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ORIGINAL ARTICLE

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Class-1 hemoglobin and antioxidant metabolism in alfalfa roots

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Abstract In the course of nitric oxide (NO) scavenging, hemoglobin (Hb) turnover is linked to antioxidant metabolism and affects the cellular redox level. The influence of Hb presence on the ascorbate-glutathione cycle enzymes and the levels of H_2O_2 and ascorbate was investigated in alfalfa root cultures transformed to over-express (Hb+) or down-regulate (Hb–) class-1 Hb. Hb+ lines had substantially increased ascorbate levels as well as elevated monodehydroascorbate reductase and ascorbate peroxidase activities. Hb– lines showed significant increases in dehydroascorbate reductase and glutathione reductase activities. The observed changes in ascorbate and ascorbate-glutathione cycle enzymes were pronounced both at high (40 kPa) and low (3 kPa) O_2 pressures. Hb– lines had significantly reduced levels of the NO- and H_2O_2-sensitive enzyme, aconitase, as compared to Hb+ lines. This reduced activity was likely due the higher levels of NO in Hb– lines, as treatment of plant extracts with the NO-donor DEANO also affected aconitase activity. The H_2O_2 levels were not significantly different amongst the lines and showed no variation with change in oxygen partial pressure. In conclusion, the expression of class-1 Hb improves the antioxidant status through increased ascorbate levels and increased activity of enzymes involved in H_2O_2 removal.

Keywords Class-1 hemoglobin · Nitric oxide · Ascorbate · Ascorbate-glutathione cycle · Hypoxia

Abbreviations APX: Ascorbate peroxidase · DEANO: Sodium 2-(N, N-diethylamino)-diazenolate-2-oxide · DHA: Dehydroascorbate · DHAR: Dehydroascorbate reductase · GR: Glutathione reductase · GSH: Reduced glutathione · GSSG: Oxidized glutathione · Hb: Hemoglobin · MDHA: Monodehydroascorbate (ascorbate free radical) · MDHAR: Monodehydroascorbate reductase · MetHb: Methemoglobin · NO: Nitric oxide · ROS: Reactive oxygen species

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Introduction

Class-1 hemoglobins function to remove nitric oxide (NO) synthesized during oxygen deficiency in plants (Dordas et al. 2003). This can occur at O_2 partial pressures two orders of magnitude lower than the levels required for saturation of cytochrome *c* oxidase (Hill 1998; Igamberdiev and Hill 2004). In the reaction, NO is converted to nitrate and, in the process, the heme iron of the Hb molecule is oxidized to the ferric form methemoglobin (metHb). The reduction of metHb to ferrous Hb is facilitated by ascorbate (Sullivan and Stern 1982; Igamberdiev et al. 2006). There is also the possibility that the reaction of metHb with ascorbate produces hydroxyl radicals, which disproportionate to H_2O_2 (Benatti et al. 1983).

The ascorbate-glutathione cycle is one of the major pathways, by which reactive oxygen species (ROS) are degraded in the cell (Noctor and Foyer 1998). During this cycle, ascorbate peroxidase (APX) catalyzes the reaction of ascorbate with H_2O_2, forming MDHA. MDHA can be reduced back to ascorbate by monodehydroascorbate reductase (MDHAR) using NAD(P)H as a reducing agent. Alternatively, MDHA may non-enzymatically disproportionate to ascorbate and dehydroascorbate (DHA). The latter is reduced back to ascorbate by dehydroascorbate reductase (DHAR). This reaction uses reduced glutathione (GSH). The oxidized glutathione (GSSG) produced is then reduced to GSH, using NADPH as a reducing source, catalyzed by glutathione reductase (GR). Participation of ascorbate in metHb reduction suggests that it could be informative to determine the effect of varying expression of the Hb gene and the levels of NO on the components of the ascorbate-glutathione cycle.

High levels of NO and H_2O_2 can strongly affect the respiratory metabolism and antioxidant capacity of plant cells. One of the tricarboxylic acid cycle enzymes, aconitase, is inhibited by NO and/or H_2O_2 (Navarre et al. 2000). NO also inhibits cytochrome *c* oxidase and hence mitochondrial electron transport, leading to the production of ROS including H_2O_2 (Møller 2001). Class-1 Hb involvement in regulating cellular levels of NO has been demonstrated (Dordas et al. 2003; Perazzolli et al. 2004) and expression of the gene has been shown to improve the energy status of cells under hypoxic conditions (Sowa et al. 1998), suggesting that Hb presence, through regulation of ROS, may influence both respiratory metabolism and antioxidant capacity in the cell.

In this study, we investigated how different levels of class-1 Hb expression in alfalfa root cultures affect antioxidant levels and the enzymes associated with antioxidant metabolism. Marked increases in ascorbate levels, H_2O_2-scavenging enzymes and MDHAR were observed in root cultures over-expressing class-1 Hb. On the other hand, DHAR and GR exhibited higher activities in Hb down-regulating lines. Aconitase activity decreased rapidly in the presence of an NO donor or H_2O_2.

Materials and methods

Plant material

Cultured alfalfa roots either over-expressing class-1 hemoglobin (Hb+, lines 3 and 209) or down-regulating hemoglobin (Hb–, lines 24 and 44) were transformed from the control line (C) using *A. rhizogenes* A4 containing the appropriate constructs and maintained as described earlier (Dordas et al. 2003). The levels of Hb in Hb+ lines was 5–20 times those of Hb– lines and roughly twice as high as those in control lines exposed to hypoxic conditions (Dordas et al. 2003). Roots were put into syringes with 20 ml of culture medium, pretreated under normoxic conditions (40 kPa O_2) overnight, and then incubated either at 40 (normoxia) or 3 (hypoxia) kPa O_2 for 24 h. When indicated, antimycin A (50 μM) was added to block the respiratory chain in mitochondria at the level of complex III (Møller 2001). After the treatments, the roots were weighed, immediately frozen in liquid nitrogen, and stored at –80°C until assayed.

Determination of ascorbate and dehydroascorbate

Samples were ground in a mortar pre-chilled with liquid N_2, immediately transferred to 6% trichloroacetic acid, and kept on ice until assayed. Ascorbate and DHA were measured as described in Kampfenkel et al. (1995).

Enzyme assays

APX (EC 1.11.1.11) and DHAR (EC 1.8.5.1) activities were measured at 265 nm (Arrigoni et al. 1997). MDHAR (EC 1.6.5.4) (Hossain et al. 1984) and GR (EC 1.8.1.7) (Osswald et al. 1992) activities were determined at 340 nm. Catalase (EC 1.11.1.6) activity was measured at 240 nm (Aebi 1974). Tissue homogenization was performed according to the corresponding references (Aebi 1974; Hossain et al. 1984; Osswald et al. 1992; Arrigoni et al. 1997).

For aconitase (EC 4.2.1.3) measurements, plant material (250 mg) was homogenized with 1 ml of 25 mM imidazole buffer, pH 7.4, containing 2 mM citrate, 1 mM EDTA, 2 mM DTT, 2 mM $MgCl_2$, and 10% glycerol. The extract was centrifuged at 10,000 g. Aconitase in the supernatant was measured at 340 nm using *cis*-aconitate as a substrate coupled to $NADP^+$ reduction by isocitrate dehydrogenase (Rose and O'Connel 1967). The stability of aconitase towards NO was checked by incubating plant extract at 23°C with freshly prepared 200 μM DEANO. The stability of aconitase towards H_2O_2 was investigated by incubating plant extract at 4°C with 100 μM H_2O_2.

H_2O_2 measurements

H_2O_2 was measured using a fluorescence procedure (Guilbaut et al. 1967), modified as described in Creissen et al. (1999). Root tissue (200 mg) was homogenized under liquid nitrogen, extracted with 1 ml of 25 mM HCl in a mortar and centrifuged at 8,000 g for 5 min at 4°C. For the assay, 100 μl of the supernatant was mixed in a 3 ml fluorescence cuvette with 2.87 ml of 50 mM Hepes, pH 7.5, 30 μl of 50 mM homovanillic acid in the same buffer and 3 μl of 40 μM horseradish peroxidase. The H_2O_2 concentration was obtained by measuring fluorescence (excitation at 315 nm; emission at 425 nm) against a standard curve.

Statistical analysis

All the experiments were repeated at least three times and the figures shown represent a typical experiment. Data in the text and figures are expressed as means ± SD of three replicates. Statistically significant differences according to the non-parametric unpaired *t*-test are discussed. In all cases the confidence coefficient was set at 0.05.

Results

Ascorbate and dehydroascorbate levels

Hb+ alfalfa roots maintain a significantly higher ascorbate pool under normoxic and hypoxic conditions (Fig. 1) as compared to the control and Hb– lines. There were no significant differences between ascorbate levels in control and Hb– lines. Treatment with antimycin A resulted in increased ascorbate in Hb– and control lines,

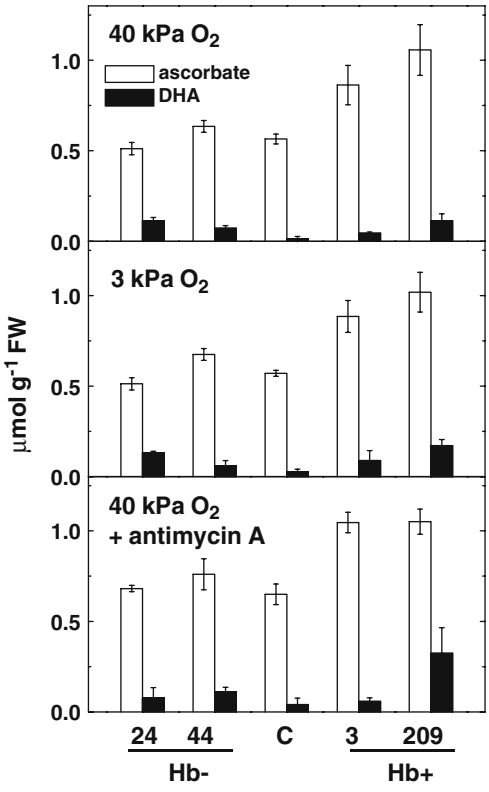

Fig. 1 Ascorbate and dehydroascorbate (DHA) levels in alfalfa lines with differential expression of barley Hb exposed to two O_2 partial pressures (40 and 3 kPa O_2) or antimycin A. *Abbreviations*: 3 and 209—Hb+ lines, 24 and 44—Hb– lines, C—control line

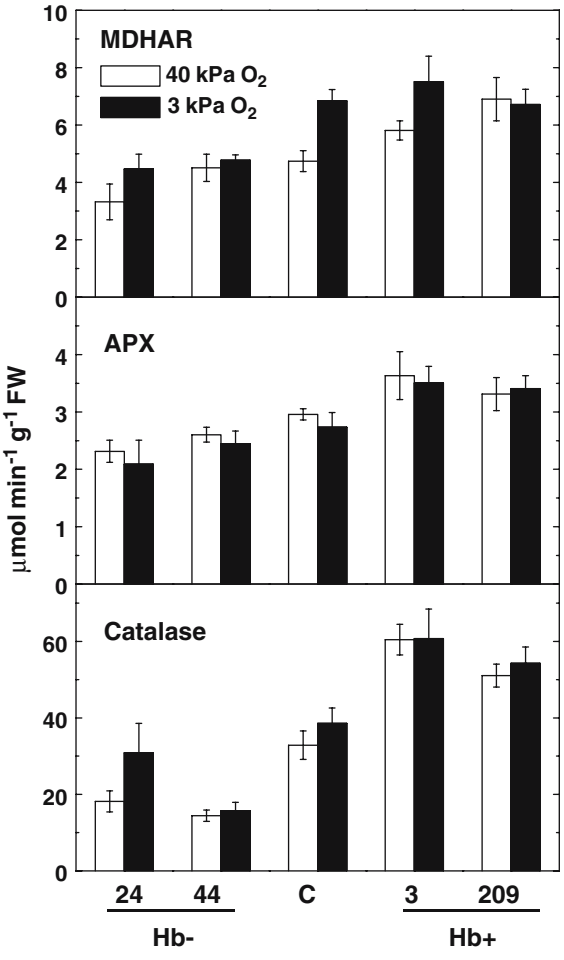

Fig. 2 Activities of monodehydroascorbate reductase (MDHAR), ascorbate peroxidase (APX) and catalase in different alfalfa lines. Abbreviations of alfalfa lines are as in Fig. 1

while the levels in Hb+ lines remained unchanged. DHA levels were considerably lower than ascorbate levels in all lines, but tended to be higher both in Hb– and Hb+ lines as compared to the control line.

Activities of the ascorbate-glutathione cycle enzymes and catalase

Within each oxygen treatment condition, the activities of MDHAR, APX and catalase were generally higher as the level of Hb increased in the line (Fig. 2). With the exception of the MDHAR activity in the control line, there were no significant differences in the activity of the three enzymes as a result of changing the oxygen partial pressure from 40 to 3 kPa. The total activity of MDHAR in the extracts of alfalfa roots was reduced in Hb– lines as compared to Hb+ and wild-type. The reduction was statistically significant at 40 kPa O_2. The differences in activity between Hb+ and Hb– lines were significant for MDHAR, APX and catalase within each treatment condition. DHAR and GR displayed opposite trends to those of APX, MDHAR and catalase, generally decreasing as Hb increased (Fig. 3). Hb– lines had significantly higher DHAR than control lines, which, in turn, were significantly higher than Hb+ lines, regardless of oxygen pressures. This was confirmed using the statistical *t*-test. GR activity was significantly higher in Hb– lines than in control or Hb+ lines. With the exception of the GR activity in the control line and DHAR in line 24, there was no significant difference in activity between oxygen treatments within lines.

Aconitase activity and stability

Aconitase activity was significantly higher in Hb+ lines than in all other lines under both oxygen treatment conditions (Fig. 4a). Aconitase activity usually exhibited lower values under hypoxic conditions, but this tendency was not statistically significant.

To determine the effect of NO on aconitase activity and the protective effect of Hb presence on the enzyme, the NO donor, DEANO, was added to extracts of an Hb+ line (3) and an Hb– line (24) (Fig. 4b). The loss of activity was significantly faster in the Hb– line. Similar effects were noted when H_2O_2 was added (Fig. 4c), although the difference in activity loss between lines was less pronounced, primarily due to a more rapid loss of

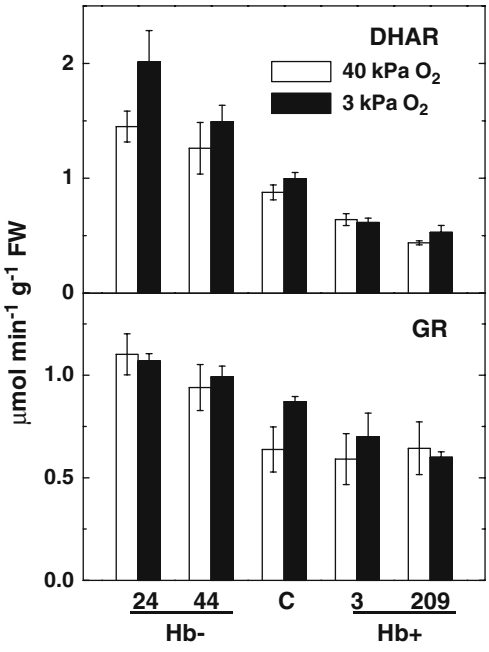

Fig. 3 Activities of dehydroascorbate reductase (DHAR) and glutathione reductase (GR) in different alfalfa lines. Abbreviations of alfalfa lines are as in Fig. 1

activity in the Hb+ line. The control line showed an intermediate rate of activity loss as compared to Hb+ and Hb–lines.

H_2O_2 in all lines was similar both at 40 and 3 kPa O_2 (Fig. 5).

Discussion

Hemoglobin expression in plants has a significant role in lowering internal NO levels (Dordas et al. 2003; Igamberdiev et al. 2004). MetHb is a product of the reaction of oxyHb with NO and for continuous NO removal regeneration of oxyHb is necessary. This can be accomplished by metHb reduction with ascorbate (Sullivan and Stern 1982; Igamberdiev et al. 2006). MDHA is formed in this reaction, which is removed via the ascorbate-glutathione cycle. The results obtained for ascorbate in Hb+ and Hb– alfalfa lines (Fig. 1) show a relationship between Hb expression and ascorbate in the tissue. The reported K_m value (0.6 mM) for ascorbate in the reaction of metHb reduction (Igamberdiev et al. 2006) is of the same order of magnitude as ascorbate concentration in the investigated lines, assuming that the water content of 1 g of tissue fresh weight is ~0.8–0.9 ml.

There are no significant shifts in the ascorbate level as the tissue is made hypoxic. DHA, however, exhibited a tendency to increase under these conditions. There were also statistically significant increases in DHA/ascorbate ratios in lines 24, control and both Hb+ lines under hypoxic as compared to normoxic conditions (not

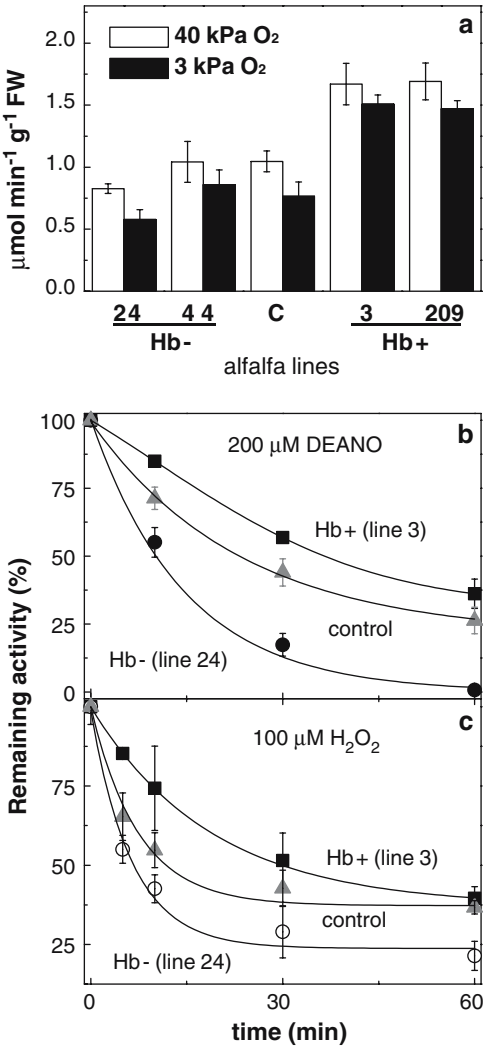

Fig. 4 Aconitase activity in different alfalfa lines (**a**) and stability towards NO (supplied by 200 µM DEANO) (**b**) and H_2O_2 (100 µM) (**c**) in extracts of Hb+ (3), control and Hb– (24) lines of alfalfa roots. Experimental conditions are described in the text

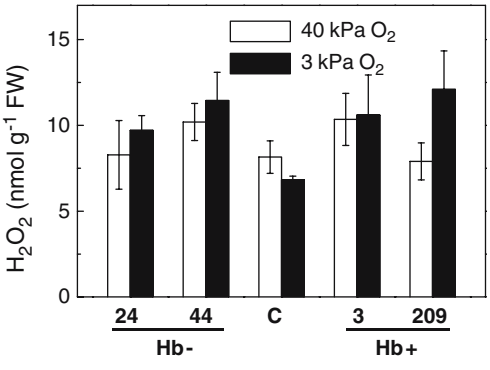

Fig. 5 H_2O_2 levels in Hb+ and in Hb– lines at different partial O_2 pressures. Abbreviations of alfalfa lines are as in Fig. 1. The results of two independent experiments (n = 3 in each) and standard deviations are presented

shown). The final step of ascorbate synthesis is linked to the mitochondrial electron transport chain (Bartoli et al. 2000). The enzyme catalyzing this step, β-galactonolactone dehydrogenase supplies electrons to cytochrome *c*. The blockage of complex III by antimycin A (Fig. 1) will inhibit the flux from respiratory substrates and correspondingly increase the capacity of complex IV to transfer electrons from galactonolactone. There is a significant increase in vitamin C (ascorbate plus DHA) upon treatment with antimycin in Hb– lines (24 and 44), while in control and Hb+ lines the effect is minor and not statistically significant. Furthermore, ascorbate synthesis appears not to be affected by increased NO as an oxygen partial pressure of 3 kPa, a concentration shown to increase root NO levels (Dordas et al. 2003), had no significant effect on tissue ascorbate. It is therefore unlikely that a change in flux through the mitochondrial portion of the ascorbate synthesis pathway due to removal of NO by Hb is responsible for the increase in ascorbate. The changes are, therefore, associated with metabolic effects of Hb expression and are independent of the events related to hypoxia. Interference with NO-mediated signal transduction (Beligni and Lamattina 2001) is one possible explanation for the observed changes.

There are also increases in MDHAR activity in Hb+ lines. MDHAR, by regenerating ascorbate, facilitates metHb reduction (Igamberdiev et al. 2006). Expression profile analysis has shown increases in MDHAR gene expression with class-1 Hb expression (Klok et al. 2002), suggesting that the changes observed in activity (Fig. 2) represent increases in enzyme protein rather than effects on enzyme kinetics.

The enzymes of the ascorbate-glutathione cycle are commonly regarded as indicators of oxidative stress, a condition that causes an increase in ascorbate and GSH oxidation (Foyer and Halliwell 1976; de Pinto et al. 2002). The enzymes responsible for the recycling of the oxidized forms of ascorbate (MDHAR and DHAR plus GR) are regulated in order to maintain the ascorbate redox state in its reduced form.

In the transformed lines, the route to recycling of the oxidized forms of ascorbate appears to be dependent on Hb expression: in Hb–, a decrease in MDHAR, relative to Hb+, is accompanied by an increase in DHAR and GR; whereas, in Hb+ an increase in MDHAR (Figs. 2 and 3) probably makes DHAR and GR less relevant for ascorbate recycling. The importance of MDHAR in Hb+ lines may also be important for MDHA radical removal to maintain Hb in its reduced form (Igamberdiev et al. 2006).

There have been reports that Hb protects plants not only from NO but also from H_2O_2 (Masuoka et al. 2003; Sakamoto et al. 2004; Yang et al. 2005). The question of the relation between Hb and H_2O_2 in vivo is unclear. On the one hand, it has been suggested that Hb can act as a peroxidase by removing H_2O_2 (Sakamoto et al. 2004). It has also been shown that H_2O_2 levels are lower when Hb is expressed (Masuoka et al. 2003). On the other hand, Hb itself can produce H_2O_2 and other ROS during its redox transitions (Benatti et al. 1983; Membrillo-Hernández et al. 1996). The reactions of Hb with ascorbate and H_2O_2 production/scavenging strongly depend on its redox state (Moreau et al. 1995). We found no significant changes in H_2O_2 related to effects of hypoxia or Hb expression (Fig. 5), so the effect of Hb on H_2O_2 levels in the cell remains questionable.

Aconitase is inactivated by NO and H_2O_2 (Navarre et al. 2000) due to the presence of FeS clusters in the molecule (Verniquet et al. 1991). Aconitase activity was higher and the decline rate in enzyme activity in the presence of either an NO donor or H_2O_2 was lower in Hb+ lines (Fig. 4). A similar tendency has been noted for plant extracts overexpressing *Vitreoscilla* Hb that was attributed to the increased ability to deplete NO with Hb present (Frey et al. 2004). The expression of *Vitreoscilla* Hb protected tobacco cells from NO but not from H_2O_2. While our experiments show an improved resistance of aconitase to H_2O_2 in Hb+ lines, this may be the result of higher catalase and APX activities rather than a direct participation of Hb in H_2O_2 removal.

In conclusion, we demonstrate that increased expression of class-1 Hbs improves the antioxidant status through increased ascorbate levels and increased activity of enzymes involved in H_2O_2 metabolism.

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