

Comparative transcriptome analysis reveals the potential mechanism of seed germination promoted by Trametenolic acid in *Gastrodia elata* Blume

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Abstract

Background

Gastrodia elata Blume (GEB) is a potential medicinal and edible plant with several active components and pharmacological activity that has a high application value in medicine and the food business. However, in natural conditions, GEB seed has a very low germination rate and depends on two specific fungi, germinal and nutritive fungi, to complete the germination process and growth. *Armillaria mellea*, while acting as a nutrient supplier, actually inhibits the germination of GEB seeds. *Mycena* strains, as the main germinating fungi, can facilitate germination but cannot support the subsequent growth and development of GEB. It requires symbiotic interactions with *Mycena* and *Armillaria mellea* to obtain nutrients for its complex life cycle. Our previous studies have shown that Trametenolic acid (TA) can effectively promote seed germination of GEB. The aim of this study was to use transcriptome sequencing to further understand the potential mechanism of seed germination triggered by TA in GEB, in order to lay the groundwork for developing a new germination-growth system for GEB with *Armillaria mellea*.

Results

The untreated symbiotic group (Group A₀) did not germinate in the seed germination test. The high-dose TA-treated symbiotic group (Group B), the low-dose TA-treated symbiotic group (Group C), and the non-symbiotic untreated germination group (Group A) had germination rates of 85.01%, 61.18% and 27.39%, respectively. This indicates that TA treatment can induce symbiosis with *Armillaria mellea* in GEB seeds and significantly increase germination rates. Transcriptome sequencing (RNA-seq) of Groups A, B, and C identified 86843 annotated genes. There were more down-regulated genes than up-regulated genes, with 3912, 2518, and 814 differentially expressed genes (DEGs) between B and A, C and A, and B and C, respectively. The DEGs were mainly involved in DNA transcription factors, cell wall actions, plant-pathogen interactions, phenylpropanoid biosynthesis, phytohormone signal transduction, and starch-sucrose metabolism pathways. Six genes were confirmed using qRT-PCR: Down-regulated genes in the lignin biosynthesis pathway include MYB4 and 4CL, while GA20ox1 in the gibberellin biosynthesis pathway was also down-regulated. Up-regulated genes in the plant-pathogen interaction pathway are AIB and WRKY51, with MYB44 in the lignin biosynthesis pathway showing up-regulation. The transcriptomics results supported these expression patterns. Lignin, GA, and abscisic acid (ABA) levels were analyzed in GEB protocorms to understand how TA promotes germination. Results showed that groups B and C had lower lignin and ABA levels, but higher GA levels compared to group A. The study revealed that certain genes play a crucial role in promoting GEB seed germination through TA, by regulating gene expression to alter lignin content and hormone levels, breaking seed dormancy, facilitating seed-fungus interactions, and promoting symbiotic relationships with *Armillaria mellea*.

Conclusion

TA can regulate genes related to lignin and hormones, leading to an increase in GA content and a decrease in ABA and lignin content. This helps seeds break dormancy and promote germination. Additionally, TA can enhance GEB's defense response against fungi by regulating plant-pathogen interaction genes. It also improves the interactions between GEB and *Armillaria mellea*, overcoming the technical challenges associated with using *Armillaria mellea* as a germinating fungus. This establishes a new symbiotic germination-growth system between *Armillaria mellea* and GEB, laying the foundation for further research on the molecular mechanisms of GEB seed germination.

Background

Orchideaceae originated in the Laoya Ancient land during the Late Cretaceous [1], making it one of the most diverse and widely distributed families of angiosperms. *Gastrodia elata* Blume (GEB), a perennial herb and saproparasitic plant belonging to the genus *Gastrodia* in the Orchidaceae family, is predominantly distributed in Yunnan, Sichuan, Hubei, and other areas across China [2, 3]. Currently, 134 compounds including phenolic compounds, polysaccharides, glycosides, organic acids, and sterols have been extracted or characterized from GEB [4–7]. Modern pharmacological studies have proved that isolated components or crude extracts of GEB possess neuroprotection, learning and memory improvement effects, cardioprotection, vaso-modulatory effect, anti-depression, anti-cancer, and other activities [8–12]. Currently, GEB has been widely applied to clinics and foods. The natural germination rate of GEB seeds is very low because of their small size, simple structure, lack of endosperm, poor permeability of seed coat, and presence of inhibitory substances in the seeds. Consequently, the germination of GEB is significantly reliant on the specific fungi of *Mycena* [13, 14], and the saprophytic plant of GEB requires *Armillaria mellea* for nutrients to grow, along with both germinating fungi and nutritive fungi to finish its entire life cycle [15].

The germination of GEB seed is a complex process with various steps and factors. Currently, there are two main germination patterns: symbiotic germination and non-symbiotic germination. Symbiotic germination involves GEB seeds interacting with specific fungi, particularly from the genus *Mycena*, to provide essential materials for germination, increase resistance to environmental stress, and enhance growth and survival rates [16]. However, only a few germinating strains are utilized in real production, which solely facilitate the germination of GEB seeds without aiding in its subsequent growth and development [17, 18]. In addition, germplasm degradation is prone to occur during the application of germinating fungi, which affects the yield and quality of GEB [19]. Non-symbiotic germination, unlike symbiotic germination, does not require symbiotic fungi and involves the use of nutrients or chemicals to stimulate GEB seed germination [20]. Fine-tuning the medium composition and environmental conditions can greatly enhance the germination rate. Non-symbiotic germination is simple to execute, regulate, and replicate, resulting in lower production costs [21]. It is imperative to develop new propagation technology for GEB in order to overcome the drawbacks of both symbiotic and non-symbiotic germination methods. The time required for non-symbiotic germination is longer than that for

symbiotic germination, and the adaptability of the interaction between the germinated protocorms and *Armillaria mellea* is less than that of symbiotic germination, so these methods have not been widely used in practical production [22]. Hence, merging the strengths of both methods is necessary to fulfill the needs of large-scale production and preserve germplasm resources. This study aimed to combine the benefits of both symbiotic and non-symbiotic germination by replacing germinating fungi with *Armillaria mellea*. By promoting eruption substances, the inhibition of *Armillaria mellea* on GEB seed germination can be alleviated, leading to the establishment of a new integrated system for GEB seed germination and growth. This system aims to simplify the cultivation process and provide technical support for GEB plant production.

Trametenolic acid (TA) is a bioactive triterpenoid compound. Its isolation and purification primarily employ solvent extraction combined with column chromatography techniques, while structural elucidation is achieved through multidimensional techniques including spectral analysis, mass spectrometry, X-ray crystallography, and infrared spectroscopy [23]. This compound is widely found in traditional germination fungi of GEB (such as *Mycena dendrobii* of the genus *Mycena*). However, recent studies have revealed that *Trametes lactinea* (Berk.) Pat. of the Polyporaceae family can also serve as a germination fungus for GEB and efficiently synthesize TA [24]. This discovery challenges the traditional understanding that germination fungi are limited to the genus *Mycena*, thereby expanding the symbiotic fungal resource pool for GEB.

This study investigated the effects of different treatments on GEB seeds, including a control group without TA and with *Armillaria mellea* (A_0 group), a non-symbiotic germination control group (A group), a high dose TA treatment group (B group), and a low dose TA treatment group (C group). The A, B, and C groups were used for RNA-seq analysis. The germination rates of the three groups were compared, and differentially expressed genes (DEGs) and transcription factors (TFs) related to ginseng seed germination pathways were identified. Key genes were validated using qRT-PCR. The study aims to uncover the mechanisms and regulatory networks of TA in promoting GEB seed symbiotic germination with *Armillaria mellea*, providing evidence for the release of germination stimulants from *Armillaria mellea* and supporting the establishment of a symbiotic germination-growth system for GEB and *Armillaria mellea*.

Results

Effect of TA treatment on the germination rate of GEB seeds

The viability of GEB seeds was assessed using the 2,3,5-triphenyltetrazolium (TTC) method before the germination test, with all seeds showing a viability of over 90%. Treatment with TA was found to enhance the symbiosis between GEB seeds and *Armillaria mellea*, leading to a significant increase in the germination rate of the seeds. While GEB seeds could germinate non-symbiotically on 1/2MS medium (Group A), the process was slow, taking at least 120 days to reach the protocorm stage with a germination rate of 27.39%. However, GEB seeds were unable to germinate symbiotically on 1/2MS

medium with *Armillaria mellea* (Group A₀), suggesting that *Armillaria mellea* had an inhibitory effect on the germination process. On the other hand, GEB seeds treated with varying doses of TA were able to germinate symbiotically with *Armillaria mellea* on 1/2MS medium, reaching the protocorm stage in just 30 days. The germination rates for these groups were 85.01% (Group B) and 61.18% (Group C), respectively (Fig. 1).

Overview of transcriptome data quality assessment

The sequencing data was preprocessed by removing sequencing connectors with cutadapt and filtering out unqualified sequences with fqtrim. The statistics of raw sequencing amount, valid sequencing amount, valid data amount, Q20, Q30, and GC content were analyzed. The data volume of valid data for each sample was 5.20 G or higher, with Q30 bases at 92.45% or higher, and GC% at 46.68% or higher (Table 1). This indicates high quality sequencing data for further experiments and analysis. The data mentioned in the following text all come from Group A of the germination test, not from Group A0.

Table 1
Overview of sequencing quality control

Sample	Raw_Reads	Valid_Reads	Valid_Bases	Q20%	Q30%	GC%
A1	41027612	38401314	5.35G	97.48	92.65	47.03
A2	39251404	37260058	5.20G	97.55	92.77	47.40
A3	41005782	39630296	5.53G	97.50	92.68	47.64
B1	38956882	37451040	5.22G	97.40	92.49	49.49
B2	40514910	39011522	5.44G	97.53	92.70	46.68
B3	39839350	38681088	5.39G	97.46	92.59	47.58
C1	42313644	40688658	5.67G	97.51	92.66	46.68
C2	42127594	40779152	5.68G	96.80	97.37	48.99
C3	40182148	38709336	5.39G	96.33	97.41	47.16

In the table, Raw_Reads: the number of reads of downlinked data; Valid_Reads: the number of reads of valid data; Valid_Bases: the amount of data of valid data; Q20%: the proportion of bases with quality value ≥ 20 ; Q30%: the proportion of bases with quality value ≥ 30 ; GC%: the proportion of GC content.

Overview of Trinity Assembly Results

We adopted the strategy of assembling all samples in a mixed manner and finally normalized all samples to obtain Unigene. The assembly quality of the obtained Unigene was evaluated, including the length, GC content and N50 of Unigene (Table 2). The data indicated that the assembly result of this time

had a high degree of completeness and accuracy, providing a reliable foundation for the subsequent gene function annotation and expression analysis.

Table 2 Overview of trinity assembly results

Index	All	GC%	Min Length	Median Length	Max Length	Total Assembled Bases	N50
Transcript	150430	41.36	201	570.00	17258	153498870	1760
Gene	86843	42.13	201	419	17258	66413039	1295

Table parameter description:

Term	Annotation
Index	It refers to the level of genes (Gene) or transcripts (Transcript).
All	It refers to the total quantity of the assembled products.
GC%	The proportion of GC content.
Min Length	The shortest length of the transcript or gene.
Median Length	Median length of transcripts or genes.
Max Length	The maximum length of the transcript or gene.
Total Assembled Bases	Total number of assembled bases.
N50	That is, the N50 length. After arranging all the assembled results in descending order of length, when the total length is reached, the length at which it is half of the total length.

Functional annotation of transcripts

Upon comparing the sequence annotation of unigene with GO classification database, KEGG classification database, and NR database, the gene functional annotations of GEB in the three databases were acquired. In this transcriptome analysis, a total of 86,843 genes were successfully annotated, with more than 3,000 genes participating in the biological processes of GEB (Fig. 2). The KEGG classification revealed that the genes annotated in the metabolic functions were predominantly engaged in carbon metabolism, amino acid metabolism, and lipid metabolism.

Analysis of DEGs

Overall analysis of transcriptome gene expression

The gene expression trends were consistent within each group, with the majority of expression log₁₀ (TPM) values falling between 0–2 (Fig. 3a). The overall low dispersion of sample expression demonstrated high sensitivity in gene expression detection and provided a favorable environment for differential gene screening (Fig. 3b).

Differential gene volcano map between groups

Comparing the sequencing results between groups, with $FDR < 0.05$ and $|\log_2 FC| > 1$, red represents up-regulated significant DEGs, blue represents down-regulated significant DEGs, and gray represents non-significant DEGs (Fig. 4). In group B vs A, there were 3,912 significant DEGs, with 1,212 up-regulated genes and 2,700 down-regulated genes. In group C vs A, there were 2,518 significant DEGs, with 904 up-regulated genes and 1,614 down-regulated genes. In group B vs C, there were 814 significant DEGs, with 310 up-regulated genes and 504 down-regulated genes.

GO classification of DEGs

Combined with the results of GO enrichment analysis, a threshold of $p < 0.05$ was set, and genes were found to be significantly enriched in this condition. The enriched genes were visualized by a scatter plot, in which the shade of the ball color indicated the confidence of the data (redder color indicated higher confidence), and the size of the ball indicated how many DEGs were enriched in that aspect (larger ball indicated more DEGs). The GO analysis showed (Fig. 5) that in the group of A_vs_B_vs_C, DEGs were significantly enriched in the following categories: structural composition of ribosomes, activity of DNA-binding transcription factors, cytoplasmic large ribosomal subunits, salicylic acid response, methyl jasmonate response, cellular components, and cell wall. DEGs in group B_vs_A are enriched in DNA-specific binding, DNA transcription factor activity, fungal defense response, cell fraction, and cell wall. In group B_vs_C, DEGs are more prevalent in DNA-binding transcription factor activity, cell wall, and plasma membrane. Group C_vs_A shows enrichment in the structural composition of ribosomes, transcriptional regulation, DNA-binding transcription factor activity, and ABA-activated signaling pathway. The focus can be on shared DEGs-enriched aspects such as those related to the cell wall, DNA, ribosomes, and transcription.

KEGG functional analysis of DEGs

KEGG analysis showed (Fig. 6) that DEGs in group A_vs_B_vs_C were significantly enriched in starch and sucrose metabolism, ribosomes, plant-pathogen interactions, phenylpropanoid biosynthesis, pentose and glucuronide interconversion, and the plant MAPK signaling pathway. In group B_vs_A, DEGs were significantly enriched in plant-pathogen interactions, phenylpropanoid biosynthesis, pentose and glucuronide interconversion, and the flavonoid biosynthesis pathway. DEGs in group B_vs_C were significantly enriched in starch and sucrose metabolism. DEGs in group C_vs_A were significantly enriched in ribosomes, plant-pathogen interactions, phytohormone signaling, phenylpropanoid biosynthesis, and plant MAPK signaling. DEGs in all three groups were significantly enriched in plant-

pathogen interactions, starch and sucrose metabolism, phytohormone signaling, and phenylpropanoid biosynthetic pathway.

Screening of candidate genes

The combination of results from differential gene function annotation and enrichment analysis identified 25 DEGs associated with four main pathways: plant-pathogen interactions, lignin synthesis pathways, transcription factors controlling lignin synthesis, and ABA and GA synthesis.

Plant-pathogen interactions

Four DEGs were identified for their roles in plant-pathogen interactions: WRKY51 gene (TRINITY_DN63725_c4_g1), AIB gene (TRINITY_DN54289_c0_g1), Hsp83A gene (TRINITY_DN65338_c4_g1), and Hsp90-2 gene (TRINITY_DN65338_c3_g1). These genes were found to be up-regulated and involved in various biological functions. The addition of TA was shown to enhance the expression of these genes. The heatmap in Fig. 7 visualizes the gene expression in different treatments, with the differential gene TPM represented using the Z-value method.

Aspects of lignin synthesis pathways and transcription factors related to the regulation of lignin synthesis

Seven DEGs related to the lignin synthesis pathway were identified (Fig. 8a), including one PAL-related gene (TRINITY_DN62379_c0_g9), two 4CL-related genes (TRINITY_DN61606_c0_g2, TRINITY_DN47157_c0_g1), two CCR-related genes (TRINITY_DN64289_c0_g3, TRINITY_DN59141_c1_g1), one CAD-related gene (TRINITY_DN62669_c2_g2), and one POX/LAC-related gene (TRINITY_DN56331_c0_g2). All of these DEGs showed down-regulation in expression. MYB transcription factors play a significant role in regulating lignin biosynthesis. Six DEGs were chosen for demonstration based on their differential expression folds, with the differential gene TPM shown using the Z-value approach for gene expression (Fig. 8b).

ABA and GA synthesis pathways are related

Three DEGs involved in GA anabolism were identified (Fig. 9a). Two genes (TRINITY_DN61474_c0_g2 and TRINITY_DN60725_c0_g2) showed up-regulated expression, potentially enhancing the enzyme activity of GA20ox and increasing GA content accumulation. On the other hand, the expression of the gene TRINITY_DN62957_c2_g1 was reduced, with a 2.64-fold decrease in group B versus group A, and a 10.18-fold decrease in group C versus group A. This gene is thought to have a negative feedback effect on GA20ox enzyme activity, thereby enhancing GA synthesis.

Five DEGs related to ABA anabolism were primarily identified (Fig. 9b), consisting of one NCED-related gene (TRINITY_DN62764_c1_g1), three CYP707A-related genes (TRINITY_DN59854_c1_g9, TRINITY_DN59854_c1_g2, and TRINITY_DN59854_c1_g4), and one BG1-related gene

(TRINITY_DN48977_c0_g1). The gene expression of group B showed a 3.17-fold decrease compared to group A, while the gene expression of group C exhibited a 4.39-fold decrease compared to group A.

Validation by qRT-PCR

To confirm the reliability of the RNA-seq data, the genes GA20ox1 (TRINITY_DN62957_c2_g1) involved in GA biosynthesis in the seed germination-related pathway, 4CL (TRINITY_DN47157_c0_g1) for lignin biosynthesis, and the TFs of the related pathway MYB4 (TRINITY_DN59408_c5_g1) and MYB44 (TRINITY_DN62886_c4_g3), the genes WRKY51 (TRINITY_DN63725_c4_g1) and AIB (TRINITY_DN54289_c0_g1) of the plant-pathogen interactions, and six genes in the three major pathways were validated through qRT-PCR. The $2^{-\Delta\Delta C_t}$ method was used to calculate the expression levels of the selected genes. Results from Fig. 10 indicated that the qRT-PCR results aligned with the transcriptome sequencing results. Specifically, GA20ox1 (TRINITY_DN62957_c2_g1), MYB4 (TRINITY_DN59408_c5_g1), and 4CL (TRINITY_DN47157_c0_g1) were down-regulated, while MYB44 (TRINITY_DN62886_c4_g3), WRKY51 (TRINITY_DN63725_c4_g1), and AIB (TRINITY_DN54289_c0_g1) were up-regulated.

Determination of physiological indexes of GEB under TA treatment

The results of RNA-seq analysis showed that TA treatment could regulate lignin content and hormone levels. In order to further elucidate the physiological mechanism of TA to promote the germination of GEB seeds, in this study, the lignin content and the levels of GA and ABA of GEB samples from Groups A, B and C were determined, using Group A as the control. The results showed that the GA content of the TA-treated symbiotic germination groups (Groups B and C) was significantly increased compared with that of the non-symbiotic germination group (Group A) (Fig. 11b), while the ABA and lignin contents were significantly decreased (Fig. 11c and Fig. 11a). It was suggested that treating GEB with TA helped it overcome seed coat barrier, leading to increased germination and better interaction with *Armillaria mellea*. As a result, the TA-treated seeds sprouted faster and at a higher rate compared to group A seeds.

Discussion

GEB is a fully heterotrophic plant belonging to the orchidaceae family. Its growth primarily relies on specific fungi from the *Mycena* and *Armillaria mellea* genera to obtain essential nutrients [14]. The symbiotic relationship between GEB and *Mycena* fungi can enhance seed germination rate, stress resistance, and growth capabilities [25, 26]. However, there are dependencies and potential risks associated with this relationship [27, 28]. To develop a new breeding technique for GEB, researchers have experimented with a non-symbiotic germination method. This method offers advantages in simplifying the breeding process and reducing reliance on fungi, but it requires high technical expertise and may lead to issues such as post-germination growth. Prolonged separation of GEB from fungi can also impact its survival in the wild. While progress has been made in non-symbiotic germination technology for GEB, resulting in successful germination in 1/2 MS medium, the process is time-

consuming and lacks effective interaction between the formed protocorm and *Armillaria mellea*. As a result, this method has not been widely adopted in practical production.

Some researchers have found that *Armillaria mellea* inhibits the germination of GEB seeds. Water-soluble metabolites of *Armillaria mellea* have been shown to significantly inhibit the germination of GEB seeds. To address this issue, a new integrated system for GEB seed germination and growth with *Armillaria mellea* has been established. This system combines the advantages of both germination methods, using 1/2MS culture medium for nutrients, enzymes from *Armillaria mellea* to overcome seed coat barriers, and exogenous substances to counteract inhibitory factors. The new system is easy to use, stable, and has a short germination time with good interaction between GEB and *Armillaria mellea*.

The alcohol extracts of germinated fungi like *Mycena dendrobii* and *Trametes lactinea* (Berk.) Pat were found to contain substances that promote germination, including terpenoids and phytosterols. TA, the main active ingredient for promoting germination, has been isolated from *Trametes lactinea* (Berk.) Pat and has been shown to significantly improve the germination rate of GEB seeds and facilitate the interaction between GEB and *Armillaria mellea*. TA is a triterpenoid compound found in various fungi like *Trametes lactinea* (Berk.) Pat and *Poria cocos*(Schw.) Wolf., making it widely available and promising for use in GEB and other orchid production.

In the germination experiment of GEB seeds, four treatment groups were set up: A₀ group (GEB and *Armillaria mellea* symbiotic untreated), A group (non-symbiotic untreated germination), B group (symbiotic high-dose TA treatment), and C group (symbiotic low-dose TA treatment). The results showed that GEB seeds did not germinate in the A₀ group, possibly due to inhibitory metabolites from *Armillaria mellea*. Treatment with TA promoted seed germination, shortened germination time, and increased germination rate. RNA-seq analysis of GEB samples from groups A, B, and C revealed that TA had different effects on gene expression. Enrichment analysis of DEGs showed that TA treatment affected genes related to DNA transcription factor activity and cell wall, potentially increasing cell wall permeability, facilitating fungal infection, and promoting nutrient digestion by GEB.

The KEGG database shows that DEGs functional annotations focus on metabolic pathways such as plant-pathogen interaction, starch sucrose metabolism, plant hormone signaling, and phenylpropanol biosynthesis pathway in response to TA treatment. This suggests that TA treatment enhances the interaction between GEB and *Armillaria mellea* infestation, accelerates starch and sucrose metabolism for energy provision, adjusts plant hormone levels to promote germination by increasing GA content and decreasing ABA content, and reduces lignin accumulation to facilitate GEB seed germination. The results were validated by determining lignin, GA, and ABA levels, confirming that TA treatment regulates gene expression to decrease lignin content during seed germination. This process overcomes seed coat barriers, breaks dormancy, promotes germination, and enhances the interaction between GEB and *Armillaria mellea*. Seed germination, growth, and culture conditions can be further optimized for practical production and application.

In this study, four genes related to plant-pathogen interaction were identified as being up-regulated and involved in various biological functions, including plant defense response to fungi. These genes include WRKY51 (TRINITY_DN63725_c4_g1), AIB (TRINITY_DN54289_c0_g1), Hsp83A (TRINITY_DN65338_c4_g1), and Hsp90-2 (TRINITY_DN65338_c3_g1). WRKY transcription factors, such as WRKY51, play a crucial role in regulating plant responses to biotic and abiotic stresses by controlling the expression of target genes [29–31]. This regulation can enhance or weaken the plant's resistance to pathogens. bHLH transcription factors are crucial for plant growth, development, and stress response. They can bind to specific promoters and interact with other transcription factors like MYB and WRKY to regulate the expression of defense genes and enhance disease resistance in plants [32, 33]. Heat shock proteins (HSP) are synthesized in response to stress and help protect cell structure and function [34]. It is believed that TA may boost GEB's defense response to fungi by increasing the expression of genes involved in plant-pathogen interactions, facilitating a beneficial symbiotic relationship between GEB and *Armillaria mellea*. However, further research is needed to determine if *Armillaria mellea* directly influences the expression of plant-pathogen interaction genes.

Thirteen genes related to lignin synthesis were identified in this study, including seven genes related to key enzymes in the lignin synthesis pathway and six genes related to transcription factors that regulate lignin synthesis. The expression of key enzyme genes in the lignin synthesis pathway was found to be down-regulated by TA treatment, leading to reduced lignin accumulation, increased cell wall permeability, and enhanced susceptibility to foreign substances and fungi in GEB seed skin. Higher lignin content in seeds is known to be associated with lower germination rates due to its impact on cell wall solubility and permeability [35, 36]. Regulating the expression of genes such as 4CL [37], CCoAOMT [38], CAD [39], and CCR [40], which are involved in lignin synthesis, can significantly affect seed germination rates.

Six key genes belonging to MYB transcription factors and involved in regulating lignin synthesis were identified from DEGs. Two of these genes were MYB4 genes (TRINITY_DN55958_c1_g9, TRINITY_DN59408_c5_g1), both down-regulated. Two MYB44 genes (TRINITY_DN62886_c4_g3, TRINITY_DN62886_c4_g1) were up-regulated. The remaining three DEGs belonged to other MYB transcription factor families. MYB transcription factors are known to bind to the promoter region of key enzyme genes in the lignin biosynthesis pathway, affecting lignin accumulation [41]. The up-regulation and down-regulation of these transcription factor genes may enhance the activity of negatively regulated MYB transcription factors, reducing lignin accumulation. This could improve seed coat permeability, nutrient absorption, defense response against fungi, and seed germination rate. The exact mechanism of lignin reduction during seed germination and the role of TA in reducing lignin content require further investigation.

GA and ABA are important plant hormones that regulate seed germination. GA promotes seed germination, while ABA inhibits it. This ensures that seeds germinate under suitable conditions for plant survival. In higher plants, GA synthesis occurs in three stages: the plastid, endoplasmic reticulum, and cytoplasmic matrix. Enzymes like gibber 20-oxidase catalyze the transformation of GA intermediates into biologically active forms like GA1 and GA4 [42]. Three DEGs related to the key enzyme GA20ox were

identified in this study. The expression of two genes (TRINITY_DN61474_c0_g2 and TRINITY_DN60725_c0_g2) was up-regulated, potentially enhancing the activity of GA20ox enzyme and increasing GA content. Conversely, the expression of TRINITY_DN62957_c2_g1 gene was down-regulated, indicating a negative regulation on GA20ox enzyme activity and a decrease in GA content in seeds [43]. Overall, up-regulation of GA20ox enzyme-related genes may have a more significant impact on GA enzymatic reaction than down-regulation, leading to increased synthesis rate and content of GA in GEB, thereby effectively regulating its germination.

Five genes related to key enzymes of ABA anabolism were initially identified. These included one NCED gene (TRINITY_DN62764_c1_g1) and three CYP707A genes (TRINITY_DN59854_c1_g9, TRINITY_DN59854_c1_g2, TRINITY_DN59854_c1_g4), as well as one BG1 gene (TRINITY_DN48977_c0_g1). NCED plays a crucial role in regulating seed germination by controlling ABA synthesis [44]. CYP707A, on the other hand, encodes ABA 8'-hydroxylase, an enzyme responsible for ABA degradation in plants. Decreased CYP707A activity can result in elevated ABA levels in seeds, leading to inhibition of seed germination [45]. BG1, a β -glucosidase, can increase the concentration of active ABA in plants by hydrolyzing ABA-glucose ester (ABA-GE) in response to abiotic stress [46, 47]. In the experiment, the expression of NCED and BG1-related genes in GEB seeds decreased after TA treatment, while the expression of CYP707A-related genes increased. This suggests that TA may enhance seed germination by inhibiting NCED and BG1 gene expression and enzyme activity, promoting CYP707A gene expression, reducing ABA synthesis, accelerating ABA degradation, and ultimately lowering ABA levels in seeds.

This study investigated the impact of TA on gene expression during the symbiotic germination of GEB seeds and *Armillaria mellea*. Transcriptome analysis identified 25 key DEGs related to pathways involved in the germination process. In groups treated with TA, DEGs related to plant-pathogen interaction were up-regulated, indicating that TA may enhance symbiosis by increasing stress resistance in GEB seeds. DEGs associated with lignin, GA, and ABA synthesis were down-regulated. It was proposed that TA regulates gene expression, increases GA levels, decreases ABA and lignin content, and promotes germination by overcoming seed coat barriers and dormancy, facilitating a beneficial interaction between *Armillaria mellea* and GEB.

Conclusion

This study identified that TA promotes the symbiotic germination and growth of GEB seeds and *Armillaria mellea* by regulating plant-pathogen interaction, lignin, ABA, and GA pathways. TA increases GA content, decreases ABA and lignin content, breaking seed coat barrier, promoting germination, and establishing a good interaction between *Armillaria mellea* and GEB. These findings enable the use of *Armillaria mellea* as germination fungi, enhance understanding of their symbiotic interaction, and establish a new symbiotic germination-growth system for *Armillaria mellea* and GEB.

Materials and Methods

Seed germination test

TA: It was isolated and purified from the alcoholic extract of *Trametes lactinea* (Berk.) Pat by our laboratory (Hubei Key Laboratory of Natural Products Research and Development, Three Gorges University). Specific methods: the dried mycelium of *Trametes lactinea* (Berk.) Pat was crushed and powdered, 20 times of 95% ethanol was added, and reflux extraction was carried out for three times to obtain the alcoholic extract of the fungus, which was separated by silica gel column chromatography to obtain the crude TA, which was prepared by preparative HPLC according to the condition of acetonitrile: water (90:10), i.e., the content of TA was obtained at 98%. The molecular formula and the high performance liquid chromatography (HPLC) diagrams are shown in Fig. 12.

Mature and unopened ubiquitous red hybrid GEB capsules were provided by Yichang City, Hubei Province, Wufeng Niuzhuang Alpine Eco-Medicine Base Co. The GEB capsules were sterilized with 75% ethanol for 30s. Filter paper was used to absorb the surface moisture, and the pods were dissected using a sterile scalpel, and the seeds were placed in 1.5mL centrifuge tubes with silica gel desiccant and stored at 4°C. The plant seed germination test was briefly operated as follows: for the germination experiment, TA was dissolved using a solution of DMSO : anhydrous ethanol = 1:9, filtered through 0.22µm microporous filter membrane, and finally made into the desired concentration of mother liquor. When used, it was then diluted with sterile water to the desired concentration. The seeds of GEB. treated with TA for 48h were evenly sown onto 1/2MS medium with *Armillaria mellea* with a sterile brush, and cultured in the dark at a constant temperature of 25°C to obtain GEB protocorms. There were three biological replicates for each treatment and 300 seeds for each replicate. All biological replicates were from the same seed bank. The samples were snap-frozen with liquid nitrogen and stored in the refrigerator at -80°C for transcriptome sequencing and physiological indexes.

Determination of lignin, GA and ABA content in GEB samples

Seed vigor was determined by TTC method, which was used to determine the content of trichothecene tetrazolium (TTF). The colorless oxidized TTC is reduced to red TTF by hydrogen gas produced by dehydrogenase in the living cellular tissues of the seed embryo [48]. Lignin content was determined using a lignin content assay kit (UV cuvette method) (Sangong Bioengineering Co., Ltd.), and GA and ABA content was determined using a plant GA ELISA assay kit and a plant plant ABA ELISA assay kit (Jiangsu Enzyme Immunity). For lignin determination, 5mg of GEB protocorm sample dried to constant weight at 80°C was weighed, and other methods were carried out according to the instructions. For the determination of GA and ABA, samples were prepared as follows: The fresh sample was not less than 50mg, the homogenate ratio was 10%, the homogenate was PBS (pH = 7.2–7.4, concentration was 0.01mmol/L), the whole grinding process was carried out in an ice bath or in liquid nitrogen, and the centrifugation was performed at 5000r/min for 15min. The supernatant was taken for measurement. The culture period of GEB protocorms used for the above experiments was 120 days for group A and 30 days for group B/C.

Construction and quality control of cDNA library, sequencing and annotation

Due to incomplete annotation of the GEB reference genome, a reference-free transcriptome analysis was conducted. After quality control of extracted total RNA, eukaryotic mRNA was enriched using Oligo(dT)-attached magnetic beads. The mRNA was then fragmented and used as a template to synthesize one-stranded cDNA with random hexamers. Following synthesis, end repair, A-addition, adapter ligation, and PCR amplification were performed to create a sequencing library with an average insert size of 300 ± 50 bases. After passing quality control, the library was sequenced using Illumina Novaseq™ 6000 with 2*150 bp (PE150) reads.

Screening, functional annotation and enrichment analysis of DEGs

Based on the results of enrichment analysis of DEGs in GO and KEGG databases, the p-value values and the apparent results of the germination rate of the samples A<C<B, combined with the relevant published literature and KEGG databases, etc., the present study focuses on the DEGs that are highly enriched in the two databases and are common to the B and C groups, and conducts basic acquisition and screening.

Differential expression analysis of genes between the two groups was performed with DESeq2 software (differential expression analysis between the two samples was performed with Edger software). genes with $FDR < 0.05$ and $FC \geq 2$ were DEGs. Where FC denotes the ratio of gene expression in the two samples, FDR was obtained by correcting the P-value (P-value) for the significance of differences. The screened DEGs were then subjected to BLAST comparison with known gene databases (mainly GO and KEGG databases, etc.) with gene function annotation, and DEGs enrichment analysis, so as to obtain the results of the functional annotation and enrichment analysis of DEGs. The P -value was calculated by the following formula:

$$P = 1 - \sum_{i=0}^{m-1} \frac{\binom{M}{i} \binom{N-M}{n-i}}{\binom{N}{n}}$$

In the formula: n: number of all genes with GO or KEGG annotations;

n: number of DEGs in N;

M: number of all genes annotated to a specific GO term or KEGG pathway;

m: number of DEGs in M.

N represents total background genes (number of TB genes);

n represents total significant genes (number of TS genes);

M represents background genes (number of B genes);

m represents significant genes (number of S genes).

qRT-PCR analysis

Total RNA was reverse transcribed into cDNA using HiScript III 1st Strand cDNA Synthesis Kit (+ gDNA wiper) (Nanjing Novozymes Biotechnology Co., Ltd.). qRT-PCR analysis was performed using SGExcel FastSYBR Master (Sangong Bioengineering Co., Ltd.). The cDNA was analyzed by qRT-PCR using SGExcel FastSYBR Master (Sangong Biological Engineering Co. The results were analyzed by the relative quantitative $2^{-\Delta\Delta C_t}$ method using β -actin as an internal reference. The primer design was commissioned to Sangong Biological Engineering Co. The gene sequences shown in Table 3 were used in qRT-PCR assays to detect and quantify the expression levels of specific genes under different conditions.

Table 3 Primer sequences

Gene ID	Primer sequences (5'-3')
TRINITY_DN47157_c0_g1(4CL)	Forward primer: GCCGCACCGACGACGAG Reverse primer: GCCACTTCTCCAACGCCTTAATC
TRINITY_DN62886_c4_g3(MYB44)	Forward primer: GGAACTGGTCTCTGATAAGCAAATCG Reverse primer: GTCCTCGGCGGTCGTGAAG
TRINITY_DN59408_c5_g1(MYB4)	Forward primer: CCGCCTCATCGCCCATATCC Reverse primer: CTCTTCCCGCACCGCAATAATC
TRINITY_DN54289_c0_g1(AIB)	Forward primer: CGTATATTACCGAACTCCAGAAGAAGC Reverse primer: CCCGCCCGCACCTCAAC
TRINITY_DN63725_c4_g1(WRKY51)	Forward primer: CTCCTACAATTGCGAGACCAACAG Reverse primer: GTGGTGGGCGGAAGAGAAGAG
TRINITY_DN62957_c2_g1(GA20ox1)	Forward primer: TGGCTGAAGGGCTGGGATTG Reverse primer: CGGGCAGGTCGGGTAATGG
β -actin	Forward primer: GGGGATGAAGCACAGTCCAA Reverse primer: GCCGTGGTTGTGAAGGAGTA

Table parameter description:

Term	Annotation
Gene ID	Is a unique identifier for each gene, generated by the bioinformatics tool Trinity.
Gene Name	Names in parentheses such as 4CL, MYB44, MYB4, AIB, WRKY51, GA20ox1 are functional annotations of genes or known gene names.
Primer sequences (5'→3')	Primer sequences used for PCR amplification of specific gene segments, oriented from the 5' end to the 3' end.
Forward primer	The forward primer binds to the antisense strand of the target DNA
Reverse primer	The reverse primer binds to the sense strand of the target DNA
β-actin	Commonly used reference genes for standardization in gene expression analyses.

Statistical analysis of data

Data were analyzed by ANOVA using Excel and SPSS followed by Dunnett's test of significant difference, $p < 0.05$ was considered significant, and all data were replicated three times biologically.

Abbreviations

TA: trametenolic acid; ABA: abscisic acid; GA: gibberellin; NCED: 9-cis-epoxycarotenoid dioxygenase; GA20ox: gibberellin 20-oxidase; DEGs: differentially expressed genes; MAPK: mitogen-activated protein kinase; Hsp90: heat shock protein 90; PAL: phenylalanine ammonia lyase; 4CL: 4-coumaroyl-coenzyme A synthetase; CCR: cinnamoyl coenzyme A reductase; CAD: cinnamyl alcohol dehydrogenase; POX/LAC: peroxidase/laccase; TPM: percentage of a given transcript per million reads; CYP707A: enzyme responsible for catalyzing the hydroxylation of ABA at the 8'-position, a member of the cytochrome P450 superfamily; BG1: encoding the β-1,3- glucanase gene; CCoAOMT: Caffeoyl CoA O-methyltransferase; ABA-GE: ABA-glucose ester; DMSO: dimethyl sulfoxide; TTC: 2,3,5-triphenyltetrazolium chloride; TTF: triphenylmethyl dirty

Declarations

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Availability of data and materials

The datasets generated and/or analysed during the current study are available in the Genome Sequence Archive (Genomics, Proteomics & Bioinformatics 2021) in National Genomics Data Center (Nucleic Acids Res 2024), China National Center for Bioinformation / Beijing Institute of Genomics, Chinese Academy of Sciences (GSA: CRA021984) that are publicly accessible at <https://ngdc.cncb.ac.cn/gsa> [49, 50].

Authors' contributions

WJZ, ZHQ, WDY and LJ conceived and designed the experiments. WJZ provided seeds and treatment suggestions. LJ and WDY treated the seeds and measured the physiological parameters. LJ and LDW collected data. LJ, WDY and YWY analyzed the data. LJ, WDY, ZHQ and WJZ wrote the paper. All authors read and approved the final draft.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

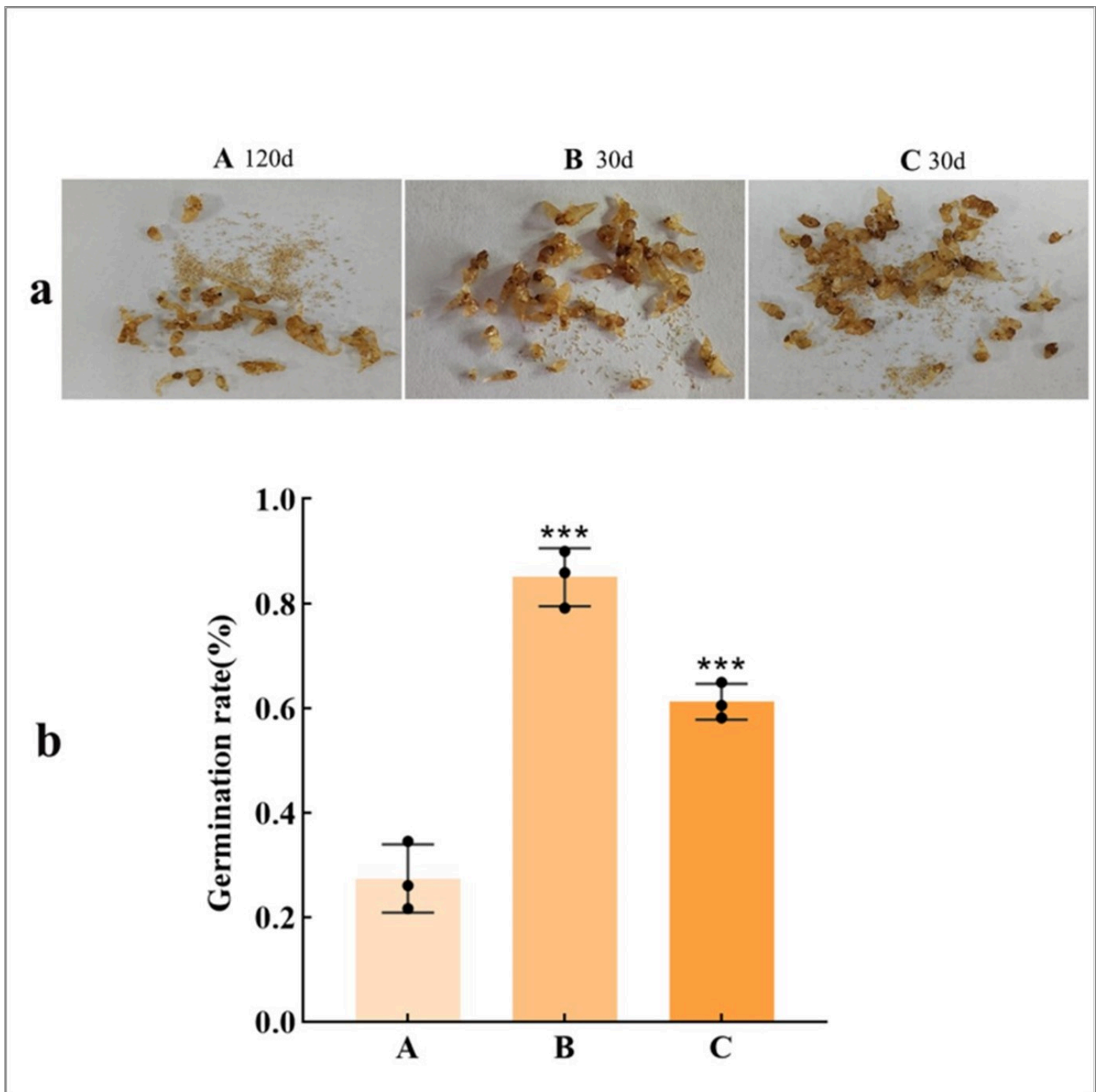


Figure 1

Germination of GEB seeds under TA treatment. **a** Comparison of GEB seed germination. **b** Comparison of GEB seed germination rate. Data are expressed as mean $\pm$ standard deviation ($\pm$ s) with three replications ($n = 3$). “***” indicates significant difference compared with group A. $p^* < 0.05$, $p^{**} < 0.01$, $p^{***} < 0.001$. “.” indicates the repeated data of each group.

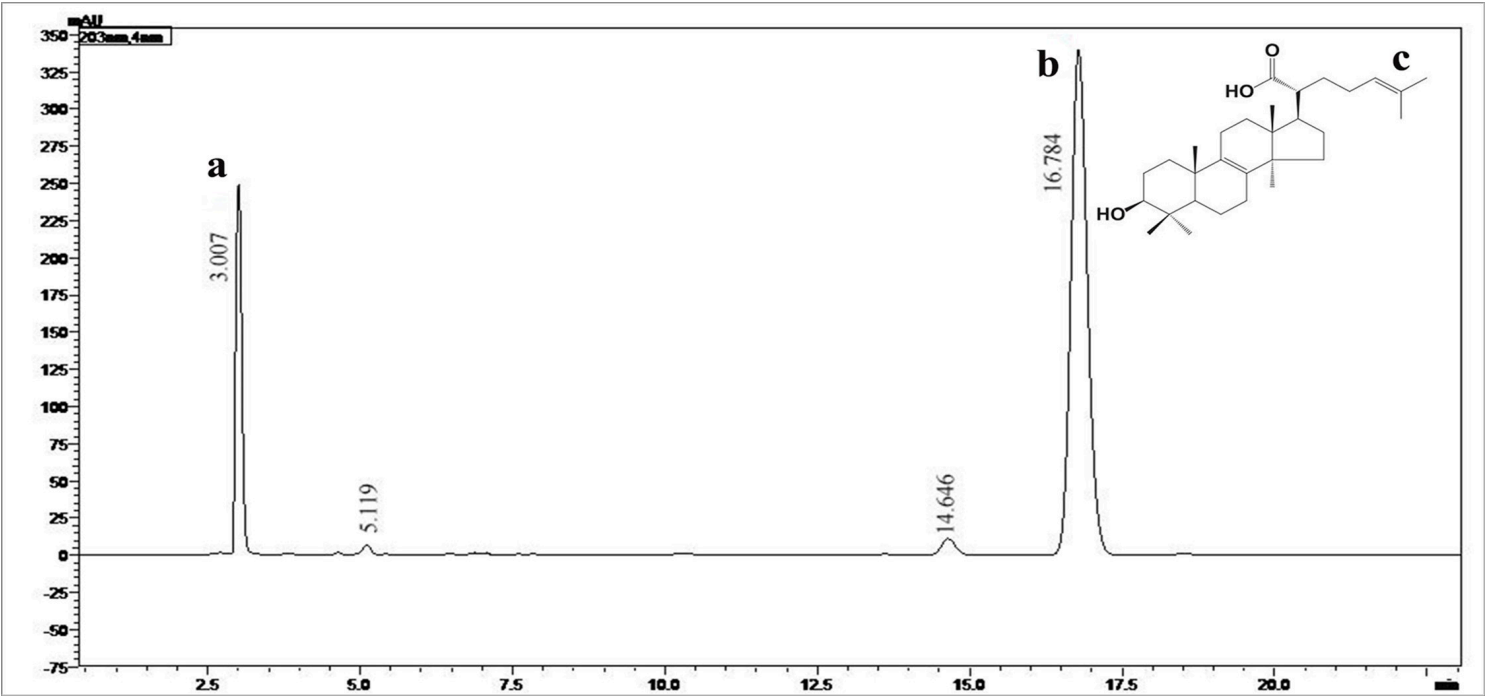


Figure 2

Statistical map of functional annotations of genes in each database of transcripts. **a** GO classification annotations. **b** KEGG classification annotations.

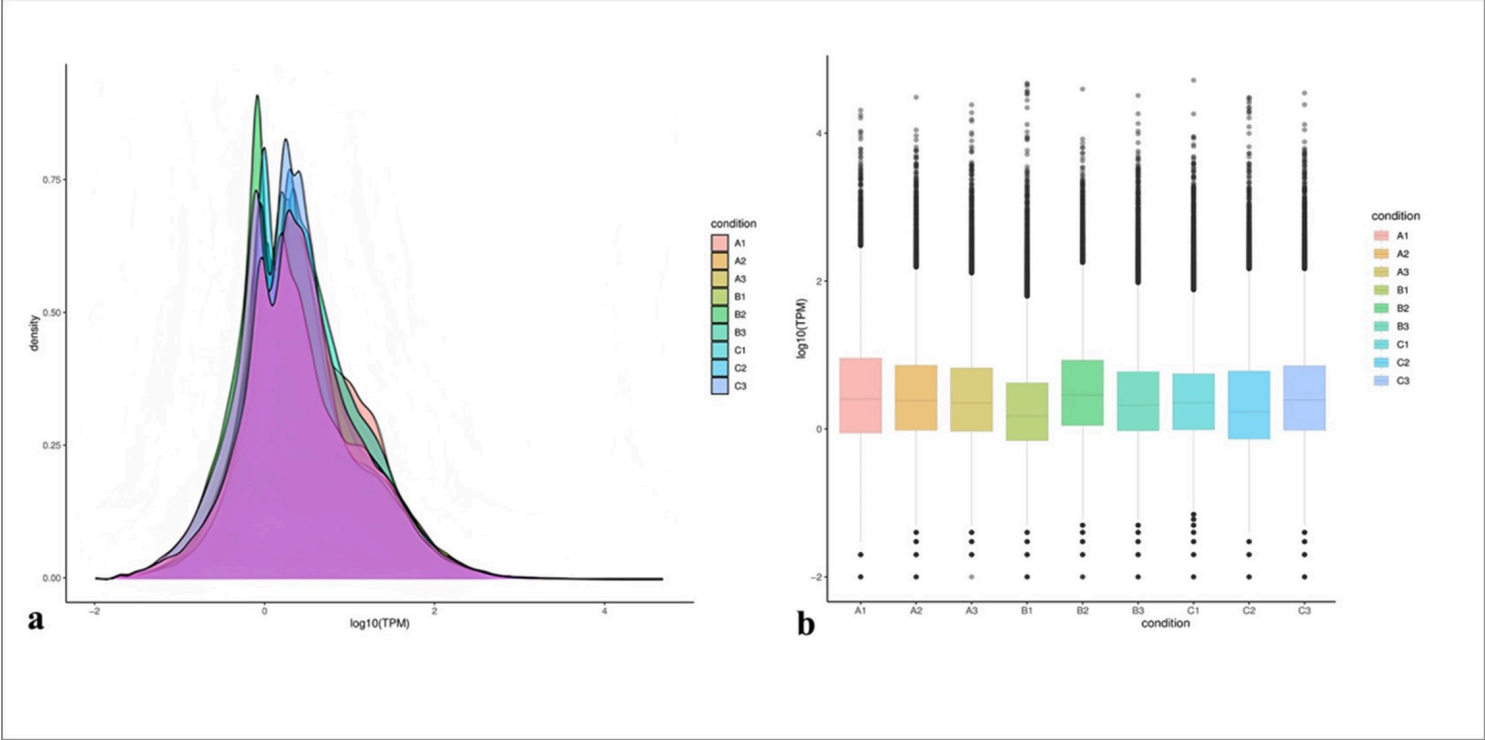


Figure 3

Transcriptome sample density distribution and box-and-line plots. **a** Sample density distribution. **b** Box-and-line plots.

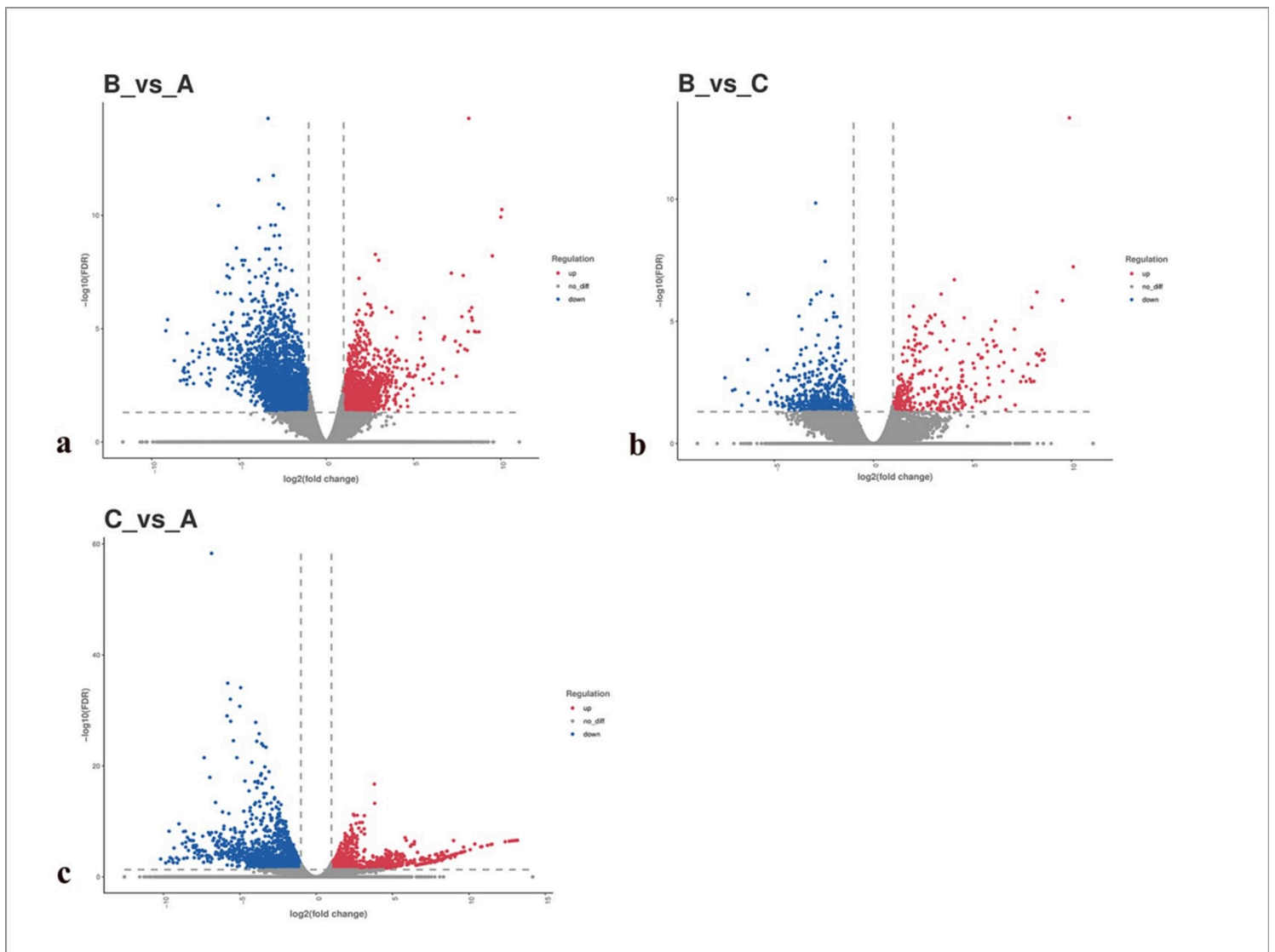


Figure 4

Volcano plot of DEGs among groups. Where $\log_2(\text{fold change})$ is the horizontal coordinate, representing the differential expression fold change of genes in different samples; $-\log_{10}(\text{FDR})$ is the vertical coordinate, representing the statistical significance of the difference in gene expression change.

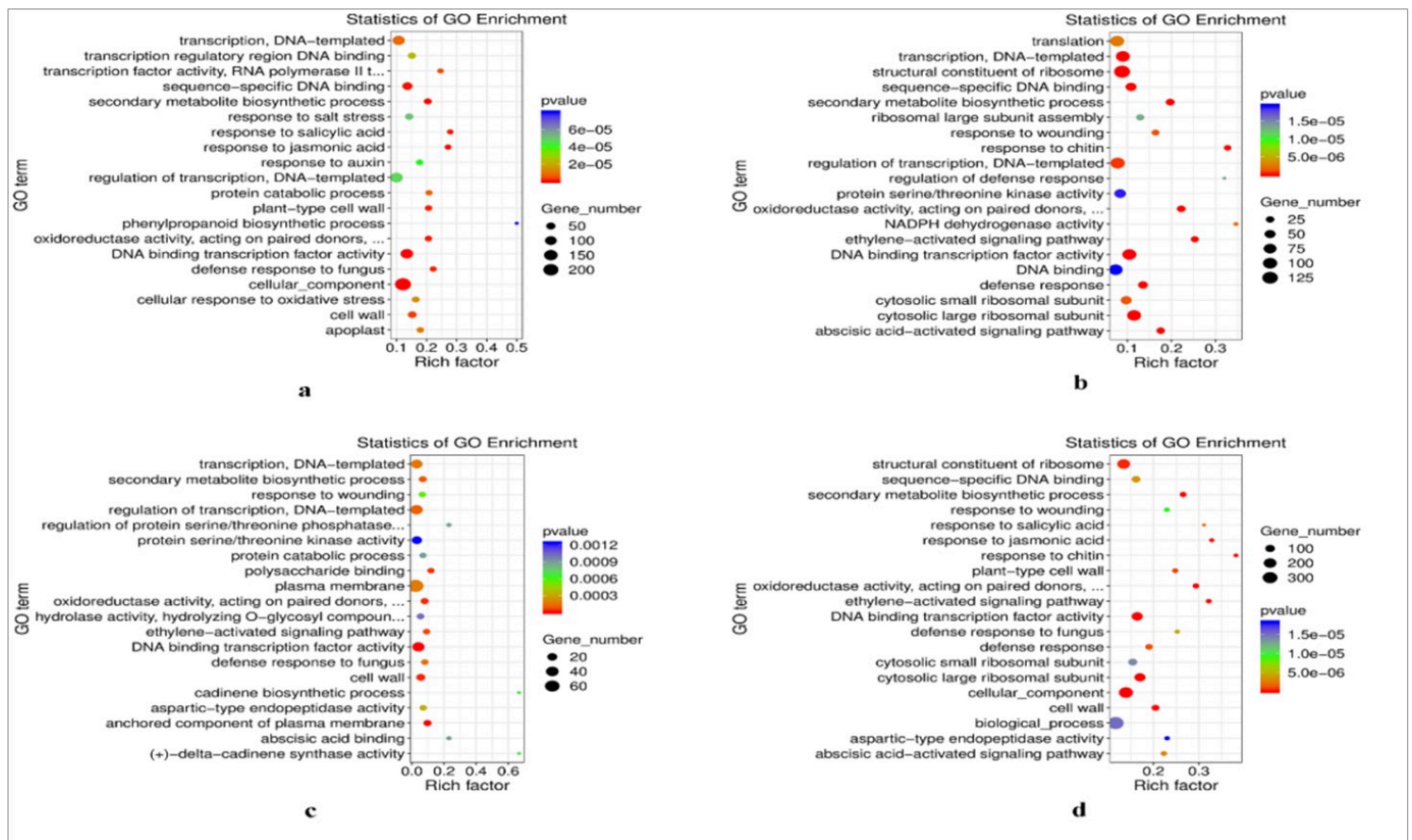


Figure 5

Scatterplot of GO enrichment of differential genes among groups. rich factor indicates the number of differential genes located in the GO/total number of genes located in the GO, the larger the rich factor, the higher the GO enrichment. **a** B_vs_A. **b** C_vs_A. **c** B_vs_C. **d** A_vs_B_vs_C.

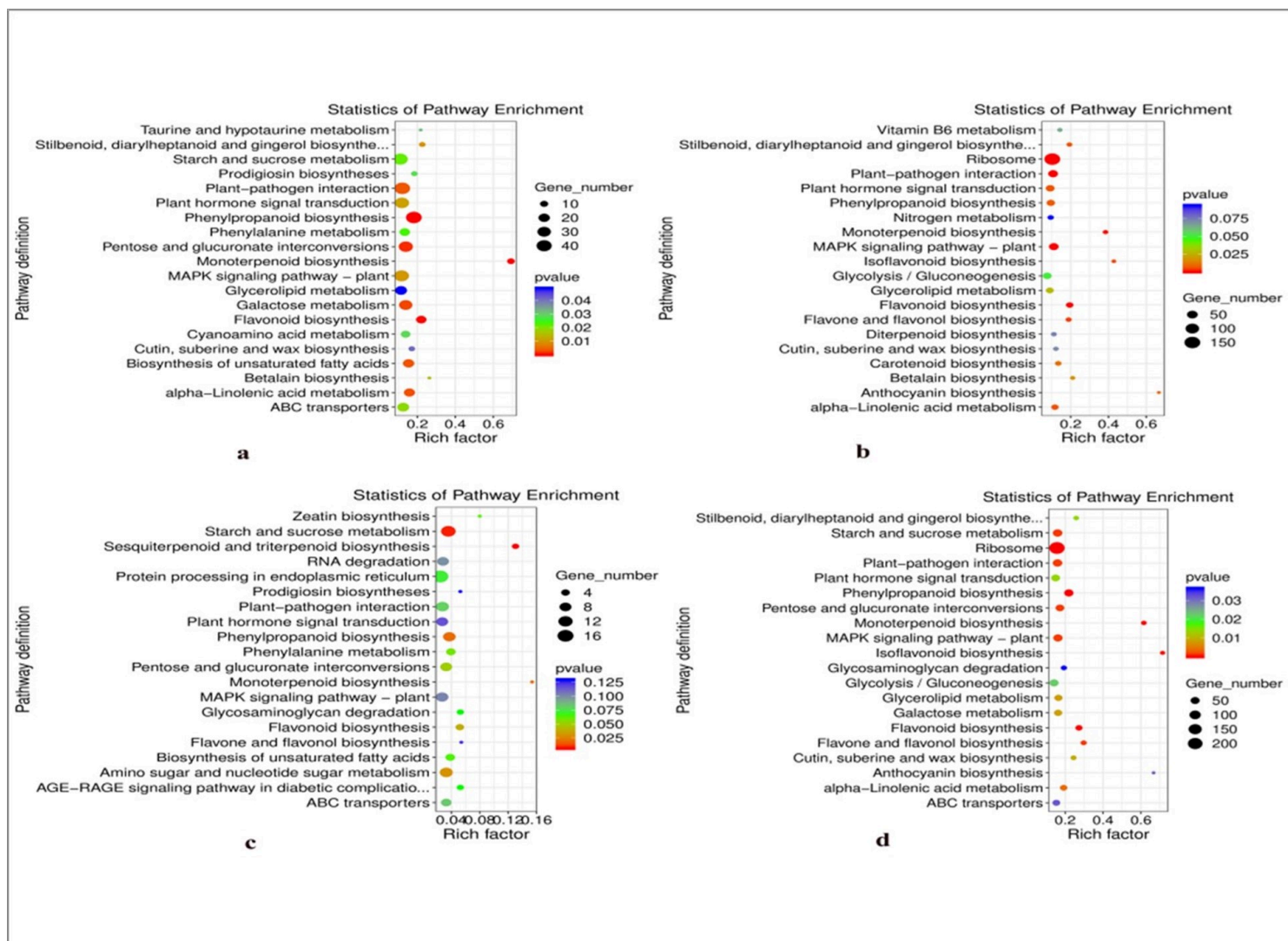


Figure 6

Scatter of KEGG enrichment of differential genes among groups. richFactor indicates the number of differential genes located in that KEGG/the total number of genes located in that KEGG, and the larger the RichFactor value, the greater the KEGG enrichment. **a** B_vs_A. **b** C_vs_A. **c** B_vs_C. **d** A_vs_B_vs_C.

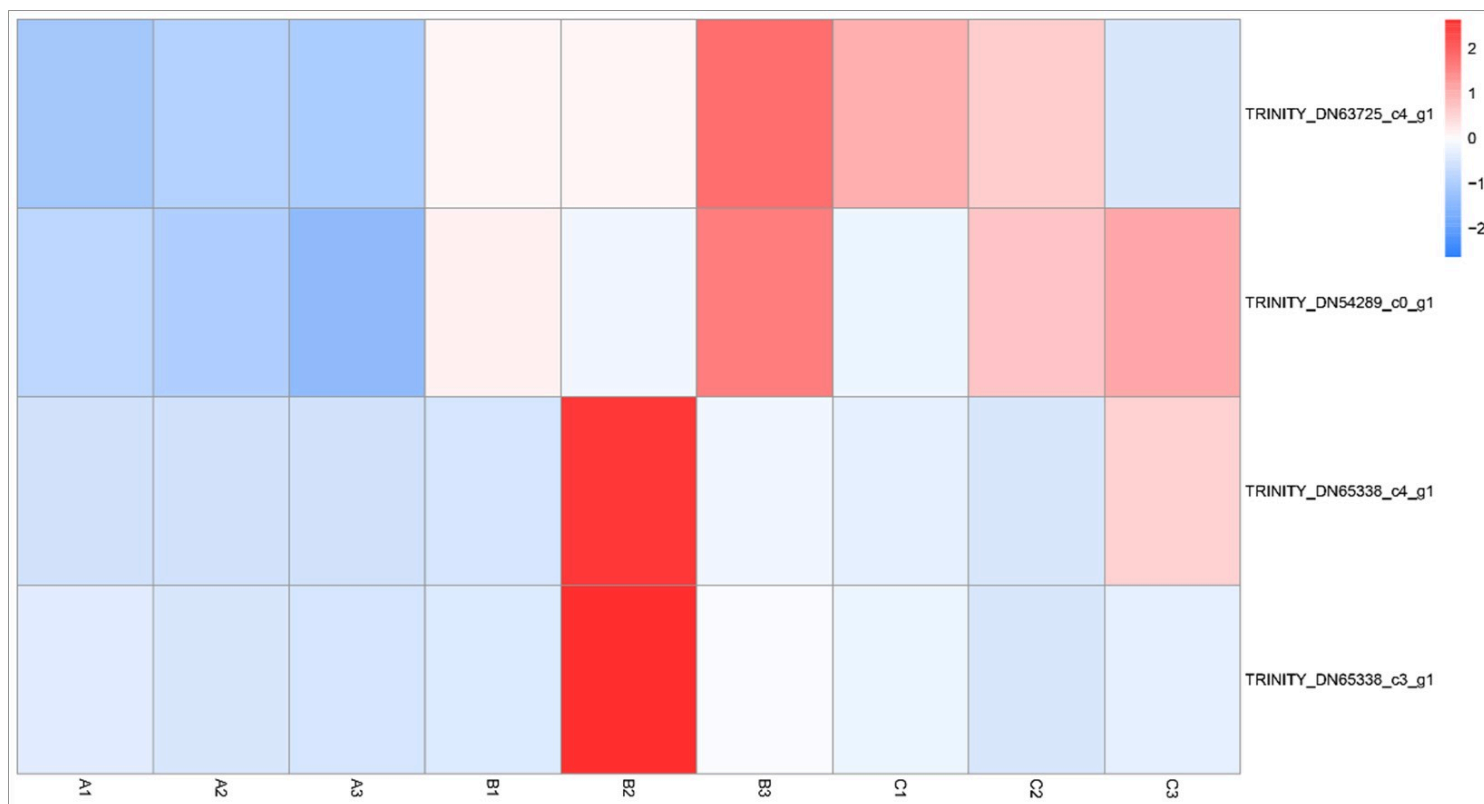


Figure 7

Heat map of gene expression of differential genes screened in terms of plant-pathogen interactions. Where the horizontal coordinates are samples and the vertical coordinates are genes, different colors indicate different gene expression levels, and blue to red indicates an increase in gene expression levels.

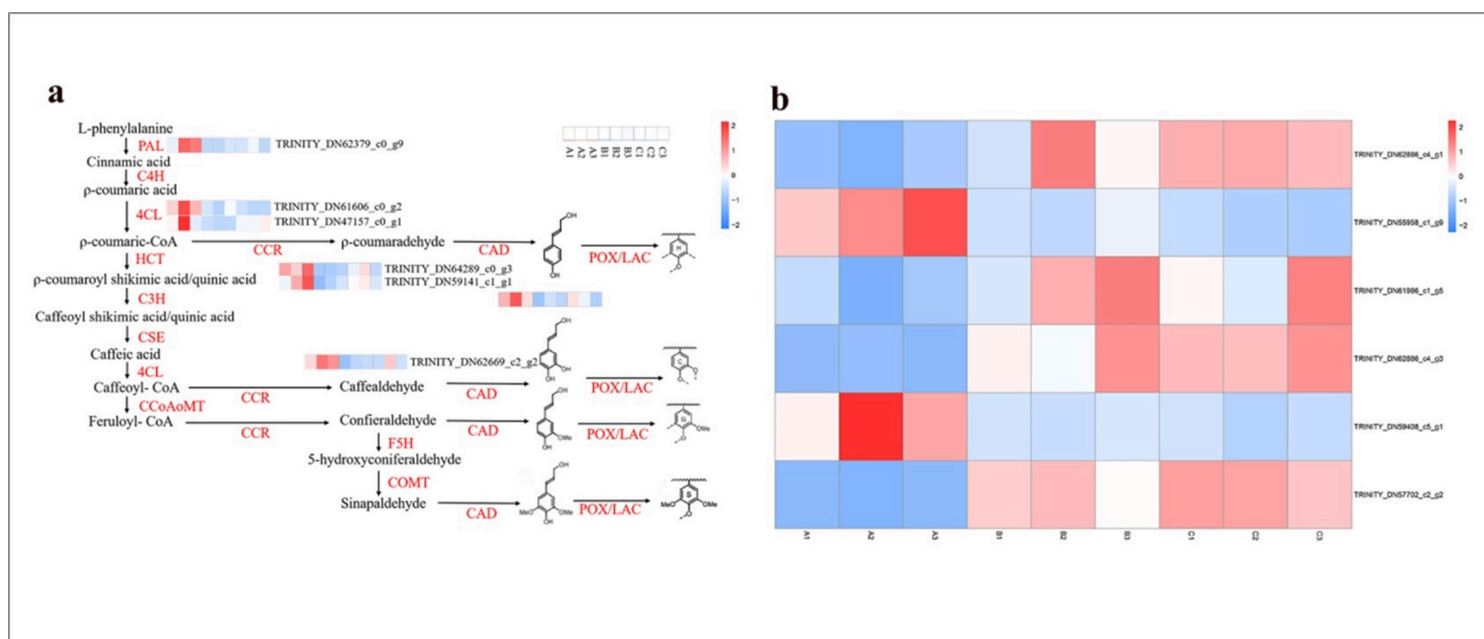


Figure 8

Heat map of differential gene expression related to lignin synthesis pathway and transcription factors regulating lignin synthesis. Where the horizontal coordinates are samples and the vertical coordinates are genes, different colors indicate different gene expression levels, and blue to red indicates the increase of gene expression levels. a Lignin synthesis pathway. b Heat map of differential gene expression related to transcription factors regulating lignin synthesis.

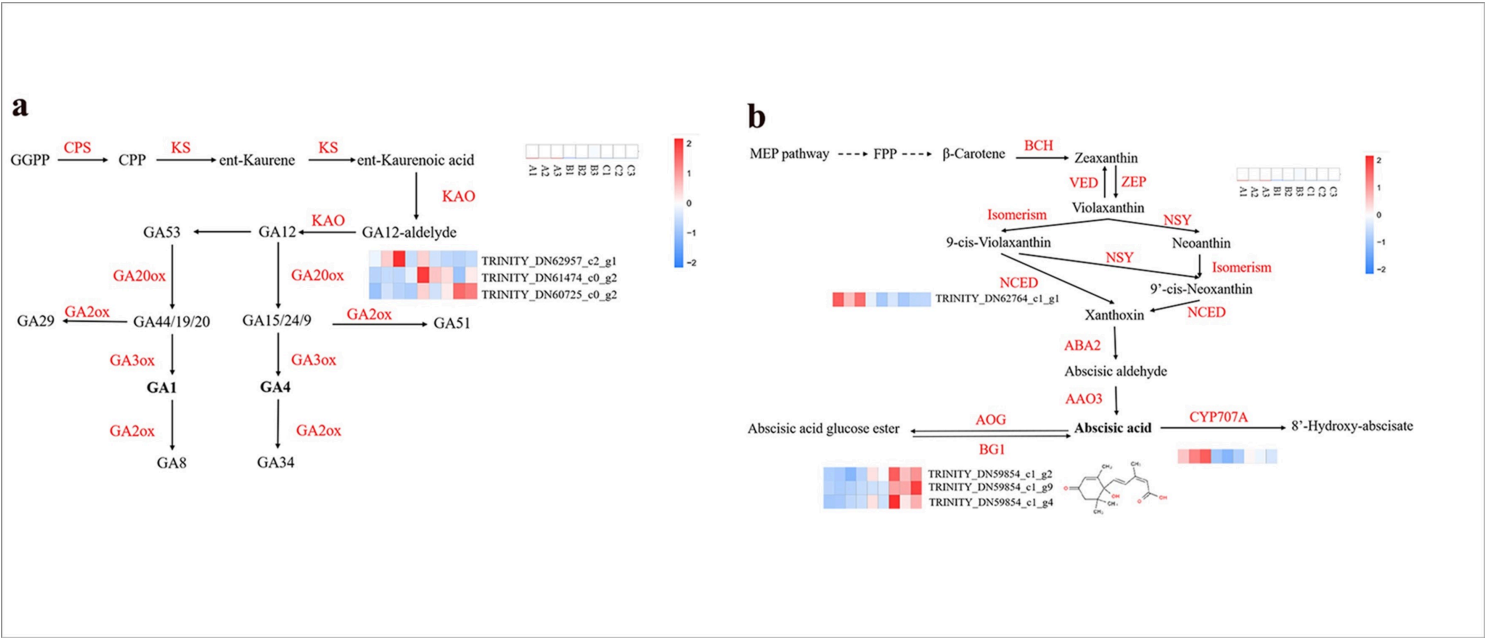


Figure 9

GA and ABA anabolic pathways. The differential gene TPM will be demonstrated by Z value way of gene expression. Where the horizontal coordinate is the sample and the vertical coordinate is the gene, different colors indicate different gene expression levels, and from blue to red indicates the increase of gene expression level. a GA anabolic pathway. b ABA anabolic pathway.

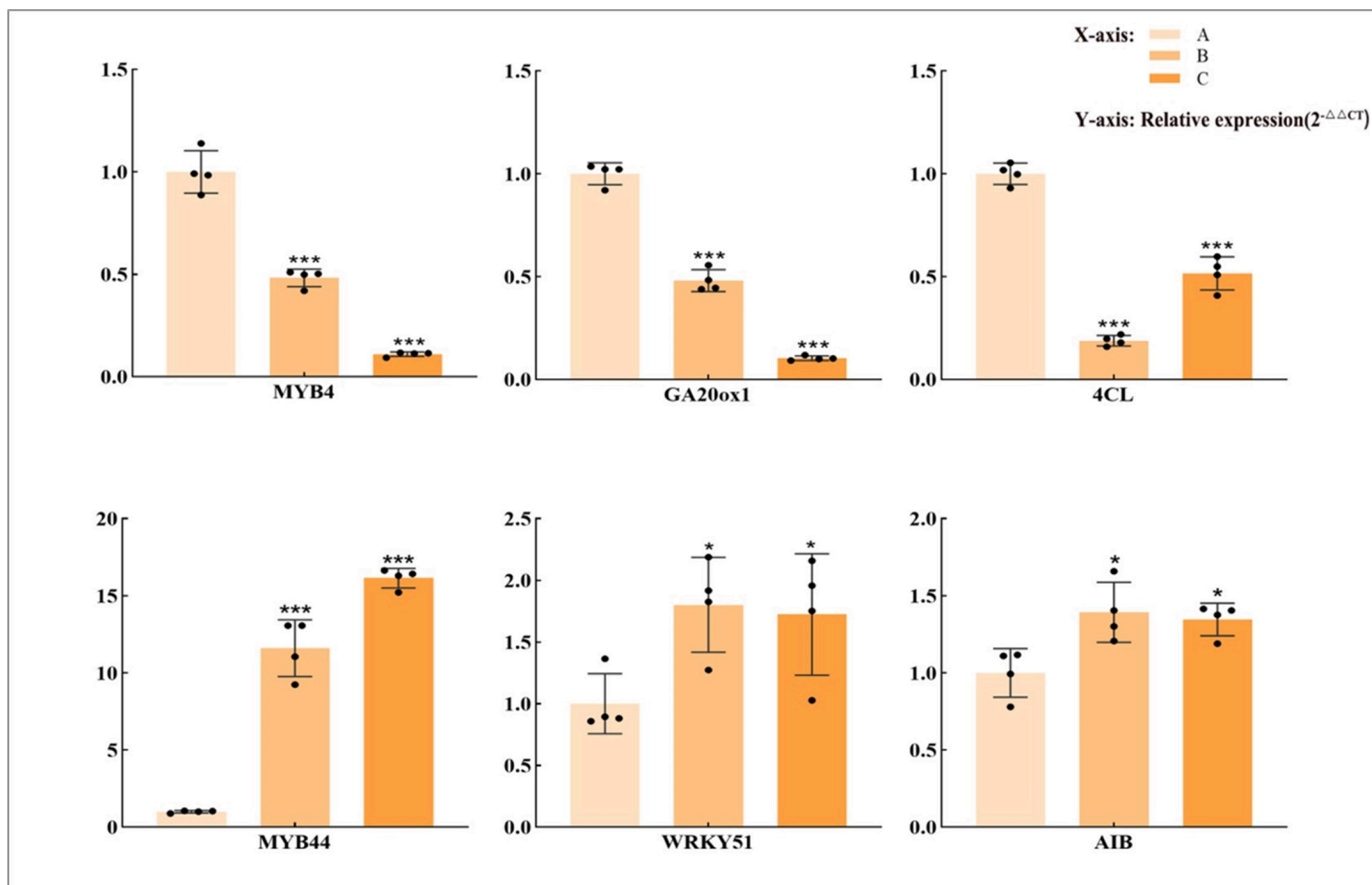


Figure 10

qRT-PCR analysis of the expression of differential genes in GEB under TA treatment. ^{***} indicates significant difference compared with group A, $p^* < 0.05$, $p^{**} < 0.01$, $p^{***} < 0.001$. ^{***} indicates the repeated data of each group.

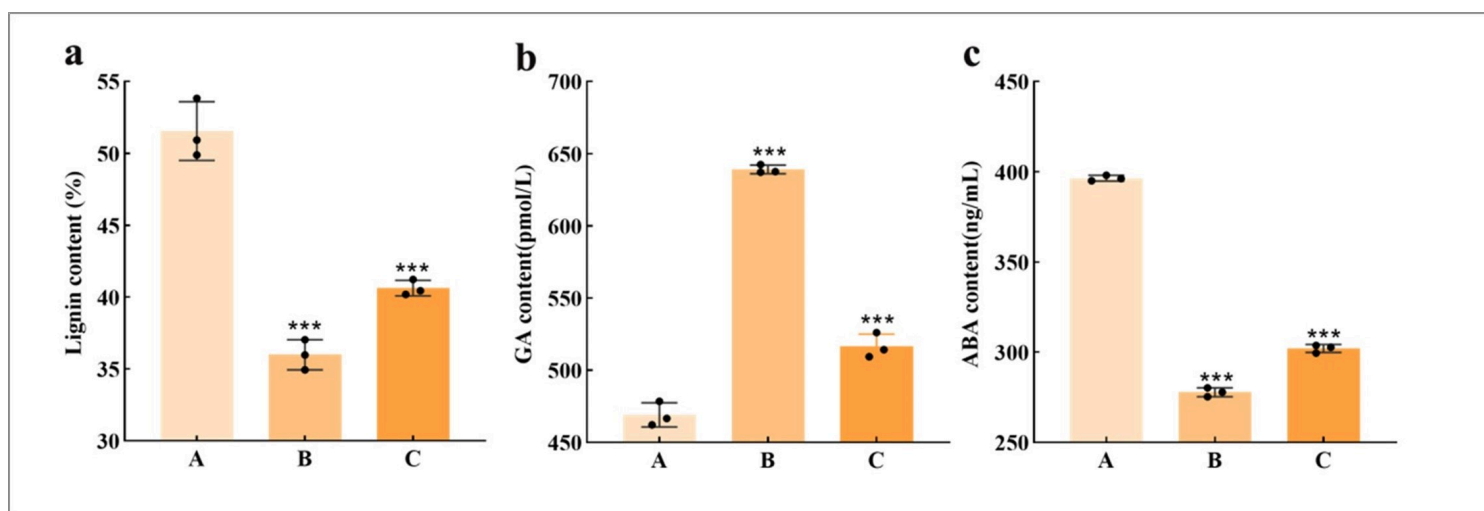


Figure 11

Comparison of physiological indexes during germination of GEB seeds under TA. **a** Comparison of lignin content in GEB seeds. **b** Comparison of GA content in GEB seeds. **c** Comparison of ABA content in GEB seeds. Data are expressed as “mean $\pm$ standard” deviation ($\pm$ s) with three replications (n = 3). “*” indicates significant difference compared with group A. $p^* < 0.05$, $p^{**} < 0.01$, $p^{***} < 0.001$. “.” indicates the repeated data of each group.

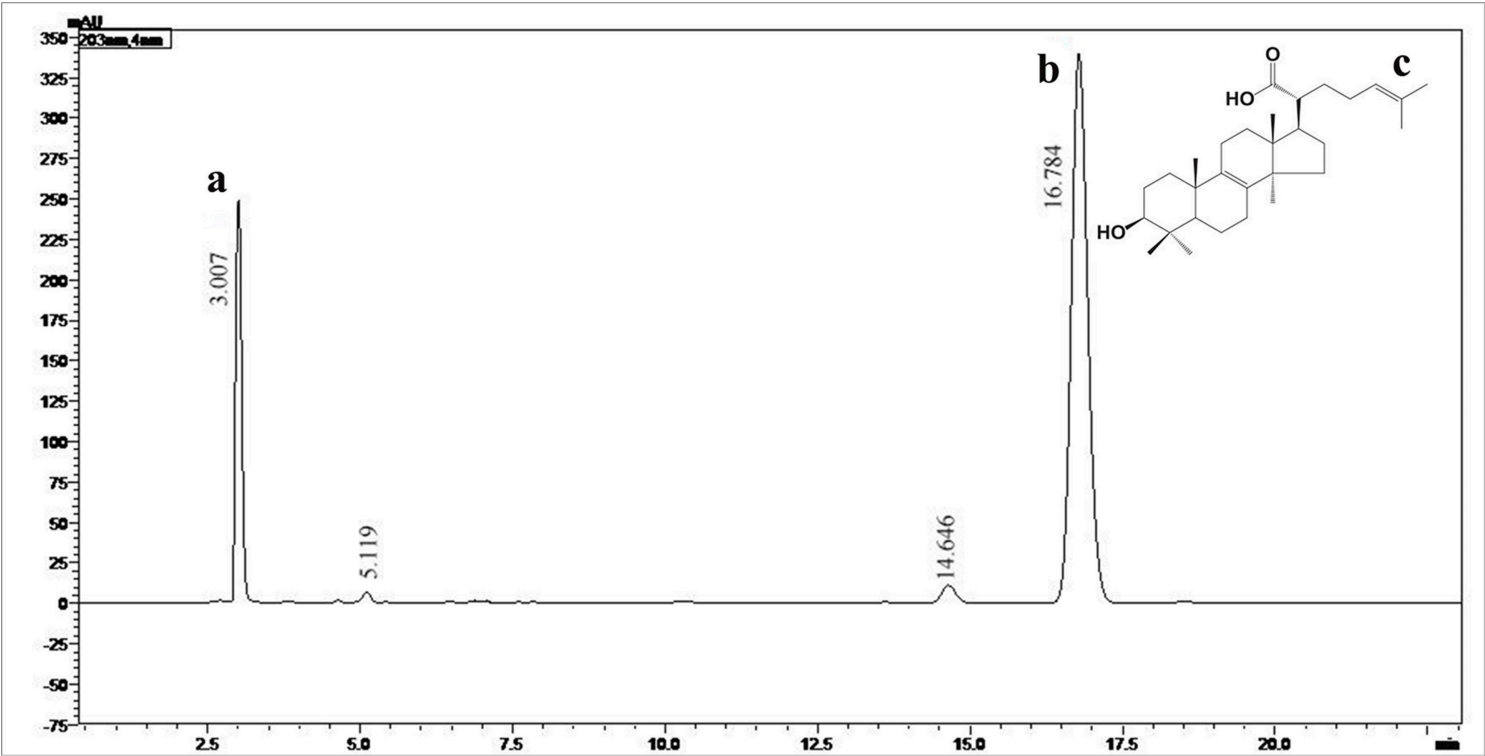


Figure 12

TA high performance liquid chromatogram and molecular structure. **a** Solvent peak. **b** TA peak. **c** TA molecular formula.