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# Comparative transcriptome analysis between high- and low-growth genotypes of Eucalyptus in response to nutrient stress

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**Abstract**

**Background:** The role of macro and micro nutrients have been reported in various plant. However, in perennial trees, the function of multiple elements to different genotypes remain to be elucidated. This study aimed at deciphering the genetic architecture of physiological differences under different nutrient levels between the high- and low-growth genotypes.

**Results:** Our results showed that growth traits of both genotypes at nutrient starvation treatment was much lower than that at nutrient abundant treatment, indicating that nutrient is essential for plant growth. However, tree height, crown width, and fresh weight of different tissues of ZQUA44 were much higher than that of ZQUB15 at the three nutrient levels, implicating the genetic basis of different trees is another factor that varied the phenotypic performance. The differentially expressed genes (DEGs) were clustered into 6 subclusters depending on different expression pattern. Functional annotation of DEGs from different subclusters showed that DEGs involved

in glutathione metabolism, flavonoid biosynthesis and stilbenoid, diarylheptanoid and gingerol biosynthesis may responsible for nutrient starvation across different genotypes, while DEGs involved in carotenoid biosynthesis and starch and sucrose metabolism may have diverse function in different genotypes. The DEGs encoding MYB_related may responsible for nutrient deficiency of all the genotypes, while B3 may play different functions in different genotypes.

**Conclusion:** Our results demonstrate that different genotypes may form different metabolic pathways to coordinate plant keeping survival when they face abiotic stresses. Furthermore, we elucidate DEGs that may widely responsible for nutrient deficiency in different treatments and DEGs that play different functions in different genotypes. Finally, our funding provide adequate nutrient supply for *Eucalyptus*.

**Keywords:** Nutrient starvation; Comparative transcriptome analysis; Stress resistance; *Eucalyptus*

## Background

Water and mineral nutrient as well as light are the main external factors needed during the process of plant growth. Nonetheless, in the area with plenty of rainfall, such as Southern China, the mineral nutrient is the essential factor for plant growth and production. For example, the macroelements nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and sulfur (S) constitute the most fundamental elements in plant cell, such as protein, genetic materials and membranes[1]. The macroelements P, Mg, and Ca also play important roles in energy metabolism, enzyme activity regulations and phytohormone signal transduction[1]. Therefore, the absorption of sufficient mineral nutrient is essential for plant growth. However, investigation on soils showed that many areas of the world, for agricultural, horticultural, forestry or herbecious plantation used, are elemental deficiencies for fundamental macro and micro nutrients to support healthy and productive plant growth. Therefore, fertilizers are applied in many parts of the world to maximum yields of various crop products. But, the growth traits are divergent between different genotypes of individuals according to the variation of mineral nutrient use efficiency

(NUE), even though the same level of nutrient were applied. The fact that trees with high-growth performance are more efficient in absorption and utilization of nutrients than the low-growth individuals under the same cultivation conditions may due to their divergence in genetic signature [2, 3]. Consequently, developing tree species with higher NUE, will greatly increase the proportion of input-output of plantation, and preventing nutrients from releasing to ecosystems. Thus, knowledge on the underlying genetic basis of the high- and low-growth trait of trees in response to nutrient treatment are of great importance to develop strategies in breeding program.

For the past few years, tremendous progress in next generation sequencing accompanying bioinformatics analysis softwares allows us to approach key genes and critical pathways on various plant species under nutrient deviation[4]. In Arabidopsis, the response to nutrient deficiency involves complex network combining different regulatory levels, including arrangement of gene expression, hormone signal transduction, and metabolism for physiological and morphological modifications [5]. Tao et al. (2003) globally studied the effects of nutrient starvation to starch accumulation in duckweed, and found that nutrient starvation inhibits the universal metabolic status. Furthermore, genes encoding key enzymes involved in starch biosynthesis process were promoted, while genes play important role in starch consumption were inhibited in nutrient starvation treatment in *L. punctata*[6]. However, few studies were found on the perennial plant species, whose genomic sizes are much bigger than herbaceous plant. The genetic mechanism for phenomenon of "stay-green" in *Litchi chinesis* pericarp under Mg foliar treatment was studied by de novo transcriptome sequencing. Further study on transcriptomic data showed that DEGs were enriched in pathways of flavonoid biosynthesis, anthocyanin biosynthesis, and ABA signal pathway, indicating the role of Mg involved in many metabolic pathways in litchi[7]. Nevertheless, the molecular basis between high- and low-growth genotypes of lignocellulose utility tree species in response to nutrient stress is so far obscure. In this study, we aim to identify nutrient-responsive genes, and to deeply clarify the regulatory and metabolic mechanisms that allow tree species

to adapt to environment changes under nutrient deficiency.

*Eucalyptus* is one of the most important economical tree species that widely grown around the world for pulp production[8]. Till 2017, about 4.6 million $hm^2$ of *Eucalyptus* were planted in South China, accounting for 6.5% of the total forestry plantation, and producing 30 million $m^3$ of wood products, which accounts for 26.9% of the country's annual wood production. *E. urophylla* is one of the most widely planted *Eucalyptus* species in South China, due to its high growth rate. High growth rate demands high nutrient availability, therefore, essential nutrient is one of the most crucial factors determining the wood product of tree species all around the world . Previous study showed that the seedling stage is the critical period for survival and establishment of trees[9]. Thus, we analyzed the phenotypic responses of *E. urophylla* seedlings subjected to nutrient stress in this study. Transcriptomic analysis was performed to study gene expression patterns under different nutrient stressed conditions using RNAs extracted from leaves of 18-year-old seedlings. The main objectives of this study is to identify genes differentially expressed under control and stress conditions, which should provide a framework of molecular mechanism involved in nutrition treatment and can be studied in more detail in future.

## Results

### *Different nutrient treatment affects tree growth characteristics*

To examine the effects of different nutrient treatment on the high- and low-growth genotypes of *E. urophylla* under long term of nutrient treatment, we measured tree growth trait, including tree height (H), ground diameter (GD) and crowd width (CW), and biomass of different tissues, such as dry weight of branches, leaves, stems and roots, under 18-month treatment of different nutrient levels. The results showed that all of the growth traits changed significantly between different nutrient treatment ($P < 0.05$, Fig. 1). For example, the H of T2 (281.00 cm and 194.67 cm respectively) and CK-treated plants (289.00 cm and 229.00 cm respectively) was significantly higher than T1-treated plants (178.00 cm and 133.00 cm respectively) for both of the

119 genotypes (Fig. 1a, $P < 0.05$). Moreover, the leaves at T1 were much smaller than that
120 at T2 and CK, and symptoms were found in the leaves at T1 (Fig. S1). No significant
121 difference was found in H between CK and T2-treated plants for ZQUA44, while a
122 significant difference was observed between CK and T2-treated plants for ZQUB15
123 (Fig. 1a). The same situation was also found in the increment of GD (Fig. 1b) and CW
124 (Fig. 1c). To further evaluate the effects of nutrient on plant growth, the fresh weight
125 of different tissues under different treatment were measured (Fig. 1d). The results
126 showed that the fresh weight of stems, branches, roots, leaves and the total fresh
127 weight at T1 were much lower than that at T2 and CK in both of the genotypes,
128 indicating that nutrient was the most important factor that restrict plant growth.
129
130 However, the tree growth traits of different genotypes showed a various performance
131 at the same level of nutrient treatment. For example, the H of ZQUA44 were much
132 higher than that of ZQUB15 at all the three treatment levels. The same situation was
133 also observed GD. Interestingly, the CW showed a similar situation at T1 and T2 of
134 CW, while a much bigger CW was observed at CK. The fresh weight of different
135 tissues showed that all of the four tissues including stems (362.67 and 232.00 g
136 respectively), branches (61.00 and 55.00 g respectively), roots (371.72 and 270.58 g
137 respectively), leaves (190.33 and 176.5 g respectively) and the total fresh weight
138 (985.72 and 734.00 g respectively) of ZQUA44 were much bigger than that of
139 ZQUB15 at T1, indicating that the genetic basis of different genotype is another
140 important factor that varied the phenotypic performance. The difference between CK
141 and T1 was much bigger in ZQUA44 than that at ZQUB15. In addition, no significant
142 difference was observed at H between T2 and CK in ZQUA44, indicating a higher
143 nutrient use efficiency in ZQUA44.
144
145 **RNA sequencing, assembling, quantification, identification and clustering of**
146 **DEGs responding to nutrient treatment**
147 To evaluate the genetic variation of the two genotypes under different treatment,
148 transcriptome profiling of 18 samples of the two genotype were evaluated by an

Illumina Hiseq 2500 platform. In total, 8.42-11.11 million of 125 bp pair-end reads were generated for the 18 samples (Tab. 1). After removing adapter, ploy-N and low quality reads through in-house perl scripts, 8.15-10.72 million clean reads were obtained. Then the clean data with the high percentage of Q20 (94.43-98.41%) and Q30 (85.65-95.71), and low percentage of error rate (<0.03%) were used for alignment to the reference genome using TopHat v2.0.9. A range of 3.21-5.72 million reads were mapped to the reference genome, and over 96% of them were uniquely mapped. In total, 35,488 unique transcripts were obtained for both genotypes, with the average length of 7,043 and 10,563 nucleotides in ZQUA44 and ZQUB15, respectively. The expression level of protein-coding gene were represented by FPKMs (fragments per kilo-base of exon per million fragments mapped). The genes with FPKMs under 0.1 in all samples were filtered out, and the remained genes were used for further analysis. To detect genes that specifically expressed at different treatment, degR softwares were used for analysis of samples under different treatment of the two genotypes. In total, 1,239 DEGs were detected in ZQUA44 and ZQUB15 at T1 and T2, as compared with those at CK using a threshold of 2-fold change in gene expression as previously mentioned ($P < 0.01$, and $Q < 0.15$, Fig. 2).

To study the expression trend of DEGs, cluster software implemented in R was used to explore the expression pattern of DEGs from different treatment in genotype ZQUA44 and ZQUB15 (Fig. 3). The DEGs at different nutrient treatment were clustered into six subclusters, and the subclusters displayed a considerable difference and consistency in gene expression trend between the two genotypes. For example, the DEGs at subcluster 1 and 2 had the same expression trends that these DEGs inhibited at T1 in genotype ZQUA44 and activated at T1 in genotype ZQUB15. However, the $\log_2$ratio of subcluster 2 was much higher than that at subcluster 1. The genes expression pattern of DEGs at subcluster 3 of ZQUA44 was consistent with ZQUB15 that they both activated at T1, while these genes at ZQUA44 had higher expression level. In subcluster 4, DEGs were greatly inhibited at T1 in ZQUA44, while no significant difference was observed in ZQUB15. On the contrast, 170 DEGs

of subcluster 5 in ZQUB15 were greatly inhibited at T1 while no significant difference was observed in ZQUA44. In subcluster 6, DEGs were activated at T1 in both genotypes, while a little lower expression level at T2 in ZQUB15, as compared with that in ZQUA44.

**Functional Enrichment of DEGs responding to nutrient deficiency**

To elucidate the functions of the DEGs that responding to low nutrient application, GO classifications were implemented to analyze DEGs with different expression trends. In total, 190 GO terms were obtained, including 97 GO terms for biological process, 48 GO terms for molecular function and 45 GO terms for cellular components (Fig. 3). In the GO term of biological process, DEGs from subcluster 1 were significantly enriched into 80 GO terms, including response to stimulus, response to abiotic stimulus, phenylpropanoid biosynthetic process, secondary metabolic process, phenylpropanoid metabolic process, flavonoid biosynthetic process and so on. DEGs in subcluster 6 were significantly enriched in 41 GO terms, while DEGs in subcluster 5 were significantly enriched in 10 GO terms. DEGs involved in photosynthesis were significantly enriched in subcluster 5, while DEGs involved in secondary metabolic process and response to stimulus were significantly enriched in subcluster 6. DEGs from all of the five subclusters were significantly enriched in response to stimulus, except subcluster 5. In the term of molecular function, DEGs from subcluster 1, 3, 4 and 6 were significantly enriched in catalytic activity and oxidoreductase activity. DEGs involved in transferase activity, UDP-glycosyltransferase activity and so on were significantly enriched in subcluster 1. DEGs from subcluster 5 were significantly involved in 44 cellular components.

KEGG analysis showed that the DEGs with the similar expression patterns were prone to clustered into the same group (Fig. 4). For example, the DEGs from subcluster 1 and 4, which inhabited at T1 in ZQUA44 and activated at T1 in ZQUB15, were clustered into the same group. The same situation was also observed at subcluster 3 and 6. DEGs of subcluster 1 were significantly enriched in carotenoid

biosynthesis, starch and sucrose metabolism, arginine and proline metabolism, ascorbate and aldarate metabolism, and inositol phosphate metabolism ($P < 0.05$, $Q < 0.20$). DEGs of subcluster 3 were significantly enriched in glutathione metabolism, stilbenoid, diarylheptanoid and gingerol biosynthesis, and flavonoid biosynthesis. DEGs of subcluster 4 were significantly enriched in metabolic pathways, galactose metabolism, diterpenoid biosynthesis, biosynthesis of secondary metabolites and fatty acid biosynthesis. DEGs of subcluster 5 were significantly enriched in ribosome and monobactam biosynthesis. The DEGs that were both activated at the two genotyppes were grouped into subcluster 6, and play important roles in cutin, suberine and wax biosynthesis, nitrogen metabolism and phenylalanine, tyrosine and tryptophan biosynthesis. The pathview was used for visualization of specific pathways. The results showed that three significant KEGG pathways were obtained, including phenylpropanoid biosynthesis, plant hormone signal transduction and plant-pathogen interaction. A total of 28 DEGs were enriched in phenylpropanoid biosynthesis pathway, with divergent expression in ZQUA44 and ZQUB15. Six of the eight hormone signal transduction pathways were identified in this study, and much more DEGs were enriched in abscisic acid and salicylic acid signal transduction. Ten DEGs were identified in the plant-pathogen interaction, including WRKY33, RBOH_F, KCS2, PR1 and so on.

**Differentially expressed genes involved in plant hormone signal transduction**

In total, 38 DEGs were identified to be involved in plant hormone signal transduction, including P2C (protein phosphatase 2C), AB (Auxin-binding protein), SAU (Auxin-responsive protein), GASA (Gibberellin-regulated protein), ERF (Ethylene-responsive transcription factor), PYL (Abscisic acid receptor), ARR (Two-component response regulator) and so on. One DEG (Eucgr.I01276) encoded SAU61 (SAUR-like auxin-responsive protein) was down-regulated at T1 in ZQUB15, while no significant difference was observed at T1 in ZQUA44. The same situation was observed at Eucgr.D00606 (encoding auxin-repressed 12.5 kDa protein). Eucgr.C03337 and Eucgr.C03536, both encoding auxin-binding protein ABP19a, were

activated at T1 in ZQUA44, while no significant difference were observed at ZQUB15. Six genes (from subcluster 1, 4 and 6) encoding P2C were identified, with five of them were inhibited at T1 and only one of them activated at T1 in ZQUA44. However, only two of them were activated at T1 in ZQUB15, and no significant difference were observed at the other three genes. A total of 11 DEGs were identified to encode ethylene-responsive transcription factors, and nine of them were activated at T1 in ZQUB15, the other two were activated at T1 in ZQUA44. Six of the DEGs were inhibited at T1 in ZQUA44, while no significant difference was observed at other three genes. Interestingly, three of the DEGs from subcluster 4 were all down-regulated at T1 in ZQUA44 and up-regulated in ZQUB15. Two DEGs from subcluster 6 encoded abscisic acid receptor, and all of them were activated at T1 in both genotype. In addition, gene (Eucgr.F03208) encoding gibberellin 2-beta-dioxygenase 1 was activated at T1 in both genotypes.

**TFs responding to nutrient deficiency**

To identified important transcriptional elements that responsible for nutrient deficiency, DEGs from different subclusters were annotated in the plant transcription factor database (Table 2). In total, 90 DEGs encoding 21 transcription factor (TF) families were obtain in all the subclusters in ZQUA44 and ZQUB15. Subcluster 1 had the most abundant TFs, including 44 DEGs encoding 16 TF families, including ERF (6), MYB (6), NAC (6), and WRKY (5) and so on. DEGs encoding MYB-related were more abundant in subcluster 3, while DEGs encoding HSF were more abundant in subcluster 4. DEGs encoding AP2 and MIKC_MADS were only found in subcluster 6, CPP was only found in subcluster 5, and DEGs encoding NF-YA and TCP were unique in subcluster 4. Though no significant KEGG pathways or TFs were found in the subcluster 2, the DEGs in this subcluster were worth to pay attentions. For example, 11 of the 25 DEGs were involved in plant defense process, such as gene encoding germin-like protein subfamily 1 member 13, defensin-like protein 2, thaumatin-like protein, superoxide dismutase, basic endochitinase A and so on. And three of them were involved in xylem development, such as genes encoding

expansin-like B1 and cellulose synthase-like protein G1. One gene encoding outer envelope pore protein 16-2, chloroplastic, which is envolved in translocation between chloroplast and cytoplasm of phosphorylated carbohydrates. The function of the other eight genes were not annotated.

**RNA Sequencing Validation by qPCR**

To validate the RNA sequencing results, ten genes with different expression patterns were randomly selected to perform quantitative real-time PCR (qRT-PCR) using ABI7500. The results indicated that there was a strong correlation between the data of qRT-PCR and RNA sequencing ($R^2 = 0.919$, Fig. 3). Similar expression pattern was found at all the three nutrient gradient of the two genotypes comparing with the results of qRT-PCR and the data of RNA sequencing, indicating that the expression results generated by RNA sequencing were reliable for further study.

**Discussion**

Comparative transcriptome analysis is an essential and powerful strategy for the demanding analysis of genotypes of plants with different phenotypic performance under various external treatment[10]. In this study, we compared the transcriptomic changes between two Eucalyptus individuals with divergent phenotypic performance under different nutrient application levels. In overall, phenotypic characteristics exhibited a similar tendency in the two Eucalyptus genotypes during the nutrient treatment, which agree with previous studies that nutrient limitation significantly restrict plant growth and physiological metabolism[11]. For example, the leaves of ZQUA44 and ZQUB15 showed symptoms on foliage at treatment T1, compared with that at T2 and CK (Fig. S1). What's more, the H, GD, CW, and fresh weight of different tissues of both genotypes were much lower at treatment T1, as compared with that at T2 and CK, indicating that nutrient was the most important factor that restrict plant growth. However, even at T1, almost all of the growth trait and fresh weight of different tissues of ZQUA44 was much higher than ZQUB15, indicating that trees of different genotype may form different metabolism process or pathways to

adapt to nutrient starvation on the long term nutrient starvation treatment.

Phytohormones are the key regulators of plant growth and development and act as mediators of environmental stress responses [5]. Plenty study showed that jasmonic acid (JA) was involved in various processes such as plant growth, reproductive output as well as stress resistance[12, 13]. Our study showed that two genes from ZQUA44 encoding lipoxygenase (LOX), an important enzyme catalyzing linolenic acid into 12-oxo-phytodienoic acid during JA biosynthesis[14], were significantly up-regulated in the nutrient-deficiency treatment and absent in the nutrient abundant treatment, and either profiles in genotype ZQUB15. Interestingly, one DEG (Eucgr.B02620) from ZQUA44 and one DEG (Eucgr.H05052) from ZQUB15, both encoding defensin-like protein, showed a low fold change (0.2 and 0.27, respectively) in nutrient-starved plants compared with that at nutrient abundant plants, which was inconsistent with previous study that these genes show high expression level in K-starved plants of Arabidopsis[5], revealing a divergent function possibility of herbaceous plant and long term perennial plant species. Except for jasmonic acid, auxin essentially plays a role in virtually every aspect of growth and development, as well as in response to stress resistant process in various plant species[15, 16]. In our study, three DEGs encoding auxin-binding protein and auxin-responsive protein were all activated at T1 in ZQUA44 and inhibited in ZQUB15, indicating a divergent function in different genotypes. Furthermore, P2C, a negative regulator of ABA signaling pathways, have been reported to positively regulate abiotic stress signaling pathway in herbaceous plants [17]. However, only one DEG were upregulated at T1 in both genotypes, the other five were down-regulated at T1 in ZQUA44. The reason for the inconsistent maybe we sampled after a long term of treatment instead of sampled immediately after treatment. These results indicate that different genotypes of Eucalyptus had formed a relatively stable internal system to maintain normal life processes under nutrient starvation conditions by regulating divergent hormone pathways under long term treatment.

The GO term analysis showed that DEGs of subcluster 1, 2, 3, 4 and 6 with various expression trend were all significantly enriched in response to stimulus (Figure 3), indicating their role in the nutrient starvation. DEGs in subcluster 1 and 4, which down-regulated at T1 in ZQUA44 and up-regulated in ZQUB15, were all significantly enriched in biological process of response to abiotic stimulus, response to abscisic acid stimulus, response to endogenous stimulus, response to hormone stimulus and response to oxidative stress. The DEGs of these subclusters may responsible for nutrient starvation, and we speculate that, the divergent of the expression pattern between the two genotype may due to the individual genetic signature. The DEGs in subcluster 5, whose expression were inhibited at T1 in both genotypes, were significantly enriched in photosynthesis, tetrapyrrole biosynthetic process, tetrapyrrole metabolic process, generation of precursor metabolites and energy, heterocycle biosynthetic process and cellular nitrogen compound metabolic process. The DEGs of subcluster 1, 3, and 6 were significantly enriched in flavonoid biosynthesis, flavonoid metabolic process, phenylpropanoid biosynthetic process and so on. These DEGs were up-regulated at T1 in subcluster 3 and 6 of the both genotypes and subcluster 1 in ZQUB15, while down-regulated in subcluster 1 of ZQUA44. The result is consistent with mounting evidence that DEGs involved in these pathways play important roles in plant development and response to various external stress, including UV light protection and resistance to pathogens[18-20]. In addition, our study reveal that the DEGs involved in the flavonoid biosynthesis may extensively participate in the stress resistance process of various plant species. The KEGG pathway enrichment analysis in our study showed that much more pathways were found in subcluster 4, whose DEGs were greatly inhibited at T1 in ZQUA44, and DEGs involved in metabolic pathways and biosynthesis of secondary metabolites were much more abundant than other pathways. The result is consistent with the fact that the phenotypic performance of the trees in nutrient abundant treatment were much better than that at nutrient deficiency treatment, indicating that nutrient was an essential element for tree growth and development[7]. Furthermore, DEGs from subcluster 5 that significantly inhibited by nutrient starvation in ZQUB15 were

enriched in pathways involved in ribosome (Fig. 4), indicating that the two Eucalyptus genotypes have formed different pathways to adapt to the nutrient deficiency. DEGs from subcluster 1 were significantly enriched in carotenoid biosynthesis, which is responsible for biosynthesis of abscisic acid, may play important role in response to abiotic stress[17]. DEGs involved in diterpenoid biosynthesis (responsible for biosynthesis of gibberellin) were significantly enriched in subcluster 4, were down-regulated in the nutrient starvation treatment in ZQUA44, indicating a lower gibberellin level in the plants that undergo abiotic stress. In total, 24 significant KEGG pathways were identified, while 11 significant KEGG pathways were identified in subcluster 4, whose expression levels were reduced greatly in ZQUA44 compared with that in ZQUB15, indicating that the genotype ZQUA44 may undergo higher pressure at the nutrient starvation treatment than the genotype ZQUB15. These results is consistent with the fact that a higher tree growth traits and bigger biomass decrease in ZQUA44. Interestingly, genes involved in plant hormone signal transduction pathway was observed in ZQUA44 and ZQUB15 with divergent expression pattern, and DEGs mainly enriched in the abiscisic acid (ABA) signal transduction pathway, such as DEGs encoding PYR/PYL, PP2C, SnRK2 and ABF, revealing that same DEGs of differnet genotypes may play different functions during the nutrient treatment. However, the roles of hormones in nutrient resistance are still not well understood in higher plant species. Whether the high-biomass growth genotype has a more complicated mediated network responsive to nutrient needs to be studied by further work.

Aside from phytohormones, plentiful of families of TFs are known to cope the signals transduction when plants are tolerating with numerous biotic and abiotic stresses, including the WRKY, MYB, MYB-related, MYC, NAC, ERF, and bHLH families, of which members have been revealed to promote or suppress abiotic stress responses [21-25]. For example, members of WRKY family are essential regulators of plant innate immunity, and play key roles in regulating biotic and abiotic stress reactions in various plant species[6, 26]. Furthermore, a gene encoding WRKY in *Larrea*

*tridentata* was proved to be an activator of ABA signalling, which plays essential role in plant stress resistance process[27]. In our study, seven transcripts encoding WRKY were observed in subcluster 1, 4 and 6 respectively, and five of them were significantly up-regulated in ZQUB15, while the other two were significantly down-regulated in ZQUA44. In addition, one DEG encoding WRKY50 was found up-regulated in both genotypes, indicating that this gene maybe an widely distributed factor responding to nutrient starvation treatment of different genotypes in Eucalyptus. However, much more members of WRKY were divergent between the two genotypes, indicating the difference between the two genotypes responding to the same abiotic stress. One genes encoding TCP, which play key roles in cell proliferation in developing tissues and predicted to be related to tree height growth, was observed specifically inhibit at T1 in ZQUA44, which may explain the fact that the tree height growth of ZQUA44 was hampered greater than that in ZQUB15 at T1[28].

Except for phytohormone-related genes and transcription factors, other genes encoding F-box protein, late embryogenesis abundant (LEA) protein, ammonium transporter 2, Cytochrome P450, UDP-glycosyltransferase (UGT), phenylalanine ammonia-lyase and so on, the expression of which were divergent at T1 in both genotypes. F-box protein was reported to related with cell cycle regulation and signal transduction[29]. LEA proteins has been studied to be related to water deficient resistance by protecting cytoplasmic components[30]. The identification of this genes in our study provided a new insight into the possible functions in nutrient deficiency resistance. Genes encoding ammonium transporter 2 were identified to be enriched in nutrient starvation treatment in both genotypes, indicating that plant try to maintain its growth by increasing the ammonium level by promoting the transport ability of this mineral elements. UGTs could catalytically transfer glycosyl group from activated donor molecules to specific receptor molecules, and play important roles in various progresses, such as hormone signal transduction, secondary metabolites, and cell wall polysaccharide synthesis[31]. UGTs together with phytohormones play pivotal role in stress resistance, by changing the water solubility of the receptor molecule[32, 33]. In

our study, eight genes from subcluster 1 and two genes from subcluster 3 were identified, eight of which were activated in ZQUB15, and down-regulated in ZQUA44, indicating that these genes may be responsible for genotype-specific phenotypic difference. Two of the DEGs (Eucgr.D02610 and Eucgr.J01008) were activated at T1 in both genotypes, indicating that these genes may responsible for stress resistance across different genotypes.

## Conclusion

The changes of H, GD, CW and biomass of different tissues clearly demonstrated that normal metabolism was restrained in the two genotypes of *Eucalyptus* under nutrient starvation, implicating that the environmental factors are essential for plant growth despite of the genetic basis. However, high-growth genotype ZQUA44 and low-growth genotype ZQUB15 did exhibit significant differences in their responses to nutrient starvation at H, CW, and biomass of different tissues. Transcript profiling analysis showed that some of the DEGs had similar expression trends at different treatments in ZQUA44 and ZQUB15, such as DEGs in subcluster 3 and 6, indicating their role in response to nutrient starvation. However, many other DEGs, such as those in subcluster 1 and 2, had converse expression trends at different treatment between the two genotypes in response to nutrient stress. These findings will provide genetic basis for breeding of *Eucalyptus* under nutrient tolerance.

## Methods

### Plant materials and nutrient treatment

The plants used in this study were grown from seeds, which were obtained commercially from Commonwealth Scientific and Industrial Research Organisation (CSIRO), Australia. All of the plants were cloned by tissue culture. Based on the field studies of different nutrient levels for 100 genotypes, two identified *Eucalyptus* genotypes, a high-growth cultivar (ZQUA44) and a low-growth cultivar (ZQUB15), were used in the present study (Yang et al., unpublished). Healthy seedlings with similar size of each genotype were selected and cultured in cylindrical containers with

black-and-white film for water controlled and nutrient gradients (T1, T2 and CK) under natural conditions in March 2015. T1 represents the only application of base fertilizer (including 250 g calcium-magnesium phosphate fertilizer). T2 indicates the application of base fertilizer and after fertilizer (including the application of 250 g and 100 g compound fertilizer in August and next March respectively). CK stands for the application of base fertilizer (including 250 g calcium-magnesium phosphate fertilizer and 100 g compound fertilizer) and after fertilizer (including the application of 100 g urea in May, 150 g and 100 g compound fertilizer in August and next March respectively). Each treatment was replicated for three times. After 18 months' culture, leaves of all treatments were sampled and stored in liquid nitrogen.

**Determination of growth characteristics**

A total of three features were quantified in 18-month-old *Eucalyptus* seedlings, and three biological replicates were used for each treatment. H refers to the distance of trees from the rhizome on the ground to the shoot apex, and was measured by height gauge. GD was determined at the basal of tree shoot by caliper. CW is the width of the north-south or east-west direction of trees, and scaled by scaled stick. Biomass of different treatment referred to the dry weight of different tissues, including leaves, stem, branch and root. All of the tissues were sampled and dried in a drying oven. The weight of each sample was recorded till the weight no longer decreased.

**RNA isolation, sequencing and Assembling**

Total RNA was isolated from leaves of the three plants as replicates for each of T1, T2 and CK separately using the Qiagen RNAeasy kit (Qiagen China, Shanghai, China), and purified using RNAclean Kit (TIANGEN BIOTECH (BEIJING) CO., LTD) following the manufacturer's instructions. The integrity of RNA was monitored on 1% agarose gels and the purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). RNA concentration was assessed using Qubit® RNA Assay Kit in Qubit® 2.0 Flurometer (Life Technologies, CA, USA). A total amount of 3 ug high-quality RNA per samples was used for the subsequent RNA

sequencing. The cDNA library was constructed for each of the nine RNA samples and sequenced on the Illumina HiSeq 2500 platform (Illumina Inc., CA, USA). Before assembly, adapter sequences, poly-N and low quality reads were removed from the raw data. Index of the reference genome (directly downloaded from https://phytozome.jgi.doe.gov/pz/portal.html#!bulk?org=Org_Egrandis) was built using Bowtie v2.0.6 and the paired-end clean reads were aligned to the reference genome using TopHat v2.0.9 (Kim et al, 2012). Then, the mapped reads of each sample were assemble by both Scripture (beta2) (Guttman et al. 2010) and Cufflinks (v2.1.1) (Trapnell et al. 2010).

**Normalization of Gene Expression Levels and Identification of Differentially Expressed Genes**

To evaluated gene expression levels, the paired-end clean reads that mapped to the reference genome were used for FPKM calculation of each sample using Cuffdiff (v2.1.1, Trapnellet al. 2010). FPKM represents fragments per kilo-base of exon per million fragments mapped reads, calculated based on the length of the fragments and reads count mapped to this fragment.

To distinguish the transcriptional changes over different treatment in the two genotypes, DEGs under varied nutrient conditions were identified by comparing the expression levels at T2 with those at T1 and the levels at CK with those at T2 in ZQUA44 and ZQUB15 respectively using degR package. To eliminate false positives, the false discovery rate (FDR) was calculated to adjust the threshold of *P*-value. Transcripts with a minimal 2-fold difference in expression ($|\log_2\text{Fold change}| \geq 1$) and a FDR $\leq 0.01$ were considered as differentially expressed between the three treatment (Audic and Claverie 1997). For convenience, DEGs with higher expression levels at T2 than those at T1, as well as those higher at CK than those at T2, were donated as "up regulated", whereas those in opposition were donated as "down regulated".

To assess the gene expression patterns over different nutrient conditions within each

genotype, expression pattern analysis were performed, which assigned all the DEGs of ZQUA44 and ZQUB15 across the two treatment levels to nine expression profiles, using Short Timeseries Expression Miner (STEM) version 1.3.8 (Ernst and Bar-Joseph, 2006). DEGs belonging to the same cluster were proposed to have similar expression pattern with each other. For each genotype, the clustered profiles of DEGs with $P < 0.05$ were considered as significantly different from the reference set.

**Validation of expression level**

Ten genes with different expression patterns revealed by RNA sequencing were randomly selected for validation by reverse transcription quantitative real-time PCR (qRT-PCR). RNA extracted from the leaves of the three independent biological replicates for each of T1, T2 and CK were employed for qRT-PCR validation. cDNA was synthesized using Tiangen FastKing RTKit (TIANGEN BIOTECH (BEIJING) CO., LTD.BEIJIN China). Gene specific primers for qRT-PCR were designed based on the corresponding sequence on NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC) and listed in Table S1 (Table S1). Actin (EF145577) was used as an internal control. The qRT-PCR was carried out using FastKing RTKit (TIANGEN BIOTECH (BEIJING) CO., LTD.BEIJIN China) and determined in 7500 Fast Real-time PCR System following the manufacturer's instructions. Three technical replicates were performed for each gene. A regression analysis was performed between qRT-PCR and RNA sequencing including all genes of the two genotypes at the three different treatments using r package (version3.1.3, http://cran.r-project.org/).

**Co-expression network construction, Functional annotation and GO and KEGG classification**

The Hmisc package implemented in r were used to calculate the correlation coefficient (PCC, r) of all expressed mRNAs using 18 expression profiles, including three nutrient gradient from the two genotypes of high biomass and lower biomass with three biological replicates. Subclusters of DEGs with same expression trend

under different treatment were analyzed by Cluster (Rousseeuw and Kaufman, 1990). The *P*-value < 0.01 and r > 0.8 or r < -0.8 were used as thresholds. The identified genes were annotated by *E. grandis* (https://phytozome.jgi.doe.gov/pz/portal.html#!bulk?org=Org_Egrandis) (Myburg et al., 2014) and TAIR database (https://www.arabidopsis.org/). Then GO terms were determined by AgriGO (http://bioinfo.cau.edu.cn/agriGO/index.php) with Arabidopsis as backgrounds, and the FDR < 0.05 was set as threshold. The KOBAS 3.0 (http://kobas.cbi.pku.edu.cn/index.php) was used to analyze the potential functions of the target genes in the pathways under three different nutrition treatment respectively ($P$ < 0.01). Pathview (https://pathview.uncc.edu/analysis) was used for visualization of significantly enriched KEGG pathways.

## Abbreviations

NUE: nutrient use efficiency; N: nitrogen; P: phosphorus; K: potassium; Ca: calcium; Mg: magnesium; S: sulfur; DEGs: differentially expressed genes; H: Tree height; GD: ground diameter; CW: crown width; qRT-PCR: reverse transcription quantitative real-time polymerase chain reaction; P2C: protein phosphatase 2C; AB: Auxin-binding protein; SAU: Auxin-responsive protein; GASA: Gibberellin-regulated protein; ERF: Ethylene-responsive transcription factor; PYL: Abscisic acid receptor; ARR: Two-component response regulator; LEA: late embryogenesis abundant protein; UGT: UDP-glycosyltransferase.

## Declarations

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Availability of data and materials

The data obtained and used in this study are available from the corresponding author on reasonable request.

## Competing interests

The authors declare that they have no competing interests.


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## Authors' contributions

WP and FQZ conceived and designed the experiments; HXY, XHY, WHZ, HQL, FX, BZZ, BX, YXW and XYC performed the experiments; XHY and HXY analyzed the data; XHY wrote the manuscript. All authors have read and approved the manuscript.


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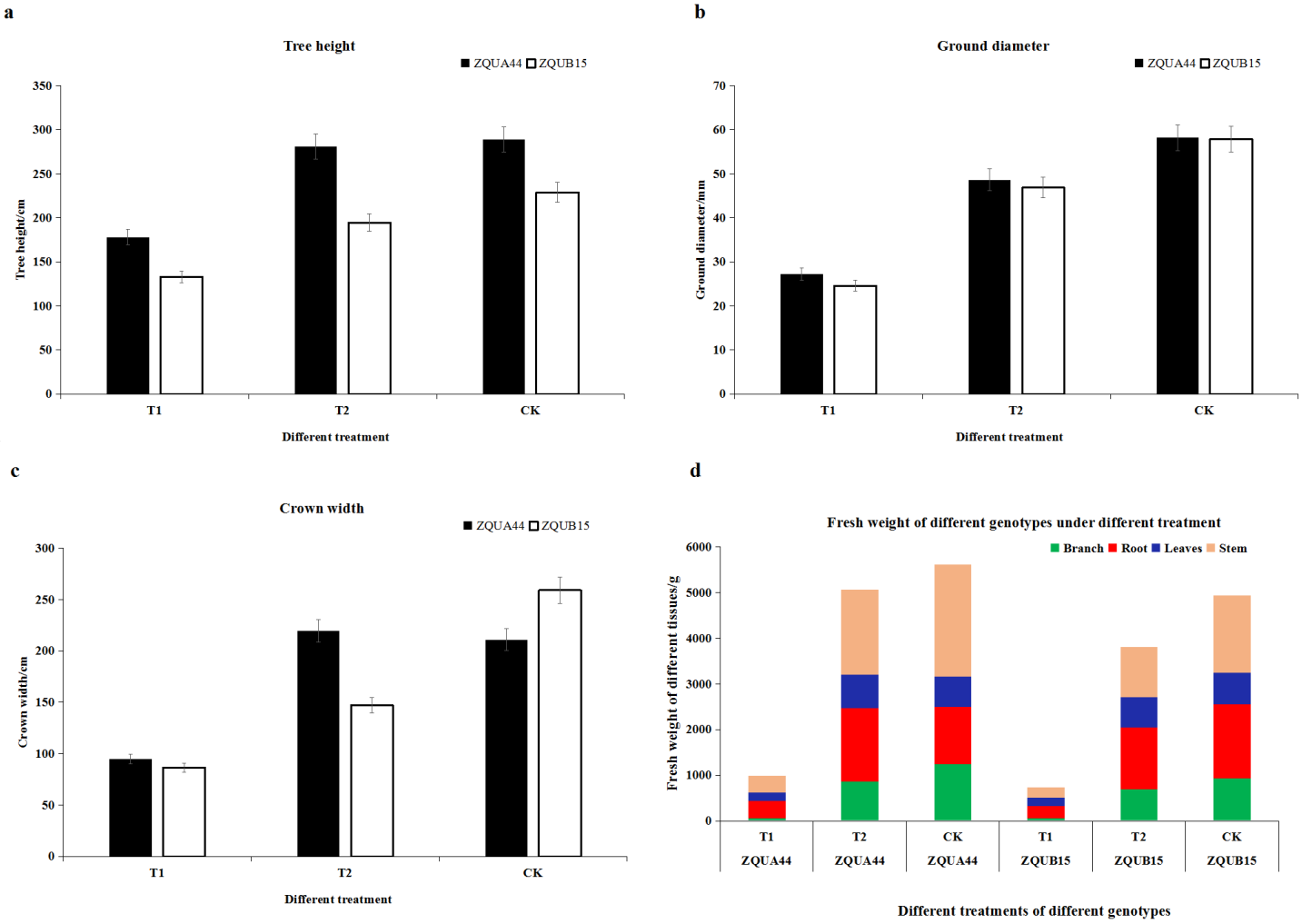
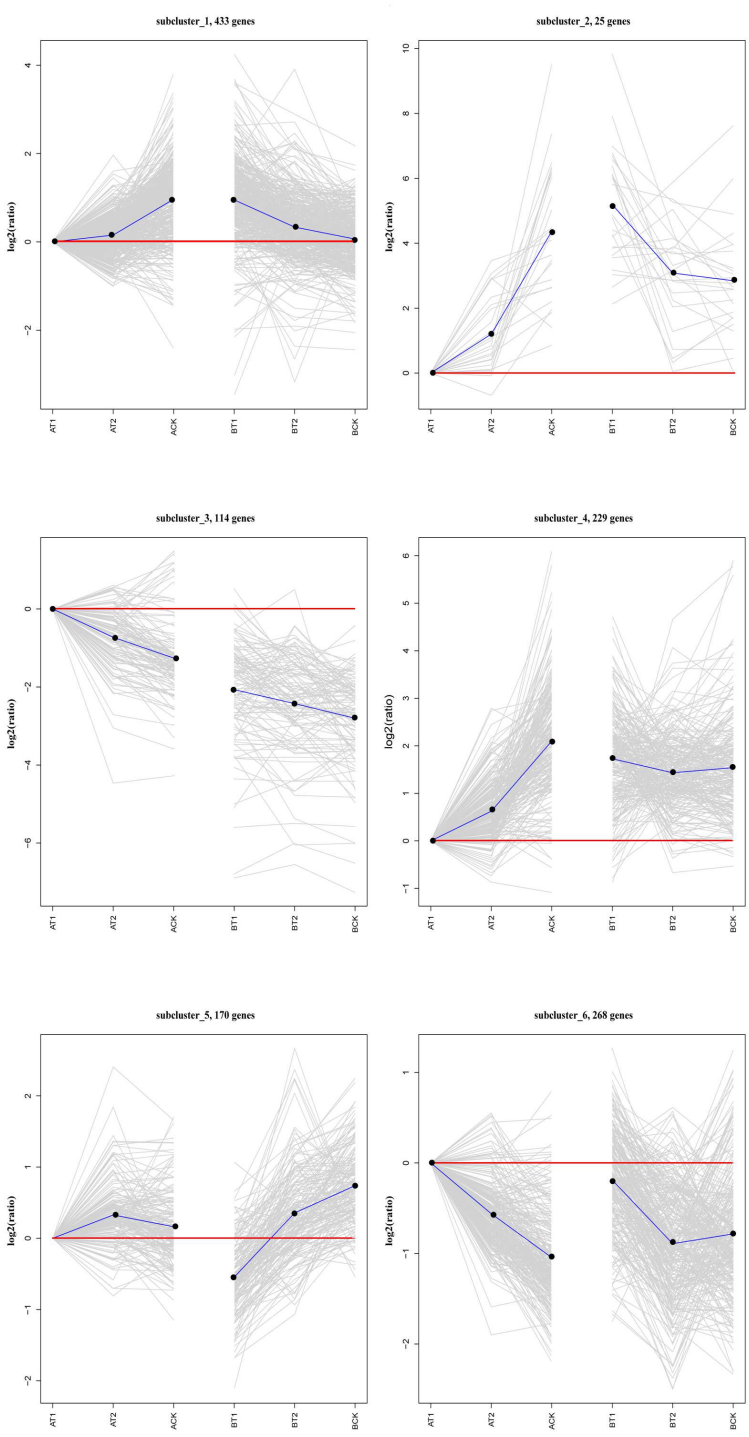
# Figures



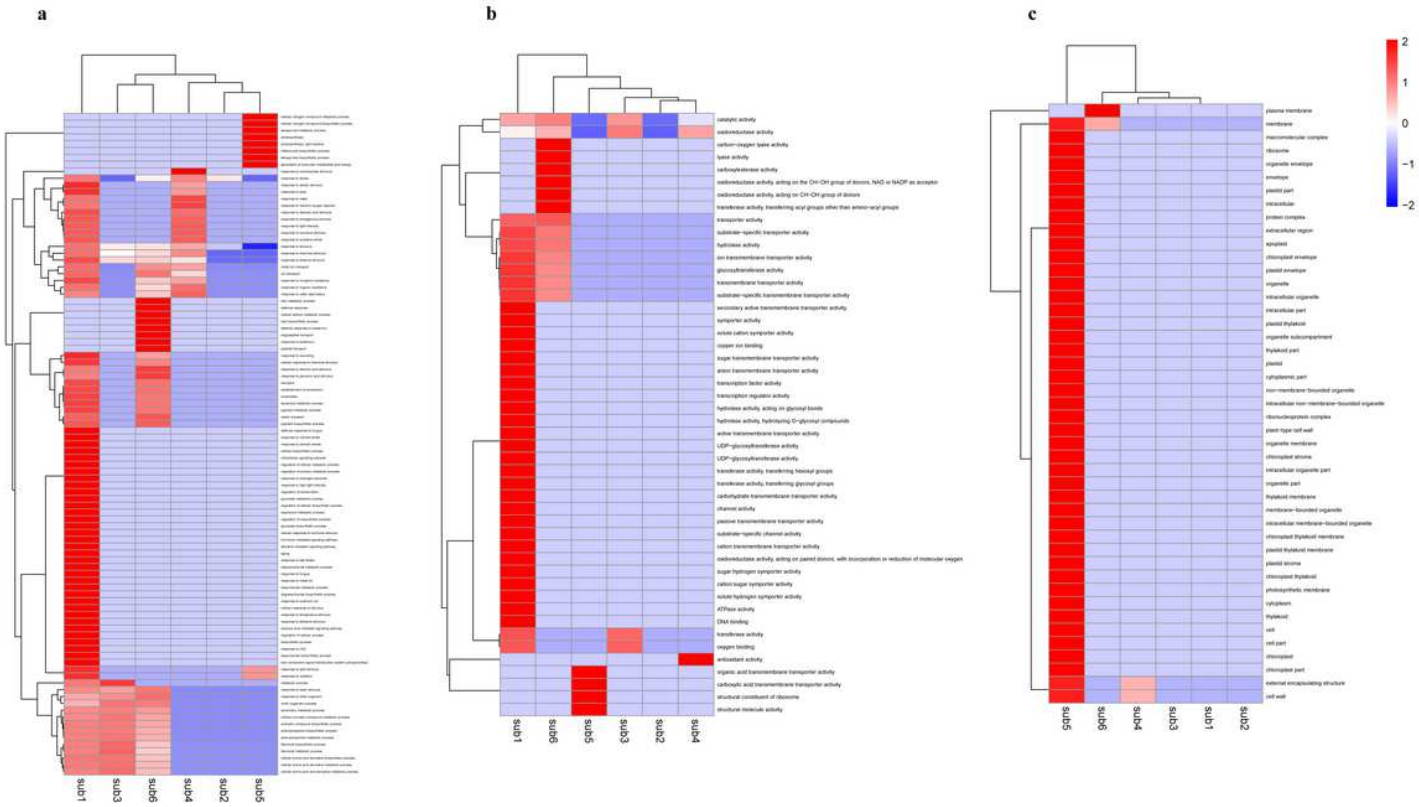

## Figure 1

All of the growth traits changed significantly between different nutrient treatment




## Figure 2

In total, 1,239 DEGs were detected in ZQUA44 and ZQUB15 at T1 and T2, as compared with those at CK using a threshold of 2-fold change in gene expression as previously mentioned




## Figure 3

To study the expression trend of DEGs, cluster software implemented in R was used to explore the expression pattern of DEGs from different treatment in genotype ZQUA44 and ZQUB15

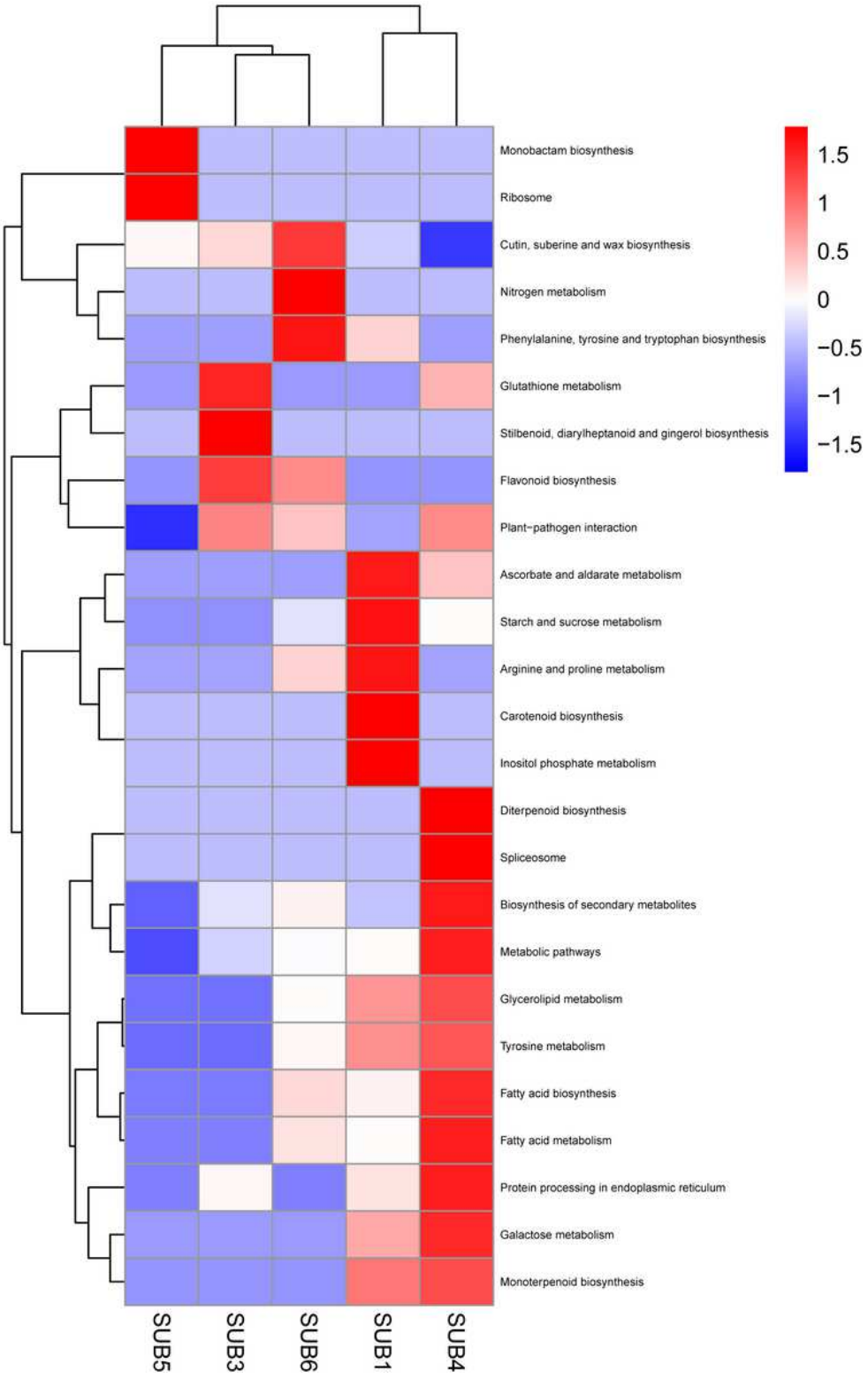
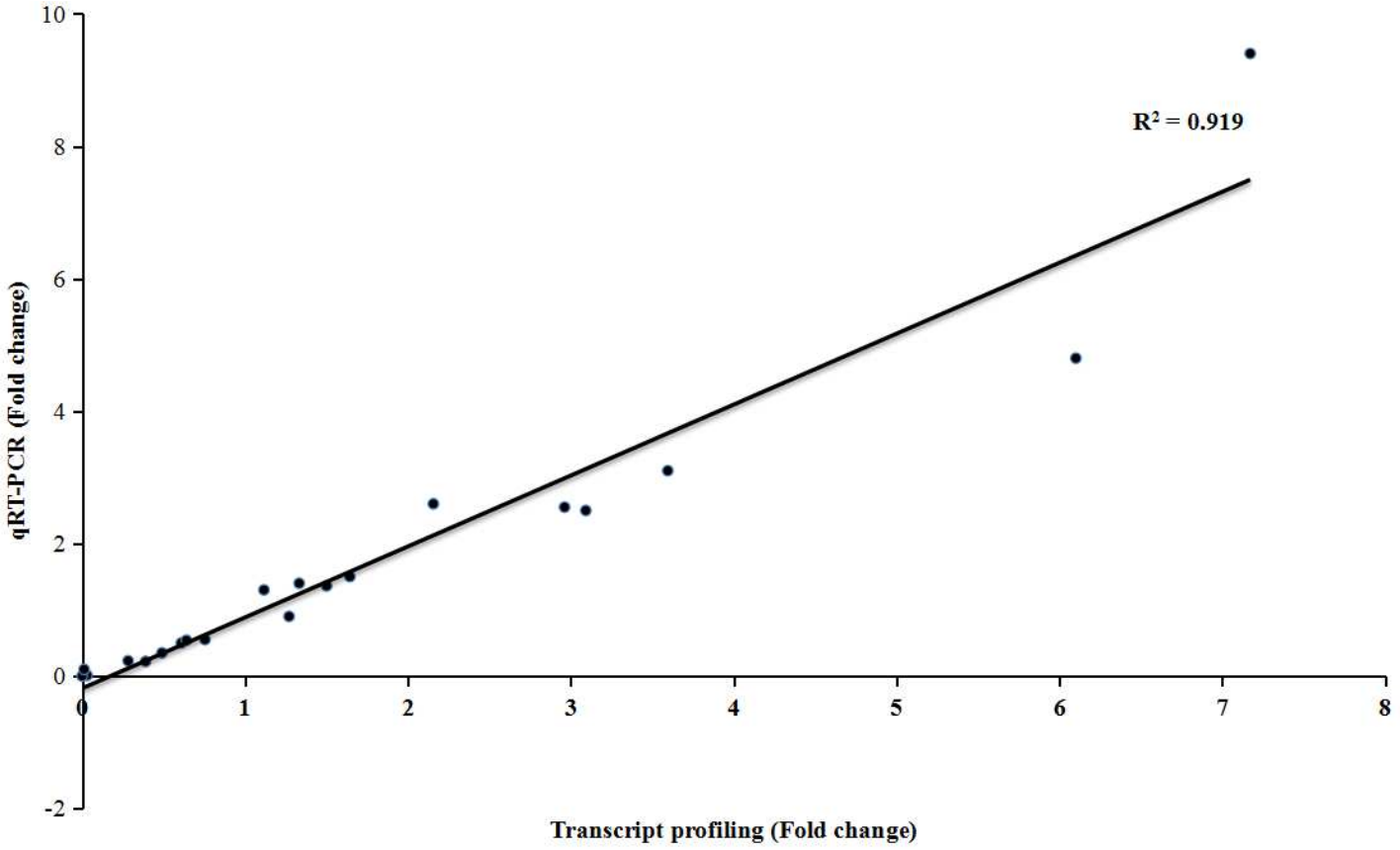



**Figure 4**

KEGG analysis




## Figure 5

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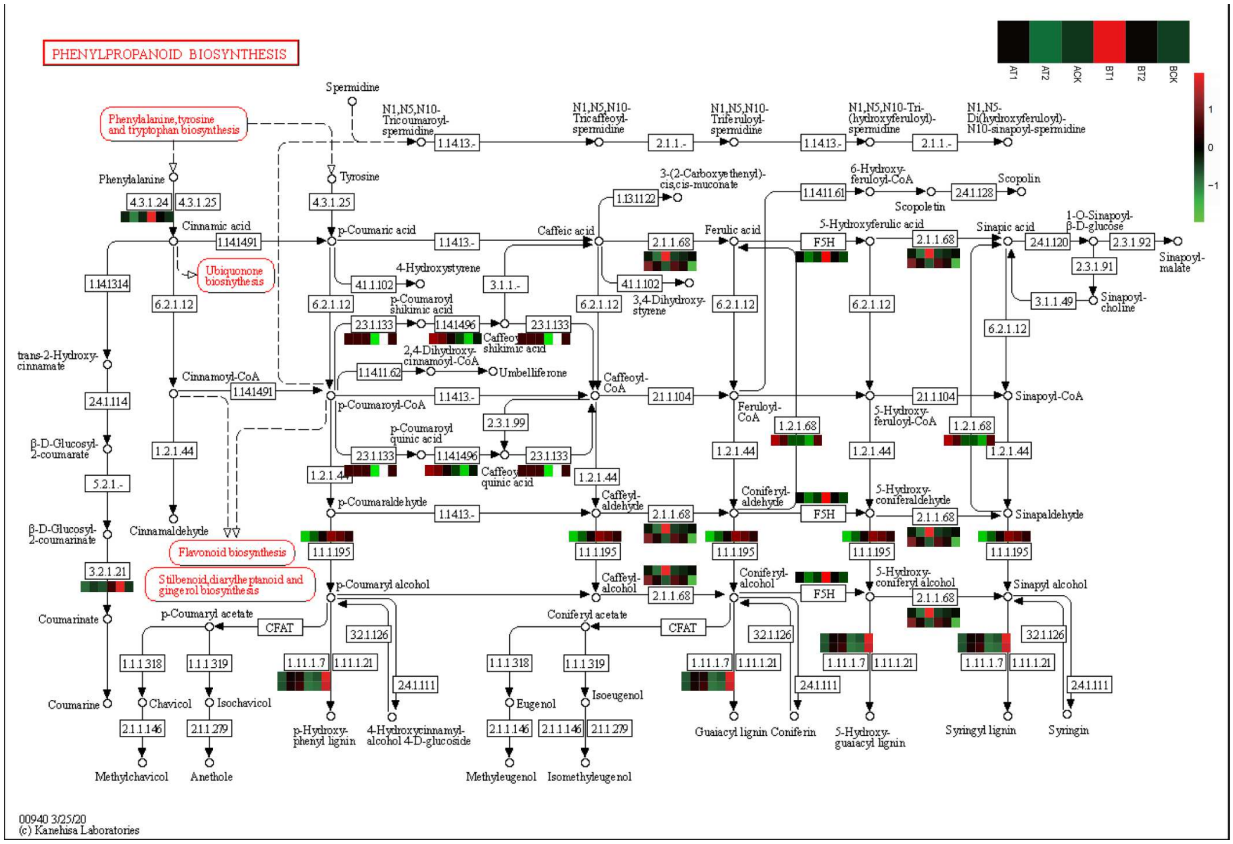

## Figure 6

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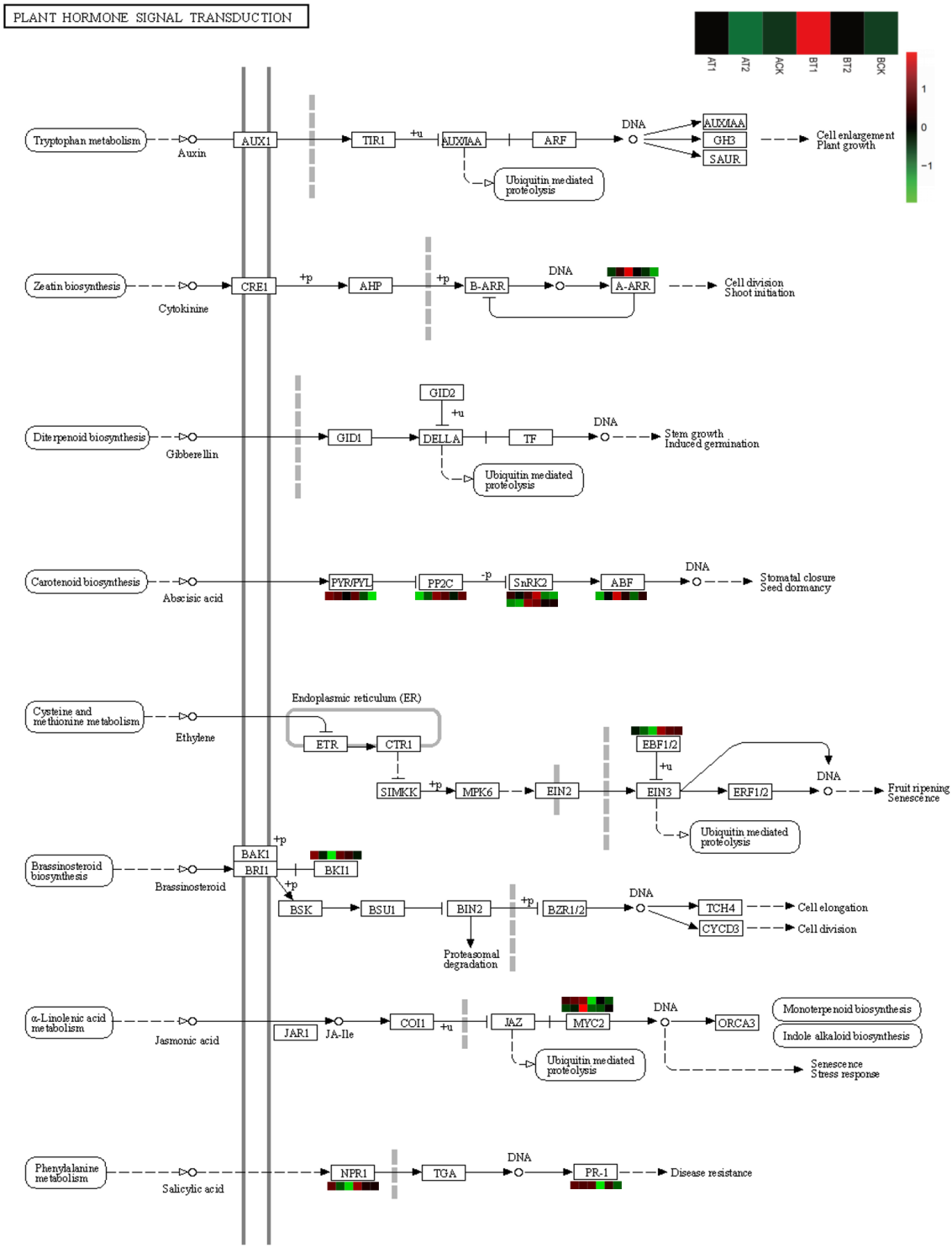


## Figure 7

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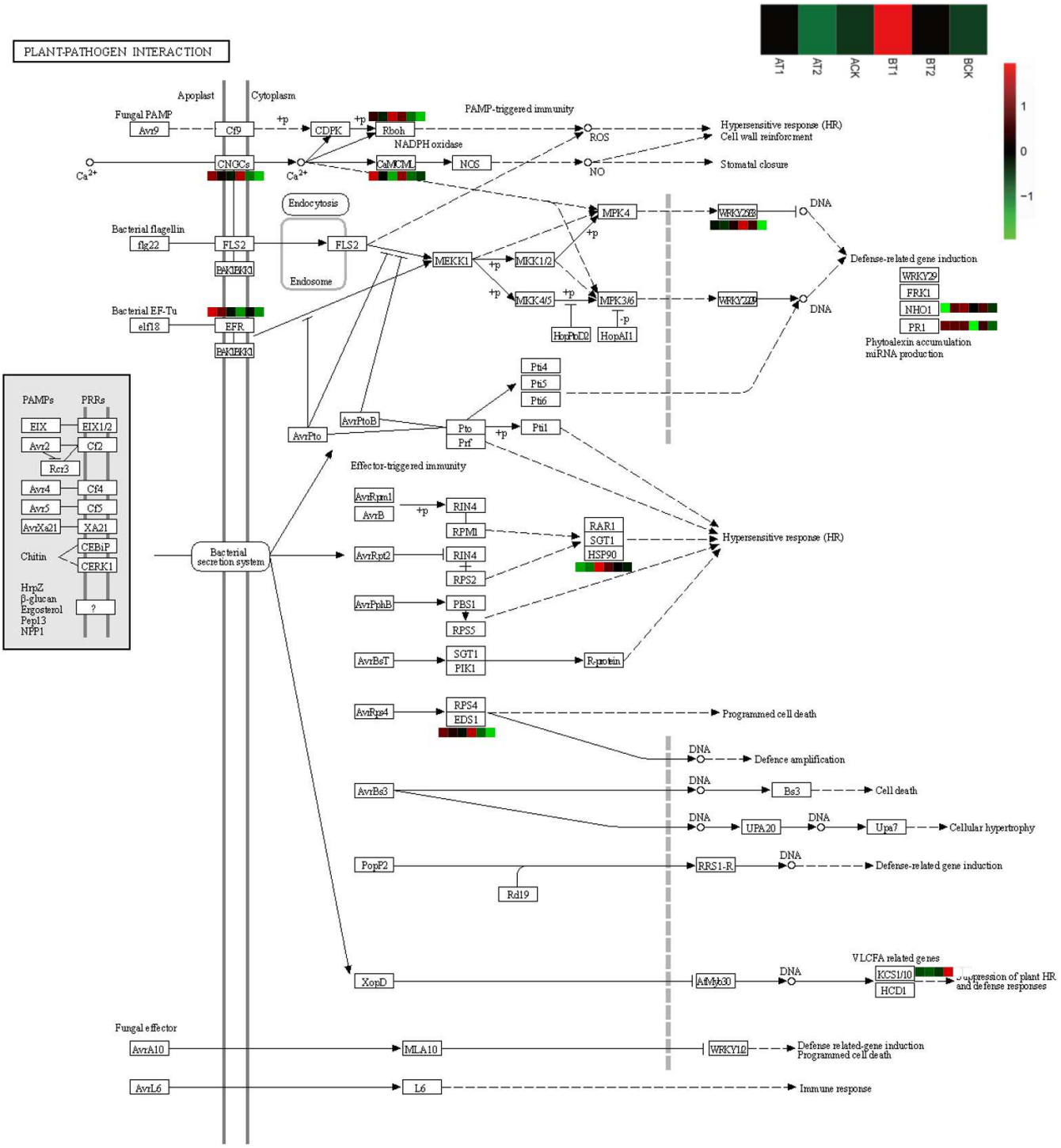


**Figure 8**

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# Supplementary Files

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