Hemoglobin expression affects ethylene production in maize cell cultures

Nathalie Manac’h-Little, Abir U. Igamberdiev, Robert D. Hill *

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

Received 21 September 2004; accepted 9 March 2005

Abstract

The formation of ethylene under different O2 concentrations and upon addition of nitric oxide (NO) donor, sodium nitroprusside (SNP), was determined using maize (Zea mays L.) cell lines over-expressing (Hb+) or down-regulating (Hb–) hypoxically inducible (class-1) hemoglobin (Hb). Under all treatments, ethylene levels in the Hb– line were 5 to 6.5 times the levels in Hb+ and four to five times the levels in the wild type. Low oxygen partial pressures impaired ethylene formation in maize cell suspension cultures. 1-Amino-cyclopropane-1-carboxylic acid (ACC) oxidase (E.C. 1.14.17.4) mRNA levels did not vary, either between lines or between treatments. There was, however, significantly enhanced ACC oxidase (ACO) activity in the Hb– line relative to the wild type and the Hb+ line. ACO activity in the Hb– line increased under hypoxic conditions and significantly increased upon treatment with NO under normoxic conditions. The results suggest that limiting class-1 hemoglobin protein synthesis increases ethylene formation in maize suspension cells, possibly via the modulation of NO levels.

© 2005 Published by Elsevier SAS.

Keywords: ACC oxidase; Ethylene; Hemoglobin; Hypoxia/nitric oxide; Zea mays

1. Introduction

Various stress situations can trigger significant changes in ethylene production and accumulation in plant cells [25]. Elevated ethylene concentrations have been reported in aerial parts of flooded plants in a variety of species including Lycopersicon esculentum [16,32] and Rumex palustris [4,30]. Flooding can result in partial or complete submergence of plant organs leading to a decrease in oxygen availability. Diffusion of gases is 10,000 times slower in water compared with air; therefore, submergence of plant organs results in restricted gas exchange between plant tissues and their surrounding environment [14,23]. Under low oxygen pressures, there is a shift to anaerobic fermentation from respiration causing ATP levels to drop considerably. Plant survival requires regulation of the processes supplying the energy (e.g. carbohydrate catabolism) and those involving a consumption of ATP (e.g. substrate biosynthesis and phosphorylation) [17].

Ethylene is a gaseous hormone involved in plant maturation, aging, cell development, cell elongation and response to stress. Under low oxygen pressures, ethylene can be responsible for triggering processes such as aerenchyma formation, root and stem elongation or adventitious root growth [23] that assist the plant in avoiding the low oxygen pressure [1]. The key enzymes involved in ethylene biosynthesis have been isolated and studied in a number of higher plant species. The formation of 1-aminocyclopropane-1-carboxylic acid (ACC) from S-adenosylmethionine is catalyzed by ACC synthase (ACS; E.C. 4.4.1.14). ACC is then oxidized to ethylene by ACC oxidase (ACO; E.C. 1.14.17.4) [33]. An increase in ethylene levels has been observed in plant tissues under hypoxia as a result of ACC-enhanced biosynthesis and accumulation in submerged tissues [19,23]. The gaseous hormone is involved in the formation of aerenchyma in the hypoxic roots of maize, facilitating the diffusion of O2 throughout the plant [15].

Two major metabolic events have been linked to Hb expression: the first, prevention of NO accumulation [12] and the second, maintenance of the energy charge [29]. In alfalfa roots and maize cell suspension cultures, nitric oxide (NO) levels increase under low oxygen pressure. A possible role for Hb in metabolizing NO in the plant cell has been suggested [22].
Nitric oxide has been shown to act as a signal molecule in animal cells as well as in plants in response to biotic and abiotic stresses and in relation to other messengers such as reactive oxygen species (ROS) or ethylene [5,26]. Ethylene has been implicated in the programmed cell death response leading to root aerenchyma formation during hypoxia [15]. Interestingly, alfalfa roots over-expressing barley Hb exhibited almost no sign of cell disruption under 3 kPa O₂, whereas Hb– lines showed strong evidence of cell death characteristic of aerenchyma formation [12], which was correlated to a significant drop in energy charge.

We wished to further investigate the relationship between Hb expression, NO and plant cell response to low oxygen partial pressures by examining ethylene formation under the same conditions that have been used to examine energy status and NO formation. We measured the formation of ethylene in maize cells experiencing different oxygen concentrations, with or without addition of exogenous NO via sodium nitroprusside (SNP) treatment. We also determined the levels of expression of the ACO transcript, as well as the activity of the enzyme. Using maize cell lines expressing various levels of hemoglobin, we also determined the effect of Hb on the ethylene production under low oxygen.

2. Results

2.1. Varying the levels of barley class-I hemoglobin in maize influences the ethylene levels in cell suspension cultures in oxygenic and hypoxic conditions

Hb– maize suspension cells produced significantly more ethylene after 6 h than either WT or Hb+ cells (Fig. 1), with levels being 4 to 6.5-fold higher. The Hb+ line generally had lower levels of ethylene than the WT (3–38%) but the difference between these two lines was significant only when SNP was added at 3 kPa O₂.

Low oxygen partial pressure (3 kPa O₂) caused a decrease of approximately 37–40% in ethylene production in all maize lines (Fig. 1), regardless of the presence of SNP. In most instances, the effect of treatment with SNP on ethylene levels was insignificant, although there appeared to be a slight positive effect in WT and Hb– cells.

2.2. ACC oxidase gene expression is not affected in hypoxic and SNP-treated maize cells

To determine whether the changes in the levels of ethylene were a result of variations in ACO gene expression, transcript levels of ACO were measured by RT-PCR using total RNA isolated from the cell samples. An actin probe was used as an internal control. Two sets of oligonucleotides were used in the RT-PCR reactions (see Materials and Methods). No significant changes were observed in ACO mRNA levels from any of the maize cells lines regardless of the treatments (Fig. 2).

Because ACO gene transcript levels were unaffected by low oxygen or SNP treatments (Fig. 3), variation in the enzyme activity was investigated as a possible explanation for the observed changes in ethylene levels. Exposure to hypoxia or addition of exogenous NO had no significant effect on ACO enzyme activities in the WT and the cells over-expressing barley hemoglobin (Fig. 3). Enzyme activities were significantly higher in the Hb– line than in the WT or Hb+ line. Hypoxia tended to increase ACO activity, while SNP treatment caused a significant 2.5-fold increase in activity in the cells down-regulating hemoglobin levels.
Two key enzymes, ACS and ACO, regulate ethylene biosynthesis [16,35]. After a few hours of flooding, root tissues experience an increase in ACS activity resulting in the accumulation of ACC but its conversion to ethylene is suppressed due to oxygen unavailability [24]. On this basis, in a hypoxic cell suspension culture with a controlled and restricted oxygen supply, the limiting step in ethylene formation will likely be the oxidation of ACC rather than its production. ACO mRNA levels in the maize cell suspensions after 6 h of treatment did not significantly vary regardless of the cell line, the available oxygen or the presence of SNP (Fig. 2). Lower ACO enzyme activities are observed when the enzyme is assayed in the presence of 3 kPa O₂ [7,34], a consequence of the enzyme’s dependence on oxygen. The $K_m$ for oxygen of the reaction is 10–11 kPa [3] indicating that the enzyme activity will be negatively affected even at moderate oxygen concentrations.

The significant difference between the ACO enzyme activity between the Hb– line and either the WT or Hb+ line, as well as the significant increase in ACO activity of the Hb– line upon SNP treatment suggest a link between NO and increased ACO enzyme activity. There are several pieces of evidence that support this argument. Dordas et al. [13] have demonstrated that NO accumulation during hypoxia is greater in Hb– maize cell lines than in WT or Hb+ lines. Addition of NO to the Hb– maize cells triggered a 2.5-fold increase in ACO activity (Fig. 3), in agreement with the data that show hypoxia contributes to the increased levels of ACO activity [20,21]. The effect of externally added SNP on ethylene levels was not always detectable. It was small even in the wild type lines, where some stimulation of the ethylene production could be seen (Fig. 1).

Ethylene production was always high in Hb– lines, even under oxygenic conditions when NO does not accumulate in significant amounts [13]. This suggests that the relationship between NO levels and ethylene are complex. Ethylene, auxin, abscisic acid and gibberellin have been shown to be involved in regulating hyponastic growth in submerged $R$. palustris petioles [10]. With each of these hormones, nitric oxide is purported to be a component of the hormone signal transduction pathway [11,18,27]. The ACC limitation in conditions of high ACC activity and high ethylene production as in Hb– lines (Figs. 1,3) could be an important factor explaining why e.g. higher ACO activity in SNP-treated Hb– cells does not lead to a significant increase of ethylene production. Limitation by ACC is an essential factor of post-transcriptional regulation of ethylene biosynthesis and ethylene synthesis is dependent on locally generated ACC [9] when it is not restricted by ACO activity.

We have shown a positive influence of Hb under-expression on ethylene biosynthesis during hypoxic stress that may be, at least in part, due to regulatory effects of NO on ethylene biosynthesis and ethylene perception. Further work is needed to assess the mechanisms by which hemoglobin expression mediates ethylene formation under hypoxia, contributing to the adaptation of plants to low oxygen partial pressures.

4. Methods

4.1. Cell cultures and experimental treatments

Maize (Zea mays) cells (BMS) over-expressing (Hb+) and down-regulating (Hb–) class-1 non-symbiotic barley hemoglobin were maintained as suspension cultures as previously described [29]. Before treating the cells, the flasks were flushed overnight with 40 kPa O₂ to ensure normoxic conditions.
tions in the culture medium. All treatments were performed under sterile conditions. One to 1.5 ml of cells was transferred with 3 ml of incubation buffer (50 mM Tris–HCl pH 5.8 and 20 g 1−1 sucrose) to an 8-ml vial sealed with a rubber serum stopper. Normoxic treatments consisted of flushing the vials with 40 kPa O2 for 60 s every hour, via a needle inserted through the septum, with another needle providing an outflow. For hypoxic treatments, vials were flushed with 3 kPa O2. Treatment with NO was performed by adding SNP to the incubation buffer at a final concentration of 100 µM. Vials were placed on a rotator (10 rpm) for 6 h at room temperature and illuminated to promote NO release from SNP. Cells were collected by vacuum filtration at the end of the treatment. The fresh weight of each sample was determined before freezing the cells in liquid nitrogen and tissue was stored at −80 °C. All cultures were vigorously shaken throughout the treatments (6 h) to minimize the limitation of oxygen diffusion in cells. This was important since the degree of O2 deficiency critically depends on the turbulence [2].

### 4.2. Ethylene measurements

A gas sample (1 ml) was withdrawn from the vial headspace with a syringe at 1 h intervals up to 6 h and assayed for ethylene by gas chromatography (Poropak T column and a flame ionization detector, Carle Analytical Gas Chromatograph, USA) for ethylene measurement. After ethylene was measured, the vials were purged with 40 kPa or 3 kPa O2 as described above. This allowed renewal of the gas mixture inside the vial, preventing ethylene accumulation and positive or negative feedback of its own biosynthesis.

### 4.3. RNA isolation and RT-PCR

Total RNA was isolated from maize cell samples according to a modified version of the Tri-reagent method [8]. Cells (100 mg FW) were ground in liquid nitrogen with a prechilled mortar and pestle and transferred to a microcentrifuge tube. After addition of 1 ml of extraction buffer (2 M guanidine thiocyanate, 12.5 mM sodium citrate pH 7.0, 0.25% sarcosyl, 50 mM β-mercaptoethanol, 50% water saturated phenol, 200 mM potassium acetate pH 4.8), the content of the tube was homogenized by vortexing and left at room temperature for 3 min. The tube was centrifuged at 10,000 × g at 4 °C for 15 min. The aqueous supernatant was collected and transferred to a new microcentrifuge tube and RNA was precipitated by the addition of 0.5 ml isopropanol. The RNA pellet was recovered after centrifugation at 10,000 × g at 4 °C for 5 min and washed with 70% ethanol, dried and resuspended in 20 µl of diethylpyrocarbonate-treated water. Total RNAs were treated with proteinase K (Sigma) and DNase I (Gibco BRL).

Using 1 µg of total RNA sample, RT-PCR reactions were carried out according to the manufacturer’s instructions (Superscript One-Step RT-PCR with Platinum Taq, Invitrogen) in a Programmable Thermal Controller (PTC-100, MJ Research, Inc., USA) The following oligonucleotide sequences were used: ACCOXF 5′gtgccgagaactgggg3′ and ACCOXR 5′ccactcgccgtctc3′ for ACO, ACTINF 5′gcctgttccctgta3′ and ACTINR 5′ctctgggacctgaacc3′ for actin. The first set of primers was designed from conserved areas identified on the alignment of ACO gene sequences from \( Z. mays \) and \( Oryza sativa \) (Genebank accession numbers \( \text{AY359573} \) and \( \text{X85747} \)).

### 4.4. In vitro ACC oxidase enzyme activity

ACO enzyme activity was determined according to a modified protocol from Vriezen et al. [31]. Frozen maize cells (200–300 mg FW) were ground and homogenized with 750 µl of extraction buffer (300 mM Tris–HCl pH 7.2, 30 mM ascorbic acid, 10% glycerol). The homogenate was transferred to a microcentrifuge tube and 750 µl of buffer were added. The tube was centrifuged at 12,000 × g at 4 °C for 10 min. The supernatant (crude protein extract) was recovered and placed on ice.

In an 8-ml vial, 200 µl of crude extract was added to 1.7 ml of incubation buffer (100 mM Tris–HCl pH 7.2, 30 mM ascorbic acid, 10% glycerol, 5 mM DTT) with 50 µl of 80 mM ACC, 50 µl of 3 mM FeSO4, and 100 µl of 1 M NaHCO3. The vial was hermetically sealed and incubated for 1 h at 28 °C.

Ethylene was measured from a 1 ml sample taken from the head space in the vial. A correction was applied for each measurement to account for the formation of ethylene by the sample in the absence of ACC.

Protein concentration was measured using the method described by Bradford [6] using BSA as a standard.

When ACO enzyme activity was measured under different oxygen atmospheres, sealed vials were purged as described for ethylene measurements with either 3 kPa O2 or 40 kPa O2 prior to incubation at 28 °C.

### Acknowledgments

The authors wish to thank Doug Durnin for helpful technical assistance. Financial support from the Natural Sciences and Engineering Research Council of Canada and Genome Canada is gratefully acknowledged.

### References

4. In vitro ACC oxidase enzyme activity


