

# Identification of Potential Shade Response Regulators in Endangered Species *Magnolia Sinostellata*

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## Research Article

**Keywords:** Magnolia sinostellata, potential shade response, endangered species, plants, Magnoliaceae plants

**Posted Date:** July 21st, 2021

**DOI:** <https://doi.org/10.21203/rs.3.rs-701102/v1>

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**Additional Declarations:** No competing interests reported.

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**Version of Record:** A version of this preprint was published at Scientific Reports on December 29th, 2022. See the published version at <https://doi.org/10.1038/s41598-022-25393-x>.

# Abstract

*Magnolia sinostellata* is one of the endangered species in China and largely grows under canopy shade. However, the shade response molecular mechanism remains unclear. To explore potential regulators in the mechanism, weighted gene co-expression network analysis (WGCNA) was performed to analysis the transcriptome data of *M. sinostellata* leaves subjected to shading with different time. Gene co-expression analysis illustrated that lightsteelblue1, paleturquoise, darkolivegreen modules are closely associated with shade treatment. Gene ontology and KEGG analyses showed that genes in lightsteelblue1 module mostly participated in amino and nucleotide metabolism, genes in paleturquoise module mostly involved in carbon fixation and genes in darkolivegreen module mainly participated in photosynthesis related pathways. Through Cytoscape3.8.2, we identified 6, 7 and 8 hub genes in lightsteelblue1 module, paleturquoise module and darkolivegreen module, respectively. This study found that shading impacted photosynthesis, carbon assimilation and flowering of *M. sinostellata*. In addition, key shade response regulators identified in this study have laid a firm foundation for further investigation of shade response molecular mechanism and protection of shade sensitive plants.

## Introduction

Records on Magnoliaceae plants can be traced back to the Mesozoic era. Until now, many Magnoliaceae plants have been favored by people for their important ornamental characteristics. However, with the changes of the climate, forest community composition, and the succession of forests, deciduous Magnoliaceae species are facing endangerment in the wild<sup>1</sup>. The growth and distribution of endangered species of Magnoliaceae are mostly confined to narrow areas. Magnoliaceae plants was mainly adapted to coniferous and broad-leaved mixed communities, while declined to evergreen broad-leaved communities<sup>2-4</sup>. *Magnolia sinostellata* is an endangered species belonging to family Magnoliaceae. It is a deciduous shrub which can grow to 3 m in height and blossom in early spring (February and March) in subtropical regions<sup>5</sup>. *M. sinostellata* is endemic to a narrow area of China (mainly distributed in Jingning, Wenzhou counties, Zhejiang Province), with extremely small population. Pre-investigations indicated that *M. sinostellata* mainly distributed in coniferous forests, sparsely distributed in broad-leaved forests and mixed forests<sup>4</sup>. The upper tree layer of forest community determines the light intensity in the community. The light intensity of coniferous communities is higher than that of evergreen broad-leaved communities<sup>6</sup>.

Canopy shade is the major factor that affect plant growth and development in understories in natural environment<sup>7-10</sup>. The primary effect of shading on plants is weakened photosynthesis efficiency<sup>11</sup>. Under shady conditions, light captured by light harvesting complex (LHC) is limited in plants<sup>12</sup>. Electron transport rate through PS I (ETR I) is significantly reduced in Rice due to the defect in chloroplast<sup>13</sup>. Moreover, in *Zea mays*, photochemical quenching coefficient(qp) and effective quantum yield of PSII photochemistry (ΦPSII) were significantly reduced under shade, which indicating that the photosynthesis ability weakened<sup>14</sup>. In photosynthetic plants, Ribulose 1,5-bisphosphate carboxylase/oxygenase

(Rubisco) is the key enzyme in Calvin cycle, which transforms CO<sub>2</sub> into carbohydrates for further metabolism<sup>15</sup>. Rubisco activity is repressed and the content of Rubisco enzyme is decreased under shade in plants<sup>16</sup>, showing that the fixed carbon for further carbon assimilation in plants was reduced. In addition, the activity of sucrose phosphate synthase and sucrose synthase are limited in wheat, indicating that its starch and sucrose metabolism was inhibited<sup>17</sup>. With the increase of shading degree, the carbon assimilation and metabolism capacities of seedlings decreases<sup>18</sup>, water usage and transpiration are also affected<sup>19</sup>, which inhibit plant growth and community regeneration. At the same time, with the growth of seedling age and the development of plant reproduction, the demand for light of light-needing species will also increase. The lack of light intensity will inevitably reduce the number of flowers and affect the fruiting of plants. Shading dramatically reduced flower quality, alkaloid yield and seed number of *Papaver somniferum*<sup>20</sup>. In *Paeonia lactiflora*, shading led to delayed flowering date, reduced flower fresh weight and faded flower color<sup>9</sup>.

Transcriptome data analysis is a sharp tool to analyze and reveal underlying molecular mechanism in response to various abiotic stress in plants<sup>21-23</sup>. In recent studies, various shade-responsive genes involved in photosynthesis, plant hormone signal transduction, circadian rhythm and metabolic pathways have been identified through RNA-seq<sup>24-26</sup>. However, the shade response molecular mechanism in plants remains unclear. More importantly, the key shade response regulatory genes in *M. sinostellata* and other shade sensitive plants remains unknown. Weighted gene co-expression network analysis (WGCNA) is an effective tool to identify co-expressed modules and hub genes<sup>27</sup>, which has been applied in various plants including *Camellia sinensis*<sup>28</sup>, hot peppers<sup>29</sup>, rice<sup>30</sup> and wheat<sup>27</sup> to explore various abiotic stresses response molecular mechanisms. It is a significant way to understand gene function and association from the transcriptome-wide<sup>31-33</sup>. However, there is no previous report of using WGCNA to identify shade response hub genes in *M. sinostellata*.

In this study, transcriptomes of shade treated and untreated *M. sinostellata* leaves were firstly analyzed to identify core differential expression genes (DEGs) under shade. Further, WGCNA was applied to detect key modules involved in shade response. Finally, hub genes in response to shade were pinpointed, which can be breakthrough points to understand shade response molecular networks in *M. sinostellata*. Our results provide a theoretical basis for further understanding of shade response molecular system in plants and provide essential information for protection of shade sensitive species.

## Results

### Identification of the shade-responsive core DEGs in *M. sinostellata*

A total of 181,902 genes were detected in this transcriptome sequencing, and the average comparison rate with the compared gene set was 80.49%. 246481 non-redundant transcript sequences were obtained. The total length of these sequences is 270,112,156, the longest sequence is 20445bp, the shortest sequence is 199bp, and the average sequence length is 3420.95bp. In this study, we analyzed the global

gene expression profiles of *M. sinostellata* for shade response using five datasets, including CK-D0, CK-D5, ST-D5, CK-D15 and ST-D15, the details of which are shown in Table S2. Each dataset contains 3 replicates. Correlation analysis was performed among these 15 samples, which showed good reproductivity in the same group and a significance difference between control groups and shade treated groups (Figure S1). This analysis suggests that these samples can be used for subsequent screening and analysis.

From a total of 181,902 expressed genes, 4734 core DEGs were identified using cross-compared venn diagrams including CK-D0 vs ST-D5, CK-D0 vs ST-D15, CK-D5 vs ST-D5 and CK-D15 vs ST-D15 ( $\text{Log}_2\text{FC} > 1$ ,  $\text{Qvalue} < 0.05$ ) (Fig. 1). Then, we characterized these 4734 core DEGs to get more insight into their contributing molecular pathways. First, 4734 DEGs were subjected to GO analysis, which were classified into three groups and 33 subgroups (Fig. 2A). The biological process group can be divided into 18 subgroups, among which cellular process and metabolic process were the top two sub-groups involved the most genes. Four subgroups were related to cellular component, among which cellular anatomical entity and intracellular were the main subgroups involved most genes. Eleven subgroups constitute the molecular function group, and the catalytic activity and binding involved the most genes. GO enrichment analysis showed that most significant GO terms are related to photosynthesis. The top five enriched GO terms were (GO:0009522); photosynthesis, light harvesting (GO:0009765); photosystem (GO:0009521); photosynthesis (GO:0015979) and photosystem II (GO:0009523) (Fig. 2B). Then, we performed KEGG analysis to explore contributing pathways of 4734 DEGs (Fig. 3A). For KEGG classification, genes were annotated into five groups and 19 subgroups. Among metabolism group, global and overview maps, carbohydrate metabolism and energy metabolism subgroups were significantly enriched. Photosynthesis-antenna proteins was the most significantly enriched KEGG pathway. The top five frequently enriched pathways were as follows: photosynthesis-antenna proteins, galactose metabolism, phenylpropanoid biosynthesis, starch and sucrose metabolism and alanine, aspartate and glutamate metabolism (Fig. 3B).

### **WGCNA of core shade responsive DEGs in *M. sinostellata***

To identify key shade responsive genes in *M. sinostellata*, WGCNA was performed to analysis 4734 core DEGs, which can identify modules of highly related genes. The selection of an optimal soft threshold power is an essential step to construct WGCNA. A network topology research of 1–20 was executed, and the scale independence and the average connectivity of the WGCNA relative equilibrium were determined. Threshold 18 was selected to construct a hierarchical clustering tree of DEGs (Fig. 4A). MEDiss Thres was set to 0.22 to merge similar modules and 4 modules was generated (Fig. 4B). Genes in grey module that cannot be assigned to any modules were not analyzed in the further study. Four different modules were generated by WGCNA including lightsteelblue1, paleturquoise, darkolivegreen and violet. The darkolivegreen module, lightsteelblues1 module, paleturquoise module and violet module including 2481, 2008, 75 and 71 DEGs, respectively (Table 1). To identify co-expression similarity of modules, characteristic genes were calculated and clustered according to their correlation (Fig. 4C). We found that these 4 modules are divided into two categories: the first included lightsteelblue1 and paleturquoise modules; the second included darkolivegreen and violet modules. Gene modules of the same category

may have similar functions or contributing to the same regulatory mechanism. To investigate modules associated with shade treatment, we plotted module-trait relationship heat map (Fig. 4D). This result shows that lightsteelblue1 module positively correlated with two shade-treated groups (ST-D5,  $r = 0.7$ ; ST-D15,  $r = 0.49$ ) but negatively correlated with control groups (CK-D0,  $r = -0.40$ ; CK-D5,  $r = -0.38$ ; CK-D15,  $r = -0.41$ ). Similarly, paleturquoise module had a strong correlation with ST-D15 ( $r = 0.68$ ), while had a negative correlation with CK-D0 ( $r = -0.26$ ), CK-D5 ( $r = -0.25$ ) and CK-D15 ( $r = -0.25$ ). In contrast, darkolivegreen showed a positive correlation with control groups (CK-D0,  $r = 0.38$ ; CK-D5,  $r = 0.35$ ; CK-D15,  $r = 0.49$ ) but illustrated a significant negative correlation with treated groups ( $\text{cor} < -0.5$ ,  $p < 0.05$ ). However, genes in violet module showed no significant correlation with treated groups or control groups. These results suggest that DEGs in lightsteelblue1 and paleturquoise modules mainly up-regulated and DEGs in darkolivegreen module down-regulated under shade treatment. These results showed that lightsteelblue1, paleturquoise and darkolivegreen modules were significantly correlated with shade treatment. To obtain further understand of the expression pattern of these three modules, heat maps of gene expression for these modules were generated along with eigengene expression values (Fig. 5). We observed that lightsteelblue1 module genes were significantly responsive to shade treatment and mainly up regulated. In paleturquoise module, genes were slightly induced in ST-D5 while markedly up-regulated in ST-D15. In contrast, darkolivegreen genes mainly downregulated under shade treatment.

Table 1  
Details of four detected modules

Module	Gene number
lightsteelblues1	2008
paleturquoise	75
darkolivegreen	2481
violet	71

### GO and KEGG pathways analysis of DEGs in key modules in *M. sinostellata* associated with shade

The functions of genes under shade treatment in specific modules and its contributing regulatory pathways were revealed by GO and KEGG analysis. GO analysis suggested that 'N-acetyltransferase activity' and 'acetyltransferase activity' as the most significantly enriched GO terms in lightsteelblue1 module, which means genes in this module mainly response to shade by regulating acetyltransferase activity (Fig. 6A). KEGG analysis in lightsteelblue1 module identified 'Valine, leucine and isoleucine degradation' and 'Amino sugar and nucleotide sugar metabolism' as the most enriched regulatory pathways (Fig. 6B). Concerning paleturquoise module, GO analysis displayed that 'hydrolase activity' and 'hydrolyzing O-glycosyl compound' were the top two enriched GO terms indicating that these genes regulating hydrolase-related metabolism (Fig. 6C). KEGG analysis demonstrated that 'Starch and sucrose metabolism' pathway was appreciably influenced by shade (Fig. 6D). These results matched GO and

KEGG analysis conclusion of 4734 core DEGs, indicating that the results are reliable. In darkolivegreen module, GO analysis indicated that enriched GO terms of cellular component, biological process and molecular function were mainly related to photosynthesis, among which the top five GO terms were 'photosystem I', 'photosynthesis, light harvesting', 'photosystem', 'photosynthesis' and 'thylakoid' (Fig. 6E). KEGG analysis showed that 'Photosynthesis-antenna proteins' and 'Photosynthesis' were the most enriched pathways (Fig. 6F). These indicate that genes in darkolivegreen module mainly involved in photosynthesis.

### **Hub genes identification in key Co-Expressed modules in *M. sinostellata* under shade**

To identify hub genes in these three modules, genes with a weight parameter over 0.4 were analyzed and visualized through Cytoscape 3.8.2 to construct interaction networks (Fig. 7). We identified 6, 7 and 8 hub genes in lightsteelblue1, paleturquoise and darkolivegreen modules respectively via integrated analysis results of MCODE, cytoHubba and Centiscape2.2 in Cytoscape3.8.2 (Table 2). Isoform\_16555 (Anthesis Promoting Factor 1, *MsAPF1*), isoform\_15622 (DUF1644 domain-containing protein, *MsSIZ1*), isoform\_210768 (Acyl-CoA N-acyltransferase protein, *MsGNAT6*), isoform\_13861 (Detoxification 21, *MsHMP21*), isoform\_16567 (Mitogen-activated protein kinase 10, *MsCXIP4*), and isoform\_107196 (Unknown) were identified as hub genes in lightsteelblue1 module, indicating that these genes have significant functions in Amino acids and nucleic acids metabolism under shade. In paleturquoise module, isoform\_10150 (Beta-glucosidase 18, *MsBGL18*), isoform\_92874 (Basic 7S globulin, *MsBg7S*), isoform\_192429 (Cytochrome P450 710A11, *MsCYP710A11*), isoform\_238198 (Transcription factor TGA2.2, *MsTGA2*), isoform\_55976 and isoform\_152869 (Pathogenesis-related protein P2, *MsPR4*) might play central roles in carbon fixation related pathways under shade.

Table 2  
the hub genes detected in three modules

Modules	Gene ID	Gene name	Arabidopsis Orthologs	Predicted functions
Lightsteelblue1	isoform_16555	<i>MsAPF1</i>	AT5G66240.1	Anthesis pomoting factor 1
	isoform_15622	<i>MsSIZ1</i>	AT3G25910.1	DUF1644 domain-containing protein
	isoform_210768	<i>MsGNAT6</i>	AT2G06025.5	Acyl-CoA N-acyltransferase protein
	isoform_13861	<i>MsHMP21</i>	AT1G33110.1	Detoxifiction 21
	isoform_114887	<i>MsMAPK10</i>	AT4G21970.1	Mitogen-activated protein kinase 10
	isoform_16567	<i>MsCXIP4</i>	AT2G28910.2	Zinc finger protein
	isoform_107196	<i>unknown</i>	AT1G23440.1	Unknown
paleturquoise	isoform_10150	<i>MsBGL18</i>	AT1G61820.1	Beta-glucosidase 18
	isoform_92874	<i>MsBg7S</i>	AT1G03220.1	Basic 7S globulin
	isoform_192429	<i>MsCYP710A11</i>	AT2G34500.1	Cytochrome P450 710A11
	isoform_238198	<i>MsTGA2</i>	AT1G68640.1	Transcription factor TGA2.2
	isoform_55976	<i>unknown</i>	AT5G57123.1	Unknown
	isoform_152869	<i>MsPR4</i>	AT3G04720.1	Pathogenesis-related protein P2
darkolivegreen	isoform_10052	<i>MsFLA15</i>	AT3G52370.1	FAS1 domain-containing protein
	isoform_12760	<i>MsUGT73C7</i>	AT3G53160.1	UDP-rhamnose: rhamnosyltransferase 1
	isoform_121918	<i>MsUGT91C1</i>	AT5G49690	UDP-rhamnose: rhamnosyltransferase 1
	isoform_108687	<i>MsSBT3</i>	AT1G66220.1	Subtilisin-like protein protease SBT3.3
	isoform_11567	<i>MsFLA17</i>	AT5G06390.1	FAS1 domain-containing protein
	isoform_28177	<i>MsLECRK-V.1</i>	AT1G70110.1	L-type lectin-domain-containing protein
	isoform_119775	<i>MsFMN</i>	AT4G27270.1	NADPH-dependent FMN reductase

Modules	Gene ID	Gene name	Arabidopsis Orthologs	Predicted functions
	isoform_47022	<i>MsGHL</i>	AT4G31500.1	Geraniol 8-hydroxylase-like protein

In darkolivegreen module, isoform\_10052 (FAS1 domain-containing protein, *MsFLA15*), isoform\_12760 (UDP-rhamnose:rhamnosyltransferase1, *MsUGT73C7*), isoform\_121918 (UDP-rhamnose:rhamnosyltransferase1, *MsUGT71C1*), isoform\_108687 (Subtilisin-like protein protease SBT3.3, *MsSBT3*), isoform\_11567 (FAS1 domain-containing protein, *MsFLA17*), isoform\_28177 (L-type lectin-domain-containing protein, *MsLECRK-V.1*), isoform\_119775 (NADPH-dependent FMN reductase, *MsFMN*) and isoform\_47022 (Geraniol 8-hydroxylase-like protein, *MsGHL*) were identified as hub genes, suggesting that these genes exert vital functions in photosynthesis in *M. sinostellata* in response to shade (Table S3).

### Verification of hub genes in *M. sinostellata* under shade by RT-qPCR

We executed a quantitative reverse-transcription PCR (RT-qPCR) analysis to verify the expression level of 21 hub genes under shade treatment after 0d, 5d and 15d in *M. sinostellata*. These results suggested that the expression level of all the hub genes were significantly changed during shade treatment, which indicating that these genes were all shade-responsive (Fig. 8). Interestingly, hub genes in lightsteelblue1 and paleturquoise modules were all significantly up regulated during shade treatment, while 8 hub genes in darkolivegreen modules were all down regulated under shade in *M. sinostellata*.

## Discussion

Canopy shade is a major abiotic stress that affect plants growth and reproduction under natural environment. Various studies have investigated on shade response molecular mechanism in plants<sup>22,26</sup>, but few of them have revealed key shade response regulators. In this study, we identify lightsteelblue1, paleturquoise and darkolivegreen modules that closely related with shade treatment. Genes in lightsteelblue1 were all up regulated and mainly regulate plant flowering. In contrast, genes in paleturquoise and darkolivegreen modules were all downregulated, and contributing to carbon fixation and photosynthesis related pathways, respectively. Then, we have detected potential shade response regulators of *M. sinostellata* through a comprehensive analysis of transcriptome data.

Hub genes in lightsteelblue1 module controlled flowering related genes. DUF1644 domain-containing proteins are abiotic stress responsive in rice<sup>34</sup> and Anthesis promoting factor 1 (*AtARF1*) have essential roles in plant flowering in Arabidopsis<sup>35</sup>. In *M. sinostellata*, *MsSIZ1* (DUF1644 domain-containing protein, isoform\_15622) controls flowering related pathways by up regulated the expression level of *MsAPF1* (isoform\_16555). *MsHMP21* (Detoxification 21 isoform X1, isoform\_13861) regulating flowering in *M. sinostellata* together with *MsAPF1* by enhanced the expression of Constans (isoform\_25713 and

isoform\_21721) under shade. Acyl-CoA N-acyltransferase protein was reported regulating the reproductive growth and flower bud differentiation in *Hordeum vulgare*<sup>36</sup>. In *M. sinostellata*, *MsGNAT6* (Acyl-CoA N-acyltransferase protein, isoform\_210768) was regulated by hub gene isoform\_107196 (Unknown). The expression of seven hub genes in lightsteelblue1 module were all increased, showing that flowering was promoted under shading. However, early flowering was harmful for accumulation of reserves for resume growth in the next season for perennial plants<sup>37</sup>. In paleturquoise module, six identified hub genes were all up regulated and mainly interacted with genes involved in carbon fixation, showing that carbohydrate metabolism was enhanced in *M. sinostellata* under shade. Basic 7S globulin is essential in starch and sucrose pathways<sup>38</sup>. *MsBGL18* regulated the expression of *MsBg7S* (Basic 7S globulin, isoform\_92874) and *MsCYP710A11* (Cytochrome P450 710A11, isoform\_192429) in the sucrose and starch metabolism pathway in *M. sinostellata*. *MsBGL18* can also controls starch and sucrose metabolism through beta-glucosidase in *M. sinostellata*, which homology was reported involved in various pathways, including activation of chemical defense compounds, phytohormones, and metabolites<sup>39</sup>. Interestingly, *MsPR4* (Pathogenesis-related protein, isoform\_152869) regulated key enzymes such as beta-glucosidase, beta-amylase and trehalose 6-phosphate synthase to promote the metabolism of sucrose and starch under shade. Unknown protein (isoform\_55976) could regulate the metabolism of sucrose and starch by activating the activity of beta-glucosidase and threonine aldolase.

Previous study have shown that the photosynthesis efficiency of *M. sinostellata* weakened under shady environment<sup>5</sup>, which was consistent with findings in this research. In darkolivegreen module, eight hub genes were downregulated and mainly regulated genes in photosynthesis and stress response related pathways, illustrating that photosynthesis and stress resistance in *M. sinostellata* were impaired. *MsLECRK-V.1* (Lectin-domain-containing protein, isoform\_28177) weakened photosynthetic efficiency of *M. sinostellata* by downregulated *MsFMN*. Through inhibited the expression of cellulose synthase synthesis genes (isoform\_48903), it reduces the transport and utilization efficiency of photosynthetic products in plants. The stress response and stress signal transduction abilities are essential for plant adaptation and survival. Subtilisin-like protein protease and Geraniol 8-hydroxylase-like protein is associated with plant pathogen resistance and MAPK signaling pathways respectively<sup>40,41</sup>. *MsSBT3* (Subtilisin-like protein protease, isoform\_108687) can attenuate the expression of the *MsGHL* (Geraniol 8-hydroxylase-like protein, isoform\_47022). Therefore, the ability of sensing stress signals in *M. sinostellata* was weakened under shade treatment. FAS1 domain-containing proteins have important function in plant development and abiotic stress response<sup>42</sup>, among which *MsFLA15* (isoform\_10052) and *MsFLA17* (isoform\_11567) inhibited transfer efficiency of the electron transport chain in the photosystem I of *M. sinostellata* under shade by repressed the activity of *MsFMN* (NAD(P)H dehydrogenase, isoform\_119775). Lectin-domain-containing protein and NADPH-dependent FMN reductase were reported to have essential roles in stress response<sup>43,44</sup>. *MsUGT73C7* (isoform\_12760) and *MsUGT91C1* (isoform\_121918) belongs to UDP-glucose: glycosyltransferase (UGT) family, which acatalyzed the steps from derhanmosyl acteoside to acteoside and from hydroxytyrosol glucoside to decaffeoylacteoside<sup>45</sup>.

*MsUGT73C7* and *MsUGT91C1* reduced the expression of superoxide dismutase synthesis genes (isoform\_15962) to weaken the stress resistance ability of *M. sinostellata*.

In general, under shading conditions, the photosynthesis and stress resistance ability of *M. sinostellata* were weakened, leading to diminished growth potential. Due to the weakening of photosynthesis, *M. sinostellata* can only support its life activities by consuming the carbohydrate in the plant. The flowering-related genes and pathways of *M. sinostellata* were enhanced, which might lead to earlier flower bud differentiation and flowering. In natural environment, the early flowering of plants can lead to excessive consumption of nutrients and affect its population renewal in the next year. The hub genes identified in this research have set a solid foundation for further research on the mechanism of shade response in plants.

## Methods

### Plant material and experimental design

The *M. sinostellata* seedlings used in this study were collected by Zhejiang Agriculture & Forestry University, Hangzhou, Zhejiang Province, China (latitude 30°26' N, longitude 119°73'E) and cultivated in Qingshan Lake Garden Center, Hangzhou, Zhejiang Province, China (latitude 30°25' N, longitude 119°81'E), which both have been approved by Forestry Bureau of Zhejiang Province, P.R.China. In addition, experimental research on *M. sinostellata* was strictly comply with the IUCN Policy Statement on Research Involving Species at Risk of Extinction and the Convention on the Trade in Endangered Species of Wild Fauna and Flora. The seedlings were placed in artificial climate room (luminance  $1400 \pm 30 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , photoperiod 14 h light, temperature  $25 \pm 2^\circ\text{C}$ , humidity 40%-60%) in Zhejiang Agriculture & Forestry University throughout the experiment. In order to simulate the closed canopy of forest, the shade experimental set-up was built with three layers of black shade net (25% full light, luminance  $350 \pm 30 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) and bamboo poles. The control group seedlings were unsheltered (100% full light, luminance  $1400 \pm 30 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ), and the other conditions remained unchanged. There were no leaves or stems overlapping between plants, each treatment had 3 replicates. Leaves were collected from the plants in both the treated and control groups after shade treatment for 0d, 5d and 15d, among which the samples of 0d were evenly pooled samples of control and treated groups, then immediately frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  for further experiments and RNA-seq analyses. At sampling, collections were performed from 3 plants, and each sample collection was repeated 3 times for biological replicates.

### Transcriptome sequencing and data analysis

To identify shade responsive genes, RNA sequencing was performed to analyze transcriptome gene expression profiles in 15 samples. These 15 samples were divided into five groups, including three control groups (CK-D0, CK-D5 and CK-D15) and two shade-treated groups (ST-D5 and ST-D15). Total RNA of 15 samples were proceed by mRNA enrichment method or rRNA removal method. The purified RNA was fragmented with the interrupted buffer and reversed with random N6 primer, and then synthesized into

cDNA two-strand to form double-stranded DNA. The ends of synthetic double-stranded DNA are filled in and the 5' end is phosphorylated. The 3' end forms a sticky end with an 'A' protruding, and then a bubbly linker with a protruding 'T' on the 3' end is connected. The ligation product is amplified by PCR with specific primers. The PCR product is heat-denatured into single-stranded, and then the single-stranded DNA is circularized with a bridge primer to obtain a single-stranded circular DNA library. DNBSEQ platform was employed to sequence the libraries. The R package (edgeR v3.16) was employed to identify the differentially expressed genes (DEGs) between shade-treated and control samples. Genes fulfilled stringent criteria were identified as DEGs (fold-change > 2 and q value < 0.05, with false discovery rate (FDR) less than 0.05). The function and involved pathways of the DEGs were classified according to the GO and KEGG annotation results and official classification. Phyper function in R software was used for enrichment analysis.

### **Weighted gene co-expression network construction and hub genes detection**

The weighted gene co-expression network was constructed using WGCNA package in R software to further analysis gene functions and contributing pathways in response to shade in *M. sinostellata*. The 4734 core DEGs of 15 samples were used to construct this network. The similarity matrix was calculated by identifying the Pearson correlation coefficient between all gene pairs. The correlation matrix was transformed by soft-thresholding process to mimic the scale-free topology. The adjacency matrix was converted into a topological overlap matrix (TOM), and all coding sequences were hierarchically clustered by TOM similarity algorithm. The co-expression gene modules of the gene dendrogram were detected by the dynamic tree cut method, which using a height-cut less than 0.22. The module membership (MM) and the significance gene (GS) were calculated and used to confirm the distinguished modules. Module network visualization was performed by Cytoscape 3.8.2 with a cut-off of weight parameter set at 0.4. Using MCODE, cytoHubba and Centiscape2.2 in Cytoscape3.8.2 to identify hub genes.

### **RT-qPCR analysis of hub genes expression**

Total RNA was extracted from the leaves of *M. sinostellata* in treated and control groups of 0 d, 5 d and 15 d using ultraclean polysaccharide and phenol plant RNA purification kit. 1 µg total RNA was converted to first-strand cDNA using the Prime Script RT master Mix. RT-qPCR were performed with Light Cycler 480 II (Roche) using BCG qPCR Master Mix. Results were analyzed with the software Light Cycler 480 SW 1.5.1. Experiments was performed under the following conditions: 95°C for 30 s; 95°C for 5 s, and 58°C for 30 s. A total of 40 cycles were performed. After the end of the program, melting curves were generated (65–95°C, 0.2°C increment). Relative gene expression was calculated using  $2^{-\Delta\Delta Ct}$  method and *M. sinostellata EF1-a* was employed as the reference gene<sup>5</sup>. DNA primers used are listed in Table S1. Each sample testing was repeated at three times.

## **Declarations**

### **Author contribution**

D.L. contributed to writing, statistical analysis and visualization of this article. Z.L., Y.W., S.Z., Y.S. put forward and carried out this study, and Y.S. was the corresponding author. Q.Y., Z.L., M.R. and C.W. investigated and collected data.

## Funding

This research was funded by Key R&D project of Shanghai Municipal Science and Technology Commission 'R&D and demonstration application of new varieties of white magnolia cultivation technology' (Grant No. 20DZ1203700), Thirteenth Five-Year Plan of Forest Tree Variety Breeding in Zhejiang Province 'Special Advantage Woody Flower Resource Conservation and Germplasm Innovation' (Grant No. 2016C02056-1) and Key R&D Project in Zhejiang Province 'R&D and Promotion Demonstration of Rural Ecological Landscape Construction Technology in Zhejiang Province' (Grant No. 2019C02023).

## Competing interests

The authors declare that there are no competing interests in the research.

## Statements

*Magnolia sinostellata* seedlings collected from Qingshan Lake Garden Center in Hangzhou, Zhejiang Province, China, have both been approved by Forestry Bureau of Zhejiang Province, P.R.China. In addition, experimental research on *M. sinostellata* was strictly comply with the IUCN Policy Statement on Research Involving Species at Risk of Extinction and the Convention on the Trade in Endangered Species of Wild Fauna and Flora.

## Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.XXXX/sXXXXX-XXX-XXXXX-X>.

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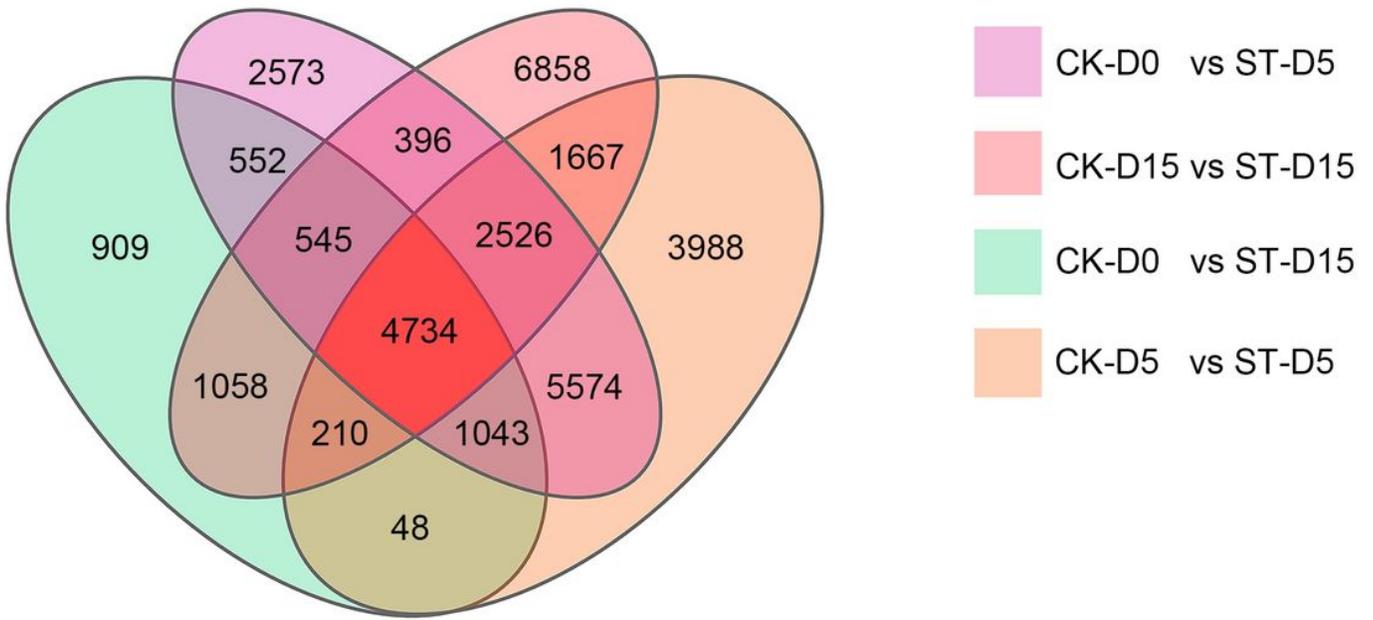
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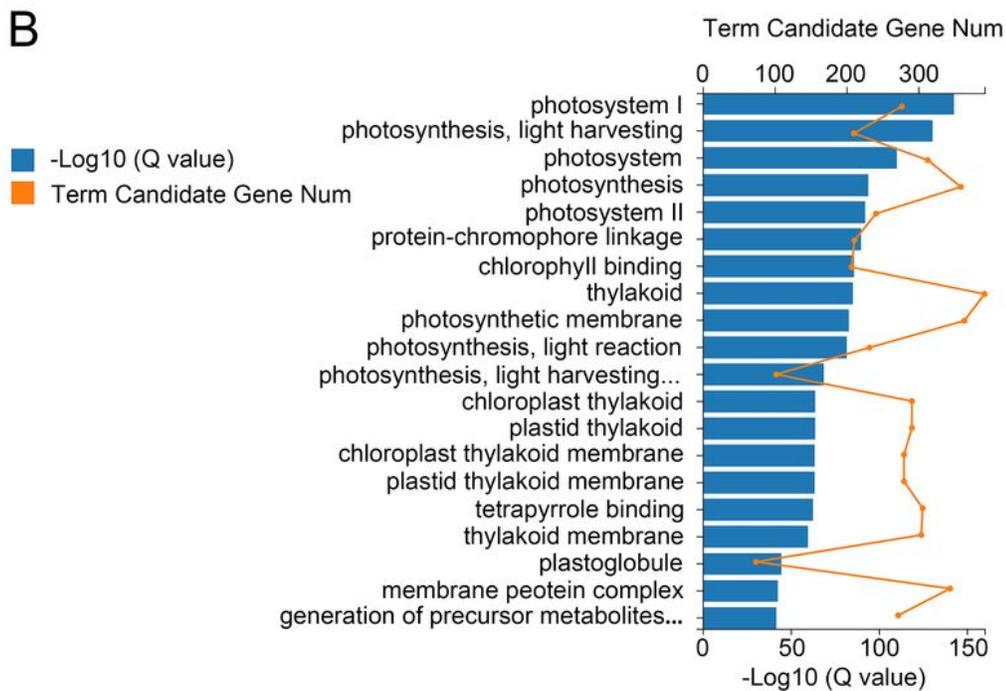
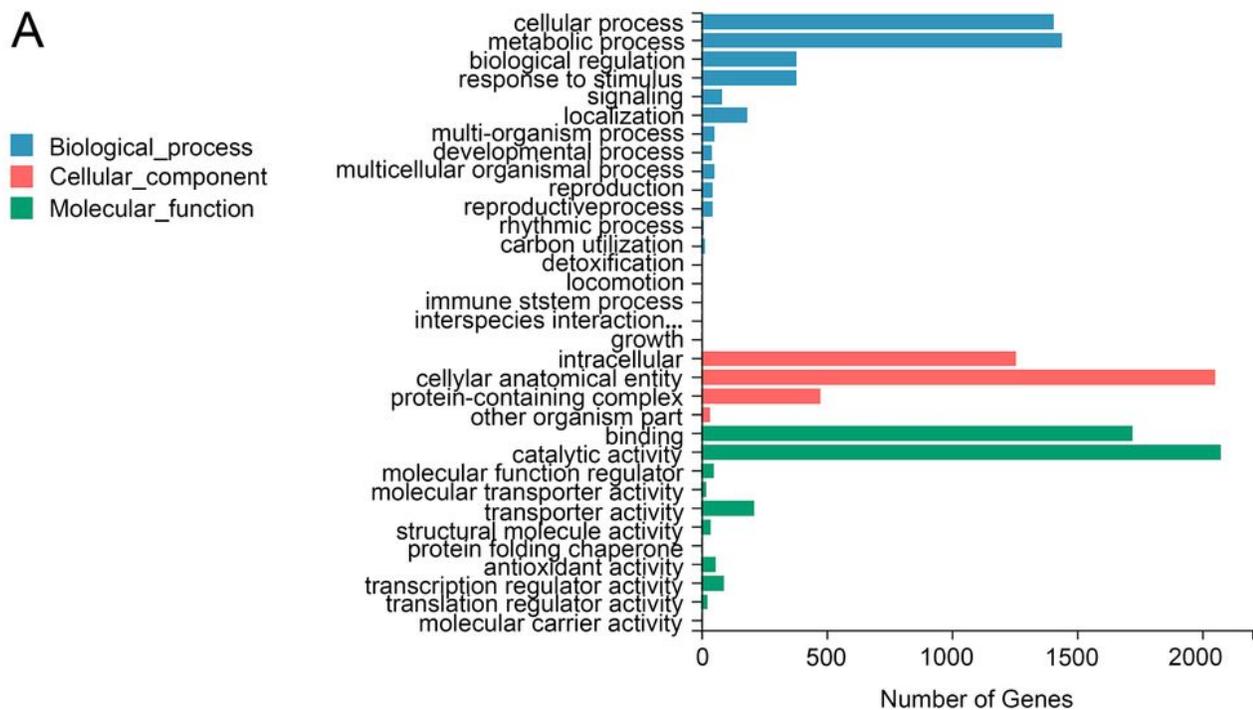
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## Figures



**Figure 1**

Venn diagram of differentially expression genes in treated and control groups in *M. sinostellata*. Venn diagram showing 4734 core shade responsive DEGs among treated and control groups used in this study.

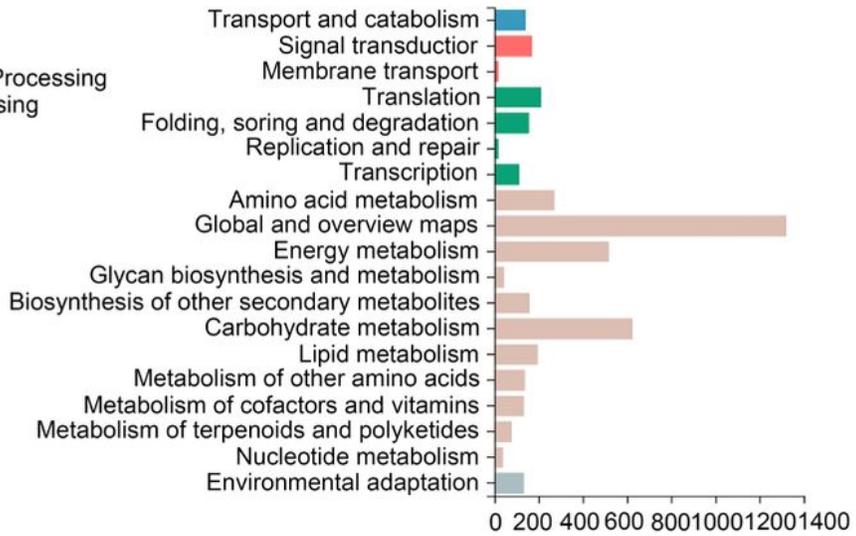


**Figure 2**

Gene ontology (GO) analysis of 4734 core DEGs. (A) GO classification of 4734 core DEGs. 4734 core DEGs were divided into three main categories and 36 subgroups. (B) GO enrichment of 4734 core DEGs. The top 20 GO Terms with the smallest Qvalue were selected to plot the chart.

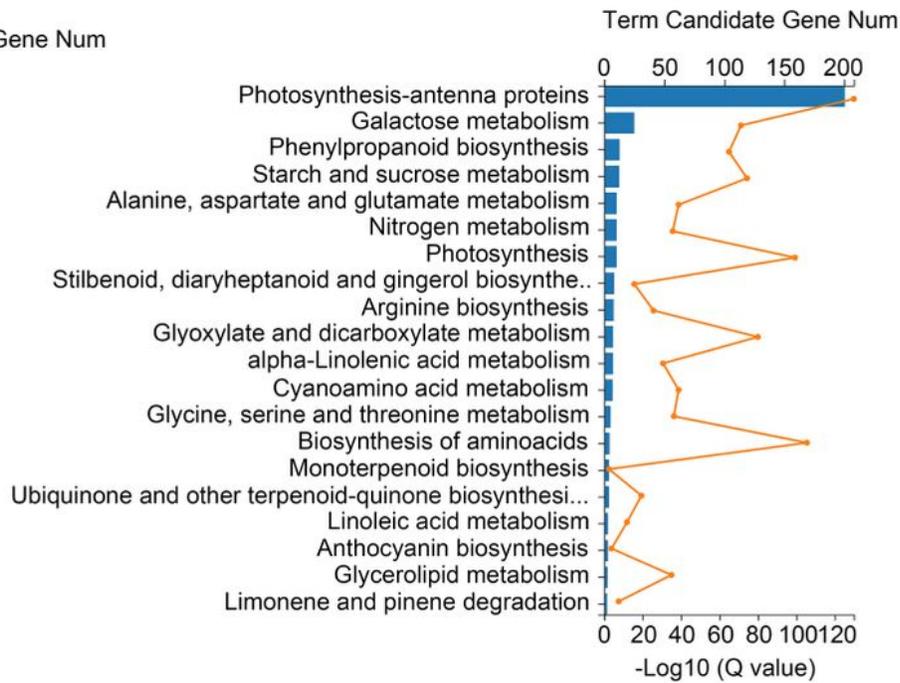
A

- Cellular Processes
- Environmental information Processing
- Genetic Information Processing
- Metabolism
- Organismal Systems



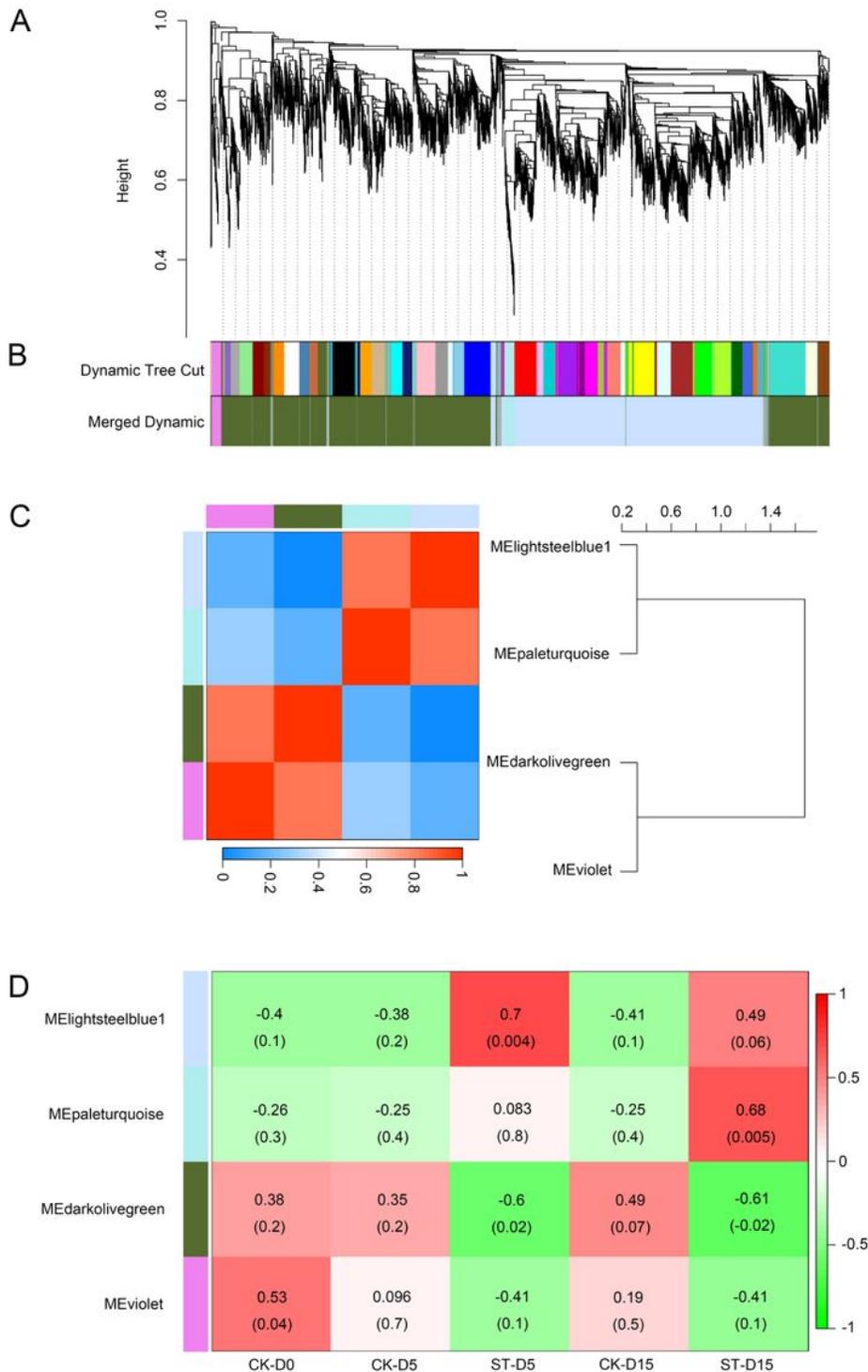
B

- -Log<sub>10</sub> (Q value)
- Term Candidate Gene Num



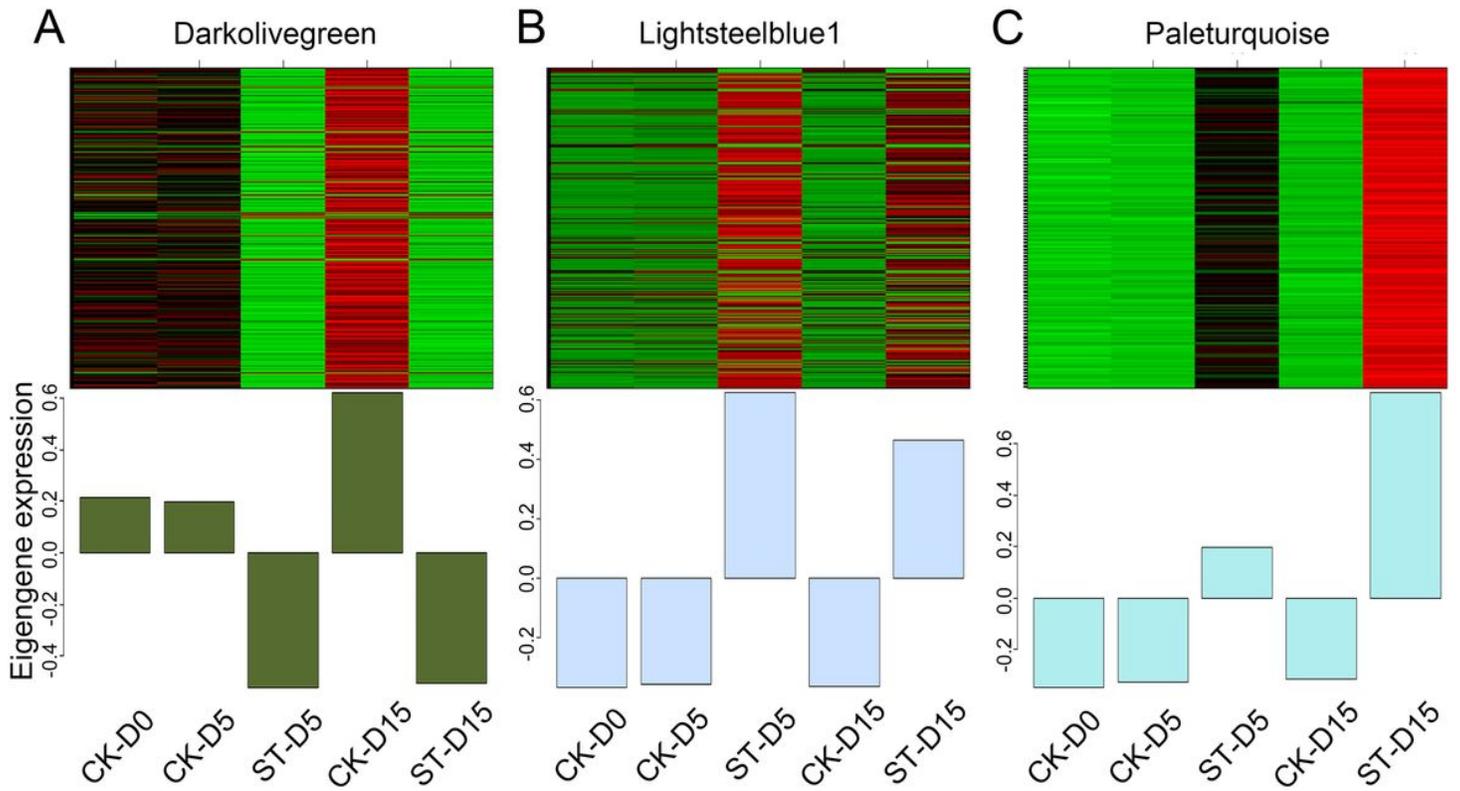
**Figure 3**

KEGG analysis of core DEGs in *M. sinostellata*. (A) KEGG classification of core DEGs. The metabolism pathways contributed by core DEGs were divided into five groups and 19 subgroups. (B) KEGG enrichment of 4734 core DEGs. The top 20 KEGG pathways with the smallest Qvalue were selected to plot the chart, among which 'Photosynthesis-antenna proteins' was the most enriched pathway.



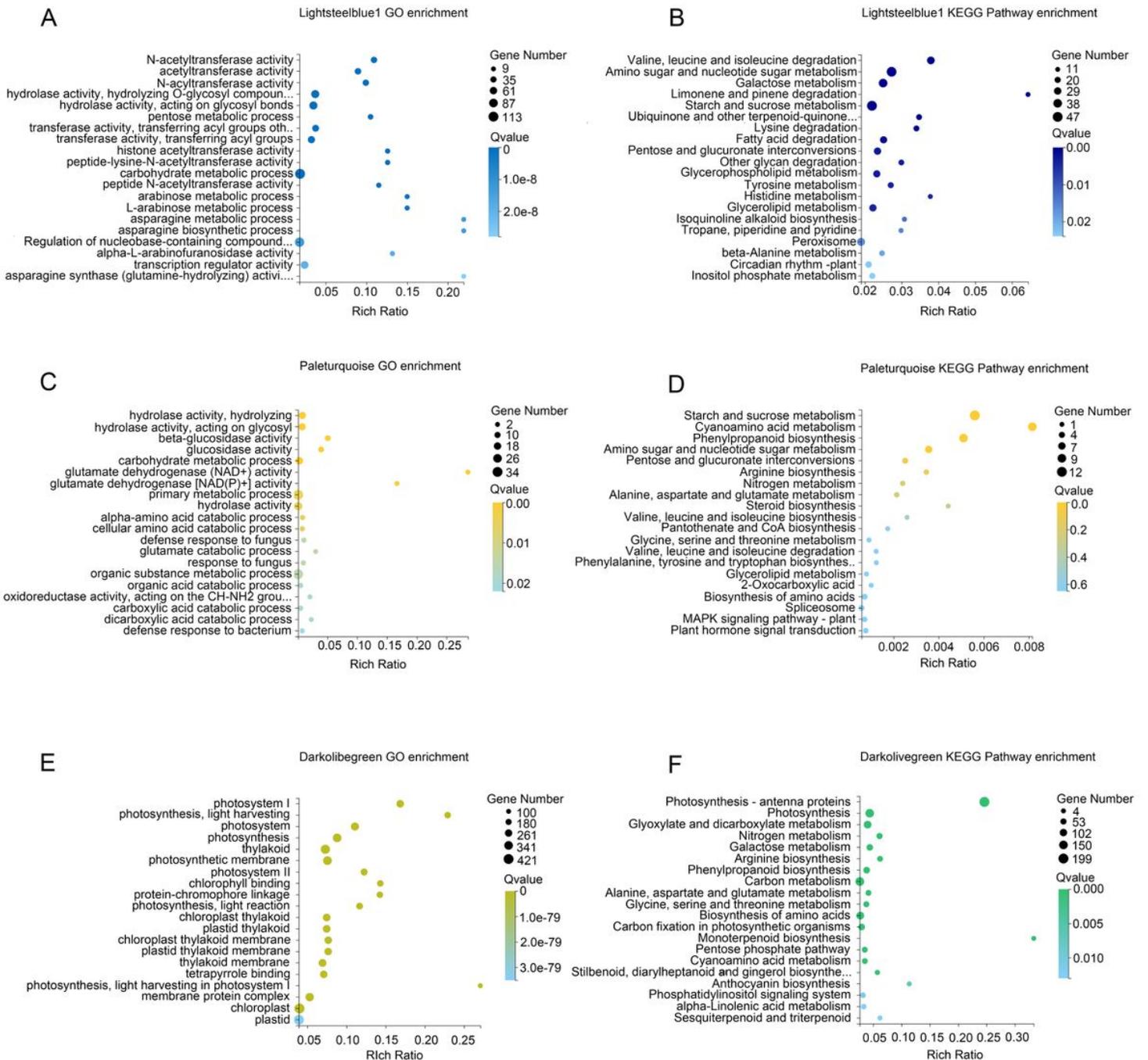
**Figure 4**

WGCNA module identification of 4734 core DEGs in *M. sinostellata*. (A) hierarchical clustering tree of 4734 core DEGs in *M. sinostellata*. (B) Dynamic tree cut and merged modules. (C) Dendrogram heatmap of four merged modules. (D) Module-trait relationship heat map. the correlation of the identified modules in control and shade treated groups. Red and green color illustrated positive and negative correlation with gene expression, respectively.



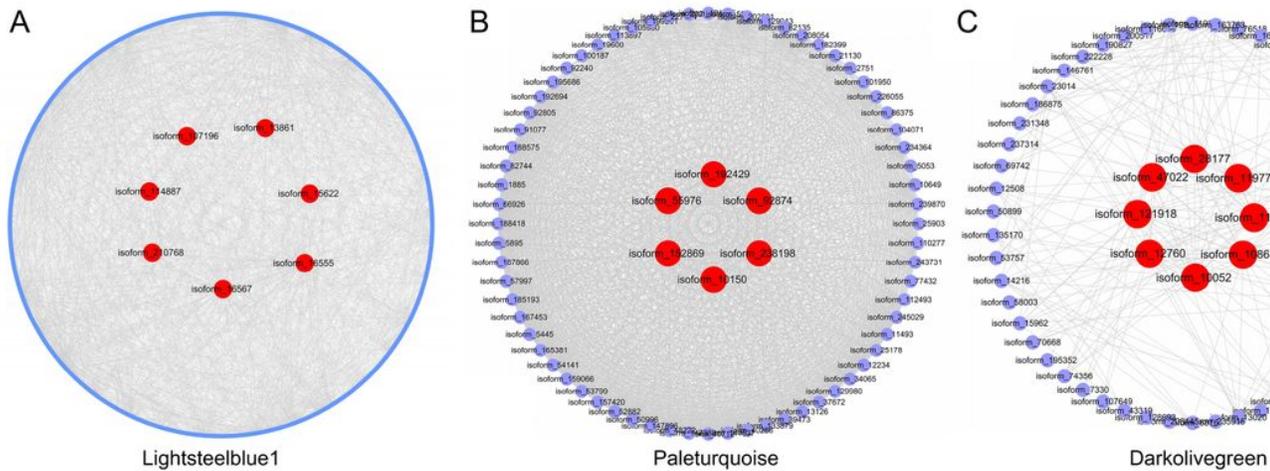
**Figure 5**

Eigengene expression pattern of key shade associated modules. (A) Eigengene expression pattern of Lightsteelblue1 module. (B) Eigengene expression pattern of Paleturquoise module. (C) Eigengene expression pattern of Darkolivegreen module. Eigengene expression pattern is the optimal tool to summarize expression pattern of specific module.



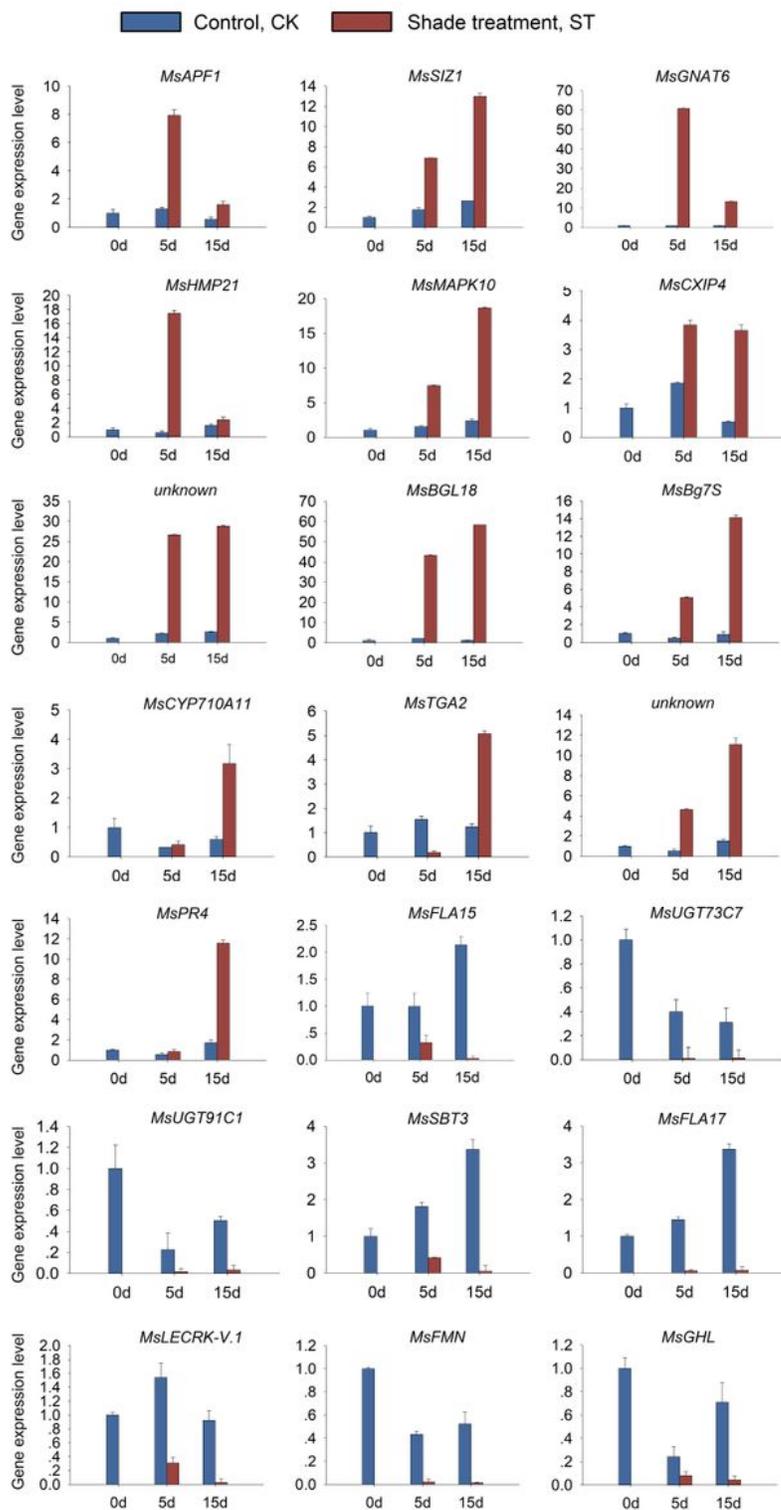
**Figure 6**

GO and KEGG enrichment analysis of Lightsteelblue1, Paleturquoise and Darkolivegreen modules. (A) GO enrichment analysis of Darkolivegreen module. (B) KEGG pathway enrichment analysis of Darkolivegreen module. (C) GO enrichment analysis of Paleturquoise module. (D) KEGG pathway enrichment analysis of Paleturquoise module. (E) GO enrichment analysis of Darkolivegreen module. (F) KEGG pathway enrichment analysis of Darkolivegreen module. The top 20 GO terms or KEGG pathways with the smallest Qvalue were selected to plot these charts.



**Figure 7**

Co-expression network analysis of core shade responsive modules. (A) Co-expression network of Lightsteelblue1 module. Eight hub genes were identified in this module. (B) Co-expression network of Paleturquoise module. There are seven hub genes detected in this module. (C) Co-expression network of Darkolivegreen module. Six hub genes were found in this module.



**Figure 8**

Relative expression of 21 hub genes in *M. sinostellata* under shade and control conditions. Data are the means of three biological replicates and three technical replicates. The  $2^{-\Delta\Delta Ct}$  method was employed to conduct the gene differential expression analysis.

## Supplementary Files

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