CHARACTERIZING A NEW ACTIVITY OF GLUTATHIONE IN THE REGULATION OF REDOX METABOLIC PATHWAYS.

by

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ABSTRACT

As sessile organisms, plants employ an intricate signaling network to interconnect and orchestrate multiple layers of defense mechanism to overcome environmental stresses. In this sense, cyclophilins (CYPs) belong to a peptidyl-prolyl *cis-trans* isomerase family are known to be key players on regulating growth and defense on plants. CYPs are found across all subcellular compartments and are involved in various physiological processes including transcriptional regulation, organogenesis, photosynthetic and hormone signaling pathways, stress adaptation and defense responses. These important and diverse activities of CYPs must be reflected by their versatility as cellular and molecular modulators. In the chloroplast, for instance, CYP20-3 is a regulatory hub that activates defense by interaction with SAT1, but also growth by interaction 2-Cys peroxiredoxins (2CPs) proteins. Therefore, the overall goal of this study was to investigate the interaction of plants CYP20-3 with peroxiredoxins proteins. As a result, it was found that under heat shock conditions CYP20-3 inhibit 2CPs. In addition to that, we found that 2CPs conformation is regulated by a post-translation modification, named S-glutathionylation, that great changes 2CPs metabolic pathway.

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1.1. ABSTRACT

Cyclophilins (CYPs) belong to a peptidyl-prolyl *cis-trans* isomerase family, and were first characterized in mammals as a target of an immunosuppressive drug, cyclosporin A, preventing proinflammatory cytokine production. In *Arabidopsis*, 29 CYPs and CYP-like proteins are found across all subcellular compartments, involved in various physiological processes including transcriptional regulation, organogenesis, photosynthetic and hormone signaling pathways, stress adaptation and defense responses. These important but diverse activities of CYPs must be reflected by their versatility as cellular and molecular modulators. However, our current knowledge regarding their mode of actions is still far from complete. This review will briefly revisit recent progresses on the roles and mechanisms of CYPs in *Arabidopsis* studies, and information gaps within, which help understanding the phenotypic and environmental plasticity of plants.

Keywords: Cyclophilin; PPlases; *Arabidopsis*; organogenesis; defense responses

1.2. INTRODUCTION

Cyclophilins (CYPs) are members of, namely, immunophilins that possess binding abilities towards immunosuppressive drugs such as cyclosporine A (CsA), FK506 and rapamycin. CsA binds to a group of CYP proteins, and FK506 and rapamycin bind to a distinct set of receptors, called FKBPs (FK506 and rapamycin-binding proteins), of which complexes (CsA-CYPs or FK506-FKBPs) inhibit nuclear translocation of NF-AT (nuclear factor of activated T-cells), and

prevent the release of a proinflammatory cytokine, interleukin-2, and subsequent activation of immune responses, engendering immunosuppressive effects. Both CYPs and FKBPs exhibit a characteristic peptidyl-prolyl *cis-trans* isomerase (PPlase) activity which catalyzes the rotation of X-Pro peptide bonds from a *cis*to *trans* conformation, a rate-limiting step in protein folding or the assembly of protein complexes, tuning the roles and activities of a wide variety of proteins containing *trans*-prolyl imide bonds [1,2]. Note that *cis*-prolyl bonds are uncommon, most likely because of unfavorable contacts between adjacent amino acid residues in this isomeric form [3]. These post-translational modifications in turn coordinate a layer of primary and secondary metabolic pathways in diverse cellular processes. Hence, alteration of immunophilins' functions and expressions render not only the loss of innate immunity, but also various diseases such as cancer, neurodegeneration, diabetes, asthma, rheumatoid arthritis, and cardiovascular, Parkinson's and Alzheimer's diseases, urging us to revisit their potential importance as drugs targets and pharmacological uses [1,2,3,4,5].

CYPs are structurally and evolutionally conserved PPIases found in all types of life including mammals, plants, insects, fungi and bacteria. They are categorized as single- and multi-domain PPlases; single-domain CYPs encode only a catalytic (PPlase) domain, referred to as CYP-like domain (CLD), whereas multi-domain CYPs include additional domains—in general—involved in protein and protein, or protein and nucleic acid interactions such as WD40 repeat, tetratricopeptide repeat, U-box, RNA recognition motif, Zn-finger, α-helical bundle, Leu-zipper, Ser/Lys and/or Arg/Glu-rich domains [1,2,6,7]. Reportedly, all CLD shares a common folding architecture consisting of eight antiparallel β-sheets, capped by three α-helices [1,2,3,6]. The second α-helix, placed between the β6 and β7 loop region, possesses an active site residue, Trp;

the most conserved and critical amino acid (aa) for both catalytic and substrate/inhibitor binding activities. Shifting of the Trp to Ala or Phe showed a negative impact on PPIase activity and CsA-binding affinity [1,7,8,9,10]. In addition, three catalytic aa residues (Arg, Phe and His) are found across β3, β4 and β7 sheets which form a so-called 'active pocket' and facilitate the substrate bindings and metabolisms [1,7,11,12].

In plants, CYPs were first isolated in 1990 concomitantly from tomato (*Lycopersicon esculentum*), maize (*Zea mays*) and oilseed rape (*Brassica napus*) [13]. Since then, major efforts have been made to identify and characterize CYPs from *Arabidopsis*, a model plant system (designated as AtCYPs) [6,14,15,16,17,18,19,20]. In particular, two pioneering studies carrying out the comprehensive analyses of *Arabidopsis* genomics databases revealed 29 AtCYPs and CYP-like proteins. The surprisingly large number of AtCYPs along with their ubiquitous localizations across all subcellular compartments and widespread expressions throughout all major organs (e.g., flowers, leaves, stems and roots; except a specific expression of AtCYP26-1 in flowers) proposed that CYPs' activities must be intrinsic in the growth and survival of *Arabidopsis* [1,7,21]. In agreement, several studies have unveiled the putative substrates, interacting partners, as well as biochemical and physiological activities of AtCYPs, corroborating the multifaceted roles of AtCYPs in broad ranges of cellular processes including transcriptional regulation, organogenesis, photosynthetic and hormone signaling pathways, stress adaptation and defense responses [22]. Now, this review will revisit the recent advances and working models of the functional circuitry of AtCYPs, and information gaps within, in effort to further understand the versatile activities of plant CYPs, and help delineating the phenotypic and environmental plasticity of plants.

1.3. ACTIVITIES OF CYCLOPHILINS IN PLANT GROWTH AND DEVELOPMENT

Recently, emerging evidences have elucidated that CYPs are important regulators in various metabolic pathways controlling organellar housekeeping, temporal and spatial specific metabolisms, as well as organismal development and growth in plants (Figure 1) [1,7,22]. These roles and activities of CYPs must be closely associated with their subcellular localizations. Especially, most CYPs share the same enzymatic (PPlase) activity and inhibitor (CsA), highlighting that their locations are the key limit factors of accessible substrates and interacting partners which in turn reflect their cellular activities and functions. This chapter thus will discuss recent advances in our understanding of CYPs in *Arabidopsis* growth and developmental processes in comparison with their subcellular locations.

1.3.1. NUCLEAR LOCALIZED AtCYPs

In *Arabidopsis*, four multi-domain AtCYPs (i.e., AtCYP59, AtCYP63, AtCYP71 and AtCYP95) were predicted to target the nucleus [1,7]. Among these, AtCYP63 and AtCYP95 harbor a C-terminal RS (Arg-Ser) rich domain, known to regulate protein and protein interactions in the formation of the spliceosomal complex and the activation of RNA polymerase II [1,23,24,25,26,27], suggesting their potential activities in RNA metabolisms [1]. In fact, their human counterparts such as SR-CYP, Matrin-CYP and hCYPH actually demonstrated binding affinity to a splicesomal snPNP complex and/or RNA polymerase II [28,29,30] (Figure 1).

AtCYP59 is another nuclear AtCYP that contains a C-terminus RS rich domain, along with an N-terminal CLD, an RNA recognition motif and a zinc finger domain. The RS rich domain enables AtCYP59 to interact with a number of SR proteins (e.g., SR28, SR33 and SR35)

involved in RNA splicing during various plant growth and developmental processes [31,32]. However, AtCYP59 appeared not to colocalize with those SR proteins in nuclear speckles, instead it showed a punctuate localization pattern resembling transcription initiation sites. In line with this scenario, in vitro protein and protein interaction assays exhibited the binding affinity of AtCYP59 to the nascent transcript of mRNA, as well as the C-terminal domain (CTD) of RNA polymerase II that is a binding platform of the transcription and splicing factors, and the nascent transcripts [31,33,34,35]. Perhaps, PPIase activity of AtCYP59 can modulate the structure and phosphorylation states of CTD, which in turn controls the transcription of selective mRNA associated with cell growth and development [31]. However, mRNA-binding spontaneously inhibits the PPlase activity of AtCYP59 [35], further suggesting that AtCYP59 is positioned at the interface of splicing and transcription, perhaps tuning the elongation of RNA polymerase, from where they might translocate to the nascent transcripts to ensure efficient splicing, concomitant with transcription [28,35].

AtCYP71, a highly conserved eukaryotic CYP, is an important regulator of organogenesis in *Arabidopsis* (Figure 1). Disruption of *AtCYP71* mRNA hence demonstrated the drastic disfiguration of the shape and size of leave, as well as petioles, via upregulating the expressions of a class I *KNOTTED-like* homeobox (*KNOX*) gene family including *SHOOT MERISTERMLESS* (*STM*), *KNOTTED-1-LIKE 1/2* (*KNAT1/2*) and *ASYMMETRIC LEAVES 1/2* (*AS1/2*), which is required for the initiation and maintenance of the shot apical meristem (SAM) [36,37]. These results suggest that AtCYP71 regulates negatively, or fine-tunes the expression of *KNOX* family genes. Interestingly, an N-terminus region of AtCYP71 possesses WD40 repeat domains, interacting with histone H3, chromatin assembly factor-1 and like-

heterochromatin protein1, suggesting a potential role of AtCYP71 in the chromatin remodeling [37,38]. Histone modifications (i.e., methylation and acetylation) are lined to transcriptional controls. In particular, methylation of Lys^4 in H3 is associated with activation of gene expressions, whereas methylation of Lys^9 or Lys^{27} in H3 (H3K9/27) leads to gene silencing. In line with this scenario, AtCYP71 can binds and reinforces the methylation of H3K27 in the coding regions of e.g.,) *STM* and *KNAT1*, thereby maintaining the silenced state of those genes, and regulating proper SAM development in *Arabidopsis* [37].

1.3.2. CYTOSOL LOCALIZED AtCYPs

Screening a series of T-DNA insertion KO *Arabidopsis* mutants has disclosed that AtCYP40 plays intrinsic roles in the organogenesis of plants [20,37]. AtCYP40 is a unique multi-domain AtCYP, containing tetratricopeptide repeat domains that are able to bind ARGONAUTE 1 (AGO1) and HSP90 in the formation of an intermediate assembly of RNA-induced silencing complex (RISC) [39,40]. RISC is an effector complex of post-transcriptional gene silencing (PTGS), consisting of a single-stranded (ss) small RNA such as small interfering RNA (siRNA) and microRNA (miRNA) that is bound to an AGO family protein, which prevents the production of proteins from mRNAs that contain sequences complementary to the ss small RNAs, through cleavage or translational repression [41]. In this system, AtCYP40 promotes binding of AGO1 with a molecular chaperon, HSP90, to facilitate RISC assembly via an ATP-dependent chaperone cycle [40,42], which in turn stimulates the production of miRNAs (e.g., miR156) [39]. Constitutive expression of miR156 then prolongs the juvenile phase of vegetative development and increases the rate of leaf initiation [43,44,45]. Thus, *AtCYP40* KO mutant plants showed an

alteration of leaf numbers, leading to a precocious expression of adult vegetative traits without induction of the reproductive maturation of shoots [20,39].

Two single-domain, AtCYP18-3 and AtCYP19-1 are highly homologous (95% aa sequence similarity) AtCYPs, but have displayed distinctive activates. Firstly, AtCYP18-3 is a multi-functional protein involved in plant growth, hormone signaling, and defense responses against biotic and abiotic stresses [46,47,48,49,50,51,52]. In the context of plant growth, AtCYP18-3 is positioned at the interface between light and brassinosteroid (BR) signaling pathways. BR signal antagonizes light-dependent seedling development, switching etiolation to de-etiolation by inhibiting cell elongation and promoting chloroplast development [23]. Hence, partial loss-of-function *AtCYP18-3*alleles displayed elevated sensitivity to BR in the light, which subsequently arrested de-etiolation processes (photomorphogenesis) [50]. On the other hand, AtCYP19-1 is considered to control seed development as the promoter trapping detected its expressions predominantly in the peripheral endosperm and in the late heart stage of embryo development [53]. However, earlier RNA blotting assays argued that AtCYP19-1 is expressed also in seedlings, stems and leaves of *Arabidopsis* [15], suggesting that it acts in diverse physiological functions beyond seed organogenesis (e.g., immune responses; see Section 3) (Figure 1).

1.3.3. CHLOROPLAST LOCALIZED AtCYPs

Arabidopsis chloroplasts include five AtCYPs in the thylakoid lumen (i.e., AtCYP20-2, AtCYP26-2, AtCYP28, AtCYP37 and AtCYP38) and one in the stroma (AtCYP20-3), of which AtCYP20-2 and AtCYP38 showed the functional involvements in the assembly and maintenance of photosystem (PS) components [54,55,56] (Figure 1). For instance, AtCYP20-2 showed a

physical association with thylakoid membrane-embedded NAD(P)H dehydrogenase (NDH) complexes that mediates cyclic electron (e^-) transport in photosynthesis, and chlororespiration [54,57]. In fact, the level accumulations of AtCYP20-2 were strongly reduced in *NDH*-defective mutant plants, suggesting that its functions as an auxiliary protein in the biogenesis of NDH complexes [54,58]. Besides, *AtCYP38*-deficient mutants exhibited significant reduction of the biogenesis and the half-life of PSII complexes, which in turn rendered PSII centers extremely susceptible to photoinhibition [55,59], indicating that AtCYP38 is necessary for the assembly of PSII and stabilization of light-dependent reactions of photosynthesis. It is worth nothing that a recent report has revealed that AtCYP20-2 can also bind and stimulate a BR signaling component, a BRASSINAZOLE RESISTNAT1 (BZR1) transcription factor (TF) in activating the expression of *FLOWERING LOCUS D* and promoting early flowering [56]. However, further studies are necessary to define (a) how plastidic AtCYP20-2 can interact with nuclear BZR1 TF, and (b) if expressions of *AtCYP20-2* are differentially regulated in leaf plastids (perhaps constitutive) vs. flowers (temporal)—if so, how?

1.3.4. ENDOPLASMIC RETICULUM (ER) Localized AtCYPs

Thus far, the TargetP has identified that five single-domain AtCYPs (i.e., AtCYP19-4, AtCYP20-1, AtCYP21-1, AtCYP21-2 and AtCYP23) are located to the endoplasmic reticulum (ER), a protein secretory pathway [1,7,60,61] (Figure 1). Indeed, the subcellular distribution of green fluorescence proteins fused with a signal peptide of AtCYP19-4 confirmed the ER localization of AtCYP19-4, especially in the apical cells of young stem and peduncle tissues [17,62,63], where it can physically bind a GNOM protein. GNOM is an ADP ribosylation factor-guanine-nucleotide exchange factor, that fine-tunes vesicular formations in membrane

trafficking, and a cellular polarity along the apical-basal embryo axis control [17]. These interactions suggested that AtCYP19-4 may chaperone the activity of GNOM in the endosomal recycling of the auxin-efflux carrier PINFORMED1 to the basal plasma membrane in provascular cells, which in turn is required for the accumulation of the plant hormone auxin at the future apical meristems through polar auxin transport (Figure 1) [17,63].

AtCYP20-1 belongs to a family of unfolded protein response (*UPR*) genes, responsive to ER stress [64]. In this context, a promoter region of AtCYP20-1 contains a X-box binding protein 1 (XBP1). During ER stresses, an ER transmembrane protein kinase/riboendonuclease (Ire 1p) is activated and splices the mRNA of XBP1. Matured XBP1 is then translocated to the nucleus where it binds to and activates the *cis*-acting element of *AtCYP20-1*(Figure 1) [64,65]. Once expressed, AtCYP20-1 binds to PP2A, ubiquitous Ser/Thr protein phosphatase, that regulates multiple pathways in plant growth and defense responses [16,66]. In fact, T-DNA insertion KO mutant *Arabidopsis*of *AtCYP20-1* (*rcn1*) exhibited the drastic reduction of root and hypocotyl growth under ER stress, mimicked by toxic cantharidin treatments [16,67], suggesting that AtCYP20-1 play a critical role in proper protein synthesis and folding, as well as a removal of misfolded proteins during the life cycle of plants [68].

1.3.5. MITOCHONDRIA AND GOLGI LOCALIZED AtCYPs

Previously, two homologous AtCYP21-3 and AtCYP21-4 were predicted as mitochondrial AtCYPs [1,7]. A recent study, however, showed that AtCYP21-4 is likely localized at the Golgi apparatus [69] carrying out various post-translational modification processes including the glycosylation of proteins, producing glycoproteins (Figure 1) [70,71] . In plants, glycoproteins

play crucial roles in a variety of processes, e.g.,) forming cell wall matrixes, and optimizing morphogenesis under resting and stressed states [72,73]. Indeed, transgenic potatoes overexpressing *AtCYP21-4*demonstrated increased glycoprotein contents in all tissues, as well as higher yields (size and number of tubers), substantiating the intrinsic roles of AtCYP21-4 in plant growth and development via stimulating glycoprotein synthesis or glycan processing in the Golgi apparatus [69,74].

1.4. ROLES OF CYCLOPHILINS IN DEFENSE REPONSES AGAINST ABIOTIC STRESSES

Environmental stresses such as heat, cold, drought, salt and excess water are major limiting factors in plant growth and productivity. As sessile organisms, plants employ elaborate regulatory pathways that rapidly rearrange the temporal and spatial profiles of gene expressions in responding and adapting those abiotic stresses [75,76,77] . Over recent decades, a large number of studies have utilized various transcriptome and bioinformatics analyses to delineate the genetic and functional circuitry of plant stress defense responses [78]. Of these studies, five *AtCYP*transcripts were found to be stress responsive; the heat shock-dependent induction of cytosolic *AtCYP18-1*, cold-dependent upregulation of plastidic *AtCYP19-2*, salt-dependent induction of cytosolic *AtCYP18-3* and ER *AtCYP19-4*, salt-responsive downregulation of cytosolic *AtCYP18-4* [7,79,80], but further investigations are needed to understand their roles in plant stress physiology .

Intrinsic activities of AtCYPs in the activation of plant stress response machineries have been further substantiated by the analyses of *Arabidopsis* KO mutant plants. For instance, disruption

of *AtCYP20-3* and *AtCYP21-2* demonstrated enhanced hypersensitivity towards abiotic environmental stresses such as high light, oxidative, salt and/or water stresses [81,82]. Interestingly, expression of *AtCYP21-2* is highly upregulated during ER stresses that can be caused by various endogenous and exogenous stresses [64,65], suggesting that ER stress-responsive genes such as *UPR* genes play potentially important roles in a broad range of stress defense responses. However, the same states of ER stress did not induce the other *UPR, AtCYP20-1* (Section 2.4), discerning *AtCYP21-2* as a defense responsive gene while *AtCYP20-1* as plant growth regulators. Besides, ER stress showed little effect on *AtCYP19-4* transcripts, but salt stress caused the moderate level increases (~2-folds) in *AtCYP19-4* mRNA [7,82]. As alluded, AtCYP19-4 is involved in, unlike AtCYP21-2, the regulation of ER-mediated secretory system (Figure 1), perhaps explaining the need and roles of distinct metabolic pathways for the resolution processes of comparatively reverse stresses (i.e., salt/drought vs. excess water stresses). Note that AtCYP20-3 is the best-characterized AtCYP, and we will discuss its possible mode of actions during stressed and resting states in the Section 4 and Section 5.

1.5. ROLES OF CYCLOPHILINS IN DISEASE RESISTANCE AGAINST PATHOGEN INFECTIONS

To understand the potential roles of AtCYPs in the plant and microbe interactions, two recent studies have carried out meta-analyses and found the activation of *AtCYP19-1* and *AtCYP57* expressions by the infection of pathogenic bacteria, *Pseudomonas syringae* and *Xanthomonas campestris* [83,84]. Pogorelko's group [83] has then followed up to show that the disruption of *AtCYP19-1* and *AtCYP57* expressions enhance susceptibility, whereas the overexpression of *AtCYP19-1* and *AtCYP57* can promote disease resistance against *P.*

syringae infections, providing solid evidence that AtCYP19-1 and AtCYP57 play intrinsic roles in the activation of immune responses (Figure 1). In parallel, they have utilized the yeast two-hybrid assays to probe the interactions of AtCYP19-1 with antioxidant regulators such as ENGD1 (GTPase/GTP-binding protein) and Rm1C like cupins [84], hypothesizing that the upregulation of *AtCYP19-1* expression is lined with the temporal modulation of antioxidant and detoxification systems to increase ROS accumulations shown in the *AtCYP19-1*-overexpression plants [83,85]. On the other hand, the overexpression of *AtCYP57* induced callose depositions, which is perhaps via binding and stimulating the activity of pyruvate decarboxylase I whose overexpression demonstrated increased callose depositions and expression of defense genes, in conjunction with anaerobic alcohol formation and soluble sugar formation (Figure 1) [83,85,86].

Recently, emerging evidences have proposed that plants possess several restriction factors, being able to interfere with the viral replications by directly targeting viral replicase complexes (VRC) in the cytoplasm of infected cells [87,88]. Among the plant restriction factors are two cytosolic AtCYPs (i.e., AtCYP18-3 and AtCYP19-3), which showed binding affinity to the (+)-stranded RNA and/or replicase of tomato bushy stun tombusvirus (TBSV). These interactions then impeded the *de novo* replication of TBSV RNA via the inhibition of viral RNA recruitment, subsequently blocking the VRC assembly. In line with this scenario, the overexpression of *AtCYP18-3* and *AtCYP19-3* in plants, *Nicotiana benthamiana*, manifested the significant reduction of TBSV RNA accumulations, and the suppression of disease symptom development [51].

It is worth noting that AtCYP18-3 along with AtCYP18-4 and AtCYP20-3 were reported to interact with a virulence gene (VirD2) of *Agrobacterium tumefaciens*, a causative pathogen of a crown gall tumor disease on a wide variety of dicotyledonous plants by transporting a transfer (T)-DNA, a ss DNA segment of the tumor-inducing plasmid, from the bacterium to the plant cell [46,89,90]. These interactions hypothesized that AtCYPs are involved in maintaining the correct structural and/or functional states of VirD2. Indeed, incubation of *Arabidopsis* and tobacco cells with the CsA showed decreased T-DNA translocations, and perhaps disease establishment [46]. However, a recent finding by van Kregten et al. [91] that the VirD2 CYP-binding domain is not necessary for the T-DNA transformation suggests that CYPs may not be absolute requirement for VirD2 activity in the plant cells [90]. Alternatively, AtCYP18-3 may act as a negative regulator in defense activation, targeting and inhibiting the receptor (i.e., RPS2 and RPM1)-mediated recognition of pathogens (e.g., *P. syringae* DC3000 *avrRpt2*, *avrB* and *avrRpm1*). Therefore, the gain-of-function mutation of *AtCYP18-3* demonstrated the loss of receptor (collectively called resistance (*R*)-gene)-mediated disease resistance (Figure 1) [52].

Lately, affinity screening has identified AtCYP20-3 as a signal receptor of plant defense hormone, (+)-12-oxo-phytodienoic acid (OPDA), belonging to jasmonate family of hormones which includes jasmonic acid, its precursors and derivatives [92,93]. OPDA is an autonomous signaling molecule that regulate unique subsets of jasmonate-responsive genes in activating and fine-tuning defense (adaptive) responses against necrotrophic fungi and insect herbivores, as well as growth processes [94,95]. When OPDA is produced under stress states, it binds and stimulates AtCYP20-3 to form a complex with serine actyltransferase1 (SAT1), which triggers the formation of a hetero-oligomeric Cys synthase complex (CSC) with *O*-actylserine(thiol)lyase B

[92,96,97]. CSC formation then leads to the production of CYS (sulfur assimilation) and subsequently thiol metabolites, which increases cellular reduction potentials. The enhanced reduction capacity in turn coordinates the expression of a subset of OPDA-responsive genes that actuate and calibrate immune responses. Hence, the disruption of *AtCYP20-3* expression concurred with the enhanced disease susceptibility against necrotrophic fungal (e.g., *Alternaria brassicicola* and *Botrytis cinerea*) and oomycete (*Pythium irregulare*) pathogens (Figure 1) [92,98].

1.6. ROLES OF CYCLOPHILIN AT THE INTERFACE BETWEEN PLANT GROWTH AND DEFENSE; A CASE STUDY OF ATCYP20-3

Emerging outcomes from a number of recent studies on AtCYPs have underpinned that CYPs are versatile metabolites in plants regulating various processes in growth and survival. In particular, AtCYP20-3 is found to be positioned within multiple signaling and metabolic pathways, binding with several interacting partners including SAT1, thioredoxins (Trxs) and 2-Cys peroxiredoxin (2-CysPrxs) in the chloroplasts [81,92,93,99,100,101], which propose AtCYP20-3 to be a key regulator in controlling the interface between OPDA (defense) and light-dependent redox (growth) signaling. The latter, also referred to as the electron (e^-) transport chain (ETC) of PSI, is a primary metabolism converting solar energy into biologically useful chemical energies, which is a source of the overall biomass of plants and living organisms [93,102]. When PSI antenna captures solar energy, it excites e^- that reduces Trxs via a ferredoxin (Fd) and a Fd-Trx reductase. Trxs, small oxidoreductases, then delivers e^-, and activates target enzymes in the Calvin cycle (carbon fixation) that balances consumption in photosynthesis [103,104,105]. Recent studies however have started to unveil that Trxs also target other, Calvin

cycle-unrelated proteins, including AtCYP20-3 [106], a key regulator of (a) OPDA signaling (see above) and (b) photosynthetic pathway as an e^- donor of 2-CysPrxs which metabolize the detoxification of a toxic byproduct in photosynthesis (e.g., H_2O_2), and the activation of Calvin cycle enzymes [99,100,101,107]. The interaction of Trxs with AtCYP20-3, thereby, positioned AtCYP20-3 as a redox sensor of ETC, transferring e^- from Trxs towards 2-CysPrxs and/or SAT1. Reduction (activation) of 2-CysPrxs then continues peroxide detoxification and activates photosynthetic 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 [92,108,109,110,111]. In line with this scenario, stress-induced OPDA binds and, perhaps, modulates the functional and conformational states of AtCYP20-3 to which adjusts its subsequent binding and electron transfer between 2-CysPrxs and/or SAT1 [92,93], hypothesizing that AtCYP20-3 is a unique player in controlling the interface between OPDA signaling and photosynthesis. This interplay thus enables plants to make an adaptive decision in allocating resources (e^-) between growth and defense responses (e.g., fitness tradeoffs) towards constant environmental challenges such as pathogens, pests, tissue injury, as well as light and oxidative stresses [110,112,113]—in the end—ensuring optimal growth, reproduction and survival of plants.

1.7. CONCLUSIONS

Plants constantly cope with a vast array of environmental challenges whilst concurrently optimizing their fitness by reprogramming the growth and reproduction processes. Towards that, plants employ a number of primary and secondary metabolites, and intricate signaling network to interconnect and orchestrate multiple layers of complex cellular mechanisms. As discussed in

this review, a growing number of studies have espied that plant CYPs are highly versatile protein regulators involved in a variety of metabolic signaling and pathway during plants growth and survival, suggesting that the activity or activities of each CYP and their functional crosstalk play intrinsic roles in controlling many of key regulatory hubs (e.g., AtCYP20-3) that coordinate the growth, development, as well as immune and defense responses in plants. Noticeable, CYPs are structurally conserved PPIases, and thus molecular components and mechanisms in which are involved their activities, likely share common ancestry and evolutionary processes across the plant Kingdom. Therefore, furthering our understanding of functional and biological activities between, and within plant CYPs will not only: (i) provide new insights into the cellular mechanisms that plants use to make adaptive decisions when challenged by multiple stressors; and (ii) can enrich plant breeding and engineering strategies for selection of elite genetic traits that will maximize plant fitness; but also (iii) assist understanding the immune activation of a mammalian system; and (iv) help improving drug developments through facilitating the rational design of more potent and safe reagents.

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CHAPTER 02: ROLE OF *S*-GLUTATHIONYLATION ON PLANTS PEROXIREDOXINS

2.1. ABSTRACT

S-glutathionylation is a post-translation modification (PTM) known to regulate proteins containing thiol groups. In this context, the overall goal of this research was to investigate the role of *S*-glutathionylation on plants 2Cys-Peroxiredoxins (2CPs) protein. Towards that, we cloned and expressed the two isoforms of *Arabidopsis thaliana* 2CPs, namely 2CPA and 2CPB proteins and tested their interaction with glutathione (GSH). As a result, GSH binding regulates both 2CPs quaternary structure and activity. Moving forward, *in vitro* studies were carried out to elucidate the role of this PTM on 2CPs interaction with known enzymatic partners NTRC and CYP20-3. Interestingly we found that *S*-glutathionylation greatly impaired the NTRC reduction of 2CPs. Additionally, our data propose that under heat shock stress, CYP20-3 plays a role in inactivated *S*-glutathionylated 2CPA, while maintaining 2CPB chaperone activity. Moreover, our study unveils that the physiological ratio of oxidized (GSSG) and reduced (GSG) glutathione forms are major players in regulating the function of 2CPs proteins.

2.2. INTRODUCTION

As sessile organisms, in order to overcome environmental stresses, plants employ an intricate signaling network to interconnect and orchestrate multiple layers of cellular mechanisms. In this context, the plant defense hormone, (+)-12-oxo-phytodienoic acid (OPDA), belonging to jasmonate family of hormones (Park et al., 2013, Cheong et al., 2017), is an autonomous signaling molecule that regulate unique subsets of jasmonate-responsive genes fine-tuning defense response against necrotrophic fungi and insect herbivores, as well as growth

processes (Pieterse et al., 2012, Acosta, et al. 2010). Lately, affinity screening has identified CYP20-3 as the OPDA signal receptor.

Hence, under stress conditions OPDA is produced and binds and stimulates CYP20-3 to form a complex with serine actyltransferase1 (SAT1), triggering the formation of a hetero-oligomeric Cys synthase complex (CSC) with O-actylserine(thiol)lyase B (Park et al., 2013, Takahashi et al 2011, Wirtz and Hell, 2006). CSC formation leads to the production of CYS (sulfur assimilation) and subsequently thiol metabolites, which increases cellular reduction potentials. The enhanced reduction capacity in turn coordinates the expression of a subset of OPDA-responsive genes that calibrate immune responses. Accordingly, the disruption of CYP20-3 expression concurred with the enhanced disease susceptibility against necrotrophic fungal (e.g., *Alternaria brassicicola* and *Botrytis cinerea*) and oomycete (*Pythium irregulare*) pathogens (Park et al., 2013, Liebthal et al., 2016).

During photosynthesis process, the electron (e−) transport chain (ETC) of PSI , the primary metabolism converting solar energy into biologically useful chemical energy, which is a source of the overall biomass of plants and living organisms (Cheong et al., 2017, Jensen et al., 2007). When PSI antenna captures solar energy, it excites e− that reduces thioredoxins (Trxs) proteins via a ferredoxin (Fd) and a Fd-Trx reductase. In *Arabidopsis*, the chloroplasts contain ~10 Trxs isoforms that are categorized into five types (f, m, x, y, z), that relay light-dependent activation of enzymes in the Calvin cycle (carbon fixation) such as glyceraldehyde-3-phosphate dehydrogenase, fructose 1,6-bisphosphatase, sedoheptulose 1,7-bisphosphatase and phosphoribulokinase, which balance energy conversion and consumption in photosynthesis

(Meyer et al., 2009, Serrato et al., 2013, Nikkanen et al., 2014).

Recent studies, however, have started to unveil that Trxs also target Calvin cycle-unrelated proteins, including CYP20-3 (Motohashi et al., 2011). Upon receiving electrons from Trxs, the reduced CYP20-3 may then transfer those e− towards SAT1, activating CSC formation and defense mechanism. Moreover, transcriptional coexpression (Muthuramalingam et al. 2009) and DNA-protection assay (Laxa et al., 2007) have pinpointed the interaction of CYP20-3 with 2-Cys peroxiredoxins proteins (2CPS) proteins, in which, reduced CYP20-3 is hypothesized to transfer electrons to 2CPs, thus regulating plants growth mechanism (Liebthal et al.,2018)

The *A. thaliana* genome encodes two plastidic 2CPs (denoted A and B), thiol-based peroxidases that function in protecting and modulating photosynthesis. Typically, 2CPs form an obligatory homodimer as the peroxidatic Cys (Cys_P) from one monomer is linked via a redox-active disulfide to the resolving Cys (Cys_R) located at a complementary polypeptide. The dimerization however inactivates the enzymatic activity of 2CPs.

Therefore, NADPH-dependent thioredoxin reductase C (NTRC) reduces (activates) them to monomers capable of metabolizing the removal of a toxic byproduct in photosynthesis (i.e., H_2O_2), and the activation of Calvin cycle enzymes such as a fructose 1,6-bisphosphatase. On the other hand, oxidative stress (overoxidation) prompts 2CPs to assemble a homodecamer that disables peroxidase activity but gains chaperon activity to protect cellular molecules against oxidative damages (Aran et al., 2009, Liebthal et al., 2018).

In line with this scenario, our preparatory pull-down assays showed that Arabidopsis 2-Cys peroxiredoxin A (2CPA) can directly bind GSH-agarose beads (Fig. 3A), underpinning a potential *S*-glutathionylation of 2CP-family proteins (Liebthal et al., 2018). Furthermore, recent studies have elucidated that members of 2CPs such as human PrxI, and pea 2CP and PrxIIF are glutathionylated by GSSG under oxidative stress, rather shifting its quaternary structure from decamers to dimers, and concomitantly inactivates its molecular chaperone activity (Park et al., 2009, 2011, Calderón et al., 2017).

Glutathione (GSH; y-L-glutamyl-L-cysteinyl-glycine) is the most abundant nonprotein thiol in plants, playing a pivotal role in maintaining cellular redox homeostasis under different ecological conditions. GSH reduces reactive species and other peroxides by donating its electron (H^+, e) and subsequently being oxidized to a disulphide form (GSSG). Therefore, cellular increases in GSH production and reduction capacity concurs with the generation of free radicals and oxidants, supporting a notion that GSH status is involved in transmitting oxidative stress signaling (Noctor et al., 2011).

Oxidative stress signaling renders a decrease in GSH:GSSG ratios and/or depletion of GSH, which in turn induces the reversible formation of mixed disulfides between protein sulfhydryl groups (PSH) on cysteine (Cys) residues and GSSG (i.e., glutathionylation) on multiple proteins. *S*-glutathionylation then conveys the regulation of signaling and metabolic pathways via the enzymatic modification of redox-sensitive proteins such as oxidoreductases (Grek et al., 2013). In this sense, our goal is to investigate *S*-glutathionylation of chloroplast 2-Cys Prx proteins.

2.3. MATERIALS AND METHODS

Recombinant Proteins. Preparation of recombinant proteins were performed as described previously (Park et al., 2013). Briefly, gene fragments encoding the mature protein region (determined by Edman sequencing or predicted by TargetP, Emanuelsson et al., 2000) of 2CPA (At3g11630), 2CPB (At5g06290), NTRC (At2g41680) and SRX (At1g31170) were cloned into the pET28a expression vector (Novagen) to obtain N-terminus 6x HIS tagged proteins. Point mutations of Cys residues in 2CPs were introduced using the QuikChange II Site-directed Mutagenesis Kit (Agilent) according to the manufacturer's instructions. These recombinant proteins were then expressed in *E. coli* BL21(DE3), and purified using a nickel- (Ni-NTA, Qiagen) column as previously described (Cheong et al., 2017). Primers used for plasmid constriction and site directed mutagenesis are listed in Table 1 and 2, respectively.

S-glutathionylation of 2CPs. Typically, a S-glutathionylation reaction was conducted by incubating 10 μM 2CPs with 1.0 mM GSH at ambient temperature (~25 ^{o}C) for 30 min in 50 mM Tris-HCl buffer (pH 7.5), with some varied GSH concentrations (0.5 to 10 mM), incubation times (0.5 to 30 min) or buffer pH (7.1 to 8.1).

Preparation of S-glutathionylated, Oxidized and Reduced 2CPs. In 50 mM Tris-HCl (pH 7.5) buffer, 10 μM 2CPs were S-glutathionylated for 30 min by 10 mM GSH, oxidized for 15 min by 0.1 mM H_2O_2, or reduced for 30 min by 1 mM (for 2CPA) and 20 mM (for 2CPB) TCEP-HCl. Following the reactions, excess GSH, H_2O_2 and TCEP-HCl were removed using size exclusion chromatography (Sephadex G-25 medium, GE Healthcare), and stored at 4 ^{o}C until use.

Protein Extraction. Leaf tissues of three-weeks-old-Arabidopsis were immersed in liquid N_2 and ground to a powder using a mortar and pestle. Ground leaf tissue was dissolved into two

volumes of 50 mM potassium phosphate buffer (pH 7.2), containing protease inhibitor cocktails (Sigma-Aldrich), agitated for 60 min, and centrifuged for 30 min at 10,000*g*. The supernatant was collected and stored at 4°C until use.

Immunoblot (IB) analysis. To assess the quaternary structure of 2CPs, total protein was resolved by SDS/PAGE and electroblotted onto PVDF membranes (Millipore). The blot was probed with protein G- purified polyclonal rabbit anti-2CPA antibody (1:8000, 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). Coomassie blue staining was used to verify equal loading by assessing the abundance of the large subunit (LSU) of ribulose bisphosphate carboxylase/oxygenase.

Peroxidase Activity Assay. Reduction of H_2O_2 by 2CPs was quantified using the eFOX assay method (Chesseman 2007). The assay was carried out at 37 ^oC in 50 mM potassium phosphate buffer (pH 7.2) with 5 µM 2CPs. A reaction was started by addition of 50 µM H_2O_2, incubated for 10 min, and terminated by 2% (v/v) TCA. A volume of 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 then mixed with 100 µL of the reaction solution, and its decrease in H_2O_2 levels was determined spectrophotometrically by measuring absorbance at 550 nm.

Chaperone Assay. The chaperone activity of 2CPs was measured using citrate synthase as substrate (Bhuwan et al., 2017). Briefly, 10 µM 2CPs was incubated at 45 ^oC in 50 mM potassium phosphate buffer (pH 7.2). After temperature adjustment for 15 min, citrate synthase (1 µM, Sigma-Aldrich) was added and the increase in absorption at 360 nm was monitored with a spectrophotometer.

Semiquantitative RT-PCR. Total leaf RNA was prepared using TRIzol reagent (Invitrogen) and the Direct-zol RNA Kit (Zymo Research) according to the manufacturer's instructions. RNA qualities were assessed by agarose gel electrophoresis and NanoDrop (A_{260}/A_{280} > 1.8 and A_{260}/A_{230} > 2.0) (Udvardi et al., 2008). RT reactions were performed by using an oligo(dT) reverse primer and a qScript reverse transcriptase (Quantabio). Semiquantitative RT-PCR was then performed using 2 μL cDNA with *Taq* 2X master mix (New England BioLabs). The annealing temperature for primer pairs was 55 °C. The PCR profiles were 40 cycles (for *2CP*s) or 25 cycles (for *POLYUBIQUITIN* [*UBC*]). Each cycle consisted of 94 °C for 30 s, annealing temperature for 30 s, and 72 °C for 1 min; the final step occurred at 72 °C for 10 min.

Plant growth conditions. A. *thaliana* wild type (WT) Columbia (Col-0) were grown in a growth chamber with 12 hours of day and 12 hours of night (100-120 μE/m^2/s) at 22 ºC and 25 ºC with 60 % to 80 % relative humidity.

***In vitro* Protein-protein interaction assay.** The His-fused GSTL2, His-fused 2CPA proteins where immobilized on GSH-affinity beads (Sigma) for 1 h at room temperature, washed with 50 mM sodium phosphate buffer (pH 7.5, containing 150 mM NaCl), and eluted with and with GSH. As a negative control, MBP protein was incubated with GSH-affinity beads. Next, all pull-downed proteins were resolved by SDS/PAGE and probed with a monoclonal anti-His antibody (Invitrogen).

2.4. RESULTS AND DISCUSSION

In our previous study Cheong et al (2017) proposed that OPDA signaling stimulates the buildup of reduction potential through CYP20–3, which activates SAT1-dependent sulfur assimilation and regulates defense (OPDA responsive) gene expressions. As CYP20–3 is also reported to target 2CPS, Cheong et al (2017) investigated the potential effects of OPDA on the interactions of CYP20–3 with 2CPs. Unexpectedly, OPDA promoted only CYP20–3–2CPB, but not CYP20–3–2CPA, interaction (Fig. 2A, B, upper panels). In line with this scenario, OPDA enhanced the CYP20–3– 2CPB, as well as CYP20–3-SAT1 interactions simultaneously, but promoted only the CYP20–3-SAT1 interaction when they were co-incubated with 2-CPA (Fig. 2A, B, lower panels). Unlike Trxs, the expression of both 2-CPA and -B (Fig. 2C), as well as SAT1 , was constitutive regardless of wounding, suggesting that OPDA signals the post-translational modification of, perhaps structural and/or functional states of, CYP20–3, which subsequently enhances its binding capacities to the downstream target proteins (i.e., 2CPB and SAT1) in two distinctive cellular processes; plant photosynthesis (growth) and OPDA signaling (defense) pathways.

Moving forward, our preparatory pull-down assays showed an interaction of 2CPs binding to GSH-agarose beads (Fig. 3A), hence we assessed a possible *S*-glutathionylation of 2CPA via probing its oxidized (^{ox}) and reduced (^{red}) forms with anti-GSH antibody following the preincubation with GSH, GSSG or GSNO (Fig. 3B). Surprisingly, 2CPA^{ox} and 2CPA^{red} appeared to bind only GSH, but not GSSG and GSNO. GSH-binding then modulated the quaternary structure of 2CPA to muster tripartite conformations (Fig. 3C and Fig. 4A). Upon binding to GSH, a majority of dimeric 2CPA^{ox} became split into monomers, while some of them were merged to assemble decamers (Fig. 3C lanes 1, 2 and 6). Likewise, *S*-glutathionylation of mono-

/dimeric 2CPA^{red}, evinced by slight increases in their molecular masses (Fig. 3C lanes 3 and 4), formed triple conformations (e.g., monomers, dimers and decamers) (Fig. 3C lanes 4 and 8). Since GSH selectively targeted the Cys_R^{53} out of two Cys residues in 2CPA (Fig. 4B), the binding ratios of the Cys_R^{53} to GSH vs. the Cys_P^{175} of a complementary 2CPA likely contributes to forming the different quaternary structures of 2CPA^{GS}.

To understand the physiological significance of GSH-dependent *S*-glutathionylation, we first examined the active concentrations of GSH, and their reaction rates in binding 2CPA (Fig.4C and D). Indeed, the physiological concentration of GSH in the chloroplasts to where 2CPs are localized (~1 to 1.5 mM, Koffler et al., 2013) was sufficiently able to *S*-glutathionylate 2CPA, and tripartition its quaternary structures. These modifications occurred rapidly (≤60 sec) to reach the steady states and remained stable over a month at 4 ºC (Fig.4E), indicating that *S*-glutathionylation determines the intrinsic quaternary structure of 2CPA, and subsequently their cellular activities in the chloroplasts. In line with this scenario, *S*-glutathionylated (^{GS}) 2CPA rendered dual functionalities; a monomeric peroxidase that reduces H_2O_2, and a decameric chaperone activity protecting citrate synthases against thermal aggregations (Figs. 3D and E).

On the other hand, previous studies have proposed that pH gradients and peroxides are cellular switches capable of controlling the quaternary structures and activities of 2CPA, as the supplementation of HCl (pH 8.0 to 7.0) or H_2O_2 fostered its dimerization and/or oligomerizations and resulting changes in enzymatic activities (Barranco-Medina et al., 2008, Morais et al., 2015). Note that chloroplasts maintain pH gradients from 8.0 to 7.0, and H_2O_2 levels between 0.4 and 0.6 mM (Rejeb et al., 2015, Höhner et al., 2016). The cellular acidification and oxidation, however, are likely of no effect on 2CPA^{GS} (Figs. 3F and G). A basal level concentration of GSH

(1 mM) effectively *S*-glutathionylated 2CPA and released tripartite conformations throughout pH gradients, 8.0 to 7.0 (Fig. 3F). Furthermore, the quaternary structures of $2CPA^{GS}$ were stable against excess oxidation up to 5 mM of H_2O_2 Fig. 3G), elucidating that *S*-glutathionylation is a predominant mode of post-translational modifications to 2CPA in the chloroplasts, and it determines and protects the structures and activities of 2CPA from various ecological conditions.

We next investigated whether 2CPA is *S*-glutathionylated *in planta* by comparatively probing its molecular masses to those of GSH-binding proteins from total protein extracts, using anti-2CPA and anti-GSH antibodies (Fig. 3H). As expected, one of major bands cross-reacted with anti-2CPA antibody were visualized at ~20 kDa, corresponding a monomeric size of 2CPA, in both nonreducing and reducing conditions, while two bands paralleled to di-/decameric sizes (~40 and ~200 kDa) of 2CPA were markedly detectable only in a nonreducing condition. These three protein signals by 2CPA-immunoblotting (IB) were then mainly paralleled to those from GSH-IB, underpinning that GSH indeed binds 2CPA and determines its tripartite structures and dual enzymatic activities in vivo.

The ex vivo IBs corroborated an intrinsic role of *S*-glutathionylation in governing the cellular functions of 2CPA, which are considered to be vital in plant growth and survival, and further insured by its isoform 2CPB (Dietz et al., 2006, Liebthal et al., 2018). We thus assessed if GSH is also able to bind 2CPB and determine its quaternary structures and activities (Fig.5A). However, a caveat was that the quaternary structure of 2CPB responds differently to redox agents, comparing to 2CPA (Figs. 3C and 5A). In fact, 2CPB constituted the same, dipartite conformations (i.e., dimers and decamers) regardless of being oxidized or reduced with the lower concentrations (≤0.1 mM) of H_2O_2, TCEP-HCl and DTT (Fig. 5A and Fig. 6A). Further oxidation and reduction then principally accelerated the decamerization of 2CPB (Figs.6A and

B), except that excess reduction by 20 mM TCEP-HCl could completely monomerize 2CPB (Fig. 5A and Fig. 6A). In this context, GSH-binding largely fostered the decamer-/icosamerization of 2CPB (Fig. 5A and 6B and C), and subsequently stimulated its chaperone activity while inactivating its peroxidase activity (Figs. 6 B and C).

As shown in 2CPA, *S*-glutathionylation of 2CPB rapidly (≤30 sec) occurred at the Cys_R^{53} under the physiological concentrations of GSH (~1 mM, Fig. 7A and B), and protect also its molecular activity from cellular stresses such as overoxidation (Fig. 6A). Noticeably, $2CPB^{GS}$ predominantly formed decamers and, to a lesser extent, icosamers which were perhaps marginally tapered to decamers in a time-dependent manner (Fig. 7A). However, increases in GSH concentrations (≥4 mM) and/or HCl gradients (pH 8.0 to 7.0) could equilibrate reduced decamer/icosamer ratios of $2CPB^{GS}$ (Figs. 5D and 5E). Taken together, our results a) illuminated a new role of GSH as a functional group of protein post-translational modification, which conveys the target-specific regulation of molecular structure and/or functional activity, as well as prevails other cellular homeostasis and stress constrains; b) Suggested that regardless the high similarity on the amino acid sequence (>96 %) (Fig. 8) 2CPA and 2CPB, *S*-glutathionylation differently regulates the quaternary structure and function of 2CPs, in which $2CPA^{GS}$ possess peroxidase and chaperone activities, whereas $2CPB^{GS}$ are stable chaperone.

In line with this scenario, site direct mutagenesis was used to generate 2CPB mutagenic proteins: $2CPBE^{33}D$, $2CPBY^{64}H$:$E^{65}S$, $2CPBV^{106}I$:$I^{109}V$, $2CPBP^{122}H$, and $2CPBV^{167}I$. As a result, $2CPBV^{106}I$:$I^{109}V$ mutation drastically changes the 2CPB dipartite conformations rendered to quaternary structure mostly composed by dimers that upon *S*-glutathionylated released dimers and monomers, but no decamers and icosamers where observed (Fig. 9). Highlighted that

decamer-/isocamerization of 2CPB is mostly stabilized by to valine and isoleucine amino acids interaction.

Moreover, S-glutathionylation has shown to be critical in the enzymatic reactions and their metabolic pathways. For instance, an activation (reduction) of 2CPs has reported to be metabolized by NTRC pathways (Liebthal et al., 2018). NTRC reduced disulfide bonds in homodimeric 2CPs, promoting either the monomerization of 2CPA or the decamerization of 2CPB (Fig.10A and B). These reactions however needed, on our hands, an excessive reducing power, ≥1 mM NADPH. Note that the physiological concentrations of total NADPH ranged only <1.5 µM (Maruta et al., 2016). Nonetheless, our assays under 1 mM NADPH observed little effect of NTRC on the quaternary structure of $2CPs^{GS}$ (Fig. 11), indicating that S-glutathionylation reduces the kinetic accessibility of 2CPs towards NTRC, as well as the presence of alternative metabolic pathways recharging the activity of 2CPs, especially considering the inconceivably low physiological concentrations of NADPH for the NTRC activity. In line with this scenario, our data suggest that S-glutathionylation utilize GSH to control the direct reduction (activation) mode of 2CPs.

As *S*-glutathionylation of 2CPs greatly changes the enzymatic activity of NTRC towards 2CPs, we investigated whether it also affects 2CPs interaction with CYP20-3. First, 2CPs were incubated at 25^0C with an increased concentration of CYP20-3 but no activity was observed on either 2CPs (Fig. 12A and B). However, increased temperature was able to dimerize or inactivate 2CPA (Fig. 13A), whereas no activity was observed on 2CPB (Fig. 13B). Unveiling a possible role of CYP20-3 on plants defense mechanism during heat shock stress, in which CYP20-3

inhibit 2CPA reductase activity whereas maintaining 2CPB chaperone activity that protects other molecules against heat shock damages.

Moreover, the glutathione pool on plants cells is composed of the reduced (GSH) and oxidized (GSSG) glutathione forms. Under non-stress conditions, GSH concentration ranges from 1 to 4.5 mM and GSSG representing 10% of this pool (Noctor and Foyer 1998). However, stress conditions may directly affect GSH:GSSG ratio by increasing GSH concentration, such as (+)-12-oxo-phytodienoic acid (OPDA)/CYP20-3 signaling pathway, (Park. et al 2013) or increase GSSG due to oxidative stress signaling. Hence, multiple signaling trigger a rapid adjustment of redox homeostasis and GSH:GSSG ratios (Park et al., 2013).

To investigate the effect of redox homeostasis adjustment on *S*-glutathionylation of peroxiredoxins we incubate 2CPs with different ratio of GSH:GSSG. As a result, increase of GSH:GSSG ratio promote formation of decamers and monomers of 2CPA, thus enhancement of chaperone and reductase activity whereas decrease of GSH:GSSG leaded to inactivation (dimerization) of 2CPA. Similarly, increased of GSH:GSSG ratio leaded to decamerization/ icosamerization of 2CPB but oxidation slightly increase on dimers (Fig. 14). In this sense, although $2CP^{GS}$ have a reduced regulation by known enzymatic proteins, it is directly regulates by the GSH:GSSG pool.

2.5. CONCLUSION

Taken together our study propose that GSH, the most abundant form free-thiol of cells, conveys reductant signaling via finetuning and stabilizing the activities of redox regulators such as plastid 2CPs. Upon binding to 2CPs cysteine 53, GSH differently regulates those proteins quaternary structure (and activity), in which $2CPA^{GS}$ exhibit a tripartite conformation composed of monomers (peroxidase), dimers (inactive form) and decamers (chaperone) whereas $2CPB^{GS}$ quaternary structure is stabilized as decamers/isocamers (chaperone). Additionally, *S*-

glutathionylation greatly affects 2CPs interaction with known protein partners (i.e. NTRC). Furthermore, during heat shock stresses CYP20-3 inactivates (dimerized) 2CPA but no activity is observed towards 2CPB chaperone activity. Lastly, although *S*-glutathionylation impaired NTRC regulation of 2CPs, the GSH:GSSG ratio strongly modulates 2CPA peroxidase and chaperone activity.

As an overall conclusion, we have uncovered a new mechanism inside of plants cells that are regulates by the reducing agent GSH. Additionally to maintaining the redox homeostasis, GSH also modulate 2CPs activities independently of oxidative signaling.

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Table 1. Oligonucleotides used for cloning proteins from *A. thaliana*.

GENE	DIRECTION	SEQUENCE (5′-3′)
2CPA	FORWARD	tcgatcggatccgcccaggccgatgatcttccac
	REVERSE	gatctgaagcttctaaatagctgagaagtactc
2CPB	FORWARD	tcgatcggatccgctcaggctgatgatttaccac
	REVERSE	gatctgaagcttctagatagctgaaaagtattc
CYP20-3	FORWARD	catatgtccatggctgctgagga
	REVERSE	gaattctcaagcatctaacgggagctc
GSTL2	FORWARD	tcgatcccatggctgttgtagagtcaagtcg
	REVERSE	gatctgggatccttagagacgtgcttctgcttgg
NTRC	FORWARD	caaggatccatggctgcgtctccca
	REVERSE	cttaagctttcatttattggcctca
SRX	FORWARD	tcgatcggatccggaggatctagcggcggtgta
	REVERSE	gatctggcggccgctcagcgaagatgatgcctga

Table 2. Oligonucleotides used for site-direct mutagenesis of 2CPs

MUTATION	DIRECTION	SEQUENCE (5′-3′)
2CPAC53S	SENSE	ttggactttactttcgtcagcccaacagagattactg
	ANTISENSE	cagtaatctctgttgggctgacgaaagtaaagtccaa
2CPAC175S	SENSE	cccggatgaagtcagcccagcaggatg
	ANTISENSE	catcctgctgggctgacttcatccggg
2CPBC53S	SENSE	tggacttcacttttgtcagccccactgagattac
	ANTISENSE	gtaatctcagtggggctgacaaaagtgaagtcca
2CPBC175S	SENSE	cccggatgaagtgagccctgcgggatg
	ANTISENSE	catcccgcagggctcacttcatccggg
2CPBE33D	SENSE	ttcataaaggtgaagctctctgattacattggcaaaaagtatgttat
	ANTISENSE	ataacatacttttgccaatgtaatcagagagcttcacctttatgaa
2CPBY64H:E65S	SENSE	actgagattactgccttcagtgaccgtcattcagaatttgagaagctaaacac
	ANTISENSE	gtgtttagcttctcaaattctgaatgacggtcactgaaggcagtaatctcagt
2CPBV106I:I109V	SENSE	ctcggtgatctgaattatcctcttatttcggatgtcactaaatccatttcaaaatc
	ANTISENSE	gattttgaaatggatttagtgacatccgaaataagaggataattcagatcaccgag
2CPBP122H	SENSE	gtttggagtgctcatccatgatcagggcattgcac
	ANTISENSE	gtgcaatgccctgatcatggatgagcactccaaac
2CPBV167I	SENSE	cctccaggcattacagtatattcaagaaaacccggatg
	ANTISENSE	catccgggttttcttgaatatactgtaatgcctggagg

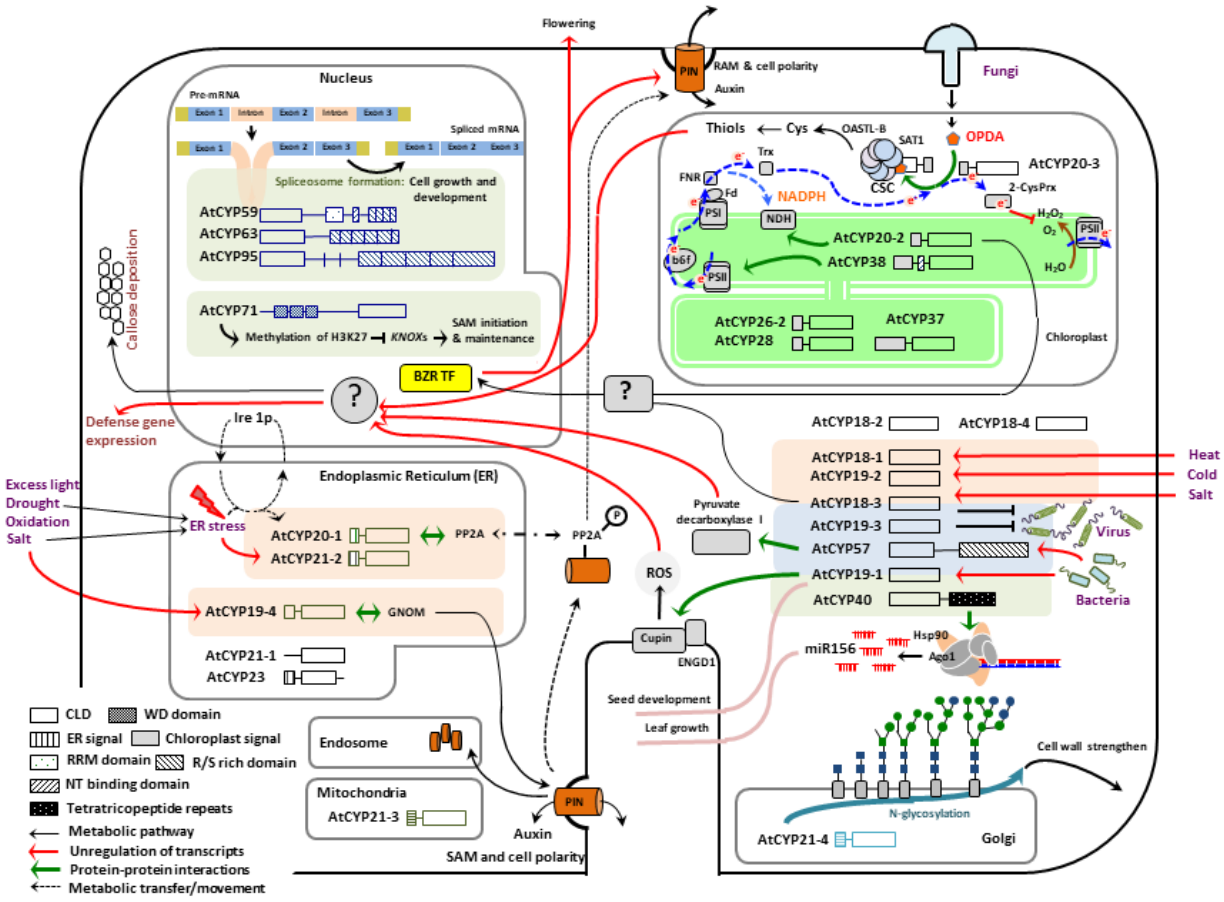

Figure 1. A working model; the metabolic and signaling pathways of AtCYPs in plant growth and defense responses. See the context for detailed explanation. Abbreviations: Ago1 (ARGONAUTE 1), BZR TF (BRASSINAZOLE RESISTNAT1 transcription factor), B_6f (Cytochrome b_6f complex), CSC (Cysteine synthase complex), Cupin (RmlC-like cupin superfamily), Cys (cysteine), ENGD1 (GTPase/GTP-binding protein), Fd (Ferredoxin), FNR (Ferredoxin NADPH reductase), GNOM (ADP ribosylation factor-guanine-nucleotide exchange factor), Hsp90 (Heat shock protein 90), H3K27 (Lysine 27 on histone H3 protein), Ire 1p (Inositol-requiring enzyme-1), KNOXs (KNOTTED-like homeoboxes), MiR156 (MicroRNA 156), NADPH (Nicotinamide adenine dinucleotide phosphate), NDH (NAD(P)H dehydrogenase), OASTL-B (*O*-actylserine(thiol)lyase B), OPDA [(+)-12-oxo-phytodienoic acid], P (Phosphorus), PIN (Pin-formed 1 auxin efflux carrier proteins), PP2A (Ser/Thr Protein Phosphatase 2), PSI & II (Photosystem I & II), RAM (Root apical meristem), ROS (Reactive oxygen species), SAM (Shot apical meristem), SAT1 (Serine actyltransferase1), Trx (Thioredoxin), and 2-CysPrx (2-Cys peroxiredoxin).

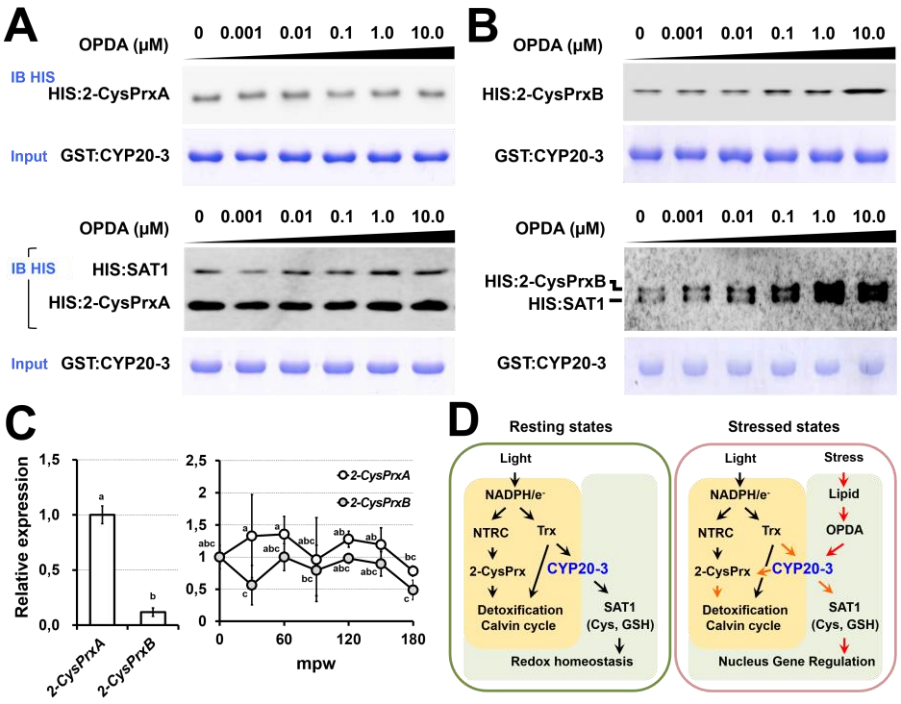

Figure 2. OPDA stimulates the CYP20–3 and 2CPB interaction. (A and B) In vitro pull-down assays between CYP20–3, SAT1, 2CPA (A) and/or 2CPB (B) in the presence of various concentrations of OPDA. GST:CYP20–3 fusion protein was used as a bait to pull-down 2-CysPrxs and/or HIS:SAT1. Coomassie blue-stained gels indicate the amount of bait proteins used in each pull-down assay (Input), while parallel immunoblots for proteins that co-purified with GST:CYP20–3 were probed with monoclonal anti-His antibody (IB HIS). (C) The basal levels of (Left chart), and the wound-responsive changes in (Right chart) 2-CysPrxA and B transcripts, determined by the high-resolution qRT-PCR in Col-0 WT plants. Total RNAs were prepared from wounded leaves at 0, 30, 60, 90, 120, 150 and 180 mpw, and results were normalized to the expression levels of UBC gene (means § SD; n D 3). Statistical analysis was performed with the Student's t-test. (D) Proposed model of CYP20–3 as a regulatory hub in the interplay between light and OPDA signaling. In resting states, CYP20–3 relays light signaling in buffering cellular redox homeostasis, whereas in stressed states CYP20–3 interfaces light and OPDA signaling, which fine-tune plant fitness between growth (light-dependent detoxification and Calvin cycle) and defense response (reduction-mediated retrograde signaling). Colored arrows indicate the heighten passage of electron transfers, proposed in this (orange) and previous3 (red) studies, during stress defense responses.

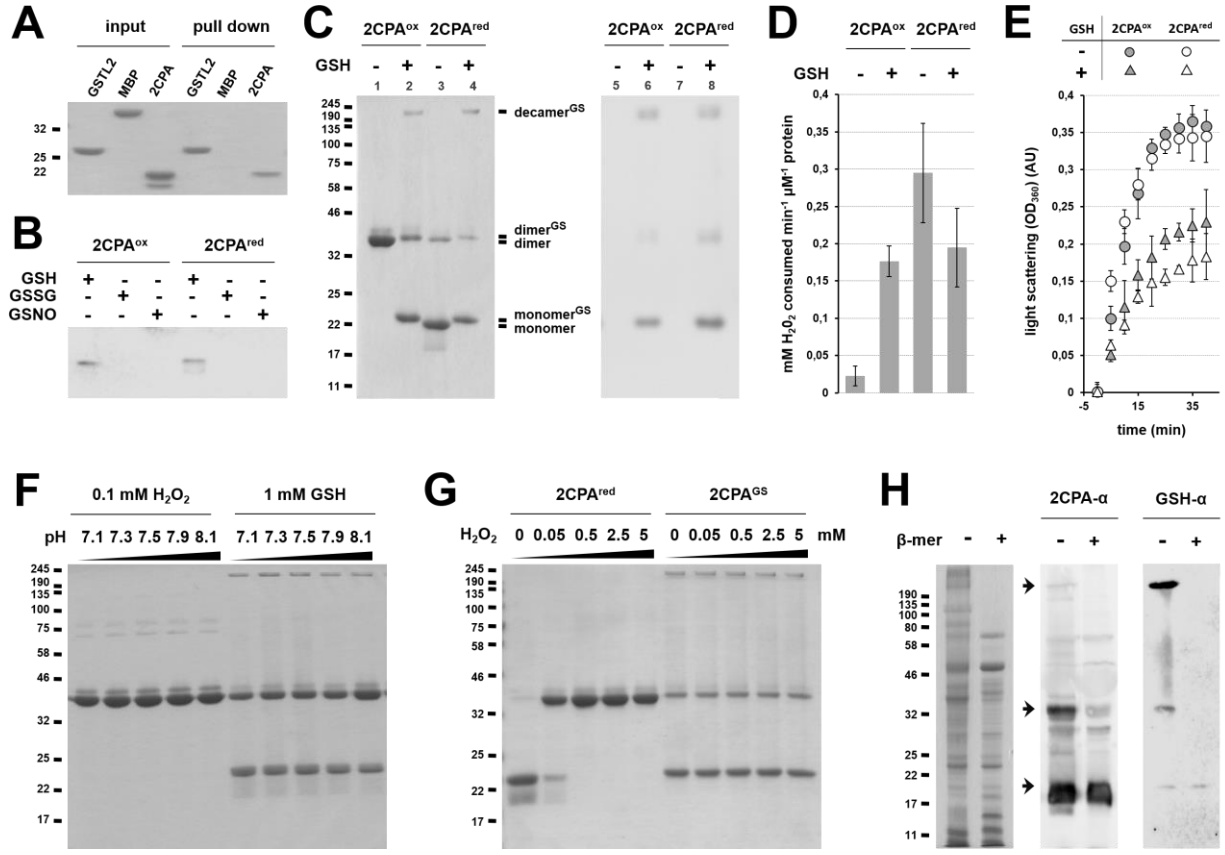

Figure 3: GSH-binding determines the quaternary structures and enzymatic activities of 2CPA. (**A**) Binding affinity of 2CPA to GSH-agarose beads. The same amount of 2CPA, GSH S-transferase λ2 (GSTL2, positive control) and maltose-binding protein (MBP, negative control) (input) was subjected to GSH-affinity column, and bound proteins were eluted competitively with 10 mM GSH and visualized by SDS/PAGE. (**B**) GSH-dependent S-glutathionylation of 2CPA. Oxidized (^{ox}) and reduced (^{red}) 2CPAs were incubated with 1 mM GSH, GSSH or GSNO at 25 ^{o}C for 30 min, and subjected to immunoblot (IB) analysis with anti-GSH antibody. (**C**) Quaternary structures of S-glutathionylated (^{GS}) 2CPA. The 2CPA$^{ox/red}$ incubated with/without 1 mM GSH were subjected to nonreducing SDS/PAGE (*left panel*) and IB analysis with anti-GSH antibody (*right panel*). (**D**) Peroxidase activity of 2CPA^{GS}. Reduction of H_2O_2 was measured using the eFOX reagents by a spectrophotometer at 560 nm. (**E**) Chaperone activity of 2CPA^{GS}. The light scattering due to thermal aggregation at 45 ^{o}C of citrate synthase (1 µM) was visualized spectrophotometrically at 360 nm. (**F**) Oxidation and S-glutathionylation of 2CPA^{red} at various pH conditions (7.1 to 8.1) for 30 min at 25 ^{o}C. (**G**) Oxidation rates of 2CPA^{red} and 2CPA^{GS} at various concentrations of H_2O_2 for 10 min at 25 ^{o}C. (**H**) IB analyses of total protein extracts against anti-2CPA (*middle panel*) and anti-GSH (*right panel*) antibodies. (**F** to **H**) Proteins were subjected to nonreducing SDS/PAGE. β-mer, β-mercaptoethanol. α, antibody.

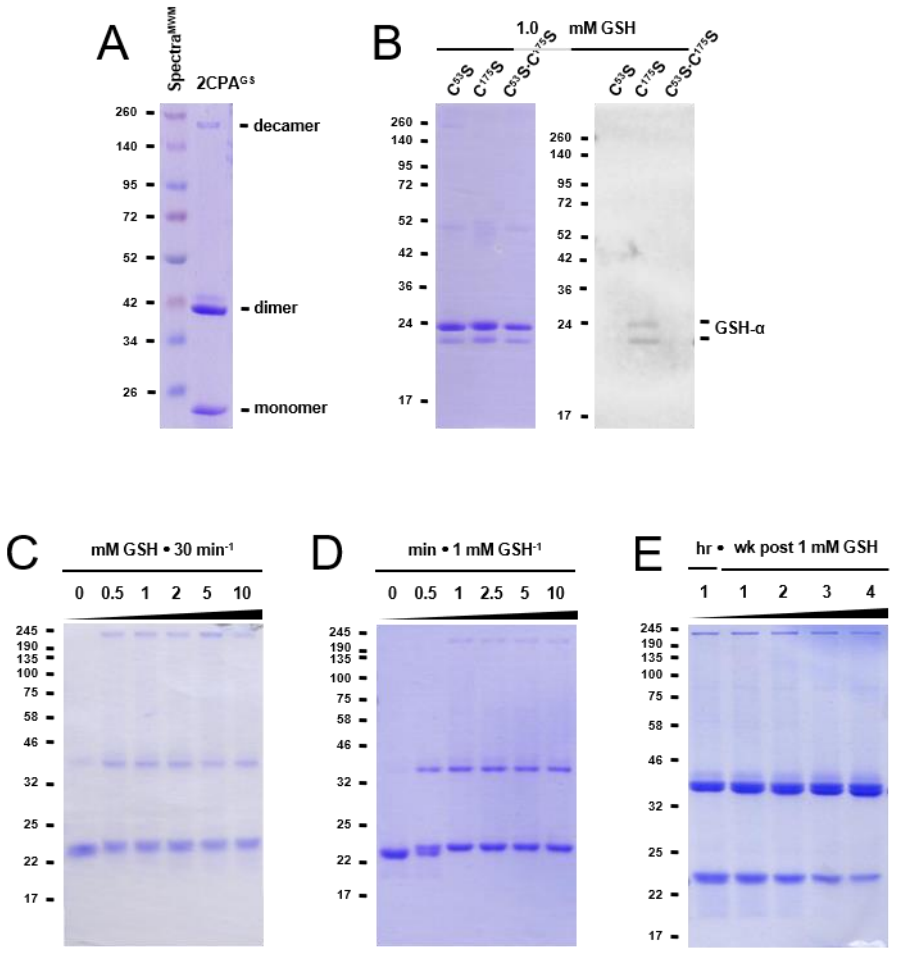

Figure 4. GSH-dependent S-glutathionylation of 2CPA. **(A)** Determination of the quaternary structure of $2CPA^{GS}$. Molecular weights of three major quaternary structures, monomer (~20 kDa), dimer (~40 kDa) and decamer (~200 kDa), of $2CPA^{GS}$ were comparatively determined in reference to the Spectra Broad Range Protein Ladder (left lane, Thermo Scientific) in non-reducing SDS/PAGE. (B) Determination of GSH-binding Cys residue(s) in 2CPA. Cys mutagenized 2CPAs ($C^{53}S$, $C^{175}S$ and $C^{53}S{\cdot}C^{175}S$) incubated with 1 mM GSH at 25 ºC for 30 min were separated in nonreducing SDS/PAGE (*left panel*) and im-munoblot (IB) analysis with anti-GSH antibody (*right panel*). (C) S-glutathionylation rates of $2CPA^{red}$ at various GSH concentrations for 30 min at 25 ºC. (D) S-glutathionylation rates of $2CPA^{red}$ at various times by 1 mM GSH at 25 ºC. (E) Stability of the quaternary structure of $2CPA^{GS}$ over time. After being incubated with 1 mM GHS for 30 min at 25 ºC, $2CPA^{GS}$ was di-alyzed against 50 mM Tris-HCl, pH 7.5, and stored at 4 ºC for 1 hr to 4 wks. (C to D) The $2CPAs^{GS}$ were subjected to nonreducing SDS/PAGE.

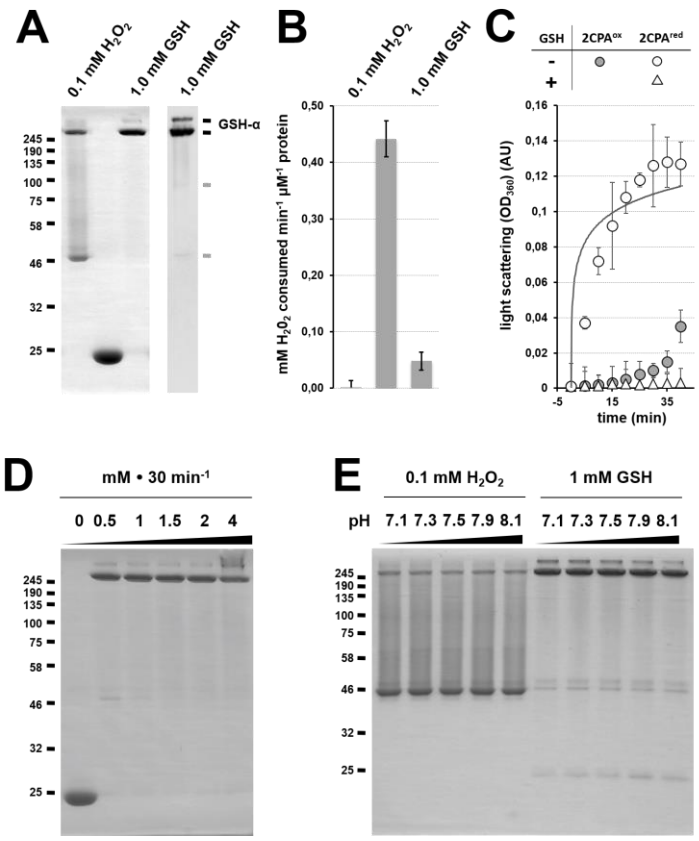

Figure 05: GSH-binding stimulates the oligomerization and chaperone activity of 2CPB. (**A**) Quaternary structures of $2CPB^{GS}$. At 25 ºC, 2CPB was incubated with 0.1 mM H_2O_2 for 10 min, or 20 mM TCEP-HCl or 1 mM GSH for 30 min (*left panel*). The $2CPB^{GS}$ was subjected to IB analysis with anti-GSH antibody (*right panel*). (**B**) Peroxidase activity of $2CPB^{GS}$. Reduction of H_2O_2 was measured using the eFOX reagents by a spectrophotometer at 560 nm. (**C**) Chaperone activity of $2CPB^{GS}$. The light scattering due to thermal aggregation at 45 ºC of citrate synthase (1 µM) was visualized spectrophotometrically at 360 nm. (**D**) S-glutathionylation of 2CPB at various GSH concentrations for 30 min at 25 ºC. (**E**) Oxidation and S-glutathionylation of 2CPB at various pH conditions (7.1 to 8.1) for 30 min at 25 ºC.

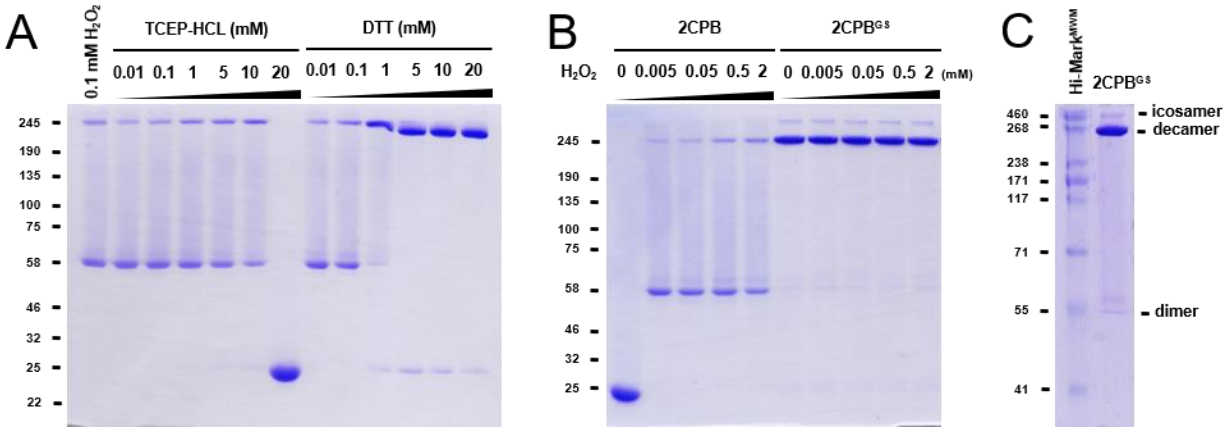

Figure 06: GSH-dependent S-glutathionylation oligomerizes 2CPB. (A) Rearrange of quaternary structure of 2CPB^{ox} upon the increased concentrations of reducing agents, tri(2-caboxyethyl)phosphine hydrochloride (TECEP-HCl, *left*) and dithiothreitol (DTT, *right*) for 30 min at 25 ^{o}C. (B) Oxidation rates of 2CPA^{red} and 2CPA^{GS} at various concentrations of H_2O_2 for 10 min at 25 ^{o}C. (C) Determination of the quaternary structure of 2CPB^{GS}. Molecular weights of three major quaternary structures, di-mer (~46 kDa), decamer (~250 kDa) and icosamer (~500 kDa), of 2CPB^{GS} were comparatively determined in reference to the HiMark Protein Standard (left lane, Invitrogen) in nonreducing SDS/PAGE. (A to C) Proteins were subjected to nonreducing SDS/PAGE

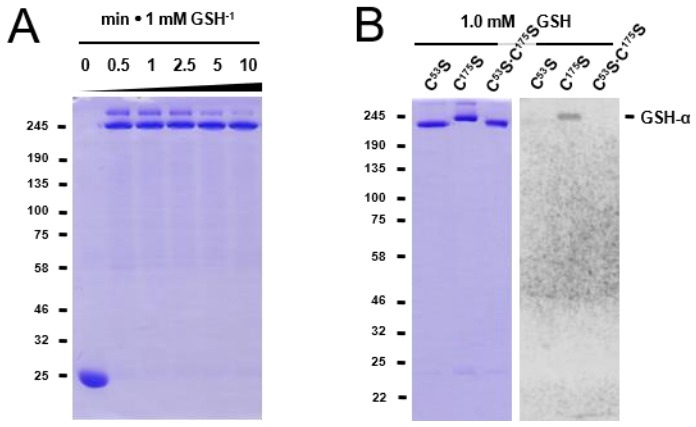

Figure 07: GSH binds to the Cys_R^{53} residue of 2CPB. (**A**) S-glutathionylation rates of $2CPB^{red}$ at various times by 1 mM GSH at 25 ºC. (**B**) Determination of GSH-binding Cys residue(s) in 2CPB. Cys mutagenized 2CPBs ($C^{53}S$, $C^{175}S$ and $C^{53}S{\cdot}C^{175}S$) incubated with 1 mM GSH at 25 ºC for 30 min were separated in nonreducing SDS/PAGE (*left panel*) and IB analysis with anti-GSH antibody (*right panel*). The $2CPBs^{GS}$ were subjected to nonreducing SDS/PAGE.

```
2CPA      AQADDLPLVGNKAPDFEAEAVFDQEFIKVKLSDYIGKKYVILFFYPLDFTFVCPTEITAF 60
2CPB      AQADDLPLVGNKAPDFEAEAVFDQEFIKVKLSEYIGKKYVILFFYPLDFTFVCPTEITAF 60
          ********************************:***************************

2CPA      SDRHSEFEKLNTEVLGVSVDSVFSHLAWVQTDRKSGGLGDLNYPLISDVTKSISKSFGVL 120
2CPB      SDRYEEFEKLNTEVLGVSVDSVFSHLAWVQTDRKSGGLGDLNYPLVSDITKSISKSFGVL 120
          ***:.*****************************************:**:************

2CPA      IHDQGIALRGLFIIDKEGVIQHSTINNLGIGRSVDETMRTLQALQYIQENPDEVCPAGWK 180
2CPB      IPDQGIALRGLFIIDKEGVIQHSTINNLGIGRSVDETMRTLQALQYVQENPDEVCPAGWK 180
          * ****************************************:**************

2CPA      PGEKSMKPDPKLSKEYFSAI    200
2CPB      PGEKSMKPDPKLSKEYFSAI    200
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Figure 08: Clustal omega amino acids sequence comparassion of 2CPs proteins.

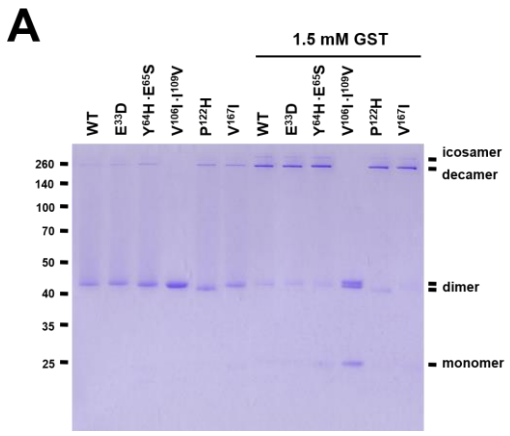

Figure 9: The amino acids V^{106} and I^{109} are responsible for 2CPB decamer/icosamerization: Mutagenized 2CPB (2CPB$E^{33}D$, 2CPB$Y^{64}H$:$E^{65}S$, 2CPB$V^{106}I$:$I^{109}V$, 2CPB$P^{122}H$, and 2CPB$V^{167}I$) with (right*)* and without (l*eft*) 1.5 mM GSH incubation at 25 ^{o}C for 30 min were separated in nonreducing 10% SDS/PAGE

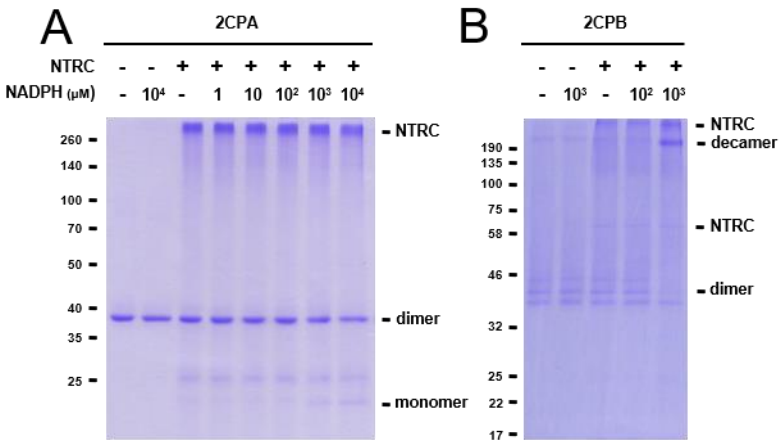

Figure 10: Free 2CPs reduction by NTRC. (A) Coomassie blue-stain gel of 2CPA (A) and 2CPB (B) incubated for 1 hour with NTRC with various NAPH concentrations at 25 ^{o}C.. Proteins were subjected to nonreducing 10% SDS/PAGE

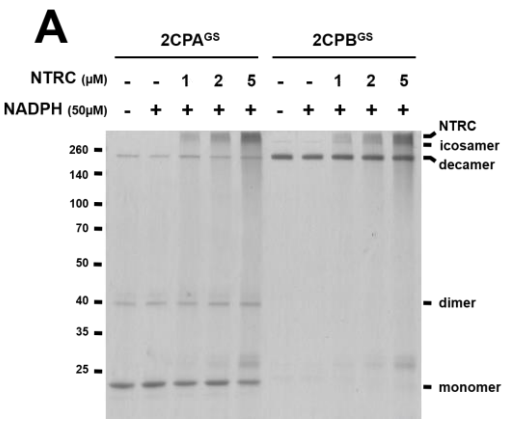

Figure 11: S-glutathionylation regulates enzymatic activity of NTRC towards 2CPs. (A) 2CPs were incubated with various concentration of NTRC and 50 uM NADPH for 1 hour at 25 ^{o}C and proteins were subjected to nonreducing 10% SDS/PAGE.

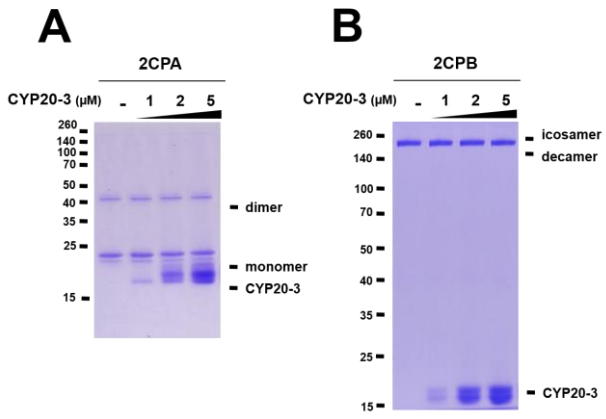

Figure 12: Enzymatic activity of CYP20-3 towards 2CPs. S-glutathionylated 2CPA and 2CPB proteins were incubated with various concentration of CYP20-3 at 25^0C and subjected to nonreducing SDS/PAGE gel.

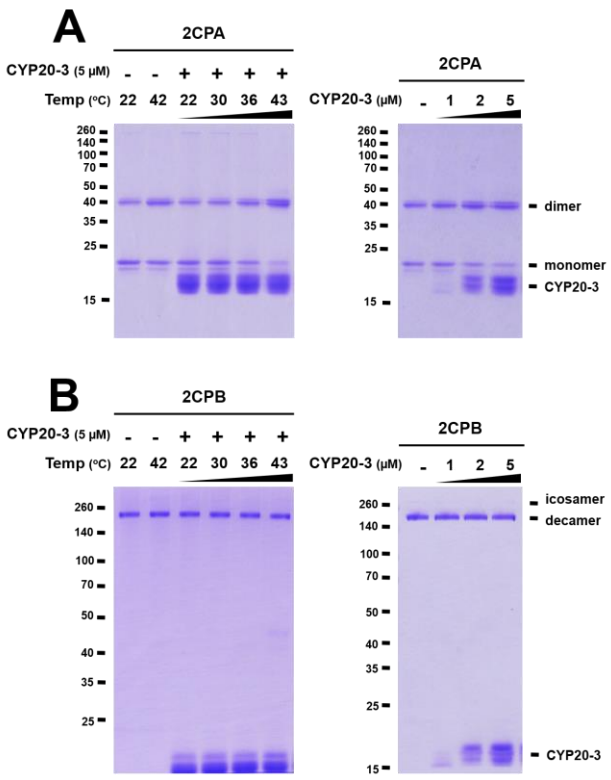

Figure 13: Heat-shock activity of CYP20-3 towards 2CPs. S-glutathionylated 2CPA (A) and 2CPB (B) proteins with 5 uM CYP20-3 were incubated in a gradient of temperature of 22, 30, 36, and 43 ^{0}C and subjected to nonreducing SDS/PAGE gel.

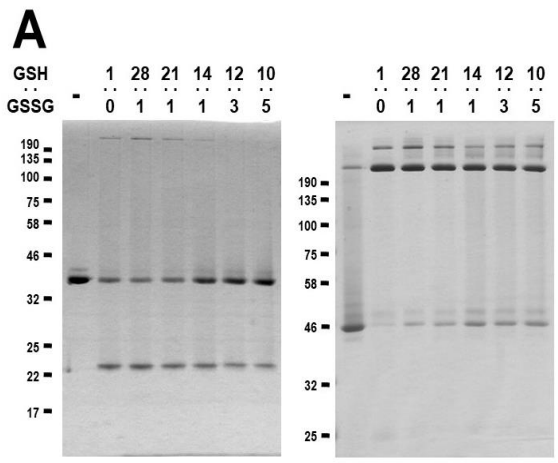

Figure 14: GSH:GSSG -dependent regulation of 2CPs quartenary structure. 2CPA and 2CPB were incubated with various GSH:GSSG ratio for 30 min at 25 ^{0}C and subjected to nonreducing SDS/PAGE gel.