Quantitative Redox Proteomics Revealed Molecular Mechanisms of Salt Tolerance in the Roots of Sugar Beet Monomeric Addition line M14

He Liu  
Heilongjiang University of Science and Technology

Xiaoxue Du  
Heilongjiang University of Science and Technology

Jialin Zhang  
Heilongjiang University of Science and Technology

Jinna Li  
Heilongjiang University of Science and Technology

Sixue Chen  
University of Florida

Huizi Duanmu  
Heilongjiang University of Science and Technology

Haiying Li (lvzh3000@sina.com)  
Heilongjiang University of Science and Technology

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Abstract

Background: Salt stress is often associated with excessive production of reactive oxygen species (ROS). Oxidative stress caused by the accumulation of ROS is a major factor that negatively affects crop growth and yield. Root is the primary organ that senses and transmits the salt stress signal to the whole plant. How oxidative stress affect redox sensitive proteins in the roots is not known.

Results: In this study, the redox proteome of sugar beet M14 roots under salt stress was investigated. Using iTRAQ reporters, we determined that salt stress caused significant changes in the abundance of many proteins (2305 at 20 min salt stress and 2663 at 10 min salt stress). Using iodoTMT reporters, a total of 95 redox proteins were determined to be responsive to salt stress after normalizing again total protein level changes. Notably, most of the differential redox proteins were involved in metabolism, ROS homeostasis, and stress and defense, while a small number play a role in transport, biosynthesis, signal transduction, transcription and photosynthesis. Transcription levels of 14 genes encoding the identified redox proteins were analyzed using qRT-PCR. All the genes were induced by salt stress at the transcriptional level.

Conclusions: Based on the redox proteomics results, we construct a map of the regulatory network of M14 root redox proteins in response to salt stress. This study further refines the molecular mechanism of salt resistance at the level of protein redox regulation.

Background

Salt stress is often associated with excessive production of reactive oxygen species (ROS). Oxidative stress caused by the accumulation of ROS is a major factor that negatively affects crop growth and yield. Root is the primary organ that senses and transmits the salt stress signal to the whole plant. How oxidative stress affect redox sensitive proteins in the roots is not known.

In this study, we used the iodoTMTRAQ dual-labelling technology to investigate changes in redox proteins and total protein levels in a single experiment. This study revealed different functions of the differential redox proteins and the different pathways involved. Combined with the analysis of the changes at the transcript level of the genes encoding the differential proteins, it has provided insight into the physiological response strategies and molecular regulatory mechanisms of salt stress tolerance in sugar beet M14. The knowledge forms a theoretical basis for the use of genetic engineering and/or molecular breeding tools for improving crop resilience.
Materials And Methods

Plant material, salt stress treatment and physiological indicators measurement

The M14 seeds were soaked in water for 4 h, disinfected with 70% ethanol for 1 min, soaked for 15 min using 0.1% HgCl₂, treated with TMTD (1:500) for 12 h and rinsed in water. The treated seeds were sown in white porcelain trays lined with vermiculite and incubated at 25°C/ 20°C (day/night) in a light chamber with a light intensity of 450 µmolm⁻²s⁻¹, a light duration of 14 h and relative humidity of 65%. After 7 days, the seedlings were transferred into a half strength Hoagland's nutrient solution (Cherki et al. 2002) for hydroponics, and then treated with salt stress when the fifth real leaf emerged. BvM14 seedlings were treated with 0 mM NaCl as a control and the final concentration of NaCl was added to the nutrient solution up to 200 mM and 400 mM as salt stress treatments. Root samples from three individual plants (each as a biological replicate) were snap frozen in liquid nitrogen after harvesting and stored at -80°C till further use. Free sulfhydryl group of cysteine, AsA and GSH content was measured following a manufacturer protocol (Comin Biotechnologies, Suzhou, China). Three biological repeats were used for each analysis.

Protein sample preparation

The root samples were ground to a powder in liquid nitrogen with cysteine alkylation reagent N-Ethylmaleimide (NEM), and the total protein was extracted by phenol extraction. In particular, equilibrated phenol (pH=7.8) was added to the samples contained in the tubes, mixed thoroughly and then a phenol extraction buffer (900 mM sucrose, 100 mM Tris-HCl (pH8.8), 1 mM PMSF, 20 mM N-Ethylmaleimide (NEM), 10mM EDTA) was added, mixed well and centrifuged. To the protein fraction, 5 times the volume of 100% methanol containing 0.1M ammonium acetate was added. The the mixture was incubated overnight at -20°C. After centrifugation at 20,000 r/min for 20 minutes at 4°C, the pellet was collected and washed with pre-cooled 80% and 100% acetone respectively. A protein lysis buffer (0.5% SDS, 6 M Urea, 30 mM Tris-HCl, pH 8.5) was added to solubilize the pellet. Protein concentration was determined using a bicinchoninic acid (BCA) kit according to the manufacturer's instructions (TAKARA, Beijing China).

iodoTMTRAQ labeling, strong cation exchange fraction and LC-MS/MS

The reversibly oxidized cysteine thiols in the protein samples were firstly reduced for reverse labelling by incubating the protein samples with 5 mM of tris(2-carboxyethyl) phosphine at 50°C for 1 hour. We labelled control samples with 126, 128 and 130 TMT reagents for 0, 10 and 20 minutes and salt-treated samples with 127, 129 and 131 reagents, respectively. The labelling was performed for 2 hours at 37°C in the dark, followed by quenching with 0.5 M DTT for 15 minutes at 37°C in the dark. Trypsin (sequencing grade, Promega, Madison) was added at an enzyme to protein ratio of 1:50 (w/w) and digested overnight at 37°C(Parker et al. 2012). Peptides were cleaned up using a C18 desalting column (The Nest Group Inc., Southborough, MA) and lyophilized to dryness. The C18 cleaned peptides were labelled with iTRAQ reagent according to the manufacturer's protocol (AB Sciex Inc., Framingham, MA, USA). The control samples at 0, 10 and 20 min were labelled with reporter labels 113, 115 and 117, respectively, while treatment samples were labelled with reporter labels 114, 116 and 119. Labelling was maintained at 37°C for 2 h and labelled peptides were desalted according to published procedures (Parker et al. 2012, Yu et al. 2016). LC-MS/MS was connected to an Easy-nLC 1000 on a Q-Exactive Plus MS/MS system (Thermo Fisher Scientific, Bremen, Germany). Tandem mass spectrometry was performed following the method of Yu et al. (Yu, et al. 2016).

Bioinformatics analysis

Data analysis for peptide MS2 spectra was performed by Thermo Fisher's Proteome Discoverer 2.1, searching the combined Sugar Beet Protein Database and the Green Plant Protein Database from NCBI (with a total of 6255663 entries). Oxidatively modified protein and total protein data were normalized to the 126 tag in the iodoTMT reporter and the 113 tag in the iTRAQ reporter, respectively. The control group was used as a criterion to screen peptides with P-values <0.05, while fold-change analysis was performed to select peptides with fold-change >1.2 and <0.8 as significant peptides on the redox level and protein abundance level. The full sequences of the differential proteins were queried in the Protein Data Bank of NCBI (http://www.ncbi.nlm.nih.gov/protein/), UniProt database (http://www.ebi.uniprot.org/) using Gi numbers. Functional annotations of redox proteins were obtained using GO (http://geneontology.org/) and combined with relevant literature, and KEGG pathways (https://www.kegg.jp/). Subcellular localization was predicted using online analysis tools (YLoc, LocTree3, ngLOC, TargetP). The redox protein network of sugar beet M14 roots under salt stress was mapped using Adobe Illustrator 2021. Physiological and biochemical index data and qRT-PCR results were analyzed and data processed using GraphPad Prime 6 software. Significant differences were analyzed with * indicating P<0.05 and **indicating P<0.01.

qRT-PCR

The genes encoding differential redox proteins were selected for real-time quantitative PCR (qRT-PCR) in order to test possible correlation between the transcription level and protein level under 200 mM and 400 mM NaCl treatment conditions. A total of 14 differential redox proteins involved in ROS homeostasis and signal transduction, and differential redox proteins in roots and leaves were selected. Total RNA from sugar beet M14 roots was extracted with Trizol, cDNA templates were obtained using a reverse transcription kit (TAKARA) and qRT-PCR was performed using the SYBR dye method with the 18S rRNA reference gene (Zhang et al. 2015). Each reaction consisted of three biological replicates and three technical replicates. The relative expression levels of the target genes were calculated by normalizing against an internal standard 18S by the -ΔΔCt method.

Results

1. Changes of cysteine free sulfhydryl, AsA and GSH contents in roots of sugar beet M14 treated with salt stress

The changes in cysteine free sulfhydryl, ASA and GSH over a 90 min time-course of treatment with different salt concentrations are shown in Fig. 1. Under control conditions, the lowest levels of cysteine free sulfhydryl were reached at 20 min (200 mM NaCl) and 10 min (400 mM NaCl) in response to the salt stress (Fig. 1A). Excessive accumulation of ROS in plants induced by salt stress prompted oxidative modification of cysteine sulfhydryl groups and a decrease
in free sulfhydryl content, indicating the highest level of oxidative modification of proteins at this time. Further studies revealed that the levels of AsA and GSH in the sugar beet M14 roots remained stable. Their levels peaked at 20 min (200 mM NaCl) and 10 min (400 mM NaCl) under salt stress (Fig. 1B, Fig. 1C). The results clearly indicate that salt stress caused significant changes in cellular redox status as early as 10 min after treatment. Based on these results, we selected samples collected at 200 mM NaCl for 20 min and 400 mM NaCl for 10 min for iodoTMTARQ-based redox proteomics studies.

2. LC-MS/MS analyses of root proteins and redox proteins in BvM14 response to salt stress

LC-MS/MS quantitative analysis identified 2305 proteins (20 min) (Additional file 2: Table S1) and 2663 proteins (10 min) (Additional file 3: Table S2) with iTRAQ tags. There were 462 (20 min) and 279 (10 min) proteins that showed significant changes in protein abundance. A total of 260 (20 min) (Additional file 4: Table S3) and 249 (10 min) (Additional file 5: Table S4) proteins with iodoTMT tags were identified as having significant changes in redox levels. Among them, 42 (20 min) and 63 (10 min) proteins screened by bioinformatic analysis showed significant changes in redox levels, while 41 (20 min) and 61 (10 min) of these proteins did not exhibit significant changes in protein abundance (Fig. 2A). A total of 95 redox proteins were identified under 200 mM and 400 mM NaCl stress (Table 1). There was also variable expression among the identified redox proteins, with 54 proteins oxidized (FC>1.2) and 48 proteins reduced or irreversibly oxidized (FC<0.8) (Fig. 3B). Notably, there were seven redox proteins under salt stress, three of which had the same total protein level and significantly increased oxidation levels. They were identified as proteasome subunit beta-6 type (PBA6), protein P21 (P21) and basic 7S globulin (Bg7s). Bioinformatic analysis indicated that these proteins are important oxidative sensors of root responses to salt stress in M14.
Table 1
A list of 95 differential redox proteins in BvM14 roots between control and NaCl-treated groups.

| No | Protein ID | Description | Abbreviation | Sequence with modification | Plant species | salt200/control Ratio |
|----|------------|-------------|--------------|---------------------------|--------------|----------------------|
| 1  | A0A2H5P1K5 | 6-phosphogluconate dehydrogenase, decarboxylating | PGDH | IC2SYAQGMN | Citrus unshiu | — |
| 2  | 731322678 | Beta-fructofuranosidase, soluble isoenzyme I | β-FFase | NWFC4TDQR | Beta vulgaris subsp. vulgaris | — |
| 3  | Q41140    | Pyrophosphate-fructose 6-phosphate 1-phosphotransferase subunit alpha | PFP1 | SLYKPELPPC10LQGTTVR | Ricinus communis | — |
| 4  | 1108966238 | Sucrose synthase isoenzymes X2 | SUS | LLPDAVGTTT10QQR | Beta vulgaris subsp. vulgaris | — |
| 5  | 731323052 | Probable fructokinase-4 | FRK | LLLVTLDQG11R | Beta vulgaris subsp. vulgaris | 0.75 |
| 6  | A0A0S3T1M9 | UDP-glucose 6-dehydrogenase | UGDH | VFDC4MQPAFVDGR | Vigna angularis var. angularis | — |
| 7  | 731364471 | Trypsin inhibitor BvTI | TI | NPELPC6PYYITR | Beta vulgaris subsp. vulgaris | 0.30 |
| 8  | 731344067 | Kunitz trypsin inhibitor 1-like | KTI | C1PYYSVVQSQDDR | Beta vulgaris subsp. vulgaris | — |
| 9  | 731331165 | Alpha-amylose/trypsin inhibitor α-TI | AMP | ANGGC5NNAYNSY | Beta vulgaris subsp. vulgaris | 0.52 |

**Metabolism**

**Carbohydrate metabolism**

|   |   |   |   |   |   |   |
|---|---|---|---|---|---|---|
| 10| 731353768 | Aspartate aminotransferase | Aps | VASAQC6LSGTGLR | Beta vulgaris subsp. vulgaris | — |
| 11| A0A2P5X5J0 | Aspartate aminotransferase | Aps | IAAVQALSGTAC13R | Gossypium barbadense | — |
| 12| 731351009 | Aspartic proteinase A1-like | AP | VGEPAAC5ISGFTALVPPP | Beta vulgaris subsp. vulgaris | 1.35 |
| 13| 731353609 | 3-hydroxyisobutyryl-CoA hydrolase-like protein 3, mitochondrial isoform X1 | H2BCH | C1VLIESSSPR | Beta vulgaris subsp. vulgaris | — |
| 14| A0A2I0XB93 | Aspartate-semialdehyde dehydrogenase | ASDH | IRQDLSEGHNGLDIFVC18GDQIR | Dendrobium catenatum | — |
| 15| A0A0M3TGF7 | Acetolactate synthase | ALS | C1GISDVFAYPGGASMEIQLT | Poa annua | — |
| 16| 731325199 | Serine hydroxymethyltransferase 4 | SHMT | MLIC4GGSAYPR | Beta vulgaris subsp. vulgaris | 0.59 |
| 17| 731317741 | LL-diaminopimelate aminotransferase, chloroplastic | DAPL | TELIFFC7SPNPTGAAATR | Beta vulgaris subsp. vulgaris | — |

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a Protein ID, gi number of NCBI  
b Sequence with modification, the lower case letter are phosphorylation site in each peptide  
c salt200/control Ratio, a relative abundance of proteins at redox peptide level (200 mM NaCl treatment versus control), P-value < 0.05  
d salt400/control Ratio, a relative abundance of proteins at redox peptide level (400 mM NaCl treatment versus control), P-value < 0.05  
e Protein location, refer to subcellular location prediction website (YLoc, LocTree3, ngLOC, TargetP)
| No | Protein IDa | Description | Abbreviation | Sequence with modificationb | Plant species | iodoTMT salt200/control Ratioc |
|----|-------------|-------------|--------------|-----------------------------|--------------|-------------------------------|
| 18 | A0A0K9RN52  | Glutamate-1-semialdehyde 2,1-aminomutase | GSAM | FVNSGTEAC9MGVLR | Spinacia oleracea | 1.21 |
| 19 | A0A0B2RAS0  | Proteasome subunit alpha type-5 | PSAM5 | FSYGEPMTVESTTQAIC17DLALR | Glycine soja | 0.76 |
| 20 | 731363918   | Proteasome subunit alpha type-5 | PSAM5 | FSYGEPMTVESTTQALC17DLALR | Beta vulgaris subsp. vulgaris | - |
| 21 | 731361751   | Proteasome subunit alpha type-5 | PSAM5 | FSYGEPMNVESTTQALC17DLALR | Beta vulgaris subsp. vulgaris | - |
| 22 | A0A287HD16  | Proteasome subunit beta type-6 | PSAM6 | QLTDNYYVC9R | Hordeum vulgare subsp. vulgare | 1.23 |
| 23 | M0UCJ4      | ATP synthase subunit beta | ATPsny | VC2VQIGAVVDR | Musa acuminate subsp. malaccensis | 0.74 |
| 24 | M8C108      | ATP synthase subunit alpha, mitochondrial | ATPsny | MTNFC5TNFQVDEIGR | Aeglops tauschii | - |
| 25 | A0A287X935  | Peroxidase | POD | ASVEAVC7PGVVC13ADILAITAR | Hordeum vulgare subsp. vulgare | - |
| 26 | A0A2G9HTZ9  | Peroxidase | POD | QAVEAQC7PGVVC13SDILAAAR | Handroanthus impetiginosus | - |
| 27 | A0A1S2YYJ3  | Peroxidase | POD | SDLENAC7PSTVC13ADILLAAR | Cicer arietinum | - |
| 28 | A0A2G2WVY9  | Peroxidase | POD | IKTMC6PGAAVSC12ADILALAR | Capsicum baccatum | 0.46 |
| 29 | J3L3F3      | Peroxidase | POD | LEAAC6PKTVSC11ADILLAAR | Oryza brachyantha | - |
| 30 | A0A0J8CS88  | Peroxidase | POD | QC2PAGNAGANIVVPMDPISPTISDTAYYR | Beta vulgaris subsp. vulgaris | - |
| 31 | 731316487   | Peroxidase 4 | POD4 | TC2PQLFPTIR | Beta vulgaris subsp. vulgaris | - |
| 32 | 731313635   | Peroxidase 12 | POD12 | VVSC4ADITSLAAR | Beta vulgaris subsp. vulgaris | 0.42 |
| 33 | 731313633   | Peroxidase 12 | POD12 | VVSC4ADITTLAAR | Beta vulgaris subsp. vulgaris | - |
| 34 | 731313639   | Peroxidase 12 | POD12 | VVSC4ADITLALAR | Beta vulgaris subsp. vulgaris | 0.64 |
| 35 | A0A0A9MG34  | Peroxidase 72 | POD72 | AALEAAC7PSTVC13ADILALAR | Arundo donax | - |
| 36 | 731337443   | Peroxidase 72 | POD72 | AAVEQC7PHTVC13ADILALAR | Beta vulgaris subsp. vulgaris | - |
| 37 | 731331163   | Protein P21 | P21 | TDNYC5CNSGSC11GPTDYSR | Beta vulgaris subsp. vulgaris | 4.09 |

a Protein ID, gi number of NCBI
b Sequence with modification, the lower case letter are phosphorylation site in each peptide
c salt200/control Ratio, a relative abundance of proteins at redox peptide level (200 mM NaCl treatment versus control), P-value < 0.05
d salt400/control Ratio, a relative abundance of proteins at redox peptide level (400 mM NaCl treatment versus control), P-value < 0.05
e Protein location, refer to subcellular location prediction website (YLoc, LocTree3, ngLOC, TargetP)
| No. | Protein ID | Description | Abbreviation | Sequence with modification | Plant species | iodoTMT / control Ratio |
|-----|------------|-------------|--------------|---------------------------|--------------|-------------------------|
| 38  | A0A1S3TTL2 | DSBA domain-containing protein | DSBA | NVGLEYC\(^2\)MSGLTGNTIDSHR | Vigna radiata var. radiata | 0.55 |
| 39  | 731399890 | EG45-like domain containing protein 2 | EG45 | VTDLC\(^6\)DSCA\(^8\)AGDLNLSQEAFNVIADTR | Beta vulgaris subsp. vulgaris | -- |
| 40  | 731352762 | EG45-like domain containing protein | EG45 | VTC\(^3\)VSGTNQGVQPQC\(^15\)R | Beta vulgaris subsp. vulgaris | -- |
| 41  | A0A0J8B2W2 | Fe2OG dioxygenase domain-containing protein | Fe2OG | VAIYPEC\(^7\)PNPELVR | Beta vulgaris subsp. vulgaris | -- |
| 42  | M0RV51 | Glutathione S-transferase DHAR2 | GST | AAVGAPDVLGDC\(^{12}\)PFSQR | Musa acuminata subsp. malaccensis | 0.64 |
| 43  | A0A199U4J8 | 3-ketoacyl-CoA thiolase 2, peroxisomal | HT | IELFAQARDC\(^{10}\)LLPMGIKENVAHR | Ananas comosus | -- |
| 44  | 731355863 | L-ascorbate oxidase-like | AOX | QLGPWADGTASISQC\(^{16}\)PINPGETFTYR | Beta vulgaris subsp. vulgaris | 0.51 |
| 45  | A0A151QMI1 | Nitrile reductase [NADH] | NR | QSGALHV\(^5\)FEGAEDLPGGSGKYGTSVTR | Cajanus cajan | -- |
| 46  | 731357289 | NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 8-B | NADH | C\(^1\)VFSLLR | Beta vulgaris subsp. vulgaris | -- |
| 47  | 731359814 | Peptide methionine sulfoxide reductase B5-like | MSR | FDSGC\(^6\)GWPAYEGLPGAISTR | Beta vulgaris subsp. vulgaris | -- |
| 48  | 731312054 | Cysteine protease RD19A | RD19A | LVSLSEQQLVDC\(^{12}\)DHEC\(^{16}\)DPEER | Beta vulgaris subsp. vulgaris | 1.63 |

**Stress and Defense**

| No. | Protein ID | Description | Abbreviation | Sequence with modification | Plant species | iodoTMT / control Ratio |
|-----|------------|-------------|--------------|---------------------------|--------------|-------------------------|
| 49  | 731330989 | Probable polygalacturonase | PGs | VIDNFEYSAINC\(^{12}\)R | Beta vulgaris subsp. vulgaris | 1.5 |
| 50  | 731338906 | PLAT domain-containing protein 3 | PITI | GPC\(^3\)LNAPVC\(^3\)AMR | Beta vulgaris subsp. vulgaris | -- |
| 51  | A0A166FTZ6 | Heat shock cognate 70 kDa protein | Hsp70 | MDIC\(^4\)SVHDVVLVGGSTR | Daucus carota subsp. sativus | -- |
| 52  | Q9XF7W | Chitinase | - | FGFC\(^4\)GSTDAYC\(^{11}\)GEGC\(^{15}\)R | Beta vulgaris subsp. vulgaris | 2.05 |
| 53  | 731352263 | Endochitinase EP3 | EP3 | VGYYTQYC\(^8\)QQLGVSPGNNLR | Beta vulgaris subsp. vulgaris | -- |
| 54  | 731352251 | Endochitinase EP3 | EP3 | AINGGEC\(^7\)GGNTPAVNR | Beta vulgaris subsp. vulgaris | -- |
| 55  | 731352259 | Endochitinase EP3 | EP3 | LEC\(^3\)DGGNPATVNAR | Beta vulgaris subsp. vulgaris | 0.71 |
| 56  | 731329194 | Pathogenesis-related protein PR-4 | PR-4 | NQYGWTACF\(^9\)PGAGPTGQASC\(^{20}\)GR | Beta vulgaris subsp. vulgaris | 1.64 |
| 57  | 731326017 | Jasmonate-induced protein homolog | JIP | LDASHDRESH\(^{10}\)PGAAAR | Beta vulgaris subsp. vulgaris | -- |

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\(^{a}\) Protein ID, gi number of NCBI

\(^{b}\) Sequence with modification, the lower case letter are phosphorylation site in each peptide

\(^{c}\) salt200/control Ratio, a relative abundance of proteins at redox peptide level (200 mM NaCl treatment versus control), P-value < 0.05

\(^{d}\) salt400/control Ratio, a relative abundance of proteins at redox peptide level (400 mM NaCl treatment versus control), P-value < 0.05

\(^{e}\) Protein location, refer to subcellular location prediction website (YLoc, LocTree3, nLoc, TargetP)
| No | Protein ID* | Description | Abbreviation | Sequence with modification^b | Plant species | IodoTMT salt200/control Ratio^c |
|----|-------------|-------------|--------------|-----------------------------|--------------|--------------------------------|
| 58 | 731332586   | Jasmonate-induced protein homolog | JIP | LEINSGNC\(^3\)SYDVDYETR | Beta vulgaris subsp. vulgaris | 0.36 |
| 59 | 731312253   | Jasmonate-induced protein homolog | JIP | C\(^1\)GPAAEFNNWNWTQVR | Beta vulgaris subsp. vulgaris | — |
| 60 | A0A2P4NB14  | Flavonoid 3',5'-methyltransferase | GIP | IESSLSIGGITLC\(^\circ\)R | Quercus suber | — |
| 61 | 731357526   | LysM domain-containing GPI-anchored protein 2 | STC\(^3\)AVGYNR | Beta vulgaris subsp. vulgaris | 0.53 |

**Transport**

| 62 | A0A0K9RCQ9  | Purple acid phosphatase | PAP | FLEEC\(^5\)LASANR | Spinacia oleracea | 0.40 |
| 63 | 731352863   | Probable inactive purple acid phosphatase 29 | PAP | QEEVIC\(^6\)PGVNGSFFTMR | Beta vulgaris subsp. vulgaris | 0.68 |
| 64 | 731320622   | Importin subunit alpha | IMP | NATWTLSNFC\(^\circ\)R | Beta vulgaris subsp. vulgaris | 1.21 |
| 65 | A0A061E090  | Vacuolar sorting receptor 3 isoform 1 | VSR | VC\(^2\)EC\(^4\)PLVDGVQFR | Theobroma cacao | 0.70 |
| 66 | 731352092   | Vacuolar-sorting receptor 4 | VSR | YC\(^2\)APDPEQDFS | Beta vulgaris subsp. vulgaris | 0.61 |
| 67 | A0A2N9HVW5  | Mitochondrial import receptor subunit TOM40-1-like protein | TOM40 | EEEKVDYFNLPC\(^\circ\)PIPYEEIHR | Fagus sylvatica | — |

**Cellular structure**

| 68 | 731336429   | Actin-depolymerizing factor | ADP | TGTPAESYDDFLAVPGND\(^2\)R | Beta vulgaris subsp. vulgaris | — |
| 69 | 731320854   | Actin-depolymerizing factor | ADP | TGGPAESYDDFLASPD\(^2\)R | Beta vulgaris subsp. vulgaris | — |
| 70 | 731375712   | Basic 7S globulin | Bg7s | TIAPFNVC\(^6\)VDPSTFPASR | Beta vulgaris subsp. vulgaris | 10.20 |
| 71 | 731317399   | Prolin-3 | Pfn | TQGALVIGLYDEPVTGQC\(^1\)NMIVER | Beta vulgaris subsp. vulgaris | 1.29 |
| 72 | A4GDT3      | Prolin-1 | Pfn | TQGALVFIYEEVSPTGQC\(^1\)NMVVER | Olea europaea | 1.53 |
| 73 | 731354018   | Prolin | Pfn | TQGALVFIYDEPVPAGQC\(^1\)NMVVER | Beta vulgaris subsp. vulgaris | 1.40 |

**Signal transduction**

| 74 | 731337809   | Protein TAPETUM DETERMINANT 1 | TPD | C\(^1\)LGFSVTQPVNR | Beta vulgaris subsp. vulgaris | — |
| 75 | 731357482   | Ubiquitin domain-containing protein DSK2b | DSK2b | SLVAQNC\(^7\)DVPAEQQR | Beta vulgaris subsp. vulgaris | — |
| 76 | A0A287MC57  | Ubiquitin-like domain-containing protein | Uds | LMNAYC\(^6\)DR | Hordeum vulgare subsp. vulgare | — |
| 77 | 731354496   | Ribosome-inactivating protein PD-L1/PD-L2 | Ubls | NQVEAPIRIC\(^1\)GLPSTR | Beta vulgaris subsp. vulgaris | 2.04 |

*Protein ID, gi number of NCBI

^b Sequence with modification, the lower case letter are phosphorylation site in each peptide

^c salt200/control Ratio, a relative abundance of proteins at redox peptide level (200 mM NaCl treatment versus control), P-value < 0.05

^d salt400/control Ratio, a relative abundance of proteins at redox peptide level (400 mM NaCl treatment versus control), P-value < 0.05

^e Protein location, refer to subcellular location prediction website (YLoc, LocTree3, ngLOC, TargetP)
| No | Protein ID | Description | Abbreviation | Sequence with modification | Plant species | iodoTMT salt200/control Ratio |
|----|------------|-------------|--------------|---------------------------|--------------|-----------------------------|
| 78 | 731345483 | Auxin-binding protein ABP19a | ABP | GPEGYAC\textsuperscript{7}RDPATLTDDFVTGFR | Beta vulgaris subsp. vulgaris | 0.42 |
| 79 | A0A2K1KH59 | Protein kinase domain-containing protein | AMPK | C\textsuperscript{1}IPYLTR | Physcomitrium patens | 0.76 |
| 80 | 731370564 | Receptor-like serine/threonine-protein kinase SD1-8 isoform X1 | RIPK | TAFVNDGLNLDO\textsuperscript{13}R | Beta vulgaris subsp. vulgaris | 0.70 |
| 81 | 731348205 | Cell wall / vacuolar inhibitor of fructosidase 1 | C/VIF1 | FGEQAMVDAGNEAEGC\textsuperscript{16}R | Beta vulgaris subsp. vulgaris | — |

**Transcription**

| No | Protein ID | Description | Abbreviation | Sequence with modification | Plant species | iodoTMT salt200/control Ratio |
|----|------------|-------------|--------------|---------------------------|--------------|-----------------------------|
| 82 | 731323512 | Transcription elongation factor TFIIS | TFIIS | IC\textsuperscript{2}NLTAEMASEQR | Beta vulgaris subsp. vulgaris | 0.62 |
| 83 | 731358684 | Glycine-rich RNA-binding protein | RBP | C\textsuperscript{1}FVGGLAWATDDR | Beta vulgaris subsp. vulgaris | 0.72 |
| 84 | 731363127 | KH domain-containing protein | KHP | IGTVPGC\textsuperscript{6}DER | Beta vulgaris subsp. vulgaris | 0.76 |
| 85 | 731317968 | RNA-binding KH domain-containing protein PEPPER | RBP | VSGVGDEVSADAAYC\textsuperscript{17}SIR | Beta vulgaris subsp. vulgaris | — |
| 86 | A0A1D1Z0S0 | U6 snRNA-associated Sm-like protein LSm7 | - | SLGLIVC\textsuperscript{2}R | Anthurium amnicola | — |
| 87 | A0A0C9S8X9 | Transcribed RNA sequence | - | C\textsuperscript{1}GNVNFSFR | Wollemia nobilis | 1.72 |

**Biosynthesis**

| No | Protein ID | Description | Abbreviation | Sequence with modification | Plant species | iodoTMT salt200/control Ratio |
|----|------------|-------------|--------------|---------------------------|--------------|-----------------------------|
| 88 | A0A0J8C157 | Eukaryotic translation initiation factor 6 | elf6 | NC\textsuperscript{2}LPDSVVVQR | Beta vulgaris subsp. vulgaris | — |
| 89 | 731369461 | Eukaryotic translation initiation factor 3 subunit D | elf3 | C\textsuperscript{1}ELQASLDINNQR | Beta vulgaris subsp. vulgaris | — |
| 90 | 1108926884 | Elongation factor Tu, chloroplast | EF-TU | MEVELIHPVAC\textsuperscript{11}EEGMR | Beta vulgaris subsp. vulgaris | — |

**Photosynthesis**

\(a\) Protein ID, gi number of NCBI

\(b\) Sequence with modification, the lower case letter are phosphorylation site in each peptide

\(c\) salt200/control Ratio, a relative abundance of proteins at redox peptide level (200 mM NaCl treatment versus control), P-value < 0.05

\(d\) salt400/control Ratio, a relative abundance of proteins at redox peptide level (400 mM NaCl treatment versus control), P-value < 0.05

\(e\) Protein location, refer to subcellular location prediction website (YLoc, LocTree3, ngLOC, TargetP)
3. Functional classification and subcellular localization of root redox proteins

The 95 redox proteins under salt stress were divided into nine functional groups (Fig. 3A). A large proportion of redox proteins were involved in the regulation of ROS homeostasis (25.3%), carbohydrate, amino acid and basal metabolism (24.2%), stress and defence (21.1%), and signal transduction (8.4%). A small number of proteins are involved in transport (6%), transcription (5%), photosynthesis (2%), and some proteins are of unknown functions (3%). Subcellular localization showed that the majority of proteins were localized in the cytoplasm (25.3%), extracellular (22.1%), nucleus (12.2%) and others in the cell wall (7.4%), chloroplasts (7.4%), plasma membrane (7.4%) and vacuole (7.4%), mitochondria (5.1%), Golgi apparatus (2%) and peroxisomes (2%) and endoplasmic reticulum (1.1%) (Fig. 3B). We found that more proteins were increased than decreased in oxidative levels in each functional group under salt stress (Fig. 3C). Notably, most of the proteins involved in metabolism and maintenance of ROS homeostasis were oxidized. In contrast, more proteins were reduced or irreversibly oxidized in other processes. GO enrichment results were further analysed in terms of biological processes, molecular function and cellular composition for 95 differential redox proteins (Supplementary file 1: Figure S1). The biological processes involved are metabolic process, cellular process, response to stimulus, developmental process, etc. The cellular components were catalytic activity, binding, antioxidant activity, etc. These results suggest that proteins with elevated levels of oxidation in metabolism and maintenance of ROS homeostasis have a dominant role in the tolerance of sugar beet M14 roots to salt stress. In contrast, decrease of protein oxidation levels in other processes was more favorable for salt stress response in sugar beet M14 root systems.

4. Transcriptional analysis of differential redox proteins and differential proteins

Key redox proteins were selected for transcript level analysis according to the following criteria. First, we selected three proteins whose oxidation levels were significantly increased after both 200 mM and 400 mM salt stress. Second, proteins specifically involved in maintaining ROS homeostasis, signal transduction, stress and defense regulation and metabolism were selected whose oxidation levels were significantly altered under 200 mM or 400 mM salt stress. Finally, proteins that were identified in both roots and leaves after salt stress, as well as those with significantly altered redox levels, were selected. The expression patterns of these 14 functional genes under salt stress were analyzed by qRT-PCR using the primers in Additional file 6: Table S5. As shown in Fig. 5, of the 14 genes encoding differential proteins, the transcript levels of four genes coincided with the corresponding redox level trends (Additional file 7: Table S6). This suggests that key genes encoding redox proteins can be induced at the transcriptional level by salt stress, and then function through the redox post-translational modifications.

5. Overview of potential salt stress response mechanisms in sugar beet M14

Based on the redox proteomics results including functional classification, KEGG pathway as well as relevant literature, a preliminary network map of redox proteins in response to salt stress in the roots of sugar beet M14 strain was developed (Fig. 6). The redox proteins are marked with yellow and green representing proteins with significantly increased or decreased oxidation levels under 200 mM NaCl treatment. Red and blue represent proteins with significantly increased or decreased oxidation levels under 400 mM NaCl treatment. Plant roots sense salt stress signals and then transmit the signals to the cells via ion signaling and ROS accumulation, leading to oxidative stress. In the roots of sugar beet M14, 25% of the 95 redox proteins identified were involved in maintaining ROS homeostasis, and most of these redox proteins were directly involved in the ROS scavenging process. A small number of redox proteins also provided reducing power to the ROS scavenging system and accelerated the scavenging of ROS in plants under salt stress. In addition, significant changes in the redox levels of protein subunits involved in the ubiquitin-proteasome system were also identified (Fig. 6). Such redox modifications may affect the degradation of oxidatively modified proteins under salt stress, thus contributing to the protein turnover and resistance of plants to salt stress.
Discussion

Salt stress leads to changes in the levels of PTMs in plants, which regulate the localization, accumulation and activity of proteins. Therefore, studying differential PTM proteins in plants under salt stress will contribute to understanding the complex adaptive mechanisms of plants under adverse environmental conditions. Here we used an iodoTMT-TRAP double-labeling approach to study changes in redox modifications of sugar beet M14 root proteins in response to salt stress. Our goal was to compare and contrast the differential redox proteins in sugar beet M14 roots under salt stress with those in the leaves, to ultimately understand sugar beet salt tolerance mechanisms.

Roots maintain ROS homeostasis through redox modification of antioxidant enzymes and antioxidants

In this study, the number of proteins with increased oxidation was significantly higher in roots of sugar beet M14 under high salt treatment (400 mM NaCl) than that at moderate salt treatment (200 mM NaCl). Some proteins were also found to be almost entirely decreased under the 200 mM salt concentration, while oxidation levels were significantly increased at 400 mM salt. Changes in the oxidation levels of several antioxidant enzymes, including ascorbate oxidase (AOX), dehydroascorbate reductase (DHAR) and peroxidase (POD), were found in the antioxidant system. This caught our attention, and we hypothesize that BvM14 initiates plant defense mechanisms in extreme environments by regulating protein oxidation levels in roots. It enhances the ROS scavenging capacity of plants, repairs oxidatively modified proteins under salt stress and regulates various metabolic pathways.

AOX and DHAR promote the regeneration of AsA (Yu et al. 2021). AOX catalyzes the oxidation of AsA to dehydroascorbic acid (DHA) via a monodehydroascorbic acid (MDHA) intermediate, which produces AsA following DHAR (Farida et al. 2020). AOX can undergo reversible oxidative modifications and can promote the accumulation of AsA. This could explain the decreased AOX oxidation levels under 200 mM salt stress and the apparently increased oxidation under 400 mM salt stress treatments. The enzymatic activity of DHAR is regulated by reduced sulphydryl groups in Arabidopsis (Tullio et al. 2013). In the present study, Cys12 of DHAR was identified to be decreased in oxidation levels under salt stress. This indicates that the elevated catalytic activity of DHAR is induced under salt stress, which promotes the regeneration of AsA to scavenge ROS in plants and thus improves the tolerance of the BvM14 roots to salt stress.

The main function of POD is to reduce H₂O₂ to H₂O and to scavenge ROS in plants (Bodra et al. 2017). Salt stress treatment of sugar beet M14 roots revealed altered redox levels of 12 PODs. Further multiple comparisons of amino acid sequences revealed that oxidative modifications occurred at eight conserved Cys sites and were mainly concentrated at two of these Cys sites (Fig. 7). It was found that POD was able to sense the level of ROS based on the oxidation status of Cys (Liu et al. 2014), indicating that the catalytic activity of POD may be induced by high salt stress. The results suggest that changes in the redox status and enzymatic activity of various antioxidant enzymes can regulate and scavenge ROS, which in turn promotes plant tolerance to salt stress.

Salt stress induces significant changes in protein redox levels in protein degradation systems

The ubiquitin-proteasome system (UPS) is the main pathway for protein degradation in eukaryotic cells (Xu et al. 2019). Ubiquitin domain-containing protein (Uds) and ubiquitin-like domain-containing protein (Ubls) were decreased at the oxidation level in roots of salt-stressed sugar beet M14. Four proteasomes (three proteasome subunit alpha type-5 and one proteasome subunit beta-6) were identified, three of which had significantly increased oxidation levels. Ubiquitin modified proteins are transported to the proteasome via ubiquitin structural domain proteins, and proteins with ubiquitin tags are recognized by 19S regulatory particles to enter the 26S protease for hydrolysis (Genschik et al. 1994). Redox proteomic findings suggest that the protein degradation system itself may be regulated by redox. How redox and ubiquitination crosstalk in the sugar beet M14 roots to confer salt stress response and tolerance is not known (Harshbarger et al. 2015, Roos et al. 2011)

Salt stress affects redox state of proteins in glucose metabolism and amino acid metabolism

Redox proteomics studies have identified significantly increased expression levels of two sucrose synthase isoform (SUS) proteins under salt stress. The SUSs are widely distributed glycosyltransferases in plants and catalyze the catabolism of sucrose. The accumulation of SUS in plant roots under abiotic stresses has been identified several times (Liu et al. 2019, Orłowski et al. 2008, Sasaki et al. 2001, Sharif et al. 2019). SUSs were shown to be involved in osmoregulatory processes, and the sucrose breakdown products promoted cell wall biosynthesis or glycolysis (Albrecht et al. 2003). In this study, SUS oxidation levels were found to be significantly decreased. This suggests that it may act as an osmoregulatory substance to promote plant root tolerance to salt stress by redox activation. In addition, significant changes in the redox levels of four key enzymes (6-phosphogluconate dehydrogenase (PGDH), UDP-glucose 6-dehydrogenase (UGDH), beta-fructofuranosidase, soluble isoenzyme I (FFase) and Pyrophosphate-fructose 6-phosphate 1-phosphotransferase subunit alpha (PFP1)) involved in the sugar metabolism pathway were determined. The redox levels of four enzymes that catalyze aspartate synthesis and metabolism (Aspartate-semialdehyde dehydrogenase (ASDH), Aspartate aminotransferase (AST), Aspartic proteinase A1-like (Aps) and Diaminopimelate aminotransferase (DapL)) were significantly altered, with increased expression of ASDH, AST and Aps. In subsequent studies, the glucose and aspartate contents in the roots of sugar beet M14 strain could be measured to further verify the effects of redox modifications on the activities of key enzymes in the sugar and amino acid metabolism pathways.

Relationship between redox proteins and phosphorylation-modified proteins

Protein phosphorylation modifications are one of the most fundamental and important post-translational modifications. In eukaryotes, phosphorylation modifications occur mainly on residues of serine, threonine and tyrosine. Phosphorylated proteins are intricately linked to the regulation of intracellular kinases and phosphatases and are involved in a variety of cellular processes, such as transmembrane or intracellular signaling, conformation change of proteins, and subcellular trafficking (Hsu et al. 2009, Jørgensen et al. 2008, Zhou et al. 2018). For example, it was found that the phosphorylation of the Ser534 site of Arabidopsis nitrate reductase (NR) is sensitive to exogenous H₂O₂. Interestingly, the Met538 site of NR acts as a recognition element for Ser534 phosphorylation. The Met538 site is oxidized to methionine sulfoxide (MetSO), and this redox modification oxidation significantly inhibits the phosphorylation.
modification of the Ser534 site. Coupling redox signal to changes in protein phosphorylation is important (Hardin et al. 2009). Receptor-like serine/threonine-protein kinase (RSTK) was decreased at phosphorylation levels and significantly increased at oxidation levels in previous studies (Tyler et al. 2004, Wang et al. 2014, Yu, et al. 2016). RSTK belongs to the receptor-like kinase (rlk/pelle) family. Rlk/pelle family proteins can interact with other proteins and play an important signal role in pathogen recognition, activation of plant defense mechanisms and developmental regulation (Li et al. 2002). RSTK may contribute to the tolerance of sugar beet M14 lines to salt stress by regulating the levels of redox and phosphorylation modifications, while the effect of oxidation on phosphorylation levels needs to be further investigated.

**Different strategies employed in salt stress responses in roots and leaves of sugar beet M14**

Under salt stress, signals are sensed by the cell membrane and transmitted to organelles such as chloroplasts, mitochondria and the nucleus in plant leaves (Fig. 6). Redox levels of proteins involved in photosynthesis are significantly altered and play a dominant role in salt stress. The leaves regulate the redox levels of photosynthesis-related proteins and influence protein conformation, thereby regulating protein function to ensure that plants receive the energy they need to survive salt stress. Unusually, roots accelerate the rate of ROS scavenging and maintain ROS homeostasis in plants under salt stress, mainly through significant changes in the redox levels of antioxidant enzymes and related proteins that provide reducing power to the ROS scavenging system, thereby improving the salt tolerance. Ten redox proteins from leaves and roots were found to respond synergistically to salt stress (Fig. 4B). Among them, the oxidation levels of POD and Hsp70 were significantly increased, while VSR, Fd and GPI were significantly decreased. VSR is a transmembrane receptor protein involved in the targeted transport of soluble vesicular proteins to the vesicle (Kang et al. 2014, Soares et al. 2019). In leaves, Fd is the major protein involved in the last step of the photosynthetic electron transport reaction (Hanke et al. 2004). However, Fd is mainly reduced under non-photosynthetic conditions in roots, allowing the reduced Fd state to transfer electrons to NADP+, and the resulting NADPH reducing power may be used in roots or transported to leaves for carbon fixation in the Calvin cycle and other metabolic processes in the chloroplasts. In addition, the reduced state of Fd can also use electrons for other reactions such as nitrogen assimilation, sulphur assimilation, lipid and chlorophyll synthesis, and it also participates in metabolic processes such as the AsA-GSH cycle, thus indirectly regulating ROS homeostasis (Hanke, et al. 2004). LysM-GPI was identified in the secretome of grapes in response to cyclodextrin and methyl jasmonate, but the role of LysM-GPI in plant is not known. The specific functions of LysM-GPI in plant resistance pathways have not been reported and need to be further investigated.

**Conclusions**

In this study, the root redox proteomics of sugar beet M14 seedlings under salt stress was analysed using iodoTMTRAQ double-labelling technique combined with LC-MS/MS proteomics. A total of 95 redox proteins exhibiting different redox levels were identified. These proteins were involved in metabolism, ROS homeostasis, stress and defense, transport, cell structure, protein folding and degradation, signal transduction, transcription, photosynthesis and some unknown functions. It is clear that while the potential salt response mechanisms involve many different components, pathways and processes, root redox proteins are central to those involved in the regulation of ROS homeostasis (Fig. 6). Interestingly, crosstalk between redox and phosphorylation was noted. Subcellular localization predictions showed that most redox proteins were predicted to be localized in the cytoplasm and extracellular compartments. Combined analysis of the differential redox proteins in M14 leaves, we can achieve a comprehensive understanding of the mechanisms of post-translational modifications under salt stress in the special BvM14, which is conducive to a profound analysis of the salt tolerance mechanism in sugar beet. Real-time PCR of genes encoding 14 important redox proteins showed that four proteins had consistent expression at the transcript level and protein level. Based on the experimental results, a working model to guide future functional studies was proposed for the potential involvement of redox proteins and phosphoproteins in response to salt stress in the roots of sugar beet M14.

**Declarations**

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**Authors’ contributions**

HL: conducted proteomics experiments and written the first draft; XD: conducted biochemical experiments and assisted with draft editing; LJ and JZ: conducted gene transcription analysis; SC: assisted with mass spectrometry and editing of the manuscript; HD: assisted with experimental design, data analysis and supervision of experiments; HL: funding acquisition, project supervision and finalized the manuscript. All authors have read and agreed to the published version of the manuscript.

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**Availability of data and materials**

The data and materials used and analyzed in the current study can be provided by the corresponding author for scientific, non-profit purposes.
Ethics approval and consent to participate

Not applicable, the study involves no human participants.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

1Key Laboratory of Molecular Biology of Heilongjiang Province, College of Life Sciences, Heilongjiang University, Harbin 150080, China 2Engineering Research Center of Agricultural Microbiology Technology, Ministry of Education, Heilongjiang University, Harbin 150080, China 3Heilongjiang Provincial Key Laboratory of Ecological Restoration and Resource Utilization for Cold Region, School of Life Sciences, Heilongjiang University, Harbin 150080, China 4Proteomics and Mass Spectrometry, Interdisciplinary Center for Biotechnology Research, University of Florida, Gainesville, FL 32610, USA. 5Department of Biology, Genetics Institute, Plant Molecular and Cellular Biology Program, University of Florida, Gainesville, FL 32610, USA.

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Figures

![Figure 1](image-url)

Figure 1

Temporal changes in cysteine free sulphydryl, AsA, and GSH contents in BvM14 roots under salt stress. (A) Cysteine free sulphydryl content under 200 mM and 400 mM NaCl stress. (B) ASA content under 200 mM and 400 mM NaCl stress. (C) GSH content under 200 mM and 400 mM NaCl stress. These values are the means of three biological replicates from different samples with standard errors. *, p < 0.05; **, p < 0.01.
Figure 2

Visualization of redox protein profile data from BvM14 roots under salt stress. (A) iTRAQ-labeled total protein and iodoTMT-labeled redox protein of BvM14 under 200 mM and 400 mM NaCl stress. (B) Significant changes in protein redox levels in BvM14 roots under salt stress. (C) Comparison of the number of differential redox proteins identified under 200 mM NaCl and 400 mM NaCl treatments.

Figure 3

Functional classification and subcellular localization of the differential redox proteins. (A) Functional classification of the differential redox proteins. (B) Subcellular localization prediction of the differential redox proteins. (C) Number of redox proteins in each function.
Figure 4

Comparative analysis of differential redox proteins in sugar beet M14 roots and leaves under salt stress. (A) Comparative analysis of redox protein functions under salt stress in roots and leaves. (B) Comparison of protein redox levels under salt stress in roots and leaves of the M14. Abbreviations: EG45: EG45-like domain containing protein, RD19A: Cysteine protease RD19A, NADH: NADH dehydrogenase [ubiquinone] 1 alpha, VSR: Vacuolar-sorting receptor, GSAM: Glutamate-1-semialdehyde 2,1-aminomutase, Fd: Ferredoxin, root R-B1, Pfn: Profilin, POD: Peroxidase, Hsp: Heat shock cognate protein.
Figure 5

Real-Time PCR assays of genes encoding differential redox proteins and differential proteins in different pathways. (A) Real-Time PCR assays of genes encoding redox proteins common to roots and leaves under salt stress, (B) Real-Time PCR assays of genes encoding redox proteins specific to 200 mM or 400 mM salt stress condition, and (C) Real-Time PCR assays of genes encoding redox proteins common to 200 mM and 400 mM salt stress. The x-axis is the salt concentration. y-axis is the relative expression of each gene ($2^{-\Delta\Delta CT}$). Please refer to Table 1 for abbreviations.
Figure 6

The metabolic networks of the redox protein in sugar beet M14 roots under salt stress. Under 200 mM NaCl treatment, the reduced protein is orange colors and the oxidized protein is green colors. Under 400 mM NaCl treatment, the reduced protein is red colors and the oxidized protein is blue colors. Please refer to Table S6 for abbreviations.

Figure 7

Alignment of amino acid sequence of different expression of peroxidase in salt stress response. Black boxes indicate conserved Cys sites and red boxes indicate Cys sites that undergo redox modifications.

Supplementary Files

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