



Transcriptomic Analysis Reveals That Exogenous Indole-3-Butyric Acid Affects the Rooting Process During Stem Segment Culturing of *Cinnamomum camphora* Linalool Type

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Abstract

Cinnamomum camphora linalool type (*C. camphora* linalool type) is an economically important plant that contains abundant linalool. To explore the optimal concentration of exogenously applied indole-3-butyric acid (IBA) for root formation and development, morphological analyses were performed with plants after a 1-min soaking treatment with five different concentrations of IBA. When grown on the medium (laterite with plant ash) for 60 days after treatment with 3000 mg/L IBA, the root ratio and the number of roots were significantly higher than those obtained with 0, 1000, 2000, or 4000 mg/L IBA treatment. After soaking with 3000 mg/L IBA, the stem bases at five key stages of root development (0, 14, 21, 28, and 42 days) were selected for global transcriptome analyses. IBA treatment modified the expression of genes related to the auxin, brassinosteroid, and abscisic acid signalling transduction pathways. The rooting process reduced the accumulation of starch and the synthesis of linalool but promoted the metabolism of D-glucose. Our study identified the optimum concentration of IBA for a short treatment of *C. camphora* linalool type stem segments to regulate root formation and development at the transcriptional level.

Keywords *Cinnamomum camphora* linalool type · Rooting · Transcriptomics analysis · Linalool · IBA

Introduction

Cinnamomum camphora (Linn.) Presl (*C. camphora*) is one of the oldest herbal medicines used as in traditional medicine (Chen et al. 2020). It can be divided into the isoborneol, camphora, cineole, linalool, and borneol types according to the composition of the essential oils of the branches and leaves (Guo et al. 2017). Most of these types have been used

in biomedicines, spices, cosmetics, and forest planting (Chen et al. 2018; Liu et al. 2019; Wu et al. 2019).

Cinnamomum camphora var. *linaloolifera* Fujita is the linalool-type *C. camphora* and is an evergreen broadleaved tree with the advantages of rapid growth, dense branches, and straight trunks. It is also an excellent urban green-tree species in southern China and is cultivated as an economically important tree for its medicinal and aromatic properties (Chen et al. 2017; Li et al. 2018). The volatile oil, based on the GC–MS analysis, in *C. camphora* linalool type leaves is mainly composed of linalool (60.97%, the highest in all *C. camphora*), camphor (7.13%), caryophyllene (5.17%), and eucalyptus oleol (3.8%) (Guo et al. 2016; Xinyu Fu et al. 2016; Wang et al. 2019). Linalool extracted from the leaves, twigs, and seeds shows insecticidal and repellent activities against cotton aphid (Jiang et al. 2016), *Schistosomiasis japonicum* (Yang et al. 2014), *Pseudomonas aeruginosa*, and *Escherichia coli* (Wang et al. 2019). With the increasing interest in naturally occurring essential oils with insecticidal and insect repellent activity, including linalool, *C. camphora* linalool type has gained the attention of researchers.

Key Message

- Exogenous IBA modifies carbohydrate secondary metabolism and the auxin and BR and ABA signalling transduction pathways to assist the regulation of the rooting and stress response of *Cinnamomum camphora* linalool type

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At present, cultivation of *C. camphora* plants is mainly through cutting, grafting, and tissue culture seedlings (Wu Youmei et al. 2009). Previous research found that the growth and biomass of tissue culture seedlings were higher than that of cutting seedlings and grafting seedlings, with more adventitious roots with high quality. And the number of thick roots of cuttings was less and the quality of adventitious roots was lower than that of tissue culture seedlings (Yue-Qiu and Wang 2015). Cutting seedlings also has the disadvantages of low survival rates, slow growth rates and other limitations to rapid propagation without any treatment with exogenous phytohormones (over 50 days for the annual branches on May) (Guorong Huang 2020). Auxin, abscisic acid (ABA), brassinosteroid (BRs), and other phytohormones play important roles in the rooting process of *C. camphora* linalool type and show a dynamic equilibrium (Lal et al. 2007; Du et al. 2012; Gonzalez et al. 2015; Jamro et al. 2018). ABA plays a crucial role in root architecture (Dong et al. 2018), while auxin plays a key role in lateral root formation (Xie et al. 2000) and BRs determine the root hair specification by regulating the fate of the root epidermal cell (Cheng et al. 2014). Considering that the cost and it is difficult to popularize tissue culture on a

large scale, exogenous application of plant hormone (such as IBA) is the most suitable way to address disadvantages of traditional cutting method.

In this study, stem segments of the annual shoot were used for morphological analysis to identify the optimal exogenous concentration (3000 mg/L) of β -oxidation of indole-3-butyric acid (IBA) to maximize the rooting potential of *C. camphora* linalool type. The rooting process of stems after the treatment of 3000 mg/L IBA was observed for 6 weeks, and five root development key nodes (0, 14, 21, 28, and 42 days) were selected for the determination of the transcriptome. According to the transcriptome analysis results, auxin and BR signalling were found to be involved in the regulation of root development, linalool biosynthesis, and carbohydrate metabolism. RT-PCR of core DEGs and the content determination of linalool, starch, and soluble sugar were also performed to provide further evidences. We propose that a combination of the metabolic regulation of core substances and the signal transduction of plant hormones provides the molecular basis for the development of *C. camphora* linalool type roots and rapid seeding.



Fig. 1 Root development under the different concentration of IBA. **A** Phenotypes of the stem sections before and 60 days after a 1-min soaking treatment of 0, 1000, 2000, 3000, and 4000 mg/L IBA solutions. White bar=2 cm. **B** Root ratio, number of root, root length, and root diameter after a 60-day stem section cultures after a 1-min soaking treatment of 0, 1000, 2000, 3000, and 4000 mg/L IBA. **C** Phenotypes of the stem sections with a 0-day, 7-day, 14-day, 21-day,

28-day, 35-day, and 42-day stem section cultures after the treatment of 3000 mg/L IBA. The 0-day, 14-day, 21-day, 28-day, and 42-day plants were chosen and named as F1 to F5 respectively. White bar=2 cm. All bars represent means $\pm$ s.d, and those labelled with a letter are significantly different at $P < 0.05$ by Duncan's test. Three biological replicates in the experiment were performed, and each biological replicate consists of 450 stems

Results

The Optimum Concentration of Exogenous IBA in Stem Segment Culture

To explore the optimum concentration of exogenous IBA in the stem segment culture of *C. camphora* linalool type, different concentrations of IBA solutions (1000, 2000, 3000, and 4000 mg/L) were used to soak the stem bases for 1 min, which were then grown on culture medium (laterite with plant ash) for 60 days (Fig. 1A). The root ratio of the stem segments treated with IBA was significantly higher than that of the control group (0 mg/L). The

highest rooting rates were found after 3000 mg/L IBA treatment ($67.67 \pm 3.52\%$). The root number of stem segments after the treatment with 3000 mg/L IBA was also the highest (3.27 ± 0.08), followed by 2000 mg/L IBA, and all of them were significantly higher than that of control. The root length of stem segments after treatment with 4000 mg/L IBA was the longest (4.20 ± 0.87 cm) but showed no significant difference from that of 3000 mg/L IBA (3.89 ± 0.43 cm). There were no significant differences in root diameters among the treatments (Fig. 1B). In summary, 3000 mg/L IBA is the optimum concentration to promote the rooting of *C. camphora* linalool type stem segments.

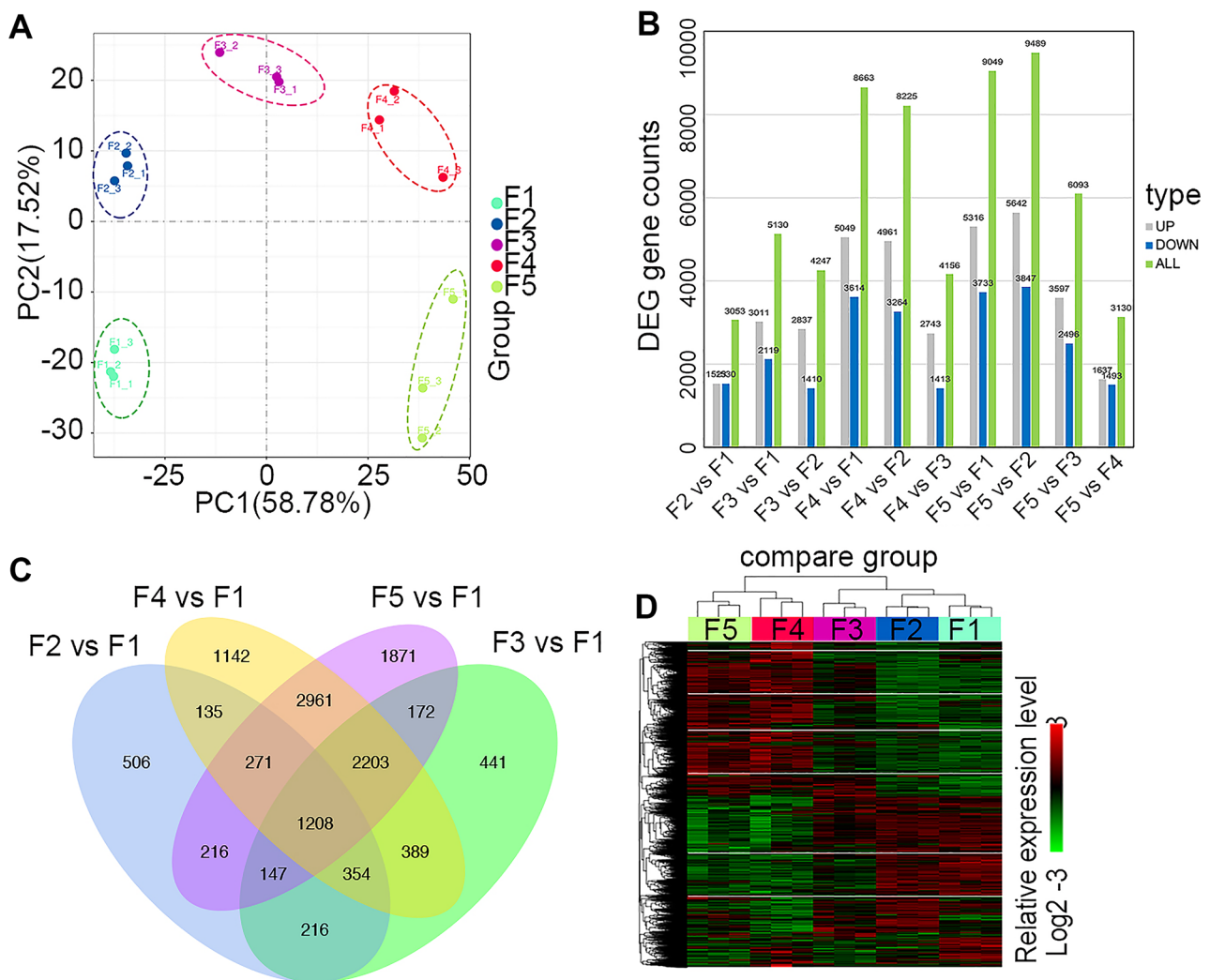


Fig. 2 Overall differentially expressed genes (DEGs) during the stem segment culturing. **A** Principal component analysis (PCA) of differentially expressed genes ($\log_2$ (relative expression level) ≥ 1 and ≤ -1 , $P < 0.05$, DEGs) in F1, F2, F3, F4, and F5. **B** The histogram of the statistical results for the DEGs in 5 groups. The blue for down-expressed genes and the gray for up-expressed genes. **C** Venn

diagram of the overlap between the DEGs between F2 and F1, F3 and F2, F4 and F3, F5 and F4. **D** The clustering analysis results of the relative expression levels of DEGs between five different groups. The abscissa is the sample name, and the ordinate is the normalized value of the differential gene FPKM. The redder the colour, the higher the expression level. The more green the colour, the lower the expression

Phenotypes of the Stem Segment Base

The phenotypes of the stem sections after 0, 7, 14, 21, 28, 35, and 42 days of stem section culture after treatment with 3000 mg/L IBA are shown in Fig. 1C. There were no obvious morphological changes at the base of the cuttings from the first week, and the cuttings healed slowly in the second week. The incision swelling was more obvious during the third week and more callus appeared at the base. The callus area was enlarged in the fourth week. A large number of adventitious roots appeared after 6 weeks of culture accompanied by fibrous roots. Considering that the base of the stem is the part of direct contact and absorption of exogenous IBA, and is the active and core area of cell differentiation during rooting, the base of the cuttings at five time points (0, 14, 21, 28, and 42 days) representing the major stages in root development was chosen for global transcriptome analysis and were respectively named F1, F2, F3, F4, and F5.

Differentially Expressed Genes During Root Development

To understand the molecular mechanism and regulation networks of *C. camphora* linalool type during its root developmental periods, differentially expressed genes (DEGs; $\log_2$ (relative expression level) ≥ 1 and ≤ -1 , $p \leq 0.05$) in F2, F3, F4, and F5 compared with F1 were identified by RNA-Seq (Supplemental Tables S1, S2, S3 and S4). Principal component analysis (PCA) of the DEGs revealed the separation of gene expression profiles among F1 to F5 was different from each other. The distribution of the three biological replicates was aggregated (Fig. 2A). As summarised in Fig. 2B, compared with F1, 1523 up- and 1530 down-regulated genes were observed in F2, 3011 up- and 2119 down-regulated genes were observed in F3 compared with F1, 5049 up- and 3614 down-regulated genes were observed in F4 against F1, and 5316 up- and 3733 down-regulated genes were observed in F5 compared with F1 (Fig. 2B). When comparing the DEGs of different groups, 1208 genes were consistently up- or down-regulated during the developmental periods and had a large number of specifically expressed genes (Fig. 2C). The clustering analysis of the relative expression levels of all of the DEGs (union of 10 comparative combinations in Fig. 2B) is shown in Fig. 2D.

Gene ontology (GO) enrichment analyses were performed with the DEGs in F2 to F5 compared with F1 (Fig. 3A). The up-regulated genes were enriched in the metabolism of cellular glucan and polysaccharides, as well as the biosynthesis of cellular carbohydrate and ATP, especially in response to external stimuli (such as pathogenesis-related protein CKAN_01791200 / CKAN_01791000) and oxidative stress (such as

peroxidase CKAN_00579200/CKAN_00756800) (Fig. 3A, red dotted box). Down-regulated genes were enriched in the metabolism of coenzymes, cofactors and nucleotides (Fig. 3A, green dotted box), glycolysis, and the response to chemicals. Each pair of genes (red and green points in Fig. 3A) with significant changes in the expression of pathogenesis-related protein and peroxidase families were selected for real-time RT-PCR analysis. The time-specific expression assay results (Fig. 3B) are in agreement with the RNA-seq results. The activity of peroxidase enzyme (POD) was consistent with the expression trend (Fig. 3C). The KEGG pathway enrichment assay was also performed with these DEGs, and both the up- and down-regulated genes were enriched in starch and sucrose metabolism, while the up-regulated genes were specifically enriched in plant hormone signal transduction and the down-regulated genes were specifically enriched in linalool biosynthesis (Fig. 3C, orange or blue squares, respectively, for the up- and down-regulated genes).

Transcriptome Differences in Plant Hormone Signal Transduction Pathways During Root Development

Both the GO and KEGG pathway of DEGs were significantly enriched in the response to extremal stimuli and plant hormone signal transduction (Figs. 4A and C). By comparison, auxin and brassinosteroid (BR) were the two most altered hormone signalling cascades, as demonstrated by the following: down-regulated expression of eight *AUX/IAA* (auxin-responsive protein) genes; increased expression of auxin response genes, including one *ARF* (Arf GTPase activating protein), two *GH3* (beta-glucosidase), and six *SAUR*-like auxin-responsive family genes; increased expression of five *BR11* (*BRASSINOSTEROID INSENSITIVE 1*) genes, three *BZR1/2* (*BRASSINAZOLE-RESISTANT 1/2*) genes, one *CYCD3* (*cyclin D-type protein 3*) gene, and five *TCH4* (*xyloglucan:xyloglucosyl transferase 4*) genes. Concomitantly, each pair of genes with significant changes in the expression of *AUX/IAA* and *SAUR*, *BR11*, *BZR1/2*, and *TCH4* family members were selected for real-time RT-PCR analysis. The time-specific expression assay results (Fig. 4B) were consistent with the RNA-seq results.

During the formation of adventitious roots, there are contrasting changes in the amount of endogenous IAA and ABA in the roots. The expression of the ABA signalling pathway transduction genes in the transcriptome data conforms to this, as demonstrated by the down-regulated expressions of four *PYR/PYL* (*REGULATORY COMPONENT OF ABA RECEPTOR*) genes and four *ABF* (*ABA responsive element binding factor*) genes, as well as the up-regulated expressions of nine *PP2C* (*type 2C protein phosphatases*) genes (Fig. 4C).

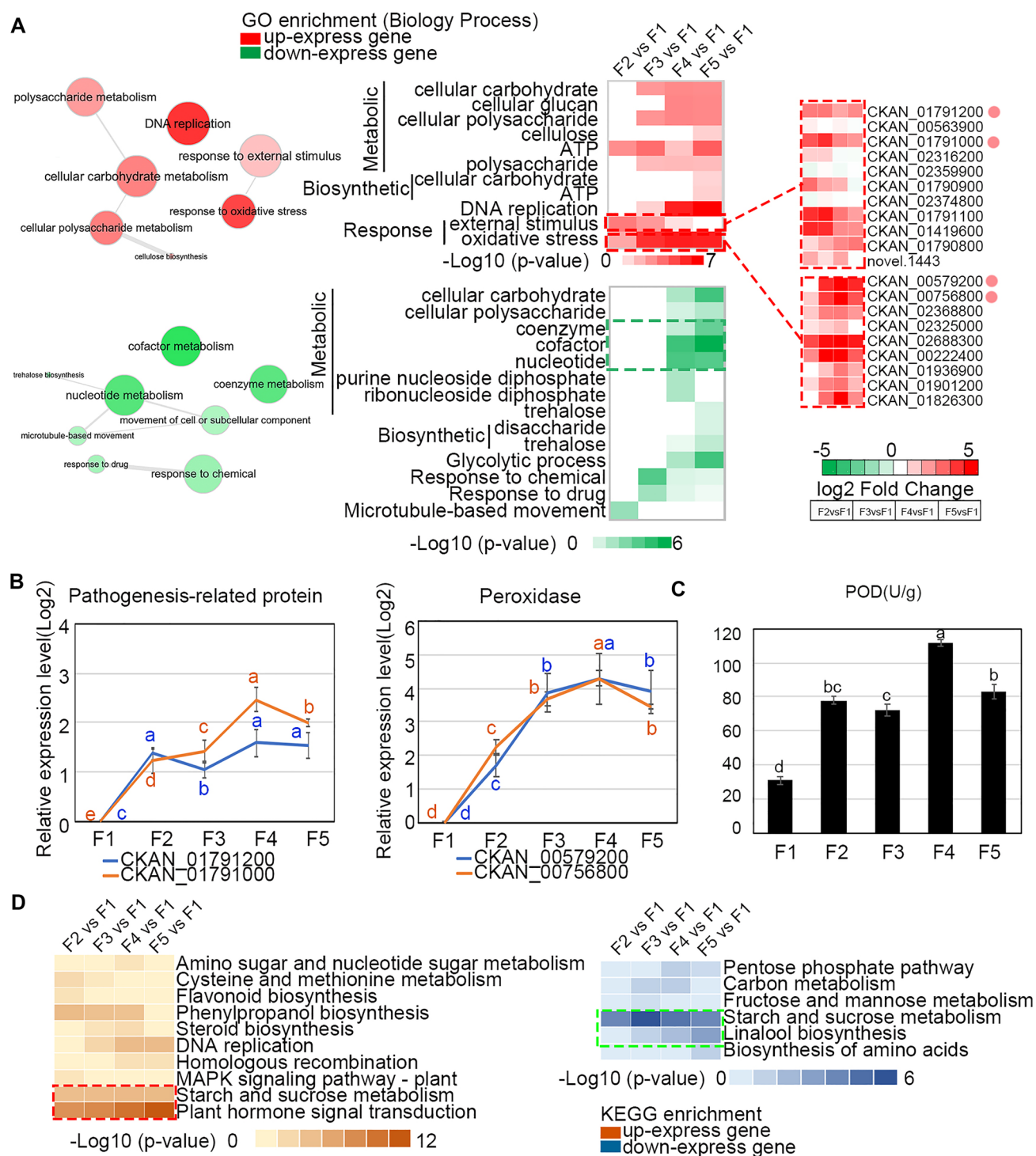


Fig. 3 GO and KEGG enrichment of the DEGs during the stem segment culturing. **A** GO (biological process) enrichment analysis (P -value for significance test between GeneRatio and BgRatio) clustering results and the interactive graph analysis results of the up-expressed (red) and down-expressed (green) gene of F2, F3, F4, and F5 in contrast to F1. The clustering analysis results of genes, which belonged to the response to external stimulus and oxidative stress, the metabolic of trehalose, relative expression levels of the different

groups based on the results of RNA-seq analysis. **B** Expression profiles of genes (real-time RT-PCR results) selected in **A** (red and green points). **C** The POD enzyme activity determination results. **D** KEGG pathway enrichment analysis clustering results of the up-expressed (orange) and down-expressed (blue) gene of F2, F3, F4, and F5 in contrast to F1. All bars represent means $\pm$ s.d. (three biological replicates, each biological replicate consists of 5 stems) and those labelled with a letter are significantly different at $P < 0.05$ by Duncan's test

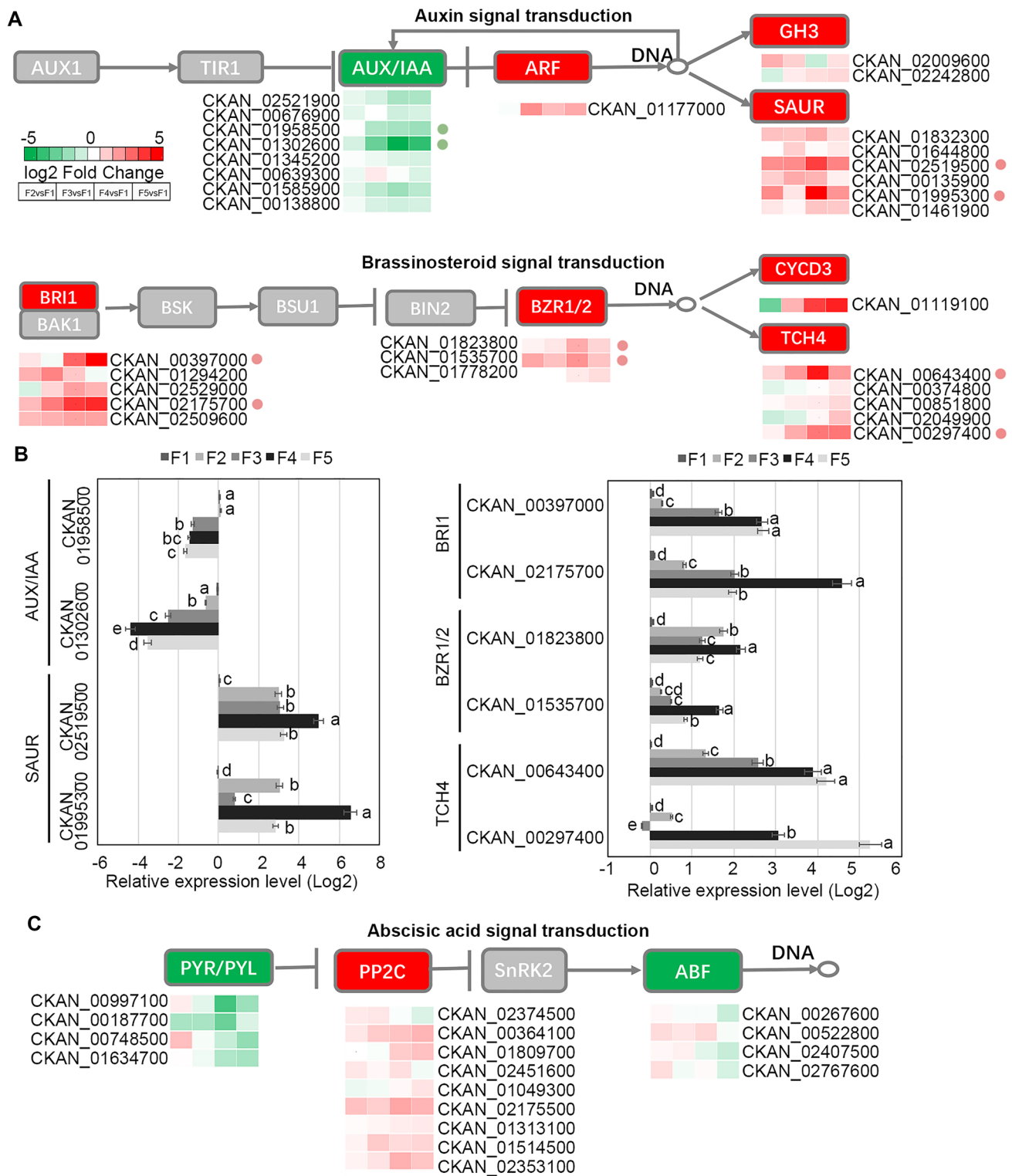


Fig. 4 Differential expression of representative genes involved in plant hormone signal transduction. **A** DEGs in auxin and brassinosteroid signal transduction pathway. **B** Expression profiles of genes involved in auxin and brassinosteroid signal transduction pathway from F1 to F5. **C** DEGs in abscisic acid signal transduction pathway. The absolute values of log₂ (F2, 3, 4, 5/F1) ≥ 1 and FDR < 0.001

were used as the criteria for DEGs. The colour of the box represents up (red) and down (green)-regulated genes. All bars represent means ± s.d (three biological replicates, each biological replicate consists of 5 stems) and those labelled with a letter are significantly different at $P < 0.05$ by Duncan's test

Expression Profiles of Carbohydrate Metabolism Pathway Genes

DEGs were significantly enriched in the linalool biosynthesis, as well as starch and sucrose metabolic pathways, and we, therefore, compared the expression trends of core genes in these pathways. Significant repression of genes involved in the linalool biosynthesis process was observed, including three (3S)-linalool synthases and five linalool 8-monoxygenases (Fig. 5A). Genes in the starch and sucrose metabolic pathway showed significant changes in expression during the culture (Fig. 5B). Genes encoding four sucrose synthases, three trehalose phosphatases, five glycosyl hydrolases, five β -glucosidases, four β -amylases, two starch synthase, one 4- α -glucanotransferase, and five glycosyltransferases

were significantly up-regulated in F2 to F5 compared with F1 (Fig. 5B). Genes encoding four sucrose synthases, one cell wall invertase, two fructokinases, two glucose-6-phosphate isomerases, and two phosphoglucomutases were repressed in F2 to F5 compared with F1 (Fig. 5B). Genes with significant changes in expression of (3S)-linalool synthase and linalool 8-monoxygenase, glycosyl hydrolase, β -glucosidase, 4- α -glucanotransferase, and glycosyltransferase families were also selected for real-time RT-PCR analysis to confirm the RNA-Seq assay results (Fig. 5C). The linalool, starch, and soluble sugar contents in stems during the five periods were determined after 12-h light exposure. The content of soluble sugars was increased, while the content of starch and linalool were decreased with the time of culture (Fig. 5D).

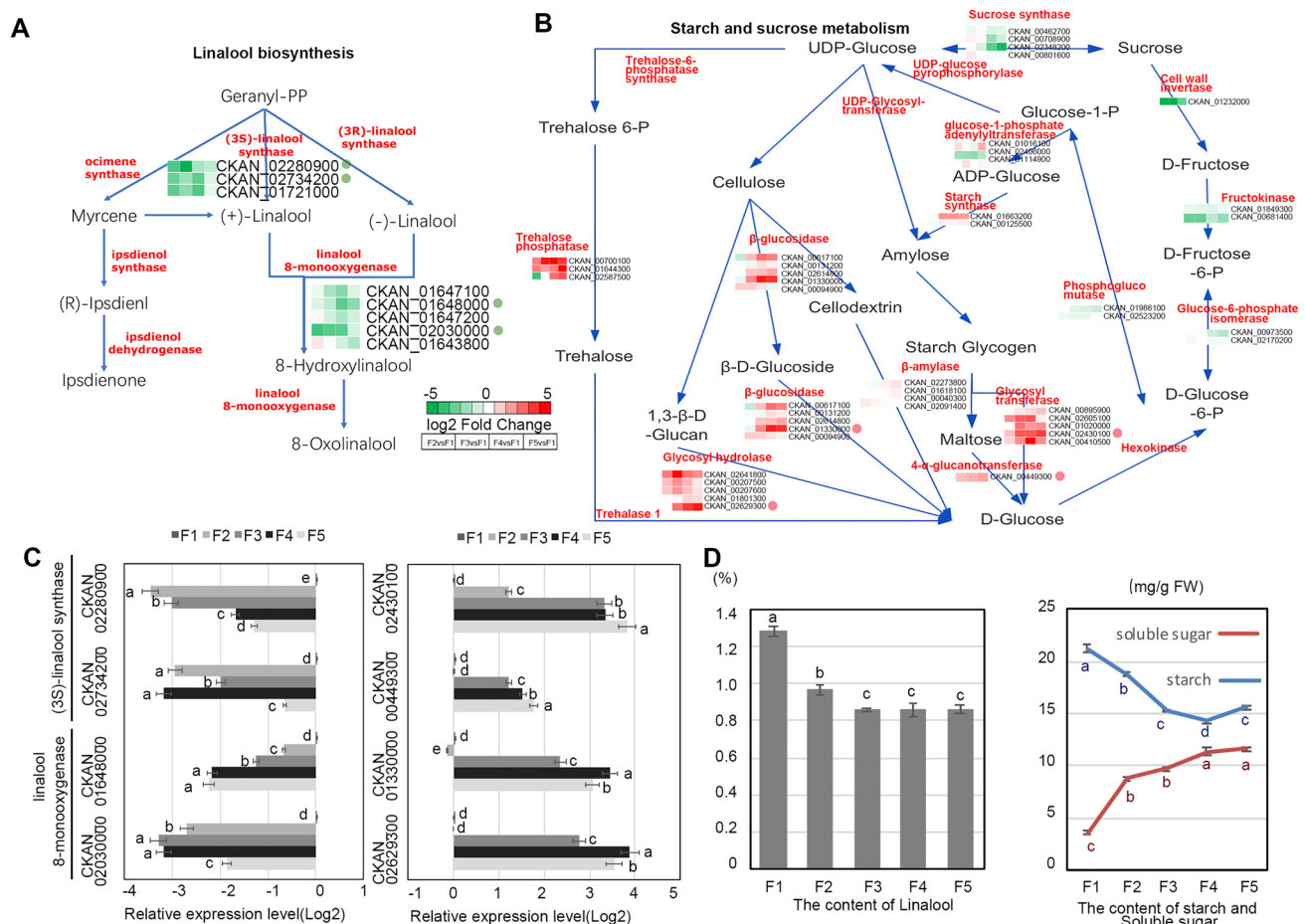


Fig. 5 Differential expression of representative genes involved in linalool biosynthesis and starch and sucrose metabolism. **A** DEGs in linalool biosynthesis. **B** DEGs in starch and sucrose metabolism. **C** Expression profiles of representative genes involved in linalool biosynthesis and starch and sucrose metabolism. **D** Changes of starch, soluble sugar and linalool contents in F1, F2, F3, F4, and

F5. The absolute values of $\log_2 (F2, 3, 4, 5/F1) \geq 1$ and $FDR < 0.001$ were used as the criteria for DEGs. The colour of the box represents up (red)- and down (green)-regulated genes. All bars represent means $\pm$ s.d (three biological replicates, each biological replicate consists of 5 stems) and those labelled with a letter are significantly different at $P < 0.05$ by Duncan's test

Discussion

Accuracy and Representativeness of Transcriptome Data

Indole-3-butyric acid plays a role in the root regeneration of different plant species (Pan and Tian 1999; Rout 2006; Ali et al. 2009), and its physiological role is two-fold: a low concentration of IBA promotes plant growth, while a high concentration retards development through regulating the proliferation and expansion of cells (Björkman 2004; Iqbal et al. 2016). Based on the root ratios, the number of roots, and root length and diameter, 3000 mg/L IBA was considered to be the optimum concentration for further transcriptome measurements. Plant tissue cultivation always begins with the process of the stress response. Based on the GO enrichment results (Fig. 3A), the expression of genes involved in reactive oxygen stress peroxidases (Fu 2014), salicylic acid, and auxin response pathogenesis-related protein (Mitsuhara et al. 2008) were significantly induced. The peroxidase enzyme activity measured during the five stages was similar with the trend in the level of transcription (Fig. 3C). This result illustrates the accuracy of transcriptome sampling and the reliability of transcriptome data from the side.

Dynamic Homeostasis of Plant Hormones During Root Development

By comparing the expression of key genes in various plant hormone signal transduction pathways, it was found that the auxin, BR, and ABA signal transduction pathway genes were significantly altered during root development (Figs. 4A and C). Auxin plays a crucial role in root growth and developmental regulation by regulating the expression of *AUX/IAA*, *GH3*, and *SAUR* et al. Two Indole-3-pyruvate monooxygenase (*CKAN_01183000* and *CKAN_00588900*), which involved in auxin biosynthesis, were repressed in F2 to F5 when compared with F1. That is consistent with previous studies that exogenous IBA inhibits endogenous auxin biosynthesis. The *AUX/IAAs* are also repressed by treatment with exogenous auxin and encode short-lived nuclear proteins (Park et al. 2002). In our study, *CKAN_01958500*, *CKAN_01302600*, and another six genes were significantly repressed in F2 to F5 compared with F1. The *AtIAA2/3* orthologs in *Arabidopsis* regulate the development of lateral roots through activating the expression of *LBD16/18* (Lee et al. 2015a, b). As the downstream regulating gene of *AUX/IAA*, *CKAN_01177000* encodes AUXIN RESPONSE FACTOR (ARF) and was up-regulated in the later stages of

tissue culture when *AUX/IAA* may retard the expression of ARF TFs in the absence of auxin (Korasick et al. 2014). *CKAN_02009600* and *CKAN_02242800* are orthologs of *AtGH3.17* in *C. camphora* linalool type and encode IAA-amino synthases (group II family) (Pierdonati et al. 2019). There was increased expression of *CKAN_02519500*, *CKAN_01995300*, and another four genes, which are the orthologs of *SAUR41* (*SMALL AUXIN UPREGULATED 41*) in *C. camphora* linalool type and encode clade III SAUR proteins that contribute to radial root pattern formation (Qiu et al. 2013, 2019). Transcriptome data also revealed that a large number of auxin efflux carriers (*PIN*, involved in the root-specific auxin transport, such as *CKAN_01601500*, *CKAN_00099200*, and *CKAN_00765500*) and auxin-induced proteins 6B (*CKAN_00774200*) and X15 (*CKAN_00761400*) were significantly up-regulated after IBA treatment.

In other species, the content of IAA and ABA show a contrasting trend; i.e., the content of ABA is lowest when the IAA content is highest, during the development of seeds and roots (García-Martín et al. 2005; Albacete et al. 2008; Bosco et al. 2014). In this study, the expression of the core genes in the ABA signal transduction pathway, such as the *PYR/PYL* and *ABF*, were just repressed gradually (Choi et al. 2000; Kim et al. 2004; Fujii et al. 2007; Ma et al. 2009). *PYR/PYLs* are ABA-receptors that function at the apex of a negative regulatory pathway by inhibiting *PP2Cs* (Park et al. 2009; Hao et al. 2011), which is similar to the gradually increasing expression of *CKAN_02175500* and another eight *PP2Cs*. The increased expression of *PP2C* inactivates SnRK2 by direct dephosphorylation to retard the expression of *ABF* indirectly (Umezawa et al. 2010).

Brassinosteroid is a plant growth-promoting steroid that was first isolated from the pollen of *Brassica napus* (Grove et al. 1979). *BR11* is an important component of the BR receptor that is required for systemin-induced root elongation (Holton et al. 2007). *BZR1/2* controls root meristem development by regulating the cell division (Hak-Soo et al. 2015; Espinosa-Ruiz et al. 2017) and increasing the expression of *CYCD3* and *TCH4*. *CYCD3* positively affects root development and cell division (Li et al. 2008; Randall et al. 2015), while *TCH4* encodes one of the xyloglucan endo-transglycosylase (XET) enzymes to increase cell elongation and induced expressed by the auxin (Xu et al. 1996; Koka et al. 2000; Friedrichsen and Chory 2001).

In summary, the expression trend of auxin signalling transduction pathway genes positively correlated with that of BRs and negatively correlated with that of ABA. Together, these indicate a trend towards the occurrence of root primordia and the development of roots.

Changes in Carbon Metabolism and Secondary Metabolism During Rooting

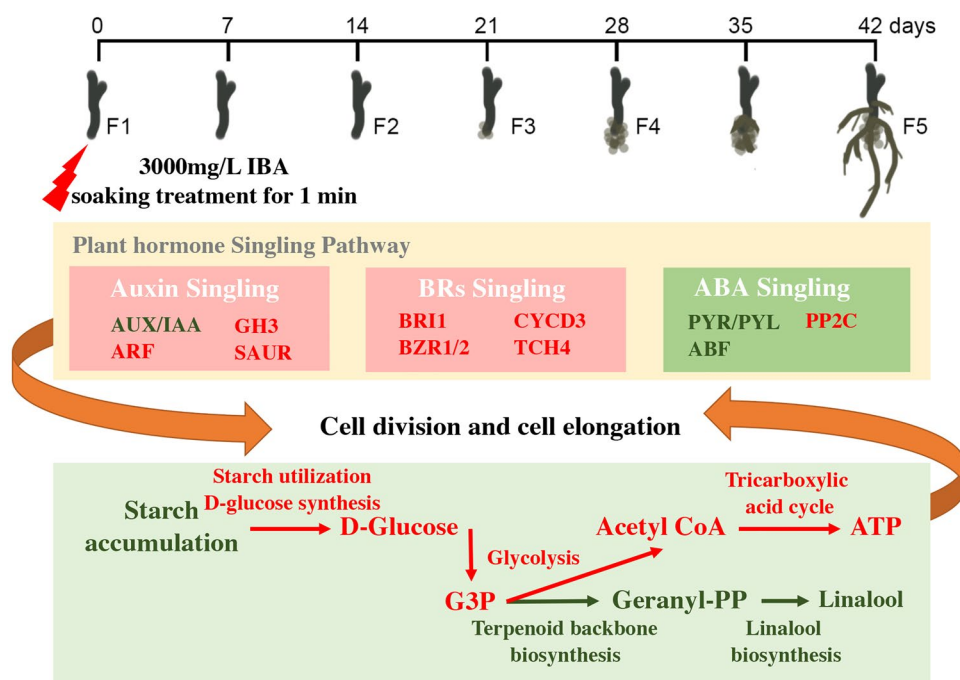
As a crucial secondary metabolite of *C. camphora* linalool type, linalool has insecticidal and insect repellent activity. (3S)-Linalool synthase (TPS14) synthesises linalool from geranyl diphosphate (Cseke et al. 1998; Aubourg et al. 2002). Geranyl diphosphate is an intermediate product of the terpenoid backbone biosynthesis process, whose substrate is glyceraldehyde 3-phosphate (G3P, intermediate product of the glycolysis pathway), and G3P forms acetyl-CoA via glycolysis (Lehninger et al. 2008; Hu et al. 2017). Linalool is then metabolised by 8-monooxygenase (CYP111A) into 8-hydroxylinalool and 8-oxolinalool (Bell et al. 2010; Ilc et al. 2017). Both *TPS14* and *CYP111A* were repressed during the rooting process, which suggests that the decline in linalool levels might be caused by a decrease in biosynthesis rates. It is worth noting that the KEGG pathway genes were enriched in the starch and sucrose metabolism process (Fig. 3C). By comparing the expression of key genes based on both the RNA-seq and real-time RT-PCR data, a large amount of starch and sucrose can be converted into D-glucose (Figs. 5B and C). We also found that genes encoding two glyceraldehyde-3-phosphate dehydrogenase (*CKAN_01457400* and *CKAN_01754000*, 2.67- and 1.13-fold, respectively), one pyruvate kinase (*CKAN_01349800*, 2.59-fold) and one enolase (*CKAN_01387600*, 0.36-fold expression level in F5 compared with F1), were up-regulated. All of these genes

encode key rate-limiting enzymes in the glycolysis pathway that converts glucose into pyruvate and acetyl-CoA (Chuang et al. 2005; Piast et al. 2005). Therefore, we speculate that during rooting, *C. camphora* linalool type plants accumulated glucose as a substrate to produce a large amount of acetyl-CoA to provide energy (such as ATP) through the tricarboxylic acid cycle for the rooting and stress response processes. At the same time, the reduction of the G3P content retarded the synthesis of linalool.

Conclusion

Based on transcriptome data and physical and chemical indicators, a proposed model for the stem segment cultures after the treatment of exogenous IBA can be proposed: exogenous IBA modifies carbohydrate, secondary metabolism and the auxin, and BR and ABA signalling transduction pathways (Fig. 6). Auxin and BRs play a positive and synergistic role in the rooting process, but this is in contrast to the expression trend of ABA signalling transduction pathway-related genes. On the other hand, the accumulation of starch gradually decreases and a large amount of D-glucose is used to synthesise pyruvate and acetyl-CoA through the glycolysis pathway to provide more energy for the rooting and stress responses. In consequence, the synthesis of linalool was slowed down by retarding the expression of terpenoid backbone biosynthesis pathway genes.

Fig. 6 The proposed model for the stem segment cultures after the treatment of exogenous IBA. Exogenous IBA promotes the rooting of *Cinnamomum camphora* linalool type cuttings by regulating carbohydrate, secondary metabolism and the auxin, BR, and ABA signalling transduction pathways



Materials and Methods

Plant Materials

The branches were from cuttings of 4 years old *Cinnamomum camphora* linalool type ‘Ganfang No. 1’ (Gan-S-SC-CC-001–2020). The average oil yield of fresh weight, dry weight, and L-linalool content of leaves were 2.53%, 6.03%, and 93.50% respectively. The mother tree of Ganfang No. 1 is from Xikou Village, Datong Town, Jiande City, Zhejiang Province, with a diameter at breast height of 25 cm, a height of 12 m, and a north–south crown width of 10 m. In the experiment, 1-year semi-lignified softwood twigs with one leaf and one bud were selected as cuttings. Stem segment cutting were performed by randomizing block arrangement after the 1-min soaking treatment of 0, 1000, 2000, 3000, and 4000 mg/L IBA solutions on the medium (Laterite: plant ash, v:v, 4:1). Use plastic film to maintain humidity above 80%, the temperature to 15 ~ 20 °C (night)/25 ~ 30 °C (day), and to avoid direct light (shading 70 ~ 75%). Before cutting, 0.1% carbendazim solution was used to sterilize the mediums and cuttings, and once a week after cutting. Root ratio, number of root, root length, and root diameter were calculated after a 60-day culturing. Three biological replicates in the experiment were performed, and each biological replicate consists of 450 stems. For RNA extraction and content measurement, fresh tissues of the stem base (15 mm, 5 plants per sample) taken 0 day, 14 days, 21 days, 28 days, and 42 days after planting on the media were collected.

Library Construction

All samples were sent to Novogene (Beijing, China) for extraction, sequencing, and bioinformatics analyses. The total RNA was extracted using Trizol reagent (Takara, Japan) and treated with 1U DNase I (Life Technologies). Total RNA integrity, RIN = 7.4–8.8, 28S/18S = 1.3–1.4; total RNA purity, OD260/OD280 = 1.88–1.97, OD 260/OD230 = 1.93–2.04; total RNA amount = 89.6–162.4 µg were quantified by Agilent 2100 bioanalyzer and Nanodrop spectrophotometer (Thermo Scientific). mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5X). First-strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase (RNase H-). Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. Remaining overhangs were converted into blunt ends

via exonuclease/polymerase activities. After adenylation of the 3' ends of the DNA fragments, NEBNext Adaptor with a hairpin loop structure was ligated to prepare for hybridization. In order to select cDNA fragments that were preferentially 250–300 bp in length, the library fragments were purified with an AMPure XP system (Beckman Coulter, Beverly, USA). Then, 3 µl USER Enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA at 37 °C for 15 min, followed by 5 min at 95 °C before PCR analysis. Then, PCR was performed with Phusion High-fidelity DNA polymerase, Universal PCR primers, and Index (X) Primer (14 cycles). Finally, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system (OD260/280 = 1.79–1.84, OD260/230 = 1.71–1.78).

Clustering and Sequencing

Clustering of the index-coded samples was performed on a cBot Cluster Generation System using a TruSeq PE Cluster Kit v3-cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina Novaseq platform and 150-bp paired-end reads were generated. The range of raw reads of these 15 samples was from 46,066,820 to 53,058,852, with the error rates from 0.2 to 0.3, Q30 from 92.39 to 94.51. Reads with adapter, N (undetermined base) and reads with low quality (over 50% bases showed $Q_{phred} \leq 20$) were filtered out, and the clean reads were from 43,920,844 to 51,980,346. Exactly 83.55 to 87.24% of the clean reads were mapped on the genome of a total of 83.55 to 87.24% of the sequences that were associated with the reference genome of *Cinnamomum kanehirae* (Asbrckan_0, BioProject: PRJNA477266), and 73.51–75.12%, 2.73–6.21%, and 22.15–20.25% of the mapped reads were mapped on the exon, intron, and intergenic respectively.

Differential Expression Analysis

Differential expression analysis of five groups (three biological replicates per condition) was performed using the R (3.5.1) package DESeq2_1.20.0 (Love et al. 2014). DESeq2 provides statistical routines for determining differential expressions in digital gene expression data using a model based on the negative binomial distribution. The resulting *P*-values were adjusted using Benjamini and Hochberg's approach for controlling the false discovery rate. Genes with an adjusted $P < 0.05$ found by DESeq2 were assigned as differentially expressed. The selection of differentially expressed genes was based on $FDR \leq 0.001$ and an absolute value of Log2 (relative expression level, compared with WT) ≥ 1 .

GO and KEGG Enrichment Analysis of Differentially Expressed Genes

Case special deep analysis based on DEGs, including GO and KEGG pathway enrichment analysis, was done by our group. Gene ontology (GO) enrichment analysis of differentially expressed genes was implemented by the cluster Profiler R(3.5.1) package (Young et al. 2010), in which gene length bias was corrected. GO terms with corrected $P < 0.05$ were considered significantly enriched by differentially expressed genes. KEGG is a database for understanding the high-level functions and utilities of biological systems, such as cells, organisms, and ecosystems, from molecular-level information, especially large-scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies (<http://www.genome.jp/kegg/>). We used the cluster Profiler R(3.5.1) package to test the statistical enrichment of differentially expressed genes in KEGG pathways (Kanehisa et al. 2008).

Plant RNA Extraction and Real-Time RT-PCR

The total RNA was extracted using Trizol reagent (Takara, Japan) and treated with 1U DNase I (Life Technologies). Total RNA integrity, $RIN = 7.1–8.2$, $28S/18S = 1.2–1.4$; total RNA purity, $OD_{260}/OD_{280} = 1.82–1.98$, $OD_{260}/OD_{230} = 1.89–2.01$; and total RNA amount = $102.13–245.80 \mu\text{g}$ were quantified by Agilent 2100 bioanalyzer and Nanodrop spectrophotometer (Thermo Scientific). An amount of 500 ng total RNA was used for inverse transcription with a Transcriptor First Strand cDNA Synthesis Kit (Takara, Japan). We diluted the cDNA 10 times and then used it as the template for real-time PCR (RT-PCR). The RT-PCR reaction system comprised SYBR® Premix Ex Taq™ (2×) 5 μl (Takara, Japan), PCR Forward Primer (10 μM) 0.2 μl , PCR Reverse Primer (10 μM) 0.2 μl , cDNA template 1 μl , and distilled deionized water up to 10 μl . The PCR process used the following conditions: 95 °C for 15 s, 58.2–66.8 °C for 30 s, and 72 °C for 30 s for 40 cycles. *CKAN_02474600* which encoded Actin was selected as the stably expressed reference genes for the normalization of the RT-qPCR data. Transcriptome data indicated that the expression level of *CKAN_02474600* showed no significant difference between these five groups ($\log_2 \text{FC} < 0.01$), and the amplification efficiency of its primer was 103.97% with $R^2 = 0.9867$. Melt curves of 22 genes were all unimodal, which indicates that the primer specificity is high. Details of primers sequences, melting temperature, primer efficiency (E, %), and R^2 were showed in Supplemental Table S5.

Determination of POD Enzyme Activity and the Content of Soluble Sugars, Starch, and Linalool

The activity of peroxidase enzyme (POD) were determined according to the method of Vetter (Vetter et al. 1958). Starch and soluble sugars were determined with Anthrone reagent (Hansen and Moller 1975; Taylor et al. 1982) with the stems (1.5 cm to the end) as the materials (three biological replications, 5 plants for each biological replication). Linalool were determined with gas chromatography (Wang et al. 2006).

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Declarations

Conflict of Interest The authors declare no competing interests.

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