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Class-1 hemoglobins, nitrate and NO levels in anoxic maize cell-suspension cultures

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Abstract Nitric oxide (NO) is a reactive gas involved in many biological processes of animals, plants and microbes. Previous work has demonstrated that NO is formed during hypoxia in alfalfa (*Medicago sativa* L.) root cultures and that the levels of NO detected are inversely related to the levels of expression of class-1 hemoglobin expressed in the tissue. The objectives of this study were: to examine whether NO is produced in transgenic maize (*Zea mays* L.) cell-suspension cultures exposed to anoxic growth conditions; to determine whether a similar relationship existed between a class-1 hemoglobin and the amount of NO detected under these conditions; and, to estimate the route of formation and breakdown of NO in the tissue. Maize cell-suspension cultures, transformed to express the sense or antisense strands of barley hemoglobin were used to overexpress or underexpress class-1 hemoglobin. A maize cell-suspension culture transformed with an empty vector was used as a control. Up to 500 nmol NO $(g\ FW)^{-1}$ was detected in maize cells exposed to low oxygen tensions for 24 h. The steady-state levels of NO in the different cell lines under anoxic conditions had an inverse relationship to the level of hemoglobin in the cells. There was no detectable NO produced under aerobic growth conditions. Spectroscopic data demonstrated that recombinant maize hemoglobin reacted with NO to form methemoglobin and NO_3^-. Nitrate was shown to be a precursor of NO in anoxic maize cell-suspension cultures by using $^{15}NO_3^-$ and electron paramagnetic resonance spectroscopy, suggesting that NO is formed via nitrate reductase during hypoxia. The results demonstrate that NO is produced in plant tissues grown under low oxygen tensions and suggest that class-1 hemoglobins have a significant function in regulating NO levels.

Keywords Anoxia · Hemoglobin · Nitric oxide · Signalling · Stress · Zea

Abbreviations DEANO: 2-(N,N-Diethylamino)-diazenolate-2-oxide · EPR: Electron paramagnetic resonance · Hb: Hemoglobin · MGD: N-(Dithiocarbamoyl)-N-methyl-D-glucamine · NOS: Nitric oxide synthase · WT: Wild type

Introduction

Nitric oxide (NO) is an active oxygen species that plays an important role in many physiological and pathological processes in animals, plants and microbes (Wendehenne et al. 2001). In mammals NO is involved in several physiological processes such as relaxation of smooth muscle, inhibition of platelet aggregation, neural communication, immune regulation and apoptosis (Schmidt and Walter 1994). NO is produced by nitric oxide synthase (NOS) in mammals, which oxidizes L-arginine to L-citrulline and NO by using 2 moles of O_2 and 1.5 moles of NADPH. In bacteria, NO is produced through the nitrite reductase pathway (Chen and Rosazza 1994). In plants, NO can be formed via NAD(P)H-dependent nitrate reductase (Dean and Harper 1988; Yamasaki and Sakihama 2000) and by NOS (Delledonne et al. 1998).

NO reacts with transition metals, the most studied reaction being that with iron. It reacts very rapidly with oxyhemoglobin (Eich et al. 1996; Minning et al. 1999), forming nitrate and methemoglobin. It reacts with a number of other iron-containing proteins such as guanylate cyclase, cytochrome *c*, cytochrome oxidase, aconitase and NADH dehydrogenase (Cooper 1999). The interaction of hemoglobin with NO has an important function in most organisms. In bacteria,

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flavohemoglobin detoxifies NO by converting it to nitrate, utilizing NADH to regenerate oxyhemoglobin (Gardner et al. 1998; Hausladen et al. 1998). NO is believed to be involved in plant growth and development, signal transduction and disease resistance (Durner and Klessig 1999).

Hemoglobins are ubiquitous proteins found in organisms from bacteria through to animals and plants (Weber and Vinogradov 2001). There are two main types of hemoglobin found in plants. One type of hemoglobin, termed 'symbiotic' is found only in nodules of plants capable of symbiotic nitrogen fixation and its function is to regulate oxygen supply to nitrogen-fixing bacteria (Appleby 1992). A second type of hemoglobin, consisting of two distinct classes, is expressed in seed, root and stem tissue of both dicot and monocot plants (Hill 1998; Arredondo-Peter et al. 1998). Class-1 hemoglobins have an extremely high affinity for oxygen (approx. 2–3 nM; Duff et al. 1997; Arredondo-Peter et al. 1997) and are induced in plants during hypoxic stress (Taylor et al. 1994; Lira-Ruan et al. 2001). A class-2 hemoglobin isolated from *Arabidopsis* has a lower affinity for oxygen and is inducible by low temperature (Trevaskis et al. 1997) or cytokinin treatment (Hunt et al. 2001). It has been suggested that monocots possess only class-1 hemoglobins (Hunt et al. 2001).

Hemoglobins are most commonly known for their ability to act either as oxygen carriers or stores to facilitate O_2 delivery, or oxygen sensors to regulate gene expression at low oxygen tensions. Since class-1 hemoglobins have very low oxygen dissociation constants, they deliver oxygen only at very low oxygen tensions and remain oxygenated at oxygen partial pressures far below the levels at which they would be useful as oxygen sensors (Hill 1998). Class-1 hemoglobins are generally found at low concentrations [1–20 μmol (kg FW)$^{-1}$] in plant organs (Duff et al. 1997). They are expressed in root and aleurone tissue of barley under anoxic conditions (Taylor et al. 1994), but are also present in rapidly growing tissues such as root tips of germinating seeds (Hill 1998; Duff et al. 1998). Class-1 hemoglobin gene expression is not directly influenced by O_2 usage or availability, but ATP or some consequence of ATP action is involved in regulating expression (Nie and Hill 1997).

NO is intimately involved with hemoglobins in other organisms (Dean and Harper 1986; Stamler et al. 1997) and is produced by a variety of different conditions in plants (Durner and Klessig 1999), including hypoxic stress in root cultures of alfalfa (Dordas et al. 2003a), a dicot species. Dordas et al. also showed that a class-1 hemoglobin influenced the levels of detectable NO in the roots. An objective of the present study was to determine whether a similar phenomenon existed in cell-suspension cultures of maize, a monocot species. In addition, we wished to determine the source of nitrogen for NO formation and the likely products of the breakdown of NO.

Materials and methods

Materials

The expression vector pPROEXHTa, control vector pUC19 plasmid, the *Escherichia coli* strain DH5α used as the host for both expression vectors and the control plasmid, enzymes for DNA manipulation, PCR primers and agarose were from Invitrogen Life Technologies (Burlington, ON, Canada). GeneClean II Kit was from Bio-Can Scientific (Mississauga, ON, Canada). All other chemicals were obtained from Sigma (St. Louis, MO, USA) or Invitrogen Life Technologies. MGD (N-(dithiocarbamoyl)-N-methyl-D-glucamine) and DEANO (2-(N,N-diethylamino)-diazenolate-2-oxide) were purchased from Alexis Biochemicals (San Diego, CA, USA).

Anoxic treatment of maize cells and determination of NO production using electron paramagnetic resonance (EPR)

Transgenic maize (*Zea mays* L.) cell-suspension culture lines were obtained as described previously (Sowa et al. 1998). Maize cell-suspension lines overexpressing barley hemoglobin (Hb^+), wild type (WT) and underexpressing barley hemoglobin (Hb^-) were used in the experiments. For treatments under low oxygen tension, the flasks were closed with serum caps and flushed with N_2 for 2 min (Sowa et al. 1998). For the aerobic treatment, the flasks were covered with aluminum foil to allow the exchange of oxygen. All the treatments were done under sterile conditions and in the presence of 500 mg l^{-1} carbenicillin to inhibit any bacterial growth during the course of the experiments. All treatments under an N_2 atmosphere were for 24 h.

The NO spin trap Fe^{2+}–$(MGD)_2$ complex was used to measure NO production by EPR spectroscopy. The complex was prepared immediately before each experiment by reacting 10 mM MGD with fresh $FeSO_4$ (0.5 mM). Briefly a solution of MGD with a concentration of 200 mM was prepared in 0.1 M Hepes (pH 7.0) buffer. $FeSO_4$ (10 mM in 1 mM H_2SO_4) was prepared at the same time. The Fe^{2+}–$(MGD)_2$ complex was prepared by adding 500 μl of $FeSO_4$ to 500 μl of MGD. After a few minutes, when the reaction was completed, 100 μl of the solution containing the Fe^{2+}–$(MGD)_2$ was added to the samples to give a final concentration of 10 mM MGD and 0.5 mM $FeSO_4$ (Kotake 1996). For the time-course study of NO production from maize cells, 15 μl of sample was taken with a gas-tight Hamilton syringe at time intervals of 0, 6, 12, 18 and 24 h and placed in a capillary tube. The capillary tube was placed in Suprasil synthetic quartz EPR tubes (Wilmad, Buena, NJ, USA) and the EPR tube was placed in the EPR sample cavity for measurement. Special care was taken to measure the NO that was trapped immediately after sampling. NO reacts with the spin-trap complex to produce a spin adduct (Kotake 1996) that has a characteristic three-line EPR spectrum with hyperfine coupling constant $a_N = 12.6$ G and $g_{iso} = 2.04$. The EPR spectra were recorded at room temperature on an EMX spectrometer (Bruker, Billerica, MA, USA). Ten spectra were recorded over a period of 8 min and their signals were averaged. The instrument settings were as follows: microwave power 20 mW, modulation amplitude 4 G, modulation frequency 100 kHz, microwave frequency 9.24 GHz, receiver gain of 2×10^4 and magnetic field centered at 3,255 G with a 100-G scan range. NO quantification of the Fe^{2+}–$(MGD)_2$(NO) complex was accomplished by measuring the peak-to-peak signal of the lowest field peak in the EPR spectrum compared to NO spin-trapped by [Fe^{2+}–$(MGD)_2$] that was rapidly produced by the addition of a known amount of DEANO. The detection limit of the assay was 1.5 nmol NO (g FW)$^{-1}$.

Protein immunoblotting

One gram of maize cells was ground in liquid nitrogen with a mortar and pestle. Ice-cold extraction buffer [0.2 ml of 50 mM

Tris–HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 1 mM DTT and 1 mM phenylmethylsulfonyl fluoride (PMSF)] was added and the proteins were separated by SDS–PAGE. Proteins were electroblotted onto polyvinylidenedifluoride (PVDF) membranes and detected by using a polyclonal antibody raised against recombinant barley (*Hordeum vulgare* L.) hemoglobin. The immunoblot protein levels were calculated by densitometric comparison with a set of standards of known hemoglobin concentrations and the use of the software program, Image J (http://rsb.info.nih.gov/ij/). Recombinant barley hemoglobin (BHb) was used as a standard for the Hb^+ line since this line was transformed with the sense strand of the barley hemoglobin gene. Recombinant maize hemoglobin (MHb) was used for the remaining samples.

Expression and purification of recombinant maize hemoglobin from *E.coli*

cDNA encoding a maize hemoglobin gene was inserted into a pBluescript vector SK + (Accession number AF236080). A one-step polymerase chain reaction (PCR) was used to amplify the coding region of the hemoglobin gene with two different restriction sites with a forward primer (5′-CGGGATCCATGGCACTCGCGGAGGCCGACGA) complimentary to the extreme 5′-end of the coding sequence of hemoglobin that also provided a *Bam*HI restriction site. The reverse primer (5′-CCCAAGCTTCTACTAGGCATCGGGCTTCATCTCC) was complimentary to the extreme 3′-end of the coding region with a *Hin*dIII restriction site. The PCR product was separated on an agarose gel, digested with the restriction enzymes *Bam*HI and *Hin*dIII, ligated into pPROEXHTa and then transformed into *E. coli* strain DH5α using standard protocols (Sambrook et al. 1989). Positive clones were selected on Luria-Bertani (LB) ampicillin plates and were screened for expression of maize hemoglobin with the 6×His antibody (Qiagen, Mississauga, ON, Canada) and a polyclonal antibody against barley hemoglobin. Cultures overexpressing maize hemoglobin were grown and used for protein purification following the instructions of the manufacturer (Invitrogen Life Technologies). The 6×His tag was then removed by digesting the recombinant maize hemoglobin with TEV protease following the instruction of the manufacturer (Invitrogen Life Technologies).

Protein concentration was measured by the method of Bradford (1976) using the Bio-Rad prepared reagent and BSA as standard. Hemoglobin concentration was determined by Bradford protein assay or by A_{412} measurements.

SDS–PAGE was performed using a Bio-Rad Miniprotean II gel apparatus following standard Bio-Rad protocol. Final acrylamide monomer concentration in the 0.75-mm-thick slab gels was 15% (w/v) for the separating gel and 4% (w/v) for the stacking gel.

Properties of maize hemoglobin

The different forms of MHb were prepared as described previously (Duff et al. 1997) by incubating recombinant MHb solutions with excess of sodium dithionite to convert all MHb to MHbFeII. The solution was then desalted by chromatography through a Sephadex G-25 column (Amersham Pharmacia Biotech, Chicago, IL, USA). The $MHbO_2$ was formed from MHbFeII after exposure to air. For the formation of MHbFeIII the MHb solution was incubated with excess K_3FeCN_6 for more than 10 min, then desalted as described previously.

The optical spectra were acquired using a microplate reader (Molecular Devices, San Diego, CA, USA). Different forms of MHb (HbO_2, HbFeII, and HbFeIII; 12 μM) were titrated with different concentrations (3, 6, 9, 12, 15, 18 μM) of NO. Air-oxygenated Hb was titrated with 3 μM NO. Samples were analyzed after NO addition by UV-visible spectrophotometry.

To follow nitrate formation from the reaction of HbO_2 with NO, aliquots of DEANO were added to solutions of recombinant maize Hb to give final concentrations of 5, 10 and 15 μM DEANO and 10 μM Hb in a final volume of 100 μl. Following the addition of DEANO, the reaction mixture was left for 30 min at room temperature and 30 μl of the reaction was removed and analyzed for nitrate, subtracting the absorbance at 250 nm from the absorbance at 202 nm and using KNO_3 as a standard (Weger and Turpin 1989).

Determining the nitrogen source for NO formation

Since plant cells accumulate nitrate in the vacuoles, the maize cell cultures (Hb + , WT, Hb−) were incubated in MS2D medium in the absence of a source of nutrient nitrogen for 48 h in order to deplete the internal pools of nitrate. The maize lines were then transferred into MS2D medium with Mes (pH 5.8) where N was provided as $(NH_4)_2SO_4$. The culture was grown for 1 week and the pH was monitored to make sure that the pH of the medium did not decline due to the NH_4^+ uptake. The cell cultures were then washed in MS medium with no N and approximately 0.1 g of maize cell cultures was placed in vials with 0.5 ml MS2D medium (with 500 μg ml^{-1} carbenicillin) containing 10 mM $^{15}NO_3^-$ or NH_4^+ as the source of N. The cultures were divided into anoxic and aerobic treatments and incubated for 24 h. For the anoxic treatment the samples were flashed with N_2 for 2 min according to Sowa et al. (1998). The aerobic samples were left with the aluminum foil cover to allow free exchange of O_2. After treatment, the spin trap Fe–$[MGD]_2$ was added and the vials were placed on a rotary shaker shaking at 150 rpm.

Statistics

All experiments were performed three times with four replications in each treatment. Values are given as mean ± SE.

Results

The maize cells cultures have a respiration rate of 600 nmol $(g\ FW)^{-1}\ min^{-1}$. At that rate, they will consume all the oxygen in solution under a nitrogen atmosphere within a few minutes. They, therefore, more closely approximate an anaerobic environment than a hypoxic one. The lines had a 10-fold range in hemoglobin content under low oxygen tension (Fig. 1) with a maximum level of 2.6 nmol $(g\ FW)^{-1}$ for the Hb^+ line.

Production of NO under anoxic stress

Spin trapping and EPR spectroscopy were used to determine the presence of NO in the cell lines exposed to

Fig. 1 Immunoblot of hemoglobin protein levels in control maize (*Zea mays*) cell lines (*WT*), and in lines overexpressing (Hb^+) and underexpressing (Hb^-) barley (*Hordeum vulgare*) hemoglobin. 50 μg soluble protein was loaded on each lane. The proteins were separated by SDS–PAGE, and they were electroblotted onto PVDF membranes and detected by using a polyclonal antibody raised against recombinant barley hemoglobin

various oxygen tensions (Figs. 2, 3). NO reacted with the spin trap [Fe^{2+}–$(MGD)_2$] and gave a characteristic three-peak EPR signal with hyperfine coupling constant $a_N = 12.7$ G and $g_{iso} = 2.04$ (Fig. 2), similar to values reported previously (Kalyanaraman 1996). NO was not detected in aerobically grown samples (Figs. 2, 3). NO levels were low in the first 6 h of treatment under low oxygen tension, after which there was a significant increase in NO in all samples (Fig. 3). The amount of NO continued to accumulate under low oxygen tension during the course of the study, reaching a level of 495 nmol $(g\ FW)^{-1}$ after 24 h for the Hb^- line. The amount of NO trapped under 3% oxygen varied with the maize cell line. Hb^+ had the lowest levels while Hb^- had the highest levels of NO, with the WT line being intermediate.

Characteristics of maize recombinant hemoglobin and reaction with NO

The spectral properties of recombinant maize hemoglobin and selected derivatives are shown in Table 1. The properties of the ferrous and ferric forms and oxyhemoglobin are essentially identical to those of barley hemoglobin (Duff et al. 1997), confirming the existence

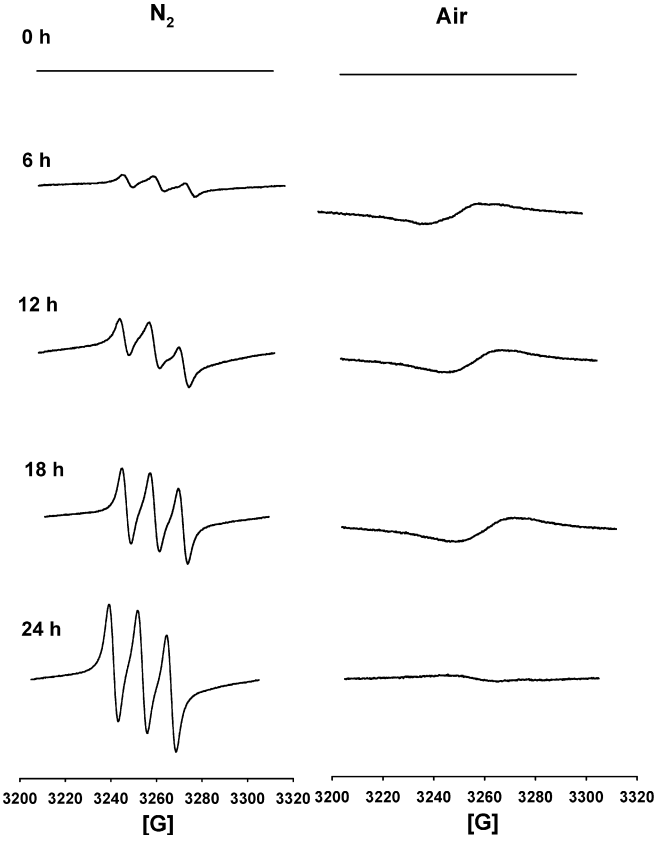

Fig. 2 EPR spectra of NO trapped by Fe^{2+}–$(MGD)_2$. Maize control cultures were exposed to anaerobic (N_2) and aerobic (Air) growth conditions for 24 h. At intervals (0, 6, 12, 18, 24 h), a 15-µl sample was taken for EPR analysis

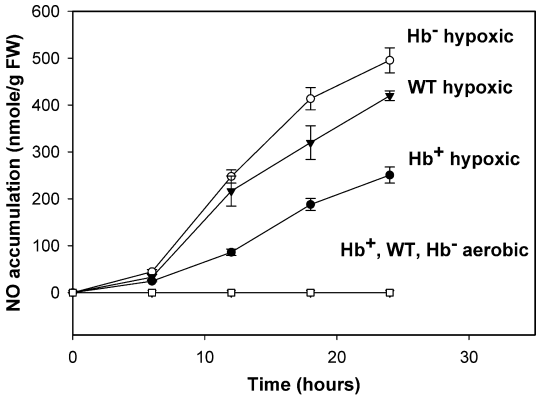

Fig. 3 NO in maize cell lines grown under anaerobic (N_2) and aerobic (Air) growth conditions. Maize lines overexpressing (Hb^+) and underexpressing (Hb^-) barley hemoglobin, and the control (WT) line were exposed to anaerobic and aerobic growth conditions for 24 h. At intervals (0, 6, 12, 18, 24 h), a 15-µl sample was taken for EPR analysis

Table 1 Spectral properties of recombinant maize (*Zea mays*) hemoglobin

Derivative	γ_{max}
Ferrous Hb	425
	535
	563
HbO_2	412
	540
	576
Ferric Hb	411
	534
	565
Ferrous Hb–NO	427
	534
	563
Ferric Hb–NO	418
	540
	670

of low-spin, six-coordinate (6C) species in class-1 hemoglobins. The optical spectra of the nitrosyl derivatives are also indicative of the presence of 6C species.

To determine whether class-1 oxyhemoglobins, like other oxyhemoglobins (Gardner et al. 1998; Minning et al. 1999), oxygenate NO and are oxidized to methemoglobin, recombinant maize oxyhemoglobin was titrated with NO and the amount of nitrate formed by the reaction determined (Figs. 4, 5). There were stoichiometric amounts of nitrate formed for the first two aliquots of NO added (Fig. 4). At this point, all of the oxyhemoglobin should have been consumed if it was converted to methemoglobin and, in fact, no further change in nitrate occurred upon the addition of a third aliquot of NO. Absorption spectra of the titration of a 12 mM $MHbO_2$ solution with NO are shown in Fig. 5. With increasing addition of NO to the $MHbO_2$ solution there was a spectral shift from the characteristic peaks at 412, 540 and 576 nm for $MHbO_2$ to peaks at 411, 534, and 565 nm, characteristic of MHbFeIII. There was no

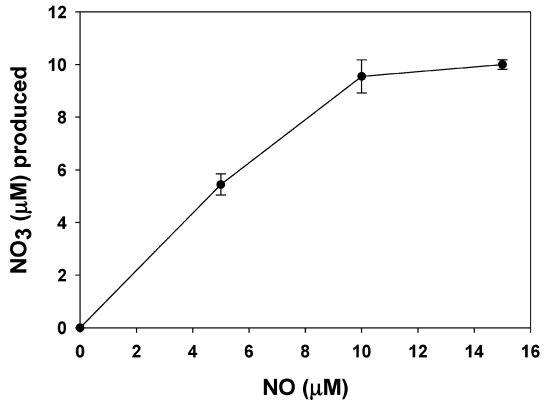

Fig. 4 Production of NO_3 by HbO_2 and NO. Aliquots of DEANO, to give final NO concentrations of 5, 10, 15 μM, were added to a 10-μM solution of HbO_2 and nitrate determined

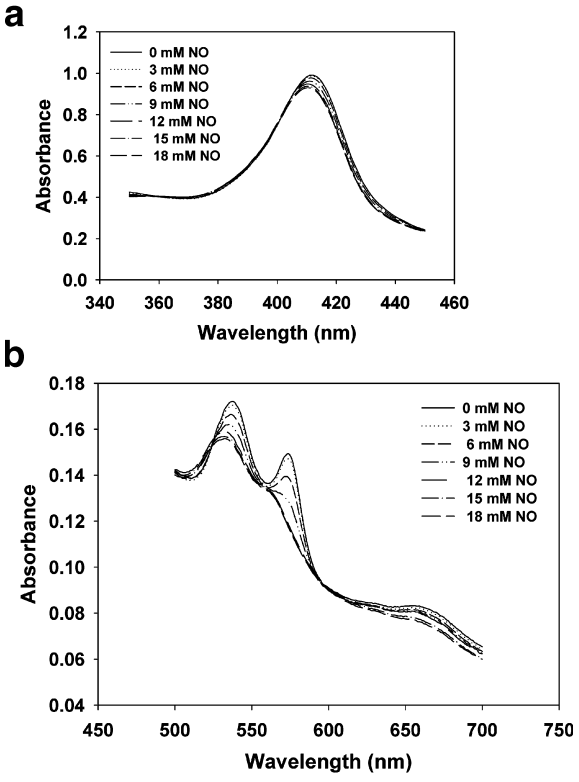

Fig. 5a,b Visible spectra of the reaction of recombinant maize $MHbO_2$ with NO. Absorption spectra were recorded after repeated addition of 3 μM NO, in the form of DEANO, to a 12-μM hemoglobin solution. **a** 350–450 nm; **b** 500–700 nm

appreciable difference in the spectra in the presence or absence of NADH, in contrast to observations with *Ascaris* Hb (Minning et al. 1999).

Nitrogen source for NO formation

NO synthesis in plants has been shown to occur both via NOS (Cueto et al. 1996; Chandok et al. 2003) and nitrate reductase (Dean and Harper 1986). The substrates

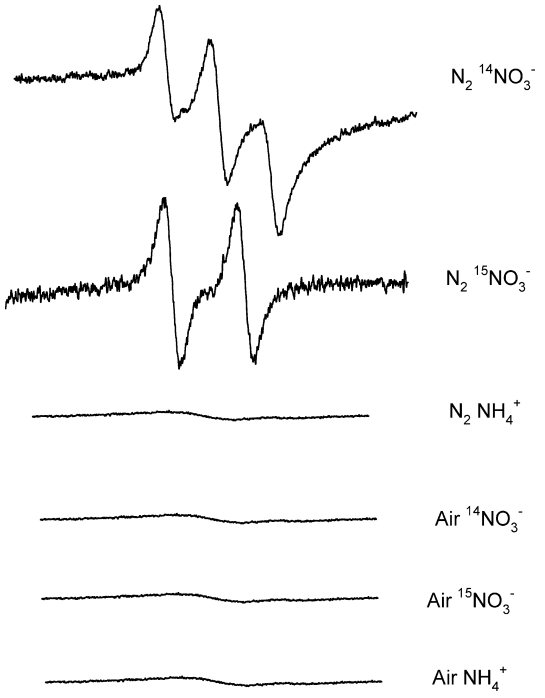

Fig. 6 EPR spectra of NO trapped by Fe^{2+}–$(MGD)_2$. Maize cell cultures were pre-cultured in medium containing NH_4^+ as the only source of N. After the cells were washed they were divided into three groups, one in which the medium contained $^{14}NO_3^-$, one with $^{15}NO_3^-$ and the other with NH_4^+ as N source. Maize cell cultures were exposed to an N_2 atmosphere or to air for 24 h. After 24 h a 15-μl sample was taken for EPR analysis

for the two enzymes differ considerably. This difference was used to determine whether nitrate, via nitrate reductase, was a substrate for NO formation during hypoxia. There was no evidence of NO formation during hypoxia in the transgenic maize suspension cultures when they were pre-cultured in NH_4^+ medium lacking nitrate. When the pre-cultured NH_4^+ cell cultures were exposed to $^{15}NO_3^-$, however, the characteristic three-line spectrum of ^{14}NO was replaced by the two-line spectrum characteristic of ^{15}NO (Fig. 6).

Discussion

NO involvement in mammalian metabolism has been known for a number of years. Its involvement in plant biology, however, has only recently been investigated, with the majority of the studies being related to plant–microbe interactions (Durner and Klessig 1999). Previous work has shown that NO is formed under anoxic conditions in alfalfa root cultures. The relatively large amounts of NO produced over a period of 24 h in maize cell-suspension cultures grown under anoxic conditions (Fig. 3) demonstrate that this phenomenon occurs both in dicots and monocots and is likely a common occurrence in plants undergoing hypoxic and anoxic stress. The levels found represent only NO trapped by the trapping agent and, therefore, likely represent a lower limit of the rates of

production. Between 6 h and 24 h after exposure to hypoxia, the minimum rate of formation of NO, based on the Hb^- cells, is about 0.4 nmol (g FW)$^{-1}$ min^{-1}. This is lower than the 1.0–1.5 nmol (g FW)$^{-1}$ min^{-1} observed upon bacterial treatment of *Arabidopsis* cell-suspension cultures (Clarke et al. 2000), but it is within the range where the NO effect on plant tissue might be similar. Clarke et al. (2000) propose that NO is acting as a signal molecule, through its action on guanylate cyclase, to initiate programmed cell death. Programmed or selective cell death is a process that may also be involved in root aerenchyma formation (Drew 1997). Inhibitors of signal transduction pathways leading to cGMP formation have been shown to inhibit aerenchyma formation (Drew et al. 2000). The experiments with alfalfa root cultures gave evidence of root cell death in association with high levels of NO in the tissue.

The levels of NO under low oxygen tension in the various cell cultures were inversely related to the hemoglobin content. The Hb^+ line, expressing high levels of hemoglobin, had the lowest accumulated NO after 24 h under low oxygen tension, whereas the Hb^- line, with the lowest hemoglobin after 24 h, had the highest NO levels. These results are consistent with previous findings with alfalfa root cultures and suggest that the NO is reacting with hemoglobin. NO reacts rapidly with oxyhemoglobin forming nitrate and methemoglobin (Doyle and Hoekstra 1981), a reaction that also occurs with class-1 hemoglobins (Fig. 4, 5). We have evidence of an NO dioxygenase-like activity in alfalfa root cultures (Igamberdiev et al. 2004) that may be responsible for the catalysis of this reaction.

How NO is formed during hypoxia or anoxia and its fate relative to hemoglobin are critical questions for elucidating the function of these hemoglobins in metabolism. NO synthase (NOS) has been detected in plants (Ribeiro et al. 1999; Caro and Puntarulo 1999). NO generation by NOS, however, may be limited under hypoxic or anoxic conditions by its requirement for oxygen and L-arginine. The NOS K_m for oxygen can, however, be as low as 5 μM (Le Cras and McMurtry 2001). Nitrate reductase is another potential contributor to NO production (Yamasaki and Sakihama 2000), and is also activated during hypoxia (Glaab and Kaiser 1993). Adding further support to this argument is the observation that nitrate has been shown to impart protection to a number of higher plants under hypoxic conditions (Arnon 1937; Fan et al. 1988). Nitrate reductase has the further advantage that oxygen is not required in the reaction and two moles of NAD(P)H are consumed per mole of NO formed. The absence of NO production in anoxic maize cell-suspension cultures utilizing NH_4^+ in place of NO_3^- as the nitrogen source and the observation that ^{15}NO is produced from $^{15}NO_3^-$ under the same conditions (Fig. 6), are evidence that NO is derived from NO_3^- probably through nitrate reductase and not from an NOS.

Both NO (Figs. 2, 3) and hemoglobin (Taylor et al. 1994) are increased in tissues in a similar time frame after exposure to hypoxia or anoxia. It has also been shown that the presence of hemoglobin in hypoxic maize cells has a positive effect on their energy charge (Sowa et al. 1998). Mitochondria are one of the earliest targets of NO in mammalian tissue, interfering with cytochrome oxidase (Cooper and Davies 2000), and it has been shown that NO affects plant mitochondrial function in a similar fashion (Caro and Puntarulo 1999; Zottini et al. 2002). The observed effect of the presence of hemoglobin on energy charge may reflect the modulation of NO levels by hemoglobin, resulting in reduced inhibition of mitochondrial respiration. Transformed alfalfa roots, constitutively expressing hemoglobin, maintained growth during short-term hypoxia, whereas WT roots or roots transformed to reduce hemoglobin expression failed to do so. Hemoglobin and NO may also contribute to the maintenance of glycolytic flux through regeneration of NAD^+ (Hill 1998; Dordas et al. 2003b). NO formation from nitrate reductase utilizes two moles of NAD(P)H per mole of NO formed. Breakdown of NO by oxyhemoglobin produces methemoglobin. The methemoglobin can be regenerated to hemoglobin by methemoglobin reductase (Bashirova et al. 1979) and NAD(P)H to take part in another cycle of NO breakdown. In the synthesis and breakdown of one mole of NO with regeneration of hemoglobin, therefore, three moles of NAD(P)H would be utilized. We have evidence (Igamberdiev et al. 2004) that alfalfa root cultures overexpressing hemoglobin have lower NADH/NAD and NADPH/NADP ratios than control or underexpressing lines, which is consistent with this hypothesis.

The combined evidence suggests that hemoglobin modulation of NO levels may be intimately linked in the short-term survival of plant tissue under hypoxic or anoxic conditions.

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References

Appleby CA (1992) The origin and functions of haemoglobin in plants. Sci Prog 76:365–398

Arnon DI (1937) Ammonium and nitrate nutrition of barley at different seasons in relation to hydrogen-ion concentrations, manganese, copper and oxygen supply. Soil Sci 44:91–113

Arredondo-Peter R, Hargrove MS, Sarath G, Moran JF, Lohrman J, Olson JS, Klucas RV (1997) Rice hemoglobins: gene cloning, analysis and oxygen-binding kinetics of a recombinant protein synthesized in *Escherichia coli*. Plant Physiol 115:1259–1266

Arredondo-Peter R, Hargrove MS, Moran JF, Sarath G, Klucas RV (1998) Plant hemoglobins. Plant Physiol 118:1121–1126

Bashirova NF, Melik-sarkisian SS, Kretovich VL (1979) Methemoglobin reductase from lupine nodules. Purification and properties (in Russian). Biokhimiya 44:1240–1245

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 72:248–254

Caro A, Puntarulo S (1999) Nitric oxide generation by soybean embryonic axes. Possible effect on mitochondrial function. Free Rad Res [Suppl] 31:205–212

Chandok MR, Ytterberg AJ, van Wijk KJ, Klessig DF (2003) The pathogen-inducible nitric oxide synthase (iNOS) in plants is a variant of the P protein of the glycine decarboxylase complex. Cell 113:469–482

Chen Y, Rosazza JP (1994) A bacterial nitric oxide synthase from a *Nocardia* species. Biochem Biophys Res Commun 203:1251–1258

Clarke A, Desikan R, Hurst RD, Hancock JT, Neill SJ (2000) NO way back: nitric oxide and programmed cell death in *Arabidopsis thaliana* suspension cultures. Plant J 24:667–677

Cooper CE (1999) Nitric oxide and iron proteins. Biochim Biophys Acta 1411:290–309

Cooper CE, Davies NA (2000) Effects of nitric oxide and peroxynitrite on the cytochrome oxidase *K*(m) for oxygen: implications for mitochondrial pathology. Biochim Biophys Acta 1459:390–396

Cueto M, Hernandez-Perera O, Martin R, Bentura ML, Rodrigo J, Lamas S, Golvano MP (1996) Presence of nitric oxide synthase activity in roots and nodules of *Lupinus albus*. FEBS Lett 398:159–164

Dean JV, Harper JE (1986) Nitric oxide and nitrous oxide production by soybean and winged bean during the in vivo nitrate reductase assay. Plant Physiol 82:718–723

Dean JV, Harper JE (1988) The conversion of nitrite to nitrogen oxide(s) by the constitutive NAD(P)H-nitrate reductase enzyme from soybean. Plant Physiol 88:389–395

Delledonne M, Xia Y, Dixon RA, Lamb C (1998) Nitric oxide functions as a signal in plant disease resistance. Nature 394:585–588

Dordas C, Hasinoff BB, Igamberdiev AU, Manach N, Rivoal J, Hill RD (2003a) Expression of a stress-induced hemoglobin affects NO levels produced by alfalfa root cultures under hypoxic stress. Plant J 35:763–770

Dordas C, Rivoal J, Hill RD (2003b) Plant haemoglobins, nitric oxide and hypoxic stress. Ann Bot 91:173–178

Doyle MP, Hoekstra JW (1981) Oxidation of nitrogen oxides by bound dioxygen in hemoproteins. J Inorg Biochem 14:351–358

Drew MC (1997) Oxygen deficiency and root metabolism: injury and acclimation under hypoxia. Annu Rev Plant Physiol Plant Mol Biol 48:223–250

Drew MC, He II, Morgan PW (2000) Programmed cell death and aerenchyma formation in roots. Trends Plant Sci 5:123–127

Duff SMG, Wittenberg JB, Hill RD (1997) Expression, purification, and properties of recombinant barley (*Hordeum* sp.) hemoglobin: optical spectra and reactions with gaseous ligands. J Biol Chem 272:16746–16752

Duff SMG, Guy PA, Nie X, Durnin DC, Hill RD (1998) Haemoglobin expression in germinating barley. Seed Sci Res 8:431–436

Durner J, Klessig DF (1999) Nitric oxide as a signal in plants. Curr Opin Plant Biol 2:369–374

Eich RF, Li T, Lemon DD, Doherty DH, Curry SR, Aitken JF, Mathews AJ, Johnson KA, Smith RD, Phillips GN Jr, Olson JS (1996) Mechanism of NO-induced oxidation of myoglobin and hemoglobin. Biochemistry 35:6976–6983

Fan TWN, Higashi RM, Lane AN (1988) An in vivo ^{1}H and ^{31}P NMR investigation of the effect of nitrate on hypoxic metabolism in maize roots. Arch Biochem Biophys 266:592–606

Gardner PR, Gardner AM, Martin LA, Salzman AL (1998) Nitric oxide dioxygenase: an enzymic function for flavohemoglobin. Proc Natl Acad Sci USA 95:10378–10383

Glaab J, Kaiser WM (1993) Rapid modulation of nitrate reductase in pea roots. Planta 191:173–179

Hausladen A, Gow AJ, Stamler JS (1998) Nitrosative stress: metabolic pathway involving the flavohemoglobin. Proc Natl Acad Sci USA 95:14100–14105

Hill RD (1998) What are hemoglobins doing in plants? Can J Bot 76:707–712

Hunt PW, Watts RA, Trevaskis B, Llewelyn DJ, Burnell J, Dennis ES, Peacock WJ (2001) Expression and evolution of functionally distinct haemoglobin genes in plants. Plant Mol Biol 47:677–692

Igamberdiev AU, Seregélyes C, Manac'h N, Hill RD (2004) NADH-dependent metabolism of nitric oxide in alfalfa root cultures expressing barley hemoglobin. Planta (in press)

Kalyanaraman B (1996) Detection of nitric oxide by electron spin resonance in chemical, photochemical, cellular, physiological, and pathophysiological systems. Methods Enzymol 268:168–187

Kotake Y (1996) Continuous and quantitative monitoring of rate of cellular nitric oxide generation. Methods Enzymol 268:222–229

Le Cras TD, McMurtry IF (2001) Nitric oxide production in the hypoxic lung. Am J Physiol Lung Cell Mol Physiol 280:575–582

Lira-Ruan V, Sarath G, Klucas RV, Arredondo-Peter R (2001) Synthesis of hemoglobins in rice (*Oryza sativa* var. Jackson) plants growing in normal and stress conditions. Plant Sci 161:279–287

Minning DM, Gow AJ, Bonaventura J, Braun R, Dewhirst M, Goldberg DE, Stamler JS (1999) *Ascaris* haemoglobin is a nitric oxide-activated 'deoxygenase'. Nature 401:497–502

Nie XZ, Hill RD (1997) Mitochondrial respiration and hemoglobin gene expression in barley aleurone tissue. Plant Physiol 114:835–840

Ribeiro EA Jr, Cunha FQ, Tamashiro WM, Martins IS (1999) Growth phase-dependent subcellular localization of nitric oxide synthase in maize cells. FEBS Lett 445:283–286

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Schmidt HH, Walter U (1994) NO at work. Cell 78:919–925

Sowa A, Duff SMG, Guy PA, Hill RD (1998) Altering hemoglobin levels changes energy status in maize cells under hypoxia. Proc Natl Acad Sci 95:10317–10321

Stamler JS, Jia L, Eu JP, McMahon TJ, Demchenko IT, Bonaventura J, Gernert K, Piantadosi CA (1997) Blood flow regulation by S-nitrosohemoglobin in the physiological oxygen gradient. Science 276:2034–2037

Taylor ER, Nie XZ, MacGregor AW, Hill RD (1994) A cereal haemoglobin gene is expressed in seed and root tissues under anaerobic conditions. Plant Mol Biol 24:853–862

Trevaskis B, Watts RA, Andersson C, Llewellyn D, Hargrove MS, Olson JS, Dennis ES, Peacock WJ (1997) Two hemoglobin genes in *Arabidopsis thaliana*: The evolutionary origins of leghemoglobins. Proc Natl Acad Sci 94:12230–12234

Weber RE, Vinogradov SN (2001) Nonvertebrate hemoglobins: functions and molecular adaptations. Physiol Rev 81:569–628

Wendehenne D, Pugin A, Klessig DF, Durner J (2001) Nitric oxide: comparative synthesis and signaling in animal and plant cells. Trends Plant Sci 6:177–183

Weger HG, Turpin DH (1989) Mitochondrial respiration can support NO^{3-}, and NO^{2-} reduction during photosynthesis. Plant Physiol 89:409–415

Yamasaki H, Sakihama Y (2000) Simultaneous production of nitric oxide and peroxynitrite by plant nitrate reductase: in vitro evidence for the NR-dependent formation of active nitrogen species. FEBS Lett 468:89–92

Zottini M, Formentin E, Scattolin M, Carimi F, LoSchiavo F, Terzi M (2002) Nitric oxide affects plant mitochondrial functionality in vivo. FEBS Lett 515:75–78