Detection of S-nitrosated nuclear proteins in pathogen-treated *Arabidopsis* cell cultures using biotin switch technique

Azam Shekariesfahlan[1] and Christian Lindermayr[2]

[1] Iranian Research Institute of Plant protection, Agricultural Research, Education and Extension Organization (AREEO), Tehran, Iran

[2]Institute of Biochemical Plant Pathology, Helmholtz Zentrum München – German Research Center for Environmental Health, Munich/Neuherberg, Germany

Corresponding author:

PD Dr. Christian Lindermayr

Tel.: +49(0)89 3187 2285

Fax.: +49(0)89 3187 3383

Email: lindermayr@helmholtz-muenchen.de

Running head: Detection of S-nitrosated nuclear proteins

Abstract

Nitric oxide (NO) is an important signaling molecule involving in various plant physiological processes. The main effect of NO arises from its reaction with proteins. S-Nitrosation is the most studied NO-mediated protein post-translational modification in plant field. Via S-nitrosation, NO derivatives react with thiol groups (SHs) of protein cysteine residues and produce nitrosothiol groups (SNOs). From the time of discovering the biological function of NO in plants, an interesting case of study has been the detection of the endogenous S-nitrosated proteins in different plants, tissues, organelles and various conditions. Maps of S-nitrosated proteins provide hints for deeper studies on the function of this modification on specific proteins, biochemical pathways and physiological processes. Many functions of NO have been found to be related to plant defense; on the other hand the involvement of nuclear proteins in regulation of plant defense reactions is well studied. Here, an approach is described in which the *Arabidopsis* cell cultures first are treated with *P. syringae*, afterward their bioactive nuclear proteins are extracted, then the nuclear proteins are subjected to biotin switch assay in which S-nitrosated proteins are specifically converted to S-biotinylated proteins. BST which was introduced by Jaffrey et al [1] solves the instability issue of SNOs. Additionally, it provides detection and purification of biotinylated proteins by anti-biotin antibody and affinity chromatography, respectively.

Key words

Nitric oxide, S-nitrosation, biotin switch assay, suspension cell culture, nuclear proteins, *P. syringae*, *Arabidopsis thaliana*

1. Introduction

Nitric oxide is a radical gas which is known as an important regulatory molecule in many different physiological processes such as growth, development and defense. Many regulatory roles of NO are the consequence of its direct or indirect reaction with proteins [2]. In the animal field, the most known example is cyclic guanosine monophosphate (cGMP)-dependent signalling; in which binding of NO to the heme center of soluble guanylate cyclase (sGC) activates its catalytic domain. This leads to the production of cGMP from guanosine triphosphate; and cGMP in turn acts as a second messenger [3]. In the plant field, several studies in various pathways have shown the involvement of metal nitrosylation (direct binding of NO to transition metal ions of proteins) and tyrosine nitration (covalent modification of tyrosine residues by reactive nitrogen species) in NO-dependent signalling. However, S-nitrosation (covalent attachment of an NO moiety to the SH group of a cysteine residue) has grown into the most relevant NO-mediated protein modification in plants. S-Nitrosothiols are highly unstable due to the low binding energy of SNOs [4]; this property grants them to provide a sensitive mechanism in signalling processes. However, the labile nature of SNOs causes the detection of endogenously S-nitrosated proteins turns into a challenging process. Besides direct methods of SNO detection, such as mass spectrometry and X-ray crystallography; indirect methods have been established at the base of either measuring NO levels after breaking off the S-NO bonds or altering nitrosothiols to a detectable tag [5]. BST - the most commonly used method for indirect detection of S-nitrosated proteins - was invented by Jaffrey et al [1] and further adopted for plants by Lindermayr et al [6]. BST contains three steps: 1) the free thiols are blocked by methyl methanethiosulfonate (MMTS) and remaining of MMTS is removed, 2) the nitrosothiols are selectively reduced by ascorbate and 3) labelled by biotin-HPDP. Afterwards the biotin-labeled proteins are detected by an antibiotin antibody, purified by avidin affinity and identified by mass

spectrometry [1,6]. The specificity of BST to S-nitrosothiols is on the base of the fact that ascorbate can convert SNOs to SHs but not SSGs and other S-oxides [7]. Thermodynamic measurements support this specificity [1], however some controversial reports exist about the specificity of ascorbate [5]. Another important challenge of BST is producing false-positives due to non-adequate blocking by MMTS [4]. Finally, although different treatments and controls are conducted with the same amount of starting protein, the high number of chemical steps and acetone precipitations causes to count on BST rather as a qualitative method.

Using BST combined with mass spectrometry, hundreds of S-nitrosation candidates have been identified in several organelles such as mitochondria [8,9], peroxisomes [10], and the apoplast [11]. The nucleus contains the hereditary information of the cell and its function is to maintain the probity of the genes and to regulate their expression. The main nuclear events are transcriptional programming, ribosome biogenesis and nuclear transport. Those events are facilitated with large number of proteins and enzymes. One mean to regulate the function of those proteins could be by post-translational modifications such as S-nitrosation. Indications for accumulation of NO in the nucleus of stomatal and epidermal cells in response to various stress conditions have been reported [12-14]. Moreover, S-nitrosation has been studied in plants, in some nuclear-localized proteins such as Non-expressor of Pathogenesis-related genes 1 (NPR1), transcription factor TGA1, glyceraldehyde-3-phosphate dehydrogenase, aldolase, and MYB transcription factors [15-18]. Here a method of infection of *Arabidopsis* cell culture with bacteria is described which is followed by nuclear protein extraction and biotin switch assay and further with purification of biotinylated proteins.

2. Materials

2.1 Inoculation of *Arabidopsis* cell suspension cultures with *P. syringae* isolates

1. *Arabidopsis* cell suspension culture.
2. Sterile hood.
3. 2 mg/mL 2,4-Dichlorophenoxyacetic acid (2,4-D) in methanol; store at 4 °C.
4. MS (Murashige & Skoog) vitamin solution: 2.5 mg/mL nicotin acid, 2.5 mg/ml pyridoxine.HCl, 0.53 mg/mL thiamine HCl.H_2O (vitamin B1) and 500 mg/mL M-inositol in ddH_2O; store at -20 ºC.
5. AS medium: 4.3 g/L MS (Murashige & Skoog) basal medium with vitamins, 30 g/L sucrose; 1 mg/L 2,4-D and 1% (v/v) MS-Vitamin in ddH_2O. Adjust the pH to 5.7 with 1M KOH. Transfer 40 mL of medium into 200 mL glass flasks, sterilize by autoclaving and store at 4 ºC.
6. A shaking incubator or a rotary shaker placed in a growth chamber.
7. 50 mg/mL Rifampicin stock solution in methanol; store at -20 ºC. In case of precipitation during storage, re-dissolve before use.
8. 50 mg/ml Kanamycin stock solution in ddH_2O. Sterilize using 0.22 µm syringe filters and store at -20 ºC.
9. King's B medium: 20 g/L peptone, 1.5 g/L K_2HPO_4, 1.5 g/L $MgSO_4.7H_2O$, 1.6 % (v/v) glycerol and 1.5 % (w/v) agar for solid medium in ddH_2O. Adjust the pH to 7.2-7.4 with HCl and sterilize by autoclaving. Add 50 µg/mL rifampicin and 50 µg/mL kanamycin into the solid medium before pouring the plates and into the liquid medium before use. Store at 4 ºC.
10. *Pseudomonas syringae pv. tomato (Pst)* DC3000 containing avirulent *Pst* DC3000 *avrRpm1* (*Pst avr*).

11. 1 M $MgCl_2$ in ddH_2O. Sterilize by autoclaving and store at RT.
12. Spectrophotometer.
13. Nylon mesh filters (150 µm).

2.2 Isolation of *Arabidopsis* nuclei and extraction of nuclear proteins

1. *Arabidopsis* cell suspension cultures (pathogen-treated samples and controls) (*see* **section 3. 1**).
2. Vacuum pump.
3. Filter device.
4. Extraction buffer: 20 mM piperazine-N,N′-bis (2-ethanesulfonic acid) (PIPES)-KOH, pH 7.0, 2 M hexylene glycol, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol and 1 % (w/v) protease inhibitor cocktail; store at 4 ºC (add β-mercaptoethanol and protease inhibitor cocktail just before use).
5. Plastic sticks.
6. Nylon mesh filters (80 µm).
7. 10 % (v/v) Triton-X-100 in ddH_2O.
8. Gradient buffer: 5 mM PIPES-KOH, pH 7.0, 0.5 M hexylene glycol, 10 mM $MgCl_2$, 1 % (v/v) Triton-X-100 and 1 % (w/v) protease inhibitor cocktail; store at 4 ºC (add protease inhibitor cocktail just before use).
9. 30 % (v/v) percoll in gradient buffer; prepare just before use.
10. 80 % (v/v) percoll in gradient buffer; prepare just before use.
11. A refrigerated centrifuge containing angle rotor for 50 mL falcons.
12. A refrigerated centrifuge containing angle rotor for 1.5/2 mL micro tubes.

13. Nuclear lysis buffer: 10 mM tris((hydroxymethyl)aminomethane) (Tris)-HCl, pH 7.5, 500 mM NaCl, 1% (v/v) Triton-X-100, 10% (v/v) glycerol, 1 mM tetrasodium diphosphate and 1% (w/v) protease inhibitor cocktail; prepare the buffer just before use.
14. 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI).
15. Microscopic slides.
16. Coverslips.
17. A fluorescence microscope (λ_{ex} ~395 nm, λ_{em} ~461 nm).
18. A tip sonicator system.

2.3 Determination of protein concentration by Bradford assay

1. Bradford reagent (5X).
2. Vortex mixer.
3. Cuvettes for spectrophotometer.
4. Spectrophotometer.

2.4 Generation of S-nitrosated proteins and biotin labelling of S-nitrosothiols

1. 0.5 M Ethylenediaminetetraacetic acid (EDTA) in ddH_2O. During stirring the solution, add ~20 g NaOH pellets; this makes EDTA to dissolve and adjusts the pH to 8.0. Sterilize by autoclaving and store at RT.
2. HEN buffer: 25 mM 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-NaOH, pH 7.7, 1 mM EDTA, 0.1 mM neocuproine; sterilize by autoclaving and store at RT. Neocuproine decomposes in light; dissolve it just before use.
3. 10 mM S-Nitrosoglutathione (GSNO) in ddH_2O (*see* **Note 1**).

4. 10 mM Glutathione (GSH) in ddH_2O. If possible, use only freshly, otherwise dispense in aliquots and store at -20 ºC.
5. 25 % (w/v) Sodium dodecyl sulfate (SDS) in ddH_2O; store at RT.
6. 2 M Methyl methanethiosulfonate (MMTS) in dimethylformamide (DMF); store at 4 ºC.
7. Vortex mixer.
8. Acetone; pre-cooled at -20 ºC.
9. A refrigerated centrifuge containing angle rotor for 1.5/2 mL micro tubes.
10. HENS buffer: HEN buffer supplemented with 1 % (w/v) SDS; store at RT.
11. 50 mM ascorbate in ddH_2O; store at -20 ºC.
12. 4 mM N-[6-(Biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide (Biotin HPDP) in DMF; store at -20 ºC.

2.5 SDS polyacrylamide gel electrophoresis

1. 70 % (v/v) ethanol.
2. Isopropanol.
3. Separation buffer (4 X): 1.5 M Tris-HCl, pH 8.8 and 0.4 % (w/v) SDS.
4. 10 % (w/v) Ammonium persulfate (APS) in ddH_2O; store at -20 ºC.
5. Separation gel (12 %) (7.5 mL): 1.87 mL of separation buffer, 3 mL of 30 % acrylamide, 75 µL of 10 % (w/v) SDS, 3.75 µL tetramethylethylenediamine (TEMED), 37.5 µL of 10 % (w/v) APS and 2.51 mL ddH_2O (*see* **Note 2**). Since TEMED and APS catalyze polymerization of acrylamide, add them only before use.
6. Stacking buffer (4X): 0.5 M Tris-HCl, pH 6.2 and 0.4 % (w/v) SDS.

7. Stacking gel (~1.5 mL): 390 µL of stacking buffer, 313 µL of 30 % acrylamide, 15.6 µL of 10 % (w/v) SDS, 6.25 µL of TEMED, 12.5 µL of 10 % (w/v) APS and 781 µL ddH_2O (add TEMED and APS only before use).
8. SDS-PAGE running buffer (10X): 250 mM Tris base, 2 M glycine and 1 % (w/v) SDS; store at 4 ºC.
9. Loading buffer (5X): 250 mM Tris-HCl, pH 6.8, 10 % (w/v) SDS, 50 % (v/v) glycerol, 0.05 % (w/v) bromophenol blue. For separation of proteins in reducing condition, add 1mM DTT just before use.
10. Pre-stained protein ladder (10-170 kDa).
11. A protein electrophoresis system consisting of glass plates (10 ×10 cm; thickness 1.0 mm), spacers, casting frames, casting stands, combs, electrophoresis tank, running module, lid with power cables and electrophoresis power supply.

2.6 Western blot analysis

1. Polyvinylidene difluoride (PVDF) or nitrocellulose membranes (0.2 µm).
2. Whatman 3 MM papers.
3. Semidry transfer apparatus.
4. Transfer buffer: 80 % (v/v) SDS-PAGE running buffer (1X) (*see* **step 8 in section 2.6**) and 20 % (v/v) methanol; prepare freshly.
5. Tris-buffered saline (TBS) (10 X): 100 mM Tris-HCl, pH 7.5, 9 % (w/v) NaCl and 10 mM $MgCl_2$; sterilize by autoclaving and store at RT.
6. Tris-buffered saline with Tween (TBS-T) (10X): 0.05 % (w/v) Tween 20 in TBS.
7. Ponceau-S staining solution.

8. Orbital shaker.
9. Blocking buffer: 1 % (w/v) bovine serum albumin (BSA), 1 % (w/v) milk powder and 0.05 % (v/v) Tween 20 in TBS (1X); prepare freshly.
10. Mouse monoclonal antibiotin alkaline phosphatase-conjugated antibody.
11. Antibody incubation buffer: 1 % (w/v) BSA in TBS-T (1X); prepare freshly.
12. Alkaline phosphatase (AP)-buffer: 100 mM Tris-HCl, pH 9.5, 100 mM NaCl and 5 mM $MgCl_2$; sterilize by autoclaving and store at RT.
13. 5 % (w/v) 5-Bromo-4-chloro-3-indolyl phosphate disodium salt (BCIP) in 100 % DMF; store at -20 ºC.
14. 10% (w/v) nitro blue tetrazolium (NBT) in 70 % (v/v) DMF; store at -20 ºC.

2.7 Purification of biotinylated proteins

1. Neutravidin agarose slurry (50 %).
2. Empty columns with ~ 1-2 cm diameter and ~5 -10 cm length.
3. Neutralization buffer: 20 mM HEPES-NaOH, pH 7.7, 100 mM NaCl, 1 mM EDTA and 0.5% (v/v) Triton X-100; store at RT.
4. Micro tube rotator.
5. A refrigerated centrifuge containing angle rotor for 1.5/2 ml micro tubes.
6. Washing buffer: 20 mM HEPES-NaOH, pH 7.7, 600 mM NaCl, 1 mM EDTA and 0.5% (v/v) Triton X-100; store at RT.
7. β-Mercaptoethanol.
8. Acetone; pre-cooled at -20 ºC.

2.8 Silver staining of polyacrylamide gels

1. Clean tray.
2. Orbital shaker.
3. 50% (v/v) ethanol.
4. Fixation solution: 50% (v/v) methanol, 12% (v/v) acetic acid; and 0.05% (v/v) formaldehyde (37%) (*see* **Note 3**) in H_2O (Milli-Q).
5. Sensitizer: 0.2 g/L $Na_2S_2O_3$ in H_2O (Milli-Q); prepare freshly. Ensure that sodium thiosulfate is not exhausted.
6. Staining solution: 2 g/L $AgNO_3$ and 0.075% (v/v) formaldehyde (37%) in H_2O (Milli-Q) (*see* **Note 3**). Ensure that silver nitrate is not exhausted. Prepare freshly and use at darkness.
7. Developing solution: 60 g/L Na_2CO_3, 5 mg/L $Na_2S_2O_3$ and 0.05% (v/v) formaldehyde (37%) in H_2O (Milli-Q) (*see* **Note 3**). Ensure that sodium thiosulfate is not exhausted. Prepare freshly.
8. Stopping solution: 50% (v/v) methanol and 12% (v/v) acetic acid in ddH_2O.
9. Storage solution: 20% (v/v) ethanol and 2% (v/v) glycerol in ddH_2O.

3. Methods

3.1 Inoculation of *Arabidopsis* cell suspension cultures with *P. syringae*

1. Transfer 2.2-2.4 g of *Arabidopsis* cell suspension cultures - under a sterile hood - into 200 ml flasks which contain 40 ml AS medium (*see* **Note 4**). Grow and maintain the cell cultures at 25-26 °C on a rotary shaker (120 rpm) in darkness and sub-culture them into a fresh medium weekly.

2. Grow *Pst avr* on King's B medium containing rifampicin and kanamycin at 28 °C for 2 days.
3. Prepare suspension of bacteria from fresh colonies in 5 mM $MgCl_2$ under a sterile hood.
4. Dilute the bacterial suspension until achieving $OD_{600\ nm}$ of 0.2 using spectrophotometer. Use 5 mM $MgCl_2$ as the blank. The OD_{600nm} of 0.2 is approximately equal to 1×10^8 CFU/ml of fresh colonies of *P. syringae* isolates.
5. Take 1 ml of the bacterial suspension (OD_{600nm}= 0.2) and dilute it with 9 ml of 5 mM $MgCl_2$ (1×10^7 CFU/ml).
6. Inoculate 40 ml of 6-days old *Arabidopsis* cell suspension cultures with 0.4 ml of diluted bacterial suspension to achieve 1×10^6 CFU/ml. Treat the control samples with 5 mM $MgCl_2$.
7. Incubate the cell cultures at 25-26 °C on a rotary shaker (120 rpm) in darkness for desired time point(s) (2 to 13 h) (*see* **Note 5**).
8. Before nuclei isolation, wash the cell cultures twice through a 150 µm pore size membrane with AS medium to remove the bacteria (*see* **Note 4**).

3.2 Isolation of *Arabidopsis* nuclei and extraction of nuclear proteins

This protocol uses a hexylene glycol-based extraction buffer with further percoll-density centrifugation to isolate active nuclei from cellular debris and other organelles. The advantage of hexylene glycol is its less viscosity in compare with other nuclear extraction buffers. And percoll is a biologically inert compound which also leads to a clear concentration of nuclei [19,20].

1. Connect a vacuum pump to a filter device and harvest *Arabidopsis* cells in AS medium by vacuum drying.

2. Weigh 7 g of *Arabidopsis* cells by transferring into a 50 mL falcon tube and keep the sample cold during the next steps.
3. Re-suspend the cells in 2 volumes of cold extraction buffer; homogenize gently using a clean plastic stick.
4. Filter through a nylon mesh filter (80 µm).
5. Dilute the lysate into a total volume of 30 ml by extraction buffer, add 1 % (v/v) Triton X-100 and incubate for 30 min on ice.
6. Prepare 6 ml of 80 % percoll and 12 mL of 30 % percoll suspensions in gradient buffer.
7. Prepare percoll density gradient by transferring 6 ml of 30 % percoll to a 50 mL falcon tube and further by gently pipetting 6 ml of 80 % percoll into the bottom of 30 % percoll (by passing the pipette through 30 % percoll).
8. Pipette the lysate very gently onto the top of the 30 % percoll. Ensure that the percoll layers are not disturbed.
9. Centrifuge at 2,000 x g for 30 min at 4 ^{o}C. The nuclear fraction will appear as a white band at the interface between the 30 % and 80 % percoll fractions.
10. Place the tip of pipette on the top of white band in the interface of two percoll fractions. Pipette the nuclear fraction very gently on the top of 6 ml of 30 % percoll in a new 50 ml falcon tube.
11. Centrifuge at 2,000 x g for 10 min at 4 ^{o}C to wash the nuclei. The pellet will be separated in multiple layers in which the nuclei will reside as a gray band in the top layer.
12. Re-suspend the top layer of the pellet very carefully in 1.5 ml of gradient buffer.
13. Centrifuge at 800 x g for 10 min at 4 ^{o}C. The nuclei again will appear in the top layer of the pellet.

14. Re-suspend the nuclei gently in 350 µL of nuclear lysis buffer.
15. To observe the intact nuclei, take 1 µL of the sample and pipette on a microscopic slide, and then mix with 1 drop of DAPI (*see* **Note 6**). Place a coverslip on the stained nuclei and observe under a microscope using fluorescent light (λ_{ex} ~395 nm, λ_{em} ~461 nm) (Fig. 1) [20].
16. Sonicate the isolated nuclei using 5 mm sonicator tip of a sonicator system for 7 times with 1 min intervals with 10 % power and 5 cycles for 25 s.
17. Centrifuge the lysate at 12,000 x g for 15 min at 4 °C. The supernatant will contain nuclear protein enriched fraction.
18. Determine the protein concentration by Bradford assay and use it freshly for the biotin switch assay.

3.3 Determination of protein concentration by Bradford assay

The Bradford assay is based on binding the dye Coomassie blue G250 to lysine and arginine residues of proteins. This is achieved by measuring the absorbance of the solution at 595 nm (*see* **Note 7**).

1. Prepare 10 concentrations of bovine serum albumin (BSA) standard with a range from 1-9 µg/µL in 1 µg/µL increments.
2. Transfer 1 µL of each standard and 799 µL ddH_2O into a 1.5 mL micro tube.
3. Add 200 µL of Bradford reagent (5X) and mix very well by vortexing.
4. Incubate the samples for 10 min at RT and then transfer into the cuvettes for spectrophotometer.

5. Measure the absorbance at 595 nm. Collect the data of 3 independent replicates and use for producing the standard curve.
6. For estimating the concentration of protein samples; perform the preparation, incubation and measuring of the samples in similar way and employ the equation of the standard curve on the data. Use the corresponding buffer as the blank.

3.4 Generation of S-nitrosated proteins and biotin labelling of S-nitrosothiols

1. Adjust the concentration of nuclear enriched fractions to 0.8-1µg/µL (*see* **Note 8**) in HEN buffer (*see* **Note 9**).
2. For trans-nitrosating of redox-sensitive cysteine residues, treat ~1 mL of the samples containing ~0.8-1 mg of nuclear proteins with 250 µM GSNO (*see* **Note 10**) in darkness for 20 min at RT. To avoid decomposition of GSNO, continue also the next steps in dark. Treat control samples with the same volume of ddH_2O. Use 1 mM GSH as a negative control.
3. Denature the proteins by adding 2.5 % SDS (using 25 % SDS (w/v)) to improve the access of MMTS to the free thiols. For the next calculations, consider the new volume of sample after adding SDS.
4. Block the free thiols by adding 20 mM MMTS and incubate at 50 °C for 20 min with frequent vortexing.
5. Precipitate the proteins with 2 volumes of ice cold acetone for at least 20 min.
6. Centrifuge at 10,000 x g for 20 min at 4 °C.
7. Rinse the pellet again with ice acetone by centrifuging at 10,000 x g for 2-3 min at 4 °C.
8. Dry the pellet for a few minutes at RT; ensure that the proteins do not get over-dried.

9. Re-suspend the pellet in 50 μL of HENS buffer per 1 mg or lesser amount of starting protein material.
10. Treat the protein sample with 1 mM ascorbate (*see* **Note 11**) and 2 mM biotin-HPDP for 1 h at RT. In this step, the S-nitrosated cysteine residues are reduced selectively with ascorbate (*see* **Note 11**) and the freshly generated free thiols are S-biotinylated by biotin-HPDP. Since, S-nitrosothiols have been biotinylated; it is no longer necessary to protect the samples from light.
11. For detection of S-nitrosated proteins, take 5 % of the samples, separate proteins by a 12 % self-cast gel in non-reducing condition (*see* **section 3.5**); and detect biotinylated proteins via western blotting using mouse monoclonal antibiotin alkaline phosphatase-conjugated antibody (*see* **section 3.6**) (Fig. 2) [20].
12. Add 2 volumes of ice cold acetone to the rest (95 %) of samples and precipitate the proteins at -20 °C overnight; perform affinity purification by neutravidin agarose (*see* **section 3.7**) [6,20].

3.5 SDS polyacrylamide gel electrophoresis

1. Set the casting frames in the casting stand. Clean the glasses with water and then 70 % (v/v) ethanol. Place the spacers between the glass plates. Transfer the glass plates into the casting frames.
2. Pipette appropriate amount of separation gel into the gap between the glasses. Fill the rest of the gap with isopropanol in order to make the top of the gel horizontally. Wait for 20-30 min to polymerize the gel. Exchange isopropanol with water. The gel can be stored overnight at 4 ºC.
3. Discard the water from the top of separation gel.

4. Pipette the stacking gel on the top of separation gel. Insert a 10-well comb into the stacking gel without making any bubbles. Wait for 20-30 min to polymerize the gel and then take out the comb.
5. Take the glass plates out of the casting frames and set into the running module inside the electrophoresis tank.
6. Pour running buffer (1X) into the inner chamber until the buffer surface in outer chamber reaches to the required level.
7. Add loading buffer (2X) to the protein samples (*see* **Note 12**).
8. Load 5 μL of pre-stained protein marker (10-170 kDa) into the first well. Load the samples into the wells. The samples should not be overloaded.
9. Cover the lid and set 25 mA per gel. Run the electrophoresis until the bromophenol blue dye front reaches the bottom of the gel (needs around 1 h).

3.6 Western blot analysis

1. Cut a PVDF or a nitrocellulose membrane and 9 Whatman papers with similar size of the gel.
2. Activate the membrane in transfer buffer for 5 min. Soak the gel and filter papers in transfer buffer.
3. Make a blotting unit consisted of six sheets of Whatman paper, the activated membrane, the gel and again three sheets of Whatman paper. No bubbles should be trapped in the blotting unit.
4. Connect the trans-blotter to power supply, set the blot from anode to cathode and perform the transfer based on the size of membrane (2.5 mA per cm^2) for 45 min.

5. After transfer, disconnect the power and remove the lid and 3 paper sheets.
6. Transfer membrane to a clean tray and stain with Ponceau-S staining solution for 5 min with gentle shaking (*see* **Note 13**).
7. Document the stained membrane and de-stain by water for 30 min with gentle shaking.
8. Block the membrane with 30 ml of blocking buffer for 30 min at RT with gentle shaking.
9. Incubate the membrane with 1:10,000 dilution of monoclonal antibiotin alkaline phosphatase antibody in 20 ml TBS-T (1X) with gentle shaking at 4 °C for 4 h or at 25 °C for 1 h.
10. Wash the unbound antibody using two washing steps of TBS-T (1X) (50 mL) and one washing step of TBS (1X) (50 mL) for 10 min with gentle shaking at 4 °C.
11. Perform colorimetric detection of marked proteins by incubating the membrane in 3 ml AP-buffer supplemented with 10 µL of 5 % (w/v) NBT solution and 10 µL of 10 % (w/v) BCIP solution for a maximum of 10 min. The longer the development, the stronger the background will appear. Stop developing by washing with water and then document the stained membrane.

3.7 Purification of biotinylated proteins

1. Shake the neutravidin agarose slurry (50 %) thoroughly and transfer 30 µl per 1mg/ml of protein starting amount to a 1.5 ml micro tube.
2. To equilibrate the neutravidin matrix, add 5-6 volumes of neutralization buffer.
3. Centrifuge the matrix at 200 x g for 1 min and discard the supernatant gently.
4. Repeat steps 2-3.
5. Add 100 µl neutralization buffer to each matrix sample and incubate at RT for 30 min.

6. Centrifuge the precipitated proteins in acetone (*see* **Step 12 in section 3.4**) at 10,000 x g and 4 °C for 20 min to pellet the proteins.
7. Discard the supernatant. Take care not to aspirate off the pellet.
8. Centrifuge again at 10,000 x g and 4 °C for 1 min to discard the rest of acetone.
9. Re-suspend the proteins in 50 µL of HENS buffer per 1 mg or lesser amount of protein. Add at least 2 volumes of neutralization buffer.
10. Add the sample to equilibrated neutravidin matrix and incubate at 25 °C for 1-2 h with gentle shaking.
11. Wash the matrix 5 times with 10 volumes of washing buffer by centrifuging at 200 x g for 1 min and discarding the supernatant by gentle pipetting.
12. Close a new empty column using its cap.
13. Resuspend the matrix in 150 µL of washing buffer and transfer to the column.
14. Add 100 mM β-mercaptoethanol in neutralization buffer and boil at 95 °C for 5 min.
15. Add 100 µL of boiled neutralization buffer supplemented with β-mercaptoethanol to the matrix and incubate for 20 min at RT.
16. Open the cap and elute the bound proteins to a new 1.5 mL microtube.
17. Add 2 volumes of ice acetone to the purified biotinylated protein sample and precipitate at -20°C overnight.
18. Centrifuge at 10,000 x g and 4 °C for 20 min to pellet the proteins.
19. Centrifuge again at 10,000 x g and 4 °C for 1 min to discard the rest of acetone.
20. Separate the proteins by 12 % self-cast gels in reducing condition (*see* **section 3. 5**) and visualize the purified proteins by silver-staining of the gels (*see* **section 3. 8**) (Fig. 3) [20]

(Due to existing very low amounts of purified proteins, coomassie staining is not sensitive enough to detect them (*see* **Note 14**)).

3. 8 Silver staining of polyacrylamide gels

Silver staining is a widely used protein detection method on polyacrylamide gels. It is sensitive to law nano grams of proteins. Perform all the steps of staining at RT by gentle shaking on an orbital shaker. The duration of incubations are for small (10×10 cm) gels, for staining big gels one may need more time.

1. Put the gel in a clean tray and fix with 20 ml of fixation solution for 30 min.
2. Wash with 20 ml of 50 % (v/v) ethanol for 30 min.
3. Sensitize with 20 ml of 0.2 g/L $Na_2S_2O_3$ for 1 min and wash shortly with ddH_2O.
4. Stain with 20 mL of staining solution for 20 min and wash shortly (*see* **Note 15**).
5. Develop using 20 mL of developing solution up to 10 min until sufficient coloring. Discard the developing solution.
6. Terminate the developing by incubation with 20 mL of stop solution for 10 min.
7. Document the gel and keep in storage solution at 4 °C.

4. Notes

1. GSNO decomposes very fast when exposed to light; therefore, prepare in dark and place in dark-coloured micro tubes. If possible, use only freshly, otherwise dispense in 100µl aliquots and store at -20 ºC. Thaw on ice and use immediately. GSNO aliquots should not be freeze-thawed more than once.
2. 12 % Polyacrylamide gel is suitable for separation of 10 to 200 kDa proteins.

3. Formaldehyde is toxic and must be handled under a fume hood.
4. Before culturing and washing the bacteria, transfer AS medium from 4 ºC to the room temperature and incubate for 1 h to avoid from cold shock of the cells. A cold shock or any other stress may induce some un-purposed S-nitrosation of *Arabidopsis* nuclear proteins.
5. The time points of 2 and 13 h are chosen based on the NO production levels in *Arabidopsis* after treatment with *Pst avr*. The highest level of NO production in *Pst avr*-treated *Arabidopsis* cell cultures occurred 2 h post-treatment and NO production was continued until 14 h [20].
6. DAPI is a fluorescent stain. When it binds to double-stranded DNA, fluorescence increases around 20 folds.
7. Bradford assay is relatively free from interference by many commonly used compounds except some detergents and ampholytes. These can be removed from the sample by gel filtration, etc before performing the assay. Alternatively, these compounds can be included in the blanks and calibration standards.
8. Higher concentration of proteins can reduce the efficacy of GSNO to S-nitrosate all NO-sensitive thiol groups and later on can lead to incomplete blocking of free thiols.
9. S-Nitrosothiols are highly labile, especially when exposed to light and metal ions like Cu^{2+}. Therefore, it is necessary that the buffers are supplemented with the metal-chelating compounds like EDTA and neocuproine.
10. Since GSNO is a physiological NO-donor; it is appropriate for *in-vitro* S-nitrosation experiments. Alternatively, other NO donors like S-Nitroso-N-acetyl-DL-penicillamine (SNAP) and sodium nitroprusside (SNP) can be used.

11. Ascorbate is a physiological antioxidant which reacts as an SNO-specific reducing agent and produces new thiol groups. Avoid from incubation with higher concentrations of ascorbate for a longer time; since, it can lead to false-positive signals.
12. Biotinylated proteins should be separated in non-reducing condition; otherwise the biotin linker will be lost. Therefore, the sample buffer should not contain β-mercaptoethanol or DTT and also the sample should not be boiled. For separation of non-biotinylated proteins a reducing sample buffer is needed followed by heating the samples at 95 ºC for 5-10 min.
13. Ponceau-S provides a quick staining method for detection of proteins on membranes. It gives general information about location and approximate amount of transferred proteins.
14. Silver staining is not a very appropriate staining method to downstream LC-MS/MS analysis. Therefore, it is highly suggested to perform the exact experiment once again with western blot detection and further purification, but without silver staining of the samples and further continue with mass spectrometry analysis.
15. Silver nitrate is toxic; discard the solution to the silver waste container.

References

1. Jaffrey SR, Erdjument-Bromage H, Ferris CD, Tempst P, Snyder SH (2001) Protein S-nitrosylation: a physiological signal for neuronal nitric oxide. Nat Cell Biol 3 (2):193-197

2. Lindermayr C, Sell S, Durner J (2008) Generation and detection of S-nitrosothiols. Methods in molecular biology (Clifton, NJ) 476:217-229

3. Francis SH, Busch JL, Corbin JD, Sibley D (2010) cGMP-dependent protein kinases and cGMP phosphodiesterases in nitric oxide and cGMP action. Pharmacological reviews 62 (3):525-563. doi:10.1124/pr.110.002907

4. Chen YJ, Ching WC, Lin YP, Chen YJ (2013) Methods for detection and characterization of protein S-nitrosylation. Methods (San Diego, Calif) 62 (2):138-150. doi:10.1016/j.ymeth.2013.04.016

5. Kovacs I, Lindermayr C (2013) Nitric oxide-based protein modification: formation and site-specificity of protein S-nitrosylation. Frontiers in Plant Science 4:137. doi:10.3389/fpls.2013.00137

6. Lindermayr C, Saalbach G, Durner J (2005) Proteomic identification of S-nitrosylated proteins in Arabidopsis. Plant physiology 137 (3):921-930. doi:10.1104/pp.104.058719

7. Forrester MT, Foster MW, Benhar M, Stamler JS (2009) Detection of protein S-nitrosylation with the biotin-switch technique. Free Radic Biol Med 46 (2):119-126. doi:10.1016/j.freeradbiomed.2008.09.034

8. Palmieri MC, Lindermayr C, Bauwe H, Steinhauser C, Durner J (2010) Regulation of plant glycine decarboxylase by s-nitrosylation and glutathionylation. Plant physiology 152 (3):1514-1528. doi:10.1104/pp.109.152579

9. Camejo D, Romero-Puertas Mdel C, Rodriguez-Serrano M, Sandalio LM, Lazaro JJ, Jimenez A, Sevilla F (2013) Salinity-induced changes in S-nitrosylation of pea mitochondrial proteins. Journal of proteomics 79:87-99. doi:10.1016/j.jprot.2012.12.003

10. Ortega-Galisteo AP, Rodriguez-Serrano M, Pazmino DM, Gupta DK, Sandalio LM, Romero-Puertas MC (2012) S-Nitrosylated proteins in pea (Pisum sativum L.) leaf peroxisomes: changes under abiotic stress. Journal of experimental botany 63 (5):2089-2103. doi:10.1093/jxb/err414

11. Sehrawat A, Deswal R (2014) S-Nitrosylation Analysis in Brassica juncea Apoplast Highlights the Importance of Nitric Oxide in Cold-Stress Signaling. Journal of proteome research. doi:10.1021/pr500082u

12. Foissner I, Wendehenne D, Langebartels C, Durner J (2000) In vivo imaging of an elicitor-induced nitric oxide burst in tobacco. The Plant journal : for cell and molecular biology 23 (6):817-824

13. Gould K, Lamotte O, Klinguer A, A P, D aW (2003) Nitric oxide production in tobacco leaf cells: a generalized stress response? Plant Cell Environment 16

14. Vitecek J, Reinohl V, Jones RL (2008) Measuring NO production by plant tissues and suspension cultured cells. Molecular plant 1 (2):270-284. doi:10.1093/mp/ssm020

15. Holtgrefe S, Gohlke J, Starmann J, Druce S, Klocke S, Altmann B, Wojtera J, Lindermayr C, Scheibe R (2008) Regulation of plant cytosolic glyceraldehyde 3-phosphate dehydrogenase isoforms by thiol modifications. Physiologia plantarum 133 (2):211-228. doi:10.1111/j.1399-3054.2008.01066.x

16. Lindermayr C, Sell S, Muller B, Leister D, Durner J (2010) Redox regulation of the NPR1-TGA1 system of Arabidopsis thaliana by nitric oxide. The Plant cell 22 (8):2894-2907. doi:10.1105/tpc.109.066464

17. van der Linde K, Gutsche N, Leffers HM, Lindermayr C, Muller B, Holtgrefe S, Scheibe R (2011) Regulation of plant cytosolic aldolase functions by redox-modifications. Plant Physiol Biochem 49 (9):946-957. doi:10.1016/j.plaphy.2011.06.009

18. Tavares CP, Vernal J, Delena RA, Lamattina L, Cassia R, Terenzi H (2014) S-nitrosylation influences the structure and DNA binding activity of AtMYB30 transcription factor from

Arabidopsis thaliana. Biochim Biophys Acta 1844 (4):810-817. doi:10.1016/j.bbapap.2014.02.015

19. Folta KM, Kaufman LS (2006) Isolation of Arabidopsis nuclei and measurement of gene transcription rates using nuclear run-on assays. Nature protocols 1 (6):3094-3100. doi:10.1038/nprot.2006.471

20. Chaki M, Shekariesfahlan A, Ageeva A, Mengel A, von Toerne C, Durner J, Lindermayr C (2015) Identification of nuclear target proteins for S-nitrosylation in pathogen-treated Arabidopsis thaliana cell cultures. Plant science : an international journal of experimental plant biology 238:115-126. doi:10.1016/j.plantsci.2015.06.011

Figure legends

Fig 1. Isolated nuclei of *Arabidopsis* cell suspension cultures visualized with DAPI under fluorescent light.

Fig 2. Detection of S-nitrosated proteins in pathogen-treated *Arabidopsis* nuclear enriched extracts. *Arabidopsis* cell cultures were treated with 10 mM $MgCl_2$ and *Pst* DC3000 avrRpm1 for 2 h and 13 h. Extracted proteins from nuclei enriched fractions were treated with either ddH_2O or 250 µM GSNO and subjected to the biotin switch assay. Biotinylated proteins were separated with SDS-PAGE and visualized by immunoblotting using anti-biotin antibody. The Ponceau S-stained membrane is shown in the bottom of the figure. The relative masses of protein standards are shown on the right.

Fig 3. Purification of S-nitrosated proteins in pathogen-treated *Arabidopsis* nuclear enriched extracts. *Arabidopsis* cell cultures were treated with 10 mM $MgCl_2$ and *Pst* DC3000 avrRpm1 for 2 h and 13 h. Extracted proteins from nuclei enriched fractions were treated with either ddH_2O or 250 μM GSNO and subjected to the biotin switch assay. Biotinylated proteins were separated with SDS-PAGE and visualized by immunoblotting using anti-biotin antibody. After immuonoblot detection, the rest of biotinylated samples were purified by neutravidin agarose beads, separated by SDS-PAGE and visualized by silver staining. The relative masses of protein standards are shown on the right.