of Hb in isolated aleurone layers, unless induced by anoxia using a nitrogen environment. The aleurones in these half-seeds may well be experiencing anoxia due to entrapment in the endosperm and seed coat.

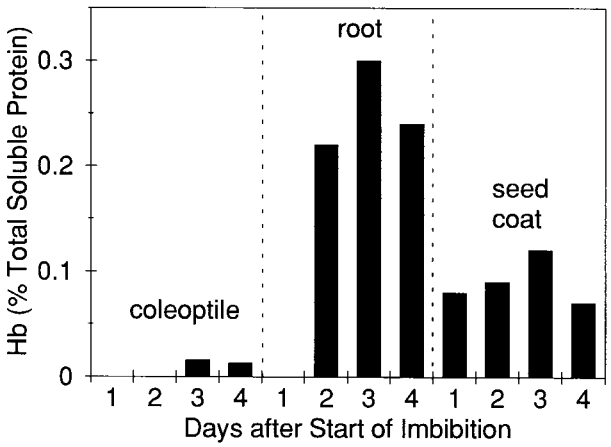

Figure 4. Amounts of Hb in tissues dissected from germinating barley seeds. Hb amount was determined and expressed as in Figure 3. Coleoptiles and roots were separated from the seed coat (containing the aleurone tissue) and Hb was measured for each tissue as indicated.

The high oxygen avidity of Hb (Arredondo-Peter *et al*., 1997; Duff *et al*., 1997; Trevaskis *et al*., 1997) argues against Hb functioning to facilitate diffusion of oxygen. Because the Hb will be induced intracellularly in a highly reductive environment with low energy charge it is possible that Hb functions as an electron transport protein similar to cytochrome c. We have discussed this possibility in a previous study (Duff *et al*., 1997; Sowa *et al*., 1998). Further work is now being carried out to examine more closely the potential effect of oxygen limitation and Hb expression during germination.

The function of this enigmatic protein is still far from certain. We have observed Hb gene expression (or increases in Hb expression) unequivocally in at least 4 cases: (1) in intact whole seeds during germination; (2) in excised embryos and embryo-less half-seeds imbibed in water; (3) in aleurone layers which have been stressed by a low oxygen environment or respiratory inhibitors (Nie and Hill, 1997); (4) in barley roots after flooding (Taylor, *et al*., 1994). In every situation it is likely that the ATP requirement of the cell exceeds the ATP supply either because of low oxygen supply (such as in the case of the flooded plants or stressed seed tissue) or due to high metabolic rates (such as likely to be the case during germination). Hb expression seems to be both a normal event during seed germination as well as an adaptation of plants to low oxygen environments.

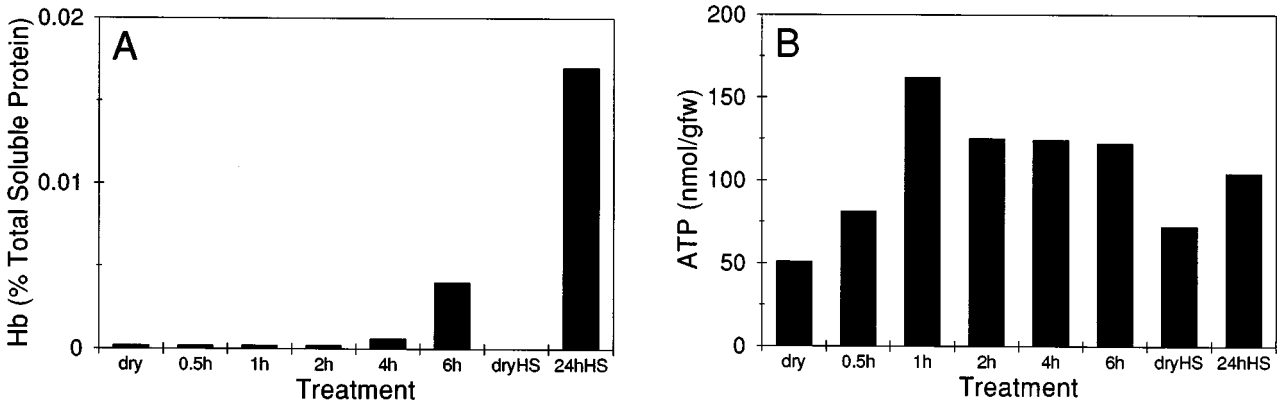

Figure 5. Amounts of Hb and ATP in excised embryos and half-seeds. Treatments, dry–6 h are for excised embryos. Dry embryos were prepared as described in Materials and methods and were imbibed at 22ºC for up to 6 h. Dry half-seeds (HS) were imbibed for 24 h. **A.** Hb amounts; determined and expressed as in Figure 3. **B.** ATP amounts; determined as described in Materials and methods.

Table 1. Maximum relative Hb amounts in the germinated seeds of various species. All amounts are expressed in terms of percentage total soluble protein using recombinant barley Hb as the standard. Seeds were germinated for 3 days (7 days in the case of maize)

Species	Amount of Hb
wheat	0.01
maize	0.04
rice	0
wild oat	0.004
E. crus-galli	0.01

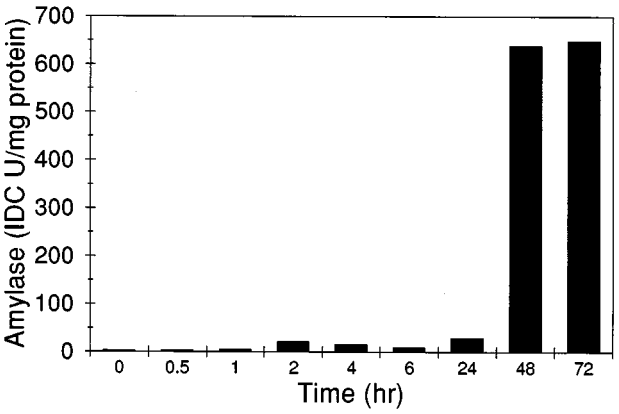

Figure 6. α-Amylase activity in embryos. α-Amylase activity was determined in the extracts of embryos imbibed for the lengths of time indicated on the figure.

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