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Identification of endogenously S-nitrosylated proteins in Arabidopsis plantlets: Effect of cold stress on cysteine nitrosylation level

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ABSTRACT

S-nitrosylation is a nitric oxide (NO)-based post-translational modification regulating protein function and signalling. We used a combination between the biotin switch method and labelling with isotope-coded affinity tag to identify endogenously S-nitrosylated peptides in *Arabidopsis thaliana* proteins extracted from plantlets. The relative level of S-nitrosylation in the identified peptides was compared between unstressed and cold-stress seedlings. We thereby detected 62 endogenously nitrosylated peptides out of which 20 are over-nitrosylated following cold exposure. Taken together these data provide a new repertoire of endogenously S-nitrosylated proteins in Arabidopsis with cysteine S-nitrosylation site. Furthermore they highlight the quantitative modification of the S-nitrosylation status of specific cysteine following cold stress.

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1. Introduction

Nitric oxide (NO) is involved in a range of physiological functions associated with plant development and adaptative responses to biotic and abiotic stresses [1,2]. The biological effects of NO are determined by its chemical reactions with a variety of molecules e.g. proteins, lipids and nucleic acids. In particular NO affects the activity of proteins by promoting post-translational modifications (PTM) through S- or metal nitrosylation and tyrosine nitration. Out of these PTM, S-nitrosylation which affects the thiol group of cysteines has emerged as an important PTM in physiological and in pathophysiological processes [3,4]. Recent reports have identified an array of plant S-nitrosylated proteins in tissues, cell suspensions or isolated organelles. Nevertheless, this information mainly concerns plant materials treated with NO donors (mostly nitrosoglutathione, GSNO) as S-nitrosylating agents. Using such approaches ≈150 proteins have been identified as potentially S-nitrosylated in *Arabidopsis thaliana* [5,6]. Many of them have also been reported as S-nitrosylated in animal cells [7–9]. Indeed, these proteins were involved in key processes such as metabolism, signalling, redox homeostasis and cellular architecture. In contrast, only few studies reported the identification of proteins undergoing S-nitrosylation in physiological contexts [10–13].

Being a reversible and specific mechanism, S-nitrosylation shares commons features with other PTM involved in signal transduction and therefore recently gained major interest. Nevertheless as the S-NO bond is extremely labile, light- and redox-sensitive, and the S-nitrosylation status is also governed by enzymatic (thioredoxines, GSNO reductase) and non-enzymatic (glutathione, ascorbate, etc.) processes, this PTM is difficult to analyze. Moreover, the study of S-nitrosylated proteins is slowed by the lack of conserved consensus motif surrounding the nitrosylated cysteine residue. Indeed, although animal studies suggested that an acid-base motif is located in the nearby of the S-nitrosylated cysteine,

Abbreviations: NO, nitric oxide; PTM, post-translational modification; BS, Biotin Switch; ICAT, isotope-coded affinity tag; MMTS, methyl methanethiosulfonate; MS/MS, tandem MS; HPDP, N-(6-(biotinamido)hexyl)-3′-(2′-pyridyldithio)propionamide; SNO, S-nitrosothiols; TFA, trifluoroacetic acid; ACN, acetonitrile; PSI, Photosystem I; PSII, Photosystem II; CBF, C-repeat Binding Factors genes; ARR, type-A Arabidopsis response regulator genes; Q-TOF, quadrupole time-of-flight; SDS, sodium dodecylsulfate; EDTA, ethylenediaminetetraacetic acid; HENS, Hepes buffer; EDTA, neocuproine, SDS; BCA, bicinchoninic acid; DAF2-DA, 4,5-diaminofluorescein diacetate; MES, 2-(N-morpholino)ethanesulfonic acid; GSNO, S-nitrosoglutathione; Cys, cysteine; SNO-Cys, S-nitrosylated cysteine; RuBP, ribulose 1,5-bisphosphate; SAHH, S-adenosylhomocysteine hydrolase.

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thereby contributing to trans-nitrosylation [14,15], this structural requirement is not stringent enough to unequivocally identify putative target cysteines in silico. Furthermore, whether such features are conserved in plant proteins is currently unknown, mainly due to the low number of S-nitrosylated sites reported so far.

We recently evidenced that a rapid formation of NO occurred in *A. thaliana* plantlets after 4 h exposure at 4 °C [16]. Strikingly NO formation was required for proper induction of key cold-responsive genes including CBF transcription factors [16,17]. Furthermore, Zhao et al. [17] demonstrated that NO participates in cold acclimation and freezing tolerance. Therefore NO appears as an important element of a cold-activated signalling network. Nevertheless, the downstream targets of NO signal in chilled Arabidopsis are currently unknown. Abat and Deswal [18] pointed out that some proteins became S-nitrosylated in *Brassica juncea* after 6 h exposure at 4 °C, suggesting that S-nitrosylation might be involved in NO signal transduction during cold stress. Nevertheless, this analysis did not provide informations on the level of S-nitrosylation upon stress. Moreover, it did not identify the Cys residue(s) nitrosylated for all protein target which might be a valuable information when addressing the functional outcome of S-nitrosylation. Thus, we used a quantitative method combining Biotin Switch assay (BS) and Isotope-Coded Affinity Tag (ICAT) labelling recently developed by Fares et al. [11] on *A. thaliana* suspension cells. This approach allowed us: (i) to identify new endogenously S-nitrosylated proteins, (ii) to determine for each candidate the cysteine residue(s) modified and (iii) to compare the level of S-nitrosylation of these residues in unstressed and cold-stressed plantlets. We report here that specific proteins undergo modifications of the S-nitrosylation level following cold exposure, with particular quantitative patterns.

2. Materials and methods

2.1. Plant culture

A. thaliana Columbia-0 seeds were sterilized twice with ethanol-diluted bleach and rinsed with absolute ethanol. When dry, seeds were sown on basic half-strength Murashige and Skoog medium, pH 5.7 (Sigma–Aldrich, L'Isle d'Abeau Chesnes, France), supplemented with 0.5 $g\,L^{-1}$ MES, 10 $g\,L^{-1}$ sucrose and 8 $g\,L^{-1}$ agar and stratified for 3 days at 4 °C. Experiments were performed on 14 days old seedlings. Seedlings were cultivated under continuous 100 $\mu E\,m^2\,s^{-1}$ white light at 22 °C. For cold treatment, plants were transferred to a cold room set to 4 °C for 4 h under the same light conditions. Analysis was carried out on two independent biological experiments. For each experiment, three independent cultures of *A. thaliana* plantlets (5 g) were pooled and ground in a frozen mortar. The proteins were then extracted with a 150 mM Hepes buffer (pH 7.7) containing 5 mM EDTA, 0.5 mM neocuproine (HEN buffer) and 1% SDS (HENS buffer). After acetone precipitation, pellets were dried and resuspended in HENS buffer (containing 0.1% SDS) and proteins were quantified using the BCA assay (Pierce).

2.2. Detection of nitrosylated peptides

2.2.1. Biotin switch assay

After cold treatment, seedling were harvested, frozen in liquid nitrogen and ground in a mortar.

The BS technique was performed according to Astier et al. [12]. Proteins were extracted in HEN buffer with 0.5% CHAPS. After centrifugation at 14,000 × *g* for 15 min 4 °C, the protein concentration in the supernatant was quantified according to Bradford [19] and adjusted to 0.8 $\mu g\,\mu l^{-1}$. Samples were then treated by the addition of freshly prepared MMTS (20 mM final concentration), SDS (2.5%, v/v) and incubated for 20 min at 50 °C. MMTS was removed by acetone precipitation. Pellets were resuspended in 10 μl of HENS buffer (containing 1% SDS) and mixed with 1 mM biotin-HPDP {*N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide} and 1 mM ascorbate. Labelling reactions were performed at room temperature in the dark for 90 min. Proteins were then precipitated by acetone at −20 °C for 20 min. The pellets were resuspended in 62 μl of 25 mM Tris/HCl (pH 6.8), buffer containing 1% SDS. The proteins were quantified by Bradford assay before the addition of 60 μl of 2× modified Laemmli buffer (126 mM Tris/HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol and 0.12% Bromophenol Blue). Proteins (100 μg) were then analyzed by Western blot with streptavidine peroxydase detection.

2.2.2. Labelling with ICAT reagents and purification of S-nitrosylated peptides

Proteins (2 mg) were incubated with 100 mM MMTS in HENS buffer (1 h, 37 °C). For UV control, proteins were first incubated 1 h under illumination at 312 nm, 8 W, in HENS buffer without MMTS in a cold room and then incubated with MMTS. Free MMTS was removed by centrifugation on Amicon filter devices and proteins were washed three times with HENS buffer without SDS. Nitrosothiols were reduced by 5 mM ascorbic acid (2 h, 37 °C) and free thiols were labelled with ICAT reagents (Applied Biosystems) as described by Fares et al. [11]. Excess reagent was removed by centrifugation and washes with 50 mM ammonium carbonate. Proteins were finally trypsin-digested overnight, and heavy- and light-labelled peptides mixed before avidin purification. After evaporation, the biotin tag was cleaved with TFA (37 °C, 2 h) and peptides were dried, resuspended in 0.1% formic acid and desalted on a C18-precolumn (C18 PepMap100, Dionex).

2.3. Mass spectrometry and data analysis

Peptides were resolved on an Ultimate 3000 HPLC (Dionex) using C18-PepMap100 phase for column (75 μm diameter, 250 mm length) and a 140 min linear gradient (solvent A: 0.1% formic acid; 2–27% of solvent B: 90% ACN, 0.1% formic acid) for elution into a Q-TOF mass spectrometer (Maxis, Bruker). For peptide identification, raw data were processed using the DataAnalysis software (Bruker) and the Tair9 database was searched through the Mascot engine, both in target and decoy mode, using 20 ppm and 0.05 Da mass tolerance for peptides and fragments, respectively, methionine oxidation and ICAT light or heavy as variable modification and allowing one missed-cleavage. Significant matches ($p < 0.05$) were selected according to Mascot score. The average FDR (false discovery rates) over all experiments was below 1%. For quantitative analysis, extracted ion chromatograms were calculated using data analysis and processed (Warp-LC software, Bruker) to determine the ratios of intensities for light and heavy ICAT-labelled peptides. Ratio data were normalized to the mean of all ratios within each replicate and averaged for each peptide across replicates.

2.4. Determination of S-nitrosothiols content

S-nitrosothiols (SNO) content was measured using the Saville–Griess assay [20] with modifications. Plantlets were grown in liquid nitrogen and plant tissues powder was lysed in 600 μl of extraction buffer (50 mM Tris–HCL, pH 8.0, and 150 mM NaCl) containing 1 mM PMSF and incubated on ice for 20 min. After centrifugation (10,000 × *g*, 15 min, 4 °C), 50 μl of supernatant was incubated with the same volume of 1% sulfanilamide with or without the addition of 0.2% (w/v) $HgCl_2$ for 20 min in dark. Then, NED 0.02% (100 μl) was added and incubated 5 min. SNO content was measured photometrically at 540 nm using 96-well plates. The SNO

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