



Nitric oxide and iron modulate heme oxygenase activity as a long distance signaling response to salt stress in sunflower seedling cotyledons



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ABSTRACT

Nitric oxide is a significant component of iron signaling in plants. Heme is one of the iron sensors in plants. Free heme is highly toxic and can cause cell damage as it catalyzes the formation of reactive oxygen species (ROS). Its catabolism is carried out by heme oxygenase (HOs; EC 1.14.99.3) which uses heme both as a prosthetic group and as a substrate. Two significant events, which accompany adaptation to salt stress in sunflower seedlings, are accumulation of ROS and enhanced production of nitric oxide (NO) in roots and cotyledons. Present investigations on the immunolocalization of heme oxygenase distribution in sunflower seedling cotyledons by confocal laser scanning microscopic (CLSM) imaging provide new information on the differential spatial distribution of the inducible form of HO (HO-1) as a long distance in response to NaCl stress. The enzyme is abundantly distributed in the specialized cells around the secretory canals (SCs) in seedling cotyledons. Abundance of tyrosine nitrated proteins has also been observed in the specialized cells around the secretory canals in cotyledons derived from salt stressed seedlings. The spatial distribution of tyrosine nitrated proteins and HO-1 expression further correlates with the abundance of mitochondria in these cells. Present findings, thus, highlight a link among distribution of HO-1 expression, abundance of tyrosine nitrated proteins and mitochondria in specialized cells around the secretory canal as a long distance mechanism of salt stress tolerance in sunflower seedlings. Enhanced spatial distribution of HO-1 in response to NaCl stress in seedling cotyledons is in congruence with the observed increase in specific activity of HO-1 in NaCl stressed conditions. The enzyme activity is further enhanced by hemin (HO-1 inducer) both in the absence or presence of NaCl stress and inhibited by zinc protoporphyrin. Western blot analysis of cotyledon homogenates using anti-HO-1 polyclonal antibody shows one major band (29 kDa) of HO-1. NaCl-modulated HO-1 activity correlates with endogenous NO content in the cotyledons. Increased NO accumulation by hemin treatment also correlates with enhanced activity of HO-1 in both control and NaCl stress conditions. Present work indicates that NO positively modulates HO-1 activity in sunflower seedling cotyledons. NaCl stress tends to antagonize NO action on HO-1 activity. NO (from sodium nitroprusside; SNP) is probably positively modulating HO-1 activity by way of its interaction/binding with heme group. Present work also shows enhanced NO accumulation in seedling cotyledons both in the absence or presence of iron in the growth medium, in response to NaCl stress. Thus, a probable link between endogenous NO, NaCl stress and iron-homeostasis by way of modulation of HO-1 activity at early stage of sunflower seedling growth has been proposed.

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Abbreviations: NO, nitric oxide; CLSM, confocal laser scanning microscopic; SC, secretory canal; SNP, sodium nitroprusside.

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1. Introduction

Investigations on the characterization of iron signals in plants are a recent area of research [1]. Nitric oxide is a significant component of iron signaling in plants [2]. In tomato plants, rapid NO accumulation has been observed under iron deficiency. When subjected to as S-nitrosoglutathione (NO donor) treatment, iron-

deficient roots of tomato exhibit enhanced expression of ferric reduction oxidase 1 (FRO1) coupled with alleviation of oxidative damage. A down regulation of this enzyme in iron-deficient roots of tomato mutant (*fer*), however, can not be reversed by the addition of NO. This suggests that NO acts upstream of *fer* to initiate adaptation to iron-deficiency. NO might also play a role as a long distance signaling molecule between shoot and root under iron-deficiency conditions [2]. Iron-deficiency in *Arabidopsis* leads to enhanced accumulation of NO in roots accompanying an up-regulation of root ferric-chelate reductase (FCR) activity [3]. Most of the iron available in the tissue system is thought to be chelated by various biomolecules to maintain its solubility and prevent toxicity. Fe^{2+} is also incorporated into iron-proteins. Heme is one of the iron sensors in plants which is synthesized in plastids [1]. It is a ubiquitous, lipophilic and iron-containing porphyrin complex which is conjugated to numerous proteins, such as hemoglobin, peroxidases, cytochromes, various oxidases, nitric oxide synthase, prostaglandin synthase and guanylate cyclase [4]. Free heme is highly toxic and can cause cell damage as it catalyzes the formation of reactive oxygen species (ROS) [5]. It has been observed to accumulate in tissues under pathological conditions [6]. Heme catabolism is carried out by heme oxygenase (HOs; EC 1.14.99.3) which uses it both as a prosthetic group and as a substrate [7]. Among photosynthetic organisms, HO was first reported in red algae-*Cyanidium caldarium* [8]. HOs are involved in the biosynthesis of phytochrome chromophore [9,10]. Four isoforms of HOs have been identified in *Arabidopsis thaliana*. HO-1 is the inducible and highly expressed form. HO-2, HO-3 and HO-4 are constitutive forms and are expressed at low levels [11]. HO genes have also been characterized in various plant species [12–18]. HO-1 is well known to be localized in plastids [9]. Recent investigations on *Glycine max* have also revealed its mitochondrial localization [19]. Using O_2 and NADPH, heme oxygenase removes excess heme (pro-oxidant) via oxidative degradation of Fe-porphyrin ring to produce equimolar amount of biliverdin, free iron (Fe^{2+}) and carbon monoxide (CO) [5]. Biliverdin is subsequently converted to bilirubin by biliverdin reductase. CO liberated following the HO-1 catalyzed reaction, acts as a gaseous signaling molecule and has been reported to mediate induction of growth elongation of wheat root segments by IAA [20,21]. Both in animals and plants, HO-1 also imparts protection against oxidative stress through its catalytic product-biliverdin and byproduct CO [5,22,23].



Several abiotic stress factors against which HO-1 has been reported to deliver protection to plant cells include salt [17,24–26], low temperature [27] and heavy metals [16,28,29]. In *Brassica napus*, enhanced HO-1 activity correlates with NaCl stress and polyethylene glycol (PEG)-induced lateral root formation. Role of HO-1 in salt stress acclimation has also been observed in wheat and sunflower [17,30]. Investigations have also suggested the role of ROS in HO-1 mediated salinity acclimation in wheat and *Arabidopsis* [30,31]. It has been suggested that in *Arabidopsis* roots, HO-1 contributes to salinity acclimation through the regulation of plasma membrane H^+ -ATPases [32]. Upregulation of HO-1 accompanying

alleviation of salt toxicity in alfalfa plants has also been observed in response to spermidine application which mimics the effect of heme (HO-1 inducer) [26]. A probable crosstalk between HO-1 and NO against various abiotic stress factors, namely UV-B radiation, salt stress and cadmium toxicity, has also been observed [24,29,33–35]. Plant processes, like adventitious root formation, lateral root formation in response to auxin and seed germination, are also known to be modulated by an interaction between HO-1 and NO [36,37].

Understanding the physiological and biochemical basis of salt tolerance mechanisms in sunflower seedlings has been a major area of research in the author's laboratory in recent past [38–41]. Seed germination in sunflower is relatively more tolerant to salt stress than seedling growth [39]. Effect of salt stress (120 mM NaCl) is evident during the extension growth of seedlings through a reduction of hypocotyl elongation, primary root growth and proliferation of lateral roots. Two significant events, which accompany adaptation to salt stress in sunflower seedlings, are accumulation of ROS and enhanced production of NO in roots and cotyledons. A transient NO accumulation has been observed in roots of sunflower seedlings at early stage of seedling development (2 d) in response to NaCl stress [38]. Salt stress has further been observed to significantly enhance tyrosine nitration of cytosolic and oil body membrane proteins in seedling cotyledons [42]. Seedling cotyledons have also been observed to accumulate lipid peroxides coinciding with high specific activity of phospholipid hydroperoxide glutathione peroxidase [43]. Superoxide dismutase (SOD) isoforms exhibit differential induction by NO as an early response to NaCl stress in seedling cotyledons [44]. Melatonin (a well-known indoleamine) has also been reported to regulate salt tolerance in plants through its crosstalk with various ROS scavenging enzymes [45].

Based on the available literature on the modulation of HO-1 activity in response to a variety of stress factors and signaling molecules, attempts have been made in the present work to examine HO-1 activity and its expression in sunflower seedling cotyledons in response to salt stress (120 mM NaCl). This work also examines the significance of differential spatial distribution of HO-1 in the cells of seedling cotyledons. Present findings provide significant evidence for NaCl stress-induced modulation in HO-1 activity and its spatial distribution and a possible crosstalk between HO-1 and NO in relation with NaCl stress and iron availability. The data have been discussed with reference to the long distance

signaling mechanisms operative in sunflower seedlings subjected to NaCl stress, highlighting the role of the above-stated signaling molecules.

2. Material and methods

2.1. Plant growth and treatments

Sunflower (*Helianthus annuus* L. cv., KBSH 53) seeds were washed with a liquid detergent under running tap water, disinfected using 0.005% mercuric chloride (HgCl_2) and placed under

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