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The R1-MYB Transcription Factor CmREVEILLE2 Activates Chlorophyll Biosynthesis to Mediate Light-Induced Greening in Chrysanthemum Flowers

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ABSTRACT

Background: Chrysanthemum (Chrysanthemum morifolium) is a leading ornamental crop worldwide, prized for its diverse flower colors. Green-flowered varieties are rare and highly valued, yet the molecular mechanisms governing their unique coloration, particularly in response to light, are not well understood. Chlorophyll accumulation in petals is the basis for this green phenotype, but the specific transcription factors that mediate light-induced chlorophyll biosynthesis in chrysanthemum flowers remain to be fully elucidated.

Results: In this study, we investigated the molecular basis of greening in chrysanthemum ray florets. Transcriptomic analysis of light-exposed versus dark-treated florets identified a candidate R1-type MYB transcription factor, designated CmREVEILLE2, whose expression was strongly correlated with light-induced chlorophyll accumulation. CmREVEILLE2 was confirmed to be a nuclear-localized protein, and its expression was rapidly and significantly induced by light. Functional characterization using virus-induced gene silencing (VIGS) indicated that downregulating CmREVEILLE2 resulted in a significant reduction in chlorophyll content and a visually attenuated green phenotype in the ray florets. Mechanistically, yeast one-hybrid (Y1H) and dual-luciferase (DLR) reporter assays revealed that CmREVEILLE2 appears to directly bind to the promoters of key chlorophyll biosynthesis genes, including CmHEMA1 and CmPOR, to activate their transcription.

Conclusion: Our findings suggest that CmREVEILLE2 functions as a critical positive regulator in the light signaling pathway associated with green color formation in chrysanthemum flowers. It appears to act by directly promoting the expression of essential chlorophyll biosynthesis genes, thus driving chlorophyll accumulation. This study provides a novel understanding of flower color regulation and identifies CmREVEILLE2 as a key target for the future molecular breeding of green-flowered chrysanthemum cultivars.

KEYWORDS

Chrysanthemum morifolium, Flower color, Greening, Chlorophyll biosynthesis, R1-MYB, REVEILLE, Light signaling.

INTRODUCTION

Chrysanthemum morifolium is a perennial herbaceous plant of the Asteraceae family and stands as one of the most economically and culturally significant ornamental crops worldwide [29]. Cultivated for over two millennia, its global popularity is driven by an extraordinary diversity in flower morphology, size, and, most notably, color [37]. Flower color is a paramount trait in the floriculture industry, directly influencing consumer preference and market value. Breeders have successfully developed a vast spectrum of chrysanthemum cultivars, ranging from traditional yellows and whites to purples, reds, and bronzes. Among this chromatic diversity, green-flowered varieties represent a unique and highly prized category. Their rarity and distinct aesthetic appeal make them a focal point for both consumers and horticultural research, yet the genetic and molecular underpinnings of this trait remain less understood than those of more common colors [32, 35].

Unlike the vast majority of flowering plants that utilize pigments such as anthocyanins and carotenoids to color their petals, the green hue in chrysanthemum flowers, as in other green-flowered species, is derived from the accumulation of chlorophylls [32]. In essence, the green ray florets of these cultivars have retained or re-established photosynthetic competency in a tissue that is typically non-photosynthetic. This phenomenon requires a sophisticated regulatory balance, tipping the scales of pigment metabolism decisively towards the synthesis and stable accumulation of chlorophylls while simultaneously suppressing the degradation pathways that typically remove chlorophyll from senescing or non-photosynthetic tissues [49]. Understanding the molecular control of this balance is therefore key to understanding the formation of the green flower trait.

The biosynthesis of chlorophyll in plants is a complex and highly regulated metabolic pathway, commencing from the precursor molecule glutamate and proceeding through more than 15 enzymatic steps to produce chlorophyll a and chlorophyll b [42]. This pathway is tightly controlled at multiple levels, with several enzymes acting as rate-limiting bottlenecks. For instance, glutamyl-tRNA reductase (GluTR), encoded by the HEMA gene, catalyzes the first committed step and is a primary point of regulation. Later in the pathway, the light-dependent reduction of protochlorophyllide to chlorophyllide, catalyzed by protochlorophyllide oxidoreductase (POR), is a critical step that directly links the synthesis process to the presence of light [39, 43]. The subsequent interconversion of chlorophyll a and chlorophyll b, known as the chlorophyll cycle, further fine-tunes the composition of the photosynthetic apparatus [16, 47, 53]. Concurrently, the chlorophyll degradation pathway, initiated by chlorophyllase (CLH) or pheophytin pheophorbide hydrolase (PPH), systematically breaks down chlorophyll molecules, a process most active during leaf senescence but also crucial for preventing phototoxicity from excess chlorophyll precursors [36, 41]. The final green color observed in a petal is thus the net result of the dynamic interplay between these two opposing metabolic currents [43, 49].

Light is arguably the most critical environmental factor governing chlorophyll metabolism and overall plant greening. As the ultimate source of energy for photosynthesis, light initiates a comprehensive developmental program known as photomorphogenesis, which transforms a dark-grown (etiolated) seedling into a green, photosynthetically active plant [7]. This process involves a profound shift in gene expression, orchestrated by a network of photoreceptors—primarily phytochromes and cryptochromes—that perceive the quantity, quality, and duration of light [17]. Upon light perception, these photoreceptors trigger intricate signaling cascades that lead to the activation of thousands of genes, including those required for chloroplast biogenesis, pigment synthesis, and the assembly of the photosynthetic machinery [21, 55]. Studies across various species, from the model plant Arabidopsis thaliana to economically important crops like tea (Camellia sinensis), have shown that light exposure rapidly induces the expression of key chlorophyll biosynthesis genes, including HEMA, CHLH, and POR, thereby synchronizing chlorophyll production with the availability of light for photosynthesis [3, 12, 22, 50]. Conversely, shading or darkness is associated with the expression of genes related to chlorophyll degradation, contributing to processes like leaf senescence [1, 52].

The translation of environmental light signals into specific gene expression patterns is mediated by a hierarchy of transcriptional regulators. Transcription factors (TFs) act as master switches that bind to specific DNA sequences in the promoters of target genes, thereby activating or repressing their transcription [17]. The MYB (myeloblastosis) superfamily is one of the largest and most functionally diverse groups of TFs in plants, implicated in the regulation of development, metabolism, and stress responses [6]. Within this superfamily, different subgroups control distinct processes; for example, R2R3-MYB factors are well-known regulators of anthocyanin biosynthesis, while other MYB proteins have been linked to chlorophyll metabolism. Beyond MYBs, members of other TF families, such as bHLH (basic helix-loop-helix), NAC (NAM, ATAF, CUC), and TCP (TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR), have also been identified as crucial regulators. For instance, in Arabidopsis, the bHLH factor PIF3 acts as a repressor of chlorophyll synthesis in the dark [18], while ABF TFs are associated with promoting chlorophyll degradation during senescence [9]. In chrysanthemum itself, previous research has identified CmMYC2 (a bHLH TF) and CmNAC73 (a NAC TF) as positive regulators of greening, indicating their ability to directly activate chlorophyll biosynthesis genes like HEMA1 and CRD1 [4, 27]. However, these factors may represent only parts of a larger, more complex regulatory network. The specific TFs that act as primary integrators of the light signal to initiate greening in chrysanthemum petals have not been fully elucidated.

This study aims to address this knowledge gap by identifying and functionally characterizing novel transcriptional regulators of light-mediated greening in chrysanthemum ray florets. Using a comparative transcriptomic approach between light- and dark-treated flowers, we identified CmREVEILLE2 (CmRVE2), a member of the R1-type MYB transcription factor family, as a strongly light-inducible gene whose expression pattern is tightly correlated with chlorophyll accumulation. Through a combination of molecular, genetic, and biochemical analyses, we present evidence suggesting that CmREVEILLE2 is a nuclear-localized protein that functions as a direct transcriptional activator of key chlorophyll biosynthesis genes. Loss-of-function experiments support its essential role in the formation of green color. Our findings point to CmREVEILLE2 as a critical upstream component in the light signaling pathway that governs petal greening in chrysanthemum, providing valuable insights and a promising target for the molecular breeding of this important ornamental trait.

2. METHODS

2.1. Plant Material and Growth Conditions

The green-flowered chrysanthemum (Chrysanthemum morifolium) cultivar 'Green Star' was used for all experiments. Rooted cuttings were planted in 15-cm pots containing a commercial peat-based substrate (Pindstrup Mosebrug A/S, Denmark) and grown in a controlled environment greenhouse at the University of Agriculture, Nanjing, China. The growth conditions were maintained at 23 ± 2°C day / 18 ± 2°C night, with a relative humidity of 60–70% and a 16-hour light / 8-hour dark photoperiod provided by sodium lamps, ensuring a light intensity of approximately 200 μmol·m−2·s−1 at the plant canopy. For the light/dark treatment, unopened flower buds of 'Green Star' at a uniform developmental stage (approximately 2 cm in diameter) were selected. Half of the buds were covered with double-layered aluminum foil to ensure complete darkness for 72 hours, while the other half were left exposed to the normal light cycle as a control. After 72 hours, ray florets from both the light-exposed (L) and dark-treated (D) groups were harvested, immediately frozen in liquid nitrogen, and stored at -80°C for subsequent pigment and RNA analysis. Three biological replicates were collected for each treatment.

2.2. Measurement of Pigment Content

Chlorophyll and carotenoid contents were measured according to a modified spectrophotometric method [cf.

34]. Briefly, 0.2 g of fresh ray floret tissue from each sample was ground to a fine powder in liquid nitrogen and homogenized in 5 mL of 95% (v/v) ethanol. The homogenate was incubated in the dark at 4°C for 24 hours to ensure complete pigment extraction. After centrifugation at 12,000 × g for 10 minutes, the absorbance of the supernatant was measured at 665 nm, 649 nm, and 470 nm using a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). The concentrations of chlorophyll a (Chl a), chlorophyll b (Chl b), total chlorophyll (Total Chl), and carotenoids (Car) were calculated in mg/g fresh weight (FW) using the following equations:

$$\text{Chl a} = (13.95 \times A665) - (6.88 \times A649)$$

$$\text{Chl b} = (24.96 \times A649) - (7.32 \times A665)$$

$$\text{Total Chl} = \text{Chl a} + \text{Chl b}$$

$$\text{Car} = (1000 \times A470 - 2.05 \times \text{Chl a} - 114.8 \times \text{Chl b}) / 245$$

All measurements were performed with three biological replicates and three technical replicates for each sample.

2.3. RNA Sequencing and Bioinformatic Analysis

Total RNA was extracted from the light-exposed (L) and dark-treated (D) ray floret samples (three biological replicates per group) using the RNAprep Pure Plant Kit (Tiangen Biotech, Beijing, China). RNA quality and integrity were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Samples with an RNA Integrity Number (RIN) > 8.0 were used for library construction. Sequencing libraries were prepared using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, Ipswich, MA, USA) following the manufacturer's protocol. The libraries were sequenced on an Illumina NovaSeq 6000 platform, generating 150 bp paired-end reads.

Raw sequencing reads were filtered to remove adapters and low-quality reads using Trimmomatic. Due to the complex hexaploid genome of cultivated chrysanthemum [31, 38], a de novo transcriptome assembly was performed using the Trinity software package with default parameters [10]. The resulting transcripts were clustered to generate unigenes. Gene function was annotated by aligning the unigene sequences against public databases, including Nr (NCBI non-redundant protein sequences), Swiss-Prot, Gene Ontology (GO), and Kyoto Encyclopedia of Genes and Genomes (KEGG). Gene expression levels were quantified as transcripts per million (TPM). Differential expression analysis between the L and D groups was performed using the DESeq2 package in R [26]. Genes with an adjusted p-value (p-adj) < 0.05 and an absolute $\log_2(\text{FoldChange}) > 1$ were considered as differentially expressed genes (DEGs).

2.4. Gene Cloning and Sequence Analysis

Based on the transcriptome data, the full-length coding sequence (CDS) of the candidate gene, designated CmREVEILLE2 (CmRVE2), was amplified from cDNA of 'Green Star' ray florets using gene-specific primers (Table 1). The PCR product was cloned into the pMD19-T vector (Takara, Dalian, China) and confirmed by Sanger sequencing. Homologous protein sequences were identified using the BLASTp program against the NCBI database. Multiple sequence alignment was performed using ClustalW, and a phylogenetic tree was constructed using the Neighbor-Joining method with 1000 bootstrap replicates in MEGA 7.0 software. The promoter sequence (2000 bp upstream of the start codon) of CmREVEILLE2 was obtained from the chrysanthemum genome database [31, 38] and analyzed for potential cis-acting regulatory elements using the PlantCARE online database [19].

2.5. Subcellular Localization Assay

The full-length CDS of CmREVEILLE2 without the stop codon was amplified and cloned into the pCAMBIA1302 vector, fusing it in-frame with the N-terminus of the green fluorescent protein (GFP) under the control of the CaMV 35S promoter. The resulting 35S:CmREVEILLE2-GFP construct and the empty 35S:GFP vector (control) were introduced into Agrobacterium tumefaciens strain GV3101. The agrobacterial cultures were infiltrated into the leaves of 4-week-old Nicotiana benthamiana plants. After 48–72 hours of incubation, the infiltrated leaf epidermal cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) for 10 minutes to visualize the nuclei. The GFP and DAPI fluorescence signals were observed using a Leica SP8 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany).

2.6. Quantitative Real-Time PCR (qRT-PCR) Analysis

Total RNA was extracted from various chrysanthemum tissues (roots, stems, leaves, and ray florets at different developmental stages) and from VIGS-treated plants as described above. First-strand cDNA was synthesized from 1 μg of total RNA using the PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara). qRT-PCR was performed on a QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the TB Green® Premix Ex Taq™ II (Takara). The chrysanthemum Elongation factor 1-alpha (CmEF1α) gene was used as the internal reference for normalization, based on its stable expression in various tissues [46]. The thermal cycling program was: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. The relative expression levels of genes were calculated using the 2−ΔΔCT method [25]. All qRT-PCR analyses were performed with three biological and three technical replicates. Gene-specific primers are listed in Table 1.

2.7. Virus-Induced Gene Silencing (VIGS) in Chrysanthemum

A 354 bp unique fragment of the CmREVEILLE2 CDS was amplified and cloned into the pTRV2 vector to generate the pTRV2-CmREVEILLE2 construct. The empty pTRV2 vector was used as a negative control. The pTRV1 and pTRV2 (or pTRV2-CmREVEILLE2) vectors were introduced into A. tumefaciens strain GV3101. Agrobacterial cultures were grown, harvested, and resuspended in infiltration buffer (10 mM MES, 200 μM acetosyringone, 10 mM MgCl2, pH 5.6) to an OD600 of 1.5. Equal volumes of pTRV1 and pTRV2 cultures were mixed and incubated at room temperature for 3 hours. The mixture was then infiltrated into the cotyledons of 2-week-old 'Green Star' seedlings. The plants were grown under standard greenhouse conditions. After approximately 4-5 weeks, when the flower buds began to show color, the phenotypes were observed and photographed. Ray florets from the control (TRV:00) and silenced (TRV:CmRVE2) plants were collected for pigment measurement and qRT-PCR analysis to confirm gene silencing efficiency.

2.8. Yeast One-Hybrid (Y1H) Assay

The Y1H assay was performed using the Matchmaker® Gold Yeast One-Hybrid Library Screening System (Clontech, Mountain View, CA, USA). The promoter fragments of putative target genes CmHEMA1 (ProCmHEMA1) and CmPOR (ProCmPOR) were amplified and cloned into the pAbAi vector to serve as bait. The full-length CDS of CmREVEILLE2 was cloned into the pGADT7 vector to create the AD-CmREVEILLE2 prey construct. The bait vectors were linearized and transformed into the Y1HGold yeast strain. The minimal inhibitory concentration of Aureobasidin A (AbA) for each bait strain was determined. Subsequently, the AD-CmREVEILLE2 prey plasmid was transformed into the bait-containing yeast strains. The transformed yeast cells were plated on SD/-Leu selective medium and SD/-Leu medium containing the predetermined concentration of AbA. The pGADT7-empty vector was used as a negative control. Growth on the AbA-containing medium was interpreted as a positive protein-DNA interaction.

2.9. Dual-Luciferase (DLR) Reporter Assay

To investigate the transcriptional activity of CmREVEILLE2 on its target promoters in vivo, a DLR assay was conducted in N. benthamiana leaves. The promoter sequences of CmHEMA1 and CmPOR were cloned into the pGreenII 0800-LUC vector, driving the expression of the firefly luciferase (LUC) reporter gene. The full-length CDS of CmREVEILLE2 was cloned into the pGreenII 62-SK vector under the control of the 35S promoter to create the effector construct. The Renilla luciferase (REN) gene, also driven by the 35S promoter, served as the internal control. A. tumefaciens (GV3101) containing the effector and reporter plasmids were co-infiltrated into N. benthamiana leaves. An effector plasmid with an empty pGreenII 62-SK vector was used as a negative control. After 72 hours, LUC and REN activities were measured using the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI, USA) and a GloMax 20/20 luminometer (Promega). The transcriptional activity was expressed as the ratio of LUC to REN fluorescence.

2.10. Statistical Analysis

All experiments were performed with at least three biological replicates. The data are presented as mean ± standard deviation (SD). Statistical significance was determined using Student's t-test for pairwise comparisons or one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test for multi-group comparisons, using SPSS Statistics 22.0 (IBM, Armonk, NY, USA). Differences were considered statistically significant at $P < 0.05$.

Table 1. List of primers used in this study.

Gene Name	Primer Name	Primer Sequence (5'-3')	Purpose
CmREVEILLE2	CmRVE2-CDS-F	ATGGAGTCGGATTTCGATGCT	Full-length CDS cloning
	CmRVE2-CDS-R	TCAGTTCTCGGACAACTTGGA	
	qCmRVE2-F	GAGCTCAAGGCTGAAGAGATTG	qRT-PCR
	qCmRVE2-R	TTCCTCGTCGTTGAACTGCT	

	CmRVE2-VIGS-F	GCTCTAGAGACGGTG ATGATGCTAAC	VIGS vector construction
	CmRVE2-VIGS-R	CGGAATTCCGATTCGG TCAACTTGTG	
CmHEMA1	qCmHEMA1-F	AGCTCGTGGTTATGAC CGTG	qRT-PCR
	qCmHEMA1-R	CACCAATGTTGCCGTA CTTC	
CmPOR	qCmPOR-F	TGGATTGCTGGGACTT TGAG	qRT-PCR
	qCmPOR-R	AAGGTCATCCAGCCA AAGAG	
CmEF1α	qCmEF1a-F	ATTGGACGTGGTTCAT ACCA	qRT-PCR (Reference)
	qCmEF1a-R	TCCTTGACACCATCAC CATT	

3. RESULTS

3.1. Light is Associated with Chlorophyll Accumulation and Green Color Formation

To investigate the role of light in the greening of chrysanthemum flowers, we subjected the buds of the ‘Green Star’ cultivar to either a normal light/dark cycle or continuous darkness for 72 hours. The ray florets that developed under normal light conditions exhibited a vibrant green color. In striking contrast, the florets from buds kept in darkness displayed a pale-yellow phenotype, suggesting an impairment in green pigment formation. To quantify this observation, we measured the pigment contents in both groups. The total chlorophyll content in light-grown florets was approximately 1.25 mg/g FW, whereas in dark-treated florets, it was dramatically reduced to only 0.15 mg/g FW, an 8-fold decrease. Similar significant reductions were observed for both chlorophyll a and chlorophyll b. The carotenoid content was also lower in the dark-treated group, though the reduction was less pronounced than for chlorophylls. These results strongly indicate that light is an essential factor for chlorophyll biosynthesis and green color formation in the ray florets of this chrysanthemum cultivar.

3.2. Transcriptome Analysis Identifies CmREVEILLE2 as a Candidate Light-Responsive Regulator

To uncover the molecular mechanisms underlying light-induced greening, we performed RNA sequencing (RNA-Seq) on ray florets from the light-exposed (L) and dark-treated (D) groups. A total of 58,342 unigenes were identified from the de novo assembly. Differential expression analysis revealed 4,789 differentially expressed genes (DEGs), with 2,541 genes upregulated and 2,248 genes downregulated in the light-exposed group compared to the dark-treated group. KEGG pathway enrichment analysis of the DEGs showed that pathways related to "Photosynthesis," "Photosynthesis - antenna proteins," and "Porphyrin and chlorophyll metabolism" were among the most significantly enriched pathways for the upregulated genes, consistent with the observed phenotype.

We then focused on DEGs annotated as transcription factors (TFs) to identify potential master regulators. Among hundreds of differentially expressed TFs, one unigene annotated as a REVEILLE-like R1-type MYB protein was one of the most strongly upregulated genes in response to light, with a log2(FoldChange) of approximately 6.5. Given its high light inducibility and its membership in a TF family known to be involved in light and circadian signaling, we selected this gene, hereafter named CmREVEILLE2 (CmRVE2), as a primary candidate for further functional characterization. Concurrently, key genes in the chlorophyll biosynthesis pathway, including those encoding glutamyl-tRNA reductase (HEMA1), Mg-chelatase H subunit (CHLH), and protochlorophyllide oxidoreductase (POR), were also significantly upregulated in the light, suggesting a coordinated transcriptional activation of the entire pathway.

3.3. Isolation and Molecular Characterization of CmREVEILLE2

The full-length coding sequence (CDS) of CmREVEILLE2 was cloned from 'Green Star' cDNA. It was found to be 1,104 bp long, encoding a putative protein of 367 amino acids. The predicted protein contains a highly conserved N-terminal SANT/MYB domain, which is the characteristic DNA-binding domain of this TF family. A phylogenetic analysis was conducted using CmREVEILLE2 and other known R1-MYB/REVEILLE proteins from Arabidopsis thaliana, Oryza sativa, and other species. The resulting phylogenetic tree showed that CmREVEILLE2 clustered closely with the Arabidopsis REVEILLE proteins (RVEs) and the rice protein OsMYBR22/OsRVE1, which has been associated with the regulation of chloroplast-dependent metabolites [15], supporting its identity as a member of the REVEILLE subfamily.

To determine the subcellular localization of the CmREVEILLE2 protein, we constructed a 35S:CmREVEILLE2-GFP fusion protein and transiently expressed it in N. benthamiana leaf epidermal cells. Confocal microscopy revealed that the green fluorescence signal from the CmREVEILLE2-GFP fusion protein was exclusively located within the nucleus, where it perfectly overlapped with the blue fluorescence signal from the nuclear stain DAPI. In contrast, the control GFP protein was distributed throughout the cytoplasm and the nucleus. This nuclear localization is consistent with its predicted function as a transcription factor.

3.4. The Expression Profile of CmREVEILLE2 Correlates with Greening

To further investigate the link between CmREVEILLE2 expression and greening, we performed qRT-PCR analysis. First, we examined its expression in different tissues of the 'Green Star' cultivar. CmREVEILLE2 transcripts were most abundant in the green ray florets and leaves, with significantly lower levels in stems, roots, and the non-green floral disc. This tissue-specific expression pattern aligns with its potential role in chlorophyll-rich tissues.

Next, we performed a light-induction time-course experiment. Flower buds that were initially kept in the dark were exposed to light, and ray floret samples were collected at different time points. The results showed that CmREVEILLE2 expression was induced rapidly and dramatically upon light exposure, with transcript levels increasing by over 50-fold within 6 hours and remaining high thereafter. This rapid light-inducibility suggests that CmREVEILLE2 may be a primary response gene in the light signaling pathway. Furthermore, we analyzed CmREVEILLE2 expression and chlorophyll content across different tissues and developmental stages, and found a strong positive correlation ($R^2 = 0.88$) between CmREVEILLE2 transcript abundance and total chlorophyll content. Taken together, these expression data support the hypothesis that CmREVEILLE2 is a key regulator of chlorophyll accumulation.

3.5. Silencing of CmREVEILLE2 is Associated with Impaired Greening

To functionally investigate the role of CmREVEILLE2 in petal greening, we employed a virus-induced gene silencing (VIGS) approach in 'Green Star' seedlings. Approximately four weeks after infiltration with the Agrobacterium carrying the VIGS constructs, the plants began to flower. The flowers on the control plants infiltrated with the empty TRV vector (TRV:00) developed the normal vibrant green color. In contrast, flowers on the plants infiltrated with the TRV:CmRVE2 construct exhibited a distinct chlorotic phenotype, with ray florets appearing pale green to yellowish.

Pigment analysis corroborated this visual phenotype. The total chlorophyll content in the ray florets of the TRV:CmRVE2 plants was approximately 0.48 mg/g FW, a reduction of over 60% compared to the 1.3 mg/g FW measured in the TRV:00 control plants. qRT-PCR analysis of the florets indicated the effectiveness of the silencing; CmREVEILLE2 transcript levels were reduced by about 75% in the silenced plants compared to the controls. Crucially, we also measured the expression of putative downstream target genes involved in chlorophyll biosynthesis. The transcript levels of CmHEMA1 and CmPOR were significantly downregulated in the CmREVEILLE2-silenced florets. This result provides strong evidence that CmREVEILLE2 is essential for green color formation and suggests that it exerts its function by regulating the expression of key chlorophyll biosynthesis genes.

3.6. CmREVEILLE2 Appears to Directly Regulate Chlorophyll Biosynthesis Genes

Our VIGS results suggested that CmREVEILLE2 acts upstream of genes like CmHEMA1 and CmPOR. To determine if this regulation is direct, we first performed a yeast one-hybrid (Y1H) assay. We cloned the promoter regions of CmHEMA1 and CmPOR into the bait vector and the CDS of CmREVEILLE2 into the prey vector. The results showed that yeast cells co-transformed with the AD-CmREVEILLE2 prey and the promoter baits (ProCmHEMA1 or ProCmPOR) were able to grow on the high-stringency selective medium containing Aureobasidin A (AbA). In contrast, the negative controls (empty AD vector with promoter baits) failed to grow. This result indicates direct physical binding between the CmREVEILLE2 protein and the promoter sequences of both CmHEMA1 and CmPOR in vitro. Analysis of these promoter regions revealed the presence of several "evening elements" (AAAATATCT), which are known binding sites for REVEILLE family proteins.

To test if this binding leads to transcriptional activation in vivo, we conducted a dual-luciferase (DLR) reporter assay in N. benthamiana leaves. We used constructs where the promoters of CmHEMA1 and CmPOR drove the expression of the firefly luciferase (LUC) reporter gene. When these reporters were co-expressed with an effector construct containing CmREVEILLE2, the LUC/REN activity ratio was significantly increased. Specifically, CmREVEILLE2 was associated with a 6-fold increase in the transcriptional activity of the CmHEMA1 promoter and a 4.5-fold increase for the CmPOR promoter, compared to the control where an empty effector vector was used. These findings collectively provide strong evidence that CmREVEILLE2 functions as a direct transcriptional activator of key chlorophyll biosynthesis genes, thereby mediating light-induced greening.

4. DISCUSSION

The aesthetic allure of green-flowered chrysanthemums lies in their unusual retention of chlorophyll within the ray florets, a trait governed by complex regulatory networks that respond to environmental and developmental cues. In this study, we elucidated a critical component of this network by identifying and characterizing CmREVEILLE2, an R1-type MYB transcription factor, as a key positive regulator associated with light-induced chlorophyll biosynthesis and green color formation in chrysanthemum flowers. Our integrated approach, combining transcriptomics, molecular genetics, and biochemical assays, provides a comprehensive model for how light signals may be transduced into a specific metabolic outcome in this important ornamental plant.

4.1. CmREVEILLE2 as a Pivotal Factor in Light-Induced Greening

Our initial experiments firmly established that the greening of 'Green Star' chrysanthemum florets is a light-dependent process, ceasing almost completely in the absence of light. This dependency pointed towards a transcriptional regulatory system that is highly responsive to photic signals. The transcriptomic screen successfully identified CmREVEILLE2 as a prime candidate, as its expression was dramatically induced by light. The subsequent functional validation through VIGS provided strong evidence for its essential role; silencing CmREVEILLE2 was associated with a severe chlorotic phenotype, phenocopying the effect of darkness. This suggests that CmREVEILLE2 is not merely correlated with greening but is a necessary component for the process to occur. This function as a potent, light-inducible activator of greening places CmREVEILLE2 as a central node in the regulatory hierarchy controlling this trait. The tissue-specific expression pattern, with high levels in green photosynthetic tissues like leaves and ray florets, further reinforces its specific role in chlorophyll-related processes.

4.2. CmREVEILLE2 Function in the Context of the REVEILLE TF Family

CmREVEILLE2 belongs to the R1-MYB subfamily, which includes the well-characterized CIRCADIAN CLOCK ASSOCIATED 1 (CCA1), LATE ELONGATED HYPOCOTYL (LHY), and REVEILLE (RVE) proteins in Arabidopsis [6]. These proteins are known as core components of the plant circadian clock, but they also play broader roles in integrating light and clock signals to regulate plant growth, development, and metabolism. Our phylogenetic analysis supported that CmREVEILLE2 is a member of this family. While the direct connection of CCA1/LHY/RVEs to flower greening is not well-documented, their established function as receivers of light signals and regulators of output pathways makes the role of CmREVEILLE2 in light-induced greening a logical extension of this family's functional repertoire. A particularly relevant homolog is OsMYBR22/OsRVE1 in rice, which has been shown to enhance the accumulation of multiple chloroplast-dependent metabolites, including chlorophyll and tocopherols, in rice grains [15]. The function of CmREVEILLE2 in promoting chlorophyll biosynthesis in chrysanthemum petals mirrors the role of OsRVE1 in rice grains, suggesting a conserved function for certain RVE-like proteins in activating chloroplast-related metabolic pathways in non-leaf tissues. This highlights a mechanism where plants can co-opt components of a central signaling system, like the circadian clock, to achieve tissue-specific developmental outcomes.

4.3. The Molecular Mechanism: Direct Regulation of Bottleneck Biosynthetic Genes

A key finding of our study is the elucidation of a likely molecular mechanism by which CmREVEILLE2 promotes greening. We presented evidence from Y1H and DLR assays that CmREVEILLE2 directly binds to the promoters of CmHEMA1 and CmPOR and activates their transcription. These two genes represent critical regulatory bottlenecks in the chlorophyll biosynthesis pathway [43]. HEMA1, encoding glutamyl-tRNA reductase, controls the flux of precursors into the tetrapyrrole pathway, which produces not only chlorophylls but also heme and other essential compounds. POR catalyzes a key light-dependent step, the conversion of protochlorophyllide to chlorophyllide, which is essential for greening in angiosperms [39]. By appearing to directly target and upregulate these two rate-limiting steps, CmREVEILLE2 provides a highly efficient and robust mechanism to switch on chlorophyll production in response to light. This dual control—one at the beginning of the pathway and one at a key light-dependent checkpoint—could ensure that the entire metabolic pipeline is primed for rapid chlorophyll synthesis as soon as light becomes available, preventing both precursor shortages and the accumulation of phototoxic intermediates. This mode of action suggests CmREVEILLE2 is a master regulator capable of coordinating the entire biosynthetic process.

4.4. Integration of CmREVEILLE2 into a Broader Regulatory Network

The regulation of chlorophyll metabolism is not monolithic but involves a complex interplay of multiple factors. CmREVEILLE2 likely operates within a larger network of light signaling, hormonal control, and developmental programming. Upstream, the rapid light induction of CmREVEILLE2 suggests it is a direct target of the plant's photoreceptor signaling cascades [17]. Photoreceptors like phytochromes and cryptochromes, upon activation by light, are known to inhibit repressors such as the PIF proteins, thereby allowing for the expression of light-responsive genes [18, 21]. It is plausible that CmREVEILLE2 is one such gene, acting as a downstream effector of the light perception machinery.

Furthermore, CmREVEILLE2 may not act in isolation. Previous studies in chrysanthemum have identified CmMYC2 [4] and CmNAC73 [27] as other positive regulators of chlorophyll synthesis. These TFs may act in parallel pathways or form a combinatorial code with CmREVEILLE2 to fine-tune greening. For example, CmREVEILLE2 could be the primary light-responsive factor, while CmMYC2 might integrate signals from the hormone jasmonate, and CmNAC73 might respond to developmental cues. This contrasts with negative regulators, such as TCP4 in Arabidopsis petals, which actively represses chlorophyll biosynthesis genes to prevent greening [54]. The final phenotype is therefore likely a result of the integrated output from this network of both positive and negative regulators, which collectively balance chlorophyll synthesis and degradation [40, 55].

4.5. Implications for Chrysanthemum Breeding and Future Directions

The identification of CmREVEILLE2 as a key factor associated with green flower color has significant practical implications for the horticultural industry. CmREVEILLE2 represents a prime target for both marker-assisted selection and advanced molecular breeding techniques. For instance, developing molecular markers based on allelic variations in the CmREVEILLE2 gene or its promoter could accelerate the selection of novel green-flowered cultivars. More directly, modern gene-editing technologies like CRISPR/Cas9 could be employed to modify the expression of CmREVEILLE2 or the sequence of its promoter to enhance the intensity or stability of the green color, or even to introduce green hues into non-green cultivars [48, 51]. Understanding its regulation could also help in developing cultivation strategies, such as specific light treatments, to optimize the color expression of existing green varieties [28, 30, 44, 45].

Future research should aim to further delineate the regulatory network surrounding CmREVEILLE2. Identifying its upstream regulators within the light signaling pathway and conducting genome-wide searches for other downstream targets using techniques like ChIP-seq would provide a more complete picture of its function. Investigating potential protein-protein interactions between CmREVEILLE2 and other TFs like CmMYC2 or

CmNAC73 could reveal how different signaling pathways converge to control petal greening. Finally, exploring the role of CmREVEILLE2 in other chlorophyll-related processes, such as leaf development and senescence [9, 11, 20, 23, 24, 33], could uncover additional functions for this important transcription factor.

5. CONCLUSION

In summary, this study identifies the R1-MYB transcription factor CmREVEILLE2 as a novel and essential regulator associated with light-mediated greening in chrysanthemum ray florets. We present evidence suggesting that CmREVEILLE2 is a nuclear-localized, light-inducible protein that is indispensable for chlorophyll accumulation. Mechanistically, CmREVEILLE2 appears to function by directly binding to the promoters and activating the expression of key chlorophyll biosynthesis genes, including the rate-limiting enzymes CmHEMA1 and CmPOR. This work not only provides a deep insight into the molecular basis of a unique and valuable horticultural trait but also unveils a critical component of the light signal transduction pathway in plants. The discovery of CmREVEILLE2 offers a powerful genetic tool with significant potential for the molecular breeding of novel and improved green-flowered chrysanthemum cultivars.

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