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Identification and functional analysis of soybean *GmSBH1* gene promoter conferring high temperature- and humidity-induced expression

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Abstract: Soybean homeobox gene *GmSBH1* has previously been proven to be involved in response to high temperature and humidity (HTH) stress. To investigate its expression patterns and active cis-elements, a 2040-bp 5'-upstream genomic DNA fragment of *GmSBH1*, named *GmSBH1P*, was isolated by PCR walking and characterized. Sequence analysis revealed that the fragment contains a series of cis-acting elements related to stress responses. The transient expression assay in the leaves of *Nicotiana benthamiana* and in the cotyledonary nodes of soybean indicated that the *GmSBH1P* strongly and rapidly mediates the induction of GUS expression under HTH stress. Deletion and mutation analysis of the promoter indicated that the cis-acting HSE (GAACTTTC) in GmSBH1P is essential for the promoter in response to HTH stress. In addition, 82 different proteins were identified to bind the cis-element by a yeast one-hybrid system. These results indicated that the cloned promoter *GmSBH1P* could be applied to enhance the resistance to HTH stress, and the HSE would be an ideal candidate for mediating the expression of HTH-responsive genes in plants.

Key words: Soybean, promoter, high temperature and humidity stress, cis-acting elements, yeast one-hybrid

1. Introduction

A promoter is a DNA sequence to which RNA polymerase and transcription factors bind to initiate gene transcription. Promoters play important roles in regulating the transcription level (Schwechheimer et al., 1998). The control of transcription is fundamental for the regulation of plant gene expression and involves a variety of trans-acting factors and cis-acting elements. Many functional elements have been isolated and identified as promoter constituents for precise and regulated transcriptional initiation, such as the TFIIB-recognition element (BRE), TATA box, downstream promoter element (DPE), initiator (Inr) motif, and so-called cis-regulatory elements (Yamamoto et al., 2007).

The promoters of plants have been generally divided into three categories, including inducible, constitutive, and tissue-specific promoters (Cornejo et al., 1993; Hou et al., 2016). The expression of genes with constitutive promoters is not restricted in time or space, nor it is induced by endogenous or exogenous substances/agents. Constitutive promoters are those of cauliflower mosaic virus gene (*CaMV35S*) and of rice actin gene (*ActinI*) (Bruce et al., 1989; Seagull and Gunning, 1989; Christensen et al., 1992; Ince and Karaca, 2016). The expression of a gene with a tissue-specific promoter occurs only in a particular tissue or organ of the plant, such as the specific expression in vascular tissue (*PAL*) (Osakabe et al., 2009; Ince and Karaca, 2016). The expression of a gene with an inducible promoter is induced by some physical or chemical signals. In the absence of the inducer, the gene expression level is very low or absent (Deng et al., 2012; Chai et al., 2013; Ince and Karaca, 2016).

Plants under different signal stimulations can be induced to express some genes of certain signal responses, which could resist and ward off harmful environmental factors so as to achieve the goal of healthy growth in an unfavorable environment. It involves inducible promoters with certain specific physical or chemical stimulation signals, and these types of promoters can greatly increase the level of transcription of the genes (Deng et al., 2012; Chai et al., 2013). So far, people have separated the gene promoters of heat-induced expression (Haralampidis et al., 2002), such as the cis-element HSE, necessary for the promoter of sunflower heat shock gene (*Ha hspl 8.6G2*) to respond to high temperature (Coca et al., 1996), and the AAT-box, a core cis-element of soybean gene *Gmhspl7.3-B* promoter responding to high temperature (Prändl and Schöffl, 1996). Some studies showed that cis-elements AT-

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rich and CCGAC-motif also respond to high temperature (Rojas et al., 1999; Navarre et al., 2011). However, there were no reports on cis-elements in response to the combination of high temperature and humidity (HTH) stress. A promoter contains many different cis-acting elements, and under stress, these cis-elements may be combined with some common proteins, regulating the expression of the downstream gene (Li et al., 2015).

Due to the high content of oil and protein, soybean (*Glycine max* L.) seed is very susceptible to HTH stress before harvesting, resulting in seed deterioration (Wang et al., 2007). This deterioration includes the production of abnormal seeds and the reduction of germination rate, vigor, storability, and nutritional quality of the seed (Zanakis et al., 1994; Spears et al., 1997; Wang et al., 2007). Our previous study indicated that *GmSBH1* was related to growth and development and responded to HTH stress in soybean (Shu et al., 2015). Based on the results, the upstream promoter of *GmSBH1* was speculated to have one or several key elements in response to HTH stress. Here, we proved the existence of a core HTH-responsive cis-element in the promoter by 5'-terminal deletion and then revealed that the GAACTTTC fragment was required for its activity. Moreover, 82 various proteins were identified by yeast one-hybrid system to be able to interact with the GAACTTTC fragment under HTH stress combination. The results will enhance our understanding of how *GmSBH1* responds to HTH stress in the growth and development of soybean.

2. Materials and methods

2.1. Plant materials and growing

Cotyledonary nodes of soybean cultivar Xiangdou No. 3 and leaves of *N. benthamiana* were used for transient expression. The plant seeds were grown at 25 °C in a 16:8-h light/dark cycle in a growth chamber (Shu et al., 2015). The cotyledonary nodes of 1-week-old soybean were immersed by agroinfiltration (Gao et al., 2015). The leaves of *N. benthamiana* (6 weeks old) were injected by agroinfiltration (Chai et al., 2013). Treated soybean cotyledonary nodes and *N. benthamiana* leaves were grown at 40 °C and 100% relative humidity in a growth chamber (Shu et al., 2015). The samples used were collected at 1 and 2 h, while the corresponding tissues without treatment were sampled as controls.

2.2. Isolation and analysis of *GmSBH1P* sequence

Genomic DNA was extracted from the seeds at physiological maturity (R7 period), based on the hexadecyl trimethyl ammonium bromide (CTAB) isolation procedure (Saghai-Maroof et al., 1984; Chai et al., 2013). *GmSBH1P* was isolated with the GmSBH1 promoter (FL)FR primers (Table 1). The 2040-bp full length of the promoter was obtained, which included the transcription start site (Figure 1). The 2040-bp (from –1250 bp to +790 bp) sequence of *GmSBH1P* was used to identify the potential cis-acting elements by PLACE (http://www.dna.affrc.go.jp/PLACE/) and PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Higo et al., 1999; Lescot et al., 2002; Hou et al., 2016).

2.3. Construction of transformation vector

To study the functional regions of the *GmSBH1* promoter, 5'-end deletion analysis was carried out (Park et al., 2007; Li et al., 2012), with the forward primers possessing a *Hind* III restriction site and a reverse primer possessing an *Xba* I restriction site. The deletion promoter fragments were amplified using a series of promoter sequences (Hisada et al., 2008; Crinelli et al., 2015). P2FR primers were used to obtain P2 deletion fragment (–1024/+790 bp), P3FR primers to obtain P3 deletion fragment (–638/+790 bp), P4FR primers to obtain P4 deletion fragment (–336/+790 bp), P5FR primers to obtain P5 deletion fragment (+119/+790 bp), P2-P3FR primers to obtain P2-P3 deletion fragment (–1024/-638 bp), P2-P3-MFR primers to obtain HSE mutation fragment (–1024/-638), and P2-P3-DFR primers to obtain HSE deletion fragment (–1010/-638 bp). All the promoter fragments were cloned into the *Hind* III-*Xba* I sites of PBI121-GUS (Supplemental Figure 1), a plant expression vector fused with the *GUS* reporter gene. The *CaMV 35S* minimal promoter (*35S min*) without any cis-elements was cloned into the PBI121 vector as a negative control by using 35S minFR primers, whereas the PBI121 vector was applied as a positive control (Chai et al., 2013). All the recombinants were confirmed by sequencing and enzyme digestion and introduced into *Agrobacterium* strains by freeze-thaw method (Hou et al., 2016). All the primers used are listed in Table 1.

2.4. Agroinfiltration of leaves of *N. benthamiana*

All the recombinants were transformed into *A. tumefaciens* strain *EHA105* by freeze-thaw method (Chattopadhyay et al., 1998). The transformation of *N. benthamiana* leaf discs was conducted according to Llave et al. (2000). The positive agrobacteria clones were coated on YEB plates with kanamycin (50 mg L^{-1}) and rifampicin (25 mg L^{-1}) for 2 days at 28 °C. Bacteria were collected, resuspended in infiltration medium [150 μM acetosyringone (pH 5.7), 10 mM MES-KOH (pH 5.7), 10 mM $MgCl_2$] to an $OD_{600\ mm}$ of 0.6, and then incubated at 25 °C for 3 h in the dark. A needleless syringe was used to penetrate bacterial suspensions into the abaxial side of fully expanded leaves of 6-week-old *N. benthamiana*. For each experiment, 35S-GUS was applied as a positive control while 35S min-GUS was a negative control, and all of the promoter fragments were infiltrated in leaves of *N. benthamiana*. After infiltration, plant leaves were kept for 24 h at 25 °C for further HTH stress combination.

Table 1. Sequences of primers used in this study. The underlined sites are the sites for the digestion of restriction enzyme *Hind* III. The underlined italicized sites are the sites for the digestion of restriction enzyme *Xba* I.

Name	Forward (5' to 3')	Reverse (5' to 3')
GmSBH1PFR	CCCAAGCTTATAAAGATGCAGAAAT	GCTCTAGAGATAAGAAGTGCCATTA
GmSBH1 promoter(FL)FR	GACCATGATTACGCCAAGCTTATAAAGATGCAGAAATCGG	ACTACCACCCTCCATTCTAGAAGATAAGAAGTGCCATTAG
P2FR	GACCATGATTACGCCAAGCTTTTAGTAGAACTTTCAAGAA	ACTACCACCCTCCATTCTAGAAGATAAGAAGTGCCATTAG
P3FR	GACCATGATTACGCCAAGCTTCTCAAGAAAAAAAGAAGAG	ACTACCACCCTCCATTCTAGAAGATAAGAAGTGCCATTAG
P4FR	GACCATGATTACGCCAAGCTTTCTACTATATCGTACGTAT	ACTACCACCCTCCATTCTAGAAGATAAGAAGTGCCATTAG
P5FR	GACCATGATTACGCCAAGCTTGATTTGATCGATCGTTCCT	ACTACCACCCTCCATTCTAGAAGATAAGAAGTGCCATTAG
P2-P3FR	GACCATGATTACGCCAAGCTTTTAGTAGAACTTTCAAGAA	ACTACCACCCTCCATTCTAGAAGATAAGAAGTGCCATTAG
P2-P3-MFR	GACCATGATTACGCCAAGCTTTTAGTAGAGCTGTCAAGAA	ACTACCACCCTCCATTCTAGAAGATAAGAAGTGCCATTAG
P2-P3-DFR	GACCATGATTACGCCAAGCTTAAGAACTAAGCCTACGTAC	ACTACCACCCTCCATTCTAGAAGATAAGAAGTGCCATTAG
35S minFR	GACCATGATTACGCCAAGCTTATCTCCACTGACGTAAGGG	ACTACCACCCTCCATTCTAGATTCTCTCCAAATGAAATGA
p35SFRFR	AGATTAGCCTTTTCAATTTCAGAAA	AAGGGACTGACCACCCGGGGATCC
GUSFR	ATGGAGGGTGGTAGTAGTA	GAGCATGGGATGGGAAAGA
PFR	AATTCGAACTTTCGAACTTTCGAACTTTCGAGCT	CGAAAGTTCGAAAGTTCGAAAGTTCG

2.5. Soybean transformation

The soybean genetic transformation system was used in this study (Song et al., 2013; Gao et al., 2015), with minor modifications. Soybean seeds were disinfected by chlorine gas, which was the reaction of NaOH with $NaClO_4$. The disinfected seeds were placed on germination medium (0.3% Phytagel, 2% sucrose, 3 mM MES, 3.21 g/L B5 salts with vitamins, pH 5.8) and germinated at 25 °C in the dark overnight. One day later, the imbibed soybean seeds were cut along the hilum into two explants, each explant including the cotyledons and hypocotyls. The cotyledonary nodes were cut three to four times to form a wound, and then the wounded nodes were immersed in the suspension of *A. tumefaciens* at 25 °C for 30 min with slight agitation. After inoculation, the explants were cultured on cocultivation medium with one piece of sterile filter paper at 25 °C for 3 days in darkness for further HTH treatment.

2.6. Histochemical and fluorometric analysis of the *GUS* gene

The expression level of the *GUS* reporter gene was analyzed at 0, 1, and 2 h under HTH stress. The methods of GUS histochemical and fluorometric assays were conducted based on the description of Jefferson (1991) with minor modifications. For histochemical staining, the tissues of plants were incubated for 24 h at 37 °C in the dark in staining solution [1 mM potassium ferrocyanide, 10 mM EDTA (ethylenediaminetetraacetic acid), 100 mM PBS (sodium phosphate, pH 7.0), 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-b-D-glucuronide, dissolved in DMSO), 1 mM potassium ferricyanide, 0.1% (v/v) Triton X-100]. After 24 h of staining, the chlorophyll of leaves was removed by 10% ethanol solution of acetic acid (v/v) (Llave et al., 2000; Deng et al., 2012).

For fluorometric assays (Rushton and Somssich, 2002; Hiroyuki et al., 2008), the *N. benthamiana* leaves were ground with liquid nitrogen in extraction buffer (4 °C) [100 mM PBS (sodium phosphate, pH 7.0), 10 mM EDTA (ethylenediaminetetraacetic acid), 10% (w/v) sodium lauryl sarcosine, 0.1% (v/v) Triton X-100, 10 mM β-mercaptoethanol]. The samples were centrifuged at 12,000 × *g* and 4 °C for 5 min. Total protein was measured by the Bradford method, using bovine serum albumin (BSA) as a standard, and 250 μL of supernatant was mixed with 450 μL of 2 mM 4-methylumbelliferyl-β-d-glucuronide (4-MUG) at 37 °C. Then 50 μL of mixed solution was transferred rapidly to a clean EP tube containing 950 μL of GUS stop buffer (0.2 M Na_2CO_3) to be used as a control. GUS activity analysis was measured at 37 °C for 15, 30, 45, and 60 min. The mixed solution (50 μL) was transferred rapidly to a clean EP tube containing 950 μL of GUS stop buffer (0.2 M Na_2CO_3), respectively. The fluorescence of each sample was determined at excitation of 365 nm and

Figure 1. Nucleotide sequence of the promoter of *GmSBH1* (*GmSBH1P*). A: The transcription start site of *GmSBH1P*, and designated as "+1". Putative cis-acting elements were underlined or shown in boxes. See Table 2 for descriptions of the elements.

emission of 455 nm with a SpectraMax M5. The GUS activity was calculated according to Chai et al. (2013).

2.7. Screening of yeast one-hybrid library

When the plants of soybean cultivar Xiangdou No. 3 reached the physiological maturity stage (R_7), they were treated for 6, 12, 24, 48, 96, and 168 h with HTH stress (Wang et al., 2012). Finally, the seeds sampled at each time point were mixed to build a HTH cDNA library (Shu et al., 2015). The library was constructed with the SMARTTM cDNA library construction kit and then the cDNA fragments were cloned into pGADT7 (Supplemental Figure 2A) by double enzyme digestion method. The fragment of GAACTTTC was constructed 3 times into the bait vector (pHIS2) (Supplemental Figure 2B) to generate pHIS2-24-bp (3 × GAACTTTC). The plasmids containing the transformed bait vectors (pHIS2) were transformed into yeast strain Y187. The pHIS2-24-bp (3 × GAACTTTC) was used for transcriptional activation function. Then CLONTECH yeast one-hybrid system was used to screen the soybean HTH yeast one-hybrid library (Chen et al., 2008; St-Jean et al., 2013; Walhout et al., 2016).

2.8. Statistical analysis

Analysis of variance (ANOVA) was performed for the data and the least significant difference (LSD, $P < 0.05$) was used for multiple comparisons. Each column indicated the fold of change in HTH stress at 1 h and 2 h compared to 0 h, normalized to 35S min. Each experiment was conducted in triplicate and error bars represent the standard errors.

3. Results

3.1. Isolation and sequence analysis of *GmSBH1P*

A sequence of 2040 bp in length containing the transcription initiation site of the promoter of the *GmSBH1* gene, named *GmSBH1P*, was isolated from soybean genomic DNA. The sequence of *GmSBH1P* was further analyzed by the online software PLACE and PlantCARE. Eighteen kinds of potential cis-acting elements were found to exist in *GmSBH1P* (Figure 1; Table 2). Among them, 6 × CAAT and 3 × TATA boxes existed at numerous positions. A series of putative cis-regulatory elements that enable the tissue-specific or inducible expression of *GmSBH1* were identified, including four types of light-responsive elements (2 × E-BOX, G-BOX, CA-element, and I-BOX), three kinds of ABA-responsive elements (3 × MYB, DPBF, 5 × WRKY), two cis-acting elements involved in pathogen-induced expression (3 × GT1-BOX, BIND10S), a drought-inducible element (3 × MYB), several elements required in tissue-specific expression (5 × OSE, 2 × E-BOX, SEF4, SURE), two heat-inducible elements (HSE, STRE), one of plant proteins gather at ACGT element (3 × A-BOX), and a zinc protein binding site (DOF-cores).

3.2. *GmSBH1P* mediates rapid and strong expression of GUS in soybean cotyledonary nodes and *N. benthamiana* leaves under HTH stress

To investigate the inducibility of *GmSBH1* promoter, a recombinant containing it fused with the *GUS* reporter gene (GmSBH1P-GUS) was generated. The *CaMV 35S* minimal promoter (35S min-GUS) without any cis-elements and *CaMV 35S* promoter (35S-GUS) served as negative and positive controls, respectively (Figure 2A). The expression levels of GUS were examined by *Agrobacterium*-mediated transient expression system in the leaves of *N. benthamiana* and the cotyledonary nodes of soybean under HTH stress, respectively. Histochemical staining revealed that *GmSBH1P* was more strongly induced both in the leaves of the transformed *N. benthamiana* and in the cotyledonary nodes of soybean under HTH treatment than in those of the controls (Figures 2B and 2C). Further enzyme activity assays of GUS indicated that *GmSBH1P* showed almost 6.3- and 7.6-fold induction by HTH stress at 1 and 2 h compared to 0 h in the leaves of *N. benthamiana* (Figure 2D) and 5.6- and 7.3-fold by HTH stress at 1 and 2 h compared to 0 h in the cotyledonary nodes of soybean (Figure 2E). As expected, the negative control infiltrated into *N. benthamiana* leaves and soybean cotyledonary nodes displayed very low levels of GUS activity, whereas the positive control exhibited high levels of GUS activity regardless of the treatment (Figures 2D and 2E). Taken together, the induction activity levels of GUS by *GmSBH1P* were markedly increased under HTH stress (Figure 2), implying that *GmSBH1P* responded rapidly and strongly to HTH treatment.

3.3. Deletion assay identifies an important HTH-stress responsive region in *GmSBH1P*

To identify the cis-regulatory regions in response to HTH stress, a series of progressive 5' truncated *GmSBH1P* were obtained and fused to the *GUS* reporter gene to generate P2 (–1024/+790 bp), P3 (–638/+790 bp), P4 (–336/+790 bp), and P5 (+119/+790 bp) constructs (Figure 3A). With these constructs, *Agrobacterium*-mediated transient expressions in the leaves of *N. benthamiana* and in the cotyledonary nodes of soybean were conducted, respectively. Their potential expression patterns and induced activities were determined under HTH stress. P2 was found to increase GUS activity by 4.2- and 5.3-fold at 1 and 2 h under HTH stress compared to 0 h in the leaves of *N. benthamiana* (Figure 3B) and by 4.7- and 5.6-fold at 1 and 2 h under HTH stress compared to 0 h in soybean cotyledonary nodes (Figure 3C), while P3, P4, and P5 did not increase GUS enzyme activity under HTH stress (Figures 3B and 3C). The results of GUS activity were further confirmed by histochemical staining analysis (Figures 3D and 3E). Our results indicated that the fragment (from –1024 to –638 bp) was essential for *GmSBH1P* in response to HTH treatment.

3.4. Cis-acting HSE is a vital element in response to HTH stress in the *GmSBH1* promoter

A cis-acting HSE (GAACTTTC) was found to exist in the sequence from –1024 to –638 bp in *GmSBH1P* by website analysis (Figure 1; Table 2), which was proven above to be essential for the promoter in response to HTH treatment. To investigate whether the HSE was involved in mediating *GmSBH1P* in response to HTH stress, it was substituted with an irrelevant sequence to generate a mutated construct P-M (GAgCTgTC) and deleted to generate a construct P-D, respectively (Figure 4A). The construct containing the 8-bp fragment (P2-P3) was used as a control. Specific primers were used to construct these recombinant plasmids by PCR. Then all the constructs were used to infect the leaves of *N. benthamiana* and the cotyledonary nodes of soybean using the *Agrobacterium*-mediated method, respectively. The GUS enzyme activity in *N. benthamiana* leaves transformed by construct P2-P3 was increased by 3.2-fold and 3.9-fold upon the induction of HTH for 1 h and 2 h, while for the transformants with P-M and P-D, the GUS enzyme activity remained unchanged (Figure 4B). In the transformed soybean cotyledonary nodes, similar results were obtained (Figure 4C). Moreover, histochemical staining also showed similar results (Figures 4D and 4E). All the results indicated that the cis-acting HSE (GAACTTTC) was essential for *GmSBH1P* in response to HTH stress.

3.5. Screening of interaction proteins using yeast one-hybrid library under HTH stress

It is known that the expression of the downstream gene is promoted by the coregulation of the upstream promoter

Table 2. Identification of cis-acting elements in the *GmSBH1P* sequence using the PLACE and PlantCARE databases.

Cis-element	Sequence	Function	Position from ATG
OSE	*AAAGAT/CTCTT*	Organ-specific elements in infected cells of Root nodules	–1242 +67 +110 +269 +545
G-BOX	*GATA*	Response light signal	–1165
E-BOX	*CACCTG*	Light-responsive and tissue-specific activation of phenylpropanoid biosynthesis genes	–1142 –372
CAAT-BOX	*CAAT*	Sequences responsible for the tissue promoter activity	–1088 –977 –941 –596 –587 –140
BIND 10S	*TGTCA*	Disease-responsive element	–1073
HSE	*GAACTTTC*	Cis-element for heat induction	–1018
A-BOX	*TACGTA*	Plant proteins gather at ACGT elements	–993 –580 –324
DOF-cores	*AAAG*	Zinc protein binding site	–952 –174 +222 +386 +436 +534 +743
MYB	*CCTACC/CTGTTA/AACCA*	In response to drought and ABA signal	–903 –808 +452
CA-element	*CTAACAC*	Response light signal	–830
GT1-BOX	*GAAAAA*	Cis-acting element involved in pathogen- and salt-induced expression	–763 –627 –435
WRKY	*TGAC*	Response ABA signal	–705 –642 +164 +493 +734
SURE	*AATAGAAAA*	Separate cis-sequences and trans-factors direct metabolic and developmental cis-element for heat induction	–675
STRE	*AGGGGG*	Involved in multiple signaling pathways in plants	–607
CGCG-BOX	*GCGCGC*	Response light signal	–396 +311
I-BOX	*GATAAG*	Essential for promoter recognition	+690
TATA-BOX	*TTATTT/ TATTAA*	Response ABA signal	–22 –375 –135
DPBF	*ACACAAG*		+663

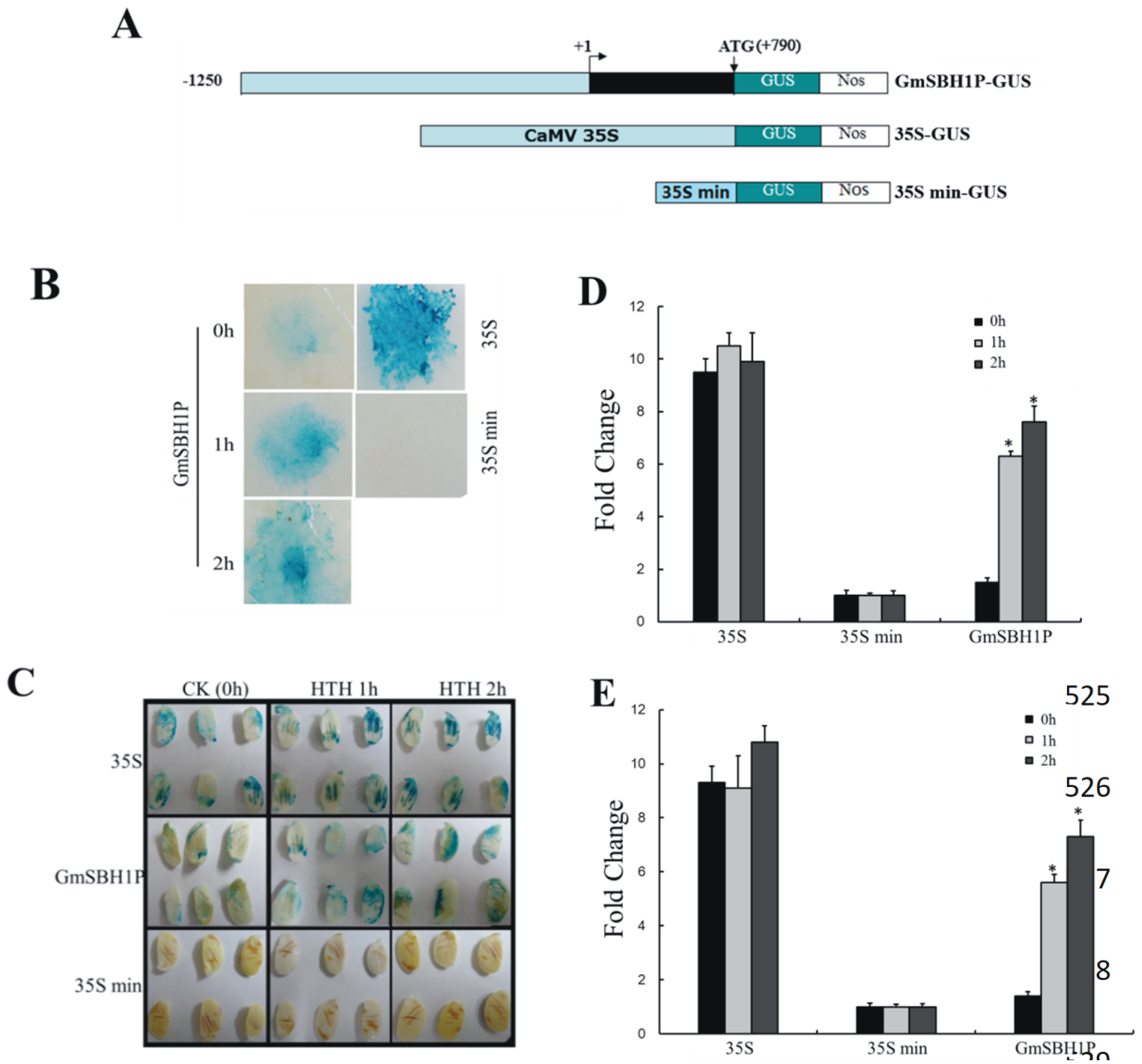

Figure 2. Induction of the *GmSBH1P* in *N. benthamiana* leaves and soybean cotyledonary nodes under HTH treatment. A) GmSBH1P-GUS, a construct containing the *GmSBH1* promoter (*GmSBH1P*) fused with the GUS reporter gene; 35S min-GUS, a PBI121 vector containing CaMV 35S minimal promoter (35S min) without any cis-elements, served as a negative control; 35S-GUS, PBI121 containing *GUS* gene under the control of the *CaMV 35S* promoter (35S), used as a positive control. B, C) Histochemical GUS staining of GmSBH1P-GUS constructs in the leaves of *N. benthamiana* (B) and in the cotyledonary nodes of soybean (C) under HTH stress for 0, 1, and 2 h, respectively. D, E) Enzymatic determination of GUS activity in expanded leaves of *N. benthamiana* (six weeks old) (D) and soybean cotyledonary nodes (E). Each column indicates the fold change of HTH treatment at 1 h and 2 h compared to 0 h and then is normalized to 35S min. Each experiment was conducted in triplicate and the error bars display the standard error.

and its element binding protein. To identify the proteins that can interact with the cis-acting HSE (GAACTTTC) in GmSBH1P in response to HTH stress, a bait vector, pHIS2-24-bp (with three copies of the core 8-bp sequence of 3 × GAACTTTC), was constructed, and then the plasmids were transformed into Y187 (yeast strain). The existence of transcriptional activation of the bait sequence was verified, and the results showed that the pHIS2-24-bp had transcriptional activation function (Supplemental Figure 3).

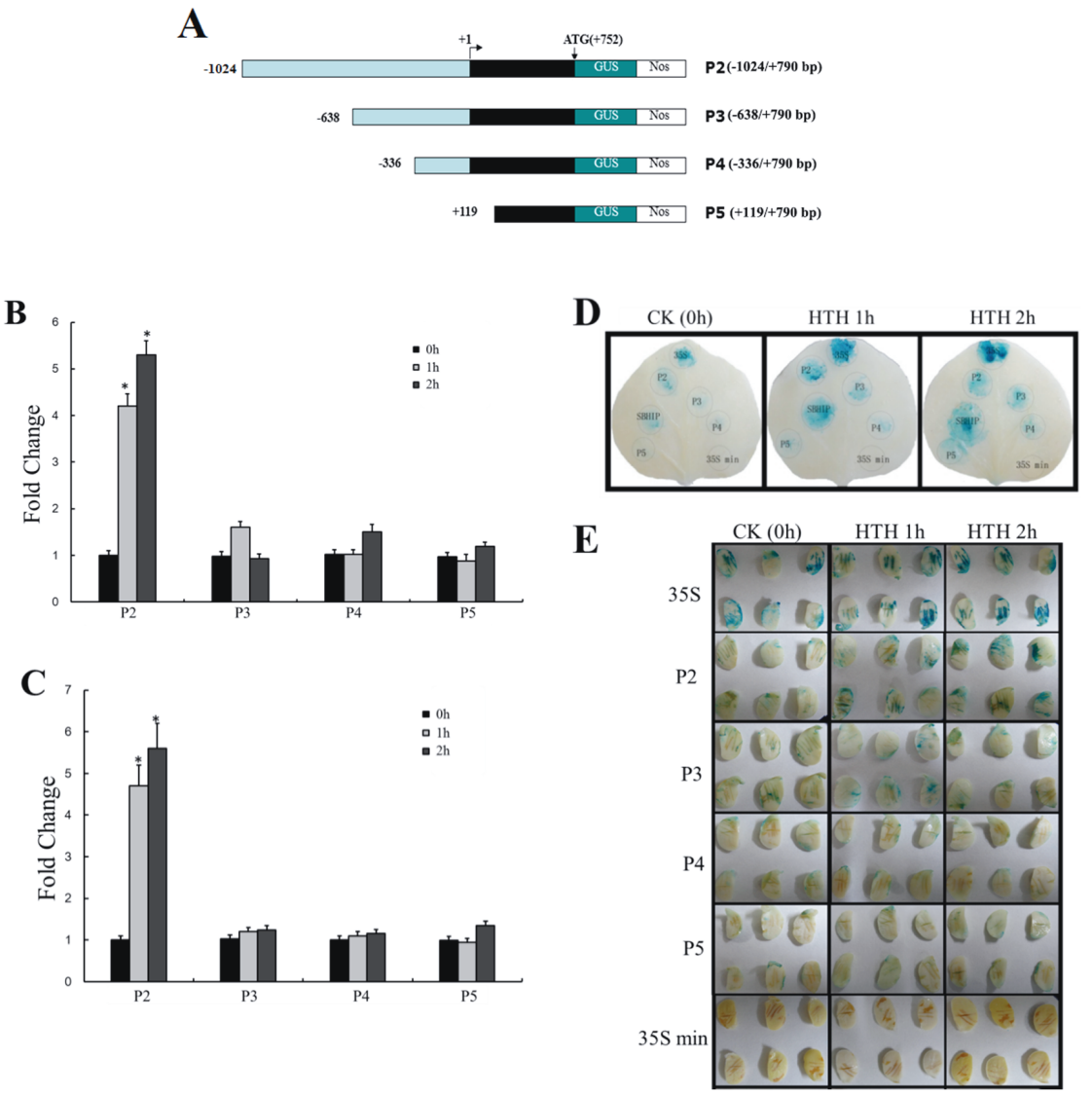

Figure 3. The 5' deletion assay of *GmSBH1P* activity in transiently expressing soybean cotyledonary nodes and *N. benthamiana* leaves. A) Constructs produced using a series of progressive 5' truncated *GmSBH1P* fused to the GUS reporter gene. P2, containing the sequence of –1024/+790 bp of *GmSBH1P*; P3, –638/+790 bp; P4, –336/+790 bp; P5, +119/+790 bp. B, C) Enzymatic determination of 5' deletion constructs in transiently expressed leaves of *N. benthamiana* (B) and cotyledonary nodes of soybean (C). The expanded leaves of *N. benthamiana* (six weeks old) and the cotyledonary nodes of soybean were infiltrated with *Agrobacterium*-harboring constructs and inoculated under HTH after infiltration, respectively. Each column indicates the fold of change in HTH stress at 1 h and 2 h compared to 0 h and normalized to 35S min. Each experiment was conducted in triplicate and the error bars represent the standard error. D, E) Histochemical GUS staining of *GmSBH1P*, P2, P3 P4, and P5 in the leaves of *N. benthamiana* (D) and the cotyledonary nodes of soybean (E). 35S is a positive control while 35S min is a negative control.

Using pHIS2-24-bp as bait, 169 positive clones were obtained from the cDNA library of soybean seed by yeast one-hybrid assay (Figure 5A). To determine whether there existed true interaction between the 24-bp fragment and 169 positive clones, the 24-bp fragment and 169 positive clones were cotransformed into yeast cells and selected on SD-TL (–Trp and –Leu) (Figure 5B) and SD-TLH (–Trp, –Leu, and –His) plates with 350 mM 3-amino-1,2,4-triazole (3AT) (Figure 5C), respectively. The results showed that the positive control was able to grow normally on the screening medium with or without 3AT (Figure 5D), while the negative control could only grow on the screening

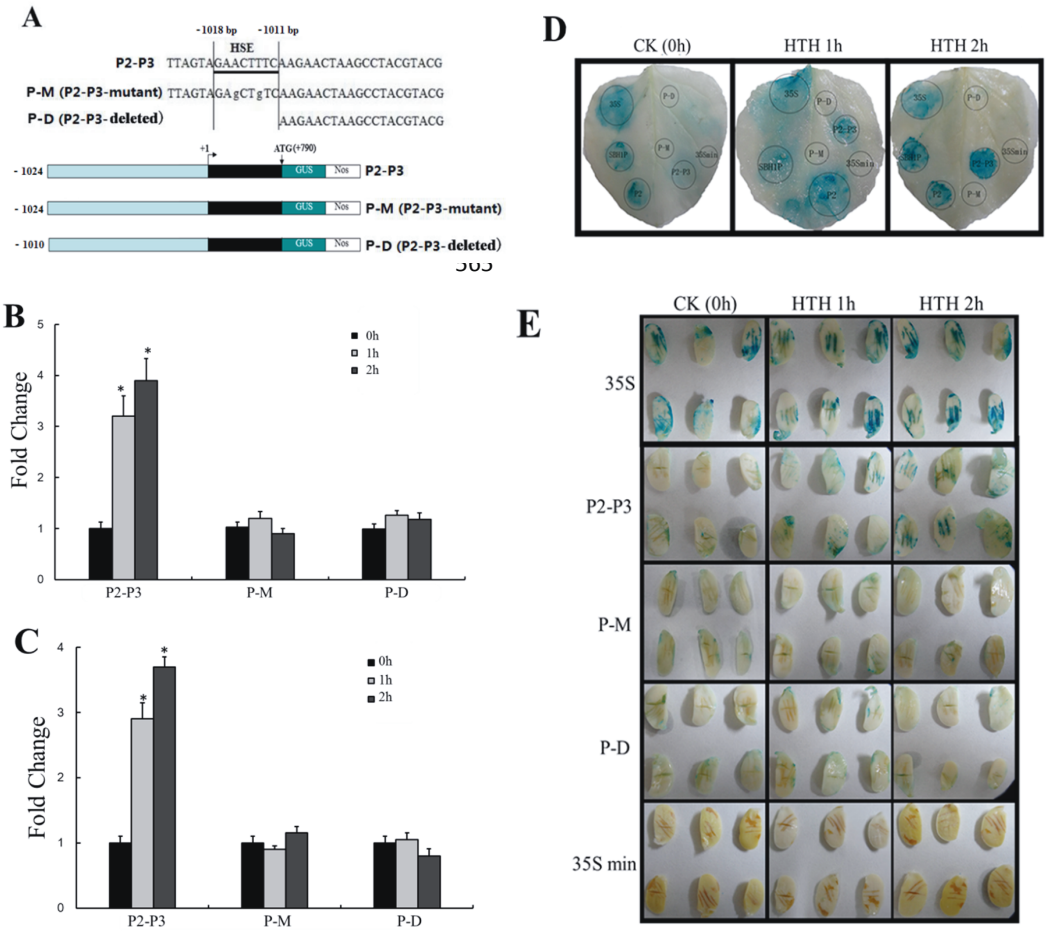

Figure 4. The activity analysis of HSE (GAACTTTC) in *GmSBH1P* under HTH stress. A) The 8-bp HSE was substituted with an irrelevant sequence to generate a mutated P-M construct and deleted to generate a P-D construct, respectively. The construct P2-P3 containing the 8-bp element was used as a control. Lowercase letters indicate the nucleotide substitutions. B, C) Enzymatic determination of GUS activity in expanded leaves of *N. benthamiana* (six weeks old) (B) and the cotyledonary nodes of soybean (C) under HTH stress for 0, 1, and 2 h, respectively. Each column shows the fold of change under HTH stress at 1 h and 2 h compared to 0 h and normalized to 35S min. Each experiment was conducted in triplicate and the error bars represent the standard error. D, E) Histochemical GUS staining of different constructs in transiently expressing *N. benthamiana* leaves (D) and soybean cotyledonary nodes (E) under HTH stress for 0, 1, and 2 h. 35S is a positive control and 35S min is a negative control.

medium without 3AT, due to be unable to activate the His3 reporter gene. Only 126 initial positive clones were found to be able to grow normally on the medium with 3AT. From them, 82 proteins were identified through blasting with the GenBank database (Supplemental Table 1).

4. Discussion

The combination of HTH stress is a serious stress for spring soybean during seed development, resulting in the reduction of seed vigor (Wang et al., 2012; Shu et al., 2015). Previously, *GmSBH1* was proven to be strongly induced by HTH stress (Shu et al., 2015). Here, its promoter (*GmSBH1P*, 2040-bp) was isolated and characterized. *GmSBH1P* contains many known cis-acting elements (Figure 1; Table 2). Deletion and mutation analysis indicated that its cis-acting HSE (GAACTTTC) was essential to confer itself activity in rapid and strong response to HTH stress (Figure 4). These results suggested that *GmSBH1P* could be applied

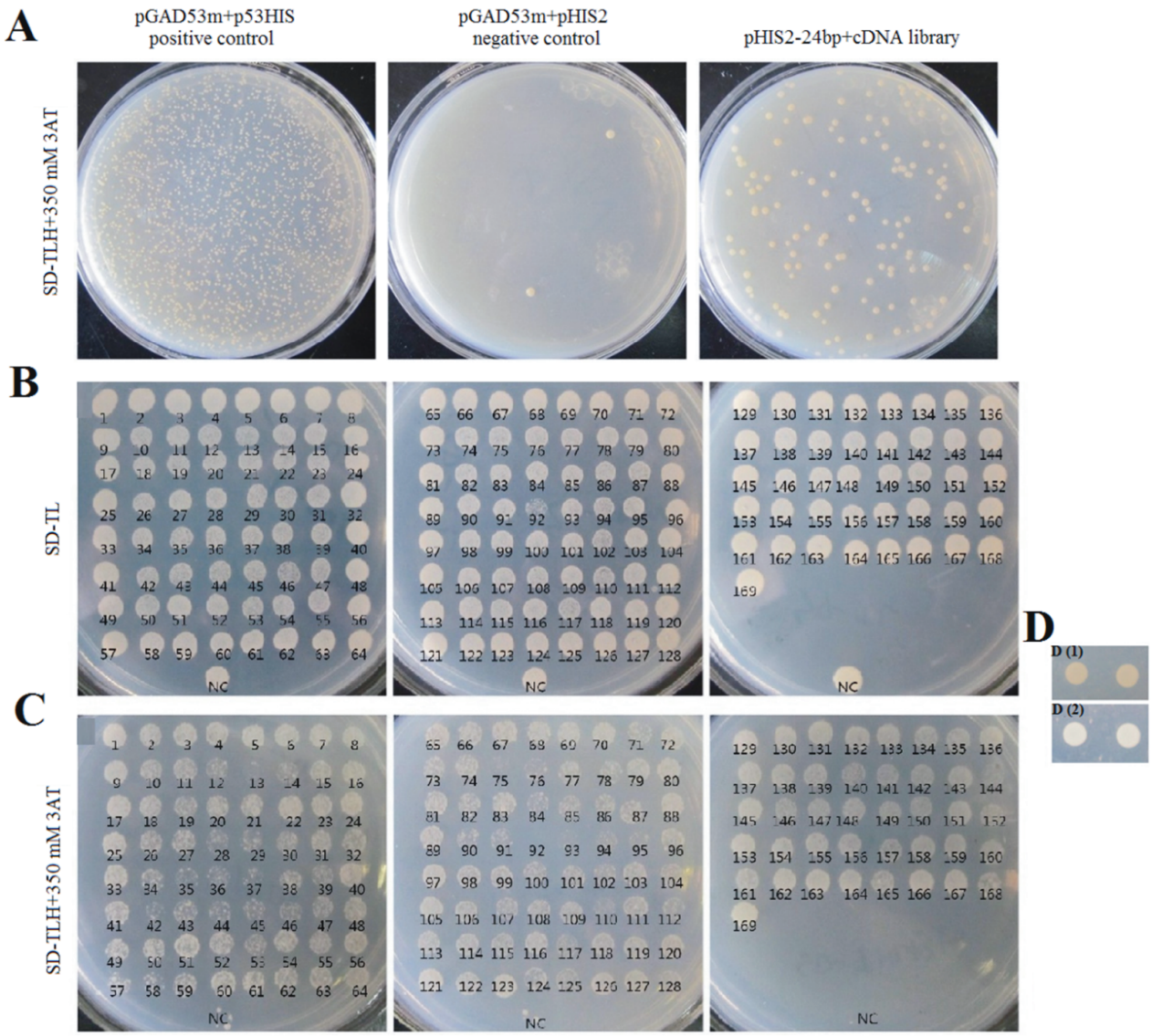

Figure 5. Screening yeast one-hybrid library. A) Using pHIS2-24-bp (3 × GAACTTTC) as bait, interacting proteins were screened using yeast one-hybrid from cDNA library of soybean seed. B) The growth of 169 initial positive clones and negative controls on defect type filter plate (SD-TL). C) The growth of the initial positive clones and negative controls on the defect type filter plate (SD-TLH+350 mM 3 AT). NC, Negative control. D) The growth of the positive control in SD-LH (D(1)) and SD-LH+350 mM 3 AT (D(2)).

in enhancing the resistance to HTH stress, and the HSE (GAACTTTC) would be an ideal candidate in mediating the expression of HTH-responsive genes in plants.

Agroinfiltration is a simple and reliable transient expression to study the expression of plant promoters (Jang et al., 2004; Hong et al., 2005; Gao et al., 2015). In the present study, to further understand the cis-acting elements of *GmSBH1P* in response to HTH, first GmSBH1P-GUS was expressed in the leaves of *N. benthamiana* and in the cotyledonary nodes of soybean, respectively. The results indicated that the GUS enzyme activity was rapidly and highly induced by HTH (Figure 2). Second, successive deletion of *GmSBH1P* in the leaves of *N. benthamiana* and the cotyledonary nodes of soybean showed that the fragment from –1024 to –638 bp (containing a HSE) was essential for *GmSBH1P* in response to HTH treatment. Moreover, the fragment from –638 to –336 bp in *GmSBH1P* (containing a STRE) was not induced by HTH (Figure 3). All results indicated that cis-acting HSE might respond to HTH stress, whereas cis-acting STRE would not. To further verify the results, we carried out mutation and deletion analyses and found that the cis-acting HSE

(GAACTTTC) could strongly respond to HTH in the leaves of *N. benthamiana* and in the cotyledonary nodes of soybean (Figure 4). Previous studies showed that cis-acting HSE responds to heat stress (Coca et al., 1996). However, in the present study, it was further found in response to HTH stress combination. The HSE could thus be used as a candidate to produce HTH-inducible promoters or identify novel HTH-responsive cis-elements.

Regulation of gene expression at the level of transcription controls many biological processes. It is the prevalent model of control in activities as diverse as cell cycle regulation, metabolic balance, and responses to the environment. To achieve this regulation, transcription factors act in several mechanisms, including the interactions between DNA and proteins as well as proteins and proteins (Schwechheimer et al., 1998). Plants constantly recognize, measure, and react to changes in the environment by multiple signaling pathways that lead to repression or activation of gene transcription (Lee et al., 2016). In response to stress, cis-acting elements of promoters and their associated binding protein partners play a crucial role (Shen et al., 2015; Harris et al., 2016). Yeast one-hybrid analysis has been widely applied in identification and characterization of DNA-protein interactions and for searching interaction networks in pathways and genomes (Li et al., 1993). The DNA sequence as the bait with no transcriptional activation function is a prerequisite for the yeast one-hybrid (Park et al., 2004; Chen et al., 2008; Shen et al., 2015). To identify the proteins interacting with cis-acting HSE (GAACTTTC), we constructed bait vector pHIS2-24-bp with three copies of HSE to screen the yeast one-hybrid library. The cis-acting HSE was verified to have transcriptional activation function (Supplemental Figure 3) and interacted with 82 kinds of different proteins under HTH stress (Figure 5; Supplemental Table 1). The function of these 82 proteins could be roughly divided into 8 categories of seed storage proteins, seed mature proteins (Shewry et al., 1995; Natarajan et al., 2006), defense proteins (Lee et al., 2016), ribosomal proteins (Mager, 1988), signal transduction proteins (Zeng et al., 2017), enzyme and protease inhibitors (Tormo et al., 2006), other functional proteins, and unknown functional proteins. These proteins can provide a basis for studying the regulatory mechanism of GmSBH1 under HTH stress.

In conclusion, all the above results suggest that cis-acting HSE (GAACTTTC) is a key component of the *GmSBH1* promoter in response to HTH.

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