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Delineating genetic regulation of cannabinoid biosynthesis during female flower development in *Cannabis sativa*

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Abstract

Cannabinoids are predominantly produced in the glandular trichomes on cannabis female flowers. There is little known on how cannabinoid biosynthesis is regulated during female flower development. We aim to understand the rate-limiting step(s) in the cannabinoid biosynthetic pathway. We investigated the transcript levels of cannabinoid biosynthetic genes together with cannabinoid contents during 7 weeks of female flower development. We demonstrated that the enzymatic steps for producing cannabigerol (CBG), which involve genes *GPPS*, *PT*, *TKS*, and *OAC*, could rate limit cannabinoid biosynthesis. Our findings further suggest that upregulation of cannabinoid synthases, *CBDAS* and *THCAS* in a commercial hemp and medical marijuana variety, respectively, is not critical for cannabinoid biosynthesis. The cannabinoid biosynthetic genes are generally upregulated during flower maturation; increased expression occurs coincident with glandular trichome development and cannabinoid production in the maturing flower. The results also suggest that different cannabis varieties may experience discrete transcriptional regulation of cannabinoid biosynthetic genes. In addition, we showed that methyl jasmonate (MeJA) can potentially increase cannabinoid production. We propose that biweekly applications of 100 μM MeJA starting from flower initiation would be efficacious for promoting cannabinoid biosynthesis. Our findings provide important genetic information for cannabis breeding to generate new varieties with favorable traits.

KEYWORDS
cannabinoid biosynthesis, cannabinoid biosynthetic genes, *Cannabis sativa*, methyl jasmonate, transcriptional regulation

1 | INTRODUCTION

Cannabis sativa (cannabis or *C. sativa*) is currently garnering attention for its important chemical constituents known as cannabinoids, which have a number of pharmacological properties, including chronic pain treatment, seizure mitigation, spasticity reduction, and a host of others (Brodie & Ben-Menachem, 2018). Female plants of dioecious cannabis varieties are favored for cannabinoid production because their unfertilized flowers produce cannabinoids in the highest concentration in the capitate-stalked glandular trichomes (GSTs). Terminal cannabinoids such as cannabidiolic acid (CBDA), tetrahydrocannabinolic acid (THCA), and cannabichromenic acid (CBCA) are the products of a biosynthesis pathway (Figure S1). Cannabinoid synthase enzymes are generated in the rosette of gland cells of GSTs and secreted into the extracellular cavity at the top of the trichome where terminal cannabinoid synthesis and

accumulation takes place (Balcke et al., 2017; Rodziewicz et al., 2019).

Up to now, little is known on the regulation of cannabinoid biosynthesis in *C. sativa*. We aimed to elucidate (a) how cannabinoid biosynthesis is regulated, (b) which of the enzymatic step(s) in the biosynthetic pathway rate-limit the production of cannabinoids, and (c) what developmental programming factors could be manipulated to improve cannabinoid production and/or prevent occurrences such as high THCA accumulation in hemp varieties cultivated for CBD production. Our experimental work brings to light new information and understanding to these questions by monitoring cannabinoid content and expression of key biosynthesis genes as the female flower matures. Another factor that may be important for cannabinoid production is trichome development. Jasmonic acid has been shown to increase trichome density and secondary metabolite production in other plant species containing glandular trichomes (Boughton et al., 2005; Yan et al., 2017). However, little is known about the effect of methyl jasmonate (MeJA) on cannabinoid production in cannabis.

In this report, we monitored the expression pattern of cannabinoid biosynthetic genes during 7 weeks of female flower development in a hemp variety, "Cherry Wine" (CW), and a medical marijuana variety, "Gorilla Glue" (GG). We also monitored coincident cannabinoid contents at the same time points. In general, cannabinoid biosynthesis showed a positive correlation with the biosynthetic genes. In addition, we examined the effect of MeJA on cannabinoid production. The results showed that MeJA increased THC content by about 21%. The rate limiting step(s) of cannabinoid biosynthesis has not been heretofore examined; results presented here address this issue.

2 | MATERIALS AND METHODS

2.1 | Plant materials

Cuttings of 5-month-old GG mother plants were taken and rooted in an EZ-Cloner Classic ChamberTM (Sacramento, California) under aeroponic conditions. Rooted cuttings were grown under 18-h light/6-h darkness for 9 weeks in the closed-loop commercial facility, CTPharma LLC. Plants were then grown under 12-h light/12-h darkness for reproductive growth.

Cuttings of 5-month-old CW mother plants grown in the greenhouse at University of Connecticut were taken to produce new clones for experiments. Plants were grown under 18 h light/6 h darkness for vegetative growth. After 3 weeks of vegetative growth, plants shifted to a new cycle of 12-h light/12-h darkness to initiate floral development. The plants were maintained in this environment for 7 weeks.

2.2 | Sample collection, RNA isolation, and cDNA synthesis

Approximately 0.1 g of flower material was collected and then immediately placed into liquid nitrogen for flash freezing. Samples were then stored in a −80°C freezer until RNA extraction. Macherey-Nagel Plant and Fungi Mini kit for RNA (Düren, Germany) was used for RNA extraction according to the manufacturer's protocol with modifications. Single-stranded cDNA was synthesized from 1 μg of the isolated RNA using the Bio-Rad iScript™ cDNA synthesis kit (Hercules, California).

2.3 | Cannabinoid extraction and HPLC analysis

Approximately 0.5 g of flower material was collected and oven-dried at 30°C. This dried material was added to individual 50-ml Falcon® tubes, and then 20 ml of 25°C 9:1 methanol:dichloromethane (v/v) was added to tubes. Each tube was vortexed vigorously for 3 s, and then tubes were placed on a shaker plate for 90 min. Tubes were then vortexed vigorously again for 3 s before transferring 1–2 ml of extract into a syringe and pressing the liquid through 0.45-μm filter into a 2-ml microcentrifuge tube. Following filtration, 100 μl of extract was added to 900 μl of methanol in autosampler vials. The extracts were stored at −20°C until further analysis.

Cannabinoids were resolved on a 1260 Agilent High Pressure Liquid Chromatograph (HPLC) instrument (Santa Clara, California) using the Restek Raptor ARC C18 column (150 × 4.6 mm, 2.7-μm particle size) (Bellefonte, Pennsylvania). Samples were eluted at the rate of 1.5 ml/min with an isocratic 25% mobile phase A (water, 5-mM ammonium formate, 0.1% [v/v] formic acid) and 75% mobile phase B (acetonitrile, 0.1% [v/v] formic acid) at 30°C. A UV detector at 228 nm was implemented for the analysis. Peaks on the chromatograms were identified through comparison of retention times of cannabinoid reference RestekTM (Bellefonte, Pennsylvania) standards. Cannabinoid quantification was performed by comparing values to a calibration curve created with cannabinoid reference standards. The cannabinoid content represents the total amount of carboxylated (CBGA, CBDA, and THCA) and decarboxylated cannabinoids following the formulas presented in Stack et al. (2021), which is presented as the percentage of total flower dry weight.

2.4 | Real-time PCR

Reactions were performed with the Bio-Rad iTaq Universal SYBR Green Supermix (Hercules, California). Primers were chosen based off what other authors have used in other publications (Table S1). Ubiquitin (UBQ) was used as a reference gene. RT-qPCR analysis was performed using four biological replicates.

2.5 | Methyl jasmonate (MeJA) treatment

Different concentrations of MeJA (Food Grade, Sigma Aldrich) were prepared from a stock 95% MeJA solution: 100 μM, 500 μM, 1000 μM, and a water control. In CTPharma LLC., MeJA was applied to three biological replicates of a different medical marijuana variety "White

Tangy Haze," which was available to use at the time of experiment, for each concentration to the point of runoff after they had developed flowers for 2 weeks. Each plant received approximately 1 L of MeJA solution. For the following 4 weeks, four samples were harvested from each biological replicate to be used in cannabinoid analysis.

3 | RESULTS

3.1 | The correlation between cannabinoid production and the expression of biosynthetic genes

To gain insight into the regulation of cannabinoid production during flowering development, we examined flowers starting from the transition to reproductive growth for 7 weeks (Figure S2). In both varieties, CBG content represented the residual amount but not the produced amount in the trichome cells (Figure 1, black lines) because the majority has been used for the biosynthesis of end-point cannabinoids (Figure 2, black lines). Therefore, CBG content was much less than THC or CBD. The enzyme prenyltransferase (PT) catalyzes the alkylation of GPP and OA to create the precursor cannabinoid, cannabigerolic acid (CBGA). PT1 was initially found to catalyze CBGA biosynthesis (Page & Boubakir, 2012). Most recently, PT4 was reported to have higher enzymatic activity than PT1 (Blatt-Janmaat & Qu, 2021). Both genes are enriched in glandular trichomes and localized in the chloroplast (Gülck et al., 2020; Page & Boubakir, 2012). We examined the expression of both genes to learn how these two genes are transcriptionally regulated during female flower development.

In both varieties, there was a general trend of increased expression of PT1 and PT4 (Figure 1, bars) as the flower matured and CBG levels rose (Figure 1, black line); PT1 showed higher upregulation than PT4. In CW, PT1 expression increased during the last 3 weeks, while PT4 was not much upregulated throughout the whole period (Figure 1a,c). In GG, peak PT1 expression is much higher than PT4 (Figure 1b,d), and expression of both genes abolished as the flowers further matured.

We next examined end-point cannabinoids and their corresponding synthase genes. CBD accumulation was steadily increased from week 1 to week 7 in CW (Figure 2a, black line). CBDAS showed a similar expression pattern to that of PT1 in CW, indicating that upregulation of CBDAS is necessary for increased CBD production. However, because the residual CBG content is very low (Figure 1a), further increase of CBDAS expression in CW presumably would not result in more CBD generation due to the lack of substrates. In the marijuana variety GG, no CBD was detected despite that CBDAS transcripts could still be measured (Figure 2b). Unlike the upregulation of CBDAS in CW, CBDAS expression in GG was maintained at similar levels during the whole period of flower development. One possibility for the absence of CBD in the quantification assay is that there are mutations in the CBDAS gene that result in a nonfunctional protein. The other may be that the amount of CBDAS enzyme is very low and the synthesized CBD is under the detectable level.

In CW, THC biosynthesis showed a very similar trend to that of CBD production (Figure 2a,c). However, THCAS gene expression had a descending trend during flower development in GG (Figure 2d, bars). THC content in both CW and GG increased steadily (Figure 2c,d, black lines). Importantly, the findings on THC production indicate that in marijuana GG, increased THC biosynthesis does not rely on the upregulation of THCAS gene expression during flower development. THCAS transcript may have been at higher levels at the beginning of
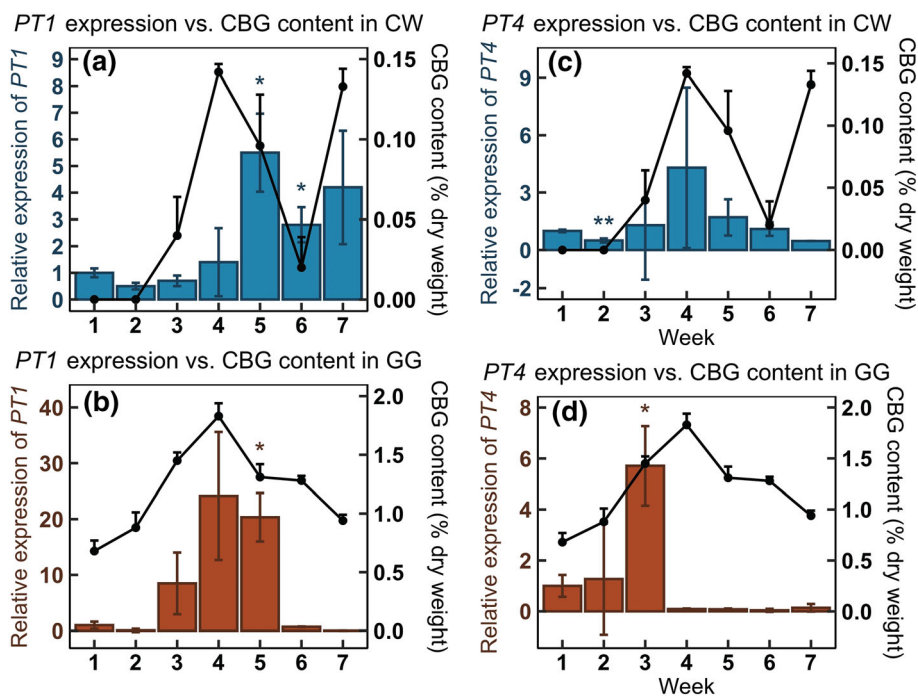

FIGURE 1 The remaining CBG amount and gene expression of PT1 and PT4 over the course of 7-week flower development in CW (a, c) and GG (b, d). Y axis on the left show relative expression of PT1 and PT4. Y axis on the right show the percentage of CBG amount in dry flower samples. Data are presented as means ± SE (n = 4). Positive direction error bars are shown for the line. Significant difference of P < .05 (*) and P < .01 (**) was determined by comparing to week 1 using Student's t test

flower development in GG and/or the dramatic increase in THC levels as marijuana flowers mature may not be rate-limited by the level of *THCAS*. Decreased *THCAS* expression in later stages in GG might be a feedback regulation leading to diminished *PT* expression, and then resulted in CBG reduction (Figure 1b,d).

Even though CBC levels are typically very low compared to the other two end-point cannabinoids, *CBCAS* was still highly upregulated

in both varieties (Figure S3). CBC content did not rise above 0.5% dry weight in CW.

GPPS is an enzyme that produces geranyl pyrophosphate (GPP), the precursor for both terpenes and cannabinoids in cannabis (Allen et al., 2019) (Figure S1). In CW, *GPPS* showed a similar expression pattern to the synthase genes despite that *GPPS* expression was abolished in week 7 (Figure 3a). We further examined *TKS* and *OAC*
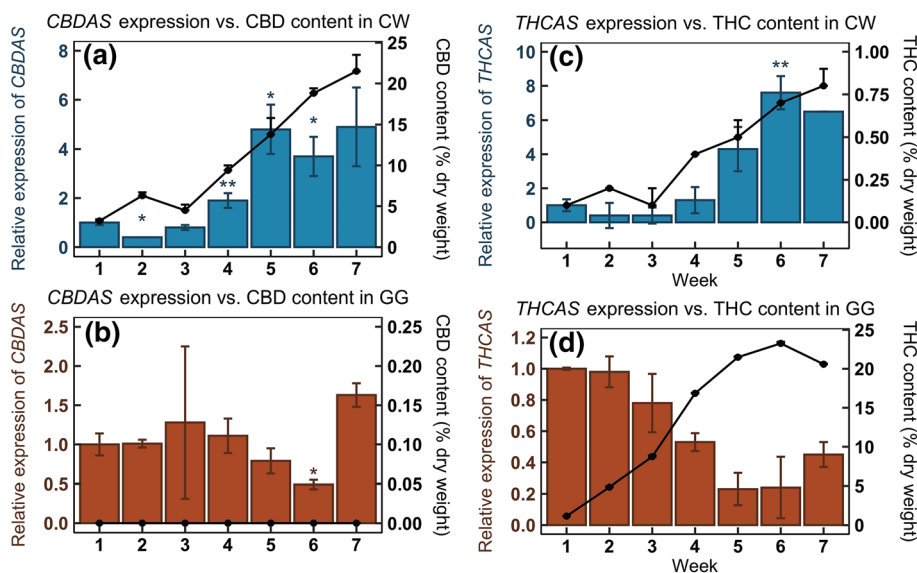

FIGURE 2 Analysis of CBD and THC accumulation with the expression of cannabinoid synthase genes over the course of 7-week flower development. (a, b) Superimposition of CBD content with *CBDAS* expression in CW (a) and GG (b). (c, d) Superimposition of THC content with *THCAS* expression in CW (c) and GG (d). Data are presented as means ± SE (n = 4). Positive direction error bars are shown for the line. Significant difference of $P < .05$ (*) and $P < .01$ (**) was determined by comparing to week 1 using Student's *t* test
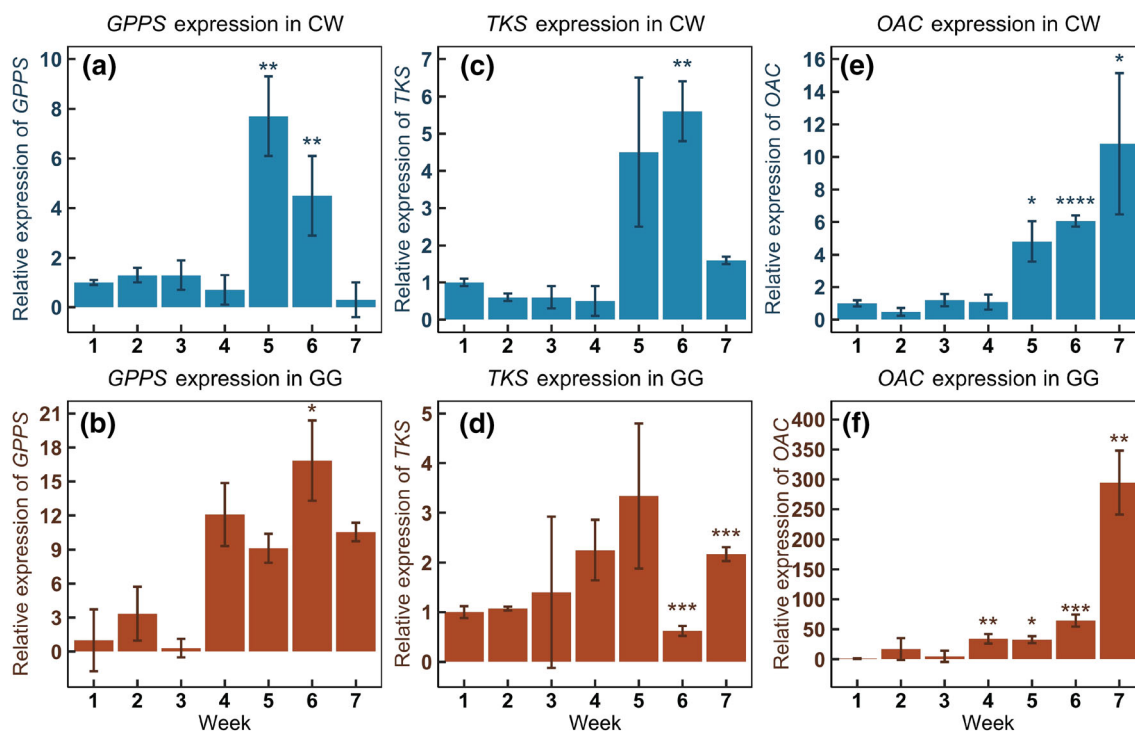

FIGURE 3 Expression of upstream genes in the cannabinoid biosynthetic pathway over the course of 7-week flower development. (a, c, e) Expression of *GPPS*, *TKS*, and *OAC* in CW. (b, d, f) Expression of *GPPS*, *TKS*, and *OAC* in GG. Data are presented as means ± SE (n = 4). Significant difference of $P < .05$ (*), $P < .01$ (**), and $P < .001$ (***) was determined by comparing to week 1 using Student's *t* test

expression, the genes that produce OA. *TKS* expression showed a similar pattern to that of *GPPS* in CW (Figure 3c). *OAC* expression showed a more similar trend to the synthase genes (Figure 2) but not *GPPS* and *TKS*. *OAC* expression continued increasing till week 7 (Figure 3e).

In GG, *GPPS* expression was highly upregulated starting in week 4 (Figure 3b). *TKS* expression did not increase as much as that of *GPPS* but still demonstrated a similar expression pattern (Figure 3d). It is possible that *TKS* transcripts are already abundant in GG to facilitate sufficient OA production for cannabinoid synthesis. Interestingly, unlike other genes, *OAC* expression was highly induced with a dramatic 300-fold increase in week 7 compared with week 1 (Figure 3f). Because GPP is also the precursor for monoterpenes (Allen et al., 2019), greater upregulation of *GPPS* is still necessary to produce ample amount of GPP. The different expression patterns of *GPPS* in CW and GG also suggest discrete regulation of monoterpene production in the two varieties.

3.2 | Correlation analysis of cannabinoid biosynthetic genes and cannabinoid production

Overall, the expression of cannabinoid biosynthesis genes follows a similar trend during flower development. The genes were upregulated in the middle stages of flower development, and cannabinoid accumulation increased steadily as flowers develop. We analyzed the correlation between the gene expression and cannabinoid content using Pearson's correlation matrix; this analysis integrates the expression patterns and cannabinoid production across the entire flowering cycle to evaluate any potential associations between expression patterns and metabolite production.

In CW, correlation matrix analysis clearly showed that the expression of *PT1* and *PT4* was positively correlated with cannabinoid content. In general, *PT1* had stronger and significant positive correlations

with genes and contents of end-point cannabinoids than *PT4* did (Figure 4a). In addition, expression of both *CBDAS* and *THCAS* was positively correlated with CBD and THC production respectively in CW (Figure 4a). *GPPS* has positive but weak correlation with cannabinoid production in CW.

Interestingly, in GG, there was a strong negative correlation between the expression of *THCAS* and THC (Figure 4b), suggesting that increasing *THCAS* transcript levels would not promote THC synthesis. *PT1* expression had a weak positive correlation with THC content, while *PT4* expression had a negative correlation with THC production. *GPPS* expression was significantly associated with THC production in GG. THC production in GG has positive correlations with the steps for CBG production but not with *THCAS*, suggesting that CBG production but not *THCAS* transcript level is critical for THC biosynthesis. OA is a special component in cannabinoid biosynthetic pathway, and there is a positive correlation between *OAC* expression level and cannabinoid production in both varieties.

3.3 | Effects of MeJA on cannabinoid production

MeJA has been shown to enhance secondary metabolites production in various plant species (Chen et al., 2006; Choi et al., 2005; Jiang et al., 2017; Kim et al., 2006; Yan et al., 2017). We examined how MeJA treatment could regulate cannabinoid production in a marijuana variety, "White Tangy Haze."

One week following application, only plants sprayed with 1-mM MeJA had significantly higher THC (Figure 5a) and total cannabinoid levels (Figure S4A) than those treated with water. All the treatments in the second week ameliorated THC production compared with the water control. More interestingly, plants treated with 100-μM MeJA produced a similarly high level of THC (Figure 5b) and total cannabinoid contents (Figure S4B) to those treated with 1-mM MeJA. The
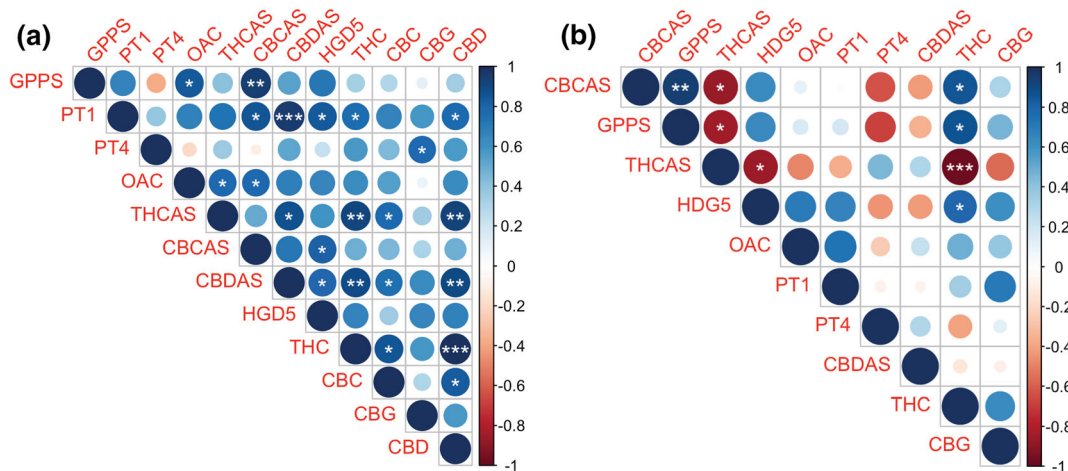

FIGURE 4 Graphic representation of correlation matrices demonstrates the correlation plot of cannabinoid biosynthetic genes and metabolites for CW (a) and GG (b). Blue indicates a positive correlation, while red indicates a negative correlation. Size and color of circle represent strength of correlation. Figures were generated using the R corrplot package https://github.com/taiyun/corrplot. (*, P < .05; **, P < .01; ***, P < .001). Percentage data was arcsine transformed prior to statistical analysis
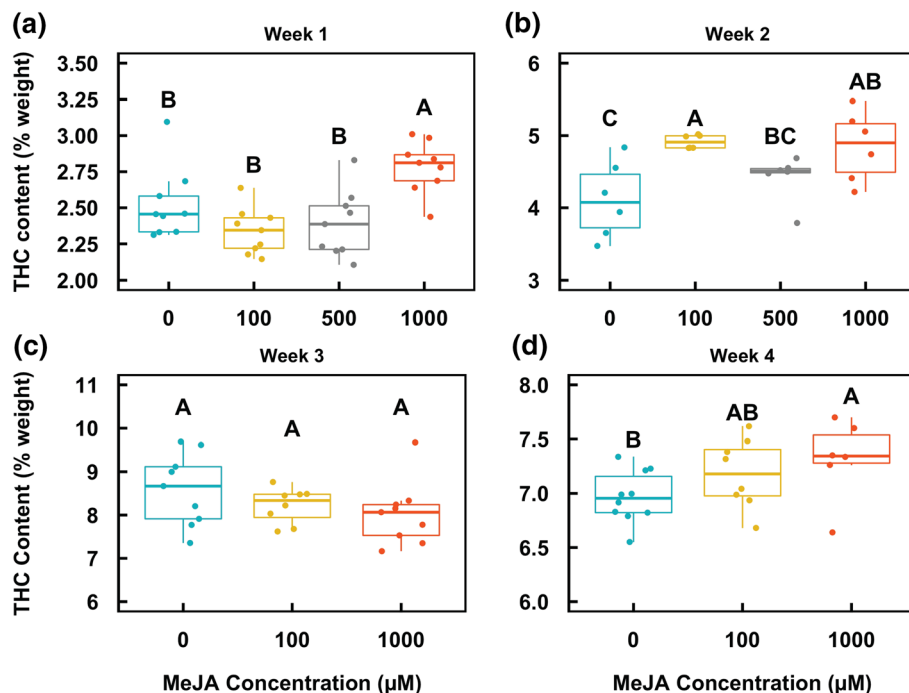
FIGURE 5 THC content in White Tangy Haze flowers over the period of 4 weeks (a–d) following application of MeJA. The box whiskers indicate variability outside the upper and lower quartiles (the upper and lower lines of the box) ($n = 3$). Different letters indicate statistical significance ($P < .05$) determined by one-way ANOVA followed by Fisher's LSD. Percentage data were arcsine transformed prior to statistical analysis. The untransformed data are presented

effect of MeJA was diminished 3 weeks after application (Figure 5c). The positive effects of 100-μM MeJA on cannabinoid production was no longer evident 4 weeks after treatment, while 1-mM MeJA could still significantly enhance THC production compared with control (Figure 5d). The results of this experiment suggest that MeJA may be efficacious for enhancing cannabinoid production and could be used to increase cannabinoid production in cannabis growth industry.

4 | DISCUSSION

4.1 | CBGA biosynthesis is the rate-liming step in the cannabinoid biosynthetic pathway

We provide an analysis of the correlation between cannabinoid production and expression of the biosynthetic genes in both hemp and medical marijuana. In general, both hemp CW and medical marijuana GG showed gene upregulation during female flower maturation, indicating that the regulation of cannabinoid biosynthetic genes is also associated with trichome development. Livingston et al. (2020) showed that cannabinoids are primarily accumulated in the GSTs, which are abundant during the later stages of flower development. Our analysis showing increased expression of the biosynthetic genes during mid to late stages of flower development is consistent with their observation that cannabinoids are primarily accumulated in the later GST stage. Some genes were substantially reduced in week 6 and/or week 7. However, it may not indicative of protein level or enzyme activity, and the enzymes may remain active even though there is no more mRNA generated.

Our findings on the negative correlation between *THCAS* gene expression and THC production is consistent with previous limited

analyses of this relationship that *THCAS* was downregulated during flower development despite the increase in THC levels in the latter stages (Liu et al., 2021; Richins et al., 2018). In addition, Muntendam et al. (2012) did not find strong correlations between synthase enzymes and their corresponding products. Our and other studies imply that transcript level of the synthase genes is not critical for cannabinoid production in the commercial strains. This is possibly because that the targeted breeding for high cannabinoid production has led to generation of cannabis strains containing higher synthase transcript levels throughout the flowering cycle. Figure 1 showed that the remaining CBG was extremely low because the majority has been rapidly used for terminal cannabinoid biosynthesis, suggesting that this step is unlikely to be rate-limiting. Therefore, increasing the synthase levels as future breeding targets may not enhance cannabinoid production.

We further showed that the expression levels of *PT* genes, *GPPS*, *TKS* and *OAC* were also significantly increased (Figures 1 and 3) as the flowers matured. Based on our findings in GG, we propose that THCA production is mainly determined by the expression and activity of *PT* and *GPPS*, the steps leading to CBGA production. It has been shown in several plant species that overexpression or upregulation of GPPS increased monoterpenes (Chuang et al., 2018; Xi et al., 2016; Yin et al., 2017). Pearson's matrix further demonstrated that *GPPS* is highly correlated with THC in GG (Figure 4b). It is reasonable to propose that increasing GPP production could enhance CBGA amount and eventually cannabinoid production in cannabis. In addition, due to the dual function of GPP as a precursor for both monoterpenes and cannabinoids, increasing *PT* expression would allow more GPP to be allocated for CBGA production.

We included *HDG5*, a TF that we believe may be involved in glandular trichome initiation, in the correlation analysis (Figure 4). *HDG5*

expression showed positive correlation with cannabinoid contents, suggesting that glandular trichome development could also be essential for cannabinoid production. The discussed genes could be targets for breeding.

4.2 | Hemp and marijuana differ in transcriptional regulation of cannabinoid biosynthetic genes

Our transcript analysis suggests different transcriptional regulation of cannabinoid biosynthesis in hemp and medical marijuana. *PT1* expression in CW increased in week 5 and was maintained at a similar level (Figure 1a), while *PT1* expression in GG increased week 3 to week 5 but was completely abolished in later weeks (Figure 1b). These findings suggest that the *PT1* may be distinctly regulated in hemp and marijuana. In addition, *GPPS* expression in GG and CW also showed temporally different regulation (Figure 3a,b). Distinct monoterpene profiling could also be a feedback regulation of *GPPS* expression in the two varieties.

We showed that the change of *THCAS* transcription in CW follows the general upregulation trend, while CW does not produce as much THC as GG. To learn if there are mutations in *THCAS*, we sequenced CW *THCAS* and no mutation was found. By comparing *THCAS* transcripts in CW and GG, we found that GG *THCAS* expression was hundreds of folds higher than CW *THCAS* (Figure S5), indicating that CW *THCAS* is tremendously suppressed. However, *THCAS* in CW was still upregulated and contained a problematic level of THC. Our work does not identify a causal relationship between *THCAS* expression in hemp with the problematically high THC level, although this may be a reasonable assertion. Therefore, suppression or knockout of *THCAS* in commercial hemp strains with viable *THCAS* genes could be an important breeding goal for high CBD hemp.

4.3 | MeJA regulates cannabinoid biosynthesis

MeJA is the plant hormone that plays important roles in secondary metabolite production and glandular trichome development (Boughton et al., 2005; Montiel et al., 2011; Shen et al., 2016; Wang et al., 2010; Yan et al., 2017). However, there is to date no publication examining the effect of MeJA on cannabinoid production in cannabis flowers. We showed that 100-μM MeJA was as effective as 1-mM MeJA to enhance THC production 2 weeks after treatment (Figure 5b).

Studies have shown that MeJA controls secondary metabolite biosynthesis by modulating TFs that regulate glandular trichome formation and biosynthetic genes. MeJA upregulated TFs that induce glandular trichome formation and increased artemisinin content in *Artemisia* and terpene production in tomato (Chalvin et al., 2020; Schuurink & Tissier, 2020; Yan et al., 2017). Furthermore, JA responsive TFs in various plant species directly or indirectly regulated transcription of terpene biosynthetic genes (Li et al., 2015; Nolan et al., 2017; Shen et al., 2016; Van Der Fits & Memelink, 2000). The

alteration of these MeJA upregulated TFs resulted in changes in secondary metabolite levels. Based on our findings, MeJA could also play vital roles in cannabinoid and secondary metabolite biosynthesis in cannabis. It would be critical to identify TFs in cannabis that are responsible for glandular trichome development and transcriptional regulation of cannabinoid biosynthetic genes. The further characterization of HDG5 initially reported here in preliminary fashion may identify the first native cannabis TF associated with trichome morphogenesis.

CONFLICT OF INTEREST

The authors declare no conflict of interest associated with the work described in this manuscript.

AUTHOR CONTRIBUTIONS

PVA, YM, and GAB designed the research; PVA and LBS performed experiments and analyzed data; PVA, YM, and GAB wrote the manuscript; GAB and YM supervised the research.

DATA AVAILABILITY STATEMENT

The data presented in this paper will be publicly available after publication without further permission from the authors.

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