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Original Article

Physiological and transcriptomic analyses of leaves from *Gardenia jasminoides* Ellis under waterlogging stress

Análises fisiológicas e transcriptômicas de folhas de *Gardenia jasminoides* Ellis sob estresse hídrico

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Abstract

Gardenia jasminoides Ellis is a Chinese herbal medicine with medicinal and economic value, but its mechanism of response to waterlogging stress remains unclear. In this study, the "double pots method" was used to simulate the waterlogging stress of *Gardenia jasminoides* Ellis to explore its physiological and transcriptomic response mechanism. We found no significant damage to *Gardenia jasminoides* Ellis membrane lipid during stress. POD played a vital antioxidant role, KEGG enrichment showed that secondary metabolites such as flavonoids might also play an antioxidant role, and PRO played a significant osmotic adjustment. Endogenous hormones regulate the *Gardenia jasminoides* Ellis's growth and development and play a role in signal transduction. Among them, light waterlogging stress is delayed. At the same time, there were 19631, 23693, and 15045 differentially expressed genes on the 5th, 10d, and 15d of *Gardenia jasminoides* Ellis under waterlogging stress. These genes were closely associated with the proteasome, endopeptidase, ribosome, MAPK signal transduction, and endogenous hormone signal transduction, plant-pathogen interaction and phenylpropanoid biosynthesis and other physiological and metabolic pathways, which regulate the turnover and transportation of protein, the reinforcement and adhesion of cell walls, the induction of stomatal closure, allergic reactions, defense reactions, leaf movements and others. It also can absorb ultraviolet rays to reduce the generation of oxygen free radicals, change the way of energy utilization and adjust the osmotic pressure of plant cells.

Keywords: *Gardenia jasminoides* Ellis, waterlogging stress, physiological response, RNA-seq, DEGs.

Resumo

Gardenia jasminoides Ellis é um fitoterápico chinês com valor medicinal e econômico, mas seu mecanismo de resposta ao estresse hídrico permanece obscuro. Neste estudo, o "método de potes duplos" foi usado para simular o estresse hídrico de *G. jasminoides* Ellis e explorar seu mecanismo de resposta fisiológica e transcriptômica. Não encontramos danos significativos aos lipídios da membrana de *G. jasminoides* Ellis durante o estresse. POD desempenhou um papel antioxidante vital, o enriquecimento KEGG mostrou que metabólitos secundários, como flavonoides, também podem desempenhar um papel antioxidante e PRO desempenhou um ajuste osmótico significativo. Os hormônios endógenos regulam o crescimento e o desenvolvimento de *G. jasminoides* Ellis e desempenham um papel na transdução de sinal. Entre eles, o estresse hídrico leve é retardado. Ao mesmo tempo, havia 19631, 23693 e 15045 genes diferencialmente expressos de *G. jasminoides* Ellis nos dias 5, 10 e 15 sob estresse hídrico. Esses genes foram intimamente associados ao proteassoma, endopeptidase, ribossomo, transdução de sinal MAPK e transdução de sinal de hormônio endógeno, interação planta-patógeno e biossíntese de fenilpropanoides e outras vias fisiológicas e metabólicas, que regulam o turnover e transporte de proteínas, o reforço e adesão das paredes celulares, a indução do fechamento estomático, reações alérgicas, reações de defesa, movimentos foliares, entre outros. Também podem absorver os raios ultravioleta para reduzir a geração de radicais livres de oxigênio, alterar a forma de utilização da energia e ajustar a pressão osmótica das células vegetais.

Palavras-chave: *Gardenia jasminoides* Ellis, estresse hídrico, resposta fisiológica, RNA-seq, DEGs.

1. Introduction

Together with abiotic factors such as drought, high salinity and extreme temperatures, waterlogging, one of the principal abiotic stresses suffered by plants, determines the composition and distribution of species (van Bodegom et al.,

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2008; Tan et al., 2009). With global climate weirding, some areas appear, and natural resources were utilized unreasonably. Due to these, waterlogging has become one of the most severe natural disasters currently facing (Zhihuan, 2008). After a plant is under waterlogging stress, a series of physiological and biochemical responses occur in the body to maintain growth and development, which involves the expression and regulation of many stress response genes (Zhang, 2016). The analysis of the physiological response and transcriptome changes in plants under waterlogging stress, especially the DEGs (differential expression genes) in plants under waterlogging stress, is of great significance to reveal the mechanism of plant waterlogging resistance and to construct a transcriptional regulatory network of the genome in adversity. With the development and maturity of high-throughput transcriptome sequencing technology, it has been reported that high-throughput sequencing technology has been used to analyze the molecular response mechanism of plants to waterlogging stress from the transcription level in rape (Zou et al., 2015), cucumber (Xu et al., 2017) and soybean (Chen et al., 2016), which is significant for the deep analysis of the molecular response mechanism of plant waterlogging tolerance.

In China, *Gardenia jasminoides* Ellis has a long history of medicinal use. It is the first batch of medicinal and edible plants released by the Chinese Ministry of Health. Its fruit has anti-inflammatory, analgesic, liver-protecting, choleretic, hypolipidemic, anti-thrombotic, Neuroprotection and other effects (Wang, 2015). At the same time, rich in pigments, *Gardenia jasminoides* Ellis's fruits are widely used in beverages, food, daily chemicals and cosmetics. Because *Gardenia jasminoides* Ellis is widely used and in great demand. It has been planted and produced massively in Jiangxi, Hunan, Hubei, Fujian, Zhejiang, Anhui, Sichuan and Guizhou of China, and Anhui Jiangsu, Henan and Shandong (Liu, 2010). At present, the area of artificially planted *Gardenia jasminoides* Ellis in China is up to 17,000 hm^2, with an annual output of nearly 40,000 t (Pan et al., 2019). In this study, the "double pots method" was used to simulate *Gardenia jasminoides* Ellis being subjected to different levels of waterlogging stress. Various physiological indicators and endogenous hormone contents were measured to study the characteristics of *Gardenia jasminoides* Ellis's physiological changes in response to waterlogging stress. Meanwhile, high-throughput sequencing was used to obtain *Gardenia jasminoides* Ellis transcriptome data under waterlogging stress, and DEGs were analyzed through various software and techniques. The purpose was to explore the molecular mechanism of *Gardenia jasminoides* Ellis in response to waterlogging stress, providing guidance or ideas for other research on anti-waterlogging stress.

2. Materials and Methods

2.1. Test materials and treatment

In the experiment, one-year-old *Gardenia jasminoides* Ellis seedlings were selected as materials provided by the breeding base established by Jiangxi Puzheng Pharmaceutical Co., Ltd. The seedlings were transplanted from the soil in the nursery to the white polyethene plastic flowerpot whose specification was 29cm×23cm, and the cultivation medium was the ordinary garden soil. After 30 days of normal water and fertilizer management for potted seedlings, 40 pots of *Gardenia jasminoides* Ellis seedlings with consistent growth and no pests and diseases were selected. Every ten pots were a treatment group. The "double pots method" was used to simulate the waterlogging stress of *Gardenia jasminoides* Ellis, that is, the flowerpot planted with *Gardenia jasminoides* Ellis was placed in a plastic bucket, whose water surface was 5cm below the soil surface, which was a light waterlogging (LW) stress when the simulated groundwater level was too high. The soil moisture content was utterly saturated, a moderate waterlogging (MW) stress when the water damage was simulated in a natural state. The soil water content is supersaturated, and the water surface is about 5cm higher than the soil surface, which simulates waterlogging, which is a severe waterlogging (SW) stress. Moreover, the average water and fertilizer management was used as the standard control (CK). The mature leaves of *Gardenia jasminoides* Ellis at the same leaf position were picked every five days, placed in liquid nitrogen for quick freezing, and then stored in a refrigerator at -80 ºC for subsequent research.

2.2. Measurements of various physiological indicators

The drying and weighing method measure the relative moisture content of the leaves,and the chlorophyll content was determined with reference to the acetone extraction method of Shu et al. (2010). SOD activity was measured by the NBT riboflavin microplate method. POD activity is measured by the guaiacol method. The MDA content is measured by TBA colorimetry. PRO content was measured by the ninhydrin microplate method.

2.3. Measurements of endogenous hormone content

The endogenous hormones of *Gardenia jasminoides* Ellis leaves were crudely extracted with 80% methanol solution (containing 0.5% BHT antioxidant). Then the extract was purified by C-18 solid-phase extraction cartridge and dried by nitrogen and then obtained sample to be measured by the constant volume of sample diluent. The content of the four endogenous hormones (ABA, IAA, GA and ZR) in the *Gardenia jasminoides* Ellis leaves was measured by enzyme-linked immunoassay.

2.4. Transcriptome sequencing analysis

The *Gardenia jasminoides* Ellis leaves samples (named CK5, CK10, CK15, WS15, WS5, WS10 and WS15, respectively) of the CK and SW groups at 5d, 10d and 15d were selected and sent to Shanghai Shengong Biological Engineering Co., Ltd. for total RNA extraction and cDNA library construction and sequencing, quality control and De Novo assembly operations. On the one hand, Blast software is used to compare the All-Unigene sequence with the six significant databases (NR, Swiss-prot, Pfam, COG, GO and KEGG databases), the critical value is E<10-5, and the statistics of the annotations of each database are counted.

On the other hand, Bowtie and RSEM software are utilized to estimate the expression level, then standardize the raw counts between different samples based on the TMM method, and subsequently use DEGseq software to perform differential expression analysis between groups, default parameters: p-adjust<0.001, |log2FC|≥1. Gene sets were created for DEGs between different *Gardenia jasminoides* Ellis samples, enriched and analyzed using GO and KEGG databases. At the same time, DEGs were analyzed by a protein-protein interaction network (PPI) to screen the crucial genes of *Gardenia jasminoides* Ellis in response to waterlogging stress.

2.5. DEGs verification

In order to verify the reliability of this transcriptome data, we selected 6 DEGs that played a role in *Gardenia jasminoides* Ellis response to waterlogging stress, including three up-regulated genes and three down-regulated genes, with GU797554.1 as a housekeeping gene, using real-time fluorescence quantitative PCR (qRT-PCR) technology to verify the expression of these genes. Primer Premier 5.0 was used to design primers. See Table 1 for specific information.

3. Results and Analysis

3.1. The changes of various physiological indexes of Gardenia jasminoides Ellis under waterlogging stress

As shown in Figure 1, compared with *Gardenia jasminoides* Ellis leaves in the CK group, the relative water content decreased overall under waterlogging stress, and there was a significant difference(P<0.05).

The overall decrease in chlorophyll content was observed and was greatest at the tenth day of stress. Furthermore, the chlorophyll content per unit leaf area of *Gardenia jasminoides* Ellis decreased by 56.33%, 45.20%, and 63.97% in LW, MW and SW groups. The SOD activity first increased slightly and then dropped significantly, SOD activity declined by 10.91%, 18.18% and 19.85% in LW, MW and SW group compared with the CK group on the 20th day, and the difference between each group and CK group was significant (P<0.05). The POD activity rose first and then decreased, the maximum value of LW increased by 48.33% compared with the CK group on the 15th day, the maximum value of the MW and SW groups increased by 112.38% and 173.59% compared with the CK group on the 10th day. The MDA content did not change much, and there was no significant difference compared with the CK group. The PRO content climbed rapidly and then gradually decreased; the PRO of LW, MW, and SW groups increased by 152.17%, 104.14%, and 80.07% compared with the CK group with the maximum value at the fifth day of waterlogging stress. The change of soluble sugar was not significant, the LW group increased first and then decreased than the CK group, and the MW and SW groups decreased first and then increased than the CK group.

3.2. The changes of endogenous hormone content of Gardenia jasminoides Ellis under waterlogging stress

As shown in Figure 2, compared with *Gardenia jasminoides* Ellis leaves in the CK group, the ABA content in the LW group dropped at first and then rose, while that in MW and SW groups increased at first and then decreased. The ABA content in LW, MW and SW groups increased by 38.26%, 31.96% and 5.60% compared with the CK group on the 15th

Table 1. Primer sequences used in qRT-PCR.

Gene ID	Gene annotation	Primer F(5'-3')	Primer R(5'-3')	Expression level
TRINITY_DN154598_c0_g1	oxidoreductase activity、acting on single donors with incorporation of molecular oxygen、incorporation of two atoms of oxygen	TAATCTCAGCGTCTTCGTCTCC	CTGTTGCTGTTGTTGAATTACCTAA	Up-regulate
TRINITY_DN148369_c2_g1	maturation of SSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)	TCGTTGTTAGAGGGATGGAAA	GTGGCGGAAACCGAAAGT	Up-regulate
TRINITY_DN142518_c0_g1	cell wall、apoplast	GTGGTCAGATTCCGCAAGG	CGACAGCCAGAAAATAAGTGAA	Up-regulate
TRINITY_DN155770_c0_g5	glutathione transferase activity、auxin-activated signaling pathway	ACGTTTTGGCTAAATGGGG	CCAAGCAACGAAACTAAGGATT	Down-regulate
TRINITY_DN163369_c0_g1	defense response、ATP binding、ADP binding	ACTTCTTTTCTTCCTCAAATCCTAT	CACCGTTTCTGACTCATCCAC	Down-regulate
TRINITY_DN132608_c0_g2	protein serine/threonine kinase activity	GCCAAGGAAATCATCAGAGGA	TTCAATGGCAGAAGTGGTTAGA	Down-regulate

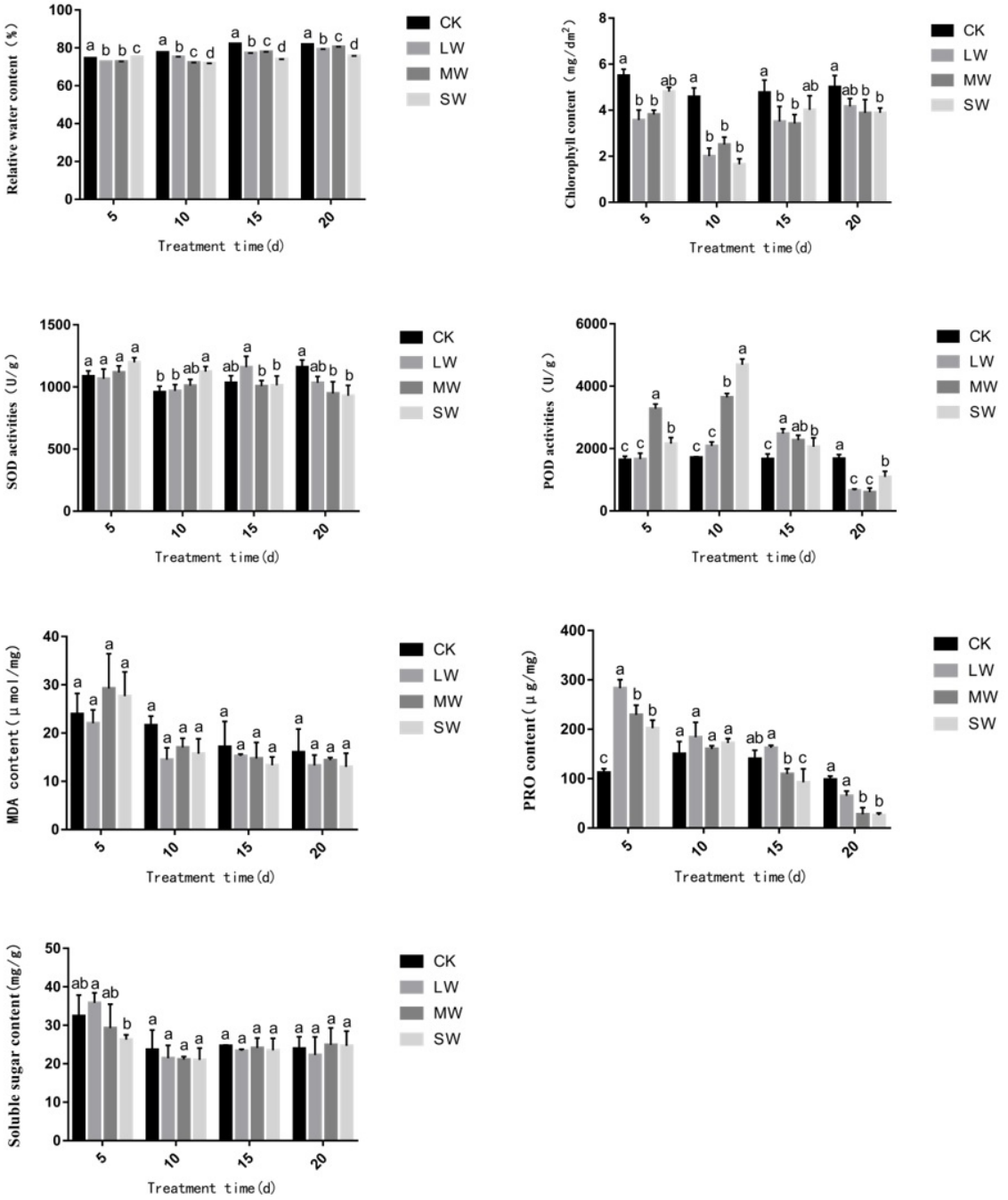

Figure 1. Changes of physiological indexes in *Gardenia jasminoides* Ellis leaves under waterlogging stress. Note: different letters indicate that $P<0.05$ is statistically significant between different treatments at the same treatment time.

day of stress. The IAA content of *Gardenia jasminoides* Ellis leaves in the LW group increased initially and then decreased gradually. The IAA content in MW and SW groups was higher than that in the CK group on the 5th day, then decreased gradually, and was significantly lower than that in the CK group on the 15th day of stress($P<0.05$). The GA content of *Gardenia jasminoides* Ellis leaves in the LW and MW groups showed a low-high-low trend, and the GA content in the SW group increased at first and then decreased. It could be seen that the GA content in *Gardenia jasminoides* Ellis leaves decreased in the later stage of waterlogging stress. The ZR content in *Gardenia jasminoides* Ellis leaves dropped at first and then increased in the LW group, and the ZR content in MW and SW groups rose at first and then decreased gradually.

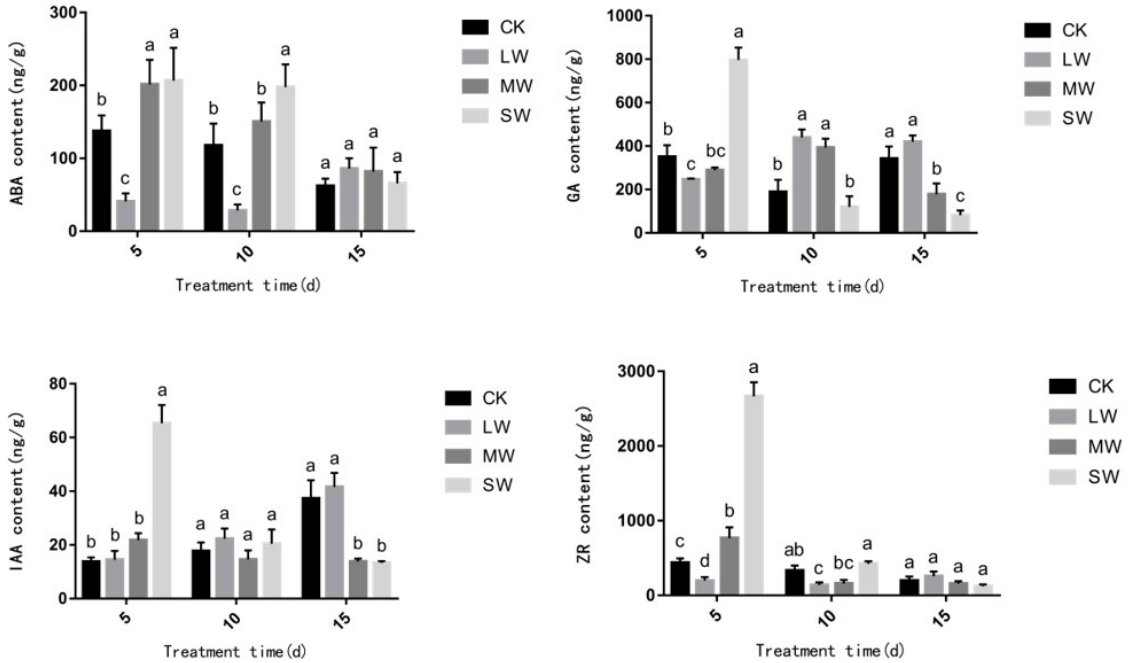

Figure 2. Changes of endogenous hormones in *Gardenia jasminoides* Ellis leaves under waterlogging stress. Note: different letters indicate that $P<0.05$ is statistically significant between different treatments at the same treatment time.

3.3. All-unigene analysis

3.3.1. Analysis of transcriptome sequencing and assembly results

After completing the sequencing of six cDNA libraries (CK5, CK10, CK15, WS5, WS10 and WS15), the clean reads statistics of each sample are shown in Table 2. After quality control, clean reads were obtained, in which the GC content was between 42.96%-45.87%, and the Q20 value was greater than 97%, indicating that the data obtained was of good quality and could be used for subsequent analysis.

Because *Gardenia jasminoides* Ellis does not have a reference genome, De Novo assembly of clean reads is required to obtain a reference sequence for subsequent analysis. The assembly results are shown in Table 3. It could be found that a total of 247,678 Transcripts and 124,192 Unigenes were obtained. The average length of the Transcript is 812.54bp, the maximum length and the minimum lengths are 14176bp and 201bp, respectively. The Transcript length is mainly distributed between 200bp-500bp, with a total of 144180, N50 and N90 are 1505bp and 289bp, respectively. The average length of a single Unigene is 537.88bp, and the maximum and minimum lengths are consistent with the Transcript. The length of Unigene is also mainly distributed between 200bp-500bp, a total of 92715, N50 and N90 are 753bp and 235bp, respectively.

3.3.2. All-unigene annotation statistics

Blast alignment of All-Unigene sequence with NR, Swiss-prot, Pfam, COG, GO, and KEGG databases, a total of 74556 annotation information was obtained, accounting for 60.03%. The results are shown in Table 4. 68,648 Unigenes were annotated in the NR database, 56,039 Unigenes in the Swiss-Prot database, 52,627 Unigenes in the Pfam database 1,1971 Unigenes in the COG database, 37,676 Unigenes in the GO database and 39,811 Unigenes in the KEGG database.

3.4. Analysis of differentially expressed genes

3.4.1. Screening of differentially expressed genes

DEGseq software was used to analyze the DEGs between groups, and the BH method was adopted for multiple test correction. The statistical results of DEGs are shown in Table 4 and Figure 3. Compared with the CK group at the same time, there were 14410 up-regulated and 5221 down-regulated genes in *Gardenia jasminoides* Ellis on the 5th day after waterlogging stress, 15354 genes up-regulated and 8339 genes down-regulated on the 10th day of waterlogging stress, 5872 genes up-regulated and 9173 genes down-regulated on the 15th day of waterlogging stress.

3.4.2. DEGs GO enrichment

Differential genes or gene products were enriched in 211 sites, among which 17 were significantly enriched on the fifth day of waterlogging stress. Differential genes or gene products were enriched in 139 sites, and nine were significantly enriched on the 10th day of waterlogging stress. Differential genes or gene products were enriched in 157 locations, among which 21 were significantly enriched on the 15th day of waterlogging stress. The specific information is shown in Figure 4. At the same time, it was found that the differential genes or gene

Table 2. Overview of the sequencing result.

Sample	Raw reads	Raw bases	Q20*(%)	GC content(%)	Clean reads	Clean bases	Q20(%)	GC content(%)
CK5	55668944	8350341600	95.84	44.03	54675502	7340888801	97.82	42.96
CK10	37620388	5643058200	96.17	44.09	36935234	5295819936	97.84	43.55
CK15	42857196	6428579400	96.30	44.16	42274124	6071098218	97.92	43.73
WS5	42751408	6412711200	95.28	44.46	41694182	5907846285	97.45	43.7
WS10	43987130	6598069500	96.52	46.48	43033270	6138624477	98.09	45.87
WS15	51895910	7784386500	96.80	44.33	51210314	7321068827	98.10	43.96

*Q20 represents the percentage of bases whose data quality value is greater than or equal to 20; GC: Guanine and Cytosine.

Table 3. Overview of the assembly.

	Transcript	Unigene
Total number	247678	124192
Length≥500bp	103498	31477
Length≥1000bp	58686	14083
N50	1505	753
N90	289	235
Max Length	14176	14176
Min Length	201	201
Total Length	538512587	193874222
Average Length	812.54	537.88

N50 and N90 refer to the length of Transcript/Unigene sorted from small to large and then accumulated to 50% and 90% of the total length.

Table 4. Annotation of the transcriptome.

	Unigene number	Percent(%)
Annotated in NR	68648	55.28
Annotated in Swiss-Prot	56039	45.12
Annotated in Pfam	52627	42.38
Annotated in COG	11971	9.64
Annotated in GO	37676	30.34
Annotated in KEGG	39811	32.06
Total annotation	74556	60.03
Total	124192	100

products that were significantly enriched together on the 5th, 10th, and 15th day of the waterlogging stress were peptidase regulator activity, peptidase inhibitor activity, endopeptidase regulator activity, endopeptidase inhibitor activity, enzyme inhibitor activity. The differential genes or gene products significantly enriched on the 5th and 10th day of waterlogging stress were xyloglucans: xyloglucosyl transferase activity. The differential genes or gene products that were significantly enriched on the other the 10th and

Figure 3. The statistics of differentially expressed genes.

15th days of stress were cysteine-type endopeptidases inhibitor activity.

3.4.3. DEGs KEGG enrichment

DEGs were enriched in 127 pathways, and there appeared five significantly enriched pathways on the fifth day of waterlogging stress. DEGs were enriched in 128 pathways and five significantly enriched pathways on the 10th day of stress. DEGs were enriched in 128 pathways, and 12 pathways were significantly enriched on the 15th day. The information on the pathways for the significant enrichment of DEGs in each group is shown in Table 5. Plant-pathogen interaction is the pathway for the *Gardenia jasminoides* Ellis to be significantly enriched on the 5th, 10th and 15th day under waterlogging stress, and phenylpropanoid biosynthesis is the pathway for the 10th and 15th-day stress to be significantly enriched together. In addition, glycolysis/gluconeogenesis, circadian rhythm-plant, circadian rhythm-plant and flavonoid biosynthesis pathways may play an essential role in *Gardenia jasminoides* Ellis response to waterlogging stress. MAPK signalling pathway-plant and plant hormone signal transduction belong to the signal transduction pathway, and ribosome belongs to the genetic regulatory substance. Proteasome controls the degradation and renewal of protein. They play a role in stress signal transduction, genetic material regulation and degradation of redundant and damaged proteins to generate new proteins.

3.4.4. The analysis of protein interaction network

The proteins encoded by 28 genes in the protein interaction network of *Gardenia jasminoides* Ellis DEGs

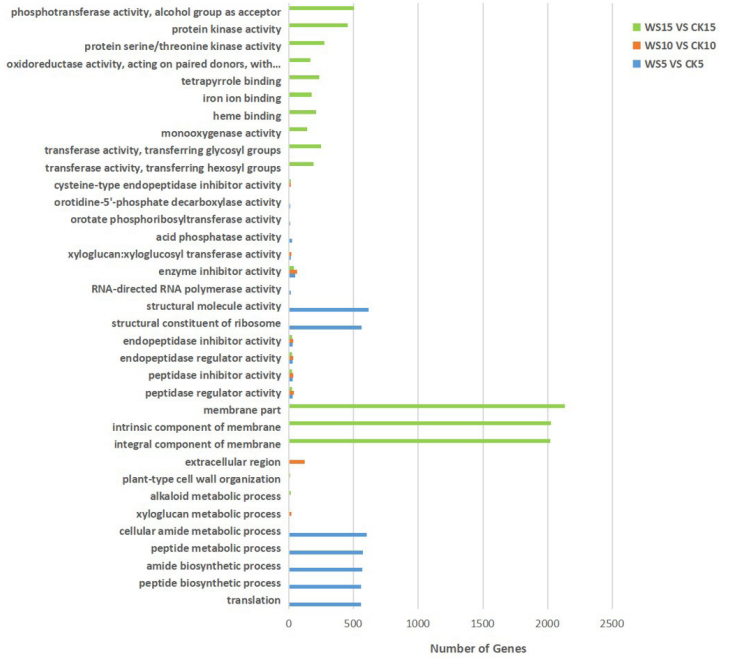

Figure 4. The bar graph of significant GO enrichment of DEGs. GO: Gene Ontology.

Table 5. The pathways were enriched by differentially expressed genes.

Group	Pathway ID	KEGG description	Gene number	Q value
WS5 VS CK5	map03010	Ribosome	712	1.24415E-09
	map03050	Proteasome	154	0.000985689
	map04626	Plant-pathogen interaction	199	0.021613488
	map04016	MAPK signaling pathway-plant	129	0.033747127
	map00010	Glycolysis / Gluconeogenesis	258	0.041066867
WS10 VS CK10	map00940	Phenylpropanoid biosynthesis	116	2.06058E-05
	map03050	Proteasome	173	6.26594E-05
	map03010	Ribosome	754	0.000128457
	map04626	Plant-pathogen interaction	227	0.001190977
	map04712	Circadian rhythm-plant	27	0.001840331
WS15 VS CK15	map00940	Phenylpropanoid biosynthesis	94	5.16291E-12
	map04626	Plant-pathogen interaction	174	2.3978E-11
	map04016	MAPK signaling pathway-plant	104	2.01935E-06
	map00592	alpha-Linolenic acid metabolism	38	6.42831E-05
	map04075	Plant hormone signal transduction	53	0.00028909
	map00901	Indole alkaloid biosynthesis	15	0.00026099
	map00960	Tropane, piperidine and pyridine alkaloid biosynthesis	30	0.00044388
	map00500	Starch and sucrose metabolism	137	0.000408926
	map00350	Tyrosine metabolism	76	0.000836549
	map00460	Cyanoamino acid metabolism	42	0.002182674
	map00030	Pentose phosphate pathway	93	0.003063753
	map00941	Flavonoid biosynthesis	12	0.003442877

KEGG: Kyoto Encyclopedia of Genes and Genomes.

had high connectivity on the fifth day (Figure 5a). The proteins encoded by 26 genes in the protein interaction network of *Gardenia jasminoides* Ellis DEGs had high connectivity on the 10th day (Figure 5b). The proteins encoded by 31 genes in the protein interaction network of *Gardenia jasminoides* Ellis DEGs had high connectivity on the 15th day (Figure 5c).

Among these genes, 12 genes in *Gardenia jasminoides* Ellis DEGs have high connectivity on the 5th, 10th and 15th day of waterlogging stress. The encoded proteins and functional annotation information are shown in Table 6. Most of them are structural components of protease, endopeptidase and ribosome, which have the functions of protein turnover and transport, ribosome formation and metal ion binding.

3.4.5. qRT-PCR verification results

In order to verify the accuracy of the expression data obtained in RNA-seq, six differentially expressed genes (including three up-regulated expression genes and three down-regulated expression genes) are closely related to *Gardenia jasminoides* Ellis response to waterlogging stress were selected in this study. We used qRT-PCR technology to verify these differential genes. The qRT-PCR results of the selected genes tend to be consistent with the expression data obtained by the corresponding RNA-seq, indicating the accuracy of the RNA-seq results in this study, which can be seen in Figure 6.

4. Discussion

Plants have evolved and formed highly complex mechanisms to respond to environmental stress (Yang et al., 2015). Since waterlogging disasters frequently occur in *Gardenia jasminoides* Ellis planting areas, exploring the mechanisms of *Gardenia jasminoides* Ellis and other Chinese medicines in response to waterlogging stress can promote the development of Chinese medicines to a certain extent. For the cultivation and production of *Gardenia jasminoides* Ellis and other traditional Chinese herbs, we measured the changes of various physiological indexes and the content of four endogenous hormones of *Gardenia jasminoides* Ellis under waterlogging stress, analyzed the transcriptome data, and conducted a preliminary study on the differentially expressed genes.

4.1. The changes of various physiological indexes of Gardenia jasminoides Ellis under waterlogging stress

Under waterlogging stress, the plant's water content and chlorophyll content will change, which is most obviously reflected in the plant leaves. Plants must have

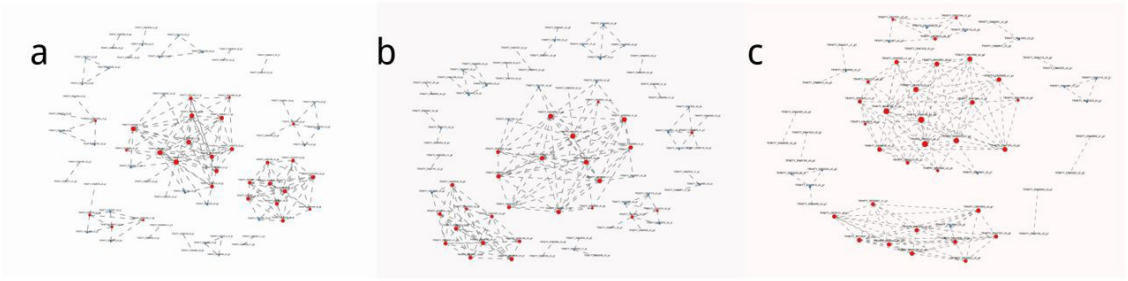

Figure 5. Protein interaction network analysis of DEGs. a: At the fifth day of flooding stress. b: At the tenth day of waterlogging stress. c: At the day fifteen of waterlogging stress.

Table 6. Key genes screened by protein interaction network analysis.

Gene name	Encoded protein	Description
TRINITY_DN35681_c1_g1	AT1G04810	26s proteasome non-atpase regulatory subunit 1 like b
TRINITY_DN12547_c0_g1	PRC3	threonine-type endopeptidase activity
TRINITY_DN35451_c0_g1	ATS9	proteasome complex
TRINITY_DN50809_c0_g1	RPT5B	proteasome-activating ATPase activity;;
TRINITY_DN47437_c0_g2	PBG1	threonine-type endopeptidase activity
TRINITY_DN46105_c0_g2	PAE1	threonine-type endopeptidase activity
TRINITY_DN42033_c0_g4	PBC1	proteasome subunit beta type-3-like
TRINITY_DN42762_c1_g1	EMB2207	structural constituent of ribosome
TRINITY_DN50543_c1_g1	AT1G15930	structural constituent of ribosome
TRINITY_DN41545_c1_g1	AT1G52300	metal ion binding; structural constituent of ribosome
TRINITY_DN39915_c1_g1	AT1G08360	RNA binding; structural constituent of ribosome
TRINITY_DN41968_c0_g6	AT1G07070	structural constituent of ribosome

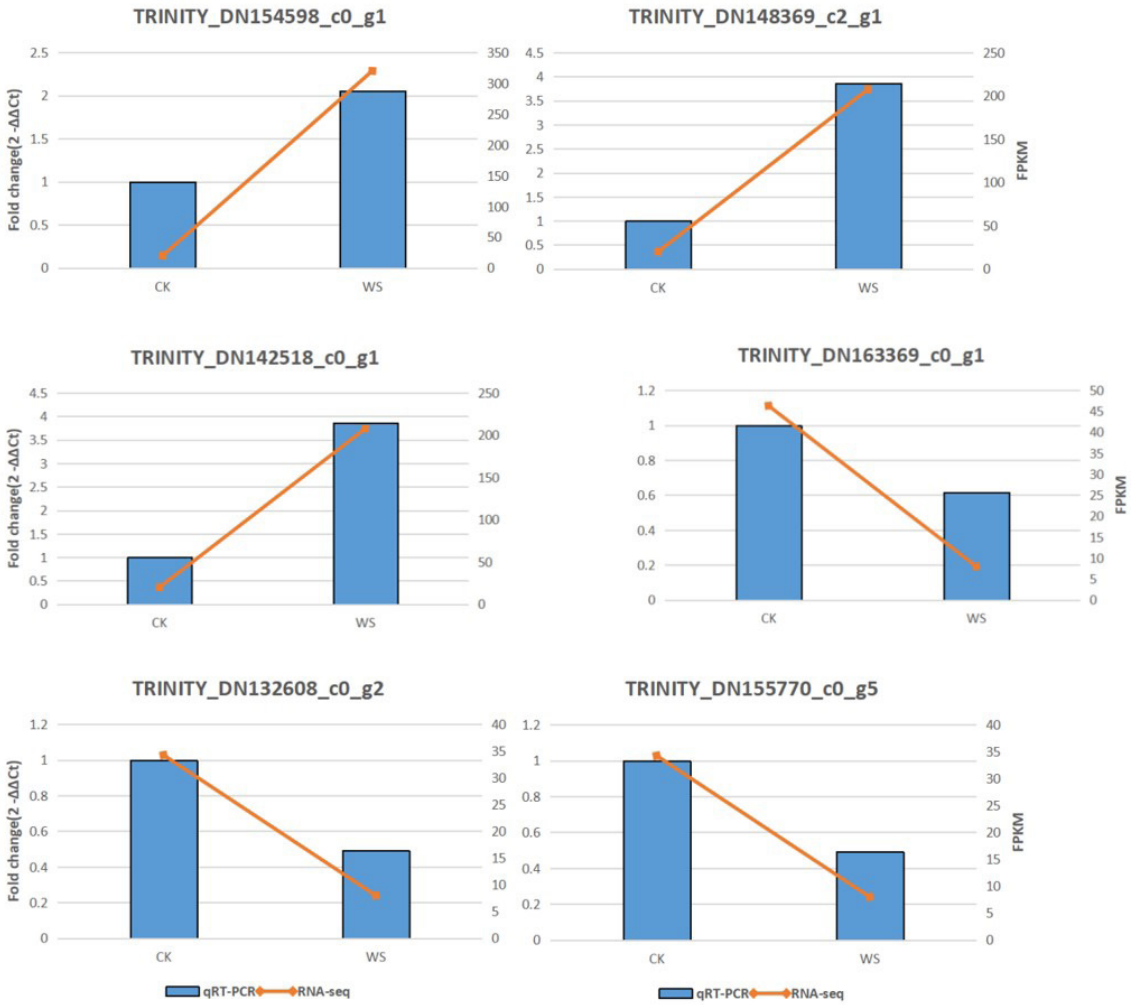

Figure 6. qRT-PCR validation of differential gene expression levels.

a certain water content to maintain normal physiological activities (Wang et al., 2012). Chlorophyll content can reflect the absorption and transformation of light energy by plants to a certain extent. This study found that the relative water content and the chlorophyll content per unit area of *Gardenia jasminoides* Ellis decreased under waterlogging stress and increased slightly with the extension of stress time, while still lower than that of the CK group. This shows that *Gardenia jasminoides* Ellis responds to waterlogging stress by reducing the relative water content and chlorophyll content and maintaining it at a certain level, which is similar to the research results of Chen et al. (2018a).

Waterlogging stress will destroy the balance between the production and removal of reactive oxygen species in the plant, resulting in increased membrane lipid peroxidation, increased cell membrane permeability, and damage to plant cell membranes increased levels of active oxygen and free radicals, and increased membrane peroxidation products (Chen et al., 2018b). It has been shown that plants mainly scavenge cellular reactive oxygen species through enzymatic and non-enzymatic reactions (Liang, 2018). The enzymatic reaction system mainly includes superoxidase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX) and glutathione peroxidase (GPx) (Zhong, 2016); The non-enzymatic reaction system mainly includes cysteine, reduced glutathione and ascorbic acid (ASA) (Zhu et al., 2011). This study found that the SOD activity in *Gardenia jasminoides* Ellis under waterlogging stress increased slightly and then decreased significantly compared with the CK group, indicating that SOD played a role in the early stage of stress, which is consistent with the results of Xia et al. (2019). The POD activity increased significantly in the first 15 days, indicating that *Gardenia jasminoides* Ellis had anaerobic resistance, and then significantly decreased on the 20th day, indicating that the POD has a specific limit to remove active oxygen. MDA is one of the final decomposition products of membrane lipid peroxidation, and its content can reflect the degree of damage to plants (Zhang et al., 2016). There was no significant change of MDA content in *Gardenia jasminoides* Ellis under waterlogging stress, which indicated that

Gardenia jasminoides Ellis had strong waterlogging resistance and an excellent antioxidant system, which could reduce MDA content and oxidative damage.

The way of plant water absorption is mainly osmotic action, i.e., from the parts with high water potential to the low water potential parts. Under the stress of adversity, plants maintain the cell swelling pressure and osmotic potential by accumulating small molecular organic substances and inorganic ions, stabilizing the internal structure of cell membrane and related proteins, and ensuring the normal physiological function of plants (Martínez et al., 2007). The main substances that play the role of osmoregulation are PRO, soluble sugar, betaine and polyols (Long, 2013). PRO and soluble sugar are important Osmoregulation Substances and response substances to water stress. This study found that the PRO in the *Gardenia jasminoides* Ellis increased first and then decreased gradually compared with the CK group under waterlogging stress, indicating that the PRO in the *Gardenia jasminoides* Ellis played an important osmotic adjustment role in the early stage of the stress. The soluble sugar content of the LW group increased first. It then decreased compared with the CK group, indicating that *Gardenia jasminoides* Ellis could rapidly increase soluble sugar under light waterlogging to regulate the osmotic pressure to maintain the internal environment's stability. The soluble sugar content of the MW and SW groups decreased and then increased compared with the CK group, indicating that the moderate and severe waterlogging inhibited the aerobic respiration of the *Gardenia jasminoides* Ellis due to hypoxia and consumed a large amount of soluble sugar, resulting in a weakened osmotic adjustment ability, and a later recovery, which may be an adaptive reflection of waterlogging stress.

4.2. The changes of endogenous hormones contents in Gardenia jasminoides Ellis under waterlogging stress

ABA is a plant hormone with critical regulatory functions on plant stress resistance, stomatal movement, and gene expression (Sun, 2007). It is the most apparent change among many endogenous hormones in plants and is often used as an essential indicator to evaluate plant stress resistance (Liu et al., 2012). This study found that the ABA content of *Gardenia jasminoides* Ellis leaves in the LW group decreased first and then increased compared with the CK group, while the ABA content of the MW and SW groups increased first and then decreased. This is consistent with the results obtained by Li (2017), who flooded the river bamboo. It shows that LW stress reduces the ABA content of *Gardenia jasminoides* Ellis in the short term, promotes growth and metabolism, but with the extension of stress time, *Gardenia jasminoides* Ellis adapts to the environment by slowing down growth and closing stomata. It also shows that *Gardenia jasminoides* Ellis under MW and SW stress rapidly increased ABA content in the body to carry out stress signalling, regulate gene expression and physiological state to resist waterlogging. With the extension of stress time, ABA content gradually dropped, *Gardenia jasminoides* Ellis's ability to resist stress gradually weakens.

The three hormones IAA, GA and ZR can promote the growth and development of plants. The distribution and content of IAA play a vital role in plant cell polarity development, cell elongation and others (Liu et al., 2011), which is also closely related to stress (Park et al., 2007). This study found that the IAA content of *Gardenia jasminoides* Ellis under waterlogging stress showed a declining trend after increasing compared with the CK group. The IAA content of the LW group was slower than that of the MW and SW groups, and the IAA content of the MW and SW groups was higher than that of the CK group on the fifth day the largest, the LW group reached the highest level at 10d. It also found that the IAA content of *Gardenia jasminoides* Ellis under waterlogging stress showed a declining trend after increasing compared with the CK group. The IAA content of the LW group was slower than that of the MW and SW groups. Compared with the CK group, the IAA content of the MW and SW groups increased the most on the 5th day, and the LW group had the most significant increase on the 10th day. GA can promote plant stomata closure, reduce plant transpiration, and promote plant growth (Li et al., 2014). In this experiment, the GA content of the *Gardenia jasminoides* Ellis leaves in the LW and MW groups decreased first and then increased and then decreased compared with the CK group, and the SW group first increased and then decreased. It can be found that the GA content in the *Gardenia jasminoides* Ellis has a gradually decreasing trend in the later stage of waterlogging stress. ZR can promote cell division and expansion and delay leaf senescence. The ZR content of *Gardenia jasminoides* Ellis leaves in the LW group gradually decreased and then increased compared with the CK group. The increase of ZR content in the later period may delay leaf senescence shedding to maintain life activities. The ZR content of the MW and SW groups increased first. It then decreased, indicating that *Gardenia jasminoides* Ellis accelerated cell division and growth in response to the stress of the early stage.

4.3. Analysis of RNA-Seq data of Gardenia jasminoides Ellis

Transcriptome research has been widely used in medicine and biology, and other disciplines, but data distortion may occur during the research process, resulting in questionable reliability of subsequent results (Yan et al., 2018). The research object of transcriptomics should include all the mRNA in the cell, and the ideal transcriptome data should also contain all the full-length cDNA sequences (Wei et al., 2011; Wu et al., 2012). However, the introduction to wrong bases may occur during sequencing and assembling (Wang et al., 2010). Together with homologous gene sequences and variable splicing present in eukaryotes, they may cause assembly sequences to be non-full-length sequences (Xiao et al., 2013). In this study, the quality of raw and clean reads was evaluated, and information such as unigenes length distribution, N50 length, and gene saturation was comprehensively analyzed. It was determined that *Gardenia jasminoides* Ellis sequencing data under waterlogging stress met the requirements. Based on the excellent quality of sequencing data, this study still selected six differentially expressed genes for

qRT-PCR verification, whose results showed that the gene expression trends between qRT-PCR and RNA-seq are consistent, proving that the transcriptome data obtained in this study is accurate and reliable, and can be used for subsequent in-depth mining and research. Meanwhile, we annotated All-Unigene in different databases. We found that 60.03% of the genes were successfully annotated, and the remaining 39.97% of the genes may be new genes or specific genes unique to *Gardenia jasminoides* Ellis under waterlogging stress.

4.4. DEGs analysis of Gardenia jasminoides Ellis

After stress, the cell wall of plants plays a vital role in the activation of metabolic sites and can produce mitogen-activated protein kinase (MAPK) associated with stress signal molecules (Opdenakker et al., 2012). MAPK plays an essential role in plant signal transduction. It has a three-stage kinase mode that is activated sequentially and jointly regulates critical cellular physiological processes such as cell growth, differentiation, and environmental stress adaptation (Li et al., 2004). Plant hormones function in regulating the growth and development of plants and work in signal transduction when plants respond to external stress. It means that hormones in plant cells can regulate the metabolism of plants by binding to receptor proteins under stress, producing a series of physiological and biochemical reactions, thus forming an efficient and orderly regulatory network to reduce the damage of stress to plants (Liu, 2016). This study found that MAPK signalling pathway-plant and plant hormone signal transduction were significantly enriched in the KEGG analysis. Both played a signal transduction role in *Gardenia jasminoides* Ellis in response to waterlogging stress and regulated the metabolic process and biochemical physiology level.

Plant-pathogen interaction responds to plant susceptibility and during plant cutting and adversity stress. This pathway is closely related to cell wall reinforcement, induction of stomatal closure, allergic reactions and defence-related genes (Xu et al., 2012). This pathway was significantly enriched in *Gardenia jasminoides* Ellis on 5d, 10d, and 15d under waterlogging stress. The Phenylpropanoid biosynthesis pathway is the leading way to produce phenolic compounds in higher plants. Phenolic compounds can participate in the strengthening, adhesion, defence of plant cell walls, absorb ultraviolet rays, and reduce the generation of oxygen free radicals (Yoshitama, 2000). The pathway is significantly enriched after 10d and 15d waterlogging stress, combined with the MDA activity changes. This pathway is vital for *Gardenia jasminoides* Ellis to protect the cell membrane and reduce ultraviolet radiation.

Glycolysis refers to the process by which organisms obtain energy from glycolysis and the metabolism of sugar. The key enzyme is Phosphofructokinase (PFK). Gluconeogenesis refers to the process by which organisms convert various non-sugar substances into glucose and glycogen. The key enzyme is fructose-1,6-diphosphate (FDP) (Gao et al., 2019). These two metabolic processes are coordinated with each other, which can provide energy in the process of plants responding to adversity stress, adjusting the osmotic pressure of plant cells and maintaining the normal shape of cells. Taking the *Gardenia jasminoides* Ellis on the fifth day of waterlogging stress as an object, it was found that there are 14 genes related to PFK, 8 of which are up-regulated and two related to FDP are all up-regulated. The glycolysis/gluconeogenesis metabolic pathway is systemic, including starch, cellulose, sucrose, and other substances, and is closely related to lipid metabolism, protein metabolism, and tricarboxylic acid cycle.

Circadian rhythm-plant refers to the plant's adjustment to many critical physiological processes such as stomatal switch, hypocotyl elongation, and leaf movement to cope with environmental changes and coordinate plant physiological activities and development processes to occur at an appropriate time of the day (Gao et al., 2017). Flavonoids and flavonol secondary metabolites can participate in removing plant oxidation products in a variety of abiotic stress environments and are non-enzymatic antioxidant components in plant cells (Nakabayashi et al., 2014; Martinez et al., 2016). Under waterlogging stress, the activities of SOD and POD in *Gardenia jasminoides* Ellis showed an increasing trend and then a decreasing trend. The increasing changes were insignificant, and the cell lipid membrane was not damaged too much. We believe that there are other ways to remove oxygen free radicals to reduce oxidation in *Gardenia jasminoides* Ellis. In addition to glutathione, phenols and terpenes, flavonoids and flavonol secondary metabolites may also play an important role.

Protein degradation and renewal runs through the entire life process of the plant to meet the needs of different growth and development stages or growth conditions. The degradation of protein requires the participation of many proteases (Wang, 2010). Proteolytic enzymes (peptidases) can be divided into endopeptidases and exopeptidases, of which endopeptidases have a vigorous activity and are divided into serine endopeptidases, cysteine endopeptidases, aspartate endopeptidase Enzymes, metal endopeptidases and threonine endopeptidases. Endopeptidases in plants mainly belong to the class of cysteine proteases, and the primary function is to turn around and transport proteins. As early as 2001, Cruz de Carvalho et al. (2001) found that drought-sensitive soybeans had significantly increased peptidase activity and reduced protein content within the leaves after drought stress (Zhong, 2008). At the same time, studies have shown that endopeptidases play the role of signal recognition, transduction and execution in the process of plant disease resistance. This study found that the genes or gene products in the significant analysis of GO are mainly reflected in the activity regulation of peptidases, endopeptidases and cysteine endopeptidases. Proteasome in KEGG is significantly enriched after 5d and 10d waterlogging stress. Most of the genes encoding highly connected proteins in PPI are related to proteases and endopeptidases. To conclude, we believe that proteases, endopeptidases and other substances play an essential role in *Gardenia jasminoides* Ellis's resistance to waterlogging stress.

In general, after being subjected to waterlogging stress, *Gardenia jasminoides* Ellis regulates various physiological and metabolic pathways through MAPK signalling

pathway-plant and plant hormone signal transduction. These pathways involve the strengthening and adhesion of cell walls, the induction of stomatal closure, allergic reactions, defence reactions, leaf movements and others. They can absorb ultraviolet light to reduce the generation of oxygen free radicals, increase secondary metabolites such as flavonoids to remove intracellular oxidation products, change the ways of energy utilization and regulate osmotic pressure of plant cells. At the same time, a variety of proteasomes, proteases and endopeptidases are used to turn around and transport proteins to maintain *Gardenia jasminoides* Ellis stability.

5. Conclusion

In summary, the mechanism of *Gardenia jasminoides* Ellis in response to waterlogging stress can be briefly summarized as follows: waterlogging can activate or inhibit the expression of related genes through MAPK signal transduction and endogenous hormone signal transduction pathways after experiencing stress, adjust the turnover and transportation of proteins in the body, regulate the physiological changes, and change the metabolism of secondary products to resist flooding stress. This study investigated the physiological response of Gardenia jasminoides to waterlogging stress and analyzed its transcriptomic, which provides reference data and theoretical support for future comprehensive understanding of plant response to other adversity stresses such as waterlogging.

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