Functional Characterization of PpGS2 Promoter in Response to Hormones and Low Nitrogen in Kentucky Bluegrass

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Abstract

Kentucky bluegrass (*Poa pratensis* L.) is an important cool-season turfgrass with high nitrogen (N) fertilizer requirements. *PpGS2* is a key gene for nitrogen uptake assimilation, but the core regulatory region of its promoter remains unclear. To characterize the transcriptional regulation of the *PpGS2* promoter, a series of 5'-terminal deletion constructs were generated, each fused to the β-glucuronidase (GUS) reporter gene and transformed into Arabidopsis and tobacco. All fragments exhibited transcriptional activity, with activity declining as fragment length decreased. The full-length promoter drove GUS expression most strongly in the leaves of transgenic Arabidopsis. In addition, exogenous hormones, low nitrogen, and light stress all significantly enhanced promoter activity, with methyl jasmonate (MeJA) showing the strongest induction. Deletion analysis further revealed the distribution of response elements: Gibberellin (GA)-responsive elements were predominantly localized between − 2000 bp and − 1600 bp; Abscisic acid (ABA)- and light-responsive elements were mainly concentrated within the − 1200 bp to -800 bp and − 1600 bp to -800 bp ranges, respectively; Auxin (IAA) and MeJA responses relied on the synergy of multiple regions; The low-nitrogen response was mediated by both positive and negative cis-acting elements. The − 2000bp to -1200 bp region likely contained inhibitory elements, whereas the − 1200bp to -500 bp region harbored enhancing elements. In conclusion, this study systematically elucidated the regulatory mode of the *PpGS2* promoter for the first time, thereby enabling a better understanding of the synergistic regulatory mechanism of *PpGS2* under hormonal and environmental signals.

1. Introduction

The promoter is located upstream of the gene's 5' end and functions to activate RNA polymerase, facilitating its accurate binding to the template DNA. A promoter typically consists of a core promoter and a proximal promoter, though the exact boundaries of the promoter region remain undefined. For most genes, the promoter region extends approximately 2000 bp upstream of the transcription start site (TSS) [1]. Relative to the TSS, the minimal promoter region capable of initiating basal transcription (-60 to + 40 bp) is termed the core promoter, while the proximal promoter extends further upstream [2]. Promoters regulate the initiation of gene transcription, thereby influencing the timing, location, and intensity of gene expression [3]. Transfection of the *PsSUT2* promoter expression vector into tree peony (*Paeonia suffruticosa* A) increased the expression level of the *PsSUT2* gene [4]. Similarly, introduction of the *TkSRPP* promoter into tobacco (*Nicotiana tabacum* L.) elevated *TkSRPP* expression and subsequently modulated hormone stress responses [5]. Therefore, functional characterization of promoters is crucial for elucidating the molecular mechanisms underlying the regulation of downstream gene expression.

Nitrogen (N) is a vital component of amino acids, proteins, nucleic acids, chlorophyll, and various primary and secondary metabolites [6], and thus represents an essential nutrient for plant growth and development [7]. Critically, nitrogen availability and signaling are known to interact extensively with phytohormone networks, forming an integrated system that coordinates the expression of genes

involved in nitrogen metabolism [8–9]. In soils, inorganic nitrogen typically exists as ammonium (NH_4^+) and nitrate (NO_3^-) ions, however, plants are unable to directly utilize inorganic nitrogen in these forms for metabolic processes [7]. Consequently, inorganic nitrogen must be assimilated into organic nitrogen compounds to support plant growth and development, a process in which glutamine synthetase (GS) plays a central role. Plants GS enzymes are categorized into GS1 and GS2. GS1 is located in the cytoplasm and mainly exists in non-photosynthetic tissues such as roots and stems of plants, responsible for primary nitrogen assimilation and long-distance transportation and redistribution of nitrogen [10]. GS2 is primarily localized in chloroplasts (plastids) and plays a crucial role in the efficient assimilation of excess ammonia, including primary ammonia generated during nitrate reduction as well as ammonia released through photorespiration [11]. Therefore, the activity and expression level of GS2 directly affect the nitrogen utilization efficiency and photosynthetic capacity of plants. Overexpression of *GS2* in rice (*Oryza sativa* L.) has been shown to enhance nitrogen uptake and significantly improve nitrogen use efficiency (NUE) [12]. Overexpression of *NtGS2* in tobacco promotes amino acid accumulation, enhances NUE, accelerates seedling growth, and increases plant fresh weight by 20–30% [13]. Similarly, under low-nitrogen conditions, *Arabidopsis thaliana* plants overexpressing *AtGS2* exhibit increased leaf area and elevated levels of total protein, chlorophyll, sucrose, and glucose compared with wild-type plants, reflecting improved nitrogen use efficiency [14]. Under low-nitrogen conditions, transgenic wheat (*Triticum aestivum* L.) overexpressing *TaGS2-2A* exhibited enhanced nitrogen uptake efficiency and increased grain nitrogen remobilization capacity [15]. Overexpression of GS2 in duckweed (*Lemna minor* L.) increased biomass and protein content by 10% and 24% respectively, with this effect being conserved in rice, tobacco and Arabidopsis [16]. Collectively, these findings indicate that elevated *GS2* expression enhances nitrogen assimilation and metabolic efficiency, thereby promoting biomass accumulation and improving plant tolerance to abiotic stress. As *GS2* expression is regulated by its upstream promoter region, elucidating the functional characterization of the *GS2* promoter is essential for a comprehensive understanding of the molecular mechanisms of GS2-mediated nitrogen assimilation and utilization in plants.

The promoter region of the *GS2* gene contains light-responsive, low-nitrogen-responsive, and various stress-responsive elements [17–20]. In barley (*Hordeum vulgare* L.), *HvGS2* mutants exhibit a reduced maximum quantum efficiency of photosystem II (Fv/Fm) under high light conditions, suggesting that the *GS2* promoter, through its light-responsive elements, participates in abiotic stress response processes [21]. When the *GS2* gene promoter from rice was introduced into tobacco, exogenous NH_4^+ was found to positively regulate *GS2* promoter activity [22]. The expression of *OsGS2* in the drought-tolerant rice variety Khitish was stable under water shortage conditions, while its expression decreased in the sensitive variety IR-64, indicating the variety specificity of the GS2 promoter in response to drought [18]. The induced expression of *CsGS2* in Tea plant (*Camellia sinensis* L.) leaves is dependent on light exposure, with darkness significantly inhibiting its transcriptional activation, suggesting the presence of light-responsive elements within the promoter [19]. However, previous studies have primarily elucidated the *GS2* promoter's responses to light signals, nitrogen sources, and certain abiotic stresses. In contrast, the

specific hormone-responsive elements within the *GS2* promoter and the mechanisms by which they mediate hormone signalling and synergistically regulate transcription with environmental signals remain largely unexplored.

Kentucky bluegrass (*Poa pratensis* L.), native to Eurasia, is a globally important perennial cool-season turfgrass species [23]. Owing to its desirable characteristics, including strong cold tolerance, dark green foliage, and rapid establishment, it is widely used in golf courses, sports fields, and recreational green spaces [24]. However, Kentucky bluegrass exhibits a high demand for nitrogen fertilizer during growth, excessive nitrogen application not only increases turf maintenance costs but also leads to a range of environmental issues [25]. Therefore, developing nitrogen-efficient cultivars is essential for achieving sustainable maintenance practices. In this study, we aimed to elucidate the functional characteristics of the *PpGS2* promoter in Kentucky bluegrass. The upstream promoter at the 5’ end of the *PpGS2* gene was cloned, and its cis-acting elements was analyzed. Subsequently, a series of *GUS* reporter constructs containing truncated promoter fragments were generated and introduced into *Arabidopsis thaliana* and tobacco. By integrating GUS histochemical staining, quantitative real-time PCR (qRT-PCR), and GUS enzymatic activity assays, we systematically characterized the promoter activity, tissue-specific expression patterns, and transcriptional responses to various phytohormones, low-nitrogen stress and light stress. Collectively, the study could provide a theoretical basis for elucidating transcriptional regulatory networks underlying nitrogen metabolism in turfgrass species and identify potential molecular targets for the breeding of nitrogen-efficient turfgrass germplasm.

2. Materials and Methods

2.1 Plant Materials, Growth Conditions and Treatment

Two-year-old ‘Maoershan’ Kentucky bluegrass turf was collected from the experimental field of Northeast Agricultural University (Harbin, China; 44°29'N, 127°17'E) and transplanted into plastic pots (9.5 cm length × 11.3 cm width × 10.5 cm height). Each pot contained 30 seedlings and 500 g of growth substrate composed of black soil (pH = 7.0, organic matter content 16.8 g/kg), vermiculite and at a ratio of 3:1:1 (v/v/v). Plants were grown in a controlled-environment growth chamber under the following conditions: temperature 25°C ± 2°C/18°C ± 2°C, relative humidity 65% ± 5%, photoperiod 13 h, light intensity 700 ± 10 μmol·m^{-2}·s^{-1}. Plants were irrigated every 2 dto maintain adequate moisture conditions and trimmed every 3 d to maintain a turf height of 4–5 cm. After 45 d of growth, aerial tissues were harvested, immediately frozen in liquid nitrogen, and stored at -80°C for promoter cloning.

Wild-type *Arabidopsis thaliana* (Col-0) was used for genetic transformation of *PpGS2* promoter. Seeds were sterilized, rinsed in sterile distilled water, and sown on solid Murashige and Skoog (MS) medium. After stratification at 4°C for 3 d, seedlings were grown for 12 d under controlled conditions (22 ± 1°C, 70% relative humidity, 16-h light/8-h dark photoperiod). Seedlings were then transplanted into pots containing a mixture of perlite and peat moss (1:2 ratio) (top diameter 9.8 cm×bottom diameter 6.5 cm×height 8.2 cm), with 120 g of substrate per pot. These were placed in an artificial climate chamber

(temperature 23°C, light intensity 700 ± 10 μmol $m^{-2}·s^{-1}$, 16/8-hour photoperiod, humidity 70%), and watered regularly to keep the soil moist. Plants were used for genetic transformation approximately four weeks after germination. *Nicotiana benthamiana* culture methods were identical to those for *Arabidopsis thaliana*.

Homozygous T3 transgenic plants were cultured under the same conditions as wild-type plants. After 4 weeks of growth, plants were subjected to low-nitrogen, high-light, and hormone treatments. For low-nitrogen treatment, plants were cultured in Hoagland nutrient solution containing 0.5 mM $NaNO_3$ for 7 d, while plants grown in standard Hoagland solution served as controls. For high-light stress, plants were exposed to a light intensity of 1000 ± 10 μmol m^{-2} s^{-1} for 4 h at 22°C; control plants were maintained at a light intensity of 700 ± 10 μmol m^{-2} s^{-1} under the same temperature conditions. For hormone treatments, leaves were sprayed with 100 μM abscisic acid (ABA), 1 mM indole-3-butyric acid (IAA), 1 mM gibberellic acid (GA), or 1 mM methyl jasmonate (MeJA), with deionized water used as the control. Following treatment, plants were cultivated for an additional 24 h. *GUS* transcript levels and GUS enzymatic activity were subsequently analyzed.

2.2 Cloning of Promoters and Bioinformatics Analysis

Genomic DNA was extracted from frozen aboveground tissues of Kentucky bluegrass using a plant genomic DNA extraction kit (Tiangen, China) according to the manufacturer's protocol. Based on the *PpGS2* coding sequence available in NCBI (https://www.ncbi.nlm.nih.gov/) and the published Kentucky bluegrass reference genome, the promoter region was defined as the 2,000 bp sequence immediately upstream of the translation start codon. Primers specific to the putative promoter region were designed for PCR amplification (Table S1). PCR products were purified and sequenced, and the correctly assembled promoter sequence (PR0) was analyzed for cis-acting elements using the PlantCARE online tool (http://www.example.com/webtools/plantcare/html/).

2.3 Promoter Truncation and Vector Construction

PCR amplification yielded promoter fragments with 5'-end deletions (Table S1). The resulting fragments were designated PR1, PR2, PR3, and PR4, with lengths of 1600 bp, 1200 bp, 800 bp, and 500 bp, respectively. The full-length promoter (PR0) and truncated fragments were individually cloned upstream of the GUS reporter gene in the pCAMBIA3301 vector, replacing the CaMV 35S promoter, using homologous recombination (Table S2). The ligation products were transformed into DH5α competent cells, and positive clones were screened on plates containing 100 mg/L kanamycin (Kan) and verified by colony PCR. Sequencing (Kumei, Jilin) confirmed the correct recombinant plasmids, designated pCAMBIA3301-PR0, pCAMBIA3301-PR1, pCAMBIA3301-PR2, pCAMBIA3301-PR3, and pCAMBIA3301-PR4.

2.4 Acquisition and Identification of Transgenic Plants

The recombinant plasmids were introduced into *Agrobacterium tumefaciens* strain GV3101 and subsequently transformed into *Arabidopsis thaliana* using the floral dip method[5]. First, T0 seeds were

disinfected and sown on medium containing 0.001% Basta for preliminary screening. Healthy green seedlings were transplanted, and T1 seeds were harvested from individual plants. Subsequently, PCR molecular validation was performed on T1 plants to confirm transgenic positivity, and their T2 seeds were collected. Subsequently, T2 seeds were sown for Basta resistance screening. Lines exhibiting a 3:1 resistance segregation ratio were selected, cultured, and harvested for T3 seeds. Finally, T3 seeds underwent resistance validation. Those with 100% seedling survival were identified as homozygous transgenic lines for subsequent experiments.

Transient expression assays were performed in *Nicotiana benthamiana* leaves using *Agrobacterium*-mediated infiltration. *Agrobacterium* cultures harboring the indicated constructs were resuspended in infiltration buffer and introduced into the abaxial surface of fully expanded leaves using a needleless syringe until the leaf tissue was completely infiltrated. Four to five leaves per plant were infiltrated, with five biological replicates per treatment. Leaves infiltrated with *Agrobacterium* carrying the empty pCAMBIA3301 vector were used as negative controls. After infiltration, plants were maintained in darkness for 2–3 d and subsequently transferred to normal growth conditions for further analyses.

2.5 GUS Staining and Activity Assay

Transgenic tobacco leaves and 4-week-old PR0 transgenic *Arabidopsis* plants were immersed in GUS staining solution. The reaction proceeded continuously at 37°C in a constant-temperature metal bath for 8–12 h. Subsequently, the staining solution was removed and 75% ethanol was added for decolorization. The ethanol solution was replaced every 5–10 min until endogenous pigments in the plant tissues were completely removed. After decolorization, samples were prepared for temporary mounting. Specific blue precipitate distribution patterns were observed and documented using an E200MV optical microscope (Nikon, Japan).

GUS enzyme activity was measured using an ELISA kit (SolaBio, Beijing). Proteins extracted from test samples were mixed with the GUS detection buffer. After incubation at 37°C, the reaction was terminated with 0.2 M termination buffer ($NaCO_3$). Fluorescence intensity was measured using a Varioskan Flash (Thermo, USA) at an excitation wavelength of 365 nm and an emission wavelength of 455 nm. Protein concentration was determined by the Bradford protein assay [26].

2.6 RNA Extraction and qRT-PCR Validation

Total RNA was extracted from transgenic *Arabidopsis* using the EasyPure® Plant RNA Kit (TianGen, Beijing). cDNA was synthesized using the PrimeScript First-Strand cDNA Synthesis Kit (Takara, Japan). Gene expression levels were measured on a Bio-Rad CFX 96 real-time PCR system using Vazyme's ChamQ SYBR Color qPCR Master Mix (Low ROX). Primers used are listed in Table S3. The reaction program was set as follows: 10 min pre-denaturation at 95°C; followed by 40 cycles of amplification, each cycle comprising 10 sec denaturation at 95°C, 30 sec annealing at 58°C, and 10 sec extension at 72°C; post-cycle extension at 72°C for 10 min, then final storage at 4°C. Experiments were conducted

with three independent biological and technical replicates. Relative gene expression levels were calculated using the $2^{(-\Delta\Delta CT)}$ method with *AtACTIN* as the internal control [27].

2.7 Data Processing and Analysis

Statistical analyses were performed using SPSS software (version 22.0; SPSS Inc., Chicago, IL, USA). Data were analyzed by one-way analysis of variance (ANOVA), followed by Student's t-test where appropriate. Differences were considered statistically significant at $p < 0.05$ and highly significant at $p < 0.01$. All graphical representations were generated using GraphPad Prism version 10 (GraphPad Software, San Diego, USA).

3. Result

3.1 Cloning and Bioinformatics Analysis of the *PpGS2* Promoter

To investigate the transcriptional regulation mechanism of the *GS2* gene, a 2000 bp promoter fragment upstream of the translation start site (ATG) was cloned from genomic DNA and designated PR0 (Fig. 1). Analysis of this sequence via the PlantCare online platform revealed that, in addition to containing core promoter elements such as the TATA-box and CAAT-box, it also contained multiple functional cis-acting elements associated with stress response, hormone signaling, and light response. Among these, abiotic stress-related elements included the drought response element DRE, the low temperature response element LTR, the TATABOX5 involved in low nitrogen response, and multiple MYB/MYC binding sites. Hormone response elements comprise the abscisic acid (ABA) response element ABRE, the gibberellin (GA) response element GARE-motif, and the auxin (IAA) response element AuxRR-core. Additionally, multiple light response elements such as G-Box, GATA-motif, and GT1-motif were identified (Table 1). These findings suggest that *PpGS2* gene expression may be co-regulated by multiple environmental stresses, plant hormones, and light signals.

Table 1
Analysis of cis-acting elements of the *PpGS2* promoter

cis-Elements	Number	Function	Motif
A-box	1	sequence conserved in alpha-amylase promoters	AATAACAAACTCC
A-box	2	cis-acting regulatory element	CCGTCC
AACA-motif	1	involved in endosperm-specific negative expression	TAACAAACTCCA
ABRE	2	cis-acting element involved in the abscisic acid responsiveness	AACCCGG
AuxRR-core	1	cis-acting regulatory element involved in auxin responsiveness	GGTCCAT
CAAT-box	22	common cis-acting element in promoter and enhancer regions	CCAAT
CAT-box	3	cis-acting regulatory element related to meristem expression	GCCACT
CCAAT-box	1	MYBHv1 binding site	CAACGG
CGTCA-motif	8	cis-acting regulatory element involved in the MeJA-responsiveness	CGTCA
G-box	2	cis-acting regulatory element involved in light responsiveness	GCCACGTGGA
GARE-motif	1	gibberellin-responsive element	TCTGTTG
GATA-motif	3	part of a light-responsive element	GATAGGG
GC-motif	1	enhancer-like element involved in anoxic-specific inducibility	CCCCCG
GT1-motif	2	light-responsive element	GGTTAA
HD-Zip 1	1	element involved in differentiation of the palisade mesophyll cells	CAAT(A/T) ATTG
LTR	2	cis-acting element involved in low-temperature responsiveness	CCGAAA
MBS	1	MYB binding site involved in drought-inducibility	CAACTG
RY-element	1	cis-acting regulatory element involved in seed-specific regulation	CATGCATG
Sp1	1	light-responsive element	GGGCGG
TATA-box	6	core promoter element around − 30 of transcription start	TATA
TGA-element	1	auxin-responsive element	AACGAC

cis-Elements	Number	Function	Motif
TGACG-motif	8	cis-acting regulatory element involved in the MeJA-responsiveness	TGACG
W box	2	WRKY binding wite involved in salicylic acid (SA)-induced responsiveness	TTGACC
MYB	4	Unknown	CAACAG
MYC	4	Unknown	CATTTG
Myb	1	Unknown	CAACTG
Myb-binding site	2	Unknown	CAACAG
STRE	4	Unknown	AGGG
TATABOX5	2	cis-acting regulatory element involved in the low-nitrogen responsiveness	TTATTT

3.2 Analysis of the transcriptional activity of the *PpGS2* promoter

To evaluate the transcriptional activity of the *PpGS2* promoter, the full-length PR0 fragment was used as a template to generate a series of 5'-end deletion constructs, designated PR1, PR2, PR3, and PR4, with lengths of 1,600 bp, 1,200 bp, 800 bp, and 500 bp, respectively (Fig. 2A). These constructs were introduced into tobacco leaves by *Agrobacterium*-mediated transient transformation, followed by histochemical GUS staining. Leaves expressing the full-length PR0 promoter exhibited strong blue staining compared with the empty-vector control, indicating robust activation of GUS expression by PR0. In contrast, PR1, PR2, and PR3 drove progressively weaker GUS staining as promoter length decreased, whereas the shortest fragment, PR4, displayed only faint staining (Fig. 2B). Collectively, these results demonstrate that the five *PpGS2* promoter fragments (PR0-PR4) exhibit distinct transcriptional activities, and that promoter activity is positively correlated with fragment length, suggesting that upstream regulatory regions are required for full promoter function.

A. Schematic illustration of 5' end deletions of different lengths of the *PpGS2* promoter; B. Negative control (injection of empty vector, I), PR0 (II), PR1 (III), PR2 (IV), PR3 (V) and PR4 (VI) promoter fragments driving GUS histochemical staining in tobacco leaves (Scale bar: 1 mm).

3.3 Characterization of *PpGS2* promoter expression in different tissues of *Arabidopsis thaliana*

To investigate the expression properties of the *PpGS2* promoter in different tissues of *Arabidopsis*, histochemical staining was performed on 4-week-old WT and PR0 transgenic *Arabidopsis* plants. The experimental results showed that the *PpGS2* full-length promoter drove the transcriptional activity of the

GUS gene in all tissues examined compared with WT, but it was particularly strong in leaves, followed by stems and roots (Fig. 3A). Quantitative analysis of *GUS* gene expression driven by the *PpGS2* full-length promoter by qRT-PCR showed that the *GUS* gene was expressed at the highest level in transgenic *Arabidopsis* leaves, which was about 1.41-fold higher than that in stems(Fig. 3B), and this result was in agreement with the results of the histochemical staining, which indicated that the *PpGS2* promoter drives *GUS* reporter gene expression primarily in *Arabidopsis* leaves and secondarily in stems.

A. Histochemical staining of GUS in wild-type *Arabidopsis* and *PpGS2* full-length promoter transgenic Arabidopsis; B. Detection of the relative expression of *GUS* genes in different tissues of transgenic *Arabidopsis* by qRT-PCR.

Vertical bars indicate the standard deviation of the mean (n = 6) and * indicates statistical significance as determined by t-test (**p < 0.01).

3.4 Expression analysis of *GUS* genes driven by *PpGS2* promoter under abiotic stresses

To further investigate the response of the *PpGS2* promoter to exogenous hormones, low nitrogen stress and light stress, PR0 transgenic *Arabidopsis thaliana* plants were used as materials to detect the relative expression levels of *GUS* genes after treatment with different exogenous hormones, low nitrogen and light stress. The results showed that all four phytohormones (GA, IAA, ABA, and MeJA), low-nitrogen stress and light stress all significantly induced the expression of *GUS* genes compared with the control, but there were differences in the intensity of induction among the different treatments (Fig. 4). The highest expression of *GUS* genes was observed under the MeJA treatment, which was about 10.12-fold higher than that of the control. Secondly was the low-nitrogen treatment, which increased *GUS* gene expression by about 4.32-fold compared with the control. The *PpGS2* promoter was slightly less sensitive to IAA, ABA and light stress, and the *GUS* gene expression levels were about 2.24-fold, 2.35-fold and 1.76-fold of the control, respectively, while the *PpGS2* promoter was the weakest in response to GA, which was about 1.42-fold of the control. The above results indicated that the *PpGS2* promoter could respond to a variety of hormone signals, low-nitrogen stress and light stress, among which the response to MeJA was particularly significant.

Vertical bars indicate the standard deviation of the mean (n = 6), and different letters indicate significant differences ($P < 0.05$).

3.5 Analysis of transgenic *Arabidopsis* GUS enzyme activity under abiotic stresses

To identify the key cis-regulatory regions within the *PpGS2* promoter that mediate responses to phytohormones, low-nitrogen stress, and light signals, GUS enzymatic activity was quantified in PR0-PR4 transgenic *Arabidopsis thaliana* lines following treatments with GA, IAA, ABA, MeJA, low nitrogen, and altered light conditions (Fig. 5). Following GA treatment, a pronounced increase in GUS activity was

observed exclusively in the PR0 transgenic line, with an approximately 1.27-fold induction relative to the control. In contrast, no significant changes were detected in PR1, PR2, PR3, or PR4 (Fig. 5A). This pattern suggests that GA responsiveness of the *PpGS2* promoter is primarily dependent on cis-acting elements located within the − 2,000 to -1,600 bp region. IAA (Fig. 5B) and MeJA (Fig. 5D) treatments significantly enhanced GUS activity in all transgenic lines compared with the control. However, GUS activity progressively declined with successive promoter truncation, indicating that IAA and MeJA responsive regulation involves multiple cis-acting elements distributed across the promoter and that these elements likely function synergistically to regulate *PpGS2* transcription. Under ABA treatment, GUS activity was significantly increased in PR0, PR1, and PR2 transgenic lines, whereas no significant differences were detected in PR3 and PR4 compared with the control (Fig. 5C). These results indicate that ABA responsive cis-acting elements are mainly localized within the − 1,200 to -800 bp region of the *PpGS2* promoter.

Low-nitrogen stress led to increased GUS activity in all transgenic lines; however, the strongest induction was observed in PR2 and PR3, with approximately 0.63-fold and 0.72-fold increases, respectively, relative to the control (Fig. 5E). This pattern suggests a bidirectional regulatory mechanism underlying the low-nitrogen response, in which the − 2,000 to -1,200 bp region may harbor negative regulatory elements, while the − 1,200 to -500 bp region likely contains positive regulatory elements that enhance transcription under nitrogen-deficient conditions. In response to altered light conditions, no significant change in GUS activity was detected in the PR0 transgenic line compared with the control. In contrast, PR1 and PR2 lines, corresponding to the − 1,600 to -800 bp region, exhibited significantly elevated GUS activity (Fig. 5F), indicating that this region likely represents the core light-responsive domain of the *PpGS2* promoter.

A-F. Response of five PpGS2 promoter fragments of different lengths to gibberellin (GA), auxin (IAA), abscisic acid (ABA), methyl jasmonate (MeJA), low nitrogen (LN) and light treatments.

Vertical bars indicate the standard deviation of the mean (n = 6) and * indicates statistical significance as determined by t-test (*$p < 0.05$, **$p < 0.01$).

4. Discussion

Glutamine synthetase 2(GS2) plays an important role in plant nitrogen metabolism, and its expression regulation involves multiple environmental factors, such as light, nitrogen sources, and developmental signals [11]. Increasing evidence indicates that transcriptional regulation mediated by promoter sequences is a key determinant of gene expression dynamics [3]. Most of the reported studies on the functions of *GS2* promoters have focused on food crops such as rice and barley, while there are relatively few studies on important turfgrasses and forages like Kentucky bluegrass [21, 28]. In this study, we successfully cloned the promoter sequence (PR0) 2000 bp upstream of the *PpGS2* coding region from Kentucky bluegrass, and bioinformatic analyses revealed that it contains a variety of cis-acting elements related to hormone response, light signaling, and abiotic stress (Table 1). In addition to core promoter components such as TATA-box and CAAT-box, we identified functional components related to abiotic

stress responses such as LTR, DRE and TATABOX5, as well as the component TATABOX5 involved in low-nitrogen responses. This is consistent with the regulatory characteristics of the *GS2* promoter in other plant species [17–20]. In addition, we identified hormone response elements such as ABRE, GARE-motif, and AuxRR-core. These results suggest that *PpGS2* transcription may be synergically regulated by multiple endogenous and environmental signals.

The number, type and location of response elements contained in the promoter affect the promoter activity [29–30]. Previous studies have shown that promoter truncation can result in either enhanced or reduced transcriptional activity, depending on the functional organization of regulatory elements. For instance, the activity of the *GI* promoter in sweet potato (*Ipomoea batatas* L.) initially increased and then decreased following progressive 5'-end deletions [31], whereas the promoter activity of the *F3H* gene in mountain grape increased with promoter shortening [32]. In this study, by constructing 5'-end deletion fragments (PR1-PR4) and transforming tobacco, we found that the intensity of GUS staining was gradually weakened with the shortening of the promoter length (Fig. 2B), suggesting that the transcriptional activity of the *PpGS2* promoter is dependent on its integrity, and fragment deletion may result in the loss of key or synergistic elements, which may diminish its driving ability. Previous studies have demonstrated that GS2 expression is predominantly localized in green tissues, particularly leaves [33–34]. Consistent with these findings, the *PpGS2* promoter drove *GUS* expression in roots, stems, and leaves of transgenic *Arabidopsis*, with the strongest activity observed in leaves. This expression pattern aligns well with the established role of GS2 in chloroplasts, where it functions in photorespiratory ammonium reassimilation and nitrogen metabolism associated with photosynthesis [11]. These results further support the critical contribution of *GS2* to leaf nitrogen assimilation [33–34].

Gene expression is often dynamically regulated by phytohormones and environmental stresses [27, 35–36]. In wheat, NH_4^+ preferentially induces *GS* expression in roots, whereas NO_3^- promotes *GS* expression in leaves [37], and nitrate signaling mediated by the transcription factor *NLP7* can directly induce *GS2* expression [38]. In the present study, transgenic *Arabidopsis* plants harboring the full-length *PpGS2* promoter exhibited significant induction of *GUS* expression in response to GA, IAA, ABA, MeJA, low-nitrogen stress and light stress. Among these treatments, MeJA exerted the strongest inductive effect (Fig. 4), suggesting a prominent role for jasmonate signaling in regulating *PpGS2* transcription. In addition, low-nitrogen stress markedly enhanced promoter activity, implying that *PpGS2* may be transcriptionally upregulated to facilitate nitrogen reassimilation under nitrogen-limited conditions. These findings are consistent with the presence of multiple hormone and stress-responsive elements within the promoter and further confirm the responsiveness of the *PpGS2* promoter to diverse regulatory cues.

Promoter regions often exhibit functional partitioning, with distinct segments responsible for specific regulatory responses. For example, in moso bamboo, the − 578 to -493 bp region of the *PheMADS47a* promoter was identified as critical for MeJA responsiveness [39]. Similarly, analysis of the *Jatropha curcas JcGASA6* promoter revealed discrete regions harboring negative regulatory elements responsive

to SA and ABA [40]. Consistent with these observations, our deletion analysis revealed clear regional functional differentiation within the *PpGS2* promoter (Fig. 5). The GA response was primarily dependent on the − 2,000 to -1,600 bp region, suggesting the presence of cis-acting elements involved in gibberellin signal perception. ABA responsiveness was mainly associated with the − 1,200 to -800 bp region, which may function in integrating stress-related signals. In contrast, IAA and MeJA responses showed a gradual decline with promoter truncation, indicating that their regulation likely relies on the synergistic action of multiple cis-acting elements distributed across the promoter. Under low-nitrogen conditions, GUS activity exhibited bidirectional regulation: the − 1,200 to -500 bp region appeared to contain positive regulatory elements enhancing transcription, whereas the − 2,000 to -1,200 bp region may harbor repressive elements that modulate transcriptional output under nitrogen deficiency. Additionally, the core light-responsive region was localized to -1,600 to -800 bp, which may be enriched in light-responsive motifs such as G-box and GT1-motif and associated with light-regulated transcription factor binding. Together, these results provide important insights into the modular organization of the *PpGS2* promoter.

5. Conclusion

In summary, this study systematically analyzed the modular regulatory characteristics of the *PpGS2* promoter of Kentucky Sedum for the first time. Through the deletion cloning at the 5’ end, the specific distribution regions of GA, ABA, IAA, MeJA, low nitrogen and light response elements were clarified, which revealed the multi-regional synergy of hormone responses and the new positive and negative regulatory model of low nitrogen responses. At the same time, it was confirmed that the tissue specificity of its preferred expression in Arabidopsis thaliana leaves was highly consistent with the chloroplast localization function of *GS2*. These findings address a research gap regarding the synergistic regulation of the *PpGS2* promoter by hormones and environmental signals in turfgrass. Furthermore, they provide a molecular basis for breeding turfgrass varieties with improved nitrogen use efficiency and stress resistance.

Declarations

Ethics approval, **guidelines and consent to participate**

Not applicable.

Consent for publication

Not applicable.

Declaration of Competing Interest

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

Author Contribution

Shasha Liu: Writing - original draft, Investigation. Xiashun Liu: Writing - original draft, Investigation. Chen Wang: Writing - original draft, Investigation. Mengyao Sang: Investigation. Qinyi Wang: Investigation. Qiyun Wei: Investigation. Yajun Chen: Writing - review & editing. Jia Jiang: Writing - original draft, Formal analysis. Fuchun Xie: Writing - review & editing, Writing - original draft, Project administration.

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Data Availability

Data will be made available on request.

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Figures

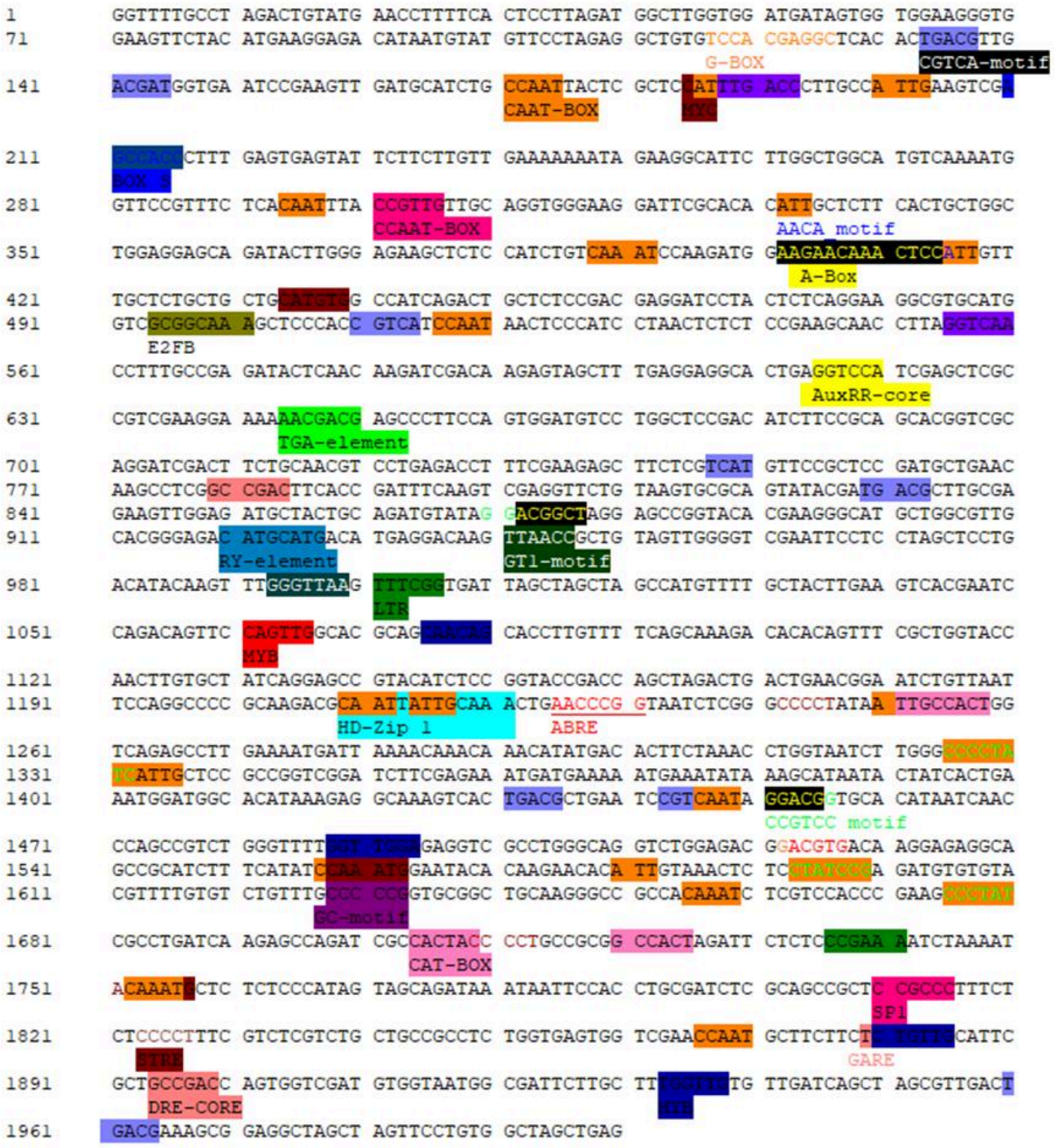

Figure 1

PpGS2 promoter sequence and cis-acting elements distribution

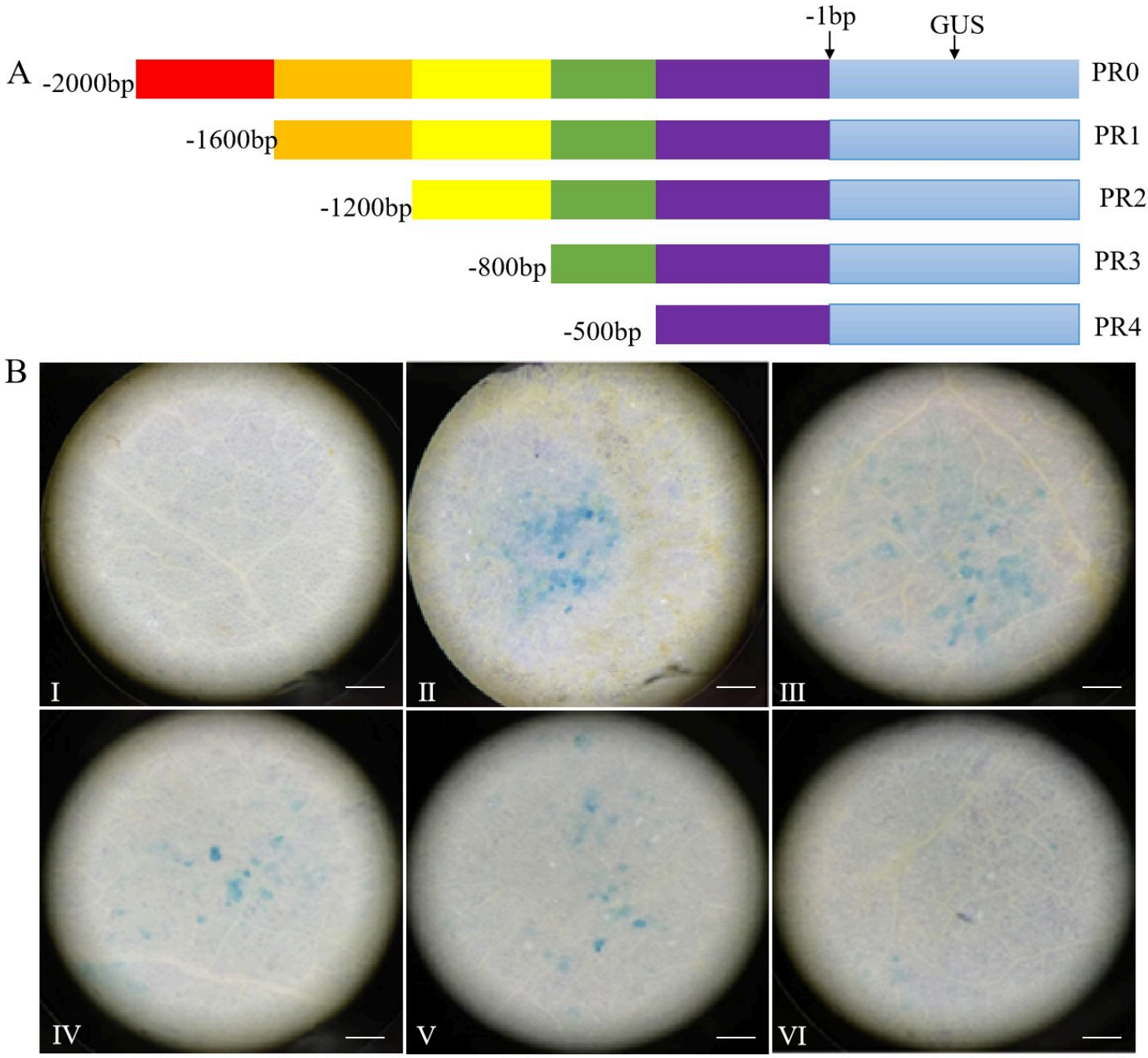

Figure 2

Activity analysis of different deletion fragments of the *PpGS2* promoter

A. Schematic illustration of 5’ end deletions of different lengths of the *PpGS2* promoter; B. Negative control (injection of empty vector, I), PR0 (II), PR1 (III), PR2 (IV), PR3 (V) and PR4 (VI) promoter fragments driving GUS histochemical staining in tobacco leaves (Scale bar: 1 mm).

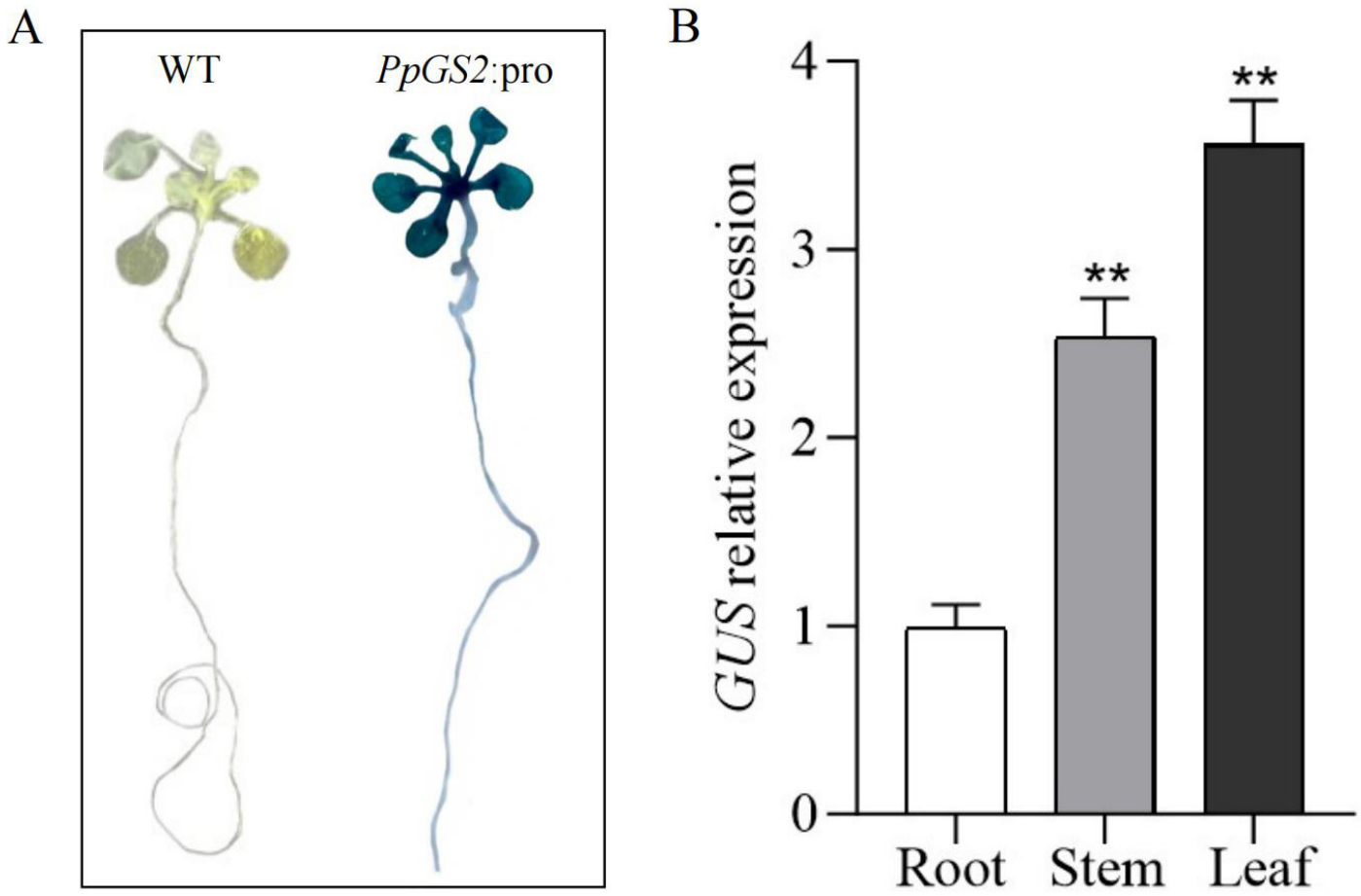

Figure 3

Tissue-specific analysis of the *PpGS2* promoter in *Arabidopsis thaliana*

A. Histochemical staining of GUS in wild-type *Arabidopsis* and *PpGS2*full-length promoter transgenic Arabidopsis; B. Detection of the relative expression of *GUS* genes in different tissues of transgenic *Arabidopsis*by qRT-PCR.

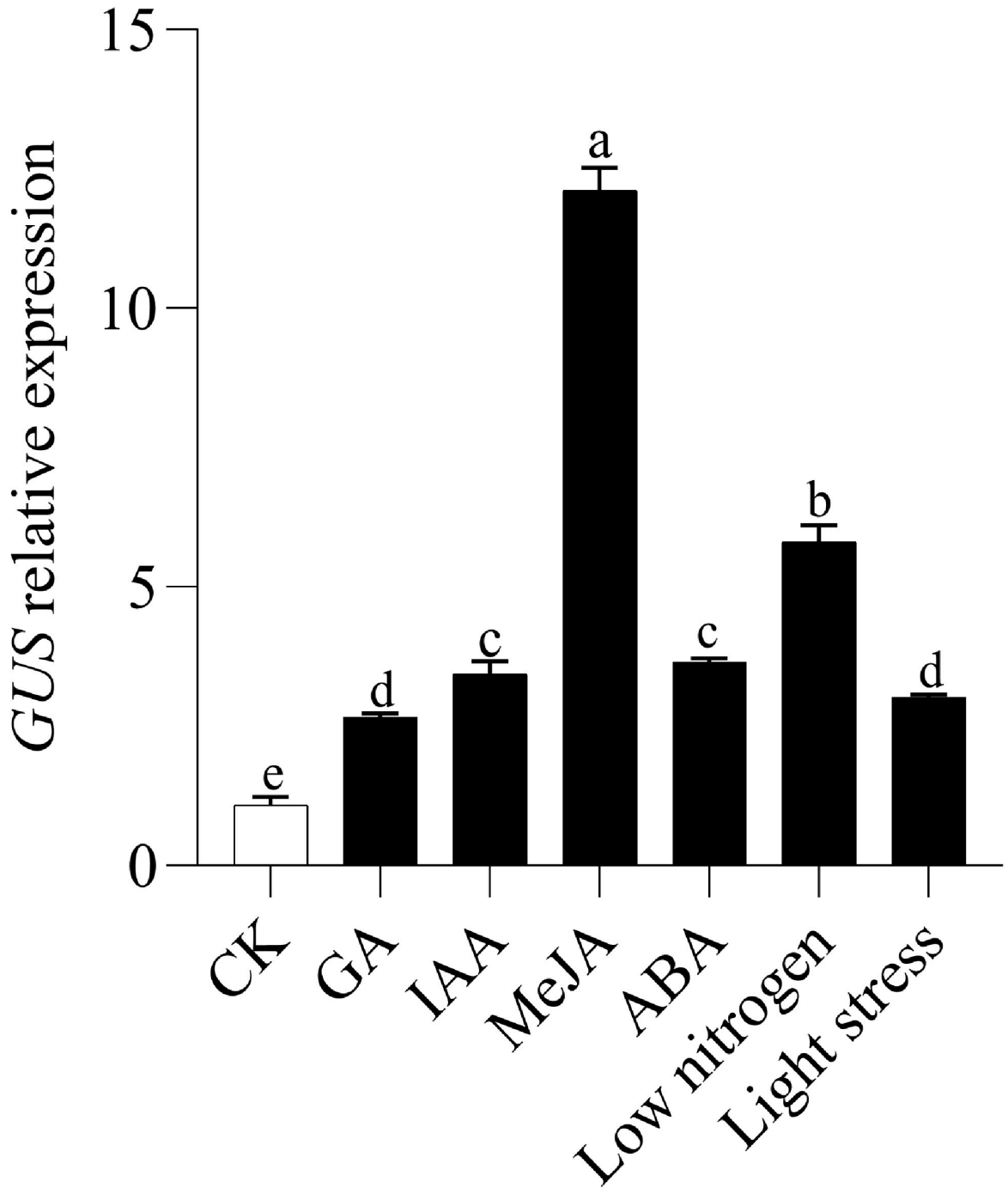

Figure 4

Analysis of *GUS* gene expression in PR0 transgenic *Arabidopsis thaliana*under different phytohormones and stress

Vertical bars indicate the standard deviation of the mean (n=6), and different letters indicate significant differences (P<0.05).

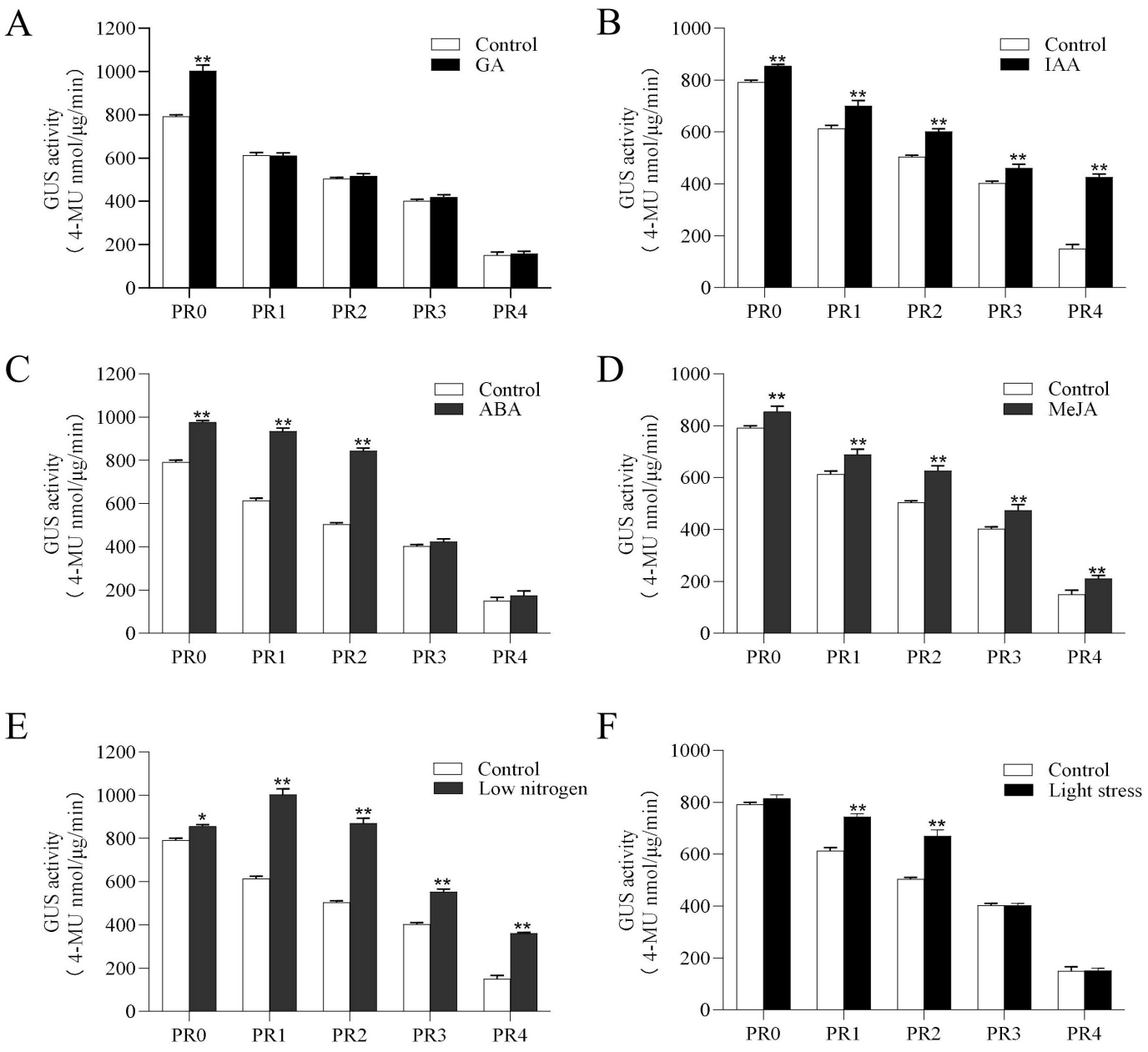

Figure 5

Analysis of GUS enzyme activity in transgenic *Arabidopsis* under abiotic stresses

A-F. Response of five PpGS2 promoter fragments of different lengths to gibberellin (GA), auxin (IAA), abscisic acid (ABA), methyl jasmonate (MeJA), low nitrogen (LN) and light treatments.

Vertical bars indicate the standard deviation of the mean (n=6) and * indicates statistical significance as determined by t-test (*p<0.05, **p<0.01).

Supplementary Files

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