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Studies of the Major Gene Expression and Related Metabolites in Cannabinoids Biosynthesis Pathway Influenced by Ascorbic Acid

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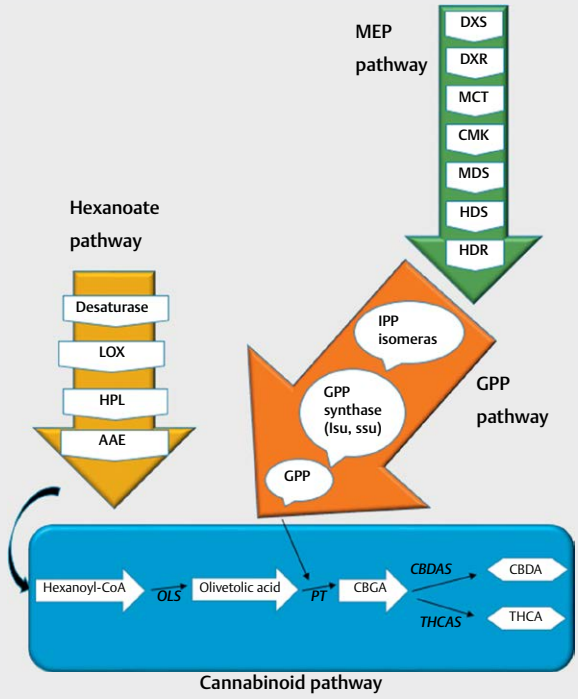
ABSTRACT

Cannabis sativa L. is an annual dioecious plant that belongs to the Cannabaceae family and is essential for different pharmaceutical and nutritional properties. The most important and prevalent cannabinoids in cannabis are cannabidiol and delta-9-tetrahydrocannabinol. The application of elicitors is an effective method to improve secondary metabolite production, leading to a whole spectrum of molecular, genetic, and physiological modifications. Therefore, the expression changes of four key genes (*THCAS*, *CBDAS*, *PT*, and *OLS*) of the cannabinoids pathway along with the delta-9-tetrahydrocannabinol and cannabidiol metabolites fluctuation were surveyed following the application of ascorbic acid as an elicitor. Cannabis was sprayed immediately before flowering with ascorbic acid. Treated and untreated (control) plants were sampled in different time courses for real-time PCR and HPLC experiments. Results showed significant increases in *THCAS*, *CBDAS*, *PT*, and *OLS* expression after ascorbic acid treatments. The results of metabolite quantification also indicated that secondary metabolites, especially delta-9-tetrahydrocannabinol and cannabidiol, increased after the ascorbic acid application. This study contributes to the growing body of knowledge of the functions of key genes in the cannabinoids pathway to the engineering of cannabis for improving the production of delta-9-tetrahydrocannabinol and cannabidiol metabolites in this plant.

ABBREVIATIONS

AA	ascorbic acid
AIDS	acquired immune deficiency syndrome
GABA	γ-aminobutyric acid
CBD	cannabidiol
CBDA	cannabidiolic acid
CBDAS	cannabidiolic acid synthase
CBGA	cannabigerolic acid
GPP	geranyl pyrophosphate
CsOAC	*Cannabis sativa* olivetolic acid cyclase
CsPT4	*Cannabis sativa* prenyltransferase
CsTKS	*Cannabis sativa* tetraketide synthase
MEP	methylerythritol 4-phosphate
OA	olivetolic acid
OAC	olivetolic acid cyclase
OLS	olivetol synthase
PT	aromatic prenyltransferase
RT-qPCR	real-time quantitative PCR
ROS	reactive oxygen species
THC	delta-9-tetrahydrocannabinol
THCA	tetrahydrocannabinolic acid
THCAS	tetrahydrocannabinolic acid synthase
TKS	tetraketide synthase



▶ **Fig. 1** Cannabinoids biosynthetic pathway.

Introduction

Cannabis sativa L. is a source of food, fiber, and drugs in Asia and Europe. Cannabis is a native plant of countries in Central, South East, and Western Asia as well as Eurasian countries, including Russia, and in China, India, Pakistan, and Iran. In particular, cannabis is a native plant of the Indian Himalayan Region and this plant grows abundantly in nonarable lands [1]. Medicinal plants such as cannabis have been used as an important pharmaceutical source. In the past, cannabis (Cannabaceae, hemp, marijuana) was consumed as a psychoactive herbal and medicinal drug [2, 3], but currently, among illegal drugs, it has the highest consumption around the world [4]. There are growing activities to legalize marijuana because of its medicinal advantages [5]. So far, more than 545 different compounds such as CBDA and THCA have been isolated and identified from cannabis. Despite intoxication effects, THCA and CBDA compounds in marijuana show various therapeutic activities such as neuroprotective effects, analgesia, antipyretic, and appetite stimulation in AIDS patients, and is used in the treatment of multiple sclerosis, Parkinson's disease, and various cancers [6–9].

MEP and hexanoate pathways are the two possible pathways that have been proposed for the biosynthesis of cannabinoids using isoprenoid precursors and fatty acids (▶ **Fig. 1**). The first pathway is conducted by olivetolic acid synthase that catalyzes the hexanoyl-CoA condensation using three malonyl-CoA molecules to generate OA [10–12]. Next, GPP pentylates olivetolic acid [13, 14]. Decarboxylation is the last step in the first pathway [15]. The second pathway is hexanoyl-CoA biosynthesis, and the substrate in this pathway is hexanoate [16]. In the next step, OAC and TKS/OLS coordinatedly catalyze the conversion of hexanoyl-CoA into olivetolate [17]. Gene expression encoding CsTKS and CsOAC produces OA, prenylated by CsPT4. CBGA transformation to cannabinoid acids THCA and CBDA is mediated by cannabinoid synthases THCAS and CBDAS. Heat exposure decarboxylates THCA and CBDA to THC and CBD, respectively [18].

Biosynthesis of a cannabinoid mainly occurs in female flower glandular trichomes [19]. Low-density glandular trichomes are observed in male flowers in which trichomes are in a row on the inner surfaces of anthers [20]. Low concentration levels of active constituents in medicinal plants such as cannabis result in a major challenge since the syntheses of metabolites are usually complicated and costly [21]. There are several strategies to increase the concentrations of secondary metabolites, such as manipulation of biosynthesis pathways by genetic engineering approaches, stimulation of secondary metabolite production by various biotic and abiotic elicitors as well as integrated methods.

The application of elicitors, including jasmonic acid, AA, phenylalanine, nitric oxide, and chitosan, is a safe and inexpensive approach that has been used to accumulate different secondary metabolites [22, 23]. Specifically, the application of jasmonic acid increases production of triterpenic saponins, leading to decreases in sterols [24]. In addition, the application of elicitors such as salicylic acid can alleviate toxicity in plants [25]. AA plays major roles in different plants' molecular and biological processes, such as protein synthesis [26], production of defense compounds [27], senescence [27], ozone tolerance [28], ROS detoxification [26], cellular growth and division, electron donation, cellular H_2O_2 regulation, and phenolic compounds annihilation [23]. Understanding the mo-

lecular basis of the elicitors' effects in the molecular and metabolic levels is a crucial step for improving the production of the metabolites. To this effect, this study analyzes the key gene expression changes of the cannabinoids pathway and quantifies metabolite fluctuations following the application of AA.

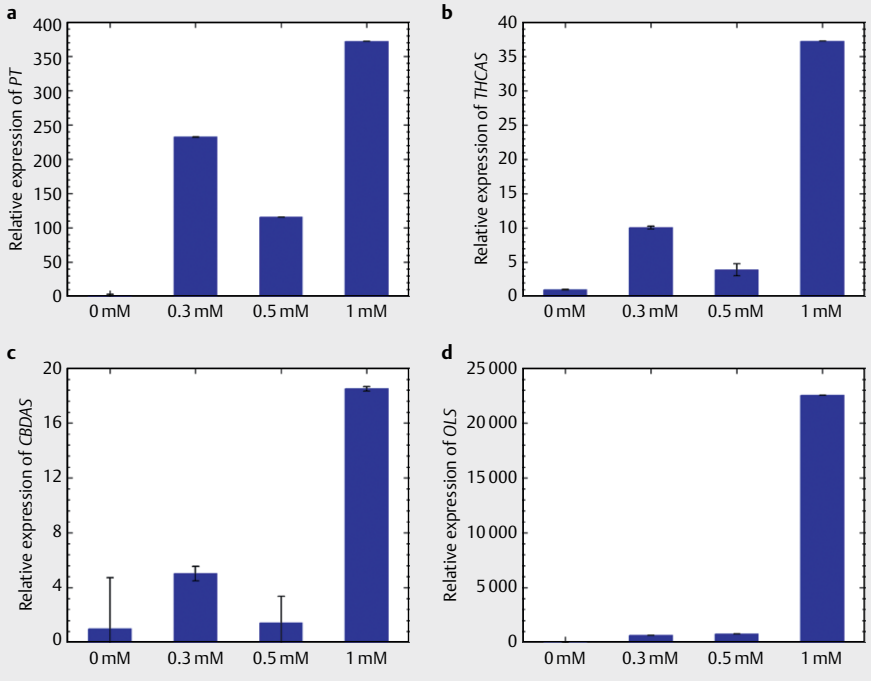
Results and Discussion

Cannabinoids are unique plants with polyketide biosynthesized from hexanoate or hexanoate-CoA precursors. Fatty acyl-CoAs are considered polyketide primers in many microbes and plants. However, only a few examples of hexanoate/hexanoyl-CoA originating from polyketides exist in fungi and plants [29]. A promising substitute is the application of exogenic derivers to produce scaleable amounts of cannabinoids [30]. Therefore, the identification of key gene functions is a crucial step to improve cannabinoid biosynthesis by application of elicitors. In this study, cannabis plants were sprayed with 0–1 mM AA. For commercial purposes, the best time to spray AA on cannabis is before flowering time. Here, experiments were performed on four main gene expressions, and active ingredients in cannabis were measured.

▶ **Fig. 2** shows the effects of AA on the expression of four selected genes. ▶ **Fig. 2a** shows that treatment with 0.3 and 0.5 mM AA increases *PT* expression approximately 232 and 115 times, respectively, more than the control group. The highest level of *PT* expression occured after the 1 mM AA treatment (▶ **Fig. 2a**). Similarly, ▶ **Fig. 2b** shows the increased expression of *THCAS* to be ten and five times that of the control group after treatment with 0.3 and 0.5 mM AA, respectively. At the 1 mM AA treatment, the expression of *THCAS* increased 37 times more than the control group (▶ **Fig. 2b**).

▶ **Fig. 2c** shows AA significantly increases *CBDAS* expression at the 0.3 and 1 mM treatments. ▶ **Fig. 2d** shows that treatment with 1 mM AA increases the *OLS* expressions about 20000 times higher than the control group. AA in low concentrations does not have a significant effect on *OLS* expression (▶ **Fig. 2d**). Similarly, Jalali et al. [31] investigated the target genes' relative expression, including *THCAS*, *OLS*, *CBDAS*, and *PT*, in addition to compounds CBD and THC with quantitative analysis using HPLC, which revealed the highest activation of *THCAS* was 0.1 mM GABA and 1 mM of salicylic acid concentration. They also showed an increase of approximately 90 % in the THC level and a decrease in the CBD level 72 h after treatments with 1 mM salicylic acid. The *THCAS* and *CBDAS* genes play key roles in the polyketide (OA) pathway, and *PT* and *OLS* genes play important roles in the hexanoate pathway.

Oxidocyclase THCAS converts *CBGA* to *THCA*, while CBDAS produces *CBDA* [17, 32]. Increases in the expression of key genes of two pathways indicate that AA simultaneously activates the two pathways for cannabinoids biosynthesis. As shown in ▶ **Fig. 2**, the *OLS* and *PT* expression changes are significantly greater than *THCAS* and *CBDAS* gene experessions. To our knowledge, there is not any report that describes the effects of AA on key gene expression processes in cannabinoid flowers. A former study analyzed the whole flower with trichomes and showed readily detectable levels of hexanoyl-CoA and the highest concentrations in the female flowers in comparison to their other tissues, including leaves, roots, and stems [16]. The members of the superfamily of the acyl-activating enzyme forming hexanoyl-CoA caused carboxylic acid activation via the intermediary of an adenylate [33]. Plants contain various substrates, including fatty



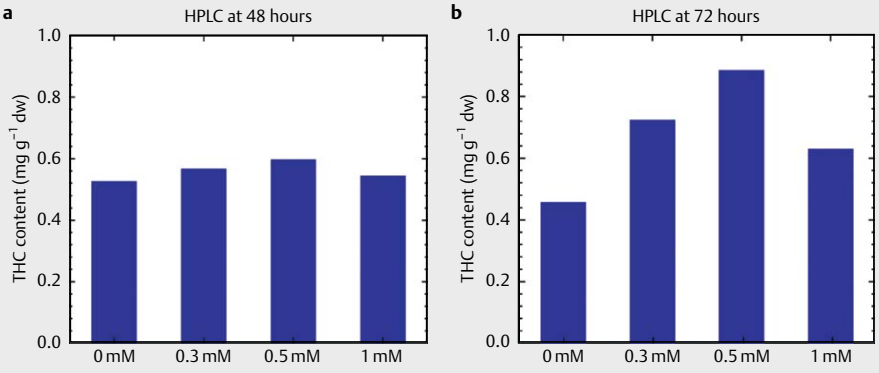
▶ **Fig. 2** Expression changes of *THCAS*, *CBDAS*, *OLS*, and *PT* following the treatments with different concentrations of ascorbic acid. Values represented the means of 3 replications ± standard deviation.

acids, phenylpropanoids, and jasmonate precursors, to activate carboxylic acids. The enzyme 4-coumarate:CoA ligase (4 CL) engages in the metabolism of phenylpropanoid in addition to the long-chain acyl-CoA synthetases, which are well-identified plant-activated carboxylic acids [34].

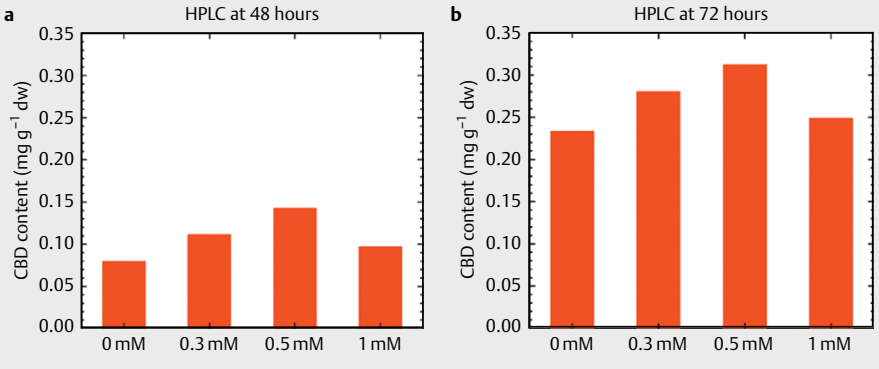
The analysis of gene expression along with the metabolic change improves our understanding of elicitors' underlying mechanisms. This study quantifies the metabolic changes of CBD and THC following AA treatment. ▶ **Fig. 3a**, **b** shows THC levels at 48 and 72 h, respectively. At 48 h, THC content levels are in the range of 0.53–0.6 mg.g^{-1} d.w. or all AA treatment concentrations. As the time increases to 72 h, the THC metabolite concentration increases by 91.3 % with respect to no treatment. The maximum THC content is 0.88 mg.g^{-1} d.w. and occurs at the 0.5 mM AA treatment. ▶ **Fig. 4a**, **b** shows CBD levels at 48 and 72 h, respectively. At 48 h, CBD levels are between 0.08 and 0.14 mg.g^{-1} d.w. for all AA treatment concentrations. At 72 h, the CBD concentration increases by 34.7 % for the 0.5 mM AA treatment. In general, the maximum level of THC and CBD occurs at the 0.5 mM AA treatment (▶ **Figs. 3** and ▶ **4**). Furthermore, the THC and CBD levels at the 1 mM AA treatment are lower than the 0.5 mM treatment. It is expected that the THC and CBD levels increase for the 1 mM AA treatment over longer time frames.

The trends observed here are in agreement with the literature. Secondary metabolite biosynthesis induction by elicitors has been reported in *Arachis hypogaea, Vitis vinifera,* and *Papaver somniferum* [35–37]. The accumulation of the secondary metabolites, primarily flavonoids, phenolic compounds, hydroxycinnamate esters, and anthocyanins, have been shown in the plant cells. As a result of the accumulation of these secondary metabolites, the penetration of UV-B radiation into deeper cell layers and ROS detoxication decrease [38, 39]. ROSs rise due to UV-B, jasmonic acid, and salicylic acid, which can affect the secondary metabolite biosynthesis. Therefore, one may also expect that UV radiation induces secondary metabolite biosynthesis in cannabis. Increased irradiance leads to an elevated total THC concentration in the plants [40] along with an increased photosynthetic rate and water use efficiency [41]. Specifically, it has been reported that the amount of THC and CBD dramatically increased in cannabis leaves following treatment with 100 mM GA3 [42].

Existing data reveals that abiotic and biotic derivatives may elevate vitamin C concentrations in the plant tissues. One study shows that vitamin C level increases in lettuce due to jasmonic acid



▶ **Fig. 3** Effect of ascorbic acid on THC metabolites following the treatments with different concentrations of ascorbic acid in cannabis plants at 48 hours (**a**) and 72 hours (**b**).



▶ **Fig. 4** Effect of ascorbic acid on CBD metabolites following the treatments with different concentrations of ascorbic acid in cannabis plants at 48 hours (**a**) and 72 hours (**b**).

▶ **Table 1** Sequences of the primer genes used to analyze qPCR gene expression.

Primers	Amplicon length	Forward primer (5'→3')	TM (°C)	Reverse primer (5'→3')	TM (°C)	Accession Number
THCAS	148	GATCAGCTGGGAAGAAGACG	60.5	ATACCACCGTAAGGGTACAACA	60.1	AB057805
CBDAS	188	CAATTCCAGAATCTGTATTTGTCC	60.3	TTCTTGCTTCTCCCAACTACATA	59.2	AB292682
OLS	145	TTCTTGCTTCTCCCAACTACATA	59.2	ACGCACGACCACTCTTTCG	59.5	AB164375.1
PT	150	CTCGTAAAATCTTCTGCCTGCT	60.1	TTCTTGCTTCTCCCAACTACATA	59.2	AB164375.1
rbcl	106	CTACTGGTACATGGACAACTG	59.5	AATTGATTTTCTTCTCCAGCAACG	60.3	AJ402933
TM: primer melting temperature						

treatment [43]. On the other hand, chitosan and salicylic acid are known to be associated with indirect vitamin C stimulation in broccoli through carbohydrate formation, including glucose and sucrose, which are vital players in the l-ascorbate biosynthetic pathway [44]. Increased CBD and THC levels confirm the expression changes of cannabinoid biosynthetic genes after AA treatment. These findings suggest that after AA treatment, the majority of THC and CBD metabolites are produced from the hexanoyl-CoA pathway.

Overall, the findings demonstrate that AA increases THC, CBD, and secondary metabolite production. Also, the results highlight the efficiency of AA as an elicitor for the scalable increase of cannabinoid production from *C. sativa* L. as a natural source for products with pharmaceutical applications.

Materials and Methods

Plant material and ascorbic acid treatment

C. sativa L. seeds with accession number 891385 were obtained from the CGN seed bank located in the Netherlands. After sterilization using sodium hypochlorite 1.5 % solution for 3 min, seeds were transferred to a mixture of soil, peat, and perlite (2:1:1) containing pots. The female plants of cannabis were selected for AA treatment. A total number of 500 seeds was sown and 80 % of these seeds were germinated. Among the germinated plants, 30 % were female plants. To prevent the reduction of metabolite concentrations following the pollination, male plants were removed immediately after the appearance of early flowers. Immediately before flowering, plants were treated by spraying with 0.3, 0.5, and 1 mM AA. Moreover, some seedlings were treated with distilled water as a control group. Seventy-two hours later, flower samples were taken, immediately frozen in liquid nitrogen, and kept at a temperature of −80°C.

RNA extraction and cDNA synthesis

RNAs were extracted using frozen female flower samples using bioZOL-G RNA Isolation Reagent (Biofluxbioer) following the instructions provided by the manufacturer. RNA quantity and quality were analyzed using agarose gel electrophoresis and NanoDrop spectrophotometry ND1000. DNase I was applied to remove genomic DNA contamination (Fermentase) following the instructions provided by the manufacturer. cDNA was produced using a iScript cDNA synthesis kit manufactured by Bio-Rad.

Gene expression analysis by real-time quantitative PCR

RT-qPCR on a Light Cycler 96 system (Roche Co.) was conducted using a HiFi SYBR Green Master Mix (5 ×). The 20 µL RT-qPCR reaction was comprised of 4 µL SYBR R PremixEx TaqTM (2 ×), 0.5 µL of each forward and reverse primer (10 Pmol/mL), and 4 µL of 1:5 diluted cDNA. The cycling parameters used were 15 min at 95°C as initial activation, 45 cycles of 15 sec at 95°C, 20 sec at 60°C, and 20 sec at 72°C. The $2^{-\Delta\Delta CT}$ method was performed to analyze the expression data [45]. Three biological replicates were used as a data source and are presented as mean values ± standard deviation. To assess the significance level, Student's t-tests were used. P values lower than 0.05 were used as a baseline of significance. Gene expression levels were normalized to *rbcl* as housekeeping genes. Based on the available sequences in NCBI, specific primers for the candidate genes, including the *CBDAS*, *THCAS*, *PT, OLS*, and *rbcl*, were developed using Plus Primer 3 software (▶ **Table 1**). Subsequently, the physical parameters of the primers were confirmed by Oligo Calculator software.

Analysis of cannabidiol and tetrahydrocannabinol

Female plants (0.1 mg) were extracted and mixed with 2 mL methanol/chloroform (V/V: 9/1) by centrifugation at a speed of 10000 rpm and a temperature of 10 °C for 15 min followed by ultrasonication at a temperature of 50°C for 40 min. The upper phase was separated and filtered. Subsequently, the solution was dried, and the residue was dissolved in 1 mL of methanol and centrifuged at a speed of 13000 rpm for 10 min. The solutions were subjected to reversed-phase HPLC analyses. The standard solutions of CBD and THC (0.1 mg/mL) were supplied from Carillion Corporation, and the standards were derived based on the areas under the curves measured with HPLC (Agilent 1260 UV/Vis detector) at concentrations of 12.5, 25, and 50 µg/mL. The HPLC device was equipped with C18 LC columns. Plant solutions with concentrations of 12.5, 25, and 50 µg/mL were used to derive the standard curves of the THC and CBD. Twenty microliters of the solutions were injected into the HPLC instrument. Considering the standard samples' retention time, CBD and THC peaks were observed at the time of 13.6 and 28.7, respectively.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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