Foliar application of kinetin modulates the cannabinoid content and transcriptome of *Cannabis sativa* L.

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Abstract

*Cannabis sativa* is highly cultivated owing to its secondary metabolites, especially cannabinoids, which possess several medicinal effects. This study aims to examine the effect of foliar application of kinetin (KT) on the growth, cannabinoid content, and transcriptome of *C. sativa* at the flowering stage. A pot experiment was conducted in a greenhouse under a 16h/8h light/dark cycle for growing, and the photoperiod was adjusted to 10h/14h to induce flowering. The plant was sprayed with 0, 20, 40, and 100 mg/L KT every 2 days for a total of six times. Phenotypic parameters (fresh weight, cannabinoid content, and enzyme activity) and metabolome were analyzed. Foliar application of 20 and 40 mg/L of KT increased the growth and cannabinoid content of *C. sativa*, particularly that of cannabidiol. Similarly, treatment with 20 mg/L of KT increased the Δ⁶-tetrahydrocannabinol content to 0.24%, which is below the legal value of 0.30% in North American countries. Plants treated with 20 and 40 mg/L of KT showed decreased activities of 3-hydroxy-3-methylglutaryl coenzyme A reductase and 1-deoxy-D-xylulose 5-phosphate synthase. KEGG pathway analysis showed that differentially expressed genes (DEGs) in the KT-treated cannabis were mainly enriched in 12 pathways, including sesquiterpenoid and triterpenoid biosynthesis, linoleic acid metabolism, flavonoid and phenylpropanoid biosynthesis, and other secondary metabolic pathways; 14DEGs (including eight upregulated and six downregulated genes) were enriched in sesquiterpene and triterpene biosynthesis pathways. Overall, these findings showed that KT plays a significant role in regulating terpenoid biosynthesis and the cannabinoid content of *C. sativa*.

Keywords: cannabinoids; *Cannabis sativa*; KEGG pathway analysis; kinetin; terpenoid biosynthesis; transcriptome.

Abbreviations: KT, kinetin; CBD, cannabidiol; THC, Δ⁶-tetrahydrocannabinol; MVA, mevalonate; CBDA, cannabidiolic acid; THCA, tetrahydrocannabinolic acid; CBGA, cannabigerolic acid; GPP, geranyl diphosphate.

Introduction

As an annual herb, *Cannabis sativa* L. belongs to the Cannabaceae family (Flemming et al., 2007) and is used as a source of oil, food, fiber, medicine, and inebriants. However, its best known for the production of cannabinoids, a unique class of terpenoids that can be used for chemical defense but also have pharmaceutical and psychiatric activities (Laverty et al., 2019).

There are over 100 known cannabinoids; the two most abundant natural derivatives are Δ⁹-tetrahydrocannabinol (THC) and cannabidiol (CBD). The THC causes psychoactive effects upon cannabis consumption. Although CBD is nonintoxicating, it has therapeutic properties and is currently being studied as a treatment for schizophrenia and Alzheimer's disease (Osborne et al., 2017; Watt and Karl, 2017). Cannabinoids are predominantly concentrated in the secretory cavity of glandular trichomes and mainly occur in the female flowers of cannabis plants. Cannabis species with a high CBD content are being used for medical purposes.

Terpene biosynthesis in plants occurs through the mevalonate (MVA) and 1-deoxy-D-xylulose-5-phosphate/2-methyl-D-erythritol-4-phosphate (DOX/MEP) pathways (Mansouri et al., 2011). Cannabinoids and terpenes share common isoprenoid precursors (Fig.1). Phytocannabinoids, including tetrahydrocannabinolic acid (THCA) and cannabidiolic acid (CBDA), are synthesized from cannabigerolic acid (CBGA) by cannabinoid synthases. CBGA is synthesized by condensation of the monoterpene precursor geranyl diphosphate (GPP) with aromatic polyketo acetic acid (OA) (Booth et al., 2020). THC and CBD are formed during storage by decarboxylation of their acidic forms or interaction with heat and light (Happyana et al., 2013).

The production of plant secondary metabolites (such as essential oils and aromatic compounds) is regulated by many biochemical, physiological, genetic, and metabolic factors. As a mature inducer, plant growth regulator stimulates the production of secondary metabolites during the cultivation of medicinal plants (Jamwal et al., 2018). Kinetin (N⁶-furfuryladenine; KT) is a growth regulator that affects
several physiological development processes, including apical dominance, nutrient mobilization, leaf senescence, and meristem formation and activity. Recent studies have shown that the application of growth regulators can increase the yield of several Solanaceae species (Trethewey et al., 2016) and the initial growth of maize (Zhang et al., 2022). Exogenous foliar application of KT (0.01–2 mM) enhanced the growth rate, yield, crop quality, and abiotic stress tolerance in several crops (Hamayun et al., 2015; Kaya et al., 2018; Kaya et al., 2010). KT also upregulated both enzymatic and nonenzymatic antioxidative systems, thereby increasing the reactive oxygen species scavenging ability of plants (Ahanger et al., 2018). Exogenous leaf application of KT can activate and stimulate the biosynthesis of several plant secondary metabolites by upregulating the corresponding genes or increasing the activity of biosynthetic enzymes (Jalal-ud-Din et al., 2015; Weremczuk-Lezyna et al., 2018). However, the effect of KT application on the cannabinoid content of C. sativa has not yet been investigated.

Recent advances in high-throughput sequencing technology have facilitated studies on the molecular mechanisms of specific process in plants. Transcriptome analysis of glandular trichomes of peppermint, hops, and other plants has resulted in the identification of enzymes involved in specific metabolic pathways (Dai et al., 2010; Lange et al., 2000). Stout et al. (Stout et al., 2012) analyzed the transcriptome of glandular trichomes of female Alfalfa flowers and identified an acyl-activating enzyme capable of synthesizing hexanoyl-CoA from hexanoate. Additionally, higher expression of terpene biosynthesis-related genes was observed in the trichomes of cannabis hemp than in non-resin-producing tissues (Booth et al., 2017). This study aimed to elucidate the influence of foliar application of KT on the growth of C. sativa, as well as the mechanism of KT in cannabinoid biosynthesis in female cannabis plants, using transcriptomic techniques.

Results and discussion

Effect of KT on plant growth

The effect of KT on the growth of cannabis plants was assessed in terms of whole-plant fresh weight (FW). Foliar application of 20, 40, and 100 mg/L of KT increased the FW of whole cannabis plants by 25.45%, 27.35%, and 23.69%, respectively, compared with the control (Fig.2). The increase in plant growth after foliar application of KT was consistent with previous findings in black cumin plants (Shah, 2007). KT, a cytokinin, plays a crucial role in inducing cell division, synthesis of secondary metabolites, regulation of nutrient absorption, and stimulation of RNA synthesis (Barciszewski et al., 1999; Mok and Mok, 2001) and may be responsible for the increase in the growth of KT-treated plants. Li et al. (2018) found that 40 mg/L KT significantly increased the plant height and FW of P. cretica var. nervosa and attributed this to the regulation of several developmental processes.

Effect of KT on cannabinoid content

CBD and THC are important medicinal compounds in cannabis, therefore, the effect of foliar application of KT on the CBD and THC of ca (Fig.3). The foliar application of 20 and 40 mg/L of KT increased the CBD content of the plants by 69.08% (from 3.50%) and 50.72% (from 3.13%), respectively, compared with the control group (2.07%). However, treatment with 100 mg/L of KT decreased the CBD content of the plants (1.21%). Similarly, foliar application of 20 and 40 mg/L of KT increased the THC content of the plants, with plants treated with 20 mg/L of KT having a THC content of 0.24%, which is below the legal value of 0.30% in North American countries (Schluttenhofer and Yuan, 2017). However, treatment with 100 mg/L of KT inhibited THC accumulation. Mansouri et al. (2013) reported an increase in the CBD content in male flowers following treatment with low concentrations of ethephon (1 and 5 μM) and a decrease upon treatment with high concentrations (10 and 100 μM) compared with the control group.

Danova et al. (2016) reported that the endogenous application of cytokinin mediates the biosynthesis of terpenoids by affecting the thylakoid morphology and altering the efficacy of biogenic pathways. Treatment with cytokinin increased the monoterpenoid/sesquiterpenoid ratio in the aerial parts of Artemisia alba Turra (Danova et al., 2018). Cannabinoid compounds possess antimicrobial, anti-desiccation, antifeedant, and UV-B protection activities (Mansouri et al., 2013). Additionally, KT is involved in regulating plant defense and stress responses. KT application enhanced the photosynthesis rate and antioxidant system of Vigna angularis exposed to cadmium by increasing the content of non-enzymatic components and activity of antioxidant enzymes (Ahanger et al., 2020). Similarly, KT application decreased the levels of the reactive free radical hydrogen peroxide and lipid peroxidation in Solanum lycopersicum plants exposed to NaCl (Ahanger et al., 2018). Overall, these findings indicate that KT plays a significant role in the mediation of terpenoid biosynthesis.

Effect of KT on DXS and HMGR activity

The effect of foliar application of KT on the activities of key enzymes (HMGR and DXS) involved in terpenoid biosynthetic pathways are presented in Fig.4. There was a linear decrease in HMGR activity with an increase in KT concentration. Additionally, there was a decrease in DXS activity in plants treated with 20 and 40 mg/L of KT compared with the control group; however, an increase in DXS activity was observed in cannabis plants treated with 100 mg/L of KT.

HMGR is a key enzyme that regulates the MVA pathway in plants. HMGR activity was inversely correlated with the levels of exogenous plant hormones in C. sativa at the flowering stage (Mansouri et al., 2009b). DXS was the first enzyme identified in plastidal terpenoid biosynthesis in plants. Moreover, the limiting effect of DXS on the production of MEP-derived isoprenoids in all systems and the regulatory role of this enzyme in controlling the flow of the MEP pathway in plants have been reported (Rodriguez-Concepcion and Boronat, 2015). In this study, KT treatment at low concentrations inhibited DXS activity. However, treatment at a high concentration (100 mg/L) increased DXS activity. Unlike HMGR activity, DXS activity was independent of changes in the levels of MEP-derived isoprenoid end-products, such as CBD and THC. These data support the hypothesis that metabolite biosynthesis via the MEP pathway is regulated by several enzymes. Mansouri et al. reported that gibberelic acid treatment decreased DXS activity, which was not related to the reduction in the levels of end-products in the MEP pathway (Mansouri et al., 2009a). Therefore, we inferred that the MEP pathway may be regulated at several control points to compensate for fluctuations in the precursor/product balance; thereby
Table 1. Main KEGG enriched pathways of differentially expressed genes (DEGs) in KT-treatment Cannabis sativa.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>KEGG pathway ID</th>
<th>DEGs number</th>
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<tbody>
<tr>
<td>Starch and sucrose metabolism</td>
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<tr>
<td>Phenylpropanoid biosynthesis</td>
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<td>Plant hormone signal transduction</td>
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<td>Flavonoid biosynthesis</td>
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<td>Amino sugar and nucleotide sugar metabolism</td>
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</tr>
<tr>
<td>Pentose and glucuronate interconversions</td>
<td>ko00040</td>
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</tr>
<tr>
<td>MAPK signaling pathway - plant</td>
<td>ko04016</td>
<td>17</td>
</tr>
<tr>
<td>Sesquiterpenoid and triterpenoid biosynthesis</td>
<td>ko00909</td>
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<td>Plant-pathogen interaction</td>
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<td>Galactose metabolism</td>
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<td>Glycerolipid metabolism</td>
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<tr>
<td>DNA replication</td>
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<tr>
<td>Phenylalanine metabolism</td>
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<td>Cysteine and methionine metabolism</td>
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<td>Linoleic acid metabolism</td>
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</table>

Figure 1 Terpene and cannabinoid biosynthetic pathways. Precursors and intermediates are shown in black, final product classes in green, and enzymes in purple. The IPP and DMAPP are precursors for terpenoid synthesis and are produced via the MVA pathway localized in the cytoplasm and the MEP pathway in plastids in plants. The MVA pathway generally supplies DMAPP and IPP for sesquiterpene synthesis, whereas the MEP pathway provides the same five-carbon prenyl diphosphates for the synthesis of monoterpenes, diterpenes, and tetraterpenes. MEP pathway: MEP, 2-C-methyl-D-erythritol 4-phosphate; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; DXP, 1-deoxy-D-xylulose 5-phosphate; DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; HMB-PP, 4-hydroxy-3-methylbut-2-en-1-yl diphosphate; MEP, dimethylallyl diphosphate; GPPS, geranyl pyrophosphate synthase; TPS, terpene synthases, MVA pathway: MVA, mevalonic acid; HMGS, 3-hydroxy-3-methyl glutaryl coenzyme A synthase; HMGR, 3-Hydroxy-3-methylglutaryl coenzyme A reductase; IPP, isopentenyl diphosphate isomerase; FPPS, farnesyl diphosphate synthase; CBDGA, cannabigerolic acid; CBDAS, cannabigerolic acid synthase; CBDA, cannabidiolic acid; CBD, cannabidiol; THCAS, tetrahydrocannabinolic acid synthase; THCA, tetrahydrocannabinolic acid; Δ⁹-tetrahydrocannabinol.
Table 2. Functional annotation of differentially expressed genes in sesquiterpene and triterpene biosynthesis.

<table>
<thead>
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<th>KO entry</th>
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<th>Regulation</th>
<th>Annotation</th>
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<td>5.4.99.39</td>
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</tr>
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</table>

Figure 2. Effect of KT on fresh weight.

Figure 3. Effect of KT on CBD and THC contents.
Figure 4. Effect of KT on the activities of HMGR and DXS.

Figure 5. Cluster analysis of differential gene expression profiles in KT-treatment and control. Red represents high gene expression, and blue represents low gene expression.
transcriptome analysis
Transcriptome sequencing and the analysis of the samples treated with 20 mg/L KT and control samples were conducted to explore the influence of exogenous hormones on secondary metabolites in industrial cannabis. We obtained clean reads with high base quality scores (Q30 > 98%), and the percentage of GC ranged between 43% and 44%. After cleaning the sequencing data and the reference genome according to the gene location information specified in the genome annotation file, and the comparison rate was > 89%. Differential gene expression analysis identified 1,190 differentially expressed genes (DEGs) in KT versus control groups, including 1,027 upregulated and 163 downregulated genes. Cluster analysis showed that the DEGs formed two distinct clusters according to the treatment conditions (Fig.5).

Functional annotation using the gene ontology (GO) database showed that 1,106 DEGs (956 upregulated and 150 downregulated) were enriched in GO terms. Specifically, DEGs were predominantly enriched in the following biological processes: transcription, DNA-templated, defense response, and protein phosphorylation. They were enriched in the following cellular components: nucleus, cytoplasm, plasma membrane, cytosol, chloroplast, and extracellular region. Lastly, they were enriched in the following molecular functions: protein binding, DNA binding, molecular function, and DNA binding transcription factor activity (Fig.6). We speculated that exogenous KT treatment may affect the translation of proteins in ribosomes.

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis showed that the DEGs were mainly involved in starch and sucrose metabolism, phenylpropanoid biosynthesis, plant hormone signal transduction, flavonoid biosynthesis, amino sugar and nucleotide sugar metabolism, pentose and glucuronate interconversions, MAPK signaling pathway plant, sesquiterpenoid and triterpenoid biosynthesis, plant pathogen interaction, galactose metabolism, endocytosis, and glycerolipid metabolism (Table 1). A total of 14DEGs were identified in the sesquiterpene and triterpene biosynthesis pathways (Table 2), among which five were upregulated, all of which were genes involved in α-humulene synthase. One significantly downregulated gene was annotated as the (E, E)-α-farnesene synthase gene. Five genes were annotated as β-amyrin synthase genes, three of which were upregulated and two were downregulated. Three genes were annotated as lupeol synthase genes, two of which were upregulated and one was downregulated.

α-humulene synthase (α-HS) is a common terpene synthase and is widely distributed among plant species. In plant cells, terpenoids are biosynthesized by terpene synthases, which are localized in the cytoplasm, to produce sesquiterpenes through the mevalonate-dependent pathway, or in the chloroplast, to produce monoterpines and diterpenes through the 2-C-methyl-D-erythritol-4-phosphate pathway (Lee et al., 2015). In this study, five upregulated genes were related to α-HS. Therefore, we speculated that foliar application of KT increased terpene synthase expression, thus activating enzymes related to cannabinoid synthesis and increasing the CBD and THC contents. α-farnesene synthase is the final restriction enzyme in the sesquiterpene α-farnesene synthesis pathway and regulates α-farnesene synthesis. The synthetic precursor of α-farnesene is farnesyl pyrophosphate (FPP). Similar to the synthetic precursor gerany1 pyrophosphate (GPP) of cannabinoids, FPP is synthesized from isomalyl pyrophosphate (Liu et al., 2016). In this study, the (E, E)-α-farnesene synthase gene was significantly downregulated. Therefore, we speculated that downregulating the α-farnesene synthase gene inhibits the synthesis of α-farnesene, thus promoting the synthesis of GPP and cannabinoids.

Figure 6. Gene ontology analysis showed the enrichment categories of differentially expressed genes (DEGs) in 20 mg/LKT-treatment vs. control.
Lupineol is a triterpenoid compound, and lupineol synthase is responsible for the formation of lupineol in the cuticle, which leads to an increase in the number of glandular trichomes on the stem surface of castor plants and formation of glandular trichome crystals on the stem surface of plants (Guhling et al., 2006). Cannabinoids exist mainly in the glandular trichomes of cannabis plants. KT treatment may increase the glandular trichomes of cannabis plants and promote the accumulation of cannabinoids. β-amylase synthetase and luteop synthetase are two branches of the 2,3-oxidosqualene metabolic pathway responsible for the synthesis of triterpenoids and sterols in birch, which can promote the synthesis of triterpenoids in cannabis.

Materials and methods

Plant cultivation and treatment
The industrial cannabis strain DMG227 used in this study was provided by the Institute of Bast Fiber Crops, Chinese Academy of Agricultural Sciences, under a Health China research license, and the plants were grown in their laboratory. The branches of a robust DMG227 mother plant were selected for cutting and cloning to generate a clone population with a consistent genetic background. For clone propagation of plants, cuttings were collected from the mother plant during the vegetative period, surface sterilized with 5% (v/v) bleaching agent, and placed in a Jiffy seedling block soaked for 30 min in water (pH=6). The cuttings were then stored in trays under a transparent plastic dome to maintain humidity and promote rooting. Once rooted, the cuttings in the Jiffy seedling block were moved to potted soil. After growing for 4 weeks under a 16 h/8 h light/dark cycle, the photoperiod was adjusted to 10 h/14 h to induce flowering. After growing for 4 weeks under a 16 h/8 h light/dark cycle, the photoperiod was adjusted to 10 h/14 h to induce flowering. At 2 weeks of flowering the whole plant was sprayed with 0, 2, 20, 40, and 100 mg/L of KT and subsequently sprayed once every 2 d (40 mL/plant each time) for a total of six times. All plants were cultured in a warm room at 24±2°C. The industrial cannabis strain DMG227 used in this study was provided by the Institute of Bast Fiber Crops, Chinese Academy of Agricultural Sciences, under a Health China research license, and the plants were grown in their laboratory. The branches of a robust DMG227 mother plant were selected for cutting and cloning to generate a clone population with a consistent genetic background. For clone propagation of plants, cuttings were collected from the mother plant during the vegetative period, surface sterilized with 5% (v/v) bleaching agent, and placed in a Jiffy seedling block soaked for 30 min in water (pH=6). The cuttings were then stored in trays under a transparent plastic dome to maintain humidity and promote rooting. Once rooted, the cuttings in the Jiffy seedling block were moved to potted soil. After growing for 4 weeks under a 16 h/8 h light/dark cycle, the photoperiod was adjusted to 10 h/14 h to induce flowering. At 2 weeks of flowering the whole plant was sprayed with 0, 2, 20, 40, and 100 mg/L of KT and subsequently sprayed once every 2 d (40 mL/plant each time) for a total of six times. All plants were cultured in a warm room at 24±2°C. Each treatment was repeated three times, and plants were watered and fertilized regularly. The plants were harvested four weeks after the end of the last treatment.

Cannabinoid extraction and content analysis
Three inflorescences (approximately 15 cm each) from different female plants were collected, mixed, and dried overnight at 35°C, and the seeds and stems were removed. Thereafter, the samples were ground and sieved using a 0.45-µm filter. The samples (200 mg) were extracted in a glass tube containing 4 mL of methanol and incubated for 10 min in an ultrasonic bath at 30°C. After standing for 1 h, the supernatant was centrifuged at 2900 × g for 5 min and diluted to 10 mL with methanol. The supernatant was filtered using a 0.45-µm filter in a high-performance liquid chromatography (HPLC) vial for analysis. HPLC analysis was performed using an Agilent modular model 1260 system equipped with a constant-temperature chromatographic column (ZORBAX SB-C18, 250 mm × 4.6 mm) and a PDA detector. The mobile phase was acetonitrile and 0.1% acetic acid; the flow rate was 0.8 mL/min; the injection volume was 10 µL. CBD and THC detection was performed at 220nm using a PDA detector.

Enzyme activities
Briefly, 0.2 ± 0.0020 g of samples (fresh weight of inflorescence) were treated with liquid nitrogen and stored at −80°C in a freezer for further analysis. The liquid nitrogen-treated samples were ground and transferred to centrifuge tubes, PBS was added at a ratio of 1:9 (g/mL), and the samples were centrifuged for 20 min at 8000 × g. The supernatant was discarded, and the activities of DXS and HMGR were measured using an enzyme kit.

mRNA library construction and sequencing
TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from tissue samples according to the manufacturer’s protocol. The total RNA amount and purity were analyzed using a Bioanalyzer 2100 and RNA 6000 Nano LabChip kit (Agilent, CA, USA), respectively, with an RIN value > 7.0. Approximately 10 µg of total RNA, representing a specific adipose tissue type, was subjected to poly(A) mRNA isolation using polyT oligo-ligated magnetic beads (Invitrogen). After purification, mRNA was fragmented using divalent cations at high temperatures. The cut RNA fragments were reverse-transcribed using an mRNA-Seq sample preparation kit (Illumina, San Diego, USA), according to the manufacturer’s instructions to create the final cDNA library with an average insertion size of 300 bp (±50). Paired-end sequencing was performed on an Illumina HiSeq4000 platform (LC Sciences, USA) according to the manufacturer’s instructions.

Transcriptome sequencing and preliminary analysis
A cDNA library, constructed using pooled RNA technology from porcine brain samples, was sequenced using the Illumina 4000 sequence platform. Using the Illumina paired-end RNA-seq method, the transcriptome was sequenced to generate a million paired-end reads of bp length. This generated gigabases (Gbs) in the sequence. Low-quality reads were removed before assembly. Subsequently, a total of G bp of cleaned paired-end reads was generated.

Transcript abundance estimation and differential expression analysis
StringTie software was used to assemble the mapped reads of each sample. All transcriptomes in the samples were merged, and a comprehensive transcriptome was reconstructed using Perl scripts. After generating the final transcriptome, the relative expression levels of genes were determined using StringTie and edgeR and expressed as fragments per kilobase of transcription per million fragments mapped. DEGs were screened based on log2 (fold change) ≥ 1 and p < 0.05 using the R package. The sequences obtained in this study were aligned with the reference genome of C. sativa available from the NCBI repository (https://www.ncbi.nlm.nih.gov/genome/browse#l/eukaryotes/11681/) (Laverty et al., 2019).

Statistical analysis
Statistical analyses were performed in SPSS version 19.0. The data from three replications (n = 3) per treatment were analyzed using one-way analysis of variance (ANOVA), followed by Duncan’s multiple interval test for multiple mean comparisons. Statistical significance was set at p < 0.05. Data are expressed as mean ± standard deviation (SD).
Conclusion
Foliar application of KT improved the growth parameters, including FW, height, and stem diameter, of cannabis plants. Application of 20 and 40 mg/L of KT increased the CBD and THC contents of the in florescences C. sativa. However, HMGR and DXS activities were not positively affected by KT treatment. KEGG pathway analysis showed that DEGs in KT-treated cannabis were mainly enriched in 12 pathways, including sesquerpenoid and triterpenoid biosynthesis, linoleic acid metabolism, flavonoid and phenylpropanoid biosynthesis, and other secondary metabolic pathways. Overall, our findings showed that KT plays an important role in modulating cannabinoid content and regulating terpenoid biosynthesis in C. sativa.

Acknowledgments
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