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Identification of dimedone-trapped sulfenylated proteins in plants under stress

Salma Akter[a,b,c,d,e,f], Sebastien Carpentier[g,h], Frank Van Breusegem[a,b,*], Joris Messens[c,d,e,**]

[a] Department of Plant Systems Biology, VIB, 9052 Ghent, Belgium
[b] Department of Plant Biotechnology and Bioinformatics, Ghent University, 9052 Ghent, Belgium
[c] Structural Biology Research Center, VIB, 1050 Brussels, Belgium
[d] Brussels Center for Redox Biology, 1050 Brussels, Belgium
[e] Structural Biology Brussels, Vrije Universiteit Brussel, 1050 Brussels, Belgium
[f] Department of Fisheries, Faculty of Biological Sciences, University of Dhaka, 1000 Dhaka, Bangladesh
[g] Division of Crop Biotechnics, KU Leuven University, 3001 Leuven, Belgium
[h] Facility for Systems Biology based Mass Spectrometry, KU Leuven University, 3000 Leuven, Belgium

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ABSTRACT

In stressed plants, the reactive oxygen species (ROS) levels rise. Key to ROS signaling research are detection and identification of the protein cysteine sulfenylation (-SOH), the ROS-mediated oxidative product of a thiol (-SH). *Arabidopsis thaliana* seedlings were stressed with hydrogen peroxide (H_2O_2) and the sulfenylated proteins were tagged with dimedone. Dimedone-tagged sulfenic acid proteins were visualized on a two-dimensional electrophoresis (2DE) immunoblot with an anticysteine sulfenic acid antibody and were subsequently detected by mass spectrometry. We optimized the detection method for protein sulfenylation in *Arabidopsis*. We conclude that dimedone can penetrate the cell wall, does not stress plants, and can "read" the changes in the protein sulfenylation pattern under oxidative stress. We observed that the number of sulfenylated proteins in plants treated with 10 mM H_2O_2 was higher than that in untreated plants. A total of 39 sulfenylated protein spots were found on 2DE immunoblots. By means of mass spectrometry, 11 sulfenylated proteins were discovered involved in primary metabolism, redox regulation, translation and signaling pathways. Hence, by combining an immunochemical 2DE strategy with mass spectrometry, we were able to identify sulfenylated proteins in H_2O_2-stressed *Arabidopsis* seedlings. The sulfenylated proteins can be considered for further validation as redox regulators in plants.

1. Introduction

In response to a changing abiotic and biotic environment, plants produce reactive oxygen species (ROS), including superoxide ($O_2^{\bullet-}$), hydroxyl radicals ($OH^{\bullet}$), and hydrogen peroxide (H_2O_2). The subsequent perturbation of ROS homeostasis can trigger posttranslational modifications in signaling proteins, ultimately leading to the expression of genes and the synthesis of proteins to protect against ROS [1–3]. One of the major challenges in redox biology is the discovery of the proteins that sense ROS and transduce these stimuli into downstream biological effects. Nowadays, it is a well-recognized concept that detection of plant protein sulfenylation under oxidative stress is a validated method to find potential redox sensors of ROS signaling pathways [4–7]. Cysteine thiols are prone to oxidize to a diverse range of oxidative modifications [8,9], of which one is sulfenylation, the formation of a sulfenic acid (-SOH) on a cysteine thiol (-SH).

Recently, we have applied two approaches to detect sulfenylated proteins in *Arabidopsis thaliana* cell suspensions under H_2O_2 stress: a YAP1C-based genetic probe and a DYn-2-based chemical probe [4,5]. YAP1 is a yeast AP-1 based genetic probe [10,11], whereas DYn-2 is a chemical probe based on dimedone [12,13]. With both approaches, -SOH proteins were successfully identified under oxidative stress conditions. As such, 97 and 226 sulfenylated proteins were discovered with the YAP1C-based genetic probe and the DYn-2 chemical probe, respectively. Comparison of the list of proteins detected with the YAP1C-based genetic probe (95 cytoplasmic sulfenylated proteins) and DYn-2 based approach (123 cytoplasmic sulfenylated proteins) revealed that 16 proteins were common between both strategies [4–6]. The discrete sensitivity of both probes in cell suspension cultures motivated us to look into a third approach for sulfenome mining.

* Corresponding author at: Department Plant Systems Biology, VIB-Ghent University, Technologiepark 927, 9052 Gent, Belgium.
** Corresponding author at: Structural Biology Research Center, VIB-Vrije Universiteit Brussel, Pleinlaan 2, 1050 Brussels, Belgium.
E-mail addresses: frank.vanbreusegem@psb.vib-ugent.be (F. Van Breusegem), joris.messens@vib-vub.be (J. Messens).

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The DYn-2 chemical probe consists of two functional units: a dimedone scaffold for sulfenic acid recognition and an alkyne chemical handle for enrichment [13]. The chemistry between the electrophilic sulfenic acid and the nucleophile dimedone (5,5-dimethyl-1, 3-cyclohexanedione) is highly selective and has been exploited to detect dimedone-modified sulfenic acids with mass spectrometry [14]. We selected the chemical compound dimedone to explore the sulfenome of seedlings of the model plant *Arabidopsis thaliana*, which is a more complex system than cell suspension cultures. Dimedone is a cell-permeable, cheap, and small molecule with a relative molecular weight of 140.18. Its reaction rate with dipeptide-SOH is 25.5 M^{-1} s^{-1}, whereas for DYn-2, it is 11 M^{-1} s^{-1} and for disulfide bond formation, such as with YAP1C, it is 21.6 M^{-1} s^{-1} [6,15]. As YAP1C is a protein-based probe, it needs to recognize its target sulfenic acids within a huge variety of structural conformations surrounding the modified cysteines. The YAP1C probe makes complexes through protein-protein interactions with exposed sulfenic acids, whereas the relatively smaller dimedone-based probes and dimedone are able to penetrate cavities within proteins independently of the target structure [5,16].

An anti-cysteine sulfenic acid antibody exhibiting high specificity and sensitivity for dimedone-tagged sulfenic acids was used to detect sulfenylated proteins on immunoblots and to monitor changes in the sulfenylation status [17,18] (Fig. 1). After two-dimensional electrophoresis (2DE) immunoblots, the sulfenylated protein spots were visualized with an anti-rabbit antibody conjugated with horseradish peroxidase (HRP). Here, we optimized the conditions to trap sulfenic acids in *Arabidopsis* seedlings with dimedone after H_2O_2 stress treatment. By combining detection of 2DE immunoblots and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis with mass spectrometry; we could identify 11 sulfenylated proteins in *Arabidopsis* seedlings.

2. Materials and methods

2.1. Plant material, stress treatment and dimedone labeling

Arabidopsis thaliana (L.) Heynh., accession Columbia-0 seeds were germinated and grown in liquid Murashige and Skoog (MS) medium (2.15 g MS salts, 500 mg 2-(*N*-morpholino) ethanesulfonic acid buffer, 100 mg myo-inositol, 2 g sucrose, pH 5.7) in a 6-well plate under controlled environmental conditions (16 h/8 h light/dark regime, 100 µmol m^{-2} s^{-1} light intensity, 21 °C, 70% relative humidity). Seeds were first surface-sterilized by fumigation overnight and cold-treated at 4 °C for 3–4 days before germination. Ten-day-old seedlings were treated with 0, 5 and 10 mM H_2O_2 for 1 h to induce oxidative stress, whereafter 5 mM dimedone was supplemented to the H_2O_2-triggered sulfenic acids either for 15 min or 60 min. Both H_2O_2 and dimedone were added directly to the liquid MS culture medium in which *Arabidopsis* seedlings were grown. After treatment, plants were washed with culture medium to remove excess H_2O_2 and dimedone. Dimedone was prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich). Before each experiment, the H_2O_2 concentration was determined at 240 nm with 43.6 M^{-1} cm^{-1} as the molar extinction coefficient.

2.2. Photosynthetic performance

Data for photosystem II (PSII) maximum efficiency (Fv′/Fm′) were recorded with an Imaging-PAM-Series chlorophyll fluorescence system (Heinz Walzy). Fv′ and Fm′ denote variable fluorescence (photochemical ability of PSII) and maximal fluorescence (closed PSII centers) from light-adapted leaves, respectively [19]. The Fv′/Fm′ ratios were measured in 10-day-old wild-type (Col-0) *Arabidopsis* seedlings, before and after 10 mM H_2O_2 treatment as well as after 15 min of 5 mM dimedone incubation of both non-stressed and 10 mM H_2O_2-treated seedlings.

2.3. Protein extraction, SDS-PAGE, and protein gel blot analysis

After treatment, the plants were harvested, dried on Whatman® blot paper, and frozen in liquid nitrogen. The frozen plants were ground on ice with sand in the presence of ethylenediaminetetraacetic acid-free extraction buffer (25 mM Tris-HCl, pH 7.6, 15 mM $MgCl_2$, 150 mM NaCl, 15 mM $pNO_2phenylPO_4$, 60 mM β-glycerolphosphate, 0.1% NP-40, 0.1 mM Na_3VO_4, 1 mM NaF, 1 mM phenylmethanesulfonyl fluoride, 1 µM E64, 1× protease inhibitor cocktail [Roche], 5% [w/v] ethylene glycol) supplemented with 10 mM IAM and 10 mM NEM. The lysates were centrifuged at 16,100×*g* for 30 min at 4 °C to clear cell debris. Protein content from the soluble fractions was quantified with a standard DC Protein Assay (Bio-Rad). Protein samples were denatured for 5 min at 96 °C. From each sample, 25 µg of proteins was evaluated on SDS-PAGE gel and transferred to a polyvinylidene difluoride (PVDF) membrane. The blotted PVDF membrane was blocked with 2% (w/v) nonfat dry milk for 1 h at room temperature or at 4 °C

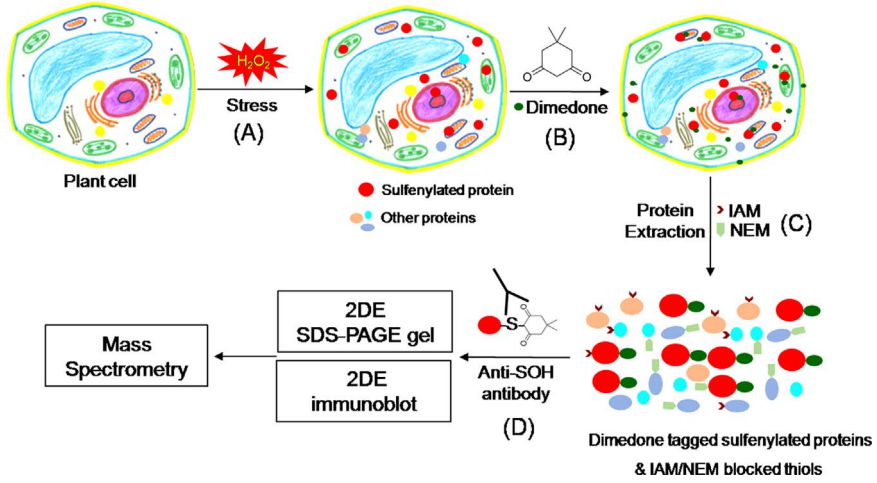

Fig. 1. Schematic representation of the method to identify dimedone-tagged sulfenic acid proteins. Upon H_2O_2 stress, sulfenic acids are formed on specific cysteine thiols of plant proteins. (A) Penetration of dimedone into the plant cells and reaction with the sulfenic acid proteins. (B) Extraction of the proteins in the presence of iodoacetamide (IAM) and *N*-ethylmaleimide (NEM) to block all free thiols and to prevent aspecific oxidation during the extraction procedure. (C) Formation by the dimedone-tagged sulfenylated proteins of a unique epitope for recognition by anti-cysteine sulfenic acid antibodies. (D) Detection of spots of sulfenylated proteins by combining the information of the two-dimensional immunoblots with the SDS-PAGE, and identification with mass spectrometry.