Activity and Function Studies of The Promoter Cis-Acting Elements of The Key Enzymes in Saponins Biosynthesis of DS From Panax Notoginseng

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Abstract

Background *Panax notoginseng* is a traditional Chinese medicine for the treatment of blood diseases, in which saponins were the main active components. Dammarenediol synthase (DS) is a key enzyme in the saponin synthesis pathway of *P. notoginseng*. The promoter is an important region to regulate gene expression, and the study of the promoter sequence provides important evidence for revealing the mechanism of gene expression regulation.

Methods: However, there was still little research on the promoter function of *P. notoginseng*. In this study, the 1382 bp promoter upstream of *DS* from *P. notoginseng* was cloned and sequenced. The promoter sequence was analyzed by online databases. The plant expression vector fused with the β -glucuronidase gene was constructed, transferred into *Agrobacterium tumefaciens*. Then tobacco was injected, and its response to exogenous hormones (gibberellin and abscisic acid) was studied by transient expression to verify its unique action elements.

Results: The results showed that the tobacco leaves transferred with *DS* promoter had significantly increased GUS protease activity after spraying GA and ABA, indicating that both *DS* promoter can specifically and significantly respond to exogenous GA and ABA signal.

Conclusions: These findings will help us to better understand the regulatory mechanisms of the upstream region of the *DS* gene and provide a basis for future research on the interaction of cis-acting elements of promoters with related transcription factors.

Introduction

The promoter is a DNA sequence located upstream of a structural gene and can recognize and bind to RNA polymerase and start transcription. It contains a conserved sequence required for RNA polymerase specific binding and transcription initiation (Lu et al., 2014; Zhang et al., 2004; Yang et al., 2010). The promoter is an important part of genes, which controls the time and degree of gene expression in organisms (Nie et al., 2008). The promoter is mainly composed of the core promoter region, upstream control elements, and response elements (Zhu et al., 2018). In recent years, the regulation of the transcription level of target genes has been a hot research direction, and the study of promoter function has become an important part of the study of expression regulation. By fusing part of the promoter fragment with a reporter gene (including β -glucuronidase gene and green fluorescent protein gene, etc.) and constructing a plant expression vector, and then transforming a model plant (*Arabidopsis thaliana* or *Nicotiana tabacum*), the expression of the reporter gene in transgenic plants was detected to confirm promoter function (Zhao et al., 2013; Ma et al., 2018; Zeng et al., 2018).

Panax notoginseng (Burk.) F.H.Chen, belonging to Araliaceae, is a traditional Chinese medicine with a medicinal history of nearly 500 years in China. Modern pharmacological studies found that *P. notoginseng* has pharmacological activity in the immune system, cardiovascular system, nervous system, anti-tumor, and anti-aging (Zhang et al., 2018; Ng, 2010; Xia et al., 2014; Xia et al., 2017). Saponin

is the main active ingredient of *P. notoginseng*, which has the effects of improving myocardial ischemia, lowering blood pressure, and lowering blood fat, etc., and has a significant effect in preventing and treating cardiovascular and cerebrovascular diseases (Ning et al., 2017; Wang et al., 2016). The saponins in *P. notoginseng* are all dammarane type, and no oleanane-type and ocotillol-type saponins are found. From the perspective of aglycone classification, the saponins in *P. notoginseng* can be divided into protopanaxadiol type and protopanaxatriol type saponins. Usually, the five main saponins— notoginsenoside R₁ (7–10%), ginsenosides Rb₁ (30–36%), Rg₁ (20–40%), Rd (5–8.4%) and Re (3.9–6%)—constitute up to 90% of the total *P. notoginseng* saponins. The structure of the main saponins in *P. notoginseng* was shown in Figure 1.

The synthesis of saponins is generally through the mevalonate (MVA) pathway and/or the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway, with MVA being the main pathway (Kim et al., 2015). Isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) catalyze the synthesis of 2,3-oxidosqualene by isopentenyl transferase and anthracycline cyclase, involving several enzymes, including geranyl pyrophosphate synthase (GPS), farnesyl pyrophosphate synthase (FPS), squalene synthase (SS) and squalene epoxidase (SE) (Wu et al., 2009; Suzuki et al., 2002). 2,3-oxidosqualene is catalyzed by different enzymes to synthesize different components. Oleanane-type saponins are catalyzed by β -amyrin synthase (β -AS), while dammarane-type saponins are synthesized under dammarenediol-II synthase (DS) catalysis. Cycloartenol synthase (CAS) can catalyze the synthesis of phytosterols from 2,3-oxidosqualene (Fig.2). Some related enzyme genes in the saponin synthesis pathway have been cloned, such as *GPS*, *FPS*, *SS*, *SE*, and *DS* (Xia et al., 2019). Their functions have been verified in prokaryotic and eukaryotic cells. Studies have shown that overexpression of *FPS*, *SS*, *DS* genes can increase the content of saponins in *P. notoginseng* (Yu 2014; Yang 2015). However, there are not many studies on the transcriptional regulation of the saponin synthesis pathway of *P. notoginseng*.

Many key enzyme promoter regions contain multiple cis- or trans-acting elements in response to biological and non-biological signal regulation. Among them, the regulation of hormone signals plays an important role in plant growth and regulation (Bai, 2018). The promoter of the *ZmGols2* gene in *Zea mays* had a cis-acting element HSE that interacted with nuclear proteins (Gu et al., 2014). The *SLARF2* gene promoter of *Solanum lycopersicum* contained cis-acting elements that responded to hormones such as MeJA, ethrel (ETH), gibberellin (GA), and salicylic acid (SA), and GUS staining showed strong expression in young tissues (Ren et al., 2017; Xu et al., 2014). Dammarenediol synthase (DS) **whose genes were cloned and studied in previous study** are key enzymes in the saponin synthesis pathway of *P. notoginseng* (Xia et al., 2019). DS can catalyze 2,3-oxidosqualene to form dammarenediol-II, and the precursor substance of various saponins in *P. notoginseng* is dammarenediol type. Therefore, the *DS* gene plays an important regulatory role in the synthesis of notoginsenosides. However, there is still little research on its promoter function. In this study, we cloned and sequenced the upstream sequence of the *DS* gene from *P. notoginseng*. The promoter sequences were analyzed by online databases. The plant expression vector fused with the β -glucuronidase gene was constructed, transferred into *Agrobacterium tumefaciens*. Then tobacco was injected, and its response to exogenous hormones (gibberellin and

abscisic acid) was studied by transient expression to verify its unique action elements. Our results could provide a basis for future research on the interaction of cis-acting elements of promoters with related transcription factors.

Materials And Methods

2.1 Materials

Three-year-old *P. notoginseng* samples were collected from Kunming University of Science and Technology, which were identified by Prof. Xiu-ming Cui. All samples were thoroughly rinsed first with tap water and then with distilled water. After cleaning, samples were taken for the extraction of genomic DNA. *Nicotiana tabacum*, *Escherichia coli*, and *Agrobacterium tumefaciens* were kept by our laboratory. PCAMBIA0390::GUS was provided by Dr. Zhen-qing Bai. *E. coli* strain DH5 α (Shanghai Weidi Biotechnology Co., Ltd) was used for the cloning and propagation of all recombinant vectors. *A. tumefaciens* strain EHA105 (Shanghai Weidi Biotechnology Co., Ltd) was used for plant transformations.

2.2 Cloning of PnDS promoter

Genomic DNA was extracted using the CTAB method (Li et al., 2009). DNA samples were qualified photometrically, checked by electrophoresis on a 1% agarose gel, and stored at -20 °C for use. According to the published complete genome sequence of *Panax notoginseng*, 1500 bp upstream of the *DS* genes were used as the research object. A pair of primers, PnDS-F (5'-TGTCGTTTTTTTTCAATCC-3') and PnDS-R (5'-TTTTCAACCTCTTCCCTCT-3'), designed with primer premier 5.0 (Premier Biosoft, CA, USA), were used for PCR amplification under the following conditions: 94°C for 2 min; 35 cycles of 98°C for 10 s, 51°C for 30 s, 72°C for 2 min; 72°C for 5 min. The PCR products were analyzed by electrophoresis on 1.0% agarose gel. The PCR products were purified using a Gel Extraction Kit (TIANGEN, DP190123, Beijing, China) according to the manufacturer's instruction, and then ligated into pMD19-T vector and transformed into DH5 α competent cells. Based on the antibiotic selection (50 mg/L Amp), the positive clones were sequenced by Hangzhou Youkang Biological Technology Company (Hangzhou, China).

2.3 Promoter sequence analysis

The online program PlantCARE database (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html>) and online program PLACE database (<https://www.dna.affrc.go.jp/PLACE/?action=newplace>) were used to predict and analyze cis-regulatory elements (Prestridge, 1991; Higo et al., 1999). The online website (http://www.fruitfly.org/seq_tools/promoter.html) was used to predict the transcription initiation site.

2.4 Vector construction

The Plant binary expression vector PCAMBIA0390::GUS was used for vector construction. A sequence of about 300 bp in the *DS* promoter was selected to study its response to exogenous hormones. The promoter of *DS* was amplified with forward and reverse primers using KOD-Plus-Neo DNA Polymerase. A

forward primer carrying the *Pst*I restriction site (5'-AACTGCAGTTTCAATCCGACAATAATTCCGTTG-3') and a reverse primer carrying *Bam*HI restriction site (5'-CGGGATCCACACTCACATAAGAGAAAGAGGCAG-3') were designed. The PCR reaction was performed as follows: 94 °C for 2 min; 35 cycles of 98°C for 10 s, 63.5°C for 30 s, 68 °C for 1 min; 68 °C for 5 min.

The PCR products were detected by 1% agarose gel electrophoresis. After product purification, the product and PCAMBIA0390 vector were double-digested, respectively. The double-digested reaction mixture contained 5 µL of 10×NEBuffer 3.1, 1 µL of *Pst*I, 1 µL of *Bam*HI, ≤1.0 µg of *DS*/PCAMBIA0390 plasmid and finally the total volume was supplemented to 50 µL with ddH₂O. The purified target gene fragments were ligated with the PCAMBIA0390 vector. The reaction mixture of T4 DNA ligase was as follows: 1.0 µL of 10×T4 DNA ligase Buffer; 2.0 µL of PCAMBIA0390 vector plasmid fragment; 6.0 µL of purified target gene fragments; 1.0 µL of T4 DNA ligase. The above reaction system was reacted at 16°C overnight. The ligated product was transformed into *E. coli* DH5α. Based on the antibiotic selection (50 mg/L Kan), the positive clones were sequenced by Hangzhou Youkang Biological Technology Company (Hangzhou, China). The recombinant plasmid was extracted using a TIANpure Mini Plasmid Kit II (TIANGEN, DP107-02, Beijing, China), and then transferred the recombinant plasmid into *A. tumefaciens* strain EHA105.

2.5 Injection of tobacco leaves

The strain EHA105 containing the recombinant plasmid was cultured to OD₆₀₀=0.4~0.6, then detoxified in injection buffer (2 mL of 0.5 M MES buffer (pH 5.6), 2 M MgCl₂, 0.2 M acetosyringone and the total volume was supplemented to 100 mL with ddH₂O) for 2 h, and adjusted the OD₆₀₀ to 0.8 to inject tobacco leaves. The tobacco was cultivated under 16 h of light and 8 h of darkness at 25 °C for 72 h. Then spray gibberellin (GA) (200 mg/L) and abscisic acid (ABA) (100 mg/L), respectively. Tobacco leaves were collected after 24 hours of cultivation in a transparent plastic bag and placed in liquid nitrogen for quick freezing. Uninjected tobacco leaves were used as negative controls.

2.6 Quantitative detection of GUS activity

The total tobacco protein was extracted with GUS extracting solution, which contained 50 mL of 0.1 M PBS (pH7.0), 1 mL of 10% SDS (pH7.2), 2 mL of 0.5 M EDTA (pH 8.0), 100 µL of Triton X-100, 100 µL of β-mercaptoethanol and the total volume was supplemented to 100 mL with ddH₂O. The absorbance was measured at 595 nm using an enzyme-labeled instrument to detect the protein content. Added 400 µL GUS extracting solution, 100 µL protein samples, 500 µL 2mM MUG in a 1.5 mL centrifuge tube, mixed well, and placed in 37°C water bath. At five time points (0 min, 15 min, 30 min, 45 min, and 60 min), 200 µL of the reaction liquid was added to 800 µL of 0.2 M Na₂CO₃ solution and stored at room temperature in the dark. Fluorescence scanning (excitation light: 360 nm, emission light: 460 nm) was performed with an enzyme-labeled instrument, and the rate of change in fluorescence intensity per unit time was calculated. The rate of change divided by the total amount of protein was the GUS protease activity level.

2.7 Statistical Analysis

The data were analyzed by SPSS 21.0 software for calculating and significance test of difference.

Results

3.1 Cloning of promoters

Using the genomic DNA of *P. notoginseng* as a template, according to the 1500 bp sequence region upstream of the *DS* genes found in the *P. notoginseng* genome, the 1476 bp of *DS* of the PCR products were cloned. The sequencing results were compared with the genome information of *P. notoginseng*, and the results showed that the sequences were correct. Among them, the 1348 bp promoter upstream of *DS* were obtained.

3.2 Analysis of promoter cis-acting elements

The sequence of *DS* promoters was predicted and analyzed in PLANTCARE and PLACE, and it was found that *DS* promoters contained two main types of cis-acting elements, one was growth regulating elements and light response elements, and the other was the stress response elements (Table 1). The distribution of cis-acting elements of the *DS* promoter was shown in Figure 3. Multiple light-responsive motifs were identified, including GT1-motif, Sp1, GATA-motif, LAMP-element, AT1-motif, AE-box, and ATCT-motif. The presence of these light-responsive elements indicated that the expression of *DS* genes may be greatly affected by light regulation. In the *DS* promoter, the -1079 bp position contained an MBS cis-acting element which was involved in drought induction. At -815 bp of *DS* promoter, there was an AT-rich element which was a binding site of AT-rich DNA binding protein. Cis-acting elements related to external stress were also predicted. The cis-acting element involved in the SA responsiveness was located at -915 and -241 bp. The PYRIMIDINEBOXHVEPB1 element responsive to GA and the TGA-element responsive to auxin were found at -937 bp and -1347 bp, respectively. The cis-acting element involved in salt induction was located at -356 bp and -916 bp. In addition, the *DS* promoter contained the basic cis-acting elements including TATA-box and CAAT-box. The transcription initiation site was predicted by online software and found that the *DS* promoters contained 3 possible transcription initiation sites (Fig. 4).

Table 1. Cis-acting elements of *DS* promoter.

Cis-acting elements	Position	Sequence	Function
GT1-motif	-822(+)	GGTTAA	light responsive element
Sp1	-341(-)	GGGCGG	light responsive element
GATA-motif	-715(-)	AAGATAAGATT	part of a light responsive element
LAMP-element	-334(-)	CTTTATCA	part of a light responsive element
AT1-motif	-466(+)	AATTATTTTTTATT	part of a light responsive module
AE-box	-484(-)	AGAAACTT	part of a module for light response
Box 4	-584(-)	ATTAAT	part of a conserved DNA module involved in light responsiveness
ATCT-motif	-1067(-); -676(+)	AATCTAATCC	part of a conserved DNA module involved in light responsiveness
AT-rich element	-815(+)	ATAGAAATCAA	binding site of AT-rich DNA binding protein (ATBP-1)
TGA-element	-1347(-)	AACGAC	auxin-responsive element
CAT-box	-148(-)	GCCACT	cis-acting regulatory element related to meristem expression
ARE	-600(-)	AAACCA	cis-acting regulatory element essential for the anaerobic induction
CCAAT-box	-1319(-)	CAACGG	MYBHv1 binding site
MBS	-1079(+)	CAACTG	MYB binding site involved in drought-inducibility
GT1GMSCAM4	-916(+); -356(+)	GAAAAA	cis-acting element involved in salt induction
PYRIMIDINEBOXHVEPB1	-937(+)	TTTTTTCC	cis-acting element involved in the gibberellin responsiveness
TCA-element	-915(-); -241(-)	CCATCTTTTT	cis-acting element involved in salicylic acid responsiveness
CAAT-box	-1335(+); -774(+); -413(+); -77(+)	CAAT	common cis-acting element in promoter and enhancer regions

TATA-box	-1208(+); -375(+); -209(+)	TTATTT	core promoter element around -30 of transcription start
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3.3 Analysis of promoter response to exogenous hormones

GA is a growth regulator that promotes seed germination, plant growth and early flowering, and ABA is a hormone related to plant stress and stress response. The cis-acting element involved in the gibberellin responsiveness were predicted in the *DS* promoter, and in order to explore whether cis-elements predicted in *DS* promoter specifically respond to exogenous GA and ABA, the recombinant plasmid was constructed (Fig. 5). We injected the recombinant vector into tobacco leaves, and after 72 h of cultivation, GA and ABA were sprayed. Samples were collected after 24 h of treatment and quickly frozen them with liquid nitrogen for GUS analysis. Tobacco leaves without injection of agrobacterium were used as blank control, while those with empty vector PCAMBIA0390 were used as control. The result of the GUS enzyme activity analysis was shown in Fig. 6. Compared with the control, the tobacco leaves transferred with *DS* promoter had significantly increased GUS protease activity after spraying GA and ABA, indicating that *DS* promoter can specifically and significantly respond to exogenous GA and ABA signal.

Discussion

DS is a key enzyme in the saponin synthesis pathway of *P. notoginseng*, which can catalyze the formation of 2,3-oxidosqualene to dammarenediol. DS plays a crucial role in regulating the saponin synthesis of *P. notoginseng*. The functions of DS were preliminarily verified by techniques such as overexpression, RNA silencing, and RNA interference (Han et al., 2010; Han et al., 2006; Kim et al., 2014). The promoter is an important region to regulate gene expression, and the study of the promoter sequence provides important evidence for revealing the mechanism of gene expression regulation. The promoter sequence of *DS* was cloned and predicted through online software. The results showed that they contained basic elements, including CAAT-box, TATA-box, and contained cis-acting elements that respond to exogenous ABA, SA, GA, and some anaerobic induction, and the binding site of MYB transcription factor. In addition, the promoter contained some growth regulation and light-responsive elements. It showed that the promoter of the *DS* gene played an important role in the growth and development of *P. notoginseng* and metabolic regulation. We speculated that it might be inducible promoters. Sequence analysis also found that the *DS* promoter had a CACNTG sequence in the complementary strand at -283 bp and -174 bp, which is consistent with the recognition sequence of the core domain of the MYC2 transcription factor reported in the literature (Li et al., 2019). MYC2 transcription factor is an important member of the MYC family, a core regulator of a large number of JA signaling pathway branches, and has a certain regulatory role in the growth and development of plant tissues and organs. Therefore, it is inferred that MYC2 may bind to the *DS* promoter and respond to hormone signals, thereby regulating gene expression.

The results of the predictive analysis of *DS* promoters showed that it contained cis-acting elements involved in the hormone responsiveness. Therefore, a plant expression vector fused with the GUS reporter

gene was constructed, *A. tumefaciens* was used to transform tobacco and transient expression was conducted, and whether the *DS* promoter regions responded to exogenous GA and ABA signals was studied. The results showed that the *DS* promoter can respond to exogenous GA and ABA signals, and GUS protease activity was significantly improved. Therefore, the cis-acting elements in the *DS* promoter that responded to GA and ABA had a positive regulatory effect on the promoter. ABA treatment had an effect on the accumulation of saponins in Ginseng plants. Studies have shown that after processing *Panax quinquefolium* hairy root with exogenous ABA (1 mg/L), the contents of ginsenoside Rg1 and Re increased (Kochan et al., 2019). Our research laid the foundation for screening transcription factors that interact with *DS* promoter and for in-depth analysis of saponin synthesis pathways in *P. notoginseng*.

Conclusion

In this study, the promoters of the *DS* gene in *P. notoginseng* were cloned and sequenced, and the 1348 bp promoter upstream of *DS* was analyzed by the online database. The predicted results showed that the promoter contained growth regulating elements, light-responsive elements, hormone-responsive elements, etc. Besides, the cis-acting elements involved in the GA and ABA responsiveness were presented in the *DS* promoter. Thus, through the transient expression experiment of tobacco, the response of *DS* promoter to exogenous GA and ABA was studied. The results indicated that the *DS* promoter can specifically and significantly respond to exogenous GA and ABA signal. Our study laid the foundation for a deeper exploration of the functions of the *DS* gene and the analysis of the saponin synthesis pathways they regulate, providing a basis for the further verification of gene functions.

Declarations

Contributors

Yujie Zheng and Rongyu Shao performed the experiments and data analysis, wrote the manuscript. Pengguo Xia conceived the study and contributed to the study design, discussion, and revision of the manuscript. Zongsuo Liang contributed to study design and revision of the manuscript. Kaijing Yan contributed to data verification, revision and proofreading of the manuscript. All authors have read and approved the final manuscript and, therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

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Availability of data and materials

The datasets used in this study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

The manuscript is approved by all authors for publication.

Competing interests

The authors declare no competing financial interests.

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Figures

Figure 1

The structure of the main saponins in *Panax notoginseng*.

Figure 2

Biosynthetic pathway of saponins. Notes: MVA: mevalonate; MEP: 2-C-methyl-D-erythritol 4-phosphate; IPP: isopentenyl pyrophosphate; DMAPP: dimethylallyl pyrophosphate; GPS: geranyl pyrophosphate synthase; GPP: geranyl pyrophosphate; FPS: farnesyl pyrophosphate synthase; FPP: farnesyl pyrophosphate; SS: squalene synthase; SQ: squalene; SE: squalene epoxidase; β -AS: β -amyrin synthase; DS: dammarenediol-II synthase; CAS: cycloartenol synthase.

TGA-element CAAT-box CCAAT-box
 -1348 bp TGTCTGTTTTTCAATCCGACAATAATTCCGTTGTGGGGTCCACCAATGATTATACCGTCGTACGGATATCCCCGATAAAAAAATATTCTCCGACC
 -1248 bp AATCGAATATTAATAACAACCTGCATACTTTATAAAATTATATAATATTACCCACAACATAACATTATATCAAATCCATAACATAATATAAAAAAAT
 TATA-box
 -1148 bp ATTCGAGAATATTAAATATTTATTCATAAAATCTGGGGTCTCACACTTTGTGTTCAAATGAACATGATGCAACTGTGTCGAATAGATTGTGTCAAA
 MBS ATCT-motif
 -1048 bp TTATTTTATCTGTAGTTCTGCCTCTTCTCTTATGTGAGTGTCAATGAGAGTTGTGTTCTCTAAAAAGATAAAGATATGACACTGGAATAAAAAATTAGA
 -948 bp ACTGTATGCATTTTTTCCATACAATCATACAGGAAAAAGTTGGACCAAGTGTATTTTTTCAAGATTTCTTCCAATACTTGTAGTTTTGTGATTTTCCAAA
 PYRIMIDINEBOXHVEPB1 GT1GMSCAM4 TCA-element
 -848 bp TTTGACAGCTAGTAGGAGAACTAGGTGGTTAACTAGAAATCAAATATTTTCTTAACTGCACAATCTGCATAACAATAGCCCTACAAGAAGCAATAGT
 GT1-motif AT-rich element CAAT-box
 -748 bp AAATCATTATAGTACTGATTACTACTCTCAATTTATCTTTTATAGGACAAATAAAATAATTATCATAATCAATCCCAAAAATGGAATAATAAA
 GATA-motif ARE box 4 ATCT-motif
 -648 bp AAAAAAGAAACATTAAAATTTATTTGAAGCTTATAAAGATACCAAAACGGTTTATCTTTGAAATTAATTCATGAGTTTGTGTTGAATATAGAAAAATAAA
 -548 bp TTTGGATCGAGGCTTAAGCATTTTGGGAATCATTCAAATTGAATTATGACAAATAACCGAAGTAAGTTTCAATTGTATCTAAATATTTTATTGGAA
 AE-box AT1-motif
 -448 bp ATTTCTTTTATAGTTTATGATTATGAATATTTATGCAATTGTAACATTCTTATGATTAGTTTAAATTTTATTTAAGTTTAAATAAAAGAAAAATT
 Sp1 LAMP-element CAAT-box TATA-box GT1GMSCAM4
 -348 bp CCTAGATCCGCCCCTGATAAAGAGTACAATTATATTTTCTAGAAGGAATAATTTTAAATAATGCAGGTGTATAATTCTACAAAAAAGGAGTATAATT
 TCA-element TATA-box
 -248 bp TTTTATTTAAAAAGGATGGGCACATTTTAAACGTTGTACGTTATTTCTTCCATGTTTATATCGTGCCTAAATATACAAGTGGACTTTATGTCTAGATCTAG
 -148 bp AGTGGCTAGATGTTTATATATTATCAGCTCCACAACATTTGGGTGGTATATAAATAATGGTTCAACTACAATAATATAATATATATATATA
 CAT-box translation start site
 -48 bp GTAGATAATTAAGCCATCTATGTACACCACATAACCACTAGTAGAAGAATGTTGGAAGCTGAAGGTTGCTCAAGGAAATGATCCATATTGTATAGCACTA
 +53 bp ACAACTTTGTGGCAGACAATATTGGGAGTTTCAGCCCGATGCTGGTACTCCAGAAGAGAGGGAAGAGGTTGAAAA

Figure 3

Distribution of cis-acting element of DS promoter. Notes: The name of the cis-acting element is consistent with the name in the table 1.

Start	End	Score	Promoter Sequence
1097	1147	0.99	ATTTTTTTTTTAAAAAGGATGGGCACATTTTAAACGTTGTACGTTATTTT
1205	1255	0.91	GCTAGATGTTTATATATTATCAGCTCCACAACATTTGGGTGGTATATAAT
1281	1331	0.97	TAATATAATATATATATAGTAGATAATTAAGCCATCTATGTACACCAC

Figure 4

Transcription initiation sites analysis of DS promoter.

Figure 5

Recombinant vector structure map.

Figure 6

DS promoter in response to exogenous GA and ABA treatment. (A) The injection of tobacco. (B) All repeated 3 times (n=3), analysis of variance ($p < 0.05$), control: without injection of bacteria liquid, PC0390: PCMBIA0390 empty vector, PC0390-DS is the abbreviation of PCAMBIA0390-DS.

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