Identification of NO-sensitive cysteine residues using cysteine mutants of recombinant proteins

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Abstract

Nitric oxide (NO) is a free radical gas regulating a wide range of biological processes in plants. Proteins are the main reaction target of NO inside the cells. The relevance of S-nitrosation as one of the NO-mediated protein post-translational modifications has been studied in detail. S-nitrosyalation causes alterations of the activity/function, sub-cellular localization or interaction partners of proteins. Up to present, a large number of S-nitrosation candidates have been detected in plants. Recombinant proteins are widely used to show or confirm the protein post-translational modifications. Here, using recombinant proteins subjected to biotin switch assay, the S-nitrosation of some nuclear candidates of *Arabidopsis* is verified. Proteins usually contain several cysteine residues which each might involve in structure of protein active sites. So, an important question is: which cysteine residue is the target of S-nitrosation and does it belong to an active site? Here, using the approach of substitution of cysteines by serines on recombinant proteins, the NO-sensitive cysteine residue of an *Arabidopsis* nuclear protein is identified. The next step could be to investigate the effect of S-nitrosation on protein activity/function and further to test the role of target cysteines and S-nitrosation of them in protein activity/function.

Key words

Nitric oxide, S-nitrosation, biotin switch assay, *Arabidopsis* nuclear proteins, recombinant proteins, cysteine mutation.

1. Introduction

Nitric oxide is a redox-active molecule which is known as an important secondary messenger. It plays significant role in many different physiological processes such as growth, development and defense. Many regulatory roles of NO are the result of its direct or indirect reaction with proteins [1]. S-nitrosation is the covalent attachment of an NO moiety to a thiol group of cysteine residues of proteins which produces an S-nitrosothiol group (SNO). This form of post-translational modification is the most relevant NO-mediated mechanism in plants. During the last two decades more than 3,000 candidates of S-nitrosation have been identified in plants using direct and indirect methods of detection [2]. Biotin switch technique (BST) - the most widely used method for indirect detection of S-nitrosated proteins - was invented by Jaffrey et al [3] and further adopted for plants by Lindermayr et al [4]. BST contains three steps in which first the free thiols are blocked by MMTS and the residual of MMTS is removed, afterwards the nitrosothiols are selectively reduced by ascorbate and finally are labelled by biotin-HPDP. The biotin-labeled proteins can be detected by antibiotin antibody, purified by avidin affinity and finally identified by mass spectrometry [3,4]. Candidate proteins identified from proteomic screens often need to be validated using the BST on the recombinant proteins. In addition, by performing site-directed mutagenesis, this allows for the identification of the NO-targeted cysteine residues. Here, we describe the cloning, site-directed mutatgenesis of cysteines, recombinant purification and in vitro biotin-switch of targets of S-nitrosation.

2. Materials

2.1 Total RNA extraction from *Arabidopsis* cell suspension cultures

1. *Arabidopsis* cell suspension culture.
2. 2 mg/ml 2,4-Dichlorophenoxyacetic acid (2,4-D) in methanol; store at 4 °C.

3. MS (Murashige & Skoog) vitamin solution: 2.5 mg/ml nicotinic 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.
4. 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 each 200 ml glass flask, sterilize by autoclaving and store at 4 ºC.
5. Sterile hood.
6. Rotary shaker.
7. Vacuum pump.
8. Liquid nitrogen.
9. Mortars and pestles.
10. Tri-reagent: 38 % (v/v) phenol, 0.8 M guanidine thiocyanate, 0.4 M ammonium thiocyanate, 0.1 M sodium acetate, pH 5.2 and 5 % (v/v) glycerol; store at 4 ºC.
11. Fume hood.
12. Micro tube rotator.
13. Chloroform; pre-cool at -20 ºC.
14. Isopropanol; pre-cool at -20 ºC.
15. 70% ethanol; pre-cool at -20 ºC.
16. A refrigerated centrifuge containing angle rotor for 1.5/2 mL micro tubes.
17. Nuclease-free H_2O.
18. NanoDrop spectrophotometer.

2.2 Total cDNA synthesis from total RNA

1. Random hexamer primers.
2. 1M DTT in ddH_2O.
3. A refrigerated centrifuge containing angle rotor for 1.5/2 mL micro tubes.
4. First strand buffer (5X): 250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$, 0.1 M DTT (add DTT just before use).
5. Deoxynucleotide triphosphates (dNTPs).
6. RNaseOUT.
7. Superscript II reverse transcriptase.
8. Thermocycler.

2.3 Polymerase chain reaction (PCR) amplification of cDNA of candidate genes

1. PCR reaction buffer (10 X): 200 mM Tris-HCl, pH 8.4 and 500 mM KCl; store at -20 ºC.
2. 50 mM $MgCl_2$ solution for PCR; store at -20 ºC.
3. 10 mM dNTPs (deoxynucleotides); store at -20 ºC.

   attB1-forward primer (*see* **Note 1**). After receiving the ordered primers, prepare 100 μM stock solutions of oligonucleotides in ddH_2O and store at -20 °C.

   attB2-reverse primer (*see* **Note 1**). Prepare 100 μM stock solutions in ddH_2O and store at -20 °C.
4. Taq DNA polymerase or a High-Fidelity DNA polymerase; store at -20 ºC.
5. Thermocycler.

2.4 DNA gel electrophoresis

1. 0.5 M Ethylenediaminetetraacetic acid (EDTA) in ddH_2O. Add ~20 g NaOH pellets during stirring the solution to make EDTA to dissolve and adjust the pH to 8.0; sterilize by autoclaving and store at RT.
2. Tris-acetate-EDTA (TAE) running buffer (50X): 2 M Tris base, 5.71 % (v/v) glacial acetic acid and 50 mM EDTA.
3. DNA loading buffer (6 X): TAE buffer (1X), 30 % (v/v) glycerol and 0.25 % (w/v) Orange G.
4. 1% agarose gel: 1 % (w/v) agarose in TAE buffer (1X). Before pouring the gel, add 0.5 μg/mL ethidium bromide.
5. A DNA gel electrophoresis system consisting of a UV-transparent gel tray, a gel caster, a DNA electrophoresis tank, an electrophoresis power supply and a gel Doc system.

2.5 Purification of PCR products

1. UV-transluminator.
2. UV blocking eye glasses.
3. Scalpel blades.
4. DNA gel extraction kit.

2.6 Gateway® cloning for producing N-terminal 6X-His tagged recombinant proteins in *E.coli*

1. Gateway® donor vector pDONR™221 (Gateway® entry cloning by BP recombination); store at -20 ºC.

2. Gateway® destination vector pDEST™ 17 (Protein expression in *E.coli*, N-terminal 6X His-tag); store at -20 ºC.
3. Gateway® BP Clonase™ enzyme mix; store at -80 ºC.
4. BP clonase™ reaction buffer (5X) supplied with BP Clonase™ enzyme mix; store at -20 ºC.
5. TE buffer: 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA.
6. 2 µg/µl Proteinase K solution; store at -80 ºC.
7. *Escherichia coli* DH5α™ competent cells; store at -80 ºC.
8. Refrigerated centrifuge containing angle rotor for 1.5/2 mL micro tubes.
9. Thermoblock.
10. Vortex mixer.
11. Water-bath.
12. Lysogeny broth (LB) medium: 1% (w/v) tryptone, 0.5 % (w/v) yeast extract, 0.5 % (w/v) NaCl and 1.5 % (w/v) agar for solid media. Adjust the pH to 7.0 using NaOH. Sterilize by autoclaving and store at 4 ºC.
13. 50 mg/ml Kanamycin stock solution in ddH_2O. Sterilize using 0.22 µm syringe filters and store at -20 ºC.
14. Shaking incubator.
15. Incubator.
16. Dental sticks.
17. Plasmid purification Kit.
18. Nanodrop spectrophotometer.
19. Restriction endonucleases.

20. Gateway® LR Clonase™enzyme mix; store at -80 ºC.
21. LR clonase™ reaction buffer (5X), supplied with LR Clonase™ enzyme mix; store at -20 ºC.
22. 50 mg/mL Ampicillin stock solution in ddH_2O. Sterilize using 0.22 µm syringe filters and store at -20 ºC.

2.7 Site-directed mutagenesis of cysteine residues

1. Plasmid DNA: The donor vector pDONR 221 containing the gene of interest.
2. 20 mM dNTPs; store at -20 ºC.
3. Mutated forward primer (*see* **Note 2**). After receiving the ordered primers, prepare 100 µM stock solutions of oligonucleotides in ddH_2O and store at -20 °C.
4. Mutated reverese primer (*see* **Note 2**). Prepare 100 µM stock solutions of oligonucleotides in ddH_2O and store at -20 °C.
5. High-Fidelity DNA polymerase and a PCR reaction buffer supplemented with it; store at -20 ºC.
6. Thermocycler.
7. DpnI enzyme.
8. *Escherichia coli* DH5α™ competent cells; store at -80 ºC.

2.8 Recombinant protein production in *E.coli*

1. The destination vector containing the gene of interest.
2. *Escherichia coli* BL21 (DE3).
3. ZY medium: 1 % (w/v) tryptone and 0.5 % (w/v) yeast extract; prepare freshly.

4. M stock solution (50X): 25 mM Na_2HPO_4, 25 mM KH_2PO_4, 50 mM NH_4Cl and 5 mM Na_2SO_4; sterilize by autoclaving and store at RT.
5. 5052 stock solution (50X): 0.5 % (v/v) glycerol, 0.05 % (w/v) glucose and 0.2 % (v/v) A-lactose; sterilize by autoclaving and store at RT.
6. 1 M $MgSO_4$ in ddH_2O; sterilize by autoclaving and store at RT.
7. Trace elements (1,000X): 50 mM $FeCl_3$, 20 mM $CaCl_2$, 10 mM $MnCl_2$, 10 mM $ZnSO_4$, 2 mM $CoCl_2$, 2 mM $CuCl_2$, 2 mM $NiCl_2$, 2 mM Na_2MoO_4, 2 mM Na_2SeO_3 and 2 mM H_3BO_3; store at 4 ºC.
8. ZYM-5052 medium: 95.8 % (v/v) ZY medium, M solution (1X), 5052 solution (1X), 2mM $MgSO_4$, trace elements (0.2 X); sterilize by autoclaving and store at 4 ºC (add 100 µg/mL ampicillin just before use).
9. 1 L flasks.
10. Shaking incubator.
11. Spectrophotometer system.
12. Refrigerated centrifuge containing angle rotor for up to 200 mL tubes.
13. Bacteria lysis buffer: 50 mM Tris-HCl pH 8.0, 300 mM NaCl, 20 mM imidazole, 10 mM $MgSO_4$, 10 mM β-mercaptoethanol, and 1 % (w/v) protease inhibitor cocktail (add β-mercaptoethanol and protease inhibitor cocktail just before use).
14. Sonicator.

2.9 Purification of 6X-His-tagged recombinant proteins by Ni^{2+}-affinity chromatography

1. Ni-NTA agarose beads.
2. Empty columns with ~2cm diameter and ~20 cm length.

3. Buffer A: 50 mM Tris-HCl pH 8.0, 300 mM NaCl, 20 mM imidazole and 10 mM β-mercaptoethanol (add β-mercaptoethanol just before use).
4. Parafilm bands.
5. 5 M NaCl in ddH_2O; sterilize by autoclaving and store at RT.
6. Buffer A supplemented with 1 M NaCl.
7. Buffer B: 300 mM imidazole in buffer A.
8. Glycerol.
9. Liquid nitrogen.

2.10 Determination of protein concentration by Bradford assay

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

2.11 SDS polyacrylamide gel electrophoresis

1. 70 % (v/v) ethanol.
2. Isopropanol.
3. Separation buffer (4X): 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 of tetramethylethylenediamine (TEMED), 37.5 µL of

10 % (w/v) APS and 2.51 mL ddH_2O (*see* **Note 3**). 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 of ddH_2O (add TEMED and APS directly 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. 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.

2.12 Coomassie staining of polyacrylamide gels

1. Orbital shaker.
2. Coomassie staining solution: 40 % (v/v) methanol, 10 % (v/v) acetic acid and 0.1 % (w/v) brilliant blue R-250 in H_2O (Milii-Q).
3. Coomassie de-staining solution: 40 % (v/v) methanol and 10 % (v/v) acetic acid in ddH_2O.

2.13 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* s**tep 8** in **Section 2. 11**) and 20 % (v/v) methanol; prepared freshly.
5. Tris-buffered saline (TBS) (10X): 100 mM Tris-HCl, pH 7.5, 9 % (w/v) NaCl and 10 mM $MgCl_2$; sterilize by autoclaving and store at RT (dilute 100 ml with 900 ml H_2O for use).
6. Tris-buffered saline with Tween (TBS-T): 0.05 % (w/v) Tween 20 in TBS.
7. Ponceau-S staining solution.
8. Blocking buffer: 1 % (w/v) BSA, 1 % (w/v) milk powder and 0.05 % (v/v) Tween 20 in TBS; prepare freshly.
9. Mouse monoclonal antibiotin alkaline phosphatase-conjugated antibody.
10. Poly-histidine monoclonal antibody produced in mouse.
11. Anti-mouse IgG-alkaline phosphatase-conjugated secondary antibody.
12. Antibody incubation buffer: 1% (w/v) BSA in TBS-T; prepare freshly.
13. 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.
14. 5 % (w/v) 5-bromo-4-chloro-3-indolyl phosphate disodium salt (BCIP) in 100 % DMF; store at -20 ºC.
15. 10 % (w/v) nitro blue tetrazolium (NBT) in 70 % (v/v) DMF; store at -20 ºC.

2.14 Biotin switch assay on recombinant proteins

1. Micro biospin columns.
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 Glutathione (GSH) in ddH_2O. If possible, use only freshly, otherwise dispense in 100µL aliquots and store at -20 ºC.
4. 2 M Methyl methanethiosulfonate (MMTS) in dimethylformamide (DMF); store at 4 ºC.
5. 25 % (w/v) sodium dodecyl sulfate (SDS) in ddH_2O; store at RT.
6. Vortex mixer.
7. Acetone; pre-cooled at -20 ºC.
8. Refrigerated centrifuge containing angle rotor for 1.5/2 mL micro tubes.
9. HENS buffer: HEN buffer supplemented with 1% (w/v) SDS; store at RT.
10. 4 mM N-[6-(Biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide (Biotin HPDP) in DMF; store at -20 ºC.
11. 50 mM Ascorbate in ddH_2O; stored at -20 ºC.

3. Methods

3.1 Total RNA extraction from *Arabidopsis* suspension cell cultures

1. Transfer 2.2-2.4 g of *Arabidopsis* cell suspension cultures into 200 mL flasks containing 40 mL of AS medium, under a sterile hood. Grow the cell cultures at 25-26 °C on a rotary shaker (120 rpm) in darkness and maintain them by sub-culturing into fresh medium weekly.

2. Connect a vacuum pump to a filter device and harvest *Arabidopsis* cells in AS medium by vacuum drying.
3. Weigh 100-150 mg of cells and ground in liquid nitrogen using a mortar and a pestle.
4. Transfer the cell powder in a cold 2 mL micro tube and immediately add 1 mL of Tri-reagent (*see* **Note 5**).
5. To homogenize the sample, rotate at 200 x g for 5 min at RT.
6. Add 200 µL of ice cold chloroform (*see* **Note 5**) to the homogenized sample, under a fume hood.
7. Shake overhead for 15 sec and incubate for 2-3 min at RT.
8. Centrifuge the sample at 18,800 x g for 20 min at 4 ºC. Take the upper phase (RNA fraction) very gently and pipette into a new 2 mL micro tube. Discard the lower phase (DNA and protein fraction).
9. Add 500 µL of ice cold isopropanol to the extracted RNA. Shake overhead for 15 sec and incubate for 30-60 min at 4 ºC.
10. Wash the RNA pellet twice with 1 ml of ice cold 70 % (v/v) ethanol by centrifuging at 17,500 x g for 10 min at RT. Float the pellet in the ethanol between two washing steps.
11. Dry the RNA pellet for 30 min at RT and then dissolve in 25-50 µL of nuclease-free H_2O. Determine the RNA concentration by NanoDrop spectrophotometer (*see* **Note 6**) and store at -80 ºC.

3.2 Total cDNA synthesis from total RNA

1. Pipette 2.5 µg total RNA and 250 ng/ µL random hexamer primers into a 0.5 ml micro tube. Reach the final volume to 15 µL by adding ddH_2O and centrifuge briefly. To

examine DNA contamination of the RNA sample, set a control sample which will not contain reverse transcriptase.

2. For denaturing RNA, incubate the sample at 65 °C for 5 min.
3. Incubate the sample on ice for 1 min and centrifuge briefly.
4. Add first strand buffer (1X), 2.5mM dNTPs, 0.1M DTT, 1 µL of RNaseOUT and ddH_2O to reach the final volume of 25 µL and centrifuge briefly.
5. Incubate the sample in a thermocycler at 25 °C for 10 min and at 42 °C for 2 min.
6. Add 1 µL of superscript II reverse transcriptase and incubate at 42 °C for 60 min.
7. Inactivate the reaction at 70 °C for 15 min.
8. Mix the cDNA sample and incubate on ice for 1 min; store at -20 °C.

3.3 Polymerase chain reaction (PCR) amplification of cDNA of candidate genes

1. Prepare PCR mixture for a Taq DNA polymerase on ice: 2-20 ng template DNA, PCR reaction buffer (1X), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM attB1-forward primer, 0.5 µM attB2-reverse primer, 1 unit DNA polymerase; and ddH_2O up to final volume of 20-50 µL. For a High-Fidelity DNA polymerase, prepare PCR mixture using the manufacturer's manual.
2. Mix the reaction gently and spin down in centrifuge.
3. Perform PCR by 25-35 cycles of denaturing, primer annealing and elongation to amplify DNA sequences from template double-stranded cDNA. Set the annealing temperature and the duration of elongation step according to the melting temperature of primers and the size of the DNA fragment of interest, respectively. Carry the PCR reaction using a thermocycler with standard programs of High-Fidelity DNA polymerases or the

following program for a Taq DNA polymerase: 1 (denaturing): 94 ºC for 1-2 min, 1 cycle. 2 (denaturing): 94 ºC for 15-30 sec, (annealing): X ºC for 15-30 sec; and (elongation): 72 ºC, for 1 min per each kb length of target DNA, for 25 to 35 cycles. 3 (elongation): 72 ºC for 10 min, 1 cycle. 4 (storage): at 4 ºC.

4. Detect the PCR products by DNA gel electrophoresis.

3.4 DNA gel electrophoresis

1. Prepare 1 % agarose liquid matrix. Set a UV-transparent gel tray on a gel caster. Pour the liquid matrix on the gel tray and immediately place an appropriate comb. Take out the comb after solidifying the gel.
2. Transfer required volume of TAE running buffer (1X) inside the electrophoresis tank. Place the gel tray gently inside the buffer.
3. Determine the sizes of nucleic acids by loading 2 µL of an appropriate standard size marker in the first well.
4. Mix 10 µL of the DNA samples with DNA loading buffer (1X) and load into the wells.
5. Run the gel at 5-10 V/cm for 30 - 60 min. Determine the progress of running by the front dye.
6. Record the gel with a gel Doc system following the manufacturer's manual.
7. Purify PCR products using a gel extraction Kit.

3.5 Purification of PCR products

1. Put the gel on a UV-transluminator and visualize the DNA bands using UV light (310 nm).

2. Cut the target DNA band from the gel using a new scalpel blade and transfer into a sterile micro tube. Wear UV blocking eye glasses during cutting the gel.
3. Perform the extraction using a DNA gel extraction Kit following the manufacturer's manual.

3.6 Gateway® cloning for producing N-terminal 6X His-tagged recombinant proteins in *E.coli*

Gateway® technology is a cloning method based on site-specific recombination properties of bacteriophage lambda recombinase. Via BP cloning, an attB-flanked DNA fragment and an attP-containing donor vector generate an entry clone. Afterwards, LR cloning generates an expression clone from an attL-contaning entry clone and an attR-containing destination vector.

3.6.1 BP reaction

1. Add the following reagents to a 0.5 mL micro tube on ice and mix: 1-5 µL of attB-PCR product (10 ng/µL; final amount ~15-150 ng), 1 µL of pDONR™ 221 vector (150 ng/µL) (*see* **Note 7**), 4 µL of BP clonase™ reaction buffer, TE buffer up to 4 µL.
2. Add 1 µL of BP Clonase™ enzyme mix to the reaction and mix by briefly centrifuging.
3. Recombine PCR fragment into the donor vector pDONR™ 221 by incubating the reaction in a thermoblock at 25 °C for 4 h or overnight.
4. Add 1 µL of proteinase K solution to terminate the reaction, vortex briefly and incubate at 37°C for 10 min.
5. Transfer 1 µL of each reaction into 50 µl of DH5α™ competent cells.
6. Incubate at ice for 10 min.

7. Heat-shock transform the cells by incubating in a water-bath at 42 °C for 30 sec.
8. Incubate at ice for 2 min.
9. Add 250 µL of LB medium and incubate at 37 °C for 1 h with gentle shaking.
10. Plate 100 µL of each transformation into plates containing LB + 100µg/mL kanamycin (selection marker of pDONR™221).
11. Incubate plates at 37 °C overnight.
12. Select single colonies using dental sticks and grow in liquid LB + 100µg/mL kanamycin.
13. Extract plasmids from entry clones by a plasmid purification kit following the manufacturer's manual.
14. Determine the plasmid DNA concentration using Nanodrop spectrophotometer (*see* **Note 5**).
15. Verify the insertion of target DNA fragments into the donor vector using plasmid DNA digestion.

3.6.2 Verification of insertion of target DNA fragments into the donor vector

1. For the verification of presence of target DNA fragments in the donor vector, use the restriction digestion performed with restriction enzymes using the appropriate buffer and temperature according to the manufacturer's recommendations.
2. After digestion, deactivate the enzymes for 10 min at 65 °C and check the size of fragment(s) by 1 % agarose gel electrophoresis (*see* **Section 3. 4**).

3. Process DNA by a DNA sequencing company and align the obtained sequences of PCR products with those from the original databases to prove their complete similarity (*see* **Note 8**).

3.6.3 LR reaction

1. Add the following reagents to a 0.5 mL micro tube at ice and mix: 1-5 µL of entry clone (50-150 ng), 1 µL of pDEST™17 vector (150 ng/µL) (*see* **Note 7**); 4 µL of LR reaction buffer and TE buffer up to 4 µL. Add 1µL of LR Clonase enzyme mix into the reaction and mix very well by pipetting.
2. Incubate the reaction at 25 °C overnight to recombine entry clone into the destination vector pDEST™17 which represents a 6X His-tag in N-terminal region of the produced proteins in *E. coli*.
3. Repeat the steps 4-14 of BP reaction except using LB + 100µg/mL ampicillin (selection marker of pDEST17).
4. Verify insertion of target DNA fragments into the vectors using plasmid DNA digestion (*see* **section 3. 6. 2**).

3.7 Site-directed mutagenesis of cysteine residues

Site- directed mutagenesis is widely used to study protein function. Using this tool, mutation is introduced by a PCR using a pair of partly complementary primers containing the mutation of interest. In case of producing a wild type recombinant protein, skip this section; otherwise perform it before LR reaction (*see* **section 3.6.3**).

1. Prepare the PCR mixture on ice by adding 50 ng of plasmid DNA, 1 µM of mutated forward primer, 1 µM of mutated reverse primer, PCR reaction buffer (1X), 0.2 mM dNTPs, 1 unit High-Fidelity DNA polymerase; and ddH_2O up to final volume of 15 µL into 0.2 mL micro tube.
2. Perform PCR by one cycle of denaturation (30 s at 98 °C) and 20 cycles of 25 sec at 98 °C, 25 sec at around 60 º C, and 10 min at 72 °C which followed by a final extension step at 72 °C for 10 min.
3. Digest the parental and hemiparental template DNA with 1 µL of DpnI by incubating at 37 °C for 2 h.
4. Heat-shock transform 1 µl of final reaction to 50 µL of DH5α™ competent cells (repeat **steps 5-15 in section 3.6.1**).
5. Verify the mutation by sequencing (*see* **Note 8**).

3.8 Recombinant protein production in *E.coli*

1. Thaw a 50 µL of competent *E.coli* cells (BL21 (DE3)) (*see* **Note 9**) on ice and mix with approximately 100-200 ng of plasmid DNA (pDEST17 containing the target DNA) (1-2 µL) and incubate on ice for 10 min.
2. Heat-shock transform by incubating at 42 °C water bath for 45 sec and subsequently cool at ice for 2 min.
3. Add 500 µl of LB medium and incubate the cells for 1 h at 37 °C with gentle agitation. Centrifuge the cells at 4,000 x g for 2 min at RT.
4. Re-suspend the pellet with 1 mL of LB medium containing 100 µg/mL ampicillin (selection marker of pDEST17) and further culture into a 1 L shake flask containing 250

mL of ZYM-5052 auto inductive medium [5] supplemented with 100 µg/mL ampicillin (selection marker of pDEST17) (*see* **Note 10**).

5. Incubate the bacteria at 37 °C in a shaking incubator (230 rpm) until O.D $_{600}$ nm reaches 1 to 2.
6. Incubate at 18 °C (230 rpm) overnight.
7. Centrifuge the bacteria at 8,000 x g for 20 min at 4 °C.
8. Re-suspend the pellet with 4 volumes of cold bacterial lysis buffer (*see* **Note 11**). Place the rest of samples at ice or 4 ºC during re-suspending time.
9. Sonicate the lysate 3 times with 1 min intervals using 5mm sonicator tip of a tip sonicator system with 60 % power, 3 cycles for 3 min. Place the sample on ice during sonication.
10. Centrifuge the lysate at 17,000 x g for 1 h at 4 °C.
11. Recover the supernatant containing the soluble proteins (*see* **Note 12**) and purify the Histidine-tagged recombinant proteins using Ni^{2+}-affinity chromatography.

3.9 Purification of His-tagged recombinant proteins by Ni^{2+}-affinity chromatography

6X Histidine-tag displays high affinity to metals such as Nickel. Use the Ni^{2+}-affinity chromatography, to purify the recombinant proteins from the bacterial lysate as follow:

1. Shake the Ni-NTA agarose beads thoroughly and transfer 250 µl into an empty column (*see* **Note 13**).
2. Close the column and add 10 volumes of buffer A and incubate at 4 ºC for 30 min to equilibrate the beads.
3. Open the column to discard buffer A and close again.
4. Load the lysate from **step 11 in section 3.8** on the column.

5. Cover the lid of the column with a parafilm band carefully and incubate at 4 °C for 30 min with overhead mixing in 5 min intervals. By binding the proteins, the color of beads will turn to white.
6. Open the column to discard the supernatant containing unbound proteins.
7. Wash the column with 30 ml of buffer A.
8. Wash the column with 30 ml of buffer A supplemented with 1 M NaCl.
9. Wash the column with 30 ml of buffer A.
10. Close the column and elute the bound proteins by adding 1 ml of buffer B and incubating at 4 °C for 5 min. After a successful eluting, the color of beads will turn to blue again.
11. Determine the protein concentration by Bradford assay (*see* **section 3.10**).
12. Take a 25 µl aliquot of the eluted sample and keep for analysis on a 12 % self-cast gel (*see* **section 3.11**). Transfer the eluted sample to 1.5 mL micro tubes containing 50 µL of glycerol and mix very well. Freeze the purified recombinant proteins in liquid nitrogen and store at -80°C.
13. Detect the production of target protein by coomassie staining of the gel (*see* **section 3. 12**) (*see* Fig. 1) and the presence of the histidine-tag on the recombinant protein via western blotting using poly-histidine monoclonal antibody produced in mouse (*see* **section 3.13**) (*see* Fig. 1).

3.10 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 which results in a shift of the absorbance maximum of this dye to 595 nm (*see* **Note 14**).

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. To estimate the concentration of protein extracts and recombinant proteins; 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.

3.11 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.
3. Exchange isopropanol with water. The gel can be stored overnight at 4 ºC.
4. Discard the water from the top of the separation gel.
5. Pipette the stacking gel on the top of the separation gel until there is an overflow.
6. Insert an appropriate comb into the stacking gel without making any bubbles.

7. Wait for 20-30 min to polymerize the gel and then take out the comb.
8. Take the glass plates out of the casting frames and set into the running module inside the electrophoresis tank.
9. Pour running buffer (1X) into the inner chamber until the buffer surface in outer chamber reaches the required level.
10. Add loading buffer (2X) to the protein samples (*see* **Note 15**). Load 5µL of pre-stained protein marker (10-170 kDa) into the first well. Load the samples inside the wells. The samples should not be overloaded.
11. Cover the lid and set 25 mA per gel. Run the electrophoresis until the bromophenol blue dye front reaches to the bottom of the gel (this takes around 1 h).

3.12 Coomassie staining of polyacrylamide gels

Coomassie staining is sensitive to around 50 ng protein bands.

1. Stain the gels in coomassie staining solution for 1 to 2 h at RT.
2. De-stain with coomassie de-staining solution for a few h or overnight until the background color is completely cleared.

3.13 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.
3. Soak the gel and filter papers in transfer buffer.

4. Make a blotting unit consisting of 6 sheets of Whatman paper, the activated membrane, the gel and again 3 sheets of Whatman paper. No bubbles should be trapped in the blotting unit. Transfer the separated proteins on the gel to the membrane using a semidry transfer apparatus.
5. 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.
6. After transfer, disconnect the power and remove the lid and 3 paper sheets.
7. Transfer membrane to a clean tray and stain with Ponceau-S solution for 5 min with gentle shaking (*see* **Note 16**).
8. Document the stained membrane and de-stain by water for 30 min with gentle shaking.
9. Block the membrane with 30 mL blocking buffer for 30 min at RT with gentle shaking.
10. Incubate the membrane with 1:3,000 dilution of monoclonal anti-poly-histidine primary antibody in 20 ml of TBS-T with gentle shaking at 4 °C for 4 h or at 25 °C for 1 h.
11. Wash the unbound antibody using two washing steps of TBS-T (50 ml) and one washing step of TBS (50 mL) for 10 min with gentle shaking at 4°C.
12. Incubate the membrane with 1:7,000 dilution of anti-mouse IgG-alkaline phosphatase conjugated secondary antibody in TBS-T (20 mL) with gentle shaking at 4 °C for 2 h.
13. Wash the unbound antibody using two washing steps of TBS-T (50 mL) and one washing step of TBS (50 mL) for 10 min with gentle shaking at 4 °C.
14. 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 maximum 10 min. The longer the development, the stronger the

background will appear. Stop developing with washing by water and then document the stained membrane.

3.14 Biotin switch assay on recombinant proteins

1. Re-buffer recombinant proteins (*see* **Note 17**) for removing the reducing agents using micro biospin columns following the manufacturer's manual (*see* **Note 18**).
2. Use 1 to 5 µg of each recombinant wild type or mutant protein as starting material. Adjust concentration of recombinant proteins to 0.8-1µg/µL (*see* **Note 19**) in HEN buffer (*see* **Note 20**).
3. For trans-nitrosating of redox-sensitive cysteine residues, treat the samples with 250 µM GSNO (*see* **Note 21**) for 20 min in darkness 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.
4. 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.
5. Block free thiols by adding 20 mM MMTS and incubation at 50 °C for 20 min with frequent vortexing.
6. Discard the residual MMTS by precipitation with 2 volumes of ice cold acetone for at least 20 min at -20 °C.
7. Centrifuge at 10,000 x g for 20 min at 4 °C.
8. Rinse the pellet again with ice acetone and centrifuge at 10,000 x g for 2-3 min at 4 °C.

9. Discard the supernatant carefully and dry the pellet for a few minutes, ensure that the proteins do not get over-dried.
10. Re-suspend the pellet in 50 µL of HENS buffer per 1 µg of starting recombinant protein.
11. Treat the sample with 1 mM ascorbate (*see* **Note 22**) 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 22**) 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.
12. For verification of S-nitrosation of wild type and mutant recombinant proteins, take 5 % of the samples, separate proteins by a 12 % self-cast gel in non-reducing condition (*see* **section 3. 11**); and detect biotinylated proteins via western blotting (*see* **section 3.13**) using mouse monoclonal antibiotin alkaline phosphatase-conjugated antibody (*see* **Note 23**) (Fig. 2 [6] and Fig. 3).

4. Notes

1. For single fragment cloning via Gateway® technology, the full-length cDNAs from total cDNA should be amplified by PCR using gene specific primers containing specific attachment sites allowing recombination reactions. So, download the required cDNA sequences of the genes of interest from The Arabidopsis Information Resource (TAIR) database or National Center for Biotechnology Information (NCBI) database. Design the primers using the Gateway® technology manual and according the form of protein (native, N-terminal fusion or C-terminal fusion) and the organism(s) which you would

like to express. The following is the primer design for producing N-terminal fusion protein in *E.coli*:

attB1-forward primer: GGGG-attB1(ACA AGT TTG TAC AAA AAA GCA GGC T), 2 bases to keep the reading frame of the gene in-frame of attB1 (eg. TC), ATG; and 18-24 gene specific primer.

attB2-reverse primer: GGGG-attB2 (AC CAC TTT GTA CAA GAA AGC TGG GT), 1 base (eg. T), stop codon; and 18-24 gene specific primer (reverse and complement).

2. Primer pairs should contain mutations in bases encoding target cysteine to bases encoding another amino acid such as serine. Here, a modified version of QuickChange™ primer design is employed in which the primer pairs do not have a complete complementation; and at least 8 non-overlapping bases are presented in 3'- terminus of each primer [7]. Moreover, the mutations are introduced at both primers and at least one G or C is placed at each terminus of primer pairs [7].
3. 12 % Polyacrylamide gel is suitable for separation of 10 to 200 kDa proteins.
4. GSNO decomposes very fast when exposed to light; therefore, prepare the solution 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.
5. Perform any step in which Tri-reagent or chloroform involved under a fume hood. These compounds are toxic; discard the waste in their special container.
6. Determine concentration of DNA and RNA samples by measuring the absorption using a Nanodrop spectrophotometer at 260 and 280 nm, respectively. Use only 1 µL of RNA or DNA extract for measuring concentration. For measuring a new sample, clean the lower

and upper optical surface with adding 2 µL ddH_2O to lower optical surface and close-opening the lever arm for a few times. Use the corresponding buffer or ddH_2O as the blank. For evaluating the purity of total DNA or RNA, use the ratio of A_{260}/A_{280} which yields information about the contaminants which could be absorbed at 280 nm. A ratio of approximately 1.8 and 2.0 is considered to be an indication of high quality of DNA and RNA, respectively. Control the purity further by the ratio of A_{260}/A_{230}, which has to be in the range of 2.0 to 2.2 in the loss of contaminants absorbing at 230 nm.

7. Keep a 1: 1 molar ratio between concentration of PCR product and donor vector and that of entry clone and destination vector.
8. Prepare the sequencing samples by mixing the pDONR221 containing the DNA fragment with M13 forward primer (5′-GTAAAACGACGGCCAG-3') (for sequencing around first 600-700 nucleotides) or with M13 reverse primer (5'-CAGGAAACAGCTATGAC-3') (for sequencing around last 600-700 nucleotides) according to the manufacturer's instructions and process by a DNA sequencing company. For DNA fragments longer than 1200 nucleotides, a gene specific primer should be designed for sequencing the middle nucleotides. For validating of mutations, use gene specific primers upstream of the mutations. Align the sequences of all target DNAs with those from the original databases to prove their complete similarity.
9. Alternatively various *E. coli* competent cells can be used such as BL21 (DE3) pLysS and BL21 (DE3) cc4. In our experiments, *E. coli* BL21 (DE3) cc4 could produce more recombinant protein with better solubility. BL21 (DE3) cc4 contains plasmids encoding bacterial chaperones; these chaperones enhance the solubility of produced recombinant proteins; however it is not commercially available.

10. After adding lysis buffer, the pellet might be very sticky and hard to be dissolved. Use clean plastic sticks to dissolve the pellet, pipetting is not recommended, since it will make lots of foam.
11. For an appropriate aeration, the volume of the medium inside the flask should not exceed one fourth of the whole volume.
12. Producing recombinant proteins in *E. coli* is very widely used in molecular biology, due to its big advantages such as simplicity, low cost and rapid cell growth. However, prokaryotes do not contain similar post-translational modification of proteins with eukaryotes. Therefore, many proteins will produce insoluble aggregates in *E. coli* (inclusion bodies). This is the reason that *E. coli* expression system is not recommended for proteins $\geq$ 50 kDa.
13. 250 µl of Ni- NTA agarose is appropriate volume of beads for purification of the recombinant protein yielded from 250 ml bacterial culture.
14. Bradford assay is relatively robust concerning interference by commonly used compounds except some detergents and ampholytes. These can be removed from the sample by gel filtration, before performing the assay. Alternatively, they can be included in blanks and calibration standards.
15. 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 the sample should not be boiled. For separation of non-biotinylated proteins in this chapter a reducing sample buffer is needed followed by heating the samples at 95 ºC for 5- 10 min.

16. Ponceau-S is a quick staining method for detection of proteins on membranes and gives general information about location and approximate amount of transferred proteins.
17. Lacking disulfide bands, glycosylation and phosphorylation in bacterial system leads to improper protein folding; in some cases this will consequently change the bioactivity of the produced recombinant proteins. The same reason may cause the false-positive results in verification of S-nitrosation or defining the target cysteine(s). Therefore, it is suggested that the results on *E. coli* recombinant proteins examined by other tools. For instance, transgenic lines in which the wild type gene and the cysteine mutated genes are introduced to a knockout line can be used.
18. To avoid unspecific S-nitrosation, it is important that the *in-vitro* experiments are carried on native proteins; so, the buffers should not contain any SDS. Moreover, reducing agents (eg. DTT and β-mercaptoethanol) should be removed for effective S-nitrosation.
19. 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.
20. 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 metal-chelating compounds like EDTA and neocuproine.
21. 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.
22. Ascorbate is a physiological antioxidant which reacts as an SNO-specific reducing agent and produces reduced, unmodified thiol groups. Avoid from incubation with higher concentrations of ascorbate for a longer time; since, it can lead to false-positive signals.

23. For biotinylated proteins, incubate the membrane with 1:10,000 dilution of monoclonal anti-biotin alkaline phosphatase antibody in 20 ml TBS-T with gentle shaking at 4 °C for 4 h or at 25 °C for 1 h. Wash the unbound antibody using two washing steps of TBS-T (50 mL) and one washing step of TBS (50 mL) for 10 min with gentle shaking at 4 °C.

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Figure legends

Fig 1. Analysis of purified recombinant proteins of *Arabidopsis* HD2 proteins and HD2C with replaced cysteine(s) by serine(s). 5 µg of purified 6X His-tagged recombinant proteins were loaded in the SDS-PAGE and the gel was then visualized using a) coomassie blue staining solution or followed by b) immunoblotting using monoclonal anti-polyhistidine primary antibody and anti-mouse IgG-alkaline phosphatase secondary antibody. The relative masses of protein standards are shown on the left.

Fig 2. Immunoblot analysis of S-nitrosylation of *Arabidopsi*s HD2 recombinant proteins treated with GSNO *in-vitro*. 5 µg of recombinant HD2 proteins were used as the starting material and underwent the biotin switch assay by treating either with ddH_2O, 250 µM GSNO or 1 mM GSH. Biotinylated proteins were visualized by immunoblotting using monoclonal antibiotin alkaline phosphatase antibody.

Fig 3. Immunoblot analysis of S-nitrosylation of *Arabidopsis* HD2C recombinant proteins with replaced cysteine(s) by serine(s) using SNAP *in-vitro*. 5 µg of recombinant protein was

used as the starting material for biotin switch assay by treating either with ddH_2O or 10 and 100 μM SNAP. The whole sample was loaded in the gel. Biotinylated proteins were visualized by immunoblotting using monoclonal anti-biotin alkaline phosphatase antibody. The ponceau S-stained membranes are shown in the right.