Excess Copper-Induced Alterations of Protein Profiles and Related Physiological Parameters in Citrus Leaves

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Abstract: This present study examined excess copper (Cu) effects on seedling growth, leaf Cu concentration, gas exchange, and protein profiles identified by a two-dimensional electrophoresis (2-DE) based mass spectrometry (MS) approach after Citrus sinensis and Citrus grandis seedlings were treated for six months with 0.5 (control), 200, 300, or 400 µM CuCl₂. Forty-one and 37 differentially abundant protein (DAP) spots were identified in Cu-treated C. grandis and C. sinensis leaves, respectively, including some novel DAPs that were not reported in leaves and/or roots. Most of these DAPs were identified only in C. grandis or C. sinensis leaves. More DAPs increased in abundances than DAPs decreased in abundances were observed in Cu-treated C. grandis leaves, but the opposite was true in Cu-treated C. sinensis leaves. Over 50% of DAPs were associated with photosynthesis, carbohydrate, and energy metabolism. Cu-toxicity-induced reduction in leaf CO₂ assimilation might be caused by decreased abundances of proteins related to photosynthetic electron transport chain (PETC) and CO₂ assimilation. Cu-effects on PETC were more pronounced in C. sinensis leaves than in C. grandis leaves. DAPs related to antioxidation and detoxification, protein folding and assembly (viz., chaperones and folding catalysts), and signal transduction might be involved in Citrus Cu-toxicity and Cu-tolerance.

Keywords: Citrus grandis; Citrus sinensis; CO₂ assimilation; copper-toxicity; 2-DE; leaves

1. Introduction

Microelement copper (Cu) is highly toxic to plants when in excess. Cu-containing fungicides and bactericides are widely used in agriculture to control fungal and bacterial diseases in crops including Citrus in order to improve crop production and quality. Cu contamination in agriculture soils is on the rise all over the world [1,2]. Cu accumulation in soils can cause Cu-toxicity and related nutritional disorders, resulting in a series of adverse effects on plants ranging from morphological and physiological to molecular levels [1,3]. In old Citrus orchards, the excess accumulation of Cu in soils is a common phenomenon because of the extensive and continued use of Cu-containing agricultural chemicals against fruit and foliage diseases such as anthracnose and canker [3,4]. Cu concentration and availability in soils under continuous Citrus production orchards increase with increasing production
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period [2]. In Citrus, the common Cu-toxic symptoms include leaf iron (Fe) chlorosis, poor growth, and stunted, and discolored root systems [3,5].

Cu, which can act as a cofactor for over 100 proteins including plastocyanin, laccase, cytochrome c oxidase, Cu/zinc (Zn) superoxide dismutase (SOD), ethylene receptors, amino oxidase, polyphenol oxidases, ascorbate (ASC) oxidase, diamine oxidases, and phytocyanin, is involved in photosynthesis, respiration, ATP biosynthesis, ethylene reception, reactive oxygen species (ROS) metabolism, cell wall formation, and carbon, lipid, and nitrogen metabolisms [6]. Accordingly, a lot of researchers have examined the toxic effects of Cu on the uptake of nutrients and water [1,5], growth [1,3], photosynthetic pigment production [7], photosynthetic electron transport [5,8], CO₂ assimilation [8], carbohydrate and nitrogen (N) metabolism [7,9], respiration [10], hormonal status [11], cell wall metabolism [12], phenolic metabolism [13], as well as ROS generation and detoxification [8].

Although Cu-toxic effects on plant growth and physiology have been investigated in some details [2,14], little is known about Cu-toxicity-induced alteration of protein profiles in plants. Proteomics is a powerful approach to elucidate the complicated responses of plants to unfavorable environments [15,16]. Recently, there have been several reports investigating Cu toxicity responsive proteins. Most reports, however, have focused on herbaceous plants, including rice [17–19], Allium cepa [20], Oenothera glazioviana [21], Arabidopsis [22], Cannabis sativa [23], Agrostis capillaris [24], Elsholtzia splendens [25,26], sorghum [27,28] and wheat [29], while only one study investigated Cu-toxic effects on protein profiles in leaves of woody plant Eucalyptus camaldulensis [30]. Also, most of the above studies mainly focused on Cu-toxicity-responsive proteins occurring in roots because Cu is preferentially accumulated in Cu-stressed roots, while only few studies investigated Cu-toxic effects on protein profiles in leaves [25,27,29,30]. Evidence shows that the toxic effects of Cu on plant proteomics vary with Cu concentration, plant species, populations and/or cultivars, and plant tissues [17,18,24,25,27–29]. Therefore, more extensive proteomic research on the leaves of woody plants is needed to elucidate the molecular mechanisms of plants under Cu-toxicity.

Here, a two-dimensional electrophoresis (2-DE) based mass spectrometry (MS) approach was used to examine Cu-toxicity-responsive proteins in Citrus leaves. Meanwhile, we examined excess Cu effects on seedling growth, and leaf Cu concentration and gas exchange. The objectives were (a) to identify Cu toxicity responsive proteins in Citrus leaves and (b) to screen the candidate proteins possibly responsible for Cu tolerance in Citrus.

2. Results

2.1. Excess Cu-Effects on Seedling Growth, Leaf Cu and Gas Exchange

As shown in Supplementary Figures S1 and S2, C. sinensis (C. grandis) biomass remained little changed as Cu concentration in the nutrient solution elevated from 0.5 to 300 (200) μM, then declined at 400 (300–400) μM Cu. Biomass was lower in C. sinensis seedlings than that in C. grandis seedlings at each given Cu supply.

Leaf Cu concentration increased with Cu supply and did not differ between the two Citrus species with the exception that its concentration in leaves was higher in C. sinensis than that in C. grandis at each given Cu supply.

Leaf Cu concentration increased with Cu supply and did not differ between the two Citrus species with the exception that its concentration in leaves was higher in C. sinensis than that in C. grandis at each given Cu supply (Figure 1A).

Leaf CO₂ concentration and stomatal conductance (gₛ) kept unchanged or increased as Cu concentration in the nutrient solution rose from 0.5 to 200 μM, then declined with further rise in Cu concentration. Cu supply had little influence on the ratio of intercellular to ambient CO₂ concentration (Cᵢ/Cₐ) except for that Cᵢ/Cₐ in C. grandis leaves was slightly higher at 200 μM Cu than that at 300–400 μM Cu. No significant differences were observed in the three parameters between the two Citrus species over the range of Cu supply (Figure 1B–D).

Based on these results, seedlings that received 300–400 μM Cu were regarded as Cu excess.
2.2. Protein Yield and Cu-responsive Proteins in Leaves

Three biological replicates were performed in order to obtain reliable data. No significant differences were observed in protein yields and the number of protein spots per gel among eight means (Table 1, Figure 2, Supplementary Figures S3 and S4).

As shown in Tables 1 and 2, Supplementary Table S1 and Figure S5, a total of 42 and 45 differentially abundant protein (DAP) spots were obtained from Cu-treated C. grandis and C. sinensis leaves, respectively. All of these DAP spots were submitted to matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry (MALDI-TOF/TOF-MS) based identification. In total, 41 and 37 DAP spots were identified in 200, 300, and/or 400 µM Cu-treated C. sinensis and C. grandis leaves, respectively. Most of these DAP spots only presented in Cu-treated C. sinensis or C. grandis leaves. Only seven DAPs with the same accession number [viz., Orange1.1t05091.1, S-adenosyl-L-homocysteine (AdoHcy) hydrolase (Orange1.1t01892.1), chaperonin CPN60-1, (Orange1.1t01459.2), major allergen Pru ar 1 (Cs9g03630.1), ribulose bisphosphate carboxylase/oxygenase (Rubisco) activase 1 (Cs7g31800.3), sedoheptulose-1,7-bisphosphatase (Cs7g31640.4), and 29 kDa ribonucleoprotein A (CP29A; Cs6g11900.1)] presented in the two Citrus species. Fifteen, 12 and 12 (2, 4, and 5) spots increased in abundances and 4, 2, and 9 (9, 12, and 23) spots decreased (including disappeared) in abundances were identified in 200, 300, or 400 µM Cu-treated C. grandis or C. sinensis leaves. For C. grandis, 10, 6, or 14 DAP spots were identified only in 200, 300 or 400 µM Cu-treated leaves, respectively, only 2 DAP spots with the same accession number (viz., malate dehydrogenase (MDH, Cs9g10470.1) and glutathione S-transferase (GST, Cs5g32800.1)) were shared by the three. For C. sinensis, 3, 3, or 15 DAP spots were identified only in 200, 300, or 400 µM Cu-treated leaves, respectively, only 2 DAP spots with the same accession number (viz., ferritin-3 (Cs6g09150.2) and enolase (Cs6g15540.1)) were shared by the three.
Figure 2. Representative 2-DE images of proteins extracted from 0.5 (A,E), 200 (B,F) 300 (C,G) and 400 (D,H) Cu-treated *Citrus grandis* (A–D) and *Citrus sinensis* (E–H) leaves.
Table 1. Protein yield, number of differentially abundant protein (DAP) spots and number of identified DAP spots in 0.5 (control), 200 (Cu200), 300 (Cu300) or 400 (Cu400) Cu-treated *Citrus grandis* and *C. sinensis* leaves.

|                      | Citrus grandis | Citrus sinensis |
|----------------------|----------------|-----------------|
|                      | Control | Cu200 | Cu300 | Cu400 | Total | Control | Cu200 | Cu300 | Cu400 | Total |
| Protein yield (mg g\(^{-1}\)DW) | 49.4 ± 5.1a | 45.3 ± 0.7a | 47.6 ± 1.2a | 44.7 ± 8.3a | 44.4 ± 5.0a | 43.8 ± 5.4a | 40.9 ± 5.4a | 35.6 ± 3.1a |
| Number of spots per gel | 613 ± 4a | 627 ± 8a | 621 ± 12a | 621 ± 22a | 614 ± 7a | 625 ± 15a | 617 ± 12a | 618 ± 9a |
| Number of DAP spots | | | | | | | | |
| Increased in abundances | 15 | 12 | 13 | 2 | 4 | 5 |
| Decreased in abundances | 4 | 2 | 7 | 8 | 12 | 18 |
| Disappeared | 2 | 2 | 1 | 13 |
| Total | 19 | 14 | 22 | 42 | 12 | 17 | 36 | 45 |
| Number of identified DAP spots | | | | | | | | |
| Increased in abundances | 15 | 12 | 12 | 2 | 4 | 5 |
| Decreased in abundances | 4 | 2 | 7 | 7 | 11 | 12 |
| Disappeared | 2 | 1 | 11 |
| Total | 19 | 14 | 21 | 41 | 11 | 16 | 28 | 37 |

Note: Means (±SE, n = 3) with a row followed by the same letter are not significant different at p < 0.05.
### Table 2. Differentially abundant protein (DAP) spots and their identification by MALDI-TOF/TOF-MS in 0.5 (control), 200 (Cu200), 300 (Cu300) or 400 (Cu400) Cu-treated Citrus grandis and C. sinensis leaves.

| Spot No. | Protein Identity | Accession No | Mr(kDa)/PI Exp. | Mr(kDa)/PI Theor. | Protein Score | Peptide Ions | NMP | Cu5 | Cu200 | Cu300 | Cu400 | CS (%) | Charge |
|----------|------------------|--------------|-----------------|------------------|---------------|-------------|-----|-----|-------|-------|-------|--------|--------|
| G1       | 29 kDa ribonuclease A, chloroplastic | Cs6g11930.1 | 30.37/5.17 | 36.20/4.33 | 358 | 186 | 9 | 1.00 ± 0.11c | 1.68 ± 0.19ab | 1.20 ± 0.14bc | 2.24 ± 0.26a | 8 | 1 |
| G20      | Photosystem II stability/assembly factor HCF136, chloroplast, putative | Cs7g13970.1 | 45.06/8.46 | 48.11/5.91 | 902 | 42 | 22 | 1.00 ± 0.11b | 1.19 ± 0.20d | 1.17 ± 0.13b | 1.91 ± 0.12a | 19 | 1 |
| G31      | Photosystem II stability/assembly factor HCF136, chloroplast, putative | Cs7g13970.1 | 53.11/5.75 | 59.08/5.94 | 684 | 172 | 28 | 1.00 ± 0.23b | 1.25 ± 0.25b | 2.40 ± 0.19a | 1.58 ± 0.21b | 24 | 1 |
| G30      | Ferredoxin—NADP reductase, leaf-type isozyme, chloroplastic | Cs1g25510.4 | 55.49/5.09 | 62.32/4.92 | 74 | 47 | 14 | 1.00 ± 0.11a | 0.53 ± 0.08b | 0.69 ± 0.10ab | 0.36 ± 0.16b | 12 | 1 |
| G42      | Ferredoxin—NADP reductase, leaf-type isozyme, chloroplastic | Cs1g25510.4 | 40.48/6.68 | 35.76/6.62 | 458 | 122 | 26 | 1.00 ± 0.23b | 3.66 ± 0.24a | 1.64 ± 0.10b | 1.54 ± 0.32b | 23 | 1 |
| G8       | RrafusCO subunit binding protein alpha subunit, chloroplast, putative, expressed; Chaperonin 60 subunit alpha 1, chloroplastic | Cs8g16040.3 | 61.50/5.23 | 68.61/4.72 | 1350 | 169 | 47 | 1.00 ± 0.22b | 0.31 ± 0.06c | 1.86 ± 0.24a | 1.06 ± 0.13b | 41 | 1 |
| G9       | Ribulose bisphosphate carboxylase/oxygenase activase 1, chloroplastic | Cs7g31803.1 | 47.86/6.29 | 53.65/5.10 | 617 | 119 | 17 | 1.00 ± 0.20ab | 1.38 ± 0.05a | 0.80 ± 0.08bc | 0.48 ± 0.08c | 24 | 1 |
| G6       | Sedoheptulose-1,7-bisphosphatase, chloroplastic | Cs6g16144.0 | 42.40/5.82 | 48.51/4.64 | 489 | 84 | 29 | 1.00 ± 0.22b | 1.66 ± 0.20a | 1.09 ± 0.18ab | 1.32 ± 0.10b | 25 | 1 |
| G28      | Glycolaldehyde-3-phosphate dehydrogenase B, chloroplastic | Cs3g27520.2 | 48.00/7.10 | 52.45/6.38 | 515 | 104 | 30 | 1.00 ± 0.27b | 2.43 ± 0.31a | 1.42 ± 0.08b | 1.34 ± 0.14b | 26 | 1 |
| G29      | Triosephosphate isomerase, cytosolic (Fragment) | Cs5g16459.1 | 26.96/5.73 | 32.66/6.15 | 326 | 129 | 7 | 1.00 ± 0.04b | 1.58 ± 0.36a | 0.63 ± 0.12b | 0.18 ± 0.10c | 6 | 1 |
| G35      | Triosephosphate isomerase, cytosolic | Cs5g32500.1 | 26.96/5.73 | 31.99/6.16 | 236 | 125 | 14 | 1.00 ± 0.34a | 0.69 ± 0.14a | 0.51 ± 0.14a | 0 | 11 | 1 |
| G41      | Triosephosphate isomerase, cytosolic | Cs8g18560.2 | 27.24/5.75 | 33.66/6.67 | 428 | 102 | 17 | 1.00 ± 0.14b | 1.97 ± 0.27a | 1.49 ± 0.05ab | 1.16 ± 0.19b | 15 | 1 |
| G4       | Probable 6-phosphoglucuronolactone 4, chloroplastic | Orange1g1805242.1 | 35.38/6.24 | 34.54/4.72 | 1050 | 179 | 25 | 1.00 ± 0.10b | 2.05 ± 0.35a | 2.04 ± 0.26a | 1.30 ± 0.20b | 20 | 1 |
| G26      | Fructose-1,6-biphosphatase, cytosolic | Cs5g21280.1 | 57.65/5.95 | 47.35/6.37 | 408 | 80 | 22 | 1.00 ± 0.42b | 4.05 ± 1.70a | 2.31 ± 0.11ab | 1.63 ± 0.16b | 19 | 1 |
| G17      | Malate dehydrogenase [NADP], chloroplastic | Cs5g10470.1 | 55.54/6.10 | 48.01/6.44 | 212 | 83 | 15 | 1.00 ± 0.28b | 1.66 ± 0.13a | 1.01 ± 0.01b | 0.82 ± 0.15b | 13 | 1 |
| G27      | Malate dehydrogenase, cytoplasmic | Cs5g10470.1 | 42.35/5.94 | 56.49/6.33 | 193 | 144 | 11 | 1.00 ± 0.21b | 2.76 ± 0.46a | 2.11 ± 0.20a | 2.35 ± 0.48b | 10 | 1 |
| G18      | ATP synthase subunit beta, mitochondrial | Cs2g13550.1 | 58.85/6.06 | 61.56/6.45 | 1240 | 203 | 21 | 1.00 ± 0.11bc | 0.73 ± 0.20c | 1.66 ± 0.12a | 1.40 ± 0.11b | 18 | 1 |
| G23      | ATP synthase gamma chain, chloroplastic | Cs2g20880.1 | 40.62/5.08 | 45.19/5.67 | 394 | 115 | 25 | 1.00 ± 0.08bc | 0.64 ± 0.20b | 1.65 ± 0.09a | 1.38 ± 0.04b | 22 | 1 |
| G26      | Bus5'-adenylyltransferase II, mitochondrial | Cs1g13060.1 | 17.37/5.94 | 18.09/5.99 | 216 | 105 | 7 | 1.00 ± 0.17a | 1.03 ± 0.37b | 1.47 ± 0.09ab | 1.86 ± 0.14a | 14 | 1 |
| G24      | Glucose-1-phosphate adenylyltransferase small subunit 2, chloroplastic | Cs2g18880.1 | 57.08/6.74 | 58.03/5.66 | 1120 | 121 | 45 | 1.00 ± 0.12b | 1.97 ± 0.42a | 2.24 ± 0.27a | 1.64 ± 0.31b | 39 | 1 |
| G25      | Glucose-1-phosphate adenylyltransferase small subunit 2, chloroplastic | Cs2g18880.1 | 65.86/8.50 | 68.69/5.67 | 751 | 139 | 37 | 1.00 ± 0.14b | 2.11 ± 0.43a | 1.09 ± 0.08b | 1.36 ± 0.19ab | 32 | 1 |
| Spot No. | Protein Identity                                      | Accession No       | MrkDal/PI Exp. | MrkDal/PI Theor. | Protein Score | Peptide Ions | NMP | Ratio   | CS (%) | Charge   |
|--------|--------------------------------------------------------|-------------------|----------------|-----------------|---------------|--------------|-----|---------|--------|----------|
| G40    | Glutathione-S-transferase                              | Cs5g32800.1       | 23.83/6.17     | 30.07/6.75      | 389           | 100          | 20  | 1.00 ± 0.13 | 78.9 ± 28.10a | 93.20 ± 15.40a | 47.10 ± 8.39a | 17  | 1       |
| G34    | Glutathione-S-transferase DHARK1, mitochondrial        | Cs7g28340.4       | 23.85/6.18     | 30.34/6.46      | 544           | 127          | 21  | 1.00 ± 0.41a| 0.57 ± 0.33a  | 0.26 ± 0.06a  | 0    | 18      |
| G21    | Copper/zinc superoxide dismutase (Fragment)           | Cs3g12000.1       | 15.09/5.47     | 19.86/5.80      | 83            | 46           | 7   | 1.00 ± 0.07b| 1.03 ± 0.41b  | 1.18 ± 0.16b  | 2.43 ± 0.51a | 12  | 1       |
| G33    | Manganese superoxide dismutase (Fragment)             | Cs7g29850.1       | 25.29/6.79     | 28.64/6.34      | 520           | 107          | 19  | 1.00 ± 0.08b| 1.51 ± 0.15ab | 1.47 ± 0.16b  | 2.02 ± 0.47a | 17  | 1       |
| G16    | Quinone oxidoreductase-like protein At1g23740, chloroplastic | Cs7g08640.2       | 41.88/8.77     | 48.90/5.38      | 728           | 189          | 27  | 1.00 ± 0.12a| 1.17 ± 0.08a  | 1.01 ± 0.04a  | 0.66 ± 0.03b | 23  | 1       |
| G15    | Probable protein disulfide-isomerase A6                | Cs5g33860.2       | 41.75/6.91     | 44.67/5.58      | 522           | 130          | 28  | 1.00 ± 0.11b| 1.45 ± 0.18ab | 1.49 ± 0.03ab | 1.60 ± 0.21a | 24  | 1       |
| G12    | 20 kDa chaperonin, chloroplastic                       | Cs4g07030.2       | 26.59/8.89     | 30.93/5.32      | 874           | 186          | 27  | 1.00 ± 0.18b| 0.69 ± 0.13b  | 1.66 ± 0.08a  | 0.73 ± 0.19ab| 23  | 1       |
| G11    | Heat shock cognate 70 kDa protein 2                   | Cs7g29030.1       | 70.99/5.09     | 74.6/4.89       | 794           | 113          | 5   | 1.00 ± 0.18a| 0.81 ± 0.12ab | 0.52 ± 0.12b  | 0.56 ± 0.04b | 4    | 1       |
| G19    | Chaperonin CPN60-1, mitochondrial, putative, expressed  | Orango1.181459.2   | 61.73/5.85     | 68.57/5.40      | 632           | 60           | 46  | 1.00 ± 0.23b| 0.46 ± 0.04c  | 1.76 ± 0.06a  | 0.81 ± 0.22bc| 40  | 1       |
| G3     | Calreticulin-1                                         | Cs3g15060.3       | 52.52/6.29     | 62.84/4.12      | 122           | 99           | 11  | 1.00 ± 0.33a| 0.72 ± 0.17ab | 0.54 ± 0.08ab | 0.19 ± 0.02b | 15  | 1       |
| G27    | Major allergen Pru ar 1 (Major pollen allergen Bet v 1-DH4, Major pollen allergen Bet v 1-A) | Cs9g03660.1       | 17.60/5.67     | 21.51/6.05      | 253           | 104          | 19  | 1.00 ± 0.09c| 3.02 ± 0.42a  | 1.66 ± 0.28bc | 2.17 ± 0.49ab| 17  | 1       |
| G8     | 14-3-3 protein 7 (14-3-3-like protein CF14 epsilon)    | Cs6g18200.2       | 28.86/4.92     | 37.48/4.61      | 670           | 174          | 23  | 1.00 ± 0.20b| 1.36 ± 0.08ab | 1.39 ± 0.13ab | 1.83 ± 0.16a | 21  | 1       |
| G22    | Annexin D1                                            | Cs3g18360.1       | 35.88/5.17     | 46.63/5.66      | 784           | 138          | 31  | 1.00 ± 0.72c| 3.18 ± 2.11ab | 4.77 ± 0.99a  | 2.03 ± 0.43b | 27  | 1       |
| G28    | Ferritin-2, chloroplastic                              | Cs7g03600.1       | 29.47/5.41     | 32.63/5.90      | 499           | 155          | 27  | 1.00 ± 0.12a| 0.55 ± 0.12b  | 0.88 ± 0.05ab | 1.21 ± 0.16a | 23  | 1       |
| G7     | Tubulin beta-6 chain                                   | Cs3g26180.1       | 50.38/4.75     | 62.28/4.68      | 773           | 162          | 30  | 1.00 ± 0.10b| 3.48 ± 0.55a  | 1.59 ± 0.09b  | 1.23 ± 0.39b | 26  | 1       |
| G2     | Endochitinase 1                                        | Cs6g01850.1       | 35.39/4.85     | 52.52/4.16      | 75            | 33           | 9   | 1.00 ± 0.16a| 0.55 ± 0.27ab | 0.44 ± 0.05b  | 0.75 ± 0.09ab| 8    | 1       |
| G14    | Abscisic stress-ripening protein 1-like               | Cs5g21500.1       | 17.88/6.00     | 30.30/5.56      | 323           | 123          | 8   | 1.00 ± 0.35b| 1.75 ± 0.48b  | 1.97 ± 0.10b  | 5.75 ± 0.59b | 29  | 1       |
| G20    | Orango1.181590.1                                       | 157.30/6.83       | 19.28/5.70     | 161             | 12            | 31          | 1.00 ± 0.16c| 1.38 ± 0.32bc | 1.80 ± 0.26ab | 2.31 ± 0.19a | 30  | 1       |
| G32    | S-adenosyl-L-homocysteine hydrolase (adensylhomocysteinase) | Orango1.181892.1   | 80.71/6.26     | 77.69/6.12      | 577           | 97           | 35  | 1.00 ± 0.11b| 2.72 ± 0.72a  | 0.87 ± 0.09b  | 0.79 ± 0.10b | 3   | 1       |
| G13    | Receptor serine-threonine protein kinase, putative     | Cs9g04750.2       | 25.57/8.87     | 23.24/5.56      | 45            | 109          | 13  | 1.00 ± 0.15b| 1.06 ± 0.28b  | 1.13 ± 0.25b  | 2.12 ± 0.35a | 22  | 1       |
### Table 2. Cont.

| Spot No. | Protein Identity | Accession No | Mr(kDa) | PI | Peptide | NMP | Ratio | CS (%) | Charge |
|----------|------------------|--------------|---------|----|---------|-----|-------|--------|--------|
| S2       | 29 kDa ribonucleoprotein A, chloroplastic; Ribonucleoprotein At2g37220, chloroplastic | Cs6g11900.1 | 30.37/5.17 | 45/18.21 | 418 | 195 | 13 | 1.00 ± 0.08a | 0.82 ± 0.16ab | 0.43 ± 0.09c | 0.53 ± 0.06bc | 11 | 1 |
| S3       | 29 kDa ribonucleoprotein A, chloroplastic; Ribonucleoprotein At2g37220, chloroplastic | Cs7g01430.1 | 28.53/7.78 | 33.75/11.11 | 392 | 114 | 19 | 1.00 ± 0.20a | 0.76 ± 0.02ab | 0.49 ± 0.14b | 0 | 23 | 1 |
| S17      | Oxygen-evolving enhancer protein 1, chloroplastic | Cs1g23450.1 | 35.38/5.83 | 24.74/5.56 | 261 | 116 | 7 | 1.00 ± 0.05c | 1.06 ± 0.03bc | 1.34 ± 0.10ab | 1.54 ± 0.14a | 19 | 1 |
| S3       | Carbomyl carboxylase, chloroplastic | Cs2g28090.1 | 36.77/6.66 | 53.09/8.25 | 171 | 162 | 5 | 1.00 ± 0.26c | 3.02 ± 0.38ab | 3.14 ± 0.23a | 1.18 ± 0.35bc | 5 | 1 |
| S11      | Rubisco subunit binding protein alpha subunit, chloroplast, putative, expressed; Chaperonin 60 subunit alpha 1, chloroplastic | Cs8g16400.1 | 61.50/5.23 | 99.02/5.94 | 1250 | 182 | 39 | 1.00 ± 0.1a | 0.70 ± 0.16a | 0.80 ± 0.17a | 0.27 ± 0.04b | 34 | 1 |
| S9       | Ribulose biphosphate carboxylase/oxygenase activase 1, chloroplastic | Cs7g31800.3 | 50.90/5.33 | 81.78/6.10 | 505 | 107 | 21 | 1.00 ± 0.23a | 0.75 ± 0.10ab | 0.66 ± 0.10ab | 0.51 ± 0.04b | 28 | 1 |
| S14      | Ribulose biphosphate carboxylase/oxygenase activase 1, chloroplast | Cs7g31800.3 | 46.96/5.94 | 75.75/6.55 | 505 | 107 | 21 | 1.00 ± 0.11a | 0.65 ± 0.02b | 0.48 ± 0.05b | 0.45 ± 0.05b | 28 | 1 |
| S4       | Ribulose biphosphate carboxylase/oxygenase activase 1, chloroplastic | Cs7g31800.3 | 46.96/5.94 | 83.26/6.21 | 597 | 139 | 19 | 1.00 ± 0.46a | 0 | 0.38 ± 0.03a | 0.77 ± 0.06a | 17 | 1 |
| S10      | Ribulose biphosphate carboxylase/oxygenase activase 1, chloroplastic | Cs7g31800.3 | 46.96/5.94 | 79.78/6.02 | 641 | 175 | 24 | 1.00 ± 0.16b | 1.38 ± 0.30b | 2.16 ± 0.23a | 1.05 ± 0.04b | 21 | 1 |
| S21      | Phosphotriboilokinase, chloroplastic | Cs3g08480.1 | 45.19/5.97 | 67.41/5.56 | 686 | 137 | 31 | 1.00 ± 0.05a | 1.01 ± 0.04a | 1.02 ± 0.15b | 0 | 27 | 1 |
| S33      | Sedoheptulose-1,7-biphosphatase, chloroplastic | Cs7g31640.4 | 36.77/6.66 | 43.56/4.96 | 576 | 101 | 27 | 1.00 ± 0.06a | 1.02 ± 0.06a | 0.75 ± 0.24a | 0 | 23 | 1 |
| S44      | Malate dehydrogenase, mitochondrial | Cs7g25390.1 | 35.40/6.52 | 66.81/4.32 | 613 | 144 | 24 | 1.00 ± 0.02b | 1.18 ± 0.21ab | 0.93 ± 0.15b | 1.64 ± 0.26a | 21 | 1 |
| S45      | Malate dehydrogenase, mitochondrial | Cs7g25390.3 | 37.65/5.95 | 63.69/5.53 | 287 | 171 | 9 | 1.00 ± 0.16bc | 1.15 ± 0.2b | 0.46 ± 0.12c | 1.85 ± 0.22a | 8 | 1 |
| S30      | Enolase | C6g15340.1 | 15.09/5.47 | 18.14/4.99 | 928 | 154 | 30 | 1.00 ± 0.12a | 0.53 ± 0.07b | 0.63 ± 0.09b | 0.22 ± 0.08c | 26 | 1 |
| S36      | Enolase | C6g15340.1 | 47.79/5.54 | 89.59/5.04 | 531 | 136 | 15 | 1.00 ± 0.25b | 1.04 ± 0.22b | 1.79 ± 1.3a | 1.92 ± 0.11a | 47 | 1 |
| S43      | Dihydroxyproline-residue succinyltransferase component of 2-oxoglutamate dehydrogenase complex 2, mitochondrial | Cs2g21190.3 | 40.39/6.95 | 87.84/4.79 | 268 | 144 | 8 | 1.00 ± 0.26a | 0.56 ± 0.20ab | 0.30 ± 0.06b | 0 | 25 | 1 |
| **Antioxidant and Detoxification** | | | | | | | | | | | | |
| S1       | 2-Cys peroxiredoxin BAS1, chloroplastic | C6g313880.1 | 29.49/7.65 | 30.56/5.65 | 465 | 161 | 17 | 1.00 ± 0.1a | 0.89 ± 0.11a | 0 | 0.81 ± 0.19a | 15 | 1 |
| S20      | Cysteine synthase, chloroplastic/chloroplastic | Orange1.1829144.1 | 41.35/8.29 | 56.87/5.55 | 880 | 197 | 28 | 1.00 ± 0.14a | 0 | 0.91 ± 0.09a | 1.17 ± 0.06a | 21 | 1 |
| S39      | Cysteine synthase | C9g06970.1 | 29.29/6.78 | 60.56/4.69 | 114 | 49 | 6 | 1.00 ± 0.26a | 1.04 ± 0.12a | 0.79 ± 0.15a | 0 | 17 | 1 |
| S34      | L-ascorbate peroxidase 1, cytosolic | Cs8g17370.1 | 28.68/4.52 | 46.45/5.05 | 392 | 114 | 19 | 1.00 ± 0.05a | 0.82 ± 0.18a | 0.77 ± 0.14a | 0.35 ± 0.01b | 23 | 1 |
| S24      | Glutathione peroxidase (Fragment) | Cs5g07830.1 | 18.58/5.72 | 25.08/5.15 | 646 | 135 | 21 | 1.00 ± 0.14a | 1.02 ± 0.07a | 0.88 ± 0.16ab | 0.55 ± 0.12b | 30 | 1 |
### Table 2. Cont.

| Spot No. | Protein Identity | Accession No. | MrkDa(PI) Theor. | MrkDa(PI) Exp. | Peptide Score | Peptide Ions | NMP | Ratio Cu0.5 | Ratio Cu200 | Ratio Cu300 | Ratio Cu400 | CS (%) | Charge |
|----------|------------------|---------------|-----------------|---------------|--------------|--------------|-----|-----------|-----------|-----------|-----------|--------|--------|
| **Chaperones and Folding Catalysts** | | | | | | | | | | | | | |
| S16 | Luminal-binding protein 5 | Cs5g01840.2 | 73.56/5.09 | 108.09/5.72 | 578 | 112 | 27 | 1.00 ± 0.18b | 1.07 ± 0.21b | 2.57 ± 0.19a | 2.39 ± 0.23a | 23 | 1 |
| S8 | Peptidyl-prolyl-cis-trans isomerase CYP37, chloroplastic | Cs1g07611.1 | 50.39/6.42 | 58.95/5.92 | 109 | 92 | 5 | 1.00 ± 0.13a | 1.03 ± 0.07a | 0.73 ± 0.05ab | 0.66 ± 0.12b | 2 | 1 |
| S26 | Chaperonin CFN60-1, mitochondrial, putative, expressed | Orange1.101459.2 | 46.12/8.24 | 52.38/5.28 | 727 | 152 | 39 | 1.00 ± 0.13a | 0.90 ± 0.14a | 1.16 ± 0.12a | 0 | 34 | 1 |
| **Signal Transduction** | | | | | | | | | | | | | |
| S35 | Major allergen Pru a 1 (Major pollen allergen Bet v 1-D/H, Major pollen allergen Bet v 1-A) | Cs9g02630.1 | 48.33/6.19 | 89.59/5.09 | 230 | 94 | 15 | 1.00 ± 0.07a | 0.57 ± 0.15b | 1.20 ± 0.13a | 0.91 ± 0.05ab | 13 | 1 |
| S7 | 14-3-3 protein 6 | Orange1.101399.1 | 29.44/4.84 | 45.11/6.09 | 439 | 137 | 18 | 1.00 ± 0.18a | 0.28 ± 0.02b | 0.51 ± 0.13ab | 0.54 ± 0.04ab | 16 | 1 |
| **Cellular Transport** | | | | | | | | | | | | | |
| S5 | Ferritin-3, chloroplastic | Cs6g09150.2 | 28.97/5.46 | 41.03/5.97 | 406 | 129 | 13 | 1.00 ± 0.03a | 0.65 ± 0.03b | 0.61 ± 0.12b | 0.46 ± 0.03b | 33 | 1 |
| **Nucleic acid Metabolism** | | | | | | | | | | | | | |
| S42 | RuvB-like helicase 1 | Cs6g16920.1 | 38.08/6.90 | 90.61/4.73 | 268 | 99 | 16 | 1.00 ± 0.17a | 0.82 ± 0.33a | 0.56 ± 0.08a | 0 | 25 | 1 |
| **Others** | | | | | | | | | | | | | |
| S27 | Orange1.105091.1 | 53.64/5.26 | 85.72/5.32 | 246 | 73 | 19 | 1.00 ± 0.38a | 0.15 ± 0.01b | 0.23 ± 0.04b | 0.44 ± 0.16ab | 17 | 1 |
| S28 | Orange1.105091.1 | 61.73/5.85 | 95.17/5.28 | 212 | 70 | 16 | 1.00 ± 0.23a | 0.91 ± 0.15a | 0.32 ± 0.05b | 0.60 ± 0.07ab | 14 | 1 |
| S31 | Orange1.105091.1 | 177.77/7.13 | 24.86/4.86 | 170 | 45 | 15 | 1.00 ± 0.07a | 0.75 ± 0.15ab | 0.53 ± 0.12b | 0 | 14 | 1 |
| S23 | Anthranilate N-methyltransferase | Cs5g24940.1 | 39.48/5.20 | 23.56/5.23 | 300 | 146 | 17 | 1.00 ± 0.03a | 0.93 ± 0.02a | 0.27 ± 0.10b | 0 | 15 | 1 |
| S37 | S-adenosyl-L-homocysteine hydrolase (adenosylhomocysteine) | Orange1.101892.1 | 17.60/4.67 | 26.91/4.83 | 776 | 172 | 24 | 1.00 ± 0.12a | 1.18 ± 0.07a | 0.73 ± 0.24a | 0 | 21 | 1 |
| S38 | 14-3-3 protein 6 | Orange1.101399.1 | 29.44/4.84 | 45.11/6.09 | 439 | 137 | 18 | 1.00 ± 0.18a | 0.28 ± 0.02b | 0.51 ± 0.13ab | 0.54 ± 0.04ab | 16 | 1 |
| **Unidentified Protein Spots** | | | | | | | | | | | | | |
| S6 | Light-harvesting chlorophyll-a/b-binding protein Lhca6 (Fragment) | Cs7g27930.1 | 26.56/5.43 | 35.60/5.91 | 105 | 50 | 4 | 1.00 ± 0.24a | 0.87 ± 0.06ab | 0.71 ± 0.09ab | 0.49 ± 0.02b | 31 | 1 |
| S12 | Thioredoxin M-type, chloroplastic | Cs3g02063.1 | 19.91/8.83 | 16.79/5.85 | 60 | 43 | 4 | 1.00 ± 0.18a | 0.95 ± 0.13a | 0.80 ± 0.08ab | 0.40 ± 0.17b | 7 | 1 |
| S15 | Nicotinate-nucleotide pyrophosphorylase [carboxylating], putative | Orange1.1014780.1 | 55.48/5.09 | 91.64/5.82 | 50 | 131 | 26 | 1.00 ± 0.26a | 0.79 ± 0.18ab | 0.59 ± 0.04ab | 0.33 ± 0.02b | 58 | 1 |
| S18 | Disease resistance protein RFL1, putative | Cs3g08210.1 | 49.77/9.44 | 31.35/5.42 | 50 | 18 | 10 | 1.00 ± 0.21a | 0.39 ± 0.13b | 0.33 ± 0.04b | 0.19 ± 0.1b | 17 | 1 |
| S22 | Dehydration-responsive family protein, putative, expressed | Orange1.1083088.3 | 49.93/5.04 | 88.43/5.57 | 57 | 117 | 24 | 1.00 ± 0.19a | 0.94 ± 0.06ab | 0.87 ± 0.02ab | 0.65 ± 0.07b | 57 | 1 |
| S25 | Transducin/WD40 domain-containing protein-like protein | Cs9g09840.1 | 29.76/6.18 | 31.98/5.16 | 67 | 41 | 9 | 1.00 ± 0.14a | 0.83 ± 0.15a | 0.51 ± 0.12a | 0 | 8 | 1 |
| S29 | ATPase 8, plasma membrane-type | Cs4g03730.1 | 14.72/5.41 | 22.10/5.07 | 68 | 98 | 11 | 1.00 ± 0.09a | 0.90 ± 0.07ab | 0.72 ± 0.09bc | 0.50 ± 0.09c | 21 | 1 |
| S40 | 4-hydroxy-3-methylbut-2-enyl diphosphate reductase | Cs5g28200.1 | 52.22/6.00 | 81.69/4.72 | 63 | 49 | 5 | 1.00 ± 0.16a | 0.68 ± 0.06a | 0.66 ± 0.14a | 0 | 17 | 1 |

Note: Spot number corresponds to the 2-DE images in Figure 2. Ratio means the ratio of 0.5, 200, 300 or 400 μM Cu-treated leaves to 0.5 μM Cu-treated leaves. NMP: the number of matched peptides; CS: covered sequence. Means (±SE, n = 3) with a row followed by different letters are significantly different at p < 0.05.
DAPs were mainly involved in photosynthesis, carbohydrate and energy metabolism, antioxidation and detoxification, protein folding and assembly (viz., chaperones and folding catalysts), and others. Cell wall, cytoskeleton (G7 and G2), and stress response (G14) related DAPs were obtained only in Cu-treated *C. grandis* leaves, but nucleic acid metabolism related DAP (S42) was identified only in Cu-treated *C. sinensis* leaves (Table 2 and Figure 3).

**Figure 3.** Differentially abundant proteins (DAPs) in 200, 300 and 400 μM Cu-treated *Citrus grandis* (A–C) and *Citrus sinensis* (D–F) leaves. PHE: photosynthesis, carbohydrate and energy metabolism.

### 2.3. KEGG Pathway Analysis of DAPs

For total DAPs in *C. grandis* leaves, there were eight significantly enriched KEGG pathways—namely carbon fixation in photosynthetic organisms (ko00710), exosome (ko04147), glycolysis/gluconeogenesis (ko00010), fructose and mannose metabolism (ko00051), photosynthesis (ko01951), chaperones and folding catalysts (ko03110), photosynthesis proteins (ko01941) and inositol phosphate metabolism (ko00562). Four, six, and ten KEGG pathways were significantly enriched by DAPs in 200, 300, and 400 μM Cu-treated *C. grandis* leaves, respectively. For total DAPs in *C. sinensis* leaves, carbon fixation in photosynthetic organisms, photosynthesis proteins, exosome, tricarboxylic acid (TCA) cycle (ko00020) and photosynthesis were the significantly enriched KEGG pathways. One [photosynthesis-antenna proteins (ko00196)], one (exosome) and five KEGG pathways were significantly enriched by DAPs in 200, 300 and 400 μM Cu-treated *C. sinensis* leaves, respectively (Supplementary Figure S6).
2.4. PCA Loading Plots and Correlation Matrices of DAPs

As shown in Figure 4 and Supplementary Tables S2 and S3, PC1 and PC2 accounted for 30.5% and 26.5%, and 45.8% and 17.4% of the total variation in *C. grandis* and *C. sinensis* leaves, respectively. The association patterns of DAPs were more obvious in *C. sinensis* leaves than those in *C. grandis* leaves. Similarly, more positive and negative relationships between DAP spots existed in *C. sinensis* leaves than those in *C. grandis* leaves (Supplementary Figure S7).

![Figure 4](https://via.placeholder.com/150)

**Figure 4.** Principal component analysis (PCA) of differentially abundant proteins (DAPs) in Cu-treated *Citrus grandis* (**A**) and *Citrus sinensis* (**B**) leaves. PHE: photosynthesis, carbohydrate and energy metabolism.

2.5. qRT-PCR Analysis of Genes for DAPs

The expression levels of genes for 22 DAPs from 400 μM Cu-treated *C. grandis* (viz., G3, G9, G10, G11, G14, G26, G29, G33, G34, and G35) and *C. sinensis* (viz., S2, S5, S9, S16, S17, S23, S24, S30, S32, S33, S37, and S43) leaves were analyzed by qRT-PCR. With the exceptions of G26, G33, S9, S16, S23, S32, and S33, the abundances of the other 16 DAPs matched well with the expression levels of the corresponding genes regardless of whether *PRPF31* or *actin* served as an internal standard (Table 2 and Supplementary Figure S8).

3. Discussion

3.1. DAPs Related to Photosynthesis, Carbohydrate and Energy Metabolism

Excess Cu-treated *C. grandis* and *C. sinensis* leaves had lower CO₂ assimilation (Figure 1) and higher concentrations of nonstructural carbohydrates relative to controls [5]. Accordingly, many
Cu-toxicity-responsive proteins related to photosynthesis, carbohydrate and energy were identified in these leaves (Table 2 and Figure 3). Damkjær et al. reported that *Arabidopsis* mutants lacking light-harvesting chlorophyll (Chl) α/b binding protein *Lhcb3* had a lower maximum photosystem (PSII) efficiency of dark-adapted leaves (F<sub>v</sub>/F<sub>m</sub>) than wild type under high light condition and still displayed a lower F<sub>v</sub>/F<sub>m</sub> after 7 d of recovery under normal light, implying that PSII in these plants suffered from photoinhibition under high light [31]. The abundance of Chl a-b binding protein 8 (Lhca3; S19) was increased and decreased in 200 and 400 μM Cu-treated *C. sinensis* leaves, respectively. Thus, the decreased abundance of Lhca3 in 400 μM Cu-treated *C. sinensis* leaves might contribute to the Cu-induced photoinhibition. This could explain why photoinhibition was slightly greater in 400 μM Cu-treated *C. sinensis* leaves than that in 400 μM Cu-treated *C. grandis* leaves [5]. Also, the abundance of protease D olig-1 (DEGP1; S41) was decreased in 200 and 400 μM Cu-treated *C. sinensis* leaves. DEGP1, an enzyme responsible for the degradation of damaged proteins, plays a role in photoinhibition repair of PSII in *Arabidopsis* [32]. Also, the abundance of PsbP domain-containing protein 3 (PPD3, S13) involved in PSII light reaction was decreased in 300 and 400 μM Cu-treated *C. sinensis* leaves.

Phosphorylation of PSII antenna protein RNA-binding protein CP29, localized in chloroplasts, was induced under conditions of decreased photosynthetic capacity and excess light. Maize plants lacking the ability to perform the phosphorylation of CP29 were more sensitive to cold-induced photoinhibition [33]. CP29 phosphorylation has been indicated to play a role in lowering O<sub>2</sub> generation and improving excess energy dissipation [34]. The abundance of CP29A (G1) was increased in 200 and 400 μM Cu-treated *C. grandis* leaves, while the abundances of CP29A (S2 and S32) were decreased in 300 and 400 μM Cu-treated *C. sinensis* leaves. The different response of CP20A to excess Cu between the two agreed with the report that excess Cu had less influence on Chl a fluorescence (OJIP) transients in *C. grandis* leaves than those in *C. sinensis* leaves [5]. Similarly, the abundance of PSII stability/assembly factor HCF136, an essential protein for the stability/assembly of PSII, was increased in 300 (G31) and 400 (G30) μM Cu-treated *C. grandis* leaves, but not in Cu-treated *C. sinensis* leaves. Increased abundance of HCF136 has been obtained in cadmium (Cd) treated *Arabidopsis* shoots [35]. However, the abundances of oxygen-evolving enhancer protein 1 (PSBO2, S17) were enhanced significantly in 400 μM Cu-treated *C. sinensis* leaves. PSBO2 is required for the stability of the photosynthetic water-splitting complex [36]. Interestingly, the damage of the oxygen evolving complexes (OEC) was greater in *C. sinensis* leaves than that in *C. grandis* leaves under 400 μM Cu [5]. Evidently, other factors play a role in stabilizing the water-splitting complex.

The abundance of G10 (ferredoxin-NADP reductase, leaf-type isozyme (LFNR2)) was decreased in 200 and 400 μM Cu-treated *C. grandis* leaves, and of G42 (LFNR2) was increased in 200 μM Cu-treated *C. grandis* leaves. LFNR oxidizes ferredoxin (Fd) to yield NADPH, which is utilized in various reactions such as lipid and Chl biosynthesis, CO<sub>2</sub> fixation and stomatal redox regulation. *Arabidopsis* fnr2 RNAi mutants had decreased concentrations of photosynthetic thylakoid proteins and Chls, and rate of carbon fixation relative to the wild type plants [37]. The abundances of Rubisco subunit binding-protein α subunit [chaperonin 60 subunit α 1 (Cpn60α1); S11] involved in protein folding and Rubisco activase 1 (G4, S4, and S14) involved in the activation of Rubisco were decreased in 400 μM Cu-treated *C. sinensis* and/or *C. grandis* leaves. The abundance of Rubisco activase 1 (S14) was also decreased in 200 and 300 μM Cu-treated *C. sinensis* leaves. Cpn60α1 is necessary for the folding of Rubisco large subunit (rbcL) and proper chloroplast development [38]. Rubisco activase-deficient transgenic tobacco plants had decreased Rubisco carboxylation and CO<sub>2</sub> assimilation [39]. Also, the abundances of sedoheptulose-1,7-bisphosphatas (SBPase; S33) and phosphoribulokinase (PRK; S21) involved in Calvin cycle were decreased in 400 μM Cu-treated *C. grandis* leaves. The lower abundances of LFNR2 (G10), Cpn60α1 (S11), Rubisco activase 1 (G4, S4, S9 and S14), PRK (S21) and SBPase (S33) agreed with our finding and previous report that excess Cu-treated *Citrus* leaves had reduced CO<sub>2</sub> assimilation and Chl concentrations (Figure 1) [5]. However, the abundances of LFNR2 (G42), SBPase (G6) and glyceraldehyde-3-phosphate dehydrogenase B (GAPB, G38) were increased in 200 μM Cu-treated *C. grandis* leaves. This was agreement with the finding that CO<sub>2</sub> assimilation
displayed an upward trend in 200 µM Cu-treated *C. grandis* leaves relative to controls (Figure 1). Similarly, the abundance of fructose-1,6-bisphosphatase, cytosolic (cyFBPase; G36), a major site for controlling sucrose synthesis, was increased in 200 µM Cu-treated *C. grandis*. Strand et al. reported that photosynthesis was inhibited in antisense cyFBPase *Arabidopsis* mutants [40]. Also, the abundance of Cpn60α1 (G8) in *C. grandis* leaves was decreased and increased at 200 and 300 µM Cu, respectively, and the abundance of Rubisco activase 1 (S10) was increased in 300 µM Cu-treated *C. sinensis* leaves.

Carbonic anhydrase (CA, a Zn-metalloenzyme) is required for CO2 assimilation in cotyledons. The abundance of CA (S3) was increased or unaffected by Cu supply in *C. sinensis* leaves. However, CA activity was reduced in Cu excess *Brassica juncea* [41]. The difference between CA abundance and activity could be explained by the Cu-induced decrease in Zn level in *C. sinensis* [5], because its activity is regulated by Zn availability.

Mitochondrial MDH (mMDH) is necessary for CO2 and energy partitioning in leaves. Antisense mMDH tomato plants displayed increased photosynthetic electron transport rate, CO2 assimilation, gS, and growth rate, but decreased respiration rate [42]. The increased abundances of mMDH (S44 and S45) in 400 µM Cu-treated *C. sinensis* leaves agreed with the report that 400 µM Cu-treated *C. sinensis* seedlings had decreased growth, leaf CO2 assimilation and gS, and impaired photosynthetic electron transport chain (PETC) [5]. Chloroplastic NADP-MDH, which catalyzes the excess NADPH produced through PETC and oxaloacetate to malate and NADP+, plays a key role in countering PETC over-reduction and in H2O2 signaling by exporting chloroplast NADPH to other cell compartments. *Arabidopsis* nadp-mdh mutants lacked the reversible inactivation of catalase activity and the concomitant accumulation of H2O2, but had a higher reduction state of the plastoquinone (PQ) pool when exposed to high light [43]. The decreased abundance of NADP-MDH (G17) in 400 µM Cu-treated *C. grandis* leaves might contribute to the Cu-induced inhibition of photosynthesis and the increased reduction of the PSII acceptor side, as indicated by the positive ΔΙ and ΔI-bands in 400 µM Cu-treated *C. grandis* leaves [5]. However, Cu treatments increased or did not alter the abundance of cytosolic MDH (cyMDH; G37 and G39) in *C. grandis* leaves. cyMDH plays a key role in the transport of chloroplast or mitochondria NADPH to other cell compartments. Transgenic apple plants overexpressing an apple cyMDH gene displayed a higher stress-tolerance accompanied by increased reducing power, as indicated by increased concentrations of ASC and reduced glutathione (GSH) and ratios of ASC/dehydroascorbate (DHA), GSH/GSSG and NAD(P)H/NAD(P)+ [44]. Thus, the Cu-toxicity-induced increases of cyMDH abundances in *C. grandis* leaves might be an adaptive strategy.

Pentose phosphate pathway (PPP) provides NADPH for biosynthesis of GSH and maintenance of cellular redox state necessary to deal with oxidative stress. *Arabidopsis* PGL3 T-DNA insertion mutants with decreased flux through the plastidial PPP displayed a decrease in plant size and a lower cellular redox potential [45]. The increased abundance of probable 6-phosphogluconolactonase 4 (PGL4, an enzyme involved in PPP; G4) in 200 and 300 µM Cu-treated *C. grandis* leaves agreed with the increased need for ROS scavenging [5].

Triosephosphate isomerase (TPI), which catalyzing the reversible interconversion of glyceraldehydes 3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP), may prevent the spontaneous degradation of DHAP into methylglyoxal (MG, a cytotoxic metabolite). TPI-deficiency led to increased generation of MG in red blood cells [46]. The decreased abundances of TPI (G29) in 400 µM Cu-treated *C. grandis* leaves implied that MG formation was increased in these leaves, thus increasing ROS generation and lipid peroxidation [5].

The increased abundances of glucose-1-phosphate adenylyltransferase (APS, G24, and G25) in 200 and 300 µM Cu-treated *C. grandis* leaves implied that starch biosynthesis was enhanced in these leaves. However, this way could not explain starch accumulation in 400 µM Cu-treated *C. grandis* leaves, because APS abundance was not increased in these leaves. A weaker sink for the photosynthetic requirement due to Cu toxicity-induced inhibition of growth has been suggested to be responsible for the accumulation of nonstructural carbohydrates including starch in Cu-toxic *Citrus* leaves [5].
There is a close relation between energy availability and stress-tolerance [47]. An extra energy supply is necessary for stressed plants to fortify their tolerance. The increased abundances of ATP synthase subunit β (G12) and ATP synthase γ chain (G23) 300 µM Cu-treated C. grandis leaves and bis(5'-adenosyl)-triphosphatase (Ap3A, G26) in 400 µM Cu-treated C. grandis leaves suggested that ATP biosynthesis was enhanced in these leaves to meet the increased energy needs. Similar result has been obtained in Cu-stressed Elsholtzia splendens leaves [25].

To conclude, Cu-toxicity might affect the abundances of proteins involved in PETC and CO₂ assimilation, thus decreasing electron transport rate and CO₂ assimilation. Cu-toxic effects on PETC were more pronounced in C. sinensis leaves than those in C. grandis leaves.

3.2. DAPs Related to Antioxidation and Detoxification

Five (five) DAP spots involved in antioxidation and detoxification were identified in Cu-treated C. sinensis (C. grandis) leaves (Table 2). The striking Cu-mediated alteration was the big increase in GST (G40) abundance in Cu-treated C. grandis leaves. Dianthus superbus plants overexpressing GST were observed to biosynthesize phytochelatins (PCs), thus sequestering and detoxifying excess Cu [48]. Lambda class of GSTs could be used to enhance plant tolerance against various stresses including heavy metals [49]. However, the abundance of GST DHAR1 (G34), an enzyme having glutathione-dependent thiol transferase and DHA reductase (DHAR) activities, was decreased in 400 µM Cu-treated C. grandis leaves. SOD can rapidly dismutate O₂⁻ to H₂O₂ and protect organisms against oxidative damage. The increased abundances of Cu/Zn SOD (G21) and manganese (Mn) SOD (G33) in 400 µM Cu-treated C. grandis leaves agreed with the report that Cu stress increased Cu/Zn SOD and Mn SOD activity in Arabidopsis leaves [50]. Cu/Zn SOD abundance increased and Fe SOD abundance decreased in Cu-sufficient Arabidopsis leaves, but the reverse was true in Cu-limited leaves, which could save Cu for the biosynthesis of plastocyanin necessary for photosynthesis [51]. Thus, excess Cu increased the biosynthesis of Cu/Zn SOD by a direct effect of Cu on the gene for SOD, hence preventing a Cu-toxic effect on photosynthesis. Methyl viologen (mainly to enhance PSI-originated ROS formation) induced decrease of Fv/Fm was more severe in aor [a chloroplastic NADPH-dependent alkenal/one oxidoreductase (AOR, At1g23740)] Arabidopsis mutants than in Col-0 plants, concluding that AOR played a role in the scavenging of stromal reactive carbonyls (RCs) generated under oxidative stress [52]. Therefore, the decreased abundance of quinone oxidoreductase-like protein At1g23740 (G16) in 400 µM Cu-treated C. grandis might contribute to the Cu-induced inhibition of photosynthesis by lowering the photosynthetic electron transport rate.

The abundances of all the five DAP spots were decreased in Cu-treated C. sinensis leaves. The decreased abundances of three H₂O₂ detoxifying enzymes in 300 (S1) and 400 (S24 and S34) µM Cu-treated C. sinensis leaves agreed with the report that H₂O₂ production was increased in these leaves [5]. Cysteine (Cys) synthase (CS) catalyzes the final step for Cys biosynthesis in plants. The overexpression of CS conferred tolerance to Cd and selenium (Se) by over-production of Cys, GSH and presumably PCs, but not to Cu in transgenic tobacco plants [53]. PCs have been proven not to be the major factor responsible for plant Cu-tolerance [54]. Thus, the Cu-induced decrease of CS abundance (S20 and S39) might not lower the tolerance of C. sinensis seedlings to Cu.

To conclude, the antioxidation and detoxification system as a whole could not effectively protect Citrus leaves from Cu-toxicity-induced oxidative stress, as indicated by the increased H₂O₂ production and electrolyte leakage [5].

3.3. Chaperones and Folding Catalysts

Luminal binding protein (BiP) functions in both protein folding and endoplasmic reticulum (ER) quality control mechanism. Heterologous expression of an ER BiP gene alleviated Cd-induced ER stress and programmed cell death in transgenic tobacco BY-2 cells [55]. Transgenic tobacco plants overexpressing an ER chaperone BiP gene had enhanced Cd-tolerance accompanied by decreased level of ROS and increased level of GSH [56]. Thus, the increased abundance of luminal-binding
protein 5 (BiP5, S16) might contribute to Cu-tolerance of *C. sinensis*. Protein disulfide isomerase (PDI), which catalyzes thiol-disulfide interchange, is the most abundant oxidative protein folding catalyst and a multifunctional protein chaperone. PDI could serve as a Cu chelator or Cu delivering protein to protect cells against Cu-toxicity [57]. The increased abundance of probable PDI A6 (G15) in 400 µM Cu-treated *C. grandis* leaves might play a role in preventing these leaves from Cu-toxicity by binding Cu and/or decreasing oxidative damage. Like Cpn60x1 (G8), the abundance of 20 kDa chaperonin (Cpn20, a co-chaperonin of CPN60; G12) was increased in 300 µM Cu-treated *C. grandis* leaves. Cpn20 played a role in oxidative stress protection and chloroplast development via positively regulating the activation of Fe SOD [58]. Interestingly, the abundance of chaperonin CPN60-1 (G19 and S26) involved in the correct folding of imported proteins was decreased and increased in 200 and 300 µM Cu-treated *C. grandis* leaves, respectively, but was decreased in 400 µM Cu-treated *C. sinensis* leaves. Also, the abundance of heat shock cognate 70 kDa protein 2 (HSP70-2, G11) involved in the folding of de novo translocation of precursor proteins into organelles, and degradation of damaged protein under disadvantaged conditions was decreased in 300 and 400 µM Cu-treated *C. grandis* leaves. These results demonstrate the involvement of chaperones and folding catalysts in the Cu tolerance and Cu toxicity of *Citrus*.

### 3.4. DAPs Related to Signal Transduction

Plant plasma membrane (PM) H\(^+\)-ATPase activity can be regulated by 14-3-3 proteins involved in brassinosteroid (BR)-mediated signaling pathway [59]. The increased abundance of 14-3-3 protein 7 (G5) in 400 µM Cu-treated *C. grandis* leaves agreed with the increased expression of a 14-3-3 gene in *Fucus vesiculosus* in response to moderately elevated level of Cu [60], and the increased activity of PM H\(^+\)-ATPase in Cu-treated cucumber roots [61]. However, the abundance of 14-3-3 protein 6 (S7) was reduced in 200 µM Cu-treated *C. sinensis* leaves.

Major pollen allergen, which is involved in abscisic acid (ABA)-activated signaling pathway, have high sequence homology to pathogenesis related (PR) proteins. The increased or unaltered abundance of major allergen Pru ar 1 (G27) in 200–400 µM *C. grandis* leaves agreed with the elevated abundances of Bet v 1-Sc3 (PR-10c) and PvPR1 in Cu-stressed *Betula pendula* and bean leaves, respectively [62,63]. Annexins, a key element of Ca\(^{2+}\)-signaling pathways, are involved in counteracting oxidative stress. Transgenic tobacco plants overexpressing an annexin displayed elevated total peroxidase activity, improved tolerance/resistance to Cd, oxidative stress and diseases, and increased message levels for several PR proteins [64]. The increased or unchanged abundance of annexin D1 (G22) in Cu-treated *C. grandis* leaves agreed with the increased abundance of annexin D1 in Cu-stressed *Allium cepa* roots [20]. Thus, Cu supply might enhance the resistance of *C. grandis* to diseases [65]. However, the abundance of major allergen Pru ar 1 (S35) was decreased in 200 µM Cu-treated *C. sinensis* leaves.

Calreticulin (CRT), a crucial Ca\(^{2+}\)-binding protein mainly in the ER, functions in Ca\(^{2+}\) signaling in response to stress in plants. The decreased abundance of CRT-1 (G3) agreed the decreased abundance of CRT in excess Cu-treated *Ectocarpus siliculosus* [66] and the decreased expression level of CRT in Mg-deficient *Citrus reticulata* leaves [67], because Mg concentration was decreased in Cu-stressed *C. grandis* leaves [5].

To conclude, hormone (ABA and BR)- and Ca\(^{2+}\)-mediated signaling pathways might function in *Citrus* Cu-tolerance and Cu-toxicity. This was also supported by data suggesting that 28-homobrassinolide [41] and Ca [68] could alleviate plant Cu-toxicity, and that a reciprocal cross-talk existed between Cu status and ABA metabolism and signaling in *Arabidopsis* [69].

### 3.5. DAPs Related to Cellular Transport, Nucleic Acid and Cell Wall Metabolisms, and Cytoskeleton

Ferritin can protect plant cells from Fe-toxicity by storing excess Fe in a non-toxic form in plant cells [70]. A characteristic of Cu-toxicity in *Citrus* leaves is Fe chlorosis [5,71]. The decreased abundance of ferritin-3 (S5) in 200–400 µM Cu-treated *C. sinensis* leaves agreed with the report that ferritin
accumulation in plant cells increased under high Fe concentrations [72]. The decreased abundance of ferritin-3 might contribute to Fe homeostasis by lowering the chelation of Fe to ferritin.

Both α- and β-tubulins are the primary constituents of microtubules (MTs), one of the cytoskeletal components. MTs have been proposed to function in plant Cu-toxicity and Cu-tolerance. Song et al. found that the abundances of three protein spots—namely tubulin α-1 chain, putative tubulin α-1 chain and tubulin α-2 chain, were decreased in excess Cu-treated rice roots, concluding that the decreased accumulation of α-tubulin might impair MT polymerization and alignment, thus influencing MT functions [18]. However, the abundance of tubulin β-6 chain (G7) in C. grandis leaves increased or did not alter in response to Cu supply, implying that MTs might be not impaired in these leaves. This might be related to the preferential accumulation of most Cu in the roots under Cu-stress [5].

DNA helicases, which are ATP-dependent DNA unwinding enzymes, are involved in DNA repair, replication and recombination. Ectopic expression of a Medicago sativa helicase 1 (a homolog of the pea DNA helicase 4) gene conferred Arabidopsis tolerance to drought, salt and oxidative stress [73]. The decreased abundance of RuvB-like helicase 1 (S42) in 400 µM Cu-treated C. sinensis leaves implied that DNA repair was impaired in these leaves.

The decreased or unaltered abundance of endochitinase 1 (G2) related to cell wall polysaccharide (macromolecule) catabolic process in Cu-treated C. grandis leaves implied that the level of cell wall polysaccharides might be increased in these leaves because of decreased degradation. This agreed with the increased concentration of total polysaccharide in the cell walls of Cu-treated Elsholtzia splendens roots [26]. However, the abundance of chitinase was enhanced in rice leaves treated with 100 µM Cu for 72 h [74]. Chitinase activity was not altered in pepper roots, stems, and cotyledons after 28 days of treatment with 50 µM Cu [65]. Thus, it seems that the effects of Cu on chitinase vary with plant species, Cu concentration, and time of exposure to Cu.

3.6. Other DAPs

AdoHcy hydrolase, which catalyzes the reversible hydrolysis of AdoHcy to L-homocysteine and adenosine, plays a crucial role in maintaining methyl cycling via the removal of AdoHcy. Taddei et al. observed that AdoHcy hydrolase was induced by Cu stress in in vitro-cultured pith explants of Nicotiana glauca, suggesting that AdoHcy hydrolase played a crucial role in regulating Cu level and intracellular distribution [75]. B-induced alleviation of C. grandis Al-toxicity was accompanied by increased root expression of adenosylhomocysteinase-like [76]. The increased abundance of AdoHcy hydrolase (G32) in 200 µM Cu-treated C. grandis leaves might contribute to their Cu-tolerance. However, its (S37) abundance was decreased in 400 µM Cu-treated C. sinensis leaves.

Flavonoids can act as ROS scavengers, and inhibit ROS production by chelating metals. The decreased abundance of dihydroflavonol-4-reductase (DFR, S38) in 400 µM Cu-treated C. sinensis leaves suggested that anthocyanin biosynthesis might be decreased in these leaves. This disagreed with the increased expression level of DFR in Cu-stressed rice leaves [77].

4. Materials and Methods

4.1. Plant Materials

Seedling culture and Cu treatments were made according to Li et al. [5]. Briefly, 6-week-old uniform seedlings of ‘Xuegan’ (Citrus sinensis) and ‘Shatian pummelo’ (Citrus grandis) were transported to 6 L pots (two plants per pot) filled with sand thoroughly washed with tap water, then grown in a greenhouse under natural conditions at Fujian Agriculture and Forestry University. Six weeks after transporting, seedlings were watered daily with freshly papered nutrient solution at a Cu concentration of 0.5 (Cu0.5, control), 200 (Cu200), 300 (Cu300), or 400 (Cu400) µM from CuCl2 until nutrients begin to flow out of the bottom hole of the pot (~500 mL per pot). Nutrient solution pH was adjusted to 4.8 with 1 M HCl before supply. Six months after Cu treatments, the fully expanded (about 7-week-old) leaves were used for all measurements. Firstly, leaf gas exchange was measured. Then, leaves (winged
leaves, petioles and midribs removed) were taken at a sunny noon and immediately frozen in liquid N\textsubscript{2}. All samples were stored at \(-80^\circ\text{C}\) until extraction of proteins and total RNA. These seedlings unused for the collection of leaves were used for the measurements of plant dry weight (DW) and leaf Cu.

4.2. Measurements of Plant DW, and Leaf Gas Exchange and Cu Concentration

Root, stem, and leaf DW were weighted after being washed with tap water and dried to a constant weight at 70 °C (~48 h) [78].

Gas exchange was measured with a CIARS-2 portable photosynthesis system (PP systems, Herts, UK) at a controlled light intensity of ~1000 \(\mu\text{mol m}^{-2} \text{s}^{-1}\) and a controlled CO\textsubscript{2} concentration of ~380 \(\mu\text{mol mol}^{-1}\) between 9:30 and 12:30 a.m. on a sunny day [79].

Leaf Cu was determined with a NexION 300X Inductively Coupled Plasma Mass Spectrometer (ICP-MS, PerkinElmer, Shelton, CT, USA).

4.3. Leaf Protein Extraction, 2-DE and Image Analysis

About 1 g of frozen leaves harvested equally from four seedlings (one seedling per pot) was mixed as one biological replicate. There were three biological replicates per treatment (a total of 12 seedlings from 12 pots). Proteins were extracted using a phenol extraction procedure [80] and their concentration was measured as described by Bradford [81]. 2-DE was performed according to Sang et al. [82]. Stained gels were scanned with an Epson Scanner (Seiko Epson Corporation, Japan) at a resolution of 300 dpi. Images were analyzed with PDQuest version 8.0.1 (BioRad, Hercules, CA, USA), including background subtraction, normalization, spot detection, matching, Gaussian fitting and gel alignment [83]. A fold change of >1.5 or <0.67 was set to determine DAP spots in addition to a \(p\)-value < 0.05. After being visually checked and manually excised from gels, all DAP spots were submitted to MALDI-TOF/TOF-MS-based identification.

4.4. MALDI-TOF/TOF-MS-Based Protein Identification and Bioinformatic Analysis

Peptide identification was carried out on an AB SCIEX 5800 TOF/TOF plus MS (AB SCIEX, Shanghai, China) as described by Peng et al. [83]. After being processed with TOF/TOF Explorer™ Software (AB SCIEX, Shanghai, China) in a default mode, all acquired spectra were submitted to MASCOT (Version 2.3, Matrix Science Inc., Boston, MA) by GPS Explorer (Version 3.6) for the search of \textit{C. sinensis} databases (http://citrus.hzau.edu.cn/orange/index.php) using following search parameters: trypsin cleavage with one missed, MS tolerance of 100 ppm and MS/MS tolerance of 0.6 Da. At least two of matched peptides were necessary for each protein. Protein identifications were accepted if MASCOT score was ≥ 70, and the sequence coverage was ≥ 20% or the number of matched peptides (NMP) was ≥ five [84,85]. DAPs were classified according to KEGG (http://www.kegg.jp/), GO (http://www.geneontology.org/) and Uniprot (http://www.uniprot.org/) databases [86,87].

4.5. KEGG Pathway Analysis of DAPs

KEGG pathway was analyzed using KOBAS 3.0 (Peking University, Beijing, China). Pathways were considered as significantly enriched if the corrected \(p\)-value was less than 0.05

4.6. qRT-PCR Analysis

Total RNA were extracted from ~300 mg frozen of leaves (mixed sample from four seedlings, one seedling per pot) using Recalcitrant Plant Total RNA Extraction Kit (Bioteke Corporation, Beijing, China). There were three biological replicates per treatment (a total of 12 seedlings from 12 pots). The sequences of specific primers designed using Primer Primver Version 5.0 (PREMIEr Biosoft International, CA, USA), were listed in Table S4. qRT-PCR was performed with three biological and two technical replicates [86]. Two \textit{Citrus} genes: \textit{U4/U6 small nuclear ribonucleoprotein PRP31} (PRP31,
Cs7g08440.1) and *actin* (Cs1g05000.1) were used as internal standards and 0.5 µM Cu-treated leaves were used as reference (set as 1).

### 4.7. Data Analysis

There were 15 pots (30 seedlings) per treatment in a completely randomized design. Results were presented as the mean ± SE for *n* = 3–10. Eight means [two (species) × four (Cu levels)] were tested by two ANOVA followed by the least significant difference at *p* < 0.05 level.

Pearson correlation analysis and principal component analysis (PCA) for all identified DAP spots were made using SPSS (version 17.0, IBM, NY, USA) [88].

### 4.8. Data Deposit

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD017049.

### 5. Conclusions

In this study, a 2-DE based MS approach was used to investigate Cu-toxicity-responsive proteins in *Citrus* leaves. Forty-one and 37 DAP spots were identified in 200, 300 and/or 400 µM Cu-treated *C. grandis* and *C. sinensis* leaves, respectively. Over 50% of these DAPs were involved in photosynthesis, carbohydrate, and energy metabolism, followed by antioxidation and detoxification, protein folding and assembly (viz., chaperones and folding catalysts), and signal transduction. More than 80% of these DAPs were identified only in *C. grandis* or *C. sinensis* leaves. More (Less) DAPs increased in abundances than DAPs decreased in abundances were identified in Cu-treated *C. grandis* (*C. sinensis*) leaves. Impaired PETC and decreased abundances of proteins involved in CO₂ assimilation might be responsible for the Cu-induced inhibition of photosynthesis. Cu-toxicity affected the PETC more in *C. sinensis* leaves than in *C. grandis* leaves. DAPs related to antioxidation and detoxification, protein folding and assembly (viz., chaperones and folding catalysts), and signal transduction might be involved in *Citrus* Cu-toxicity and Cu-tolerance. Also, we identified some new DAPs (viz., LFNR2, SBPase, probable PGL4, ferritin, AdoHcy hydrolase and abscisic stress-ripening protein 1-like) that were not reported in leaves and/or roots (Figure 5). In conclusion, this study revealed some novel mechanisms on Cu-toxicity and Cu-tolerance in plants.
Figure 5. A possible model displaying the differentially abundant proteins (DAPs) in 400 μM Cu-treated *Citrus grandis* leaves (CGL) and *Citrus sinensis* leaves (CSL). In this Figure, plain format and italics were used for Cu-toxic CGL and CSL, respectively. Red: DAPs increased in abundance; Black: DAPs decreased in abundances; APX, L-ascorbate peroxidase; DLST: dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex 2; GIPX: glutathione peroxidase; Pn: photosynthesis; RA1: Rubisco activase A1.

**Supplementary Materials:** The following are available online at http://www.mdpi.com/2223-7747/9/3/291/s1, Figure S1: Excess Cu effects on growth of *Citrus grandis* (A) and *Citrus sinensis* (B), Figure S2: Excess Cu effects on root (A), stem (B), leaf (C) and whole plant (D) dry weight (DW) in *Citrus grandis* and *Citrus sinensis* seedlings, Figure S3: 2-DE images of proteins extracted from 0.5 (A, É, I, M), 200 (B, F, J, N) 300 (C, G, K, O) and 400 (D, H, L, P) Cu-treated *Citrus grandis* (A-D and I-L) and *Citrus sinensis* (E-H and M-P) leaves for the other two replicates, Figure S4: Close-up views of 24 DAP spots in 200, 300 and 400 μM Cu-treated *Citrus sinensis* and *Citrus grandis* leaves, Figure S5: Venn diagram analysis of Cu-responsive proteins in *Citrus grandis* and *Citrus sinensis* leaves, Figure S6: Significantly enriched (A-E and H) and the most enriched (F-G) KEGG pathways for annotated differentially abundant proteins (DAPs) in Cu-treated *Citrus grandis* (A-D) and *Citrus sinensis* (E-H) leaves, Figure S7: Matrices of Pearson correlation coefficients for differentially abundant proteins (DAPs) in *Citrus grandis* (A) and *Citrus sinensis* (B) leaves, Figure S8: Relative expression levels of genes encoding 22 differentially abundant proteins (DAPs) identified in 400 μM Cu-treated *Citrus grandis* (G3, G9, G10, G11, G14, G26, G29, G32, G34 and G35) and *Citrus sinensis* (S2, S5, S9, S16, S17, S23, S24, S30, S32, S33, S37 and S43) leaves using qRT-PCR analysis, Table S1: Master list of proteins identified in MALDI TOF/TOF MS from 200, 300 and or 400 μM Cu-treated *Citrus citron*, *Citrus sinensis* and *Citrus grandis* leaves using 2DE and DIGE experiments, Table S2: Principal component analysis (PCA) for copper-responsive proteins in *Citrus sinensis* leaves, Table S3: Principal component analysis (PCA) for copper-responsive proteins in *Citrus grandis* leaves, Table S4: Specific primer pairs used for qRT-PCR analysis.

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preparation, W.-L.H.; writing—review and editing, L.-S.C.; supervision, L.-T.Y. and L.-S.C.; project administration, L.-S.C.; funding acquisition, L.-S.C. All authors have read and agreed to the published version of the manuscript.

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