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ARTICLE

Expression Analysis and Functional Validation of Lily *LoWRKY22*

Ling He, Shun Tao, Qian Wang, Yu-Pei Yin, Xin-Yu He, Shuo Shi and Chun-Yan Wang*

College of Horticulture and Landscape Architecture, Jinling Institute of Technology, Nanjing, China
*Corresponding Author: Chun-Yan Wang. Email: wcy@jit.edu.cn

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ABSTRACT: As essential regulatory proteins, WRKY transcription factors participate in the regulation of plant growth, development and stress resistance; however, the functions of *LoWRKY22* in the 'Siberia' cultivar of *Lilium* remain uncharacterized. In this study, *LoWRKY22* was cloned and subjected to comprehensive functional analyses. Phylogenetic analysis revealed that *LoWRKY22* belongs to the WRKY-IIe type subgroup, featuring a conserved WRKY domain and a C2H2-type zinc finger motif, indicating evolutionary conservation with WRKY homologs from *Arabidopsis thaliana*. Subcellular localization and transactivation assays confirmed its nuclear localization and transcriptional activation activity, supporting its role as a transcriptional regulator. Structural characterization of the *LoWRKY22* promoter identified multiple *cis*-elements responsive to light, low temperature, and gibberellin, suggesting its involvement in environmental and hormonal signaling pathways. Tissue expression analysis demonstrated predominant expression of *LoWRKY22* in leaves, consistent with its proposed roles in photosynthesis- and senescence-related processes. The heterologous overexpression of *LoWRKY22* in *Arabidopsis thaliana* induced early flowering, reduced plant height, and accelerated silique maturation relative to those in wild-type plants. Quantitative real-time polymerase chain reaction (PCR) revealed that *LoWRKY22* overexpression significantly upregulated senescence-associated genes (*AtWRKY53*, *AtSAG12*, and *AtSAG13*) and flowering-promoting genes (*AtFT* and *AtAP1*), while downregulating the floral repressor *AtFLC*. These findings indicate that *LoWRKY22* functions as a positive regulator of flowering and senescence, potentially coordinating the transition from vegetative to reproductive growth via the modulation of key regulatory networks. Collectively, this research firstly identified and explored the functions of *LoWRKY22* in the'Siberia' cultivar of *Lilium*, elucidating its regulatory role in growth-phase transitions and establishing a molecular foundation for its potential utilization as a candidate gene in genetic breeding programs aimed at optimizing growth, flowering time, and developmental traits in lilies.

KEYWORDS: *Lilium* 'Siberia'; *LoWRKY22*; WRKY transcription factor; flowering; senescence; *Arabidopsis*; heterologous transformation; phylogenetic analysis

1 Introduction

Lilium spp., belonging to the Liliaceae family, comprise over 110 species that are predominantly distributed across the temperate and frigid regions of the Northern Hemisphere. China represents a principal center for the cultivation and production of lilies [1]. Owing to their diverse colors and distinctive floral morphologies, lilies possess significant ornamental value and are consistently ranked among the five most commercially important cut flowers worldwide. They are extensively utilized in horticultural cut-flower production and indoor decoration, and also exhibit considerable medicinal and edible potential. In China, lilies are cultivated extensively and utilized across diverse applications, supported by a broad consumer market [2]. At present, driven by technological innovation and supportive policy frameworks, the industry is increasingly focusing on the ‘Siberia’ cultivar, accompanied by steady growth in market demand and the increasing diversification of consumption scenarios.

Functional elucidation of the genes in *Lilium* sp. is essential for understanding the molecular mechanisms underlying plant growth and development, stress tolerance, and flower color regulation. In recent years, numerous studies have employed gene cloning techniques to isolate members of the WRKY, MYB, bZIP, and NAC transcription factor families from diverse lily cultivars, with primary emphasis on gene isolation, expression profiling, and functional characterization [3–10]. The identified transcription factors, including LoMYB21 [3], LoTDF1 [4], LiWRKY33 [5], LibZip11 [6], LpNAC48 [7], LdHSFB2a [8], and LzSCL9 [9], have been shown to regulate plant growth and stress responses. Functional studies on *Lilium* sp. genes have predominantly focused on transcriptome analyses, flower development, floral color regulation, and stress tolerance. By employing Virus-Induced Gene Silencing (VIGS), He et al. investigated gene function in the ‘Siberia’ cultivar of *Lilium* and elucidated the mechanism underlying anther dehiscence [3]. By analyzing the physicochemical properties and expression patterns of the protein encoded by *LoTDF1* in the ‘Siberia’ cultivar, Sui et al. elucidated its role during the pollen mother cell stage [4]. During trichome development in *Lilium pumilum*, the bZIP transcription factor LpbZIP29 binds to the promoter region of *LpNAC48* to activate its expression and promote trichome formation [7]. Anthocyanin biosynthesis has also emerged as a key research focus in lilies, and recent findings by Yang et al. demonstrated that *LhERF061* represses anthocyanin synthesis via downregulating *LhMYBSPLATTER* and *LhDFR* transcription levels, as well as by interacting with *LhMYBSPLATTER* [10]. Additionally, studies on stress tolerance in lilies have identified a *WRKY–HSF* regulatory module involving *WRKY33* and *HSFB2a*, which enhances stress resistance by regulating the expression of heat-responsive genes, suppressing cell death, and reducing the accumulation of reactive oxygen species (ROS). Furthermore, whether trichokonins were applied or not, *LzSCL9* expression under heat stress showed a strong positive association with *LzHsfA2a-1*, a well-documented pivotal regulator in trichokonin-enhanced high temperature tolerance [9]. Collectively, these studies underscore the critical roles of transcription factors in growth, development, and stress tolerance in lilies. However, reports on transcription factors that simultaneously modulate plant growth and development as well as stress resistance remain scarce.

Previous studies have demonstrated that *LlWRKY22* from *Lilium longiflorum* positively regulates plant tolerance to heat, salt, and osmotic stresses, functioning as a broad-spectrum abiotic stress-responsive factor [11]. Wu et al. found that LlWRKY22 activates *LlDREB2B* expression through targeting two consecutive W-box sequences in the promoter. Meanwhile, it regulates its own transcription by binding to its native promoter, which constructs a positive regulatory cycle and further strengthens high-temperature stress tolerance [12]. Furthermore, *WRKY22* is widely acknowledged as a marker gene for pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) in model plant species [13], and its upregulation under dark conditions induces leaf senescence [14]. In contrast, the overexpression of *BrWRKY22* in *Brassica rapa* has been shown to delay flowering and leaf senescence by inhibiting gibberellin (GA) biosynthesis [15]. Collectively, these findings indicate that *WRKY22* plays critical roles in stress tolerance as well as growth and development in lilies, although its functions may be species-specific, with potential functional divergence across plant species. To date, the functions of *LoWRKY22* in the ‘Siberia’ cultivar of *Lilium* remain to be elucidated, and the molecular mechanisms by which it influences plant growth and development have not been thoroughly investigated.

2 Materials and Methods

2.1 Plant Materials and Growth Conditions

Lilium 'Siberia' bulbs used in this study were purchased from Hongyue Horticultural Corporation (Zhejiang, China; official website: https://www.hongyue.com). *Lilium* 'Siberia' plants were cultivated at the Horticultural Experiment Station of Jinling Institute of Technology. When the flower buds reached a length of approximately 8 cm, the root, stem, leaf, perianth, anther, pollen, and pistil tissues were harvested, immediately frozen in liquid nitrogen, and stored for subsequent analyses. Three biological replicates were prepared for each sample.

Arabidopsis thaliana seeds (ecotype Columbia) were surface-sterilized by soaking in 1% sodium hypochlorite for 10 min, sown on Murashige and Skoog (MS) medium supplemented with 3% sucrose and 0.8% agar (pH 5.8). After stratification at 4°C in darkness for 3 days, the seedlings were then cultured in a light incubator at 22°C under a 16 h/8 h light/dark photoperiod.

2.2 Gene Cloning and Vector Construction of LoWRKY22

Total RNA was extracted from the leaves of *Lilium* 'Siberia' Plants using the TRIzol method [3], and first-strand cDNA was synthesized using the HiScript II kit (Vazyme, Nanjing, China). The open reading frame (ORF) of *LoWRKY22* was subjected to polymerase chain reaction (PCR) amplification using cDNA extracted from the mature leaves of potted *Lilium* 'Siberia' plants as the template and *LoWRKY22*-F/R as gene-specific primers (Table S1). The PCR reaction procedure was as follows: initial denaturation at 98°C for 30 s; 35 cycles of 98°C for 10 s, 60°C for 30 s and 72°C for 60 s; followed by a final extension at 72°C for 10 min. The amplified products were purified and ligated into the pMD18-T vector (Takara, Japan), and the recombinant plasmids were transformed into *Escherichia coli* DH5α competent cells. Positive clones were screened and verified by sequencing.

2.3 Multiple Sequence Alignment and Phylogenetic Analysis of LoWRKY22

Multiple sequence alignment of the LoWRKY22 protein was performed using Clustal W (version 1.81) and BioEdit software (version 7.0) with default parameters. The alignment results were imported into MEGA (version 7), and a phylogenetic tree was constructed using the neighbor-joining (NJ) method with 1000 bootstrap replicates for statistical validation, and other parameters were kept as default.

2.4 Analysis of Transactivation Activity of LoWRKY22

pGBKT7 (BD) vector (CLONTECH Laboratories, USA) was used in the experiment. The BD-LoWRKY22 recombinant expression vector was constructed by homologous recombination using the ClonExpress II One Step Cloning Kit (Vazyme, Nanjing, China). The sequences of the primers used for vector construction are provided in Table S1. A GAL4 vector (positive control), an empty BD vector (negative control), and the recombinant BD-LoWRKY22 vector were independently transformed into *Saccharomyces cerevisiae* AH109 competent cells. The transformed yeast cells were plated on SD/-Trp solid medium and incubated in an inverted manner at 30°C for 2–3 days. Single colonies were selected and verified by PCR and sequencing. The confirmed positive yeast strains were resuspended in 100 μL of sterile water. Then, 5 μL aliquots of the yeast (*Saccharomyces cerevisiae*) suspension were spotted onto solid culture plates including SD/-Trp, SD/-Trp-His, SD/-Trp-His containing 5 mM 3-AT and SD/-Trp-His with 15 mM 3-AT, followed by incubation at 30°C for 2–3 days. After incubation at 30°C for 2–3 days, the growth status of yeast colonies was observed and photographed to identify the self-transactivation activity of BD-LoWRKY22 fusion protein.

The growth of the yeast colonies was observed and documented photographically. The β-galactosidase chromogenic assay was performed as follows: a sterile filter paper was placed flat on SD/-Trp plates containing fresh yeast colonies, and the colonies were transferred onto the filter paper. The filter paper was then clamped with tweezers, rapidly frozen in liquid nitrogen for 1 min, thawed at 15–30°C, and this freeze-thaw cycle was repeated 3–5 times. The filter paper was subsequently placed in a clean Petri dish, with the yeast colonies facing upward, and 1 mL of Z buffer/X-Gal solution was evenly applied. The plate was then incubated in the dark at 30°C, following which color development was monitored and β-galactosidase activity was recorded photographically.

2.5 Subcellular Localization Analysis of LoWRKY22

The full-length coding sequence of LoWRKY22 without stop codon was inserted into the expression vector by homologous recombination. Competent cells of *Agrobacterium tumefaciens* strain GV3101 were transformed with the the constructed fusion plasmid. The transformed bacterial cells were cultured in liquid Luria-Bertani (LB) medium supplemented with kanamycin and rifampicin, with shaking at 200 rpm and 30°C for 14 to 16 hours, until the OD_{600} absorbance value reached 1.5 to 2.0. Bacterial cells were gathered by centrifugal treatment and re-dissolved in infiltration buffer consisting of 10 mM $MgCl_2$ and 10 mM 2-(N-morpholino)ethanesulfonic acid, 200 μM acetosyringone, and pH = 5.6. The bacterial suspension was adjusted to an OD_{600} value of 1.0, followed by dark static incubation at 15–30°C for 4 h. A needleless 1 mL syringe was used to inject the agrobacterial suspension into tobacco leaf mesophyll tissues from the abaxial side. The infiltrated tobacco plants were cultured in darkness for 1 day and then under normal light conditions for another day. Leaf tissues around the injection sites were sampled, and the fluorescence signals of target protein were observed and photographed using a laser scanning confocal microscope (Zeiss LSM780, Germany).

2.6 Cloning and Characterization of the LoWRKY22 Promoter

The *LoWRKY22* promoter was cloned using the FPNI promoter cloning method [16]. Three reverse primers (R1, R2, and R3) were designed to amplify the 5′ region located within 500 bp upstream of the start codon of the *LoWRKY22* ORF in the *Lilium* 'Siberia' cultivar. The first primer (R1) was positioned farthest from the start codon. Each primer was 20–25 bp in length, spaced 60–100 bp apart, and exhibited a GC content of 45–55%. The sequences of the three reverse primers are provided in Table S1. Following confirmation of the PCR-positive amplicons by sequencing, new specific primers were designed, and the *LoWRKY22* promoter was re-amplified using high-fidelity PCR with 2× Super Pfx Master Mix (CWBIO, Beijing, China). The re-sequencing data were compared with the primary cloning sequences and calibrated to ensure accuracy.

The structural organization of the *LoWRKY22* promoter was visualized using TBtools [17]. The *LoWRKY22* promoter sequence was submitted to the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) for predicting the *cis*-acting regulatory elements, and the results were visualized and annotated using TBtools.

2.7 Heterologous Transformation of Arabidopsis thaliana and Gene Expression Analysis

The ORF sequence of LoWRKY22 excluding the stop codon was inserted into the expression vector via homologous recombination, and the resultant recombinant plasmid was transformed into *A. tumefacien* strain GV3101 competent cells. The bacterial cells were harvested and resuspended in 10 mM $MgCl_2$ solution to adjust the OD_{600} value to 0.6, and wild-type *A. thaliana* was transformed using the floral dip technique [18]. After obtaining transgenic *Arabidopsis* lines, seedlings of wild-type and transgenic plants were transplanted into potted soil. The bolting time of each seedling was recorded 14 days after transplantation, and six uniformly growing plants from each line were selected for statistical investigation. At 60 days post-transplantation, five representative plants per line were chosen to measure plant height and count the number of dehiscent siliques. All statistical analyses were performed using SPSS 17.0 software, and Duncan's new multiple range test was applied for significance difference analysis. GraphPad Prism software was used for graph plotting. All experimental data were presented as mean ± standard deviation (SD).

For quantitative real-time PCR (qRT-PCR) assays, seeds of wild-type and transgenic *A. thaliana* were surface-sterilized with 1% sodium hypochlorite and sown on solid MS medium containing 3% sucrose and 0.8% agar. After stratification treatment, the seeds were transferred to a plant growth chamber at 25°C with a 16 h light/8 h dark photoperiod. Whole seedlings were harvested 10 days after sowing for total RNA extraction and subsequent cDNA synthesis. Specific qRT-PCR primers were designed as shown in Table S1. Actin was used as the internal reference gene for *A. thaliana*, and 18S rRNA was selected as the reference gene for *Lilium 'Siberia'*. The qRT-PCR reactions were carried out using ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China) with the following program: pre-denaturation at 95°C for 10 min; 40 cycles of 95°C for 15 s, 55°C for 15 s and 72°C for 20 s. The relative gene expression levels were calculated according to the $2^{-\Delta\Delta Ct}$ method. Each sample was prepared with three independent biological replicates, and all PCR primer sequences are shown in Table S1.

3 Results

3.1 Multiple Sequence Alignment and Phylogenetic Analysis of LoWRKY22

The *TRINITY_DN192_c0_g1_i1_3_1* gene was isolated from the *Lilium* 'Siberia' cultivar and encodes a Trinity transcript derived from the RNA-seq dataset associated with NCBI BioProject PRJNA767274. It contains a full-length cDNA sequence of 834 bp (Table S1) and an ORF encoding 277 amino acids. Phylogenetic analysis revealed that this gene formed a cluster with the *AtWRKY22*, *AtWRKY29*, and *AtWRKY27* genes of *Arabidopsis thaliana* (Fig. 1). Amino acid sequence alignment of LoWRKY22 with AtWRKY22, AtWRKY27 and AtWRKY29 revealed that it shares the closest evolutionary relationship with AtWRKY22 (Fig. S1). Further sequence alignment analysis revealed that the protein encoded by *LoWRKY22* contains a canonical WRKY repeat domain at the N-terminus, which mediates DNA binding, as well as a conserved C2H2-type zinc finger domain at the C-terminus (Fig. 2).

3.2 Transactivation Activity of LoWRKY22

As depicted in Fig. 3, the transcriptional activation assays revealed that yeast colonies transformed with the GAL4 vector (positive control), the empty BD vector (negative control), and the recombinant BD-LoWRKY22 vector grew normally on—Trp medium. Yeast cells transformed with GAL4 or BD-LoWRKY22 exhibited normal growth on—Trp-His medium, whereas those transformed with the empty BD vector (negative control) failed to grow. The addition of 5 mM or 15 mM 3-AT to the—Trp-His medium did not inhibit the growth of yeast colonies transformed with the GAL4 or BD-LoWRKY22 vectors. β-galactosidase staining assays further confirmed that both GAL4 and BD-LoWRKY22 exhibit transactivation activity.

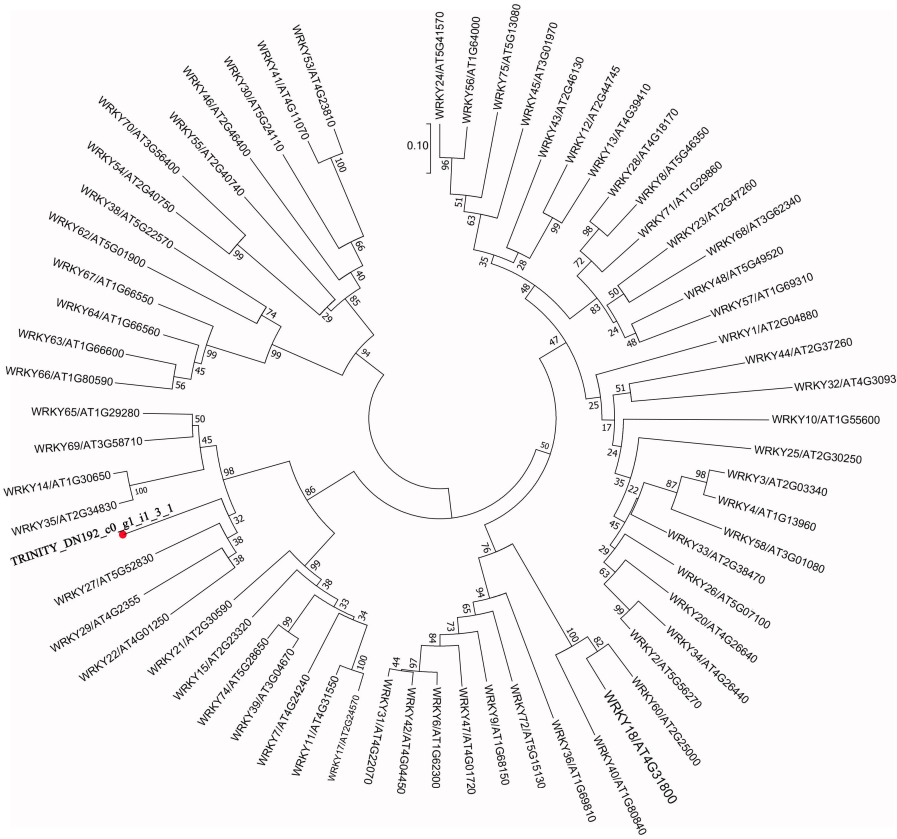

Figure 1: Phylogenetic analysis of *LoWRKY22* and its homologous genes in *Arabidopsis thaliana*.

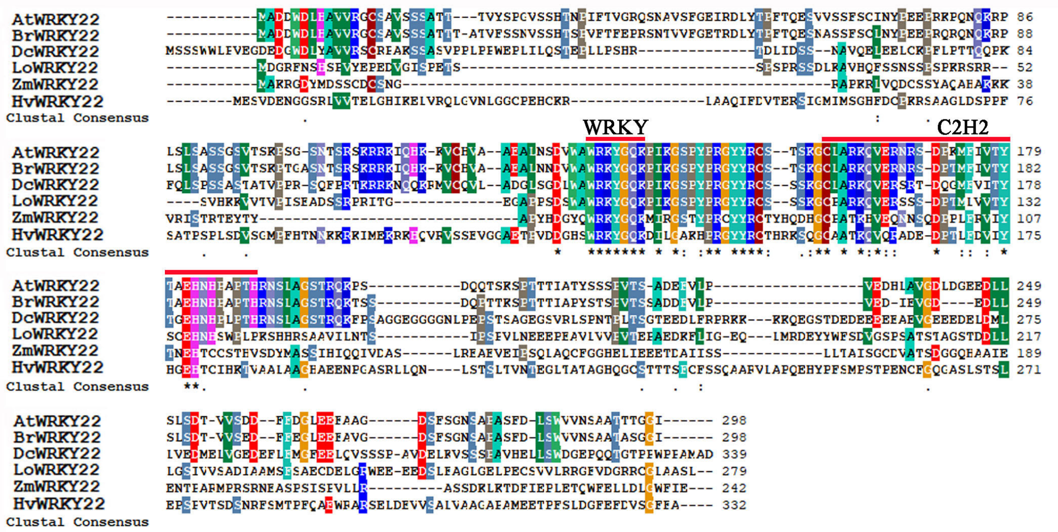

Figure 2: Multiple sequence alignment of LoWRKY22 and homologous WRKY proteins from other plant species. The conserved domains at the N-terminal and C-terminal are indicated by red lines above the aligned sequences. Species abbreviations: At, *Arabidopsis thaliana* (AT4G01250); Br, *Brassica rapa* (NP_001288962.1); Dc, *Dendrobium catenatum* (XP_020684026.1); Zm, *Zea mays* (NP_001147816.1); and Hv, *Hordeum vulgare* (XP_044946498.1).

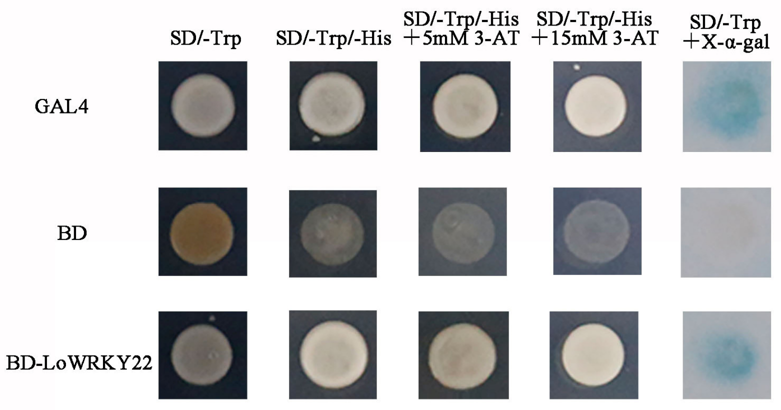

Figure 3: Transactivation activity analysis of LoWRKY22. The GAL4 and empty BD vectors, transformed into the AH109 yeast strain, served as the positive and negative controls, respectively. The full-length sequence of *LoWRKY22* was cloned into the BD vector to create the recombinant BD-LoWRKY22 construct.

3.3 Subcellular Localization of LoWRKY22

To determine the subcellular localization of LoWRKY22 fused with green fluorescent protein (GFP)—specifically, its distribution within the cytoplasm, nucleus, or other cellular compartments—*Agrobacterium tumefaciens* harboring the recombinant plasmid was infiltrated into tobacco leaf epidermal cells using a needleless syringe. The fluorescence signals were visualized using a Zeiss LSM800 laser scanning confocal microscope following 48 h of incubation. Bright-field images confirmed that the cells remained spherical and structurally intact, indicating that the observed green fluorescence originated from intracellular protein expression rather than cellular damage or extraneous contaminants. These findings collectively demonstrate that LoWRKY22 localizes to the nucleus (Fig. 4).

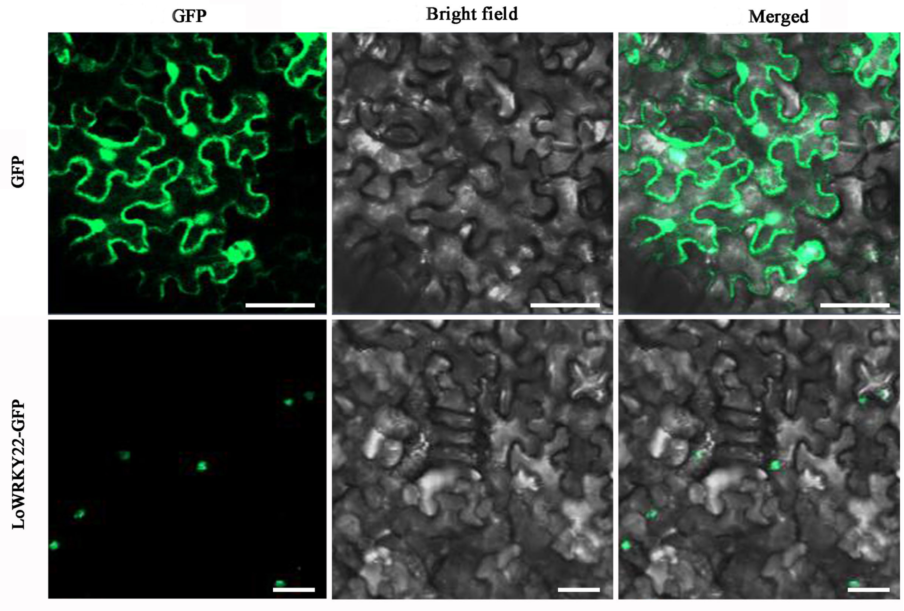

Figure 4: Subcellular localization of LoWRKY22 in tobacco leaf epidermal cells. Free GFP was detected in both the nucleus and cytoplasm, whereas the LoWRKY22-GFP fusion protein localized exclusively to the nucleus. Scale bar = 50 μm.

3.4 Cloning and Analysis of Cis-Acting Elements in LoWRKY22 Promoter

To clarify the specific regulatory basis governing *LoWRKY22* expression, we conducted an in silico prediction of cis-regulatory motifs in the promoter region upstream of its transcription start site (Fig. 5). The analysis revealed that, besides canonical core promoter features such as the TATA box (positioned approximately 30 bp upstream of the transcription start site) and the CAAT box (a ubiquitous cis-regulatory motif in promoters and enhancers), the LoWRKY22 promoter also contains two light-responsive motifs (GT1-motif and Box 4), one low-temperature-responsive motif (LTR), and one gibberellin-responsive motif (P-box) (Table 1).

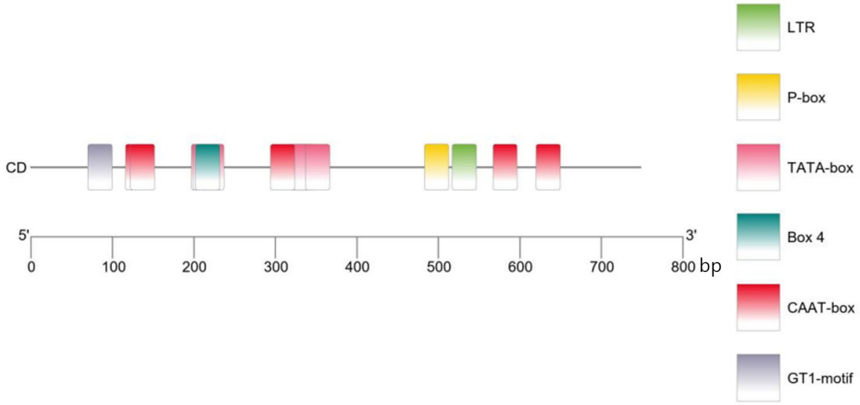

Figure 5: Cloning and structural visualization of the *LoWRKY22* promoter.

Table 1: *Cis*-acting elements in the *LoWRKY22* promoter.

Element Name	Core Sequence	Function	Number of Elements
LTR	CCGAAA	*Cis*-acting element involved in low-temperature responsiveness	1
P-box	CCTTTTG	Gibberellin-responsive element	1
TATA box	ATTATA	Core promoter element located ~30 bp upstream of the transcription start site	2
TATA box	TATA	Core promoter element located ~30 bp upstream of the transcription start site	5
TATA box	ATATAA	Core promoter element located ~30 bp upstream of the transcription start site	1
TATA box	TATATA	Core promoter element located ~30 bp upstream of the transcription start site	1
TATA box	ATATAT	Core promoter element located ~30 bp upstream of the transcription start site	1
Box 4	ATTAAT	Part of a conserved DNA module involved in light responsiveness	1
CAAT box	CCAAT	Common *cis*-acting element in promoters and enhancers	1
CAAT box	CAAAT	Common *cis*-acting element in promoters and enhancers	3
CAAT box	CAACCAACTCC	Common *cis*-acting element in promoters and enhancers	1
GT1-motif	GTGTGTGAA	Light-responsive element	1

3.5 Tissue-Specific Expression of LoWRKY22

Analysis of the tissue-specific expression of *LoWRKY22* (Fig. 6) revealed that its relative expression levels were highest in leaves (63.36). *LoWRKY22* expression did not differ significantly between the roots and perianths, which exhibited the second-highest expression levels (13.13 and 14.61, respectively; mean = 13.87). The relative expression of *LoWRKY22* was lowest in the stems, anthers, pollen, and pistils, with no significant differences in expression among these tissues (2.67, 2.40, 0.93, and 2.21, respectively; mean = 2.05). The relative expression level of *LoWRKY22* in the leaves was significantly higher than that in the roots and perianths, representing a 356.81% increase. Furthermore, the mean relative expression level of *LoWRKY22* in the roots and perianths was significantly higher than that in the stems, anthers, pollen, and pistils, with a 576.59% increase. The relative expression level of *LoWRKY22* in the leaves was 6.77-fold higher than its mean expression in stems, anthers, pollen, and pistils.

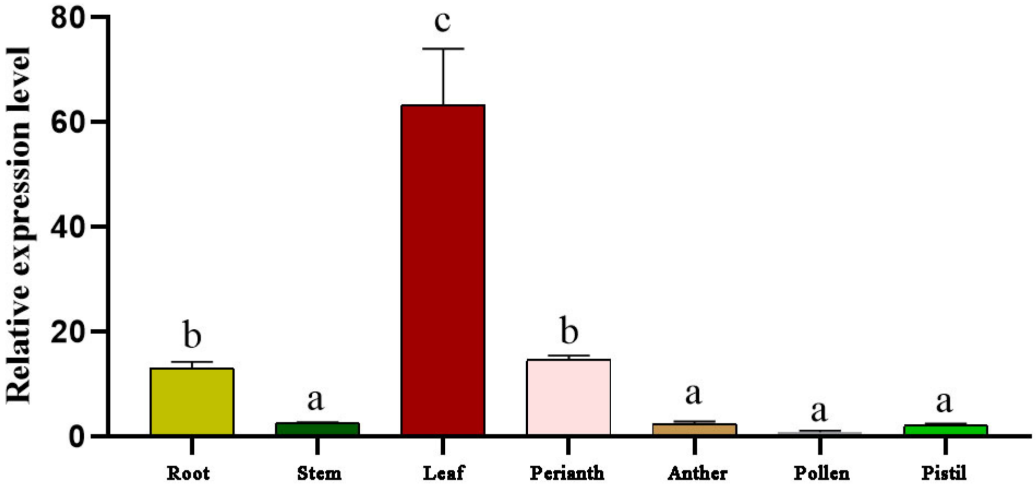

Figure 6: Tissue-specific expression pattern of *LoWRKY22* in the *Lilium* 'Siberia' cultivar. Transcript levels of *LoWRKY22* were measured in various tissues of potted 'Siberia' plants when flower buds reached 8 cm in length. Error bars denote the mean ± standard deviation (SD) from three independent biological replicates. Different lowercase letters (a, b, c) indicate significant differences at $p < 0.05$, as determined by Duncan's multiple range test.

3.6 Phenotypic Analysis of Arabidopsis Plants Heterologously Expressing LoWRKY22

For functional characterization, *LoWRKY22* was heterologously expressed in *Arabidopsis thaliana*. Three independent transgenic lines (OE-4, OE-5, and OE-8) were confirmed by RT-PCR and selected for subsequent functional analyses. Under normal growth conditions, the flowering phenotype of seedlings expressing *LoWRKY22* was recorded two weeks following transplantation from MS medium to soil. The transgenic plants exhibited significantly earlier flowering than the WT *Arabidopsis* plants ($p < 0.05$) (Fig. 7A). At 60 days post-transplantation, the transgenic lines expressing *LoWRKY22* displayed a dwarf phenotype, characterized by reduced plant height (Fig. 7B) and premature silique maturation compared to that observed in WT plants.

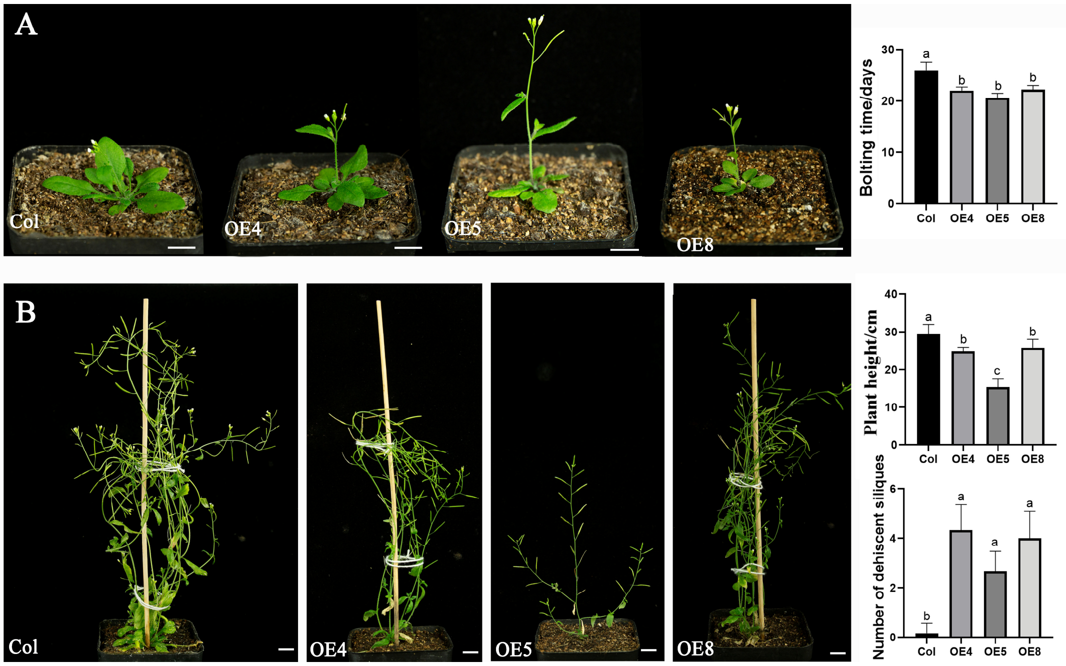

Figure 7: Phenotypic characterization of LoWRKY22-overexpressing transgenic *Arabidopsis* lines compared with WT plants. (**A**) Morphology of WT and transgenic seedlings grown in soil for 20 days (scale bar = 1 cm) and their bolting times. Data are presented as the mean ± SD of six independent experiments, with different lowercase letters indicating significant differences at $p < 0.05$. (**B**) Phenotypes of WT and transgenic plants grown in soil for 60 days (scale bar = 1 cm), including plant height and the number of dehiscent siliques. Values represent the mean ± SD of five replicates; distinct lowercase letters denote significant differences ($p < 0.05$).

3.7 LoWRKY22 Regulates the Expression of Genes Related to Plant Growth and Development

To determine the effects of *LoWRKY22* overexpression on the expression of genes involved in plant growth and development, 10-day-old whole seedlings of transgenic *Arabidopsis thaliana* were subjected to qRT-PCR analysis. As depicted in Fig. 8, *LoWRKY22* overexpression in *Arabidopsis thaliana* significantly upregulated the genes associated with plant senescence ($p < 0.05$). Specifically, the expression levels of *AtWRKY53*, *AtSAG12*, and *AtSAG13* increased by 4.24-, 2.75-, and 6.47-fold, respectively, in transgenic lines, compared to those in WT plants. Furthermore, analysis of flowering-related genes revealed that *LoWRKY22* overexpression significantly altered their expression. Compared to those in WT plants, the expression levels of *AtFT* and *AtAP1* increased by 2.75- and 2.74-fold, respectively, in transgenic lines, whereas *AtFLC* levels decreased by 0.71-fold.

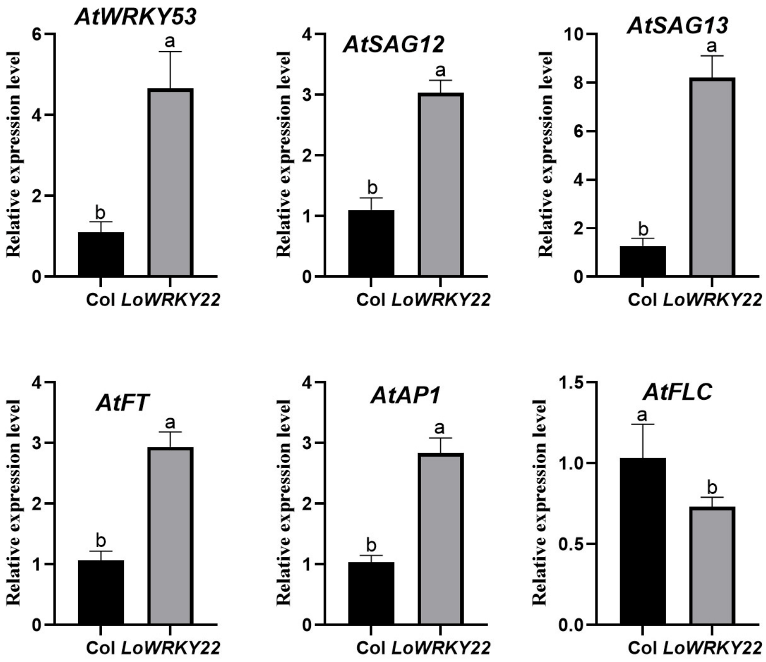

Figure 8: *LoWRKY22* modulates the expression of genes involved in senescence and flowering in *Arabidopsis thaliana*. Gene IDs: *AtWRKY53* (AT4G23810), *AtSAG12* (AT5G45890), *AtSAG13* (AT2G29350), *AtFT* (AT1G65480), *AtAP1* (AT1G69120), and *AtFLC* (AT5G10140). All values are presented as mean ± SD from three biological replicates. Significant differences ($p < 0.05$) are indicated by distinct lowercase letters.

4 Discussion

It has been nearly two decades since the first WRKY transcription factor, SPF1, was identified in sweet potato (*Ipomoea batatas*). Since then, substantial progress has been made in elucidating the diverse biological functions of WRKY transcription factors, which are widely implicated in regulating plant growth, development, and responses to biotic and abiotic stresses [19,20]. Members of the WRKY protein family have been successively identified across a wide range of plant species, with 74, 129, and 111 *WRKY* genes documented in *Arabidopsis thaliana*, *Oryza sativa* subsp. *japonica* (japonica rice), and *Oryza sativa* subsp. *indica* (indica rice), respectively [21,22]. WRKY Group II proteins are characterized by a single WRKY domain coupled with a C2H2-type zinc finger domain and are further classified into five subgroups (IIa–IIe) [23]. In the present study, phylogenetic analysis revealed that *LoWRKY22* formed a cluster with *AtWRKY22* and *AtWRKY27* from *Arabidopsis thaliana*. Multiple sequence alignment further demonstrated that LoWRKY22 contains a highly conserved WRKYGQK motif and a C2H2-type zinc finger-like motif (C-X5-C-X23-H-X-H) (Figs. 1 and 2). Based on these findings, LoWRKY22 was classified as a member of the WRKY-IIe subgroup [24], consistent with the observations of Wu et al. for LiWRKY22 in *Lilium longiflorum* [12].

As depicted in Fig. 4, subcellular localization assays revealed that LoWRKY22 localizes to the nucleus, consistent with the distribution of homologous WRKY proteins and their established function as nuclear transcription factors in modulating plant responses to biotic and abiotic stresses. Additionally, LoWRKY22 exhibited autonomous transactivation activity in yeast cells (Fig. 3), suggesting its potential to activate the transcription of downstream target genes within the nucleus, thereby amplifying its regulatory functions [12]. Tissue-specific expression analysis revealed that the relative expression levels of *LoWRKY22* were highest in the leaves, perianths, and roots of the *Lilium* 'Siberia' cultivar (Fig. 6).

Analysis of the *cis*-acting elements in the *LoWRKY22* promoter further revealed multiple light-responsive elements (Table 1). Previous studies in *Arabidopsis thaliana* have reported that WRKY22 participates in dark-induced leaf senescence [14]. Consistent with these findings, our results demonstrated that the overexpression of *LoWRKY22* in *Arabidopsis thaliana* significantly upregulated the expression of leaf senescence-related genes and induced an early flowering phenotype in transgenic lines (Figs. 7 and 8). Similar regulatory mechanisms have been partially reported in other plant species. For instance, AEL (*Arabidopsis EL1-like*)-mediated phosphorylation modulates ethylene biosynthesis and accelerates leaf senescence by enhancing the transactivation activity of *WRKY22*, thereby providing insights into the finely regulated ethylene biosynthetic pathway and the regulatory cascade underlying leaf senescence [25]. Additionally, the heterologous expression of *GhWRKY27* from cotton in *Arabidopsis thaliana* has been reported to promote leaf senescence, as evidenced by the reduction in chlorophyll content and upregulation of senescence-associated genes (*SAGs*) [26]. Although the biological function of *LoWRKY22* has been elucidated in *Arabidopsis thaliana*, its transient overexpression has not yet been performed in lily leaves or tepals for phenotypic validation. Future studies will conduct transient overexpression assays in lily native systems to systematically evaluate the immediate effects of *LoWRKY22* in its highly expressed tissues, thereby providing more direct experimental evidence for deciphering the tissue-specific regulatory network in lily.

We propose a working model: in *Lilium* 'Siberia', light signals may activate the promoter of *LoWRKY22* via abundant light-responsive cis-elements; nucleus-localized LoWRKY22 functions as a transcriptional activator to promote leaf senescence and accelerate flowering; its relatively high expression in tepals further implies a potential role in flower tissue aging and nutrient redistribution during the transition from vegetative to reproductive growth. Although the precise molecular mechanism by which *LoWRKY22* regulates plant senescence was not investigated in this study, previous findings provide valuable insights into the potential regulatory pathways. In the monocarpic species *Arabidopsis thaliana*, WRKY1 directly represses the expression of *FLC* to promote flowering and induces leaf senescence by activating the genes in the salicylic acid (SA) biosynthetic pathway. Furthermore, *WRKY1* has been shown to directly upregulate the genes involved in nitrogen assimilation and transport, thereby facilitating nitrogen remobilization from senescing leaves to developing seeds [27]. Notably, WRKY transcription factors not only mediate the regulation of plant development and senescence but also play a central role in plant responses to environmental stresses [19,20]. For instance, the majority of the 70 identified *VbWRKY* genes in the cold-sensitive ornamental plant *Verbena bonariensis* contain low-temperature-responsive *cis*-elements, and more than half are differentially expressed under cold stress, indicating their extensive involvement in plant cold stress responses [28]. Multiple *WRKY* genes, including *WRKY40*, *WRKY51*, and *WRKY53*, are co-upregulated with jasmonic acid (JA) defense-related genes during the interaction between lily and Cucumber mosaic virus (CMV), thereby forming a core immune module in leaves that contributes to resistance against viral infection [29,30]. These findings collectively suggest that *LoWRKY22* may play a pivotal role in balancing flowering and leaf senescence during the transition from vegetative to reproductive growth in the 'Siberia' cultivar of *Lilium*, thus providing valuable insights into the regulatory mechanisms underlying plant life cycle progression.

5 Conclusions

In this study, the *LoWRKY22* gene from the 'Siberia' cultivar of *Lilium* was cloned and functionally characterized to investigate its expression profile and biological functions. The evolutionary position of LoWRKY22 within the WRKY family was elucidated through multiple sequence alignment of LoWRKY22 with WRKY22 homologs from the model plant *Arabidopsis thaliana* and other species, followed by phylogenetic analysis. Subcellular localization assays in tobacco leaf cells confirmed the nuclear localization of LoWRKY22, and transcriptional activation assays verified its autonomous transactivation activity. Analysis of *cis*-acting elements within the *LoWRKY22* promoter identified multiple functional motifs, including light-responsive elements, and tissue-specific expression analysis revealed elevated *LoWRKY22* expression in the leaves, perianths, and roots of the 'Siberia' cultivar of *Lilium.* The heterologous expression of *LoWRKY22* in *Arabidopsis thaliana*, combined with phenotypic characterization and qRT-PCR analysis of flowering- and senescence-related gene expression profiles, further elucidated the potential role of *LoWRKY22* in regulating the transition between vegetative and reproductive growth in lilies. Collectively, these findings serve as a valuable reference for further investigations into the functions of *LoWRKY22* as a growth-regulatory gene in lilies and establish a preliminary molecular basis for the genetic improvement of *Lilium* with optimized growth and developmental traits.

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