Chapter 12

Quantification and Localization of *S*-Nitrosothiols (SNOs) in Higher Plants

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Abstract

S-nitrosothiols (SNOs) are a family of molecules produced by the reaction of nitric oxide (NO) with –SH thiol groups present in the cysteine residues of proteins and peptides caused by a posttranslational modification (PTM) known as *S*-nitrosylation (strictly speaking *S*-nitrosation) that can affect the cellular function of proteins. These molecules are a relatively more stable form of NO and consequently can act as a major intracellular NO reservoir and, in some cases, as a long-distance NO signal. Additionally, SNOs can be transferred between small peptides and protein thiol groups through *S*-transnitrosylation mechanisms. Thus, detection and cellular localization of SNOs in plant cells can be useful tools to determine how these molecules are modulated under physiological and adverse conditions and to determine their importance as a mechanism for regulating different biochemical pathways. Using a highly sensitive chemiluminescence ozone technique and a specific fluorescence probe (Alexa Fluor 488 Hg-link phenylmercury), the methods described in this chapter enable us to determine SNOs in an nM range as well as their cellular distribution in the tissues of different plant species.

Key words Chemiluminescence, Confocal laser scanning microscope, Fluorescent probes, Nitric oxide, Reactive nitrogen species, *S*-nitrosothiol

1 Introduction

Nitric oxide (NO) is a gaseous free radical that affects a wide variety of physiological and pathological aspects of higher plants [1–5]. Nitric oxide can interact with many molecules that affect the function of target molecules in many cases. For example, the chemical reaction between NO and the superoxide radical ($O_2^{\bullet -}$) generates peroxynitrite ($ONOO^-$) which is the most powerful oxidant that mediates PTM through nitration reactions [6, 7]. On the other hand, the interaction of NO with –SH thiol groups present in the cysteine residues of peptides and proteins generates a group of molecules called *S*-nitrosothiols (SNOs) [8–10]. This NO, which

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binds with thiols to form *S*-nitrosothiols, is biologically reversible [11]. In addition, its molecular family is regarded as one of the most important mechanisms for transducing signals mediated by NO in biological systems [11–13]. This is explained by the fact that a process of *S*-nitrosylation—strictly speaking *S*-nitrosation [11]—is capable of regulating the function of target proteins [14–18].

In general, one group of SNOs contains designated high-molecular-mass SNOs produced by NO binding to sulfhydryl (–SH) groups present in specific cysteine residues of proteins. Another group is composed of low-molecular-mass SNOs, the most important one being *S*-nitrosoglutathione (GSNO) which is generated by NO's *S*-nitrosylation reaction with the thiol tripeptide, γ-glutamyl cysteinyl glycine (glutathione, GSH), one of the major low-molecular-weight soluble antioxidants in plant cells [19, 20]. This group includes other molecules such as *S*-nitrosocysteine (CySNO) and *S*-nitrosocysteinylglycine (GlyCySNO) [21], which have been studied to a lesser degree in the field of plant research.

The detection and quantification of SNOs in biological systems and specifically in higher plants is a major challenge as the presence of artifacts can quite easily produce misleading information (reviewed in ref. [22]). This chapter provides two complementary approaches which can be useful for studying the content and distribution of *S*-nitrosothiols in different plant tissues and species under physiological and stress conditions and confirms the involvement of these molecules in the response and signaling mechanism in different situations [23–28].

2 Materials

2.1 Specific Equipment

1. Nitric oxide analyzer (NOA): Sievers, model 280i, Sievers Instruments.
2. Preparative centrifuge (Sorvall RC-5 and rotors).
3. Vibratome (Leica Microsystems, Germany).
4. Confocal laser scanning microscope (CLSM) system (Leica TCS SL, Leica Microsystems, Wetzlar, Germany).

2.2 Reagents

1. Nitrite (NO_2^-) standard stock solution: 100 mM.
2. *N*-ethylmaleimide (NEM) stock solution: 100 mM.
3. Sulfanilamide stock solution: 100 mM.
4. Mercury (II) chloride ($HgCl_2$): $HgCl_2$ (molecular weight = 271.5) in 10 ml ultrapure water: 13.7 mg.
5. Reaction solution (iodine/triiodide mixture) for the NOA purge vessel: 4.5 ml of glacial acetic acid and 500 μl aqueous mixture containing 450 mM potassium iodide (KI) and 100 mM iodine (I_2). Dissolve 108 mg KI in 1 ml ultrapure

water. Add 38 mg I_2 and mix at room temperature. Mix 1 ml of this solution with 9 ml glacial acetic.

6. Alexa Fluor 488 Hg-link phenylmercury (cat. no. H30462, Molecular Probes, Eugene, OR, USA).

2.3 Solutions

1. The extraction medium (1:5; w/v) was made up of 100 mM Tris–HCl buffer (pH 7.5) containing 5 % (w/v) sucrose, 7 % (w/v) PVPP, 0.05 % (v/v) Triton X-100, 0.1 mM EDTA, 15 mM DTT, 1 mM PMSF, 100 μM DTPA, and a protease inhibitor cocktail.

3 Methods

3.1 Ozone Chemiluminescence Detection of Total and Low-Molecular-Mass SNOs

The ozone-based chemiluminescence technique has become a reliable method to detect and quantify NO and other related molecules including SNOs as it is highly sensitive, having in some cases a concentration range of nM. The method used in plants to quantify total SNOs is based on the technique described by Valderrama et al. [23] with some modifications [26]. The detection of SNOs is based on the reductive decomposition of nitroso species by an iodine/triiodide mixture to release NO, which is then measured with the aid of gas-phase chemiluminescence in reaction with ozone [29]. Unlike other nitroso species such as nitrosamines and nitrosyl hemes, SNOs are sensitive to reductants and mercury-induced decomposition.

3.1.1 Plant Extracts

1. During all procedures, the samples were kept at 4 °C and protected from the light.
2. Plant samples were grounded using a mortar and pestle in liquid nitrogen.
3. The resulting coarse powder was transferred to 1/5 (w/v) extraction buffer, which can change depending on the plant species and tissue involved. For example, for pea leaf samples, we successfully used a medium (1:3; w/v) containing 100 mM Tris–HCl, pH 7.6, 1.5 mM dithiothreitol (DTT), 5 % sucrose (w/v), 0.005 % Triton X-100 (v/v), and 100 μM diethylenetetraminepentaacetic acid (DTPA).
4. The extract was then centrifuged at 3000 × *g* for 10 min at 4 °C.
5. The supernatant was used for SNO determination. In the case of sunflower hypocotyls, the extraction medium is more complex as this tissue can oxidize very rapidly (brown color).
6. The supernatant was used to determine total SNOs and the level of low-molecular-mass SNOs was determined in a filtered supernatant fraction that had previously passed through a 10 kDa cutoff membrane.

Note: For the chemiluminescence detection of total SNOs, the whole procedure needs to be performed under a red safety light in order to protect SNOs from light-dependent decomposition.

3.1.2 Treatment of Plant Samples to Block Free Thiols and to Eliminate Nitrite Content

Add 10 mM NEM to the plant samples (for each 1.5 ml sample, add 167 μl of 100 mM NEM) and incubate for 15 min at 4 °C in order to block free thiols.

For each sample, three 1.5 ml aliquots were prepared:

1. Aliquot A: Inject directly into the nitric oxide analyzer (NOA) with its corresponding blank.
2. Aliquot B: Add 10 mM sulfanilamide and incubate for 15 min at 4 °C. This will eliminate nitrite from the samples.
3. Aliquot C: Add 10 mM sulfanilamide and 7.3 mM $HgCl_2$. Incubate for 30 min at room temperature. This will eliminate nitrite and SNOs, respectively.

Calculations

Nitrite concentration = Aliquot A – Aliquot B

S-nitrosothiol concentration = Aliquot B – Aliquot C

Other nitroso species = Aliquot C

Set up the NOA and samples.

1. Prepare samples and treatments (aliquots A, B, and C).
2. Prepare purge chamber of the NOA containing 5 ml iodine/triiodide mixture at 95 °C under a nitrogen stream.
3. Prepare nitrite standard curve between 1 nM and 100 mM prepared from a standard 100 mM nitrite stock solution.
4. Set up the NOA.
   (a) Set up a water bath at 70 °C and open the NOA refrigeration system.
   (b) Open the N_2 and O_2 system and maintain a constant flux (0.5 bar).
   (c) Add 5 ml reaction solution (potassium iodide/iodine mix) to the purge vessel. It is recommended to change this solution every 4–5 injections.
5. Inject samples (40 μl or more depending on the signal obtained) into the NOA purge chamber.

Figure 1a represents total SNO content in pea leaf samples from plants exposed to different abiotic stress situations [24]. Figure 1b shows total SNO content in the hypocotyls of two sunflower cultivars after infection by *Plasmopara halstedii* [25].

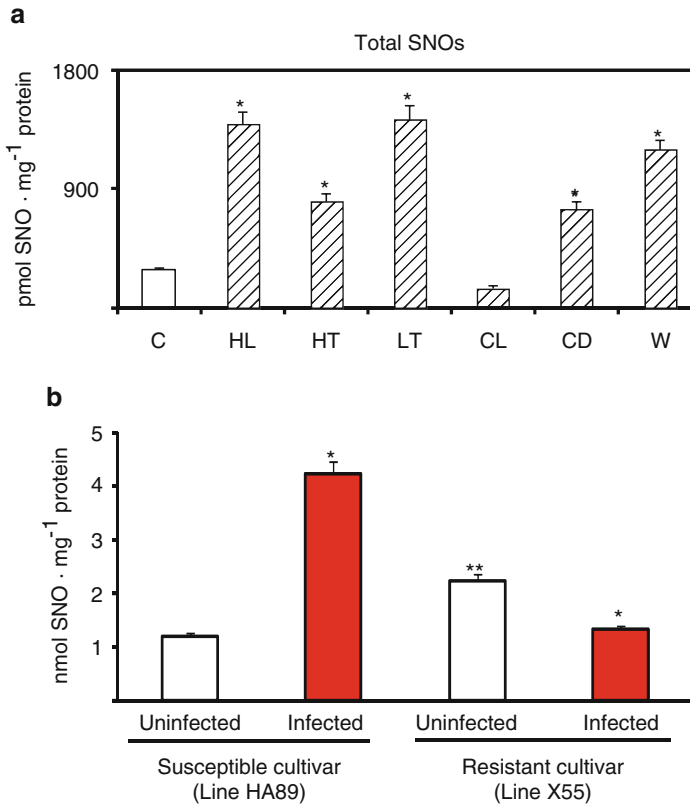
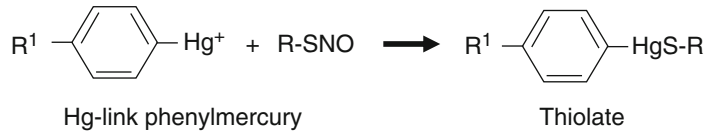


Fig. 1 Analysis of total *S*-nitrosothiols (SNOs) determined by ozone chemiluminescence in (**a**) pea leaves of plants exposed to different abiotic stress conditions and (**b**) sunflower hypocotyls after infection by *Plasmopara halstedii*. (**a**) Leaf extracts from pea plants subjected to different abiotic stress conditions. *C* control, *HL* high light intensity, *HT* high temperature, *LT* low temperature, *CL* continuous light, *CD* continuous dark, *W* wounding. Results are means ± SEM of samples from at least three different experiments. *Asterisks* indicate that the increase in SNOs was statistically significant ($P > 0.005$; $n \geq 3$) as compared with control plants (C). Reproduced with permission from Corpas et al. [24]. (**b**) Hypocotyl extracts from two sunflower cultivars (HA89 and X55, susceptible and resistant cultivars, respectively), uninfected and after infection by *P. halstedii*. Results are the mean of four different experiments ± SEM. *Differences between uninfected and infected values were significant at $P < 0.05$. **Differences between control (uninfected) values were significant at $P < 0.05$. Reproduced with permission from Chaki et al. [25]

3.2 Localization of SNOs with the Aid of a Confocal Laser Scanning Microscope

In plant tissues, SNOs can also be detected using the fluorescent reagent Alexa Fluor 488 Hg-link phenylmercury. The method used to study the cell distribution of total SNOs in plants is based on the technique described by Valderrama et al. [23] with some modifications [30]. This dye, which is a new generation of fluorescent probes, has certain advantages over other fluorescent dyes; for example, it is highly fluorescent over a broad pH range (pH 4–10), has a higher level of photostability, allows more time for image capture, has good water solubility, and is less affected by autofluorescence from

proteins and other biomolecules. Thus, Alexa Fluor 488 Hg-Link phenyl mercury can be used for direct *S*-nitrosothiol detection in plant tissues when samples are preincubated with *N*-ethylmaleimide (NEM) to block free sulfhydryl groups. Under these conditions, the fluorescent Hg-Link reagent labels R-SNO-modified proteins and peptides according to the following equation:



3.2.1 Procedure

1. Plant tissue is cut into segments of approximately 25 mm^2 and is incubated at 25 °C for 2 h in darkness, with 100 μM (DTPA) plus 10 mM NEM prepared in ethanol, which blocks free sulfhydryl groups.
2. Each segment is washed three times in 10 mM Tris–HCl buffer, pH 7.4, for 15 min.
3. Segments are incubated with 10 μM Alexa Fluor 488 Hg-Link phenyl mercury for 1 h at 25 °C in darkness.
4. Segments are washed again three times in the previous buffer.
5. Plant segments are embedded in order to obtain appropriate sections to be observed under a confocal laser scanning microscope in a 15 % acrylamide solution prepared in 0.01 M phosphate-buffered saline (PBS) including 0.3 % TEMED for 4 h.
6. Place each plant segment in inclusion containers (1.5 × 0.9 × 0.5 mm; Sorvall Instruments) and add 0.5 ml of a fresh 15 % acrylamide stock solution and 50 μl of 2 % persulfate ammonium (PSA) to be polymerized. Plant segments must be quickly oriented after PSA is added, and the containers are covered with parafilm in order to improve polymerization.
7. After the acrylamide block containing the plant segments is polymerized, latter are removed.
8. Sections measuring 80–100 μm thick are cut, as indicated by the Vibratome scale, under 10 mM PBS. The sections are then soaked in a glycerol:PBS mixture containing azide (1:1, v/v) and mounted in the same medium on a slide for examination with the aid of a confocal laser scanning microscope (CLSM) system (Leica TCS SL) using standard filters for Alexa Fluor 488 green fluorescence (excitation 495 nm; emission 519 nm).

   All procedures must be performed under a red safety light.

3.2.2 Additional Controls

SNOs are capable of releasing NO in a controlled manner into the living system either spontaneously or through interaction with various biological reductants such as glutathione and ascorbate. In addition, the reduced metal ion (e.g., Cu^+) breaks SNOs down more rapidly than the oxidized metal ion (e.g., Cu^{2+}), indicating

that reducing agents such as glutathione and ascorbate can cause the breakdown of *S*-nitrosothiol by the chemical reduction of contaminating transition-metal ions [31–33]. To confirm the effect of these reductants, as a control, the detection of SNOs in plant samples can be carried out in the presence of reductants. Although the procedure used is similar to the previous one, in this case, the plant tissue segments are preincubated in a reductant solution containing 1 mM ascorbate, 10 μM CuCl, and 200 μM cPTIO in 10 mM phosphate-buffered saline (PBS) at room temperature for 1 h. Afterwards, each sample is washed three times for 15 min (two washes with PBS and one with 10 mM Tris–HCl buffer, pH 7.4). The procedure then proceeds from **steps 1** to **8** as described above.

Figure 2 shows an example of the cellular localization of SNOs in hypocotyl sections of sunflower seedlings growing under optimal

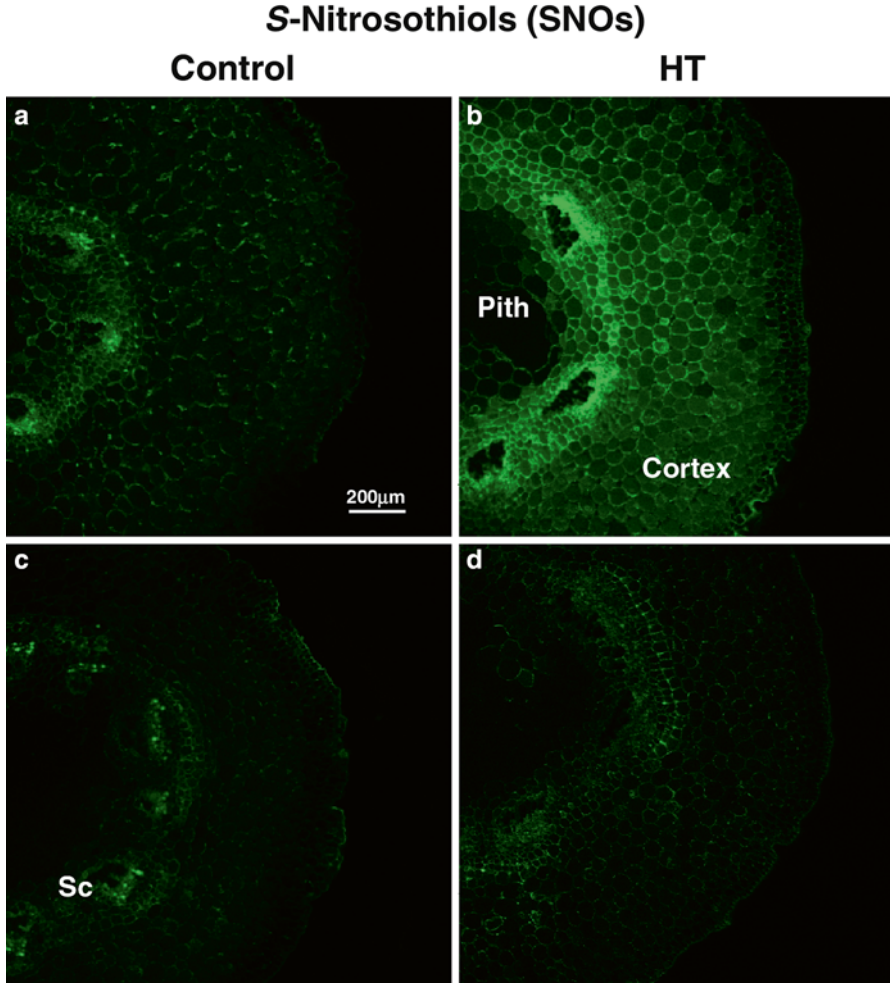


Fig. 2 Images illustrating the CLSM detection of SNOs in cross sections of hypocotyls in sunflower seedlings from control and subjected to high temperature (HT) in the absence of reductants (A and B) and in the presence of reductants (D and E). Bright green fluorescence corresponds to detection of SNO with 10 μM Alexa Fluor 488 Hg-link. *Sc* sclereids. Reproduced with permission from Chaki et al. [26]

conditions (Fig. 2a) and exposed to high temperature (HT) where green fluorescence indicates the presence of SNOs [26]. It can thus be observed how HT stress provokes a significant increase in SNO content in cortex cells and sclereids. On the other hand, Fig. 2c, d shows the additional internal controls where the hypocotyl section from sunflower seedlings growing under both optimal and stress conditions was preincubated with a reductant solution which causes a significant reduction in SNO content.

Acknowledgments

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