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Exposure to nitric oxide increases the nitrosyl-iron complexes content in sorghum embryonic axes

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ABSTRACT

This work was aimed to investigate nitrosyl-Fe complexes formation by reaction of endogenous ligands and Fe, in sorghum embryonic axes exposed to NO-donors. Electron paramagnetic resonance (EPR) was employed to detect the presence of nitrosyl-Fe complexes in plant embryos, as well as changes in labile iron pool (LIP). Nitrosyl-Fe complexes formation was detected in sorghum embryonic axes homogenates incubated *in vitro* in the presence of 1 mM of NO donors: diethylenetriamine NONOate (DETA NONOate), S-nitrosoglutathione (GSNO) and sodium nitroprusside (SNP). In axes isolated from seeds incubated *in vivo* in the presence of 1 mM SNP for 24 h, the content of NO was increased by 2-fold, and the EPR spectrum from mononitrosyl-Fe complexes (MNIC) was observed with a concomitant increase in the fresh weight of sorghum axes. The simultaneous exposure to deferoxamine and the NO donor precluded the increase in fresh weight observed in the presence of excess NO. While total Fe content in the axes isolated from seeds exposed to 1 mM SNP was not significantly affected as compared to control axes, the LIP was increased by over 2-fold.The data reported suggest a critical role for the generation of complexes between Fe and NO when cells faced a situation leading to a significant increase in NO content. Moreover, demonstrate the presence of MNICs as one of the important components of the LIP, which could actively participate in Fe cellular mobilization.

1. Introduction

Plants can produce and release significant amounts of NO, especially under stress or in certain physiological processes [1], mainly in actively growing tissues such as embryonic axes [2,3]. Besides endogenous production, plants are in contact with atmospheric NO [4], provided by various sources [5] such as combustion of fossil fuels, lightening and many biological processes [6]. In addition, soils can provide environmental NO, and contribute to almost 20% of the global atmospheric NO budget [7], being the major greenhouse pollutant.

In animals NO has been shown to act as intracellular or transcellular signal, and as a cytotoxic host defense compound [8]. In spite of its high chemical reactivity the NO molecule can be transported to a distance several folds exceeding cell sizes [9] to exert its actions as a cellular signalling molecule. Moreover, it is capable of binding Fe with high stability constants (e.g. $K_{Fe2+\text{-citrate:NO}} = 2.1 \times 10^4\ M^{-1}$) [10], and it was even described that NO could act as an antioxidant in Fe-mediated oxidative stress in rat hepatocytes by reacting with Fe low molecular weight complexes to form inactive Fe complexes unable to induce oxidative stress [11].

NO binds Fe^{2+} to form Fe-nitrosyl complexes. Depending on the nature of the attached ligand, both mononitrosyl- (MNIC) and dinitrosyl-Fe complexes (DNIC) were reported to be formed [12], with the general formula L_2–Fe–NO and L_2–Fe–$(NO)_2$, respectively. From the physicochemical and functional properties of the Fe-nitrosyl complexes, it was postulated that these compounds can function in NO transportation in animals and humans, acting as forms for NO stabilization which provide NO transport over various cells and tissues [13]. DNICs were found in animal tissues or isolated cells treated with gaseous NO or NO donors, by their characteristic electron paramagnetic resonance (EPR) spectra centred at about 2.03 g [13]. In these complexes, Fe^{2+} ion coordinates two NO molecules together with two other ligands, the thiol-containing

Abbreviations: DFO, deferoxamine; DETA NONOate, diethylenetriaamine NONOate; DETC, diethylenetriamine; DNIC, dinitrosyl-Fe complexes; EPR, electron paramagnetic resonance; LIP, labile iron pool; MNIC, mononitrosyl-Fe complexes; NO, nitric oxide; GSNO, S-nitrosoglutathione; MGD, N-methyl-D-glucamine dithiocarbamate; SNP, sodium nitroprusside; TEMPOL, 4-hydroxy-2,2,6,6-tetramethyl piperidine-N-oxyl.

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groups which could belong to proteins or low molecular weight compounds [13]. The physiological role of DNICs is still controversial, because in spite of acting as stable natural NO carriers, they are also known to have toxic effects in biological systems [14,15].

On the other hand, it has been generally accepted that MNICs are highly stable in animal organisms and cultured cells, which results in accumulation of these compounds upon formation, as it was shown by the addition of N-methyl-D-glucamine dithiocarbamate (MGD) or diethyl dithiocarbamate (DETC) [16]. MNICs can be converted, under certain conditions, e.g. the presence of oxidizing species, into an EPR-silent form [17], showing that they are redox active compounds.

Previous studies also linked NO with Fe homeostasis in plant tissues, since it was shown that NO participates in ferritin expression in Arabidopsis [18] and prevented Fe-deficiency-induced chlorosis in maize [19]. Recently, it was observed that GSNO treated tomato plants improved their capacity to survive under low-Fe conditions without increasing total Fe content in the leaves, supporting the hypothesis for a relation between NO and Fe availability [20]. In this regard, it was also found that incubation of sorghum seeds in the presence of NO donors drove to a protective effect over protein and lipid oxidation accompanied by an increase on Fe availability [21].

The hypothesis of this work was that nitrosyl-Fe complexes could be formed *in vivo,* by reaction of endogenous ligands and Fe, in embryonic axes exposed to NO-donors. The nitrosyl-Fe complexes were detected employing EPR techniques, and their presence was linked to the intracellular labile iron pool (LIP).

2. Materials and methods

2.1. Plant material and treatments

Sorghum bicolor (L.) Moench seeds (germination over 98%) were placed in the dark at 26 °C over distilled water saturated filter paper, after 24 h of imbibition embryonic axes were excised from seeds, washed several times with distilled water, and used for further assays. Incubations were carried out either in the presence or the absence of the NO donors, diethylenetriamine NONOate (DETA NONOate), S-nitrosoglutathione (GSNO) or sodium nitroprusside (SNP) up to a concentration of 1 mM. The water used to prepare all solutions was passed through columns containing Chelex 100 resin (Sigma Chemical Co.) to remove metal contaminants.

2.2. Synthesis of dinitrosyl diglutathionyl Fe complex

The complex of dinitrosyl-Fe, with glutathione as the ligand, was employed as a model for DNIC. The synthesis was performed according to Cesareo et al. [22], after incubation on ice in the dark, the strong yellow solution (ε = 3390 M^{-1} cm^{-1} at 340 nm) [23] was kept in liquid nitrogen.

2.3. Synthesis of mononitrosyl Fe complex

The complex of mononitrosyl-Fe, with dithiocarbamate as the ligand, was employed as a model of MNIC. The synthesis was performed as follows: to 100 μl of 10 mM N-methyl-D-glucamine dithiocarbamate (MGD) prepared in 100 mM MES buffer, pH 7.0, 10 μl of 10 mM $FeSO_4$ prepared in HCl 10 mM, and 100 μl of 100 mM GSNO were added [24].

2.4. DNIC detection by EPR

Either samples of plant tissues or DNIC solutions, were immediately frozen and measured at −196 °C as previously described [25]. The samples were cooled employing a finger Dewar flask filled with liquid nitrogen. The spectra were recorded with a Bruker ECS 106 EPR spectrometer, operating at 9.5 GHz. Measurements were performed employing the following parameters: microwave frequency 9.40 GHz, microwave power 5 mW, center field 3292 G, time constant 0.5 s, modulation amplitude 6.3 G, and modulation frequency 100 KHz [25].

2.5. MNIC detection by EPR

Either samples of plant tissues or MNIC solutions, were placed in bottom-sealed Pasteur pipettes for EPR measurements at room temperature. The spectra were recorded with a Bruker ECS 106 EPR spectrometer, operating at 9.5 GHz with the following instrument settings: 200 G field scan, 83.886 s scan time, 327.68 ms time constant, 5.983 G modulation amplitude, 50 kHz modulation frequency and 20 mW microwave power [24].

2.6. NO detection

Sorghum embryonic axes were excised from seeds, homogenized in 100 mM phosphate buffer, pH 7.4, and supplemented with the spin trap solution (10 mM MGD, 1 mM $FeSO_4$) [24]. The homogenates were immediately transferred to pasteur pipettes for EPR spin trapping measurements. The spectra were recorded at room temperature (18 °C) with a Bruker ECS 106 EPR spectrometer, operating at 9.5 GHz. Instrument settings include 200 G field scan, 83.886 s scan time, 327.68 ms time constant, 5.983 G modulation amplitude, 50 kHz modulation frequency and 20 mW microwave power. Quantification of the spin adduct (MGD_2–Fe^{2+}–NO) was performed using as standard an aqueous solution of TEMPOL, a stable free radical, introduced into the same sample cell used for spin trapping measurements. The TEMPOL solutions were standardized spectrophotometrically at 429 nm (ε = 13.4 M^{-1} cm^{-1}), and the concentration of MGD_2–Fe^{2+}–NO adduct was obtained by double integration of the three lines spectra and cross-checked with the TEMPOL spectra.

The kinetic of NO release from NO-donor solutions was carried out employing electrochemical detection. NO was detected at room temperature and recorded as a function of time with a selective electrode (ISO-NO Mark II WPI).

2.7. Determination of labile iron pool (LIP)

Either samples of plant tissues or chemical solutions, were added with deferoxamine mesylate (DFO, 1 mM), frozen, and transferred to a finger Dewar flask containing liquid nitrogen for EPR examination at 77 K [26]. Measurements were performed using the following instrument settings: modulation frequency 50 kHz, microwave power 20 mW, microwave frequency 9.45 GHz, centred field 1600 G, time constant 81.92 ms, modulation amplitude 4.759 G and sweep time with 800 G. The concentration of the DFO–Fe complex [DFO–Fe (III)] in the samples was obtained by comparison of the signal height to a standard curve where 1 mM solution of DFO was added to solutions of known concentrations of Fe [27].

2.8. Statistical analyses

Data in the text, figures, and tables are expressed as means ± SE of three to six independent experiments, with two replicates in each experiment. Effect of treatments on measured parameters was tested for significance using single-factor ANOVA. Significantly different means were evaluated using the Tuckey post test (GraphPad InStat for Windows Version 3.0; GraphPad Software Inc.).