Proteomic analysis of glucohexaose induced resistance to downy mildew in Cucumis sativus

Yuhan HAO¹,⁸, Chunfei WU¹,⁸, Dawei ZHAO¹, Leena Thung³, Yang YU¹, Haiyan FAN¹,²,*.¹

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
³School of Food and Agriculture Science, The University of Queensland, Brisbane 4072, Australia

*Corresponding author: hyfan74@163.com
#Co-first authors with equal contributions

Abstract

Glucohexaose, as one of synthetic oligosaccharides, induces the resistance response to protect plants from pathogen infection by inducing the systemic acquired resistance-like (SAR-like) response. To study the molecular mechanism of glucohexaose induced resistance, we investigate the physiological, biochemical and proteomic changes after glucohexaose treatment. The results shows cucumber plants had the highest protection level of 66.79% 48 h after the third times of 10 μg mL−1 glucohexaose treatment. Significant increases in chlorophyll, photosynthetic rate, soluble sugar, leave dry weight and H₂O₂ were observed after glucohexaose treatment. Eighteen up-regulated proteins were identified by MALDI-TOF/TOF in glucohexaose-treated plants, predicted to be involved in photosynthesis, photosynthesis, oxidative burst, transcriptional regulation, signal transduction and pathogen defense processes. The identification of up-regulated proteins involved in photosynthetic processes is a significant finding which suggests that a boost in metabolites is required for reparation of resources towards defense mechanisms. The proteins which responded to glucohexaose also included those associated with oxidative burst response, such as APX and isocitrate dehydrogenase. More comprehensive studies about the link between the molecular mechanisms regulated by ROS mediated photosynthesis and cucumber induced resistance by glucohexaose, are necessary in the future to broaden our understanding of induced resistance in plants.

Keywords: glucohexaose; cucumber; induced resistance; 2-DE; MALDI-TOF/TOF.

Abbreviation: APX_cytosolic ascorbate peroxidase; BRs_Brassinosteroids; GO_glycolate oxidase; H₂O₂_hydrogen peroxide; HPR_NADH-dependent hydroxypyruvate reductase; HR_hypersensitive defense response; ICDH_NADP(+) dependent isocitrate dehydrogenase; PKC_protein kinase C; ROS_reactive oxygen species; RBP_RNA binding protein; RubPCase_ribulose bisphosphate carboxylase; sHsp_small heat shock proteins.

Introduction

Cucumber (Cucumis sativus L.) is an important vegetable crop grown worldwide. Cucumber downy mildew, caused by Pseudoperonospora cubensis, is an important disease in most cucumber production areas. It adversely affects plant growth and causes severe yield losses. Spraying the plants with various chemicals, including oligosaccharides, induces resistance to the fungus infection by activating pathogenesis-related proteins. Oligosaccharides are believed to be elicitors of the plant’s natural defense mechanisms. When pathogens such as bacteria, fungi, and viruses challenge a plant cell, it develops complex defense mechanisms. Numerous studies have shown that the cell wall of plants can be digested into oligosaccharides by specific enzymes (Fry et al., 1993). These oligosaccharides act as signaling molecules to induce early defense systems (Montesano et al., 2003). Glucohexaose is a synthetic oligosaccharide which was synthesized by Research Center for Eco-Environmental Science, Chinese Academy of Science. The Structural formulas showed in Fig. 1. Previously, we detected the activity of glucohexaose and analyzed its resistance to some plants. The study had shown that glucohexaose can protect plants from various pathogen, such as Erysiphe cichoracearum, Pseudomonas syringae pv. Lachrymans, Botrytis cinerea and Xanthomonas campestris pv. Viscaria, and protects plants from pathogen infection by inducing the systemic acquired resistance-like (SAR-like) response (Li et al., 2001). To date, only a few studies investigated oligosaccharides elicitors. The existence of homologous elicitor-binding sites within a plant taxonomic family may provide preliminary evidence for putative evolutionary relationships in pathogen perception mechanisms among plants (Cosio et al., 1996). A 75kDa protein identified by affinity labeling represents a functional receptor for an N-acylchitooligosaccharide elicitor (Ito et al., 1997). Highly conserved signaling elements also appear to be employed in elicitor signal transduction, such as G-proteins, calcium transients, ion channels, reactive oxygen species, nitric oxide, protein kinases and phosphatases, cyclic GMP, cyclic ADP ribose and fatty acid derivatives (Scheel, 1998). In addition, ion-related changes specifically in the efflux of Na⁺ and K⁺ ions might be closely associated with the signal transduction system for defense responses at the tissue level (Amano et al., 1997). Finally, expression in soybean of a yeast cDNA encoding the cinnamate 4-hydroxylase P450 induced the glyceollin biosynthesis pathway (Schopter et al., 1998). Thus, although genetic
studies have identified some important components of resistance pathways, the mechanisms involved in the glucohexaose induced SAR-like response in cucumber remain unclear. Based on the concept and experimental evidence that mRNA levels do not always correspond with protein levels (Gygi et al., 1999; Ideker et al., 2001), and proteins ultimately carry out the functions, so proteomic analysis through protein profiling becomes equally important to understand how genes/proteins are regulated. Plant proteomics as a tool to compare protein changes has rapidly developed over the last several years and has provided a large amount of information about the individual proteins involved in specific biological responses. However, there is a very limited number of published proteomic studies on cucumbers (C. sativus L.) including: cucumber leaf proteomics in response to Acibenzolar-S-Methyl (ASM) (Saligrama et al., 2008); trichoderma-treated plants becoming more resistant to pathogen attacks (Segarra et al., 2007); the responses of cucumber seedlings to salt stress (Du et al., 2010); β-amylase isozymes in dehydrated cucumber cotyledons (Todaka et al., 2007); phloem exudates (Walz et al., 2004) and xylem sap (Buhtz et al., 2004); and the response of cucumber seedlings to cucumber powdery mildew fungus (Fan et al., 2009; Fan et al., 2009). As a result, several confirmed and putative defense-related proteins were identified. To date, however, no proteomic studies have been undertaken to reveal protein abundance regulated by glucohexaose. Here we investigated the effect of glucohexaose induction on cucumber resistance against P. cubensis. Differential proteomics of the C. sativus L. cv. Shandong mici leaf are reported to reveal the metabolic reprogramming following glucohexaose induced treatment and discuss how it prevents damage in cucumber plants after pathogen inoculation. We also report the physiological and biochemical changes in cucumber plants as a response to fungus infection.

Results and discussion

Relative efficacy of glucohexaose-mediated resistance to P. cubensis in cucumber

Firstly, the relative efficacy of the glucohexaose treatments to protect cucumber from fungal infection was determined. The data showed that the number of glucohexaose sprays had a significant influence on cucumber resistance to P. cubensis. As shown in Table 1, the relative efficacy of the glucohexaose induced protective mechanisms gradually increased with the number of glucohexaose treatments. The relative efficacy of the treatment reached a maximum of 65.40% after three treatments with 10 μg.mL\(^{-1}\) glucohexaose. As shown in Table 2, the degree of resistance also increased with the length of time between treatments. In the first investigation, the relative efficacy of seven day treatment intervals reached a maximum of 66.49%, which was higher than that for two or five day treatment intervals (41.4% and 20.64%, respectively). In the second investigation, two days after the first investigation, the relative efficacy of the seven day treatment intervals remained at 64.33%, but the relative efficacies of the two and five day treatment intervals declined to 19.28% and 29.69%, respectively, implying that the time interval between glucohexaose applications has a strong effect on cucumber resistance. The effect of the time elapsed between treatment and inoculation on induced resistance against P. cubensis is shown in Fig. 2. The result shows that cucumber plants had the highest protection level of 66.79% 48 h after the last glucohexaose treatment. The level of resistance declined gradually after 48 h, and protection against P. cubensis was reduced to only 11.11% at 144 h after the final glucohexaose treatment. The present study investigated the relative efficacy of inducing resistance to downy mildew fungus infection in cucumber by glucohexaose treatment. This results indicated that the concentration of glucohexaose, the number of glucohexaose sprays and the length of time between treatments have significant influence on cucumber resistance to P. cubensis. And the level of resistance decline gradually followed the time elapsed after glucohexaose treatment. The most effective condition is three treatments with 10 μg.mL\(^{-1}\) glucohexaose and seven day treatment intervals, we use this condition for studies below.

Physiological and biochemical changes in response to glucohexaose treatment

In this study, a significant increase in total chlorophyll content was observed in the glucohexaose treated plants (Table 3), and the photosynthetic rate was always significantly higher than the control (Fig. 3). A dramatic decline in chlorophyll and photosynthetic activity were described for several plant species infected by oomycetes causing downy mildew (Ingram, 1981; Lindenthal et al., 2005), and the chlorophyll content of cucumber was positively correlated with resistance to downy mildew (Sharma et al., 2004). Thus, treatment with 10 μg.mL\(^{-1}\) glucohexaose appears to elevate chlorophyll contents and photosynthetic rate to compensate for the decrease caused by fungus infection. There was no significant change in seedling height, however, leave dry weight increased in the induced compared to control plants (Table 3). Lower concentrations of soluble sugars in downy mildew infected leaves have also been reported (Mandal et al., 2009; Yun, 1993). An increase in soluble carbohydrate contents was noted in glucohexaose treated plants (Table 3). Thus, treatment with glucohexaose prior to fungus inoculation may prevent decreases in the soluble carbohydrate content. These results indicate the potential to enhance tolerance of cucumber plants to P. cubensis through induction of natural resistance metabolites.

Changes in protein abundance in response to glucohexaose treatment

To elucidate the proteomic response to glucohexaose treatment in downy mildew susceptible C. cucumis plants, a comparative study of the cucumber leaf proteome of un-inoculated and 10 μg.mL\(^{-1}\) glucohexaose treated plants (proteins isolated 48 h after treatment) was performed. Representitive 2-DE gels are shown in Fig. 3. Over 800 protein spots per gel were revealed for each treatment and 45 protein spots showed expression changes (>2-fold or <−2-fold). Twenty five up-regulated spots were analyzed by MALDI-TOF/TOF MS. Among these proteins, 18 were well matched to proteins in the NCBI nr database (Fig. 4, Table 4). Spots which showed up at the same relative positions in different gels were regarded as the same protein. The differentially expressed proteins identified could be divided into six functional groups based on homologies, consisting of proteins involved in photosynthesis (protein 3, 7, 12, 13, 15, 2, 9, 14 and 16), photosynthesis (protein 2, 8, 11 and 14), oxidative burst (protein 4 and 10), transcriptional regulation (protein 6 and 18), signal transduction (protein 17) and other pathogenesis-related processes (protein 1 and 5). Among the proteins identified, four are involved in light-
dependent reactions of photosynthesis, including ATP synthase (protein 3), Chlorophyll a-b binding protein (protein 7 and 12), oxygen-evolving complex protein (protein 15) and Chromoplast-specific carotenoid-associated protein (protein 13). The accumulation of these four proteins suggests that glucohexaose treatment up-regulates photosynthetic activity in cucumber leaves (Fig.3). The chlorophyll a-b binding proteins are involved in harvesting light energy and transferring it to photochemical reaction centers (Green et al., 1991). The oxygen-evolving complex, along with manganese, chloride and calcium, appears to form the simplest structure, involved in the photooxidation of water during the light reactions of photosynthesis (Ghanotakis et al., 1987). The chloroplast-specific carotenoid-associated protein is a component of the pigment complex in chromoplasts which is involved in chromoplast biogenesis and carotenoid biosynthesis. During the first stages of photosynthesis, light energy is converted into chemical energy, in the form of the energy-carriers ATP and NADPH (Smirra et al., 1993). The accumulation of Chlorophyll a-b binding proteins, oxygen-evolving complex proteins and Chromoplast-specific carotenoid-associated proteins would enhance the light reaction and transfer more photon energy to chemical energy. The increase in ATP synthase may be an indication that the light reaction has been up-regulated to produce more energy to counter pathogen-induced stress. Three proteins were found to be involved in the dark reaction of photosynthesis, including ribulose bisphosphate carboxylase (RuBPCase; protein 2, 14), sedoheptulose-1,7-bisphosphatase (protein 9) and chloroplast latex aldolase-like protein (protein 16). These proteins were up-regulated 48 h after glucohexaose treatment (Fig.3). RuBPCase is the primary enzyme of the carbon fixation pathway in the chloroplast of C3 plants. Sedoheptulose-1,7-bisphosphatase and the chloroplast latex aldolase-like protein are two important enzymes in the Calvin cycle. Up-regulation of these three proteins may boost carbon dioxide fixation and increase the plant’s capacity to resist pathogen invasion. Downy mildew disease reduces chlorophyll substantially, resulting in severe disruptions to photosynthesis in cucumber and significant metabolic changes following P. cubensis infection have been reported (Sun et al., 2009; Sharma et al., 2004). In the study, maintaining relatively high chlorophyll content in glucohexaose-treated plants could ensure that plants can efficiently capture and convert light energy, and then improve photosynthetic rate. Proteomic analysis of glucohexaose treated plants showed enhancement of several proteins involved in the light and dark reactions of photosynthesis in the chloroplast. The revival of the photosynthetic machinery could be ensured even after the initial chlorophyll degradation caused by the fungus infection. Reactive Oxygen Species (ROS) are continuously produced as byproducts of aerobic metabolism or in response to biotic and abiotic stresses. Furthermore, they are considerably more abundant at the site of attempted invasion during the early stages of plant defense reactions which include the oxidative burst (Nanda et al., 2010). The extracellular hydrogen peroxide (H$_2$O$_2$) generated during oxidative burst, has been shown to play an essential role in signaling the development of the hypersensitive defense response (HR). Two processes induce reactive oxygen generation, aerobic metabolism and oxidation of NADPH. Protein 4, identified as an isocitrate dehydrogenase in this study, is an important source of NADPH. Recently, a study showed that isocitrate dehydrogenase activity increased after pathogen invasion (Cozzone, 2005). One of the primary functions of NADP(+) dependent isocitrate dehydrogenase (ICDH) is the control of cytosolic and mitochondrial redox balance and the cellular defense against oxidative damage.

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**Table 1.** The effect of the number of glucohexaose treatments on induced resistance against *P. cubensis*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of sprays</th>
<th>First investigation</th>
<th>Second investigation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Disease index</td>
<td>Relative efficacy*</td>
<td>Disease index</td>
</tr>
<tr>
<td>Control</td>
<td>--</td>
<td>28.82</td>
<td>34.99</td>
</tr>
<tr>
<td>10μg.mL$^{-1}$</td>
<td>1</td>
<td>24.47</td>
<td>15.00±4.08b</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>21.83</td>
<td>24.25±3.35c</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>9.97</td>
<td>65.40±7.69c</td>
</tr>
<tr>
<td>1μg.mL$^{-1}$</td>
<td>1</td>
<td>26.21</td>
<td>8.97±2.41b</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>24.75</td>
<td>14.12±3.61c</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>18.91</td>
<td>34.32±3.86d</td>
</tr>
</tbody>
</table>

Disease seedlings were treated once, twice, or three times with 1 μg.mL$^{-1}$ and 10 μg.mL$^{-1}$ glucohexaose, with 7 days between treatments. Deionized water was used to spray control plants. *Data are the mean of independent measurements of three replicates ± standard deviation (SD). Values followed by different letters are significantly different at 0.05% level.

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**Fig 1.** Structure pattern of glucohexaose.
Protein 10, identified as a cytosolic ascorbate peroxidase (APX), is the cytosolic H₂O₂-scavenging enzyme. In chloroplasts, H₂O₂ is reduced by APX using ascorbate as an electron donor. Oxidized ascorbate is then eliminated by monodehydroascorbate reductase in a series of catalytic reactions known as the Halliwell-Asada pathway (Davletova et al., 2005). In our study, the level of APX and ascorbate dehydrogenase increased considerably following glucohexaose treatment. Thus the production and the scavenging of ROS appear to be one of the important events which occur during glucohexaose-induced systemic acquired resistance in cucumber. To verify our conjecture that the oxidative burst is one of the major events which occurs during glucohexaose-induced responses in cucumber plants, the result shows that H₂O₂ concentration increased intensively 3h after glucohexaose treatment (Fig.5). This result suggested glucohexaose can induce oxidative burst in cucumber leaves which looks worthy of further research. Two proteins involved in photosrespiration: NADH-dependent hydroxypyruvate reductase (HPR; protein 8) and glycolate oxidase (GO; protein 11), were identified. HPR and GO are key enzymes in photosrespiration. Photosrespiration occurs when the CO₂ levels inside a leaf become low, it enables the plant to recruit CO₂ molecules lost during photosynthesis (Somerville et al., 1980; Somerville, 2001). It is widely accepted that photosynthesis influences a wide range of processes from bioenergetics, photosystem II function, and carbon metabolism to nitrogen assimilation and respiration. In particular, H₂O₂ mainly comes from the photosynthetic pathway in photosynthetic cells, photosrespiration makes a key contribution to cellular redox homeostasis. Formerly H₂O₂ was considered as a toxic molecule which can causes oxidative stress, but now it is recognized as a major signaling molecule in plant stress responses, particularly fungus infection (Moreno et al., 2005; Foyer et al., 2005). Chlorophyll damage caused by pathogenic attack could be prevented by glucohexaose treatment as stated above, and possibly metabolic reprogramming in chloroplast led to changes in redox state. The hypothesis is supported from the result that HPR and glycolate oxidase which are involved in photosrespiration, showed up-regulated in glucohexaose treated plants. It has been reported that photosrespiration plays a protective role against biotic stresses (Fujita et al., 2006; Noctor et al., 2002). Based on the data presented here, we suggest that some peroxisomal photosespiratory enzymes may be induced by glucohexaose and may provide protection against disease. Protein 6 was identified as a RNA binding protein (RBP). RBPs play major roles in post-transcriptional control of RNAs, which is a major way to regulate patterns of gene expression during development (Lee et al., 2006). Although it was not clear which gene encoded the RNA binding protein identified, it may activate the transcription of an important gene involved in defense responses in cucumber. Protein 18 has 100% identity with a retrotransposon protein identified as a putative polyprotein. Plant retrotransposons could be stress-induced generators of genomic diversity. In this study, we found that the retrotrans-
Table 3. Effects of glucohexaose on height, leaf dry weight, total chlorophyll and soluble carbohydrate of cucumber seedlings

<table>
<thead>
<tr>
<th>Time</th>
<th>Treatment</th>
<th>Height (cm)</th>
<th>% change</th>
<th>Dry weight (g·cm⁻²)</th>
<th>% change</th>
<th>Total chlorophyll (mg·g⁻¹fw)</th>
<th>% change</th>
<th>Soluble carbohydrate (mg·g⁻¹fw)</th>
<th>% change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-7d</td>
<td>Control</td>
<td>9.85±0.06a</td>
<td>0.039±0.004a</td>
<td>1.18±0.07a</td>
<td>0.75±0.07a</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>glucohexaose</td>
<td>9.93±0.07a</td>
<td>0.81</td>
<td>0.042±0.005a</td>
<td>1.94±0.09b</td>
<td>64.41</td>
<td>1.08±0.06a</td>
<td>44.00</td>
<td></td>
</tr>
<tr>
<td>2-7d</td>
<td>Control</td>
<td>15.58±0.09a</td>
<td>1.54</td>
<td>0.048±0.007a</td>
<td>1.09±0.09a</td>
<td>84.40</td>
<td>1.01±0.09a</td>
<td>144.55</td>
<td></td>
</tr>
<tr>
<td></td>
<td>glucohexaose</td>
<td>15.82±0.09b</td>
<td>0.063±0.007a</td>
<td>31.25</td>
<td>2.01±0.08b</td>
<td>144.55</td>
<td>2.47±0.12b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-7d</td>
<td>Control</td>
<td>24.95±0.09a</td>
<td>0.299±0.005a</td>
<td>0.96±0.11a</td>
<td>0.48±0.04a</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>glucohexaose</td>
<td>25.00±0.10a</td>
<td>0.20</td>
<td>0.037±0.006a</td>
<td>1.58±0.11b</td>
<td>65.10</td>
<td>0.77±0.10b</td>
<td>60.42</td>
<td></td>
</tr>
</tbody>
</table>

The first leaves of cucumber seedlings were treated with 10 μg.mL⁻¹ glucohexaose three times every seven days. Experiments were conducted after 7d of each glucohexaose treatment. 1-7d: the 7th day after the first treatment, 2-7d: the 7th day after the second treatment, 3-7d: the 7th day after the third treatment. *Data are the mean of independent measurements of three replicates ± standard deviation (SD). Values followed by different letters are significantly different at 0.05% level.

Fig 3. Effects of glucohexaose on the rates of photosynthesis in cucumber seedlings. The first leaves of cucumber seedlings were sprayed with 10 μg.mL⁻¹ glucohexaose every seven days for 21 days. Experiments were conducted after 1d and 7d of each glucohexaose treatment. 1-7d: the 1st day after the first treatment, 1-7d: the 7th day after the first treatment, 2-1d: the 1st day after the second treatment, 2-7d: the 7th day after the second treatment, 3-1d: the 1st day after the third treatment, 3-7d: the 7th day after the third treatment. Data are the mean of three independent measurements ± standard deviation shown by vertical error bars.

Protein 5 was the hypothetical protein OsI_19374, and it had high similarity (97%) with chloroplast heat shock protein 70 (gi|145388994). Small heat shock proteins (sHsps) are a diverse group of heat induced proteins that are especially abundant in plants and are known to be induced in response to short-term stress. HSP70 and related genes are involved in protein folding, modulation of signal transducers and controlling the biological activity of folded regulatory proteins, facilitating refolding and proteolytic degradation of non-native proteins. HSP70 may play a key role in disease resistance by acting as co-chaperones associated with stabilization of Rx protein levels (Lee et al., 2000). Our findings suggest that a high abundance of proteins related to light-dependent reaction and dark reaction of photosynthesis provides the plant with increased primary metabolites to reallocate to secondary metabolism, it is same as the report on SA induced resistance to Mungbean Yellow Mosaic India Virus in Vigna mungo (Kundu et al., 2011). The abundance of ROS related proteins, as observed in this study, demonstrates that the SAR may be induced by ROS generation after glucohexaose treatment. Involvement of photosynthesis in glucohexaose mediated resistance to downy mildew is indicated by the abundance of HPR and glycolate oxidase. H₂O₂ is generated as a by-product of photosynthesis and is recognized as a signaling molecule to trigger defense response. High abundance of several proteins...
involved in signal transduction, transcriptional regulation and plant defense responses may have contributed further to reduce the susceptibility of glucohexaose treated cucumber plants compared to the untreated plants. Overall, our results suggest that the enhancement of photosynthesis and production and scavenging of ROS are the major events which occur during glucohexaose induced systemic resistance acquired in cucumber. H$_2$O$_2$ has been found to enhance plant photosynthesis (Ozaki et al., 2009), elevated H$_2$O$_2$ levels resulting from enhanced NADPH oxidase activity are involved in the BR-induced stress tolerance and H$_2$O$_2$ plays a key role in BRs induced photosynthesis (Jiang et al., 2012). It is possible that glucohexaose can activate the continuous production of H$_2$O$_2$, and glucohexaose-induced H$_2$O$_2$ may induce changes in expression of photosynthetic genes. Further investigations of the link between the molecular mechanisms that ROS mediated photosynthesis regulate and glucohexaose induced resistance could broaden our understanding of induced resistance in plants.

### Materials and methods

#### Materials

*C. sativus* L. cv. Shandong mici, a cucumber cultivar known for its susceptibility to *P. cubensis*, was grown in humus pots (Peat moss: vermiculite=1:2) in the greenhouse for all experiments. Glucohexaose was synthesized at the Research Center for Eco-Environmental Science, Chinese Academy of Science. Following glucohexaose treatment, plants were inoculated with *P. cubensis*, the most important pathological fungus of cucumber.

#### Glucohexaose treatments

To determine the effect of the number of glucohexaose treatments on cucumber resistance, samples consisting of 30 one-month-old seedlings were treated once, twice, or three times with 1 μg.mL$^{-1}$ and 10 μg.mL$^{-1}$ glucohexaose, with 7 days between treatments.

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**Table 4.** Glucohexaose induced cucumber leaf proteins identified by MALDI MS and MS/MS analysis.

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>NCBI Accession</th>
<th>Protein Name [Species]</th>
<th>MW/ pl theor.</th>
<th>MW/ pl obs.</th>
<th>Score</th>
<th>NM/SC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>gi</td>
<td>175831161</td>
<td>hypothetical protein LOC_Os1g37630 [Oryza sativa Japonica Group]</td>
<td>94240/8.53</td>
<td>75293/5.62</td>
<td>75</td>
</tr>
<tr>
<td>2</td>
<td>gi</td>
<td>108951104</td>
<td>ribulose-1,5-biphosphatase carboxylase/oxygenase large subunit [Bornerosicosus simplex]</td>
<td>51370/6.00</td>
<td>61505/5.30</td>
<td>218</td>
</tr>
<tr>
<td>3</td>
<td>gi</td>
<td>146317661</td>
<td>ATP synthase subunit alpha.chloroplastic [Cucumis sativus]</td>
<td>55405/5.13</td>
<td>62877/5.29</td>
<td>218</td>
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<tr>
<td>4</td>
<td>gi</td>
<td>19171610</td>
<td>isocitrate dehydrogenase [Cucumis sativus]</td>
<td>46432/6.00</td>
<td>62727/5.61</td>
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<td>gi</td>
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<td>hypothetical protein OsL_19374 [Oryza sativa Indica Group]</td>
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<td>6</td>
<td>gi</td>
<td>25574263</td>
<td>RNA binding protein, putative [Ricinus communis]</td>
<td>65723/8.45</td>
<td>52594/5.08</td>
<td>86</td>
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<tr>
<td>7</td>
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<td>9587207</td>
<td>LHCII type I chlorophyll a/b-binding protein [Vigna radiata]</td>
<td>27950/5.13</td>
<td>52752/4.87</td>
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<tr>
<td>8</td>
<td>gi</td>
<td>118664</td>
<td>NADH-dependent hydroxypyruvate reductase [Cucumis sativus]</td>
<td>41908/5.95</td>
<td>51658/6.50</td>
<td>175</td>
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<tr>
<td>9</td>
<td>gi</td>
<td>229597543</td>
<td>sedoheptulose-1,7-bisphosphatase [Cucumis sativus]</td>
<td>42532/5.96</td>
<td>50918/5.09</td>
<td>126</td>
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<td>10</td>
<td>gi</td>
<td>1669585</td>
<td>cytosolic ascorbate peroxidase[Cucumis sativus]</td>
<td>27549/5.43</td>
<td>49000/5.56</td>
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<td>11</td>
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<td>260268351</td>
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<td>45612/6.53</td>
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a Numbers correspond to the 2-DE gels shown in Figs. 3. b Theoretical and observed MW(kDa) and pl values. c Number of mass values matched(NM) and the percentage of sequence coverage (SC).
Fig 4. Representative 2-DE gels and close-up views of gel regions showing differences in protein abundance following glucohexaose treatment compared to the control. Identified glucohexaose induced proteins are indicated by circles and numbers. These numbers on the 2D-gel correspond to the spot number in Table 4.
The whole plant was sprayed until runoff was seen. Deionized water was used to spray control plants. On the 2nd day after the final treatment, cucumber leaves were challenged with a suspension of 1×10⁶ conidia/mL of \textit{P. cubensis}. The first disease index was determined when the control groups were obviously infected and a second investigation was carried out two days later. To determine the effect of the interval between two glucohexaose treatments on resistance, 30 one-month-old seedlings were sprayed three times with 10 μg.mL⁻¹ glucohexaose with either two, five, or seven days between treatments. On the 2nd day after the final treatment, cucumber leaves were challenged as above. To investigate the time post-treatment when glucohexaose confers the most resistance, 30 one-month-old seedlings were sprayed with 10 μg.mL⁻¹ glucohexaose every 7 days for 21 days. Then, at 1d, 2d, 3d, 4d, 5d and 6d after the last treatment, cucumber leaves were challenged as above.

**Disease symptoms**

The disease progress of cucumber downy mildew was estimated on the basis of the severity of leaf scabs as follows: Grade 0: no scabs observed; Grade 1: less than 1/10 of leaf infected; Grade 3: 1/10 -1/4 of leaf infected; Grade 5: 1/4 -1/2 of leaf infected; Grade 7: 1/2 -3/4 of leaf infected; Grade 9: more than 3/4 of leaf infected.

Disease index= 100×(no. of diseased leaves of each grade×disease grade) / (total no. leaves×9)

Relative efficacy (%) = (disease index of control—disease index of treated plant) / disease index of control×100

**Physiological and biochemical experiments**

Physiological and biochemical experiments were conducted one and seven days after each glucohexaose treatment, the first leaves were used for experiments. Total chlorophyll, chlorophyll a and chlorophyll b were determined spectrophotometrically. Leaves were extracted in ethanol and acetone (v/v=1:1) and centrifuged at 4000 rpm for 10 min. After suitable dilution, the extinction coefficient of the supernatant was measured at wavelengths of 645 and 663 nm using a Hitachi U-3900 spectrophotometer (Japanese).

The soluble carbohydrate content was determined in the aqueous solution with anthrone sulfuric acid reagent. The rates of photosynthetic were determined using a Beckman 865 Analyzer (USA). The intracellular accumulation of H₂O₂ was determined using the fluorescent probes H₂DCFDA. Briefly, freshly cut, cucumber leaf epidermis were incubated with a solution containing Tris buffer (10mM Tris, 50mM KCl, pH 7.2), and 50 mM H₂DCFDA. The tissues were incubated for 20 min at room temperature in the dark and fluorescence was measured with excitation wavelength 488 nm and emission wavelength 525 nm by fluorescence spectrophotometer. Control groups were subjected to the same manipulation, except for the treatment with leaf epidermis.

**Protein extraction**

Cucumber leaves were treated every seven days with 10 μg.mL⁻¹ glucohexaose for 21 days. The first leaves were used for protein extraction 48 h after the final treatment. Cucumber leaves were ground in liquid nitrogen in a prechilled mortar, and then finely ground powder was collected in a 50 mL Falcon tube. 5 mL of 2% w/v DTT was added to 1 g of ground tissue. The homogenate was centrifuged for 30 min at 1000 rpm and the supernatant was removed to a new tube. The proteins in the supernatant were precipitated with cold 80% acetone at -20°C for 12 hours, then centrifuged at 12 000 rpm for 30 min. The pellet of precipitated proteins and debris was washed three times with 5 mL of cold 80% acetone. After centrifugation at 12 000 rpm for 20 min, pellets were air dried and stored at -80°C.

**Two-dimensional gel electrophoresis and Image analysis**

1 mL of lysis buffer (9.5 M Urea, 2 mol L⁻¹ Thiourea, 1% TBP, 1% DTT, 4% CHAPS, 1% Cock tail, 0.001% Bromophenol blue, 2% IPG buffer) was added to the 400 mg pellets. The protein concentration of the final supernatant was measured using BSA as the standard according to the Bradford method. Samples were used to rehydrate IPF strips, 24 cm in length with pH ranges of 4-7. Rehydration was done in a rehydration tray at 22°C under a layer of mineral oil. Strips were then assembled onto IEF and focused for 1 h at 250 V, 1 h at 500 V, 1 h at 1000 V, 4 h at 10000 V, and then holding at 10000V until 100 000 Vh was reached. The current was monitored and did not exceed 50mA/strip throughout the run. Before loading in the second dimension the strips were equilibrated for 2×10 min in 2×120 mL equilibration buffer containing 50 mM Tris-HCl (pH 8.8), 6 M Urea, 30% Glycerol, 2% SDS. DTT (10 mg/mL) was added to the first equilibrated buffer and iodoacetaamide (5 mg/mL) was added to the second equilibration. For the second dimension, the strips were loaded onto second dimension 12.5% SDS-PAGE gels. The gels were silver-stained according to Yan et al. (2000) with modifications. The silver-stained gels were digitalized with UMAX Power Look 2100XL (Maxium Tech.) and analyzed using PDQuest Advanced™ 2-D Analysis software, version 8.0.1(Bio-Rad). Only spots present in each of the three replicate gels of both groups were considered for subsequent analysis. Quantity changes were estimated for the spots between inner limit (+2-fold) and outer limits (>+2-fold or <-2-fold). Selected protein spots were subjected to in-gel digestion for identification by MALDI-TOF MS and MS/MS.
MALDI-TOF MS and MS/MS analysis and database search

Interesting spots were excised from gels with pipette tips and placed in Eppendorf tubes and washed three times with milliQ water. Then the gel was washed with 100 μl of 100 mM NH₄HCO₃ for 5 min, followed by 100 μl of Acetonitrile for 10 min, until an opaque suspension was formed. After air drying at 56-60°C for 15-45 min, the gel plugs were rehydrated with 10 ng/μl of trypsin solution in digest buffer containing 50 mM NH₄HCO₃. Peptides were extracted with 20 μl of 100 mM NH₄HCO₃, followed by 20 μl of 50% ACN/0.5% TFA at least twice and once with 10 μl of ACN. The extracted peptides were purified using ZipTip C18-microcolumns (Millipore), according to the manufacturer’s instructions. Protein MS was conducted on a 4700 MALDI-TOF mass spectrometer (Applied Biosystems). The digested protein samples were mixed (1:1 v/v) with a saturated solution of recrystallized CHCA matrix dissolved in 0.1% TFA/50%, then spotted on a MALDI plate. Data Interpretation and automated database searching were carried out using the GPS Explorer Software (Applied Biosystems) and the MASCOT program (Matrix) respectively. Combined MS-MS/MS searches were conducted with the following criteria: NCBI nr database (http://www.ncbi.nlm.nih.gov), all entries; MS/MS mass tolerance of 0.15 Da; Carbamidomethyl-Cys (variable modification). Calculation by the software was used as criteria for correct identification.

Statistical analysis

All these experiments were repeated three times, and 30 plants were used each treatment. Its result describe by average mean ± standard error. Tukey’s test was used for testing the mean divergence between treatments by using the SPSS statistical software package.

Conclusion

The present study investigated the relative efficacy of inducing resistance to infection by downy mildew fungus in cucumber by treatment with the glucohexaose. Treatment of glucohexaose 48 h prior to inoculation with P. cubensis decreased disease symptoms in a concentration and interval-dependant manner. We observed that glucohexaose treatment induced changes in energy metabolism with increases in chlorophyll levels and the rates of photosynthesis as well as the soluble carbohydrate contents of leaves. Comparative analysis of the C. sativus L. cv. Shandong mici leaf proteome between non-inoculated control plants and 10 μg.mL⁻¹ glucohexaose treated plants showed relative changes in abundances of several proteins. The predicted functional annotations of the identified proteins suggested their involvement in photosynthesis, photorespiration, oxidative burst, transcriptional regulation, signal transduction and pathogen defense processes. Oxidative burst and photosynthesis related proteins indicated important molecular mechanisms need further investigation.

Acknowledgements

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