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Alternative fluorimetric-based method to detect and compare total *S*-nitrosothiols in plants

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ABSTRACT

Nitric oxide (NO) is an important signaling molecule occurring in virtually all organisms, whose mechanism of action relies mainly on its interaction with proteins or peptides by nitrosylation, forming compounds such as *S*-nitrosothiols (SNO). The Saville reaction and the ozone-based chemiluminescence method are the main techniques used for nitrosylated protein quantification. While the Saville assay is not very sensitive, the ozone-based chemiluminescence is expensive and time-consuming. Here we propose a method in which the protein-bound NO groups are exposed to UV light, cleaving the bond and allowing the quantification of the derived NO molecules by diamino-rhodamine (DAR) dyes. The DAR-based method was shown to be specific in plant tissues by pre-treatment of the samples with reducing agents and parallel EPR analysis. Spike-and-recovery assays revealed 72% recovery after a GSNO spike. Moreover, the method was significantly more sensitive than the Saville reaction, and this increase in sensitivity was crucial for detecting the reduced levels of nitrosylated proteins in plant species other than *Arabidopsis*. The method presented here is a suitable alternative to compare plant samples, allowing simple and fast detection of nitrosylated proteins.

1. Introduction

Nitric oxide (NO) is an important signaling molecule occurring in virtually all biological systems, from bacteria to animals and vascular plants [1]. Instead of relying on dedicated receptors, NO action critically depends on the post-translational modifications of peptides and proteins via either nitrosylation (technically a nitrosation process, but the term “nitrosylation” is widely used by plant biologists) or nitration reactions [2]. Of these two processes, nitrosylation appears to play important roles in regulating protein activity, stability and even cellular localization [3]. Cysteine residues are one of the most important sites of nitrosylation in proteins and peptides, and this process is specifically referred to as *S*-nitrosylation [4]. Heme-nitrosylation is also an important process in NO signal transduction and could represent a primary reaction in many cases [2]. Besides being formed *de novo*, *S*-nitrosothiols (SNOs) can also be transferred to free thiols in other molecules or even in the same molecule through a process called *trans*-nitrosylation [5]. Also, glutathione (GSH), an important and remarkably abundant molecule in the redox system of eukaryotic cells, can be nitrosylated, yielding *S*-nitrosoglutathione (GSNO [6,7]). Due to its central role in NO metabolism, SNOs quantification and/or identification have been a common goal of many research publications [5,8–18]. Not surprisingly, distinct methods have been developed so far to facilitate identifying and/or quantifying SNO and other nitrosylated proteins, each of them presenting some positive and negative features, as discussed in several reviews [10,11,14,18].

In plant research, two quantitative techniques have been frequently employed over the years to determine the levels of nitrosylated proteins: the Saville reaction [19] and reduction of the S-NO group followed by detection of emitted NO by

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chemiluminescence [5,20,21]. The Saville reaction is a relatively easy and low-cost method to assess the contents of protein nitrosylation, which is based on measuring the nitrite formed by the release of NO from SNOs upon the incubation with $HgCl_2$. In this method, nitrite formed is spectrophotometrically detected using the Griess colorimetric reaction [22]. The difference between absorbance with and without $HgCl_2$ indicates the levels of nitrosylated proteins in the initial sample. However, the Saville reaction lacks sensitivity when compared to other methods [14]. In contrast, the chemiluminescence-based detection of NO after the reduction of the S-NO group is very sensitive, but requires a chemiluminescence-based NO monitoring system coupled to a glassware reaction chamber, which can be rather expensive. The reducing medium used in this method may vary, but it usually involves iodine [5] or copper/cysteine [14].

King et al. [8] proposed a method that allowed detection of endogenous nitrosylated proteins in animal samples on polyacrylamide gels, but its sensitivity was limited [10,11,14]. This method is based on cleaving the S-NO bond upon UV light exposure and detection by a fluorescent dye [8]. Very recently this method has been evaluated in plant samples allowing a qualitative and comparative analysis of endogenous nitrosylated proteins during pepper (*Capsicum annuum* L.) fruit ripening [23].

By using the same general principle (i.e., UV-dependent photolysis of S-NO bonds), in the present work we developed and thoroughly tested an alternative method for detection and quantification comparison of nitrosylated protein and peptides in plant samples from different origins such as arabidopsis, tomato, sorghum or maize. Overall, this alternative method proved to be sensitive, reproductive and easy-to-perform, dispensing chemiluminescence-based detectors and not influenced by nitrite contamination.

2. Material and methods

2.1. Plant material and experimental conditions

Most of the experiments were conducted with wild-type (WT) *Arabidopsis thaliana* Columbia (Col-0). *A. thaliana* plants were germinated in a Stender® substrate (Latvia) and maintained for approximately 45 days in a growth chamber at 25 ± 2 °C, 70 ± 10% relative humidity, 12 h photoperiod and light intensity of approximately 100 μmol m^{-2} s^{-1}. Plants were fertilized once a week, with Murashige and Skoog nutrient solution [24]. When applicable, NO fumigation (10,000 ppm) was applied for 15 min as described previously [25].

To test the applicability of this method to species other than arabidopsis, either tomato (*Solanum lycopersicum* cv. Micro-Tom), sorghum (*Sorghum bicolor* cv. CSMXS 101B) or maize (*Zea mays*) plants were used. Tomato and maize were cultivated for approximately 2 months and 2 weeks, respectively, in a growth chamber with the following environmental conditions: day/night temperature of 27/23 °C, humidity of 50/70%, 12 h photoperiod and light intensity of approximately 250 μmol m^{-2} s^{-1}. Sorghum plants were grown in a greenhouse under automatic irrigation (four times a day) in an average mean temperature of 25 °C, 11.5/13 (winter/summer) light hours and incident photo-irradiance of approximately 300 μmol m^{-2} s^{-1}. We also performed a spike-and-recovery assay in *Arabidopsis* samples, by adding 20 μM GSNO, prior to the measurements.

2.2. SNO extraction

Leaf samples were immediately ground to powder with liquid nitrogen and extracted in a buffer containing 50 mM phosphate buffer (pH 7.2) and 80 mM of *S*-methyl methanethiosulfonate (MMTS). Samples were centrifuged at 20,000 g for 20 min at 4 °C and the supernatants were collected and added to chilled acetone (3:1, chilled acetone: extract volume). Samples were then incubated for 60 min at −20 °C and centrifuged again at 20,000 g for 20 min at 4 °C. Acetone was discarded and the pellets were resuspended in the same extraction buffer. These procedures were performed in partial darkness and samples were maintained on ice and protected from light whenever possible. Protein concentration was measured after acetone precipitation using the Bradford method [26].

2.3. SNO measurements

2.3.1. Saville method

The Saville reaction was performed as described previously [10]. Briefly, 300 μL of plant extracts or commercial standards were incubated for 20 min with 300 μL of 3.4% (w/v) sulfanilamide (in 0.4 M HCl) and 250 μL of 0.1% (w/v) *N*-(1-naphthyl) ethylenediamine, with or without 0.1% (w/v) of $HgCl_2$. After incubation for 20 min at room temperature, absorbance was measured at 540 nm. The difference in absorbance between samples treated and untreated with $HgCl_2$ were used to estimate SNO concentration in the samples.

2.3.2. Chemiluminescence-based method

The detection of GSNO was performed as Feelisch et al. [27] with some modifications. Plant extracts or commercial standards were injected in a reaction chamber with a reducing mixture of 225 mM KI and 30 mM I_2 dissolved in 90% (v/v) acetic acid. The reaction chamber was maintained at 60 °C. Emitted NO was measured using an Ecophysics CLD88 NO analyzer as described previously [28]. NO-free nitrogen was used as carrier (100 mL min^{-1}).

2.3.3. Diamino-rhodamine (DAR)-based method

An aliquot of 200 μL of extract or standard was mixed with 1.5 μL of a 5 mM diaminorhodamine-4M (DAR-4M) solution in DMSO and then divided into two aliquots of 100 μL. One of these aliquots remained in absolute darkness at room temperature for 5 min, while the other one was transferred into a well in a 0.2 mL PCR plate and placed face-down in a transilluminator (Loccus Biotecnologia, Cotia, Brazil), where it remained for 5 min exposed to UV light (302–312 nm provided by 8 W lamps). Afterward, the volume of both aliquots was adjusted to 1 mL with 50 mM phosphate buffer (pH 7.2) and fluorescence was measured in a Perkin-Elmer LS-55 spectrofluorometer with excitation and emission recorded at 560 and 575 nm, respectively. The difference between fluorescence in UV-treated and -untreated aliquots was used to estimate the concentration of nitrosylated proteins in the extracts by comparing to a standard curve of GSNO.

2.3.4. Electron paramagnetic resonance (EPR) method

For this approach, GSNO was used as standard prepared in the lab based on previously published data [25,29], using both $Na^{14}NO_2$ and $Na^{15}NO_2$. The resulting GSNO was washed with acetone to remove the unreacted nitrite or other reaction components. The concentration of the GSNO was confirmed each time by using the extinction coefficients ε_{335nm} = 0.922 mM^{-1} cm^{-1} and/or ε_{545nm} = 0.015 mM^{-1} cm^{-1}. Aliquoted stocks of GSNO were stored in −80 °C freezer. In each measurement, the GSNO solutions of different concentrations were mixed with 350 μL of 1 mM deoxyhaemoglobin (Hb) in 50 mM potassium phosphate buffer (pH 7.2), in sealed special glass vials that were degassed with nitrogen for 5 min to ensure complete lack of oxygen. The vials were then exposed to UV light or remained in darkness as mentioned above