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Accumulation of Anthocyanin and Associated Gene Expression in Radish Sprouts Exposed to Light and Methyl Jasmonate

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ABSTRACT: Radish (*Raphanus sativus*) sprouts have received attention as an important dietary vegetable in Asian countries. The flavonoid pathway leading to anthocyanin biosynthesis in radishes is induced by multiple regulatory genes as well as various developmental and environmental factors. This study investigated anthocyanin accumulation and the transcript level of associated genes in radish sprouts exposed to light and methyl jasmonate (MeJA). The anthocyanin content of sprouts exposed to light and treated with MeJA was higher than that of sprouts grown under dark conditions without MeJA, and the highest anthocyanin content was observed within 6−9 days after sowing (DAS). Transcript levels of almost all genes were increased in radish sprouts grown in light conditions with 100 μM MeJA relative to sprouts grown under dark conditions with or without MeJA treatment, especially at 3 DAS. The results suggest that light and MeJA treatment applied together during radish seedling development enhance anthocyanin accumulation.

KEYWORDS: *anthocyanin, radish sprouts, gene expression, light, methyl jasmonate*

INTRODUCTION

Anthocyanins are natural pigments responsible for the blue, purple, red, and orange colors in all higher plants.[1−3] Anthocyanins have important functions, such as insect attraction,[4] and are produced in response to UV irradiation.[5] In addition, anthocyanins have beneficial effects on human health, including protection against cancer, inflammation, coronary heart disease, and other age-related diseases.[6−8] The antioxidant and tumor-arresting activities of anthocyanins have been widely studied.[9−11]

The red-skinned radish (*Raphanus sativus* L.) belongs to the Brassicaceae family and possesses the same basic glycosidic pattern as anthocyanins of red cabbage; however, the radish also contains pelargonidin derivatives.[12] Moreover, the red radish predominantly contains pelargonidin, whereas other plants contain cyanidin or delphinidin as their respective aglycons.[13,14] Sprouts have attracted attention as functional vegetables because of their beneficial nutritional components, including amino acids, fiber, minerals, carbohydrates, and protein.[15] Seed sprouts of common and tartary buckwheat sprouts are considered to be an excellent dietary source of phenolic compounds.[16] Martinez-Villaluenga et al.[17] have reported on the bioactive compounds and antioxidant capacity of broccoli and radish cultivars during germination. Recently, our group observed that the duration and amount of light applied during sprouting strongly affected the mechanisms of flavonoid and anthocyanin biosynthesis.[18]

The anthocyanin biosynthetic pathway is regulated by environmental factors such as light and temperature as well as internal factors such as plant hormones, other secondary metabolites, and nutrients.[19] Light acts as an essential stimulus and also modulates the intensity of the pigment by affecting the regulatory and structural genes involved in anthocyanin biosynthesis.[20]

Methyl jasmonate (MeJA) has been identified as a vital cellular regulator that mediated diverse developmental processes and defense responses against biotic and abiotic stresses.[21] MeJA and its associated free acid, jasmonic acid (JA), are important cellular regulators involved in diverse developmental processes, such as seed germination, root growth, fertility, fruit ripening, and senescence.[22,23] Genes involved in JA biosynthesis, secondary metabolism, and cell-wall formation, and those encoding stress-protective and defense proteins, are up-regulated by MeJA treatment.[21] Many studies have shown that MeJA enhances anthocyanin synthesis in various plants such as soybean seedlings,[24] *Arabidopsis* seedlings,[25] tulip bulbs,[26] and peach shoots.[27]

In radishes, genes in the flavonoid pathway are responsible for anthocyanin biosynthesis and play an important role in the biosynthesis of various secondary metabolites (Figure 1).[28,29] The initial three steps of the pathway catalyzed by phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), and 4-coumaroyl:CoA-ligase (4CL) are mandatory and provide the basis for all subsequent branches and resulting metabolites.[30] Anthocyanins are then synthesized by chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), DFR, and anthocyanidin synthase (ANS).[31,32]

Several studies have addressed factors that affect the extent of anthocyanin accumulation. However, no paper has evaluated

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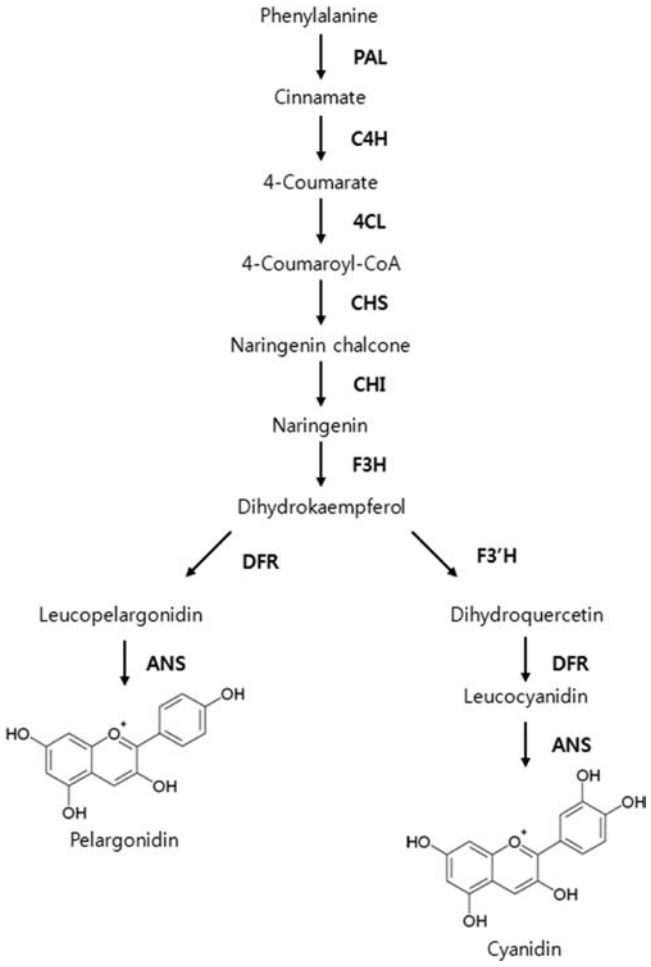

Figure 1. Flavonoid pathway in *Raphanus sativus*. PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumaroyl:-CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavone 3-hydroxylase; DFR, dihydroflavonol reductase; ANS, anthocyanidin synthase.

light exposure and MeJA treatment under light and dark conditions during the development of radish sprouts. Therefore, we investigated mRNA levels of genes involved in anthocyanin biosynthesis and also determined the anthocyanin content during the development of radish sprouts in response to MeJA treatment under light and dark conditions.

MATERIALS AND METHODS

Chemicals. HPLC- grade acetonitrile (CH_3CN), mobile phase solvent, was obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ, USA). For separation, 5% (v/v) formic acid (Kanto Chemical Co., Inc., Tokyo, Japan) was added to mobile phase solvents A and B. Two anthocyanin external standards (cyanidin-3-*O*-glucoside chloride and pelargonidin-3-*O*-glucoside chloride) were purchased from Extrasynthèse (Genay, France).

Plant Materials. *R. sativus* L. (cultivar Jukwhan 21 Moo) seeds were purchased from Asia Seed Co. (Korea). Seeds were surface-sterilized using 70% (v/v) ethanol for 30 s and 4% (v/v) sodium hypochlorite solution for 10 min and then rinsed five times with distilled water. The sterilized seeds were divided into two sets for treatment with or without 100 μM MeJA and then germinated on 1/2 MS medium in a growth chamber under light (16 h light/8 h dark) or dark conditions (24 h darkness) at 25 °C, 60% humidity, and 440 μmol/m^2/s light intensity. For biological replicates, we used three plastic boxes for each single treatment, placing 100 seeds per box and harvested sprouts without roots in each plastic box at 0, 3, 6, 9, and 12 days after sowing (DAS). Fifteen seedlings per replication were used to measure the length and weight of seedling. Sprouts were ground with a mortar and pestle under liquid nitrogen for gene expression studies and anthocyanin analysis.

RNA Isolation and Quantitative Real-time RT-PCR. Total RNA was isolated from each radish sprout using Tri-Reagent (MRC, USA) and a Plant Total RNA Mini kit (Geneaid, Taiwan) together. The quality and concentration of the total RNA were determined by 1.2% formaldehyde agarose gel electrophoresis and Nanodrop spectrophotometry, respectively. Using 1 μg of total RNA, reverse transcription was performed according to the manufacturer's instructions (ReverTra Ace-á, Toyobo, Japan) using an oligo dT_{20} primer. The cDNA was diluted 20-fold for real-time RT-PCR. Transcript levels of genes involved in anthocyanin biosynthesis were analyzed by real-time PCR (Bio-Rad, USA) using SYBR Green Real-Time PCR Master Mix (Toyobo, Japan). Real-time PCR was conducted in a 20 μL reaction volume containing 0.5 μM primer (Table 1) and 2× SYBR Green Real-Time PCR Master Mix (Toyobo, Japan). The PCR reaction performed was as follows: initial denaturation at 95 °C for 3 min followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 55 °C for 10 s, and extension at 72 °C for 30 s. The real time RT-PCR results were obtained as the mean of three replicates, and statistical differences were evaluated according to the standard deviation. The 26S ribosomal gene (Genbank accession no. AY366932) was used as an internal control.

Table 1. Real-Time PCR Primers Used in This Study

primer name	sequence (5′ → 3′)	accession no.
RsPAL-RT(F)	AATAACGGTCTTCCGTCGAATTT	AB087212
RsPAL-RT(R)	TAACCGGATTGGCTAGGTACTGA	
RsC4H-RT(F)	AATCATGACGGTTCCTTTCTTCA	HQ641568
RsC4H-RT(R)	TCAGGATTCTTCTTCACGTCCTC	
Rs4CL-RT(F)	AGGTATGATCTGAGCTCGGTGAG	HQ641569
Rs4CL-RT(R)	CTTGGTCTTGAATGGGTTCTTTG	
RsCHS-RT(F)	GAGATCAGAAAGGCACAGAGAGC	AF031922
RsCHS-RT(R)	TAGTCAGGATACTCGGCTTGGAG	
RsCHI-RT(F)	TCTTCCGTGAAATCGTCATAGGT	AF031921
RsCHI-RT(R)	CCAAGAACCTCTCCACTGCTTTA	
RsF3H-RT(F)	TCCTGAGGAGAAACTGAAGTTC	AB087211
RsF3H-RT(R)	CGTCACGATCTCTCTCCAATCTT	
RsDFR-RT(F)	ACCGGATGGATGTATTTCATGTC	AB087210
RsDFR-RT(R)	ATGATGGAGTAATGTGCCTCGTT	
RsANS-RT(F)	GAGCCTGACCGTATTGAGAAAGA	AB087206
RsANS-RT(R)	CAAACCTGGAACCATGTTGTGTA	
RsMYB-RT(F)	TTGAGGCGATGCATTGATAAGTA	DQ538391
RsMYB-RT(R)	TATGAAGCCGGAGAAGAAGATCA	
Rs26S-RT(F)	AACACCCTTTGTGGGTTCTAGGT	AY366932
Rs26S-RT(R)	GCCCTCGACCTATTCTCAAACTT	

Extraction and High-Performance Liquid Chromatographic (HPLC) Analysis of Anthocyanidin. A fine radish powder (100 mg) was weighed into a 2 mL Eppendorf tube, to which was added 2 mL of water/formic acid (95:5, v/v), and the extract solution was then vigorously vortexed for 5 min and sonicated for 20 min. After centrifugation (at 8000 rpm for 15 min), the supernatant was filtered through a 0.45 μm PTFE syringe filter (Advantec DISMIC-13_{HP}, Toyo

Roshi Kaisha, Ltd., Tokyo, Japan). The filtrate was then analyzed using a Perkin-Elmer Flexar HPLC system (Shelton, CT, USA) equipped with a PDA LC detector. Individual anthocyanins within the extract solution were separated on a Synergy 4 μ Polar-RP 80A (250 × 4.6 mm, i.d.) column with a Security Guard AQ C18 (4 × 3 mm, i.d.) both purchased from Phenomenex (Torrance, CA, USA). The detection wavelength and temperature of the column oven were set at 520 nm and 40 °C, respectively. The injection volume was 10 μL with automatic sampling, and the solvent system was run at a flow rate of 1 mL/min.

The mobile phase solvents consisted of a mixture of (A) water/formic acid (95:5, v/v) and (B) acetonitrile/formic acid (95:5, v/v). The gradient program was largely modified from those described by Giusti et al.[33] and Tatsuzawa et al.[34] to the following: 0–2 min, 5–18% B; 2–4 min, 18% B; 4–9 min, 18–20% B; 9–14 min, 20% B; 14–19 min, 20–21% B; 19–24 min, 21% B; 24–24.1 min, 21–5% B; and 24.1–30 min, 5% B (total 30 min). The data were recorded using a computer (Samsung DM-V599, South Korea) and analyzed using Perkin-Elmer Flexar HPLC Chromera software. Each anthocyanin was identified by comparison with the retention times of the HPLC chromatograms and our previous paper.[35] Quantification of the different anthocyanins was based on peak areas and calculated as equivalents of two representative standard compounds. All contents were expressed as milligrams per gram dry weight.

RESULTS AND DISCUSSION

Radish Sprout Growth under Light and Dark Conditions. The length and fresh weight of radish seedlings after MeJA treatment were measured every 3 days until 12 DAS (Figure 2). Seedlings grown under light conditions were green, whereas seedlings grown under dark conditions were yellow. The length and fresh weight were lower in MeJA-treated seedlings than in the untreated controls. The length and seedling weight increased with increasing age; the increase initially occurred slowly but then accelerated rapidly. The length and seedling weight were 2-fold higher at 9 DAS than at 3 DAS under dark conditions. The growth trend observed in this study was similar to that previously observed for buckwheat sprout.[36] The fresh weight did not vary significantly between 6 and 12 days. In buckwheat, Li et al.[36] noted that maximum biomass for commercial purposes could be achieved for buckwheat sprouts cultured under darkness for approximately 8 days. Similar to buckwheat sprouts, the maximum biomass of radish sprouts used for commercial purposes can be obtained in 9 days under dark conditions.

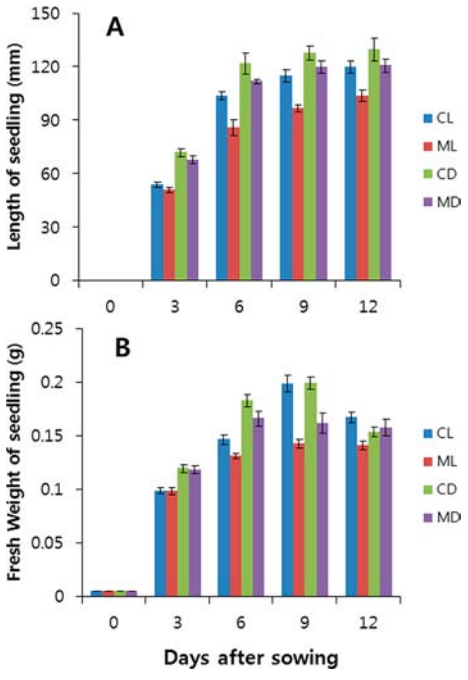

Figure 2. Growth of radish sprouts from 0 to 12 days: (A) average sprout length (n = 45 per treatment); (B) average fresh weight of radish sprouts from 0 from 12 days (n = 45 per treatment); blue, CL (control light); red, ML (MeJA + light); green, CD (control dark); purple, MD (MeJA + dark).

Transcript Level of Anthocyanin Biosynthetic Genes in Radish Sprouts. The mRNA transcript levels of genes involved in anthocyanin biosynthesis were investigated by real-time PCR in sprouts grown under light and dark conditions with and without MeJA treatment (Figure 3). The transcript levels were higher in MeJA-treated (ML) sprouts grown under light conditions than in the untreated control (CL) for most of the genes. The transcript levels were highest in the ML condition for nearly all of the genes at 3 DAS; that is, they were 2–104-fold higher than transcript levels in the CL condition. At 3 DAS, the transcript levels for *Rs4CL* and *RsDFR* were 103- and 71-fold higher, respectively, than that for the 26S ribosomal gene in ML-treated sprouts and 28- and 6-fold higher, respectively, than that for 26S in the CL condition. Song et al.[37] reported that *RsCHS*, *RsCHI*, and *RsDFR* mRNAs were not expressed in the dark, but their expression was induced in seedlings exposed to white light up to day 6. *FtPAL*, *Ft4CL*, *FtF3H*, *FtDFR*, and *FtANS* show higher transcript levels in both seeds and sprouts from 2 to 10 DAS relative to the transcript level of the housekeeping gene (histone H3), and most genes in the flavonoid biosynthetic pathway are up-regulated at 2 DAS under light/dark or dark culture,[36] whereas carotenogenesis genes reach the maximum transcription at 8 DAS.[38] The transcription of carotenogenesis genes in seedlings does not vary widely between dark and light conditions.[38]

In the present study, in the dark condition after MeJA treatment (DM), transcript levels of all genes, except for *RsC4H*, decreased gradually after 3 DAS. The expression pattern for all genes in the dark condition was similar to that in the light condition, as the transcript levels were highest at 3 DAS. Transcript levels of *Rs4CL* and *RsDFR* were higher at 3 DAS than those of other genes in the dark condition (CD). Transcript levels of *Rs4CL* and *RsDFR* under CD and MD conditions were 72- and 75-fold higher, respectively, than those of the internal control gene. The radish transcription factor RsMYB, as reported previously,[35] was induced in the ML condition. Park et al.[35] reported that RsMYB overexpression may regulate the transcription of anthocyanin-biosynthetic genes to increase anthocyanin production. Like MeJA, JA has also been shown to enhance pigmentation in plants.[24,25,39] Recently, Shan et al.[40] reported that JA induction enhanced transcription of downstream anthocyanin biosynthetic genes, *DFR*, *leucoanthocyanidin dioxygenase* (*LDOX*), and *UDP-Glc:flavonoid 3-O-glucosyltrans ferase* (*UF3GT*), and also accumulated anthocyanin content. It was also found that *Arabidopsis* mutant *coronatine insensitive1* (*COI1*) is essential for the expression of DFR, the transcription factors PAP1 (MYB75) and PAP2 (MYB90), and GL3 expression in JA-induced anthocyanin biosynthesis.[40] Qi and colleagues[41] reported that the WD-repeat/basic helix loop helix (bHLH)/MYB complex is involved in JA-regulated anthocyanin accumulation and trichome initiation in *Arabidopsis thaliana*. In addition, Loreti et al.[42] pointed out that sucrose induction of

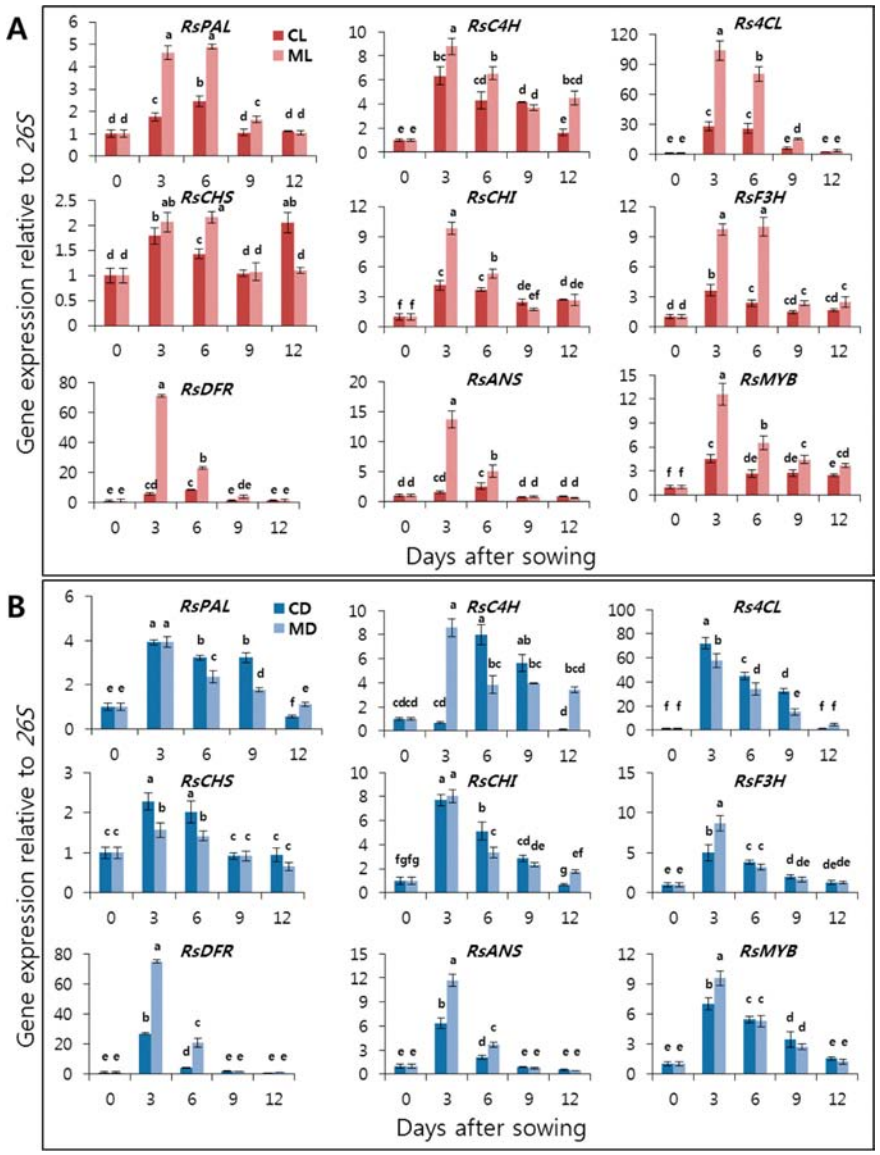

Figure 3. Transcript level of flavonoid and anthocyanin biosynthetic genes during the development of radish sprouts under dark and light conditions: (A) CL and ML indicate control light and MeJA + light conditions, respectively; (B) CD and MD indicate control dark and MeJA + dark conditions, respectively. The longitudinal axis indicates the transcript levels of genes relative to the level of 26S. The values and error bars represent the mean and standard deviation, respectively, of three biological repeats. Different letters represent the LSD (p = 0.05).

the anthocyanin synthesis pathway was repressed by the addition of gibberellic acid, whereas JA and abscisic acid have a synergic effect with sucrose. They also suggested the existence of crosstalk between the sucrose and hormone signaling pathways in the regulation of the anthocyanin biosynthetic pathway.[42]

Identification of Anthocyanidins in Radish Sprouts. The composition of anthocyanidins in radish sprouts treated with 100 μM MeJA during development under light or dark conditions was analyzed by HPLC (Figure 4). Cyanidin and pelargonidin were identified as the major anthocyanidins in radish sprouts. The anthocyanidin content of sprouts grown under light conditions with (ML) or without (CL) MeJA treatment was higher than that of sprouts grown under dark conditions without MeJA treatment (CD). Anthocyanins were not detected in seeds used as a control (0 DAS). At 6 DAS, the cyanidin content [0.03 mg/g dry weight (DW)] in radish sprouts grown under light conditions was 3-fold higher than that in sprouts grown under dark conditions (0.01 mg/g DW). In the ML condition, the cyanidin and pelargonidin contents were highest at 9 and 6 DAS, respectively. From the previous study it was shown that secondary metabolite content varies among the treatments; for example, Bakhshi and Arakawa[43] reported that the content of phenolic acids, anthocyanin, and flavonols increased rapidly by irradiation, whereas flavanols, procyanidins, and dihydrochalcones did not change in either mature or ripe apple fruits. According to Bakhshi and Arakawa's result, we can assume that two major anthocyanidins do not respond equally to the ML treatment, so a different amount of accumulation has occurred in our study. We are not sure but assume that the trend of anthocyanidin levels reduced with time, because MeJA is a volatile compound.

Anthocyanidin levels in the MD condition were similar to or slightly higher than those in the CD condition. The flesh of the Man Tang Hong red radish and the skin of the Hong Feng No. 1 red radish were previously found to have higher total

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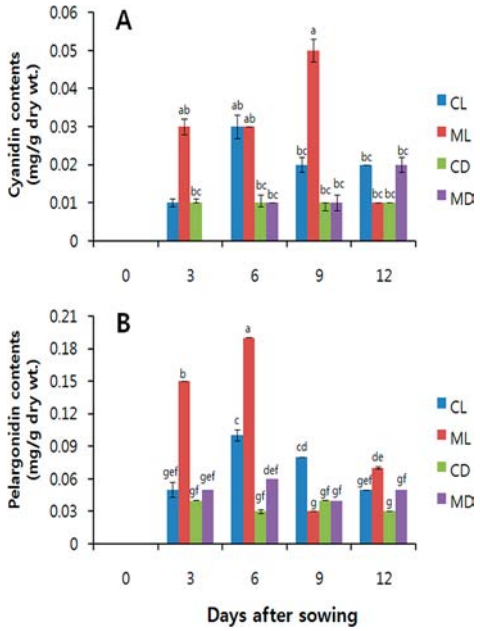

Figure 4. Anthocyanidin contents (mg/g dry weight) during the development of radish sprouts under dark and light conditions with or without MeJA treatment. The values and error bars represent the mean and standard deviation, respectively, of three biological repeats. Different letters represent the LSD (p = 0.05).

anthocyanin amounts (4.69 and 4.39 mg/g DW, respectively)[35] than we observed in the present study. In addition, previously our group[35] described that almost all anthocyanin modifications were detected to be acylated pelargonidin of anthocyanidins, except for two glycosides, such as pelargonidin 3-diglucoside-5-glucoside and cyanidin 3-(glucosyl) rhamnoside in three radish cultivars. Our result was in accord with a previously reported finding.[35] Politis[44] described that the anthocyanins of *R. sativus* seedlings are primarily located in the cells of the subepidermal layer of the cotyledons and the hypocotyl axis. Phytochrome mechanisms as well as light intensity, quality, and duration are the predominant factors affecting the extent of anthocyanin accumulation.[45] Grisafi and Venturella[46] obtained increased anthocyanin production in seedlings grown on 10^{-4} M chloramphenicol. Additionally, MeJA has been shown to induce gum production and stimulate anthocyanin accumulation in peach shoots.[27] Our results agree with previously published data on anthocyanins in soybean seedlings,[24] *Arabidopsis* seedlings,[25] tulip bulbs,[26] and peach shoots.[27] These findings suggest that simultaneous light and MeJA treatment induce higher production of anthocyanins than dark, light, or non-MeJA treatments alone during radish sprout development.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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