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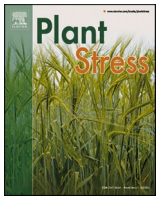

Effect of low root-zone temperature and UV radiation on growth and gene expression of secondary metabolite pathways in *Nicotiana benthamiana*☆

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ABSTRACT

The current study explored the effects of low root-zone temperature (LT) and UV radiation (UV) alone and combined on changes in growth, transcription, and gene expression related to secondary metabolite production. *Nicotiana benthamiana* plants were grown in a controlled environment (25/20°C, 16/8 h [light/dark], 70% relative humidity, 1,000 μmol·mol^{-1} CO_2 with photosynthetic photon flux densities of 100 and 200 μmol·m^{-2}·s^{-1} for 10 and 18 d, respectively). Twenty-eight days after sowing, these plants were treated with LT (15°C), 0.3 W·m^{-2} of UV radiation, and a combined treatment with LT and UV (LT*UV) for 3 d. Results found that the treatment with UV alone decreased the quantum efficiency of photosystem II by approximately 1.5 times, and most growth characteristics decreased under the UV (approximately 1.5 times) and LT*UV (approximately 2 times) treatments. Treatment with LT*UV significantly inhibited the growth characteristics and photosynthetic rates compared to those under the single LT and UV treatments. Changes in the transcriptome of plants in response to different environmental conditions were determined based on the expression levels of genes related to secondary metabolites and microarrays. In particular, the transcriptome levels of phenylpropanoid and flavonoid biosynthesis were the most significantly affected by LT*UV. This suggests the potential of using LT treatment in hydroponic systems and UV radiation to control the synthesis of health-promoting compounds of secondary metabolites in greenhouses and controlled-environment agricultural facilities.

1. Introduction

Secondary metabolism in plants encompasses biochemical metabolites that are non-essential for the survival of plants but play a significant role in the interactions between plants and the environment, facilitating plant adaptation to biotic and abiotic stresses (Bennett and Wallsgrove, 1994). Secondary plant metabolites can be classified into three major groups: terpenoids, phenolic compounds, and nitrogen-containing compounds, which are economical food sources, traditional medicines, perfumes, and industrial raw materials (Yang et al., 2012). Environmental factors (light, temperature, humidity, air, and water) considerably affect the synthesis and accumulation of secondary metabolites. Controlling environmental stresses during cultivation may change the accumulation of bioactive compounds and improve the quality traits of plants (Edreva et al., 2008).

Nicotiana benthamiana is an essential crop because of its potential as a natural product in the cosmetic and medicinal industries. It is widely used as a model plant in plant-based production systems (Reed and Osbourne, 2018). It is grown in closed-type plant production systems such as greenhouses and growth chambers for research and industrial use. The studies of *N. benthamiana* as a model plant for plant molecular biology have recently increased, and it has been used to study plant–microbe and protein–protein interactions (Bally et al., 2018). Commercially, it offers speed, mass production, and a low risk of contamination by pathogens in molecular farming and biopharmaceutical production (Stoger et al., 2014). However, studies on the transcriptome of *N. benthamiana* under various environmental conditions need more attention.

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Among the abiotic factors, low temperature (chilling) stress is one of the main factors affecting the growth, development, and quality of crops, as it causes oxidative damage at the cellular level (Gechev et al., 2003). The root-zone temperature (RZT) exerts various effects on developmental and physiological processes, such as photosynthesis, plant growth, and metabolism in plants (Stoltzfus et al., 1998). Previous studies on RZT on growth and physiological processes explored the accumulation of secondary metabolites in tobacco species. Low temperatures (cold, ranging from 0°C to 15°C) may affect the activity of antioxidant enzymes, such as peroxidase, superoxide dismutase, and catalase, in tobacco seedlings (Xu et al., 2010).

Whereas an abiotic stressor, ultraviolet (UV) radiation may affect the plant metabolites and nutritional components, such as carbohydrates, amino acids, vitamins, hormones, flavonoids, phenolics, and glucosinolates (Hounsome et al., 2008). Within the UV wavelength range of 280–400 nm, UV-B (280–315 nm) may impair plant growth and development, and enhanced levels of UV radiation inhibit plant physiological processes and growth (Day and Neale, 2002). UV-B can increase the non-enzymatic total antioxidant capacity and peroxidase activity in *N. benthamiana* leaves (Mátai et al., 2019).

Under abiotic stresses, the effect of RZT and UV radiation on the secondary metabolite biosynthesis and transcriptional profiling of leaf tissues of *N. benthamiana* at a high throughput level are vital. Therefore, the objectives of this study were (i) to identify the functions of genes affected by RZT and UV radiation and (ii) to study the induced or suppress expression of beneficial secondary metabolites under RZT and UV radiation.

2. Methods

2.1. Plant materials

N. benthamiana seeds obtained from National Institute of Advanced Industrial Science and Technology, Japan. The seeds were sown into a urethane foam cube (M urethane, 2.3 × 2.3 × 2.7 cm, L × W × H, M Hydroponic Research Co., Aichi, Japan) on a plastic tray (30 × 25 × 5 cm, L × W × H) and maintained in a closed-type plant production system (March - December 2017), Chiba University (35°46'35.8"N, 139°54'11.2"E, 28m), under controlled environmental conditions (air temperature of 25/20°C, relative humidity of 70%, CO_2 concentration of 1,000 μmol·mol^{-1}, photosynthetic photon flux density (PPFD) using white fluorescent lamps of 100 μmol·m^{-2}·s^{-1}, and photoperiod of 16 h) for 10 d (Supplementary Table S1). Ten days after sowing (DAS), twenty-four seedlings per treatment were transplanted into a 64-well polystyrene foam plate (normal type, M Hydroponic Research Co.). Each plate was floated in a plastic tray with 11 L of nutrition solution (38.5 × 25.5 × 12 cm, L × W × H) using a deep-flow technique system. After transplanting, the *N. benthamiana* seedlings were grown for 15 d (Supplementary Table S1). The nutrient solution (half-strength Otsuka-A nutrient solution, OAT Agrio Co., Ltd., Tokyo, Japan, 6.4 pH, 1.8 EC dS·m^{-1}) was replenished every 3 d throughout the growth stage.

2.2. LT and UV treatments

Twenty-five DAS *N. benthamiana* plants were grown under different treatments (Supplementary Table S1). The treatments included low-RZT (15°C, LT), UV radiation (UV) of 0.3 W·m^{-2} (daily dose 17.28 kJ m^{-2}), and combined (LT*UV) treatments according to Son et al. (2020). The RZT used in this study was determined from the growth and accumulation of antioxidants through preliminary experiments using 10, 15, and 20°C with 25/20°C (day/night) air temperature. The UV energy level used in this study was determined from the result of growth through preliminary experiments using 0.0, 0.3, and 0.6 W·m^{-2}. The roots of *N. benthamiana* plants were exposed to an RZT of 15°C for 3 d at 25 DAS to determine the effect of LT while the shoots remained at 25°C. The nutrient solution temperature of the root zone was controlled by a compact handy cooler (TRL-107NHF, Thomas Kagaku Co., Tokyo, Japan), and the temperature of each nutrient solution was recorded using a data logger (GL820, Graphtec Corporation, Kanagawa, Japan) at 10 min intervals. The *N. benthamiana* plants subjected to UV treatment were irradiated by a combination of UV-rich fluorescent lamps (improved model of FHF 32-EX-N-H, Panasonic Corp., Osaka, Japan) and white LED lamps (LDL40S-N/19/21; Panasonic Corp., Osaka, Japan) at 25 DAS. The spectral distribution and intensity were measured using a spectroradiometer (USR-45D, USHIO Inc., Tokyo, Japan). The UV irradiation at 200 μmol·m^{-2}·s^{-1} of PPFD included various wavelengths of UV-A (0.14 W·m^{-2}, daily dose 8.06 kJ m^{-2}), UV-B (0.08 W·m^{-2}, daily dose 4.61 kJ m^{-2}), and UV-C (0.08 W·m^{-2}, daily dose 4.61 kJ m^{-2}).

2.3. Growth characteristics

Plants were separated into shoots and roots after 3 d of treatment, and their fresh and dry weights were measured using an electronic scale (ASP2102, AS ONE Co., Osaka, Japan). The samples were dried for 74 h at 80°C in an oven (MOV-202F, Sanyo Electric Co., Osaka, Japan). Tissue samples were collected from the third leaf position, frozen in liquid N_2, and stored at -80°C for microarray analysis.

2.4. Net photosynthetic rate and chlorophyll fluorescence measurements

The net photosynthetic rate (Pn) was determined as outlined by Bremer and Ham (2005), and measured using a portable photosynthesis system (LI-6400, Li-Cor Inc., Lincoln, NE, USA) using the fully expanded third leaves of four plants from each treatment. A standard leaf chamber was used for all measurements. The PPFD was approximately 200 μmol·m^{-2}·s^{-1}, and the leaf temperature and CO_2 concentration in the leaf chamber were 25°C and 1000 μmol·mol^{-1}, respectively. The relative humidity ranged from 70% to 80%. Data were collected when Pn reached a steady state.

Chlorophyll fluorescence was measured to evaluate the potential quantum yield of photosystem II (Fv/Fm), which indicated the stress level of plants subjected to environmental stress (Maxwell and Johnson, 2000). Fv/Fm was determined from the fully expanded third leaves of four plants from each treatment using a PAM fluorometer (Mini-PAM/L, Heinz Walz GmbH, Effeltrich, Germany). A saturating pulse of 0.8 s at a PPFD of 8000 μmol·m^{-2}·s^{-1} was applied to determine the maximum fluorescence yield.

2.5. RNA preparation

RNA extraction was performed as described by Abuqamar et al. (2013). The third leaves of the *N. benthamiana* plants grown under each treatment (five replicates per treatment) were freeze-dried and crushed using a mixer ball mill (MM400, Retsch GmbH, Haan, Germany) with zirconia beads for 1.5 min at 30 Hz for microarray analysis and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) at 1, 2, and 3 d after the start of treatment. Total RNA was isolated using an RNeasy Plant Mini Kit (Qiagen N.V., Venlo, The Netherlands) following the manufacturer's instructions. RNA quantity and purity were determined by evaluating the absorbance at 260 nm and 260/280 nm absorbance ratio, respectively, using a spectrophotometer (NanoDrop Lite, Thermo Fisher Scientific, Waltham, MA, USA).

2.6. Microarray and data analysis

Microarray analysis was conducted using approximately 5 μg of total RNA isolated from the plants of each treatment using an Agilent Quick Amp Labeling Kit. Agilent one-color gene expression microarray was analyzed using an Agilent Tobacco 4 × 44 K format *N. tabacum* Array. Accurate gene information from a 60-base-long probe sequence was obtained from the National Center for Biotechnology Information using

the Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the DAVID database (Laboratory of Human Retrovirology and Immunoinformatics; https://david.ncifcrf.gov/). Following fragmentation, 1.65 μg of cRNA was hybridized to the Agilent expression microarray following the protocols provided by the manufacturer. The arrays were then scanned using Agilent Technologies G4900DA SG12494263. Raw data were extracted using Agilent Feature Extraction Software (v11.0.1.1). Raw data for the same gene were then automatically summarized following the Agilent feature extraction protocol to generate a raw data text file containing expression data for each gene probed on the array. Array probes were normalized and separated using the flags ("present," "marginal," or "absent"), and only "present" or "marginal" transcripts were taken and analyzed. The selected gProcessedSignal value was then logarithmically transformed and normalized using the quantile method (Fu et al., 2009).

The gene information such as Locus, UniGene ID, and KEGG orthology (KO) were obtained from the Universal Protein Resource (UniProt, https://www.uniprot.org/). All processes were conducted using Python (Python 3.7.4), Visual Studio Code (Microsoft), and Pandas (Python Data Analysis Library). Hierarchical cluster analysis was conducted using MultiExperimentViewer software (http://www.tm4.org/mev.html) using Euclidean distance with a complete linkage setting. The GO functional annotation describing the biological process, molecular function, or cellular components (Supplementary Fig. S1) was obtained from the online tool WEGO (Ye et al., 2018). Gene enrichment and functional annotation analyses were conducted using KEGG (http://kegg.jp) for the significant probe list. KEGG functional classification and pathway analysis were conducted using the KO. All data analysis and visualization of differentially expressed genes were conducted using R 3.0.2 (www.r-project.org). Genes with $p < 0.05$ and changes in expression of 2-fold or greater were considered and gathered for analysis to compare differentially expressed genes.

2.7. Reverse transcription-polymerase chain reaction analysis

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) analysis was conducted in five biological replicates using the same RNA sample as that used in the microarray analysis. Complementary DNA was synthesized from RNA isolated using a PrimeScript RT Reagent Kit (Perfect Real Time; Takara Bio Inc., Shiga, Japan). The PCR was conducted in a Thermal Cycler Dice Real-Time System (TP800, Takara Bio Inc.) using a QuantiTect SYBR Green PCR kit (Takara Bio Inc.). The PCR conditions were as follows: initial denaturation step (9°C, 10 min), followed by 40 denaturation cycles at 95°C for 10 s, annealing at 72°C for 30 s, and a final extension at 65°C for 40 s. The relative gene expression of RNA was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The data obtained were normalized based on the expression of the housekeeping gene *UBQ*. The primers used for RT-PCR are listed in Supplementary Table S2.

2.8. Statistical analyses

The experiment was repeated three times to verify the reproducibility of each experimental condition. Growth characteristic measurements and RT-PCR were conducted on five plants per treatment. Statistical analyses were conducted using the Statistical Products and Service Solutions for Windows, ver. 24.0 (SPSS Inc., Chicago, IL, USA). The data obtained for each parameter were analyzed by one-way analysis of variance (ANOVA) and means comparison among treatments using Duncan's test at 5% probability.

3. Results

3.1. Growth characteristics and photosynthetic parameters

Three d of LT and UV treatments significantly affected the growth characteristics of the *N. benthamiana* plants (Table 1, Fig. 1). The fresh shoot and root weights after 3 d of treatments decreased in the following order: LT (shoot 1.24 times; root 1.11 times), UV (shoot 1.54 times; root 1.17 times), and LT*UV (shoot 1.95 times; root 1.45 times) compared to the control. Significant differences in shoot fresh and dry weights were observed between the LT and UV treatments. The LT*UV treatment inhibited all growth characteristics more significantly than the other treatments, excluding shoot dry weight. The fresh root weight, dry shoot, and root weights did not significantly differ between the control and the LT treatment, whereas the UV treatment inhibited the dry shoot weight (1.42 times). The LT*UV treatment yielded the lowest values for all growth characteristics among all treatments. The dry root weight was not significantly affected by the LT and UV treatments, whereas the fresh root weight was significantly affected by LT*UV (1.45 times). Treatment with UV radiation (UV and LT*UV) induced the production of epicuticular wax (Fig. 1A).

Table 1
Growth characteristics of *N. benthamiana* plants grown under LT and UV treatments. Data were obtained 3 d after treatment. Values are means ± SE (n = 5). LT – treatment of low root-zone temperature, UV – UV treatment, LT*UV – combined treatment with LT and UV.

Treatment	Fresh weight (g)		Dry weight (g)	
	Shoot	Root	Shoot	Root
Control	5.98 ± 0.31 a[1]	2.79 ± 0.16 a	0.51 ± 0.03 a	0.39 ± 0.01 a
LT	4.82 ± 0.25 b	2.51 ± 0.09 ab	0.47 ± 0.02 a	0.37 ± 0.01 a
UV	3.89 ± 0.29 c	2.38 ± 0.06 b	0.36 ± 0.03 b	0.37 ± 0.01 a
LT*UV	3.06 ± 0.12 d	1.92 ± 0.04 c	0.30 ± 0.01 b	0.35 ± 0.00 b
Significance[2]	***	***	***	**

[1] Means ± standard error marked with different letters differ significantly at p = 0.05.
[2] ** and *** indicate significance at p = 0.01 and 0.001, respectively.

The net photosynthetic rate followed different trends on days one and three of the treatment (Fig. 2A). A significant decrease in the net photosynthetic rate from that of the control was observed in the UV treatment group on day one of treatment. However, no significant decrease was observed in the LT and LT*UV treatment groups. On day three of treatment, the highest value was observed in control, and the values significantly decreased in the order of LT- (approximately 1.19 times), UV- (approximately 1.63 times), and LT*UV- (approximately 3.02 times) treated plants. The most significant reduction in Pn (3-fold) was observed in the LT*UV treatment group.

The maximum quantum yield of photosystem II did not significantly differ among the three stress treatments on day one (Fig. 2B). However, on the third day of treatment, statistically significant decreases were observed in the UV (approximately 1.18 times) and LT*UV (approximately 1.07 times) treatments. Unlike the net photosynthetic rate, the Fv/Fm value of the UV treatment decreased by 1.2 times to that of the control and exhibited the largest decrease among those of the other treatment groups.

3.2. Overall gene upregulation and downregulation by the LT and UV treatments

The expression levels and patterns of genes in *N. benthamiana* plants were compared among the LT, UV, and LT*UV treatments to understand how LT and UV affect the overall gene expression (Fig. 3 and Supplementary Fig. S2). The expression levels and patterns of genes varied among treatments and were also affected by the treatment day. A large portion of the differentially expressed genes was observed on 2 d of LT (Up 2,141; Down 2,583), 1 d of UV (Up 2,262; Down 2,313), and 2 d of LT*UV (Up 3,949; Down 3,798) treatments (Fig. 3A). Overall, the genes

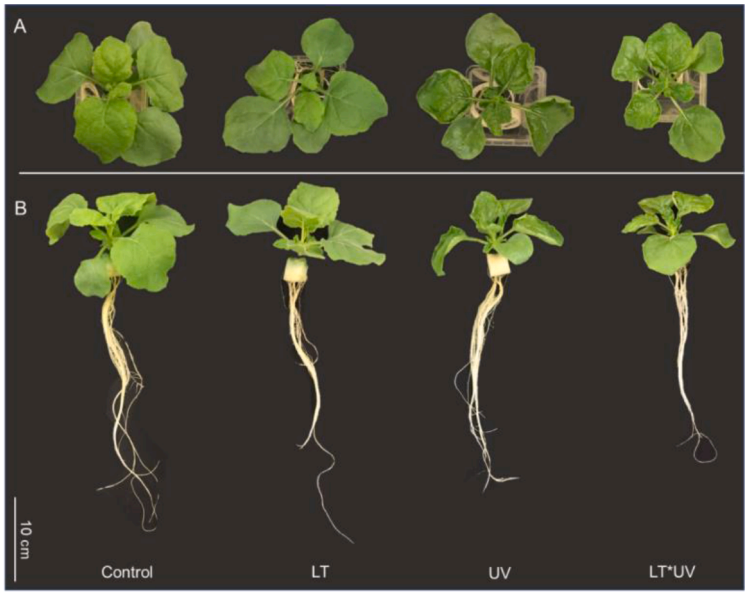

Fig. 1. Top (A) and front (B) view of *N. benthamiana* plants grown under LT and UV treatments. LT – treatment of low root-zone temperature, UV – UV treatment, LT*UV – combined treatment with LT and UV.

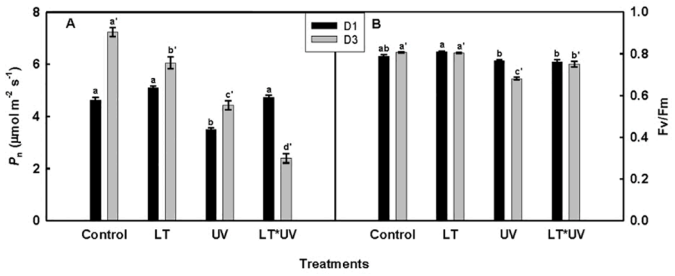

Fig. 2. Pn (net photosynthetic rates, A) and Fv/Fm (maximum quantum yield of PSII, B) of *N. benthamiana* plants grown under each treatment at 1 and 3 d. The bars represent mean ± standard error (n = 5). Statistical analyses were performed at 1 and 3 d of treatment, respectively. Different letters indicate significant differences among treatments ($p < 0.05$). LT – treatment of low root-zone temperature, UV – UV treatment, LT*UV – combined treatment with LT and UV.

were more regulated under the LT*UV (Up 1,724; Down 2,529) combined treatment than under the other treatments, particularly after 2 d of treatment (Fig. 3B).

The Venn diagram in Fig. 4 shows the number of up-, down-, or contraregulated genes after 3 d of treatment. Genes that co-expressed among the treatments varied. Specifically, 657 genes were contra-regulated between LT and LT*UV (Fig. 4B), while only 69 genes were contra-regulated between UV and LT*UV (Fig. 4C). The LT*UV treatment exhibited the highest number of upregulated genes (1195) (Fig. 4D).

3.3. Two-dimensional distribution of the LT and UV treatments

Multidimensional scaling (MDS) analysis of the *N. benthamiana* transcripts indicated differences between the LT, UV, and LT*UV treatments (Fig. 5). The MDS analyses of all expression data indicated that 70.8% of the variation could be explained by two components. The clear separation of the LT and UV data on days 1, 2, and 3 indicated that the environmental conditions mainly determined component 1. On day one of treatment, UV and LT*UV were distinguished from the control and LT treatments at component 1. In each treatment, UV and LT*UV were separated from the control at component 1, while LT was separated from the control at component 2. On day 3 of treatment, UV and LT*UV were separated from the control and LT treatments at component 1, while the UV treatments were separated from the LT treatments at component 1. Based on the effect of the number of days of treatment, UV and LT*UV treatments stimulated components 1 and 2, while LT stimulated only component 2. These MDS data confirmed that the environmental conditions and the number of days of treatment might change gene expression patterns in different ways.

3.4. Differences in the gene expression levels of N. benthamiana under LT and UV treatments

Environmental factors largely affect secondary metabolism in plants, resulting in the stimulation of alkaloid, phenylpropanoid, flavonoid, anthocyanin, and terpenoid biosynthesis pathways. Supplementary Fig. S3 shows major secondary metabolite biosynthesis pathways, including the genes targeted in this study.

The effects of LT and UV after 3 d of treatment on the expression of phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxylase (C4H), chalcone synthase (CHS), and ascorbate peroxidase (APX) in *N. benthamiana* are shown in Fig. 6. LT significantly increased *C4H* (approximately 1.35 times), *CHS* (approximately 2.35 times), and *APX* (approximately 1.59 times) gene expression levels compared to those of the control. In comparison, UV treatment significantly decreased the *C4H* (approximately 1.74 times) and *APX* (approximately 2.43 times) gene expression levels compared to the control. Additionally, LT*UV treatment significantly increased the gene expression levels of *PAL* (approximately 3.15 times) and *CHS* (approximately 2.08 times) and decreased that of *APX* (approximately 1.71 times) compared to those of the control.

The expression levels of alkaloid-related genes, such as aspartate oxidase (*AO*), quinolinate synthetase (*QS*), quinolinate phosphoribosyl transferase (*QPT*), omithine decarboxylase (*ODC*), putrescine N-methyltransferase (*PMT*), N-methylputrescine oxidase (*MPO*), and isoflavone reductase-like protein A622 (*A622*), were observed in *N. benthamiana* (Fig. 7). The relative expression levels of *QPT, PMT, MPO*, and *A622* were significantly higher in the LT group than in the control group. Meanwhile, UV treatment significantly decreased the *AO* (approximately 2.95 times), *QS* (approximately 3.09 times), and *QPT* (approximately 3.27 times) expression levels compared to those in control. LT*UV treatment significantly decreased the expression levels of *AO*

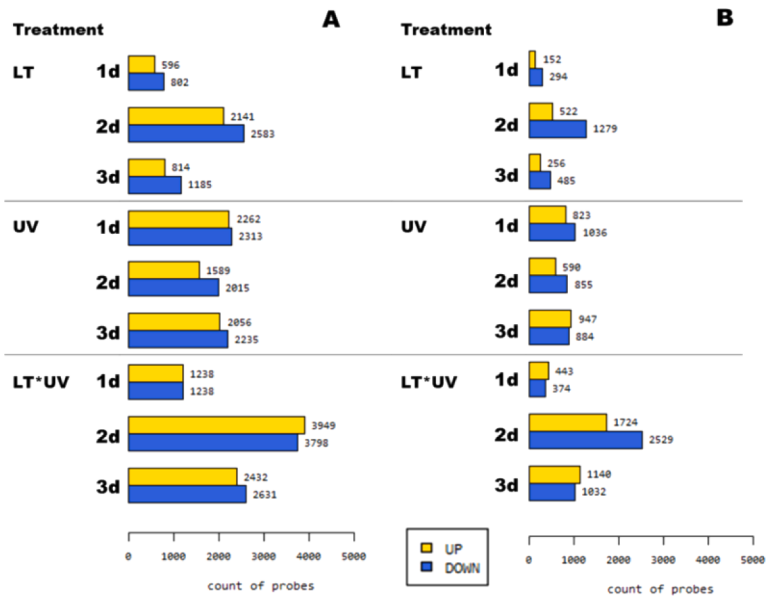

Fig. 3. Gene expression changes of *N. benthamiana* plants grown under LT and UV treatments at 1, 2, and 3 d. Up and downregulated genes were counted using bar plot-significant genes. Genes with statistically different expression ($p < 0.05$) and 2FC (A) and 3FC (B) were considered to be upregulated genes, and below -2 (A) and -3 (B) were considered to be downregulated genes. LT – treatment of low root-zone temperature, UV -/– UV treatment, LT*UV – combined treatment with LT and UV.

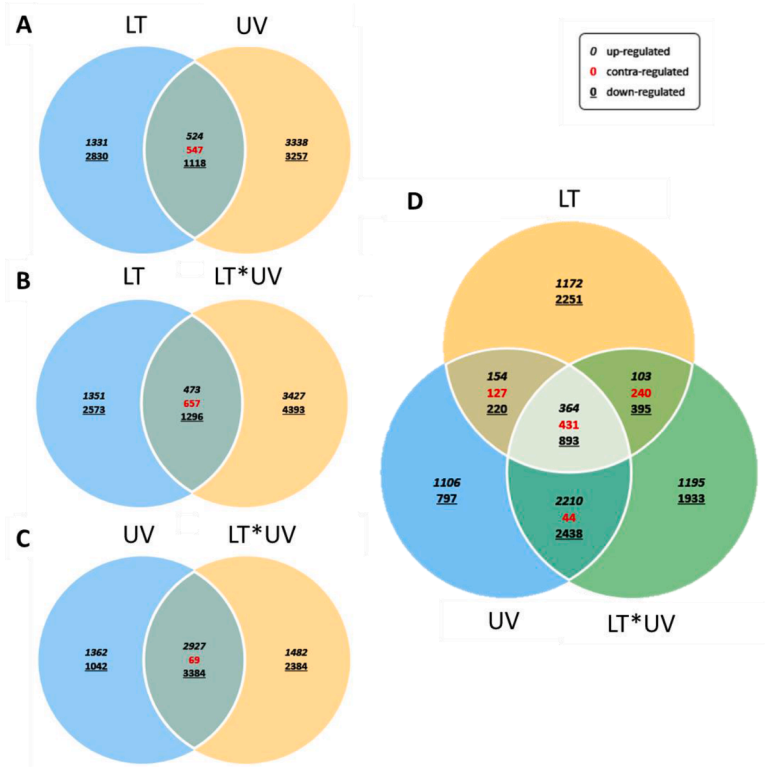

Fig. 4. Venn analysis of differentially expressed (DE) genes (up-, contra-, and downregulated) in *N. benthamiana* grown under LT and UV treatments (A), LT and LT*UV treatments (B), UV and LT*UV treatments (C), and all treatments (D). LT – treatment of low root-zone temperature, UV – UV treatment, LT*UV – combined treatment with LT and UV.

(approximately 2.61 times), *QS* (approximately 3.76 times), and *QPT* (approximately 2.35 times) and increased the expression of *ODC* (approximately 6.76 times) compared to those of the control.

To examine the terpenoid biosynthesis pathway, the mevalonate (3-hydroxy-3-methylglutaryl coenzyme A reductase (*HMGR*), squalene synthase (*SQS*), squalene epoxidase (*SQE*), cycloartenol synthase (*CAS*), and lanosterol synthase (*LAS*) and plastidic methylerythritol phosphate (MEP) pathway [farnesyl diphosphate synthase (*FPPS*), 1-deoxy-D-xylulose 5-phosphate synthase (*DXS*), deoxyxylulose 5-phosphate reductoisomerase (*DXR*), geranyl diphosphate synthase (*GPPS*), and geranylgeranyl diphosphate synthase (*GGPPS*)] were observed in *N. benthamiana* (Fig. 8). LT significantly induced *CAS* (approximately 2.07 times), *LAS* (approximately 3.56 times), *DXS* (approximately 1.90 times), and *GGPPS* (approximately 1.27 times), while UV treatment

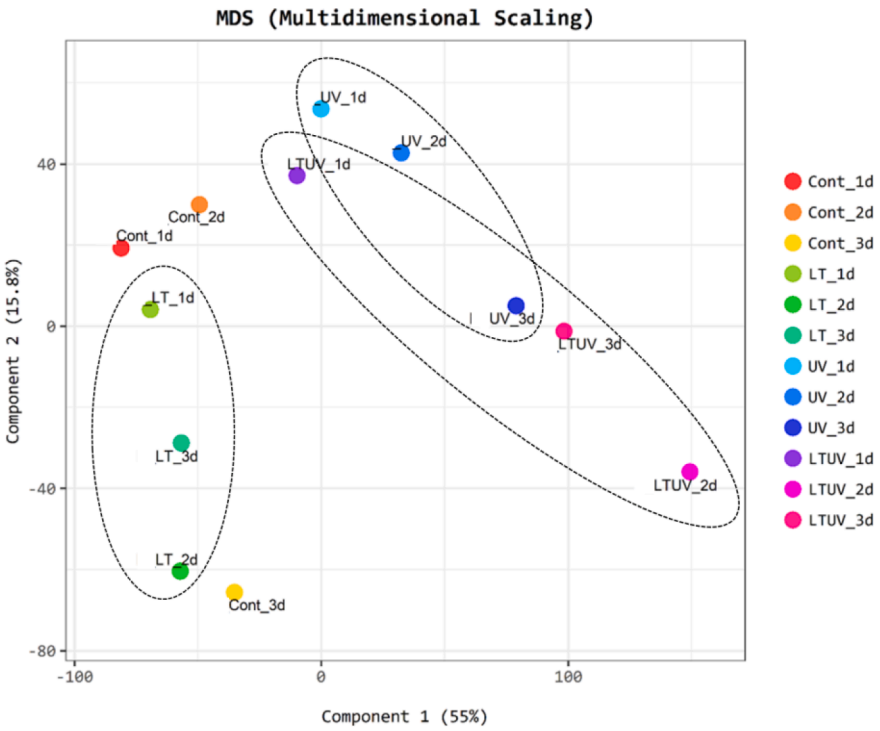

Fig. 5. Multidimensional Scaling (MDS) plots for the transcript of *N. benthamiana* plants grown under each treatment on days 1, 2, and 3. Cont – Control, LT – treatment of low root-zone temperature, UV – UV treatment, LT*UV – combined treatment with LT and UV.

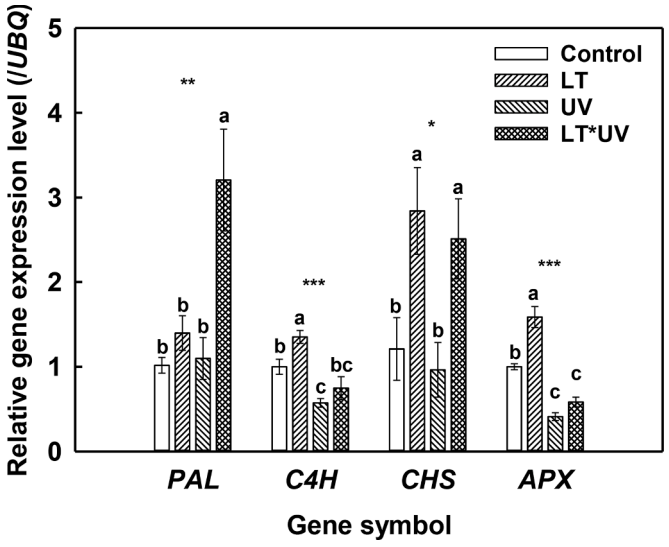

Fig. 6. Relative gene expression levels of *PAL* (phenylalanine ammonium lyase), *C4H* (cinnamate-4-hydroxylase), *CHS* (chalcone synthase), and *APX* (ascorbate peroxidase) of *N. benthamiana* plants grown under each treatment after 3 d. The bars represent mean ± standard error (n = 5). Different letters indicate significant differences among treatments (*p < 0.05, **p < 0.01, and ***p < 0.001). LT – treatment of low root-zone temperature, UV – UV treatment, LT*UV – combined treatment with LT and UV.

significantly induced *HMGR* (approximately 5.65 times). The expression levels of *SQS* (approximately 3.11 times), *CAS* (approximately 2.83 times), *DXS* (approximately 5.54 times), *DXR* (approximately 5.80 times), and *GPPS* (approximately 1.57 times) were significantly decreased by UV treatment compared to those of the control. Meanwhile, LT*UV treatment significantly increased the gene expression levels of *HMGR* (approximately 10.01 times), *SQE* (approximately 1.90 times), and *FPPS* (approximately 2.06 times) compared to those of the control. However, the gene expression levels of *DXS* (approximately 2.32 times) and *DXR* (approximately 3.26 times) were significantly decreased by LT*UV treatment.

3.5. KEGG pathway enrichment analyses of differentially expressed genes

KEGG annotations were distributed across 82 KEGG pathways. Metabolic and secondary metabolite biosynthesis pathways were the most highly enriched KEGG pathways (Supplementary Table S3 and Fig. 9). The biosynthesis of secondary metabolites in *N. benthamiana* was greatly affected by LT, UV, and LT*UV treatments. The significant alterations in gene expression in each pathway followed different patterns, indicating that environmental factors affect the metabolic pathways differently. The most significant differences in gene expression between the treatments used in this study were observed in the metabolic pathways (168 transcripts) and secondary metabolism (108 transcripts).

4. Discussion

In this study, transcriptomic and gene expression analysis regarding the pathway of secondary metabolites in *N. benthamiana plants* provide a further understanding of the detailed mechanisms in response to LT and UV radiation. The current study determined the environmental stress conditions for regulating secondary metabolites. The growth parameters of *N. benthamiana* plants were significantly decreased by RZT treatment at 15°C and UV treatment at 0.3 $W \cdot m^{-2}$ from those of the control plants (Table 1). The fresh and dry shoot and root weights decreased in the order of LT*UV > UV > LT > control group, suggesting that stress treatments for 3 d caused photosynthesis inhibition and different reaction steps in *N. benthamiana* plants. Although tolerance and resistance to low temperatures depend on the species, plants suffer physiological dysfunction when the temperature decreases below 10−15°C (Saltveit and Morris, 1990). Moreover, RZT can affect the mineral nutrient and water uptake rates from the roots to the leaves (Díaz-Pérez, 2009), which then causes a decline in photosynthetic rate and affects plant

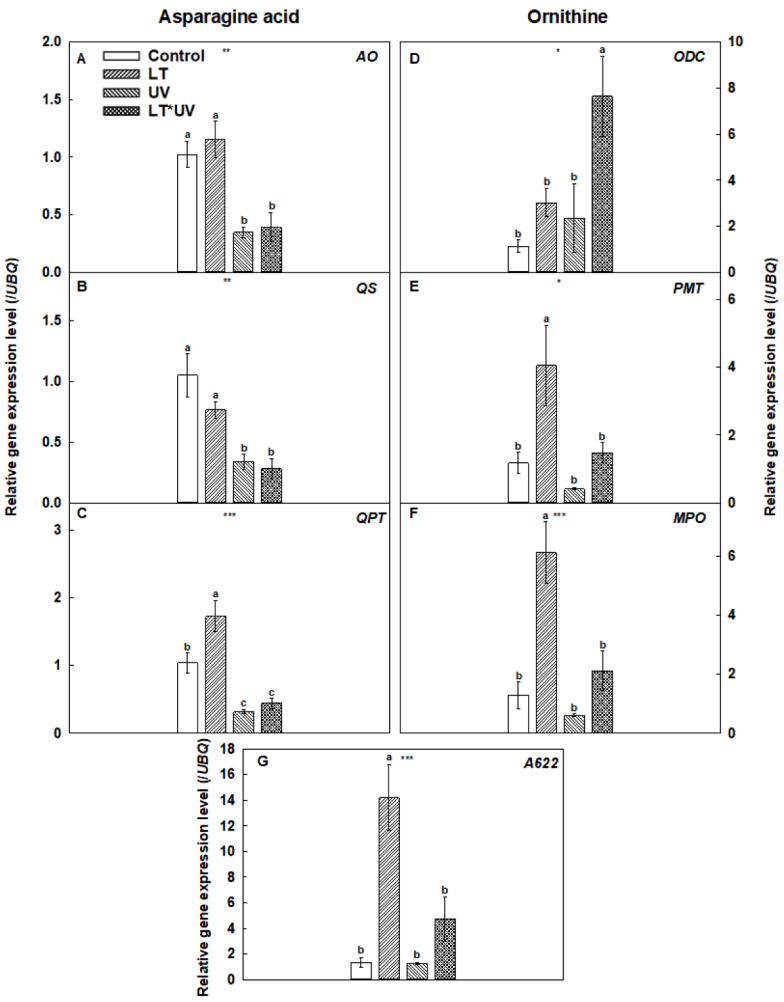

Fig. 7. The relative gene expression level of *AO* (aspartate oxidase, A), *QS* (quinolinate synthetase, B), *QPT* (quinolinate phosphoribosyltransferase, C), *ODC* (omithine decarboxylase, D), *PMT* (putrescine N-methyltransferase, E), *MPO* (N-methylputrescine oxidase, F), and *A622* (isoflavone reductase-like protein A622) of *N. benthamiana* plants grown under each treatment for 3 d. The bars represent mean ± standard error (n = 5). Different letters indicate significant differences among treatments (*p < 0.05, **p < 0.01, and ***p < 0.001).

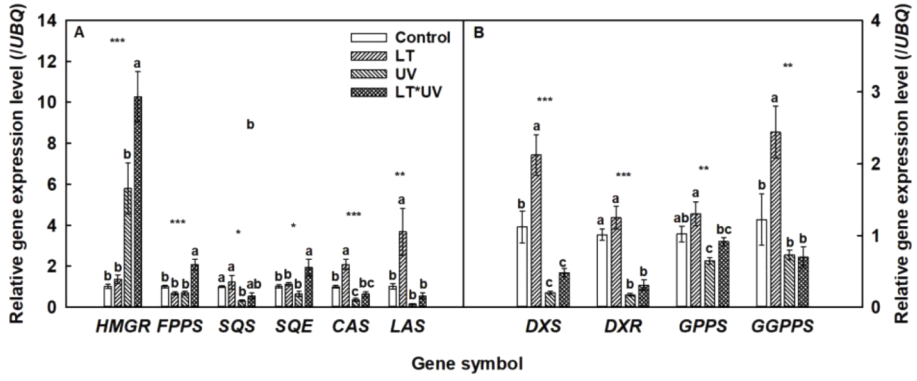

Fig. 8. Relative expression levels of genes related to the lanosterol and cycloartenol biosynthetic pathway (A) and isoprenoid biosynthesis (B) of *N. benthamiana* plants grown under each treatment for 3 d. The bars represent mean ± standard error (n = 5). Different letters indicate significant differences among treatments (*p < 0.05, **p < 0.01, and ***p < 0.001). *HMGR* (3-hydroxy-3-methylglutaryl coenzyme A reductase), *FPPS* (farnesyl diphosphate synthase), *SQS* (squalene synthase), *SQE* (squalene epoxidase), *CAS* (cycloartenol synthase), *LAS* (lanosterol synthase), *DXS* (1-deoxy-D-xylulose 5-phosphate synthase), *DXR* (deoxyxylulose 5-phosphate reductoisomerase), *GPPS* (GPP synthase), *GGPPS* (geranylgeranyl diphosphate synthase). LT – treatment of low root-zone temperature, UV – UV treatment, LT*UV – combined treatment with LT and UV.

growth (Fig. 2 and Table 1). Similar observations have been reported for several horticultural plants. In a soil-less culture, a low-nutrient solution and RZT inhibited the growth of spinach (Chadirin et al., 2011) and bell pepper (Aidoo et al., 2017). The LT treatment did not result in photosynthetic disorders after 1 d of treatment compared to the control; however, the value significantly decreased after 3 d of treatment (Fig. 2A). These are attributed to the cumulative effects of physiological disorders caused by chilling (Allen and Ort, 2001).

UV radiation may damage higher plants in various ways, affecting their DNA (Britt et al., 1993), photosynthetic apparatus (Greenberg et al., 1996), and plant growth (Day and Neale, 2002). UV-B reduces plant growth; however, the response of plants depends on the level of UV energy. Within the same species, UV-B treatment of 5.40 $kJ \cdot m^{-2}$.d reduced plant growth in wheat plants, such as the plant height and fresh and dry weight, while treatment of 3.24 $kJ \cdot m^{-2}$.d did not decrease growth (Lv et al., 2013). Here, our findings indicated that UV-A (8.06 $kJ \cdot m^{-2}$.d) and B (4.61 $kJ \cdot m^{-2}$.d) radiation intensity could exert slight stress on *N. benthamiana* plants (Table 1). UV-B radiation may directly affect photosystem II. Therefore, the net photosynthetic rate significantly decreased from day one of treatment (Fig. 2A). On the third day of

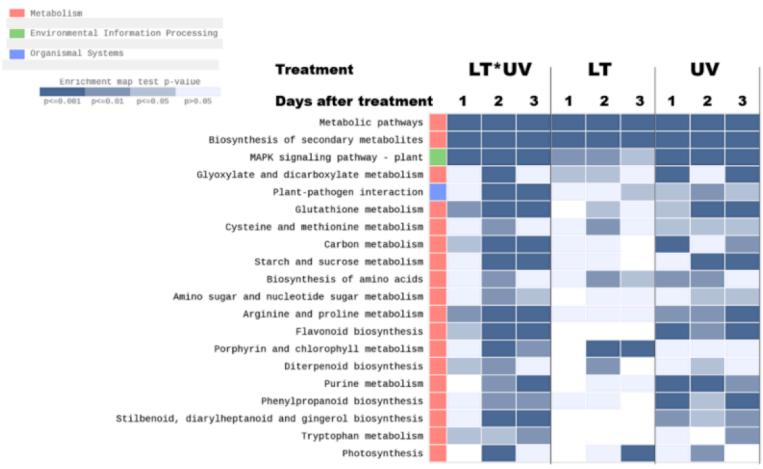

Fig. 9. The 20 high-ranking enriched KEGG pathways. The 20 high-ranking terms shown in the heatmap in the enrichment test. LT – treatment of low root-zone temperature, UV – UV treatment, LT*UV – combined treatment with LT and UV. The values of each enriched pathway in each treatment are based on the control.

treatment, UV treatment significantly decreased the Fv/Fm and Pn values (Fig. 2B). The rapid decrease in the photosynthetic parameters supported the growth results.

The most rapid decline occurred in LT*UV, which could be attributed to the synergistic effect (Son et al., 2020). In a previous study, combined treatment with 12°C (LT) and 10 kJ·m^{-2}·d (UV) significantly reduced the growth parameters (plant height, leaf area, and total dry weight) of cotton plants (Brand et al., 2016). Additionally, the combined stress resulted in a sharp decrease in Pn compared to that of single stress treatments after 3 d of treatment, thereby rapidly decreasing the growth of *N. benthamiana* plants (Fig. 2A).

The heatmap for the photosynthesis pathway in *N. benthamiana* indicates increased and decreased metabolite levels shown in green and red, respectively (Supplementary Fig. S4). The treatments with LT and UV were separated, and it was found that the levels of enzymes involved in the photosynthesis pathway fluctuated between treatments. The heatmap also revealed that the genes involved in photosynthesis such as photosystem II and I were differentially regulated at different times during LT and UV radiation treatments. Therefore, the expression of most genes involved in the photosynthesis pathway was decreased by LT and UV radiation.

Environmental factors primarily affect secondary metabolism in plants, stimulating the biosynthetic pathways of alkaloids, phenylpropanoids, flavonoids, and terpenoids. According to previous studies, PAL and C4H catalyze the first reaction of the phenylpropanoid and flavonoid metabolite pathway in plants (Ferrer et al., 2008). CHS is the first enzyme in the flavonoid pathway, producing several compounds, such as flavanones, flavanols, and anthocyanins (Albert et al., 1997). LT*UV treatment significantly increased the activities of both *PAL* and *CHS* compared to those in control (Fig. 7 and Supplementary Fig. S5). Additionally, the LT treatment group showed significantly increased gene expression of *CHS*. These results suggest that LT and LT*UV treatments can increase the synthesis of flavonoids. However, there was a small decrease or no significant effect on the expression of any genes related to the phenylpropanoid pathway for the UV-treated plants. The UV intensity of 0.3 W·m^{-2} may be sufficient to block flavonoid biosynthesis during the 3 d treatment. Tobacco growth was significantly decreased when the plants were exposed to UV treatment for 3 d (Table 1). Alternatively, the relative gene expression levels may have increased on days 1 or 2 of treatment rather than on the third day. Some studies have reported similar results; when the rice plants were treated with 0.15 W·m^{-2} UV-B light for 24 h, the expression of *PAL* and *CHS* significantly increased after 6 h and 2 h treatment, respectively (Kim et al., 2009). Generally, low temperatures cause imbalances in the metabolite pathway and accelerate the accumulation of additional reactive oxygen species (Apostolova et al., 2008). The expression level of the *APX* gene was only significantly increased in the LT treatment group (Fig. 7), which is one of the main pathways of ascorbate metabolism in plants and detoxifies hydrogen peroxide into the water using ascorbic acid (Ahmad et al., 2016). Therefore, the accumulation of *APX* can lead to the suppression of oxidative stress at low temperatures. This is similar to the results found by Janda et al. (2007), who reported that the expression of *CAT* and *APX* significantly increased in wheat plants treated at low temperatures.

Tobacco plants contain numerous alkaloids structurally related to nicotine (Cai et al., 2003), the most predominantly accumulated alkaloid in the leaves of most tobacco varieties, and accounts for 90%–95% of the total alkaloid content (Miceli et al., 2005). The *NAD* (nicotinamide adenine dinucleotide)*, AO, QS, QPT, ODC, PMT, MPO*, and *A622* are involved in nicotinic acid biosynthesis (Imanishi et al., 1998; Sinclair et al., 2000). Among them, *AO, QS*, and *QPT* lead to the production of nicotinic acid, and *ODC, PMT,* and *MPO* lead to the production of 4-methyl aminoethanol. Both the aspartic acid and ornithine pathways induce *A622* and ultimately biosynthesize nicotine. In *N. benthamiana* plants, a set of nicotine biosynthesis genes was activated by LT treatment at 15°C; however, their activation was effectively suppressed by UV radiation at 0.3 W·m^{-2} (Fig. 8). In particular, the upstream genes of nicotine biosynthesis, such as *AO, QS, PMT*, and *MPO*, were highly expressed, and the downstream gene *A622* was specifically expressed in the LT treatment (Fig. 8). Similar results were reported by Malik et al. (2013), who found that the expression of *PMT* and *A622* in tobacco plants treated at 12 and 25°C exceeded that of plants treated at 30°C. Our results suggest that low-temperature treatment can promote the genes related to nicotine accumulation more effectively than UV treatment (Supplementary Fig. S6).

The expression of genes related to terpenoid biosynthesis showed various responses to single and complex stress treatments. The expression of downstream genes of terpenoids, that is, *CAS, LAS, DXS*, and *GGPPS*, were significantly increased by LT treatment at 15°C. However, the expression of upstream genes, such as *HMGR* and *FPPS*, was not substantially different from that of the control. *HMGR* and *FPPS* may have been expressed before day 3 of treatment (days 1 or 2). Under UV treatment, there was no significant difference in the expression of most of the genes from that of the control, while the expression of *SQS, CAS, DXS*, and *GPPS* significantly decreased. However, according to the results of previous studies, some results are contradictory. UV radiation increased the expression of terpenoid-related genes (such as DXS and GPPS) from that of the controls in rice, cumin, and pyrethrum (Dabiri et al., 2020; Ghasemi et al., 2019; Kim et al., 2005), suggesting that 0.3 W·m^{-2} of UV treatment exerted extreme stress on tobacco plants in our

experiment. These results are consistent with those of the previous study (Jiao et al., 2018). The expression of *HMGR* and *FPPS* significantly increased in the LT*UV treatment. However, there was no significant difference in the expression of all genes except that of the *SQE* gene, which significantly decreased. The increase in *HMGR* and *FPPS* expression may respond to the intense stress from the combination treatment. Like the phenylpropanoid and alkaloid pathways, the low temperature was an effective treatment for activating terpenoid biosynthesis (Supplementary Fig. S7).

5. Conclusions

The main scope of this study was to determine the growth and pathways related to secondary metabolites by environmental stress using LT and UV in *N. benthamiana*. Many gene transcripts were discovered in response to LT and UV. The current study revealed that RZT and UV radiation affect the growth, transcription, and many pathways of *N. benthamiana*. LT and UV radiation had different effects on the pathways related to secondary metabolites. In contrast, the expression of most genes involved in the photosynthesis pathway was decreased by LT and UV irradiation. Meanwhile, the low temperature was an effective treatment for activating terpenoid and nicotine biosynthesis genes. Moreover, the combined treatment with LT and UV provides novel information to improve the quality of *N. benthamiana*; however, a detailed study on the synergistic effects under two environmental factors, RZT and UV radiation should be considered. The results of this study may improve the research on the enrichment of gene pathways in secondary metabolites, which would necessarily research environmental stresses in *N. benthamiana*.

Author contributions

K.-H. S. carried out the measurements and data analysis and drafted the manuscript. R. K. participated in microarray analysis. J.-H. L. contributed to gene expression and wrote the manuscript. J. W. completed the research and data analysis. E. G. made a substantial guide on the experimental design and critically revised the manuscript. We thank all our members for their technical support.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-10871.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.stress.2023.100136.

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