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The Deep Thioredoxome in *Chlamydomonas reinhardtii*: New Insights into Redox Regulation

María Esther Pérez-Pérez[1,3,4], Adeline Mauriès[1,3], Alexandre Maes[1], Nicolas J. Tourasse[2,5], Marion Hamon[1,2], Stéphane D. Lemaire[1,*] and Christophe H. Marchand[1,2,*]

[1]Institut de Biologie Physico-Chimique, UMR8226, CNRS, Sorbonne Universités, UPMC Univ Paris 06, 13 rue Pierre et Marie Curie, 75005 Paris, France

[2]Institut de Biologie Physico-Chimique, Plateforme de Protéomique, FRC550, CNRS, 13 rue Pierre et Marie Curie, 75005 Paris, France

[3]These authors contributed equally to this article.

[4]Present address: Instituto de Bioquímica Vegetal y Fotosíntesis, Consejo Superior de Investigaciones Científicas (CSIC)-Universidad de Sevilla, Seville, Spain

[5]Present address: ARNA Laboratory, INSERM U1212, CNRS UMR5320, Université Bordeaux 2, Bordeaux, France

***Correspondence: Stéphane D. Lemaire (stephane.lemaire@ibpc.fr), Christophe H. Marchand (christophe.marchand@ibpc.fr)**

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ABSTRACT

Thiol-based redox post-translational modifications have emerged as important mechanisms of signaling and regulation in all organisms, and thioredoxin plays a key role by controlling the thiol-disulfide status of target proteins. Recent redox proteomic studies revealed hundreds of proteins regulated by glutathionylation and nitrosylation in the unicellular green alga *Chlamydomonas reinhardtii*, while much less is known about the thioredoxin interactome in this organism. By combining qualitative and quantitative proteomic analyses, we have comprehensively investigated the *Chlamydomonas* thioredoxome and 1188 targets have been identified. They participate in a wide range of metabolic pathways and cellular processes. This study broadens not only the redox regulation to new enzymes involved in well-known thioredoxin-regulated metabolic pathways but also sheds light on cellular processes for which data supporting redox regulation are scarce (aromatic amino acid biosynthesis, nuclear transport, etc). Moreover, we characterized 1052 thioredoxin-dependent regulatory sites and showed that these data constitute a valuable resource for future functional studies in *Chlamydomonas*. By comparing this thioredoxome with proteomic data for glutathionylation and nitrosylation at the protein and cysteine levels, this work confirms the existence of a complex redox regulation network in *Chlamydomonas* and provides evidence of a tremendous selectivity of redox post-translational modifications for specific cysteine residues.

Key words: *Chlamydomonas reinhardtii*, disulfide bond, isotope-coded affinity tag, redox proteomics, redox regulation, thioredoxin targets

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INTRODUCTION

Living cells rely on a complex interplay among thousands of different molecules that maintain cellular integrity and morphology and perform numerous biological functions. Although protein functions are encoded in genes, the actual regulation of protein structure and function is generally executed by specific post-translational modifications (PTMs), which enable a gigantic heterogeneity and diversity of gene products. Cell and tissue types as well as environmental stimuli influence the way proteins are post-translationally modified. Thus, cell-specific patterns of PTMs will determine protein structure, subcellular localization, protein function, and interactions with other proteins. To cope with the numerous environmental challenges they may encounter, living cells have developed complex signaling networks and adaptive responses for which PTMs are key players (Choudhary et al., 2014). Consequently, studying the nature and mechanisms of signaling events is a large and crucial part of biological and medical research. A major challenge of contemporary biology is to map out, understand, and model, in quantifiable terms, the topological and dynamic properties of the protein networks that control the behavior of the cell (Barabasi and Oltvai, 2004; Pieroni et al., 2008; Kandpal et al., 2009).

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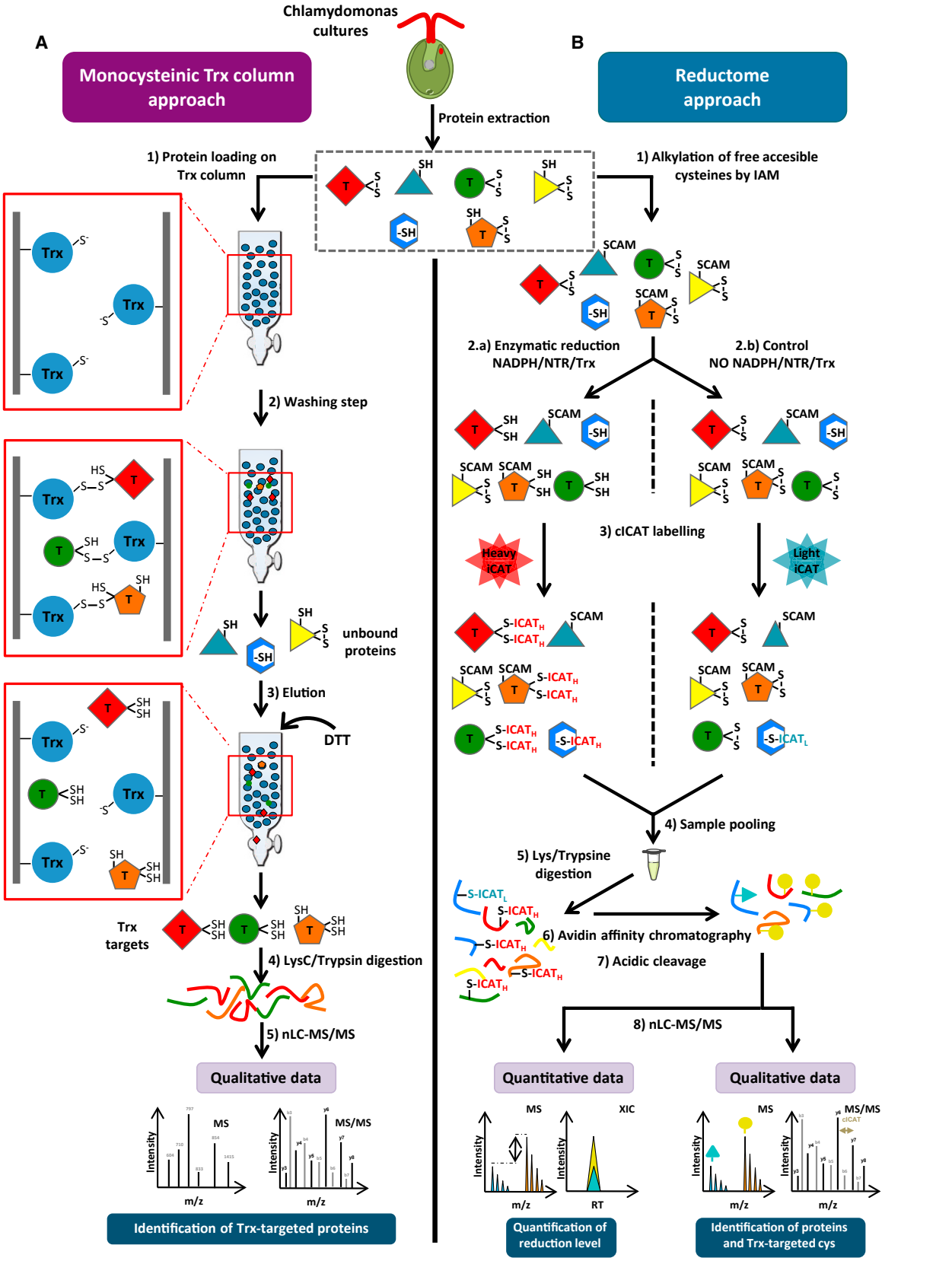

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Redox PTMs have emerged as important mechanisms of signaling and regulation in all organisms. Indeed, the thiol moiety of cysteine residues can evolve toward reversible redox states (e.g., sulfenic acid, intra- or inter-subunit disulfide bonds, S-cysteinylation, S-glutathionylation, S-nitrosylation) or can be irreversibly damaged into sulfinic and sulfonic acids (Couturier et al., 2013; Go et al., 2015). These thiol-based redox PTMs constitute molecular switches regulating protein functions and are themselves under the control of small oxido-reductases called thioredoxins (Trxs) and glutaredoxins (Hancock, 2009; Paulsen and Carroll, 2010; Go et al., 2015).

Trxs are evolutionary conserved proteins exhibiting a characteristic three-dimensional structure denominated "Trx fold" composed of five β strands surrounded by four α helices (Martin, 1995). Trxs contain a canonical disulfide active site (-WCG(P)PC-), which is at the origin of their thiol/disulfide oxidoreductase activity. Since its discovery in 1964 as hydrogen donor for ribonucleotide reductase in *Escherichia coli* (Laurent et al., 1964; Sengupta and Holmgren, 2014), the Trx system has been extensively studied and recognized as having multiple roles in cellular processes and human diseases (Buchanan et al., 2012; Hanschmann et al., 2013; Lee et al., 2013; Toledano et al., 2013). Non-photosynthetic organisms contain a limited number of Trxs (two in *E. coli*; three in *Saccharomyces cerevisiae*, and two in *Homo sapiens*) that are localized in the cytosol and mitochondria and are reduced by the NADPH-dependent flavoenzyme thioredoxin reductase (NTR). In photosynthetic organisms, Trxs are part of a large multigenic family (four Trxs in *Synechocystis*, 21 in the plant model *Arabidopsis thaliana* (At), and 10 in *Chlamydomonas reinhardtii*). They are classified according to their subcellular localization: f-, m-, x-, y-, and z-type Trxs are chloroplastic while o-type and h-type are found in mitochondria and cytosol (Lemaire et al., 2007; Meyer et al., 2012; Serrato et al., 2013). Whereas cytosolic and mitochondrial Trxs are reduced by NTRs, chloroplastic Trxs are specifically reduced by the ferredoxin-thioredoxin reductase which derives electrons from ferredoxin and the photosynthetic electron transfer chain (Schürmann and Buchanan, 2008; Jacquot et al., 2009; Michelet et al., 2013; Balsera et al., 2014). In photoautotrophic organisms, Trxs were originally highlighted for their capacity to modulate the activity of chloroplastic enzymes involved in carbon metabolism such as the Calvin–Benson cycle (CBC) enzyme fructose-1,6-bisphosphatase (FBPase) (Wolosiuk and Buchanan, 1977), NADP-malate dehydrogenase (NADP-MDH) (Jacquot et al., 1978), or glucose-6-phosphate dehydrogenase (Scheibe and Anderson, 1981). In the following years, a few additional proteins were also recognized as Trx-regulated targets such as proteins involved in the CBC or in its regulation, in light-dependent ATP production, or in diverse carbon metabolism pathways (Lemaire et al., 2007; Schürmann and Buchanan, 2008). All these enzymes are regulated by light through Trx-dependent reduction of disulfide bonds. Trxs were also recognized as electron donors for the regeneration of major antioxidant enzymes such as peroxiredoxins or methionine sulfoxide reductases (Perez-Perez et al., 2009; Lu and Holmgren, 2014; Kaya et al., 2015; Sevilla et al., 2015). The mechanisms of these Trx-dependent processes were investigated in detail at the molecular and structural level in different model systems. However, during the last 15 years, the development of proteomic studies considerably expanded the repertoire of Trx-target proteins. These studies were initiated within the field of plant biology and are mostly focused on photosynthetic organisms. We and other groups participated in this effort by developing proteomic approaches that led to the identification of hundreds of putatively redox-regulated proteins (Montrichard et al., 2009; Lindahl et al., 2011; Buchanan et al., 2012). Two main approaches were employed. The first one relies on our knowledge of the reaction mechanism of thiol-disulfide interchange occurring between reduced Trx and its oxidized target protein. It has been established that the N-terminal and most reactive cysteine of the Trx active site performs an initial nucleophilic attack on the disulfide bond of the target protein, leading to the formation of a transient mixed disulfide, i.e., a covalent disulfide-bonded heterodimer between the Trx and its target (Brandes et al., 1993; Holmgren, 1995). This heterodimer is further resolved by a second nucleophilic attack involving the C-terminal Trx active site cysteine. This attack allows releasing the oxidized Trx and the reduced target protein. Therefore, mutation of the second active site cysteine (resolving cysteine), into serine or alanine, allows to stabilize the heterodimer. Such a monocysteinic Trx variant can be used as bait to trap covalently bound targets. This strategy was used to purify Trx targets *in vivo* in yeast (Verdoucq et al., 1999) and more recently in human cells (Wu et al., 2014) and *E. coli* (Arts et al., 2016). The monocysteinic Trx can also be grafted on a chromatographic resin, and Trx-bound targets can be specifically eluted by adding a chemical reductant such as DTT and identified by mass spectrometry (MS) (Figure 1A). This approach was applied to numerous protein extracts from different photosynthetic prokaryotes (Lindahl and Florencio, 2003; Pérez-Pérez et al., 2006, 2009) and eukaryotes (Motohashi et al., 2001; Goyer et al., 2002; Balmer et al., 2003; Lemaire et al., 2004; Wong et al., 2004; Yamazaki et al., 2004; Balmer et al., 2004a, 2004b; Hosoya-Matsuda et al., 2005; Marchand et al., 2006; Balmer et al., 2006b; Alkhalfioui et al., 2007; Bartsch et al., 2008; Hall et al., 2010; Marchand et al., 2010; Yoshida et al., 2013). The second main strategy for proteomic analysis of Trx targets is based on the *in vitro* reconstitution of the enzymatic Trx system (NADPH, NTR, Trx) within a cell-free extract (Figure 1B). This system allows *in vitro* reduction of Trx-regulated cysteines that are labeled with thiol-specific probes, allowing detection of Trx-targeted proteins and their identification by MS. Initially developed by Buchanan and co-workers on proteins from peanut seeds using the monobromobimane fluorescent probe (Yano et al., 2001), this strategy

Figure 1. Schematic Overview of the Experimental Workflow Used to Identify both Thioredoxin Targets and Their Redox-Regulated Cysteines.

The experimental workflow proceeds through two procedures.

(A) Monocysteinic Trx column approach: identification of Trx targets using the affinity chromatography approach with the cytosolic CrTrxh1-C39S mutant.

(B) Reductome approach: identification of the redox-regulated cysteines after *in vitro* reduction by the cytosolic CrTrxh1 system and labeling of reduced cysteines with cleavable ICAT.

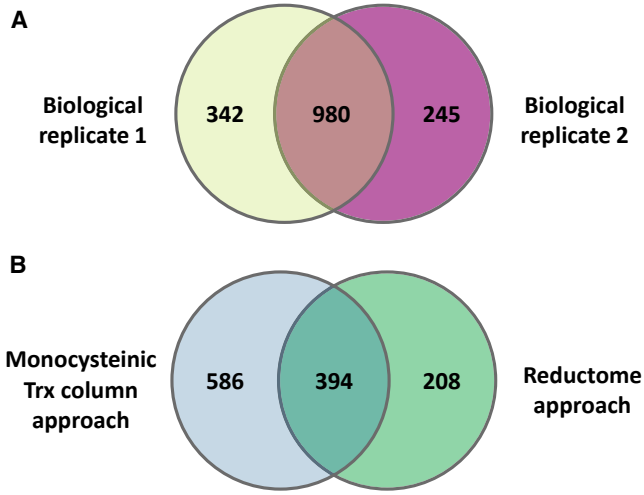

Figure 2. Distribution of Thioredoxin Targets.
(A) Venn diagram showing the distribution of proteins identified by the monocysteinic Trx column approach between two independent biological samples.
(B) Venn diagram showing the distribution of proteins identified by the two proteomic approaches used in this study.

was rapidly applied to different total or subcellular soluble protein extracts from different land plants (Marx et al., 2003; Wong et al., 2003, 2004; Maeda et al., 2004; Maeda et al., 2005; Yano and Kuroda, 2006; Balmer et al., 2006a, 2006b; Alkhalfioui et al., 2007; Hall et al., 2010) and more recently to Archaea (Susanti et al., 2014). This strategy was also extended in terms of detection to fluorescent (Maeda et al., 2004), radioactive (Marchand et al., 2004), or biotinylated probes (Marchand et al., 2006). More recently, quantitative adaptations of this approach were developed for MS analyses based on isotopic labeling with cleavable isotope-coded affinity tag reagents (cICAT) (Hagglund et al., 2008, 2014) or with cysteine-reactive tandem mass tag (Cys-TMT) (Zhang et al., 2016). Both of these cysteine-specific reagents allowed the enrichment of cysteine-containing peptides by affinity or immuno-purification, respectively, and the identification of Trx-targeted cysteines.

In the green biflagellate microalga *Chlamydomonas reinhardtii* (Cr), we previously identified 55 putative targets of Trx by coupling the monocysteinic Trx-based approach with protein separation on 2D gels and identification by MALDI-TOF MS (Lemaire et al., 2004). Considering the number of Trx targets identified later in other photosynthetic organisms and the results from our own studies in *Chlamydomonas*, which identified 225 proteins regulated by S-glutathionylation (Michelet et al., 2008; Zaffagnini et al., 2012a) and 492 nitrosylated proteins (Morisse et al., 2014b), the number of 55 Trx targets appears likely underestimated. Taking advantage of the latest improvements in MS sensitivity and capacity to deal with complex samples (Hebert et al., 2014; Doll and Burlingame, 2015; Riley et al., 2016) we not only comprehensively reinvestigated the "thioredoxome" in *Chlamydomonas* but also performed an in-depth characterization of Trx-reduced cysteines by combining qualitative and quantitative MS analyses. This study broadens the landscape of redox regulation in photosynthetic organisms by identifying more than 1000 Trx targets and sheds light on the underlying molecular mechanisms, thereby providing a valuable resource for future functional studies. Moreover, this work confirms the existence of a complex redox regulation network and provides evidence of a tremendous selectivity of redox PTMs for specific cysteine residues.

RESULTS AND DISCUSSION

Identification of Trx Targets by Affinity Chromatography

To broaden the landscape of proteins targeted by Trx, we took advantage of the enrichment capacity of the affinity chromatography approach. As in our pioneering study in *Chlamydomonas* (Lemaire et al., 2004), we grafted the monocysteinic variant of the cytosolic Trx, CrTrxh1-C39S, on a Sepharose chromatographic resin. *Chlamydomonas* contains only two cytosolic Trx but CrTrxh1 is by far the most highly expressed and the most abundant (Lemaire et al., 2003a, 2003b; Lemaire and Miginiac-Maslow, 2004). Moreover, CrTrxh1 does not contain any extra cysteine in its primary sequence, meaning that covalent binding between the C39S mutant Trx and a target protein occurs exclusively through the nucleophilic cysteine. Finally, CrTrxh1 exhibits a broad substrate specificity, as it is able, *in vitro*, to reduce most known cytosolic and chloroplastic Trx targets efficiently, including antioxidant enzymes and Calvin–Benson cycle enzymes (Rivera-Madrid et al., 1995; Stein et al., 1995; Goyer et al., 2002; Fischer et al., 2009). Soluble protein extracts from two independent *Chlamydomonas* cultures grown under standard laboratory growth conditions in Tris–acetate–phosphate medium were subjected to the classical workflow depicted in Figure 1A. For each biological replicate, soluble proteins retained on the column were selectively eluted with DTT and further analyzed in duplicate by liquid chromatography tandem mass spectrometry (nanoLC-MS/MS) without any prior separation on SDS–PAGE or 2D gels. Moreover, to go deeper into this sub-proteome and identify less-abundant Trx targets, flow-through fractions were kept, concentrated, and loaded again onto the column. Raw MS data from analytical replicates were concatenated before identification, and the results obtained from successively eluted protein fractions of the same biological replicate were merged. Each biological replicate allowed us to identify 1322 and 1225 cysteine-containing proteins, respectively. To generate robust data, we considered only proteins identified in both biological replicates, restricting the landscape of Trx targets to almost 1000 proteins (980 proteins) (Figure 2A).

From the Trx-target proteome identified, only 31 supplemental proteins lacking cysteine in their primary NCBI sequence were also identified by this approach. Using the primary sequence of these 31 proteins, we performed a BLAST search against the latest *Chlamydomonas* genome annotation available at the Phytozome website (https://phytozome.jgi.doe.gov/pz/portal.html). Seven of these NCBI sequences corresponded to partial sequences or wrong gene models since the corresponding proteins contain at least one cysteine in their Phytozome full-length sequences. Therefore, they were finally reintegrated in our Trx-target dataset. For the 24 remaining cysteine-lacking proteins, we hypothesized that their presence likely reflects their natural abundance in our soluble protein extracts rather than a lack of specificity. To confirm this hypothesis, we experimentally characterized the soluble proteome of *Chlamydomonas* using extracts

from cells grown under conditions similar to those employed for identification of Trx targets. The 24 proteins were almost all identified among the most abundant proteins (according to their sequence coverage) (Supplemental Table 2). This suggests that, under our experimental conditions, false-positive contaminants were limited to a very small number of highly abundant proteins.

The affinity chromatography approach allowed us to finally identify 980 putative Trx targets (Figure 2A) with low contamination (Supplemental Table 1). We also noted that 142 proteins identified as putative Trx targets by affinity chromatography were not identified in the soluble proteome, confirming the enrichment capacity of this approach. Among these 980 proteins, we found 54 of the 55 previously identified Trx targets (Lemaire et al., 2004). In fact, the missing CPN20 chaperonin was identified in only one of the two biological replicates and thus was not taken into account in our dataset. Moreover, almost all proteins known to be regulated by Trx in other photosynthetic organisms, but not yet established in *Chlamydomonas*, were identified by this approach. These include methionine sulfoxide reductases (Vieira Dos Santos et al., 2005; Tarrago et al., 2009), ACCase (Sasaki et al., 1997; Hunter and Ohlrogge, 1998; Kozaki et al., 2001), APS reductase (Bick et al., 2001), DAHP synthase (Entus et al., 2002), glucan water dikinase (Mikkelsen et al., 2005), uricase (Du et al., 2010), magnesium chelatase CHLI subunit (Ikegami et al., 2007), cytosolic NAD-MDH (Hara et al., 2006), and ADP-glucose pyrophosphorylase (Ballicora et al., 2000). This suggests that other proteins identified in this dataset are also relevant. Thus, the Trx-affinity strategy combined with modern MS is a powerful method to identify proteins forming mixed disulfides with thioredoxins. However, one major drawback of this approach is that the exact cysteine targeted by Trx within the protein remains undetermined. Therefore, in addition to the Trx-affinity chromatography, we developed a quantitative reductome approach in order to gain further insights into the thioredoxome of *Chlamydomonas*.

Identification of Trx-Targeted Cysteines by the Reductome Approach

Combination of the reductome approach with differential labeling was proven to be efficient to characterize cysteines targeted by Trxs at a proteome scale (Marchand et al., 2006; Hagglund et al., 2008; Zhang et al., 2016). In this study, we used cICAT reagents, commercially available in light ($ICAT_L$) and heavy ($ICAT_H$) forms, to label cysteines of soluble proteins after *in vitro* incubation in the presence or absence of the Trx system composed of NADPH, AtNTR-B, and cytosolic CrTrxh1 (Figure 1B). The cICAT-labeled proteins were digested, and the corresponding peptides were recovered after avidin affinity purification and acidic cleavage of the biotin tag. The peptide mixtures were then analyzed by nanoLC-MS/MS. For each cysteine-containing peptide, the determination of cICAT ratio by quantitative MS can be assimilated to a reduction level allowing discrimination between truly Trx-reduced proteins and false positives.

First, we sought to determine whether the acidic cleavage of the biotin moiety of cICAT-labeled peptides was required since this supplementary step was a potential source of material loss. For this purpose, *Chlamydomonas* protein extracts were labeled either with light or heavy cICAT reagents and mixed together in equal ratio. After trypsin digestion and avidin affinity purification, eluted peptides were analyzed by MS on a Q-Exactive instrument before or after cleavage of the biotin moiety by acidic treatment. The base peak chromatogram revealed that retention times of biotinylated peptides were clearly delayed toward the end of the gradient, suggesting that biotinylated peptides are more hydrophobic (Supplemental Figure 1A). Moreover, fragmentation spectra of cICAT-labeled peptides generated by HCD with a normalized collision energy of 27 appeared dominated by an intense fragmentation pattern (m/z 167.07, 232.15, 258.17, 284.14, 515.29 Da) due to the cICAT moiety rendering them much less informative about the peptide sequence (Supplemental Figure 1B and 1C). By comparison, fragmentation spectra of peptides for which the biotin moiety was removed are much more informative. Finally, the presence of the biotin moiety also appeared to alter the elution profiles of heavy and light peptides and could therefore significantly alter MS quantification. Indeed, the extracted ion chromatograms for heavy and light peptides differed significantly when the biotin moiety was present, whereas co-elution of isotopes was achieved when the biotin tag had been removed (Supplemental Figure 1D). Altogether, under our experimental MS conditions, these multiple issues led to dramatic losses in terms of identification (data not shown), and we decided to systematically cleave the biotin moiety for subsequent experiments.

To establish thresholds below and above which a cICAT ratio determines that cysteines are Trx targeted, we reduced a protein extract either chemically, using Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) as strong reducing agent, or enzymatically, with the thioredoxin system. In each case, protein extracts were divided equally into two fractions for labeling with the light or heavy form of cICAT and mixed again in equal ratio. After digestion, avidin affinity purification, and removal of the biotin tag, peptides were analyzed and quantified by MS. For both experiments, the logarithmized $ICAT_H/ICAT_L$ ratio showed a symmetric distribution centered on a value close to zero, as expected for samples mixed in equal ratio (Supplemental Figure 2A and 2B). Moreover, calculated standard deviations for both conditions were similar, suggesting that the workflow was reproducible. To generate results with high confidence, we calculated logarithmized thresholds based on the global mean ratio and three standard deviations as shown in the following equation:

$$\log_2(\text{thresholds}) = \pm|\text{global mean ratio}| \pm 3 \times \text{standard deviation}.$$

Application of this formula to our experimental dataset revealed that peptides with $ICAT_H/ICAT_L$ ratios below 0.46 and above 2.18 could be considered as having a cysteine whose redox state is significantly reduced (ratio above 2.18) or oxidized (ratio below 0.46) by Trx. Both distributions failed the Shapiro statistical test, indicating that they do not strictly follow a normal distribution. Thus, a precise false-positive rate for these thresholds cannot be statistically extrapolated but was estimated to remain below 1.5% based on our experimental data (1.27% for TCEP reduction and 1.37% for enzymatic reduction).

Finally, we also decided to check whether an isotopic effect could be observed in our workflow. Therefore, on the same biological sample, we performed two reductome experiments in parallel that were similar in all aspects except that the heavy and light

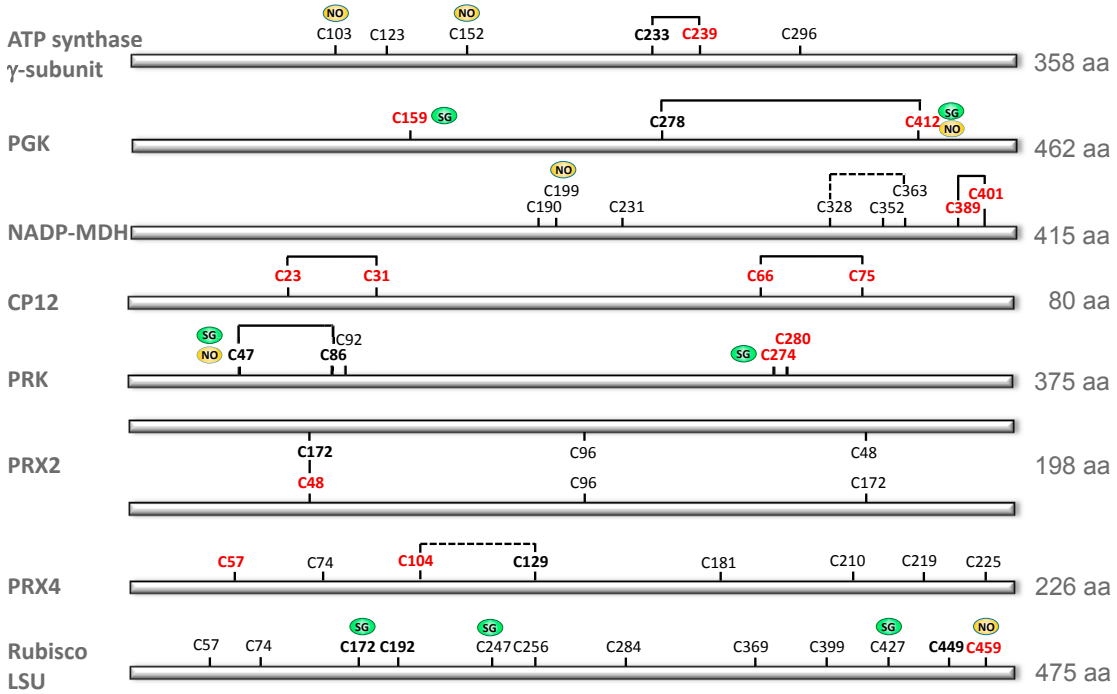

Figure 3. Redox-Regulated Cysteines in *Chlamydomonas* Enzymes.

Eight redox-regulated proteins are schematically and linearly represented: ATP synthase γ-subunit (Cre06.g259900); PGK (chloroplastic phosphoglycerate kinase, Cre22.g763250); NADP-MDH (chloroplastic NADP-malate dehydrogenase, Cre09.g410700); CP12 (chloroplastic protein 12, Cre08.g380250); PRK (chloroplastic phosphoribulokinase, Cre12.g554800); PRX2 (cytosolic 2-cys peroxiredoxin; Cre02.g114600); PRX4 (type-II peroxiredoxin; Cre02.g080900); Rubisco LSU (chloroplastic ribulose bisphosphate carboxylase/oxygenase; rbcL). The numbering corresponds to the full-length sequence. Lines between cysteines indicate confirmed (plain) or suggested (dashed) disulfide bonds in *Chlamydomonas* enzymes. Cysteines identified as nitrosylated (Morisse et al., 2014b) and glutathionylated (Zaffagnini et al., 2012a) by proteomic approaches in *Chlamydomonas* are labeled with NO (yellow) and SG (green), respectively.

forms of cICAT reagents were interchanged. We performed comprehensive MS analyses (five technical replicates) associated with stringent criteria for MS identification (peptide false discovery rate <0.1%) and quantification (peptide quantified at least three times over the five replicates). Frequency profiles of cICAT ratios from both experiments appeared similar, indicating that enzymatically reduced and control protein samples can be indifferently labeled by light or heavy cICAT (Supplemental Figure 3).

The quantification results from the two experimental datasets obtained after incubation in the presence or absence of the Trx system (Figure 1B) were taken into consideration and concatenated. Among peptides identified in our analysis, only around 6% did not contain cysteine in their sequence (data not shown), underlining the specificity of our approach. Moreover, among cysteine-containing peptides, 977 peptides had a significant $ICAT_H/ICAT_L$ ratio (Supplemental Table 3). Actually, only one peptide belonging to the β subunit of the T-complex protein 1 had inconsistent quantitative data and thus was not included in the final list. Almost all identified cysteines showed a higher reduction level and only 25 cysteines appeared unexpectedly oxidized after the Trx reduction treatment. The appearance of oxidized proteins after Trx reduction was previously observed by Zhang and colleagues using a Cys-TMT approach (Zhang et al., 2016). These cysteines belong to 14 different proteins. For two of these, at least another peptide having a Trx-reduced cysteine was also identified, and eight proteins were also retained on the affinity chromatography column. Altogether, this suggests that detection of such an oxidized redox state in the presence of the Trx system could originate from complex mechanisms involving conformational changes and/or disulfide bond isomerization as established for chloroplastic NADP-malate dehydrogenase (Ruelland et al., 1997). Thus, we decided to consider these 14 proteins as putative Trx targets. Almost all peptides exhibiting a significant H/L ratio were proteotypic, and only 18 peptides could map to more than one possible protein. As these proteins are closely related since they are encoded by duplicated genes such as tubulin α, they were grouped together and counted as only one isoform. Overall, these 977 peptides allowed us to identify 1052 Trx-targeted cysteines spread over 602 proteins.

Validation of Trx-Reducible Cysteines Identified by the Reductome Approach

This fruitful identification of putative Trx-targeted cysteines prompted us to evaluate the relevance of the cysteines identified. Interestingly, the two active site cysteines of CrTrxh1, CrNTR3, and AtNTR-B were identified. To validate our data, we focused on eight other *Chlamydomonas* enzymes known to be activated by Trx and for which we have structural information about Trx-dependent cysteines (Figure 3).

Chloroplastic ATP-Synthase

Chloroplast ATPase (CF_0CF_1) is activated both by the electrochemical proton potential difference (Strotmann and Bickel-Sandkötter,

1984) and by reduction of a disulfide bond located in the γ subunit of the CF_1 domain (Moroney et al., 1984; Yu and Selman, 1988) under the control of m- and f-type thioredoxin (Schwarz et al., 1997; He et al., 2000). In *Chlamydomonas*, the regulatory disulfide occurs between Cys198 and Cys204, whose mutation to serine completely abolished redox regulation (Ross et al., 1995). In this study, we identified Cys204, the cysteine corresponding to the second regulatory cysteine.

NADP-Malate Dehydrogenase

This enzyme is involved in the export of reducing power from the chloroplast to the cytosol through the malate valve. Chloroplastic NADP-MDH from land plants are redox regulated through two disulfide bonds present in N- and C-terminal extensions (Issakidis et al., 1996). By contrast, *Chlamydomonas* NADP-MDH contains only one C-terminal Trx-regulated disulfide bond and a potential second internal structural disulfide bridge with no regulatory function (Lemaire et al., 2005). Interestingly, in the present study, we identified the two C-terminal Cys involved in the regulatory disulfide bond but not the cysteines involved in the structural disulfide bond, suggesting that the reductome approach is selective for redox-modified cysteines.

Phosphoglycerate Kinase

This Calvin–Benson cycle enzyme is not known to be redox regulated in land plants. By contrast, a Trx-dependent redox activation was reported in *Chlamydomonas*, *Synechocystis*, and *Phaeodactylum tricornutum* (Bosco et al., 2012; Tsukamoto et al., 2013; Morisse et al., 2014a). In *Chlamydomonas* chloroplastic PGK, the regulatory disulfide bond was shown to involve the two C-terminal cysteines (Morisse et al., 2014a), one of which, Cys412, was identified in our reductome approach.

CP12/Phosphoribulokinase/GAPDH Complex

In photosynthetic organisms, Trxs are known to modulate independently PRK and GAPDH activities but also to initiate the disassembly of the $(A_4\text{-GAPDH})_2\text{-CP12}_4\text{-PRK}_2$ complex through the reduction of CP12 (Marri et al., 2009; Lopez-Calcagno et al., 2014). CP12 contains four well-conserved cysteines that are involved in two Trx-controlled disulfide bonds (Lopez-Calcagno et al., 2014) (Avilan et al., 2000). In the present study, CP12 was retained on the Trx column and the four cysteines were clearly identified as reduced by Trx, thereby validating the two approaches. In PRK, the two N-terminal cysteines form a Trx-regulated disulfide bond in higher plants (Porter et al., 1988; Milanez et al., 1991; Brandes et al., 1996) and in *Chlamydomonas* (Lebreton et al., 2003). Surprisingly, instead of these two cysteines, the reductome approach identified two other well-conserved cysteines, Cys243 and Cys249. Interestingly, these cysteines were suggested to participate in a disulfide whose formation alters the physical interaction of PRK with GAPDH and CP12 (Thieulin-Pardo et al., 2015). A_4-GAPDH is likely regulated by glutathionylation (Zaffagnini et al., 2007) but was not shown to be controlled by Trx. Intriguingly, we identified the A_4-GAPDH active site cysteine Cys149 and its vicinal cysteine Cys153 as both labeled by cICAT reagents, suggesting that they form a Trx-reduced disulfide which was also detected in human GAPDH (Rinalducci et al., 2015). Moreover, we also identified ADK3, an adenylate kinase having a C-terminal domain similar to that of CP12 (Thieulin-Pardo et al., 2016). Chloroplastic ADK3 was identified by both approaches, and we identified the two cysteines of the disordered CP12-like domain, suggesting that ADK3 could be regulated by chloroplastic Trxs through its CP12 tail.

Peroxiredoxins

Peroxiredoxins constitute a family of enzymes reducing peroxides and peroxynitrites and present in the chloroplast, mitochondria, and cytosol (Dietz, 2011). Oxidized Prxs can be regenerated in their active form by different electron donors such as Trxs, glutaredoxins, glutathione, and ascorbate. In *Chlamydomonas*, the reductome approach identified CrPrx2, a 2-cys type Prx located in cytosol or flagella and CrPrx4, which belongs to the group of type II Prxs and is localized in mitochondria (Dayer et al., 2008). CrPrx1, the chloroplastic counterpart of Prx2, has been shown to be dependent on Trxs for its regeneration (Goyer et al., 2002). Interestingly, in both Prx2 and Prx4, we identified the peroxidatic cysteine responsible for the reactivity of Prxs with peroxides. For Prx4, we also identified Cys57 as a cysteine putatively reduced by Trxs.

Ribulose-1,5-bisphosphate Carboxylase/Oxygenase

This Calvin–Benson cycle enzyme catalyzes the first step of photosynthetic CO_2 fixation through its carboxylase activity. Despite the fact that ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) was systematically identified among putative Trx targets in redox proteomic studies performed in photosynthetic cells (Michelet et al., 2013), a direct effect of Trx on Rubisco has not yet been reported. In *Chlamydomonas* Rubisco large subunit, seven of the 12 cysteines are highly conserved among photosynthetic eukaryotes (Moreno et al., 2008) and among them, oxidation of Cys172, Cys192, Cys449, and Cys459 was reported to play a prominent role either in Rubisco inactivation and degradation through conformational modulation (Marin-Navarro and Moreno, 2006; Garcia-Murria et al., 2008) or chloroplastic mRNA binding (Yosef et al., 2004; Uniacke and Zerges, 2008). Interestingly, we identified Cys459 as a putative Trx target. Cys449 and Cys459 are located close to the surface and in a flexible region, since the distance between the two residues seems to fluctuate (Garcia-Murria et al., 2008). Moreover, Cys459 was recently identified as nitrosylated in *Chlamydomonas* (Morisse et al., 2014b). Altogether, these data are compatible with the proposed role of Cys449 and Cys459 as a redox sensor (Garcia-Murria et al., 2008) in which chloroplastic Trxs could play a direct regulating role.

Other Proteins

Numerous other enzymes of the dataset were also studied at the molecular level in other species but not in *Chlamydomonas*. In this case, comparison of the position of target cysteines is much less informative. Indeed, redox regulatory mechanisms may vary significantly between species. For example, the activity of the autophagy cysteine protease Atg4 is regulated by Trx in both yeast (Pérez-Pérez et al., 2014) and *Chlamydomonas* (Pérez-Pérez et al., 2016) but through distinct disulfide bonds, i.e., located in completely different regions of the enzyme. The fact that *Chlamydomonas* Atg4 was not identified among Trx targets in the present study may be linked to its low abundance, as suggested by its absence from our soluble proteome of *Chlamydomonas* cells (Supplemental Table 2) but also by the fact that this protease is regulated by Trx under conditions of autophagy induction, whereas our extracts were prepared from non-autophagic conditions. Some enzymes may also be regulated only in some species as established for GAPDH (Trost et al., 2006) or PGK (Morisse et al., 2014a). Another typical example is Rubisco activase (RCA). RCA is a molecular chaperone of the AAA+ family that uses the energy from ATP

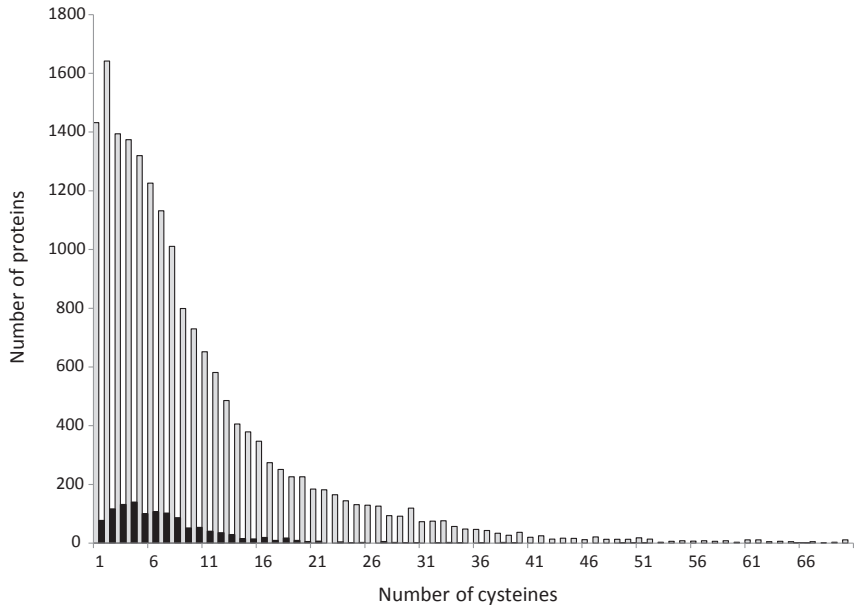

Figure 4. Distribution of *Chlamydomonas* Proteins According to the Number of Cysteines.

Distributions of *Chlamydomonas* Trx targets identified in this work (black bars) and proteins in the total proteome derived from the genome sequences (white bars) according to the number of cysteines present in their sequence.

hydrolysis to release tight binding inhibitors from the active site of Rubisco (reviewed in Portis, 2003; Portis et al., 2008). *Chlamydomonas* only contains a short β isoform of RCA that does not contain TRX-regulated cysteines found in α isoforms (Zhang and Portis, 1999; Zhang et al., 2001) but still exhibit light-dependent regulation of RCA activity (Salvucci and Anderson, 1987; Michelet et al., 2013; Gontero and Salvucci, 2014). Nevertheless, *Chlamydomonas* RCA contains four cysteines (Cys148, Cys196, Cys255, Cys289) that are highly conserved in all photosynthetic organisms and were all identified by our reductome approach. This suggests that Trx may also play some role in the regulation of β isoforms of RCA. Interestingly, the α isoform of *Arabidopsis* RCA was detected as nitrosylated in *Arabidopsis* leaves (Lindermayr et al., 2005) and Cys196 and Cys148 from *Chlamydomonas* and *Arabidopsis* β-RCA, respectively, were detected as S-nitrosylated (Fares et al., 2011; Morisse et al., 2014b). Altogether, these data suggest that the redox regulation of RCA is likely more complex than currently established and is similar in some aspects to the redox regulation of A-type and B-type chloroplastic GAPDHs. Nevertheless, for some proteins, the position of cysteines reduced by Trx is conserved between land plants and algae as confirmed by our *Chlamydomonas* reductome. For example, this is the case for the Calvin–Benson cycle enzymes FBPase and SBPase (Jacquot et al., 1997; Rodriguez-Suarez et al., 1997; Chiadmi et al., 1999; Gutle et al., 2016), chloroplastic magnesium chelatase CHLI (Ikegami et al., 2007), 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase (Entus et al., 2002), or glucose-6-phosphate dehydrogenase (Wenderoth et al., 1997; Nee et al., 2014). For some of these enzymes, the reductome approach suggests, in addition to known target cysteines, the existence of additional sites of redox regulation. Interestingly, some of these sites were found to undergo glutathionylation (Zaffagnini et al., 2012a) or nitrosylation (Morisse et al., 2014b).

Altogether, the *Chlamydomonas* reductome recapitulates most of the previously established sites of Trx-dependent regulation, strongly suggesting that other sites identified in our dataset are also relevant. Therefore, this study unravels the existence of hundreds of new Trx-dependent regulatory sites.

Chlamydomonas Thioredoxome: More than 1000 Targets, More than 1000 Cysteines

Mass spectrometers have improved tremendously over the last 10 years in terms of mass accuracy, resolution, sensitivity, and scan speed, and they are now able to deal with highly complex biological samples even without fractionation (Hebert et al., 2014; Doll and Burlingame, 2015; Riley et al., 2016). The use of such up-to-date instruments is surely at the origin of our fruitful harvest of Trx-regulated proteins, but the development of complementary biochemical approaches performed at the protein and peptide levels also contributed. In this report, the two strategies, namely the Trx-affinity column and the reductome, allowed the identification of 980 and 602 Trx targets, respectively, with a global overlap of ca. 33% (Figure 2B). This indicates that the two methods are complementary. A similar limited overlap between these two approaches was previously reported in wheat (Wong et al., 2004) and *Arabidopsis* (Marchand et al., 2006), two studies exclusively conducted at the protein level, or in *Chlamydomonas* for S-nitrosylation where both proteins and cysteinyl-peptides were identified (Morisse et al., 2014b). In the present study, the combination of both approaches allowed us to identify 1188 putative Trx targets in *Chlamydomonas*.

This number may seem high but only represents, at the proteome scale, 6.5% of cysteine-containing proteins, a value consistent with previous studies (Susanti et al., 2014; Morisse et al., 2014b; Arts et al., 2016). Moreover, distribution of *Chlamydomonas* proteins according to their number of cysteines does not show a significant enrichment for proteins containing a higher number of cysteines (Figure 4). In the reductome approach, 92% of identified peptides contain only one cysteine and 7.5% contain two cysteines in their sequence. When compared with the theoretical proteome (Supplemental Figure 4), this distribution suggests that peptides containing at least two cysteines could be underestimated, a limitation that may be linked to the MASCOT identification software (Garcia-Santamarina et al., 2014).

The depth of proteomic analyses dedicated to PTMs is generally hampered by both modification levels and protein relative abundance. Without the use of robust depletion/equalization or enrichment methods, low-abundant proteins are often missed (Doll and Burlingame, 2015; Riley et al., 2016). Interestingly,

18.6% of Trx targets present in our dataset (142 and 118 proteins in the affinity chromatography and the reductome approaches, respectively) are likely low-abundant proteins. Indeed, these proteins were not identified in the soluble proteome but were identified as Trx targets most likely thanks to the enrichment capacity of our approaches (Supplemental Table 4). However, other Trx-regulated proteins probably remain to be identified in *Chlamydomonas*, especially those that are induced under specific growth conditions (strictly photoautotrophic conditions, nutrient starvation, light/dark cycles) or those that are expressed at very low levels, such as nuclear proteins for which dedicated and exhaustive analyses of Trx targets will be required. Nevertheless, by comparison with all previously published data, our study considerably broadens the landscape of redox regulation in photosynthetic organisms since our *Chlamydomonas* thioredoxome contains 1188 Trx targets and 1052 redox-regulated cysteines.

Subcellular Localization

The subcellular distribution of the 1188 proteins identified was determined using the PredAlgo software, a multi-subcellular localization prediction tool dedicated to algae (Tardif et al., 2012). As shown in Supplemental Figure 5A, Trx targets are predicted to be distributed for 30% to the chloroplast, 9% to mitochondria, 6% to the secretory pathway, and 55% to other compartments. Such a distribution is not surprising despite the fact that we used a cytosolic Trx. Indeed, it is recognized that redox proteomic studies performed *in vitro* lack specificity, and similar targets are identified regardless of the Trx isoform employed (Montrichard et al., 2009; Buchanan et al., 2012); this is all the more true for CrTrxh1, which is able to reduce *in vitro* most cytosolic and chloroplastic Trx targets (Rivera-Madrid et al., 1995; Stein et al., 1995; Goyer et al., 2002; Fischer et al., 2009).

Proteins present in the *Chlamydomonas* soluble proteome (Supplemental Figure 5B) exhibited, in percentage, a subcellular distribution similar to that of Trx targets, while a slight enrichment for chloroplastic proteins was observed with the total theoretical proteome encoded by the three genomes (Supplemental Figure 5C). These data suggest that the redox regulation mediated by Trx plays an important role in the different subcellular compartments and that the chloroplast is a metabolic hub for redox regulation even under mixotrophic growth conditions where photosynthesis is dispensable for *Chlamydomonas* cells. These results are consistent with the established importance of thioredoxins in multiple subcellular compartments, organs, and developmental stages of photosynthetic organisms (for detailed reviews, see (Gelhaye et al., 2005; Lemaire et al., 2007; Konig et al., 2012; Meyer et al., 2012; Michelet et al., 2013; Traverso et al., 2013; Zaffagnini et al., 2013a; Balsera et al., 2014; Nikkanen and Rintamaki, 2014; Rouhier et al., 2015; Sevilla et al., 2015; Delorme-Hinoux et al., 2016; Hagglund et al., 2016; Kang and Wang, 2016)).

Functional Annotation

Functional annotation according to the *Kyoto Encyclopedia of Genes and Genomes for Chlamydomonas* revealed that Trx-targeted enzymes participate in a wide variety of metabolic pathways and cellular processes (Figure 5). The major categories correspond to proteins involved in metabolic functions (30.3%), genetic information processing (22.1%), cellular processes (8.3%), stress response and redox homeostasis (4.2%), environmental information processing (4.2%), and unknown functions for 30.9%.

The implication of Trxs in metabolic functions (essentially carbon metabolism, energy metabolism, and lipid metabolism) is not surprising. Initially discovered in plant chloroplasts as light-dependent activators of Calvin–Benson cycle enzymes, the functional role of Trxs was rapidly extended to other metabolic pathways such as ATP synthesis, starch synthesis and degradation, and lipid and amino acid synthesis (Geigenberger et al., 2005; Lemaire et al., 2007; Meyer et al., 2009; Geigenberger and Fernie, 2014). Interestingly, some metabolic pathways appear highly controlled by Trxs. Among these, it is not surprising to find the Calvin–Benson cycle, for which all 11 enzymes were identified in the present study. Redox regulation of the Calvin–Benson cycle by Trxs has been well established for five of the 11 enzymes, and all enzymes were also detected as glutathionylated or nitrosylated in *Chlamydomonas* and other species (Michelet et al., 2013; Morisse et al., 2014b). This tight redox regulation may be required to allow fine-tuning of the Calvin–Benson cycle for a transient redistribution of the energy (in the form of ATP) and reducing power (in the form of NADPH) within chloroplasts to cope with stress conditions (Michelet et al., 2005; Lemaire et al., 2007). Redox PTMs may also divert these abundant enzymes to moonlighting functions, i.e., new functions unrelated to their metabolic role in carbon metabolism as well established for glycolytic GAPDH (Zaffagnini et al., 2013a). The biosynthesis pathway for aromatic amino acids also appeared as a major Trx target, since almost all the enzymes of the pathway (14 out of 20) were identified in the present study, including seven out of eight enzymes of the shikimate pathway. In *Arabidopsis*, the first enzyme of this pathway, DAHP synthase, was suggested to be redox regulated since its activity was shown to be Trx f-dependent *in vitro* (Entus et al., 2002). For all other enzymes, very little is known about the functional role of oxidative modifications on their regulation (Maeda and Dudareva, 2012). It should be noted that some of these enzymes were also detected as glutathionylated and nitrosylated in *Chlamydomonas* (Zaffagnini et al., 2012a; Morisse et al., 2014b). Interestingly, higher contents of tyrosine and phenylalanine were observed in illuminated poplar leaves compared with darkened ones (Noctor et al., 1998) or under biotic and abiotic oxidative stress conditions (Maeda and Dudareva, 2012; Sadeghnezhad et al., 2016). Altogether, these data suggest that redox regulation of the biosynthesis pathway for aromatic amino acids should be carefully studied, and our putative Trx-targeted sites could be a good starting point to evaluate the redox dependency of these enzymes.

A total of 263 proteins are functionally annotated as participating in genetic information and processing. Among these, 41 proteins (16%) are involved in DNA-related functions: transcription, replication and repair, retrotransposon element. By contrast, 222 proteins (84%) participate in protein metabolism: translation, folding, sorting, and degradation. This suggests that, in *Chlamydomonas*, Trx-dependent regulation of protein expression might occur mainly at the translational and post-translational levels. The 64

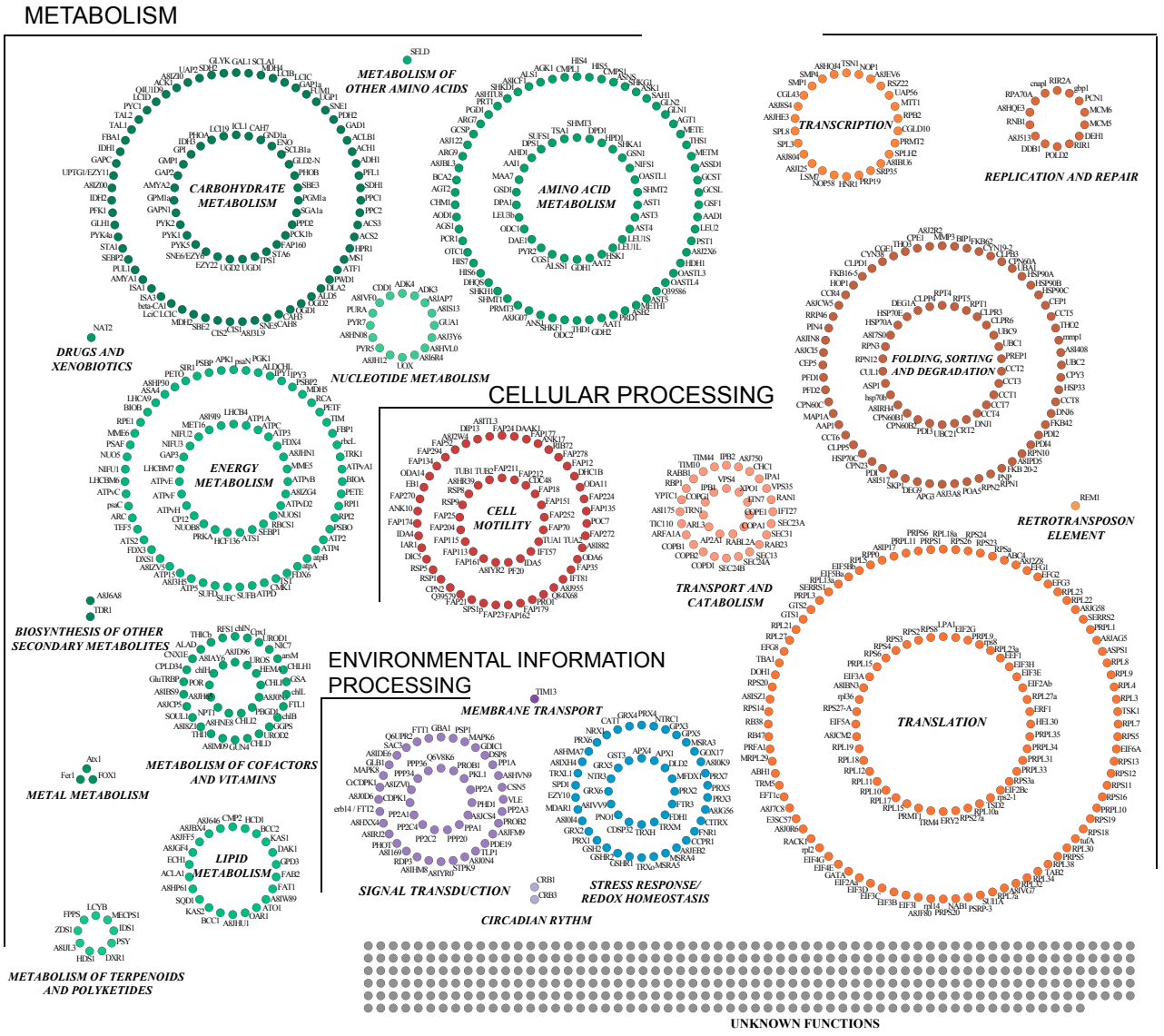

Figure 5. Functional Annotation of the *Chlamydomonas* Thioredoxome.

Proteins were classified according to the *Kyoto Encyclopedia of Genes and Genomes for Chlamydomonas* and using a limited number of Gene Ontology annotation levels. Nodes are labeled either with the gene name or with the UniprotKB identifier. Proteins are grouped according to their role in metabolic pathways or cellular processes with the following color code: metabolism, green; genetic information processing, brown and tawny; cellular processes, red and light red; environmental information processing, purple; stress response/redox homeostasis, light blue; unknown functions, gray.

proteins involved in cell motility (5.4%) mainly correspond to flagellar proteins, a result consistent with the existence of two specific Trx isoforms in the flagella, absent in *Arabidopsis* (Patel-King et al., 1996). These Trxs were proposed to participate in a redox regulatory pathway that affects outer dynein arm function and influences flagellar motility (Harrison et al., 2002; Wakabayashi and King, 2006). Among transport and catabolism proteins (35 proteins, 2.9%), we identified several proteins involved in nuclear transport, mitotic spindle formation, and nuclear envelope assembly (reviewed in Quimby and Dasso, 2003), such as the RAN1 protein, also identified in our pioneering study (Lemaire et al., 2004). This underscores the importance of redox regulation of nuclear protein trafficking and the nuclear functions of Trxs that have recently emerged in both photosynthetic and non-photosynthetic eukaryotes (Go et al., 2015; Delorme-Hinoux et al., 2016). The identification of 47 proteins (4.0%) involved in signaling is consistent with the central role of oxidative PTMs of cysteine residues in plant signal transduction (Waszczak et al., 2015). The two subunits of the circadian RNA-binding protein CHLAMY1 were also recovered among Trx targets, consistent with the suggested existence of a close coupling of redox processes and the circadian clock in *Chlamydomonas* (Voytsekh et al., 2008; Filonova et al., 2013). Similarly, 50 proteins (4.2%) are not surprisingly involved in stress responses and redox homeostasis. Most of these proteins correspond to established partners of Trx, including peroxiredoxins, glutathione peroxidases, or methionine sulfoxide reductases (Navrot et al., 2006; Fischer et al., 2009; Tarrago et al., 2009; Dietz, 2011). Finally, the most prominent category corresponds to the 368 proteins of unknown function

that represent 30.9% of the Trx targets. This high proportion of proteins of unknown function illustrates our limited understanding of biological systems as recently illustrated by studies on the minimal genome by the Craig Venter Institute that assembled the smallest genome of any self-replicating organism that contains 473 genes including 149 genes (31.5%) of unknown function (Hutchison et al., 2016). However, the percentage in our dataset is lower than in the theoretical proteome where more than 50% of the proteins have unknown functions, suggesting that Trx targets primarily map to major functional networks.

Protein and Cysteine Specificity in a Complex Network of Redox Modifications

Emerging data indicate that redox networks coordinate large numbers of redox elements involved in a multitude of pathways and cellular processes to allow resistance and adaptation to environmental challenges (Go et al., 2015). These networks involve multiple redox PTMs, the best studied being oxidoreduction of disulfide bonds, glutathionylation, nitrosylation, and sulfenylation (Couturier et al., 2013; Yang et al., 2016). This cysteine proteome (Cys proteome) can be considered as an interface between the functional genome and the external environment (Go and Jones, 2014). It is a highly dynamic network of protein thiols with flexible reactivities (Weerapana et al., 2010; Paulsen and Carroll, 2013; Poole and Schoneich, 2015). Therefore, combinations of multiple redox PTMs act in concert throughout the cell and act as a network rather than as insulated elements. Gaining insights into the functioning of redox networks will require the determinants of the specificity of the diverse redox PTMs for specific proteins and cysteines to be unraveled.

Numerous proteins are known to be regulated by multiple redox PTMs. A typical example is the *E. coli* transcription factor OxyR, which can be nitrosylated, glutathionylated, and sulfenylated and undergoes reversible disulfide bond formation under the control of Trx (Choi et al., 2001; Kim et al., 2002; Seth et al., 2012). The Calvin–Benson cycle constitutes an extreme example since all 11 enzymes of the pathway were identified as glutathionylated (Zaffagnini et al., 2012a), nitrosylated (Morisse et al., 2014b), and were also all found here to be regulated by Trx. However, such multiple redox targeting may not be necessarily true for other proteins. In other words, we can wonder whether multiple redox PTMs occur on a limited number of proteins containing reactive cysteines or if each modification targets a distinct redox network. The redox proteomic datasets we have generated in *Chlamydomonas* provide a unique opportunity to further explore the specificity of redox PTMs at the proteome scale. Indeed, besides the extended thioredoxome reported here, we have previously identified 492 nitrosylated proteins (Morisse et al., 2014b) and 225 glutathionylated proteins (Michelet et al., 2008; Zaffagnini et al., 2012a). These studies were performed on the same strain of *Chlamydomonas* using highly similar growth conditions, i.e., starting from a comparable proteome. Although some proteins are clearly targeted by multiple PTMs, the overlap appears limited since 68.8% appear regulated by a single type of modification (Figure 6). This result may appear surprising considering that *Chlamydomonas* Trxh1 was reported to catalyze denitrosylation (Berger et al., 2016) and deglutathionylation (Bedhomme et al., 2012). However, they are consistent with the fact that (1) Trx targets were analyzed in conditions where nitrosylation and glutathionylation are limited or absent (Zaffagnini et al., 2012a; Morisse et al., 2014b); (2) Trx-dependent denitrosylation is considered to affect a limited fraction of nitrosylated proteins since a vast majority (more than 80%) of nitrosylated proteins are denitrosylated by GSH rather than Trx (Paige et al., 2008; Romero and Bizzozero, 2009; Benhar et al., 2010; Zaffagnini et al., 2013b); (3) the mechanisms of denitrosylation and deglutathionylation in photosynthetic organisms are considered to yield Trx-SNO and Trx-SSG rather than a mixed disulfide between the Trx and its target (Bedhomme et al., 2012; Zaffagnini et al., 2012c; Kneeshaw et al., 2014), suggesting that the Trx-affinity column identifies proteins containing a Trx-reducible disulfide rather than nitrosylated or glutathionylated proteins. A high specificity, comparable with that found in *Chlamydomonas* (Figure 6), was already observed when comparing 193 sulfenylated proteins with previously identified targets of disulfide bond formation, nitrosylation, and glutathionylation (Leonard et al., 2009). This limited overlap suggests that the different redox modifications are specific toward different subsets of the proteome.

Moreover, when a given protein is regulated by multiple PTMs, this does not necessarily imply that the same cysteine is targeted. The ability of a given cysteine to undergo a specific type of redox PTM is governed by multiple factors. Some cysteines undergo multiple redox modifications, as demonstrated for Cys178 of *Chlamydomonas* isocitrate lyase (Bedhomme et al., 2009) and Cys149 of *Arabidopsis* GAPC1 (Bedhomme et al., 2012), which both undergo nitrosylation and glutathionylation. In the case of other enzymes, such as the *Chlamydomonas* Calvin–Benson cycle enzymes fructose-1,6-bisphosphatase and transketolase (Michelet et al., 2013); *E. coli* OxyR (Choi et al., 2001; Kim et al., 2002; Seth et al., 2012), or human Trx (Casagrande et al., 2002; Hashemy and Holmgren, 2008; Sengupta and Holmgren, 2012; Benhar, 2015), the different modifications occur on distinct cysteine residues. The specificity primarily depends on the biochemical properties of the cysteine residue, which are largely linked to its microenvironment within the folded protein and can notably influence the accessibility, the acidity, and the nucleophilicity of the residue (Reddie and Carroll, 2008; Winterbourn and Hampton, 2008; Zaffagnini et al., 2012b). Our *Chlamydomonas* proteomic datasets allow the site specificity of the different redox PTMs to be explored at the proteome scale. These comparisons revealed a strikingly high specificity of each modification for specific cysteine residues. Indeed, considering all cysteines experimentally identified as redox regulated, more than 75% undergo a single modification, while less than 1% are regulated by nitrosylation, glutathionylation, and disulfide bond formation (Figure 7A). To avoid any bias due to the performance of the mass spectrometers used in these three studies, a second comparison was performed by restricting this cysteine dataset to *Chlamydomonas* proteins undergoing at least two different redox PTMs and again, similar proportions were observed (Figure 7B). These results indicate that the Cys proteome does not represent a small subset of highly reactive cysteines that are modified through indiscriminate interaction with the molecules they encounter but represent a complex organized network of redox PTMs. The different redox PTMs appear to control different subnetworks that are strongly interconnected. Strikingly, a recent analysis of 1319 mouse

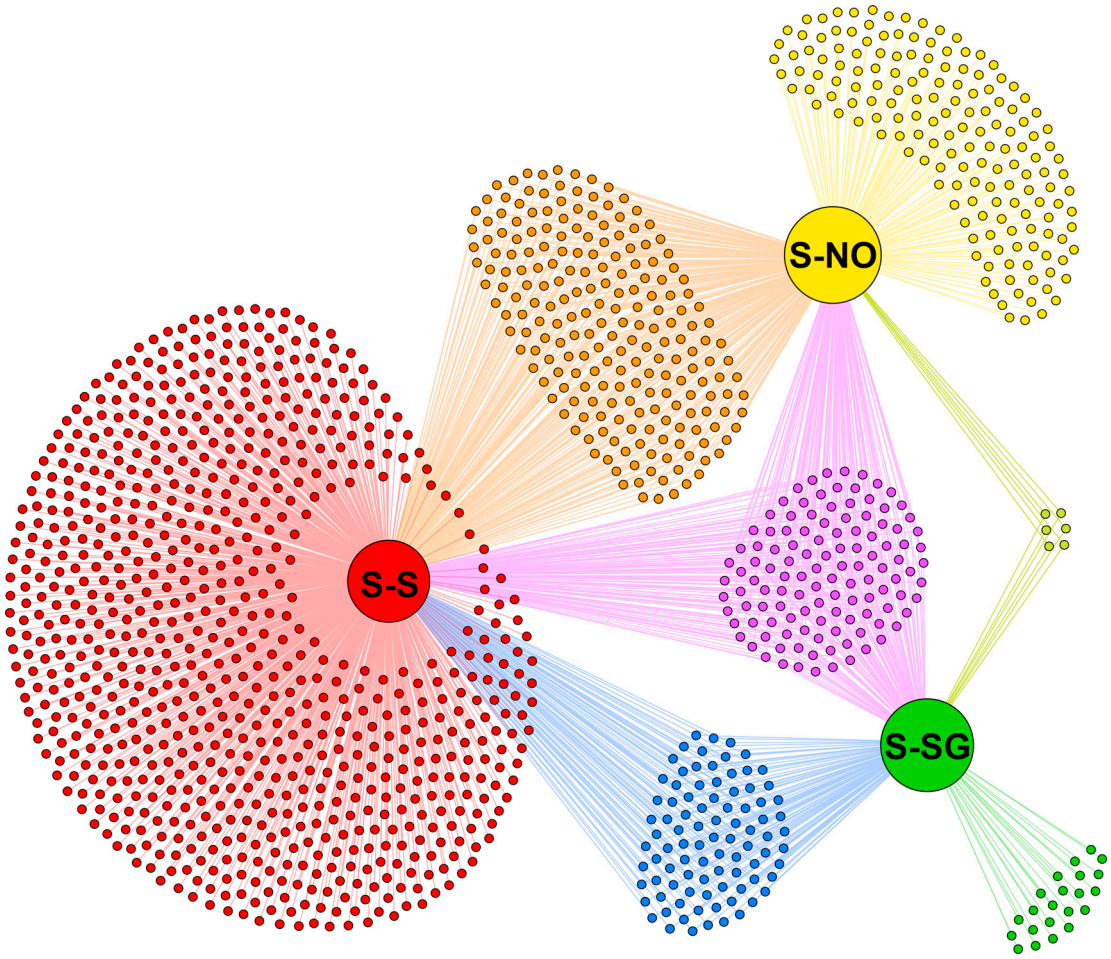

Figure 6. Global Network of Redox Post-Translational Modifications in *Chlamydomonas* Cells.
Chlamydomonas proteins are represented as nodes in a network depending on proteins targeted by thioredoxins (S–S) (this study and Lemaire et al., 2004), S-nitrosylation (S-NO) (Morisse et al., 2014b), or S-glutathionylation (S-SG) (Michelet et al., 2008; Zaffagnini et al., 2012a) using in-house software and D3.js.

liver proteins and four cysteine modifications (nitrosylation, glutathionylation, sulfenylation, and S-acylation) also revealed a very high specificity of redox PTMs with limited overlap (Gould et al., 2015). These results suggest that the different redox modifications are specific toward distinct interconnected protein networks.

Our proteomic datasets provide a unique and comprehensive map of this network, the Cys proteome, in the model alga *Chlamydomonas reinhardtii* (Figure 6). However, this view is rather static, while the redox network probably involves spatial and temporal regulation of several redox PTMs on hundreds of proteins in a highly dynamic manner. This complexity is likely providing the robustness and specificity required to allow a limited number of simple molecules such as reactive oxygen and nitrogen species (ROS/RNS) to play a signaling role. The redox network is likely a major component of signal integration and constitutes the molecular signature of the ROS/RNS crosstalk whose importance in cell signaling has been recognized (Gross et al., 2013; Frederickson Matika and Loake, 2014; Sevilla et al., 2015; Foyer and Noctor, 2016; Mock and Dietz, 2016). Understanding this complex network requires the stoichiometry and dynamics of multiple redox PTMs to be determined under diverse physiological conditions or in different genetic backgrounds. This should be favored in the future by the development of sensitive and accurate redox quantitative MS approaches combined with the development of new selective chemical probes (Yang et al., 2016). In addition, computational structural genomic approaches will be required to integrate the Cys proteome at the structural level in order to get insights into the molecular mechanisms and the structural determinants governing each type of redox modification.

Besides redox PTMs, the integration of the signal implicates a myriad of other molecules and processes acting at the translational, transcriptional, and post-transcriptional levels (Mastrobuoni et al., 2012). In photosynthetic organisms, numerous redox PTMs are clearly linked to signaling pathways controlled by hormones (Terrile et al., 2012; Yu et al., 2012; Feng et al., 2013; Kneeshaw et al., 2014; Wang et al., 2015; Waszczak et al., 2015) or calcium (Trapet et al., 2015), and in mammals, nitrosylation was reported to interfere with signaling processes mediated by phosphorylation, ubiquitylation, sumoylation, acetylation, or palmitoylation (Held and

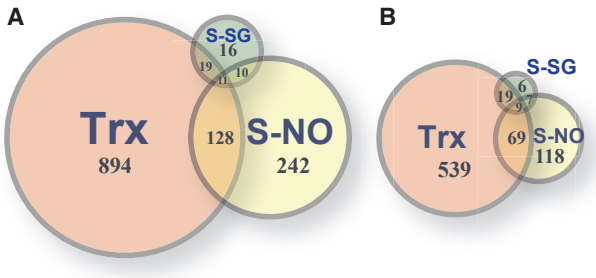

Figure 7. Specificity of Redox Post-Translational Modifications in *Chlamydomonas*.
(A) Venn diagram showing the distribution of cysteines experimentally identified as reduced by thioredoxins (Trx; this study) or modified by S-nitrosylation (S-NO; Morisse et al., 2014b), or by S-glutathionylation (S-SG; Zaffagnini et al., 2012a).
(B) Venn diagram showing the distribution of cysteines experimentally identified as redox modified after dataset restriction to *Chlamydomonas* proteins undergoing at least two of these three redox modifications (Trx reduction, S-nitrosylation, S-glutathionylation).

Gibson, 2012; Hess and Stamler, 2012). An intensive effort is therefore required to integrate redox signaling networks with other signaling pathways and to analyze their impact on the cellular responses at multiple levels. This will require large-scale systems biology approaches and the development of innovative computational methods to analyze and explore the massive datasets generated. Such approaches will certainly be crucial to unravel how environmental challenges are encoded into a biochemical signal that can be exploited to trigger the appropriate responses in terms of localization, duration, and intensity, at the genome, transcriptome, proteome, and metabolome level to allow adaptation and survival.

METHODS

Detailed descriptions of experimental and analytical approaches are found in Supplemental Materials and Methods

All MS proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomeexchange.org) via the PRIDE partner repository (Vizcaino et al., 2013) with the dataset identifiers PXD006097 (reviewer account username, reviewer97461@ebi.ac.uk; password, pM7i6HgK) and PXD006116 (reviewer account username, reviewer69485@ebi.ac.uk; password, YuEMZT17).

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant Online*.

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AUTHOR CONTRIBUTIONS

S.D.L. and C.H.M. designed the research. M.E.P.P., A. Mauries, M.H., and C.H.M. performed research. A. Maes and N.J.T. performed bioinformatic analyses. M.E.P.P., A. Mauries, S.D.L., and C.H.M. analyzed data. S.D.L. and C.H.M. wrote the article with input from the other authors.

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