Sulfenome mining in *Arabidopsis thaliana*

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Reactive oxygen species (ROS) have been shown to be potent signaling molecules. Today, oxidation of cysteine residues is a well-recognized posttranslational protein modification, but the signaling processes steered by such oxidations are poorly understood. To gain insight into the cysteine thiol-dependent ROS signaling in *Arabidopsis thaliana*, we identified the hydrogen peroxide (H_2O_2)-dependent sulfenome: that is, proteins with at least one cysteine thiol oxidized to a sulfenic acid. By means of a genetic construct consisting of a fusion between the C-terminal domain of the yeast (*Saccharomyces cerevisiae*) AP-1–like (YAP1) transcription factor and a tandem affinity purification tag, we detected ~100 sulfenylated proteins in *Arabidopsis* cell suspensions exposed to H_2O_2 stress. The in vivo YAP1-based trapping of sulfenylated proteins was validated by a targeted in vitro analysis of DEHYDROASCORBATE REDUCTASE2 (DHAR2). In DHAR2, the active site nucleophilic cysteine is regulated through a sulfenic acid-dependent switch, leading to S-glutathionylation, a protein modification that protects the protein against oxidative damage.

oxidative stress | redox regulation | cysteine oxidation

Numerous posttranslational modifications (PTMs) have been discovered within proteomes, creating a complex landscape of protein diversity and function (1). One of the recognized reversible redox-based PTMs is the oxidation of a cysteine thiol group to a sulfenic acid (Cys-SOH) (2) that acts as regulatory switch in several oxidative stress signal transduction pathways (3). Sulfenic acids, unless they are stabilized into the protein environment, can react rapidly with other protein thiols or with low-molecular weight thiols to form intramolecular and intermolecular disulfides. These mechanisms protect the sulfenic acids against overoxidation to sulfinic (SO_2H) or sulfonic (SO_3H) acid and allow sulfur oxygen signaling (2).

In plants, the best-known redox regulation mechanisms are the light-dependent thiol-disulfide exchange switches in chloroplast proteins (4). Examples of other redox-regulated proteins are the transcription coactivator NONEXPRESSER OF PR GENES 1 (5), the vacuolar H^+-ATPase (6), and several transcription factors (TFs), such as the AP2-type RAP2.4a (7), the G-group of basic leucine zipper TFs (8), and the TEOSINTEBRANCHED1/CYLOIDEA/PROLIFERATING CELL FACTOR class I TFs (9). The redox relay mechanisms that bridge the signal perception to the final oxidative stress response are largely unknown.

Some thiol peroxidases have an H_2O_2-dependent signaling function and can act as receptor and transducer (10). In yeast (*Saccharomyces cerevisiae*), the H_2O_2 sensor oxidant receptor peroxidase1 (ORP1/glutathione peroxidase 3) controls, together with the transcription factor YAP1 (yeast AP-1–like), a redox regulon via a sulfenic acid thiol-disulfide relay mechanism (11). Upon reaction with H_2O_2, the peroxidatic cysteine of ORP1 is oxidized to a sulfenic acid that reacts with the YAP1 C-terminal cysteine-rich domain (cCRD) and forms a disulfide (Fig. S1A). This specific mixed disulfide formation has prompted us to

develop a YAP1-based probe for trapping plant sulfenylated proteins in vivo (12).

To categorize the sulfenome, which is the set of proteins with at least one sulfenic acid, and its dynamics at the proteome level in *Arabidopsis thaliana* cells upon oxidative stress, we implemented the YAP1-based sulfenic acid trapping method coupled to a tandem affinity purification (TAP) tag (13). We identified 97 sulfenylated proteins during the early and late oxidative stress responses, of which 67 had previously not been recognized to undergo oxidative PTMs. Validation of sulfenylation on DEHYDROASCORBATE REDUCTASE2 (DHAR2) demonstrates the importance of a glutathione (GSH)-dependent redox switch on its sulfenylated nucleophilic cysteine that reversibly regulates the DHAR activity.

Results and Discussion

H_2O_2 Triggers the Formation of YAP1C Heterocomplexes in a Time- and Dose-Dependent Manner. To apply the YAP1-TAP approach, we synthesized a YAP1-cCRD construct with adapted codons for proficient expression in plants and mutated Cys620 and Cys629 to alanine and threonine (Fig. S1B and Table S1), retaining only the redox-active cysteine Cys598. Then, we fused this construct at its N terminus to a GS tag that combines two IgG-binding domains of protein G with a streptavidin-binding peptide (SBP), separated by a tobacco etch virus (TEV) protease cleavage site (13). The Cys598 of YAP1C-GS is essential for the formation of mixed disulfides with sulfenylated proteins (12). In addition, we constructed a similar control version, YAP1A-GS, in which

> ### Significance
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> When oxygen gets incompletely reduced, reactive oxygen species (ROS) are generated. These ROS molecules can harm the building blocks of the cell but are also important signaling molecules. Until now, the ROS language of the cell has not been understood and a clear view is needed on how the cell differentiates metabolic ROS noise from ROS that allows signaling, regulation, and protection. To address this question, we focused on *Arabidopsis thaliana* and identified the proteins that react with hydrogen peroxide on the thiol of the amino acid cysteine, which after reaction forms a sulfenic acid. The characterization of the plant sulfenome improves the understanding of important ROS signaling pathways.

PLANT BIOLOGY

all cysteines were mutated (Fig. S1*B* and Table S1). The cauliflower mosaic virus 35S promoter-driven constructs were transformed in *Arabidopsis* cell suspensions. Western blot analysis with a specific antibody complex to detect the G moiety of the tag [peroxidase-antiperoxidase (PAP) antibody complex] revealed that the yield of the two fusion proteins YAP1C-GS and YAP1A-GS is comparable and that they migrate as a single band at 35 kDa (Fig. S1*C*).

Previously, a 20-mM H_2O_2 treatment of *Arabidopsis* cells had been found to provoke oxidative stress signaling (14). We treated the transformed *Arabidopsis* cell suspension cultures with 0, 0.1, 1, 5, 10, and 20 mM H_2O_2 at the mid-log phase for 1 h. To block all free thiols, we extracted the soluble protein in the presence of iodoacetamide and *N*-ethylmaleimide (NEM) (Fig. S2) and analyzed the disulfide bond formation on a nonreducing Western blot with the PAP antibody complex. In untreated cells and in cells treated with 0.1 mM H_2O_2, YAP1C-GS migrated at 35 kDa, but H_2O_2 treatments ranging between 1 and 20 mM resulted in a proportional increase in the number of high-molecular weight YAP1C-GS complexes (Fig. 1*A*). The disulfide nature of the interactions has been proven by the disappearance of most of the high-molecular weight bands on a reducing Western blot (Fig. 1*A*). In cells producing YAP1A-GS, only the 35-kDa monomer is detected, even after a 20-mM H_2O_2 treatment, strengthening that Cys598 is essential for the YAP1-disulfide formation in plant cells under stress.

Next, we checked the transient dynamic character of the intermolecular disulfide bond formation by YAP1C in a time-course experiment. Cell cultures were pulse-treated with 1 mM H_2O_2 and harvested 5, 10, 30, 60, and 120 min after treatment. The YAP1C complexes were most abundant after 10 min (Fig. 1*B*), but almost undetectable after 2 h. This decreased number of mixed disulfide bonds between YAP1C and target proteins could result from the protection by a resolving cysteine present in the target proteins, from the activation of the reducing systems [glutaredoxin (Grx)/GSH/glutathione reductase (GR), or thioredoxin (Trx)/Trx reductase system], or from the proteolytic degradation of proteins inactivated by sulfonic acid formation. Taken together, these results indicate that a YAP1-based sulfenic acid-trapping methodology is a solid tool to study time- and dose-dependent H_2O_2 stress responses in plant cells.

Unique Sulfenylated Proteins Are Selectively Trapped with YAP1C-GS. To evaluate the early signaling events in the presence of 1 mM H_2O_2, we decided to focus on cysteine oxidation 10 min after stress. Protein extracts of cells containing YAP1C-GS and YAP1A-GS were purified by TAP (Fig. 2). Briefly, first, YAP1-GS complexes were captured on IgG-Sepharose. Second, after a TEV protease cleavage step, the YAP1 fused to the SBP tag together with several mixed disulfide complexes and all possible interacting proteins were eluted. In a subsequent SBP purification step, the mixed disulfide YAP1 complexes were enriched. The sulfenylated proteins were released by selective elution of the disulfide-bonded proteins with 5 mM DTT followed by a 20-mM desthiobiotin elution. The majority of interactors (41 of 46) eluted with DTT (Dataset S1). To guarantee a complete interactor recovery, we decided to use desthiobiotin for all of the TAP purifications.

Hereafter, we focused on the late-response sulfenome observed 1 h after an oxidative stress pulse with 0, 1, and 20 mM H_2O_2. In agreement with the increased mixed-disulfide complex formation that positively correlates with the H_2O_2 dose (Fig. 1*A*), we identified 4, 11, and 70 YAP1C-specific interactors (Fig. 1*C*, Table 1, and Dataset S1). Based on the background YAP1A datasets, 215 of the interactors can be regarded as nonspecific. Furthermore, almost all of the specific interactors contain at least one cysteine, except the histone superfamily protein, which might be a nondisulfide interactor of one of the YAP1C mixed-disulfide target proteins. The majority (8 of 11) of the specific interactors detected after treatment with 1 mM H_2O_2 are also present in the set of 70 proteins identified with 20 mM H_2O_2 (Fig. 1*C*). Nine of the 11 interactors derived from cells treated with 1 mM H_2O_2 for 1 h occur also at the 10-min early time point (Fig. 1*D*), emphasizing their sensitivity toward oxidation and suggesting an important function in oxidative stress sensing.

Functional Categorization of the *Arabidopsis* H_2O_2-Dependent Sulfenome. In the *Arabidopsis* sulfenome we detected 67 proteins that, until now, had not been classified as sensitive to H_2O_2 and, additionally,
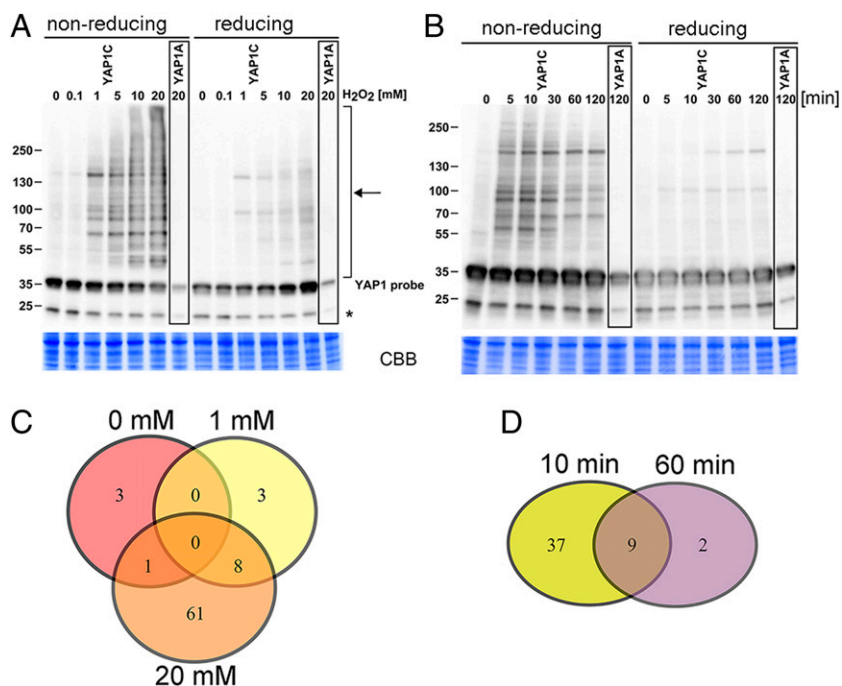
Fig. 1. Dose- and time-dependent formation of YAP1C-involving complexes. (*A*) Cell cultures overproducing the YAP1C/YAP1A-GS probe treated with 0, 0.1, 1, 5, 10, and 20 mM H_2O_2 for 1 h. Complexes (marked with an arrow) are visualized with the PAP antibody complex. The H_2O_2 concentration and the signal intensity clearly correlate. Treatment of protein samples with 50 mM *tris*(2-carboxyethyl)phosphine led to reduction of the complexes. (*B*) Cell cultures treated with 1 mM H_2O_2. The time course was taken after 0, 5, 10, 30, 60, and 120 min. The initial signal intensity peak returns to a near basal level after 120 min of treatment. The asterisk denotes an unknown protein recognized by the antiserum. (*C* and *D*) Schematic comparison of datasets identified after treatment of cultures with 0, 1, and 20 mM H_2O_2 for 1 h (*C*) and 1 mM H_2O_2 for 10 min (early response) and 1 h (late response) (*D*).
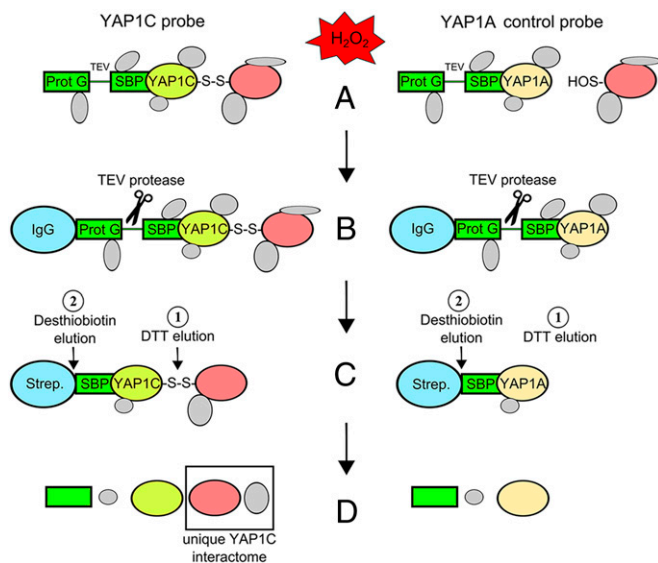
Fig. 2. Experimental set-up for in vivo identification of the *Arabidopsis* sulfenome. (*A*) Cell cultures overproducing the YAP1C/YAP1A probes treated with H$_2$O$_2$ as described (see main text). (*B* and *C*) Proteins isolated and subjected to a two-step purification procedure based on IgG-protein G and SBP affinity. Numbers indicate the sequence of elution steps. (*D*) LC-MS/MS analysis of proteins after elution. Comparison of interactors between the negative control probes of YAP1C and YAP1A potentially undergoing cysteine sulfenic acid (-SOH) formation under oxidative stress.

30 proteins that had previously been reported to have oxidative modifications, such as disulfides, S-glutathiolynation, S-nitrosylation, and sulfenic acids, and some to be Trx/Grx substrates (Table S2). A first step in the redox-dependent signaling pathway involves reversible sulfenic acid formation that later rapidly reacts with other thiols to form intra- or intermolecular disulfides, for example by S-glutathionylation. In a next step, specific redox enzymes, such as Trxs and Grxs, reduce these disulfides. The identification of S-glutathiolated proteins or Trx/Grx target proteins in the *Arabidopsis* sulfenome opens an interesting route for further investigation of the physiological consequence related to the pathways in which these enzymes operate.

Sixty-six of the proteins in the identified sulfenome could be functionally categorized: 13 are involved in signal perception and transduction, 19 in protein degradation, 7 in RNA-binding and translation, 6 in primary metabolism, 4 in hormone homeostasis, 5 in protein transport, 5 in amino acid metabolism, 7 are redox-related enzymes, and 31 have miscellaneous and unknown functions. For the complete list of interactors and the corresponding reference classification, see Table S2. When we focus on the 46 sulfenylated proteins within the first 10 min after the oxidative stress (Table 1), we find several signal perception and transduction proteins, of which almost one-fourth has proteasomal activities; moreover, some of several identified redox-related enzymes are detected at an early (10 min) as well as a late (1 h) oxidative stress response, whereas additional members of these functional classes are present within the late response (Table S2).

Signal Perception and Transduction. Three different MAPKs have been found to be sulfenylated: MAPK2, MAPK4, and MAPK7. The MAPK signaling cascades, including MAPKs, are integral parts of plant biotic and abiotic stress signaling pathways and are activated by H$_2$O$_2$ (15, 16). In yeast and human model systems, MAPKs are redox-regulated through upstream regulators and through direct cysteine oxidation events (17). A cysteine oxidation event in the human p38 MAPK had been shown to act as a

functional regulatory switch (18), whereas in plant MAPK modules, such a thiol modification has not yet been reported. The fast sulfenylation after an oxidative stress stimulus suggests that MAPK2, MAPK4, and MAPK7 could function as redox sensors downstream of a reactive oxygen species (ROS)-producing event. The MAPK activity is also controlled through dephosphorylation by redox-sensitive phosphatases (17). Sulfenylation of the catalytic nucleophilic cysteine leads to the inhibition of protein tyrosine phosphatases (PTPs) (19). The identified *Arabidopsis* AtPTP1 undergoes cysteine-dependent inhibition by H$_2$O$_2$ and negatively regulates the MAPKs (20), suggesting that the oxidation-dependent AtPTP1 inhibition might be a primary step in the oxidative stress response leading to MAPK signaling derepression (21). Furthermore, at least two members of the plant-specific SNF1-related protein kinase2 (SnRK2) family are rapidly sulfenylated. The plant stress response SnRK2 pathways are regulated by direct phosphorylation of various downstream targets, including the respiratory burst oxidase homolog RbohF and TFs, which are required for the expression of numerous stress response genes (22). Only recently, a redox-regulated rapeseed (*Brassica napus*) SnRK2 was shown to be involved in guard cell signaling (23).

Protein Degradation. Approximately 20% of the YAP1C-GS interactors are involved in proteolysis, with a clear enrichment for proteins participating in proteasome-mediated degradation, among which five control ubiquitination, such as the UBIQUITIN-CONJUGATING ENZYME27 (UBC27), two subunits of the Skp/Cullin/F-box (SCF) E3-ubiquitin ligase complex (ASK1 and ASK2), and the 3 and 5A subunits of the constitute photomorphogenic9 (COP9) signalosome. In the 26S proteasome, we identified the REGULATORY PARTICLE NON-ATPASE 12A (RPN12A) as a potentially H$_2$O$_2$-modified protein. Interestingly, RPN12A has been established as a cytoplasmic thioredoxin h (Trxh) target protein (24) that acts as a potential cross-talk point in cytokinin signaling (25). The removal of oxidatively damaged proteins is an important event in the stress responses (26), but oxidative modifications of the proteasome and the ubiquitin-proteasome system itself trigger changes in activities in such a manner that it manages both the removal of oxidized proteins and the adaptation of the cellular metabolism to the stress situation (27). In maize (*Zea mays*), sugar starvation-triggered oxidative stress leads to oxidative modifications of the 20S proteasome, modulating its proteolytic activity (28). In addition, our dataset includes a number of de-ubiquitinating enzymes (DUBs), such as UBIQUITIN C-TERMINAL HYDROLASE 3 (UCH3) and the ubiquitin-specific proteases UBP12, UBP13, and UBP24. Redox regulation of multiple ovarian tumor DUBs has been reported to occur via reversible sulfenylation of a catalytic cysteine residue (29), but until now this inhibition mode has not been described for plant DUBs.

Redox Proteins. At least four redox-related proteins have been detected: MONOTHIOL GLUTAREDOXIN17 (GRXS17), THIOREDOXIN-DEPENDENT PEROXIDASE1 (TPX1), GLUTAREDOXIN C2 (GRXC2), and a DHAR2. Other well-documented ROS-scavenging enzymes of plants, such as glutathione peroxidases, peroxiredoxins, and methionine sulfoxide reductases are not present in the list, possibly because of their highly specialized redox mechanisms that often involve resolving cysteines for the rapid conversion of sulfenic acids (2), hence impeding the formation of mixed disulfides with YAP1C. TPX1 had been identified as a target of cytosolic Trxh3 (30) and also in a subset of early-responsive redox-sensitive proteins (31). GRXC2 and GRXS17 function in early plant development during embryonic (32) and temperature-dependent postembryonic (33) growth, respectively, but their specific substrate proteins are unknown. DHAR2 was sulfenylated in both the early and late oxidative stress responses (Table S2). DHAR plays an important role in counterbalancing oxidative stress by

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Table 1. YAP1C-specific interactors within the first 10 min after the oxidative stress trigger

AGI code	Description	H₂O₂ dynamics after 1 h			Cys residues	Cys PTM
		0 mM	1 mM	20 mM		
Signal perception and transduction						
AT1G59580	Map kinase 2 (MPK2)		1	1	8	
AT4G01370	Map kinase 4 (MPK4)			1	8	
AT2G18170	Map kinase 7 (MPK7)		1	1	8	
AT3G50500	SNF1-related protein kinase 2-2 (SNRK2-2)				7	
AT1G60940	SNF1-related protein kinase 2-10 (SNRK2-10)			1	6	
AT2G18790	Phytochrome B (PHYB)				25	
AT2G43980	Inositol 1,3,4-trisphosphate 5/6-kinase 4 (ITPK4)			1	9	
AT1G71860	Protein tyrosine phosphatase 1 (PTP1)				7	
AT1G51690	Protein phosphatase 2A 55-kDa regulatory subunit (PP2A-b55α)		2	2	15	
AT2G46900	bHLH protein			2	6	
Protein degradation						
AT1G64520	Regulatory particle non-ATPase 12A (RPN12A)				4	Trxs target
AT1G75950	S phase kinase-associated protein 1 (SKP1)		2	2	3	
AT2G47790	CUL4-RING ubiquitin ligase complex subunit (GTS1)				12	
AT5G06600	Ubiquitin-specific protease 12 (UBP12)				11	
AT3G11910	Ubiquitin-specific protease 13 (UBP13)				10	
AT4G30890	Ubiquitin-specific protease 24 (UBP24)				3	
AT4G17510	Ubiquitin C-terminal hydrolase 3 (UCH3)		1		4	SOH
AT5G50870	Ubiquitin-conjugating enzyme 27 (UBC27)			1	4	
AT4G17830	Peptidase M20/M25/M40 family protein			1	8	S-SG
AT5G14250	COP9 signalosome subunit 3 (CSN3)			1	8	
AT5G42190	SKP-like protein 1B (SKP1B)		1	2	3	
Redox related						
AT1G65980	Thioredoxin-dependent peroxidase 1 (TPX1)	1			2	SNO
AT1G75270	Dehydroascorbate reductase 2 (DHAR2)			1	2	S-SG; Trxs target; S-S
AT4G04950	Monothiol glutaredoxin 17 (GRXS17)		2	2	6	
AT3G44190	FAD/NAD(P)-binding oxidoreductase family protein			2	3	
AT1G01800	NAD(P)-binding Rossmann-fold superfamily protein				4	
RNA Binding translation						
AT4G27000	RNA-binding family protein (ATRBP45C)				3	
AT4G31120	Protein arginine methyltransferase 5 (PRMT5)				12	
Primary metabolism						
AT1G78900	Vacuolar ATP synthase subunit A (VHA-A)			2	6	S-S
AT5G11670	NADP-malic enzyme 2 (NADP-ME2)			2	7	S-SG
Amino acid metabolism						
AT5G01410	Pyridoxine biosynthesis 1.3 (PDX1.3)			2	5	
AT5G17330	Glutamate decarboxylase 1 (GAD1)		1	2	7	
Protein transport						
AT3G56190	α-Soluble NSF attachment protein 2 (ASNAP)				8	
AT4G30550	γ-Glutamyl peptidase 3 (GGP3)				8	
Miscellaneous and unknown functions						
AT1G69800	Cystathionine β-synthase (CBS) protein			2	6	
AT2G42910	Phosphoribosyltransferase family protein		1	2	7	
AT3G07720	Galactose oxidase/kelch repeat superfamily protein				6	
AT3G16520	UDP-glucosyl transferase 88A1 (UGT88A1)			1	9	
AT3G53180	Nodulin/glutamine synthase-like protein (NODGS)				9	S-SG
AT3G63000	NPL4-like protein 1 (NPL41)				5	
AT4G14710	Acireductone dioxygenase 2 (ATARD2)				5	
AT4G29350	Profilin 2 (PFN2)				2	Trxs target
AT5G13050	5-Formyltetrahydrofolate cycloligase (5-FCL)				5	
AT5G17270	Protein prenyltransferase superfamily protein			2	20	
AT4G27450	Unknown protein				7	Trxs target
AT3G29280	Unknown protein				6	

Numbers indicate the occurrence of proteins in two independent experiments after 1 h.

catalyzing the regeneration of ascorbate, a major antioxidant in plants. In *Arabidopsis*, three isoforms are present: the mitochondrial DHAR1, the cytosolic DHAR2, and a chloroplastic DHAR3. In planta, perturbation of DHAR has been shown to limit ascorbate recycling, consequently influencing the rate of plant growth and leaf aging due to ROS-mediated damage (34).

DHAR2 Kinetics Are Affected by H₂O₂ Treatment. DHAR2 catalyzes the reduction of oxidized ascorbate with a concomitant oxidation of GSH to GSSG; therefore, it is one of the core enzymes of the GSH/ascorbate cycle that maintains reduced ascorbate pools (35). We recombinantly produced and purified His-tagged DHAR2. Recombinant DHAR2 eluted as a monomer from the

Ni^{2+}-immobilized metal-affinity column and migrated as a single band at 25 kDa on a SDS-PAGE gel. Its molecular weight was confirmed by MS with a total mass of 26,748 Da after loss of the N-terminal methionine (Fig. S3).

We analyzed the activity of recombinant DHAR2 by following the ascorbate formation in progress curves in function of time at 265 nm. The initial velocities were measured at varying dehydroascorbate (DHA) concentrations in a 5-mM excess of GSH. Plots of the initial velocities versus the DHA concentrations revealed a sigmoidal curve with a Hill factor of 2.65, indicative for positive cooperativity and hinting at the positive influence of the GSH binding at increasing DHA concentrations. For DHAR2, a $k_{cat}/K_{0.5}$ value of 9.3×10^5 M$^{-1} \cdot$s^{-1} was determined with a $K_{0.5}$ of 23.8 ± 1.2 μM, whereas for DHAR1 and DHAR3, K_M values of 260 μM and 500 μM had been reported (36). To understand the possible role of the cysteine thiols of DHAR2 in its catalytic cycle, we modified the free thiols with 1 mM iodoacetamide and oxidized DHAR2 with increasing concentrations of H$_2$O$_2$ (Fig. 3A). In both cases, the activity is affected, indicating that cysteines are essential for catalytic DHAR2 activity, as observed previously for DHAR1 (36).

DHAR2 Is Sulfenylated and S-glutathionylated on Its Nucleophilic Cysteine. DHAR2 contains two cysteines (Cys6 and Cys20). To prove the sulfenylation of DHAR2 by H$_2$O$_2$, we used 5,5-dimethyl-1,3-cyclohexadione (dimedone), a chemical compound that forms a thioether bond with the electrophilic sulfur atom of sulfenylated proteins (37). Previously, dimedone and its derivatives have successfully been applied to study sulfenylation in many important physiological pathways in various organisms and in recombinant proteins (38). We analyzed the H$_2$O$_2$-induced DHAR2 sulfenylation in the presence and absence of GSH on Western blots with antibodies that specifically recognize dimedone-tagged sulfenic acids (Fig. 3B). After a 30-min treatment with 100 μM H$_2$O$_2$ in the presence of dimedone, DHAR2 was sulfenylated. Remarkably, sulfenylation was lower in H$_2$O$_2$-treated than in nontreated samples, possibly because of a rapid rescue of sulfenic acids by resolving cysteines or overoxidation to sulfinic or sulfonic acids (2). In the presence of 1 mM GSH, the sulfenylation signal did not depend on the H$_2$O$_2$ treatment, suggesting that the majority of the formed sulfenic acid is unavailable for dimedone because a mixed disulfide is formed with GSH. To confirm this observation, we analyzed H$_2$O$_2$-treated DHAR2 in the presence of dimedone or GSH by LC-MS/MS (Table S3). After treatment with 100 μM H$_2$O$_2$ in the presence of dimedone, we blocked all free thiols. A tryptic digest revealed a dimedone adduct on the Cys20 peptide, resulting in a 138-Da mass increase of this peptide compared with the parent peptide (Fig. 3C), confirming that Cys20 is sulfenylated. Cys20 is also partially overoxidized to sulfonic acid (48-Da larger than the parent peptides). In contrast, in the absence of dimedone but in the presence of GSH, MS data clearly show S-glutathionylation at Cys20 (Fig. S4).

To test the 1-mM GSH protection on the DHAR2 activity, we added increasing concentrations of H$_2$O$_2$ to DHAR2 in the presence of 1 mM GSH and determined the initial velocities (Vi) of the progress curves (Fig. 3A). The Vi of the GSH-pretreated DHAR2 sample is slightly higher than that of the nontreated sample, which is, at least, partially sulfenylated after purification (Fig. 3B). At low H$_2$O$_2$ concentrations a disulfide is formed between C6 and C20 together with partial overoxidation of C20 (Table S3), without affecting the DHAR2 activity (Fig. 3A). However, at higher H$_2$O$_2$ concentrations (1 and 5 mM), sulfonic
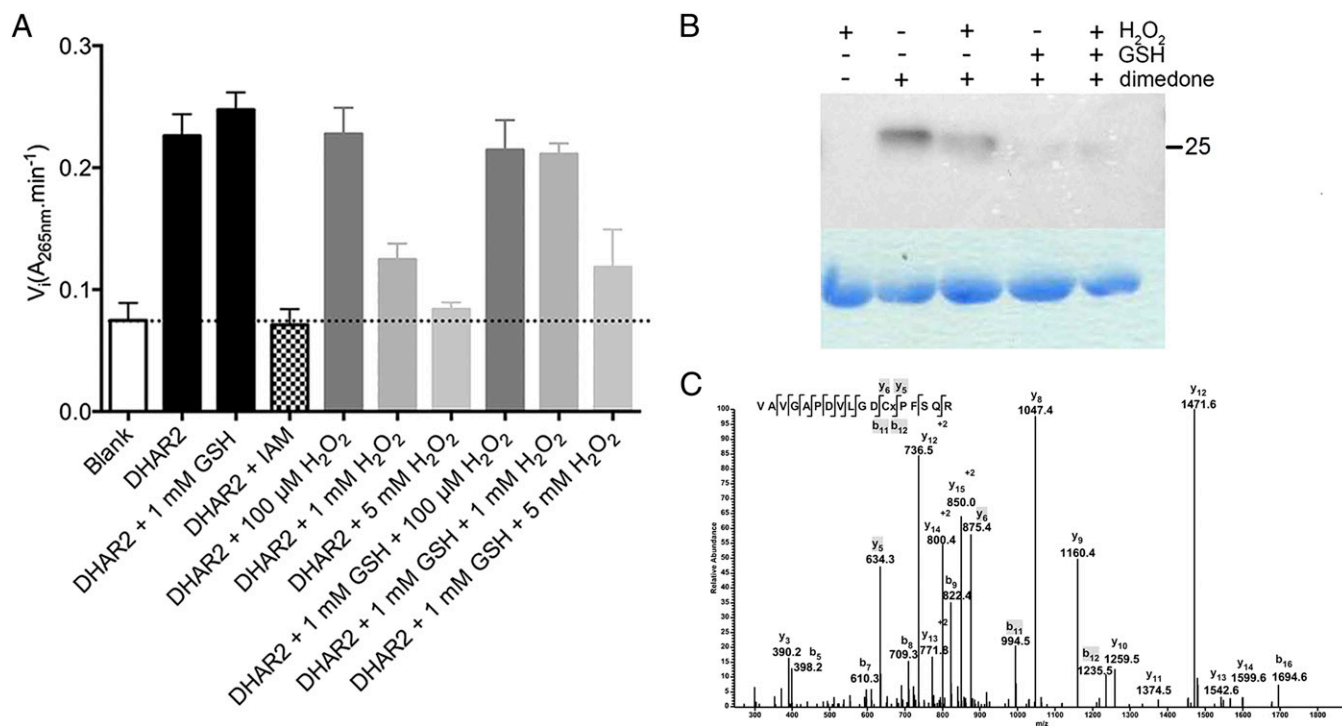
Fig. 3. Requirement of DHAR2 cysteines and GSH protection against overoxidation. (A) Mean initial velocities $\pm$ SD of three independent measurements of DHAR2 determined on progress curves under different conditions. The reaction was started under V_{max} conditions. (B) Dimedone labeling of DHAR2-SOH in vitro. DHAR2 (20 μM) nontreated or treated with 1 mM GSH either not or incubated with 100 μM H$_2$O$_2$ in the presence or absence of dimedone (1 mM). DHAR2-SOH formation was analyzed by immunoblot with an anticysteine sulfenic acid antibody. (C) Identification of the dimedone modification Cys20 of DHAR2. The LC-MS/MS spectrum shows data obtained from a +2 parent ion with m/z 935.5. The cysteine residue corresponds to a dimedone-modified sulfenic acid, which produces a +138-Da mass increment.

PLANT BIOLOGY

acid is more probably formed (Table S3) and associated with decreased activity (Fig. 3*A*). This progressive oxidation is further supported by the fact that GSH (1 mM) rescues the 1-mM H_2O_2 sulfenylation of DHAR2 from overoxidation, whereas at 5 mM H_2O_2, the DHAR2 activity could only partially be rescued by GSH (Fig. 3*A*). Possibly, the overoxidation rate is too fast for 1 mM GSH to react with the sulfenic acid to recover all activity (Fig. 3*A*). Thus, in the absence of substrate, S-glutathionylation occurs after sulfenylation of Cys20 as a reversible protection mechanism, recovering DHAR2 activity, which results in increased initial velocities. Taking these data together, we show that the nucleophilic Cys20 in DHAR2 is vulnerable to oxidation and becomes sulfenylated under H_2O_2 stress. The formation of a Cys20–Cys6 disulfide bond or the Cys20 S-glutathionylation might protect DHAR2 against irreversible overoxidation.

Materials and Methods

A full discussion of materials and methods can be found in *SI Materials and Methods.*

***Arabidopsis* Suspension Cultures.** The *Saccharomyces cerevisiae* YAP1-coding region corresponding to the Asn565-to-Asn650 region was codon optimized for expression in *A. thaliana* (L.) Heynh., synthesized, and mutated to create the YAP1C and YAP1A probes, as described previously (12).

Stress Treatments. Mid-log phase cell cultures expressing the YAP1C/YAP1A N-terminal GS-tag fusions were treated with 0.1, 1, 5, 10, and 20 mM H_2O_2. Cells were harvested after 1 h.

Protein Extractions, Western Blot Analysis, and TAP. Plant material was ground on ice in the presence of sand and TAP extraction buffer (39). For the Western blot analysis, soluble proteins were separated on SDS-PAGE gradient gel (Bio-Rad), blotted, and hybridized with a 1:5,000 dilution of PAP-soluble complex (Sigma-Aldrich) to detect the GS tag. The TAP protocol was adapted to allow the use of the redox-active baits YAP1A-GS and YAP1C-GS.

For details on design and cloning of YAP1A-GS and YAP1C-GS and on cloning and purification of recombinant DHAR2, DHAR2 activity/inhibition assays and in vitro sulfenic acid labeling, and MS on DHAR2, see *SI Materials and Methods*. PCR primers used for cloning of probes are listed in Table S4.

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