The CsHSFA-CsJAZ6 module mediated high temperature regulates flavonoid metabolism in Camellia sinensis

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Abstract

High temperatures (HT) seriously affect the yield and quality of tea. Catechins, derived from the flavonoid pathway, are characteristic compounds that contribute to the flavor of tea leaves. In this study, we first showed that the flavonoid content of tea leaves was significantly reduced under HT conditions via metabolic profiles; and then demonstrated that two transcription factors, CsHSFA1b and CsHSFA2 were activated by HT and negatively regulate flavonoid biosynthesis during HT treatment. Jasmonate (JA), a defensive hormone, plays a key role in plant adaption to environmental stress. However, little has been reported on its involvement in HT response in tea. Herein, we demonstrated that CsHSFA1b and CsHSFA2 activate *CsJAZ6* expression through directly binding to HSE elements in its promoter, and thereby repress the JA pathway. Most secondary metabolites are regulated by JA, including catechin in tea. Our study reported that CsJAZ6 directly interacts with CsEGL3 and CsTTG1 and thereby reduces catechin accumulation. From this, we proposed a CsHSFA-CsJAZ6 mediated HT regulation model of catechin biosynthesis. We also determined that negative regulation of the JA pathway by *CsHSFAs* and its homologues is conserved in *Arabidopsis*. These findings broaden the applicability of the regulation of JAZ by HSF transcription factors and further suggest the JA pathway as a valuable candidate for HT-resistant breeding and cultivation.

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ABSTRACT

High temperatures (HT) seriously affect the yield and quality of tea. Catechins, derived from the flavonoid pathway, are characteristic compounds that contribute to the flavor of tea leaves. In this study, we first showed that the flavonoid content of tea leaves was significantly reduced under HT conditions via metabolic profiles; and then demonstrated that two transcription factors, CsHSFA1b and CsHSFA2 were activated by HT and negatively regulate flavonoid biosynthesis during HT treatment. Jasmonate (JA), a defensive hormone, plays a key role in plant adaption to environmental stress. However, little has been reported on its involvement in HT response in tea. Herein, we demonstrated that CsHSFA1b and CsHSFA2 activate *CsJAZ6* expression through directly binding to HSE elements in its promoter, and thereby repress the JA pathway. Most secondary metabolites are regulated by JA, including catechin in tea. Our study reported that CsJAZ6 directly interacts with CsEGL3 and CsTTG1 and thereby reduces catechin accumulation. From this, we proposed a CsHSFA-CsJAZ6 mediated HT regulation model of catechin biosynthesis. We also determined that negative regulation of the JA pathway by *CsHSFAs* and its homologues is conserved in *Arabidopsis.* These findings broaden the applicability of the regulation of JAZ by HSF transcription factors and further suggest the JA pathway as a valuable candidate for HT-resistant breeding and cultivation.

Keywords: *Camellia sinensis* , catechin biosynthesis,*CsJAZ6* , heat shock factor (HSF), high temperatures (HT), JA, molecular regulation

INTRODUCTION

Temperature is one of the most important environmental factors affecting the seasonal growth and geographic distribution of plants (Li et al., 2018). Tea (*Camellia sinensis*) is an economic crop with wide popularity around the world for its stimulating health effects and mellow flavor, which are attributable to the remarkable composition of flavonoids, caffeine, and theanine it contains. Nowadays, tea-growing countries include 64 countries spread across five continents of the world spanning tropical and subtropical as well as temperate regions (Wei et al., 2018). In China, the cultivated area occupied by tea trees comprises 3.059 million hectares sited in 22 of the country's 35 provinces. Notably, the temperature range suitable for tea-growing is 20 °C to 30 °C, thus the increased prevalence and frequency of high temperature (HT) conditions around the world have seriously affected the yield and quality of tea.

The catechin biosynthetic pathway has been identified as producing the primary characteristic flavonoid components of tea. The enzymes of this pathway include chalcone synthase (CHS), chalcone isomerase (CHI), and flavanone 3-hydroxylase (F3H), which sequentially catalyze chalcone to produce the intermediate naringenin. Subsequently, dihydroflavonol 4-reductase (DFR), leucoanthocyanidin 4-reductase (LAR), anthocyanidin synthase (ANS), and anthocyanidin reductase (ANR) are responsible for the formation of final products under the control of the MBW complex (Baudry et al. 2004; Zhao et al. 2013; Li. 2014; Huang et al. 2016). This complex consists of R2R3 MYB transcriptional factors (MYB21, MYB75, MYB90, MYB113, or MYB114) (Borevitz et al., 2000; Zimmermann et al., 2004; Stracke et al., 2007; Allan et al., 2008; Gonzalez et al., 2008; Rowan et al., 2009; Shan et al., 2020; Zhang et al., 2021), and basic-helix-loop-helix (bHLH) transcription factors (Toledo-Ortiz et al., 2003) such as enhancer of glabra3 (EGL3) (Zhang et al., 2003) and the WD-repeat protein transparent testa glabra1 (TTG1) (Walker et al., 1999). Investigation of the MBW complex has suggested that additional regulators which influence its formation and function would also participate in catechin biosynthesis.

HT conditions refer to the ambient temperature rising above the optimum temperature for a period, which causes reversible damage to plant growth and development (Alcázar and Parker. 2011). Recent studies have demonstrated that various signaling pathways are integrated to regulate the plant heat stress response, including the heat shock transcription factor-heat shock protein pathway, calcium ion-calmodulin (Ca^{2+}-CaM) pathway, reactive oxygen pathway, and hormone pathways (Mittler et al., 2012). As the terminal components of heat shock signal transmission, heat shock factors (HSFs) directly regulate thermo-responsive gene expression after perception of ambient HT and as such play a central role in the heat shock response process. Most HSFs recognize heat shock elements (HSEs; nGAAnnTTCn or nTTCnnGAAn) in the promoters of heat-inducible genes (Xiao et al., 1988; Ohama et al., 2017). HSF proteins are divided into A, B, and C classes, among which members of the A class (HSFA) act as master regulators in the heat stress response (HSR);

these mainly include HSFA1 and HSFA2 (Scharf et al., 2011). Based on recent research, HSFA1 can activate 65% of heat stress-inducible genes (Liu et al., 2011), while HSFA2 has been identified to act downstream of HSFA1 and regulate a subset of genes involved in thermotolerance (Charng et al., 2007, Yoshida et al., 2011; Liu et al., 2018; Friedrich et al., 2021). Heterologous overexpression of *HSFA2* from *Lilium longiflorum* , *Zea mays,* and*Oryza sativa* in *Arabidopsis* has been shown to confer heat tolerance (Xin et al., 2010; Yokotani et al., 2008; Gu et al., 2019). While several CsHSF genes in the tea genome have been predicted based on bioinformatics studies, the involvement of CsHSFs in the regulation of secondary metabolism in tea plants under HT yet remains unclear (Zhang et al., 2020b).

JA is an oxylipin phytohormone that acts as a defensive signal to protect plants from biotic and abiotic stress, and in this role regulates most secondary metabolites (Zhai et al., 2015; Mao et al., 2017; Wasternack et a., 2019). Recently, the JA pathway and its components have been identified. In the absence of bioactive JA, JAZ proteins interact with MYC transcription factors to block downstream processes (Katsir et al., 2008; Wasternack et al., 2013). JA transduces external signals to reprogram metabolic pathways that initiate the production of defense compounds against biotic and abiotic stress (Mao et al., 2017; Chen et al., 2019; Jing et al., 2021). Furthermore, JA can induce flavonoid accumulation by increasing the expression of members of the MBW complex (Qi et al., 2011; An et al., 2015; Wang et al., 2019). However, whether JA-mediated flavonoid metabolites are involved in the adaption of tea plants to HT stress has not been examined.

In this study, we explored the HT-regulatory networks of tea metabolism and found that HT decreases both JA biosynthesis and catechin accumulation in tea leaves. Based on the involvement of JA in HT-regulated catechin biosynthesis, we further demonstrated that CsJAZ6, through integrating HT signal and JA pathway, acts as an important negative regulator in catechin accumulation. Using a combination of biochemical and genetic analysis, we determined HT to activate CsHSFA1b and CsHSFA2 and those proteins to directly bind the HSE*cis* -element in the promoter of *CsJAZ6* , thereby up-regulating *CsJAZ6* transcription. More importantly, our data further suggested that CsJAZ6 could repress catechin accumulation through direct interaction with components of the catechin biosynthetic regulator complex, CsEGL3 and CsTTG1. Altogether, our work builds a bridge between plant internal hormones and the external environment to offer a good guide for tea cultivation.

MATERIALS AND METHODS

Plant materials

One-year-old tea plants (*Camellia sinensis* cv. Longjing 43) were placed in plastic pots and grown in a plant growth room at 25 with a 14-h-light/8-h-dark cycle for two months. The Columbia-0 (Col-0) ecotype of *Arabidopsis* was grown in a growth chamber at 22 degC, and the parameter 16-h-light/8-h-dark cycle was used. The *athsfa2* mutant was provided by Dr. Yee-Yung Charng. Transgenic plants Col-0 and*athsfa2* overexpressing *CsHSFA1b/CsHSFA2* were generated by cloning the corresponding cDNA fragment into pCAMBIA2300 (kanamycin-resistant) and the 35S promoter was used as control. These constructs were transformed into *Agrobacterium* strain GV3101. Using the floral dip method, the bacteria were subsequently introduced into *Arabidopsis* plants (Clough and Bent, 1998).

Plant treatments

For hormone treatments, MeJA (Sigma-Aldrich), ibuprofen, and MG132 were dissolved in absolute ethanol. Attached tea plant leaves and detached*Arabidopsis* leaves were then sprayed with MeJA (100 μM), ibuprofen (100 μM), or MeJA (100 μM) + MG132 (100 μM), with the same concentration of absolute ethanol used as a mock treatment. The leaves were harvested at time points appropriate to the experiment with three repetitions.

Metabolomic profiling

Metabolomic profiling was conducted using a LC-ESI-MS/MS system (HPLC, UFLC SHIMADZU CBM30A system; MS, Applied Biosystems 6500 Q TRAP). Chromatographic separation was performed on an ACQUITY UPLC HSS T3 C18 column (2.1 mm X 100 mm X 1.8 μm; Waters) using a mobile phase A of 0.04%

acetic acid in deionized water and mobile phase B of 0.04% acetic acid in acetonitrile with flow rate 0.4 mL min^{-1}. The fold-change for each metabolite was calculated as the ratio of the amount in stress-treated plants relative to that in control plants. According to the statistically significant threshold (P <0.05) and variable influence on projection (VIP) values (VIP>1.0), differential metabolites were selected.

Anthocyanin and Catechin determination

Arabidopsis leaves were weighed (0.1 g) and incubated overnight in extraction buffer (methanol containing 1% HCl). Anthocyanins were separated from the chloroform by adding 200 μl distilled water and 500 μl chloroform, as previously described by He et al[47]. Spectrophotometer was used to measure the total anthocyanins in the aqueous phase with the absorbance at 530 and 657 nm using. The number of anthocyanins per gram fresh weight was calculated as (A_{530}-0.25×A_{657}). A modified protocol was further used to detect the Catechin content (Su et al., 2020).

Measurement of MeJA concentration

MeJA profiling was carried out using reversed-phase chromatography on a Waters ultra-performance liquid chromatography (UPLC) system (Absciex 5500). As described in previous report (Mao et al., 2017; Kong et al., 2021), 700 μl of methanol was used to dissolve dried pellets of MeJA extracts. An Agilent Eclipse Plus C18 column (3.0 mm×100 mm×1.8 μm) was used for UPLC at 30 °C with a flow rate of 0.4 ml min^{-1}. Gradient elution with solvent A (0.02 mM ammonium formate and 0.01% formic acid in water) and solvent B (0.02 mM ammonium formate and 0.01% formic acid in methanol) were used to separate the compounds, using the following elution profile: 0-1 min 60% A, 40% B; 1-8 min 60-5% A, 40-95% B; 8-10 min 5% A, 95% B; 10-10.1 min 5-60%A, 95-40% B; 10.1-12 min 60% A, 40% B.

Transient gene suppression in tea plants

Gene suppression assays were according to previous study from Hu et al (Hu et al., 2022). *CsHSFA1b* , *CsHSFA2* , and *CsJAZ6*were used as input sequences to design candidate antisense oligonucleotides (AsODNs) with the Soligo software (Ding and Lawrence, 2003) (Table S1). At least ten individual plants were infiltrated with gene-specific AsODNs; as controls, untreated plants grown under the same conditions and infiltrated with corresponding sense oligonucleotides (sODNs). The leaves were harvested after 24 h treatment.

Gene expression analysis

Total RNA was extracted from tea leaves using Trizol reagent (Invitrogen). Purified RNA (1 μg) was subsequently reverse transcribed using a first-strand cDNA synthesis kit to generate cDNA. ABI7900HT Sequence Detection System (Applied Biosystems, CA, USA) was used to measure the gene expression levels. The determined gene expression levels in tea plants were normalized to *GAPDH* (TEA003029.1) and in *Arabidopsis* were normalized to *UBQ10* (At4g05320). Primers sequence required for gene expression are listed in Table S1.

Yeast one-hybrid (Y1H) assay

PCR products of *CsHSFA1b* and *CsHSFA2* were amplified from tea leaves (Longjing 43) and inserted into the pGAD vector with EcoR I and BamH I restriction sites, and full-length and truncated derivatives of the *CsJAZ6* promoter were inserted into the pABAi vector with Pst I and Nco I sites. The *CsJAZ6pro* -pABAi construct was then linearized by digestion with BstB I, introduced into the Gold Y1H strain, and plated on SD/-Ura for three days. Colonies harboring the*CsJAZ6* promoter were used to prepare competent cells that were transformed with the CsHSFA1b/A2-pGAD constructs (or pGAD plasmid as the control) and plated on SD/Leu. Then colonies were moved to SD/Leu with supplemented 200 ng aureobasidin A (AbA).

Electrophoresis mobility shift assay (EMSA)

The CDS of *CsHSFA1b* and *CsHSFA2* were cloned into pCold vector to construct the fusion protein with His-tag fusion vector and then introduced into BL21 (DE3) *Escherichia coli* . His-beads (70501, Beaver, China) were used to purify the fusion protein His-CsHSFA1b/CsHSFA1bA2 in accordance with the manufacturer's

instructions. DNA fragments from the *CsJAZ6* promoter that contained HSE motifs were amplified using biotin-labeled primers (Table S1). His-tagged CsHSFA1b and CsHSFA2 proteins were incubated in binding buffer with biotin-labeled probes for 20 min at room temperature. The LightShift Chemiluminescent EMSA kit (Thermo Scientific, Waltham, MA, USA) was used to detect the igration of biotin-labeled probes.

Dual-luciferase assay

Dual-luciferase (Dua-Luc) assays were carried out as described by He et al (2021). The promoter regions of *CsJAZ6* and *CsANS* were sub-cloned into the pGreenII 0800-LUC vector as reporters. The cDNA sequences of *CsHSFA1b* , *CsHSFA2* , *CsJAZ6* ,*CsAN1* , *CsEGL3* , and *CsTTG1* were inserted into the pCambia2300 vector as effectors. The pCambia2300 empty vector was used as a negative control. Tobacco leaves were sprayed with 100 mmol/L fluorescein (Promega) and left in the dark for 5 min. Then, a low-light cooled charge-coupled device imaging device (NightOWL II LB983) were used to capture Luc images. LUC and REN activities of the *CsJAZ6*and *CsANS* promoters were measured with the Dual-Luciferase Reporter Assay kit (Promega Corporation, Wisconsin, USA).

Bimolecular fluorescence complementation (BiFC) assay

The full-length cDNA sequences of *CsJAZ6* , *CsEGL3* , and*CsTTG1* were cloned into the cYFP and nYFP vectors. Six fusion constructs obtained, including CsJAZ6-YFP^{N}, CsJAZ6-YFP^{C}, CsEGL3-YFP^{N}, CsEGL3-YFP^{C}, CsTTG1-YFP^{N}, and CsTTG1-YFP^{C}, were transformed into *Agrobacterium tumefaciens* strain GV3101. CsJAZ6-YFP^{N} was paired with CsEGL3/CsTTG1-YFP^{C} and CsJAZ6-YFP^{C} with CsEGL3/CsTTG1-YFP^{N}for transient coexpression in tobacco leaves. The YFP fluorescence signal for each combination was imaged at 44-48 h after infiltration using a Leica TCS SP5 confocal laser scanning microscope system (Leica Microsystems, Bannockburn, IL, USA). An excitation wavelength of 488 nm and fluorescence detection at 500-542 nm were used.

Yeast three-hybrid (Y3H) assay

CsEGL3-CsJAZ6/pBridge and CsAN1/pGAD constructs were generated for yeast three-hybrid analyses. The pBridge vector in the Y3H system utilizes an inducible Met25 promoter, while *CsJAZ6* was subcloned into the pBridge vector and driven by the Met25 promoter. Co-transforming the constructs into AH109 yeast cells and selected on SD/-Leu/-Trp plates. Then colonies were moved to selective dropout liquid medium (SD/-Met/-Leu/-Trp/-His) with different concentrations of Met, and the β-galactosidase activity associated with CsAN1-CsEGL3 binding activities was quantified using the O-nitrophenyl-β-D-galactopyranoside (ONPG) liquid assay according to the manufacturer's protocol (Clontech).

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The yeast colonies were suspended in SD/-Met/-Leu/-Trp/-His with different concentrations of methionine and cultured at 30 with 200 rpm shaking until the OD_{600} reached 0.8. After centrifuging, the precipitate was dissolved in 1.5 ml z-buffer, centrifuged again, and the resulting cell pellet finally re-suspended in 300 μL z-buffer. Liquid nitrogen was used to break down the yeast cell walls, and the solution was incubated at 37 with added 700 μL z-buffer and 160 μL o-nitropheyl-β-D-galactopyranoside (OPNG). 400 μL stop buffer (100 mM Na_2CO_3) was added once the reaction had clearly turned yellow. The average β-galactosidase activity was determined based on the wavelength of the supernatant was at OD_{420}.

Immunoblot analysis

CsJAZ6 protein fused to the Myc tag and CsEGL3 and CsTTG1 proteins fused to the Flag tag were prepared for co-immunoprecipitation assays. Transiently transfected tobacco leaves expressing either CsEGL3-Flag or CsTTG1-Flag with CsJAZ6-Myc were lysed with a buffer. The buffer contained 50 mM Tris, 50 mM NaCl, 1 mM EDTA 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 100 mM MG132 (Sigma-Aldrich), and protease inhibitor cocktail (Roche). Lysates were added the Anti-Flag Magnetic Beads (Sigma-Aldrich) and incubated for 4 h at 4 °C. Then, the beads were washed three times, then solubilized sodium dodecyl sulfate (SDS) sample buffer and boiled for 5 min. Samples were subsequently analyzed by

4%-12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot with anti-Myc peroxidase antibody. ECL Plus Western Blotting kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used to detect the bound antibody.

RESULTS

HT inhibits flavonoid biosynthesis in tea leaves

HT is known to influence secondary metabolism in various plant species. To explore its effect and associated regulation mechanism in tea, we compared the metabolites from tea leaves grown under normal (25) and HT (40) conditions. The results showed that 58 metabolites were up-regulated and 53 down-regulated in HT conditions (Figure 1a). It is worth noting that 11 of the 15 detected flavonoids were significantly decreased, including catechin which are featured compounds in tea. HPLC analysis further confirmed that leaves from tea plants exposed to HT accumulate less catechin than those grown in normal conditions (Figure 1b).

Next, we examined the expression levels of catechin biosynthetic genes through qRT-PCR. Consistent with the changes in metabolic products, the transcript levels of genes encoding pertinent enzymes including *CsPAL* (*TEA023243.1*), *CsCHS* (*TEA023340.1*),*CsCHI* (*TEA034003.1*), *CsF3H* (*TEA023790.1*),*CsF3'H* (*TEA006847.1)* , *CsDFR* (*TEA022775.1*),*CsFLS* (*TEA033416.1)* and *CsANS* (*TEA015769.1*) were lower in tea leaves treated with HT than those grown under normal conditions (Figure 1c). The qRT-PCR results also showed that eight of the 25 *CsHSFs* identified in tea were responsive to HT in different degrees (Figure 1d). Among those, *CsHSFA1a* and *CsHSFA2* exhibited especially altered expression, climbing up to more than 50-fold above normal (Figure 1d).

CsHSFA1b and ***CsHSFA2*** are involved in the heat stress inhibition of catechin biosynthesis

To investigate the function of *CsHSFA1b* and *CsHSFA2* in catechin biosynthesis, we transiently decreased the expression of both these genes in tea leaves using the antisense oligodeoxynucleotide (AsODN) suppression strategy. Firstly, to determine the efficiency of gene suppression at each sampling, we measured the respective transcript levels of *CsHSFA1b* and *CsHSFA2* (Figure 2a). The qRT-PCR results revealed an average inhibition of 49% for *CsHSFA1b* and 53% for *CsHSFA2* (Figure 2a). Relative to the sODN group, the expression levels of catechin biosynthetic genes, including *CsCHS* , *CsDFR* , and *CsANS* , increased in *CsHSFA1b*- silenced and *CsHSFA2*- silenced plants (Figure 2b). Accordingly, the quantification of catechin contents by HPLC showed that leaves from *CsHSFA1b* -silenced plants accumulated 21% more and those from *CsHSFA2*- silenced plants accumulated 17% more catechins than control leaves (Figure 2c). Collectively, our results suggest that *CsHSFA1b* and *CsHSFA2* negatively regulate catechin biosynthesis.

Roles of JA in regulating catechin biosynthesis under HT

It has been demonstrated that JA plays an important role in the regulation of flavonoid biosynthesis, including proanthocyanidins and anthocyanins (An et al., 2015). When tea plants were exposed to HT, we noticed that JA-responsive genes including *CsLOX1* , *CsAOS* , and *CsMYC2* were down-regulated, suggesting impaired JA signaling to be associated with HT exposure (Figure 3a). Given the lower content of MeJA in HT-treated tea leaves (Figure 3b), we wondered whether the JA pathway mediates the inhibiting effect of HT on catechin biosynthesis. After spraying tea leaves with MeJA, ibuprofen (a JA synthesis inhibitor), and MeJA plus MG132, we then transferred pre-treated plants into growth chambers to grow at 25 or 40 for 3 d. Subsequent HPLC analysis revealed that HT significantly reduced catechin content with an average reduction of 15.5%, and ibuprofen treatment also repressed catechin accumulation by 32.8%, whereas treatment with MeJA alleviated the inhibitory effect of HT on catechin biosynthesis, resulting in catechin levels about equal to those in plants grown at normal temperature (Figure 3c). Consistent with the observed changes in catechin levels, expression of biosynthetic genes including *CsCHS* , *CsDFR* , and *CsANS* was significantly inhibited in plants sprayed with ibuprofen and the expression patterns resembled those in plants treated with HT, whereas MeJA was able to partly counteract the inhibition caused by HT (Figure 3d).

CsHSFA1b and CsHSFA2 regulate ***CsJAZ6*** expression

Distinct from the down-regulation of JA-responsive genes, expression of *CsJAZ6* , an inhibitor of the JA signaling pathway, was significantly induced by HT treatment (Figure 3a). As indicated in Figure 4a, three HSEs were identified in the *CsJAZ6* promoter region (˜2 kb upstream of the start codon). The presence of these sequence elements encouraged us to investigate whether CsHSFA1a and CsHSFA2 could directly bind to the *CsJAZ6* promoter. Firstly, yeast one-hybrid assays demonstrated that CsHSFA1b could recognize all three HSE elements, whereas CsHSFA2 only bound to the segment containing HSE1 (Figure 4a and Figure S1). Similarly, EMSA using a 50-bp fragment containing the HSE1 (TTCNNGAA) motif as a probe suggested that both CsHSFA1b and CsHSFA2 could bind directly to the HSE1 element in the *CsJAZ6* promoter (Figure 4b, c). Subsequent competitive experiments showed that the addition of unlabeled wild-type probes at 1X concentration could partly prevent the binding of CsHSFA1b and CsHSFA2 to labelled probes; adding 10X enhanced the effect of this competition (Figure 4b, c). Based on the *in vitro* observation that both CsHSFA1b and CsHSFA2 bind directly to HSE1 in the *CsJAZ6* promoter, we subsequently explored their capacity for transcriptional activation of *CsJAZ6* in tobacco leaves. Dual-Luc assays revealed that compared with the individual expression of corresponding negative controls, CsHSFA1b and CsHSFA2 activated the *CsJAZ6* promoter by 3.3-fold and 2.1-fold, respectively (Figure 4d, e). This finding indicates that HT promotes CsHSFA1b and CsHSFA2 and thereby activates *CsJAZ6* transcription *in planta* . Together, our data suggest that CsHSFA1b and CsHSFA2 could repress JA signaling mainly through their binding to the *CsJAZ6* promoter and activating its transcription.

CsJAZ6 represses catechin biosynthesis though physically interacting with CsEGL3 and CsTTG1

To investigate whether CsHSFs-regulated *CsJAZ6* participates in catechin biosynthesis, we injected AsODN-*CsJAZ6* solution into tea leaves. Compared with sODN treatment, the expression of *CsJAZ6* was significantly reduced (Figure 5a). Also, we found that in *CsJAZ6* -silenced leaves, the expression of almost all flavonoid biosynthetic genes was up-regulated (Figure 5b). This finding was supported by HPLC analysis, which consistently showed the silenced leaves to accumulate more catechin than the control leaves (Figure 5c).

To decipher the molecular mechanisms underlying CsJAZ6-mediated regulation of catechin biosynthesis, we utilized CsJAZ6 protein as a bait to screen a yeast library constructed from tea leaves. Among the screened candidates, CsEGL3 and CsTTG1 stood out as known important components of a catechin biosynthesis regulator complex. After first verifying the CsJAZ6-CsEGL3 and CsJAZ6-CsTTG1 interactions by Y2H assay using full-length CsEGL3 and CsTTG1 (Figure 6a), we confirmed them in tobacco leaves by BiFC assay. Co-expression of CsJAZ6-YFP^N and CsEGL3/CsTTG1-YFP^Cor CsJAZ6-YFP^C and CsEGL3/CsTTG1-YFP^N resulted in strong signals in the nucleus, while the negative control combinations, including CsJAZ6-YFP^N/YFP^C and CsJAZ6-YFP^C/YFP^N, did not produce any detectable fluorescence signal (Figure 6b). We then performed an *in vivo* co-immunoprecipitation assay by co-transforming CsJAZ6-Myc with CsEGL3-Flag and CsTTG1-Flag into tobacco leaves, with a GFP-Myc construct as control. This revealed CsJAZ6 to co-precipitate specifically with CsEGL3 and CsTTG1 (Figure 6c, d). Collectively, these results imply that CsJAZ6 physically interacts with both CsEGL3 and CsTTG1. It has been reported that CsEGL3 and CsTTG1 couple with CsAN1 and form the MBW complex to regulate the expression of genes involved in the catechin biosynthesis pathway (Sun et al., 2016; Wei et al., 2019). To answer whether the interactions of CsJAZ6 with CsEGL3 and CsTTG1 would interfere with MBW complex formation, we performed a yeast three-hybrid assay. CsEGL3 was pre-inserted in the pBridge vector as the bait, and CsJAZ6 was integrated into the CsEGL3/pBridge vector under control of the pMET25 promoter. This assay revealed that, with increasing methionine concentration, CsJAZ6 gradually reduced and thereby caused growth of the CsEGL3-CsAN1 yeast colonies (Figure 6e). These findings suggest that CsJAZ6 mediates catechin biosynthesis, at least in part, through affecting formation of the MBW complex.

To further elucidate the regulatory impact of the CsJAZ6-CsEGL3/CsTTG1 interaction on catechin biosynthesis, the *CsANS* promoter was fused to a luciferase gene to generate the *CsANSpro-LUC* reporter. In the subsequent Dual-Luc assay, CsAN1, CsEGL3, CsTTG1, and CsJAZ6 were used as effectors (Figure 6f). Compared to the control, the promoter activity was significantly elevated by 2.7-fold when co-expressed with

MBW complex (CsAN1, CsEGL3 and CsTTG1). However, the addition of CsJAZ6 reduced the activation effect of the MBW complex on the *CsANS*promoter by 48% (Figure 6g). These results hint that CsJAZ6 negatively regulates *CsANS* gene expression as well as anthocyanin biosynthesis by interfering with MBW formation.

Conserved roles of the HSFA-mediated JA pathway in regulating anthocyanin biosynthesis under HT in *Arabidopsis* and tea

Numerous studies have reported that JA participates in the heat stress response; however, its specific physiological role in heat response remains ambiguous in plants (Pan et al., 2019; Wu et al., 2022). Here, we applied the same JA-related treatments described above in tea plants (MeJA, ibuprofen, or MeJA + MG132) to *Arabidopsis* in order to explore the conserved roles of JA in plant HSR. Following application of MeJA, there was a clear increase in the transcript abundance of*AtCHS* , *AtDFR* , and *AtANS* ; moreover, treatment with ibuprofen decreased expression of these genes much as HT did, whereas MeJA partially rescued the influence of HT (Figure 7a, b). In addition, the patterns of anthocyanin content observed in *Arabidopsis*leaves were consistent with the patterns of catechin content in tea leaves. As expected, anthocyanin accumulation was severely reduced upon treatment with ibuprofen (67%), whose effect was similar with that of HT (75%), whereas JA could partially relieve the impact of HT (Figure 7c). These results suggest that JA also acts as a core mediator in the regulatory effect of HT on anthocyanin synthesis, another important branch of the flavonoid pathway.

Previous studies have indicated that HT decreases the expression of*Arabidopsis* genes required in both early and late steps of the anthocyanin biosynthetic pathway; consequently, HT represses anthocyanin biosynthesis (Kim et al., 2017; Chen et al., 2017). However, the potential roles of HSFs in regulating anthocyanin biosynthesis under HT have not been revealed. It has been reported that mutation of*AtHSFA2* , which is homologous to *CsHSFA1b* and*CsHSFA2,* causes greater sensitivity to heat stress in*Arabidopsis* (Charng et al., 2007; Zhang et al., 2020a). Accordingly, we analyzed the expression of flavonoid biosynthesis genes in Col-0 and the *athsfa2* mutant. The qRT-PCR results revealed that transcript levels of *AtDFR* and *AtANS* were significantly up-regulated in the *athsfa2* mutant. Furthermore, the expression of these genes in both Col-0 and the *athsfa2*mutant was obviously changed by HT Figure S2). It has been reported that detached leaves of mature *Arabidopsis* exhibit purple-red coloration due to excessive anthocyanin accumulation (Chen et al., 2017). Observations of detached leaves of Col-0 and *athsfa2*plants exposed to HT (40 , 48 h) revealed Col-0 leaves to have significantly lighter purple-red color than those of *athsfa2*following HT treatment (Figure 8a). This is consistent with the anthocyanin content in Col-0 was greatly reduced by HT, whereas that in*athsfa2* was less affected (Figure 8a, Fig S4a).

To further define the conserved roles of HSFs, at least for those HSFA family members that mediate the effect of HT on anthocyanin biosynthesis, we over-expressed *CsHSFA1b* and *CsHSFA2*driven by the 35S promoter in Col-0 and the *athsfa2* mutant. Two overexpression lines for each combination of construct and background with" (implies 8 lines total, 2 constructs x 2 backgrounds x 2 replicates) with relatively higher expression levels were then selected for further analysis (Figure S3). As expected, anthocyanin content was significantly reduced in these overexpression lines (Figure 8b). Correspondingly, the transcript levels of anthocyanin biosynthetic genes such as *AtCHS* , *AtDFR,* and *AtANS* were lower in*CsHSFA1bOE3* /Col-0, *CsHSFA2OE3* /Col-0,*CsHSFA2OE3/athsfa2* , and *CsHSFA2OE3/athsfa2* (Figure 8c, Figure S4), and HT treatment exacerbated this down-regulation (Figure 8c, Figure S4). These findings are further supported by the purple-red color of HT-treated *CsHSFA1bOE3/* Col-0 and *CsHSFA2OE3/* Col-0 leaves being significantly lighter upon visual inspection than in leaves of Col-0.

To verify that HSFs participate in regulating MeJA biosynthesis, we then examined MeJA in the overexpression lines by LC-MS. Compared to WT, MeJA content was significantly reduced in *CsHSFA1bOE3/* Col-0,*CsHSFA2OE3/* Col-0, *CsHSFA1bOE3/athsfa2* , and*CsHSFA2OE3/athsfa2* , and decreased even further when exposed to HT (Figure 8d). Taken together, these findings suggest that HSFA family members are activated by HT and negatively regulate JA as well as flavonoid biosynthesis, and this working model is conserved between*Arabidopsis* and tea.

DISCUSSION

Plants encounter natural seasonal changes in temperature and respond by optimizing their growth and development. For tea, an unsuitable growth temperature seriously affects yield and quality, causing enormous economic losses. Adaption to low temperature and frost have been extensively reported in tea plants mainly through increasing the enzymatic activity responsible for free radical scavenging and accumulating high levels of indole and sucrose (Hao et al., 2018a,b; Wang et al., 2013; Shen et al., 2015; Zhou et al., 2020). With the presently climbing global temperature, the mechanism by which HT affects tea quality has gradually become of considerable interest. Therefore, we investigated the physiological response of tea plants to heat stress and explored ways by which their heat tolerance might be improved. Previous work has shown that anthocyanin-related genes and anthocyanin contents are down-regulated under HT (Shen et al., 2019). In this study, we focused on the influence of extreme temperature (40) on metabolites in tea leaves, and found that 11 of 15 detected flavonoids were significantly reduced after HT treatment. HPLC further verified that HT inhibited the accumulation of catechins in tea leaves. Finally, in parallel with the metabolic changes in tea flavonoids, expression levels of flavonoid-related genes were repressed by HT.

The effect of HT on secondary metabolism remains largely unexplored in tea leaves, including the effect on tea's characteristic compounds, catechins. Increasing evidence supports that HSFs play a critical role in heat stress tolerance. HSFs are divided into three main classes, HSFA, HSFB, and HSFC, and plants have multiple different HSFs: there are 21 total in *Arabidopsis* , 25 in rice, 28 in *Populus trichocarpa* , and 25 in tea, (Scharf et al., 2011; Zhang et al., 2015; Muthuramalingam et al., 2020; Zhang et al., 2020a). AHA motifs are usually present in HSFA subfamily members and confer transcriptional activator functionality. Within this subfamily, HSFA1 proteins function as "master regulators" by activating HSR genes and enhancing thermotolerance, while HSFA2 proteins have been identified as coactivators of HSFA1-targeted transcription (Busch et al., 2005; Charng et al., 2007).

As we observed levels of *CsHSFA1b* and *CsHSFA2* to be more responsive to HT treatment than those of other transcripts, we further investigated the effect of CsHSFA1b and CsHSFA2 on the HSR in tea. Although the heat-stress-activated transcriptional network that regulates thermo-responsive gene expression has been characterized in many species (Liu et al., 2013), evidence concerning the interaction between metabolites and CsHSF-involved heat stress responses is yet lacking in tea. Here, we transiently silenced *CsHSFA1b* and*CsHSFA2* in tea plants, and found that catechin content was much higher than in controls; in addition, flavonoid-related genes were up-regulated. We concluded that CsHSFA1b and CsHSFA2 could negatively regulate expression levels of flavonoid-biosynthesis-related genes and ultimately impair flavonoid production.

As JA signaling is known to regulate HT-mediated flavonoid synthesis, we further investigated this pathway as a prospective molecular mechanism and explored the involved components. In tea plants, a series of CsMYBs has been identified to play key roles in flavonoid biosynthesis, including CsAN1, CsMYB1, CsMYB2, CsMYB26, CsMYB5a, CsMYB5e, and CsMYB184 (Li et al., 2022; Wang et al., 2018; Wei et al., 2019; Jiang et al., 2018; Wei et al., 2018; Sun et al., 2016). Among those, CsAN1 in particular has been demonstrated to regulate catechin accumulation by forming the MBW complex through interaction with bHLH transcription factors (CsGL3 and CsEGL3) and recruitment of a WD-repeat protein CsTTG1 (Zhao et al., 2013; Sun et al., 2016). Herein, our findings further highlight CsJAZ6 as influencing formation of the MBW complex through competing with CsEGL3 and CsTTG1, and thereby inhibiting catechin biosynthesis. Our previous study also demonstrated CsJAZ6 to interact with CsMYC2 (Chen et al., 2021), the core mediator of the JA pathway. Thus, it is also possible that CsJAZ6 could indirectly regulate catechin biosynthesis through repressing the CsMYC2-mediated JA pathway.

JA is an emerging player in alleviating the deleterious effects of adverse heat stress conditions on plants (Balfagon et al., 2019). Although there remains much to be elucidated regarding the function of JA in the HSR, a series of findings give us hints that JA could contribute to plant adaption to HT. In *Arabidopsis* , the*cpr5-1* mutant, which exhibits constitutive activation of the JA signaling pathway, displays enhanced heat tolerance, whereas the*coi1* mutant, which is deficient in JA biosynthesis, is more sensitive to HT (Clarke et al., 2009). Warm temperature has also been shown to increase levels of $12HSO_4$-JA and consequently

alter levels of bioactive JA-Ile; this leads to the stabilization of JAZ proteins, which in turn facilitates plant growth (Zhu et al., 2021). In tomato and rice, HT also inhibits JA accumulation (Pan et al., 2019; Wu et al., 2022), with the rice protein OsHTG3 conferring heat tolerance through activating expression of two JAZ genes (Wu et al., 2022). Exogenous application of JA has proved effective in improving plant stress tolerance under adverse temperature conditions, whether low or high (Du et al., 2013; Pan et al., 2019). Here, we observed that HT reduced JA content in tea leaves as well as the expression levels of JA biosynthesis and responsive genes. Meanwhile, we noticed that*CsJAZ6* , the expression of which was activated, showed an opposite pattern in responding to HT. Importantly, we further identified that CsHSFA1b and CsHSFA2 positively regulate the expression of *CsJAZ6*by directly binding to the HSE1 *cis* -element in its promoter. Specifically, CsHSFA1b could bind all three HSEs in the promoter of CsJAZ6, whereas CsHSFA2 could only bind the HSE1 element. In addition, we noticed that HSE1 had a gap of two bases between TTC and GAA, whereas HSE2 and HSE3 had a gap of three bases between two core*cis* -elements. Thus, our data suggest that CsHSFAs may recognize HSE elements in the form of "nGAAnnTTCn", which is consistent with previous reports (Xiao et al., 1988; Ogawa et al., 2007). Altogether, our data indicate the involvement of JAZ proteins in the heat stress response, namely by acting as inhibitors of JA signaling.

Flavonoid biosynthesis is affected by multiple environment factors and regulated by plant hormones (Liang et al., 2020; Zhou et al., 2020). JA, classified as a defensive hormone, plays important roles in the regulation of flavonoid biosynthesis when plants face adversities (An et al., 2021; Ding et al., 2022). The metabolic profiling in this study revealed MeJA content to be reduced under HT and encouraged us to investigate whether JA is involved in HT-induced regulation of flavonoid accumulation. Our results suggest that JA treatment could increase catechin content in tea leaves under HT conditions to levels paralleling those in plants grown under normal conditions. Conversely, treatment with ibuprofen, a JA synthesis inhibitor, repressed flavonoid accumulation under normal conditions. However, the promotive effect of JA was not enhanced under normal conditions, nor was the inhibitory effect of ibuprofen enhanced under heat stress. To further distinguish the effect of JA biosynthesis and JA signaling on HT-regulated flavonoid production, we treated tea plants with JA plus MG132, a specific inhibitor of JA signal transduction. We found that the addition of MG132 could abolish the effect of JA on flavonoid synthesis under HT condition. Altogether, our findings highlight impaired JA signaling, not JA biosynthesis, as a key hub in HT-regulated flavonoid synthesis. In parallel, JA treatment was able to partially alleviate the inhibitory effect of HT on anthocyanin synthesis in *Arabidopsis* . Based on these findings, we further developed a mechanistic model of JA-mediated HT modulation of flavonoid metabolite biosynthesis (Figure 9). Comprehensively, CsHSFAs mediate the JA pathway in regulating secondary metabolism under HT conditions, and this mechanism is conserved in*Arabidopsi* s and tea plants.

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CONFLICT OF INTERESTS

There are no conflict of interests to declare

DATA AVAILABILITY STATEMENT

All the experimental data are available and accessible via the main text and the supplemental data.

AUTHOR CONTRIBUTIONS

XYZ and GJH designed the research and performed most of the experiments. LYL, YQH, YZ, HT, and QSL performed some of the experiments. XYZ and GJH were responsible for data analysis and wrote the manuscript. The authors agree with the content of the manuscript.

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Figure legends

Figure 1. HT inhibits catechin biosynthesis in tea leaves.

(a) Counts of different metabolites s identified in tea plants under HT. **(b)** Catechin levels in tea plants under HT.**(c)** The effect of HT on expression of genes in the flavonoid biosynthetic pathway. Transcripts were analyzed by qRT-PCR and GAPDH was used as the internal standard. Error bars indicate the SD of three biological replicates. **(d)** Expression of HSF genes in tea plants under HT. Error bars indicate the SD of three biological replicates.

Figure 2. Suppression of *CsHSFA1b* and *CsHSFA2*increases catechin accumulation.

(a) , *CsHSFA1b* and *CsHSFA2* expression in control (sODN), *CsHSFA1b/AsODN* , and *CsHSFA2/AsODN* tea leaves. Error bars indicate the SD of three biological replicates. **(b)**Expression of catechin-related genes in control (sODN),*CsHSFA1b/AsODN* , and *CsHSFA2/AsODN* tea leaves. Error bars indicate the SD of three biological replicates. **(c)**Quantitative analysis of catechins in control (sODN),*CsHSFA1b/AsODN* , and *CsHSFA2/AsODN* tea leaves. Error bars indicate the SD of three biological replicates.

Figure 3. Effect of JA treatment on catechins in tea leaves under HT.

(a) , The effect of HT on expression of genes in the JA synthesis and signaling pathways. Error bars indicate the SD of three biological replicates. **(b)** MeJA accumulation in tea leaves under HT. Error bars indicate the SD of three biological replicates.**(c)** Catechin content in tea leaves treated with MeJA, Ibu, or MeJA+MG132 and then transfer to grown under normal (25) or high (40) temperature. Different letters above the bars indicate significant differences between groups (P<0.05; ANOVA with Fisher's LSD test). **(d)** qPCR determination of the transcript levels of*CsCHS* , *CsDFR* , and *CsANS* in tea leaves treated with MeJA, Ibu, or MeJA+MG132 and then transfer to grown under normal (25) or high (40) temperature. Different letters above the bars indicate significant differences between groups (P<0.05; ANOVA with Fisher's LSD test).

Figure 4. CsHSFAs regulate *CsJAZ6* expression.

(a) Yeast one-hybrid assay to confirm binding of CsHSFAs to the*CsJAZ6* promoter. **(b-c)** EMSA assay to validate the binding of CsHSFA1b and CsHSFA2 to HSE motifs (TCCNNGAA) in the*CsJAZ6* promoter. **(d)** Structural schematics of the effector (*35S:CsHSFA1b* and *35S:CsHSFA2*) and reporter (*CsJAZ6Pro::LUC*) constructs used in the dual-luciferase transient expression assay. **(e)** CsHSFA1b and CsHSFA2 activate the transcription activity of the *CsJAZ6* promoter. Relative firefly luciferase (LUC) to Renilla luciferase (REN) ratios in tobacco leaves co-transfected with the *CsJAZ6* reporter and CsHSFA1b/CsHSFA2 effector constructs. Error bars indicate the SD of three biological replicates.

Figure 5. Suppression of *CsJAZ6* increases catechin accumulation.

(a) Expression of *CsJAZ6* in control (sODN) and*CsJAZ6/AsODN* tea leaves. Error bars indicate the SD of three biological replicates. **(b)** Expression of catechin-related genes in control (sODN) and *CsJAZ6/AsODN* tea leaves. Error bars indicate the SD of three biological replicates. **(c)**Quantitative determination of catechins in control (sODN) and*CsJAZ6/AsODN* tea leaves. Error bars indicate the SD of three biological replicates.

Figure 6. CsJAZ6 regulates catechin biosynthesis through physically interacting with CsEGL3 and CsTTG1.

(a) Yeast two-hybrid assay to confirm the interaction of CsJAZ6 and CsEGL3/CsTTG1. The co-transfected yeast competent cells were grown on SD/-LT medium with or without ABA. The empty vector pGADT7 was used as a negative control. **(b)** BiFC analysis of the interaction between CsJAZ6 and CsEGL3/CsTTG1 *in vivo* . Leaves from*tobacco* were infiltrated with agrobacteria as indicated. The N- and C-terminal fragments of YFP (nYFP and cYFP) were fused to the C-terminus of CsJAZ6 and CsEGL3/CsTTG1, respectively. Combinations of nYFP or cYFP with the corresponding CsJAZ6 or CsEGL3/CsTTG1 constructs were used as negative controls. Bars, 20 μm. **(c-d)** Co-IP assay of CsJAZ6-CsEGL3/CsTTG1 binding. Recombinant CsEGL3/CsTTG1-Flag protein was incubated with total proteins isolated from tobacco leaves expressing CsJAZ6-Myc driven by the 35s promoter. Anti-Flag antibody was used to detect CsEGL3 or CsTTG1 fusion proteins in solutions before (Crude) and after (Pull-down) immunoprecipitation. KD, Kilodalton.**(e)** Yeast three-hybrid assay investigating the influence of CsJAZ6 on the CsAN1-CsEGL3 interaction. CsAN1-CsEGL3 binding activity is represented by β-galactosidase activity, and the promoter driving*CsJAZ6* expression was suppressed by increasing Met concentration. Error bars indicate the SD of three technical replicates; the results were consistent across the three biological replicates. Error bars indicate the SD of three biological replicates. **(f)** Structural schematics of the effector (*35S:CsHSFA1b* and *35S:CsHSFA2*) and reporter (*CsJAZ6Pro::LUC*) constructs used for the dual-luciferase transient expression assay. **(g)** Relative firefly luciferase (LUC) to Renilla luciferase (REN) ratios from transient expression assays. These represent the activity of the*CsANS* promoter in the absence/presence of MBW (CsAN1-CsEGL3-CsTTG1) complex and the combination of MBW-CsJAZ6. Error bars indicate the SD of three biological replicates. Different letters above the bars indicate significant differences between groups (P<0.05; ANOVA with Fisher's LSD test). Error bars indicate the SD of three biological replicates.

Figure 7. Effect of JA treatment on anthocyanin in*Arabidopsis* leaves under HT.

(a) Phenotypes of detached *Arabidopsis* leaves treated with MeJA, Ibu, or MeJA+MG132 and then transfer to grown under normal (25) or high (40) temperature. **(b)** qPCR analysis of the transcript levels of *AtCHS* , *AtDFR* , and *AtANS* in*Arabidopsis* leaves treated with MeJA, Ibu, or MeJA+MG132 and then transfer to grown under normal (22) or high (40) temperature. Different letters above the bars indicate significant differences between groups (P<0.05; ANOVA with Fisher's LSD test).**(c)** Anthocyanin content of detached *Arabidopsis* leaves treated with MeJA, Ibu, or MeJA+MG132 and then transfer to grown under normal (22) or high (40) high temperature. Different letters above the bars indicate significant differences between groups (P<0.05; ANOVA with Fisher's LSD test).

Figure 8. CsHSFAs mediate the effect of HT on anthocyanin biosynthesis in *Arabidopsis.*

(a-b) Phenotype and anthocyanin content of detached leaves of Wild-type (Col-0), *Pro35S:CsHSFA1b* (*CsHSFA1bOE2/OE3*), and*Pro35S:CsHSFA2* (*CsHSFA2OE2/OE3*) plants that grown under normal (22) or high (40) temperature. Different letters above the bars indicate significant differences between groups (P<0.05; ANOVA with Fisher's LSD test). **(c)** Gene expression of*AtCHS* , *AtDFR* and *AtANS* in Wild-type (Col-0),*Pro35S:CsHSFA1b* (*CsHSFA1bOE2/OE3*), and*Pro35S:CsHSFA2* (*CsHSFA2OE2/OE3*) plants that grown under normal (22) or high (40) temperature. Different letters above the bars indicate significant differences between groups (P<0.05; ANOVA with Fisher's LSD test). **(d)** MeJA content of detached leaves in Wild-type (Col-0), *Pro35S:CsHSFA1b*(*CsHSFA1bOE2/OE3*), and *Pro35S:CsHSFA2*(*CsHSFA2OE2/OE3*) plants that grown under normal (22) or high (40) temperature. Different letters above the bars indicate significant differences between groups (P<0.05; ANOVA with Fisher's LSD test).

Figure 9. A model of JA-mediated high temperature (HT) modulation of flavonoid metabolite biosynthesis.

Under HT (40), CsHSFA1b and CsHSFA2 are activated and form trimers. Active CsHSFAs promote expression of *CsJAZ6* through directly binding to HSE elements in the promoter region of *CsJAZ6* . CsJAZ6 can then directly interact with CsEGL3 and CsTTG1, two components of the catechin biosynthetic regulator complex. The CsJAZ6-CsEGL3 interaction interferes with formation of that complex, resulting in repressed catechin accumulation. While spraying tea leaves with MeJA, CsJAZ6 is degraded and released CsEGL3. CsEGL3 can then form the MYB-bHLH-WD40 transcriptional activation complex with CsAN1 and CsTTG1, which directly promotes catechin biosynthesis. CsHSFA and its homologues in*Arabidopsis* negatively regulate JA and anthocyanin accumulation, which regulation is conserved in tea and *Arabidopsis.*

Supplemental Data

Supplemental Figure S1. Yeast one-hybrid assay to confirm binding of CsHSFAs to the *CsJAZ6* promoter.

Supplemental Figure S2. Gene expression of *AtCHS* ,*AtDFR* , and *AtANS* in leaves of Arabidopsis. **(a)**qPCR analysis of the transcript levels of *AtCHS* , *AtDFR* , and *AtANS* in leaves of Col-0 and *athsfa2* plants. Error bars indicate the SD of three biological replicates. **(b)** qPCR analysis of the transcript levels of *AtCHS* , *AtDFR* , and*AtANS* in leaves of Col-0 that grown under normal (22) or high (40) temperature. Error bars indicate the SD of three biological replicates.

Supplemental Figure S3. Gene expression of *CsHSFA1b* and*CsHSFA2 in* over-expressed lines Col-0, *athsfa2* and over-expressed plants. **(a)** qPCR analysis of the transcript levels of *CsHSFA1b* in Col-0, *athsfa2,* and*Pro35S:CsHSFA1b/* Col-0 *(CsHSFA1b/* Col-0 *T2 1-3)* and*Pro35S:CsHSFA1b/athsfa2 (CsHSFA1b/athsfa2 T2 1-3)* transgenic plants. Error bars indicate the SD of three biological replicates.**(b)** qPCR analysis of the transcript levels of *CsHSFA2* in Col-0, *athsfa2,* and *Pro35S:CsHSFA2/* Col-0*(CsHSFA2/* Col-0 *T2 1-3)* and *Pro35S:CsHSFA2/athsfa2 (CsHSFA2/athsfa2 T2 1-3)* transgenic plants. Error bars indicate the SD of three biological replicates.

Supplemental Figure S4. CsHSFAs mediate the effect of HT on anthocyanin biosynthesis in *Arabidopsis* .

(a-b) Phenotype and anthocyanin content of detached leaves of*athsfa2* , *Pro35S:CsHSFA1b/athsfa2* (*CsHSFA1b/athsfa2 T2 2-3*) and *Pro35S:CsHSFA2/athsfa2* (*CsHSFA2/athsfa2 T2 2-3*) plants that grown under normal (22) or high (40) temperature. Different letters above the bars indicate significant differences between groups (P<0.05; ANOVA with Fisher's LSD test).**(c)** Gene expression of *AtCHS* , *AtDFR* , and*AtANS* in *athsfa2* , *Pro35S:CsHSFA1b/athsfa2*(*CsHSFA1b/athsfa2 T2 2-3*), and *Pro35S:CsHSFA2/athsfa2*(*CsHSFA2/athsfa2 T2 2-3*) plants that grown under normal (22) or high (40) temperature. Different letters above the bars indicate significant differences between groups (P<0.05; ANOVA with Fisher's LSD test). **(d)** MeJA content in detached leaves of*athsfa2* , *Pro35S:CsHSFA1b/athsfa2* (*CsHSFA1b/athsfa2 T2 2-3*), and *Pro35S:CsHSFA2/athsfa2*(*CsHSFA2/athsfa2 T2 2-3*) plants that grown under normal (22) or high (40) temperature. Different letters above the bars indicate significant differences between groups (P<0.05; ANOVA with Fisher's LSD test).

Table S1 Oligonucleotide primer sequences