CaNAC2c acts modulator in the tradeoff among pepper growth, adaptation to high temperature stress and resistance to Ralstonia solanacearum infection

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Abstract

In their natural habitats, plants are inevitably exposed to different biotic and abiotic stresses, to maximize fitness, their adaptation to these stresses should be appropriately coordinated by trade-offs among growth and stress response. However, the involved players and their possible mode of action remain to be investigated. Herein, by approaches of reverse genetics including virus induced gene silencing (VIGS), transient overexpression in pepper or ecoptic overexpression in Nicotiana benthamiana, ChIP-PCR and physiology, CaNAC2c was functionally characterized in trade-offs between growth and immunity against Ralstonia solanacearum inoculation (RSI) or thermotolerance. Our results demonstrate that CaNAC2c remains lower level of transcripts in absence of stress and functions negatively in pepper growth, but is upregulated and functions positively in pepper response to high temperature stress (HTS) and to RSI probably via signaling mediated by ABA and JA, respectively. CaNAC2c functions by directly targeting CaHSFA5 and decreasing H2O2 accumulation upon HTS. On the other hand, it acts positively in pepper response to RSI by upregulating JA- and enhancing accumulation of H2O2, while downregulating SA-signaling mediated PR genes, but does not target or regulate CaHSFA5. In addition, CaNAC2c exhibits redundancy with CaNAC2d in response to challenge of RSI but not to that of HTS, indicating that immunity against RSI is more robust than thermotolerance. These findings collectively unveil that tradeoff between growth and thermotolerance/immunity mediated by CaNAC2c is mainly determined by its differential transcription, while tradeoff between thermotolerance and immunity mediated by CaNAC2c is conferred by its context dependent post-translational regulation.

Introduction

In their natural inhabits, plants are inevitably exposed to different biotic and abiotic stresses individually or simultaneously and have to appropriately respond to the diverse stresses to maximize fitness. As the resource is limited, and the stress response is costly, the defense response to the stress with the greatest threat is usually prioritized by repressing growth and unnecessary defense response to the stresses that are absent or not so serious(Lozano-Duran et al., 2013), thus result in extensive tradeoff among plant growth and response to different stresses (Berens et al., 2019; Huot, Yao, Montgomery, & He, 2014). HTS and pathogen attack are two closely related stresses frequently encountered by plants in tropical or subtropical climates, plant immune responses to pathogens are generally dampened(Hua, 2013) but in some case promoted(Onaga et al., 2017) by HTS. ABA, a crucial regulator in plant thermotolerance(Y. C. Huang, Niu, Yang, & Jinn, 2016), plays a role in inhibition of disease resistance by HTS(Mang et al., 2012). Despite the distinct nature of pathogen infection and HTS and their different modes of perception by plant cells(Jones & Dangl, 2006; Mittler, Finka, & Goloubinoff, 2012), signaling initiated by HTS and pathogen attack share components such as reactive oxygen species (ROS), phytohormones including JA and salicylic acid (SA), and transcription factors such as WRKY33(Li, Fu, Chen, Huang, & Yu, 2011; J. Liu, Feng, Li, & He, 2015; S. Liu, Kracher,

Ziegler, Birkenbihl, & Somssich, 2015). However, how plants coordinately respond to HTS and pathogen attacks remain elusive.

As plant response to various stresses are largely regulated at transcriptional level mediated by transcription factors(TFs)(Moore, Loake, & Spoel, 2011). NAC [No apical meristem (NAM), Arabidopsis transcription activation factor (ATAF), Cup-shaped cotyledon (CUC)] constitute one of the largest plant TF families, they generally contain a well conserved N-terminal NAC domain and a diversified C-terminal transcription regulatory region, and were classified comprehensively into eight subfamilies(Puranik, Sahu, Srivastava, & Prasad, 2012). NAC transcription factors have been implicated in the regulation of plant response to stress conditions by binding specific recognition site [CGT(G/A)] in their target genes(L. Fang et al., 2016; Khedia, Agarwal, & Agarwal, 2018). The stress response related NACs fall into one subgroup(Y. Fang, You, Xie, Xie, & Xiong, 2008; Nakashima, Takasaki, Mizoi, Shinozaki, & Yamaguchi-Shinozaki, 2012; Negi, Tak, & Ganapathi, 2018), these stress response related NACs might be regulated at multiple levels including transcriptional, post-transcriptional and post-translational level (W. Huang et al., 2013; Puranik et al., 2012; Tran et al., 2004). In addition, a single NAC TF might act as convergent node of plant response to different stress conditions(McGrann et al., 2015). However, the role of these NAC TFs and how they operate in the coordination of different plant biological processes currently remains poorly understood.

Pepper (*Capsicum annuum*) is a solanaceous vegetable of great economic importance worldwide. Bacterial wilt caused by root infection of *R. solanacearum* , a soil borne pathogen with extremely versatile lifestyle (Jiang et al., 2017; Mansfield et al., 2012), is one of the most frequently occurred diseases(Du et al., 2017). The bacterium attacks pepper plants by penetrating the root system, proliferating and spreading through xylem vessels, causing the disruption of the plant vascular system and eventual plant wilting(Digonnet et al., 2012; Mansfield et al., 2012; Turner et al., 2009). HTS is another important abotic stress frequently encountered by pepper in tropical and subtropical climates usually cause retard in pepper growth and development. To survive these stresses, the response of pepper to these stresses and growth should be appropriately coordinated. However, how pepper coordinately respond to RSI and HTS currently remain elusive. In the present study, we found that CaNAC2c play a crucial role in the tradeoffs among pepper growth and its response to RSI and HTS.

Materials and methods

Plant materials and growth conditions

The seeds of the pepper inbred line HN42 and *N. benthamiana* were sown in a soil mix [peat moss: perlite, 2:1 (v/v)] in plastic pots and were placed in a growth room under condition of 28, 60–70 mmol photons m^{-2} s^{-1}, a relative humidity of 70%, and a 16-h light/8-h dark.

The vectors construction

To construct vectors for overexpression, the full-length ORF of*CaNAC2c* was cloned into the entry vector pDONR207 by BP reaction with appropriate primers (Table S1) and then cloned into destination vectors pEarleyGate103, pDEST-15 and PK7WG2 by LR reaction, using Gateway cloning techniques (Invitrogen, Carlsbad, CA, USA). Two specific 300-400 bp fragments in the ORF and 3'UTRs of *CaNAC2c /d*and *CaHSFA5,* which were confirmed by BLAST searching against genome sequence in the databases of Zunla-1 (http://peppersequence.genomics.cn/page/species/blast.jsp), were used for vector construction for *CaNAC2c /d* or*CaHSFA5* VIGS silencing. The specific fragments were cloned into the entry vector pDONR207 and then into the PYL279 vector. To construct vectors for 5' series deletions assay of *pCaNAC2c,* the full length *pCaNAC2c* and its 5' series deletions were first cloned into pDONR207 by BP reaction with appropriate primers (Table S2) and the into the pMDC163 destination vector by LR reaction using Gateway cloning techniques for assaying expression of β-glucuronidase (GUS) driven by*pCaNAC2c* or its 5'series deletions in leaves of pepper plants by transient overexpression through agro-infiltration.

Fluorometric GUS enzymatic assay

A fluorometric GUS enzymatic assay for measuring GUS activity in pepper plant extract was performed

as described previously (RA, TA, & MW, 1987). Leaf samples used for GUS assay were harvested and then frozen with liquid nitrogen, and then were ground into fine powder using a pestle and mortar. The samples were lysed in extraction buffer (50 mM phosphate buffer, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 0.1% sodium lauryl sarcosine, and 10 mM β-mercaptoethanol), and then collect the supernatant by centrifugation. Aliquots of the extracts (100 μl) were added to 1 ml of assay buffer (extraction buffer containing 1 mM MU), pre-warmed, and incubated at 37 . After 0, 5, and 20 min of incubation, 100 μl samples were removed and placed in 1.9 ml of stop buffer (200 μM sodium carbonate). Fluorescence was measured using a Multi Detection Microplate Reader (Bio-TEK Synergy HT, Bad Friedrichshall, Germany). The total protein content in plant extracts was estimated by the Bradford method using BSA as a standard (MM, 1976).

Plant treatment with HTS

HTS treatment of pepper plants was performed by placing 8-leaf stage pepper plants under conditions of 42 and 50% humidity, while the control plants were placed under condition of 28 and 50% humidity.

Pathogens and *R. solanacearum* inoculation

The highly virulent *R. solanacearum* strain FJC100301 (F. F. Dang et al., 2013) was used in the present study. The bacterial cell solution used for inoculation was diluted to 10^8cfu ml^{-1} (OD_{600} = 0.8) or 10^3cfu ml^{-1} (OD_{600} = 0.3). For root inoculation of pepper or *N. benthamiana* plants planted in pots, the soil was watered sufficiently and the roots were wound by scissors and then 1 ml of suspension containing *R. solanacearum* (OD_{600} = 0.8) was irrigated into the soil. For leaf inoculation with *R. solanacearum* , 100 μl suspension of*R. solanacearum* (OD_{600} = 0.3) was inoculated with a needle-free syringe at each inoculating site. We counted the disease index of plants in the whole infection cycle of pathogens by observation (Table S4). There were 36 plants in each population, and the average value of every three plants was taken for a group.

DAB and NBT staining

The accumulation of H_2O_2 and active oxygen species (Papageorgiou et al.) were assessed by staining the leaves, root or stems of pepper or *N. benthamiana* plants with 1 mg ml^{-1} diaminobenzidine (DAB) or Nitrotetrazolium Blue chloride (NBT). After overnight treatment with DAB and NBT, the stained leaves were cleared by boiling in [lactic: glycerol: absolute ethanol (1: 1: 3, V: V: V)] and then destained overnight in absolute ethanol.

VIGS of *CaNAC2c/d* and *CaHSFA5* in pepper plants

To silence *CaNAC2c(d)* or *CaHSFA5* in pepper plants by virus induced gene silencing (VIGS), GV3101 cells containing PYL192 and PYL279-*CaNAC2c* (*CaNAC2d* , *CaHSFA5*), or PYL279 (as a negative control) were mixed and resuspended in the induction medium at 1:1 ratio to OD_{600} = 0.6, which were infiltrated into cotyledons of 2-week-old pepper plants. To simultaneously silence*CaNAC2c* and *CaNAC2d* in pepper plants, GV3101 cells containing PYL192 and PYL279-*CaNAC2c* and cells containing PYL192 and PYL279-*CaNAC2d* were mixed at ratio 1:1 and resuspended in the induction medium to OD_{600} = 0.6. 100 μl were infiltrated into cotyledons of 2-week-old pepper plants, which were placed in dark at 16 for 56 h, and then moved to a growth room under conditions of 28 , 60–70 mmol photons m^{-2} s^{-1}, a relative humidity of 70%, and a 16-h light/8-h dark. At 15 days post inoculation (dpi), the success and specificity of gene silencing were assayed in pepper plants challenged with HTS by measuring the transcript level of *CaNAC2c* (*CaNAC2d* , *CaHSFA5*) or the transcript level of *CaNAC2c* and *CaNAC2d* .

Transient expression of *CaNAC2c* in pepper leaves

For transient expression analysis, GV3101 cells harboring*35S:CaNAC2c-GFP* (using *35S:GFP* as control) was grown overnight and then resuspended in induction medium (10 mM MES, 10 mM $MgCl_2$, 200 μM acetosyringone, pH 5.6) to OD_{600} = 0.8, approximately 1 ml was infiltrated into the leaves of pepper plants

at the eight-leaf stage using a syringe without a needle, and then the infiltrated leaves were collected at the indicated time points for further use.

Generation of transgenic *CaNAC2c*-overexpressing *N. benthaminana* plants

Leaf discs of *N. benthamiana* were transformed with GV3101 harboring *35S:CaNAC2c-GFP* vector followed the method of Regner*et al* (F et al., 1992). and Bardonn*et al* (N, F, MA, & L, 1994). 19 independent T_0 transgenic *N. benthamiana* lines were selected by hygromycin (5 mg l^{-1}) selection and further confirmed by PCR and quantitative real-time RT-PCR (qRT-PCR). The T_0 plants were then self-pollinated and their seeds individually harvested. Similarly, corresponding seeds of T_2 and T_3 were acquired. Two T_3 transgenic lines that exhibited moderate levels of *CaNAC2c* transcripts without phenotypic abnormality were selected for further analysis.

qRT-PCR

To determine the relative transcript levels of selected genes, qRT-PCR was performed with specific primers (Table S1) according to the manufacturer's instructions for the BIO-RAD Real-time PCR system (Foster City, CA, USA) and the SYBR Premix Ex Taq II system (TaKaRa). Total RNA preparation and real-time RT-PCR were carried out following procedures described in our previous studies (Cai et al., 2015; F. Dang et al., 2013; Zhang et al., 2015). Total RNA was isolated from the pepper samples using TRIzol Reagent according to manufacturer's protocol (Invitrogen, Canada). The mRNA was reverse-transcribed into cDNA using the reverse transcription system (Takara Biotechnology, Japan). Four replicates of each treatment were performed. Data were analyzed by the Livak method (Livak & Schmittgen, 2001) and expressed as a normalized relative expression level ($2^{-\Delta\Delta^{\tau}T}$) of the respective genes. The relative transcript level of each sample was normalized to*CaActin* (GQ339766) and 18S ribosomal RNA (EF564281), respectively.

Fluorescence spectrophotometry

A MINI Imaging PAM instrument (Heinz Walz GmbH, Effeltrich, Germany) was used to measure the F_v/F_m and $^F/F_m{}'$*ofpepperandN. benthamianaleaves.Theplantswereadaptedtodarknessfor*15*minandthenplaceddirectlyintotheins*

Immunoblot analysis

Protein extraction buffer [(10% glycerol, 25 mM Tris-HCl pH 7.5, 150 mM sodium chloride, 1 mM ethylenediamine tetraoxalic acid (EDTA), 1% Triton X-100, 10 mM dithiothreitol, 1 × plant protease inhibitor, 2% (w/w) poly (ethylene pyrrolidone) (PVPP)] was used to extract the total protein of pepper samples. At 4 , the extracted protein was incubated with anti-hemagglutinin (anti-GFP) agarose (Thermo Fisher Scientific, Waltham, MA, USA) overnight. Beads were collected using a magnetic rack and washed three times with Tris-buffered saline and Tween-20 (0.05%). Eluted protein was examined by immunoblotting using anti-GFP–peroxidase antibodies (Abcam, Cambridge, UK).

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed according to the protocol of previous study Khan et al. (2018). Three fully extended pepper leaves at six-leaf stage were inoculated with GV3101 cells harboring *35S:CaNAC2c-GFP* . The infiltrated leaves were harvested at 48 hpi and crosslinked with 1% of formaldehyde; chromatins were isolated and sheared into fragments of 300 to 500 bp length; and DNA-protein complexes were immunoprecipitated using anti-GFP antibodies. Finally, the DNAs were decrosslinked, purified and used as template for PCR using primer pairs specific to a CATGTG-box containing fragment or CATGTG free fragment in *CaHSFA5*promoter by semiquantitative PCR using specific primer pairs (Supplemental Table S1).

Prokaryotic expression of CaNAC2c-GST and its purification

In order to obtain plenty of soluble CaNAC2c proteins, a pDEST-15 plasmid harboring *T7:CaNAC2c-GST* was transformed into the*Escherichia coli* (*E.coli*) strain BL21 (DE3). Expression of the fusion protein was induced with 1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) at 20 for 12 h, and an SDS-PAGE assay was performed to confirm whether the soluble fusion protein was present in the supernatant of the *E.*

coli strain BL21 (DE3) cell lysate. For purification of the recombinant proteins with GST tag, the Beaver Beads^{TM} GSH (Beaver Biosciences, China) were washed three times by buffer A (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4), and then mixed with protein liquid slowly incubated for 3 h at 4. The beads were washed five times by buffer A and the target proteins were eluted by buffer B (50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0).

Electrophoretic mobility shift assay (EMSA)

Cy5-labelled double strand DNA fragments containing CATGTG motif and its mutated version (GGGGGG) were artificially synthesized as probes in EMSA. CaNAC2c-GST or GST proteins were incubated with wild-type or mutated probe which was labelled with Cy5 fluorochrome in 5×binding buffer (1 M Tris-HCl pH 7.5, 5 M NaCl, 1 M KCl, 1 M $MgCl_2$, 0.5 M EDTA pH 8.0, 10 mg ml^{-1}BSA). The mixture was separated by PAGE gal and then scanned by Odyssey$^{@}$ CLX (LI-COR, USA).

Interaction of Protein with Promoter Fragment by MST in Solution

The interaction of CaNAC2c protein with the promoter fragment of a*CaHSFA5* was analyzed by microscale thermophoresis (MST) in solution (Zillner et al., 2012). GFP in the fused protein TF-GFP was used as fluorescent label, and a CATGTG motif containing fragment within the promoter of *CaHSFA5* , which was amplified by PCR with a specific primer pair and was further purified. The fragment containing the mutant version of CATGTG motif (GGGGGG) was amplified by PCR by conventional overlapping PCR-based site-directed mutagenesis. The two DNA fragments were used as the nonfluorescent molecules. The protein-DNA interactions were measured following the method of our previous study (Qiu et al., 2018). The Nano Temper Analysis 1.2.20 software was used to fit the data and determine the apparent K_d values (Papageorgiou et al., 2016; Zillner et al., 2012).

Statistical analyses

Statistical analyses of the bioassays were performed with DPS software package. Data represented the means ± SD obtained from three or four replicates; different uppercase letters above the bars indicated significant differences among means ($P < 0.01$), as calculated with Fisher's protected least-significant-difference (LSD) test.

Results

The response of pepper NAC2Cgenesto attack of HTS and RSI

It was previously found that NAC TFs that exhibit inducible expression upon heat stress or pathogen generally play roles during plant response to these stresses(Nakashima et al., 2012). Based on the public annotated information of pepper genome, we found that there are 90 NAC transcription factors in genome of pepper lineZunla-1 (Qin et al., 2014).To find pepper NACs involved in coordination of pepper response to HTS and RSI, we identified NAC genes upregulates in pepper plants against HTS or RSI based on the public available data in Pepper Hub (http://www.hnivr.org/)(F. Liu et al., 2017) and our unpublished data from RNA-seq assay, we found that a NAC gene (Capana06g001739) that exhibits the highest sequence identity to NAC2 in Arabidopsis was upregulated by both HTS and RSI (Figure S1A and Table S3). In addition, this NAC gene was promoted by exogenous application of ABA or JA but inhibited by IAA or GA3(Figure S1B). To confirm the transcriptional response of this NAC gene to HTS or RSI, its transcript level in roots of pepper plants challenged with RSI or HTS were measured by qRT-PCR, the result showed that it was upregulated by HTS or RSI (Figure S1C).

The sequence analysis of pepper NAC2 genes

By sequence analysis and genome wide assay, the HTS and RSI responsive NAC and the other three NACs all contain a well conserved N-terminal NAC domain and a diversified C-terminal transcription regulatory (TR) region (Figure S2A) and share the highest sequence identity to NAC2 in Arabidopsis (Figure S2B), so they were named as *CaNAC2a* to*CaNAC2d* , and the HTS and RSI responsive NAC gene was named*CaNAC2c* . Among these four genes, *CaNAC2c* and*CaNAC2d* share the highest sequence identity (Figure S2C and

D). In addition, the response of the four NAC2 genes to HTS and RSI was assayed based on the data of RNA-seq with pepper plants challenged with RSI, *CaNAC2c* is was found to be upregulated by HTS and RSI,*CaNAC2d* was only induced by RSI in pepper roots (Figure S2E).

The promoter activity analysis of *CaNAC2c* in response to HTS and exogenous applied hormones

As *cis* -elements such as TGACG and ABRE responsive for JA and ABA responsiveness, respectively, are present in the promoter of*CaNAC2c* (*pCaNAC2c*), to assay whether stress induction of*CaNAC2c* is determined by its promoter, we assayed activity of*pCaNAC2c* against HTS and exogenous application of ABA, jasmonic acid methyl ester (MeJA), indoleacetic acid (IAA) and gibberellic acid 3 (GA3) and the possible regions responsible for the responsiveness by 5' series deletion analysis. We found that the response of *CaNAC2c*to HTS is regulated by ABA via the ABRE (ABA response element) containing $pCaNAC2c^{261}$. In addition,*CaNAC2c* was upregulated by exogenous applied MeJA, which was attributed to the TGACG-box locates -1381 to -632bp in *pCaNAC2c* . Furthermore, *CaNAC2c* was downregulated by exogenous applied GA3 or IAA, and the *cis* -elements responsible for GA3or IAA responsive might locate within -1381 to -935bp in *pCaNAC2c* (Figure S3). These data imply that *CaNAC2c* is transcriptional regulated by HTS or RSI via signaling mediated by JA, ABA, IAA or GA3.

CaNAC2c is a nuclear protein

To assay the subcellular localization of CaNAC2c, it was transiently overexpressed in *N.benthamiana* leaves by agro-infiltration with GV3101 cells containing *35S:CaNAC2c-GFP* (using *35S:GFP* as a control) and its location was assayed by GFP signal observation. The result showed that, at 48 hpi, GFP signal exclusively occurred in the nucleus of epidermal cells in leaves infiltrated with*35S:CaNAC2c-GFP* , while that with *35S:GFP* was observed in the whole epidermal cell including plasma membrane, cytosol, and the nucleus, indicating that CaNAC2c is a nuclear protein(Figure S4).

The effect of *CaNAC2c*silencing on pepper growth, thermotolerance and response to RSI

To assay the possible role of *CaNAC2c* in pepper response to HTS or RSI, we assayed the effect of *CaNAC2c* silencing by VIGS on response of pepper plants to HTS or RSI. The results showed that*CaNAC2c* was successfully silenced by the two vectors (Figure 1A and B). It was found that upon HTS, the *CaNAC2c* silenced plants exhibited decreased basal and acquired thermotolerance, displayed with serious wilt phenotype, high levels of plant mortality from 1 to 42 hpt with or without pretreatment of nonlethal high temperature (Figure 1C and D). The decreased thermotolerance was accompanied with significant downregulation of thermotolerance related*CaHSP24, CaHSFB2a* and *CaHSP70* (Ashraf et al., 2018) (Figure1E-G).We also found that ion leakage upon HTS displayed by conductivity in *CaNAC2c* silenced plants was significantly higher than that of the control plants (Figure 1H and I). The DAB and NBT staining representing H_2O_2 and ROS accumulation, which were previously found to be negatively related to thermotolerance(Yu et al., 2019; Zhuang et al., 2020), were much darker in the leaves and stems of *CaNAC2c* -silenced plants than that in the control plants (Figure 1J and K).We also found*CaNAC2c* -silenced plants exhibited lower levels of Fv/Fm and actual photochemical efficiency of PSII in the light (ϕPSII), indicator of thermotolerance and thermostability of the photosynthetic apparatus, respectively(Guan et al., 2018; Wang, Zhang, Goatley, & Ervin, 2014; Yan et al., 2008), immediately after the HTS treatment(Figure 1L and M). All these data indicate that *CaNAC2c* acts as positive regulator in basal and acquired thermotolerance. By contrast, the *CaNAC2c*silenced pepper plants were inoculated with *R. solanacearum* , but no difference was found between the *CaNAC2c* silenced and the control plants (Data not shown). In addition, the *CaNAC2c*silenced pepper plants exhibited promotion not only in plant height and size of roots, stems and leaves, but also in leaf and flower number (Figure 2), indicating that CaNAC2c acts negatively in pepper growth and development.

Transient overexpression of *CaNAC2c*promoted thermotolerance and hypersensitivity reaction (HR) mimic cell death and expression of defense related genes

To confirm the result from VIGS, we assayed the effect of *CaNAC2c*transient overexpression(*CaNAC2c* -TO)

on Fv/Fm and $^{F}/Fm'$ and expression of thermotolerance related genes, the results showed that CaNAC2c was successfully expres
TO under HTS (Figure 3B and C), coupled with enhanced expression of thermotolerance related genes including CaHSFB2A, Ca

Since resistance to RSI and thermotolerance are two different processes, we study the switching of CaNAC2c function in different treatments. In parallel, *CaNAC2c* -TOwas found to trigger clear HR mimic cell death at room temperature, increased accumulation H_2O_2 displayed with darker DAB staining upon challenge of RSI, while the exogenous applied ABA blocked the cell death. By contrast, at 37, *CaNAC2c* -TO did not trigger any cell death, but the exogenous application of fluridon, an inhibitor of biosynthesis of ABA, restored the cell death triggered by *CaNAC2c* -TO at 37 (Figure 3E and F), indicating that ABA might confer the repression of cell death induction of *CaNAC2c* at 37. In addition, it was found that both SA signaling dependent *CaNPR1* and JA signaling dependent *CaDEF1* were depressed by HTS (Figure 3G), although *CaNPR1* was depressed, *CaDEF1* and HR related *CaHIR1* was induced by *CaNAC2c* -TO at room temperature (Figure 3G), indicating that *CaNAC2c* acts as positive regulator in JA-dependent immunity and HR but a negative regulator in SA-dependent immunity. These results suggest that CaNAC2c might have different transcriptional regulation directions under RSI and HTS.

Overexpression of *CaNAC2c* enhanced basal and acquired thermotolerance, resistance to RSI but decreased growth of *N.benthamiana* plants

To further confirm the results from *CaNAC2c* silencing and transient overexpression, we assayed the effect of ectopic overexpression of *CaNAC2c* (*CaNAC2c* -OE) in *N.benthamiana* plants on thermotolerance and RSI resistance. We generated transgenic *N.benthamiana* plants of *35S:CaNAC2c* , a total 9 T_0 plants were acquired, by strict self-pollination, their corresponding T_1 and T_2 lines were acquired. Two T_2 lines, *CaNAC2c-OE1#* and *CaNAC2c-OE2#* , with high levels of *CaNAC2c* transcripts were selected for further assay (Figure 4A). It was found that compared to the wild type plants, the growth and development of two lines were significantly inhibited, manifested by their smaller size in leaves, roots and stems, fewer numbers of leaves and flowers (Figure S5), confirming the result from *CaNAC2c* silencing in pepper plants that CaNAC2c acts as negative regulator in pepper growth and development.

In parallel, it was also found that *CaNAC2c* -OE *N. benthamiana* plants exhibited significantly enhanced tolerance to HTS (Figure 4B), displayed by their significantly lower mortality rate calculated after 1–42 h of HTS with or without pretreatment of nonlethal HTS (Figure 4C). In addition, Fv/Fm and $^{F}/Fm'$ were found to be enhanced, ion leak
OE (Figure S6). Consistently, the transcript levels of thermotolerance related NbAPX, NbHSP18 and NbsHSP were up regulated
OE in N. benthamiana plants compared to the control plants, and this up regulation was amplified by HTS (Figure 4D). These resul
OE upon HTS (Figure S9I).

To assay the effect of *CaNAC2c* -OE on immunity against RSI, the *CaNAC2c* -OE *N. benthamiana* plants were challenged with RSI, an obvious wilt symptoms in the wild type plants were observed at 7 dpi, while the transgenic plants exhibited only slight wilt symptoms, and lower levels of disease index and population of *R.solanacearum* in transgenic plants were found (Figure 4E, F and G). Consistently, enhanced transcript levels of immunityrelated genes such as *NtDEF1* were found in the *CaNAC2c* -OE *N. benthamiana* plants upon RSI, while thermotolerance related genes did not alter in their transcript levels in the transgenic plants upon RSI (Figure S9I).

***CaHSFA5* is directly targeted by CaNAC2c upon HTS but not upon RSI**

By co-expression assay in pepperhub, it was found *CaHSFA5* was co-expressed with *CaNAC2c* under HTS (Table S5). To test if *CaHSFA5* is one of target genes of CaNAC2c, we study the binding of CaNAC2c to promoter of *CaHSFA5* by ChIP-PCR. The result showed that the specific primer pair of *CaHSFA5* promoter produced clear DNA band, while the control primer pair did not produce any DNA band, indicating that CaNAC2c directly targets *CaHSFA5* (Figure 5A). Moreover, the binding of CaNAC2c to the promoter fragment of *CaHSFA5* was significantly enhanced by HTS (Figure 5B and C). MST confirmed the binding of CaNAC2c to its putative binding site in the *CaHSFA5* promoter *in vitro* (Figure 5D). By EMSA, prokaryotically expressed CaNAC2c-GST bound promoter of *CaHSFA5,* importantly, *cis* -element CATGTG was found to be responsible for the binding of CaNAC2c to the promoter of *CaHSFA5* , since its mutation

version GGGGGG blocked the binding (Figure 5E). In addition, *CaHSFA5* was found to be repressed by *CaNAC2c*silencing in pepper plants challenged with HTS, while upregulated by*CaNAC2c* -TO in pepper plants. Similarly, *NbHSFA5* was upregulated by *CaNAC2c* -OE in NB plants (Figure 5F and G). All these results indicate that *CaHSFA5* is directly and positively regulated by CaNAC2c.

To assay the role of CaHSFA5 in pepper thermotolerance and immunity against RSI, the effect of *CaHSFA5* silencing was studied. We successfully and specifically silenced *CaHSFA5* by VIGS in pepper plants (Figure 6H). Similar to CaNAC2c, CaHSF5A acts negatively in pepper growth, since the*CaHSF5A* silenced pepper plants exhibited an enlarged size in leaves, stems and roots (Figure S7). In addition, the *CaHSFA5* silenced plants exhibited thermo sensitiveness compared to the wide type plants(TRV:*00*) with or without pretreated with nonlethal HTS, displayed with higher mortality rate as well as decreased Fv/Fm and $^{F}/Fm'(Figure 6B-E).Consistently, CaHSFA5silencingsignificantlyimpairedupregulationofCaHSP24andCaHSP70upon$
$K).Bycontrast,thesilencingofCaHSFA5didnotaffecttheresistanceofpepperplantstoRSI(Figure 6FandG).$

To further assay the role of CaHSFA5 in the function of CaNAC2c, NbHSFA5, the ortholog of CaHSFA5 in *N. benthamiana* genome, was specifically and successfully silenced in *CaNAC2c* overexpressing*N. benthamiana* plants by VIGS, its effect on growth and response to RSI or HTS were checked. The result showed that the silencing of*NbHSFA5* significantly restored the growth inhibited (Figure S8) in *CaNAC2c* overexpressing *N. benthamiana* plants, but did decreased its thermotolerance (Figure S9B-D) and not affected its*R. solanacearum* resistance (Figure S9E and F).

Simultaneous silencing of *CaNAC2c* and *CaNAC2d*significantly inhibited resistance of pepper plant to RSI

The silencing of *CaNAC2c* did not produced obvious symptoms upon RSI, while its overexpression in pepper and *N. benthamiana* plants did promoted immunity against RSI, implying that CaNAC2c may function redundantly with other genes in response to RSI. As *CaNAC2d*shares the highest sequence identity to *CaNAC2c* and exhibited similar upregulation in response to RSI (Figure S2), we speculate that it might act redundantly with *CaNAC2c* in pepper response to RSI. To test this hypothesis, we assay the effect of simultaneous silencing of *CaNAC2c* and *CaNAC2d* on response of pepper plants to RSI and HTS. The result showed that the silencing of *CaNAC2d* alone did not significantly affect either pepper thermotolerance or immunity against RSI (Figure 7A), while *CaNAC2c* and *CaNAC2d*simultaneously silenced pepper plants exhibited obvious wilt soymptoms at 192 hpi when inoculated with *R. solanacearum* , and the highest disease index from 2 to 12 dpi, while their individually silenced plants did not exhibit obvious wilt soymptoms (Figure 7B-E).The simultaneous silencing of *CaNAC2c* and *CaNAC2d* and their silencing alone pepper plants exhibited enlarged size compared to the control plants(Figure S10). Consistently, the thermotolerance related*CaHSP24* , *CaHSP70* and *CaHSFB2a* were not downregulated by silencing of *CaNAC2d* , their transcript levels in*CaNAC2c* silencing were similar to that in *CaNAC2c* and*CaNAC2d* simultaneously silenced pepper plants (Figure 7F). By contrast, transcript levels of *CaDEF1* were not downregulated by silencing of either *CaNAC2c* or *CaNAC2d* , but was significantly decreased by simultaneous silencing of *CaNAC2c* and*CaNAC2d,* indicating that *CaNAC2c* and *CaNAC2d*function redundancy in pepper immunity against RSI mediated by JA signaling.

Discussion

To maximize the fitness, Plant response to these stresses and growth should be appropriately coordinated, but the related players and underlying mechanisms remains poorly understood. In the present study, we provided evidence that CaNAC2c act as crucial regulator in coordinating pepper growth and response to HTS and RSI.

Our data demonstrated that upon HTS or RSI, *CaNAC2c* is transcriptionally upregulated, leading to enhanced basal and acquired thermotolerance or resistance to RSI, but repression of growth and development, indicating role of CaNAC2c as negative regulator in pepper growth but positive regulator in pepper thermotolerance and defense response to RSI. Noticeably, *CaNAC2c* silencing did not produced any effect on pepper resistance to RSI, and its simultaneous silencing with *CaNAC2d* significantly decreased resistance

to RSI, indicating that *CaNAC2c* acts redundantly with *CaNAC2d* in pepper immunity. Similar functional redundancy has been extensively found in immunity associated genes such as basic Helix-loop-Helix (bHLH) type transcription factor(Xu et al., 2014). It can be speculated that functional redundancy in NAC2 subfamily might enable more robust transcriptional reprogramming upon pathogen recognition. The difference that CaNAC2c acts specifically in thermotolerance but in redundancy with other genes in response to pathogen attack might be due to the different nature of selection pressure derived from HTS and pathogen attack. During arm race between plants and pathogens, plant immunity has been frequently overcome by the newly occurred effectors in the pathogens via targeting specific immune components including transcription factors(Canonne et al., 2011). Thus this redundancy might be an important mechanism for the robust of immunity, removal or repression of a part of the redundant components can be functionally compensated for by others.

In addition, our data indicated that CaNAC2c acts as a regulator in the trade-off between pepper growth and thermotolerance or immunity. Growth-defense trade-offs are thought to occur in plants to maximize their fitness upon different stresses when resources are limited(Berens et al., 2019; Huot et al., 2014). The transcriptional levels of *CaNAC2c* is lower in the absence of stress in pepper plants, and is upregulated by RSI or HTS, indicating that the transcriptional levels *CaNAC2c* are crucial for derepression of pepper growth in absence of stress and enhanced defense response upon stress. The transcriptional modification of *CaNAC2c* was found to be determined by signaling mediated by JA, ABA, IAA or GA3, and the data from 5' series deletion assay further related the transcriptional modification of *CaNAC2c*by exogenous applied JA, ABA, IAA or GA3 to specific regions of *CaNAC2c* promoter (Figure S3), indicating that ABA and JA are involved in pepper response to HTS and to RSI, respectively. Similar observations have been found previously that ABA is involved in plant response to heat stress(Y. C. Huang et al., 2016), and JA signaling contribute to plant immunity towards necrotrophic pathogens(Campos, Kang, & Howe, 2014), and signaling mediated by auxin or gibberellins (GA3) is involved in the regulation of growth(Denance, Sanchez-Vallet, Goffner, & Molina, 2013). So, it can be speculated that tradeoff between growth and immunity or thermotolerance mediated by CaNAC2c is mainly regulated at transcriptional level through crosstalk between signaling mediated by ABA, JA, IAA or GA3.

Furthermore, our data showed that CaNAC2c acts as a positive regulator in thermotolerance and immunity against RSI. This result is similar to that of our previous studies that *CaWRKY6* (Cai et al., 2015),*CaWRKY40* (F. F. Dang et al., 2013) and *CabZIP63* (Shen et al., 2016) act positively in pepper response to both RSI and HTS, supporting the notion that pepper response to RSI is closely linked to its response to HTS. Although, *CaNAC2c* is upregulated by both HTS and RSI, its targeting and transcriptional regulation might be different. For instance, *CaHSFA5* is directly targeted and regulated by CaNAC2c upon HTS, but not upon RSI (Figure 5, 6 and S7). Similarly, thermotolerance related *CaHSP24* , *CaHSP70* and *CaHSF2A* were upregulated by *CaNAC2c* upon HTS, but not upon RSI (Figure 6F and G). By contrast, immunity related *DEF1* was upregulated by *CaNAC2c* upon RSI but not upon HTS (Figure 6K). Importantly, immunity positively related(Alvarez et al., 1998; Yoshioka et al., 2003) and thermotolerance negatively related H_2O_2 accumulation(Yu et al., 2019; Zhuang et al., 2020) (Figure 1 and S6) was decreased by CaNAC2c upon HTS but enhanced upon RSI (Figure 3E-F). All these data indicate a trade-off between thermotolerance and immunity against RSI mediated by CaNAC2c, and that the targeting of CaNAC2c and its function are modified in a context dependent manner. It can be speculated that unlike that trade-offs between growth and disease resistance or thermotolerance mediated by CaNAC2c is determined by its transcription, tradeoff between thermotolerance and immunity against RSI mediated by CaNAC2c might be mainly determined by its post-translational regulation, probably in protein-protein interaction manner(Alves et al., 2014; Chi et al., 2013). As upregulation of *CaNAC2c* upon RSI and HTS is regulated by JA- and ABA-signaling, respectively, potential immune reaction during HTS response or thermotolerance during RSI response might be blocked by the antagonism between ABA- and JA-signaling(Anderson et al., 2004; S. Liu, Ziegler, Zeier, Birkenbihl, & Somssich, 2017; Nahar, Kyndt, Nzogela, & Gheysen, 2012). HTS and to RSI are most frequently occurred stresses in subtropical or tropical climates where pepper originally distributed, the post-translational regulation might benefit rapid and precise defense response to a specific stress, in

particular, from one stress to another.

Collectively, our data indicate that CaNAC2c acts positively in thermotolerance and in immunity against RSI while negatively in pepper growth, CaNAC2c functions differentially in a context dependent manner, tradeoffs between growth and thermotolerance or immunity mediated by CaNAC2c is determined by its transcription, while tradeoffs between thermotolerance and immunity mediated by CaNAC2c is conferred by its post-translational regulation.

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Author contributions

S.L.H. ,R.H.H. ,D.Y.G. and W.W.C. conceived the research and designed the experiments. W.W.C., S.Y., R.J.W., J.S.C. and Z.W. performed the experiments. W.W.C., S.Y., and L.S. analyzed the data. S.L.H.

wrote the manuscript.

Data Availability Statement

All data, models, and code generated or used during the study appear in the submitted article.

Figures

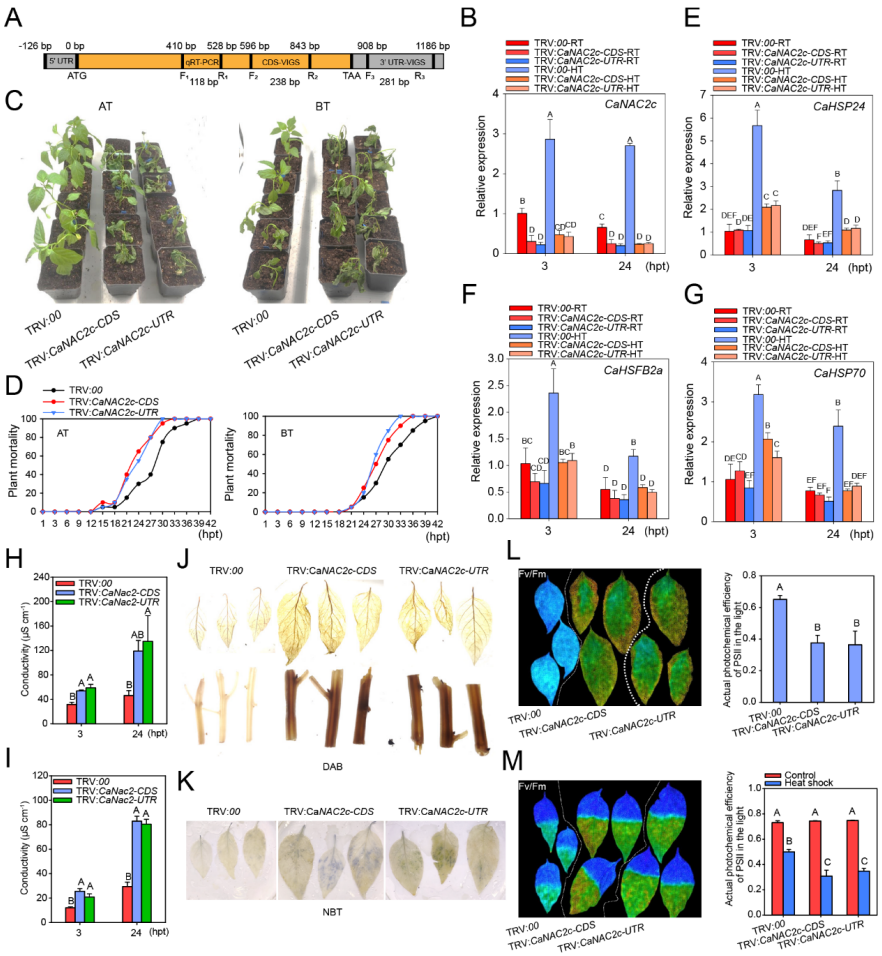

Figure.1 The effect of *CaNAC2c* silencing in pepper plants on thermotolerance. A. Schematic diagram of the fragments used for VIGS vectors construction, a specific fragment within the CDS (TRV:*CaNAC2c-CDS*) and a fragment within 3'UTR(TRV:*CaNAC2c-3'UTR*) were used for vectors construction. B. The success of *CaNAC2c* silencing by VIGS by measuring the transcript level of *CaNAC2c* in HTS challenged TRV:*CaNAC2c-CDS* and TRV:*CaNAC2c-3'UTR* pepper plants at 3 and 24 hpt.C.*CaNAC2c*silenced pepper plants displayed significantly decreased basal(BT) and acquired (AT) thermotolerance(pretreated with 37 for 12 h and recovered for 12 h).D. Dynamic plant mortality under HTS from 1 to 42 hpt. E-G. The transcript level of thermotolerance related marker genes in*CaNAC2c* silenced pepper plants challenged with HTS at 3 and 24 hpt. H. ion leakage displayed by conductivity in CaNAC2c silenced pepper plants pretreated with nonlethal high temperature (37 for 12 h, and recovered for 12 h). I. ion leakage displayed by conductivity in CaNAC2c silenced pepper plants without pretreatment with nonlethal high temperature stress. J. H2O2 accumulation displayed by DAB staining of different organs in CaNAC2c silenced pepper plants challenged with HTS. K. ROS accumulation displayed by NBT staining of leaves in CaNAC2c silenced pepper plants challenged with HTS. L. Fv/Fm and $^{F}/Fm'inleavesofCaNAC2csilencedpepperplantschallengedwithHTS.M.Fv/Fmand^{F}/Fm$

Figure.2 CaNAC2c silencing promoted the growth of pepper plants. A. The dynamic leaf number of *CaNAC2c* silenced pepper. B. the size of leaves, stem and root of *CaNAC2c* silenced and the control pepper plants. C. The dynamic flower number of *CaNAC2c*silenced and the control pepper plants. D. The height of *CaNAC2c*silenced and the control pepper plants. E. The root length of*CaNAC2c* silenced and the control pepper plants. F. The phenotype of *CaNAC2c* silenced and the control pepper plants 35 days after sowing. In A, C, D and E, data presented are means ± standard error (SE) of twelve replicates. In E different letters above the bars indicated significant differences among means ($P < 0.01$), as calculated with Fisher's protected LSD test.

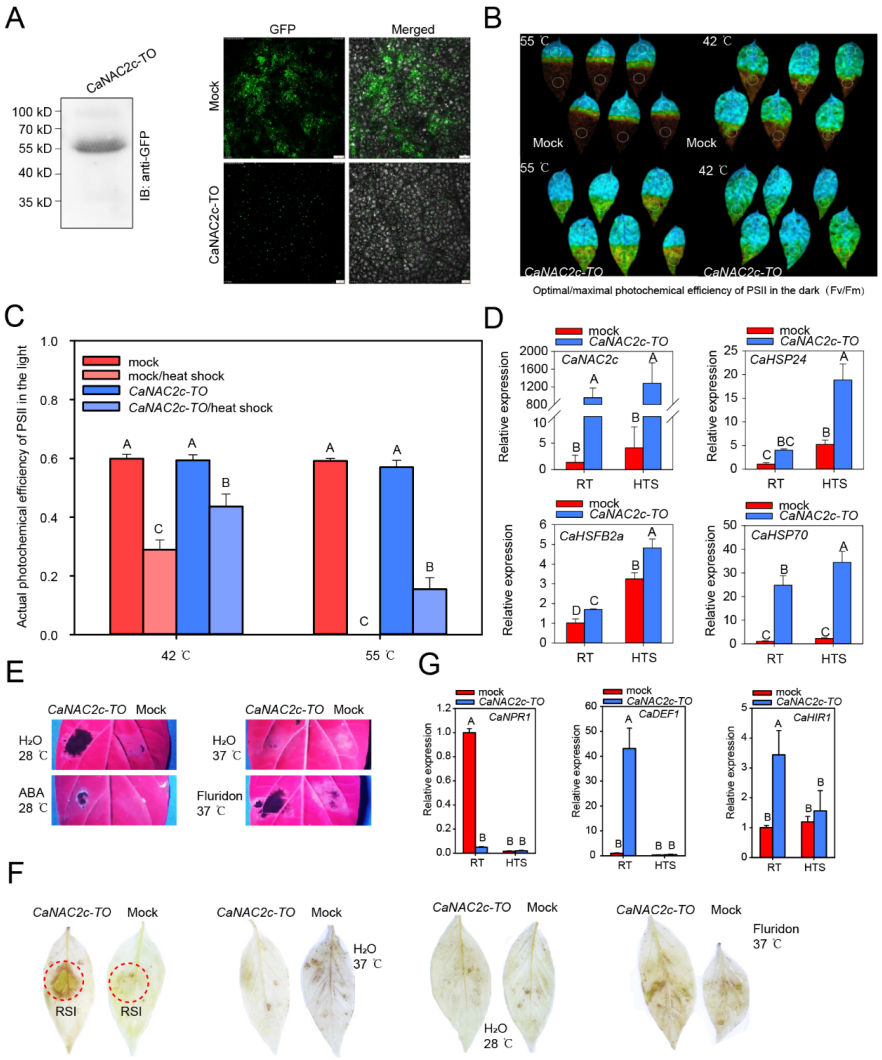

Figure.3 The effect of *CaNAC2c* transient overexpression on thermotolerance and triggered HR mimic cell death antagonized by ABA in pepper leaves. A. the success of *CaNAC2c* transient overexpression in pepper leaves displayed by western blotting using antibodies against GFP and GFP signal observed under fluorescent confocal microscope at 48 hpi. B. the *CaNAC2c* transiently overexpressed pepper leaves exhibited an enhanced Fv/Fm compared to that in the control leaves upon HTS.C. the *CaNAC2c* transiently overexpressed pepper leaves exhibited an enhanced $^{F}/Fm'comparedtothatinthecontrolleavesuponHTS.D.thermotole$

Figure.4 *CaNAC2c* overexpressing *N. benthamiana*plants exhibited enhanced resistance to HTS and RSI. A. The transcript level of*CaNAC2c* in plants of two T2 transgenic *N. benthamiana*lines. B. CaNAC2c overexpressing *N. benthamiana* plants displayed significantly increased basal (BT) and acquired thermotolerance (AT) (pretreated with 37 for 12 h and then recovered 12 h). C. The plant mortality of the two *CaNAC2c* overexpressing *N. benthamiana*lines, *CaNAC2c-OE1* and *CaNAC2c-OE2* under challenge of HTS with or without pretreatment of nonlethal high temperature stress. D. Thermotolerance related *NtAPX* , *NtHSP18* and *NtsHSP*were transcriptionally upregulatd by overexpression of *CaNAC2c* in*N. benthamiana* plants challenged by HTS. E. The resistance of*CaNAC2c* overexpressing *N. benthamiana* plants to RSI was significantly higher than the control plants. F. *CaNAC2c*overexpressing *N. benthamiana* plants exhibited lower dynamic disease index than the control plants. G. The *R.solanacearum*inoculated *CaNAC2c* overexpressing *N. benthamiana* plants supported lower population of *R.solanacearum* compared to the control plants. In A, D, F, and G data presented are means ± standard error (SE) of twelve and three replicates, respectively. In A, D and G different uppercase letters above the bars indicate significant differences among means ($P < 0.01$), as calculated with Fisher's protected LSD test.

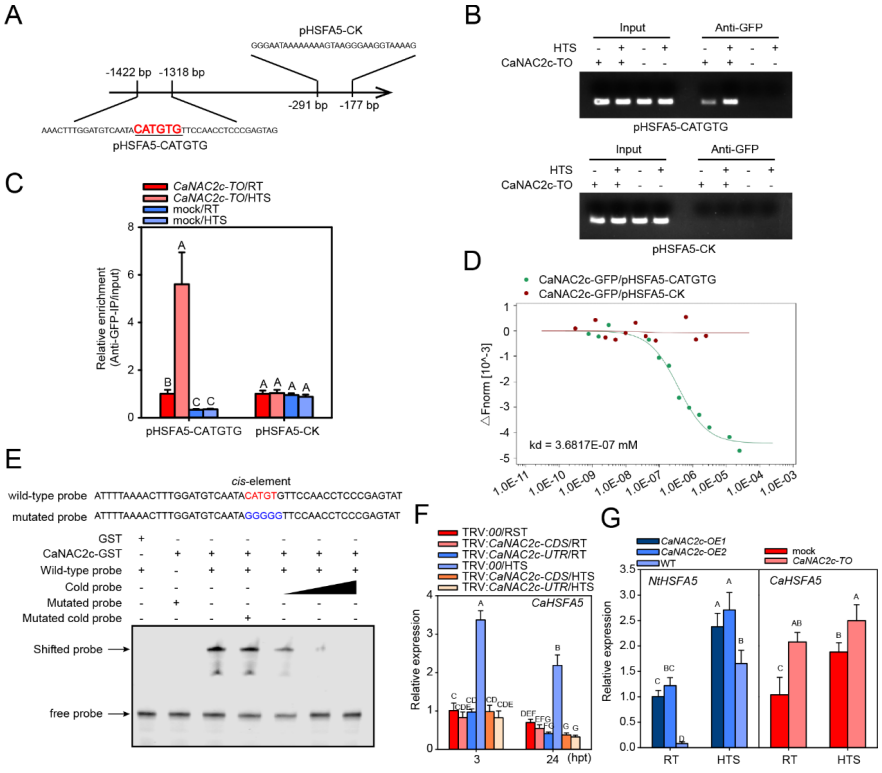

Figure.5 CaHSFA5 was directly targeted and transcriptionally regulated by CaNAC2c under HTS. A. The CATGTG containing fragment and a CATGTG free fragment within the *CaHSFA5* promoter used for primer pairs design for ChIP-PCR to assay the direct targeting of*CaHSFA5* by CaNAC2c. B. ChIP-PCR showed that *CaHSFA5* was directly targeted by CaNAC2c. Chromatins were isolated from*CaHSFA5-GFP* transiently overexpressed pepper leaves, which was sheared into fragment of 300-500 bps in length. The DNA was immunoprecipited with antibodies of GFP, the acquired DNA was used as template with specific primer pair of CATGTG containing promoter region. C. ChIP-qPCR showed that enhanced ability of *CaNAC2c* to bind to*CaHSFA5* promoter under HTS. D. the binding of promoter fragment of *CaHSFA5* by CaNAC2c was confirmed by MST using CaNAC2c-GFP fusion protein transiently overexpressed in pepper leaves and immunoprecipited by antibody of GFP and a CATGTG containing promoter fragment. E. the binding of promoter fragment of *CaHSFA5* by CaNAC2c was confirmed by EMSA using prokaryotically

expressed CaNAC2c-GST and promoter fragment of CaHSFA5 containing CATGTG or its mutant version (GGGGGG). F. *CaHSFA5* was downregulated by *CaNAC2c* silencing under HTS.G. *CaHSFA5* was upregulated by *CaNAC2c* overexpression in *N. benthamiana* plants or by *CaNAC2c* transient overexpression in pepper leaves. In C, F and G, data presented are means ± standard error (SE) of four replicates, different uppercase letters above the bars indicated significant differences among means ($P < 0.01$), as calculated with Fisher's protected LSD test.

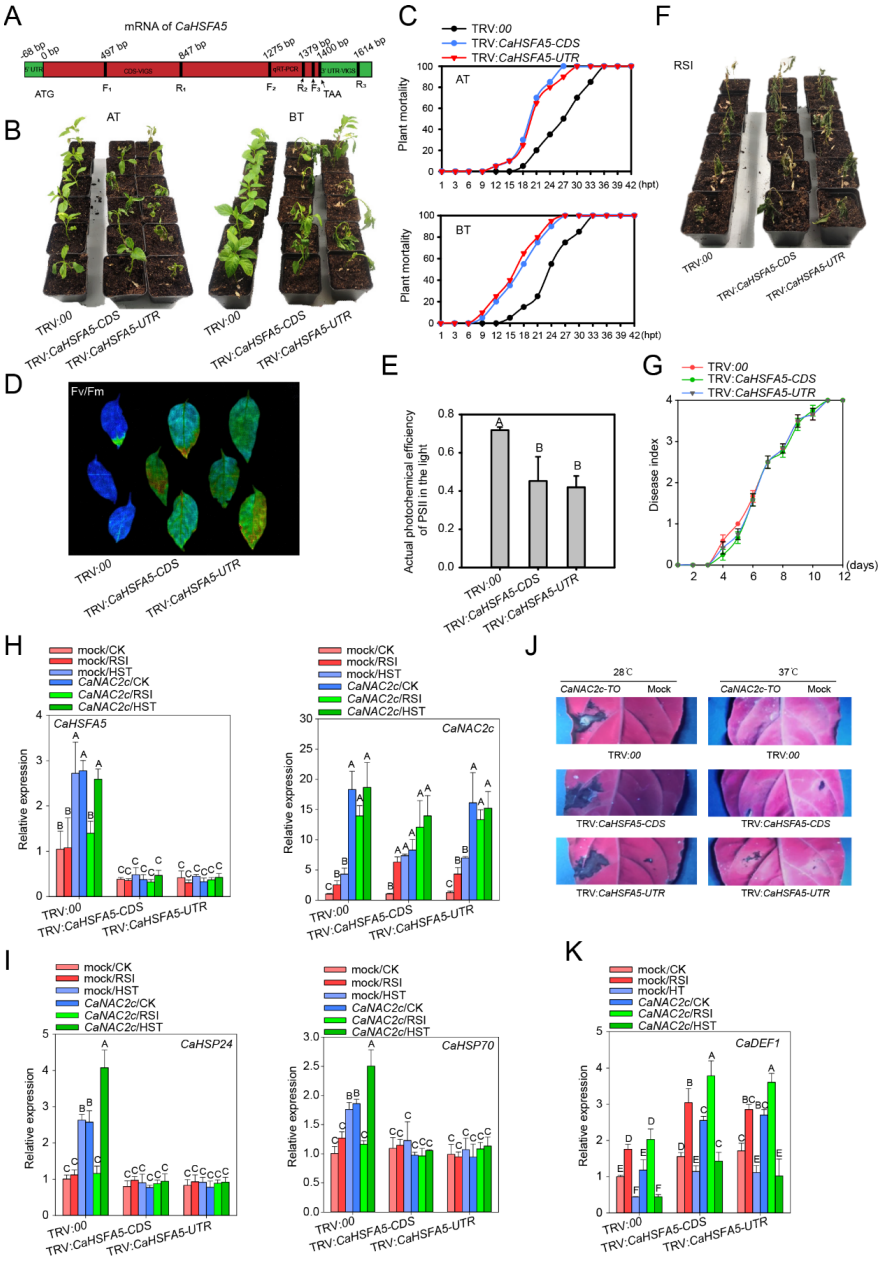

Figure.6 The silencing of *CaHSFA5* decreased thermotolerance of pepper plants but did not significantly affect the resistance of pepper plants to RSI. A. Schematic diagram of the fragments in the CDS or 3'UTR of *CaHSFA5* used for VIGS vectors (TRV:*CaHSFA5-CDS* and TRV:*CaHSFA5-3'UTR*) construction. B. The silencing of *CaHSFA5* decreased basal and acquired thermotolerance of pepper plants.

C. The silencing of *CaHSFA5*decreased basal and acquired thermotolerance of pepper plants. D. The silencing of *CaHSFA5* decreased Fv/Fm of pepper leaves upon HTS.E. The silencing of *CaHSFA5* decreased $^{F}/Fmofpepperleavesupon HTS.F.Thesilencingof CaHSFA5didnotaffecttheresistanceofpepperplantstoRSI.G.Thesilenci$

Figure.7 Simultaneous silencing of *CaNAC2c* and *CaNAC2d* did not produce additive decrease in thermotolerance but significantly decreased the resistance of pepper plants to RSI. A. Simultaneous silencing of *CaNAC2c* and *CaNAC2d* did not produced additive decrease in thermotolerance of pepper plants. B. Simultaneous silencing of *CaNAC2c* and *CaNAC2d* did not produced additive decrease in resistance of pepper plants to RSI at 96 and 192 hpi. C. Simultaneous silencing of *CaNAC2c* and *CaNAC2d* did not produced additive decrease in Fv/Fm in pepper plants. D. Simultaneous silencing of *CaNAC2c* and *CaNAC2d* did not produced additive decrease in $^{F}/Fmofpepperleavesupon HTSinpepperplants.E.Simultaneoussilencingof CaNAC2c$

Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article:

Supporting information for Figures

Figure. S1. The transcriptional response of members of pepper NAC family and CaNAC2c to HTS or RSI.

Figure. S2. Deduced amino acid sequences of pepper NAC2 proteins and their phylogenic relationship.

Figure. S3. The promoter activity of *pCaNAC2c* and its series 5' deletions to exogenous application of MeJA, ABA, IAA and GA3.

Figure. S4. Nuclear localization of the CaNAC2c in *N. benthamiana* epidermal cells.

Figure.S5 . The CaNAC2c overexpressing *N. benthamiana*plants inhibited growth and retarded development compared to the wild type control plants.

Figure. S6. The *CaNAC2c* overexpressing *N. benthamiana* plants exhibited enhanced Fv/Fm, $^{F}/Fmanddecreasedionleaka$

Figure. S7. *CaHSFA5* acts as negative regulator in pepper growth.

Figure. S8. The silencing of *CaHSFA5* significantly promoted the growth of *CaNAC2c* overexpressing *N. benthamiana* plants.

Figure. S9. The silencing of *NbHSFA5* significantly decreased thermotolerance but not affect the resistance of*CaNAC2c* overexpressing *N. benthamiana* plants to RSI.

Figure. S10. The silencing of *NtHSFA5* did not affect transcription of *NbDEF1* but significantly down-regulated*NtsHSP* , *NtAPX* in CaNAC2c overexpressing *N. benthamiana* plants.

Supporting information for Tables

Table S1. Primers used in this study.

Table S2. Primer pairs used in assay on promoter activity of*pCaNAC2c* and its 5'series deletions.

Table S3. Sequence similarity of *CaNAC2c* induced amino acid to its orthologs from other plant species.

Table S4. Disease index for *Ralstonia solanacearum*infected pepper plants.

Table S5. Results of *CaNAC2c* co-expression analysis under HTS in pepperhub.