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.
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 such as CBDA and THCA has been isolated and identified from cannabis. Despite intoxication effects, THCA and CBDA compounds in marijuana show various therapeutic activities such as neuroprotective effects, analgesia, antiinflammatory, 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 coordinately catalyze the conversion of hexanoyl-CoA into olivetolate [17]. Gene expression encoding CsTKS and CsOAC produces OA, prenylated by CsPT4. CBG transformation to cannabinoid acids THCA and CBDA is mediated by cannabinoid synthases THCAS and CBDA. 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 [27], cellular growth and division, electron donation, cellular H2O2 regulation, and phenolic compounds annihilation [23]. Understanding the mo-
lecular basis of the elicitors’ effects in the molecular and metabol-
ic levels is a crucial step for improving the production of the me-
tabolites. 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. How-
ever, only a few examples of hexanoate/hexanoyl-CoA originating
from polyketides exist in fungi and plants [29]. A promising substi-
tute is the application of exogenic derivers to produce scalable
amounts of cannabinoids [30]. Therefore, the identification of key
gene functions is a crucial step to improve cannabinoid biosynthe-
sis 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 ingre-
dients in cannabis were measured.

Fig. 2 shows the effects of AA on the expression of four select-
ed genes. Fig. 2a shows that treatment with 0.3 and 0.5 mM AA
increases PT expression approximately 232 and 115 times, respec-
tively, more than the control group. The highest level of PT expres-
sion occurred 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 expres-
sion 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, includ-
ing THCAS, OLS, CBDAS, and PT, in addition to compounds CBD
and THC with quantitative analysis using HPLC, which revealed the high-
est 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 treat-
ments 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 pro-
duces CBDA [17, 32]. Increases in the expression of key genes of two
pathways indicate that AA simultaneously activates the two path-
ways for cannabinoids biosynthesis. As shown in Fig. 2, the OLS
and PT expression changes are significantly greater than THCAS and
CBDAS gene expressions. 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 mem-
bers of the superfamily of the acyl-activating enzyme forming hex-
anoyl-CoA caused carboxylic acid activation via the intermediary
of an adenylate [33]. Plants contain various substrates, including fatty

![Fig. 2](image-url) Expression changes of THCAS, CBDAS, OLS, and PT following the treatments with different concentrations of ascorbic acid. Values repre-
sented 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
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 concentration following the pollination, male plants were removed immediately after the appearance of 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 ºC.

#### 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 an 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 ºC as initial activation, 45 cycles of 15 sec at 95 ºC, 20 sec at 60 ºC, and 20 sec at 72 ºC. The 2^ΔΔCT 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 ºC for 15 min followed by ultrasonication at a temperature of 50 ºC 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.

### Acknowledgments

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<table>
<thead>
<tr>
<th>Primers</th>
<th>Amplicon length</th>
<th>Forward primer (5′→3′)</th>
<th>TM (°C)</th>
<th>Reverse primer (5′→3′)</th>
<th>TM (°C)</th>
<th>Accession Number</th>
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<td>CBDAS</td>
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<td>AJ402933</td>
</tr>
</tbody>
</table>

TM: primer melting temperature
Conflict of Interest

The authors declare that they have no conflict of interest.

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