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Heme oxygenase-1 is involved in the cytokinin-induced alleviation of senescence in detached wheat leaves during dark incubation

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ABSTRACT

This study tested whether an inducible isoform of heme oxygenase (HO, EC 1.14.99.3), HO-1, is involved in the cytokinin (CTK)-induced alleviation of senescence in detached wheat (*Triticum aestivum* L.) leaves during dark incubation. We discovered that exogenous supplement of 6-benzylaminopurine (6-BA) at 10 μM for 48 h not only delayed the dark-induced loss of chlorophyll and protein contents in detached wheat leaves, but also significantly increased HO activity in a time-dependent manner. This induction reached a maximum within 3 h of 6-BA supply, which was further confirmed by using semi-quantitative RT-PCR and protein gel blot analysis. Furthermore, the decreases in intracellular thiobarbituric acid reactive substances (TBARS) content, and the increases in the transcript level, total and isozymatic activities of some important antioxidant enzymes, such as catalase (CAT, EC 1.11.1.6), peroxidase (POD, EC 1.11.1.7), superoxide dismutase (SOD, EC 1.15.1.1), and ascorbate peroxidase (APX, EC 1.11.1.11), were observed. Reversed responses of chlorophyll, protein and TBARS contents, HO activity, and the expression of above antioxidant enzymes were observed when zinc protoporphyrin-IX (ZnPPIX), a potent HO-1 inhibitor, was added together with 6-BA. In contrast, HO-1 inducer hemin could partially mimic the effects of 6-BA. Together, the results suggest that HO-1 might be involved in the CTK-induced alleviation of senescence and lipid peroxidation in detached wheat leaves.

Introduction

Cytokinins (CTKs) play a major role in many different developmental and physiological processes in plants, such as cell division, root and shoot growth and branching, chloroplast development, stress response and pathogen resistance. In particular, it has been proposed that CTK induces the delay of dark-associated leaf senescence. For example, the application of CTK usually slows senescence-induced decreases in protein and chlorophyll contents, and photosynthetic parameters such as the rate of CO_2 assimilation (Selivankina et al., 2001; Vlčková et al., 2006), and delays the senescence-induced increase in lipid peroxidation by the up-regulation of antioxidative enzyme expression (Zavaleta-Mancera et al., 2007). However, the molecular mechanisms of these CTK responses are poorly understood.

In animals, heme oxygenase (HO, EC 1.14.99.3) is known to catalyze the oxidative cleavage of the α-mesocarbon of Fe-protoporphyrin-IX yielding equimolar amounts of biliverdin-IXα (BV), free divalent iron, and carbon monoxide (CO). Among the three isoforms identified to date, only HO-1 is a stress-responsive protein induced by hypoxia, endotoxic shock, atherosclerosis, inflammation, and other oxidative stimuli, conferring protection against oxidative stress in a variety of tissues (Bauer and Bauer, 2002). Previous results have illustrated that the antioxidant mechanism of HO-1 might involve its enzymatic reaction products, including BV and CO. In plants, interestingly, the first role attributed to HOs was their participation in the biosynthetic pathway leading to phytochrome chromophore formation or functioning in light signaling (Muramoto et al., 1999; Davis et al., 2001; Shekhawat and Verma, 2010). Furthermore, the involvement of HO-1, BV, and CO in the antioxidant defense system was confirmed in soybean and alfalfa plants (Noriega et al., 2004; Balestrasse et al., 2005; Han et al., 2008). Recently, we provided a series of pharmacological, physiological, and molecular evidence that HO-1 was involved in the auxin-induced adventitious rooting in cucumber

Abbreviations: ABA, abscisic acid; APX, ascorbate peroxidase; 6-BA, 6-benzylaminopurine; BV, biliverdin-IXα; CAT, catalase; cGMP, cyclic guanosine monophosphate; CO, carbon monoxide; CTK, cytokinin; H_2O_2, hydrogen peroxide; HO, heme oxygenase; HO-1, heme oxygenase-1; NO, nitric oxide; POD, peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; ZnPPIX, zinc protoporphyrin IX.

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explants (Xuan et al., 2008). The induction of HO-1 by osmotic stress, glutathione (GSH) depletion, cobalt chloride ($CoCl_2$), abscisic acid (ABA), nitric oxide (NO), and hydrogen peroxide (H_2O_2) was also confirmed (Yannarelli et al., 2006; Cao et al., 2007; Noriega et al., 2007; Chen et al., 2009; Cui et al., 2010; Liu et al., 2010; Xu et al., 2010). For example, HO-induced CO production was involved in ABA-induced stomatal closure in *Vicia faba*, and NO and cyclic guanosine monophosphate (cGMP) might function as downstream intermediates in the CO signaling responsible for stomatal closure (Cao et al., 2007). However, the role of HO in CTK-induced cytoprotective effects is still unclear, and there is no published study on CTK-induced up-regulation of HO isoforms in wheat plants.

In fact, detached wheat leaf is a good biological system for senescence bioassays of CTK activity. In this context, detached wheat leaves were treated with 6-benzylaminopurine (6-BA), HO-1 inducer hemin, or the potent inhibitor of HO-1 zinc protoporphyrin IX (ZnPPIX) alone, or 6-BA plus ZnPPIX. Thus, the relationship between CTK-induced *HO-1* expression and the CTK responses, such as the delay of leaf senescence and the alleviation of lipid peroxidation in detached wheat leaves, were studied. The results suggest that HO-1 might be involved in CTK responses by the modulation of antioxidative enzyme expression, such as catalase (CAT, EC 1.11.1.6), peroxidase (POD, EC 1.11.1.7), superoxide dismutase (SOD, EC 1.15.1.1), and ascorbate peroxidase (APX, EC 1.11.1.11).

Materials and methods

Plant material and growth condition

Seeds of wheat (*Triticum aestivum* L., Yangmai 13) were surface-sterilized with 2% NaClO for 10 min, rinsed extensively in distilled water and germinated at 25 °C in the darkness for 1 d. Then, the uniform buds were selected. Roots were gently washed and transferred to separate containers for hydroponics. Plants were germinated and grown in a controlled climate chamber (12 h light period, 25 °C, humidity 50 ± 4%; 12 h dark period, 18 °C, humidity 56 ± 5%, MGC-300B, Shanghai Yiheng Technology Co., Ltd., Shanghai, China) with modified Hoagland solution containing 3 mM KNO_3, 1 mM $NH_4H_2PO_4$, 0.5 mM $MgSO_4$, 5.5 mM $Ca(NO_3)_2$, 50 mg of FeEDTA/l (10% iron), 25 μM KCl, 12.5 μM H_3BO_3, 1 μM $MnSO_4$, 1 μM $ZnSO_4$, 0.25 μM $CuSO_4$, and 2 μM H_2MoO_4 (Gulick and Dvořák, 1987). The irradiance was approximately 300 μmol m^{-2} s^{-1} provided by fluorescent lamps. The culture solution was renewed every other day until two fully expanded leaves appeared.

Chemicals

All chemicals were obtained from Sigma (St Louis, MO, USA). 6-BA was used at a concentration of 10 μM. ZnPPIX, and a specific inhibitor of HO-1 (Lamar et al., 1996; Xuan et al., 2008), was used at 100 μM. Hemin was used at 1 μM as the HO-1 inducer (Xuan et al., 2008).

Treatments

The first fully expanded leaves, which have exposed ligulae, were cut off 1 cm below the leaf tips. Leaf segments (4 cm) were detached and then floated abaxial side down in a plastic chamber containing 100 mL of distilled water and kept in darkness at 25 °C for 72 h to induce senescence. Subsequently, wheat leaf segments were exposed to 100 mL of distilled water (Con), 100 μM ZnPPIX, 10 μM 6-BA, 1 μM hemin alone, or 6-BA plus ZnPPIX respectively, for another 48 h in the dark. After various treatments, leaf segments were immediately frozen in liquid nitrogen, and stored at −80 °C until further analysis. At least three replicates were carried out for each treatment, and at least fifteen detached leaves were in each replicate.

Determination of chlorophyll, soluble protein, and thiobarbituric acid reactive substances (TBARS) content

Chlorophyll content was determined according to the method described by Wintermans and De Mots (1965). Soluble protein content was determined according to the method of Bradford (1976) using bovine serum albumin (BSA) as the standard. Lipid peroxidation was estimated by measuring the amount of TBARS as described previously by Liu et al. (2007).

HO activity determination

Heme oxygenase (HO, EC 1.14.99.3) activity was analyzed following the method described by Xuan et al. (2008). For the HO activity assay, the concentration of BV was estimated using a molar absorption coefficient at 650 nm of 6.25 mM^{-1} cm^{-1} in 0.1 M HEPES–NaOH buffer (pH 7.2). One unit of activity (U) was calculated by taking the quantity of the enzyme to produce 1 nmol BV per 30 min.

Protein gel blot of HO-1

Rabbit polyclonal antibody against the mature wheat HO-1 (TaHO1) expressed in *Escherichia coli* was used (Wu et al., 2011). Homogenates obtained for HO activity assays were also analyzed by protein gel blot analysis. Sixty micrograms of protein from homogenates were subjected to SDS-PAGE using a 12.5% acrylamide resolving gel (Mini Protean II System, Bio-Rad, Hertz, UK). Separated proteins were then transferred to polyvinylidene difluoride (PVDF) membranes, and non-specific binding of antibodies was blocked with 5% non-fat dried milk in phosphate-buffered saline (PBS, pH 7.4) for 2 h at room temperature. Membranes were then incubated overnight at 4 °C with primary antibodies diluted 1:200 in PBS buffer plus 1% non-fat dried milk. Immune complexes were detected using horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G. The color was developed with a solution containing 3,3′-diaminobenzidine tetrahydrochloride (DAB) as the HRP substrate. Additionally, the films were scanned (Uniscan B700$^+$, Tsinghua Unigroup Ltd., Beijing, China) and analyzed using Quantity One v4.4.0 software (Bio-Rad, USA).

Antioxidative enzyme activity assays

Frozen wheat leaf segments (0.5 g) were homogenized in 10 mL of 50 mM PBS (pH7.0) containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 1% polyvinylpyrrolidone (PVP) for catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD) total activity assays, or combinations with the addition of 1 mM ascorbic acid (ASC) in the case of ascorbate peroxidase (APX) determination. The homogenate was centrifuged at 10,000 × *g* for 30 min at 4 °C, and the supernatant was used for the four antioxidative enzyme determinations.

CAT activity was spectrophotometrically measured by monitoring the consumption of H_2O_2 (extinction coefficient 39.4 mM^{-1} cm^{-1}) at 240 nm. POD was determined by measuring the oxidation of guaiacol (extinction coefficient 26.6 mM^{-1} cm^{-1}) at 470 nm. Total SOD activity was assayed by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) according to the method described by Beauchamp and Fridovich (1971). One unit of SOD (U) was defined as the amount of crude enzyme extract required to inhibit the reduction rate of NBT by 50%. APX activity was determined by monitoring the