# Isolation and functional characterization of cold-induced gene (AmCIP) promoter from Ammopiptanthus mongolicus

**Bohao Ge**

1020421013@qq.com

Beijing Forestry University College of Biological Sciences and Biotechnology https://orcid.org/0009-0006-9350-8584

**Kuo Dong**

Beijing Forestry University College of Biological Sciences and Biotechnology

**Rongchen Li**

Beijing Forestry University College of Biological Sciences and Biotechnology

**Xiaorui Bi**

Beijing Forestry University College of Biological Sciences and Biotechnology

**Qianru Liu**

Beijing Forestry University College of Biological Sciences and Biotechnology

**Weiwei Zhang**

Beijing Forestry University College of Biological Sciences and Biotechnology

**Yuzhen Chen**

Beijing Forestry University College of Biological Sciences and Biotechnology https://orcid.org/0000-0002-3391-8841

**Cunfu Lu**

Beijing Forestry University College of Biological Sciences and Biotechnology

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# Abstract

AmCIP is a dehydrin-like protein which involved in abiotic stress tolerance in xerophytes evergreen woody plant *A. mongolicus*. *AmCIP* could be induced in the cotyledon and radicle during cold acclimation. To further elucidate the regulation of the upstream region of the gene, we isolated and characterized the promoter of *AmCIP*. Herein, a 1048 bp 5'-flanking region of *AmCIP* genomic DNA was isolated and cloned by Genome walking from *A. mongolicus* and the segment sequence was identified as "PrAmCIP" promoter. Analysis of the promoter sequence revealed the presences of some basic cis-acting elements, which were related to various environmental stresses and plant hormones. GUS histochemical staining showed that *PrAmCIP* was induced by 4℃, 55℃, NaCl, drought and ABA, whereas it could hardly drive GUS gene expression under normal conditions. Furthermore, we constructed three deletion fragments and genetically transformed them into *Arabidopsis thaliana*. GUS histochemical staining showed that the MYCATERD1 element of the CP7 fragment(-122~-1)may be a key element in response to drought. In conclusion, we provide an inducible promoter, *PrAmCIP*, which can be applied to the development of transgenic plants under abiotic stresses.


# Introduction

Dehydrins (DHNs) belongs to the late embryonic abundance proteins (LEA), which are usually abundantly expressed in the late stage of seed growth and development (Koag et al. 2009). Dehydrins can be classified into five subfamilies based on their conserved motifs: Kn, SKn, KnS, YnKn, and YnSKn (Close 1996). It has been shown that different types of dehydrins can respond to different external factors, for example, YnSKn dehydrins can be induced by salt and drought (Wang et al, 2014), and KnS, Kn, SKn and YnKn can be induced by cold stress (Graether and Boddington 2014). Dehydrins play important functions in ROS detoxification systems, biotic and abiotic stresses (Riyazuddin et al. 2021; Yang et al. 2012; Cui et al. 2020). Nowadays, dehydrin genes have been isolated and identified in different plants, such as rice (Verma et al. 2017; Kuma et al. 2018; Ganguly et al. 2020), wheat (Brini et al. 2007; Saibi et al. 2015), tomato (Guo et al. 2017). Most studies have shown that dehydrin genes are respond to biotic and abiotic stresses (Abdul Aziz et al. 2021).

As functional elements that participate in gene expression and regulation, promoters serve as transcriptional regulatory centers that control temporal and spatial gene expression patterns. Currently, the promoters of several dehydrin genes have been reported to have many cis-acting elements in the promoter region for both adversity and hormone response (Zhu et al. 2014; Qin and Qin 2016; Lv et al. 2017). DREB/CBF transcription factors are able to enhance plant resistance to cold stress by binding to CRT sequences in the dehydrin promoter region and thereby regulating the expression of dehydrin genes (Vazquez Hernandez et al. 2017). H3K4me3 positively regulates the binding of OsbZIP23 to the dehydrin promoter by modification thereby enhancing the expression of the dehydrin gene under drought stress (Zong et al 2020). Despite of significant progress in the analysis of the structure of dehydrin gene promoters, revealing the specific regulation and function of dehydrin gene promoters in different plants is an actual challenge.

*Ammopiptanthus mongolicus* as the only evergreen broad-leaved shrub in the deserts of eastern China, can survive in -30°C or even lower in the winter and is an ideal shrub for the investigation in the cold resistance mechanism (Xu et al. 2002; Liu et al. 2006; Liu et al. 2013). In previous studies, we have identified a cDNA, *AmCIP*, encoding a novel 15-mer K-segment of HKEGLVDKIKDKVHG similar to the consensus sequence EEKKGIMDKIKELPG of typical dehydrins protein from a cold-acclimated *A. mongolicus* seedling (Liu et al. 2006). We found that AmCIP enhanced cold resistance not only in transformed *E.coli* but also in transgene tobacco (Shi et al. 2016). However, little is known about the promoter information of *AmCIP* and the functional elements.

In this work, we isolated and characterized the promoter sequence of *PrAmCIP* and also analyzed its activity in heterologous systems under various stress conditions. Furthermore, the key regulatory element, which was necessary for promoter core sequence has been confirmed. Our results will not only provide the basis for the identification of *AmCIP* gene expression regulatory networks, but also provide a favorable tool for plant genetic engineering.

# Materials and methods

### Plant material and growth conditions

*A. mongolicus* seeds were obtained from Alashan Desert in Inner Mongolia, China. Healthy seeds were sterilization with 75% ethanol for 30 s and then with 10% sodium hypochlorite for 15 min. After rinsed thoroughly with distilled water three times, the seeds were germinated and grown on the 200 mL pots containing hormone-free Murashige and Skoog medium, which was autoclaved at 121°C for 20 min. The seedlings were incubated with a photoperiod of 16 h light and 8 h dark in a culture room.

# Isolation and amplification of the PrAmCIP promoter

Two-week-old cotyledons of *A. mongolicus* were used for promoter cloning. Genomic DNA was extracted and used as templates for Genome Walking PCR amplification (Xu et al. 2002). For PCR reaction, the *AmCIP* gene specific primers CSP1, CSP2, CSP3 (sequences shown in Table S1) and the adaptor primers AP1 (Siebert et al. 1995) were used. Amplification procedures are described in Figure S2. The nucleotide sequence of the *AmCIP* was registered in the GenBank nucleotide sequence databases with the accession number AY590122. Putative functional cis-acting elements of the PrAmCIP promoter were identified by the PLACE (http://www.dna.affrc.go.jp/PLACE/) and PLANTCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) database.

# Construction of reporter plasmids

To construct reporter plasmids fusing the *PrAmCIP* promoter or its derivatives with β-glucuronidase (GUS) reporter gene cassette, PCR was performed to generate sequential *PrAmCIP* promoter as *Hind* III and *Xba* I segment at corresponding restriction sites just upstream of the GUS gene in the binary vector pCP-GUS binary vector to analyze the expression pattern of the induced promoter in vivo. Three expression vectors

containing various lengths of *PrAmCIP* promoters were individually obtained and designated CP2, CP4, and CP7. All primers information were listed in Table S2. Then the CaMV35S promoter of pBI121 was separately replaced by the above released segments. *Escherichia coli* JM109 were used in all construction experiments and Agrobacterium tumefaciens LBA4404 and GV3101 were used for the purpose of plant transformation. All constructs were verified by sequencing. In addition, the CaMV35S promoter was the positive control and wild-type tobacco/ wild-type *Arabidopsis* the negative control.

# Arabidopsis and tobacco transformation

The recombinant plasmid was transfected into Agrobacterium tumefaciens LBA4404 by freeze-thaw method. *PrAmCIP* promoter::GUS chimeric construct was transferred to Tobacco (N. tabacum cv. K326) by the leaf discs method (Horsch 1985) and Arabidopsis was transformed by floral dip method (Clough and Bent 1998). Transformants were screened for integration of the intact *PrAmCIP* promoter::GUS chimeric gene into the genome DNA on MS agar plates containing 50 μg/ml kanamycin further confirmed by PCR.

# Identification of transgenic plants and PCR analysis

PCR analysis was carried out using the primer pair: CIP_GUSSF:5‘-GTCACTCATTACGGCAAAGT-3'// CIP_GUSSR: 5‘-CAGCAGCAGTTTCATCAATC-3'. Cycling conditions were 94°C, 5 min; 35 cycles of 94°C, 30 s; 55°C, 30 s; 72°C, 1 min followed by a final extension of 72°C for 10 min. The total genome DNA was isolated from leaves of kanamycin-resistant plants using the Tiangen plant DNeasy kit and was used as a template. PCR products were analyzed on 1% (w/v) agarose gel containing ethidium bromide. Kanamycin-resistant T1 transgenic seedlings were transplanted into soil and allowed to self-fertilize and produce T2 generation.

# Histochemical GUS staining

For GUS histochemical staining assays, two-week-old transgenic tobacco plants containing *PrAmCIP* promoter::GUS fusion constructs were vacuum infiltrated in a solution containing 50 mM NaP buffer (pH 7.0), 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, 0.1% Triton X-100, 10 mM $Na_2EDTA$, 20% methanol and 10 mg/ml X-Gluc (5-bromo-4-chloro-3-indole-β-D-glucuronide cyclohexyl ammonium salt) for several minutes and incubated overnight at 37°C (Jefferson et al. 1987). The GUS-positive plant individual’s tissues were followed by 70% ethanol fixation and then examined with a light microscope (Leica) at a low magnification and photographed with a digital camera (Jefferson 1987). GUS blue stained tissues and plants assessment represent the typical results of at least three independent transgenic lines for each construct.

# Protein extraction and fluorometric GUS assays

GUS activity of transgenic tobacco in seedling protein extracts was compared quantitatively by monitoring cleavage of the β-glucuronidase substrate 4-methyl-umbelliferyl β-D-glucuronide (MUG) for the fluorescent assay (Bradford, 1976). Briefly, two hundred milligrams of 3-week-old tissues from T2 transgenic plant were ground in a mortar and pestle pre-cooled with liquid nitrogen to powder. The

powder was transferred to a microcentrifuge tube containing 0.6 ml of extraction buffer (50 mM $NaHPO_4$ (pH 7.0), 10 mM $Na_2EDTA$, 0.1% Triton X-100, 0.1% (w/v) Sarcosyl, 10 mM β-Mercaptoethanol), and then performed with the method of Vortex oscillation and subsequently placed on ice for 10 min, centrifuged in a microfuge at 13, 000 g for 10 min at 4°C and at last the supernatant was transferred to a clean tube on ice. The total protein concentrations of the extracts were measured by Bradford method. Fluorescence intensity was measured on a GENios (Tecan, Männedorf, Switzerland) with emission at 465 nm and excitation at 340 nm. Fluorescence units were plotted against 4-MU concentration to create a standard curve ($R^2 > 0.99$). The reaction consisted of 20 µl sample extract and 480µl assay buffer (GUS extraction buffer containing 1M MUG). The reaction was incubated at 37°C water bath in the dark. After 20 min, 100 µl reaction mixes was added to the 900 µl stop buffer (0.2 M $Na_2CO_3$); mixed, and 200 µl mixes above was transferred into each well of an opaque 96-well plate. GUS activity was expressed as nmol of 4-methylumbelliferone (4-MU) produced per minute per milligram of soluble protein, which has been normalized to protein concentration in each of the tissue homogenates.

# Abiotic stress and hormone treatment

Histochemical GUS staining assay was used to characterize the PrAmCIP promoter. T2 transgenic tobacco plants were transferred to either normal MS agar plates or MS agar plates supplemented with 200 mM D-mannitol, 200 mM NaCl, or 20% PEG, respectively for 5 days. Besides, for cold stress and heat shock stress, plants growing on MS agar plates were exposed to 4°C and 40°C for 2 days, respectively. In fluorometric GUS assays, transgenic tobaccos were transferred to the MS agar plates supplemented with 0 mM, 50 mM, 100 mM, 150 mM and 200 mM NaCl. To induce low-temperature stress, the plants were transferred to an incubator at 4°C with 0 h, 4 h, 8 h, 12 h, 16 h, 20 h, 24 h, 36 h, 48 h, 1 week or 2 weeks, respectively. Seedlings of transgenic plants without any induction were used as controls. All the above treatments were carried out under a growth regime of 16/8 h light/dark at 25 ± 1°C unless otherwise mentioned.

# Data analysis

The results of GUS activity measurements were all expressed as mean values with ± SD. The standard deviations (SD) of the experimental data are showed as Error bars in figures. Each assay was repeated at least three times. The data presented were collected from at least three independent lines for each construct. The least significant difference (LSD) was performed at 1% probability level with the IBM SPSS 19.0 software package (IBM Corporation, Armonk, NY, USA).

## Results

# Isolation and sequence analysis of the PrAmCIP promoter

Based on our previous study, the 5'-flanking region of the *AmCIP* gene was isolated and amplified from genomic DNA of seedlings in *A. mongolicus* via Genome Walking PCR amplification (Fig. S1). Sequencing resulted in a final fragment of 1158bp, sequence alignment demonstrated that a 110 bp

sequence of overlapping compared with *AmCIP* gene DNA sequence has been obtained by BioEdit (Hall, 1999). In addition, sequence analysis of the 1048 bp 5' UTR region showed that the transcription start site (TSS) of *AmCIP* is located probably 67 bp upstream of its ATG codon, and predicted as "A" via TSSP-TCM (Shahmuradov et al. 2005), according to the general rules for a transcription start site (Joshi, 1987). A putative 'TATA' box was found and located at -29 bp from the putative transcription start site (Fig. 1). This study also revealed that the A + T percentage was 70.6% (35.3% of A; 35.3% of T), while G + C percentage was 29.4% (11.7% of G and 17.7% of C), which is consistent with the composition character of the promoter sequence (Aozasa et al. 2001). We named this 1048 bp promoter as *PrAmCIP* (GenBank: AY590122)

### Cis-elements in PrAmCIP promoter sequence

Analysis of the *PrAmCIP* promoter sequence using PLACE and PlantCARE databases has been performed in this work. Meanwhile, a mass of regulatory motifs involved in the activation of abiotic stress and hormone-responsive (ABA, GA, IAA, CTK and MeJA) genes were predicted and located in the sequence of *PrAmCIP* promoter.

The analysis showed that a TATA-box was detected in the site – 29 upstream of transcription start site, which is the most basic cis-acting element in eukaryotic promoter region. It often determines the selection of the gene transcription start, as one site of the junction of RNA polymerase. Certainly, the CAAT-box was also found in -332 bp, it may be the other site of junction of RNA polymerase; it mainly controls the transcription start frequency. In addition, several environmental-related and hormone-related stress cis-acting elements has been also examined, such as ABRE/EBOX, which is responsive to ABA signal cis-element and identified in plant promoters. An ACGT core characteristic sequence of G-boxes exists in this cis-acting element, which is mainly used in recognizing the sequences of leucine zipper (bZIP) transcription factors. The homologue cis-acting elements sequences of DRE could be induced by low temperature or salt (Kazuko and Kazuo 1994). Furthermore, the homologue sequences of DREC/LTRE, GT1-motif, MYBCORE, MYCATERD1, GGATA, MYC, CANNTG, NTB-motif, ACTTTA, TATCCA-motif, TATCCA, and WBOX were also generally typical cis-acting elements in activating the transcription and expression of resistant genes responding to abiotic stresses and all kinds of hormonal stresses, respectively (Table 1). More interestingly, most of these function cis-regulatory elements could be mainly induced by cold and drought stresses.

# Vector construction of transgenic plants and PCR identification

To study the function of *PrAmCIP*, we constructed the reporter plasmid fusing the *PrAmCIP* promoter and derivatives with GUS reporter gene. But beyond that, we also performed a comparative transgenic analysis of four different PrAmCIP promoter deletion segments to drive GUS fusion constructs (CP2, CP4, and CP7). The schematic illustrations of the 35S-GUS and *PrAmCIP* Promoter -GUS binary vectors were showed in Fig. 2. After two weeks, there are lots of resistant shoots appearing on leaf disc edges, constantly. Kan-resistant groups were transferred to the regeneration medium, and multiple green shoots

have been produced. After another two weeks, the transformed plants grew vividly and formed strong root systems. A total of 30 transgenic plant lines were obtained from the independent Kan resistant shoots. We collected the seeds of T1 and T2 generation transgenic plants and validated the positive groups of by PCR (Fig. 3). The results confirmed that foreign promoter DNA sequence had been integrated into the genome of three individual positive transgenic plants.

# Histochemical analysis of GUS activities in transgenic tobacco seedlings

In order to better analyze activity and inducibility of the cis-acting elements in plant, a GUS staining experiment was performed in transgenic tobacco seedlings. It is interesting that no GUS staining was observed in young seedlings of the transgenic tobacco carrying the construct PrAmCIP-GUS under the normal growth conditions. However, intense GUS staining was found in the cotyledons, young leaves and roots after various biotic stresses treatment. More importantly, the GUS expression level of transgenic tobacco seedlings showed diversity and specificity under abiotic stress (Fig. 4). Clearly, *PrAmCIP* promoter could be induced in response to low temperature (4°C), heat shock (55°C), D-Mannitol, NaCl, 20% PEG, and ABA.

# Fluorometric analysis of GUS activities in transgenic tobacco

To further verify the functions of *PrAmCIP* promoter in response to drought, NaCl, and cold stresses, GUS assay was performed from transgenic tobacco seedlings at different temperatures and concentrations (Fig. 5), and the GUS activity increased gradually with the increase of mannitol concentration and reached a maximum value of 101.4 nmol 4MU/min/g, which was 6.14 times higher than of the control.

Similarly, *PrAmCIP* was also induced by NaCl, and GUS viability was slowly increasing with increasing NaCl concentration. Under 200 mM NaCl treatment, GUS viability reached a maximum value of 40.2 nmol 4 MU/min/g, which was 2.47 times higher than of the control.

However, unlike mannitol and NaCl, PrAmCIP was more strongly induced by 4°C. Under cold stress, GUS activity increased gradually from 0 to 36 h and reached a maximum at 36 h, 245 nmol 4 MU/min/g, which was 14.26-fold higher than that the control group. But the GUS viability tended to decrease with the prolongation of the cold treatment. In all treatments, GUS activity was at a stable level in both the negative control (wild-type tobacco) and the positive control (35S), proving the reliability of our data.

# The GUS activity driven by three promoter deletion segments

To further clarify the key action elements in the *PrAmCIP* promoter in response to drought and cold stress, three 5' deletion fragments of different lengths were designed based on the known distributional features of cis-acting elements in the *PrAmCIP* promoter sequences, and each of them was transfected into

Arabidopsis thaliana and subjected to GUS histochemistry staining using the T2 generation of Arabidopsis thaliana (Fig. 6).

We observed that a cis-element (LTRE) involved in cold response exists the region between − 1048 and − 778 of *PrAmCIP* promoter. The results clearly showed that PrAmCIP, CP2 and CP4 fragments were able to drive GUS gene expression under treatments subjected to cold stress, and the staining results were not significantly different, whereas CP7 could not be induced by cold stress (Fig. 6). This result suggests that the LTRE element may function in the *PrAmCIP* promoter region in response to cold stress, but is not required for the promoter to respond to cold stress, and that MYC may be a key element in response to cold stress.

Based on the results of GUS staining, we found that CP2, CP4, CP7 were able to be induced by drought. Segment construct CP7 is the shortest sequence, which only contains one MYCATERD1 element. MYCATERD1 is the major cis-acting regulatory elements involved in drought stress response (Tran et al. 2004). Accordingly, GUS activity signal still could be detected in construct CP7 under drought treatment but not under the cold (Fig. 6).

# Discussion

Previous research has shown that AmCIP is a KnS-type dehydrin-like protein, in *A. mongolicus* and plays important roles in abiotic stress responses, especially in cold/ dehydration conditions (Liu et al. 2006; Shi et al. 2016). Herein, the 1048 bp promoter sequence of *AmCIP* was amplified from *A. mongolicus* genome by Genome Walking. Transcription start sites (TSS) are essential for the regulation of RNA molecules (Kapranov 2009), the TSS of the *AmCBL1* and *AmDUF1517* gene promoters are located between − 80 and − 60 bp upstream of the ATG codon (Guo et al. 2010; Zhou et al. 2020). The TSS of YnSKn-type wzy2 gene is “A”, located 75 bp upstream of the ATG codon (Zhu et al. 2014), and our study showed that the TSS of AmCIP gene was located 66 bp upstream of the ATG codon, also “A”.

Although KnS-type dehydrin are expressed in response to cold and dehydration, KnS dehydrin gene promoters typically contain GATA core motifs and do not have cis-acting elements typical of cold or drought response (Zolotarov and Strömvik 2015). However, our study revealed the presence of some cis-acting elements of cold stress and drought response in the *AmCIP* promoter region, such as DREB/LTRE, MYC, MYBCORE. DRE or C-repeats element is able to bind to the CBF/DREB or ERF transcription factor, thereby increasing the cold stress capacity of plants, and it was found that one or more of these elements were present in the promoter region of some dehydrin genes, such as *PpDHN1*, *VviDHN2*, *TdCor410b* (Wisniewski et al. 2006; Vazquez-Hernandez et al. 2017; Eini et al. 2013). MYC elements can be bound by ICE, which regulates the expression of CBF genes, and finally leads to the up-regulation of dehydrin genes under cold stress (Peng et al. 2014). In addition, we predicted a number of light-responsive elements and hormone-responsive elements in the *AmCIP* promoter region (Table 1). These results imply that *AmCIP* gene is regulated by multiple factors.

Current research shows that the promoter of the dehydrin gene is an inducible promoter, which can only activate gene expression in response to hormonal and abiotic stresses (Zhu et al. 2014; Qin and Qin 2016; Lv et al. 2017). Our results similarly demonstrated that *PrAmCIP* is also a inducible promoter, *PrAmCIP* was able to drive GUS gene expression in tobacco under 4°C, 55°C, drought, high salt, and ABA treatments, in contrast, was barely functionalunder normal conditions (Fig. 4). GT-1motif, LTRE, MYBCORE and MYCATERD1 are key core components in response to salt, cold and drought, respectively (Terzaghi et al. 1995; Baker et al. 1994; Urao et al. 2003; Tran et al. 2004), and they are also present in the *PrAmCIP* region.

Li et al. (2023) found that LTRE1HVBLT49 at -526 bp to -429 bp in the promoter region of *PLkF3H2* was a key component in response to low temperature. In our result, CP2 and CP4 were able to be induced by cold stress as well as PrAmCIP, whereas CP7 did not respond to cold stress (Fig. 7), suggesting that the DREB/LTRE elements present in the *PrAmCIP* sequences are not required for *PrAmCIP* to respond to cold stress, and that the MYC elements may be the key elements for *PrAmCIP* to respond to cold stress. In addition, we found that CP7 was able to respond to drought stress but not to cold stress, implying that the MYCATERD1 element in the − 122bp~-1 region of PrAmCIP may play an important role in response to drought stress (Fig. 7).

In summary, we identified an inducible promoter *PrAmCIP*, which can respond to drought and cold stress, and these results provide a theoretical basis for explaining the expression and regulatory mechanism of *AmCIP*. Meanwhile, this inducible promoter can be used for the development of abiotic tolerant transgenic plants.

# Declarations

**Supplementary Information** The online version contains supplementary material available at

### Author contributions

BG analyzed data and drafted the manuscript. KD, RCL, QRL, WWZ participated in the sample preparation and performed experiments. YZC and CFL planned and designed the research and approve the final manuscript.


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**Data Availability** All data generated or analysed during this study are included in this published article (and its additional files). Requests for material should be made to the corresponding authors.

**Conflict of Interest** The authors declare that the research was conduct-ed in the absence of any potential conflict of interest.

**Informed consent** Informed consent was not required as no human or animals was involved.

**Research involving human and animal rights** Our study has no re-search involved human participants or animals.

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# Tables

Table 1 is available in the Supplementary Files section.

# Figures


## Figure 1

**Nucleotide sequence of the *AmCIP* promoter.** The numbers above the nucleotide sequence indicate the positions of the nucleotides (in bold and italics) corresponding to the boundaries of the truncated promoter segments (5'-, 3'-end and internal deletions). The transcriptional start site (+1) is in bold. The start codon ATG is also in bold. The predicted cis-acting elements are underlined and named. The region

necessary for abiotic stress induction is underlined with double lines, and the cis-acting elements mentioned for hormone-related are boxed.

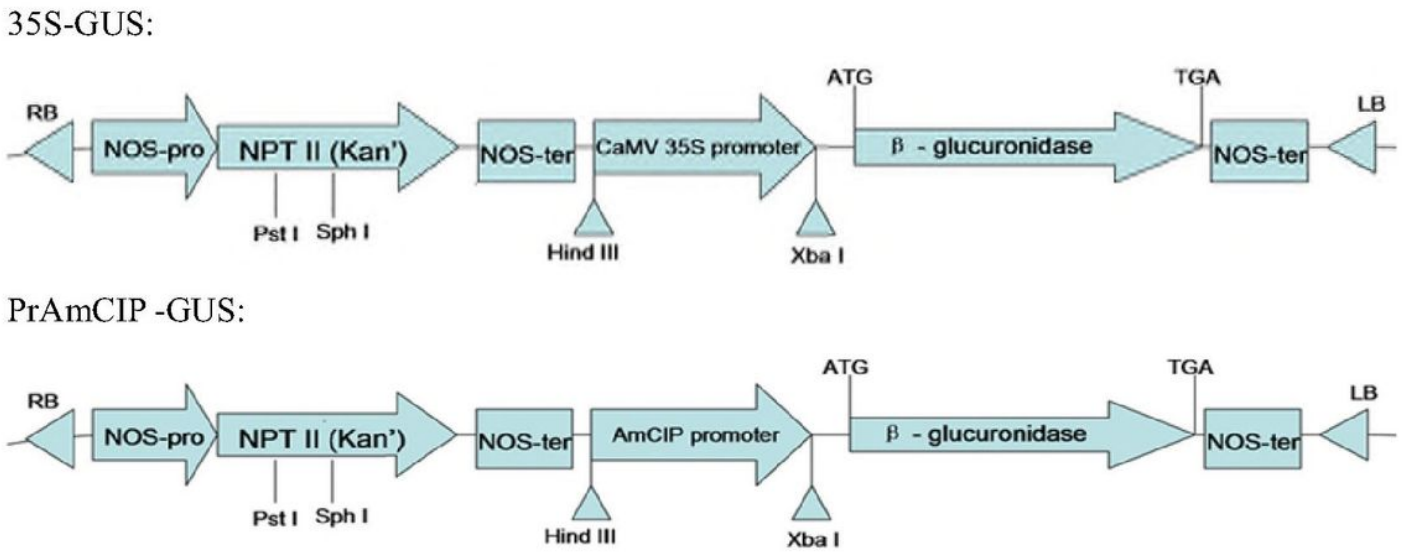



**Figure 2**

**Schematic illustrations of the CaMV35S-GUS and PrAmCIP -GUS binary vectors used for Agrobacterium-mediated transformation.**

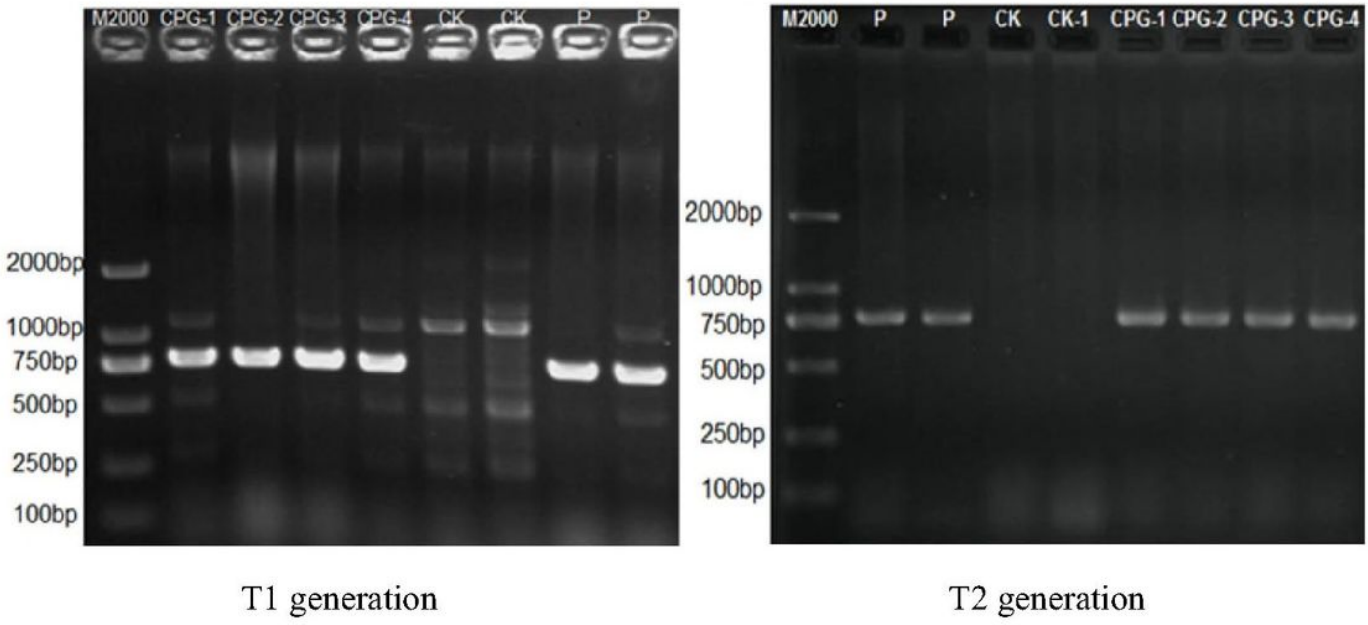



**Figure 3**

**PCR verification of TI and T2 generation tobacco.** M2000: Marker 2000; CK: Wild type; CPG-1-CPG-4: *PrAmCIP* lines; P: 35S lines.

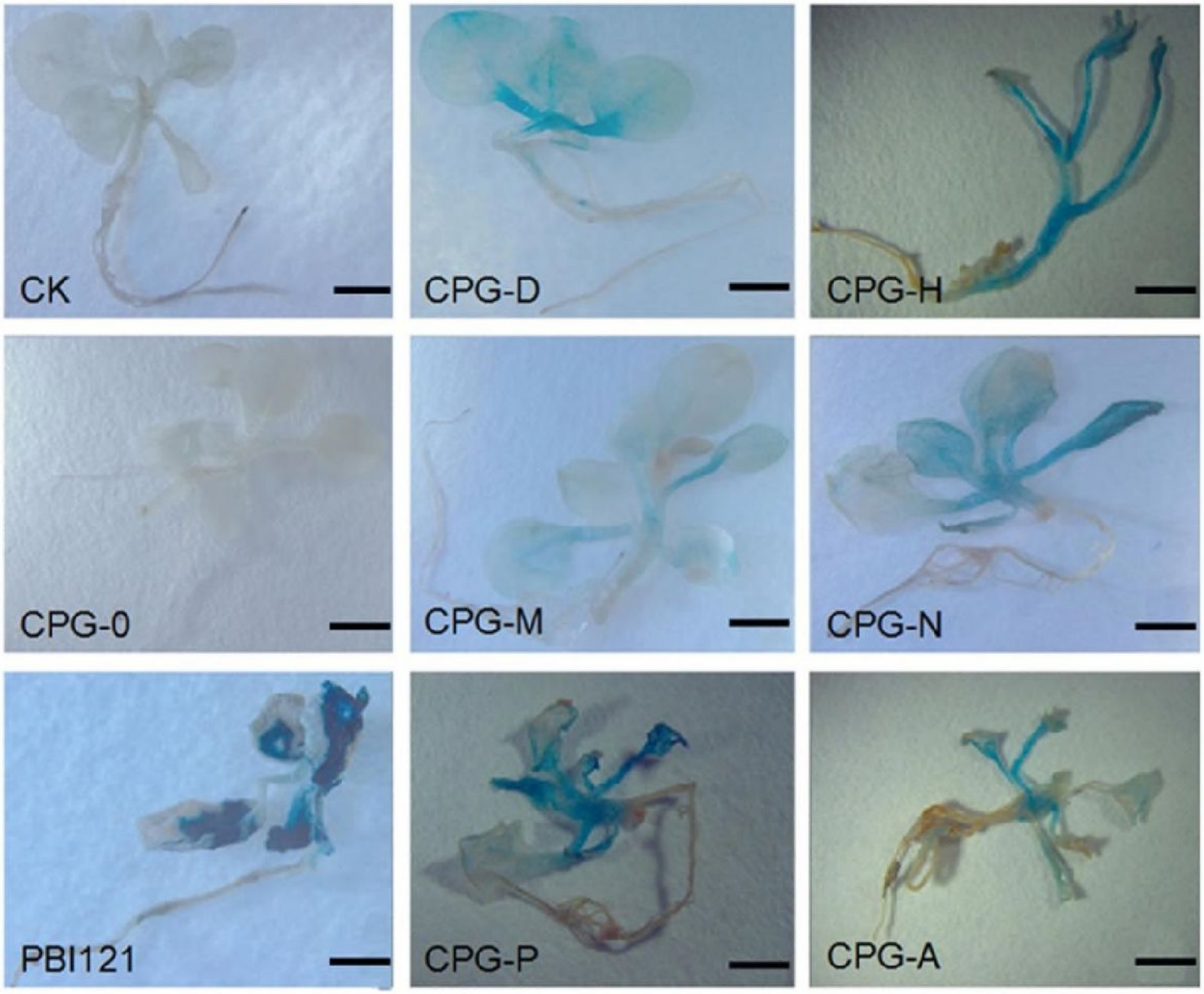
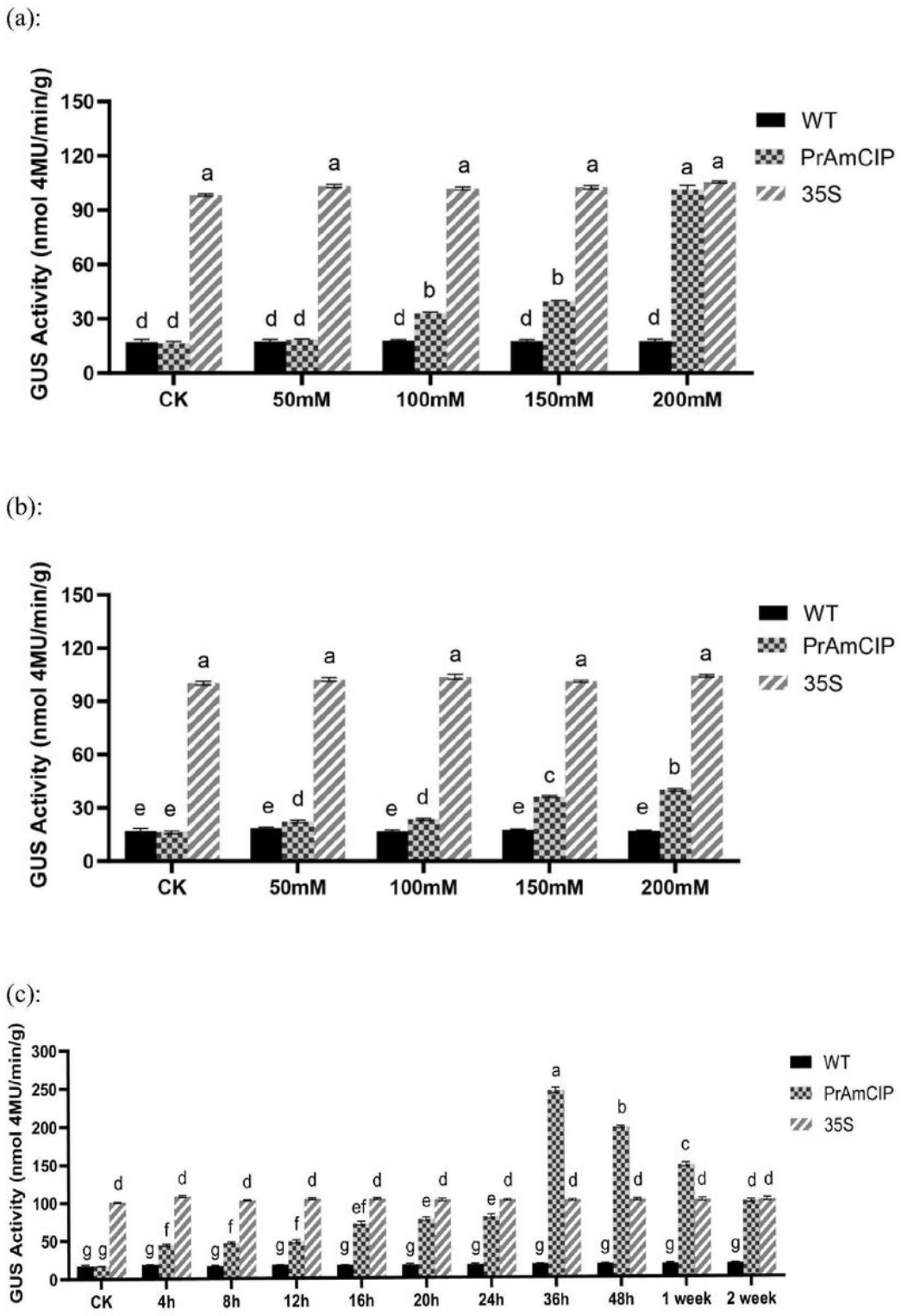



**Figure 4**

**Histochemical analysis of GUS activities of transgenic tobacco seedlings containing the CPGUS or pBI121GUS construct.** CK:wild-type tobacco (negative control); pBI121: CaMV35S (positive control);CPG-D: transgenic tobacco containing *PrAmCIP* promoter::GUS construct with 4°Ctreatment; CPG-H: transgenic tobacco containing *PrAmCIP* promoter::GUS construct with heat shock(55 °C) treatment; CPG-0: transgenic tobacco containing *PrAmCIP* promoter::GUS construct without any treatment; CPG-M: transgenic tobacco containing *PrAmCIP* promoter::GUS construct with D-Mannitol treatment; CPG-N: transgenic tobacco containing *PrAmCIP* promoter::GUS construct with NaCl treatment; CPG-P: transgenic tobacco containing *PrAmCIP* promoter::GUS construct with 20% PEG treatment; CPG-A: transgenic tobacco containing *PrAmCIP* promoter::GUS construct with ABA treatment. Bar= 2mm.




## Figure 5

**Fluorometric quantification GUS activity assay.** (a) GUS enzyme activity of the seedlings of transgenic plants driven by *PrAmCIP* promoter under different drought (D-mannitol) pretreatment concentration group; (b) GUS enzyme activity of the seedlings of transgenicplants driven by *PrAmCIP* promoter under different NaCl pretreatment concentration group; (c) GUS enzyme activity of the seedlings of transgenic plants driven by *PrAmCIP*promoter under different cold(4 °C) treatment time group.CK: seedlings without

any treatment. Bars represent standard deviations of three replicates, different letters indicate significant differences ($p<0.01$).

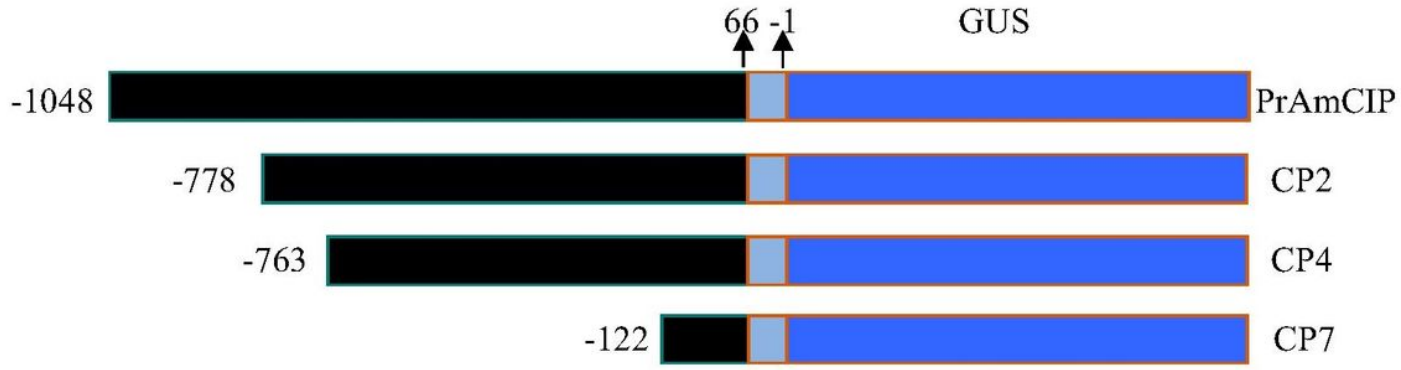



**Figure 6**

***PrAmCIP* promoter deletion analyses.** A in translation start site ATG represents +1; the first base before ATG represents -1; PrAmCIP-P: full-length of PrAmCIP promoter; CP2: AmCIP-P 5'-end deletion of 270 bp; CP4: CP2 5'-end deletion of 15 bp; CP7: CP4 5'-end deletion of 641 bp.

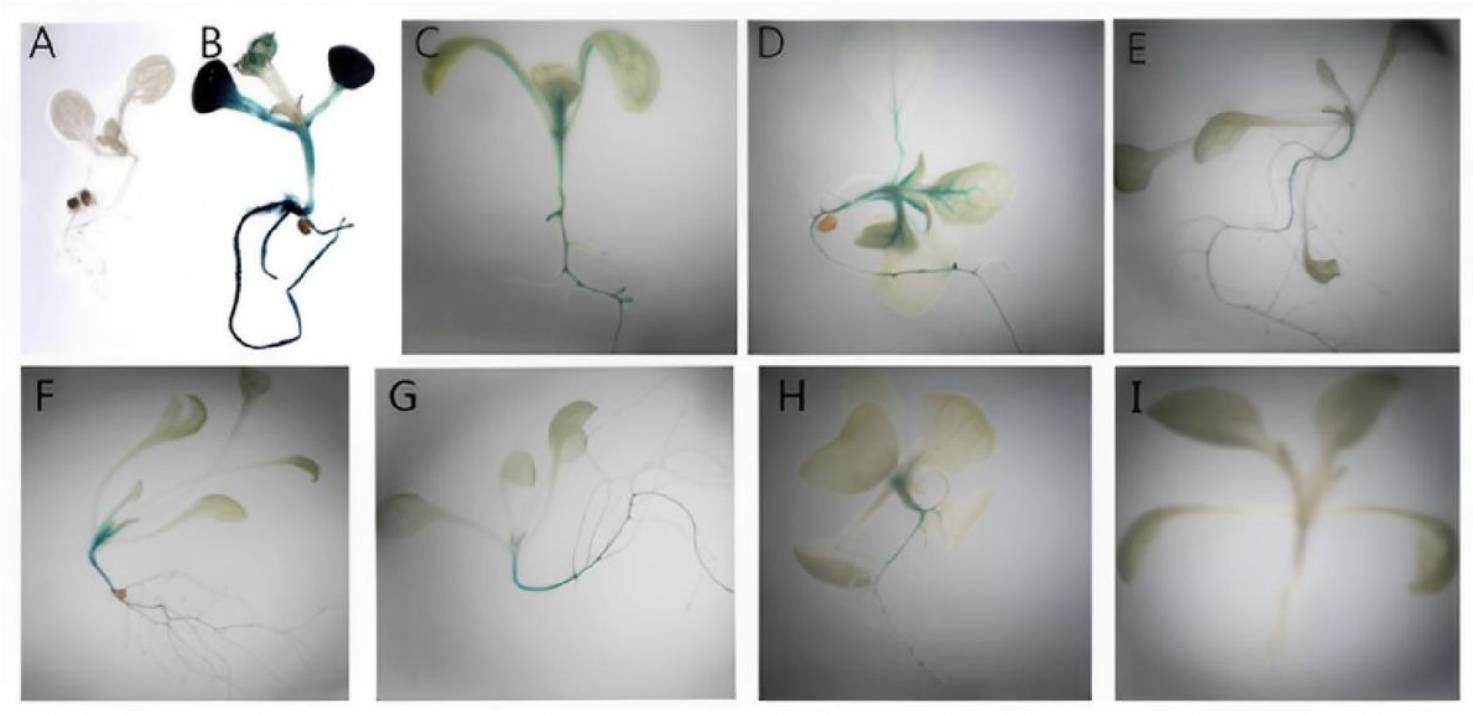



**Figure 7**

**Histochemical analyses of GUS activities driven by *PrAmCIP* promoter deletion segment.** GUS activity was detected by vacuuming one-week-old transgenic *Arabidopsis* seedlings at 37 °C overnight into X-Gluc solution. A: negative controls (WT); B: positive controls (CaMV35S); C:full length of *PrAmCIP*promoter treated with cold (4°C) stress; D: *PrAmCIP*promoter deletion segments construct CP2 treated with cold (4

°C) stress; E: *PrAmCIP* promoter deletion segments construct CP2 treated with drought (D-Mannitol) stress;F: *PrAmCIP* promoter deletion segments construct CP4 treated with cold (4 °C) stress; G: *PrAmCIP* promoter deletion segments construct CP4 treated with drought (D-Mannitol) stress; H: *PrAmCIP* promoter deletion segments construct CP7 treated withdrought (D-Mannitol) stress; I: *PrAmCIP* promoter deletion segments construct CP7 treated with cold (4 °C) stress.

## Supplementary Files

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