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RESEARCH ARTICLE

Transcription factor CsHSFB2c suppresses *CsTS1* and *CsGS1* expression to reduce theanine biosynthesis in tea plants under heat stress

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Highlights

- Heat stress led to the unique accumulation patterns of theanine and catechins in tea plants: Theanine content decreased significantly with the increasing temperature (peaking at 20°C), while catechins levels increased with increasing temperature (peaking at 30°C).
- RNA-seq identified a class B heat shock transcription factor CsHSFB2c, whose expression was negatively correlated with theanine content.
- CsHSFB2c directly binds to the promoters of *CsTS1* and *CsGS1* and inhibits their transcription, thereby reducing theanine biosynthesis.

Abstract

Heat stress reduces theanine content in tea plants, but the underlying molecular mechanism remains unclear. In this study, a temperature gradient treatment (20°C, 25°C, 30°C, and 35°C) was performed to unveil the effect of heat stress on biosynthesis and accumulation of theanine. We found that heat stress induced metabolic changes, characterized by decreased theanine content and increased catechin levels. In addition, heat stress up-regulated the expression of the class B heat shock transcription factor gene *CsHSFB2c*, while significantly suppressing the transcription of key theanine biosynthetic genes *CsTS1* and *CsGS1*. Functional studies showed that silencing *CsHSFB2c* increased theanine content, while its overexpression significantly decreased theanine levels. Consistent with these changes, silencing *CsHSFB2c* upregulated the expression of *CsTS1* and *CsGS1*, while overexpression of *CsHSFB2c* downregulated their expression. Yeast one-hybrid (Y1H) and dual-luciferase reporter gene (Dual-LUC) assays showed that CsHSFB2c directly binds to the promoters of *CsTS1* and *CsGS1* and inhibits their expression. These results demonstrate that CsHSFB2c mediates heat-induced suppression of theanine biosynthesis by directly inhibiting the expression of *CsTS1* and *CsGS1*. This study provides a theoretical basis for improving the heat resistance and quality of tea plants *via* molecular breeding.

Keywords: tea plant, high temperature, heat shock transcription factor (HSF), glutamine synthetase, theanine synthetase

1. Introduction

Camellia sinensis (L.) O. Ktze. belongs to Theaceae and is a *Camellia* shrub or small tree. It originates in the Yunnan-Guizhou Plateau of China and is cultivated as a perennial evergreen leaf crop (Chan *et al.* 2007). The young shoots of tea are rich in various metabolites, such as L-theanine, catechins, caffeine and aroma compounds (Fu *et al.* 2015; Wei *et al.* 2018). Among them, theanine (L-theanine) is a non-protein amino acid unique to tea plants and a key component in numerous tea products. It can significantly reduce the bitter and astringent taste of tea soup and elevate the drinking experience (Feng *et al.* 2014). This amino acid

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is present in the roots, buds, and cotyledons of tea seedlings, and the content of theanine in young stems is significantly higher than that in buds (Deng *et al.* 2008; Zhang *et al.* 2025). Theanine content in the tea plant is regulated by genetic factors. Key enzymes such as theanine synthase (CsTS1) and glutamine synthetase (CsGS1) play vital roles in its biosynthesis. The former is responsible for catalyzing the conversion of glutamate and ethylamine into theanine, and the latter provides a key precursor for the synthesis of glutamic acid by catalyzing the formation of glutamine, thereby indirectly regulating the biosynthesis of theanine (Cheng *et al.* 2017; Fu *et al.* 2021).

Theanine content is also affected by various environmental factors. Temperature, as a key factor for the growth environment of tea plants, is believed to have effects on theanine synthesis. Tea shoots grown in warm climates exhibit a characteristic metabolic profile with elevated polyphenol and reduced theanine levels (Xu *et al.* 2012). This leads to increased bitterness and astringency in the summer and autumn teas, reducing the economic value of the product and resulting in significant waste of resources (Dai *et al.* 2015). Theanine content is the highest in spring tea (harvested in cooler months) and declines as temperatures rise, signifying the role of high temperatures in negatively affecting theanine levels in tea plants (Li *et al.* 2016). The gene expression levels of *CsFd-GOGAT* were positively correlated with L-theanine content, whereas the protein level of CsFd-GOGAT was negatively correlated with the theanine content except under the shading treatment. Additionally, CsFd-GOGAT interacted directly with aCsNADH-GOGAT, bCsNADH-GOGAT, CsTS1, or CsNiR in yeast (Liu *et al.* 2017). Another study has shown that high temperature negatively affects theanine accumulation, with content being highest at 20°C and decreasing progressively as temperature rises beyond this point (Zhu *et al.* 2023). These studies signify that the heat stress has significantly affects the hydrolysis and catabolism of L-theanine. For instance, high temperature leads to the accumulation of nitrogen-containing compounds in tea plants, stimulates the activity of hydrolases and accelerates the hydrolysis of L-theanine, resulting in a decrease in its content (Liu *et al.* 2016). The activity and expression of L-theanine synthetase were significantly decreased at 38°C, decreasing the theanine biosynthesis (Liu *et al.* 2016). In addition, high temperature degrades key synthetases such as GOGAT and GS, down-regulates theanine biosynthesis genes, reduces glutamate levels, and weakens theanine synthesis (Liu *et al.* 2017; Li *et al.* 2018).

Heat shock transcription factors (HSFs) and heat shock proteins (HSPs) play dominant roles in the activation of the heat shock response (HSR), with heat-responsive signaling molecules (such as Ca^{2+}, NO, and hormones) acting upstream of HSFs (Li *et al.* 2018). HSFs function by inducing the highly conserved heat shock elements (HSEs) in promoters, which contain alternating motifs. Shim *et al.* (2009) found that Class A (HSF) is involved in transcriptional activation and environmental stress response, while Class B (HSF) act as repressors of gene expression. HSFB members (except HSFB5) contain a characteristic tetrapeptide LFGV in the C-terminal domain, which functions as a repressor domain (Fragkostefanakis *et al.* 2015). Class C (HSF) has neither activation motif nor inhibition motif (Bakery *et al.* 2024). It has been found that HSFA1s, as key regulators of integrating light and temperature signals in *Arabidopsis thaliana*, can induce a large number of *HSFA1s* gene expression under high temperature treatment at 28°C, so that plants can better adapt to high temperature in summer (Tan *et al.* 2023; Li *et al.* 2024). As a heat-inducible transactivator, HsfA2 maintains the expression of *Hsp* genes and prolongs the duration of heat stress tolerance of *Arabidopsis* (Charng *et al.* 2007). A previous study showed that HSFA1d induces *HSFA2* expression to enhance plant heat tolerance (Li *et al.* 2024; Raturi and Zinta 2024). HSFA1a regulates plant heat stress response and is an essential heat tolerance factor for tomato stress (Huang *et al.* 2023). TaHsfA1 is a core regulatory factor necessary for basic heat tolerance in wheat, and *Tahsfa1*-KO is highly heat-sensitive compared to the wild type (Wang *et al.* 2023). The heterologous expression of *CsHsfA2* improved the heat resistance of transgenic yeast (Zhang *et al.* 2020). In addition, studies have shown that tomato *HsfB1* can cooperate with class A HSFs and may help maintain or restore the expression of certain viruses or housekeeping genes during continuous heat stress (Bharti *et al.* 2004). HSFB1 and HSFB2b are transcriptional repressors, and their loss of function can slightly elevate the heat resistance of *A. thaliana* at 42°C above that of the wild type (Ikeda *et al.* 2011). HSFB2b promotes flavonoid accumulation by activating a subset of flavonoid biosynthesis-related genes and inhibiting the repressor gene *GmNAC2* to release another gene subset in the flavonoid biosynthesis pathway, thereby improving salt tolerance of soybean (Bian *et al.* 2020). The above results showed that HSFs are the core regulatory elements of plant heat stress response. HSFAs are well-studied positive regulatory factors under plant heat stress. However, studies on the molecular regulation mechanism of HSFBs as inhibitors in plant heat stress are rare. Therefore, the regulatory mechanism of theanine biosynthesis at high temperatures in tea plants needs elucidation.

In this study, we found that theanine content in tea plants decreases with increasing temperature within the 20°C–35°C range. Transcriptome analysis showed that the expression level of CSS0006648 (*CsHSFB2c*) was significantly increased at 30°C and 35°C, while the expression of *CsTS1* and *CsGS1* was significantly decreased. After silencing the expression of *CsHSFB2c*, theanine content increased significantly, while transient overexpression of *CsHSFB2c* decreased theanine content. In addition, the results exhibited that CsHSFB2c directly binds to the promoter region of *CsTS1* and *CsGS1*, inhibits the expression of *CsTS1* and *CsGS1*, and then reduces the synthesis of theanine. This study revealed the molecular mechanism of CsHSFB2c negatively regulating theanine biosynthesis by inhibiting the expression of *CsTS1* and *CsGS1* under heat stress. It provides a theoretical basis for stress-resistant cultivation and quality improvement of tea plants.

2. Materials and methods

2.1. Materials and treatments

One-year-old cuttings of the tea plant 'Fuding Dabaicha' were selected as materials for gradient temperature treatment. Subsequently, changes in metabolites within young shoots were measured, and RNA-seq analysis was performed. The cuttings were cultivated under a photoperiod of 16 h light (25°C)/8 h darkness (20°C) in a controlled environment chamber for 15 d prior to treatment. Four distinct temperature regimes were applied: 20°C, 25°C, 30°C and 35°C. After 20 d, the young shoots (one bud and two leaves) were collected and immediately placed in liquid nitrogen, then stored at −80°C for RNA extraction. The artificial climate chamber was set with a photosynthetic photon flux density of 200 μmol m^{-2} s^{-1}, a relative humidity of 70%, and a CO_2 concentration of 750 μmol mol^{-1}. Samples were pooled from three plants per replicate, with three biological replicates conducted throughout the study (Chen F *et al.* 2024).

2.2. Identification and phylogenetic analysis of CsHSFB2c homologs

The protein sequence of CsHSFB2c (CSS0006648) was retrieved using the Tea Plant Information and Analysis (TPIA) database (http://tpia.teaplants.cn). Homologous and orthologous protein sequences from *Camellia sinensis*, *Arabidopsis*, and *Oryza sativa* were collected and further confirmed by scanning against PROSITE (http://prosite.expasy.org/prosite.html) and NCBI (www.ncbi.nlm.nih.gov). The software MEGA11 was employed for the analysis of phylogenetic relationships.

2.3. Transcriptome sequencing, RNA extraction, cDNA preparation, and qRT-PCR analysis

Transcriptome sequencing was performed on three biological replicate samples (one bud and two leaves) using the Illumina Nova Seq 6000 platform through Novogene (Beijing, China). In this study, the FPKM values of key differentially expressed genes were analyzed. For tea shoot samples with gene silencing and transient expression, total RNA was extracted by the CTAB method (Lu *et al.* 2021), cDNA was synthesized according to Prime Script RT Master Mix (TaKaRa, Beijing), and then qRT-PCR was performed using gene-specific primers (Appendix A). The Bio-Rad CFX Connect™ real-time quantitative PCR instrument was used for qRT-PCR analysis. *CsActin* served as an internal reference gene. Relative expression was determined from cDNA synthesized from three independent biological replicates, and analyzed using the $2^{-\Delta\Delta C_T}$ method. Prior to correlation analysis, items with FPKM values<10 were removed.

2.4. Analysis of theanine content

As previously reported (Tsushida and Takeo 1984), the theanine content in tea plant tissues was analyzed by hot water extraction, with the following modifications: Initially, freeze-dried tea tissue samples weighing 0.1 g were meticulously ground into a fine powder. This powder was then dissolved in 4 mL of distilled water and subjected to hydrolysis in a hot water bath at 100°C for 30 min. Following this step, the extract underwent centrifugation at a speed of 12,000 r min^{-1} for 15 min to separate the solid and liquid components. The resultant supernatant was further purified by passing it through a 0.22 μm membrane filter. Subsequently, the filtered liquid extract was analyzed using high-performance liquid chromatography (HPLC). The entire process adhered to the parameters and methodologies outlined by Dong *et al.* (2020) ensuring accuracy and reliability in the determination of theanine levels in tea plant tissues (Dong *et al.* 2020).

2.5. Determination of caffeine content

The fresh tea samples were de-enzymed by steaming for 1 min, then dried at 80°C and ground into a fine powder. According to the national standard GB/T8312-2013 (slightly modified), the content of caffeine was determined by HPLC. Specifically, 1.0 g sample was weighed in a 500 mL flask, followed by the addition of 4.5 g magnesium oxide and 300 mL ultrapure water. The mixture was then boiled and extracted in a constant temperature water bath for 20 min, with oscillation applied once every 5 min. After extraction, the solution was filtered under reduced pressure while still hot and immediately transferred into a 500 mL volumetric flask. Subsequently, it was allowed to cool, diluted to the mark, and mixed thoroughly. Finally, the sample was filtered through a 0.45 μm membrane before being tested on the machine. The HPLC parameters and methods refer to the description outlined by Li G D *et al.* (2022).

2.6. Determination of catechins content

The content of various catechins was determined by HPLC (Hu *et al.* 2024; Yuan *et al.* 2025). The detailed steps are in accordance with the national standard GB/T8313. A 0.2 g aliquot of the freeze-dried sample was weighed, crushed and extracted with 5 mL 70% methanol in a 70°C water bath. The extract was centrifuged at 4°C, and the upper solution was transferred to a 10 mL volumetric flask. Repeat the above operation. The extract was finally diluted to 10 mL, and filtered with a 0.45 μm filter membrane. Three biological replicates were set for each sample.

2.7. Subcellular localization of CsHSFB2c in *Nicotiana benthamiana*

The open reading frame (ORF) of CsHSFB2c was cloned from a cDNA library of tea leaves and inserted into the GFP fusion expression vector pCAMBIA1300 to yield the chimeric construct 35S::*CsHSFB2c*-GFP. This construct was transiently expressed in *N. benthamiana* epidermal cells in conjunction with a nuclear marker through *Agrobacterium*-mediated transformation. Post-infiltration, tobaccos were maintained under low-light conditions for a period of 2–3 d. Prior to the examination of fluorescence, the epidermal cells were prepared as slides. The distribution of the green fluorescent signal was then visualized using a laser scanning confocal microscope, and fluorescent images were subsequently captured. Primers used in this study are detailed in Appendix A.

2.8. Transient overexpression of *CsHSFB2c* in tea leaves

Referring to previous studies (Li H *et al.* 2022), the experimental method was optimized to achieve transient overexpression of *CsHSFB2c* in tea leaves. The *CsHSFB2c* gene was cloned into pCAMBIA1300 to obtain the overexpression vector, which was transformed into *Agrobacterium tumefaciens* cells of the GV3101A strain and cultured overnight. A resuspended medium containing 10 mmol L^{-1} MES and 10 mmol L^{-1} $MgCl_2$ was prepared, adjusted to pH 5.6, and 200 μmol L^{-1} acetosyringone was added. The bacteria after centrifugation were suspended by using the suspension medium. When the OD_{600} value reached 1.0, it was injected into the second leaf. *Agrobacterium* with an empty vector carrying a 35S promoter was injected into the second leaf as a control. The samples of the treated leaves were collected after 4 d. The theanine content of the samples was determined, and the expression levels of *CsHSFB2c*, *CsTS1* and *CsGS1* were confirmed by qRT-PCR analysis.

2.9. Virus-induced gene silencing (VIGS) of *CsHSFB2c* in tea plants

The tea plant variety 'Fuding Dabaicha' served as the experimental material, with its cuttings being trimmed to 20 cm in length. For the VIGS of *CsHSFB2c* in tea plants, *A. tumefaciens* strain GV3101 carrying pTRV1 and pTRV2-*CsHSFB2c* or pTRV2 was resuspended in induction medium at a 1:1 ratio to an optical density (OD_{600}) of 0.6 and co-infiltrated into the cuttings, with pTRV2 serving as a negative control. The detailed procedure adhered to the protocols established in previous studies (Li G D *et al.* 2022). Following 3 d in darkness, the inoculated tea cuttings were cultivated in a greenhouse under a 16 h/8 h light/dark photoperiod at 25°C. After 40 d of culture, one bud and two leaves of the new shoots were collected for further analysis.

2.10. Yeast one-hybrid (Y1H) analysis

The Y1H test was performed as described previously (Lin *et al.* 2021), under instructions of the Matchmaker™ Gold Yeast One-Hybrid Kit (Clontech, San Francisco, USA). Full-length CDS of *CsHSFB2c* was cloned into pGADT7 and digested with *Eco*RI and *Bam*HI as a prey vector. The sequences of *CsTS1* and *CsGS1* promoters were cloned into the pAbAi vector, digested with *Kpn*I and *Sal*I, as bait vectors. The pAbAi vectors containing *CsTS1* and *CsGS1* promoters were linearized and transformed into Y1H Gold yeast strains, respectively. The minimum inhibitory concentration of AbA (CA2332, Coolaber, Beijing, China) was screened to remove the promoter self-activation. The yeast cells containing the bait vector were transformed with pGADT7-CsHSFB2c or pGADT7 empty vector, respectively. The transformed yeast strains were cultured on SD–Leu–Ura medium at 30°C for 3 to 4 d and their growth was monitored.

2.11. Dual-luciferase reporter assay system

Transcriptional regulation was assessed in *N. benthamiana* leaf cells *via* a dual-luciferase assay (Grentzmann *et al.* 1998; Wu *et al.* 2020). Promoter sequences of *CsTS1* and *CsGS1* were cloned into the pGreenII 0800-LUC vector to serve as the reporter, whereas the ORF of *CsHSFB2c* was inserted into the pGreenII 62-SK vector to act as the effector. The constructs were introduced into *A. tumefaciens* EHA105. Transient expression of the recombinant plasmids occurred in leaf cells following infection by EHA105. The inoculated leaf areas were uniformly sprayed with 0.2 mg mL^{-1} D-luciferin potassium salt and then incubated in darkness for 10 min. Luminescent images were captured and fluorescent activity were measured using the Fusion FX7 system (VILBER, France). Primer sequences are detailed in Appendix A.

2.12. Statistical analysis

All experiments were conducted with three biological replicates. Data are presented as means±standard deviations and were subjected to analysis of variance (ANOVA). ANOVA and multiple comparisons were performed using SPSS version 22.0 software (Statistical Product and Service Solutions). Significance at the $P<0.05$ level was analyzed using Duncan's multiple range test.

3. Results

3.1. Changes of tea plant metabolites under different temperature treatments

To investigate the impact of heat stress on the flavor components of tea, one-year-old tea plant cuttings were subjected to different temperature treatments (20°C, 25°C, 30°C, and 35°C), and then the levels of metabolites were detected. The results revealed profound variations in the concentration of various flavor compounds in tea shoots exposed to diverse temperature conditions (Fig. 1). Specifically, the content of theanine peaked at 20°C treatment. As the temperature increased, the theanine content decreased gradually (Fig. 1-A). The contents of total catechins (Fig. 1-B), caffeine (Fig. 1-C), gallated catechins (Fig. 1-D) including epicatechin gallate (ECG) (Fig. 1-I) and epigallocatechin gallate (EGCG) (Fig. 1-J), trihydroxylated catechins (TRIC) (Fig. 1-M) and dihydroxylated catechins (DIC) (Fig. 1-N) increased significantly with the increase of temperature gradient, peaked at 30°C and then remained stable. The content of non-gallated catechins (Fig. 1-E), gallocatechin (GC) (Fig. 1-F), epicatechin (EC) (Fig. 1-G), and gallocatechin gallate (GCG) (Fig. 1-H) also increased significantly with the increase of temperature gradient, and reached the peak at 30°C treatment. At 20°C, the content of catechin (C) (Fig. 1-K) and epigallocatechin (EGC) (Fig. 1-L) was significantly lower than at the other three temperatures. The catechin index (CI) (Fig. 1-O) was not affected by temperature changes. These results suggest that temperature treatments differentially regulate the metabolic accumulation of theanine and catechins, thereby significantly shaping the flavor profile of tea shoots.

3.2. Heat stress induced the expression of *CsHSFB2c*

In order to explore the role of HSFs in tea plants under heat stress, one-year-old tea cuttings were subjected to different temperature treatments, and young shoots (one bud and

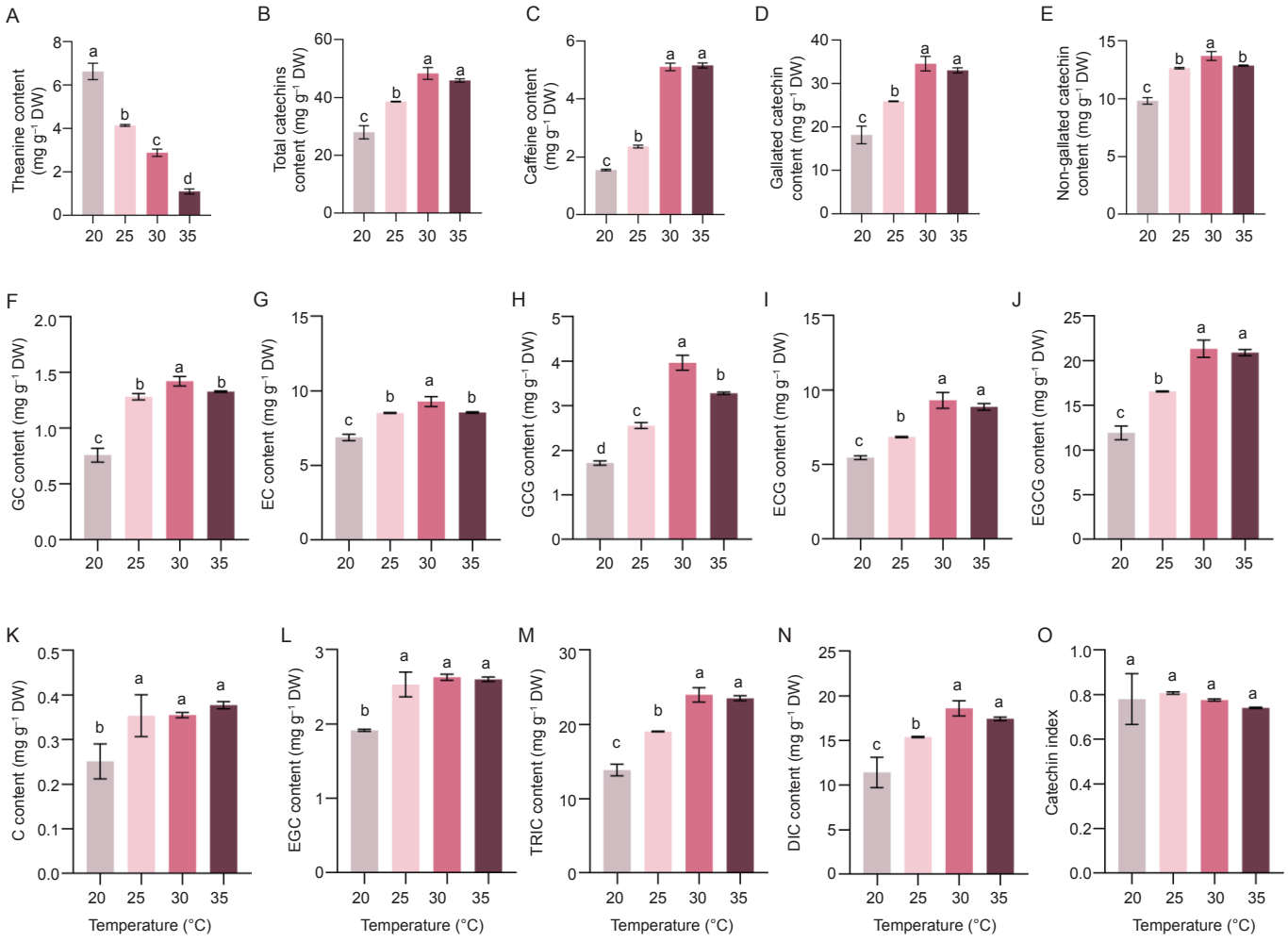


Fig. 1 The content or index of flavor-related metabolites was measured in tea plants under different temperature treatments. A, theanine content. B, total catechins content. C, caffeine content. D, gallated catechins content. E, non-gallated catechins content. F, gallocatechin (GC) content. G, epicatechin (EC) content. H, gallocatechin gallate (GCG) content. I, epicatechin gallate (ECG) content. J, epigallocatechin gallate (EGCG) content. K, catechin (C) content. L, epigallocatechin (EGC) content. M, trihydroxylated catechins (TRIC) content. N, dihydroxylated catechins (DIC) content. O, catechin index. DW represents dry weight. Data are presented as the mean±SD (n=3). Different lowercase letters above the bars indicate significant differences among means (P<0.05).

two leaves) were collected for RNA-seq analysis (Appendix B). We found that the expression levels of the 10 HSF family genes at 30°C or 35°C were higher than those at 20°C and 25°C (Fig. 2-A). A Pearson correlation analysis was then performed between the expression of these 10 genes and theanine content across these four temperature treatments. The results showed that the expression level of CSS0006648 showed a strong negative correlation with L-theanine content under 20°C–30°C treatment (Fig. 2-B).

In order to further study the function of CSS0006648 gene, we performed homologous sequence alignment of its protein sequence. The results showed that CSS0006648 had the highest sequence similarity with CsHSFB2b (CSS0037241 and CSS0026973), and also had high similarity with AtHSFB2b (AT4G11660.1) (Fig. 2-C). Therefore, we named it CsHSFB2c. In addition, we found that the expression levels of *CsTS1* and *CsGS1* at 30°C and 35°C were lower than those at 20°C and 25°C (Fig. 2-D and E). The correlation analysis showed that CsHSFB2c was negatively correlated with the expression of *CsTS1* and *CsGS1* (Fig. 2-F). These results indicated that the identified CsHSFB2c, a heat-induced HSF transcription factor, may function as a negative regulator of theanine accumulation under heat stress by repressing the expression of key biosynthetic genes, *CsTS1* and *CsGS1*.

3.3. Silencing of *CsHSFB2c* increased the theanine content in tea shoots

The *CsHSFB2c* gene was silenced in tea by VIGS method. Gene expression and theanine content were assessed at 72 h post-infiltration. The results showed that theanine content in VIGS-mediated *CsHSFB2c*-silenced tea plants was significantly higher (increases of 3.55-, 1.78- and 2.76-fold across three biological replicates) than that of the control (Fig. 3-A). qRT-PCR analysis indicated that the expression levels of *CsHSFB2c* in silenced plants were 64, 62, and 84% lower than in control plants (Fig. 3-B). The expression of *CsTS1* and *CsGS1* in leaves of *CsHSFB2c*-

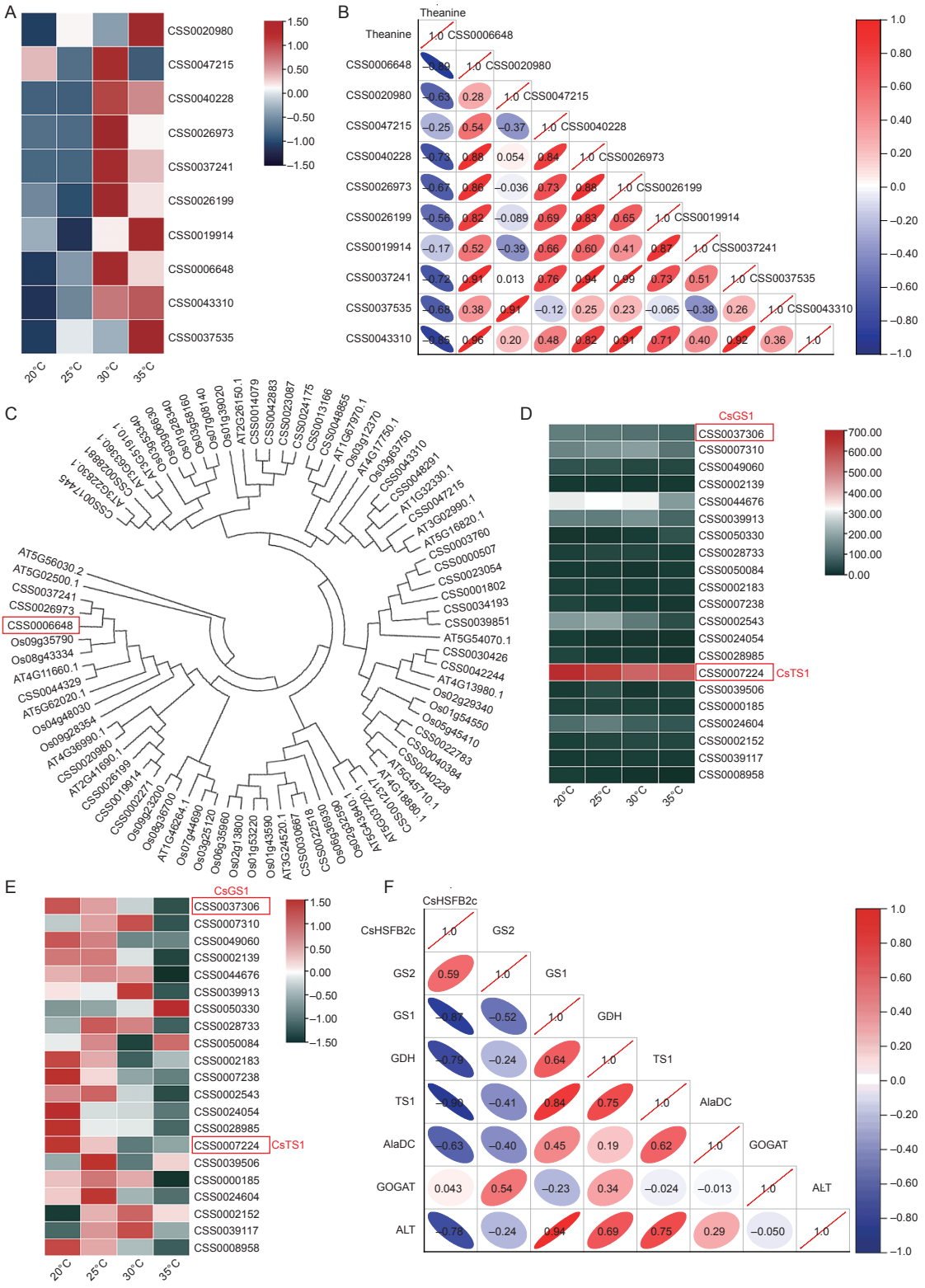


Fig. 2 The expression and correlation analysis of genes under different temperature treatments, as well as the phylogenetic analysis of heat shock transcription factors (HSFs) in different species. A, heatmap of normalized gene expression pattern of *CsHSFs*. B, correlation analysis between the expression level of *CsHSFs* and theanine. C, phylogenetic analysis of HSFs in *Camellia sinensis*, *Arabidopsis thaliana* and *Oryza sativa*. D, heatmap of expression profiles of theanine synthesis related genes. E, heatmap of normalized gene expression pattern of theanine synthesis related genes. F, correlation analysis of genes related to theanine synthesis. The size of circles indicates the degree of correlation. Blue means negative correlation, and red means positive correlation.

silenced plants (Fig. 3-C and D) was significantly up-regulated. These results indicate that silencing *CsHSFB2c* can enhance the expression of *CsTS1* and *CsGS1* in tea leaves and increase theanine content.

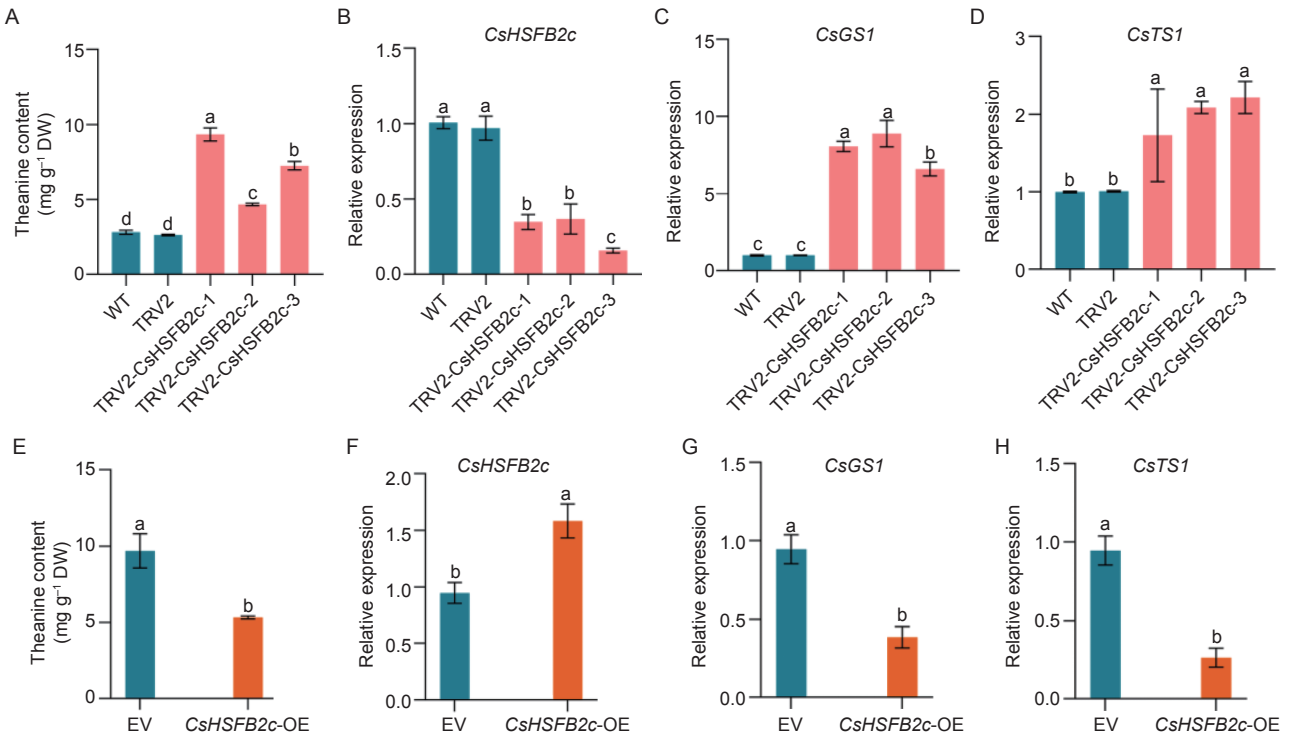


Fig. 3 Effects of *CsHSFB2c* silencing and overexpression on theanine content and expression levels of key biosynthetic genes in tea plants. A, the changes of theanine content in *CsHSFB2c*-silenced plants. B, C, and D, relative expression levels of *CsHSFB2c*, *CsGS1*, and *CsTS1* in *CsHSFB2c*-silenced plants. E, theanine content after overexpression of *CsHSFB2c*. F, G, and H, relative expression levels of *CsHSFB2c*, *CsGS1*, and *CsTS1* in *CsHSFB2c*-overexpressed plants. Data are presented as the mean±SD (n=3). Different lowercase letters above the bars indicate significant differences among means (P<0.05).

3.4. Transient overexpression of *CsHSFB2c* in tea leaves decreased the content of theanine

CsHSFB2c was transiently overexpressed in tea. Gene expression and theanine content were assessed at 72 h post-infiltration. Notably, theanine content decreased to 55.6% of that in plants transformed with the empty vector (EV) (Fig. 3-E). In overexpressed tea leaves, the expression level of *CsHSFB2c* was 1.58 times higher than that in EV (Fig. 3-F). In contrast, the expression levels of *CsTS1* and *CsGS1* were significantly lower than that in EV (Fig. 3-G and H). The findings indicate that transient overexpression of *CsHSFB2c* inhibits the expression levels of *CsTS1* and *CsGS1*, and negatively regulates theanine biosynthesis.

3.5. CsHSFB2c is localized in the nucleus

The 35S::CsHSFB2c-GFP fusion protein was constructed and co-transformed with the p2300-35S-H2B-mcherry plasmid as a nuclear localization marker into the *N. benthamiana* leaves. The GFP signal was then observed using confocal laser scanning microscopy at 50 to 60 h post-infiltration. The results showed that the CsHSFB2c-GFP recombinant plasmid only induced fluorescence in the nucleus. In contrast, the fluorescence signal from the GFP-only empty plasmid was observed in the cytoplasm and nucleus (Fig. 4). The results indicated that CsHSFB2c was localized in the nucleus.

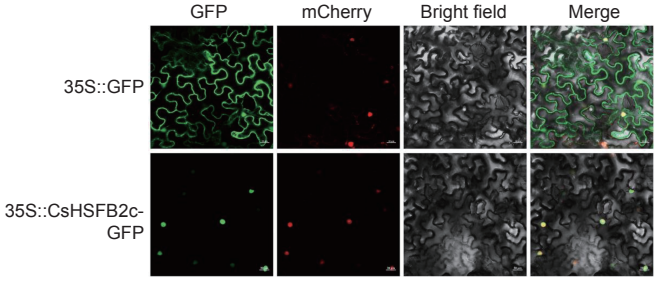


Fig. 4 Subcellular localization of CsHSFB2c-GFP fusion protein.

3.6. CsHSFB2c directly binds to the promoters of *CsTS1* and *CsGS1* and inhibits their expression

To determine whether CsHSFB2c directly binds to the promoters of *CsTS1* and *CsGS1*, the prey vector pGADT7-CsHSFB2c and bait vectors Pro*CsTS1*-pAbAi and Pro*CsGS1*-pAbAi were constructed. The result of yeast one-hybrid (Y1H) assays revealed that yeast co-transformed with pGADT7-CsHSFB2c+Pro*CsTS1*-pAbAi or pGADT7-CsHSFB2c+Pro*CsGS1*-pAbAi grew normally on SD–Leu/Ura medium containing 600 ng mL^{-1} AbA, whereas yeast containing pGADT7+Pro*CsTS1*-pAbAi or pGADT7+Pro*CsGS1*-pAbAi did not grow under the same conditions, indicating that CsHSFB2c interacts with the promoters of both *CsTS1* and *CsGS1* (Fig. 5-A).

To further test whether CsHSFB2c can bind to the

promoters of *CsTS1* and *CsGS1*, transient expression was performed in *N.benthamiana* leaves (Fig. 5-B). Compared to leaves co-transformed with the 35S::CsHSFB2c and empty vector of *N. benthamiana* leaves, the luminescence signal was significantly reduced in leaves co-transformed with 35S::CsHSFB2c and pro*CsTS1*::LUC. In addition, the co-transformatio n of pro*CsGS1*::LUC and 35S::CsHSFB2c in *N. benthamiana* leaves also obtained weaker fluorescence. The results showed that CsHSFB2c could bind to the promoter of *CsTS1* and *CsGS1* and negatively regulate the transcription of the two genes (Fig. 5-C and D).

4. Discussion

4.1. Theanine content decreased under high temperature conditions

With the continuous challenge of global warming, the yield and quality of tea are facing more severe challenges (Chen *et al.* 2023). Theanine is a unique non-protein amino acid in tea plants (Juneja *et al.* 1999) and is a key flavor component of tea products. Theanine content is regulated by genetic factors and environmental factors. Temperature is an important environmental factor affecting theanine content and negatively regulates theanine content. Previous studies have shown that both CsTS and CsGS have the activity of synthesizing theanine *in vivo* (Fu *et al.* 2021). It has been reported that low temperature can induce the up-regulation of theanine synthesis gene *CsTS1* and the increase of theanine content, but long-term low temperature environment will lead to metabolic disorders in tea plants and inhibit theanine synthesis (Li *et al.* 2019). Under 35°C treatment, the expression abundance of theanine synthase (TS) and glutamine synthetase (GS) decreased, and theanine content in tea leaves decreased significantly (Li *et al.* 2018). Our results showed that under the treatment of 20°C–35°C, the accumulation of theanine was the highest at 20°C, and then its content gradually decreased with the increase of temperature (Fig. 1-A). In addition, previous studies have shown that under heat stress, antioxidant enzyme activity is activated and catechin content is significantly increased (Ren *et al.* 2021). Under the same temperature treatment, our study found that the content of caffeine and various catechins increased with the increase of temperature (Fig. 1-B–O). This is basically consistent with previous research results. This antagonistic relationship between the decrease of theanine content and the increase of catechin content may reflect the resource allocation strategy of tea plants under heat stress: sacrificing flavor substances (theanine) to enhance stress resistance (catechins act as antioxidants).

4.2. Heat stress induced the heat shock transcription factor CsHSFB2c

When plants are subjected to heat stress, HSFs are highly expressed in a short period and respond to heat stress by activating the HSR. HSFs in plants are divided into three categories: A, B, and C (Scharf *et al.* 2012). The C-terminus of class B (HSF) contains a repressor domain (RD) of the tetrapeptide motif LFGV, which is identified as inhibitory in transcription factors (TFs) (Lü *et al.* 2022). It has been reported that HSFA1s are key regulators of integrating light and temperature signals in *Arabidopsis*. High temperature

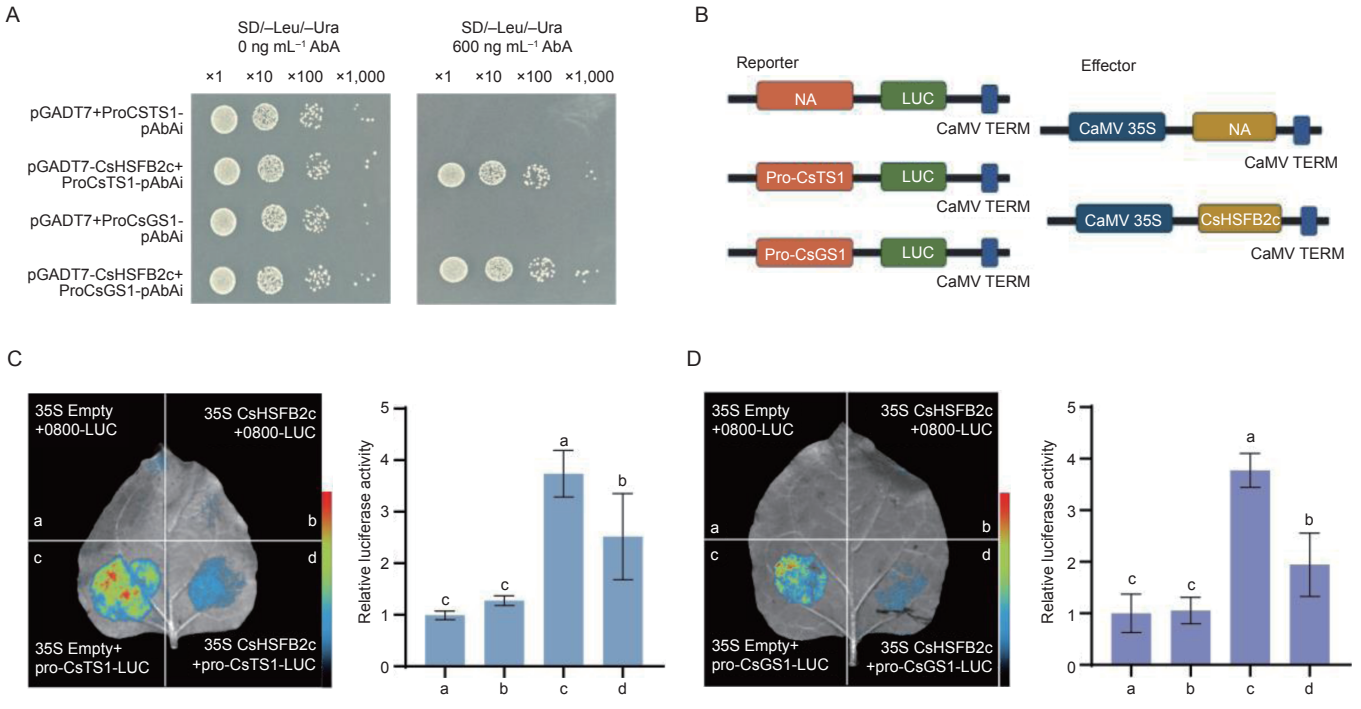


Fig. 5 CsHSFB2c directly binds to the promoters of *CsTS1* and *CsGS1*. A, the interaction between CsHSFB2c and the *CsTS1/CsGS1* promoter in the Y1H assay. B, the vector diagram constructs used in the Dual-LUC experiment. C, CsHSFB2c inhibits the activity of *CsTS1* promoter and attenuates the LUC signal observed in tobacco leaves. D, CsHSFB2c inhibits the activity of *CsGS1* promoter and attenuates the LUC signal observed in tobacco leaves. Different lowercase letters above the bars indicate significant differences (P<0.05).

treatment at 28°C can induce a large number of *HSFA1s* gene expression, which makes plants better adapt to high temperature in summer (Tan *et al.* 2023; Li *et al.* 2024). TaHSFA1 is a core regulatory factor necessary for basic heat tolerance in wheat. Knockout TaHSFA1 is highly heat-sensitive compared to the wild type (Wang *et al.* 2023). In *Arabidopsis*, HSFB1 and HSFB2b are transcriptional repressors. After the loss of hsfb1-hsfb2b function, the heat tolerance of *Arabidopsis* at 42°C is slightly higher than that of wild type (Ikeda *et al.* 2011). Many CsHSFs in tea plants are differentially regulated by drought, salt and heat stresses, as well as exogenous abscisic acid (ABA) and Ca^{2+} (Zhang *et al.* 2020). For example, the heterologous expression of *CsHSFA2* improved the heat resistance of transgenic yeast (Zhang *et al.* 2020). In this study, we identified a class B heat shock transcription factor CsHSFB2c (CSS0006648) (Fig. 2-B) by analyzing the RNA-seq data obtained after treatment of tea plants at 20°C–35°C (Fig. 2-A). We found that the expression of *CsHSFB2c* at 30 and 35°C was significantly higher than that at 20°C, indicating that *CsHSFB2c*, as a member of plant HSFs, could be induced by high temperature.

4.3. CsHSFB2c inhibits the expression of *CsTS1* and *CsGS1*, and negatively regulates the biosynthesis of theanine

Although there have been many reports on theanine synthase and glutamine synthetase in the theanine biosynthesis pathway, little is known about how they are regulated. Current reports have shown that CsMYB6 directly binds to the *CsTS1* promoter to regulate the biosynthesis of theanine in roots (Zhang *et al.* 2021). CsMYB73 binds to the *CsGS1* and *CsGS2* promoter sequences through the MYB recognition sequence to inhibit theanine biosynthesis (Wen *et al.* 2020). Phosphorous signaling factors CsSPX3 and CsPHL7 reduce the accumulation of theanine in tea roots and leaves by directly inhibiting theanine synthesis-related gene *CsGS1* and *CsTS1* (Chen Z Z E *et al.* 2024). In the present study, down-regulation of *CsHSFB2c* significantly elevated theanine content (Fig. 3-A), whereas its overexpression significantly decreased theanine content (Fig. 3-E). In addition, we detected that the expression of *CsTS1* and *CsGS1* was significantly up-regulated in the leaves of *CsHSFB2c*-silenced plants (Fig. 3-C and D), while the trend was opposite in the leaves of *CsHSFB2c*-overexpressing plants. These results indicate that CsHSFB2c may negatively regulate theanine biosynthesis by inhibiting the expression of *CsGS1* and *CsTS1*. In order to further prove this conclusion, we conducted Y1H and Dual-LUC experiments, and the results showed that CsHSFB2c can bind to the promoters of *CsGS1* and *CsTS1*, respectively (Fig. 5-A and B). This indicates that CsHSFB2c specifically binds to the promoters of *CsGS1* and *CsTS1*, inhibits their expression, and negatively regulates the biosynthesis of theanine.

In this study, high temperature (30°C–35°C) significantly induced the expression of class B heat shock transcription factor CsHSFB2c. CsHSFB2c directly binds to the promoter regions of the key genes *CsTS1* and *CsGS1* for theanine biosynthesis and inhibits their expression, resulting in a decrease in theanine content. Therefore, we established a regulatory model of CsHSFB2c inhibiting the expression of *CsTS1* and *CsGS1* to reduce theanine biosynthesis under heat stress (Fig. 6).

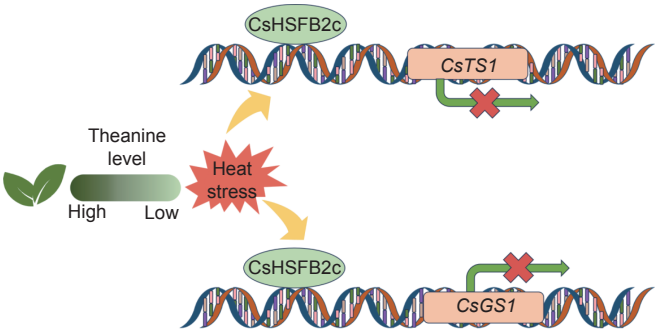


Fig. 6 The regulatory mechanism model of CsHSFB2c-*CsTS1*/*CsGS1* in regulating theanine biosynthesis under heat stress.

5. Conclusion

In this study, we screened the *CsHSFB2c* gene from RNA-seq data and verified its inhibitory effect on theanine accumulation in the tissues of new shoots and true leaves of tea cuttings by VIGS technology and transient transformation leaf technology. Our findings uncovered that CsHSFB2c, as a transcriptional repressor, affects theanine biosynthesis by inhibiting the transcription of glutamine synthase gene *CsGS1* and theanine synthase gene *CsTS1*, which provides a new perspective for understanding the regulatory mechanism of theanine biosynthesis in tea plants.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this manuscript, the authors used Deepseek to assist in improving the clarity and readability of the language. All content generated was carefully reviewed and revised by the authors as necessary, and the authors take full responsibility for the final content of the article.

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