Posttranslational Modification of 2-CysPrxs by Reduced Glutathione Interplays with CYP20-3-Dependent OPDA Signaling Pathways

by

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Abstract

Glutathione, a tripeptide (GSH; a 307 Da γ-L-glutamyl-L-cysteinyl-glycine peptide) is the most abundantly present, thiol in plants and animals, and considered as a master antioxidant. It prevents damage to cells caused by reducing reactive oxygen species (ROS) and other peroxides. It is capable of donating electron (H^+, e-) to ROS and is itself oxidized to glutathione disulphide (GSSG). Reduced GSH, beside GSSG, can directly crosslink with the sulfhydryl group of protein cysteine residues (e.g. 2-cysteine peroxiredoxins, 2CPs), hereafter referred to as 'GSH-glutathionylation'. Arabidopsis contains 2 isoforms of 2CPs, called A and B, that are 96.5 % identical in amino acid sequences. There are only 7 amino acid differences between them and, because of this similarity, most studies have considered them as functionally redundant enzymes. However, we observed that GSH, as a posttranslational modifier, is able to form mixed disulfide bond with, and differentially regulates 2CPs; monomerizing and enhancing peroxidase activity of 2CPA, whereas decamerizing and showing chaperone activity of 2CPB.

Plants constantly encounter a wide range of environmental stresses, and thus have developed efficient defense mechanisms to overcome them. (+)-12-*oxo*-phytodienoic acid (OPDA) is an important biologically active jasmonates, able to trigger and cause signaling pathway on its own and control the expression of selective genes, as well as activate plant defense and growth processes. Here we assessed if (**1**) OPDA signaling enhances an electron transfer (govern energy allocation) from thioredoxins (TRXs, photosystem) to cyclophilin 20-3 (CYP20-3, OPDA receptor) in plastid sulfur assimilation (defense activations), and (**2**) OPDA signaling stimulates CYP20-3-mediated reduction of 2CPs. Our in vitro and ex vivo protein analyses illuminated that CYP20-3 relays an OPDA signal during stress responsive regulation of cellular redox homeostasis. OPDA signaling stimulates an electron transfer from TRX to CYP20-3. Once activated, CYP20-3

target-reduces (deglutathionylates) and inactivates $2CPA^{GS}$, suppressing the peroxide detoxification in photosynthesis.

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List of Abbreviations

12-OPDA	12-oxophytodienoic acid
2-CysPrxs	2-cysteine peroxiredoxins
CYP20-3	cyclophilin 20-3
OPDA	12-oxo-phytodienoic acid
FA	fatty acid
ATP	adenosine triphosphate
JA	jasmonic acid
WT	wild type
SAT1	serine acetyltransferase1
CSC	cysteine synthase complex
OASTL-B	O-acetylserine(thiol)yase B
ORGs	OPDA-responsive genes
KO	knock-out
ETC	electron transport chain
PSI	photosystem I
TRXs	thioredoxins
Fd	ferredoxin
FTR	Fd-TRX reductase
FBPase	fructose bisphosphatase
2CPs	2-Cys peroxidoxins
GSH	glutathione
GSSG	glutathione disulfide

DNA	deoxyribonucleic acid
ROS	reactive oxygen species
PSH	protein sulfhydryl groups
GSNO	S-Nitrosoglutathione
PTM	posttranslational modification
HIS	histidine
μM	micromole
mM	millimole
pH	potential of hydrogen
Pad2	phytoalexin deficient 2
Cad2	cadmium-sensitive 2
μmol	micromole
g	gram
SDS/PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
PVDF	polyvinylidene difluoride
H_2SO_4	sulfuric acid
nM	nanomole
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
μL	microliter
^{ox}	oxidized
^{red}	reduced
Cys^R	resolving cysteine

Cys^P	peroxidatic cysteine
^{GS}	glutathionylation
TCEP-HCL	tri(2-caboxyethyl)phosphine hydrochloric acid
DTT	dithiothreitol
SA	salicylic acid
PBS	phosphate-buffered saline
WB	western blot
kDa	kilodalton
MWS	molecular-weight sizes
CS	citrate synthase
UBC	polyubiquitin
MeJA	methyl jasmonate
PPIase	peptidyl-prolyl *cis-trans* isomerases

Chapter 1

Literature Review

1.1. Oxylipins

Oxylipins, oxygenated derivatives of fatty acids (FA), are important signaling molecules in various physiological processes in plants and animals [1]. Oxylipins operate cellular defense responses in plants, while in mammals they control complex regulatory mechanisms in immunity, functioning as messengers in the central nervous system and participating in healing processes after tissue injury [2,3]. Recent studies have highlighted the medicinal value of phytooxylipins, emphasizing their anticancer and anti-inflammatory activities [4,5]. Molecular components and pathways involved in oxylipin synthesis and signaling share common ancestry across plant and animal kingdoms [1]. Hence, uncovering the modes of actions associated with plant oxylipins will not only help in the development of agricultural strategies for improving disease resistance and stress adaptation resulting in yield and biomass increases in plants, but also the advancement of drug development by facilitating the rational design of more powerful and safer anticancer and anti-inflammation reagents. However, our current knowledge regarding oxylipin signaling is still incomplete, despite years of investigation [2,3].

1.2. Jasmonates and its biosynthesis

Plant hormone, (-)-jasmonic acid [JA; (1*R*,2*R*)-3-oxo-2-(2Z)-2-pentenyl-cyclopentaneacetic acid] and its precursors/derivative, known in collective term as jasmonates, are derived from trienoic-FA via the octadecanoid pathway in the chloroplasts. Lipase-mediated oxidation of trienoic-FA leads to the release of OPDA that travels to the peroxisomes through ATP-binding transporters (e.g. COMATOSE) [6] and undergoes β-oxidations to form JA. JA is then exported to the cytosol,

and further metabolized to various derivatives including JA-isoleucine (JA-Ile), JA-tryptophan, hydroxyl-JA and methyl-JA. JA-Ile then can signal and control the expression of a number of nuclear genes, and exhibit defense (adaptive) responses towards various forms of biotic and abiotic stresses including microbial pathogens, insect herbivores, tissue injury, and light damage. Jasmonate signaling also plays major roles in reproduction and other developmental processes such as senescence, root growth and tuberization, fruit ripening, and tendril coiling [7,8].

1.3. OPDA as one of the biologically active jasmonate signals

(+)-12-oxo-phytodienoic acid (OPDA) is the primary precursor of the jasmonate family of phyto-oxylipins [9,10]. OPDA is the biologically active jasmonates, able to trigger signaling pathway on its own that regulates jasmonate-responsive genes, activating plant defense responses, as well as growth processes [11,12]. Its unique activity was described for the first time by the analyses of a mutant Arabidopsis (*opr3*) where conversion of OPDA to JA was arrested [13]. Wild type (WT)-like resistance of *opr3*, highlighted important roles of OPDA signaling in plant defense responses in the absence of JA and JA-Ile [13-15]. Studies with several mutant plants suppressing or impairing JA production (e.g. *siOPR3*, *OPR3-RNAi*, *cts-2*/*opr3* and *acx1*) or OPDA signaling (*cyp 20-3*) further proved that OPDA signaling is important in basal defense responses against a variety of pathogenic fungi and insects such as *Alternaria brassicicola*, *Botrytis cinerea*, *Sclerotinia sclerotiarum*, *Nilaparvata lugens*, *Manduca sexta* and *Bradysia impatiens*, as well as seed germination, embryogenesis and balancing abscisic acid signaling [9,16-20].

1.4. Cyclophilin 20-3 dependent OPDA signaling during stress responses

Cyclophilin 20-3 (CYP20-3) is the most important regulator of OPDA signaling that conveys stress-responsive regulation of cellular redox homeostasis [9]. CYP20-3 is a dual function enzyme in the chloroplasts. It basically has two activities which are chaperoning protein folding (peptidyl-

prolyl isomerase; PPlase) or transferring electrons (e^-) to peroxide substrates (reductase) [21-26]. During infection or insect predation (Fig. 1.1, left), OPDA binds to CYP20-3 and stimulates the formation of a complex with serine acetyltransferase1 (SAT1), which triggers the formation of a Cysteine (Cys) synthase complex (CSC), a hetero-oligomeric complex with O-acetylserine(thiol)lyase B (OASTL-B). Formation of this complex then leads to the production of amino acid Cys and then thiol metabolites (sulfur assimilation). This further builds up the reduction potential of cells. The increased redox capacity in turn coordinates the expression of a subset of OPDA-responsive genes (ORGs) that activate pathogen defense and stress adaptation processes. Thus, the deletion mutant (*cyp20-3*) plant displays enhanced susceptibility to pathogen infection (e.g. *A. brassicicola*, *B. cinerea* and *Pythium irregulare*) compared with WT. These results support: **a**) that OPDA is a biologically active jasmonate signal, connecting stress cues to sulfur assimilation and cellular redox homeostasis in plant defense mechanisms, and **b**) that sulfur metabolism has a role in transmitting signal messages from the chloroplasts to the nucleus (referred to as 'retrograde signaling'), activating defense gene expressions.

1.5. Cyclophilin 20-3 is positioned as a regulatory hub between OPDA and light-dependent redox signaling

On the other hand, CYP20-3 is positioned as a redox sensor of the light-dependent redox reactions (also known as an electron transport chain; ETC) of photosystem I (Fig. 1.1) that plays major roles in the conversion of solar energy into biologically useful chemical energies, necessary for the production of overall biomass [27,28]. When photosystem (PSI) captures solar energy, it excites electrons ($e^–$) which causes reduction of thioredoxins (TRXs). This reduction is caused by ferredoxin (Fd) and Fd-TRX reductase (FTR). TRXs, small oxidoreductases, when reduced deliver $e^–$ and activate enzymes of their target in the Calvin cycle. These enzymes include fructose

bisphosphatase (FBPase), glyceraldehyde-3-phosphate dehydrogenase, sedoheptulose bisphosphatase and phosphoribulokinase [29-31]. Recent studies have shown that TRXs can also reduce other proteins which are not related to Calvin cycle like CYP20-3, a key regulator of **i**) OPDA defense signaling and **ii**) photosynthetic ETC as an e^- donor of 2-Cys peroxiredoxins (2CPs) [23,30-32]. Plastid 2CPs are thiol-based peroxidases involved in protecting and optimizing photosynthesis. Once activated (reduced) by NADPH-dependent TRX reductase C (NTRC) and/or other e^- donors such as TRXs and CYP20-3, they catalyze the reduction of toxic byproducts (e.g. H_2O_2) of photosynthesis, or activate Calvin cycle enzymes such as a FBPase [23-25, 33-36]. The interaction of TRXs with CYP20-3, thereby, places CYP20-3 as a redox regulatory hub, transferring e^- from TRXs to 2CPs and/or SAT1 (Fig. 1.1). Reduction of 2CPs then continues peroxide detoxification and carbon metabolisms, whereas the activation of SAT1 stimulates sulfur assimilation which coordinates redox-resolved nucleus gene expressions in defense responses against biotic and abiotic stresses [34, 37-39]. In line with this scenario, stress-induced OPDA binds and, perhaps, modulates the functional and conformational states of CYP20-3 which adjusts its subsequent binding and e^- transfer between 2CPs and/or SAT1 [9,10]. CYP20-3 is hypothesized as a unique player in controlling the interface between OPDA signaling and photosynthesis [10]. This interplay enables plants to make an adaptive decision in allocating resources (e^-) between growth and defense responses (e.g. fitness tradeoffs or balances) [40,41] towards constant environmental challenges such as pathogens, pests, tissue injury as well as light and oxidative stresses [38,42,43] in the end, ensuring optimal growth, reproduction and survival of plants.

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1.7. Figures

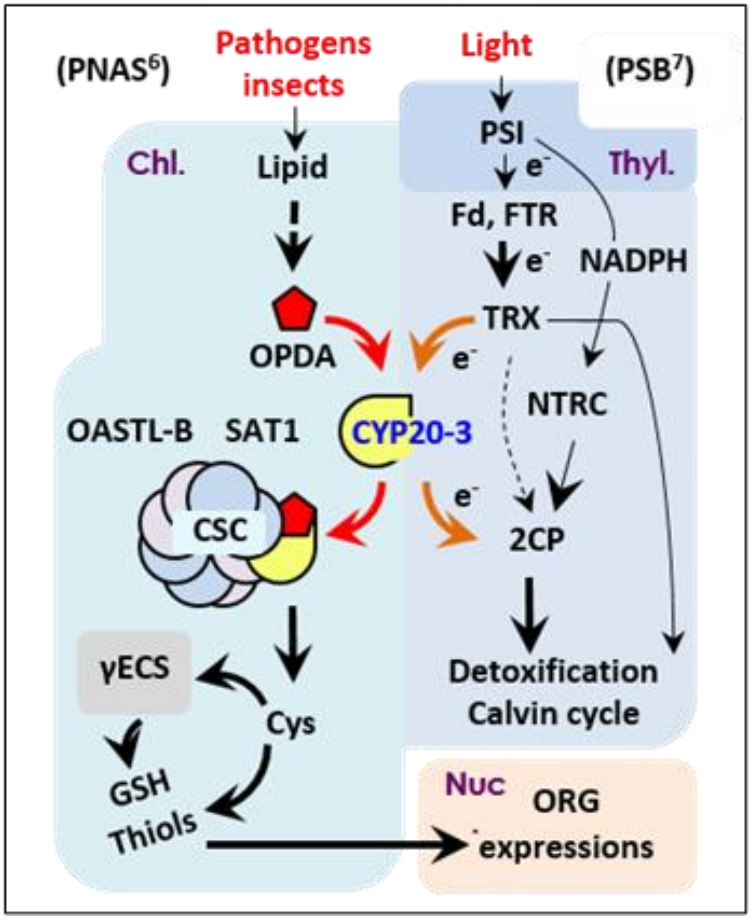

Figure 1.1: Proposed roles of CYP20-3 as a hub linking OPDA signaling and redox reaction chain. Chl., chloroplasts; Thyl., thylakoid; Nuc., nucleus

Chapter 2

Posttranslational modification by a reduced glutathione determines the structure and function of 2-cysteine peroxiredoxins in the chloroplasts

2.1. Abstract

Glutathione (GSH) is one of the most important intracellular thiols in all aerobic organisms. Until recently, it has been characterized as a master antioxidant that cleans-up a broad range of toxic peroxides and peroxynitriles. Now, we demonstrate a unique, regulatory activity of GSH that operates autonomous redox metabolic and signaling pathways. Reduced GSH, not oxidized GSSG, as a target selective posttranslational modifier, can directly crosslink with the sulfhydryl group of protein cysteine residues (GSH-glutathionylation). GSH-crosslinking then modulates the structure and function of redox-sensitive enzymes such as 2-cysteine peroxiredoxins (2CPs). The nuclear genome of Arabidopsis consists two plastid 2CPs (denoted A and B) isoforms, described as redundant, thiol-based peroxide reductases. However, a series of our assays redefine that GSH-glutathionylation renders distinctive activities of 2CPs. When arrived at the chloroplasts, 2CPs immediately interact with GSH, this in turn causes monomerization and peroxidase activity of 2CPA, whereas decamerizing and highlighting the chaperone activity of 2CPB. The posttranslational modification is further stimulated by the systematic induction of GSH and attendant reduction capacity, occurring independently of oxidant generations. Thus, we conclude that GSH can act as a signal cue, beyond its general antioxidant (electron-donor) activity, capable of controlling a large number of cellular metabolisms and processes.

2.2. Introduction

The tripeptide glutathione is the most important thiol in plants and animals, playing important roles in regulating cellular redox homeostasis and events including gene expression, DNA and peptide biosynthesis, cell proliferation and death, signal transduction, and immune response. GSH acts as a master antioxidant that interacts, rather nonspecifically, with different metabolites. Most importantly, it prevents damage to cells caused by reducing reactive oxygen species (ROS) and other peroxides. It is then oxidized to glutathione disulphide (GSSG) [1].

Presence of oxidative stress results in GSSG accumulations and increase in GSSG to GSH ratio, which in turn stimulates mixed disulfides formation. This is a reversible reaction and occurs between protein sulfhydryl groups (PSH) and GSSG (i.e., S-glutathionylation). PSH are oxidized to thiyl radicals under stress condition which have strong reactivity with oxygen, leading to the spontaneous buildup of disulfide bridges with vicinal thiols such as GSSG, or GSNO [2, 3]. S-glutathionylation then causes the structural and functional modulation of redox-sensitive enzymes, triggering the redox regulation of both signal transductions and metabolic pathways during cell growth, migration and recycle, immune response, injury recovery, and neurogenesis in mammals [3], but very little is known about its formation and function in plants. To date, only a few proteins have been tested as the target of S-glutathionylation in plants, which include peroxiredoxins, thioredoxins, and a few glutaredoxin-target proteins such as glyceraldehyde 3-phosphate dehydrogenase, protein tyrosine phosphatase, and BR1-assciated receptor like kinase 1 [4-8]. It has been proposed that GSSG-crosslinking then protects those target proteins against oxidative (peroxide) toxicity, or conversely disrupts their cellular (molecular) activities [2-8]. Further studies will be needed to assess the comprehensive scope, and role of S-glutathionylation. However, previous proteomics from model systems such as Arabidopsis and Chlamydomonas identified a

list of proteins to be targeted by S-glutathionylation [9-11]. These targets constitute multiple key mechanisms of redox regulations and signaling transductions in controlling energy (e^-) transfers across various metabolic pathways and cell processes, implying the crucial role of protein S-glutathionylation in plant growth and defense responses against various environmental stresses.

Pull-down assays [12] we performed detected that Arabidopsis 2-Cys peroxiredoxin A (2CPA) can directly bind to GSH-agarose beads (Figure 2.1), underpinning a potential S-glutathionylation of 2CP-family proteins that contain two Cys; the peroxidatic Cys (Cys^P) and the resolving Cys (Cys^R) [4]. The Arabidopsis genome encodes two plastid 2CP (denoted A and B) isoforms, both are highly abundant thiol-based peroxidases that function in protecting and modulating photosynthesis. However, 2CPs are known to typically form obligatory homodimers as the Cys^P from one monomer is connected via a disulfide bond to the Cys^R located within its complementary monomer. The oxidation of Cys^P then, in consequence, deactivates the catalytic activity of 2CPs. Thus, 2CP dimers require electron donors such as an NADPH-dependent thioredoxin reductase (NTRC), which reduces (activates) them to be able to metabolize the detoxification of a toxic by product in photosynthesis (i.e., H_2O_2), and the activation of Calvin cycle enzymes such as a fructose 1,6-bisphosphatase [13, 14]. On the other hand, under oxidative stress, 2CPs could be overoxidized and form a homodecameric complex that disables their peroxidase activity, but instead gains a chaperone activity to protect cellular molecules against oxidative damage [13]. However, recent studies with human PrxI (huPrxI) and pea 2CP have argued that 2CPs are rather S-glutathionylated by GSSG during oxidative stress, protecting their quaternary structures to remain as dimers and, as a result, enzymatically inactivated status [4, 15, 16].

To better understand the significance of S-glutathionylation in plant cells and for the cellular activity of 2CPs, in this study we studied the structural and functional dynamics of 2CPs in

conjunction with cellular redox states (esp., GSH, GSSG, and H_2O_2) and metabolisms. A series of in vitro and in-vivo analyses identified that 2CPs are indeed S-glutathionylated in the chloroplasts. However, unlike an earlier hypothesis [13], this posttranslational modification (PTM) *i*) differentially modulates and activates 2CPs; monomerizing and ensuring the peroxidase activity of 2CPA, while decamerizing and increasing the chaperone activity of 2CP, as well as *ii*) suggests that 2CPA and B are not functional homologues, involved in unique physiological processes. More importantly, the S-glutathionylation of 2CPs occurs with reduced GSH, but not oxidized GSSG. The systematic induction of GSH productions via stress hormone signaling, occurring independently of free radicals and oxidant generations, and attendant reduction capacity (increased GSH:GSSG ratio) shows the crosslinking GSH to 2CPs. These combined results shed new light on a unique, regulatory activity of GSH as a functional group of posttranslational modifier, apart from its antioxidant activity, that turns on GSH (reductant) signaling for maintaining cellular metabolisms, as well as activating defense responses.

2.3. Materials and methods

2.3.1 Cloning, Expression and Purification of Proteins

Recombinant proteins were prepared according to the previous description in the literature [17]. Mature protein region [18] of 2CPB (At5g06290) was cloned into Pet28a vector (Novagen) and N-terminus 6x HIS tagged proteins were obtained. 2CPA (At3g11630) were harbored in pCRT7/NT-TOPO expression vector (Invitrogen) and it was provided to us by Prof. K. J. Dietz (University of Bielefeld, Germany). Point mutations of 2 CPs were induced by QuikChange II Site-directed Mutagenesis Kit (Agilent). Recombinant proteins thus prepared were expressed in *E. coli* BL21(DE3). Purification was done using a nickel- (Ni-NTA, Qiagen) column [12].

2.3.2. S-Glutathionylation of 2CPs

S-glutathionylation of proteins was done by incubation of 1 or 2 µM 2CPs with 1.0 mM GSH, GSSG, or GSNO in 50 mM Tris-HCl buffer (pH 7.5) at 25 °C for 30 min. There existed varying GSH concentrations (0.5 to 10 mM), time of incubation (0.5 to 30 min), or pH (7.0 to 8.0) in some reactions.

2.3.3 Preparation of GSH-Glutathionylated, Oxidized and Reduced 2CPs

S-glutathionylation of 10 µM of 2CPs was done in 50 mM Tris-HCl (pH 7.5) buffer by GSH (10mM) for 30 min, oxidized by 0.1 mM H_2O_2 for 15 minutes, or reduced by TCEP-HCl for 30 min. Concentration for reduction of 2CPA was 1 mM and for 2CPB was 20 mM. GSH, H_2O_2 and TCEP-HCl in excess were removed with the use of size-exclusion chromatography (Sephadex G-25 medium, GE Healthcare), and stored at 4 °C.

2.3.4. Plant Materials

WT plants of *A. thaliana* (Col-0) and mutants *2CPA* (CS875813 [19]), *2CPB* (SALK_017213 [19]), *2CPA*/*2CPB* (SALK_065264/SALK_017213 [20]), *PHYTOALEXIN DEFICIENT 2* (*pad2* [21]), and *CADIMIUM-SENSITIVE 2* (*cad2* [22]) were used for our study. Plants were grown in growth chamber. Growth conditions were maintained at 12-h day cycle (80-100 μmol photons m^{-2} s^{-1}) at 22 ^{o}C and relative humidity of 60% to 80%.

2.3.5. Total Protein Extraction

Leaves of Arabidopsis were immediately kept in liquid N_2 after harvest and were made to powder form with the use of mortar and pestle. Powdered sample was kept in two volumes of 50 mM potassium phosphate buffer (pH 7.2) with protease inhibitor cocktails (Sigma-Aldrich). Agitation was done for 60 min, and centrifugation was carried out for 30 min at the speed of 10,000*g*.

Supernatant after the centrifugation step was collected, and was subjected to western blot. Extraction steps in all our experiments were carried out at the temperature of 4 °C.

2.3.6. In-vivo Western Blot Analysis

For the assessment of the quaternary structure of 2CPs, protein samples thus prepared were run in SDS/PAGE (10% to 12%), immunoblotting was done using PVDF membranes (Millipore). Probing of blotted membranes was carried out for 2 hours using polyclonal rabbit anti-2CPA antibody (1:7500, MyBioSource) or for 16 hours using monoclonal mouse anti-GSH antibody (1:3000, Enzo Life Science). Visualization of the membranes carried out by chemiluminescence (ECL kit; GE Healthcare). Equal loading was verified using coomassie blue staining.

2.3.7. Peroxidase Activity Assay

Quantification of H_2O_2 reduced by 2CPs was done using the eFOX assay [23] which was carried out at 37 °C using 50 mM potassium phosphate (pH 7.2) having 5 µM 2CPs. Reaction was started by adding 50 µM H_2O_2, following 10 min incubation, and terminating by 2% (v/v) TCA. 500 µL eFOX reagent (250 µM $Fe(NH_4)_2(SO_4)_2$, 100 µM sorbitol, 100 µM xylenol orange, and 1% (v/v) in 20 mM H_2SO_4) was mixed with 100 µL of solution. Reduction in H_2O_2 was measured by spectrophotometer at 550 nm.

2.3.8. Chaperone Activity Assay

Citrate synthase was used as a substrate to study the chaperone activity of 2CPs [24]. 10 µM 2CP at 45 °C was added in 50 mM potassium phosphate buffer (pH 7.2). Temperature was stabilized for 15 min. Increase in absorption (360 nm) after the addition of citrate synthase (1 µM, Sigma-Aldrich) was studied with spectrophotometer.

2.3.9. Semiquantitative RT-PCR

TRIzol reagent (Invitrogen) and Direct-zol RNA Kit (Zymo Research) were used for RNA extraction from the leaves. Quality assessment of RNA was done with agarose gel and quantification was done using NanoDrop (A_{260}/A_{280} > 1.8 and A_{260}/A_{230} > 2.0 [25]). Reactions for RT were performed using an oligo(dT) reverse primer and qScript reverse transcriptase (Quantabio). 2 µL CDNA and *Taq* 2X master mix (New England BioLabs) were used for semi-quantitative RT-PCR. PCR conditions were annealing at 55 °C for 30 s, 27 cycles (*2CPA*), 30 cycles (*2CPB*), 31 cycles (*UBC* [26]) at 94 °C for 30 s, elongation at 72 °C for 1 min, and final elongation was at 72 °C for 10 min.

2.4. Results

2.4.1. Reduced GSH, but not GSSG, Targets and Modifies the Quaternary Structure of 2CPA

To further depict the role and modus operandi of 2CPs in a redox reaction circuitry, potential S-glutathionylation was studied. For this, oxidized (^{ox}) and reduced (^{red}) 2CPA were probed using anti-GSH antibody after prior incubation with all the three forms i.e. GSH, GSSG or GSNO. Both oxidized and reduced 2CPA crosslinked with GSH. No crosslinking was found with GSSG or GSNO (Figure 2.2). It further gave rise to the quaternary structure of 2CPA to display mono-, di- and decamers (Figure 2.3). After binding to GSH, most of the 2CPAs^{ox} in dimeric form changed to monomers, some were still dimers and others formed decamer (Figure 2.3 lane 1, 2 and 6, and Figure 2.4). S-glutathionylation of 2CPAs, is showed by shift in the gel which means increase of the molecular weights (Figure 2.3 lane 3 and 4). There is small amounts of decameric complex formation as well. There are basically 3 structures mono-, di- and decamers (Figure 2.3 lane 4 and 8). GSH targeted the Cys^{R}_{53}, but not the Cys^{P}_{175}, of 2CPA (Figure 2.4), indicating that the crosslinking potential of GSH to the Cys^{R}_{53} is a key determinant of the quaternary structures of S-

glutathionylated (^{GS}) 2CPA. While the S-glutathionylation of Cys^{R}_{53} breaks off its disulfide bond with the Cys^{P}_{175} of a complementary $2CPA^{GS}$ and releases monomeric $2CPAs^{GS}$, interactions between the Cys^{P}_{175} of $2CPA^{GS}$ and the Cys^{R}_{53} of free 2CPA likely results in the dimeric complex of $2CPA^{GS}$.

These results unveil a unique target-specific activity of GSH, besides its general antioxidant (e^- donor) activity, directly crosslinking with the sulfhydryl group of Cys residues, which in turn shapes protein structures (hereafter, called GSH-glutathionylation).

Physiological relevance of GSH-glutathionylation was studied by assessing the reaction concentration and rate of GSH when crosslinking with 2CPA (Figure 2.5 and 2.6). Chloroplastic concentration of GSH (~1 mM [27]) was enough to cause their S-glutathionylation and lead to their quaternary structures to show mono-, di- and decamers (Figure 2.6). This modification was rapid and occurred in less than 60 sec and remained stable for weeks at 4 ^{o}C (Figure 2. It suggests that GSH can act as the determinant of the quaternary structure of 2CPA. Monomers of $2CPA^{GS}$ showed peroxidase activity, detoxifying H_2O_2 (Figure 2.9), while decamers of $2CPA^{GS}$ exhibited chaperone activity (Figure 2.9).

2.4.2. GSH-Glutathionylation Protects the Quaternary Structure of 2CPA against pH Neutralization and Overoxidation

Earlier studies suggested that cellular pH and peroxide contents could also influence the quaternary structure and activity of 2CPs [28, 29]. Neither pH neutralization nor the overoxidation however demonstrated any effect on the GSH-glutathionylation of 2CPA, and $2CPA^{GS}$ (Figure 2.10 and 2.11). In a pH range of 7.0 to 8.0, the plastid concentration of GSH (ca. 1 mM) performed equally well in S-glutathionylating $2CPA^{ox}$ assembling mono-, di-, and decameric structures (Figure 2.10).

It remained stable in overoxidation up to 5 mM H_2O_2 (Figure 2.11), consistent with GSH being a key regulator, crosslinking with 2CPA in the chloroplasts, and protecting its structures and functions against various ecological constrains that cause changes in pH and/or oxidative stresses.

2.4.3. S-Glutathionylated 2CPs form Mono-, Di- and Decameric Structures in Planta

Our results showed that GSH can act a functional group of PTM, which refines 2CPA to a mature and active form upon its arrival at the chloroplasts after being synthesized. To further justify our hypothesis, we examined whether 2CPA is S-glutathionylated in vivo by comparing the molecular sizes of 2CPs with those of S-glutathionylated proteins (protein^{GS}) from total Arabidopsis extracts, using anti-2CPA and anti-GSH antibodies (Figure 2.12). As per our expectation, in nonreducing conditions, both antibodies cross-reacted with several proteins, including three major bands corresponding to the molecular sizes of mono-, di- and decameric 2CPs (Figure 2.12 lane 1 and 3). However, an anti-2CPA antibody detected only monomeric 2CPs when proteins were reduced (Figure 2.12 lane 2), since their di- and decamers were cleaved to monomers by β-mercaptoethanol. Simultaneously, β-mercaptoethanol led to the deglutathionylation of most protein^{GS} (Figure 2.12 lane 4), inferring that 2CPs are indeed S-glutathionylated in planta through a disulfide bridge, and thus constitute mono-, di-, and decameric structures.

Next, we examined whether T-DNA insertion mutants in *2CPs* (e.g., *2cpa*/*b* and *2cpb* [19, 20]) impair forming of the mono-, di- and decameric structure of 2CPs^{GS}. As reported [20], *2cpa*/*b* showed the residual-level expression of *2CPA* (Figure 2.13). Similarly, 2*cpb* appeared to markedly attenuate *2CPA* expression, while both mutants completely knocked out *2CPB* expression (Figure 2.13). As a result, *2cpa*/*b* and *2cpb* displayed a considerable decrease in the accumulated level of the three major protein bands cross-reacting with anti-2CPA and anti-GSH antibodies (Figure 2.14 right).

2.4.4. GSH-Glutathionylation Decamerizes 2CPB, and Accentuates its Chaperone Activity

Our western analyses confirm that S-glutathionylation is intrinsic in resolving the cellular structure and function of 2CPA under basal conditions, which are crucial for plant growth and development [13, 30]. We studied if GSH can also crosslink with 2CPB to determine the formation of quaternary structure in them similar to 2CPA. However, 2CPB behaved different than that of 2CPA (2.15). The 2CPB formed preferably a stable dual-structure (i.e., di- and decamer), when oxidized or reduced by (≤0.1 mM) H_2O_2, TCEP-HCl or DTT used for 2CPA (Figure 2.16). Moreover, increases in the concentration of redox agents showed decamerization of 2CPB (Figure 2.16), except that the overreduction by 20 mM TCEP-HCl resulted in the complete monomerization of 2CPB (Figure 2.16 and 2.17). In these states, GSH was able to crosslink and stimulate the readjustment of 2CPB structures, constituting predominantly a decamer and to a lesser extent an icosamer (Figure 2.15, and Figure 2.17). Increasing the concentration of GSH (over ≥4 mM) or from 8.0 to pH 7.0 promoted icosamerization of $2CPB^{GS}$ (Figure 2.18 and 2.19).

As expected from these results, a T-DNA insertion mutation in *2CPA* (CS875813 [19]), partly suppressing its expression (Figure 2.20), was still able to accumulate wild type (WT)-level 2CP (Figure 2.21 lane 8). In comparison, *2cpb* and *2cpa*/*b* mutants knocking-out *2CPB* (Figure 2.13*)* mostly disappeared decameric $2CPs^{GS}$ (Figure 2.14), showing that 2CPB assembles a homodecameric complex in planta. On the other hand, the decreased amount of total 2CPs in CS871813 (see monomers in Figure 2.21 lane 7 vs. 5), likely caused by the downregulation of *2CPA* (Figure 2.20), reflected the reduction of mono- and dimeric $2CPs^{GS}$ (Figure 2.21 lane 8 vs. 6). These results concur with the conclusion that in planta S-glutathionylation differentially modulates the structure and function of two plastid 2CPs; monomerizing and ensuring the

peroxidase activity of 2CPA^{GS}, while decamerizing and increasing the chaperone activity of 2CPB^{GS}.

2.4.5. GSH and its Metabolisms Control the S-Glutathionylation Kinetics of 2CPs in Planta

Next, we investigated whether there may be an alternative mechanism that could be controlling the activity and/or GSH-glutathionylation of 2CPs^{GS}.

Depletion of GSH accumulations in *pad2* and *cad2* mutants [22, 31] paralleled the impairment of S-glutathionylation of 2CPs (Figure 2.22, right panel). Note that *PAD2* and *CAD2* encode γ-glutamylcysteine synthetase (γ-ECS [22, 31]), the enzyme that catalyzes the first step of de novo GSH biosynthesis [32]. As a result, both mutants largely lacked monomeric (peroxidatic) 2CPs (Figure 2.22, middle panel) that mainly accounts for 2CPA^{GS} and increased the dimerization (inactivation) of 2CPs. On the other hand, *pad2* and *cad2* accumulated WT-like level 2CP decamers (Figure 2.22, middle panel), which likely represent a typical, obligatory form of free 2CPB's quaternary structure, a decamer. The need of γ-ECS, GSH-producing enzyme, in the S-glutathionylation of 2CPs decisively underpins the intrinsic role of GSH as a posttranslational modifier, apart from its antioxidant activity [1] and/or GSSG-dependent S-glutathionylation [2, 3], that carries out a target-specific PTM, modifying redox-related proteins (e.g., 2CPs) toward the mature and active form upon being synthesized.

2.5. Discussion

Recently, GSSG-mediated PTM (called, S-glutathionylation) has emerged as an important mechanism that conveys oxidative stress, ROS, signaling [2, 3]. ROS, considered as highly toxic molecules, are generally produced as the byproduct of aerobic metabolisms but also induced in response to various types of endo- or exogenous signals (e.g., biotic and abiotic stresses). ROS

accumulations in turn rapidly actuate an effective scavenger system that employs a master antioxidant, GSH [33, 34]. GSH reduces ROS by donating its electron (H^+, e^-), and is oxidized to a disulfide form (GSSG). Most GSSG are afterwards recharged back to the sulfhydryl form (GSH) by an enzyme GSH reductase, but some GSSG bind and modify the shape and activity of thiol-containing proteins. This GSSG-mediated PTM then gives rise to the regulation of redox metabolic cascades (referred to as ROS signaling) which, allow adaptation of organisms to new environmental stresses [2,3]. However, the role and formation of GSSG-dependent S-glutathionylation have not yet been well understood in plants. Furthermore, most in vitro assays have indicated that S-glutathionylation requires ~10 to 50-folds higher than the cellular concentration of GSSG [4, 5, 7, 27], changing its actual, physiological activities [9, 10, 27]. Our in vitro and in-vivo analyses here explain a unique, regulatory activity of GSH, besides its antioxidant activity (see above), that directly binds to PSH and controls the structure and/or function of target proteins (e.g., 2CPs). Notably, GSH - unlike GSSG - is not only able to bind oxidized PSH but also target and form a S-S bond with reduced PSH, even without an enzymatic reaction. More details of binding chemistry between GSH and $PSH^{ox/red}$ are needed to be investigated in future work. Nonetheless, this GSH-mediated PTM (i.e., GSH-glutathionylation) explain that GSH not only conveys ROS scavenging mechanisms and signaling transductions, but also carries its own, autonomous, and vital messages in a number of cellular processes contributing to plant growth and survival.

In the chloroplasts, 2CPs thiol-based peroxidases are positioned as a regulatory hub interacting with multiple proteins in diverse cellular processes including photosynthesis, hormone and redox signaling, as well as amino acid and peptide biosynthesis [35]. These housekeeping activities were proposedly protected by the redundant function of two isoforms, 2CPA and B [36], which share

high amino acid sequence identity (>96 %). Indeed, deficiency of a single 2CP, *2cpa* or *2cpb*, exhibited little effect on its phenotypes as compared with WT, while a double mutant *2cpa*/*b* showed slightly lower weight and photosynthetic pigments than WT [20, 36]. However, our in-vivo western blots revealed that 2CPA and B are not in fact functional homologues. Upon the arrival at the chloroplasts, 2CPs are GSH-glutathionylated and form distinctive structural conformations; *i*) largely monomerizing and ensuring the peroxidase activity of $2CPA^{GS}$, while *ii*) decamerizing and enhancing the chaperone activity of $2CPB^{GS}$. This discovery compels to reassess functional dynamics between two $2CPs^{GS}$. The mechanistic details of $2CPA^{GS}$ and B^{GS} will have to be further investigated.

In the current literature, a series of biochemical analyses have suggested that 2CPs play rather restricted roles in plants, since their structures and peroxidase activity are overly sensitive to various internal and external factors, circadian dynamics, as well as the availability of electron donors. Hence, 2CPs are presumed to be active only in a resting condition but supplemental under stressed conditions [13, 20]. If so, why do plants consistently produce large amounts of 2CPs (>1.2 % of stomatal proteins [37]), even though they, are coping with constant encounters with a wide range of environmental stresses? Here, our results explain that 2CPs are the intrinsic target of GSH, principally activated by GSH-glutathionylation. GSH-binding then protects the structures and activities of $2CPs^{GS}$ against various cellular and ecological constraints, suggesting that $2CPs^{GS}$ are, unlike a present model [13, 20], functionally stable and ubiquitous throughout different physiological stages and states in plants.

GSH (GSH: GSSG) status is central in the maintenance of cellular redox homeostasis. As a master antioxidant (redox buffer), GSH is mostly produced for counteracting inter- and external free radicals, and environmental toxins [1]. However, several studies have indicated an alternative,

systematic induction of GSH, independently of oxidant generations, during different physiological processes including light signaling, cell growth, and defense against abiotic and biotic stresses [17, 32, 38-42], though very little is understood about their roles and significances. The present study uncloses, for the first time, *i*) a target-specific role of GSH as a post-translational modifier, and *ii*) that the increased level of GSH further fine-tunes the cellular activities of redox-sensitive proteins (e.g., 2CPs). This GSH-mediated PTM (GSH-glutathionylation) thus is able to support an earlier hypothesis [17] that GSH can *iii*) act as a signal cue and *iv*) autonomously trigger redox metabolic cascades in a retrograde direction (chloroplasts to nucleus) to modify nuclear transcription (i.e., retro-grade signaling) [43, 44]. For instance, under stressed states, OPDA - accumulates in the chloroplasts - binds and stimulates its receptor, cyclophilin 20-3, to form a complex with serine acetyltransferase 1, which triggers the formation of a hetero-oligomeric Cys synthase complex (CSC) with *O*-acetylserine(thiol)lyase B in the chloroplasts [17, 38]. CSC formation then leads to the production of Cys (sulfur assimilation) and subsequently GSH, building up reduction capacity, which in turn activates a subset of OPDA-responsive genes [15], possibly via target-specific GSH-glutathionylation that modulates the cellular activity of oxidoreductase cascades [45] in controlling transcriptional regulators (17). Further, more detailed work to identify the GSH-target proteins will be necessary to study the precise roles and functions of GSH-glutathionylation and reductant signaling in cellular mechanisms.

2.6. References

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2.7. Figures

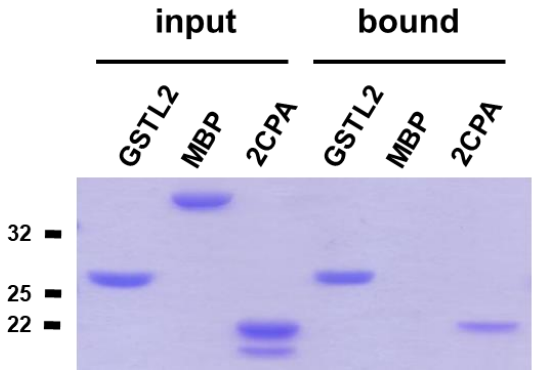

Figure 2.1: Affinity of 2CPA to GSH. Equal amounts of 2CPA, GSH S-transferase λ2 (GSTL2, positive control) and maltose-binding protein (MBP, negative control) were incubated with GSH-agarose resins (input) and washed using PBS buffer. Resin-retained proteins (bound) were then fractionated by SDS/PAGE.

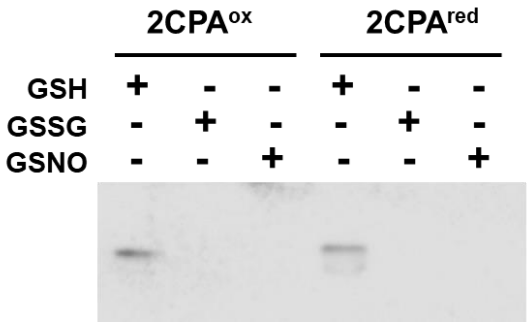

Figure 2.2: GSH-glutathionylation of 2CPA. The 1 µM oxidized (^{ox}) and reduced (^{red}) 2CPAs were incubated with 1 mM GSH, GSSG or GSNO for 30 min, and analyzed by western blot (WB) using a monoclonal anti-GSH antibody.

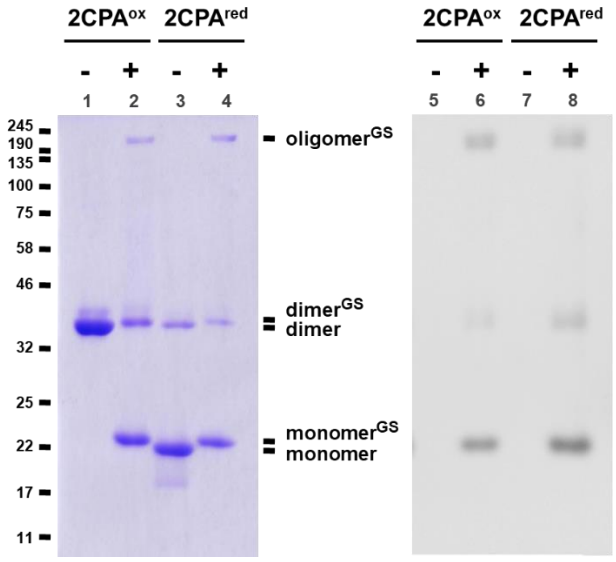

Figure 2.3: Quaternary structure of 2CPA. GSH-glutathionylated (^{GS}) 2CPA forms mono-, di- and decameric structures.

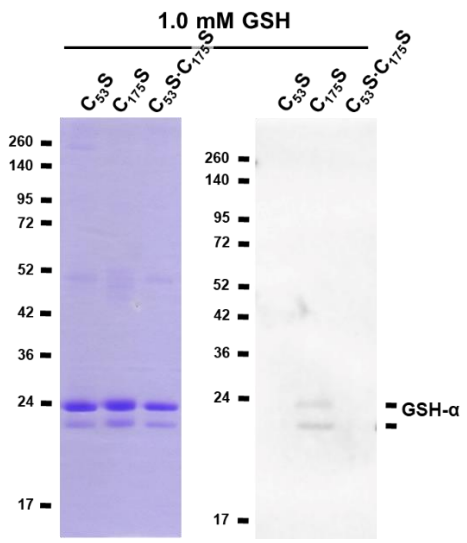

Figure 2.4: Site-specific crosslinking of GSH with the Cys^{R}_{53} of 2CPA.1 µM $2CPA^{ox/red}$ or Cys to Ser mutant 2CPAs ($C^{53}S$ and/or $C^{175}S$), incubated with/without 1 mM GSH, were subjected to nonreducing SDS/PAGE (Left), and analyzed by WB using a monoclonal anti-GSH antibody (Right). Gels were stained with Coomassie Brilliant Blue, and the standard molecular-weight sizes (MWS, kDa) were indicated in the left of gels.

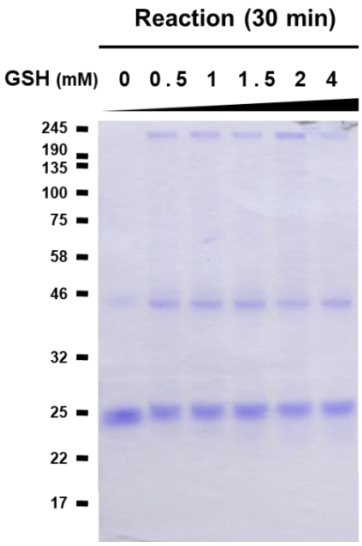

Figure 2.5: GSH concentration dependent glutathionylation activates the catalytic activity of 2CPA. GSH concentration-dependent (0 to 4 mM for 30 min; S-glutathionylation of $2CPA^{red}$. $2CPAs^{GS}$ were separated by nonreducing SDS/PAGE and stained with Coomassie Brilliant Blue. MWS were indicated in the left of gels.

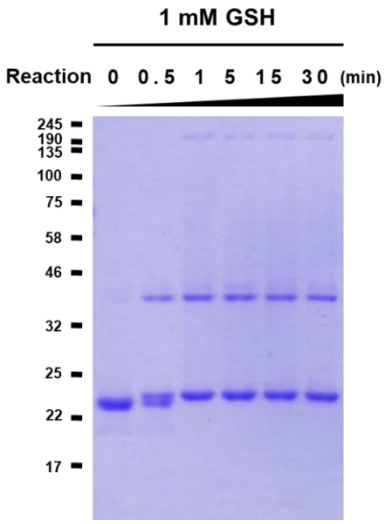

Figure 2.6: GSH time dependent glutathionylation activates the catalytic activity of 2CPA.Time-dependent (0 to 30 min with 1 mM GSH; B) S-glutathionylation of $2CPA^{red}$. $2CPAs^{GS}$ were separated by nonreducing SDS/PAGE and stained with Coomassie Brilliant Blue. MWS were indicated in the left of gels.

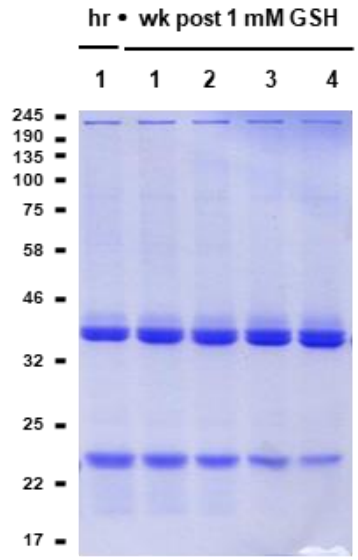

Figure 2.7: Stability of $2CPA^{GS}$. After treating 1 mM GSH for 30 min at 25 ^{o}C, $2CPA^{GS}$ was dialyzed against 50 mM Tris-HCl (pH 7.5), stored at 4 ^{o}C for the duration of 1 hour to 4 week and subjected to nonreducing SDS/ PAGE. Gels were stained with Coomassie Brilliant Blue, and the standard molecular-weight sizes (kDa) were indicated in the left of gels.

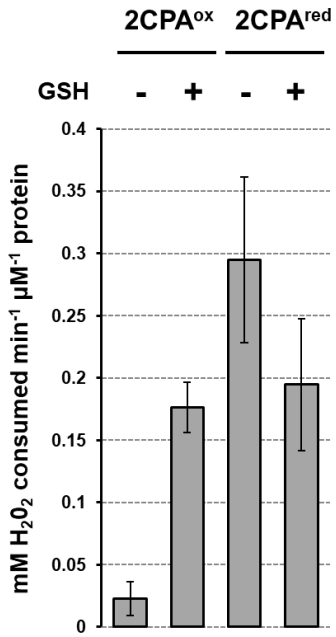

Figure 2.8: Peroxidase activities of glutathionylated 2CPA. Peroxidase activity was measured in $2CPA^{ox/red}$ and $2CPA^{GS}$ by incubating with H_2O_2 for 10 min. H_2O_2 was then quantified using the eFOX method.

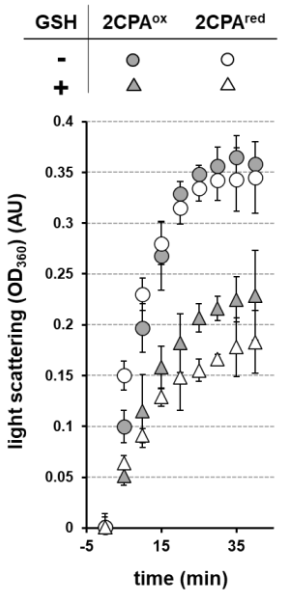

Figure 2.9: Chaperone assay of glutathionylated 2CPB. Chaperone function was determined using a citrate synthase (CS) as a substrate. The light scattering of CS due at 45 ˚C was visualized using a spectrophotometer at 320 nm.

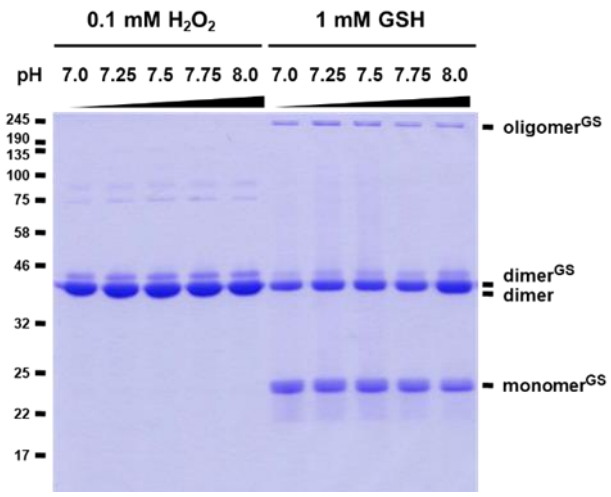

Figure 2.10: GSH-glutathionylation against pH neutralization. Effect of pH changes (7.0 to 8.0) on the oxidation (0.1 mM H_2O_2), and GSH-glutathionylation (1 mM GSH) of 2CPA.

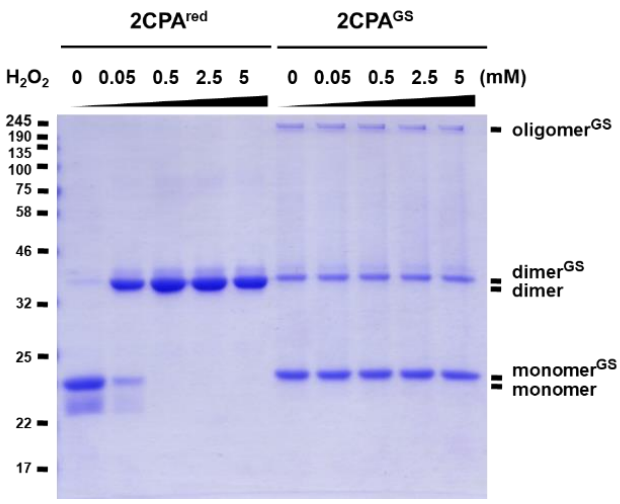

Figure 2.11: Effect of overoxidation on $2CPA^{GS}$. Upto 5 mM H_2O_2 was subjected on $2CPA^{red}$ and $2CPA^{GS}$. All reactions (oxidation and GSH-glutathionylation) were carried out for 10 min, and resulted proteins were separated in nonreducing SDS/PAGE. Gels were stained with Coomassie Brilliant Blue, and MWS were indicated in the left of gels.

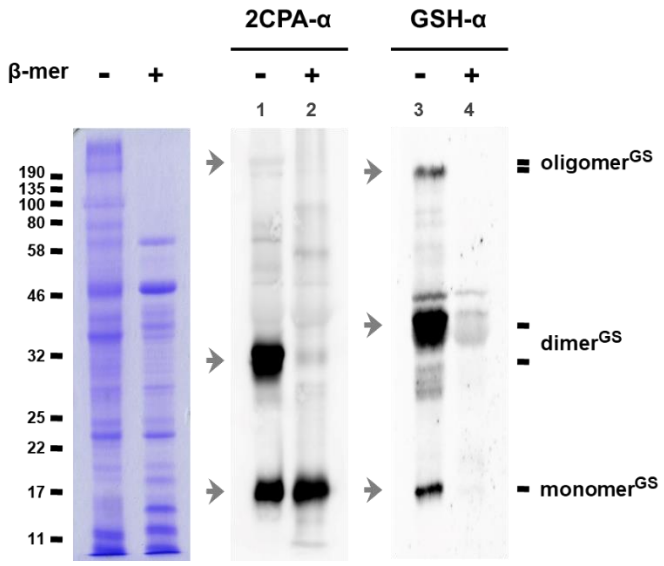

Figure 2.12: 2CPs are S-glutathionylated in planta. Western blot assays detecting the quaternary structures mono-, di- and decamers in planta of 2CPs, in WT (Col-0) plants. Equal amounts of protein extracted from plants were exposed to nonreducing (-β-mercaptoethanol; -β-mer) or reducing (+β-mer) SDS/PAGE (Left), and analyzed by WB using a polyclonal anti-2CPA antibody (2CPA-α, Middle), and a monoclonal anti-GSH antibody (GSH-α, Right).

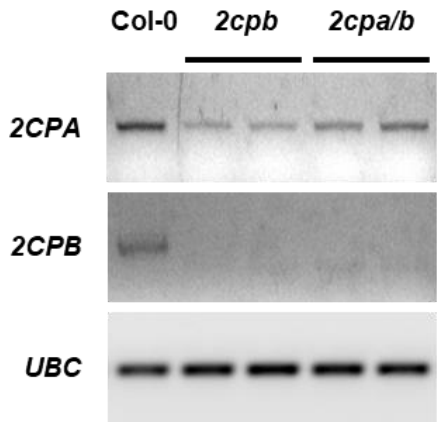

Figure 2.13: Both 2cpb and 2cpa/b attenuate the level expression of 2CPA. Semiquantitative RT-PCR analyses of 2CPA and 2CPB in WT (Col-0), 2cpb and 2cpa/b mutant plants. Total RNAs were prepared from the leaves of each plant, and transcript levels of POLYUBIQUITIN (UBC) were used as an equal loading control.

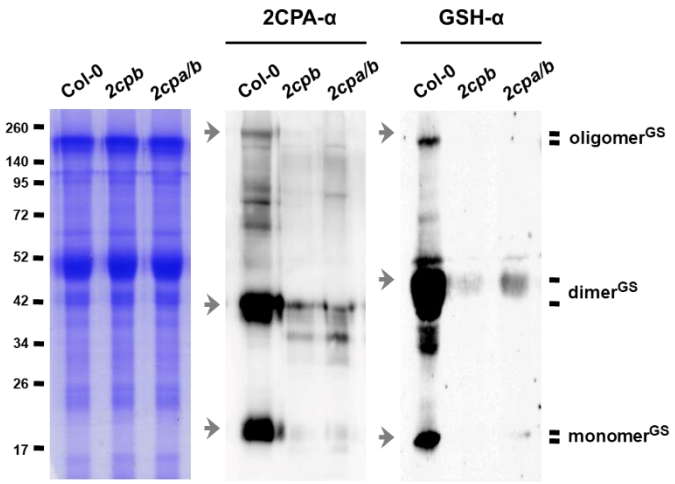

Figure 2.14: In-vivo WB assays in *2CPB* KO mutant plants. In-vivo WB assays show the quaternary structure of 2CPs, and proteins^{GS} in *2CPB* KO mutant (*2cpb* and *2cpa/b*) plants. Equal amounts of total protein extracts were separated in nonreducing SDS/PAGE (Left), and WB analyzed by using 2CPA-α (Middle) and GSH-α (Right). Grey arrows indicate three major bands corresponding to the molecular sizes of mono-, di and decameric 2CPs. All proteins were separated in nonreducing SDS/PAGE, and gels were stained with Coomassie Brilliant Blue. MWS were indicated in the left of gels.

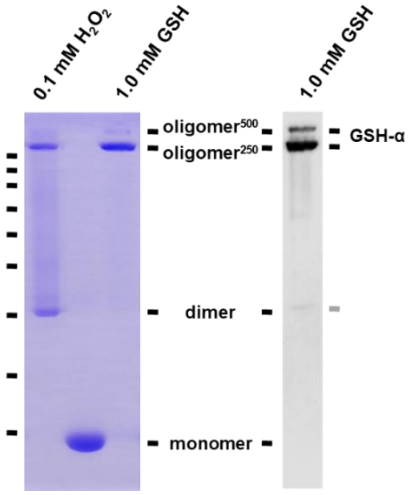

Figure 2.15: GSH decamerizes and shows chaperone function of 2CPB. Oligomerization of 2CPB by GSH-glutathionylation. 1 μM of 2CPB, incubated with 0.1 mM H_2O_2, 20 mM TCEP-HCl or 1.0 mM GSH, was separated in nonreducing SDS/PAGE (Left), and its GSH-binding was probed by WB using a monoclonal anti-GSH antibody (Right).

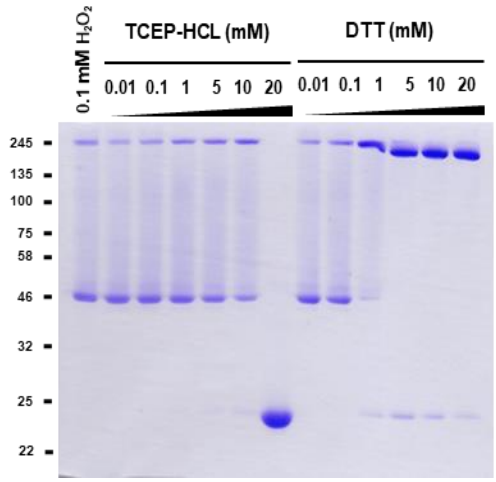

Figure 2.16: Changes in the quaternary structure of 2CPB^{ox} with increased concentration of TCEP-HCl. Upon incubation with increased concentrations (0 to 20 mM) of reducing agents, tri(2-caboxyethyl)phosphine hydrochloride (TCEP-HCl, Left) and dithiothreitol (DTT, Right) at 25 ^{o}C for 30 min, there is change in structure of 2CPB protein.

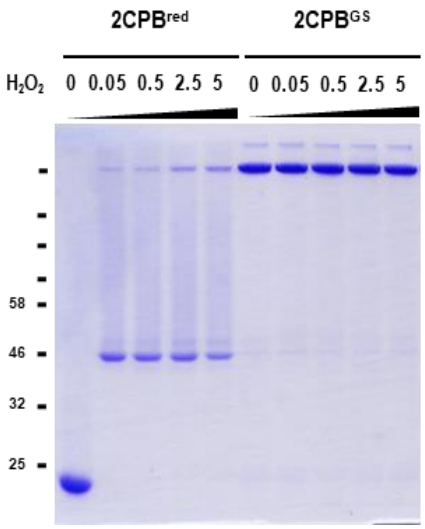

Figure 2.17: Effects of overoxidation on 2CPB. Overoxidation up to 5 mM H_2O_2 on the quaternary structure of $2CPB^{red}$ and $2CPB^{GS}$. $2CPB^{red}$and $2CPB^{GS}$were incubated with H_2O_2 (25 ^{o}C, 10 min).

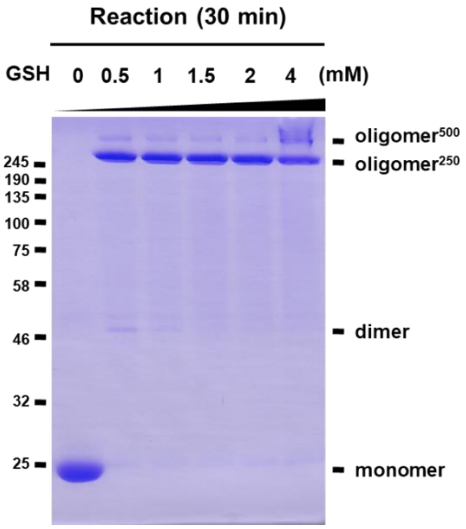

Figure 2.18: GSH concentration-dependent (0 to 4 mM for 30 min), glutathionylation of 2CPB^{red}.

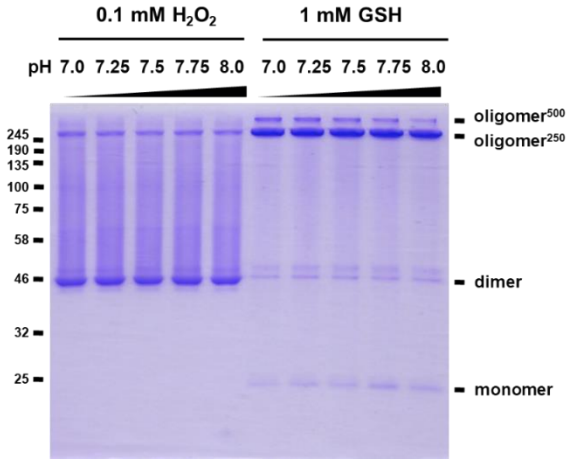

Figure 2.19: Effects of pH change (7.0 to 8.0) on the oxidation (0.1 mM H_2O_2) on 2CPB.

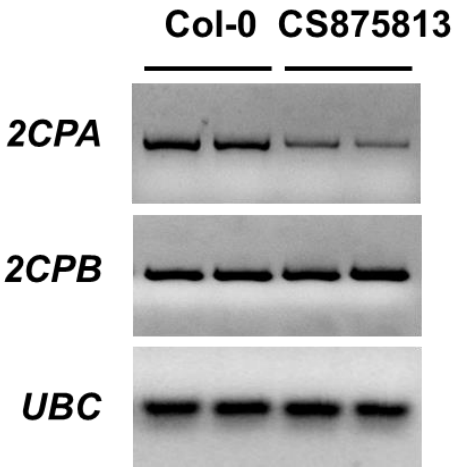

Figure 2.20: Semiquantitative RT-PCR analyses of 2CPs in WT (Col-0) and CS871813(2CPA KD) mutant plants. Transcript levels of UBC [26] were used as an equal loading control.

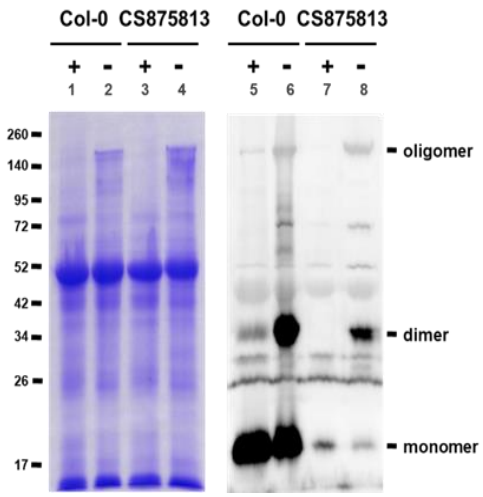

Figure 2.21: In-vivo WB assays surveying the quaternary structure of 2CPs between WT (Col-0) and CS875813 (*2CPA* KD) mutant plants. Equal amounts of extracted protein were subjected to nonreducing (- β-mer) or reducing (+ β-mer) SDS/PAGE (Left) and analyzed by WB using a polyclonal anti-2CPA antibody (Right). All proteins were separated in nonreducing SDS/PAGE, and gels were stained with Coomassie Brilliant Blue.

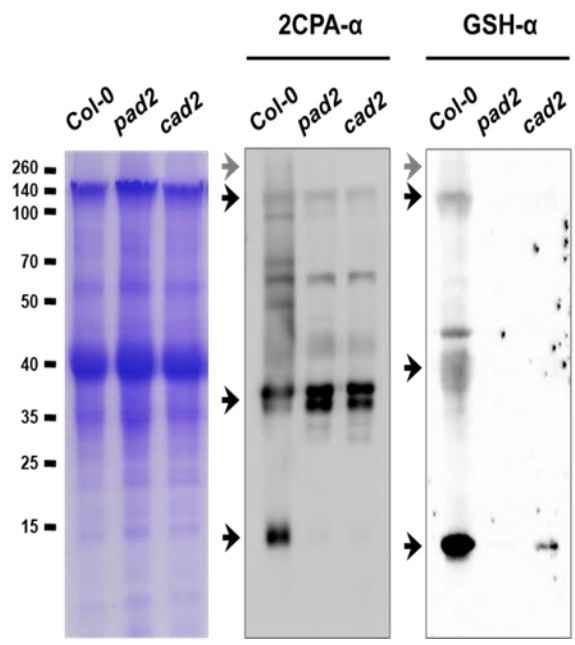

Figure 2.22: In-vivo WB assays comparing quaternary structures of $2CPs^{GS}$ between WT (Col-0) and GSH-imbalanced mutant (*pad2* and *cad2*) plants. Equal amounts of total protein extracts were subjected to nonreducing SDS/PAGE (Left) and analyzed by WB using a polyclonal anti-2CPA antibody (2CPA-α, Middle), and a monoclonal anti-GSH antibody (GSH-α, Right). Black arrows indicate three major bands corresponding to the molecular sizes of mono-, di- and decameric 2CPs, and grey arrows point the molecular size of icosameric 2CPs.

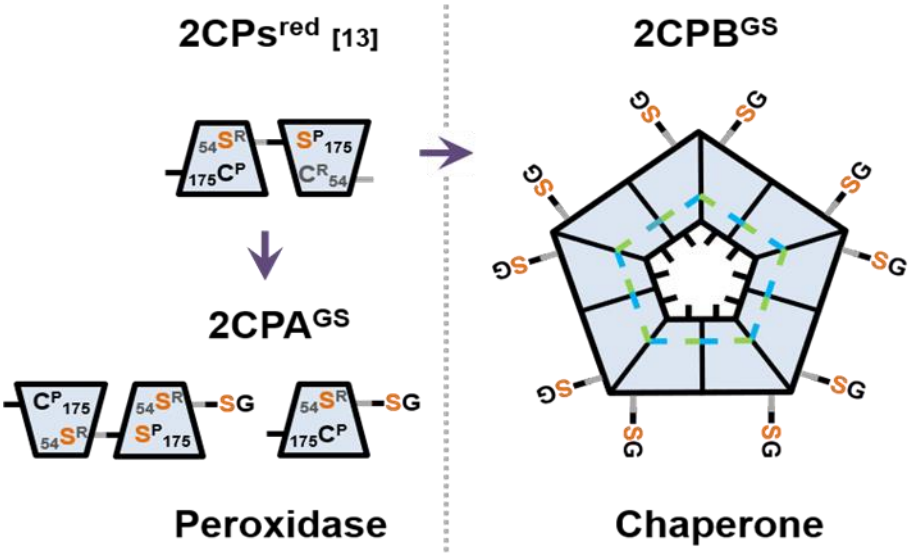

Figure 2.23: Structural and functional modifications of 2CPs by GSH-glutathionylation. Until recently, reduced 2CPA and 2CPB ($2CPs^{red}$) both have reported to form dimers, show peroxidase activity (Left top, [13]). However, the Cys $(C)^{R}_{54}$ of $2CPs^{red/ox}$ is cross-linked with GSH (GSH-glutathionylation; purple arrows), monomerizes and ensures the peroxidase activity of $2CPA^{GS}$ (Left bottom), whereas decamerizing and enhancing the chaperone activity of $2CPB^{GS}$ (Right).

Chapter 3

Posttranslational modification by a reduced glutathione determines the structure and function of 2-cysteine peroxiredoxins

3.1. Abstract

As sessile organisms, plants cope with constant encounters with a wide range of biotic consumers by mobilizing the intricate signaling network of small hormone molecules, which orchestrates large-scale expression of defense genes. In particular, (+)-12-oxo-phytodienoic acid (OPDA) hormone coordinates the signaling of defense activations against diseases and injuries caused by a diverse array of necrotrophic microbial pathogens and insect herbivores. OPDA binds and activates cyclophilin 20-3 (CYP20-3), a regulatory hub between redox-defense signaling and 2-cysteine peroxiredoxins (2CPs)-mediated photosynthetic pathways. The goal of this research was to assess (**a**) if OPDA signaling enhances an electron transfer (allocates chemical energy) from photosystem via thioredoxins (TRX) to CYP20-3 in sulfur assimilation (defense responses) and (**b**) if OPDA signaling stimulates CYP20-3-mediated reduction of 2CPA. Our in vitro protein analysis and ex vivo western blot analysis showed that OPDA signaling is able to enhance electron transfer from TRX to CYP20-3. Also, CYP20-3 deglutathionylates $2CPA^{GS}$, reducing (cleaving) a disulphide bond between glutathione (GSH) and a cysteine residue of proteins. Thus, the reductase activity of CYP20-3 results in the inactivation of $2CPA^{GS}$, but not the activation of 2CPA.

3.2. Introduction

Oxylipins, the oxygenated derivatives of fatty acids (FA), are important signaling molecules in a number of physiological processes in life. In plants, oxylipins are involved in a layer of defense and ontogenetic pathways, while oxylipins in mammals (eicosanoids) control complex regulatory mechanisms in immunity, functioning as messengers in the central nervous system and

participating in the resolution process following tissue injury. In addition, recent studies have shown the medicinal values of plant oxylipins, presenting their anticancer and anti-inflammatory activities. Noticeably, the molecular components and metabolic pathways, which are involved in oxylipin biogenesis and signaling, share common ancestry and evolutionary processes across kingdoms. Hence, uncovering the modes of actions associated with oxylipins will not only help in the development of agricultural strategies in enhancing disease resistance and stress adaptation, as well as yield and biomass increases in plants, but also the improvement of drug development through facilitating the rational design of more potential and safer anti-cancer (and anti-inflammation) drugs. However, our current knowledge regarding oxylipin signaling is still incomplete, despite decades of investigations [1,2,3,4,5].

Jasmonic acid (JA) is a well-characterized member of jasmonate family. It was first isolated from the culture of fungus *Lasiodiplodia theobromae* [6,7]. Jasmonate biosynthesis is initiated at the chloroplast by degradation of a lipid, linolenic acid, which results in the production of (+)-12-oxo-phytodi-enoic acid (OPDA) that moves to the peroxisome, where it is beta-oxidized to JA. JA is then released to cytosol and derivatized to methyl-JA and jasmonate isoleucine (JA-Ile) [8,9], which are involved in a number of physiological processes in plants [9]. They also confer a broad range of defense against wide range of stresses including nectrotrophic pathogens, mostly fungi, insects, as well as abiotic stresses such as UV damage [10,11].

JA, produced in the peroxisome, has long been believed to be an active molecule, but recent studies show that JA-Ile and OPDA are actually, two biologically active signaling molecules [12,13]. In particular, OPDA triggers signaling transduction pathways that activates a subset of jasmonate-responsive genes (i.e. ORGs), actuating defense responses and growth processes in plants. OPDA produced in the chloroplast binds cyclophilin 20-3 (CYP20-3). This binding causes CYP20–3 to

form a complex with serine acetyltransferase1 (SAT1), which causes the formation of Cysteine (Cys) synthase complex (CSC) with O-acetylserine (thiol)lyase B. CSC then produces Cys and in turn thiol metabolites, increasing the reduction potential of the cells which helps in expressing a number of ORGs that activates defense responses against pathogen infections and abiotic stresses [14,15].

CYPs belong to the family of peptidyl-prolyl cis-trans isomerases (PPIases). In Arabidopsis, 29 CYPs and CYP-like proteins have been found, and their transcriptomic analyses have suggested five of them are involved in stress defense processes [15,16, 17]. CYP20-3 is located in the stroma of the chloroplast. It is a single-domain isoform of CYP. It is encoded by the nuclear genome and functions in a number of physiological processes that include defense responses, signaling pathways of hormones, transcriptional regulation and adaptation to various environmental stresses like heat, cold, salt and water stresses. CYP20-3 is bifunctional enzymes conferring PPIase and reductase activities. These enable CYP20-3 to interact with various enzymes such as thioredoxins (TRX) and 2-cysteine peroxiredoxins (2CPs) in the light-dependent redox reactions (also known as an electron transport chain; ETC) of photosystem I. As alluded, CYP 20-3 also plays an important role in Cys biosynthesis taking place in the stroma of chloroplasts [17].

The *CYP20-3* gene is only expressed in the photosynthetic tissues of plants, where are induced by light. Analysis of Arabidopsis mutants have further helped understand the intrinsic activities of CYP20-3. These studies showed that when *cyp20-3* mutant plants and wild-type (WT) plants were grown in normal conditions, they didn't exhibit any phenotypic difference. But when they were grown under stress conditions *cyp20-3* mutant plants showed hypersensitivity to high light intensity that cause damage to the plants by producing reactive oxygen species (ROS). There was

inhibition in the growth of both WT and *cyp20-3* mutant plants when they were grown in increased salt concentrations or osmotic shock, but *cyp20-3* mutants suffered the most [17].

TRXs are small redox proteins (12 to 14 kDa) and present across all organisms. They are disulfide oxidoreductases, meaning that they transfer electron and restructure the target proteins, that play vital roles in various physiological processes including photosynthesis, growth, flowering and seed germination. Chloroplasts of *Arabidopsis* contain more than 10 isoforms of Trx that are broadly divided into five types (f, m, x, y and z) [18]. These isoforms cause the activation of Calvin cycle enzymes such as fructose 1,6-bisphosphatase [19]. Although they share little homology in their sequence, they are often redundant in functional and biochemical properties. All plastid Trxs have binding affinity to CYP20-3. Moreover, some of these interactions like CYP20-3 with Trx-f2, -m1 or -m4 is promoted by OPDA [16,20,21].

2CPs are peroxidase enzymes with the size of 20 kDa, and also are located in the chloroplasts. There are 2 isoforms of 2CPs in Arabidopsis chloroplast, namely A and B. These 2 proteins share 96.5% amino acid sequence identity; there are just 7 amino acids differences. Until now, they have been considered as redundant enzymes [22].

Photosynthesis accumulates toxic compounds, H_2O_2, as a byproduct, which is then detoxified by the peroxidase activity of 2CPs [23,24]. As per their name sake, 2CPs have 2 Cys residues at position 53 and 175. They form dimers through the formation of disulfide bonds. However, when 2CPs are overoxidized, they are known to become decamers, an association of 10 subunits. Changes in the quaternary structure of 2CPs are associated with their functions. Monomers have peroxidase (reductase) activity, dimers are the inactive form while decamers function as chaperone

proteins (but not peroxidase). In their basal state, they are dimers, inactive structures. When they accumulate as dimers, they should be reduced to activate their peroxidase activity [23].

So, when 2CPs assemble as inactive dimers they need to be reduced or recharged to reactivate as peroxidase. When reduced by reductases like CYP20-3, 2CPs metabolize the detoxification of a toxic byproduct in photosynthesis (i.e. H_2O_2) and the activation of Calvin cycle enzymes such as fructose 1,6-bisphosphate, enhancing photosynthetic pathways (energy production).

3.3. Objectives

The main objective of this research was to delineate whether CYP20-3-dependent OPDA signaling regulates energy (electron) transfer from photosystem (sources) to defense activation (sulfur assimilation; i.e. if OPDA signaling stimulates TRX-mediated activation of CYP20-3, which in turn activates 2CPs to detoxify peroxide byproducts and control carbon metabolism in photosynthesis. This main objective was achieved by accomplishing two specific objectives mentioned below.

3.3.1. Objective 1

To assess if OPDA signaling enhances an electron transfer from TRX to CYP20-3; i.e. activation of mode of CYP20-3.

3.3.2. Objective 2

To determine if OPDA signaling stimulates CYP20-3-mediated reduction of 2-CPA; i.e. activating the mode of 2CPA, or peroxide detoxification.

3.4. Materials and methods

3.4.1. Cloning, Expression and Purification of Proteins

Recombinant proteins were prepared according to the previous description in the literature. CYP 20-3 was cloned into pET28a vector (Novagen) and N-terminus 6x HIS tagged proteins were obtained. These recombinant proteins were expressed in *E. coli* BL21(DE3) and purified using a nickel- (Ni-NTA, Qiagen. 2CPA (At3g11630) was provided by Prof. K. J. Dietz (University of Bielefeld, Germany).

3.4.2. Plant Materials

A. thaliana WT plants (Col-0) and *TRX* mutants were used in this study. TRX mutants were provided by Dr. Francisco Javier Cejudo. Plants were grown in a growth chamber with a 12-h day cycle at 22 ^{o}C.

3.4.3. Total Protein Extraction

Leaves of Arabidopsis were harvested and immediately kept in liquid N_2 and were made to powder form with the use of mortar and pestle. Powdered sample was kept in two volumes of 50 mM potassium phosphate buffer (pH 7.2) with protease inhibitor cocktails (Sigma-Aldrich). Agitation was done for 60 min, and centrifugation was carried out for 30 min at the speed of 10,000*g*. Supernatant after the centrifugation step was collected, and was subjected to western blot. Extraction steps in all our experiments were carried out at the temperature of 4 °C.

3.4.4. In vivo Western Blot Analysis

To study interaction of OPDA, TRX and CYP20-3 in in-vivo condition, the prepared protein samples were run in 13.5% SDS/PAGE and electroblotted onto PVDF membranes (Millipore). The resulting blots were probed with polyclonal rabbit anti-2CYP20-3 antibody (1:7500,

MyBioSource) for 2 h, or monoclonal mouse anti-GSH antibody (1:3000, Enzo Life Science) for 16 h, and visualized by chemiluminescence (ECL kit; GE Healthcare). *Arabidopsis thaliana* WT plants (Col-0) and Trx mutants were used in this study, Plants were grown in a chamber with a 12-h day cycle (80-100 μmol photons m^{-2} s^{-1}) at 22 ^{o}C and 60% to 80% relative humidity. 4 weeks old plants were subjected to wounding to initiate OPDA signaling and protein was extracted at 0, 3 and 6 hours of wounding and were immediately used for western blot analyses. All extraction steps were carried out at 4 °C.

3.5. Results

3.5.1. OPDA signaling is able to enhance electron transfer from TRX to CYP20-3 (activation of CYP20-3) in in vitro conditions.

To see the interaction between OPDA, TRX and CYP20-3, we performed an in vitro redox-shift gel-assay (figure 3.1). We incubated CYP20-3 with TRX in the absence (lane 2) or presence of OPDA (lane 3 and 4) and separated by non-reducing SDS/PAGE. As we increased the concentration of OPDA from 1 to 5 µM, CYP20-3 became reduced, suggesting that **a**) TRX is able to reduce CYP20-3 and **b**) this reduction is further enhanced with the increasing concentrations of OPDA.

3.5.2. OPDA signaling is able to enhance electron transfer from TRX to CYP20-3 (activation of CYP20-3) in ex vivo conditions.

Upon wounding in WT plants, we detected the clear reduction of CYP20-3, which is indicated by reduced its mobility in nonreducing SDS-PAGE (figure 3.2). However, *trx* double KO mutants did not show any change in CYP20-3's mobility in nonreducing SDS-PAGE after wounding, further validating that TRXs and OPDA are essential to activate CYP20-3 during defense activation processes (figure 3.2).

3.5.3. CYP20-3 deglutathonylates 2-CysPrxA^{GS}; reducing a disulfide bond between GSH and 2CPs

Our redox-shifting assay (figure 3.3) displayed that CYP20-3 is able to convert monomeric 2CPAs ^{GS} to dimers (left panel). When incubated, CYP20-3 was able to reduce a mixed GS-S bond (i.e. deglutathionylation), and led 2CPA^{GS} to form an obligatory dimer (right panel). Thus, the reductase activity of CYP20-3 results in the inactivation of 2CPA^{GS}, but not the activation of 2CPA.

3.6. Conclusion

Plants optimize their fitness against a wide range of environmental stresses by employing intricate signaling network. To better understand whether CYP20-3-dependent OPDA signaling regulates electron transfer from photosystem to defense activation and to access the interaction of OPDA with TRX and CYP20-3, series of in vitro and ex vivo analyses were performed. We found that OPDA signaling enhances an electron transfer from TRX to CYP20-3; i.e. activation of mode of CYP20-3. Thus, CYP20-3 relays OPDA signal during stress responsive regulation of cellular redox homeostasis. Although we tested multiple conditions, we were not able to validate that CYP 20-3 can reduce 2CPs. In the meantime, we found that CYP20-3 deglutathionylates 2CPA^{GS}, reducing a disulphide bond between GSH and 2CP (not between 2CP proteins). Thus, the reductase activity of CYP20-3 results in the inactivation of 2-CysPrxA^{GS}, but not the activation of 2CPA.

3.7. References

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3.8. Figures

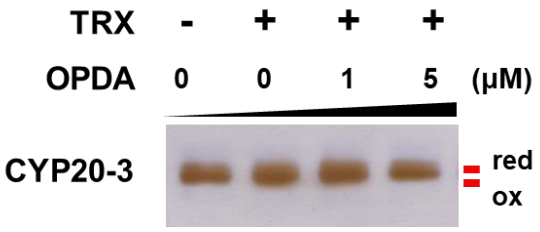

Figure 3.1: Interaction of OPDA, Trx and CYP20-3. Trx is able to reduce CYP20-3 and this reduction is further enhanced with increasing concentration of OPDA. In in vitro condition, CYP20-3 were subjected to different concentrations of OPDA (0-5 µM) and were separated by nonreducing SDS/PAGE and were analyzed using silver staining.

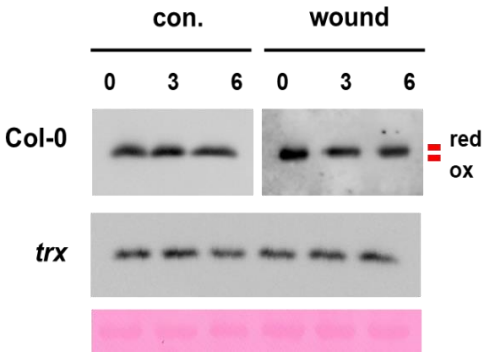

Figure 3.2: OPDA mediated reduction of CYP 20-3 by Trx. Trx is able to reduce CYP20-3 and this reduction is further enhanced with increasing concentration of OPDA. Arabidopsis WT plants (Col-0) and *trx* KO mutants were used in this study, plants were grown in a chamber with a 12-h day cycle (80-100 μmol photons m^{-2} s^{-1}) at 22 ºC and 60% to 80% relative humidity. 4 weeks old plants were subjected to wounding to initiate OPDA signaling and protein was extracted at 0, 3 and 6 hours of wounding and were immediately used for western blot analyses. All extraction steps were carried out at 4 °C.

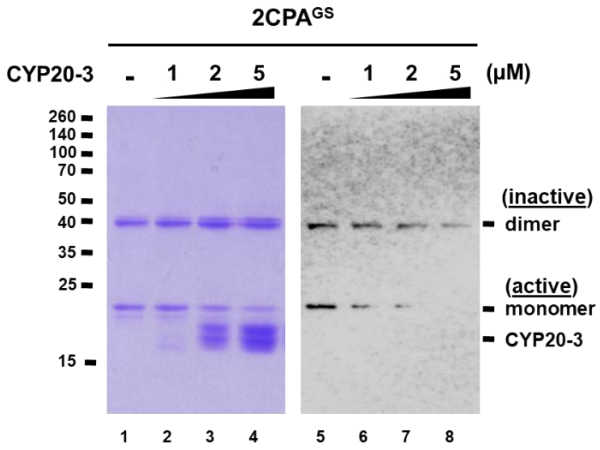

Figure 3.3: Interaction of 2CPA (GS) and CYP20-3. CYP20-3 deglutathionylates $2CPA^{GS}$ reducing a disulphide bond between GSH and 2CP (not between two 2CP proteins). $2CPA^{GS}$ were incubated with various concentrations of CYP20-3 (0–5 μM) and were subjected to nonreducing SDS/PAGE and probed by western blot analyses using a monoclonal anti-GSH antibody (right panel).