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Quality of ornamental crops: Effect of genotype, preharvest, and improved production chains on quality attributes of ornamental crops, volume II

Edited by
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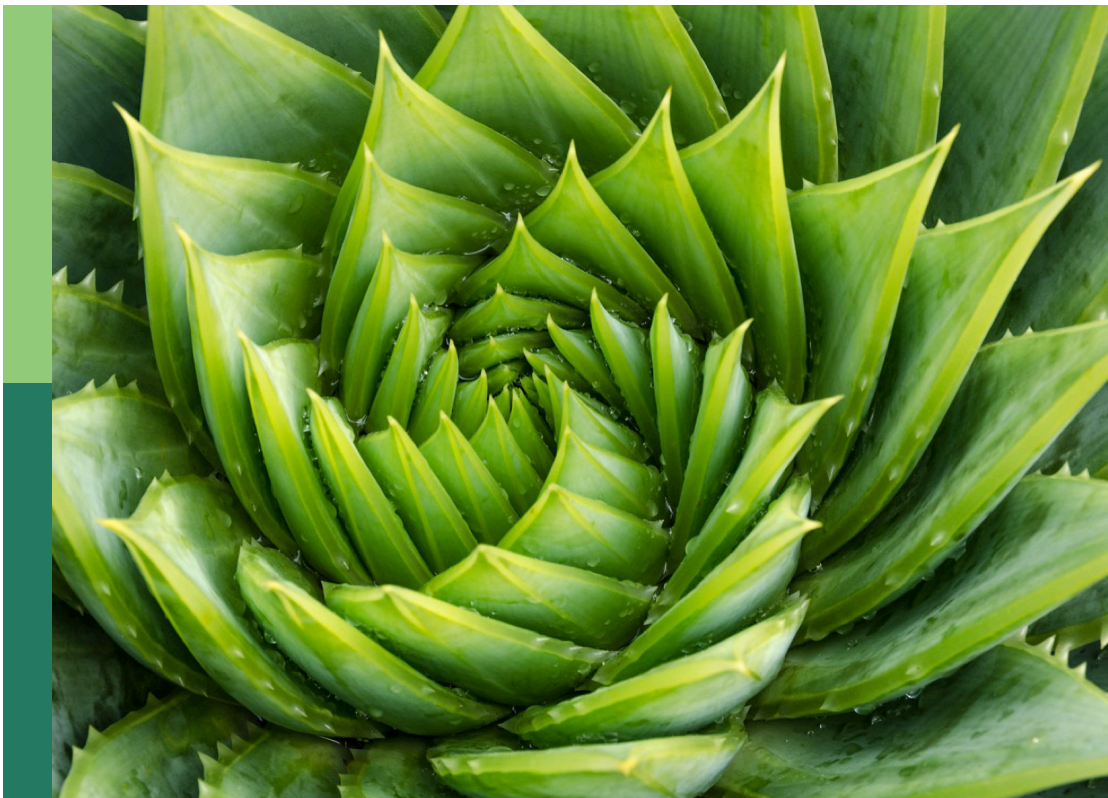

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Quality of ornamental crops: Effect of genotype, preharvest, and improved production chains on quality attributes of ornamental crops, volume II

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Table of contents

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Editorial: Quality of ornamental crops: effect of genotype, preharvest, and improved production chains on quality attributes of ornamental crops, volume II

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Editorial on the Research Topic

Quality of ornamental crops: effect of genotype, preharvest, and improved production chains on quality attributes of ornamental crops, volume II

Considering the interest in the release of a first volume on the Research Topic "Quality of Ornamental Crops: Effect of Genotype, Preharvest, and Improved Production Chains on Quality Attributes of Ornamental Crops", the Editorial team wanted to collect further findings in a second volume to highlight innovation, technology, and sustainability of ornamental plant production. Indeed, the diversified ornamental sector is characterized by a fast-changing evolution and technological advancements appear to be one of the most important driving forces of the ornamental industry.

This Research Topic collected 10 articles to provide readers with findings aimed to clarify important challenges faced during ornamental plant production. The research and review articles published included information and strategies to set a sustainable production and to get innovative products, including new uses of ornamentals in the urban context. The ornamental sector is considered in its complexity and the published research aims to involve the different levels of production from breeding to postharvest. In the following paragraphs, we summarize the findings of the published articles according to three main topics.

Production and development aspects of ornamental crops

Urban green spaces are becoming increasingly popular. It is expected that their impact on the population will increase in the coming years because by 2050, 68% of the global population will live in cities. Creating green spaces in urban or peri-urban areas poses challenges related to the condition of the selected space prior to plantation (e.g., physico-chemical characteristics of soil and quality of the environment) and the appropriate land planning and management. Moreover, the choice of appropriate ornamental plants has a pivotal role. The selection of ornamental plants should be based on their aesthetic value and ability to adapt to conditions such as the shading of buildings and trees. A non-destructive leaf gas exchange, chlorophyll a fluorescence, and chlorophyll content are proposed as useful tools for selecting suitable ornamental plants under diverse shading conditions (Francini et al.).

The ornamental sector needs to be fully prepared for high-standard production. The control of environment in greenhouses allows a consistent and high-quality cut-flower production. In particular, photoperiod manipulation is important in managing the production of flowers and improving the productivity of many floriculture crops. Since there was a lack of information on this subject, the research developed by Spall and Lopez indicated a suitable photoperiod process for marigold (*Tagetes erecta*) and witchgrass 'Frosted Explosion' (*Panicum capillare*) production, varying the period of the light in different phases of plant development. Moreover, light and gravity can affect plant development by influencing the movements of plant hormones. Transcriptome analysis revealed that stem bending in water lily (*Nymphaea tetragona*) cut flowers is regulated by multiple factors and genes. Differentially expressed genes (DEGs) were associated with the dorsal and ventral stems of the water lily, suggesting a transcription-based regulation of stem bending. Significant differences in genes associated with plant hormones, calcium ions, glucose metabolism, and photosynthesis pathways were observed and related to stem curving, in addition to changes in the cell morphology during the vase life (Li et al.). The authors highlighted that the higher concentration of the auxin indole-3-carbosaldehyde on the dorsal part compared to the ventral part, due to gravity and light, can account for the bending of water lily cut-flower stem (Li et al.).

Regarding abiotic stress factors, drought may be considered one of the most relevant, affecting plant growth and development, and providing serious losses. Transcriptome analysis using an RNA-Seq approach identified unigenes and differentially expressed genes (DEGs) in drought stress in *Heimia myrtifolia*, a native tropical plant. The most prevalent was abscisic acid signal transduction although other plant hormone signal transductions were also involved in the drought stress response. The balance and stability maintenance of the metabolic processes involved many photosynthesis-related and antioxidant enzyme genes (Lin et al.). The findings in this research provide important information for future studies of the drought resistance in other ornamental plants.

Germplasm characterization and phylogeny

The ornamental sector is characterized by the need for new products. In the next years, it is expected that the demand for floriculture products will increase, and it becomes more evident that consumers value the novelty and sustainability of floriculture products (Gabellini and Scaramuzzi, 2022). In this light, germplasm collection and characterization are of great importance in breeding new cultivars. Some germplasms do not need further breeding and can be used by local growers mainly for the market focused on specialty crops (Darras, 2021). Germplasm characterization refers to the agronomic description of plant materials including traits concerning their aesthetic features and other issues concerning crop improvement (e.g., disease resistance, suitability for a certain production area, or adaptability to climate change). To ensure the best utilization of the germplasm collection for breeding purposes, genetic approaches are also fundamental. In this regard, a study investigated the potential of start codon targeted (SCoT) markers for analyzing genetic diversity among *Cyclamen* species and/or genotypes. Sequence-related amplified polymorphisms (SRAP) markers proved to be useful tools for their separation particularly when combined with phenotypic data. The unweighted pair group method with arithmetic mean (UPGMA) method for hierarchical clustering technique was used to generate the dendrograms. Comparison between the color measurements of flowers and leaves with SCoT analysis revealed differences at species level, discriminating between similar genotypes (Cornea-Cipcigan et al.).

Concerning the transcription factors, the MADS (MCM1, AG, DEFA, and SRF (serum response factor) transcription factors) box transcription factor family and its members play critical roles during plant growth and development. For example, the MIKC^{C}-type gene family plays important roles in plant growth, especially during floral organ differentiation. Despite this importance, studies on MIKC^{C}-type genes available for roses focused only on transcriptome data, providing an inaccurate mapping and incomplete characterization of these genes. The results of this study provide new insights into the functions of MIKC^{C} genes in rose and provide the basis for future work to explore the evolution of *Rosaceae* (Wang et al.).

Genome editing (GE) methods, particularly those mediated by CRISPR/Cas-related tools are a successful strategy used to alter the function of key genes, regulating biological processes including plant male sterility (MS). These precision breeding technologies can accelerate the line development of new genetic variability with the accumulation of favorable alleles. Farinati et al. provided a general overview of insights and advances in the mechanisms underlying the recent CRISPR technology focusing on plant male sterility applied to main crops and ornamental species.

Preharvest to postharvest advances in cut flowers

The ornamental industry strongly relies on its final products that are recognized and valued for their aesthetic qualities, thus, it is

mandatory for the products to keep their attractiveness also during the postharvest period. After harvesting, different practices have been proposed to prolong the shelf life of ornamentals. This step in the production flow of ornamentals remains challenging. For this reason, it is important to explore the underlying mechanisms related to this delicate phase by promoting a wide perspective on the whole cultivation practices and molecular approaches. Vase-life issues are often linked to only postharvest conditions although preharvest conditions may also affect and reduce the quality of cut flowers. In this sense, Verdonk et al. pointed out the importance of encouraging studies focused on providing information gathered during the cut-flower production process. The role of growth conditions such as irrigation, air humidity, and light quantity and quality, which is essential to increase quality and longevity, is discussed in a review (Verdonk et al.). Studying the complex effects of preharvest conditions on quality illustrates the need for more rapid and reliable phenotyping methods (Verdonk et al.). The use of QTL analysis, candidate gene (CG) mapping, and virus-induced gene silencing (VIGS) proved to be a useful combination to identify possible causal genes and for understanding the molecular mechanisms of the resistance of *Botrytis cinerea* in gerbera's (*Gerbera hybrida*) preharvest and postharvest conditions (Fu et al.).

Regarding tropical flower postharvest, the 'low-temperature sensitivity' restricts transportation together with other traditional cut flowers. Packaging is another issue hampered by shape, size, and weight. The limited advancements in *Heliconia* research highlighted the need for a comprehensive review that consolidates all available information on this species,emphasizing chemical treatments, nano-based technology, and advanced packaging techniques (Malakar et al.).

Future and outlook

This Research Topic outlined some aspects concerning the ornamentals production. Although many issues still need to be covered in the future, all the publications in this Research Topic present novelty and contribute outstanding information for the ornamental plant development.

In particular, we would like to empathize that important contributions for flower postharvest have been made, and the articles in this Research Topic show that there is a need for research on preharvest, and anatomical and biochemical aspects should be the focus in the future. The results of a meta-analysis on tropical flowers noted that research on identifying solutions to deepen understanding and improve techniques, consequently improving postharvest quality, remains the focus of this industry (Cunha Neto et al., 2023). A future outlook focusing on new phenotyping tools is necessary to quantify the complex interactions between cultivation factors and postharvest performance of cut flowers as indicated by (Verdonk et al.).

Moreover, in this Research Topic, transcriptomics was shown to be a powerful tool in gene identification (Lin et al.; Wang et al.; Li et al.). Future challenges include addressing new potential applications of CRISPR/Cas systems, especially for MS mutant production and transgenerational gene editing for precision-breeding strategies.

We hope that readers find interesting ideas for their research, thus contributing to increasing knowledge on the production of ornamentals, a smaller sector compared to the main agricultural crops but with a significant economic impact.

Author contributions

PP: Writing – review & editing, Writing – original draft, Supervision, Conceptualization. MB: Writing – review & editing, Supervision, Conceptualization. RP: Writing – review & editing, Supervision, Conceptualization. JV: Writing – review & editing, Supervision, Conceptualization.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Transcriptome Approach Reveals the Response Mechanism of *Heimia myrtifolia* (Lythraceae, Myrtales) to Drought Stress

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Drought is a major environmental condition that inhibits the development and cultivation of *Heimia myrtifolia*. The molecular processes of drought resistance in *H. myrtifolia* remain unknown, which has limited its application. In our study, transcriptome analyzes were compared across three treatment groups (CK, T1, and T2), to investigate the molecular mechanism of drought resistance. Plant leaves wilted and drooped as the duration of drought stress increased. The relative water content of the leaves declined dramatically, and relative electrolyte leakage rose progressively. Using an RNA-Seq approach, a total of 62,015 unigenes with an average length of 1730 bp were found, with 86.61% of them annotated to seven databases, and 14,272 differentially expressed genes (DEGs) were identified in drought stress. GO and KEGG enrichment analyzes of the DEGs revealed significantly enriched KEGG pathways, including photosynthesis, photosynthetic antenna proteins, plant hormone signal transduction, glutathione metabolism, and ascorbate and aldarate metabolism. Abscisic acid signal transduction was the most prevalent in the plant hormone signal transduction pathway, and other plant hormone signal transductions were also involved in the drought stress response. The transcription factors (including MYB, NAC, WRKY, and bHLH) and related differential genes on significantly enriched pathways all played important roles in the drought process, such as photosynthesis-related genes and antioxidant enzyme genes. In conclusion, this study will provide several genetic resources for further investigation of the molecular processes that will be beneficial to *H. myrtifolia* cultivation and breeding.

Keywords: RNA-Seq, ornamental plant, drought tolerance, plant hormone, transcription factor

INTRODUCTION

Drought is a key abiotic stress factor affecting plant growth and development and threatening worldwide agricultural production, which restricts economic development and environmental governance (Fahad et al., 2017; Shah et al., 2017; Scharwies and Dinnen, 2019). Since the problem of serious global drought has increased with higher frequencies, longer durations, and wider

ranges (Basu et al., 2016; Gupta et al., 2020), it has gradually become a common concern for an increasing number of plant species (Zhao X. et al., 2019; Qi et al., 2021). With the improvement in the quality of people's life, the vision of building a garden city is strengthened, but urban population growth and industrialization have led to a prominent urban heat island effect, which exacerbates urban water shortages and high plant maintenance costs (Mccarthy et al., 2010; Ruosteenoja et al., 2018). Therefore, choosing drought-tolerant ornamental plants for a reasonable configuration would be very beneficial for water-saving garden construction (Zhang et al., 2019; Li et al., 2021). *Heimia myrtifolia* is a native tropical plant that grows beside streams and has good adaptability to high temperatures. After its introduction in China, it was used for garden applications and breeding germplasm. However, under seasonal drought with the absence of water, the growth of *H. myrtifolia* is significantly restricted and pollen vigor is reduced, which limits the progress of hybrid breeding and promotion. Therefore, it is necessary to explore the drought response mechanism of *H. myrtifolia* to provide a theoretical reference for subsequent breeding work and application.

Drought will cause a wide range of plant responses, such as decreasing cell osmotic potential, increasing reactive oxygen species (ROS), and cell membrane damage, inhibiting photosynthesis, and activating a large number of metabolic processes (Guo et al., 2018b; Kamanga et al., 2018; Dani and Siswoyo, 2019). During long-term natural selection and evolution, plants have formed a comprehensive regulatory mechanism for adaptation and resistance to drought stress, involving morphological, physiological, biochemical, and molecular mechanisms (Zhao D. et al., 2019). Plants will modify root architecture to absorb more moisture from the environment, and leaf stomata will be closed to reduce water evaporation (Buckley, 2019; Uga, 2021). Regarding physiological and biochemical processes, the enzymatic scavenging system can eliminate ROS caused by drought stress, as well as enzymes and reducing substances in the antioxidant defense system, including catalase (CAT), superoxide dismutase (SOD), and ascorbate peroxidase (APX) (Mittler et al., 2011; Fei et al., 2020). At the same time, photosynthesis is an important link for plants to synthesize organic compounds and conduct energy metabolism; they can adapt to arid environments by regulating stomata, enzyme activities, and photosynthetic pigments (Martin et al., 2017). When plants encounter drought stress, cells produce stimulatory responses, and the stress signals are transduced through transcription factors, protein kinases, and plant hormones. Then, a series of responses, such as osmotic regulation and photosynthesis, are activated (Wang et al., 2016; Cai et al., 2019). Signal transduction requires joint participation and regulation of a variety of hormones (Osakabe et al., 2014), and abscisic acid (ABA) is a well-studied plant hormone messenger molecule that is translocated from the sites of biosynthesis to guard cells; it further activates downstream signaling components, such as SnRK2s and MAPKs, which regulate stomatal closure (Kuromori et al., 2018). Most responses are normally controlled by transcriptional regulation involving transcription factors (TFs), which bind to the promoter regions of target genes and activate downstream gene responses. TFs closely related to drought stress include ABRE-binding factors (ABRE/ABF), WRKY, ABA-independent AP2/ERF, and NAC families (Takahashi et al., 2018; Yao et al., 2021). In conclusion, the response of plants to drought stress is an integrated process involving multiple signaling and gene expression changes that require comprehensive analysis.

With the development of sequencing technology and decreasing costs, transcriptomes play an important role in extensive areas, including abiotic stress resistance (Singh et al., 2017a). From the transcriptional level, it can be revealed that differential gene expression in plant signal transduction, endogenous hormones, photosynthesis, and other pathways under drought stress will deepen the comprehensive understanding of the mechanism of drought stress resistance (Sprenger et al., 2016). In recent years, an increasing number of studies have used sequencing technology to explore the molecular mechanisms of garden plants under drought stress, such as *Salix babylonica* and *Chrysanthemum rhombifolium*, and these results have greatly promoted drought resistance research on garden plants (Xu et al., 2021; Zhang et al., 2021). In research on *Pinus massoniana*, several transcription factor genes associated with the circadian rhythm (HY5 and LHY), signal transduction (ERF), and defense responses (WRKY) have been identified as playing a key role in adapting to drought stress (Du et al., 2018). Transcriptional regulation analyzes in peony (*Paeonia suffruticosa*) revealed that the synergy of the expression of *ERF* and *MYB* genes contributed to its resistance to varying degrees of drought stress (Guo et al., 2018a). Research has shown that rose (*Rosa chinensis*) transcription factors balance growth and drought survival, and the physiological and molecular mechanisms under drought stress have also been explored (Li et al., 2020b). Current drought resistance research mainly focuses on crops, while the research on ornamental plants is relatively rare, although it deserves in-depth attention and analysis.

Heimia myrtifolia (Lythraceae) is a deciduous shrub native to South America, ranging from Brazil to Uruguay, and is commonly known as "sun opener" or "shrubby yellow crest" (Rawat et al., 2007). It is of good ornamental value and breeding potential for its bright yellow petals and longstanding flowering period from August to September (Malone and Rother, 1994). *Heimia myrtifolia* can also be used as a medicinal plant with several pharmacologically active alkaloids and phenolics (Yang et al., 2000), which exhibit diuretic and strong anti-inflammatory activity (Lema et al., 1986; Ayoub et al., 2010). Despite its huge potential, *H. myrtifolia* is mainly restricted by seasonal drought and less moisture against its origin. Based on previous research, the physiological regulation mechanism of *H. myrtifolia* to resist drought stress has been revealed, but the molecular mechanisms that account for the adaptation of *H. myrtifolia* to drought remain unclear, limiting its introduction and application. Herein, we used RNA-Seq to explore the molecular mechanism and identify the metabolic pathways and genes enabling resistance to drought to comprehensively explain the drought resistance mechanism. This research will

provide extensive genetic resources for further molecular biology research, drought-resistant cultivation, and breeding.

MATERIALS AND METHODS

Plant Material, Treatments, and Physiological Analysis

Uniformly sized 1-year-old cut seedlings of *H. myrtifolia* were obtained from the Zhejiang A&F University intelligent greenhouse, Hangzhou, Zhejiang province, China (30°13′48″N, 119°43′12″E). Each pot contained a single plant and was filled with 4 kg of soil. The soil water content was maintained at field capacity (33.5%). Before the water-deficit treatments, the seedlings were incubated in an artificial climate room with 70% humidity and light (14 h, 28°C)/dark (10 h, 25°C). Three days before the drought stress treatment, all materials were watered thoroughly every day. The natural drought method without watering was adopted using a TDR100 portable soil moisture meter (Spectrum, Aurora, IL, United States) to measure the soil moisture content. The seedling pots were weighed and watered at 8:00 a.m. every day to maintain each treatment within the control range. The drought treatment continued for 12 days. The relative soil water content of the control treatment (CK) was 65–75%, and the relative soil water content of the two stress treatments was maintained at 30–45% (T1) and 15–25% (T2). The phenotype, relative water content (RWC), and relative electrolyte leakage (REL) of the leaves (mature leaves at the same height and the same orientation in the upper part) were determined using the method presented by Galmés et al. (2007) and Sun et al. (2020). The leaves were collected for physiological experiments and transcriptome sequencing, and three biological replicates for each sampling time were immediately collected and stored at –80°C.

RNA Extraction, cDNA Library Construction, and Transcriptome Sequencing

Total RNA was extracted from the leaves using TRIzol reagent (TaKaRa, Inc., Dalian, China) according to the manufacturer's instructions (Zhang et al., 2016). The total RNA was quantified using NanoDrop and an Agilent 2100 bioanalyzer (Thermo Fisher Scientific, Waltham, IL, United States). To prepare sequencing libraries, oligo(dT)-attached magnetic beads were used to purify mRNA. Fragmentation buffer was added to break the mRNA into short fragments. mRNA was used as a template, and random hexamers were used to synthesize the first-strand cDNA. Then, buffer, dNTPs, RNase H, and DNA polymerase I were added to synthesize the second-strand cDNA. The cDNA fragments obtained from the previous step were amplified by PCR, and products were purified by AMPure XP Beads and then dissolved in EB solution. The double-stranded PCR products from the previous step were heated denatured and circularized by the splint oligo sequence to obtain the final library. Single-stranded DNA was used as the format for the final library.

De novo Assembly, Functional Annotation, and Classification of Unigenes

We used Trinity software (v2.8.0[1]) with default settings for *de novo* transcriptome assembly. The two obtained contigs were connected into a single scaffold to generate unigenes (Grabherr et al., 2011). These unigenes were further spliced to generate longer complete consensus sequences, and redundant sequences were removed with Tgicl (v2.1[2]) (Pertea et al., 2003). The unigene sequences were aligned to seven functional databases (KEGG, GO, NR, NT, Swiss-Prot, Pfam, and KOG), employing BLAST (v2.2.23) with system default parameters and a threshold of $e \leq e^{-10}$ (Zhou et al., 2016). Based on the NR annotation, GO functional annotation was obtained using Blast2GO software (Conesa and Götz, 2008). The pathway assignments were conducted by performing sequence searches against the KEGG database using the BLASTX algorithm with an E value threshold of 10^{-5} (Mcginnis and Madden, 2004).

Analysis of Differentially Expressed Genes (DEGs)

Bowtie2 was used to align clean reads to the reference sequences that were composed of all obtained transcript sequences, and RSEM was used to calculate the gene expression level of each sample (Li and Dewey, 2011; Langmead and Salzberg, 2012). The relative gene expression level between different samples was calculated using the log2 ratio. Fragments per kilobase of exon model per million mapped fragments (FPKM) of each gene were calculated based on the length of the gene and reads count mapped to this gene. Differential expression between the two conditions was analyzed using the DESeq2 method (Love et al., 2014). Genes, with $|\log2FC| > 1$ and a p-value < 0.05, were considered as DEGs (Anders and Huber, 2010). The DEGs were analyzed for GO functional classification and KEGG pathway enrichment analysis, and P-value ≤ 0.05 was the threshold of significance for the GO terms and KEGG pathways (Minoru et al., 2004).

The qRT-PCR Validation for Differentially Expressed Genes (DEGs)

According to the gene annotation results, nine DEGs were selected for qRT-RCR validation experiments. These genes were randomly selected among the DEGs that were closely related to drought stress and had large differential expression fold changes. The primers were designed using Primer Premier 5.0 software (**Supplementary Table 1**). cDNA was transcribed from 5 μg of total RNA using the PrimeScript^{TM} First-Strand cDNA Synthesis Kit in 20 μL of the reaction mixture. RT-qPCR was performed in an ABI7500 Real-Time System (Applied Biosystems) using SYBR Green I (Roche). The amplification procedures were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 58°C for 10 s, and 72°C for 25 s. Three biological and technical replications were performed for each sample. The relative expression levels

[1]http://trinityrnaseq.sourceforge.net/

[2]http://sourceforge.net/projects/tgicl/

were normalized to the expression of the *GAPDH* gene (Chen et al., 2021). Data are presented as relative transcript levels based on the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Statistical Analysis

Data were analyzed with the R software[3] using the one-way analysis of variance (ANOVA) for significant difference. The error bars were calculated with data from three replicates. ANOVA results were considered significant at $p < 0.05$, and mean comparisons were made using the Tukey HSD test.

RESULTS

Phenotypic and Physiological Responses

As the primary organ for photosynthesis, leaves can effectively reflect a plant's drought adaptability. Three treatment groups of *H. myrtifolia* leaves were photographed and collected (CK, T1, and T2). The healthy CK leaves were lanceolate, and the leaf color was bright green. The edges of the leaves were slightly curled, but the branches were significantly drooping in T1. The leaves were severely curled, and chlorosis of the leaves was obvious at T2 (**Figure 1A**). Plant morphology was also affected; growth and development were inhibited, and the leaves withered and fell off. The RWC gradually decreased from 92.18% in CK to 60.45% (T1) and 35.54% (T2) in the treatments (**Figure 1B**). Drought stress can damage plant cell membranes, cause changes or loss of plant cell membrane permeability, and cause electrolyte extravasation (Susilo et al., 2019). In this experiment, with drought increases, the REL of the leaves showed an increasing trend, reaching a peak at 38.23% in T2 (**Figure 1C**).

Overall Summary of Transcriptome Data

The sample sequencing quality evaluation results showed that nine samples obtained 43.02–49.08 M raw reads (**Table 1**). Raw reads removed linker sequences, duplicates, and low-quality fragments. A total of 37.63–43.46 M high-quality clean reads were obtained, and the GC content was 46.81% on average. After filtering out the unqualified reads in the original data, the effective data volume was 56.44 Gb, and the effective rate was between 86.89 and 89.41%. The proportion of data with data quality ≥ Q20 was above 93.77%.

Using Trinity software to *de novo* assemble the high-quality data of nine samples, 62,015 unigenes were obtained. The total number of unigenes was 107,289,516 bp with an average length of 1730 bp. The N50 length was 2510 bp, and the N90 length was 948 bp (**Supplementary Figure 1**). To fully understand gene function, the transcriptome sequences were compared to seven functional databases (**Supplementary Table 2**), and 62,105 unigenes were annotated. The number of unigenes compared with the NR database was the largest, with 51,982 (83.82%) unigenes. The number of unigenes successfully annotated to the NT, Swiss-Prot, KEGG, KOG, Pfam, and GO databases was 45,155 (72.81%), 40,806 (65.80%), 42,435 (68.43%), 42,089 (67.87%), and 39,212 (63.23%), respectively. Among them, there were 23,739 (38.28%) unigenes that could be successfully

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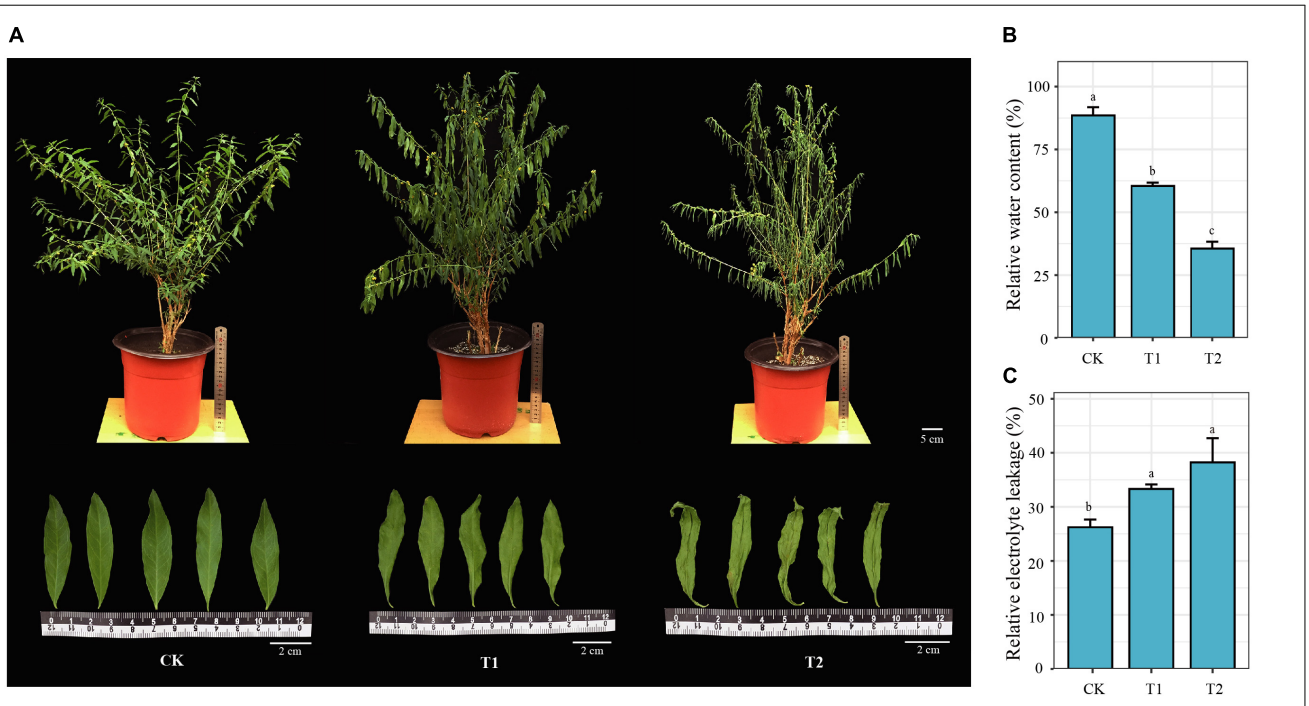

FIGURE 1 | Phenotypic and physiological responses of *Heimia myrtifolia*. **(A)** Morphological observation of *H. myrtifolia* in response to different drought stress treatments. **(B)** Relative water content (%). **(C)** Relative electrolyte leakage (%). Different letters indicate the result is significantly different ($p < 0.05$).

TABLE 1 | Summary of RNA-sequencing data under drought stress.

Sample	Total raw reads (M)	Total clean reads (M)	Total clean bases (Gb)	Clean reads ratio (%)	Q20 (%)	GC (%)
CK_1	49.08	42.94	6.44	87.50	95.65	46.80
CK _2	49.08	42.68	6.40	86.96	95.52	47.00
CK _3	47.33	42.31	6.35	89.41	95.28	47.27
T1_1	49.08	43.46	6.52	88.55	95.41	46.73
T1_2	43.02	37.63	5.65	87.48	94.1	47.29
T1_3	48.95	42.72	6.41	87.28	93.77	46.69
T2_1	49.08	42.65	6.40	86.89	93.78	46.55
T2_2	44.53	38.81	5.82	87.15	93.88	46.69
T2_3	49.08	43.03	6.45	87.67	93.77	46.30

≥ Q20 (%) indicates the percentage of bases with the quality value ≥ 20 in clean data.

annotated in the seven major databases, and the number of genes annotated in at least one database was 53, 433 (86.16%).

Compared with the NR database, the species with the greatest homologous sequence alignments was *Punica granatum* of the Lythraceae family, with 43,143 (83%) homologous sequences (**Supplementary Figure 2**). The KEGG annotation results (**Figure 2A**) showed that the most represented metabolic pathway was carbohydrate metabolism (3764, 10.03%). The main basic metabolic pathways included amino acid metabolism (1916, 5.10%), lipid metabolism (1675, 4.46%), and transportation and catabolism (1608, 4.28%). Using the KOG database to classify the proteins (**Supplementary Figure 3**), 25 KOG functional categories were annotated. Among these functional categories, general function predictions only category when compared with 8761 genes (20.65%), accounted for the largest proportion, showed that some new proteins with unknown functions were found. This was followed by signal transduction (5752, 13.5%) and translation modification (3627, 8.55%). The analysis of GO enrichment was performed using Blast2GO software (**Figure 2B**), and GO functions were mainly enriched in three aspects: biological process, cellular component, and molecular function (Harris et al., 2004). The distribution of GO functions involved 43 functional subcategories, and the biological process contained 25 functional subcategories, of which the most functional subcategory was cellular anatomical entity and binding, with 27,870 (17.41%) and 24,356 (15.22%) unigenes, respectively. GO subclass functions were related to the drought stress response, which are mainly related to antioxidant activity, transcription regulation, and signal transmission.

Expression and Functional Analysis of Differentially Expressed Genes (DEGs)

By comparing the number of independent fragments in the three libraries to reflect the relative abundance of mRNA, using the control treatment (CK) as the reference value, pairwise comparisons were made between the two treatment groups (T1 and T2) and CK. The threshold of DEGs was $|\log2 \text{ fold change}| \geq 1$ and $P\text{-value} \leq 0.05$. DEGs that met the criterion $\log2 \text{ fold change} > 0$ were considered upregulated, and others were considered downregulated (Anders and Huber, 2010). From the Venn diagram and histogram (**Figure 3**), a total of 14,272 DEGs were detected, of which 277 genes were differentially expressed among the two treatment groups, and 358 and 5448 DEGs were specifically expressed in T1 and T2, respectively. There were 2021 DEGs (1030 were upregulated and 991 were downregulated) between CK and T1, and a total of 11,432 DEGs (5593 were upregulated and 5839 were downregulated) were detected between CK and T2. Furthermore, principal component analysis showed good intergroup correlation and high repeatability (**Supplementary Figure 4**); therefore, the samples were reliable for use in subsequent data analysis.

GO enrichment analysis (**Supplementary Figure 5**) showed that DEGs were enriched in 41 GO terms (**Supplementary Table 3**), and the least GO terms enriched to cell components, which was consistent with the result that unigenes were successfully annotated into the GO database. "Cellular anatomical entities" were enriched with the largest number of genes, with 787 and 4592 genes enriched in T1 and T2, respectively. "Catalytic activity" (733 DEGs in T1, 4173 DEGs in T2) and "binding" (727 DEGs in T1, 4197 DEGs in T2) in the "molecular function" category were significantly enriched. In the "biological process" category, "cellular process" and "metabolic process" were significantly enriched. The top GO terms enriched at the two time points were roughly similar, but the number of differential genes enriched in T2 increased significantly. The GO terms related to drought stress were "antioxidant activity," "cell redox homeostasis," "photosynthesis," "response to heat," and "signaling." DEGs involved in these terms were worthy of follow-up attention.

The KEGG pathway enrichment analysis (**Figure 4**) found that the plant hormone signal transduction pathway (KO: 04075) was significantly enriched in T1 (**Supplementary Table 4**). Three pathways closely related to drought resistance were significantly enriched in T2 (**Figure 4B**): photosynthesis–antenna proteins (KO: 00196), photosynthesis (KO: 00195), and glutathione metabolism (KO: 00480). Amino acid metabolism, biosynthesis of other secondary metabolites, carbohydrate metabolism, and lipid metabolism were the top four enriched pathways with 400, 219, 104, and 68 DEGs enriched in T1, respectively, and 2223, 946, 683, and 401 DEGs enriched in T2, respectively. The DEGs enriched in these four metabolic pathways may be involved in *H. myrtifolia*'s drought response. The top 20 KEGG pathway results revealed that plant hormone signal transduction pathways, photosynthesis pathways, photosynthesis–antenna proteins pathways, glutathione metabolism pathways, and ascorbate and aldarate metabolism pathways were significantly enriched, which played an important role in the process of drought stress and mainly regulated the expression of downstream functional genes in response.

Regulation of the Plant Hormone Signal Transduction Pathway to Drought Stress

The plant hormone signal transduction pathway (ko04075) was significantly enriched ($P < 0.05$) in T1, and most DEGs were involved in the abscisic acid (ABA) and auxin (AUX) pathways (**Supplementary Table 5**). In the ABA signal

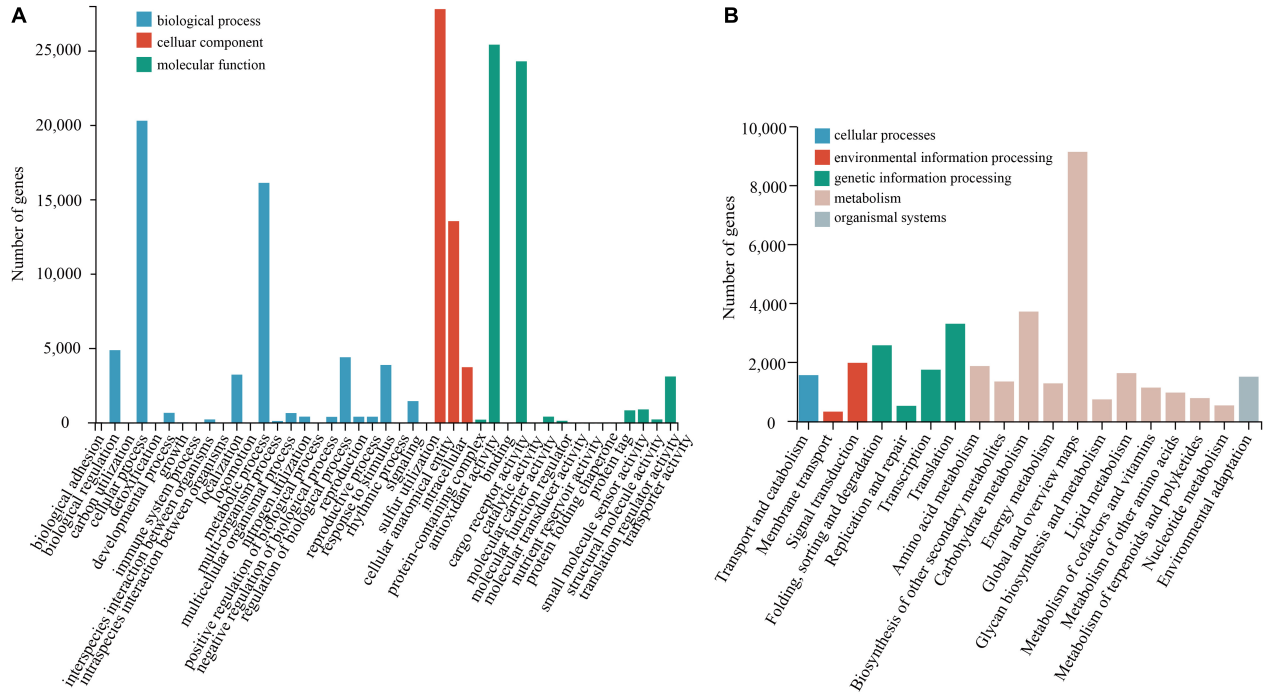

FIGURE 2 | Functional and pathway enrichment of unigenes. **(A)** GO enrichment with three primary classifications of biological process, cellular component, and molecular function. **(B)** Functional classification and pathway assembled unigenes by KEGG.

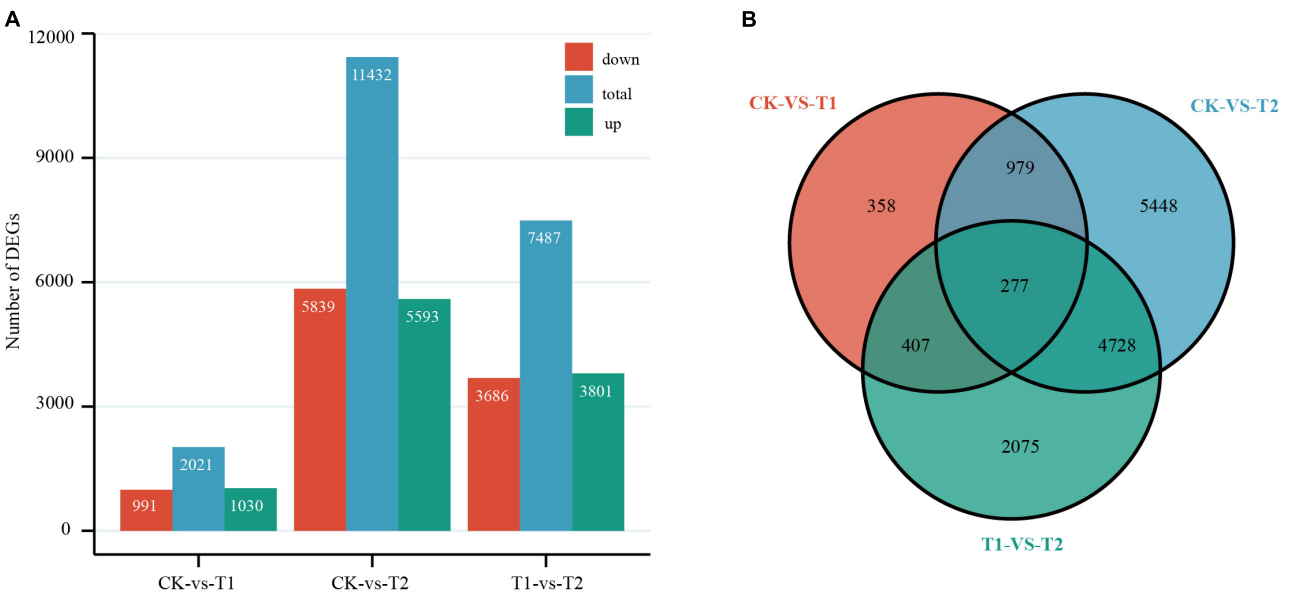

FIGURE 3 | Differentially expressed genes (DEGs) in *Heimia myrtifolia* during drought stress. **(A)** The number of DEGs between CK and drought treatment group in T1 and T2. **(B)** Venn diagram representing DEGs expressed after drought treatment.

transduction pathway (**Figure 5A**), ABA receptor PYR/PYL was downregulated in T1, while two (*CL2679.Contig1_All* and *CL2679.Contig2_All*) were upregulated and one (*Unigene417_All*) was downregulated in T2. The activity of PYR/PYL enhanced the downstream negative regulatory protein factor PP2C. Eighteen DEGs were annotated as PP2C; PP2Cs were all upregulated in T1, most were downregulated in T2, and only one PP2C (*CL6736.Contig5_All*) was continuously upregulated in both treatments. Protein kinase SnRK2 was peculiarly expressed in T2, except for one

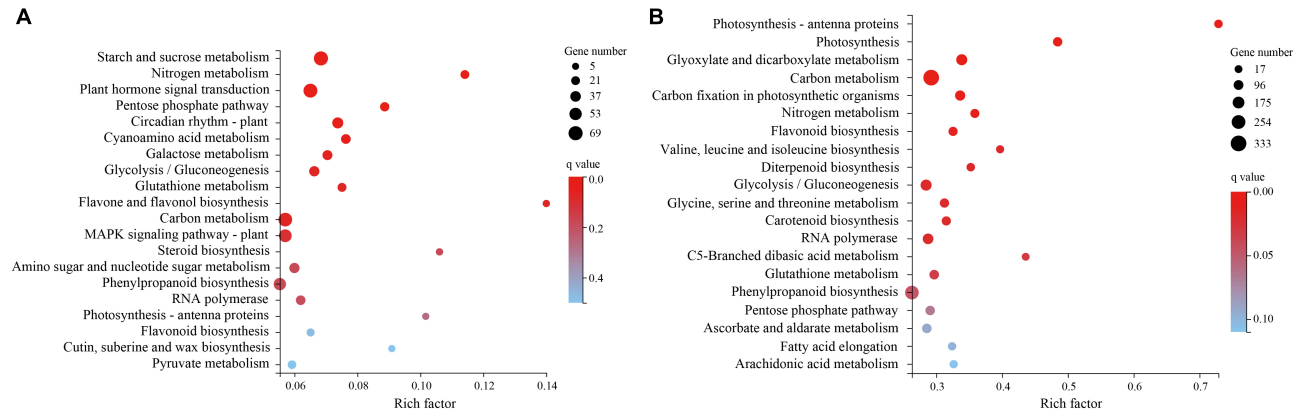

FIGURE 4 | KEGG pathway enrichment with differentially expressed genes (DEGs) between different droughts treated. **(A)** KEGG pathway enrichment of DEGs to drought stress in T1. **(B)** KEGG pathway enrichment of DEGs to drought stress in T2.

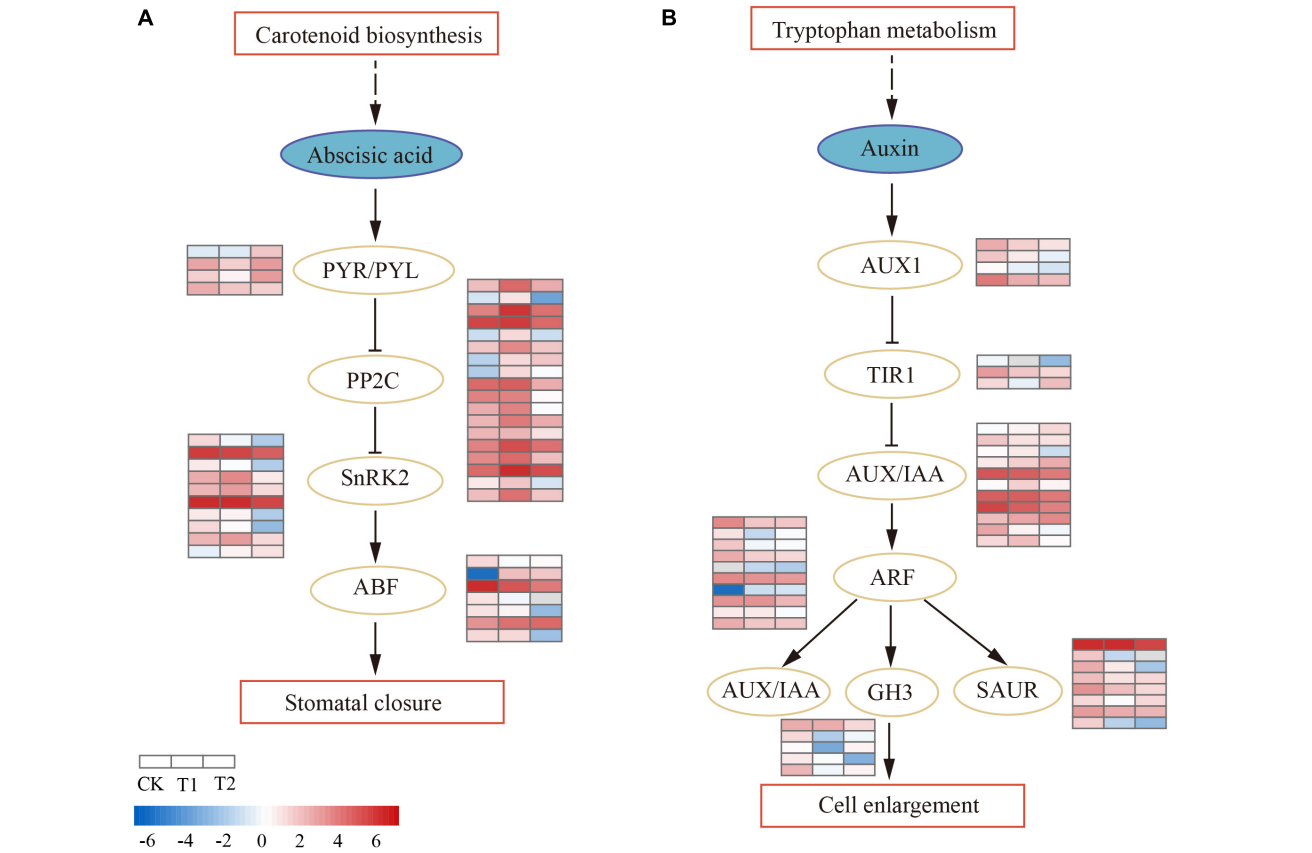

FIGURE 5 | Differential regulation of plant hormone signal transduction pathway in drought treatments. The heatmaps show log_2FPKM values of the differentially expressed genes (DEGs). **(A)** Abscisic acid signal transduction pathway. **(B)** Auxin signal transduction pathway.

SnRK2 (*CL1848.Contig8_All*) that was upregulated in T2; nine SnRK2s were downregulated. The downstream ABA-responsive transcription factor ABF decreased activity and inhibited the expression of ABA-responsive genes. In auxin hormonal signaling (**Figure 5B**), there were 11 *AUX/IAA* genes. Only one (*CL7309.Contig2_All*) was specifically upregulated in T1, most were downregulated in T2 (three upregulated, seven downregulated), and one *AUX/IAA* gene (*Unigene9101_All*) was downregulated in both treatments. A total of 10 differentially expressed auxin regulator ARFs (two upregulated and eight downregulated) were identified. ARF downregulated downstream early auxin response genes *SAUR*, *TIR1*, and *GH3* in

the drought process. The brassinosteroid biosynthesis pathway was enriched ($P < 0.05$) in T2. In brassinosteroid (BR) signaling, the membrane receptors BRI1 and BAK1 had enhanced activity, both of which were upregulated in T1. BSK (BR-signaling kinase) was mainly upregulated in T2, which eventually led to the downregulation of *TCH4* genes and D-type cyclin (CYCD3) expression (**Supplementary Table 6**).

Regulation of Photosynthesis to Drought Stress

The photosynthesis–antenna protein pathway (ko00196) was significantly enriched in both T1 and T2. Many photosystem II light-harvesting chlorophyll a/b-binding proteins, such as *Lhcb* genes, were differentially expressed, and most of them were activated at T2 (**Supplementary Table 7A**). There were two and 37 *Lhcb* genes downregulated in T1 and T2, respectively. Fifty-nine DEGs were enriched in the photosynthesis pathway (ko00195) in T2. These DEGs were mainly composed of the photosystem I protein *Psa* gene, photosystem II protein *Psb* gene, photosystem I reaction center protein gene, photosystem I chlorophyll apolipoprotein gene, and photosystem II protein gene. A total of four *Psa* genes and eight *Psb* genes were found, and the FPKM values of some photosynthesis-related DEGs were up to 1000 or higher (**Supplementary Table 7B**). The pathways of porphyrin and chlorophyll metabolism (ko00860) were also significantly enriched in T2, and 45 DEGs were enriched. Chlorophyll was catalyzed by a series of enzymatic reactions, and the final step was chlorophyll synthase (CHLG), which catalyzed the synthesis of chlorophyll a/b. In our study, two chlorophyll degradation key enzymes, *CAO* (*CL2647.Contig2_All* and *CL2647.Contig3_All*), were downregulated in T2, and one *CHLG* gene (*Unigene5893_All*) was also differentially downregulated in T2 (**Supplementary Table 7C**).

Regulation of Active Oxygen-Scavenging System to Drought Stress

In our study, glutathione metabolism (ko00480) and ascorbate and aldarate metabolism pathways (ko00053) were significantly enriched in T2, which were enriched 87 and 89 DEGs, respectively. A total of 21 glutathione-S-transferase (GST) DEGs were identified (**Supplementary Table 8A**). Five *GST* genes were upregulated and seven were downregulated in T1, while in T2, most *GST* genes were upregulated. Two differentially expressed glutathione peroxidase (GSH) genes were also identified during drought stress, and both *GSH* genes were upregulated in T2. Five DEGs of dehydroascorbate reductase (DHAR) were identified in the ascorbate and aldarate metabolism pathways. The expression of five *DHAR* genes was significantly changed in T2 (three upregulated and two downregulated), one (*Unigene10653_All*) of which was upregulated in both drought periods. Four of the six ascorbate peroxidase (APX) genes were downregulated in T2, and *APX* genes that were upregulated in T1 had a higher expression than that observed in T2 (**Supplementary Table 8B**). These two pathways were significantly enriched, suggesting that related antioxidants play an important role in eliminating oxidative damage caused by drought stress. In our study, 26 differentially expressed antioxidant enzyme genes were also identified, including 14 *POD* genes, 10 *CAT* genes, and two *SOD* genes (**Supplementary Table 8C**). Both *SOD* genes were upregulated in T1. The *CAT* genes were mostly downregulated in T2, and two were upregulated (*CL654.Contig3_All* and *CL654.Contig14_All*) in both periods. Fourteen *POD* genes (among them four upregulated in T1 and one downregulated in T1) were identified. The *POD* genes mainly changed in T2, seven were upregulated, and five were downregulated.

Major Transcription Factors Differentially Regulated Under Drought Conditions

Using the PlantTFDB plant transcription factor database, 1901 encoding transcription factors (TFs) belonging to 56 gene families were identified in *H. myrtifolia*'s transcriptome database (**Figure 6A**). The top five TF families were MYB (287, 15.10%), C3H (171, 9.01%), bHLH (129, 6.79%), WRKY (101, 5.31%), and AP2-EREBP (98, 5.16%). Differentially expressed TFs were displayed in an expression heatmap (**Figure 6B**), including the common TF families MYB, bHLH, AP2-EREBP, NAC, and C2H2. In our study, the DEGs of the MYB family accounted for the largest proportion. A total of 93 MYB TFs were differentially expressed under drought stress, with 25 MYB TFs (10 upregulated and 15 downregulated) expressed in T1. A large number of MYB TFs (42 upregulated and 43 upregulated) were specifically expressed in T2, but the downregulated MYB TFs were generally higher in expression. Most of the NAC TFs were upregulated, with nine being upregulated in T1. A total of 27 NAC TFs were upregulated in T2, and six were upregulated in both treatments. Similarly, WRKY TFs were upregulated during drought stress, and 39 were differentially expressed in T2, of which 35 were significantly upregulated. Only one WRKY TF was upregulated, but eight WRKY TFs were downregulated in T1. Ten bHLH TFs were upregulated, and 16 were downregulated in T1. A total of 12 bHLH TFs were upregulated and 22 were downregulated in T2, of which five were upregulated in both treatments. Most of the AP2-EREBP TFs were upregulated, five were upregulated, and five were downregulated in T1. In T2, 15 were downregulated and 35 were upregulated. The upregulated AP2-EREBP TFs maintained a high expression level. Thirteen differentially expressed bZIP TFs were identified, of which three and nine were upregulated in T1 and T2, respectively. HSPs changed significantly and played an important role under drought stress, while the heat shock transcription factor (HSF) regulated the expression of HSPs. Twelve differentially expressed HSF TFs were found in both treatments and were mostly upregulated.

Real-Time qPCR Validation

To verify the reliability of the transcriptome results, nine DEGs related to drought stress were selected and the specific primers were designed for qRT-PCR. The relative expression of the genes in the qRT-PCR analysis results and the FPKM value in RNA-Seq were used for the validation of sequencing data. The qRT-PCR results of nine genes (**Figure 7**) were slightly different from the expression in the sequencing data, but the

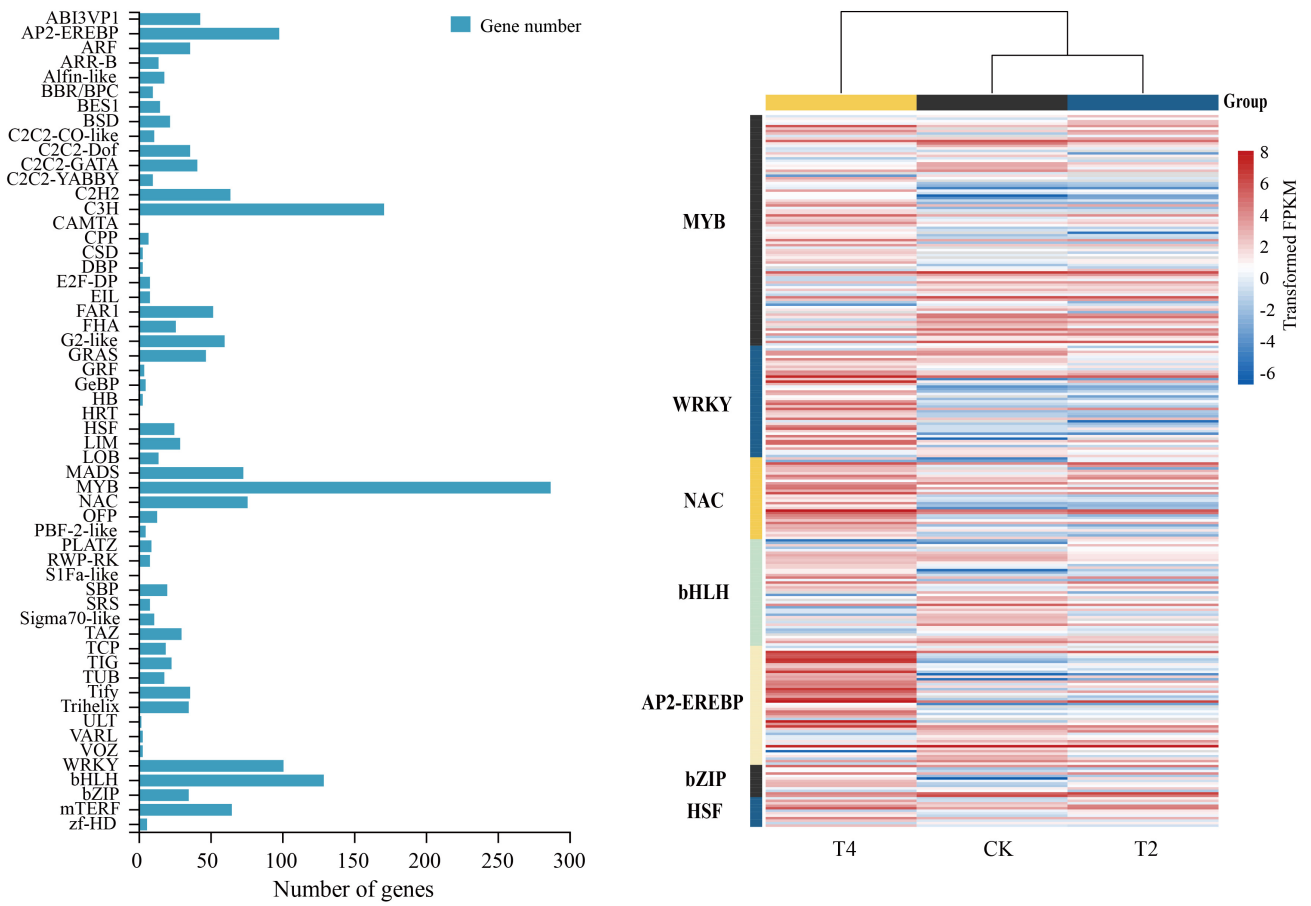

FIGURE 6 | Differential regulation of transcription factors in drought treatments. **(A)** The number of differentially expressed genes (DEGs) of transcription factors after drought treatment. **(B)** Heatmap of seven classes of transcription factors (MYB, WRKY, NAC, bHLH, AP2-EREBP, bZIP, and HSF) closely associated with drought; the heatmap shows $\log_2$FPKM values of the differentially expressed TFs.

overall expression trend was basically the same. The correlation of the linear regression analysis was 0.8132, which further verified the accuracy and credibility of the transcriptome data (**Supplementary Figure 6**).

DISCUSSION

Key Pathways and Drought Tolerance-Related Genes Under Drought Stress

Among the KEGG pathways enriched under drought stress, the photosynthesis–antenna protein pathway was significantly enriched in both periods, and the photosynthesis pathway and porphyrin and chlorophyll metabolism pathway were also significantly enriched in T2, indicating that the regulation of the photosynthetic system plays an important role under drought stress. Plant photosynthesis is a complex physiological process and an important pathway for plant energy synthesis and metabolism, and drought stress affects stomatal opening and the CO_2 transfer rate, limiting plant photosynthesis (Banks, 2018). Photosystem II is a critical mechanism for photosynthesis and energy conversion in plant chloroplasts, converting electrical energy into active chemical energy (Hu et al., 2018). In our study, the photosystem I and II protein genes and light-harvesting pigment protein genes were downregulated during drought stress. The photosystem II light-harvesting chlorophyll a/b-binding protein *Lhcb* gene was significantly downregulated in T2, which maintained a high expression level before downregulation. The results indicated that the light-responsive process was disrupted to a certain extent, and the roles of *Lhcb* genes in regulating drought-induced responses are relatively active. Similar to the results of our study, the downregulation of DEGs related to chlorophyll degradation and photosynthetic capacity in *Paeonia lactiflora* severely damaged the photosynthetic system and reduced the ornamental value (Li et al., 2020a). In our study, the photosynthetic protein genes *Psa* and *Psb* were also downregulated under drought stress, and the D1 protein in the light reaction center was damaged. Therefore, the photosynthetic

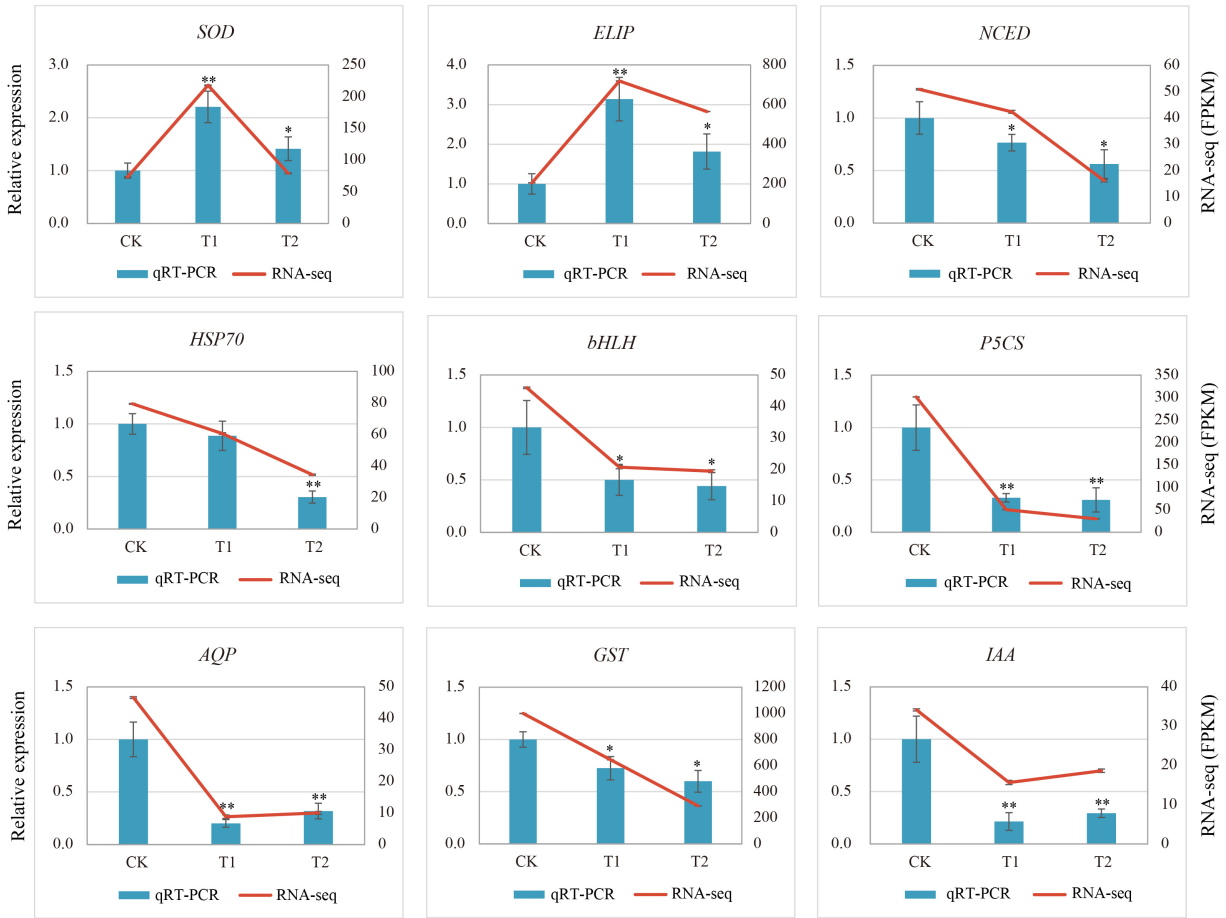

FIGURE 7 | Relative gene expression of nine differentially expressed genes (DEGs) in response to drought stress. Vertical bar charts with error bars (left y-axis) represent the quantification results of nine DEGs using RT-qPCR, and the values are means ± SE (n = 3). Line graphs (right y-axis) represent the transcript abundance (FPKM) of each gene detected by RNA-Seq. Asterisks (* or **) represent the significant differences at $p < 0.05$ or $p < 0.01$ when compared with the control, respectively.

system may be inhibited by non-stomatal factors. Photosynthetic pigments are a significant medium for plants to convert inorganic substances into organic substances, and the excitation of chlorophyll molecules by light will transfer electrons and synthesize ATP (Lang et al., 2018; Wang et al., 2018). In our study, two chlorophyll b synthesis *CAO* genes and one chlorophyll synthesis *CHLG* gene were downregulated in T2. The results may indicate that the chlorophyll synthesis process was hindered to some extent, and the leaf color gradually became lighter during the stress process, from tender green to yellow–green. In this study, the photoreaction system of *H. myrtifolia* was destroyed under drought stress, and the expression of many photosynthesis-related genes was downregulated, resulting in a decrease in the photosynthetic rate and blocking chlorophyll synthesis. However, the energy absorbed by photosynthesis still plays a protective role and maintains metabolism.

Drought stress increased the production rate of reactive oxygen species (ROS), and excessive ROS induced oxidative damage in plant membrane systems; thus, the balance of ROS was closely related to peroxidase pathways (Zhang et al., 2019). In our study, the relative conductivity (REL) of *H. myrtifolia* gradually increased with drought stress, indicating that the membrane system was damaged by oxidation. The ascorbic acid–glutathione system is an important mechanism of the antioxidant system in plants, and these two pathways were also significantly enriched in our study (Aguiar et al., 2016). Glutathione-S-transferase in the glutathione pathway is a multifunctional enzyme that can degrade harmful substances and reduce cell damage. The *APX* genes and *GST* genes help remove excess reactive oxygen species (Dalal et al., 2018; Rehman et al., 2021). In our study, 17 *GST* genes were identified, most of which had high expression levels, which were consistent with a previous study on *Masson pine*, with an upregulated expression of many genes in the antioxidant defense system (Haas et al., 2021). The results showed that the glutathione metabolic pathway played an active and synergistic role in scavenging excess reactive oxygen species. DHAR is a plant-specific glutathione-S-transferase that catalyzes the reduction in dehydroascorbic acid to ascorbic acid (Li et al., 2018). In our study, five differentially expressed *DHAR* genes were identified and were shown to assist plants in

producing ascorbic acid to enhance their antioxidant capacity. SOD, POD, and CAT enzymes are also important antioxidant enzymes for free radical scavenging in plants, and the genes regulating these enzymes were also differentially expressed in our study (Rahimi et al., 2021). Two *SOD* genes in our study were upregulated in T2, but most of the 10 *CAT* genes were downregulated in T2. *POD* genes were also mainly differentially expressed in T2, indicating that the generation and clarity of hydrogen peroxide during the stress process were basically the same. The antioxidant enzyme genes actively participate in the stress resistance response and jointly remove superoxide-free radicals and H_2O_2 in plants, alleviating the damage caused by drought stress.

Drought Stress Regulates the Plant Hormone Signal Transduction Pathway

Plant hormones are key regulators of plant growth and development. Plant hormones can mediate signal transduction, which is directly involved in regulating the drought response process (Gupta et al., 2020; Sun et al., 2020). The plant hormone signal transduction pathway was significantly enriched ($P < 0.05$) in T2. ABA is an important plant hormone in the abiotic stress response, and drought stress can induce the production of ABA in the root system. ABA signal transduction to the leaves causes the stomata to close, which inhibits photosynthesis in plants (Kuromori et al., 2018). A study found that ABA signaling-related genes were susceptible to drought stress in *Pinus massoniana*, such as PYL and PP2C, which regulates the expression of stress-responsive genes (Péret et al., 2012). ABA signaling is needed to recognize ABA and initiate to receive the subsequent signal transduction process. The ABA receptor PYR/PYR protein was located the most upstream of the ABA signal transduction pathway, and the transcription efficiency of PYL significantly affected the rate of ABA signal transduction (Peleg and Blumwald, 2011; Takahashi et al., 2018). PYR/PYL and PP2C, as part of the ABA signaling pathway, were upregulated in our study. Between the two treatments, four *PYR/PYL* genes were differentially expressed. The PYR/PYL genes formed a complex with PP2Cs, and the expression of PP2Cs was negatively regulated, which was consistent with the results from previous studies on *Oryza sativa* (Ramachandran et al., 2021). The decreased activity of PP2C led to the downregulation of the protein kinase SnRK2, which ultimately led to increased ABA signaling intensity, activation of stomatal closure, and restricted root growth. The results suggest that ABA signaling plays an active role in improving drought resistance in *H. myrtifolia*. Auxin signal transduction, which can regulate root growth, leaf development, and phototropic growth, among other processes, also played an important role in our study (Liang et al., 2019). The auxin-responsive genes *AUX/IAA* in our study were mainly downregulated in T2. The auxin regulatory factor (ARF) and the auxin early-responsive genes *SAUR* and *GH3* were mainly downregulated under drought stress. Similar to the results obtained in *Benincasa hispida*, *SAUR* genes were less expressed in drought-tolerant cultivars, reflecting better regulation of penetration (Wang et al., 2019). ARF TFs can regulate hormonal signaling to maintain normal plant growth under stress (Péret et al., 2012). AtARF2 in *Arabidopsis thaliana* is involved in the regulation of ethylene and auxin signaling pathways (Singh et al., 2017b). In this study, the downregulated expression of *SAUR*, AUX/*IAA*, and *GH3* genes may be due to the inhibition of auxin synthesis during drought stress resistance, which improved the drought resistance of *H. myrtifolia* and slowed the growth of plants to adapt to the external environment. In BR signaling, the expression of membrane receptor proteins BRI1 and BAK1 was activated, and the activity of D-type cyclin was inhibited, which affected plant cell division (Ye et al., 2010). The enhanced activity of membrane receptors BRI1 and BAK1 in this study positively regulated plant adaptation to drought. In this study, plant hormones actively transmitted stress signal molecules, regulated downstream gene expression with transcription factors, and participated extensively in the adaptation of *H. myrtifolia* to the environment.

Role of Transcription Factors in the Drought Stress of *Heimia myrtifolia*

In our study, 1901 encoding TFs belonging to 56 gene families were identified. MYB, WRKY, and bHLH TFs are among the top five gene families that have been identified to be closely related to hormone regulation and play an important role in the drought tolerance process of plants (Wang et al., 2016). MYB is an important TF involved in cell differentiation and root growth and induces the expression of ABA-related genes under drought stress (Imran et al., 2018). Overexpressing MYB2 transgenic Arabidopsis had enhanced osmotic stress tolerance, and MYB TFs were induced by exogenous ABA to regulate stomatal movement (Yu et al., 2019; Dossa et al., 2020). In our study, the MYB family contained the largest number of TFs. MYB TFs were abundantly expressed in T2, and downregulated MYB showed higher expression, indicating that the negative regulation of MYB was more obvious. These MYB TFs may play key roles in the regulation of drought resistance. The bHLH TF family is the second largest transcription factor family in plants, among which MYC2 can activate the expression of JA-related genes (Xu et al.). In our study, MYC2 TFs were downregulated, which inhibited the expression of downstream genes, slowing down the senescence process of plants. Most of the bHLH TFs downregulated in T2 showed that bHLH TFs mainly played a negative regulatory role under drought stress. WRKY TFs also directly participate in the drought response process through phytohormone and osmotic regulation under drought stress (Maleck et al., 2000). The overexpression of loquat EjWRKY17 enhanced the drought tolerance of transgenic lines, which showed lower water loss and electrolyte leakage (Wang et al., 2021). In our study, WRKY TFs were abundantly expressed in T2, and 35 WRKY TFs were upregulated. TFs can interact with plant hormones to jointly regulate the stress resistance process of plants. In our study, the NAC and AP2-EREBP TF families were mainly upregulated during drought stress, activating the expression of downstream genes. The large upregulated expression of TFs indicated that the drought environment transmitted the stress

signal to the aerial parts, which played a positive role in the regulation of the stress environment. Overall, TFs clearly play a crucial role in the responses of *H. myrtifolia* to drought stress.

CONCLUSION

The morphological observation and physiological determination in our study revealed that following dehydration, leaf margins curl and become lighter, and the RWC decreased and REL increased. The transcriptome analysis of drought-stressed *H. myrtifolia* detected 62,015 unigenes and 14,272 DEGs, revealing a molecular-level drought resistance mechanism. The functional annotation of DEGs found that drought significantly affects plant hormone signal transduction, photosynthesis, glutathione metabolism, and ascorbate and aldarate metabolism, among other processes. Plant hormone signaling molecules and many transcription factors, such as MYB, NAC, bHLH, WRKY, and HSF, were activated to induce the expression of key downstream genes involved in drought response. Many photosynthesis-related genes were downregulated, and antioxidant enzyme genes were also regulated to protect the balance and maintain the stability of metabolic processes. The results of this study will fill the gap in research on abiotic stress molecules in *H. myrtifolia*; it will provide abundant genetic resources for subsequent research and breeding work. It also provides a theoretical basis for the study of the drought resistance of other ornamental plants and promotes the construction of water-saving and drought-resistant gardens.

DATA AVAILABILITY STATEMENT

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 below: https://www.ncbi.nlm.nih.gov/, PRJNA804698.

AUTHOR CONTRIBUTIONS

LL and JW performed the experiments. LL, JW, QW, and QM performed transcriptomic analysis. LL, MJ, SH, LS, GZ, and YZ wrote and reviewed the manuscript. CG provided the funds. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.877913/full#supplementary-material

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Daily light integral and/or photoperiod during the young plant and finishing stages influence floral initiation and quality of witchgrass and marigold cut flowers

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To produce consistent and high-quality specialty cut flowers throughout the year, growers in temperate climates must utilize controlled environment greenhouses. Research-based information on photoperiod management and supplemental lighting for specialty cut flowers is limiting. Therefore, our objectives were (1) to determine the effect of photoperiod during the young-plant and finishing stages on floral initiation and morphology of witchgrass 'Frosted Explosion' (*Panicum capillare*) and marigold 'Xochi' (*Tagetes erecta*) and (2) to quantify the effect of daily light integral (DLI) on floral initiation and morphology of witchgrass during the finishing stage. Seeds of marigold and multi-seed pellets of witchgrass were sown and placed under 9-, 11- (marigold only), 12-, 13-, 14-, 15-, 16-, 18-, or 24-h photoperiods or a 9-h short day with a 4-h night interruption (NI) from 2200 to 0200 h. Plugs were distributed among 10-, 11-, 12-, 13-, 14-, 15-, or 16-h photoperiods or a 4-h NI, for finishing. Witchgrass was finished under a very low or moderate DLI of ≈3 or 10 mol·m^{-2}·d^{-1}, respectively, while marigold was finished under a DLI of ≈10 mol·m^{-2}·d^{-1}. Marigold grown under a photoperiod ≥ 11 h or a 4-h NI during the young-plant stage and finished under an 11- or 12-h photoperiod had thick stems and consistently met the marketable stem length of ≥ 65 cm. Up to 29% and 107% more stems were harvestable under 11- and 12-h finishing photoperiods, respectively, compared to a 10-h finishing photoperiod. Marigold visible buds were delayed, and stems were not harvestable under photoperiods ≥ 13 h or a 4-h NI after 8 weeks. Young witchgrass plants grown under a photoperiod between 14- and 24-h or a 4-h NI and finished under photoperiods ≥ 14 h or a 4-h NI, and at least a moderate DLI, were reliably harvestable (≥ 50 cm long with a fully developed panicle). Witchgrass finished under day lengths < 13 h (rep. 1) or < 14 h (rep. 2) flowered prematurely and were roughly one-sixth the length of harvestable stems at an open flower. All witchgrass stems grown under a very low DLI were shorter and thinner than those grown under a moderate DLI, and none were harvestable. Therefore, we recommend growing marigold 'Xochi' young

plants under a photoperiod ≥ 11 h or a 4-h NI and finishing under a 12-h photoperiod. Additionally, witchgrass 'Frosted Explosion' young plants should be grown under a photoperiod ≥ 14 h or a 4-h NI and finished under photoperiods ≥ 14 h or a 4-h NI to prevent premature flowering. Witchgrass and marigold cut flowers should be finished under a DLI of ≥ 10 $mol \cdot m^{-2} \cdot d^{-1}$ for consistent production of high-quality stems.

KEYWORDS

light-emitting diodes, *Panicum*, specialty cut flowers, supplemental lighting, *Tagetes*, young plants

Introduction

Year-round demand for locally sourced specialty cut flowers continues to increase in the United States [Faust and Dole, 2021; Produce Marketing Association and Food Marketing Institute [PMAFMI], 2016]. From 2015 to 2018, the number of domestic cut flower producers with annual sales ≥ $100,000 increased by 20%, and producers reported a wholesale value of $374 million in 2018 [US Department of Agriculture [USDA]., 2019]. Of this, California accounted for $288 million (77%) of domestic production at least partly because of the coastal climates of its central and southern counties (Carman, 2007). However, demand persists across the nation, and growers in northern latitudes cannot produce specialty cut flowers outdoors year-round due to low temperatures and solar radiation during the winter and early spring. Thus, controlled-environment greenhouses must be utilized to produce high-quality specialty cut flowers year-round.

Many varieties of specialty cut flowers are categorized as short-day plants (SDPs), including marigold (*Tagetes erecta*), celosia (*Celosia* spp.), and zinnia (*Zinnia elegans*) (Craig and Runkle, 2013; Dole, 2015). Young plants with a short day (SD) flowering response may flower prematurely if grown during periods with natural SDs, resulting in short, unmarketable stems (Dole and Warner, 2017). Therefore, photoperiodic lighting techniques such as low-intensity day extension (DE), night interruption (NI), or high-intensity cyclic lighting can be utilized to create long days during the beginning of the production cycle (Meng and Runkle, 2016), ensuring that plants do not flower prematurely, and thereby preventing inferior cut flower quality (Currey et al., 2013).

Preventing premature flowering through photoperiod manipulation may also reduce the need for plant growth regulator applications. Once flower initiation has occurred, it is rarely possible to revert plants to a vegetative state by placing them under non-inductive photoperiods (Runkle, 2008) or by removing flower buds. Thus, flower-aborting plant growth regulators such as ethephon must be applied, and multiple applications may be necessary over the duration of the crop cycle (Styer, 2002). Additionally, such plant growth regulators can inhibit internode elongation and suppress apical dominance (Runkle, 2013). Therefore, it is recommended that cut flowers be grown under non-inductive photoperiods for several weeks before flower initiation (Porat et al., 1995; Dole and Warner, 2017).

Limited research-based information detailing photoperiodic lighting applications for greenhouse-grown SDP cut flowers exists. Blacquière (2002) reported that a low-intensity 2-h NI was effective at inhibiting the flowering of chrysanthemum 'White Reagen' and 'Majoor Bosshardt' (*Chrysanthemum* × *morifolium* Kitamura) by 28 and 30 d, respectively. Furthermore, Park and Jeong (2019) demonstrated the efficacy of a 16-h photoperiod and 4-h NI of various light qualities at inhibiting the flowering of chrysanthemum 'Gaya Yellow' for the duration of the study (46 d) when applied at intensities of 180 and 10 $\mu mol \cdot m^{-2} \cdot s^{-1}$, respectively, whereas SD conditions promoted flower bud initiation after 21 d. In addition, LDs and NIs provided by red (R; 600–700 nm), white (W; 400–700 nm), and far-red (FR; 700–800 nm) radiation resulted in crops that were 6–8 cm taller than those grown under SDs (Park and Jeong, 2019). In a separate study, pinched celosia 'Rocket' (*Celosia argentea* var. *plumosa*) grown under a 16-h photoperiod for 3 weeks, and then an 8-h photoperiod for 29 d, had four times as many stems per plant and were ≈183% taller than pinched plants grown under continuous SDs for 50 d (Porat et al., 1995).

In addition to regulating photoperiod, growers must maintain sufficient radiation intensities through the use of supplemental lighting (SL) when solar radiation intensities are low (Wollaeger and Runkle, 2014) to consistently produce high-quality cut flowers. This is especially important in northern latitudes as the outdoor daily light integral (DLI) can fall to 5 to 10 $mol \cdot m^{-2} \cdot d^{-1}$ during the winter and early spring (Korczynski et al., 2002), and can drop further to < 5 $mol \cdot m^{-2} \cdot d^{-1}$ in greenhouses due to reflection of incoming radiation from greenhouse glazing and shading from the greenhouse superstructure (Lopez and Runkle, 2008). Increasing the DLI with SL to produce greenhouse crops other than specialty cut

flowers is well-documented. Generally, a moderate to high DLI (e.g., 8 to 12 mol·m^{-2}·d^{-1}) during the young-plant and finishing stages elicits favorable growth responses, including a reduction in time to flower and an increase in biomass and finished plant quality (Faust et al., 2005; Pramuk and Runkle, 2005; Owen et al., 2018). Research documenting the use of SL to increase the DLI during specialty cut flower production is limited. By reducing the time to flower, growers gain the potential for more production cycles per season, and thus, the potential for increased annual income. For instance, godetia (*Clarkia amoena*) 'Satin White,' 'Salmon,' 'Rose Pink,' and 'Red' flowered ≈41, 94, 98, and 114 d faster, respectively, when grown under SL providing 79 μmol·m^{-2}·s^{-1} from 1800 to 2400 h in comparison to those grown without SL in autumn (Anderson, 1993). Although the finished stem length of godetia was 19% to 33% shorter when grown under SL compared to those grown without SL (Anderson, 1993), the finished stems were still of sufficient length for sale. Similarly, time to flower and height of oriental lily (*Lilium* spp.) 'Laura Lee' were reduced by an average of 21 d and 20%, respectively, when grown under SL providing 60 μmol·m^{-2}·s^{-1} for 5 h per day, compared to those grown without SL (Treder, 2003).

High DLIs also have the potential to increase harvestable cut flower yields (Dole and Warner, 2017). Stem yield of gerbera 'Estelle' and 'Ximena' (*Gerbera* × *cantabrigensis*) increased by 13 and 10 stems, respectively, when grown under a DLI of 6.5 mol·m^{-2}·d^{-1} in comparison to 3.2 mol·m^{-2}·d^{-1} (Autio, 2000). The stem yield of gerbera 'Panama' increased by 40% as the DLI increased from 5.3 to 11.3 mol·m^{-2}·d^{-1} (Llewellyn et al., 2020). Increased cut flower yield under higher DLIs is partly due to increased branching for some varieties. Lim et al. (2022) reported that mountain spike speedwell (*Veronica rotunda* var. *subintegra*) and long-leaf spike speedwell (*Veronica longifolia*) had 331% and 308% more branches when grown under a DLI of 18.3 mol·m^{-2}·d^{-1} compared to 6.6 mol·m^{-2}·d^{-1} for 12 weeks.

Additional research quantifying the influence of photoperiod and DLI on the growth and development of greenhouse-grown specialty cut flowers is needed for growers in northern latitudes. Therefore, the objectives of this study were to (1) determine how various photoperiods during the young-plant and finishing stages interact to influence floral initiation and morphology of witchgrass 'Frosted Explosion' (*Panicum capillare*) and marigold 'Xochi' (*Tagetes erecta*) and (2) quantify how DLI influences floral initiation and morphology of witchgrass during the finishing stage. We hypothesized that both witchgrass and marigold would exhibit a facultative SD response, characterized by delayed flowering and longer stem lengths as the young-plant (seedling stage) and finishing (remainder of the crop cycle after transplant) photoperiods increased. We also hypothesized that witchgrass grown under a moderate DLI would be of higher quality, although shorter, compared to those grown under a very low DLI.

Materials and methods

Young plant material, culture, and lighting treatments

Multi-seed pellets of witchgrass 'Frosted Explosion' (PanAmerican Seed, West Chicago, IL, United States) and seeds of marigold 'Xochi' (PanAmerican Seed) were sown in 288-cell (7 mL individual volume) trays by a commercial propagator (Raker-Roberta's Young Plants, Litchfield, MI, United States). These genera and varieties were selected as they were recent introductions with reports of premature flowering. Nine plug trays of witchgrass and 10 plug trays of marigold were received on 15 September 2020 (Rep. 1) and 8 September 2021 (Rep. 2), 1 day after sowing. Each tray was divided into two blocks of 144 cells. The blocks were randomly and equally distributed in a greenhouse at the Michigan State University (East Lansing, MI; lat. 43°N) under various photoperiodic treatments. Photoperiodic treatments consisted of a 9-h SD (0800 to 1700 h) or a 9-h SD extended with four R + W + FR light-emitting diode (LED) lamps (Arize Greenhouse Pro; General Electric, Boston, MA, United States) on each bench to create 9-, 11- (marigold only), 12-, 13-, 14-, 15-, 16-, 18-, or 24-h photoperiods or a 4-h NI from 2200 to 0200 h. Each LED lamp was covered with multiple layers of aluminum wire mesh (General purpose aluminum; New York Wire, Grand Island, NY, United States) to achieve an average total photon flux density (TPFD) of 2 to 3 μmol·m^{-2}·s^{-1} between 400 and 800 nm. The 100-nm waveband ratios (%) emitted by the LED lamps, defined by their B (400–500 nm), green (G; 500–600 nm), R, and FR photon flux densities (PFDs), were 6:19:45:30.

Young plant greenhouse environment

Young plants were grown in a glass-glazed greenhouse with exhaust fans, evaporative-pad cooling, radiant hot-water heating, and SL controlled by an environmental control system (Priva Integro 725; Priva North America, Vineland Station, ON, Canada). High-intensity LED fixtures (Philips GP-TOPlight DRW-MB; Koninklijke Philips N.V., Eindhoven, Netherlands) provided a supplemental photosynthetic photon flux density (PPFD) of 120 ± 10 μmol·m^{-2}·s^{-1} [as measured with a quantum sensor (LI-190R; LI-COR Biosciences, Lincoln, NE, United States)] when the ambient PPFD dropped below ≈400 μmol·m^{-2}·s^{-1} between 0800 and 1700 h. On each bench, a line quantum sensor (LI-191R, LI-COR, Lincoln, NE, United States) or a quantum sensor (LI-190R, LI-COR, Lincoln, NE, United States) positioned horizontally at plant height measured PPFD every 10 s and a datalogger (CR1000; Campbell Scientific, Logan, UT, United States) recorded hourly averages. The actual DLIs during the young-plant stages of the two replications of the experiment were 10.3 to 11.7 mol·m^{-2}·d^{-1}

(Table 1). The 100-nm waveband ratios (%) emitted by the LED fixtures, defined by their B, G, and R photon flux densities, were 10:5:85.

The greenhouse air average daily temperature (ADT) set point was 20°C (12 h day/12 h night at 22/18°C), with daytime and nighttime temperatures maintained from 0500 to 1700 h and 1700 to 0500 h, respectively. An aspirated thermocouple [36-gauge (0.127 mm diameter) type E, Omega Engineering, Stamford, CT] positioned in the middle of each bench measured the air temperature at plant height every 10 s, and the data logger recorded hourly means. The data logger controlled a 1,500-W electric heater underneath each bench to provide supplemental heat when the nighttime temperature was < 19.8°C. The actual air ADT and average day and night temperature at plant height of each treatment during the young-plant stages are provided in Table 1.

Young plants were irrigated as needed with MSU Plug Special [13N-2.2P-10.8K water-soluble fertilizer containing ($mg \cdot L^{-1}$) 61 nitrogen, 10 phosphorus, 50 potassium, 28.1 calcium, 4.7 magnesium, 1.3 iron, 0.6 manganese, 0.6 zinc, 0.6 copper, 0.4 boron, and 0.1 molybdenum; (GreenCare Fertilizers Inc., Kankakee, IL, United States)] blended with reverse-osmosis water and applied with a mist nozzle (Super Fine Fogg-It Nozzle; Fogg-It Nozzle Co., Inc., Belmont, CA, United States).

Finished plant lighting treatments, greenhouse environment, and culture

The same high-intensity LED fixtures described above provided a supplemental PPFD of 120 ± 10 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (as measured with a quantum sensor) from 0800 to 1700 h. Additionally, a combination of whitewash applied to the exterior of the greenhouse (KoolRay Classic Liquid Shade, Continental Products, Euclid, OH, United States) and shade cloth surrounding benches (Harmony 5120 OE, Ludvig Svensson Inc., Charlotte, NC, United States) was utilized to create DLIs of ≈3 (very low) and ≈10 $mol \cdot m^{-2} \cdot d^{-1}$ (moderate). The actual DLIs on each bench during the finishing stages of the two replications of the experiment were calculated and are provided in Tables 2, 3. For both witchgrass and marigold, photoperiods of 10-, 11-, 12-, 13-, 14-, 15-, or 16-h, or a 9-h SD with a 4-h NI from 2200 to 0200 h, were maintained with the same methods and equipment described in the section "young plant greenhouse environment". Greenhouse temperature set points during the finishing stage were identical to those in the young-plant stage. The actual air ADT and average day and night temperature at plant height of each treatment during the finishing stages are provided in Tables 2, 3.

A total of 160 bulb crates (39.3 cm wide × 59.7 cm long × 17.8 cm tall; 0.23 m^2) were filled with a soilless medium containing (by volume) 70% peat moss, 21% perlite, and 9% vermiculite (Suremix; Michigan Grower Products Inc., Galesburg, MI, United States). After 14 and 19 d under young-plant photoperiods for the first rep. (29 September 2020) and second rep. (27 September 2021), respectively, 160 witchgrass young plants were randomly selected for transplant from each treatment: 9-, 12-, 13-, 14-, 16-, 18-, and 24-h photoperiods or the 4-h NI (1,280 young plants total). For marigold, 80 young plants were randomly selected for transplant from each treatment: 11-, 13-, 14-, 15-, 16-, and 24-h photoperiods or the 4-h NI (560 young plants total). Eight bulb crates designated for witchgrass seedlings were placed under each photoperiod under both the very low and moderate DLI treatments, and four bulb crates designated for marigold seedlings were placed under each photoperiod under the moderate DLI treatment. Each of the eight or four bulb crates was divided into two or four sections, respectively, yielding 32 sections total per bench (16 sections each for witchgrass and marigold). Five witchgrass or marigold seedlings from one of the aforementioned young-plant treatments were transplanted into a block at a density of 43 or 97 plants per m^2, respectively. This was repeated randomly across the sections until 10 seedlings from each aforementioned witchgrass or marigold young-plant treatment were transplanted per bench.

One layer of 15 cm supportive netting (HGN32804; Hydrofarm, Petaluma, CA, United States) was positioned ≈15 cm above the bulb crates on each bench. Plants were irrigated as needed with MSU Orchid RO Special [13N-1.3P-12.3K water-soluble fertilizer containing ($mg \cdot L^{-1}$) 125 nitrogen, 13 phosphorus, 121 potassium, 76 calcium, 19 magnesium, 1.7 iron, 0.4 copper and zinc, 0.9 manganese, 0.2 boron, and 0.2 molybdenum; (GreenCare Fertilizers Inc.)] blended with reverse-osmosis water.

Data collection and analysis

Ten randomly selected young plants from each treatment were monitored daily for the presence of the first visible flower bud (VB). After 14 or 19 d for reps. 1 and 2, respectively, fully-expanded leaf number, node number, and height from the bottom of the media to the tallest point of the seedling were recorded for these young plants. Additionally, root dry mass (RDM) and shoot dry mass (SDM) were assessed after gently rinsing media from the roots and drying the plant material in an oven for a minimum of 3 d at 70°C.

During the finishing stage, the individual stems of each witchgrass plug were monitored daily for the presence of VB. On this date, the node number below the first VB was recorded. Individual stems of each witchgrass seedling were also monitored daily for the presence of the first open flower (OF) and the date was recorded. On the date of OF, stem length from the media to the tallest point of the most developed stem, branch number, and stem caliper at the thickest point of the stem was recorded with a digital caliper (3-inch carbon fiber

digital caliper, General Tools and Instruments, LLC, New York, NY, United States). For witchgrass, the date of harvest (indicated by plants becoming ≥ 50 cm tall with a fully developed panicle) was recorded for the most developed plant in each plug, and for marigold, the date of harvest (indicated by plants becoming ≥ 65 cm tall and terminal blossom 50% open) was recorded for each plant. On the date of harvest, stem length from the media to the tallest point of the inflorescence, stem caliper at the thickest point of the stem, branch number, and the total number of initiated inflorescences were recorded for marigold. Data were analyzed using SAS (version 9.4; SAS Institute, Cary, NC, United States) mixed model procedure (PROC MIXED) for analysis of variance (ANOVA), and means were separated by Tukey's honest significant difference (HSD) test at $P \leq 0.05$.

Results

Young plant morphology and dry mass

Neither witchgrass nor marigold young plant node or leaf number were influenced by photoperiod (data not reported). Additionally, no plants initiated VBs during the young-plant stage. However, as the young-plant photoperiod increased from 9 to 16 h, the height of witchgrass and marigold increased by 0.8 cm and 1.9 cm, respectively, and then decreased over 16 h (Figures 1A,D). As the photoperiod increased from 9 to 18 h for witchgrass and 10 to 16 h for marigold, RDM increased by up to 14% and 52%, respectively. However, as the photoperiod increased to 24 h, RDM decreased by 9% and 22% for witchgrass and marigold, respectively (Figures 1B,E). The SDM of witchgrass decreased by 41% as the photoperiod increased from 9 to 24 h. In contrast, as the photoperiod increased from 9 to 16 h, the SDM of the marigold increased by up to 32%, after which the SDM decreased by 13% as the photoperiod increased to 24 h (Figures 1C,F).

Time to visible flower bud

The young-plant and finishing photoperiods interacted to control the time to VB (TVB) of witchgrass ($P < 0.0001$). TVB increased quadratically by 18 or 14 d, for rep. 1 and 2, respectively, when the young-plant photoperiod increased from 9 to 24 h and plants were finished under a 10-h photoperiod and a moderate DLI (Figures 2A,C). This relationship was further accentuated under a longer finishing photoperiod; TVB increased quadratically by an average of 38 d as the young-plant photoperiod increased from 9 to 24 h under a 16-h finishing

TABLE 1 Actual daily light integral (DLI) [mean ± SD ($mol \cdot m^{-2} \cdot d^{-1}$)], air average daily temperature (ADT), day temperature, and night temperature [mean ± SD (°C)] throughout the duration of the witchgrass and marigold young-plant stage for reps. 1 and 2.

Photoperiod (h)	DLI ($mol \cdot m^{-2} \cdot d^{-1}$)	ADT (°C)	Day (°C)	Night (°C)
Rep. 1				
9	–[z]	20.1 ± 1.0	21.6 ± 2.5	18.6 ± 2.5
11	–[z]	20.1 ± 1.0	21.6 ± 2.5	18.6 ± 2.5
12	–[z]	–[z]	–[z]	–[z]
13	–[z]	–[z]	–[z]	–[z]
14	10.6 ± 3.6	21.5 ± 1.0	22.8 ± 2.1	20.1 ± 2.0
15	11.1 ± 1.9	–[z]	–[z]	–[z]
16	10.9 ± 3.7	21.4 ± 1.1	23.1 ± 3.0	19.7 ± 3.1
18	10.5 ± 3.6	21.2 ± 0.9	22.8 ± 2.7	19.5 ± 2.8
24	10.7 ± 3.8	22.0 ± 1.3	23.7 ± 3.9	20.3 ± 4.3
4-h NI	10.3 ± 3.2	21.0 ± 1.0	22.0 ± 1.8	19.9 ± 1.5
Rep. 2				
9	10.5 ± 5.4	21.1 ± 2.0	21.5 ± 4.1	20.6 ± 3.5
11	–[z]	20.4 ± 2.1	21.0 ± 4.1	19.7 ± 3.1
12	11.3 ± 6.2	20.6 ± 2.1	21.3 ± 4.3	19.8 ± 3.1
13	10.6 ± 4.9	20.5 ± 2.3	21.0 ± 4.1	19.8 ± 3.2
14	–[z]	20.4 ± 2.2	20.9 ± 4.0	19.8 ± 3.1
15	10.4 ± 6.7	20.9 ± 2.5	21.4 ± 4.4	20.4 ± 3.7
16	11.7 ± 6.4	20.4 ± 2.3	20.9 ± 4.1	19.8 ± 3.2
18	10.8 ± 5.6	19.8 ± 2.1	19.8 ± 3.5	19.9 ± 3.3
24	10.7 ± 5.9	20.5 ± 2.0	20.8 ± 3.7	20.2 ± 3.2
4-h NI	11.6 ± 5.9	21.1 ± 2.1	22.0 ± 5.0	20.1 ± 3.3

[z]No data recorded.

TABLE 2 Actual DLIs [mean ± SD (mol·m^{-2}·d^{-1})], air ADTs, mean day temperature, and mean night temperature [mean ± SD (°C)] throughout the duration of the witchgrass finishing stage for reps. 1 and 2.

	Moderate DLI				Very low DLI			
Photoperiod (h)	**DLI (mol·m^{-2}·d^{-1})**	**ADT (°C)**	**Day (°C)**	**Night (°C)**	**DLI (mol·m^{-2}·d^{-1})**	**ADT (°C)**	**Day (°C)**	**Night (°C)**
Rep. 1								
10	10.0 ± 4.8	19.7 ± 1.4	22.1 ± 3.3	17.2 ± 3.2	2.9 ± 1.0	20.3 ± 1.2	22.3 ± 2.4	18.3 ± 2.4
11	–[z]	19.9 ± 1.2	22.0 ± 2.7	17.9 ± 2.5	2.7 ± 1.0	20.6 ± 0.9	22.7 ± 2.3	18.5 ± 2.1
12	10.1 ± 4.8	20.9 ± 1.2	23.3 ± 3.0	18.5 ± 2.9	2.7 ± 0.9	20.3 ± 1.1	22.1 ± 2.2	18.5 ± 2.2
13	–[z]	–[z]	–[z]	–[z]	2.9 ± 0.9	20.4 ± 1.0	22.2 ± 2.3	18.5 ± 2.1
14	10.6 ± 4.9	20.8 ± 1.3	23.0 ± 3.2	18.6 ± 2.5	–[z]	19.9 ± 1.3	21.9 ± 2.6	17.9 ± 2.7
15	11.6 ± 4.7	–[z]	–[z]	–[z]	3.1 ± 0.9	20.7 ± 0.9	22.6 ± 2.6	18.8 ± 2.2
16	10.8 ± 4.4	20.3 ± 1.0	22.2 ± 2.8	18.4 ± 2.4	2.9 ± 1.1	20.6 ± 1.0	22.6 ± 2.2	18.6 ± 2.0
4-h NI	10.3 ± 3.9	20.4 ± 0.9	21.8 ± 2.2	19.0 ± 1.7	–[z]	20.3 ± 1.4	22.4 ± 2.6	18.1 ± 2.6
Rep. 2								
10	10.0 ± 3.5	20.1 ± 1.8	22.2 ± 3.1	18.0 ± 3.2	3.0 ± 1.6	19.7 ± 1.6	21.8 ± 2.6	17.6 ± 3.2
11	9.8 ± 3.1	20.1 ± 1.3	22.2 ± 2.7	18.0 ± 2.8	3.6 ± 1.8	19.6 ± 1.2	22.0 ± 2.5	17.3 ± 2.7
12	10.0 ± 3.2	20.3 ± 1.1	22.3 ± 2.7	18.4 ± 2.5	3.4 ± 1.8	19.8 ± 1.9	21.9 ± 3.0	17.7 ± 3.2
13	–[z]	19.1 ± 2.0	20.6 ± 3.8	17.1 ± 3.7	2.9 ± 1.8	19.6 ± 1.2	21.9 ± 2.5	17.3 ± 2.7
14	10.1 ± 3.0	19.1 ± 1.9	20.6 ± 3.8	17.3 ± 3.4	3.1 ± 2.0	19.4 ± 1.8	21.6 ± 2.9	17.2 ± 3.5
15	9.8 ± 2.9	20.4 ± 1.6	22.4 ± 2.7	18.4 ± 3.2	3.2 ± 1.7	19.5 ± 1.4	21.6 ± 2.5	17.3 ± 2.9
16	10.2 ± 4.2	–[z]	–[z]	–[z]	2.9 ± 1.4	19.5 ± 1.6	21.6 ± 2.5	17.4 ± 3.2
4-h NI	–[z]	18.9 ± 1.9	20.9 ± 2.9	16.8 ± 3.4	3.6 ± 1.9	19.2 ± 1.4	21.5 ± 2.5	17.0 ± 3.0

[z]No data recorded.

TABLE 3 Actual DLIs [mean ± SD (mol·m^{-2}·d^{-1})], air ADTs, mean day temperature, and mean night temperature [mean ± SD (°C)] throughout the duration of the marigold finishing stage for reps. 1 and 2.

Photoperiod (h)	**DLI (mol·m^{-2}·d^{-1})**	**ADT (°C)**	**Day (°C)**	**Night (°C)**
Rep. 1				
10	10.6 ± 4.2	19.5 ± 1.4	22.1 ± 3.5	17.0 ± 3.3
11	–[z]	20.1 ± 1.3	22.1 ± 2.7	18.0 ± 2.6
12	10.6 ± 4.8	21.0 ± 1.2	23.4 ± 3.0	18.7 ± 2.9
13	–[z]	–[z]	–[z]	–[z]
14	11.0 ± 4.8	21.0 ± 1.3	23.2 ± 3.2	18.8 ± 2.5
15	11.9 ± 4.6	–[z]	–[z]	–[z]
16	11.2 ± 4.4	20.4 ± 1.1	22.3 ± 2.9	18.4 ± 2.5
4-h NI	10.8 ± 3.8	20.5 ± 0.9	21.9 ± 2.2	19.1 ± 1.7
Rep. 2				
10	9.8 ± 2.0	19.8 ± 1.9	22.0 ± 3.3	17.7 ± 3.5
11	9.0 ± 1.7	20.4 ± 1.3	22.3 ± 2.6	18.6 ± 2.7
12	10.0 ± 2.1	20.4 ± 1.2	22.3 ± 2.8	18.6 ± 2.7
13	9.8 ± 1.5	19.3 ± 2.1	20.6 ± 4.1	17.6 ± 4.0
14	10.1 ± 2.6	19.4 ± 2.0	20.5 ± 4.1	17.9 ± 3.6
15	10.0 ± 1.7	20.8 ± 1.5	22.7 ± 2.7	18.9 ± 3.2
16	10.4 ± 2.9	–[z]	–[z]	–[z]
4-h NI	–[z]	19.1 ± 2.2	20.8 ± 3.3	17.2 ± 3.8

[z]No data recorded.

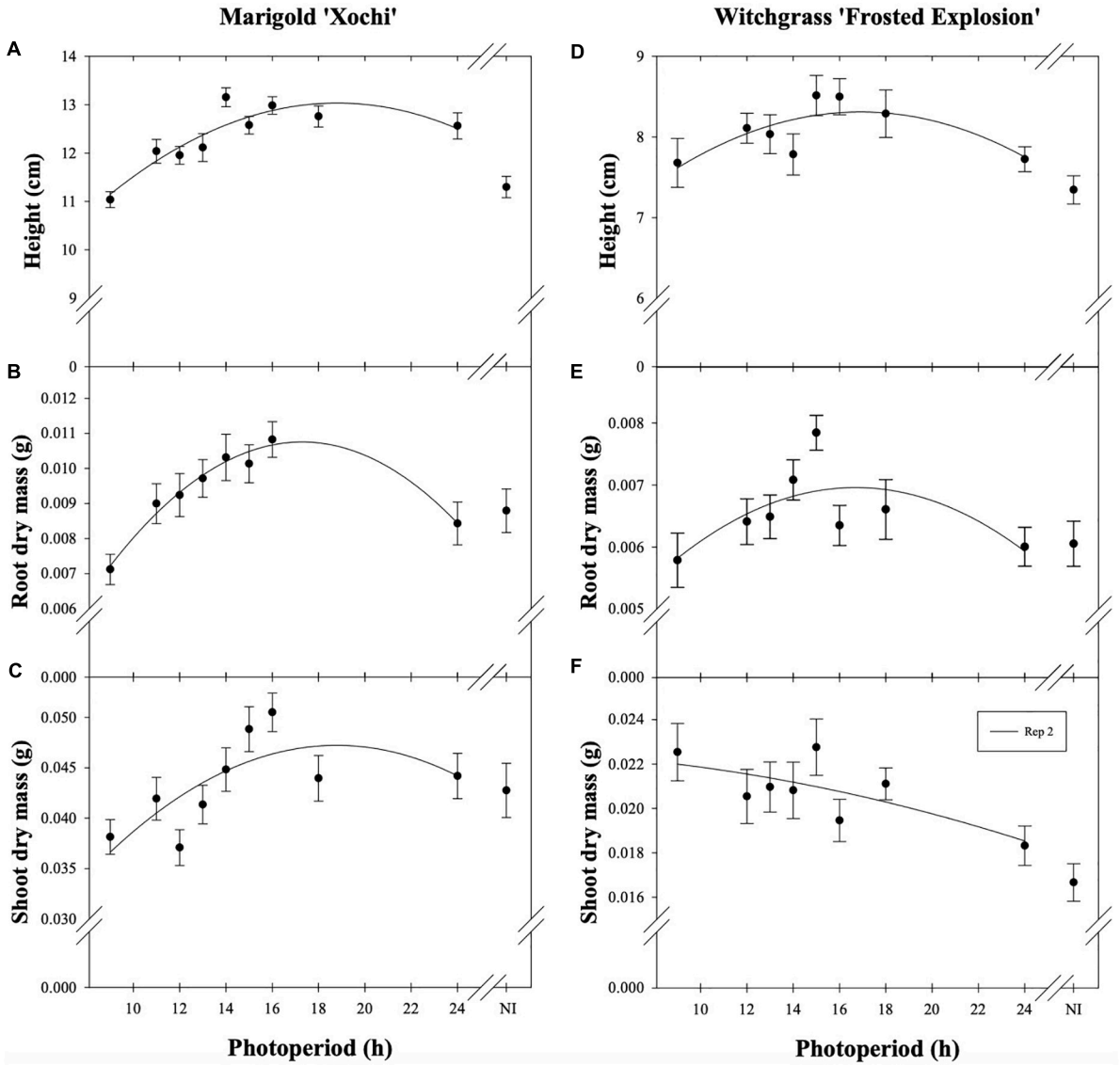

FIGURE 1
Effect of photoperiod [9, 11, 12, 13, 14, 15, 16, 18, and 24 h, or a 4-h night interruption (NI)] on the height **(A,D)**, root dry mass **(B,E)**, and shoot dry mass **(C,F)** of marigold 'Xochi' (*Tagetes erecta*) and witchgrass 'Frosted Explosion' (*Panicum capillare*) young plants. Panel **(F)** presents data from replication 2 as trends from replication 1 were not significant. Coefficients are presented in **Table 7**.

photoperiod. TVB was also influenced by finishing photoperiod, particularly as the young-plant photoperiod increased. For example, TVB of plants grown under a 9-h young-plant photoperiod was delayed by ≈1 d as the finishing photoperiod increased from 10 to 16 h. However, TVB of plants grown under a 24-h young-plant photoperiod was delayed by ≈23 d as the finishing photoperiod increased from 10 to 16 h. Similar trends, although attenuated, were seen for plants finished under a very low DLI (**Figures 2B,C**).

Young-plant and finishing photoperiod interacted to influence TVB of marigold during rep. 1 ($P < 0.0001$). However, young-plant photoperiod did not commercially influence TVB and finishing photoperiod had the dominant effect. For instance, TVB of plants finished under 10-h photoperiods increased by only ≈1 d as the young-plant photoperiod increased from 11 to 24 h (**Figure 3A**). TVB increased by only ≈2 d as the young-plant photoperiod increased from 11 to 24 h when plants were finished under a 16-h photoperiod. In comparison, TVB increased by ≈18 d as the finishing photoperiod increased from 10 to 16 h for plants grown under 11-h young-plant photoperiods. Conversely, young-plant and finishing photoperiod independently influenced TVB of plants grown during the rep. 2 ($P = 0.23$). As the young-plant photoperiod increased from 11 to 24 h, TVB decreased by ≈1 d (**Figure 3B**).

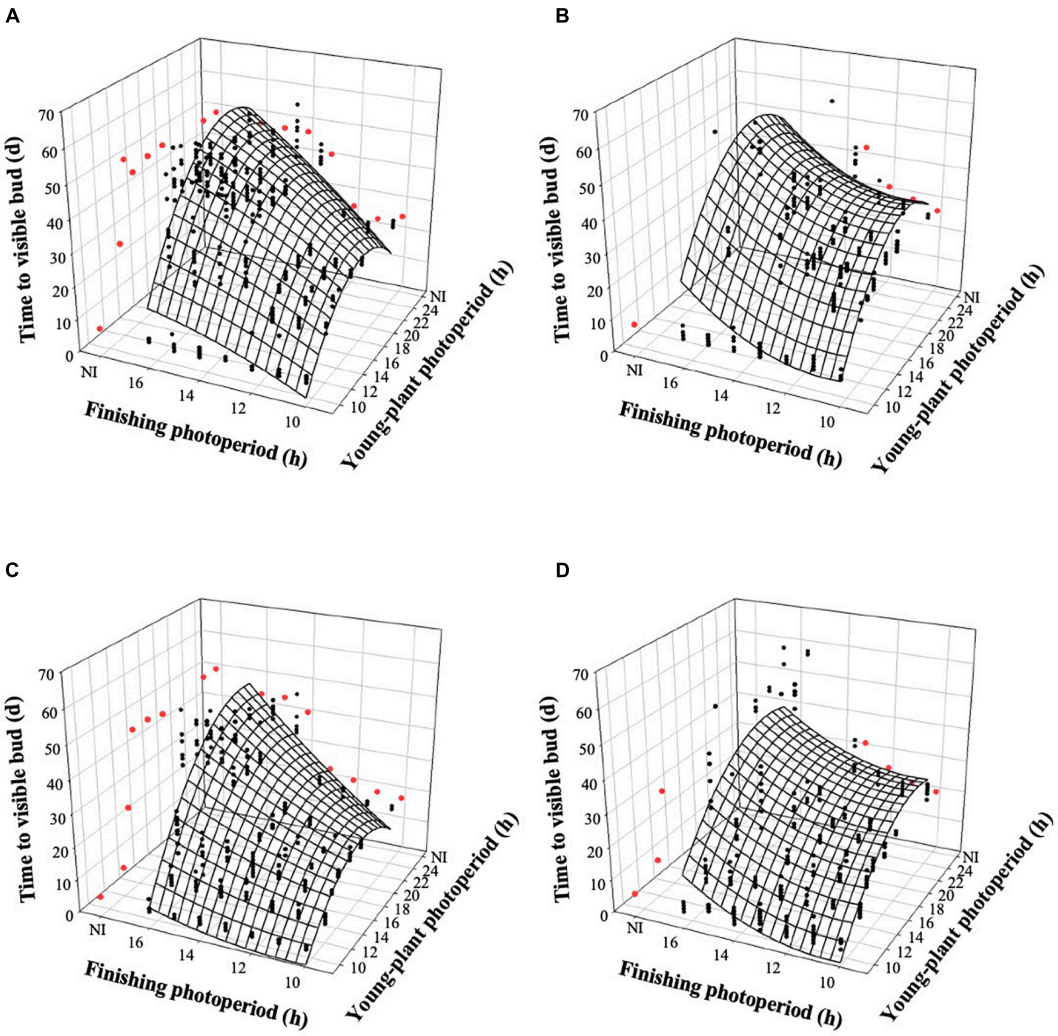

FIGURE 2
Effects of young-plant photoperiod (9, 12, 13, 14, 16, 18, and 24 h, or a 4-h NI) and finishing photoperiod (10, 11, 12, 13, 14, 15, and 16 h, or a 4-h NI) on time to visible bud of witchgrass 'Frosted Explosion' (*Panicum capillare*). Figures represent **(A)** moderate-DLI-grown (≈10 mol·m^{-2}·d^{-1}) cut flowers from replication 1, **(B)** very-low-DLI-grown cut flowers from replication 1, **(C)** moderate-DLI-grown cut flowers from replication 2, and **(D)** very-low-DLI-grown (≈3 mol·m^{-2}·d^{-1}) cut flowers from replication 2. Black circles represent individual data points for sequential photoperiods; red circles represent averages from NI treatments. Model predictions are represented by response surfaces; coefficients are presented in Table 8.

As the finishing photoperiod increased from 10 to 16 h, TVB increased by ≈16 d (Figure 3C).

Node number below the visible bud

Witchgrass seedlings grown under 9- to 12-h or 9- to 13-h photoperiods during reps. 1 and 2, respectively, developed ≈4 nodes below the first VB regardless of finishing photoperiod or DLI. For plants grown under longer young-plant photoperiods, node number increased proportionally with the finishing photoperiod. Plants grown under 13- (rep. 1) or 14-h (rep. 2) young-plant photoperiods had up to ≈2 more nodes below the first VB as the finishing photoperiod increased from 10 to 16 h, or a 4-h NI, under a moderate DLI. A similar trend was observed for very low DLI grown plants (data not reported). Marigold grown under a 10-h finishing photoperiod formed VBs after a minimum of six nodes had developed, and node count increased up to nine nodes as the finishing photoperiod increased to 16 h (data not reported).

Time to open flower of witchgrass

The time to open flower (TOF) of witchgrass was influenced by the interaction between the young-plant and finishing

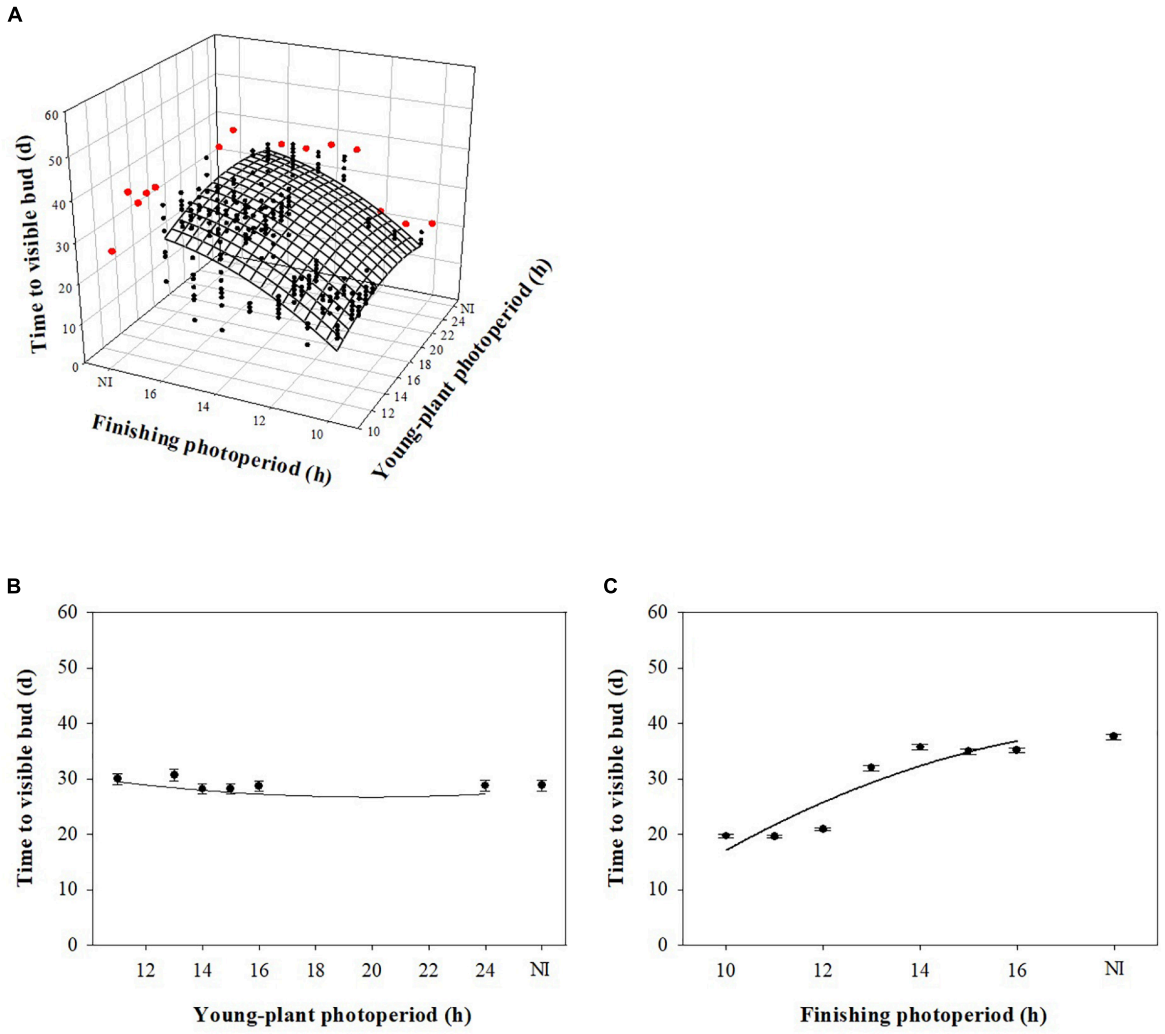

FIGURE 3
Effect of young-plant photoperiod (11, 13, 14, 15, 16, and 24 h, or a 4-h NI) and/or finishing photoperiod (10, 11, 12, 13, 14, 15, and 16 h, or a 4-h NI) on time to visible bud (TVB) of marigold 'Xochi' (*Tagetes erecta*). Figures represent **(A)** the interaction between young-plant and finishing photoperiod on TVB of plants from replication 1, **(B)** the effect of young-plant photoperiod on TVB of plants from replication 2, and **(C)** the effect of finishing photoperiod on TVB of plants from replication 2. In panel **(A)**, black circles represent individual data points for sequential photoperiods; red circles represent averages from NI treatments. Coefficients are presented in Table 9.

photoperiods, following a trend similar to TVB. TOF increased by up to 22 or 15 d, for reps. 1 and 2, respectively, as the young-plant photoperiod increased from 9 to 24 h under a finishing photoperiod of 10 h and a moderate DLI (Figures 4A,C). This effect was stronger under a longer finishing photoperiod. For instance, under a finishing photoperiod of 16 h, TOF increased quadratically by 38 or 34 d, for reps. 1 and 2, respectively, as the young-plant photoperiod increased from 9 to 24 h. The effect of finishing photoperiod on TOF accentuated as the young-plant photoperiod increased. For example, TOF of plants grown under a 12-h young-plant photoperiod was delayed by ≈5 or 1 d as the finishing photoperiod increased from 10 to 16 h for reps. 1 and 2, respectively. However, flowering of plants grown under a 24-h young-plant photoperiod was delayed by ≈9 or 19 d as the finishing photoperiod increased from 10 to 16 h for reps. 1 and 2, respectively. Plants finished under a very low DLI experienced a similar trend, although fewer plants flowered when grown under ≥ 13- (rep. 1) or ≥ 14-h (rep. 2) young-plant photoperiods, or a 4-h NI, and ≥ 13- (rep. 1) or ≥ 14-h (rep. 2) finishing photoperiods or a 4-h NI (Figures 4B,D).

Witchgrass stem length, caliper, and branch number at open flower

The stem length of witchgrass at OF was proportional to the TOF and was influenced by the interaction of young-plant and finishing photoperiods. As the young-plant photoperiod

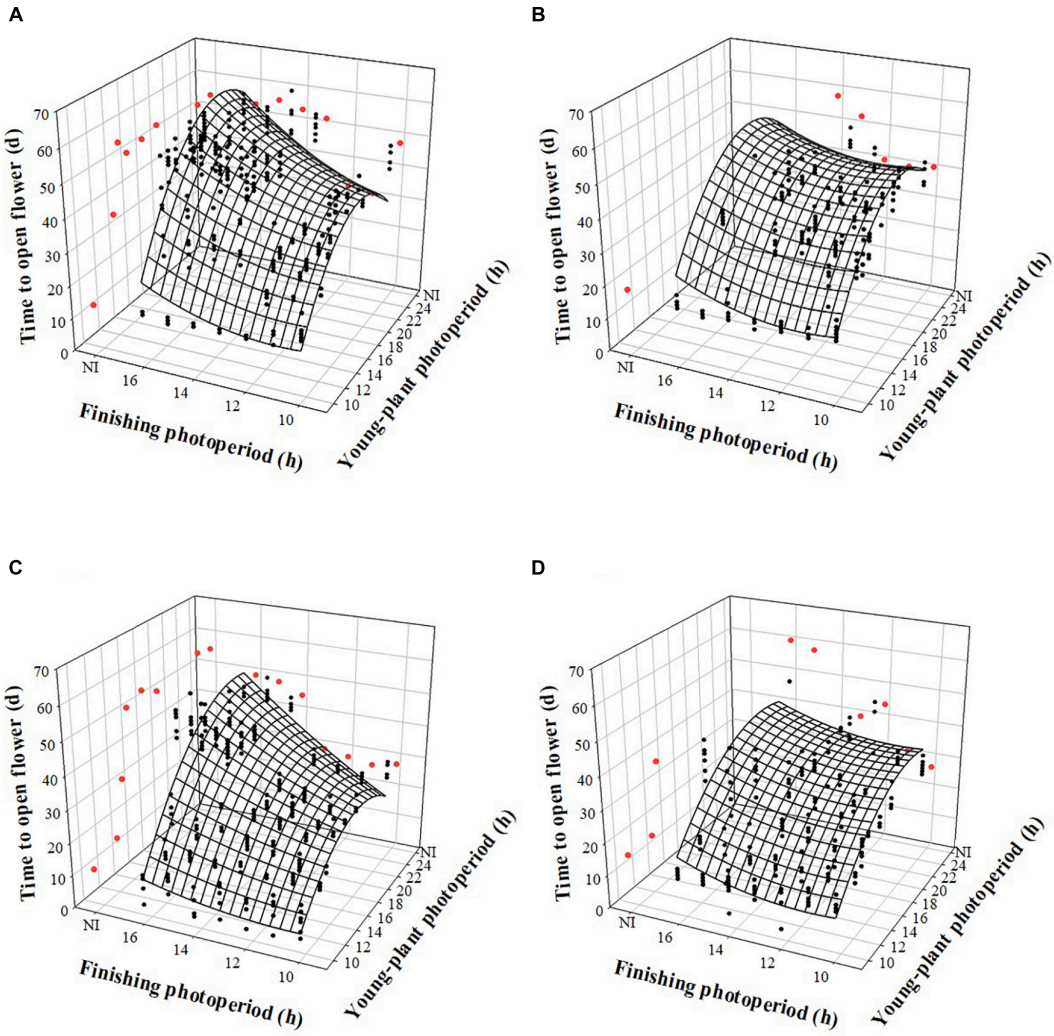

FIGURE 4
Effects of young-plant photoperiod [9, 12, 13, 14, 16, 18, and 24 h, or a 4-h night interruption (NI)] and finishing photoperiod (10, 11, 12, 13, 14, 15, and 16 h, or a 4-h NI) on time to open flower of witchgrass 'Frosted Explosion' (*Panicum capillare*). Figures represent **(A)** moderate-DLI-grown (≈10 mol·m^{-2}·d^{-1}) cut flowers from replication 1, **(B)** very-low-DLI-grown (≈3 mol·m^{-2}·d^{-1}) cut flowers from replication 1, **(C)** moderate-DLI-grown cut flowers from replication 2, and **(D)** very-low-DLI-grown cut flowers from replication 2. Black circles represent individual data points for sequential photoperiods; red circles represent averages from NI treatments. Model predictions are represented by response surfaces; coefficients are presented in Table 8.

increased from 9 to 24 h, under a finishing photoperiod of 10 h, stem length increased by an average of 19.5 and 11.0 cm for reps. 1 and 2, respectively (Figures 5A,C). This effect was strengthened as the finishing photoperiod increased; the stem length of plants finished under a 16-h photoperiod and grown under a young-plant photoperiod of 9 h was 71.1 and 42.0 cm shorter than those grown under a 24-h young-plant photoperiod for reps. 1 and 2, respectively. Furthermore, the stem length of witchgrass increased by 1.0 and 2.0 cm for reps. 1 and 2, respectively, when seedlings were grown under 9 h photoperiods and the finished plant photoperiod increased from 10 to 16 h. Conversely, when seedlings were grown under a 24-h young-plant photoperiod and finished under a 16-h photoperiod, stems were 51.6 or 33.0 cm longer than those finished under a 10-h photoperiod for reps. 1 and 2, respectively. Similar trends were seen for the very-low-DLI-grown plants that reached OF, although stem lengths were shorter than the plants finished under the moderate DLI treatment (Figures 5B,D). None of the plants finished under the very low DLI were long enough or developed enough by the end of the study (≈62 d) to be considered harvestable.

Young-plant and finishing photoperiod interacted to influence stem caliper of witchgrass. The stem caliper of plants grown under a 10-h finishing photoperiod was 0.8 or 1.0 mm thicker for reps. 1 and 2, respectively, as the young-plant photoperiod increased from 9 to 24 h (Table 4). This effect

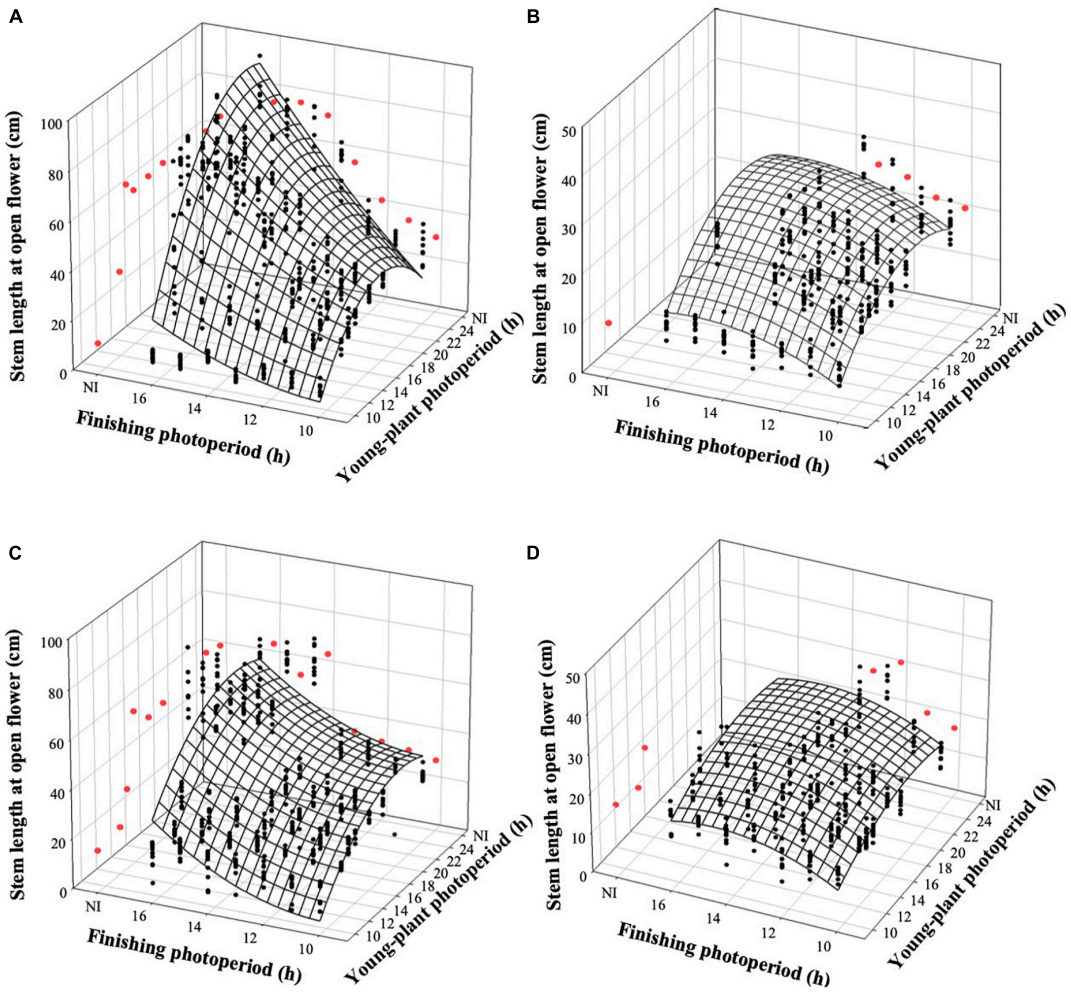

FIGURE 5
Effects of young-plant photoperiod [9, 12, 13, 14, 16, 18, and 24 h, or a 4-h night interruption (NI)] and finishing photoperiod (10, 11, 12, 13, 14, 15, and 16 h, or a 4-h NI) on stem length of witchgrass 'Frosted Explosion' (*Panicum capillare*) at the open flower. Figures represent **(A)** moderate-DLI-grown (≈10 mol·m^{-2}·d^{-1}) cut flowers from replication 1, **(B)** very-low-DLI-grown (≈3 mol·m^{-2}·d^{-1}) cut flowers from replication 1, **(C)** moderate-DLI-grown cut flowers from replication 2, and **(D)** very-low-DLI-grown cut flowers from replication 2. Black circles represent individual data points for sequential photoperiods; red circles represent averages from NI treatments. Model predictions are represented by response surfaces; coefficients are presented in Table 8.

was accentuated as the finishing photoperiod increased; the stem caliper of plants finished under a 16-h photoperiod was 3.5 or 4.0 mm thicker when the young-plant photoperiod was 24 h compared to 9 h. Thicker stem calipers were recorded for plants finished under 16-h photoperiods compared to 10-h photoperiods when young plants were grown under 9-h photoperiods. This effect strengthened as the young-plant photoperiod increased. The stem caliper of plants grown under a 24-h young-plant photoperiod was 2.8 or 3.2 mm greater for reps. 1 and 2, respectively, as the finishing photoperiod increased from 10 to 16 h (Table 4). Similar trends, although attenuated, were seen for the very-low-DLI-grown plants that reached OF. However, stem caliper measurements generally ranged only from 0.4 to 2.3 mm for rep. 1 and from 1.2 to 2.5 mm for rep. 2. Plants grown under a moderate DLI had one to three branches at OF, regardless of young-plant or finishing photoperiod, while those grown under a very low DLI had none to two branches at OF.

Time to harvest

During rep. 1, witchgrass stems were only harvestable when seedlings were grown under a photoperiod ≥ 13 h and finished under a photoperiod ≥ 13 h and a moderate DLI. Plants finished under photoperiods < 13 h flowered prematurely and were unmarketable. Generally, plants grown under 13-h young-plant and finishing photoperiods became harvestable the fastest, whereas those grown under a NI during the young-plant and finishing stages were the slowest

to reach harvest (Table 5). During rep. 2, plants grown under young-plant photoperiods < 14 h and finishing photoperiods < 14 h flowered prematurely. All harvestable plants were harvested within a 10- to 13-d timeframe, depending on reps. (Table 5). Plants finished under a very low DLI did not yield harvestable stems.

Only marigolds grown under 10- to 12-h finishing photoperiods were harvestable by the end of the study (≈50 d). However, up to 29% and 107% more stems were harvested under 11- and 12-h finishing photoperiods, respectively, compared to the 10-h photoperiod (data not reported). Time to harvest of marigold finished under 10-, 11-, and 12-h photoperiods ranged from 40 to 48 d after transplant (Table 6).

Marigold stem length, caliper, branch, and inflorescence number at harvest

Finishing photoperiod had the dominant effect on marigold stem length at harvest, and young-plant and finishing photoperiods did not interact to influence the length of the stems that became harvestable. As the finishing photoperiod increased from 10 to 12 h, stem length at harvest increased from 70 to 74 cm. While plants grown under photoperiods > 12 h were not harvestable at the end of the study, they were at least 65 cm long, regardless of finishing photoperiod (data not reported), indicating the potential for all stems to eventually reach marketability. Stem caliper and branch and inflorescence numbers at harvest of plants grown under finishing photoperiods of 10 to 12 h were not significantly different (data not reported).

Discussion

The results of this study further support that photoperiod manipulation during the young-plant and finishing stages, in addition to maintaining or increasing the DLI, can aid in producing high-quality specialty cut flowers while reducing crop time. Growers can manipulate these environmental parameters to improve finished cut flower quality and reduce the time to harvest. These techniques are particularly useful when the natural photoperiod is not conducive to the photoperiodic responses of the crop to be grown, or when solar radiation is limiting.

When grown under inductive conditions, specialty cut flowers can flower prematurely with unmarketable stem lengths (Dole and Warner, 2017). Witchgrass demonstrated this phenomenon, which is consistent with several other publications on photoperiodic lighting of SDPs. Plants in this study grown under 9- to 12-h (rep. 1) or 9- to 13-h (rep. 2) young-plant photoperiods flowered prematurely, regardless of finishing photoperiod. Premature flowering was also seen for witchgrass finished under 10- to 12-h (rep. 1) or 10- to 13-h (rep. 2) finishing photoperiods, regardless of young-plant photoperiod. These findings indicate that the critical photoperiod of witchgrass 'Frosted Explosion' is 12 to 13 h. During rep. 2, the stem length of plants grown under photoperiods ≤ 13 h were only 10 to 32 cm long at OF, which is below the market minimum of 50 cm (BloomStudios, 2020, personal communication). Similarly, marigolds finished under inductive photoperiods were shorter than those under non-inductive photoperiods, although all treatments would have yielded marketable stem lengths upon flowering.

Jensen et al. (2012) reported similar trends to witchgrass after investigating the photoperiodic response of Amur silvergrass (*Miscanthus sacchariflorus*), a grass used as a biofuel. The flowering of *Miscanthus* was delayed by 83 d under LDs (15.3-h photoperiods) compared to gradually decreasing SDs (15.3-h photoperiods for 21 d, followed by 119 d of a decreasing photoperiod consistent with that at 34.1°N), designating it as a facultative SDP. Plants grown under LDs accumulated ≈52% more biomass (stem and leaf tissue) compared to plants grown under SDs, aligning with the witchgrass stem caliper increase in the present study. The authors hypothesized that stem length under LDs would likely have been longer than those grown under SDs if their experiment ran longer, as *Miscanthus* exhibits rapid stem elongation during the emergence of flag leaves, which is ≈18 d after floral initiation (Jensen et al., 2012). However, the experiment was terminated before LD-mediated plant elongation would have occurred. During rep. 2 of the present study, witchgrass finished under a 14-h LD were up to 224% longer at OF than plants finished under a 13-h SD, suggesting a similar stem elongation response.

In the present study, witchgrass may have a similar sensitivity to inductive photoperiods as celosia (*Celosia argentea* var. *plumosa*) during the young-plant stage. Warner (2009) reported that the SDP celosia 'Gloria Scarlet' overcame juvenility and perceived inductive treatments 9 to 12 d after cotyledon emergence. Plants were exposed to 12 9-h SDs before cotyledon emergence, then placed under a 4-h NI from 2200 to 0200 h, and had ≈7 fewer nodes below the terminal inflorescence than plants grown under continuous LDs. Moreover, plants exposed to 12 SDs at the beginning 3 d after cotyledon emergence had a similar node number below the terminal inflorescence compared to those under continual SDs (Warner, 2009). This could explain why plants grown under inductive conditions during the young-plant stage flowered prematurely, even when transferred to non-inductive finishing conditions. Further experimentation to determine when witchgrass begins reproductive development may be necessary. However, growers should avoid premature flower-inductive conditions to ensure proper market specifications are met.

TABLE 4 Effects of young-plant photoperiod (9, 12, 13, 14, 16, 18, and 24 h, or a 4-h NI) and finishing photoperiod (10, 11, 12, 13, 14, 15, and 16 h, or a 4-h NI) on stem caliper (mm) of witchgrass 'Frosted Explosion' (*Panicum capillare*) at the open flower.

	Young-plant photoperiod (h)							
Finishing photoperiod (h)	**9**	**12**	**13**	**14**	**16**	**18**	**24**	**NI**
Rep. 1								
10	0.61	1.05	1.31	1.17	1.68	1.41	1.43	1.41
11	0.69	0.97	2.00	2.06	2.35	2.35	2.41	2.08
12	0.74	1.61	2.36	2.26	2.58	2.50	2.61	2.70
13	0.58	2.66	3.77	4.10	3.59	4.01	4.11	3.64
14	0.82	3.06	3.96	4.62	4.30	4.19	3.73	4.05
15	0.63	3.22	3.98	4.61	3.91	4.35	4.43	3.94
16	0.67	3.59	4.45	4.40	4.12	4.38	4.25	3.89
NI	0.88	3.37	3.89	4.38	3.96	3.93	4.23	4.04
Rep. 2								
10	1.42	1.74	2.34	2.31	2.33	2.63	2.39	2.39
11	1.53	1.82	2.40	2.54	2.61	2.66	2.52	2.53
12	1.65	1.74	2.43	2.26	2.80	2.78	2.68	2.46
13	1.53	1.89	2.58	3.16	3.25	3.47	3.46	3.46
14	1.60	1.88	3.37	5.30	5.61	5.10	5.29	4.88
15	1.64	1.97	3.20	4.83	5.56	5.18	5.41	5.17
16	1.56	1.95	3.63	5.75	5.75	5.91	5.61	5.40
NI	1.73	1.87	3.86	5.28	5.91	5.41	5.62	5.28

Cut flowers were finished under a moderate DLI of ≈ 10 $mol \cdot m^{-2} \cdot d^{-1}$.

TABLE 5 Effects of young-plant photoperiod (9, 12, 13, 14, 16, 18, and 24 h, or a 4-h NI) and finishing photoperiod (10, 11, 12, 13, 14, 15, and 16 h, or a 4-h NI) on time to harvest (d) from the date of transplant of witchgrass 'Frosted Explosion' (*Panicum capillare*) grown under a moderate DLI of ≈ 10 $mol \cdot m^{-2} \cdot d^{-1}$.

	Young-plant photoperiod (h)							
Finishing photoperiod (h)	**9**	**12**	**13**	**14**	**16**	**18**	**24**	**NI**
Rep. 1								
10	–[z]	–[z]	–[z]	–[z]	–[z]	–[z]	–[z]	–[z]
11	–[z]	–[z]	–[z]	–[z]	–[z]	–[z]	–[z]	–[z]
12	–[z]	–[z]	–[z]	–[z]	–[z]	–[z]	–[z]	–[z]
13	–[z]	–[z]	53	53	53	51	53	54
14	–[z]	–[z]	54	53	53	54	56	56
15	–[z]	–[z]	58	57	56	58	53	55
16	–[z]	–[z]	55	55	57	58	58	55
NI	–[z]	–[z]	60	55	55	56	56	58
Rep. 2								
10	–[z]	–[z]	–[z]	–[z]	–[z]	–[z]	–[z]	–[z]
11	–[z]	–[z]	–[z]	–[z]	–[z]	–[z]	–[z]	–[z]
12	–[z]	–[z]	–[z]	–[z]	–[z]	–[z]	–[z]	–[z]
13	–[z]	–[z]	–[z]	–[z]	–[z]	–[z]	–[z]	–[z]
14	–[z]	–[z]	–[z]	49	48	47	47	48
15	–[z]	–[z]	–[z]	51	49	53	51	51
16	–[z]	–[z]	–[z]	52	50	48	48	51
NI	–[z]	–[z]	–[z]	60	58	57	57	60

[z]No harvestable stems by the end of study.

TABLE 6 Effects of young-plant photoperiod (11, 13, 14, 15, 16, or 24 h, or a 4-h NI) and finishing photoperiod (10, 11, 12, 13, 14, 15, and 16 h, or a 4-h NI) on time to harvest (d) of marigold 'Xochi' (*Tagetes erecta*).

	Young-plant photoperiod (h)						
Finishing photoperiod (h)	**11**	**13**	**14**	**15**	**16**	**24**	**NI**
Rep. 1							
10	46	48	43	47	44	44	44
11	41	43	43	44	43	45	40
12	43	46	42	43	46	46	45
13	–[z]	–[z]	–[z]	–[z]	–[z]	–[z]	–[z]
14	–[z]	–[z]	–[z]	–[z]	–[z]	–[z]	–[z]
15	–[z]	–[z]	–[z]	–[z]	–[z]	–[z]	–[z]
16	–[z]	–[z]	–[z]	–[z]	–[z]	–[z]	–[z]
NI	–[z]	–[z]	–[z]	–[z]	–[z]	–[z]	–[z]
Rep. 2							
10	45	44	42	45	42	43	43
11	42	41	41	42	44	44	42
12	44	44	41	41	42	42	41
13	–[z]	–[z]	–[z]	–[z]	–[z]	–[z]	–[z]
14	–[z]	–[z]	–[z]	–[z]	–[z]	–[z]	–[z]
15	–[z]	–[z]	–[z]	–[z]	–[z]	–[z]	–[z]
16	–[z]	–[z]	–[z]	–[z]	–[z]	–[z]	–[z]
NI	–[z]	–[z]	–[z]	–[z]	–[z]	–[z]	–[z]

[z]No harvestable stems by the end of study.

TABLE 7 Regression analysis equations and r^2 or R^2 for height, root dry mass, and shoot dry mass in response to photoperiod (P; 9-, 11-, 12-, 13-, 14-, 15-, 16-, 18-, 24-h photoperiods or a 4-h NI) of marigold 'Xochi' (*Tagetes erecta*) or witchgrass 'Frosted Explosion' (*Panicum capillare*).

Parameter	**y0**	**a**	**b**	**R^2 or r^2**
	Marigold 'Xochi'			
Height (cm)	6.09[z]	0.74	−0.02	0.240
Root dry mass (g)	−0.00	0.00	−5.14E-05	0.174
Shoot dry mass (g)	−0.01	0.00	0.00	0.102
	Witchgrass 'Frosted Explosion'			
Height (cm)	5.14	0.38	−0.01	0.049
Root dry mass (g)	0.00	0.00	−1.92E-05	0.070
Shoot dry mass (g)	0.02	−5.27E-06	-6.83E-06	0.075

[z]Coefficients for model equations were used to generate Figure 1A through Figure 1F.
All models are in the form of: $f = y0 + a^*P + b^*P^2$.

TVB of marigold was negligibly influenced by young-plant photoperiod, suggesting marigold was not induced to flower during the first 2 weeks of growth. This is inconsistent with Warner (2006), who identified the photoperiod-sensitive stages of the SDPs cosmos 'Sonata White' (*Cosmos bipinnatus*) and signet marigold 'Tangerine Gem' (*Tagetes tenuifolia*). It was reported that both species were receptive to inductive conditions after 1 to 2 leaf pairs had unfolded, with five 9-h SDs delivered after cotyledon emergence promoting flowering of cosmos by 23 d compared to a constant 4-h NI treatment. Furthermore, marigolds exposed to 5 SDs after cotyledon emergence flowered ≈10 d faster than plants grown under continual LDs (Warner, 2006).

TABLE 8 Regression analysis equations and r^2 or R^2 for time to visible bud; time to open flower; and stem length at the open flower in response to young-plant photoperiod (YP; 9-, 12-, 13-, 14-, 16-, 18-, or 24-h, or a 4-h night interruption; NI) and finishing photoperiod (FP; 10-, 11-, 12-, 13-, 15-, or 16-h, or a 4-h NI) of witchgrass 'Frosted Explosion' (*Panicum capillare*).

Parameter	y0	a	b	c	d	e	R^2 or r^2
Time to visible bud (d)							
Rep. 1 Moderate DLI	−100.65[z]	7.86	5.09	−0.29	−0.18	0.25	0.774
Rep. 1 Very Low DLI	[y]	11.17	−13.33	−0.29	0.64		0.638
Rep. 2 Moderate DLI		4.56	−7.53	−0.23	0.20	0.36	0.761
Rep. 2 Very Low DLI		7.67	−10.27	−0.18	0.49		0.589
Time to open flower (d)							
Rep. 1 Moderate DLI		9.05	−9.41	−0.30	0.36	0.19	0.652
Rep. 1 Very Low DLI		10.26	−9.07	−0.27	0.40		0.745
Rep. 2 Moderate DLI		4.72	−5.98	−0.21	0.16	0.29	0.742
Rep. 2 Very Low DLI		6.70	−6.39	−0.16	0.29		0.585
Stem length at open flower (cm)							
Rep. 1 Moderate DLI		8.17	−13.32	−0.41	0.43	0.62	0.783
Rep. 1 Very Low DLI	−96.37	5.42	9.84	−0.14	−0.32		0.686
Rep. 2 Moderate DLI		10.08	−13.50	−0.24	0.70		0.589
Rep. 2 Very Low DLI	−90.87	2.91	12.44	−0.07	−0.43		0.478

[z]Coefficients for model equations were used to generate Figure 2 through Figure 4.
[y]Blank cells = 0.
All models are in the form of: $f = y0 + a*YP + b*FP + c*YP^2 + d*FP^2 + e*(YP*FP)$.
Cut flowers were finished under a moderate DLI of ≈ 10 mol·m^{-2}·d^{-1} or a very low DLI of ≈ 3 mol·m^{-2}·d^{-1}.

TABLE 9 Regression analysis equations and r^2 or R^2 for time to visible bud in response to young-plant photoperiod (11, 13, 14, 15, 16, 24 h, or a 4-h NI) and/or finishing photoperiod (10, 11, 12, 13, 14, 15, 16 h, or a 4-h NI) of marigold 'Xochi' (*Tagetes erecta*).

Figure	y0	a	b	c	d	R^2 or r^2
3A	−94.22[z]	3.26	11.72	0.09	−0.33	0.797
3B	40.34	−1.36	0.03	[y]	[y]	0.098
3C	−57.28	10.05	−0.26	[y]	[y]	0.836

[z]Coefficients for model equations were used to generate Figure 3A through Figure 3C.
[y]Blank cells = 0.
Models 3A is in the form of: $f = y0 + a*YP + b*FP + c*YP^2 + d*FP^2$ and models 3B and 3C are in the form of: $f = y0 + a*P + b*P^2$

Although the variables interacted, finishing photoperiod had a greater effect on TVB of marigold than young-plant photoperiod. Plants that finished under 10- to 12-h photoperiods had faster TVB than those finished under photoperiods ≥ 13 h (Figure 3C). Therefore, the critical photoperiod of marigold 'Xochi' was determined to be ≈12 h. Marigold's flowering responses align with other studies detailing photoperiodic responses of SDPs (Park et al., 2013; Kang et al., 2019). TVB of zinnia 'Dream Land' decreased by 10 d when finished under 9-h SDs instead of 4-h NI (Park et al., 2013). Similarly, Kang et al. (2019) demonstrated that 8-h SDs promoted flowering of kalanchoe 'Lipstick' (*Kalanchoe blossfeldiana*), while 16-h photoperiods or 4-h NIs inhibited flowering, regardless of NI TPFDs. Unlike marigold 'Xochi', kalanchoe 'Lipstick' was ≈45 or 33% taller under SDs compared to plants grown under a 16-h LD or a 4-h NI, respectively (Kang et al., 2019). However, this may have been due to the absence of stem elongation associated with flowering, as plants grown under LDs or NIs did not transition from vegetative to reproductive growth.

Absorbed radiation is the driving force for photosynthesis and subsequent plant growth and development. Thus, SL must be utilized when solar radiation is limited to produce high-quality cut flowers year-round. SL had a substantial effect on the growth and development of witchgrass. TVB and TOF were hastened for plants grown under a moderate DLI compared to those grown under a very low DLI. Similarly, Faust et al. (2005) reported that the time to flower of vinca 'Pacific Lilac' (*Catharanthus roseus*) and zinnia 'Dreamland Rose' decreased by 3 and 10 d, respectively, when grown under a DLI of 43 mol·m^{-2}·d^{-1} compared to 5 mol·m^{-2}·d^{-1}. In a separate study, jasmine tobacco 'Domino White' (*Nicotiana alata* Link

and Otto) and helipterum (*Helipterum roseum* Hook.) flowered 17 and 6 d faster, respectively, when grown under SL providing 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 18 h compared to those grown without SL (Erwin and Warner, 2002).

One hundred percent of witchgrass plants grown under young-plant photoperiods between 13 and 24 h or a 4-h NI (rep. 1) or 14 and 24 h or a 4-h NI (rep. 2), and finished under photoperiods $\geq$ 13 h, or a 4-h NI (rep. 1) or $\geq$ 14 h, or a 4-h NI (rep. 2), and a moderate DLI yielded harvestable stems. Conversely, no plants finished under a low DLI-yielded harvestable stems. Likewise, Furufuji et al. (2014) reported that cut rose 'Tint' (*Rosa* spp.) yield was 101% higher when grown with a supplemental DLI of 5.8 $mol \cdot m^{-2} \cdot d^{-1}$ compared to those grown without SL. Moreover, greenhouse-grown lisianthus 'Echo Champagne' and 'Rosita White' (*Eustoma* spp.) produced 12 and 2 more stems per m^2, respectively, when grown under 67% shade compared to 88% shade for 5 weeks (Lugasi-Ben-Hamo et al., 2010). Furthermore, witchgrass stem length, caliper, and branch number improved when grown under moderate DLIs. Similarly, Torres and Lopez (2011) found that stem caliper of yellow trumpet bush 'Mayan Gold' (*Tecoma stans*) seedlings increased by 133% as the DLI increased from 0.8 to 25.2 $mol \cdot m^{-2} \cdot d^{-1}$. In another study, stem caliper and height of mountain spike speedwell (*Veronica rotunda* var. *subintegra*) increased by 110% and 77%, respectively, as the DLI increased from 3.6 to 18.3 $mol \cdot m^{-2} \cdot d^{-1}$ (Lim et al., 2022).

In conclusion, the present study indicates that high-quality marigold 'Xochi' cut flowers can be produced in a timely fashion when young plants are grown under any photoperiod between 11 and 24 h, or a 4-h NI, and finished under a 12-h photoperiod, as stem yield was highest under this finishing photoperiod compared to 10- or 11-h finishing photoperiods. The interactions between young-plant and finishing photoperiods were not commercially impactful for the stems of marigold that reached VB or were harvestable. While marigolds finished under photoperiods > 12 h did not reach harvestability during the study, they all developed flower buds and were likely to have become harvestable, after a delay, compared to marigolds finished under photoperiods < 13 h. As such, the influence of young-plant and finishing photoperiod was not empirically quantified for these plants and could be investigated further in another study. Moreover, high-quality witchgrass 'Frosted Explosion' cut flowers can be grown under any photoperiod between 14 and 24 h, or a 4-h NI, during the young-plant stage, and finished under photoperiods equal to or greater than 14-h, or a 4-h NI, to prevent premature flowering and subsequent inferior quality. While these photoperiods yielded cut flowers of similar thickness, 16-h photoperiods can be maintained to produce longer witchgrass stems. Witchgrass should be grown under at least a moderate DLI of $\geq$ 10 $mol \cdot m^{-2} \cdot d^{-1}$ during the finishing stage to produce cut flowers with sufficient stem lengths and calipers for market. Growers once limited to producing witchgrass and marigold outdoors or in high tunnels during warm and temperate seasons may use these recommendations to produce these varieties in greenhouses during the winter and early spring, allowing for consistent production.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

CS and RL conceptualized and designed the study. CS performed the experiments, conducted data analysis, and prepared the manuscript. RL obtained funding and revised the manuscript. Both authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Genome-wide identification and expression analysis of MIKC^C genes in rose provide insight into their effects on flower development

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The MIKC^C-type gene family plays important roles in plant growth, development, and tolerance of biotic and abiotic stress, especially during floral organ differentiation. However, there have been no studies of MIKC^C-type genes in rose, and functional differentiation of family members has not been explored. In this study, we identified 42 MIKC^C-type genes in rose, classified the genes into 12 subfamilies, and constructed a phylogenetic tree. We performed expression analysis of these genes, and found that expression patterns correlated with the predicted subfamily, indicating that the features of MIKC^C-type genes were broadly conserved during evolution. Collinear analysis of MIKC^C genes among Rosaceae species confirmed the occurrence of whole genome duplications (WGD) and revealed some species-specific MIKC^C genes. Transcriptome analysis showed that the expression of some MIKC^C-type genes responded to low temperatures (4°C, 24 h) during flower organ differentiation. These conserved, duplicated, and novel expression patterns of MIKC^C-type genes may have facilitated the adaptation of rose to various internal and external environmental changes. The results of this study provide a theoretical basis for future functional analysis of the MIKC^C genes in rose and investigation of the evolutionary pattern of the MIKC^C gene family in the Rosaceae genome.

KEYWORDS

MIKC^C, gene family, rose, expression analysis, low temperature

Introduction

The MADS-box transcription factor family is one of the largest families of transcription factors, and its members play critical roles during plant growth and development, especially in the development of root, flower, and fruit (Shore and Sharrocks, 1995; Causier et al., 2010). The MADS family was named for four DNA-binding proteins: MCMI (*Saccharomyces*), AGAMOUS (AG) (*Arabidopsis*), DEFICIENS (*Antirrhinum majus*), and SERUM RESPONSE FACTOR (SRF) (*Homo sapiens*) (Sommer et al., 1990; Alvarez-Buylla et al., 2000). MADS-box family genes can be divided into type I and type II classes. The type I MADS-box genes contain ARG80/SRF-like genes (animals and fungi) and diverse MADS-box genes (plants), which lack a 'K' domain and are categorized into Mα, Mβ, and Mγ classes (Günter et al., 1996; Parřenicová et al., 2003). The type II MADS-box genes include MEF2-like genes (animals and fungi) and MIKC-type genes (plants) (Alvarez-Buylla et al., 2000). MIKC-type proteins are conserved in structure, with a highly conserved DNA-binding MADS (M) domain, an intervening (I) domain, a keratin-like (K) domain, and a C-terminal (C) domain (Günter et al., 1996). The I domain is crucial to the formation of DNA dimers, the K domain is involved in protein-protein interaction *via* a coiled-coil structure, and the C domain is required for transcriptional activation and ternary complex formation (Wang et al., 2020). MIKC genes can be further classified into two categories, MIKC^C and MIKC*. During the evolution of land plants, a gene duplication event occurred in the MADS-box genes; MIKC^C-type proteins retained the original domain structure, while MIKC*-type proteins acquired an extended 'I' domain (Becker and Theißen, 2003; Kaufmann et al., 2005). MIKC^C-type genes have been phylogenetically and functionally characterized in various plant systems, including Arabidopsis, rice, wheat, and litchi (Becker and Theißen, 2003; Arora et al., 2007; Schilling et al., 2019; Guan et al., 2021). MIKC^C-type genes play vital roles in flowering time, floral organ characteristics, and fruit maturity in plants, especially during floral transition and development (Theissen and Melzer, 2007; Li et al., 2016; Barrero-Gil et al., 2021).

The ABCDE model was established by analysis of homeotic floral mutants. Class A and E genes specify the identity of sepals; Class A, B, and E genes specify the identity of petals; class B, C, and E genes specify the identity of stamens; class C and E genes specify the identity of carpels; class D and E genes specify the identity of ovules (Causier et al., 2010; Silva et al., 2016). In angiosperms, class A genes include *SQUAMOSA/APETALA1* (*AP1*) and *APETALA2* (*AP2*); class B genes include *APETALA3* (*AP3*) and *PISTILATA* (*PI*); class C genes include *AGAMOUS* (*AG*); class D genes include *SHATTERPROOF* (*SHP*) and *SEEDSTICK/AGAMOUS-LIKE11* (*STK*); class E genes include *SEPALLATA1*, *2*, *3*, *4* (*SEP1*, *2*, *3*, *4*) (Wang et al., 2020). All the genes in the ABCDE model are MADS-box family members except *AP2*. MIKC^C-type genes are also involved in the regulation of floral transition. Previous studies found that *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) regulates flowering time and flower initiation (Barrero-Gil et al., 2021); FLOWERING LOCUS C (FLC) targets *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 15* (*SPL15*) and *SHORT VEGETATIVE PHASE 1* (*SVP1*) to produce different flowering phenotypes and regulate floral transition mechanisms (Madrid et al., 2020); *AGL17* is a key regulator of floral transition (Shu et al., 2020). MIKC^C-type transcription factors play crucial roles in regulating gene expression under various abiotic stress conditions (Saha et al., 2015; Guo et al., 2016; Wang et al., 2018; Chen et al., 2019). In *Brassica rapa*, 19 *BrMADS* genes were analyzed, and expression of eight and six genes were induced by drought and salt stress, respectively. In *Solanum lycopersicum*, MADS-box protein SlMBP11 is a stress-responsive transcription factor and plays an important role in the positive regulation of salt stress (Saha et al., 2015; Guo et al., 2016). However, our understanding of the potential cold stress-related functions of MIKC^C transcription factors remains limited.

Currently, we have only a general understanding of the functions of MIKC^C-type genes in rose from analysis of transcriptome data (Liu et al., 2018; Raymond et al., 2018). However, analysis based solely on transcriptome data can be limited by inaccurate mapping and incomplete characterization of MIKC^C-type genes. The goal of this study was to better understand the evolution of MIKC^C-type genes in Rosaceae and to facilitate future research on this important transcription factor family. The results of our work provide a detailed overview of the number, phylogeny, and expression of MIKC^C-type genes in *Rosa chinensis*. We found that a large number of MIKC^C orthologous genes were retained during the differentiation of Rosaceae, and we speculate that the functions of these genes may be relatively conserved. Interestingly, MIKC^C-type genes of rose showed novel expression patterns under low temperatures. Collinear analysis of MIKC^C genes among Rosaceae species (*Rosa chinensis*, *Fragaria vesca*, *Rubus chingii*, *Prunus persica*, *Pyrus pyrifolia*, and *Malus domestica*) confirmed the occurrence of whole genome duplications (WGD) and revealed species-specific MIKC^C genes. We hypothesized that these conserved, duplicated and novel expression patterns of MIKC^C-type genes may have facilitated the adaptation of rose to various internal and external factors. The results of this study provide new insights into the functions of MIKC^C genes in rose,

and provide the basis for future work to explore the evolution of Rosaceae.

Materials and methods

Plant materials and low temperature treatment of rose floral buds

Rose (*Rosa hybrida* cv 'Samantha') plants were cultivated and grown as described previously (Zhang et al., 2019). Briefly, rose plantlets were propagated by tissue culture. Rose shoots approximately 2 cm in length and with at least 1 axillary bud were used as explants and cultured on Murashige and Skoog (MS) medium supplemented with 1.0 mg/L 6-benzyl aminopurine (6-BA) and 0.05 mg/Lα-Naphthaleneacetic acid (NAA) for 30 days at 23°C, under a 16-h light/8-h dark photoperiod (Wu et al., 2016). Rose seedlings were then transferred to 1/2 MS medium supplemented with 0.1 mg/L NAA for 20-30 days for rooting. After rooting, plantlets were transferred to pots containing peat moss: vermiculite (1:1) and cultured at 23°C with a relative humidity of ~60% and 16-h light/8-h dark photoperiod. After the emergence of floral buds, the rose plants were incubated at 4°C for 24 h (low temperature treatment) (Lee et al., 2021).

Genome-wide identification and phylogenetic analysis of MIKC^C-type genes in *Rosa chinensis*

The Rosa chinensis OldBlush Hm r2.0 genome was downloaded from the Lipme database (https://lipm-browsers.toulouse.inra.fr/pub/RchiOBHm-V2/). The sequences of the previously identified MIKC^C genes of *Arabidopsis thaliana* (Becker and Theißen, 2003) were obtained and confirmed in the TAIR database (https://www.arabidopsis.org/). The HMM models of the SRF-TF (PF00319) and K-box (PF01486) were obtained from the Pfam database (http://pfam.xfam.org/). The MIKC^C candidate genes were screened using HMMER3 (E-value = 0.01) and BLASTP (E-value = 1e-5) software (Camacho et al., 2009; Finn et al., 2011). The sequences of the putative MIKC^C genes were confirmed to contain the complete MADS domain by searching the conserved domain database (CDD) (Lu et al., 2019) for further analysis. MADS genes in *Arabidopsis* were used to classify the RcMADS genes. Multiple sequence alignment of the full-length protein sequences was achieved using MUSCLE software with default parameters (Edgar, 2004). A phylogenetic tree of the AtMADS and RcMADS genes was subsequently generated using IQ-TREE 2 (Minh et al., 2020). The same strategy was used for the construction of the phylogenetic tree of the MIKC^C genes of Rosaceae.

Conserved motif analysis of the RcMIKC^C genes

The full-length protein sequences of the RcMIKC^C genes were obtained and the conserved motifs were analyzed. To do this, we used the Multiple Em for Motif Elicitation (MEME) online program (v5.5.0; http://meme-suite.org) with the following parameters: the number of repetitions was set to zero or one and the maximum number of motifs was 20. The conserved motifs were visualized with TBtools software (Chen et al., 2020).

Collinearity analysis and chromosomal location of RcMIKC^C genes

We downloaded the genome sequences and annotation information from the Genome Database for Rosaceae (GDR) (https://www.rosaceae.org/) containing strawberry, raspberry, peach, pear, and apple. The GFF annotation files of Rosaceae species were used to retrieve gene location information. Construction of orthologous gene collinearity maps was based on inferred differentiation times for Rosaceae species from previous studies (Xiang et al., 2016). Gene duplication and collinearity relationships were produced by default parameters using the multicollinearity scanning toolkit (MCScanX) (Wang et al., 2012), and these results were visualized by Circos (http://circos.ca/) with a minimum block size of 30.

The positions of RcMIKC^C genes on the choromosome were mapped using MapChart (https://www.wur.nl/en/show/Mapchart.htm).

Transcriptome sequencing and differential expression analysis

A total of 1.5 μg RNA per sample was used as input material for rRNA removal, using the RiboZero rRNA Removal Kit (Epicentre, Madison, WI, USA). Five sequencing libraries (Supplementary Table S1) were generated using the NEBNext UltraTM Directional RNA Library Prep Kit for Illumina (NEB, Ipswich, MA, USA), according to the manufacturer's recommendations. The libraries were sequenced on an Illumina NovaSeq 6000 platform (Illumina, San Diego, CA, USA), and paired-end reads were generated. Differential expression analysis of two groups of floral bud (three biological replicates per group) (Supplementary Table S2) was performed using the DESeq2 R package. Genes with an adjusted p-value < 0.05 and an absolute value of $\log_2$ (FoldChange) > 1, were designated as being differentially expressed. The transcriptome data for other tissues (L-leaves, FB-floral buds, UP-upper of petals, MP-middle of petals, BP-bottom of petals)

were previously measured in our lab (not uploaded), and the transcript fragments per kilobase (FPKM) values are shown in Supplementary Table S1.

RhMIKC^C gene expression profiles in different tissues were converted to transcript fragments per kilobase (FPKM) in millions of mapped reads and the results are displayed as a heatmap using TBtools. Expression correlations between individual genes are also shown.

RNA extraction and qRT-PCR

Samples were ground in liquid nitrogen, then Total RNA was extracted by RNA extraction kit (TaKaRa, Ohtsu, Japan). The quality of extracted RNA was measured by determining the 260/280 absorbance ratio in the range of 1.8-2.0, and the 260/230 absorbance ratio is more than or equal to 2.0. The purified RNA samples were reversely transcribed into cDNA using HiScript® II Q RT SuperMix for qPCR (+gDNA wiper) (Vazyme, Nanjing, China) according to the manufacturer's instructions. Next, qRT-PCR was conducted using the SYBR FAST qRT-PCR universal kit (mei5bio) in a Roche LightCycler 96 according to the manufacturer's instructions. For each sample, qRT-PCR was performed with six biological replicates. *RhUBI2* was used as an internal control. The primers used in the experiments are shown in Supplementary Table S3. The data were analyzed by correcting to the signal for the internal control and the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

In situ hybridization

In situ hybridization method of floral bud tissue samples was as described previously (Ma et al., 2015; Zhang et al., 2019; Chen et al., 2021). *In situ* hybridization probes were synthesized by PCR amplification of the cDNAs of *RhAP1*, *RhFUL*, *RhMADS6-1*, *RhSOC1*, *RhAGL42-1*, and *RhAGL24-3* using gene-specific primers containing T7 and SP6 RNA polymerase binding sites, with T7 RNA polymerase binding site in the antisense probe and SP6 RNA polymerase binding site in the sense probe. The floral bud tissue samples were treated at 4°C and 23°C in a plant chamber, and then samples were fixed in 3.7% FAA overnight at 4°C, followed by gradient dehydration, embedding, sectioning, hybridizing, and observation by microscope.

Gene structure and cis-acting element analyses of RcMIKC^C genes

The structure of the MIKC^C genes, including exons, introns, and UTRs, were obtained from the genome GFF3 file. The Gene Structure Display Server (http://gsds.gao-lab.org/) tool was used to visualize the gene structures. The cis-acting elements in the promoter of the RcMIKC^C genes (in the 2 kb sequences upstream from the start codon) were predicted using the PlantCARE (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/). Finally, TBtools software was used to display the cis-acting elements on promoters (Chen et al., 2020).

Interaction networks of MIKC^C proteins in rose

Orthologous genes in *Arabidopsis thaliana* corresponding to the RcMIKC^C genes were used to predict protein interaction networks. The protein sequences of AtMIKC^C genes were used to search in STRING 11.0 (https://string-db.org/).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.4.3 (GraphPad Software Inc., USA: http://www.graphpad.com/). Experimental data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple range test to compare different groups among the experimental sites at $P < 0.05$. Student's *t*-test was used to assess differences between two sets of data. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Results

Identification and conserved motif analysis of MIKC^C genes in *Rosa chinensis*

The candidate genes with typical SRF-TF and K-box domain (PF00319 and PF01486) were preliminarily screened from the *Rosa chinensis* 'Old Blush' Hm r2.0 genome according to the Hidden Markov Model (HMM). The domain of MIKC^C is conserved, and the *Arabidopsis* MIKC^C genes were used as a reference to identify potential MIKC^C genes in rose. A total of 80 genes were initially identified in *Rosa chinensis*, as the overlap of HMM and BLASTP results (Figure 1A; Supplementary Table S4). The MADS domains of these genes were further classified by constructing a maximum-likelihood (ML) phylogenetic tree. We identified 42 conserved MIKC^C genes containing complete SRF-TF (MADS-box) domains in *Rosa chinensis*, including genes of the ABCDE model, flowering-related genes, and other genes (Figure 1B). The remaining 38 genes were clearly classified into MADS Type I (33 genes) and MIKC* (5 genes) families (Figure 1B). The MIKC^C genes distributed on seven chromosomes of rose, with two genes on chromosome 3 and fourteen genes on chromosome 7 (Supplementary Table S5). To further understand the characteristic motifs of the RcMIKC^C

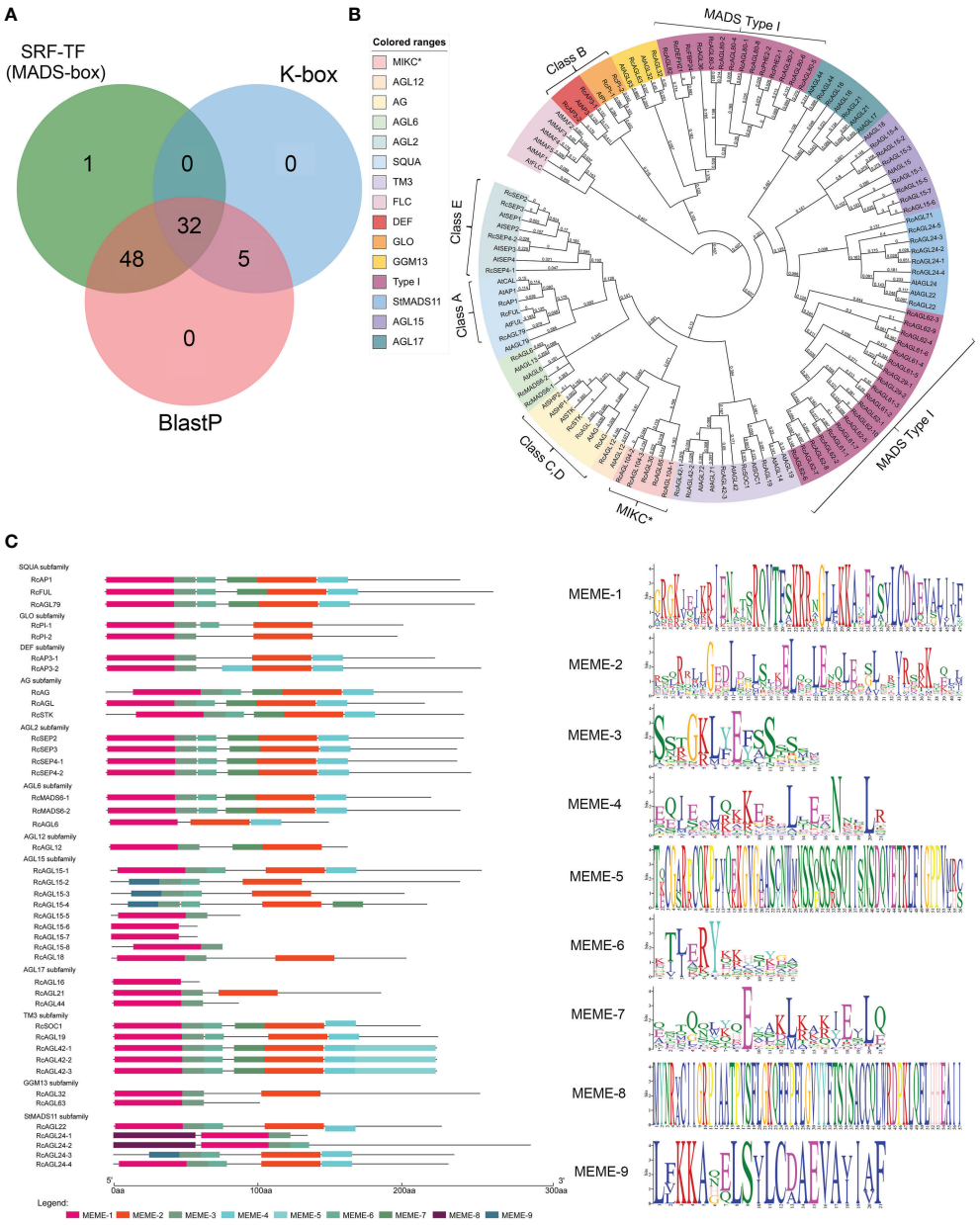

FIGURE 1
Identification of RcMIKC^C genes by HMM and BLASTP and analysis of their conserved domains. **(A)** Genes with MADS domains predicted by HMM and BLASTP. **(B)** Phylogenetic relationships among MADS genes in *Rosa chinensis* and *Arabidopsis thaliana*. **(C)** Analysis of the conserved domains of the RcMIKC^C genes identified by the phylogenetic tree.

genes, Motif Elicitation (MEME) analysis was performed. The 42 MIKC^C genes contain nine motifs that are conserved in the same subfamily. Among them, The SQUA and AGL2 subfamilies contain six identical motifs; and the other subfamilies contain 1-7 motifs. MEME-1 and MEME-9 motifs had similar protein sequences. MEME-1 or MEME-9 motifs were found in all 42 RcMIKC^C genes, indicating high conservation of these motifs (Figure 1C). The MEME-5 motif is unique to the TM3 subfamily and is located at the end of the protein. Our analysis found that rose genes of the model share conserved domains, including

MEME-1, MEME-2, and MEME-3. Class A, C, D, and E genes also contain the MEME-7 motif, which is not present in class B genes.

We also found five atypical MIKC^{C} genes that contained only the K-box domain. These were classified by constructing phylogenetic tree with the full-length protein sequences of the identified MIKC^{C} genes (Supplementary Figure S1). Subsequent analyses removed these atypical genes, and we focused on the MIKC^{C} genes with an intact SRF-TF (MADS-box) domain.

Homology identification of MIKC^{C} genes in *Rosa chinensis*

Collinearity between MIKC^{C} genes was examined by MCScanX to identify paralogous genes. The potential collinearity results are shown in a circle plot, and the collinearity between genes is indicated with a red signal (Figure 2A). *RcAP1* is located on chromosome 4 (class A gene) of rose and has a paralogous gene *RcFUL* on chromosome 7.

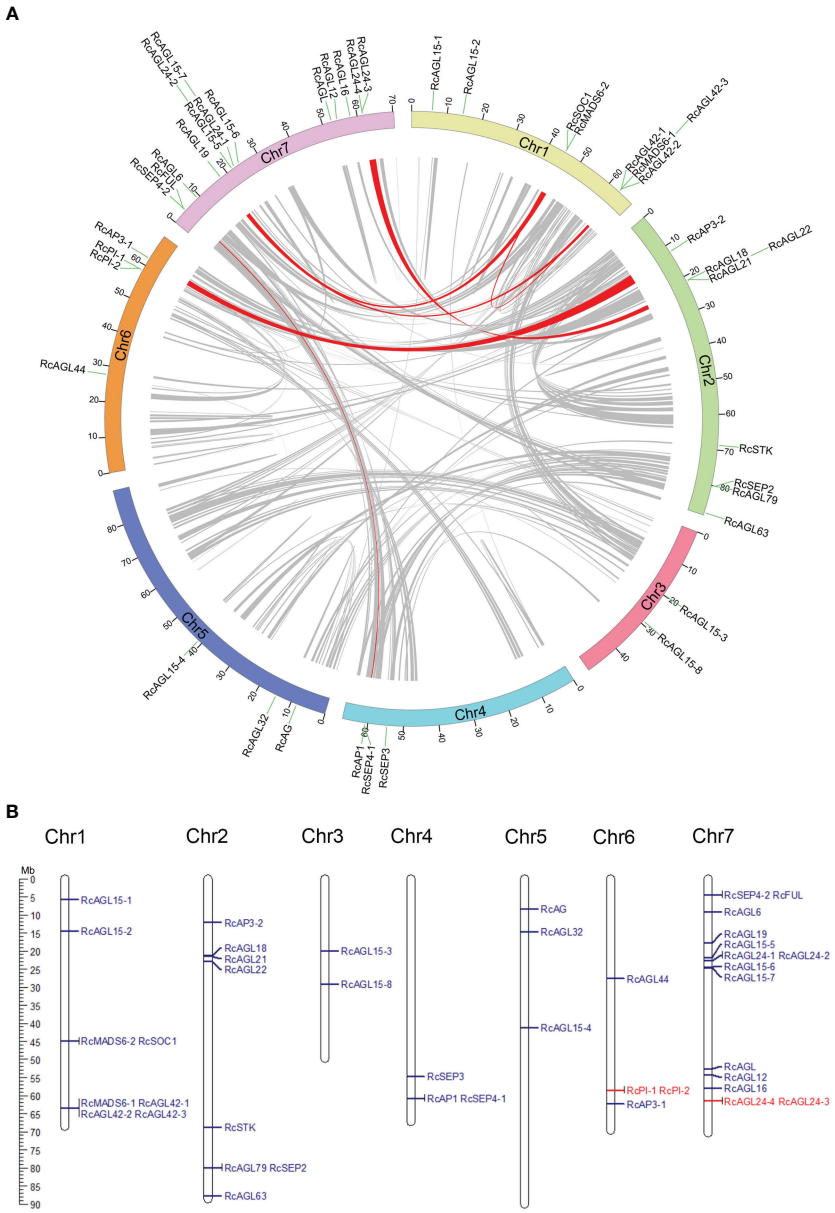

FIGURE 2
Identification of paralogous and tandem duplication genes of RcMIKC^{C}. **(A)** Paralogous gene pairs in genome were analyzed by MCScanX and connected with red lines. **(B)** Karyotype diagram shows tandemly duplicated RcMIKC^{C} genes are marked in red.

RcAP3-1 is located on chromosome 6 (class B gene) of rose and has a paralogous gene, *RcAP3-2*, on chromosomes 2. *RcSEP4-1* (class E gene) on chromosomes 4 has a paralogous gene, *RcSEP4-2*, on chromosome 7. These highly similar class A, B, and C genes may be redundantly involved in the development of the four types of floral organs (sepals, petals, stamens, and carpels). There may be other paralogous genes, such as *RcMADS6-1* and *RcMADS6-2*, *RcSOC1* and *RcAGL42-1*, *RcAGL42-1* and *RcAGL19*, *RcSOC1* and *RcAGL19*, and *RcAGL22* and *RcAGL24-4*. The sequence similarity of these paralogous genes suggests they may have similar functions.

In *Rosa chinensis*, the number of singleton genes is 10,618, accounting for 24% of the total genes. The largest proportion of dispersed genes is 46%, and the number of tandem genes is 4,068, accounting for 9% (Supplementary Figure S2). Tandem duplications are multiple members of a family that occur in the same intergenic region. Tandem duplications are mainly caused by chromosomal recombination. In the 42 conserved MIKC^C genes of rose, *RcPI-1* and *RcPI-2*, and *RcAGL24-4* and *RcAGL24-3* resulted from tandem duplication as indicated by schematic representation of the chromosomal location (Figure 2B). The tandem duplication of these coding genes during evolution could generate novel paralogous genes with new or enhanced functions.

Gene structure and promoter motif analysis in *Rosa chinensis*

To obtain a more comprehensive understanding of the structure of RcMIKC^C genes, we next analyzed the exon/intron organization of RcMIKC^C genes. Some of the RcMIKC^C genes have multiple exons, and the exons are distributed unevenly. AGL15, AGL17, and GGM13 subfamilies include genes with only one exon, and the AGL15 subfamily includes seven single-exon genes. The genes within the SQUA, AG and AGL2 subfamilies had eight exons and the genes within the GLO, DEF and TM3 subfamilies had 7 exons. The RcAGL6 and RcAGL12 genes had 5 exons. The number of exons in StMADS11 subfamily genes ranged from 3 to 9. The total gene length of RcAGL24-4 reached 18 kb, due to a large intron (Figure 3A). The varying gene structure indicated that RcMIKC^C genes may differ in many aspects, including gene function, evolution, mRNA process, and transcriptional regulation.

Significant regulation of gene expression can occur at transcription initiation with multiple regulatory elements in the promoter sequences. The general structure of a promoter includes core promoter elements and upstream regulatory elements. We extracted the promoter sequences of RcMIKC^C

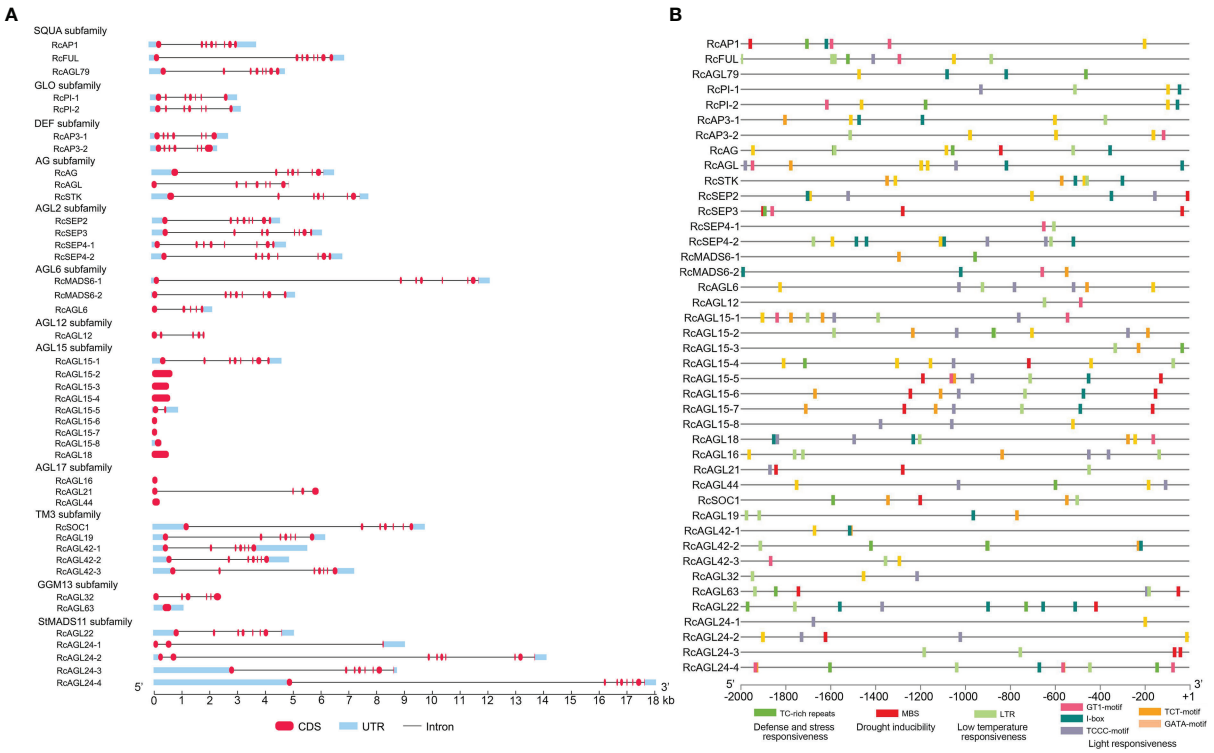

FIGURE 3
Gene structure and promoter response element analysis of RcMIKC^C genes. **(A)** Gene structure analysis of the RcMIKC^C family. The red labels, blue boxes, and black lines indicate exons, untranslated regions, and introns, respectively. **(B)** Analysis of hormone and stress response elements of the RcMIKC^C promoters.

genes (2 kb) to search for potential regulatory motifs using the PlantCARE database (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/). Many hormone-responsive elements were identified: auxin-responsive elements (AuxRR-core), SA-responsive elements (TCA elements), MeJA-responsive elements (CGTCA and TGACG motifs), gibberellin-responsive elements (TATC-box), and ABA-responsive elements (ABRE) were found in the promoter sequences (Supplementary Table S6; Supplementary Figure S3). Additionally, many abiotic response elements were identified, such as defense and stress-responsive elements (TC-rich repeats), drought-responsive elements (MBS), low temperature-responsive (LTR) elements, and light-responsive elements (GT1-motif; I-box; TCCC-motif; TCT-motif; GATA-motif). Of these, LTR elements were widely distributed in RcMIKC^C promoters (Supplementary Table S6; Figure 3B), so we inferred that low temperature may be an important signal regulating the expression of these genes.

Transcriptional abundance and correlation of MIKC^C genes in *Rosa hybrida*

As one of the most important classes of MADS family, MIKC^C genes play vital roles in plant growth and flower organ development (Li et al., 2016; Barrero-Gil et al., 2021). To further investigate the differential expression of the MIKC^C genes in different tissues, transcriptome analysis was performed on different tissues (L-leaves, FB-floral buds, UP-upper of petals, MP-middle of petals, and BP-bottom of petals). The ABCDE genes were specifically expressed in floral buds or petals, consistent with their function of regulating flower organ development. The class A gene *RhAP1* was highly expressed in early floral buds, and another A class gene, *RhFUL*, was highly expressed in both floral buds and petals. There are two Class B genes, *PI* and *AP3*, in rose, and both were expressed in floral buds and petals. C genes *RhAG* and *RhAGL* were significantly expressed in early floral buds. The class D gene *RhSTK* exhibited greater transcript accumulation in floral buds compared to leaves and petals. As functionally redundant subfamily members (AGL2), class E genes are directly involved in multifaceted floral organ identity, and *RhSEP2*, *RhSEP3*, and *RhSEP4-2* were all detected in both early floral buds and petals. However, *RhSEP4-1* was highly expressed in floral buds, and not expressed in petals and leaves. *RhSOC1*, *RhAGL19*, *RhAGL22*, *RhAGL24-2*, and *RhAGL24-4* showed a trend of high expression in leaves (Figure 4A). Expression was not detected for additional 13 MIKC^C genes in leaves, floral buds, or petals. These included seven genes in the AGL15 subfamily, suggesting that these genes may not be directly involved in flower and leaf development. Gene ontology analysis of the 29 expressed MIKC^C genes indicated that 27 of them may have transcription factor activity and all may have protein dimerization activity (Supplementary Figure S4).

To study the expression correlation between MIKC^C genes, the expression matrix of the transcriptome was used as the original data to analyze the correlation (Figure 4B). In the SQUA subfamily, all genes except *RhFUL* showed a highly positive trend in expression. Class B genes and class C/D genes showed high positive correlation. Inconsistency in expression results in poor correlation of *RhSEP4-1* with other class E genes. There is a high positive correlation between the SQUA subfamily and the AG subfamily because the genes in these groups were expressed in floral buds. Genes among several subfamilies of AGL12, AGL15, TM3 and GGM13 were expressed in leaves, resulting in a positive correlation, as shown in the heatmap. The FPKM values of ABCE genes obtained from the transcriptome were used to visually display the expression trends in leaves, floral buds, and petals (Figure 4C). These genes showed low expression levels in leaves and high expression in floral buds, confirming the specificity of expression of these floral organ development genes.

Expression patterns of MIKC^C genes in rose floral buds at low temperature

Low temperature usually results in flower malformation (Ma et al., 2015), which seriously compromises ornamental quality and economic value. Our analysis revealed that LTR elements are widely distributed in promoters of the RcMIKC^C genes (Supplementary Table S6; Figure 3B). Therefore, we next investigated the effect of low temperature on floral bud differentiation. Many genes regulating floral organ development are MIKC^C genes, so we focused on their expression after 24 h of treatment at 4°C by heatmap analysis (Figure 5A; Supplementary Table S2). *RhAP1*, *RhFUL*, *RhMADS6-1*, *RhSOC1*, *RhAGL42-1*, and *RhAGL24-3* were significantly up-regulated under low temperature. These six differentially expressed genes were further tested qRT-PCR and *in situ* hybridization to validate the accuracy of the RNA-Seq data (Figures 5B, C). Surprisingly, the expression of *RhMADS6-1*, *RhSOC1*, *RhAGL42-1*, and *RhAGL24-3* were not detected in floral buds at normal temperature, but increased expression was detected at low temperature. These results suggest that the MIKC^C genes of rose induced by low temperature may be involved in the development of flower organs under short-term low temperature stress.

Interaction network analysis of the MIKC^C proteins in rose

Previous studies reported that MADS proteins can form protein dimers (Kaufmann et al., 2005). The moderately conserved K-box domain of MADS proteins is important for protein-protein

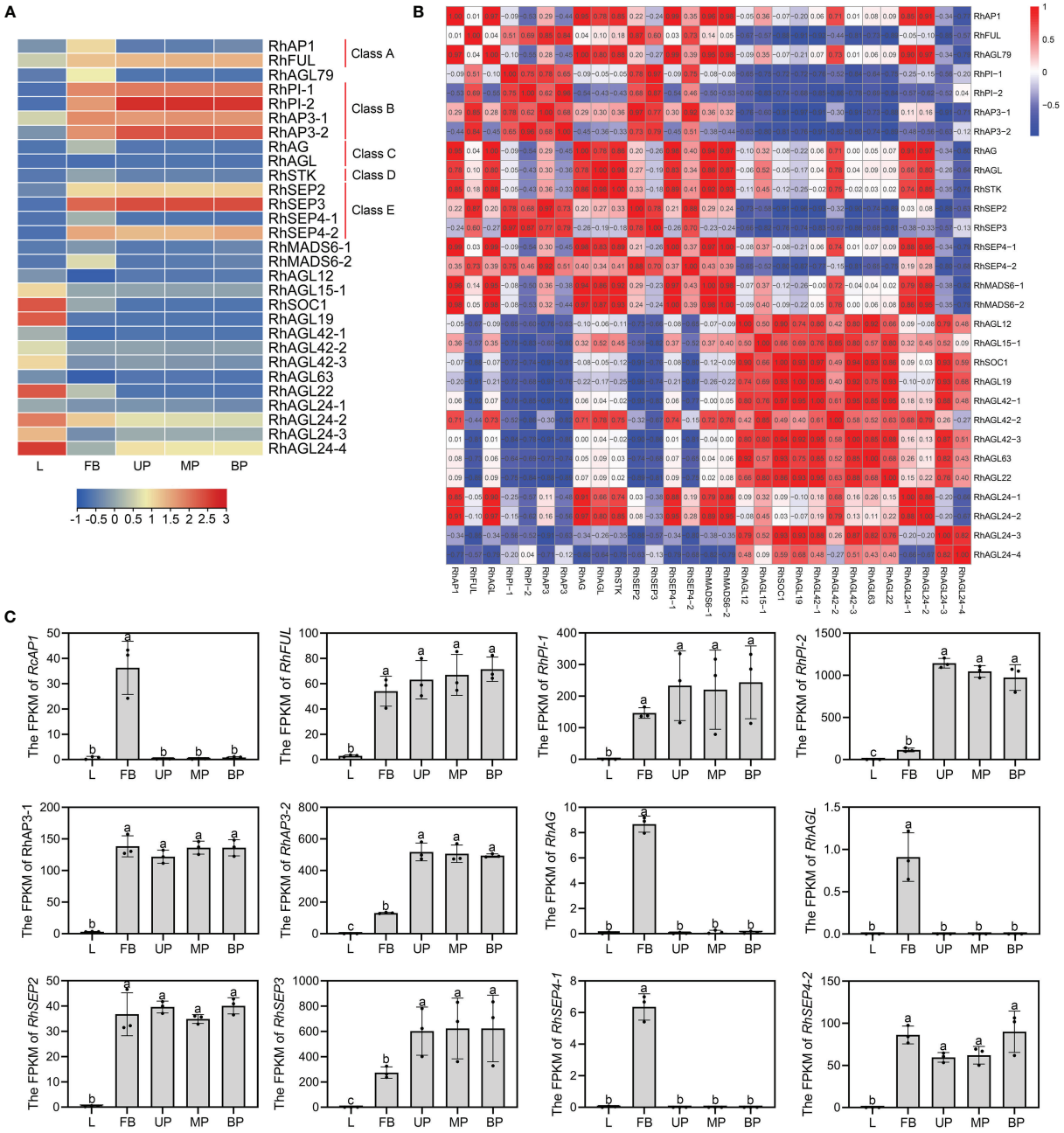

FIGURE 4
Expression patterns of RhMIKC^C genes by transcriptome analysis of rose leaves, floral buds, and petals. **(A)** Heatmap of RhMIKC^C gene expression in leaves (L), FB (floral buds), UP (upper of petals), MP (middle of petals), and BP (bottom of petals). Red color represents increasing gene expression and blue color indicates decreasing gene expression. **(B)** Expression correlation between individual genes is displayed in a heatmap. **(C)** The FPKM values of class ABCE genes in the transcriptome. Error bars indicate the standard error of the mean ± SD. Different letters above the bars indicate significantly different values ($P < 0.05$), calculated using one-way analysis of variance (ANOVA) followed by a Tukey's multiple range test.

interactions and promotes the formation of α-helical structures. The orthologous genes of *Arabidopsis thaliana* were used to construct the RcMIKC^C protein interaction network (Figure 6; Supplementary Table S7). In the constructed network, class A genes (RcAP1 and RcFUL), class B genes (RcPI and RcAP3), class C genes (RcAG and RcAGL), and class D genes (RcSTK) may interact with the class E genes, respectively. Additionally, RcAP1 may interact with RcAGL22, and RcFUL may interact

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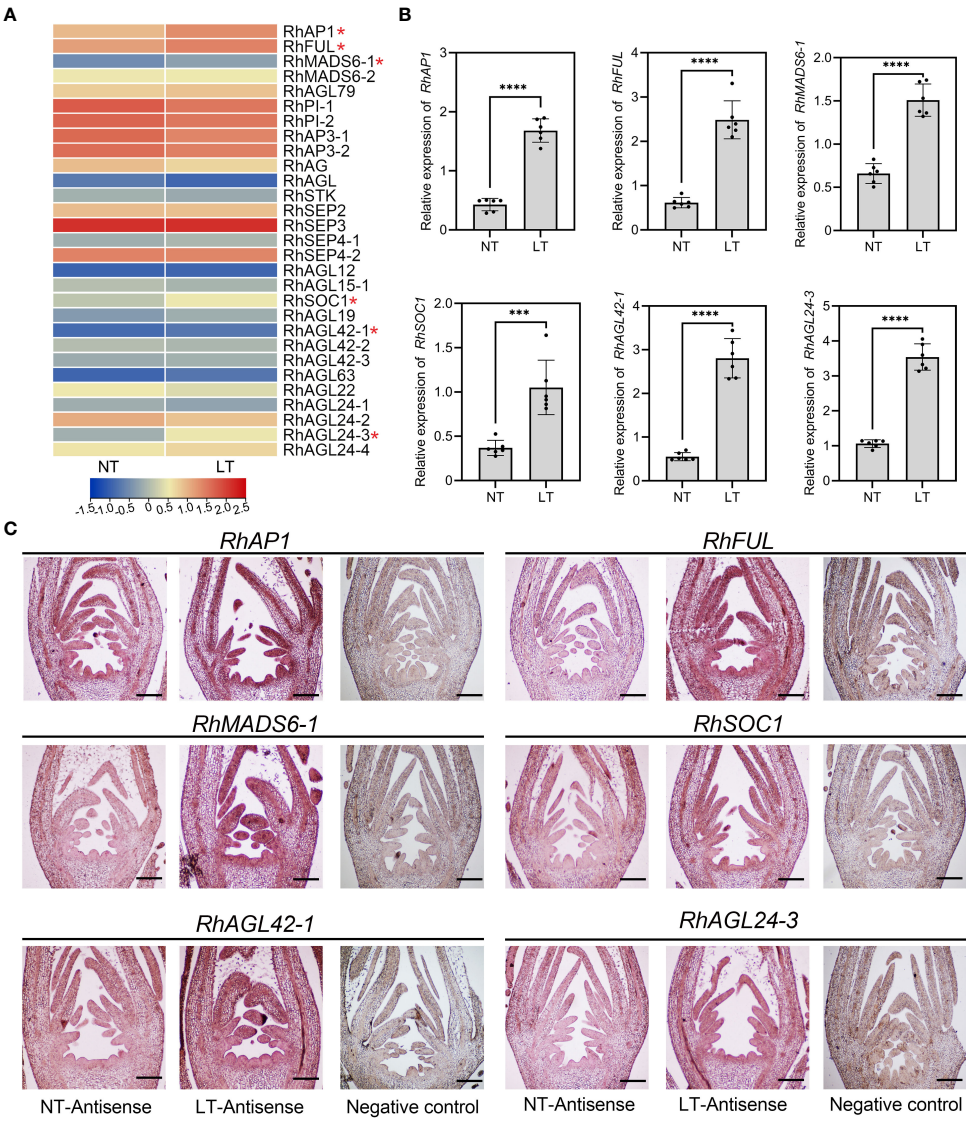

FIGURE 5
Transcriptome analysis of RhMIKC^C genes in rose floral buds at low temperature. **(A)** Heatmap of RhMIKC^C genes expression in transcriptome of floral buds treated with low temperature. Red color represents increasing level of gene expression and blue color indicates decreasing gene expression. **(B)** qRT-PCR analysis of the up-regulated RhMIKC^C genes expression in floral buds treated at 4°C for 24 h *RhUBI2* was used as an internal control. Asterisks indicate significant differences (two-sided Student's *t*-test, ***$P < 0.001$, ****$P < 0.0001$). **(C)** *In situ* hybridization of the up-regulated RhMIKC^C genes in floral buds under normal temperature and low temperature (4°C for 24 h) floral buds. The sense probe was used as a negative control. Images show vertical sections of floral buds. Scale bar, 200 μm.

with RcSOC1. Interestingly, RcPI may interact with RcAP3, and RcPI and RcAP3 may both interact with RcAG. RcSTK may interact with RcAGL32. RcSEP2 and RcSEP3 may interact with RcSOC1, RcMADS6, and RcAGL32 (Figure 6; Supplementary Table S7). These protein interactions may provide clues to the novel functions of RhMIKC^C genes.

Identification and collinearity analysis of MIKC^C genes in rosaceae

The Rosaceae family is widespread worldwide, with more than 3,000 species, three subfamilies, 16 tribes, and 88-100 genera (Xiang et al., 2016). As a member of the Rosaceae

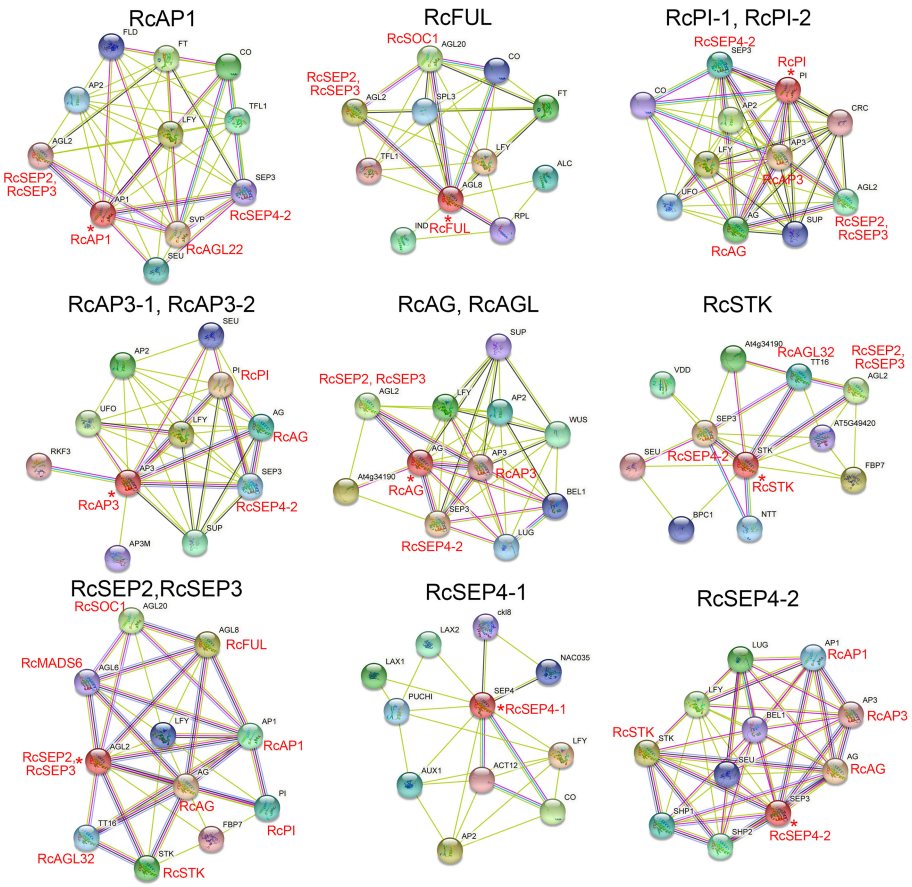

FIGURE 6
Interaction networks of RcMIKC^C proteins according to orthologs in *Arabidopsis*. Asterisks (*) represent core genes in the interaction network.

family, rose is an important ornamental crop. To explore the evolution of Rosaceae MIKC^C genes, rose (*Rosa chinensis*), strawberry (*Fragaria vesca*), raspberry (*Rubus chingii*), peach (*Prunus persica*), pear (*Pyrus pyrifolia*), and apple (*Malus domestica*) were selected as comparative genomes, and the sequences of the MIKC^C genes in these genomes were retrieved. All the identified genes were combined for multiple sequence alignment, and an ML phylogenetic tree was constructed to classify the MIKC^C genes of each species. A total of 306 MADS genes were used to construct this phylogenetic tree (Figure 7A), and 13 subfamilies of MIKC^C were definitively identified (FLC subfamily genes are not present in Rosaceae). By comparison of Rosaceae species, the orthologous genes of MIKC^C with complete SRF-TF domains were effectively identified. We found that there were differences in the number of MIKC^C identified in the different species, and there are significantly more MIKC^C genes in pear and apple (Supplementary Table S8; Supplementary Figure S6). Both pear and apple experienced whole genome duplications (WGD) in the process of Rosaceae differentiation, resulting in multiple paralogous genes for many genes in the genome, and the number of chromosomes is increased to 17.

The order of colinearity analysis is arranged according to the time of differentiation of Rosaceae and revealed that some MIKC^C genes are conserved in Rosaceae. *RcAGL15-1*, *RcMADS6-2*, *RcSOC1*, *RcMADS6-1*, *RcAP3-2*, *RcAGL21*, *RcAGL22*, *RcSTK*, *RcAGL79*, *RcSEP2*, *RcSEP3*, *RcAP1*, *RcAG*, *RcAGL32*, *RcSEP4-2*, *RcFUL*, *RcAGL19*, *RcAG,L* and *RcAGL24-4* are conserved in Rosaceae (Supplementary Table S9). These conserved MIKC^C genes may be directly involved in plant growth and floral organ development (Figure 7B). We found *RcAGL42-3*, *RcPI-2*, *RcAGL15-5*, *RcAGL24-1*, *RcAGL24-2*, *RcAGL15-6*, and *RcAGL15-7* only present in rose, with no homologous genes in strawberry, raspberry, peach, pear, and apple genomes (Supplementary Table S9). These genes in rose may have contributed to the unique traits of this plant, and also suggest that rose may have multiple evolutionary branches.

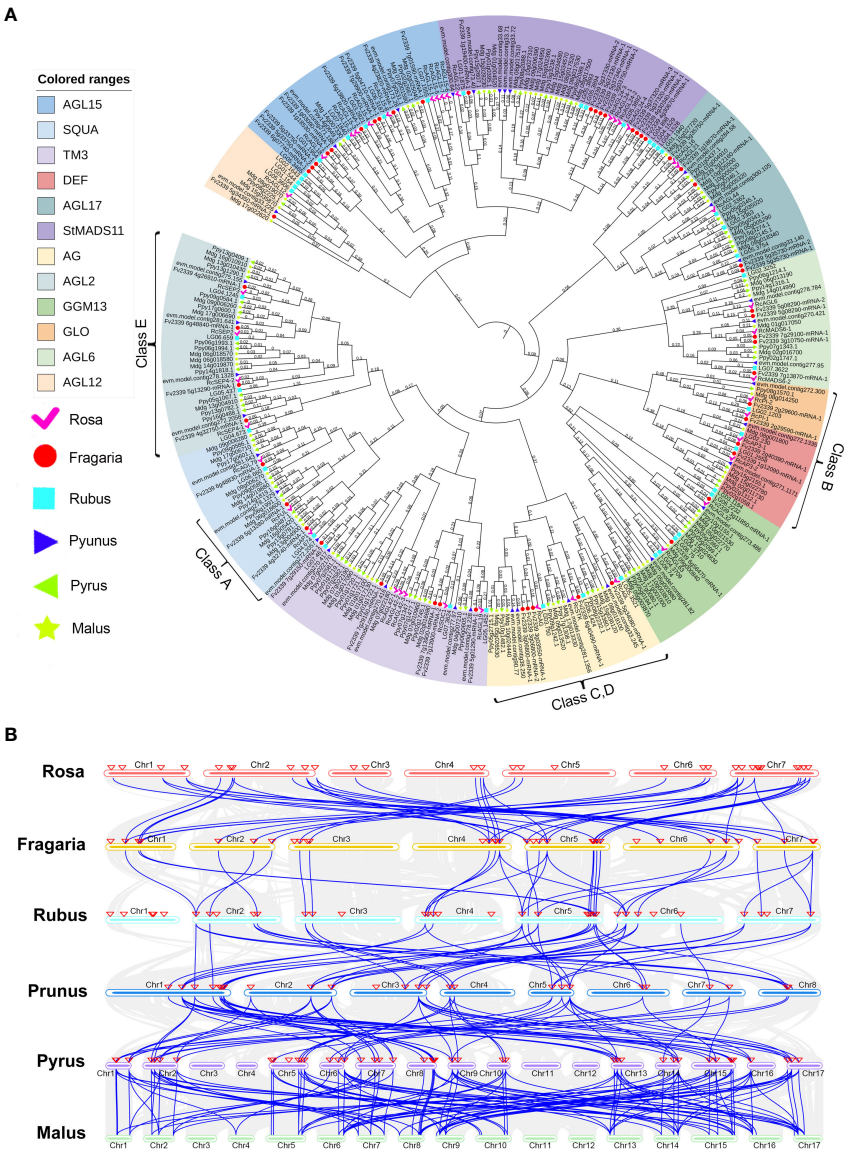

FIGURE 7
Identification of orthologous genes of MIKC^C in Rosaceae. **(A)** Phylogenetic tree of MIKC^C genes in Rosaceae. The MIKC^C gene members of rose (*Rosa chinensis*), strawberry (*Fragaria vesca*), raspberry (*Rubus chingii*), peach (*Prunus persica*), pear (*Pyrus pyrifolia*), and apple (*Malus domestica*) were analyzed separately. **(B)** Collinear analysis of MIKC^C genes among six Rosaceae species. The blue lines represent the collinearity between species, and the red triangles indicate the location of the MIKC^C genes.

Discussion

MIKC^C genes play important roles in plant growth and developmental processes, as well as responses to biotic and abiotic resistance. In recent years, the expression of MIKC^C genes has been analyzed for many plants, such as *Solanum lycopersicum*, *Vitis vinifera* (Díaz-Riquelme et al., 2008), and *Rosa chinensis* (Liu et al., 2018). However, the evolutionary dynamics and functional analysis of the MIKC^C gene family in *Rosa chinensis* at the genomic level are still largely unknown. Here, a total of 42 MIKC^C genes were identified from the genome of *Rosa chinensis*, and these genes were assigned to 12 conserved subfamilies (but not the FLC subfamily) (Figure 1). There were similar total number of MIKC^C genes in different plants: *Rosa chinensis* (42), *Arabidopsis thaliana* (39), *Oryza sativa* (37) (Parřenicová et al., 2003; Arora et al., 2007), *Vitis vinifera* (32) (Díaz-Riquelme et al., 2008), and *Litchi chinensis* (37) (Guan et al., 2021). Almost all characterized MIKC^C genes are involved in flower transition and flower development (Causier et al., 2010; Smaczniak et al., 2012). We found that

the AGL6, StMADS11 and AGL15 subfamilies in rose were significantly expanded from their corresponding clades in *Arabidopsis* (Figure 1A), suggesting that enhanced or novel roles of these genes might be required for specific aspects of floral transition and development in rose. In Wintersweet, AGL6 clade genes may positively regulate FT and negatively regulate FLC (Wang et al., 2011). In rice, members of the AGL17 clade may regulate flowering (Puig et al., 2013). In Arabidopsis, ALG15 clade genes may act in endosperm or gametophyte development (Adamczyk et al., 2007). AG, SQUA, AGL2, 406 DEF, and GLO subfamilies genes are specifically expressed in the reproductive organs (Causier et al., 2010; Smaczniak et al., 2012). Overall, the results suggest that MIKC^C genes play an extremely critical role in flower transition and flower organ development. Interestingly, there is no *FLOWERING LOCUS C* (*FLC*) and *MADS AFFECTING FLOWERING* (*MAF*) genes of the FLC subfamily in *Rosa chinensis*, as reported in previous studies (Liu et al., 2018; Raymond et al., 2018). The FLC subfamily genes play important roles in vernalization of some plants (Ratcliffe et al., 2001). Deletion of *FLC-like* genes in rose may result in a different flowering mechanism from Arabidopsis. Most modern roses have a continuous flowering trait, suggesting a novel regulatory mechanism.

Whole genome duplication (WGD) events are key drivers of gene evolution and expansion in many plants. These events can facilitate the emergence of new functional genes and species and make plants more tolerant to adverse environmental conditions (Cannon et al., 2003; Song et al., 2014). Previous results showed that MIKC^C genes are closely related to floral transition and floral organ development. According to the paralogous analysis of RcMIKC^C genes (Figure 2), both *RcPI* and *RcAP3* have paralogous genes, directly leading to the doubling of the B class genes in rose. Some RcMIKC^C genes were identified as tandem duplication events, such as *RcPI-1*, *RcPI-2*, *RcAGL24-3*, and *RcAGL24-4*, by co-linear analysis and chromosome localization. These results suggest that some RcMIKC^C genes may have emerged through gene duplication in *Rosa chinensis*, which further explains the mechanism of MIKC^C gene expansion. Collinearity analysis revealed differences in the duplication of MIKC^C genes (Figure 2), suggesting differences in retention and loss across subfamilies.

Phylogenetic analysis showed that RcMIKC^C genes can be divided into 12 subfamilies, and the five genes containing only the K-box domain were named *RcFUL-like*, *RcAGL6-like*, *RcAGL21-like*, *RcAGL24-3-like*, and *RcAGL24-4-like*. Since the predicted proteins lack the MADS domain, these are special MIKC^C genes (Supplementary Figure S1). The emergence of these special genes may be due to gene duplication in the distal telomeric segment, as previous studies have shown that gene duplication in the distal telomeric segment may lead to truncated genes lacking MADS or K-box domains. These sequences may encode proteins with impaired function (Schilling et al., 2019). Other examples were previously found, such as *TaSEP1-A2* (*WLHS1-A*), which lacks the 'K' domain and protein-protein interaction *in vivo* could not be detected (Shitsukawa et al., 2007). MIKC^C proteins lacking the K-box domain may theoretically be able to bind DNA and compete with other proteins for target sites, thus repressing transcription (Ferrario et al., 2004; Seo et al., 2012). The basic helix-loop-helix protein structure of the K-box domain may be important in the evolution and development of transcription factors (Jones, 2004). In this study, *RcFUL-like*, *RcAGL6-like*, *RcAGL21-like*, *RcAGL24-4-like*, and *RcAGL24-3-like* were identified with only the K-box domain may have new functions in the process of rose evolution. The diversity of sequences of these genes can help determine the evolutionary origin and functional relevance of RcMIKC^C gene duplication. Comparison of Rosaceae species reveals that MIKC^C genes in rose and strawberry are highly homologous, but raspberry lacks many of these homologous genes. There are no orthologous MIKC^C genes on chromosome 1 of raspberry, indicating that their production is independent.

Gene structure analysis revealed many MIKC^C genes with only one exon, and these are concentrated in the AGL15, AGL17, and GGM13 subfamilies (Figure 3A). The expression of these intron-less genes was not detected in leaves, floral buds, or petals of rose (Figure 4). In mammals, intron-less genes are expressed at lower levels, tend to be tissue specific, and evolve significantly faster than intron-containing genes (Shabalina et al., 2010). Intron-less genes often act in processes related to nucleosome assembly, signal transduction, and immunity (Louhichi et al., 2011). Introns increase the length of genes and the frequency of genetic recombination; although intron-less genes have no advantage in species evolution or genetic recombination, they can respond quickly to external abiotic stress (Shabalina et al., 2010; Fan et al., 2021). Currently, whether these rose MIKC^C family intron-less genes are functionally important remains an open question. We also found a gene, *RcAGL24-4*, 18 Kb in length due to a large intron insertion, which may explain its multiple paralogous genes. We classified 42 rose MIKC^C genes into five different categories according to the duplication type in the genome, namely singleton, dispersed, proximal, tandem, WGD or segmental (Supplementary Figure S2). Together, these results revealed that most rose genes have dispersed paralogous genes, indicating the high divergences of RcMIKC^C genes. This may be due to the functional diversity of gene families caused by chromosome rearrangement and fusion (Xu et al., 2012).

Low temperature an important environmental factor affecting floral transition and development (Acosta-Rangel et al., 2021; Jin et al., 2021). Therefore, we further investigated the effect of short-term low temperature on floral organ differentiation. We found that *RhAP1*, *RhFUL*, *RhMADS6-1*, *RhSOC1*, *RhAGL42-1*, and *RhAGL24-3* were significantly up-regulated under short-term low temperature treatment. Of all the RhMIKC^C genes, only significantly up-regulated genes were found, suggesting these genes respond to low temperature and

regulate the development of floral organs. In *Arabidopsis*, the floral meristem recognition genes *AtAP1* and *AtLFY* are required for the transformation of inflorescence meristems into floral meristems (Gregis et al., 2008). Interestingly, *RhLFY* is also up-regulated in the low-temperature transcriptome data, and it regulates *RhAP1* expression as a pioneer transcription factor (Supplementary Figure S5). This may explain the absence of a low temperature response element (LTR) in the *RhAP1* promoter sequence. Some differentially expressed MIKC^C genes lack low temperature response elements, so differential expression may be due to the rapid cascade response of upstream transcription factors.

Previous studies have shown that *RhAG* (class C gene) expression was significantly reduced under low temperature treatment, promoting the stamen to petal transition (Zhang et al., 2019). However, the significantly inhibition of *RhAG* by low temperature was not detected in our results (Figure 5A; Supplementary Figure S5), which may reflect a difference in the treatment time or temperature. The up-regulation of class A genes (*RhAP1* and *RhFUL*) may play a key regulatory role in floral organ identity and development. Interestingly, a complex interaction network was constructed through orthologous genes among RhAP1, RhFUL, RhMADS6-1, RhSOC1, RhAGL42-1 and RhAGL24-3 (Figure 6). Dimerization between proteins can promote their ability to bind as transcription factors. These results provide a reference to explore the mechanisms related to floral organ differentiation and development under low temperature.

To understand in more detail the process of genome duplication in Rosaceae, we determined the number of MIKC^C genes in the AG, SQUA, AGL2, DEF and GLO subfamilies (ABCDE genes) of Rosaceae. Except for the B class, ACDE class genes showed significant gene duplication in *Pyrus* and *Malus* compared to the other Rosaceae species (*Rosa*, *Fragaria*, *Rubus*, and *Prunus*). This analysis indicated significantly increased numbers of MIKC^C genes in pear and apple. Peach, pear, and apple have experienced whole genome duplication events, and the collinearity relationship was observed from raspberry to peach with doubling of orthologous genes (Figure 7B). Although gene duplication occurred, the MIKC^C genes were highly conserved compared to rose and strawberry.

MIKC^C genes play central regulatory roles in flower transition and flower development in many plant species. Although MIKC^C genes have been studied for many years, the biological functions and regulatory mechanisms of these genes in the processes of flowering transition and floral organogenesis are not fully understood, especially in Rosaceae. Here, the MIKC^C genes in rose were comprehensively analyzed from gene structure, promoter elements, expression patterns and protein interaction to explore the roles of these genes in flower organogenesis and development in Rosaceae. This study provides a basis for marker development and targeted gene editing for future breeding efforts to enhance the ornamental value of rose.

Data availability statement

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 below: https://www.ncbi.nlm.nih.gov/, SRR18440429-SRR18440424.

Author contributions

XZ conceived and designed the experiments. YW, TY, YL and JlH performed the experiments and analyzed the data. JnH and NM provided technical support and conceptual advice. YW, TY and XZ wrote the article. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.1059925/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Classification of K-box MIKC^C genes by phylogenetic tree. The full-length protein sequences of the RcMIKC^C genes were used to construct the phylogenetic tree using the maximum-likelihood method in IQ-TREE.

SUPPLEMENTARY FIGURE 2
Duplicated genes in rose.

SUPPLEMENTARY FIGURE 3
Gene structure and promoter response element analysis of RcMIKC^C genes.

SUPPLEMENTARY FIGURE 4
Gene Ontology molecular function annotation of RhMIKC^C genes identified in leaves, floral buds, and petals transcriptomes.

SUPPLEMENTARY FIGURE 5
The expression levels of *RhAG* and *RhLFY* under short-term low temperature stress.

SUPPLEMENTARY FIGURE 6
Statistics on the number of ABCDE genes in Rosaceae.

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Characterization of *Cyclamen* genotypes using morphological descriptors and DNA molecular markers in a multivariate analysis

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Morphological and molecular characterization of germplasm is essential for the improvement of cultivated plants efforts. This study investigated the genetic diversity of 32 *Cyclamen* genotypes comprising 16 *C. persicum* varieties and 16 *Cyclamen* species using multivariate analysis for 36 morphological traits (19 quantitative and 17 qualitative) and molecular characterization (SRAP and SCoT markers). The color CIELab parameters were collected *via* PCE-CSM7 that separately measured the leaves dark and silvery patterns and the flower's slip (region of the petal top), eye (the region of the petal base) and sepal. Genetic diversity was also evaluated using Shannon Wiener (H′) and Simpson's (λ) Indices, and Pilou evenness (J) using the library vegan from R software. According to the principal component analysis, the variables that contributed the most were leaf pattern color, leaf abaxial surface color, pedicel coiling, leaf and stem number. The color indicators of *Cyclamen* leaves showed decreased L* values in darker colored genotypes, whereas increased a* values were noticed in flower eye and lower in slip. Molecular characterization was based on 26 SRAP and 12 SCoT markers that produced clearly repeatable DNA bands and exhibited significant levels of polymorphism. Based on the morphological traits and molecular markers data, the UPGMA method for hierarchical clustering technique was used to generate the dendrograms, and their entanglement was obtained using the Tanglegram algorithm from the dendextend package with the R software. Entanglement analysis (0.30) between dendrograms obtained from the morphological and genetic analysis using SRAP markers showed a high association. Comparison between color measurements of flowers (entanglement=0.45) and leaves (entanglement=0.47) with SCoT analysis revealed differences at species level, discriminating between similar genotypes. Combined phenotypic and molecular analysis improved the comprehensive estimation of real diversity in the investigated *Cyclamen* genotypes. The findings of the present study are useful for quantifying diversity and genetic variability in *Cyclamen* breeding and genetic investigations.

KEYWORDS

***Cyclamen*, cluster analysis, genetic diversity, morphological characterization, molecular markers**

1 Introduction

Cyclamen sp. are part of the Primulaceae family, being widely cultivated throughout the Mediterranean area as an ornamental plant, but also for its pharmaceutical properties. Of the 24 known species, *Cyclamen persicum* Mill. is distributed from south-central Turkey to Lebanon-Syria, being the most significant in terms of production, because of its adaptability to new ecological conditions. *C. persicum* is a much admired ornamental plant of high economic importance, especially in the Netherlands, Germany and Italy. *Cyclamen* is particularly essential in traditional and modern medicine, in addition to its decorative and commercial importance (Sarikurkcu, 2011; Turan and Mammadov, 2018; Cornea-Cipcigan et al., 2019). *Cyclamen* possesses cultural and religious relevance, symbolizing empathy and devotion in the Mediterranean culture, is cultivated in Islamic churchyards and monasteries, and is a consecrated flower in Japan (Grey-Wilson, 2015). In addition to their ornamental importance, *Cyclamen* sp. also possesses strong antioxidant properties due to the phenolic composition, and anti-cancer activities reported in HeLa, non-small cell lung cancer H1299 cells, human colorectal cancer cells (HCT 116 and HT-29), human breast adenocarcinoma (MDA-MB-231) and human fibroblasts BJ cells (Mihci-Gaidi et al., 2010a; Mihci-Gaidi et al., 2010b; Yildiz et al., 2013; Cornea-Cipcigan et al., 2022a). Due to a shortage of plant material, their use for the production of pharmaceuticals has become a severe problem, potentially leading to the loss of plant populations and variety, natural habitat deterioration, and/or species extinction. Classification of morphological traits has been done in only a few *Cyclamen* species endemic to Turkey (Curuk et al., 2015; Curuk et al., 2016).

The materials preserved in gene banks, such as wild, cultivated and selected accessions represent important sources of variability for breeders. Thus, breeders have used a variety of approaches throughout the years to investigate and assess the level of diversity in plant populations (Bhandari et al., 2017). In *Cyclamen*, genetic variation has been evaluated through morphological characters and molecular markers, such as the internal transcribed spacer regions (ITS) (Anderberg et al., 2000), random amplified polymorphic DNAs (RAPD) (Naderi et al., 2009; Taşkin et al., 2012), and sequence-related amplified polymorphisms (SRAP) (Simsek et al., 2017) (Robarts and Wolfe, 2014; Yagi et al., 2014; Samarina et al., 2021).

As the availability of specific molecular markers for *Cyclamen* is limited, SRAP markers represented the basis of the molecular investigations performed in this study. Also the PCR-based start codon targeted (SCoT) markers which were only used previously on other species were included in the analysis. Both types of markers constitute simple, reproducible and low cost techniques for characterizing germplasm collections (Collard and Mackill, 2008), with the aim of genetic diversity screening (Igwe et al., 2017), species identification or phylogenetic analysis (Jalilian et al., 2018).

Although molecular markers have been extensively used to evaluate species variety, extremely low or insignificant correlations have been observed among dissimilarity matrices generated using both phenotypic and molecular data (Gupta et al., 2018). As a result, if the non-overlapping data is derived from phenotypic and genotypic divergence matrix, combining them may offer a full picture of a population's variety (Houmanat et al., 2021). Multivariate analysis approaches (HCA or PCA), are commonly used to precisely classify various plants based on their agro-morphological, molecular, chemical composition, or bioactivities that are regularly compared with correlation coefficients (Granato et al., 2018). To accurately evaluate the genetic variation among individuals, it is important to choose a suitable dissimilarity coefficient and hierarchical clustering method since both have an impact on the outcomes of genetic diversity analysis (Darkwa et al., 2020). Using both agronomic variables and molecular markers may contribute to a more comprehensive genetic diversity analysis within and among species, phylogenetic investigations, and fingerprinting in various plants (Jalilian et al., 2018; Long et al., 2020). Consequently, to evaluate the genetic diversity of *Cyclamen* genotypes, joint hierarchical cluster analysis was constructed between phenotypic and molecular data. Choosing the most suitable clustering method is significant to determine proper genetic dissimilarity and diversity between and within populations and clustering, since different correlations may give inconclusive or dissimilar results. To best of our knowledge, comparison between clustering methods based on phenotypic and molecular markers (SRAP and SCoT markers) has not been employed in *Cyclamen* until now. Our studies objectives were (1) the comparison of several correlation matrices and HCA for investigating genetic diversity in *Cyclamen*, and (2) the evaluation of genetic diversity and divergence in *Cyclamen* genotypes by the use of morphological, molecular, and combined data.

2 Materials and methods

2.1 Plant material

Seeds of *Cyclamen* genotypes were germinated on two-layer filter paper in Petri dishes with distilled water, as previously reported (Cornea-Cipcigan et al., 2020). After germination (~5 weeks), the seedlings were transplanted into pots and watered when necessary. The growing substrate (50/20/20/10 *v/v*) was a mixture of sowing and propagation soil (pH = 6.0) with NPK (0.1:0.01:0.03 m/m%), *Cyclamen* substrate (pH = 6.2) with NPK (1.0:0.1:0.3 m/m%), organic substances (70%) and perlite. The average greenhouse temperature was 18-22°C and 60% relative humidity. The quantitative variables were scanned and measured using the ImageJ Programme (v1.52a, Wayne Rasband, National Institutes of Health Bethesda, Maryland, USA) for image processing after plants reached maturity. Details regarding breeding companies and genotype characteristics are shown in Supplementary Table S1.

2.2 Phenotyping

Thirty-six morpho-agronomic characters were measured for the 32 *Cyclamen* genotypes (Figure 1), from which 19 were quantitative and 17 qualitative. The quantitative variables assessed included number of stems/plant (NS), petiole diameter (PD), petal length (PL), petal width (PW), flower area (FL), pistil length (PIL), stamen length (SL), flower numbers (FN), pedicel length (PEDL), leaf number (LN), lamina length (LL), lamina width (LW), leaf length/width ratio (LL/W ratio), petiole length (PETL), leaf shape (LS), leaf margin

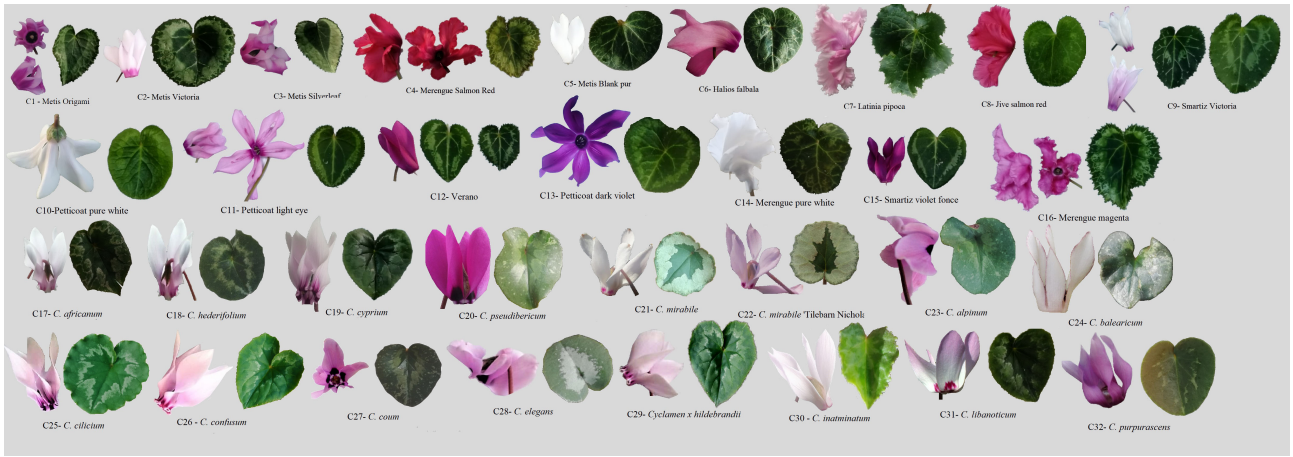

FIGURE 1
Cyclamen genotypes used in the study. Lane C1-C16, *C. persicum* cultivars, C17-C32, different *Cyclamen* species.

(LM), leaf pattern area (LPA), leaf area (LA) and canopy area (CA) were recorded at maturity. The qualitative variables included the petiole color (PETC), plant vigor (PV), flowering intensity (FI), petal color at flowering (PCF), petal color at senescence (PCS), basal corolla ring color (BCPR), color of upper corolla (CUP), color of lower corolla (CLC), basal corolla ring diameter (BCRD), pedicel coiling (PEC), darker petal margin (DPM), stigma position (SP), lamina (degree of lobbing, LD), leaf pattern (LP), leaf patter color (LPC), leaf abaxial surface color (LASC), canopy architecture (CAR) were recorded at maturity. The quantitative and qualitative variables are shown in Supplementary Table S2.

2.3 Color measurements

To estimate the color of *Cyclamen* flowers and leaves a portable colorimeter PCE-CSM7 (PCE instruments, Meschede, Germany) was used. According to the International Commission of Illumination's (CIE), the color spaces were expressed as L^*, a^*, b^*, color intensity and shade, where L^* expresses the sample's lightness (100) to darkness (0), a^* represents the greenness (negative) to redness (positive) degrees and b^* the blueness (negative) to yellowness (positive) degrees. Intensity of color (C, chroma) and shade ($H°$, hue angle) (Clydesdale and Ahmed, 1978) were generated by the use of the equations shown below:

$$C = \left[(a^*)^2 + (b^*)^2\right]^{0.5}$$

$$H° = tan^{-1}\frac{b^*}{a^*}$$

DNA extraction was carried out using lyophilized *Cyclamen* leaves with the CTAB-based method by Lodhi et al. (1994) with slight modifications. NanoDrop-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA) was used for evaluating the purity and concentration of genomic DNA (absorbance ratios 260/280, 260/230).

2.4.1 SRAP analysis

A total of 36 primer combinations were tested, from which 26 combinations showed higher polymorphism levels and were selected for further analysis. Forward and reverse SRAP primer sequences are shown in Table 1. For PCR reactions were used 5x green PCR buffer, 0.6 M of each primer (Bioline GmbH, Luckenwalde, Germany), $MgCl_2$ (1.5 mM), dNTPs, Taq DNA polymerase (1 U) (Promega, Madison, Wisconsin, USA) and genomic DNA (50 ng). Amplification of DNA was performed in a 96-well Eppendorf Mastercycler Nexus PCR Cycler (Sigma-Aldrich GmbH, Darmstadt, Germany) as described by (Li and Quiros, 2001) and (Simsek et al., 2017) with minor changes: denaturation at 94°C for 5 min, followed by five cycles at 94°C, annealing at 35°C and elongation at 72°C for 60 s, followed by 35 cycles at 94°C, 50°C and 72°C for 60 s with a final elongation at 72°C. Separation of amplicons was performed on agarose gels (2%) (Promega, Madison, Wisconsin, USA) in 1 x TAE, at 0.29 V/cm^2 for 1.5 hours, and detected by EtBr staining (Sigma-Aldrich GmbH, Darmstadt, Germany), using a 50 bp DNA ladder (GeneDireX, Inc., Taoyuan, Taiwan). PCR amplifications were performed in duplicate.

2.4.2 SCoT analysis

A total of 20 SCoT primers were evaluated, from which 12 generated clear bands and enabled the selection of consistent and rich polymorphisms. SCoT primer sequences are shown in Table 2. PCR reaction mixtures comprised Green Master Mix (Promega), 20 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 0.24 mM dNTPs, Taq polymerase (0.5 U) (Promega), primer (0.8 µm) and template DNA (25 ng). Amplification was performed using the protocol described by (Collard and Mackill, 2008), with few modifications: denaturation at 94°C for 3 min, followed by 35 cycles of 94°C, 50°C, and 72°C for 1 min, and a final extension at 72°C for 5 min. PCR amplification products were separated as described in the previous sub-section, using a 100 bp DNA ladder (GeneDireX, Inc. USA).

TABLE 1 Forward and reverse SRAP primer sequences used in the study.

SRAP forward primer	Sequence (5′-3′)	Tm (°C)	GC content (%)
me1	TGAGTCCAAACCGGATA	48.8	47.1
me2	TGAGTCCAAACCGGAGC	54.7	58.8
me3	TGAGTCCAAACCGGAAT	49.8	47.1
me4	TGAGTCCAAACCGGACC	54.4	58.8
me5	TGAGTCCAAACCGGAAG	51.2	52.9
me6	TGAGTCCAAACCGGACA	52.9	52.9
SRAP reverse primer			
em1	GACTGCGTACGAATTAAT	45.6	38.9
em2	GACTGCGTACGAATTTGC	51.3	50.0
em3	GACTGCGTACGAATTGAC	50.4	50.0
em4	GACTGCGTACGAATTTGA	49.0	44.4
em6	GACTGCGTACGAATTGCA	52.5	50.0
ba1	GTCGAGCTGCCAATTATA	48.3	44.4

2.5 Data analysis

2.5.1 Molecular marker analysis

Clearly and repeatable SCoT and SRAP segments were evaluated as present (1) or absent (0). The polymorphic bands number (PB) and the ability to reveal dissimilarity in SRAP and SCoT markers were evaluated by calculating the polymorphism, diversity index (H), polymorphic information content (PIC), effective multiple ratio (E), marker index (MI), discriminating power (DP), band informativeness (Ib) and resolving power (RP).

H was calculated as follows:

$$H = 1 - \sum \; p_i^2$$

where p_i is the allele frequency for the i^{th} allele (Liu, 1998).

PIC of each SRAP and SCoT primers pair was estimated accordingly:

$$PIC = 1 - \left(\sum_{i=1}^{n} p_i^2\right) - \left(\sum_{i=1}^{n=1}\sum_{j=i+1}^{n} 2q_1^2 q_j^2\right)$$

where n is allele number (marker), q_i is the i^{th} allele frequency, and q_j is the j^{th} allele frequency (Botstein et al., 1980).

The effective multiple ratio (E) was calculated according to (Powell et al., 1996), as follows:

$$E = n\frac{n_p}{n_p + n_{np}}$$

TABLE 2 SCoT primer sequences used in the study.

SCoT primer	Sequence (5′-3′)	Tm (°C)	GC content (%)
SCoT 1	CAACAATGGCTACCACCA	52.0	50
SCoT 2	CAACAATGGCTACCACCC	53.5	55.6
SCoT 3	CAACAATGGCTACCACCG	53.7	55.6
SCoT 4	CAACAATGGCTACCACCT	51.8	50.0
SCoT 5	CAACAATGGCTACCACGA	52.0	50.0
SCoT 6	CAACAATGGCTACCACGC	54.3	55.6
SCoT 7	CAACAATGGCTACCACGG	53.7	55.6
SCoT 8	CAACAATGGCTACCACGT	52.3	50.0
SCoT 13	ACGACATGGCGACCATCG	58.3	61.1
SCoT 16	ACCATGGCTACCACCGAC	57.5	61.1
SCoT 30	CCATGGCTACCACCGGCG	62.8	72.2
SCoT 33	CCATGGCTACCACCGCAG	59.5	66.7

where p and np represent the polymorphic and non-polymorphic markers fraction.

MI was calculated (Powell et al., 1996; Amiryousefi et al., 2018) as follows:

$$MI = PIC \ x \ PB$$

DP was calculated as follows:

$$DP = 1 - \sum \ p_i \ \frac{Np_i - 1}{n - 1}$$

where the i^{th} pattern of the particular j^{th} primer, present at frequency p_i in a set of varieties, and N as individuals, according to (Tessier et al., 1999).

The band informativeness (Ib) was calculated accordingly:

$$Ib = 1 - (2 \ x \ |0.5 - p_i|)$$

where p_i is the band frequency amplification (Prevost and Wilkinson, 1999).

RP was calculated as follows:

$$R = \sum \ Ib$$

Additionally, the Shannon Wiener (H′) (Shannon, 1948), Simpson's Indices (λ) and Pilou evenness (J) were calculated to evaluate the genetic diversity and assessed using library vegan from R (version 4.2.2) (Oksanen et al., 2019).

2.5.2 Multivariate analysis of phenotypic, colorimetric, and genotypic data using hierarchical clustering.

Principal component analysis (PCA) was performed using the FactoMiner factoextra package (Lê et al., 2008). The unweighted pair group method with arithmetic mean (UPGMA) was used to construct hierarchical cluster analysis (HCA) based on similarity matrices (Euclidean distance) between the morphological, colorimetric and genetic analysis. Euclidean distance was estimated using the Cluster R package (Maechler et al., 2019). The dendextend program was used to display tanglegrams, visual approaches for comparing two trees with the identical set of labels connected by lines (Galili, 2015). The entanglement coefficient ranges between 0 (complete entanglement) and 1 (no entanglement), and was used to evaluate the effectiveness of the alignment of the two trees; a lower coefficient corresponds to a good alignment. Using color gradients for dissimilarity determination across individuals, visual representations of matrices for the morphological and molecular data were generated using the corrplot package in R.

3 Results

3.1 Principal component analysis and clustering pattern based on morphological diversity

The PCA results revealed that the first ten PC, with eigenvalues ranging from 1.19 to 7.89, were significant in explaining the variation between the evaluated *Cyclamen* species, accounting for 82.98% of the total variation. (Supplementary Table S3; Figure 2). The 1st PC accounted for 21.79% variation and pointed out dissimilarities in NS, FI, FN, PL, SL, PEL, PEC, FA, BCRD, and LN, representing traits associated to flower development, along with CAR and CA associated to plant development. The 2nd PC accounted for 13.78% of the variance and correlated with PCF, PCS, BCRC, CUP, CLC and LPC, suggesting the relation with flora color traits. The 3rd component highly correlated with leaf characteristics, mainly LL, LPA and LA and explained 9.76% of the total variance. The 4th and 5th PCs accounted for 9.31% and 6.59% of the total variance, and explained variation in LL, LW, LM, DPM and LP, respectively. Phenotypic variations of the 36 evaluated traits were assessed (minimum, maximum, median, mean, and Kurtosis variation) and are presented in Supplementary Table S4. As seen in Figure 1A, the first quadrant highlights the LPC in genotypes 21 (*C. mirabile*), 24 (*C. balearicum*), and 25 (*C. cilicium*) with a silver green pattern. The second quadrant highlights samples 7 and 10 that presented the highest pedicel length. The third emphasized the light purple upper corolla of *C. persicum* cv. Petticoat Dark Violet (13). The last quadrant, emphasized the genotypes 27 (*C. coum*), 28 (*C. elegans*) and 32 (*C. purpurascens*) with a red-purple abaxial surface color.

HCA was performed to better discriminate the genotypes based on their morphology. Thus, the grouping pattern of the *Cyclamen* species for morphological diversity using Euclidean distance with UPGMA algorithm for clustering revealed four major clusters (Figure 2C). Cluster I (red) highlights four *C. persicum* genotypes (4, 6, 7 and 13) with *C. africanum*, *C. hederifolium*, *C. pseudibericum*, *C. alpinum* and *C. hildebrandii*. These genotypes presented medium canopy architecture, a similar petal color (light carmine), leaf width/width ratio, medium pedicel length and a high plant vigor. Cluster II (brown) highlights genotypes 14, 15 and 16 (Merengue group and Smartiz type) with the highest canopy area, petiole diameter and similar flower area dimensions. Cluster III (green) comprised a single *C. persicum* genotype (12-Verano) and five species, namely *C. coum*, *C. elegans*, *C. intaminatum*, *C. libanoticum*, and *C. purpurascens*. These genotypes presented a light purple color at flowering and a deep purple at senescence, similar color of upper and lower corolla, lower canopy area and the absence of leaf pattern. The largest number of genotypes was identified in cluster IV (purple) with 14 genotypes. Out of them, eight were *C. persicum* accessions comprising the Metis group (1-3, 5), Victoria (9), and Petticoat group (10, 11), whereas the remaining genotypes were *C. balearicum*, *C. cilicium*, *C. confusum*, *C. cyprium*, *C. mirabile*, and *C. mirabile* 'Tilebarn Nicholas'. Genotypes in cluster IV presented a paler-pink petal color and upper corolla, along with a lower flower number, but a medium to strong degree of lobbing in leaf lamina.

3.2 Color distribution among different parts of Cyclamen leaves and flowers

A phenotypic trait that commonly reflects the plants physiological condition is the color of the flowers and leaves. Furthermore, the variation in pigment provides information on the genetic intra- and interspecific taxa and/or populations variability, recognizing the advancement of evolutionary ideas from a phylogenetic perspective. The color indicators of the green, silvery lamina and petiole are presented in Supplementary Figure 1, whereas the indicators of the

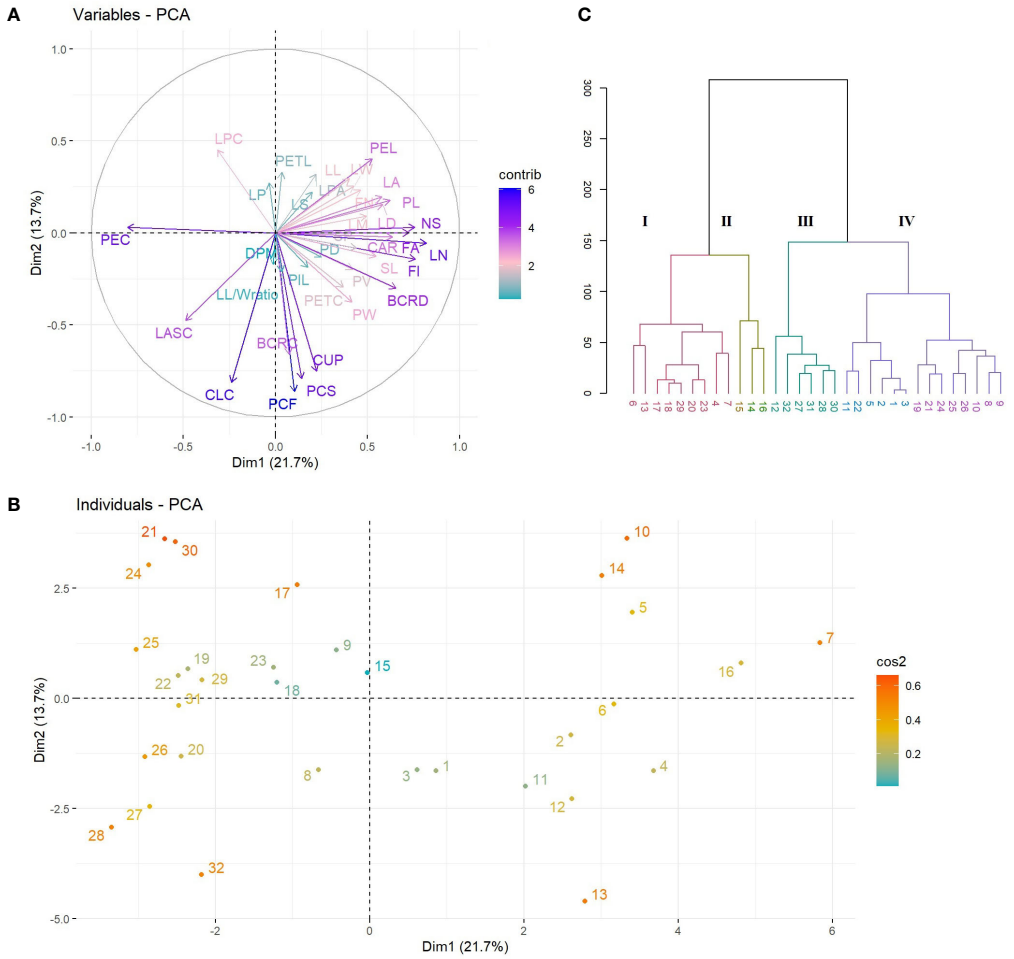

FIGURE 2
PCA plots of variables **(A)** and species **(B)**. The first two dimensions accounted for 35% of full variance. HCA of *Cyclamen* genotypes based on morphological data (Euclidean distance, r=0.73); each color represents a different cluster).

slip, eye, and petiole are presented in Supplementary Figure 2. Variation in color parameters were assessed and are presented in Supplementary Tables S5, S6. In flowers, the luminance L* ranged between 10.0 (eye, C20) and 99.7 (slip, C24). The redness a* between -8.6 in sepal in C6 and 72.8 in C9 (slip) denoting a tendency of red and purple pigment accumulation, and the yellowness b* with the lowest value of -39.3 (C28) in slip and the highest in sepal 43.8 in C30. Regarding the leaves, the L* ranged between 12.9 (C5) and 87.6 (C24). The redness a* varied between -32.2 (C7) and 33.8 (dark pattern, C22), whereas b* ranged between -8.2 (C22) and 42.6 (petiole of C18).

The grouping pattern of *Cyclamen* flowers (Figure 3A) revealed four major clusters, where the first cluster (red) grouped *C. mirabile* (21) and *C. purpurascens* (32). These genotypes presented similarities in terms of L* and hue in the upper part of the petal (slip), a* and hue in sepal and b* and hue in petiole. The following cluster (brown) comprised the largest number of genotypes which presented similar a* and b* values in slip, higher hue level in the lower part of the petal (eye), higher a*, lower b* in sepal and lower hue in petiole. The subsequent cluster (green) comprised lighter-colored samples as seen by the higher values in L*. The last cluster (purple) highlighted two *C. persicum* genotypes, *C. cilicium* and *C. hildebrandii*. These genotypes had the lowest values in hue angle and the lowest in a* and b* in the eye of flower, and similar b* values in flower pedicel. The last two clusters outlined the dark red *C. persicum* genotypes (8 and 4), that were closely followed by the grey-white genotypes 10 and 14 due to their distinct color characteristics compared with the other genotypes with pink and purple color patterns.

The *Cyclamen* leaves (Figure 3B) were organized into two major clusters except *C. mirabile* 'Tilebarn Nicholas' (22), considered an 'outlier' due to its distinct purple-green color pattern. The first major branch (cluster I, brown) includes genotypes *C. persicum* 'Petticoat pure white' (10) and *C. cilicium* (25) with similar L*, b* and C* of the green and light-green patterns. Subsequently, cluster II (olive) comprised the majority of *C. persicum* genotypes, with similarities in a* and hue in darker green lamina, together with b*, C* and hue values in silvery lamina. The following cluster III (green), mainly grouped the genotypes with similar a* (darker green lamina), chroma and shade in leaves, along with similarities in terms of petiole hue. The last, cluster IV (purple) emphasized *C. elegans* (28) and *C. libanoticum* (31) that presented similar color in petiole and in light silver and green patterns. *C. cyprium* (19), *C. balearicum* (24), *C. confusum* (26), and *C. coum* (27) presented similar petiole color characteristics in terms of L*, a* and C*.

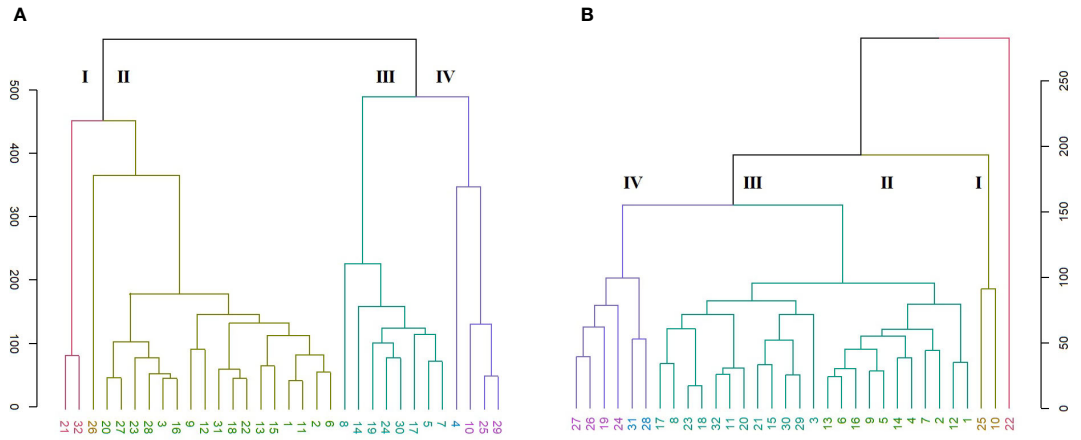

FIGURE 3
HCA of color characteristics of *Cyclamen* genotypes flowers (A; *r*=0.92) and leaves (B; *r*=0.96).

3.3 Summary statistics and clustering pattern of Cyclamen genotypes based on molecular diversity indices

3.3.1 SRAP analysis

Among the evaluated 36 primer combinations, 26 produced higher polymorphism, with a total of 349 bands of which 264 showed higher polymorphism. The polymorphic bands per primer combination ranged from 5 (me1-em3) to 15 (me2-em4, me4-em6 and me4-em6), with an average of 10.1. The percentage of PB in each primer varied between 56.25 and 88.23%, with an average of 75.0%. Three representative profiles (me1-em4, me1-em6, me2-em3) are shown in Figure 4.

Indicators including H, PIC, E, MI, D, and R were used to evaluate the polymorphisms and discriminatory capacity of the tested markers. H had an average value of 0.30 with the highest observed in me1-em2. The mean values of E, MI, D and R were 2.91, 2.78, 0.93 and 5.35, respectively (Supplementary Table S7).

3.3.2 SCoT analysis

The selected 12 primers generated 204 reliable SCoT bands, from which 169 proved to be polymorphic. The PB per primer ranged from 4 (SCoT5) to 19 (SCoT13 and SCoT30), with an average of 14.1. PB percentage varied between 71.42 and 90.0%, with a mean value of 82.43%. Two representative profiles (SCoT 6, and SCoT 13) are shown in Figure 5. The mean value of H was 0.38, with a range between 0.499 in SCoT4 and 0.173 in SCoT5. PIC had a mean value of 0.33 with the highest in SCoT 2. The average values of E, MI, D and R were 3.19, 4.93, 0.93 and 4.35, respectively (Supplementary Table S8).

In the SRAP analysis, the HCA organized the genotypes in four main clusters (Figure 6A). A clear discrimination is seen between the *C. persicum* genotypes and other *Cyclamen* species. Cluster I (red) comprised *C. elegans* (28) as an 'outlier', mainly due to its silvery patterned leaves, closely followed by *C. purpurascens* (32), *C. alpinum* (23), *C. coum* (27), *C. x hildebrandii* (29) and *C. libanoticum* (31) with pink colored flowers, magenta blotches and silver speckled leaves. This cluster also comprised *C. mirabile* (21), *C. mirabile* 'Tilebarn Nicholas' (22) and *C. intaminatum* (30), with similar flowers and lower corolla diameter, followed by *C. balearicum* (24), *C. cilicium* (25), and *C. confusum* (26), with similar white to pinkish flowers and morphology. Cluster II (brown) consisted of Metis and red-colored *C. persicum* types. Cluster III (green) comprised the Petticoat types (10, 11, 13) and *C. cyprium* (19). The last cluster (IV, purple) included two *C. persicum* genotypes (7 and 15) and *C. pseudibericum* (20).

The SCoT analysis organized the genotypes mostly by their color characteristics, but also on their habitat as described by breeding companies (Figure 6B). Thus, cluster I (red) comprised genotypes 22 (*C. mirabile* 'Tilebarn Nicholas'), 31 (*C. libanoticum*), and 32 (*C. purpurascens*), probably due to their distinct leaf color pattern (22 and 32), redish eye and pink-colored petal margins. The *C. persicum* genotypes 4, 5, 7 presented dissimilar flower characteristics and leaf color, but proved to be resistant to *Botrytis cinerea* (gray mold). Cluster II (brown) comprised *C. pseudibericum* (20) and *C. coum* (27), with large carmine flowers,white rim and a dark stain around the mouth and leaves marked with a hastate pattern, speckling, or blotching of silver and green. Genotypes *C. africanum* (17), *C. mirabile* (21), and *C. balearicum* (24) presented similarities in flowers with white to pale pink. Furthermore, these genotypes bloom prior to leaf emergence particularly in autumn and spring. Cluster III (green) comprised genotypes *C. alpinum* (23), *C. elegans* (28) with pink flowers, a deep magenta blotch and round leaves with silver markings, along with *C. x hildebrandii* (29), *C. intaminatum* (30) and *C. persicum* accessions 2, 6, and 12 with pink hues. Genotypes 2, 6 and 12 are resistant to both winter and summer conditions and according to breeding companies exhibit outstanding outdoor performances. Cluster IV (purple) comprised the Merengue and Petticoat *C. persicum* accessions, with fringed and fragrant flowers. Furthermore, genotypes 1, 9, 14 and 15 are resistant to high temperatures. The following *C. hederifolium* (18), *C. cilicium* (25) and *C. confusum* (26) with similar flower colors and leaf patterns, bloom in the winter and are resistant to rainy days.

In order to obtain more accurate genetic estimates, combined analysis was carried out using all molecular data (Figure 6C). The dendrogram grouped the individuals into four main clusters. Cluster I (red) comprised genotypes 22, 31 and 32 similar to SRAP analysis. Clutser II (olive) organized the genotypes that are part of the

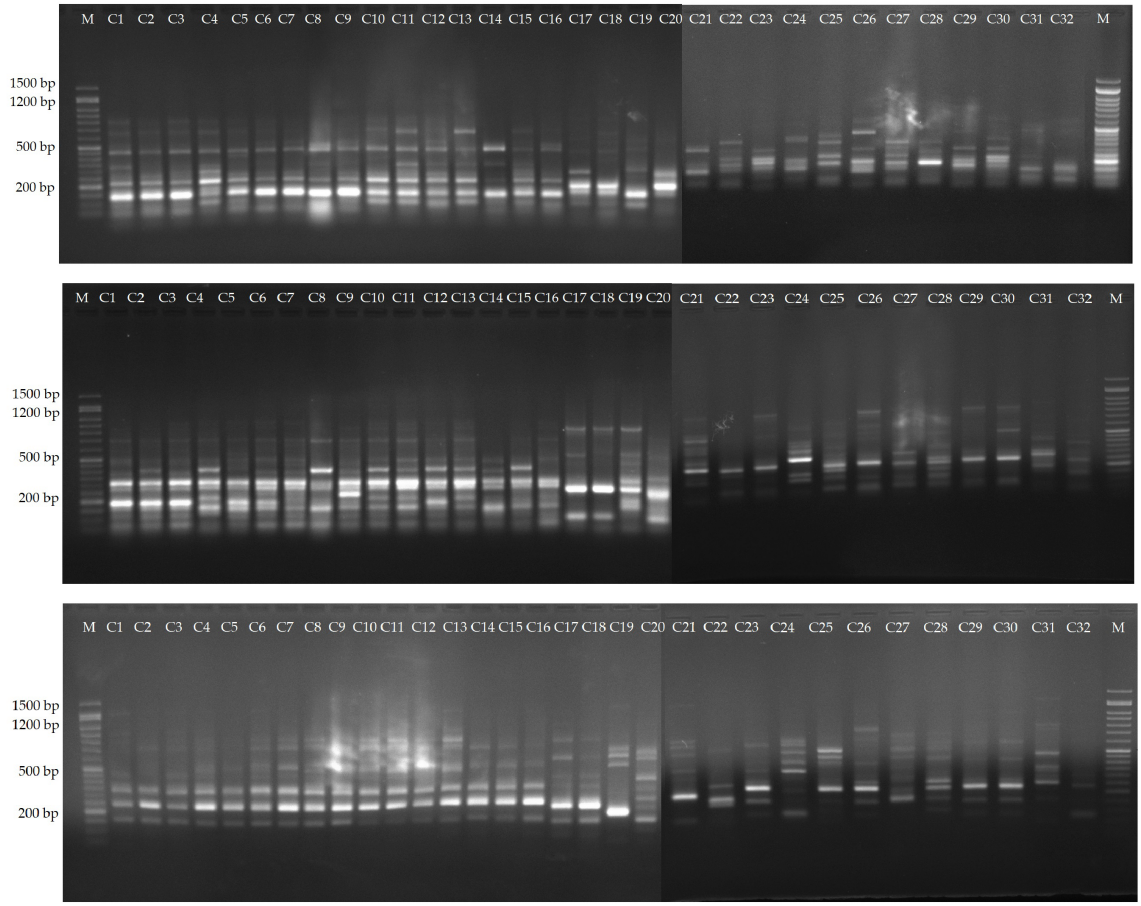

FIGURE 4
Amplification profiles of markers me1-em4, me1-em6 and me2-em3. Lane 1-16, *C. persicum* cultivars, 16-32, different *Cyclamen* species.

subgenus *Gyrophoebe*, except *C. balearicum* (24) (subgenus *Psilanthum*). Cluster III (green) comprised genotypes *C. africanum* (17) and *C. hederifolium* (18) part of subgenus *Cyclamen* together with two *C. persicum* accessions (Merengue type) and *C. pseudibericum* (20) that corresponds to subgenus *Gyrophoebe*. Cluster IV (purple) comprised *C. persicum* genotypes with Metis and Smartiz types in the same sub-cluster followed by the Petticoat types in the following sub-cluster.

Table 3 displays the most frequently used indexes calculated for 32 *Cyclamen* genotypes based on morphological and molecular data. The Shannon-Wiener index was generally high in the SRAP analysis, with the highest value in *C. libanoticum* (31) and the lowest in *C. persicum* accession Smartiz (15), along with the color characteristics that had the highest value in *C. persicum* Metis Vitoria (2) and the lowest in *C. purpurascens* (32). The combined molecular data revealed the highest *H'* value in *C. persicum* Jive (8) and the lowest in *C. purpurascens*. Comparatively, the phenotypic data and SCoT analysis presented relatively close lower values. The same similarities were observed in the Simpson's indices. Conversely, the Pilou evenness index was generally high in color parameters in *C. purpurascens* (32) with the lowest values observed in *C. hederifolium* (18).

Assessment of genetic data revealed low variability among the evaluated genotypes with few exceptions' seen by the different color gradient (Figure 7A). Conversely, the genetic data was high among *Cyclamen* genotypes and lower among *C. persicum* accessions as revealed by the higher ginger squares number in Figure 7B.

3.3.3 Genetic diversity using linkage analysis for morphological, colorimetric and molecular data

Entanglement analysis (0.30) among dendrograms obtained from the morphological and SRAP anlayses showed a high association. The highest similarities in joint analysis of morphological data and SRAP analysis were observed in accessions 1 and 3, Merengue types 14 and 16, along with *C. cilicium* (25) and *C. confusum* (26). Accession 6 (Halios falbala) along with *C. africanum* (17) and *C. hederifolium* (18) maintained their position in the same cluster with slight order modifications (Figure 8).

The HCA generated from the phenotypic data were compared with the color characteristics. Higher similarities were noticed when separating the colorimetric data by flowers and leaves. In terms of color characteristics of leaves and phenotypic data (entanglement=0.49), two genotypes maintained the same position in the cluster as seen by the colored lines between the dendrograms in *C. persicum* Halios falbala (6) and Petticoat dark violet (13). Furthermore, similarities were also noticed in Merengue types (4, 7, 15 and 16) that grouped in the same sub-cluster even though not in the same order (Supplementary Figures 3A, B). Most similarities to SCoT analysis were noticed in the colorimetric data for leaves, (entanglement=0.57) in *C. x hildebrandii* (29) and *C.*

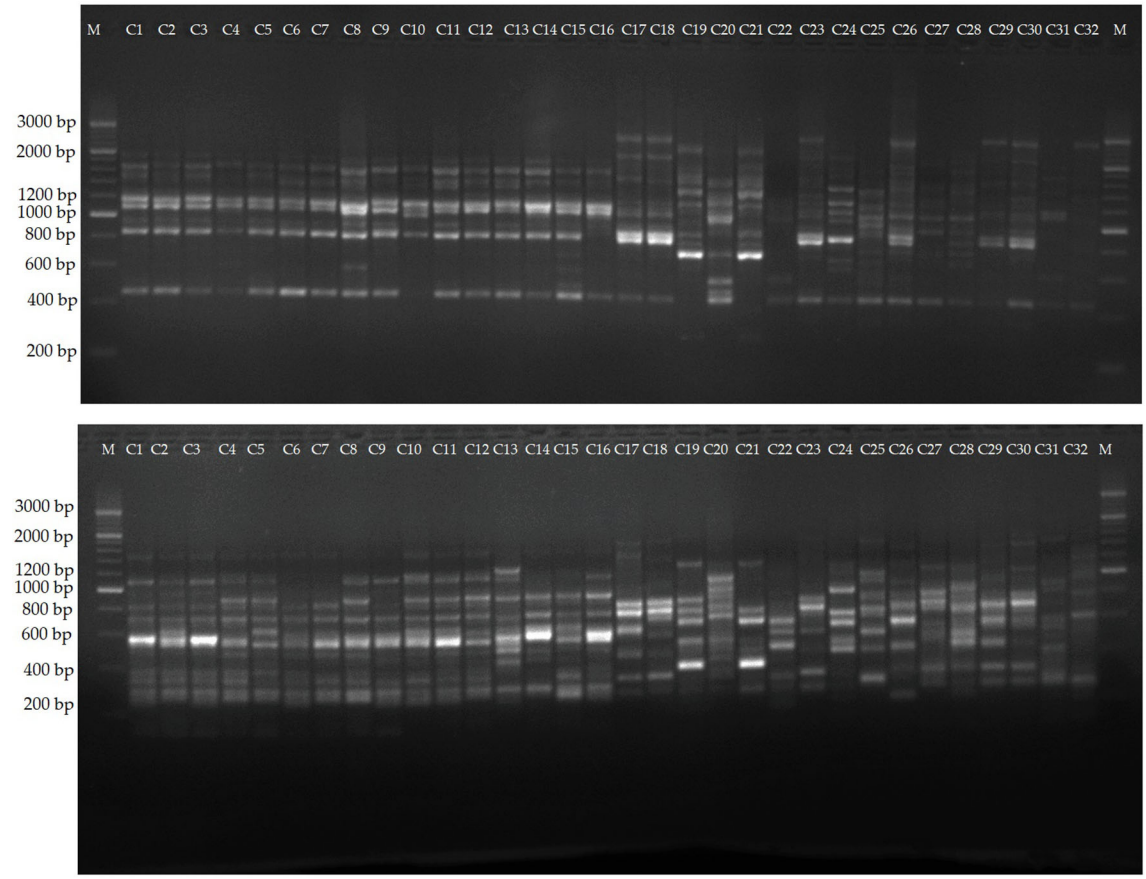

FIGURE 5
Amplification profiles of markers SCoT 6, and SCoT 13. Lane 1-16, different *C. persicum* genotypes, 16-32, different *Cyclamen* species.

intaminatum (30) (Supplementary Figures 3C, D), and flowers, respectively, where two *C. persicum* accessions (2 and 6) maintained the same cluster position (Supplementary Figures 4A, B). Also, genotypes *C. alpinum* (23), and *C. elegans* (28) presented similarities and were grouped in the same cluster even though not in the same order. The HCA generated from the colorimetric analysis of *Cyclamen* flowers compared with SRAP data (entanglement=0.55) presented similarities in two *C. persicum* accessions (9 and 12) which maintained in the same cluster positioning. Genotypes *C. alpinum* (23), *C. coum* (27), *C. elegans* (28), and *C. purpurascens* (32) grouped in the same cluster with small differences in their position (Supplementary Figures 4C, D). The grouping pattern of the molecular markers used (SRAP and SCoT) presented low similarities with no entanglement (data not shown), which is not unexpected as these primers are not specific for *Cyclamen*.

4 Discussion

Prior research has found considerable morphological differences between closely related accessions or populations, due to the climatic conditions of various habitats, and lower heredity of reproductive and vegetative traits in *Cyclamen* (Yesson and Culham, 2011; Cornea-Cipcigan et al., 2022b). In the present study, PCA was performed to assess the similarities and differences between genotypes using morpho-agronomic characters. PCA generally supported the assemblage of phenotypic traits into species-specific clusters, as seen by *C. persicum* genotypes that mainly grouped in the 2nd and 3rd quadrants. Significant variables composing PC1 were NS, PL, FA, LN, CA, and BCRD as seen by the highest eigenvalues, whereas PC2 accounted for the color characteristics of flowers (Supplementary Table S3). Using the 36 morphological parameters from the first ten PCs, the UPGMA grouped the genotypes based on their canopy architecture and plant vigor in cluster I, lower canopy area and absence of leaf pattern in cluster II. Highest canopy area and similar flower area dimensions were grouped in cluster III, whereas the IVth cluster comprised paler pink colored genotypes with lower flower number, and strong degree of lobbing. These are in accordance with morphological descriptions of different *Cyclamen* species, as the most distinctive variables were found to be quantitative with the highest eigenvalues in the first PCs (Curuk et al., 2015). Several researchers claim that morphological characters are less effective for assessing genetic diversity owing to the shift in environmental conditions and plant growth development; HCA groups the samples mainly based on their morphological characters rather than geographical origin (Shinwari et al., 2014; Curuk et al., 2015; Curuk et al., 2016). Nonetheless, morphological and agronomic characterization is significant for describing plant populations and for selecting varieties with favorable traits for crop development that is of great

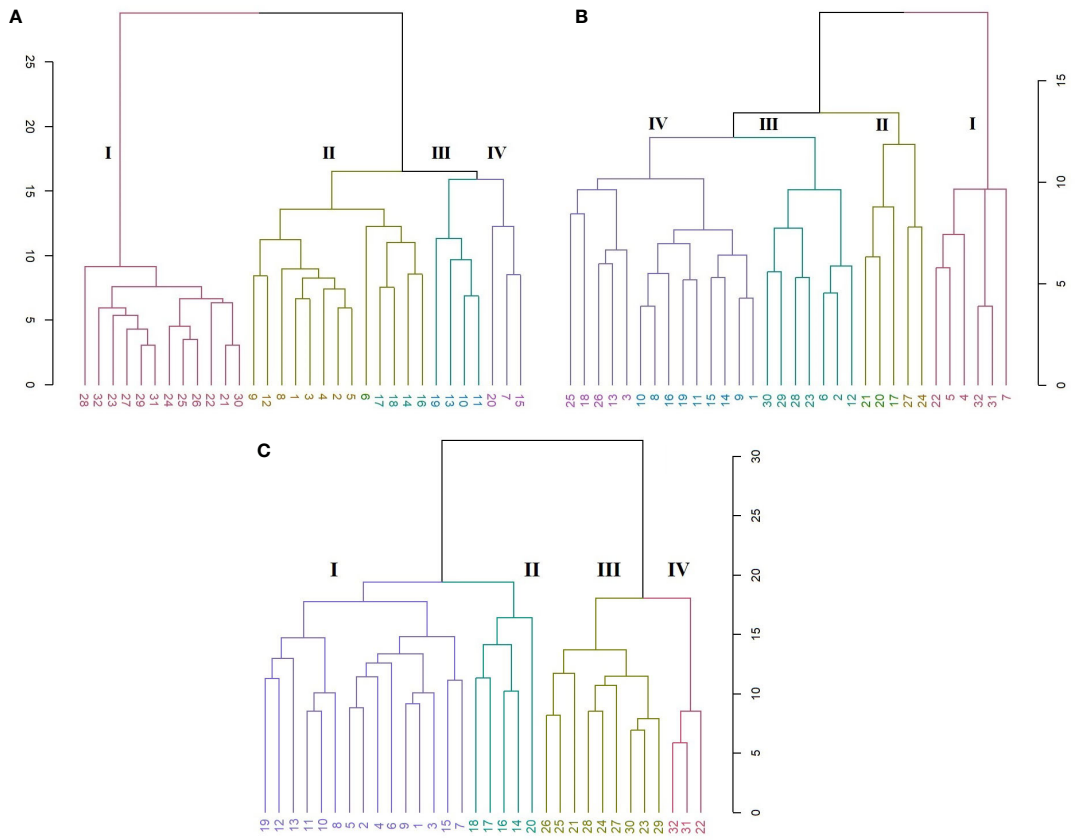

FIGURE 6
HCA representing the genetic relationships among the 32 *Cyclamen* genotypes from the SRAP (**A**; *r*=0.95), SCoT (**B**; *r*=0.82) and combined (**C**; *r*=0.93) analysis. Each color represents a different cluster.

interest for plant breeders. Other classifications, such as chromosome number, duration of green period, early or late blossoming period and flowering habit, disease spot detection and fruit color were successfully assessed in different ornamental and medicinal crops that may demonstrate more complex distribution classification (Chaudhary et al., 2012; Cui et al., 2019; Lu et al., 2021).

A phenotypic trait that often reflects the physiological status of the plant is the color of the flowers and leaves (Noman et al., 2017; Cui et al., 2019). The concentration of secondary metabolites with considerable chromatic elements, that may have decorative, medicinal, and economic relevance, is indicated by the color of the flowers and leaves (Cornea-Cipcigan et al., 2022a). Color variation also gives data on the genetic inter- and intraspecific diversity of populations and/or species, recognizing the progress of evolutionary principles from a phylogenetic standpoint (Tanaka et al., 2008; Klančnik et al., 2016). Color parameters were assessed to evaluate the color of *Cyclamen* flowers and leaves that may aid in differentiating similar varieties or species. Genotypes that presented higher values in L* grouped in the same cluster (cluster II, Figure 3A). Furthermore, distinct color characteristics were observed in dark red and grey-white genotypes that grouped in the same cluster (III and IV, Figure 3A). Regarding the leaves, *C. mirabile* 'Tilebarn Nicholas' (C22) is considered an 'outlier' due to its purple-green leaf pattern. Genotypes *C. persicum* 'Petticoat pure white' (10) and *C. cilicium* (25) presented similar L*, b* and C* in the dark and silver patterns (cluster I, Figure 3B). *C. persicum* genotypes presented similarities in a* and hue in dark lamina and b* and C* in silver pattern, whereas the following cluster comprised genotypes with similar a* (darker green lamina), C* and shade in leaves, together with similarities in terms of petiole hue (II and III, Figure 3B). The genotypes with similar petiole color characteristics grouped in cluster IV. Similar findings were observed in *C. purpurascens*, where semi-silvery and patterned leaves had higher L* values compared with green and silver lamina. According to the study's findings, all genotypes showed positive b* values, indicating that the leaves had a faint yellow tint (Osterc et al., 2014; Cornea-Cipcigan et al., 2019). Additionally, higher hue levels were noted in *C. purpurascens* flowers and lower values in the leaves (Osterc et al., 2018). Consistent with previous reports, the values of b* were significantly lower in dark-colored flowers and higher in leaves (Cui et al., 2019).

Genetic diversity analysis using the Euclidean distance generated with the SRAP (r = 0.95) and SCoT (r= 0.82) markers partitioned the *Cyclamen* genotypes into four groups. This study firstly evaluated the effectiveness of SCoT markers to determine genetic variation between *Cyclamen* genotypes. SCoT markers have been chosen based on their successful use for analysis of genetic diversity and cultivar identification in multiple plants. A relatively high percentage of polymorphic bands (82%) was detected, consistent with the proportion of polymorphism reported in other medicinal and ornamental species, such as sage (Etminan et al., 2018), chrysanthemum (Feng et al., 2016) and orchids (Tikendra et al., 2021). In the SRAP analysis, a high percentage of polymorphic bands

TABLE 3 Genetic diversity indices based on phenotypic, color and genotypic data of *Cyclamen* genotypes.

	Phenotypic data				Color characteristics				Genotypic data									
									SRAP markers				SCoT markers				Combined data	
Diversity indices	Min	Median	Max	Average	Min	Median	Max	Average	Min	Median	Max	Average	Min	Median	Max	Average	Median	Max	Average
Shannon–Wiener Index (H')	1.467	2.247	2.96	2.24	1.9869	3.2604	3.3142	2.9022	1.9869	3.2604	3.314	2.902	2.04134	2.32273	2.4575	2.3	3.577517	3.646	3.3483
Simpson's Index (λ)	0.4876	0.7575	0.919	0.742	0.8512	0.9583	0.9583	0.9328	0.8512	0.9584	0.9617	0.932	0.8531	0.896	0.9125	0.89	0.969894	0.9726	0.9593
Pilou evenness (J)	0.1382	0.21368	0.2677	0.211	0.28843	0.2855	0.4093	0.3184	0.1323	0.2046	0.2695	0.2045	0.2613	0.2645	0.3268	0.28	0.264574	0.3268	0.2796

(75%) and a clear discrimination between genotypes was observed, as shown by the high cophenetic correlation coefficient (r=0.95). SRAP primers have been chosen due to their high reproducibility compared to RAPDs and ease of assay performance than AFLPs. Previously, UPGMA dendrograms were constructed to assess the similarities and differences between *Cyclamen* species form different regions of Turkey showing a clear discrimination between *C. persicum* genotypes and other species. Furthermore, *C. pseudibericum* and *C. alpinum* genotypes clustered in different groups based on their geographic origin, demonstrating their narrow genetic diversity (Taşkin et al., 2012; Simsek et al., 2017). Consistent with previous studies on SRAP markers (Simsek et al., 2017), *C. hederifolium* and *C. persium* clustered in the same group (Figure 6A). Furthermore, *C. intaminatum* and *C. cilicium* grouped in the same sub-cluster, while *C. alpinum* was closely followed by *C. coum*, possibly due to the same chromosome number (2n = 30). These genotypes are genetically related as already reported (Compton et al., 2004). The combined SRAP and SCoT analysis revealed a more accurate genetic estimate between the genotypes. For example, the 2nd cluster organized the genotypes that mainly correspond to subgenera Gyrophoebe with few exceptions, whereas 3rd cluster comprised genotypes corresponding to *Cyclamen* subgenus. This is in accordance to previous studies that revealed a similar phylogeny using cluster analysis (Anderberg et al., 2000; Compton et al., 2004).

Plants that present similarities in terms of agro-morphological characters may have significantly different molecular characteristics, and vice versa. Discrepancies in genotypic and phenotypic data revealed in clusters might be due to the effects of environment-genotype interaction often noticed in quantitative inherited characteristics. Understanding the nature and degree of genetic variety in crops requires non-overlapping and complementary morphological and molecular data that are highlighted by the absence of relationship between the diversity matrices (grey lines). Several researches investigated the discrepancies between phenotypic and genotypic characteristics, such as maize (Hartings et al., 2008), wheat (Soriano et al., 2016), sweet sorghum (Da Silva et al., 2017), kalanchoe (Al-Khayri et al., 2022), sesame (Stavridou et al., 2021). An entanglement rate of 0.65 was obtained among 77 passion fruit genotypes showing dissimilarities between the molecular and agronomic data (Preisigke et al., 2020). The same divergence in sample distribution in dendrograms was identified in 65 hot pepper accessions using genetic and agronomic data (Moreira et al., 2018). The dendrogram's irregular genotype ordering might be explained by the lack of phenotypic trait specificity of the used molecular markers. They amplify more stable parts of the genome but are unable to relate a genetic marker to a particular phenotypic characteristic.

The present study assessed the variation between clustering methods based on phenotypic and molecular markers (SRAP and SCoT markers) which have not been employed in *Cyclamen*. Several HCA for evaluating genetic diversity in *Cyclamen* were compared, followed by combined analysis using morphological and molecular data. With a few outliers for morphological data (r = 0.73), high cophenetic correlation coefficients were noticed for the others. UPGMA method was demonstrated to generate the highest coefficient values for the majority of matrices between morphological, colorimetric, and molecular data, implying that these matrices and distances are well represented as dendrograms.

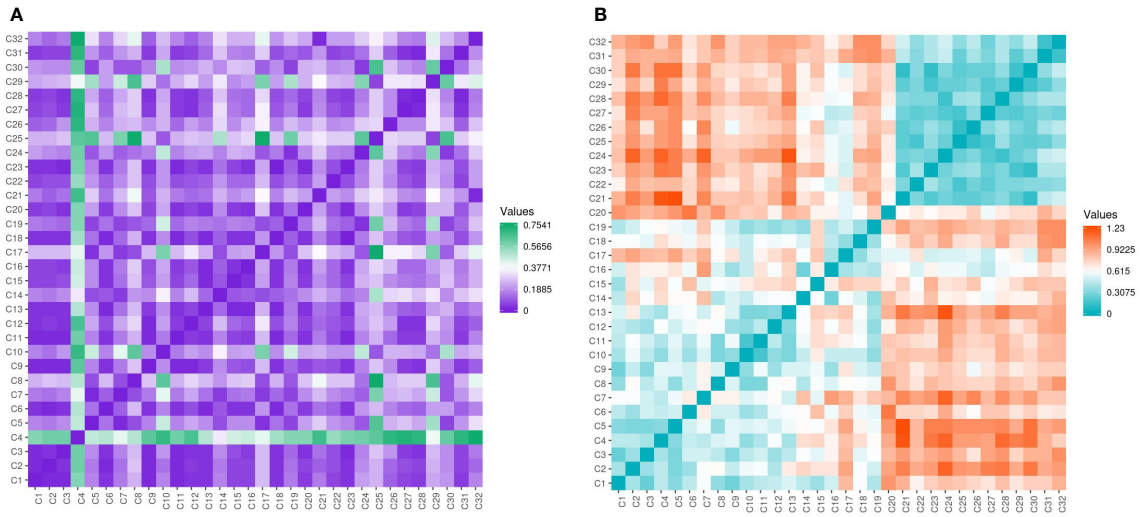

FIGURE 7
Gower's dissimilarity matrix from the morphological data **(A)** and Pearson's correlation matrix **(B)** from the molecular data of the *Cyclamen* genotypes. For the morphological data, green represents similarities, while purple represents dissimilarities. For the molecular data, orange represents similar genotypes, whereas turquoise represents the most dissimilar genotypes. As the matrices were symmetrical, the values below the crossways correspond to the values above.

In line with the current study's findings compared to other methods, the UPGMA clustering approach was shown to provide significant correlation coefficients for genetic diversity investigations in safflower (Houmanat et al., 2021), yam (Granato et al., 2018), sweet potato (Paliwal et al., 2022), and gladiolus (Singh et al., 2018).

To evaluate genetic diversity in plant populations, a method that creates a single matrix from the phenotypic and genotypic dissimilarity matrices was presented (Da Silva et al., 2017). Combined analysis of morphological and molecular data particularly with SRAP markers, showed the highest performance and similarities, whereas SCoT markers and color characteristics demonstrated moderate similarities. For a better classification of genotypes, more complex and significant correlations may be revealed using molecular approaches. However, evaluation of diversity using only molecular markers is insufficient. As a result, the molecular assessment of genetic diversity may adequately describe genetic variability, when combined with phenotypic variance. In *Cyclamen*, genetic biology advances have permitted the development of correlations between molecular markers and morphological characteristics (Yesson and Culham, 2006; Naderi et al., 2009). In this aspect, the combination of agronomic parameters, color characteristics and molecular markers is efficient for assessing genetic diversity across and among species with greater precision, taxonomic study, and genotyping.

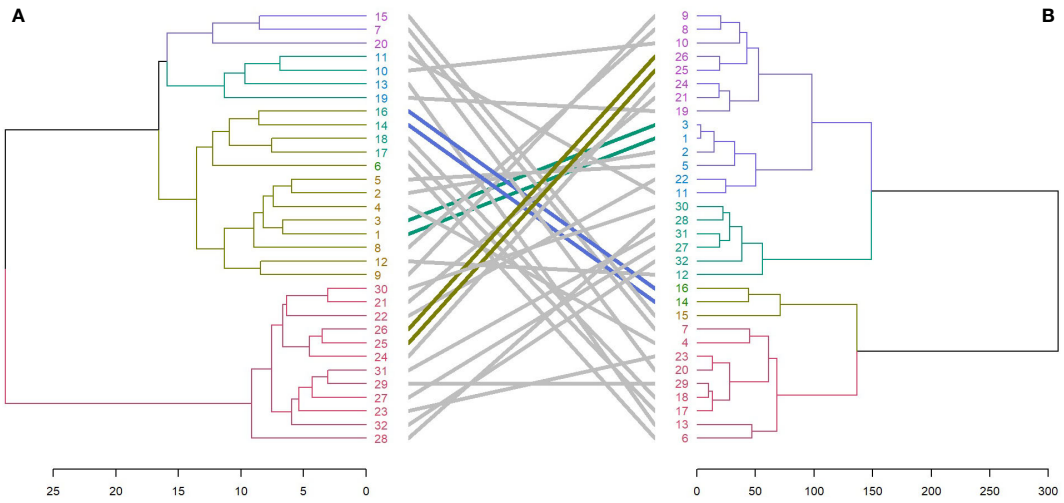

FIGURE 8
Comparison of dendrograms for the 32 *Cyclamen* genotypes from SRAP markers **(A)**, and phenotypic data **(B)** with entanglement = 0.30. The grey lines in connecting the dendrograms correspond to mismatched genotypes whereas the colored lines are genotypes that maintained their position between clusters.

5 Conclusions

Color indicators of leaves, petioles, flowers and pedicel were measured separately for color phenotypic data. The present study also attempted to establish a more precise characterization of leaf and flower color in order to be used as a framework for *Cyclamen* breeding. Furthermore, this study firstly evaluated the potential of SCoT markers for analyzing genetic diversity among *Cyclamen* genotypes. SRAP markers proved to be useful tools for the separation of *Cyclamen* species and/or genotypes, particularly when combined with phenotypic data, as seen by the high entanglement analysis (0.30) among dendrograms. Conversely, similarities were noticed in the colorimetric data for leaves and SCoT data (entanglement=0.47), compared with the colorimetric analysis of flowers and SCoT markers (entanglement=0.45). Thus, combined HCA of phenotypic and molecular data, particularly with SRAP markers, showed the highest performance and similarities, whereas SCoT markers and color characteristics demonstrated moderate similarities. As a result, we underline that SCoT markers along with color characters are suggested for the characterization of germplasm banks with a large number of genotypes in order to detect duplicate accessions since they are more efficient and economically feasible. Conversely, phenotypic data along with SRAP markers more accurately describe in terms of genotype evaluation for later use in breeding programs.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author contributionss

MC-C, RM and CS: conceptualization. CS and RM: methodology. MC-C: software. MC-C: formal analysis. MC-C and CS: investigation. CS and RM: resources. MC-C, CS and RM: writing—original draft preparation. MC-C, CS, RM and DP: writing—review and editing. DP: supervision. RM and CS: funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1100099/full#supplementary-material

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The use of a candidate gene approach to study *Botrytis cinerea* resistance in *Gerbera hybrida*

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Candidate genes (CG) for *Botrytis cinerea* resistance described in literature were mapped on gerbera linkage maps for which several QTL for Botrytis resistance had been found previously using a rapid, low-cost platform for SNP genotyping. In total, 29 CGs were mapped in either of two mapping populations. Four CGs were mapped within the previous identified QTL intervals and three co-localized with QTL. Two of these CGs for resistance against *B. cinerea*, *PG1* (polygalacturonase gene) and *sit* (*sitiens*, ABA-aldehyde oxidase gene) that mapped in QTL regions for the ray floret disease resistance test were studied in detail. Virus-induced gene silencing (VIGS) was used for gene function analysis to determine the CGs' role in gerbera resistance to Botrytis. Ray florets, of which the CGs were silenced, showed a significantly delayed growth of lesions upon Botrytis infection compared to controls. Combining QTL analysis, candidate gene mapping and VIGS showed to be an useful combination to identify possible causal genes and for understanding the molecular mechanisms of Botrytis resistance in gerbera. The two genes seem to act as partial S-genes and are likely among the determining genes leading to the variation observed for *B. cinerea* resistance in gerbera.

KEYWORDS

HRM, QTL haplotypes, MAS, gene localization, functional allele, TRV, susceptibility genes

Introduction

Gerbera is an economically important ornamental plant, which is mainly used as cut flower. In pre-harvest and post-harvest processes, high relative humidity regularly occurs, which can lead to grey mold infections and subsequent major losses in gerbera production. For instance, economical loss was estimated at € 2.5 million in 2007 in The Netherlands alone (Marcelis, unpublished). Gerbera grey mold may be suppressed by management practices and fungicide application during production in greenhouses, yet prevention and control of this disease during transportation or after sales is difficult.

Traditionally, breeding in gerbera is performed by F1 crossing and (recurrent) selection in a number of generations. Although this traditional method is considered time and labor consuming, it is still widely in use for gerbera and ornamental plant breeding in general since lots of horticultural traits (e.g. flower color, flower pattern and shape), as well as agronomical traits (e.g. multiplication rate and flower production) are segregating due to the high variation in parental genotypes of these outcrossing crops (Arens et al., 2012; Smulders et al., 2012). These traits can also be easily selected as they are either visible traits (color, pattern, shape) or easily quantifiable traits (multiplication rate and flower production). However, selection for genetic and phenotypic complex quantitative traits like disease resistances, which are often also influenced by environment and present themselves in continuous distributions (Paterson et al., 1988), is very difficult using only visual selection schemes.

Resistance to *B. cinerea*, the cause of gerbera grey mold, is such a quantitative trait where the contributions of multiple loci are needed to reduce disease severity (Mengiste et al., 2003; Poland et al., 2009). In our previous study, 20 QTLs for Botrytis resistance in ray floret, whole and bottom of inflorescence trait were detected in two gerbera mapping populations (Fu et al., 2017). Genetic linkage between a marker and a QTL allele of the trait of interest, can be broken by recombination (Andersen and Lübberstedt, 2003). Furthermore, in cross-pollinating highly diverse ornamental species, there is often an ascertainment bias (Kumar et al., 2000). Observed linked marker alleles which were detected in single F1 outbred populations might not be directly applicable to predict the association with the preferred QTL allele in another population (Williams, 1997; Liu et al., 2011). For ornamental crops, with increasing EST sequence data availability the use of a candidate gene (CG) approach (Debener, 2009; Smulders et al., 2011; Arens et al., 2012) might improve the accuracy of Marker Assisted Selection (MAS) and circumpass the mentioned problems. Developing molecular markers which are derived directly from polymorphic loci in functional CGs will be in complete linkage (Andersen and Lübberstedt, 2003). Through co-localization of CGs with QTLs, promising genes underlying the QTLs could be quickly pinpointed (Pflieger et al., 2001; Decroocq et al., 2005; Norelli et al., 2009; Kawamura et al., 2011; Gardner et al., 2016) and used for understanding molecular mechanisms for traits of interest (Smulders et al., 2011). Like many other ornamental plants, gerbera has a heterozygous genetic background and a lack of adequate genetic information. Instead of going through the process of high-resolution fine mapping and identifying tightly-linked markers for MAS, we developed a CG approach searching for possible causal genes for Botrytis resistance in gerbera.

The infection process of the necrotrophic pathogen, *B. cinerea*, is described by the three stages: penetration of host epidermal cell surface, primary lesion formation, lesion expansion/tissue maceration and sporulation (Jarvis, 1962; van Kan, 2006; Choquer et al., 2007; Valero-Jiménez et al., 2019). During the infection, a series of plant genes, such as genes in cell wall biosynthesis or affecting cell wall composition, in signaling pathways, and in the complex pathways of plant natural product biosynthesis, can be involved in resistance to the pathogen.

Using literature on genes involved in Botrytis resistance and the availability of transcriptome data (Fu et al., 2016) from which a random set of SNP markers was previously used to perform a QTL study for Botrytis resistance (Fu et al., 2017), we now specifically targeted genes known from other species to test a CG approach for Botrytis resistance is an functional option. The first step was to map SNPs found in CGs from literature to look for co-localization of the CG gene positions with previously found QTL positions. In order to validate the approach we also used virus induced gene silencing (VIGS) for two genes to see if silencing of genes found to co-localize with QTL for Botrytis resistance would influence Botrytis disease severity. VIGS systems (Baulcombe, 1999; Becker & Lange, 2010) have been successfully applied in a large number of ornamental plants and as such considered an attractive approach for gene characterization in ornamentals (Jiang et al., 2011), particularly those not amenable to tissue culture or genetic transformation.

Materials and methods

Plant materials and Botrytis disease test data

Plant materials were composed of the parents and two gerbera populations previously used for QTL mapping (Fu et al., 2017). In brief, population S containing 276 offspring, was obtained from a cross between breeding lines SP1 and SP2 whereas population F consisting of 270 progeny was derived from a cross between FP1 and FP2. Genomic DNA of the two populations and the four parents were isolated following the DNA isolation protocol of Fulton et al. (1995) with some adaptations. Phenotypic data available for these two F1 progenies were based on three tests of whole inflorescence, bottom (of disc floret) and ray floret, respectively with scores ranging from 0 (no symptom) to 5 (very serious) as described by Fu et al. (2017). All tests were performed with *B. cinerea* (strain B05.10) which was kindly provided by J. van Kan (Wageningen University). In brief, for the Botrytis disease test on whole inflorescence and bottom, a spore suspension of 1×10^5/ml in water was sprayed on the inflorescence with a fine plant sprayer and incubated for 5 days in a climate cell at 20°C and a R.H. of 90%. Whole inflorescences were visually evaluated to score, after which, the bottom of the capitulum was cut (horizontal cross section) to check (score) fungal growth inside the capitulum for the bottom test. The response to Botrytis infection on whole inflorescence and bottom was scored ranging from 0 (no symptom) to 5 (completely rotten). For the ray floret test, inoculation was performed by pipetting 2 µl of a 3×10^5/ml spore suspension in potato dextrose on the upper surface of ray florets and incubated for 48 h at 20°C and nearly 100% relative humidity. Disease scoring was assessed as follows: 0 no visible symptoms; 1 infection limited in inoculation droplet size; 2 lesion extended twice to four times the droplet size; 3 large lesion area but still smaller than half of the ray floret; 4 lesion area larger than half of the ray floret; and 5 complete necrosis. Botrytis scores varied between parents and populations means. Scores in population S for whole inflorescence, bottom and ray floret were 2.42 ± 0.55 (SP1 =

1.0/SP2 = 2.2), 2.96 ± 0.63 (SP1 = 1.7/SP2 = 2.9) and 2.98 ± 0.79 (SP1 = 1.9/SP2 = 1.8) and in population F 3.64 ± 0.40 (FP1 = 3.4/FP2 = 3.6), 3.80 ± 0.40 (FP1 = 3.4/FP2 = 4.1) and 3.14 ± 0.80 (FP1 = 4.2/FP2 = 2.3), respectively.

Candidate gene genotyping

Literature was screened for genes involved in Botrytis resistance. Sequences of CGs associated with Botrytis resistance were run against the gerbera ESTs database (Fu et al., 2016) using tBLASTn in the blast-2.2.28+ program. Within the selected best-hit gerbera EST sequence, heterozygous SNPs in just one parent were identified as potential marker and Open Reading Frames (ORFs) were identified to avoid SNPs close to intron/exon boundaries. High Resolution Melting (HRM, LightScanner, Idaho Technology) analysis was used for genotyping. Primer pairs were designed to amplify 80 to 150 bp fragments using Primer3 online (Untergasser et al., 2012) and first tested on the parents and four randomly selected offspring. Only when the melting curve of the two parents and selected individuals could be clearly distinguished, the markers were used for whole population genotyping.

Segregation ratios of the two allelic offspring groups (heterozygous vs homozygous) were tested by χ^2 (1:1) statistics (95% CI) and the mean of the disease scores between these two groups from all three disease tests was tested for significance ($P<0.05$) by T-test using SPSS software (Version 21). CGs were mapped on the individual parental linkage maps previously constructed (Fu et al., 2017) by using JoinMap®4 (van Ooijen, 2006). QTL analyzes, with CG positions added to the map, were repeated as described earlier (Fu et al., 2017). Because not all individuals from the original mapping population were available, results were slightly different from previous results of Fu et al. (2017) and only used for confirmation.

Allele identification of candidate genes

Haplotype information of promising CGs in the four parents was obtained using the sequence and SNP positions in their transcriptome data (Fu et al., 2016) and these were supplemented by Sanger sequencing to acquire a longer and if possible full-length gene sequence. Primers for amplifying CG fragments from genomic DNA for Sanger sequencing were designed by Primer3 online. PCR conditions were in Fu et al. (2017). PCR products, showing single bands with expected size, were purified using the QIAquick PCR Purification Kit (Qiagen) and cloned into the pGEM®-T Easy Vector System I (Promega). Multiple positive clones were sequenced using Sanger sequencing

Candidate gene expression analysis after Botrytis inoculation

B. cinerea spore suspension (3×10^5/ml) was inoculated on the upper side of ray florets. Infected ray florets were collected at different time points (0 hpi, 6 hpi, 12 hpi, 24 hpi, 36 hpi, 48 hpi and 72 hpi at initial experimental setup) whereas mock samples were taken at 24 hpi only. Single ray floret (for FP2 due to smaller size three ray florets) were put in an Eppendorf with two metal balls frozen in liquid nitrogen and stored at -80 ^{0}C upon gene expression analysis.

RNA from infected ray florets was extracted using Trizol (Life Technologies) according to the manufacturer's protocol with a small modification (Fu et al., 2016). Quality and quantity of RNA samples was checked on 1% agarose gel and by NanoDrop. cDNA synthesis according to the iScript™ cDNA Synthesis Kit (Bio-Rad) was used for Real time quantitative PCR (RT-qPCR). The same primers as used for HRM analysis and reference gene GAPDH obtained from Deng et al. (2014) were used for SYBR green qRT-PCR. Reference and CGs were always run together with three biological replicates and three technical replicates.

Relative quantification method (Livak and Schmittgen, 2001) was used to analyze data. The change in expression of candidate (target) gene was normalized to the reference gene expression and presented as fold change.

VIGS vector construction

Sequences of CGs *ghPG1* and *ghsit* were retrieved from the gerbera EST database and the supplementary Sanger sequencing whereas reporter genes were retrieved from the gerbera EST database only. Gene-specific primers, added with *att*B1 and *att*B2 adapter, were designed using Primer3 online (listed in Table S1). The expected fragments of target genes were divided into 20 bp sequences to blast against our gerbera EST database to check whether they may trigger an off-target gene silencing. The fragments of the CGs (*ghPG1*, *ghsit*) and three reporter genes (*ghPDS*, *ghCHS1*, *ghCHS4*) for VIGS were amplified from gerbera cDNA. Furthermore to co-suppress the two CGs (*ghPG1+ghsit*) simultaneously a construct was made consisting of both gene fragments.

The Gateway-compatible Tobacco rattle virus (TRV) two-component Agrobacterium mediated expression system was used for gene silencing as previously described (Liu et al., 2002). DNA fragments of reporter genes and CGs were individually cloned into pDONR207 to generate entry vectors. Entry vectors with the CG fragment insertion, were verified by sequencing using primers *att*L1 and *att*L2 listed in Table S1. Target gene fragments were subsequently cloned into the destination vector pTRV2 to generate constructs *TRV2::GOI* (Gene Of Interest). A negative control TRV2 construct (*TRV2::ghGUS*) carrying a 648 bp *GUS* fragment was also used as described by Song and Thomma (2016). All *TRV2* constructs were confirmed by DNA sequencing (using TRV2 primers; Table S1).

All *TRV1* and *TRV2::GOI* constructs were transformed to *Agrobacterium tumefaciens* strain GV3101 by electroporation. Transformed agrobacteria were inoculated on LB agar selection media and cultured in the LB liquid media with antibiotics. Constructs were confirmed by PCR and used for the agro-infiltration.

Agrobacterium infiltration and disease testing

Parental lines grown in greenhouse chambers (Unifarm, Wageningen UR, the Netherlands, under 16h light/8h dark photoperiods with 21°C/19°C day/night temperature in relative humidity of ~75%) were used for infiltration. TRV constructs were agro-infiltrated as described previously (Deng et al., 2012). Briefly, flower stem (scape) was scratched for around 1 cm length and covered with an *Agrobacterium*-soaked cotton pad. To test the Botrytis infection on TRV-treated gerbera plants, flowers were harvested at around 2 to 3 weeks post agro-infiltration. Ray florets (in the sector right above the scar due to the scratching) from each inflorescence were collected for Botrytis inoculation and for total RNA isolation.

To analyze the efficiency of CG silencing, cDNA was synthesized for RT-qPCR. Target gene and reference gene (*GAPDH*) were amplified using the cDNA of *TRV2::GOI* plants and *TRV2::GUS* plants. The change in expression of the target gene in silenced plants was normalized to the reference gene (*GAPDH*) relative to control plants (*TRV2::GUS* plant in this experiment) and represented as fold change. The method is called $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001), where $\Delta\Delta CT = (C_{T,\ Target} - C_{T,\ GAPDH})_{\ TRV2::GOI} - (C_{T,\ Target} - C_{T,\ GAPDH})_{\ TRV2::GUS}$. Three ray florets from different silenced inflorescences and three technical replications for each treatment were used.

For Botrytis resistance testing, single fresh ray florets were inoculated with 2µl of *B. cinerea* (strain B05.10) spore suspension (5×10^5/ml). From individual inflorescences, for each treatment 5-6 ray florets were collected for the inoculation. Lesion sizes on inoculated ray florets were obtained 24 hours post infection from images using ImageJ. Data from different gene silenced treatments were analyzed by one-way ANOVA in SPSS (Version 21).

Results

Selection of candidate genes and homologues of gerbera

A list of 71 genes was identified from literature as potential CGs for Botrytis resistance in gerbera (Table S2). These CGs belong to genes affecting cell wall composition, signal transduction or secondary metabolism. Most of the CGs with confirmed involvement in Botrytis resistance were derived from Arabidopsis, grapevine and tomato being the most studied plants. Some genes in the phenylpropanoid and flavonoid biosynthetic pathway derived from the Asteraceae family, like sunflower, Artemisia and gerbera itself, were also included in the CG list as they are important for flavonoid phytoalexin accumulation during Botrytis infection.

For all potential CGs the corresponding homologous sequence of gerbera was searched for in the *Gerbera ESTs database* (Fu et al., 2016). At least one hit could be found for all CGs in the Gerbera ESTs, the contigs with the highest hit score, lowest e-value and highest identity percentage were identified as the homologous CG sequence (Table S2). Over half of the identified homologs (42/71) were with an e-value < 1E-180 and the identities of most of the contigs (64/71) are above 50%. Frames showing the longest ORF were used for re-BLAST analysis. Identified gerbera CG sequences compared (blastp) against the NCBI database, showed hits to annotated CG with even higher hit scores (Table S2). In general, almost full-length gerbera ORFs were identified based on the coding regions of other species.

Genotyping by HRM analysis, mapping and co-localization

Primers were designed to flank a targeted SNP in the CG ORF region with expected PCR products sizes of 80-150 bp. Only primers amplifying single band amplicons were chosen for further analysis (Table S3 and Figure S1). Primers designed on 29 CGs with clear grouping results in the parents test were used for genotyping the whole population and homoduplex (from homozygous SNP) and heteroduplex offspring (from heterozygous SNP) could be easily distinguished by the change in the normalized melting curve (Figure S2).

All 29 tested CG markers showed the expected 1:1 segregation ratio. The difference in phenotypic means in Botrytis infection disease score on whole inflorescence, bottom and ray floret test, respectively, between the two genotypic groups from these CG markers was tested by Student's t-tests for significance. Seventeen CG markers showed a significant difference in at least one test at the level of ≤ 0.05, of which nine showed an even higher threshold value (≤ 0.01 or ≤ 0.001). Interestingly, the CG marker for *CHI* showed a highly significant association (P≤ 0.01) in both populations for whole inflorescence (Table S4).

All 29 CGs could be mapped to at least one of the linkage maps, and seven CGs (*ghCHI*, *ghDND*, *ghPER21*, *ghPG1, ghPG10, ghsit*, and *ghSS*) mapped to linkage maps in both populations (Table S4). CGs *ghsit*, *ghPG1* and *ghCHI* mapped in previously detected QTL regions being RBQRF2 on LG5, RBQRF8 on LG21 and RBQWI4/6 on LG23 (Table 1 and Figures 1A-C), respectively. CGs *ghPG9* and *ghcutin* mapped on the paternal linkage map SP2_02 close to markers WGC23656_151_S1F1 and WGC11243_647_S2F1. On the maternal linkage map SP1 QTL RBQB1 was located between these two markers (Figure S3). Also CGs *ghPER62* and *ghSS* mapped in close proximity to QTLs; RBQRF7 on LG18 and RBQB2 on LG 16, respectively (results not shown).

Compared with the previous QTL analysis (Fu et al., 2017), a number of small differences were detected (Table S5). First of all, two new QTLs were detected, RBQB7 in SP1_06 (LOD = 5.33) with CGs *ghPG7* and *ghPG10* mapped in the vicinity; and RBQB8 in SP2_02 with CG marker (PG9_25150_888) from *ghPG9*. This latter QTL was at a similar position as the previous identified QTL RBQB1 on the S maternal map (SP1_02). CG marker PG1_15001_1052 from *ghPG1* was mapped on linkage group FP2_21 in a 13 cM marker interval (Fu et al., 2017) and re-analyzes of the QTL lead to a shift of the location of the maximum LOD score to this position. Variance explained by this QTL (RBQRF8) increased from 8.0% to 10.7%. Adding CG marker

TABLE 1 CGs co-localized with QTLs regions.

CGs	CGs marker	Mapped inside QTL region	Maps near to QTL region
ghsit	sit_19807_6510	RBQRF2	
ghPG1	PG1_15001_1052	RBQRF8	
ghCHI	CHI_22447_421	RBQWI4/RBQWI6	
ghPG9	PG9_25150_888	RBQB1/RBQB8	
ghcutin	cutin_4918_3081		RBQB1 (2.6 cM)
ghPER62	PER62_31925_540		RBQRF7 (4.9 cM)
ghSS	SS_5198_1668		RBQB2 (4.98 cM)

PER62_31925_540 showed that the LOD score of the previous detected QTL RBQRF7 (4.09) was just a fraction below the GW threshold (4.1).

Allelic diversity of candidate genes

Using a single bi-allelic SNP marker, CG alleles having a positive contribution on the QTL can only be identified from the parent in which the particular marker was polymorphic. To have a better understanding of the variation within genes, allelic diversity was assessed for the four parents for the CGs indicated (Figure S4). As a diploid heterozygous crop, at most 8 haplotypes/alleles per gene can be expected in the four parents. Allelic diversity ranged from at least 3 haplotypes (*ghSS*) to at most 7 haplotypes (*ghPG9*) in the CGs loci of the four parents. In all analyzed CGs, unique haplotype(s) which were not shared with other parents existed. In two CGs (*ghPER62*, *ghPG9*), the unique haplotype was linked to the resistance effect. The seven CGs loci from parent FP1 are all heterozygous, SP1 has six heterozygous loci (except *ghcutin*) and

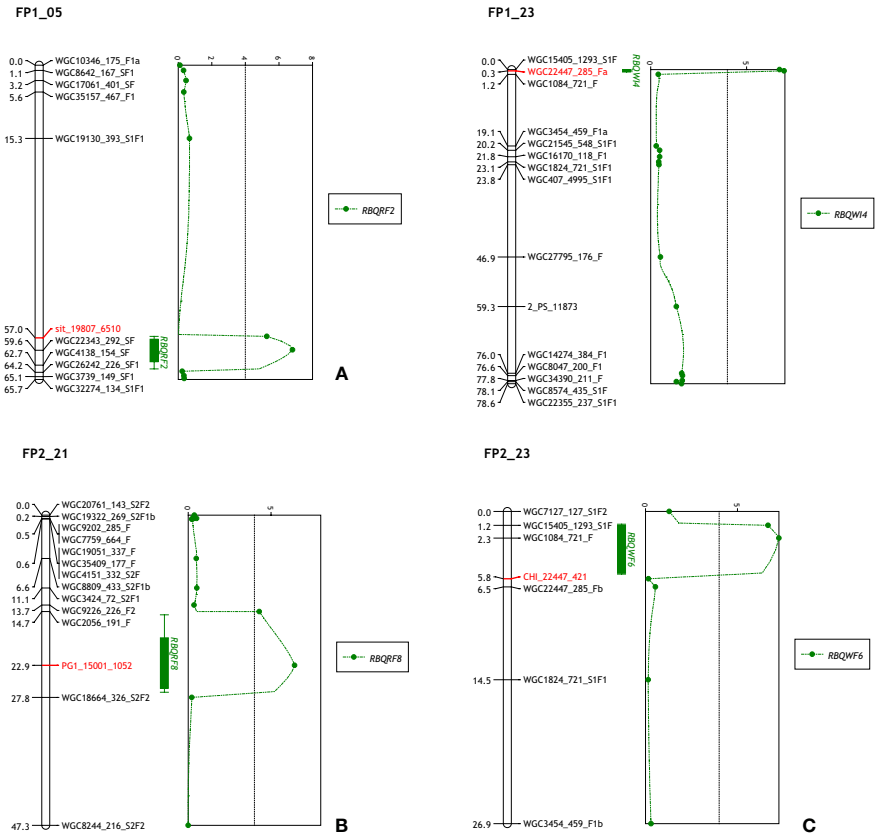

FIGURE 1
Mapped CGs and QTL positions. **(A)** *ghsitiens* mapped on FP1_05 in the region of QTL RBQRF2. **(B)** *ghPG1* mapped on FP2_21 in the region of QTL RBQRF8. **(C)** The QTLs RBQWI4 and RBQWI6 in the maternal and paternal LG23 of F population. The marker WGC22447_285_Fa is a KASP marker from contig22447 and CHI_22447_421 is a marker from the same transcriptome contig used in HRM for validation of contig.

FP2 five heterozygous loci (except *ghsit* and *ghcutin*), while in SP2, only three loci contained two distinctive alleles in the CGs loci (ghPG9, *ghPER62* and *ghcutin*).

More than half of the SNPs were present in the third base of the codon. Overall, 57% of the SNPs were synonymous SNPs. Two alleles from *ghPER62*, *ghSS*, and three alleles from *ghcutin, ghPG9* encoded the same protein (Figure S4). Interestingly, a 36 bp insertion–deletion (InDel) was found in the first intron of *ghsit*, while a 1 bp InDel in the second exon region was found for an *ghCHI* allele which would lead to an early stop codon.

Expression of candidate genes

Expression levels of *ghsit*, *ghPG1*and *ghCHI* were analyzed on ray florets. In the initial experimental setup, inoculated ray florets were sampled for expression analysis at 0 hpi, 6 hpi, 12 hpi, 24 hpi, 36 hpi, 48 hpi and 72 hpi. Spores of Botrytis already germinated on ray florets 6 hpi (Figure S5). The initial stages of Botrytis infection resulted in necrotic lesions which were clearly visible on ray florets and then lesions expanded quickly from the initial necrotic lesions to the whole ray floret. Expression of gerbera house-keeping gene at later stages of infection (36 h, 48 h and 72 h after inoculation) was already absent because florets had become necrotic. So final expression analyzes were performed on ray florets at 0, 6, 12 and 24 hrs after inoculation, with a control sample with mock inoculation sampled after 24 hrs (Table S6).

Expression of all three CGs was detected in the four parents except for FP1 for which no expression of *ghPG1* and *ghCHI* was found. The expression pattern of studied CGs showed variation at different time points but with significant up-regulation after Botrytis infection. In general, gene expression levels reached their peak at 12h or 24 h after Botrytis inoculation. There was no significant difference in gene expression between time point 0h and 24h in the mock samples.

VIGS construction and indications for successful silencing of reporter genes

To characterize the function of CGs *ghsit* and *ghPG1*, we constructed a TRV-based VIGS system (Liu et al., 2002) which carried the target CG fragments in *pTRV2*, to suppress the level of plant endo-gene expression. Fragments of *PDS* (phytoene desaturase) and *CHS* (Chalcone synthase) that both are widely used as reporter genes to recognize the silenced phenotypes in gerbera (Deng et al., 2014; Deng et al., 2012) and other crops, and a β-glucuronidase (*GUS*) gene fragment were also cloned into the TRV2 vectors for control experiments.

Target gene fragments with lengths varying from 274 bp to 428 bp were amplified from parental gerbera cDNAs. Furthermore, a fragment with a length of 722 bp combining two gene fragments (*ghPG1* 428 bp + *ghsit* 294 bp) was developed by overlapping PCR. To confirm that fragments might not trigger any other unexpected non-target gene silencing; entire fragments of CGs and reporter genes were divided into a series of continuous 20 bp sub-sequences that were used as queries for BLASTn. In BLAST output, all 20 bp-subsequences of target genes only aligned to the original contigs indicating no other off-target hits were found that could lead to possible non-target gene silencing based on the currently available transcriptome information. Since only a few SNPs were found between the target gene fragments in the four parents, target gene fragments from SP1 were used for subsequent TRV-VIGS vector construction. All entry vectors and TRV2 constructs that were generated in the Gateway reactions have been sequenced and confirmed carrying the right target gene fragments.

Reporter genes (*ghPDS*, *ghCHS1* and *ghCHS4*) were used to identify in which sectors of the inflorescences the silencing phenotype was present. Since *ghCHS1* and *ghCHS4* silenced plants did not give visible indications of gene silencing, they were not used in further tests. TRV2 constructs with the *GUS* gene fragment were used as negative control.

In *TRV2::ghPDS* silenced plants of SP2, a visible color change emerged above the scar by scratching for agro-infiltration and along on the green trunk of the elongated scapes until the bottom of the flower head that could be easily followed (Figures S6A-C, as the arrows shown). Three of the six PDS silenced SP2 plants exhibited a changed pink color in ray florets of the inflorescences with varying ray floret numbers (Figure S6D). The ray florets from the original white inflorescences that were on sectors of the same side of the scar from the scratching and also the sectors of the opposite side of the scar turned to pink. Those pink ray florets/sectors defined in which parts of the flower head *PDS* had been silenced. The phenotype of PDS silencing in SP1 plants varied, showing a diluted orange color in parts of the inflorescences (Figure S7A) or few petals (Figures S7B, C) on the scratching side, or bleaching on ray florets (Figures S7D-F). Quite a few flower scapes of FP1 broken at scape scratching position as the stem extended (Figures S8A, B) and few even fell off, before the flower blossoming. Out of the scapes without stem breaking after flowering, no detectable phenotype was found in the reporter gene silenced inflorescences indicating that gene silencing in FP1 was likely ineffective.

Gene expression in silenced ray florets and disease testing on candidate gene silenced ray florets

To quantify the suppression of the candidate and reporter gene expression after VIGS, we used a relative quantification method to determine the efficiency of silencing. The relative expression of target gene in negative control *TRV2::GUS* plants were set on 1. The change in expression of target genes was normalized to the reference gene (*ghGAPDH*) relative to *TRV2::GUS* silenced plant and represented as fold change.

The expression levels of *PDS* in *TRV2::ghPDS* plants showed a significant decrease ($P<0.05$) compared to *PDS* expression in control *TRV2::GUS* plants of SP1 and SP2 (Figure 2A). PDS silenced FP2 showed a lower but not statistically significant difference in expression. The expression of *PG1* and *sit* were analyzed in the two-gene-silenced SP1 plant, and both genes were successfully silenced, albeit to a different extent (Figure 2B).

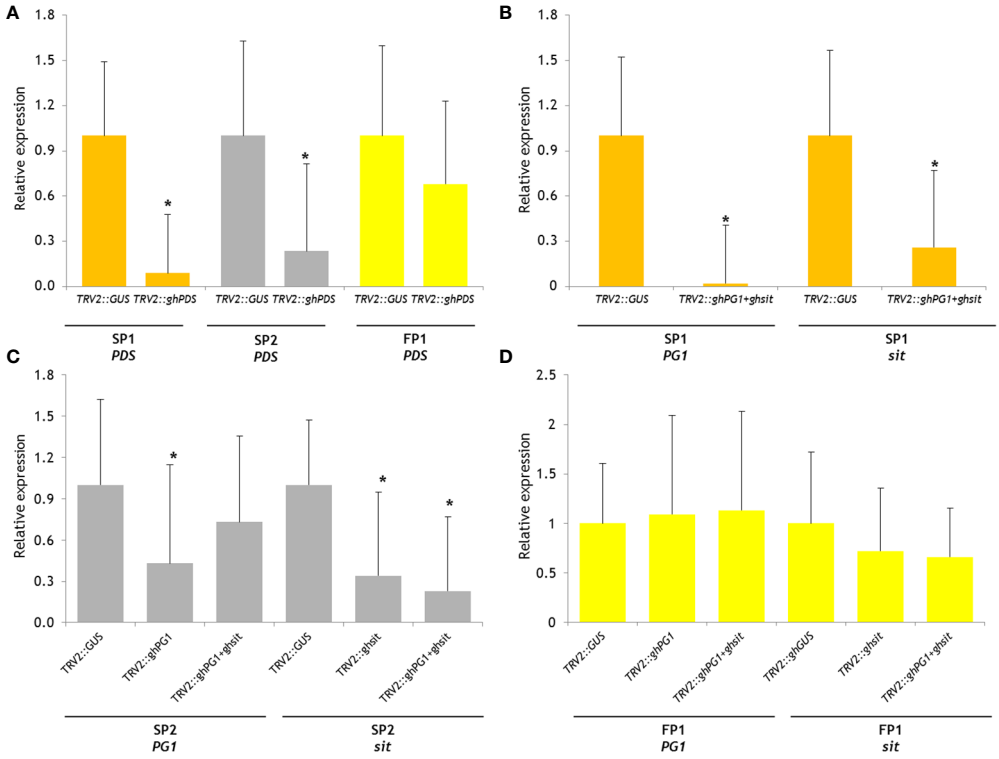

FIGURE 2
Relative gene expression level of reporter gene PDS and CGs in silenced plants using $2^{-\Delta\Delta CT}$ method. The change in expression of target gene in silenced plant which was normalized to reference gene (GAPDH) relative to control plant (as 1, *TRV2::GUS* plant in this experiment) and represented as fold change. The aster indicate significant difference compared with gene expression on control TRV2::*GUS* plants (P<0.05). **(A)** Relative *PDS* expression on three parental *TRV2::ghPDS* silenced plants; **(B)** relative *PG1* and *sit* expression on SP1 *TRV2::GUS* silenced plants and *TRV2::ghPG1+ghsit* silenced plants; **(C)** relative *PG1* expression on SP2 *TRV2::GUS* silenced plants, *TRV2::ghPG1* silenced plants and *TRV2::ghPG1+ghsit* silenced plants; relative *sit* expression on SP2 *TRV2::GUS* silenced plants, *TRV2::ghsit* silenced plants and *TRV2::ghPG1+ghsit* silenced plants; **(D)** relative *PG1* expression on FP1 *TRV2::GUS* silenced plants, *TRV2::ghPG1* silenced plants and *TRV2::ghPG1+ghsit* silenced plants; relative *sit* expression on FP2 *TRV2::GUS* plants, *TRV2::ghsit* silenced plants and *TRV2::ghPG1+ghsit* silenced plants. * Significant difference compared to the control TRV2::GUS plant.

Significant silencing of *PG1* expression was only in SP2 *TRV2::ghPG1* plants, but not in TRV::*ghPG1+ghsit* plants. While the relative *sit* expression in SP2 in both TRV2::*ghsit* and *TRV2::ghPG1+ghsit* plants decreased significantly (Figure 2C). The expression levels of neither *PG1* nor *sit* were found statistical significantly decreased on any gene silencing treatment in FP1 (Figure 2D).

To test Botrytis resistance on ray florets after VIGS, single ray florets were collected for Botrytis inoculation. According to the results from the indicator gene, we collected 5-6 ray florets in the sectors just right above the scratching scar around or less than 1/4 of the total ray florets. Lesion sizes 24 h post inoculation, of each ray florets from target gene silenced plant and *GUS* gene silenced plants were compared. The number of ray florets sampled from each parent and each silencing treatment for disease test is shown in Table S7.

Compared with the mean lesion size of control treatment (*TRV2::GUS*), the mean lesion size on ray florets from TRV2::*GOI* silenced plants showed a decrease on the three genotypes used (Figures 3A-C). Due to the material limitations, SP1 was only used for two genes (*TRV2::ghPG1+ghsit*) co-suppression. The lesion sizes on *TRV2::ghPG1+ghsit* co-silenced plants were reduced by 80% compared to *TRV2::GUS* silenced plants. The same amount of reduction (81%) was also observed in SP2 with *TRV2::ghPG1+ghsit* co-silencing. There were significant differences (P<0.05) between *TRV2::ghPG1+ghsit* and *TRV2::ghsit* silenced plants compared to *TRV2::GUS* silenced SP2 plants, while no significant difference between *TRV2::PG1+sit* co-silenced plants compared to *TRV2::PG1* silenced plants was found. Although the lesion size on FP1 ray florets showed less reduction and more variation than of other parents, it seems a bigger lesion reduction in FP1 came from the *TRV2::PG1* silenced plants. Significant differences could be observed in the mean of lesion size between the four treatments of FP1, yet in each treatment, large variations existed.

Discussion

Genotyping, mapping and the co-localization with QTLs

In this study, we selected putative CGs involved in plant resistance against Botrytis from literature and screened a gerbera

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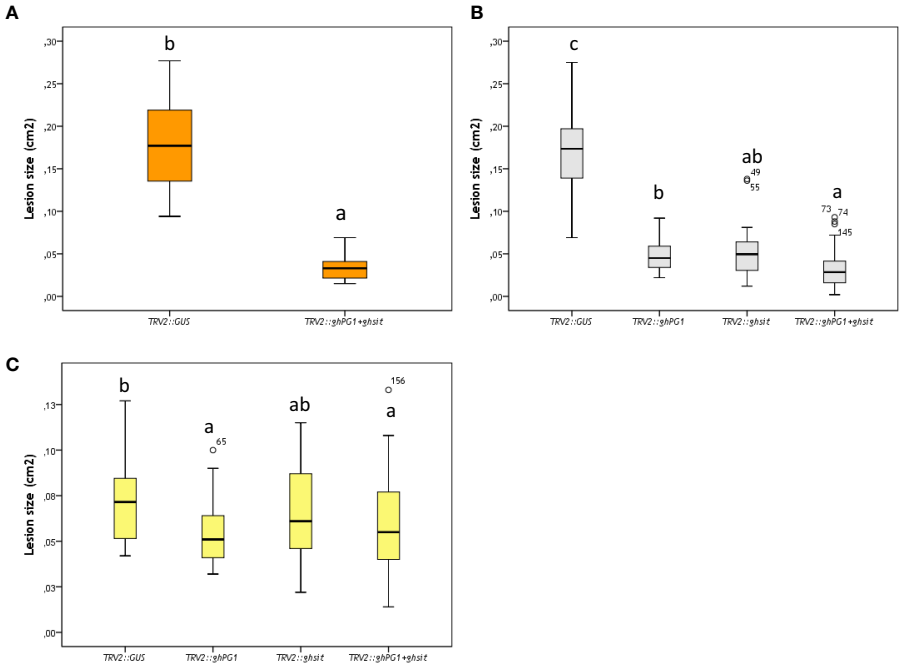

FIGURE 3
The lesion size (cm2) of Botrytis inoculation at 24hpi on different ray florets of parental silenced plants. **(A)** SP1; **(B)** SP2; **(C)** FP1. Different letters indicate significant difference (P<0.05). Every single ray floret for ImageJ measurement was coded with a number, and ray florets with number such as 49, 55, 73, 74 etc were indicated the outliers of box-plots.

EST database (Fu et al., 2016) for homologous genes. After alignment of the homologous gene sequences found among the parents and selection of suitable SNP markers, CGs were mapped on the genetic maps of two populations used for QTL mapping previously (Fu et al., 2017).

For good HRM results several criteria need to be satisfied in order to generate amplicons with just single SNPs producing easy to distinguish single-base differences on the melting curve. In such cases, all homozygotes show a single melt transition whereas heterozygotes produce a deviating melting curve arising from the integrated melting curves of two homoduplexes and two heteroduplexes (Gundry et al., 2003; Reed and Wittwer, 2004). First, PCR conditions must be optimized and primers checked to amplify a single PCR fragment, as non-specific bands can significantly reduce HRM performance (Lehmensiek et al., 2008). For 14 CG primers (12 in both populations and 2 in one population) multiple bands after amplification have been found. It might be mainly because these genes, such as *DELLA* and *CHS*, belong to gene families with homologues genes existing in the genome.

Another criterion is the amplicon size. Product size should be below 300 bp and preferably much smaller to keep a high sensitivity and specificity to detect the possible heterozygotes by HRM without error (Reed and Wittwer, 2004). When product length increases, the difference between homozygote and heterozygote curves will become smaller making SNP calling more difficult (Reed and Wittwer, 2004). Moreover, gerbera is a highly heterozygous ornamental crop and if the target sequence is larger the potential for inclusion of other unexpected SNPs is increasing. Amplicons with several SNPs always result in a complex situation with several melting curves. According to our previous study (Fu et al., 2016), there is a SNP in every 200~250 bp in each parental genotype. Thus, we produced small fragments to avoid additional SNPs.Primers were designed taking into account intron exon structure from the homologous gene coding sequence and avoiding additional SNPs as detected in the gerbera EST database.

In total, we designed 89 pairs of primers for 71 CGs, and 29 CGs with a clear 1:1 segregation in the offspring were mapped in at least one population. The percentage of genes successfully mapped was 41%, other gene primer pairs dropped out mainly because of additional SNPs and multiple bands.

Out of the 29 mapped CGs, several genes are from the same gene family. For example, several poly-galacturonase genes (*ghPG1*, *ghPG2*, *ghPG7*, *ghPG9*, *ghPG10*) could be mapped. Those genes were mapped on different linkage groups, except *PG7* and *PG10* which were mapped on the same linkage group close to each other (1 cM apart).

Using a CG approach for identifying Botrytis resistance genes in gerbera seems effective. Several mapped CG alleles showed significantly difference in resistance for the traits whole inflorescence, bottom and ray floret in the two populations, and several CGs were found co-localized with identified QTLs. Few CGs showed allelic variation that gave a high significance level (P<0.01) in the t-test, but couldn't be detected as a QTL. This might be due to

the environmental conditions which could influence the power of detecting QTLs. The detection of two new QTLs after addition of specific CGs to the map also indicated that marker density may also play a role.

Allelic variation and expression of the candidate genes

Allelic diversity was assessed mainly for genes co-localized with detected QTLs and statistically correlated with phenotypic variation. The sequence polymorphisms of these selected CGs offer a glimpse of the heterozygosity of gerbera. Although only two species are considered to be involved in the origin of modern gerbera cultivars with a possible bottleneck at the moment of hybridization (Hansen, 1999), genetic diversity is relatively high in gerbera germplasm. The SNP density in the specific genes involved in Botrytis resistance varied from 5.7 to 27.2 SNPs/kb which is higher than the average SNP density identified within the four parental overall ESTs (from 3.7 ~ 4.8 SNPs/kb) (Fu et al., 2016). It is also higher compared to the SNP density of 10.5 SNP/kb found in the exons and introns of 7 genes in eleven safflower (*Carthamus tinctorius*) individuals (Chapman and Burke, 2007).

Multiple alleles existed at the candidate loci of the (four) parental genotypes and all genotypes are unique. Acquaah (2012) implied that for the improvement of cross-pollinated species breeding has to focus on increasing the frequency of favorable alleles. QTL analysis from bi-parental populations of gerbera in our previous study (Fu et al., 2017) only indicated the favorable segregating alleles present in our populations. Considering the heterozygous and heterogeneous situation in the four parents, it could be a practicable start to screen possible alleles focusing on these CGs in a broader gene pool and linking these to botrytis resistance.

The upregulation of expression levels of the CGs upon Botrytis infection, given the function of these genes in other species, is a clear implication that these CGs are involved in Botrytis response in gerbera. Tracking the Botrytis infection process on gerbera ray florets of parents till 72 hpi, we found that all the ray florets were infected eventually whereas the speed of disease development varied. For some CGs expression was not detected in SP2 and FP1 and that might be because of the genetic variation resulting in no expression or other genes playing a role in these two genotypes. All the studied CGs expressed in FP2 and the highest level of gene expression were at or before 12 hpi which was in line with this genotype FP2 having a relative high resistance to Botrytis in ray florets. A quick response of disease-related genes reaching the highest expression level as early as possible seems important to resist the attack of Botrytis.

The CG approach as used in this study in gerbera, for which crop no genome sequence is available, can efficiently pinpoint a number of potential causal genes. Whereas using QTL regions in outcrossing crops possess practical problems in the implementation for MAS, finding causal genes involved in a trait would be a major step and can also help in understanding the molecular interactions between Botrytis and gerbera.

Possible mechanisms for Botrytis resistance in gerbera

Several CGs with statistical associations with the whole inflorescence, bottom and ray floret tests might be involved in Botrytis resistance under multiple mechanisms. Plant cuticle and cell wall are constituted as the first protective barriers to defense against Botrytis invasion (Curvers et al., 2010). *B. cinerea* secretes at least 6 genes polygalacturonases (PGs) to decompose plant cell walls (van Kan, 2006; Ferrari et al., 2003). However, fungal PGs can be inhibited by plant polygalacturonase-inhibiting proteins (PGIPs) whereas these proteins may not inhibit a plant's own endo-PGs. Blanco-Ulate et al. (2014) suggested that *B. cinerea* might be able to manipulate plants to produce endo-PGs in order to degrade plant cell walls. From our study, two gerbera endo-PGs (ghPG1 and ghPG9) were found associated with Botrytis resistance on gerbera ray floret and bottom test respectively showing high statistical significance in the disease tests. The two candidate loci were detected as QTLs and explained 10.7% and 6.1% of the phenotypic variation. We assume *B. cinerea* might indeed be manipulating endo-PGs in gerbera plants to take the advantage of this in the infection process.

Plant hormones are considered to play an essential role in defense against Botrytis, especially the Ethylene (ET) and Jasmonic acid (JA) pathways. The ethylene responsive transcription factor (ERF) family encode proteins in disease resistance regulation pathways (Gutterson and Reuber, 2004) and their binding target sequence is the GCC box which is found in several promoters of pathogen related and ET- or JA-induced genes. Overexpression of ERF1 in Arabidopsis is sufficient to enhance tolerance to *B. cinerea* (Berrocal-Lobo et al., 2002). Also in our study, the ghERF is related to the phenotypic variation in the bottom test ($P = 0.0075$) and could be a promising candidate locus. Abscisic acid (ABA) signaling is believed to play an important role in *B. cinerea* resistance as shown in tomato mutant *sitiens* (Asselbergh et al., 2007; Curvers et al., 2010). ABA signaling regulated the cuticle and pectin composition which affect Botrytis resistance. The last step of ABA biosynthesis (ABA-aldehyde oxidation) in *sitiens* is blocked and leads to accumulation of trans-ABA instead of ABA (Rock et al., 1991). The difference of *sitiens* wild type allele (*sit*+) and mutant allele (*sit*) in tomato is in the deletion of intron 1 and division of exon 2 (Harrison et al., 2011). In our study, the two groups sorted by gerbera sit gene allelic variation showed significant difference in the ray floret test (at $p<0.001$ level) and the gene mapped in the QTL interval of RBQRF2. Interestingly, like the tomato sit allele, we found a 36 bp InDel in intron 1 of the four gerbera parental sit alleles (data not shown).

Phenylpropanoid compounds are natural secondary products which are derived from the general phenylpropanoid pathway and the consecutive flavonoid pathway. These derivatives, like anthocyanins, are known for the origin of flower pigmentation (Winkel-Shirley, 2001), but other derivates in the pathways like isoflavonoid phytoalexins are active in plant defence (Dixon et al., 2002). Enzymes in the phenylpropanoid/flavonoid pathway have been well studied (Elomaa et al., 1993; Helariutta et al., 1995; Ainasoja, 2008; Deng et al., 2014) in some crops and include

phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), chalcone isomerase (CHI), dihydroflavonol reductase (DFR), flavanone 3-hydroxylase (F3H) on the main phenylpropanoid pathway, and 2-pyrone synthase (2-PS) and stilbene synthase (SS) on the branch for flavonoid production. Several of these metabolites were confirmed to be involved in Botrytis resistance (Dixon et al., 2002; Laquitaine et al., 2006; Koskela et al., 2011) or their expression was enhanced by Botrytis infestation (Blanco-Ulate et al., 2015). Our study confirms that for a number of genes they might play a similar role in botrytis defence in gerbera., CG marker SS_5198_1668, the homologous sequence of stilbene synthase from grapevine, is found to be co-localized with the bottom test QTL RBQB2. Alignment of the ORF region of this contig5198 with the GhCHS4 (AM906210.1), a gerbera chalcone synthase gene showed that the two sequences are identical. Deng et al. (2014) found that GhCHS4 is highly expressed in carpels. The bottom test is in accordance with "heart rot", which is describing the disc florets infection by Botrytis. Interestingly, the disk florets color of SP1 which contributes to the QTL is black, while the other three parents have green/yellowish disk florets.

The polymorphisms of ghCHI in the two gerbera populations are associated with whole inflorescence test and the ghCHI gene might be the causal gene underlying the QTLs which were identified in the F population (RBQWI4, RBQWI6). After the Botrytis inoculation, ghCHI expression in SP1, SP2 and FP2 increased but no ghCHI expression was detected in FP1. Yellow-pigmented carnation, cyclamen and antirrhinum were all identified as due to the absence or reduction of CHI activity (Forkmann and Dangelmayr, 1980; Takamura et al., 1995; Ono et al., 2006). Considering that parent FP1 is yellow colored, it might also have lost its CHI activity. Interestingly, we found a SNP deletion in the two ghCHI alleles of FP1. The loss-of-function allele might contribute to Botrytis resistance in gerbera.

Validation of candidate genes with VIGS

Two CGs, *ghPG1* and *ghsit*, which were mapped in QTL regions from the *ray floret* test were further characterized in this study. The two CGs are the homologs of the genes responsible for Botrytis infection in tomato. Polygalacturonases (*PGs*) are cell-wall-degrading enzymes and participate in tomato ripening and have been found to facilitate Botrytis susceptibility (Cantu et al., 2009). The ABA-deficient *sitiens* mutant in tomato is impaired in the last step of ABA biosynthesis (Taylor et al., 1988; Rock et al., 1991) and the mutant is more resistant to Botrytis than wild type tomato plants that have higher amounts of ABA (Audenaert et al., 2002). The *sit* mutant also accumulates H_2O_2 and changes cell walls timely and efficiently to resistance Botrytis infection (Asselbergh et al., 2007).

The normal function of these two genes, *ghPG1* and *ghsit*, might be beneficial to Botrytis infection, while when the gene expressions are suppressed, Botrytis might fail to take advantage of the genes for infection. Plant genes that facilitate pathogen infection are defined as susceptibility (S) genes and an exclusive S-gene list is given by van Schie and Takken (2014). These two genes are part of that list. Silencing S-genes can limit the ability of the pathogen for infection and whether our found CGs might function as S-genes in gerbera was tested in this study for *ghPG1* and *ghsit*. A remarkable reduction of lesion size was found in these S-gene silenced gerbera ray florets and resulted from slowing the spreading of Botrytis on ray florets for 24hpi. Denby et al. (2004) considered that the variations in lesion size on Arabidopsis ecotypes for Botrytis infection were caused by either time prior to lesion initiation or the lesion's growth rate. Suppression of these two genes might postpone the lesion initiation and presented a delayed invasion at 24hpi on the ray florets. The two genes interacted with Botrytis in a somehow similar way which might be the reason that no further decrease of the lesion size on SP2 for the two gene silenced constructs was found compared with *TRV2::ghsit* alone.

Based on the visible indications from *TRV2::PDS* gene silenced plants of SP2, there may only be a small part, of the ray florets on the flower inflorescence be silenced. We also constructed TRV2 vectors carrying the CG *ghCHI* that mapped in a Botrytis resistance QTLs from whole flower test. However, when we sprayed the Botrytis spore on the whole flower, no visible difference was found (data not shown). The phenotypes after Botrytis infection may be difficult to detect as whole inflorescences are sprayed for the Botrytis test, but only a small part of the ray petals are silenced based on the reporter gene. To explore the function of CGs that were found with the whole inflorescence or bottom test, a stable transformation may be needed for confirming their role in Botrytis resistance.

This study confirmed that *ghsit* and *ghPG1* are involved in variation of Botrytis resistance on gerbera. After 24h of Botrytis inoculation on ray florets, a significantly delayed spread of lesions was observed on CG silenced ray florets compared to controls. For crops without genome sequence, using a CG approach could be an efficient method to pinpoint possible causal genes. VIGS here provided a rapid way to study the relationship between gene expression and susceptibility to Botrytis. Using markers developed from the causal genes themselves could make marker-assisted selection more accurate and can avoid the risk of loss of linkage due to recombination when using more distant markers. Further research will have to show the value of the combined effects of the best alleles for the two genes *ghsit* and *ghPG1* in gerbera with respect to Botrytis incidence and resistance under normal greenhouse and postharvest conditions.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

YF conducted the study, and generated and analyzed data. YS was involved in the construction of the VIGS constructs. JT

supervised progress and was involved in discussions. RV and PA initiated the research and did conceptualization and supervision including discussion on methodology. YF and PA wrote the original draft. YF, JT, RV, and PA reviewed and edited. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1100416/full#supplementary-material

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Current insights and advances into plant male sterility: new precision breeding technology based on genome editing applications

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Plant male sterility (MS) represents the inability of the plant to generate functional anthers, pollen, or male gametes. Developing MS lines represents one of the most important challenges in plant breeding programs, since the establishment of MS lines is a major goal in F1 hybrid production. For these reasons, MS lines have been developed in several species of economic interest, particularly in horticultural crops and ornamental plants. Over the years, MS has been accomplished through many different techniques ranging from approaches based on cross-mediated conventional breeding methods, to advanced devices based on knowledge of genetics and genomics to the most advanced molecular technologies based on genome editing (GE). GE methods, in particular gene knockout mediated by CRISPR/Cas-related tools, have resulted in flexible and successful strategic ideas used to alter the function of key genes, regulating numerous biological processes including MS. These precision breeding technologies are less time-consuming and can accelerate the creation of new genetic variability with the accumulation of favorable alleles, able to dramatically change the biological process and resulting in a potential efficiency of cultivar development bypassing sexual crosses. The main goal of this manuscript is to provide a general overview of insights and advances into plant male sterility, focusing the attention on the recent new breeding GE-based applications capable of inducing MS by targeting specific nuclear genic loci. A summary of the mechanisms underlying the recent CRISPR technology and relative success applications are described for the main crop and ornamental species. The future challenges and new potential applications of CRISPR/Cas systems in MS mutant production and other potential opportunities will be discussed, as generating CRISPR-edited DNA-free by transient transformation system and transgenerational gene editing for introducing desirable alleles and for precision breeding strategies.

KEYWORDS

male sterility, precision breeding, genome editing, CRISPR/Cas system, DNA free, transgenerational gene editing, food crops, ornamental species

1 Introduction

Plant male sterility (MS) refers to the inability of the plant to generate functional anthers, pollen, or male gametes, although female fertility remains unaffected (Kaul, 1988). Therefore, male sterile plants cannot undergo self-pollination, but they can be fertilized by male fertile plants. The establishment of MS lines is a major goal in F_1 hybrid production and marketing because by disabling self-fertilization, it is possible to facilitate the exploitation of heterosis in predominantly autogamous species (Longin et al., 2012; Kim and Zhang, 2018; Li et al., 2022; Ramlal et al., 2022). In the past, the main way to avoid considerable shares of progeny derived from self-pollination, even in species with predominantly allogamous fertilization, was to perform physical emasculation with chemical, mechanical or even manual methods. The main advantage in the use of MS lines is the reduction of costs, time and energy related to these emasculation procedures (Colombo and Galmarini, 2017). For these reasons, MS lines have been developed in several species of economic interest (Abbas et al., 2021; Wang et al., 2023), particularly in horticultural crops and ornamental plants (Yamagishi and Bhat, 2014; Barcaccia et al., 2016; Khan and Isshiki, 2016; Jindal et al., 2019; Singh and Khar, 2021). MS exhibits, in most cases, Mendelian inheritance, which is controlled either by the coordinated action of nuclear and cytoplasmatic genes or exclusively by nuclear genes (Kaul, 1988). The first scenario, defined as *cytoplasmic male sterility* (CMS), or three-line breeding system, relies on loci localized within the mitochondrial genome (Rogers and Edwardson, 1952; Chen and Liu, 2014). Cytoplasmic maternal inheritance causes all progeny derived from plants carrying the S locus (*sterile*) cytoplasm to inherit the male sterility trait (Budar and Pelletier, 2001; Yamagishi and Bhat, 2014; Xu et al., 2022). This condition can be overcome by nuclear genes that are functional in dominant conditions (Jindal et al., 2019); they are defined as *restorers of fertility* (*Rf*) and can suppress or downregulate the CMS genes and revert male sterility (Schnable and Wise, 1998; Ning et al., 2020). In contrast, *genic male sterility* (GMS), also reported as *nuclear male sterility* (NMS), or two-lines breeding system, is generally controlled by single nuclear genes, mostly by recessive alleles (*ms*) (Colombo and Galmarini, 2017; Manjunathagowda, 2021).

Although genes involved in MS have not yet been characterized in many species, the complex molecular mechanisms at the bases of GMS and CMS have been studied in the most important crops at the transcriptomic, biochemical and epigenetic levels (Fan et al., 2016; Li et al., 2019; Liu et al., 2022; Nie et al., 2023). Potential limitations in the application of MS systems in agricultural species reside first in the availability of MS resources, in the difficulty encountered when introgressing the trait into commercial varieties (e.g., linkage drag issues), and in the maintenance of the MS lines. Furthermore, considering that MS is detectable only during flowering stages, selecting plants characterized by GMS systems may be a challenge for preventing self-pollination. In addition, MS can be influenced by environmental conditions, resulting in instability and being a major issue for conducting crosses. Nevertheless, external conditions have been successfully exploited in rice and wheat, manipulating temperature or photoperiod to guarantee alternating cross-pollination or self-pollination (*environmental genic male sterility* – EGMS) (Zhou et al., 2012; Meng et al., 2016).

Developing MS lines therefore represents one of the most important challenges in plant breeding programs. Over the years, MS has been accomplished through many different techniques ranging from cross-mediated breeding to advanced methods based on knowledge of genetics and genomics to the most advanced molecular technologies based on genome editing (GE). If MS sources have not been found in the species of interest or if their transfer to the productive varieties is hindered by technical limitations, MS can also be induced by mutagenesis with chemical or physical agents (Hawkes et al., 2011). At present, the ability to precisely recognize and edit DNA sequences can have a significant impact on functional genomics and crop advancement studies. The recent development of GE-based technologies has provided researchers with powerful tools not only for decoding gene functions but also for improving or introducing new plant traits. This progress offers an increasing number of approaches considered revolutionary in molecular biology since it allows modifications at genomic loci in a precise and efficient manner (Malzahn et al., 2017). GE methods, being less time-consuming, can accelerate the creation of new genetic variability with the accumulation of favorable alleles, able to dramatically change the biological process and resulting in a potential efficiency of cultivar development bypassing sexual crosses (Chen and Gao, 2014; Gao, 2015; Arora and Narula, 2017; Scheben et al., 2017). Furthermore, since the development of a new commercial male-sterile line using traditional breeding systems usually takes several years or decades, these modern genetic engineering techniques can reduce dramatically the breeding time (Zhou et al., 2016).

Starting from these assumptions, the aim of this manuscript is to provide a general overview of insights and advances into plant male sterility, first providing a brief description of conventional breeding programs, and then focus attention on the recent new breeding GE-based applications capable of inducing MS by targeting specific nuclear genic loci. In particular, a summary of the mechanisms underlying the recent CRISPR technology and relative success applications will be described for the main crop and ornamental species. Finally, we discuss the future challenges and potential opportunities of such technologies for introducing desirable alleles and improving many traits for precision breeding strategies.

2 MS based-conventional breeding methods

The selection of improved varieties through conventional breeding primarily relies on phenotypic observations and the breeder's experience. For planning a promising plant breeding program, the following association establishment between phenotype and relative genotype results is fundamental (Chen and Lubberstedt, 2010). As reported in the Introduction section, MS is an important trait for different purposes, primarily for the

production of hybrid seeds. The development and propagation process of an F1 hybrid obtained through an MS system involves multiline maintenance strategies (Khan and Isshiki, 2016; Kim and Zhang, 2018; Xu et al., 2022; Scariolo et al., 2023). Various agronomic strategies can be used to introduce the MS trait in a commercial line of interest through conventional breeding approaches: after identifying naturally occurring male sterility within a species, the MS trait may be transferred to elite germplasm by cross pollination (Yamagishi and Bhat, 2014; Bruns, 2017; Zheng et al., 2020). Briefly, as schematically reported in Figure 1, CMS bases on a three-line system that includes, in addition to the MS line, a fertility restorer line and a maintainer line (Chen and Liu, 2014). Conversely, the use of the Mendelian recessive genes of GMS requires the discrimination of male fertile and sterile progeny prior to anthesis to ensure the maintenance of the MS line. This task can be challenging unless functional molecular markers associated with the MS locus are available (Wu et al., 2016). EGMS could overcome this problem by altering specific environmental conditions to make MS lines either male fertile or sterile (Sun et al., 2022).

The identification of functional molecular markers linked to specific traits can be of primary importance to allow future selection programs mediating marker-assisted selection (MAS), which identifies the following mapping gene responsible for the observed phenotype (Page and Grossniklaus, 2002; Schneeberger, 2014; Aklilu, 2021). The use of MAS results in fact in a useful predictive tool for the identification of male sterile genotypes, mapping markers closely associated with the MS locus (Mackenzie, 2012). The complexity and long times of these breeding strategies make the exploration of the molecular mechanisms a key feature to improve productivity and other traits of interest (Bohra et al., 2016; Van Ginkel and Ortiz, 2018; Yu et al., 2021a). On the basis of these articulated schemes of MS maintenance, defining the conditions in which the markers can be predictive molecular tools of genotype is a key point of each program. Furthermore, starting from the knowledge of a well-characterized mechanism of the MS system in model species, substantial genetic resources can be used for the discovery of homologous ms-related genes in other species (Leino et al., 2003; Fernandez Gomez and Wilson, 2014; Morales et al., 2022).

2.1 CMS system

Four different models have been described to explain how CMS can produce male sterility Chen and Liu (2014):

i) *Cytotoxicity model*: the proteins encoded by the CMS genes directly cause the death of the cells involved. At the basis of the

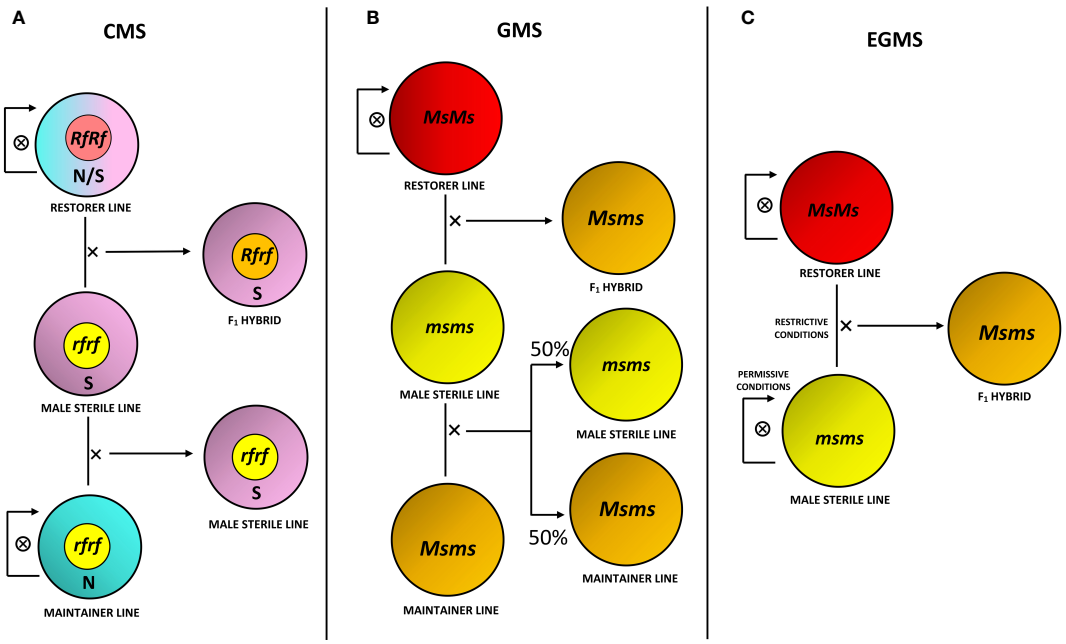

FIGURE 1
Production and maintenance strategies for MS systems. **(A)** Three-line system for *cytoplasmic male sterility* (CMS), involving an MS line with sterile cytoplasm (S) and restorer gene in recessive homozygous condition (*rfrf*), crossed with a maintainer line carrying normal fertile cytoplasm (N) and *rf* alleles for its maintenance, in addition to a fertility restorer line with N or S cytoplasm without distinction and restorer alleles in dominant homozygous, hence functional, condition (*RfRf*), crossed with the MS line for F_1 hybrid production. The F_1 hybrid consequently brings S cytoplasm and is heterozygous for the restorer gene (*Rfrf*), hence male fertility. Maintainer and restorer lines are self-pollinated for their maintenance. **(B)** Three-line system for *genic male sterility* (GMS), involving a recessive homozygous MS line for the MS gene (*msms*), crossed with a heterozygous maintainer line (*Msms*) for maintenance, producing half recessive homozygous and half heterozygous progeny, in addition to a dominant homozygous restorer line (*MsMs*), crossed with the MS line for heterozygous and fertile F_1 hybrid production. **(C)** Two-line system for *environmental genic male sterility* (EGMS), involving a recessive homozygous MS line, kept in permissive environmental conditions (i.e., low temperatures or short-day photoperiod) in order to make it become male fertile and self-pollinate for its maintenance, while kept in restrictive conditions (i.e., high temperatures or long-day photoperiod) to make it male sterile and to cross it with a dominant homozygous restorer line for F_1 hybrid production.

mechanisms by which this occurs it has been hypothesized that there is mitochondrial dysfunction, but a well-defined model has not yet been developed, lacking molecular evidence of cytotoxicity itself. As a result, a simple explanation for CMS in these systems is that the CMS proteins cause mitochondrial malfunction in the anthers' sporophytic or gametophytic cells, resulting in male abortion (Levings, 1993).

ii) *Lack of energy model*: the cellular respiration process is altered. In fact, CMS proteins can act as dysfunctional homologues of parts of complexes forming the electron transport chain, or changing proton gradients critical to the cellular respiration process, resulting in no ATP production. The molecular evidence supports the concept that some CMS are caused by an energy deficit in growing anthers, which demand more energy (Sabar et al., 2003; Wang et al., 2013).

iii) *Asynchronous programmed cell death* (PCD) *model*: PCD is induced in tapetum cells earlier than its normal course. It is implemented through the release of cytochrome C, a protein complex of the electron transport chain, and by increasing the production of reactive oxygen compounds (ROS, from Reacting Oxygen Species). By starting the autolysis before the pollen is mature, the tapetum cannot continue to nourish it and this does not complete its development. Plant male gametophytes form in anthers through cooperative contacts between sporophytic (anther wall) and gametophytic (microspore) cells, as well as correct PCD-controlled cellular degeneration of the tapetum, the deepest cell layer of the anther wall tissue (Mah, 2005).

iv) *Retrograde regulation model*: some CMS proteins are able to regulate the expression of nuclear genes, including some involved in the processes for correct reproduction. For example, they can disturb the formation activity of the stamens, in place of which carpels or petals develop. Or they can nullify the action of fertility restorative genes, when they are in their recessive allelic/haplotype form (Linke et al., 2003).

By comparing the proteomes of CMS and fertile lines, some CMS causative proteins, such as URF13 of maize CMS-T (Forde et al., 1978) and truncated COX2 of sugar beet CMS-G (Ducos et al., 2001), were discovered in other CMS systems. The CMS candidate genes were found in a few cases, such as radish CMS-Ogu (Bonhomme et al., 1991) and wheat alloplasmic CMS-AP (Rathburn and Hedgcoth, 1991), by analyzing the mitochondrial DNAs of segregating somatic hybrids (cybrids) produced from protoplast fusion between CMS-carrying lines and normal fertile lines. However, owing of the difficulties in acquiring cybrids and the uncertainty of recombination events between the mitochondrial DNAs of the fusion lines, this strategy is ineffective for most crops. Several methodologies can be used to identify CMS candidate genes. The most common approach is to look for changes in mitochondrial gene organization and/or mitochondrial transcriptome or proteome differences in CMS cytoplasm lines with and without the Rf gene(s). Nuclear Rf genes perform their action at different levels, involving various steps of protein synthesis or cellular metabolism (Chen and Liu, 2014).

Since in the case of CMS, 100% of offspring individuals will be MS, the use of molecular markers results a strategic key if identified and mapped in association with Rf genes. Table 1 shows main examples of the important crop classes for which mapped and retrievable information regarding reproducible, codominant molecular markers linked to Rf genes, offering fast and reliable detection tools to select, by MAS, parental lines for production of the desired progeny (Jordan et al., 2010; Yan et al., 2017).

In addition to major cereal crops and species, a great interest in mapping CMS-related loci has also been reflected in ornamental plants such as sunflower and petunia (Bentolila et al., 1998; Gentzbittel et al., 1999; Feng and Jan, 2008; Yue et al., 2010; Liu et al., 2012; Qi et al., 2012; Liu et al., 2013; Talukder et al., 2019).

2.2 GMS system

Several molecular mechanisms underlie GMS in different species, in many of them genes coding transcription factors are capable of modifying the expression of genes involved in reproductive processes. The result is disturbance of gamete formation, due to failure of homologous chromosome separation in meiotic anaphase I and delayed of programmed cell death in tapetum (Jeong et al., 2014). In particular, several nuclear genes have been found responsible for MS, causing arrest of microspore development. Furthermore, as mentioned in Introduction section, the male sterility can also depend on environmental conditions, and in this case the GMS is define as EGMS. Temperature-sensitive genic male sterile (TGMS) and photoperiod-sensitive genic male sterile (PGMS) lines were developed especially in cereals crops like rice and wheat. TGMS lines are sterile at high temperatures and fertile at low temperatures, while PGMS lines can either be sterile when the day is longer than the night and fertile when it is shorter, or vice versa. In China, EGMS lines occupy 20% of the area dedicated to the cultivation of hybrid rice (Li et al., 2007). Also in this case, the molecular markers result strategic keys if identified and mapped in association with the ms locus, as testified in past (Barcaccia et al., 2016). However, to date, in more than 610 species of flowering plants the MS trait is under investigation, and specifically in the past few decades, at least 40 GMS genes have been identified by MAS and characterized in model Arabidopsis and rice (Chen and Liu, 2014; Singh et al., 2019; Wan et al., 2019). As similarly reported in Tables 1, 2 shows main examples of the several crop classes for which mapped and retrievable information regarding reproducible, codominant molecular markers linked to nuclear male sterility genes. Molecular markers such as SSR, RFLP, SCAR, and SNP were fully employed for mapping male sterility genes, while insertion–deletion (InDel), target region amplification polymorphism (TRAP), sequence-related amplified polymorphism (SRAP), high resolution melting (HRM), and conserved orthologous set (COS) markers were sporadically used among these research studies. However, the data availability of mapped genes was correlated with species of great agronomic and economic importance: studies on cereals and other horticulture crop were prevalent, resulting in numerous mapped markers and associated genes involved in both MS systems, which offer open access for hybrid production using male-sterile lines (Li et al., 2007; Rout et al., 2021; Morales et al., 2022). These insights were interesting because proper MAS application could offer competitive phenotypes for market demand and contribute to reducing production costs, which is also fundamental for ornamental plant companies.

TABLE 1 Male sterility-related molecular markers mapped to Rf genes in CMS system.

	Species	Gene locus	CMS type	LG/Chrom	Molecular markers	Reference(s)
Cereals	Maize	Rf1	CMS-T (S)	3	RFLP	Schnable and Wise, 1994
		Rf2	CMS-T (S)	9	RFLP	Schnable and Wise, 1994
		Rf8, Rf*	CMS-T (S)	2L	RFLP	Dill et al., 1997
		Rf3	CMS-S (G)	2L	SSR, AFLP	Zabala et al., 1997; Zhang et al., 2006
		Rf4	CMS-C (S)	8	CAPS	Dewey et al., 1991; Liu et al., 2022
		Rf5	CMS-C(S)	5	RFLP	Sisco, 1991
	Soybean	Rf3	CMS (G)	9	CAPS, SSR	Sun et al., 2022
		Rf-m	CMS-M(G)	16	SSR	Wang et al., 2016
	Wheat	Rf1	CMS-T (S)	1A	SNP	Melonek et al., 2021
		Rf3	CMS-T (S)	1B	SNP	Geyer et al., 2016; Melonek et al., 2021
		Rf9	CMS-T (S)	6AS	SNP	Shahinnia et al., 2020
	Rice	Rf1	CMS-BT (G)	10	RFLP	Komori et al., 2004
		Rf4	CMS-WA (S)	10	SSR, SNP, InDel	Tang et al., 2014
		Rf2	CMS-LD (G)	2	CAPS, SNP	Itabashi et al., 2011
		Rf5	CMS-HL (G)	10	SSR	Hu et al., 2012
		Rf17	CMS-WA (S)	4	SNP	Fujii and Toriyama, 2009
		Rf98	CMS-RT98(G)	10	SSR	Igarashi et al., 2016
		Rf3	CMS-WA (S)	1	RAPD, RFLP, SSR	Zhang et al. 1997; Ahmadikhah and Karlov, 2006
		Rf6	CMS-BT (G)	8	SNP	Liu, 2004; Zhang et al., 2017; Zhang et al., 2019
Horticultural	Pepper	Rf	CMS-Peterson	6	SCAR, CAPS	Jo et al., 2016; Kang et al., 2022
Non-food	Rapeseed	Rf1	CMS-Pol (S)	18	RFLP	Jean et al., 1997
	Cotton	Rf2	CMS-D8(G)	19	RAPD, CAPS, AFLP, SSR	Wang et al., 2007
		Rf1	CMS-D2-2(S)	D5	SNP, InDel	Cheng et al., 2023; Wu et al., 2017
Ornamental	Petunia	Rf	NR	4	SSR	Bentolila et al., 1998
	Sunflower	Rf1	CMS-PET1(S)	13	SSR, TRAP	Yue et al., 2010
		Rf3	CMS-PET1(S)	7	SSR	Liu et al., 2012
		Rf5	CMS-PET1(S)	13	SSR	Qi et al., 2012
		Rf7	CMS-PET1(S)	13	SSR, SNP	Talukder et al., 2019
		Rf4	CMS-GIG2	3	SSR	Feng and Jan, 2008;
		Rf6	CMS-514A	4	SSR	Liu et al., 2013
		Msc1	CMS-PET1(S)	12	RFLP	Gentzbittel et al., 1999

The class to which each species taken into account belongs is reported alongside. The linkage group (LG) or chromosome (chrom), and available molecular markers for MAS application are indicated. CMS-type: S, sporophytic; G, gametophytic, indicates where the CMS acts. NR, Not reported.

3 A new frontier of precision plant breeding technology: GE mediated by the CRISPR/Cas system

Novel GE technologies have been intensively developed through diverse biological systems depending on sequence-specific nucleases (SSNs). Upon induction, all SSNs may detect a specific DNA fragment and cause double-stranded breaks (DSBs), repaired by two endogenous repair machinery of plant. Initially, ZFNs (zinc-finger nucleases) and TALENs (transcription activator-like effector nucleases) were the two systems primarily employed in genome editing techniques (Smith et al., 2006; Petolino, 2015; Malzahn et al., 2017). However, the difficulties of array and vector design in each of these methods, as well as the time-consuming work necessary to construct vectors for each new DNA sequence target, have hampered their widespread usage for plant genome editing. In contrast, with subsequent scientific breakthroughs, CRISPR/Cas-

TABLE 2 Male sterility-related molecular markers mapped to relative genes associated with the GMS trait.

	Species	Gene locus	Type	Gene function	Role in ms	LG/ Chrom	Molecular markers	Reference(s)
Cereals	Maize	Ms30	GMS	GDSL Lipase	pollen exine formation, anther cuticle development	4	SNP	An et al., 2019
		ms39	GMS	callose synthase12 (ZmCals12)	pollen development, plant height, tassel length, tassel branch number	3	InDel, SSR, SNP	Zhu et al., 2018; Niu et al., 2023
		Ms28	GMS	ZmAGO5c protein	regulation of the tapetum development	5	InDel, SSR, SNP	Li et al., 2021
		ms40	GMS	bHLH transcription factor	tapetum degeneration retardation	4	InDel	Liu et al., 2021
		ms32	GMS	bHLH transcription factor	regulator of both division and differentiation during anther development	2	RFLP	Moon et al., 2013; Chaubal et al., 2000
		Ms33	GMS	glycerol-3-phosphate acyltransferase (GPAT)	tapetum development and metabolism disruption	2L	SSR	Xie et al., 2018
		Ms7	GMS	PHD finger transcription factor	abnormal microspore wall and tapetal cell development	7	SSR	Zhang et al., 2017
		Ms20	GMS	irregular pollen exine1 (ipe1)	anther cuticle and pollen exine formation	1	SSR	Wang et al., 2019
	Soybean	mst-M	GMS	NA	NA	13	CAPS, SSR	Zhao et al., 2019
		ms1	GMS	kinesin protein	cell plate formation in male gametogenesis	13	SSR	Yang et al., 2014b; Fang et al., 2021
		ms6	GMS	R2R3 MYB (GmTDF1-1) transcription factor	anther development regulator	13	SSR	Yang et al., 2014b; Yu et al., 2021
		ms4	GMS	PHD protein	failure of cytokinesis after telophase II, coenocytic microspores	2	SSR	Yang et al., 2014b; Thu et al., 2019
	Wheat	ms5	GMS	GPI-anchored nsLTP	pollen development	3AL	SNP	Pallotta et al., 2019
		ms1	GMS	GPI-anchored nsLTP	pollen exine development	4BS	SSR, CAPS, SNP	Yang et al., 2021; Tucker et al., 2017; Wang et al., 2017
		Ms3	GMS	NA	NA	5A	centromere-related	Maan et al., 1987
	Rice	rpms1	rPGMS	NA	NA	8	SSR	Peng et al., 2008
		rpms2	rPGMS	NA	NA	9	SSR	Peng et al., 2008
		tms12-1	P/ TGMS	small RNA osa-smR5864w	regulator of the development of the male reproductive organ	12	SSR	Zhou et al., 2012
		tms2	TGMS	alpha-galactosidase precursor (ORMDL)	sphingolipid homeostasis, pollen development	7	SSR	Lopez et al., 2003; Chueasiri et al., 2014
		tms3(t)	TGMS	NA	NA	6	RAPD, RFLP	Subudhi et al., 1997
		pms2	PGMS	NA	NA	3	RFLP	Zhang et al., 1994
		pms4	PGMS	NA	NA	4	SSR	Huang et al., 2008
		pms1	PGMS	21-PHAS gene	differential accumulation of the phasiRNAs	7	SSR, CAPS, InDel	Fan et al., 2016
		tms5	TGMS	RNase Z-S1	defective pollen production	2	CAPS, AFLP	Wang et al., 2003; Yang et al., 2007; Zhou et al., 2014
		tms4(t)	TGMS	NA	NA	2		Dong et al., 2000

(Continued)

TABLE 2 Continued

	Species	Gene locus	Type	Gene function	Role in ms	LG/ Chrom	Molecular markers	Reference(s)
							AFLP, RFLP, SSR	
		rtms1	rTGMS	NA	NA	10	AFLP	Jia et al., 2001
		tms6	TGMS	NA	NA	5	STS, SSR	Lee et al., 2005
		pms3	PGMS	long noncoding RNAs (lncRNA)	pollen development of plants grown under long-day conditions	12	RFLP	Mei et al., 1999; Ding et al., 2012
Horticultural	**Chicory**	ms	GMS	MADS-box gene	flower development	4	SSR	Cadalen et al., 2010
		ms-myb80	GMS	Myb 80 transcription factor	anther development	9	SSR, CAPS, SNP	Barcaccia and Tiozzo, 2012; Palumbo et al., 2019
		NMS	GMS	hypothetical S-domain RLK gene	anther development	5	SSR, SCAR	Cadalen et al., 2010; Gonthier et al., 2013
	Pepper	ms1	GMS	PHD finger transcription factor	sporophytic factor controlling anther and pollen	5	HRM	Jeong et al., 2018
		ms3, msw	GMS	NA	NA	1, 5	CAPS	Naresh et al., 2018
		ms8	GMS	NA	abortion of microspore formation	P4	SCAR	Bartoszewski et al., 2012
		ms10	GMS	NA	NA	1	SSR	Aulakh et al., 2016
	Tomato	ps2	GMS	polygalacturonase gene (PG)	blocking anther dehiscence, fruit ripening	4	COS	Gorguet et al., 2006; Gorguet et al., 2009
		ms10	GMS	anthocyanin-related GST gene (SlGSTAA)	role in anthocyanin transport	2	InDel	Zhang et al., 2016
		ms32	GMS	bHLH transcription factor	pollen and tapetum development	1	InDel	Liu et al., 2019
		ms15^{26}	GMS	B-class MADS-box TM6	stamen development	2	InDel	Cao et al., 2019
Non-food	**Rapeseed**	ftms	GMS	putative β-(1,3)-galactosyltransferase (Bra010198)	microspore development	A05	SSR	Tan et al., 2019
		ms3	GMS	Tic40 protein	tapetal function and pollen development	N19	SCAR	Huang et al., 2007; Zhou et al., 2012
		Ms-cd1	GMS	*SALT-INDUCED AND EIN3/EIL1-DEPENDENT 1 (SIED1)*	primary pollen mother cell (PMC) and microspore formation	3	SRAP	Zhang et al., 2011; Liang et al., 2017
	Cotton	ms5, ms15	GMS	NA	NA	12	SSR	Chen et al., 2009
		ms6	GMS	NA	NA	26	SSR	Chen et al., 2009
Ornamental	**Marigold**	Tems	GMS	B class MADS-box genes	floral organ homeotic conversion of the petals and stamens	NR	AFLP, SCAR	He et al., 2010

The class to which each species taken into account belongs is reported alongside. The linkage group (LG) or chromosome (chrom), and available molecular markers for MAS application are indicated. NA, Not Applicable. rPGMS, reverse photoperiod-sensitive genic male sterility. rTGMS, reverse temperature-sensitive genic male sterility. P/TGMS, photoperiod, temperature-sensitive male sterility.

based genome editing systems (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein) have been increasingly employed in the last decade (Li et al., 2013; Nekrasov et al., 2013; Shan et al., 2015). Their use is constantly expanding in numerous applications, resulting in a wider array of editing tools developed for several purposes (Figure 2). CRISPR/Cas-based systems are considered more robust and simpler for targeting gene editing since they present a significant advancement over previous systems, such as the simplicity and versatility in vector design and construction for subsequent plant transformation (Chen et al., 2019; Bhat et al., 2020; Nadakuduti and Enciso-Rodriguez, 2020; Zhu et al., 2020). An increasing number of studies attest to the

expanded applications of Cas9 nuclease for editing beyond double strand breaks, and the accompanying benefits of those systems have resulted in quick, widespread acceptance for editing applications in a diverse range of plant species. Cas9-related nuclease, if associated with an RNA guide (single guide RNA, sgRNA), is able to identify a special site PAM (Protospacer Adjacent Motif) in the host DNA and cut the target sequence recognized, mediated by the complement to which the sgRNA binds (Mojica et al., 2009; Jinek et al., 2012), inducing the development of modified and improved forms of Cas9 and Cas9-like nucleases. In addition to Cas9, other related enzymes (Cas12a, CasΦ, and Cms1), derived from other CRISPR systems, have been implemented since they are potentially useful for editing approaches, each with slightly different capabilities to recognize and modify PAM sites (Zetsche et al., 2015; Begemann et al., 2017; Gao et al., 2017; Li et al., 2018; Pausch et al., 2020).

This approach is defined as a precision-type plant breeding technology, and it is currently being utilized to change the characteristics of various plants, including important crops, as well as to produce new germplasm resources. (Gaillochet et al., 2021). The applications have been primarily focused on traits related to stress tolerance, disease resistance, quality improvement, and higher yields with minimal input (Liu et al., 2022) (Figure 2A). In particular, the CRISPR/Cas system has been widely employed to edit plant genomes to modify genes in various ways, e.g., gene knockout, gene knock-in, gene regulation, base editing, and prime editing (Zhang et al., 2021a). Gene knockout/-in and base and/or prime editing mediated by CRISPR/Cas-related tools have resulted in flexible and successful

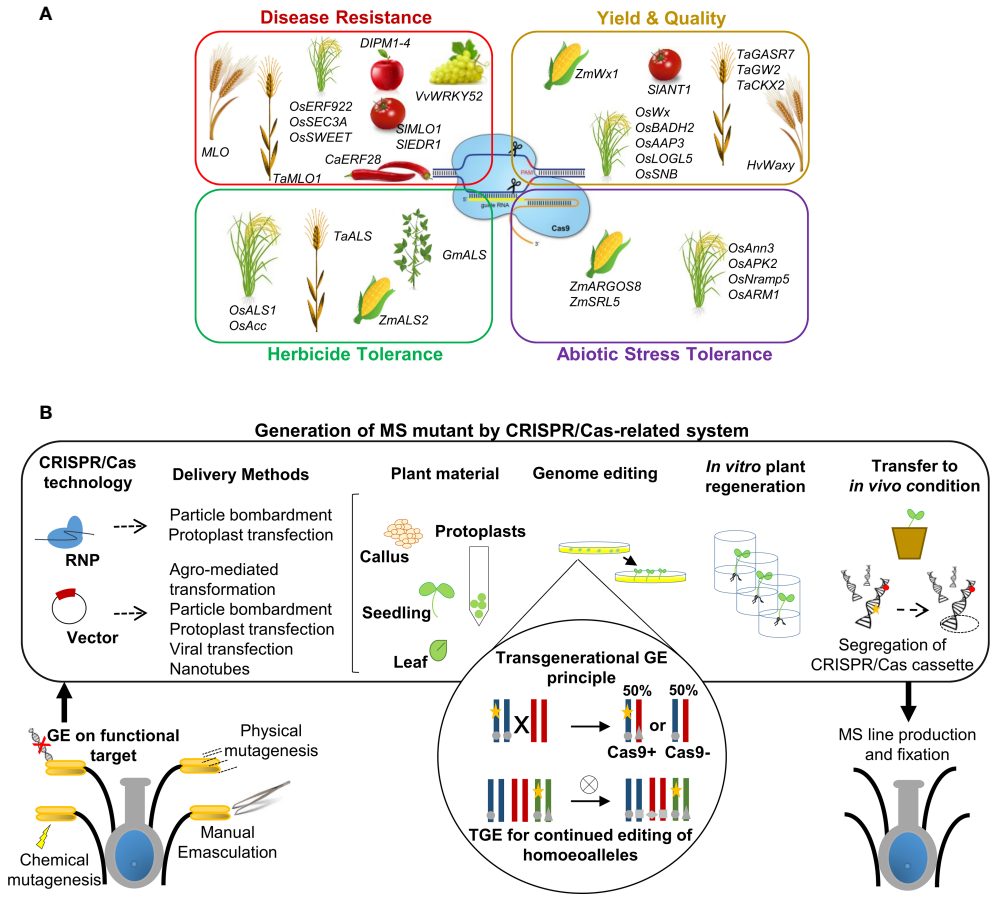

FIGURE 2
GE applications in precision plant breeding approaches. **(A)** Schematic representation of the main applications of GE for crop improvement through CRISPR/Cas and related systems. Examples of genes modified for improving specific traits are listed in each specific box for various reference crops. **(B)** Simplified representation of the workflow for MS generation mediated by CRISPR/Cas technology applied to target genes. In summary, gene editing is induced by transient or stable expression of a Cas nuclease and gRNA through the transformation/transfection of the ribonucleoprotein (RNP) complex or DNA vector. Both CRISPR machinery reagents can be delivered into plant cells using biolistic transformation or other methods, according to suggested transformation/transfection protocols related to species, plant tissues and the methodological approach followed. Such events can lead to the generation of edited whole plants. The transgene locus is usually heterozygous in the first generation of transgenic plants (T0). Afterwards, elimination of the CRISPR/Cas cassette transgene (yellow star) by genetic segregation, according to Mendelian genetics, occurred to obtain transgene-free material. Transgene-free and edited T1 plants can be identified by PCR-based genotyping. The transgenerational GE principle and potential applications in plants are highlighted schematically in circles: transgenic plants represented as a chromosome pair are hemizygous for a CRISPR/Cas9-containing T-DNA locus (yellow stars) and edited in both alleles (grey circles). When crossed with a WT, the resulting progeny either lacks the T-DNA and inherits a single edited allele or inherits the T-DNA, resulting in (transgenerational) editing of the inherited WT allele. TGE for continued editing of homoeoalleles in polyploids species: a transgenic line may have edits only in a subset of homoeoalleles at the homologous chromosomes. After self-crossing and selecting plants that inherited the T-DNA, all homoeoalleles may now be edited. The details can be found within the text.

strategic ideas used to alter the function of key genes and their associated networks, regulating complicated crop traits (Gaillochet et al., 2021). The successes of CRISPR/Cas application in crop improvement have been reviewed in many papers, which are focused on the improvement of crop resistance to fungi, bacteria, and viruses, typically mediating targeting susceptibility systems to increase resistance (Wang et al., 2014; Malnoy et al., 2016; Wang et al., 2016; Nekrasov et al., 2017; Borrelli et al., 2018; Ma et al., 2018; Pu et al., 2018; Wang et al., 2018b; Dong and Ronald, 2019; Oliva et al., 2019; Mishra et al., 2021), resistance to an assortment of herbicides (Li et al., 2015; Chen et al., 2017; Zong et al., 2018; Zhang et al., 2019; Kuang et al., 2020), and abiotic stresses such as drought, salinity, high temperatures, and soil pollution (Lou et al., 2017; Nieves-Cordones et al., 2017; Shi et al., 2017; Tang et al., 2017; Pan et al., 2020). In particular, the main purposes of food crop improvement research using GE technology are to raise yield (e.g., grain size/weight/number per panicle) and crop quality traits determined by internal (e.g., contents of nutrients and bioactive substances) and external (e.g., size, color, and texture) factors related to a response to adverse surrounding environments (Shan et al., 2015; Zhang et al., 2016b; Lou et al., 2017; Dahan-Meir et al., 2018; Wang et al., 2018c; Chen et al., 2019; Ma et al., 2019; Voss-Fels et al., 2019; Wang et al., 2020; Zhu et al., 2020; Liu et al., 2021b). To simplify the overview of this complicated scenario, Figure 2A summarizes the main traits that can be enhanced by genome editing, with a list of example genes edited by the CRISPR/Cas system to improve related plant species.

4 CRISPR/Cas system applications for producing MS

In addition to stress response, and traits related to quality and yield, CRISPR/Cas-based technology offers a new strategic tool to affect other crop traits associated to fertility/sterility (Figure 2B). As described in section 2, researchers have employed several strategies for integrating MS traits into genomes of interest using information and methods arising from conventional breeding approaches, with the aim to guarantee high varietal purity breeding and to have better offspring in terms of uniformity, yield, and stress tolerance (Bao et al., 2022). Thanks to important forward genetic tools, as mutagenesis approaches and TILLING populations, it has been possible to discover and investigate new candidate genes controlling male sterility. Furthermore, the increasing number of transcriptomic and proteomic studies in recent decades, mostly on crop species, has allowed us to characterize an emergent number of genes with different roles in the development of male reproductive organs and consequently with a putative role in MS induction. If the role of these genes is confirmed as influencer of MS trait, they could be potential targets for subsequent gene editing strategies (Carroll, 2011; Li et al., 2012). The elucidation of molecular processes regulating anther and pollen development has increased the identification and characterization of new putative candidate male-sterility genes (MSGs) in several species, allowing the development and effective use of numerous biotechnology-based male-sterility systems for crop hybrid breeding (Perez-Prat and Van Lookeren Campagne, 2002; Whitford et al., 2013; Wu et al., 2016; Zhang et al., 2019). As reported in more detail in following sections, CRISPR/Cas technology is resulted a novel, rapid and alternative method for the generation of MS lines through target gene editing, both in food crops (monocots and horticultural dicots) and in the increasing ornamental sector, implementing also the knowhow underlying male sterility in plants (Cong et al., 2013; Wang et al., 2018a).

4.1 Generation of MS mutant by CRISPR/Cas-related system

The CRISPR/Cas system, especially based on Cas9, has been successfully applied for the generation of male sterile lines in important worldwide food crops (Barman et al., 2019; Okada et al., 2019). Studies in main crops, such as rice, soybean, maize, and tomato, have reported that pooled CRISPR/Cas9 methods can result in valid strategies to generate a population of mutants for the MS trait (Jacobs et al., 2017; Meng et al., 2017; Liu et al., 2019; Liu et al., 2020). The application of CRISPR/Cas technologies for generating mutants with a male sterile phenotype is an effective tool, mainly mediating a knock-out approach towards target GMS genes with nuclear origin since, compared to cytoplasmic male sterile lines, it is much easier and more useful to produce hybrid seeds (Qi et al., 2020).

Generally, MS mutants result from mutations in target genes involved in microsporogenesis and/or microgametogenesis. Meiosis-related, tapetum-specific and transcription regulatory genes, such as eme1/exs, tpd1, ams and ms1, have been elucidated as key candidate genes involved in these biological processes (Canales et al., 2002; Zhao et al., 2002; Yang et al., 2003). Furthermore, many Arabidopsis transcription factors (TFs) genes, such as MYB103, DYT1, TDF1, AMS, bHLH10, bHLH89 and bHLH91, have been investigated as direct controllers of pollen development (Sorensen et al., 2003; Zhang et al., 2006; Zhang et al., 2007; Zhu et al., 2008; Zhu et al., 2015; Pan et al., 2020). The molecular and functional information has been then easily transferred from models to crops, as reported for Arabidopsis and rice, in which two analogous pathways regulating pollen and tapetum development have been identified in previous research (Fu et al., 2014; Jeong et al., 2014; Zhu et al., 2015; Mishra et al., 2018). In tomato, two homologous genes have been identified as regulators of tapetum and pollen formation. The first, *SlMS10* (Solyc02g079810_*ms10*^{35}) gene, encoding a basic helix-loop-helix TF (bHLH) and homologue to *AtDYT1* and *OsUDT1*, carries both PCD and meiosis alteration in the tapetum during microsporogenesis (Jeong et al., 2014). Its editing has confirmed that *SlMS10* is a possible good target candidate for male sterility induction since its knockout mediated by the CRISPR/Cas9 system conferred a male sterility phenotype (Jung et al., 2020). Recently, Liu and colleagues demonstrated that the creation of a mutation in *ms10*^{35} by CRISPR/Cas9 technology in association with its linkage marker genes led to marker use for creating mutants exhibiting complete male sterility and recognition during the early developmental stage, confirming promising application possibilities in the production of hybrid seeds

(Liu et al., 2021a). The second, Solyc01g081100, homologous to the AtbHLH10/89/90 and *OsEAT1* genes, is a candidate gene for the *male sterile 32* (*ms32)* mutant, a locus affecting tapetum and pollen development, and for this reason, it is suggested as a good target for gene editing to quickly develop such lines of interest (Liu et al., 2019). Furthermore, knockout by CRISPR/Cas9 of the *SlAMS* gene, encoding another basic helix-loop-helix (bHLH) TF, caused downregulation leading to abnormal pollen development, which in turn decreased pollen viability and subsequently generated a male-sterile phenotype (Bao et al., 2022). Recently, other tomato CRISPR/Cas9-edited lines with male sterility phenotypes were obtained by knock-out of *SlPHD_ms1* (Solyc04g008420), encoding a PHD-type TF involved in pollen formation and tapetum development, suggesting a key role for SlPHD in male sterility and aiding research into the regulatory processes of pollen and tapetum growth in tomato (Gökdemir et al., 2022). With analogous purposes, CRISPR/Cas technology was also applied in other horticultural crops, as demonstrated in cucurbit species. For example, the knockout of *eIF4E* by CRISPR/Cas9 in melon highlighted for the first time the association between *eIF4E* editing and the development of male sterility (Pechar et al., 2022). In watermelon (*Citrullus lanatus* L.), knockout of *ClATM1* by CRISPR/Cas9 causes male sterility, confirming its self-regulatory activity and providing new insights into the molecular mechanism underlying anther development (Zhang et al., 2021b).

In monocots, several CRISPR/Cas systems for producing MS have been reported as successful applications in precision breeding. An improved CRISPR/Cas9 system was driven by the TaU3 RNA polymerase III U3 promoter, and three homologous alleles expressing the wheat redox enzyme NO POLLEN 1 (NP1) were altered to produce totally male-sterile wheat mutants (Li et al., 2020). Furthermore, with recent molecular identification of the *Ms1* gene and exploiting strategies related to transgenerational gene editing (see below section 5.2), it has been possible to extend the use of the CRISPR/Cas9 system to generate *Ms1* knockout wheat lines that exhibit male sterility in the first generation, demonstrating the utility of the CRISPR/Cas9 system for the rapid generation of nuclear male sterility in hexaploid species like wheat (Okada et al., 2019). Chen and colleagues created a CRISPR/Cas9 vector in maize to target the male sterility gene 8 (Ms8). The resulting mutant was male-sterile, which was compatible with Mendelian genetic rules and was stably acquired by subsequent generations (Chen et al., 2018b). Furthermore, editing *ZmMTL* (ZmPLA1) with the CRISPR/Cas9 system has produced maternal haploid inducers with powerful haploid identification markers useful for breeding doubled-haploid crops, such as maize itself (Dong et al., 2018). Additionally, *ZmMS26*, a known nuclear fertility gene (Loukides et al., 1995; Djukanovic et al., 2013) that is conserved in other monocots, like rice, wheat, and sorghum (Cigan et al., 2017), was subjected to precision editing: targeted mutagenesis of MS26 utilizing the modified I-CreI homing endonuclease or CRISPR/Cas9 resulted in the generation of new ms26 male sterile lines (Djukanovic et al., 2013; Svitashev et al., 2015; Qi et al., 2020). In rice, gene knockout by CRISPR/Cas9 of the *OsHXK5* gene resulted in male sterility, contributing to demonstration that OsHXK5 contributes to a large portion of the hexokinase activity necessary for the starch utilization pathway during pollen germination and tube growth, as well as for starch biosynthesis during pollen maturation (Lee et al., 2020).

4.2 Environmental genic male sterility

The success of CRISPR/Cas technology application has also been provided in EGMS conditions, or rather the ability to switch from fertile to sterile conditions and vice versa, by adjusting environmental variables such as temperature and photoperiod. Great progress has been recently achieved in the understanding of PGMS or TGMS traits in cereal crops, and several genes controlling P/TGMS traits have been investigated and transferred, mediating conventional breeding and/or biotechnological transformation, in specific lines on which more than 30% of cereal hybrid production depends in China (Ding et al., 2012; Zhou et al., 2012; Zhang et al., 2013; Huang et al., 2014; Zhou et al., 2014). Several studies elucidated the molecular genetic mechanisms at the base of EMGS, confirming also the interesting role assumed by phasiRNAs (phased small-interfering RNAs) generated by long-noncoding RNAs. In rice, for example, the phasiRNAs originated from PMS1T locus regulates PSMS in rice (Fan et al., 2016). Especially in rice, in the last few years, many genes influencing PGMS or TGMS traits have been discovered and cloned, and several reports describe different CRISPR/Cas-based approaches to obtain photo- and thermosensitive male-sterile lines. For example, a simple and efficient rice TGMS cultivation system using CRISPR/Cas9 editing technology was proposed to knock out the *TMS5* (*thermosensitive genic male-sterile 5*) gene target, with great value in new commercial "transgene free" TGMS rice lines (Zhou et al., 2016). *TMS5* is a nuclear recessive gene that controls the TGMS trait and extensively used in two-line hybrid rice breeding. It was the first spontaneously mutated *Oryza sativa* ssp. indica, identified more than 30 years ago, and encodes an RNase ZS1 endonuclease, able to degrade the temperature-sensitive ubiquitin fusion ribosomal protein L40 (UbL40) mRNA (Zhou et al., 2014). A study found that when plants were grown under a high temperature regime, several tms5 mutants developed in a background of the japonica type showed a high degree (85.3%) of pollen sterility (Zhou et al., 2016), confirming that targeted modification of TMS5 by the CRISPR/Cas9 system is a successful approach to develop TGMS lines for hybrid rice production. Huang et al. targeted the TMS5 gene, producing a mutant that was entirely male-sterile at high temperatures but male-fertile at low temperatures, with a pollen fertility transition temperature fixed at 28°C. (Huang et al., 2014). Recent studies revealed the molecular mechanism of *tms5* leading to male sterility in rice to easily obtain excellent TGMS lines (Fang et al., 2022) and potentially applicable in other crops. CRISPR/Cas9-engineered mutation of *TMS5* also resulted in the formation of thermosensitive male sterility in maize (Li et al., 2017). In addition, Li et al. altered the carbon starvation *CSA* gene in pollen grains of the rice variety 'Kongyu 131' and found that the *csa* mutant had a male-sterile phenotype in short-day and a male-fertile phenotype in long-day conditions. (i.e., photosensitive nuclear male sterile mutant) (Li et al., 2016), whereas in tomato, Shen and colleagues generated photosensitive/thermosensitive

male-sterile lines by using CRISPR/Cas9 modifying the genic male-sterile 2-2 (PTGMS2-2) gene (Liu et al., 2019).

5 New potential applications of CRISPR/Cas systems in MS mutant production

5.1 Generating CRISPR-edited DNA-free by transient transformation system

As described in the previous sections, site-specific genome editing by CRISPR/Cas9 technology is becoming a progressively more successful tool for functional, basic and applied plant research because it can generate a high rate of mutation while being relatively easy to use (Zhang et al., 2013; Lowder et al., 2015; Ma et al., 2015; Wang et al., 2016). Numerous methods have been used to create CRISPR-edited plants devoid of CRISPR constructs and other transgenes because the lack of any transgenes in gene-edited plants is a requirement for the commercialization of any CRISPR-edited plants with stable valuable traits. For public approval, gene elimination or bypassing alien elements to edit endogenous genes is fundamental and could be a strategic approach, even if transgenic intermediates are transiently necessary (Figure 2B). The main different strategies useful to avoid the maintenance of transgene integration have been deeply described by He and Zhao, 2020 (He and Zhao, 2020). Commonly, after CRISPR-mediated mutagenesis, the Cas9 gene and associated DNA sequences are eliminated through genetic segregation, which frequently allays public concerns about genetically modified individuals. The biggest advantage of the method is that it could allow the selection of plants that no longer contain the T-DNA sequence, producing plant materials not containing any foreign DNA even though they were produced using transgenic technology mediating stable transformation methodologies. However, the fact that many commercial crop varieties are polyploid, heterozygous, or asexually reproduced complicates these efforts. Many commercial cultivars' genome complexity, long juvenile phase, and/or self-incompatibility limit the development of CRISPR-mediated transgenic crops since backcrossing is required to remove the CRISPR transgene.

In the past, plant transient transformation technology has been widely used as an alternative approach to facilitate rapid and efficient gene function analysis (Sheen, 2001; Chen et al., 2006). Using transient transformation methods, such as particle bombardment (Romano et al., 2003), transient transformation by *Agrobacterium* sp. (Cui et al., 2017) and polyethylene glycol (PEG)-mediated protoplast transfection (Cankar et al., 2022), excellent results in plant research have been achieved. Among these, the protoplast transient expression system has played a relevant role in genomics and proteomics research, resulting in a potential, rapid, and convenient technique for testing new technologies, such as GE approaches. In general, transient expression methods for protoplasts have been designed for many crop species, including monocots, dicots, herbaceous and woody species, such as rice (Yang et al., 2014a), barley (Bai et al., 2014), corn (Cao et al., 2014), apple (Maddumage et al., 2002), and grapevine (Zhao et al., 2016). These findings demonstrate the possibility and feasibility of utilizing protoplasts for CRISPR-mediated gene editing, particularly in species with a protracted juvenile phase, heterozygosity, or asexual propagation. Likewise, this strategy could represent the most feasible way to directly apply CRISPR-mediated DNA-free genome editing technologies for improving traits and increasing commercial value, as already experimentally confirmed for food and non-food crops, such as strawberry (Martin-Pizarro et al., 2019; Wilson et al., 2019), potato (Gonzalez et al., 2019; Nicolia et al., 2021; Zhao et al., 2021), lettuce (Woo et al., 2015), chicory (De Bruyn et al., 2020; Cankar et al., 2022), *Nicotiana tabacum* (Lin et al., 2018; Hsu et al., 2019) and *Brassica oleracea* (Lee et al., 2020; Hsu et al., 2021), and ornamental species, as petunia (Yu et al., 2021b). For these reasons, protoplast transient expression systems represent a promising and valid approach for generating CRISPR-edited DNA-free plant material and MS mutant production (Figure 2B). Numerous studies describe the different gene modification methods using transient expression of the Cas protein and associated sgRNA, mediating the main delivery methods into somatic plant cells, which may be done either as DNA vectors, through Agrobacterium infiltration (Chen et al., 2018a), or as ribonucleoprotein (RNP), using biolistic delivery (Liang et al., 2018), nanotubes (Demirer et al., 2019), virus transfection (Ellison et al., 2020), PEG-calcium (PEG–Ca^{2+}) (Toda et al., 2019). Because there is no foreign DNA present during transfection, direct transfection of the RNP complex eliminates the risk of plasmid DNA insertions into the plant genome (Andersson et al., 2018). Genome editing is realizable utilizing protoplasts without the insertion of foreign CRISPR DNA and without the necessity for hybridization, introgression, or back-crossing of progeny in the T0 generation. Furthermore, protoplasts are single cells that are edited before the first cell division: new plants grow from a single modified protoplast, ensuring that all cells share the same genetic background and that edited alleles are passed down to the next generation However, RNP-mediated genome editing has been employed successfully in many plant species, targeting genes with agronomic interest, involved disease resistance (Malnoy et al., 2016), in grain yield (Toda et al., 2019), nutritional composition (Andersson et al., 2018), and male fertility (Svitashev et al., 2016). MS induction, using an analogous approach, has been successfully achieved only in maize. Svitashev and colleagues demonstrated the success of their research, in which two male fertility nuclear genes (*MS26* and *MS45*) were targeted by purified Cas9 protein preassembled with *in vitro* transcribed gRNAs, demonstrating DNA-free genome editing in a major crop species using biolistically delivered Cas9–gRNA RNPs on immature embryos and subsequent plant regeneration (Svitashev et al., 2016). These positive results suggest the potential of applying similar methodologies in other large crops to increase the number of examples of male sterile lines CRISPR-edited DNA-free by transient transformation system by RNP complex.

5.2 Transgenerational gene editing

Because CRISPR/Cas9 expression cassettes and target sites are distributed throughout the genome, segregation and deletion of CRISPR/Cas9 cassettes is conceivable through subsequent selfing or crossing (Figure 2B). However, in crops with a high level of genome complexity, highly heterozygous, polyploid genomes, and usually propagated vegetatively, this is not easily achieved. Specifically, efficient propagation and stacking of first-generation mutations becomes increasingly difficult or nearly impossible with polyploidy.

Numerous new strategies have been developed to extend the CRISPR toolbox, and many of these new schemes could also take advantage from *transgenerational gene editing* (TGE)-based strategies, defined as the continued ability of Cas9 to edit also after cross: this means that if the Cas9 nuclease is still active, after cross it will encounter a new WT allele, which can be edited to create independent alleles. TGE has been utilized for a variety of applications, some of which are not always defined as TGE, such as the editing new alleles in polyploid crops, the creating allelic variation, and the editing target genes in refractory genetic backgrounds (Impens et al., 2022). Mutations are frequently found only in a fraction of the homoeoalleles targeted by the same sgRNA in polyploid crops such as hexaploid common wheat (*Triticum aestivum*) and tetraploid cotton (*Gossypium hirsutum*) (Wang et al., 2018a; Wang et al., 2018b; Wang et al., 2018c). While expressing CRISPR/Cas9 for more than one generation during TGE promotes on-target homoeoallele editing, it does not always boost off-targeting.

On the basis of TGE, with the purpose of accelerating the understanding of MS and ensuring speedy improvement, a new approach (Ramadan et al., 2021) was tested for example in cotton system, in which the use of pooled sgRNAs targeting single or duplicated genes belonging to different families provided a large number of intentional mutants that would help us know male sterility in cotton itself. Furthermore, this strategy ensured a rapid characterization of the key genes which may influence fertility in cotton, with important consequences for cotton future genetic improvement (Ramadan et al., 2021). Furthermore, as previously mentioned, a TGE-based methodology was implemented to facilitate the ongoing modification of homoeoalleles in species like hexaploid wheat, which is not easily amenable to conventional mutagenesis techniques. In this approach, a transgenic line may exhibit modifications in only a subset of homoeoalleles. However, through self-crossing and careful selection of plants inheriting the T-DNA, it becomes possible to modify all homoeoalleles. Singh and colleagues proposed an effective utilization of the CRISPR/Cas system and next-generation sequencing for mutant analysis in wheat. They successfully established the role of TaMs26 in wheat pollen generation by combining mutations in TaMs26 from the A-, B-, and D-genomes through crossing, resulting in the development of male sterile plants (Singh et al., 2017). Orthologous Ms26 mutations in rice and sorghum plants, as in maize, confer a recessive male sterile phenotype, and restoration of fertility in these mutant sorghum plants was achieved by a copy of maize Ms26 (Cigan et al., 2017). Afterwards, with recent molecular identification of the male fertility Ms1 gene, it has been possible to extend the use of the CRISPR/Cas9 system to generate Ms1 knockout wheat lines with male sterility in the first generation, demonstrating the potential of the CRISPR/Cas9 system for the fast generation of GMS in hexaploid wheat (Singh et al., 2018; Okada et al., 2019).

This evidence on transgenerational gene editing activity demonstrates that TGE can contribute to novel variation in the offspring of CRISPR/Cas9-expressing plants, and that Cas9-inducible trait can be transferred by crossing the plants expressing the gene editing constructs with the lines of interest.

6 Potential application of the CRISPR/Cas system in MS ornamental species: open perspectives

The interest in obtaining MS lines by molecular precision breeding mediated by the CRISPR/Cas system has been generally described as a fundamental step for the production of F_1 hybrids in horticultural crops. In contrast, in ornamental plant research, this aspect has not been deeply investigated to date, despite an increasing number of studies on potential CRISPR/Cas system applications in precision breeding in ornamental plants being continually tested for improving several traits. In fact, in ornamental species, where traits such as high heterozygosity, large genomes, high chromosome numbers, polyploidy, long life cycles, self-sterility, or the inability to produce seeds frequently limit the applicability of conventional breeding methods, genome editing approaches are particularly desirable (Azadi et al., 2016; Sharma and Messar, 2017). Furthermore, obtaining nontransgenic first-generation altered plants and permitting the development of foreign DNA-free editing approaches would be extremely beneficial in such instances. However, the potential of using such methodologies in ornamental species breeding is dependent on information on the availability of efficient transformation and regeneration protocols, as well as the structure of plant genomes and function of genes. In recent decades genome sequencing technology played a significant role, allowing site-specific mutagenesis approaches on several key genes controlling traits of high interest and suggesting that CRISPR/Cas9-induced mutagenesis is effective also in ornamental sector (Zhang et al., 2016a; Kishi-Kaboshi et al., 2017; Yan et al., 2019; Yu et al., 2021b). In fact, it has been successfully employed to create gene knockouts and induce genetic alterations in ornamental *Petunia inflate* and *Petunia hybrid* (Subburaj et al., 2016; Zhang et al., 2016a; Sun and Kao, 2018; Yu et al., 2021b; Xu et al., 2022), *Chrysanthemum morifolium* (Kishi-Kaboshi et al., 2017), *Dendrobium officinale* (Kui et al., 2017), *Ipomoea nil* (Watanabe et al., 2017), *Lilium longiflorum* and *Lilium pumilum* (Yan et al., 2019), and *Phalaenopsis equestris* (Tong et al., 2020). In particular, in polyploid species, such as chrysanthemum, the possibility of mutating multiple copies of a target gene has been indirectly shown, as demonstrated in other polyploid crops, *e.g.*, hexaploid wheat (Wang et al., 2014; Mekapogu et al., 2022).

Regarding MS induction, the production of male-sterile ornamental plants is of great interest for many purposes, such as

facilitating hybrid seed production, eliminating pollen allergens (i.e., gene escape), reduce the need for deadheading to extend the flowering period, redirect resources from seeds to vegetative growth and increase flower longevity and self-life (Garcia-Sogo et al., 2010). In past decades, the production of engineered male sterile plants by canonical transgenesis approaches was documented in ornamental *Kalanchoe blossfeldiana* through the directed expression of the ribonuclease Barnase gene under control of the PsEND1 promoter, which determines tissue-specific expression of the Barnase gene in anther tissues (epidermis, endothecium, middle layer, connective). The Barnase gene affected normal anther development, inducing the ablation of specific tissues at early stages of anther development with a consequent lack of pollen at anthesis in transgenic flowers (Garcia-Sogo et al., 2010). The use of this technology was especially useful to produce environmentally friendly transgenic ornamentals carrying new traits, as this modification would prevent gene flow between the genetically modified plants and related species (Roque et al., 2007; Gardner et al., 2009). A similar approach was used to efficiently create male sterile versions of existing *Pelargonium* spp. cultivars, which represent one of the most popular garden plants around the world, have considerable economic importance in the market of ornamental plants. Using a cotransformation protocol, two new traits were introduced in *P. zonale*, one to produce long-life plants by inducing the *IPT* gene during plant senescence and the other to produce male sterile plants without pollen (Garcia-Sogo et al., 2012). With similar molecular strategies and related purposes, male sterility was induced in *Chrysanthemums* spp. In this specific case, since many wild chrysanthemum relatives in the *Compositae* family are cross-compatible with chrysanthemum cultivars, to reduce the possibility of transgene flow into wild relatives, a male sterility trait using the mutated ethylene receptor gene Cm-ETR1/H69A was introduced into chrysanthemum cultivars (Shinoyama et al., 2012). Recently, thanks to the release of whole genome sequence information (Hirakawa et al., 2019), Shinoyama et al. (Shinoyama et al., 2020) reported an important example of MS induction in *Chrysanthemums* spp. by a genome editing approach targeting the *CmDMC1* gene through the use of TALENS technology to knock out all six identified *CmDMC1* genes. Two chrysanthemum cultivars with the TALEN expression vector resulted in the development of lines with disruption of all *CmDMC1* loci, successfully inducing male and female sterility (Shinoyama et al., 2020). The interest in creating MS lines in ornamental species, together with the positive results obtained in some of them, supports the idea of implementing CRISPR/Cas-based technologies as a potential tool for genetic improvement in floricultural research.

7 Concluding remarks and future perspective

Conventional breeding approaches still depend on breeders choosing materials based on phenotypic analyses. Breeders and scientists choose purposefully different parents to produce crop varieties that combine the desired characteristics of both parents. However, the usefulness of traditional breeding methods may be restricted to complex traits. To complement traditional breeding techniques, molecular breeders have developed and applied GE technologies, which should supplement rather than replace traditional breeding methods. Generally, two major criteria should be considered while assessing the applicability and future development of GE technology. Firstly, the development of cost-effective, low-risk, and efficient transformation systems that align with agricultural requirements is crucial in expanding the utilization of this molecular techniques. Secondly, the regulatory practices implemented by governments play a pivotal role. Currently, there is a global debate around whether CRISPR-edited lines should undergo similar regulations as conventional genetically modified (GM) plants, or if they should be allowed to enter the market without regulation once the CRISPR-cassette mediating segregating cycles have been removed (Chen and Gao, 2014; Voytas and Gao, 2014; Gao, 2015).

This review aims to emphasize that these approaches could make available potential and alternative methods for many breeding purposes. Several examples report that CRISPR/Cas technology has thus far been proven to be successful in genome editing of numerous food and non-food crops, as well as ornamental plants, whose genomes have been efficiently modified to induce genetic variability, resulting in a strong tool in plant genetics and precision breeding. The use of these modification tools, in comparison to their adaptability and final use, has provided a remarkable breakthrough in biological applications thanks to a growing number of accessible genome sequencing data related to the reduction in sequencing costs. In this intricate scenario, this review provides an overview of recent successes for MS induction based on GE applications, accelerating and lowering the cost of male sterility induction by targeting known candidate functional loci. The following development of male sterility, especially in food crops, has been greatly investigated for seed hybrid production. Conditional MS mutants, for example, created through genome editing, are particularly useful in major crops such as rice and maize, opening the possibility for applying the idea to many other crops Interestingly an increasing number of new additional applications of GE technology for MS producing have been reported, especially in promising ornamental species, in which the final goal of obtaining a MS ornamental species arises from the need to have allergenic free plant material. Furthermore, because only a few nucleotides are changed to modify the genome, the new improved methods based on simultaneous editing of gene sequences could be an important starting point for the development of new elite varieties by utilizing efficient and specific modifications at genomic loci, offering advantages over GM crops. Many of these new tools also benefit from TGE-based methods for editing additional alleles in polyploid species. Additionally, according to the studies mentioned above, the transient expression of the CRISPR/Cas cassette, and in particular the direct transfection of the RNP complex, exhibits a number of benefits compared to DNA plasmid delivery, followed by stable integration. With a transient approach, we have a DNA-free transfer, eliminating the possibility of unintended recombinant DNA insertion into the plant genome, a bypass of the cell's transcriptional and translational machinery, with an immediate activity of the RNP complex per single cell, and finally a quick breakdown of complexes after delivery, which lowers the incidence of mosaicism effects. For these reasons, they are regarded as the most innovative and the new frontier of precision plant breeding programs,

and this is the strategic direction that breeding could take in the future, supporting the idea that these approaches could be the new strategic assisted evolution technology towards reproductive systems, with potential to form new varieties.

Author contributions

Conceptualization, SF and GB. Investigation and resources, SF, SD, and AB. Data curation, SF and FP. Writing—original draft preparation, SF, SD, and AB. Writing—review and editing, SF, FP, and AV. Visualization, SF, FP, AV, and GB. Supervision, ML and GB. Project administration, GB. Funding Acquisition, GB. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Review of recent advances in post-harvest techniques for tropical cut flowers and future prospects: *Heliconia* as a case-study

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Aesthetic attributes and easy-to-grow nature of tropical cut flowers (TCFs) have contributedto their potential for increased production. The dearth of information regarding agronomic practices and lack of planting materials are the key hindrances against their fast expansion. Unconventional high-temperature storage requirements and the anatomy of the peduncle contribute topoor vase life performance, while troublesome packaging and transport due to unusual size and structureprimarily cause post-harvest quality deterioration. Nonetheless, the exotic floral structuresconsequently increase market demand, particularly in temperate countries. This boosts studies aimed at overcoming post-harvest hindrances. While a few TCFs (*Anthurium, Strelitzia, Alpinia*, and a few orchids) are under the spotlight, many others remain behind the veil. *Heliconia*, an emerging specialty TCF (False Bird-of-Paradise, family Heliconiaceae), is one of them. The structural uniquenessand dazzling hues of *Heliconia* genotypes facilitate shifting its position from the back to the forefrontof the world floriculture trade. The unsatisfactory state-of-the-art of *Heliconia* research and the absence of any review exclusively on it are the key impetus for structuring this review. In addition to the aforementioned setbacks, impaired water uptake capacity after harvest, high chilling sensitivity, and the proneness of xylem ducts to microbial occlusion may be counted as a few additional factors that hinder its commercialization. This review demonstrates the state-of-the-art of post-harvest research while also conceptualizing the implementation of advanced biotechnological aid to alleviate the challenges, primarily focusing on *Heliconia* (the model crop here) along with some relevant literature on its other allied members. Standard harvesting indices, grading, and packaging are also part of the entire post-harvest operational chain, but since these phases are barely considered in *Heliconia* and the majority of tropical ornamentals except a few, a comprehensive account of these aspects has also been given. The hypothesized cues to nip chilling injury, resorting to different bio-chemical treatments, nano-based technology, and advanced packaging techniques, may

help overcome preservation difficulties and propel its transition from niche to the commercial flower market. In a nutshell, readers will gain a comprehensive overview of how optimum post-harvest handling practices can rewardingly characterize this unique group of TCFs as the most remunerative component.

KEYWORDS

Heliconia, post-harvest, longevity, tropical ornamental cut flowers, preservation, packaging

1 Introduction

The climate of tropical region (starts from Tropic of Cancer to the North to the Tropic of Capricorn to the South) gifts brightly hued tropical flowers (considered as niche products) which since time immemorial, receiving huge admiration (Yue and Hall, 2010) for their shape, symbolic as well as aesthetic significances. Traditionally, these plants are used in gardens and also as cut flower. The current scenario, post-pandemic, is associated with the increasing popularity of these segments among consumers in pan world (Paull, 1990; Paull and Chantrachit, 2001; Jaroenkit and Paull, 2003; Reis et al., 2020a and Reis et al., 2020b; Malakar et al., 2022). The main pros of tropical flowers are the eco-friendliness characteristics as mentioned by Darras (2021) while the current global floral trends i.e 'Sustainable' and 'Wellbeing' (Kaishita, 2022) also endorse the significance of its cultivation. These could reinforce their growing as mentioned by several researchers (cited herein). Hence, the consistent production of tropical flowers under tropical and sub-tropical regions not only foster the aesthetic and environmental significance but could also revive the tradition of their worthwhile usages with remunerative potential eulogistically. However, tropical flowers represent only approximately 4%-5% of all cut flowers traded (Laws, 1998 and Laws, 2005).

Tropical flowers consist of a diversified group of flowers that are native to tropical and subtropical climates (Paiva and Beckmann-Cavalcante, 2023). The most commonly known tropical flowers in the markets are cut orchids (*Cymbidium*) and *Anthurium*, but this product group also includes many other species belonging to the monocotyledonous taxonomic order of Zingiberales (Scitaminae, Clade - Commelinids) (Kress, 1990). This order includes eight families: Musaceae, Strelitziaceae, Lowiaceae, Heliconiaceae, Zingiberaceae, Costaceae, Cannaceae, and Marantaceae.The respective genus and species of these families usually grow in tropical regions (Cronquist, 1978).

A recent classification, elaborated by International Association of Horticultural Producers (AIPH) (2019), describes the production and consumption markets at global level, divided into four homogenous geographical groups: (1) Mature domestic producer countries (Europe, Canada, US, China, and Japan); (2) Emerging domestic producer countries (India, Mexico, and Brazil); (3) Mature exporting producer countries (Colombia, Kenya, and Ecuador); (4) Emerging exporting producer countries (Ethiopia and Vietnam). Producers and exporters of tropical cut flowers are present in these different groups, although it is often difficult to obtain separate market statistics for each product (Silva et al., 2019).

Mexico, Colombia, Ecuador, and Costa Rica are also considered as major exporters to USA and Canada, thanks to the benefits of NAFTAAlliance(North American Free Trade Agreement). Tropical flowers represent a relatively small segment of the European flower market and are traded at flower auctions in Holland (Royal Flora Holland) and Germany (Veiling Rhein-Maas), where a small number of merchants specialize in tropical flowers. However, important companies like Dutch Flower Group BV ([e-source: https://dfg.nl/en/] and Dümmen Orange [e-source: https://na.dummenorange.com/site/en] are involved in this market. The most commonly known tropical flowers in European markets are cut orchids (*Cymbidium*) and *Anthurium*. The product group also includes many other species, such as *Strelitzia*, *Heliconia*, *Protea*, *Leucadendron*, *Leucospermum*, *Ananas*, *Calathea* and Zingiberales (*Ginger*).In the South-East Asian province, China is the largest producer and exporter of tropical cut ornamentals. *Anthurium*, *Dahlia*, Lily, orchids and *Zantedeschia* (Calla Lilly) are the major tropical cut flowers exported to Dutch flower auctions from this province. Europe (mainly Germany and Italy), Japan and US are the largest importers of tropical flowers (Laws, 1998; COLEACP (Europe-Africa-Caribbean-Pacific Liaison Committee), 2002; Pizano, 2005; Linares-Gabriel et al., 2020; Chandel et al., 2022; Nzomoi et al., 2022).

In Europe, tropical flowers are primarily sold in mixed and colorful bouquets. Consumers appreciate these exclusive tropical bouquets and are willing to pay extra for them.In the Netherlands, a tropical bouquet consisting of*Anthurium*, *Heliconia*, *Celosia* and *Ananas*can cost around € 40/50 at the florist shop. In supermarkets, a single tropical flower, such as a smaller *Strelitzia* or Protea, is usually combined with cheaper flowers and fillers, and a small bouquet may cost as little as € 5 (CBI Product Factsheet, 2016). Recent consumption trends, marketing strategies, and governance settings in ornamental horticulture have been elucidated by Gabellini and Scaramuzzi (2022), but no mention of tropical ornamentals has been found.

According to the report of CBI Market Intelligence (2016) on the 'Tailored study of tropical flowers and foliagefrom Colombia, UAE, and other Middle East markets', *Heliconia* and gingers are of great importance. Both of these flowers originate from Asian countries such as Thailand and Malaysia. However, the main producing and exporting countries of tropical ornamentals of the

Zingiberalesorder are Colombia and Costa Rica. Although no quantitative data related to the export, import, and detailed statistics on national and international trade of *Heliconia*have been found through bibliographic searches, it is reported that Brazil (54.5%), Colombia (15.4%) and Mexico (10.9%) are at the forefront of its production (Linares-Gabriel et al., 2020). India has been identified as the emerging domestic producer of this Specialty Tropical Cut Flower (STCF) among the Southeast Asian countries (Malakar et al., 2022).The price of a cut stem is approximatelyUS$2-3 or US$3-5 for the erected (80-100 cm length) or the pendent helicônias (e.g.*H. rostrata*)respectively (e-Source: https://www.cbi.eu/events/royal-floraholland-trade-fair-rfhtf-2016; no recent data are available).

Taking into consideration the great potential and burgeoning demand as a cut flower, our review will focus on *Heliconia*(>250 inter and intra-specific varieties are existing), commonly known as 'False Bird-of-Paradise', which belongs to the family Heliconiaceae (Abalo and Morales, 1982; Kress, 1990; Berry and Kress, 1991; Criley and Broschat, 1992; Abadie and Berreta, 2001; Urooj-UI-Nissa et al., 2015; Malakar et al., 2016; Avendaño-Arrazate et al., 2017; Krause et al., 2023). This review will specifically focus on the pre- and post-harvest factors that affect the desired attributes of heliconia cut flowers as expected by consumers. To the best of our knowledge, there is currently no dedicated review exclusively focused on post-harvest practices in Heliconia.

The state-of-the-art of scientific investigations on Heliconia reveals that research is mainly carried out in Brazil (67%), Colombia (19%),with a focus on production-related aspects (75%) rather than post-harvest (16%), marketing (7%), and industry-based (2%) research areas(Linares-Gabriel et al., 2020). On the contrary, scientific literature on post-harvest management practices for common tropical ornamentals (*Strelitzia*, *Anthurium*, Ornamental gingers, *Cymbidium, Dendrobium* etc.) is available and accessible. Therefore, in this review, we have considered the available literature on post-harvest practices in Heliconia, but we have also provided a brief overview of other related tropical members to present a comparative view of advancements in post-harvest measures and to support certain concepts. On the other hand, Jaroenkit and Paull (2003) stated that post-harvest management practices in all tropical ornamentals are more or less similar, possibly due to the structural anatomical similarities in peduncles (for inflorescences) or pedicels (for flowers). However, a detailed literature survey challenges the retention of this view.

In the following sections, we will take into account the factors that could affect the quality of Heliconia cut flowers throughout the entire production chain, from 'field to consumer.' This includes pre-harvest factors such as agronomic practices (Criley and Paull, 1993; De et al., 2014), harvest factors as harvesting maturity and seasons (Pompodakis et al., 2005) and post-harvest factors such as chain management and post-harvest handling sequences (Dolan and Sutherland, 2002; Collins and Dent, 2022). In a meta-analysis study on tropical plant postharvest, it was identified that the addition of preservatives to solutions, variations in storage temperature, and the use of electromagnetic fields are, currently, the most effective techniques in extending the shelf life of cut flowers (Cunha Neto et al., 2023). We will also consider post-harvest physiology determinants, including multiple genetic factors, maintenance of water balance components, and respiratory substrates (Onozaki et al., 2001; Fanourakis et al., 2013).

In summary, this review will highlight the challenges faced and effective measures to enhance the growing impact of tropical Specialty Cut Flowers (SCFs), along with providing directional hypothetical views on the necessary advancement for future perspective.

2 Post-harvest quality in *Heliconia*

Flowers are ephemeral organs (Smyth, 2005; Costa et al., 2021) due to their highly perishable nature, which results in a short post-harvest life. The quality of the final products plays a crucial role in determining the acceptability of cut flowers. Ensuring marketable post-harvest quality, which is a basic requirement for different market channels, necessitates maintenance at both pre- and post-harvest stages. Generally, the factors that affect the flowers physiology are classified as 'Pre' (including all aspects of agronomic practices) (Criley and Paull, 1993) and 'Post-harvest'(abiotic factors such as storage temperature, relative humidity [RH], atmospheric gas composition, and biotic factors such as microbial occlusion and deterioration of physiological mechanisms) (Fernandes et al., 2020; Costa et al., 2021). Harvesting (considering maturity and the season/time of cutting) and handling (including sorting, storage, packing, and transport) of harvested produce are also vital factors to consider.

Among the most common drawbacks that can affect post-harvest quality, there are several factors that apply to various tropical species. These factors include the lack of standardization of harvest indices, inadequate handling facilities, negligence during handling, sharing of storage space with other traditional cut flowers, and more. Additionally, structural differences in floral taxonomy, sensitivity to chilling, large size, special packaging requirements, and anatomical specifications can also contribute to these challenges. Due to these factors, post-harvest practices cannot be generalized, and special attention should be given to the specific species' post-harvest requirements (Jaroenkit and Paull, 2003).

In *Heliconia*, its inflorescences (bold cincinnus type; with either an upright or pendant posture) consisting of multihued bracteate structures with spiral or distichous aestivation.) (Kress, 1984; Criley and Broschat, 1992; Criley and Paull, 1993; Castro et al., 2007; Rodríguez, 2013; Costa et al., 2015; Loges et al., 2016; Souza et al., 2016). These inflorescences are popularly used as cut flowers, unlike its true flowers, which are inconspicuous in nature, usually white, yellow or orange in color, numbering from 2 to 20 and remaining enveloped by bracts (Criley and Paull, 1993, Criley and Broschat, 1992, Krause et al., 2023). The importance of Heliconia as a cut flower lies in the bright hues, sturdiness, and freshness of its inflorescences. However, ill-developed or deteriorated chromatic features and desiccated or browning symptoms of the boat-shaped bract tips categorize them as non-acceptable cut units. To overcome these drawbacks, the role of several factors mentioned in the preceding paragraph will be delineated in the following sections.

2.1 Pre-harvest factors

Very few research reports have been obtained on the role of pre-harvest factors in determining the post-harvest quality of cut *Heliconia*. Similarly, when considering other allied cut tropical ornamentals such as *Strelitzia* sp. and ornamental gingers (OGs), apart from Anthurium, the status of research investigations on pre-harvest factors as indicators of after-harvest quality of cut flowers is very limited. Nonetheless, achieving a high yield of quality cut units is the main objective of manipulating pre-harvest factors. In this section, the roles of light, temperature, and fertilizers as optimum standards for boosting yield and determining post-harvest quality will be discussed.

Light: Optimum light condition impacts on the phenotypic features of plants, hence, it is considered as one of the crucial factors during the agronomic practices. Concerning tropical ornamentals, generally, the light requirement varies from genotype to genotype. According to Broschat and Donselman, 1983a; Broschat and Donselman, 1983b) *Heliconia* can thrive best under the semi-shaded condition while excessive shade and crowding can cause the reduced yield with weak, lanky stems as most evident in *H. psittacorum* groups. Malakar et al. (2016) had also corroborated the same based on the outcomes of their experiment on evaluation of agro-morphological attributes of diversified *Heliconia* genotypes under West Bengal, India, condition but any standardized range of shade percentage yet to be made. The other tropical cut flowers also prefer semi-shaded condition like in *Alpinia* 30% (Criley, 1988), *Curcuma* 50-70% (Criley, 2014), *Anthurium* 30%-75% (Kamemoto, 1962) while according to Ismail et al. (2019), *Etlingera* needs partial shade (standardized percentage not found) but the empirical views of the authors of this review are paradoxical to the view of these researchers.

The flowering in *Heliconia* is seasonal and depends on certain growth condition and physiognomic features (eg. in *H. angusta*, pseudostem bearing 3 unfurled leaf blades can only cause bud emergence, in *H. chartacea*, number of emerged shoots and leaves determine the onset flowering) as well as place of growing (Criley and Lekawatana (1994) but a few research reports on roles of photoperiod for growth and flowering have been noticed. According to Criley et al. (1999), the photoperiodic requirement, for flowering in *Heliconia* genotypes, is species-specific. They found *H. Wagneriana* and *H. stricta* var. 'Dwarf Jamaican' as short day (SD) species while *H. angusta* as long day (LD). They also mentioned that in *H. angusta*, if exposure to LD condition for>13.3 h for the duration of 15 to 17 weeks takes place, then only the anthesis can happen while in rest 2 species, anthesis takes place after 15 to 19 weeks of exposure to SD condition. Again Geertsen (1990) found that to get early, abundant flowering along with shortened peduncle length in *H. aurantiaca* Ghiesbr. ex. Lemaire, exposure at 16h of LD condition is optimum while to get off-season blooming in *H.rostrata*, critical day length condition of 11.5-12h; in other words, SD condition, is needed (Maciel, 2000). The photoperiodic requirement of other tropical ornamentals is basically LD while some exceptions also could be found (Table 1).

Temperature: Concerning *Heliconia* cultivation, like light intensity, temperature is also another major factor but dearth of scientific investigations in this regard have been evidenced; hence, only a handful of old scientific reports have been cited here.

TABLE 1 Pre-harvest photoperiodic requirement in some tropical cut ornamentals.

Names of genotypes	Common names	Photoperiodic requirement	Remarks	Sources
Alpinia sp.	Red ginger	Long day (LD)	–	Criley and Maciel, 2002; Criley, 2014
Hedychium sp.	White ginger lily	Long day	–	Agboka and Criley, 2002; Changjeraja et al., 2008
Curcuma sp.	Curcuma	Long day (>13h) either by night interruption lighting or day length extension	–	Hagiladi et al., 1997; Sarmiento and Kuehny, 2003; Criley, 2013 and Criley, 2014)
Etlingera sp.	Torch ginger	Short day (SD)	Based on the practical experience, it may be said that it is LD photoperiodic plant because a few of the places in Brazil where high temperature and LD condition prevails perpetually, there round-the-year flowering can be experienced while in South-Eastern part of Brazil where low temperature (average 10-15°C) as well as SD conditions occur during winter season, flowering found absent. In nutshell, the influence of temperature factor rather than the light factor is more pronounced.	Criley, 2011 and Criley, 2014, Castro et al., 2011
Strelitzia sp.	Bird-of-Paradise	Photoperiod insensitive	–	Halevy et al., 1976
Anthurium sp.		LD (16h)	–	Budiarto, 2010

-, No information.

According to Broschat and Donselman (1987), the air temperature below 10-12.5°C could be harmful for *H. psittacorum* while harvesting season also matters as reported by Bredmose (1986). Both the researchers mentioned about the vase life (V) difference in *Heliconia* genotypes grown in Denmark due to different harvesting season (winter harvesting V_{short} and summer harvesting V_{long}). Again Geertsen (1990) had been found to be reported that the temperature range between 15 to 21°C could cause increase in flowering percentage by >20% along with the peduncle length of 40 cm while the number of leaves subtending the inflorescence was increased by 2.5% in *H. psittacorum*. Any report on frost sensitiveness of *Heliconia* has yet be found while the impact of exposing its inflorescence under varied degree of light intensity is also absent. The state-of-the-art on this aspect regarding other tropical ornamentals like *Alpinia*, Anthurium, Strelitzia, Curcuma etc. are also unsatisfactory (Table 2); apparently proves the research negligence on current date, which embody the importance of conductance of research investigation on them including *Heliconia*.

Fertilizer: In 1987, Broschat and Donselman worked on the effect of NPK on qualitative and quantitative evaluation of cut stems of *H. psittacorum* and found that K does not have any impact while N up to 600gm^{-2} could augment the yield and quality attributes both. Recently, Sardinha et al. (2019) had carried out an investigation on the influence of phyto-stimulants (like Bion, Agro-MOS Quartz, Ca-Fosfitotal (Ca-Phosphite), K-Fosfitotal (K-Phosphite)), applied in the field condition, on the stem quality of *H. psittacorum* cv. 'Golden Torch' during vase life. They found the positive implications of them while the best outcomes had been obtained in K-Fosfitotal since electrolyte leakage (EL) and lipid peroxidation scores were low in this case. Other associated post-harvest parameters like visual aspect, fresh matter loss, water balance in cut stems were improved too by due application of phyto-stimulants during cultivation phase as pre-harvest management strategy (Sardinha et al., 2019).

Loss of cell membrane integrity generally is triggered by electrolyte's leakage which consequently causes the cell death. (Hatsugai and Katagiri, 2018). Electrolytes like Ca^{2+}, K^{+}, Mg, Chlorite, Ph, Na^{2+} etc. play vital role in determining the post-harvest physiology of cut floral units. High concentration of electrolytes aids to withstand various abiotic and biotic post-harvest stresses (like water stress condition, pathogen attack etc.) while its low quantity causes the reverse (Hatsugai and Katagiri, 2018). On other side, peroxidation of lipids can disturb the assembly of the membrane by causing changes in fluidity and permeability, alter the ion transport and inhibition of metabolic processes. High scores of it, cause injured mitochondria inducing higher rate of ROS generation (Pourzarnegar et al., 2020) and consequently affect the post-harvest phase of ornamental cut units. Different enzymatic activities like peroxidase (POD), polyphenol oxidase, and superoxide dismutase (SOD) had been also influenced by application of phyto-stimulants (Sardinha et al., 2019). Optimum agronomic practices reduce the post-harvest oxidative stress by increasing anti-oxidant enzyme i.e. SOD and catalase (CAT) activities as reported by Zulfiqar et al. (2022).

Based on the bibliographic searches, it may be said that the nutritional requirement and its impact on post-harvest quality vary in different tropical cut flowers. The curated information(s) have been tabulated (Table 3). Furthermore, bract's hue determines the value of tropical flowers; hence, the impact of fertilizers on maintaining the bract color is another vital aspect of investigation. In *Heliconia* and any other tropical ornamentals except Anthurium (Table 3), any such reports have not been identified.

Other factors: Any scientific reports on standardized spacing, irrigation and plant growth regulators (PGRs) regime for *Heliconia* cut flower production have not yet found. But in other tropical cut flowers, meager information(s) have been obtained. As for example, for *Alpinia* and *Etlingera*, optimum plant-to-plant (P/P) spacing are

TABLE 2 Pre-harvest temperature requirement in some tropical cut ornamentals. .

Names of genotypes	Temperature requirement	Impacts	Remarks	Sources
Alpinia sp.	<16 and >10°C[1]; 21°C[2]	[1]Optimum for high yield; [2]Early emergence of flowering stalk and diminish of frost sensitiveness but exposure to temperature >25^{0}C cause the onset of 'Tip burning disorder'.	The empirical views of authors of this review cannot fully endorse these results.	[1]Criley, 1984 and Criley, 1988; [2]Criley, 1988
Curcuma sp.	Tropical temperature (temperature specifications did not find)	Seasonal blooming	To have perpetual blooming, forcing technique which involves the low temperature (15°C) storage of rhizomes followed by warmer storage (25-32°C) for 15days, found effective.	Criley et al., 2003; Sarmiento and Kuehny, 2003; Paz et al., 2005; Roh et al., 2005; Criley, 2014
Anthurium sp.	<25°C during day and 18°C during night	Improved postharvest quality	Temperature should be critically maintained since temperature and deterioration in post-harvest quality of it are inversely co-related with each other	Paull, 1991
Strelitzia sp.	–	–	Standardized threshold range is yet to determine but PGRs, especially GA, plays significant role to reduce its temperature sensitivity; cause increased length and number of emerged flowering stalk along with early inflorescences' maturation during off-season cultivation.	Halevy et al. (1976), Criley (2015)

-, No information.

TABLE 3 Status of research on pre-harvest nutritional requirement in some tropical cut ornamentals.

Names of genotypes	Fertilizer dose rates	Impacts	Remarks	Sources
Strelitzia sp.	–	–	Varied dose rates owing to the adoption of region-specific diverse cultural practices	Criley, 2014 and Criley, 2005
Anthurium sp.	N:P:K: 312:448:375	Quantitative (high yield) and qualitative (increased peduncle length) improvement;	Moderate to high level of K can improve the quality of spathe; P has no effect; high level of N impairs the quality but the combination of N and K, at high dose rate (rate did not found), cause the linear increase in spathe size may be due to the synergistic interaction effect;	Higaki et al. (1992); Paull (1991); Criley and Paull (1993)
Anthurium var. 'Kamuna' & 'Ozaki'	Osmocote: 13.5:13.5:13.5	Alleviation of 'Spathe bleaching disorder'	Temperature range should be 21-26°C for 9 weeks duration cause the optimum release of NH_4-N and NO_3-N	Mills, 1981; Leonhardt and Woomer, 1991
Alpinia sp.	N:P:K level ranges from 1:1:1 to 3:1:5 [under Florida condition] while 300kg N ha^{-1}[under Venezuela condition])	Optimum yield	Place of growing affect the dose rates.	González and Mogollón, 2001; Criley, 2014 and Criley, 1988
*Etlingera*sp.	NPK: 200g $plant^{-1}$; organic compost of 1kg $month^{-1}$;	Improved quantitative and qualitative attributes	–	Criley and Maciel, 2002; Araújo et al., 2018,
Hedychiumcoronarium	NPK: 19:19:19 along with garlic extract	Improved flowering attributes like main stalk length and diameter, inflorescence number $clump^{-1}$, rachis length, florets number $inflorescence^{-1}$ and fresh and dry weight of flowering spikes	–	Attia et al., 2020
Curcuma sp.	N:K: 200 ppm each	Improved quantitative and qualitative attributes	–	Ruamrungsri et al., 2006
C. alismatifolia x *C. cordata* cv. 'Laddawan'	N:P:K: 20:20:20 combining with chitosan: 20mgL^{-1}	Improved post-harvest quality	Foliar spray application	Uthairatanakij et al. (2007)

-, No information.

1.2-2.0m (Criley, 1988), and 1.5 x 1.5m (Criley, 2011 and Criley, 2014, Castro et al., 2011) respectively while irrigation of 25mm $week^{-1}$in *Alpinia*(Criley, 2014) and withholding of irrigation in scheduled manner for about 2.0-2.5 months, had been found beneficial in early flowering in *Strelitzia* (Criley, 2005). In case of *C. alismatifolia*, *C. gracillima* and *C. thorelli*, pre-soaking of rhizomes in GA_{4+7}solution (concentration did not found), for 10mins duration, during pre-planting stage may cause the dwarfing of floral stem length (Sarmiento and Kuehny, 2003) which consequently make these cut blooms more feasible to use and transport also. Again, according to Hagiladi et al. (1997), the planting of propagules with >5 t-roots (tuberous egg-shaped root ends of rhizome) as compare to<2 t-roots, result in early flowering in ornamental *Curcuma*.

Surprising augmentation in demand of tropical flowers in international floral market has alarmed to be mindful about the quality and phyto-sanitary aspects of cut flowers. Several fungal causative agents like *Calonectria spathiphylli* (susceptible genotypes: *H. angusta* cv. 'Holiday', *H. bihai* cv. 'Lobster Claw'), *Bipolaris incurvata* (susceptible genotypes: *H. stricta* var. 'Dwarf Jamaican Red', *H. chartacea*), *Cercospora* sp. and *Pseudocercospora* sp. (susceptible genotypes: *H. psittacorum* cv. 'Andromeda', *H. wagneriana*) cause severe foliage diseases while *Rhizoctonia solani* (susceptible genotypes: *H. bihai* cv. 'Lobster Claw', *H. caribaea*), *Phytophthora nicotianae* (susceptible genotype(s): *H. caribaea* and highly tolerant genotype(s): *H. mutisiana*), *Pythium* sp.(susceptible genotypes: *H. psittacorum* var. 'Bengal', *H. indica* cv. 'Spectabilis') - these fungal strains found responsible for the rhizome and root diseases (Ferreira et al., 1991 and Sewake and Uchida (year missing, e-source: http://www.extento.hawaii.edu/kbase/reports/heliconia_pest.htm). Barring these, several bacterial diseases like 'Leaf Rolling', 'Wilting' and 'Die back' also found to be caused by *Pseudomonas solanacearum*; *H. psittacorum* and *H. rostrata* show the high susceptibility (Ferreira et al., 1991) towards it while 'burrowing' nematode (*Radopholus similis*), 'root-knot' nematode (*Meloidogyne* sp.), and 'lesion' nematode (*Pratelenchus* sp.) have also been reported to infect *H. angusta, H. chartacea, H. stricta* (Sewakeand Uchida (year missing, e-source: http://www.extento.hawaii.edu/kbase/reports/heliconia_pest.htm). Any such viral diseases have yet to be reported in *Heliconia* but Hamim et al. (2017) had reported about the occurrence of BBTV (Banana Bunchy Top Virus), for the first time ever, in *H. aurantiaca* in Hawaii. Positive result of triple-antibody

sandwich (TAS)-ELISA and PCR tests had confirmed the infestation but any remedial measures had not been stated by the researchers. In case of *Anthurium, Xanthomonas* bacterial blight led lesions on spathe often impairs the quality of cut units (Norman and Ali, year missing; e-source: https://edis.ifas.ufl.edu/publication/PP292; Hara et al., 2004).

Generally, several pre-harvest management practices determine the occurrence and spreading of diseases; Heliconia is also not exceptional. The mode of spreading is significantly diverse; as for example, may be through soil contamination, contact infestation, water stagnation condition (synonymously poor drainage), influence of various abiotic factors etc. In most cases, 'rhizome rotting', caused by bacterial and nematodal infestations; mainly spread from soil to roots, results in dying of Heliconia plants while the degree of foliage's infestation also directly affects the plant health and consequences into low yield with impaired quality's inflorescences (Berry and Kress, 1991).

To date, barring the conventional measures viz. optimum cleaning of rhizomes, controlled soil moisture level, appropriate spacing between plants for good air movement, sanitation of growing substrates, enhancement of soil organic matter content, use of resistant genotypes, application of insecticides and pesticides, soil fumigation processes, any modern approaches to mitigate the said menace have not been devised concerning our discussed ornamental's group. El-Baky and Amara (2021) mentioned about some novel advanced approaches towards controlling phytopathogenic issues. Several strategies like biofumigation (employs organic material's fermentation to develop anaerobic condition and toxic metabolites to make phytopathogens non-functional), use of antagonistic microorganisms in virgin soils, use of microbial fungicides by unique application method via honey bees, known as 'flying doctors', as they deliver bacterial fungicide *Bacillus subtilis* during pollination, use of agronanotechnology. Fungal cell deactivation and evacuation using 'Ghost' technique, use of UV light and floral extracts like Chammomile tea and modern breeding strategies like RNA interference (RNAi) (i.e host-induced gene silencing [HIGS] and spray-induced gene silencing [SIGS]) to develop phytopathogenic resistant plants have been mentioned by them which are based on 'Green principles' and are already in practice in case of other horticultural crops. However, the conductance of trial tests of these said techniques aiming to minimize not only the occurrence of phytopathogenic interference but also to produce sustainably export quality's cut inflorescences. A few tropical flowers importing countries after disembarkation of the cut produces, clean (either by simple hot water treatment or by foamy soap water cleaning) them on-site (Berry and Kress, 1991) rather following any stringent phytosanitary regulations.

Summarily, it may be said that the optimum field management practices could necessarily ascertain the qualitative and quantitative features of post-harvest products. So, resorting to afore cited reports, some ventures might would be also taken in *Heliconia* for further future investigations.

2.2 Harvesting factors

Generally, *Heliconia* inflorescences are harvested by cutting near the stalks (Criley and Paull, 1993; Criley, 1995) while on the contrary, Broschat and Donselman (1983b) found satisfactory keeping quality with the pulling method of harvesting. Harvesting time (in terms of season and hours) and stage of harvesting are key factors determining the post-harvest quality of cut flowers. Generally, bract opening after harvesting does not occur in *Heliconia* inflorescences (Criley, 1995). To achieve the best post-harvest quality, harvesting cut units from well-irrigated plants could be a way to reduces the chance of desiccation to some extent (Dolselman and Broschat, 1986). Harvesting indices are usually genus and species-specific. For large-sized *Heliconia* inflorescences, harvesting at the stage of ½ or 2/3rd open bracts, while for a few 1 small-sized cultivars of *H. psittacorum*, harvesting at the stage of 1 or 2 open bracts or tight bud stage, has been found to yield optimum shelf life and post-harvest performance, as stated by Broschat and Donselman (1983b) and Criley (1995). In Strelitzia sp. and *Alpinia* sp., the harvesting indices are almost similar, while in *Etlingera*, 'tight bud stage' and 'torch stage' are considered as the optimum stage of harvesting (Criley, 2014), although it may vary from species to species (Table 4). According to Liju (2013), harvesting the inflorescences at the 1-2 open bracts stage in general increases the vase life. Tjia and Sheehan (1984) stated that harvesting immature inflorescences could extend the keeping

TABLE 4 Harvesting indices of some tropical ornamentals.

Names of genotypes	Optimum harvesting condition/indices	Sources
Strelitzia sp.	Tight bud condition (orange colored tepals start to peep from the spathe)	Criley and Paull, 1993; Jaroenkit and Paull, 2003; Bayogan et al., 2008; Koley, 2013; Criley, 2014
*Alpinia*sp.	½ to 2/3rd or 3/4th number of open bracts with 2 or 3 attached foliage(s)	Broschat and Donselman, 1988; Criley, 1996 and Criley, 2014
Etlingeraelatior	Semi-opened inflorescence [initiation of unfurling of basal bracts and visible central portion of inflorescence]	Castro et al., 2013; Hintze, 2013, and Hintze, 2014
E. haemispherica	Do	Do
E. corneri	Tight bud stage	Do
Hedychium	Shape and arrangement of bracts of cone to terminal' and 'tight to loose' respectively	Wood, 1999; Criley, 2014

"Do" refers to same as above.

quality by an extra 42% compared to mature harvesting (3-4 bracts open stage). The retention of foliage in harvested inflorescences generally does not affect their post-harvest life (Ka-ipo et al., 1989; Criley and Broschat, 1992; Criley, 1996). Generally, for large and small types of *Heliconia* (eg.*H. psittacorum*'s cultivars), a total stem length of 150 cm and 60-90cm, is maintained (Criley and Paull, 1993), while for *Alpinia* and Etlingera, peduncle length of 60-150cm (Criley and Paull, 1993) and 50-70cm (Araújo et al., 2018; Baskaran, 2022), respectively, are maintained. The standardization of maturity threshold for harvesting allied tropical ornamentals like *Globba*, *Curcuma*, and *Zingiber* species is yet to be made.

Several physiological reasons lie behind harvesting at the optimum maturity stage, but there is no evidence of any investigations concerning this in *Heliconia.* To support this, it may be mentioned that the activities of cellulase and pectin methyl esterase enzymes, which principally regulate flowering and post-harvest abscission, respectively, are determined by the optimum harvesting stage, as evidenced in *Etlingera* by Wang (2017). High cellulase activity has been found in involucral bracts of *Etlingera* from the 'tight bud' to the '6-tip opened stage', but its activity deteriorates from '6-tip opened stage' to the 'torch stage', while the activity of pectin methyl esterase increase (Yeat, 2016). The same researcher reported that from the 'tight bud' to the 'bloom stage', a high content of ethanol insoluble residue and cellulose content in the peduncle are usually found. Taken together, it may be said that further insight into the physiological basis of determining the harvesting stage of tropical cut flowers may be the timely need.

2.3 Post-harvest factors

The *Heliconia*, as a cut flower, is highly accepted by customers due to its diversity in bract color and exotic appearance (Loges et al., 2005 and Costa et al., 2011; Loges et al., 2016). Therefore, preserving the hue of its bracts is one of the primary goals during their presence with consumers. The hardy nature, firmness of the peduncle, and natural durability (Castro et al., 2006, Reid, 2001) can also be considered as additional features that contribute to their high demand. However, to maintain the post-harvest quality of cut units, several factors need to be carefully taken into account. Temperature, water balance, carbohydrate supply, and growth regulators (Reid and Kofranek, 1980; Halevy and Mayak, 1981) are key factors that play a crucial role in the storage of cut items and in extending their vase life. In the following sections, we will discuss the role of each factor in the post-harvest vase life of *Heliconia* cut inflorescences, as well as other related tropical cut flowers.

Temperature: The recommended storage temperature for cut *Heliconia* is >12°C (Broschat and Donselman, 1983b; Criley and Paull, 1993) since all species and cultivars of this genus are highly sensitive to low temperature condition. chilling injury (CI) such as depressions, browning, or dark spots on the bracts and flowers can occur if they are stored at temperatures below 10°C for 2 days, as reported by several researchers (Markhart, 1986; Paull, 1991; Darras, 2020 and Carrera-Alvarado et al., 2021). Silva et al. (2019) evaluated the optimum storage temperature of *H. densiflora* and *H. psittacorum* (in three different color varieties: 'Red' [5R 4/10], 'Orange' [7.5Y 7/10], and 'Yellow' [2.5Y 7/10]) under controlled temperature condition. The samples were stored at three different temperatures (14°C, 18°C, and 22°C) inside a cold chamber and under controlled ambient conditions (26°C). They found that 14°C (storage period up to 9 days) was optimal for the 'Red' variety, and 14-22°C (for a storage period of up to 6 days) was optimal for both the 'Yellow' and 'Orange' varieties of *H. psittacorum*, with no signs of senescence or necrosis. For *H. densiflora*, a temperature of 18°C was recommended for storing healthy cut stems for up to 6 days. Controlled ambient conditions were found to be unsuitable for storage (maximum storage period recorded was 1-2 days). Costa et al. (2011) also studied the storage of *H. bihai* cv. 'Lobster Claw' at low temperatures (12°C and 19°C). The development of CI symptoms was observed from the 3rd day onwards when stored at 12°C, while storage at 19°C for up to 8 days was recommended for maintaining visual quality and fresh weight of the cultivar.

CI is a physiological malfunction of plants caused by exposure low temperatures rather than freezing temperatures, rendering the product unusable (Markhart, 1986; Parkin et al., 1989). Loss of membrane integrity due to low-temperature-induced membrane lipid phase transitions and physiological dysfunctions are the fundamental biochemical causes of CI (Bhattacharya and Bhattacharya, 2022). The high concentration of high melting phospholipids, membrane retailoring, increased fluidity at low temperatures, direct or indirect impact on intrinsic enzymes due to membrane perturbations, and redistribution of cellular Ca (which acts as a secondary messenger of many cellular functions) may also be considered as primary transducer of CI (Parkin et al., 1989; Chen and Ko, 2021; Tian et al., 2022; Olmedo et al., 2023). Maintenance of membrane integrity enables the electron transport chain system and oxidative phosphorylation to produce ATP and supports the normal functioning of pathways like TCA cycle, glycolysis, β-oxidation, etc (Parkin et al., 1989; Darras, 2020), but disruption of membrane structure impacts the entire physiology. According to Lukatkin et al. (2012), cyto-physiological changes such as distorted cell membranes leading to loss of cell compartmentation, swelling and rupture of plasmalemma, destruction of the endoplasmic reticulum and vesiculation of its membranes, changes in the Golgi apparatus, swelling and degeneration of mitochondrial structure, matrix enlightenment, and cristae shortening also occur due to CI. CI also affects photosynthesis, as reported by Markhart (1986) and Lukatkin et al. (2012). They mentioned that chilling-induced water stress decrease the quantum yield of whole-plant photosynthesis and also cause direct injury to the chloroplast (the oxidative side of photosystem II is the site of injury). Furthermore, decreased mitotic cell index (Strauss et al., 2007), cessation of cell growth (Rymen et al., 2017), decreased cytoplasm viscosity (Lukatkin et al., 2012), coagulation of structural proteins (Zhang et al., 2021), low soluble protein content, shift in intracellular pH (Kasamo et al., 2000), cessation of cytoplasmic streaming (Lukatkin et al., 2012), and changes in ATP (adenosine-5'-triphosphate) levels (Minorsky, 1985) are also part of the implications. Parkin et al. (1989) hinted at

the role of lipid peroxidation in causing irreversible damage, during low-temperature storage, in the form of free radical-induced damage to tissues and progressive membrane rigidification.

Generally, all tropical ornamentals except *Strelitzia* (Table 5) are susceptible to low-temperature storage. In this context, *Anthurium* should be mentioned first (Promyou et al., 2012). The key CI symptoms of *Anthurium*are 'Browning' and 'Blueing' of the spathes and wilting of spadixes (Promyou et al., 2012; Aghdam et al., 2015). Generally, storing Anthurium at an optimum temperature of 10-12°C controls these symptoms (Reid and Wu, 1992; Balas et al., 2006; Aliniaeifard et al., 2020). However, Paull (1987) found exceptions regarding the storage of some other Anthurium varieties at higher temperatures (14-17°C). Chemical solutions, such as salicylic acid and GABA-based treatments, have also been shown to alleviate CI issues (Promyou et al., 2012, Soleimani Aghdam et al., 2015 and Aghdam et al., 2016). However, none of these strategies completely eliminate browning of the spathe. Recently, Aliniaeifard et al. (2020) conducted an experiment to evaluate the role of different post-harvest light spectral compositions (Red [R], Blue [B], R & B@ 70:30% and White [W]; intensity - 125µmol $m^{-2}S^{-1}$) in alleviating chilling injury symptoms in 'Calore' (red-colored spathe) and 'Angel' (white-colored spathe). They observed that protection from blue light could reduce chilling injury, as indicated by lower electrolyte leakage, and water loss percentage during storage under blue light exposure (storage temperature was 4^0C). They also found that the short vase life of the blue light-treated cultivar may be attributed to the effect of this light spectrum on oxidative stress and membrane integrity. Despite categorization Heliconia as a highly chilling-sensitive crop, no investigations on the role of light spectrum in alleviate this issue have taken place yet. However, a few investigations on this aspect have been conducted on other related tropical ornamentals, and the findings are summarized in Table 5.

Principally, the major post-harvest deteriorations occur during the shipment of the produces, and the temperature conditions at the destination also play a vital role in this case (Paull, 1991). Since our target crop originates from tropical and sub-tropical regions, when it is shipped to temperate regions, the sudden low-temperature shock poses a vulnerability. However, prolonged exposure of the cartons to scorching temperatures due to unprofessional handling can also cause injury (Reid and Kofranek, 1980; Heliconia Society International (HSI), 1989). Leite et al. (2015) evaluated the optimum storage conditions for *H. stricta* var. 'Bucky'. They stored the cut inflorescences in cardboard boxes for 2, 4, 6 and 8 days under three different conditions: 'in box at room temperature (23°C and 89% relative humidity)'; 'in box under refrigerated storage (17°C and 94% relative humidity)'; and 'control treatment' i.e at room temperature condition (25°C and 77% relative humidity).They found that the optimum post-harvest attributes along with a shelf life of 7 days, were achieved under the first storage condition. However, the development of chilling symptoms (dark brown spots at the center and apex of bracts and at the conjunction of rachis and bracts) was noticed in the second storage condition, emphasizing the sensitivity of Heliconia to chilling. Paradoxically, Liju (2013) reported that a storage temperature of 17°C under packed conditions (details not found) is best for all Heliconia varieties, resulting in a shelf life of 8.6 to 11.6 days. Furthermore, brief storage of 4h at 20°C temperature before final storage also has positive effect on the post-harvest life of *Heliconia* (Liju, 2013). Darras (2020) also mentioned the significant problem of transporting *H. bihai* and other tropical ornamentals like *Alpinia, Dendrobium, Phalaenopsis, Strelitzia reginae*, and *Anthurium andraeanum* in mixed cargo with other low-temperature tolerant traditional cut flowers.

Very limited recent investigations and a few old investigations from several decades ago on Heliconia highlight the need for further research. In summary, storing of chilling-sensitive *Heliconia*at low temperature may disrupt various physiological processes such as water regulation, mineral nutrition, respiration, and overall metabolism. Therefore, novel techniques, should be developed to either breed chilling-resistant varieties or reduce their sensitivity to low temperatures in order to support the commercialization of Heliconia (Figure 1).

Water Balance: The water balance and post-harvest physiology of cut units are intimately related. The fundamental components of water balance include water uptake, loss, and accumulation, as well as the potential interactions between them (Sankat and Mujaffar, 1994). Water uptake plays a vital role in maintaining optimal hydration levels in cells (Costa et al., 2021), reducing abscission, senescence, and wilting (Seman and Rafdi, 2019; Costa et al., 2021), preserving the turgidity of floral units (Halevy and Mayak, 1981), and slowing down respiratory and metabolic activities (Kamemoto, 1962).

The water uptake capacity of *Heliconia* cut stems is relatively poor (Dolselman and Broschat, 1986; Criley and Paull, 1993). Therefore, it is crucial to carefully manage the water status in cut stems. According to Criley and Paull (1993), the water uptake rate of *H. psittacorum* var. 'Lady Di' and 'Sassy' decreases significantly after harvesting. However, the same researchers found that the average water uptake rate of the 'Parakeet' cut stem (without any foliage) was 3 mL after 15 days of harvesting. This result suggests that there may be a genotypic variation in the water uptake capacity of Heliconia. The findings of Carrera-Alvarado et al. (2021) also support these findings, as they observed differences in the water uptake potential of *H. wagneriana* (as low as 6%) and *H. psittacorum* x *H. spathocircinata* cv. 'Tropics' (0.08 - 0.11 mL g-1). It is characteristic of Heliconia stems to have low solution absorption (Jaroenkit and Paull, 2003; Carrera-Alvarado et al., 2020). The impaired solution uptake may be due to a close relationship between the diameter of the xylem vessels and susceptibility to embolism, where wider vessels are more prone to cavitations compared to narrow vessels (Arriaga-Frías et al., 2016).

Generally, in *Heliconia* cut flowers, the leaves are removed from the stem before commercialization because they tend to dehydrate quickly due to the high stomatal density. However, Ka-ipo et al. (1989) stated that there is a positive correlation between the number of attached foliage and water uptake in cut stems. This view is supported by several researchers (Broschat and Donselman, 1983b; Tjia and Sheehan, 1984; Criley and Broschat, 1992; Criley and Paull, 1993). They mentioned that removing all foliage and leaf sheaths from cut inflorescences significantly reduces water uptake rates,

TABLE 5 Research status on impact of post-harvest factors on qualitative attributes of some tropical ornamentals.

Names of genotypes	Purpose of evaluation(s)	Factors				Impacts	Remarks	Sources
		[A]Storage Temperature	[B]Water balance	[C]Preservative solutions				
				[C1]Chemical (s) based	[C2]GR based			
Strelitzia sp.	[A]Standardization of temperature to retain post-harvest quality; [C1f]Efficacy of GO andAgNPs as anti-microbial agents & to extend the vase life longevity [C1g]Efficacy of Ce $(NO_3)_3$ as pulsing and holding solution	>8^0C[a]; ≤7-15^0C[b]; 6-7^0C[e]; 10-13^0C[c]	–	[f]GOandAgNPs: 1μML^{-1}(each) (holding treatments); [g]Pulsing (24h) by 300μM of $Ce(NO_3)_3$ &holding solution using 300-600μM of $Ce(NO_3)_3$	–	[A]Confusional; [C1f]Vase-life extended >6 days over control along with improved RWU and FW retention, reduced rate of microbial blockage, decreased rate of electrolyte leakage and better enzymatic activities (high SOD, POD and low MDA); [C1g]Pulsing treatment caused longer longevity of 11.68 days along withincreasedwater uptake, dry matter content, & FW &reduced bacterial populations at the cut stem end and in vase solution too, utmost POD (0.147nmol g^{-1} FW) and catalase activities (1.02nmol g^{-1} FW) along with lowest MDA accumulation (0.09 nmol g^{-1} FW) had also been recorded.	[A]Little tolerant to low temperature condition; in fact, may be at 0^0C temperature for short term (7days) without occurrence of chilling injury (CI) symptoms (i.e bract and sepal discoloration)[d]	[Aa]Jaroenkit and Paull, 2003, [a]Nowak and Rudnicki, 1990, [b]Rudnicki et al., 1991, [c]Vieira et al., 2012; [d]Reid and Jiang, 2012; [e]Reid, 2004; [C1f]Thakur et al., (2022); Yan and Chen (2019) and Sharma et al., (2022) [C1g]Azarhoosh et al., (2021)
Alpinia	[A]Standardization of temperature to retain post-harvest quality; [B]Any investigations yet to be conducted; [C1]Vase life extension; [C2kl]Impact of GRs alone & in combination with other chemicals to extend vase life;	>12^0C temperature and >80% of RH	–	0.1% of ascorbic acid treatment (as vase solution)	[k]BA: 100mgL^{-1} (dipping or spray) [l]Combination of 5% of sucrose + 3mgL^{-1} of BA + 200mgL^{-1} of 8-HQC (holding solution)	[A]Minimization of CI symptoms; [C1]It had protracted the longevity for 11.60days over control (8 days); [C2k]Extended the vase life of 11.60days over control (8 days); [C2l]Caused optimum enzymatic activities with reduced oxidative stress	[B] To minimize BD disorder owing to high post-harvest transpiration water loss, optimum balance b/w all water balance components seems vital.	[A]Broschat and Donselman, 1988; Paull, 1991; [B]Criley, 2014; [C1]Morais et al., 2012; Islam et al., 2013; [C2k]Paull and Chantrachit, 2001; [C2l]Morais et al., 2012
Etlingeraelatior	[A]Any investigations yet to be conducted; [C2hij]Impact of GRs in combination with other chemicals to extend vase life& to improve postharvest quality;	–	–	–	[h]1-MCP treatment (holding solution) (concentration did not mention); [i]Combination of 8-HQ: 100mgL^{-1} + GA: 50mgL^{-1}+ sucrose: 2% + BA: 50mgL^{-1}(as	[C2 hi]Both found beneficial to get the vase life of 23.63 days over control (18 days). [C2j]Improved vase life by 18.7% (over the control 10-15days), reduced senescence symptoms *viz.* browning of bracts, loss in gloss and hues.	[A]Onset of BB disorder (CI symptom) takes place for low temperature storage, depletion of soluble sugar and cellulose level, along with high respiration rate; [C2j]Peduncle length of 65cm had been maintained	[A]Yeat, 2016; [C2h]Bayogan and Gratuito, 2013; [C2i]Chaudhari et al., 2016; [C2j]Choresca et al., 2019

(Continued)

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TABLE 5 Continued

Names of genotypes	Purpose of evaluation(s)	Factors				Impacts	Remarks	Sources
		[A]Storage Temperature	[B]Water balance	[C]Preservative solutions				
				[C1]Chemical (s) based	[C2]GR based			
					holding solution); [j]Pulsing (30 min) using 200mgL^{-1} of BA (under ambient temperature of 27^{0}C, 72% RH)			
Costus	[A]Standardization of temperature to retain post-harvest quality	5-7^{0}C; 25^{0}C (temperature) & 75-80% (RH)	–	–		[A]Protraction of longevity at low temperature, like the higher one, without emergence of any CI symptoms	[A]Confusing outcomes and paradoxical in terms of empirical views.	[A]Criley, 2014,
Curcuma	[C2mnop]Efficacy of GRs on vase life extension	–	–	–	[m]1-MCP: 900ppb (pulsing); [n]100ppm of GA_3 (pulsing); [o]Combination of 1.8% of both GA_{4+7} and BA (each 2mgL^{-1}) (dipping for 15hrs) [p]1μM of STS (pulsing)(anti-ethylene growth regulator)	[C2mnop]useful for vase life extension	–	[C2m]Chutichudet et al., 2011; Bunya-atichartet al. (2004), and Criley (2014); [C2n]Kjonboon and Kanlayanarat, 2004; [C2o]Favero et al., 2017; [C2p]Chanasut, 2004
Anthurium x ferrierense	[B]Impact of refrigerated condition on water balance components & the subsequent vase life.	–	Best T: WU ranged from 0.86-1.04	–	–	[B]Optimum water balance & extended vase life of 30days.	[B]13 and 18^{0}C temperatures (refrigerated conditions) found most appropriate than ambient temperature (28^{0}C) for maintaining balanced T: WU ratio.	[B]Sankat and Mujaffar (1994)
*A. andreanum*cv. 'Spirit',	[B]Evaluation of the impact of RWC on eradication of SN disorder	–	Positive relation b/w high RWC and	–	–	[B]Extended vase life & alleviation of SN disorder.	[A]Normal range of storage temperature of cut blooms is 14-17^{0}C. [B]Low ion leakage, intact	[B]Farrell et al. (2012)

(Continued)

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TABLE 5 Continued

Names of genotypes	Purpose of evaluation(s)	Factors				Impacts	Remarks	Sources
		[A]Storage Temperature	[B]Water balance	[C]Preservative solutions				
				[C1]Chemical (s) based	[C2]GR based			
'Success', and 'Hondurus'			low intensity of SN				membrane integrity and optimum hydraulic conductance in spathe had been found.	
Anthurium var. 'Ozaki'	[C1]Impact of ammonium salts on color retention during after harvest period	–		NH_4NO_3 (9 and 15meq. NH_4^+) and $(NH_4)_2SO_4$ (9meq. NO_3 and NH_4^+) as pulsing solution	–	[C1]Retention of spathe's hue (temporary effect)	–	[C1]Leonhardt and Woomer, 1991

*T; WU, Transpiration & water uptake ratio; RWC, Relative Water Content; SN, Spadix Necrosis; BD, Bract Desiccation; BB, Bract Browning; GR, Growth Regulator; RWU, Relative water Uptake; FW, Fresh Weight;GO, Graphene oxide; AgNPs, Silver Nano Particles; MCP, Methyl cyclopropane; STS, Silver thiosulfate; MDA, Malondialdehyde -, No information.

leading to a deterioration in fresh weight. The presence of leaves influences water uptake in plants, and the absorption rate is proportional to the number of leaves attached to the flower stem. However, in *Heliconia*, there are no vascular connections between the flower stalk and the leaves, unlike in other floral species. This contributes to the low water uptake after harvest (Carrera-Alvarado et al., 2020). Criley and Broschat (1992) also mentioned the physiological explanation that there are no upper ground vascular connections between the flower, peduncle, and leaves, which prevents water translocation and accumulation in the flower, thus not affecting its vase life. It is hypothesized that the immaturity of the basal intercalary meristem of the inflorescence peduncle at the cutting base may be the reason for this (Criley and Broschat, 1992). However, Ka-ipo et al. (1989) found that the intercalary meristem is located near the flower and not close to the basal end of the peduncle, except for the 'Parakeet' cultivar. Therefore, this hypothetical explanation is not suitable for this particular variety of *H. psittacorum*.

Whatsoever, water uptake may get hampered due to water quality, xylem occlusion by microorganisms, and deposition of pectin and phenols (Jedrzejuk et al., 2012). In general, the tap water is used as an easily available vase liquid medium and contains the common salts such as $Ca(HCO_3)_2$and $CaSO_4$, which determine water hardness. Van Meeteren et al. (1999) mentioned a threshold value of 60 mg/L for Ca, <25 mgL^{-1} for sulfate, and 30 mg/L for HCO3- in tap water. These researchers had raised an argument regarding the beneficial roles of distilled or deionized (DI) water as a control in post-harvest physiological studies. In their experiment on Chrysanthemum cut flowers, they found that DI water caused a sharp decrease in fresh weight of cut blooms after 1-3 days, while tap water did not show such an issue. Several researchers (Kirst and Bisson, 1979; Navarro et al., 2003) stated that DI water may mimic the normal physiological situation of an intact flower because xylem sap in intact plants contains various cations, anions, amino acids, and organic acids. However, Zimmermann (1978), in line with Van Meeteren's argument, stated that drawing distilled water through stem segments progressively decreases the rate of conductance, but this can be eliminated by using tap water or a dilute osmoticum (e.g., 10 mM NaCl). Hutchinson et al. (2004) also confirmed the negative impacts of DI water on the post-harvest physiology of tuberose (Polianthes tuberosa) cut flowers. According to Halevy and Mayak (1981) and van Doorn (2012), this phenomenon may make water conductance in Heliconia stems more complex. Hence, this aspect should caution post-harvest researchers before using DI water for vase life improvement in Heliconia.

Different plant growth regulators (PGRs) such as BA and GA, as well as chemical substances like NaOCl, citric acid, sucrose, STS, HQC, and HQS, may also be used to maintain the components of water balance, as proven in *Lisianthus* (Musembi et al., 2013) and tuberose (Hutchinson et al., 2004) cut flowers. Tjia and Sheehan (1984) reported on the inefficiency of 8-HQC (antimicrobial agent) to improve the water uptake and prolong vase life in Heliconia. Microbial-induced stem plugging, often known as 'physiological' stem-plugging, occurs due to wounds or injuries at the stem end (Rogers, 1973). According to Rogers, the plugging may be caused by oxidative exudates resulting from the phosphorylation process of

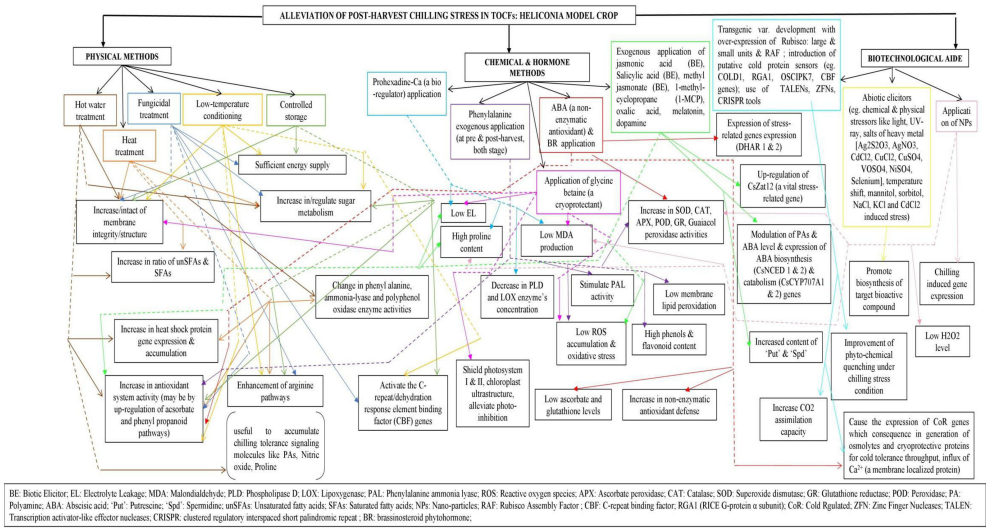

FIGURE 1
Schematic form of hypothetical strategies to obviate chilling injury (CI) in *Heliconia*. Arrows with black colored solid line indicate different treatments under principal methods. Multicolored treatment boxes have been used for segregation and easy identification purpose. Arrows with multicolored solid lines indicate the physiological impacts of individual treatments while the multicolored dotted lines only serve the purpose of connectors to reach different target distant square boxes. (Sources: Mirdehghan and Rahemi, 2004; Erkan et al., 2005; Nayyar et al., 2005; Kagale et al., 2007; Marton et al., 2010; Guo et al., 2012; Aghdam and Bodbodak, 2014; Gupta and Musunuru, 2014; Kim and Kim, 2014; Zhu et al., 2016; Zhao et al., 2017; Shi et al., 2018; Aghdam et al., 2019; Golding, 2019; Hahne et al., 2019; Halder et al., 2019; Vats et al., 2019; Zhang et al., 2019a; Ahn et al., 2020; Ahn et al., 2020; Darras, 2020; Ding et al., 2020; Liu et al., 2020; Salesse-Smith et al., 2020; Sogvar et al., 2020; Giovannini et al., 2021; Jiao et al., 2021; Song et al., 2022; Wei et al., 2022; Castro-Cegrí et al., 2023; Hui et al., 2023).

damaged cells, and the plugs may consist of pectin degradation products (Burdett, 1970). Furthermore, microorganisms can secrete enzymes (tannins, peroxidase) or other metabolic products that form viscous materials composed of calcium and magnesium salts of oxidized tannins. These materials move with the transpirational stream, causing pectin degradation and subsequent plugging (Rogers, 1973). This issue can be addressed by using an acidic preservative solution with a pH of 3-4.

Tjia and Sheehan (1984) found that the increased rate of water uptake during the nighttime is higher than during the daytime. This may be due to the high root hydrostatic pressure, which influences the flow of water uptake to the flower. For example, in *H. psittacorum*, a 2-fold increase has been observed. Folha et al. (2016) evaluated the benefits of periodic cutting (at intervals of 24 hours and 48 hours) of the base (1 cm in length) of peduncles of *H. psittacorum* cv. 'Golden Torch', as well as the renewal of vase water (deionized), in maintaining water balance components, fresh and dry mass, and post-harvest longevity. They found that these two strategies, at a 24-hour interval, optimize the restoration of water potential, leading to greater tissue hydration and maintenance of post-harvest quality. Similar results were also obtained in *H. wagneriana* by cutting the inflorescence peduncle every 2 days (Costa et al., 2015). After being removed from the plant, the flower starts to experience moisture stress. At this stage, the upward movement of the xylem water column draws air bubbles to accumulate at the cut end. These air bubbles, lodged against the cross wall of one of the xylem vessels, impede moisture flow and consequently reduce water uptake. Vacuum infiltration of the stem end, the use of acidified water (pH 3.5), calcium nitrate, and enzyme inhibitors (such as azide and DNP) promote normal water uptake and help regain turgidity (Rogers, 1973). Except for Anthurium, no significant investigations regarding the linkage between water balance and vase life improvement have been reported for other tropical ornamental plants. However, relevant reports are summarized in Table 5 to maintain the breadth and conciseness of the article.

3 Preservative solutions

The detachment of cut blooms, the actively metabolizing plant part, from the mother plant deprives them of the raw materials that are essential for a myriad of metabolic processes. Therefore, the need arises to externally supply these naturally available essentials. Generally, water, anti-senescent substances (e.g., benzimidazole, kinetin, benzyladenine, etc.), and respirable substrates (i.e., sucrose, glucose) are highly demanded substances required to maintain excised blooms in a decorative form (Rogers, 1973). Additionally, various chemicals (e.g., STS [silver thiosulfate], AgNO3 [silver nitrate], citric acid, 8-HQ [hydroxyquinoline], boric acid, Al2(SO4)3 [aluminum sulfate], sodium hypochlorite, NO [nitric oxide]-based donor compounds, 1-MCP [1-methyl cyclopropane], etc.) and growth regulators (e.g., gibberellins, cytokinins, auxins, etc.) can be provided through pulsing (momentary treatment), holding (vase) solutions, or postharvest fumigation treatments (Badiyan et al., 2004; Janowska and Andrzejak, 2023) with the aim of improving the post-harvest life of cut blooms. In the following sections, the functions of the aforementioned substances to prolong the post-harvest life of Heliconia blooms are delineated.

3.1 Chemicals based preservatives

Based on the bibliographic searches, the overall idea of a low water uptake capacity by the *Heliconia* inflorescence's peduncle has been acknowledged. It has been found that this problem cannot be improved by the use of various vase life-extending chemical components, either alone or in combination. However, exceptional cases have also been discovered. The research outcomes of Broschat and Donselman (1983a); Ka-ipo et al. (1989), and Tjia and Sheehan (1984) can support the aforementioned statement.

8-hydroxyquinoline citrate or sulfate (8-HQC or S), sucrose, silver thiosulfate (STS), dithiothreitol (DTE), citric acid, etc., are commonly used preservatives in Heliconia (Broschat and Donselman, 1983b; Bredmose, 1986; Ka-ipo et al., 1989; Whittaker, 1993; Malakar et al., 2019). Among these, 8-HQ salts and sucrose possess multifarious potential properties, such as anti-desiccant and anti-microbial components, and the ability to maintain balanced water content. The preservative-based solutions prepared using non-deionized water containing less than 200 ppm of total dissolved salts usually result in satisfactory water uptake, as reported by Rogers (1973). Costa et al. (2015) found improved post-harvest longevity in *H. wagneriana* after pre-treatment with 10% and 20% sucrose solutions and holding solutions of 30 and 75 mg/L of AgNO3. Liju (2013) discovered that pulsing with a solution of 5% sucrose + 200 ppm 8-HQ for 6 hours, followed by a holding solution of 5% sucrose + 100 ppm 8-HQ, extended the post-harvest period in seven varieties of Heliconia. Sarkar et al. (2022) also obtained similar results for different Heliconia genotypes using 8-HQC-based holding solutions. Recently, Malakar et al. (2019) evaluated the consequences of different vase solutions on the post-harvest durability and quality of Heliconia cut inflorescences available in West Bengal, India. They used silver nitrate (AgNO3): 1500 ppm, calcium chloride (CaCl2): 750 mg/L, citric acid: 200 mg/L in combination with 8-HQC: 500 mg/L and sucrose: 2%. The treatment combinations of AgNO3, CaCl2, and citric acid, along with 8-HQC and sucrose, were found to be the most effective for all the Heliconia genotypes, prolonging vase life (up to 7 days on average, compared to the control of 7-8 days) and improving other features such as pigment retention, solution uptake rate, and enzymatic activities (high catalase [CAT], peroxidase [POD], and low lipid peroxidation). However, they mentioned that the performance may vary depending on the species and variety. Additionally, the spray of bovine serum albumin (BSA): 50 mg/L was found to prolong vase life by almost 2-fold in 'Golden Torch', as reported by Mangave et al. (2013).

Holley (1960) estimated that as much as one-third of a flower's shelf life may be influenced by its pre-harvest environment, while the remaining two-thirds are determined by postharvest maintenance. One of the reasons for the qualitative deterioration of cut blooms is the depletion of respirable substrates, although there is a connection between the amount of reserved dry matter content and the shortage of respirable substrates (Rogers, 1973). According to the same researcher, exposing the excised units to incidental light increases the photosynthetic capacity and production of photosynthates, indirectly alleviating the scarcity of respirable substrates. A carbon dioxide-enriched growing environment (pre-harvest) also influences the storage of more photosynthates (Shaw and Rogers, 1964; Mattson and Widmer, 1971).The physiological process known as "respiratory metabolism" is closely associated with the post-harvest improvement of cut blooms. A shift in respiratory quotient is observed in isolated blooms, with Hew and Yip (1987) estimating the highest respiratory quotient of 1.0 (in excised petals) compared to 0.5 (in *in situ* blooms at the tight bud stage) in Aranda orchid petal cells. They also mentioned that in cut blooms, carbohydrate metabolism predominantly occurs via the EMP pathway (the synthetic conversion of glucose to pyruvate), and there is a shift towards cyanide-sensitive respiration. Additionally, sucrose not only serves as a source of carbohydrates (Malakar et al., 2019) but also helps protect the ultrastructure of chromoplasts, resulting in pigment retention (Singh et al., 2008). Sucrose also aids in maintaining water balance, keeping the bract cells turgid by influencing osmotic pressure (Halevy and Mayak, 1981).While Tjia and Sheehan (1984) mentioned that 8-HQC or 8-HQS does not have any impact on solution uptake or the elimination of microbial occlusion, Malakar et al. (2019) found the beneficial impact of this germicide on extending vase life.

According to Subhashini et al. (2011), Ag++ ions help to prolong the vase life, while Ca++ ions reduce the respiratory rate and contribute to cell wall toughness, preventing cell breakage or collapse. The broad antimicrobial effects of AgNO3 are well-known, as Ag++ ions replace the hydrogen cations (H+) of sulfhydryl or thiol groups (-SH) on the cell membranes of bacteria, resulting in membrane integrity loss and cell death (Feng et al., 2000; Li et al., 2020; Elatafi and Fang, 2022). Several researchers (Jiang et al., 2004; Foldbjerg et al., 2009; Elatafi and Fang, 2022) have revealed that Ag nanoparticles (Ag-NPs) are more effective than other forms of Ag because they possess a larger surface area-to-volume ratio, making them more efficient as a biocide. Additionally, Ag-NPs have lower toxicity effects and optimize the content of soluble solids (SSC), titratable acidity (TA), malondialdehyde (MDA), as well as the activities of polyphenol oxidase (PPO), pyrogallol peroxidase (POD), and pectin methylesterase (PME).

The pH of the solution also has a significant impact on vase life. A neutral or alkaline pH is not suitable for maintaining satisfactory post-harvest quality (Reid and Kofranek, 1980). The pH of plant cell sap is generally between 3-3.5 (Khan et al., 2009; Gupta and Dubey, 2018); therefore, an acidic solution or the addition of organic acids like citric acid can yield good results. Low-pH water (pH 3.5) travels faster in the water-conducting system (xylem), thereby preventing or reducing wilting during the post-harvest stage. Commercial rehydration solutions, such as Hydraflor, often contain sufficient citric acid to lower the pH of the vase solution to 3.5 (source: https://ag.umass.edu/greenhouse-floriculture/fact-sheets/sugar-acidity-in-preservative-solutions-for-field-grown-cut).

Some eco-friendly solutions could also serve the purpose of extending the vase life, as evidenced in the case of Heliconia 'Golden Torch'. Shokalu et al. (2021) studied the impact of aloe vera (*Aloe barbadensis* Miller.) and moringa (*Moringa oleifera* Lam.) solutions on prolonging the display life of the said Heliconia variety. They

found that the combination of aloe vera solution (5%) along with 4% sucrose could improve the water balance components, resulting in a 67.4% increase in open bracts and 78.9% relative water content (RWC). In the case of Anthurium, the use of *Stevia rebaudiana* extract at a concentration of 0.1 mgL^{-1}along with 10 mgL^{-1} of nano-silver had been found to enhance the water uptake rate after fifteen days of placing the cut bloom in the solution (Amin, 2017). The scientific and commercial approaches towards using organic extracts as potent preservatives for cut tropical ornamentals are still in the early stages.

Regarding other tropical ornamentals, except for *Strelitzia* and *Anthurium* (Table 5), very limited published reports have been observed. For example, in the case of *Globba*, several researchers (Criley, 2014; Branney, 2005; Chuengpanya et al. 2016) have mentioned that the longevity of its bracts may last up to 1-1.5 months, but scientific authentication is lacking. Despite having significant cut flower attributes, there are no scientific reports available, although an article by Aung et al. (year missing) on "post-harvest quality and vase life of ornamental cut flower *G. orixensis*Roxb." has been found, but its accessibility is limited. The status of Hedychium is also the same; the frequent emergence of new flowers and its limited use as a cut flower may have hindered post-harvest researchers from conducting investigations on standardizing chemical formulations for bud opening and extending vase life. The current scenario emphasizes the importance of conducting research on Hedychium to bring it to the forefront and establish its use as a commercial tropical cut bloom.

3.2 Growth regulators based preservatives

Mangave et al. (2013) investigated the role of growth regulators spray (GA, BA, and Alar [synonymously Daminozide]) in extending the quality and post-harvest life of the 'Golden Torch' cultivar of *H. psittacorum x H. spathocircinata*. The spray of GA: 100 mg/L had yielded satisfactory effects in this case by decreasing enzymatic activity and lipid peroxidation, while also improving factors such as the percent absolute integrity (PAI) of the bract cell membrane (which delays bract cell death), enhancement in inflorescence fresh weight, vase life (2-fold increase over control), and removal of oxidative stress. Malakar et al. (2019) also found the positive impact of GA3 at a dose rate of 80 ppm on vase life extension of different genotypes of Heliconia.

BA is a synthetic cytokinin (CK) (Paull and Chantrachit, 2001; e-source: https://www.acs.org/molecule-of-the-week/archive/b/6-benzyladenine.html) that has been reported to increase the vase life of diverse tropical ornamentals, including Heliconia. Paull and Chantrachit (2001) stated that the use of BA, in both spray and dipping forms, could extend the vase life of *H. psittacorum* var. 'Sexy Pink' inflorescences up to 21 and 18 days, respectively. Similarly, the use of BA in these forms could retain the keeping quality of ornamentally significant leaves and flowers of the 'Andromeda' variety for up to 32 and 31 days, respectively. BA helps delay both bract darkening and abscission. Another study on the effect of BA was carried out by de Moraes et al. (2005) in *H. latispatha*. They found a linear increase in vase life with increasing concentrations of BA spray (100, 200, and 300 mg/L). The longest vase life, 1.85-fold increase, was obtained at a concentration of 300 mg/L of BA. The dipping or spray of BA at a dose rate of 200 mg/L also extended the vase life of the 'Nickeriensis' variety of Heliconia by about 8 days, as reported by Whittaker (1993). According to Whittaker (1993) and Costa et al. (2021), the treatment using BA at a concentration of 200 mg/L increased the vase life of Heliconia genotypes (var. 'Sexy Pink' and 'Andromeda') by 1.2 to 2.5-fold.

GA reduces senescence rates by regulating cell membrane permeability and protein degradation (Shaul et al., 1996) and by influencing the action of ABA (Kumar et al., 2014; Costa et al., 2016). Cytokinins (CKs) and GAs are considered inhibitors of aging; however, unfortunately, their content in plant tissues decreases during the aging process, while the levels of regulators that accelerate aging, such as ethylene, salicylic acid (SA), brassinosteroids (BR), abscisic acid (ABA), and jasmonic acid (JA), increase (Asami and Nakagawa, 2018; Janowska and Andrzejak, 2022). During the aging of petal or perianth cells, active membrane-damaging enzymes, proteolysis, accelerated breakdown of pigments, and a large amount of free radicals contribute to the destruction of cell components (Rogers, 1973). A high content of reactive oxygen species (ROS) causes oxidative stress, leading to damage to cellular macromolecules and membranes, as well as increased lipid peroxidation (Janowska and Andrzejak, 2022). Application of BA and GA3 significantly reduces protein degradation (Hayden, 2003), and both act as pigment protectors (Rabiza-Świder et al., 2012). Moreover, pre-soaking plant parts in GA3 solution before planting and harvesting stages noticeably enhances the content of hydrocarbons, especially fructose and glucose, thereby improving post-harvest life (Janowska et al., 2022; Janowska and Andrzejak, 2022). In other related tropical ornamentals, the use of growth regulators has been found to be limited (Table 5).

4 Other strategies to improve after-harvest life

Since water balance is the ultimate factor in preventing desiccation injury and subsequent senescence, maintaining the rate of water uptake and minimizing evaporative water loss are two vital factors (Criley and Broschat, 1992). Given the circumstances, the use of anti-transpirants can be a viable solution.

Ka-ipo et al. (1989) mentioned that dipping 'Parakeet' types of *H. psittacorum* in a 'Wilt Pruf' solution (dilution rate 1:10) or 'Wax' solution (dilution rate 1:4) could increase the vase life by up to 36%. Dipping 'Nickeriensis' type in a 'Folicote' solution (dilution rate 1:40) could also extend the vase life by around 26% as reported by Whittaker (1993). Carrera-Alvarado et al. (2021) conducted a study on the impact of waxing and salicylic acid (1mM) treatment on the post-harvest life of H. wagneriana under low temperature (13°C and 84% RH) conditions. They found that the bract tissue of waxed peduncles showed optimal enzymatic activity, low oxidative stress, good water accumulation (maintenance of bract turgidity), and a satisfactory shelf life of an extra 2-3 days compared to 9 days under

control conditions. In the case of the 'Golden Torch' cultivar, graded concentrations of wax emulsions (0.25%, 0.50%, 0.75%, and 1%) were found to enhance the shelf life from 10-11.78 days, while the fresh weight increased by 48% (Powar et al., 2014). However, several researchers [Criley and Broschat, 1992, Broschat and Donselman (1987)] expressed concerns about the limited efficacy of waxing in extending the vase life of Heliconia genotypes. This may be due to the inability to cover the entire bract surface, especially the groove areas, while the waxy surface of the bract cuticle could be another reason, as reported by Criley and Paull (1993).

Another effective strategy may be hot water treatment, as the deterioration in keeping quality can also be caused by disease infestation. Therefore, this treatment may minimize the problem. In the case of *H. chartacea* var. 'Sexy Pink', *H. caribaea* (Red), and *H. psittacorum* (Red), hot water treatment (49°C temperature for 12 minutes) has been found to increase the keeping quality for a few days (Criley and Broschat, 1992). However, no further details regarding the threshold level of temperature and duration have been reported by any researchers for Heliconia.

Among other tropical ornamentals, the application of waxing treatment has been noticed in Strelitzia and Etlingera. For example, de Paula et al. (2021) evaluated the impact of canauba wax coating (20% and 40%) on the foliage of *S. juncea* and *S. reginae*, resulting in an extended post-harvest longevity of 16th day for *S. reginae* and 18th day for *S. juncea*, low leaf mass loss percentage, and minimal visual quality impairment. Additionally, the application of 3% carnauba wax on the bracts of *E. elatior* var. Porcelana (at semi-open and fully open stages) effectively maintained water balance, carbohydrate content (starch and total soluble sugar), promoted bract expansion, and flower opening (Mattos et al., 2017; Mattos et al., 2018).

In Strelitzia, the major post-harvest problem is the development of saprophytic mold, leading to floret desiccation and bract darkening (Jaroenkit and Paull, 2003; Balas et al., 2006; Koley, 2013; Criley, 2014). The secretion of mucilage during anthesis encourages Botrytis growth, while post-harvest nectar and slime production facilitate saprophytic mold growth (Jaroenkit and Paull, 2003; Criley, 2014). Jaroenkit and Paull (2003) suggested dipping Strelitzia inflorescences in a solution of benomyl or thiobendazole (200 mg/L concentration) to address this issue.

'Geotropic curvature' has been mentioned as a serious concern in *Alpinia* (Criley and Paull, 1993). Dipping red ginger inflorescences in TIBA (tri-iodobenzoic acid; an auxin movement inhibitor) could be useful in controlling the geotropic curvature, as reported by Chantrachit (1999). Hot water treatment (49°C for 12 minutes) and maintaining a vertical posture during shipment may be other viable remedies (Hara et al., 1997). In the case of Heliconia, no such post-harvest issues have been documented.

To restrict insect and pest infestations, irradiation treatment of 250 Gy may be useful (Sangwanangkul et al., 2008). To minimize minimal irradiation injury, hot water treatment (40.0-47.5°C) for a duration of 20-30 minutes may be applied, although the physiological basis for improvements after heat treatment is yet to be unraveled.

Topolines (Ts), ionic liquids, and quaternary ammonium salts with selected organic cations and GA3 anions have been used in florist greens (ornamental foliage) to improve post-harvest longevity (Janowska and Andrzejak, 2022). Therefore, their implementation may also be introduced in Heliconia.

The Controlled Atmospheric Storage (CA) system, which relies on low levels of O2 (0.5-1% for flowers, but not 0, as it may cause undesirable anaerobic breakdown reactions) and increased levels of CO2, can be implemented to reduce respiration rates and preserve respirable substrates during post-harvest storage (Rogers, 1973). This approach may also be applied to Heliconia. All tropical ornamentals, including Heliconia, are ethylene insensitive, but an exception has been found in Strelitzia. According to Bayogan et al. (2008), Strelitzia is not highly sensitive to ethylene, but its longevity may be affected by exogenous ethylene exposure. Pre-treatment using 500 ml L-1 of 1-MCP and 0.2 mM STS for 6 hours at a temperature of 20°C can reduce ethylene-related injuries, as reported by Macnish et al. (2009), who exposed Strelitzia inflorescences to 1 µL L-1 of ethylene for 24 hours. This may be useful in preventing any undesirable physiological changes that may occur in tropical ornamentals.

4.1 Post-harvest handling

At this phase, the harvested produce is subjected to cleaning and grading processes, which are considered preceding steps to final packaging. Cleaning is a crucial step in post-harvest handling, particularly during export. In Heliconia, insect disinfection and additional cleaning and treatment steps are typically followed because the floral structures (bracts) of Heliconia provide natural hiding sites for many insects (Jaroenkit and Paull, 2003). In Hawaii, the conventional hand wash along with insecticidal soap is usually employed, and washing in a detergent solution followed by rinsing with pressurized water flow can be useful not only for cleaning purposes but also to remove field heat. For *Heliconia* inflorescences' disinfection, a solution of 50% Diazinon 40 WP (160g/100 L-1 of water) + 0.18 L of light volck oil or a solution of 57 EC malathion (1 ml L-1) can be used (Nowak and Rudnicki, 1990; Criley and Paull, 1993). Hot water treatment has also been found to be effective for disinfection purposes (Hara et al., 1997). However, recent advanced research approaches in tropical ornamentals, such as the usage of ultraviolet-C radiation for sanitation purposes, are not yet available, although they are being used to characterize the effects on the sanitary quality of castor oil seeds (de Araujo et al., 2019).

Regarding *Strelitzia*, cleaning before the packaging of the inflorescence is another essential step. The structural characteristics of *Strelitzia* inflorescence, which are inappropriate as habitats or resting places for various insects and pests, have minimized the need for mandatory cleaning, except for simple washing with water to remove field dust and heat (Jaroenkit and Paull, 2003).

In terms of grading standards for *Heliconia*, to meet the "Hawaii Fancy Grade" standards, each inflorescence must have at least 2

open bracts, while other units must be well-formed, and the minimum length of the inflorescence peduncle must be 15 cm (Hawaii Department of Agriculture, 1972). For *Anthurium*, grading is related to descriptive and visual aspects of the spathe and spadix, including color, shine, turgidity, and the occurrence of spots and necrosis (Cuquel and Polack, 2010). The standard grading of *Anthurium* cut blooms based on spathe width is as follows: >15 cm - Grade (G1), 13-15 cm - G2, 11-13 cm - G3, 9-11 cm - G4, 7-9 cm - G5, 5-7 cm - G6, and >5 cm - G7 (Ng et al., year missing). Several scientific reports on digital vision-based grading systems for *Anthurium* have been observed (Hemming et al., 2010; Soleimanipour et al., 2019; Soleimanipour and Chegini, 2020), while this aspect is completely unknown for other tropical ornamentals. In summary, there is a lack of investigation reports on the characterization of mandatory export grading systems for tropical ornamental cut flowers, highlighting the importance of conducting intensive research on this topic in the near future.

Overall, proper post-harvest handling can affirm the retention of all essential aesthetic attributes which along with increase in market importance could also broaden the participation potential of group of tropical flowers' in value-added based enterprises like in bio-color and alternative dietary sources [i.e edible ornamentals; eg. edible status of bracts of 'Torch Ginger' (Lekawatana and Pituck, 1998)] sectors, in 'green chemistry' industries [eg. use of *Alpinia* plant part's extract for 'green synthesis' of nano-particles (Zhang et al., 2019b; Shinde et al., 2021)] etc. But the negligence has impeded the involvement of this specialty cut flowers' group in value-added studies as mentioned by Kreissig (2019) and Quinaya and d'Almeida (2019).

5 Advancement in packaging: potent shield of post-harvest quality

Packaging acts as a protective cover to absorb shock during the transport of cut flowers. Not only that, it helps maintain the optimum physiological condition of the flowers throughout the transportation distance, ensuring their quality remains intact. Appropriate packaging, combined with pulsing, is helpful in ensuring fresh quality for consumers and extending the vase-life of the flowers (Senapati et al., 2016). However, the unusual structure, extravagant size, and weight of tropical ornamentals, including *Heliconia*, pose significant challenges in terms of packaging and transport. Viable measures to meet this challenge are almost absent, with only a handful of reports available (Table 6). Due to the large size and heavy weight of Heliconia inflorescences, large containers packed with moistened shredded papers are used to maintain humidity and prevent bruising (Criley and Paull, 1993). Smaller *Heliconia* species like *H. psittacorum* and *H. angusta* are usually packed in bunches of 5 and 10, respectively, while larger and heavier species are packed individually in cartons, with plastic film or net sleeves used to minimize bruising (Criley and Paull, 1993). Liju (2013) reported that packing Heliconia inflorescences with a wet cotton plug at the peduncle end and using polythene lining yields the best results. For Strelitzia, cardboard containers sized 102x43x28 cm are generally used for packaging cut units (Criley and Paull, 1993). To keep pace with the recent demand for tropical cut flowers, the adoption of advanced packaging systems such as Modified Atmosphere Packaging (MAP), Controlled Atmosphere Packaging (CAP),

TABLE 6 State-of-art of packaging and transport in tropical cut flowers.

Name of flowers	Packaging methods adopted	Packaging materials	Details of packaging	Impacts of packaging	Mode of transport& other details	Remarks	Source(s)
Alpiniapurpurata	MAP	*PP plastic; large Containers*	Packed with moistened shredded newspaper to prevent bruising and to maintain high humidity within the carton	*Longer freshness period (7.77 days), lowest weight loss (0.71%), optimum freshness (sensory score of 4), capacity of holding solution uptake increased (4.81mL/stalk/day)*	Air freight	The text is illegible	Marsetyowati, 2014; Akamine, 1976; Kobayashi et al., 2007
Anthurium	Cartoon packaging (size 21.6 x 50.8 x 91.4 cm; 27.9 x 43.2 x 101.6 cm)	Corrugated cardboard	The basal stem end of cut units are inserted in water filled rubber balloon to render optimum hydration; Wax paper sleeving is used	Prolonged freshness	Surface transportation;	considered as hard ornamentals than tropicals, especially tropical orchids like Vanda; hence, surface transport had been preferred over air transport from Hawaii to US	Akamine, 1976

(Continued)

TABLE 6 Continued

Name of flowers	Packaging methods adopted	Packaging materials	Details of packaging	Impacts of packaging	Mode of transport& other details	Remarks	Source(s)
Strelitzia	*Containers packing*	-	packed with moistened shredded newspaper to prevent bruising and to maintain high humidity within the carton	Prolonged freshness	Surface transportation& Air freight both	Considered as hard ornamentals than tropicals, especially tropical orchids like Vanda; hence, surface transport had been preferred over air transport from Hawaii to US	Akamine, 1976
Vanda	MAP	PP plastic; sometimes corrugated cardboard cartoons	reduced pressures; gases used N2 or CO2;atmospheric pressures 125-190 mm Hg;O2 concentration ranges from 3.45%-5.25%, 3% O2 in air may be modified with N2 or 3% CO2 in air	Long shelf life	Air freight; shipped as intact flowers or as leis;	Ethylene producer; packaging materials must be gas impervious and sufficiently durable to withstand any "ballooning" effect due to decrease in atmospheric pressures in flight.	Akamine, 1976
Cattleya	Cartoonpackaging of variable sizes	Corrugated cardboard, Foam box & Plastic vials	–	Long shelf life	Air freight	–	Akamine, 1976; De, 2020
Cymbidium	Carton packaging of variable sizes	Corrugated cardboard, Polypropylene-150 gauge; Cellophane; LDP-100 gauge; HDP -150 gauge; Newspaper	–	Long vase life	Air freight	–	Akamine, 1976; De, 2020
Dendrobium	Carton packaging of variable sizes	Corrugated cardboard, Foam box & Plastic vials, Cellophane	–	Long shelf life	Air freight		Akamine, 1976, De, 2020
Etlingeraelatior (Torch zinger)	–	–	–	–	–	packaging & transportation are very limited for high mass of inflorescences (over 1 kg)	Gonçalves et al., 2014; Araújo et al., 2018; Loges et al., 2008
Zingiberspectabile (Beehive zinger)	Carton packaging of variable sizes	Corrugated cardboard	Individual cut units are wrapped by plastic bag or mesh; lining by polyethylene film in b/w the cut flowers are made; bactericidal treatment at pre-packaging phase	Better shelf life	Refrigerated (15-18^{0}C & 90% RH) surface transport	–	da Silva Veira et al.,2014
Strelitziareginae	102x43x28cm sized cardboard containers	Corrugated cardboard	Usually packed in bunch of 5; newspaper used as wrapper material;	Long shelf-life	Air freight	–	Criley and Paull, 1993; Jaroenkit and Paull, 2003

(Continued)

TABLE 6 Continued

Name of flowers	Packaging methods adopted	Packaging materials	Details of packaging	Impacts of packaging	Mode of transport& other details	Remarks	Source(s)
			pre-packing and transport dipping treatment in benomyl: 200mgL^{-1} solution to curb nectar and slime production & saprophytic mould growth				
Curcuma cv. 'Chiang Mai Pink'	MAP, carton packaging	PP film; fiberboard cartons	Coating of cut stem end by mixture of 25 ppm BA & GA3 each before packaging	Long shelf life	No information given	–	Yimphak and Chanasut, 2009
*Globbabulbifera*Roxb.	Carton packaging	wooden box, newspaper, the leaves banana	–	Better longevity	Surface transportation in Malaysia	–	Win, 2020; Criley, 2014

-, No information.

Composite Packaging, Antimicrobial/Antifungal Packaging (AP), Edible Packaging (EP), and Nano Packaging (NP) is crucial (Yadav et al., 2022). However, none of these packaging systems are currently in practice for tropical cut flowers, although they are commonly used in fruit crops. These advanced packaging systems rely on the principles of reducing the rate of oxygen consumption, maintaining high CO2 concentration to prevent ethylene production, and extending the shelf life of the produce (Murmu and Mishra, 2018a and Mangaraj et al., 2014; Murmu and Mishra, 2018b; Etemadipoor et al., 2019; Wang et al., 2020). They also maintain the proper gaseous environment around the packed produce, reduce respiration and biochemical reaction rates, and impede transpiration through the use of packaging materials such as polymeric films like PP, LDPE, etc. (Forato et al., 2015; Mamede et al., 2016; González-Reza et al., 2018; Teixeira, 2020), thus keeping the packed produce fresh and turgid (Yadav et al., 2022). Due to stringent environmental legislations, the use of plastic-based films has been minimized, and the usage of biodegradable films or edible films or coatings (commonly used in fruits like guava) is being encouraged (Yadav et al., 2022). The implementation of these techniques for Heliconia and other related species appears to be absent, possibly due to a lack of knowledge, negligence, and suppression, in addition to other physiological bottlenecks mentioned in preceding sections. Since chilling stress is one of the major setbacks in the export of tropical cut flowers, the use of novel or advanced packaging systems resistant to chilling stress should be explored for their export worldwide (Figure 2). Furthermore, the weight of tropical flowers, especially *Heliconia*, significantly restricts their export due to increased air freight charges. To maintain an optimum cost-benefit ratio, air transport is found to be nonviable, and the adoption of surface or marine transport is unquestionable due to the prolonged duration. To overcome these hindrances, weight reduction methods, the development of low-weight *Heliconia* varieties, or the use of advanced and equipped means of transport need to be devised. In this context, the use of a novel dried form of cut units, known as 'Dehydrated cut inflorescences,' may be a viable way to reduce the weight of *Heliconia* cut flowers, although this aspect remains unexplored. Additionally, the employment of cutting-edge molecular aids and functional technological advancements may facilitate the export of *Heliconia* seamlessly. Summarily, significant improvement in packaging of tropical flowers ought to be one of the imperative aspects of several future thrust areas (Figure 3) while active utilization of frontier bio-technological aides may also be a viable option to achieve the target in totality (Figure 4).

6 Conclusion

Tropical cut flowers are vital components of the floriculture industry. However, their commercialization has been hindered by non-commercialization, unprofessionalism, and various loopholes in research and extension activities, as well as in physiological studies related to these flowers even after possessing unique appearance and diverse range of colors. Moreover, the unconventional structure of tropical cut flowers, along with environmental filters and physiological intricacies, has also limited their widespread cultivation.

These have caused the impetus to pen up all available published scientific information(s) as well as knowledge-based views explicitly on Heliconia which is a chosen model crop as well as the ambassador of TCFs group. This review summarizes all possible pre-harvest, harvest and post-harvest factors which are the potent determiner of after-harvest life of them. The major issues regarding tropical flowers are the 'low temperature sensitivity' which restricts to ferry with other traditional cut flowers and 'packaging' for the out of the turn shape, size and weight. The hypothetical measures to rid both the said concerns have also been highlighted which may hint some useful cues for the conductance of further investigations. Furthermore, the cursory overview on contextual aspects of other related tropical ornamental members may also assist the readers to associate with the current realistic research scenario. Summarily, our approach may be considered as an initiative for the promotion of this specialty group of flowers.

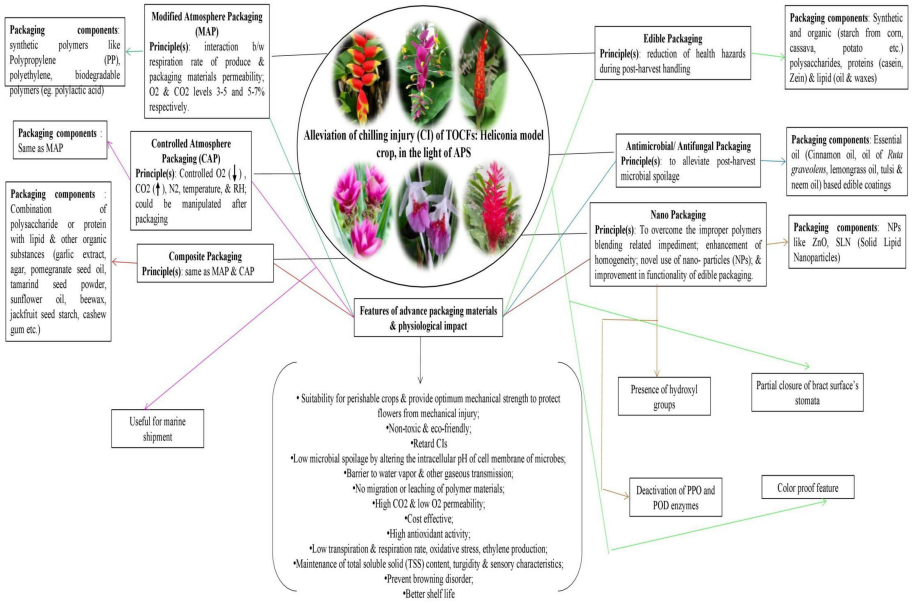

FIGURE 2
Graphical abstract of hypothetical strategies to alleviate CI in Heliconia resorting advance packaging system (APS) Source: Etemadipoor et al., 2020; Yadav et al., 2022. The 'Solid' lines are merely the connecting lines. Black solid lines indicate the key advanced packaging systems. Other multi-colored solid lines show the packaging components of the respective packaging systems. Other multi-colored arrows show the other details of the respective packaging systems.

Successful mitigation of all concerns and the realistic application of all associated endeavors, from cultivation to effective post-harvest measures, should be prioritized as future thrust areas. Moreover, the utilization of high-throughput genetic tools could be a significant boon for tropical ornamentals and researchers, facilitating exemplary improvements in post-harvest quality and addressing specific issues related to after-harvest handling of tropical cut flowers. This paradigm shift could position tropical cut flowers favorably in the global flower trade. Fruitful implementation, professional conduct, and openness to embrace innovation may transform this specialty group of cut flowers

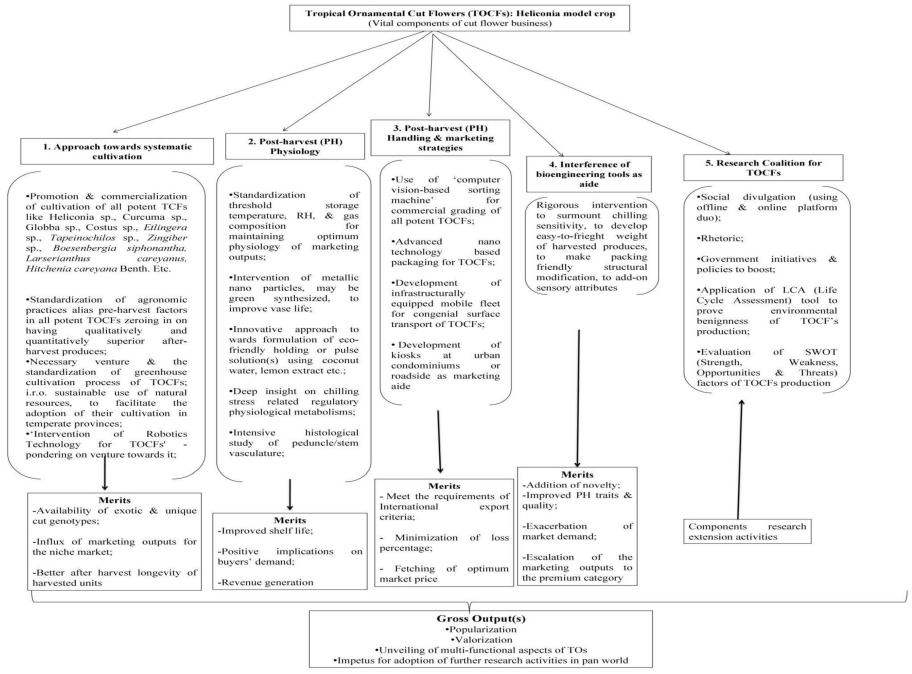

FIGURE 3
Future thrust areas on the Tropical Ornamental Cut Flowers (TOCFs) and its subsequent expected outcomes.

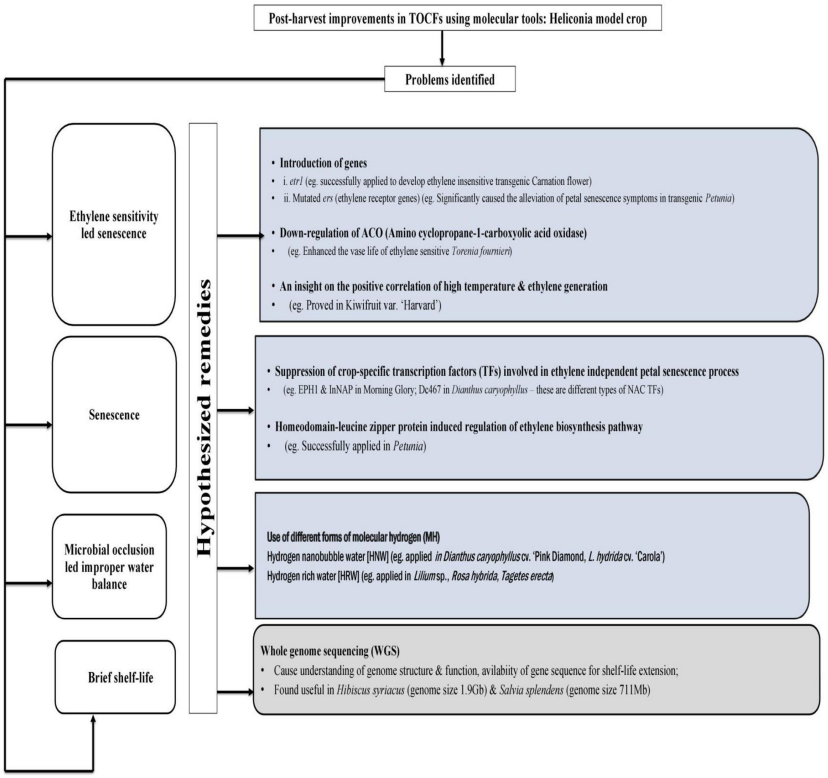

FIGURE 4
Hypothesized strategies for post-harvest improvement by biotechnological interventions in Heliconia. Sources: Bleecker and Schaller, 1996; Aida et al., 1998; Antunes and Sfakiotakis, 2000; Kosugi et al., 2002; Shibuya et al., 2014; Yin et al., 2015; Kim et al., 2017; Ren et al., 2017; Zhu and Liao, 2017; Deng et al., 2018; Woo et al., 2018; Yagi, 2018; Ahn et al., 2020; Carrera-Alvarado et al., 2021; Costa et al., 2021; Giovannini et al., 2021; Verma et al., 2021; Fang et al., 2021; Nguyen and Lim, 2022.

from an "unprofitable niche" into a remunerative and commercially viable component of the flower market.

The paved position of tropical flowers beyond boosting the iterated fact i.e global floral trade, can potently be useful in value-addition sector. Of late value-addition is one of the cardinal sectors of post-harvest industries, especially during the current 'Go Green' era. However, the multihued floral structures (like, inflorescences of tropical ornamental genotypes', bi-color foliage of *H. metallica* etc.) and characteristics of a few botanical plant parts (like, fibrous pseudostem of *H. bihai* etc.) can endorse their eligibility to produce value-added products; as for example the production of eco-colorants and sources of natural cellulosic fibers. Furthermore, the botanical extracts of them may also be used for green synthesis of metallic nanoparticles (NPs) while for edible ornamental's sector, they are almost unexplored. *Hitherto*, the rarest utilization of the members of specialty cut flowers in the said fields has been evidenced. In nutshell, considering the potential future prospects of these specialty TCFs, intensive scientific investigation may be a laudable approach.

Author contributions

MM, PDOP, and MB conceived and conceptualized the article. The overall review of the entire paper has been made by MB, PDOP, and ARCN. Final compilation and editing were done by MM and MB. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Transcriptome and hormone metabolome reveal the mechanism of stem bending in water lily (*Nymphaea tetragona*) cut-flowers

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Water lilies are popular ornamental cut-flowers with significant economic and cultural value. However, stem bending affects the preservation of cut-flowers during their vase life. To gain further insights into the molecular mechanisms of stem bending, transcriptome profiling, hormone measurement, and morphological analysis were performed using the stems of the 'Blue Bird' water lily. Transcriptome analysis revealed that 607 differentially expressed genes (DEGs) were associated with the dorsal and ventral stems of the water lily, of which 247 were up-regulated and 360 were down-regulated. Significant differences in genes associated with plant hormones, calcium ions, glucose metabolism, and photosynthesis pathways genes involved in the dorsal and ventral areas of the curved stem. In particular, DEGs were associated with the hormone synthesis, gravity response, starch granules, Ca^{2+} ions, and photosynthesis. The results of qRT-PCR were consistent with that of the transcriptome sequence analysis. A total of 12 hormones were detected, of which abscisic acid, indole-3-carboxaldehyde, indole-3-carboxaldehyde and jasmonic acid were significantly differentially expressed in the dorsal and ventral stems, and were significantly higher in the dorsal stem than in the ventral stem. The cell morphology in the dorsal and ventral areas of the curved stem clearly changed during vase life. The direction of starch granule settlement was consistent with the bending direction of the water lily stem, as well as the direction of gravity. In conclusion, stem bending in water lily cut-flowers is regulated by multiple factors and genes. This study provides an important theoretical basis for understanding the complex regulatory mechanism of water lily stem bending.

KEYWORDS

water lily, cut-flowers, stem bending, transcriptome, hormone level, paraffin section

1 Introduction

Water lilies (*Nymphaea tetragona*, *Nymphaea* L.) are a perennial aquatic flower plant of the order Nymphaeaceae, and have high ornamental, edible, and medicinal value (Yu et al., 2018). Based on the adaptability of water lilies to climatic conditions, the Nymphaea water lily may be divided into two ecological types: tropical water lily and hardy water lily (Xie et al., 2022). Water lilies are known as the "sleeping beauty of flowers". They have colorful and fragrant flowers with numerous petals and long stalks, with considerable potential to be used as cut-flowers (Samarakoon, 2005). However, stem bending is one of the primary factors affecting the preservation of cut-flowers during their vase life. Thus, it is important to identify the mechanism of water lily stem bending to prolong the vase life of water lilies and improve their ornamental value.

The gravitropic response is a dynamic process in which plants perceive gravitropic stimuli and change their growth direction during development. This process may be divided into four stages, including the sensing of gravitropic signals, transmission of gravitropic signals, asymmetric distribution of auxin, and curved growth of gravitropic responsive organs (Strohm et al., 2012; Wu et al., 2016; Wang et al., 2018). Sensing and backward transmission of gravity signals into second messengers occur by signaling molecules such as Ca^{2+}, InsP3, and PIN. Subsequent asymmetric transport of auxin transmits these signals to various parts of the gravity responsive organs, which results in differences in downstream substrate responses, such as curved growth of the gravity responsive organs (Strohm et al., 2012; Baldwin et al., 2013). Thus, proper bending requires the coordination of various cellular processes, including signal transduction, plant hormone transport, and cell expansion, of which multiple genes are involved (Strohm et al., 2012; Eng and Sampathkumar, 2020; Jonsson et al., 2023).

Studies have confirmed that curved growth caused by gravity is the result of the asymmetric growth of plant organs (Strohm et al., 2012; Jonsson et al., 2023). When plant organs are stimulated by gravity, the amyloplast shifts and converts the physical signal into a biological signal. A previous study showed that *lazy2* regulates the rice tiller angle by specifically regulating starch biosynthesis in gravity sensing cells (Huang et al., 2021). Ca^{2+} acts as a signaling molecule, and Ca^{2+} channels are opened to transmit information from the sedimenting amyloplasts within the gravity-sensing cells (Nakamura et al., 2019). Studies have shown that cytoplasmic free Ca^{2+} concentrations increase in the petiole and hypocotyl of Arabidopsis seedlings expressing apoaequorin during gravity stimulation (Toyota et al., 2008; Toyota and Gilroy, 2013; Tatsumi et al., 2014). Ca^{2+} content is closely related to the flexibility of the peonies peduncle, which is an important indicator of the quality of cut-flowers. Calcium chloride treatment significantly improved the mechanical strength of peonies peduncle (Li et al., 2012). Plants use signaling molecules to transmit gravity signals to phytohormones, which mediate the gravity bending response. Studies have shown that various hormones, such as auxin, ethylene, gibberellin, brassinosteroid (BR), jasmonic acid (JA), abscisic acid (ABA), and cytokinin (CK), are involved in this process; however, auxin plays a leading role (Mazzella et al., 2014; Gerttula et al., 2015; Ajala and Hasenstein, 2022; Wang et al., 2023). Plant hormones can be transported between plant tissues (Philosoph-Hadas et al., 2005) and accumulate in target tissues, which results in their asymmetric distribution (Friml, 2003). Several studies have indicated that ABC transporters function in the auxin transport phase of gravitropism (Lewis et al., 2007; Strohm et al., 2012). In addition to gravity, several environmental factors affect the growth direction of plants, such as nutrients, light, temperature, water, and minerals, which interact with gravity to regulate the growth and development of plants (Song et al., 2019). The expression of the *rice morphology determinant* gene has been reported to be inhibited by the phytochrome-interacting factor-like protein OsPIL16, and the overexpression of OsPIL16 resulted in gravisensing and actin patterning defects (Song et al., 2019).

Currently, the molecular mechanism of stem bending during the vase life of water lily cut-flowers is unknown. Therefore, we conducted transcriptomic analysis, hormone measurements, and morphological observations to examine stem bending in the dorsal and ventral stems of the water lily 'Blue bird'. The curved stems of water lily cut-flowers were analyzed at the molecular, hormone metabolism, and cellular levels to understand and address the stem bending problem.

2 Materials and methods

2.1 Plant material

Water lily (Nymphaea 'Blue Bird') plants were collected from DaDao lake, DaZhiPo town, Haikou city, Hainan, P.R. China (19.82 N, 110.63 E). The water lilies, which were collected on the first day of opening, exhibited erect stems and a largely consistent growth status. The pedicels were cut to a length of 25 cm, and inserted into a bottle (height, 22 cm) filled with ddH_2O water. After the stems of cut-flowers were naturally bent on the fifth day, the bent area was cut with a scalpel and bisected into two parts by length cutting: the dorsal and ventral stems (Figure 1A). Some of the samples were used for cell morphology observation, whereas the remaining samples were rapidly frozen in liquid nitrogen and stored at −80° C for subsequent transcriptome analysis and hormone measurements. All RNA-seq and hormone analyses of the dorsal and ventral stems were performed in triplicate.

2.2 Paraffin section observation

Samples of the dorsal and ventral bent stems of 'Blue bird' water lily were fixed in FAA (37% formaldehyde, glacial acetic acid, 70% ethanol; 1:1:18 v:v:v) (Johansen, 1940). The FAA-fixed samples were dehydrated and embedded in paraffin using an embedding machine (JB-L5, Junjie Electronics Co., Ltd, Wuhan, China). Samples were sliced from the modified tissue wax block using a paraffin slicer (RM2016, Leica Instrument Co., Ltd, Shanghai, China). Dewaxing was performed by rinsing the sections in the

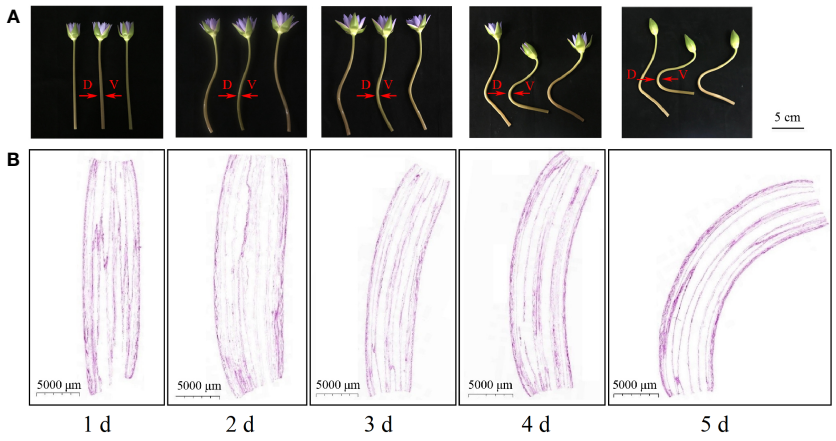

FIGURE 1
Phenotypes and longitudinal section of 'Blue bird' water lily flower stems during vase life. **(A)** phenotypes of the 'Blue bird' water lily stem, bar represents 5 cm. **(B)** longitudinal section of 'Blue bird' water lily flower stems, bar represents 5000 μm. D: dorsal stem; V: ventral stem.

following solutions: xylene I for 20 min, xylene II for 20 min, 100% ethanol I for 5 min, 100% ethanol II for 5 min, 75% ethanol for 5 min, and tap water. The sections were stained with PAS (G1008, Servicebio, Wuhan, China) dye solution B for 10–15 min, rinsed with distilled water, and with PAS A for 25–30 min in the dark. After rinsing for 5 min in tap water, the slides were stained with PAS C for 30 s and rinsed with tap water. The slices were subsequently treated with hydrochloric acid solution and ammonia, and washed with water between steps. Dehydration was performed using100% ethanol I for 5 min, 100% ethanol II for 5 min, 100% ethanol III for 5 min, xylene I for 5 min, and xylene II for 5 min. The samples were sealed with neutral gum (Sinopharm Chemical Reagent Co., Ltd). Finally, the cell morphology was observed via light microscopy using a NIKON ECLIPSE E100 microscope. Images were visualized using the NIKON DS-U3 imaging system.

2.3 Total RNA isolation and illumina sequencing

Total RNA of stems on the fifth day was extracted using a Trizol reagent, and mRNA was enriched using Oligo (dT) beads (Invitrogen, CA, USA). The enriched mRNA was fragmented into short fragments using a fragmentation buffer and reverse transcribed into cDNA with random primers. Second-strand cDNA was synthesized by adding DNA polymerase I, RNase H, dNTP, and buffer. The cDNA fragments were purified using the QiaQuick PCR extraction kit, repairing the ends, adding poly (A), and ligating to Illumina sequencing adapters. Finally, the ligation products were size selected by agarose gel electrophoresis, and PCR amplified. RNA-seq was performed using an Illumina HiSeq platform by Mega Genomics Health Science and Technology (Beijing) Co., Ltd. (Beijing, China).

2.4 Sequence filtration, assembly, unigene expression analysis and functional annotation

Raw reads obtained from the Illumina sequencing analysis were filtered to obtain high quality clean reads by removing reads containing adapters, 10% of the unknown nucleotides and >40% of low-quality (Q-value ≤ 10) bases. Transcriptome assembly of the clean reads from all sequenced samples was performed using Trinity (Grabherr et al., 2011). Firstly, the sequencing reads are fragmented into shorter fragments K-mers, then extends these small fragments into longer contiguous sequences Contigs, and utilizes the overlaps between these segments to generate a collection of fragments called Components. Finally, employing the De Bruijn graph method and sequencing read information, transcript sequences are identified within each fragment collection. The expression level of each transcript was calculated and normalized to FPKM (expected number of Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced) (Trapnell et al., 2010). The default parameter of Subread package featureCounts 2.0.3 is used to calculate the FPKM quantitative calculation of the gene (Liao et al., 2014). DESeq (http://www.bioconductor.org/packages/release/bioc/html/DESeq.html) package was used to identify transcripts with a fold-change≥2, fold-change ≤ 0.5 and an *FDR*<0.01 in the dorsal and ventral stems were considered differentially expressed genes (DEGs). The identified genes were annotated using the following databases: NCBI non-redundant protein (NR), Swiss-Prot protein, Kyoto Encyclopedia of Genes and Genomes (KEGG), euKaryotic orthologous groups of proteins (KOG), and gene ontology (GO) using the BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) program with an e-value ≤ 10^{-5} (Conesa et al., 2005).

$$\text{FPKM} = \frac{\text{mapped fragments of transcript}}{\text{Total Count of mapped fragments (Millions)} \times \text{Length of transcript (kb)}}$$

2.5 qRT-PCR validation

Total RNA was isolated from the dorsal and ventral stems of the bent water lily stems using a Plant RNA Kit (R6827, Omega) according to the manufacturer's protocol. The integrity of the total RNA was assessed on 1% agarose gels (Figure S1). Primer sequences for the selected DEGs (Table S1) were designed using the NCBI primer blast program (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and Primer Premier 5.0. The primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). Reverse transcription was performed using the PrimeScript RT kit with gDNA Eraser (TaKaRa, Shanghai, China) based on the manufacturer's protocol. qRT-PCR was performed with three technical and three biological replicates. qRT-PCR analysis was performed using Luna Universal qPCR Master Mix (New England Biolabs, Ipswich, MA) according to the manufacturer's instructions with denaturation at 95°C for 60 s and 40 cycles of amplification (95°C for 15 s and 60°C for 30 s). *Actin* was used as a reference control. The $2^{-\triangle\triangle Ct}$ method (Ct, cycle threshold value of target gene) was used to calculate relative mRNA expression levels (Willems et al., 2008).

2.6 Hormones identification and quantification

2.6.1 Sample preparation and extraction

Plant materials (120 mg fresh weight) were ground to a fine powder in liquid nitrogen, extracted with 1.2 mL 80% methanol by vortexing briefly, and incubated for 16 h at 4°C. The extract was centrifuged for 15 min (12,000 g, 4°C) to remove solid particles. The resulting supernatant was evaporated to dryness under nitrogen gas stream, and reconstituted in 30% methanol (Li et al., 2014). The solution was centrifuged for 15 min (12,000 g, 4°C) and the supernatant was analyzed by LC-MS.

2.6.2 LC/MS/MS analysis

The extracts were analyzed using an LC-ESI-MS/MS system (Ultra Performance Liquid Chromatography, UPLC, Shim-pack UFLC SHIMADZU CBM30A system; Tandem mass spectrometry, MS/MS, Applied Biosystems 6500 Quadrupole Trap). Hormone separation was performed with a HPLC Waters ACQUITY UPLC HSS T3 C18 column (1.8 µm, 2.1 mm × 100 mm) using UP water with 0.04% acetic acid (solvent A) and acetonitrile with 0.04% acetic acid (solvent B) as mobile phases. The following parameters were used for HPLC: flow rate of 0.35 mL/min; column temperature 40°C; 5 µL injection; method: 0 min (95% A: 5% B), 0–11 min linear gradient to 5% A:95% B, 11–12 min (5% A: 95% B), 12–12.1 min (95% A: 5% B), and 12.1–15 min (95% A:5% B). The effluent was alternatively connected to an ESI-triple quadrupole-linear ion trap (Q TRAP)-MS. The ESI source operation parameters were as follows: ion source, turbo spray; source temperature, 500°C; ion spray voltage (IS), 5500 V; curtain gas, 35.0 psi; and collision gas medium. DP and CE for individual MRM transitions were performed with further DP and CE optimization. A specific set of MRM transitions was monitored for each period according to the plant hormones eluted during this period.

2.7 Statistical analysis

All measurements and qRT-PCR analyses were performed in triplicate. Univariate values were analyzed using ANOVA and mean values were compared using Duncan's new multiple range test ($P< 0.05$) with SPSS 22.0 software (IBM Corporation, United States).

3 Results

3.1 Microscopic observation of cut-flowers

3.1.1 Observation of longitudinal flower stem sections during vase life

Paraffin sections of the longitudinal stem samples of the 'Blue bird' water lily cut-flowers (Figure 1B) revealed that the pedicels were upright on the first day during vase life. The pedicel appeared to show a trend toward bending on the second day, but the bending angle was small. The cut-flower stems were significantly bent on the third day compared with the first day. The flower stem bending angle continued to increase on the fourth and fifth day, and was the greatest on the fifth day. The results indicated that the cut-flower stems were clearly bent on the third day during vase life. With an increase in time, the level of the bending of the cut-flowers increased.

3.1.2 Cell morphology of the dorsal and ventral stems

Microscopy of the dorsal and ventral stem cells of cut-flowers on the first- and fifth-days during vase life revealed no significant differences in the morphology of cells (Figures 2A, B). However, the dorsal stem cells were larger, and the ventral cells were smaller on the fifth day (Figures 2C, D).

3.1.3 Starch granules of the dorsal and ventral stems

Examination of the starch granules revealed that there were a large number on the dorsal and ventral stems of the water lilies during vase life (Figure 3). The distribution of starch granules on the dorsal and ventral stems on the first day was similar and primarily on the left side or below the cell (Figure 4). The distribution of starch granules on the dorsal and ventral stems changed over time. On the second day, the distribution of starch granules was different between the dorsal and ventral stems; however, the distribution of starch granules on the dorsal areas 1 and 2, and ventral areas 1 and 2 was essentially the same (Figure 4). On the third day, the distribution of starch granules in different areas of the dorsal and ventral stems was relatively concentrated (Figure 4). On the fourth day, the distribution of starch granules in different areas of the cell continued to change (Figure 4). By the fifth day, the starch granules were mostly concentrated on the right side of the cell (Figure 4).

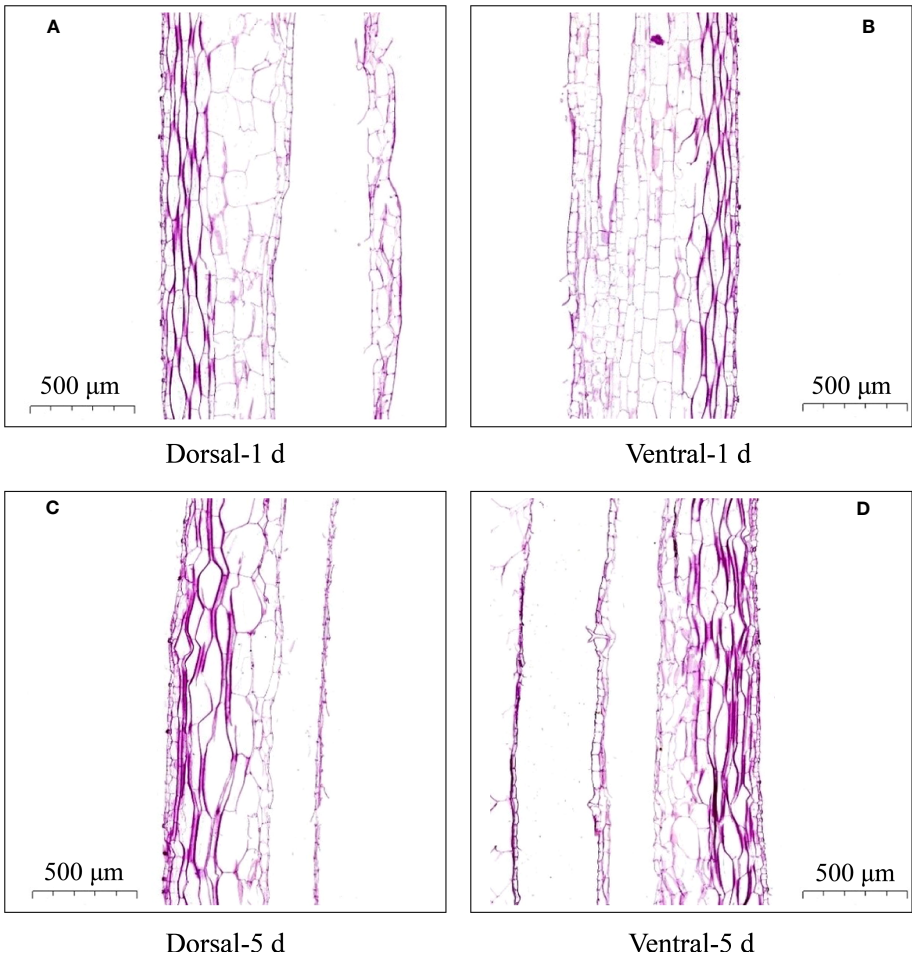

FIGURE 2
Cell morphology of the dorsal and ventral stems of the 'Blue bird' water lily. **(A)** dorsal stem on the first day, **(B)** ventral stem on the first day, **(C)** dorsal stem on the fifth day, **(D)** ventral stem on the fifth day.

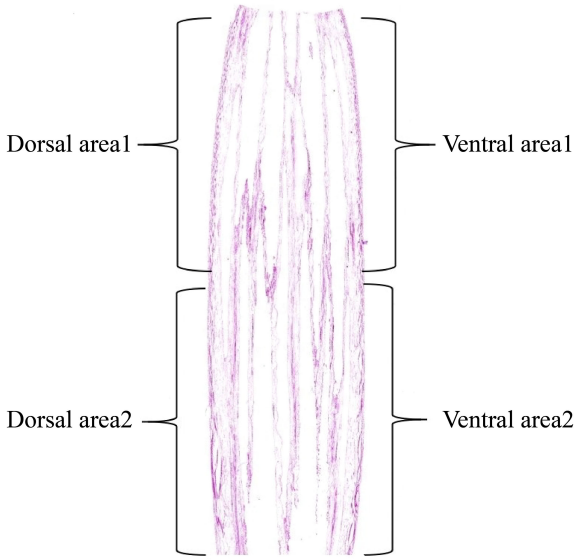

FIGURE 3
Division of the stem area in the 'Blue bird' water lily.

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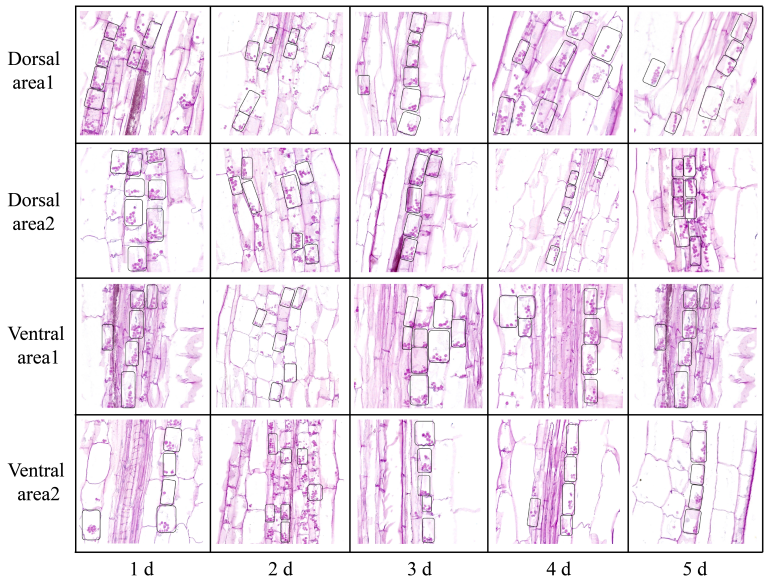

FIGURE 4
The intracellular distribution of starch granules in different areas of the dorsal and ventral stems of the 'Blue bird' water lily. The small black boxes are starch granules.

Figure 5 shows a schematic representation of the intracellular distribution of starch granules in the dorsal and ventral stems of the 'Blue bird' water lily cut-flowers. The starch granules distributed in the cells exhibited a 90° clockwise rotation from the first day to the fifth day during vase life, and the direction of rotation was consistent with the bending direction of the water lily stem (Figure 5). The intracellular distribution of starch granules in the ventral stems from the second day onward was different from that in the dorsal stems. Overall, a 90° clockwise rotation occurred in the intracellular distribution of starch granules from the first to fifth

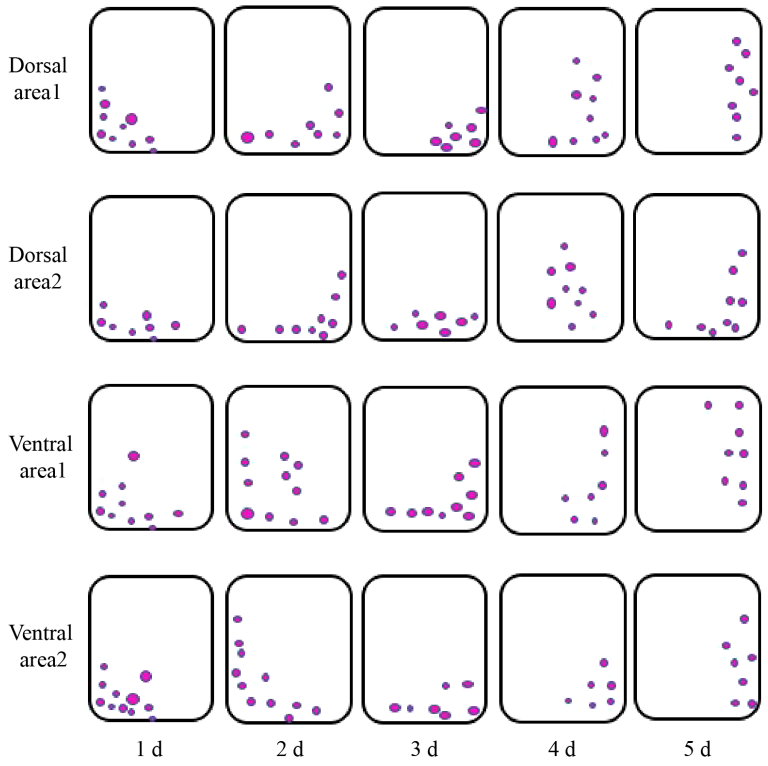

FIGURE 5
Schematic representation of the intracellular distribution of starch granules in different areas of the dorsal and ventral stems of the 'Blue bird' water lily.

day, which was consistent with the direction in which the stems appeared to bend (Figure 5).

3.2 RNA sequencing, gene functional annotation, and classification

To analyze the gene expression profiles in the dorsal and ventral stems of the 'Blue bird' water lily during stem bending, RNA-seq was performed using the Illumina platform. A total of six cDNA libraries, three from the dorsal stems and three from the ventral stems were constructed and sequenced. A total of 41.05 GB of clean data were obtained, and the ratios of Q20, Q30, and GC contents were greater than 97.77%, 94.23%, and 48.16%, respectively (Table S2). A total of 214.53 million reads were mapped. The proportion of reads that were mapped to unique locations and multiple locations in the genome were 0.06%–0.07% and 76.54%–79.61%, respectively (Table S3). This indicates that the quality of the transcriptome data for each sample was high and confirmed that our transcriptome data were accurate and reliable.

A total of 95,337 unigenes were generated, of which 42,866 were annotated against public databases including COG, GO, KEGG, KOG, Pfam, Swiss-Prot, eggnog, and NR, and 10,568, 25,432, 24,386, 26,807, 38,156, and 40,871 genes were annotated using the COG, KOG, Pfam, Swiss-Prot, eggnog, and NR databases, respectively. GO annotations (11,685 genes) and KEGG pathway annotations (14,902 genes) were also obtained to gain more insights into putative gene function (Table S4, Figures S2, S3).

3.3 Identification of DEGs

The DEGs in the comparison group Dorsal_vs_Ventral were analyzed. From the clustering heatmap in Figure 6A, it can be observed that the sample replicates exhibit good reproducibility. And 607 DEGs were identified, including 247 up-regulated genes and 360 down-regulated genes (Figures 6B, C, Table S5), of which 226 DEGs were annotated in the GO database. GO analysis, revealed the terms metabolic process, catalytic activity, cellular process, binding, and single-organism process as the top five annotations containing the largest number of genes (Figure 7A). KEGG analysis indicated that 212 DEGs were significantly associated with 61 metabolic pathways. The top five metabolic pathways were photosynthesis (27), carbon metabolism (23), photosynthesis-antenna proteins (21), plant hormone signal transduction (16) and amino acid biosynthesis (16) (Figure 7B).

3.4 DEGs involved in hormone signaling

A total of 23 DEGs involved in hormone signaling in Dorsal_vs_Ventral were identified, including auxin, zeatin (ZT), JA, ABA and BR metabolic pathways genes (Table 1). RNA-seq showed that the relative expression of other genes was increased in the Dorsal_vs_Ventral except *IAA26*, *AXX6B*, *ARG7*, *AHP*, *BR6OX2*, *LUT5*, and *CYP707A1* (Table 1). qRT-PCR revealed that the trend in expression of 15 genes was consistent with the transcriptome data, of which *AUX22D*, *SAUR*, *AXX15A*, and *AXX6B* were highly significantly different in the Dorsal_vs_Ventral. *AUX22D*, *SAUR*, and *AXX15A* were increased 11.90-, 30.69-, 11.73-fold in the ventral stems, compared with the dorsal stems, respectively, and *AXX6B* was decreased 9.10-fold. *IAA26*, *GH3.1*, *BZR1/2*, *BR6OX2* and *LOX2S* were not significantly different, whereas all other genes were significantly different (Table 1, Figure 8).

3.5 DEGs involved in gravity, starch granules, and Ca^{2+} signaling

A total of 12 DEGs were involved in gravity, starch granules, and Ca^{2+} signaling (Table 2). RNA-seq analysis revealed the DEGs *CML42* and *CML49*, which are associated with the major calcium

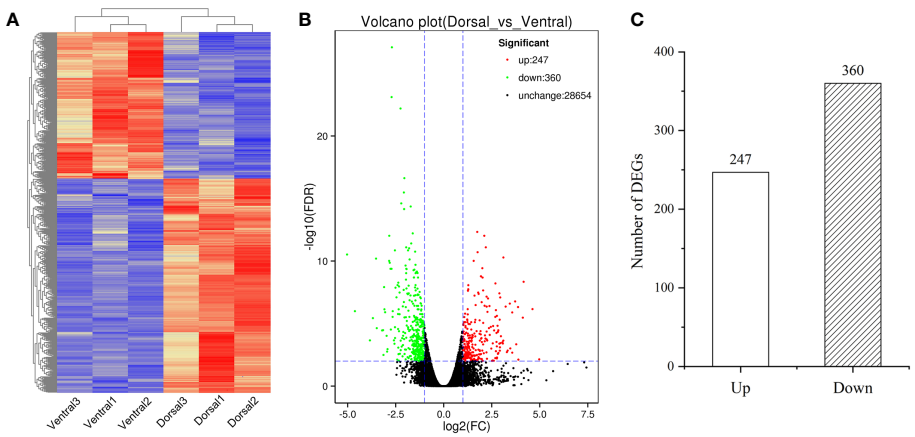

FIGURE 6
Transcriptomics analysis of the dorsal and ventral stems associated with 'Blue bird' water lily stem bending. **(A)** Heatmap of all DEGs. **(B)** Volcano plot of all DEGs with red representing the up-regulated genes, green representing the down-regulated genes, and black representing the genes that did not significantly change. **(C)** The number of up- and down-regulated genes in the Dorsal_vs_Ventral.

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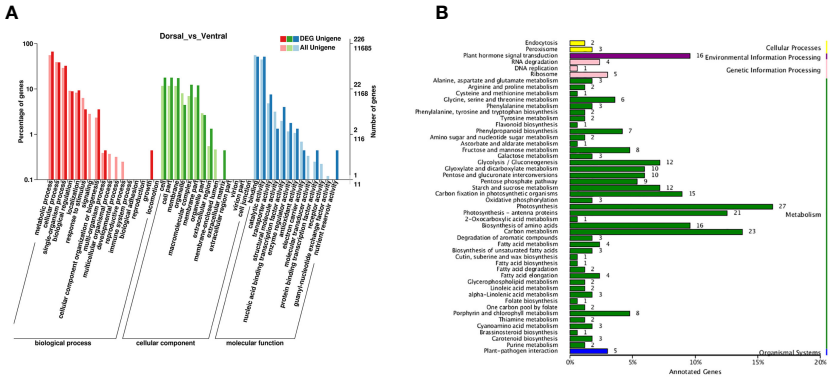

FIGURE 7
Enrichment analysis of DEGs. **(A)** GO enrichment analysis. The red, green, and blue columns indicate biological process, cellular components, and molecular function, respectively. **(B)** KEGG pathway enrichment analysis.

receptor CaM-like protein (CML) in plants, were up-regulated in the ventral stems, compared with the dorsal stems. KIC, a calcium binding protein functioning in trichomes was also up-regulated. The transcription factors WRKY and MYB, which are associated with the synthesis of lignin and celluloses and thus affect the thickness of secondary cell walls and the mechanical strength of plant stems, were also significantly differentially expressed. Of these, the expression of the WRKY genes *WRKY75*, *WRKY18*, and *WRKY40* was up-regulated. The expression of the MYB genes *MYB44* and *MYB330-like* was also up-regulated. *LPA2* senses the gravity signals by affecting the synthesis and sedimentation of starch granules in plants. And RNA-seq showed that its expression was significantly down-regulated in the ventral stems, compared with the dorsal stems (Table 2). The expression of six other genes was consistent with the transcriptome data; *LPA2* was down-regulated 2.13-fold, and all other genes were up-regulated 2.55–6.42-fold as determined by qRT-PCR (Table 2, Figure 9).

3.6 DEGs involved in photosynthesis, peroxidase, and ABC transporters

A total of 24 DEGs were involved in photosynthesis, peroxidase, and ABC transporters (Table 3). RNA-seq analysis revealed the expressions of *LHCA1*, *LHCA2*, *LHCA3*, *LHCA4*, *LHCA5*, *LHCB1*, *LHCB1-like*, *LHCB2*, *LHCB3*, *LHCB4*, *LHCB5*, *LHCB6*, *CAB1D*, *CAB1D-like*, and *CAB5* in pathways related to photosynthesis were significantly down-regulated in the ventral stems, compared with the dorsal stems. The expression of the peroxidase gene *HAO* and the ABC transporters genes were also down-regulated. The expression trend of

TABLE 1 DEGs and RNA-seq expressions involved in hormone signaling in the dorsal and ventral stems of the water lily.

Gene name	Gene ID	FDR	Log_2FC	up/down
IAA31	TRINITY_DN31826_c0_g1	0.000673	1.13	up
AUX22D	TRINITY_DN32832_c0_g1	0.001375	1.88	up
IAA16	TRINITY_DN34432_c0_g2	0.002277	1.28	up
IAA17	TRINITY_DN34432_c0_g4	0.003804	1.07	up
AUX22	TRINITY_DN38270_c1_g1	0.000469	2.05	up
IAA1	TRINITY_DN42468_c2_g1	2.09E-09	2.02	up
IAA26	TRINITY_DN40610_c0_g2	0.007946	−1.49	down
GH3.1	TRINITY_DN38483_c0_g4	0.000235	1.36	up
SAUR	TRINITY_DN30526_c0_g1	3.38E-06	4.12	up
AXX15	TRINITY_DN33080_c0_g1	0.001443	2.01	up
AXX15A	TRINITY_DN33931_c1_g2	0.007298	4.97	up
AXX15A	TRINITY_DN46640_c0_g4	0.000103	2.98	up
AXX6B	TRINITY_DN30678_c0_g1	3.01E-11	−5.03	down

(Continued)

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TABLE 1 Continued

Gene name	Gene ID	FDR	Log_2FC	up/down
ARG7	TRINITY_DN41116_c0_g1	0.004078	−1.77	down
AHP	TRINITY_DN43746_c1_g1	0.001355	−1.23	down
BZR1/2	TRINITY_DN46154_c0_g1	0.001924	1.14	up
BR6OX2	TRINITY_DN28579_c0_g1	0.000119	−1.38	down
LUT5	TRINITY_DN41861_c0_g2	3.17E-06	−1.44	down
CYP707A1	TRINITY_DN38908_c0_g1	0.000443	−1.43	down
LOX2S	TRINITY_DN43196_c0_g1	3.98E-07	2.38	up
AOS	TRINITY_DN30976_c0_g1	4.05E-05	1.92	up
OPCL1	TRINITY_DN41043_c0_g1	0.001989	1.53	up
CISZOG	TRINITY_DN43968_c0_g1	0.004797	1.81	up

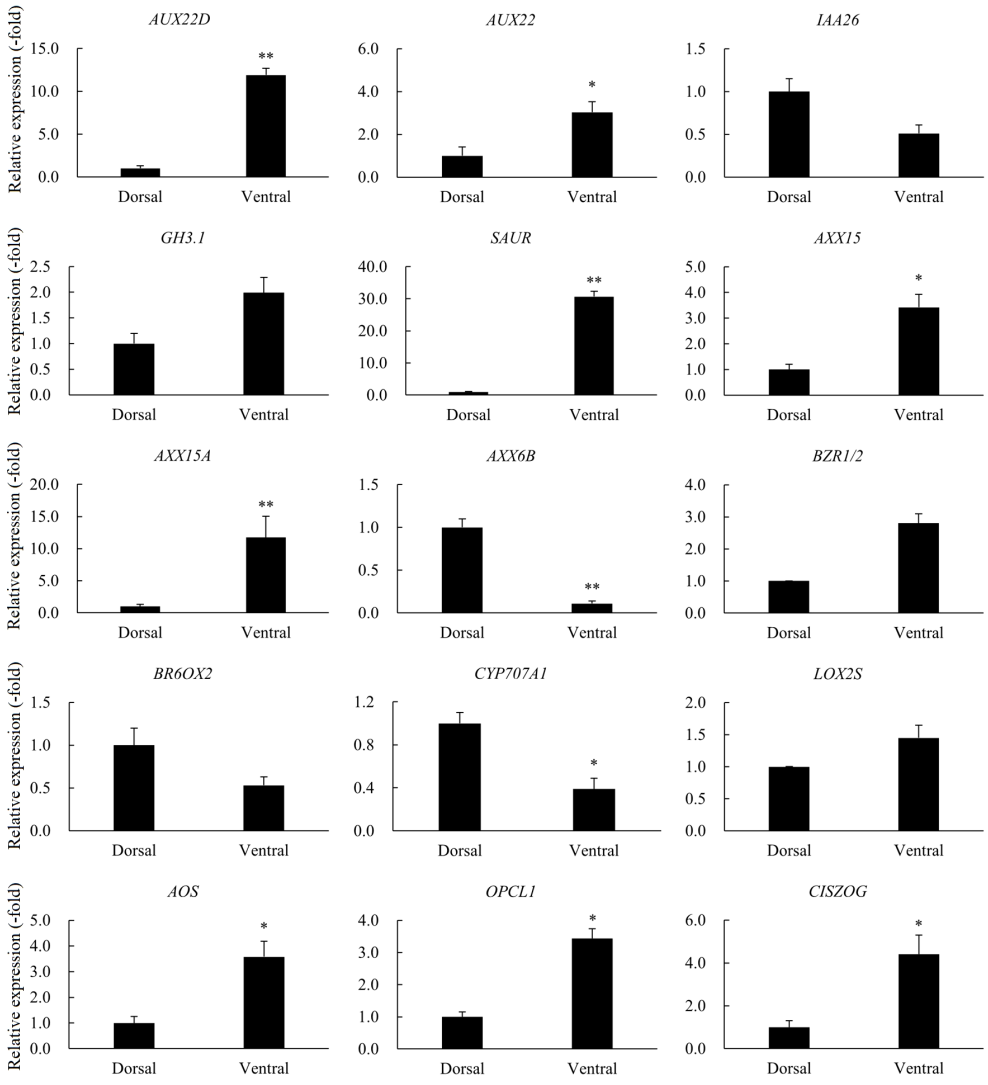

FIGURE 8
The relative expression of DEGs involved in hormone signaling in the dorsal and ventral stems of the 'Blue bird' water lily during stem bending, as determined by qRT-PCR. "*" and "**" represent significant difference level ($P< 0.05$ and $P< 0.01$) between the dorsal and ventral stems.

TABLE 2 DEGs and RNA-seq expressions involved in gravity, starch granules, and Ca^{2+} signaling in the dorsal and ventral stems of the water lily.

Gene name	Gene ID	FDR	Log_2FC	up/down
CML42	TRINITY_DN41755_c0_g3	0.00021	1.08	up
CML49	TRINITY_DN48382_c0_g1	0.000252	1.01	up
KIC-1	TRINITY_DN46389_c0_g1	0.000317	3.06	up
KIC-2	TRINITY_DN34835_c0_g1	1.11E-05	1.3	up
WRKY75	TRINITY_DN44747_c1_g1	0.008794	1.75	up
WRKY18	TRINITY_DN35911_c0_g2	0.000114	1.01	up
WRKY40-1	TRINITY_DN35911_c0_g1	0.002113	1.23	up
WRKY40-2	TRINITY_DN39344_c0_g1	7.75E-06	1.09	up
MYB44-1	TRINITY_DN40100_c0_g1	0.00654	2.2	up
MYB44-2	TRINITY_DN41812_c0_g1	9.57E-13	2.11	up
MYB330-like	TRINITY_DN31403_c6_g2	0.008376	1.72	up
LPA2	TRINITY_DN40026_c0_g1	4.84E-06	−1.38	down

nine other genes was consistent with that of the transcriptome data, and all genes were down-regulated 1.85–26.39-fold as determined by qRT-PCR (Table 3, Figure 10).

3.7 Changes in hormone concentrations in the dorsal and ventral stems

The levels of 15 hormones were measured in the dorsal and ventral areas of the 'Blue bird' water lily during stem bending, including indole-3-acetic acid (IAA), methyl indole-3-acetate (ME-IAA), 3-indolebutyric acid (IBA), indole-3-carboxaldehyde (ICA), N6-isopentenyladenine (IP), trans-zeatin (tZ), cis-zeatin (cZ), dihydrozeatin (DZ), methyl jasmonate (MEJA), JA, dihydrojasmonic acid (H2JA), jasmonoyl-L-isoleucine (JA-ILE), methylsalicylate (MESA), salicylic acid (SA) and ABA, using LC/MS/MS (Table S6, Figure S4). The results indicated that 12 hormones were detected, but 3 hormones (MEJA, H2JA, and MESA) were not detected (Figure 11). Of the hormones detected, the levels of ABA, SA, JA-ILE, ICA, IBA, ME-IAA, and IAA were relatively high. Moreover, the levels of ABA, ICA, JA-ILE, and JA were significantly different ($P< 0.05$) in the dorsal and ventral stems, with the levels in the dorsal stems being significantly higher than those in the ventral stems. There was a little difference in the levels of SA, cZ, tZ, DZ, IP, ME-IAA, IBA, and IAA between the dorsal and ventral stems (Figure 11).

4 Discussion

4.1 Effect of gravitropic response on stem bending

The gravitropic response of plants refers to a phenomenon in which plants perceive gravistimulation to relocate their own growth

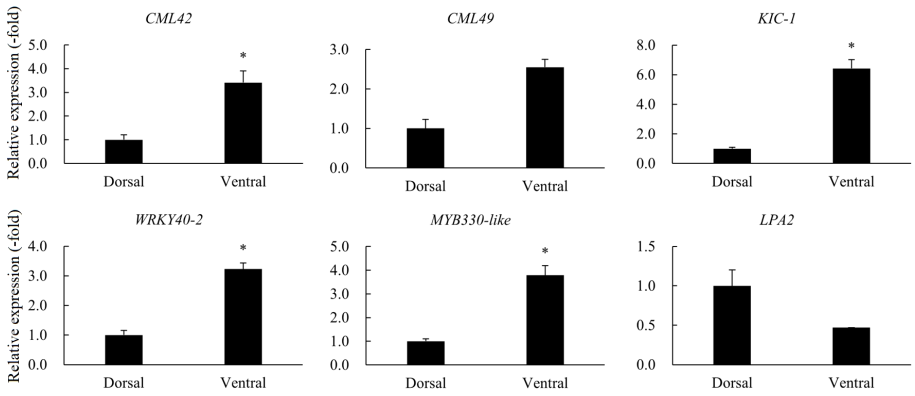

FIGURE 9
The relative expression of DEGs involved in gravity, starch granules, and Ca^{2+} signaling in the dorsal and ventral stems of the 'Blue bird' water lily during stem bending, as determined by qRT-PCR. "*" represents significant difference level ($P < 0.05$) between the dorsal and ventral stems.

TABLE 3 DEGs and RNA-seq expressions involved in photosynthesis, peroxidase, and ABC transporters in the dorsal and ventral stems of the water lily.

Gene name	Gene ID	FDR	Log_2FC	up/down
LHCA1	TRINITY_DN36720_c0_g1	1.41E-11	−1.86	down
LHCA2-1	TRINITY_DN33142_c0_g1	6.37E-23	−2.25	down
LHCA2-2	TRINITY_DN33142_c0_g2	5.37E-09	−2.37	down
LHCA2-3	TRINITY_DN37431_c1_g1	0.000147	−2.16	down
LHCA3	TRINITY_DN33438_c0_g1	0.000103	−1.46	down
LHCA4	TRINITY_DN38058_c1_g1	4.01E-08	−1.90	down
LHCA5	TRINITY_DN35522_c1_g2	0.000525	−2.12	down
LHCB1	TRINITY_DN34054_c0_g1	2.34E-05	−3.49	down
LHCB1-like1	TRINITY_DN38243_c1_g1	9.58E-05	−2.97	down
LHCB1-like2	TRINITY_DN39580_c0_g1	3.68E-06	−3.69	down
LHCB1-like3	TRINITY_DN49673_c1_g1	7.26E-07	−3.09	down
LHCB1	TRINITY_DN49673_c1_g2	3.30E-16	−2.07	down
CAB1D	TRINITY_DN49673_c1_g3	0.002611	−2.28	down
CAB1D-like	TRINITY_DN49673_c1_g7	8.01E-08	−2.46	down
LHCB2	TRINITY_DN38243_c1_g2	1.99E-05	−1.85	down
CAB5	TRINITY_DN49673_c1_g5	9.32E-09	−1.83	down
LHCB3	TRINITY_DN36703_c0_g1	0.000234	−2.24	down
LHCB4	TRINITY_DN31522_c0_g1	2.19E-05	−1.63	down
LHCB5	TRINITY_DN41916_c0_g1	3.84E-08	−1.82	down
LHCB5	TRINITY_DN41916_c0_g2	4.37E-15	−1.72	down
LHCB6	TRINITY_DN42125_c0_g2	3.99E-05	−1.80	down
ABC-1	TRINITY_DN33803_c1_g3	0.001748	−2.95	down
ABC-2	TRINITY_DN40514_c0_g1	1.77E-06	−1.11	down
HAO	TRINITY_DN44068_c0_g1	9.01E-05	−1.08	down

morphology and maintain the optimal angle of various organs in the direction of gravitropism (Firn and Digby, 1997; Perera et al., 2006; Song et al., 2021). Starch granule displacement and Ca^{2+} signals both affect the gravitropic response, because Ca^{2+}, as an important second messenger, is involved in various steps of the response (Aarrouf and Perbal, 1996; Philosoph-Hadas et al., 1996; Sinclair and Trewavas, 1997; Toyota et al., 2008; Zhang et al., 2011). In this study, the starch granules settlement in pedicel cells of water lily was consistent with the bending direction of the water lily stem, as well as the direction of gravity (Figures 4, 5), indicating the gravitropic response is an important cause of the stem bending of water lily cut-flowers.

Studies have shown that starch granules in endodermal cells stimulated by gravity precipitate along the direction of the stimulation. The *Loose Plant Architecture 1* (*LPA1*) gene can sense gravity signals by regulating the synthesis and sedimentation of starch granules in plants. *LPA1* mutation affects the perception of gravistimulation, resulting in a loose shoot architecture in rice, which is in contrast to the rather compact shoot system of the wild-type plant (Wu et al., 2013). Secondary signaling molecules, such as Ca^{2+}, are activated, which transform gravity signals into physiological and biochemical signals (Wu et al., 2013; Wu et al., 2016; Jiao et al., 2021). Kinesin-like calmodulin binding protein (KCBP) is a microtubule motor protein involved in the regulation of cell division and trichome morphogenesis. Reddy et al. (2004) have shown that calcium can bind to KCBP-interacting Ca^{2+} binding protein (KIC), then interact with KCBP and inhibits KCBP microtubule binding activity and microtubule stimulated ATPase activity. Poovaiah et al. (2013) suggested that calmodulin (CaM) and CaM-like protein (CML) are the primary calcium receptors in plants. The study found intracellular calcium receptor perception of changes in intracellular calcium ion concentration, in response to the NAC, MYB (such as MYB330), and WRKY (such asWRKY40) transcription factors, which affected the biosynthesis of the secondary cell wall, resulting in changes in lignin and cellulose accumulation. This affects the thickness of the secondary cell wall and attenuates stem mechanical strength (Tang et al., 2019). Lear et al. (2022) used RNA-seq to analyze gene

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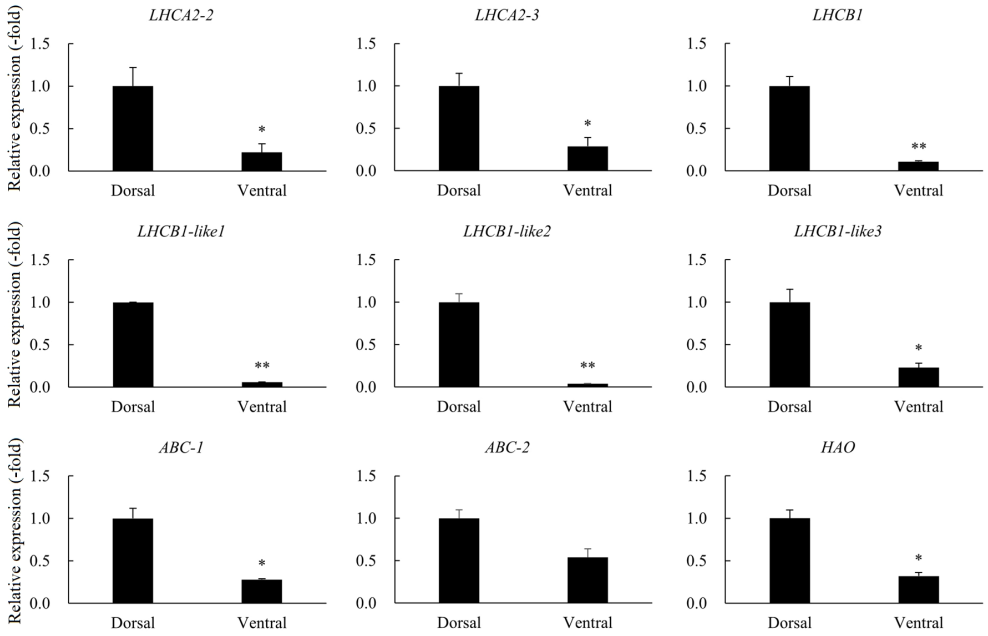

FIGURE 10
The relative expression of DEGs involved in photosynthesis, peroxidase, and ABC transporters in the dorsal and ventral stems of the 'Blue bird' water lily during stem bending, as determined by qRT-PCR. "*" and "**" represent significant difference level ($P< 0.05$ and $P< 0.01$) between the dorsal and ventral stems.

expression at three stages of pedicel necking in *Rosa hybrida* (straight,<90° and >90°) and found that more genes down-regulation than up-regulation during necking; moreover, most NAC and WRKY transcription factors involved in stress and senescence were up-regulated.

In this study, the calcium signal-related genes *CML* and *KIC* as well as some WRKY and MYB transcription factors related to the calcium signal-related genes were up-regulated in the 'Blue bird' water lily during stem bending (Figure 9). These results are in general agreement with those of Lear et al. (2022). At the same time the *LPA2*, which senses the effect of gravity by affecting the settling of starch granules in the dorsal and ventral stems, was down-regulated. These results indicated the starch granules settlement to the ventral parts of the cells activated the calcium signal-related genes and transcription factors, which resulted in an uneven distribution of auxin in the plant, and finally induced a curved growth of stem of water lily.

On the other hand, during the vase life, because of the heavy head of the water lily flower, the stem is squeezed against the bottle wall, resulting in different squeezing forces on the dorsal and ventral areas. This affects the growth and arrangement density of the cells in the dorsal and ventral areas during stem bending, which results in a

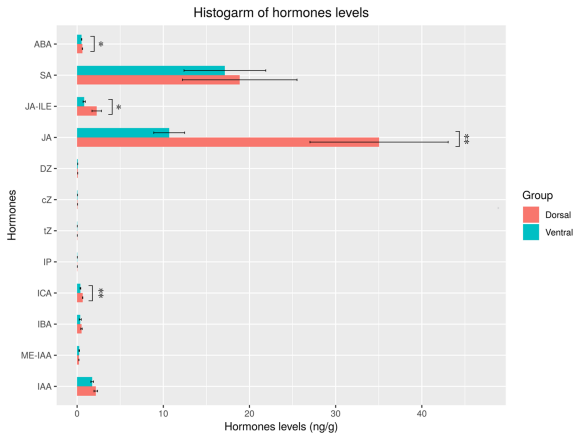

FIGURE 11
Hormone contents in the dorsal and ventral stems of the water lily. "*" and "**" represent significant difference level ($P< 0.05$ and $P< 0.01$) between the dorsal and ventral stems.

change in the mechanical force on the stem. Longitudinal paraffin sections prepared from bent stems showed no significant difference in the cell morphology of the dorsal and ventral areas on the 1st day. However, on the 5th day, the cell size and arrangement density in the dorsal and ventral areas were different (Figure 2). Thus, the change of cell morphology by gravity may also another cause of stem bending of water lily flower stem.

4.2 Effect of hormone signaling on stem bending

Plants perceive stimulatory signals and respond rapidly by remodeling intracellular processes and rebalancing the utilization of limited resources between growth and defense responses (Ning et al., 2017; Ye et al., 2018; Lakehal et al., 2019). Among them, phytohormones act as mediators that sense gravity. They are transported between tissues and accumulate in target tissues, causing an asymmetric distribution of phytohormones (Philosoph-Hadas et al., 2005). The Cholodny-Went model suggests that auxin is the primary mediator of gravitropism (Went and Thimann, 1937). In this study, as for the auxin, the content of ICA was significantly higher ($P< 0.05$) in the dorsal part than that of ventral part, while IBA, ME-IAA, and IAA were not significantly higher in the dorsal part, which could explain the pedicel bending because the dorsal part grew more quickly than the ventral part (Figure 11).

Studies have shown that other hormones act synergistically with auxin to participate in the gravitropic bending process (Wang et al., 2015). ABA, JA, GA_3, BR and CK have all been demonstrated to participate in the regulation of plant gravity response. The interactions among these hormones determine the overall response of the stem to external stimuli or internal signals, regulating the growth and development processes that influence stem bending. Study has shown that the IAA and ABA amounts were larger on the tension wood side than on the opposite wood side and in upright trees after 3 weeks of bending, and found that the distribution patterns of IAA and ABA might have important roles in tension wood formation (Kijidani et al., 2023). ABA can weaken the root gravitropic response under conditions of water stress (Taniguchi et al., 2010). MeJA was found to be a key phytohormone for determining the branching angle of *Arabidopsis* lateral roots, which is dependent on canonical JAR1-COI1-MYC2,3,4 signaling to incline lateral roots to a more vertical orientation. JA can synergistically interact with auxin, and light signals were found to enhance JA biosynthesis leading to the erect root architecture, whereas glucose induced wider branching angles (Sharma et al., 2022). GA_3 is typically involved in cell elongation in conjunction with IAA. Muday's research has shown that after gravity treatment, gibberellins exhibit uneven distribution on both sides of the stem reaction site, participating in the regulation of uneven growth along with auxin (Muday, 2001). BR biosynthesis and signal transduction play important roles in regulating plant agronomic traits, including plant height, leaf angle, grain size, and flowering (Tong and Chu, 2018; Nolan et al., 2020). BR can induce high expression of *PIN2*, facilitating polar auxin transport to establish concentration gradients, thereby accelerating the gravity response (Kim et al., 2000; Li et al., 2005). High concentrations of BR treatment inhibit plant gravity response, whereas low concentrations of BR can promote gravity response (Vandenbussche et al., 2011). Tian et al. (2021) found that the rice bHLH transcription factor OsBIM1 functions as a positive regulator in BR signaling and its overexpression significantly increases rice leaf angles by enhancing BR sensitivity and response. Low-concentration ABA increases leaf inclination in rice through a synergistic effect by inducing the expression of the BR-biosynthesis regulatory gene *OsGSR1*, to activate the BR signal in a fast, limited and short-term manner (Li et al., 2021). CKs are phytohormones involved in shaping rice architecture. Huang et al. (2023) used a phenotypic analysis to show that the leaf angle of a rice *OCSKX3* mutant was smaller, whereas the leaf angle of overexpressed lines (*OcSKX3-OE*) was larger. Histological sections showed that the changes were caused by an asymmetric proliferation of ganglion cells and vascular bundles, which indicates that enhancing CK levels in the lamina joint by disrupting *OsCKX3* negatively regulates leaf angle. In this study, in all phytohormones, the levels of ABA, JA-ILE, and JA were significantly different ($P< 0.05$) in the dorsal and ventral stems, suggesting that ABA and JA exert certain effects on water lily stem bending, but the specific mechanism still needs to be elucidated.

4.3 Expression of hormone-related DEGs on stem bending

Ge et al. (2019) found that the expression of many unigenes involving in signaling of phytohormones, such as auxin, CTK, GA, ABA, ethylene, BR, and SA showed significantly changes during the stem bending process of gerbera. In our study, 23 DEGs involved in hormone signaling in Dorsal_vs_Ventral were identified, including auxin, zeatin (ZT), JA, ABA and BR metabolic pathways genes (Table 1). *IAA26*, *AXX6B*, *ARG7*, *AHP*, *BR6OX2*, *LUT5*, and *CYP707A1* were down-regulated, and the other DEGs were up-regulated (Table 1). As for the auxins, previous studies have indicated that changes in auxin biosynthesis, transportation, and signal transduction lead to abnormal gravity, and finally changes in the branch angle (Roychoudhry et al., 2013; Roychoudhry and Kepinski, 2015; Zhang et al., 2018; Li et al., 2019; Ke et al., 2021; Zhu et al., 2021). Our study revealed differential expression of auxin-related genes in the dorsal and ventral parts of the water lily stem, such as the Aux/IAA proteins are short-lived transcription factors that repress early auxin response genes at low auxin concentrations (Liscum and Reed, 2002). *GH3.1* encodes IAA-amido synthetases, which help to maintain auxin homeostasis by conjugating excess IAA with amino acids (Staswick et al., 2005). Our transcriptome and qRT-PCR studies revealed that these genes presented significantly expression difference between the dorsal part and the ventral part, which were consistent with hormone contents (Table 1, Figure 8). Thus, we speculate that these differentially expressed genes may regulate stem bending in the water lily by regulating the expression of auxin, but further verification is needed for their specific functions.

Moreover, our study revealed that other plant hormones also exhibited differential expression between the dorsal and ventral parts of the water lily stem. For example, as for the BR-related DEGs, *BZR1* coordinates BR homeostasis and signaling by playing dual roles in mediating the downstream growth response and negative feedback regulation of BR biosynthesis (He et al., 2005). *BR6OX2* catalyzes the C6-oxidation step and lactonization during BR biosynthesis (Shimada et al., 2001). *LUT5* and *CYP707A1* are involved in carotenoid biosynthesis and ABA catabolism (Kim and Dellapenna, 2006; Saito et al., 2004). *LOX2S*, *AOS*, and *OPCL1* are involved in the biosynthesis of JA, thereby regulating a wide variety of growth development and defense-related processes (Royo et al., 1996; Koo et al., 2006). *CISZOG* is involved in the CK metabolic process and encodes zeatin o-xylosyltransferase, which is considered important for storage and protection against degradative enzymes (Martin et al., 1999). qRT-PCR validation was consistent with the transcriptome results, leading us to propose that these differentially expressed genes may play important roles in stem bending in the water lily by participating in the metabolism of plant hormones.

4.4 Effect of light on stem bending

The plant branching angle is regulated by several factors, including gravity stimulation, phytohormones, and the external environment, such as light and water (Bai et al., 2013; Roychoudhry et al., 2017; Sharma et al., 2022). The light-harvesting complex (LHC), as a type of photoreceptor, is involved in regulating plant growth and development (Wientjes et al., 2011). Light serves as a critical environmental signal, playing a significant role in shaping plant architecture. Our study revealed that the expressions of 15 DEGs (*LHCA1*, *LHCA2*, *LHCA3*, *LHCA4*, *LHCA5*, *LHCB1*, *LHCB1-like*, *LHCB2*, *LHCB3*, *LHCB4*, *LHCB5*, *LHCB6*, *CAB1D*, *CAB1D-like*, and *CAB5*) related to photosynthesis were significantly down-regulated in the ventral stems, compared with the dorsal stems (Table 3). Extensive research has shown that under different light conditions, plant growth direction and branching angles are induced through different pathways to varying degrees. For instance, studies have demonstrated that the *Tillering Angle control 1* (*TAC1*) plays a role in the lateral branch orientation of different plant species. Waite and Dardick (2018) found that *TAC1* expression is light-dependent, and *TAC1* overexpression partially prevents the reduction of the branch angle under dark or far-red light conditions. In addition, studies have found that changes in *TAC1* in other plant species are also related to upright tillers or branch angles (Jiang et al., 2012; Dardick et al., 2013; Hollender et al., 2018). Rice *OsPIL15* integrates light and gravity signals to negatively regulate the tiller angle in rice (Xie et al., 2019). Furthermore, a study found that ABC transporters function in the auxin transport phase of gravitropism (Strohm et al., 2012). Another study showed that ABC transporters (*abcb19*) hypocotyls respond to gravistimulation twice as quickly as wild-type plants, and they also exhibit an enhanced phototropic response (Noh et al., 2003).

Water lilies are very sensitive to light. During vase life, the pedicel ventral is close to the bottle wall, so the light intensity at the ventral area is lower than that at the dorsal area. This difference in light may also affect the growth of the dorsal area. In this study, many genes related to photosynthesis, carbon metabolism, and photosynthesis-antenna proteins were identified (Figures 7B, 10). The expression levels of photosynthesis-related genes in the dorsal area were significantly higher than those in the ventral area; these findings were consistent with those of a study by Lear et al. (2022), who found that photosynthetic, starch, and lignin biosynthesis genes were all down-regulated during pedicel necking in *Rosa hybrida*. This indicated that light response and photosynthesis in the pedicel ventral were significantly lower than those in the dorsal area due to weak light exposure. As a result, more photosynthetic products accumulate in the dorsal area, resulting in relatively stronger dorsal growth and stem bending. In summary, although in this study the differential expression of photosynthesis, peroxidase, and ABC transporters related DEGs in the dorsal and ventral area suggested they may play important roles in the development of water lily stems bending, the specific regulatory mechanism is still unclear, and the further verification of the functions of these genes should be carried out in the future.

4.5 Speculated pathways of DEGs regulating stem bending

Cai (2014); Kruse and Wyatt (2022) summarized the possible pathways of the direction of tropic movement relating to the direction of the stimulus, which can be induced by gravity, light, moisture, and chemicals. In this study, according to the results of stem bending at the transcriptional, metabolic and cellular level in water lily cut-flowers, we hypothesized that gravity, light and the uneven distribution of hormones affects cell growth in the dorsal and ventral parts, which causes stem bending (Figure 12). At the transcriptional level, genes related to plant hormone synthesis pathways, gravity, starch granules, Ca^{2+} signaling, and photosynthesis were differentially expressed in the dorsal and ventral parts of the bent water lily stems. Gene expression measured by qRT-PCR was consistent with the transcriptome sequencing results. At the metabolic level, significant differences in hormone content were observed between the dorsal and ventral areas, and ABA, ICA, JA-ILE and JA were significantly levels. At the cellular level, during vase life, the water lily stem was significantly bent, the cell morphology of the dorsal and ventral areas was changed, and starch granules in the bent stem cells were precipitated in the same direction as the bent stem and the direction of gravity. In conclusion, during vase life of water lily cut-flowers, the deposition of starch granules in the bent stem cells resulted in asymmetric auxin distribution and a change in the secondary cell wall, which affected the mechanical strength of the stem. Moreover, the higher weight of the water lily flowers head results in different forces on the dorsal and ventral areas, resulting in changes in cell size and arrangement density and affecting the mechanical strength of the stem. Uneven light exposure to the dorsal and ventral areas resulted in the downregulation of gene expression related to photosynthesis, differences in the accumulation of photosynthetic products, and growth rate, which contributed to stem bending.

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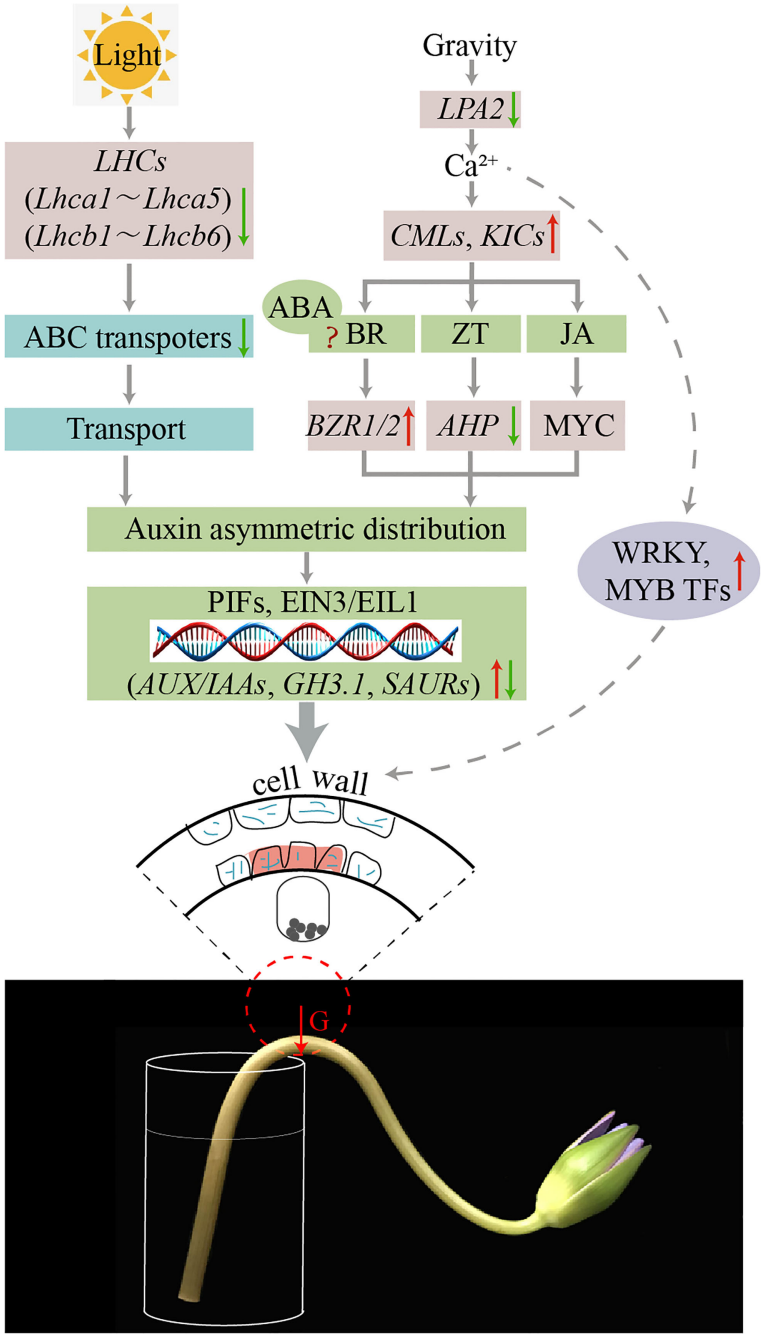

FIGURE 12
Schematic representation of putative pathways of DEGs that regulate stem bending in the 'Blue bird' water lily.

5 Conclusion

In this study, the DEGs identified in different areas of the water lily cut-flowers suggest transcription-based regulation of stem bending. This comparative transcriptome analysis focused on the pathways mediating the occurrence of cut-flowers stem bending including hormone signaling, gravity response, and light pathways. This was confirmed by the measurement of hormone levels in the stem and dynamic changes in cell morphology and starch granules. According to the results, the main possible causes of the bending of water lily cut-flower stems are the higher concentration auxin of ICA on the dorsal part to the ventral part due to gravity and light. In this progress, calcium signaling may play an important role as the second messenger. Therefore, auxin transport inhibitors and calcium channel blockers could be used to treat the cut-flowers, reducing the asymmetric distribution of auxin and relieving the bending phenomenon. At the same time, ABA, MeJA and JA also had the higher concentrations on the dorsal part compared to the

ventral part, so the antagonistic substances of these hormones such as gibberellin may also alleviate the bending phenomenon.

The results of this study can provide ideas for subsequent treatment of water lily cut-flowers with various fresh-keeping methods, but what kind of exogenous hormones can effectively prolong the vase life of water lily cut-flowers needs further research and will be the focus of our next research. Meanwhile, although the genes associated with stem bending have been identified and mapped in this study, the role of these genes in regulating stem bending needs to be elucidated in future studies. Ideally, it would be desirable to develop varieties less prone to stem bending through molecular breeding. Therefore, we expect that the findings of this study will be valuable for prolonging the occurrence of cut-flowers that experience early stem bending issues.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA934415 (to be released on acceptance).

Author contributions

JL, YS, YaZ, YiZ, XS, and JW participated in the experimental design and data analysis. JL, YS, HX, QL, and XL performed the experiments. JL, YS, and JW wrote the paper. All authors contributed to the article and approved the submitted version. JL and YS contributed equally to this paper.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1195389/full#supplementary-material

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Method for selecting ornamental species for different shading intensity in urban green spaces

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In urban areas, ornamental plants face different constraints, such as the shading of buildings and trees. Therefore, the selection of suitable species and their integration or combination with pre-existing plants is very important. Trees, shrubs, and herbaceous plant species must be distributed according to plant light requirements and shading intensity. Ornamental plants are classified into two groups based on their light intensity or shade tolerance: sun and shade species. To properly position the plants, especially in the immediate vicinity of buildings, it is necessary to study the projection of shadows during the year and the most critical periods, such as July and August. The position of ornamental species with different shading tolerances can be obtained by characterizing the leaf gas exchange for each species. Among the physiological parameters, the most important is the light compensation point, which is the light intensity corresponding to a net photosynthesis equal to zero. This means that the assimilation of carbon dioxide through photosynthesis is equal to the carbon dioxide emitted by respiration. This steady state represents the most critical condition for plants to endure the summer. The distribution of species inside a green area should be determined by considering the minimum light intensity that allows sufficient photosynthesis to compensate for the respiration rate. In this context, non-destructive leaf gas exchange, chlorophyll a fluorescence, and chlorophyll content can be useful tools for selecting suitable ornamental plants under diverse shading conditions.

KEYWORDS

light compensation point, shadow projection, shade plants, sun plants, chlorophyll *a* fluorescence, photosynthesis

1 Introduction

The selection of ornamental plants for building a green urban or peri-urban area is based on the aesthetic value of the plants and their ability to adapt to suboptimal conditions. Together with the experimental values, the level of maintenance requirements must also be carefully considered, particularly if the maintenance is under municipal administration. Plant maintenance includes watering, fertilization, and pruning (Aronson et al., 2017). Annual, perennial, woody shrubs, and tree species were also selected, considering their tolerance to biotic and abiotic stresses (Toscano et al., 2019). Ornamental plants are resistant to pests and diseases. This characteristic is important to reduce the need for pesticides and ensure that plants remain healthy and visually appealing. Moreover, treatment in urban areas is not permitted. The plants were selected based on their seasonal interest, including the flowering period, foliage color, and unique features, such as bark or berries. Select a combination of plants that will provide visual interest throughout the year, ensuring a vibrant and dynamic urban landscape. Urban environmental tolerance is therefore an important issue. Some species are more tolerant to urban conditions such as compacted soil, air or soil pollution, low nutrient availability, and limited space. In the selection of plants, it is also important to use non-invasive species that have the potential to escape cultivation and negatively impact native ecosystems (Leotta et al., 2023). Plants must be chosen based on their interactions with the inhabitants. In particular, plants must be safe; therefore, potential hazards associated with certain plants must be appropriately evaluated, such as thorns, toxic berries or leaves, or allergies, and plants that may pose a risk to people or pets in urban areas should be avoided. Selecting ornamental plants for urban green spaces requires careful consideration of various factors to ensure that the plants thrive and to enhance the aesthetic appeal of the area. The selection of plants can consider the most important factors that can compromise the ornamental value of the area over a long period. Under low maintenance management conditions, plants must tolerate the most frequent abiotic stresses (Francini and Sebastiani, 2019).

A combination of plants of diverse heights and those close to tall buildings should be carefully planned to avoid the negative effects of shading. Light intensity and duration are important parameters for photosynthesis and plant morphology (Cocetta et al., 2017). The lack of light can reduce the growth rate and ornamental quality. Accordingly, the combination and integration of plants must be conducted by considering the shading intensity created by buildings or other plants. Based on light requirements, ornamental species are classified as shade and sun (Bohning and Burnside, 1956). Shade plants do not require a large amount of light and grow underbrushes naturally. These plants have low light compensation points and are rich in chlorophylls. Sun plants require high light intensity and leaves with high light-compensation points. Light intensity must be sufficient to provide sufficient photosynthetic activity to accumulate sufficient sugar to satisfy basal metabolism.

The shadows in the green areas can be classified into three levels.

- Light or bright shade: The area of interest can be completely shaded for a few hours per day. The sun's rays are blocked from buildings or vegetation for several hours during the day, but there are hours when the area is exposed to the full sun.
- Partial shade: The area is shaded for most of the day, but at least early in the morning or evening, the plants are reached by the sun's rays.
- Complete or total shade: The area is shaded daily.

This classification system provides a simple and general guide for the selection and positioning of ornamental plants in the proposed green area.

2 Eco-physiological plant responses under urban environments

Light or shade intensity and temperature directly affect photosynthesis, whereas respiration is regulated only by temperature. The difference between photosynthesis and respiration represents net photosynthesis. Because photosynthesis produces sugars and respiration uses them, growth occurs when the balance between photosynthesis and respiration on a 24 h basis is positive. These physiological processes can change during different seasons, leading to the loss of ornamental quality. Therefore, they must be carefully studied to avoid positioning unsuitable plants in terms of adaptation to shade. The compensation point of different species could be a criterion for selecting ornamental plants for use in urban and peri-urban green areas. Based on these considerations, shade projections, which are the patterns of light intensity resulting from the shading of buildings and other objects, must be identified year-round, although most importantly, during spring and summer. Furthermore, temperature must also be accounted for, as net photosynthesis is temperature-dependent; increasing temperature enhances the respiration rate and reduces net photosynthesis (Evensen and Olson, 1992). Consequently, during the hottest weeks of summer, plants with a high light compensation point may produce insufficient carbohydrates for storage or growth, leading to plant death.

The relationship between the plant respiration rate and environmental temperature is well known. As the temperature increased, the respiration rate of plants also increased. This relationship can be explained by the basic principles of biochemical reactions as well as physiological and metabolic processes in plants (Atkin and Tjoelker, 2003). Plant respiration involves the breakdown of organic molecules such as sugars and carbohydrates to release energy for cellular activities. It occurs in both leaves and roots; therefore, soil temperature in urban environments is also a critical issue because of the low water availability, especially in the summer period. Several studies have investigated the relationship between plant respiration rate and temperature in urban environments (Lambrecht et al., 2016). The most evident effect was the altered phenology. Plants in urban areas

can affect the timing of life cycle events including flowering, leaf emergence, and senescence. Urban plants may exhibit earlier flowering or extended growing seasons because of higher temperatures or altered light conditions in cities.

Studies performed on *Crepis sancta* (L.) Babc. plants grown in urban and rural areas showed that plants in different environments differed in phenological, morphological, and physiological traits. Plants grown in urban environments exhibit delayed flowering and senescence (Lambrecht et al., 2016). Delaying the phenological stages can be a plant strategy to accumulate sufficient resources to allow seed production for survival purposes.

3 Ornamental plant selection and placement

3.1 Photosynthesis and respiration of ornamental plants

As established above, ornamental plants used in urban and peri-urban green areas should be adapted to shade and light conditions. Green shaded areas can be generated by buildings or other plants. As a general guide, shade-tolerant plants should be used in shaded areas and sun-tolerant plants in areas of full sunlight exposure. However, shade is not constant throughout the day or during the different seasons. Therefore, it is important to identify the shade intensity of trees and buildings around green areas will be planned. The correct selection of plants in the area should be carried out by matching the light compensation point of each species with the light intensity and photoperiod throughout the daily and seasonal cycles. Figure 1 illustrates the distribution of plants with different light compensation point values.

In summer, more extensive plant canopies and higher temperatures can reduce photosynthetic activity and increase respiration, resulting in a negative net photosynthesis. Under these conditions, plants used more energy than they accumulated over 24 h. Therefore, the selection of plants should account for this, ensuring that the amount of light is sufficient to compensate for the respiration rate within a 24 h interval. The problem is less acute in spring, as plants typically do not show symptoms of shading' however, with an increase in temperature, plants start to suffer from limited light conditions.

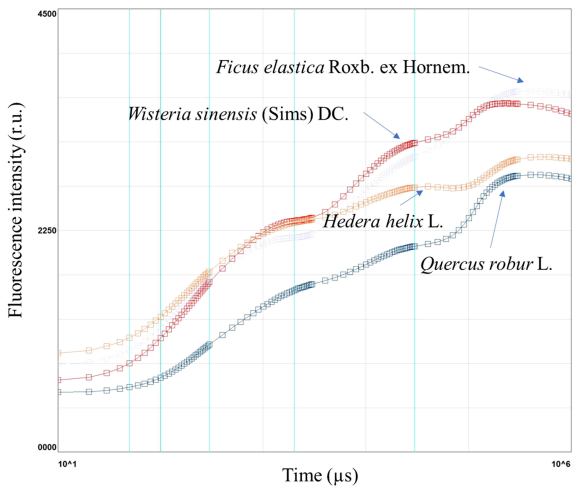

FIGURE 1
Chlorophyll *a* fluorescence induction curve in *Ficus elastica* Roxb. ex Hornem., *Wisteria sinensis* (Sims) DC., *Hedera helix* L., and *Quercus robur* L., in plants under different light conditions.

3.2 Chlorophyll *a* fluorescence measurement

Chlorophyll *a* fluorescence is a non-destructive measurement that allows the evaluation of light use efficiency, leaf functionality, and the overall leaf health status, under optimal or stress conditions. Chlorophyll *a* fluorescence is widely used to establish stressful conditions in plants. For example, it has been used to measure the chlorophyll a fluorescence in *Ficus elastica* Roxb. ex Hornem., *Wisteria sinensis* (Sims) DC., *Hedera helix* L., *Quercus robur* L.

The results showed that *F. elastica* had the highest induction curve, whereas *Q. robur* had the lowest. *W. sinensis* had a similar trend *F. elastica* and *H. helix* to *Q. robur* (Figure 1).

The JIP test allows the calculation of several indices from the data obtained from the data points (Tsimilli-Michael, 2020). These indices can help identify species with the highest variability. The performance index (PI), time to reach the maximal fluorescence (Tfm), and reaction centers per PSII antenna Chl a (RC/ABS) are the parameters that could greatly show the differences in plants under different light conditions (Figure 2). An expanded list of the JIP indices is provided in Supplementary Table 1.

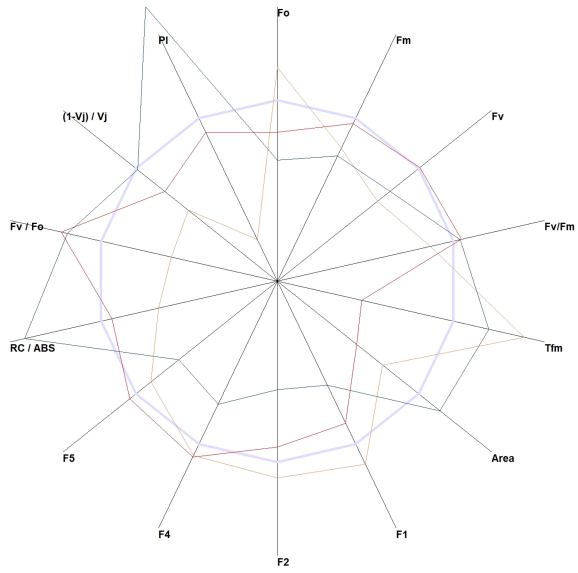

FIGURE 2
The JIP test-derived parameters were obtained from the elaboration of the chlorophyll *a* fluorescence induction curve. All parameters of *Wisteria sinensis* (Sims) DC. (red line), *Hedera helix* L. (orange line), and *Quercus robur* L. (green line) were standardized to *Ficus elastica* Roxb. ex Hornem (light blue line).

3.3 Adaptation of plants to light conditions

Plants can adapt to suboptimal conditions. In light compensation points of ornamental plants under low- or high-light conditions are reported in Table 1. Plants can alter their light compensation points by adapting to various environments. For example, *Pseudomonas scutellarioides* R. Br. reported an increase in the light compensation points in plants grown under high-pressure sodium lamps (HPS) or light-emitting diodes (LEDs) (Domurath et al., 2012). Lower compensation points are required for plants used in indoor environments, such as *Philodendron erubescens* K. Koch & Augustin and *Dracaena surculosa* Lindl. (Tan et al., 2017), whereas an analogous experiment simulating indoor conditions by lowering the light intensity resulted in reduced light compensation points in *Leea coccinea* Planch and *L. rubra* Blume ex Spreng. (Sarracino et al., 1992). Different light compensation points can also be observed in the same

TABLE 1 Light compensation points (LCPs) of different species and light intensities are recommended in the area during the day of the most critical season.

Species	Light compensation point (LCPs) (μmol m^{-2} s^{-1})	Light intensity recommended (μmol m^{-2} s^{-1}) × 12 h	Reference
Aeschynanthus longicaulis Wall. ex R.Br.	9.1–21.9	>11–24	Li et al. (2014)
Chlorophytum comosum (Thunb.) Jacques	10	>12	Torpy et al. (2017)
Epipremnum aureum (Linden ex André) G.S.Bunting	10	>12	Torpy et al. (2017)
Ficus benjamina L.	5.9	>6	Scuderi et al. (2012)
Gibasis Raf. spp.	10	>12	Torpy et al. (2017)
Heuchera americana L.	3.47–4.96	>4.2–5.9	Garland et al. (2012)
Leea coccinea Planch.	52–88	>63–106	Sarracino et al. (1992)
Leea rubra Blume ex Spreng.	67–84	>81–101	Sarracino et al. (1992)
Neomarica Sprague spp.	15	>18	Torpy et al. (2017)
Paeonia lactiflora Pall.	7–13	>8.4–16	Zhao et al. (2012)
Passiflora morifolia Mast.	36–39	>43–47	Pires et al. (2011)
Passiflora palmeri var. *sublanceolata* Killip	16–21	>19–25	Pires et al. (2011)
Passiflora suberosa subsp. *litoralis* (Kunth) Port.-Utl. ex M.A.M.Azevedo, Baumgratz & Gonç.-Estev.	27–32	>32–38	Pires et al. (2011)
Physocarpus amurensis (Maxim.) Maxim.	20	>24	Zhang et al. (2016)
Physocarpus opulifolius (L.) Maxim.	20	>24	Zhang et al. (2016)
Peperomia Ruiz & Pav. spp.	13	>16	Torpy et al. (2017)
Philodendron erubescens K.Koch & Augustin or *Dracaena surculosa* Lindl.	6–12	>7.2–14.4	Tan et al. (2017)
Philodendron xanadu Croat, Mayo & J.Boos	14	>17	Torpy et al. (2017)
Plectranthus scutellarioides R.Br.	15	>18	Domurath et al. (2012)

The recommended light intensity should be 20% higher than the LCP within the range of 12 h.

plant under different light-exposure conditions (Zhao et al., 2012). Leaves of *Paeonia lactiflora* Pall. exposed to the sun showed double the light compensation point compared with those under shade. This information can be useful when selecting plants for use in shaded locations, as adaptation may be used to lower the light composition of a species at the nursery level by applying different shading nets.

Aeschynanthus longicaulis Wall. ex R.Br., the morphological and photosynthetic responses were measured under high or low irradiance (Li et al., 2014). Under high irradiance, the photosynthetic photon flux density (PPFD) was maintained under 650 μmol·m^{-2}·s^{-1} by internal and external shading of the greenhouse. The light intensity of the low irradiance treatment was maintained under 150 μmol·m^{-2}·s^{-1} by extra shading on the bench (Li et al., 2014). The LCP of *Athyrium pachyphlebium* C. Chr. at four different shade ranges was 813 μmol m^{-2} s^{-1} to 886 μmol m^{-2} s^{-1}, 576 μmol m^{-2} s^{-1} to 633 μmol m^{-2} s^{-1}, 335 μmol m^{-2} s^{-1} to 402 μmol m^{-2} s^{-1}, 134 μmol m^{-2} s^{-1} to 175 μmol m^{-2} s^{-1} and 1,846 μmol m^{-2} s^{-1} to 1,914 μmol m^{-2} s^{-1}. The LCP under the highest light regime was 33 μmol m^{-2} s^{-1} to 16 μmol m^{-2} s^{-1} (Huang et al., 2011). Similar study was performed on *Passiflora morifolia* Mast., *P. suberosa* subsp. *litoralis* (Kunth) Port.-Utl. ex M.A.M.Azevedo, Baumgratz and Gonç.-Estev., and *P. palmeri* var. *sublanceolata* Killip (Pires et al., 2011). These ornamental plants showed different LCPs ranging from 36 μmol m^{-2} s^{-1} to 21 μmol m^{-2} s^{-1} under reduced light intensities of 25%, 50%, and 75% solar radiation for evaluating the adaptation ability (Pires et al., 2011). Artificial shade was provided using different shading nylon nets, fixed in wooden frames with dimensions of 5 m × 5 m × 2 m, under field conditions, which allowed a 25% reduction (1,000 μmol m^{-2} s^{-1}–1,400 μmol m^{-2} s^{-1}, min–max), 50% reduction (600 μmol m^{-2} s^{-1} –900 μmol m^{-2} s^{-1}), and 75% reduction (200 μmol m^{-2} s^{-1} –400 μmol m^{-2} s^{-1}) of solar radiation, along with a control treatment under full sunlight (1,500 μmol m^{-2} s^{-1}–1,900 μmol m^{-2} s^{-1}). The reduced light intensity by 75% allowed the LCP reduction in all three species and the highest plasticity was observed in *P. suberosa* subsp. *litoralis*, and *P. palmeri* var. *sublanceolata* (Pires et al., 2011). The shading effect of LCP and plant growth were studied in *Ficus benjamina* L. grown under five shading conditions during two growing periods (Scuderi et al., 2012). The control (0% shading) and 80% shading levels had light intensities ranging from 880 μmol·m^{-2}·s^{-1} to 142 μmol·m^{-2}·s^{-1} in the first growing period and from 1,531 μmol·m^{-2}·s^{-1} to 173 μmol·m^{-2}·s^{-1} in the second growing period. Under these shading regimes, the LCP of *F. benjamina* was 17 μmol m^{-2} s^{-1} and 5.9 μmol m^{-2} s^{-1} (Scuderi et al., 2012).

Two species, *Physocarpus amurensis* (Maxim.) Maxim. and *P. opulifolius* (L.) Maxim. were exposed to low (100 μmol m^{-2} s^{-1}) or high light intensity (1,000 μmol m^{-2} s^{-1} –1,500 μmol m^{-2} s^{-1}), and their LCP respected the two conditions. The LCP values were 26 μmol m^{-2} s^{-1} and 28 μmol m^{-2} s^{-1} for *P. amurensis* and *P. opulifolius*, respectively, under high-irradiance conditions. At low irradiance, both species have an LCP of 20 μmol m^{-2} s^{-1} (Zhang et al., 2016).

Shade and sun exposure conditions were studied in *Calophyllum inophyllum* L. (Clusiaceae), *Inga spectabilis* (Vahl) Willd. (Fabaceae), and *Ormosia macrocalyx* Ducke (Fabaceae). *C. inophyllum* and *I. spectabilis* under shade showed a LCP three times lower than plants exposed to sun. *O. macrocalyx* only halved the LCP when exposed to shade (Slot et al., 2019).

Several plants have been adapted to indoor conditions to evaluate the ability of some species to remove carbon dioxide from the environment (Treesubsuntorn and Thiravetyan, 2018). The LCP of these species ranges from 10 μmol·m^{-2}·s^{-1} to 15 μmol·m^{-2}·s^{-1} (Torpy et al., 2017).

Based on experimental or literature information (Ignatieva, 2021), plants can be planted in various green areas. If we consider plants with LCP 25 μmol·m^{-2}·s^{-1}, 50 μmol·m^{-2}·s^{-1}, and 75 μmol·m^{-2}·s^{-1} (Figure 3A) the possible planting position/zone in the area mapped for the shade/light intensity are reported in Figure 3B.

Considering the information reported in Table 1 in the shade area with light intensity below 10 μmol·m^{-2}·s^{-1} can be planted the *Heuchera americana* L. In areas comprised between 10 and 15 can be planted species such as *Chlorophytum comosum* (Thunb.) Jacques, *Epipremnum aureum* (Linden ex André) G.S.Bunting, *Ficus benjamina* L., and *Gibasis* Raf. spp. In areas with higher light intensities, the number of species that can be used greatly increased (Table 1). Of course, species must be selected based on the temperature of the geographical area in winter and these temperatures must be compatible with species tolerance.

4 Materials and methods

4.1 Protocol and procedures for the identification of LCP and chlorophyll *a* fluorescence measurements

Ornamental plants must be maintained under optimal nutrition and water conditions. The light saturation curve and light compensation point should be measured under specific light intensities that should be set from 1,000 μmol m^{-2} s^{-1} to 1,600 μmol m^{-2} s^{-1} for sun plants, 100 μmol m^{-2} s^{-1}–200 μmol m^{-2} s^{-1} for shade plants and of 500 μmol m^{-2} s^{-1}–700 μmol m^{-2} s^{-1} for intermediate conditions. The plasticity of plants to adapt to low light regimes must be evaluated by transferring plants under shading and reducing the light intensity using nets with a reduction of 25%, 50%, or 75% solar radiation. Measurements should be carried out after one week of adaptation.

4.2 Light saturation curve and light compensation point determinations

The light saturation curve and light compensation point of a plant can be measured using a leaf gas exchange analyzer. This instrument allows for the identification of the light compensation point and light intensity that allows the achievement of the highest net photosynthesis. The light saturation curve can be determined by measuring the CO_2 assimilation by decreasing the light intensity from 1,600 μmol m^{-2} s^{-1} to 0 μmol m^{-2} s^{-1}. The CO_2 concentration should be at ambient level (400 μL L^{-1}). Readings were taken

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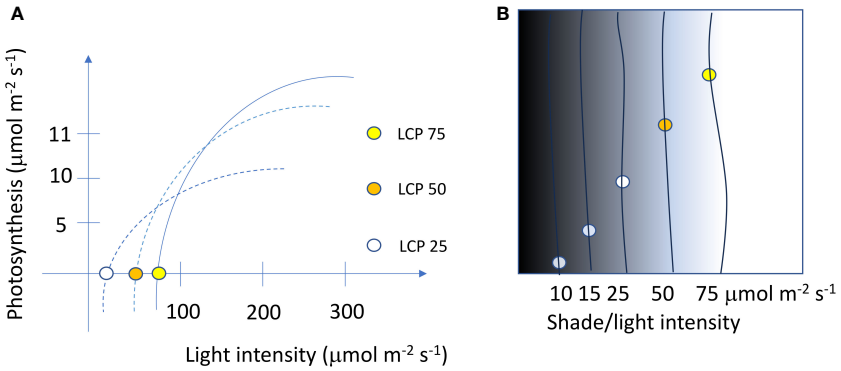

FIGURE 3
Light saturation curves and light compensation points (LCPs) of three different ornamental species **(A)** and the positioning of plants in different shade or light intensities that match the requirement of LCP on a 24 h basis **(B)**.

between 9:30 A.M. and 11:30 A.M. from the fully expanded leaves of the three plants.

4.3 Chlorophyll *a* fluorescence determination

Chlorophyll *a* fluorescence can be measured using a portable fluorimeter that allows the determination of plant health status, light-use efficiency, and plant adaptability to different light conditions. The chlorophyll *a* fluorescence induction curve was measured using a Handy Plant Efficiency Analyzer (PEA, Hansatech, UK). Chlorophyll *a* fluorescence can be measured in leaves that must be dark-adapted for 30 min by using a leaf clip (4 mm diameter). A rapid pulse of high light intensity of 3,300 µmol m^{-2} s^{-1} (600 W m^{-2}) was emitted on the leaf area shaded by the leaf clip (Figure 4). The sensor recorded the leaf fluorescence. Chlorophyll a fluorescence parameters, such as Fo (minimal fluorescence), Fm (maximum fluorescence), Fv (variable fluorescence), and Fv/F_M ratio (maximum quantum efficiency of photosystem II), were automatically calculated. JIP analysis can be performed to determine several indices related to light use efficiency and plant adaptation (Supplementary Table 1).

4.4 Distribution of plants in the green area

Based on the data collected regarding the light compensation point, it was possible to distribute the plants in the green areas. Light availability at the planting position must be higher than the light intensity of the light compensation point under the worst conditions, such as in summer with high temperatures.

5 Conclusion

The success of building a green area depends on many environmental factors including soil properties, temperature, relative humidity, and light or shade intensity. In particular, the low-light conditions that plants can be exposed to during the summer season must be considered. Correct ornamental plant species selection should be performed considering the light compensation point of the species and the shade intensity in the green area generated by tall trees or buildings.

FIGURE 4
Leaf clip used for dark incubation of leaf area. The leaf clip should be placed on fully expanded and mature leaves. The incubation period should be at least 40 min to allow complete oxidation of PSII.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author/s.

Author contributions

AlF: Writing – original draft. ST: Writing – review & editing. AnF: Conceptualization, Writing – original draft, Writing – review & editing. DR: Supervision, Writing – review & editing.

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Conflict of interest

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Supplementary material

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Effect of preharvest conditions on cut-flower quality

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The cut flower industry has a global reach as flowers are often produced in countries around the equator and transported by plane or ship (reefer) mostly to the global north. Vase-life issues are often regarded as linked to only postharvest conditions while cultivation factors are just as important. Here, we review the main causes for quality reduction in cut flowers with the emphasis on the importance of preharvest conditions. Cut flower quality is characterised by a wide range of features, such as flower number, size, shape, colour (patterns), fragrance, uniformity of blooming, leaf and stem colour, plant shape and developmental stage, and absence of pests and diseases. Postharvest performance involves improving and preserving most of these characteristics for as long as possible. The main causes for cut flower quality loss are reduced water balance or carbohydrate availability, senescence and pest and diseases. Although there is a clear role for genotype, cultivation conditions are just as important to improve vase life. The role of growth conditions has been shown to be essential; irrigation, air humidity, and light quantity and quality can be used to increase quality. For example, xylem architecture is affected by the irrigation scheme, and the relative humidity in the greenhouse affects stomatal function. Both features determine the water balance of the flowering stem. Light quality and period drives photosynthesis, which is directly responsible for accumulation of carbohydrates. The carbohydrate status is important for respiration, and many senescence related processes. High carbohydrates can lead to sugar loss into the vase water, leading to bacterial growth and potential xylem blockage. Finally, inferior hygiene during cultivation and temperature and humidity control during postharvest can lead to pathogen contamination. At the end of the review, we will discuss the future outlook focussing on new phenotyping tools necessary to quantify the complex interactions between cultivation factors and postharvest performance of cut flowers.

KEYWORDS

vase life, ornamental crops, pathogens, water balance, stomata, xylem architecture, carbohydrate starvation

Introduction

Vase life of cut flowers is the time flowers have a good appearance. Vase life depends on genotype, preharvest and postharvest conditions. End of vase life can be determined by discolouration of flowers, yellowing of leaves, wilting of leaves or flowers, bending of stems, abscission of flower-(petals) or leaves, presence of pathogens, turbid vase water, inflorescence blackening, drooping of the flower head, and often a combination of these processes (van Meeteren, 1992; van Doorn and Cruz, 2000). Although breeding has led to a spectacular variety in phenotypes, improving postharvest keeping quality is still a challenge. This is because harvesting leads to dramatic changes in environment; especially when plant products such as cut flowers, are transported and/or stored. Temperature, light, CO_2 level, air velocity and humidity change considerably, influencing photosynthesis, respiration, and transpiration.

The carbohydrate level of cut-flowers can be strongly influenced by cultivar, growth practices and postharvest treatments (Zieslin et al., 1975; Rajapakse and Kelly, 1995; Eason et al., 1997; Han, 2003; Kazuo et al., 2005; van Geest et al., 2017). A negative carbohydrate balance may lead to carbohydrate starvation, which may lead to induction of senescence. Hormone levels have a striking effect on flower longevity and senescence; important hormones such as ethylene, gibberellins, auxins, and abscisic acid have been implicated in flower longevity and keeping quality (Reid and Jiang, 2012). For example, ethylene can lead to flower senescence and leaf abscission, in climacteric species such as carnations and rose. Harvest is the onset of potential water loss and a reduction of water content in cut flowers (Halevy and Mayak, 1979; van Doorn, 1996). This occurs through continued transpiration through stomata: openings between two guard cells that control gas exchange between inner and outer environment of the plant. To prevent water loss, stomata have to close, triggered by environmental conditions (Fanourakis et al., 2013b). Pathogen pressure can shorten vase-life greatly, by negatively affecting the water balance, e.g., by bacteria in vase water (van Doorn, 1996). In addition, various phytopathogenic fungi, amongst others, *Botrytis cinerea* is an ever-present disaster risk that creates leaf spots, blight in leaves, stems, and flowers, sepal yellowing, and peduncle bending (Bika et al., 2021).

This mini review aims to provide an overview of the effects of cultivation factors on the (postharvest) quality of cut flowers. Better understanding of these effects will be useful to grow higher quality cut flowers and speed up the selection of new genotypes with improved quality characteristics. The aim of this mini review is not to describe cut flower quality issues in detail as many other reviews have described these in great detail and are referenced in the text where relevant.

Water balance

In cut flowers, the negative effects of water loss through transpiration are large when water uptake is limited. When a flower is cut, the water content of the flowering stem is at risk: water uptake through xylem is obstructed whereas evaporation through stomata may continue (Fanourakis et al., 2013b; Schouten et al., 2018a). Water content is an important factor for vase life of cut flowers. The water content is commonly measured by following the fresh weight over time, as well as the water uptake and transpiration of water (van Meeteren et al., 1999). Although end of vase life is not only dependent on water content, end of vase life is often correlated to the point where fresh weight drops below a certain threshold of the initial fresh weight (van Meeteren, 1992; van Meeteren et al., 1999). Genotype and environment during preharvest and postharvest are both important. The effect of preharvest factors such as harvest date, and cultivation conditions determine water balance between uptake and transpiration (Halevy and Mayak, 1979; Torre and Fjeld, 2001; van Meeteren et al., 2005).

Water transport occurs through the xylem: a water transport network of long, dead, tracheary cells that facilitates mostly transpiration but also is important maintaining the water content in the stem. It consists of vessels and tracheid, both interconnected through perforations in their common walls. The interconnection is achieved through pit pores; thinner portions of the cell wall that allow fluids movement. The rate of water transport, the hydraulic conductance (K_h), is proportional to the radius of the vessel (Zimmermann, 1983; van Doorn, 1996; van Doorn, 2012). Because water transport is facilitated by the xylem, blockage and resistance to water flow, can lead to a negative water balance and subsequent wilting (Aarts, 1957; Halevy and Mayak, 1979; Halevy and Mayak, 1981). Blockage occurs through microbial growth in the vase water, the occurrence of air emboli, or a wound response.

Microbial growth in the vase water causes xylem blockage limiting water uptake leading to premature wilting: addition of bacteria into vase water shortened vase life (van Doorn, 1996; Schouten et al., 2018b). Vase water bacteria likely originate from the hairy surface of the stem, and take advantage of carbohydrates that leak from wounded surfaces induced by harvesting and postharvest removal of leaves and thorns (Woltering, 1987; Put, 1990). Xylem architecture determines bacterial colonization as bacteria cannot pass the pit pores. Therefore, vessel length is a limiting factor. In rose, bacteria did not travel further than 50 mm from the cut end of the stem (Robinson et al., 2007).

Air emboli are the result of air entering the xylem at the cut end (van Doorn, 1990; van Meeteren, 1992) or higher up in the xylem caused by cavitation which results from strong negative xylem pressure due to strong transpiration. During harvest, the xylem vessels are cut from the root, exposing them to the air, which is sucked into the vessels due to the negative pressure in the xylem. This causes complete embolization. Air does not pass to adjacent non-cut xylem vessels because air cannot pass the pit membranes. The air is trapped between the entering water column and the pit membranes and high surface tension prevent water transport (Zimmermann, 1983; Nijsse et al., 2000; Nijsse et al., 2001; van Ieperen et al., 2001). Large diameter xylem vessels are more susceptible to air emboli than narrow ones. Smaller, less developed vessels are less susceptible to air embolism because they have smaller pit pores (Sperry and Tyree, 1988; Hargrave et al., 1994; van Ieperen et al., 2002). Cutting of the stem under water is the best way to prevent air emboli. Cold water is also

advised, because it reduces bacterial growth, and air has a higher solubility in cold water (van Meeteren, 1992). Reducing the surface tension by addition of surfactant can also reduce air emboli (van Doorn, 2012; Schouten et al., 2018a).

Mechanical damage of the tissue can induce a wound response: the deposition of materials such as gums and mucilage in the lumen of xylem vessels, and formation of tyloses on the cut stem surface (van Doorn, 1996). These water-uptake restricting depositions are considered a physiological (oxidative) wound response to cutting, depending both on peroxidase and catechol oxidase activity. Inhibitors of oxidative enzymes can prevent these responses (van Doorn and Cruz, 2000; Çelikel et al., 2011).

Removal of 2.5 to 5 cm of the stem improves water balance, removing air emboli, bacterial colonization, and wound induced depositions. However, there is hesitance to remove too much because stem length determines value (Moody et al., 2014). Also, further wounding of the tissue could lead to the additional biosynthesis of blocking depositions. Commercial vase solutions contain carbohydrates to prevent early senescence, and in addition, compounds that prevent bacterial growth, air emboli and wound response (Vaslier and van Doorn, 2003).

Role of cultivation on xylem architecture

For most cut flower quality related factors, the xylem architecture is important. Although wide vessels are responsible for most of the water transport, the presence of narrow shorter vessels is needed to overcome air emboli (Zimmermann, 1983; van Doorn, 1996). In grape, it was shown that bacterial growth is restricted in cultivars with more narrow xylem vessels (Chatelet et al., 2011). Nevertheless, dedicated experiments linking growth conditions with bacterial xylem blockage have not been carried out, perhaps due to the complexity of the interacting factors that not only affect the risk of cavitation but also affect the type of bacteria growing on stems (Carlson et al., 2015). Experiments with varying light intensity, and cultivation temperatures led to only minor changes in the ability to restore K_h after induction of air emboli. This was observed when conditions were rather extreme (van Meeteren et al., 2005). However, the water content of the root substrate was shown to have a significant effect on restoration of induced air emboli in chrysanthemum flowering stems. Without air emboli, stems grown at perlite with a 70% water content had a better K_h that those grown at 20% (van Meeteren et al., 2005). In grape and zinnia, the effect of reduced irrigation and water stress during growth was also shown to lead to reduced xylem diameter (Lovisolo and Schubert, 1998; Twumasi et al., 2005). This might be employed to optimize water transport during postharvest. However, effect of growth conditions on xylem architecture is anecdotal and limited to few species. The limiting factor is quick and non-destructive quantification of the xylem architecture. Recent work has demonstrated the visualization of xylem architecture with micro-CT images and ultrasound (Schneider et al., 2021; Wason et al., 2021; Dutta et al., 2022). Also helpful is that the xylem architecture can be simulated using water flow equations (Nijsse et al., 2001; van Ieperen et al., 2002; Couvreur et al., 2021). These new non-destructive phenotyping methods can be used to study plants grown under highly controlled growing conditions, allowing the study of the effects of cultivation on xylem architecture (Rosenqvist et al., 2019; van Delden et al., 2021).

Stomata

A stoma consists of two guard cells that control opening and closing of the stomatal pore by swelling and shrinking, respectively (Schroeder et al., 2001a). Stomata form the connection between internal leaf space and aerial environment around the leaf controlling CO_2 entry for photosynthesis and outflow of transpired water (Tallman, 2004). Stomatal guard cells are regulated by signals as diverse as light (spectrum, intensity, and photoperiod), relative air humidity (RH), temperature, air velocity, nutrition, leaf water status, and carbon dioxide concentration (Tallman, 2004; Reynolds-Henne et al., 2010; Feller and Vaseva, 2014). In addition, stomatal functioning is also regulated through abscisic acid (ABA) and nitric oxide (NO), and mediated by gene expression and protein activity (Assmann, 1993; Schroeder et al., 2001a; Schroeder et al., 2001b; Cutler et al., 2010; Kline et al., 2010; Assmann and Jegla, 2016; van Meeteren et al., 2020). Recent work in arabidopsis suggests an involvement of ethylene in stomata response, by tuning/accelerating stomatal conductance responses to CO2 and ABA (Azoulay-Shemer et al., 2023).

To reduce energy use in greenhouses, growers are stimulated to grow plants at high RH (>85% RH) (de Gelder and Dieleman, 2012; de Gelder et al., 2012; Marcelis et al., 2014). Growing at high RH often leads to malfunctioning stomata, meaning that they do not respond well to closing stimuli such as light-dark transition, ABA, or desiccation (Torre and Fjeld, 2001; Rezaei Nejad and van Meeteren, 2006; Fanourakis et al., 2011; Arve et al., 2013; Fanourakis et al., 2013a; Aliniaeifard et al., 2014). The consequence of this stomatal malfunctioning is a high transpiration rate after harvest leading to early onset of wilting (Lange et al., 1971; Fanourakis et al., 2012; Arve et al., 2013). A significant shortening (9-80%) of vase life in roses, bouvardia, and chrysanthemum was observed (Torre and Fjeld, 2001; Fanourakis et al., 2013a; Aliniaeifard and van Meeteren, 2016; Fanourakis et al., 2016; van Meeteren and Aliniaeifard, 2016; Aliniaeifard and Van Meeteren, 2018; Schouten et al., 2018a). Interestingly, depending on the species, exposure time to high RH leading to stomatal malfunctioning can vary from less than one day to more than four days (Aliniaeifard and van Meeteren, 2013). There is genotypic variation in stomatal malfunctioning, at least in arabidopsis and rose (Aliniaeifard and van Meeteren, 2014; Carvalho et al., 2016). In arabidopsis, there is a variation in sensitivity to growth at high RH; and in rose, a segregating population was phenotyped for stomatal responsiveness to which led to the identification of genomic regions (Carvalho et al., 2015). This suggests that breeding can be used to reduce the effects of stomatal malfunction.

Stomata respond strongly to light. Continuous light can worsen the negative effects of high RH in stomatal functioning in rose (Mortensen and Gislerød, 1999). High light can alleviate the

stomata function, cultivation at PPFD of >200 µmol·m^{-2}·s^{-1} led to more responsive stomata and decreased cuticular permeability (Fanourakis et al., 2019). It was shown that a dark period is important to develop functional stomata (Arve et al., 2013). Also, in well-watered plants, blue light suppressed signalling of ABA-induced stomatal closure and promoted stomatal opening (Boccalandro et al., 2012). A recent publication demonstrated that growing chrysanthemum plants under red light reduced postharvest water loss from leaves. Possibly, this is caused by generation of small/fast acting stomata during the growth of plants (Seif et al., 2021). At this time, it is not understood how high relative humidity causes stomata to lose the ability to respond to closing stimuli (Aliniaeifard and van Meeteren, 2014; Arve et al., 2014). In rose, multiple genes involved in ABA pathway form a highly complex regulatory network acting together towards tolerance to high RH (Carvalho et al., 2016). Plants grown at high RH have reduced ABA content through upregulated ABA catabolism genes, or increased derivatization, however this does not entirely explain the reduced response to ABA (Schwartz et al., 2003; Lee et al., 2006; Rezaei Nejad and van Meeteren, 2006; Seki et al., 2007; Giday et al., 2013; Arve et al., 2015).

Several strategies are known to alleviate of stomatal malfunctioning in plants developed under high RH: increased salinity, ABA application, soil water deficit, increased air movement, grafting, increased blue light, temporary increase of temperature and temporary decrease of RH (Fanourakis et al., 2016). Some of those strategies could have a direct implication in increasing the vase life of cut roses (Fanourakis et al., 2013a).

Carbohydrates

At harvest, cut flowers are excised from organs that are the source of carbohydrates: bulbs, tubers, roots, and stems. Cut flowers are required to metabolize and grow during the postharvest phase. Harvest and distribution often have a negative effect on the carbohydrate status of cut flowers. During transportation and storage, plant produce is usually kept in the dark in which photosynthesis is restricted, and the carbohydrate reserves are depleted by metabolic processes. Finally, during vase life, limited light, nutrient and water availability strongly impair photosynthesis, and carbohydrate availability is further reduced. These negative circumstances for maintaining carbohydrate reserves can strongly reduce postharvest quality of cut-flowers.

Carbohydrate starvation often leads to senescence-like symptoms, such as flower wilting, loss of chlorophyll in leaves and loss of chlorophyll and colour in flowers (van der Meulen-Muisers et al., 2001; Han, 2003; Ichimura et al., 2003; Buchanan-Wollaston et al., 2005; Kazuo et al., 2005; Trivellini et al., 2012; van Geest et al., 2016). However, carbohydrate starvation also leads to symptoms that are not observed during normal developmental senescence such as leaf blackening and reduced flower opening (Bieleski et al., 1992; van der Meulen-Muisers et al., 2001; Han, 2003; van Geest et al., 2016). The molecular pathways associated with developmental senescence and carbohydrate-starvation induced deterioration have many analogies, but there are also essential differences (Buchanan-Wollaston et al., 2005; Trivellini et al., 2012). It can therefore be difficult to separate postharvest problems associated with carbohydrate starvation or developmental senescence, because they might have the same initial trigger (van Doorn, 2004).

In chrysanthemum, cultivar differences in carbohydrate content of disk florets are related to susceptibility to disk floret degreening, with high light increasing the carbohydrate content of disk florets (van Geest et al., 2017). Choice of cultivar and light intensity can therefore significantly affect postharvest performance through carbohydrate content. Also altering the sink-source balance, by pruning, can strongly affect the amount of storage carbohydrates, and with that the postharvest performance (Zieslin et al., 1975; Kool et al., 1996).

Carbohydrates play an important role in the postharvest performance in cut-flowers; the addition of sugars to the vase water is usually one of the most effective measures to improve vase-life. Cut flowers that can better cope with carbohydrate starvation during the postharvest chain will be less perishable. This notion brings interesting opportunities to improve postharvest performance by breeding, improved growing practices and postharvest technology.

Botrytis

B. cinerea ('grey mould') is a constant and costly threat to the ornamental industry with various attack modes, and the ability to survive in favourable and unfavourable conditions (Elad, 2016). Even some *B. cinerea* species that grow as harmless endophytes might turn into 'necrotrophic thugs' due to increased inbreeding and reduced genetic diversity in ornamental crops (van Kan et al., 2014). In addition, *B. cinerea* has the ability to quickly develop fungicide resistances (Li et al., 2014). *B. cinerea* isolates were found resistant to many single, and sometimes, several classes of fungicides in commercial rose shipments (Muñoz et al., 2019). Biological control agents such as fungi or bacteria are also alternatives as they disturb *B. cinerea* hyphae development and induce systemic resistance (Zhao et al., 2018; Calderón et al., 2019; Nakkeeran et al., 2020; South et al., 2020; Motlagh and Jafari, 2022).

Managing *B. cinerea* during ornamental production starts with proper sanitation protocols. Infections in cut roses has been correlated with the spore density in glasshouses with dead leaves as sources of inoculum (Kerssies et al., 1995; Dik and Wubben, 2007). The other key issue is to limit dew point temperatures to prevent free water on the crop by e.g., drip irrigation, reducing plant density and avoiding harvest on rainy days (Daughtrey and Benson, 2005; Elad, 2016). Fortification against *B. cinerea* is also a good strategy, by e.g., calcium sprays. Fortification against *B. cinerea* can also be elicited by phytohormones such as brassinosteroids, salicylic-, jasmonic- and abscisic acid (AbuQamar et al., 2017; Liu et al., 2018; Shafiee-Masouleh, 2018; Bennett et al., 2020). An interesting, additional tool, for *B. cinerea* management is the use of UV-C radiation. UV-C has shown to limit *B. cinerea* development in gerbera and freesia flowers (Darras et al., 2010). UV-C has recently been applied commercially in rose greenhouse

cultivation as an end of day treatment and during sorting. It is likely a *B. cinerea* containment strategy can only be attained by both fortifying the ornamental crop and limiting the number of spores, either dormant or active, in a systemic management approach (Bika et al., 2021). Perhaps the toughest hurdle to overcome is that treatments published in literature are not necessarily effective in the ornamental industry due to ever changing cultivars, cultivation practises and global chains.

Conclusions and future perspectives

The quality of cut flowers is difficult to predict because it is dependent on several interacting processes that depend on carbohydrate status, xylem architecture, stomatal behaviour, and microbial (botrytis) pressure; factors that are all shaped during cultivation. The question is whether the continuing accumulation of knowledge of important processes that shape quality is currently sufficient to provide a strategy to create the best conditions for the best cut flower quality. We mention a number of important factors; (1) good hygiene is important both for pathogenic and nonpathogenic microorganisms, (2) light conditions determine photosynthesis and the carbohydrate status, and (3) the importance for RH control is emphasized because high RH could lead to stomatal malfunction.. We feel that we are currently not able to do provide such a strategy and would like to emphasize gathering more information during the cultivation phase, for instance by embracing new phenotyping tools. For example, the influence of root substrate on xylem architecture is not well understood, and more research is needed. The use of novel non-destructive methods opens new possibilities in this topic. Recent developments in new phenotyping tools such as xylem architecture measurements with sound, and with 3D high-resolution X-ray micro-computed tomography (micro-CT) images have recently been reported (Wason et al., 2021; Dutta et al., 2022).

Complete control of growth conditions does not necessarily improve quality; vase life of zinnia grown in natural photoperiods from May to June was better than that of those grown under artificial long days in the greenhouse in February and April (Stimart and Brown, 1982). During field production, plants are grown under varying environmental conditions from spring to fall and this influences quality (Kalinowski et al., 2022). Dry periods can be good for xylem architecture, and high light improves carbohydrate deposition, although dynamic control of these factors is possible in protected horticulture.

Recent developments such as increases in gas prices and the implementation of LED lights to replace traditional HPS lamps in greenhouses can lead to changes in cut flower quality, especially in winter. Lower quality could be caused by a lower carbohydrate load, caused by a reduced photosynthesis, and reduced growth under lower temperatures. However, it is also possibly due to stomatal malfunctioning in plants developed under high RH. Strategies to prevent this, are difficult to implement in horticultural practices (e.g., ABA application is expensive) and therefore a more permanent solution is required. Identification of the molecular mechanism that leads to stomatal malfunction can lead to genetic markers that can be used to select for better stomatal function. A better understanding of optimal conditions for quality would allow for the application of dynamic growth conditions that yield this quality.

To study the complex effects of preharvest conditions on quality, illustrates the need for more rapid and reliable phenotyping methods. Hydration status might also be evaluated non-destructively by applying spectral information and artificial neural network. The water status at harvest of leaves of two ornamental species (*Spathiphyllum wallisii, Chrysanthemum morifolium*) was analysed by multi spectral imaging (Fanourakis et al., 2023). During growth, it might be possible to monitor stomatal function and crop health and performance using thermal cameras, or by spectral imaging (Stamford et al., 2023). Thermal camaras could be used to monitor stomatal behaviour and photosynthetic activity (Vialet-Chabrand and Lawson, 2020). In addition, currently, the development of complete controlled growth conditions in conditioned environmental agriculture and vertical farms, as well as monitoring of the plants during their growth has grown tremendously (van Delden et al., 2021). These high tech growth conditions offer the possibility to study the effect of growth conditions on postharvest quality in more detail (Rosenqvist et al., 2019; Hall et al., 2022).

Author contributions

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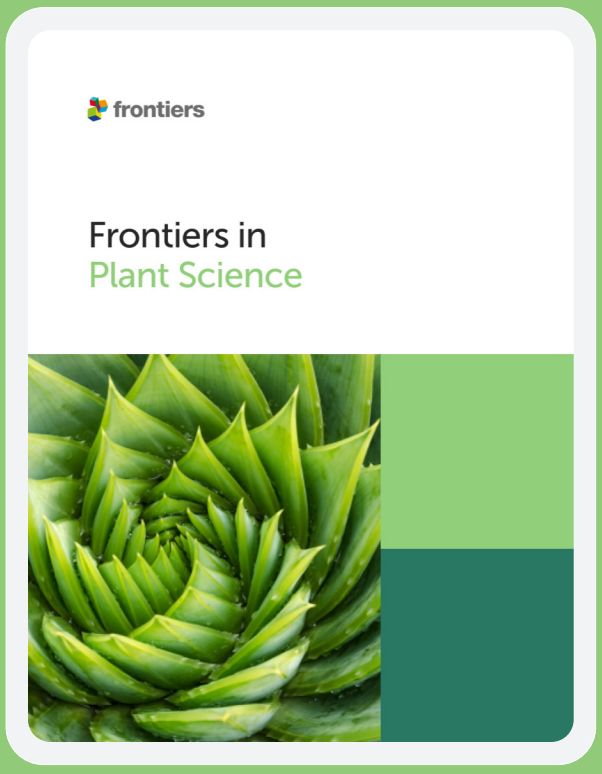

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