



Identification of dimedone-trapped sulfenylated proteins in plants under stress



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ARTICLE INFO

Keywords:

Arabidopsis
Oxidative stress
Sulfenylation
Dimedone

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 (H₂O₂) 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 H₂O₂ 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 H₂O₂-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 (O₂^{•-}), hydroxyl radicals (OH[•]), and hydrogen peroxide (H₂O₂). 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 H₂O₂ 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.

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<http://dx.doi.org/10.1016/j.bbrep.2016.11.014>

Received 27 July 2016; Received in revised form 12 October 2016; Accepted 21 November 2016

Available online 30 November 2016

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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 $25.5 \text{ M}^{-1} \text{ s}^{-1}$, whereas for DYn-2, it is $11 \text{ M}^{-1} \text{ s}^{-1}$ and for disulfide bond formation, such as with YAP1C, it is $21.6 \text{ M}^{-1} \text{ s}^{-1}$ [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, $100 \mu\text{mol m}^{-2} \text{ s}^{-1}$ 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 $43.6 \text{ M}^{-1} \text{ cm}^{-1}$ as the molar extinction coefficient.

2.2. Photosynthetic performance

Data for photosystem II (PSII) maximum efficiency (F_v'/F_m') were recorded with an Imaging-PAM-Series chlorophyll fluorescence system (Heinz Walzy). F_v' and F_m' denote variable fluorescence (photochemical ability of PSII) and maximal fluorescence (closed PSII centers) from light-adapted leaves, respectively [19]. The F_v'/F_m' 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_2 phenyl IP_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 \times protease inhibitor cocktail [Roche], 5% [w/v] ethylene glycol) supplemented with 10 mM IAM and 10 mM NEM. The lysates were centrifuged at $16,100\times g$ 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 .

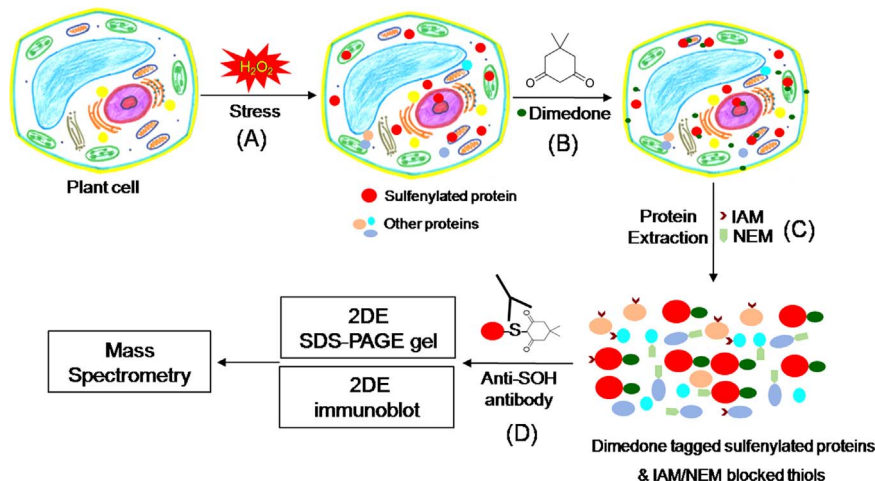


Fig. 1. Schematic representation of the method to identify dimedone-tagged sulfenic acid proteins. Upon H_2O_2 stress, sulfenic acids are formed on specific cysteine thiols of plant proteins. (A) Penetration of dimedone into the plant cells and reaction with the sulfenic acid proteins. (B) Extraction 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.

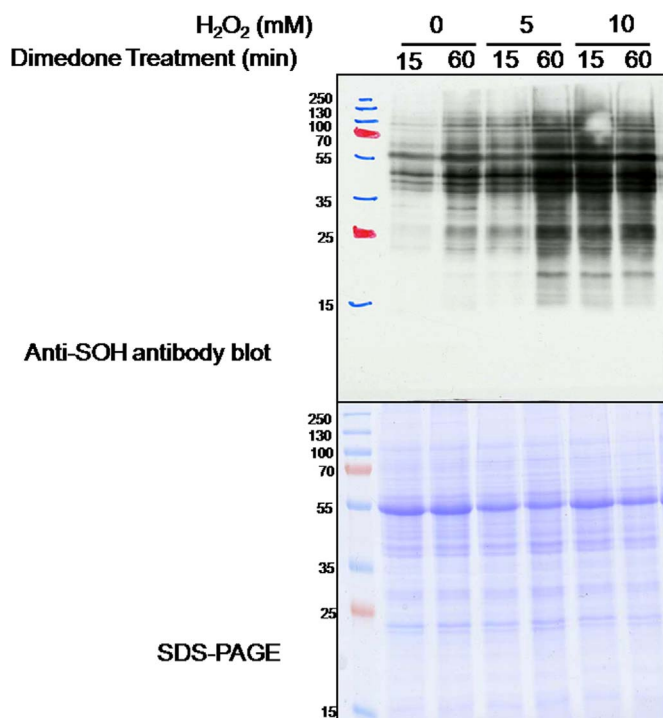


Fig. 2. Detection by dimedone of dose-dependent changes in H_2O_2 -induced protein sulfenylation in *Arabidopsis* seedlings. Ten-day-old *Arabidopsis* seedlings were treated with 0, 5, and 10 mM H_2O_2 for 1 h following 5 mM dimedone incubation for 15 and 60 min. The H_2O_2 dose-dependent sulfenylation was visualized on an anti-cysteine sulfenic acid immunoblot and equal protein loading was confirmed on a CBB-stained SDS-PAGE gel.

overnight with constant agitation. The membrane was washed 3 times (10 min each) with phosphate buffered saline with 0.1% (v/v) Tween-20. After the membrane had been washed, it was incubated with a 1/10,000 dilution of an anti-cysteine sulfenic acid antibody (Millipore) for 1 h at room temperature, whereafter, washed again and treated with a 1/5000 dilution of an anti-rabbit antibody-HRP for 1 h at room temperature. Finally, the membrane was washed and developed with ECL Plus detection reagent (GE Healthcare). Equal loading was confirmed on a Coomassie Brilliant Blue (CBB)-stained SDS-PAGE gel.

2.4. 2DE analysis

Proteins were extracted and analyzed by means of the phenol extraction/ammonium acetate precipitation protocol as described [20]. A total of 300 μ g of protein was estimated with the 2D Quant Kit (GE Healthcare), loaded per 2DE gel, and separated. The protein spots were visualized with colloidal G250 CBB staining [21]. To detect the dimedone-tagged protein spots, the protein spots were first transferred to PVDF membranes and detected with 1/10,000 diluted anti-cysteine sulfenic acid antibodies. The transfer of the protein spots from the 2DE gel to the PVDF membrane was quantitated with Fast Green FCF staining (Sigma-Aldrich).

2.5. Mass spectrometry

2DE gels were ran in duplicate: one gel was used for immunoblotting and the other gel for mass spectrometry after colloidal G250 CBB staining. The sulfenylated protein spots we found on the immunoblot (protein spots transferred from the first gel) were analyzed with the help of Image Master 2D platinum software and detected on the colloidal G250 CBB-stained gel (the second gel). The selected protein spots were collected, washed and subsequently digested in gel with trypsin. For spot picking, the gel pieces were picked manually and

treated as described [22]. The samples were re-suspended in Milli-Q (MQ) water containing 5% ACN and 0.1% FA. Capillary liquid chromatography (LC)/tandem MS experiments were done with an Ultimate 3000 system run in nano-LC set-up (Dionex) coupled to a Q-TOF hybrid quadrupole time-of-flight MS (Micro-tofQ). Separation was conducted using a linear gradient from 95% solvent A, 5% solvent B to 5% A, 45% B in 40 min (solvent A: water, acetonitrile, formic acid; 94.9, 5, 0.1 (v/v/v); solvent B: water, acetonitrile, formic acid; 19.9, 80, 0.1 (v/v/v)). The flow rate was set at 200 nl/min. All MS and MS/MS spectra were automatically processed (background subtraction, smoothing and peak picking) using the Flex Analysis software (Bruker Daltonics) to generate peak list files in MGF format. MS/MS peak list files were submitted to Mascot (Matrix Science) against the Swiss Protspot_h (517,802 sequences; 182,492,287 residues) Taxonomy: *Arabidopsis thaliana* (thale cress) (9099 sequences). Cysteine_Dimedone_136 (C), Cysteine_Dimedone_138 (C), Oxidation (M) were set as variable modifications. Peptide Mass Tolerance was set at ± 100 ppm. Fragment Mass Tolerance was set at: ± 0.1 Da. The max number of allowed missed cleavages was 2. All results from MASCOT were imported to Scaffold version 3.6.3. The parameters used in Scaffold for protein identification was to retain proteins containing at least one identified peptide with confidence level 95%. The resulting false discovery rate on the protein level was 0%.

3. Results

3.1. Dimedone specifically detects protein sulfenylation in a H_2O_2 dose-dependent manner

Sulfenic acids are highly reactive, transient, and are easily over-oxidized to sulfinic and sulfonic acids in the presence of an excess of ROS or they can react with another cysteine to form a disulfide [8]. Additionally, during protein extraction procedures, the intracellular compartmentalization of the redox state might be disrupted, resulting in artificial non-native protein oxidations [15,23]. Therefore, control of the factors that influence the basal cysteine oxidation levels is of crucial importance, while trapping the sulfenylated proteins in cells [5]. For *Arabidopsis* seedlings growing on MS liquid medium in a 6-well plate, these factors might be changes in the physicochemical parameters of the culture medium, in growth chamber settings, in nutrient deficiency, etc. First, we set out an experiment to optimize the incubation period of the dimedone treatment required for sulfenome trapping (Fig. 2). We treated the *Arabidopsis* seedlings with 0, 5 and 10 mM H_2O_2 for 1 h and trapped sulfenylated proteins with 5 mM dimedone for 15 min and 60 min. To avoid possible dimedone tagging of *de novo* sulfenylated proteins generated during the extraction process, the excess of H_2O_2 and dimedone was washed away with culture medium. Moreover, the free thiols were blocked by supplementing the extraction buffer with 10 mM of the alkylating agents IAM and NEM (Fig. 1), hence, avoiding *de novo* sulfenylation during the extraction procedure. On an anti-cysteine sulfenic acid immunoblot, we observed that dimedone was able to penetrate plant cells and protein sulfenylation could be detected with 5 mM dimedone. We also found that dimedone reacts with sulfenylated proteins in a H_2O_2 dose-dependent manner. Non-stressed plants (not treated with H_2O_2) displayed only low levels of basal sulfenic acid labeling, whereas an increased signal was observed with 5 mM and 10 mM H_2O_2 , both for a dimedone incubation period of 15 min and 60 min. Thus, dimedone responded to a changing protein sulfenic acid pattern with increasing H_2O_2 concentrations. Furthermore, the response in function of the duration of the dimedone treatment differed: in H_2O_2 -untreated samples and under 5 mM H_2O_2 stress, the intensity of the sulfenylation signal changed (Fig. 2).

In contrast, in samples stressed with 10 mM H_2O_2 , the intensity of the sulfenylation signal did not change in function of the dimedone treatment length. This observation indicates that a 15-min dimedone incubation is sufficient to trap the sulfenylated oxidation state in

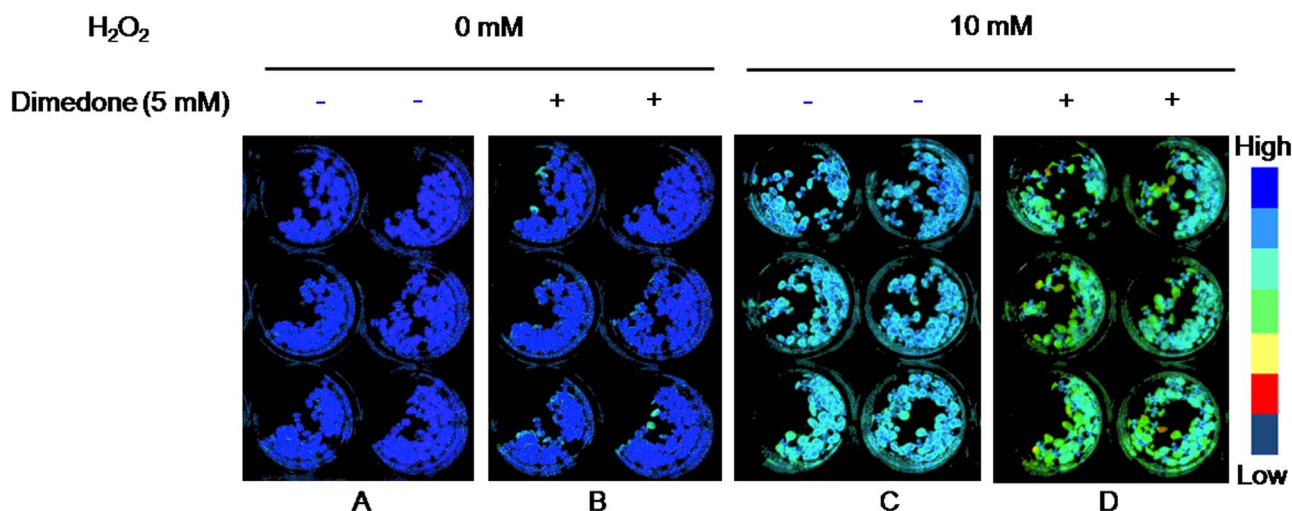


Fig. 3. Oxidative stress induction by H_2O_2 treatment. Both H_2O_2 and dimedone treatment influenced the efficiency of PSII. F_v'/F_m' levels were measured in 10-day-old *Arabidopsis* seedlings grown in a 6-well plate before and after dimedone treatment under both non-treated (A, B) and H_2O_2 -treated conditions (C, D). F_v'/F_m' levels are depicted by color codes, with blue and yellow-green representing high and low values, respectively. F_v'/F_m' levels were high in non-stressed and dimedone-treated plants (A and B), indicating that dimedone did not interrupt the PSII efficiency in the control plants, but were lower under 10 mM H_2O_2 stress, indicating that dimedone blocked sulfenylated proteins with an effect on the F_v'/F_m' readout.

Arabidopsis seedlings induced with 10 mM H_2O_2 .

3.2. Dimedone does not induce stress and allows detection of sulfenylated proteins in H_2O_2 -stressed plants under oxidative stress

After showing that dimedone penetrates plant cells and that this small chemical compound is able to specifically trap sulfenylated proteins under oxidative stress, we decided to identify the dimedone-tagged sulfenylated proteins. First, we checked whether dimedone itself caused stress to plants and thereby affected the sulfenome artificially. Therefore we performed a chlorophyll fluorescence analysis that measures the PSII maximum efficiency (F_v'/F_m') (Fig. 3A and B), with F_v' and F_m' the variable and the maximal fluorescence from light-adapted leaves, respectively, denoting the light energy that is not absorbed by chlorophyll molecules [19]. Non-stressed plants are able to absorb the light energy and a low fluorescence signal, as indicated by the blue color, whereas stressed plants reflect much more fluorescence, indicated by orange/red (Fig. 3). Data for the F_v'/F_m' levels were recorded in non-stressed 10-day-old *Arabidopsis* seedlings, before and after incubation with 5 mM dimedone for 15 min (Fig. 3A and B).

By measuring the fluorescence yield, we observed that the F_v'/F_m' level was high in non-stressed plants before and after dimedone treatment (Fig. 3A and B), indicating that a 15-min dimedone incubation had no influence on the chlorophyll fluorescence under nonstressed conditions, but, as such, a 15-min dimedone treatment itself did not stress plants. A similar result had been obtained with *Arabidopsis* cell suspension cultures on a Strep-HRP blot with the dimedone-based DYn-2 probe [5], in which DYn-2 did not create stress for an incubation period of 15–120 min.

Once we knew that dimedone did not affect the sulfenome, we aimed to identify the dimedone-tagged sulfenylated proteins. As dimedone does not contain a functional purification handle like DYn-2 [13] and the anti-cysteine sulfenic acid antibody could not immunoprecipitate the dimedone-tagged proteins (unpublished data), no enrichment was possible from the protein lysates. Therefore, we decided to separate the proteins on 2DE SDS-PAGE gels and to visualize the sulfenylated spots on a corresponding 2DE immunoblot. Under the optimized conditions, 10-day-old *Arabidopsis* seedlings were treated with 10 mM H_2O_2 for 1 h, followed by a 5 mM dimedone incubation for 15 min.

To confirm that H_2O_2 induced stress in the experimental plants, we

analyzed the chlorophyll fluorescence before and after dimedone incubation (Fig. 3C and D). Plants treated with 10 mM H_2O_2 for 1 h were stressed, because the F_v'/F_m' level was low. Interestingly, a 15-min dimedone incubation, in which sulfenylated proteins were trapped, influenced the fluorescence emission, indicating that sulfenylation of proteins involved in PSII photochemistry, are a necessary signaling event; hence, tagging these proteins with dimedone blocked the signal and affected the F_v'/F_m' level.

3.3. Identification of 11 sulfenylated proteins in seedlings under H_2O_2 stress

For sulfenylated protein identification, proteins were extracted from 10 mM H_2O_2 -treated and non-treated samples and separated on 2DE gels. After confirmation of the transfer of the protein spots on the PVDF membrane by Fast Green FCF staining (data not shown), the transferred protein spots were immunoblotted with anti-cysteine sulfenic acid antibodies. On the non-treated immunoblot, four sulfenylated proteins were spotted, whereas a total of 39 sulfenylated spots were detected under the 10-mM H_2O_2 stress (Fig. 4).

The H_2O_2 -treated immunoblot and the corresponding CBB-stained 2DE gel were analyzed by using the Image Master 2D platinum software. A total of 39 sulfenylated protein spots (pick 1 to pick 39) were selected on the 2DE gel, corresponding to the 39 sulfenylated protein spots on the immunoblot (Fig. 5A). After mass spectrometry analysis, we were able to confidently identify 11 proteins (Table 1). Based on the GO annotation (TAIR 10 database), dimedone penetrates the cell wall and reacts with cytoplasmic sulfenylated proteins, even trapping chloroplastic sulfenylated proteins as well as mitochondrial and peroxisomal sulfenic acids (Table 1). Similarly, with the dimedone-based DYn-2 probe, sulfenylated proteins were trapped in different subcellular compartments, such as cytoplasm, plastid, mitochondria, nucleus, endoplasmic reticulum, Golgi apparatus, plasma membrane, and peroxisomes [5].

The identified proteins were found to be involved in primary enzyme metabolism, such as NADP-DEPENDENT MALATE DEHYDROGENASE, FRUCTOSE-1,6-BISPHOSPHATASE, PHOSPHOGLYCERATE KINASE 1, GLUTAMINE SYNTHETASE; redox-related enzymes, such as MONODEHYDROASCORBATE REDUCTASE 1 and 4; CO_2 -fixing enzyme, such as RIBULOSE BISPHOSPHATE CARBOXYLASE; proton transport protein ATP SYNTHASE SUBUNIT BETA; gene expression-regulating

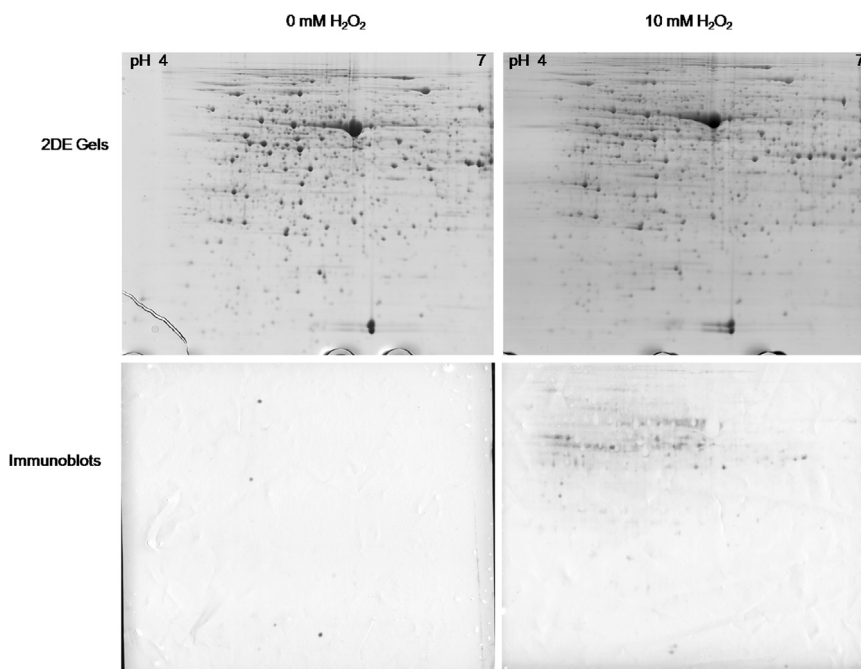


Fig. 4. Protein pattern visualized on 2DE gels and the corresponding sulfenylation pattern on the respective immunoblots. Ten-day-old *Arabidopsis* seedlings were treated with 0 and 10 mM H_2O_2 for 1 h and subsequently treated with 5 mM dimedone for 15 min. After extraction, the proteins were separated on 2DE SDS-PAGE and the gels were stained with G250 CBB or immunoblotted with anti-cysteine sulfenic acid antibodies, followed by visualization with anti-rabbit-HRP. The number of sulfenylated proteins was higher in 10-mM-stressed plants than in non-stressed plants.

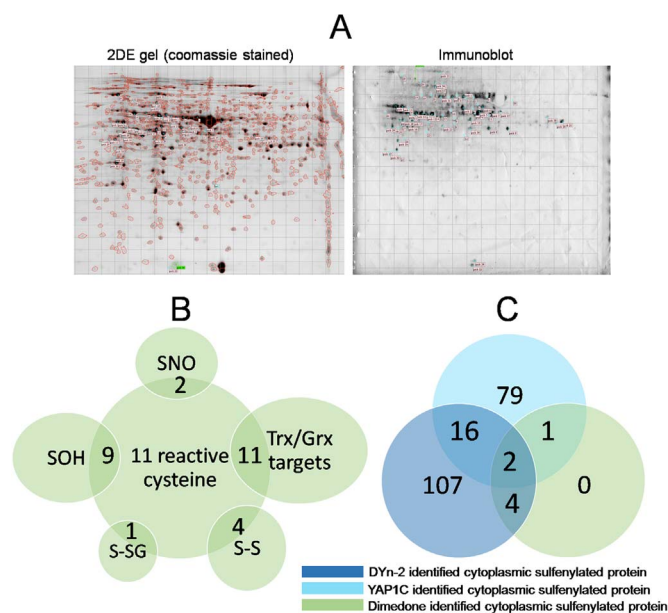


Fig. 5. Analysis of the sulfenylated proteins in *Arabidopsis* seedlings identified by dimedone under 10 mM H_2O_2 stress. (A) A total of 39 sulfenylated protein spots detected in 10-mM H_2O_2 -stressed plants after 2DE SDS-PAGE and immunoblot analysis with Image Master 2D platinum software. These dimedone-labeled sulfenylated proteins were identified by mass spectrometry. (B) The 11 identified sulfenylated proteins, of which 11 had previously been reported for reactive cysteine containing sulfenic acid (SOH), disulfide (S-S), S-glutathionylation (S-SG), S-nitrosylated (SNO), and Trx/Grx target cysteine proteins. (C) Seven cytoplasmic sulfenylated proteins identified by dimedone are common proteins with either cytoplasmic YAP1C (1) or cytoplasmic DYN-2 (4) or both YAP1C and DYN-2 (2).

enzyme, such as EUKARYOTIC TRANSLATION INITIATION FACTOR 4A-1, and proteins involved in signaling processes, such as ADENOSINE KINASE 1.

4. Discussion

A chemical compound that can detect sulfenylated proteins should be sufficiently reactive and selective under physiological conditions, compatible with an aqueous environment, and have minimal impact on the redox balance and cell viability. The chemistry between the electrophilic sulfenic acid and the nucleophile dimedone is highly selective [14] and relatively fast compared to the DYN-2 chemical probe and the YAP1C genetic probe [5,15]. For example, for the sulfenylated human serum albumin (HSA-SOH) the second order rate constant for the reaction with dimedone in competition with mixed disulfide formation with glutathione (GSH) has been reported to be $0.027 M^{-1} s^{-1}$ and to be approximately 100 times slower than the $2.9 M^{-1} s^{-1}$ with glutathione. However, we found that addition of an extracellular concentration of 5 mM dimedone is sufficient for sulfenic acid trapping. Similarly, modifications of the protein sulfenic acid have been profiled in human breast cancer cells after 5 mM dimedone incubation [18]. In addition, the lack of enrichment or visualization “handle” on dimedone makes the chemical compound more compact, facilitating a better cell-permeability than the dimedone-biotin/fluorophores conjugates [15]. However, due to the absence of an enrichment handle, the number of sulfenylated proteins for mass spectrometry identification becomes limited. Furthermore, a critical step in this dimedone-based approach is not only the lack of an enrichment step, but also the selection of the protein spots of the stained 2DE gel, corresponding to the immunoblot visualized by the anti-cysteine sulfenic acid antibody. Moreover, the abundance of proteins in single 2DE protein spots is crucial to be a significant hit in mass spectrometry analysis.

This dimedone approach is a relatively fast and cheap method to identify sulfenylated proteins. For example, we have used dimedone for

Table 1
Protein spots analyzed by mass spectrometry with different subcellular localizations in *Arabidopsis thaliana*.

| Protein spot (pick) | AGI code | Description | Max protein identification probability | Subcellular localization | Functional categorization | # Cys | Redox modification ^a | Reference |
|-----------------------------------|--------------------|---|--|--------------------------------------|---|-------|---|--------------------|
| 1, 3 | ATCG00480 | ATP SYNTHASE SUBUNIT | 1 | Cell wall, chloroplast | Proton transport | 1 | S-S, Trx target, H ₂ O ₂ sensitive proteome, SOH | [26,34,5]* |
| 1 | AT2G36530 | ENOLASE 2 | 0.995 | Cytoplasm, mitochondria | Stress responsive protein | 5 | SOH, S-SG, Grx target, salt/osmotic stress responsive, early responsive redox sensitive | [5,24,31,36,41]* |
| 2 | AT3G13920 | EUKARYOTIC TRANSLATION INITIATION FACTOR 4A-1 | 1 | Cytoplasm | Regulation of gene expression | 5 | SOH, salt and osmotic stress responsive | [4,5,36] |
| 6 | ATCG00490 | RIBULOSE BIPHOSPHATE CARBOXYLASE | 1 | Cell wall, chloroplast | CO ₂ fixing, photochemistry II | 7 | S-S, SNO; Trx target, H ₂ O ₂ sensitive | [26,27,33,34] |
| 9 | AT5G58330 | NADP-DEPENDENT MALATE DEHYDROGENASE | 0.95 | Chloroplast, cytoplasm, mitochondria | Primary metabolism | 9 | SOH; Trx target | [4,5,27,28] |
| 12 | AT3G12780 | PHOSPHOGLYCERATE KINASE 1 | 0.987 | Cytoplasm, mitochondria, chloroplast | Primary metabolism | 2 | SOH; Trx target | [5,27] |
| 24 | AT3G09820 | ADENOSINE KINASE 1 | 0.95 | Cytoplasm | AMP biosynthesis via salvage pathway | 8 | SNO, Trx target | [29,33] |
| 17 | AT1G43670 | FRUCTOSE-1,6-BISPHOSPHATASE | 0.976 | Cytoplasm | Fructose metabolic process, | 7 | SOH; Trx target | [4,27,28] |
| 23 | AT5G35630 | GLN2, GLUTAMINE SYNTHETASE 2 | 0.983 | Chloroplast/mitochondria | primary metabolism | 7 | SOH; Trx-target, Grx target, H ₂ O ₂ sensitive proteome | [4,5,25–28,31,34]* |
| 26 | AT3G09940 | MONODEHYDROASCORBATE REDUCTASE 1 | 0.95 | Cytoplasm, peroxisome | Redox related | 3 | SOH, Trx target; S-S | [5,30,32]* |
| 26 | AT3G27820 | MONODEHYDROASCORBATE REDUCTASE 4 | 0.997 | Cytoplasm, peroxisome | hydrogen peroxide catabolic process | 5 | SOH, Trx target; S-S | [5,30,32]* |
| [4,5,7,8,10,11,13–16,18–23,27–37] | No Significant hit | | | | | | | |

Abbreviations of redox modifications are as follows: SNO, S-nitrosothiol; SOH, sulfenic acid; S-SG, S-glutathionylation; S-S, disulfide bridge; Trx/Grx target, thioredoxin/glutaredoxin target proteins. References describing identification of homologs are marked with an asterisk.

the *in vitro* validation of sulfenic acid formation on the recombinant protein dehydroascorbate reductase 2 (DHAR2), a YAP1C-trapped sulfenylated protein [4]. However, the number of proteins identified in the present study is really low compared to the 226 and 97 proteins identified with DYn-2 and with YAP1C approaches, respectively [4,5]. We detected only 11 sulfenylated proteins. All had previously been reported for redox modification such as -SOH, S-S, S-SG, -SNO, or as Trx/Grx targets (Table 1; Fig. 5B). Comparison of the seven cytoplasmic sulfenylated proteins discovered by dimedone with the YAP1C probe (95 cytoplasmic sulfenylated proteins) [4] and the DYn-2 probe (123 cytoplasmic sulfenylated proteins) [5] revealed that all the seven proteins had already been reported as sulfenylated proteins of *Arabidopsis*: two proteins EUKARYOTIC TRANSLATION INITIATION FACTOR 4A-1 and NADP-DEPENDENT MALATE DEHYDROGENASE occur in all three approaches (Yap1C, DYn-2 and dimedone); FRUCTOSE-1,6-BISPHOSPHATASE was also identified with the YAP1C probe approach; and four proteins, PHOSPHOGLYCERATE KINASE 1, and the isoforms of MONODEHYDROASCORBATE REDUCTASE 1 and 4 and ENOLASE 2 were also found with the DYn-2 approach (Table 1; Fig. 5C). The identified sulfenylated ENOLASE 2 had been previously reported to be S-glutathionylated (S-SG) [24], a ubiquitous redox-sensitive and reversible modification of a cysteine that protects against overoxidation and regulates the protein activity. Dimedone also tagged two chloroplastic proteins, GLUTAMINE SYNTHETASE 2, of which isoforms have already been found with the YAP1C and DYn-2 approaches, and the isoform of ATP SYNTHASE SUBUNIT present in the DYn-2 sulfenome (Table 1).

Checking the redox functions of the identified proteins, we discovered that ATP SYNTHASE SUBUNIT BETA and GLUTAMINE SYNTHETASE 2 are reported as interactor proteins of thioredoxin (Trx) [25–28] as well as RIBULOSE BISPHOSPHATE CARBOXYLASE, NADP-DEPENDENT MALATE DEHYDROGENASE, PHOSPHOGLYCERATE KINASE 1, ADENOSINE KINASE 1 and FRUCTOSE-1,6-BISPHOSPHATASE [27–29]. We also identified sulfenylated MONODEHYDROASCORBATE REDUCTASE 1 and 4, which isoforms have been found as Trx target in *Arabidopsis* roots [30], and sulfenylated ENOLASE 2 and GLUTAMINE SYNTHETASE 2 as glutaredoxin (Grx) targets [31]. Glutaredoxins are small ubiquitous proteins with a thioredoxin (Trx) fold, that catalyze disulfide (S-S) exchange reactions or reduce protein-mixed disulfides (S-SG). We postulate that prior to disulfide bond formation, the relatively unstable sulfenic acids, which we detected in this study, react with thiols to form disulfides (S-SG and S-S). As such, the Trx interactor proteins should have a disulfide. If we look into the disulfide proteome in cyanobacteria under oxidative stress [26] and in *Arabidopsis* [32], we find that the isoforms of MONODEHYDROASCORBATE REDUCTASE, and also ATP SYNTHASE SUBUNIT BETA and RIBULOSE BISPHOSPHATE CARBOXYLASE have been identified with redox-dependent disulfides [26]. So, our data together with the published disulfide data suggest the postulated reaction mechanism for these proteins. Whether this reaction really takes place needs to be validated in a case-by-case study.

Other proteins with redox-sensitive cysteines are definitely ADENOSINE KINASE 1 and RIBULOSE BISPHOSPHATE. We detected a sulfenic acid on one of their cysteines, but also cysteine nitrosylation (-SNO), another reversible posttranslational modification of cysteines, was present on these proteins [33].

Stress-responsive proteins are ATP SYNTHASE SUBUNIT BETA, RIBULOSE BISPHOSPHATE CARBOXYLASE and GLUTAMINE SYNTHETASE 2. They are H₂O₂-sensitive and play a role under different stress conditions [34]. Also the EUKARYOTIC TRANSLATION INITIATION FACTOR 4A-1 that promotes or inhibits translation of specific mRNAs [35] and ENOLASE 2 are salt and osmotic stress-responsive proteins [36]. Further, we found MONODEHYDROASCORBATE REDUCTASE, an important H₂O₂-scavenging enzyme, that is active in the ascorbate-glutathione cycle

[37], and NADP-DEPENDENT MALATE DEHYDROGENASE that plays an important role in the short-term adjustment of the stromal NADP(H) redox state in response to changing environmental conditions, and as such ensures maintenance of the redox homeostasis [38]. The latter enzyme also regulates the catalase activity and the accumulation of H₂O₂ in peroxisomes under light stress. NADP-DEPENDENT MALATE DEHYDROGENASE could transmit the reducing equivalents from the chloroplast to the peroxisomes, thereby partially inhibiting the catalase activity leading to H₂O₂ accumulation [39].

RIBULOSE BISPHOSPHATE CARBOXYLASE (Rubisco) is an important player in the regulation of CO₂ assimilation and the PSII activity. Oxidation of Rubisco cysteines contributes to a sequence of conformational changes and triggers its catabolism under increasing oxidative conditions [40]. In plants under 10 mM H₂O₂ stress after a 15-min dimedone treatment, we observed that the PSII maximum efficiency (Fv'/Fm') level decreases (Fig. 3C and D). This observation can be correlated with the sulfenylation state of this enzyme (Table 1). Rubisco sulfenylation could be a signal event for PSII photochemistry under 10-mM H₂O₂ stress, hence affected after a reaction with dimedone, which calls for a follow-up study.

Altogether, all 11 sulfenylated proteins identified with the dimedone approach (Table 1) can be connected to redox signaling events. Further, comparison of the data from three independent approaches (Yap1C, DYn-2 and dimedone) showed only a limited overlap of sulfenylated proteins (Fig. 5C). Important to note, these overlapping sulfenylated proteins are common in both *Arabidopsis* cell suspension cultures and plants. Finally, chemical and genetic probe-based approaches can be considered as complementary to cover the complete plant sulfenome.

5. Conclusion

Dimedone is a nontoxic chemical compound that functions as a plant cell-permeable sulfenome “reader”. The dimedone approach is a fast and cheap method to trap and identify sulfenylated proteins. Important to note, to apply this dimedone-based sulfenome identification method in a more effective way, an enrichment step will be needed. In this study, we could only identify 11 proteins from the 39 sulfenylated protein spots. Therefore, development of antibodies capable of immunoprecipitating dimedone-tagged proteins would be invaluable for the improvement of this dimedone-based sulfenome-trapping method.

Acknowledgements

The authors thank Wesley Vermaelen (KU Leuven) for mass spectrometry analysis, Annick De Troyer (KU Leuven) for helping in 2DE technique, Khadija Wahni for her excellent technical assistance, Amna Mhamdi (VIB-Ghent University) and Alexandra Lewandowska (VIB-Ghent University and Vrije Universiteit Brussel) for insightful comments, and Martine De Cock for help with preparing the manuscript. This work was supported by VUB Grant (Horizontal Research Action HOA 22) (to J.M.), the European Co-operation in Science and Technology (COST Action BM1203 EU-ROS) and the Research Foundation-Flanders [G0D7914N (to F.V.B.) and G0D70149 (to J.M.)], S.A. is indebted to the Erasmus Mundus External Cooperation Window for a predoctoral fellowship.

Transparency document. Supporting information

Transparency document associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bbrep.2016.11.014>.

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