Abstract

Carbon nanomaterials (CNMs) have attracted a great deal of research interest for their potential application in plants. Some types of CNMs have exhibited the ability to regulate plant growth, which showed a promising future in agriculture. However, detailed mechanism of their regulation on plant growth is still not well characterized especially at the molecular level. In this study, our objective is to study the regulation mechanism of carbon nanoparticles (CNPs) on tobacco callus growth at protein level. During the incubation period, proteomic profiling of tobacco callus was investigated by using the isobaric tags for relative and absolute quantitation labelling (iTRAQ) coupled with 2D-LC MS/MS. The function of differentially expressed proteins was achieved by using a gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) analysis with InterproScan, and qRT-PCR was used to support the results of the proteomic data. The results showed that the growth of tobacco callus was enhanced by CNPs, with the optimal concentration at 50 mg/L. The expression levels of 198 cellular proteins were significantly up- or down-regulated after CNPs treatment. Some differentially expressed proteins were located in mitochondria and involved in calcium-mediated signaling pathway. The proteomic profile changes provided a more deeper understanding of the highly complex regulatory mechanisms in tobacco callus exposed to CNPs.

Keywords  Carbon nanoparticles · Tobacco callus · iTRAQ · Proteomics

Introduction

Nanotechnology focuses on studying the properties and applications of materials in the nanoscale (mainly 1–100 nm) range. Since the discovery of fullerene, many carbon nanomaterials (CNMs) including carbon nanotubes (CNTs), graphenes, and other CNMs with different structural features have been discovered and applied. With the development of nanotechnology, the application of some CNMs attracted the attention of plant biologists, especially for some beneficial regulation on plant growth (Cañas et al. 2008; Lahiani et al. 2013; Verma et al. 2019). Some CNMs have been applied to agricultural purpose and demonstrated their potential to promote agricultural production (Kole et al. 2013; Lijuan et al. 2020; Mukherjee et al. 2016; Zhang et al. 2013). Carbon-based nanomaterials, such as single-walled carbon nanotubes (SWCNTs), multi-walled carbon nanotubes (MWCNTs), single-walled carbon nanohorns (SWCNHs), water-soluble carbon nano-onions, and carbon nano-dots, have been investigated for their positive effects on plant growth. Although some researches showed the adverse reaction on plants from exposure to a range of CNMs, the effects exerted by the CNMs always involve a high exposure concentration specific to plant species and growth environment (Mukherjee et al. 2016). Plenty of studies reported the positive effects of low-dose CNMs on
plant growth and development (Tiwari et al. 2013). The positive morphological effects include the promotion of the seed germination process, root elongation, and the accumulation of biomass (Ali et al. 2020; Kim et al. 2019; Rico et al. 2011; Saxena et al. 2014). However, the mechanism behind this is still not clarified well. More studies would be necessary to reveal the regulation mechanism of CNMs on plants.

To explore the effects of CNMs on plant growth and development, a lot of researches have been conducted. Among them, the reports of carbon nanotubes appeared relatively early, and reports on the regulation of plant growth were relatively abundant. Khodakovskaya et al. (2015) found that SWCNTs could significantly promote the growth of tobacco callus, the highest increase of fresh weight reached 64%. Three genes were up-regulated in cells after callus was exposed to MWCNTs. These genes are involved in water transport, cell wall extension, cell division, etc. In the rhizobium-plant interaction, MWCNTs could improve the activity of nitrogenase and increase the level of the key gene (NIN) which regulates the development of nodules (Yuan et al. 2017). In some commercial crops, oxidized MWCNTs could penetrate the cell wall and enter the cytoplasm in roots, which promote cell elongation and dehydrogenase activity in the root system (Tiwari et al. 2013). There are also reports about the changes of plant hormones in rice seedlings under carbon nanotube treatment, which indicated some possible relationships with reactive oxygen species (Zhang et al. 2017). Some raw CNPs isolated from biochar also can enhance the growth rate of wheat plants, and the optimum growth occurs at concentration of 50 mg/L in seeds (Saxena et al. 2014).

The current studies mainly focused on the physiological changes, activity of enzymes, and some gene expression in mRNA level in plants. However, they are far from being able to reveal the detailed physiological and molecular mechanism of plants in response to CNMs. Proteomics, a powerful tool for investigating changes in proteomes, could achieve a role evaluation of proteins in a global scale (Anderson and Anderson 1998). Given the crucial role of proteins in almost all cellular functions, it is necessary to study plants exposed to CNMs at the proteomic level. Proteomic profile analysis of mammalian cells exposed in SWCNTs was reported previously (Yuan et al. 2012). By studying changes in plants at the protein level, it will help to reveal the action mechanism of CNMs on plants.

CNPs are a promising carbonaceous nanosized material. It is prepared by the electrolytic graphite method, which has the advantages of green and high efficiency (Chen et al. 2020). CNPs also are different from carbon nanotubes and graphite oxide. It retains the sp2 structure of graphite and contains functional groups such as hydroxyl, carboxyl, and ester groups. Previous studies have shown that CNPs have good biocompatibility with mammalian cells (6–18 μg/mL) and plant cells (75–600 μg/mL) (Chen et al. 2018). The CNPs prepared by this method can promote growth and improve nutrient utilization efficiency in plants. There have been reports on improving the utilization rate of nutrient and improving root vitality and crop quality (Jian et al. 2015). The aim of this study is to assess the promoting effect of CNPs on tobacco callus and using iTRAQ coupled with 2D-LC MS/MS to identify the proteins differentially expressed after exposed to CNPs.

Materials and Methods

Synthesis and Characterization of CNPs

CNPs were prepared as described in Chen et al. (2018). The morphology of CNPs was observed by a JEOL 2011 transmission electron microscope (TEM) at an accelerating voltage of 200 kV. Fourier transform infrared (FTIR) spectroscopy of the CNPs was recorded by Thermo (USA) FTIR spectrophotometer.

The Culture of Plant Materials and Experimental Design

Tobacco callus (Nicotiana tabacum L. Bright Yellow-2 cell) and tissue culture bottles were used as the material to carry the experiment, and tobacco callus was cultured in Murashige and Skoog (MS) medium agar plates (Murashige and Skoog 1962). The method used for tobacco callus culture was the same as described by Khodakovskaya et al. (2015). The control medium was MS medium containing phytagel (2.5 g/L) and 2, 4-Dichlorophenoxyacetic acid (1 mg/L). The CNPs medium was made up of control medium added CNPs at the concentrations of 0, 12.5, 25, 50, and 100 mg/L. Three equal amounts (300 mg) of initial calluses (inoculum) were placed in each tissue culture bottle and the calluses were cultured in the dark condition at 26–28 °C for 25 days. The samples were collected and weighed, then dried in an oven at 55 °C for 24 h, and the dry weight was measured.
Transmission Electron Microscopy Analysis of Tobacco Cell and CNPs

After 25 days incubation, tobacco callus exposed to CNPs and control were dissected, and the upper cells in callus, which is away from the surface of phytagel, were carefully collected. The samples were fixed in phosphate buffered 2% glutaraldehyde, and postfixed with 1% osmium tetroxide for 2 h. Then, the samples were dehydrated in a graded series of ethanol (30%, 50%, 70%, and 90%) and acetone (90% and three times with 100%), and embedded in Epon resin. Then, the samples were sectioned with a Leica ultrathin microtome (50–70 nm thickness). Finally, it was stained with a Leica EM AC20 automatic stainer and dyed with uranyl acetate-lead citrate for about 1 h. TEM samples were imaged by Hitachi HT7700. CNPs were observed by TEM on a copper grid after dilution. Image J software was used to calculate the diameter of CNPs.

Protein Extraction and Digestion

Tobacco callus was collected on the 8th day after treatment of CNPs. The samples were immediately frozen in liquid nitrogen and stored at −80 °C. Samples were extracted with SDT (4% (w/v) SDS, 100 mM Tris/HCl pH 7.6, 0.1 M DTT) lysis method, and protein quantification was performed using the BCA method. Protein digestion was conducted according to the filter-aided sample preparation procedure (Wiśniewski et al. 2009). For each sample, 200 μg of protein was incorporated into 30 μL SDT buffer (4% SDS, 100 mM DTT, 150 mM Tris–HCl pH 7.6), boil it in a water bath for 5 min, and cool to room temperature. The detergent, DTT, and other low-molecular-weight components were removed using UA buffer (8 M Urea, 150 mM Tris/HCl pH 8.0) by repeated ultrafiltration (Microcon units 10 kD), which was centrifuged at 14,000 g for 15 min. Then, 100 μL iodoacetamide buffer (100 mM IAA in UA buffer) was subsequently added to the filter in order to block the reduced cysteine residues, and the samples were incubated for 30 min in darkness at room temperature followed by centrifugation at 14000 g for 10 min. The filters were washed with 100 μL UA buffer three times and then 100 μL dissolution buffer (AB SCIEX) twice. Finally, the protein suspensions were digested with 4 μg trypsin (Promega) in 40 μL dissolution buffer overnight at 37 °C, and the resulting peptides were collected as a filtrate. The C18 Cartridge was used to desalt the enzymatic peptide fragment. The peptide was lyophilized in a centrifugal vacuum concentrator and then a 40 μL dissolution buffer was used to reconstitute the peptide. The peptide content was calculated by UV light spectral density at 280 nm.

iTRAQ Labelling and LC–MS/MS Analysis

One hundred micrograms peptide mixture of each sample was labelled using iTRAQ Reagent-8 plex Multiplex Kit (AB Sciex U.K.) according to the manufacturer’s instructions. Each tagged set of peptides was pooled and fractionated using AKTA Purifier 100. The absorbance at 214 nm was monitored during the elution and fractions were collected every 1 min. The collected fractions were desalted on C18 Cartridges and concentrated by vacuum centrifugation. Each graded sample was separated using the Easy nLC HPLC system in nanoliter flow. Samples were chromatographed and analyzed by Q-Exactive mass spectrometry. The precursor ion scan range is 300–1800 m/z. The first MS resolution is 70,000 at 200 m/z, and the second MS resolution is 17,500 at 200 m/z.

Database Search and Protein Quantification

Mass spectrometry raw data was screened and quantified using software Mascot2.2 and Proteome Discover1.4. The data was searched against the UniProt Nicotiana tabacum database (released in July 2017). Search parameters were set as follows: Trypsin was used to conduct the search which allowed for two missed cleavages per peptide. Peptide and fragment tolerance were ± 20 mg/L and 0.1 Da respectively. Only unique peptides which was reported with a cumulative confidence > 95% were identified. The quantification of protein was that the protein ratios were calculated as the median of only unique peptides of the protein. Then, all peptide ratios were normalized by the median protein ratio. The median protein ratio should be 1 after the normalization.

Bioinformatics Functional Analysis

The function of differentially expressed proteins was annotated based on sequence similarities to sequences in public UniProt database (Uniprot Consortium 2015). A standard T-test was used for the statistical analysis to determine the significance ($P < 0.05$) of changes in each protein between biological conditions in the software R (Team CR 2013). Functional classification of differentially expressed proteins was conducted based on gene ontology (GO) analysis (Alex et al. 2015).

RNA Extraction and qRT-PCR Analysis

Total RNA was extracted from tobacco callus by using RNA iso plant Plus reagent (TaKaRa Bio, Otsu, Japan) and then cDNA was synthesized in 20 μL reaction solution by using Transcriptor First-Strand cDNA Synthesis Kit (Roche, Switzerland) according to the manufacturer’s instructions. All qRT-PCR amplification reactions were performed by using LightCycler 96 Real-Time PCR System (Roche, Switzerland). PCR reactions were run in triplicate. Actin
gene (Accession number: AB158612) was used as internal reference to normalize the expression data (Khodakovskaya et al. 2015). Relative quantification of the transcription level was determined by using the 2-ΔΔCT method (Livak and Schmittgen 2001). The specific primers and the annealing temperatures are presented in Table 1.

Results

Characterization of CNPs

The morphology and size distribution of CNPs were characterized by TEM. As shown in Fig. 1B, CNPs were quasi-

| Uniprot ID | Gene symbol | Forward primer (5’→3’) | Reverse primer (5’→3’) |
|------------|-------------|------------------------|------------------------|
| A0A1S3Z1V3 | Tim9        | GCCATGATTGACAGCTCCA     | CCTGACACAGGTCTCCCTC    |
| A0A1S4BA7  | Cox6b       | GCACGTGGTTACACGCTACG   | GGAAGTGCCGTTCTCCCTC    |
| A0A1S3YMB4 | Cytc        | TGAACAGGCTCAGACGGTTGA  | CTGTTTGTGACACCACCTT    |
| A0A1S3ZUS5 | Cbi3        | GTTGTACGTGCCTAGACGTA   | AGAGCTTCAATCTCACTGACAC |
| Q76ME6     | Ncam10      | GACGGCGATGTTGCTTAC     | GCTCCCTCCTCCAGTCTGTA   |
| A0A1S4DQT4 | Cbs         | ATGAAGAGACGGAGTGC      | TGAGGAAGATTGTCCCAGT    |

Fig. 1  A CNPs were dispersed into water. B TEM images of the CNPs on Cu grid coated with ultrathin amorphous carbon film. C Statistical distribution of nano-carbon particle size; the black line was the Gaussian fitting curve. D FTIR spectra of CNPs.
spherical. Diameter distribution of CNPs showed that the size ranged from 28 to 77 nm (Fig. 1C) agrees well with the Gaussian distribution. As shown in Fig. 1D, FTIR also was used to identify the functional groups of CNPs. A broad peak centered at 3420 cm\(^{-1}\) associated with the stretching vibrations of O–H. The existence of a sharp absorption peak at 1100, 1640 cm\(^{-1}\) associated with C-O, C = O stretching was observed, revealing the presence of COOH.

**Growth Enhancement of Tobacco Callus Induced by CNPs and its Biodistribution Based on TEM Analysis**

After 25 days of cell incubation treated by CNPs, the promotion effects of CNPs on tobacco callus growth were clearly observed (Fig. 2). Fifty milligrams per liter and 100 mg/L CNPs enhanced cell growth with fresh weight by 30.1% and 18.4%, and the dry weight increased by 33.9% and 17.8%, respectively (Fig. 2A). At the concentration of 50 mg/L, it indicated a significant increase in tobacco callus (Fig. 2B). CNPs in cell were observed by TEM, and CNPs were located around the cell wall (Fig. 2C).

**Identification of Differentially Expressed Proteins in Tobacco Callus in Response to CNPs Treatment**

iTRAQ-based comparative proteomic analysis was used to enable comparison of the proteomic of tobacco callus in response to CNPs treatment. Proteins were extracted from the above-mentioned tobacco callus of 50 mg/L CNPs treatment (CNPs-50) and untreated control. Proteins were identified and quantified by iTRAQ labelling and LC–MS/MS analysis, respectively. A total of 6711 proteins were detected in these samples (S1 Table). Among these proteins, 198 displayed significant differences in expression levels, accounting for 2.95% of the total number of proteins (S2 Table). In between, the expression of 89 proteins increased and 109 decreased (CNPs-50: Control ratio <0.71 or ratio > 1.4, q-value <0.05), respectively (Fig. 3A). The fold variation

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**Fig. 2** Effect of the CNPs on the biomass accumulation and the biodistribution of the CNPs in tobacco cells. Biomass accumulation of tobacco callus cultured in MS medium and medium supplemented with CNPs. A Fresh weight and dry weight were determined (average ± SE, n = 18). Asterisks indicate significant differences between control and treatment determined by Student’s t-test (*, P < 0.05, **, P < 0.01). B Optical image of the control (left) and the tobacco callus incubated with 50 mg/L CNPs for 25 days (right). C TEM image of untreated tobacco callus and treated with CNPs after 25 days at the concentrations of 50 mg/L.
was calculated as the average value obtained from three replicates. Hierarchical clustering of all differentially regulated proteins indicated that CNPs induced great changes in protein levels (Fig. 3B).

**GO and Pathway Enrichment Analysis of Differentially Expressed Proteins**

In order to evaluate the biological significance of 198 differential proteins and their functional enrichment, the function of differentially expressed proteins was achieved by using a gene ontology (GO) and KEGG analysis by InterproScan. Results showed that the differentially expressed proteins were mainly involved in “ubiquinol-cytochrome-c reductase activity,” “protein domain specific binding,” “plant-type secondary cell wall biogenesis,” “cytochrome-c oxidase activity,” and “calcium-dependent phospholipid binding” in GO enrichment (Fig. 4A). Interestingly, plenty of GO items were related to mitochondrion. Furthermore, after treated with CNPs, many proteins located in mitochondrion were viewed at a higher level. This was especially true for mitochondrial import inner membrane translocase and cytochrome c.

Complex biological functions of protein were also classified by KEGG pathway analysis to get valuable information. The enrichment item of KEGG showed that differentially expressed proteins were mainly involved in oxidative phosphorylation, calcium signaling pathway, and carbon metabolism (Fig. 4B). Checking the details of proteins, such as calmodulin, may play an important role in plants under treatment of CNPs.

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Fig. 3 General overview of quantitative proteomic profiles. 
A Venn diagram of differentially expressed proteins for the CNPs-50 vs. Control comparison. 
B Hierarchical clustering of all the differentially expressed proteins, sample tree (CNPs-1, CNPs-2, CNPs-3, three replicates treated with 50 mg/L CNPs treatment; Control-1, Control-2, Control-3, three replicates of controls). The yellow and blue scale bar at the top indicate decreased and increased levels respectively, and white indicates no significant change in proteins.

Fig. 4 GO and KEGG pathway enrichment analysis of the 198 differentially expressed proteins based on their functional annotations. 
A Top 25 enriched GO items for the differentially expressed proteins. 
B Top 20 enriched pathways for the differentially expressed proteins.
qRT-PCR Analysis of Genes Encoding the Differentially Expressed Proteins

Results showed that the significant changes in specific proteins also occurred at the level of gene expression in response to CNPs. Six proteins whose expression changed significantly, and with different biological functions and cellular locations, were selected to evaluate the gene changes by qRT-PCR. The genes Tim9, Cox6b, Cbl3, Ntcam10, Cytc, and Cbs of selected proteins showed the same trend with the proteomic changes (Fig. 5).

Discussion

The phenomenon of CNPs promoting the growth of tobacco callus was similar to that of MWCNTs (Khodakovskaya et al. 2015) and SWCNHs (Lahiani et al. 2015). The stimulating effect of CNPs has been reported previously. In this study, CNPs significantly promoted the growth of tobacco callus. Meanwhile, CNPs have a concentration effect, and suitable concentration was beneficial to the growth of tobacco callus. Lahiani et al. (2016) demonstrated that CNMs with different morphologies can promote cell growth, seed germination, and plant growth. Hence, the positive effect may share similar mechanisms in plants. Previous research suggested that CNPs were biocompatible with BY-2 cells (Chen et al. 2018). The phenomenon that some CNMs are absorbed by plants and then transported to the cell gap or into the cell interior has been observed in many electron microscope experiments (Wang et al. 2012; Yan et al. 2013). In this study, CNPs particles were observed to adhere in the cell wall. This is different from a previous report that CNPs were found inside of tobacco cell in suspension cell culture (Chen et al. 2018). The reason could be due to different effects of different culture conditions and culture cycles on the growth of tobacco cells.

In this study, we performed a general assessment of differential proteins by using the iTRAQ technology. Sodium dodecyl sulfate extraction method is one of the major strategies for the extraction of proteins from biological material for mass spectrometry, which has been used in plenty of experiments (Wisniewski et al. 2009). The iTRAQ-based protein analysis is a well-established and validated methodology for identifying differentially regulated proteins. Attribute to the nature of its ultra-sensitivity and high-throughput, iTRAQ coupled with two-dimensional liquid chromatography and tandem mass spectrometry (LC–MS/MS) analysis has been considered to be one of the most reliable methods for quantitative proteomic analysis, which is more accurate and easier to identify a greater number and low-abundance proteins (Martinez-Esteso et al. 2014; Elliott et al. 2009). One hundred ninety-eight proteins were up- or down-regulated after the exposure of CNPs, indicating that CNPs strongly influenced the physiological process of tobacco callus. The items of KEGG and GO enrichment were associated with mitochondria and calcium-mediated signaling. Mitochondria are important organelles inside plant cells for producing energy via ATP production as part of the Krebs cycle. Among the top 20 up- and 20 down-regulated proteins, our study found that a large portion of these proteins were mitochondria-related such as membrane transferase and cytochrome c oxidase. Therefore, it is likely that CNPs strongly affect the function of mitochondria. It
was previously reported that some of the SWCNTs crossed the membrane and were located inside the mitochondria, stimulating cell growth in *Arabidopsis* mesophyll cells only at a low dose (15 μg/mL) (Yuan et al. 2011). In this study, we identified several members in TIM protein family including TIM9, TIM10, and TIM13, which increased dramatically after treatment. TIM complex is a part of the chain for transferring proteins from cytoplasm to mitochondrial matrix (Schwarzländer and Fuchs, 2017). The gene *Tim9* showed the same trend with the proteomic changes. TIM9 play critically important roles during sporophyte initiation, which performs non-redundant regulation of early embryonic cells and endosperm-free nuclear division and development (Deng et al. 2014). A previous report also showed that mitochondrial protein TIM50 affected hypocotyl cell elongation through affecting intracellular ATP level in *Arabidopsis* (Kumar et al. 2012) and TIM21 affected seedling development in *Arabidopsis* (Hamasaki et al. 2012). This may be associated with increased protein transport to the mitochondrial matrix.

Intracellular energy is crucial for the growth of plant cell. The level of energy is expected to have an important effect on cell proliferation and cell elongation. Among the top 20 up-regulated proteins, cytochrome c oxidase and cytochrome c were two other increased expressed proteins. They are located in the inner mitochondrial membrane and are involved in the electron transport chain. This may suggest that energy processing activities of tobacco callus were increased to meet the enhanced energy requirement after treated by CNPs. The analysis of GO function enrichment also revealed plenty of items in the mitochondria. Therefore, we presumed that the effect of CNPs treatment on mitochondrial function of callus was closely related to its growth.

The analysis of proteomics data showed that some differentially expressed proteins were annotated as calmodulin (CaM), and the expression level was significantly up-regulated (up-regulated by 1.5 times). The calcium signaling pathway was significantly enriched among the differently expressed proteins. Calmodulin is a calcium-binding protein, which is only activated after binding to Ca\(^{2+}\), and hormones can regulate calmodulin activity by affecting intracellular Ca\(^{2+}\) concentration changes. It has been reported that the Ca\(^{2+}\)-CaM signaling system mediates auxin-mediated plant cell signaling and regulates the growth of corn sheaths (Yang and Poovaiah 2000). Some researches have shown that graphene oxide induces the increase of auxin efflux vector transcription level (Feihong et al. 2018). However, we did not detect the changes in some hormone-related proteins, which may be due to different cell culture periods and type of carbon nanomaterials. Therefore, further experiment study is very necessary in the subsequent study. In addition, CaM-like protein (CML) also showed a 1.5-fold up-regulation difference in this study. It has been reported that CML regulates plant cell morphology and division (Pina et al. 2005). Exploring the changes of calmodulin in callus treated by CNPs provides a new visual angle for studying the mechanism of this promoting effect.

## Conclusion

CNPs have the ability to enhance the growth of tobacco callus. The proteome profile changes of tobacco callus after CNPs treatment were compared using iTRAQ-based proteomics technology, and 198 proteins with significant changes regulated by adding CNPs-50 in expression levels were identified. The differentially expressed proteins were involved in mitochondrial function and calcium signaling fluxes of callus, which provided new insights into the molecular mechanism of CNPs. These data can help us to understand the regulation mechanism of CNPs on plant growth at the protein level.

## Abbreviations

CNMs: Carbon nanomaterials; CNPs: Carbon nanoparticles; GO: Gene ontology; iTRAQ: Isobaric tag for relative and absolute quantitation; KEGG: Kyoto encyclopedia of genes and genomes; TBY-2 cell: *Nicotiana tabacum* L. Bright Yellow-2 cell; TEM: Transmission electron microscopy

## Supplementary Information

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## Author Contribution

QY and TL conceived and designed the experiments. ZZ, QC, and XM performed the experiments. ZZ, LH, and HD participated in the data analysis. ZZ and TL drafted the manuscript. TL provided guidance for preparing the manuscript. All authors read and approved the final manuscript.

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## Declarations

### Conflict of Interest

The authors declare no competing interests.

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