

GENE EXPRESSION MARKERS FOR PREDICTIVE CANNABIS BREEDING THROUGH GAMMA RADIATION-INDUCED MUTAGENESIS

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Received: February 15, 2024; Revised: January 24, 2025; Accepted: February 28, 2025

Abstract

Enhancing cannabis varieties through plant breeding plays a pivotal role in meeting market demands. Identifying gene markers associated with THC and CBD synthesis could streamline the early selection process within a plant breeding program. To assess the efficiency of radiation-induced breeding of the Thai ‘Hang Kra Rog’ marijuana, two methods of radiation exposure are employed: exposing young seedlings to low doses of radiation and exposing seeds to high doses of radiation. The expression of the *THCAs* gene in seedlings exposed to 20 Gy and 40 Gy increased to 4.0 and 2.5 times as high as control, respectively. Seeds exposed to 200 Gy of gamma radiation exhibited a substantial increase in *THCAs* gene expression compared to control (2.4 times as high). Meanwhile, no evidence of *CBDAs* gene expression was observed. In chemical analysis using HPLC, seedlings exposed to a dose of 40 Gy showed a THC content of 3.9%, which was higher than control (2.8%). This trend was similar to the group of seeds irradiated with 200 Gy, which had a THC content of 2.7%, also higher than control (2.3%). Meanwhile, no detectable CBD presence was observed. There is a correlation between *THCAs* and *CBDAs* gene expression and their respective cannabinoid concentrations. In conclusion, the study outlines its potential contribution to cannabis breeding practices. Gamma radiation exposure is an effective method for inducing mutations in cannabis breeding. The gene expression markers can be used to predict cannabinoid profiles and support the selection of desirable traits of cannabis.

Keywords: Breeding; Cannabis; Gamma Irradiation; Gene Expression; Mutation

Introduction

Cannabis is an economically valuable plant that is legal in some countries. Its main compounds, Tetrahydrocannabinol (THC) and Cannabidiol (CBD), are found in the inflorescence and play an important role in medicine and pharmaceuticals (Chayasirisobhon, 2020). The concentration of THC and CBD in cannabis can vary widely depending on

factors such as the genetics, growing conditions, and processing methods (Naim-Feil *et al.*, 2023). Plant breeding is an important means to improve cannabis varieties and supporting the demand for medical use. Irradiation techniques are used for inducing mutations to create genetic diversity in plants for a long time. Gamma rays are most commonly used in

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DOI: <https://doi.org/10.55766/sujst3610>

Suranaree J. Sci. Technol. 32(2):020036(1-6)

plant breeding due to their high penetrating power through various kinds of plant samples. This method has been used for breeding various plants, including examples like bean (Ezzat *et al.*, 2021), rice (Morita *et al.*, 2009), potato (Roer, 1967), and corn (Killion and Constantin, 1972). Gamma rays offer a simple and efficient approach for plant breeding. These high-energy rays induce mutations by causing changes in the plant's DNA, leading to genetic variability. This variability is a valuable resource for breeders aiming to enhance specific traits, such as the production of secondary metabolites in annual vinca (*Catharanthus roseus*). Vinblastine and vincristine yield was quantified in *in vitro* grown tissues and leaves of embryo regenerated annual vinca after gamma ray treatment (Mujib *et al.*, 2022). The chemistry of THC and CBD synthesis in this plant is evidently reflected by the expression of related enzymes such THCA synthase (*THCAs*) and CBDA synthase (*CBDAs*). The *THCAs* gene comprises a 1635-nucleotide that is found in all marijuana cultivars (Sirikantaramas *et al.*, 2004). The *CBDAs* gene comprises a 1635 bp intronless open reading frame that is found in all CBDA-producing cultivars (Taura *et al.*, 2007). The *THCAs* and *CBDAs* are responsible for tetrahydrocannabinolic acid (THCA) and cannabidiolic acid (CBDA) synthesis, respectively. The amino acid sequences are also highly similar, with *THCAs* and *CBCAs* being 92% identical to each other (Sirikantaramas *et al.*, 2004). Besides, the acyl-activating enzyme 1 (*AAE-1* gene) (Stout *et al.*, 2012) and *Bet-v1* gene (Gagne *et al.*, 2012) have also been discovered to be involved in the biosynthesis of cannabinoids, which is a crucial substrate for the production of THC and CBD. Furthermore, reports indicate that the expression of the *THCAs* gene is only slightly detected in leaf samples from young plants, and the expression of the gene increases in correlation with the THC content in more mature plants (Cascini *et al.*, 2013).

The aim of this study was to identify potential genes that could predict a plant's capacity for THC and CBD synthesis, as well as to monitor genetic enhancements through radiation using gene markers. Candidate gene markers based on THC and CBD synthesis can facilitate the early identification process for a plant breeding program.

Materials and Methods

Plant Materials

The Thai cannabis samples, including, marijuana 'Hang Kra Rog', were obtained from Rajamangala University of Technology Isan Sakon Nakhon campus. There were three replicates for

each radiation dose and each replicate contained ten seeds.

Acute Gamma Irradiation and Planting

The 7-days-old seedling and seeds were exposed to acute gamma radiation using a Gamma chamber - GC 5000 with Co-60 source at Thailand Institute of Nuclear Technology (Public Organization), Nakhon Nayok, Thailand at a dose rate of 26.95 Gy/min. Each of the 10 seedlings, 7 days old, was exposed to gamma radiation at doses of 0, 20, and 40 Gy. Each of the 10 cannabis seeds was exposed to gamma radiation at doses of 0, 100, and 200 Gy. After irradiation, seeds were sowed in pots with mixed material used for planting consisted of equal parts of the following components: soil, peat moss, perlite, vermiculite, coconut coir, and organic soil, with a ratio of 1:1:1:1:1:1. and placed in a greenhouse. The water was applied daily to maintain moisture, and fertigation was employed to ensure proper nutrient delivery. The pots were maintained at a temperature of 25-30°C and a photoperiod of 12:12 (Light:Dark). Pruning techniques were also applied to encourage healthy growth. The plant were growth in the greenhouse of the institute during September 2021 - April 2022. Six months after planting, the leaves and inflorescence were collected for gene expression and High-performance liquid chromatography (HPLC) determination.

RNA Isolation and cDNA Synthesis

The mature leaves from the inflorescence were selected, with one plant chosen from each treatment. The leaves were bulked and ground in a mortar with liquid nitrogen, then 50-80 mg was taken for RNA extraction. Total RNA extraction from leaves were extracted using RNeasy Plant Mini Kit (Qiagen, Germany). RNA quality and purity were determined using spectrophotometer and gel electrophoresis. The 20 µg of total RNA was used as template for cDNA using QuantiTect® Reverse Transcription (Qiagen, Germany) according to the manufacturer's instructions and store at -20°C

Gene Expression by PCR

The gene expression as *THCAs*, *CBDAs*, *AAE-1*, *Bet-v1*, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) of the cannabis were analyzed using PCR (C1000™ Thermal Cycler, Bio-Rad, USA). The primer sequences used for indicate the cannabinoid content in the cannabis are shown in Table 1 The reaction for PCR contained OnePCR Ultra Supermix 6 µL, 10 pmol of each primer 1 µL, RNase free water 3.5 µL and cDNA template 1 µL. The positive control contained DNA with the gene of interest, and the negative control

Table 1. The sequences of primers used for PCR

Gene	Primer sequence (5'→3')	Amplicon size (bp)	Accession no.
<i>THCAs</i>	F: ATTATGGCCTTGCGGCTGAT R: TTCTCCTCCACCACCACGTA	122	MN422091.1
<i>CBDAs</i>	F: TGATAGATCCGCTGGGCAGA R: ACCGTAAGGGTACAACGCAT	148	NM001397936.1
<i>AAE-1</i>	F: GCGGTGGACCTGAGCAATTA R: AGAGGCACAACACGAGTGAC	148	JN717233.1
<i>Bet-v1</i>	F: CCGAAAATCGCTCCTCAAGC R: GTCGATCGCCTCGATCTTGT	280	JN679225.1
<i>GAPDH</i>	F: AATCGCAACCCAACTCTGC R: AGTGGCCGTTGCTTTAATGG	123 °	XM030632832.1 °

contained RNase-free water without the DNA template. A PCR was performed according to the optimum conditions to amplify the specific genes. Thermal cycling for *THCAs* and *CBDAs* at 94°C (10 min) for initial DNA denaturation, followed by 30 cycles of 94°C (1 min) for DNA denaturation, 60°C (1 min) for primers annealing and 72°C (2 min) for primer extension, with a final extension of the cycle at 72°C for 5 min. Thermal cycling for *AAE-1* and *Bet-v1* at 94°C (10 min) for initial DNA denaturation, followed by 30 cycles of 94°C (1 min) for DNA denaturation, 55°C (1 min) for primers annealing and 72°C (2 min) for primer extension, with a final extension of the cycle at 72°C for 5 min. Thermal cycling for *GAPDH* at 95°C (5 min) for initial DNA denaturation, followed by 30 cycles of 95°C (30 S) for DNA denaturation, 55°C (1 min) for primers annealing and 72°C (1 min) for primer extension, with a final extension of the cycle at 72°C for 7 min. The amplified PCR products were separated on 2% agarose gels. Voltage and time for gel electrophoresis 100V for 30 min to prove the amplification of each gene.

Gel Electrophoresis Image Analysis

To quantitate the band intensity, a gel electrophoresis image was captured and analyzed using the Scion Image program. The program was calibrated to convert the pixel values of the bands to signal intensities, which were expressed as relative optical densities (ROD). The ROD values were then used to compare the band intensities between different samples. The relative gene expression of *THCAs*, *CBDAs*, *AAE-1* and *Bet-v1* was normalized to *GAPDH* gene.

THC and CBD Determination by HPLC

The dried inflorescence samples were extracted and subjected to THC and CBD analysis according to In-house Method SOP 22 02 231 by HPLC of Thai herbal pharmacopoeia at the Bureau of Drug and Narcotic of Thailand.

Results and Discussion

Effect of Gamma rays on gene expression in Cannabis

In this study, the response of cannabis plants to gamma radiation and its effects on gene expression and cannabinoid content were investigated. The cannabis plants presented in Figure 1 compose of those grown from 7-days-old seedlings exposed with gamma ray at 0 (Figure 1(a)), 20 (Figure 1(b)), and 40 Gy (Figure 1(c)), and seeds exposed with 0 (Figure 1(d)), 100 (Figure 1(e)), and 200 Gy (Figure 1(f)). In terms of morphological, there is no difference between the irradiated and non-irradiated groups.



Figure 1. The inflorescence of marijuana 'Hang Kra Rog' at 5 months after planting. (A) seedling_control, (B) seedling_20 Gy, (C) seedling_40 Gy, (D) seed_control, (E) seed_100 Gy and (F) seed_200 Gy

The results highlighted the complexity of the relationship between radiation doses and gene expression of *THCAs*, *CBDAs*, *AAE-1*, and *Bet-v1* were analyzed using PCR (Figure 2). Out of the 4 genes studied, expression was observed in only 3 genes, namely *THCAs*, *AAE-1*, and *Bet-v1*. However, no expression of *CBDAs* was found in any of the samples. The presence of each gene in the agarose gel shown in Figure 2 was quantified based on band intensity using Scion image software.

The expression of different genes was then normalized to the housekeeping gene (*GAPDH*) and expressed as a ratio (Figure 3).

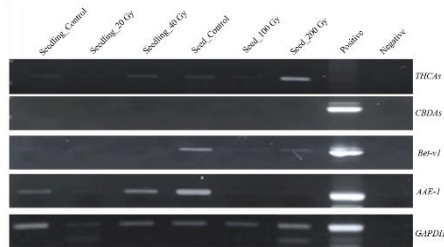


Figure 2. Agarose gel electrophoresis for PCR products, showing the expression of *THCAs*, *CBDAs*, *Bet-v1*, *AAE-1*, and *GAPDH* (housekeeping gene) in female leaves of cannabis

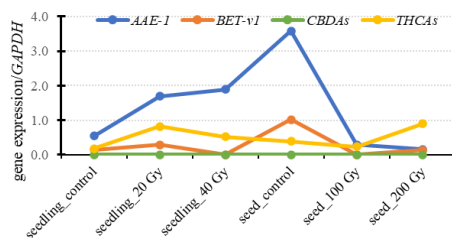


Figure 3. Relative gene expression of *THCAs*, *CBDAs*, *Bet-v1*, and *AAE-1* gene in female leaves of cannabis. The expression was calculated the band intensity using Scion image software

In irradiated seedlings, the groups exposed to 20 Gy and 40 Gy of radiation exhibited higher expression ratio of the *THCAs* and *AAE-1* genes compared to the control. The expression ratios for *THCAs* in irradiated seedlings at 20 Gy and 40 Gy were 0.8 and 0.5, respectively, which were higher than the control (0.2). The expression of the *THCAs* gene in seedlings exposed to 20 Gy and 40 Gy were 4.0 and 2.5 times as high as the control, respectively. Similarly, a consistent trend was observed for the *AAE-1* gene, with the gene ratios in irradiated seedlings at 20 Gy and 40 Gy being 1.7 and 1.9, respectively, which were also higher than the control value of 0.5. Relatively, the expression of the *AAE-1* gene in seedlings exposed to 20 Gy and 40 Gy were 3.4 and 3.8 times as high as the control, respectively. However, differences were noted for the *Bet-v1* gene, as the expression ratios for irradiated seedlings at 20 Gy and the control were 0.3 and 0.1, respectively, which exceeded the value observed in the irradiated seedlings at 40 Gy (0.0). The expression ratios of *THCAs* and *AAE-1* genes were found to be significantly elevated in irradiated

seedlings exposed to 20 Gy and 40 Gy of radiation, indicating a potential correlation between radiation exposure and cannabinoid synthesis-related gene expression. Additionally, the expression of the *Bet-v1* gene showed variations among different radiation doses, suggesting its role in the modulation of cannabinoid synthesis.

In irradiated seeds, the *THCAs* gene expression differed across radiation doses. At 200 Gy and in the control group, gene ratios were 0.9 and 0.4 respectively, higher than 100 Gy group. Seeds exposed to 200 Gy exhibited a significant increase in *THCAs* gene expression compared to controls (2.4 times as high). Contrastingly, *AAE-1* and *Bet-v1* genes in control showed higher expression than irradiated groups. *AAE-1* gene expression in control was 3.6, while at 100 Gy and 200 Gy, the values were 0.3 and 0.2, respectively. *Bet-v1* gene expression in control was 1.0, while at 100 Gy and 200 Gy, the values were 0.0 and 0.1 respectively. *AAE-1* and *Bet-v1* gene expressions were strongest in normal seeds, while *CBDAs* expression was absent across all samples. This absence could be attributed to genetic variability, as previous studies have shown that *CBDAs* expression is often linked to specific cannabis chemotypes (Bakel *et al.*, 2011). Alternatively, it may suggest a potential inhibitory effect of gamma radiation on the transcriptional activity of the *CBDAs* gene.

Effect of Gamma rays on cannabinoids content in Cannabis

The content of THC and CBD in six cannabis inflorescence samples were measured using HPLC and the data are summarized in the Table 2. The THC content was detected in all samples. On the other hand, no CBD was detected in any samples, consistent with the expression results of the *CBDAs* gene mentioned above. In the group of seedlings exposed to a dose of 40 Gy, the THC content was 3.9%, which was higher than the control (2.8%). This trend was similar to the group of seeds irradiated with 200 Gy, which had a THC content of 2.7%, also higher than the control (2.3%). However, during the irradiation of seedlings at 20 Gy and seeds at 100 Gy, lower THC levels were observed compared to the control.

Table 2. Cannabinoids content in cannabis samples analyzed by HPLC

Sample	%THC	%CBD
Seedling (control)	2.8	ND
Seedling_20 Gy	2.4	ND
Seedling_40 Gy	3.9	ND
Seed (control)	2.3	ND
Seed_100 Gy	2.1	ND
Seed_200 Gy	2.7	ND

This study's findings are consistent with the principles of radiation-induced plant breeding in other plant species, indicating that radiation-induced mutagenesis is a random method. Radiation can disrupt the process of producing secondary metabolites in plants by generating free radicals (ROS) through the radiolysis of water (Ma *et al.*, 2021). Gamma radiation has been reported to induce phytochemical changes in plants of commercial or medical value. The concentration of phytochemicals in plants can be altered after gamma radiation exposure. The seedlings of the accession CA23 of *Centella asiatica* (L.) Urb., irradiated with 20 and 30 Gy doses, contained the highest concentrations of total flavonoids by 54.7% and 46.8%, respectively more than the control (Moghaddam *et al.*, 2011). The calluses of *Ferula gummosa* Boiss., irradiated with 20 and 25 Gy doses, showed an increase in phenolic content by 36.5% and 38.9%, respectively (Ashouri *et al.*, 2016). The shoots of *Sesuvium portulacastrum* (L.) L. exposed to low doses of gamma rays (20 Gy) had 66% more ecdysteroid (20-hydroxyecdysone) content (0.321 mg dry weight of the plant⁻¹) than the control (Kapare *et al.*, 2017).

Relationship between gene expression and cannabinoid content in Cannabis

The monitoring of genotype changes using molecular techniques is commonly employed to enhance plant breeding through radiation (Riviello-Flores *et al.*, 2022). The presence of THC and CBD measured by HPLC reveals *THCAs*, *CBDAs* gene expression by agarose gel electrophoresis as Figure 4. In the irradiated seeds, *THCAs* gene expression were correlated with THC content. The seed control, irradiated seed 100 Gy, and irradiated seed 200 Gy had relative *THCAs* gene expression values of 0.4, 0.2, and 0.9, respectively, which correlated to THC content of 2.3, 2.1, and 2.7% by dried weight. Meanwhile, no correlation was discovered in seedling treatments. Seedlings are more radio-sensitivity (Amirikhah *et al.*, 2021) and these variables might influence the expression of genes involved in cannabinoid biosynthesis, leading to inconsistent results. On the other hand, CBD and *CBDAs* were not detected in any samples, which might be attributed to the lower sensitivity of the detection methods used or the genetic makeup of cannabis. The findings of this study are consistent with previous findings of increased relative expression in samples containing the highest percentage of THC (Cascini *et al.*, 2013). The *THCAs* and *CBDAs* genetic markers have been widely used in cannabis plants to distinguish THC-type cannabis from CBD-type cannabis, *THCAs* gene increased in THC-type, while *CBDAs* gene was lost of function,

resulting in marijuana containing much more THC and much less CBD (Cascini *et al.*, 2019). Previous research studies have explored the correlation between radiation dosage and gene expression in plant species. In the case of *C. asiatica*, studies have reported the upregulation of the enzyme chalcone synthase (CHS) and an increase in flavonoid content in response to gamma radiation (Moghaddam *et al.*, 2011). However, findings from research on Yard long bean indicate that phenotypic and genotypic variations did not follow a uniform trend of increasing or decreasing with different doses of gamma radiation (Ezzat *et al.*, 2021).

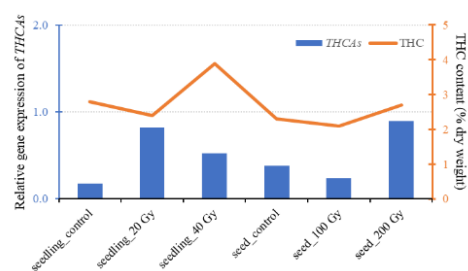


Figure 4. Correlation between *THCAs* gene expression and THC concentration in female cannabis

Conclusions

The findings of this study contribute to identifying gene markers for THC and CBD synthesis, which streamline early selection in cannabis breeding programs using gamma irradiation. The study found a correlation between *THCAs* and *CBDAs* gene expression in mature leaves and their respective cannabinoid concentrations of THC and CBD in inflorescences. Irradiation of seedlings at low doses of gamma rays and higher doses for seeds seems to increase *THCAs* gene expression in 'Hang Kra Rog' cannabis. However, further research is necessary to explore the broader implications of radiation-induced mutagenesis on the complex biochemical pathways involved in cannabinoid synthesis. This research not only demonstrates the effectiveness of gamma radiation but also underscores the importance of gene expression markers in optimizing breeding strategies for cannabis cultivation.

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