A cyclophilin links redox and light signals to cysteine biosynthesis and stress responses in chloroplasts

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Cyclophilins belong to a large family of enzymes called "peptidyl prolyl isomerases" that assist protein folding and assembly. The cyclophilin CYP20–3 (also known as "ROC4") is the only member of this group located in the stroma (soluble phase) of chloroplasts. In the present study we isolated mutant *Arabidopsis* plants defective in the *CYP20–3* gene and found them to be hypersensitive to oxidative stress conditions created by high light levels, rose bengal, high salt levels, and osmotic shock. Chloroplast serine acetyltransferase (SAT1), a rate-limiting enzyme in cysteine biosynthesis, was identified as an interacting partner for CYP20–3 by protein interaction analyses. In the present experiments, SAT1 activity increased significantly under conditions of light and oxidative stress in concert with total thiols in wild-type plants. By contrast, these parameters changed only marginally in experiments with the *cyp20–3* mutant, suggesting that CYP20–3 links light and stress to SAT1 activity and cysteine biosynthesis. In further support of this conclusion, our analyses showed that the salt-hypersensitive phenotype of the mutant developed under illumination and not in the dark. Together with the earlier report that CYP20–3 foldase activity is enhanced by thioredoxin-mediated reduction, our findings suggest that CYP20–3 links photosynthetic electron transport and redox regulation to the folding of SAT1, thereby enabling the cysteine-based thiol biosynthesis pathway to adjust to light and stress conditions.

immunophilin | oxidative stress | photosynthesis | thioredoxin

Originally defined as receptors for immunosuppressants, immunophilins consist of two large groups of proteins called "cyclophilins" (receptors for cyclosporine A, CYPs) and FK506-binding proteins (FKBPs) (1). Immunophilins later were found in a wide range of organisms, from bacteria to plants and mammals, and are located in virtually all subcellular compartments. Most immunophilins possess peptidyl prolyl isomerase activity, catalyzing a rate-limiting step in protein-folding processes (2).

The *Arabidopsis* immunophilin family consists of 29 cyclophilin and 22 FKBP isoforms distributed in various cellular locations (3). Perhaps most striking is the finding that the largest group of immunophilins is located in the chloroplast, a plant-specific organelle, thereby explaining why plants have many more immunophilins than animals. Interestingly, a large majority of chloroplast immunophilins (6 CYPs and 11 FKBPs) are predicted to be located in the thylakoid lumen. However, one member, CYP20–3, designated as "ROC4," is found in the stroma (4). CYP20–3 is a single-domain cyclophilin isoform encoded by the nuclear genome. The *CYP20–3* gene is expressed only in photosynthetic tissues and is strongly induced by light, consistent with earlier observations on a chloroplast CYP of *Vicia faba* (5). In a biochemical analysis, the PPIase activity associated with CYP20–3 was shown to depend on reduction by thioredoxin (Trx) after the enzyme was treated with an oxidant ($CuCl_2$) (6). The results suggest that CYP20–3 may be regulated via thiol–disulfide exchange and play a role in protein folding and assembly in response to light and redox changes. In pursuing its function, we obtained evidence for a role for CYP20–3 in cysteine biosynthesis taking place in the chloroplast stroma.

In the cysteine biosynthesis pathway, inorganic sulfate is acquired by roots and is translocated to leaves where it is reduced and metabolized (7). The assimilation of sulfate results in the synthesis of L-cysteine, the precursor for the biosynthesis of a numerous sulfur-containing metabolites such as methionine and glutathione. The rate-limiting step of cysteine biosynthesis is accomplished in two sequential reactions catalyzed by the terminal enzymes of the pathway, serine acetyltransferase (SAT, EC 2.3.1.30) and O-acetylserine(thiol)lyase (OASTL, EC 4.2.99.8). SAT is responsible for the acetylation of L-serine by acetyl-CoA to produce O-acetyl-serine (OAS), whereas OASTL catalyzes the subsequent formation of cysteine from OAS and hydrogen sulfide. In land plants, the SAT and OASTL enzymes associate to form the cysteine synthase complex that has been identified in several plant species, including *Arabidopsis* (7).

The *Arabidopsis* SAT family consists of five members, three in the cytosol, one in chloroplast stroma, and one in mitochondria (8–10). It has been proposed that the SAT activity of chloroplasts and mitochondria is regulated through the assembly and maintenance of the cysteine synthase complex (11). The SAT protein is unstable when not associated with OASTL, thus enabling the formation of the complex to serve as a mechanism for regulating cysteine biosynthesis (7). We now report evidence that the stromal cyclophilin CYP20–3 interacts with the chloroplast isoform of SAT and is required for a fully functional enzyme and normal accumulation of thiol compounds. Our findings suggest that CYP20–3 provides a mechanism for linking cysteine biosynthesis to light, redox and stress.

Results and Discussion

The *cyp20–3* Mutant is Hypersensitive to Oxidative Stress. To examine the function of CYP20–3 in chloroplasts, we analyzed a transferred DNA (T-DNA) insertional mutant in the *CYP20–3* gene (At5g62030). Sequence analysis showed the T-DNA insertional site to reside in the fifth exon, 491st bp after ATG (Fig. 1*A*). This insertion disrupted the expression of *cyp20–3* (Fig. 1*B*). Homozygous *cyp20–3* mutant plants were transformed with a 3.1-kb genomic DNA fragment containing the complete coding region and putative promoter of the *cyp20–3* gene (Fig. 1*A*). Restored expression of the *cyp20–3* gene is shown in homozygous complemented plants (Fig. 1*B*).

To evaluate the consequences of inactivation of the gene, we examined the *cyp20–3* mutant and wild-type plants under normal growth conditions throughout their life cycle and observed no significant phenotypic differences (data not shown). We found, however, that *cyp20–3* mutant plants when grown under stress conditions were hypersensitive to various stress treatments, including high light that damages plants by producing reactive oxygen species (ROS) (Fig. 1*C*).

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The authors declare no conflict of interest.

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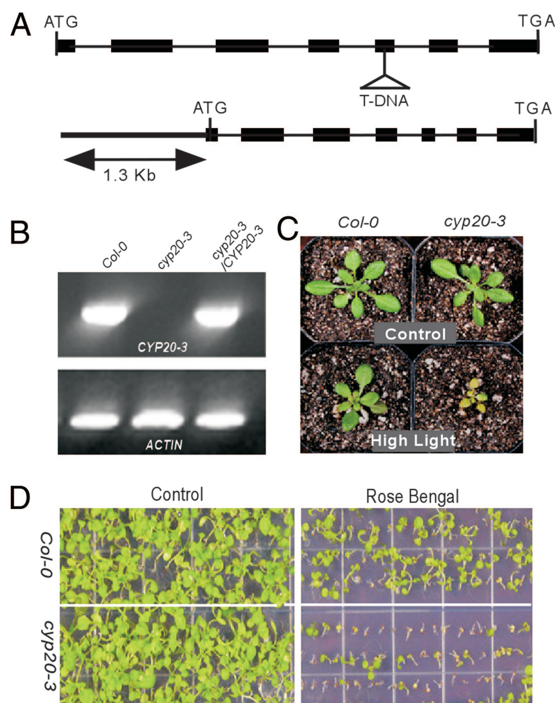

Fig. 1. The *cyp20–3* mutants are hypersensitive to high light and rose bengal. (*A Upper*) Intron–exon organization of the *Arabidopsis CYP20–3* coding region and T-DNA location. (*A Lower*) The genomic DNA fragment used for complementation. Solid boxes and lines indicate exons and introns, respectively. The position of the T-DNA insertion is indicated by a triangle (not to scale). The promoter region is highlighted by a thicker line. (*B*) RT-PCR analysis of *CYP20–3* transcripts in wild-type (*Col-0*), mutant (*cyp20–3*), and a complementation line (*cyp20–3/CYP20–3*). *ACTIN-2* expression level was determined as a quantification control. (*C*) Inhibition of growth of young *cyp20–3* mutant and (*Col-0*) wild-type seedlings by high light. (*D*) Effect of ROS generated by rose bengal on growth of wild-type (*Col-0*) and *cyp20–3* mutant plants. Seeds were plated on MS agar medium without or containing 3 μM rose bengal and were incubated at 4°C for 4 days before being transferred to 23°C. The photographs were taken on the seventh day after the transfer.

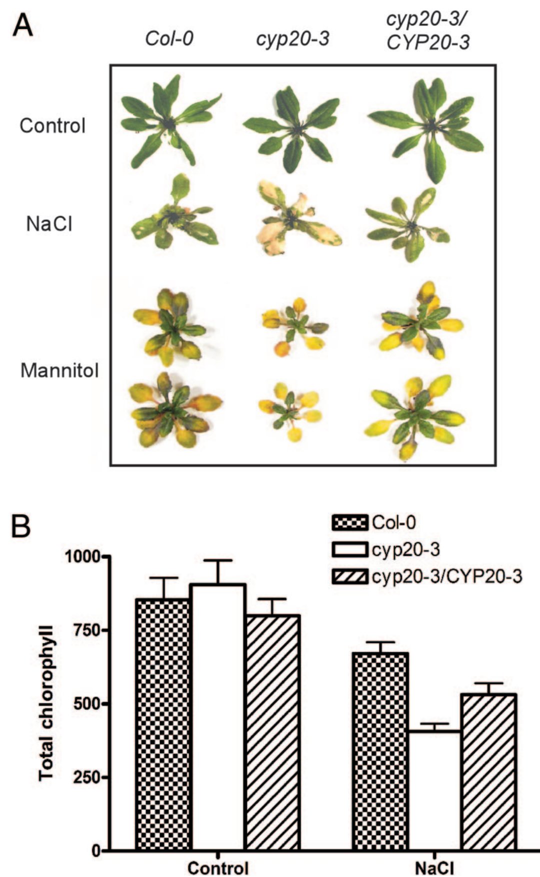

Fig. 2. *cyp20–3* Mutant plants are hypersensitive to salt and mannitol. (*A*) Plants were watered with NaCl or mannitol and photographed on the seventh day. (*B*) Chlorophyll content was measured with NaCl-treated plants in three independent experiments.

We therefore analyzed the *cyp20–3* mutant under other conditions that produce oxidative stress. First, we tested response to rose bengal, a light-dependent inducer of ROS. Rose bengal is a water-soluble xanthene dye that forms singlet oxygen after absorbing light of a certain wavelength (12). Fig. 1*D* shows that germination and growth of *cyp20–3* mutant seedlings were inhibited significantly when seeds were plated on Murashige and Skoog (MS) medium supplemented with 3 μM rose bengal. Such a result is consistent with a defect in response to oxidative stress.

Abiotic stress conditions such as elevated salt levels and osmotic shock also affect plant growth by producing ROS. When subjected to these stresses, the *cyp20–3* mutant showed, relative to the wild-type and complemented lines, a response similar to that observed with high light levels. When 2-week-old soil-grown plants were treated with 250 mM NaCl, loss of chlorophyll was observed in the leaves of all plants. However, the leaves of the *cyp20–3* mutant were most severely damaged, as indicated by loss of chlorophyll (Fig. 2*A*, *B*). Similarly, osmotic stress treatment (350 mM mannitol) severely inhibited growth and induced chlorophyll loss in all plants (Fig. 2*A*). Once again, however, the *cyp20–3* mutant suffered the most.

CYP20–3 Physically Interacts with SAT1. In their function as chaperones (or in related regulatory capacities), immunophilins typically interact with their target proteins (13, 14). To test this possibility with CYP20–3, we used a yeast two-hybrid screening system that earlier proved successful in the identification of an AtFKBP13 target protein (15). Several positive clones were isolated and sequenced. Interestingly, these clones represented different-length versions of a single cDNA species encoding the chloroplast isoform of serine acetyltransferase (SAT1), a regulatory member of the cysteine biosynthesis pathway (16). To confirm the interaction between CYP20–3 and SAT1, we cloned the full-length SAT1 cDNA into the pAD vector and conducted yeast two-hybrid assays. Yeast cells that carried both the pBD-CYP20–3 and pAD-SAT1 plasmids grew well on the selection medium (synthetic medium lacking Leu and Trp, SC-LTH), indicating interaction of SAT1 with CYP20–3 (Fig. 3*A*). In contrast, cells co-transformed with the empty pBD vector and pAD-SAT1 or with the empty pAD vector and pBD-CYP20–3 failed to express the reporter gene (Fig. 3*A*). These results indicate that the full-length SAT1 interacts with CYP20–3 in the yeast two-hybrid system.

To corroborate the interactions observed in the yeast two-hybrid assays, we performed an *in vitro* protein–protein interaction assay. CYP20–3 and SAT1 were co-expressed in *Escherichia coli* as a GST fusion protein and a 6xHis-tagged protein, respectively. For the interaction assay, the two proteins were coexpressed in the same *E. coli* strain, and both were immobilized on glutathione beads. Using anti-His antibody as a probe, we found that the SAT1 protein co-purified with the GST-CYP20–3 fusion protein but not with GST (Fig. 3*B*, lanes 2 and 3 vs. lane 4), suggesting interaction between CYP20–3 and SAT1. This co-purification procedure confirmed the yeast two-hybrid results in demonstrating interaction between CYP20–3

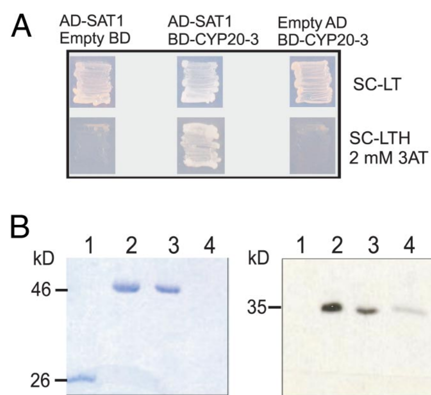

Fig. 3. The CYP20–3 protein physically interacts with SAT1. Interaction of CYP20–3 and SAT1 proteins as determined by yeast two-hybrid system (*A*) or by *in vitro* protein-protein interaction assay (*B*). pBD and pAD empty vectors were used as negative controls in the yeast-growth assay, and results from one representative experiment of three duplicates are shown in A. The left panel of B shows a Coomassie blue-stained gel indicating the amount of bait proteins used in each pull-down assay. The GST-CYP20–3 fusion protein was used as a bait to pull down the SAT1-His protein (lanes 2 and 3 are duplicates). For a negative control (lane 1), the GST protein was used as a bait. Purified SAT1 protein was loaded as positive control (lane 4). The right panel of B shows a parallel immunoblot of proteins that co-purified with GST or GST-CYP20–3 probed with rabbit anti-His antibody. The approximate molecular mass (kD) of SAT1-His is shown on the left (35 kD).

and SAT1 proteins, both of which are located in the chloroplast stroma.

Disruption of CYP20–3 Reduces SAT Activity and Thiol Content in Mutant Plants. Because of its role in catalyzing the final step of cysteine biosynthesis, SAT is located at a critical junction connecting sulfur assimilation and amino acid metabolism in leading to the synthesis of key sulfur metabolites such as glutathione (γ-Glu-Cys-Gly tripeptide). As the most abundant soluble thiol metabolite in plants, glutathione serves as both a storage and transport form of sulfur. Glutathione also functions in enzyme regulation via redox change and in protection of proteins against oxidative damage brought about by different stresses that produce ROS (17). The combined finding of an interaction between CYP20–3 and SAT1 and of the stress sensitivity of the *cyp20–3* mutant suggested that the association of the two proteins could influence SAT1 activity and thus the ability of chloroplasts to accumulate thiols and respond to oxidative stress. We tested this possibility by measuring SAT1 activity in the three types of plants grown under normal and stress conditions.

Under normal conditions, the *cyp20–3* mutant showed a modest reduction in SAT activity compared with wild-type and complemented lines (Fig. 4*A*). When subjected to salt stress, each of the three types of plants showed an increase in activity of SAT (Fig. 4*A*). However, the increase in activity was much less in the *cyp20–3* mutant than in wild-type and complemented lines.

It is known that change in SAT activity often leads to alteration in thiol content (18). This change affects the ability of plants to cope with oxidative stress resulting from abiotic stresses, including those tested in this study. In view of this relationship, we measured the total thiol content of mutant, wild-type, and complemented plants and observed differences similar to those seen with SAT activity (Fig. 4*B*). The results of both the SAT enzyme and thiol analyses suggested that the sensitivity of the *cyp20–3* mutant to salt can be explained, at least in part, by a reduction in its ability to accumulate essential

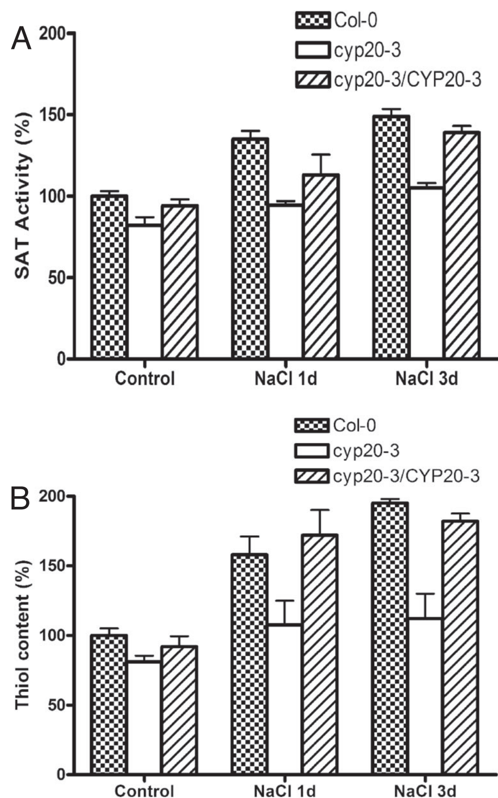

Fig. 4. Serine acetyltransferase activity and thiol contents are altered in the *cyp20–3* mutant plants. Serine acetyltransferase (SAT) activity (*A*) and total non-protein thiol content (*B*) were measured in protein fractions extracted from wild-type (Col-0), mutant (*cyp20–3*), and complemented (*cyp20–3*/CYP20–3) plants. Total protein extracts were prepared from leaves of 2-week-old plants before and 1 and 3 days after treatment with 250 mM NaCl. The values shown represent the averages of three independent experiments; the error bars represent standard deviations.

soluble thiols to mitigate the induced stress. These findings, which are consistent with earlier work linking salt stress with increased thiols (19, 20), also provide evidence that the observed interaction of CYP20–3 with SAT1 is functionally relevant. Finally, although mitochondria have been reported to contain the bulk of the cell's SAT activity (21, 22), the present work suggests that the enzyme residing in chloroplasts (~10% of total) is required for response to stress.

CYP20–3 Is Required for Light-Dependent Stress Response. The synthesis and accumulation of soluble thiols during a normal day/night cycle is induced by light in addition to other stress conditions (23). An earlier study (6) showed that, after oxidation with $CuCl_2$, chloroplast CYP20–3 is reductively activated by Trx *m*. Because Trx *m* is linked to light, we suspected that CYP20–3 also might function in regulating thiol accumulation during the day/night cycle. We monitored thiol content in wild-type and *cyp20–3*-mutant plants diurnally. Leaves from 3-week-old plants were collected at several time points during a 16/8-h light/dark cycle (with light intensity of 150 μmol m^{-2} sec^{-1}) and were analyzed for soluble non-protein thiols. A diurnal pattern of thiol accumulation was observed in both the *cyp20–3* and wild-type plants, i.e., thiol content was higher in the light than in the dark (Fig. 5). The light-induced increment was compromised in mutant as compared with wild-type plants, however. The results suggest that CYP20–3 is required for the light-induced increase of thiol accumulation.

If CYP20–3 is, indeed, required for light-dependent thiol

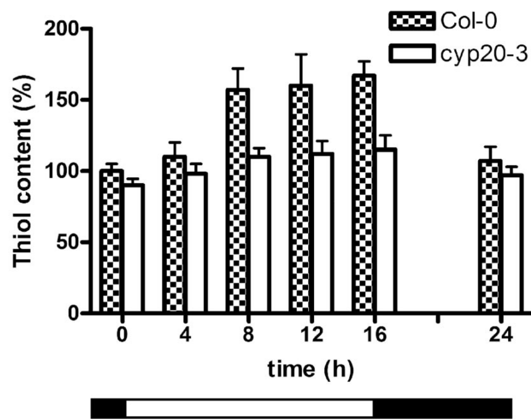

Fig. 5. Diurnal fluctuation of thiol content in wild-type and mutant plants. Leaves of 3-week-old wild-type (Col-0) and mutant (*cyp20–3*) plants grown in chambers with long-day conditions (16 h light/8 h dark) were used for measuring thiol content. Samples were harvested at the beginning (0 h) and at 4-h intervals during the day period. A sample also was harvested at the end of the dark cycle (24 h). The white and black bar lines below the figure indicate light and dark periods, respectively. The values shown represent the averages of three independent experiments; error bars represent standard deviations.

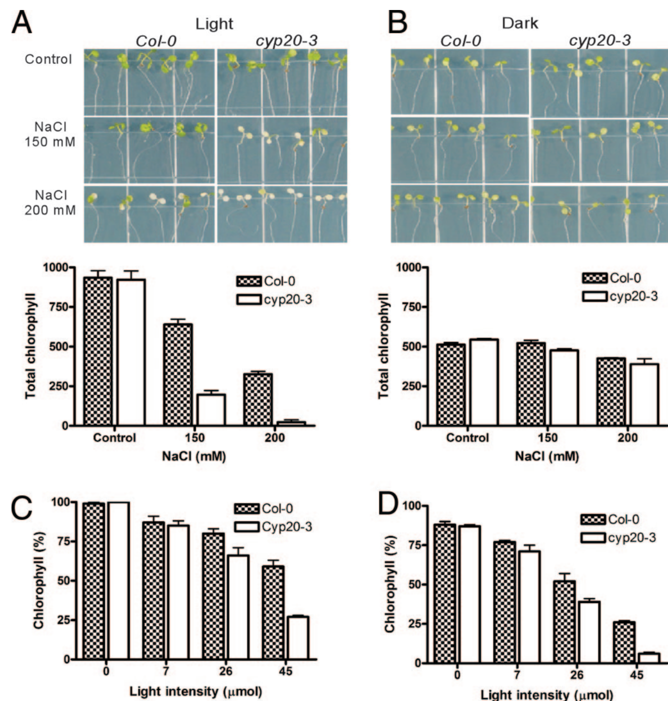

Fig. 6. Sensitivity of plants to NaCl is light regulated. Five-day-old wild-type and *cyp20–3* mutant seedlings grown on MS agar medium were transferred to the same medium containing 150 mM or 200 mM NaCl and grown under moderate light (45 μmol m^{-2} sec^{-1}) (*A*) or in darkness (*B*). The photographs were taken on the fifth day after transfer to the salt medium. Total chlorophyll content is shown below the photographs. (*C* and *D*) Five-day-old wild-type seedlings were transferred to MS medium with 150 mM NaCl (*C*) or 200 mM NaCl (*D*) and grown under different light intensities for 3 days. The bar graphs summarize data from three independent experiments; the error bars represent standard error. Ten plants were used to measure chlorophyll for each sample.

accumulation, the appearance of the stress-sensitive phenotype should depend on light (24). In an attempt to confirm this response, we determined the effect of light intensity on the salt tolerance of 5-day-old *cyp20–3* mutant and wild-type seedlings grown in MS medium (Fig. 6). When incubated for 5 d under low light (7 μmol m^{-2} sec^{-1}) or in darkness, neither the wild-type nor the mutant was significantly affected by the salt stress (Fig. 6*B–D*). However, under moderate light conditions (26 and 45 μmol m^{-2} sec^{-1}), the *cyp20–3* seedlings were significantly more sensitive to salt than their wild-type counterparts at either 150 mM NaCl (Fig. 6*C*) or 200 mM NaCl (Fig. 6*D*). When chlorophyll was measured in seedlings treated with the same concentration of NaCl but maintained under different light regimens (Fig. 6*C*, *D*), we found that light intensity affected the salt sensitivity of both wild-type and *cyp20–3* seedlings, but *cyp20–3* seedlings were significantly more salt sensitive than wild-type seedlings under moderate light conditions (Fig. 6*C*, *D*).

Concluding Remarks. It is well established that productive protein folding requires the assistance of foldases and molecular chaperones and that, in some cases, immunophilins serve in both capacities in the maintenance or assembly of "supermolecular complexes." Thus, in *Saccharomyces cerevisiae*, the FKPB protein, Fpr4, forms a complex with histones and facilitates nucleosome assembly in regulating rDNA silencing (25, 26). In the case of plants (*Arabidopsis*), immunophilins residing in the chloroplast thylakoid lumen, FKBP20–2 and CYP38, are required for accumulation of the PSII supercomplex (27, 28). The current study adds another immunophilin representative to the supercomplex list: CYP20–3 of the chloroplast stroma was found to function *in vivo* in assisting the folding or assembly of SAT1 enzyme to form the well-known hetero-oligomeric complex between cysteine synthase and OASTL, the cysteine synthase complex (7). In studying this complex, we uncovered a regulatory mechanism by which an immunophilin links light and redox signals to the regulation of cysteine biosynthesis in response to stress (Fig. 7). Our findings are consistent with the conclusion that ROS elicits two responses: the expression of SAT1 and, possibly in association with a peroxiredoxin (29), the oxidation of CYP20–3. Photoreduced Trx then reduces and activates CYP20–3, which, through its protein-folding capability, is needed for the formation of a functional cysteine synthase complex. Once a part of the complex, SAT1 is stabilized and in this way facilitates the biosynthesis of cysteine. Along with other antioxidants, the newly formed cysteine is essential for the biosynthesis of glutathione that enables the chloroplast to mitigate detrimental effects of ROS. It remains to be seen whether this mechanism applies to plants such as soybean in which chloroplast SAT is regulated by phosphorylation, a mechanism apparently absent in *Arabidopsis* (30). Another question that remains unanswered is whether, as indicated by proteomic studies (31), Trx also can interact directly with OASTL and in this way provide an alternate and more direct regulatory link to light.

Materials and Methods

Plant Materials. Seeds (*Arabidopsis* ecotype Col-0) were sown in soil and kept in darkness for 72 h at 4°C before being transferred to a greenhouse. The plants were grown under long-day conditions (16 h light/8 h dark) to flowering stage for the transformation procedure.

Atcyp20–3 insertional mutant (SALK_001615) was acquired from the Arabidopsis Biological Resource Center. Confirmation of null mutant was carried out by RT-PCR, using the gene-specific primer set. Expression levels of *ACTIN2* were monitored as a quantifying control using a pair of gene-specific primers.

For complementation of the *cyp20–3* mutant, a 3.1-kb genomic DNA fragment including the coding region of *CYP20–3* and 1.3 kb of the 5′ flanking region upstream from the starting codon was amplified by PCR from *Arabidopsis* genomic DNA and cloned into the binary vector pCAMBIA1300 (CAMBIA GPO). The constructs were introduced into the *Agrobacterium* strain GV3101 and then into the *Arabidopsis* plants by the floral-dip method (32). Transgenic seeds were plated on half-strength MS (33) medium containing 0.8% (wt/vol) agarose, 112 mg/L Gamborg's B5 vitamin mixture, 50 mg/ml kanamycin, and 15 mg/ml hygromycin. The resistant seedlings were transplanted to soil and grown in a greenhouse to produce seeds. Homozygous

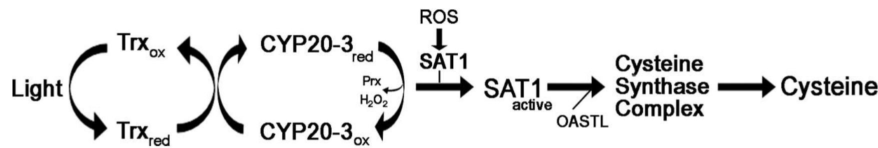

Fig. 7. A mechanism linking CYP20–3 to redox regulation and abiotic stress. Trx reduced by light via ferredoxin and ferredoxin-thioredoxin reductase reduces CYP20–3 which, in turn, interacts with SAT1, thereby enabling it to form a stable, catalytically active complex (cysteine synthase complex) with OASTL. The sequence of events is accentuated when SAT1 or another component of the system is oxidized by ROS resulting from abiotic stress. CYTP20–3 also can be oxidized catalytically by 2-Cys peroxiredoxin B in the presence of H_2O_2, thereby down-regulating the system.

complemented lines (designated ''*cyp20–3*/CYP20–3'') were used for further analysis. All PCR procedures were performed using Pfu DNA polymerase (Stratagene) to enhance fidelity. In each case constructs were verified by DNA sequencing.

Stress Treatments. To study the diurnal changes in thiol content, leaf samples from 3-week-old plants were collected, frozen with liquid nitrogen, and stored at −80°C until use. For salt stress assays carried out under different light conditions, sterilized seeds were plated on MS medium solidified with 0.8% (wt/vol) agar. After 5 days' growth in the vertical position under white light (45 μmol m^{-2} sec^{-1}), seedlings were transferred to MS medium supplemented with NaCl and kept under the indicated light conditions. Seedlings were photographed and chlorophyll content was measured on the third or fifth day. For high-light treatments, 3-day-old seedlings grown under normal light were transferred to high-intensity light (300 μmol m^{-2} sec^{-1}) for 6 days. For the salt and mannitol assays, 2- and 3-week-old plants, grown in the soil, were watered with 250-mM NaCl or 350-mM mannitol solutions, respectively, every 3 days and monitored for 2 weeks for loss of chlorophyll in the leaves. Leaves were weighed and photographed and chlorophyll content was measured on the seventh day. For rose bengal treatment, ≈100 seeds from the wild-type (*Col-0*), and *cyp20–3* mutant lines were plated on MS medium supplemented with 3 μM of rose bengal, incubated at 4°C for 4 days, and then placed at 23°C under long-day conditions. Photographs were taken on the seventh day. For the salt/mannitol and rose bengal treatments, light intensity was 45 μmol m^{-2} sec^{-1}.

SAT Activity Assay, Thiol Content Measurement, and Chlorophyll Extraction. Serine acetyltransferase activity was assayed as described earlier (34). Briefly, plant material was ground using a mortar and pestle with liquid nitrogen followed by homogenization in 25 mM phosphate buffer (pH 7.5). The homogenate was centrifuged at 15,000 *g* for 15 min at 4°C, and the resulting supernatant was assayed for enzyme activity.

Water-soluble thiols were determined as described (35). Chlorophyll was extracted according to (36) and quantified using equations by Arnon (37).

Yeast Two-Hybrid Screening and Assays. The *Arabidopsis* λ-ACT cDNA expression library (CD4–22) was obtained from the Arabidopsis Biological Resource Center. The two-hybrid library screening was carried out according to an earlier procedure (38). The plasmid library was obtained from the phage library by *in vivo* excision and was used to transform the Y190 strain that expressed a bait protein (CYP20–3). Transformants were plated onto the selection medium (SC-LTH). To inhibit background growth of yeast cells, 2.5 mM 3AT also was added to the medium. Colonies that appeared within a 5-day incubation period were selected for further analysis. After identifying the partial cDNAs of SAT1 as a positive clone from the library, interaction between the full-length CYP20–3 and SAT1 mature proteins was confirmed further by yeast two-hybrid assay. The cDNA regions encoding the mature CYP20–3 and SAT1 proteins were amplified from total leaf mRNA with Pfu polymerase and cloned into the binding domain (pGBKT7) and the activation domain (pGADT7-Rec) vectors, respectively, producing the plasmids pBD-CYP20–3 and pAD-SAT1. The pBD-CYP20–3 and the pAD-SAT1 plasmids were introduced into yeast strain Y190 by the lithium acetate method (39). After selection of transformants on the selection medium, three colonies were streaked on the SC-Leu-Try-His agar medium supplemented with 1.5 mM 3AT to score growth as an indicator of protein–protein interaction.

Expression of Recombinant Proteins and Protein–Protein Interaction Assays. The cDNA regions encoding the CYP20–3 and SAT1 mature proteins were amplified from total leaf mRNA and cloned into the pGEX-4T-3 (Amersham Biosciences) and pET28 vector, respectively, to produce GST-CYP20–3 fusion and His-tagged SAT1. The resulting clones were sequenced and co-transformed into the BL21 DE3 strain of *E. coli* (Novagen). Expression of both proteins was induced by 0.5 mM isopropyl-β-D-thiogalactopyranoside at 30°C for 4 h, and the proteins were co-purified using glutathione beads (15). Recovered proteins were subjected to SDS/PAGE and Western blot analysis using anti-His antibody. A chemiluminescence kit (Amersham Pharmacia Biotech) was used to detect the His-tagged SAT1 that co-purified with the CYP20–3-GST.

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