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Nitric oxide accumulation and protein tyrosine nitration as a rapid and long distance signalling response to salt stress in sunflower seedlings

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ABSTRACT

Sensing of salt stress by sunflower seedlings accompanies temporal and spatial modulation of intracellular nitric oxide (NO) accumulation and protein tyrosine nitration as markers of nitrosative stress. Employing a novel NO-specific probe for NO localization (a copper derivative of 4-methoxy-2-(1H-naphtho(2,3-*d*)imidazol-2-yl)phenol; MNIP-Cu) synthesized in author's laboratory, immunological analysis of tyrosine nitrated proteins by confocal laser scanning microscopy (CLSM) and Western blot analysis, these rapid signalling events have been investigated. MNIP-Cu reveals the distribution of NO in whole seedlings. Preferential and enhanced NO localization around oil bodies (OBs) in cotyledons within 48 h of salt-stressed seedlings exhibits rapid transport of nitrosative stress signal from roots to the cotyledons. Immunological analysis reveals enhanced gradient of tyrosine nitrated proteins in salt-stressed roots from tip to the differentiating zone and from columella to the deep-seated cells. Western blot analysis shows that at least eight major cytosolic proteins exhibit enhanced tyrosine nitration in seedling roots in response to salt stress. Present observations provide strong evidence for rapid NO accumulation in salt stressed sunflower seedling roots and cotyledons and its impact on enhanced tyrosine nitration of cytosolic and OB proteins, as a mechanism to provide longevity to OBs for seedling survival under the salt stress.

1. Introduction

Plants undergo nitrosative stress accompanying de-regulated synthesis or overproduction of nitric oxide (NO) and other reactive nitrogen species (RNS), such as peroxynitrite ($ONOO^-$) and dinitrogen trioxide (N_2O_3), which can have toxic physiological consequences. Salinity has also been reported to cause a general increase in the endogenous levels of NO, nitrosoglutathione (GSNO) and RSNO (S-nitrosothiols) in some plants [1,2]. A general increase in NO in palisade and spongy mesophyll cells has been observed along with that in the vascular cells. This appears to coincide with the enzymatic production of NO from L-arginine (NOS activity) in olive leaf extracts from salt stressed plants [3]. A rapid and significant increase in NO accumulation has also been reported in the leaf cells of tobacco plants subjected to heat, salt and osmotic stresses [4]. Detection of RSNO and GSNO in the spongy mesophyll and vascular tissues of olive leaves and the sensitivity of RNS to salt stress suggest that the redistribution of GSNO-derived NO from the vascular tissues can have a significant role in nitrosative stress. The work from Valderrama et al. (2007) suggested the induction of oxidative stress along with nitrosative stress in olive plants and also that, vascular tissues could play an important function in the redistribution of RNS during normal and stress conditions.

NO is synthesized through enzymatic [nitrate reductase (NR), nitrite-NO reductase (Ni-NOR) [5] and presumptive nitric oxide synthase (NOS)] and non-enzymatic (under acidified conditions, like in apoplast) reactions [6]. NO synthesized by putative NOS activity has been reported in olive plants treated with 200 mM NaCl [3], in pea leaf extracts [2], sunflower hypocotyl extracts [7], in *Lupinus albus* nodules [8], in sunflower seedlings subjected to 120 mM NaCl stress [9] and during sunflower–mildew interaction [7], as established by the application of L-NG-monomethyl arginine citrate (L-NMMA) and aminoguanidine, the well known inhibitors

Abbreviations: OB, oil body; CLSM, Confocal laser scanning microscopy; RNS, reactive nitrogen species; GSNO, nitrosoglutathione; RSNO, S-nitrosothiols; DAF, diaminofluorescein; PTIO, 2-phenyl-4,4,5,5-tetramethyllimidazoline-1-oxyl-3-oxide; MNIP-Cu, 4-methoxy-2-(1H-naphtho(2,3-*d*)imidazol-2-yl)phenol (a copper derivative).

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of inducible and constitutive NOSs in animal cells [10]. Using *Atnoa1* mutants of *Arabidopsis* (reduced endogenous NO level), direct evidence for a putative NOS-dependent NO production has been provided in *Arabidopsis thaliana* in relation with salt tolerance [11]. The AtNOS1 protein is not a real L-arginine-dependent NOS enzyme [12,13]. The expression of rice gene *OsNOA1*, homologous to *AtNOA1*, re-establishes NO synthesis in *Atnoa1* mutants by inducing the expression of PM Na^+/H^+ antiporter gene (*AtSOS1*) and H^+-ATPase genes [14]. This suggests that NO may enhance salt tolerance by inducing the ion transporter genes that enable the required Na^+ homeostasis and K^+ acquisition.

NO-mediated regulation of protein function is a recent area of research in plant biology [15–17]. It has been established that superoxide radicals and NO can react to form peroxynitrite (a very powerful antioxidant), which can subsequently react with cysteine, methionine and tryptophan residues [18]. In animal cells, peroxynitrite can modify a number of biological molecules, including proteins, lipids, and nucleic acids, thereby modulating cellular oxidative damage [18,19]. One of the changes produced by peroxynitrite is the irreversible nitration of tyrosine residues of proteins at the ortho position, leading to loss of function, which can be used as a biomarker of nitrosative stress [3,19,20]. In plants, the effect of salinity on tyrosine nitration of proteins has been reported in olive leaves and citrus plants [3,21]. Salt stress produces an increase in the number and intensity of tyrosine-nitrated proteins of 44–60 kDa. Work on *Arabidopsis* seedlings subjected to salt stress has shown tyrosine nitration of an additional protein of 42 kDa and enhanced accumulation of a 55 kDa protein in salt stressed seedling roots [22]. NO can react reversibly with thiol groups on the cysteine residues of proteins (S-nitrosylation), thereby modulating their functions. This covalent modification is not a direct reaction and could probably be performed through the formation of N_2O_3 in the presence of oxygen, by nitrosonium ions (NO^+), or by a process of trans-nitrosylation from S-nitrosoglutathione (GSNO) [23–25]. GSNO is formed by the reaction of NO with reduced glutathione (GSH) in the presence of oxygen [26]. Very limited number of nitrated proteins with varied functions have been identified so far [27,28]. Some common targets of S-nitrosylation of proteins in plants include cytoskeleton, stress-related, redox-related, metabolic and signalling proteins [29]. GSNO serves as an intracellular reservoir of NO and also a vehicle of NO transport throughout the cell [30]. In the presence of reductants like GSH, ascorbate and Cu^+, GSNO gets broken down to release NO. Fifty two S-nitrosylated proteins have been identified in *Arabidopsis* plants treated with gaseous NO [29].

Sunflower seedlings grown in dark in presence of 120 mM NaCl seem to adopt various biochemical mechanisms to tolerate salt stress. These include enhanced expression of ouabain-sensitive putative sodium efflux pumps [31], accumulation of nitric oxide in the columella, actively dividing cells and vascular parenchyma of the seedling roots [9] enhanced expression of laccase activity in roots, leading to lignification of dividing cells, thereby slowing down the growth of roots under salt stress in view of the enhanced accumulation of NO in roots as well as on the oil body surface in seedling cotyledons. Tyrosine nitration of proteins in the specified regions could probably be one of the mechanisms of NO-mediated signalling mechanisms operating in salt-stressed seedlings to modulate their growth pattern. Observation of NO signal in 2 d old seedling cotyledons (on the oil body surface) further indicates the role of NO in the signalling mechanism for long distance transport (from root to cotyledon) of the salt stress signals. Based on these observations, present work highlights changes in protein tyrosine nitration analysis in the 2d old seedling roots and cotyledons in response to salt stress. The analysis has been carried out both using immunohistochemical and western blot analysis and highlights the significant changes in the spatial distribution of tyrosine nitrated proteins and their electrophoretic pattern.

2. Materials and methods

2.1. Detection of NO in salt-stressed seedlings, oil bodies and protoplasts using a NO-specific fluorescent probe (MNIP-Cu)

MNIP-Cu has been used for the detection of NO signal in macrophages and endothelial cells in animal system [32]. Recently, its synthesis and application for NO detection has been reported from the author's laboratory, using protoplasts from sunflower seedling hypocotyls [33,34].

OBs were isolated from the cotyledons of 2d old dark grown seedlings of sunflower (*Helianthus annuus* L., cv. Morden) irrigated with half-strength Hoagland solution and medium containing 120 mM NaCl. OBs incubated with 25 μM of MNIP-Cu for 10 min were visualized at 492 nm using epifluorescence microscope (Axioskop from Zeiss, Germany). To confirm that the fluorescence is specifically due to NO, OB suspension was incubated in dark for 1 h at 25 °C in a solution of 1 mM PTIO (prepared in 10 mM Tris–HCl buffer, pH-7.4), followed by incubation in 25 μM MNIP-Cu for another 10 min and observed for fluorescence due to NO and its quenching by PTIO.

For isolation of protoplasts, tissues (roots/cotyledons) were chopped and incubated in dark for 4 h at 25 °C on a rocker shaker (12 revolutions min^{-1}) in the protoplast isolation medium [50 mM MES, pH 5.5, 0.7 M mannitol, 20 mM $CaCl_2$, 2% (w/v) cellulase and 1% (w/v) pectinase], its volume being 10 mL g^{-1} FW and 5 mL g^{-1} FW for cotyledons and roots, respectively. Protoplasts were washed and made debri-free, according to Gupta et al. (2003) [35]. Briefly, the protoplast preparations in enzyme solutions were passed through a nylon mesh of 70 μm porosity to obtain a debri-free preparation. The protoplasts were pelleted by centrifugation at 1000 g for 10 min and resuspended in washing buffer (50 mM MES, 0.7 M mannitol, 20 mM $CaCl_2$, pH-7.5). Control protoplasts were washed once more and concentrated by centrifugation at 1000 g. For salt shock treatment, purified protoplasts were resuspended in NaCl-containing buffer (50 mM MES, 20 mM $CaCl_2$, 460 mM mannitol and 120 mM NaCl, pH 7.5) for 10 min. Following the salt shock treatment, protoplasts were washed in washing buffer to remove NaCl. Protoplast viability was tested with 0.01% fluorescein diacetate (FDA).

In order to visualize the salt stress-associated NO expression, both the control and NaCl-treated protoplast preparations were incubated with 25 μM of MNIP-Cu for 10 min and visualized at 492 nm using epifluorescence microscope (Axioskop from Zeiss, Germany) fitted with a digital camera (Axiocam, Zeiss, Germany). Specificity of the probe for NO was established, both in control and NaCl-stressed protoplasts by incubating them with 1 mM PTIO (NO scavenger) for 30 min, followed by co-incubation with MNIP-Cu for 10 min, and subsequent visualization of fluorescence.

2.2. Sample preparation for the detection of tyrosine-nitrated proteins from oil body membrane proteins

Oil body membrane proteins were obtained from cotyledons of 2 d old dark-grown seedlings, grown in the absence and presence of NaCl (120 mM), by grinding them to fine powder in liquid nitrogen and homogenization in the homogenization buffer (pH-7.5; 0.1 M HEPES-KOH, 0.4 M sucrose, 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM PMSF and 0.2% mercaptoethanol) in a proportion of 3 mL g^{-1} FW. The homogenates were filtered through 4 layers of muslin cloth and centrifuged at 10,000 g for 20 min at 4 °C. The oil body pad was collected and subsequently washed with sodium bicarbonate