Proteome-wide identification of S-sulfenylated cysteines in Brassica napus

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Abstract

Deregulation of reduction-oxidation (redox) metabolism under environmental stresses results in enhanced production of intracellular reactive oxygen species (ROS), which ultimately leads to posttranslational modifications (PTMs) in structure and molecular function of responsive proteins. Redox PTMs are important mediators of cellular signalling and regulation and several proteomic approaches attempted to quantify them under various stresses in plants. We aimed to generate large-scale redox proteomics data in response to short-term salt stress in Brassica napus by analyzing reversible cysteine modification. We employed iodoTMT approach to analyze the redox proteome of Brassica napus seedlings under control and salt stress conditions. We identified 2,010 peptides in 1,017 proteins, of which 1,821 sites in 912 proteins had oxidative modification. The redox homeostasis of biology processes in chloroplast and cytoplasm were mainly affected and the modification levels of proteins involved in photosynthesis and glycolysis pathways were significantly changed. Two oxidatively modified proteins were selected from the candidates and their in vitro activity under oxidative stress was assayed and validated the findings of this proteomics study. This targeted approach should contribute to the understanding of redox-based molecular changes prevailing in Brassica napus proteome subjected to salt stress and the mechanism adopted to cope with it.

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mediators of cellular signalling and regulation and several proteomic approaches attempted to quantify them under various stresses in plants. We aimed to generate large-scale redox proteomics data in response to short-term salt stress in *Brassica napus* by analyzing reversible cysteine modification. We employed iodoTMT approach to analyze the redox proteome of*Brassica napus* seedlings under control and salt stress conditions. We identified 2,010 peptides in 1,017 proteins, of which 1,821 sites in 912 proteins had oxidative modification. The redox homeostasis of biology processes in chloroplast and cytoplasm were mainly affected and the modification levels of proteins involved in photosynthesis and glycolysis pathways were significantly changed. Two oxidatively modified proteins were selected from the candidates and their in vitro activity under oxidative stress was assayed and validated the findings of this proteomics study. This targeted approach should contribute to the understanding of redox-based molecular changes prevailing in *Brassica napus*proteome subjected to salt stress and the mechanism adopted to cope with it.

KEYWORDS: *Brassica napus* , iodoTMT, reactive oxygen species (ROS), redox, proteomics

1 | INTRODUCTION

Exposure to abiotic stress fosters accumulation of reactive oxygen species (ROS) in plants, perturbing cellular homeostasis, where ROS act as key signalling molecules in various physiological processes. ROS have been shown to mediate post-translational modifications (PTMs) of proteins by oxidation of cysteine residues (Navrot et al., 2011). Thiol side chains (R-SH) of cysteine are the most sensitive targets of ROS, as they contain electron rich sulfur atom with various oxidation states (–2 to +4). Moreover, their nucleophilic and electrophilic behaviour makes them the major sites of oxidative modification (Wouters et al., 2010). Oxidation of cysteine thiols results in the formation of intra or intermolecular protein disulfide bridge or organosulfur oxoacids, which sets the grounds for reduction-oxidation (redox) regulation by bringing change in the activity and function of affected proteins which can be either reversible or irreversible. The generation of sulfenic acid (R-SOH) is an intrinsically reversible modification, which occurs as result of prompt oxidation of RSH with electron rich oxidant (H_2O_2). R-SOH is the first product of cysteine residue oxidation in most cases, which functions as redox sensor and protects the affected protein from irreversible oxidation by regulating enzymatic activities of signalling proteins, but further oxidation of cysteine thiols to sulfinic acid (R-SO_2H) or sulfonic acid (R-SO_3H) often leads to permanent loss of function of proteins, as these modifications are irreversible (Wang et al., 2012). Some known redox regulated proteins include glyceraldehyde 3-phosphate dehydrogenase (GAPDH), peroxiredoxin (PRDX), glutaredoxin (GLRX) and thioredoxins (TXN). These enzymes behave as housekeeping proteins involved in redox responses and metabolic regulation and are frequently reported in redox proteomic studies (Araki et al., 2016; Lindahl et al., 2011; Zaffagnini et al., 2012).

Several proteomic methodologies attempted to investigate the reversible cysteine oxidation through the identification of oxidation sensitive thiols within proteins (Lo Conte and Carroll, 2013). Gel based proteomic approaches employ thiol reactive reagents to label both reduced and oxidized thiols, then two-dimensional electrophoresis (2DE) is used for protein separation and differentially labelled proteins are identified according to their weight and charge (Alvarez et al., 2009; Wang et al., 2012). In addition to gel-based methods, gel-free high-throughput screening approaches are available for absolute measurement of abundance of known proteins in multiple samples. These include isotope coded affinity tags (ICAT) (Fu et al., 2008), OxICAT (Leichert et al., 2008), multiple reaction monitoring (MRM) (Held et al., 2010), thioredoxin affinity chromatography and some others as wells (Picotti and Aebersold, 2012). Although these methods can detect oxidized proteins, their extent of multiplexing in terms of simultaneous quantitative protein profiling of multiple samples and enrichment of oxidized cysteines is limited. Quantitative proteomic techniques based on stable isotope labelling offers many advantages in terms of ease and accuracy (Tambor et al., 2012). Some of the examples include isobaric tags for relative and absolute quantitation (iTRAQ) and its modifications such as oxiTRAQ (Liu et al., 2014), cysTRAQ (Zhang et al., 2016) etc. The major shortcoming is that they cannot distinguish the exact modified site in a peptide having multiple cysteine residues. Recently, an approach in combination of BTD-based probe with quantitative chemoproteomics workflow has been applied in site-specific mapping and quantification of cysteine S-sulfenylation in Arabidopsis cells (Jingjing Huang et al., 2019). Despite the

development of various proteomic methodologies over the past decade, identification of multiplexed cysteine modification remained technically challenging as these are diverse and less abundant under endogenous conditions. Moreover, thiol redox reactions are very complex as the number of modified cysteine residue per protein is fairly higher than other PTMs (Gu and Robinson, 2016).

Iodoacetyl tandem mass tags (iodoTMT) based proteomic approach is considered as the most efficient high-throughput strategy for the analysis and quantification of reversible cysteine oxidation as it allows to determine the position of modified site and quantify the level of oxidized protein based on cysteine specific tags (Wojdyla et al., 2015). These isobaric tags are composed of sulfhydryl-reactive iodoacetyl group, a mass normalizing spacer arm and a mass reporter and have identical overall mass, but a different distribution of isotopes around their structures with different masses which are detected during mass spectrometry analyses and are employed for quantification of the change of modified protein (Shakir et al., 2017). To quantify cysteine oxidations in proteomes, unmodified cysteines are blocked with S-Methyl methanethiosulfonate (MMTS) in cell lysates, reversibly oxidized cysteine residues and blocked cysteines are first reduced by tris (2-carboxyethyl) phosphine (TCEP) and arsenite respectively, and then labelled by iodoTMT-labels with different mass. Anti-TMT antibody is used to purify these labelled cysteine residues by immunoprecipitation, which are later quantified in tandem MS through comparison of the intensity of reporter ion. This technique offers sample multiplexing coupled with a higher sensitivity level. A combination of iodoTMT and iTRAQ strategies was employed to identify 47 redox regulated proteins in Arabidopsis suspension culture subjected to bicarbonate (Yin et al., 2017). Another TMT based labelling approach, cysTMT (cysteine reactive tandem mass tag) identified 80 thioredoxin targeted cysteine residues in the epidermal peels of *Brassica napus* (*B. napus*) (Zhang et al., 2016). This iodoTMT technique has also been used in animal studies, where 1754 cysteine containing peptides were identified in mammalian cell treated with DTT and H_2O_2(Araki et al., 2016). Another study using OxiTMT approach detected 1229 iodoTMT-labelled cysteine residues in*E.coli* cultures subjected to oxidative stress treatment with H_2O_2(Shakir et al., 2017).

Although cysteine oxidative modifications in plants have been investigated by several studies, the oxidation is usually induced by chemical treatment of suspension cells. The obtained results may not reflect physiological status of cysteine oxidative modifications in proteins. In our study, indigenous ROS levels in *B. napus*seedlings were made to rise by the application of 200 mM salt stress. Elevated H_2O_2 content in leaf samples led us to hypothesize that cysteine-containing thiols would have been oxidized. We aimed to quantify reversible cysteine modification and resulting oxidized protein abundance changes. Here, we present the identification of 1821 oxidative modification sites in 912 proteins by iodoTMT labelling approach under control and salt stress conditions. This study demonstrates the efficacy of iodoTMT labelling in the identification and quantification of hundreds of redox modified proteins in *B. napus* under salt stress. These results will help unveil the mechanism of redox-mediated PTM in *B. napus* .

2 | METHODS AND MATERIALS

2.1 | Plant material, salt stress treatment and H_2O_2 measurement

Seeds of *B. napus* variety Zhongshuang 11 were first disinfected with 10% sodium hypochlorite for 10 min, then washed thrice with distilled water and later put on gauze partially dipped in water for germination. After 7 days, the seedlings with uniform size were shifted to pots with Hoagland medium and kept under the conditions of 16 h light/8 h dark and temperatures of 23/19°C with 120 μmol m^{-2} s^{-1} light intensity. The nutrient solution was replaced every 7 days. After two weeks, the plants were treated by 200 mM sodium chloride. ROS levels in the third leaf (from top) were selected for sample collection for proteomic analysis. Leaf samples from three individual plants were snap frozen in liquid nitrogen after harvesting and stored at -80°c till further use. H_2O_2 content was measured following manufacturer protocol (Comin Biotechnologies, Suzhou, China). Three biological repeats were used for each analysis.

2.2 | Protein sample preparationand iodoTMT labelling

Frozen leaf samples (three biological replicates) were ground to fine powder using liquid nitrogen followed by addition of lysis/blocking buffer containing150 mM HEPES (pH 7.3), 1 mM EDTA, 2% SDS, 50 mM S-

Methyl methanethiosulfonate and 1% protease inhibitor. The lysate was sonicated and centrifuged at 20,000 g for 10 min at 4 °C). The resulting supernatant was precipitated with 4 volumes of ice-cold acetone and was kept at -20degC for about 4 hours. Another step of centrifugation using the same conditions was carried out to produce the protein pellet that was washed thrice with cold acetone. Washed pellets were dissolved in a buffer of 50 mM ammonium bicarbonate, 1 mM EDTA, 2% SDS, pH 8.0 and protein concentration was determined using bicinchoninic acid (BCA) kit according to the manufacturer's instructions.

iodoTMT Label Reagent Set was purchased from Thermo Scientific and iodo-TMT labelling was performed following Thermo Scientific's protocol with modifications. For total cysteine labelling, reduction of each sample (100 µg, 1 µg μl^{-1}) was carried out using 10 mM TCEP for one hour at 37 °C followed by alkylation with 0.9 mM iodoTMT-128 (control) and iodoTMT-130 (salt-stressed). For S-sulfenylation labelling, 100 µg of each sample was reduced with 10 mM sodium meta arsenite and alkylated with 0.9 mM iodoTMT-129 and iodoTMT-131 for control and salt-stressed samples, respectively. IodoTMT labelling for all samples continued for 2 h with occasional shaking at 37 °C. Subsequently, 500 mM dithiothreitol (DTT) was added to quench the reaction and samples were incubated for 15 min, pooled and precipitated with 4 volumes of chilled acetone overnight at -20 °C. Resulting pellets were washed thrice with ice cold acetone to remove excess iodoTMT reagents and dissolved in a buffer of 50 mM ammonium bicarbonate, 8 M urea, pH 8.0. About 4 ug proteins were separated by discontinuous SDS-PAGE with a 12% separating gel and transferred to nitrocellulose membrane to verify the enrichment of proteins treated with TCEP and arsenite. Proteins were detected using anti-TMT antibody. Final samples were reduced using 10 mM DTT, alkylated with 20 mM iodoacetamide (IAM) for 45 min at room temperature in the dark and then urea concentration was adjusted below 2 M using 50 mM ammonium bicarbonate. First enzymatic digestion with trypsin was carried out overnight at 37degc using 1:50 trypsin-to-protein mass ratio followed by another 4 h digestion employing 1:100 ratio. The resulting peptide solution was acidified with 0.5% trifluoroacetic acid (TFA) followed by a desalination procedure using Strata X C18 SPE column (Phenomenex) and later vacuum-dried.

2.3 | HPLC fractionation and affinity enrichment

Peptides were separated into 80 fractions over 80 minutes using a gradient of 2% to 60% acetonitrile in 10 mM ammonium bicarbonate (pH 10) by high pH reverse-phase HPLC using Agilent 300 Extend C18 column (5 µm particles, 4.6 mm ID, 250 mm length). Peptides were combined into 6 fractions and vacuum-dried. For affinity enrichment, anti-TMT Resin (100 ul/100 µg peptide) was washed thrice with 1×TBS buffer (Tris-buffered saline) and incubated with peptides for 2 h at room temperature or overnight at 4 °C. The supernatant was discarded and the resin was washed with TBS buffer for 5 times followed by washing three times with 1 column volume of water. Peptides were eluted using 4 column volumes of TMT elution buffer (40% ACN, 5% TFA) and lyophilized.

2.4 | LC-MS/MS analysis

Peptides were dissolved in 0.1% formic acid and loaded onto a reversed-phase pre-column (Acclaim PepMap 100, Thermo Scientific). Reversed-phase analytical column (Acclaim PepMap RSLC, Thermo Scientific) was used for peptide separation. The gradient consisted of an increase from 5-25% using solvent (0.1% FA in 98% ACN) for 26 min, 25-40% for 8 min and a final rise to 80% in 3 min, followed by holding for the last 3 min at a constant flow rate of 400 nl/min on an EASY-nLC 1000 UPLC system. Peptides were analyzed by Q Exactive^{TM} hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific).

2.5 | Database search

The resulting MS/MS data were searched against *B. napus* database (http://www.genoscope.cns.fr/brassicanapus/) using MaxQuant with an integrated Andromeda search engine (v.1.4.1.2). The following parameters were employed. Trypsin as cleavage enzyme, allowing 2 missing cleavages, 5 modifications per peptide, 5 charges, 10 ppm precursor mass tolerance ions and 0.02 Da fragment mass tolerance. Variable modifications were specified as Carbamidomethylation (Cys), oxidation (Met), and acetylation on protein N-terminus. Minimum peptide length was set at 7 and false discovery rate (FDR) thresholds for protein, peptide and modification site were set at 1%. IodoTMT-6plex was

selected as a quantification method and site localization probability was 0.75. All the other parameters were specified to their default values. QC validation of MS data was also conducted. The lengths of most peptides distributed between 8 and 20, which depicts the property of tryptic peptides, hence authenticating the sample preparation procedure.

2.6 | Bioinformatics analysis

Several annotation methods were used to predict the functional characteristics of identified proteins. Gene ontology was checked using UniProt-GOA database (www. http://www.ebi.ac.uk/GOA/). Domain annotation search was conducted using both InterProScan and InterPro (http://www.ebi.ac.uk/interpro/). KEGG pathway Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to annotate protein pathway and subcellular localization was analyzed using Wolfpsort program (http://wolfpsort.org). Soft motif-x was used to analyze the specific positions of modified amino acids in protein sequences. A two-tailed Fisher's exact test was used to test the enrichment of the modified versus identified proteins and annotation with corrected p-value < 0.05 was considered significant. The Z scores collected from one-way hierarchical clustering i.e Euclidean distance, average linkage clustering was clustered and then visualized by a heat map using the "heatmap.2" function from the "gplots" R-package.

2.7 | Enzyme activity assay

Evaluation of the effect of H_2O_2 on the enzyme activity was carried out by assaying activity of cytosolic fructose 1-6, bisphosphatase (FBPase) and phosphoglycerate kinase 3 (PGK3) *in vitro* . Total RNA was extracted from the leaves of Zhongshuang 11 using the reagent Transzol (Sigma). After DNase treatment (Thermo Scientific), extracted RNA was reverse transcribed into cDNA using Revert Aid First strand cDNA synthesis kit (Thermo Scientific) following the manufacturer's protocol. The coding region of fructose 1-6, bisphosphatase was amplified using gene specific primers F:5´GCG*GAATTC* ATGGATCACGAAGCAGATGC3´; R:5´GCG*GTCGAC* TTAGTTATTCTCCTCCGCAGC3´ with EcoRI and SalI sites (underlined). For phosphoglycerate kinase, primers F: 5´GCG*GGATCC* ATGGCGACGAAGAGAAGC3´ and F:5´GCG*GTCGAC* TCAAGCGTCGTCGAGAG3´with BamHI and SalI sites (underlined) were used. I-5 2X Hi-Fi PCR Master Mix (Molecular cloning laboratories) was used for PCR under the condition of 98 degC 2 min, 35 cycles of 98 degC 10s, 61 degC 15 s, and 72 degC 45 s with a final extension step of 5 minutes. The PCR products were ligated with pET-28a-c (+) vector with 6xHis tag at the N terminus. Both vectors were sequenced before use. The recombinant plasmids were transformed into *E. coli* BL21 (DE3) pLysS. Protein expression was induced by adding 0.8 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at 28 °C for 8 hours. Bacterial cultures were pelleted at 4000 rpm at 20 °C for 10 min and subsequently resuspended in 15 ml lysis buffer (50mM NaH_2PO_4, 300 mM NaCl, pH 8) containing 1 mM phenylmethanesulfonyl fluoride (PMSF). The lysate was centrifuged at 12,000 *g* at 4 °C for 20 min and proteins were purified by using Ni-NTA resin Kit (Sangon Biotech) according to the manufacturer's protocol. Enzyme activity was assayed following the previously described methods (Ganapathy et al., 2015; Reddy and Wendisch, 2014).

3 | RESULTS

3.1 | Workflow for proteome-wide identification of protein S-sulfenylation by iodoTMT-based proteomic approach

The workflow of iodoTMT-based proteomic approach, shown in Figure 1, was used to quantify cysteine oxidation in control and salt treated samples. Proteins are extracted from control and salt-treated samples in the presence of 50 mM MMTS to block the free thiols (SH). Reversible oxidized thiols (SOH) are specifically reduced by arsenite, while blocked SH together with disulfide bonds (S-S) are reduced by TCEP followed by labelling proteins in the samples with different TMT-labels. After tryptic digestion, the multiple labelled peptides were enriched by anti-TMT antibody and analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). Relative abundance of peptides in salt-treated samples are compared to that in control samples and the ratio is calculated to evaluate the differentially expressed proteins and differentially oxidized proteins under salt stress. The quantitative ratio over 1.2 was considered up-regulation while quantitative ratio below 0.833 was considered as down-regulation ($p < 0.05$).

3.2 | Quantitative identification of oxidized proteins

We detected the H_2O_2 level in the leaves after salt stress treatment at different time points to help us determine the appropriate time point for sampling. H_2O_2 level significantly increased after 0.5 h salt treatment and reached the highest level at 12 h (Figure 2a). Redox status in plant cells is dynamic under stress conditions and we decided to collect the leaves at 4 h for proteomics analysis. After labelling, the labelled proteins treated with TCEP and arsenite were confirmed by western blot using anti-TMT antibody. The results showed the proteins were successfully labelled and the pattern of protein bands was generally different between SH and SOH (Figure 2b). The quality control and distribution of peptide length indicated that the mass error and sample preparation reach the standard (Figure S1).

Altogether, 1,338 unique oxidized peptides were identified in our data, containing 1,821 quantifiable sites in 912 proteins (Table S1). The abundance of reduced cysteine sites can be quantified via the labelled RSH. There were 1,809 oxidized sites in 909 proteins shared by both control and salt treated samples (Figure 2c). Among these 912 oxidized proteins, 48% were located in chloroplast and 29% in cytoplasm, some other subcellular localizations include nucleus, extracellular, mitochondria and also several proteins were located in plasma membrane, cytoskeleton, vacuolar membrane, endoplasmic reticulum, peroxisome and golgi apparatus (Figure 2d). The length of modified peptides distributes between 7 to 42 (Figure S1c), and the number of modified sites on proteins were counted, most of the oxidized proteins have one to three oxidized cysteine sites, and there were up to 14 oxidized sites in one protein (Figure 2e). These results suggest that there was little difference in the types of oxidized proteins after salt treatment compared with control. Most of these oxidized proteins were distributed in chloroplast and cytoplasm and 90% of these protein peptides have one to three modified cysteines.

We performed Gene ontology (GO) enrichment analysis for these oxidized proteins. These proteins were enriched in biological processes such as photosynthesis, pyruvate metabolic process and glycolytic process, and molecular functions such as catalytic activity, oxidoreductase activity, lyase activity and hydrolase activity and DNA binding (Figure 3a). We also compared these oxidized peptides with reported oxidized peptides identified from H_2O_2 treated suspension cells of *Arabidopsis thaliana* (Huang et al., 2019). Of the 1338 unique peptides we identified, 161 can matched to *Arabidopsis thaliana* which contain 1526 unique peptides, suggesting a different selection of modification between the two different species under the different treatments (Figure 3b).

3.3 | Salt treatment caused an increased %oxidation level *in vivo*

Among these oxidized proteins, many have been reported as redox sensitive proteins previously, and a few of them were indicated in the volcano plot, such as glutathione S-transferase (GST), Chloroplast protein 12 (CP12) and Phosphoglycerate kinase (PGK) (Figure 4a). In order to distinguish the real oxidation level of single protein, we calculated %oxidation of proteins, the percentage of modified state to total state of proteins, to estimate the impact of protein modification, due to small change of low %oxidation proteins is unlikely to cause changes in plant physiological level, the average %oxidation as well as the total %oxidation of proteins in salt-treated sample was significantly higher than that in control (Figure 4b and Table S2). In order to make a thorough inquiry of the change of %oxidation under salt stress, we divided the oxidized proteins into four groups depending on their %oxidation value, group 1 (0-10%), group 2 (10%-20%), group 3 (20%-50%) and group 4 (50%-100%). The distribution of four groups showed an obvious difference between salt treated and control sample. The proteins in salt sample showed a decreased count in group 1 and group 2 with an increased count in group 3 (Figure 4c). In order to observe the %oxidation of proteins in different subcellular locations, we found that the increased high %oxidation proteins were mainly in chloroplast, cytoplasm, and nucleus (Figure 4d, e). These results point to a trend of proteins from low %oxidation (0-20%) to high %oxidation (20-50%) change under treatment, which occurred extensively in chloroplast, cytoplasm and nucleus.

3.4 | The landscape of oxidation fluctuation *in vivo* under salt stress

The differentially expressed and modified sites were identified with a threshold value of 1.2 folds for increase

of oxidation level and 0.83 folds for decrease of oxidation level. There was no correlation between the change of protein abundance and the change of oxidation level (Figure S2a). The number of identified oxidized cysteine of the differentially oxidized proteins is between 1 and 5 while 67% of the proteins have only one identified oxidized cysteine (Figure S2b). Totally, 127 differentially oxidized proteins were identified (Table S3), which mainly participate in processes such as carbon metabolism, photosynthesis and amino acid metabolism (Figure S2c). Among these salt-induced modified proteins, only 85 proteins (less than 10%) containing 96 sites were up-regulated and 43 proteins containing 98 sites were down-regulated in oxidative level. Meanwhile, the abundance of 97 sites in 52 proteins was up-regulated and 19 sites in 13 proteins were down-regulated under salt stress (Figure 5a). GO enrichment analysis of differentially expressed proteins (DEPs) showed that the up-regulated proteins were involved in various biological processes such as metabolic process of xyloglucan and glucan, cellular components such as microtubule and supramolecular fiber, and molecular functions such as transferase activity and calcium ion binding. The down-regulated proteins were involved in biological processes such as carbon utilization and photosynthesis, cellular components such as thylakoid and photosystem, and molecular functions such as carbon dehydratase activity and chlorophyll binding (Figure S2d).

Representative differentially modified peptides are shown in Table 1. According to the distribution pattern of differential abundance at expression and oxidation level, we found just 5 common proteins were up-regulated both in total protein level and oxidized level, whereas 5 proteins were down-regulated in oxidized level but up-regulated in expression level and 2 proteins were up-regulated in oxidized level but down-regulated in expression level (Figure 5b). According to the prediction of subcellular localizations, most of the differentially oxidized proteins were located in chloroplast and cytoplasm, and some other proteins were located in nucleus, mitochondria, membrane, and cytoskeleton (Figure 5c). Motif analysis revealed the occurrence of three main motifs in differentially oxidized proteins (DOPs), a glycine (G) following the oxidized cysteine (cys-SOH), a glutamate (E) in the two positions before cys-SOH, and a lysine (K) in the eighth position before cys-SOH, each having varying levels of fold change (Figure 5d). Average ratios of these modified sites were plotted (Figure S3a).

Based on GO enrichment analysis, the up-regulated DOPs were enriched in biological processes involved in metabolic processes, biosynthetic process, photorespiration, carbon utilization, fructose 1,6-bisphosphate metabolic process, carbon fixation, and carboxylic acid metabolic process, and molecular functions controlling catalytic activity, alanine-oxo-acid transaminase activity, lyase activity, transaminase activity, GTPase activity, carbonate dehydratase activity, vitamin binding, ion binding, small molecule binding, GTP binding, nucleoside binding (Figure 5e). The down-regulated DOPs mainly function in methionine adenosyl-transferase activity and oxidoreductase activity and controlling the biological processes of S-adenosylmethionine biosynthetic process, S-adenosylmethionine metabolic process, metabolic process (Figure 5f). These results suggest that protein activity of multifarious metabolic processes in chloroplast and cytoplasm are mainly affected by salt stress. KEGG enrichment analysis showed that oxidation level of proteins involved in carbon fixation during photosynthesis, protein processing in endoplasmic reticulum, arginine biosynthesis and nitrogen metabolism were enhanced under salt stress, whereas proteins involved in photosynthesis and nitrogen metabolism had reduced oxidation level (Figure S3b).

3.5 | Enzymatic activity of fructose 1-6 bisphosphatase (FBPase) and phosphoglycerate kinase 3 (PGK3) are regulated by hydrogen peroxide

GO enrichment analysis of oxidized proteins showed that these proteins mainly participate in metabolic pathway of glycolytic, pyruvate and photosynthesis and have molecular function of catalytic activity and binding (Figure 3a). Biochemical assays based on activity change of purified thiol-containing enzymes in response to oxidants like H_2O_2 have been considered a powerful tool to demonstrate redox status and switching of thiol residues*in vitro* (Nietzel et al., 2017). Enzyme activity of cytosolic fructose 1-6 bisphosphatase (cyFBPase) and phosphoglycerate kinase 3 (PGK3) under control and oxidative stress conditions was assayed to validate the results of proteomic study. After these two proteins were successfully expressed and purified from *E . coli* (Figure S4), the enzyme assay was performed by adding different concentrations of H_2O_2 (0

mM, 0.2 mM, 0.5 mM, 1 mM, 2 mM, 5 mM, 10 mM) in reaction buffer. The activity of cyFBPase was continuously enhanced with an increasing H_2O_2 content, while PGK3 displayed decreased activity after treatment with different concentrations of H_2O_2 (Figure 6a,b). Mass spectrometry showed that the enriched peptides of these two enzymes were modified*in vivo* , and the modified sites were cyFBP-cys48 and PGK3-cys62 (Figure 6c,d). These results indicated that oxidation of protein by H_2O_2 could directly affect the enzyme activity of cyFBPase and PGK3, supporting our iodoTMT-based redox proteomic findings.

4 | DISCUSSION

Understanding the complex adoptive mechanisms by which plants mediate their signal transduction and cellular regulation under salt stress will help the development of elite varieties that are more capable of tolerating abiotic stress. Different from the chemical treatment on*Arabidopsis* suspension cells, we employed iodoTMT labelling based approach to study reversible oxidized proteins in the leaves of *B. napus* modified by H_2O_2 produced*in vivo* under salt treatment, which can reflect the true redox event happening in plants under stress. Our goal was to use iodoTMT to map the cysteine PTMs and identify specific sites of protein S-sulphenylation in response to salt stress in *B. napus* . This method proved to be very efficient for analyzing multiplex redoxome under salt stress, which generated proper information about *in vivo* thiol status of 912 modified proteins containing 1338 unique peptides under control and salt stress. Some reported redox modified proteins in *Arabidopsis* , such as GST, CP12, protein tyrosine phosphatase 1 (PTP1), elongation factor Ts (EF-Ts), nitrilase 4 (NIT4), citrate synthase (CS), PGK and so on (Liu et al., 2014; Liu et al., 2015; Wang et al., 2012) were also detected (Figure 4a). . Besides these reported proteins, a large number of novel redox-sensitive peptides were also identified by our data, hence increasing the scope of our understanding of redox regulatory network. Under salt stress, 128 proteins were differentially oxidized. Among these DOPs we find some known proteins have function in mediating salt tolerance, such as Phospholipase D α (PLDα) and lipid transfer proteins (LTPs) (Table 1). PLDα1 was known to play a crucial role in mediating salt tolerance by the pH-modulated interaction with PA (Li et al., 2019). LTPs are widespread stress regulatory proteins in plants and LTP4 has been reported as a positive regulator during salt stress in *N. tabacum*(Xu et al., 2018).

Previous research showed that the oxidized proteins induced by H_2O_2 treatment were mainly located in cytoplasm (Huang et al., 2019). In this study, we found the most oxidized proteins and DOPs were mainly located in chloroplast, and the increased %oxidation of protein also mainly happened in chloroplast (Figure 2d, 4e, 5c). This corresponds to the knowledge that chloroplast is one of the primary cellular structure of endogenous ROS generation (Farooq et al., 2019). And H_2O_2 could accumulates in the cytoplasm during transport from organelles and outside the cell (Waszczak et al., 2018), which could be the main reason of the different subcellular localizations of oxidized proteins aggregation. It may be the same reason that the oxidative modified peptides induced by H_2O_2treatment in Arabidopsis suspension cells *in vitro* are not as much as those identified in our data (Figure 3b).

We identified up to 14 modification sites belonging to the same protein (Figure 2e), but these sites do not represent all cysteine sites on the protein, because the enriched products are in the form of peptides distribute in length from 7 to 42 (Figure 1, Figure S1c), this still reflects the existence of some proteins that are rich in redox sensitive sites. The results from the motif enrichment analysis showed that the characteristics of oxidized sequence displayed three amino acid preferences near the modification sites. It seems that the sensitivity of cysteine to H_2O_2 is related to the changeable polarity and charge of around amino acids caused by disturbed intracellular environment, as the positively charged side group stabilizes the negatively charged sulphide (S^-) (Xiao et al., 2020).

The oxidized proteins were enriched in glycolytic, pyruvate and photosynthesis processes (Figure 3a). The pathway of carbon fixation in photosynthetic organisms was also enriched as up-regulated proteins in oxidized level (Figure 5e). The detail proteins identified in these pathways and processes were mapped out (Figure S5). After an encounter with stress, carbohydrate metabolism and energy production are rapidly adjusted as plant needs more energy to maintain its routine activities (Ghosh and Xu, 2014). Up-regulation in oxidized level of enzymes like enolase (ENO), fructose bisphosphate aldolase (ALDO), phosphoglycerate kinase (PGK)

and fructose-1.6-bisphosphatase (FBPase) suggested strengthened glycolysis pathway and neoglycogenesis pathway in response to salt stress leading to the generation of more ATP and NADPH to meet energy demands under oxidative stress. ALDO is a key role player enzyme in signal transduction and production of water soluble carbohydrates (Schaeffer et al., 1997). We observed an enhanced activity of cyFBPase and a decreased activity of PGK under oxidative stress, which suggests a way of plant adapting to an adverse environment by lowering their own metabolic levels and adjusting glucose allocation.

CONCLUSION

IodoTMT labelling based approach proved to be a powerful strategy for the quantification of cysteine modification and identification of proteins in *B. napus.* We identified 909 redox-modified proteins at varying fold change in oxidized level under salt stress and observed an increased oxidation level *in vivo* during salt stress. These oxidized proteins contained a large number of metabolic enzymes and were involved in various biological processes such as photosynthesis and glucose metabolism. We also provided a vitro evidence of enzyme activity altered by hydrogen peroxide modification. Our study explored the response to salt stress in *B. napus* using iodoTMT method. The discovery of these redox responsive proteins in *B. napus* lays a foundation for future studies directed toward functional characterization of these previously unknown redox responsive proteins and increases the understanding of molecular mechanisms underlying the role of redox regulatory network in stresses tolerance.

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AUTHOR CONTRIBUTIONS

LG and XY designed this study. YL, SI and G.Z performed the experiments. YZ and YL did bioinformatics analysis. SI, YL and YZ wrote the manuscript. LG, XY, YL and UA revised the manuscript. All authors read and approved the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. The quality control test and distribution of peptide length.

Figure S2. Differentially expressed and differentially modified proteins.

Figure S3. Enrichment of differential modification sequence and protein function.

Figure S4. Purification of proteins from *E.coli.*

Figure S5. Redox modified key enzymes involved in carbon metabolism.

Table S1. Quantification and annotation of oxidized sites and proteins.

Table S2. %oxidative of protein sites in control and salt treated samples.

Table S3. Differentially oxidized protein information.

FIGURE LEGEND

FIGURE 1 Workflow of the iodoTMT method for identification of redox-sensitive proteins. MMTS, S-Methyl methanethiosulfonate; SH, reduced thiols; SOH, reversible oxidized thiols; TCEP, tris (2-carboxyethyl) phosphine.

FIGURE 2 Proteomic identification of redox sensitive proteins in rapeseed. (a) H_2O_2 content in rapeseed leaves under 200 mM NaCl treatment. Values are means ± SD of three biological replicates (n = 6-8. Student's t-test was used for statistical analysis (*, $P < 0.05$; **, $P< 0.01$). (b) Labeled proteins detected by anti-TMT antibody. Total proteins (-SH) and oxidized proteins (S-OH) were labeled with iodoTMT-128 and iodoTMT-129 respectively. Proteins (4 μg) of each sample were separated by SDS-PAGE followed by western blot using anti-TMT antibody. (c) Venn diagram showing shared modified sites and proteins identified under control and salt stress conditions. The numbers of sites and proteins are listed in each diagram component, respectively. (d) The distribution of predicted subcellular localizations of oxidized proteins. (e) The number of oxidized sites in the oxidized proteins.

FIGURE 3 Proteomic identification of redox sensitive proteins in rapeseed. (a) GO enrichment analysis of oxidized proteins. The FDR values are plotted on the x-axis and the dot size indicates the number of the oxidized proteins. (b) Venn diagram showing the overlapped oxidized peptides between *B.napus* and *Arabidopsis* .

FIGURE 4 Salt treatment caused an increased %oxidation (oxidative cys/total cys) of proteins in *B. napus* . (a) Volcano plot showing statistical significance (-$\log_{10}$ of P-value) on the y-axis and differentially oxidation level ($\log_2$ of ratio) on the x-axis. Blue dots represent the proteins with up-regulated oxidation level and green dots represent the proteins with down-regulated oxidation level ($P < 0.05$, n = 3, ANOVA). Oxidized proteins identified in *Arabidopsis* are marked in the volcano plot. (b) %oxidation level of sites under control and salt stress conditions. (c) %oxidation distribution of oxidized sites under control and salt stress conditions. (d) and (e) %oxidation distribution of oxidized sites in different subcellular compartments.

FIGURE 5 Differentially modified protein analysis. (a) Number of sites and proteins in differentially expression level (total) and differentially oxidation level (oxidative). (b) Venn diagram showing the overlapped proteins between differentially oxidized proteins and differentially expressed proteins including up-regulated and down-regulated proteins, respectively. (c) Subcellular localizations of the differentially oxidized proteins. (d) Sequence motif analysis of differentially oxidized proteins (n = 3,$P < 0.05$). (e) and (f) GO enrichment analysis of differentially oxidized proteins, including up-regulated proteins (e) and down-regulated proteins (f). The FDR values are plotted on the x-axis and the dot size indicates the number of the oxidized proteins.

FIGURE 6 Effect of H_2O_2-induced modification on enzyme activity. (a) Fructose-1,6-bisphosphatase (FBP) activity under H_2O_2 treatment. (b) Phosphoglycerate kinase (PGK) activity under H_2O_2 treatment. Values are means ± SD (n=3). *, $P < 0.05$; **, $P < 0.01$ based on t-test. (c) Mass spectrograms of FBP-C48. (d) Mass spectrograms of PGK-C62.

FIGURE S1 The quality control test and distribution of peptide length. (a) Reproducibility test between control and salt treated samples used in proteomics study. Three biological replicates were tested under both control and oxidative conditions. (b) Mass error of all identified peptides. (c) Distribution of peptide segment length generated by mass spectrometry.

Figure S2 Differentially expressed and differentially modified proteins. (a) Correlation analysis of the changes of protein abundance and oxidation level. (b) Number of differentially oxidized sites per protein in response to salt stress. (c) Metabolic pathway distribution of differentially modified proteins. (d) GO enrichment analysis of differentially expressed proteins.

Figure S3 Enrichment of differential modification sequence and protein function (a)Sequence motif analysis of differentially oxidized proteins. (b) KEGG pathway enrichment analysis of differentially oxidized proteins.

Figure S4 Purification of proteins from *E.coli.* (a) Fructose 1-6 bisphosphatase (FBP). (b) Phosphoglycerate kinase (PGK). FBP and PGK were expressed in *E.coli* and purified by Ni-NTA resin Kit. Elution buffer having 250 mM imidazole was used to elute the purified protein, which was further used in activity assay.

Purified proteins were visualized by coomassie blue staining and western blot using anti-His antibody. M, protein marker; S, soluble fraction; FT, flow through fraction; W1, first wash. Values are means ± SD (n = 3). *,$P < 0.05$; **, $P < 0.01$ based on t-test.

Figure S5 Redox modified key enzymes involved in carbon metabolism. SOH-up and SOH-down indicate up and down-regulated oxidation modification of proteins under salt stress, respectively. SOH-proteins indicate oxidized protein identified in this study. SOH-previous indicates redox proteins identified in previous studies. PGP, phosphoglycolate phosphatase; PGIp, polygalacturonase-inhibiting proteins; PGMp, phosphoglucomutase; GLYK, d-glycerate 3-kinase; GAP, glyceraldehyde-3-phosphate dehydrogenase; ENO, enolase; PGK, phosphoglycerate kinase; PK, pyruvate kinase; TPI, triose-phosphate isomerase; PGAM, phosphoglycerate mutase; PGAM, phosphoglycerate mutase; PEP, phosphoenolpyruvate; PGA, phosphoglyceric acid; PGI, phosphoglucose Isomerase; FBP, fructose 1,6-bisphosphatase; Glu-6p, glucose 6-phosphatase; DHAP, dihydroxyacetone phosphate; 1,3-BPGA, 1,3-bisphosphoglyceric acid; RuBP, ribulose biphosphate; Ru-5P, ribulose-5-phosphate; AGPase, ADP-glucose pyrophosphorylase.

Table 1 Representative differentially modified peptides

Protein description	**Gene name**	**Modified peptide**	**Modified peptide**
Lipoxygenase 2	BnaC06g18870D	BnaC06g18870D	YEC*VFDMPEDFGTV
Glutathione peroxidase 1	BnaAnng31150D	BnaAnng31150D	SQGFEILAFPC*NQFG
Rotamase CYP 4	BnaA09g39610D	BnaA09g39610D	IYAC*GELPLDA
Phosphoenolpyruvate carboxylase 2	BnaC04g02430D	BnaC04g02430D	EFVQEC*YEVAADYD
Tyrosine transaminase family protein	BnaC03g65220D	BnaC03g65220D	LSPDDVFMTVGC*K
Lipid-transfer protein	BnaC09g08390D	BnaC09g08390D	ATC*PIDTLK
Thioredoxin superfamily protein	BnaC05g41590D	BnaC05g41590D	TLQALQYIQENPDEV
Cell division cycle 48	BnaA05g28710D	BnaA05g28710D	GVLFYGPPGC*GK
Lactoylglutathione lyase	BnaC03g13130D	BnaC03g13130D	TDYGVVGVHHVGLL
Cyclophilin 20-2	BnaA03g04200D	BnaA03g04200D	VVIADC*GQLPMSEA
Phospholipase D alpha 2	BnaAnng18990D	BnaAnng18990D	C*WEDIFDAITNAK
Pseudouridine synthase	BnaCnng69640D	BnaCnng69640D	TGESPPDGFMC*PSG
Ribulose bisphosphate carboxylase	BnaAnng36210D	BnaAnng36210D	LPLFGC*TDSAQVLK
Glycine decarboxylase P-protein 1	BnaA01g03860D	BnaA01g03860D	DLSLC*HSMIPLGSC*T
Glycine decarboxylase P-protein 1	BnaA01g03860D	BnaA01g03860D	NVC*IIPVSAHGTNPA
Lipid transfer protein 5	BnaA09g32110D	BnaA09g32110D	GC*C*SGVR
Rubisco activase	BnaA03g18710D	BnaA03g18710D	MC*C*LFINDLDAGAG
Glutamate synthase 1	BnaCnng10340D	BnaCnng10340D	IC*HTNNC*PVGVASQ
Glyceraldehyde-3-phosphate dehydrogenase B subunit	BnaC08g46180D	BnaC08g46180D	GVLEVC*DTPLVSC*D
Sedoheptulose-bisphosphatase	BnaA09g35380D	BnaA09g35380D	TASC*GGTAC*VNSFG
ATPase, F1 complex, gamma subunit protein	BnaA03g25540D	BnaA03g25540D	GEIC*DINGNC*VDAA
Ketol-acid reductoisomerase	BnaC08g29320D	BnaC08g29320D	DFETAYSASFYPC*ME
2Fe-2S ferredoxin-like super family protein	BnaA06g06760D	BnaA06g06760D	EVEC*DGDTYVLDAA
Methionine adenosyltransferase 3	BnaC04g08360D	BnaC04g08360D	LC*DQVSDAILDAC*LE
S-adenosylmethionine synthetase	BnaA03g34510D	BnaA03g34510D	LC*DQISDAILDAC*LE
S-adenosylmethionine synthetase 2	BnaCnng75920D	BnaCnng75920D	LC*DQISDAVLDAC*LE
Chloroplast stem-loop binding protein	BnaC04g20890D	BnaC04g20890D	DLSSMLTSAVSNPEAA

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