Faculty of Science and Bio-engineering Sciences,
Department of Bio-engineering Sciences
Vrije Universiteit Brussel (VUB), Belgium

Faculty of Sciences, Department of Plant
Biotechnology and Bioinformatics
Ghent University (UGent), Belgium

Mining the reactive-oxygen-species signal transduction proteins in *Arabidopsis thaliana*: a sulfenomic approach

Mosammat Salma Akter

Thesis submitted in partial fulfillment of the requirements for the degree of

Doctor (PhD) in Bio-engineering Sciences | Doctor (PhD) in Sciences, Biotechnology

Academic year: 2014-2015

Promoter:
Prof. Dr. Joris Messens
Faculty of Science and Bio-engineering Sciences
Department of Bio-engineering Sciences
Vrije Universiteit Brussel (VUB), Belgium

VIB Structural Biology Research Center
(SBRC)
Oxidative Stress Signaling Lab
Pleinlaan, 2 B-1050 Brussel, Belgium

Co-Promoter:
Prof. Dr. Frank Van Breusegem
Faculty of Sciences, Department of Plant
Biotechnology and Bioinformatics
Ghent University (UGent), Belgium

VIB Department of Plant Systems Biology
(PSB)
Oxidative Stress Signaling Lab
Technologiepark 927, 9052 Ghent, Belgium

This work was conducted at the Structural Biology Research Center (SBRC) and Department of Plant Systems Biology (PSB) of the Flanders Institute for Biotechnology (VIB), and at the Vrije Universiteit Brussel (VUB) and Ghent University (UGent).

Mosammat Salma Akter is indebted to an Erasmus Mundus External Cooperation Window predoctoral fellowship.

This work was supported by grants from Vrije Universiteit Brussel, and from Ghent University Multidisciplinary Research Partnership ["Ghent BioEconomy" (Project 01MRB 510W)] and Bijzondere Onderzoeksfonds (BOF 01J11311)]; the Interuniversity Attraction Poles Program (Grant IUAP VII/29), initiated by the Belgian State, Science Policy Office; Research Foundation-Flanders (Projects G.0D.79.14N and G.0038.09N); the European Cooperation in Science and Research (COST Action BM1203/EU-ROS); the FWO research grant (G. 0D70.149); the VUB grant (HOA 22).

MEMBERS OF THE EXAMINATION BOARD

Prof. Dr. Joris Messens (Promoter)
Faculty of Science and Bio-engineering Sciences
Department of Bio-engineering Sciences
Vrije Universiteit Brussel, Belgium

Prof. Dr. Frank Van Breusegem (Co-Promoter)
Faculty of Sciences
Department of Plant Biotechnology and Bioinformatics
Ghent University, Belgium

Prof. Dr. ir. Geert ANGENON (Voorzitter)
Faculty of Science and Bio-engineering Sciences
Department of Bio-engineering Sciences
Vrije Universiteit Brussel, Belgium

Prof. Dr. Henri De Greve (Secretaris)
Faculty of Science and Bio-engineering Sciences
Department of Bio-engineering Sciences
Vrije Universiteit Brussel, Belgium

Prof. Dr. Kris Gevaert
Faculty of Medicine and Health Sciences
Department of Biochemistry
Ghent University, Belgium

Prof. Dr. Geert De Jaeger
Faculty of Sciences
Department of Plant Biotechnology and Bioinformatics
Ghent University, Belgium

Prof. Dr. Jean-Pierre Hernalsteens
Faculty of Science and Bio-engineering Sciences
Department of Biology
Vrije Universiteit Brussel, Belgium

Prof. Dr. Lars Leichert
Faculty of Medicine
Institute for Biochemistry and Pathobiochemistry
Microbial Biochemistry
Ruhr-Universität Bochum, Germany

Prof. Dr. Jean-Pierre Jacquot
Faculty of Sciences and Technologies
University of Lorraine
Nancy, Lorraine, France

Table of contents

Summary

Under stress, living cells produce 'reactive oxygen species' (ROS), which may transmit signal messages into biological defense responses through the formation of sulfenic acids on cysteines (-SOH) in signal transducing proteins. Profiling the sulfenome, the set of proteins with at least one sulfenic acid modified cysteine residue, has emerged as one of the central strategic approaches to understand and identify new ROS signal perception mechanisms. To get insight into this perception process in plants, we focused on the proteomic identification of the sulfenome in *Arabidopsis thaliana* cell suspensions exposed to H_2O_2 stress.

In the first part of this research work, we optimized cytoplasmic *in vivo* trapping of sulfenylated proteins 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. As such, we identified 97 cytoplasmic sulfenylated proteins with mass spectrometry, which might be involved in H_2O_2 signal transduction, redox homeostasis, and other metabolic pathways. The *in vivo* YAP1-based trapping of sulfenylated proteins was validated by a targeted *in vitro* analysis of DEHYDROASCORBATE REDUCTASE 2 (DHAR2). We found that 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.

In the second part of this study, we report the first successful application of a dimedone-based chemical probe (DYn-2) to map the sulfenome in plants at subcellular level. We optimized an *in vivo* trapping method of sulfenic acids in H_2O_2 stressed Arabidopsis cell suspension using the DYn-2 probe. With mass spectrometry, we identified 226 sulfenylated proteins from different cellular compartments including the cytoplasm (123), plastid (68), mitochondria (14),

nucleus (10), endoplasmic reticulum & Golgi (7) and the peroxisomes (4). About one fifth of these proteins had already been reported as sulfenylated proteins in plants, thereby technically validating this approach. Remarkably, with this DYn-2 trapping approach, we identified 123 sulfenylated proteins never reported before.

This thesis provides two comprehensive methods for mining the sulfenome in plants using two different trapping approaches. With the identification of proteins from the sulfenome, we have opened new directions to understand complex redox signal perception and transduction mechanisms in plants. Future research has to address the detailed redox properties and the mode of action of the newly identified proteins in order to assess their role in oxidative stress signal transduction events.

Samenvatting

Om zich te verdedigen tegen omgevingsstress produceren cellen reactieve zuurstofmoleculen (RZ). Deze RZ zijn signaalmoleculen die de biologische afweermechanismen van de cel op de hoogte te brengen van de stress. Dat gebeurt onder meer doordat cysteïnes van signaaleiwitten oxideren tot sulfeenzuren (-SOH). De analyse van het *sulfenoom*, de verzameling eiwitten waarin minimaal één cysteïneresidu geoxideerd is tot sulfeenzuur, laat zich bijgevolg gebruiken als een geschikte strategie om nog onbekende mechanismen van RZ-signaalontvangst te identificeren en te leren begrijpen. Om een inzicht te krijgen in de werking van dit signaalontvangstsysteem bij planten hebben we ons onderzoek toegespitst op de identificering van het eiwitsulfenoom bij de zandraket *Arabidopsis thaliana.* We gebruikten daarvoor celsuspensies die we aan H_2O_2-stress blootstelden.

In het eerste deel van dit werk bespreken we de optimalisering van de cytoplasmatische *in-vivo* trapping van gesulfeneerde eiwitten met behulp van een genetisch construct bestaande uit een fusie van het C-terminusgebied van de op AP-1-gelijkende transcriptiefactor van bakkersgist (*Saccharomyces cerevisiae*) (YAP1) met een dubbel zuiveringshandvat. Zo identificeerden we met behulp van massaspectrometrie 97 cytoplasmatische gesulfeneerde eiwitten die mogelijk betrokken zijn bij de signalering van H_2O_2, de redoxhomeostase en andere stofwisselingsroutes. We valideerden de op YAP1 gebaseerde *in-vivo* trapping van gesulfeneerde eiwitten door middel van gerichte *in-vitro* analyse van DEHYDROASCORBAATREDUCTASE 2 (DHAR2). We achterhaalden dat het nucleofiele cysteïne van DHAR2 gereguleerd wordt middels een sulfeenzuurafhankelijke schakelaar die tot S-glutathionylatie aanleiding geeft. Deze eiwitmodificatie beschermt het eiwit tegen oxidatieve schade.

In het tweede deel van dit werk melden we het voor het eerst met succes toegepast gebruik van een chemische dimedonesonde (DYn-2) bij het in kaart

brengen van het plantensulfenoom op subcellulair niveau. We optimaliseerden een *in-vivo* trapping methode voor sulfeenzuren in Arabidopsis-celsuspensies onder H_2O_2- stress, gebruikmakend van de DYn-2-sonde. We identificeerden met behulp van massaspectrometrie 226 gesulfeneerde eiwitten, afkomstig van diverse celonderdelen waaronder het cytoplasma (123), de plastiden (68), de mitochondriën (14), de kern (10), het endoplasmatisch reticulum & Golgi-apparaat (7) en de peroxisomen (4). Een vijfde van deze eiwitten stond voorheen al bekend als gesulfeneerd planteneiwit, hetgeen de door ons gekozen aanpak valideert. Op te merken valt dat we met de DYn-2-trappingtechniek 123 voorheen onbekende gesulfeneerde eiwitten hebben geïdentificeerd.

Deze thesis schuift twee alomvattende methoden voor ontginning van het plantensulfenoom naar voren, daarbij gebruikmakend van twee verschillende detectiemethoden. Door deze sulfenoomeiwitten te hebben geïdentificeerd, hebben we een nieuwe weg ingeslagen waarlangs de ingewikkelde redoxsignaalontvangst- en overdrachtsmechanismen bij planten kunnen worden begrepen. Vervolgonderzoek moet duidelijk maken wat de precieze redoxeigenschappen en het werkingsmechanisme zijn van de nieuw geïdentificeerde eiwitten, zodat hun rol bij de signaaloverdracht bij oxidatieve stress kan worden opgehelderd.

Chapter 1

Introduction

Chapter 1

Introduction

Two review articles will be published from a modified version of this chapter as

1. **Salma Akter**, Cezary Waszczak, Jingjing Huang, Silke Jacques, Kris Gevaert, Frank Van Breusegem and Joris Messens (2014). Cysteines under ROS attack in plants: a proteomics view (review paper submitted to J Exp Bot, Journal of Experimental Botany)

2. Cezary Waszczak, **Salma Akter**, Jingjing Huang, Silke Jacques, Kris Gevaert, Frank Van Breusegem and Joris Messens (2014). Oxidative post-translational modifications of cysteine residues in plant signal transduction (review paper submitted to J Exp Bot, Journal of Experimental Botany)

Author contributions: Mosammat Salma Akter wrote this chapter with the help of Cezary Waszczak, Jingjing Huang, Silke Jacques, Kris Gevaert, Frank Van Breusegem and Joris Messens.

Chapter 1

Introduction

1.1 Abstract

Plants accumulate reactive oxygen species (ROS) during stress conditions. ROS have a dual face as they can both damage cellular components, inducing cell death, and act as signal molecules (Miller *et al*., 2010; Huang *et al*., 2012). Despite progress over the last years, the understanding of ROS signaling is still far of being achieved. Exploring which and how proteins sense ROS and transduce these stimuli into downstream biological effects is one of the major challenges in redox biology. One way for proteins to sense ROS is by targeting the sulfur containing amino acids cysteine and methionine. The reactivity of Cys residues toward ROS and the possibility to be present in different oxidation states permit them to appear on the crossroad of highly dynamic oxidative events. Nowadays, it is a well-recognized concept that profiling of ROS modified protein thiols (-SH), can serve as a key way to discover ROS signal transduction pathways. This PhD thesis focuses on mining the Arabidopsis sulfenomes, the first oxidation products of cysteine residues, which might sense and transmit ROS signals under oxidative stress. The introductory chapter summarizes the background information to understand the research gap, which is aimed to address by this PhD work. First, the reader will be introduced to ROS signaling in plants and the fate of cysteine residues under ROS attack. Next, the involvement of ROS-mediated cysteine posttranslational modifications (Cys Ox-PTMs) in different signaling pathways in plants will be described. Further, we give an overview of the proteomic techniques applied to identify different redox modified cysteines in plants. Special focus goes to the identification of sulfenylated proteins, which have the potential to be involved in plant signal transduction.

1.2 ROS in plants

Reactive oxygen species (ROS) production is considered as an unavoidable metabolic consequence of aerobic life. As part of their aerobic life, plants produce ROS; mainly superoxide $O_2^{\bullet -}$, hydroxyl radicals OH• and hydrogen peroxide H_2O_2 in subcellular compartments like chloroplast, mitochondria, peroxisomes etc. Importantly, the level of ROS production is enhanced in response to different abiotic and biotic stress factors. Plants appear to be tolerant to ROS due to well-controlled antioxidant systems that maintain a steady redox state. In contrast to earlier views, ROS production is not necessarily a symptom of cellular dysfunction. It is rather the fine tuned balance of the cellular redox status that allows ROS to function as a necessary signal leading to a wide range of physiological changes and defense responses. Genetic evidences suggest that ROS influence the expression of several genes, which indicates that ROS act as a messenger in regulating stresses (Neill *et al*., 2002; Laloi *et al*., 2004).

Stress mediated ROS signaling is highly integrated with, and regulated through hormonal signaling networks. In response to drought stress, plants produce abscisic acid (ABA) which induces ROS production in guard cells and triggers a signaling cascade resulting in stomata closure and the reduction of water loss (Pei *et al*., 2000; Kwak *et al*., 2003). Ethylene is a gaseous plant hormone, able to control the closure of stomata (Desikan *et al*., 2006). Perception system of ethylene triggers ROS accumulation, which is essential for the ethylene-induced stomata closure (Desikan *et al*., 2006). Brassinosteroids (BRs) play important roles in the complex network of plant signal transduction regulating plant growth and development. Exogenous BRs can also improve plant tolerance to abiotic and biotic stress and ROS play a critical role in BR-induced stress tolerance (Xia *et al*., 2009). Elevation of ABA and BR levels results in increased ROS production together with increased tolerance against a subset of stresses (Xia *et al*., 2009; Zhang *et al*., 2009). Recently, the role of BR in the regulation of stomatal opening or closure was studied and it was reported that ROS production is essential in BR-induced stomata movements (Xia *et al*., 2014).

Apart from abiotic stress, ROS are proposed to coordinate plant defense responses following successful pathogen recognition (Apostol *et al*., 1989; Levine *et al*., 1994). Similar to ROS, salicylic acid (SA), a signaling molecule, is involved in pathogen related defense responses (Durrant and Dong, 2004). Interestingly, ROS act synergistically in a signal amplification loop with SA to establish systemic defenses

(Torres *et al*., 2006). Moreover, SA accumulation can also down-regulate ROS scavenging systems that, in turn, can contribute to the increased ROS levels leading to pathogen recognition (Klessig *et al*., 2000). In addition, ROS signaling has been linked with nitric oxide signaling in trigger the pathogen-induced cell death (Delledonne et al., 1998; Delledonne et al., 2001).

1.3 Cysteines under ROS attack

ROS interact with multiple signaling pathways and transmit signals through the oxidative modifications of signaling proteins. Cysteine is one of the sensitive targets of ROS, as it contains the electron-rich sulfur atom appearing in a wide range of oxidation states that make Cys residues the major sites of oxidative modifications within proteins (Davies, 2005). In cysteine, the thiol group (-SH) represents the -2 oxidation state of the sulfur atom, which is the fully reduced form. The reactivity of the sulfur is further enhanced and becomes a nucleophile in the deprotonated form of the thiol, known as the thiolate anion (S^-) (Roos and Messens, 2011). Initially, ROS result in the formation of reversible oxidation of a cysteine residue, known as a sulfenic acid (-SOH) (reaction 1, Figure 1.1). This transient oxo-form of a cysteine is highly reactive in which the oxidation state of the sulfur atom goes from −2 to 0, and has been shown to function as a redox sensor involved in many physiological pathways, by affecting the enzymatic and metal binding activities of crucial signaling proteins, and the activity of transcription factors modulating gene expression (Ma *et al*., 2007; Poole and Nelson, 2008; Reddie and Carroll, 2008; Roos and Messens, 2011; Oger *et al*., 2012). In the case that the structural determinants in the protein environment are not stabilizing the sulfenic acid, it can undergo an additional reaction with H_2O_2 and form sulfinic acid (SO_2H) (reaction 2, Figure 1.1) and sulfonic acid (SO_3H) (reaction 3, Figure1.1), although the rate of these reactions is slower than that of the reaction of H_2O_2 with a thiolate (Hugo *et al.*, 2009; Hugo *et al.*, 2014). The oxidation state of the sulfur atom goes from 0 to +2 in sulfinic acid and +4 in sulfonic acid. In most cases, overoxidation is irreversible and it will lead to protein degradation (Roos and Messens, 2011). However, the reversibility of a sulfinic acid ($R\text{-}SO_2H$) modification has been debated. For example, it has been shown that an

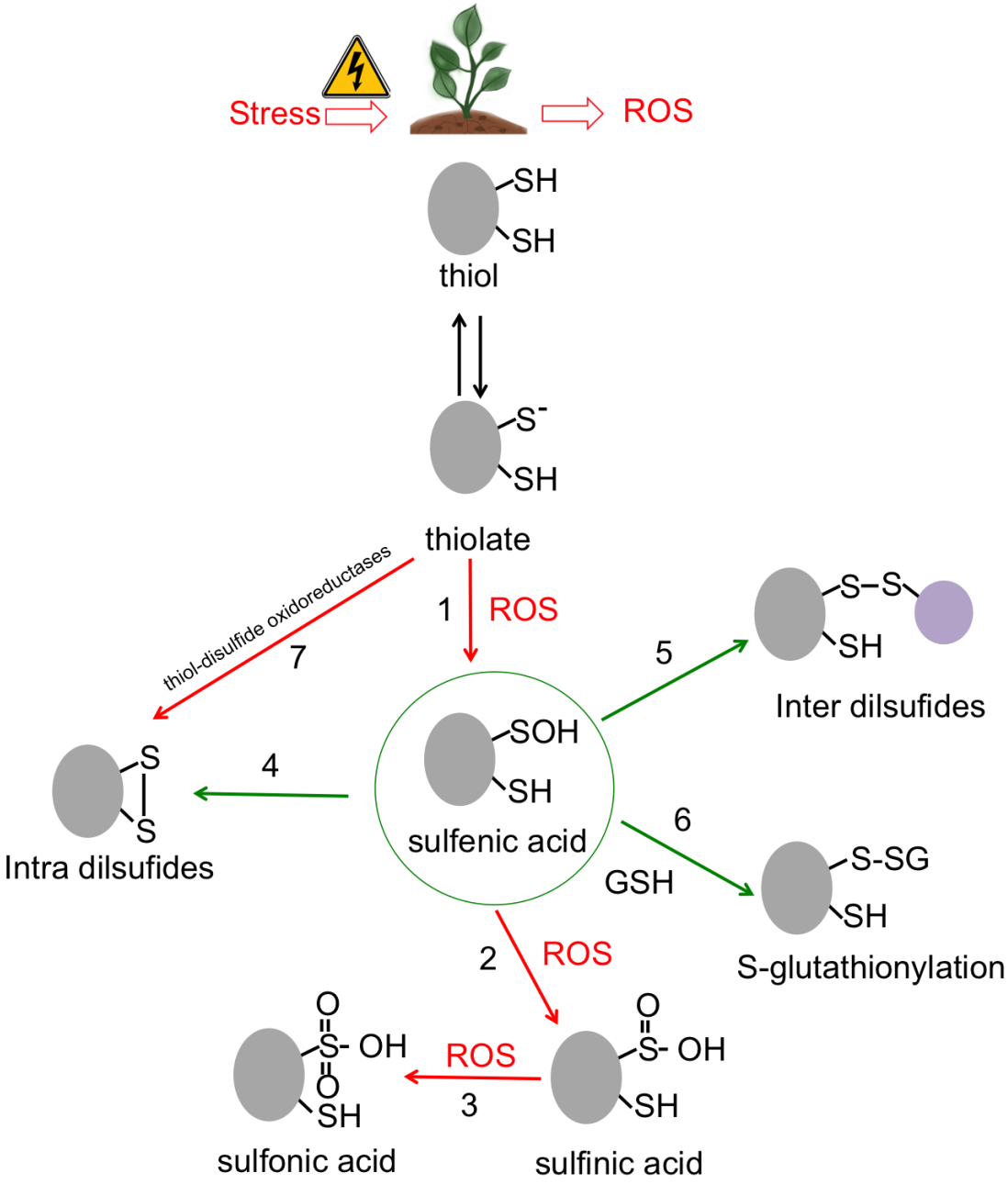


Figure 1.1 The fate of cysteine exposed to ROS. Exposure of redox-sensitive cysteine residues to ROS leads to reversible sulfenic acid formation (1). Unless sulfenic acids are stabilized within the protein environment, they readily react with nearby thiols of the same protein to form intramolecular disulfide bonds (4), or with thiols from other proteins (5) or with glutathione (GSH) to form mixed disulfide bonds (6). Disulfide bond formation might occur via a direct thiol disulfide exchange (7). In the presence of high levels of ROS, overoxidation to sulfinic (2) and irreversible sulfonic (3) acid can occur. The green arrow represents the protection of the sulfenic acid and the red arrow represents the oxidation of the thiolate or overoxidation of the sulfenic acid. Modified from (Roos and Messens, 2011).

ATP-dependent sulfiredoxin (Srx) enzyme was capable of reducing R-SO_2H in plant cells (Rey *et al.*, 2007). Since thus far the only known substrates of *At*Srx are chloroplast 2-Cys peroxiredoxins and mitochondrial PrxIIF (Rey *et al*., 2007; Iglesias-Baena *et al*., 2011), the reduction of R-SO_2H cannot be regarded as a general rule.

The kinetic stability of the sulfenic acid strongly depends on the presence of nearby cysteines and on the accessibility to low molecular weight thiols, like the tri-peptide glutathione (GSH). The electrophilic sulfenic acid will then react and form an intramolecular (reaction 4, Figure1.1) within one protein, or intermolecular disulfide (reaction 5, Figure 1.1) between proteins, or a mixed disulfide (i.e. S-glutathionylation) (reaction 6, Figure 1.1). When two thiols are oxidized to form a disulfide bond, sulfur reaches the oxidation state -1. Disulfide bond formation might occur via a direct thiol disulfide exchange (reaction 7, Figure1.1). In Arabidopsis, the thylakoid-located LUMEN THIOL OXIDOREDUCTASE 1 (LTO1/ AtVKOR-DsbA) can catalyze disulfide bond formation in PsbO (PS II OXYGEN-EVOLVING COMPLEX) and FK506 BINDING PROTEIN 13 (AtFKBP13) proteins, and in addition LOW QUANTUM YIELD OF PHOTOSYSTEM II 1 (LQY1) and SNOWY COTYLEDON 2 (SCO2) exhibit protein disulfide isomerase activity (Feng *et al*., 2011; Kieselbach, 2013; Lu *et al*., 2013).

The disulfides are reversible as they can be reduced by the GSH/Grx system or the Trx/thioredoxin reductase (TxR) systems (Collet and Messens, 2010; Messens and Collet, 2013). Plants are equipped with a complex network of these redox systems, for example, Arabidopsis genome encodes for 44 Trx and 50 Grx proteins (Meyer *et al*., 2012). These Trx and Grx proteins also need to be reduced, the major reducing equivalents are coming from NADPH, which is known to be the general electron donor for both Trx and Grx reducing systems. The regulation mechanisms of both systems are described below:

1.3.1 Grx system. Glutaredoxins (Grxs) are small ubiquitous glutathione-dependent oxidoreductases that play a crucial role in response to oxidative stress (Fernandes and Holmgren, 2004; Buchanan and Balmer, 2005). According to the amino acid sequence motif at their active sites, plant Grxs fall into three groups, the CPYC, CGFS, and CC-type classes (Rouhier *et al*., 2004). The CPYC and CGFS classes are

common to all prokaryotes and eukaryotes, whereas the CC-type class is specific for land plants (Rouhier *et al*., 2006; Xing *et al*., 2006). Grx undergoes thiol disulfide exchange reactions with a mono- or di-thiol mechanism (Figure 1.2) (Herrero and de la Torre-Ruiz, 2007; Lillig *et al*., 2008). The monothiol mechanism utilizes only the N-terminal cysteine (nCys) of CPYC motif to reduce S-glutathionylated proteins. The first step of this mechanism is a thiol disulfide exchange reaction between the nCys and S-glutathionylated protein resulting in S-gluathionylation of the nCys and releasing the target protein in its thiolate state. Further, another molecule of GSH releases the nCys of Grx and forms a GSSG complex with the first GSH.

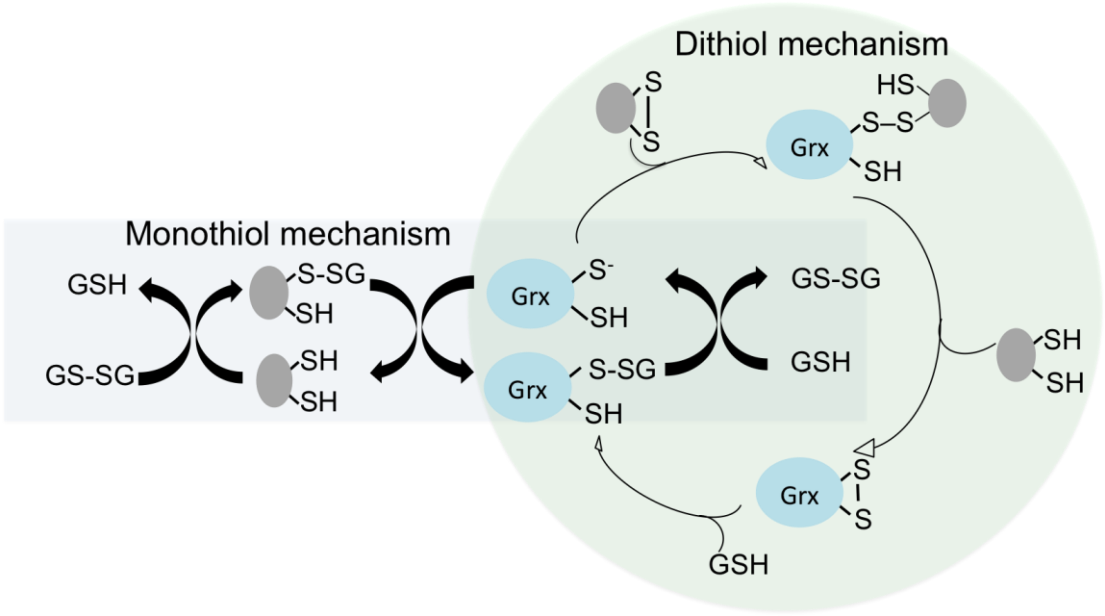
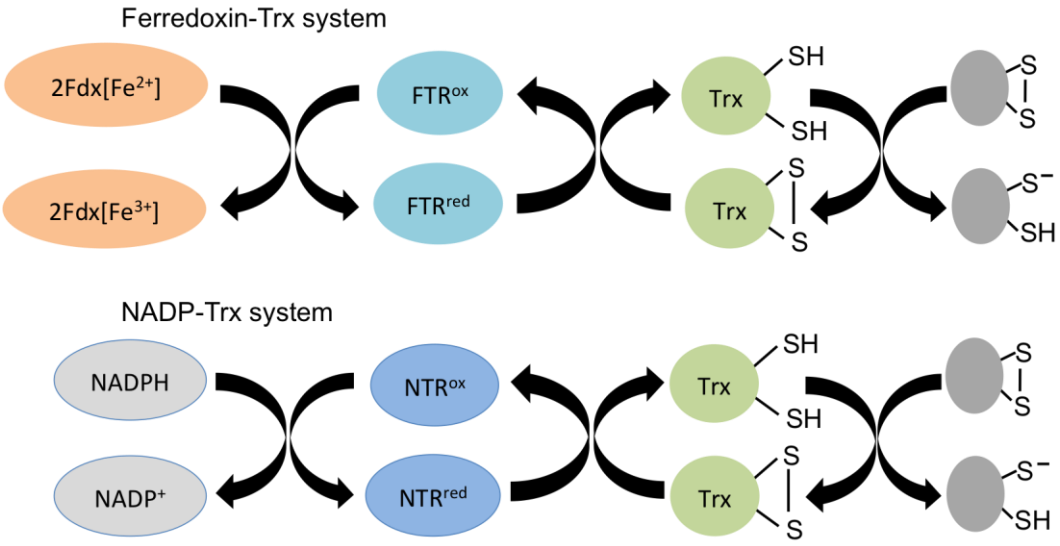


Figure 1.2 Schematic presentations of the monothiol and dithiol mechanisms of Grx system in plants. The monothiol mechanism (rectangular shaded area) utilizes single cysteine residue (N-terminal cysteine of CPYC motif) to reduce S-glutathionylated proteins while the dithiol mechanism (circular shaded area) utilizes both active site cysteines (Rouhier *et al*., 2008).

The dithiol mechanism utilizes both active site cysteines of CPYC motif, nCys and C-terminal cysteine (cCys). The first step of this mechanism is a thiol disulfide exchange reaction between the nCys and the target protein disulfide resulting in an intermolecular disulfide between the nCys of Grx and a cysteine of that target protein. Next, the cCys performs a nucleophilic attack on the nCys of Grx resulting in an intramolecular disulfide between both active site cysteines while releasing the

reduced target protein. In the following steps, two molecules of GSH reduce this oxidized form of Grx. First, a thiol disulfide exchange reaction between GSH and oxidized Grx yields a mixed disulfide between the GSH and nCys of Grx, thus releasing cCys. This mixed disulfide is reduced in the following exchange reaction with another GSH forming GSSG and reduced Grx. The GSSG formed in both reaction mechanisms is reduced by the NADPH-dependent glutathione reductase. However, the dithiol mechanism occurs probably less frequent than the monothiol mechanism (Meyer *et al*., 2012).

1.3.2 Trx system. Trxs are able to reduce disulfide bridges by using the dithiol mechanism. In plants, two redox systems of Trx are found in different cell compartments (Figure 1.3).



igure 1.3 Schematic presentations of the two kinds of Trx dependent reduction systems of the disulfide modification in plants. One, the ferredoxin-Trx system located in chloroplasts is comprised of ferredoxin (Fdx), ferredoxin-thioredoxin reductase (FTR) and Trx. The other NADP-Trx system is localized both in the cytosol and in the mitochondria. This system is comprised of NADP-Trx reductase (NTR), NADPH and Trx-h in the cytoplasm or in the mitochondria (Montrichard *et al*., 2009).

The ferredoxin-Trx system located in chloroplasts is comprised of ferredoxin (Fdx), ferredoxin-thioredoxin reductase (FTR) and Trx-f, Trx-m (Schürmann and Buchanan, 2008), Trx-x (Mestres-Ortega and Meyer, 1999) and Trx-y (Lemaire *et al*., 2003).

Electrons released in the oxidation of H_2O are transferred along the photosynthetic electron transfer chain via Photosystems I and II to ferredoxin (Fdx) which reduces either Trx via ferredoxin-thioredoxin reductase (FTR) or NADP via Fdx:NADP reductase (FNR) (Meyer *et al.*, 2009). The NADP-Trx system is localized both in the cytosol and in the mitochondria. This system is comprised of NADP-Trx reductase (NTR), NADPH and Trx-h in the cytoplasm or Trx-h/Trx-o in the mitochondria.

1.2.3 Cysteine reacts differentially to ROS. ROS-mediated modifications do not happen to all cysteine residues in an individual protein, and different thiol-proteins react with ROS at different rates (Marinho *et al.*, 2014). Table 1.1 represents examples of the rate constants for the reactions between H_2O_2 and thiol compounds.

Table 1.1 Rate constants for the reactions between H_2O_2 and several thiol-proteins at pH 7.4 −7.6 at 37ºC unless noted otherwise

Thiol compounds	Rate constant ($M^{-1}s^{-1}$)	References
GSH	0.87	(Winterbourn and Metodiewa, 1999)
Thioredoxin	1.05	(Goldman *et al*., 1995)
PTP1B	20	(Denu and Tanner, 1998)
Cdc25B	160	(Sohn and Rudolph, 2003)
GAPDH	500	(Little and O'brien, 1969)
PerR	1.0×10^5, pH 7.0	(Lee and Helmann, 2006)
Peroxiredoxin-2	1.0×10^7, 20 -25ºC	(Peskin *et al*., 2007)
Peroxiredoxin-5	3.0×10^5, 20 -25ºC	(Trujillo *et al*., 2007)

The subtle structural environment within a protein will determine the reactivity and the final fate of the cysteine, and as such also the function of the respective protein. Crucial is the kinetic stability of the first oxidation state of the Cys residue, the sulfenic acid (Figure 1.1), which makes that the Cys-SOH can be regarded as the central PTM on the crossroad toward different destinations. One key factor that determines this reactivity is the pK_a of the cysteine (Roos *et al*., 2013). In fact, the structural environment of a protein will determine the pK_a of a specific cysteine, and as such the balance between the thiol (SH) and the thiolate anion (S^-) state of the cysteine (Roos and Messens, 2011). While free cysteine has a pK_a of 8.3-8.5 *(*Tajc *et al*., 2004; Luo *et al.*, 2005), the most oxidation sensitive Cys residues described thus far have a p$K_a \leq 3.5$. For instance, the Cys26 of yeast thioltransferase was alkylated by iodoacetamide at pH 3.5. This enzyme was completely inactivated when the

Cys26 was carboxymethylated. The result suggested that Cys26 could readily initiate nucleophilic attack on disulfide substrates at physiological pH (Gan *et al*., 1990; Nelson and Creighton, 1994). Some factors affecting cysteine pK_a and its reactivity are mentioned below:

1. By the presence of polar, positively charged amino acids, or local dipoles, like at the N-terminus of an α-helix, the thiolate is stabilized through electrostatic interactions, which decrease the pK_a of the cysteine (Roos *et al*., 2013).

2. If the pK_a is lower than the pH of the solvent, the majority of the thiols will be present as a thiolate. However, the effect of lowering the pK_a on the nucleophilicity enhancement will be the most significant when pK_a values are close to the solution pH (Figure 1.4). On the other hand, the decrease of the pK_a of a cysteine functioning as a leaving group has positive effect on the rate enhancement (Figure 1.4).

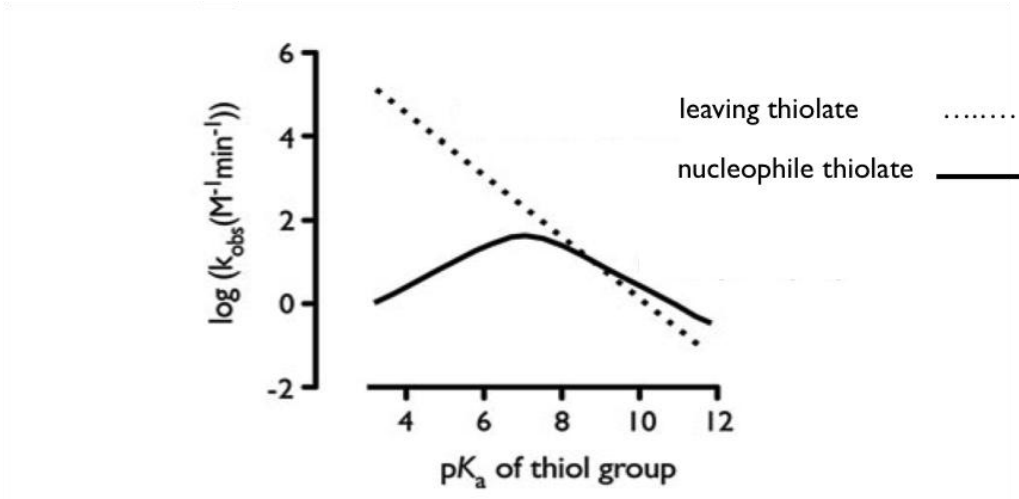


Figure 1.4 The effect of lowering pK_a on the rate constants for thiol–disulfide exchange at pH 7. If the pK_a of the nucleophile is greater that 7, lowering the pK_a will result in an increased reactivity at pH 7, whether a pK_a of the nucleophile lower than 7, lowering the pK_a results in a decreased nucleophilicity and reaction rate. On the other hand, the pK_a of the leaving thiolate is increases linearly with decreasing pK_a. Both effects are clearly visualized in the log (kobs) versus pK_a plot (Roos et al., 2013).

3. Also, hydrogen bonding has a strong influence on the pK_a of cysteines. For example in thioredoxin (Trx), the active-site nucleophilic cysteine receives two hydrogen bonds which result in a pK_a ~7.0, while the respective cysteine in the Trx-fold of glutaredoxin (Grx) receives three hydrogen bonds, which results in a pK_a of ~ 4.0 (Roos *et al*., 2013). In general, the more hydrogen bonds a cysteine-sulfur receives, the lower its pK_a, the more the thiolate form is stabilized.

4. Other factors that play a role in the reactivity of a cysteine-sulfur are the accessibility and its presence in specific binding sites (Marino and Gladyshev, 2010), like for example in peroxiredoxins (Roos and Messens, 2011).

1.4 Cys Ox-PTMs: control switches in plant signal transduction

Plants have evolved strategies to keep the ROS levels under tight control, interplaying between ROS producers and scavengers (Apel and Hirt, 2004; Mittler *et al*., 2011). This cellular regulation allows ROS to transform redox signals into biological defense responses, through the oxidation of redox sensor and signaling proteins. Here, we discuss the oxidative modifications of cysteine residues of plant signaling proteins and the functional consequences in specific pathways.

1.4.1 Cys Ox-PTMs control of transcriptional regulators

Controlling the transcriptional machinery in plants is a part of the ROS signal transduction pathways, thereby enabling rapid gene expression adjustments in response to environmental signals. This redox-dependent regulation involves conformational switching of the proteins, nucleo-cytosolic partitioning, assembly with cofactors, redox control of upstream signaling elements, and proteolysis (Dietz, 2014). In addition, the expression of different transcription factors themselves is enhanced by ROS and includes members of the WRKY, ZAT, RAV, GRAS and MYB families (Mittler *et al*., 2004). We discuss here redox control of plant transcription factors.

1.4.1.1 R2R3-MYB P1. Plant MYB (Myeloblastosis) proteins are characterized by a highly conserved MYB DNA-binding domain. MYB proteins are classified into four major groups namely, 1R-MYB, 2R-MYB, 3R-MYB and 4R-MYB based on the number and position of MYB repeats (Katiyar *et al*., 2012). Maize ZmP1 transcription factor is a typical R2R3 MYB-domain protein, which controls the expression of A1 gene required for the flavonoid biosynthesis in maize (Grotewold and Williams, 1997; Heine *et al*., 2004). Two conserved cysteines Cys49 and Cys53 located within the MYB DNA-binding domain (Figure 1.5) form a reversible intramolecular disulfide bond, which inhibits the binding of P1 to the A1 promoter (Heine *et al*., 2004).

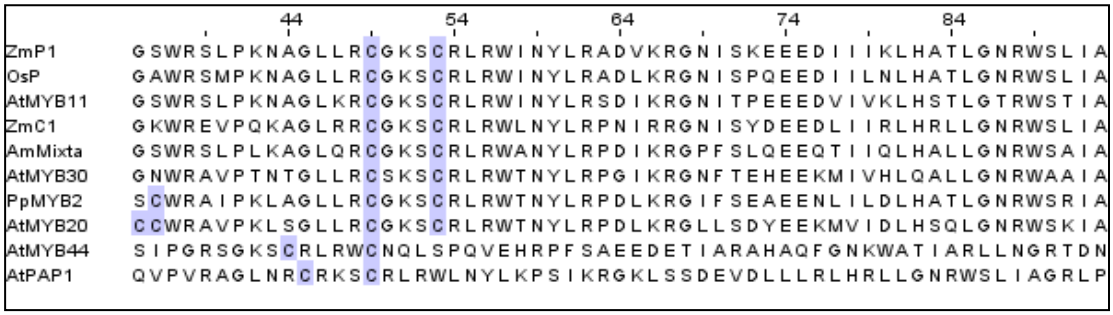


Figure 1.5 Sequence alignments of MYB domains in P1 transcription factor. The purple line at Cys-49 and Cys-53 indicates the conserved cysteines from *Zea mays* ZmP1, *Oryza sativa* OsP, *Arabidopsis thaliana* AtMYB11, *Z. mays* ZmC1, *Antirrhinum majus* AmMixta, *A. thaliana* AtMYB30, *Physcomitrella patens* PpMYB2, *A. thaliana* AtMYB20, AtMYB44, At-PAP1 (Heine *et al*., 2004).

Using EMSA (electrophoretic mobility shift assay), the DNA binding capacity of the recombinant MYB domain of P1 was analyzed after 1 mM DTT (dithiothreitol) reduction and 3 mM diamide oxidation, with the APB1 cis-regulatory element of the A1 promoter site (Heine *et al*., 2004). A DNA band shift was only observed with reduced P1, indicating that the reducing conditions are necessary for P1 to bind to the A1 promoter. Next, they checked whether the oxidized form of the recombinant MYB domain has an inter- or intramolecular disulfide bond. After diamide treatment, free thiols were blocked with the fluorescent DCIA (7-di-ethylamino-3-(4-iodoacetylaminophenyl)-4-methylcoumarin) dye. No fluorescence was detected in the oxidized recombinant MYB domain indicating that Cys49 and Cys53 of MYB form an intramolecular disulfide. Serpa *et al*. reported that NO (nitric oxide) modifies DNA-binding activity of AtMYB2, a typical R2R3-MYB from *A. thaliana*, through

S-nitrosylation of its conserved Cys53 residue detected by biotin switch assay (Serpa *et al*., 2007).

1.4.1.2 HD-ZIP. The plant specific homeodomain-leucine zipper (HD-ZIP) transcription factors control a wide range of developmental processes (Ariel *et al*., 2007). According to the evolutionary sequence analysis they can be classified into four classes (HD-ZIP I to IV) (Mukherjee *et al*., 2009), which consist of 48 proteins containing a homeodomain for DNA-binding and a leucine zipper domain that mediates dimerization necessary for DNA binding. Thus far, the redox-regulated DNA binding activity is reported for the members of HD-ZIP class II, III and IV (Tron *et al*., 2002; Comelli and Gonzalez, 2007).

HD-ZIP class II. HD-ZIP class II controls the apical embryo development and the meristem function in plants (Turchi *et al*., 2013), One of the distinct features of Arabidopsis class II HD-ZIPs is the presence of highly conserved CPSCE motif (Figure 1.6). The cysteine residues of CPSCE motif in the sunflower class II HD-ZIPprotein (Hahb-10 transcription factor) were demonstrated to exert a redox regulatory function (Tron *et al*., 2002). Oxidation and subsequent formation of intermolecular disulfide bonds, presumably occurring between the Cys residues of adjacent monomers, were shown to inhibit the DNA binding activity (Tron *et al*., 2002).

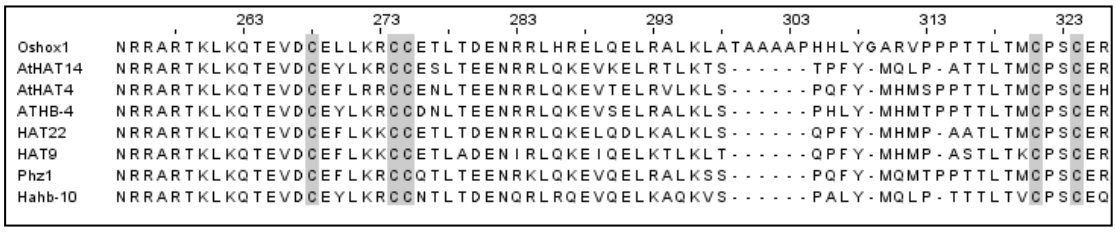


Figure 1.6 Conserved CPSCE motif of class II HD-ZIP proteins. Oshox1 from *Oryza sativa*; HAT14, HAT4, Athb-4, HAT22, and HAT9 from *A. thaliana*; Phz1 from *Pimpinellabrachycarpa* and Hahb-10 from *Helianthus annuus*. Conserved cysteines are marked in gray (Tron *et al*., 2002).

HD-ZIP class III. HD-ZIP III member proteins are involved in embryo development, organ polarity and meristem function (Prigge *et al*., 2005). Arabidopsis PHAVOLUTA (PHV, AtHB9), a member of HD-ZIP III, contains a CPILC

(CXXXC) motif (Figure 1.7) (Comelli and Gonzalez, 2007), which has been implicated as a redox switch in other proteins like the pox-viruses encoded thiol oxidoreductase A2.5L protein (Williams *et al*., 2005) and the human SCO1 protein (Senkevich *et al*., 2002). The DNA binding activity of AtHB9 is also redox dependent, as oxidation of conserved cysteine residues results in the formation of intermolecular disulfides that inhibit to interact with DNA (Comelli and Gonzalez, 2007).

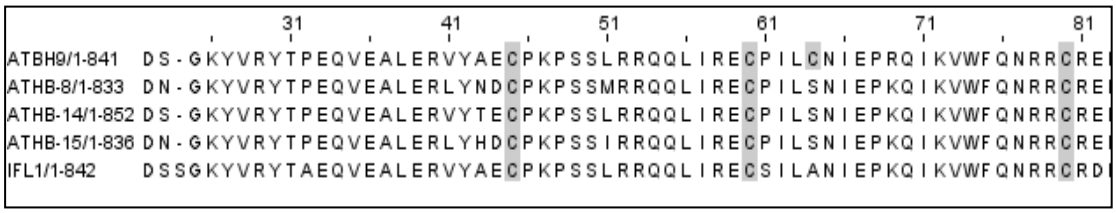


Figure 1.7 Amino acid sequence comparisons of the HD-ZIP domains of five Arabidopsis HD-ZIP III transcription factors. The presence of three conserved cysteine residues marked in gray (Comelli and Gonzalez, 2007).

HD-ZIP class IV. A similar kind of redox regulation was demonstrated for HD-ZIP class IV sunflower HAHR1 transcription factor, a homologue of Arabidopsis GLABRA2 protein, which controls the epidermal cell fate (Rerie *et al*., 1994; Masucci *et al*., 1996). A highly conserved CXXCG motif within this class (Figure 1.8) suggests the redox regulation might be conserved (Tron *et al*., 2002).

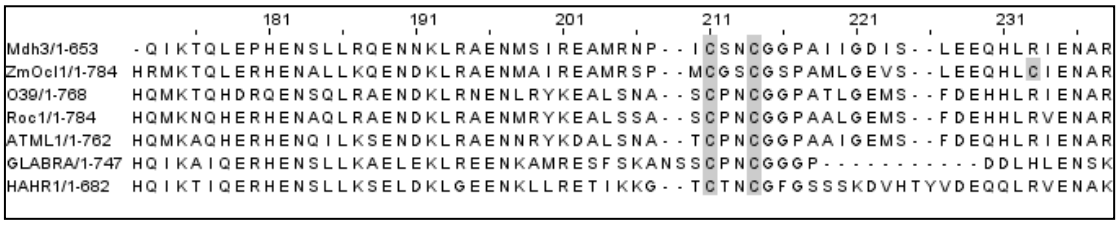


Figure 1.8 Amino acid sequence alignment of a region of the dimerization motif of seven GLABRA2 like proteins. Mdh3 from *Malusdomestica*, ZmOcl1 from *Zea mays*, O39 from *Phalaenopsis sp*, Roc1 from *O. sativa*, ATML1/GLABRA2 from *A. thaliana* and HAHR1 from *Helianthus annuus*. Conserved cysteines are marked in gray (Tron *et al*. 2002).

The redox regulated transcription activities of class II, III and IV HD-ZIP proteins were demonstrated by analyzing their interaction with specific promoters using

EMSA. DNA binding activity of DTT reduced recombinant Hahb-10, AtHB9 and HAHR1 increases compared to samples incubated with diamide, suggesting that cysteines in the reduced state are required for efficient binding (Tron *et al*., 2002; Comelli and Gonzalez, 2007). The oxidative form, which does not bind to DNA, was further analyzed. DTT-treated protein migrates as a monomer in non-reducing SDS-polyacrylamide gels; however, a significant proportion of diamide-treated protein migrates as dimer, suggesting the formation of intermolecular disulfide bonds in HD-ZIP proteins. Moreover, the thioredoxin reduction system (Trx/TrxR/ NADPH) can replace the DTT in the redox activation of all three HD-ZIP transcription factors (Hahb-10, HAHR1 and AtHB9) (Tron *et al*., 2002; Comelli and Gonzalez, 2007). Interestingly, Arabidopsis GLABRA2 has been identified as a redox regulated protein forming intermolecular disulfide bonds observed by their altered mobility in the oxidized and reduced states on diagonal 2D-PAGE (Ströher and Dietz, 2008).

1.4.1.3 bZIP. Shaikhali *et al*. identified a G-group basic leucine-zipper (bZIP) transcription factor in Arabidopsis (AtbZIP16) that binds to a high light responsive G-box-containing promoter fragment of LIGHT-HARVESTING CHLOROPHYLL A/B-BINDING PROTEIN 2.4 (LHCB2.4). The redox-dependent DNA binding activity of AtbZIP16 was observed by EMSA. The DNA binding capacity of DTT reduced AtbZIP16 was enhanced compared to H_2O_2 oxidized AtbZIP16 (Shaikhali *et al*., 2012). AtbZIP16 contains two cysteine residues, Cys330 and Cys358 (Figure 1.9). The Cys330 in AtbZIP16 forms intermolecular disulfides during oxidation thus leads to the formation of high molecular weight oligomers. An EMSA experiment was performed with AtbZIP16 single cysteine mutants (C330L, C358L) and double cysteine mutant (C330L/C358L). The DNA binding activity was greatly enhanced in both C330L and C330L/C358L mutants compared to the wild type protein. Moreover, both C330L and C330L/C358L migrated as a monomer in a non-reducing SDS-PAGE whereas the wild type and C358L migrated as oligomer and dimer respectively. Furthermore, it was demonstrated that two closely related transcription factors belonging to the same family, bZIP68 and G-BOX BINDING FACTOR 1 (GBF1) also exhibit a similar mode of redox regulation that relies on the redox status of Cys320 and Cys247 respectively (Shaikhali *et al*., 2012). GBF1 was found to bind on the CATALASE2 (CAT2) promoter site and repress its expression. This mechanism

serves to transiently elevate the H_2O_2 concentration necessary for the developmental transition to flowering (Smykowski *et al*., 2010).

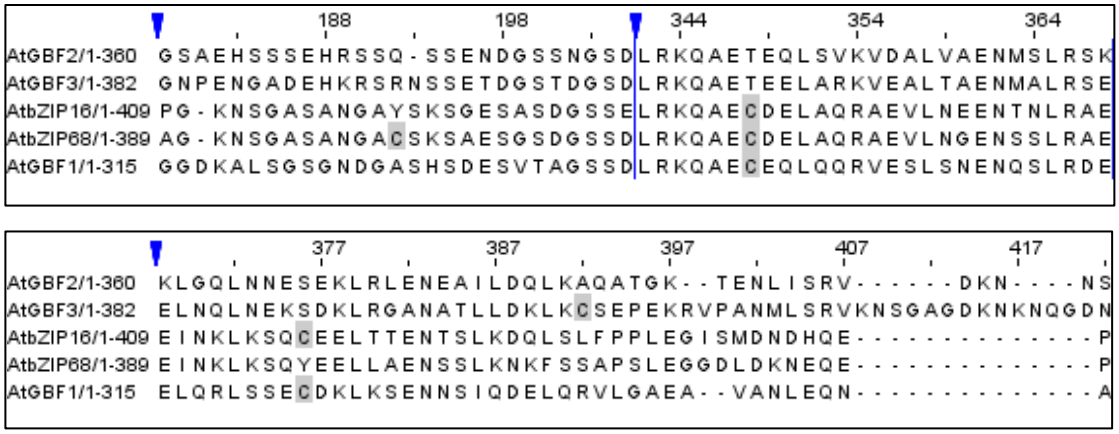


Figure 1.9 Amino acid sequence alignment of the G-group basic leucine-zipper (bZIP) transcription factors. Cysteine1 (C1) and Cysteine2 (C2) of AtbZIP16 are also conserved in AtGBF1 (marked in gray). C2 of AtbZIP68 resembles C1 in AtbZIP16 and AtGBF1. In AtbZIP68, C1 is located in the N terminus and upstream of the bZIP. The blue marks represent the hidden amino acid sequences (Shaikhali *et al*., 2012).

It is tempting to speculate that after reaching the necessary threshold concentration, H_2O_2 could de-repress the expression of CAT2by inhibiting the binding of GBF1. Moreover, cysteine residues of bZIP68 and GBF1 are reported to undergo reversible oxidative modifications in Arabidopsis under 5 mM H_2O_2 stress, identified by a differential alkylation proteomic study (Liu *et al*., 2014).

1.4.1.4 Rap2.4a. The Rap2.4a transcription factor controls the transcript level of the prominent chloroplast antioxidant enzyme, 2-Cys peroxiredoxin-A (2CPA) in a redox-dependent manner (Shaikhali *et al*., 2008). Under reducing conditions (1 mM DTT), this transcription factor is found in its inactive monomeric form (Figure 1.10) whereas mildly oxidizing conditions (1 mM H_2O_2) promote formation of dimers that bind to the 2CPA promoter, and activate its expression, while under conditions of severe oxidative stress (H_2O_2 conc. > 3 mM) transcriptionally inactive oligomers are formed (Shaikhali *et al*., 2008).

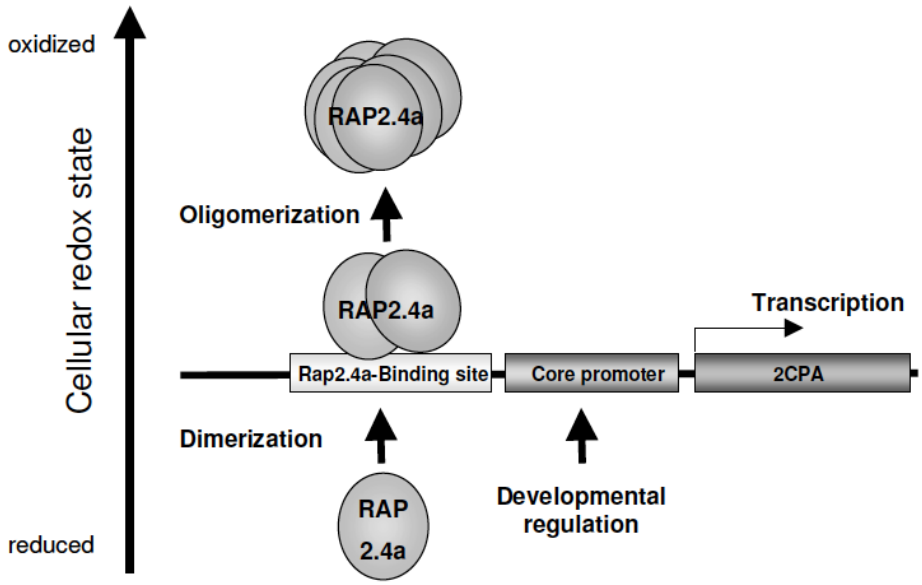


Figure 1.10 Redox regulation of 2CPA gene expression by the Rap2.4a transcription factor. The Rap2.4a dimerizes under mild oxidative stress and activates 2CPA expression. Under severe oxidative stress 2CPA expression is decreased as the Rap2.4a oligomerizes and loses its DNA affinity (Shaikhali *et al*., 2008).

1.4.1.5 NPR1. In plants, the redox changes regulate the conformation of NONEXPRESSER OF PATHOGENESIS RELATED GENES 1 (NPR1), a master regulator of salicylic acid (SA)-mediated defense genes. Under normal physiological conditions, NPR1 is localized to the cytoplasm in the form of an intermolecular disulfide oligomer involving two cysteine residues, Cys82 and Cys216 (Mou *et al*., 2003). Tada *et al*. reported that S-nitrosylation of NPR1 by S-nitrosoglutathione (GSNO) at Cys156 facilitates its oligomerization. Conversely, the SA-induced NPR1 oligomer-to-monomer reaction is catalyzed by Trx (Tada *et al*., 2008). Conversely, salicylic acid triggers the fluctuations in the cellular redox status that leads to the Trxh3 or Trxh5-dependent reduction of disulfides, monomerization of NPR1, and subsequent nuclear import as shown in figure 1.11 (Mou *et al*., 2003; Tada *et al*., 2008).

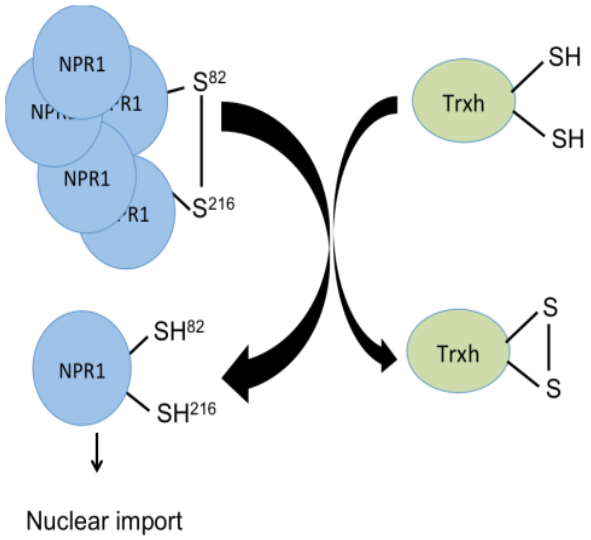


Figure 1.11 Schematic presentation of the NPR1 activation by Trx reduction system. Salicylic acid triggers the fluctuations in the cellular redox status that leads to the Trxh-dependent reduction of NPR1disulfides to monomeric NPR1, and subsequent nuclear import to induce the defense genes.

1.4.1.6 TGA. TGA are transcription factors that bind specifically to the DNA sequence 5'-TGACG-3'. TGA factors interact with NPR1, a central regulator of many SA-induced defense responses. The interaction of NPR1 with TGA transcription factors relies on their redox status. When oxidized, TGA1 and/or TGA4 form an intra-molecular disulfide bridge (Cys260 - Cys266) that hinders interaction with NPR1. Reduction of this disulfide stimulates formation of complex with NPR1, and subsequent binding to the as-1 element for activation of PR genes (Després *et al.*, 2003). As such both TGA and NPR1 function as key redox-controlled regulators of systemic acquired resistance in plants (Mou *et al.*, 2003; Tada *et al.*, 2008). In a parallel process, a subset of TGA transcription factors form disulfide bond that prevents interaction with NPR1 (Lindermayr *et al.*, 2010).

Yeast two-hybrid screens identified TGA transcription factors (TGA2, TGA3, TGA7 and TGA8) as interacting proteins with floral monothiolglutaredoxin (ROXY1, CC-type active site motif) *in planta* (Li *et al.*, 2009). These interactions are likely required for normal petal initiation and petal morphogenesis. Overlapping expression patterns of ROXY1 and TGA genes during flower development demonstrate that ROXY1/TGA protein interactions can occur *in vivo* and support their biological relevance in petal development. Deletion analysis of ROXY1 demonstrates the

importance of the C-terminus for its functionality and for mediating ROXY1/TGA protein interactions. Mutagenesis of PERIANTHIA Cys residues (PAN, TGA8) indicates that only Cys-340 (equivalent to Cys-266 in TGA1) is crucial for its function (Li *et al*., 2009). In another study, TGA9 and TGA10 are showed to interact with ROXY1 andROXY2 (Murmu *et al*., 2010), and TGA2 and TGA6 interacts with glutaredoxin GRX480 (ROXY 19) (Ndamukong *et al*., 2007) suggesting that these transcription factors function under redox control.

1.4.1.7 COI1. Plant defense responses are modulated by jasmonic acid (JA). The biologically active form of JA is JA-isoleucine conjugate, which triggers 26 proteasome-mediated proteolysis of JAZ proteins by mediating their interaction with the F-box protein CORONATINE INSENSITIVE1 (COI1), part of the Skp1/Cullin/F-box SCF^{COI1} ubiquitin E3 ligase complex. Proteolysis of JAZ proteins de-represses multiple transcription factors (i.e. MYC2) and leads to global changes in gene expression (Pauwels and Goossens, 2011). The interaction of COI1 with JAZ, depends to a great extent on a reducing environment (Yan *et al*., 2009).

1.4.1.8 TCP. TEOSINTE BRANCHED 1/ CYCLOIDEA/ PROLIFERATING CELL FACTOR class 1 (TCP) transcription factors constitute a family of plant developmental regulators (Cubas *et al*., 1999; Martín-Trillo and Cubas, 2010). The DNA-binding domain of the class I TCP transcription factors contain 94% conserved cysteine residue (Cys20) (Viola *et al*., 2013). This conserved Cys20 mediates oxidative inhibition of DNA-binding via the formation of intermolecular disulfide bonds (Viola *et al*., 2013). Using TCP15 as a model family member, it was demonstrated that the DNA-binding activity is inhibited upon treatment with hydrogen peroxide or diamide, as analyzed by EMSA *in vitro*. Under oxidizing conditions, covalently linked dimers were formed, suggesting that inactivation is associated with the formation of intermolecular disulfide bonds. Mutation of Cys20 in the class I protein TCP15 abolished its redox sensitivity. Inhibition of class I TCP protein activity was also observed *in vivo* in yeast (*Saccharomyces cerevisiae*) cells expressing TCP proteins and in plants after treatment with redox agents. Inhibition can be reversed by treatment with the reductants DTT or by incubation with the thioredoxin/thioredoxin reductase system.

1.4.2 Cys Ox-PTMs control of signaling proteins

1.4.2.1 MAPK & PTP. The mitogen-activated protein kinase (MAPK) signaling cascades regulate respective cellular processes depending on environmental and developmental stimuli. Numerous studies reported the involvement of MAPK cascades in interactions with multiple hormonal signaling pathways (Colcombet and Hirt, 2008; Hahn and Harter, 2009; Pitzschke and Hirt, 2009; Rasmussen *et al*., 2012; Danquah *et al*., 2013). Hydrogen peroxide was demonstrated to induce MAPKKK1which in turn activates MAPK3 and MAPK6 (Kovtun *et al*., 2000).

On the other hand, protein tyrosine phosphatases (PTPs) are involved in the control of MAPK associated signaling cascades such as guard cell signaling (MacRobbie, 2002), oxidative stress tolerance (Lee and Ellis, 2007), SA homeostasis (Bartels *et al*., 2009), disease responses (Lumbreras *et al*., 2010) as well as auxin signaling (Strader *et al*., 2008; Lee *et al*., 2009), and cortical microtubule functions (Walia *et al*., 2009). PTPs require a highly conserved Cys residue (Cys265 in AtPTP1) in their active site(Xu *et al*., 1998) and their activity is inhibited under oxidative stress conditions (Gupta and Luan, 2003). Interestingly, this inactivation is positively correlated to MAPK6 activation by H_2O_2. However the exact mechanism of this regulation is not known. Three members of the Arabidopsis MAPK family (MAPK2, MAPK4 and MAPK7) and AtPTP1 have been identified as H_2O_2 sensitive protein forming sulfenic acids (Wazsczak *et al.*, 2014) as discussed in chapter 2. Moreover, AtPTP1 was reported for reversible cysteine modification under H_2O_2 stress (Wang *et al*., 2012). It is appealing to speculate that MAPK might function as H_2O_2 sensors transmitting the signal through the formation of sulfenic acids and AtPTP1 inactivation through sulfenylation triggers this signaling cascade.

In contrast, the activity of PTP from maize, ZmRIP1 is insensitive to H_2O_2 and its activity decreases upon reduction with DTT. Cys181 near the active center ZmRIP1 was found to regulate this mode of redox regulation, as mutation of C181 allowed ZmRIP1 to be activated (Li *et al*., 2012). Authors suggest that Cys181 might be involved in disulfide bond formation; however, the exact mechanism of this redox regulation needs further research.

1.4.2.2 ABI1/ ABI2. Two members of the PP2C (protein phosphatase 2C) family, ABI1 (ABA intensive1) and ABI2, act as negative regulators of open stomata 1 kinase (OST1) triggered stomata closure signaling (Merlot *et al*., 2001). ABI1 and ABI2 were shown to undergo H_2O_2 dependent inhibition. This oxidizing condition leads to the inactivation of their phosphatase activity *in vitro*, probably via formation of an intramolecular disulfide bond (Meinhard and Grill, 2001; Meinhard *et al*., 2002). Therefore, redox-regulated inhibition of PP2C activates OST1, which induces the generation of H_2O_2, and then, H_2O_2 activates a Ca^{2+}channel. It is noteworthy to mention that PP2C undergoes reversible cysteine modification under H_2O_2 stress (Wang *et al*., 2012). Further studies indicated that the oxidation of these enzymes might be mediated by GPX3 in planta. As it was shown that GPX3 interacts with ABI2 *in vivo* and inhibits its PP2C activity (Miao *et al*., 2006). The reduced form of ABI2 can be converted to the oxidized form by the addition of oxidized AtGPX3. These results suggest that GPX3 might act as a sensor protein that upon perception of ROS signals relays the oxidizing equivalents to ABI2 via a thiol-disulfide exchange mechanism (Miao *et al*., 2006). A similar scenario was described in yeast cells exposed to oxidative stress (Delaunay *et al*., 2000; Delaunay *et al*., 2002) and in mammalian cells (Gutscher *et al*., 2009) suggesting that thiol peroxidases might act as universal redox sensors. It is noteworthy that several redox regulated signaling proteins discussed in the previous section have been identified by this strategy. PP2C undergoes reversible cysteine modification under H_2O_2 stress detected by blocking/IAF labeling (Wang *et al*., 2012).

1.4.2.3 CPK21. In the stomata closure signaling, the rise in Ca^{2+} concentration activates CALCIUM-DEPENDENT PROTEIN KINASE 21 (CPK21) that control the levels of osmotically active ions within guard cells under drought stress (Geiger *et al*., 2010). The redox conditions were recently shown to affect the activity of CPK21 (Ueoka-Nakanishi *et al*., 2013). CPK21 was identified in a screen for targets of cytosolic Trx-h. H_2O_2 treatment was shown to inhibit the CPK21 kinase activity both *in vitro* and *in vivo*. This inactivation was linked with the formation of an intramolecular disulfide bond (Cys97-Cys108) and was effectively restored by Trx-h (Ueoka-Nakanishi *et al*., 2013). CPK21 was identified in a screen for targets of cytosolic Trx-h. CYP20-3 was initially identified in a screen for Trx-m targets in spinach chloroplast stroma (Motohashi *et al*., 2001) and undergoes reversible cysteine

oxidation detected in blocking/IAF labeling (Wang *et al*., 2012). Reduction of cyclophilin by Trx-m recovered its isomerase activity. Two intramolecular disulfide bonds (Cys53-Cys170; Cys128-Cys175) were determined by disulfide-linked peptide mapping in oxidized inactive form (Motohashi *et al*., 2003).

1.4.2.4 ETR1. The stomata closure signaling is triggered by ETHYLENE RESPONSE 1 (ETR1) protein, a member of endoplasmic reticulum membrane-localized receptor protein family. Perception of ethylene by ETR1 triggers RESPIRATORY BURST OXIDASE HOMOLOG F (AtRbohF) mediated H_2O_2 accumulation essential for ethylene-induced stomata closure signaling (Desikan *et al*., 2006). ETR1 is also involved in ethylene independent stomata closure signaling mediated by H_2O_2 (Desikan *et al*., 2005). Cys65 of ETR1 plays crucial roles in stomata closure signaling pathway; as this signaling is inhibited in the *etr1-1* (Cys65Tyr) mutant plants. These data suggest that ETR1, particularly the Cys65 residue of ETR1 is important for H_2O_2 signaling in guard cells. Therefore, Cys65 might be involved in H_2O_2 mediated post-transcriptional modifications, and as such, might act as redox sensor.

1.3.2.5 ANNEXIN1. Annexins are characterized by a Ca^{2+}-dependent binding to lipid membranes (Laohavisit and Davies, 2011). Recent evidence suggests that the ROS dependent Ca^{2+} fluxes in Arabidopsis roots and in guard cells are also mediated by ANNEXIN1 (AtANN1) (Konopka-Postupolska *et al*., 2009; Laohavisit *et al*., 2010; Laohavisit *et al*., 2012; Richards *et al*., 2013). AtANN1 was identified as an oxidation susceptible protein which cysteine undergoes a reversible thiol modification (Muthuramalingam *et al*., 2013). Interestingly, this protein was demonstrated to undergo S-glutathionylation on both of its cysteine residues, Cys111 and Cys239, and this modification results in a 50% decrease of its Ca^{2+} affinity (Konopka-Postupolska *et al*., 2009). Most probably, this mechanism serves to restrict the AtANN1 membrane association, and to inhibit ROS-mediated Ca^{2+} fluxes in a negative feedback loop. Apart from S-glutathionylation AtANN1 was found to undergo S-nitrosylation (Lindermayr and Saalbach, 2005). ANNEXIN1 was identified as oxidation susceptible protein in Arabidopsis using a differential labeling strategy (Muthuramalingam *et al*., 2013).

1.4.2.6 CYP20-3. A recent study by Park *et al*. identified CYCLOPHYLIN 20-3 (CYP20-3) as a chloroplastic receptor of OPDA in the JA signaling (Park *et al*., 2013). Cyclophilins are characterized by a highly conserved Peptidyl-Prolyl Isomerase (PPIase) domain that assists proper folding of the target proteins (Trivedi *et al*., 2012). Thus far, in Arabidopsis, CYP20–3 was found to function *in vivo* in assisting the assembly of SERINE ACETYL-TRANSFERASE1 (SAT1) to form the hetero-oligomeric complex between cysteine synthase and *O*-ACETYLSERINE THIOL LYASE (OASTL), the cysteine synthase complex (CSC) (Leustek *et al*., 2000) (Figure 1.12). The CSC formation then activates sulfur assimilation that leads to increased levels of thiol metabolites and the buildup of cellular reduction potential. The enhanced redox capacity in turn coordinates the expression of a subset of OPDA-responsive genes. The physical interaction of CYP20-3 with SAT1 is crucial for the optimal synthesis of the amino acid cysteine. CYP20-3 was initially identified in a screen for Trx-m targets in spinach chloroplast stroma (Motohashi *et al*., 2001) and undergoes reversible cysteine oxidation (Wang *et al*., 2012).

Figure 1.12 Schematic illustration of Trx activation of CYP20-3. The physical interaction of CYP20-3 with SAT1 promotes the formation of a hetero-oligomeric complex between cysteine synthase and *O*-acetylserine thiol lyase (OASTL); the cysteine synthase complex (CSC) (Leustek *et al*., 2000), which synthesize the amino acid cysteine.

Two intramolecular disulfide bonds (Cys53-Cys170; Cys128-Cys175) were determined by disulfide-linked peptide mapping in an oxidized inactive form (Motohashi *et al*., 2003). Apart from that, *cyp20-3* mutant plants exhibit low thiol content and are impaired in light dependent stress responses (Dominguez-Solis and He, 2008). Direct binding of OPDA to CYP20-3 was shown to stimulate this interaction and ultimately promote the production of cellular antioxidants (Park *et al*., 2013). Therefore, CYP20-3 is a redox sensitive crosstalk point linking OPDA-

signaling with the maintenance of the cellular redox balance.

1.4.2.7 RB47/RB60. In *Chlamydomonas reinhardtii*, the chloroplast *psbA* gene encodes the photosystem II reaction center protein D1. Its translation process involves the redox dependent interaction of two proteins, polyadenylation-binding protein RB47 and disulfide isomerase RB60. RB60 has two Trx-like -CGHC- motifs at its N and C-terminal ends (Figure 1.13). During the photosynthetic light reactions, a reducing environment is generated by PSII through the ferredoxinFdx/ ferredoxin-thioredoxin reductase FTR system (Kim and Mayfield, 1997). In this chain reaction, RB60 interacts with RB47 and controls its reduction status. Reduced RB47 contains directly binds to the 5' untranslated region (UTR) of the *psbA* mRNA, which is required for itstranslation (Yohn *et al*., 1998).

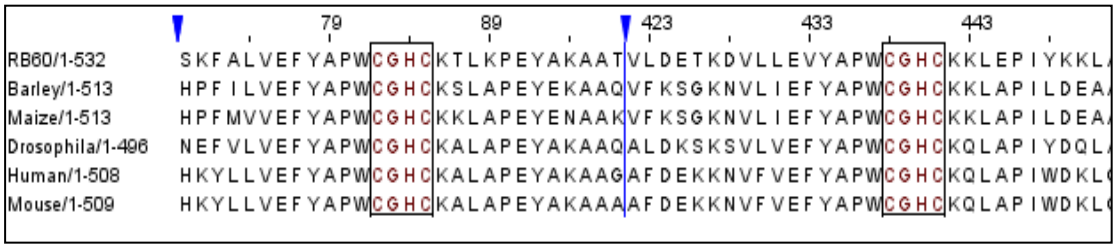


Figure 1.13 Two Trx-like conserved –CGHC- motifs at N- and C-terminal ends of RB60 from *Chlamydomonas reinhardtii* aligned with disulfide isomerases of barley, maize, drosophila, human and mouse (Kim and Mayfield, 1997). The blue marks represent the hidden amino acid sequences of the disulfide isomerases.

Based on CNBr-digestion and reducing/non-reducing SDS-PAGE gel evaluation, it was found that the intramolecular disulfide bond is possibly between Cys259 and Cys143 (Alergand *et al*., 2006). Incubating DTNB-oxidized RB47 with the reduced RB60 cysteine mutants (containing a single intact nucleophilic cysteine at any of the C-or N-terminal active sites), it was shown that RB60 forms a mixed disulfide intermediate with RB47. The identification of RB60 as a Trx target trapped on monocysteinic *Chlamydomonas* Trx-h1 column, confirms that the light control of psbA translation involves the Trx system (Lemaire *et al*., 2004).The identification of RB60 as a Trx targets trapped on monocysteinic *Chlamydomonas* Trx-h1 column, confirms that the light control of psbA translation involves Trx system.

1.5 Proteomics for studying Cys Ox-PTMs

Here, we discuss the proteomic approaches for identifying ROS mediated cysteine PTMs; special focus goes to the techniques applied for the identification of the sulfenome, disulfide bonds, and S-glutathionylation. Numerous studies focused on the identification of reversible cysteine oxidative modifications under different stress conditions. All these studies include three basic steps: I) tagging of the oxidative modifications, II) isolation or enrichment of the modified proteins and III) finally identification of the modified proteins (Table 1.1 and 1.2). A critical step in the study of cysteine oxidation is to preserve the *in vivo* thiol oxidation status and, as such, to prevent *de novo* thiol oxidation during/after lysis.

1.5.1 Differential alkylation based indirect proteomics

Currently, differential alkylation methods are the most widely used to indirectly detect and study the cysteine status in proteins, which undergo reversible oxidative modifications. This method comprises three consecutive steps: blocking, reduction, and labeling (Figure1.14). The way of ensuring the non-reactivity of free thiols is by applying alkylating reagents such as iodoacetamide (IAM) which forms an irreversible S-carbamidomethylated thiol, or N-ethylmaleimide (NEM) which results in irreversible thioether formation (Hansen *et al*., 2009). After blocking the free thiols, the reversible cysteine modifications are reduced back to their thiol status with reducing agents TCEP (Tris 2-carboxyethyl phosphine) or DTT. Subsequently, these newly introduced thiols are labeled with a tagged alkylating agent, such as biotin-conjugated IAM/NEM (BIAM/ BNEM), iodoacetamidofluorescein (IAF), or monobromobimane (mBrB) derivatives or purified by thiol affinity chromatography using CNBr activated thiol sepharose 4B. This differential labeling allows downstream analysis by Western blot or fluorescence-based visualization of the redox active protein spots on two-dimensional electrophoresis (2-DE) gel, or avidin affinity enrichment (BIAM and BNEM), and finally mass spectrometry-based identification. Quantification of the cysteine redox state of individual proteins is possible by using this differential labeling allows downstream analysis by Western blot or fluorescence-based visualization of the redox active protein spots on a two-dimensional electrophoresis (2-DE) gel, or avidin affinity enrichment, and finally mass spectrometry-based identification (Alvarez *et al.*, 2009; Bykova *et al.*, 2011b; Liu *et al.*, 2014; Parker *et al.*, 2012; Wang *et al.*, 2012). Quantification of the cysteine redox state of individual proteins is possible by using ICAT, iTRAQ, cysTMT tags, as they enable selective

labeling and relative quantitation of cysteine-containing peptides representing their redox status in the samples. Table 1.2 shows a list of studies for exploring reversible Cys Ox-PTMs under different stress condition in plants. Application of this strategy at cellular level provides a global scenario of the reversible cysteine oxidation status including disulfide bond, S-glutathionylation, and sulfenylation. However, specific reversible cysteine modifications can also be identified, and they have thoroughly been studied in plants.

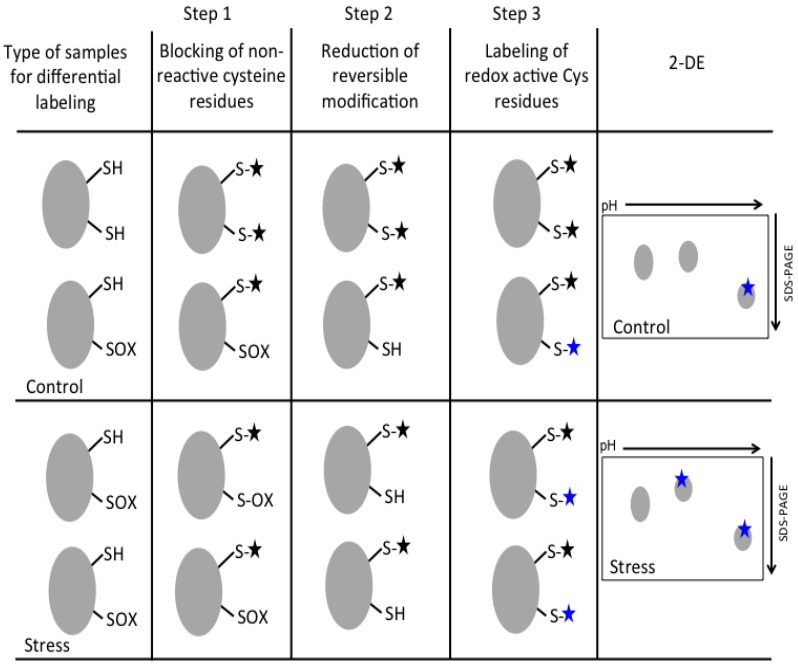


Figure 1.14 Schematic presentation of differential labeling of oxidized and non-oxidized cysteine residues. In the first step all the free thiols are blocked with IAM or NEM as marked by a yellow stars. In the second step, the thiols that are reversibly modified by ROS can be reduced back to their thiol status by DTT reduction, and then these newly introduced thiols are tagged by alkylating agent, such as biotin-conjugated IAM/NEM (BIAM/ BNEM), iodoacetamidofluorescein (IAF), or monobromobimane (mBrB) derivatives (marked by blue star). This differential labeling allows downstream analysis by Western blot or fluorescence-based visualization of the redox active protein spots on two-dimensional electrophoresis (2-DE) gel, or avidin affinity enrichment (BIAM and BNEM). Here an example of 2-DE for downstream analysis.

1.5.1.1 Sulfenylation (S-OH). Differential alkylation following the specific sulfenic acid reduction with arsenite is applied at proteome wide level to study the sulfenome, as for example in H_2O_2 treated heart tissue (Saurin *et al.*, 2004) and in rat kidney cell extracts (Tyther and Ahmeda, 2010). The peroxide inactivation of papain through sulfenic acid modification was reported by using this approach (Lin *et al.*, 1975), but has not been used in plant redox proteomics so far. However, application of this technique for sulfenome

proteomics is limited due to the highly reactive, transient nature of sulfenic acids. Although *in vitro* blocking, reduction and labeling steps are performed under acidic or anaerobic conditions, it does not completely preclude the chance of *de novo* sulfenylation due to an altered cysteine redox state in the cell lysates, or that the sulfenic acid modifications are insufficiently trapped due to protection or overoxidation (Couturier *et al.*, 2013; Leonard and Carroll, 2011).

1.5.1.2 Disulfide bonds (S-S). The application of a specific reducing enzyme such as Trx in differential alkylation approach leads to the identification of Trx target proteins. This approach has been applied in germinating seeds of barley (Marx *et al*., 2003; Maeda *et al*., 2005; Hägglund *et al*., 2008), axes and cotyledons seeds of *Medicago trunculata* (Alkhalfioui *et al*., 2007), wheat seed (Wong *et al*., 2003; Wong *et al*., 2004), *Nicotianaalata* styles (Juárez-Díaz *et al*., 2006), peanut dry seeds (Yano *et al*., 2001) and in rice bran (Yano and Kuroda, 2006). In germinating barley seeds, 16 Trx-target proteins were identified by cyanine-5-maleimide (Cy5 maleimide) labeling and have been proven more sensitive compared to mBBr labeling by which only 6 Trx-target proteins were detected (Maeda *et al*., 2004).

1.5.1.3 S-glutathionylation (S-SG). S-glutathionylated proteins can be identified by differential alkylation using Grx as a reducing agent. Forty-three proteins were identified as substrates for Grx (Lind *et al*., 2002), however this strategy has not been explored yet in plants.

1.5.2 Direct proteomics for Cys Ox-PTMs

Apart from the alkylation-based indirect technologies, direct detections have been developed for the identification of Cys Ox-PTMs. Most of these direct proteomics are based on specific probes for direct trapping or labeling of the modified proteins, or for affinity enrichment of the targets (Table 1.3).

1.5.2.1 Sulfenylation (S-OH). Due to the central role of sulfenic acids in oxidation pathways, several methods have been developed for its detection. One major advantage of detecting sulfenic acids in proteins is that this modification represents the initial product of oxidation and functions as a marker for ROS-sensitive cysteine

residues, while a potential disadvantage is the rather transient nature of this modification. Therefore, the detection and analysis of the sulfenome is one of the challenging parts of redox proteomic studies. Direct detection of the cellular sulfenome depends on chemical probes, and YAP1 (yeast *Saccharomyces cerevisiae* activator protein1) based genetic probes (Figure 1.15).

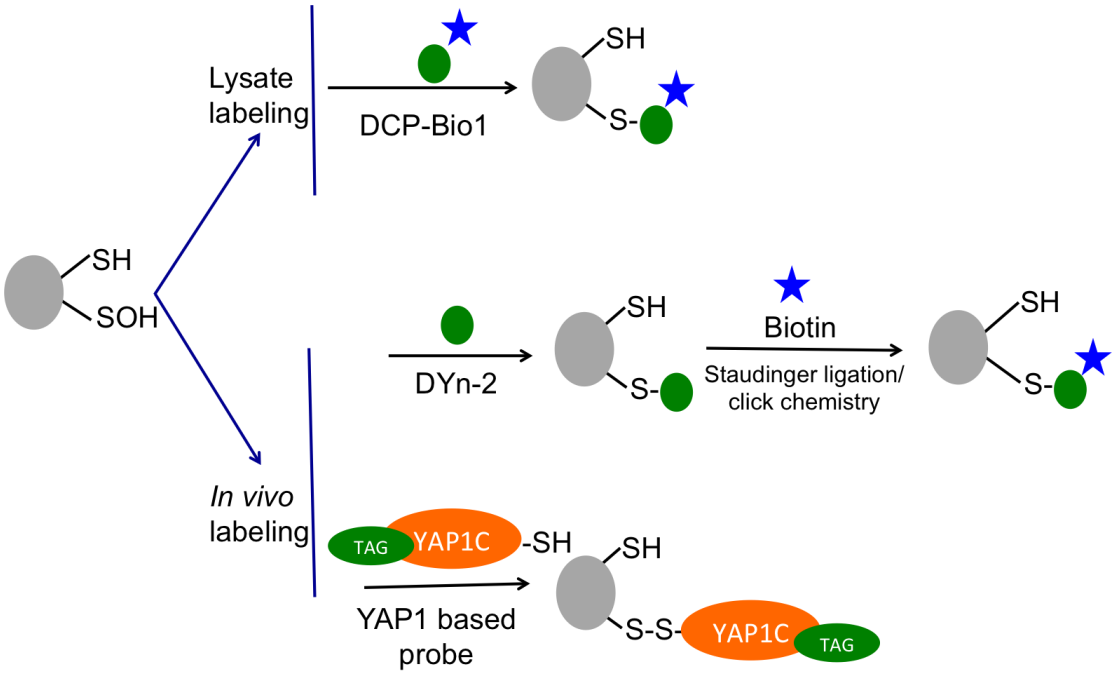


Figure 1.15 Schematic presentation of sulfenic acid trapping either using a chemical probe or a genetic probe. DCP-Bio1 chemical probe is used to trap sulfenic acids in cell lysates. However, DYn-2 chemical probe and YAP1 based genetic probe trap sulfenic acids *in vivo*. After tagging, the sulfenylated proteins are isolated, enriched by affinity purification and finally identified by mass spectrometry. Incase of DYn-2 approach, the DYn-2 tagged sulfenic acids are biotinylated with the help of click chemistry for downstream analysis.

Several chemical probes specifically react with sulfenic acids like NBD-Cl (4-chloro-7-nitrobenzo-2-oxa-1,3-diazole) (Ellis and Poole, 1997) (Poole and Claiborne, 1989, Ellis and Poole, 1997), dimedone (5,5-dimethyl-1,3-cyclohexadione) (Benitez and Allison, 1974). However, the cellular sulfenome detection methods reported to date depend on the dimedone- sulfenic acid specific reaction (Leonard and Carroll, 2011). The lack of functional groups for the enrichment or the visualization of protein-S-dimedone adducts has encouraged the development of biotinylated and fluorophore-

conjugated analogues like the DCP-bio series, the DCP-FL series, and the DCP-Rho series (Poole *et al*., 2005; Charles *et al*., 2007; Poole *et al*., 2007). Specific and sensitive antibodies have been developed for detecting protein-S-dimedone adducts by Western blot and immunofluorescence (Seo and Carroll, 2009a). Biotinylated and fluorophore-conjugated analogues of dimedone probes have been used in a proteomic study with isolated rat hearts (Charles *et al*., 2007). One potential drawback of using these probes is that the bulky chemical tags (Poole *et al*., 2005; Poole *et al*., 2007) compromise cell permeability (Seo and Carroll, 2009b). A subsequent alternative approach is the development of azido- and alkyne-functionalized dimedone analogous termed DAz-1(Reddie *et al*., 2008; Seo and Carroll, 2009b), DAz-2 (Leonard *et al*., 2009), DYn-1 and DYn-2 (Paulsen *et al*., 2012; Yang *et al*., 2014), which enable the direct trapping and tagging of protein sulfenic acid modifications in living cells. Proteins covalently modified by DAz or DYn probes can be coupled to biotin or fluorophores by the Staudinger ligationor by click chemistry reactions (Truong and Carroll, 2012). A recent report on the use of a linear β-ketoester expands the scope of the chemical probe in cellular sulfenome studies (Qian *et al*., 2012). However, caution should be taken with linear derivatives since they have been reported to cross react with amines, as such might cross react with lysine containing side chain amine (Paulsen and Carroll, 2013).Very recently T. H. Poole *et al.*, described a new chemical probe for sulfenic acid termed BCN (9- hydroxymethylbicyclo [6.1.0] nonyne) derivatives (Poole *et al*., 2014). They reported BCN reacts with sulfenic acids within purified protein, lysates and live cells at faster reaction rate (15 $M^{-1}s^{-1}$), which is more than 2 orders of magnitude faster as compared to dimedone and dimedone-based probes (0.05 $M^{-1}s^{-1}$).

The genetic probe YAP1-based strategy offers a way to trap sulfenic acids *in vivo* for the identification of redox sensitive proteins undergoing sulfenylation under oxidative stress conditions (Takanishi *et al*., 2007). In this study, the C-terminal cysteine rich domain of YAP1 was mutated leaving a single Cys598 for trapping sulfenylated proteins. This Yap1 probe equipped with an affinity tag was expressed in *Escherichia coli* and used to co-purify proteins undergoing H_2O_2 dependent sulfenylation. In a follow-up study, this technique was applied to yeast (Takanishi and Wood, 2011) to profile sulfenylated proteins under H_2O_2 stress. By combining the use of DCP-Bio1 and YAP1 based genetic probes, sulfenylated proteins were identified in

the *Medicago truncatula* and *Sinorhizobium meliloti* symbiosis. Here, 44 proteins were found sulfenylated in inoculated roots and 65 proteins in the functioning symbiotic organ (Oger *et al*., 2012). There is a crosstalk between *in vitro* and *in vivo* trapping of highly reactive sulfenic acids (Bachi *et al*., 2013; Paulsen and Carroll, 2013; Gupta and Carroll, 2014). In cells, the subcellular compartments like nucleus, mitochondria, and cytoplasm have reducing environment whereas the secretory pathway and the extracellular space are more oxidizing environments (Go and Jones, 2008). During cell lysis, the redox environment is disrupted, which can result in substantial protein oxidation artifacts, and which potentially also hamper the detection of labile cysteine modifications such as sulfenic acids. Methods to decrease these oxidation artifacts in lysates have been reported by the addition of ROS-metabolizing enzymes to the lysis buffer (Klomsiri *et al*., 2010), such as catalase which scavenges H_2O_2 (Chapter 3) or by addition of thiol blocking agents IAM/NEM.

In situ trapping approaches with these chemical probes have their own limitations, as the addition of a small membrane permeable molecule to cells might interfere with the signaling pathways. Another important consideration with chemical probes is the rate at which the probes react with the modified Cys residue. If the reaction is slow, transient cysteine oxidation events might be missed. The modest second order rate constant for the reaction of many dimedone analogs with sulfenic acid is only 2.7 x 10^{-2} $M^{-1}s^{-1}$ (Paulsen and Carroll, 2013), which might not be sufficient to trap especially transient modifications. Therefore, it will be important to develop chemical probes with a high reaction rate to trap these transiently formed sulfenic acids. The DYn-2 probe, however, is doing much better, since its reaction rate with dipeptide-SOH is estimated to be 11 $M^{-1}s^{-1}$ (Gupta and Carroll, 2014). In recent work of Poole *et al.* (2014), it has been shown that strained cycloalkynes react with sulfenic acids to yield a stable alkenyl sulfoxide with a reaction rate that is 100 times faster than that of most dimedone-based 1,3 dicarbonyl reagents. However, increasing the probe concentration can also compensate for relatively modest reaction rates, but appropriate controls must be performed to ensure that the underlying biology is not disturbed.

In contrast to chemical probes, the YAP1-based sulfenome probe is non-invasive and more physiological. The use of the YAP1 protein probe enables exploration of

organellar sulfenomes, as it can be easily modified with target peptide sequences (Takanishi *et al*., 2007; Takanishi and Wood, 2011). In addition, if we compare the rate constant of YAP1 disulfide formation with target sulfenic acids with that of the reaction of sulfenic acids with thiols to form a disulfide bond (21.6 $M^{-1}s^{-1}$) (Paulsen and Carroll, 2013; Gupta and Carroll, 2014), the YAP1 genetic probe should be more efficient in trapping sulfenic acids than the chemical probe. The drawbacks of the YAP1-based strategy are the relative low efficiency and the somewhat high risk of false identifications. The number of detected sulfenic acid-forming proteins could be underestimated due to the reduction of disulfide bonds (between YAP1 and target sulfenylated proteins) by glutathione or redox enzymes or other cysteine containing proteins, or by the resolving cysteine of the trapped protein itself. Another limitation could be the steric effects, since the Yap1-carboxy-terminal, cysteine-rich domain (c-CRD) variant must be expressed in host cells and, since it is protein-based, it may exhibit substrate bias when compared to chemical-based probes. Moreover, there is a chance of co-elution of non-sulfenic acid proteins due to complex protein-protein interactions.

At this stage, combining the data of a genetic probe and a chemical probe would be an appropriate strategy to explore the complete sulfenome in plants, because both probes have their specific advantages. Moreover, future research should be focused on mapping and quantifying the sulfenylation sites in the signal transduction proteins to assess their role in oxidative stress signal transduction events. A recent work of Yang *et al.* (2014) reports a proteomic workflow for the quantitative identification of sulfenylation sites combining DYn-2 with a functionalized biotin reagent containing a cleavable linker. In human cells, this probe allows to identify about 1,000 sulfenylation sites in more than 700 proteins (Yang *et al.*, 2014).

1.5.2.2 Disulfide bonds (S-S). A direct *in vitro* disulfide proteomics method called redox two-dimensional electrophoresis (redox 2D-E) or diagonal two-dimensional electrophoresis (diagonal 2D-E) is used to identify intra- or inter-molecular disulfides under non-reducing (first dimension) and reducing (second dimension) conditions based on the differential migration in the gel (Figure1.16) (Cumming, 2008). Non-disulfide proteins will migrate on a diagonal since they have the same electrophoretic mobility in both dimensions.

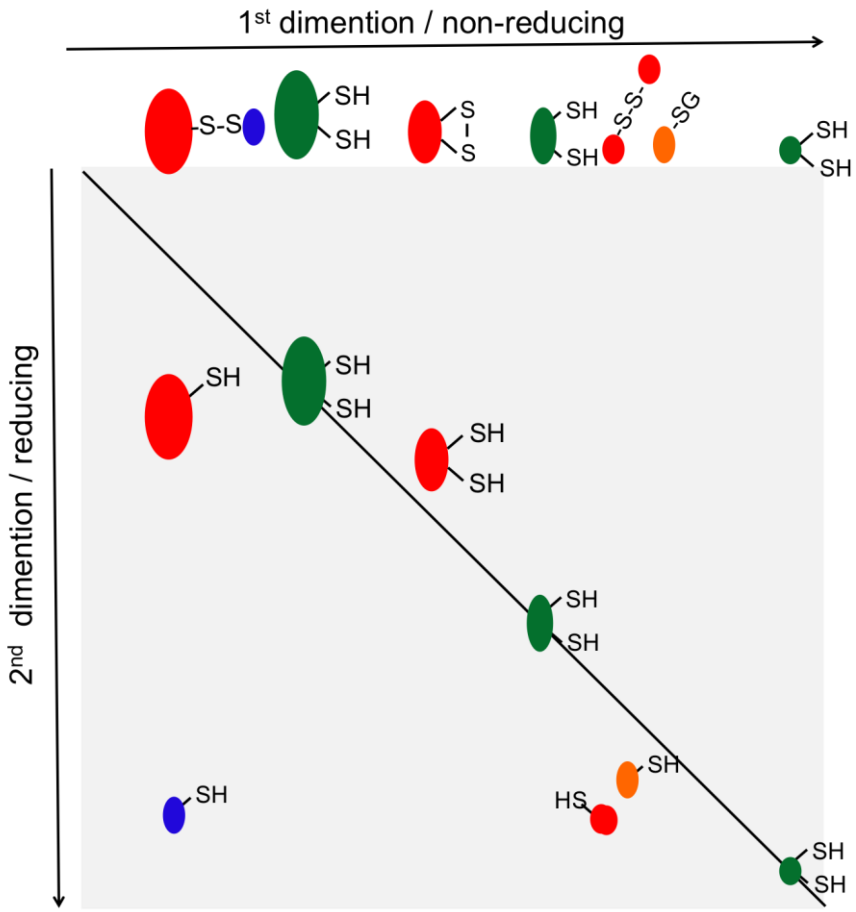


Figure 1.16 Schematic presentation of diagonal 2D-PAGE. Proteins without disulfide run at the level of the diagonal across the gel. In contrast, proteins with a disulfide migrate below the diagonal, whereas proteins with an intramolecular disulfide bridge run above the diagonal.

However, proteins containing intra-molecular disulfide bondshave lower electrophoretic mobility in their reduced state and therefore migrate above the diagonal, while cross-linked proteins with inter-molecular disulfides will pop up under the diagonal since they will migrate slower in the first dimension than in the second one. An earlier application of redox 2D-E leads to the identification of two pea leaf chloroplast thylakoid membrane proteins (Anderson and Manabe, 1979). Eighteen redox active proteins forming disulfides in *A. thaliana* mitochondria were examined using 2D-E (Winger *et al.*, 2007). Identification of 22 novel disulfide redox proteins in the stromal subproteome fractions has added new insights into the redox network of chloroplast from *A. thaliana* (Ströher and Dietz, 2008).

Disulfide bonds are targeted and reduced by Trxs. The screening of Trxs targets was used for the identification of proteins form disulfide bonds. Based on the finding that the disulfide bonds between target proteins and Txrs became stabilized when the second cysteine of Trxs was mutated, the monocysteinic Trx trapping method was developed (Brandes *et al*., 1996; Goyer *et al*., 1999). Proteomic approaches, based on Trx affinity chromatography using immobilized monocysteinic Trx as a ligand, have been extensively employed for the identification of the disulfide proteome of Trx targets from plenty of plant species (Figure 1.17).

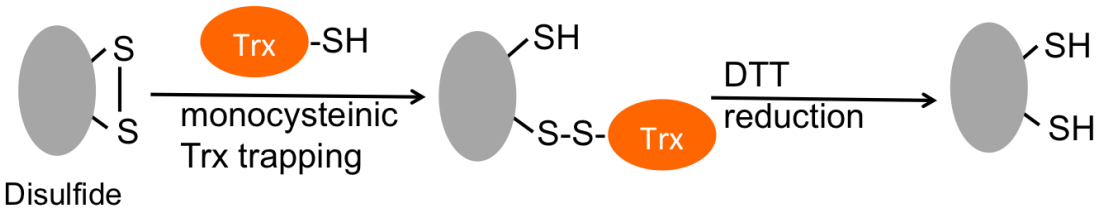


Figure 1.17 Schematic presentation of the strategy of Trx affinity chromatography. Using monocysteinic Trx as a ligand, a stable complex is formed between Trx and Trx target proteins. The bound Trx targets are eluted by DTT reduction.

In *A. thaliana* leaves, 40 Trx-target proteins were identified by ^{14}C IAM labeling (Marchand *et al*., 2004), but application of three different approaches; ^{14}C IAM labeling, PEO-iodoacetylbiotin, and mutated cytosolic Trx-h3 affinity, identified a total of 73 Trx-target proteins (Marchand *et al*., 2006). Affinity chromatography on a monocysteinic mutant of plastidial y-type Trx trapped 72 target proteins in Arabidopsis roots (Marchand *et al*., 2010). In wheat seeds, 23 Trx-target proteins were reported by mBBr labeling, whereas addition of mutant Trx-h affinity trapping further increased the identification number to 68 potential target proteins (Wong *et al*., 2003; Wong *et al*., 2004). Using Trx affinity strategy, the subcellular Trx target proteins have been identified, such as, 50 targets in mitochondria isolated from photosynthetic (pea and spinach leaves) and heterotrophic (potato tubers) sources (Balmer *et al*., 2004), 3 proteins in chloroplast envelope and stroma of barley (Bartsch *et al*., 2008), 1 protein reported in *Nicotianaalata* styles (Juárez-Díaz *et al*., 2006), 8 proteins in spinach chloroplast stroma (Motohashi *et al*., 2001a), 35 proteins in

spinach chloroplast stroma (Balmer *et al*., 2003), 19 Trx target proteins were identified in the chloroplast lumen of *A. thaliana* (Hall *et al*., 2010), 42 in amyloplast wheat (Balmer *et al*., 2006). Plasma membrane proteins from *A. thaliana* cell cultures were screened and a total of 48 candidate proteins were obtained (Ueoka-Nakanishi *et al*., 2013).

1.5.2.3 S-glutathionylation (S-SG). A number of direct methods have been developed to identify and analyze plant S-glutathionylated proteins (Figure 1.18). The most widely used techniques for S-glutathionylated proteomic analyses in plants are based on the use of ^{35}S-radiolabeled cysteine, biotinylated glutathione (GSH-biotin/GSSG-biotin), or biotinylated reduced glutathione ethyl ester (GEE-biotin) (Ito *et al.*, 2003; Dixon *et al*., 2005; Michelet *et al*., 2005; Michelet *et al*., 2008; Zaffagnini *et al.*, 2012a).

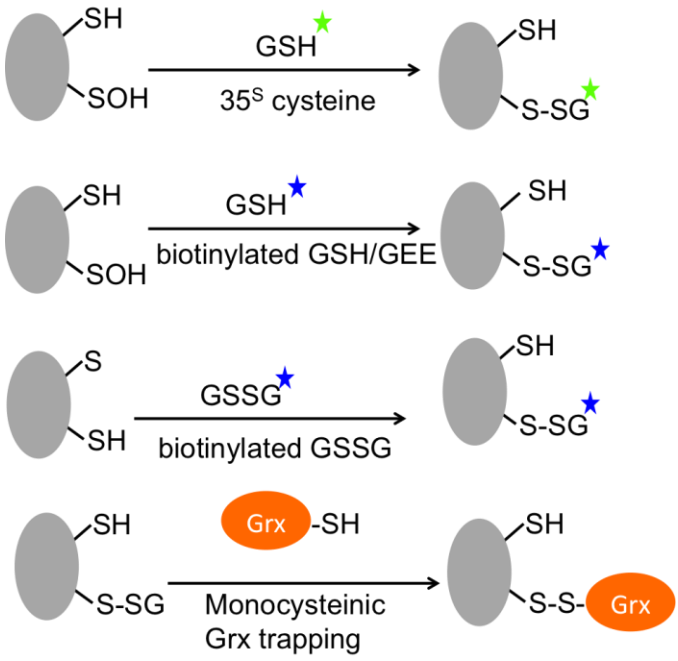


Figure 1.18 Schematic presentation of the strategies used to trap plant S-SG proteome. Radiolabeled GSH pool/ biotinylated GSH & GEE reacts with sulfenic acids to form S-SG complexes whereas biotinylated GSSG reacts with thiolates. Using monocysteinic Grx as a ligand, a stable complex is formed between Grx and Grx target proteins

With the ^{35}S-radiolabeled cysteine technique, the glutathione pool of growing cells will be labeled. The first step of this method is the inhibition of protein synthesis in

both the cytosol and organelles by cycloheximide, followed by incubation with radiolabeled cysteine for four hours, so that it enters the cell and gets incorporated into glutathione molecules (Dixon *et al*., 2005). After an oxidative stress treatment, radiolabeled -SSG proteins are visualized by fluorography of a 2D-gel. The spots in the gel that disappear after the reduction with DTT correspond to those of S-glutathionylated proteins. Subsequently, the proteins of these spots are identified by mass spectrometry.

With a biotinylated glutathione labeling technique, the biotin tag allows downstream analysis of the glutathionylated proteins by Western blot or avidin affinity chromatography (Michelet *et al.*, 2008). Based on the same principle as for monocysteinic Trx trapping, monocysteinic Grx trapping also has been developed for the identification of S-glutathionylated proteins (Nordstrand *et al*., 1999). The use of a monocysteinic mutant of Grx (poplar GrxC4 mutated on the second active-site cysteine) has led to the identification of the Grx-interacting proteins from poplar, Arabidopsis, potato and pea (Rouhier *et al*., 2005) (Table 1.2). Other strategies, like biotin labeled GST (Cheng *et al*., 2005), and immobilized GSH/GSSG affinity matrices to trap the glutathionylated proteins (Niture *et al*., 2005), have not been applied for plant proteomic studies so far.

1.5.2.4 Sulfinylation (S-O_2H) and sulfonylation (S-O_3H). Sulfinylated (-SO_2H) proteins can be detected by mass spectrometry based on a 32 Da increase in the mass (Witze *et al*., 2007). However, confusion might occur, as hydrogen sulfide leads to persulfide species (RSSH) with the same nominal mass shift. Therefore, it is necessary to develop specific methods for monitoring the formation of -SO_2H within proteins. Antibodies directed against proteins containing -SO_2H have been developed (Woo *et al*., 2003), but they are not suited for global profiling studies. Apart from that, aryl-nitroso compounds can act as chemo-selective probes for -SO_2H (Lo Conte and Carroll, 2012), which might open a new avenue for studying -SO_2H at the proteomic level. Like -SO_2H, the role of -SO_3H in redox regulation has for a long time been hindered by a lack of means to selectively detect this irreversible Cys Ox-PTMs. Recently, a method has been developed that permits selective enrichment of proteins containing -SO_3H using poly-arginine (PA)-coated nano-diamonds as high affinity probes (Chang *et al*., 2010), but due to the competition with phosphorylated peptides, the number of detected -SO_3H containing proteins is limited.

Table 1.2 Differential alkylation based indirect proteomics for reversible Cys Ox-PTMs mediated by oxidative stress

PTMs	Targeting			Isolation	Identification	Examples in plants
	Blocking	Reduction	Labeling			
All reversible PTMs (SOH, S-S, SSG)	mBBr	DTT/ TCEP	IAM	2-DE	MS	79 proteins and 193 redox active cysteines in dormant, non-dormant, abscisic, or gibberellic acid-treated wheat (Bykova *et al*., 2011)
	IAM		mBBr			22 proteins/ root and shoot of MeJA treated Arabidopsis (Alvarez *et al*., 2009)
			cysTMT	Enrichment by anti-TMT resin		Redox proteome of the tomato leaves under *Pseudomonas syringae*treatment (Parker *et al*., 2012)
	NEM		Biotin-IAM & IAF	BIAM-IP/IAF 2-DE		84 proteins in 10 min H_2O_2 treated Arabidopsis cells (Wang *et al*., 2012)
			IAF	2-DE		22 proteins in ozone treated soybean (Galant *et al*., 2012)
			Biotin-maleimide	2-DE/Streptavidin enrichment		17 chloroplast proteins in H_2O_2-treated Arabidopsis and 24 proteins in MV-treated samples (Muthuramalingam *et al*., 2013)
			Biotin-HDPD	Avidin affinity purification >iTRAQ		179 proteins in Arabidopsis cells under H_2O_2 stress, 164 cysteine in 150 proteins are found oxidized in the H_2O_2-treated sample than in the control, whereas 31 cysteines from 29 proteins are in a more reduced state in the treated sample in Arabidopsis cells (Liu *et al*., 2014)
Sufenylation (SOH)	IAM/NE	Arsenite	Biotin-NEM	Streptavidin affinity		—

	M			purification>2-DE		
	IAM/^{12}C IAM		IAM based-ICAT	2-DE		40 targets in Arabidopsis leaves (Marchand *et al*., 2004)
	IAM		TCEP >Heavy (^{13}C) ICAT & light (^{12}C) ICAT labeling	Avidin affinity purification		104 targets in germinating seed embryos of Barley (Hägglund *et al*., 2008)
Disulfide bond (S-S)		Trx	mBBr /cyanine 5 maleimide	2-DE (IEF/SDS), 2-DE (Non-reducing/ reducing)	Amino acid sequencing /MS	8 targets in germinating seeds of Barley (Marx *et al*., 2003); 6 targets in Barley seed proteome by mBBr labeling and 16 proteins by cyanine 5 maleimide labeling (Maeda *et al*., 2004); 45 targets in axes and cotyledons seeds in *Medicago trunculata* (Alkhalfioui *et al*., 2007); 23 targets in wheat seeds (Wong *et al*., 2003); 68 targets in wheat (Wong *et al*., 2004); 1 target in *Nicotianaalata* styles (Juárez-Díaz *et al*., 2006); 20 targets identified in *Arachishypogaea* dry seeds (Yano *et al*., 2001); 4 targets in rice (Yano and Kuroda, 2006).
S-glutathionylation (S-SG)	NEM	Grx	BNEM	Avidin affinity purification, 2-DE	MS	—

Table 1.3 Direct proteomics for Cys Ox-PTMs mediated by oxidative stress

Cys-PTMs	Method	Targeting	Isolation	Identification	Examples in plants
Sulfenylation (SOH)	Probe	DCP-Bio1 (*in vitro*)	Streptavidin affinity	MS	44 sulfenylated proteins 2 dpi and 65 in 4 dpi in *M. truncatula* (Oger *et al*., 2012)
		DAz-2 (*in vivo*)	Biotinylation by click reaction following Streptavidin affinity		—
		DYn-2 (*in vivo*)			276 sulfenylated proteins in H_2O_2 treated Arabidopsis cells (chapter 3)
		YAP1 based genetic (*in vivo*)	Tandem affinity purification		4 proteins in *M. Truncatula* (Oger *et al*., 2012), 97 cytoplasmic sulfenylated proteins in H_2O_2 treated Arabidopsis cells (Waszczak *et al*., 2014)
		β-ketoesters (*in vivo*)	Biotinylation by click reaction following Streptavidin -HRP		—
		BCN (*in vivo*)			—
Disulfide bonds (S-S)	Redox 2D-PAGE		2-DE gel		2 pea leaf chloroplast thylakoid membrane proteins(Anderson and Manabe, 1979); 18 proteins in Arabidopsis mitochondria (Winger *et al*., 2007); 22 proteins Arabidopsis stroma (Ströher and Dietz, 2008)
	Trx affinity trapping	Mutated Trx	Trx affinity purification >SDS-PAGE, 2-DE (IEF/SDS), 2-DE (Non-reducing/reducing)		14 targets in dark-grown *A. thaliana* (Yamazaki *et al*., 2004);19 targets in the chloroplast lumen of *A. thaliana* (Hall *et al*., 2010); 73 targets in *A. thaliana* (Marchand *et al*., 2006); 9 proteins in Arabidopsis thylakoid lumen (Motohashi and Hisabori, 2006). For all other plant examples we refer to (Montrichard*et al.*, 2009)
S-glutathionylation (S-SG)	Direct labeling	35S radiolabeling	Fluorography, 2-DE (Reducing/non-reducing)		25 chloroplastic proteins in diamide treated *Chlamydomonas reinhardtii* (Michelet *et al*., 2008); Unsuccessful in Arabidopsis cell (Dixon et al., 2005)
		GSSG-biotin	Streptavidin affinity		79 proteins in BHP (tert-butyl hydroperoxide) treated

		labeling	enrichment, 2-DE gel	Arabidopsis (Dixon et al., 2005), 225 proteins in H_2O_2/ diamide treated *Chlamydomonas reinhardtii* (Zaffagnini et al., 2012b)
		BioGEE labeling	Anion exchange chromatography, Hydrophobic interaction chromatography	20 proteins under 1 mM H_2O stress for 10 min in suspension-cultured cells of Arabidopsis (Ito *et al*., 2003)
	Grx affinity trapping	Mutated Grx	Grx affinity purification	94 targets from poplar leaves and stems, *A. thaliana* leaves, *A. thaliana* or potato mitochondria, and pea chloroplast stroma (Rouhier *et al*., 2005)

1.2 References

Alergand T, Peled-Zehavi H, Katz Y, Danon A (2006) The chloroplast protein disulfide isomerase RB60 reacts with a regulatory disulfide of the RNA-binding protein RB47. Plant Cell Physiol **47**: 540–8

Alkhalfioui F, Renard M, Vensel WH, Wong J, Tanaka CK, Hurkman WJ, Buchanan BB, Montrichard F (2007) Thioredoxin-linked proteins are reduced during germination of *Medicago truncatula* seeds. Plant Physiol **144**: 1559–79

Alvarez S, Zhu M, Chen S (2009) Proteomics of Arabidopsis redox proteins in response to methyl jasmonate. J Proteomics **73**: 30–40

Anderson LE, Manabe K (1979) Disulfide-linked peptides in the chloroplast thylakoid membrane. Biochim Biophys Acta **579**: 1–9

Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol **55**: 373–99

Apostol I, Heinstein PF, Low PS (1989) Rapid Stimulation of an Oxidative Burst during Elicitation of Cultured Plant Cells: Role in Defense and Signal Transduction. Plant Physiol **90**: 109–16

Ariel F, Manavella P, Dezar C, Chan R (2007) The true story of the HD-Zip family. Trends Plant Sci **12**: 419–26

Bachi A, Dalle-Donne I, Scaloni A (2013) Redox proteomics: chemical principles, methodological approaches and biological/biomedical promises. Chem Rev **113**: 596–698

Balmer Y, Koller A, del Val G, Manieri W, Schürmann P, Buchanan BB (2003) Proteomics gives insight into the regulatory function of chloroplast thioredoxins. Proc Natl Acad Sci U S A **100**: 370–5

Balmer Y, Koller A, Val G Del, Schürmann P, Buchanan BB (2004) Proteomics uncovers proteins interacting electrostatically with thioredoxin in chloroplasts. Photosynth Res **79**: 275–80

Balmer Y, Vensel WH, Cai N, Manieri W, Schürmann P, Hurkman WJ, Buchanan BB (2006) A complete ferredoxin/thioredoxin system regulates fundamental processes in amyloplasts. Proc Natl Acad Sci U S A **103**: 2988–93

Bartels S, Anderson JC, González Besteiro M a, Carreri A, Hirt H, Buchala A, Métraux J-P, Peck SC, Ulm R, Gonzalez Besteiro MA, et al., (2009) MAP kinase phosphatase1 and protein tyrosine phosphatase1 are repressors of salicylic acid synthesis and SNC1-mediated responses in Arabidopsis. Plant Cell **21**: 2884–97

Bartsch S, Monnet J, Selbach K, Quigley F, Gray J, von Wettstein D, Reinbothe S, Reinbothe C (2008) Three thioredoxin targets in the inner envelope membrane of chloroplasts function in protein import and chlorophyll metabolism. Proc Natl Acad Sci U S A **105**: 4933–8

Benitez L V, Allison WS (1974) The inactivation of the acyl phosphatase activity catalyzed by the sulfenic acid form of glyceraldehyde 3-phosphate dehydrogenase by dimedone and olefins. J Biol Chem **249**: 6234–43

Brandes HK, Larimer FW, Hartman FC (1996) The molecular pathway for the regulation of phosphoribulokinase by thioredoxin f. J Biol Chem **271**: 3333–5

Buchanan BB, Balmer Y (2005) Redox regulation: a broadening horizon. Annu Rev Plant Biol **56**: 187–220

Bykova N V, Hoehn B, Rampitsch C, Banks T, Stebbing J-A, Fan T, Knox R (2011) Redox-sensitive proteome and antioxidant strategies in wheat seed dormancy control. Proteomics **11**: 865–82

Chang Y-C, Huang C-N, Lin C-H, Chang H-C, Wu C-C (2010) Mapping protein cysteine sulfonic acid modifications with specific enrichment and mass spectrometry: an integrated approach to explore the cysteine oxidation. Proteomics **10**: 2961–71

Charles RL, Schröder E, May G, Free P, Gaffney PRJ, Wait R, Begum S, Heads RJ, Eaton P (2007) Protein sulfenation as a redox sensor: proteomics studies using a novel biotinylated dimedone analogue. Mol Cell Proteomics **6**: 1473–84

Cheng G, Ikeda Y, Iuchi Y, Fujii J (2005) Detection of S-glutathionylated proteins by glutathione S-transferase overlay. Arch Biochem Biophys **435**: 42–9

Colcombet J, Hirt H (2008) Arabidopsis MAPKs: a complex signalling network involved in multiple biological processes. Biochem J **413**: 217–26

Collet J-F, Messens J (2010) Structure, function, and mechanism of thioredoxin proteins. Antioxid Redox Signal **13**: 1205–16

Comelli RN, Gonzalez DH (2007) Conserved homeodomain cysteines confer redox sensitivity and influence the DNA binding properties of plant class III HD-Zip proteins. Arch Biochem Biophys **467**: 41–7

Lo Conte M, Carroll KS (2012) Chemoselective ligation of sulfinic acids with aryl-nitroso compounds. Angew Chem Int Ed Engl **51**: 6502–5

Cubas P, Lauter N, Doebley J, Coen E (1999) The TCP domain: a motif found in proteins regulating plant growth and development. Plant J **18**: 215–22

Cumming RC (2008) Analysis of global and specific changes in the disulfide proteome using redox two-dimensional polyacrylamide gel electrophoresis. Methods Mol Biol **476**: 165–79

Danquah A, de Zelicourt A, Colcombet J, Hirt H (2013) The role of ABA and MAPK signaling pathways in plant abiotic stress responses. Biotechnol Adv **32**: 40–52

Davies MJ (2005) The oxidative environment and protein damage. Biochim Biophys Acta **1703**: 93–109

Delaunay A, Isnard A-D, Toledano M (2000) H2O2 sensing through oxidation of the Yap1 transcription factor. EMBO J **19**: 5157–66

Delaunay A, Pflieger D, Barrault MB, Vinh J, Toledano MB (2002) A thiol peroxidase is an H2O2 receptor and redox-transducer in gene activation. Cell **111**: 471–81

Delledonne M, Xia Y, Dixon RA, Lamb C (1998) Nitric oxide functions as a signal in plant disease resistance. Nature **394**: 585–8

Delledonne M, Zeier J, Marocco A, Lamb C (2001) Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive disease resistance response. Proc Natl Acad Sci U S A **98**: 13454–9

Denu JM, Tanner KG (1998) Specific and reversible inactivation of protein tyrosine phosphatases by hydrogen peroxide: evidence for a sulfenic acid intermediate and implications for redox regulation. Biochemistry **37**: 5633–42

Desikan R, Hancock JT, Bright J, Harrison J, Weir I, Hooley R, Neill SJ (2005) A Role for ETR1 in Hydrogen Peroxide Signaling in Stomatal Guard Cells 1. **137**: 831–834

Desikan R, Last K, Harrett-Williams R, Tagliavia C, Harter K, Hooley R, Hancock JT, Neill SJ (2006) Ethylene-induced stomatal closure in Arabidopsis occurs via AtrbohF-mediated hydrogen peroxide synthesis. Plant J **47**: 907–16

Després C, Chubak C, Rochon A, Clark R, Bethune T, Desveaux D, Fobert PR (2003) Arabidopsis NPR1 disease resistance protein is a novel cofactor that confers redox regulation of DNA binding activity to the basic domain/leucine zipper transcription. Plant Cell **15**: 2181–2191

Dietz K-J (2014) Redox Regulation of Transcription Factors in Plant Stress Acclimation and Development. Antioxid Redox Signal. doi: 10.1089/ars.2013.5672

Dixon DPD, Skipsey M, Grundy NMN, Edwards R, Sciences B, Kingdom U (2005) Stress-induced protein S-glutathionylation in Arabidopsis. Plant Physiol **138**: 2233–2244

Dominguez-Solis J, He Z (2008) A cyclophilin links redox and light signals to cysteine biosynthesis and stress responses in chloroplasts. Proc. **105**: 16386–16391

Durrant WE, Dong X (2004) Systemic acquired resistance. Annu Rev Phytopathol **42**: 185–209

Ellis HR, Poole LB (1997) Novel application of 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole to identify cysteine sulfenic acid in the AhpC component of alkyl hydroperoxide reductase. Biochemistry **36**: 15013–15018

Feng W-K, Wang L, Lu Y, Wang X-Y (2011) A protein oxidase catalysing disulfide bond formation is localized to the chloroplast thylakoids. FEBS J **278**: 3419–30

Fernandes AP, Holmgren A (2004) Glutaredoxins: glutathione-dependent redox enzymes with functions far beyond a simple thioredoxin backup system. Antioxid Redox Signal **6**: 63–74

Galant A, Koester RP, Ainsworth E a, Hicks LM, Jez JM (2012) From climate change to molecular response: redox proteomics of ozone-induced responses in soybean. New Phytol **194**: 220–9

Gan ZR, Sardana MK, Jacobs JW, Polokoff MA (1990) Yeast thioltransferase--the active site cysteines display differential reactivity. Arch Biochem Biophys **282**: 110–5

Geiger D, Scherzer S, Mumm P, Marten I, Ache P, Matschi S, Liese a, Wellmann C, Al-Rasheid K a S, Grill E, et al (2010) Guard cell anion channel SLAC1 is regulated by CDPK protein kinases with distinct Ca2+ affinities. Proc Natl Acad Sci U S A **107**: 8023–8

Go Y-MY, Jones DDP (2008) Redox compartmentalization in eukaryotic cells. Biochim Biophys Acta (BBA)-General Subj **1780**: 1273–1290

Goldman R, Stoyanovsky DA, Day BW, Kagan VE (1995) Reduction of Phenoxyl Radicals by Thioredoxin Results in Selective Oxidation of Its SH-Groups to Disulfides. An Antioxidant Function of Thioredoxin. Biochemistry **34**: 4765–4772

Goyer A, Decottignies P, Lemaire S, Ruelland E, Issakidis-Bourguet E, Jacquot J-P, Miginiac-Maslow M (1999) The internal Cys-207 of sorghum leaf NADP-malate dehydrogenase can form mixed disulphides with thioredoxin. FEBS Lett **444**: 165–169

Grotewold E, Williams CE (1997) Differences between Plant and Animal Myb Domains Are Fundamental for DNA Binding Activity, and Chimeric Myb Domains Have Novel DNA Binding Specificities. J Biol Chem **272**: 563–571

Gupta R, Luan S (2003) Redox Control of Protein Tyrosine Phosphatases and Mitogen-Activated Protein Kinases in Plants. PLANT Physiol **132**: 1149–1152

Gupta V, Carroll KS (2014) Sulfenic acid chemistry, detection and cellular lifetime. Biochim Biophys Acta **1840**: 847–75

Gutscher M, Sobotta MC, Wabnitz GH, Ballikaya S, Meyer AJ, Samstag Y, Dick TP (2009) Proximity-based protein thiol oxidation by H_2O_2-scavenging peroxidases. J Biol Chem **284**: 31532–40

Hägglund P, Bunkenborg J, Maeda K, Svensson B, Per Hagglund, Jakob Bunkenborg, Kenji Maeda and BS, Enzyme (2008) Identification of Thioredoxin Disulfide Targets Using a Quantitative Proteomics Approach Based on Isotope-Coded Affinity Tags Per Ha research articles. J Proteome Res **7**: 5270–5276

Hahn A, Harter K (2009) Mitogen-activated protein kinase cascades and ethylene: signaling, biosynthesis, or both? Plant Physiol **149**: 1207–10

Hall M, Mata-Cabana A, Akerlund H-E, Florencio FJ, Schröder WP, Lindahl M, Kieselbach T (2010) Thioredoxin targets of the plant chloroplast lumen and their implications for plastid function. Proteomics **10**: 987–1001

Hansen RE, Roth D, Winther JR (2009) Quantifying the global cellular thiol-disulfide status. Proc Natl Acad Sci U S A **106**: 422–7

Heine GF, Hernandez JM, Grotewold E (2004) Two cysteines in plant R2R3 MYB domains participate in REDOX-dependent DNA binding. J Biol Chem **279**: 37878–85

Herrero E, de la Torre-Ruiz MA (2007) Monothiol glutaredoxins: a common domain for multiple functions. Cell Mol Life Sci **64**: 1518–30

Huang G-T, Ma S-L, Bai L-P, Zhang L, Ma H, Jia P, Liu J, Zhong M, Guo Z-F (2012) Signal transduction during cold, salt, and drought stresses in plants. Mol Biol Rep **39**: 969–87

Iglesias-Baena I, Barranco-medina S, Sevilla F, Lázaro J-J (2011) The dual-targeted plant sulfiredoxin retroreduces the sulfinic form of atypical mitochondrial peroxiredoxin. Plant Physiol **155**: 944–55

Ito H, Iwabuchi M, Ogawa K (2003) The sugar-metabolic enzymes aldolase and triose-phosphate isomerase are targets of glutathionylation in *Arabidopsis thaliana*: detection using biotinylated glutathione. Plant Cell Physiol **44**: 655–60

Juárez-Díaz JA, McClure B, Vázquez-Santana S, Guevara-García A, León-Mejía P, Márquez-Guzmán J, Cruz-García F (2006) A novel thioredoxin h is secreted in Nicotiana alata and reduces S-RNase in vitro. J Biol Chem **281**: 3418–24

Katiyar A, Smita S, Lenka SK, Rajwanshi R, Chinnusamy V, Bansal KC (2012) Genome-wide classification and expression analysis of MYB transcription factor families in rice and Arabidopsis. BMC Genomics **13**: 544

Kieselbach T (2013) Oxidative folding in chloroplasts. Antioxid Redox Signal **19**: 72–82

Kim J, Mayfield SP (1997) Protein Disulfide Isomerase as a Regulator of Chloroplast Translational Activation. Science (80-) **278**: 1954–1957

Klessig DF, Durner J, Noad R, Navarre DA, Wendehenne D, Kumar D, Zhou JM, Shah J, Zhang S, Kachroo P, et al (2000) Nitric oxide and salicylic acid signaling in plant defense. Proc Natl Acad Sci U S A **97**: 8849–55

Klomsiri C, Nelson KJ, Bechtold E, Soito L, Johnson LC, Lowther WT, Ryu S-E, King SB, Furdui CM, Poole LB (2010) Use of dimedone-based chemical probes for sulfenic acid detection evaluation of conditions affecting probe incorporation into redox-sensitive proteins. Methods Enzymol **473**: 77–94

Konopka-Postupolska D, Clark G, Goch G, Debski J, Floras K, Cantero A, Fijolek B, Roux S, Hennig J (2009) The role of annexin 1 in drought stress in Arabidopsis. Plant Physiol **150**: 1394–410

Kovtun Y, Chiu WL, Tena G, Sheen J (2000) Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. Proc Natl Acad Sci U S A **97**: 2940–5

Kwak JM, Mori IC, Pei Z-M, Leonhardt N, Torres MA, Dangl JL, Bloom RE, Bodde S, Jones JDG, Schroeder JI (2003) NADPH oxidase AtrbohD and AtrbohF genes function in ROS-dependent ABA signaling in Arabidopsis. EMBO J **22**: 2623–33

Laloi C, Apel K, Danon A (2004) Reactive oxygen signalling: the latest news. Curr Opin Plant Biol **7**: 323–8

Laohavisit A, Brown AT, Cicuta P, Davies JM (2010) Annexins: components of the calcium and reactive oxygen signaling network. Plant Physiol **152**: 1824–9

Laohavisit A, Davies JM (2011) Annexins. New Phytol **189**: 40–53

Laohavisit A, Shang Z, Rubio L, Cuin T a, Véry A-A, Wang A, Mortimer JC, Macpherson N, Coxon KM, Battey NH, et al (2012) Arabidopsis annexin1 mediates the radical-activated plasma membrane Ca^{2}+- and K+-permeable conductance in root cells. Plant Cell **24**: 1522–33

Lee J-W, Helmann JD (2006) Biochemical characterization of the structural Zn2+ site in the Bacillus subtilis peroxide sensor PerR. J Biol Chem **281**: 23567–78

Lee JS, Ellis BE (2007) Arabidopsis MAPK phosphatase 2 (MKP2) positively regulates oxidative stress tolerance and inactivates the MPK3 and MPK6 MAPKs. J Biol Chem **282**: 25020–9

Lee JS, Wang S, Sritubtim S, Chen J-G, Ellis BE (2009) Arabidopsis mitogen-activated protein kinase MPK12 interacts with the MAPK phosphatase IBR5 and regulates auxin signaling. Plant J **57**: 975–85

Lemaire SD, Collin V, Keryer E, Quesada A, Miginiac-Maslow M (2003) Characterization of thioredoxin y, a new type of thioredoxin identified in the genome of Chlamydomonas reinhardtii. FEBS Lett **543**: 87–92

Lemaire SD, Guillon B, Le Maréchal P, Keryer E, Miginiac-Maslow M, Decottignies P (2004) New thioredoxin targets in the unicellular photosynthetic eukaryote Chlamydomonas reinhardtii. Proc Natl Acad Sci U S A **101**: 7475–80

Leonard SE, Carroll KS (2011) Chemical "omics" approaches for understanding protein cysteine oxidation in biology. Curr Opin Chem Biol **15**: 88–102

Leonard SE, Reddie KG, Carroll KS (2009) Mining the Thiol Proteome for Sulfenic Acid Modifications Reveals New Targets for Oxidation in Cells. ACS Chem Biol **4**: 783–799

Leustek T, Martin MN, Bick J-A, Davies JP (2000) Pathways and Regulation of Sulfur Metabolism Revealed through Molecular and Genetic Studies. Annu Rev Plant Physiol Plant Mol Biol **51**: 141–165

Levine A, Tenhaken R, Dixon R, Lamb C (1994) H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell **79**: 583–93

Li B, Zhao Y, Liang L, Ren H, Xing Y, Chen L, Sun M, Wang Y, Han Y, Jia H, et al (2012) Purification and characterization of ZmRIP1, a novel reductant-inhibited protein tyrosine phosphatase from maize. Plant Physiol **159**: 671–81

Li S, Lauri A, Ziemann M, Busch A, Bhave M, Zachgo S (2009) Nuclear activity of ROXY1, a glutaredoxin interacting with TGA factors, is required for petal development in *Arabidopsis thaliana*. Plant Cell **21**: 429–41

Lillig CH, Berndt C, Holmgren A (2008) Glutaredoxin systems. Biochim Biophys Acta **1780**: 1304–17

Lin WS, Armstrong DA, Gaucher GM (1975) Formation and repair of papain sulfenic acid. Can J Biochem **53**: 298–307

Lindermayr C, Saalbach G (2005) Proteomic Identification of S-Nitrosylated Proteins. **137**: 921–930

Lindermayr C, Sell S, Müller B, Leister D, Durner J (2010) Redox regulation of the NPR1-TGA1 system of *Arabidopsis thaliana* by nitric oxide. Plant Cell **22**: 2894–907

Little C, O'brien PJ (1969) Mechanism of Peroxide-Inactivation of the Sulphydryl Enzyme Glyceraldehyde-3-Phosphate Dehydrogenase. Eur J Biochem **10**: 533–538

Liu P, Zhang H, Wang H, Xia Y (2014) Identification of redox-sensitive cysteines in the Arabidopsis proteome using OxiTRAQ, a quantitative redox proteomics method. Proteomics **14**: 750–62

Lu Y, Wang H-R, Li H, Cui H-R, Feng Y-G, Wang X-Y (2013) A chloroplast membrane protein LTO1/AtVKOR involving in redox regulation and ROS homeostasis. Plant Cell Rep **32**: 1427–40

Lumbreras V, Vilela B, Irar S, Solé M, Capellades M, Valls M, Coca M, Pagès M (2010) MAPK phosphatase MKP2 mediates disease responses in Arabidopsis and functionally interacts with MPK3 and MPK6. Plant J **63**: 1017–30

Luo D, Smith SW, Anderson BD (2005) Kinetics and mechanism of the reaction of cysteine and hydrogen peroxide in aqueous solution. J Pharm Sci **94**: 304–16

Ma L-H, Takanishi CL, Wood MJ (2007) Molecular mechanism of oxidative stress perception by the Orp1 protein. J Biol Chem **282**: 31429–36

MacRobbie EA (2002) Evidence for a role for protein tyrosine phosphatase in the control of ion release from the guard cell vacuole in stomatal closure. Proc Natl Acad Sci U S A **99**: 11963–8

Maeda K, Finnie C, Svensson B (2005) Identification of thioredoxin h-reducible disulphides in proteomes by differential labelling of cysteines: insight into recognition and regulation of proteins in barley seeds by thioredoxin h. Proteomics **5**: 1634–44

Maeda K, Finnie C, Svensson B (2004) Cy5 maleimide labelling for sensitive detection of free thiols in native protein extracts: identification of seed proteins targeted by barley thioredoxin h isoforms. Biochem J **378**: 497–507

Marchand C, Le Maréchal P, Meyer Y, Decottignies P (2006) Comparative proteomic approaches for the isolation of proteins interacting with thioredoxin. Proteomics **6**: 6528–37

Marchand C, Le Maréchal P, Meyer Y, Miginiac-Maslow M, Issakidis-Bourguet E, Decottignies P (2004) New targets of Arabidopsis thioredoxins revealed by proteomic analysis. Proteomics **4**: 2696–706

Marchand CH, Vanacker H, Collin V, Issakidis-Bourguet E, Maréchal P Le, Decottignies P (2010) Thioredoxin targets in Arabidopsis roots. Proteomics **10**: 2418–28

Marino S, Gladyshev V (2010) Cysteine function governs its conservation and degeneration and restricts its utilization on protein surfaces. J Mol Biol **404**: 902–916

Martín-Trillo M, Cubas P (2010) TCP genes: a family snapshot ten years later. Trends Plant Sci **15**: 31–9

Marx C, Wong JH, Buchanan BB (2003) Thioredoxin and germinating barley: targets and protein redox changes. Planta **216**: 454–60

Masucci JD, Rerie WG, Foreman DR, Zhang M, Galway ME, Marks MD, Schiefelbein JW (1996) The homeobox gene GLABRA 2 is required for position-dependent cell differentiation in the root epidermis of *Arabidopsis thaliana*. Developent **122**: 1253–1260

Meinhard M, Grill E (2001) Hydrogen peroxide is a regulator of ABI1, a protein phosphatase 2C from Arabidopsis. FEBS Lett **508**: 443–6

Meinhard M, Rodriguez PL, Grill E (2002) The sensitivity of ABI2 to hydrogen peroxide links the abscisic acid-response regulator to redox signalling. Planta **214**: 775–82

Merlot S, Gosti F, Guerrier D, Vavasseur a, Giraudat J (2001) The ABI1 and ABI2 protein phosphatases 2C act in a negative feedback regulatory loop of the abscisic acid signalling pathway. Plant J **25**: 295–303

Messens J, Collet J-F (2013) Thiol-disulfide exchange in signaling: disulfide bonds as a switch. Antioxid Redox Signal **18**: 1594–6

Mestres-Ortega D, Meyer Y (1999) The *Arabidopsis thaliana* genome encodes at least four thioredoxins m and a new prokaryotic-like thioredoxin. Gene **240**: 307–16

Meyer Y, Belin C, Delorme-Hinoux V, Reichheld J-P, Riondet C (2012) Thioredoxin and glutaredoxin systems in plants: molecular mechanisms, crosstalks, and functional significance. Antioxid Redox Signal **17**: 1124–60

Miao Y, Lv D, Wang P, Wang X-C, Chen J, Miao C, Song C-P (2006) An Arabidopsis glutathione peroxidase functions as both a redox transducer and a scavenger in abscisic acid and drought stress responses. Plant Cell **18**: 2749–66

Michelet L, Zaffagnini M, Marchand C, Collin V, Decottignies P, Tsan P, Lancelin J-M, Trost P, Miginiac-Maslow M, Noctor G, et al (2005) Glutathionylation of chloroplast thioredoxin f is a redox signaling mechanism in plants. Proc Natl Acad Sci U S A **102**: 16478–83

Michelet L, Zaffagnini M, Vanacker H, Le Maréchal P, Marchand C, Schroda M, Lemaire SD, Decottignies P (2008) In vivo targets of S-thiolation in Chlamydomonas reinhardtii. J Biol Chem **283**: 21571–8

Miller G, Suzuki N, Ciftci-Yilmaz S, Mittler R (2010) Reactive oxygen species homeostasis and signalling during drought and salinity stresses. Plant Cell Environ **33**: 453–67

Mittler R, Vanderauwera S, Gollery M, Van Breusegem F (2004) Reactive oxygen gene network of plants. Trends Plant Sci **9**: 490–8

Mittler R, Vanderauwera S, Suzuki N, Miller G, Tognetti VB, Vandepoele K, Gollery M, Shulaev V, Van Breusegem F (2011) ROS signaling: the new wave? Trends Plant Sci **16**: 300–9

Montrichard F, Alkhalfioui F, Yano H, Vensel WH, Hurkman WJ, Buchanan BB (2009) Thioredoxin targets in plants: the first 30 years. J Proteomics **72**: 452–74

Motohashi K, Hisabori T (2006) HCF164 receives reducing equivalents from stromal thioredoxin across the thylakoid membrane and mediates reduction of target proteins in the thylakoid lumen. J Biol Chem **281**: 35039–47

Motohashi K, Kondoh a, Stumpp MT, Hisabori T (2001a) Comprehensive survey of proteins targeted by chloroplast thioredoxin. Proc Natl Acad Sci U S A **98**: 11224–9

Motohashi K, Kondoh A, Stumpp MT, Hisabori T (2001b) Comprehensive survey of proteins targeted by chloroplast thioredoxin. Proc Natl Acad Sci U S A **98**: 11224–9

Motohashi K, Koyama F, Nakanishi Y, Ueoka-Nakanishi H, Hisabori T (2003) Chloroplast cyclophilin is a target protein of thioredoxin. Thiol modulation of the peptidyl-prolyl cis-trans isomerase activity. J Biol Chem **278**: 31848–52

Mou Z, Fan W, Dong X (2003) Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. Cell **113**: 935–44

Mukherjee K, Brocchieri L, Bürglin T (2009) A comprehensive classification and evolutionary analysis of plant homeobox genes. Mol Biol Evol **26**: 2775–94

Murmu J, Bush MJ, DeLong C, Li S, Xu M, Khan M, Malcolmson C, Fobert PR, Zachgo S, Hepworth SR (2010) Arabidopsis basic leucine-zipper transcription factors TGA9 and TGA10 interact with floral glutaredoxins ROXY1 and ROXY2 and are redundantly required for anther development. Plant Physiol **154**: 1492–504

Muthuramalingam M, Matros A, Scheibe R, Mock H-P, Dietz K-J (2013) The hydrogen peroxide-sensitive proteome of the chloroplast in vitro and in vivo. Front Plant Sci **4**: 54

Ndamukong I, Abdallat A Al, Thurow C, Fode B, Zander M, Weigel R, Gatz C (2007) SA-inducible Arabidopsis glutaredoxin interacts with TGA factors and suppresses JA-responsive PDF1.2 transcription. Plant J **50**: 128–39

Neill SJ, Desikan R, Clarke A, Hurst RD, Hancock JT (2002) Hydrogen peroxide and nitric oxide as signalling molecules in plants. J Exp Bot **53**: 1237–47

Nelson JW, Creighton TE (1994) Reactivity and ionization of the active site cysteine residues of DsbA, a protein required for disulfide bond formation in vivo. Biochemistry **33**: 5974–83

Niture SK, Velu CS, Bailey NI, Srivenugopal KS (2005) S-thiolation mimicry: quantitative and kinetic analysis of redox status of protein cysteines by glutathione-affinity chromatography. Arch Biochem Biophys **444**: 174–84

Nordstrand K, slund F, Holmgren A, Otting G, Berndt KD (1999) NMR structure of Escherichia coli glutaredoxin 3-glutathione mixed disulfide complex: implications for the enzymatic mechanism. J Mol Biol **286**: 541–52

Oger E, Marino D, Guigonis J-M, Pauly N, Puppo A (2012) Sulfenylated proteins in the *Medicago truncatula-Sinorhizobium meliloti* symbiosis. J Proteomics **75**: 4102–13

Park S-W, Li W, Viehhauser A, He B, Kim S, Nilsson AK, Andersson MX, Kittle JD, Ambavaram MMR, Luan S, et al (2013) Cyclophilin 20-3 relays a 12-oxo-phytodienoic acid signal during stress responsive regulation of cellular redox homeostasis. Proc Natl Acad Sci U S A **110**: 9559–64

Parker J, Zhu N, Zhu M, Chen S (2012) Profiling thiol redox proteome using isotope tagging mass spectrometry. J Vis Exp **24**: 66 doi: 10.3791/3766

Paulsen CE, Carroll KS (2013) Cysteine-mediated redox signaling: chemistry, biology, and tools for discovery. Chem Rev **113**: 4633–79

Paulsen CE, Truong TH, Garcia FJ, Homann A, Gupta V, Leonard SE, Carroll KS (2012) Peroxide-dependent sulfenylation of the EGFR catalytic site enhances kinase activity. Nat Chem Biol **8**: 57–64

Pauwels L, Goossens A (2011) The JAZ proteins: a crucial interface in the jasmonate signaling cascade. Plant Cell **23**: 3089–100

Pei ZM, Murata Y, Benning G, Thomine S, Klüsener B, Allen GJ, Grill E, Schroeder JI (2000) Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. Nature **406**: 731–4

Peskin A V, Low FM, Paton LN, Maghzal GJ, Hampton MB, Winterbourn CC (2007) The high reactivity of peroxiredoxin 2 with H_2O_2 is not reflected in its reaction with other oxidants and thiol reagents. J Biol Chem **282**: 11885–92

Pitzschke A, Hirt H (2009) Disentangling the complexity of mitogen-activated protein kinases and reactive oxygen species signaling. Plant Physiol **149**: 606–15

Poole LB, Klomsiri C, Knaggs S a, Furdui CM, Nelson KJ, Thomas MJ, Fetrow JS, Daniel LW, King SB (2007) Fluorescent and affinity-based tools to detect cysteine sulfenic acid formation in proteins. Bioconjug Chem **18**: 2004–17

Poole LB, Nelson KJ (2008) Discovering mechanisms of signaling-mediated cysteine oxidation. Curr Opin Chem Biol **12**: 18–24

Poole LB, Zeng B-B, Knaggs SA, Yakubu M, King SB (2005) Synthesis of chemical probes to map sulfenic acid modifications on proteins. Bioconjug Chem **16**: 1624–8

Poole TH, Reisz J a, Zhao W, Poole LB, Furdui CM, King SB (2014) Strained cycloalkynes as new protein sulfenic acid traps. J Am Chem Soc **136**: 6167–70

Prigge M, Otsuga D, Alonso J, Ecker J, Drews G, Clark S (2005) Class III Homeodomain-Leucine Zipper Gene Family Members Have Overlapping , Antagonistic , and Distinct Roles in Arabidopsis Development. Plant Cell **17**: 61–76

Qian J, Wani R, Klomsiri C, Poole LB, Tsang AW, Furdui CM (2012) A simple and effective strategy for labeling cysteine sulfenic acid in proteins by utilization of β-ketoesters as cleavable probes. Chem Commun (Camb) **48**: 4091–3

Rasmussen MW, Roux M, Petersen M, Mundy J (2012) MAP Kinase Cascades in Arabidopsis Innate Immunity. Front Plant Sci **3**: 169

Reddie KG, Carroll KS (2008) Expanding the functional diversity of proteins through cysteine oxidation. Curr Opin Chem Biol **12**: 746–54

Reddie KG, Seo YH, Muse Iii WB, Leonard SE, Carroll KS (2008) A chemical approach for detecting sulfenic acid-modified proteins in living cells. Mol Biosyst **4**: 521–31

Rerie WG, Feldmann K a, Marks MD (1994) The GLABRA2 gene encodes a homeo domain protein required for normal trichome development in Arabidopsis. Genes Dev **8**: 1388–1399

Rey P, Bécuwe N, Barrault M-B, Rumeau D, Havaux M, Biteau B, Toledano MB (2007) The *Arabidopsis thaliana* sulfiredoxin is a plastidic cysteine-sulfinic acid reductase involved in the photooxidative stress response. Plant J **49**: 505–14

Richards SL, Laohavisit A, Mortimer JC, Shabala L, Swarbreck SM, Shabala S, Davies JM (2013) Annexin 1 regulates the H2O2 -induced calcium signature in *Arabidopsis thaliana* roots. Plant J 136–145

Roos G, Foloppe N, Messens J (2013) Understanding the pK(a) of redox cysteines: the key role of hydrogen bonding. Antioxid Redox Signal **18**: 94–127

Roos G, Messens J (2011) Protein sulfenic acid formation: from cellular damage to redox regulation. Free Radic Biol Med **51**: 314–26

Rouhier N, Couturier J, Jacquot J-P (2006) Genome-wide analysis of plant glutaredoxin systems. J Exp Bot **57**: 1685–96

Rouhier N, Gelhaye E, Jacquot J-P (2004) Plant glutaredoxins: still mysterious reducing systems. Cell Mol Life Sci **61**: 1266–77

Rouhier N, Lemaire SD, Jacquot J-P (2008) The role of glutathione in photosynthetic organisms: emerging functions for glutaredoxins and glutathionylation. Annu Rev Plant Biol **59**: 143–66

Rouhier N, Villarejo A, Srivastava M, Gelhaye E, Keech O, Droux M, Finkemeier I, Samuelsson G, Dietz KJ, Jacquot J-P, et al (2005) Identification of plant glutaredoxin targets. Antioxid Redox Signal **7**: 919–29

Saurin AT, Neubert H, Brennan JP, Eaton P (2004) Widespread sulfenic acid formation in tissues in response to hydrogen peroxide. Proc Natl Acad Sci U S A **101**: 17982–7

Schürmann P, Buchanan BB (2008) The ferredoxin/thioredoxin system of oxygenic photosynthesis. Antioxid Redox Signal **10**: 1235–74

Senkevich TG, White CL, Koonin E V, Moss B (2002) Complete pathway for protein disulfide bond formation encoded by poxviruses. Proc Natl Acad Sci U S A **99**: 6667–72

Seo YH, Carroll KS (2009a) Profiling protein thiol oxidation in tumor cells using sulfenic acid-specific antibodies. Proc Natl Acad Sci U S A **106**: 16163–8

Seo YH, Carroll KS (2009b) Facile synthesis and biological evaluation of a cell-permeable probe to detect redox-regulated proteins. Bioorg Med Chem Lett **19**: 356–9

Serpa V, Vernal J, Lamattina L, Grotewold E, Cassia R, Terenzi H (2007) Inhibition of AtMYB2 DNA-binding by nitric oxide involves cysteine S-nitrosylation. Biochem Biophys Res Commun **361**: 1048–53

Shaikhali J, Heiber I, Seidel T, Ströher E, Hiltscher H, Birkmann S, Dietz K-J, Baier M (2008) The redox-sensitive transcription factor Rap2.4a controls nuclear expression of 2-Cys peroxiredoxin A and other chloroplast antioxidant enzymes. BMC Plant Biol **8**: 48

Shaikhali J, Norén L, de Dios Barajas-López J, Srivastava V, König J, Sauer UH, Wingsle G, Dietz K-J, Strand Å (2012) Redox-mediated mechanisms regulate DNA binding activity of the G-group of basic region leucine zipper (bZIP) transcription factors in Arabidopsis. J Biol Chem **287**: 27510–25

Smykowski A, Zimmermann P, Zentgraf U (2010) G-Box binding factor1 reduces CATALASE2 expression and regulates the onset of leaf senescence in Arabidopsis. Plant Physiol **153**: 1321–31

Sohn J, Rudolph J (2003) Catalytic and chemical competence of regulation of cdc25 phosphatase by oxidation/reduction. Biochemistry **42**: 10060–70

Strader LC, Monroe-Augustus M, Bartel B (2008) The IBR5 phosphatase promotes Arabidopsis auxin responses through a novel mechanism distinct from TIR1-mediated repressor degradation. BMC Plant Biol **8**: 41

Ströher E, Dietz K-J (2008) The dynamic thiol-disulphide redox proteome of the *Arabidopsis thaliana* chloroplast as revealed by differential electrophoretic mobility. Physiol Plant **133**: 566–83

Tada Y, Spoel SH, Pajerowska-Mukhtar K, Mou Z, Song J, Wang C, Zuo J, Dong X (2008) Plant immunity requires conformational changes [corrected] of NPR1 via S-nitrosylation and thioredoxins. Science **321**: 952–6

Tajc SG, Tolbert BS, Basavappa R, Miller BL (2004) Direct determination of thiol pKa by isothermal titration microcalorimetry. J Am Chem Soc **126**: 10508–9

Takanishi CL, Ma L-H, Wood MJ (2007) A genetically encoded probe for cysteine sulfenic acid protein modification in vivo. Biochemistry **46**: 14725–32

Takanishi CL, Wood MJ (2011) A genetically encoded probe for the identification of proteins that form sulfenic acid in response to H2O2 in Saccharomyces cerevisiae. J Proteome Res **10**: 2715–24

Torres MA, Jones JDG, Dangl JL (2006) Reactive Oxygen Species Signaling in Response to Pathogens. **141**: 373–378

Trivedi DK, Yadav S, Vaid N, Tuteja N (2012) Genome wide analysis of Cyclophilin gene family from rice and Arabidopsis and its comparison with yeast. Plant Signal Behav **7**: 1653–66

Tron AE, Bertoncini CW, Chan RL, Gonzalez DH (2002) Redox regulation of plant homeodomain transcription factors. J Biol Chem **277**: 34800–7

Trujillo M, Clippe A, Manta B, Ferrer-Sueta G, Smeets A, Declercq J-P, Knoops B, Radi R (2007) Pre-steady state kinetic characterization of human peroxiredoxin 5: taking advantage of Trp84 fluorescence increase upon oxidation. Arch Biochem Biophys **467**: 95–106

Truong TH, Carroll KKS (2012) Bioorthogonal Chemical Reporters for Analyzing Protein Sulfenylation in Cells. Curr Protoc Chem Biol **4**: 101–122

Turchi L, Carabelli M, Ruzza V, Possenti M, Sassi M, Peñalosa A, Sessa G, Salvi S, Forte V, Morelli G, et al (2013) Arabidopsis HD-Zip II transcription factors control apical embryo development and meristem function. Development **140**: 2118–29

Tyther R, Ahmeda A (2010) P1. Tyther, R. & Ahmeda, A. Proteomic profiling of perturbed protein sulfenation in renal medulla of the spontaneously hypertensive rat. J. proteome 2678–2687

Ueoka-Nakanishi H, Sazuka T, Nakanishi Y, Maeshima M, Mori H, Hisabori T (2013) Thioredoxin h regulates calcium dependent protein kinases in plasma membranes. FEBS J **280**: 3220–31

Viola IL, Güttlein LN, Gonzalez DH (2013) Redox modulation of plant developmental regulators from the class I TCP transcription factor family. Plant Physiol **162**: 1434–47

Walia A, Lee JS, Wasteneys G, Ellis B (2009) Arabidopsis mitogen-activated protein kinase MPK18 mediates cortical microtubule functions in plant cells. Plant J **59**: 565–75

Wang H, Wang S, Lu Y, Alvarez S, Hicks LM, Ge X, Xia Y (2012) Proteomic analysis of early-responsive redox-sensitive proteins in Arabidopsis. J Proteome Res **11**: 412–24

Waszczak C, Akter S, Eeckhout D, Persiau G, Wahni K, Bodra N, Van Molle I, De Smet B, Vertommen D, Gevaert K, et al (2014) Sulfenome mining in *Arabidopsis thaliana*. Proc Natl Acad Sci U S A **111**: 11545–50

Williams JC, Sue C, Banting GS, Yang H, Glerum DM, Hendrickson W a, Schon E a (2005) Crystal structure of human SCO1: implications for redox signaling by a mitochondrial cytochrome c oxidase "assembly" protein. J Biol Chem **280**: 15202–11

Winger AM, Taylor NL, Heazlewood JL, Day DA, Millar AH (2007) Identification of intra- and intermolecular disulphide bonding in the plant mitochondrial proteome by diagonal gel electrophoresis. Proteomics **7**: 4158–70

Winterbourn CC, Metodiewa D (1999) Reactivity of biologically important thiol compounds with superoxide and hydrogen peroxide. Free Radic Biol Med **27**: 322–328

Witze ES, Old WM, Resing KA, Ahn NG (2007) Mapping protein post-translational modifications with mass spectrometry. Nat Methods **4**: 798–806

Wong JH, Balmer Y, Cai N, Tanaka CK, Vensel WH, Hurkman WJ, Buchanan BB (2003) Unraveling thioredoxin-linked metabolic processes of cereal starchy endosperm using proteomics. FEBS Lett **547**: 151–156

Wong JH, Cai N, Balmer Y, Tanaka CK, Vensel WH, Hurkman WJ, Buchanan BB (2004) Thioredoxin targets of developing wheat seeds identified by complementary proteomic approaches. Phytochemistry **65**: 1629–40

Woo HA, Kang SW, Kim HK, Yang K-S, Chae HZ, Rhee SG (2003) Reversible oxidation of the active site cysteine of peroxiredoxins to cysteine sulfinic acid. Immunoblot detection with antibodies specific for the hyperoxidized cysteine-containing sequence. J Biol Chem **278**: 47361–4

Xia X-J, Gao C-J, Song L-X, Zhou Y-H, Shi K, Yu J-Q (2014) Role of H_2O_2 dynamics in brassinosteroid-induced stomatal closure and opening in Solanum lycopersicum. Plant Cell Environ **37**: 2036–50

Xia X-J, Wang Y-J, Zhou Y-H, Tao Y, Mao W-H, Shi K, Asami T, Chen Z, Yu J-Q (2009) Reactive oxygen species are involved in brassinosteroid-induced stress tolerance in cucumber. Plant Physiol **150**: 801–14

Xing S, Lauri A, Zachgo S (2006) Redox regulation and flower development: a novel function for glutaredoxins. Plant Biol (Stuttg) **8**: 547–55

Xu Q, Fu HH, Gupta R, Luan S (1998) Molecular characterization of a tyrosine-specific protein phosphatase encoded by a stress-responsive gene in Arabidopsis. Plant Cell **10**: 849–57

Yamazaki D, Motohashi K, Kasama T, Hara Y, Hisabori T (2004) Target proteins of the cytosolic thioredoxins in *Arabidopsis thaliana*. Plant Cell Physiol **45**: 18–27

Yan J, Zhang C, Gu M, Bai Z, Zhang W, Qi T, Cheng Z, Peng W, Luo H, Nan F, et al (2009) The Arabidopsis CORONATINE INSENSITIVE1 protein is a jasmonate receptor. Plant Cell **21**: 2220–36

Yang J, Gupta V, Carroll KS, Liebler DC (2014) Site-specific mapping and quantification of protein S-sulphenylation in cells. Nat Commun **5**: 4776

Yano H, Kuroda M (2006) Disulfide proteome yields a detailed understanding of redox regulations: a model study of thioredoxin-linked reactions in seed germination. Proteomics **6**: 294–300

Yano H, Wong JH, Lee YM, Cho MJ, Buchanan BB (2001) A strategy for the identification of proteins targeted by thioredoxin. Proc Natl Acad Sci U S A **98**: 4794–9

Yohn CB, Cohen A, Rosch C, Kuchka MR, Mayfield SP (1998) Translation of the Chloroplast psbA mRNA Requires the Nuclear-encoded Poly(A)-binding Protein, RB47. J Cell Biol **142**: 435–442

Zaffagnini M, Bedhomme M, Groni H, Marchand CH, Puppo C, Gontero B, Cassier-Chauvat C, Decottignies P, Lemaire SD (2012a) Glutathionylation in the photosynthetic model organism Chlamydomonas reinhardtii: a proteomic survey. Mol Cell Proteomics **11**: M111.014142

Zaffagnini M, Bedhomme M, Marchand CH, Morisse S, Trost P, Lemaire SD (2012b) Redox regulation in photosynthetic organisms: focus on glutathionylation. Antioxid Redox Signal **16**: 567–86

Zhang Y, Tan J, Guo Z, Lu S, He S, Shu W, Zhou B (2009) Increased abscisic acid levels in transgenic tobacco over-expressing 9 cis-epoxycarotenoid dioxygenase influence H2O2 and NO production and antioxidant defences. Plant Cell Environ **32**: 509–19

Chapter 2

Objectives of this thesis

2.1 Objectives

Stress in various forms such as extreme drought, temperature, salt intrusion, pathogen-infections etc. adversely affect plant growth and development; ultimately affect the global crop yield. Increasing efforts of plant biologists are being made to understand plant stress responses and tolerance mechanisms, with the objective to improve plant stress tolerance. All the primary stress factors cause secondary oxidative stress in plants producing reactive oxygen species (ROS), which is a potent signaling molecule to activate defense responses. However, the molecular mechanism of ROS signal perception and how it travels within or across different cells is still unsolved. Cysteine (Cys) residues in proteins are one of the most sensitive targets for ROS-mediated post-translational modifications (Cys Ox-PTM), and they have become key residues for ROS signaling studies. The reactivity of Cys residues toward ROS and the possibility to be present in different oxidation states permit them to appear on the crossroad of highly dynamic oxidative events. As such, a redox active cysteine can be present as S-glutathionylated (-SSG), disulfide bonded (S-S), sulfenylated (-SOH), sulfinylated ($-SO_2H$), and also sulfonylated ($-SO_3H$). The formation of a sulfenic acid (-SOH), the initial oxidation product of the Cys residue, has been considered as part of ROS sensing pathways, a potential redox sensor as it leads to further modifications which affect protein structure and function. This transient form (-SOH) has been shown to be involved in many physiological pathways, by affecting the enzymatic and metal binding activities of crucial signaling proteins, and the activity of transcription factors modulating gene expression (Oger *et al.*, 2012; Poole *et al.*, 2004; Poole and Nelson, 2008; Reddie and Carroll, 2008; Roos and Messens, 2011). So far, the main focus of ROS-triggered Cys Ox-PTM studies in plants was on reversible modification, such as disulfides and S-glutathionylated proteomes (Balmer *et al*., 2004; Marchand *et al*., 2004; Dixon *et al*., 2005; Rouhier *et al*., 2005; Marchand *et al*., 2006; Winger *et al*., 2007; Ströher and Dietz, 2008; Konopka-Postupolska *et al*., 2009; Marchand *et al*., 2010; Yoshida *et al*., 2013). These Cys Ox-PTMs play important roles in the cellular redox balance and prevent oxidative damage through the cellular redox regulation systems, like glutaredoxin (Grx) or thioredoxin (Trx) (Balmer *et al*., 2004; Fernandes and Holmgren, 2004; Buchanan and Balmer, 2005; Balmer *et al*., 2006; Marchand *et al*., 2006; Marchand *et al*., 2010; Yoshida *et al*., 2013). However, in plant redox biology, there is a missing list of the proteins forming sulfenic acids, the first

member of the redox regulatory chain. In this context, this PhD study is aimed to map the sulfenomes in *Arabidopsis thaliana* under oxidative stress to unravel potential ROS sensing and signaling proteins of plants.

In the first part, we will apply YAP1 based genetic probe approach for the *in vivo* trapping of cytoplasmic sulfenylated proteins in *A. thaliana*. *In vivo* sulfenome proteomics is crucial as they appear on the crossroad of highly dynamic oxidative events. The application of *in vitro* differential labeling strategy for sulfenome proteomics is limited (Leonard and Carroll, 2011; Couturier *et al*., 2013) as it does not completely preclude the chance of *de novo* sulfenylation due to an altered cysteine redox state in the cell lysates, or that the sulfenic acid modifications are insufficiently trapped due to protection or overoxidation event. YAP1 based genetic probe strategy offers a way to trap sulfenic acids *in vivo* for the identification of redox sensitive proteins undergoing sulfenylation under oxidative stress conditions (Takanishi *et al*., 2007). After transformation of YAP1 technology into Arabidopsis cells, the YAP1 trapped sulfenic acids formed under H_2O_2 stress will be purified by tandem affinity purification and identified by mass spectrometry. After obtaining the list of the identified sulfenylated proteins, important candidates will be selected for heterologous expression and purification for *in vitro* validation of sulfenylation events. In the second part, we will move to DYn-2 based sulfenome mining approach with an objective to uncover the complete cellular sulfenome of *A. thaliana*. DYn-2 is a membrane-permeable chemical probe and non-influencer of the intracellular redox balance, and therefore has been suggested as a global sulfenome reader in living cells (Paulsen *et al*., 2012; Truong and Carroll, 2012). After optimization of DYn-2 labeling conditions in *A. thaliana*, DYn-2 tagged sulfenylated proteins formed under H_2O_2 stress will be biotinylated with click reaction followed by streptavidin purification and mass spectrometry identification.

In conclusion, the ultimate goal of this study is to obtain sulfenylation picture in *A. thaliana* under oxidative stress, which will be useful to unravel ROS sensing and signaling events along the sulfenome of plants. Hence this study will contribute in better understanding the plant stress responses and tolerance mechanisms, which will in the long run help to improve plant stress tolerance and to increase global crop yield.

2.2 References

Balmer Y, Koller A, Val G Del, Schürmann P, Buchanan BB (2004) Proteomics uncovers proteins interacting electrostatically with thioredoxin in chloroplasts. Photosynth Res **79**: 275–80

Balmer Y, Vensel WH, Cai N, Manieri W, Schürmann P, Hurkman WJ, Buchanan BB (2006) A complete ferredoxin/thioredoxin system regulates fundamental processes in amyloplasts. Proc Natl Acad Sci U S A **103**: 2988–93

Buchanan BB, Balmer Y (2005) Redox regulation: a broadening horizon. Annu Rev Plant Biol **56**: 187–220

Couturier J, Chibani K, Jacquot J-P, Rouhier N (2013) Cysteine-based redox regulation and signaling in plants. Front Plant Sci **4**: 105

Dixon DPD, Skipsey M, Grundy NMN, Edwards R, Sciences B, Kingdom U (2005) Stress-induced protein S-glutathionylation in Arabidopsis. Plant Physiol **138**: 2233–2244

Fernandes AP, Holmgren A (2004) Glutaredoxins: glutathione-dependent redox enzymes with functions far beyond a simple thioredoxin backup system. Antioxid Redox Signal **6**: 63–74

Konopka-Postupolska D, Clark G, Goch G, Debski J, Floras K, Cantero A, Fijolek B, Roux S, Hennig J (2009) The role of annexin 1 in drought stress in Arabidopsis. Plant Physiol **150**: 1394–410

Leonard SE, Carroll KS (2011) Chemical "omics" approaches for understanding protein cysteine oxidation in biology. Curr Opin Chem Biol **15**: 88–102

Marchand C, Le Maréchal P, Meyer Y, Decottignies P (2006) Comparative proteomic approaches for the isolation of proteins interacting with thioredoxin. Proteomics **6**: 6528–37

Marchand C, Le Maréchal P, Meyer Y, Miginiac-Maslow M, Issakidis-Bourguet E, Decottignies P (2004) New targets of Arabidopsis thioredoxins revealed by proteomic analysis. Proteomics **4**: 2696–706

Marchand CH, Vanacker H, Collin V, Issakidis-Bourguet E, Maréchal P Le, Decottignies P (2010) Thioredoxin targets in Arabidopsis roots. Proteomics **10**: 2418–28

Paulsen CE, Truong TH, Garcia FJ, Homann A, Gupta V, Leonard SE, Carroll KS (2012) Peroxide-dependent sulfenylation of the EGFR catalytic site enhances kinase activity. Nat Chem Biol **8**: 57–64

Rouhier N, Villarejo A, Srivastava M, Gelhaye E, Keech O, Droux M, Finkemeier I, Samuelsson G, Dietz KJ, Jacquot J-P, et al (2005) Identification of plant glutaredoxin targets. Antioxid Redox Signal **7**: 919–29

Ströher E, Dietz K-J (2008) The dynamic thiol-disulphide redox proteome of the *Arabidopsis thaliana* chloroplast as revealed by differential electrophoretic mobility. Physiol Plant **133**: 566–83

Takanishi CL, Ma L-H, Wood MJ (2007) A genetically encoded probe for cysteine sulfenic acid protein modification in vivo. Biochemistry **46**: 14725–32

Truong TH, Carroll KKS (2012) Bioorthogonal Chemical Reporters for Analyzing Protein Sulfenylation in Cells. Curr Protoc Chem Biol **4**: 101–122

Winger AM, Taylor NL, Heazlewood JL, Day DA, Millar AH (2007) Identification of intra- and intermolecular disulphide bonding in the plant mitochondrial proteome by diagonal gel electrophoresis. Proteomics **7**: 4158–70

Yoshida K, Noguchi K, Motohashi K, Hisabori T (2013) Systematic exploration of thioredoxin target proteins in plant mitochondria. Plant Cell Physiol **54**: 875–92

Chapter 3

Sulfenome mining in *Arabidopsis thaliana*

Chapter 3

Sulfenome mining in *Arabidopsis thaliana*

This chapter is based on

Waszczak C, Akter S, Eeckhout D, Persiau G, Wahni K, Bodra N, Van Molle I, De Smet B, Vertommen D, Gevaert K, *et al* (2014) Sulfenome mining in *Arabidopsis thaliana*. Proc Natl Acad Sci U S A **111**: 11545–50

Mosammat Salma Akter and Cezary Waszczak contributed equally to this work.

Joris Messens and Frank Van Breusegem are co-corresponding authors.

Author contributions: Mosammat Salma Akter, Cezary Waszczak, Inge Van Molle, Marc Van Montagu, Joris Messens and Frank Van Breusegem designed this research. Cezary Waszczak and Mosammat Salma Akter contributed equally to the *in vivo* sulfenome mining research. Mosammat Salma Akter performed recombinant DHAR2 production, DHAR2 acitivity assay and in vitro sulfenylation validation in DHAR2; Mosammat Salma Akter, Cezary Waszczak, Dominique Eeckhout, Geert Persiau, Khadija Wahni, Nandita Bodra, Inge Van Molle and Barbara De Smet performed research; Didier Vertommen, Kris Gevaert, and Geert De Jaeger contributed new reagents/analytic tools; Mosammat Salma Akter, Cezary Waszczak, Barbara De Smet, Joris Messens and Frank Van Breusegem analyzed the data and wrote the paper.

Chapter 3

Sulfenome mining in *Arabidopsis thaliana*

3.1 Abstract

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 ex- posed 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.

3.2 Introduction

Numerous posttranslational modifications (PTMs) have been discovered within proteomes, creating a complex landscape of protein diversity and function (Garavelli, 2004). One of the recognized reversible redox-based PTMs is the oxidation of a cysteine thiol group to a sulfenic acid (SOH) (Roos and Messens, 2011) that acts as regulatory switch in several oxidative stress signal transduction pathways (Ma *et al*., 2007). 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/or intermolecular disulfides. These mechanisms protect the sulfenic acids against

overoxidation to sulfinic (SO_2H) or sulfonic (SO_3H) acid and allow sulfur oxygen signaling (Roos and Messens, 2011).

In plants, the best-known redox regulation mechanisms are the light-dependent thiol-disulfide exchange switches in chloroplast proteins (Balsera and Uberegui, 2014). Examples of other redox-regulated proteins are the transcription co-activator NONEXPRESSER OF PATHOGEN RELATED GENES 1 (NPR1) (Mou *et al*., 2003), the vacuolar H^+-ATPase (Tavakoli *et al*., 2001), and several transcription factors (TFs), such as the AP2-type RAP2.4a (Shaikhali *et al*., 2008), the G-group of basic leucine zipper (bZIP) TFs (Shaikhali *et al*., 2012), and the TEOSINTEBRANCHED1/CYLOIDEA/PROLIFERATING CELL FACTOR class I (TCP) TFs (Viola *et al*., 2013). 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 (Fomenko and Koc, 2011). In yeast, the H_2O_2 sensor oxidant receptor peroxidase1 (ORP1/glutathione peroxidase 3) controls, together with the transcription factor YAP1, a redox regulon via a sulfenic acid thiol-disulfide relay mechanism (Delaunay *et al*., 2002). 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 (Figure 3.1).

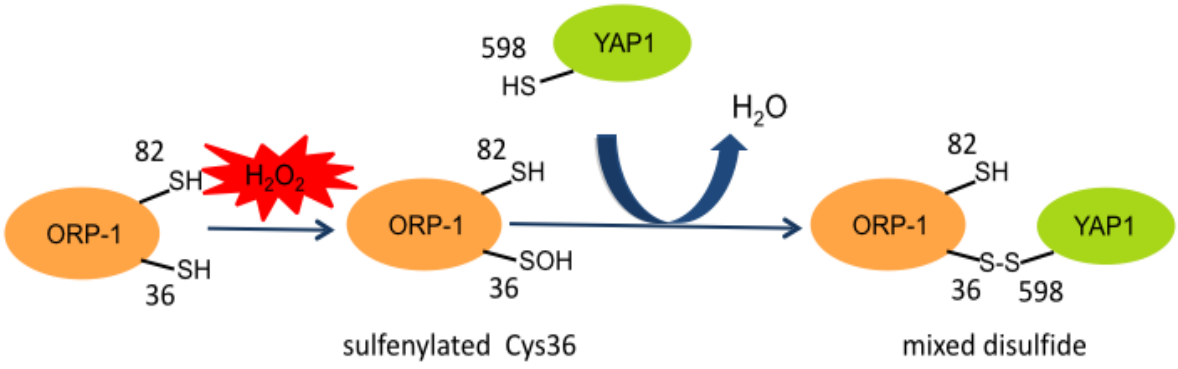


Figure 3.1 Schematic view of the molecular mechanism of the ORP-1 sulfenic acid reaction with the YAP1 transcription factor of *Saccharomyces cerevisiae*. Upon stress, ORP-1 transmits a stress signal toYAP1 through oxidation of its catalytic cysteine (Cys36) to sulfenic acid. Next, the ORP-1 Cys36 sulfenicacid condenses with Cys598 in YAP1 to formthe YAP1-Gpx3 intermolecular disulfide, which ultimately induces the signal for defense responsive gene expression.

This specific mixed disulfide formation has prompted us to develop a YAP1-based probe for trapping plant sulfenylated proteins *in vivo* (Takanishi *et al*., 2007). 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 (Leene *et al*., 2008). 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.

3.3 Materials and Methods

Arabidopsis suspension cultures. The *Saccharomyces cerevisiae* YAP1 coding region fragment corresponding to Asn565 to Asn650 was codon optimized for expression in *Arabidopsis thaliana*, synthesized, and cloned by Integrated DNA Technologies into the pIDTSmart vector with introduction of mutations to create YAP1C and YAP1A probes: YAP1C – C620A, C629T; YAP1A – C598A, C620A, C629T according to the strategy described (Takanishi *et al*., 2007). The sequences of these synthetic fragments can be found in Table 3.1.

Both synthetic DNA sequences were amplified with the specific primers (Table 3.2) to introduce the attB sites and START or STOP codon for the expression of C- and N-terminal GS tag fusions, respectively. The PCR reactions were carried out in two steps; in a second step, the initial amplicons were amplified with attB1 and attB2 primers (Table 3.2) to complete the attB sites.

Both PCR reactions were run with *pfu* proofreading polymerase (Promega). Subsequently, sequences were inserted into the pDONR221 vector by means of the Gateway$_{TM}$ technology (Life Technologies) according to the manufacturer's instructions. Vectors were sequenced with M13 primers (Table 3.2) to verify the accuracy of the gene synthesis and PCR amplifications. Expression clones were generated as previously described (Van Leene *et al*., 2007). The Arabidopsis cell suspension cultures (NASC stock no. CCL84840) were transformed and maintained as described (Van Leene *et al*., 2007).

Table 3.1 YAP1 probes used for *Arabidopsis thaliana*

Probe	YAP1-cCRD DNA sequences: codon optimized for *Arabidopsis thaliana*
YAP1C	N G S S L Q N A D K I N N G N D N D N D N AAC GGT TCT TCG CTT CAA AAC GCC GAT AAG ATA AAT AAT GGT AAC GAT AAT GAT AAC GAT AAT D V V P S K E G S L L R **C** S E I W D R I T GAC GTT GTT CCG AGC AAA GAG GGC TCC CTT TTG AGG TGT AGC GAG ATA TGG GAT CGT ATA ACC T H P K Y S D I D V D G L A S E L M A K A ACC CAC CCC AAG TAC TCT GAT ATA GAT GTC GAT GGG CTC GCC TCA GAA TTA ATG GCT AAA GCT K T S E R G V V I N A E D V Q L A L N K H AAG ACG TCT GAA AGG GGT GTA GTG ATC AAT GCT GAA GAT GTG CAG CTT GCA CTT AAT AAA CAT M N ATG AAT
YAP1A	N G S S L Q N A D K I N N G N D N D N D N AAT GGG TCG AGT CTC CAG AAT GCA GAC AAA ATT AAT AAT GGT AAT GAC AAC GAC AAT GAT AAT D V V P S K E G S L L R **A** S E I W D R I T GAC GTC GTC CCT TCA AAA GAA GGC TCA CTA TTG CGA GCG TCA GAG ATC TGG GAT CGT ATA ACG T H P K Y S D I D V D G L A S E L M A K A ACT CAT CCT AAG TAC TCA GAT ATA GAT GTC GAT GGA TTG GCA TCA GAA CTT ATG GCG AAG GCC K T S E R G V V I N A E D V Q L A L N K H AAG ACC AGT GAA AGA GGA GTT GTT ATC AAC GCT GAA GAT GTT CAA CTC GCG CTA AAT AAG CAT M N ATG AAT

Amino acid residues that differ between the two probes are marked in boldface.

Table 3.2 Primers used for cloning

Primer	Sequence (5'- 3')
Cloning – 1st PCR (YAP1(C/A)-cCRD)	
YAP1C_N_LP	AAAAAGCAGGCTTCAACGGTTCTTCGCTTCAAAACG
YAP1C_N_RP	AGAAAGCTGGGTCTTAATTCATATGTTTATTAAGT
YAP1A_N_LP	AAAAAGCAGGCTTCAATGGGTCGAGTCTCCAGAATGC
YAP1A_N_RP	AGAAAGCTGGGTCTTAATTCATATGCTTATTTAGCG
YAP1A_C_LP	AAAAAGCAGGCTCCACCATGAATGGGTCGAGTCTCCAGAATG
YAP1A_C_RP	AGAAAGCTGGGTCATTCATATGCTTATTTAGCGCGAG
Cloning – 1st PCR (DHAR2)	
DHAR2_LP	AAAAAGCAGGCTTCGAAGTGCTGTTTCAGGGCCCGATGGCTCTAGATATCTGCGTGAAG
DHAR2_RP	AGAAAGCTGGGTCTCACGCATTCACCTTCGAT
Cloning – 2nd PCR	
attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCT
attB2	GGGGACCACTTTGTACAAGAAAGCTGGGT
Sequencing	
GWM13-LP	GTAAAACGACGGCCAGTCTTA
GWM13-RP	CCAGGAAACAGCTATGACCAT

Stress treatments. Mid-log phase cell cultures (dark grown, 3 day old, approximately 10 mg fresh weight ml^{-1}) 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. For the time-dependent experiments, 1 mM H_2O_2 was added to mid-log phase cell cultures and cells were sampled after 5, 10, 30, 60, and 120 min.

Protein extractions and Western blot analysis. Plant material was ground on ice in the presence of sand and TAP extraction buffer (Van Leene *et al*., 2007) without DTT and supplemented with 10 mM iodoacetamide (IAM) and 10 mM*N*-ethylmaleimide (NEM) (unless otherwise specified) to prevent *de novo* oxidation of cysteine residues. For the Western blot analysis, soluble proteins were separated on SDS-PAGE gradient gel (Bio-Rad), blotted, and hybridized with a 1:5000 dilution of (peroxidase-anti-peroxidase [PAP] antibody complex) (Sigma-Aldrich) to detect the GS tag. Sulfenic acid residues were visualized with a 1:10000 dilution of polyclonal rabbit anti-cysteine sulfenic acid antibody (Millipore).

Tandem affinity purification. The TAP protocol was adapted to allow the use of the redox-active baits YAP1A-GS and YAP1C-GS. A soluble protein input of 25 mg was incubated with 25 µl of IgG-Sepharose 6 Fast Flow beads (GE Healthcare). The complexes eluted by cleavage with AcTEV™ Protease (Invitrogen). In a second affinity step, 25 µl of Streptavidin Sepharose High Performance (GE Healthcare) beads were used. Disulfide-bound proteins eluted with a reducing buffer containing 5 mM dithiothreitol (DTT) for 30 min at room temperature. The beads were subsequently incubated with 1× NuPAGE sample buffer containing 20 mM desthiobiotin. Both elution fractions were subjected to LC-MS/MS analysis as described (Vercruyssen *et al*., 2014). Proteins identified by at least one (for YAP1A identifications) or two (for YAP1C identifications) high-confident peptides were retained (Table 3.4).

Cloning and purification of recombinant DHAR2. The DHAR2-coding sequence was amplified by PCR (Table 3.2) from the Arabidopsis Biological Resource Center clone (stock No U25352) (Yamada *et al*., 2003). The sequence was inserted into the pDONR221 vector and subcloned in the pDEST17 expression vector by means of the Gateway technology (Life Technologies). *Escherichia coli* C41 (DE3) strain was transformed and grown aerobically overnight at 37ºC in Luria-Bertani Broth (LB)

supplemented with 100 µg/ml ampicillin. Subsequently, 1-liter LB cultures were inoculated with this overnight culture. After the culture had reached the exponential growth phase, it was cooled down to 16ºC, supplemented with 0.2 mM isopropyl β-D-1-thiogalactopyranoside, and further grown overnight. Cells were pelleted and resuspended in lysis buffer (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), pH 7.5, 1 M NaCl, 1 mM DTT, 5 mM imidazole, 1 µg/ml leupeptine, 0.1 mg/ml 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 50 µg/ml DNaseI, and 20 mM $MgCl_2$). The cells were homogenized by cell cracker at 20 kilopound per square inches and then centrifuged at 40,000$\times$g for 30 min, 4ºC to remove cell debris. The supernatant was passed through a 0.45-µm filter, and loaded onto a Ni^{2+}-Sepharose column equilibrated with 20 mM Hepes, pH 7.5, 1 M NaCl, 1 mM DTT, 5 mM imidazole. Protein peaks under the imidazole elution were pooled, concentrated by 10 kDa molecular weight cut-off (Millipore), and injected on size exclusion Superdex75 column equilibrated with 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM DTT. Protein samples analyzed by SDS-PAGE were flash-frozen for storage at -80ºC.

DHAR2 activity/inhibition assay and *in vitro* sulfenic acid labeling. DHAR2 activity was monitored by GSH-dependent reduction of DHA to ascorbate (ε = 14,000 M^{-1} cm^{-1}) by following the associated absorption increase at 265 nm (Hossain and Asada, 1984). The assay was done for 2 min at 30ºC in a buffer containing 50 mM potassium phosphate (pH 7.0), 150 mM NaCl, and 1 mM EDTA. The buffer was incubated for 1 min at 30ºC and the reaction was started by adding DHAR2 followed by the premix of DHA and GSH. In this assay, freshly prepared DHA, reduced GSH, and DTT-reduced DHAR2 were used (1 h incubation at room temperature). Excess DTT was removed by a size exclusion chromatography in a Superdex75 column equilibrated with the assay buffer. The initial velocity of the ascorbate production was measured at increasing concentrations of DHA (1, 3, 5, 10, 15, 20, 30, 40, 50, 100, 150 and 200 µM), whereas GSH was fixed at 5 mM. Non-enzymatic DHA conversion by GSH was subtracted from the corresponding enzymatic assay. To understand the role of the cysteine residue on the DHAR2 activity, the reduced enzyme was incubated 30 min in the dark with 10 mM IAM or oxidized in the presence or absence of 1 mM reduced GSH with 100 µM, 1 mM, and 5 mM H_2O_2 at room temperature. We removed excess IAM, GSH, and H_2O_2 with Micro Bio-SpinTM P-6 gel

column (Bio-Rad) equilibrated with the assay buffer. The reaction was started under V_{max} conditions by adding 100 µM DHA at a final concentration of 30 nM DHAR2 in the presence of 5 mM GSH. Initial rates of the progress curves were determined with the Cary 100 Bio UV-Visible Spectrophotometer (Agilent). For dimedone-based *in vitro* sulfenic acid labeling, DHAR2 (1 mg/ml) was reduced by 1 mM DTT and excess DTT was removed by the Micro Bio-SpinTM P-6 gel column (Bio-Rad) equilibrated with phosphate buffered saline (pH 7.4). Reduced DHAR2 (20 µM) was incubated for 30 min at room temperature with 1 mM dimedone, in the presence or absence of 100 µM H_2O_2 (dimedone stocks in dimethoxy sulfoxide: 100 mM Bis-Tris-HCl, pH 7.4 [1:1]). When GSH was added to the samples, DHAR2 was mixed with 1 mM GSH before oxidation and dimedone treatment.

MS on DHAR2. The intact mass was measured by direct infusion in a microelectrospray ionization ion trap mass spectrometer (LTQ XL; ThermoFisher Scientific). The mass spectra were deconvoluted with the ProMass Deconvolution software (ThermoFisher Scientific). To identify redox-active cysteine residues, DHAR2 was oxidized with H_2O_2 in the presence of dimedone or GSH as for the dimedone labeling experiment. All the free thiols were blocked with excess of iodoacetamide for 10 min before tryptic digestion. The peptides were analyzed by LC-MS/MS as described (Pyr Dit Ruys *et al*., 2012). The resulting peak lists were searched with SEQUEST against an Arabidopsis protein database containing the recombinant DHAR2 sequence (Uniprot Q9FRL8) and the introduced polyhistidine tag. Peptide matches were filtered by means of the PERCOLATOR program within the Proteome Discoverer software (ThermoFischer Scientific) and manually validated. The considered dynamic modifications on the cysteine residues were +138.0 Da for sulfenic dimedone, +32.0 Da for sulfinic acid, +48.0 Da for sulfonic acid, +305.1 Da for GSH, and +57.0 Da for carbamidomethyl modifications. The mixed disulfide peptide between C6 and C20 of DHAR2 was identified by means of the DBond software (Choi *et al*., 2010) and manually validated.

3.4 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 (Table 3.1, Figure 3.2A), retaining only the redox-active cysteine Cys598. Then, we fused this construct at its N-terminus to a GS tag that combines two immunoglobulin G (IgG)-binding domains of protein G with a streptavidin-binding peptide (SBP), separated by atobacco etch virus (TEV) protease cleavage site (Leene *et al*., 2008).

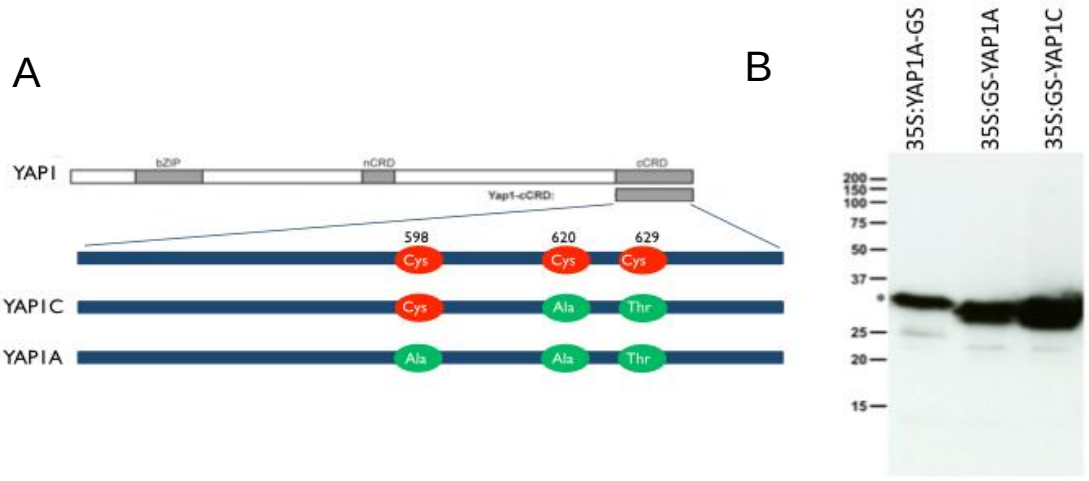


Figure 3.2 (A) Schematic presentation of YAP1-cCRD and YAP1C/YAP1A mutants. The yeast sequence corresponding to YAP1-cCRD was codon optimized for expression in Arabidopsis. For YAP1C, Cys598 was retained that is necessary for heterodimer formation, whereas in the YAP1A-negative control probe, all cysteines were mutated. **(B)** CaMV 35S promoter-driven production of recombinant YAP1-GS fusion proteins in transformed Arabidopsis cell cultures. Proteins were extracted and probes were visualized on immunoblot with the PAP antibody complex. Under reducing conditions, the probes migrate as a single band at ~35 kDa. The asterisk indicates that, because of the presence of a c-Myc tag, the C-terminal fusion is 0.7 kDa heavier.

The Cys598 of YAP1C-GS is essential for the formation of mixed disulfides with sulfenylated proteins (Takanishi *et al*., 2007). In addition, we constructed a similar control version, YAP1A-GS, in which all cysteines were mutated (Table 3.1, Figure 3.2A). The cauliflower mosaic virus (CaMV) 35S promoter-driven constructs were transformed in Arabidopsis cell suspensions. Western blot analysis with a specific antibody complex (peroxidase-anti-peroxidase [PAP] antibody complex) to detect the G moiety of the tag 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 (Figure 3.2B).

Previously, a 20-mM H_2O_2 treatment of Arabidopsis cells had been found to provoke oxidative stress signaling (Desikan *et al*., 2001). 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 the free thiols, we extracted the soluble protein in the presence of iodoacetamide (IAM) and *N*-ethylmaleimide (NEM) (Figure 3.3) and analyzed the disulfide bond formation on a non-reducing Western blot with the PAP antibody complex.

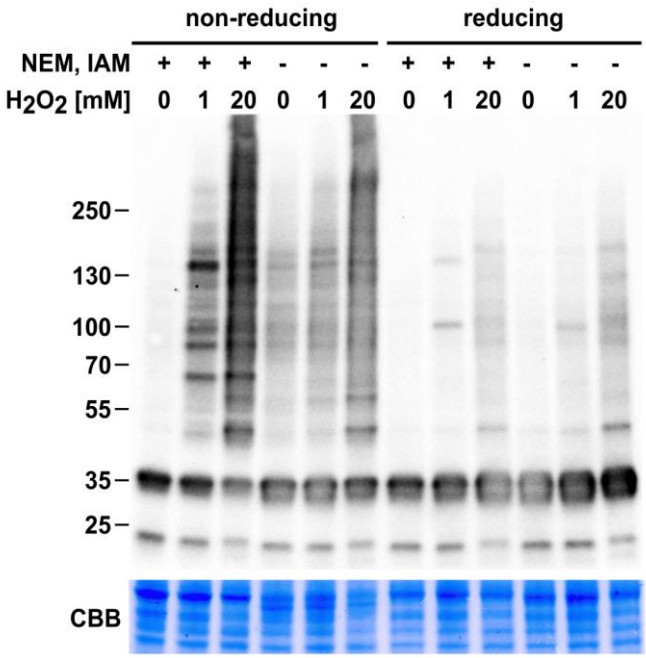


Figure 3.3 Influence of IAM and NEM on post-extraction protein oxidation. Cell cultures overproducing the GS-YAP1C probe were treated with 0, 1, and 20 mM H_2O_2 for 1 h. Proteins were extracted in extraction buffer with or without IAM and NEM and visualized on immunoblot with the PAP antibody complex. The enhanced signal intensity under control conditions without IAM and NEM indicates the formation of YAP1C heterocomplexes upon protein extraction in non-stressed samples.Under reducing conditions, the protein samples were treated with 50 mM Tris (2-carboxyethyl) phosphine (TCEP).

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 mMresulted in a proportional increase in the number of high-molecular weight YAP1C-GS complexes (Figure 3.4A). 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 (Figure 3.4A). 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

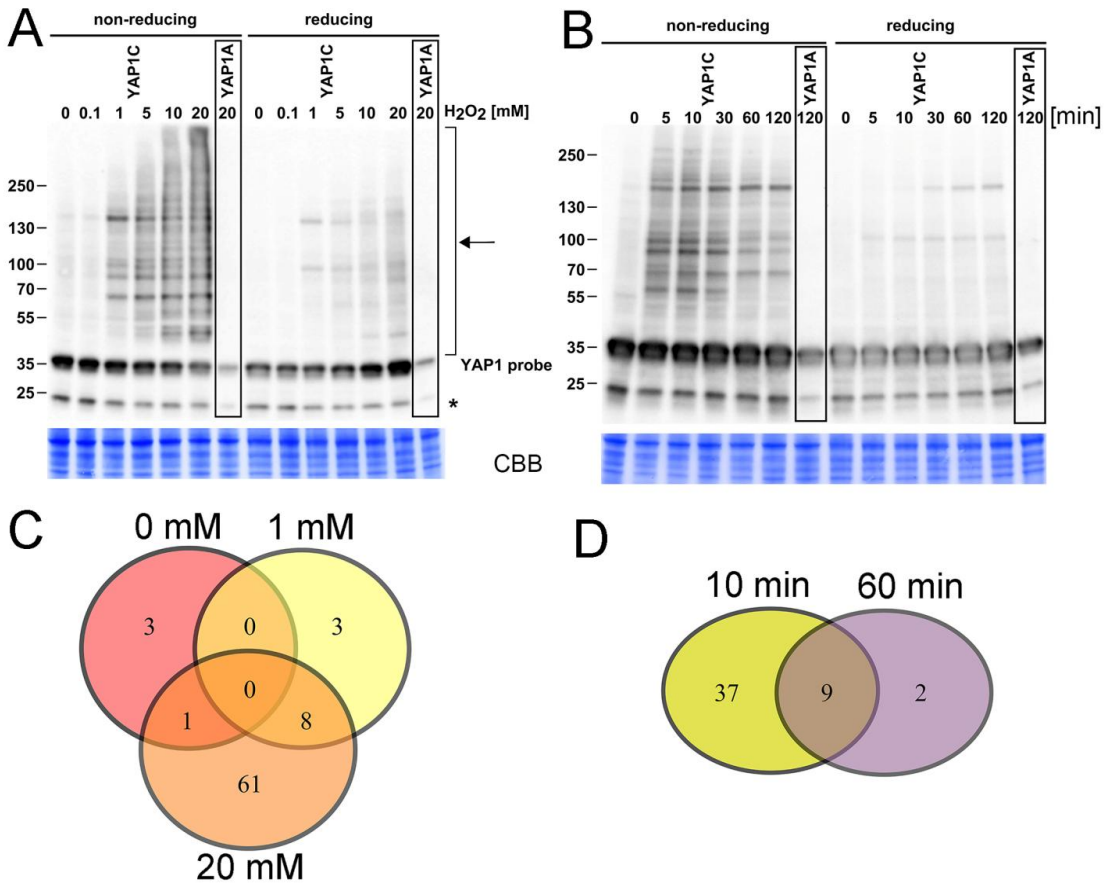


Figure 3.4 Dose- and time-dependent formations of YAP1C-involving complexes. (A) Cell cultures overproducing the GS-YAP1C/A probe treated with 0, 0.1, 1, 5, 10, and 20 mM H_2O_2 for 1 h. Complexes (marked with an arrow) are visualized with the PAP antibody complex. The H_2O_2 concentration and the amount of signal clearly correlate. Treatment of protein samples with 50 mM TCEP 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).

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 (Figure 3.4B), 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 [NTR/FTR] 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 (Figure 3.5). Briefly, first, YAP1-GS complexes were captured on IgG-sepharose. Second, with a TEV protease cleavage step, the YAP1 fused to the SBP tag together with several mixed disulfide complexes and all possible interacting proteins was eluted. In a following 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 dithiothreitol (DTT) followed by a 20 mM desthiobiotin elution. The majority of interactors (41 out of 46) eluted with DTT (Dataset S1). To guarantee a complete interactor recovery, we decided to use desthiobiotin for all the TAP purifications.

In a following experiment, 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 (Figure 3.1A), we identified 4, 11, and 70 YAP1C-specific interactors (Figure 3.4C; Table 3.4, Dataset S1). Based on the background YAP1A datasets, 215 of the interactors can be regarded as nonspecific (Dataset S1). Furthermore, almost all the specific interactors contain at least one cysteine, except the histone superfamily protein, which might be a non-disulfide interactor of one of the YAP1C mixed disulfide target proteins. The majority (8/11) 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 (Figure 3.4C). Nine out 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 (Figure 3.4D), emphasizing their sensitivity toward oxidation and suggesting an important function in oxidative stress sensing.

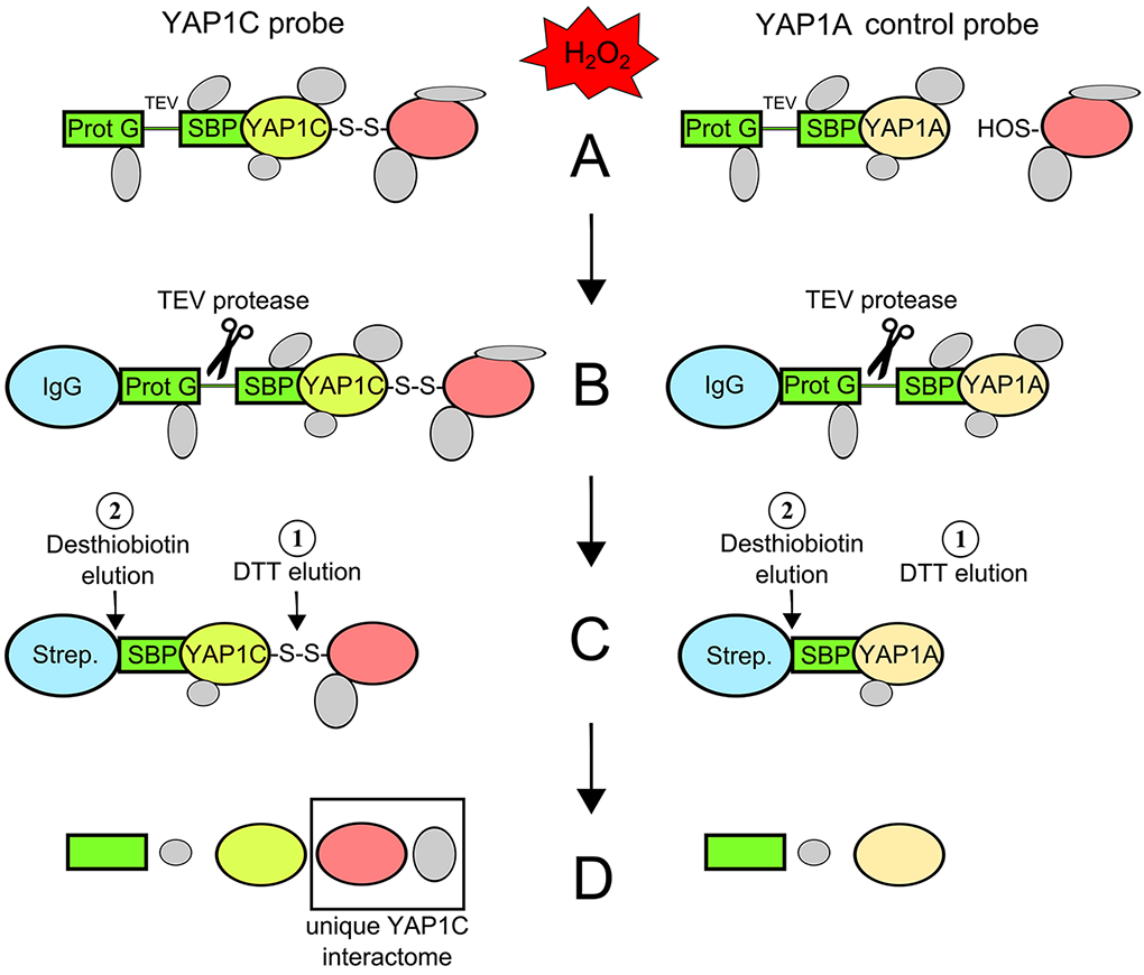


Figure 3.5 Experimental setup for *in vivo* identification of the Arabidopsis sulfenome. (A) Cell cultures overproducing the YAP1C/YAP1A probes treated with H_2O_2 as described. (B and C) Proteins isolated and subjected to a two-step purification procedure based on IgG-protein G and streptavidin–SBP 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.

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, 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 3.4). 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 instance by S-

glutathionylation. In a next step, specific redox enzymes, such as Trxs and Grxs, reduce these disulfides.

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, we refer to Table 3.4. When we focus on the 46 sulfenylated proteins within the first 10 min after the oxidative stress (Table 3.4), we find several signal perception and transduction proteins, of which almost one-fourth functions in proteasomal activities; moreover, some of several identified redox-related enzymes are found 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 3.4).

Signal perception and transduction. Three different mitogen-activated protein kinases (MAPKs) have been found to be sulfenylated: MAPK2, MAPK4, and MAPK7. The MAPK signaling cascades, including MAPKs, are integrate parts of plant biotic and abiotic stress signaling pathways and are activated by H_2O_2 (Kovtun *et al*., 2000; Zhou *et al*., 2014). In yeast and human model systems, MAPKs are redox regulated through upstream regulators and through direct cysteine oxidation events (Truong and Carroll, 2013). A cysteine oxidation event in the human p38 MAPK had been shown to act as a functional regulatory switch (Templeton *et al*., 2010), whereas, in plant MAPK modules, such thiol modification has not been reported yet. The fast sulfenylation after an oxidative stress stimulus suggests that MAPK2, MAPK4, and MAPK7 could function as redox sensors downstream of a ROS-producing event.

Sulfenylation of the catalytic nucleophilic cysteine leads to the inhibition of protein tyrosine phosphatases (PTPs) (Tanner *et al*., 2011). The identified Arabidopsis AtPTP1 undergoes cysteine-dependent inhibition by H_2O_2 and negatively regulates the MAPKs (Gupta and Luan, 2003), suggesting that the oxidation-dependent AtPTP1 inhibition might be a primary step in the oxidative stress response leading to MAPK signaling de-repression (Bartels *et al*., 2009). Furthermore, at least two members of the plant-specific SNF1-RELATED PROTEIN KINASE 2 (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 F (AtRbohF) and transcription factors, which are required for the expression of numerous stress response genes (Kulik *et al*., 2011). Only recently, a redox-regulated rapeseed (*Brassica napus*) SnRK2 was shown to be involved in guard cell signaling (Zhu *et al*., 2014).

PROTEIN PHOSPHATASE 2A (PP2A) is a holoenzyme consisting of a catalytic subunit C, a structural subunit A, and a highly variable regulatory subunit B that determines its target specificity. We identified two B subunits PP2A-b55α and PP2A-b'γ as potential redox sensitive proteins. Both subunits were found to have opposite effect on regulation of flowering time (Heidari *et al*., 2013). Moreover, PP2A-b'γ was suggested to control premature senescence and basal repression of defense responses in Arabidopsis (Trotta *et al*., 2011). PP2A-b'γ knockout plants were reported to exhibit disintegration of chloroplasts, constitutive expression of disease resistance genes and accumulation of ROS. A recent study by Li *et al*., (2014) confirmed the function of PP2A-b'γ in salicylic acid-dependent defense responses. It would be of interest to investigate the influence of the cellular redox balance as well as the role of sulfenylation modification on the substrate specificity of these B subunits.

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 ENZYME 27 (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 PHOTOMORPHOGENIC 9 (COP9) signalosome (Table 3.4). In the 26S proteasome, we identified the REGULATORY PARTICLE NON-ATPASE 12A (RPN12A) as a potentially H_2O_2-modified protein. Interestingly, RPN12A has been established as a cytoplasmic thioredoxin h (Trx-h) target protein (Yamazaki *et al*., 2004), and act as a potential crosstalk point in cytokinin signaling (Ryu *et al*., 2009). The removal of oxidatively damaged proteins is an important event in the stress responses (Jung and Grune, 2013), 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 (Höhn and Grune, 2014). In maize (*Zea mays*), sugar starvation-triggered oxidative stress leads to oxidative modifications of the 20S proteasome, modulating its

proteolytic activity (Basset *et al*., 2002). 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 had been reported to occur via reversible sulfenylation of a catalytic cysteine residue (Kulathu *et al*., 2013), but until now, this inhibition mode has not been described for plant DUBs.

Primary metabolism. Next to well established redox regulated proteins including CYTOSOLIC-NAD-DEPENDENT MALATE DEHYDROGENASE 1 (Hara *et al*., 2006), VACUOLAR ATP SYNTHASE SUBUNIT A (Seidel *et al*., 2012) and cytosolic FRUCTOSE- BISPHOSPHATE ALDOLASE 4 (van der Linde *et al*., 2011); we identified a number of new potential redox regulated enzymes involved in primary metabolism and energy homeostasis.

The properties of maize chloroplastic NADP-MALIC ENZYME (ZmC4-NADP-ME) involved in C4 photosynthesis have been explored recently (Alvarez *et al*., 2012). In this enzyme, the oxidation of cysteine residues induces conformational change that limits the catalytic process. We identified the cytosolic enzymes NADP-ME2 and in agreement with our results, NADP-ME2 was reported earlier to undergo stress induced S-glutathionylation (Dixon *et al*., 2005) and identified as a Trx-y2 target protein (Marchand *et al*., 2010). However, a recent study (Li *et al*., 2013) indicates that this enzyme is not essential for oxidative stress response.

ATP-CITRATE LYASE (ACL) is a cytosolic enzyme catalyzing ATP-dependent conversion of citrate and CoA to acetyl-CoA and oxaloacetate. This enzyme composed of two distinct subunits, ACLA and ACLB is of crucial importance for plant metabolism as acetyl-CoA serves as an initial metabolite for a plethora of natural products (Fatland *et al*., 2002; Fatland *et al*., 2005). ACL is of particular interest in modern medicine as recent studies highlight its role in metabolism of cancer cells (Zaidi *et al*., 2012; Hanai *et al*., 2013). So far, the redox-dependent modulation of ACL activity was demonstrated for rat liver enzyme (Wells and Saxty, 1992), however a similar observation for plant isoforms is still missing. Our results demonstrate a possibility for direct regulation of Arabidopsis ACL activity via redox modification of B subunit(s).

RNA binding proteins, translational machinery, post-transcriptional regulation events. Synthesis of certain proteins is activated in a redox dependent manner in response

to light exposure without an increase in the corresponding mRNA levels and therefore relays on the post-transcriptional regulation. Such mechanisms of redox-dependent translational activation were studied mostly in the context of psbA mRNA translation in algae (Kim and Mayfield, 1997; Alergand *et al*., 2006) and higher plants (Shen *et al*., 2001). In *Chlamydomonas*, this two-component system involves the POLY(A) BINDING PROTEIN RB47 and PROTEIN DISULFIDE ISOMERASE RB60. RB60 regulates the binding of RB47 to the 5'-UTR of psbA mRNA through the redox equivalents thereby providing a mechanism for its redox-dependent translation (Kim and Mayfield, 1997; Alergand *et al*., 2006).

In this study, we identified seven proteins involved in the control of protein translation mechanisms including RNA BINDING PROTEIN 45C, POLY(A) BINDING PROTEIN 8 (PAB8) and PROTEIN ARGININE METHYLTRANSFERASE 5 (PRMT5) that was shown to link the circadian clock to the control of alternative splicing in plants (Sanchez *et al*., 2010). Ample evidence supports the role of RNA binding proteins controlling plant development and stress responses (Lorković, 2009), however, further studies are necessary to investigate the potential redox dependent RNA-binding properties of proteins identified here.

Hormone homeostasis. ROS-hormonal interplay was shown to affect abscisic acid (ABA) (Suzuki *et al*., 2013; Song *et al*., 2014) and auxin (Tognetti *et al*., 2010; Tognetti *et al*., 2012) signaling pathways. Here, we identified four proteins tightly associated with hormonal homeostasis including ABA DEFICIENT 2 (ABA2) and NITRILASE 1 & 2 (NIT1, NIT2) involved in synthesis of abscisic acid (ABA) (Léon-Kloosterziel *et al*., 1996) and 3-indoleacetic acid (IAA) (Bartel and Fink, 1994) respectively. Hydrogen peroxide is involved in the ABA signaling pathway via direct post-translational modifications of ABA INSENSITIVE 1 (ABI1) and ABI2. Oxidizing conditions promote inactivation of both phosphatases, which are the negative regulators of ABA signaling pathway (Meinhard and Grill, 2001; Meinhard *et al*., 2002), thereby providing a positive feedback loop. NIT1 and NIT2 identified in this study were reported earlier as potential targets for Cys PTM (Dixon *et al* 2005; Wang *et al*., 2012). Together, our results provide a new perspective towards further investigation of ABA- and auxin-regulated pathways.

Redox proteins. At least four redox-related proteins have been detected: MONOTHIOL GLUTAREDOXIN17 (GRXS17), THIOREDOXIN-DEPENDENT PEROXIDASE1

(TPX1), GLUTAREDOXIN C2 (GRXC2), and a DEHYDROASCORBATE REDUCTASE2 (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 due to their highly specialized redox mechanisms that often involve resolving cysteines for the rapid conversion of sulfenic acids (Roos and Messens, 2011), hence impeding the formation of mixed disulfides with YAP1C.

TPX1 had been identified as a target of cytosolic Trxh3 (Marchand *et al*., 2006) and also in a subset of early-responsive redox-sensitive proteins (Wang *et al*., 2012). GRXC2 and GRXS17 function in early plant development during embryonic (Riondet *et al*., 2012) and temperature-dependent postembryonic (Cheng *et al*., 2011) growth, respectively, but their specific substrate proteins are unknown. DHAR2 was sulfenylated in both the early and late oxidative stress responses (Table 3.4). Dehydroascorbate reductase plays an important role in counterbalancing oxidative stress by 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 it had been shown that perturbation of DHAR limits ascorbate recycling, as a consequence, influences the rate of plant growth and leaf aging due to ROS-mediated damage (Chen and Gallie, 2006).

DHAR2 kinetics are affected by H_2O_2 treatment. DHAR2 catalyzes the reduction of oxidized ascorbate with a concomitant oxidation of GSH to GSSG (Figure 3.6); therefore, it is one of the core enzymes of the GSH/ascorbate cycle that maintains ascorbate pools reduced (Foyer and Halliwell, 1977). 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 sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel.

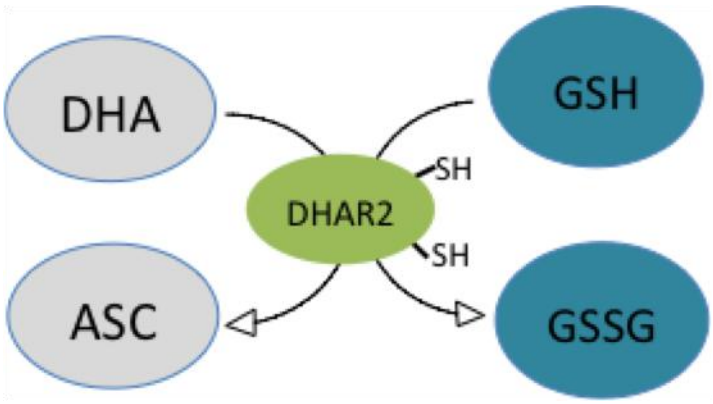


Figure 3.6 Schematic view on GSH-dependent regeneration of ascorbate (ASC) from dehydroascorbate (DHA) catalyzed by dehydroascorbate reductase (DHAR).

Its molecular weight was confirmed by mass spectrometry (MS) with a total mass of 26,748 Da after loss of the N-terminal methionine (Figure 3.7). We analyzed the activity of recombinant DHAR2 by following the ascorbate production in progress curves in function of time at 265 nm (Figure 3.8A). The initial velocities were measured at varying concentrations of dehydroascorbate (DHA) in a 5-mM excess of GSH.

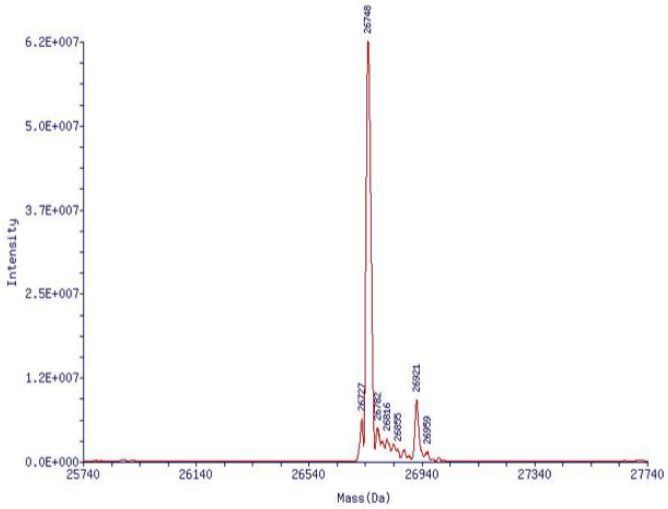


Figure 3.7 Recombinant DHAR2 identity confirmed by MS. Total mass equals 26,748 Da after loss of the N-terminal methionine.

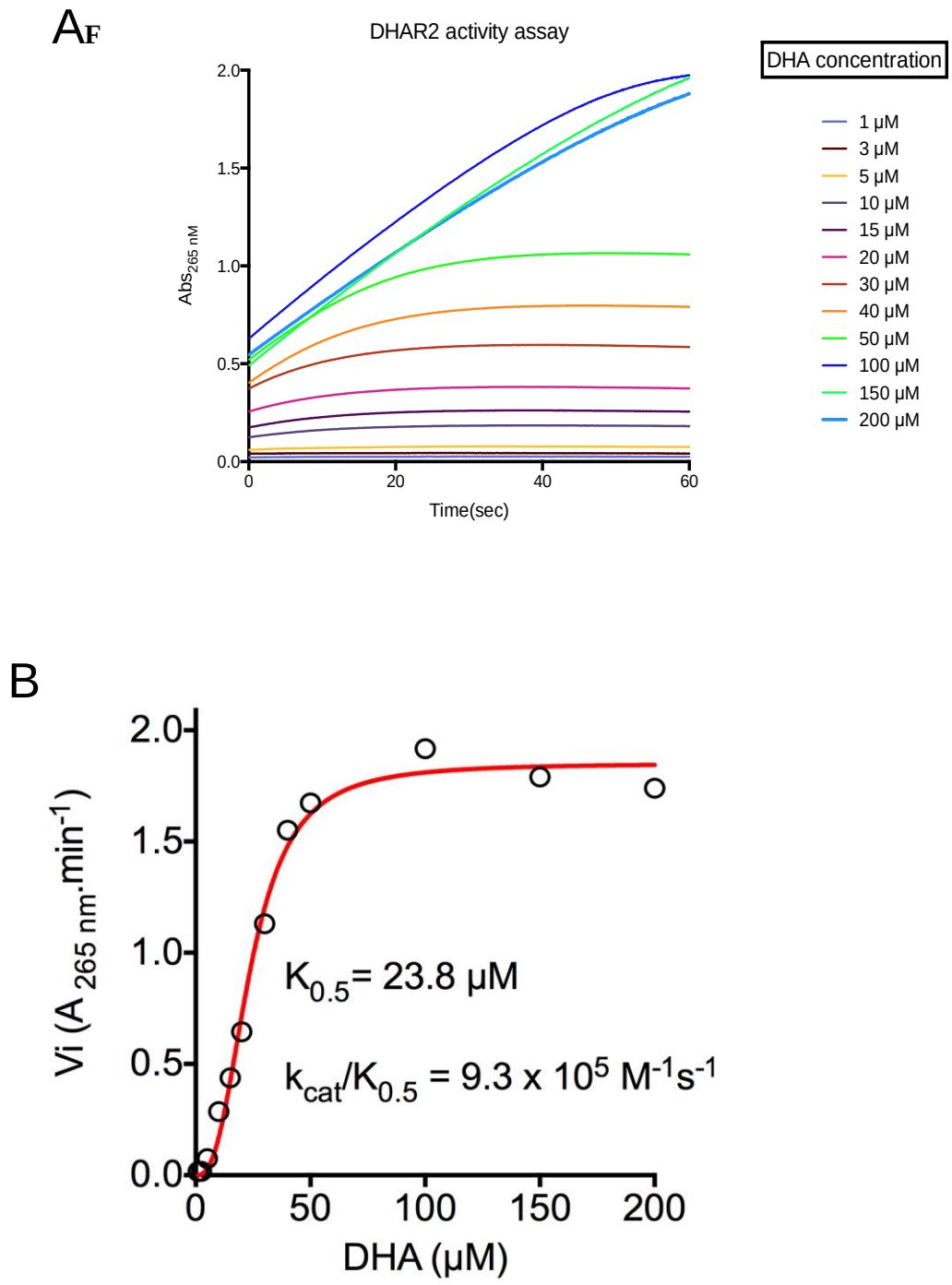


Figure 3.8 Recombinanlty expressed DHAR2 is active. (A) The activity of recombinant DHAR2 was analyzed by following the ascorbate production in the progress curves in function of time at 265 nmat increasing concentrations of DHA (1, 3, 5, 10, 15, 20, 30, 40, 50, 100, 150 and 200 µM), whereas electron donor of this reaction GSH was fixed at 5 mM. Non-enzymatic DHA conversion to ascorbate was subtracted from the corresponding enzymatic assay. (B) Kinetic constant $K_{0.5}$ and $k_{cat}/K_{0.5}$ values of DHAR2 (100 nM) determined from the Hill plot in which the initial velocity of the ascorbate production (A/min) vs. DHA concentration at a fixed GSH concentration (5 mM).

Plots of the initial velocities versus the DHA concentrations revealed a sigmoidal curve (Figure 3.8B) with a Hill factor of 2.65, indicative for positive co-operativity and hinting at the positive influence of the GSH binding at increasing DHA concentrations. We determined for DHAR2 a $k_{cat}/K_{0.5}$ value of 9.3×10^5 $M^{-1}s^{-1}$ with $K_{0.5}$ of 23.8 ± 1.2 µM, whereas for DHAR1 and DHAR3, K_M values of 260 µM and 500 µM had been reported (Dixon *et al*., 2002).

To understand the possible role of the cysteine thiols of DHAR2 in its catalytic cycle, we modified the free thiols with 1 mM IAM and oxidized DHAR2 with increasing concentrations of H_2O_2 (Figure 3.9A). In both cases, the activity is affected, indicating that cysteines are essential for catalytic DHAR2 activity, as observed previously for DHAR1 (Dixon *et al*., 2002).

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_2O_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 (Benitez and Allison, 1974). Previously, dimedone and its derivatives have successfully been applied to study sulfenylation in many important physiological pathways in various organisms and in recombinant proteins (Paulsen and Carroll, 2009). We analyzed the H_2O_2-induced DHAR2 sulfenylation in the presence and absence of GSH on Western blots with antibodies that specifically recognize dimedone-tagged sulfenic acids (Figure 3.9B).

After a 30-min treatment with 100 µM H_2O_2 in the presence of dimedone, DHAR2 was sulfenylated. Remarkably, sulfenylation was lower in H_2O_2-treated than in nontreated samples, possibly due to a rapid rescue of sulfenic acids by resolving cysteines or overoxidation to sulfinic or sulfonic acids (Roos and Messens, 2011). In the presence of 1 mM GSH, the sulfenylation signal did not depend on the H_2O_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_2O_2-treated DHAR2 in the presence of dimedone or GSH by liquid chromatography-tandem MS (Table 3.3). After treatment with 100 µM H_2O_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 when compared to the parent peptide (Figure 3.9C), confirming that Cys20 is sulfenylated. Cys20 is also partially overoxidized to

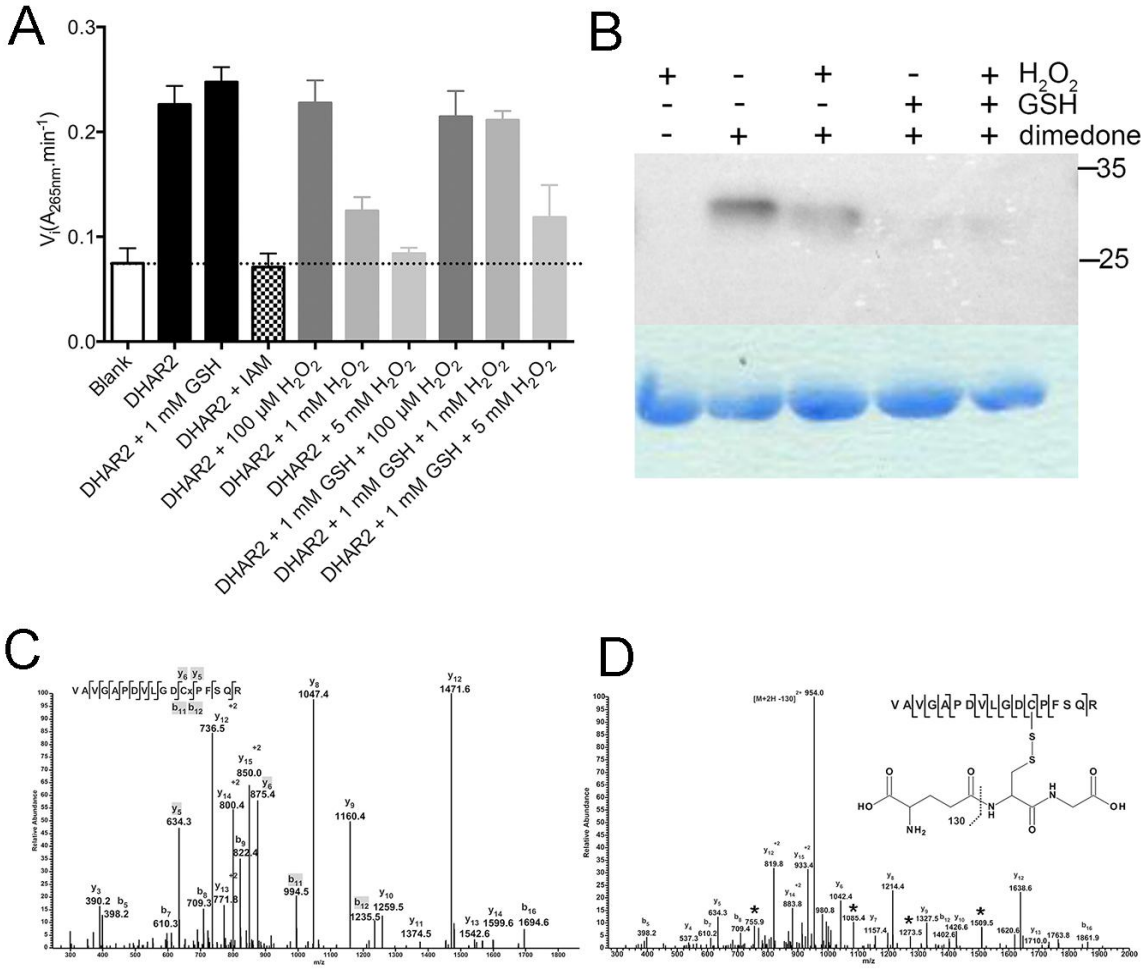


Figure 3.9 Requirement of DHAR2 cysteines and GSH protection against overoxidation. **(A)** The mean initial velocities ± S.D. of three independent measurements of DHAR2 were determined on progress curves under different conditions. DTT-reduced DHAR2 was treated with 10 mM IAM, and with 100 µM, 1 mM and 5 mM H_2O_2 in the presence or absence of 1 mM GSH, incubated for 30 min at room temperature. IAM-blocked DHAR2 was prepared by 10 mM IAM treated 30 min in the dark at room temperature. Excess of H_2O_2, IAM, and GSH was removed on a Micro Bio-Spin^{TM} P-6 gel column (Bio-Rad) equilibrated with 50 mM potassium phosphate (pH 7.0) assay buffer. The reaction was started under Vmax conditions by adding 100 µM DHA to a final concentration of 30 nM DHAR2 in the presence of 5 mM GSH. **(B)** Dimedone labeling of DHAR2-SOH *in vitro*. DHAR2 (20 µM) nontreated or treated with 1 mM GSH was either not or incubated with 100 µM H_2O_2 in the presence or absence of dimedone (1 mM). DHAR2-SOH formation was analyzed by immunoblot with an anti-cysteine 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. The *y*- and *b*-series of ions allow the modified cysteine to be localized exactly. **(D)** Identification of S-glutathionylation on Cys20 of DHAR2. The LC-MS/MS spectrum shows data obtained from a +2 parent ion with *m/z* 1019.0. The spectrum displays a major fragment ion at *m/z* 954.0, corresponding to the neutral loss of glutamic acid (130 Da) after fragmentation at a peptide bond within GSH. The mixed disulfide between GSH and the cysteine residue is located precisely by means of the *y*- and *b*-series of ions. Ions that were generated from loss of glutamic acid are marked (*).

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 (Figure 3.9D).

Table 3.3 Detection of redox PTMs of DHAR2 by MS

Samples	**Reduced (CAM)**	**Sulfenic Dimedone**	**Sulfinic**	**Sulfonic**	**GSH**	**S-S**
DHAR2 + H_2O_2	C6 (7*) C20 (18)	ND	ND	ND	ND	$^{\$}C_6$-$C_{20}$
DHAR2 + dimedone + H_2O_2	C6 (24) C20 (22)	C20 (1)	ND	C20 (2)	ND	C_6-C_6 C_6-C_{20}
DHAR2 + GSH + H_2O_2	C6 (6) C20 (18)	ND	ND	ND	C20 (13)	ND

Recombinant DHAR2 was treated with H_2O_2 in the presence of dimedone or GSH. Free thiols were blocked with excess of IAM, which results in the addition of a carbamidomethyl group. The peptides were analyzed by LC-MS/MS. Two tryptic peptides were detectable: AGFEVLFQGPMALDI***C*****$_6$**VK and VAVGAPDVLGD***C*****$_{20}$**PFSQR. Numbers in parentheses indicate the number of peptide spectral matches; $^{\$}$ marks the disulfide with the highest score in Dbond; ND, not detectable.

To test the 1-mM GSH protection on the DHAR2 activity, we added increasing concentrations of H_2O_2 to DHAR2 in the presence of 1 mM GSH and determined the initial velocities (V_i) of the progress curves (Figure 3.9A). The V_i of the GSH-pretreated DHAR2 sample is slightly higher than that of the nontreated sample, which is, at least, partially sulfenylated after purification (Figure 3.9B). At low H_2O_2 concentrations a disulfide is formed between C6 and C20 together with partial overoxidation of C20 (Table 3.1), without affecting the DHAR activity (Figure 3.9A). However, at higher H_2O_2 concentrations (1 and 5 mM), sulfonic acid is more probably formed (Table 3.3) and associated with decreased activity (Figure 3.9A). 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 (Figure 3.9A).

Possibly the overoxidation rate is too fast for 1 mM GSH to react with the sulfenic acid to recover all activity (Figure 3.9A). 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. All together, 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 (Figure 3.10).

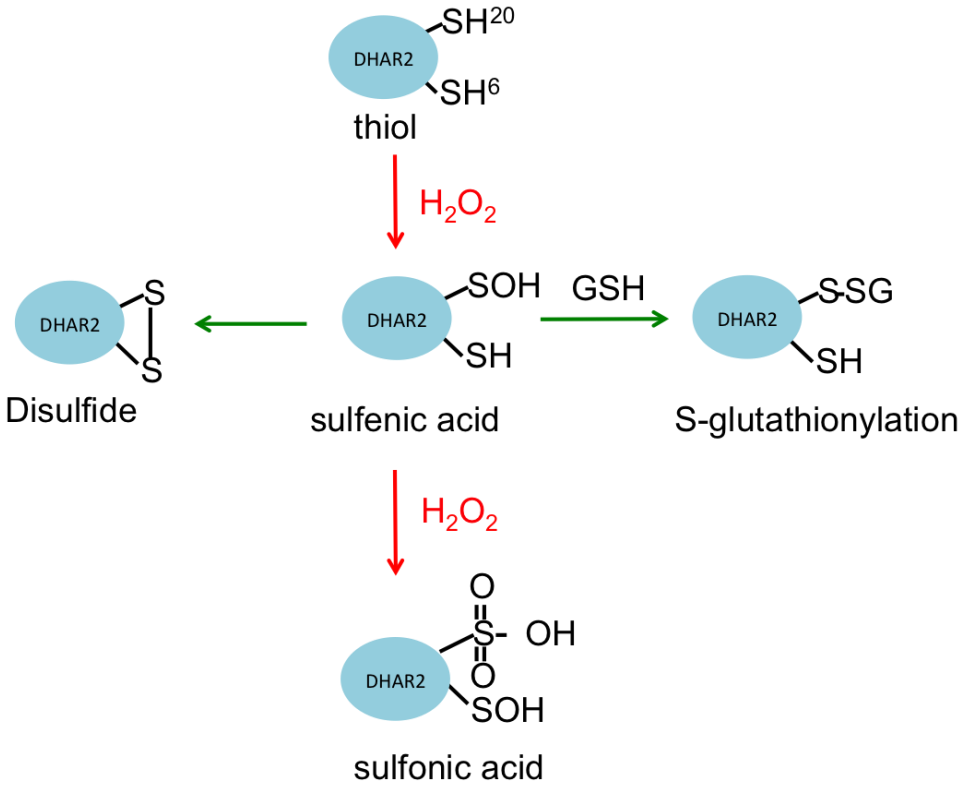


Figure 3.10 Schematic presentation of the oxidative modifications of DHAR2 treated with H_2O_2. Reversible sulfenic acid, irreversible sulfonic acid and protective mixed disulfides are shown. The green arrow represents the protection of the sulfenic acid and the red arrow represents the oxidation of the thiolate or overoxidation of the sulfenic acid.

3.5 Significance

We describe the first successful application of YAP1-based sulfenome mining strategy in *Arabidopsis thaliana*. By a unique combination of sulfenic acid trapping with tandem affinity purification, we identified a set of 97 sulfenylated proteins. The characterization of the plant sulfenome improves the understanding of important ROS signaling pathways.

The previous identification of S-glutathionylated proteins or Trx/Grx target proteins in the our identified Arabidopsis sulfenome opens an interesting route for further investigation of the physiological consequence related to the pathways in which these enzymes operate. Furthermore, studies at the whole plant level will open a possibility for the application of broad range of stress treatments, which could be related to the needs of modern agriculture, and will lead to a better understanding of the signaling events in plants under those stress treatments.

Acknowledgements

We thank Eveline Van De Slijke, Nancy De Winne, Brigitte van de Cotte, and Debbie Rombaut for excellent technical assistance. Martine De Cock for help in preparing the manuscript. This work was supported by grants from Ghent University Multidisciplinary Research Partnership ("Ghent BioEconomy" [Project 01MRB 510W]) and Bijzondere Onderzoeksfonds (BOF 01J11311), the Interuniversity Attraction Poles Program (grant no. IUAP VII/29), initiated by the Belgian State, Science Policy Office, and Research Foundation-Flanders (projects nos G.0D.79.14N and G.0038.09N), and the European Cooperation in Science and Research (COST Action BM1203/EU-ROS). C.W. and M.S.A. are indebted to the VIB International PhD Program and Erasmus Mundus External Cooperation Window for predoctoral fellowships, respectively. D.V. is "collaborateur logistique" from the FNRS-FRS Belgium and B.D.S. is a predoctoral fellow of the Research Foundation-Flanders. I.V.M. is the recipient of an Omics@vib Marie Curie COFUND fellowship.

Table 3.4 Unique YAP1C interactors identified in Arabidopsis cell cultures

	Description	Early response (10 min)	Late response (1h)			Cys residues	Cys PTM	Reference
		1 mM	0 mM	1 mM	20 mM			
SIGNAL PERCEPTION & TRANSDUCTION								
AT1G59580	MAP KINASE 2 (MPK2)	2		1	1	8		
AT4G01370	MAP KINASE 4 (MPK4)	1			1	8		
AT2G18170	MAP KINASE 7 (MPK7)	1		1	1	8		
AT3G50500	SNF1-RELATED PROTEIN KINASE 2-2 (SNRK2-2)[a]	1				7		
AT5G66880	SNF1-RELATED PROTEIN KINASE 2-3 (SNRK2-3)[a]					8		
AT4G33950	SNF1-RELATED PROTEIN KINASE 2-6 (SNRK2-6)[a]					6		
AT1G60940	SNF1-RELATED PROTEIN KINASE 2-10 (SNRK2-10)	1			1	6		
AT1G35670	CALCIUM-DEPENDENT PROTEIN KINASE 2 (CDPK2)[b]				1	9		
AT4G09570	CALCIUM-DEPENDENT PROTEIN KINASE 4 (CPK4)[b]					10		
AT2G18790	PHYTOCHROME B (PHYB)[c]	1				25	Reactive cys	(Liu *et al*., 2014)
AT4G16250	PHYTOCHROME D (PHYD)[c]					25		
AT2G43980	INOSITOL 1,3,4-TRISPHOSPHATE 5/6-KINASE 4 (ITPK4)	1			1	9		
AT2G42810	PHYTOCHROME-ASSOCIATED PROTEIN PHOSPHATASE 5 (PAPP5)				2	8		
AT1G71860	PROTEIN TYROSINE PHOSPHATASE 1 (PTP1)	1				7	Reactive cys	(Gupta and Luan, 2003; Wang *et al*., 2012; Liu *et al*., 2014)
AT1G51690	PROTEIN PHOSPHATASE 2A 55KDA REGULATORY SUBUNIT (PP2A-B55A)	2		2	2	15		
AT4G15410	PROTEIN PHOSPHATASE 2A 55 KDA REGULATORY SUBUNIT B'Γ (PP2A-B'Γ)				1	1		
AT2G46900	BHLH protein	1			2	6		
PROTEIN DEGRADATION								
AT1G22920	COP9 SIGNALOSOME 5A (CSN5A)				1	2		

AT5G14250	COP9 SIGNALOSOME SUBUNIT 3 (CSN3)	1			1	8		
AT1G64520	REGULATORY PARTICLE NON-ATPASE 12A (RPN12A)	1				4	Trx target	(Lemaire *et al*., 2004; Yamazaki *et al*., 2004)
AT1G75950	S PHASE KINASE-ASSOCIATED PROTEIN 1 (SKP1)	2		2	2	3		
AT2G45240	METHIONINE AMINOPEPTIDASE 1A (MAP1A)				2	17		
AT2G47790	CUL4-RING UBIQUITIN LIGASE COMPLEX SUBUNIT (GTS1)	1				12		
AT5G06600	UBIQUITIN-SPECIFIC PROTEASE 12 (UBP12)	1				11		
AT3G11910	UBIQUITIN-SPECIFIC PROTEASE 13 (UBP13)	1				10		
AT4G30890	UBIQUITIN-SPECIFIC PROTEASE 24 (UBP24)	1				3		
AT4G17510	UBIQUITIN C-TERMINAL HYDROLASE 3 (UCH3)	1		1		4	SOH	(Kulathu *et al*., 2013)*
AT5G50870	UBIQUITIN-CONJUGATING ENZYME 27 (UBC27)	1			1	4		
AT3G18060	Transducin family protein / WD-40 repeat family protein				1	14		
AT3G51800	ERBB-3 BINDING PROTEIN 1 (EBP1)				2	7	SNO	(Fares *et al*., 2011)
AT4G11260	ENHANCED DOWNY MILDEW 1 (EDM1)				1	4		
AT5G06600	UBIQUITIN-SPECIFIC PROTEASE 12 (UBP12)	1				11		
AT5G13520	Peptidase M1 family protein				1	7		
AT4G17830	Peptidase M20/M25/M40 family protein	2			1	8	S-SG	(Dixon *et al*., 2005b)
AT5G15400	MUTANT SNC1 -ENHANCING 3 (MUSE3)				1	12		
AT5G36210	α/β- hydrolases superfamily protein				1	13		
AT5G42190	SKP-LIKE PROTEIN 1B (SKP1B)	2		1	2	3		
REDOX RELATED								
AT1G65980	THIOREDOXIN-DEPENDENT PEROXIDASE 1 (TPX1)	1	1			2	SNO, Trx/ Grx target	(Lindermayr and Saalbach, 2005; Rouhier *et al*., 2005; Marchand *et al*., 2006; Marchand *et al*., 2010; Wang *et al*., 2012)
AT1G75270	DEHYDROASCORBATE REDUCTASE 2 (DHAR2)	1			1	2	S-SG, Trx target	(Dixon *et al*., 2005b), (Marchand *et al*., 2010), (Wang *et al*., 2012)
AT4G04950	MONOTHIOL GLUTAREDOXIN 17 (GRXS17)	1		2	2	6		
AT1G01800	NAD(P)-binding rossmann-fold superfamily protein	1				4	Reactive cys	(Wang *et al*., 2012)
AT3G44190	FAD/NAD(P)-binding oxidoreductase family protein	2			2	3		
AT5G40370	GLUTAREDOXIN C2 (GRXC2)				1	5		
AT1G37130	NITRATE REDUCTASE 2 (NR2)				1	16		
RNA BINDING- TRANSLATION								

AT1G15930	Ribosomal protein L7Ae/L30e/S12e/gadd45 family protein	1			1	6	Trx target	(Hägglund *et al*., 2008)*
AT2G32060	Ribosomal protein L7Ae/L30e/S12e/gadd45 family protein				1	6	Trx target	(Hägglund *et al*., 2008)*
AT1G49760	POLY (A) BINDING PROTEIN 8 (PABP8)				2	4		
AT3G57290	EUKARYOTIC TRANSLATION INITIATION FACTOR 3E (EIF3E)				1	5		
AT4G27000	RNA-BINDING FAMILY PROTEIN (ATRBP45C)	1				3		
AT1G70980	Class II aminoacyl-tRNA and biotin synthetases superfamily protein (SYNC3)				1	9		
AT4G31120	PROTEIN ARGININE METHYLTRANSFERASE 5 (PRMT5)	1				12		
PRIMARY METABOLISM								
AT1G04410	CYTOSOLIC-NAD-DEPENDENT MALATE DEHYDROGENASE 1 (C-NAD-MDH1)				2	6	Trx target, S-SG, SNO, SOH	(Marchand *et al*., 2004; Yamazaki *et al*., 2004; Hara *et al*., 2006), (Dixon *et al*., 2005b), (Lindermayr and Saalbach, 2005), (Oger *et al*., 2012)*
AT3G06650	ATP-CITRATE LYASE SUBUNIT B-1 (ACLB-1)[d]				1	10		
AT5G49460	ATP CITRATE LYASE SUBUNIT B 2 (ACLB-2)[d]				1	10		
AT4G35260	ISOCITRATE DEHYDROGENASE 1 (IDH1)				1	7	Trx target	(Balmer *et al*., 2006; Yoshida *et al*., 2013)
AT5G03690	FRUCTOSE-BISPHOSPHATE ALDOLASE 4 (FBA4)				1	5	S-SG, SNO, S-S; SOH	(van der Linde *et al*., 2011)*; (Oger *et al*., 2012)*
AT5G11670	NADP-MALIC ENZYME 2 (NADP-ME2)	1			2	7	S-SG, SOH, Trx targets	(Dixon *et al*., 2005b) (Oger *et al*., 2012)* (Marchand *et al*., 2010)
AT1G78900	VACUOLAR ATP SYNTHASE SUBUNIT A (VHA-A)	1			2	6	S-S	(Seidel *et al*., 2012) (Wang *et al*., 2012)
HORMONE HOMEOSTASIS								
AT1G48630	RECEPTOR FOR ACTIVATED C KINASE 1B (RACK1B)[e]				1	8	Reactive cys	(Liu *et al*., 2014)
AT3G18130	RECEPTOR FOR ACTIVATED C KINASE 1C (RACK1C)[e]					7		
AT1G52340	ABA DEFICIENT 2 (ABA2)				1	7		
AT3G44310	NITRILASE 1 (NIT1)				2	7	S-SG	(Dixon *et al*., 2005b)
AT3G44300	NITRILASE 2 (NIT2)	1			2	7	Reactive	(Wang *et al*., 2012)

							cys	
PROTEIN TRANSPORT								
AT5G58590	RAN BINDING PROTEIN 1 (RANBP1)				2	4	Trx target	(Alkhalfioui *et al*., 2007)*
AT1G07140	PUTATIVE RAN-BINDING PROTEIN (SIRANBP)				1	4	Trx target	(Wong *et al*., 2004)*
AT3G56190	ALPHA-SOLUBLE NSF ATTACHMENT PROTEIN 2 (ASNAP)	1				8		
AT3G59020	ARM repeat superfamily protein				1	16		
AT4G30550	GAMMA-GLUTAMYL PEPTIDASE 3 (GGP3)	1				8		
AMINO ACID METABOLISM								
AT5G10870	CHORISMATE MUTASE 2 (CM2)				1	3		
AT4G24830	Arginosuccinate synthase family				1	6	Trx target Reactive cys	(Hägglund *et al*., 2008)* (Wang *et al*., 2012)
AT5G17330	GLUTAMATE DECARBOXYLASE 1 (GAD1)	2		1	2	7		
AT1G49820	5-METHYLTHIORIBOSE KINASE 1 (MTK1)				1	5		
AT5G01410	PYRIDOXINE BIOSYNTHESIS 1.3 (PDX1.3)	1			2	5	Reactive cys	(Wang *et al*., 2012)
MISCELLANEOUS AND UNKNOWN FUNCTIONS								
AT3G16050	PYRIDOXINE BIOSYNTHESIS 1.2 (PDX1.2)				2	4		
AT1G04690	POTASSIUM CHANNEL BETA SUBUNIT 1 (KAB1)		1		1	4		
AT1G07660	Histone superfamily protein			1		0		
AT1G09080	BINDING PROTEIN 3 (BIP3)		1			4		
AT1G10270	GLUTAMINE-RICH PROTEIN 23 (GRP23)		1			9		
AT1G62740	HOP2				1	5		
AT1G69800	Cystathionine beta-synthase (CBS) protein				2	6		
AT1G70310	SPERMIDINE SYNTHASE 2 (SPDS2)				2	9		
AT2G27860	UDP-D-APIOSE/UDP-D-XYLOSE SYNTHASE 1 (AXS1)				2	8	S-SG	(Dixon *et al*., 2005b)
AT2G42910	Phosphoribosyltransferase family protein	1		1	2	7		
AT3G03250	UDP-GLUCOSE PYROPHOSPHORYLASE (UGP1)				1	3	S-SG; Trx target	(Dixon *et al*., 2005b) (Wong *et al*., 2004; Alkhalfioui *et al*., 2007)*
AT3G07720	Galactose oxidase/kelch repeat superfamily protein	1				6	Trx targets, reactive cys	(Marchand *et al*., 2010) (Wang *et al*., 2012)
AT3G12110	ACTIN-11 (ACT11)			1		4	Trx target; SOH	(Wong *et al*., 2004; Alkhalfioui *et al*., 2007)*; (Oger *et al*., 2012)
AT3G46010	ACTIN DEPOLYMERIZING FACTOR 1 (ADF1)				1	5		

AT3G16520	UDP-GLUCOSYL TRANSFERASE 88A1 (UGT88A1)	2			1	9		
AT3G53180	NODULIN/GLUTAMINE SYNTHASE-LIKE PROTEIN (NODGS)	1				9	S-SG	(Dixon *et al*., 2005b)
AT3G63000	NPL4-LIKE PROTEIN 1 (NPL41)					5		
AT4G02340	Alpha/beta-Hydrolases superfamily protein				1	3		
AT4G02860	Phenazine biosynthesis phzc/phzf protein				1	10		
AT4G13730	Ypt/Rab-GAP domain of gyp1p superfamily protein				2	4		
AT4G14710	ACIREDUCTONE DIOXYGENASE 2 (ATARD2)	1				5		
AT4G29350	PROFILIN 2 (PFN2)	1				2	Trx target	(Wong *et al*., 2004)*
AT4G29510	ARGININE METHYLTRANSFERASE 11 (PRMT11)				1	6		
AT5G13050	5-FORMYLTETRAHYDROFOLATE CYCLOLIGASE (5-FCL)	1				5		
AT5G17270	Protein prenylyltransferase superfamily protein	1			2	20		
AT2G42910	Phosphoribosyltransferase family protein	1		1	2	7		
AT5G17620	AUGMIN SUBUNIT 7 (AUG7)				2	2		
AT5G49650	XYLULOSE KINASE 2 (XK2)				1	8		
AT3G29280	Unknown protein	1				6		
AT3G52610	Unknown protein				2	8		
AT4G27450	Unknown protein	1				7	Trx target	(Hägglund *et al*., 2008)*
AT5G11810	Unknown protein				2	5		

Annotation according to TAIR10. Abbreviations of PTMs are as follows: SNO, S-nitrosylation; SOH, sulfenic acid; S-S, disulfide bridge; S-SG, S-glutathionylation; Trxs/Grxs target, thioredoxin/glutaredoxin target proteins. Numbers in the early response (10 min) indicate the occurrence of proteins eluted with either DTT, desthiobiotin, or both. Numbers in the late response (1 h) indicate occurrence of proteins in two independent experiments. References describing identification of homolog/ortholog are marked with an asterisk. Superscripts a, b, c, d, or e mark proteins that could not be distinguished based on the peptide identity.

3.6 References

Alergand T, Peled-Zehavi H, Katz Y, Danon A (2006) The chloroplast protein disulfide isomerase RB60 reacts with a regulatory disulfide of the RNA-binding protein RB47. Plant Cell Physiol **47**: 540–8

Alkhalfioui F, Renard M, Vensel W (2007) Thioredoxin-linked proteins are reduced during germination of Medicago truncatula seeds. Plant .**144**: 1559–79

Alvarez S, Zhu M, Chen S (2009) Proteomics of Arabidopsis redox proteins in response to methyl jasmonate. J Proteomics **73**: 30–40

Anderson LE, Manabe K (1979) Disulfide-linked peptides in the chloroplast thylakoid membrane. Biochim Biophys Acta **579**: 1–9

Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol **55**: 373–99

Apostol I, Heinstein PF, Low PS (1989) Rapid Stimulation of an Oxidative Burst during Elicitation of Cultured Plant Cells: Role in Defense and Signal Transduction. Plant Physiol **90**: 109–16

Ariel F, Manavella P, Dezar C, Chan R (2007) The true story of the HD-Zip family. Trends Plant Sci **12**: 419–26

Bachi A, Dalle-Donne I, Scaloni A (2013) Redox proteomics: chemical principles, methodological approaches and biological/biomedical promises. Chem Rev **113**: 596–698

Balmer Y, Koller A, del Val G, Manieri W, Schürmann P, Buchanan BB (2003) Proteomics gives insight into the regulatory function of chloroplast thioredoxins. Proc Natl Acad Sci U S A **100**: 370–5

Balmer Y, Koller A, Val G Del, Schürmann P, Buchanan BB (2004) Proteomics uncovers proteins interacting electrostatically with thioredoxin in chloroplasts. Photosynth Res **79**: 275–80

Balmer Y, Vensel WH, Cai N, Manieri W, Schürmann P, Hurkman WJ, Buchanan BB (2006) A complete ferredoxin/thioredoxin system regulates fundamental processes in amyloplasts. Proc Natl Acad Sci U S A **103**: 2988–93

Balsera M, Uberegui E, Schürmann P, Buchanan BB (2014) Evolutionary development of redox regulation in chloroplasts. Antioxid Redox Signal **21**: 1327–55

Bartels S, Anderson JC, González Besteiro M a, Carreri A, Hirt H, Buchala A, Métraux J-P, Peck SC, Ulm R, Gonzalez Besteiro MA, *et al* (2009) MAP kinase phosphatase1 and protein tyrosine phosphatase1 are repressors of salicylic acid synthesis and SNC1-mediated responses in Arabidopsis. Plant Cell **21**: 2884–97

Bartsch S, Monnet J, Selbach K, Quigley F, Gray J, von Wettstein D, Reinbothe S, Reinbothe C (2008) Three thioredoxin targets in the inner envelope membrane of

chloroplasts function in protein import and chlorophyll metabolism. Proc Natl Acad Sci U S A **105**: 4933–8

Basset G, Raymond P, Malek L, Brouquisse R (2002) Changes in the Expression and the Enzymic Properties of the 20S Proteasome in Sugar-Starved Maize Roots. Evidence for an *in vivo* Oxidation of the Proteasome 1. **128**: 1149–1162

Benitez L V, Allison WS (1974) The inactivation of the acyl phosphatase activity catalyzed by the sulfenic acid form of glyceraldehyde 3-phosphate dehydrogenase by dimedone and olefins. J Biol Chem **249**: 6234–43

Buchanan BB, Balmer Y (2005) Redox regulation: a broadening horizon. Annu Rev Plant Biol **56**: 187–220

Bykova N V, Hoehn B, Rampitsch C, Banks T, Stebbing J-A, Fan T, Knox R (2011) Redox-sensitive proteome and antioxidant strategies in wheat seed dormancy control. Proteomics **11**: 865–82

Carballal S, Radi R, Kirk MC, Barnes S, Freeman BA, Alvarez B (2003) Sulfenic acid formation in human serum albumin by hydrogen peroxide and peroxynitrite. Biochemistry **42**: 9906–14

Chang Y-C, Huang C-N, Lin C-H, Chang H-C, Wu C-C (2010) Mapping protein cysteine sulfonic acid modifications with specific enrichment and mass spectrometry: an integrated approach to explore the cysteine oxidation. Proteomics **10**: 2961–71

Charles RL, Schröder E, May G, Free P, Gaffney PRJ, Wait R, Begum S, Heads RJ, Eaton P (2007) Protein sulfenation as a redox sensor: proteomics studies using a novel biotinylated dimedone analogue. Mol Cell Proteomics **6**: 1473–84

Chen Z, Gallie DR (2006) Dehydroascorbate reductase affects leaf growth, development, and function. Plant Physiol **142**: 775–87

Cheng G, Ikeda Y, Iuchi Y, Fujii J (2005) Detection of S-glutathionylated proteins by glutathione S-transferase overlay. Arch Biochem Biophys **435**: 42–9

Cheng N, Liu J-Z, Liu X, Wu Q, Thompson SM, Lin J, Chang J, Whitham S a, Park S, Cohen JD, *et al* (2011) Arabidopsis monothiol glutaredoxin, AtGRXS17, is critical for temperature-dependent postembryonic growth and development via modulating auxin response. J Biol Chem **286**: 20398–406

Chiang SM, Schellhorn HE (2012) Regulators of oxidative stress response genes in Escherichia coli and their functional conservation in bacteria. Arch Biochem Biophys **525**: 161–9

Choi S, Jeong J, Na S, Lee HS, Kim H-Y, Lee K-J, Paek E (2010) New algorithm for the identification of intact disulfide linkages based on fragmentation characteristics in tandem mass spectra. J Proteome Res **9**: 626–35

Choudhury S, Panda P, Sahoo L, Panda SK (2013) Reactive oxygen species signaling in plants under abiotic stress.

Colcombet J, Hirt H (2008) Arabidopsis MAPKs: a complex signalling network involved in multiple biological processes. Biochem J **413**: 217–26

Comelli RN, Gonzalez DH (2007) Conserved homeodomain cysteines confer redox sensitivity and influence the DNA binding properties of plant class III HD-Zip proteins. Arch Biochem Biophys **467**: 41–7

Lo Conte M, Carroll KS (2013) The redox biochemistry of protein sulfenylation and sulfinylation. J Biol Chem **288**: 26480–8

Lo Conte M, Carroll KS (2012) Chemoselective ligation of sulfinic acids with aryl-nitroso compounds. Angew Chem Int Ed Engl **51**: 6502–5

Cubas P, Lauter N, Doebley J, Coen E (1999) The TCP domain: a motif found in proteins regulating plant growth and development. Plant J **18**: 215–22

Cumming RC (2008) Analysis of global and specific changes in the disulfide proteome using redox two-dimensional polyacrylamide gel electrophoresis. Methods Mol Biol **476**: 165–79

Dalle-Donne I, Rossi R, Giustarini D, Colombo R, Milzani A (2007) S-glutathionylation in protein redox regulation. Free Radic Biol Med **43**: 883–98

Danquah A, de Zelicourt A, Colcombet J, Hirt H (2013) The role of ABA and MAPK signaling pathways in plant abiotic stress responses. Biotechnol Adv **32**: 40–52

Delaunay A, Isnard A-D, Toledano M (2000) H_2O_2 sensing through oxidation of the Yap1 transcription factor. EMBO J **19**: 5157–66

Delaunay A, Pflieger D, Barrault MB, Vinh J, Toledano MB (2002) A thiol peroxidase is an H2O2 receptor and redox-transducer in gene activation. Cell **111**: 471–81

Delledonne M, Xia Y, Dixon RA, Lamb C (1998) Nitric oxide functions as a signal in plant disease resistance. Nature **394**: 585–8

Delledonne M, Zeier J, Marocco A, Lamb C (2001) Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive disease resistance response. Proc Natl Acad Sci U S A **98**: 13454–9

Denu JM, Tanner KG (1998) Specific and reversible inactivation of protein tyrosine phosphatases by hydrogen peroxide: evidence for a sulfenic acid intermediate and implications for redox regulation. Biochemistry **37**: 5633–42

Desikan R, A-H-Mackerness S, Hancock JT, Neill SJ (2001) Regulation of the Arabidopsis transcriptome by oxidative stress. Plant Physiol **127**: 159–72

Desikan R, Hancock JT, Bright J, Harrison J, Weir I, Hooley R, Neill SJ (2005) A Role for ETR1 in Hydrogen Peroxide Signaling in Stomatal Guard Cells 1. **137**: 831–834

Desikan R, Last K, Harrett-Williams R, Tagliavia C, Harter K, Hooley R, Hancock JT, Neill SJ (2006) Ethylene-induced stomatal closure in Arabidopsis occurs via AtrbohF-mediated hydrogen peroxide synthesis. Plant J **47**: 907–16

Dietz K-J (2014) Redox Regulation of Transcription Factors in Plant Stress Acclimation and Development. Antioxid Redox Signal. doi: 10.1089/ars.2013.5672

Dixon DP, Davis BG, Edwards R (2002) Functional divergence in the glutathione transferase superfamily in plants. Identification of two classes with putative functions in redox homeostasis in *Arabidopsis thaliana*. J Biol Chem **277**: 30859–69

Dixon DP, Fordham-Skelton AP, Edwards R (2005a) Redox regulation of a soybean tyrosine-specific protein phosphatase. Biochemistry **44**: 7696–703

Dixon DPD, Skipsey M, Grundy NMN, Edwards R, Sciences B, Kingdom U (2005b) Stress-induced protein S-glutathionylation in Arabidopsis. Plant Physiol **138**: 2233–2244

Dominguez-Solis J, He Z (2008) A cyclophilin links redox and light signals to cysteine biosynthesis and stress responses in chloroplasts. Proc.**105**: 16386–16391

Durrant WE, Dong X (2004) Systemic acquired resistance. Annu Rev Phytopathol **42**: 185–209

Ellis HR, Poole LB (1997) Novel application of 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole to identify cysteine sulfenic acid in the AhpC component of alkyl hydroperoxide reductase. Biochemistry **36**: 15013–15018

Fares A, Rossignol M, Peltier J-B (2011) Proteomics investigation of endogenous S-nitrosylation in Arabidopsis. Biochem Biophys Res Commun **416**: 331–6

Feng W-K, Wang L, Lu Y, Wang X-Y (2011) A protein oxidase catalysing disulfide bond formation is localized to the chloroplast thylakoids. FEBS J **278**: 3419–30

Fernandes AP, Holmgren A (2004) Glutaredoxins: glutathione-dependent redox enzymes with functions far beyond a simple thioredoxin backup system. Antioxid Redox Signal **6**: 63–74

Foloppe N, Vlamis-Gardikas A, Nilsson L (2012) The -Cys-X1-X2-Cys- motif of reduced glutaredoxins adopts a consensus structure that explains the low pK(a) of its catalytic cysteine. Biochemistry **51**: 8189–207

Fomenko D, Koc A (2011) Thiol peroxidases mediate specific genome-wide regulation of gene expression in response to hydrogen peroxide. Proc **108**: 2729–34

Foyer CH, Halliwell B (1977) Purification and properties of dehydroascorbate reductase from spinach leaves. Phytochemistry **16**: 1347–1350

Furdui CM, Poole LB, Carolina N (2014) Chemical approaches to detect and analyze protein sulfenic acids. 126–146

Galant A, Koester RP, Ainsworth E a, Hicks LM, Jez JM (2012) From climate change to molecular response: redox proteomics of ozone-induced responses in soybean. New Phytol **194**: 220–9

Garavelli JS (2004) The RESID Database of Protein Modifications as a resource and annotation tool. Proteomics **4**: 1527–33

Geiger D, Scherzer S, Mumm P, Marten I, Ache P, Matschi S, Liese a, Wellmann C, Al-Rasheid K a S, Grill E, *et al* (2010) Guard cell anion channel SLAC1 is regulated by CDPK protein kinases with distinct Ca2+ affinities. Proc Natl Acad Sci U S A **107**: 8023–8

Ghezzi P, Bonetto V, Fratelli M (2005) Thiol-disulfide balance: from the concept of oxidative stress to that of redox regulation. Antioxid Redox Signal **7**: 964–72

Go Y-MY, Jones DDP (2008) Redox compartmentalization in eukaryotic cells. Biochim Biophys Acta (BBA)-General Subj **1780**: 1273–1290

Goldman R, Stoyanovsky DA, Day BW, Kagan VE (1995) Reduction of Phenoxyl Radicals by Thioredoxin Results in Selective Oxidation of Its SH-Groups to Disulfides. An Antioxidant Function of Thioredoxin. Biochemistry **34**: 4765–4772

Grotewold E, Williams CE (1997) Differences between Plant and Animal Myb Domains Are Fundamental for DNA Binding Activity, and Chimeric Myb Domains Have Novel DNA Binding Specificities. J Biol Chem **272**: 563–571

Gupta R, Luan S (2003) Redox Control of Protein Tyrosine Phosphatases and Mitogen-Activated Protein Kinases in Plants. PLANT Physiol **132**: 1149–1152

Gutscher M, Sobotta MC, Wabnitz GH, Ballikaya S, Meyer AJ, Samstag Y, Dick TP (2009) Proximity-based protein thiol oxidation by H2O2-scavenging peroxidases. J Biol Chem **284**: 31532–40

Hägglund P, Bunkenborg J, Maeda K, Svensson B, Per Hagglund, Jakob Bunkenborg, Kenji Maeda and BS, Enzyme (2008) Identification of Thioredoxin Disulfide Targets Using a Quantitative Proteomics Approach Based on Isotope-Coded Affinity Tags Per Ha research articles. J Proteome Res **7**: 5270–5276

Hahn A, Harter K (2009) Mitogen-activated protein kinase cascades and ethylene: signaling, biosynthesis, or both? Plant Physiol **149**: 1207–10

Hall M, Mata-Cabana A, Akerlund H-E, Florencio FJ, Schröder WP, Lindahl M, Kieselbach T (2010) Thioredoxin targets of the plant chloroplast lumen and their implications for plastid function. Proteomics **10**: 987–1001

Hansen RE, Roth D, Winther JR (2009) Quantifying the global cellular thiol-disulfide status. Proc Natl Acad Sci U S A **106**: 422–7

Hara S, Motohashi K, Arisaka F, Romano PGN, Hosoya-Matsuda N, Kikuchi N, Fusada N, Hisabori T (2006) Thioredoxin-h1 reduces and reactivates the oxidized cytosolic malate dehydrogenase dimer in higher plants. J Biol Chem **281**: 32065–71

Heine GF, Hernandez JM, Grotewold E (2004) Two cysteines in plant R2R3 MYB domains participate in REDOX-dependent DNA binding. J Biol Chem **279**: 37878–85

Herrero E, de la Torre-Ruiz MA (2007) Monothiol glutaredoxins: a common domain for multiple functions. Cell Mol Life Sci **64**: 1518–30

Höhn TJA, Grune T (2014) The proteasome and the degradation of oxidized proteins: Part III-Redox regulation of the proteasomal system. Redox Biol **2**: 388–94

Hossain MA, Asada K (1984) Purification of dehydroascorbate reductase from spinach and its characterization as a thiol enzyme. Plant Cell Physiol **25**: 85–92

Huang G-T, Ma S-L, Bai L-P, Zhang L, Ma H, Jia P, Liu J, Zhong M, Guo Z-F (2012) Signal transduction during cold, salt, and drought stresses in plants. Mol Biol Rep **39**: 969–87

Ito H, Iwabuchi M, Ogawa K (2003) The sugar-metabolic enzymes aldolase and triose-phosphate isomerase are targets of glutathionylation in *Arabidopsis thaliana*: detection using biotinylated glutathione. Plant Cell Physiol **44**: 655–60

Jacques S, Ghesquière B, Van Breusegem F, Gevaert K (2013) Plant proteins under oxidative attack. Proteomics **13**: 932–40

Juárez-Díaz JA, McClure B, Vázquez-Santana S, Guevara-García A, León-Mejía P, Márquez-Guzmán J, Cruz-García F (2006) A novel thioredoxin h is secreted in Nicotiana alata and reduces S-RNase in vitro. J Biol Chem **281**: 3418–24

Jung T, Grune T (2013) The proteasome and the degradation of oxidized proteins: Part I-structure of proteasomes. Redox Biol **1**: 178–82

Katiyar A, Smita S, Lenka SK, Rajwanshi R, Chinnusamy V, Bansal KC (2012) Genome-wide classification and expression analysis of MYB transcription factor families in rice and Arabidopsis. BMC Genomics **13**: 544

Kieselbach T (2013) Oxidative folding in chloroplasts. Antioxid Redox Signal **19**: 72–82

Kim J, Mayfield SP (1997) Protein Disulfide Isomerase as a Regulator of Chloroplast Translational Activation. Science (80-) **278**: 1954–1957

Klessig DF, Durner J, Noad R, Navarre DA, Wendehenne D, Kumar D, Zhou JM, Shah J, Zhang S, Kachroo P, *et al* (2000) Nitric oxide and salicylic acid signaling in plant defense. Proc Natl Acad Sci U S A **97**: 8849–55

Klomsiri C, Nelson KJ, Bechtold E, Soito L, Johnson LC, Lowther WT, Ryu S-E, King SB, Furdui CM, Poole LB (2010) Use of dimedone-based chemical probes for sulfenic acid detection evaluation of conditions affecting probe incorporation into redox-sensitive proteins. Methods Enzymol **473**: 77–94

Konopka-Postupolska D, Clark G, Goch G, Debski J, Floras K, Cantero A, Fijolek B, Roux S, Hennig J (2009) The role of annexin 1 in drought stress in Arabidopsis. Plant Physiol **150**: 1394–410

Kovtun Y, Chiu WL, Tena G, Sheen J (2000) Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. Proc Natl Acad Sci U S A **97**: 2940–5

Kulathu Y, Garcia FJ, Mevissen TET, Busch M, Arnaudo N, Carroll KS, Barford D, Komander D (2013) Regulation of A20 and other OTU deubiquitinases by reversible oxidation. Nat Commun **4**: 1569

Kulik A, Wawer I, Krzywińska E, Bucholc M, Dobrowolska G (2011) SnRK2 protein kinases--key regulators of plant response to abiotic stresses. OMICS **15**: 859–72

Kwak JM, Mori IC, Pei Z-M, Leonhardt N, Torres MA, Dangl JL, Bloom RE, Bodde S, Jones JDG, Schroeder JI (2003) NADPH oxidase AtrbohD and AtrbohF genes function in ROS-dependent ABA signaling in Arabidopsis. EMBO J **22**: 2623–33

Laloi C, Apel K, Danon A (2004) Reactive oxygen signalling: the latest news. Curr Opin Plant Biol **7**: 323–8

Laohavisit A, Brown AT, Cicuta P, Davies JM (2010) Annexins: components of the calcium and reactive oxygen signaling network. Plant Physiol **152**: 1824–9

Laohavisit A, Davies JM (2011) Annexins. New Phytol **189**: 40–53

Laohavisit A, Shang Z, Rubio L, Cuin T a, Véry A-A, Wang A, Mortimer JC, Macpherson N, Coxon KM, Battey NH, *et al* (2012) Arabidopsis annexin1 mediates the radical-activated plasma membrane Ca^{2}+- and K+-permeable conductance in root cells. Plant Cell **24**: 1522–33

Lee J-W, Helmann JD (2006) Biochemical characterization of the structural Zn2+ site in the Bacillus subtilis peroxide sensor PerR. J Biol Chem **281**: 23567–78

Lee JS, Ellis BE (2007) Arabidopsis MAPK phosphatase 2 (MKP2) positively regulates oxidative stress tolerance and inactivates the MPK3 and MPK6 MAPKs. J Biol Chem **282**: 25020–9

Lee JS, Wang S, Sritubtim S, Chen J-G, Ellis BE (2009) Arabidopsis mitogen-activated protein kinase MPK12 interacts with the MAPK phosphatase IBR5 and regulates auxin signaling. Plant J **57**: 975–85

Van Leene J, Stals H, Eeckhout D, Persiau G, Van De Slijke E, Van Isterdael G, De Clercq A, Bonnet E, Laukens K, Remmerie N, *et al* (2007) A tandem affinity purification-based technology platform to study the cell cycle interactome in *Arabidopsis thaliana*. Mol Cell Proteomics **6**: 1226–38

Leene J Van, Witters E, Inzé D, Jaeger G De, Van Leene J, De Jaeger G (2008) Boosting tandem affinity purification of plant protein complexes. Trends Plant Sci **13**: 517–520

Lemaire SD, Collin V, Keryer E, Quesada A, Miginiac-Maslow M (2003) Characterization of thioredoxin y, a new type of thioredoxin identified in the genome of Chlamydomonas reinhardtii. FEBS Lett **543**: 87–92

Lemaire SD, Guillon B, Le Maréchal P, Keryer E, Miginiac-Maslow M, Decottignies P (2004) New thioredoxin targets in the unicellular photosynthetic eukaryote Chlamydomonas reinhardtii. Proc Natl Acad Sci U S A **101**: 7475–80

Leonard SE, Carroll KS (2011) Chemical "omics" approaches for understanding protein cysteine oxidation in biology. Curr Opin Chem Biol **15**: 88–102

Leonard SE, Reddie KG, Carroll KS (2009) Mining the Thiol Proteome for Sulfenic Acid Modifications Reveals New Targets for Oxidation in Cells. ACS Chem Biol **4**: 783–799

Leustek T, Martin MN, Bick J-A, Davies JP (2000) Pathways and regulation of sulfur metabolism revealed through molecular and genetic studies. Annu Rev Plant Physiol Plant Mol Biol **51**: 141–165

Levine A, Tenhaken R, Dixon R, Lamb C (1994) H2O2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell **79**: 583–93

Li B, Zhao Y, Liang L, Ren H, Xing Y, Chen L, Sun M, Wang Y, Han Y, Jia H, *et al* (2012) Purification and characterization of ZmRIP1, a novel reductant-inhibited protein tyrosine phosphatase from maize. Plant Physiol **159**: 671–81

Li S, Lauri A, Ziemann M, Busch A, Bhave M, Zachgo S (2009) Nuclear activity of ROXY1, a glutaredoxin interacting with TGA factors, is required for petal development in *Arabidopsis thaliana*. Plant Cell **21**: 429–41

Lillig CH, Berndt C, Holmgren A (2008) Glutaredoxin systems. Biochim Biophys Acta **1780**: 1304–17

Van der Linde K, Gutsche N, Leffers H-M, Lindermayr C, Müller B, Holtgrefe S, Scheibe R (2011) Regulation of plant cytosolic aldolase functions by redox-modifications. Plant Physiol Biochem **49**: 946–57

Lindermayr C, Saalbach G (2005) Proteomic Identification of S-Nitrosylated Proteins. **137**: 921–930

Lindermayr C, Saalbach G, Durner J (2005) Proteomic Identification of S -Nitrosylated Proteins. Plant Physiol **137**: 921–930

Lindermayr C, Sell S, Müller B, Leister D, Durner J (2010) Redox regulation of the NPR1-TGA1 system of *Arabidopsis thaliana* by nitric oxide. Plant Cell **22**: 2894–907

Little C, O'brien PJ (1969) Mechanism of Peroxide-Inactivation of the Sulphydryl Enzyme Glyceraldehyde-3-Phosphate Dehydrogenase. Eur J Biochem **10**: 533–538

Liu P, Zhang H, Wang H, Xia Y (2014) Identification of redox-sensitive cysteines in the Arabidopsis proteome using OxiTRAQ, a quantitative redox proteomics method. Proteomics **14**: 750–62

Lu Y, Wang H-R, Li H, Cui H-R, Feng Y-G, Wang X-Y (2013) A chloroplast membrane protein LTO1/AtVKOR involving in redox regulation and ROS homeostasis. Plant Cell Rep **32**: 1427–40

Lumbreras V, Vilela B, Irar S, Solé M, Capellades M, Valls M, Coca M, Pagès M (2010) MAPK phosphatase MKP2 mediates disease responses in Arabidopsis and functionally interacts with MPK3 and MPK6. Plant J **63**: 1017–30

Ma L-H, Takanishi CL, Wood MJ (2007) Molecular mechanism of oxidative stress perception by the Orp1 protein. J Biol Chem **282**: 31429–36

MacRobbie EA (2002) Evidence for a role for protein tyrosine phosphatase in the control of ion release from the guard cell vacuole in stomatal closure. Proc Natl Acad Sci U S A **99**: 11963–8

Maeda K, Finnie C, Svensson B (2005) Identification of thioredoxin h-reducible disulphides in proteomes by differential labelling of cysteines: insight into recognition and regulation of proteins in barley seeds by thioredoxin h. Proteomics **5**: 1634–44

Maeda K, Finnie C, Svensson B (2004) Cy5 maleimide labelling for sensitive detection of free thiols in native protein extracts: identification of seed proteins targeted by barley thioredoxin h isoforms. Biochem J **378**: 497–507

Marchand C, Le Maréchal P, Meyer Y, Decottignies P (2006) Comparative proteomic approaches for the isolation of proteins interacting with thioredoxin. Proteomics **6**: 6528–37

Marchand C, Le Maréchal P, Meyer Y, Miginiac-Maslow M, Issakidis-Bourguet E, Decottignies P (2004) New targets of Arabidopsis thioredoxins revealed by proteomic analysis. Proteomics **4**: 2696–706

Marchand CH, Vanacker H, Collin V, Issakidis-Bourguet E, Maréchal P Le, Decottignies P (2010) Thioredoxin targets in Arabidopsis roots. Proteomics **10**: 2418–28

Marinho HS, Real C, Cyrne L, Soares H, Antunes F (2014) Hydrogen peroxide sensing, signaling and regulation of transcription factors. Redox Biol **2**: 535–562

Martín-Trillo M, Cubas P (2010) TCP genes: a family snapshot ten years later. Trends Plant Sci **15**: 31–9

Marx C, Wong JH, Buchanan BB (2003) Thioredoxin and germinating barley: targets and protein redox changes. Planta **216**: 454–60

Masucci JD, Rerie WG, Foreman DR, Zhang M, Galway ME, Marks MD, Schiefelbein JW (1996) The homeobox gene GLABRA 2 is required for position-dependent cell differentiation in the root epidermis of *Arabidopsis thaliana*. Developent **122**: 1253–1260

Meinhard M, Grill E (2001) Hydrogen peroxide is a regulator of ABI1, a protein phosphatase 2C from Arabidopsis. FEBS Lett **508**: 443–6

Meinhard M, Rodriguez PL, Grill E (2002) The sensitivity of ABI2 to hydrogen peroxide links the abscisic acid-response regulator to redox signalling. Planta **214**: 775–82

Merlot S, Gosti F, Guerrier D, Vavasseur a, Giraudat J (2001) The ABI1 and ABI2 protein phosphatases 2C act in a negative feedback regulatory loop of the abscisic acid signalling pathway. Plant J **25**: 295–303

Mestres-Ortega D, Meyer Y (1999) The *Arabidopsis thaliana* genome encodes at least four thioredoxins m and a new prokaryotic-like thioredoxin. Gene **240**: 307–16

Meyer Y, Belin C, Delorme-Hinoux V, Reichheld J-P, Riondet C (2012) Thioredoxin and glutaredoxin systems in plants: molecular mechanisms, crosstalks, and functional significance. Antioxid Redox Signal **17**: 1124–60

Miao Y, Lv D, Wang P, Wang X-C, Chen J, Miao C, Song C-P (2006) An Arabidopsis glutathione peroxidase functions as both a redox transducer and a scavenger in abscisic acid and drought stress responses. Plant Cell **18**: 2749–66

Michelet L, Zaffagnini M, Vanacker H, Le Maréchal P, Marchand C, Schroda M, Lemaire SD, Decottignies P (2008) In vivo targets of S-thiolation in Chlamydomonas reinhardtii. J Biol Chem **283**: 21571–8

Miller G, Suzuki N, Ciftci-Yilmaz S, Mittler R (2010) Reactive oxygen species homeostasis and signalling during drought and salinity stresses. Plant Cell Environ **33**: 453–67

Mittler R, Vanderauwera S, Gollery M, Van Breusegem F (2004) Reactive oxygen gene network of plants. Trends Plant Sci **9**: 490–8

Mittler R, Vanderauwera S, Suzuki N, Miller G, Tognetti VB, Vandepoele K, Gollery M, Shulaev V, Van Breusegem F (2011a) ROS signaling: the new wave? Trends Plant Sci **16**: 300–9

Mittler R, Vanderauwera S, Suzuki N, Miller G, Tognetti VB, Vandepoele K, Gollery M, Shulaev V, Van Breusegem F (2011b) ROS signaling: the new wave? Trends Plant Sci **16**: 300–9

Montrichard F, Alkhalfioui F, Yano H, Vensel WH, Hurkman WJ, Buchanan BB (2009) Thioredoxin targets in plants: the first 30 years. J Proteomics **72**: 452–74

Motohashi K, Hisabori T (2006) HCF164 receives reducing equivalents from stromal thioredoxin across the thylakoid membrane and mediates reduction of target proteins in the thylakoid lumen. J Biol Chem **281**: 35039–47

Motohashi K, Kondoh a, Stumpp MT, Hisabori T (2001a) Comprehensive survey of proteins targeted by chloroplast thioredoxin. Proc Natl Acad Sci U S A **98**: 11224–9

Motohashi K, Kondoh A, Stumpp MT, Hisabori T (2001b) Comprehensive survey of proteins targeted by chloroplast thioredoxin. Proc Natl Acad Sci U S A **98**: 11224–9

Motohashi K, Koyama F, Nakanishi Y, Ueoka-Nakanishi H, Hisabori T (2003) Chloroplast cyclophilin is a target protein of thioredoxin. Thiol modulation of the peptidyl-prolyl cis-trans isomerase activity. J Biol Chem **278**: 31848–52

Mou Z, Fan W, Dong X (2003) Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. Cell **113**: 935–44

Mukherjee K, Brocchieri L, Bürglin T (2009) A comprehensive classification and evolutionary analysis of plant homeobox genes. Mol Biol Evol **26**: 2775–94

Murmu J, Bush MJ, DeLong C, Li S, Xu M, Khan M, Malcolmson C, Fobert PR, Zachgo S, Hepworth SR (2010) Arabidopsis basic leucine-zipper transcription factors TGA9 and TGA10 interact with floral glutaredoxins ROXY1 and ROXY2 and are redundantly required for anther development. Plant Physiol **154**: 1492–504

Muthuramalingam M, Matros A, Scheibe R, Mock H-P, Dietz K-J (2013) The hydrogen peroxide-sensitive proteome of the chloroplast in vitro and in vivo. Front Plant Sci **4**: 54

Ndamukong I, Abdallat A Al, Thurow C, Fode B, Zander M, Weigel R, Gatz C (2007) SA-inducible Arabidopsis glutaredoxin interacts with TGA factors and suppresses JA-responsive PDF1.2 transcription. Plant J **50**: 128–39

Neill SJ, Desikan R, Clarke A, Hurst RD, Hancock JT (2002) Hydrogen peroxide and nitric oxide as signalling molecules in plants. J Exp Bot **53**: 1237–47

Niture SK, Velu CS, Bailey NI, Srivenugopal KS (2005) S-thiolation mimicry: quantitative and kinetic analysis of redox status of protein cysteines by glutathione-affinity chromatography. Arch Biochem Biophys **444**: 174–84

Oger E, Marino D, Guigonis J-M, Pauly N, Puppo A (2012) Sulfenylated proteins in the *Medicago truncatula* - *Sinorhizobium meliloti* symbiosis. J Proteomics **75**: 4102–13

Palmieri MC, Lindermayr C, Bauwe H, Steinhauser C, Durner J (2010) Regulation of plant glycine decarboxylase by s-nitrosylation and glutathionylation. Plant Physiol **152**: 1514–28

Park S-W, Li W, Viehhauser A, He B, Kim S, Nilsson AK, Andersson MX, Kittle JD, Ambavaram MMR, Luan S, *et al* (2013) Cyclophilin 20-3 relays a 12-oxo-phytodienoic acid signal during stress responsive regulation of cellular redox homeostasis. Proc Natl Acad Sci U S A **110**: 9559–64

Parker J, Zhu N, Zhu M, Chen S (2012) Profiling thiol redox proteome using isotope tagging mass spectrometry. J Vis Exp. doi: 10.3791/3766

Paulsen CE, Carroll KS (2013) Cysteine-mediated redox signaling: chemistry, biology, and tools for discovery. Chem Rev **113**: 4633–79

Paulsen CE, Carroll KS (2009) Chemical dissection of an essential redox switch in yeast. Chem Biol **16**: 217–25

Pauwels L, Goossens A (2011) The JAZ proteins: a crucial interface in the jasmonate signaling cascade. Plant Cell **23**: 3089–100

Pei ZM, Murata Y, Benning G, Thomine S, Klüsener B, Allen GJ, Grill E, Schroeder JI (2000) Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. Nature **406**: 731–4

Peskin A V, Low FM, Paton LN, Maghzal GJ, Hampton MB, Winterbourn CC (2007) The high reactivity of peroxiredoxin 2 with H(2)O(2) is not reflected in its reaction with other oxidants and thiol reagents. J Biol Chem **282**: 11885–92

Pitzschke A, Hirt H (2009) Disentangling the complexity of mitogen-activated protein kinases and reactive oxygen species signaling. Plant Physiol **149**: 606–15

Poole LB, Klomsiri C, Knaggs S a, Furdui CM, Nelson KJ, Thomas MJ, Fetrow JS, Daniel LW, King SB (2007) Fluorescent and affinity-based tools to detect cysteine sulfenic acid formation in proteins. Bioconjug Chem **18**: 2004–17

Poole LB, Zeng B-B, Knaggs SA, Yakubu M, King SB (2005) Synthesis of chemical probes to map sulfenic acid modifications on proteins. Bioconjug Chem **16**: 1624–8

Poole TH, Reisz J a, Zhao W, Poole LB, Furdui CM, King SB (2014) Strained cycloalkynes as new protein sulfenic acid traps. J Am Chem Soc **136**: 6167–70

Prigge M, Otsuga D, Alonso J, Ecker J, Drews G, Clark S (2005) Class III Homeodomain-Leucine Zipper Gene Family Members Have Overlapping , Antagonistic , and Distinct Roles in Arabidopsis Development. Plant Cell **17**: 61–76

Pyr Dit Ruys S, Wang X, Smith EM, Herinckx G, Hussain N, Rider MH, Vertommen D, Proud CG (2012) Identification of autophosphorylation sites in eukaryotic elongation factor-2 kinase. Biochem J **442**: 681–92

Qian J, Wani R, Klomsiri C, Poole LB, Tsang AW, Furdui CM (2012) A simple and effective strategy for labeling cysteine sulfenic acid in proteins by utilization of β-ketoesters as cleavable probes. Chem Commun (Camb) **48**: 4091–3

Rasmussen MW, Roux M, Petersen M, Mundy J (2012) MAP Kinase Cascades in Arabidopsis Innate Immunity. Front Plant Sci **3**: 169

Reddie KG, Seo YH, Muse Iii WB, Leonard SE, Carroll KS (2008) A chemical approach for detecting sulfenic acid-modified proteins in living cells. Mol Biosyst **4**: 521–31

Rerie WG, Feldmann K a, Marks MD (1994) The GLABRA2 gene encodes a homeo domain protein required for normal trichome development in Arabidopsis. Genes Dev **8**: 1388–1399

Rey P, Bécuwe N, Barrault M-B, Rumeau D, Havaux M, Biteau B, Toledano MB (2007) The *Arabidopsis thaliana* sulfiredoxin is a plastidic cysteine-sulfinic acid reductase involved in the photooxidative stress response. Plant J **49**: 505–14

Richards SL, Laohavisit A, Mortimer JC, Shabala L, Swarbreck SM, Shabala S, Davies JM (2013) Annexin 1 regulates the H2O2 -induced calcium signature in *Arabidopsis thaliana* roots. Plant J 136–145

Riondet C, Desouris JP, Montoya JG, Chartier Y, Meyer Y, Reichheld J-P (2012) A dicotyledon-specific glutaredoxin GRXC1 family with dimer-dependent redox regulation is functionally redundant with GRXC2. Plant Cell Environ **35**: 360–73

Roos G, Foloppe N, Messens J (2013) Understanding the pK(a) of redox cysteines: the key role of hydrogen bonding. Antioxid Redox Signal **18**: 94–127

Roos G, Messens J (2011) Protein sulfenic acid formation: from cellular damage to redox regulation. Free Radic Biol Med **51**: 314–26

Rouhier N, Couturier J, Jacquot J-P (2006) Genome-wide analysis of plant glutaredoxin systems. J Exp Bot **57**: 1685–96

Rouhier N, Gelhaye E, Jacquot J-P (2004) Plant glutaredoxins: still mysterious reducing systems. Cell Mol Life Sci **61**: 1266–77

Rouhier N, Lemaire SD, Jacquot J-P (2008) The role of glutathione in photosynthetic organisms: emerging functions for glutaredoxins and glutathionylation. Annu Rev Plant Biol **59**: 143–66

Rouhier N, Villarejo A, Srivastava M, Gelhaye E, Keech O, Droux M, Finkemeier I, Samuelsson G, Dietz KJ, Jacquot J-P, *et al* (2005) Identification of plant glutaredoxin targets. Antioxid Redox Signal **7**: 919–29

Ryu MY, Cho SK, Kim WT (2009) RNAi suppression of RPN12a decreases the expression of type-A ARRs, negative regulators of cytokinin signaling pathway in Arabidopsis. Mol Cells **28**: 375–82

Saurin AT, Neubert H, Brennan JP, Eaton P (2004) Widespread sulfenic acid formation in tissues in response to hydrogen peroxide. Proc Natl Acad Sci U S A **101**: 17982–7

Schürmann P, Buchanan BB (2008) The ferredoxin/thioredoxin system of oxygenic photosynthesis. Antioxid Redox Signal **10**: 1235–74

Seidel T, Scholl S, Krebs M, Rienmüller F, Marten I, Hedrich R, Hanitzsch M, Janetzki P, Dietz K-J, Schumacher K (2012) Regulation of the V-type ATPase by redox modulation. Biochem J **448**: 243–51

Senkevich TG, White CL, Koonin E V, Moss B (2002) Complete pathway for protein disulfide bond formation encoded by poxviruses. Proc Natl Acad Sci U S A **99**: 6667–72

Seo YH, Carroll KS (2009a) Profiling protein thiol oxidation in tumor cells using sulfenic acid-specific antibodies. Proc Natl Acad Sci U S A **106**: 16163–8

Seo YH, Carroll KS (2009b) Facile synthesis and biological evaluation of a cell-permeable probe to detect redox-regulated proteins. Bioorg Med Chem Lett **19**: 356–9

Serpa V, Vernal J, Lamattina L, Grotewold E, Cassia R, Terenzi H (2007) Inhibition of AtMYB2 DNA-binding by nitric oxide involves cysteine S-nitrosylation. Biochem Biophys Res Commun **361**: 1048–53

Shaikhali J, Heiber I, Seidel T, Ströher E, Hiltscher H, Birkmann S, Dietz K-J, Baier M (2008) The redox-sensitive transcription factor Rap2.4a controls nuclear expression of 2-Cys peroxiredoxin A and other chloroplast antioxidant enzymes. BMC Plant Biol **8**: 48

Shaikhali J, Norén L, de Dios Barajas-López J, Srivastava V, König J, Sauer UH, Wingsle G, Dietz K-J, Strand Å (2012) Redox-mediated mechanisms regulate DNA binding activity of the G-group of basic region leucine zipper (bZIP) transcription factors in Arabidopsis. J Biol Chem **287**: 27510–25

Di Simplicio P, Franconi F, Frosalí S, Di Giuseppe D (2003) Thiolation and nitrosation of cysteines in biological fluids and cells. Amino Acids **25**: 323–39

Smykowski A, Zimmermann P, Zentgraf U (2010) G-Box binding factor1 reduces CATALASE2 expression and regulates the onset of leaf senescence in Arabidopsis. Plant Physiol **153**: 1321–31

Sohn J, Rudolph J (2003) Catalytic and chemical competence of regulation of cdc25 phosphatase by oxidation/reduction. Biochemistry **42**: 10060–70

Strader LC, Monroe-Augustus M, Bartel B (2008) The IBR5 phosphatase promotes Arabidopsis auxin responses through a novel mechanism distinct from TIR1-mediated repressor degradation. BMC Plant Biol **8**: 41

Ströher E, Dietz K-J (2008) The dynamic thiol-disulphide redox proteome of the *Arabidopsis thaliana* chloroplast as revealed by differential electrophoretic mobility. Physiol Plant **133**: 566–83

Tachibana T, Okazaki S, Murayama A, Naganuma A, Nomoto A, Kuge S (2009) A major peroxiredoxin-induced activation of Yap1 transcription factor is mediated by reduction-sensitive disulfide bonds and reveals a low level of transcriptional activation. J Biol Chem **284**: 4464–72

Tada Y, Spoel SH, Pajerowska-Mukhtar K, Mou Z, Song J, Wang C, Zuo J, Dong X (2008) Plant immunity requires conformational changes [corrected] of NPR1 via S-nitrosylation and thioredoxins. Science **321**: 952–6

Takanishi CL, Ma L-H, Wood MJ (2007) A genetically encoded probe for cysteine sulfenic acid protein modification in vivo. Biochemistry **46**: 14725–32

Takanishi CL, Wood MJ (2011) A genetically encoded probe for the identification of proteins that form sulfenic acid in response to H2O2 in Saccharomyces cerevisiae. J Proteome Res **10**: 2715–24

Tanner JJ, Parsons ZD, Cummings AH, Zhou H, Gates KS (2011) Redox regulation of protein tyrosine phosphatases: structural and chemical aspects. Antioxid Redox Signal **15**: 77–97

Tavakoli N, Kluge C, Golldack D, Mimura T, Dietz KJ (2001) Reversible redox control of plant vacuolar H+-ATPase activity is related to disulfide bridge formation in subunit E as well as subunit A. Plant J **28**: 51–9

Templeton DJ, Aye M-S, Rady J, Xu F, Cross J V (2010) Purification of reversibly oxidized proteins (PROP) reveals a redox switch controlling p38 MAP kinase activity. PLoS One **5**: e15012

Torres MA, Jones JDG, Dangl JL (2006) Reactive Oxygen Species Signaling in Response to Pathogens. **141**: 373–378

Trivedi DK, Yadav S, Vaid N, Tuteja N (2012) Genome wide analysis of Cyclophilin gene family from rice and Arabidopsis and its comparison with yeast. Plant Signal Behav **7**: 1653–66

Tron AE, Bertoncini CW, Chan RL, Gonzalez DH (2002) Redox regulation of plant homeodomain transcription factors. J Biol Chem **277**: 34800–7

Trujillo M, Clippe A, Manta B, Ferrer-Sueta G, Smeets A, Declercq J-P, Knoops B, Radi R (2007) Pre-steady state kinetic characterization of human peroxiredoxin 5: taking advantage of Trp84 fluorescence increase upon oxidation. Arch Biochem Biophys **467**: 95–106

Truong TH, Carroll KKS (2012) Bioorthogonal Chemical Reporters for Analyzing Protein Sulfenylation in Cells. Curr Protoc Chem Biol **4**: 101–122

Truong TH, Carroll KS (2013) Redox regulation of protein kinases. Crit Rev Biochem Mol Biol **48**: 332–56

Turchi L, Carabelli M, Ruzza V, Possenti M, Sassi M, Peñalosa A, Sessa G, Salvi S, Forte V, Morelli G, *et al* (2013) Arabidopsis HD-Zip II transcription factors control apical embryo development and meristem function. Development **140**: 2118–29

Tyther R, Ahmeda A (2010) Proteomic profiling of perturbed protein sulfenation in renal medulla of the spontaneously hypertensive rat. J. proteome **9(5)**: 2678-87

Ueoka-Nakanishi H, Sazuka T, Nakanishi Y, Maeshima M, Mori H, Hisabori T (2013) Thioredoxin h regulates calcium dependent protein kinases in plasma membranes. FEBS J **280**: 3220–31

Viola IL, Güttlein LN, Gonzalez DH (2013) Redox modulation of plant developmental regulators from the class I TCP transcription factor family. Plant Physiol **162**: 1434–47

Walia A, Lee JS, Wasteneys G, Ellis B (2009) Arabidopsis mitogen-activated protein kinase MPK18 mediates cortical microtubule functions in plant cells. Plant J **59**: 565–75

Wang H, Wang S, Lu Y, Alvarez S, Hicks LM, Ge X, Xia Y (2012) Proteomic analysis of early-responsive redox-sensitive proteins in Arabidopsis. J Proteome Res **11**: 412–24

Wang W, Hong S, Tran A, Jiang H, Triano R, Liu Y, Chen X, Wu P (2011) Sulfated ligands for the copper(I)-catalyzed azide-alkyne cycloaddition. Chem Asian J **6**: 2796–802

Waszczak C, Akter S, Eeckhout D, Persiau G, Wahni K, Bodra N, Van Molle I, De Smet B, Vertommen D, Gevaert K, *et al* (2014) Sulfenome mining in *Arabidopsis thaliana*. Proc Natl Acad Sci U S A 111: 11545–50

Williams JC, Sue C, Banting GS, Yang H, Glerum DM, Hendrickson W a, Schon E a (2005) Crystal structure of human SCO1: implications for redox signaling by a mitochondrial cytochrome c oxidase "assembly" protein. J Biol Chem **280**: 15202–11

Winger AM, Taylor NL, Heazlewood JL, Day DA, Millar AH (2007) Identification of intra- and intermolecular disulphide bonding in the plant mitochondrial proteome by diagonal gel electrophoresis. Proteomics **7**: 4158–70

Winterbourn CC, Metodiewa D (1999) Reactivity of biologically important thiol compounds with superoxide and hydrogen peroxide. Free Radic Biol Med **27**: 322–328

Witze ES, Old WM, Resing KA, Ahn NG (2007) Mapping protein post-translational modifications with mass spectrometry. Nat Methods **4**: 798–806

Wong JH, Balmer Y, Cai N, Tanaka CK, Vensel WH, Hurkman WJ, Buchanan BB (2003) Unraveling thioredoxin-linked metabolic processes of cereal starchy endosperm using proteomics. FEBS Lett **547**: 151–156

Wong JH, Cai N, Balmer Y, Tanaka CK, Vensel WH, Hurkman WJ, Buchanan BB (2004) Thioredoxin targets of developing wheat seeds identified by complementary proteomic approaches. Phytochemistry **65**: 1629–40

Woo HA, Kang SW, Kim HK, Yang K-S, Chae HZ, Rhee SG (2003) Reversible oxidation of the active site cysteine of peroxiredoxins to cysteine sulfinic acid. Immunoblot detection with antibodies specific for the hyperoxidized cysteine-containing sequence. J Biol Chem **278**: 47361–4

Xia X-J, Gao C-J, Song L-X, Zhou Y-H, Shi K, Yu J-Q (2014) Role of H2 O2 dynamics in brassinosteroid-induced stomatal closure and opening in Solanum lycopersicum. Plant Cell Environ **37**: 2036–50

Xia X-J, Wang Y-J, Zhou Y-H, Tao Y, Mao W-H, Shi K, Asami T, Chen Z, Yu J-Q (2009) Reactive oxygen species are involved in brassinosteroid-induced stress tolerance in cucumber. Plant Physiol **150**: 801–14

Xing S, Lauri A, Zachgo S (2006) Redox regulation and flower development: a novel function for glutaredoxins. Plant Biol (Stuttg) **8**: 547–55

Xu Q, Fu HH, Gupta R, Luan S (1998) Molecular characterization of a tyrosine-specific protein phosphatase encoded by a stress-responsive gene in Arabidopsis. Plant Cell **10**: 849–57

Yamada K, Lim J, Dale JM, Chen H, Shinn P, Palm CJ, Southwick AM, Wu HC, Kim C, Nguyen M, *et al* (2003) Empirical analysis of transcriptional activity in the Arabidopsis genome. Science **302**: 842–6

Yamazaki D, Motohashi K, Kasama T, Hara Y, Hisabori T (2004) Target proteins of the cytosolic thioredoxins in *Arabidopsis thaliana.* Plant Cell Physiol **45**: 18–27

Yan J, Zhang C, Gu M, Bai Z, Zhang W, Qi T, Cheng Z, Peng W, Luo H, Nan F, *et al* (2009) The Arabidopsis CORONATINE INSENSITIVE1 protein is a jasmonate receptor. Plant Cell **21**: 2220–36

Yano H, Kuroda M (2006) Disulfide proteome yields a detailed understanding of redox regulations: a model study of thioredoxin-linked reactions in seed germination. Proteomics **6**: 294–300

Yano H, Wong JH, Lee YM, Cho MJ, Buchanan BB (2001) A strategy for the identification of proteins targeted by thioredoxin. Proc Natl Acad Sci U S A **98**: 4794–9

Yohn CB, Cohen A, Rosch C, Kuchka MR, Mayfield SP (1998) Translation of the Chloroplast psbA mRNA Requires the Nuclear-encoded Poly(A)-binding Protein, RB47. J Cell Biol **142**: 435–442

Yoshida K, Noguchi K, Motohashi K, Hisabori T (2013) Systematic exploration of thioredoxin target proteins in plant mitochondria. Plant Cell Physiol **54**: 875–92

Zaffagnini M, Bedhomme M, Lemaire SD, Trost P (2012a) The emerging roles of protein glutathionylation in chloroplasts. Plant Sci **185-186**: 86–96

Zaffagnini M, Bedhomme M, Marchand CH, Morisse S, Trost P, Lemaire SD (2012b) Redox regulation in photosynthetic organisms: focus on glutathionylation. Antioxid Redox Signal **16**: 567–86

Zhang Y, Tan J, Guo Z, Lu S, He S, Shu W, Zhou B (2009) Increased abscisic acid levels in transgenic tobacco over-expressing 9 cis-epoxycarotenoid dioxygenase influence H_2O_2 and NO production and antioxidant defences. Plant Cell Environ **32**: 509–19

Zhou J, Xia X-J, Zhou Y-H, Shi K, Chen Z, Yu J-Q (2014) RBOH1-dependent H_2O_2 production and subsequent activation of MPK1/2 play an important role in acclimation-induced cross-tolerance in tomato. J Exp Bot **65**: 595–607

Zhu M, Zhu N, Song W, Harmon AC, Assmann SM, Chen S (2014) Thiol-based redox proteins in abscisic acid and methyl jasmonate signaling in Brassica napus guard cells. Plant J **78**: 491–515

Chapter 4

DYn-2 based identification of Arabidopsis sulfenomes

Chapter 4

DYn-2 based identification of Arabidopsis sulfenomes

The results of this chapter will be published as

Salma Akter, Jingjing Huang, Nandita Bodra, Barbara De Smet, Khadija Wahni, Debbie Rombaut, Jarne Pauwels, Kris Gevaert, Kate Carroll, Frank Van Breusegem and Joris Messens (2014). DYn-2 based identification of Arabidopsis sulfenomes (research article submitted to the Journal of Molecular & Cellular Proteomics)

Author contributions: Mosammat Salma Akter, Frank Van Breusegem and Joris Messens designed this research. Mosammat Salma Akter performed the experiments. Debbie Rombaut, Khadija Wahni, Jarne Pauwels, Nandita Bodra provided technical assistance. Kris Gevaert, and Kate Carroll contributed analytic tools and DYn-2 probe respectively; Mosammat Salma Akter, Jingjing Huang, Jarne Pauwels, Barbara De Smet, Joris Messens and Frank Van Breusegem analyzed the data. Mosammat Salma Akter, Jingjing Huang, Frank Van Breusegem and Joris Messens wrote the paper.

Chapter 4

DYn-2 based identification of Arabidopsis sulfenomes

4.1 Abstract

Identifying the sulfenylation state (SOH) of living cells under stress is emerging as a strategic approach for the detection of key reactive oxygen species (ROS) sensors and signaling proteins. However, the detection of this highly reactive oxidation state of cellular thiols is quite challenging, since, once formed, they might be regulated to their original thiol states (SH) or might be subject to overoxidation (SO_2H or SO_3H). After the YAP1 based cytoplasmic sulfenome mining described in Chapter 2, we aimed to uncover the complete cellular sulfenome of *Arabidopsis thaliana*. Therefore, we optimized an *in vivo* trapping method of sulfenic acids in hydrogen peroxide (H_2O_2) stressed Arabidopsis cells using the dimedone based alkyne functionalized probe DYn-2. With click chemistry the DYn-2 probe was biotinylated for subsequent streptavidin enrichment and Western blot visualization. We demonstrated that DYn-2 specifically detects sulfenylation events in a H_2O_2 dose- and time-dependent way. With mass spectrometry, we identified 226 sulfenylated proteins after H_2O_2 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 sulfenylated proteins have never been reported before to undergo cysteine oxidative post-translational modifications (PTMs) in any plant species. Amongst the previously reported targets, 48 were already reported as sulfenylated proteins in plants, thereby technically validating our approach. All in all, with this DYn-2 approach, we have identified new sulfenylated proteins, and gave a first glance on the subcellular sulfenomes of Arabidopsis.

4.2 Introduction

Among the different amino acids, the sulfur containing amino acids like cysteine residues are particularly susceptible to oxidation by ROS (Di Simplicio *et al*., 2003; Jacques *et al*., 2013). Recent studies suggest that the sulfenome, the initial oxidation products of cysteine

residues, functions as an intermediate state of redox signaling (Delaunay *et al*., 2002; Tachibana *et al*., 2009; Chiang and Schellhorn, 2012). Thus, identifying the sulfenome under oxidative stress is a potential way to detect redox sensors (Leonard *et al*., 2009; Roos and Messens, 2011).

This central role of the sulfenome in redox signaling provoked chemical biologists to develop strategies for sensitive detection and identification of sulfenylated proteins. The *in situ* trapping of the sulfenome becomes more important because of two major factors: (i) the sulfenic acids are highly reactive transient intermediates, which might be over-oxidized in excess of ROS, unless immediately protected by disulfide formation (Roos and Messens, 2011); (ii) the compartmentalization of the redox state in cells that might be disrupted during cell lysis procedures and proteins might result in artificial non-native oxidations (Go and Jones, 2008; Leonard and Carroll, 2011). 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 acids (Gupta and Carroll, 2014). In 1974, Allison and coworkers reported a condensation reaction between the electrophilic sulfenic acid and the nucleophile dimedone (5,5-dimethyl-1,3-cyclohexanedione) producing a corresponding thioether derivative (Benitez and Allison, 1974). This chemistry is highly selective and since then has been exploited to detect dimedone modified sulfenic acids using mass spectrometry (Carballal *et al*., 2003). However, dimedone has limited applications for cellular sulfenome identification due to 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 (Poole *et al*., 2005; Charles *et al*., 2007; Poole *et al*., 2007). This approach, however, was not always compatible with *in vivo* cellular sulfenome analysis, since the biotin/fluorophores conjugated dimedone is membrane impermeable (Leonard and Carroll, 2011) and endogenous biotinylated proteins might appear as false positives.

More recently, the Carroll lab has developed a sulfenic acid specific chemical probe DYn-2 (Leonard and Carroll, 2011). 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. Once the sulfenic acids are tagged with the DYn-2 probe, they are biotinylated through the copper (I)-catalyzed azide–alkyne cyclo addition, the

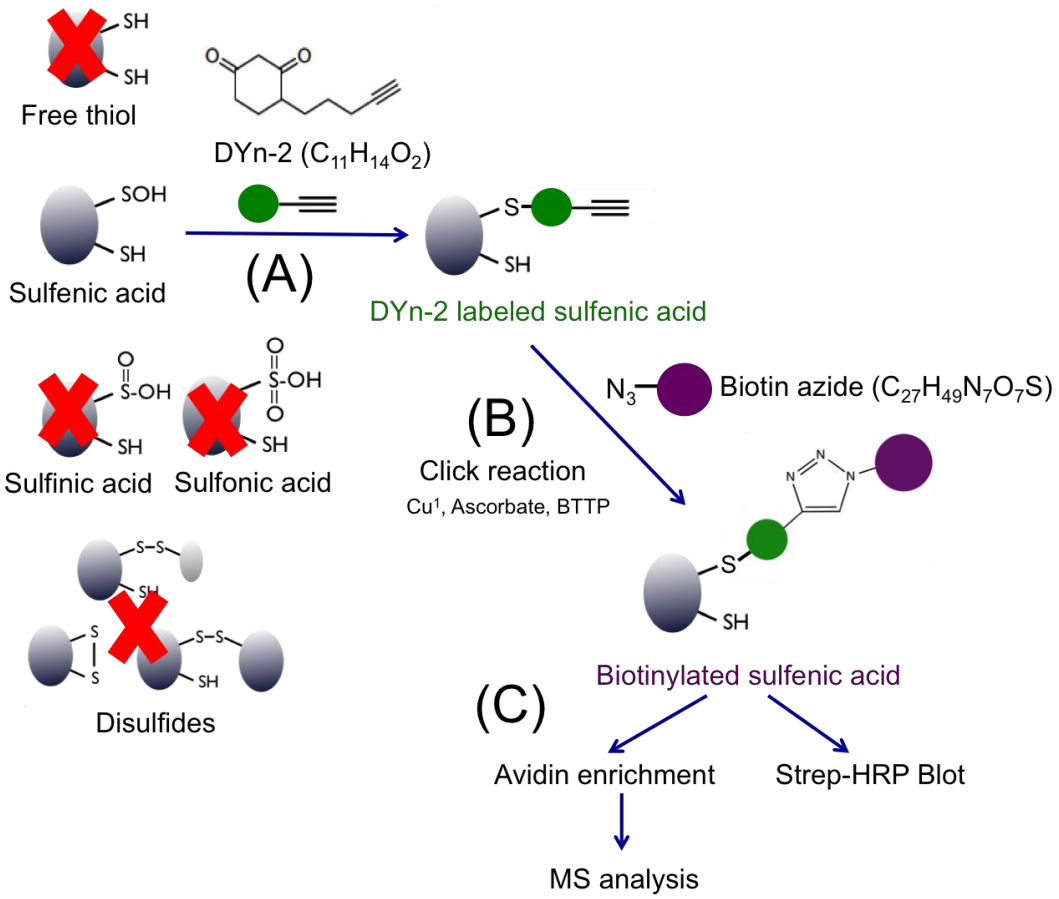


Figure 4.1 Schematic views of the molecular mechanism of the DYn-2 probe and the strategy to identify DYn-2 trapped sulfenylated proteins. (A) DYn-2 specifically detects sulfenic acid modification, not other thiol modifications. (B) Biotinylation of the DYn-2 tagged proteins by click reaction. (C) Once DYn-2 tagged proteins are biotinylated, streptavidin-HRP blot (Strep-HRP) could be applied to observe the DYn-2 tagged sulfenylation or after avidin enrichment, those proteins could be identified by mass spectrometry analysis.

typical reaction of click chemistry (Wang *et al*., 2011), facilitating downstream detection methods and mass spectrometry based identifications . A schematic presentation of the strategy is shown in figure 4.1. In an evaluation experiment, DYn-2 was found efficient to detect H_2O_2-dependent sulfenic acid modifications in recombinant glutathione peroxidase 3 (Gpx3) of budding yeast (Paulsen *et al*., 2012). Moreover, it was reported that DYn-2 is membrane-permeable, non-toxic and a non-influencer of the intracellular redox balance (Paulsen *et al*., 2012; Truong and Carroll, 2012). Therefore, DYn-2 has been suggested as a global sulfenome reader in living cells (Paulsen *et al*., 2012; Truong and Carroll, 2012), and has been applied to investigate epidermal growth factor (EGF)-mediated protein

sulfenylation in an human epidermoid carcinoma A431 cell line and to identify intracellular protein targets of H_2O_2 during cell signaling (Truong and Carroll, 2012).

Considering the successful application of DYn-2 to read the sulfenome in mammalian cell lines, we selected the DYn-2 probe to picture the sulfenome in plant cells under oxidative stress. Through a combination of biochemical, immunoblot and tandem mass spectrometry techniques, we demonstrated that DYn-2 is able to detect sulfenic acids in different subcellular compartments of *A. thaliana*. We identified 226 sulfenylated proteins in response to a H_2O_2 treatment of Arabidopsis cell suspensions, of which 123 proteins are new candidates for cysteine oxidative PTM events. Among 103 previously reported targets, 48 are already reported as sulfenylated proteins in plants (Oger *et al*., 2012; Waszczak *et al*., 2014), and importantly, they represent the validation of the sulfenylation modification of this study.

4.3 Materials and Methods

Arabidopsis cell cultures, stress treatment and DYn-2 labeling. *A. thaliana* cell suspension (PSB-D) were cultured as previously described (Van Leene *et al*., 2007). All experiments were performed on dark-grown cells to mid-log phase. The time and dose of the stress treatment as well as DYn-2 labeling were performed as follows:

(i) For optimization of DYn-2 labeling conditions, we followed two conditions: (A) 10 mL cell cultures were stressed for 1 hour by addition of 0, 0.1, 1 and 20 mM H_2O_2 in separated conical flasks (Merck, Germany). Then, the cells were harvested by filtration and rinsed with culture medium. After re-suspension of the stressed cells in the culture medium, the probe labeling was performed with 0, 0.5, 1, 2.5, 5, and 10 mM of DYn-2 for 1 hour. (B) The cell cultures were stressed for 1 hour by addition of 0, 0.1, 1 and 20 mM H_2O_2 in the presence of 5 mM DYn-2.

(ii) 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 H_2O_2 for 1 h in the presence of 1 mM DYn-2 probe.

(iii) For the optimization of DYn-2 labeling, the cells were treated with 20 mM H_2O_2 in the presence of 0, 0.5, 1, 2.5, 5, 10 mM DYn-2 for 1 hour.

(iv) For the detection of the dose dependent responses of cells to H_2O_2 treatment,

10 mL cell culture were treated with 0, 0.5, 1, 2, 5, 10 and 20 mM H_2O_2 in the presence of 500 µM DYn-2 for 1 hour.

(v) For the detection of the time dependent responses, 50 mL cell culture were treated with 0, 1 and 20 mM H_2O_2 separately in the presence of 500 µM DYn-2. After 15, 30, 60 and 120 minutes treatment, 10 mL cells were harvested at indicated time points from each H_2O_2 concentration.

(vi) For the mass spectrometry identification, 20 mL cells were treated with 0 and 10 mM H_2O_2 for 30 minutes in the presence of 500 µM DYn-2.

After stress treatment and DYn-2 probe labeling, the cells were harvested by filtration, washed 3X with culture medium and then the cells were ready for protein extraction, click reaction and following downstream analysis. Before each experiment, the concentration of H_2O_2 has been determined at 240 nm using 43.6 $M^{-1}cm^{-1}$ as the molar extinction coefficient.

Protein extraction, click reaction, Western blot analysis. For the protein extraction and biotinylation by click reaction, we followed the protocol as mentioned previously with some modifications (Truong and Carroll, 2012). Harvested cells were ground on ice using sand in the presence of EDTA free extraction buffer (25 mM Tris HCl pH 7.6, 15 mM $MgCl_2$, 150 mM NaCl, 15 mM $pNO_2PhenylPO_4$, 60 mM B-glycerolphosphate, 0.1% NP-40, 0.1 mM Na_3VO_4, 1 mM NaF, 1 mM Phenylmethanesulfonyl fluoride, 1 µM E64, 1 x Roche protease inhibitor cocktail, 5% Ethylene glycol) supplemented with catalase at 200 U/mL. The lysates were centrifuged at 16,100 x g at 4ºC for 30 min to clear cell debris. Protein content from the soluble fractions was quantified using a standard DC Protein Assay (Bio-Rad Laboratories Inc., Hercules, USA). After removing endogenous biotinylated proteins by NeutrAvidin agarose beads, click reaction was performed in 100 µg proteins for 1 hr rocking incubation at room temperature (Truong and Carroll, 2012). By incubating for 5 min with 1 mM EDTA, the click reaction was stopped. Protein samples were denatured for 5 min at 96ºC. Then 25 µg proteins from each sample were resolved by SDS-PAGE and sulfenylation were observed by streptavidin-HRP (Strep-HRP) blot with a dilution 1:80,000. Equal loading of each protein was confirmed by coomassie staining.

Affinity enrichment of DYn-2 tagged proteins. For LC-MS/MS analysis, we performed the click reaction in 1 mg 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% TCA to remove non-reacted click reagents from the lysates upon incubation overnight at -20ºC. On the second day, the precipitated proteins were pelleted by centrifugation at max speed for 30 min at 4ºC. The pellet was washed two times with ice-cold acetone containing 5 mM DTT. Then the pellet was air dried to remove the acetone from the pellet. After complete re-suspension of the precipitated proteins in PBS containing 0.2 % SDS, the biotinylated DYn-2 labeled proteins were enriched with 200 µL Neutravidin agarose beads pre-equilibrated with re-suspension buffer. The beads were collected by centrifugation at 2,800 x g for 2 min, washed with PBS, which was followed by incubation with 5 mM DTT in the same buffer for 30 min at room temperature. Then, stringent washing steps were performed: 1x PBS, 1x 1 M NaCl for 5 min, 1x PBS, 1x 4 M urea for 5 min, 1x PBS, 1x PBS containing 0.2% (w/v) SDS, 3x PBS. After each step of washing, 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, and this by boiling for 10 min. The eluted proteins were lyophilized and then re-suspensed in 15 µL/15 µL H2O/SDS loading buffer, resolved on SDS-PAGE as a single band, 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 (Thermo Scientific, Bremen, Germany), in-line connected to a Q-Exactive mass spectrometer (Thermo Fisher Scientific). 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. From the MS/MS data in each LC run, Mascot Generic Files were created using 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 5 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. These peak lists were then searched with the Mascot search engine (MatrixScience,

www.matrixscience.com) using the Mascot Daemon interface (version 2.4, Matrix Science). The MS/MS data were searched against the TAIR10 database. 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 mmu. The instrument setting was put on ESI-QUAD. Enzyme was set to trypsin, allowing for one missed cleavage. Only peptides that were ranked one 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.

We considered the total unique identifications of two independent experimental rounds of the non-treated samples as the background dataset. For the dataset of H_2O_2 treated samples, the overlapping identifications of three independent experiments were taken into account. To obtain the H_2O_2-dependent DYn-2 sulfenome, we subtracted the background datasets from the dataset of the H_2O_2 treated identifications.

4.4 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 (Truong and Carroll, 2012). For *Arabidopsis* cell suspension cultures, the factors could be the changes in physico-chemical parameters of the culture medium, nutrient deficiency, cells grown to the stationary phase etc. We performed the H_2O_2 stress treatments and DYn-2 probe labeling on dark-grown Arabidopsis cell suspension cultures grown to a mid-log phase. The culture conditions were described earlier (Van Leene *et al*., 2007).

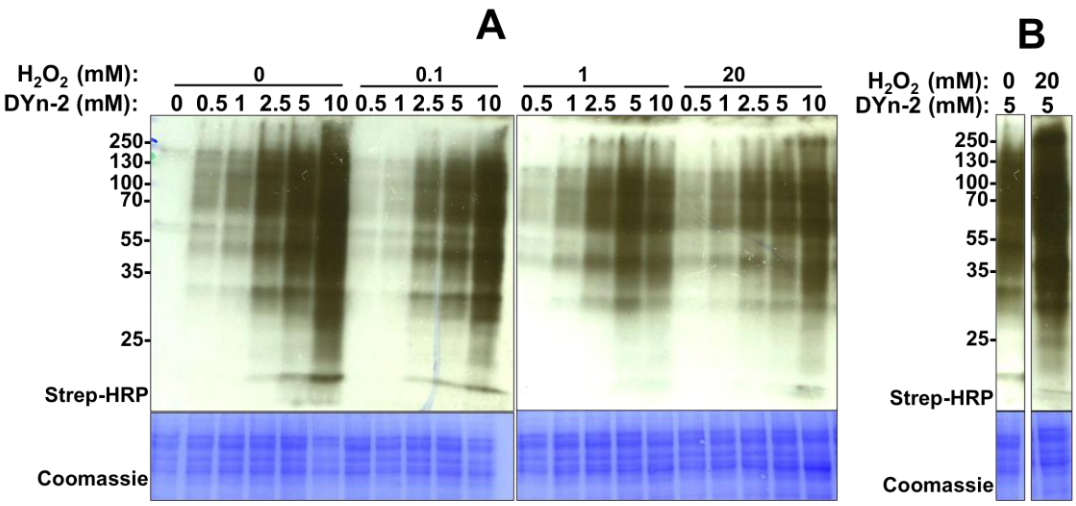


Figure 4.2 Optimization of the DYn-2 probe labeling conditions during and after H_2O_2 stress treatment. (A) The Arabidopsis cells were treated with 0, 0.1, 1 or 20 mM H_2O_2 for 1 h, and labeled with 0, 0.5, 1, 2.5, 5, or 10 mM DYn-2 for 1 h. (B) The cells were stressed by 0 or 20 mM H_2O_2 stress in the presence of 5 mM DYn-2. After click reaction and Strep-HRP Western blot analysis, the DYn-2 tagged sulfenylation signal was found to be higher in the 20-mM H_2O_2 stressed sample in comparison to non-stressed cells.

For the DYn-2 probe labeling, we tested two conditions: the DYn-2 labeling after H_2O_2 treatment (Figure 4.2A) or the DYn-2 labeling during the formation of the sulfenic acids (Figure 4.2B). The cells were harvested by filtration followed by washing with culture medium to remove excess H_2O_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 (Truong and Carroll, 2012) following proteins separation on SDS-PAGE gel and visualization of the DYn-2 tagged biotinylated proteins on Strep-HRP 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 (Truong and Carroll, 2012), we found the H_2O_2 stress treatment performed in the presence of the DYn-2 probe is an efficient approach to trap sulfenic acids in Arabidopsis cells (Figure 4.2). Important to note is that we used a catalase-supplemented extraction buffer to extract soluble protein fractions. Catalase scavenges H_2O_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 the addition of catalase showed a clear influence of catalase to control post-extraction sulfenic

acid formation at higher H_2O_2 concentrations (Figure 4.3). By incubating the lysate with NeutrAvidin agarose beads, we removed endogenous biotinylated proteins and the non-sulfenylated proteins sticking to the beads.

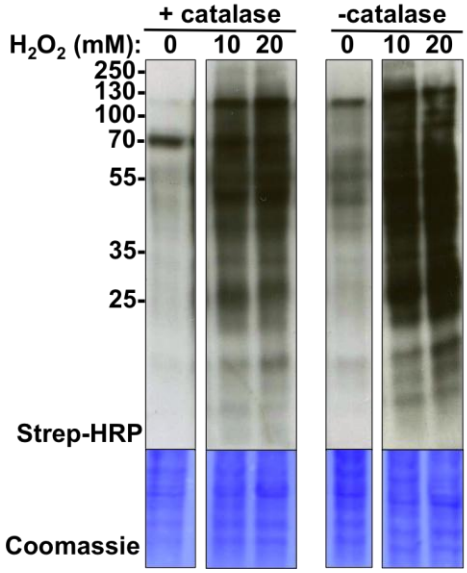


Figure 4.3 Catalase controls *de novo* sulfenylation during protein extraction. Cell cultures were treated with 0, 10 or 20 mM H_2O_2 for 1 h in the presence of 500 µM DYn-2 probe. Proteins were extracted in the extraction buffer with or without catalase, and after the click reaction, visualized on a Strep-HRP Western blot. The increased signal intensity in the absence of catalase shows the importance of the addition of catalase during protein extraction.

DYn-2 competes with YAP1C trapping. After optimizing the DYn-2 labeling conditions, 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. For this purpose, the YAP1C overexpressing cells were treated with 0 mM H_2O_2 concentration for 1 h in the presence or absence of DYn-2. As mentioned in Chapter 2, 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* (Takanishi *et al*., 2007; Takanishi and Wood, 2011). We have developed YAP1c-CRD overexpressing Arabidopsis cells to identify the cytoplasmic sulfenome under oxidative cells. Briefly, we designed two variant of YAP1 c-CRD: I) YAP1C containing redox regulatory Cys598 to trap Cys-SOH under oxidative

stress and II) YAP1A (Cys598 is mutated to Alanine) to control non-specific protein associations. YAP1 fragments were fused with GS tag moiety for downstream analysis. With the help of peroxidase-anti-peroxidase (PAP) antibody, which detect the GS tag moiety, we showed that in response to H_2O_2, YAP1C forms mixed disulfides with Cys-SOH of unknown proteins in a H_2O_2 concentration dependent manner (Waszczak *et al*., 2014). However, these complexes were absent in YAP1A control cells since the YAP1 c-CRD disulfide-bonded complexes are formed through the specific reaction of Cys598 with Cys-SOH on multiple proteins.

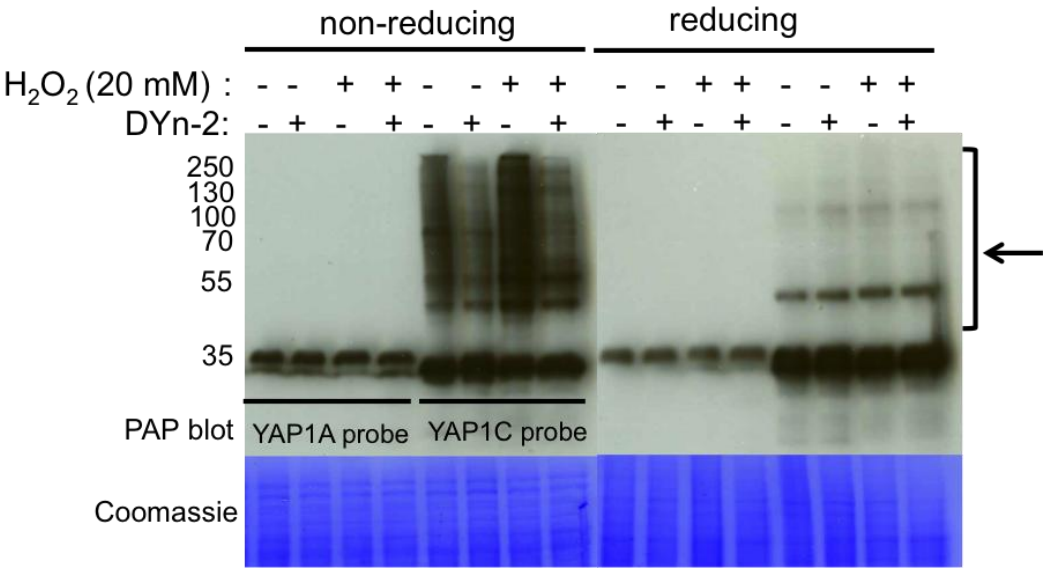


Figure 4.4 DYn-2 chemical probe is competitive to YAP1C genetic probe. YAP1C/YAP1A overexpressing cell cultures were treated with 0 and 20 mM H_2O_2 in the presence of absence of DYn-2 for 1 h. Proteins were extracted in the catalase supplemented extraction buffer, and YAP1C complexes (marked with an arrow) are visualized with the PAP antibody complex. YAP1C complex formation is reduced in the presence of DYn-2. Treatment of protein samples with 50 mM TCEP led to reduction of the complexes.

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_2O_2 for 1 h in the presence or absence of 1 mM DYn-2. As a control, we compared the response with non-stressed 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 non-stressed conditions (Figure 4.4). 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_2O_2 stressed conditions in the presence of the DYn-2 probe, YAP1C dimerization was decreased (Figure 4.4), 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 Figure 4.7F).

DYn-2 traps sulfenylated proteins under oxidative stress in a dose- and time-dependent manner. After optimizing the DYn-2 labeling conditions, we set out an experiment to optimize the dose of DYn-2 required for sulfenome trapping. Following experimental condition of B in figure 4.2, we stressed the cells with 20 mM H_2O_2 for 1 h in the absence or presence of increasing concentration of DYn-2 up to 10 mM. Sample preparation and biotinylation of the DYn-2 tagged proteins were carried according to materials and methods. On streptavidin-HRP blot, we observed that DYn-2 is able detect sulfenic acids at its lowest concentration of 500 μM (Figure 4.5). From this experiment, it was also revealed that by increasing the DYn-2 concentration we could detect more sulfenylated proteins. However, we decided to work at low concentration of DYn-2, because of probing at higher concentration might lead to the presence of non-reacted intracellular DYn-2. This is a consideration to avoid false positive sulfenylation signal as excess intracellular DYn-2 might tag if there is newly modified proteins during extraction. The sample without probe labeling was regarded as a negative control (Figure 4.5, lane 1), and showed no background signal, indicating that the click reagent biotin azide is specific to react with DYn-2 tagged proteins.

After optimization of 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_2O_2 treatment of Arabidopsis cells provokes cysteine sulfenylation (Desikan *et al*., 2001; Waszczak *et al*., 2014).

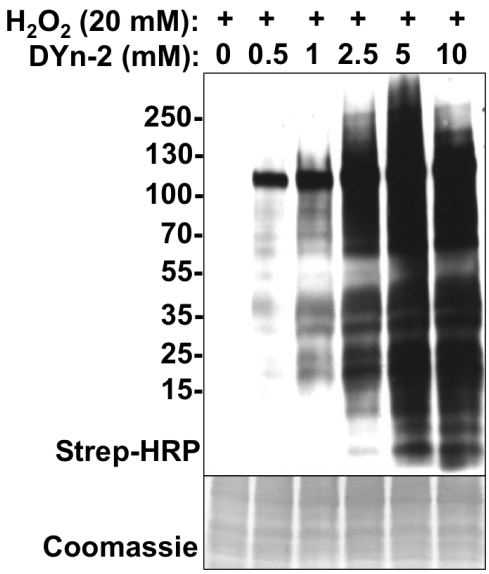


Figure 4.5 DYn-2 concentration optimization to trap sulfenic acids in Arabidopsis cell cultures. Cell cultures were treated with 20 mM H_2O_2 in the presence of 0, 0.5, 1, 2.5, 5 or 10 mM DYn-2 for 1 h. After extraction and click reaction, soluble protein fractions were visualized on a Strep-HRP Western blot. The sulfenylation signal was observed from 0.5 mM DYn-2 onward.

To evaluate the H_2O_2 dose response, we treated the cells with 0, 0.5, 1, 2, 5, 10 or 20 mM H_2O_2 for 1 h in the presence of 500 μM DYn-2 (Figure 4.6A). On Strep-HRP Western blot, we observed that sulfenic acid labeling by DYn-2 was H_2O_2 dose dependent. Non-stressed cells displayed only low levels of basal sulfenic acid labeling, whereas an increasing signal was observed from 2 mM of H_2O_2 onward. We concluded that DYn-2 responds to a changing protein sulfenic acid formation 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_2O_2 and samples were analyzed after 15, 30, 60 and 120 min of each stress treatment (Figure 4.6B). We observed a response to the changes of sulfenylation in function of time at the 20-mM H_2O_2 treatment. The time-dependent response was not significant at the 1-mM H_2O_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 non-stressed conditions remains the same in the presence of DYn-2 (Figure 4.6B). This is an important observation, since it indicates that DYn-2 itself is not generating oxidative

stress in *Arabidopsis thaliana* cells and does not disturb the basal level of sulfenylation

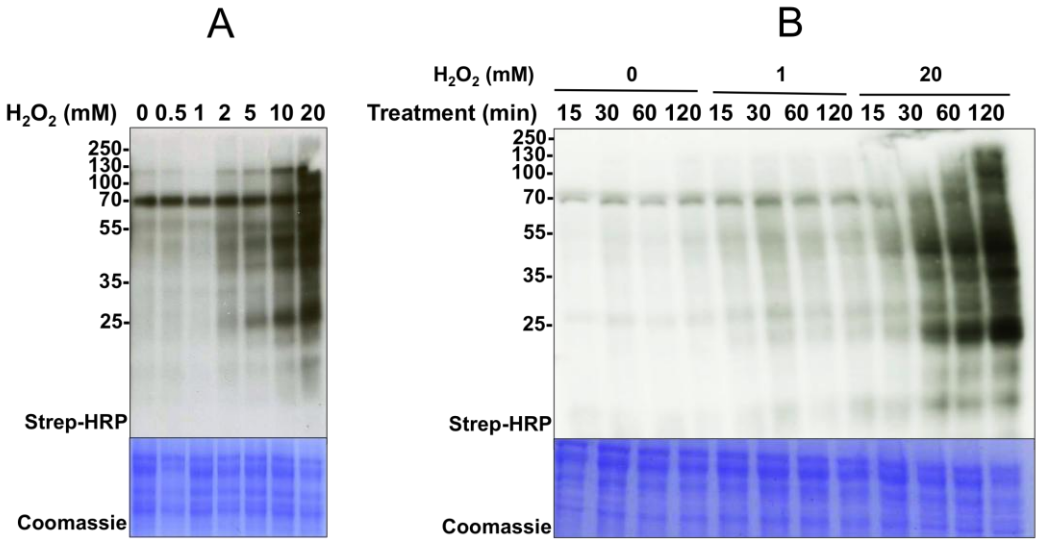


Figure 4.6 DYn-2 detects time- and dose-dependent changes of H_2O_2 mediated sulfenylation in Arabidopsis. (A) Cell cultures were treated with 0, 0.5, 1, 2, 5, 10 or 20 mM H_2O_2 for 1 h in the presence of 500 µM DYn-2 probe. After the click reaction, the H_2O_2 dose-dependent sulfenylation was visualized on a Strep-HRP developed Western blot. (B) Cell cultures were treated with 0, 1 or 20 mM H_2O_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.

under non-stressed conditions. It was also previously reported that DYn-2 does not alter cell viability and glutathione redox balance, or generate ROS in other cell types (Paulsen *et al*., 2012) .

Identification of 226 sulfenylated proteins under H_2O_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. To identify the sulfenylated proteins at subcellular level, the *Arabidopsis* cells were incubated with 0 and 10 mM H_2O_2 for a period of 30 min in the presence of 500 µM DYn-2. The 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 re-suspension of precipitated protein pellet, DYn-2 tagged proteins were trapped on NeutrAvidin beads. The high affinity of biotin-

avidin interaction (the dissociation constant, K_D is 10^{-15} M) allowed stringent washing steps like 1 M NaCl, 4 M urea to remove all non-biotinylated interactions formed during enrichment. After several intensive consecutive washing steps (for details see materials and methods), the biotinylated proteins were eluted with biotin competition under denaturing conditions. In figure 4.7A, a representative Strep-HRP developed Western blot shows an affinity purification of the DYn-2 tagged proteins of non-stressed and stressed cells. An increased sulfenylation signal in the enriched DYn-2 tagged proteins from stressed cells is observed.

After enrichment, Eluted proteins were subjected to LC-MS/MS to identify the sulfenylated proteins. The MS/MS data were searched against the TAIR10 database and we identified 420 different sulfenylated proteins from the three independent experiments of treating cells with 10 mM H_2O_2. As we wanted to focus on the sulfenylated proteins under H_2O_2 stress, the proteins identified in the absence of H_2O_2 were considered as a background dataset. As such, we identified 226 sulfenylated proteins of the H_2O_2 mediated sulfenome of *Arabidopsis thaliana* (Figure 4.7B).

DYn-2 reads the plant sulfenome in different plant organelles. We categorized the 226 H_2O_2-dependent sulfenylated proteins based on their predicted or demonstrated subcellular localization, function (Gene Ontology (GO) annotation), and reported cysteine oxidative modifications. Figure 4.7C 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 plastidal (30%); 10 nuclear (4.4%); 14 mitochondrial (6.2%), 7 from the endoplasmic reticulum, Golgi and plasma membrane (3.1 %) and 4 from the peroxisome (1.8%) (Table 4.1, Figure 4.7C). 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 SUPEROXIDE DISMUTASE 1, which might be trapped as a possible interactor of one of the identified proteins (Table 4.1). The majority of the identified proteins are 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 4.1).

Within the DYn-2 sulfenome (Figure 4.7D and 4.7E; Table 4.1), some proteins with reactive cysteines have previously been reported. As such, we confirmed 25 S-glutathionylated proteins (Dixon *et al*., 2005; Rouhier *et al*., 2005; Konopka-Postupolska *et al*., 2009), 55 proteins with a redox active disulfide bond (Balmer *et al*., 2004; Marchand *et al*., 2004; Marchand *et al*., 2006; Winger *et al*., 2007; Ströher and Dietz, 2008; Marchand *et al*., 2010; Yoshida *et al*., 2013), and 29 S-nitrosylated proteins (Lindermayr *et al*., 2005; Abat and Deswal, 2009; Tanou *et al*., 2009; Palmieri *et al*., 2010; Fares *et al*., 2011) (Figure 4.7E; Table 4.1). Apart from that, we identified 30 proteins that are in common with the sulfenome of *Medicago truncatula*, which was analyzed using Bio-DCP1, another dimedone chemistry based probe (Oger *et al*., 2012) (Table 4.2). Moreover, we also identified several established antioxidant and signaling proteins like CHLOROPLASTIC GLUTAMATE-CYSTEINE LIGASE, STROMAL ASCORBATE PEROXIDASE, GLUTATHIONE S-TRANSFERASE TAU 19, THIOREDOXIN-DEPENDENT PEROXIDASE 1, MONODEHYDROASCORBATE REDUCTASE 6, ACC OXIDASE 2, NUCLEOREDOXIN 1, ANNEXIN 1 and GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE A.

When we compare lists of proteins discovered with the YAP1C (95) (Waszczak *et al*., 2014) and DYn-2 (123; Figure 4.7F, Table 4.2) probes, only 16 proteins were common. This discrepancy is most likely due to the different mode of action and reactivity of both probes, leading to discrete sensitivities. Dimedone reacts with a sulfenic acid at a rate of 2.7×10^{-2} $M^{-1}s^{-1}$ (Paulsen and Carroll, 2013). The DYn-2 probe, however, is doing much better, since its reaction rate with dipeptide SOH is estimated to be 11 $M^{-1}s^{-1}$ (Gupta and Carroll, 2014). 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 $M^{-1}s^{-1}$) (Paulsen and Carroll, 2013; Gupta and Carroll, 2014), the YAP1C probe should be more efficient in trapping sulfenic acids compared to DYn-2. Although the dimedone 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 (Figure 4.4). Noteworthy, whether a reaction will occur does not only depend on the reaction rate, but also on the local concentration. Here, we used 500 µM DYn-2 to trap the sulfenome, and we think that it is fair to assume that the

concentration of the overexpressed YAP1C will not be in the sub-millimolar range like that of DYn-2, but rather in the nanomolar range. 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 independent 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 (Waszczak *et al*., 2014).

4.5 Significance

We report here the first successful application of the DYn-2 chemical probe for the identification of subcellular sulfenomes in plants. With an optimized DYn-2 trapping technique, cytoplasmic, plastidal, mitochondrial, nuclear, peroxisomal, endoplasmic reticulumn, Golgi and plasma membrane sulfenylated proteins were identified. Besides the identification of these sulfenomes, our efforts lead to a more complete view of the cytoplasmic sulfenome with the identification of 107 new cytoplasmic candidates, so we doubled the identified sulfenylated proteins in the cytoplasm. We strongly believe that by reading the DYn-2 sulfenome of *Arabidopsis thaliana*, an additional important piece within the cellular sulfenome jigsaw puzzle is given. On the long run, it will contribute to unraveling signaling events along the sulfenome of plants, and it will help our understanding of signaling transduction pathways under oxidative stress in general.

Acknowledgements

We thank Prof. Kate Carroll to provide us the synthesized DYn-2 probe. We are grateful to her to provide me an opportunity to be trained on DYn-2 labeling and click reaction strategies. We thank Thu H. Truong, Francisco J Garcia, Pablo Martínez-Acedo and Mauro Lo Conte for their excellent technical assistance during my training. This training

cost was provided from VUB grant (HOA 22).

Table 4.2 The identified sulfenylated candidates at different subcellular localizations in *Arabidopsis thaliana*

AGI code	Description	Subcellular localization	Functional categorization	No of Cys	Redox modification	References
Cytoplasm						
AT3G62940	OVARIAN TUMOR DOMAIN (OTU)-CONTAINING DUB (DEUBIQUITILATING 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	S-SG	(Dixon *et al*., 2005)
AT5G63680	Pyruvate kinase family protein	Cytoplasm, cytosol, plasma membrane	Primary metabolism	11		
AT1G62740	HOP2	Cytoplasm, cytosol, nucleus, plasma membrane	Miscellaneous	5	SOH	(Waszczak *et al*., 2014)
AT5G43330	CYTOSOLIC-NAD-DEPENDENT MALATE DEHYDROGENASE 2	Cytoplasm, cytosol, plasma membrane, plasmodesma, apoplast	Primary metabolism	6	Grx target; reactive cys; Trx target	(Rouhier *et al*., 2005*, Wang *et al*., 2012, Wong *et al*., 2004*)
AT2G32520	Alpha/beta-Hydrolases superfamily protein	Cytoplasm, cytosol, chloroplast	Protein degradation	1	Trx target; SNO	(Balmer *et al*., 2004a*, Tanou *et al*., 2009*)
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 motifs) domain	Cytoplasm	RNA binding-translation	3		
AT2G24050	EUKARYOTIC TRANSLATION INITIATION FACTOR ISOFORM 4G2	Cytoplasm, cytosol	RNA binding-translation	10		
AT5G10240	ASPARAGINE SYNTHETASE 3	Cytosol	Amino acid metabolism	12	SOH; reactive cys	(Oger *et al*., 2012*, Liu *et al*., 2014)
AT5G49810	METHIONINE S-METHYLTRANSFERASE	Cytoplasm, cytosol	Amino acid metabolism	20		

AT4G13930	SERINE HYDROXYMETHYLTRANSFERASE 4	Cytoplasm, cytosol,	Amino acid metabolism	8	SOH; reactive cys; Trx target; SNO	(Oger *et al*., 2012*, Liu *et al*., 2014, Tanou *et al*., 2009*, Balmer *et al*., 2004a*, Balmer *et al*., 2006*)
AT3G17820	GLUTAMINE SYNTHETASE 1.3	Cytoplasm, cytosol,, cytosolic ribosome, chloroplast	Amino acid metabolism	4	SOH; Trx target	(Oger *et al*., 2012*, Balmer *et al*., 2004a*, Tanou *et al*., 2009*)
AT2G05830	5-METHYLTHIORIBOSE KINASE 1	Cytosol, extracellular region, plasmodesma	Amino acid metabolism	4		
AT1G63660	GMP SYNTHASE (glutamine-hydrolyzing)	Cytosol, cytoplasm	Amino acid metabolism	7		
AT3G44310	NITRILASE 1 (NIT1)	Cytosol, apoplast, plasma membrane, plasmodesma	Hormone homeostasis	7	S-SG; SOH	(Dixon *et al*., 2005, Waszczak *et al*., 2014)
AT1G48630	RECEPTOR FOR ACTIVATED C KINASE 1B (RACK1B)	Cytosol, cytoplasm, cytosolic ribosome, nucleus	Hormone homeostasis	8	SOH; reactive cys	(Waszczak *et al*., 2014, Liu *et al*., 2014)
AT5G09810	ACTIN 7	Cytosol, cytoplasm, cytoskeleton, cell wall	Miscellaneous	4	S-SG; SNO; SOH; reactive cys; Trx target	(Dixon *et al*., 2005, Lindermayr *et al*., 2005, Oger *et al*., 2012*, Wang *et al*., 2012, Wong *et al*., 2004*)
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	(Waszczak *et al*., 2014)
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	7		
AT3G13460	EVOLUTIONARILY CONSERVED C-TERMINAL REGION 2	Cytosol, cytoplasm, nucleus	Unknown functions	5		
AT2G15860	Unknown protein	Cytosol, nucleus	Unknown functions	3		
AT1G77550	Tubulin-tyrosine ligases	Cytoplasm, chloroplast	Miscellaneous	14		

AT1G66680	Unknown protein	Cytosol, cytoplasm, nucleus	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		
AT5G44340	TUBULIN BETA CHAIN 4	Cytosol, cytoplasm, plasma membrane, Golgi, apoplast	Primary metabolism	10	S-SG; SNO; SOH	(Dixon *et al*., 2005, Lindermayr *et al*., 2005, Fares *et al*., 2011, Oger *et al*., 2012*)
AT5G19770	TUBULIN ALPHA-3	Cytosol, cytoplasm, plasma membrane, Golgi, apoplast	Primary metabolism	11	SOH; Trx target	(Oger *et al*., 2012*, Wong *et al*., 2004*)
AT5G12250	BETA-6 TUBULIN	Cytosol, cytoplasm	Primary metabolism	12	SOH	(Oger *et al*., 2012*)
AT4G37870	PHOSPHOENOLPYRUVATE CARBOXYKINASE 1	Cytosol, cytoplasm, nucleus	Primary metabolism	10		
AT4G16130	ARABINOSE KINASE	Cytosol, cytoplasm, plasmodesma	Primary metabolism	22		
AT4G20890	TUBULIN BETA-9 CHAIN	Cytosol, cytoplasm, plasma membrane, Golgi	Primary metabolism	12	SOH	(Oger *et al*., 2012*)
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	(Marchand *et al*., 2006, Marchand *et al*., 2004)
AT3G06650	ATP-CITRATE LYASE SUBUNIT B-1	Cytosol, cytoplasm	Primary metabolism	10	SOH	(Waszczak *et al*., 2014)
AT3G06580	GALACTOSE KINASE 1	Cytosol, cytoplasm	Primary metabolism	13		
AT2G41530	S-FORMYLGLUTATHIONE HYDROLASE	Cytosol, cytoplasm, apoplast	Primary metabolism	5	Trx target; reactive cys	(Marchand *et al*., 2010, Wang *et al*., 2012)
AT1G16350	Aldolase-type TIM barrel family protein	Cytosol	Primary metabolism	6	S-SG	(Dixon *et al*., 2005)
AT1G09780	2,3-BISPHOSPHOGLYCERATE-INDEPENDENT PHOSPHOGLYCERATE MUTASE 1	Cytosol, cytoplasm, apoplast, plasmamembrane	Primary metabolism	4	SNO; Trx target	(Fares *et al*., 2011, Wong *et al*., 2004*)
AT1G11840	GLYOXALASE I HOMOLOG	Cytosol, peroxisome, plasmamembrane, chloroplast envelope, mitochondrion	Primary metabolism	1		

AT5G13520	Peptidase M1 family protein	Cytosol, chloroplast	Protein degradation	7	SOH	(Waszczak *et al*., 2014)
AT5G60160	Zn-dependent exopeptidases superfamily protein	Cytosol, chloroplast	Protein degradation	11	Trx target	(Marchand *et al*., 2010)
AT2G24200	Cytosol aminopeptidase family protein	Cytosol, chloroplast	Protein degradation	5	S-SG; SOH; reactive cys; Trx target	(Dixon *et al*., 2005, Liu *et al*., 2014, Oger *et al*., 2012*)
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	(Waszczak *et al*., 2014, Yamazaki *et al*., 2004)
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	(Lindermayr *et al*., 2005, Oger *et al*., 2012*)
AT5G02500	HEAT SHOCK COGNATE PROTEIN 70-1	Cytosol, cytoplasm, Golgi, plasma membrane	Protein folding	7	S-SG; SNO; SOH; Trx target	(Dixon *et al*., 2005, Fares *et al*., 2011, Oger *et al*., 2012*, Balmer *et al*., 2006*, Wong *et al*., 2004*)
AT3G12580	HEAT SHOCK PROTEIN 70	Cytosol, cytosol, plasma membrane	Protein folding	7	SOH; reactive cys; SNO; Trx target	(Abat and Deswal, 2009*, Oger *et al*., 2012*, Tanou *et al*., 2009*, Wang *et al*., 2012, Balmer *et al*., 2006*)
AT1G79930	HEAT SHOCK PROTEIN 91	Cytosol, cytosol, plasma membrane	Protein folding	14	Trx target	(Balmer *et al*., 2006*)
AT1G24510	TCP-1/cpn60 chaperonin family protein	Cytosol, cytosol, plasma membrane, plasmodesma	Protein folding	9	Trx target	(Balmer *et al*., 2006*)
AT4G34450	Coatomer gamma-2 subunit, putative	Cytosol, Golgi, plasma membrane	Protein transport	12		
AT2G44100	GUANOSINE NUCLEOTIDE DIPHOSPHATE DISSOCIATION INHIBITOR 1	Cytosol, cytoplasm	Protein transport	8		
AT3G14990	DJ-1 HOMOLOG A	Cytosol, plasmamembrane, plasmodesma, nucleus, chloroplast	Redox related	7	SNO; Trx target; reactive cys	(Lindermayr *et al*., 2005, Liu *et al*., 2014, Marchand *et al*., 2010, Tanou *et al*., 2009*)
AT1G78380	GLUTATHIONE S-TRANSFERASE TAU 19	Cytosol, cytoplasm, chloroplast, plasma membrane	Redox related	1	S-SG; SNO; Trx target; reactive cys	(Dixon *et al*., 2005, Lindermayr *et al*., 2005, Marchand *et al*., 2006, Marchand *et al*., 2010, Bykova *et al*., 2011, Muthuramalingam *et al*., 2013)
AT1G65980	THIOREDOXIN-DEPENDENT PEROXIDASE 1 (TPX1)	Cytosol, cytoplasm, chloroplast, plasma membrane	Redox related	2	Trx target; reactive cysteine; SNO; SOH; Grx target	(Lindermayr *et al*., 2005, Marchand *et al*., 2006, Marchand *et al*., 2010, Rouhier *et al*., 2005*, Wang *et al*., 2012, Waszczak *et al*., 2014)

AT1G60420	ATNRX1, NRX1, NUCLEOREDOXIN 1/DC1 domain-containing protein	Cytosol	Redox related	12	reactive cys	(Wang *et al*., 2012)
AT4G14030	SELENIUM-BINDING PROTEIN 1	Cytosol, nucleus	Redox related	7		
AT4G09670	OXIDOREDUCTASE FAMILY PROTEIN	Cytosol	Redox related	6		
AT3G12290	AMINO ACID DEHYDROGENASE FAMILY PROTEIN	Cytosol	Redox related	4		
AT2G21250	NAD(P)-LINKED OXIDOREDUCTASE SUPERFAMILY PROTEIN	Cytosol, cytoplasm	Redox related	6		
AT1G59960	NAD(P)-LINKED OXIDOREDUCTASE SUPERFAMILY PROTEIN	Cytosol, chloroplast	Redox related	5		
AT1G37130	NITRATE REDUCTASE 2	Cytosol, mitochondrion, plasma membrane	Redox related	16	SOH; reactive cys	(Liu *et al*., 2014, Waszczak *et al*., 2014)
AT1G05350	NAD(P)-binding ROSSMANN-fold superfamily protein	Cytosol, cytoplasm	Redox related	10		
AT3G11940	RIBOSOMAL PROTEIN 5A	Cytosol, cytoplasm	RNA binding-translation	2	SNO	(Fares *et al*., 2011)
AT3G02760	Class II aaRS and biotin synthetases superfamily protein	Cytosol	RNA binding-translation	17	reactive cys; Trx target	(Liu *et al*., 2014, Wong *et al*., 2004*)
AT2G46280	EUKARYOTIC TRANSLATION INITIATION FACTOR 3 SUBUNIT I	Cytosol	RNA binding-translation	5	reactive cys	(Liu *et al*., 2014)
AT2G45710	Zinc-binding ribosomal protein family protein	Cytosol	RNA binding-translation	6	reactive cys	(Liu *et al*., 2014)
AT1G30580	GTP BINDING /OBG-LIKE ATPASE 1	Cytosol	RNA binding-translation	5	Trx target	(Marchand *et al*., 2006, Balmer *et al*., 2004a*)
AT1G09620	ATP binding*leucine-tRNA ligases*aminoacyl-tRNA ligases*nucleotide binding*ATP binding*aminoacyl-tRNA ligases	cytosol	RNA binding-translation	20	reactive cys	(Liu *et al*., 2014)
AT5G25780	EUKARYOTIC TRANSLATION INITIATION FACTOR 3B-2	Cytosol, cytoplasm, nucleus	RNA binding-translation	3		
AT4G39520	GTP-BINDING PROTEIN-RELATED	Cytosol, cytoplasm	RNA binding-translation	7		
AT4G31120	PROTEIN ARGININE METHYLTRANSFERASE 5 (PRMT5)	Cytosol, cytoplasm	RNA binding-translation	12	SOH	(Waszczak *et al*., 2014)
AT4G26870	Class II aminoacyl-tRNA and biotin synthetases superfamily protein	Cytosol, cytoplasm, plasmodesma	RNA binding-translation	11		
AT3G57290	EUKARYOTIC TRANSLATION INITIATION FACTOR 3E (EIF3E)	Cytosol, cytoplasm, plasma membrane	RNA binding-translation	5	SOH	(Waszczak *et al*., 2014)

AT3G04840	Ribosomal protein S3Ae	Cytosol	RNA binding-translation	4		
AT2G40660	Nucleic acid-binding, OB-fold-like protein	Cytosol, cytoplasm, plasmodesma	RNA binding-translation	4		
AT2G40290	Encodes an eIF2alpha homolog	Cytosol	RNA binding-translation	5		
AT2G23350	POLY (A) BINDING PROTEIN 4	Cytosol	RNA binding-translation	7		
AT2G15790	CYCLOPHILIN 40	Cytosol, cytoplasm	RNA binding-translation	7	Trx target	(Motohashi *et al*., 2001*)
AT1G33120	Ribosomal protein L6 family	Cytosol	RNA binding-translation	2		
AT1G10840	TRANSLATION INITIATION FACTOR 3 SUBUNIT H1	Cytosol, cytoplasm	RNA binding-translation	7		
AT3G46940	DUTP-PYROPHOSPHATASE-LIKE 1	Cytosol	Signal perception & transduction	1	reactive cys	(Liu *et al*., 2014)
AT5G20990	CO-FACTOR FOR NITRATE REDUCTASE AND XANTHINE DEHYDROGENASE	Cytosol, cytoplasm	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 L-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, nucleus	Signal perception & transduction	9	SOH	(Waszczak *et al*., 2014)
AT1G51690	PROTEIN PHOSPHATASE 2A 55KDA REGULATORY SUBUNIT (PP2A-B55A)	Cytoplasm	Signal perception & transduction	11	SOH	(Waszczak *et al*., 2014)
AT1G78300	GENERAL REGULATORY FACTOR 2	Cytosol, cytoplasm, Golgi, plasma membrane	Signal perception & transduction	2	SOH	(Oger *et al*., 2012*)
AT1G35160	GENERAL REGULATORY FACTOR 4	Cytosol, cytoplasm, Golgi, plasma membrane	Signal perception & transduction	2	SOH	(Oger *et al*., 2012*)
AT5G39570	Unknown protein	Cytosol, nucleus	Unknown functions	1		

AT5G42220	Ubiquitin-like superfamily protein	cytosol, nucleus	Protein degradation	6		
AT5G36210	Alpha/beta-Hydrolases superfamily protein	cytosol, plastid	Protein degradation	13	reactive cys; SOH	(Liu *et al*., 2014, Waszczak *et al*., 2014)
AT4G35830	ACONITASE 1	apoplast, cytoplasm, cytosol, mitochondrion, plasma membrane, plasmodesma, vacuole	Primary metabolism	12	SOH, Trx target	(Balmer *et al*., 2004a*, Oger *et al*., 2012*, Wong *et al*., 2004*)
AT3G53110	LOW EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 4	cytoplasm, nuclear envelope, nucleus, plasma membrane	Miscellaneous and unknown functions	5		
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	S-SG	(Dixon *et al*., 2005)
AT2G32730	26S PROTEASOME REGULATORY COMPLEX, RPN2	Cytosol, chloroplast	Protein degradation	8		
AT1G20200	EMBRYO DEFECTIVE 2719	Cytosol, nucleus	Protein degradation	6		
AT5G56500	CHAPERONIN-60BETA3	Cytosol, chloroplast	Protein folding	6	Grx target; Trx target	(Rouhier *et al*., 2005*, Balmer *et al*., 2006*)
AT3G59020	ARM repeat superfamily protein	Cytosol, cytoplasm, nucleus	Protein transport	16	SOH	(Waszczak *et al*., 2014)
AT3G08943	ARM repeat superfamily protein	Cytosol, cytoplasm	Protein transport	18		
AT3G44300	NITRILASE 2 (NIT2)	Cytosol, plasma membrane	Hormone homeostasis	7	SOH; reactive cys	(Wang *et al*., 2012, Waszczak *et al*., 2014)
AT4G34230	CINNAMYL ALCOHOL DEHYDROGENASE 5	Cytosol, cytoplasm	Primary metabolism	11	Trx target	(Marchand *et al*., 2010)
AT1G62380	ACC OXIDASE 2	Cytoplasm, cytosol, endoplasmic reticulum, plasma membrane, plasmodesma, Golgi apparatus, cell wall,	Hormone homeostasis	4	S-SG	(Dixon *et al*., 2005)
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	Cytoplasm, cytosol	Primary metabolism	12		

AT1G11650	RNA-binding (RRM/RBD/RNP motifs) family protein	cytoplasm, nucleus	RNA binding	3	reactive cys	(Liu *et al*., 2014)
AT4G26970	ACONITASE 2	Cytosol, mitochondrion	Primary metabolism	10	SOH; Trx target	(Oger *et al*., 2012*, Wong *et al*., 2004*)
AT5G07440	GLUTAMATE DEHYDROGENASE 2	Cytoplasm, mitochondrion, vacuolar membrane	Amino acid metabolism	6	Trx target; SNO; S-S	(Lindermayr *et al*., 2005, Winger *et al*., 2007, Yoshida *et al*., 2013, Balmer *et al*., 2004a*)
Mitochondrion						
AT1G48030	MITOCHONDRIAL LIPOAMIDE DEHYDROGENASE 1	Mitochondrion	Carbohydrate metabolism	5	Trx target; Grx target; reactive cys	(Liu *et al*., 2014, Rouhier *et al*., 2005*, Yoshida *et al*., 2013)
AT1G24180	IAA-CONJUGATE-RESISTANT 4	Mitochondrion	Primary metabolism	8	SOH; reactive cys	(Liu *et al*., 2014, Oger *et al*., 2012*)
AT5G08670	ATP SYNTHASE ALPHA/BETA FAMILY PROTEIN	Mitochondrion	Primary metabolism	3	Trx target; Grx target; S-SG; SOH; S-S	(Dixon *et al*., 2005, Oger *et al*., 2012*, Rouhier *et al*., 2005*, Winger *et al*., 2007, Yoshida *et al*., 2013)
AT5G50850	MAB1, MACCI-BOU/TRANSKETOLASE FAMILY PROTEIN/PYRUVATE DEHYDROGENASE E1 COMPONENT SUBUNIT BETA-1, MITOCHONDRIAL	Mitochondrion	Primary metabolism	5	S-S bond; reactive cys; Trx target	(Wang *et al*., 2012, Winger *et al*., 2007, Yoshida *et al*., 2013)
AT5G08300	SUCCINYL-COA LIGASE, ALPHA SUBUNIT	Mitochondrion, cell wall	Primary metabolism	8	Trx target	(Balmer *et al*., 2004a*, Yoshida *et al*., 2013)
AT1G22840	CYTOCHROME C-1	Mitochondrion, cytosol	Primary metabolism	2		
AT5G37510	NADH-ubiquinone dehydrogenase, mitochondrial,	Mitochondrion	Protein degradation	19	Trx target	(Yoshida *et al*., 2013)
AT3G62530	ARM repeat superfamily protein	Mitochondrion, nucleolus, chloroplast,	Protein transport	3	reactive cys	(Wang *et al*., 2012)
AT5G43430	ELECTRON TRANSFER FLAVOPROTEIN BETA	Mitochondrion	Redox related	3		
AT5G14040	MITOCHONDRIAL PHOSPHATE TRANSPORTER 3 (MPT3)	Mitochondrion	Signal perception & transduction	7	Trx target; SOH; SNO; S-S	(Fares *et al*., 2011, Oger *et al*., 2012*, Winger *et al*., 2007, Yoshida *et al*., 2013)
AT3G17240	LIPOAMIDE DEHYDROGENASE 2, mitochondrial	Mitochondrion,	Redox related	5	SNO; SOH; S-S	(Winger *et al*., 2007, Palmieri *et al*., 2010, Fares *et al*., 2011, Oger *et al*., 2012*)
AT1G48920	NUCLEOLIN LIKE 1	Mitochondrion, nucleolus	Protein transport	1		
AT5G14590	ISOCITRATE/ISOPROPYLMALATE DEHYDROGENASE FAMILY PROTEIN	Mitochondrion, plastid	Primary metabolism	6	Grx target; SOH	(Oger *et al*., 2012*, Rouhier *et al*., 2005*)
AT1G74260	PURINE BIOSYNTHESIS 4	Mitochondrion, plastid	Primary metabolism	24	reactive cys	(Liu *et al*., 2014)

Nucleus						
AT3G51800	ERBB-3 BINDING PROTEIN 1	Nucleolus, nucleus, plasma membrane	Protein transport	6	SOH; SNO	(Fares *et al*., 2011, Waszczak *et al*., 2014)
AT1G35780	Unknown protein*	Nucleus	Unknown function	2		
AT1G22730	MA3 domain-containing protein	Nucleus	Miscellaneous	10		
AT3G58510	DEA(D/H)-box RNA helicase family protein	Nucleus, peroxisome, plasma membrane	RNA binding-translation	6		
AT2G22400	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein	Nucleus	RNA binding-translation	14		
AT1G67680	SRP72 RNA-binding domain	Nucleus	RNA binding-translation	7		
AT2G38560	TRANSCRIPT ELONGATION FACTOR IIS	Nucleus	Transcription	11		
AT1G20110	FYVE-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		
Peroxisome						
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		
Endoplasmic reticulum/ Golgi/Plasma membrane						
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, endoplasmic reticulum lumen	Protein folding	5	SOH; SNO	(Oger *et al*., 2012*, Lindermayr *et al*., 2005)
AT1G56340	CALRETICULIN 1A	Endoplasmic reticulum, plasmodesma, apoplast	Protein degradation	3	SOH; reactive cys	(Oger *et al*., 2012*, Wang *et al*., 2012)
AT1G09210	CALRETICULIN 1B	Endoplasmic reticulum, apoplast	Protein degradation	4	SOH	(Oger *et al*., 2012*)
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, plasmodesma, clathrin coat of trans-Golgi network vesicle	Protein transport	22		
Plastid						
AT2G43750	ARABIDOPSIS CYSTEINE SYNTHASE 1	Plastid	Amino acid metabolism	5	S-S bond; reactive cys; SOH	(Ströher and Dietz, 2008, Alvarez *et al*., 2009, Oger *et al*., 2012*)
AT3G59760	O-ACETYLSERINE (THIOL) LYASE ISOFORM C	Chloroplast, chloroplast stroma, mitochondrion	Amino acid metabolism	6	Trx target; SOH; S-S; Grx target	(Marchand *et al*., 2006, Oger *et al*., 2012*, Rouhier *et al*., 2005*, Winger *et al*., 2007, Yoshida *et al*., 2013)
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; S-SG; SOH	(Dixon *et al*., 2005, Wang *et al*., 2012, Waszczak *et al*., 2014)
AT4G39980	3-DEOXY-D-ARABINO-HEPTULOSONATE 7-PHOSPHATE SYNTHASE 1, DHS1	Chloroplast, mitochondrion	Amino acid metabolism	9		
AT4G35630	PHOSPHOSERINE AMINOTRANSFERASE	Plastid	Amino acid metabolism	8		
AT4G32520	SERINE HYDROXYMETHYLTRANSFERASE 3	Plastid	Amino acid metabolism	7	Trx target, SNO	(Balmer *et al*., 2006*, Balmer *et al*., 2004a, Tanou *et al*., 2009)
AT4G29840	THREONINE SYNTHASE	Plastid, cytosol	Amino acid metabolism	11	Trx target	(Balmer *et al*., 2006*)
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-ENOLPYRUVYLSHIKIMATE-3-PHOSPHATE / EPSP synthase involved in chorismate biosynthesis	Plastid	Amino acid metabolism	10		
AT2G31810	ACT domain-containing small subunit of acetolactate synthase protein	Plastid	Amino acid metabolism	4		
AT2G29690	ANTHRANILATE SYNTHASE 2	Plastid	Amino acid metabolism	7		
AT2G22250	ASPARTATE AMINOTRANSFERASE	Plastid	Amino acid metabolism	6	Trx target; SNO	(Balmer *et al*., 2004a*, Tanou *et al*., 2009*)
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	(Liu *et al*., 2014)
AT3G53580	Diaminopimelate epimerase family protein, Chloroplastic	Plastid	Amino acid metabolism	9	reactive cys	(Liu *et al*., 2014)
AT3G23940	Dehydratase family	Plastid	Amino acid metabolism	12	Trx target	(Marchand *et al*., 2004, Marchand *et al*., 2010)
AT4G25100	FE SUPEROXIDE DISMUTASE 1	Plastid, mitochondrion	Background	0		
AT4G26300	EMBRYO DEFECTIVE 1027	Plastid, mitochondrion	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; S-SG; SOH; Grx target	(Dixon *et al*., 2005, Oger *et al*., 2012*, Rouhier *et al*., 2005*, Yoshida *et al*., 2013, Balmer *et al*., 2006*)
AT3G48000	ALDEHYDE DEHYDROGENASE 2	Chloroplast, mitochondrion	Primary metabolism	7	SOH; Grx target; reactive cys, Trx target, SNO	(Balmer *et al*., 2004a*, Oger *et al*., 2012*, Rouhier *et al*., 2005*, Tanou *et al*., 2009*, Wang *et al*., 2012, Wong *et al*., 2004*)

AT3G48990	ACYL-ACTIVATING ENZYME 3	Chloroplast, chloroplast stroma	Primary metabolism	4	reactive cys	(Liu *et al*., 2014)
AT1G35720	ANNEXIN 1	Chloroplast, chloroplast stroma, apoplast, plasmodesma, thylakoid, vacuolar membrane, vacuole	Signal perception & transduction	2	SNO; S-SG	(Lindermayr *et al*., 2005, Konopka-Postupolska *et al*., 2009, Fares *et al*., 2011)
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, cytoplasm	Primary metabolism	4		
AT3G57610	ADENYLOSUCCINATE SYNTHETASE, CHLOROPLASTIC	Plastid	Primary metabolism	8	Trx target	(Balmer *et al*., 2006*)
AT3G48730	GLUTAMATE-1-SEMIALDEHYDE 2,1-AMINOMUTASE 2	Plastid	Primary metabolism	6	SNO; Trx target	(Balmer *et al*., 2004a*, Tanou *et al*., 2009*, Wong *et al*., 2003)
AT1G74030	ENOLASE 1, CHLOROPLASTIC	Plastid	Primary metabolism	7	SOH; reactive cys; Trx target	(Oger *et al*., 2012*, Wang *et al*., 2012, Wong *et al*., 2004*)
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		
AT4G33030	SULFOQUINOVOSYLDIACYLGLYCEROL 1	Plastid	Primary metabolism	9	SNO	(Fares *et al*., 2011)
AT2G35040	AICARFT/IMPCHase bienzyme family protein	Plastid	Primary metabolism	10		
AT2G02500	HEAT SHOCK COGNATE PROTEIN 70-1	Plastid	Primary metabolism	4		
AT1G80560	ISOPROPYLMALATE DEHYDROGENASE 2	Plastid	Primary metabolism	3		
AT3G22960	PLASTIDIAL PYRUVATE KINASE 1	Plastid	Primary metabolism	9	reactive cys	(Liu *et al*., 2014)
AT1G74040	2-ISOPROPYLMALATE SYNTHASE 1	Plastid	Primary metabolism	7		

AT3G12780	PHOSPHOGLYCERATE KINASE 1	Plastid	Primary metabolism	2	Trx target; S-S	(Ströher and Dietz, 2008, Marchand *et al*., 2010, Balmer *et al*., 2006*)
AT2G21170	PLASTID ISOFORM TRIOSE PHOSPHATE ISOMERASE,	Plastid	Primary metabolism	4	Trx target; Grx target, SNO	(Wong *et al*., 2003, Tanou *et al*., 2009*, Rouhier *et al*., 2005*, Marchand *et al*., 2004, Marchand *et al*., 2006, Wong *et al*., 2004*)
AT1G43800	STEAROYL-ACYL CARRIER PROTEIN Δ9-DESATURASE6	Plastid	Primary metabolism	4		
AT1G36280	L-Aspartase-like family protein	Plastid	Primary metabolism	3		
AT1G22940	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		
AT5G49910	CHLOROPLAST HEAT SHOCK PROTEIN 70-2	Plastid	Protein folding	2	Trx target; Grx target; S-S, SNO	(Ströher and Dietz, 2008, Rouhier *et al*., 2005*, Yoshida *et al*., 2013, Tanou *et al*., 2009*, Wong *et al*., 2004*)
AT3G13470	CHAPERONIN-60BETA2	Plastid	Protein folding	7	S-S; Trx target	(Yamazaki *et al*., 2004, Ströher and Dietz, 2008)
AT5G53480	ARM repeat superfamily protein	Plastid	Protein transport	17		
AT5G50920	HEAT SHOCK PROTEIN 93-V	Plastid	Protein folding	4	S-S; Trx target	(Ströher and Dietz, 2008, Balmer *et al*., 2006*)
AT4G08390	STROMAL ASCORBATE PEROXIDASE	Plastid	Redox related	2	Trx target; SNO	(Yoshida *et al*., 2013, Abat and Deswal, 2009*, Wong *et al*., 2004*)
AT1G63940	MONODEHYDROASCORBATE REDUCTASE 6	Plastid	Redox related	5	Trx target; S-S	(Marchand *et al*., 2010, Ströher and Dietz, 2008)
AT4G16155	DIHYDROLIPOYL DEHYDROGENASES	Plastid	Redox related	9	Trx target	(Balmer *et al*., 2006*)
AT1G12900	GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE A SUBUNIT 2	Plastid	Redox related	5	Grx target; reactive cys; SNO; Trx target	(Rouhier *et al*., 2005*, Muthuramalingam *et al*., 2013, Wong *et al*., 2003, Tanou *et al*., 2009*)
AT1G79530	GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE OF PLASTID 1	Plastid	Redox related	3	S-OH; Trx target	(Oger *et al*., 2012*, Motohashi *et al*., 2001*, Balmer *et al*., 2006*)
AT3G58140	Phenylalanyl-tRNA synthetase class IIc family protein /	Plastid	RNA binding-translation	7		
AT5G65430	GENERAL REGULATORY FACTOR 8	Plastid	Signal perception & transduction	2	Grx target; SNO	(Tanou *et al*., 2009*, Rouhier *et al*., 2005*)
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 METALLOENDOPEPTIDASE 1, TOP1	chloroplast, chloroplast stroma, cytosol	Protein degradation	6	S-S; SNO	(Tanou *et al*., 2009*, Ströher and Dietz, 2008)

Annotation according to TAIR10. Abbreviations of PTMs are as follows: SNO, S-nitrosylation; SOH, sulfenic acid; S-S, disulfide bridge; S-SG, S-glutathionylation; Trx/Grx target, thioredoxin/glutaredoxin target proteins. References describing identification of homolog/ortholog are marked with an asterisk.

4.3 The 55 sulfenylated proteins previously identified validate our study

AGI code	Description	References
Signal perception & transduction		
AT2G43980	INOSITOL 1,3,4-TRISPHOSPHATE 5/6-KINASE 4 (ITPK4)	(Waszczak *et al*., 2014)
AT1G51690	PROTEIN PHOSPHATASE 2A 55 KDA REGULATORY SUBUNIT B ALPHA ISOFORM (PP2A-b55α)	
AT5G14040	MITOCHONDRIAL PHOSPHATE TRANSPORTER 3 (MPT3)	(Oger *et al*., 2012*)
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	(Waszczak *et al*., 2014)
AT1G37130	NITRATE REDUCTASE 2	
AT3G17240	LIPOAMIDE DEHYDROGENASE 2, mitochondrial	(Oger *et al*., 2012*)
AT1G79530	GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE OF PLASTID 1 (GAPCP-1)	
Protein synthesis, folding, transport,		
AT4G31120	PROTEIN ARGININE METHYLTRANSFERASE 5 (PRMT5)	(Waszczak *et al*., 2014)
AT3G57290	EUKARYOTIC TRANSLATION INITIATION FACTOR 3E (EIF3E)	
AT3G59020	ARM repeat superfamily protein	
AT3G51800	ERBB-3 BINDING PROTEIN 1 (EBP1)	
AT5G56010	HEAT SHOCK PROTEIN 81-3	(Oger *et al*., 2012*)
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	(Waszczak *et al*., 2014)
AT5G13520	Peptidase M1 family protein	
AT1G22920	COP9 SIGNALOSOME 5A (CSN5A)	
AT2G24200	Cytosol aminopeptidase family protein	(Oger *et al*., 2012*)
AT1G09210	CALRETICULIN 1B	
AT1G56340	CALRETICULIN 1A	
Primary metabolism		
AT3G06650	ATP-CITRATE LYASE SUBUNIT B-1	(Waszczak *et al*., 2014)
AT4G24830	Arginosuccinate synthase family	
AT3G48000	ALDEHYDE DEHYDROGENASE 2	(Oger *et al*., 2012*)
AT1G24180	IAA-CONJUGATE-RESISTANT 4,	
AT5G44340	TUBULIN BETA CHAIN 4	
AT5G19770	TUBULIN ALPHA-3	
AT5G14590	Isocitrate/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-ACETYLSERINE (THIOL) LYASE ISOFORM C	

AT2G43750	O-ACETYLSERINE (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)	(Waszczak *et al*., 2014)
AT3G44300	NITRILASE 2 (NIT2)	
AT1G48630	RECEPTOR FOR ACTIVATED C KINASE 1B (RACK1B)	
Miscellaneous		
AT4G27450	Aluminium induced protein with YGL and LRDR motifs	(Waszczak *et al*., 2014)
AT1G62740	HOP2, Encodes one of the 36 carboxylate clamp (CC)-tetratricopeptide repeat (TPR) proteins	
AT5G09810	ACTIN 7	(Oger *et al*., 2012*)

Annotation according to TAIR10. Reference describing identification of ortholog are marked with an asterisk

4.6 References

Abat JK, Deswal R (2009) Differential modulation of S-nitrosoproteome of Brassica juncea by low temperature: change in S-nitrosylation of Rubisco is responsible for the inactivation of its carboxylase activity. Proteomics **9**: 4368–80

Alvarez S, Zhu M, Chen S (2009) Proteomics of Arabidopsis redox proteins in response to methyl jasmonate. J Proteomics **73**: 30–40

Balmer Y, Koller A, Val G Del, Schürmann P, Buchanan BB (2004) Proteomics uncovers proteins interacting electrostatically with thioredoxin in chloroplasts. Photosynth Res **79**: 275–80

Benitez L V, Allison WS (1974) The inactivation of the acyl phosphatase activity catalyzed by the sulfenic acid form of glyceraldehyde 3-phosphate dehydrogenase by dimedone and olefins. J Biol Chem **249**: 6234–43

Bykova N V, Hoehn B, Rampitsch C, Banks T, Stebbing J-A, Fan T, Knox R (2011) Redox-sensitive proteome and antioxidant strategies in wheat seed dormancy control. Proteomics **11**: 865–82

Carballal S, Radi R, Kirk MC, Barnes S, Freeman BA, Alvarez B (2003) Sulfenic acid formation in human serum albumin by hydrogen peroxide and peroxynitrite. Biochemistry **42**: 9906–14

Charles RL, Schröder E, May G, Free P, Gaffney PRJ, Wait R, Begum S, Heads RJ, Eaton P (2007) Protein sulfenation as a redox sensor: proteomics studies using a novel biotinylated dimedone analogue. Mol Cell Proteomics **6**: 1473–84

Chiang SM, Schellhorn HE (2012) Regulators of oxidative stress response genes in Escherichia coli and their functional conservation in bacteria. Arch Biochem Biophys **525**: 161–9

Delaunay A, Pflieger D, Barrault MB, Vinh J, Toledano MB (2002) A thiol peroxidase is an H2O2 receptor and redox-transducer in gene activation. Cell **111**: 471–81

Desikan R, A-H-Mackerness S, Hancock JT, Neill SJ (2001) Regulation of the Arabidopsis transcriptome by oxidative stress. Plant Physiol **127**: 159–72

Dixon DP, Davis BG, Edwards R (2002) Functional divergence in the glutathione transferase superfamily in plants. Identification of two classes with putative functions in redox homeostasis in Arabidopsis thaliana. J Biol Chem **277**: 30859–69

Dixon DPD, Skipsey M, Grundy NMN, Edwards R, Sciences B, Kingdom U (2005) Stress-induced protein S-glutathionylation in Arabidopsis. Plant Physiol **138**: 2233–2244

Fares A, Rossignol M, Peltier J-B (2011) Proteomics investigation of endogenous S-nitrosylation in Arabidopsis. Biochem Biophys Res Commun **416**: 331–6

Go Y-MY, Jones DDP (2008) Redox compartmentalization in eukaryotic cells. Biochim Biophys Acta (BBA)-General Subj **1780**: 1273–1290

Gupta V, Carroll KS (2014) Sulfenic acid chemistry, detection and cellular lifetime. Biochim Biophys Acta **1840**: 847–75

Jacques S, Ghesquière B, Van Breusegem F, Gevaert K (2013) Plant proteins under oxidative attack. Proteomics **13**: 932–40

Konopka-Postupolska D, Clark G, Goch G, Debski J, Floras K, Cantero A, Fijolek B, Roux S, Hennig J (2009) The role of annexin 1 in drought stress in Arabidopsis. Plant Physiol **150**: 1394–410

Van Leene J, Stals H, Eeckhout D, Persiau G, Van De Slijke E, Van Isterdael G, De Clercq A, Bonnet E, Laukens K, Remmerie N, *et al* (2007) A tandem affinity purification-based technology platform to study the cell cycle interactome in Arabidopsis thaliana. Mol Cell Proteomics **6**: 1226–38

Leonard SE, Carroll KS (2011) Chemical "omics" approaches for understanding protein cysteine oxidation in biology. Curr Opin Chem Biol **15**: 88–102

Leonard SE, Reddie KG, Carroll KS (2009) Mining the Thiol Proteome for Sulfenic Acid Modifications Reveals New Targets for Oxidation in Cells. ACS Chem Biol **4**: 783–799

Lindermayr C, Saalbach G, Durner J (2005) Proteomic Identification of S -Nitrosylated Proteins. Plant Physiol **137**: 921–930

Liu P, Zhang H, Wang H, Xia Y (2014) Identification of redox-sensitive cysteines in the Arabidopsis proteome using OxiTRAQ, a quantitative redox proteomics method. Proteomics **14**: 750–62

Marchand C, Le Maréchal P, Meyer Y, Decottignies P (2006) Comparative proteomic approaches for the isolation of proteins interacting with thioredoxin. Proteomics **6**: 6528–37

Marchand C, Le Maréchal P, Meyer Y, Miginiac-Maslow M, Issakidis-Bourguet E, Decottignies P (2004) New targets of Arabidopsis thioredoxins revealed by proteomic analysis. Proteomics **4**: 2696–706

Marchand CH, Vanacker H, Collin V, Issakidis-Bourguet E, Maréchal P Le, Decottignies P (2010) Thioredoxin targets in Arabidopsis roots. Proteomics **10**: 2418–28

Muthuramalingam M, Matros A, Scheibe R, Mock H-P, Dietz K-J (2013) The hydrogen peroxide-sensitive proteome of the chloroplast in vitro and in vivo. Front Plant Sci **4**: 54

Oger E, Marino D, Guigonis J-M, Pauly N, Puppo A (2012) Sulfenylated proteins in the *Medicago truncatula-Sinorhizobium meliloti* symbiosis. J Proteomics **75**: 4102–13

Palmieri MC, Lindermayr C, Bauwe H, Steinhauser C, Durner J (2010) Regulation of plant glycine decarboxylase by s-nitrosylation and glutathionylation. Plant Physiol **152**: 1514–28

Paulsen CE, Carroll KS (2013) Cysteine-mediated redox signaling: chemistry, biology, and tools for discovery. Chem Rev **113**: 4633–79

Paulsen CE, Truong TH, Garcia FJ, Homann A, Gupta V, Leonard SE, Carroll KS (2012) Peroxide-dependent sulfenylation of the EGFR catalytic site enhances kinase activity. Nat Chem Biol **8**: 57–64

Poole LB, Klomsiri C, Knaggs S a, Furdui CM, Nelson KJ, Thomas MJ, Fetrow JS, Daniel LW, King SB (2007) Fluorescent and affinity-based tools to detect cysteine sulfenic acid formation in proteins. Bioconjug Chem **18**: 2004–17

Poole LB, Zeng B-B, Knaggs SA, Yakubu M, King SB (2005) Synthesis of chemical probes to map sulfenic acid modifications on proteins. Bioconjug Chem **16**: 1624–8

Roos G, Messens J (2011) Protein sulfenic acid formation: from cellular damage to redox regulation. Free Radic Biol Med **51**: 314–26

Rouhier N, Villarejo A, Srivastava M, Gelhaye E, Keech O, Droux M, Finkemeier I, Samuelsson G, Dietz KJ, Jacquot J-P, *et al* (2005) Identification of plant glutaredoxin targets. Antioxid Redox Signal **7**: 919–29

Di Simplicio P, Franconi F, Frosalí S, Di Giuseppe D (2003) Thiolation and nitrosation of cysteines in biological fluids and cells. Amino Acids **25**: 323–39

Ströher E, Dietz K-J (2008) The dynamic thiol-disulphide redox proteome of the Arabidopsis thaliana chloroplast as revealed by differential electrophoretic mobility. Physiol Plant **133**: 566–83

Tachibana T, Okazaki S, Murayama A, Naganuma A, Nomoto A, Kuge S (2009) A major peroxiredoxin-induced activation of Yap1 transcription factor is mediated by reduction-sensitive disulfide bonds and reveals a low level of transcriptional activation. J Biol Chem **284**: 4464–72

Takanishi CL, Ma L-H, Wood MJ (2007) A genetically encoded probe for cysteine sulfenic acid protein modification in vivo. Biochemistry **46**: 14725–32

Takanishi CL, Wood MJ (2011) A genetically encoded probe for the identification of proteins that form sulfenic acid in response to H_2O_2 in Saccharomyces cerevisiae. J Proteome Res **10**: 2715–24

Tanou G, Job C, Rajjou L, Arc E, Belghazi M, Diamantidis G, Molassiotis A, Job D (2009) Proteomics reveals the overlapping roles of hydrogen peroxide and nitric oxide in the acclimation of citrus plants to salinity. Plant J **60**: 795–804

Truong TH, Carroll KKS (2012) Bioorthogonal Chemical Reporters for Analyzing Protein Sulfenylation in Cells. Curr Protoc Chem Biol **4**: 101–122

Wang H, Wang S, Lu Y, Alvarez S, Hicks LM, Ge X, Xia Y (2012) Proteomic analysis of early-responsive redox-sensitive proteins in Arabidopsis. J Proteome Res **11**: 412–24

Wang W, Hong S, Tran A, Jiang H, Triano R, Liu Y, Chen X, Wu P (2011) Sulfated ligands for the copper(I)-catalyzed azide-alkyne cycloaddition. Chem Asian J **6**: 2796–802

Waszczak C, Akter S, Eeckhout D, Persiau G, Wahni K, Bodra N, Van Molle I, De Smet B, Vertommen D, Gevaert K, et al (2014) Sulfenome mining in *Arabidopsis thaliana*. Proc Natl Acad Sci U S A **111**: 11545–50

Winger AM, Taylor NL, Heazlewood JL, Day DA, Millar AH (2007) Identification of intra- and intermolecular disulphide bonding in the plant mitochondrial proteome by diagonal gel electrophoresis. Proteomics **7**: 4158–70

Yamazaki D, Motohashi K, Kasama T, Hara Y, Hisabori T (2004) Target proteins of the cytosolic thioredoxins in Arabidopsis thaliana. Plant Cell Physiol **45**: 18–27

Yoshida K, Noguchi K, Motohashi K, Hisabori T (2013) Systematic exploration of thioredoxin target proteins in plant mitochondria. Plant Cell Physiol **54**: 875–92

Chapter 5

General discussion & Future perspectives

Chapter 5

General discussion & Future perspectives

5.1 General discussion

Plants generate ROS either due to metabolism or various stress factors. During the course of evolution plants learned to adapt with ROS toxicity and at the same time, to use ROS as a signaling messenger under stress (Mittler et al., 2011). However, it remains mysterious how plant perceives the message of ROS that allows signaling, regulation, and protection. This signaling function depends on the oxidation of redox sensor proteins by ROS, which result in the changes in conformation and activity of these proteins. Cysteine residues in proteins are one of the sensitive targets of ROS dependent modifications, the Cys Ox-PTMs. In the first chapter, we have reviewed studies on the redox control of plant signaling proteins as well as proteomics to understand Cys Ox-PTMs. In this chapter we have addressed a research gap of sulfenome proteomics to identify the potential ROS sensors in *Arabidopsis thaliana*. This research gap is mainly a result of the lack of appropriate techniques to detect sulfenylated proteins in plants.

During my PhD, we have optimized the techniques to profile sulfenylated proteins in *Arabidopsis* based on a YAP1 genetic probe and a DYn-2 chemical probe. In chapter 2, we describe the novel strategy for the identification of sulfenome based on YAP1 genetic probe and the first successful application of this strategy in *Arabidopsis thaliana*. By a unique combination of sulfenic acid trapping with tandem affinity purification, we identified a set of 97 sulfenylated proteins. In Chapter 3, we report the first successful application of the DYn-2 chemical probe for the identification of the sulfenome at different subcellular levels in plants. Our efforts led to the optimization of this approach in *Arabidopsis* and the identification of 276 proteins that are potentially involved in redox regulated cellular processes.

During this research, we have learned about four technical issues that should be considered for a successful sulfenome study: specific recognition of the sulfenylation modification, *in vivo* trapping of the transient modification, extraction of the tagged proteins while controlling *de novo* protein sulfenylation, and finally, the specific enrichment. Our optimized methods considered these issues successfully, however, each approach is associated with its own limitations. In case of the DYn-2 chemical probe, the addition of small membrane permeable molecule to cells might interfere with the signaling pathways. Another important consideration with chemical probes is the rate at which the probes react with the modified cysteine residue. If the reaction is slow, transient cysteine oxidation events might be missed. The modest second order rate constant for the reaction of many dimedone analogues with sulfenic acid is approximately 0.027 $M^{-1}sec^{-1}$ (Paulsen and Carroll, 2013), which, might not be sufficient to trap especially transient modifications like in peroxidases where the sulfenylation has been reported to be almost ~10^5 M^{-1} s^{-1} (Hugo et al., 2009; Hugo et al., 2014). However, the rate constant of the reaction between dipeptide-SOH and DYn-2 is calculated to be 11 M^{-1} s^{-1} (Gupta and Carroll, 2014) which is faster in reaction than dimedone and GSH (HSA-SOH + GSH is 2.9 M−1 s−1) (Gupta and Carroll, 2014). Moreover, increasing the local concentration of the probe might compensate for the modest rates of reaction. These issues can be addressed, by performing appropriate control experiments to ensure that the underlying biology is not disturbed. The YAP1 based sulfenome probe in contrast is non-invasive and more physiological compared to the chemical probe. The rate constant of YAP1 disulfide formation with target sulfenic acids is not reported yet, however, if we compare it with the rate constants of sulfenic acids with thiol to form intermolecular disulfide bond formation (HSA-SOH + Cys is 21.6 M^{-1} s^{-1}) (Paulsen and Carroll, 2013; Gupta and Carroll, 2014), the YAP1C probe should be much faster in trapping sulfenic acids. However, the intracellular concentration is a matter of concern here, we used 0.5 mM DYn-2 to trap the sulfenome, and we assume the concentration of the overexpressed YAP1C might be in nano molar range. The use of protein probe enables exploration of organular sulfenome as it can be easily modified with target peptides (Takanishi et al., 2007; Takanishi and Wood, 2011). The drawbacks of the Yap1-based strategy are the relative low efficiency and the somewhat high risk of false identifications (Furdui et al., 2014). The number of sulfenic acid forming proteins detection could be under estimated due to the reduction of disulfide bonds (between YAP1 and target sulfenylated proteins) by glutathione or redox enzymes, or other cysteine containing proteins or by the resolving

cysteine of the trapped protein itself. Another limitation could be the steric effects, as the Yap1-cCRD variant must be expressed in host cells and, since it is protein-based, it may exhibits substrate bias when compared to chemical-based probes (Gupta and Carroll, 2014). Moreover, there is a chance of co-elution of non-sulfenic acids proteins due to complex protein-protein interactions. In conclusion, both chemical and genetic approaches need to be explored to identify a comprehensive family of oxidative stress induced sulfenylated proteins.

5.2 Future perspectives

Although we are making progress, we are still at the discovery phase. Our identified targets are providing the snapshots of sulfenylation modification under stress in plants and now the challenge is to make a continuous picture of how the signals pass along the sulfenome. 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. More specifically, it will be interesting to see which proteins are sensing the ROS through sulfenylation, how this signaling message is transmitting to the next signaling protein along the pathway, how the final signal goes to the nucleus to express the defense responsive proteins, these are interesting questions that need to be addressed in the near future. In signaling proteins, sulfenic acids are transiently formed. The kinetics of a probe is one issue, but many other challenges lie still ahead before we will get a clear view on the regulation of cellular networks driven by oxidative thiol modifications.

A full biochemical, functional and structural characterization of the identified sulfenylated targets are required in order to get a clear view on the ROS signal transduction events at the molecular level. First a selection of identified sulfenic acids should be made as 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. The candidate proteins could be selected based on the phenotype of loss/gain-of-function mutants, change of expression level upon stress and non-stress conditions, biological role if known, post translltional modification of cys, the feasibility in solving the protein structure etc.

Heterologous production of the candidate proteins is necessary for *in vitro* sulfenylation validation, identification of the sulfenylated cysteine, following determining the pK_a of

active site cysteine, sulfenylation reaction rate constant, regulation of the sulfenylation events through Trx/Grx pathways, protein-protein interaction assay to identify its target proteins etc. All these biochemical-characterized information might guide to understand the reaction mechanism of the candidate protein at cellular level. In parallel, a stress-related phenotyping effort of gain-and loss of function mutants of the candidate proteins should be performed. This functional characterization will guide to assess whether the specific target has physiological consequences under stress. Next the role of cysteine of the target proteins could be assess by observing the phenotype of the cysteine mutant type and manipulate them to increase plant stress tolerane.

To complete the sulfenome mining at sulcellular level, YAP-1 based genetic probe appraoch should be applied using signal peptide for specific organale. On top that, our optimized methods both YAP1-based genetic probe and DYn-2 chemical probe approach could be explored into plant system to map the sulfenomes under more physiological stress like high temperature, high light, cold stress, salinity etc.

The outcome of these experiments will assist to assess the potential for biotechnological applications of modifying the target proteins to improve abiotic stress tolerance in plants. However, we expect that future efforts will focus on translating the ROS signal transduction events. The growing knowledge on oxidative stress signaling pathways will stimulate efforts towards manipulation of plant stress tolerance and future translation of this knowledge into crop species.

5.3 References

Furdui CM, Poole LB, Carolina N (2014) Chemical approaches to detect and analyze Protein Sulfenic Acids. 126–146

Gupta V, Carroll KS (2014) Sulfenic acid chemistry, detection and cellular lifetime. Biochim Biophys Acta **1840**: 847–75

Hugo M, Van Laer K, Reyes AM, Vertommen D, Messens J, Radi R, Trujillo M (2014) Mycothiol/mycoredoxin 1-dependent reduction of the peroxiredoxin AhpE from Mycobacterium tuberculosis. J Biol Chem **289**: 5228–39

Hugo M, Turell L, Manta B, Botti H, Monteiro G, Netto LES, Alvarez B, Radi R, Trujillo M (2009) Thiol and sulfenic acid oxidation of AhpE, the one-cysteine peroxiredoxin from Mycobacterium tuberculosis: kinetics, acidity constants, and conformational dynamics. Biochemistry **48**: 9416–26

Mittler R, Vanderauwera S, Suzuki N, Miller G, Tognetti VB, Vandepoele K, Gollery M, Shulaev V, Van Breusegem F (2011) ROS signaling: the new wave? Trends Plant Sci **16**: 300–9

Paulsen CE, Carroll KS (2013) Cysteine-mediated redox signaling: chemistry, biology, and tools for discovery. Chem Rev **113**: 4633–79

Takanishi CL, Ma L-H, Wood MJ (2007) A genetically encoded probe for cysteine sulfenic acid protein modification in vivo. Biochemistry **46**: 14725–32

Takanishi CL, Wood MJ (2011) A genetically encoded probe for the identification of proteins that form sulfenic acid in response to H2O2 in Saccharomyces cerevisiae. J Proteome Res **10**: 2715–24

Yang J, Gupta V, Carroll KS, Liebler DC (2014) Site-specific mapping and quantification of protein S-sulphenylation in cells. Nat Commun **5**: 4776

Abbreviations

Abbreviations

2-DE	two-dimensional electrophoresis
ABA	abscisic acid
attB	*Escherichia coli* chromosome site-specific λ attachment (att) site
BCN	9- hydroxymethylbicyclo [6.1.0] nonyne
bHLH	basic helix-loop-helix
BIAM/ BNEM	biotin-conjugated IAM/NEM
BRs	brassinosteroids
bZIP	G-group basic leucine-zipper
CaMV 35S	cauliflower mosaic virus 35s promoter
cCRD	c-terminal cysteine-rich domain
cCys	c-terminal cysteine
Cdc25B:	cell division cycle 25 (cdc25) phosphatases
CDS	coding sequence
CNBr	cyanogen bromide
Cy5 maleimide	cyanine-5-maleimide
Cys Ox-PTMs	cysteine oxidative posttranslational modifications
cysTMT	cysteine tandem mass tag
DAz-1/ DAz-2	azido- functionalized dimedone analogous
DCIA	7-di-ethylamino-3-(4-iodoacetylaminophenyl)-4-methylcoumarin
DHA	dehydroascorbate
DNaseI	deoxyribonuclease i
DTT	dithiothreitol
DYn-1/ DYn-2	alkyne-functionalized dimedone analogous
E64	cysteine protease inhibitor
EDTA	ethylenediaminetetraacetic acid
EMSA	electrophoretic mobility shift assay
ESI	electro-spray ionization
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GEE	glutathione ethyl ester
Grx	glutaredoxin
GSH	glutathione
GSSG	oxidized form of glutathione
GST	glutathione s-transferase
HD-ZIP	homeodomain-leucine zipper
IAA	indoleacetic acid
IAM	iodoacetamide
ICAT	isotope-coded affinity tag
iTRAQ	isobaric tags for relative and absolute quantitation
LC-MS/MS	liquid chromatography–mass spectrometry
LP	forward primer
mBrB	monobromobimane

NADPH	nicotinamide adenine dinucleotide phosphate
NBD-Cl	(4-chloro-7-nitrobenzo-2-oxa-1,3-diazole)
nCys	N-terminal cysteine
NEM	N-ethylmaleimide
NP-40	nonyl phenoxypolyethoxylethanol
PA	poly arginine
PAP	peroxidase-anti-peroxidase [pap] antibody complex)
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PerR	peroxide operon regulator
Pfu	proofreading polymerase
PTMs	posttranslational protein modifications
PVDF	polyvinylidene difluoride
ROS	reactive oxygen species
RP	reverse primer
SA	salicylic acid
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SO_2H	sulfinic acid
SO_3H	sulfonic acid
SOH	sulfenic acid
Strep-HRP	streptavidin-hrp blot
SUBA3	the subcellular localisation database for arabidopsis proteins
TAIR	the arabidopsis information resource
TAP	tandem affinity purification
TCA	trichloroacetic acid
TCEP	(tris 2-carboxyethyl phosphine)
TEV	tobacco etch virus
Trx	thioredoxin
YAP1	(Yeast *Saccharomyces cerevisiae* activator protein1)

Publications & Scientific Manifestations

Publications

Cezary Waszczak[1], **Salma Akter**[1], Dominique Eeckhout, Geert Persiau, Khadija Wahni, Nandita Bodra, Inge Van Molle, Barbara De Smet, Didier Vertommen, Kris Gevaert, Geert De Jaeger, Marc Van Montagu, Joris Messens, and Frank Van Breusegem (2014). Sulfenome mining in *Arabidopsis thaliana*. Proc Natl Acad Sci U S A **111**: 11545–50

[1] Both authors contributed equally to this work.

Salma Akter, Jingjing Huang, Nandita Bodra, Barbara De Smet, Khadija Wahni, Debbie Rombaut, Jarne Pauwels, Kris Gevaert, Kate Carroll, Frank Van Breusegem and Joris Messens (2014). DYn-2 based identification of Arabidopsis sulfenomes. (research article submitted to the Journal of Molecular and Cellular Proteomics)

Salma Akter, Cezary Waszczak, Jingjing Huang, Silke Jacques, Kris Gevaert, Frank Van Breusegem and Joris Messens (2014). Cysteines under ROS attack in plants: a proteomics view. (review paper submitted to the Journal of Experimental Botany)

Cezary Waszczak, **Salma Akter**, Jingjing Huang, Silke Jacques, Kris Gevaert, Frank Van Breusegem and Joris Messens (2014). Oxidative post-translational modifications of cysteine residues in plant signal transduction. (review paper submitted to the Journal of Experimental Botany)

Scientific Manifestations

Scientific Collaboration:

- The Scripps Research Institute, Scripps Florida, Jupiter, FL 33458, U.S.
- Faculty of Bioscience Engineering Division of Crop Biotechnics, KU Leuven, Belgium.
- Faculty of Medicine and de Duve Institute, Université Catholique de Louvain (UCL), 1200 Brussels, Belgium.
- VIB Department of Medical Protein Research, Ghent University, 900 Ghent, Belgium.

Conferences and Scientific presentations

(I) 17th July 2013 Abstract no: 01.2	11th International Conference on Reactive Oxygen and Nitrogen Species in Plants, Warsaw, Poland Type: ROS production, signalling and homeostasis **Oral presentation**
(II) 5th September to 9th September 2012	16th Biennial Meeting of the Society for Free Radical Research International (SFRRI) 2012 Imperial College, London, UK **Poster presentation**
(III) 25th May 2012	206th meeting of the Belgian Society of Biochemistry and Molecular Biology 2012 University of Ghent, Belgium **Oral Presentation**
(IV) 6-8 February 2013	VIB Seminar 2013 Blankenberge, Belgium **Poster presentation**

Scientific Training

Training on **'Chemical probe labeling of sulfenic acids, click reactions, enrichment of probe labeled proteins'** from The Scripps Research Institute, Scripps Florida, Jupiter, FL 33458, U.S.

Period: 14/10/2012 to 30/11/2012

Words of thanks

I would like to acknowledge all the efforts behind the success of this research work. Firstly, I would like to acknowledge the Erasmas Mundus funding organization for providing me an opportunity to start PhD research in such excellent labs at SBRC and PSB. I would also like to acknowledge my home institute, University of Dhaka to provide me study leaves and to facilitate me to start PhD research abroad.

This research work is the success of collective efforts of many people; when I look back the 4 years of my PhD research, I can see all the faces assisted me in planning, performing, analyzing the experiments that you will find throughout this thesis. First of all, I would like to show my gratitude to my promoters Professor Joris Messens and co-promoter Professor Frank van Breusegem for giving me the opportunity to work in this excellent project. I started this research work with a lot of challenges; and their wise supervision guided me to make it possible. I can remember the encouragement and motivation I got from them from the beginning till to the end of this period. I have learned a lot from them in the past four years, from designing experiments till reporting during conferences and write scientific papers.

I would like to thank all the members of my PhD examination committee for accepting my invitation and for providing valuable comments and suggestions that greatly increased the quality of this thesis.

This work could not advance without the help of some dedicated collaborators. I am thankful to all the collaborators for sharing their excellent expertise. I am grateful to Prof. Geert De Jaeger and his lab for their assistance in YAP1 project by providing us TAP technology. I would like to thank Prof. Kate Carroll, who has been the driving force behind the DYn-2 project. When I was facing difficulty with this project, she invited me for training, and this

was a nice opportunity for me to learn all the tricks and techniques, which helped me a lot to prove DYn-2 principles in plants. I would like to thank Prof. Kris Gevaert and his lab to perform the mass spectrometry of my samples and the DYn-2 project turned back after this collaboration. I also enjoyed working together with Dr. Didier Vertommen for mass-spec analysis. He was always enthusiastic to measure one more sample, I can remember his efforts to solve the problem why we were losing 2 Da in dimedone tagged peptide. I am also thankful to Prof. Sebatien Carpentier, and his lab for their contribution in Dimedone project, guiding me to run excellent 2D-E gel and, a lot of trials to find any dimedone tagged peptides in the selected spots by mass-spec analysis.

Khadija, my special thanks to you to provide me enormous supports in the lab from the very first day of my lab work. During the last 4 years you have been not only my colleague, but also as a friend. Whenever I faced any problem in administrative and technical perspective; I always have been to you for your help and suggestions.

I am very grateful to Cezary Waszczak for guiding me in planning and performing experiments, for all the nice memories we shared in the lab. I am also thankful to other members of my lab Veronica, Wael, Koen, Mandy, Maria, Margarida, Karo, Abderahim, Brandan, Anna, Silke, Katrien, Brigitte, Debbie, Inge, Jenny, Pavel, Barbara, Aurine, Jordi, Lorin, Simon, Annelis – thank you all for your friendship, help throughout my stay at VUB and PSB, to keep me sound during the often-long days in the lab and your support to solve technical problems in the lab. I am also thankful for your suggestions and critical comments in the group meetings. Inge, thanks a lot for your support by listening and answering my questions and for all the discussion we had. During my end of my PhD research, I have met new colleagues Barbara, Nandita, Anna and Jingjing, who are now leading the plant sulfenome project, and I am quite confident that their cooperative efforts will lead them to reach the final goal. Jingjing, I am really thankful to you for all the

suggestions and comments I got from you about my thesis.

Obviously, I am thankful to the women of the secretary, Daniella, Lieve, Nadine and Maria, for their administrative help. I also wish to thank Bruno for all the technical support he gave me.

I would like to acknowledge my previous supervisors, Prof. Kawser Ahmed and Prof. Anwarul Azim Akhand – thank you Sir, for introducing me to the world of science!

My most heartfelt gratitude goes to all my family members, my parents, sisters and brothers for their love and support throughout my PhD research.

Now my thanks to all of my frineds from Bangladesh, their support and help during my Phd research….Manik Da, Gobinda Sir, Boudi, Farzana….reaserch er failure er dhakka gulu vule thaktam apnader sathe adda diye. Mila, Aziz, Sharmin…..tumader valobashay prothom Brussels e pran khuje pelam, tana 5 din er lab pressure er por tumader sathe adda diye nijeke recharge kore nitam…..r tumrai biz bopon kore gele Brussels rock on er….. Farhana, Farzana, Akhter, Naim, Muntasir, Sania, Trisna….PhD er shesh kothin somoy ta mone hoy nimishei shesh holo tumader valobashay r sohojogitay…..last 6 month amer jonno acta birat challenge cilo….tumader support seta k somvob koreche…..

Neawz r Adyan, …..ai safolota ashole tumader dujon er…..

Thank you all

Salma Akter

16th December 2014, Belgium.