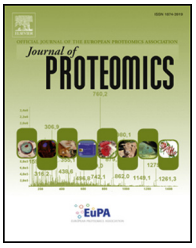
JOURNAL OF PROTEOMICS XX (2015) XXX–XXX

Available online at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/jprot



Nitration of plant apoplastic proteins from cell suspension cultures

Agnieszka Szuba[a,b,*], Anna Kasprowicz-Maluśki[c], Przemysław Wojtaszek[a,c]

[a]Institute of Bioorganic Chemistry, Polish Academy of Sciences, Noskowskiego 12/14, 61-704 Poznań, Poland
[b]Institute of Dendrology, Polish Academy of Sciences, Parkowa 5, 62-035 Kórnik Poland
[c]Department of Molecular and Cellular Biology, Adam Mickiewicz University, Umultowska 89, 61-613 Poznań, Poland

ARTICLE INFO

Article history:
Received 20 August 2014
Accepted 3 March 2015

Keywords:
Arabidopsis thaliana
Nicotina tabacum
Suspension cultures
Apoplast
Proteomics
Nitrotyrosines

ABSTRACT

Nitric oxide causes numerous protein modifications including nitration of tyrosine residues. This modification, though one of the greatest biological importance, is poorly recognized in plants and is usually associated with stress conditions. In this study we analyzed nitrotyrosines from suspension cultures of *Arabidopsis thaliana* and *Nicotiana tabacum*, treated with NO modulators and exposed to osmotic stress, as well as of BY2 cells long-term adapted to osmotic stress conditions.

Using confocal microscopy, we showed that the cell wall area is one of the compartments most enriched in nitrotyrosines within a plant cell. Subsequently, we analyzed nitration of ionically-bound cell-wall proteins and identified selected proteins with MALDI-TOF spectrometry.

Proteomic analysis indicated that there was no significant increase in the amount of nitrated proteins under the influence of NO modulators, among them 3-morpholinosydnonimine (SIN-1), considered a donor of nitrating agent, peroxynitrite. Moreover, osmotic stress conditions did not increase the level of nitration in cell wall proteins isolated from suspension cells, and in cultures long-term adapted to stress conditions; that level was even reduced in comparison with control samples. Among identified nitrotyrosine-containing proteins dominated the ones associated with carbon circulation as well as the numerous proteins responding to stress conditions, mainly peroxidases.

Biological significance

High concentrations of nitric oxide found in the cell wall and the ability to produce large amounts of ROS make the apoplast a site highly enriched in nitrotyrosines, as presented in

Abbreviations: 1DE, one-dimensional electrophoresis; 2DE, two-dimensional electrophoresis; BSA, bovine serum albumin; BY2, tobacco cultivar Bright Yellow-2; CBB, Coomassie Brilliant Blue; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; cPTIO, 4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DAPI, 4′,6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; GA, glutaraldehyde; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight; MS, mass spectrometry; PFA, paraformaldehyde; PVDF, polyvinylidene fluoride; RNS, reactive nitrogen species; ROS, reactive oxygen species; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; SIN-1, 3-morpholinosydnonimine; SNAP, S-nitroso-N-acetyl-DL-penicillamine; TBST, Tris buffered saline with Tween; TRITC, tetramethylrhodamine isothiocyanate; Tyr-NO_2, nitrotyrosine.

* *Corresponding author at:* Laboratory of Proteomics, Institute of Dendrology, Polish Academy of Sciences, Parkowa 5, 62-035 Kórnik, Poland. Tel.: +48 618170033; fax: +48 618170166.
E-mail address: agnieszkalapa@wp.pl (A. Szuba).

http://dx.doi.org/10.1016/j.jprot.2015.03.002
1874-3919/

this paper. Analysis of ionically bound fraction of the cell wall proteins indicating generally unchanged amounts of nitrotyrosines under influence of NO modulators and osmotic stress, is noticeably different from literature data concerning, however, the total plant proteins analysis. This observation is supplemented by further nitroproteome analysis, for cells long-term adapted to stressful conditions, and results showing that such conditions did not always cause an increase in nitrotyrosine content. These findings may be interpreted as characteristic features of apoplastic protein nitration.

1. Introduction

Nitric oxide, a radical molecule, modifies proteins by changing their structure and function [1,2]. One of the most biologically important chemical reactions of nitric oxide is the formation of 3-nitrotyrosines (Tyr-NO_2). This modification involves addition of $–NO_2$ groups to the aromatic ring of tyrosine residues. Despite the possibility of formation of Tyr-NO_2 in many chemical reactions [3], it is commonly believed that in living organisms 3-nitrotyrosines are produced almost exclusively in the reaction of tyrosine with peroxynitrite ($ONOO^-$), which is a product of reaction between the nitric oxide radical (NO.) and the superoxide anion ($O_2.^-$) [3,4]. Tyrosine nitration is usually associated with the activity of various ROS (reactive oxygen species) and RNS (reactive nitrogen species), and as such is regarded as a source of negative, pathological changes in the cell, often leading to apoptosis. Therefore, it is widely recognized as a marker of oxidative stress [5].

Studies on nitrotyrosines in plant tissues have been launched recently and nitration of plant proteins is known only from a few reports. In plants, nitration of tyrosine occurs under physiological conditions [6] and it may play a regulatory role in biological processes such as photosynthesis [7] or root development and senescence [8]. However, in most studies, also pertaining to animals, occurrence of nitrotyrosines is related to stress. For example, it has been shown that conditions of salt stress [9] or other abiotic [10] and biotic [11] stresses increased the amount of nitrotyrosines in plant tissues. This rise was associated with an increased production of nitric oxide [9], observed during stress, and is consistent with the assumption that NO acts via posttranslational protein modifications such as nitration of critical tyrosine residue [12,13].

Nitration of plant apoplastic proteins is still a completely unknown process. However, it was already reported that cell walls as part of an apoplastic space contain numerous sources of NO, the main factor responsible for nitration [14–16]. Moreover, it is known that cell walls are very important in plant signaling network [17] and are the first line of defense against stress conditions [18], especially against osmotic stress. To conclude, tyrosine nitration processing in the apoplast may be an important part of response to stress conditions.

This paper presents a first attempt to investigate nitration occurring in the apoplast, based on localization of nitrated proteins in plant suspension cells and identification of nitrated ionically-bound cell wall proteins from intact suspension cells. Impact of NO modulators and osmotic stress conditions on this sub-proteome was analyzed as well.

2. Materials and methods

2.1. Reagents

Rabbit polyclonal anti-nitrotyrosine antibody was from Molecular Probes. Immobilon-P membranes were from Millipore (Bedford, MA). Immobiline™ DryStrip gels were from GE Healthcare LS. Trypsin and α-cyano-4-hydroxycinnamic acid were from Promega. cPTIO (4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) and SNAP (S-nitroso-N-acetyl-DL-penicillamine) were from Calbiochem. MemCode was from Thermo Fisher Scientific Inc. Other reagents, including SIN-1 (3-morpholinosydnonimine), were purchased from Sigma-Aldrich.

2.2. Plant material

Nicotiana tabacum BY-2 (Bright Yellow-2) suspension-cultured cells were obtained from the Institute of Biochemistry and Biophysics Polish Academy of Sciences, Warsaw, Poland while *Arabidopsis thaliana* cell suspension was kindly provided by Rafael Pont-Lezica from CNRS–Université Paul Sabatier, Toulouse, France. Cells were cultured in a modified Murashige and Skoog medium [19] enriched with 3% (w/v) sucrose, *myo*-inositol (100 mg/1), and respective additional compounds: thiamine hydrochloride (1 mg/l), KH_2PO_4 (370 mg/1) and 2,4-dichlorophenoxyacetic acid (0.2 mg/l) for BY2 cells and 1-naphthaleneacetic acid (1 mg/l), kinetin (1 mg/l) and thiamine hydrochloride (1 mg/l) for *A. thaliana* suspension cells.

N. tabacum suspension-cultured cells were adapted to osmotic stress conditions in the Department of Molecular and Cellular Biology, Adam Mickiewicz University, Poznan, Poland. Cells were gradually [20] adapted to 190 mM NaCl, 180 mM KCl, 450 mM mannitol, and 450 mM sorbitol solutions.

All cultures were maintained at 22 °C with constant shaking (120 rpm, 2 in.) in the dark and were subcultured every 10 days.

2.3. Suspension cell treatments

NO modulators were used in final concentrations: 250 μM SNAP, 250 μM SIN-1 and 200 μM cPTIO. SNAP and SIN-1 were first dissolved in 20 μl DMSO (dimethyl sulfoxide; with appropriate compensation in the control probe), and next, as cPTIO, were dissolved in deionized water, all done to obtain 25 mM stocks, just before application to the suspension culture medium. Cells were incubated in the dark with constant shaking (120 rpm) for either: 30 min, 1 h, 2 h, 5 h or 24 h. After incubation, the cell wall proteins were immediately isolated or cells were used for microscopic analyses.

Please cite this article as: Szuba A, et al, Nitration of plant apoplastic proteins from cell suspension cultures, J Prot (2015), http://dx.doi.org/10.1016/j.jprot.2015.03.002