Proteomic analysis of Cucumis sativus cotyledons after glucohexaose treatment as a part of ROS accumulation related resistance mechanism

Yuhan Hao1,3, Chunmei Lin1, Haiyan Fan1,2*, Yang Yu1, Ning Li1 and Shaoli Chen4

Abstract

Background: Glucohexaose is a safe farm chemical used for pathogen prevention, which can induce systemic acquired resistance in cucumber.

Results: We found that glucohexaose treatment of cucumber plant induced an accumulation of the reactive oxidative species (ROS). Histochemistry showed sharp increases in $\text{O}_2^-$ and $\text{H}_2\text{O}_2$ 5 h after glucohexaose treatment. After 5 h, the $\text{O}_2^-$ content decreased to a normal level, but the $\text{H}_2\text{O}_2$ content remained at a high level 10 h after glucohexaose treatment. And antioxidant enzymes were also changed after glucohexaose treatment. We also investigated the relationship between ROS accumulation and glucohexaose-induced proteome alteration using 2D electrophoresis coupled with MS/MS. 54 protein spots, which enhanced expression under glucohexaose treatment but suppressed the expression by application of DPI and DMTU, have been identified.

Conclusion: Our study showed the accumulation of ROS is a part of mechanism of glucohexaose induced resistance in cucumber cotyledons. The up-regulated proteins identified by MS such as PP2C and antioxidation proteins are important in ROS signaling. It will be interesting to find out the regulatory mechanism underlying the induction of these proteins via ROS, and provide some clues to the mechanism of glucohexaose-induced resistance.

Keywords: Cucumis sativus, Glucohexaose, Reactive oxygen species, Proteome

Introduction

During vegetable production, chemical pesticides are still the main method of disease prevention. As consumers' concerns about food quality increase, how to prevent disease without pesticide residues has attracted more attention. Induced resistance by biotic and abiotic elicitors is a new method for disease resistance. Glucohexaose, synthesized by the Research Center for Eco-Environmental Science, Chinese Academy of Science, is a safe, synthetic oligosaccharide elicitor that is naturally degraded in the environment. After glucohexaose incubation, plant resistance systems were activated and plants acquired stronger resistance to many pathogens, such as Pseudoperonospora cubensis [1]. Using glucohexaose in agricultural production would be a safer and more acceptable alternative to chemical pesticides. However, the mechanism of the induced resistance remains unclear. A previous proteomic study in our laboratory using cucumber leaves after glucohexaose treatment identified certain ROS accumulation related proteins [2], which indicated that the ROS accumulation might be one of the mechanisms of glucohexaose-induced resistance.

ROS, including superoxide ($\text{O}_2^-$), hydrogen peroxide ($\text{H}_2\text{O}_2$), hydroxyl radical (OH) and singlet oxygen ($\text{O}_2^+$), play an important role in resistance to pathogens as signal molecules in plant cells [3]. The oxidative burst (OXB) was first reported by Doke in 1983 in a study of the interaction between potato tuber tissues and Phytophthora infestans [4], which involves the rapid release of ROS in the early stage of pathogen infection. Later studies indicated that in many stress conditions, such as bacterial, viral and fungal infection, the induction of elicitors and the composition of the cell wall, and mechanical stress could lead to the rapid release of ROS [5,6].
The main ROS in plant cells is H$_2$O$_2$, which can be transported across cell membranes to act as a signal molecule and is an essential signaling mediator of plant stress resistance [7-9]. There are different ROS generating mechanisms in different plants, such as germin-like oxalate oxidase, polyamine oxidase, peroxidase, thioredoxins and glutaredoxins [10-16]. However, in most plants, NADPH oxidase is the principal source of ROS induced by pathogens or elicitors [17,18]. NADPH oxidase, located in the cell membrane, is a redox enzyme containing a heme moiety and six transmembrane domains. It transfers an electron from NADPH to O$_2$, and generates large amounts of O$_3^-$ in a short time [18].

Proteomic study is a good tool to investigate the mechanism of ROS related glucohexaose induced resistance. It is used in other stress related ROS pathway studies in different species. Soares, et al. investigated wound related proteome changes in ROS pathway in Medicago and found some interesting proteins such as SODs, peroxidases and germin-like proteins [19]. Wang, et al. found AtCIAPIN1 and flg22 are early-responsive redox-sensitive proteins in Arabidopsis with proteomic studies [20]. In wheat, Bykova, et al. reported several redox-sensitive proteins functioning in seed dormancy control [21]. Therefore, we sought to use proteomic tool to further investigate the possible link between glucohexaose-induced resistance and ROS accumulation. We report that glucohexaose can induce ROS accumulation in cucumber cotyledons and provide some clues concerning the mechanism of glucohexaose-induced ROS accumulation. These results provide a theoretical basis for developing safe farm chemicals for vegetable production.

Materials and methods

Plant materials

Cucumber seeds (Jinyan No. 4) were soaked in water for 24 h and then sterilized with 75% ethanol for 30 s and 2.5% NaClO for 15 min. After washing with sterile water at least three times, sterilized seeds were placed on sterile water soaked gauze. The seeds were allowed to germinate at 25–30°C. When the cotyledons expanded, the seedlings were used for subsequent experiments.

The detection of variation of H$_2$O$_2$ and O$_3^-$ in glucohexaose-treated cotyledons

Whole plants were sprayed with 50 μg/ml glucohexaose and H$_2$O$_2$ and O$_3^-$ were detected at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 and 15 h after glucohexaose treatment. We detected H$_2$O$_2$ and O$_3^-$ using DAB and NBT staining methods, according to Zhang et al. [22] and Soares et al. [19] with modifications. Cucumber cotyledons were soaked with 1 mg/ml DAB (Sigma, St. Louis, MO, USA) for 8 h and infiltrated with 0.1% NBT (Ameresco, OH, USA) for 20 min, respectively. The cotyledons were then transferred to 95% ethanol in an 80°C water bath. After the green color of the cotyledons disappeared, the cotyledons were photographed to show the variation of H$_2$O$_2$ and O$_3^-$. Cotyledons were preserved in 95% ethanol at 4°C. Three independent replicates preformed for each assay.

To investigate the effect of DPI (an inhibitor of NADPH oxidase) and DMTU (a ROS scavenger) during the oxidative burst, we treated two groups of plants with glucohexaose after incubating them with 100 μM DPI and 5 mM DMTU for 4 h.

Determination of scavenger enzymes activity

Assay kits (Nanjing Jiancheng Bioengineering Institute, China) were used to measure SOD activity, MDA contents, POD activity, CAT activity, APX activity and GPX activity.

Protein extraction

Proteins were extracted with a PEG precipitation method according to Xi et al. [23], with modifications. Cucumber cotyledons were collected and pulverized to a fine powder with liquid nitrogen. The finely ground powder was extracted with Mg/NP-40 extraction buffer containing 0.5 M Tris–HCl (pH 8.3), 2% (v/v) NP-40, 20 mM MgCl$_2$, 2% (v/v) β-mercaptoethanol, 1 mM PMSF, 1% (w/v) PVP and 1 mM EDTA. After centrifugation at 13 000 × g for 15 min, the supernatant was precipitated with 50% PEG stock solution to adjust the final PEG concentration to 24%, which is the appropriate PEG concentration for cucumber Rubisco protein precipitation. After centrifugation at 13 000 × g for 30 min, the pellet was named as fraction F1 and the supernatant was precipitated with 10% (TCA)/acetone solution at −20°C for at least 1 h. The TCA/acetone precipitation fraction was centrifuged at 13 000 × g for 30 min and the pellet named as fraction F2. The F1 and F2 pellets were washed with TCA/acetone until they were colorless, and then they were washed three times with 80% acetone containing 0.07% β-mercaptoethanol. Proteins were freeze-dried and stored at −80°C for subsequent tests.

2-D electrophoresis

The dried proteins were redissolved in lysis buffer containing 8 M Urea, 2 M Thiourea, 4% CHAPS, 1% DTT, 1% TBP and 2% IPG buffer for 3–4 h at 30°C. The samples were centrifuged at 12 000 × g for 10 min at room temperature and the pellet was discarded. The supernatant was tested by the Bradford method to determine the protein content and loaded onto 24 cm pH 4–7 IPG stripes with 1 mg and 450 μl protein solution. The IEF conditions were as follows: 50 V for 15 h, 100 V for 1 h, 250 V for 3 h, 500 V for 3 h, 1000 V for 1 h, 10000 V for...
3 h, 10000 V for 160000 Vh and 500 V thereafter (Ettan IPGphorIII, GE Healthcare). After IEF, the focused strips were equilibrated with equilibration solutions twice. 2% DTT and 2.5% iodoacetamide were added to equilibration mother solution, which contained 6 M Urea, 0.05 M pH 8.8 Tris–HCl, 2% SDS and 20% glycerol. The second dimension SDS-PAGE was performed on an Ettan DALT six (GE Healthcare) with an 11% polyacrylamide gel. CBB R350 was used to stain the 2D gels, and max missed cleavages: 1.

**Image analysis**
A UMAX Power Look 2100XL (Maxium Tech., Taipei, China) was used to scan the 2D gels in TIF images. The spots are then analyzed by PDQuest Advanced™ 2-D Analysis software (version 8.0.1, Bio-Rad). Each image was adjusted to be the same size and the Spot Detection Parameter Wizard was used to automatically pair the spots on each image. Landmarks and manual matching helped the accuracy of the pairing. Quantitative analysis was performed by Student's t-test only for notable spots in groups of three biological replicated gels. Protein spots selected for future identification showed an increase of at least 2.0-fold in the P group compared with the CK group, and at the same time the DPI and DMTU group were decreased relative to the P group. Here, CK represents the control group and P represents the cucumber cotyledons treated with 50 μg/ml glucohexaose for 5 h. The DPI and DMTU groups represent those treated with DPI and DMTU for 4 h before 50 μg/ml glucohexaose treatment.

**Protein identification by MS**
The selected spots were excised from gels using pipette tips, placed in tubes and decolored using 200–400 μl 100 mM NH4HCO3/30% ACN. After freeze-drying, the protein spots were digested by trypsin (the ratio of trypsin to proteins was 1:20–1:100) for approximately 20 h at 37°C. The hydrolysates were then transferred to new tubes and disrupted by sonication for 15 min in a buffer containing 100 μl 60% ACN/0.1% TFA and desalinated in a ZipTip (Millipore). The in-gel digested proteins were freeze-dried and resolved by 2 μl 20% ACN for each 1 μl of protein. After air-drying, 0.5 μl of over-saturated CHCA solution was added (dissolved in 50% ACN and 0.1% TFA) and the proteins were then air-dried. A 4800 Plus MALDI TOF/TOFTM Analyzer (Applied Biosystems, USA) was used for MS analysis with the Nd:YAG lasing light emitter, 2 kV voltage, positive ion model, 800–4000 Da PMF quality scan range. MS/MS analysis was performed with parent ions with signal to noise ratios of more than 50, and excited 2500 times by the MS/MS laser with a collision energy of 2 kV and CID closure. For database searching, the conditions were set as follows: database: IPI, taxonomy: Viridiplantae (900091), type of search: Peptide Mass Fingerprint (MS/MS Ion Search), enzyme: Trypsin, Fixed modifications: Carbamidomethyl (C), mass values: Monoisotopic, protein mass: Unrestricted, peptide mass tolerance: ±100 ppm, fragment mass tolerance: ±0.8 Da, peptide charge state: 1+

**Results**

**Glucohexaose-induced ROS accumulation**
To determine whether glucohexaose can actually induce an ROS accumulation in cucumber cells and when the accumulation occurs, we detected two ROS, H2O2 and O2−, using DAB and NBT staining at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 and 15 h after glucohexaose treatment. H2O2 and O2− began to accumulate after glucohexaose treatment and peaked at 5 h after treatment (Figure 1A and B). After incubated with the inhibitor of NADPH oxidase DPI and the ROS scavenger DMTU before glucohexaose treatment, ROS failed to accumulate (Figure 1C and D). Antioxidant enzymes were also changed after glucohexaose treatment. SOD activity indicated O2− level increased after glucohexaose treatment (Figure 1E). As a main signaling messenger, H2O2 scavengers behaved differently: POD and APX activities increased and CAT and GPX activities decreased after glucohexaose treatment (Figure 2E-J). The different responses of the four H2O2 scavengers indicated a complicated mechanism of H2O2 regulation in the plant cells. Moreover, SOD, CAT, POD, APX and GPX activity returned to the control level after DPI and DMTU incubation. These results showed that ROS accumulated at 5 h after glucohexaose treatment. As ROS accumulation is an important part of plant immune action, it is also a part of mechanism of glucohexaose induced cucumber resistance.

**Proteomic analysis of cucumber cotyledons after ROS accumulation induced by glucohexaose**
Because H2O2 and O2− showed sharp increases at 5 h after glucohexaose treatment, however, DMTU or DPI pretreatment significantly abolished the effect of glucohexaose. To find out the regulatory mechanism underlying the induction of proteins via ROS, four samples were applied to 2-DE analysis. At first, sample 1 and 2 were sprayed with deionized water, sample 3 and 4 were pretreated with 5 μM DMTU and 100 μM DPI, respectively. After 4 h of pretreatment, deionized water was used to spray sample 1 (control plants), sample 2, 3, 4 were sprayed with 50 μg/ml glucohexaose. Then, at 5 h after last treatment, the cucumber cotyledons were harvested.

Total proteins extracted from cucumber cotyledons were divided into two fractions, F1 and F2, using the PEG precipitation method. After 2D electrophoresis
Figure 1 (See legend on next page.)
and image analysis by MS, we identified 55 protein spots that showed significant expression changes. Fifty-four protein spots (Figure 2, Additional file 1: Figure S1 and Additional file 1: Figure S2) corresponding to 37 proteins were identified by MALDI-TOF-TOF MS (Table 1). The protein spots chosen showed increased abundance after glucohexaose treatment and decreased when incubated with DPI or DMTU; therefore, they are possibly related to the glucohexaose induced ROS accumulation. After data analysis, sequence alignment, GO annotation and reference searching, we divided these proteins into eight groups: photosynthesis-related proteins, respiration and metabolism-related proteins, translation-related proteins, proteolytic enzymes, protein phosphatases, antioxidation proteins and unclassified proteins. Each group may play an important role in glucohexaose induced ROS accumulation.

**ROS-related proteins identified by MALDI-TOF-TOF MS**

NADPH oxidase, which transfers an electron from NADPH to O$_2$ is the principal source of ROS in a short time. In animal cells, the pentose phosphate pathway is the main source of NADPH accumulation [24]. In plant cells, the large amount of NADPH generates from both photosynthesis and pentose phosphate pathway [25]. In this study, we have identified the increase of several

![Figure 1: Glucohexaose can induce ROS accumulation. A-B. Changes of O$_2^-$ (A) and H$_2$O$_2$ (B) after treatment of glucohexaose. Cucumber cotyledons are infiltration with 0.1% NBT and 1 mg/mL DAB and decolourisation. One to fifteen hours after glucohexaose treatment indicated the plants’ early respond to glucohexaose. C-D. DPI and DMTU incubation can eliminate the ROS accumulation at five hours after glucohexaose treatment. E-J. Some important ROS scavenging enzymes activity in cucumber cotyledons with different treatment. CK represent the control group; P represent the cucumber cotyledons treated with 50 μg/mL glucohexaose for five hours; DPI and DMTU represent before treated with 50 μg/mL glucohexaose, DPI and DMTU were incubated for four hours. Error bar represent SD.](image)

![Figure 2: Proteomic analysis of glucohexaose induced ROS accumulation. A-B 2D maps of identified differential expression cucumber cotyledon proteins spots. These two maps are from the P group and other groups’ picture offered in supplemental materials. C-K Representative differential protein spots and their relative abundance. All identified protein spots’ information is supplied in supplemental materials.](image)
Table 1 Glucohxaose and ROS modulators-regulated proteins

| ID   | gi    | Protein name                                      | PI     | MW       | Protein score | Protein score C.I. % | Pep. count | E-value | Fold enhancement by P6 | Percentage of suppression by DPI/DMTU |
|------|-------|--------------------------------------------------|--------|----------|---------------|----------------------|------------|---------|------------------------|--------------------------------------|
|      |       | Photosynthesis related proteins                  |        |          |               |                      |            |         |                        |                                      |
| 1104 | gi|12620881 | Ribulose-1,5-bisphosphate carboxylase/oxygenase activase | 5.54   | 48186.1  | 284          | 100                  | 9          | 1.68    | 84.15/61.03            |                                      |
| 1111 | gi|115768  | Chlorophyll a-b binding protein of LHCl type I    | 5.14   | 27331.7  | 282          | 100                  | 7          | 2.05    | 69.18/52.48            |                                      |
| 2004 | gi|32551965 | Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit | 6.67   | 23461.8  | 651          | 100                  | 12         | 7.76    | 50.81/24.37            |                                      |
| 4102 | gi|255567170| Chlorophyll A/B binding protein                    | 6.85   | 29362    | 82           | 99.482               | 5          | 1.40    | 82.39/55.08            |                                      |
| 0401 | gi|62899808 | Chromoplast-specific carotenoid-associated protein | 5.05   | 35272.5  | 581          | 100                  | 16         | 11.35   | 86.13/83.53            |                                      |
| 3507 | gi|125578  | Phosphoribulokinase                                | 6.03   | 44485.6  | 552          | 100                  | 12         | 2.97    | 73.35/77.00            |                                      |
| 3806 | gi|12585325| Phosphoglucomutase                                 | 5.56   | 68625.8  | 322          | 100                  | 8          | 2.36    | 49.99/41.42            |                                      |
| 5104 | gi|11134156| Oxygen-evolving enhancer protein 2                 | 8.61   | 28292.3  | 786          | 100                  | 12         | 2.57    | 17.35/36.94            |                                      |
|      |      | Metabolism-related proteins                       |        |          |               |                      |            |         |                        |                                      |
| 1206 | gi|225451299| Ribose-5-phosphate isomerase                       | 6.66   | 30340.1  | 291          | 100                  | 7          | 1.58    | 49.04/61.65            |                                      |
| 1606 | gi|147838694| Chloroplast fructose-1,6-bisphosphatase            | 5.3    | 45183.4  | 898          | 100                  | 11         | 3.55    | 88.59/33.13            |                                      |
| 2406 | gi|3328122 | Phosphoglycerate kinase precursor                 | 7.68   | 50594    | 449          | 100                  | 9          | 1.87    | 57.87/50.11            |                                      |
| 2702 | gi|118721470| Vacuolar H + −ATPase subunit B                     | 5.18   | 54450.9  | 575          | 100                  | 17         | 4.21    | 81.96/47.07            |                                      |
| 5402 | gi|307136265| Fructokinase                                      | 5.61   | 35800.6  | 403          | 100                  | 16         | 2.15    | 68.33/52.23            |                                      |
| 7207 | gi|2833386 | Ribulose-phosphate 3-epimerase                    | 8.23   | 30632.2  | 299          | 100                  | 5          | 9.18    | 79.61/78.13            |                                      |
| 7904 | gi|1351856 | Aconitate hydratase                               | 5.74   | 98569.8  | 884          | 100                  | 23         | 3.60    | 90.35/74.12            |                                      |
| 7905 | gi|1351856 | Aconitate hydratase                               | 5.74   | 98569.8  | 1070         | 100                  | 26         | 7.19    | 91.27/76.31            |                                      |
| 8402 | gi|255557204| Fructose-bisphosphate aldolase, putative          | 7.59   | 38745.2  | 121          | 100                  | 3          | 4.44    | 73.13/66.04            |                                      |
|      |      | Chaperones and elongation factors                 |        |          |               |                      |            |         |                        |                                      |
| 1813 | gi|22545166 | Elongation factor ts                              | 4.78   | 123315.5 | 110          | 100                  | 6          | 7.76    | 53.68/63.39            |                                      |
| 1804 | gi|124245039| Chloroplast HSP70                                 | 5.18   | 75464.1  | 1070         | 100                  | 28         | 15.05   | 99.55/86.18            |                                      |
| 1807 | gi|124245039| Chloroplast HSP70                                 | 5.18   | 75464.1  | 1050         | 100                  | 27         | 12.82   | 97.76/79.11            |                                      |
| 2808 | gi|6911155  | Heat shock protein 50                             | 5.07   | 71843.3  | 570          | 100                  | 25         | 5.65    | 95.26/76.54            |                                      |
| 3101 | gi|255550363| Groes chaperonin                                  | 8.89   | 26582.2  | 73           | 95.384               | 2          | 17.20   | 25.06/28.38            |                                      |
| 3802 | gi|4042753  | Translation elongation factor EF-G                | 5.04   | 77865.6  | 89           | 99.894               | 14         | 5.19    | 92.84/99.06            |                                      |
| 4202 | gi|255550363| Groes chaperonin                                  | 8.89   | 26582.2  | 73           | 95.384               | 2          | 4.46    | 91.70/86.91            |                                      |
|      |      | Peptidase enzymes                                 |        |          |               |                      |            |         |                        |                                      |
| 2807 | gi|9759033  | Acyl-peptide hydrolase-like                       | 5.08   | 76116.9  | 160          | 100                  | 10         | 5.33    | 92.74/65.70            |                                      |
| 2905 | gi|297742722| Oligopeptidase B                                  | 5.21   | 79684.1  | 125          | 100                  | 9          | 2.16    | 68.83/56.15            |                                      |
| 3807 | gi|307136309| Serine-type endopeptidase                         | 5.15   | 83277.2  | 145          | 100                  | 13         | 2.55    | 84.32/37.22            |                                      |
Table 1 Glucohxaose and ROS modulators-regulated proteins (Continued)

| Protein ID | Accession | Name                        | p | IC | IC (S/S) | p | IC (S/S) |
|------------|-----------|-----------------------------|---|----|----------|---|----------|
| 3901       | gi|29747222 | Oligopeptidase B            | 5.21 | 79684.1 | 125 | 100 | 9 | 1.55 | 77.53/44.01 |
| 4801       | gi|225468332 | Similar to oligopeptidase A | 5.61 | 58732.5 | 81  | 99.234 | 9 | 2.20 | 99.63/62.75 |
| 4802       | gi|25547579 | Oligopeptidase A, putative   | 5.71 | 88118.8 | 129 | 100 | 13 | 2.13 | 99.48/44.81 |
| 4903       | gi|255537515 | Aminopeptidase, putative    | 6.04 | 98135.3 | 131 | 100 | 15 | 11.40 | 98.35/65.03 |
| 5902       | gi|255537515 | Aminopeptidase, putative    | 6.04 | 98135.3 | 304 | 100 | 15 | 10.90 | 98.02/46.32 |
| 5903       | gi|25083482 | Putative aminopeptidase     | 5.43 | 99495.2 | 252 | 100 | 15 | 15.04 | 98.38/77.61 |
| 5904       | gi|255537515 | Aminopeptidase, putative    | 6.04 | 98135.3 | 250 | 100 | 18 | 13.49 | 99.19/50.26 |

**Protein phosphatase**

| Protein ID | Accession | Name                        | p | IC | IC (S/S) | p | IC (S/S) |
|------------|-----------|-----------------------------|---|----|----------|---|----------|
| 0303       | gi|15240071 | Putative protein phosphatase 2C | 5.6 | 44302 | 72  | 94.7 | 3 | 2.46 | 99.15/49.70 |

**Antioxidation proteins**

| Protein ID | Accession | Name                        | p | IC | IC (S/S) | p | IC (S/S) |
|------------|-----------|-----------------------------|---|----|----------|---|----------|
| 1402       | gi|18874402 | Galactin synthase            | 4.81 | 38608.1 | 130 | 100 | 5 | 7.62 | 93.74/14.20 |
| 1802       | gi|11559422 | Disulfide isomerase          | 5.07 | 37249 | 589 | 100 | 20 | 3.84 | 89.41/74.09 |
| 1803       | gi|11559422 | Disulfide isomerase          | 5.07 | 37249 | 589 | 100 | 20 | 3.38 | 85.47/57.64 |
| 1805       | gi|11559422 | Disulfide isomerase          | 5.07 | 37249 | 589 | 100 | 20 | 3.01 | 91.93/51.20 |
| 1806       | gi|11559422 | Disulfide isomerase          | 5.07 | 37249 | 589 | 100 | 20 | 3.47 | 94.47/68.43 |
| 2105       | gi|240252434 | NIF-like protein             | 6.24 | 67894.1 | 69  | 89.179 | 10 | 4.07 | 11.20/34.32 |
| 2107       | gi|297842615 | Glutathione S-transferase    | 5.76 | 75307.3 | 101 | 99.993 | 8 | 2.22 | 67.69/9.46 |
| 3808       | gi|34157960 | Betaine-aldehyde dehydrogenase | 5.25 | 55339.3 | 602 | 100 | 16 | 4.58 | 77.64/59.24 |
| 5804       | gi|124057819 | Raffinose synthase           | 5.42 | 87904.5 | 96  | 99.975 | 17 | 391.29 | 99.03/60.22 |
| 7204       | gi|11763160 | Carbonic anhydrase           | 6.3 | 10976.5 | 175 | 100 | 5 | 225.27 | 80.50/99.93 |
| 7302       | gi|15222954 | Thioredoxin-like protein CDSP32 | 8.65 | 33948.5 | 253 | 100 | 8 | 1.59 | 26.05/52.03 |
| 8202       | gi|11763160 | Carbonic anhydrase           | 6.3 | 10976.5 | 257 | 100 | 7 | 6.96 | 71.19/80.24 |

**Others**

| Protein ID | Accession | Name                        | p | IC | IC (S/S) | p | IC (S/S) |
|------------|-----------|-----------------------------|---|----|----------|---|----------|
| 3803       | gi|224065421 | Peptidylprolyl isomerase     | 5.03 | 64379.7 | 105 | 99.997 | 9 | 5.19 | 94.72/75.20 |
| 5703       | gi|108710583 | Adenylosuccinate synthetase  | 9.07 | 51473.6 | 247 | 100 | 7 | 3.04 | 70.41/61.06 |
| 5711       | gi|255578102 | Imidazole glycerol phosphate synthase subunit hisf | 6.62 | 65225.3 | 496 | 100 | 16 | 1.88 | 55.65/64.14 |
| 5803       | gi|9759324 | 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase | 5.89 | 80394.2 | 700 | 100 | 25 | 3.81 | 99.30/74.98 |
| 5812       | gi|22548296 | PREDICTED: hypothetical protein | 6.55 | 106446.4 | 183 | 100 | 10 | 117.24 | 75.18/83.99 |
| 7406       | gi|18401429 | N-carbamoylputrescine amidase | 5.71 | 33683 | 242 | 100 | 6 | 6.86 | 92.78/63.85 |
| 7606       | gi|255562088 | Transaminase mtnE            | 6.95 | 50909.2 | 346 | 100 | 10 | 1.77 | 54.52/42.84 |
photosynthesis and pentose phosphate pathway proteins, which supplied enough NADPH for generating ROS by NADPH oxidase. An important protein, phosphatase PP2C, was identified in our study. Seventy-six PP2C-type phosphatase candidates were identified in Arabidopsis and divided into 10 groups [26]. The well-studied PP2C genes, ABI1 and ABI2, are associated with ABA signaling, which involves the closing of stomata by activated ICa Ca2+ Channels induced by ROS [27]. The two genes act differently: abi1-1 interrupted NADPH oxidase-related ROS generation and abi2-1 affected the activation of downstream ICa Ca2+ Channels [28]. In our study, PP2C 80 was identified as a glucohexaose-induced ROS-related protein, which may function either in processes related to ROS generation or in signal output, which should be investigated in a future study.

As ROS are toxic for pathogens and for plants themselves, plants must have antioxidant mechanisms for protecting themselves while still killing the pathogens. In our study, we identified eight antioxidation proteins: galactinol synthase, raffinose synthase, NifS-like protein, thioredoxin-like protein CDSP32, disulfide isomerase, glutathione S-transferase, betaine-aldehyde dehydrogenase and carbonic anhydrase. Galactinol synthase and raffinose synthase are important enzymes for the synthesis of the raffinose family of oligosaccharides, which are important for protecting plants during the stress response [29-31]. Nishizawa et al. found that galactinol and raffinose protected plants from oxidative stress by removing scavenging hydroxyl radicals [30]. The active center of thioredoxins comprises four amino acids, Cys-Gly-Pro-Cys, which can reduce disulfide bridges to protect plants from oxidative damage [32]. We identified a 32KDa protein thioredoxin, CDSP32. Rey et al. found that the six targets of the overexpression mutants of thioredoxin CDSP32 are involved in a strong resistance to oxidative stress. As a result, compared with other proteins in the thioredoxin family, thioredoxin CDSP32 is presumed to function mainly in antioxidative stress [33]. The reduction of disulfide bridges of thioredoxins is accompanied by an iron-sulfur cluster [34] and another identified protein, NifS-like protein, is important for iron-sulfur cluster synthesis [35]. In addition, the disulfide bridges of thioredoxins are catalyzed by an important chaperone, disulfide isomerase, which was also identified in our research [36]. We identified another three proteins in our research, glutathione-S-transferase, betaine-aldehyde dehydrogenase and carbonic anhydrase. These three are all antioxidative stress proteins, functioning through glutathione [37], betaine [38] and radical scavenging, respectively [39]. These antioxidative stress proteins are further evidence for the induction of ROS accumulation by glucohexaose treatment and may represent the plant protective mechanism induced during oxidative stress.

Discussion

ROS signaling play an important role in resistance to pathogens as signal molecules in large variety of plant species. In this study, we noticed both H2O2 and O2- accumulation in cucumber cotyledons is a part of glucohexaose-induced resistance. Normally, there are two phases of ROS accumulation induced by pathogen but only one phase of ROS accumulation induced by elicitors [40]. The glucohexaose elicitor shares similar mechanism with elicitors, which accumulated shortly after treatment and reached the peak level at about 5 h after treatment. DPI can totally repress the generation of H2O2 and O2- and proteomic analysis showed the increasing level of enzymes is relevant to synthesis of NADPH. As a result, the generation of cucumber ROS induced by glucohexaose elicitor is mainly produced by NADPH oxidase. NADPH oxidase appeared from moss and strongly expanded in vascular plants [16]. Our study indicates similar mechanism of ROS generation in cucumber.

We have identified an interesting protein PP2C 80 in our study. It is well known that PP2Cs are important negative regulators in ABA signaling. ABA is a hormone involving stress tolerance. ABA can induce stomata closing and ROS generation but NADPH oxidase double mutant atrbohD/F cannot [41]. The activity of two famous PP2Cs ABI1 and ABI2 are repressed by H2O2 in Arabidopsis [42,43]. In our study, the level of PP2C 80 increased after H2O2 accumulation. We have no idea whether the PP2C is involved in ABA signaling and why it accumulate after H2O2 accumulation. But it is an indication that glucohexaose induced resistance may have relationship with ABA signaling.

The identification of thioredoxin could contribute to the regulation of ROS level. Scavengers glutathione-S-transferase, betaine-aldehyde dehydrogenase and carbonic anhydrase are increased after glucohexaose treatment. Thioredoxin also increase after ROS accumulation in Arabidopsis, soybeans and potatoes [44-46]. Our study showed similar mechanism in cucumber. Meanwhile, SOD, POD and APX activities are increased in our study. The scavengers of ROS may function after ROS accumulation to prevent further damage to plant cells. We also found scavengers CAT and GPX decreased after glucohexaose treatment. They may have functions in ROS accumulation.

Concluding remarks

Our study detected the accumulation of ROS is a part of mechanism of glucohexaose induced resistance in cucumber cotyledons. NADPH oxidase is in charge of the main generation of the rapidly output of ROS. ROS scavengers’ activities change in the progress to regulate ROS level. Thirty-seven up-regulate proteins were identified after glucohexaose treatment and repressed
by DPI, which are involved in photosynthesis, respiration, translation, phosphorylation and antioxidation. PP2C might play a crucial role in processes related to ROS generation and signal transduction, and antioxidation proteins increased after glucosehexaose treatment, indicating the involvement of a self-protection mechanism in the process. It will be interesting to find out the regulatory mechanism underlying the induction of ROS-targeting proteins via ROS, and provide clues concerning the mechanism of glucosehexaose-induced resistance and a theoretical basis for developing safe farm chemicals for vegetable production.

Additional file

Additional file 1: Figure S1. 2D maps of cucumber cotyledon proteins with different treatment and the spots identified. A, Control group, fraction F1; B, the cucumber cotyledons treated with 50 μg/mL glucosehexaose for five hours, fraction F1; C, Before treated with 50 μg/mL glucosehexaose, DPI were incubated for four hours, fraction F1; D, Before treated with 50 μg/mL glucosehexaose, DMTU were incubated for four hours, fraction F1; E, Control group, fraction F2; F, the cucumber cotyledons treated with 50 μg/mL glucosehexaose for five hours, fraction F2; G, Before treated with 50 μg/mL glucosehexaose, DPI were incubated for four hours, fraction F2; H, Before treated with 50 μg/mL glucosehexaose, DMTU were incubated for four hours, fraction F2. Figure S2. Differential protein spots and their relative abundance. All identified protein spots’ Representative differential protein spots and their relative abundance information.

Abbreviations
H₂O₂: Hydrogen peroxide; O²−: Superoxide; DPI: Diphenyleneiodonium chloride; DMTU: Dimethylthiourea; SOD: Superoxide dismutase; CAT: Catalase; APX: Ascorbate peroxidase; GPX: Glutathione peroxidase; MDA: Malonaldehyde; POD: Peroxidase; DAB: Diaminobenzidine; ABA: Abscisic acid; SA: Salicylic acid; JA: Jasmonic acid; TCA: Tri chloroacetic acid; TBP: Tributyl phosphate; Ph: Glucosehexaose.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
HF is responsible for experimental design and manuscript revising. YH measured the H₂O₂ and O²− contents; run the 2D gels; analyzed 2D image data. NL and CL measured the contents of scavenger enzymes activity. YY and SC is responsible for mass spectrometry analysis. All authors read and approved the final manuscript.

Acknowledgements
All the authors thank Prof. J Ning for providing glucosehexaose, Dr. Haijiao Wang for revising this paper and Shanghai Applied Protein Technology Co. Ltd for protein identification with MALDI-TOF-TOF MS. At last, thank all my friends who have given us so much help.

Author details
1College of Bioscience and Biotechnology, Shenyang Agricultural University, Shenyang 110866, PR China. 2Key Laboratory of Protected Horticulture of Ministry of Education, Shenyang Agricultural University, Shenyang 110866, PR China. 3State Key Laboratory of Genetic Engineering and Institute of Plant Biology, School of Life Sciences, Fudan University, Shanghai 200433, PR China. 4Fruit and Silkworm Administrative Station of Liaoning Province, Shenyang 110866, PR China.

Received: 17 January 2014 Accepted: 1 May 2014 Published: 17 June 2014

References
1. Haiyan F, Baoy L, Chunmao L, Tianlai L, Baoli Z: Study on cucumber plant resistance introduced by glucosehexaose against Pseudoperonospora cubensis disease. Plant Prot 2003, 29:14–16.
2. Yuhun H, Chunfei W, Deliwei Z, Leena T, Yang Y: Proteomic analysis of glucosehexaoseducedresistantetodowny mildew in Cucumis sativus. Aust J Crop Sci 2013, 7(1):1235–1238.
3. Lamb C, Dixon RA: The oxidative burst in plant disease resistance. Annu Rev Plant Physiol Plant Mol Biol 1997, 48:251–275.
4. Doke N: Involvement of superoxide anion generation in the hypersensitive response of potato tuber tissues to infection with an incompatible race of Phytophthora infestans and to the hyphal wall components. Plant Pathol Lab 1983, 28:345–357.
5. Aveyanov A: Oxidative burst and plant disease resistance. Front Biosci (Elite Ed) 2009, 1:142–152.
6. Orozco-Cardenas M, Ryan CA: Hydrogen peroxide is generated systemically in plant leaves by wounding and systemic via the octadecanoid pathway. Proc Natl Acad Sci USA 1999, 96:6553–6557.
7. Wojtaszek P: Oxidative burst: an early plant response to pathogen infection. Biochem J 1997, 322(Pt 1):661–692.
8. Neill S, Desikan R, Hancock J: Hydrogen peroxide signalling. Curr Opin Plant Biol 2002, 5:388–395.
9. Moller IM, Sweetlove LJ: ROS signalling–specificity is required. Trends Plant Sci 2010, 15:370–374.
10. Bolwell GP, Davies DR, Gerrish C, Auh CK, Murphy TM: Comparative biochemistry of the oxidative burst produced by rose and French bean cells reveals two distinct mechanisms. Plant Physiol 1998, 116:1379–1385.
11. Papadalakis AK, Rou belakis-Angelakas K: The generation of active oxygen species differs in tobacco and grapevine mesophyll protoplasts. Plant Physiol 1999, 121:197–206.
12. Berna A, Bernier F: Regulation by biotic and abiotic stress of a wheat germin gene encoding oxalate oxidase, a H₂O₂-producing enzyme. Plant Mol Biol 1999, 39:539–540.
13. Yoda H, Hiroi Y, Sano H: Polyamine oxidase is one of the key elements for oxidative burst to induce programmed cell death in tobacco cultured cells. Plant Physiol 2006, 142:193–206.
14. Bindschedler LV, Dewdney J, Biele KA, Stone JM, Asai T, Plotnikov J, Denoux C, Hayes T, Gerrish C, Davies DR, Ausubel FM, Bolwell GP: Peroxidase-dependent apoplastic oxidative burst in Arabidopsis required for pathogen resistance. Plant J 2006, 47:851–863.
15. Daudi A, Cheng Z, O’Brien JA, Mammaella N, Khan S, Ausubel FM, Bolwell GP: The apoplastic oxidative burst peroxidase in Arabidopsis is a major component of pathogen-triggered immunity. Plant Cell 2012, 24:275–287.
16. Mittler R, Vanderauera S, Suzuki N, Miller G, Tognetti VB, Vandepoele K, Gollery M, Shulaev V, Breusegem F: Reactive oxygen species during plant-microorganism early interactions. J Integr Plant Biol 2010, 52:195–204.
17. Scheler C, Durner J, Astier J: Nitric oxide and reactive oxygen species in plant biotic interactions. Curr Opin Plant Biol 2013, 16:34–35.
18. Soares NC, Wojtkowska J, Jackson PA: The apoplastic oxidative burst peroxidase in Arabidopsis is a crucial component of pathogen-triggered immunity. Plant Cell 2012, 24:747–755.
19. Mittler R, Vanderauera S, Suzuki N, Miller G, Tognetti VB, Vandepoele K, Gollery M, Shulaev V, Breusegem F: Reactive oxygen species during plant-microorganism early interactions. J Integr Plant Biol 2010, 52:195–204.
20. Scheler C, Durner J, Astier J: Nitric oxide and reactive oxygen species in plant biotic interactions. Curr Opin Plant Biol 2013, 16:34–35.
21. Soares NC, Wojtkowska J, Jackson PA: The apoplastic oxidative burst peroxidase in Arabidopsis is a crucial component of pathogen-triggered immunity. Plant Cell 2012, 24:747–755.
22. Mittler R, Vanderauera S, Suzuki N, Miller G, Tognetti VB, Vandepoele K, Gollery M, Shulaev V, Breusegem F: Reactive oxygen species during plant-microorganism early interactions. J Integr Plant Biol 2010, 52:195–204.
23. Scheler C, Durner J, Astier J: Nitric oxide and reactive oxygen species in plant biotic interactions. Curr Opin Plant Biol 2013, 16:34–35.
24. Soares NC, Wojtkowska J, Jackson PA: The apoplastic oxidative burst peroxidase in Arabidopsis is a crucial component of pathogen-triggered immunity. Plant Cell 2012, 24:747–755.
25. Kruger NJ, von Schaewen A: The oxidative pentose phosphate pathway: structure and organisation. *Curr Opin Plant Biol* 2003, 6:236–246.

26. Schweighofer A, Hirt H, Meskiene I: Plant PP2C phosphatases: emerging functions in stress signalling. *Planta* 2004, 219:236–243.

27. Ma Y, Szostkiewicz I, Korte A, Moe D, Yang Y, Christmann A, Grill E: Regulators of PP2C phosphatase activity function as abscisic acid sensors. *Science* 2005, 314:1064–1068.

28. Murata Y, Pei Z-M, Morii IC, Schroeder J: Abscisic acid activation of plasma membrane Ca2+ channels in guard cells requires cystolic NAD(P)H and is differentially disrupted upstream and downstream of reactive oxygen species production in abI1-1 and abi2-1 protein phosphatase 2C mutants. *Plant Cell* 2001, 13:2513–2523.

29. Wang Z, Zhu Y, Wang L, Liu X, Liu Y, Phillips J, Deng X: Inactivation of porcine kidney NADPH oxidase AtrbohD and AtrbohF by hydrogen peroxide. *Chem Biol* 2003, 10:419–437.

30. Nishizawa A, Yabuta Y, Shigeoka S: Galactinol and raffinose constitute a novel function to protect plants from oxidative damage. *Plant Physiol* 2008, 147:1251–1263.

31. Zhao TY, Thacker R, Corum JW III, Snyder JC, Meeley RB, Obendorf RL, Downie B: Expression of the maize GALACTINOL SYNTHASE gene family: (i) Expression of two different genes during seed development and germination. *Physiol Plant* 2004, 121:634–646.

32. Bilund H, Gleason FK, Holmgren A: Structural and functional relations among thioredoxins of different species. *Proteins: Struct Funct Bioinform* 1991, 11:13–28.

33. Rey P, Cuine S, Eymery F, Garin J, Court M, Jacquot JP, Rouhier N, Philips J, Deng X: A WRKY transcription factor participates in dehydration tolerance in *Boea* hygrometrica by binding to the W-box elements of the galactinol synthase (BhGolS1) promoter. *Planta* 2009, 230:1155–1166.

34. Schurmann P: Antioxid Redox Signal 2008, 11:139–147.

35. Förster J, Büchel C, Wiedenbeck S, Jezowska-Bzdowska K, Achtelik K, Zeitler H, Budreck C: The oxidase activity of the AtNTRX1 protein is required for nodule development to reduce reactive oxygen species levels in soybean roots. *Plant Physiol* 2005, 139:1881–1889.

36. Vieira Dos Santos C, Rey P: Plant thioredoxins are key actors in the oxidative stress response. *Plants* 2014, 3:1–34.

Cite this article as: Hao et al.: Proteomic analysis of Cucumis sativus cotyledons after glucohexaose treatment as a part of ROS accumulation related resistance mechanism. *Proteome Science* 2014 12:34.