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Effects of Exogenous 24-Epibrassinolide Leaves Spraying Application on Chlorophyll Accumulation and Gene Expression Profiles of Chlorophyll Metabolism in Celery

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Abstract: Celery is an important leaf vegetable crop in *Apiaceae*, of which the petiole and leaf blade are the main edible parts. The content and proportion of photosynthetic pigments, mainly chlorophyll, have an important effect on the growth and quality of celery. As a brassinosteroid (BR) plant hormone with high physiological activity, 24-epibrassinolide (24-EBL) has the physiological functions of promoting chlorophyll accumulation and delaying leaf senescence. To investigate the effects of 24-EBL treatment on chlorophyll accumulation at different growth stages of celery, celery plants (variety Ningqin NO. 1) were treated from 45~59 days after sowing (DAS), at intervals of 7 days, with two different concentrations of 24-EBL: 1.04×10^{-6} mol·L^{-1} and 1.67×10^{-6} mol·L^{-1}. The content of chlorophyll and the expression levels of genes related to its metabolism were determined in celery leaf blades and petioles at three different stages (52, 59, 66 DAS). In the first stage (52 DAS), 1.04×10^{-6} mol·L^{-1} treatment of 24-EBL increased the expression levels of genes related to chlorophyll biosynthesis (*AgHEML*, *AgCHLG*, and *AgCAO*) to promote the accumulation of chlorophyll in leaf blades. During the second and third stages (59 and 66 DAS, respectively), 1.67×10^{-6} mol·L^{-1} 24-EBL treatment induced the expression levels of genes related to chlorophyll cyclic regeneration (*AgCLH*) and inhibited the up-regulation of genes related to chlorophyll degradation (*AgNYC*, *AgHCAR*, and *AgPPH*) to promote chlorophyll (especially chlorophyll *b*) accumulation. These treatments regulated the ratio of chlorophyll *a* content to chlorophyll *b* content and changed the leaf color of the celery. The results show that leaf spraying with an appropriate concentration of 24-EBL can facilitate chlorophyll synthesis by promoting chlorophyll synthesis and cycling-related gene expression levels and increase chlorophyll content in the leaves of celery. This study provides a reference for exploring the specific function of 24-EBL in regulating chlorophyll content during the growth and development of celery.

Keywords: 24-epibrassinolide; chlorophyll accumulation; gene expression profiles; growth period; celery

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1. Introduction

Celery (*Apium graveolens* L.) is a plant belonging to the genus *Apium* of the *Apiaceae* family, which originated from the Mediterranean coastal region and was introduced into China through the Silk Road. It is now one of the most important leaf vegetable crops with wide planting areas and market demands [1–3]. The leaves (including the petiole and leaf blade) are the main edible parts of celery, and the appropriate chlorophyll content and the content ratio of Chl *a*/Chl *b* (chlorophyll *a*/chlorophyll *b*) within celery is the key to carry out various life activities with light energy [4,5]. The celery products with higher chlorophyll content have better appearance and nutritional quality, making them more likely to be favored by consumers. Obtaining celery products with high chlorophyll content by adopting reasonable agronomic measures has become one of the research directions in high-quality celery production in recent years [6].

In higher plants, the biosynthesis and degradation of chlorophyll is a complex process regulated by multiple genes. The 5-amino-ketovaleric acid was used as a substrate for chlorophyll biosynthesis. Protoporphyrin IX was first synthesized by enzyme reaction. Then, either chlorophyll *a* were synthesized or chlorophyll *b* were transformed [7]. During chlorophyll degradation, chlorophyll *a* can be directly degraded into pheophytin *a* or transformed into chlorophyllin ester *a* into chlorophyll cyclic regeneration. During chlorophyll *b* degradation, it needs to be degraded into chlorophyll *a* before subsequent degradation or cycling regeneration [8]. At different stages of plant growth and development, the change in the expression levels of related genes in the chlorophyll metabolic pathway affects the content. Thus, the plant showed different color and photosynthetic efficiency [9]. Influenced by color of different type of chlorophyll self, with a higher content of chlorophyll and ratio of Chl *a*/Chl *b*, the color of the leaves tends to be a deeper green; conversely, with a lower chlorophyll content and Chl *a*/Chl *b* ratio, the leaves tend to be a yellow-green color [10]. During the process from maturity to senescence of the leaves, affected by the differential expression levels of genes related to chlorophyll metabolism, the total chlorophyll content tends to increase to a peak and then decrease gradually, and the ratio of Chl *a*/Chl *b* mostly fluctuates within a certain range. The content and proportion of chlorophyll become an important evaluation index in the process of leaf maturation and senescence [11,12].

Brassinosteroids (BRs) are a type of cyclopentane polyhydrophenate with high bioactivity, which have been classified as the sixth largest plant hormone [13]. BRs mainly include brassinolide (BR), epibrassinolide (EBR), and high brassinolide (HBR). Due to its strong physiological activity on plant growth and development, 24-epibrassinolide (24-EBL) has been widely used in agricultural production [14,15]. In the process of adversity or gradual senescence of plants, BRs can maintain chlorophyll content and improve crop resistance and product quality by promoting the expression levels of chlorophyll biosynthesis and recycling genes, such as *CHLD* and *CHL*, and by inhibiting the up-regulation of chlorophyll degradation genes, such as *HCAR* and *PPH* [16,17]. Under the appropriate concentration treatment of EBR, the contents of chlorophyll and carotenoid in the leaves of tomato (*Solanum lycopersicum* L.) and potato (*Solanum tuberosum* L.) were increased, which improved the contents of soluble sugar, ascorbic acid, and other nutrients in the product organs [18,19]. The optimal concentration of EBR-treated plants often varies according to crop varieties, treatment methods, and treatment sites, and there is a relatively optimal concentration range. If the treatment concentration of 24-EBL exceeds the appropriate concentration range, it will inhibit the normal growth of crops [20,21].

Leaves spraying and root irrigation with 24-EBL can promote the production of celery and improve its dietary fiber content. The appropriate treatment concentration in different varieties of vegetables have great differences; for celery leaf spraying, the suitable concentration is between 10^{-3} mol·L^{-1} to 10^{-6} mol·L^{-1} [22]. The appropriate 24-EBL leaf spraying concentration for chlorophyll accumulation in celery is unclear; for the effects and the internal mechanism of 24-EBL on chlorophyll accumulation in celery, there is a lack of systematic reports at present. The aim of this study is to help understand the appropriate

24-EBL treatment concentration for the improvement of chlorophyll content in celery which, combined with the expression profiles of genes related to chlorophyll metabolism, would be helpful to understand its internal mechanism to provide certain references for application of 24-EBL in celery production.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

Celery plants (variety Ningqin NO. 1), as the experimental material, were planted in the artificial climate growing room of the Apiaceae Vegetable Crop Laboratory, State Key Laboratory of Crop Genetics and Germplasm Enhancement and Utilization, Nanjing Agricultural University. Organic substrate, vermiculite, and perlite were mixed at a volume ratio of 2:2:1 and put into 13.5 cm × 12 cm × 12 cm seedling pots for celery planting. The appropriate temperature, humidity, light–dark period (25 °C 16 h at daytime, 18 °C 8 h at night, relative humidity 60~70%) were kept.

2.2. Exogenous 24-EBL Treatments

In experimental groups, two treatments of 24-EBL (1.04×10^{-6} mol·L^{-1} and 1.67×10^{-6} mol·L^{-1}) (100 mL) were applied by spraying on celery plants (variety Ningqin NO. 1), and the control group was treated with water. Spraying was done three times at 45, 52, and 59 DAS (days after sowing), respectively. After one week of treatment, at 52, 59, and 66 DAS, respectively, celery leaf blades and petioles were sampled to determine the chlorophyll content, proportion, and expression levels of genes related to chlorophyll metabolism in each treatment group, respectively. The chlorophyll content and the ratio of Chl *a*/Chl *b* in the leaf blades and petioles, as well as the relative expression levels of genes related to chlorophyll metabolism, were detected. The 24-EBL was purchased from Yuanye, Shanghai, China. Three biological replicates were performed. The treatments of periods and concentrations are shown in Table 1.

Table 1. Treatments of concentration, frequency, and time for celery.

Period (Stage)	**Treatment Concentration and Dosage of 24-EBL**								
	0			**1.04×10^{-6} mol·L^{-1}**			**1.67×10^{-6} mol·L^{-1}**		
45 DAS (CK)	100 mL	100 mL	100 mL	100 mL	100 mL	100 mL	100 mL	100 mL	100 mL
52 DAS (The first stage)	√	100 mL	100 mL	√	100 mL	100 mL	√	100 mL	100 mL
59 DAS (The second stage)		√	100 mL		√	100 mL		√	100 mL
66 DAS (The third stage)			√			√			√

45 DAS, 52 DAS, 59 DAS, and 66 DAS indicate days after sowing; 0, 1.04×10^{-6} mg·L^{-1}, and 1.67×10^{-6} mg·L^{-1} indicate 24-EBL treatment concentration; '√' represents the sampling date, which is one week after the last spraying treatment has finished.

2.3. Extraction and Determination of Chlorophyll Content

Chlorophyll content was determined by the acetone–ethanol mixture extraction method [23]. An amount of 0.1 g of the pre-crushed fresh celery samples was weighed and added to the extraction solution, which was obtained by mixing 10 mL ethanol with acetone with a volume ratio of 1:19, then then soaked in the dark for about 48 h to get the extraction solution of chlorophyll. When the pigment in the precipitation was completely white, the extract was filtered out. A SpectraMax enzyme marker (Molecular Devices, Santa Clara, CA, USA) was used to detect the light absorption values of the extracts at 646 nm and 663 nm, and the chlorophyll *a* (C_a), chlorophyll *b* (C_b), and total chlorophyll (C) contents of

the celery samples were calculated according to the formula as follows. Three biological replicates were set for each sample.

$$C_a\ (mg{\cdot}L^{-1}) = 2.21OD_{663} - 2.81OD_{646} \quad (1)$$

$$C_b\ (mg{\cdot}L^{-1}) = 20.13OD_{646} - 5.03OD_{663} \quad (2)$$

$$C\ (mg{\cdot}L^{-1}) = C_a + C_b \quad (3)$$

$$\text{Chlorophyll content}\ (mg{\cdot}g^{-1}) = C * V/M \quad (4)$$

2.4. Total RNA Extraction and cDNA Preparation

Total RNA from celery leaf blades and petioles were extracted according to the instructions of the Simple Total RNA Extract kit (Tiangen, Beijing, China). A NanoDrop 2000 trace ultraviolet spectrophotometer (Molecular Devices, Santa Clara, CA, USA) was used to detect the concentration and quality of RNA. Then, the Primescript RT reagent kit (TaKaRa, Dalian, China) was used to reverse-transcribe total RNA into cDNA.

2.5. Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) Analysis

The expression levels of genes related to chlorophyll metabolism in celery were determined by RT-qPCR. The total RT-qPCR system consisted of 10 µL for SYBR Premix Ex Taq, 7.2 µL for ddH_2O, 2 µL for diluted cDNA, and 0.4 µL for positive and negative primers. The reaction procedure was: predenaturation at 95 °C for 30 s, denaturation at 95 °C for 5 s, annealing at 60 °C for 30 s, for a total of 40 cycles. The celery *AgTUB-B* gene was used as the internal reference gene, and the RT-qPCR primer sequence of genes related to chlorophyll metabolism was referred to the references [24–26]. Seven genes related to chlorophyll synthesis and cyclic regeneration and degradation were selected, including *AgHEML*, *AgCAO*, *AgCHLG*, *AgCHL*, *AgNYC*, *AgHCAR*, and *AgPPH*. The relative gene expression levels were calculated based on the $2^{-\Delta\Delta Ct}$ method [27]. Three biological replicates were set in each group. Primer Premier 6.0 software (Premier Company, Oakville, ON, Canada) (Copyright (C) 2002-2006 Macrovision) was used to design primers (Table 2).

Table 2. Quantitative primer sequences of chlorophyll metabolism-related genes in celery.

Gene Name	Sequences of Forward Primers (5′–3′)	Sequences of Reverse Primers (5′–3′)
AgTUB-B	TGGTGGCACTGGATCTGGTATGG	ACTTTCGGAGGAGGGAAGACTGAA
AgHEML	GTTGTCTTATGGCGGTGCTCAAG	GTGGATTCCTGCCGTCATTGC
AgCAO	TTGGTGAATGATAGGCTGTT	GGATGGTAAGGTTGGACTG
AgCHLG	CGCCTGACATAATTGTTCTTACACTCT	CACATCAATAGCACCAACACATATCCA
AgCHL	GCTTCATTGTCATTGCTCCTCAGTTAT	CCTTCAGATAGCCACTTGGTTATTGC
AgNYC	GGATGCGAGTAGTAGTAAAGGGAAATGGAA	TGGCGTCTGTAATTGATAACCGAGTC
AgHCAR	CAGTGGACAGGCATAGTGACAACAA	ACTTCCTCTGGCGTCCTTGCTAA
AgPPH	CAAGCAGAGGCATCACAA	CCGTATAATGGAGACAACAAG

2.6. Data Analysis

SPSS 25.0 software (IBM Corporation, Armonk, NY, USA) (Copyright IBM Corp. 2006, 2016) was used for variance analysis, multiple comparison, and correlation analysis. GraphPad Prism 9.4 software (San Diego Corporation, San Diego, CA, USA) (1992–2022 GpaphPad Software, LLC) was used for charting.

3. Results

3.1. Effects of Exogenous 24-EBL on the Leaf Color of Celery

Compared with the control, exogenous 24-EBL treatment changed the appearance and leaf color of celery (Figure 1). At the first stage, 1.04×10^{-6} mol·L^{-1} and 1.67×10^{-6} mol·L^{-1} concentrations of 24-EBL treatment increased the height of celery plants. There was no

obvious difference in leaf color among the treatment and control celery plants. During the second stage, the thickening degree of the leaf blade area and petioles of the celery plants increased under each concentration of 24-EBL treatment, and the leaf blades were an oily green color. The effects of the 1.04×10^{-6} mol·L^{-1} treatment group plants were more obvious. At the third stage, celery plants under the two treatment groups showed the characteristics of tall plants, with robust root systems and dense green leaf blades, and the effects of the 1.67×10^{-6} mol·L^{-1} treatment celery plants were more obvious.

Figure 1. Effects of exogenous 24-EBL on the appearance of celery. (**A**) Control of 52 DAS; (**B**) 1.04×10^{-6} mol·L^{-1} 24-EBL treatment of 52 DAS; (**C**) 1.67×10^{-6} mol·L^{-1} 24-EBL treatment of 52 DAS; (**D**) Control of 59 DAS; (**E**) 1.04×10^{-6} mol·L^{-1} 24-EBL treatment of 59 DAS; (**F**) 1.67×10^{-6} mol·L^{-1} 24-EBL treatment of 59 DAS; (**G**) Control of 66 DAS; (**H**) 1.04×10^{-6} mol·L^{-1} 24-EBL treatment of 66 DAS; (**I**) 1.67×10^{-6} mol·L^{-1} 24-EBL treatment of 66 DAS. Scale bars = 5 cm.

3.2. Effects of Exogenous 24-EBL on Chlorophyll Content and Proportion in Leaf Blades of Celery

Compared with the control celery group plants at the same treatment date, at the first stage (52 DAS), total chlorophyll content in the 1.67×10^{-6} mol·L^{-1} treatment group decreased (Figure 2C). At the second stage (59 DAS), the contents of all types of chlorophyll increased with the 1.67×10^{-6} mol·L^{-1} treatment, with the contents of chlorophyll *a*, chlorophyll *b*, and total chlorophyll being 0.58 mg·g^{-1}, 0.23 mg·g^{-1}, and 0.81 mg·g^{-1}, which were over 1.4 times higher than those in the control group (Figure 2A–C). The content of chlorophyll *b* decreased, and the content ratio of Chl *a*/Chl *b* increased in the 1.04×10^{-6} mol·L^{-1} treatment celery group plants (Figure 2B,D). At the third stage (66 DAS), both concentrations of 24-EBL treatment promoted the accumulation of chlorophyll (Figure 2A–C). At the treatment concentration of 1.67×10^{-6} mol·L^{-1}, the content of chlorophyll *b* reached 0.26 mg·g^{-1}, 1.5 times that of the control group (Figure 2B), and the content ratio of Chl *a*/Chl *b* was lower than that of the control group (Figure 2D).

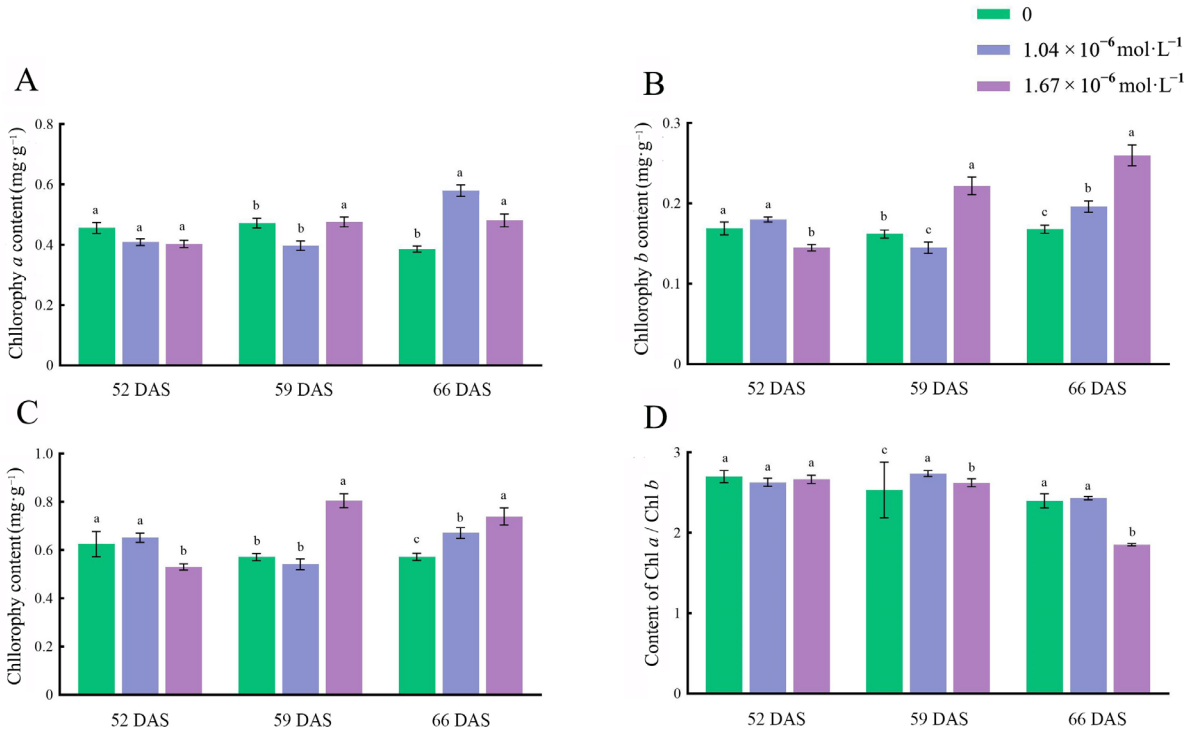

Figure 2. Effects of exogenous 24-EBL on chlorophyll content and proportion in leaf blades of celery. (**A**) Chlorophyll *a* content; (**B**) Chlorophyll *b* content; (**C**) Chlorophyll content; (**D**) The content ratio of Chl *a*/Chl *b*. The data were grouped by days after sowing and were analyzed for significance of differences. The different letters indicate significant differences at the 0.05 level. Bars are shown as the mean ± standard deviation of three biological replicates.

3.3. Effects of Exogenous 24-EBL on Chlorophyll Content and Proportion in Petioles of Celery

Compared with the control celery group plants at the same treatment date, at the first stage (52 DAS), the contents of chlorophyll *a* and total chlorophyll increased to 0.081 mg·g^{-1} and 0.117 mg·g^{-1}, respectively, in the 1.67×10^{-6} mol·L^{-1} treatment celery group plants (Figure 3A,C). The contents ratio of Chl *a*/Chl *b* in celery petioles decreased in the 1.04×10^{-6} mol·L^{-1} treatment groups (Figure 3D). At the second stage (59 DAS), the contents of all types of chlorophyll decreased with 24-EBL treatment, and the Chl *a*/Chl *b* increased with the 24-EBL treatment of 1.04×10^{-6} mol·L^{-1} (Figure 3A–D). The third stage (66 DAS) showed an increase of the contents of all types of chlorophyll with 1.04×10^{-6} mol·L^{-1} 24-EBL treatment, where the chlorophyll *b* content was 0.034 mg·g^{-1}, 1.6 times that the control group (Figure 3A–D). The content ratio of Chl *a*/Chl *b* was lower than that of the control group (Figure 3D). The contents of chlorophyll *a* and total

chlorophyll in the 1.67 × 10^{-6} mol·L^{-1} treatment group showed significant reductions (Figure 3A,C).

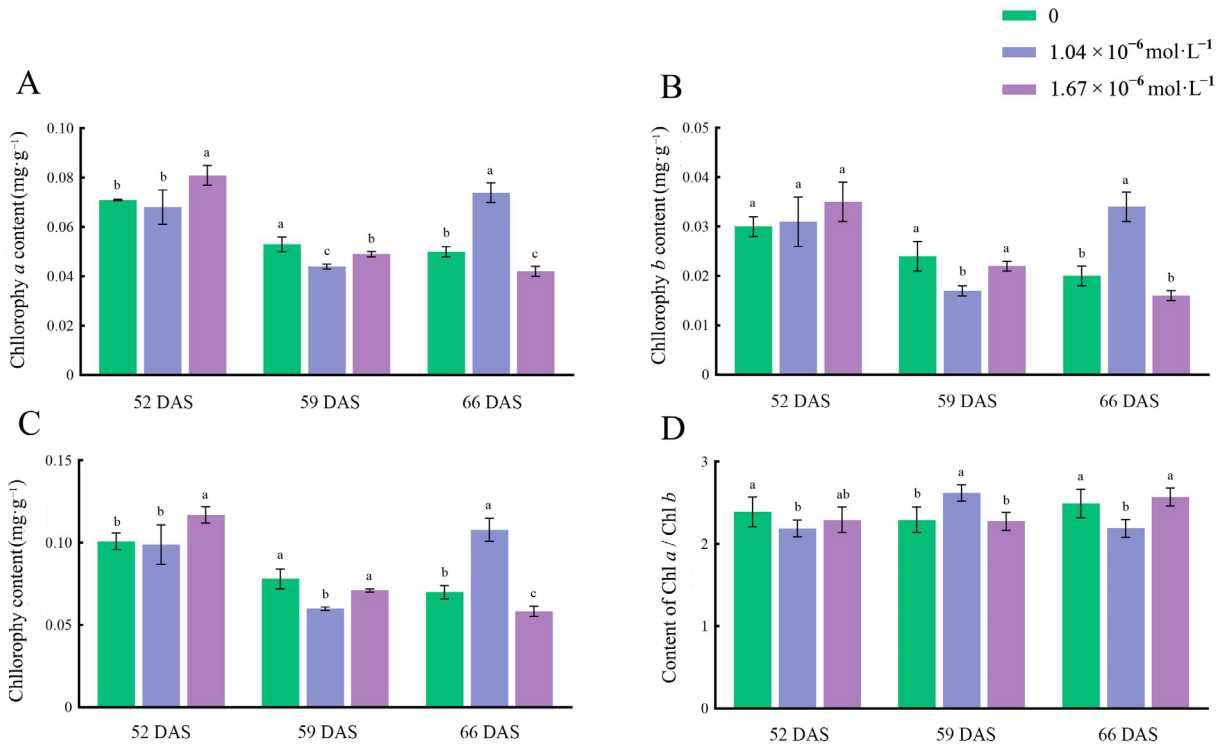

Figure 3. Effects of exogenous 24-EBL on chlorophyll content and proportion in petioles of celery. (**A**) Chlorophyll *a* content; (**B**) Chlorophyll *b* content; (**C**) Chlorophyll content; (**D**) The content ratio of Chl *a*/Chl *b*. The data were grouped by the days after sowing and were analyzed for significance of differences, with different letters indicating significant differences at the 0.05 level. Bars are shown as the mean ± standard deviation of three biological replicates.

3.4. Effects of Exogenous 24-EBL on Expression of Genes Related to Chlorophyll Metabolism in Leaf Blades of Celery

Compared with the control group at the same date, at the first stage (52 DAS), exogenous 24-EBL treatment increased the expression levels of genes related to chlorophyll synthesis (*AgCAO*) and chlorophyll degradation (*AgPPH* and *AgNYC*). The expression level of the *AgNYC* gene was more than 30 times higher than the control group. The expression level of chlorophyll degradation and the metabolism-related gene *AgHCAR* decreased (Figure 4A). At the second stage (59 DAS), the expression levels of chlorophyll synthesis-related genes, *AgCAO* and *AgCHLG*, decreased under 1.04 × 10^{-6} mol·L^{-1} treatment. At the treatment concentration of 1.67 × 10^{-6} mol·L^{-1}, the expression levels of genes related to chlorophyll synthesis and cyclic regeneration, *AgHEML*, *AgCAO*, *AgCHLG*, and *AgCLH*, were over 1.5 times that of the control group (Figure 4B). At the third stage (66 DAS), exogenous 24-EBL treatment at all concentrations increased the expression levels of the genes related to chlorophyll synthesis and cyclic regeneration, *AgCHLG* and *AgCLH*. The relative expression level of *AgCLH* gene increased 5 times more than that of the control. The expression levels of chlorophyll synthesis-related genes, *AgHEML* and *AgCAO*, decreased under 1.04 × 10^{-6} mol·L^{-1} treatment. The expression levels of chlorophyll degradation-related genes, *AgNYC* and *AgHCAR*, decreased under 1.67 × 10^{-6} mol·L^{-1} treatment (Figure 4C).

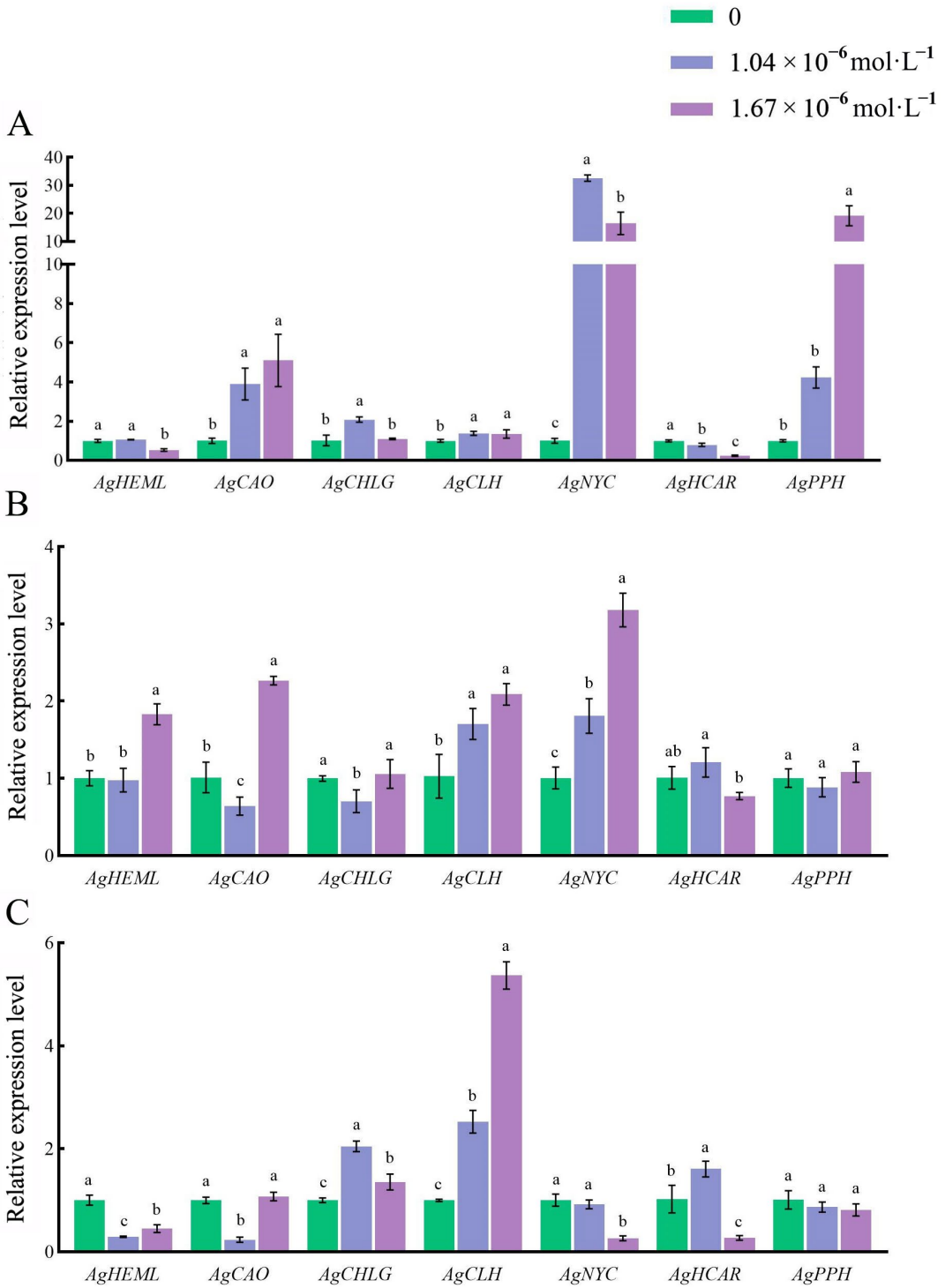

Figure 4. Effects of exogenous 24-EBL on expression levels of genes related to chlorophyll metabolism in leaf blades of celery. (**A**) 52 DAS (the first stage); (**B**) 59 DAS (the second stage); (**C**) 66 DAS (the third stage). The data were grouped by days after sowing and were analyzed for significance of differences, with different letters indicating significant differences at the 0.05 level. Bars are shown as the mean ± standard deviation of three biological replicates.

3.5. Effects of Exogenous 24-EBL on the Expression of Genes Related to Chlorophyll Metabolism in Celery Petioles

Compared with the control group at the same time, at the first stage (52 DAS), exogenous 24-EBL treatment increased the expression levels of the gene related to chlorophyll

synthesis, *AgCAO*, and the genes related to chlorophyll degradation, *AgNYC*, *AgHCAR*, and *AgPPH*. The relative expression level of the *AgCAO* gene was over 10 times that of the control the 1.67×10^{-6} mol·L^{-1} treatment, the expression levels also increased significantly compared to the control celery plants (Figure 5A). At the second stage (59 DAS), the relative expression levels of the chlorophyll synthesis-related gene, *AgCAO*, and the chlorophyll degradation-related genes, *AgNYC* and *AgHCAR*, increased under 1.67×10^{-6} mol·L^{-1} treatment. The relative expression levels of chlorophyll degradation-related genes, *AgNYC* and *AgPPH*, decreased under the treatment of 1.04×10^{-6} mol·L^{-1} 24-EBL. The relative expression levels of the gene *AgCAO* in the two treatment groups increased by over 7 times that of the control group celery plants (Figure 5B). At the third stage (66 DAS), the relative expression levels of chlorophyll synthesis and cyclic regeneration-related genes, *AgCAO* and *AgCLH*, increased under 1.67×10^{-6} mol·L^{-1} treatment compared to the control celery plants. The relative expression levels of chlorophyll degrading genes, *AgCHLG* and *AgPPH*, decreased under the treatment of 1.04×10^{-6} mol·L^{-1} 24-EBL compared to the control celery plants (Figure 5C).

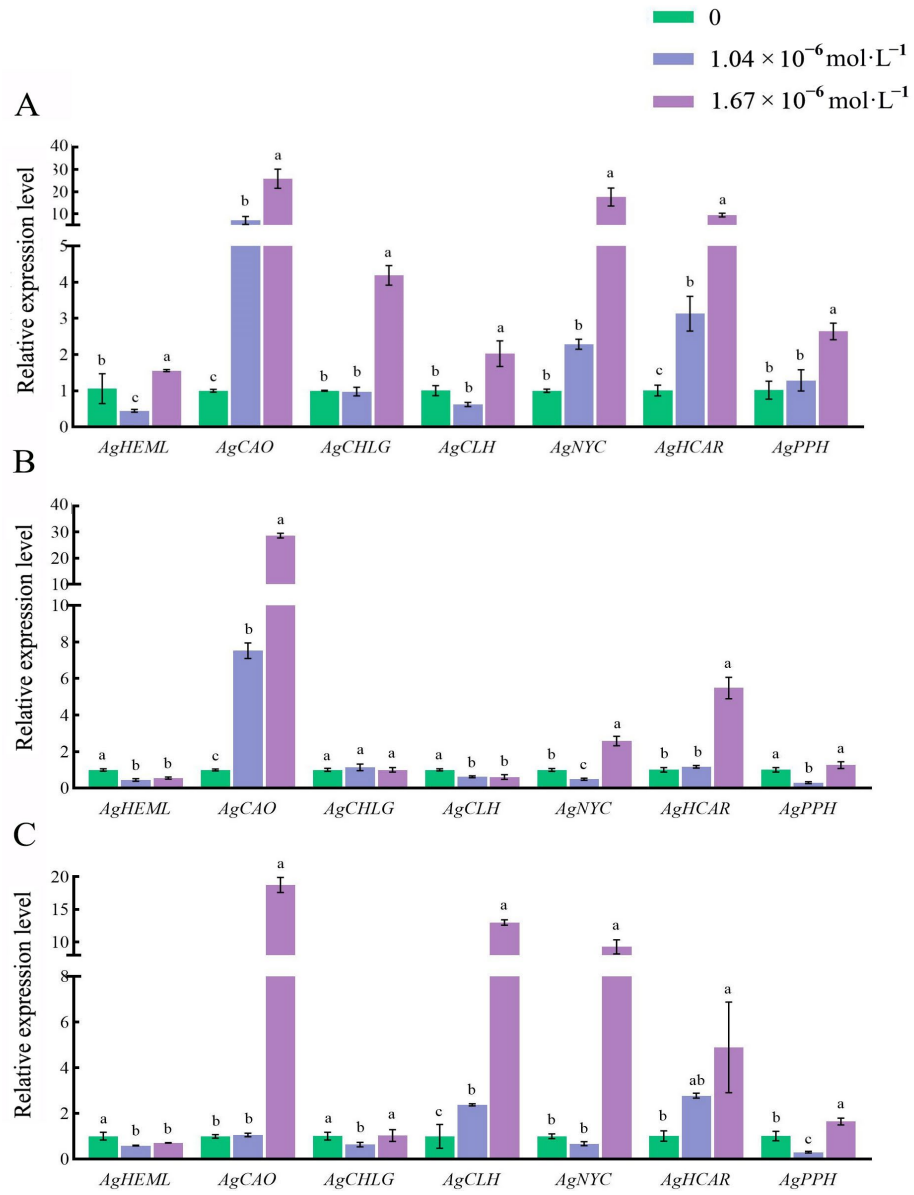

Figure 5. Effects of exogenous 24-EBL on expression levels of genes related to chlorophyll metabolism in petioles of celery. (**A**) 52 DAS (the first stage); (**B**) 59 DAS (the second stage); (**C**) 66 DAS (the third

stage). The data were grouped by days after sowing and were analyzed for significance of differences, with different letters indicating significant differences at the 0.05 level. Bars are shown as the mean ± standard deviation of three biological replicates.

4. Discussion

As a brassinosteroid, 24-Epibrassinolide (24-EBL) is a type of analogue, which is easy to synthesize with strong natural ecological activity, and has been widely applied in the production of many horticultural crops to good effects [28]. Celery is an important leaf vegetable crop, and the content and proportion of photosynthetic pigments, mainly chlorophyll, in its leaves are the key hinge for the formation of its yield and quality. Exogenous 24-EBL treatment could improve the growth of crops at different development stages and conditions by regulating the chlorophyll content and proportion [29]. BR treatment can alleviate the chlorophyll degradation and gas exchange obstacles caused by magnesium deficiency, and improve the activity of its photosynthetic system and the utilization of light energy in strawberries (*Fragaria* × *ananassa* Duch.) [28,29]. Under the condition of magnesium deficiency, 24-EBL could increase the chlorophyll content, photochemical efficiency, and gas exchange rate in the leaves of seedling soybeans (*Glycine max* L.) [30]. In the seedling stage of tomatoes (*Solanum lycopersicum* L.), 24-EBL treatment weakened the decrease of chlorophyll content in leaves under plumbeous stress. During the growth and fruit setting stage, 24-EBL treatment can alleviate the degradation of chlorophyll *b* under low-light stress to maintain the stability of chlorophyll content and ratio and increase the carbohydrate content in the tomato fruit [31,32]. Similar to the results of previous studies, the appropriate concentration of 24-EBL treatment in this study increased the chlorophyll content, and the optimal concentration was different with different treatment dates of celery. At the first stage (52 DAS) of 24-EBL treatment, 1.04×10^{-6} mol·L^{-1} of 24-EBL was more suitable for chlorophyll *b* accumulation in celery leaf blades; all the types of chlorophyll content decreased when the concentration increased to 1.67×10^{-6} mol·L^{-1}. At the second and third stages (59 and 66 DAS), the treatment concentration of 1.67×10^{-6} mol·L^{-1} was beneficial to the content of chlorophyll in leaf blades, especially the accumulation of chlorophyll *b*, whose content reached 1.4 times more than the control group at the same treatment date. The concentrations of 24-EBL suitable for chlorophyll accumulation at different stages or different acting sites were different in celery.

Furthermore, 24-EBL regulates the chlorophyll content and proportion of celery, and affects its leaf color, which may be realized by affecting the aging process of leaves. At the early stages of leaf growth, adequate glutamic acid should be used as the substrate for chlorophyll *a* synthesis. In the later stage, the relative content of chlorophyll *b* gradually increased, and the leaves' color gradually changed from light green to emerald green to yellow-green, which was an important sign of the gradual maturation of leaves [33,34]. Chlorophyll degradation was a conserved pathway during the aging leaves of green plants, and affects the side-chain modification groups of chloroplast proteins. Chlorophyll *a* and chlorophyll *b* differ only in side-chain modification groups, which can be transformed into each other in the chlorophyll metabolic pathway [35]. At the ripening stage of plants, the decrease of chlorophyll content in leaves could be inhibited with BR spraying, thereby maintaining high photosynthetic efficiency and ensuring the accumulation of nutrients in the fruits and seed [36]. The results of this study show that the appropriate concentration of 24-EBL treatment could increase the chlorophyll content in leaf blades and petioles at each stage of celery, promote the accumulation of chlorophyll *b*, and reduce the content ratio of Chl *a*/Chl *b* at a later stage. Moreover, 24-EBL may promote leaf development by promoting chlorophyll *b* accumulation and delaying leaf senescence caused by chlorophyll degradation.

The synthesis and degradation of chlorophyll is a complex pathway. Under the regulation of a series of genes, such as genes *HEML* (encoding glutamate-1-hemaldehyde transaminase) and *CAO* (encoding chlorophyll-ester a oxygen enzyme), chlorophyll-ester *a* and chlorophyll-ester *b* should be synthesized successively and then catalyzed by chloro-

phyll synthase encoded by gene *CHLG* to synthesize chlorophyll *a* and chlorophyll *b*, respectively [37]. Chlorophyll *b* can be reduced to chlorophyll *a* through the action of chlorophyll *b* reductase and 7-hydroxymethyl chlorophyll *a* reductase, encoded by genes *NYC* and *HCAR*, and then can be reduced to deenzymatic chlorophyllin *a* by the action of deenzymatic chlorophyll hydrolase encoded by gene *PPH*, and enter the process of chlorophyll degradation [38]. The chlorophyll enzyme encoded by gene *CLH* can catalyze the transformation of partially damaged chlorophyll *a* into chlorophyll-ester and promote the renewal and repair of chlorophyll by returning to the chlorophyll cycling system [39]. Both the deenzymatic chlorophyll hydrolase encoded by gene *PPH* and the chlorophyll enzyme encoded by gene *CLH* can use chlorophyll *a* as a substrate. The former catalyzes the synthesis of the first key enzyme in the process of chlorophyll degradation, which is highly expressed in aging leaves. The latter is an important gene in the cyclic regeneration pathway in chlorophyll and is one of the key genes for balancing the content ratio of Chl *a*/Chl *b* in plants and stabilizing the activity of the photosynthetic system [40,41].

In the chromium stress treatment of nightshade (*Solanum nigrum* L.), exogenous 24-EBL can up-regulate the genes expression levels of the chlorophyll biosynthesis pathway and down-regulate the genes expression levels of the degradation pathway. The chlorophyll content of leaves can be maintained at a relatively high level [42]. In horticultural crops with leaves as product organs, 24-EBL leaves spraying treatment can increase the chlorophyll content and photosynthetic system activity of leaves by up-regulating the expression levels of related genes in chlorophyll synthesis and can improve the photosynthetic efficiency and product quality [43,44]. The results of this study show that the appropriate concentration of 24-EBL treatment influence of the chlorophyll content and proportion in celery, which may be related to the induction of chlorophyll synthesis and the expression levels of related genes in the chlorophyll recycling pathway, can inhibit the expression levels of genes related to chlorophyll degradation during the growth and development process of celery leaves. The results indicate that chlorophyll content is closely related to the expression level of chlorophyll metabolism genes. At the early growth stage (52 DAS), 1.04×10^{-6} mol·L^{-1} of 24-EBL treatment promoted the expression of genes related to chlorophyll synthesis (*AgCAO* and *AgCHLG*) in celery petioles and leaf blades to promote the increase of chlorophyll content. At the late growth stage (59 and 66 DAS), 1.67×10^{-6} mol·L^{-1} of 24-EBL treatment could more effectively inhibit the expression of genes related to chlorophyll degradation (*AgPPH*) and promote the expression of genes related to chlorophyll synthesis and recycling (*AgCLH* and *AgCAO*). It increased chlorophyll (especially chlorophyll *b*) accumulation and adjusted the content ratio of Chl *a*/Chl *b* in leaves. Exogenous 24-EBL treatment can affect celery chlorophyll content by affecting the expression of genes related to chlorophyll metabolism.

5. Conclusions

Spraying an appropriate concentration of 24-EBL on celery leaves could promote the expression levels of genes related to chlorophyll synthesis and recycling, significantly increasing the content of chlorophyll (especially chlorophyll *b*) in celery, and inhibiting the expression of genes related to chlorophyll degradation during the later leaf aging process to a certain extent. At the first stage (52 DAS), the 24-EBL treatment of 1.04×10^{-6} mol·L^{-1} promoted the up-regulation of the transcription levels of chlorophyll synthesis-related genes (*AgCHLG* and *AgCAO*) and the chlorophyll recycling pathway gene (*AgCLH*), which was conducive to the accumulation of chlorophyll *b* in celery petioles. At the second and third stages (59 and 66 DAS), the expression levels of genes related to chlorophyll synthesis and recycling genes (*AgCHLG* and *AgCLH*) were induced, and the expression levels of genes (*AgNYC* and *AgPPH*) related to chlorophyll degradation pathway decreased. By analyzing the effects of 24-EBL treatment at different stages on the content and proportion of chlorophyll in leaf blades and petioles of celery, combined with the analysis of the expression levels of genes related to chlorophyll metabolism, the potential molecular mechanism of 24-EBL affecting the chlorophyll metabolism of celery was explored, providing a

reference for further research on the roles of 24-EBL in improving the chlorophyll content of celery.

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