Phytochemistry 71 (2010) 2038–2045

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

The role of 5-aminolevulinic acid in the response to cold stress in soybean plants

Karina B. Balestrasse [a], María L. Tomaro [a], Alcira Batlle [b], Guillermo O. Noriega [b,*]

[a] Departamento de Química Biológica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina
[b] Centro de Investigaciones sobre Porfirinas y Porfirias (CIPYP), CONICET, Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 4 March 2010
Received in revised form 2 June 2010
Available online 2 November 2010

Keywords:
Soybean plants
Glycine max
Fabaceae
5-Aminolevulic acid
Cold stress tolerance
Antioxidant enzymes
Heme oxygenase-1

ABSTRACT

In this study, the possibility of enhancing cold stress tolerance of soybean plants (*Glycine max* L.) by exogenous application of 5-aminolevulinic acid (ALA) was investigated. ALA was added to the Hoagland solution at various concentrations ranging from 0 to 40 μM for 12 h. After ALA treatment, the plants were subjected to cold stress at 4 °C for 48 h. ALA at low concentrations (5–10 μM) provided significant protection against cold stress compared to non-ALA-treated plants, enhancing chlorophyll content (Chl) as well as relative water content (RWC). Increase of thiobarbituric acid reactive species (TBARS) levels was also prevented, whereas exposure to higher ALA concentrations (15–40 μM) brought about a dose dependent increase of these species, reaching a maximum of 117% in plants pre-treated with 40 μM ALA compared to controls. ALA pre-treatment also enhanced catalase (CAT) and heme oxygenase-1 (HO-1) activities. These findings indicate that HO-1 acts not only as the rate limiting enzyme in heme catabolism, but also as an antioxidant enzyme. The highest cold tolerance was obtained with 5 μM ALA pre-treatment. Results show that ALA, which is considered as an endogenous plant growth regulator, could be used effectively to protect soybean plants from the damaging effects of cold stress by enhancing the activity of heme proteins, e.g., catalase (CAT) and by promoting heme catabolism leading to the production of the highly antioxidant biliverdin and carbon monoxide, without any adverse effect on the plant growth.

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1. Introduction

Plants are subjected to various biotic and abiotic stresses including water, temperature, salinity, and light. Cold stress, defined as the temperature in a range low enough to suppress growth without ceasing cellular functions, is known to induce several abnormalities at various levels of cell organization. Low-temperature effects include damaged membranes (Xing and Rajashekar, 2001), reduced cellular respiration (Lee and Lur, 1997), increased abscisic acid (ABA) levels (Nayyar et al., 2005), cryoprotectants and increased reactive oxygen species (ROS) (Lee and Lur, 1997). Taking into account the fact that soybean plants are from subtropical regions, it is not surprising that they are particularly sensitive to cold. Moreover, its cultivation is successful in climates with hot summers, and temperatures below 20 °C and over 40 °C retard its growth significantly. Following the expansion of soybean plants-growing areas towards colder climates, acclimation to cold conditions has become a major research target. Furthermore, the combination of high light intensities and low temperatures, such as those experienced on cold but sunny mornings in spring, can cause irreversible damage to young soybean plants seedlings. The sensitivity of soybean to night temperatures below 15 °C is reflected in the changes that occur in metabolism, growth, development, and yield (Musser et al., 1983, 1984; Van Heerden et al., 2003). A single night of dark cold, with minimum temperatures of 8 °C, is sufficient to inhibit pod formation (Hume and Jackson, 1981). Stress tolerance has, therefore, become a major selection criterion in current soybean plants breeding programmers.

In the last years, many reports account for the involvement of heme catabolism in the response of plants to counteract the oxidative stress elicited by UV-B and salt (Yannarelli et al., 2006; Zilli et al., 2008). Heme degradation is catalyzed by heme oxygenase (HO) (Sono et al., 1996; Ortiz de Montellano, 1998, 2000; Yoshida and Migita, 2000; Ortiz de Montellano and Wilks, 2001; Colas and Ortiz de Montellano, 2003; Unno et al., 2007). This catabolism occurs by means of an oxygen-dependent reaction mechanism converting heme, sequentially, to α-*meso*-hydroxyheme, verdoheme, and biliverdin, releasing both CO and free iron. The CO released

Abbreviations: ALA, 5-aminolevulinic acid; TBARS, thiobarbituric acid reactive species; NBT, nitro blue tetrazolium; RWC, relative water content; HO-1, heme oxygenase-1; CAT, catalase; ROS, reactive oxygen species.

* Corresponding author. Address: Centro de Investigaciones sobre Porfirinas y Porfirias, Hospital Escuela "General José de San Martín", Av. Córdoba 2351, C1120 AAR Ciudad Autónoma de Bs As, Argentina. Tel.: +54 11 4964 8237; fax: +54 11 4508 3645.
E-mail address: guiller@qb.fcen.uba.ar (G.O. Noriega).

0031-9422/$ - see front matter © 2010 Published by Elsevier Ltd.
doi:10.1016/j.phytochem.2010.07.012

during HO catalysis might well be coupled to other biological processes, because CO is one of a few small, gaseous molecules that are thought to have a signaling role *in vivo* (Verma et al., 1993). The initial step in heme degradation involves a one-electron reduction of a ferrous-oxy heme species to give a ferric-hydroperoxide intermediate, followed by formation of α-*meso*-hydroxyheme. Reaction of hydrogen peroxide (H_2O_2) with the ferric form of the enzyme, to form the same ferric-hydroperoxide intermediate, also catalyzes this first step. The conversion of verdoheme to biliverdin was initially believed to be catalyzed only by reaction with oxygen (Ortiz de Montellano, 1998), but it is now known that hydrogen peroxide can also substitute for oxygen in this reaction (Matsui et al., 2008). On the other hand, 5-aminolevulinic acid (ALA) is a key precursor in the biosynthesis of porphyrins such as chlorophyll (Chl) and heme. ALA undergoes enolization and further metal-catalyzed aerobic oxidation at physiological pH to yield superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\cdot}$). Therefore, accumulation of ALA enhances reactive oxygen species (ROS) levels leading to oxidative stress and acting as a herbicide. However, it was found that low concentrations of ALA had a promotive effect on growth and a yield of several crops and vegetables (Hotta et al., 1997a,b; Bindu Roy and Vivekanandan, 1998; Watanabe et al., 2000).

In this report, factors contributing to biochemical responses are further explored that could be involved in the protection exerted by ALA against cold stress (4 °C) in soybean plants. To this end, the study of heme oxygenase-1, a newly reported antioxidant defense in plants, was undertaken.

Here, it is shown that low ALA concentrations protect against cold not only by enhancing the activity of an antioxidant hemeprotein, such as catalase (CAT), but also by up-regulating heme catabolism.

2. Results and discussion

2.1. Low ALA concentrations increased cold tolerance in soybean plants

2.1.1. Effect of low ALA concentrations on chlorophyll and water content in leaves of soybean plants subjected to cold

Experiments were carried out in an attempt to clarify the possible adaptation mechanisms of soybean plants to cold (4 °C) promoted by ALA treatment. ALA, applied over certain concentrations, acts as an herbicide (Chakraborty and Tripathy, 1992; Kumar et al., 1999). Thus, determination of an optimum concentration is a prerequisite if this compound is to be used for improving soybean resistance to low temperatures. To this end, chlorophyll (Chl) and relative water content (RWC) were determined employing different ALA concentrations ranging from 0 to 40 μM. Cold reduced by 57% Chl content respect to controls in soybean leaves (Fig. 1). Chlorophyll loss was prevented in plants pre-treated with 5 and 10 μM ALA. Results here reported also indicate that ALA concentrations above 10 μM caused Chl reduction in a dose dependent manner. On the other hand, 5 μM ALA alone did not have any effect on Chl compared to controls (data not shown). Previous studies have shown that Chl can be bleached under oxidative stress (Noriega et al., 2007). These results can be explained on one hand from the fact that ALA, at low concentrations, acts as a regulator of Chl and heme biosynthesis and, on the other hand, oxidative stress might have occurred as a result of ROS generated by higher ALA concentrations.

ALA, is the key precursor in the biosynthesis of all porphyrin compounds such as Chl and heme and its formation in plants is the rate-limiting step in tetrapyrrole biosynthesis (Von Wettstein et al., 1995; Wang et al., 2005). It is also known that ALA biosynthesis in plants is inhibited by low and high temperatures (Hodgins and Öquist, 1989; Tewari and Tripathy, 1998). In this study, pre-treatment with low ALA concentrations (5–10 μM) enhanced Chl content compared to non-ALA-treated plants under cold stress; thus, exogenous application of low ALA concentrations prior to cold stress could be a way to overcome an inadequate biosynthesis problem.

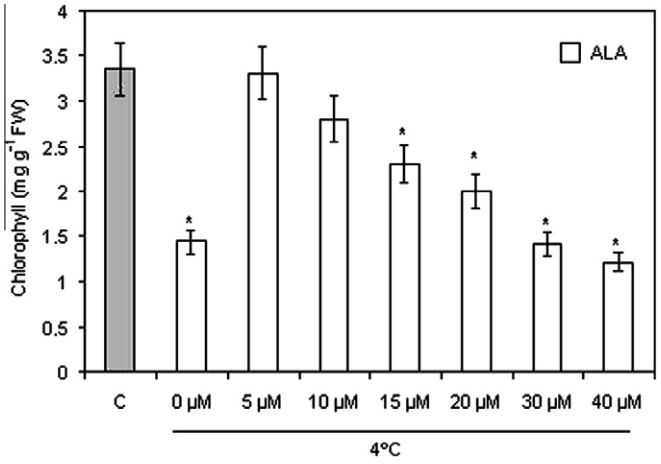

Fig. 1. Effect of 4 °C and ALA pre-treatment on chlorophyll content in soybean leaves. Experiments were performed as described in Experimental. *Significant differences ($P < 0.05$).

The RWC, as indicated by the extent of dehydration, was used to assess cellular damage. Fig. 2 shows that RWC decreased by 36% under cold respect to controls. Pre-treatment with 5, 10 and 15 μM ALA protected against this effect, whereas these concentrations alone did not have any effect on RWC when comparing to controls. Pre-treatment with 30 or 40 μM ALA showed a major RWC loss (48% and 53%, respectively) respect to controls.

2.1.2. Low ALA concentrations prevent membrane damage in leaves of soybean plants subjected to cold

Increment in thiobarbituric acid reactive species (TBARS) is a good reflection of oxidative damage to membrane lipids and other vital molecules such as proteins, DNA and RNA. In our study, TBARS levels increased 60% respect to controls under low temperature stress (Fig. 3) in agreement with results of other studies (Schwanz and Polle, 2001).

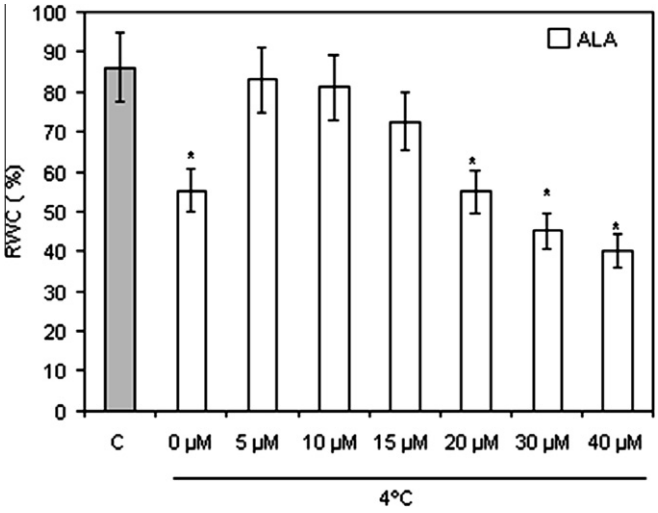

Fig. 2. Effect of 4 °C and ALA pre-treatment on RWC in soybean leaves. Experiments were performed as described in Experimental. *Significant differences ($P < 0.05$).

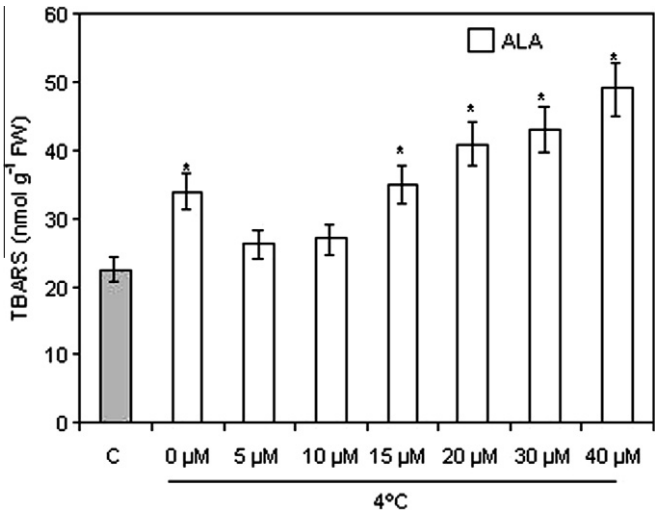

Fig. 3. Effect of 4 °C and ALA pre-treatment on soybean leaves TBARS. Experiments were performed as described in Experimental. *Significant differences ($P < 0.05$).

Peroxidation of membrane lipids may result in enhanced membrane fluidity, which may lead to enhanced electrolyte leakage and support the hypothesis that cold can induce membrane lipid peroxidation.

Pre-treatment with low ALA concentrations (5–10 μM) partially prevented the enhancement of TBARS levels, whereas exposure to higher ALA concentrations (15–40 μM) brought about a dose dependent increase, reaching a maximum of 117% in plants pre-treated with 40 μM ALA respect to controls.

2.1.3. H_2O_2 and $O_2^{\cdot-}$ determination

To obtain hints on ALA mechanism in its defense against cold, H_2O_2 and $O_2^{\cdot-}$ were determined. Table 1 shows that cold produced a 4.8-fold enhancement in H_2O_2 content respect to controls, while pre-treatment with 5 μM ALA totally prevented this effect. Nevertheless, 10 or 15 μM ALA partially avoided H_2O_2 accumulation, while higher concentrations did increase H_2O_2 formation with respect to cold treated plants. Data also show that leaves of cold treated soybean plants produced a 7.9-fold enhancement in $O_2^{\cdot-}$ content, respect to controls. Pre-treatment with 5 and 10 μM ALA completely prevented $O_2^{\cdot-}$ production induced by low temperatures, while concentrations above 15 μM ALA could only partially counteract this effect or significantly increase induced cold-$O_2^{\cdot-}$ levels (30 and 40 μM ALA).

Results here reported indicate that ROS accumulation can be induced by cold stress, which may result in injury to the plant, whereas moderate elevation of ROS could be a signal to stimulate mechanisms related to stress resistance.

Table 1
Effect of 4 °C and ALA on H_2O_2 and $O_2^{\cdot-}$ accumulation in soybean leaves.

Treatment	H_2O_2	$O_2^{\cdot-}$
Control	0.25 ± 0.02[a]	0.12 ± 0.02[a]
4 °C	1.45 ± 0.15[b]	1.07 ± 0.08[b]
5 μM ALA + 4 °C	0.24 ± 0.03[a]	0.14 ± 0.02[a]
10 μM ALA + 4 °C	0.32 ± 0.02[a]	0.17 ± 0.05[a]
15 μM ALA + 4 °C	0.77 ± 0.03[c]	0.55 ± 0.02[c]
20 μM ALA + 4 °C	1.50 ± 0.04[b]	1.08 ± 0.05[b]
30 μM ALA + 4 °C	1.85 ± 0.05[d]	1.47 ± 0.05[d]
40 μM ALA + 4 °C	2.32 ± 0.05[d]	1.52 ± 0.06[d]

Segments from the second pair of fully expanded leaves upper the cotyledons, were used for the assays. Experiments were carried out as described in Experimental. Different letters within columns indicate significant differences ($P < 0.05$), according to Tukey's multiple range test.

The present study also demonstrated that ALA is effective in enhancing cold tolerance in soybean plants up to 10 μM and that the best results were obtained in plants pre-treated with 5 μM ALA. This is the reason why the following experiments were carried out employing 5 μM ALA.

2.1.4. Low ALA concentrations effect on antioxidant enzymes activities

To further investigate the action of 5 μM ALA on cold stress in soybean plants, antioxidant enzyme activities were determined. Analyses were performed on catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione reductase (GR) enzymes in plants subjected to cold with or without ALA pre-treatment. Interestingly, Table 2 shows that the activities of CAT, SOD, and GR were increased after cold stress (26%, 35%, and 28% respect to controls) while APX was not modified.

Plants have an efficient system for decomposing ROS, using the enzymes SOD and APX in chloroplasts (Asada, 1999). SOD enzyme is located in the chloroplast, mitochondria, cytoplasm and peroxisomes, and operates as the first line of defense against ROS (Liau et al., 2007). Higher SOD activity can efficiently remove $O_2^{\cdot-}$, which leads to the production of H_2O_2, and H_2O_2 can be scavenged by CAT and GR in the Halliwell–Asada pathway. Some papers (Almeselmani et al., 2006; Wang and Li, 2006) have documented that this enzyme is involved in temperature stress tolerance. Moreover, higher SOD activity occurred in many species under stress, and this was attributed to the novo synthesis of the enzymic protein (Song et al., 2006). In general, the responses of the antioxidant system and especially those of antioxidant enzymes to low temperature stress are ambiguous and depend on plant species, cultivar, age and also on the duration of stressor action (Hodges et al., 1996).

From earlier studies, it was found that ALA stimulates activities of antioxidative enzymes including SOD in spinach seedlings subjected to salinity stress (Nishihara et al., 2003) and pakchoi seedlings grown under optimum conditions (Memon et al., 2009). Moreover, increased GR activity results in enhancement of cold-tolerance in grape seedlings (Wang and Li, 2006). Hence, the effect of 5 μM ALA pre-treatment before cold stress was examined. Results indicate that in plants pre-treated with 5 μM ALA, no significant changes in SOD, GR and APX activities were observed after 4 °C stress respect to plants subjected only to cold treatment. Furthermore, ALA did not alter their activity levels. In contrast, CAT was induced by nearly 100% in ALA treated plants followed by 4 °C stress.

Cold treatment resulted in higher enzyme activities of SOD, CAT, and GR in soybean leaves and it seems to be the physiological adaptive mechanism to regulate its redox status under low temperature stress conditions. Nevertheless, this increase appears not to be high enough to cope with the oxidative insult. In contrast, enhancement of SOD, APX and GR did not occur in plants pre-treated with ALA alone, indicating that 5 μM ALA protects against cold through another way.

2.2. Induction of heme oxygenase-1 is a defense response

2.2.1. Effect of cold on heme oxygenase-1 activity, protein content and gene expression

Taking into account the fact that ALA promotes heme biosynthesis, which in turn is the substrate of HO-1, its activity, protein content and gene expression were analyzed in soybean leaves.

When plants were subjected to cold treatment a 40% increase of HO-1 activity was found respect to controls (Fig. 4). Pre-treatment with 5 μM ALA previous to cold stress produced the highest levels of HO-1 activity (220%, respect to controls). Enzyme activity is positively correlated with protein levels (Fig. 4) and mRNA expression (Fig. 5), except from the fact that 5 μM ALA alone brings about an increase in enzyme activity (120%) without affecting protein

Table 2
Antioxidant enzyme activities in soybean leaves subjected to 4 °C and 5 μM ALA treatment.

Treatment	CAT (pmol/mg protein)	SOD (U/mg protein)	APX (U/mg protein)	GR (U/mg protein)
Control	150 ± 12[a]	17.3 ± 0.5[a]	0.004 ± 0.001[a]	0.043 ± 0.002[a]
4 °C	189 ± 15[b]	23.4 ± 0.7[b]	0.005 ± 0.001[a]	0.055 ± 0.003[b]
5 μM ALA	150 ± 12[a]	17.5 ± 0.6[a]	0.004 ± 0.001[a]	0.045 ± 0.003[a]
5 μM ALA + 4 °C	301 ± 14[c]	23.2 ± 0.5[b]	0.005 ± 0.001[a]	0.055 ± 0.004[b]

Enzymatic activities were assayed as described in Experimental. Different letters within columns indicate significant differences ($P < 0.05$) according to Tukey's multiple range test.

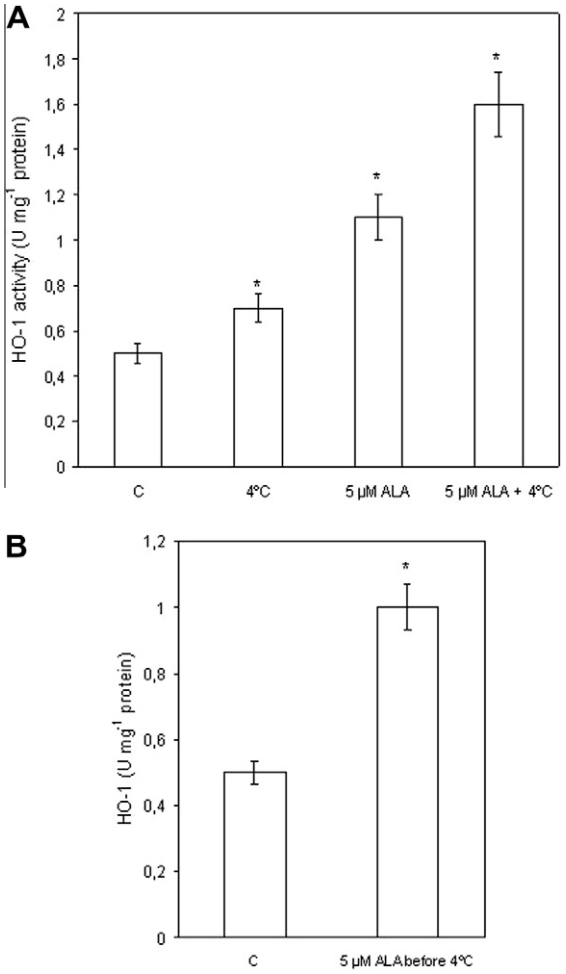

Fig. 4. Effect of 4 °C and ALA pre-treatment on soybean leaves HO activity (A). Effect of ALA before cold treatment (B). Experiments were performed as described in Experimental. *Significant differences ($P < 0.05$).

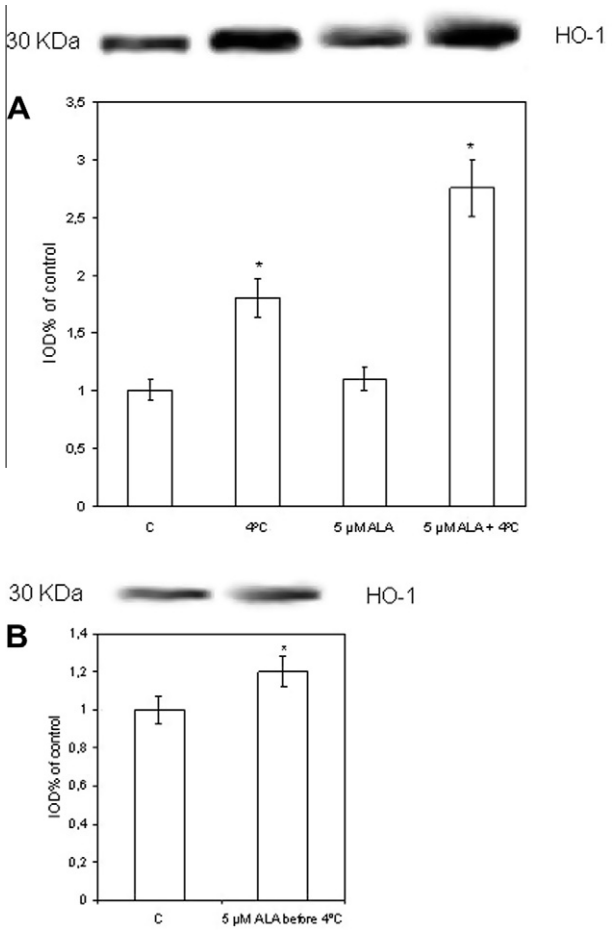

Fig. 5. Effect of 4 °C and ALA pre-treatment on soybean leaves' HO protein content (A). Densitometry was done by Gel-Pro analyzer to quantify HO-1 protein expression (B). The blot is representative of three experiments with a total of five samples/group among them.

content (Fig. 4) and mRNA expression (Fig. 5) respect to controls. These results indicated that in ALA controls, HO-1 activity could be enhanced due to an increase of the heme pool, because no gene induction was observed and we could assume that protection exerted by low ALA concentrations could be attributed to the increase of HO-1 and CAT activities. To shed light into the role played by ALA, experiments were performed immediately before cold treatment. Under this condition there is an enhancement of HO-1 activity (50%, respect to controls) (Fig. 4) that is also positively correlated with protein content (20%) (Fig. 5B) and mRNA expression (20%) (Fig. 6B). As already stated, ALA undergoes enolization and further metal-catalyzed aerobic oxidation to yield $O_2^{\cdot-}$, H_2O_2 and HO species. Therefore, accumulation of ALA might increase ROS levels leading to oxidative damage. Moreover, low concentrations could stimulate heme biosynthesis. Nevertheless, plants treated with both 5 μM ALA and cold revealed a major enhancement in HO-1 activity, protein content and gene expression. This fact indicates that ALA pre-treatment brought about a slight increase in gene expression that was not detectable when they were compared to cold treated plants, but contributed to its augmentation caused by 4 °C. In this way, ALA is not only involved in cold defense by elevating the heme pool, but also by enhancing HO-1 gene expression.

To asses whether HO-1 is involved in the protection against cold exerted by 5 μM ALA, experiments were carried out in plants treated with Zn-protoporphyrin IX (ZnPPIX), a well known irreversible HO-1 inhibitor. Table 3 indicates that HO-1 activity is inhibited in plants treated with ZnPPIX, and also shows that under this

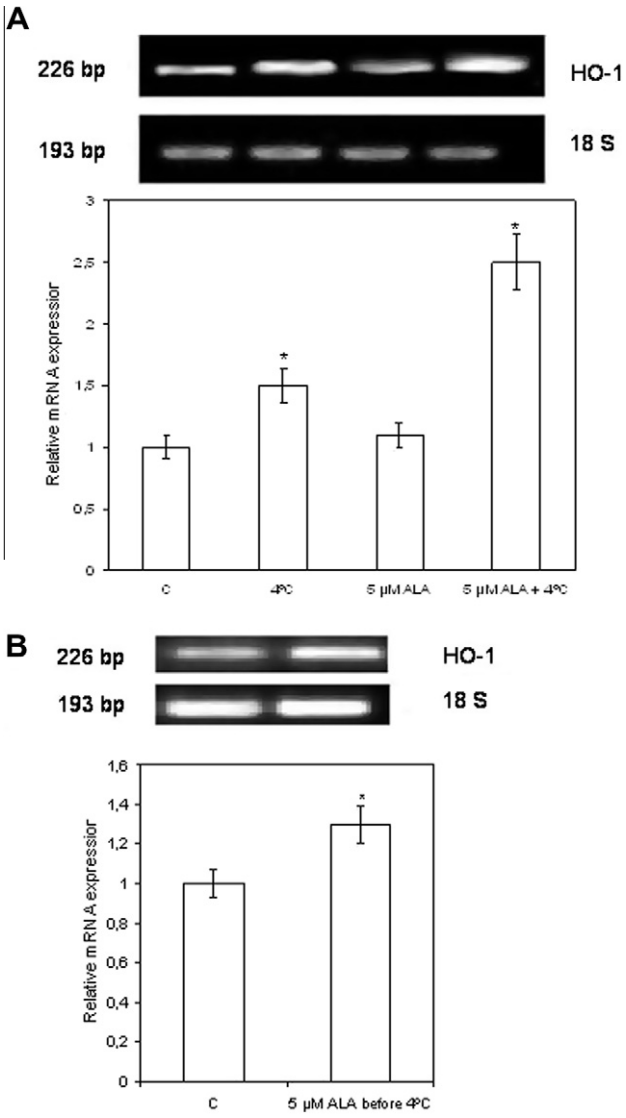

Fig. 6. Effect of 4 °C and ALA pre-treatment on soybean leaves HO gene expression. (A) HO-1 mRNA expression was analyzed by semi-quantitative RT-PCR as described in Experimental. The 18S amplification band is shown to confirm equal loading of RNA and RT efficiency. (B) Relative HO-1 transcript expression taking control as 1 U. Data are means of three independent experiments and bars indicate SE. *Significant differences ($P < 0.05$).

Table 3
Effect of ZnPPIX on HO-1 induction and on TBARS in soybean leaves subjected to 4 °C.

Treatment	HO-1 (U mg^{-1} protein)	TBARS (nmol g^{-1} FW)
Control	0.50 ± 0.02^a	22.5 ± 1.2^a
4 °C	0.70 ± 0.15^b	34.1 ± 2.8^b
ZnPPIX	0.19 ± 0.03^c	21.3 ± 2.3^a
ZnPPIX + 4 °C	0.20 ± 0.02^c	78.4 ± 5.6^c

Segments from the second pair of fully expanded leaves upper the cotyledons, were used for the assays. Experiments were carried out as described in Experimental. Different letters within columns indicate significant differences ($P < 0.05$), according to Tukey's multiple range test.

condition plants cannot cope with cold insult, as indicated by an high increase (3.5-fold) in TBARS levels.

It is well known that heme is the prosthetic group of many antioxidant enzymes, e.g., CAT, and it is subjected to degradation by the enzymatic process catalyzed by HO-1. In this way, synthesis of the potent antioxidant biliverdin is promoted. Furthermore, glutamyl-tRNA reductase catalyzes the reduction of the carboxyl group of glutamyl-tRNA in the presence of NADPH to form glutamate-1-semialdehyde. Glutamate 1-semialdehyde aminotransferase converts glutamate 1-semialdehyde to ALA by intramolecular transfer of the amino group. Under low temperature glutamyl-tRNA reductase is working slower and addition of ALA will just make up for that reduced enzymatic activity (Mohanty et al., 2006). Our data agree with previous reported results showing on one hand the protective effect of biliverdin on oxidative stress exerted by Cd (Noriega et al., 2004) and NaCl (Balestrasse et al., 2008) in soybean leaves and, on the other hand, the role of CO in the same process (Ling et al., 2009).

3. Conclusion

In summary, we provide evidence for mechanisms underlying the protective role of low concentrations of ALA against cold in soybean plants leaves. We also present data showing that ALA generates ROS which could be responsible for the activation of the enzymatic defense system. However, excess ALA suppressed these effects because the antioxidant cell system cannot cope with this oxidative insult. Interestingly, HO-1 acts not only in heme catabolism but as well as an antioxidant enzyme. Pre-treatment with low ALA concentrations before cold stress might promote HO-1 activity, protein levels and gene expression, enhancing the amount of the potent antioxidant biliverdin. Exposure of plants from subtropical origin, such as soybean to cold temperatures may stunt plant growth. We hope ALA may be useful in helping to solve serious problems occurring on a global scale, such as preventing crop losses in soybean due to low temperatures.

4. Experimental

4.1. Plant material and growing conditions

Seeds of soybean (*Glycine max* L.) were surface sterilized with 5% v/v NaOCl for 10 min and then washed with distilled H_2 (×4). The seeds were planted in vermiculite for 5 days. After germination, plants were removed from pots; roots were gently washed and transferred to separated containers for hydroponics. Plants were germinated and grown in a controlled climate room at 24 ± 2 °C and 50% relative humidity, with a photoperiod of 16 h and a light intensity of 175 µmol m^{-2} s^{-1}. The hydroponics medium was Hoagland nutrient solution (Hoagland and Arnon, 1950), this being continuously aerated and replaced every 3 days. After 4 weeks growth, plants were pre-treated for 12 h with either different 5-aminolevulic acid (ALA) concentrations (0, 5, 10, 15, 30 or 40 µM) or nutrient solution (control). Afterwards, plants were subjected to cold stress at 4 ± 0.5 °C for 48 h under the same light regime as indicated above. When the effect of Zn-protoporphyrin IX (ZnPPIX, 20 mM) was investigated, it was added to Hoagland solution alone 5 h before treatment. Determinations were carried out on one hand, immediately after ALA treatment to determine its effect before 4 °C exposure and on the other hand, 24 h after the end of cold stress .The experiments were replicated four plants (4×) in each replication and all treatments were arranged in a randomized complete block design. For comparison purposes, plants not exposed to cold stress and grown in the growth chamber at 25 °C were used as controls.

4.2. Chlorophyll content determination

Leaves (0.5 g, fresh weight) were homogenized with EtOH–H_2O (96:4) in a 1:30 w/v ratio. Extracts were heated in a boiling bath until complete bleaching occurred. After centrifugation, the

absorbance was measured in the supernatant at 665, 649 and 654 nm as described by Wintermans and de Mots (1965).

4.3. Relative water content

Relative water content (RWC), expressed as a percentage, was determined in soybean leaves according to the formula:

$$RWC\,(\%) = (FW - DW) \times 100/DW$$

where FW and DW means fresh weight and dry weight, respectively. FW was measured just after collection of the leaves at the end of the experiment and DW was measured after drying the leaves at 80 C for 48 h.

4.4. Thiobarbituric acid reactive substances determination

Lipid peroxidation was measured as the amount of TBARS determined by the thiobarbituric acid (TBA) reaction as described by Heath and Packer (1968). Fresh control and treated leaves (0.3 g) were homogenized in Cl_3CO_2H (TCA):H_2O (3 ml, 1:4, w/v). The homogenate was centrifuged at 3500*g* for 20 min. To an aliquot of the supernatant (1 ml), 20% TCA (1 ml) containing 0.5% (w/v) TBA and 4% butylated hydroxytoluene (BHT) in EtOH (100 ml) were added. The mixture was heated at 95 °C for 30 min and then quickly cooled on ice. The contents were centrifuged at 10,000*g* for 15 min and the absorbance was measured at 532 nm. Value for non-specific absorption at 600 nm was subtracted. The concentration of TBARS was calculated using an extinction coefficient of 155 $mM^{-1}cm^{-1}$.

4.5. H_2O_2 determination in leaf extracts

The H_2O_2 concentration of crude extracts from soybean leaves was determined by spectrofluorometry as described by Creissen et al. (1999). Leaves (0.5 g) were homogenized in 25 mM HCl (1.2 ml), with the crude extracts filtered through two nylon layers, and the pigments removed by mixing with charcoal (15 mg). The pigment-containing charcoal was separated by centrifugation at 5000*g* for 5 min, and the supernatants were clarified by filtration through 0.20-mm filter unit. The pH of leaf disc extracts was adjusted to 7.0 with 0.1 M NaOH and these extracts were used to measure the H_2O_2 concentration. The reaction mixtures (3 ml) contained 50 mM HEPES buffer, pH 7.6, 5 mM homovanillic acid and sample (100 ml). The reaction was started by adding 40 mM horse-radish peroxidase and the fluorescence produced was measured in a spectrofluorophotometer Shimadzu RF-540 (Kyoto, Japan), at excitation and emission wavelengths of 315 and 425 nm, respectively. The H_2O_2 concentration was determined from a calibration curve of H_2O_2 in the range of 0.1–20 mM.

4.6. $O_2^{\cdot-}$ determination in leaf extracts

The $O_2^{\cdot-}$ concentration of crude extracts from soybean leaves was determined by spectrometry as described by Boveris (1984) as above (4.6). The pH of leaf extracts was again adjusted to 7.0 with NaOH and these extracts were used to measure the $O_2^{\cdot-}$ concentration. The reaction medium consisted of 40 mM potassium phosphate buffer (pH 7.4), 120 mM KCl, 1 mM EDTA, and 1 mM epinephrine. The rate of production of $O_2^{\cdot-}$ was determined as the superoxide dismutase-sensitive rate of adrenochrome formation, measured at 485–575 nm (ε = 2.97 mM^{-1} cm^{-1}) in a Perkin–Elmer dual-wavelength spectrophotometer.

4.7. Antioxidant enzymes, preparations and assays

Extracts for determination of catalase (CAT, EC 1.11.1.6), superoxide dismutase (SOD, EC 1.15.1.1), ascorbate peroxidase (APOX, EC 1.11.1.11) and glutathione reductase (GR, EC 1.6.4.2) activities were prepared from leaves (0.3 g) homogenized under ice-cold conditions in extraction buffer (3 ml), containing 50 mM phosphate buffer (pH 7.4), 1 mM EDTA, 1 g polyvinylpolypyrrolidone, and 0.5% (v/v) Triton X-100 at 4 °C. The homogenates were centrifuged at 10,000*g* for 20 min and the supernatant fraction was used for the assays. CAT activity was determined in the homogenates by measuring the decrease in absorption at 240 nm in a reaction medium containing 50 mM potassium phosphate buffer (pH 7.2) and 2 mM H_2O_2. The pseudo-first order reaction constant ($k' = k \cdot$[CAT]) of the decrease in H_2O_2 absorption was determined and the CAT content in pmol mg^{-1} protein was calculated using $k = 4.7 \times 10^7$ M^{-1} s^{-1}) (Chance et al., 1979). Total SOD activity was assayed by the inhibition of the photochemical reduction of NBT, as described by Becana et al. (1986). The reaction mixture consisted enzyme extract (50–150 μl) and $O_2^{\cdot-}$ generating solution (3.5 ml) which contained 14.3 mM methionine, 82.5 μM nitro blue tetrazolium, and 2.2 μM riboflavin. Extracts were brought to a final volume of 0.3 ml with 50 mM K-phosphate (pH 7.8) and 0.1 mM Na_2EDTA. Test tubes were shaken and placed 30 cm from a light bank consisting of six 15-W fluorescent lamps. The reaction was allowed to run for 10 min and stopped by switching the light off. The reduction in NBT was followed by reading absorbance at 560 nm. Blanks and controls were run in the same way but without illumination and enzyme, respectively. One unit of SOD was defined as amount of enzyme which produced 50% inhibition of NBT reduction under the assay conditions. GR activity was measured by following the decrease in absorbance at 340 nm due to NADPH oxidation. The reaction mixture contained extract, 1 EDTA, 0.5 mM GSSG, 0.15 mM NADPH, 50 mM Tris–HCl buffer (pH7.5) and 3 mM $MgCl_2$ in a final volume of 200 ml. APOX activity was measured immediately in fresh extracts and was assayed as described by Nakano and Asada (1981), using a reaction mixture (1 ml) containing 50 mM K-phosphate buffer (pH 7.0), 0.1 mM H_2O_2, 0.5 mM Na-ascorbate and 0.1 mM EDTA. The hydrogen peroxide-dependent oxidation of ascorbate was followed by a decrease in the absorbance at 290 nm (ε: 2.8 mM^{-1} cm^{-1}). One unit of APOX forms 1 μmol of ascorbate oxidized per minute under the assay conditions.

GR activity was measured by following the decrease in absorbance at 340 nm due to NADPH oxidation. The reaction mixture contained tissue extract, 1 mM EDTA, 0.5 mM GSSG, 0.15 mM NADPH and 50 mM Tris–HCl buffer (pH 7.5) and 3 mM $MgCl_2$ (Shaedle and Bassham, 1977).

4.8. Heme oxygenase preparation and assay

Leaves (0.3 g) were homogenized in a Potter–Elvehejm homogenizer using 4 vol. of ice-cold 0.25 M sucrose solution containing 1 mM phenylmethyl sulfonyl fluoride, 0.2 mM EDTA and 50 mM potassium phosphate buffer (pH 7.4). Homogenates were centrifuged at 20,000*g* for 20 min and supernatant fractions were used for activity determination. Heme oxygenase activity was determined as previously described with minor modifications (Muramoto et al., 2002). The standard incubation mixture in a final volume of 500 μl contained 10 mmol potassium phosphate buffer (pH 7.4), 60 nmol NADPH, 250 ml HO (0.5 mg protein), and 200 nmol hemin. Incubations were carried out at 37 °C during 60 min. Activity was determined by measuring biliverdin formation, which was calculated using the absorbance change at 650 nm employing an a value of 6.25 mM^{-1} cm^{-1} (vis_{max} 650 nm).

4.9. Western blot analysis for HO-1

Homogenates obtained for HO activity assay were also analyzed by Western immunoblot technique. Protein (40 mg) from nodule homogenates were subjected to sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis using a 12% acrylamide resolving gel (Mini Protean II System, BioRad, Hertz, UK), according to Laemmli (1970). Separated proteins were then transferred to nitrocellulose membranes and non-specific binding of antibodies was blocked with 3% non-fat dried milk in PBS, pH 7.4 for 1 h at room temperature. Membranes were then incubated overnight at 48 °C in primary antibodies raised against *Arabidopsis thaliana* HY-1 (Muramoto et al., 1999) diluted 1:2000 in Tris–NaCl buffer plus 1% non-fat milk. Immune complexes were detected using alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G. The phosphatase labeled antigens were visualized with the colorigenic substrate 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium.

4.10. Isolation of RNA and RT-PCR analysis

Total RNA was extracted from soybean leaves by using the Trizol reagent (Gibco BRL). Total RNA (4 μg) were treated with RNase-free DNase I (Promega, CA, USA) and then 1.0 μg was reversed transcribed into cDNA using random hexamers and M-MLV Superscript II RT (Invitrogen, CA, USA). PCR reactions were carried out using *G. max* HO-1 and 18S specific primers, as previously described (Yannarelli et al., 2006). In addition, CAT gene expression was assessed by using a primer pair specific to *G. max* CAT cDNA (sense primer, 5′-CTGCTGGAAACTATCCTGAGTG-3′; antisense primer, 5′-ATTGACCTCTTCATCCCTGTG-3′). The PCR profile was set at 94 °C for 1 min and then 29 cycles at 94 °C for 0.5 min, 54 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 7 min. Primers 5′-TTCCGAATTCAAAGGTCCAG-3′ and 5′-TAAGATCAGCCACCCTCAGC-3′ were designed to amplify the *G. max* SOD cDNA. Cycling conditions were as follows: 94 °C for 1 min, then 33 cycles at 94 °C denaturing for 0.5 min, 55 °C annealing for 1 min, and 72 °C extension for 1 min, and then a final step of 72 °C for 7 min. Each primer set was amplified using an optimized number of PCR cycles to ensure the linearity requirement for semi-quantitative RT-PCR analysis. The amplified transcripts were visualized on 1.5% agarose gels with the use of ethidium bromide. Gels were then scanned (Fotodyne Incorporated, WI, USA) and analyzed using Gel-Pro Analyzer 3.1 software (Media Cybernetics, MD, USA).

4.11. Protein determination

Protein concentration was evaluated by the method of Bradford (1976), using bovine serum albumin as a standard.

4.12. Statistics

Values in the text, figures and tables indicate mean values ± SEM. Differences among treatments were analyzed by one-way ANOVA, taking $P < 0.05$ as significant according to Tukey's multiple range test.

Acknowledgments

We thank Dr. T. Kohchi for kindly providing the Arabidopsis HO-1 antibodies. This work was supported by grants from the Universidad de Buenos Aires (Argentina) and from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) (Argentina). K.B.B., M.L.T and A.B. are CONICET career Investigators from CONICET, G.O.N. holds the post of Professional Research Assistant of CONICET.

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