DYn-2 Based Identification of Arabidopsis Sulfenomes*5

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Identifying the sulfonylation state of stressed cells is emerging as a strategic approach for the detection of key reactive oxygen species signaling proteins. Here, we optimized an in vivo trapping method for cyaniste sulfenic acids in hydrogen peroxide (H2O2) stressed plant cells using a dimedone based DYn-2 probe. We demonstrated that DYn-2 specifically detects sulfonylation events in an H2O2 dose- and time-dependent way. With mass spectrometry, we identified 226 sulfonylated proteins after H2O2 treatment of Arabidopsis cells, residing in the cytoplasm (123); plastid (68); mitochondria (14); nucleus (10); endoplasmic reticulum, Golgi and plasma membrane (7) and peroxisomes (4). Of these, 123 sulfonylated proteins have never been reported before to undergo cysteine oxidative post-translational modifications in plants. In all, in this DYn-2 approach, we have identified new sulfonylated proteins, and gave a first glance on the locations of the sulfenomes of Arabidopsis thaliana. Molecular & Cellular Proteomics 14: 10.1074/mcp.M114.046896, 1183–1200, 2015.

Among the different amino acids, the sulfur containing amino acids like cysteine are particularly susceptible to oxidation by reactive oxygen species (ROS)1, 2. Recent studies suggest that the sulfenome, the initial oxidation products of cysteine residues, functions as an intermediate state of redox signaling (3–5). Thus, identifying the sulfenome under oxidative stress is a way to detect potential redox sensors (6, 7).

This central role of the sulfenome in redox signaling provoked chemical biologists to develop strategies for sensitive detection and identification of sulfonylated proteins. The in situ trapping of the sulfenome is challenging because of two major factors: (1) the highly reactive, transient nature of sulfenic acids, which might be over-oxidized in excess of ROS, unless immediately protected by disulfide formation (7); (2) the intracellular compartmentalization of the redox state that might be disrupted during extraction procedures, resulting in artificial non-native protein oxidations (8, 9). Having a sulfur oxidation state of zero, sulfenic acids can react as both electrophile and nucleophile, however, direct detection methods are based on the electrophilic character of sulfenic acid (10). In 1974, Allison and coworkers reported a condensation reaction between the electrophilic sulfenic acid and the nucleophilic dimedone (5,5-dimethyl-1,3-cyclohexanedione), producing a corresponding thioether derivative (11). This chemistry is highly selective and, since then, has been exploited to detect dimedone modified sulfenic acids using mass spectrometry (12). However, dimedone has limited applications for cellular sulfenome identification because of the lack of a functional group to enrich the dimedone tagged sulfenic acids. Later, dimedone-biotin/fluorophores conjugates have been developed, which allowed sensitive detection and enrichment of sulfenic acid modified proteins (13–15). This approach, however, was not always compatible with in vivo cellular sulfenome analysis, because the biotin/fluorophores-conjugated dimedone is membrane impermeable (9) and endogenous biotinylated proteins might appear as false positives.

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The abbreviations used are: ROS, reactive oxygen species; IAM, iodoacetamide; MMTS, S-methyl methanethiosulfonate; NEM, N-ethylmaleimide; SOH, sulfonylation state; S-S, disulfides; SSG, S-glutathionylation; SNO, S-nitrosothiol; H2O2, hydrogen peroxide; PTMs, post-translational modifications; c-CRD, carboxy-terminal cysteine-rich domain; PAP, peroxidase-anti-peroxidase; GO, Gene Ontology; YAP1, yeast AP-1 like

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More recently, the Carroll lab has developed DYn-2, a sulfinic acid specific chemical probe. This chemical probe consists of two functional units: a dimedone scaffold for sulfenic acid recognition and an alkyne chemical handle for enrichment of labeled proteins (9). Once the sulfenic acids are tagged with the DYn-2 probe, they can be biotinylated through click chemistry (16). The click reaction used here is a copper (I)-catalyzed azide-alkyne cycloaddition reaction (17), also known as azide-alkyne Huisgen cycloaddition (16). With this chemistry, a complex is formed between the alkyne functionalized DYn-2 and the azide functionalized biotin. This biotin functional group facilitates downstream detection, enrichment, and mass spectrometry based identification (Fig. 1).

In an evaluation experiment, DYn-2 was found to efficiently detect H2O2-dependent sulfenic acid modifications in recombinant glutathione peroxidase 3 (Gpx3) of budding yeast (18). Moreover, it was reported that DYn-2 is membrane permeable, non-toxic, and a non-influencer of the intracellular redox balance (17, 18). Therefore, DYn-2 has been suggested as a global sulfenome reader in living cells (17, 18), and has been applied to investigate epidermal growth factor (EGF) mediated protein sulfenylation in a human epidermoid carcinoma A431 cell line and to identify intracellular protein targets of H2O2 during cell signaling (17).

Here, we selected the DYn-2 probe to identify the sulfenome in plant cells under oxidative stress. Through a combination of biochemical, immunoblot and mass spectrometry techniques, and TAIR10 database and SUBA3-software predictions, we can claim that DYn-2 is able to detect sulfenic acids on proteins located in different subcellular compartments of plant cells. We identified 226 sulfenylated proteins in response to an H2O2 treatment of Arabidopsis cell suspensions, of which 123 proteins are new candidates for cysteine oxidative post-translational modification (PTM) events.

**EXPERIMENTAL PROCEDURES**

Arabidopsis Cell Cultures, Stress Treatments and DYn-2 Labeling—A. thaliana dark grown cell suspension line (PSB-D) was cultured as previously described (19). All experiments were performed with cells in mid-log phase (3-day old, around 10 mg fresh weight/ml). The time and dose of the stress treatment, as well as DYn-2 labeling were performed as follows:

1. For optimization of DYn-2 labeling conditions, we followed two conditions: (A) 10-ml cell cultures were stressed for 1 h by addition of 0, 0.1, 1 or 20 mM H2O2 in separated conical flasks (Merck, Germany). Then, the cells were harvested by filtration and rinsed with culture medium. After resuspension of the stressed cells in culture medium, probe labeling was performed with 0, 0.5, 1, 2.5, 5, or 10 mM of DYn-2 for 1 h. (B) The cell cultures were stressed for 1 h by addition of 0 or 20 mM H2O2 in the presence of 5 mM DYn-2. (2) For the detection of the dose-dependent responses of cells to H2O2 treatment, 10-ml cell cultures were treated with 0, 0.5, 1, 2, 5, 10, or 20 mM H2O2 in the presence of 500 μM DYn-2 for 1 h. For the detection of the time-dependent responses, 50-ml cell cultures were treated with 0, 1, or 20 mM H2O2 in the presence of 500 μM DYn-2. After 15, 30, 60, and 120 min treatment, 10 ml of cell culture were harvested at indicated time points for each H2O2 concentration. (3) For the competition study with...
the YAP1C probe, 10 ml of both YAP1C and YAP1A overexpressing *Arabidopsis* cell cultures were treated with 0 or 20 mM H2O2 for 1 h in the presence of 1 mM DYn-2 probe. For the optimization of DYn-2 labeling, the cells were treated with 20 mM H2O2 in the presence of 0, 0.5, 1, 2.5, 5, or 10 mM DYn-2 for 1 h. (4) For mass spectrometry based identification, 20-ml cell cultures were treated with 0 or 10 mM H2O2 for 30 min in the presence of 500 μM DYn-2.

After stress treatment and DYn-2 probe labeling, the cells were harvested by filtration and washed 3 times with culture medium, then the cells were ready for protein extraction and click reaction following downstream analysis. Before each experiment, the concentration of H2O2 was determined at 240 nm using 43.6 m M−1 cm−1 as the molar extinction coefficient.

**Protein Extraction, Click Reaction, Western Blot Analysis**—For protein extraction and biotinylation by click reaction, we followed the protocol as previously described (17) with some modifications. It is noteworthy to mention that the use of alkylating agents such as IAM and MMTS is not recommended, as they show reactivity with DYn-2 (unpublished data). Moreover, IAM, NEM, and MMTS are also known to form adducts with Cys-SOH, cleavable under reducing conditions (29).

Harvested cells were ground on ice using sand with extraction buffer (25 mM Tris-HCl pH 7.6, 15 mM MgCl2, 150 mM NaCl, 15 mM pNpO4Phenyl/PO4, 60 mM B-glycerophosphate, 0.1% Nonidet P-40, 0.1 mM Na2VO3, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 μM E64, 1× Roche protease inhibitor mixture, 5% ethylene glycol) supplemented with catalase (bovine liver, Sigma-Aldrich, St Louis, MO) at 200 U/ml. The lysates were centrifuged at 16,100 × g for 30 min at 4 °C to remove the cell debris. The protein content from the soluble fractions was determined using a standard DC Protein Assay (Bio-Rad Laboratories Inc., Hercules, CA). After removing endogenous biotinylated proteins by NeutrAvidin agarose beads, a click reaction was performed with 100 μg of proteins for 1 h by a rocking incubation at room temperature (17). By incubating for 5 min with 1 mM EDTA, the click reaction was stopped. Protein samples were denatured for 5 min at 96 °C, and then, 25 μg of each protein sample was resolved by SDS-PAGE. Sulfenylation was visualized by immunoblot with 1:80,000 dilution of streptavidin-HRP (Strep-HRP) antibody. Equal loading was confirmed on a Coomassie stained SDS-PAGE gel.

**Affinity Enrichment of DYn-2 Tagged Proteins**—For liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis, we performed the click reactions using 1-μg protein fractions after removing endogenous biotinylated proteins by NeutrAvidin agarose beads. Subsequently, the click reactions were stopped and proteins were precipitated in ice-cold acetone containing 10% trichloroacetic acid to remove nonreacted click reagents from the lysates upon incubation overnight at −20 °C. On the second day, the precipitated proteins were pelleted by centrifugation at 16,100 × g for 30 min at 4 °C. The pellet was washed twice with ice-cold acetone containing 5 mM dithiothreitol. Then, the pellet was air-dried to remove the acetone from the pellet. After complete resuspension of the precipitated proteins in PBS containing 0.2% SDS, the biotinylated DYn-2 labeled proteins were enriched with 200 μl NeutrAvidin agarose beads, which had been pre-equilibrated with resuspension buffer. The beads were collected by centrifugation at 2800 × g for 2 min, washed with PBS, which was followed by incubation with 5 mM dithiothreitol in the same buffer for 30 min at room temperature. Then, stringent washing steps were performed: 1× PBS, 1× NaCl for 5 min, 1× PBS, 1× M urea for 5 min, 1× PBS, 1× PBS containing 0.2% (w/v) SDS, 3× PBS. After each washing step, the beads were collected by centrifugation as described above. The biotinylated proteins were eluted in 100 μl buffer solution containing 1 mM biotin in 50 mM Tris-HCl, pH 7.1, 1% SDS by boiling for 10 min. The eluted proteins were lyophilized and then resuspended in 15 μl of 50 mM H2O/SDS loading buffer, resolved on SDS-PAGE as a single band (21), and excised for LC-MS/MS analysis.

**LC-MS/MS Analysis**—The gel bands were washed and subsequently digested in gel with trypsin. The obtained peptide mixtures were analyzed via LC-MS/MS using an Ultimate 3000 RSLC nano LC system (ThermoFisher Scientific, Bremen, Germany), in-line connected to a Q-Exactive mass spectrometer (ThermoFisher Scientific). Here, the peptides were first loaded on a trapping column (made in-house, 100 μm internal diameter (I.D.) × 20 mm, 5 μm beads C18 Reprosil-HD, Dr. Maisch, Ammerbuch-Entringen, Germany). After flushing from the trapping column, the sample was loaded on an analytical column (made in-house, 75 μm I.D. × 150 mm, 5 μm beads C18 Reprosil-HD, Dr. Maisch) packed in a needle (PicoFrit SELF/PicoTip emitter, PFS360-75-15-N, New Objective, Woburn, MA). Peptides were loaded with loading solvent (0.1% TFA in water/acetonitrile, 98/2 (v/v)) and separated using a linear gradient from 98% solvent A’ (0.1% formic acid in water) to 40% solvent B’ (0.1% formic acid in water/acetonitrile, 20/80 (v/v)) in 30 min at a flow rate of 300 nL/min. This is followed by a 5-min wash reaching 99% solvent B’. The mass spectrometer was operated in data-dependent, positive ionization mode, automatically switching between MS and MS/MS acquisition for the 10 most abundant peaks in a given MS spectrum. The source voltage was set at 3.4 kV and the capillary temperature was 275 °C. One MS1 scan (m/z 400–2000, AGC target 3 × 10^4 ions, maximum ion injection time 80 ms) acquired at a resolution of 70,000 (at 200 m/z) was followed by up to 10 tandem MS scans (resolution 17,500 at 200 m/z) of the most intense ions fulfilling predefined selection criteria (AGC target 5 × 10^5 ions, maximum ion injection time 60 ms, isolation window 2 Da, fixed first mass 140 m/z, spectrum data type: centroid, underfill ratio 2%, intensity threshold 1.7x10^4, exclusion of unassigned, 1, 5–8, >8 charged precursors, peptide match preferred, exclude isotopes on, dynamic exclusion time 20 s). The HCD collision energy was set to 275% Normalized Collision Energy and the polydimethylcyclosiloxane background ion at 445.120025 Da was used for internal calibration (lock mass).

From the MS/MS data in each LC run, Mascot Generic Files were created using the Distiller software (version 2.4.3.3, Matrix Science, www.matrixscience.com/Distiller). While generating these peak lists, grouping of spectra was allowed in Distiller with a maximal intermediate retention time of 30 s, and a maximum intermediate scan count of five was used where possible. Grouping was done with 0.005 Da precursor tolerances. A peak list was only generated when the MS/MS spectrum contained more than 10 peaks. There was no de-isotoping and the relative signal to noise limit was set at 2. These peak lists were then searched with the Mascot search engine (Matrix Science, London, UK, www.matrixscience.com) using the Mascot Daemon interface (version 2.4, Matrix Science) against the TAIR10 database containing 35,386 protein sequences. The considered variable modifications were DYn-2-cycloaddition, oxidation, dioxidation, and trioxidation of the cysteine residues; oxidation of the methionine residues; pyro-glutamate formation of amino-terminal glutamine residues; and acetylation of the protein N terminus. Mass tolerance on precursor ions was set to 10 ppm (with Mascot’s C13 option set to 1), and on fragment ions to 20 mnu. The instrument setting was put on ESI-QUAD. Enzyme was set to trypsin, allowing for one missed cleavage, and cleavage was also allowed when lysine or arginine were followed by proline. Only peptides that were ranked first and scored above the threshold score, set at 99% confidence were withheld. Furthermore, we only included peptides with a minimum length of 8 residues and with a maximum mass deviation from the calculated mass of 2 ppm. The average PSM, peptide and protein FDRs for all analyses were calculated at 0.14%, 0.31% and 0.63% respectively, using the method of Käll et al. (22).
A Proteomics View on Arabidopsis Sulfenomes

We considered the total unique identifications of two independent experimental rounds of the nontreated samples as the background dataset. For the data set of H$_2$O$_2$ treated samples, the overlapping identifications of three independent experiments were taken into account. To obtain the H$_2$O$_2$-dependent DYn-2 sulfenome, we subtracted the background data sets from the data set of the H$_2$O$_2$ treated identifications.

RESULTS AND DISCUSSION

The DYn-2 Probe is an Efficient Approach to Trap and Visualize Sulfenic Acids—For the labeling of sulfenylated proteins in living cells, it is of crucial importance to consider factors that might influence basal levels of cysteine oxidation (17). For Arabidopsis cell suspension cultures, these factors could be the changes in physico-chemical parameters of the culture medium, nutrient deficiency, cells grown to the stationary phase, etc. We performed stress treatments with increasing concentrations of H$_2$O$_2$ on the 3-day-old PSB-D Arabidopsis cell suspension cultures in the presence of DYn-2 (Fig. 3 and supplemental Fig. S1A, S1B). After harvesting, cells were washed with culture medium to remove excess H$_2$O$_2$ and DYn-2. This washing step is necessary to avoid DYn-2 tagging of de novo sulfenylated proteins generated during the extraction process. Sample preparation and biotinylation of the DYn-2 tagged proteins with click chemistry were performed as previously described (17), followed by protein separation on SDS-PAGE and visualization of the DYn-2 tagged biotinylated proteins on anti-Strep-HRP Western blots. We observed that DYn-2 is able to penetrate Arabidopsis cells and that it could detect sulfenic acids formed under stress. In contrast to mammalian cells (17), we found that the H$_2$O$_2$ stress treatment performed in the presence of the DYn-2 probe is an efficient approach to trap and visualize sulfenic acids in Arabidopsis cells (Fig. 3 and supplemental Fig. S1). Important to note is that we used a catalase-supplemented extraction buffer to extract soluble protein fractions. Catalase scavenges H$_2$O$_2$ that might be generated during the protein extraction procedure; in such a way we control de novo sulfenylation during the extraction. A pilot experiment using extraction buffer with and without catalase showed a clear influence of catalase to control post-extraction sulfenic acids formation at higher H$_2$O$_2$ concentrations (Fig. 3 and supplemental Fig. S2). By incubating the lysate with NeutrAvidin agarose beads, we removed endogenous biotinylated proteins and the nonsulfenylated proteins sticking to the beads.

DYn-2 Competes with YAP1C Trapping—After optimizing the DYn-2 labeling conditions (H$_2$O$_2$ stress treatment in the presence of 500 μM DYn-2 probe (supplemental Fig. S1 and Fig. 3), we assessed whether DYn-2 interaction with sulfenylated proteins quantitatively affects the interaction of the YAP1C genetic probe with sulfenic acids under oxidative stress conditions. YAP1C is the carboxy-terminal, cysteine-rich domain (c-CRD) of the redox-regulated yeast AP-1 like (YAP1) transcription factor that has been adapted to trap protein sulfenic acids in vivo (23–25). Briefly, we designed two variants of the YAP1 c-CRD: (1) YAP1C containing the wild-type redox regulatory Cys598 that traps CysSOH residues and (2) YAP1A, in which Cys598 is mutated to alanine as a control for nonspecific protein associations. YAP1 fragments were fused with a GS tag moiety for downstream analysis (26). With the help of a peroxidase-anti-peroxidase (PAP) antibody, which detects the GS tag moiety, we showed that in response to H$_2$O$_2$, YAP1C forms mixed disulfides with CysSOH proteins in an H$_2$O$_2$ concentration-dependent manner (25). However, these complexes were absent in YAP1A control cells, because the YAP1 c-CRD disulfide-bonded complexes are formed through the specific reaction of Cys598 with CysSOH on multiple proteins.

We performed a competitive study between the DYn-2 and YAP1C probe. Therefore, the YAP1C and YAP1A overexpressing cells were stressed with 20 mM H$_2$O$_2$ for 1 h in the presence or absence of 1 mM DYn-2. As a control, we compared the response with nonstressed cells. Analysis of the Western blots with the PAP antibody showed that the intensity of YAP1C dimerization did not increase in a DYn-2 treated sample under nonstressed conditions (Fig. 2). Further, dimerization bands disappeared under reducing conditions and ran as a monomer with similar levels of YAP1C in each lane, which confirms the redox-active disulfide nature of the interacting proteins. Further, the mixed disulfide complexes were only formed in YAP1C overexpressing cells, and were not observed with YAP1A. Under H$_2$O$_2$ stressed conditions in the presence of the DYn-2 probe, YAP1C dimerization was decreased (Fig. 2), which indicates that the DYn-2 probe is capable of competing out the reaction with YAP1C, at least for a certain number of sulfenylated proteins (see below and Fig. 4F).
**Fig. 3.** DYn-2 detects time- and dose-dependent changes of H$_2$O$_2$ mediated sulfenylation in *Arabidopsis*. A, Cell cultures were treated with 0, 0.5, 1, 2, 5, 10, or 20 mM H$_2$O$_2$ for 1 h in the presence of 500 µM DYn-2 probe. After the click reaction, the H$_2$O$_2$ dose-dependent sulfenylation was visualized on a Strep-HRP developed Western blot. B, Cell cultures were treated with 0, 1, or 20 mM H$_2$O$_2$ for 15, 30, 60, and 120 min in the presence of 500 µM DYn-2. After the click reaction, the time-dependent sulfenylation was visualized on a Strep-HRP developed Western blot.

**Fig. 4.** Analysis of the sulfenome identified in *Arabidopsis* under H$_2$O$_2$ stress. A, Enrichment of DYn-2 tagged proteins. Cell cultures were treated with 0 or 10 mM H$_2$O$_2$ for 30 min in the presence of 500 µM DYn-2 probe. After extraction, the DYn-2 tagged proteins were biotinylated and enriched using avidin beads. L: lysates, L(p): lysates after precipitation, E: eluted proteins, S: Supernatant, the nonbound part of the lysate. On a Strep-HRP developed Western blot, an increased signal was observed under stress conditions even after enrichment of the DYn-2 tagged proteins on avidin beads. B, After subtraction of the background datasets of nontreated samples, 226 proteins were identified from three independent experiments as the H$_2$O$_2$ mediated DYn-2 sulfenome. C, The number of the identified proteins predicted to be present in the different subcellular compartments. D, Percentage of the candidates previously identified as having redox-active cysteines. E, The previously reported 103 proteins contain sulfenic acids (SOH), disulfides (S-S), S-glutathionylated (SSG), and S-nitrosoylated proteins (SN). F, The 123 cytoplasmic sulfenylated proteins identified by DYn-2 contain 16 proteins in common with the YAP1C cytoplasmic sulfenome.
DYn-2 Traps Sulfenylated Proteins under Oxidative Stress in a Time- and Dose-Dependent Manner—After optimizing the DYn-2 labeling conditions, we set out an experiment to optimize the dose of DYn-2 required for sulfene trapping. We stressed the cells with 20 mM H$_2$O$_2$ for 1 h in the absence or presence of increasing concentrations of DYn-2 up to 10 mM. On Strep-HRP Western blot, we observed that DYn-2 is able to detect sulfenic acids at the lowest concentration of 500 $\mu$M DYn-2, and that by increasing the DYn-2 concentration, more sulfenylated proteins were detected (supplemental Fig. S3). Because probing at higher concentrations might lead to the presence of nonreacted intracellular DYn-2, we decided to work at the lowest possible concentration of DYn-2. In this way, we lower the possibility of detecting false positive sulfenylation signals, because excess intracellular DYn-2 might tag newly modified proteins during the extraction procedure.

After optimizing the DYn-2 dose for probing sulfenic acids, we set out an experiment to observe whether DYn-2 could detect sulfenylation patterns in a dose-dependent way. Previously, others and we have shown that a 20-mM H$_2$O$_2$ treatment of Arabidopsis cells provokes cysteine sulfenylation (25, 27). To evaluate the H$_2$O$_2$ dose response, we treated the cells with 0, 0.5, 1, 2, 5, 10 or 20 mM H$_2$O$_2$ for 1 h in the presence of 500 $\mu$M DYn-2 (Fig. 3A). On Strep-HRP Western blot, we observed that sulfenic acid labeling by DYn-2 was H$_2$O$_2$ dose-dependent. Nonstressed cells displayed only low levels of basal sulfenic acid labeling, whereas an increasing signal was observed from 2 mM of H$_2$O$_2$ onward. We concluded that DYn-2 traps the sulfenic acids in a dose-dependent way to H$_2$O$_2$ stress responses within the cells.

In the next step, the time course was evaluated. DYn-2 tagging of sulfenic acids was examined for treatment of cell cultures with 0, 1 or 20 mM H$_2$O$_2$ and samples were analyzed after 15, 30, 60, and 120 min of each stress treatment (Fig. 3B). We observed a response to the changes of sulfenylation in function of time at the 20-mM H$_2$O$_2$ treatment. The time-dependent response was not significant at the 1-mM H$_2$O$_2$ stressed sample, indicating that this concentration is too low to visualize an increase of the sulfenylation signal. In untreated samples, the intensity of the sulfenylation signal was not changing in function of time, showing that the background oxidation state under nonstressed conditions remains the same in the presence of DYn-2 (Fig. 3B). This is an important observation, because it indicates that DYn-2 itself is not generating oxidative stress in Arabidopsis cells and does not disturb the basal level of sulfenylation under nonstressed conditions. It was also previously reported that DYn-2 does not alter cell viability and glutathione redox balance, or generates ROS in other cell types (18).

Identification of 226 Sulfenylated Proteins under H$_2$O$_2$ Stress—As the previous experiments demonstrate that DYn-2 penetrates plant cells and that this small chemical probe (178.2 Da) is able to trap sulfenylated proteins under oxidative stress, we decided to map the sulfenome of Arabidopsis cells using this probe. According to the time-course and dose-response experiments, we observed that the sulfenylation signal intensity is similar between the 10- and 20-mM H$_2$O$_2$ treatment (Fig 3A and supplemental Fig. S2), and we observed a breakthrough of the signal after 30 min of H$_2$O$_2$ stress (Fig. 3B). Therefore, we decided to stress the Arabidopsis cells for 30 min with 10 mM H$_2$O$_2$ (Fig. 3A) in the presence of 500 $\mu$m DYn-2 (breakthrough detection of sulfenylation as observed in supplemental Fig. S3). DYn-2 tagged sulfenylated proteins were extracted and enriched. Before enrichment, the non-reacted click reagents were removed from the lysates by acetone precipitation to avoid competition during the enrichment process between non-clicked free biotin azide and biotinylated DYn-2 tagged proteins. After resuspension of the precipitated protein pellet, DYn-2 tagged proteins were trapped on NeutrAvidin beads. The high affinity of the biotin-avidin interaction (the dissociation constant, $K_{D}$, is $\sim 10^{-15}$ M) allowed stringent washing steps (1 M NaCl, 4 M urea) to remove all non-biotinylated interactions. After several intensive, consecutive washing steps (for details see Experimental Procedures), the biotinylated proteins were eluted with biotin competition under denaturing conditions. In Fig. 4A, a representative Strep-HRP developed Western blot shows an affinity purification of the DYn-2 tagged proteins of nonstressed and stressed cells. An increased sulfenylation signal in the enriched DYn-2 tagged proteins from stressed cells was observed. Eluted proteins were subjected to LC-MS/MS to identify the sulfenylated proteins. From the three independent experiments of treating cells with 10 mM H$_2$O$_2$ for 30 min, we identified 420 different sulfenylated proteins that are present in all rounds. As we wanted to focus on the sulfenylated proteins under H$_2$O$_2$ stress, the proteins identified in the absence of H$_2$O$_2$ were considered as a background dataset. As such, we identified 226 sulfenylated proteins of the H$_2$O$_2$ mediated sulfenome of Arabidopsis (Fig. 4B, supplemental Tables S1 and S2).

DYn-2 Reads the Plant Sulfenome in Different Plant Organelles—We categorized the 226 H$_2$O$_2$ mediated sulfenylated proteins based on their predicted or demonstrated subcellular localization, function (Gene Ontology (GO) annotation), and reported cysteine oxidative modifications. Fig. 4C displays the predicted subcellular localization of the identified proteins, which suggests the capability of DYn-2 to read the sulfenylation at different subcellular levels in vivo. DYn-2 trapped 123 cytoplasmic sulfenylated proteins (54.5%); 68 from the plastids (30%); 10 from the nucleus (4.4%); 14 from mitochondria (6.2%), 7 from the endoplasmic reticulum, Golgi and plasma membrane (3.1%) and 4 from the peroxisome (1.8%) (Table I, Fig. 4C). It is noteworthy that we did not perform a specific enrichment for the subcellular proteomes with this approach. The DYn-2 identified proteins have at least one cysteine residue, except for Fe SUPEROXIDE DISMUTASE 1, which might be trapped as a possible interactor of one of the identified proteins (Table I). The majority of the identified proteins are
This table provides the AGI code, description, subcellular localization and functional categorization as provided by the TAIR 10 DB (35,386 protein sequences) and SUBA3. In addition, we provided the number of Cys residues in the corresponding protein sequence and the type of redox modification that was found. Also, references were assigned where possible. These data can be consulted via the PRIDE partner repository with the dataset identifier PXD001562 and 10.6019/PXD001562 (username: reviewer31841@ebi.ac.uk with password: Rg04wvB) using the ProMInspect tool (48). Details on data validation and search parameters can be found in the Experimental Procedures section. Abbreviations of PTMs are as follows: SNO, S-nitrosothiol; SOH, sulfenic acid; S-S, disulfide bridge; SSG, S-glutathionylation; Trx/Gox target, thioredoxin/glutaredoxin target proteins. References describing identification of homologs/orthologs are marked with an asterisk.

| AGI code | Description | Subcellular localization | Functional categorization | No of Cys | Redox modification | References |
|----------|-------------|--------------------------|---------------------------|-----------|--------------------|------------|
| AT3G62940 | OVARIAN TUMOR DOMAIN (OTU)-CONTAINING DUB (DEUBQUITILATING ENZYME) 5 | Cytoplasm, cytosol | Protein degradation | 3 | | |
| AT2G06990 | HEN2, HUA ENHANCER 2 | Cytosol, nucleus | RNA binding-translation | 14 | | |
| AT4G24490 | RAB GERANYLGERANYL TRANSFERASE ALPHA SUBUNIT 1 | Cytoplasm, cytosol | Protein transport | 9 | | |
| AT2G45810 | DEA(D)/H-box RNA helicase family protein | Cytoplasm, cytosol | RNA binding-translation | 10 | | |
| AT4G38680 | GLYCINE RICH PROTEIN 2, GRP2 | Cytoplasm, cytosol | Signal transduction | 6 | | |
| AT3G29360 | UDP-GLUCOSE DEHYDROGENASE 2, UGD2 | Cytoplasm, cytosol, nucleus | Primary metabolism | 10 | SSG | (28) |
| AT5G63880 | Pyruvate kinase family protein | Cytoplasm, cytosol, plasma membrane | Primary metabolism | 11 | | |
| AT1G62740 | HOP2 | Cytoplasm, cytosol, nucleus, plasma membrane | Miscellaneous | 5 | SOH | (25) |
| AT5G43330 | CYTOSOLIC-NAD-DEPENDENT MALATE DEHYDROGENASE 2 | Cytoplasm, cytosol, plasma membrane, plastid, apoplast | Primary metabolism | 6 | Gox target; reactive cys; Trx target | (30*, 49, 50*) |
| AT2G32520 | Alpha/beta-Hydrolases superfamily protein | Cytoplasm, cytosol, plastid | Protein degradation | 1 | Trx target; SOH | (41*, 51*) |
| AT3G06720 | IMPORTIN ALPHA ISOFORM 1 | Cytoplasm, cytosol, cell wall, nuclear envelope, nucleolus, nucleus | Protein transport | 11 | | |
| AT1G69250 | Nuclear transport factor 2 (NTF2) family protein with RNA binding (RRM-RBD-RNP motif) domain | Cytoplasm | RNA binding-translation | 3 | | |
| AT2G24050 | EUKARYOTIC 25S RNA SYNTHETASE INITIATOR FACTOR ISOFORM AG2 | Cytoplasm, cytosol | RNA binding-translation | 10 | | |
| AT5G10240 | ASPARAGINE TRANSLATION INITIATION FACTOR ISOFORM AG2 | Cytosol | Amino acid metabolism | 12 | SOH; reactive cys | (43*, 52) |
| AT5G19810 | METHIONINE S-METHYLTRANSFERASE | Cytoplasm, cytosol | Amino acid metabolism | 20 | | |
| AT4G13930 | SERINE HYDROXYMETHYLTRANSFERASE 4 | Cytoplasm, cytosol, plastid | Amino acid metabolism | 8 | SOH; reactive cys; Trx target; SNO | (41*, 43*, 51*, 52, 53*) |
| AT3G17820 | GLUTAMINE SYNTHETASE 1.3 | Cytoplasm, cytosol, cytosolic ribosome, plastid | Amino acid metabolism | 4 | SOH; Trx target | (41*, 43*, 51*) |
| AT2G095830 | 5-METHYLTHIORIBOSE KINASE 1 | Cytosol, extracellular region, plastid | Amino acid metabolism | 4 | | |
| AT2G095830 | GMP SYNTHASE (glutamine-hydrolyzing) | Cytosol, cytoplasm | Amino acid metabolism | 7 | | |
| AT3G44310 | NITRILASE 1 (NIT1) | Cytosol, apoplast, plastid membrane, plastid | Hormone homeostasis | 7 | SSG; SOH | (25, 28) |
| AT1G48630 | RECEPTOR FOR ACTIVATED C KINASE 1 (RACK1B) | Cytosol, cytoplasm, cytosolic ribosome, nucleus | Hormone homeostasis | 8 | SOH; reactive cys | (25, 52) |
| AT5G09810 | ACTIN 7 | Cytosol, cytoplasm, cytoskeleton, cell wall | Miscellaneous | 4 | SSG; SOH; Trx target | (28, 38, 43*, 49, 50*) |
| AGI code   | Description                                                  | Subcellular localization                                      | Functional categorization                          | No of Cys | Redox modification | References |
|------------|--------------------------------------------------------------|---------------------------------------------------------------|---------------------------------------------------|-----------|--------------------|------------|
| AT5G44720  | Molybdenum cofactor sulfurase family protein                | Cytosol, mitochondrion, nucleus, plastid                      | Miscellaneous and unknown functions               | 4         |                    |            |
| AT5G43830  | Aluminium induced protein with YGL and LRDR motifs         | Cytosol, nucleus                                              | Miscellaneous and unknown functions               | 4         |                    |            |
| AT4G27450  | Aluminium induced protein with YGL and LRDR motifs         | Cytosol, nucleus, plasma membrane, plasmodesma               | Miscellaneous and unknown functions               | 7         | SOH                | (25)       |
| AT4G14930  | Survival protein SurE-like phosphatase                      | Cytosol                                                       | Miscellaneous and unknown functions               | 7         |                    |            |
| AT3G22850  | Aluminium induced protein with YGL and LRDR motifs         | Cytosol, cytoplasm, nucleus, plasma membrane,                 | Miscellaneous and unknown functions               | 7         |                    |            |
| AT3G13460  | EVOLUTIONARILY CONSERVED C-TERMINAL REGION 2               | Cytosol, cytoplasm, nucleus                                   | Unknown functions                                 | 5         |                    |            |
| AT2G15860  | Unknown protein                                             | Cytosol                                                       | Unknown functions                                 | 3         |                    |            |
| AT1G77550  | Tubulin-tyrosine ligases                                    | Cytoplasm, chloroplast                                        | Miscellaneous                                     | 14        |                    |            |
| AT1G66880  | Unknown protein                                             | Cytoplasm, chloroplast                                        | Miscellaneous                                     | 3         |                    |            |
| AT1G43690  | Ubiquitin interaction motif-containing protein              | Cytosol, nucleus                                              | Miscellaneous                                     | 12        |                    |            |
| AT5G52920  | PLASTIDIC PYRUVATE KINASE BETA SUBUNIT 1                   | Cytosol                                                       | Primary metabolism                                | 5         |                    |            |
| AT5G48180  | NITRILE SPECIFIER PROTEIN 5                                | Cytosol, cytoplasm                                            | Primary metabolism                                | 7         |                    | (28, 38, 39, 43*) |
| AT5G44340  | TUBULIN BETA CHAIN 4                                        | Cytosol, cytoplasm, plasma membrane, Golgi, apoplast         | Primary metabolism                                | 10        | SSG; SNO; SOH      | (28, 38, 39, 43*) |
| AT5G19770  | TUBULIN ALPHA-3                                             | Cytosol, cytoplasm, plasma membrane, Golgi, apoplast         | Primary metabolism                                | 11        | SOH; Trx target    | (43*, 50*) |
| AT5G12250  | BETA-6 TUBULIN                                              | Cytosol, cytoplasm                                            | Primary metabolism                                | 12        | SOH                | (43*)      |
| AT4G37870  | PHOSPHOENOLPYRUVATE CARBOXYKINASE 1                        | Cytosol, cytoplasm, nucleus                                   | Primary metabolism                                | 10        |                    | (43*)      |
| AT4G16130  | ARABINOSE KINASE                                            | Cytosol, cytoplasm, plasma membrane                           | Primary metabolism                                | 22        |                    |            |
| AT4G20890  | TUBULIN BETA-9 CHAIN                                        | Cytosol, cytoplasm, plasma membrane, Golgi                   | Primary metabolism                                | 12        | SOH                | (43*)      |
| AT3G57890  | Tubulin binding cofactor C domain-containing protein       | Cytosol, nucleus                                              | Primary metabolism                                | 9         |                    |            |
| AT5G58330  | NADP-DEPENDENT MALATE DEHYDROGENASE                         | Cytosol, cytoplasm, apoplast                                  | Primary metabolism                                | 9         | Trx target         | (32, 33)   |
| AT3G06650  | ATP-CITRATE LYASE SUBUNIT B-1                              | Cytosol, cytoplasm                                            | Primary metabolism                                | 10        | SOH                | (25)       |
| AT3G06580  | GALACTOSE KINASE 1                                           | Cytosol, cytoplasm                                            | Primary metabolism                                | 13        |                    |            |
| AT2G41530  | S-FORMYLGLUTATHIONE HYDROLASE                               | Cytosol, cytoplasm, apoplast                                  | Primary metabolism                                | 5         | Trx target; reactive cys | (31, 49) |
| AT1G16350  | Aldolase-type TIM barrel family protein                     | Cytosol                                                       | Primary metabolism                                | 6         | SSG                | (28)       |
| AT1G09780  | 2,3-BISPHOSPHOGLYCERATE-INDEPENDENT PHOSPHOGLYCERATE MUTASE 1 | Cytosol, cytoplasm, apoplast, plasmamembrane                 | Primary metabolism                                | 4         | SNO; Trx target    | (39, 50*) |
| AT1G11840  | GLYXOALASE I HOMOLOG                                        | Cytosol, peroxisome, plasmamembrane, chloroplast envelope, mitochondrion | Primary metabolism                                | 1         |                    |            |
| AT5G13520  | Peptidase M1 family protein                                 | Cytosol, chloroplast                                          | Protein degradation                               | 7         | SOH                | (25)       |
| AT5G60160  | Zn-dependent exopeptidases superfamily protein              | Cytosol, chloroplast                                          | Protein degradation                               | 11        | Trx target         | (31)       |
| AT2G24200  | Cytosol aminopeptidase family protein                      | Cytosol, chloroplast                                          | Protein degradation                               | 5         | SSG; SOH; reactive cys; Trx target | (28, 43*, 52) |
| AGI code   | Description                                              | Subcellular localization                        | Functional categorization    | No of Cys | Redox modification                      | References |
|-----------|----------------------------------------------------------|--------------------------------------------------|------------------------------|-----------|----------------------------------------|------------|
| AT2G30110 | UBIQUITIN-ACTIVATING ENZYME 1                            | Cytosol, nucleus, plasma membrane                | Protein degradation          | 18        |                                        |            |
| AT2G19520 | MULTICOPY SUPPRESSOR OF IRA1 4                           | Cytosol, cytoplasm, nucleus                      | Protein degradation          | 9         |                                        |            |
| AT1G22920 | COP9 SIGNALOSOME 5A                                      | Cytosol, nucleus                                 | Protein degradation          | 2         | Trx target; SOH                        | (25, 54)   |
| AT5G22060 | DNAJ HOMOLOGUE 2                                         | Cytosol, cytoplasm, plasma membrane              | Protein folding              | 11        |                                        |            |
| AT4G02450 | HSP20-LIKE CHAPERONES SUPERFAMILY PROTEIN                | Cytosol, cytoplasm, plasma membrane              | Protein folding              | 1         |                                        |            |
| AT5G56010 | HEAT SHOCK PROTEIN 81-3                                 | Cytosol, cytoplasm, Golgi, plasma membrane       | Protein folding              | 5         | SNO; SOH                               | (38, 43*)  |
| AT5G02500 | HEAT SHOCK COGNATE PROTEIN 70-1                          | Cytosol, cytoplasm, Golgi, plasma membrane       | Protein folding              | 7         | SSQ; SNO; SOH; Trx target              | (28, 39, 43*, 50*, 53*) |
| AT3G12580 | HEAT SHOCK PROTEIN 70                                    | Cytosol, cytosol, plasma membrane                | Protein folding              | 7         | SOH; reactive cys; SNO; Trx target     | (41*, 42*, 43*, 49, 53*) |
| AT1G79930 | HEAT SHOCK PROTEIN 91                                    | Cytosol, cytosol, plasma membrane                | Protein folding              | 14        | Trx target                             | (53*)      |
| AT1G24510 | TCP-1/cpn60 chaperonin family protein                    | Cytosol, cytosol, plasma membrane, plasmodesma   | Protein folding              | 9         | Trx target                             | (53*)      |
| AT4G34450 | Coatomer gamma-2 subunit, putative                       | Cytosol, Golgi, plasma membrane                  | Protein transport            | 12        |                                        |            |
| AT2G44100 | GUANOSEINE NUCLEOTIDE DIPHOSPHATE DISSOCIATION INHIBITOR 1 | Cytosol, cytoplasm                              | Protein transport            | 8         |                                        |            |
| AT3G14990 | DJ-1 HOMOLOG A                                           | Cytosol, plasmamembrane, plasmodesma, nucleo, chloroplast | Redox related              | 7         | SNO; Trx target; reactive cys          | (31, 38, 41*, 52) |
| AT1G78380 | GLUTATHIONE S-TRANSFERASE TAU 19                         | Cytosol, cytoplasm, chloroplast, plasma membrane | Redox related              | 1         | SSQ; SNO; Trx target; reactive cys     | (28, 31, 32, 38, 55*, 56) |
| AT1G65980 | THIOREDOXIN-DEPENDENT Peroxidase 1 (TPX1)                | Cytosol, cytoplasm, chloroplast, plasma membrane | Redox related              | 2         | Trx target; reactive cysteine; SNO; SOH; Grx target | (25, 30*, 31, 32, 38, 49) |
| AT1G60420 | ATRNRX1, NRX1, NUCLEOREDOXIN 1/DC1 domain-containing protein | Cytosol                                         | Redox related              | 12        | reactive cys                           | (49)       |
| AT4G14030 | SELENIUM-BINDING PROTEIN 1                               | Cytosol, nucleus                                | Redox related              | 7         |                                        |            |
| AT4G09670 | OXIDOREDUCTASE FAMILY PROTEIN                            | Cytosol, nucleus                                | Redox related              | 6         |                                        |            |
| AT3G12290 | AMINO ACID DEHYDROGENASE FAMILY PROTEIN                 | Cytosol, nucleus                                | Redox related              | 4         |                                        |            |
| AT3G21250 | NADP-LINKED OXIDOREDUCTASE SUPERFAMILY PROTEIN           | Cytosol, cytoplasm                              | Redox related              | 6         |                                        |            |
| AT1G59960 | NADP-LINKED OXIDOREDUCTASE SUPERFAMILY PROTEIN           | Cytosol, chloroplast                            | Redox related              | 5         |                                        |            |
| AT1G37130 | NITRATE REDUCTASE 2                                      | Cytosol, mitochondrion, plasma membrane          | Redox related              | 16        | SOH; reactive cys                       | (25, 52)   |
| AT1G05350 | NADPH-binding ROSSMANN-fold superfamily protein          | Cytosol, cytoplasm                              | Redox related              | 10        |                                        |            |
| AT3G11940 | RIBOSOMAL PROTEIN 5A                                      | Cytosol, cytoplasm                              | RNA binding-translation    | 2         | SNO                                    | (39)       |
| AT3G02760 | Class II aARS and bixin synthetases superfamily protein  | Cytosol                                         | RNA binding-translation    | 17        | reactive cys; Trx target               | (50*, 52)  |
| AT3G46280 | EUKARYOTIC TRANSLATION INITIATION FACTOR 3 SUBUNIT I     | Cytosol                                         | RNA binding-translation    | 5         | reactive cys                           | (52)       |
| AT2G45710 | Zinc-binding ribosomal protein family protein             | Cytosol                                         | RNA binding-translation    | 6         | reactive cys                           | (52)       |
| AT1G30580 | GTP BINDING /OBG-LIKE ATPASE 1                           | Cytosol                                         | RNA binding-translation    | 5         | Trx target                            | (32, 51*)  |
| AGL code     | Description                                                                 | Subcellular localization                      | Functional categorization                  | No of Cys | Redox modification | References |
|-------------|----------------------------------------------------------------------------|-----------------------------------------------|--------------------------------------------|-----------|--------------------|------------|
| AT1G09820   | ATP binding*leucine-tRNA ligases'arninoacyl-tRNA ligases'nucleotide binding*ATP binding*arninoacyl-tRNA ligases | cytosol                                       | RNA binding-transl                        | 20        | reactive cys       | (52)       |
| AT5G25780   | EUKARYOTIC TRANSLATION INITIATION FACTOR 3B-5                             | Cytosol, cytoplasm                            | RNA binding-transl                        | 3         |                    |            |
| AT4G39520   | GTP-BINDING PROTEIN-RELATED                                                | Cytosol, cytoplasm                            | RNA binding-transl                        | 7         |                    |            |
| AT4G31120   | PROTEIN ARGinine METHYLTRANSFERASE 5 (PRMT5)                               | Cytosol, cytoplasm                            | RNA binding-transl                        | 12        | SOH                | (25)       |
| AT4G26870   | Class II arninoacyl-tRNA and biotin synthetases superfamily protein        | Cytosol, cytoplasm, plasmodesma               | RNA binding-transl                        | 11        |                    |            |
| AT3G57290   | EUKARYOTIC TRANSLATION INITIATION FACTOR 3E (EIF3E)                        | Cytosol, cytoplasm, plasma membrane           | RNA binding-transl                        | 5         | SOH                | (25)       |
| AT3G04840   | Ribosomal protein S3Ae                                                     | Cytosol                                       | RNA binding-transl                        | 4         |                    |            |
| AT2G40660   | Nucleic acid-binding, OB-fold-like protein                                 | Cytosol, cytoplasm, plasmodesma               | RNA binding-transl                        | 4         |                    |            |
| AT2G40290   | Encodes an eIF2alpha homolog                                               | Cytosol                                       | RNA binding-transl                        | 5         |                    |            |
| AT2G23350   | POLY (A) BINDING PROTEIN 4                                                 | Cytosol                                       | RNA binding-transl                        | 7         |                    |            |
| AT2G15790   | CYCLOPHILIN 40                                                             | Cytosol                                       | RNA binding-transl                        | 7         | Trx target         | (57)       |
| AT1G33120   | Ribosomal protein L6 family                                                | Cytosol                                       | RNA binding-transl                        | 2         |                    |            |
| AT1G10840   | TRANSLATION INITIATION FACTOR 3 SUBUNIT H1                                | Cytosol                                       | RNA binding-transl                        | 7         |                    |            |
| AT3G49840   | DUTP-PYROPHOSPHATASE-LIKE 1                                                | Cytosol                                       | Signal perception & transduction          | 1         | reactive cys       | (52)       |
| AT5G20990   | CO-FACTOR FOR NITRATE REDUCTASE AND XANTHINE DEHYDROGENASE                | Cytosol                                       | Signal perception & transduction          | 9         |                    |            |
| AT5G16050   | GENERAL REGULATORY FACTOR 5                                                | Cytosol, cytoplasm, Golgi, plasma membrane   | Signal perception & transduction          | 2         |                    |            |
| AT4G24800   | EIN2 C-TERMINUS INTERACTING PROTEIN 1                                      | Cytosol                                       | Signal perception & transduction          | 6         |                    |            |
| AT3G15730   | PHOSPHOLIPASE D ALPHA 1                                                    | Cytosol                                       | Signal perception & transduction          | 8         |                    |            |
| AT3G02870   | Encodes a α-galactose-1-phosphate phosphatase, involved in ascorbate biosynthesis. | Cytoplasm, cytosol, plasma membrane           | Signal perception & transduction          | 5         |                    |            |
| AT2G43980   | INOSITOL 1,3,4-TRISPHOSPHATE 5/6-KINASE 4 (ITPK4)                          | Cytosol                                       | Signal perception & transduction          | 9         | SOH                | (25)       |
| AT1G51690   | PROTEIN PHOSPHATASE 2A 5KDA REGULATORY SUBUNIT (PF2A-5KDA)                 | Cytoplasm                                     | Signal perception & transduction          | 11        | SOH                | (25)       |
| AT1G78300   | GENERAL REGULATORY FACTOR 2                                                | Cytosol, cytoplasm, Golgi, plasma membrane   | Signal perception & transduction          | 2         | SOH                | (43)       |
| AT1G35160   | GENERAL REGULATORY FACTOR 4                                                | Cytosol, cytoplasm, Golgi, plasma membrane   | Signal perception & transduction          | 2         | SOH                | (43)       |
| AT5G39570   | Unknown protein                                                            | Cytosol, nucleus                              | Unknown functions                         | 1         |                    |            |
| AT5G42220   | Ubiquitin-like superfamily protein                                         | Cytosol, nucleus                              | Protein degradation                       | 6         |                    |            |
| AT5G36810   | Alpha/beta-Hydrolases superfamily protein                                 | cytosol, plastid                              | Protein degradation                       | 13        | reactive cys; SOH  | (25, 52)   |
| AT4G35830   | ACONITASE 1                                                                | apoplast, cytoplasm, cytosol, mitochondion, plasma membrane, plasmodesma, vacuole | Primary metabolism                       | 12        | SOH, Trx target    | (43*, 50*, 51*) |
| AT3G53110   | LOW EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 4                          | cytoplasm, nuclear envelope, nucleus, plasma membrane | Miscellaneous and unknown functions      | 5         |                    |            |
| AGI code       | Description                                      | Subcellular localization                                      | Functional categorization      | No of Cys | Redox modification       | References  |
|---------------|--------------------------------------------------|----------------------------------------------------------------|------------------------------|-----------|--------------------------|------------|
| AT5G19990     | REGULATORY PARTICLE TRIPLE-A ATPASE 6A           | Cytosol, cytoplasm, nucleus, plasma membrane                   | Protein degradation           | 3         |                          |            |
| AT1G56450     | 20S PROTEASOME BETA SUBUNIT G1                   | Cytosol                                                       | Protein degradation           | 1         | SSG                      | (28)       |
| AT2G32730     | 26S PROTEASOME REGULATORY COMPLEX, RPN2          | Cytosol, chloroplast                                         | Protein degradation           | 8         |                          |            |
| AT1G20200     | EMBRYO DEFECTIVE 2719                           | Cytosol, nucleus                                             | Protein degradation           | 6         |                          |            |
| AT5G56500     | CHAPERONIN-60BETA                               | Cytosol, chloroplast                                         | Protein folding              | 6         | Gox target; Trx target   | (30*, 53*) |
| AT3G59020     | ARM repeat superfamily protein                   | Cytosol, cytoplasm, nucleus                                  | Protein transport             | 16        | SOH                      | (25)       |
| AT3G09843     | ARM repeat superfamily protein                   | Cytosol, cytoplasm                                           | Protein transport             | 18        |                          |            |
| AT3G43400     | NITRILASE 2 (NIT2)                               | Cytosol, plasma membrane                                     | Hormone homeostasis          | 7         | SOH; reactive cys        | (25, 49)   |
| AT4G34230     | CINNAMYL ALCOHOL DEHYDROGENASE 5                | Cytosol, cytoplasm                                           | Primary metabolism            | 11        | Trx target               | (31)       |
| AT1G62380     | ACC OXIDASE 2                                   | Cytosol, cytoplasm, endoplasmic reticulum, plasma membrane,  | Protein homeostasis          | 4         | SSG                      | (28)       |
|               |                                                  | cytosol, nucleus, plasma membrane, plasmodesma, Ggdi apparatus, cell wall, |                              |           |                          |            |
| AT5G53400     | BOB1                                             | Cytosol, cytoplasm                                           | Protein folding              | 4         |                          |            |
| AT5G57870     | EUKARYOTIC TRANSLATION INITIATION FACTOR ISOFORM 4G1 | Cytoplasm, cytosol, nucleus                                      | RNA binding-translation      | 7         |                          |            |
| AT5G56350     | Pyruvate kinase family protein                   | Cytosol, cytosol                                             | Protein folding              | 12        |                          |            |
| AT1G16650     | RNA-binding (RRM/RDD/RNP motifs) family protein  | Cytoplasm, cytosol, nucleus                                   | RNA binding                  | 3         | reactive cys              | (52)       |
| AT4G26970     | ACONITASE 2                                      | Cytosol, mitochondrion                                       | Primary metabolism           | 10        | SOH; Trx target          | (43*, 50*) |
| AT5G07440     | GLUTAMATE DEHYDROGENASE 2                       | Cytosol, mitochondrion                                       | Amino acid metabolism        | 6         | Trx target; SNO; S-S     | (34, 35, 38, 51*) |
| Mitochondrion |                                                  |                                                                |                              |           |                          |            |
| AT1G48030     | MITOCOHAL LIPOAMIDE DEHYDROGENASE 1             | Mitochondrion                                                | Carbohydrate metabolism      | 5         | Trx target; Gox target;  | (30*, 35, 52) |
|               |                                                  |                                                                | reactive cys                  |           |                          |            |
| AT1G24180     | IAA-CONJUGATE-RESISTANT 4                       | Mitochondrion                                                | Primary metabolism           | 8         | SOH; reactive cys        | (43*, 52)  |
| AT5G08670     | ATP SYNTHASE ALPHA/BETA FAMILY PROTEIN           | Mitochondrion                                                | Primary metabolism           | 3         | Trx target; Gox target;  | (28, 30*, 35, 43*) |
| AT5G05850     | MAB1, MACC-BOU/TRANSKETOLASE FAMILY PROTEIN/PYRUVATE DEHYDROGENASE E1 COMPONENT SUBUNIT BETA-1, MITOCOHAL | Mitochondrion                                           | Primary metabolism           | 5         | S-S bond; reactive cys;  | (34, 35, 49) |
|               |                                                  |                                                                | Trx target                    |           |                          |            |
| AT5G08300     | SUCCINYL-1-COA LIGASE, ALPHA SUBUNIT             | Mitochondrion, cell wall                                      | Primary metabolism           | 8         | Trx target               | (35, 51*)  |
| AT1G22840     | CYTOCHROME C-1                                  | Mitochondrion, cytosol                                       | Primary metabolism           | 2         |                          |            |
| AT5G37510     | NADH-ubiquinone dehydrogenase, mitochondrial     | Mitochondrion                                                | Protein degradation          | 19        | Trx target               | (35)       |
| AT3G62530     | ARM repeat superfamily protein                   | Mitochondrion, nucleus, chloroplast,                         | Protein transport            | 3         | reactive cys              | (49)       |
| AT5G43430     | ELECTRON TRANSFER FLAVOPROTEIN BETA              | Mitochondrion                                                | Redox related                | 3         |                          |            |
| AT5G14040     | MITOCOHAL PHOSPHATE TRANSPORTER 3 (MPT3)        | Mitochondrion                                                | Signal perception &          | 7         | Trx target; SOH; SNO;    | (34, 35, 39, 43*) |
|               |                                                  |                                                                | transduction                 |           | S-S                      |            |
| AT3G17240     | LIPOAMIDE DEHYDROGENASE 2, mitochondrial         | Mitochondrion, plastid                                       | Redox related                | 5         | SNO; SOH; S-S            | (34, 39, 40, 43*) |
| AT1G48920     | NUCLEOUL ILE 1                                  | Mitochondrion, nucleus                                       | Protein transport            | 1         |                          |            |
| AT5G14950     | ISOCITRATE/ISOPROPYLALATE DEHYDROGENASE FAMILY PROTEIN | Mitochondrion, plastid                                     | Primary metabolism           | 6         | Gox target; SOH          | (30*, 43*) |
| AT1G74260     | PURINE BIOSYNTHESIS 4                           | Mitochondrion                                                | Primary metabolism           | 24        | reactive cys              | (52)       |
| Nucleus       |                                                  |                                                                |                              |           |                          |            |
| AT3G51800     | ERBB-3 BINDING PROTEIN 1                        | Nucleolus, nucleus, plasma membrane                         | Protein transport            | 6         | SOH; SNO                  | (25, 39)   |
| AT1G35780     | Unknown protein*                                | Nucleus                                                      | Unknown function             | 2         |                          |            |
| AT1G22730     | MA3 domain-containing protein                   | Nucleus                                                      | Miscellaneous                | 10        |                          |            |
| AGI code   | Description                                                                 | Subcellular localization                                                                 | Functional categorization       | No of Cys | Redox modification            | References |
|------------|------------------------------------------------------------------------------|------------------------------------------------------------------------------------------|---------------------------------|-----------|-------------------------------|------------|
| AT3G58510  | DEA(D/H)-box RNA helicase family protein                                      | Nucleus, peroxisome, plasma membrane                                                    | RNA binding-transcription       | 6         |                               |            |
| AT2G22400  | S-adenosyl-l-methionine-dependent methyltransferases superfamily protein     | Nucleus                                                                                  | RNA binding-transcription       | 14        |                               |            |
| AT1G67880  | SRP72 RNA-binding domain                                                      | Nucleus                                                                                  | RNA binding-transcription       | 7         |                               |            |
| AT2G38560  | TRANSCRIPT ELONGATION FACTOR IIS                                            | Nucleus                                                                                  | Transcription                   | 11        |                               |            |
| AT1G20110  | FVE-DOMAIN PROTEIN 1                                                         | nucleus                                                                                  | Miscellaneous                   | 14        |                               |            |
| AT1G50570  | Calcium-dependent lipid-binding (CaLB domain) family protein                 | nucleus                                                                                  | Miscellaneous                   | 6         |                               |            |
| AT1G45000  | AAA-type ATPase family protein                                                | Nucleolus, nucleus, plasma membrane, plasmodesma, cell wall, membrane                   | Protein degradation             | 3         |                               |            |
| AT4G16760  | ACYL-COA OXIDASE 1                                                           | Peroxisome                                                                               | Primary metabolism              | 13        |                               |            |
| AT3G24170  | GLUTATHIONE-DISULFIDE REDUCTASE                                               | Peroxisome                                                                               | Redox related                   | 8         |                               |            |
| AT2G33150  | PEROXISOMAL 3-KETOACYL-COA THIOLASE 3                                       | Peroxisome                                                                               | Signal perception & transduction| 9         |                               |            |
| AT2G42520  | P-LOOP CONTAINING NUCLEOSIDE TRIPHOSPHATE HYDROLASES SUPERFAMILY PROTEIN     | Peroxisome                                                                               | Transcription                   | 4         |                               |            |
| AT5G22770  | ALPHA-ADAPTIN                                                               | Clathrin adaptor complex, membrane, membrane coat, plasma membrane                      | Protein transport               | 15        |                               |            |
| AT1G05520  | Sec23/Sec24 protein transport family protein                                 | Endoplasmic reticulum, Golgi                                                            | Protein transport               | 20        |                               |            |
| AT5G42020  | LUMINAL BINDING PROTEIN                                                      | Endoplasmic reticulum, endoplasm reticulum lumen                                        | Protein folding                 | 5         | SOH; SNO                      | (38, 43*)  |
| AT1G58340  | CALRETICULIN 1A                                                             | Endoplasmic reticulum, plasmodesma, apoplast                                            | Protein degradation             | 3         | SOH; reactive cys              | (43*, 49)  |
| AT1G9210   | CALRETICULIN 1B                                                             | Endoplasmic reticulum, apoplast                                                         | Protein degradation             | 4         | SOH                           | (43*)      |
| AT4G23850  | LONG-CHAIN ACYL-COA SYNTHETASE 4/AMP-DEPENDENT SYNTHETASE AND LIGASE FAMILY PROTEIN | Golgi apparatus, plasma membrane, nucleus                                               | Primary metabolism              | 13        |                               |            |
| AT3G08530  | CLATHRIN, HEAVY CHAIN 2                                                     | Golgi apparatus, plasma membrane, nucleus                                               | Protein transport               | 22        |                               |            |
| AT3G34750  | ARABIDOPSIS CYSTEINE SYNTHASE 1                                              | Plastid                                                                                  | Amino acid metabolism           | 5         | S-S bond; reactive cys; SOH   | (37, 43*, 58) |
| AT3G59760  | O-ACETYL-SERINE (THIOL) LYASE ISOFORM C                                     | Chloroplast, chloroplast stroma, mitochondrion                                           | Amino acid metabolism           | 6         | Trx target; SOH; S-S; Gx target | (30*, 32, 34, 35, 43*) |
| AT5G54770  | THIAZOLE BIOSYNTHETIC ENZYME, CHLOROPLAST                                    | Plastid                                                                                  | Primary metabolism              | 2         |                               |            |
| AT5G41670  | 6-phosphogluconate dehydrogenase family protein                            | Plastid, mitochondrion                                                                   | Primary metabolism              | 6         |                               |            |
| AT4G24830  | Arginosuccinate synthase family                                              | Plastid                                                                                  | Amino acid metabolism           | 6         | reactive cys; SSG; SOH         | (25, 28, 49) |
| AT4G39980  | 3-DEOXY-1-ARABINO-HEPTULOSONATE 7-PHOSPHATE SYNTHASE 1, DHS1                 | Chloroplast, mitochondrion                                                               | Amino acid metabolism           | 9         |                               |            |
| AT4G35630  | PHOSPHOSERINE AMINOTRANSFERASE                                                | Plastid                                                                                  | Amino acid metabolism           | 8         |                               |            |
| AGI code       | Description                                                                 | Subcellular localization | Functional categorization   | No of Cys | Redox modification       | References |
|---------------|-----------------------------------------------------------------------------|--------------------------|-----------------------------|-----------|--------------------------|------------|
| AT4G32520     | SERINE HYDROXYMETHYLTRANSFERASE 3                                          | Plastid                  | Amino acid metabolism       | 7         | Trx target, SNO          | (41*, 51*, 53*) |
| AT4G29840     | THREONINE SYNTHASE                                                           | Plastid, cytosol         | Amino acid metabolism       | 11        | Trx target               | (53*)      |
| AT3G57560     | N-ACETYL-L-GLUTAMATE KINASE                                                  | Plastid, cytoplasm       | Amino acid metabolism       | 4         |                          |            |
| AT3G49680     | BRANCHED-CHAIN-AMINO-ACID AMINOTRANSFERASE 3. CHLOROPLASTIC                | Plastid                  | Amino acid metabolism       | 7         |                          |            |
| AT2G45300     | 5-ENOLPYRUVYL-L-SHIKIMATE-3-PHOSPHATE/EPSP synthase involved in chorismate biosynthesis | Plastid                  | Amino acid metabolism       | 10        |                          |            |
| AT2G31810     | ACT domain-containing small subunit of ascorbic acid oxidase synthase protein | Plastid                  | Amino acid metabolism       | 4         |                          |            |
| AT3G29690     | ANTHANILATE SYNTHASE 2                                                       | Plastid                  | Amino acid metabolism       | 7         |                          |            |
| AT2G22250     | ASPARATE AMINOTRANSFERASE                                                    | Plastid                  | Amino acid metabolism       | 6         | Trx target; SNO          | (41*, 51*) |
| AT1G80600     | HOPW1-1-INTERACTING 1                                                        | Plastid, mitochondrion   | Amino acid metabolism       | 7         |                          |            |
| AT1G58080     | ATP PHOSPHORIBOSYL TRANSFERASE 1                                             | Plastid, cytoplasm       | Amino acid metabolism       | 6         |                          |            |
| AT1G48850     | EMBRYO DEFECTIVE 1144, chorismate synthase activity                         | Plastid, nucleolus       | Amino acid metabolism       | 8         |                          |            |
| AT1G29900     | CARBAMOYL PHOSPHATE SYNTHETASE B                                            | Plastid, mitochondrion   | Amino acid metabolism       | 21        |                          |            |
| AT1G22410     | Class-II DAHP synthetase family protein                                      | Plastid                  | Amino acid metabolism       | 7         |                          |            |
| AT5G16290     | VALINE-TOLERANT 1                                                            | Plastid, cytosol         | Amino acid metabolism       | 2         | reactive cys              | (52)       |
| AT3G53580     | Diaminopimelate epimerase family protein, Chloroplastic                      | Plastid                  | Amino acid metabolism       | 9         | reactive cys              | (52)       |
| AT3G23940     | Dehydratase family                                                          | Plastid, mitochondrion   | Amino acid metabolism       | 12        | Trx target               | (31, 33)   |
| AT4G26300     | EMBRYO DEFECTIVE 1027                                                       | Plastid                  | Miscellaneous and unknown functions | 9       |                          |            |
| AT1G69740     | Encodes a putative 5-aminolevulinate dehydratase involved in chlorophyll biosynthesis | Plastid                  | Miscellaneous and unknown functions | 8       |                          |            |
| AT2G33210     | HEAT SHOCK PROTEIN 60-2                                                      | Plastid, mitochondrion, plasma membrane | Protein folding | 7         | Trx target; SSG; SOH; Gx target | (28, 30*, 35, 43*, 53*) |
| AT3G49800     | ALDEHYDE DEHYDROGENASE 2                                                    | Chloroplast, mitochondrion | Primary metabolism | 7         | SOH; Gx target; reactive cys, Trx target, SNO | (30*, 41*, 43*, 49, 50*, 51*) |
| AT3G49990     | ACYL-ACTIVATING ENZYME 3                                                    | Chloroplast, chloroplast stroma | Primary metabolism | 4         | reactive cys              | (52)       |
| AT1G35720     | ANNEXIN 1                                                                   | Chloroplast, chloroplast stroma, apoplast, plasmodesma, thylakoid, vacuolar membrane, vacuole | Signal perception & transduction | 2         | SNO; SSG                  | (29, 38, 39) |
| AT5G46290     | KETOACYL-ACYL CARRIER 3-PROTEIN SYNTHASE I                                 | Plastid                  | Primary metabolism          | 9         |                          |            |
| AT5G17530     | phosphoglucosamine mutase family protein                                     | Plastid, cytoplasm       | Primary metabolism          | 4         |                          |            |
| AT5G16440     | ISOPENTENYL-DIPHOSPHATE DELTA-ISOMERASE I, chloroplastic                    | Plastid, cytoplasm       | Primary metabolism          | 4         |                          |            |
| AT4G18440     | Plastid, cytoplasm                                                          | Plastid                  | Primary metabolism          | 4         |                          |            |
| AT3G37810     | ADENYLOSUCINATE SYNTHETASE, CHLOROPLASTIC                                   | Plastid                  | Primary metabolism          | 8         | Trx target               | (53*)      |
| AT3G48730     | GLUTAMATE-1-SEMIALDEHYDE 2,1-AMINOMUTASE 2                                  | Plastid                  | Primary metabolism          | 6         | SNO; Trx target          | (41*, 51*, 59*) |
| AT1G74030     | ENOASE 1, CHLOROPLASTIC                                                     | Plastid                  | Primary metabolism          | 7         | SOH; reactive cys; Trx target | (43*, 49, 50*) |
| AT3G25860     | PLASTID E2 SUBUNIT OF PYRUVATE DECARBOXYLASE                                | Plastid                  | Primary metabolism          | 1         |                          |            |
| AT3G21110     | PURIN 7                                                                     | Plastid                  | Primary metabolism          | 7         |                          |            |
| AT2G43710     | SUPPRESSOR OF SA INSENSITIVE 2                                              | Plastid                  | Primary metabolism          | 3         |                          |            |
| AT4G33300     | SULFOQUINOSYLDIACYLGLYCEROL 1                                               | Plastid                  | Primary metabolism          | 9         | SNO                       | (39)       |
| AT2G35040     | AICARFT/IMPCHase bienzyme family protein                                     | Plastid                  | Primary metabolism          | 10        |                          |            |
| AGI code     | Description                                                | Subcellular localization | Functional categorization | No of Cys | Redox modification                     | References       |
|-------------|------------------------------------------------------------|--------------------------|---------------------------|-----------|---------------------------------------|------------------|
| AT2G02500   | HEAT SHOCK COGNATE PROTEIN 70-1                           | Plastid                  | Primary metabolism        | 4         |                                       |                  |
| AT1G80560   | ISOPROPYLALATE DEHYDROGENASE 2                            | Plastid                  | Primary metabolism        | 3         |                                       |                  |
| AT3G22960   | PLASTIDAL PYRUVATE KINASE 1                               | Plastid                  | Primary metabolism        | 9         | reactive cys                           | (52)             |
| AT1G74040   | 2-ISOPROPYLALATE SYNTHASE 1                               | Plastid                  | Primary metabolism        | 7         |                                       |                  |
| AT3G12780   | PHOSPHOGLYCERATE KINASE 1                                 | Plastid                  | Primary metabolism        | 2         | Trx target; S-S                        | (31, 37, 53*)    |
| AT2G21170   | PLASTID ISOFORM TRIOSE PHOSPHATE ISOMERASE,               | Plastid                  | Primary metabolism        | 4         | Trx target; Grx target; SNO            | (30*, 32, 33, 41*, 50*, 59*) |
| AT1G4800    | STEAROYL-ACYL CARRIER PROTEIN Δ9-DESATURASES              | Plastid                  | Primary metabolism        | 4         |                                       |                  |
| AT1G38280   | l-Aspartate-like family protein                           | Plastid                  | Primary metabolism        | 3         |                                       |                  |
| AT1G22980   | THIAMINE REQUIRING 1                                      | Plastid                  | Primary metabolism        | 11        |                                       |                  |
| AT1G63770   | Peptidase M1 family protein                               | Plastid                  | Protein degradation       | 11        |                                       |                  |
| AT5G15450   | CASEIN LYTIC PROTEINASE B3, Encodes a chloroplast-targeted Hsp101 homologue | Plastid                  | Protein folding           | 3         |                                       |                  |
| AT1G49910   | CHLOROPLAST HEAT SHOCK PROTEIN 70-2                      | Plastid                  | Protein folding           | 2         | Trx target; Grx target; S-S, SNO      | (30*, 35, 37, 41*, 50*) |
| AT3G13470   | CHAPERONIN-60BETA2                                        | Plastid                  | Protein folding           | 7         | S-S; Trx target                        | (37, 54)        |
| AT5G53480   | ARM repeat superfamily protein                            | Plastid                  | Protein transport         | 17        |                                       |                  |
| AT5G50920   | HEAT SHOCK PROTEIN 93-V                                   | Plastid                  | Protein folding           | 4         | S-S; Trx target                        | (37, 53*)       |
| AT4G03890   | STROMAL ASCORBATE PEROXIDASE                              | Plastid                  | Redox related             | 2         | Trx target; SNO                        | (35, 42*, 50*)  |
| AT1G63940   | MONODEHYDROASCORBATE REDUCTASE 6                         | Plastid                  | Redox related             | 5         | Trx target; S-S                        | (31, 37)        |
| AT1G16155   | DIHYDRODIOXYL DEHYDROGENASES                             | Plastid                  | Redox related             | 9         | Trx target                            | (53*)           |
| AT1G12900   | GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE A SUBUNIT 2     | Plastid                  | Redox related             | 5         | Grx target; reactive cys; SNO; Trx target | (30*, 41*, 56, 59*) |
| AT1G79530   | GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE OF PLASTID 1    | Plastid                  | Redox related             | 3         | SOH; Trx target                        | (43*, 53*, 57*)  |
| AT3G58140   | Phenylalanine-tRNA synthetase class IIc family protein    | Plastid                  | RNA binding-translation  | 7         |                                       |                  |
| AT5G65430   | GENERAL REGULATORY FACTOR 8                              | Plastid                  | Signal perception & transduction | 2         | Grx target; SNO                        | (30*, 41*)      |
| AT3G56940   | COPPER RESPONSE DEFECT 1                                 | Plastid                  | Transcription             | 5         |                                       |                  |
| AT2G17630   | PHOSPHOSERINE AMINOTRANSFERASE 2                          | Plastid                  | Amino acids metabolism    | 8         |                                       |                  |
| AT1G80270   | PENTATRICOPEPTIDE REPEAT 596                             | Chloroplast envelope     | Miscellaneous and unknown functions | 6         |                                       |                  |
| AT5G65620   | THIMET METALLOENDOPETIDASE 1, TOP1                        | chloroplast, chloroplast stroma, cytosol | Protein degradation | 6         | S-S; SNO                              | (37, 41*)       |
involved in the primary metabolism of multiple pathways (pentose phosphate pathway, glycolysis, TCA cycle, shikimate, amino acid and fatty acid biosynthesis). In addition, we identified proteins involved in signal perception and transduction, hormone homeostasis, transcription/translation, protein degradation/folding (transport) (Table I).

Within the DYn-2 sulfenome (Fig. 4D, E; Table I), some proteins with reactive cysteines have previously been reported. As such, we analyzed that 25 sulfenylated proteins have been reported to be S-glutathionylated (28–30), 55 proteins with a redox-active disulfide bond (31–37), and 29 proteins for S-nitrosylation (38–42) (Fig. 4E; Table I). Apart from that, we identified 30 proteins that are in common with the sulfenome of *Medicago truncatula*, which was analyzed using Bio-DCP1, another dimeredone chemistry based probe (43) (Table II). Moreover, we also identified several established antioxidant and signaling proteins like CHLOROPLASTIC GLUTAMATE-CYSTEINE LIGASE, STROMAL ASCORBATE PEROXIDASE, GLUTATHIONE S-TRANSFERASE TAU 19, THIOREDOKIN-DEPENDENT PEROXIDASE 1, MONODEHYDROASCORBATE REDUCTASE 6, ACC OXIDASE 2, NUCLEOUREDOKIN 1, ANNEXIN 1 and GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE A.

When we compare lists of proteins discovered with the YAP1C (95 cytoplasmic sulfenylated proteins) (25) and DYn-2 (123; Fig. 4F, Table II) probes, only 16 proteins were common. This discrepancy is most likely because of the different mode of action and reactivity of both probes, leading to discrete sensitivities. Dimedone reacts with a sulfenic acid at a rate of $2.7 \times 10^{-2}$ M$^{-1}$s$^{-1}$ (44). The DYn-2 probe, however, is doing much better, because its reaction rate with dipeptide-SOH is estimated to be $11$ M$^{-1}$s$^{-1}$ (10). Although the rate constant of YAP1C disulfide formation with target sulfenic acids is not yet known, if we compare it with the rate for the reaction of sulfenic acids with thiols to form a disulfide bond (21.6 M$^{-1}$s$^{-1}$) (10, 44), the YAP1C probe should be more efficient in trapping sulfenic acids compared with DYn-2. Although the dimeredone based probe has a modest reaction rate with sulfenic acids, we observed that DYn-2 is able to trap sulfenylated proteins more specifically in vivo than YAP1C (Fig. 2). Noteworthy, whether a reaction will occur does not only depend on the reaction rate, but also on the local concentration. Apart from that, YAP1C makes complexes with sulfenic acids through protein–protein interactions, whereas the relatively small DYn-2 molecule directly reacts with the exposed sulfenic acids independently of the local protein conformation. In this way, the chance that DYn-2 is trapped within protein structural cavities will be larger than that for YAP1C. Also, DYn-2 forms a stable covalent bond with the targeted sulfur, whereas the disulfide nature of the YAP1C-target interaction is reversible and these mixed disulfides can be reduced by the cellular reduction system, leading to an underestimation of the number of sulfenylated proteins. All these reasons might account for the relatively modest number of cytoplasmic proteins identified in our previous study (25).

**Significance**—We report here the first successful application of the DYn-2 chemical probe for the identification of sulfenomes in plants. With an optimized DYn-2 trapping technique, we identified sulfenylated proteins predicted to be cytoplasmic, plastidal, mitochondrial, nuclear, peroxisomal, or residing in the endoplasmic reticulum, Golgi and plasma membrane. Besides the identification of these sulfenomes, our efforts contribute to a more complete view of the cytoplasmic sulfenome with the identification of 107 new cytoplasmic candidates, so we doubled the identified sulfenylated proteins from the cytoplasm.

Although we are making progress, we are still at the discovery phase. With the application of complementary sulfenic acid trapping techniques, the identification of additional proteins of the sulfenome does not inform us about the mechanism behind triggering oxidative stress defense signaling through sulfenylation. We are also trapping proteins in which the cysteine is damaged by oxidation, and which are prone to degradation within the cellular proteasome, or enzymes in which the formation of a sulfenic acid is part of their catalytic cycle. There is certainly room for improvement toward specificity. Future progress in understanding sulfur oxygen switches within the cell strongly depends on the chemical tools and on the technological advances that will be made in the development of new methodologies. Recent promising results have been reported. Yang *et al.* (45) detected about 1000 sulfenation sites on more than 700 proteins in human cells using a photocleavable biotin linker on a clickable chemical dimeredone probe, even though no specificity toward signaling proteins has been built in. In signaling proteins, sulfenic acids are transiently formed. Therefore, it is important to develop chemical probes with a high reaction rate to trap these transiently formed sulfenic acids. Poole *et al.* (46) have shown in their recent work that strained cycloalkynes react with sulfenic acids to yield a stable alkanyl sulfoxide with a reaction rate that is 100 times faster than that of most dimeredone based 1,3 dicarbonyl reagents. However, on the other hand, a relatively slower dimeredone based probe might facilitate selectivity toward specific stabilized sulfenic acids, which are more likely to be present in signaling pathways than on catalytically regulated active sites. The kinetics of a probe is one issue, but many other challenges lie still ahead before we get a clear view on the regulation of cellular networks driven by oxidative thiol modifications. Progress in this thiol based signaling field will dependent on combining selective chemical probes and new enrichment strategies with the latest omics technologies.

Although we are fully aware of the current technical limitations and the highly dynamic character of oxidative thiol based signaling, we strongly believe that by reading the DYn-2 sulfenome of *A. thaliana*, an additional important piece within the cellular sulfenome jigsaw puzzle is given. On the
## TABLE II

List containing 48 previously identified sulfenylated proteins in *Medicago truncatula* (42). This table provides the AGI code and description from the TAIR 10 DB with the according references. References describing identifications in plants are marked with an asterisk.

| AGI code   | Description                                                                                     | References |
|------------|------------------------------------------------------------------------------------------------|------------|
| **Signal perception and transduction**               |                                                     |            |
| AT2G43980  | INOSITOL 1,3,4-TRISPHOSPHATE 5/6-KINASE 4 (ITPK4)                                             | (25)       |
| AT1G51690  | PROTEIN PHOSPHATASE 2A 55 KDA REGULATORY SUBUNIT B ALPHA ISOFORM (PP2A-b55α)                     |            |
| AT5G14040  | MITOCHONDRIAL PHOSPHATE TRANSPORTER 3 (MPT3)                                                   | (43*)      |
| AT1G78300  | 14–3–3 PROTEIN, GENERAL REGULATORY FACTOR 2                                                     |            |
| AT1G35160  | 14–3–3 PROTEIN, GENERAL REGULATORY FACTOR 4                                                     |            |
| **Redox related**                                   |                                                     |            |
| AT1G65980  | THIOREDOXIN-DEPENDENT PEROXIDASE 1                                                             | (25)       |
| AT1G37130  | NITRATE REDUCTASE 2                                                                             |            |
| AT3G17240  | LIPOAMIDE DEHYDROGENASE 2, mitochondrial                                                       | (43*)      |
| AT1G79530  | GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE OF PLASTID 1 (GAPCP-1)                                |            |
| **Protein synthesis, folding, transport**           |                                                     |            |
| AT4G31120  | PROTEIN ARGinine METHYLTRANSFERase 5 (PRMT5)                                                   | (25)       |
| AT3G57290  | EUKARYOTIC TRANSLATION INITIATION FACTOR 3E (Eif3E)                                             |            |
| AT3G59020  | ARM repeat superfamily protein                                                                  |            |
| AT3G51800  | ERBB-3 BINDING PROTEIN 1 (EBP1)                                                                 | (43*)      |
| AT5G56010  | HEAT SHOCK PROTEIN 81–3                                                                        |            |
| AT5G42020  | LUMINAL BINDING PROTEIN                                                                         |            |
| AT5G02500  | HEAT SHOCK COGNATE PROTEIN 70–1                                                                |            |
| AT3G12580  | HEAT SHOCK PROTEIN 70                                                                           |            |
| AT2G33210  | HEAT SHOCK PROTEIN 60–2                                                                         |            |
| **Protein degradation**                             |                                                     |            |
| AT5G36210  | Alpha/beta-Hydrolases superfamily protein                                                       | (25)       |
| AT5G13520  | Peptidase M1 family protein                                                                      |            |
| AT1G22920  | COP9 SIGNALOSOME 5A (CSN5A)                                                                     | (43*)      |
| AT2G24200  | Cytosol aminopeptidase family protein                                                           |            |
| AT1G09210  | CALRETICULIN 1B                                                                                 |            |
| AT1G56340  | CALRETICULIN 1A                                                                                 |            |
| **Primary metabolism**                              |                                                     |            |
| AT3G06650  | ATP-CITRATE LYASE SUBUNIT B-1                                                                   | (25)       |
| AT4G24830  | Arginosuccinate synthase family                                                                 |            |
| AT3G48000  | ALDEHYDE DEHYDROGENASE 2                                                                        | (43*)      |
| AT1G24180  | IAA-CONJUGATE-RESISTANT 4,                                                                       |            |
| AT5G44340  | TUBULIN BETA CHAIN 4                                                                            |            |
| AT5G19770  | TUBULIN ALPHA-3                                                                                 |            |
| AT5G14590  | Ioscitrate/isopropylmalate dehydrogenase family protein                                           |            |
| AT5G12250  | BETA-6 TUBULIN (TUB6)                                                                           |            |
| AT5G08670  | Encodes the mitochondrial ATP synthase beta-subunit                                             |            |
| AT4G35830  | ACONITASE 1                                                                                     |            |
| AT4G13930  | SERINE HYDROXYMETHYLTRANSFERASE 4                                                               |            |
| AT3G59760  | O-ACETYSERINE (THIOL) LYASE ISOFORM C                                                           |            |
| AT2G43750  | O-ACETYSERINE (THIOL) LYASE B                                                                    |            |
| AT3G17820  | GLUTAMINE SYNTHETASE 1.3                                                                        |            |
| AT5G10240  | ASPARAGINE SYNTHETASE 3                                                                          |            |
| AT4G26970  | ACONITASE 2                                                                                     |            |
| AT4G20890  | TUBULIN BETA-9 CHAIN                                                                            |            |
| AT1G74030  | ENOLASE 1, CHLOROPLASTIC                                                                         |            |
| **Hormone homeostasis**                             |                                                     |            |
| AT3G44310  | NITRILASE 1 (NIT1)                                                                               | (25)       |
| AT3G44300  | NITRILASE 2 (NIT2)                                                                               |            |
| AT1G48630  | RECEPTOR FOR ACTIVATED C KINASE 1B (RACK1B)                                                     |            |
| **Miscellaneous**                                   |                                                     |            |
| AT4G27450  | Aluminium induced protein with YGL and LRDR motifs                                               | (25)       |
| AT1G62740  | HOP2, Encodes one of the 36 carboxylate clamp (CC)-tetratricopeptide repeat (TPR) proteins       |            |
| AT5G09810  | ACTIN 7                                                                                         | (43*)      |
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long run, it will contribute to the unraveling of signaling events along the sulfenome of plants, and it will help our understanding of signaling transduction pathways under oxidative stress in general.

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