

Comparative physiological and transcriptome analysis revealed the role of cerium in alleviating copper stress in *Dendrobium nobile* Lindl.

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Abstract

Low concentrations of rare earth elements can be used as mitigants to alleviate metal toxicity in plants. The aim of this experiment was to investigate the mechanism of cerium (Ce) mitigation of copper (Cu) stress in *Dendrobium nobile* Lindl. It was found that Cu treatment caused significant stress, while Ce spray significantly improved the growth under Cu stress and reduced the Cu content in the plant by 9.48%. Ce promoted photosynthesis of *D. nobile* and enhanced the ability to scavenge reactive oxygen species by increasing the activity of antioxidant enzymes. Ce combines with heavy metal ions to form chelates and transports Cu into the vesicles, thus reducing the toxicity of metals to plants. Transcriptome analysis revealed 959 differential genes in the Cu + Ce group compared to the Cu group (511up, 458down). Analysis of differential genes related to metabolic pathways for detoxification revealed that Ce can enhance tolerance of *D. nobile* under Cu stress through various pathways such as regulated metabolic pathways Isoquinoline alkaloid biosynthesis, MAPK signaling pathway, Phenylpropanoid Bioynthesis, Plant hormone signal transduction, Pyruvate metabolism pathway and ABC transporter to regulate GSH, amino acids, metallothionein, thus affecting antioxidant enzyme activity, synergistic ion transport and metal chelate synthesis. *GST, AOC3, cat, PP2C, OMT, GCSH, N4OMT, ACS, MKK9, CML* may be the key genes in *D. nobile* to resist Cu stress. This study initially reveals the mechanism of Ce alleviation of Cu stress and lays a theoretical foundation for further research on the molecular regulatory mechanism of *D. nobile* in adversity.

1 Introduction

Copper (Cu) is a naturally occurring metal and copper contamination in the environment is becoming increasingly common (Cao et al., 2018) due to geological, climatic and anthropogenic activities (Ballabio et al., 2018). Cu is an important element in plants and has a variety of functions at the physiological and molecular levels. However, in excess it may pose a risk to plant survival. Excess Cu entering plant cells causes oxidative stress, decreases chlorophyll content (Yadav, S.K, 2010) and negatively affects many metabolic pathways (Sánchez-Pardo et al., 2014). Stress induced by high levels of Cu activates intracellular signaling, leading to cellular damage (Liu et al., 2018). Li et al. found that Cu treatment retarded root and shoot growth and reduced photosynthetic pigment content in wheat (Li et al., 2023).

Rare earth elements (RRE) are a group of trivalent metal elements with similar physicochemical properties (Brewer et al., 2019), RRE are widely used in agriculture to promote processes such as plant seed germination, growth and development and plant biomass accumulation (Rehman et al., 2019). Several studies have shown that appropriate concentrations of rare earths can increase the chlorophyll content of plants (Zhang et al., 2012) and can also improve plant resistance by inducing cellular defense mechanisms, thereby reducing the phytotoxicity of environmental stressors (Liu et al., 2012).

Low RRE concentrations can promote seed germination and seedling growth, for example, related studies have shown that low concentrations of nitric acid (10 mg/L) can promote germination and growth of soybean seeds (Xu et al., 2011). Recently, it has been shown that cerium can resist heavy metal stress by enhancing ROS scavenging activity (Wang et al., 2020). Liu et al found that Ce can act as a regulator to improve copper tolerance in *P. pratensis* by affecting the regeneration and biosynthesis of ascorbic acid and glutathione in leaves (Liu et al., 2016), and Ce also alleviates stress-induced oxidative stress such as cadmium exposure (Wu et al., 2014).

Dendrobium nobile Lindl (*D. nobile*) is a perennial epiphytic herb of the orchid family, which is a traditional Chinese medicine with high medicinal value and ornamental value. It has immunomodulatory, antitumor, antidiabetic and antioxidant properties (Teixeira et al., 2017). Due to its growth habit, the main growing area of *D. nobile* is located in southwest China, and the soil copper content in this region is higher than the average soil copper content in China (Yang et al., 1985), and the cultivation of *D. nobile* is affected by copper adversity stress, and it is important to investigate how to enhance the copper tolerance of using cultivation measures for its artificial production and quality improvement. Previous studies have screened that the optimal cerium content to alleviate copper stress (CuSO_4 200mg/L) in *D. nobile* is 20mg/L. For heavy metal stress, treatment with the cerium can effectively alleviate its toxic effects on plants and also directly or indirectly affect the uptake and accumulation of heavy metal ions by plants (Wu et al., 2014).

Most of the current studies on their regulatory mechanisms have been conducted at the plant physiological level, whereas the stress effect of heavy metals on plants is a process with a transmissive nature, usually first making an adaptive response at the molecular level, followed by transcription and translation to form a series of physiological changes, and finally manifesting the stress effect morphologically (Zhang et al., 2017). In recent years, the development of transcriptomics techniques has provided an accurate method to study the molecular mechanisms of plant responses to abiotic stresses. This method has been widely used in the study of plant responses to heavy metal stress and a large number of genes related to heavy metal stress responses have been identified from a variety of plants (Wu et al., 2019).

Ce regulates the detoxification mechanism of *D. nobile* under Cu stress is not clear, therefore, this study investigated the effect of exogenous Ce on the physiology and biochemistry of *D. nobile* under Cu stress, and further explored the regulatory mechanism of Ce in molecular biology using transcriptome sequencing to reveal Ce mediated differential genes in *D. nobile* under Cu stress.

2 Materials and methods

2.1 Plant materials

D. nobile seedlings were purchased from Sichuan Lianhua Biotechnology Co. All purchased materials were planted in plastic pot containers of 13.5 cm height and 14.5 cm inner diameter, with 6-8 plants per pot. The experiments were conducted at the teaching experiment site of Sichuan Agricultural University, Chengdu campus (longitude: 103°86' 91', latitude: 30°70' 98'), with four treatment groups of CK, Cu (CuSO₄ 200mg/L), Cu+Ce (CuSO₄+ Ce (SO₄)), Ce (Ce (SO₄)), and three parallel treatments in each group. *D. nobile* was treated every two days by spraying the plants in different groups from 9 am to 10 am. All plants were collected after 20 d of incubation for index measurement and analysis.

2.2 Determination of Cu and Ce content in *D. nobile*

Weighed 0.2 g of dried plant samples passed through 100 mesh sieve, wet digested with a mixture of nitric acid-perchloric acid (4:1), treated with a graphite digester, fixed in a 25 mL volumetric flask and determined by inductively coupled plasma mass spectrometry (ICP-MS, USA) for Cu and Ce (Mongkhonsin et al., 2016).

2.3 Measuring the subcellular distribution of Cu and Ce in leaves

Subcellular Cu and Ce contents were determined by referring to the method of Li et al. (2016). 1g of leaves were taken and put into 10 mL of pre-cooled homogenate, homogenate composition: 250 mmol-L⁻¹ sucrose, 50 mmol-L⁻¹ Tris-HCl (pH 7.4), 1 mmol-L⁻¹ dithioerythritol (C₄H₁₀O₂S₂), ground into a slurry, and then ground by differential centrifugation method, the precipitate that was sequentially centrifuged at 600 r-min⁻¹ for 10 min was the cell wall, the precipitate that was centrifuged at 1000 r-min⁻¹ for 15 min was the chloroplast, the precipitate that was centrifuged at 10000 r-min⁻¹ for 20 min was the mitochondria, and the upper solution was the cytoplasmic fraction (containing vesicles, ribose, etc.), and each centrifugation was repeated three times (Tang et al., 2008). The Cu and Ce content of each fraction was also determined.

2.4 Measurement of physiological indicators

Superoxide dismutase (SOD) activity (Holm, G., 1954) photoinhibition method of nitrogen blue tetrazolium (NBT)) was determined, peroxidase (POD) activity (Tarchoune et al., 2012) was determined, catalase (CAT) was determined by the method of Grilo et al. (2020) Malondialdehyde content (MDA) and soluble sugars were determined: by barbituric acid sulfate (Schmedes A. and Hølmer G., 1989), soluble protein was determined by the Komar Brilliant Blue method (John et al., 1977).

Ascorbic acid (ASA) content was determined by referring to the method of Chen Jianxun et al, and reduced glutathione (GSH) content was determined using a kit produced by Nanjing Jiancheng Institute of Biological Engineering. Proline content was determined: the method was referred to the literature (Fu et al., 2018).

2.5 Illumina sequencing

At the end of the treatment, the leaves of *D. nobile* were taken, wrapped in tinfoil and labeled, frozen in liquid nitrogen and stored in an ultra-low temperature refrigerator at -80°C to be measured, with three biological replicates set up for each treatment. Total RNA was extracted from the tissue samples according to the method of Gu et al. (2021). The final library was obtained by PCR amplification with selected products. Finally, library fragments were double-end sequenced in Illumina Novaseq 6000 (2×150 bp).

2.6 RNA-seq data analysis

Stable quality statistics and quality assessment were performed on the raw data, including the distribution statistics of base pairs such as A, T, C and G, quality distribution statistics, and error rate distribution statistics. The raw data were filtered using Fastp software to finally obtain sequencing data with high quality. Then the assembly order was optimized and filtered by Trinity software, and the assembly results were evaluated by BUSCO. Finally, the sample data were compared with the assembled data by HMMER3 to obtain the mapping results for each sample (Xu et al., 2017)

All transcripts were matched against four major databases (NR, COG, GO and KEGG databases) with the aim of obtaining detailed annotation information in the databases and for statistical purposes. The comparison allowed for functional annotation, classification and analysis of related proteins (Li et al., 2019)

Quantitative analysis of expression levels was performed using RESM software. The standard for measuring expression levels is TPM. based on transcript expression between samples, samples are subjected to wench analysis and correlation analysis to obtain uniquely expressed genes common between samples. The differential genes can be statistically and computationally derived by DESeq2 software (Fu et al., 2018). The degree of enrichment of differential genes in GO and KEGG functions can be derived by Goatools software and R scripts. When the adjusted P-value (P-adjust) < 0.05, it can indicate a significant functional enrichment in the metabolic pathway. Finally, the Tbttools software (Diao et al., 2019) was used to screen for differential genes in key pathways and to plot heat maps.

2.7 qRT-PCR

Real-time PCR was used to detect the relative content of the 10 genes in the samples *GST* (DN4108), *AOC3* (DN11230), *cat* (DN20473), *PP2C* (DN469), *OMT* (DN2431), *GCSH* (DN6229), *N4OMT* (DN5444), *ACS* (DN81246), *MKK9* (DN10428), *CML* (DN5618), and primer sequences were designed by online programming. Based on the sample RNA, reverse transcription is used HiScript Q RT SuperMix for qPCR (+gDNA wiper), and the results of common PCR demonstrated that the electrophoretic gel plot of each primer was a single bright band, indicating no non-specific amplification, and the primers could be used for subsequent experiments. Real-time quantitative PCR experiments were performed using cDNA samples with the following reagents: ChamQ SYBR Color qPCR Master Mix (2X), and PCR cycling experiments were performed using an ABI7300 fluorescent quantitative PCR instrument with the following cycling conditions: 95°C-5s, 72°C-40s, 55°C-30s, 40 cycles (Arya et al., 2005).

2.8 Statistical analysis

Data in this experiment were calculated and plotted using Excel 2010, with standard deviation and significance analysis, one-way analysis of variance and least significant difference test (LSD) using SPSS 20.0 software, and correlation analysis using Pearson's correlation coefficient method. Significant differences were expressed as $P < 0.05$.

3 Result

3.1 Effect of Cu and Ce on growth of *D. nobile*

The effects of Cu and Ce on the growth characteristics of *D. nobile* are presented in Table 1. Cu significantly inhibited the biomass of plant. Upon the exposure of Cu, the changes of the root length, shoot height, root weight and shoot weight were significantly contributed by Cu. The reductions of the root length and shoot length from 5.37 and 22.4 cm at CK to 3.12 and 16.66 cm at Cu200 treatment, respectively. In the presence of Ce, the root and shoot weight increased significantly ($P < 0.05$). Root length and shoot length added 29.98% and 21.28% from CK.

Table.1 Effect of exogenous Ce on the biomass under Cu stress

Treatment	Root length(cm)	Shoot length(cm)	Root weight g\plant	Shoot weight g\plant)	Leaf weight g\plant	Shoot dry weight g\plant	Shoot dry weight g\plant	Leaf dry weight g\plant
CK	5.37±1.04ab	22.412±4.41a	0.497±0.46a	2.921±1.28a	0.103±0.68a	0.29±0.10a	0.205±0.10b	0.205±0.11a
Cu200	3.12±0.76d	16.66±0.61d	0.182±0.10d	1.624±0.65d	0.032±0.33c	0.172±0.01d	0.189±0.17d	0.189±0.14c
Cu200+Ce20	3.39±1.16c	19.931±4.12b	0.307±0.23b	2.599±1.54b	0.078±0.50b	0.453±0.06b	0.137±0.40a	0.137±0.04b
Ce20	6.98±2.44a	17.64±1.04c	0.282±0.01c	1.852±0.41c	0.056±0.23d	0.233±0.02c	0.102±0.10c	0.102±0.04d

Note Different lowercase letters in the same column show the significant difference among different treatments at the level of 0.05. The same below.

3.2 Effects of Cu and Ce on the antioxidant system in leaves of *D. nobile*.

To detect whether the decreased accumulation of oxidant products is associated with the antioxidant system, we next investigated the effects of Ce on the antioxidant system under Cu stress. The SOD (Fig.1a), POD (Fig.1b) and CAT (Fig.1c) activities were significantly higher in the Cu+Ce group than in the Cu group. Cu+Ce treatment significantly reduced copper stress-induced MDA accumulation in *D. nobile* leaves (Fig.3d). Thus, these results can be concluded that Ce has positive effects on the antioxidant system under copper stress.

Soluble sugar (Fig. 1e) and soluble proteins (Fig. 1f) were accumulated in the leaves, the physical efficiency was largely reduced under Cu stress, suggesting that the utilization of storage materials was inhibited. The experiments showed that with the spraying of cerium the content of soluble sugar and soluble protein of *D. nobile* leaves gradually increased.

3.3 Effects of Ce on AsA, GSH, NPT and PCs under Cu stress in leaves of *D. nobile*.

AsA content of *D. nobile* decreased significantly in the Cu group compared to the CK, but got some increase after the application of Ce, which increased by 13.07% compared to it (Fig.2a). The reduced glutathione content of *D. nobile* leaves increased with the addition of Cu and continued to increase significantly after the addition of Ce. Content of Ce+Cu increased by 30.888% compared to the Cu group (Fig.2b). The content of NPT in the leaves was significantly reduced after Cu stress, by 42.061% and 49.404% in the Cu and Cu+Ce groups, respectively (Fig. 2c). It shows that plant cell damage affects the synthesis of phytochelating peptides. The addition of Ce increased the phytochelating peptides in the Cu group by 81.5% (Fig.2d). The increase in the content of PCs complexes with more heavy metal Cu, thus inhibiting the movement of heavy metal and reducing the damage to *D. nobile*.

3.4 Effects of Cu and Ce accumulation in *D. nobile*

By analyzing the accumulation distribution of Cu and Ce in the roots, stems and leaves of *D. nobile*, it can be found that the Cu content in the plants decreased after the addition of Ce, among which the Cu content in the roots and leaves decreased significantly (Fig.3a). While observing the change of Ce content, it can be found that the Ce content in the root, stem and leaf of Cu+Ce group was lower than that of the Ce treatment group alone, indicating that some Ce and Cu reacted to prevent Cu ions from entering the plant Fig.3b

The subcellular distribution of Cu and Ce in *D. nobile* leaves was able to show that Cu and Ce had similar trends in the cell wall, cell membrane and cell soluble fraction, and the addition of Ce led to a decrease in Cu content and a decrease in Ce content itself. The increase in Cu content in plant sub-cells after Cu application indicates that the addition of Cu increased cell damage, resulting in more Cu entering the sub-cells. Cu in the Cu-treated group was mainly distributed on the cell wall (Fig. 3c), and the addition of Ce increased the mass fraction of Cu in the soluble fraction of plant sub-cells and decreased the mass fraction on the cell membrane. It suggests that Ce may be preventing more Cu ions from entering the plant by transferring Cu ions to soluble fractions such as vesicles (Fig. 3d).

3.5 RNA-seq data analysis

Based on the changes of physiological indexes of *D. nobile* under Ce mitigation of Cu toxicity, four treatment groups, CK, Cu, Cu+Ce and Ce, with a total of 12 root samples, were selected for transcriptome sequencing in this experiment. The sequencing results showed that the Q20 was greater than 97%, Q30 was greater than 92%, and GC content was greater than 44%, indicating that the sequencing results were of good quality and met the requirements of sequencing quality for subsequent analysis. All samples Clean Data were assembled from scratch, generating a total of 566388220 Raw reads and 85524621220 Raw bases. After removing splice sequencing, low quality bases, and N (modular base) containing reads, high quality 556850424 Clean reads and 81106787924 Clean bases were obtained (Table s.1)

Transcriptome sequencing obtained 123025 All-Unigene with 121942 genes expressed. The genes were compared in GO, KEGG, COG and NR databases, and the results showed that 38545 genes could be functionally annotated in GO database, and the number of effectively expressed genes was 38363, accounting for 0.31% of expressed genes; 11741 genes could be functionally annotated in KEGG database, and the number of effectively expressed genes was 11718, accounting for 0.096% of expressed 0.096% of the expressed genes; 30,408 genes were annotated in the eggNOG database, of which 30,320 were expressed, accounting for 0.25% of the expressed genes; 61,133 genes were annotated in the NR database, of which 60,809 were expressed, accounting for 0.50% of the expressed genes (Table s.2).

Further, the validity and completeness of the transcriptome data of this study were assessed by the annotation results of Unigenes in the COG database, and the obtained Unigenes were functionally classified. unknown) (17677 items, 53.9%), indicating that there are still many unexplored molecular mechanisms in the response process. The next functional types with a high distribution of Unigenes are Replication, recombination and repair, Posttranslational modification, protein turnover, chaperones, signal transduction mechanisms, Translation, ribosomal structure and biogenesis. There were 3252, 1764, 1493 and 1059 transcripts, accounting for 9.97%, 5.40%, 4.58% and 3.25% of the total, respectively. These physiological processes play an important role in plant response to external stresses, indicating that the transcriptome data are more consistent with plant response to stresses (Fig.s.1).

3.6 Different gene statistics

After obtaining the read counts of genes, differential expression analysis of genes between groups was performed on different treatment samples to identify intergroup DEGs. analysis of the differential gene Venn diagrams of different groups revealed that the copper treatment group individually regulated the most genes with 561 (Fig. 4a). Analysis of the differential gene expression volcano plot revealed that there were 824 differential genes (317 up, 507 down) in Cu vs. Ck. 959 differential genes (511 up, 458 down) were found in the Cu+Ce group compared to the Cu group. 875 differential genes (360 up, 515 down) were found in the Cu+Ce vs. Ck group. It indicates that the primary causative factor for the difference in gene expression is caused by excessive external copper toxicity, and the secondary causative factor is the addition of cerium. The genes in *D. nobile* were involved in various responses to defend the plant from metal stress as much as possible when it was stressed (Fig.3e).

3.7 GO enrichment analysis and enrichment analysis

The DEGs of CK vs Cu (Fig.3a) and Cu vs Cu+Ce (Fig.3c) were annotated into GO database separately. The GO annotation results for DEGs of all treatment groups were similar in terms of membrane part, cell part, metabolic process, cellular process, binding and catalytic activity. Among them, it can be found that the number of differential genes in binding, catalytic activity, cell part, and organelle function was larger in the Cu+Ce treatment group compared to the Cu treatment group, suggesting that the addition of Ce activated the response function and thus strengthened the ability to resist Cu stress.

To gain further insight into the potential regulation of Ce during Cu stress, we performed GO annotation analysis of the genes in the signaling pathways. We found that the DEGs after Cu stress were mainly oxidoreductase activity, hydrogen peroxide catabolic process, DNA-binding transcription factor activity, catalase activity peroxidase activity, reactive oxygen species metabolic process and other functions (Fig.4b).

While the differential genes in the Cu vs Cu+Ce group were mainly distributed in GO annotation in functions such as cellular anatomical entity, integral component of membrane, regulation of biological process, regulation of cellular process, transcription regulator activity and other functions (Fig.4d). It indicates that the effects on plants after Cu stress are mainly in hydrogen peroxide, reactive oxygen species and redox, while the alleviation of Cu stress in plants after the addition of Ce is in the effects in cellular components.

3.8 The KEGG signaling pathway of Cu stress in *D. nobile*

By comparing the KEGG functional annotation analysis between the Cu treated group and the CK group (Fig. 5a), it was found that the upregulated DEGs were mainly distributed in pathways with functions such as Transport and catalolism, Signal transmission, Biosynthesis of other secondary metabolites, Carbohydrate metabolism, etc.

Downregulated genes are mainly distributed in pathways with functions such as Transport and catalysis, Environmental adaptation, and Signal transduction. The differentially expressed genes are mainly enriched in pathways such as MAPK signaling pathway plant (map04016), Plant pathway interaction, Peroxisome, Isoquinoline alkaloid biosynthesis, etc. Cluster analysis was conducted on the key differential genes in the above pathways (Fig.5c), and it was found that *AOC3*, *N4OMT*, *cat*, *OMT*, *CP1*, *galM*, *ACS*, *treA*, *FAR*, *CYB84A*, *MKK9*, *TR1* and other genes were significantly downregulated, while *sacA*, *CALM*, *GCSH*, *TMEM222*, *SYM1*, *scrK* and other genes were significantly upregulated. These genes may be key genes in response to copper stress in *D. nobile*.

3.9 Analysis of KEGG signaling pathway and key differential genes in Ce alleviating Cu stress in *D. nobile*

By comparing the KEGG enrichment analysis between the Cu+Ce group and the Cu group, it was found that the upregulated differential genes were mainly distributed in Transport and catalysis, Environmental adaptation, Glycan biosynthesis and metabolism, while the downregulated genes were mainly distributed in functions such as Carbohydrate metabolism, Glycan biosynthesis and metabolism (Fig. 4d).

The differentially expressed genes are mainly enriched in KEGG signaling pathways such as MAPK signaling pathway plant (map04016), N-Glycan biosynthesis (map00510), Plant hormone signal transmission (map04075), Plant pathway interaction (map04626), ABC transporters (map02010), Cytosine and metabolism (map00270), Glutathione metabolism (map00480), etc. (Fig.5e). Cluster analysis was conducted on the key differentially expressed genes in the above pathways (Fig.5f), and it was found that the significantly upregulated differentially expressed genes in Cu+Ce vs Cu were *N4OMT*, *OMT*, *RPS2*, *ABC1*, *ARR-A*, *GST*, *PP2C*, etc., while the significantly downregulated genes were *RPS2*, *MDH1*, *mtnD*, *CML*, *CALM*, *ALDH*, etc.

3.10 Differentially expressed transcription factor analysis

Transcription factors (TFs) play an important role in the growth and development of plants and all living organisms in nature. Through in-depth analysis of transcription factors in each treatment group, it was found that the enriched transcription factor families (Fig.6a) in TOP20 were MYB, AP2, ERF, NAC, C2C2, bHLH, GRAS, WRKY, bZIP, C3H, B3 superfamily, LBD, TCP, MADS, GRF, FAR1, HSF, C2H2, LOB, ZF-HD, SBP. Among them, MYB was found. The superfamily, bHLH, NAC, and AP2/ERF families have the most genes, with 319, 145, 142, and 142, respectively.

We further analyzed the variations of differential genes in different transcription factor families among treatment groups (Fig.6b), and found that the differential genes were mainly *MYB78*(DN8206), *CCA1*(DN214), *RADIALIS* (DN23674), *ERF2* (DN5581), *ERF053* (DN7587), *DREB3* (DN1349), *ERF* (DN5126), *ERF9* (DN11382), *WRKY71* (DN4888), *WRKY24* (DN9847), *WRKY50* (DN31951, DN1372), *bHLH63* (DN5573), *bHLH041* (DN1951), *HAT5* (DN2227) among the MYB family, AP2 family, WRYK family, bHLH family and C2C2 family, and the differential genes were mainly *MYB78* (DN8206) among the MYB family, AP2 family, WRYK family, bHLH family and C2C2 family, and *MYB78* (DN8206) among the MYB family, AP2 family and C2C2 family.

3.11 Key gene analysis

In order to further analyze the key differential genes for copper tolerance in *D. nobile*, we performed further analysis of DEGs in Isoquinoline alkaloid biosynthesis, Peroxisome, MAPK signaling pathway-plant, Phenylpropanoid Bioynthesis, Plant Hormone Signal Transduction and Pyruvate metabolism metabolic pathways were further analyzed for DEGs that play key regulatory roles in Cu tolerance in *D. nobile*.

The most significant enrichment of differential genes into the KEGG pathway was in Isoquinoline alkaloid biosynthesis (Fig.7a), where the *N4OMT* (DN5444) and *OMT* (DN2431) genes were also present in Flavonoid biosynthesis. Phenylpropanoid biosynthesis, Stilbenoid, diarylheptanoid and gingerol biosynthesis pathways, suggesting that Cu stress affects secondary metabolite synthesis in *D. nobile*.

We found that *AOC3* (DN11230) gene was significantly down-regulated after Cu stress, but was significantly up-regulated with the application of Ce (Fig.7a). Oxidative propadiene cyclase (AOC) catalyzes the most important step in the jasmonate biosynthesis pathway, and jasmonates play a crucial role in coordinating plant defense responses to various biotic and abiotic stresses. It has been shown that overexpression of *GmAOC5* and *GmAOC* genes in transgenic tobacco enhanced tolerance to salinity and oxidative stress, respectively. Therefore, Ce is likely to alleviate oxidative stress and tolerance to Cu from Cu stress by regulating *AOC3* up-regulation.

Genes such as *SYM1*, *FAR*, *cat* in eroxisome signaling pathway were significantly regulated (Fig.7b). When plants are subjected to Cu stress, it causes damage to cell membranes, organelles, and macromolecules such as proteins and DNA, and generates ROS, which cause oxidative damage (Javed, et al., 2021), and also leads to lipid peroxidation (Fahad et al., 2019). Some studies have found that introduction of the gene *katE* can reduce ROS production and confer salt tolerance under salt stress (Nobuo et al., 2000). This study found that Cu stress significantly inhibited the entry of peroxisomes, which not only eliminated ROS in plants, but also served as receptors for ROS levels and regulators of ROS signaling networks (Wang et al., 2021) After Cu stress, the downregulation of *cat* genes indicates an imbalance of catalase, corresponding to a decrease in plant CAT activity and a weakened ability of plants to cope with stress. In addition, it is also possible to upregulate the *CALM* gene in the peroxisome pathway, leading to an increase in ROS, thereby causing stress on plants (Fig. 7b).

The mitogen activated protein kinase (MAPK) cascade signaling pathway is widely present in fungi, animals, and plants. Currently, a large number of studies have shown that the MAPK signaling pathway is closely related to plant response to abiotic stress and plays a crucial role in enhancing plant stress resistance. This pathway can gradually amplify and transmit abiotic stress signals through phosphorylation, thereby participating in biological processes such as plant growth and development and stress resistance (Kumar et al., 2020). This experiment found that genes such as *ACS*, *TMEM222*, *MKK9*, *CALM*, *cat*, etc. were significantly regulated after copper stress. After applying Ce, *CALM* and *TMEM222* were significantly downregulated, which may be the key genes for cerium to alleviate copper stress (Fig.7c).

Phenylpropanoid Bioynthesis is achieved through coordinated enzyme cascades (Koeduka et al., 2014). Benzene propylene polymers such as lignin are essential for plant growth and contribute to plant responses to abiotic and biotic stresses (Wang et al., 2019). By analyzing the phenylpropanoid pathway in the treatment group, it was found that multiple differential genes were significantly regulated. The *N4OMT* gene was significantly downregulated after copper stress, but upregulated after Ce treatment (Fig. 7d).

Nine organic based small hormone molecules are defined as plant hormones, including abscisic acid (ABA), auxin (indole-3-acetic acid [IAA]), brassinosteroids (BRs), cytokinins (CKs), ethylene (ET), gibberellins (GAs) jasmonic acid (JA), salicylic acid (SA), and stereoactones (SLs). Plant hormones are ubiquitous in angiosperms and play a crucial role in plant growth and environmental responses. Previous studies have shown that plant hormone induced ROS production regulates different cellular processes and plant hair (Mhamdi and Van Breusegem, 2018). We found that the differential genes of Cu+Ce vs Cu are heavily concentrated in this pathway, such as the *ARF* and *ARR-A* genes in the Auxin, Cytokinin, and Salicylic acid synthesis pathways being significantly upregulated, and the *PP2C* and *BZR* genes in the Abscisic acid and Brassistoid synthesis pathways being significantly downregulated (Fig.6e). In higher plants such as *Arabidopsis*, the *PP2C* gene has been shown to negatively regulate signaling pathways by antagonizing specific protein kinases. At least two PP2Cs from subgroup A in *Arabidopsis* have been characterized as key factors in ABA signal transduction. In short, subgroup APP2Cs inactivate SnRK11 through dephosphorylation, and this inactivation is inhibited by ABA receptor PYR/PYL/RCRA in an ABA dependent manner.

The differential genes in the Pyruvate metabolism pathway also regulate cysteine and methionine metabolism. Previous studies have shown that the *mMDH2* gene can respond to cadmium stress and its transcription level decreases due to cadmium stress. After overexpression of the *mMDH2*, it was found that the ROS content in plants also increases accordingly. ROS inhibits the expression of cadmium efflux proteins through signal transduction, thereby increasing the heavy metal cadmium content in the body and exhibiting a cadmium sensitive phenotype (Wu et al., 2019). This experiment found that *MDH1*, *adh*, and *ALDH* were significantly regulated after treatment, which may be key genes in response to Cu stress in *D. nobile* (Fig. 7f).

3.12 qRT-PCR validation results

Ten key differentially expressed genes, *GST* (DN4108), *AOC3* (DN11230), *cat* (DN20473), *PP2C* (DN469), *OMT* (DN2431), *GCSH* (DN6229), *N4OMT* (DN5444), *ACS* (DN81246), *MKK9* (DN10428), *CML* (DN5618) were subjected to qRT-PCR validation analysis. The trends of transcriptome expression of the ten genes and the qRT-PCR fluorescence quantification expression levels were basically consistent, which further confirmed the reliability of the transcriptome data.

4 Discussion

Agricultural rare earths are both a kind of micronutrient fertilizer and a new type of plant growth regulator, which can enhance photosynthesis of plant leaves, enhance disease and stress resistance, and have the effect of mitigating heavy metal toxicity (Xie et al., 2000). Rare earth elements have been shown to promote photosynthesis, enhance stress resistance and increase polysaccharide and dendrobate content in *Dendrobium* (Wang et al., 2014). We further analyzed its transcriptome and differential genes on the basis of previous studies, which led to the identification of key genes that Ce could alleviate copper toxicity in *D. nobile* by promoting chelation, scavenging reactive oxygen species, and mediating the transport of metal ions (Fig.7).

4.1 Physiological, biochemical and resistance genes for Cu stress alleviation by Ce

The peroxisome is actively involved in a variety of processes in plants, including abiotic stress responses, phytohormone biosynthesis, generation of signaling molecules and antioxidant enzymes, and ROS homeostasis. (Corpas, et al., 2017). We found that *D. nobile* had increased MDA content and impaired antioxidant system after Cu stress, but it was alleviated by the addition of Ce. It has been shown to respond to metal stress by regulating cat genes (Soydam-Aydin et al., 2015), and in the present experiment, SYM1 gene was found to be up-regulated and FAR and cat genes were down-regulated in Peroxisome signaling pathway (Fig.7b).

When plants are subjected to heavy metal stress, they also avoid damage to organelles by heavy metals through chelation, thus resisting stress chelates of four main types: metallothioneins, phytochelatins (PCs), amino acids and organic acids. PCs are plant polypeptides composed of cysteine, glycine, and glutamate that complex with heavy metals, and PCs are synthesized by enzymatic reactions using GSH as a substrate (Cai et al., 2003) and are important for the alleviation of heavy metal stress in plants (Yang et al., 2019). PCs form chelates with heavy metal ions and then are mainly transported into the vesicles, reducing the intracellular movement of metals to reduce toxicity. Studies have shown that appropriate concentrations of rare earths and their complexes can improve plant resilience by inducing cellular defense mechanisms, thus reducing the phytotoxicity of environmental stressors (Syta et al., 2019).

Metallothionein (MT) has the ability to chelate and detoxify heavy metals, transport trace essential metals, maintain essential metal homeostasis, and participate in plant growth and development (Qiu et al., 2021). It acts as an efficient scavenger of reactive oxygen species. Glycine (Gly) at both ends of the plant MT1 molecular structure increases the flexibility of the protein backbone in the molecule (Chen et al., 2016).

Amino acids and small-molecule organic acids can also alleviate metal toxic effects by complexing with metal ions (Chen et al., 2017). When plants are subjected to heavy metal stress, they may transport heavy metals in vivo to the outside of the body or isolate them in vesicles by the action of some membrane proteins, or they may chelate with heavy metals via some phytochelatins, amino acids, small-molecule organic acids or polyamines in vivo (Sharma, S.S. and Dietz, K., 2006). We found that a large number of differential genes were significantly regulated in pathways such as Plant Hormone Signal Transduction and Cysteine and methionine metabolism

Consistent with the above findings, the results of this experiment revealed that the application of Ce increased the content of PCs, GSH and NPT, which combined with heavy metal ions to form chelates. These compounds are transported to the vesicles for partitioning via the ABC transporter. By analyzing the distribution of metals in plant cells, it was found that the Cu+Ce treatment group showed a decrease in Cu content in the cell wall and an increase in Cu content in the vesicles compared with the Cu group, indicating that Ce can promote the formation of PCs, chelate with Cu, and reduce the effectiveness of heavy metals, thus alleviating the toxic effects. In this experiment, we found *GST*, *AOC3*, *OMT* *GCSH* and other genes were significantly up-regulated after Ce addition.

Phenylpropanoid Bioynthesis is achieved through a coordinated enzyme cascade (Koeduka et al., 2014) that scavenges reactive oxygen species and acts as an antioxidant in metal stress. Phenylpropane polymers such as lignin are essential for plant growth and contribute to plant responses to abiotic and biotic stresses (Wang et al., 2019). By analyzing the phenylpropane pathway in the treatment group, several differential genes were found to be significantly regulated. Among them, the *N4OMT* gene was significantly down-regulated after Cu stress but up-regulated after Ce treatment (Fig. 6d). We hypothesized that *Dendrobium chrysogenum* may synthesize lignin through genes such as *N4OMT* to alleviate copper stress.

4.2 Transcription factor family analysis

Increasing evidence suggests that TFs play key roles in a variety of processes in response to abiotic and biotic stresses. We analyzed the transcription factor families in each treatment group and found that the differential genes were mainly MYB family, AP2 family, WRKY family, bHLH family and C2C2 family (Fig.6).

The MYB transcription factor family is a family of proteins that is large in size and plays an important role in the control of specific functions in plants (Ambawat et al., 2013). In faba bean (*Phaseolus vulgaris*), many MYB proteins involved in ABA signaling and drought response are regulated by aluminum stress (Yang et al., 2012). In this experiment, we found that differential genes MYB78 (DN8206), CCA1 (DN214) in the MYB family were significantly regulated by copper treatment.

The AP2/ERF family is a large group of plant-specific transcription factors that are major factors involved in plant abiotic stress responses (Junya et al., 2012). Some related studies have shown that the expression of StAP2/ERF126/139 improved yeast growth under Cd stress, and the expression of StAP2/ERF075/077/126 increased tolerance to Cd (Tan et al., 2022). We found that the differential genes of this family, *ERF2* (DN5581), *ERF053* (DN7587), were significantly down-regulated after copper stress treatment. We found that DREB3 (DN1349) gene was up-regulated and *ERF* (DN5126), *ERF9* (DN11382) genes were down-regulated by spraying Ce after stress.

The WRKY gene family is thought to play an important role in regulating transcriptional reprogramming associated with plant stress responses. Individual WRKY genes usually respond to several stressors, and then their proteins may act as negative or positive regulators involved in the regulation of several seemingly distinct processes (Chen et al., 2012). *WRKY71* (DN4888), *WRKY24* (DN9847), *WRKY50* (DN31951, DN1372) genes were all significantly up-regulated after Cu treatment and tended to be down-regulated after Cu+Ce treatment.

bHLHs transcriptionally regulate their target genes by binding to specific sites on their promoters, thereby directing a variety of plant developmental and metabolic processes, such as photomorphogenesis, flowering induction, shade avoidance, and secondary metabolite biosynthesis, which are important for promoting plant tolerance or adaptation to unfavorable environments (Guo et al., 2021). We found that the differential genes *bHLH63* (DN5573), *bHLH041* (DN1951), and *HAT5* (DN2227) were significantly regulated.

4.3 Detoxification mechanism of Cu stress in *D. nobile* by Ce

Plants can regulate the distribution of heavy metals in sub-cells to achieve tolerance to heavy metal stress (Yu et al., 2013). Appropriate concentrations of rare earths can improve plant resistance to metal stress by inducing cellular defense mechanisms (Xu et al., 2015). Calcium (Ca) is an important element in plant growth, which can maintain the homeostasis of the plant internal environment and enhance the antioxidant capacity of plants (Li et al., 2016), it can repair and maintain the stability of cell walls and cell membranes and maintain the integrity of cell membranes (Hepler, P.K., 2005). To some extent, rare earth elements can take the place of Ca, and rare earth elements can enter the organism through the same biobinding sites as Ca (Goecke et al., 2015), thus reducing the toxicity of heavy metals. H₂O₂, PCs, cysteine and metallothionein, thus promoting the chelation of heavy metals by plants. In addition, Ce can replace the function of Ca in maintaining intracellular copper ions in relative equilibrium by alleviating oxidative stress, segregation of vesicles, and transporter proteins in the endoplasmic reticulum by regulating the transport of copper to the xylem. (Fig.8)

5 Conclusion

In conclusion, cerium can alleviate Cu stress in *D. nobile*, and Cu chelation by metallothionein and phytochelating peptide and scavenging of reactive oxygen species are the main mechanisms of cerium to alleviate Cu stress. Transcriptome analysis showed that cerium alleviated copper stress mainly through protein processing in Isoquinoline alkaloid biosynthesis, MAPK signaling pathway, Phenylpropanoid Biosynthesis, Plant hormone signal transduction, Pyruvate metabolism pathway and directly or indirectly regulated the expression of soluble sugars, soluble proteins and SOD, POD, CAT, MDA, GSH, PCs and NPT, which reduced oxidative damage by regulating the osmotic potential, chelated heavy metals, transported metals to the vesicles and thus reduced Cu entry into the plant to enhance copper tolerance under copper stress. In this study, we analyzed how Ce alleviates copper stress in *D. nobile* by biochemical experiments, and performed transcriptome sequencing and analysis on the treated leaves by high-throughput sequencing technology, which initially revealed the regulatory characteristics of Ce alleviating copper stress and screened the differentially expressed genes (GST, AOC3, cat, PP2C, OMT, GCSH, N4OMT, ACS, MKK9, CML) and TFs with potentially important functions, providing a basis for further research on the molecular mechanism of *D. nobile* in response to adversity stress.

Declarations

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that might influence the work reported herein.

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Figures

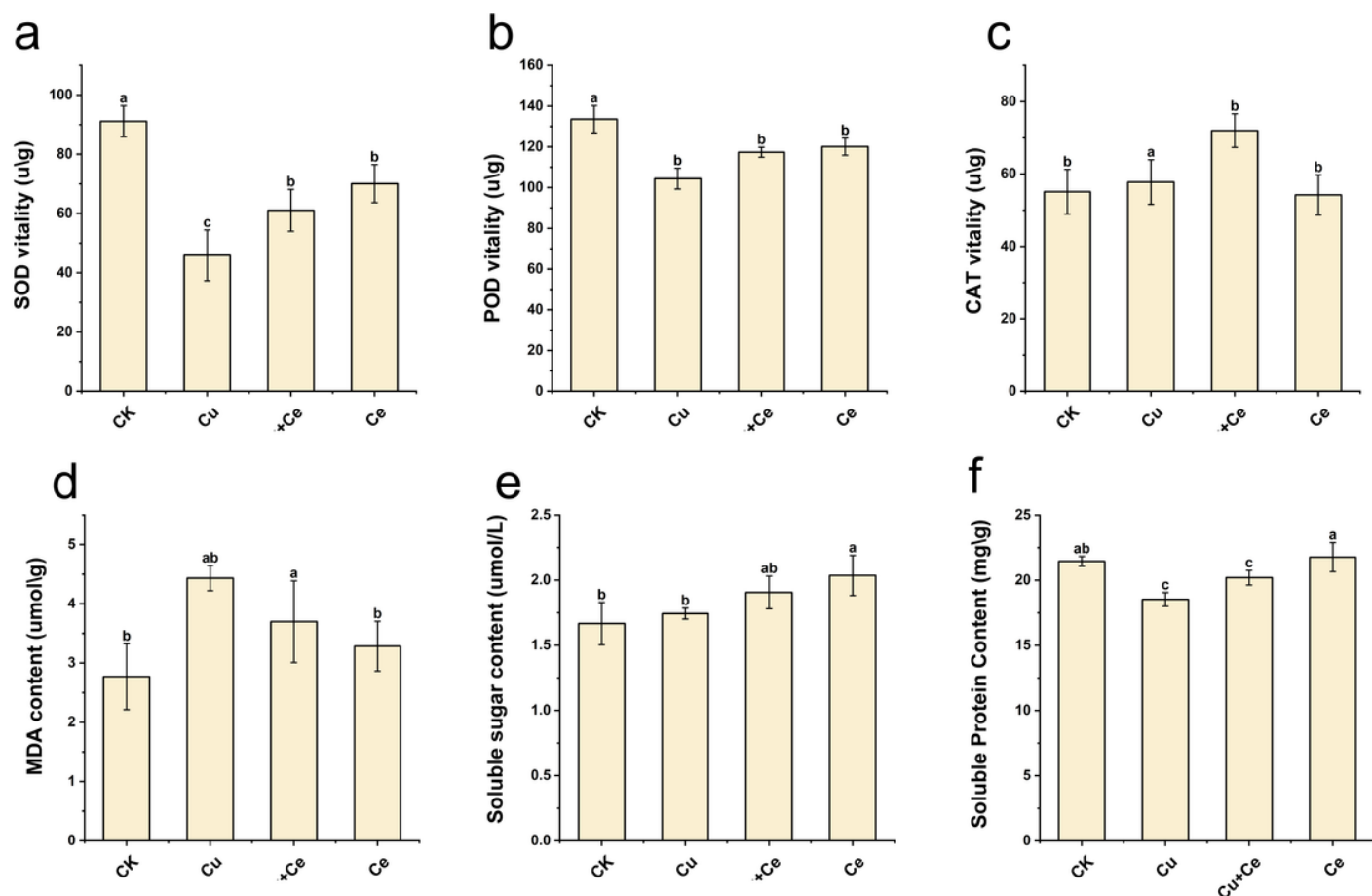


Figure 1

Effect of different concentration of Ce and Cu on SOD (a), POD (b), CAT (c), MDA (d), Soluble Sugar (e) and Soluble Protein (f) in the leaves of *D. nobile*. Means sharing similar letter(s) within a column for each parameter do not differ significantly at $P < 0.05$.

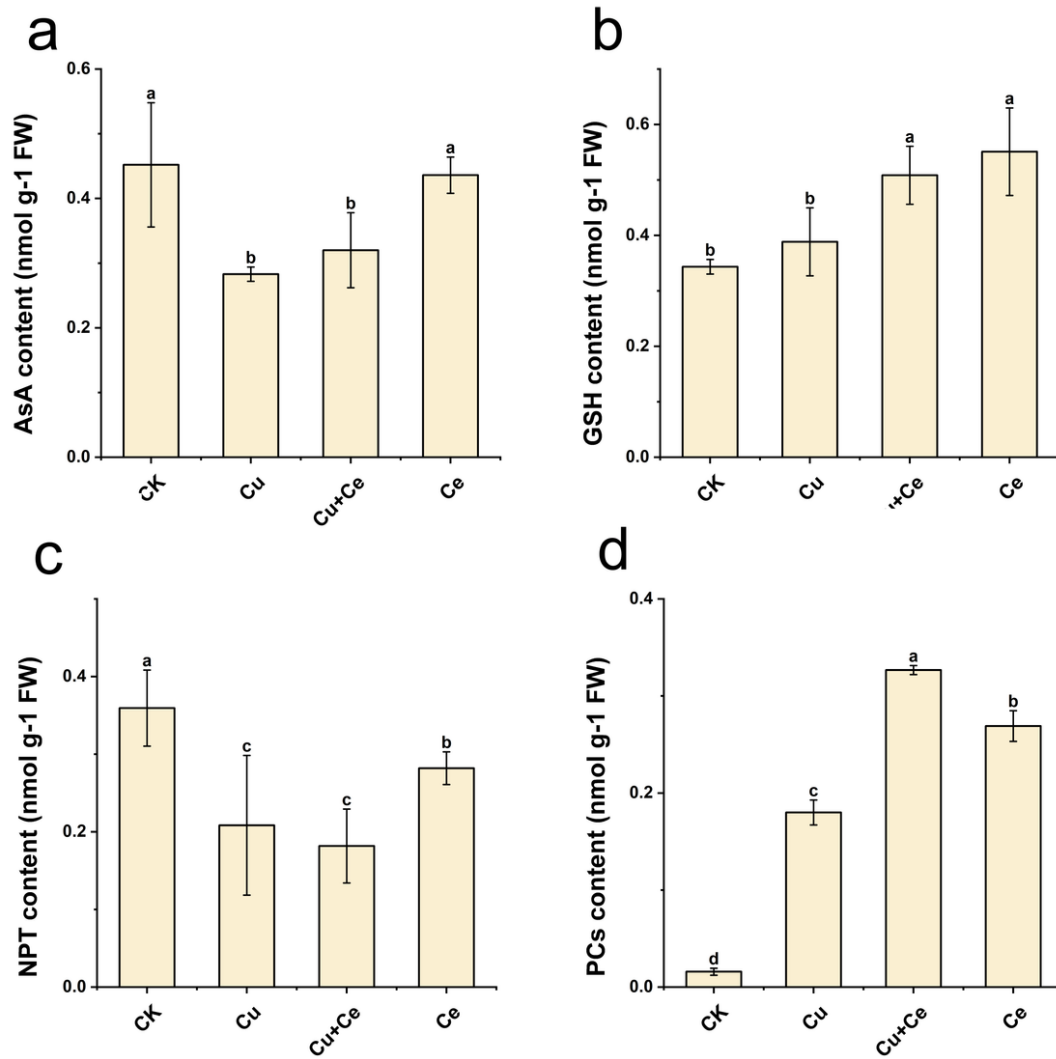


Figure 2

Effect of different concentration of Ce and Cu on AsA (a), GSH (b), NPT (c) and PCs (d) in the leaves. Means sharing similar letter(s) within a column for each parameter do not differ significantly at $P < 0.05$.

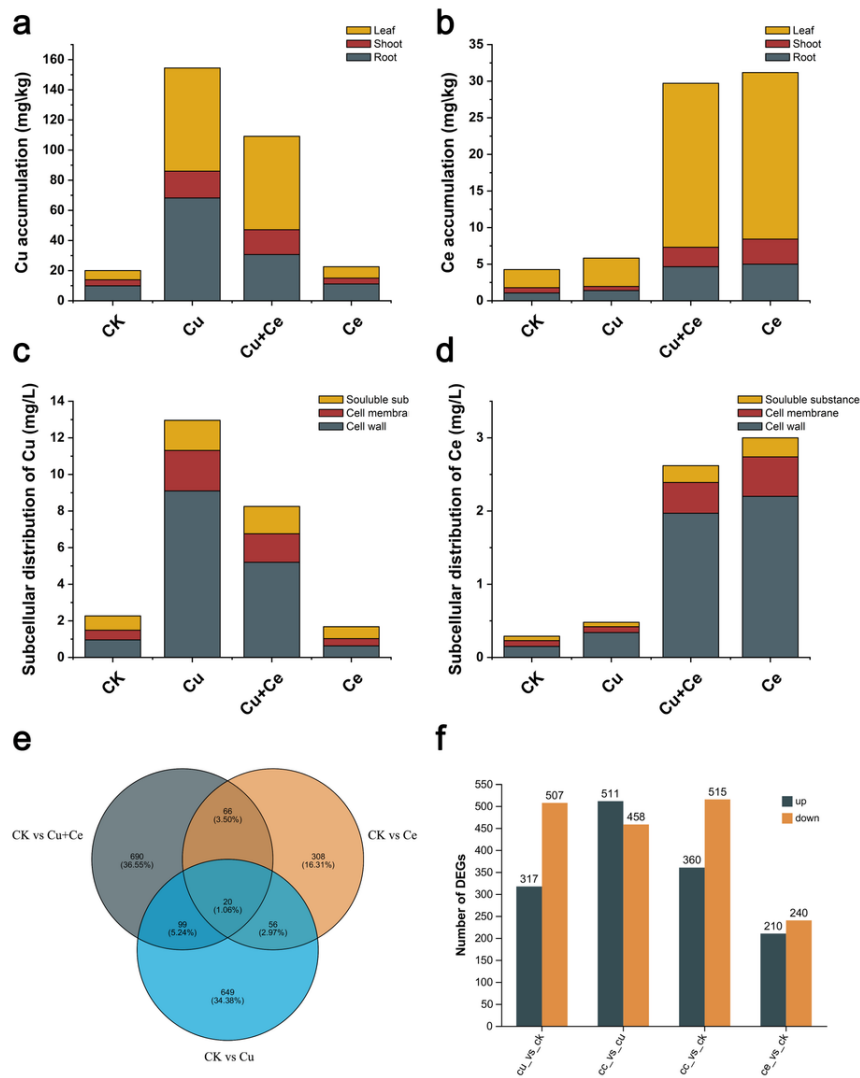


Figure 3

Cu content (a) and Ce content (b) in *D. nobile*, sub-cellular distribution of Cu (c) and Ce (d) in the leaves of *D. nobile*. Means sharing similar letter(s) within a column for each parameter do not differ significantly at $P < 0.05$. Venn diagram of the overlap between DEGs in Cu vs Ck, Cu+Ce vs Cu and Cu+Ce vs Ck (e). Number of differentially expressed genes (DEGs) in different treatments (f).

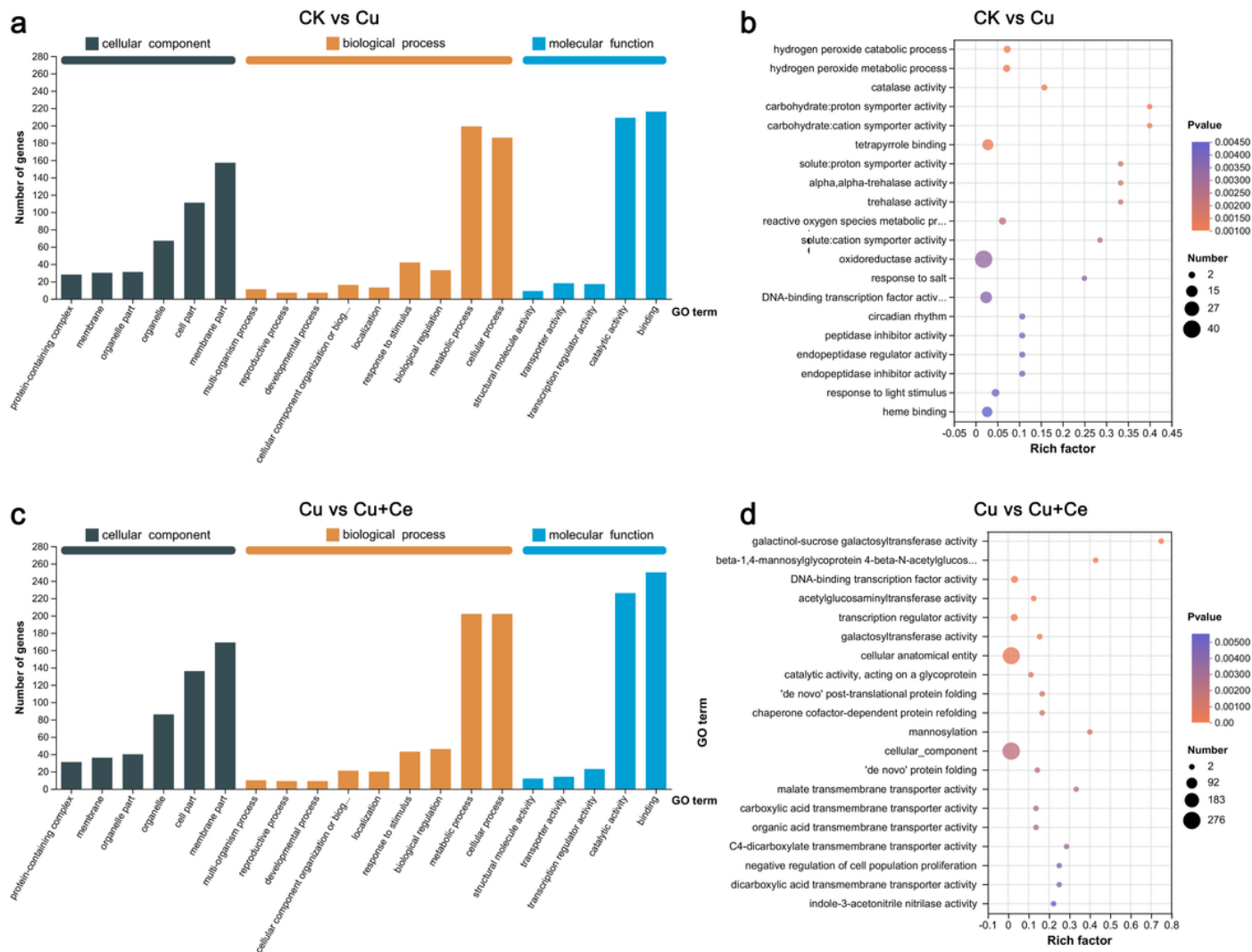


Figure 4

Classification of GO functions, a is CK vs Cu, c is Cu vs Cu+Ce. GO enrichment analysis, b is CK vs Cu and d is Cu vs Cu+Ce.

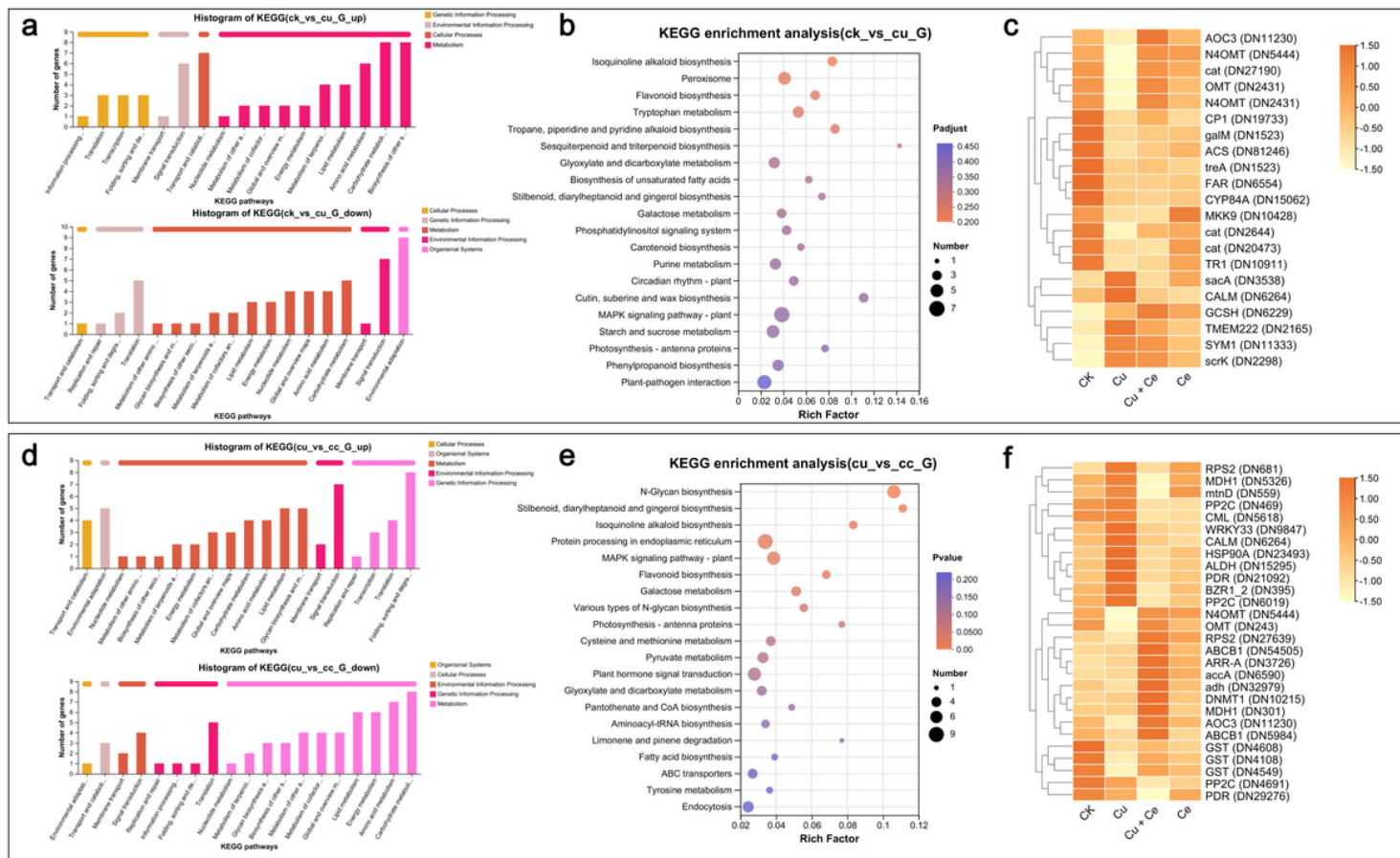


Figure 5

Differential gene up- and down-regulated KEGG function annotation analysis (CK vs Cu is a and Cu vs Cu+Ce is d). KEGG enrichment analysis (CK vs Cu is b and Cu vs Cu+Ce is e). Key differential gene heatmap (CK vs Cu is c and Cu vs Cu+Ce is e).

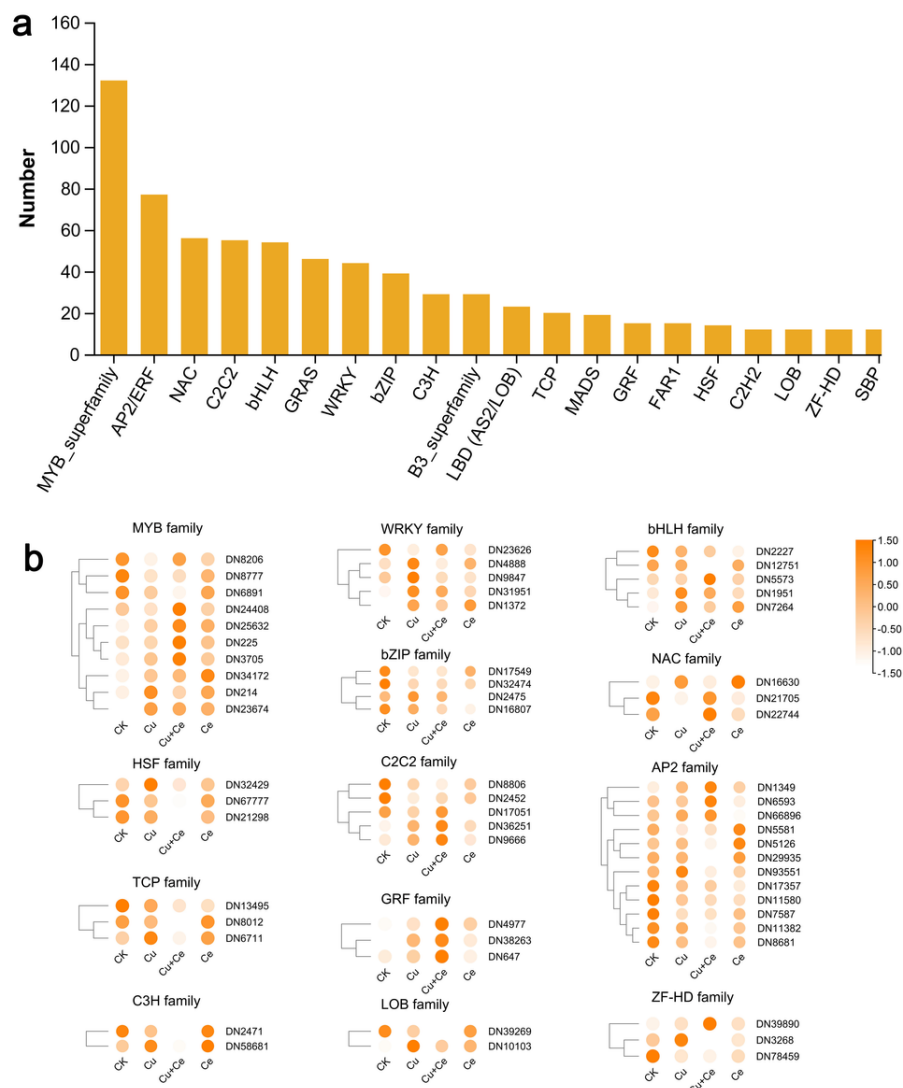


Figure 6

A summary of the number of different genes in the transcription factor family (a), Heat map of genes from different transcription factor families (b).

Figure 8

The mechanism of Ce alleviating Cu toxicity in *D. nobile*.

Supplementary Files

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- [graphicalabstract.pdf](#)
- [Appendices.docx](#)