New Insights into Regulation of Proteome and Polysaccharide in Cell Wall of *Elsholtzia splendens* in Response to Copper Stress

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**Abstract**

**Background and Aims:** Copper (Cu) is an essential micronutrient for plants. However, excess amounts of Cu are toxic and result in a wide range of harmful effects on the physiological and biochemical processes of plants. Cell wall has a crucial role in plant defense response to toxic metals. To date, the process of cell wall response to Cu and the detoxification mechanism have not been well documented at the proteomic level.

**Methods:** An recently developed 6-plex Tandem Mass Tag was used for relative and absolute quantitation methods to achieve a comprehensive understanding of Cu tolerance/detoxification molecular mechanisms in the cell wall. LC–MS/MS approach was performed to analyze the Cu-responsive cell wall proteins and polysaccharides.

**Key Results:** The majority of the 22 up-regulated proteins were involved in the antioxidant defense pathway, cell wall polysaccharide remodeling, and cell metabolism process. Changes in polysaccharide amount, composition, and distribution could offer more binding sites for Cu ions. The 33 down-regulated proteins were involved in the signal pathway, energy, and protein synthesis.

**Conclusions:** Based on the abundant changes in proteins and polysaccharides, and their putative functions, a possible protein interaction network can provide new insights into Cu stress response in root cell wall. Cu can facilitate further functional research on target proteins associated with metal response in the cell wall.

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**Introduction**

Copper (Cu) is an essential micronutrient for plants because it is a structural and catalytic component of several proteins or enzymes involved in various physiological metabolisms [1]. However, excess Cu in plants is harmful; it induces phytotoxicity [2] and disturbs the physiological metabolisms, including carbohydrate metabolism, protein metabolism, mineral nutrition, cell respiration, and photosynthesis [3]. Given its redox properties, Cu^2+ can catalyze the generation of reactive oxygen species (ROS) and Fenton reactions that damage cellular components or interfere with cellular transport processes [4]. Plants cannot avoid the stress conditions from Cu-contaminated areas. Thus, several resistance mechanisms for Cu tolerance have been developed [5], including exclusion, compartmentalization, chelation, and binding to organic ligands, which enable maintenance of Cu homeostasis for plant survival even in a highly contaminated environment [6].

Plant cell wall is the primary site for signal perception and defense response, which is significant in responding to environmental stresses [7]. The cell walls of plants that grow on heavy metal-contaminated areas provide physical barriers against toxic heavy metals and actively participate in plant defense response. The cell wall serves as a repository for high contents of metals; it senses stress signals and transmits them to the cell interior, thereby affecting the cell fate decision [8]. The main chemical components of a plant cell wall include polysaccharides and proteins. Polysaccharides represent up to 90% of the plant cell wall and constitute three different kinds of polymers, namely, cellulose, hemicelluloses, and pectins. Cell wall proteins (CWPs) of plants comprise less than 10% of the cell wall dry weight but have major roles in cell wall structure, metabolism, cell enlargement, signal transduction, defense responses, and many other physiological events [9].

Certain plants can accumulate high content of metal ions in their cell wall through various compounds. Cell wall polysaccharides possess crucial roles in heavy metal binding and accumulation. Binding of metal ions depends on the number of functional groups in the cell wall polysaccharides. Previous studies confirmed that the essential capacity of the cell wall for binding metal ions...
depends mainly on the abundance of polysaccharides in the carboxyl groups [10]. Current information about the regulation of cell wall polysaccharides under metal stress remains limited.

Abiotic stress induces expression of stress-responsive proteins, which lead to cellular adaptation in plant growth under stress conditions. Therefore, studies have been carried out to identify various CWPs involved in stress response, particularly those of regulatory or targeting function proteins [11–17]. Most reports have focused on leaf cell wall proteomes under abiotic stress, such as salt in tobacco [11]; wounding in Medicago [12]; dehydration in chickpea, rice, and maize [9,14,15]; zinc toxicity in Hordeum [18]; nickel toxicity in barley [19]; manganese toxicity in cowpea [20,21]; boron deficiency in Lupinus albus [22]; and flooding in soybean [23]. Few studies have focused on the root cell wall proteomes under abiotic stress [24–26]. All of these studies broadened our understanding of the complicated regulation of apoplast proteins. An in-depth proteome study of the cell wall can provide extensive information on the intrinsic mechanism of stress response attributed to a possible relationship between protein abundance and plant stress tolerance [27].

*Elsholtzia splendens* is a plant that can survive in a highly Cu-polluted area. This species can accumulate more than 1000 mg/ kg of Cu in the body [28], and the major proportion (70%) of Cu ions is stored in the root cell wall [29]. Nevertheless, little is known about the defense and detoxification mechanisms in the root cell wall of *E. splendens*. A recently developed 6-plex Tandem Mass Tag (TMT) was used for relative and absolute quantitation methods to gain a comprehensive understanding of Cu tolerance/ detoxification molecular mechanisms. LC–MS/MS approach was also conducted to analyze Cu-responsive CWPs and polysaccharides in this study. This proteomic study of the apoplast is the first study conducted to analyze Cu-responsive CWPs and polysaccharides compared with total soluble proteins. One unit of G6PDH activity is defined as 1 μmol of NADPH turnover per min/mg protein. Results are presented as mean ± SE of G6PDH activity from three biological replicates. The asterisks indicate significant differences in the G6PDH activity of CaCl2-extracted cell wall proteins, NaCl-extracted cell wall proteins compared with that of total soluble cytosolic proteins (**p<0.01).

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**Cu-induced changes in root CWP contents**

Plants were grown on a medium supplemented with different CuSO4 concentrations to determine changes in CWP abundance under Cu stress (Figure 2A). Cu toxicity led to a strong increase in CWP contents at low Cu concentrations. The CWP contents increased 1.39-fold at 25 μM Cu and 1.96-fold at 50 μM Cu. The highest content of the CWPs was observed at 50 μM Cu concentrations. The plant root began to decompose at 100 μM Cu (Figure 2C). The increase of Cu contents in the cell wall could proportionally increase with the Cu supply in the medium (slope = 0.3, R2 = 0.98) at Cu concentrations below 75 μM. The Cu content in the root cell wall continued to increase significantly (slope = 0.45, R2 = 0.99) above 75 μM Cu. The highest value of Cu content in the root cell wall reached 1.41 mg/g with the root cell wall dry weight at 100 μM Cu. These findings indicate an increase in Cu binding to cell walls with Cu supply in the medium (Figure 2A). Polypeptide separation by SDS-PAGE (Figure 2B) revealed about 30 polypeptide bands. Band intensities increased with the increase in Cu concentration below 50 μM (Figure S1). The staining intensity was the lowest among all the cell wall polypeptides at 100 μM Cu concentration.

**Cu-induced remodeling in root cell wall polysaccharide**

Evidence generally suggests that cell wall polysaccharides are crucial sites for Cu retention in plants. Therefore, changes in the contents of root cell wall polysaccharide of *E. splendens* were measured. Treatments with 25, 50, 75, and 100 μM Cu significantly increased the expression of CWPs and polysaccharides compared with controls (Figure 3). Cu supply enhanced the total sugar contents in various cell wall fractions. Sugar contents were higher in hemicellulose than in pectin and cellulose. Sugar contents in pectin increased 1.22-fold with 25 μM Cu. The major components of cell wall fractions are galacturonic acids.

The content of galacturonic acids was considerably higher than that in

![Figure 1. Evaluation of purity by the G6PDH activity in the cell wall proteins isolated from the roots of Elsholtzia splendens.](image)

**Figure 1. Evaluation of purity by the G6PDH activity in the cell wall proteins isolated from the roots of Elsholtzia splendens.** The activity of G6PDH was assayed in CaCl2-extracted cell wall proteins, NaCl-extracted cell wall proteins and total soluble proteins. One unit of G6PDH activity is defined as 1 μmol of NADPH turnover per min/mg protein. Results are presented as mean ± SE of G6PDH activity from three biological replicates. The asterisks indicate significant differences in the G6PDH activity of CaCl2-extracted cell wall proteins, NaCl-extracted cell wall proteins compared with that of total soluble cytosolic proteins (**p<0.01).**

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the control during Cu treatment, particularly at higher Cu supply.
The galacturonic acid values rapidly increased in pectin (1.28-fold), hemicellulose (1.22-fold), and cellulose (1.79-fold) at 100 μM Cu. A conserved disaccharide residue known as 2-keto-3-deoxyoctonic acid (KDO) is attached to C-3 of the backbone of rhamnogalacturonan II in pectin. The KDO concentration in pectin increased during Cu exposure. Thus, the highest concentration of KDO (1.49-fold) was found in the 100 μM Cu-treated group.

The results indicated that excess Cu affected the intracellular distribution of polysaccharides in the root cell wall. Given this reason, we focused on the relationship between wall polysaccharide composition and CWPs with increasing Cu intensity. The dendrogram proved the connection between the contents of polysaccharides and proteins as matched with different Cu concentrations (Figure 4). This finding suggests that Cu induced changes in the contents of root cell wall polysaccharides and proteins, which altered the intracellular distribution patterns in assigning effects on cellular activities.

**Cu-responsive CWP identification using TMT LC-MS/MS**

This study aimed to gain a better understanding of the metabolic processes and molecular mechanisms involved in Cu-responsive CWPs. A TMT-based shotgun quantitation approach was used to obtain an overall view of the proteome changes associated with Cu stress in the root cell wall of *E. splendens*. Unique proteins were successfully identified based on homology searching with a common protein confidence cutoff of 95% and relative quantitative information in control and Cu-treated samples, as indicated by the peak areas of the different TMT tags (Table 1, Table S2). A threshold of 1.5-fold change and a p, 0.05 were set as stringent criteria for significant differences between control and treatment. Fifty five proteins were classified as differentially expressed upon Cu stress using the criteria; 22 and 33 of these proteins showed increasing trends and decreasing abundance, respectively (Table 1, Table 2).

**Functional classification of identified CWPs**

Database search using Uni-Prot/NCBI accession numbers was conducted to obtain detailed information of differentially expressed proteins. These proteins were categorized into biological process and molecular function using an in-house Perl script according to the extracted GO terms from InterPro (www.ebi.ac.uk/interpro/) or Pfam (pfam.sanger.ac.uk/) (Figure 5). Functional classifications of the 55 differentially expressed proteins (Table 1, Table 2) were classified based on the GO terms that correspond to...
the biological process. The top three categories were those involved in metabolic processes (23.24%), cellular processes (16.20%), and response to stimuli (14.79%) (Figure 5A). These three categories were classified according to the GO molecular annotation into binding (41.38%), catalytic activity (31.03%), and transport activity (11.49%) (Figure 5B). The observed diversity in biological function and processes of the differentially expressed CWPs demonstrated that the response of the root cell wall of *E. splendens* to Cu stress was a complex process. Many physiological and biochemical changes were altered to counteract the adverse conditions.

Specific enzymes constituted by some small differentially expressed CWPs participated in various metabolic processes to counteract Cu stress. Classification pathways according to the KEGG database searches categorized the cell wall enzymes into 12 classes (Table 3, Table S2). The more important category pathways were involved in oxidative phosphorylation, phenylalanine biosynthesis and metabolism, ascorbate and aldurate metabolism, and citrate cycle. All these pathways suggested the relatively altered abundance of the enzymes in various metabolic pathways that probably re-optimized the metabolic processes to combat against Cu stress.

**Discussion**

**Cell wall polysaccharide remodeling in root under Cu stress**

Cell walls provide a physical barrier to plants grown in Cu-contaminated soil, and cell wall polysaccharides have a crucial role in metal binding and accumulation [7]. The binding ability depends on the number of functional groups in polysaccharides, particularly for abundant carboxyl groups [7]. In the present study, the contents of the various cell wall polysaccharides significantly increased with the increase in Cu concentration. The expression of cell wall polysaccharides, which contain more carboxyl groups, provided more binding sites for Cu ions. Excess Cu also affected the intracellular distribution of polysaccharide in the root cell walls (Figure 3). Similar situations were also described in rice roots under Cd stress [45] and *Arabidopsis* roots under Al stress [46]. The starch and sucrose metabolism-related proteins (Table 3) – Chloroplast NAD-MDH (No. 1, Table 1) – were also markedly up-regulated in response to Cu stress based on our observations. These findings suggest that Cu or Cu-induced oxidative stress may activate some crucial enzymes in the glyconeogenesis pathway, which can alter the contents of cell wall polysaccharides.

Increasing physiological, biochemical, and molecular evidence also showed that the remodeling of the binding properties of root apoplasts was attributed to Cu resistance. Enhancement at the level of low-methylesterified pectins, where the polysaccharides bound more trivalent metal ions, is one of the most remarkable alterations. Pectin methylesterases (PMEs), known as pectinesterases, catalyzed...
Table 1. Root cell wall proteins with significant increased in expression level under 50 μM Cu treatment identified by LC-ESI-MS/MS-based proteomics using SIEVE (p<0.05 and fold chang >1.5).

| Serial No. | Protein name                                      | gi number | gi number | Percent coverage a (%) | Fold Change | p valueb | Species identifiedd | MW(kDa) | p value |
|------------|---------------------------------------------------|-----------|-----------|-------------------------|-------------|-----------|---------------------|---------|---------|
| 1          | Chloroplast NAD-MDH                               | 3256066   | 8.68      | 1.00                    | 0.000000    | Arabidopsis thaliana | 49.9    | 8.2     |
| 2          | D-glyceraldehyde-3-phosphate Dehydrogenase(GAPDH) | 51849658  | 17.26     | 1.52                    | 0.000000    | Periploca sepium     | 25.3    | 6.2     |
| 3          | 40S ribosomal protein S19                         | 74231038  | 9.32      | 1.52                    | 0.00271     | Solanum peruvianum   | 51.7    | 5.5     |
| 4          | Phosphoglycerate kinase                           | 21272     | 5.08      | 1.53                    | 0.00963     | Spinacia oleracea    | 55.1    | 6.1     |
| 5          | Putative malate dehydrogenase                     | 5008249   | 15.29     | 1.55                    | 0.00346     | Oryza sativa Japonica Group | 41.6 | 5.0     |
| 6          | Cys07                                             | 1856971   | 6.12      | 1.56                    | 0.02389     | Catharanthus roseus  | 35.6    | 6.0     |
| 7          | 2,3-bisphosphoglycerate-independent phosphoglycerate mutase | 168588     | 1.61      | 1.57                    | 0.000000    | Zea mays             | 68.8    | 5.2     |
| 8          | Non-symbiotic hemoglobin                          | 11095158  | 13.75     | 1.58                    | 0.01599     | Medicago sativa      | 23.0    | 9.0     |
| 9          | P0 ribosomal protein                              | 1143507   | 6.83      | 1.59                    | 0.000000    | Lupinus luteus       | 41.0    | 9.8     |
| 10         | Putative 40S ribosomal protein s12                 | 643074    | 7.75      | 1.60                    | 0.03853     | Fragaria x ananassa  | 21.2    | 4.7     |
| 11         | Unknown                                           | 255648032 | 11.18     | 1.62                    | 0.02313     | Glycine max          | 21.3    | 6.4     |
| 12         | Mitochondrial chaperonin                          | 415733    | 7.16      | 1.68                    | 0.01830     | Bassica napus        | 74.5    | 5.4     |
| 13         | Matrasae K                                        | 3332509   | 4.13      | 1.69                    | 0.01270     | Pticaimia xanthocalyx | 48.8    | 6.9     |
| 14         | Pectin methylester                                | 1222552   | 2.23      | 1.70                    | 0.00633     | Solanum lycopersicum | 75.5    | 9.5     |
| 15         | Porin-like protein                                | 47847590  | 3.61      | 1.71                    | 0.01348     | Oryza sativa Japonica Group | 36.1 | 6.4     |
| 16         | Pollen allergenQue a 1 isoform                    | 167472851 | 7.50      | 1.72                    | 0.00062     | Quercus alba         | 21.3    | 8.8     |
| 17         | Putative 1,4-benzoquinone reductase                | 53749369  | 5.37      | 1.73                    | 0.000000    | Oryza sativa Japonica Group | 49.9 | 8.2     |
| 18         | Thoedoxin h                                       | 13624884  | 9.32      | 1.89                    | 0.02335     | Pismum sativum       | 25.3    | 6.2     |
| 19         | Predicted protein                                 | 162680354 | 5.16      | 1.91                    | 0.00523     | Physcomitrella patens subsp | 24.8 | 8.9     |
| 20         | Hypothetical protein OsI_31140                    | 12563499  | 2.51      | 1.99                    | 0.00035     | Oryza sativa Indica Group | 16.1 | 4.7     |
| 21         | Hypothetical protein OsI_07053                    | 218190658 | 1.47      | 2.70                    | 0.000000    | Oryza sativa a Indica Group | 37.3 | 5.8     |
| 22         | 60s ribosomal protein P2                          | 551267    | 7.89      | 3.31                    | 0.000000    | Parthenium argentatum | 56.7    | 5.4     |

a gi no., gene identification number as in GenBankTM.
b Coverage., sequence coverage.
c Fold change.
d p value, indicates the significance of up- or down-regulation of spots according to the F-test through analysis of variance (p<0.05).
e Species identified by Mascot search using NCBI.

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| Serial No. | Protein name                                      | gi number | Percent coverage (%) | Fold Change | p value | Species identified           | MW(kDa) | p/   |
|-----------|--------------------------------------------------|-----------|----------------------|-------------|---------|-----------------------------|---------|------|
| 23        | Predicted protein                                | 162671613 | 1.87                 | −2.68       | 0.00000 | Physcomitrella patens subsp | 71.6    | 4.9  |
| 24        | Peroxidase                                       | 17066703  | 2.71                 | −2.59       | 0.00000 | Capsicum annuum             | 39.5    | 6.9  |
| 25        | Os07g0223100                                     | 113610742 | 18.75                | −2.28       | 0.03704 | Oryza sativa Japonica Group | 6.1     | 6.0  |
| 26        | Mitochondrial phosphate translocator             | 1842188   | 2.47                 | −2.25       | 0.00466 | Betula pendula              | 45.3    | 9.4  |
| 27        | Predicted protein                                | 549049082 | 3.53                 | −2.23       | 0.01662 | Populus trichocarpa         | 30.5    | 8.9  |
| 28        | Actin                                            | 283300146 | 1.60                 | −2.17       | 0.00166 | Cleistogenes songorica      | 46.2    | 5.5  |
| 29        | Ubiquitin fusion protein                         | 149391425 | 3.60                 | −2.08       | 0.02095 | Oryza sativa Indica Group   | 16.6    | 10.0 |
| 30        | Heat shock protein Hsp70                         | 124360342 | 2.59                 | −1.98       | 0.02106 | Medicago truncatula         | 80.9    | 6.4  |
| 31        | Putative nascent polypeptide associated complex alpha chain | 20160782 | 6.93                 | −1.97       | 0.00728 | Oryza sativa Japonica Group | 26.9    | 4.4  |
| 32        | Hypothetical protein SORBIDRAFT_09g020360       | 241945088 | 8.50                 | −1.88       | 0.00003 | Sorghum bicolor             | 49.1    | 5.2  |
| 33        | Plasma membrane-type calcium ATPase              | 3335060   | 1.08                 | −1.86       | 0.03138 | Arabidopsis thaliana        | 125.3   | 8.9  |
| 34        | Cytochrome b5                                    | 2695711   | 11.94                | −1.83       | 0.01678 | Olea europaea               | 18.3    | 5.5  |
| 35        | Unknown                                          | 118482646 | 3.48                 | −1.82       | 0.00000 | Populus trichocarpa         | 41.8    | 4.9  |
| 36        | ATP synthase beta subunit                        | 8452631   | 4.35                 | −1.80       | 0.04389 | Cichorium intybus           | 18.1    | 6.2  |
| 37        | Peptidyl-prolyl cis-trans isomerase              | 118104    | 7.56                 | −1.76       | 0.00000 | Zea mays L                  | 21.8    | 9.0  |
| 38        | Ferredoxin-NADP+ reductase-like protein          | 7267299   | 8.61                 | −1.73       | 0.00081 | Arabidopsis thaliana        | 48.3    | 8.9  |
| 39        | Putative protein                                 | 5262759   | 0.82                 | −1.71       | 0.04231 | Arabidopsis thaliana        | 124.3   | 8.5  |
| 40        | Conserved hypothetical protein                   | 223517971 | 3.65                 | −1.71       | 0.00000 | Ricinus communis            | 24.2    | 4.2  |
| 41        | Unnamed protein product                          | 219742878 | 7.50                 | −1.70       | 0.01691 | Gycine max                  | 38.2    | 5.6  |
| 42        | Unknown                                          | 255634488 | 7.50                 | −1.69       | 0.01879 | Gycine max                  | 38.0    | 5.4  |
| 43        | Ferredoxin-NADP Reductase                        | 6634773   | 7.59                 | −1.69       | 0.00157 | Oryza sativa                | 50.9    | 9.2  |
| 44        | Predicted protein                                | 222844395 | 1.20                 | −1.68       | 0.00000 | Populus trichocarpa         | 72.6    | 9.2  |
| 45        | 60S ribosomal protein L25                        | 310935    | 14.94                | −1.66       | 0.00002 | Nicotiana tabacum           | 24.8    | 8.8  |
| 46        | Vacular H(+)-ATPase subunit-like protein         | 7801655   | 2.27                 | −1.64       | 0.00000 | Arabidopsis thaliana        | 58.0    | 5.9  |
| 47        | Small G protein                                  | 974780    | 4.06                 | −1.61       | 0.01530 | Beta vulgaris subsp. vulgaris | 26.3    | 10.1 |
| 48        | Elongation factor 1 subunit alpha                | 162036292 | 8.22                 | −1.58       | 0.02150 | Chenchus americanus         | 30.2    | 6.6  |
| 49        | 2-dehydro-3-deoxy phosphor heptonate aldolase    | 2546988   | 5.61                 | −1.52       | 0.01476 | Morinda citrifolia          | 65.5    | 9.3  |
| 50        | RAS-related GTP-binding protein                  | 20756     | 5.34                 | −1.52       | 0.00000 | Ptaea sativum               | 26.7    | 8.3  |
| 51        | Aspartic proteinase naphtins-1 precursor, putative| 223353578 | 3.05                 | −1.52       | 0.00000 | Ricinus communis            | 46.6    | 6.5  |
| 52        | Hypothetical protein                             | 147774267 | 0.73                 | −1.52       | 0.00000 | Vitis vinifera              | 24.1    | 4.9  |
| 53        | Hypothetical protein VITISV_025412               | 147822728 | 8.42                 | −1.52       | 0.00000 | Vitis vinifera              | 21.9    | 7.3  |
| 54        | Predicted protein                                | 222855760 | 6.33                 | −1.51       | 0.00185 | Populus trichocarpa         | 41.8    | 5.0  |
| 55        | Hypothetical protein VITISV_023718               | 147801802 | 1.87                 | −1.51       | 0.00000 | Vitis vinifera              | 71.1    | 7.8  |

*a* gi no., gene identification number as in GenBankTM.
*b* Coverage., sequence coverage.
*c* Fold change.
*d* p value, indicates the significance of up- or down- regulation of spots according to the F-test through analysis of variance (p<0.05).
*e* Species identified by Mascot search using NCBI.

Table 2. Root cell wall proteins with significant decreased in expression level under 50 μM Cu treatment identified by LC-ESI-MS/MS-based proteomics using SIEVE (p<0.05 and fold chang >1.5).
the demethylesterification of the homogalacturonan pectin domain in the cell wall. Demethylesterification of the pectin increases the abundance of free carboxylic acid groups on the galacturonic acid residue [47]. A flax PME has been recently implicated in wall remodeling following Cd treatment [48]. Interestingly, this study identified PMEs (No. 14, Table 1) as Cu-induced differentially expressed proteins; the protein had changes in abundance of 1.7-fold (p<0.5). Catalyzed ROS by excess Cu²⁺ can also damage or cause degradation of essential complex molecules in cell wall polysaccharide. Generating hydroxyl radicals from H₂O₂ possesses a direct role in cell wall loosening through polysaccharide cleavage [49,50]. Hydroxyl radicals (OH) may cause non-enzymic scission of polysaccharides in vivo (e.g., in plant cell walls) [50]. Basing on these findings, we concluded that the chemical composition and distribution of plant cell wall polysaccharides are a factor in the outcome of plant-metal interaction, which elucidated a possible role in a novel Cu-resistance mechanism.

**Cu-induced oxidative stress and antioxidant defense in cell wall**

Cu is a redox active metal that catalyzes ROS production, such as superoxide (O₂⁻), hydrogen peroxide (H₂O₂), and hydroxyl radicals (OH⁻) through Haber Weiss and Fenton reactions [51]. ROS can act as signaling molecules for stress response. However, ROS can cause damage to many cellular components above a certain threshold. Most Cu-tolerance mechanisms are primarily involved in protecting the cellular structure. An important method is the control of the level of ROS or the limitation of damage caused by ROS. In the current study, significant changes in the abundance of some antioxidant and defense-related proteins suggested that ROS can be involved in a Cu-induced oxidative stress.

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**Figure 5. Functional cataloguing of 55 differentially expressed cell wall proteins in Elsholtzia splendens’s root based on GO annotation.** The pie charts show the distribution of 55 differentially expressed cell wall proteins on of the Cu-responsive proteins into their functional classes in percentage. A: Biological Process Ontology, B: Molecular Function Ontology. doi:10.1371/journal.pone.0109573.g005
stress response. Analysis of *E. splendens* root CWPs confirmed the associated gene expression for enzymes involved in ROS scavenging and oxidative phosphorylation (Table 3), such as peroxidase (No. 24, Table 2), peptidyl-prolyl cis-trans isomerase (No. 37, Table 2), and phosphoglycerate kinase (No. 4, Table 1). Among these proteins, peptidyl-prolyl cis-trans isomerase, as a protein chaperone, possess complementary and sometimes overlapping roles in protecting the proteins [13].

Excess Cu generates oxidative stress, thereby hindering some important metabolic process, such as up-regulation of antioxidant and stress-related regulatory proteins, that help maintain cellular homeostasis [52]. Maturase K (No. 13, Table 1) changed in abundance of 1.69-fold. Maturase K has an important role in plant growth, cell division, and expansion as well as in protecting metabolic processes against H$_2$O$_2$ and other toxic derivatives of oxygen. Hydroquinone formation was accompanied by the oxidation of two moles of NADPH, and the presence of an inducible 1,4-benzoquinone reductase. Thus, putative 1,4-benzoquinone reductase (No. 17, Table 1) plays an essential role in antioxidant defense pathway [53]. Studies also reported about wall-bound malate dehydrogenase (No. 5, Table 1) [54,55], which can regenerate NADPH that is needed by cell wall peroxidases for free radical generation associated with lignin polymerization. Thioredoxin (No. 18, Table 1) is also involved in redox regulation by reducing disulfides on the target protein for detoxifying lipid hydroperoxides or repairing oxidized proteins and relaying the signal to mitogen-activated protein kinase pathway of stress signaling [56]. Phosphoglycerate kinase (No. 4, Table 1) can also interact with cytosolic catalase and has a role in relieving oxidative stress. Interestingly, down-regulation of key metabolic enzymes revealed that oxidant protection conferred by these proteins was also regulated during Cu treatment. Ferredoxin-NADP reductase (No. 43, Table 2) and ferredoxin-NADP reductase-like protein (No. 38, Table 2), known to sequester highly reactive Fe$^{3+}$ and prevent formation of toxic OH species, were also identified [57]. Vacuum H$^+$-ATPase subunit-like protein (No. 46, Table 2) was involved in ascorbate and aldarate metabolism. L-ascorbic acid is characterized by plant tissues, and ascorbate is one of the most important antioxidant molecules [58]. Furthermore, 2-dehydro-3-deoxyphosphoheptonate aldolase (No. 49, Table 2) may be involved in aromatic pathways in the secondary metabolites, which are known to act as defense responses to abiotic stress [59].

**Cu stress-activated signaling pathways**

The communication between the cytoskeleton and the CWPs is one of the most characterized features of cellular mechanisms that enable cells to respond effectively to various extracellular signals. Several candidate components involved in signal transduction were identified in this study, including Hsp70, small G protein, and stress-related regulatory proteins, that help maintain cellular homeostasis [52].

### Table 3. Category of differentially expressed cell wall proteins refers to the entry on the classification of pathways from KEGG database.

| No. | Pathway                              | Enzyme                            | Enzyme ID      | Change Folds | Protein Name                      |
|-----|--------------------------------------|-----------------------------------|----------------|--------------|-----------------------------------|
| 1   | Phenylpropanoid biosynthesis         | Lactoperoxidase                   | ec:1.11.1.7    | −2.59        | Peroxidase                        |
|     | Phenylalanine metabolism             |                                   |                | −1.69        | Ferredoxin-NADPH Reductase         |
|     |                                       |                                   |                | −1.66        | Predicted protein                 |
| 2   | Phenylalanine, tyrosine and tryptophan biosynthesis | Synthase | ec:2.5.1.54 | −1.52 | Hypothetical protein VITISV_025412 |
|     |                                      |                                   |                | 2.70         | Hypothetical protein OsI_07053    |
| 3   | Nitrogen metabolism                  | (6S)-tetrahydrofolate dehydratas  | ec:2.1.2.10    | 1.72         | Pollen allergen Que a 1 isoform   |
|     |                                       |                                   |                | 4.21         | Cytochrome b5                      |
| 4   | One carbon pool by folate            | (6S)-tetrahydrofolate dehydratas  | ec:2.1.2.10    | 1.72         | Pollen allergen Que a 1 isoform   |
| 5   | Starch and sucrose metabolism        | Synthase                          | ec:2.4.1.34    | 1.50         | Chloroplast NAD-MDH               |
| 6   | Oxidative phosphorylation            | ATPase                            | ec:3.6.3.6     | −1.76        | Peptidyl-prolyl cis-trans isomerase |
|     |                                      | Dehydrogenase                     | ec:1.3.5.1     | 1.53         | Phosphoglycerate kinase           |
| 7   | Ascorbate and aldarate metabolism    | Oxidase                           | ec:1.1.3.8     | −1.64        | Vacular H$^+$-ATPase subunit-like protein |
| 8   | Glyoxylate and dicarboxylate metabolism | (S)-synthase      | ec:2.3.3.1     | −1.86        | Plasma membrane-type calcium ATPase |
| 9   | Sphingolipid metabolism              | Phosphodiesterase                 | ec:3.1.4.12    | −1.52        | 2-dehydro-3-deoxyphosphoheptonate aldolase |
| 10  | Citrate cycle (TCA cycle)            | (S)-synthase                      | ec:2.3.3.1     | −1.86        | Plasma membrane-type calcium ATPase |
| 11  | Glycine, serine and threonine        | aldolase                          | ec:1.3.5.1     | 1.53         | Phosphoglycerate kinase           |
| 12  | Methane metabolism                  | Lactoperoxidase                   | ec:1.11.1.7    | −2.59        | Peroxidase                        |
|     |                                       |                                   |                | −1.69        | Ferredoxin-NADPH Reductase         |
|     |                                       |                                   |                | −1.66        | Predicted protein                 |

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and cytochrome. The biosynthesis and accumulation of HSP proteins (No. 30, Table 2) can generally contribute to the protection and repair of cells under stress [60]. These proteins may be involved in cell wall biogenesis [61]. Small G proteins (No. 47, Table 2) transduce signals from receptors to control a wide range of cellular functions, particularly for regulating Ca$^{2+}$ channel expression at the cell surface [62]. These proteins are clustered into distinct families but all act as molecular switches, which are active in their GTP-bound form but inactive when GDP-bound [63]. RAS-related GTP-binding protein (No. 50, Table 2) also has a primarily role in modulating cellular functions that involve actin cytoskeleton (No. 28, Table 2), such as establishing cell polarity and morphology [64]. Cytochrome has been identified in the stem cell wall of Medicago sativa [65] and germinating embryos of Oryza sativa [66]. This first proteomic study showed that cytgochromes (No. 6, Table 1; No. 34, Table 2) were Cu-responsive proteins in the root cell wall of E. splendens. More preliminary evidence suggested that porins can form aqueous transmembrane channels for transporting solutes and macromolecules across the extracellular surface [67] [68]. These pores are regulated by ATP and GTP with a gating mechanism that modulates the pore size and ion selectivity [54]. In this case, Cu resistance mechanism may be induced by active apoplastic permeability because of up-regulated porin-like proteins (No. 15, Table 1).

**Cu stress-activated energy pathways and protein synthesis**

ATP is an essential metabolite in cell walls associated with energy conversion [69]. The plasma membrane calcium ATPase (PMCA) (No. 33, Table 2) or Ca$^{2+}$ pump transports Ca$^{2+}$ ions out of the cells using the stored energy in ATP. Control of Ca$^{2+}$ concentration is significant in the cytosol [70]. PMCA down-regulation at the protein levels was observed during the experiment, which may represent an adaptive mechanism to facilitate removal of Ca$^{2+}$ in the maintenance of calcium homeostasis in abiotic stress. The vacuolar H\(\rightleftharpoons\)ATPase (No. 46, Table 2) functions as a primary proton pump that generates electrochemical gradients of protons across the transmembrane region [71], which provides the primary driving force for transporting numerous ions and metabolites against their electrochemical gradients [72]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (No. 2, Table 1), as a classical cellular enzyme involved in glycolysis, is also differentially expressed in response to Cu exposure, where GAPDH is specifically targeted at the cell wall [73]. Mitochondrial phosphate translocator (No. 26, Table 2), involved in ATP/ADP transport, is also identified in the cell wall. Amino acids and amino acid-derived molecules can be chelated with metal ions by high affinity ligands [74]. Pollen allergen Que a 1 isoform (No. 16, Table 1), which is involved in glycine, serine, and threonine metabolism (Table 3), was up-regulated in the root cell wall of E. splendens, whereas aspartic proteinase nepenthesin-1 precursor (No. 51, Table 2) was down-regulated by Cu stress. Different expression levels of amino acid-related proteins in the cell wall elucidated their possible role in Cu tolerance.

**Other Cu stress-responsive proteins and their potential functions**

The 2,3-bisphosphoglycerate independent phosphoglycerate mutase (No. 7, Table 1) was up-regulated in the study, which was also differentially expressed in rice anthers under cold stress [75]. Previous evidence indicated that the expression of non-symbiotic hemoglobin (nsHb) from cotton is induced with Verticillium wilt fungus [76]. The proteins also had a role in the defense responses against pathogen invasions in Arabidopsis [76]. Our studies showed that the nsHbs (No. 8, Table 1) were up-regulated in the cell wall of E. splendens root under Cu stress, although their physiological function is yet to be determined. A significant amount of the unidentified proteins were classified as hypothetical or predictable. These proteins were annotated in databases as unknown, hypothetical, or putative proteins because of the theoretical translation of open reading frame sequences [77]. The roles of these proteins remain to be investigated.

**Overview of regulated CWP functions**

This study provides insights into the functional role of the cell wall of E. splendens under Cu stress, where several CWPs were identified with possible roles in Cu tolerance/detoxification. Cu stress conditions can alter the composition (polysaccharide and protein) of the cell wall both qualitatively and quantitatively. Approximately 40% of the differentially expressed CWPs showed higher abundance in response to Cu stress involved in antioxidant defense, cell wall polysaccharide remodeling, and metabolism process. Up to 60% of the CWPs were in low abundance in response to Cu stress that is involved in signal, energy, and protein synthesis. Polysaccharide analysis confirmed the cell wall remodeling under Cu stress. The amount, composition, and distribution of the cell wall polysaccharides are consequential for plant adaptation to enhance Cu ion levels. Proteome analysis of cell wall confirmed that most proteins were associated with an antioxidant defense response. Hsp 70, small G protein, and RAS-related GTP-binding protein also have essential roles in signal transduction across the cell wall and through the cytoskeleton. Literature provides fundamental information about the role of polysaccharide composition of plant cell wall in metal tolerance and complementary evidence on continuous crosstalk between CWPs and the cytoskeleton. Therefore, knowledge has been expanded on plant stress-related signaling pathways in the cell wall. Cu regulation of these proteins may also not solely respond on abundance changes. Post-translational modification and dynamics are interesting subjects for future investigation. The present study is the first cell wall proteome and polysaccharide investigation of plants in response to Cu and important for the understanding of the plant cell wall response to environmental heavy metal stresses. We propose a possible protein interaction network (Figure 6) that provides new insights into Cu stress response in the root cell wall and facilitates further functional research of target proteins that are associated with Cu response based on the abundant changes in these proteins, as well as polysaccharides and their putative functions.

**Materials and Methods**

**Ethics statement**

No specific permissions were required for collecting E. splendens seeds from deposited Cu-mining soil in Zhuji County, Zhejiang Province, China. E. splendens is neither endangered nor protected. Authors maintained the population at sustainable levels. All plant work was conducted according to relevant national and international guidelines.

**Plant material and Cu treatment**

E. splendens seeds were collected from plants that grew on deposited Cu-mining soil in Zhuji County, Zhejiang Province, China. The seeds were washed with deionized water and soaked in distilled water to germinate in a controlled dark condition (25°C)
for 48 h. The solution was changed to one-quarter-strength complete nutrient solution after germination. At the fourth leaf stage, uniform seedlings were transferred into vessels filled with full-strength nutrient solution that contained macronutrients (in mM): 1.0 Ca(NO₃)₂, 0.5 MgSO₄, and 0.5 K₂HPO₄; and micronutrients (in mol/L): 27.0 Fe(III)–EDTA, 23.0 H₃BO₃, 0.8 CuSO₄, 0.5 Na₂MoO₄, 0.5 ZnSO₄, and 4.5 MnSO₄. The pH was adjusted to 5.8 with 0.1 M HCl and NaOH. Nutrient solutions were renewed every 3 d and aerated continuously through 0.2 mm filters. Plants were grown in a hydroponic nutrient solution chamber with a 16 h, 25°C day and an 8 h, 20°C night regimen at 60% to 70% relative humidity. Light conditions in the growth chamber were fixed at 5 m mol to 10 m mol photons m⁻² s⁻¹. Plants were exposed to various concentrations of Cu (25, 50, 75, and 100 μM) after 28 d of growth, which was added as sulfate for 48 h. Each treatment (15 plants) was conducted in triplicate, and the control plants (CK) were grown in pure nutrient solution for comparison. Plant roots were rinsed with distilled water at harvest and then immersed in 5 mM/L of Ca(NO₃)₂ for 20 min to remove the putative adsorbed Cu²⁺ [32]. Roots were separated, pooled, and rinsed with deionized water; plant parts were washed, immediately frozen in liquid nitrogen, and stored at −80°C for analysis [33].

Cell wall preparation

Cell wall purification and CWP extraction were prepared as described by Feiz et al. [34] with slight modifications. All procedures were conducted at 4°C unless mentioned otherwise. Fifteen independent root preparations were pooled to yield one biological replicate. Root tissues (4 g) were homogenized in pre-cold extraction buffer [5 mM acetate, 0.4 M sucrose, 1 mM phenylmethylsulfonyl fluoride (PMSF), pH 4.6, 125 mM] with a chilled mortar and pestle. After adding 0.4 g of polyvinylpolypyrrolidone (PVP), the mixture was incubated at 4°C for 30 min with stirring. Cell walls were separated from soluble cytoplasmic fluid by centrifuging the homogenate at 1,000 x g for 15 min. The pellet was washed by suspension in 125 mL of 5 mM acetate buffer at pH 4.6 with 0.6 M sucrose. The mixture was centrifuged at 1,000 x g for 15 min. The pellet was further purified by resuspension in 125 mL of 5 mM acetate buffer at pH 4.6 with

Figure 6. Pathways involved in cell defense, signaling, and cell wall remodeling under copper stress in the root cell wall of Elsholtzia splendens. Proteins identified in this study are displayed on the corresponding metabolic pathways and the number indicates the protein identification number. Gal, galacturonic acids; Methyl-gal, methylated galacturonic acids; KDO, 2-keto-3-deoxyoctonic acid; Rha, rhamnose; PME, Pectin methylesterase; Xyl, xylanase; Glu, glucose; MD, malate dehydrogenase; Trx, thioredoxin; MAPKK, mitogen-activated protein kinase kinase; PPI, peptidyl-prolyl cis-trans isomerase; PPK, phosphoglycerate kinase; MatK, maturase K; 1,4-BR, putative 1,4-benzoquinone reductase; oxidation; FNR, ferredoxin-NADP reductase; HQ, hydroquinone; DHQ, Durohydroquinone; DAHP, 3-deoxy-D-arabino-heptulosonic acid-7-phosphate; E4P, erythrose-4-phosphate; PEP, phosphoenolpyruvate; RGP, RAS-related GTP-binding protein; PLP, porin-like proteins; Cyc, cytochrome; PMCA, plasma membrane calcium ATPase; V-ATPases, vacuolar H⁺-ATPases; MPT, mitochondrial phosphate translocator; GAPDH glyceraldehyde-3-phosphate dehydrogenase; RP, ribosomal protein; UF, ubiquitin fusion protein; EF, elongation factor 1 subunit alpha; PQ, Pollen allergen Que a 1 isoform; AP, aspartic proteinase nepenthesin-1 precursor; mHBs, non-symbiotic hemoglobin; OsARF, Os070223100; IPGM, 2,3-bisphosphoglycerate-independent phosphoglycerate mutase.

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1 M sucrose and centrifuged at 1,000×g for 15 min. The residue was washed thoroughly with 750 mL of 5 mM acetate buffer at pH 4.6 while filtered on a layer of Miracloth (Merck, Darmstadt, Germany). The supernatant was discarded, and the final pellet was freeze-dried overnight. The freeze-dried cell wall materials were then stored at −80°C for further use. Most intracellular proteins were removed from the cell walls given the advantage of sucrose gradients and extensive washing with low ionic strength acidic buffer. PVPP was treated with acid to increase polymerization and to remove metal ions and contaminants.

Cell wall composition extraction and analysis

Isolation of cell wall material. Cell wall materials were extracted according to Zhong and Lauchli (1993) [35]. The pectin fraction was extracted twice with 0.5% ammonium oxalate buffer that contained 0.1% NaBH₄ (pH 4) in boiling water bath for 1 h each and pooled the supernatants. Pellets were subsequently subjected to triple extractions with 4% KOH that contained 0.1% NaBH₄ at room temperature for 24 h, followed by similar extraction with 24% KOH that contained 0.1% NaBH₄. The supernatants from the 4% and 24% KOH extractions were collected and thus yielded the hemicellulose fractions. The remaining pellet from the 24% KOH extraction was then lyophilized, weighed, and considered to be the cellulose fraction [36].

Protein extraction and digestion. A portion (2 g) of the roots was homogenized with 1 mL of PBS (pH 7.6) that contained 65 mM K₂HPO₄, 2.6 mM KH₂PO₄, 400 mM NaCl, and 3 mM NaN₃ in a mortar and pestle. The homogenate was centrifuged twice at 15,000 g for 10 min, and the supernatant was collected as the total soluble protein. CWP s were extracted from the root cell wall fraction in two successive steps. The first step used CaCl₂ solution (5 mM sodium acetate buffer, pH 4.6, 0.2 M CaCl₂, and 1 mM PMSF; CaCl₂ can efficiently extract CWP s that exert weak electrostatic interactions with other cell wall components) [37]. The second step involved two extractions with NaCl solution (5 mM sodium acetate buffer, pH 4.6, 1 M NaCl, and 1 mM PMSF; NaCl was also used to extract the strong ionically bound proteins) [34]. For each extraction, the sample was incubated with vortexing at 4°C, and the supernatant was collected after centrifugation at 4,000×g for 15 min. The supernatants from all the extraction steps were pooled and concentrated to 4 mL by centrifugation at 1,500×g at 10°C using a 3 kDa molecular weight cut-off Amicon Spin Tube (Millipore, MA). The protein sample was buffer-exchanged with ultrapure water.

CWP extracts were dissolved in 100 μL of lysis buffer (7 M urea, 2 M thiourea, 5 mM EDTA, 10 mM DTT, and 1 mM PMSF). The supernatant was transferred to a new tube, reduced with 10 mM DTT for 1 h at 56°C, and alkylated with 55 mM iodoacetamide for 45 min at room temperature in darkness. The protein was precipitated with four volumes of pre-chilled acetone for 30 min at −20°C. The pellet was dissolved in 0.5 M TEAB after centrifugation and sonicated for 5 min. The centrifugation step was repeated, and the supernatant was collected. Approximately 100 μg of proteins from each sample was digested with trypsin (Promega) overnight at 37°C in a 1:20 trypsin-to-protein mass ratio.

Analysis of Cu, protein, and cell wall polysaccharide content. Cell wall fraction was dried at 70°C to a constant weight and then digested at 145°C for 24 h in an acid mixture of HNO₃:HClO₃ (3:1, v:v). Cu concentrations were measured by Elmer flame atomic absorption spectrometry (AAS-3600).

Protein contents were determined by the Bradford method [38] with bovine serum albumin as the standard. CWP s were dissolved with 400 μL of SDS sample lysis buffer by boiling for 5 min and then loaded onto a 12% acrylamide mini-gel (5 cm to 8 cm) for 1D SDS-PAGE. The contents of total sugars, galacturonic acids, and KDO were determined colorimetrically by sulfate-phenol [39], hydroxylphenyl [40], and thiobarbituric acid [41], respectively.

Labeling TMT reagents

Peptide was desalted by Strata X C18 SPE column (Phenomenex) and vacuum-dried after trypsin digestion. Peptide was reconstituted in 0.5 M TEAB and processed according to the protocol for 6-plex TMT reagent kits. In brief, two units of TMT reagent (defined as the amount of reagent required to label 100 μg of protein) was thawed and reconstituted in 41 μL of acetonitrile. Peptide samples from 50 μM Cu were treated for 48 h, and CK samples were labeled with TMT tags 126, 128, and 130 as well as TMT tags 127, 129, and 131. Both samples were incubated at room temperature for 2 h. The peptide mixtures were then pooled, desalted, and dried by vacuum centrifugation.

LC–ESI–MS/MS analysis by Q Exactive

The labeled peptide was resuspended in buffer A (2% ACN, 0.1% FA) and centrifuged at 20,000×g for 2 min. The supernatant was transferred into a sample tube and loaded onto an Acclaim PepMap 100 C18 trap column (75 μm×2 cm; Dionex) by EASY nLC1000 nano UPLC (Thermo). The peptide was then eluted onto an Acclaim PepMap RSLC C18 analytical column (50 μm×15 cm; Dionex). An 85 min gradient program was run at 300 nL/min, which started from 3% to 35% B (80% ACN, 0.1% FA), followed by 5 min linear gradient to 90% B, and maintained at 90% B for 5 min.

The peptides were subjected to NSI source, followed by tandem mass spectrometry (MS/MS) in Q Exactive (Thermo) coupled online to the UPLC. Intact peptides were detected in the Orbitrap at a resolution of 70,000. Peptides were selected for MS/MS using 27% NCE with 12% stepped NCE; ion fragments were detected in the Orbitrap at a resolution of 17,500. A data-dependent procedure that alternated between one MS scan followed by 20 MS/MS scans was applied to the top 20 precursor ions above a threshold ion count of 3E4 in the MS survey scan with 5.0 s dynamic exclusion. The applied electrospray voltage was 1.8 kV. Automatic gain control was used to prevent overfilling of the ion trap; 1E5 ions were accumulated for generation of MS/MS spectra. The m/z scan range was 350 Da to 2000 Da for MS scans.

Proteomic database search

The instrument data file (.raw) was merged and transformed to an.mgf file by Proteome Discoverer (ver. 1.3.0.339; Thermo). Peptide and protein identifications were performed using the Mascot search engine (ver. 2.3.02; Matrix Science). Derived protein sequences from plants in the NCBI were collected, and a database containing 1,596,443 sequences was created. In the Mascot search engine version 2.3.02 software, all parameters were set as follows. Database searching was restricted to tryptic peptides. Carbamidomethyl (C), TMT 6-plex (N-term), and TMT 6-plex (K) were selected as fixed, and deamidated (Q), Gln→pyro-Glu (N-term Q), and oxidation (M) were selected as variable modifications, where two missed cleavages were allowed with precursor error tolerance at 10 ppm and fragment deviation at 0.02 Da. The Mascot search results were quantified using Mascot 2.3.02 with the following criteria: protein ratio type = median, minimum unique peptides = 1, peptide threshold type = at least homolog. Peptides were not quantified if peptide score was
too low or the deviation was too large. The final ratios of protein expression were calculated. The complete list of identified peptides was encoded in the UniProt database and quantified. The final ratios of protein expression were obtained in our bioinformatics analysis data.

Bioinformatics studies and statistical analysis

Data were the average of at least three independent experimental replicates. One-way ANOVA (Duncan’s test) and LSD test with $p<0.05$ as the significance level were performed to analyze the differences between the control and treatments. All data were presented as the mean value ± standard error and were analyzed using SPSS statistical software package (version 16.0).

The clustering of content abundance profiles was performed using Cluster 3.0. Euclidean metric was used for computing the distance between points and cluster centroids, which is a typical choice for K-means clustering analysis. The output was visualized using TreeMap after hierarchical clustering.

GO information was used to categorize the biological processes of identified proteins from TMT data sets. GO annotations were extracted from the UniProt database and were matched with corresponding gene locus identifiers embedded in the NCBI RefSeq database. Proteins were then classified based on their biological process using the Web Gene Ontology Annotation Plot (Weggo; http://wego.genomics.org.cn/cgi-bin/wego/index.pl). Functionally analyzed Cu stress-induced CWPs in biosynthesis pathways were identified through the Kyoto Encyclopedia of Genes and Genomes (KEGG) [43]. These pathways were classified into hierarchical categories according to the KEGG website (http://www.genome.jp/kegg/). Subcellular locations were predicted using TargetP (http://www.cbs.dtu.dk/services/TargetP/), Predotar (http://urgi.versailles.inra.fr/predotar/predotar.html), and WoLF PSORT (http://wolfsort.org/) using TargetP and Genomes (KEGG) [43]. These pathways were classified into hierarchical categories according to the KEGG website (http://www.genome.jp/kegg/).

Supporting Information

**Figure S1** SDS–PAGE of root cell wall proteins under different copper stress.

(DOC)

**Table S1** Protein subcellular localization and go annotation of 55 significantly differentially expressed proteins.

(XLS)

**Table S2** Details of 55 significantly differentially expressed proteins identified by TMT using ANOVA including their p–value.

(XLS)

Author Contributions

Conceived and designed the experiments: TTL JYS. Performed the experiments: TTL JYS. Analyzed the data: TTL CKH JYS. Contributed reagents/materials/analysis tools: YW. Wrote the paper: TTL.

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