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Characterization of the promoter region of the glycerol-3-phosphate-O-acyltransferase gene in *Lilium pensylvanicum*

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Abstract: Cold environmental conditions influence the growth and development of plants, causing crop reduction or even plant death. Under stress conditions, cold-inducible promoters regulate cold-related gene expression as a molecular switch. Recent studies have shown that the chloroplast-expressed *GPAT* gene plays an important role in determining cold sensitivity. However, the mechanism of the transcriptional regulation of GPAT is ambiguous. The 5'-flanking region of *GPAT* with length of 1494 bp was successfully obtained by chromosome walking from *Lilium pensylvanicum*. The *cis*-elements of *GPAT* promoters were predicted and analyzed by a plant *cis*-acting regulatory DNA element database. There exist core promoter regions including TATA-box and CAAT-box and transcription regulation regions, which involve some regulatory elements such as I-box, W-box, MYB, MYC, and DREB. Full-length and four 5'-deletion fragments linked with GUS vectors were constructed and transformed into *Nicotiana tabacum* by *Agrobacterium*-mediated transformation. Transient transformation and histochemical staining of leaves indicated that the activity of the *GPAT* promoter was strong and induced by low-temperature stress. The deletion of a –294 bp region suggested that the DRE-motif was functionally an essential element for cold induction, and the deletion of –1494 bp and –1194 bp regions suggested that negative regulation exists in the promoter. Our results show that the *GPAT* gene promoter is a key regulator under cold stress and we think that this study will have significant impact on lily molecular breeding and improving the resistance of plants.

Key words: *GPAT*, promoter, chromosome walking, *cis*-elements, 5'-deletion fragments, histochemical staining

1. Introduction

Glycerol-3-phosphate acyltransferase (GPAT) is a crucial enzyme related to plant cold resistance (Sakamoto et al., 2004; Yan et al., 2008). Previous studies have demonstrated that resistance could be improved by importing *GPAT* cDNA (Yokoi et al., 1998; Sui et al., 2007; Gupta et al., 2013; Mizoi and Yamaguchi-Shinozaki, 2013), but current studies are mostly focused on individual genes that can improve cold resistance in plants (Macková et al., 2013; Van Houtte and Vandesteene, 2013; Wang et al., 2014; Yadav et al., 2014), while associations between the promoters of these genes and resistance have not yet been reported. Plant promoters play an important role in the regulation of gene expression in plants (Kim et al., 2013; Reynolds et al., 2013). Promoters are located on the 5'-flanking region of structural genes, recognized by RNA polymerase and combined with template DNA, ensuring that transcription starts effectively and accurately. The promoter consists of two parts: the core region and upstream regulator region (Zhu and Li, 2002). There are abundant *cis*-acting elements that participate in gene regulation in the promoter (Razdan et al., 2013; Sarvestani et al., 2013). Transient transformation and histochemical staining can contribute to promoter functional analysis (Koia et al., 2013). Cloning and functional studies related to the promoters of the key genes in improving resistance of plants will aid us in understanding its signal transduction pathways and gene expression patterns.

According to gene expression, promoters can be divided into two categories: constitutive promoters and specific

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promoters. Constitutive promoters can be transcribed at any time in all cells. Specific promoters can be divided into the tissue specificity of the promoter and the induced promoter (Watanabe et al., 2014). Induced promoter transcriptional activity is restricted and very low, but the activity is significantly improved under the stimulus of signals from adverse stress (Wang et al., 2013). Transgenic plants that were imported with constitutive promoters have excessive exogenous gene expression, which causes accumulation of metabolites that could limit the growth and lead to cell death (Su and Wu, 2004). Therefore, it is best to clone and use induced promoters to cultivate stress-tolerant crops. Analyzing inducible promoters contributes to understanding the mechanism of gene expression and regulation. The application of inducible promoters has important implications for improving the resistance of *Lilium pensylvanicum*.

2. Materials and methods

2.1. Plant materials and pretreatment

Lilium pensylvanicum bulbs, which had been domesticated from wild species over 3 years, were kindly provided by Daxinganling Forestry Bureau, Inner Mongolia Province, China. *L. pensylvanicum* possesses strong cold tolerance; it was stored under 10 cm of soil in winter and could survive at temperatures from 50 °C to below 0 °C. The bulbs from *L. pensylvanicum* were separated and washed by running water, then soaked in 75% ethanol for 30 s and then placed in 0.1% $HgCl_2$ solution for 10 min. Subsequently, they were rinsed with sterilized water three times. Finally, they were placed on dry sterile filter paper for further use. The sterile bulbs were cultured on MS medium supplemented with agar powder (7 g L^{-1}) and sucrose (30 g L^{-1}), pH 5.8. The fluorescent light was set at 14 h every day with a light intensity of 1000–1200 lx. The temperature was adjusted from 23 to 26 °C. Two months later, the adventitious buds were removed from the bulbs and placed at 4 °C for 12 h; they were later used for DNA extraction or were immediately frozen in liquid N_2 and stored in an ultralow-temperature freezer at –80 °C until needed.

2.2. Construction of DNA library

Total DNA was extracted from leaf samples by the cetyltrimethylammonium bromide method (Barzegari et al., 2010). The genomic DNA was digested with restriction endonucleases *DraI*, *EcoRV*, *StuI*, and *PvuII*, followed by purification with 3 M NaOAc (pH 4.5), respectively. After that, they were ligated separately to the adaptor protein (GW-AP1, GW-AP2) to construct genomic walking libraries according to the procedure described in the Genome Walker Universal Kit User Manual (Clontech, Palo Alto, CA, USA).

2.3. Amplification of *GPAT* promoter and bioinformatics analysis

Two specific primers (GPATpR1 and GPATpR2) were designed and synthesized (Shanghai Sangon, Shanghai, China) based on the *GPAT* coding sequences, and the adapter primers (AP1 and AP2) were synthesized according to the user manual (Clontech). The primers used are shown in Table 1. The *GPAT* promoters were cloned with nested PCR (Jones et al., 1993). GPATpR1, AP1, and the constructed DNA libraries were used in the first step of PCR amplification with the following conditions: 94 °C for 5 min, followed by 7 cycles of amplification at 94 °C for 30 s and 72 °C for 3 min, followed by 32 cycles of amplification at 94 °C for 30 s, 67 °C for 3 min, and at 72 °C for 10 min. The product was diluted 50-fold and then used in nested PCR using the AP2 and GPATpR2 primers under the same conditions as in the first step. PCR products were resolved on 1.2% agarose gel electrophoresis. The second PCR product was purified, cloned into the pMD18-T vector (TaKaRa, Dalian, China), and sequenced. The DNA sequences obtained were confirmed using BLAST tools (http://blast.ncbi.nlm.nih.gov/) and sequenced (Shanghai Sangon). These sequences were analyzed using the plant *cis*-acting regulatory DNA elements database (http://www.dna.affrc.go.jp/htdocs/NewPLACE/) (Higo et al., 1999).

2.4. Construction of *GPAT* promoter deletion-GUS vectors

The transcriptional fusion of the *GPAT* promoter and the *GUS* gene was achieved using the pCAMBIA1301

Table 1. Primers used in the amplification of promoter.

Name	Sequence (5'-3')
GW-AP1	GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGT
GW-AP2	PO4-ACCAGCCC-H2N
AP1	GTAATACGACTCACTATAGGGC
AP2	ACTATAGGGCACGCGTGGT
GPATpR1	TGAATATAAGAGAGCAGTTCTT
GPATpR2	GTCCCCTCCGCCGGCTCCGCCA

GUS vector. The *GUS* reporter gene expresses under the control of the CaMV35S promoter in the vector. To construct GPAT promoter–GUS expression vectors, five 5'-fragments were isolated from the cloned promoter and amplified by PCR using five forward primers with the *BamHI* site and a reverse primer with the *BglII* site (Table 2), and the T-vector was ligated with *GPAT* promoter regions as a template. PCR amplification was done using the following program: predenaturation at 94 °C for 5 min, followed by 32 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 1 min, and extension at 72 °C for 90 s followed by a final extension at 72 °C for 10 min. The CaMV35S promoter was removed by BamHI/BglII digestion in pCAMBIA1301, followed by the insertion of five successfully amplified fragments, respectively. The vectors were named GPATp(1–5)::GUS-pCAMBIA1301, and PCR amplification by GPATpQF(1–5) and GPATpQR confirmed the validity of the recombinant vectors. PCR products were resolved by 1.2% agarose gel electrophoresis.

2.5. Agrobacterium transformation of tobacco leaf disks

Tobacco (*Nicotiana tabacum* L. variety K326) leaf disks were transformed with *Agrobacterium tumefaciens* LBA4404 (Marton et al., 1979; Bevan, 1984; Valvekens et al., 1988) containing the five constructs GPATp(1–5)::GUS-pCAMBIA1301. The tobacco leaves were cocultivated with bacteria on solid medium (1/2 MS + 2.0 mg/L 6-BA + 0.1 mg/L NAA) at 28 °C for 2 days, transferred onto the same solid medium containing 400 mg/mL cefotaxime to exclude bacteria, and cultivated in dark conditions at 28 °C.

2.6. GUS enzyme assays

GUS (β-glucuronidase) activity was assayed using the method of transient transformation with a chromogenic substrate X-Gluc (5-bromo-4-chloro-3-indyle-β-D-glucuronide) (Jefferson et al., 1987). The leaves were placed in 8 mL of solution containing 50 mM sodium phosphate buffer (pH 7.0), 10 mM EDTA-Na_2, 0.1% (v/v) Triton X-100, and 0.1% β-mercaptoethanol, supplemented with 160 µL of X-Gluc solution (50 mg/mL of X-Gluc dissolved in N,N-dimethylformamide). The reaction was incubated at 37 °C for 16 h. After that, the leaves were immersed in 75% ethanol to remove chlorophyll, until the negative control turned white, and photographed with a camera (Battraw et al., 1990). The experimental groups are shown in Table 3.

3. Results and discussion

3.1. Cloning of the 5'-flanking region of the *GPAT* gene from *Lilium pensylvanicum* by chromosome walking and sequences analysis

Chromosome walking is a cloning technology commonly used to clone flanking sequences of a known fragment (Siebert et al., 1995). This method can be divided into three categories: inverse PCR, ligation-mediated PCR such as

Table 2. Primers used in the vector construction.

Name	Sequence (5'-3')
GPATpQF1	AGCGGATCCTGAAGAGGATATACACC
GPATpQF2	CACGGATCCCTTGAAAAACAGACATTCTG
GPATpQF3	AGCGGATCCCGCCTTATATTACTATGGAG
GPATpQF4	AGAGGATCCACTCTCTACCACCACCTC
GPATpQF5	GATTGGATCCATCTTAGCTGGCCATGATCG
GPATpQR	CGCAGATCTACTAACACGTACAATCCTT

Note: Restriction site sequences are underlined.

Table 3. Groups used for GUS staining.

Temperature	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7
Low temperature (4 °C)	GPATpQ1::GUS	GPATpQ2::GUS	GPATpQ3::GUS	GPATpQ4::GUS	GPATpQ5::GUS	35s::GUS	Negative control
Room temperature (25 °C)	GPATpQ1::GUS	GPATpQ2::GUS	GPATpQ3::GUS	GPATpQ4::GUS	GPATpQ5::GUS	35s::GUS	Negative control

adaptor/linker ligation PCR, and randomly primed PCR such as tail PCR (Yan et al., 2003). In this study, the adapter PCR method was used to clone the *GPAT* promoter. Nested PCR is a method to amplify specific sequences of DNA. Two pairs of PCR primers were used in the reaction: the first pair amplified the desired sequence, while the second pair bonded with the first PCR product and yielded a shorter product than the first. This method could increase the sensitivity and reliability of the PCR (Antal et al., 2004; Shimano et al., 2009).

The full length of *GPAT* has been previously isolated (GenBank ID: JX524741). In this study, chromosome walking and nested PCR were performed on the DNA library that was digested with restriction endonuclease *PvuII*. A 5'-flanking fragment of the *GPAT* gene was amplified including 1494 bp upstream of the initiation codon (ATG) and named as GPATp, which is shown in Figure 1. The nucleotide sequence of this product is shown in Figure 2.

Using the NewPLACE database, the transcription start site was predicted based on the structure of promoter in eukaryotes (Figure 3). There are various *cis*-acting regulatory elements upstream of the translation initiation site (Figure 3). Their functions and locations in the promoter are listed in Table 4.

The *GPAT* promoter sequence contains several important *cis*-regulatory elements, such as TATA-box, CAAT-box, GATA-box, and I-boxes. Bioinformatic analysis revealed 7 TATA-boxes in the promoter region. It was usually located 25–30 bp upstream of the transcription start site, combined with RNA polymerase II, affecting the rate of transcription (Smale and Kadonaga, 2003; Hapala and Trifonov, 2013; Murakami et al., 2013; Bushnell et al., 2014). In the *GPAT* promoter, the nearest TATA-box from the forecasted transcription initiation site was 24 bp upstream. Another *cis*-element, CAAT-box, usually occurs

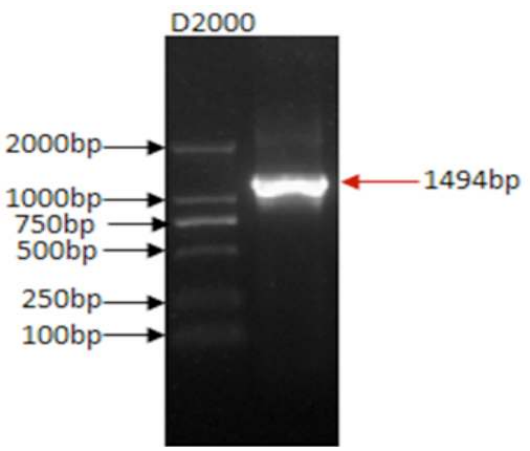

Figure 1. Agarose gel electrophoresis of 5'-flanking regions of *GPAT* gene. Products are marked by a red arrow.

75–80 bp upstream of the initial transcription site, which is also the binding site for the RNA transcription factor and typically accompanied by a conserved consensus sequence. It controls the transcription initiation frequency and impacts the conversion rates of the target gene (Edwards et al., 1998; Hogekamp et al., 2011; Singh et al., 2013).

In addition to the essential *cis*-acting elements, other corresponding stress-related *cis*-elements in the promoter region were also predicted by the NewPLACE database, such as I box, MYB-Core, MYC-Core, W-box, DRE-Core, ABRE, and others. MYB-core binding sites (T/CAACNA/G) and MYC-core recognition sites (CANNTG) were noted in the region. Both have been shown to play important roles in plant responses to salt, low temperature, and drought (Miura et al., 2012; Yang et al., 2012; Feng et al., 2013; Su et al., 2014). Furthermore, several hormones could influence the relevant promoter element (Li et al., 2013). For example, the ABA motif was induced by ABA, a classical phytohormone that plays a crucial role in plant growth and in stress or drought tolerance (Redman et al., 2002; Nakashima et al., 2014). The W-box (TGAC-motif) is a GA-responsive element and binds to WRKY proteins to regulate target gene expression. It can be induced by pathogens, wounds, signal molecules, or senescence, and it also participates in various biological processes or metabolism (Eulgem et al., 2000; Tripathi et al., 2013). DRE/CRT is a cold-induced element and binds to CBF transcriptional activators, followed by the expression of CBF-targeted genes that increase freezing tolerance. Repeated domains containing the conserved sequence CCGAC have been found in the regulation of all the cold-induced genes (Kasuga et al., 1999; Morran et al., 2011; Hudson, 2013; Li et al., 2014).

The cold-related elements in the promoter, such as MYB, MYC, and DREB, also demonstrate that the regulation of the promoter is induced by temperature; some light-responsive elements were found in the promoter, such as I-box and GATA-box. Researchers demonstrated that photoperiod was connected with plant stress tolerance (Van Huystee et al., 1967; Maibam et al., 2013). CaM can combine with Ca^{2+}, which is a messenger in multiple signaling pathways and activates or regulates gene expression. The mechanism of Ca^{2+} has been a popular research subject in recent years.

3.2. Deletion analysis of *GPAT* promoter and construction of recombinant expression vectors for *GPAT* promoter–*GUS* gene

The current method to study promoter activity is focusing on a reporter gene coding GUS or green fluorescent protein (GFP) (Chiu et al., 1996; Peach and Velten, 1996; Ducrest et al., 2002). The pCAMBIA1301 vector displays a hygromycin gene and a *GUS* gene driven by a double *35S* gene from the cauliflower mosaic virus. The promoter

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1       TGAAGAGGAT ATACACCTAT ATATATCGCT CTAGGTTTCC CCTTTGTTTT ATTCCGTTTG
61      AGTCATCCAA GAGTCAACAC ATACTCCAAA CAAACCTTGG GAAGAAGAAT AGCCAATTGT
121     TCATCCAAGA GCCATACTGA TTTTCAGATT TCTTGATATG CTGATTTGTA ACCCAAATTC
181     GAAGCTGTTG GACACGTTTT GAGGTCTTGA CCCGAACAGA TAAATTGTAG ATGAATTCCT
241     GAAGTTATCG TTACATATAT GGTACGTCAA AAAATACGTT CGGACCTGAA AGATATGCCA
301     CTTGAAAAAC AGACATTCTG TAGGAATTCA GAATGCTGAA AACAGTTTTT CTCCCCTAGA
361     AAGATAAACA TGAACCATTT TATTTACACG CATCTTGACA TGCCCTTGAA TATAATTCAC
421     TCTTTAGGGA TTTTTGTTGA TCTCTATTTC ATTTATTTGA AGGATGAGTA CTAAGACCAC
481     AGAGTGATAA ATACCACTGC TGCTTGATAG GATATAGCTA ATCTTCAGGG CCAGACAAAA
541     GTTTTTGTTG GAGAATTTCC TAAAAAAGAT CCTTTAAGTT CCTCATCACA CCGCCTTATA
601     TTACTATGGA GATTTTAAAT CATTAATAAA TTCCTCCTTT ATAATGATTT TTATTTTTTA
661     TTGTAAAACC AATATGAGTT CTAACAACCA ATAATTTCTG AGTGTTTGTT TTTCATCTTC
721     TTAGTAAGGG AAGATCACTT TGGTTGGACA AAAATAAATA CTACAAAAAT TTCAAATGGC
781     TAAGAGTAAT TGGTTGTGCA CCCACCTCTA GTTCTATGAC AAATTACTCG AATACCCCGA
841     TAAGAATGTT AGTCATGATC AGCACCTCAA CCCATGGCTC GGCGGTCATC GTCTTCCGCG
901     ACTCTCTACC ACCACCTCCC TTCCACCGGC GCCGTCCACC GACCATACTT CCGCGGCCCG
961     CCAAACCCTC ACCCCCATCC TTCCACGGGC GCCCATCGGC AGCCATCTTT TGGTGGTTCG
1021    CCAGGACCGT ACCCGCATTC TTCCACCGGC AACCATGGAT TTAGGGCTTC CGGCAGCTTT
1081    GCTGGCCAAG TTTTCTCGTA CCACTGCTCG TCCGTCAATT GTTTCAGGGA TACTGGCAAC
1141    TTTGCTGGCC ACGACCTCTC GAACCAACAC CTGTCCGTTC AGAGATTTAG GGATTCCGGC
1201    ATCTTAGCTG GCCATGATCG CTTGAACAAC CACCCATCCG TCCAAGGGTT CAGAGCTGGT
1261    GGAGGGACTG CAGCCAAAGT TGGGTCTAGC AAAGTATCCG AGACTTTGAG CTCCAAGTCT
1321    TCTGCCGTGC TCCGTCAAAA CAATGGTACC AAGAAAGAGT GGCGGCCGCG ACCTCTCGGG
1381    CCATCTTTAG TTCATGCTTT GCCAACAACA ATTTGTGACT CTGTGGCAAA GCCCGTCGTT
1441    CATATATAAC CTCGTGTGGT TGGCTCATCC AACGAAAGGA TTGTACGTGT TAGTATGCTA
1501    CGTCGGGAAC CGTGGCATTG CCCATGTGTG GCGTATAGGG CGCGGGCGAT GACGGAGCCT
1561    ACGCGGGCGA TGGCGGAGCC GGCGGAGGGG ACGGTGGCGA GGGGTTGCAG GGCGGTGCTG
1621    GAGTCGGAGG CGAGAT
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Figure 2. Nucleotide sequences of *GPAT* promoter (1–1494 bp). The translational start codon ATG is underlined and part sequences are on a gray background (1495–1637 bp).

controls the *GUS* reporter gene and a NOS terminator (Chalfun-Junior et al., 2003). The activity of the *GPAT* promoter was studied using this vector by replacing the CaMV35S promoter with the *GPAT* promoter. This recombinant expression vector and the empty plasmid were used for *Agrobacterium*-mediated transformation and infection of tobacco plants.

Sequential deletions of the promoter were cloned and sequences were 1494 bp, 1194 bp, 894 bp, 594 bp, and 294 bp in length. The schematic map of the five deleted constructs are shown in Figure 4. The constructed five *GUS* gene expression vectors of sequential segments of promoter linked with the *GUS* gene were as follows; GPATpQ1::GUS (–1494 to 28), GPATpQ2::GUS (–1194 to 28), GPATpQ3::GUS (–894 to 28), GPATpQ4::GUS (–594 to 28), and GPATpQ5::GUS (–294 to 28). PCR amplification of recombinant expression vectors using primer GPATpQF(1–5)/GPATpQR is shown in Figure 5. PCR results indicated that the gene has probably been integrated into the vector.

3.3. Transient expression of the *GUS* gene

Histochemical GUS staining on young transgenic tobacco leaves revealed GUS activity. It indicated that the *GUS* gene, driven by the *GPAT* promoter, showed expression and that there was a positive correlation between the depth of shade and gene expression quantity. Thus, GUS activity increased with deeper color, which illustrated that the activity of the promoter was stronger (Figures 6A–6N). In Figures 6C–6J, the color normally darkens along with the elongation of fragment. In some cases, however, such regularities were not observed (Figures 6A and 6B). In Figures 6A–6L, the color shows different depths at different temperatures. The color at low temperatures was deeper than at room temperature. The level of GUS activity obtained with the *35S-GUS* fusion gene was slightly lower than those obtained with the *GPATpQ-GUS* fusion gene (Figures 6K and 6L). These results indicate that the *GPAT* promoter has higher activity than *35S* in response to cold temperatures.

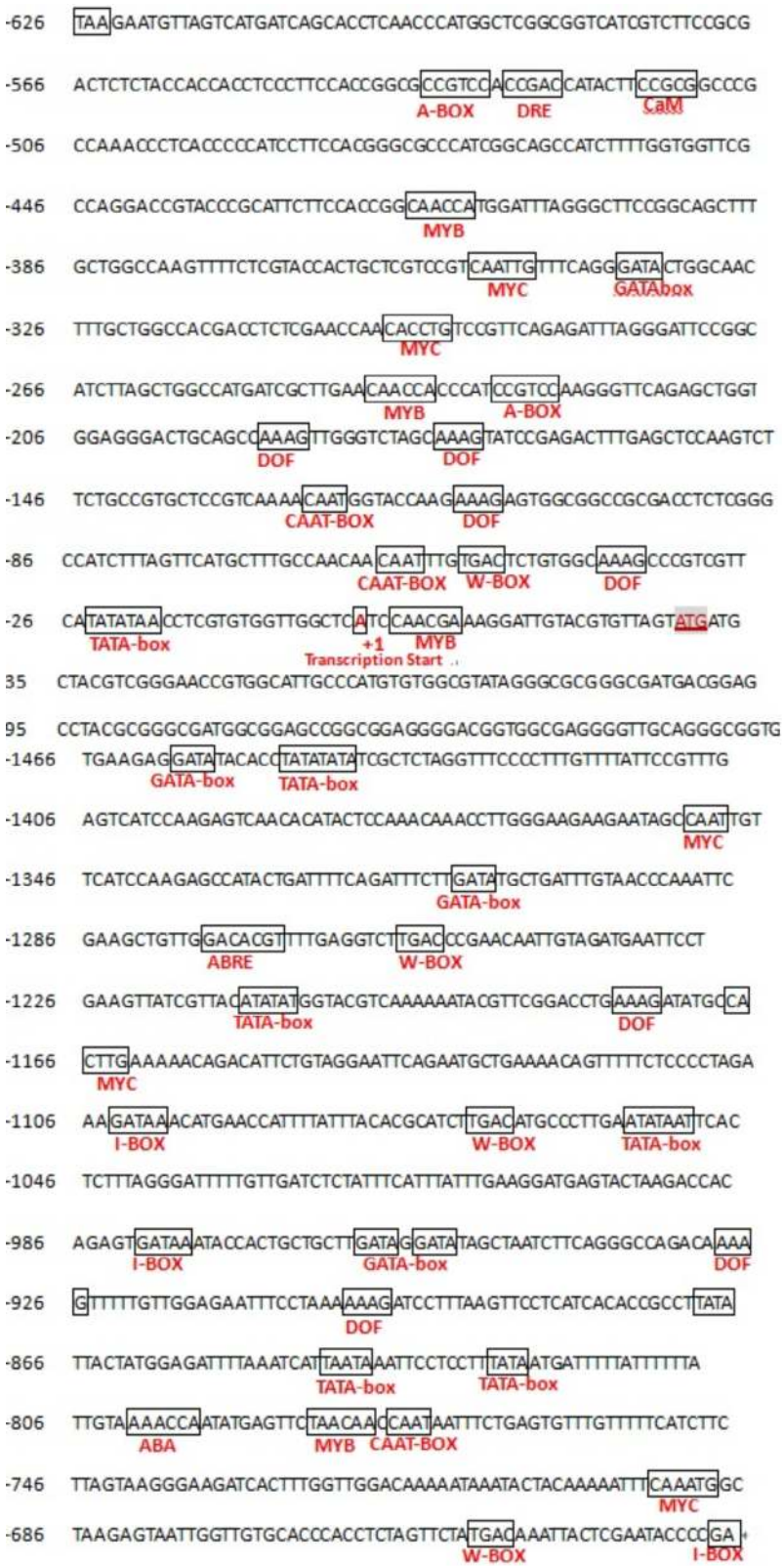

Figure 3. Sequence analysis of the *GPAT* promoter. The transcription start site is defined as +1; numbers indicate the positions relative to the transcription start site. The putative TATA-box, CAAT-box, and other elements are boxed. The start codon ATG is underlined and on a gray background.

Table 4. Putative *cis*-acting regulatory elements identified in the promoter region of *GPAT* using the NewPLACE database.

Element	Signal sequence*	Expected function	Position
MYB	T/CAACNA/G	Regulates the transcription in the abiotic stress response, related to *CBF* gene expression	+4/–240/–417/–785
MYC	CANNTG	Regulates the transcription in the cold and dehydration response	–299/–351/–694/–1168/–1353
ABRE	ACGTGG/TC	Response to dehydration and high-salinity stresses	–1271
DRE	CCGAC	Core motif of DRE/CRT	–528
CaM	VCGCGB	Ca^{2+} DNA-binding protein in multiple signaling pathways	–516
I-box	GATAA	Light-regulated element	–628/–981/–1104
W-box	TGAC	WRKY-binding site involved in pathogenesis activation and negative regulatory elements	–650/–1071/–1254
ABA-Core	WAACCA	Transcriptional activators in abscisic acid signaling	–801
Dof-Core	AAAG	Transcriptional activator.	–39/–113/–176/–191/–902/–928/–1178
GATA-box	GATA	Light-regulated and tissue-specific expression	–338/–961/–956/–1312/–1459
A-box	CCGTCC	Confers elicitor or light responsiveness	–535
TATA-box	TATA	Core promoter element around –30 of transcription start	–24/–827/–843/–870–1057/–1212/–1449
CAAT-box	CAAT	Common *cis*-acting element in promoter and enhancer regions	–58/–126/–778/–1459

*N indicates A, C, G, or T; W indicates A or T; V indicates A, C, or G.

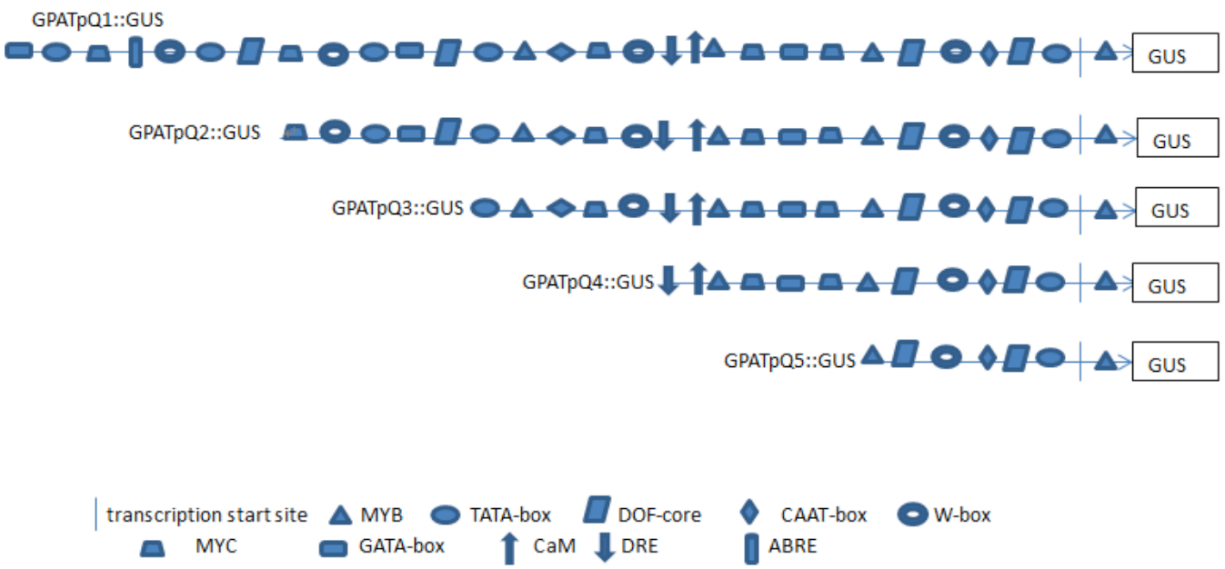

Figure 4. Deletion analysis of the *GPAT* promoter fused to the *GUS* gene. It shows a schematic map of the promoter deletion constructs. The elements are marked by different graphics.

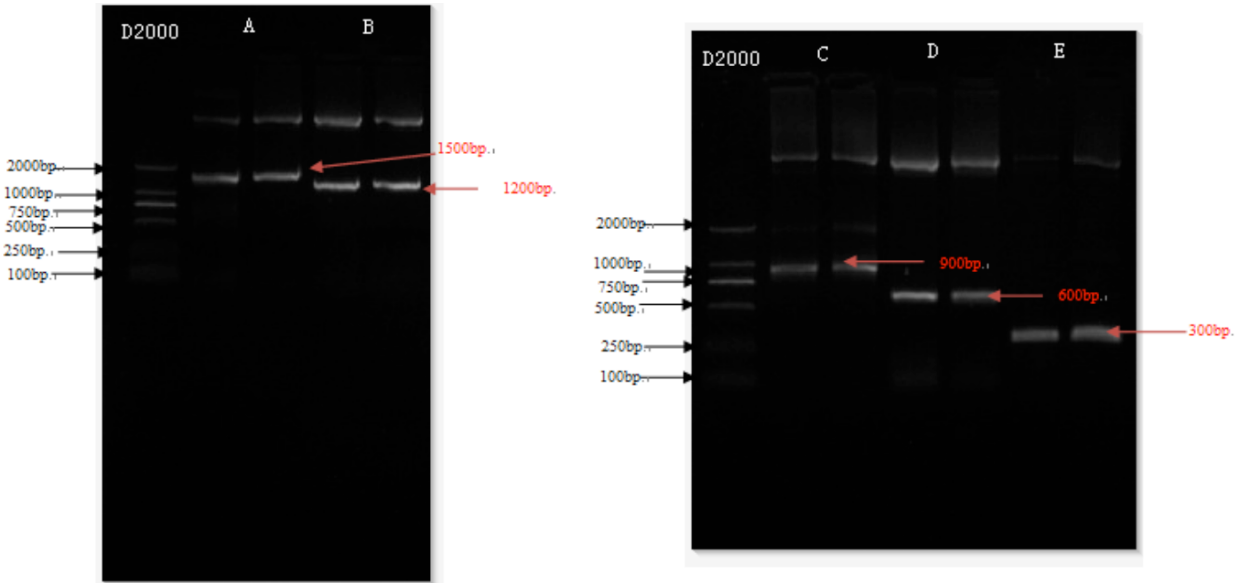

Figure 5. Agarose gel electrophoresis of PCR amplification of recombinant expression vectors. A) Products from GPATpQ1::GUS, marked at 1500 bp with a red arrow; B) products from GPATpQ2::GUS, marked at 1200 bp with a red arrow; C) products from GPATpQ3::GUS, marked at 900 bp with a red arrow; D) products from GPATpQ4::GUS, marked at 600 bp with a red arrow; E) products from GPATpQ5::GUS, marked at 300 bp with a red arrow.

GUS staining is darker with a stronger promoter (Liu et al., 2013). In this study, the darkest GUS staining was 1194 bp, while the lightest was 294 bp. Comparing the two species, there was no DRE element that was directly linked to clod resistance in the latter. This suggests that the DRE element is crucial to start the downstream gene transcription under cold treatment. The quantity of the *cis*-element was gradually increased, along with sequence length. Concerning the color of leaves in transfer of GPATpQ1::GUS and GPATpQ2::GUS, the color of the former was lighter, which showed that the activity of the larger fragment was not the strongest. There might exist negative regulatory elements in the 300 bp or upstream, which stems gene expression. *GPAT* gene expression might be regulated by the positive and negative control; the mechanism of regulation should be further studied.

The constitutive promoter *35S* is widely used as a strong promoter in plant genetic engineering, but this promoter may cause metabolic waste in the plant, because it comes from the cauliflower mosaic virus. Researchers have put forward the biological safety of this promoter. The application of a promoter from plants themselves was spontaneous (Battraw et al., 1990; Ho et al., 1999; Rezazadeh et al., 2013). This promoter from *Lilium pensylvanicum* could safely activate the gene, and *GPAT* promoter activity was slightly higher than that of the *35S* promoter under low temperature. This promoter could replace the *35S* promoter in executive function and could reduce the negative effects on plants.

Promoter function analysis methods include other experimental analyses, such as dot mutation, gel retardation assay, yeast one-hybridization, transient expression transformation, and stable expression transformation (Su et al., 2014). In this study, the promoter validation was carried out with tobacco; however, more detailed experimental verification is needed to understand the role of various *cis*-regulatory elements present on it. Fluorogenic quantitative PCR is a more powerful way to verify the accuracy of the present study. This study would provide a preliminary validation of the *GPAT* promoter.

In this study, a *GPAT* promoter was successfully cloned and analyzed using the NewPLACE database. Transient expression and histochemical staining of the *GUS* gene suggests that the *GPAT* promoter of *Lilium pensylvanicum* is a cold-inducible promoter that can regulate expression under low temperature. The deletion of a –294 bp region suggested that the DREB-motif was a functionally essential element for cold induction, and the deletion of –1494 bp and –1194 bp regions suggests that there is negative regulation in the region of 300 bp or upstream. The results of this work will help to understand the relationship between promoters and genes.

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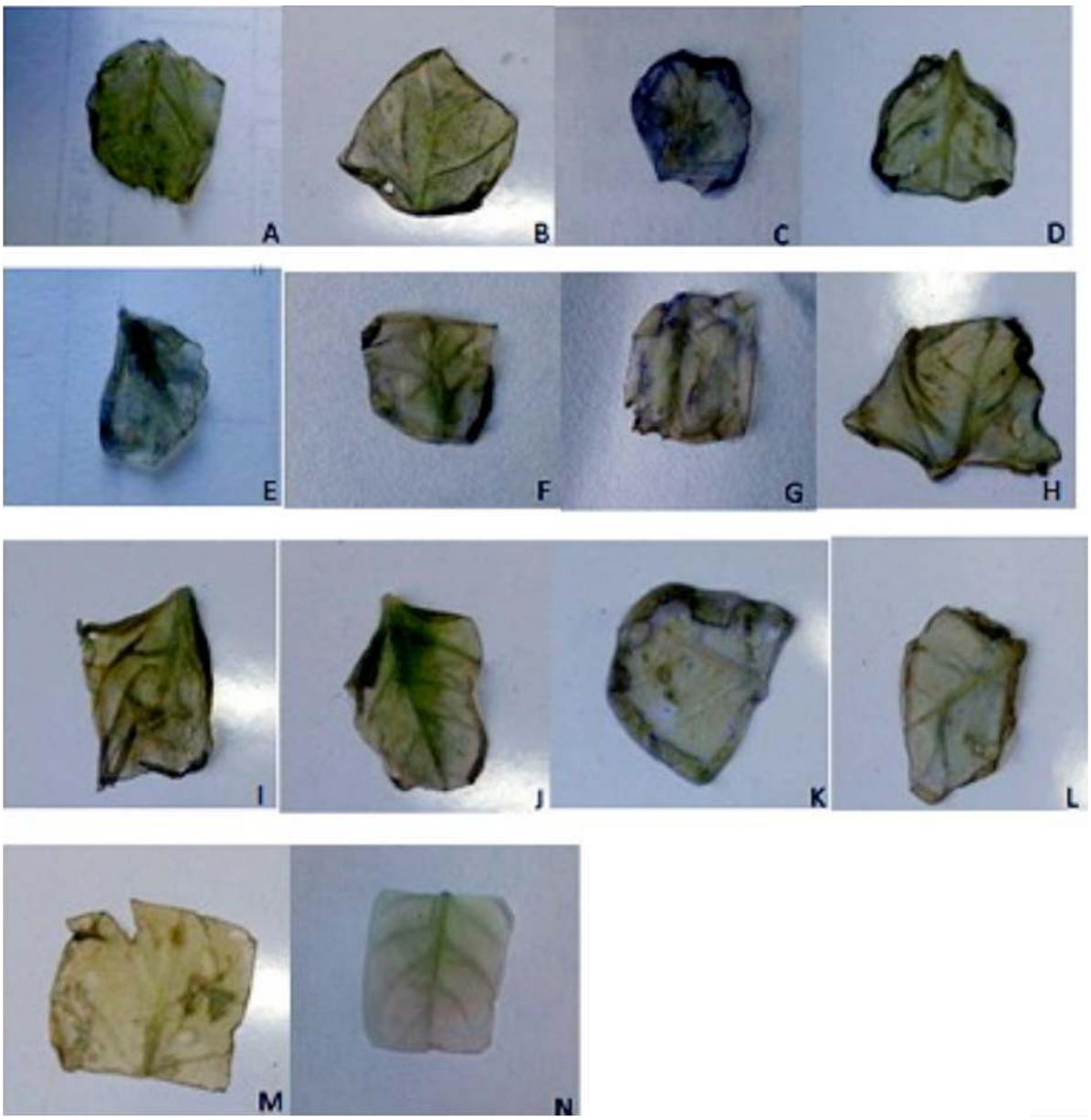

Figure 6. Histochemical analysis of *GPAT* promoter regulating GUS expression in leaves of *Nicotiana tabacum*. A) 4 °C, GUS staining result of GPATpQ1::GUS; B) room temperature, GUS staining result of GPATpQ1::GUS; C) 4 °C, GUS staining result of GPATpQ2::GUS; D) room temperature, GUS staining result of GPATpQ2::GUS; E) 4 °C, GUS staining result of GPATpQ3::GUS; F) room temperature, GUS staining result of GPATpQ3::GUS; G) 4 °C, GUS staining result of GPATpQ4::GUS; H) room temperature, GUS staining result of GPATpQ4::GUS; I) 4 °C, GUS staining result of GPATpQ5::GUS; J) room temperature, GUS staining result of GPATpQ5::GUS; K) 4 °C, GUS staining result of 35s::GUS; L) room temperature, GUS staining result of 35s::GUS; M) 4 °C, GUS staining result of negative control; N) room temperature, GUS staining result of negative control.

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