doi:10.1093/plphys/kiab190 PLANT PHYSIOLOGY 2021: 186: 2169–2189

Plant Physiology®

Research Article

Pepper NAC-type transcription factor NAC2c balances the trade-off between growth and defense responses

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The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (https://academic.oup.com/plphys/pages/general-instructions) is: Shuilin He (shlhe201304@aliyun.com).

Abstract

Plant responses to pathogen attacks and high-temperature stress (HTS) are distinct in nature but generally share several signaling components. How plants produce specific responses through these common signaling intermediates remains elusive. With the help of reverse-genetics approaches, we describe here the mechanism underlying trade-offs in pepper (*Capsicum annuum*) between growth, immunity, and thermotolerance. The NAC-type transcription factor CaNAC2c was induced by HTS and *Ralstonia solanacearum* infection (RSI). CaNAC2c-inhibited pepper growth, promoted immunity against RSI by activating jasmonate-mediated immunity and H2O2 accumulation, and promoted HTS responses by activating Heat shock factor A5 (CaHSFA5) transcription and blocking H2O2 accumulation. We show that CaNAC2c physically interacts with CaHSP70 and CaNAC029 in a context-specific manner. Upon HTS, CaNAC2c–CaHSP70 interaction in the nucleus protected CaNAC2c from degradation and resulted in the activation of thermotolerance by increasing CaNAC2c binding and transcriptional activation of its target promoters. CaNAC2c did not induce immunity-related genes under HTS, likely due to the degradation of CaNAC029 by the 26S proteasome. Upon RSI, CaNAC2c interacted with CaNAC029 in the nucleus and activated jasmonate-mediated immunity but was prevented from activating thermotolerance-related genes. In non-stressed plants, CaNAC2c was tethered outside the nucleus by interaction with CaHSP70, and thus was unable to activate either immunity or thermotolerance. Our results indicate that pepper growth, immunity, and thermotolerance are coordinately and tightly regulated by CaNAC2c via its inducible expression and differential interaction with CaHSP70 and CaNAC029.

Introduction

In their natural habitats, plants are continuously exposed to biotic and abiotic stresses, either individually or in combination. Plants, therefore, need to appropriately cope with these challenges to maximize fitness by prioritizing the allocation of limited recourses between growth and response to stress. The resulting trade-off is thought to be regulated

Received October 29, 2020. Accepted April 10, 2021. Advance access publication April 27, 2021

by crosstalk between signaling pathways (Fujita et al., 2006; Sharma et al., 2013; Nejat and Mantri, 2017) and is likely to be modulated according to dynamic changes in the severity of different stresses (Lozano-Duran et al., 2013). However, the nature of the involved factors and their possible modes of action remain elusive.

High-temperature stress (HTS) and pathogen attack are frequently encountered by plants growing in tropical or subtropical climates and lead to severe retardation in growth and development, sometimes even death. Under the constant selective pressure of these stresses, plants have evolved sophisticated defense systems. Upon pathogen infection, conserved, and ubiquitous receptors—generally termed pattern recognition receptors (PRRs)—at the plant plasma membrane and fast-evolving intracellular R proteins perceive pathogen-derived pathogen-associated molecular patterns (PAMPs) and effectors, thereby activating PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI), respectively (Jones and Dangl, 2006). Under these conditions, plant cells undergo a global reprogramming of their metabolism and adopt a defense mode rather than a growth mode. Plant immune responses generally include the production of reactive oxygen intermediates (ROIs) such as superoxide anion (O_2^-), nitric oxide (NO), and hydrogen peroxide (H_2O_2; Schreck and Baeuerle, 1991; Chandra et al., 1996; McDowell and Dangl, 2000) leading to a hypersensitive response (HR; Dangl, 1998; Delledonne et al., 2001). In addition, infected plants produce antimicrobial compounds (Jabs et al., 1997) and pathogenesis-related proteins (Fobert and Després, 2005; Breen et al., 2017) to fight off the infection.

Unlike immune responses initiated by pathogen infection mainly at the cell surface, plants perceive heat via sensors in various cellular locations such as plasma membrane-localized channels, a histone variant sensor in the nucleus, unfolded protein sensors in the endoplasmic reticulum (ER), and the cytosol, the red light photoreceptor phytochrome B (PhyB) and ER membrane-associated basic leucine zipper transcription factors (Mittler et al., 2012; Srivastava et al., 2014; Song et al., 2017). These thermosensors activate thermotolerance by inducing the production of Heat Shock Proteins (HSPs) that act as chaperones to help resolubilize protein aggregates after heat stress, as well as the biosynthesis of antioxidants or reactive oxygen species (ROS) scavengers that offer protection from oxidative damage (Kotak et al., 2007; Chen et al., 2016; Yu et al., 2019).

Although the two pathways involve distinct sensors, plant responses to pathogens and HTS share signaling components such as Ca^{2+} signaling, ROS, phytohormones such as jasmonic acid (JA) and salicylic acid (SA; Li et al., 2011; Liu et al., 2015a, 2015b) and Nucleotide Binding Site (NBS)-Leucine-Rich Repeat (LRR) proteins (Kim et al., 2015). This overlap hints at the potential for extensive trade-off between plant responses to pathogens and HTS. Indeed, these two processes have been suggested to be closely related (Lee et al., 2012): for example, plant immunity is generally dampened by HTS (Janda et al., 2019). Specific defense responses will thus require the selective activation of the appropriate shared components. However, how these crucial regulators achieve their specific regulation and maintain a balance between plant responses to HTS and pathogen attack remains largely unknown.

Given that plant defense responses against pathogen attacks or HTS generally include massive transcriptional reprogramming, transcription factors (TFs) are likely to act as crucial players in these processes (Hua, 2009; Moore et al., 2011; Fragkostefanakis et al., 2015; Liu et al., 2015a; Xue et al., 2015; Du et al., 2017; Birkenbihl et al., 2018). However, the precise roles of TFs in balancing plant responses to pathogen attacks and HTS and the underlying molecular details remain to be elucidated. The No Apical Meristem (NAM)/Arabidopsis Transcription Activation Factor (ATAF)/Cup-Shaped Cotyledon (CUC) NAC family of TFs constitute one of the largest plant TF families. They are characterized by a conserved N-terminal NAC domain and a diversified C-terminal transcription regulatory region and have been classified into eight subfamilies (Puranik et al., 2012). NAC TFs are implicated in the regulation of plant responses to stress conditions via binding a specific recognition site [CGT (G/A)] in the promoters of their target genes (Fang et al., 2016; Khedia et al., 2018). NAC TFs involved in stress responses belong to one subgroup (Fang et al., 2008; Nakashima et al., 2012; Negi et al., 2018). Although NAC proteins clearly participate in plant responses to heat stress or thermotolerance (Guan et al., 2014; Fang et al., 2015; Dong et al., 2020; Liu et al., 2020) and plant immunity (Perochon et al., 2019; Chang et al., 2020), to date, no individual NAC TF has been demonstrated to influence the balance between plant immunity and heat stress response.

A typical example of balance (or trade-off) between plant immunity and heat stress responses comes from pepper, a member of the Solanaceae family and a vegetable of great agricultural importance worldwide. Pepper originated from the tropical and subtropical regions of Central and South America and is widely grown during the warm seasons or in greenhouses, where it frequently suffers from bacterial wilt caused by *Ralstonia solanacearum* infections, a soil-borne pathogen that invades plants exclusively through their roots (Mansfield et al., 2012; Jiang et al., 2017). Pepper plants are also routinely exposed to HTS, causing severe growth retardation (Usman et al., 2014). We have shown previously that pepper responses to infection by *R. solanacearum* and HTS share a number of components, including *calcium-dependent protein kinase 15* (*CaCDPK15*; Shen et al., 2016), the WRKY TFs *CaWRKY6* (Cai et al., 2015), and *CaWRKY40* (Dang et al., 2013), as well as the TF *basic region/leucine zipper motif 63* (*CabZIP63*; Shen et al., 2016).

In the present study, we demonstrate that CaNAC2c, a member of the NAC family in pepper, not only participates in the regulation of pepper responses to RSI and HTS but also in the regulation of the trade-off between growth and responses to HTS or *R. solanacearum* infection (RSI). In addition, CaNAC2c acts as a crucial regulator coordinating

growth, immunity, and thermotolerance at both the transcriptional and post-transcriptional levels by differential and context-specific interactions with CaNAC029 and CaHSP70.

Results

Expression profiling of pepper *NAC* genes during HTS and RSI

It was previously shown that NAC TFs that play roles during plant response to heat stress or pathogen attack can be identified based on their transcriptional signature in response to these stresses (Nakashima et al., 2012). To identify candidate pepper *NAC* genes involved in coordinating responses to HTS and RSI, we explored the expression profiles of 90 *NAC* genes in pepper plants exposed to HTS and to RSI based on publicly available data from Pepper Hub (http://www.hnivr.org/; Liu et al., 2017) and our unpublished transcriptome deep-sequencing (RNA-seq) data. One *NAC* gene (Capana06g001739) was upregulated by both HTS and RSI (Supplemental Figure S1, A). We validated the observed upregulation of this gene by RT-qPCR analysis on root samples collected from pepper plants challenged with RSI or HTS (Supplemental Figure S1, B). This *NAC* and three closely related *NAC* genes encoded proteins with a conserved N-terminal NAC domain and a diversified C-terminal transcription regulatory (TR) region (Supplemental Figure S2, A). They shared the highest sequence identity with Arabidopsis (*Arabidopsis thaliana*) *NAC2* (Supplemental Figure S2, B and Supplemental Table S2). Accordingly, we named these genes *CaNAC2a* to *CaNAC2d*, *CaNAC2c* being induced by both HTS and RSI. *CaNAC2c* and *CaNAC2d* showed a sequence identity of 73%, the highest among these four *NAC* genes (Supplemental Figure S2, C and D). We explored the response of these four *NAC2*-like genes to HTS and RSI in our RNA-seq dataset of pepper plants challenged with RSI: *CaNAC2c* was strongly up-regulated by both HTS and RSI. Notably, *CaNAC2d* was only induced by RSI in roots (Supplemental Figure S2, E). NAC TFs can exhibit different subcellular localizations (Mathew et al., 2016). To determine where CaNAC2c resides in the cell, we overexpressed *35Spro:Yellow Fluorescent Protein* (*YFP*) or *35Spro:CaNAC2c-YFP* in *Nicotiana benthamiana* leaf epidermal cells. We detected YFP fluorescence exclusively in the nucleus for *CaNAC2c-YFP*, whereas free YFP was observed throughout the cell, including the cytosol and the nucleus, indicating that CaNAC2c is a nucleus-localized protein (Supplemental Figure S3).

CaNAC2c is a positive regulator of thermotolerance and *R. solanacearum* resistance but a negative regulator of plant growth

To determine the role of *CaNAC2c* in response to HTS or RSI, we first performed a loss-of-function assay by gene silencing using virus-induced gene silencing(VIGS), to avoid the possible off-targeting, we selected two specific fragments in the 3′-UTR and the open reading frame(ORF) of *CaNAC2c* for vector construction, the result showed that *CaNAC2c* transcript levels were greatly reduced by two distinct vectors targeting different portions of the *CaNAC2c* mRNA (Supplemental Figure S4, A and B). To detect the specificity of the *CaNAC2c* silencing, we tested the transcript levels of *CaNAC07*, *CaNAC08*, and *CaNAC59* that belong to different NAC subfamily in pepper genome, and found that the silencing of *CaNAC2c* did not reduce the transcript levels of the tested genes compared to the mock treatment(Supplemental Figure S5), indicating the specificity of *CaNAC2c* silencing. Upon exposure to HTS, *CaNAC2c*-silenced plants exhibited decreased basal and acquired thermotolerance, as evidenced by a pronounced wilting phenotype and high mortality rates (Figure 1, A and B). *CaNAC2c*-silenced plants also displayed increased ion leakage in response to HTS, as measured by conductivity, relative to control plants transformed with the empty pTRV vector (TRV:*00*, Supplemental Figure S4, C and D). In addition, DAB and NBT staining revealed much higher levels of H_2O_2 and ROS accumulation, respectively, in the leaves and stems of *CaNAC2c*-silenced plants compared to the control plants (Supplemental Figure S4, E and F). In agreement with these physiological responses, *CaNAC2c*-silenced plants were more affected by HTS than control plants, as seen by lower Fv/Fm and photosystem II (PSII) photochemical efficiency in the light (ΦPSII), indicator of thermotolerance and thermostability of the photosynthetic apparatus, respectively (Yan et al., 2008; Wang et al., 2014; Guan et al., 2018) immediately after HTS (Figure 1, C and D). In a complementary approach, we generated *N. benthamiana* lines overexpressing *CaNAC2c* (*CaNAC2c*-OE). We generated transgenic *N. benthamiana* plants of *35S:CaNAC2c*, a total of eight 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. In contrast to pepper plants silenced for *CaNAC2c*, these *N. benthamiana CaNAC2c*-OE plants were more tolerant to HTS compared to wild type (Supplemental Figure S6 and S7), indicating that CaNAC2c acts as a positive regulator in basal and acquired thermotolerance. To evaluate the role of *CaNAC2c* in the regulation of thermotolerance in different pepper germplasms, we silenced *CaNAC2c* comparatively in GZN13 (a heat-sensitive inbred line), HN42, and 79 (an inbred line with high level of thermotolerance), we found that these silencings all significantly reduced thermotolerance of the three pepper lines (Supplemental Figure S8).

To investigate the function of *CaNAC2c* in response to RSI, we inoculated TRV control plants and *CaNAC2c*-silenced pepper plants with *R. solanacearum*, but observed no differences (Data has been exposed to CNGB: https://db.cngb.org/search/?q=CNP0001104). By contrast, we noticed clear wilting symptoms in *N. benthamiana* plants challenged with RSI at 7 dpi (days post-inoculation), whereas *N. benthamiana CaNAC2c*-OE plants exhibited only slight wilting symptoms, as shown by the lower disease index values and lower *R. solanacearum* titer (Figure 1, E and Supplemental

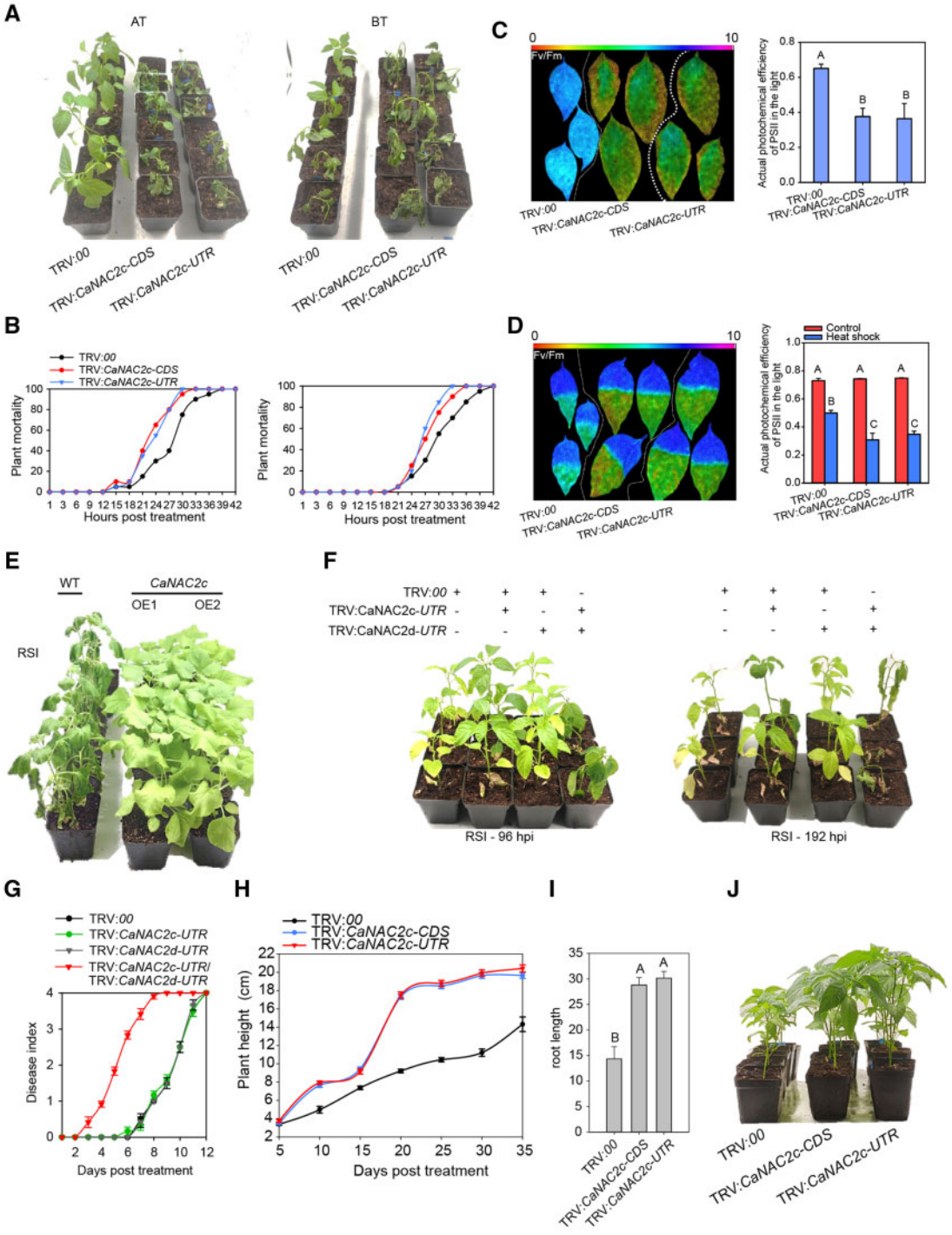

Figure 1 Phenotypes associated with *CaNAC2c* silencing in pepper plants and overexpression in *N. benthamiana* plants on thermotolerance and resistance to RSI (*R. solanacearum* infection). A, *CaNAC2c*-silenced pepper plants display reduced basal (BT) and acquired (AT) thermotolerance (pretreated with 37°C for 12 h and recovery for 12 h). B, Plant mortality (24 plants were calculated) exposed to HTS from 1 to 42 h post-treatment (hpt). C, Fv/Fm and △F/Fm′ in leaves of *CaNAC2c*-silenced pepper plants challenged with HTS. D, Fv/Fm and △F/Fm′ in leaves of *CaNAC2c*-silenced pepper plants challenged with HTS by dipping half of the leaf in water set to 42°C. E, Increased resistance of *N. benthamiana* plants overexpressing *CaNAC2c* to RSI relative to control plants. F and G, Simultaneous silencing of *CaNAC2c* and *CaNAC2d* produces an additive decrease in the resistance of pepper plants to RSI (24 plants were calculated for disease index). H, Height of *CaNAC2c*-silenced and control pepper plants. I, Root length of *CaNAC2c*-silenced and control pepper plants. J, Overall morphology of *CaNAC2c*-silenced and control pepper plants 35 d after sowing. In E, F, and J, the images were digitally extracted. In (C, D, G, H, and I), data presented are means ± standard error (SE) of four replicates, different capital letters indicate significant differences among means ($P < 0.01$), as calculated with Fisher's protected LSD test.

Figure S9). The silencing of *CaNAC2c* did not result in obvious symptoms upon RSI, while its overexpression in *N. benthamiana* plants did promote immunity against RSI, suggesting that CaNAC2c may function redundantly with other factors in response to RSI. As *CaNAC2c* was highly related to *CaNAC2d* and both genes were upregulation by RSI (Supplemental Figure S2), we hypothesized that they may act redundantly. To test this hypothesis, we silenced both *CaNAC2c* and *CaNAC2d* in pepper and measured the response of these plants to RSI and HTS. Although the silencing of *CaNAC2d* alone did not significantly affect thermotolerance or immunity against RSI, the combined silencing of *CaNAC2c* and *CaNAC2d* produced clear wilting symptoms as early as 3 dpi and reaching the highest disease index value at 8 dpi, while plants silenced for either gene individually behaved as control plants (Figure 1, F and G and Supplemental Figure S10). Collectively, these results indicate that CaNAC2c and CaNAC2d function redundantly in pepper immunity against RSI.

Notably, *CaNAC2c*-silenced plants grew larger than the control plants: *CaNAC2c*-silenced plants were taller, had longer roots, stems, and leaves, produced more leaves and flowers (Figure 1, H–J and Supplemental Figure S11). While lowering *CaNAC2c* expression improved plant fitness, raising *CaNAC2c* expression had the opposite effect, as seen in *N. benthamiana CaNAC2c*-OE plants and their smaller leaves, shorter roots and stems, fewer flowers, and leaves (Supplemental Figure S12), supporting the role of CaNAC2c as a negative regulator of plant growth.

CaNAC2c-mediated resistance to RSI is repressed upon HTS by ABA signaling

To better understand how *CaNAC2c* enhances thermotolerance and improves resistance to RSI, we measured relative transcript levels for a number of marker genes in *CaNAC2c*-silenced pepper plants, *N. benthamiana CaNAC2c*-OE plants upon HTS, and in the leaves of pepper plants transiently overexpressing *CaNAC2c* (*CaNAC2c*-TO). *CaNAC2c* silencing was accompanied by the significant down-regulation of the thermotolerance marker genes *Heat Shock Protein 24* (*CaHSP24*) and *CaHSP70*, as well as *Heat Shock Factor B2a* (*CaHSFB2a*; Ashraf et al., 2018; Supplemental Figure S13, A). In contrast, relative transcript levels of thermotolerance-related *N. benthamiana* genes *ASCORBATE PEROXIDASE* (*NbAPX*), *NbHSP18*, and *NbsHSP* rose in CaNAC2c-OE in *N. benthamiana* plants relative to control plants (Supplemental Figure S6, D), and their upregulation was amplified by HTS.

Furthermore, by exogenous application of ABA (or Furidon), SA, or JA, whose success was confirmed by transcript levels of *CaABR1* (Choi and Hwang, 2011), *CaPR1* (Kim and Hwang, 2014), and *CaCOI1* (Hu et al., 2013), respectively (Supplemental Figure S14), the association of *CaNAC2c* to signaling mediated by ABA, SA, or JA was assayed. We established that transcription of *CaNAC2c* was upregulated both by an exogenous application of abscisic acid (ABA) and by HTS, based on b-glucuronidase (GUS) activity from a *CaNAC2c pro:GUS* reporter construct. Notably, the higher transcription rate of *CaNAC2c* in response to HTS was blocked by treatment of fluridon, an inhibitor of ABA biosynthesis (Figure 2, A). These data suggest that the role of *CaNAC2c* in promoting thermotolerance is regulated by ABA signaling. In addition, *CaNAC2c* transcription was upregulated by exogenously applied methyl jasmonate (MeJA) but not by that of salicylic acid (SA; Figure 2, B). To test the possible regulation of ABA signaling in immunity and thermotolerance mediated by CaNAC2c, we detected the effect of exogenously applied ABA and fluridon on response *CaNAC2c* overexpressing *Nicotiana benthamiana* plants to RSI and to HTS, respectively, we found that exogenous application of ABA significantly increased the susceptivity of *CaNAC2c* overexpressing *Nicotiana benthamiana* plants to RSI (Figure 2, C), and the exogenous application of fluridon significantly reduced the tolerance of *Nicotiana benthamiana* plants to HTS (Figure 2, D), consistently, the exogenous application of ABA did not restore thermotolerance reduced by *CaNAC2c* silencing in pepper plants (Figure 2, E) indicating that ABA signaling acts positively in thermotolerance but negatively in immunity against RSI, and CaNAC2c might act downstream ABA signaling.

We also discovered that *CaNAC2c*-TO triggered HR-like cell death (Figure 2, F) at room temperature, resulting in the higher level of H_2O_2 accumulation upon RSI (Figure 2, G), which was also revealed by DAB staining, but this response was abolished by exogenous ABA treatment (Figure 2, F–H). In contrast, at 37°C, *CaNAC2c*-TO did not induce any cell death but reduced the accumulation of H_2O_2, although an exogenous application of fluridon restored cell death (Figure 2, F and G). These results suggest that ABA may prevent the activation of cell death induced by CaNAC2c at 37°C, and CaNAC2c-mediated resistance to RSI is repressed by ABA signaling at high temperature.

Turning to pepper plants transiently overexpressing *CaNAC2c*, JA signaling dependent gene *DEFENSIN 1* (*CaDEF1*; Choi et al., 2008; Germain et al., 2012; Choi et al., 2015; Kim et al., 2015; Zhang et al., 2018), *CaCol1* (Hu et al., 2013), two lipoxygenase genes *CaLOX14* (Sarde et al., 2018), *CaLOX31* (Sarde et al., 2019) with the most obvious response to *Ralstonia solanacearum* infection in pepper by data of our RNA-seq (Supplemental Figure S15) as well as by that of Dang et al. (2013), which encode enzyme crucial for JA biosynthesis, a hypersensitive response (HR)-related gene *hypersensitive-induced reaction 1* (*CaHIR1*; Jung et al., 2008) were all upregulated by RSI as well as by overexpression of *CaNAC2c* at room temperature, but these upregulations were blocked by the exogenous application of ABA (Figure 2, I), while *CaHSP24* that is related to thermotolerance, *CaAPX* (Wang et al., 2017) that is related to scavenger of ROS including H_2O_2, and ABA signaling related *CaABI5* (Lopez-Molina et al., 2002) were similarly upregulated by overexpression of *CaNAC2c* in the absence of *R. solanacaerum* at room temperature, and these upregulations were potentiated by exogenous application of ABA (Figure 2, I). The

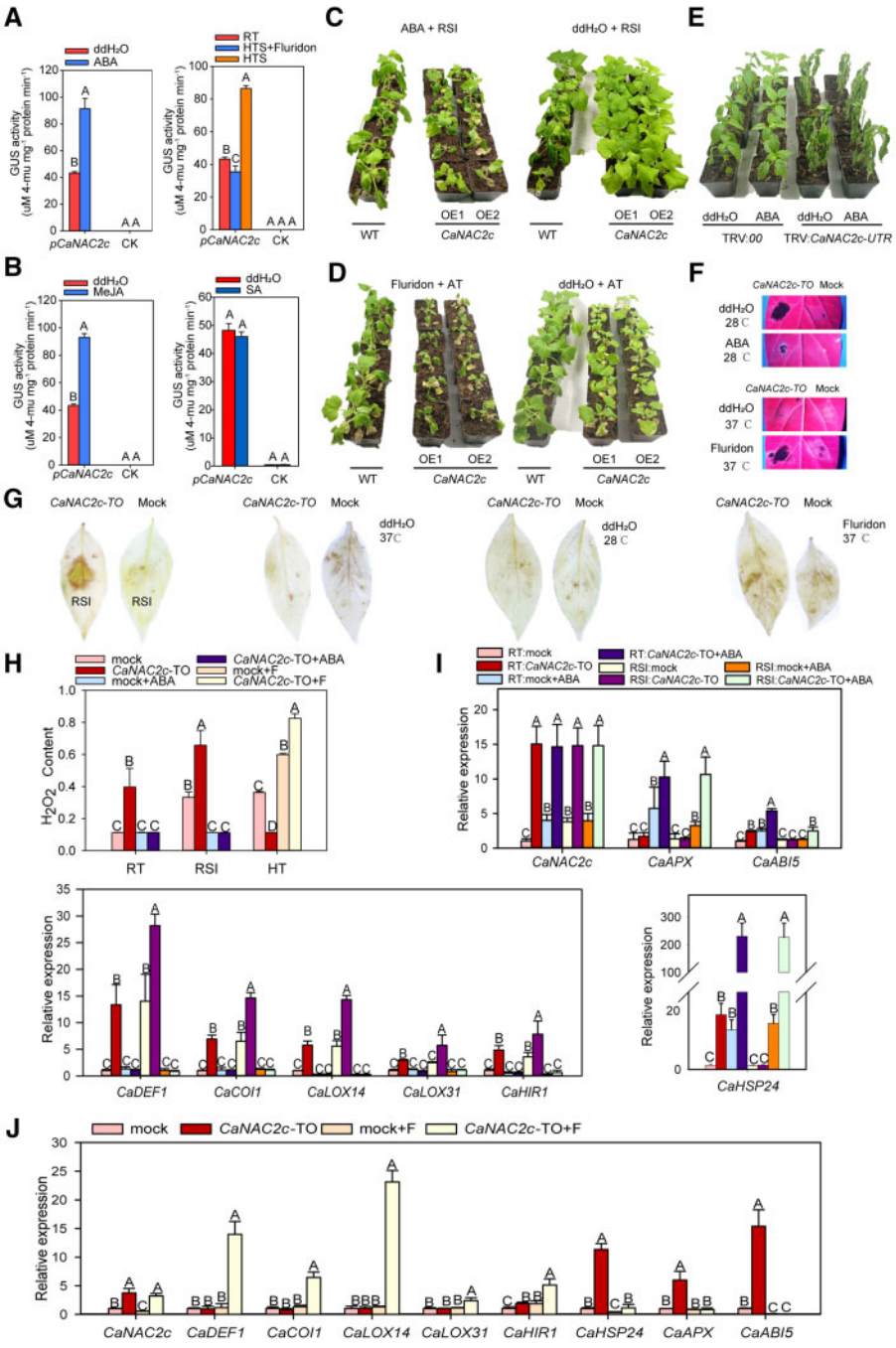

Figure 2 CaNAC2c Resistance to RSI is Repressed upon HTS. A and B, Activity of the *CaNAC2cpro:GUS* reporter to the application of SA (2 mM), ABA (30 μM), MeJA (10 μM), fluridon (10 μM) or HTS. Agrobacterium cells carrying the *CaNAC2cpro:GUS* reporter were infiltrated into pepper plants leaves, and GUS activity measured after HTS, fluridon, or phytohormones at 24 hpt (CK: pepper plants leaves infiltrated with GV3101 cells carrying *pMDC-163*). C, Effect of exogenous application of ABA on-resistance of *CaNAC2c* overexpressing *N. benthamiana* plants to *Ralstonia solanacearum* infection. D, Effect of the exogenously applied fluridon on acquired thermotolerance of *CaNAC2c* overexpressing *N. benthamiana* plants. E, Effect of exogenous application of ABA on thermotolerance of *CaNAC2c*-silenced pepper plants. F, HR-like cell death lesions at room temperature or under HTS triggered by transient overexpression of *CaNAC2c* or exogenous application of fluridon or ABA. G, H_2O_2 accumulation, displayed by DAB staining in *CaNAC2c* transiently overexpressing pepper leaves challenged with RSI, HTS, or with fluridon under HTS. H, H_2O_2 accumulation in *CaNAC2c* transiently overexpressing pepper leaves challenged with RSI, ABA, HTS, or with fluridon under HTS (F: fluridon). I, Transcript level of *CaDEF1*, *CaCOI1*, *CaLOX14*, *CaLOX31*, *CaHIR1*, *CaHSP24*, *CaAPX*, or *CaABI5* in *CaNAC2c* transiently overexpressing pepper leaves challenged with RSI, ABA, or with ABA under RSI. J, Transcript level of *CaDEF1*, *CaCOI1*, *CaLOX14*, *CaLOX31*, *CaHIR1*, *CaHSP24*, *CaAPX*, or *CaABI5* in *CaNAC2c* transiently overexpressing pepper leaves challenged with HTS or with fluridon under HTS (F: fluridon). In C–E, the images were digitally extracted. Data presented are means ± standard error (SE) of four replicates. In A, B, H, I, and J, different capital letters indicate significant differences among means ($P < 0.01$), as calculated with Fisher's protected LSD test.

upregulation of *CaLOX14* by overexpression of CaNAC2c upon RSI was much higher than that of *CaLOX31*, indicating that the biosynthesis of JA upon RSI is mainly determined by *CaLOX14*. Upon HTS plus exogenous fluridon, the overexpression of CaNAC2c failed to upregulate *CaHSP24*, *CaAPX*, or *CaABI5*, but upregulated all of the tested immunity-related marker genes (Figure 2, J). All these data suggest that ABA signaling positively regulates thermotolerance but negatively regulates immunity against RSI.

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 (Supplemental Figure S13, B). In contrast, transcript levels of *CaDEF1* were not downregulated by silencing of either *CaNAC2c* or *CaNAC2d*, but were 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.

CaHSFA5 is directly targeted by CaNAC2c upon HTS but not RSI

When we performed a co-expression analysis in Pepper Hub (http://www.hnivr.org/; Liu et al., 2017), we noticed that *CaHSFA5* was co-expressed with *CaNAC2c* under HTS (Supplemental Table S4). To test if *CaHSFA5* is a target of CaNAC2c, we determined whether CaNAC2c would bind to the *CaHSFA5* promoter by chromatin immunoprecipitation followed by PCR or qPCR (ChIP-PCR or ChIP-qPCR; Figure 3, A). Indeed, we amplified a fragment of the *CaHSFA5* promoter with a specific primer pair flanking a predicted NAC TF binding site, but not with a control primer pair directed at a region free of NAC TF binding site, indicating that CaNAC2c directly targets *CaHSFA5* (Figure 3, B and C). Moreover, HTS significantly enhanced the binding of CaNAC2c to the *CaHSFA5* promoter fragment (Figure 3, B and C). We validated the binding of CaNAC2c to its putative binding site in the *CaHSFA5* promoter *in vitro* by microscale thermophoresis (MST) assay (Figure 3, D). Likewise, recombinant CaNAC2c-GST was able to bind the *CaHSFA5* promoter in electrophoretic mobility shift assays (EMSA), via the *cis*-element CATGTG, as mutating it to GGGGGG prevented binding (Figure 3, E). As expected from their co-expression, *CaHSFA5* transcript levels decreased when *CaNAC2c* was silenced in pepper plants challenged with HTS, and was upregulated by the transient overexpression of *CaNAC2c*. Similarly, *NbHSFA5*, the presumptive *CaHSFA5* ortholog in *N. benthamiana*, was up-regulated in *N. benthamiana CaNAC2c*-OE plants (Figure 3, F and G). These results indicate that *CaHSFA5* is directly and positively regulated by CaNAC2c upon HTS.

To assay the role of CaHSFA5 in pepper thermotolerance and immunity against RSI, we generated *CaHSFA5*-silenced pepper plants via VIGS (Figure 4, A). Similar to CaNAC2c, CaHSFA5 acted as a negative regulator of plant growth, as *CaHSFA5*-silenced plants had larger leaves, as well as longer stems and roots (Supplemental Figure S16). In addition, *CaHSFA5*-silenced plants were more sensitive to heat stress compared to control plants, with or without pre-treatment with a nonlethal HTS, as evidenced by the higher mortality rates and lower Fv/Fm and $\triangle F/Fm'$ (Figure 4, B–E). Upon monitoring the expression of marker genes, we observed that silencing of *CaHSFA5* blocked the induction of *CaHSP24* and *CaHSP70* expression by HTS but did not affect *CaDEF1*, indicating that CaHSFA5 acts as a positive regulator specifically in thermotolerance (Figure 4, I and K). As might be expected from the normal transcript levels of *CaDEF1*, the silencing of *CaHSFA5* did not change the resistance of pepper plants to RSI (Figure 4, F and G).

To further characterize the role of CaHSFA5 in relation to CaNAC2c, we targeted *NbHSFA5*, the *CaHSFA5* ortholog in *N. benthamiana* by VIGS. The silencing of *NbHSFA5* in *N. benthamiana* largely rescue the growth retardation (Supplemental Figure S17) displayed by *N. benthamiana* plants overexpressing *CaNAC2c*, but at the same time lowered their thermotolerance (Supplemental Figure S18, B and D). However, resistance to *R. solanacearum* infection was not affected (Supplemental Figure S18, E and F).

CaNAC2c interacts with CaHSP70 under HTS but interacts with CaNAC029 upon RSI

The distinct behavior of CaNAC2c upon HTS or RSI suggested that its function might be modulated by additional regulatory proteins in a context-dependent manner. To isolate these possible interacting partners, we performed a GST pull-down followed by mass spectrometry identification. Accordingly, we incubated recombinant CaNAC2c-GST produced in *E. coli* with protein extracts from pepper plants challenged with HTS or RSI. All pulled-down proteins were identified by mass spectrometry, revealing a number of proteins that potentially interacted with CaNAC2c under both conditions (Supplemental Table S5). After preliminary screening by Bimolecular Fluorescent Complimentary (BiFC) assay, we selected an HSP70-type protein (XP_016569463.1) which might interact with CaNAC2c specifically under HTS and the NAC-type TF CaNAC029 (XP_016569463.1) which interact with CaNAC2c specifically upon RSI from these potential interacting partners for further analysis. As CaNAC2c regulates thermotolerance in ABA-signaling but mediate pepper immunity against RSI in JA-signaling dependent manner, we tested the response of *CaNAC029* and *CaHSP70* to the exogenous application of MeJA, SA, or ABA, the result showed that *CaNAC029* responded specifically to exogenously applied MeJA, while *CaHSP70* responded specifically to exogenous application of ABA(Supplemental Figure S19), indicating that the interactions of CaNAC2c with these two partners might play specific roles in biological processes mediated by JA and ABA signaling, respectively.

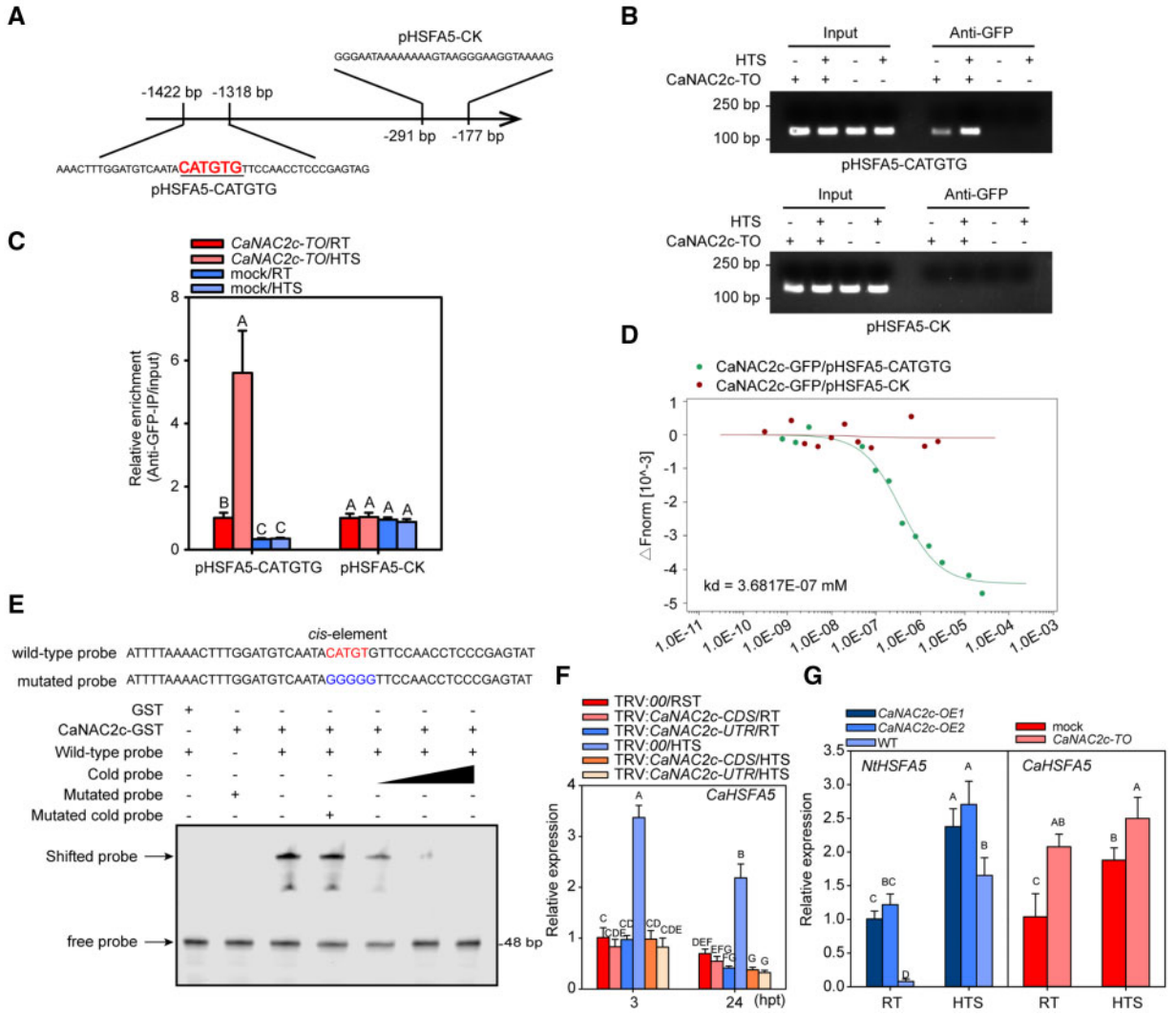

Figure 3 CaHSFA5 Is a Direct Transcriptional Target of CaNAC2c upon HTS. A, Schematics of the *CaHSFA5* promoter, with the CATGTG-containing fragment and a CATGTG-free fragment highlighted for ChIP-PCR of *CaHSFA5* by CaNAC2c. B, CaNAC2c directly targets the *CaHSFA5* promoter, as shown by ChIP-PCR. Chromatin was isolated from pepper leaves transiently overexpressing *CaNAC2c-GFP*, sheared into 300–500 bp fragments. The DNA was immunoprecipitated with antibodies against GFP, and the purified DNA was used as a template with specific primer pair of CATGTG-containing promoter region. C, CaNAC2c shows enhanced binding to the *CaHSFA5* promoter under HTS, as seen by ChIP-qPCR. D, MST analysis of CaNAC2c binding to the *CaHSFA5* promoter, using CaNAC2c-GFP fusion protein transiently overexpressed in pepper leaves and immunoprecipitated with anti-GFP antibody and a CATGTG-containing *CaHSFA5* promoter fragment. E, EMSA analysis of CaNAC2x binding to the *CaHSFA5* promoter, with recombinant CaNAC2c-GST and a *CaHSFA5* promoter fragment containing CATGTG or its mutant version (GGGGGG). F, *CaHSFA5* is downregulated by silencing *CaNAC2c* under HTS. G, *CaHSFA5* is upregulated in *N. benthamiana* plants overexpressing *CaNAC2c* or by the transient overexpression of *CaNAC2c* in pepper leaves. In C, F, and G, data presented are means ± standard error (SE) of four replicates, different capital letters indicate significant differences among means ($P < 0.01$), as calculated with Fisher's protected LSD test.

CaHSP70 interacts with CaNAC2c in the nucleus upon HTS to enhance CaNAC2c-mediated thermotolerance but represses immunity to RSI

We established by BiFC assay that CaHSP70 interacts with CaNAC2c in the cytoplasm at room temperature but in the nucleus when exposed to HTS (Figure 5, A). To validate this result, we performed co-immunoprecipitation (co-IP) assays using CaHSP70-HA immunoprecipitated from pepper leaves transiently co-overexpressing *CaNAC2c-Myc* and *CaHSP70-HA* with an anti-HA antibody. We tested for the presence of CaNAC2c in the immunoprecipitated by immunoblot analysis with an anti-MYC antibody. CaNAC2c and CaHSP70-HA interacted under both room temperature and high temperature (Figure 5, B). *CaHSP70* expression was upregulated by HTS but not by RSI (Figure 5, C). This result suggested that CaHSP70 may be involved in CaNAC2c-mediated thermotolerance. As mentioned earlier, CaHSFA5 is one of the downstream targets of CaNAC2c for mounting tolerance to high-temperature exposure. We, therefore, examined the effects associated with the overexpression of *CaHSP70* on CaNAC2c-mediated transcriptional activation of *CaHSFA5* by ChIP-qPCR. We observed that the enrichment

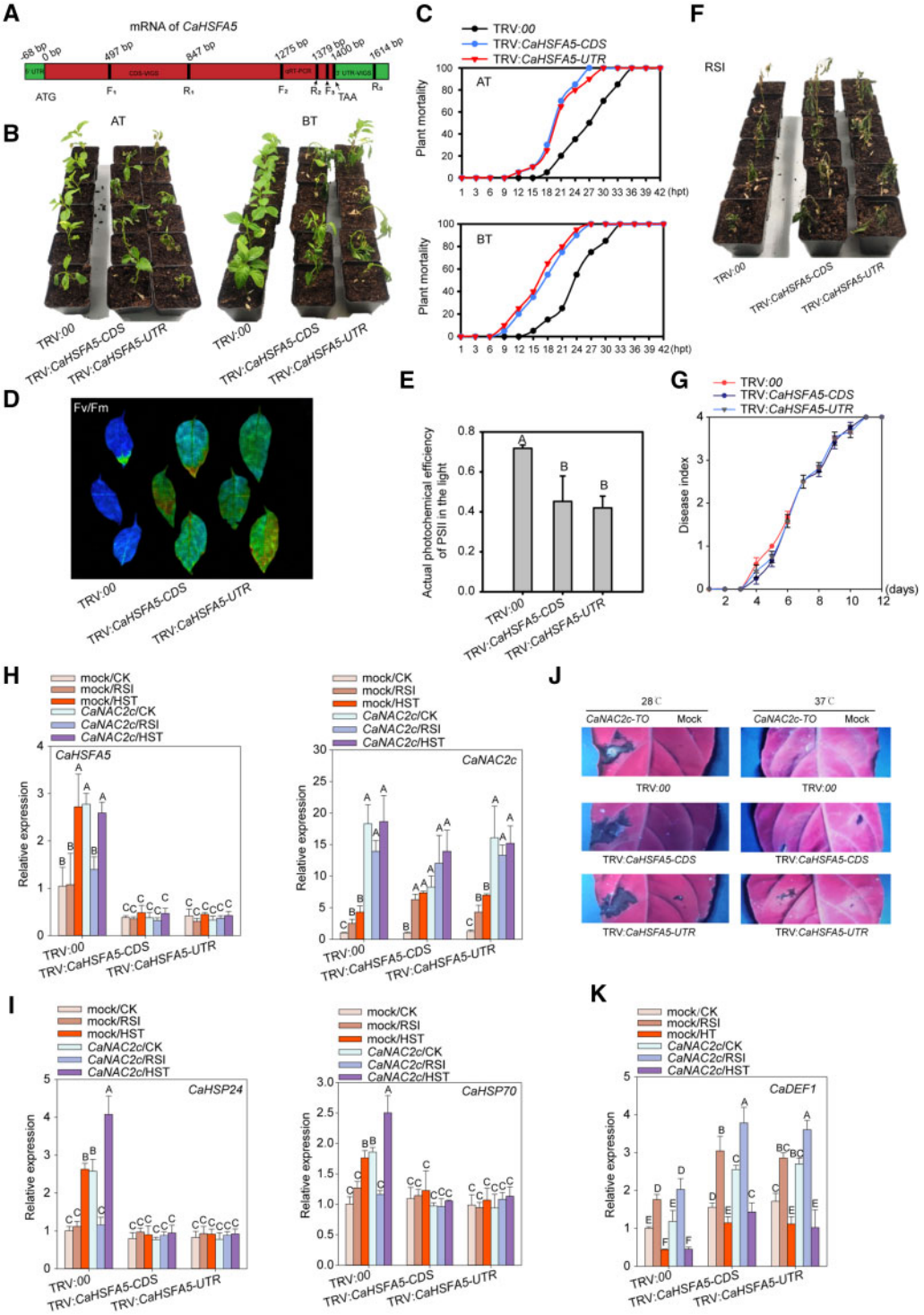

Figure 4 Silencing *CaHSFA5* Decreases Thermotolerance in Pepper Plants but does not Affect Resistance to RSI. A, Diagram of the fragments in the coding sequence (CDS) or 3′-untranslated region (UTR) of *CaHSFA5* used for VIGS (TRV:*CaHSFA5-CDS* and TRV:*CaHSFA5-3′UTR*). B and C, *CaHSFA5* silencing lowers basal and acquired thermotolerance of pepper plants (24 plants were calculated for mortality). D, *CaHSFA5* silencing decreases Fv/Fm of pepper leaves upon HTS. E, *CaHSFA5* silencing decreases △F/Fm of pepper leaves upon HTS. F, *CaHSFA5* silencing does not affect the resistance of pepper plants to RSI. G, *CaHSFA5* silencing does not affect the disease index values of pepper plants upon RSI (24 plants were calculated). H, Successful silencing of *CaHSFA5* and overexpression of *CaNAC2c*. I, *CaHSFA5* silencing significantly decreases the expression of thermotolerance-related genes *CaHSP24* and *CaHSP70* in pepper plants. J, HR-like cell death induced by *CaNAC2c-TO* at room temperature is not influenced by the silencing of *CaHSFA5*, and *CaNAC2c-TO* does not induce cell death at 37°C. K, *CaHSFA5* does not affect the induction of *CaDEF1* expression by CaNAC2c. In A and F, the images were digitally extracted. Data presented are means ± standard error of four replicates. In C, the data were counted based on 24 pepper plants. In C, E, G, H, I, and K, data presented are means ± standard error (SE) of four replicates, different capital letters indicate significant differences among means ($P < 0.01$), as calculated with Fisher's protected LSD test.

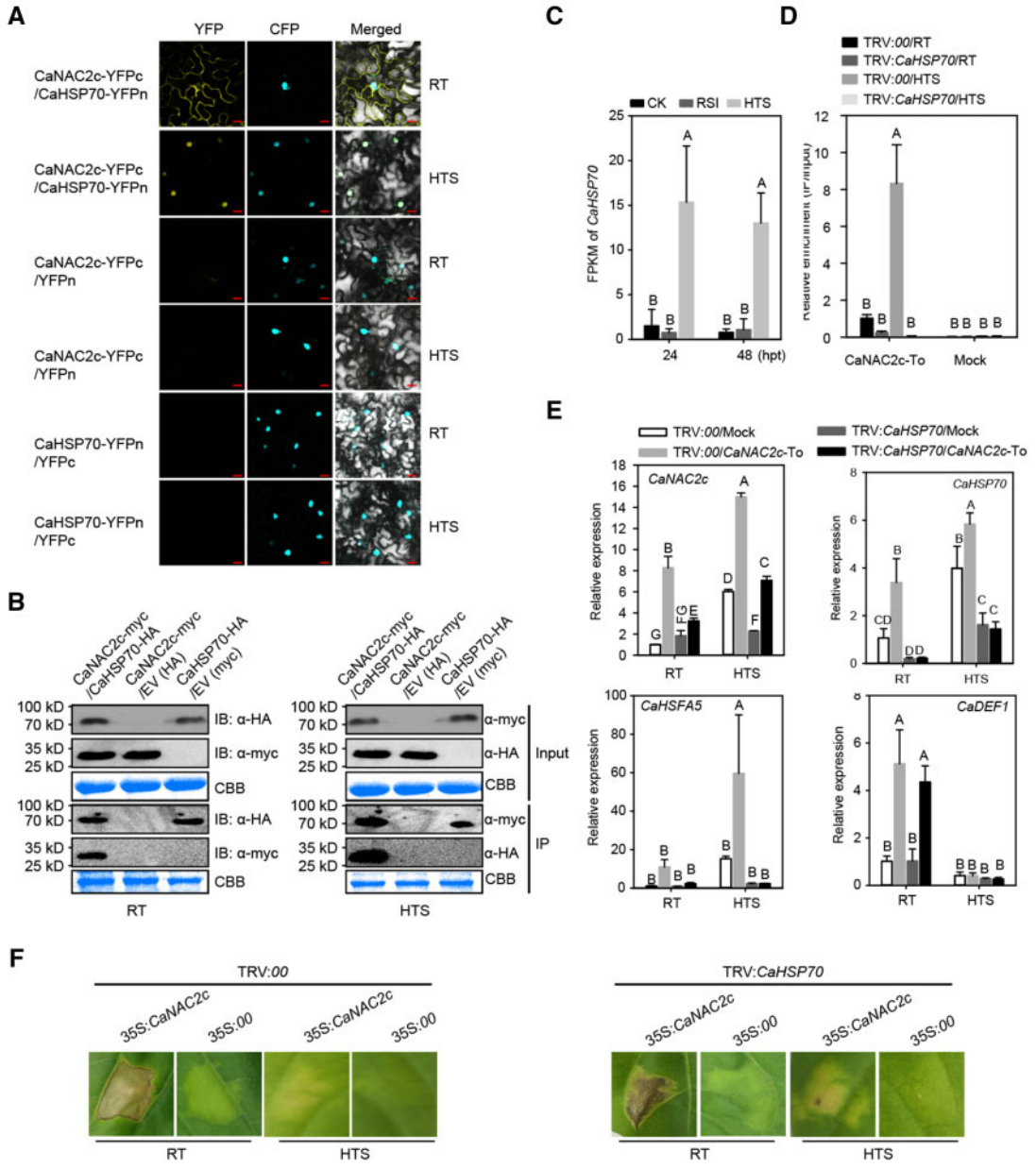

Figure 5 The interaction between CaNAC2c and CaHSP70 and its effect on transcription of immunity or thermotolerance-related genes by CaNAC2c. A, BiFC confirmation of the interaction between CaNAC2c and CaHSP70 in *N. benthamiana* leaves infiltrated with *Agrobacterium* cells bearing CaNAC2c-YFP^{C}+CaHSP70-YFP^{N} or CaNAC2c-YFP^{N}+CaHSP70-YFP^{C} constructs, NbH3 (histone H3)-CFP was used to indicate the nucleus. Cyan fluorescence and yellow fluorescence, visible light, and merged images were taken on a confocal microscope at 48 hpi. Bars = 25 μm. B, Interaction between CaNAC2c and CaHSP70 in vivo, as determined by Co-IP assay. Proteins were isolated from pepper leaves transiently overexpressing CaNAC2c-Myc and CaHSP70-HA, and CaNAC2c-Flag and its interacting partners were immunoprecipitated with antibody of Flag, the presence of CaHSP70 in the protein complex was assayed by western blotting using antibody of HA. C, Fragments per Kilobase Million (FPKM) of CaHSP70 in pepper plant challenged with HTS or RSI based the RNA-seq data set. D, The effect of CaHSP70 silencing on its deposition on the promoter of CaHSFA5 by ChIP-qPCR. E, The effect of *CaHSP70* silencing on the regulation of CaHSFA5 and CaDEF1 by transient overexpression of CaNAC2c under room temperature and upon HTS challenge by RT-qPCR. F, The effect of *CaHSP70* silencing on the HR cell death triggered by transient overexpression of CaNAC2c under room temperature and upon HTS challenge. In C, D, and E, data are shown as means ± standard error of four replicates. Different capital letters above the bars indicate significant differences between means ($P < 0.01$) by Fisher's protected least-significant-difference (LSD) test.

of CaNAC2c at the *CaHSFA5* promoter increased, as did *CaHSFA5* transcript levels, when CaHSP70 was transiently co-overexpressed with CaNAC2c (Supplemental Figure S20), but were drastically reduced when *CaHSP70* was silenced in pepper plants (Figure 5, D and E). We conclude that CaHSP70 plays a key role in ameliorating heat tolerance by upregulating *CaHSFA5* transcript levels via CaNAC2c binding to the *CaHSFA5* promoter.

We then aimed to determine the contribution, if any, of CaHSP70 to immunity against RSI. Notably, the HR-like cell death phenotype triggered by the transient overexpression of *CaNAC2c* was not affected when silencing *CaHSP70* (Figure 5, F). Although *CaDEF1* transcript levels were significantly reduced by the transient co-overexpression of *CaHSP70* and *CaNAC2c* at room temperature and high temperature (Supplemental Figure S20, B), they were not affected by the silencing of *CaHSP70* when *CaNAC2c* was transiently overexpressed (Figure 5, E). These data collectively indicate that CaHSP70 interacts with CaNAC2c in the nucleus upon HTS, thereby enhancing CaNAC2c activity toward activating thermotolerance but also repressing its function as a regulator of plant immunity.

CaNAC029 interacts with CaNAC2c to promote the regulation of CaNAC2c in pepper immunity but not thermotolerance

We performed similar BiFC and co-IP assays to validate the interaction between CaNAC2c and CaNAC029: CaNAC2c interacted with CaNAC029 under room temperature and upon RSI in the nucleus, but not upon HTS (Figure 6, A and B). Based on co-IP, CaNAC2c appeared to interact with CaNAC029 quantitatively more upon RSI than at room temperature, while we detected no interaction upon HTS (Figure 6, B).

We then explored the consequences of the interaction between CaNAC2c and CaNAC029 on the function of CaNAC2c in the induction of HR-like cell death, which play important roles in plant immunity against the pathogen (Jones and Dangl, 2006). To this end, we transient overexpressed *CaNAC2c* in the leaves of pepper plants with normal levels of *CaNAC029* (wild-type controls) or silenced for *CaNAC29*. The transient overexpression of *CaNAC2c* triggered a strong HR-like cell death response in wild-type leaves but not when *CaNAC029* was silenced (Figure 6, C), indicating that CaNAC2c positively regulates pepper immunity in a CaNAC029-dependent manner. To test the effect of the transient overexpression of *CaNAC029* on the targeting and regulation of the immunity-related gene *CaDEF1* by CaNAC2c, we performed RT-qPCR by transiently co-overexpressing *CaNAC2c* and *CaNAC029* or transiently overexpressing *CaNAC2c* alone in pepper leaves. A ChIP-qPCR assay showed that CaNAC029 failed to bind to the *CaHSFA5* promoter under RT, RSI, or HTS. In addition, overexpression of *CaNAC029* prevented the binding of CaNAC2c to the *CaHSFA5* promoter specifically at room temperature but supported full deposition of CaNAC2c at the *CaHSFA5* promoter upon HTS (Figure 6, D). The transient co-overexpression of *CaNAC029* and *CaNAC2c* induced *CaDEF1* expression to higher levels than *CaNAC2c* alone at room temperature, but this phenomenon was abolished upon HTS (Figure 6, E). In contrast, *CaNAC029* silencing significantly repressed the upregulation of *CaDEF1* by transient overexpression of *CaNAC2c* at room temperature but did not affect the positive regulation of *CaHSFA5* by CaNAC2c either at room temperature or upon HTS (Figure 6, F). In addition, transient overexpression of *CaNAC029* inhibited the increase of *CaHSFA5* transcription induced by *CaNAC2c* overexpression, but the inhibition was abolished at high temperature. These data indicate that the interaction between CaNAC2c and CaNAC029 promotes the control of gene expression mediated by CaNAC2c in pepper immunity but not in thermotolerance.

The 26S proteasome degrades CaNAC029 to direct CaNAC2c toward thermotolerance under HTS

We characterized protein stability for CaNAC2c and CaNAC29 under our experimental conditions. To our surprise, CaNAC2c-Myc remained stable under all conditions tested, including challenge by HTS (Figure 7, A). Unlike CaNAC2c, CaNAC029-HA was stable at room temperature and upon RSI, but was degraded within 2 h upon HTS (Figure 7, D). The 26S proteasome inhibitor MG132 blocked the degradation of CaNAC029 upon HTS, suggesting the involvement of ubiquitin modification and the 26S proteasome (Figure 7, D). Indeed, an immunoblot analysis with an antibody against ubiquitin revealed a ladder-like pattern upon HT, consistent with degradation through ubiquitination (Figure 7, F). Even though CaNAC2c was not degraded in response to HTS in control plants, the silencing of *CaHSP70* significantly reduced the stability of CaNAC2c (Figure 7, B). Consistently, we tested the effect of the addition of prokaryotic expressed CaHSP70-6×His on the degradation of CaNAC2c-GST at 42°C, we found that the addition of CaHSP70-6×His significantly blocked the degradation of CaNAC2c-GFP (Figure 7, C). In contrast, silencing *CaHSP70* did not affect the degradation of *CaNAC029* upon HTS (Figure 7, E), nor did it affect the HR-like cell death caused by transient overexpression of CaNAC029 in leaves (Figure 7, G). These data indicate that CaHSP70 maintains the stability of CaNAC2c upon HTS but not CaNAC029. During heat shock, the rapid elimination of CaNAC029 through the 26S proteasome will quickly target CaNAC2c to the *CaHSFA5* promoter and initiate thermotolerance responses.

Discussion

To maximize fitness, plants continuously balance their limited resources via diverse mechanisms leading to trade-offs between growth or responses to various stresses. Although TFs are crucial for plant responses to biotic and abiotic stresses, a single TF may be involved in regulating several seemingly disparate processes (Rushton et al., 2010;

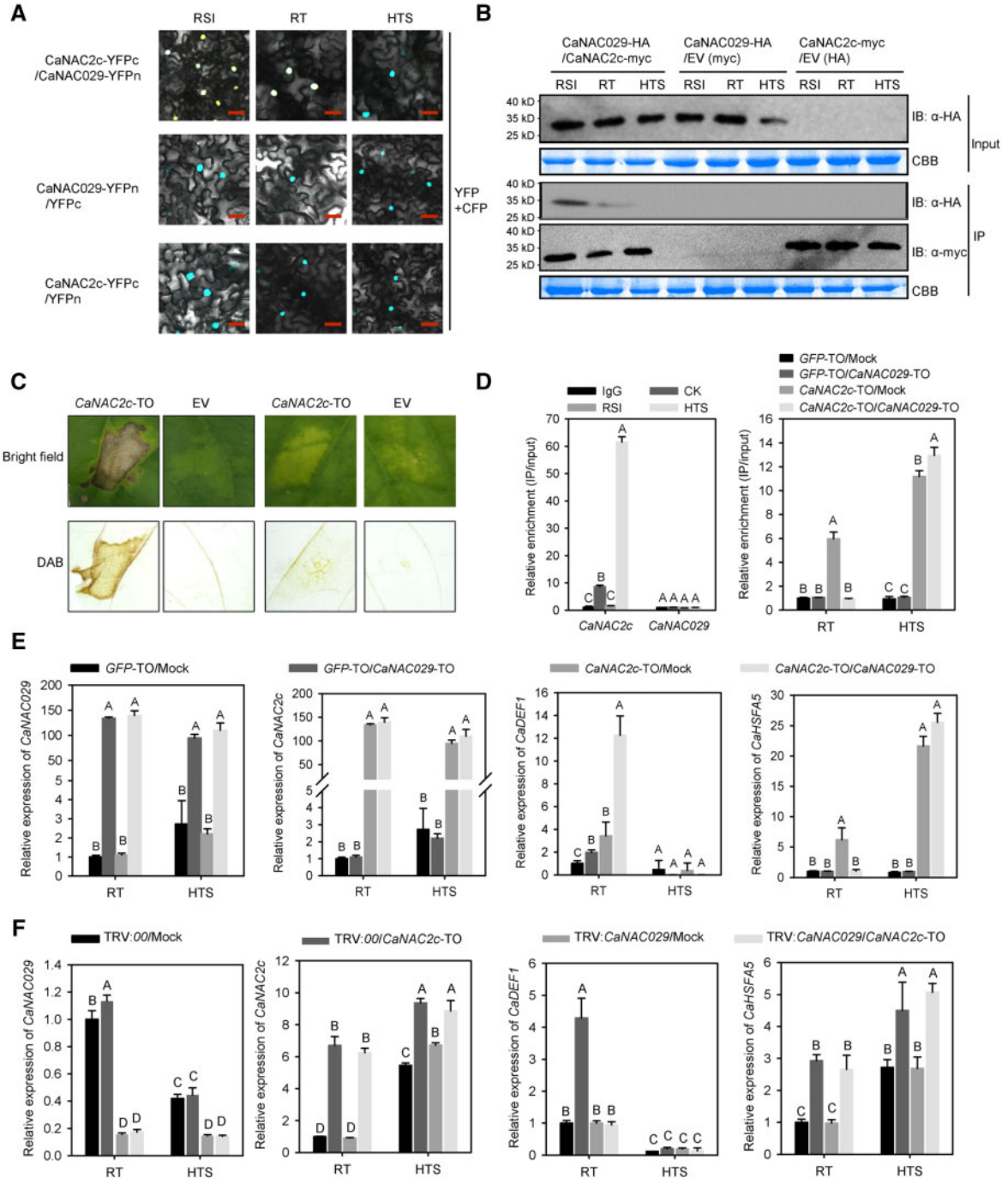

Figure 6 The interaction between CaNAC2c and CaNAC029 and its effect on transcription of immunity or thermotolerance related genes by CaNAC2c. A, BiFC confirmation of the interaction between CaNAC2c and CaNAC029 in *N. benthamiana* leaves infiltrated with Agrobacterium cells bearing CaNAC2c-YFPC+CaNAC029-YFPN or CaNAC2c-YFPN+CaNAC029-YFPC constructs, NbH3 (histone H3)-CFP was used to indicate the nucleus. Cyan fluorescence and yellow fluorescence, visible light, and merged images were taken on a confocal microscope at 48 hpi. Bars = 25 μm. B, Interaction between CaNAC2c and CaHSP70 in vivo, as determined by Co-IP assay. Proteins were isolated from pepper leaves transiently overexpressing CaNAC2c-Myc and CaNAC029-HA, and CaNAC2c-Flag and its interacting partners were immunoprecipitated with antibody of Flag, the presence of CaNAC029 in the protein complex was assayed by western blotting using antibody of HA. C, The effect of *CaNAC029* silencing on the HR cell death triggered by transient overexpression of CaNAC2c under room temperature and upon HTS challenge. D, CaNAC029 cannot bind to the promoter of CaHSFA5 upon RSI or HTS, and co-transient overexpression of CaNAC029 and CaNAC2c inhibit the binding of CaNAC2c to CaHSFA5 promoters at room temperature. E, The effect of transient overexpression of CaNAC029 on the regulation of CaHSFA5 and CaDEF1 by CaNAC2c by RT-qPCR. F, The effect of *CaNAC029* silencing on the regulation of CaHSFA5 and CaDEF1 by transient overexpression of CaNAC2c under room temperature and upon HTS challenge by RT-qPCR. In D, E, and F, data are shown as means ± standard error of four replicates. Different capital letters above the bars indicate significant differences between means ($P < 0.01$) by Fisher's protected least-significant-difference (LSD) test.

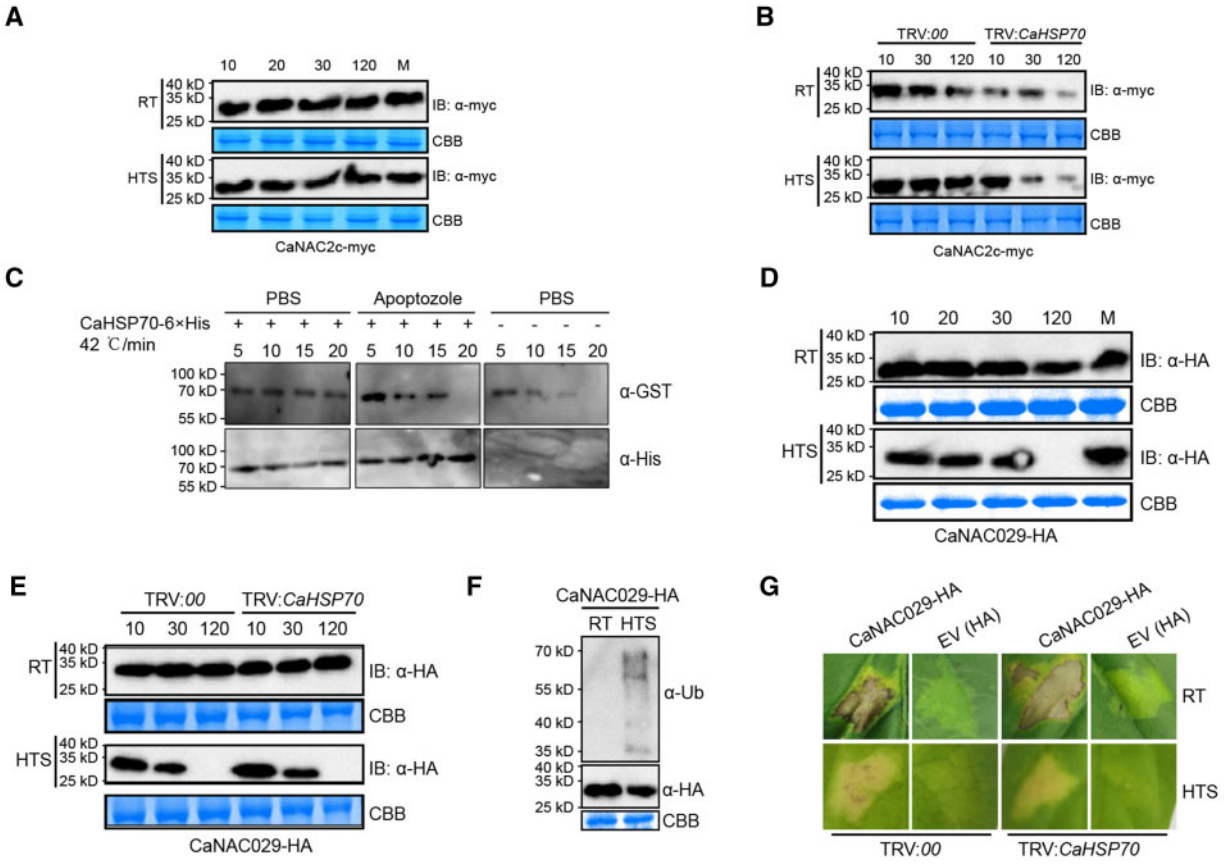

Figure 7 Effect of CaHSP70 silencing on HR-like cell death and immunity and expression of thermotolerance-related genes induced by transient overexpression of *CaNAC029*. A, Stability of CaNAC2c-myc in pepper plants transiently overexpressing *CaNAC2c-myc* at room temperature or upon HTS. Time since 10 is shown in min. B, Effect of *CaHSP70* silencing on the stability of CaNAC2c in pepper plants at room temperature or upon HTS. C, CaNAC2c was protected from degradation by CaHSP70 through in vitro assay using specific inhibitors (200 μM Apoptozole) of CaHSP70. D, Stability of CaNAC029-HA in pepper plants transiently overexpressing *CaNAC029-HA* at room temperature or upon HTS. Time since 10 is shown in min. E, Effect of *CaHSP70* silencing on the stability of CaNAC29-HA in pepper plants transiently overexpressing *CaNAC20-HA* at room temperature or upon HTS. F, Immunoblot analysis of CaNAC29-HA in pepper plants transiently overexpressing *CaNAC29-HA* upon HTS with an anti-Ub antibody. G, Effect of *CaHSP70* silencing on the HR-like cell death response triggered by the transient overexpression of *CaNAC29* at room temperature and upon HTS.

Nuruzzaman et al., 2013), making it difficult to identify the precise mode of action underlying the balance between plant growth and stress responses. In the present study, we establish that CaNAC2c acts as a negative regulator of pepper growth and as a positive regulator in defense response to HTS and RSI. Notably, whether CaNAC2c directs plant resources toward growth or response to HTS and RSI is dictated by differential and context-specific interactions with the heat shock protein CaHSP70 and the other NAC-type TF CaNAC029.

CaNAC2c acts positively in thermotolerance and in immunity against RSI but negatively in pepper growth

Our data demonstrated that upon HTS or RSI, *CaNAC2c* was transcriptionally upregulated, leading to enhanced basal and acquired thermotolerance, or enhanced resistance to RSI, depending on the stressor. At the same time, the induction of *CaNAC2c* also repressed growth, supporting a role for CaNAC2c as a negative regulator of pepper growth but as a positive regulator of thermotolerance and defense against RSI. This result is similar to our previous studies in which we had shown that *CaWRKY6* (Cai et al., 2015), *CaWRKY40* (Dang et al., 2013), and *CabZIP63* (Shen et al., 2016) act positively in pepper responses to both RSI and HTS, supporting the notion that pepper responses to RSI are closely linked to HTS responses. CaNAC2c performed its distinct functions by differential targeting and transcriptional regulation of subsets of genes: for thermotolerance, CaNAC2c directly targeted and induced the expression of thermotolerance-related genes such as *CaHSFA5* (Figures 3 and 4 and Supplemental Figure S16), *CaHSP24*, *CaHSP70*, and *CaHSFB2a* (Supplemental Figure S13, A). CaNAC2c also caused reduced H_2O_2 accumulation, which is associated with the degree of thermotolerance (Yu et al., 2019; Zhuang et al., 2020). In contrast, CaNAC2c acted as a positive regulator for immunity against RSI by upregulating *CaDEF1* expression and enhancing the accumulation of H_2O_2 (Yoshioka et

al., 2003), reflecting the different mechanisms behind heat stress response (HSR) and immune responses to pathogens. While *CaNAC2c* silencing did not affect pepper responses to RSI, this result is due to partial redundancy between *CaNAC2c* and the highly related *CaNAC2d*, as the simultaneous silencing of *CaNAC2c* and *CaNAC2d* decreased plant resistance to RSI. A comparable level of functional redundancy has been extensively described for immunity-associated genes such as bHLH-type TFs (Xu et al., 2014). That CaNAC2c acts alone in the context of thermotolerance but redundantly with other genes against pathogen attacks may stem from the varying selective pressure imposed by HTS and pathogen attack over the course of evolution. As plants and pathogens attempt to bypass each other's defense mechanisms, plant immunity has frequently been overcome by new pathogen-derived effectors that target specific plant immune components such as TFs (Canonne et al., 2011). This apparent redundancy may therefore make the immune response more robust in the face of a constant arms race against pathogens, since the removal or inactivation of a subset of redundant components may be functionally compensated for by others.

The immunity-thermotolerance trade-off mediated by CaNAC2c is tuned by differential and context-specific interactions with CaHSP70 and CaNAC029

Our data showed that CaNAC2c targets and regulates *CaHSFA5* upon HTS but regulates *CaDEF1* when pepper plants are challenged by RSI, indicating that CaNAC2c performs functions in both processes by differential targeting via context-specific interactions with other regulatory proteins (Chi et al., 2013). We tested this hypothesis by isolating possible interacting partners during pepper response to HTS or RSI by pull-down assays followed by mass spectrometry and testing these new interactors by BiFC and co-IP assays against CaNAC2c. We determined that CaNAC2c may interact with either CaHSP70 or CaNAC029 depending on the context: upon HTS, CaNAC2c interacted with CaHSP70 in the nucleus, thereby protecting CaNAC2c from degradation, promoting the targeting of CaNAC2c to the *CaHSFA5* promoter and preventing CaNAC2c from activating *CaDEF1* (Figures 5 and 7 and Supplemental Figure S18). When pepper plants were challenged by RSI, CaNAC2c interacted with CaNAC029 in the nucleus, leading to enhanced transcript levels of *CaDEF1*, while the potential activation of HSR by CaNAC2c was blocked (Figure 6), similarly, GmNAC81 in soybean interact with GmNAC30, and this interaction determines the full activation or repression of target promoters (Mendes et al., 2013), we speculate that the heterodimerization of CaNAC2c and CaNAC029 may be required for the full regulation of gene expression by CaNAC2c. To confirm this hypothesis, further study is required to determine the target genes of CaNAC2c and CaNAC029 and to study the precise molecular details behind the regulation of CaNAC2c/CaNAC029 interaction on their transcription. However, we did not observe any interaction between CaNAC2c and CaNAC029, probably due to the degradation of CaNAC029 upon HTS by the 26S proteasome (Figure 6, A, B and 7). Based on these results, we concluded that the balance between pepper responses to HTS and RSI that is mediated by CaNAC2c is modulated by CaHSP70 and CaNAC029, respectively, in a context-specific manner. As HTS and pathogen attacks such as *R. solanacearum* are two frequently co-occurring stresses in subtropical or tropical climates where pepper originated from, this post-translational regulation might benefit rapid and precise switches between defense responses to different stresses. It is worth pointing out that a subset of regulatory proteins such as MYB (Zhang et al., 2020), xylem NAC domain 1 (XND1; Zhang et al., 2020), CDPKs (Vivek et al., 2016), the phosphatidylinositol 4-kinase PI4Kgamma5 (Yong et al., 2016), radical-induced cell death 1 (RCD1; O'Shea et al., 2015), C-repeat binding factor (CBF; Shan et al., 2014), the protein phosphatase regulator of CBF gene expression 2 (RCF2; Guan et al., 2014), ring-H2 (Greve et al., 2003), RCD1 (O'Shea et al., 2015), and crowded nuclei (CRWN1; Guo et al., 2017) have been shown to interact with NAC TFs, thereby altering their targeting specificities and transcriptional activities. In contrast, to date, neither HSP protein had been demonstrated to interact with a NAC TF, nor one NAC-type TF with another NAC protein to regulate plant immunity.

The growth/defense trade-off mediated by CaNAC2c is regulated at both transcriptional and post-transcriptional levels by interaction with CaHSP70

The different roles of CaNAC2c as negative regulator in pepper growth and positive regulator in thermotolerance and immunity against RSI indicate that CaNAC2c acts as a central regulator of the trade-off between pepper growth and stress responses. Growth-defense tradeoffs are essential for optimizing plant performance and adaptation under stress conditions (de Vries et al., 2017), recent advances in plant physiology and ecology suggest that this mechanism is more complex than just a resource trade-off (de Vries et al., 2017). Signaling mediated by SA (Meldau et al., 2012; Li et al., 2019; Nakagami et al., 2020) and JA (Guo et al., 2018; Howe et al., 2018) have been found in tradeoffs between growth and defense response to biotrophic and to necrotrophic pathogen or herbivore, respectively. *CaNAC2c* expression remains low but constitutive in the absence of stress, but is upregulated by RSI or HTS, indicating that the lower transcript levels of *CaNAC2c* are crucial for the evocation of pepper growth under normal conditions. In contrast, upon HTS or RSI, *CaNAC2c* transcription was upregulated, leading to the activation of distinct defense responses via differential targeting of the encoded CaNAC2c TF. In addition, we saw that CaNAC2c interacts with CaHSP70 in nonstressed pepper plants, but outside the nucleus (Figure 5, A), indicating a possible mechanism to titrate a TF away from its target defense-related genes to prevent their untimely activation (Moore et al., 2011). These data indicate that growth/

defense trade-offs mediated by CaNAC2c are regulated at the transcriptional level and the post-translational level by interacting with CaHSP70. Since the overexpression of these TFs generally causes a growth penalty (Liu et al., 2018), it is plausible that plants have adopted reducible expression strategies for TFs involved in stress resistance (Cheng et al., 2018; Sun et al., 2019) to reduce the associated fitness cost. Since *CaNAC2c* was upregulated by exogenous application of MeJA or ABA and its expression change by overexpression altered the expression of JA- or ABA-signaling dependent marker genes(Figure 2, I and J), *CaNAC029* and *CaHSP70* were specifically upregulated by exogenous application of MeJA and ABA, respectively, and JA and ABA have been implicated in plant response to necrotrophic stage infection of *R. solanacearum*, a hemibiotrophic pathogen, and to heat stress, respectively (Kachroo et al., 2003; Hiruma et al., 2013; Huang et al., 2016), it can be speculated that the trade-off between pepper growth and immunity against RSI is mediated by JA signaling, the trade-off between pepper growth and thermotolerance is regulated by ABA signaling, and the balance between pepper immunity against RSI and thermotolerance mediated by CaNAC2c might be regulated by the antagonism between JA and ABA signaling (Robert-Seilaniantz et al., 2011; Kyndt et al., 2017).

Collectively, our data indicate that CaNAC2c acts positively in thermotolerance and immunity against *R. solanacearum* and negatively in pepper growth. Trade-offs between pepper growth and defense responses are determined by *CaNAC2c* transcription and CaNAC2c protein via its interaction with CaHSP70, while thermotolerance-immunity trade-off is regulated by CaHSP70 and CaNAC029, respectively, in a CaNAC2c interactor-dependent manner.

Materials and methods

Plant materials and growth conditions

The seeds of HN42, a pepper (*Capsicum annuum*) inbred line with middle level of thermotolerance and bacterial wilt resistance, and *Nicotiana benthamiana* were sown on a soil mixture [peat moss: perlite, 2:1 (v/v)] in plastic pots and were placed in a growth room at 28°C, 60–70 μmol photons m^{-2} s^{-1}, a relative humidity of 70%, and a 16-h light/8-h dark photoperiod.

Construction of vectors

To generate vectors for overexpression, the full-length open reading frames (ORFs) of *CaNAC2c*, *CaNAC029*, and *CaHSP70* were cloned into the entry vector pDONR207 by BP reaction with appropriate primers (Supplemental Table S1) and then cloned into the destination vectors pEarleyGate101, pDEST-15/17, and pEarleyGate103 by LR reaction, using Gateway cloning techniques (Invitrogen, Carlsbad, CA, USA). For virus-induced gene silencing (VIGS), one or two specific 300–400 bp fragments in the ORFs or 3′-untranslated regions (UTRs) of *CaNAC2c/d*, *CaHSFA5*, *CaHSP70*, and *CaNAC029* were PCR-amplified and cloned into the entry vector pDONR207 and then into the pYL279 vector. The specificity of each fragment was confirmed by Basic Local Alignment Search Tool for DNA (BLAST) searches against the pepper Zunla-1 genome (http://pepper sequence.genomics.cn/page/species/blast.jsp).

Plant treatment with HTS

Pepper plants were exposed to HTS by transferring 8-leaf stage pepper plants to 42°C with 50% humidity in a growth chamber, while the control plants were kept at 28°C and 50% humidity in another growth chamber until they are harvested for further assay.

Application of plant hormones

Application of plant hormones was carried out as described previously (Dang et al., 2013). Pepper plants at the four-leaf stage were sprayed with 5 mm salicylic acid (SA), 100 mM methyl jasmonate (MeJA; both dissolved in 1:9, v:v ethanol). Mock plants were sprayed with 10% ethanol (1:9, v:v). One-month-old pepper plants were sprayed with 100 mM abscisic acid (ABA) or 10 mM Fluridone and 10 mm ethephon in sterile ddH_2O. Control plants were sprayed with sterile ddH_2O.

Pathogens and *R. solanacearum* inoculation

The highly virulent *Ralstonia solanacearum* strain FJC100301 was used in the present study. The bacterial cell solution used for inoculation was diluted to 10^8 cfu mL^{-1} (OD_{600} = 0.8) or 10^3 cfu mL^{-1} (OD_{600} = 0.3). For root inoculation of pepper or *N. benthamiana* plants planted in pots, which was well watered before mechanically damaging the roots with scissors and irrigated with 1 mL of *R. solanacearum* cell suspension (OD_{600} = 0.8) for each pot. For leaf inoculation with *R. solanacearum*, we inoculated leaves with 100 μL of *R. solanacearum* cell suspension (OD_{600} = 0.3) at each inoculating site using a syringe without a needle. We scored the disease index of more than 24 plants at each time point over the course of the infection cycle based on visual observation (Supplemental Table S3).

DAB and NBT staining and H_2O_2 content detection

The accumulation of H_2O_2 and reactive oxygen species (Papageorgiou et al., 2016) was assessed by staining the leaves, roots, or stems from pepper or *N. benthamiana* plants with 1 mg mL^{-1} diaminobenzidine (DAB) or Nitrotetrazolium Blue chloride (NBT). After overnight incubation in 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. We used Beibo® BBcellProbe® plant hydrogen peroxide H_2O_2 detection kit to text the level of hydrogen peroxide in tissues and cells. 1:5 ratio of tissue mass (g) and reagent volume (mL; it is recommended to weigh about 0.1 g tissue and add 500 μL Buffer B) were mixed for ice bath homogenization, and then transferred to EP tube and centrifuged at 8,000 *g*, 4°C 10 min, then took the supernatant and put it on ice for testing. Then 2 μL BBcellProbe™ O11 hydrogen peroxide probe was added to the supernatant of the homogenate

and mixed well, then incubated in a 37°C cell incubator in the dark for 20 min. Then, 488 nm excitation wavelength and 525 nm emission wavelength were used to detect the intensity of the sample fluorescence.

Gene silencing by VIGS in pepper plants

To silence *CaNAC2c(d)*, *CaHSFA5*, *CaHSP70*, or *CaNAC029*, we infiltrated the cotyledons of 2-week-old pepper seedlings with Agrobacterium (*Agrobacterium tumefaciens*) strain GV3101 carrying the vector pTRV-RNA1 (pYL192) and pTRV-RNA2 (pYL279, the empty VIGS vector) or pYL279-*CaNAC2c* (*CaNAC2d*, *CaHSFA5*, *CaHSP70*, or *CaNAC029*), mixed and resuspended in induction medium (10 mM MES, 10 mM $MgCl_2$, 200 μM acetosyringone, pH 5.6) at a 1:1 ratio to a final $OD_{600} = 0.6$. To simultaneously silence *CaNAC2c* and *CaNAC2d*, Agrobacterium cells containing pTRV-RNA1 and pTRV-RNA2-*CaNAC2c* and cells containing pTRV-RNA1 and pTRV-RNA2-*CaNAC2d* were mixed at a ratio of 1:1 and resuspended in induction medium to a final $OD_{600} = 0.6$. A volume of 100 μL was infiltrated into the cotyledons of 2-week-old pepper seedlings, which were then placed in the dark at 16°C for 56 h, and then moved to a growth room at 28°C, 60–70 μmol photons m^{-2} s^{-1}, a relative humidity of 70%, and a 16-h light/8-h dark photoperiod. At 15 dpi, the success and specificity of gene silencing were assessed in pepper plants challenged with HTS by measuring the transcript levels of the gene(s) targeted for silencing.

Transient expression of *CaNAC2c* in pepper leaves

For transient expression analysis, Agrobacterium cells harboring the *35Spro:CaNAC2c-GFP* construct (or *35Spro:GFP* as control) were 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. The infiltrated leaves were collected at the indicated time points for further use.

Generation of transgenic *N. benthamiana* plants overexpressing *CaNAC2c*

Nicotiana benthamiana leaf discs were transformed with Agrobacterium strain GV3101 carrying the *35Spro:CaNAC2c-GFP* vector according to the method of Regner et al. (1992) and Bardonnet et al. (1994). Nineteen independent T_0 transgenic *N. benthamiana* plants were selected by hygromycin (5 mg L^{-1}) selection and validated by PCR and reverse transcription-quantitative PCR (RT-qPCR). The T_0 plants were then allowed to self-pollinate and set seeds. Positive transformants were propagated for two or three generations and subjected to the same selection to obtain T_2 and T_3 seeds. Two T_3 transgenic lines that exhibited moderate levels of *CaNAC2c* transcripts without phenotypic abnormality were selected for further analysis.

RT-qPCR

We performed RT-qPCR to determine the relative transcript levels of selected genes with specific primers (Supplemental Table S1) and the SYBR Premix Ex Taq II system (TaKaRa) on a BIO-RAD Real-time PCR system (Foster City, CA, USA) according to the manufacturer's instructions. Total RNA preparation and real-time qPCR were carried out following procedures described in our previous studies from four biological replicates (Cai et al., 2015). Total RNA was isolated from pepper samples using TRIzol Reagent according to the manufacturer's protocol (Invitrogen, Canada). mRNAs were reverse-transcribed into cDNA using a reverse transcription system and an oligo(dT) primer (Takara Biotechnology, Japan). Data were analyzed by the Livak method (Livak and Schmittgen, 2001; Zhang et al., 2015) and expressed as normalized relative expression level ($2^{-\Delta\Delta CT}$) of the respective genes. Relative transcript levels were normalized to *CaACTIN* (GQ339766) and *18S ribosomal RNA* (EF564281), respectively.

Chlorophyll fluorescence spectrophotometry

We used a MINI Imaging PAM instrument (Heinz Walz GmbH, Effeltrich, Germany) to measure F_v/F_m and $\triangle F/F_m'$ values from pepper and *N. benthamiana* leaves. The plants were adapted to darkness for 15 min before being placed into the instrument for measurements according to the method of Schreiber et al. (2012).

Immunoblot analysis

We extracted total protein from pepper samples by adding extraction buffer (10% glycerol, 25 mM Tris-HCl pH 7.5, 150 mM sodium chloride, 1 mM EDTA, 1% Triton X-100, 10 mM dithiothreitol, 1 × plant protease inhibitor, 2% (w/w) polyvinylpyrrolidone [PVPP]) to samples ground to a fine powder in liquid nitrogen. Protein extracts were incubated at 4°C with anti-hemagglutinin (anti-GFP) agarose beads (Thermo Fisher Scientific, Waltham, MA, USA) overnight. Beads were collected using a magnetic rack and washed 3 times with Tris-buffered saline and Tween-20 (0.05%). Eluted proteins were probed by immunoblotting using anti-GFP–peroxidase antibodies (Abcam, Cambridge, UK).

ChIP assay

ChIP assays were performed according to a previous study (Khan et al., 2018). We inoculated three fully extended pepper leaves at the six-leaf stage with Agrobacterium cells harboring the *35Spro:CaNAC2c-GFP* construct. The infiltrated leaves were harvested at 48 h post-inoculation (hpi) and crosslinked in a 1% formaldehyde solution; chromatin was isolated and sheared into 300–500 bp fragments, followed by immunoprecipitation of DNA–protein complexes using anti-GFP antibodies. The crosslinking was then reversed and DNA purified and used as a template for qPCR using primer pairs specific to a CATGTG-box-containing fragment or CATGTG-free fragment in the *CaHSFA5* promoter by semi-quantitative PCR using specific primer pairs (Supplemental Table S1).

Production and purification of recombinant *CaNAC2c-GST/6×His*

We introduced the pDEST-15/17 plasmid harboring *T7:CaNAC2c-GST/6×His* into *Escherichia coli* (*E. coli*) strain BL21 (DE3). Production of the fusion protein was induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 20°C for 12 h. To purify the recombinant proteins carrying the GST tag, Beaver Beads™ GSH (Beaver Biosciences, China) were washed thrice with 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 extract for 3 h at 4°C. The beads were washed 5 times with Buffer A and the target protein was eluted in Buffer B (50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0).

Pull-down assays

We immobilized the fusion protein CaNAC2c-6×His (0.1 mg) onto nickel Smart Beads 6FF (Smart-Life Sciences, China) and incubated the mixture under shaking for 3 h at 4°C. Proteins bound to the beads were subsequently washed with wash buffer (20 mM PBS with 0.5 mM imidazole, pH 7.4) and eluted with elution buffer (20 mM PBS with 250 mM imidazole, pH 7.4). Eluted proteins were resolved by SDS-PAGE gel electrophoresis and detected with an antibody against 6×His (PM013; 1:1,000 dilution; MBL International, Woburn, MA, USA).

Liquid chromatography-tandem mass spectrometry analysis

Isolated proteins and potential CaNAC2c interactors were analyzed on an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific), as previously described (Zuo et al., 2001; Wang et al., 2018). The samples were dissolved in 10 μL of a 10% formic acid solution, then analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) with an online sodium spray ion source. Peptide samples (5 μL) were loaded onto the trap column (Acclaim PepMapC18, 100 μm × 2 cm; Thermo Fisher Scientific) at a flow rate of 10 μL min^{-1}, and subsequently separated on a 60-min gradient on the analytical column (Acclaim PepMapC18, 75 μm × 15 cm). The column flow was controlled at 300 nL min^{-1}, and the electrospray voltage was 2 kV. Full scan spectra (*m*/*z* 350–1,550) were collected at a mass resolution of 60 K, and HCD MS/MS scans were subsequently performed at a resolution of 30 K with a dynamic exclusion for 30 s.

The original mass spectrometry collection files were imported into Proteome Discover 2.1 for retrieval. We searched peptide fragments against the Zunla pepper database and used BLAST searches at the National Center for Biotechnology Information (NCBI) database to annotate the functions of the corresponding proteins.

BiFC assay and subcellular localization

We determined the subcellular localization of CaNAC2c as described previously (Shen et al., 2016). Agrobacterium cells harboring the *35Spro:CaNAC2c-YFP* construct were infiltrated into *N. benthamiana* leaves. The YFP signal was detected 48 hpi. The open reading frames for CaNAC2c or CaNAC029/CaHSP70 in the pDONR vector were directly introduced into the destination vectors *pSPYCE* or *pSPYNE* via Gateway cloning to generate *35Spro:CaNAC2c-YFPc-HA* and *35Spro:CaNAC029/CaHSP70-YFPn-MYC*. Vectors were then introduced into Agrobacterium strain GV3101, and cells harboring *35S:CaNAC2c-YFPc-HA* and *35S: CaNAC029/CaHSP70-YFPn-MYC* were co-infiltrated into *N. benthamiana* leaves. BiFC assays were performed as described previously (Choi et al., 2012) and the fluorescent signal from Agrobacterium-infiltrated *N. benthamiana* leaves was observed at 48 hpi. YFP fluorescence was collected on a confocal microscope (YFP:527 nm/CFP:485 nm/TCS SP8, Leica Microsystems, Germany), Images were obtained at 100 Hz, 42% Exposure Rate, 1.000 Gain, and 5% Offset.

Co-IP assay

Nicotiana benthamiana leaves were infiltrated with *35Spro:CaNAC2c-YFPc-HA* and *35Spro:CaNAC029/CaHSP70-YFPn-MYC* and harvested at 48 hpi. Total protein extracts were prepared using protein extraction buffer (10% glycerol, 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2% Triton X-100, 10 mM DTT, 1× complete protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA), and 2% (w/v) PVPP). Extracted proteins were incubated with monoclonal anti-HA magnetic beads (Sigma-Aldrich) at 4°C overnight. Beads were then collected with a magnet and washed 3 times with protein extraction buffer. Eluted proteins were separated by SDS-PAGE electrophoresis and immunoblotted using anti-HA-peroxidase antibody or anti-MYC-peroxidase antibody (Sigma-Aldrich)

Electrophoretic mobility shift assay

Cy5-labeled double-stranded DNA fragments containing the CATGTG motif or its mutated version (GGGGGG) were commercially synthesized to use as probes in EMSA. CaNAC2c-GST or GST proteins were incubated with wild-type or mutated probe labeled with a 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 gel and then scanned on an Odyssey® CLX instrument (LI-COR, USA).

MST assessment of interaction of protein with promoter fragments in solution

The binding of CaNAC2c to the *CaHSFA5* promoter was analyzed by microscale thermophoresis (MST) in solution (Zillner et al., 2012). GFP fused to CaNAC2c was used as fluorescent label against a fragment containing the CATGTG motif within the *CaHSFA5* promoter, which was amplified by PCR with a specific primer pair, followed by purification. 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 in the assay. Interaction between protein and DNA was

measured as described previously (Qiu et al., 2018). We used the Nano Temper Analysis 1.2.20 software to fit the data and determine apparent K_d values (Zillner et al., 2012; Papageorgiou et al., 2016).

Statistical analyses

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

Accession numbers

CaNAC2c (XP_016575179.1); CaNAC2d (XP_016569664.1); CaHSFA5 (XM_016695662.1); CaNAC029 (A0A1U8EJR9); CaHSP70 (A0A1U8E6Q9); CaHSFB2a (XP_016564681.1); CaHSP24 (HM132040); CaNPR1 (X61679.1); CaDEF1 (AF442388); CaHIR1 (AAX20040); CaNAC07 (XM_016697660.1); CaNAC08 (XM_016725353.1); CaNAC059 (XM_016723243.1); CaLOX14 (NM_001324652.1); CaLOX31 (NM_001324819.1); CaActin (GQ339766); NbAPX (XP_016432750.1); NbHSFA5 (XM_016600975.1); NbHSP18 (XP_016481364.1); NbsHSP (XP_016463300.1); NbDEF1 (ABU40984.1); NtEF-1a (D63396).

Supplemental data

The following materials are available in the online version of this article.

Supplemental Figure S1. Transcript levels of *CaNAC2c* and pepper *NAC* family members to HTS or RSI.

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

Supplemental Figure S3. Nuclear localization of CaNAC2c in *N. benthamiana* epidermal cells.

Supplemental Figure S4. Silencing of *CaNAC2c* in pepper enhances thermotolerance.

Supplemental Figure S5. The specificity of CaNAC2c silencing in pepper plants by VIGS.

Supplemental Figure S6. Overexpression of *CaNAC2c* enhances thermotolerance in *N. benthamiana* plants.

Supplemental Figure S7. *N. benthamiana* plants overexpressing *CaNAC2c* exhibit enhanced Fv/Fm, △F/Fm and decreased ion leakage and lower ROS levels upon HTS.

Supplemental Figure S8. Silencing of CaNAC2c in different pepper germplasms.

Supplemental Figure S9. *N. benthamiana* plants overexpressing *CaNAC2c* exhibit enhanced resistance to RSI.

Supplemental Figure S10. Effect of *CaNAC2c*/2d silencing on the thermotolerance of pepper plants.

Supplemental Figure S11. *CaNAC2c* silencing promotes the growth of pepper plants.

Supplemental Figure S12. *N. benthamiana* plants overexpressing *CaNAC2c* repress growth and delay development.

Supplemental Figure S13. *CaNAC2c* influences the expression of genes related to thermotolerance and immunity.

Supplemental Figure S14. Relative transcript levels of marker genes by the exogenous application of ABA, Fluridon, SA, or MeJA.

Supplemental Figure S15. FPKMs of members in pepper LOX family in roots of pepper plants challenged with RSI.

Supplemental Figure S16. *CaHSFA5* acts as negative regulator of pepper growth.

Supplemental Figure S17. Silencing of *CaHSFA5* promotes growth of *N. benthamiana* plants overexpressing *CaNAC2c*.

Supplemental Figure S18. Silencing of *NbHSFA5* significantly decreases thermotolerance but does not affect resistance of *N. benthamiana* plants overexpressing *CaNAC2c* to RSI.

Supplemental Figure S19. CaHSP70 is upregulated by ABA and CaNAC029 is upregulated by MeJA.

Supplemental Figure S20. The effect of transient co-overexpression of *CaNAC2c* and *CaHSP70* on the enrichment of CaNAC2c at the *CaHSFA5* promoter and expression of immunity or thermotolerance-related genes at room temperature and upon HTS challenge.

Supplemental Table S1. Primers used in this study.

Supplemental Table S2. Sequence similarity between CaNAC2c predicted protein sequence and its putative orthologs from other plant species.

Supplemental Table S3. Disease index for pepper plants infected with *Ralstonia solanacearum*.

Supplemental Table S4. Results of CaNAC2c co-expression analysis under HTS in pepperhub.

Supplemental Table S5. Proteins identified as potential interaction candidates with CaNAC2c using LC-MS/MS.

Acknowledgments

The authors thank Mark D. Curtis for kindly providing the Gateway destination vectors and Dr. S. P. Dinesh-Kumar of Yale University for the pTRV1 and pTRV2 vectors.

Funding

This work was supported by the National Natural Science Foundation of China (31401890, 31401312), the Scientific Research Foundation of the Graduate School of Fujian Agriculture and Forestry University (324-1122yb068), and Development Fund Project of Fujian Agriculture and Forestry University (CXZX2016158, CXZX2017548).

Conflict of interest statement. The authors have no conflicts of interest to declare.

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