

# Pepper Heat Shock Protein 70a Interacts with the Type III Effector AvrBsT and Triggers Plant Cell Death and Immunity<sup>1[OPEN]</sup>

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Heat shock proteins (HSPs) function as molecular chaperones and are essential for the maintenance and/or restoration of protein homeostasis. The genus *Xanthomonas* type III effector protein AvrBsT induces hypersensitive cell death in pepper (*Capsicum annuum*). Here, we report the identification of the pepper CaHSP70a as an AvrBsT-interacting protein. Bimolecular fluorescence complementation and coimmunoprecipitation assays confirm the specific interaction between CaHSP70a and AvrBsT in planta. The CaHSP70a peptide-binding domain is essential for its interaction with AvrBsT. Heat stress (37°C) and *Xanthomonas campestris* pv *vesicatoria* (*Xcv*) infection distinctly induce CaHSP70a in pepper leaves. Cytoplasmic CaHSP70a proteins significantly accumulate in pepper leaves to induce the hypersensitive cell death response by *Xcv* (avrBsT) infection. Transient CaHSP70a overexpression induces hypersensitive cell death under heat stress, which is accompanied by strong induction of defense- and cell death-related genes. The CaHSP70a peptide-binding domain and ATPase-binding domain are required to trigger cell death under heat stress. Transient coexpression of CaHSP70a and *avrBsT* leads to cytoplasmic localization of the CaHSP70a-AvrBsT complex and significantly enhances *avrBsT*-triggered cell death in *Nicotiana benthamiana*. CaHSP70a silencing in pepper enhances *Xcv* growth but disrupts the reactive oxygen species burst and cell death response during *Xcv* infection. Expression of some defense marker genes is significantly reduced in CaHSP70a-silenced leaves, with lower levels of the defense hormones salicylic acid and jasmonic acid. Together, these results suggest that CaHSP70a interacts with the type III effector AvrBsT and is required for cell death and immunity in plants.

The heat shock protein HSP70 is a ubiquitous essential protein chaperone and one of the most abundant and diverse heat stress proteins in plants. HSP70s are induced by environmental stresses and are required for plants to cope with heat. HSP70s are involved in protein folding, synthesis, translocation, and macromolecular assemblies such as microtubules (Mayer et al., 2001; Hartl and Hayer-Hartl, 2002). HSP70s protect cells from heat stress by preventing protein aggregation and by facilitating the refolding of denatured proteins. Protein stability can decrease under heat stress conditions and expose hydrophobic patches that cause the aggregation of denatured proteins. HSP70s bind to hydrophobic patches of partially unfolded proteins in an ATP-dependent manner and prevent protein aggregation (Mayer and Bukau, 2005). The modular HSP70 structure consists of a N-terminal ATPase domain and a C-terminal peptide-binding domain that contains a  $\beta$ -sandwich subdomain

with a peptide-binding cleft and an  $\alpha$ -helical latch-like segment (Zhu et al., 1996; Hartl and Hayer-Hartl, 2002).

HSP70s are involved in microbial pathogenesis, cell death responses, and immune responses. Diverse RNA viruses induce HSP70 expression in *Arabidopsis* (*Arabidopsis thaliana*; Whitham et al., 2003). Cytoplasmic HSP70s enhance the infection of *Nicotiana benthamiana* by *Tobacco mosaic virus*, *Potato virus X*, *Cucumber mosaic virus*, and *Watermelon mosaic virus* (Chen et al., 2008). Recently, the coat protein of *Tomato yellow leaf curl virus* was suggested to recruit host plant HSP70 during virus infection (Gorovits et al., 2013). HSP70s appear to be involved in regulating viral reproduction, protein folding, and movement, which ultimately promotes viral infection (Boevink and Oparka, 2005; Hafrén et al., 2010). The *Pseudomonas syringae* effector protein HopI1 directly binds and manipulates host HSP70, which promotes bacterial virulence (Jelenska et al., 2010). The cytosolic/nuclear heat shock cognate 70 (HSC70) chaperone, which is highly homologous to HSP70 (Tavaria et al., 1996), regulates *Arabidopsis* immune responses together with SGT1 (for the suppressor of the G2 allele of *S-phase kinase-associated protein1* [*skp1*]; Noël et al., 2007). Cytoplasmic HSP70 is required for the *Phytophthora infestans* INF1-mediated hypersensitive response (HR) and nonhost resistance to *Pseudomonas cichorii* in *N. benthamiana* (Kanzaki et al., 2003). HSP70 is proposed to be involved in both positive and negative regulation of cell death. Selective HSP70 depletion from human cell lines activates a tumor-specific death program that is independent of known caspases

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and p53 tumor-suppressor protein (Nylandsted et al., 2000), whereas HSP70 promotes tumor necrosis factor-mediated apoptosis by binding I $\kappa$ B kinase  $\gamma$  and impairing nuclear factor- $\kappa$ B signaling in Cos-1 cells (Ran et al., 2004). In *N. benthamiana*, HSP70 is required for tabtoxinine- $\beta$ -lactam-induced cell death (Ito et al., 2014). However, HSP70 expression is shown to decrease the cell death triggered by salicylic acid (SA) in *Nicotiana tabacum* protoplasts (Cronjé et al., 2004). Overexpression of mitochondrial HSP70 suppresses heat- and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)-induced programmed cell death in rice (*Oryza sativa*; Qi et al., 2011).

The genus *Xanthomonas* YopJ-like AvrBsT protein activates effector-triggered immunity (ETI) in Arabidopsis Pitzal 0 plants (Cunnac et al., 2007). AvrBsT is a member of the YopJ/AvrRxv family identified in *Xanthomonas campestris* pv *vesicatoria* (*Xcv*; Lewis et al., 2011). AvrBsT alters phospholipid signaling and activates defense responses in Arabidopsis (Kirik and Mudgett, 2009). AvrBsT is an acetyltransferase that acetylates Arabidopsis ACETYLATED INTERACTING PROTEIN1 (ACIP1), a microtubule-associated protein required for plant immunity (Cheong et al., 2014). *Xcv* strain Bv5-4a secretes the AvrBsT type III effector protein that induces hypersensitive cell death and strong defense responses in pepper (*Capsicum annuum*) and *N. benthamiana* (Orth et al., 2000; Escobar et al., 2001; Kim et al., 2010). AvrBsT-induced HR-like cell death in pepper is likely part of the typical ETI-mediated defense response cascade (Jones and Dangl, 2006; Eitas et al., 2008; Eitas and Dangl, 2010). AvrBsT overexpression in Arabidopsis triggers plant cell death and defense signaling, leading to both disease and defense responses to diverse microbial pathogens (Hwang et al., 2012). Type III effectors such as HopI1 and AvrBsT are used to identify unknown components of plant defense cascades (Nomura et al., 2006; Block et al., 2008; Jelenska et al., 2010; Kim et al., 2014) that modulate host innate immunity to achieve disease resistance. The pepper SGT1 was identified recently as a host interactor of AvrBsT (Kim et al., 2014). Pepper SGT1 has features of a cochaperone (Shirasu and Schulze-Lefert, 2003), interacts with AvrBsT, and promotes hypersensitive cell death associated with the pepper receptor-like cytoplasmic protein kinase1 (PIK1) phosphorylation cascade.

In this study, we used a yeast (*Saccharomyces cerevisiae*) two-hybrid screen to identify the pepper HSP70a (CaHSP70a) as an interacting partner of the *Xanthomonas* spp. type III effector AvrBsT. Coimmunoprecipitation and bimolecular fluorescence complementation (BiFC) analyses verify that CaHSP70a interacts with AvrBsT in planta. Transient CaHSP70a overexpression in pepper leaves enhances heat stress sensitivity and leads to a cell death response. Cytoplasmic localization of the AvrBsT-CaHSP70a complex strongly elevates cell death. CaHSP70a expression is rapidly and strongly induced by avrBsT (for avirulent *Xcv* Dksol [Ds1]) infection in pepper. CaHSP70a silencing enhances susceptibility to *Xcv* infection, attenuates the reactive oxygen species (ROS) burst and cell death response, reduces SA and jasmonic

acid (JA) levels, and disrupts expression of the defense response genes *C. annuum* pathogenesis-related protein1 (*CaPR1*; Kim and Hwang, 2000), *CaPR10* (Choi et al., 2012), and *CaDEF1* (for defensin; Do et al., 2004). Taken together, this study demonstrates that CaHSP70a is a target of the *Xanthomonas* spp. type III effector AvrBsT and acts as a positive regulator of plant cell death and immunity signaling.

## RESULTS

### Identification of CaHSP70a

AvrBsT is an *Xcv* type III effector protein that triggers HR in pepper and *N. benthamiana* leaves (Kim et al., 2010). We performed yeast two-hybrid screens to identify proteins that interact with AvrBsT. Using AvrBsT as bait, we screened a pepper complementary DNA (cDNA) prey library generated from leaves undergoing HR to the avirulent *Xcv* Ds1 (*avrBsT*) strain. One of the AvrBsT-interacting proteins encoded HSP70 (Fig. 1A; Supplemental Figs. S1 and S2). This clone was designated *CaHSP70a*.

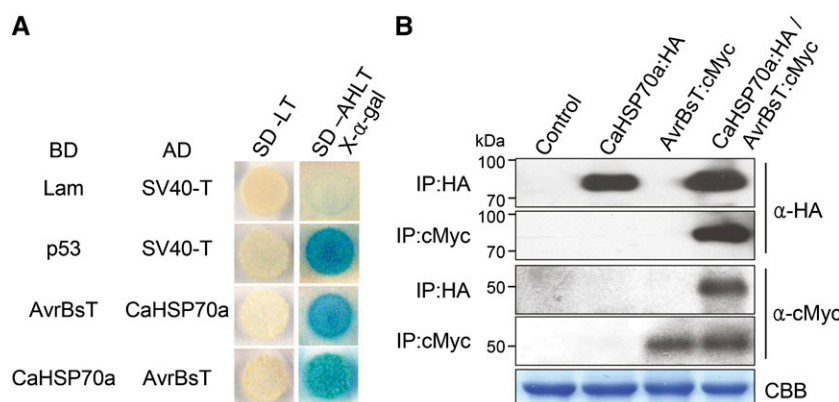
### CaHSP70a Expression Profile

RNA gel-blot analyses were performed to investigate *CaHSP70a* expression profiles in pepper plants during *Xcv* infection. *CaHSP70a* transcription was strongly induced in pepper leaves during avirulent (incompatible) *Xcv* infection compared with those of the mock control and virulent (compatible) *Xcv* infection (Supplemental Fig. S3A). In the incompatible interaction, *CaHSP70a* expression levels were significantly higher 5 h after inoculation, and the high expression levels were maintained until 20 h after inoculation (Supplemental Fig. S3A).

CaHSP70a proteins were immunodetected in the protein extracts of pepper leaves infected with *Xcv* Ds1 or *Xcv* Ds1 (*avrBsT*) using anti-CaHSP70a antibodies (Supplemental Fig. S3B). *Xcv* infection of pepper leaves significantly induced *CaHSP70a* expression at the protein level; however, CaHSP70a proteins were not detected in healthy and mock-treated leaves. Notably, the CaHSP70a protein level 5 h after avirulent *Xcv* Ds1 (*avrBsT*) infection was highest among the CaHSP70a levels at different infection time points.

### CaHSP70a Interacts with AvrBsT in Vitro and in Planta

To investigate whether CaHSP70a interacts with AvrBsT in yeast and in planta, we swapped vectors and generated a DNA-binding domain (BD) fused with CaHSP70a and an activation domain (AD) fused with AvrBsT. We transformed these constructs into yeast with positive-control and negative-control vector pairs. AD-AvrBsT and BD-CaHSP70a interacted with each other and grew on selection medium, as did BD-AvrBsT and AD-CaHSP70a (Fig. 1A). To test whether CaHSP70a and AvrBsT interact



**Figure 1.** CaHSP70a interacts with AvrBsT in yeast and plants. **A**, Yeast two-hybrid analysis of CaHSP70a and AvrBsT. CaHSP70a and AvrBsT fused with the AD or BD domain of GAL4 were cointroduced into *Saccharomyces cerevisiae* strain AH109, and reporter gene activation was monitored on SD-adenine-His-Leu-Trp (AHLT) medium containing 5-bromo-4-chloro-3-indolyl α-galactopyranoside (X-α-gal). Combinations of Lam and p53 with SV40-T were used as negative and positive controls, respectively. Lam, Laminin; p53, tumor protein p53; SV40-T, simian virus 40 large T antigen. **B**, Coimmunoprecipitation analyses of transiently expressed CaHSP70a:HA and AvrBsT:cMyc in *N. benthamiana* leaves. Extracted proteins were immunoprecipitated (IP) with anti-HA or anti-cMyc beads and immunoblotted with anti-HA and anti-cMyc antibodies.

in planta, we transiently expressed CaHSP70a:HA (for hemagglutinin) and AvrBsT:cMyc fusion proteins under the control of the cauliflower mosaic virus 35S promoter in *N. benthamiana* leaves and performed coimmunoprecipitation assays. CaHSP70a coimmunoprecipitated with AvrBsT in total protein extracts of *N. benthamiana* leaves coexpressing CaHSP70a:HA and AvrBsT:cMyc but not in extracts of leaves expressing only CaHSP70a:HA (Fig. 1B). This indicates that AvrBsT can be immunoprecipitated from the total protein extract only when CaHSP70a is present.

#### Transient CaHSP70a Overexpression Stimulates Cell Death during Heat Stress

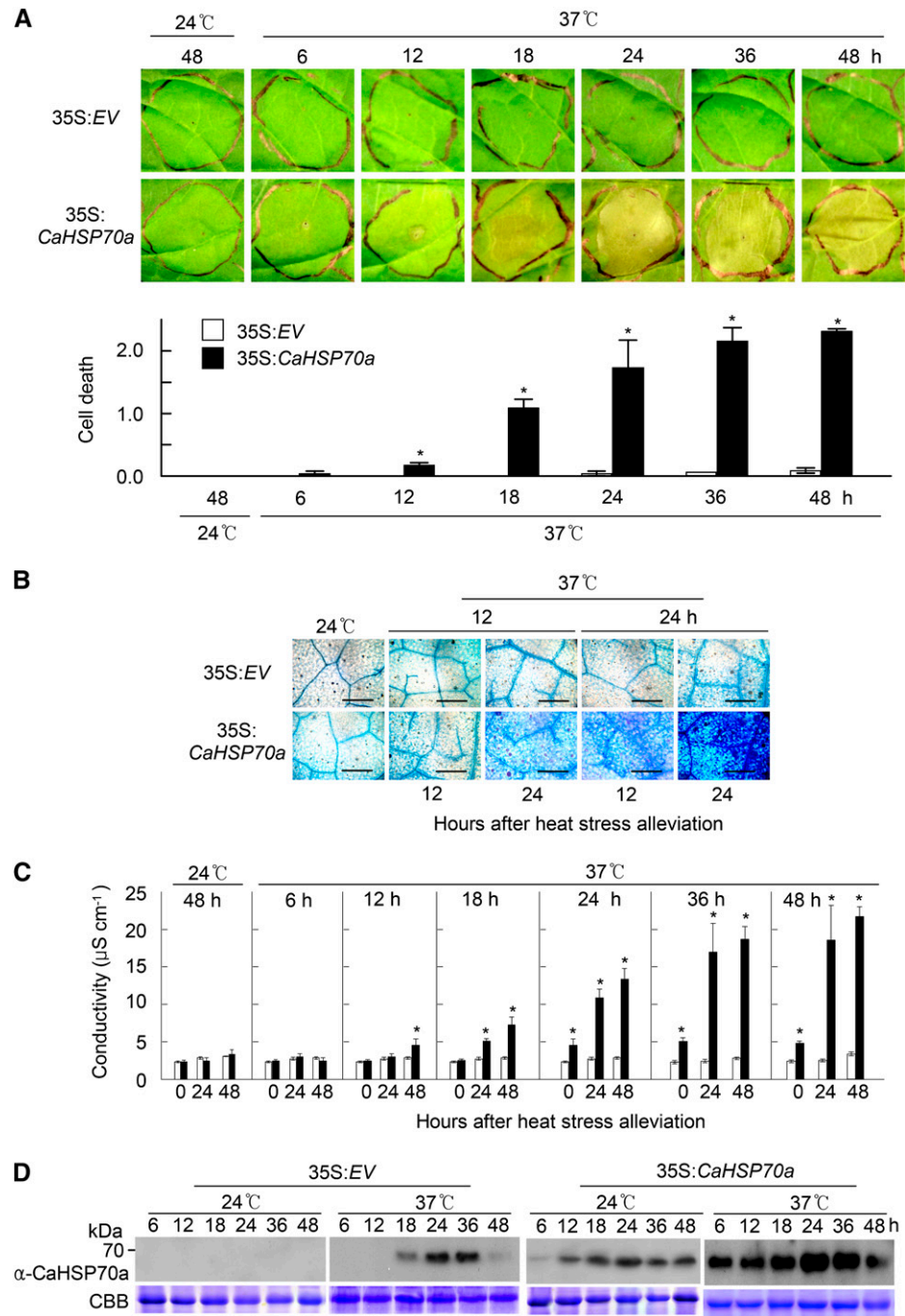
To define the role of CaHSP70a as a heat shock protein during heat stress, pepper leaves were infiltrated (optical density at 600 nm [OD<sub>600</sub>] = 0.5) with *Agrobacterium tumefaciens* expressing CaHSP70a under the control of a constitutive 35S promoter. The agroinfiltrated plants were placed at 37°C for 48 h and then returned to 24°C. Pepper leaves expressing CaHSP70a that received heat stress for 12 h started to show necrotic lesions 24 h after returning to 24°C (Fig. 2A). More prolonged exposure to heat stress exacerbated the severity of cell death, reaching maximum after 24 h of heat stress. Control plants infiltrated with the empty vector did not produce any necrotic lesions. Trypan Blue staining of leaves demonstrated that 37°C heat stress induces the cell death response in CaHSP70a-expressing leaves (Fig. 2B). The extent of cell death was quantified by measuring electrolyte leakage (Fig. 2C). By 48 h after alleviation of 12-h heat stress, electrolyte leakage was significantly greater in leaves transiently expressing CaHSP70a compared with that for the empty-vector control. This result was consistent with the observed cell death phenotypes. Heat

stress for more than 24 h caused significantly greater electrolyte leakage in leaves expressing CaHSP70a than in those expressing the empty-vector control; this result was observed as soon as the heat stress was stopped (Fig. 2C). Immunoblot analysis with anti-CaHSP70a was used to investigate whether 37°C heat stress induced CaHSP70a expression in pepper leaves (Fig. 2D). The results show that 37°C heat stress, but not 24°C, induced CaHSP70a expression 18 to 36 h after infiltration with *A. tumefaciens* carrying empty-vector control (35S:EV). In contrast, transient 35S:CaHSP70a expression at 24°C and 37°C was detected in pepper leaves 6 to 48 h after agroinfiltration. CaHSP70a protein levels were higher in CaHSP70a-expressing leaves during 37°C heat stress compared with that in leaves at 24°C (Fig. 2D).

#### ATPase- and Peptide-BDs of CaHSP70a Are Required to Trigger Cell Death during Heat Stress

To investigate whether the actin-like ATPase- and peptide-BDs of CaHSP70a are involved in triggering cell death during heat stress, we generated N-terminal and C-terminal deletion constructs under the control of the 35S promoter (Fig. 3A). The full-length and deletion constructs were agroinfiltrated in pepper leaves, and immunodetection assays were used to monitor transient gene expression (Fig. 3B). The immunoblot analysis revealed that full-length CaHSP70a was expressed during 37°C heat stress. Transient expression of 35S:CaHSP70a 195–648 induced a cell death response at 37°C similar to that induced by full-length CaHSP70a (Fig. 3C). Visual observations of cell death were substantiated by the results of electrolyte leakage analysis (Fig. 3D). Transient expression of 35S:CaHSP70a 195–648, but not other CaHSP70a deletion mutants, significantly increased electrolyte leakage from pepper leaf discs. Taken together,

**Figure 2.** Transient *CaHSP70a* expression during heat stress triggers cell death in pepper leaves. **A**, Cell death phenotypes and quantification. Pepper plants were exposed to 37°C for varying times and photographed 2 d after infiltration with *A. tumefaciens* carrying binary vector constructs ( $OD_{600} = 0.5$ ). Cell death levels were rated based on a 0 to 3 scale: 0, no cell death (less than 10%); 1, weak cell death (10%–30%); 2, partial cell death (30%–80%); and 3, full cell death (80%–100%). **B**, Trypan Blue staining of leaves infiltrated with *A. tumefaciens*. **C**, Electrolyte leakage from leaf discs infiltrated with *A. tumefaciens* carrying the indicated constructs. Data represent means  $\pm$  SD from three independent experiments. Asterisks indicate statistically significant differences (Student's *t* test;  $P < 0.05$ ). **D**, Immunoblot analyses of transient *CaHSP70a* expression. Protein loading was visualized by Coomassie Brilliant Blue (CBB) staining.



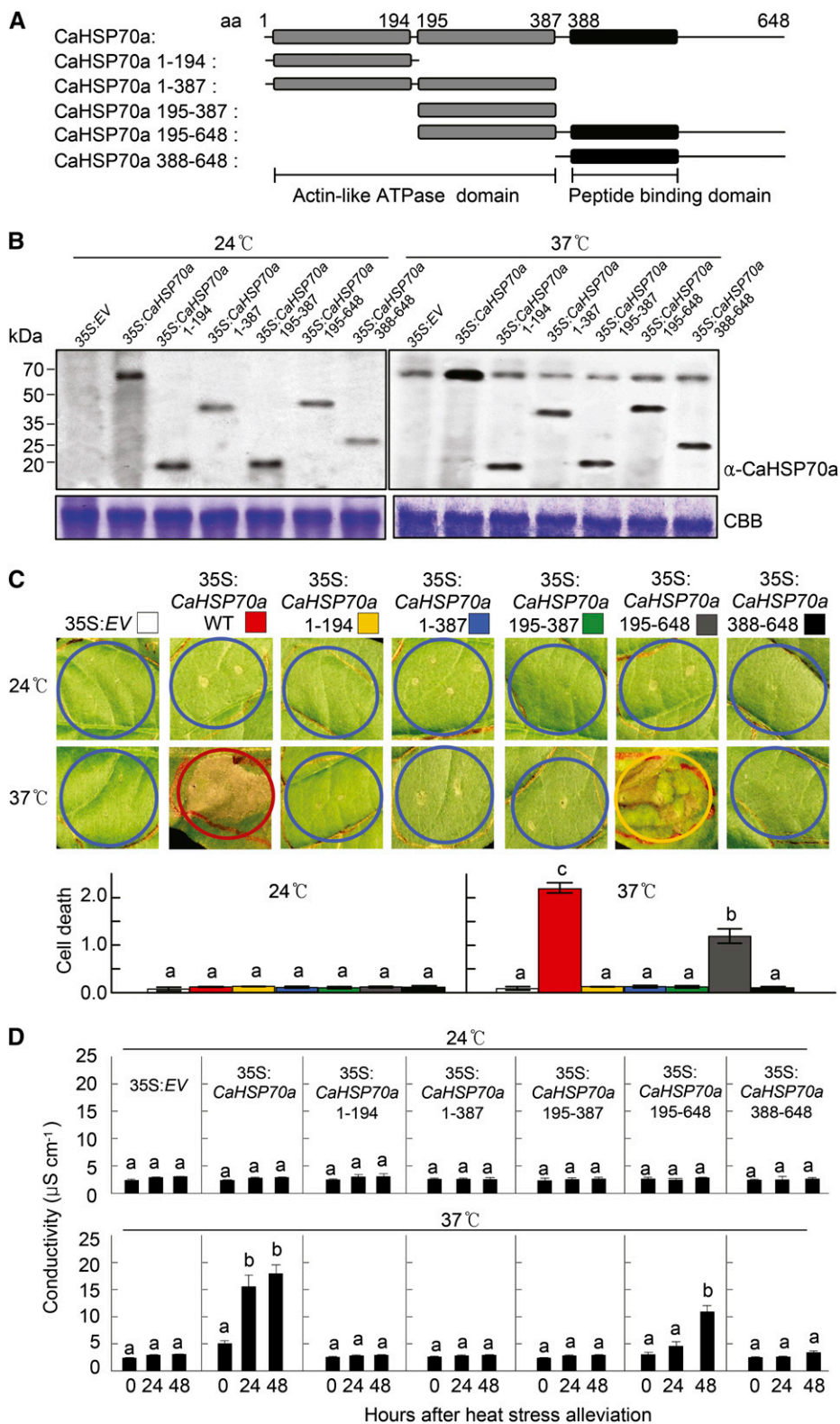
these results indicate that the actin-like ATPase- and peptide-BDs are required to trigger cell death during heat stress.

#### Cytoplasmic Localization of CaHSP70a Is Required to Induce Cell Death during Heat Stress and *Xcv* Infection

The subcellular localization of GFP-*CaHSP70a* and GFP-*AvrBsT* fusion constructs in *N. benthamiana* leaves

was visualized using a confocal microscope (Fig. 4A). *AvrBsT* and *CaHSP70a* were detected in the cytoplasm and nucleus in planta. *AvrBsT* and *CaHSP70a* fusion constructs with nuclear localization signal (NLS) or nuclear export signal (NES) were used to restrict their localization to a single cellular compartment. The confocal micrographs revealed that the NLS and NES signals targeted *AvrBsT*:NLS:GFP and *CaHSP70a*:NLS:GFP to nuclei and targeted *AvrBsT*:NES:GFP and *CaHSP70a*:NES:GFP to the cytoplasm (Fig. 4A). Immunoblots

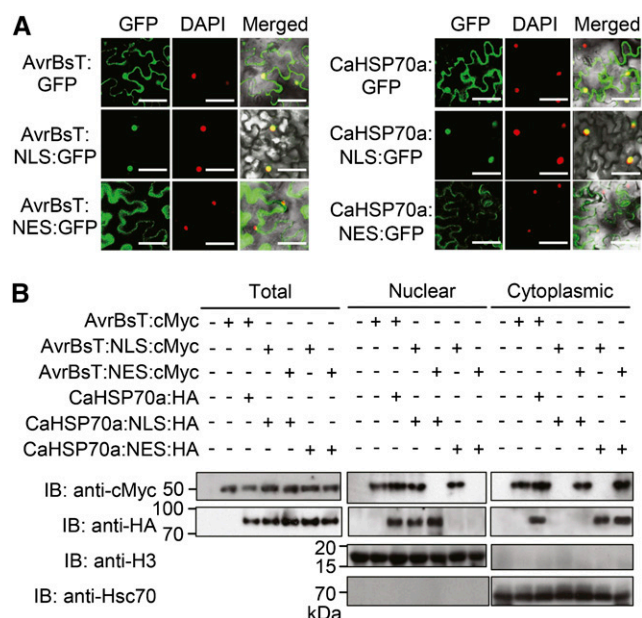




**Figure 3.** Deletion analysis of the actin-like ATPase- and peptide-binding domains of *CaHSP70a* in pepper leaves. **A**, Schematic diagrams of *CaHSP70a* deletion mutants. aa, Amino acids. **B**, Immunoblot analyses of *CaHSP70a*-deletion mutant transient expression. Protein loading was visualized by Coomassie Brilliant Blue (CBB) staining. **C**, Cell death phenotypes. Pepper plants were exposed to 37°C for varying times and photographed 2 d after infiltration with *A. tumefaciens* carrying binary vector constructs ( $\text{OD}_{600} = 0.5$ ). Cell death levels were rated based on a 0 to 3 scale: 0, no cell death (less than 10%); 1, weak cell death (10%–30%); 2, partial cell death (30%–80%); and 3, full cell death (80%–100%). WT, Wild type. **D**, Electrolyte leakage from leaf discs infiltrated with *A. tumefaciens* carrying the indicated constructs. Data represent means  $\pm$  SD from three independent experiments. Different letters indicate statistically significant differences (LSD,  $P < 0.05$ ).

showed that NLS and NES fusion constructs were detected in the nuclear and cytoplasmic fractions, respectively (Fig. 4B). HISTONE3 (H3) and HEAT SHOCK COMPLEX70 (Hsc70) were used as markers for the nuclear and cytoplasmic fractions, respectively.

NLS or NES sequences were fused to 35S:*CaHSP70a* to restrict localization to the nucleus or cytoplasm, respectively. Empty vector, *CaHSP70a*, *CaHSP70a*:NLS, and *CaHSP70a*:NES were agroinfiltrated ( $\text{OD}_{600} = 0.5$ ) and transiently expressed in pepper leaves under the control



**Figure 4.** Subcellular localization of NLS or NES fusion AvrBsT and CaHSP70a constructs in *N. benthamiana* cells. A, Confocal images show transient expression of AvrBsT:GFP, AvrBsT:NLS:GFP, AvrBsT:NES:GFP, CaHSP70a:GFP, CaHSP70a:NLS:GFP, and CaHSP70a:NES:GFP in *N. benthamiana* cells. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Bars = 50  $\mu$ m. B, Immunoblot (IB) analysis of total, nuclear, and cytoplasmic fractions of AvrBsT:cMyc, AvrBsT:NLS:cMyc, AvrBsT:NES:cMyc, CaHSP70a:HA, CaHSP70a:NLS:HA, and CaHSP70a:NES:HA proteins transiently expressed in *N. benthamiana* leaves. Anti-H3 and anti-Hsc70 antibodies were used to detect nuclear histone and cytoplasmic Hsc70 proteins, respectively.

of the constitutive 35S promoter at 37°C. Expression of these constructs was immunodetected in the subcellular fractions (Fig. 4B). Transient expression of CaHSP70a and CaHSP70a:NES induced cell death lesions in pepper leaves treated with heat stress for 24 h (Fig. 5A). However, CaHSP70a:NLS expression did not induce any necrotic lesions after 12 h of heat stress, with only a small lesion appearing after 24 h. These results suggest that cytoplasmic localization of CaHSP70a is required to induce cell death during heat stress. Trypan Blue staining showed that CaHSP70a- and CaHSP70a:NES-induced cell death was significantly greater in pepper leaves exposed to 37°C (Fig. 5B). The extent of cell death was quantified by measuring electrolyte leakage from pepper leaf discs (Fig. 5C). Transient expression of CaHSP70a and CaHSP70a:NES significantly increased electrolyte leakage compared with that of the empty-vector control and that of CaHSP70a:NLS-expressing cells 24 to 48 h after alleviation of 24-h heat stress (Fig. 5C). This result was consistent with the observed cell death phenotypes. Together, these results indicate that cytoplasmic localization of CaHSP70a is important for cell death induction during heat stress.

We next monitored CaHSP70a protein levels in nuclear and cytoplasmic fractions of the protein extracts from leaves infected with *Xcv* Ds1 or *Xcv* Ds1 (*avrBsT*;

Supplemental Fig. S3C). As positive control markers, H3 and Hsc70 proteins were detected in the nuclear and cytoplasmic fractions, respectively. CaHSP70a proteins were weakly expressed in the nuclear fractions of pepper leaves during *Xcv* infection. However, cytoplasmic CaHSP70a protein levels were much higher than the nuclear CaHSP70a ones (Supplemental Fig. S3C). Notably, high levels of CaHSP70a proteins were detected in the cytoplasmic fractions 5 and 10 h after inoculation with avirulent *Xcv* Ds1 (*avrBsT*), which causes the HR cell death response in pepper leaves. These results suggest that cytoplasmic CaHSP70a proteins contribute positively to the cell death induction by *Xcv* Ds1 (*avrBsT*) infection.

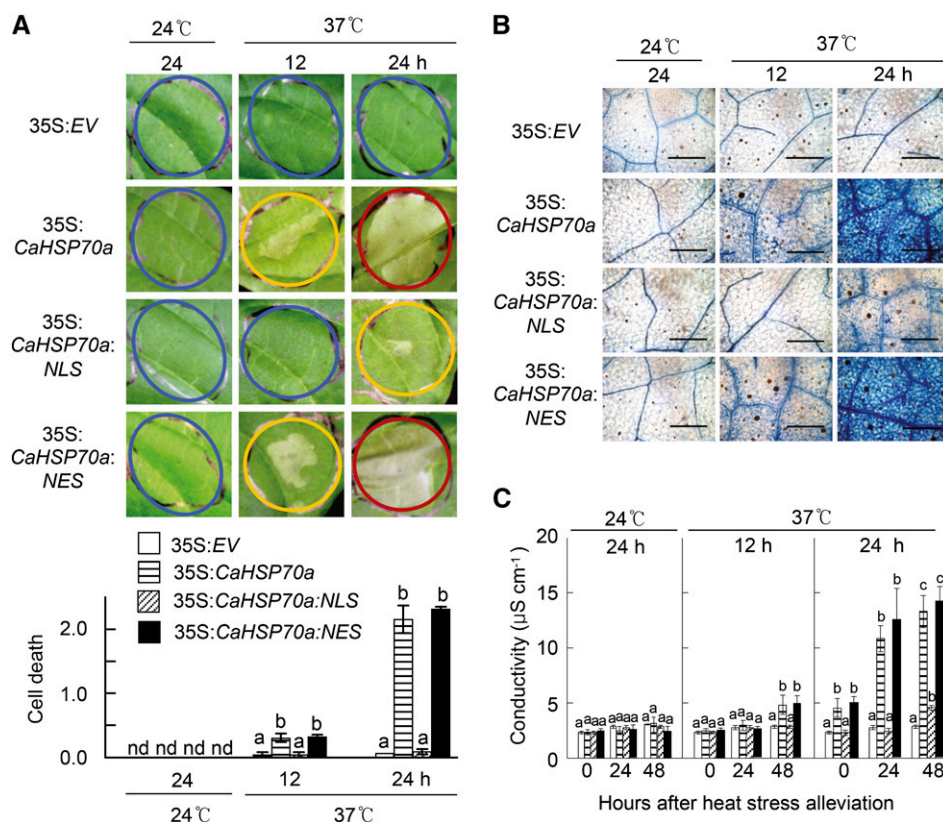
### Transient CaHSP70a Expression Induces Cell Death and Defense Response Genes

Quantitative real-time reverse transcription (RT)-PCR analyses were used to investigate whether CaHSP70a expression induces cell death and defense response genes in pepper (Fig. 6). Transient CaHSP70a expression at 37°C distinctly induced *CaPR1* (Kim and Hwang, 2000), *CaPIK1* (Kim and Hwang, 2011), and *RNA-BINDING PROTEIN1* (*CaRBP1*; Lee et al., 2012) compared with that of the empty-vector control (35:EV). *CaPR1*, *CaPIK1*, and *CaRBP1* were distinctly induced by transient CaHSP70a expression after 18 h of heat stress (Supplemental Fig. S4). These results indicate that 37°C heat stress promotes CaHSP70a-triggered expression of cell death and defense response genes.

### Transient CaHSP70a Expression Promotes an *avrBsT*-Triggered Hypersensitive Cell Death Response

CaHSP70a was constitutively expressed in healthy pepper leaves but was rapidly induced in pepper leaves during the hypersensitive cell death response to avirulent (incompatible) *Xcv* Bv5-4a carrying *avrBsT* (Supplemental Fig. S3). To define the role of CaHSP70a in AvrBsT-triggered hypersensitive cell death, CaHSP70a and *avrBsT* were agroinfiltrated into *N. benthamiana* leaves at the lower limit of *A. tumefaciens* titer ( $OD_{600} = 0.05$ ) that induces *avrBsT*-triggered cell death (Fig. 7). The B-cell lymphoma2-associated X (BAX) protein that induces plant cell death (Lacomme and Santa Cruz, 1999) was used as a positive control. Transient CaHSP70a expression resulting from infiltration with *A. tumefaciens* at  $OD_{600} = 0.05$  and  $OD_{600} = 0.2$  did not induce the cell death response (Fig. 7A). Transient *avrBsT* expression resulting from infiltration with *A. tumefaciens* at  $OD_{600} = 0.05$  did not induce typical cell death in *N. benthamiana* leaves. However, coexpression of *avrBsT* with CaHSP70a resulting from infiltration with *A. tumefaciens* at  $OD_{600} = 0.05$  produced a severe cell death response, similar to that induced by *avrBsT* expression resulting from infiltration with *A. tumefaciens* at  $OD_{600} = 0.2$  (Fig. 7A). By contrast, coexpression of Bax with CaHSP70a resulting from infiltration with *A. tumefaciens* at  $OD_{600} = 0.05$  did not produce a cell death response. The extent of cell death was quantified by





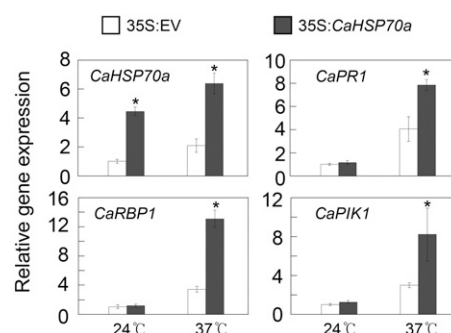
**Figure 5.** Cytoplasmic localization of CaHSP70a during heat stress triggers cell death in pepper leaves. A, Cell death phenotypes and quantification. Pepper plants were exposed to 37°C for varying times and photographed 2 d after infiltration with *A. tumefaciens* carrying binary vector constructs (OD<sub>600</sub> = 0.5). Cell death levels were rated based on a 0 to 3 scale: 0, no cell death (less than 10%); 1, weak cell death (10%–30%); 2, partial cell death (30%–80%); and 3, full cell death (80%–100%). nd, Not detected. B, Trypan Blue staining. C, Electrolyte leakage from leaf discs infiltrated with *A. tumefaciens* carrying the indicated constructs. Data represent means ± SD from three independent experiments. Different letters indicate statistically significant differences (Student's *t* test, *P* < 0.05).

measuring electrolyte leakage from *N. benthamiana* leaf discs (Fig. 7B). Transient CaHSP70a expression alone did not cause electrolyte leakage, similar to that of the empty-vector control discs. This was consistent with the observed cell death phenotypes. Leaf tissues coexpressing CaHSP70a and *avrBsT* (*A. tumefaciens* at OD<sub>600</sub> = 0.05) showed high levels of electrolyte leakage, similar to that of leaves transiently expressing *avrBsT* (*A. tumefaciens* at OD<sub>600</sub> = 0.2; 24-h agroinfiltration). Coexpression of Bax with CaHSP70a (*A. tumefaciens* at OD<sub>600</sub> = 0.05) did not produce higher levels of electrolyte leakage. Immunoblot analysis confirmed that CaHSP70a, AvrBsT, Bax, CaHSP70a/Bax, and CaHSP70a/AvrBsT proteins were distinctly expressed (Fig. 7C). Collectively, these results indicate that CaHSP70a positively regulates the AvrBsT-triggered hypersensitive cell death response.

#### Cytoplasmic Localization of CaHSP70a and AvrBsT Promotes *avrBsT*-Triggered Cell Death

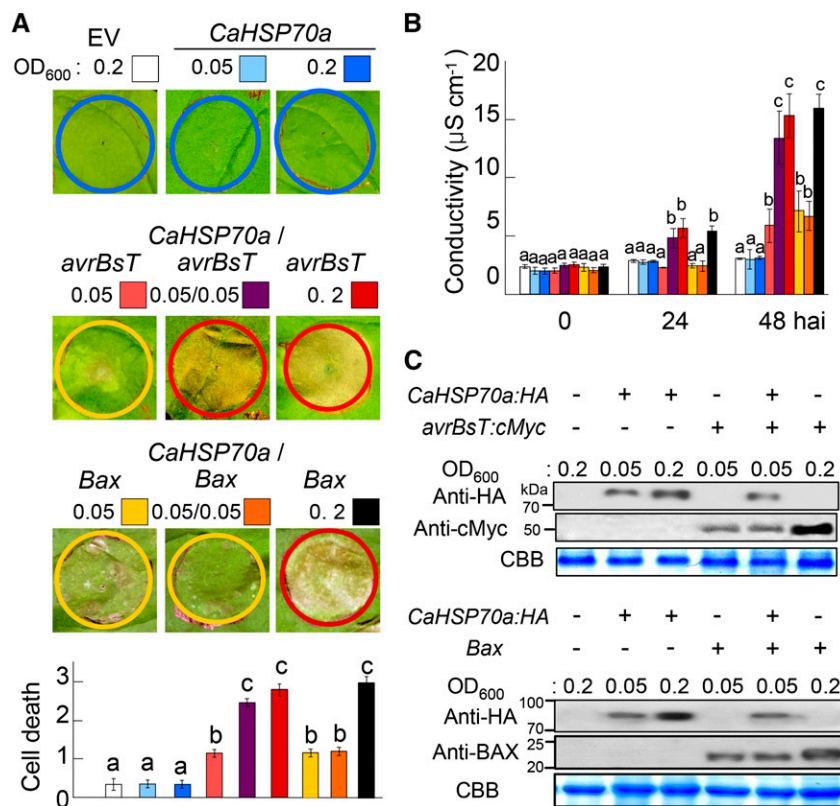
BiFC assays were used to investigate AvrBsT and CaHSP70a interaction in planta. CaHSP70a and AvrBsT were fused to the C-terminal 84-amino acid Super Cyan Fluorescent Protein 3A (SCFP3A) region and the N-terminal 173-amino acid Venus region, respectively (Waadt et al., 2008). *A. tumefaciens* cells harboring the corresponding constructs were mixed and coinfiltrated into *N. benthamiana* leaves. Confocal microscopy of *N. benthamiana* epidermal cells shows that AvrBsT and

CaHSP70a interact in the cytoplasm and nucleus in planta (Fig. 8A). To determine whether subcellular localization of CaHSP70a affects AvrBsT-triggered cell death, NES and NLS were fused to CaHSP70a and AvrBsT. When CaHSP70a:NLS:SCFP3A Y C-terminus (SCYCE) and Venus Y N-terminus (VYNE):AvrBsT:NLS were expressed, BiFC signals were localized to the nucleus. By contrast, transient expression of CaHSP70a:NES:SCYCE and VYNE:AvrBsT:NES resulted in cytoplasmic localization of the BiFC signals (Fig. 8A). Transient expression of *avrBsT*:NES, but not *avrBsT*:NLS, induced a severe cell death response similar to that induced



**Figure 6.** Quantitative real-time RT-PCR analysis of expression of CaHSP70a and defense- and cell death-related genes in pepper leaves transiently expressing CaHSP70a. Expression values are normalized by the expression level of 18S rRNA.

**Figure 7.** Transient *CaHSP70a* expression promotes *avrBsT*-triggered cell death in *N. benthamiana* leaves. **A**, Cell death phenotypes and quantification in leaves 2 d after infiltration with *A. tumefaciens* carrying empty vector (EV), *CaHSP70a*, *Bax*, and *avrBsT* at different inoculum ratios. Cell death levels were rated based on a 0 to 3 scale: 0, no cell death (less than 10%); 1, weak cell death (10%–30%); 2, partial cell death (30%–80%); and 3, full cell death (80%–100%). **B**, Electrolyte leakage from leaf discs at different time points after agroinfiltration. **C**, Immunoblot analyses of the transient expression of *CaHSP70a*, *Bax*, and *avrBsT*. Protein loading was visualized by Coomassie Brilliant Blue (CBB) staining. Data represent means  $\pm$  SD from three independent experiments. Different letters indicate statistically significant differences (LSD,  $P < 0.05$ ).



by transient *avrBsT* expression (Supplemental Fig. S5A). Figure 8B shows that nuclear colocalization of *CaHSP70A*:NLS and *AvrBsT*:NLS did not promote *AvrBsT*-triggered cell death. However, cytoplasmic colocalization of *CaHSP70a*:NES and *AvrBsT*:NES induced a severe cell death response, similar to that induced by transient *CaHSP70a* and *avrBsT* coexpression (Fig. 8B). Transient expression of *CaHSP70a*, *CaHSP70a*:NLS, and *CaHSP70a*:NES did not trigger any cell death response in *N. benthamiana* leaves (Supplemental Fig. S5B). The extent of cell death was quantified by measuring electrolyte leakage from *N. benthamiana* leaf discs transiently coexpressing *CaHSP70a* and/or *avrBsT*. Electrolyte leakage was approximately equivalent in nucleus-localized *CaHSP70A*:NLS and *AvrBsT*:NLS compared with that of cytoplasm-localized *CaHSP70a*:NES and *AvrBsT*:NES (Fig. 8C). This was consistent with observed cell death phenotypes. Cytoplasmic localization of *CaHSP70a* and *AvrBsT* resulted in high levels of electrolyte leakage from *N. benthamiana* leaf discs 24 and 48 h after infiltration with *A. tumefaciens* at OD<sub>600</sub> = 0.05. These results indicate that cytoplasmic interaction of *CaHSP70a* and *AvrBsT* enhances *AvrBsT*-triggered cell death in plants.

#### Actin-Like ATPase- and Peptide-BDs of *CaHSP70a* Are Required to Promote *avrBsT*-Triggered Cell Death

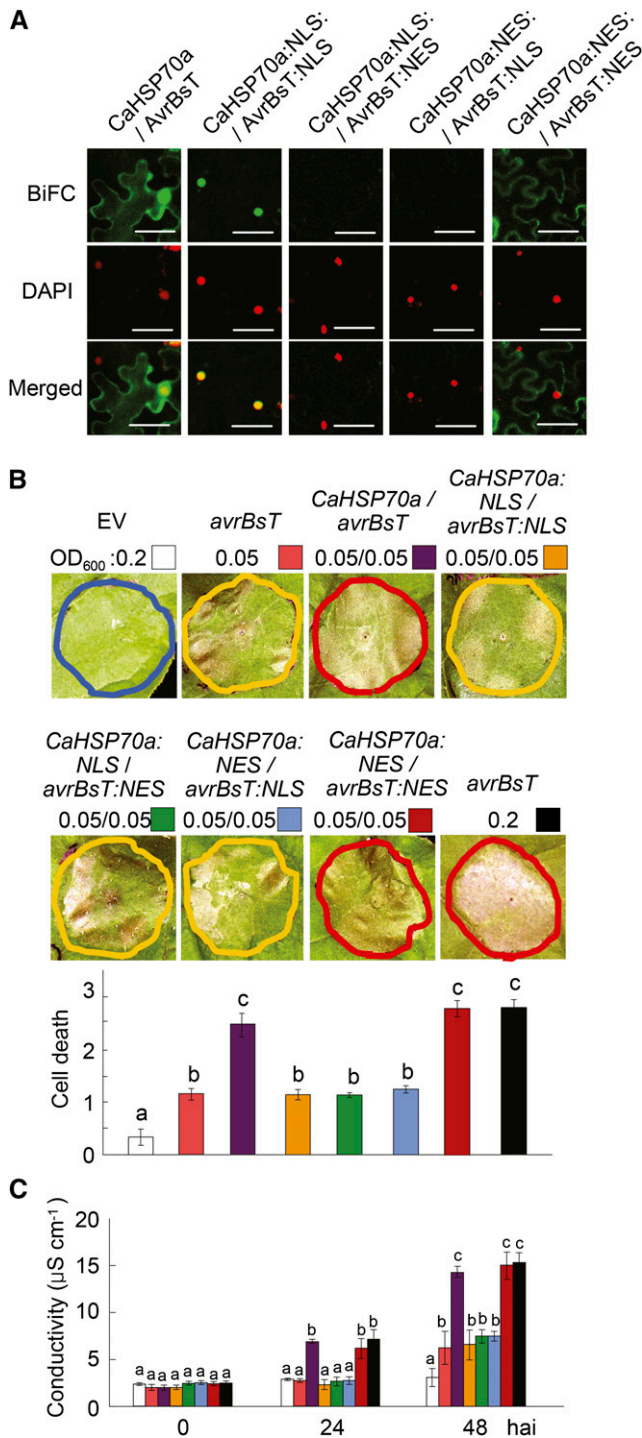
BiFC assays were used to investigate whether *CaHSP70a* domains mediate its interaction with *AvrBsT* in planta. We designed five *CaHSP70a* deletion mutants that selectively

removed the actin-like ATPase- and peptide-BDs. BiFC images showed that *CaHSP70a* 195–648 and *CaHSP70a* 388–648 interacted with *AvrBsT* in the cytoplasm (Fig. 9A), indicating that the peptide-BD was essential for *CaHSP70a* and *AvrBsT* interaction in *N. benthamiana* leaves. *A. tumefaciens*-mediated transient expression of these *CaHSP70a*-deletion mutants was immunodetected in *N. benthamiana* leaves (Fig. 9B). Coimmunoprecipitation analysis revealed that *CaHSP70a* 195–648 or *CaHSP70a* 388–648 specifically interacts with *AvrBsT* in planta. These coimmunoprecipitation data indicate that the peptide-BD of *CaHSP70a* is essential for its interaction with *AvrBsT*. Transient coexpression of *CaHSP70a* 195–648 with *avrBsT* significantly promoted the *avrBsT*-triggered cell death response in *N. benthamiana* leaves (Fig. 9C). The observed cell death phenotypes were substantiated by the electrolyte leakage assay (Fig. 9D). At 48 h after agroinfiltration, electrolyte leakage from leaves transiently expressing *CaHSP70a* 195–648 was significantly greater than that in leaves expressing other *CaHSP70a* deletion mutants. These results suggest that the actin-like ATPase- and peptide-BDs of *CaHSP70a* are required to promote *avrBsT*-triggered cell death in *N. benthamiana* leaves.

#### Silencing of *CaHSP70a* and *CaSGT1* Compromises Defense and HR-Mediated Resistance to *Xcv* Infection

The tobacco rattle virus (TRV)-based virus-induced gene silencing (VIGS) technique was used to investigate *CaHSP70A* loss of function in pepper plants (Liu





**Figure 8.** Cytoplasmic interaction between CaHSP70a and AvrBsT promotes cell death in *N. benthamiana* leaves. **A**, BiFC images of CaHSP70a/AvrBsT, CaHSP70a:NLS/AvrBsT:NLS, CaHSP70a:NES/AvrBsT:NES, and AvrBsT in leaves infiltrated with *A. tumefaciens*. CaHSP70a interacts with AvrBsT in both the cytosol and nucleus. CaHSP70a fused with the C-terminal yellow fluorescent protein (YFP) fragment and AvrBsT fused with the N-terminal YFP fragment were coexpressed in *N. benthamiana* cells. YFP signals were visualized by confocal microscopy. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Bars = 50  $\mu\text{m}$ . **B**, Cell death phenotypes and quantification in leaves 2 d after

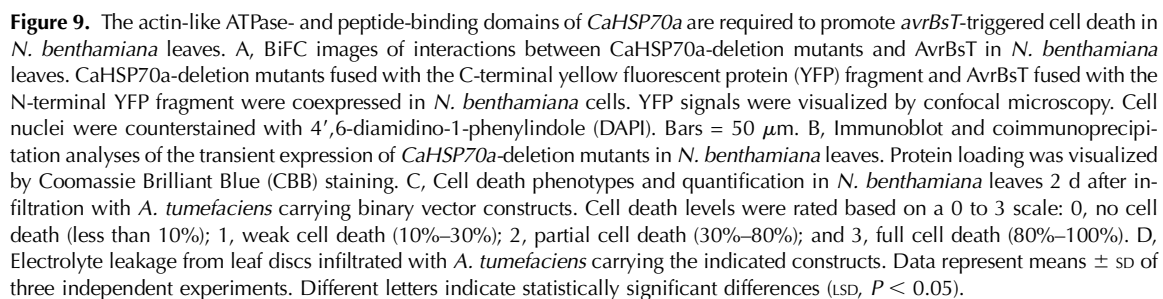
et al., 2002). Leaves of empty vector (TRV:00) and silenced (TRV:CaHSP70a) pepper plants were inoculated with virulent (compatible) Ds1 (EV) and avirulent (incompatible) Ds1 (*avrBsT*) *Xcv* strains ( $10^7$  and  $10^8$  colony-forming units [cfu]  $\text{mL}^{-1}$ ). Silencing of CaHSP70a in pepper plants led to a highly susceptible response to both compatible and incompatible *Xcv* infection (Fig. 10A). Infection with the avirulent Ds1 (*avrBsT*) *Xcv* strain ( $10^8$  cfu  $\text{mL}^{-1}$ ) caused HR in the empty-vector control leaves; however, HR-like cell death was greatly reduced in CaHSP70a-silenced leaves 2 d after inoculation. Reduced cell death was more clearly noticeable when leaves were observed under UV light (Fig. 10A), which revealed that the accumulation of autofluorescent compounds was greatly reduced in CaHSP70a-silenced leaves. *Xcv* growth in CaHSP70a-silenced leaves was approximately 10-fold higher than that in empty-vector control leaves at 3 d after inoculation (Fig. 10B). Collectively, these results indicate that CaHSP70a is required for defense and HR-mediated resistance against *Xcv* infection.

Silencing of CaHSP70a, CaSGT1, and CaHSP70a/CaSGT1 conferred enhanced susceptibility to *Xcv* infection (Supplemental Fig. S6A). *Xcv* virulent Ds1 or avirulent Ds1 (*avrBsT*) grew better in the silenced plants than in the empty-vector control (TRV:00) plants. These results suggest that CaHSP70a and CaSGT1 expression contributes independently to the resistance to *Xcv* infection.

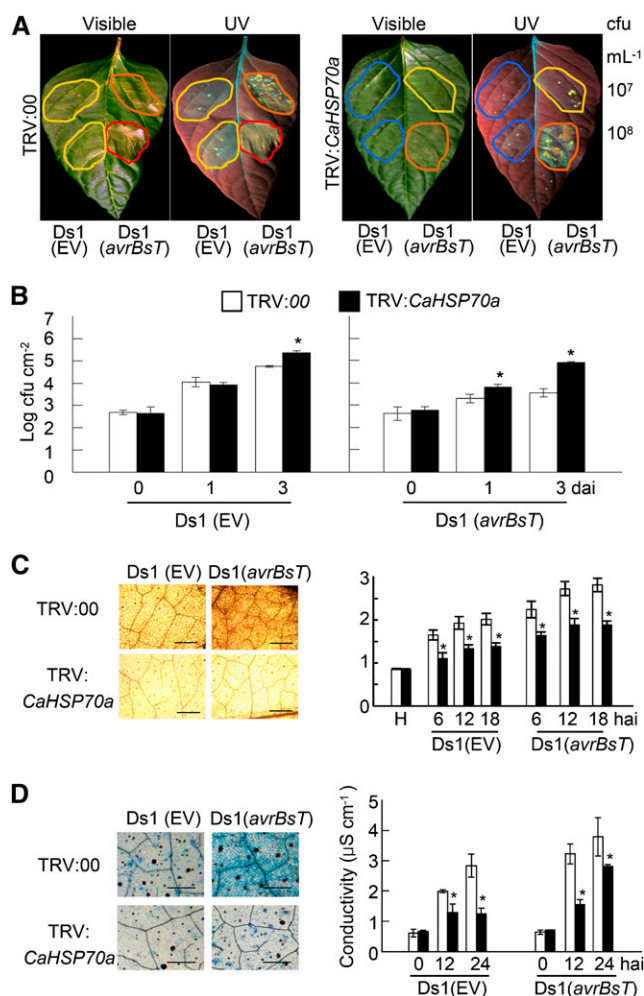
#### Cell Death and ROS Burst Are Attenuated in CaHSP70a-, CaSGT1-, and CaHSP70a/CaSGT1-Silenced Pepper

To investigate whether CaHSP70a regulates defense signaling pathways and induces early defense responses, we analyzed ROS ( $\text{H}_2\text{O}_2$ ) accumulation and the cell death response in empty-vector control and CaHSP70a-silenced pepper leaves during *Xcv* infection (Fig. 10, C and D).  $\text{H}_2\text{O}_2$  production and cell death were visualized by 3,3'-diaminobenzidine (DAB) and Trypan Blue staining, respectively.  $\text{H}_2\text{O}_2$  accumulation and the cell death response were significantly attenuated in CaHSP70a-silenced leaves inoculated with *Xcv*. Xylenol Orange assays and ion conductivity measurements were used to quantify  $\text{H}_2\text{O}_2$  production and cell death, respectively. CaHSP70a silencing significantly reduced  $\text{H}_2\text{O}_2$  accumulation and ion leakage in pepper leaves during *Xcv* infection. Infection by *Xcv* virulent Ds1 or avirulent Ds1 (*avrBsT*) induced lower  $\text{H}_2\text{O}_2$  accumulation and electrolyte leakage from CaHSP70a-, CaSGT1-, or CaHSP70a/CaSGT1-silenced leaves in comparison with empty-vector leaves (Supplemental

infiltration with *A. tumefaciens* carrying the indicated constructs. Cell death levels were rated based on a 0 to 3 scale: 0, no cell death (less than 10%); 1, weak cell death (10%–30%); 2, partial cell death (30%–80%); and 3, full cell death (80%–100%). Data represent means  $\pm$  SD from three independent experiments. Different letters indicate statistically significant differences (LSD,  $P < 0.05$ ). **C**, Electrolyte leakage from leaf discs infiltrated by *A. tumefaciens* carrying the indicated constructs. Data represent means  $\pm$  SD from three independent experiments. Different letters indicate statistically significant differences (LSD,  $P < 0.05$ ).







**Figure 10.** Enhanced susceptibility of *CaHSP70a*-silenced pepper leaves to *Xcv* infection. A, Reduction of disease symptoms on *CaHSP70a*-silenced pepper leaves 2 d after inoculation with *Xcv* Ds1 (EV) and Ds1 (*avrBsT*) ( $10^7$  cfu mL<sup>-1</sup>). Blue circles, No symptoms; yellow circles, symptoms visible only under UV light; orange circles, visible symptoms; red circles, complete cell death. B, Bacterial growth in *Xcv*-infected leaves ( $5 \times 10^4$  cfu mL<sup>-1</sup>). C, DAB staining and H<sub>2</sub>O<sub>2</sub> quantification in *Xcv*-inoculated leaves ( $5 \times 10^7$  cfu mL<sup>-1</sup>). D, Trypan Blue staining and electrolyte leakage measurement in *Xcv*-inoculated leaves ( $5 \times 10^7$  cfu mL<sup>-1</sup>). Data represent means  $\pm$  sd of three independent experiments. Asterisks indicate statistically significant differences (Student's *t* test, *P* < 0.05).

Fig. S6, B and C). Notably, reduction of H<sub>2</sub>O<sub>2</sub> accumulation and electrolyte leakage during *Xcv* infection were greater in *CaHSP70a*/*CaSGT1*-silenced leaves than in *CaHSP70a*- or *CaSGT1*-silenced leaves. Together, these results suggest that *CaHSP70a* and *CaSGT1* coexpression positively regulates cell death and ROS burst in pepper.

#### *CaHSP70a* Expression Positively Regulates Pepper Defense Response Genes

To determine whether *CaHSP70a* silencing alters the expression of pepper defense-related genes during early infection stages, real-time RT-PCR analyses were

performed using gene-specific primer pairs for *CaHSP70a*, *CaPR1*, *CaRBP1*, *CaPIK1*, *CaDEF1*, and *CaPR10* at 12 and 24 h after inoculation with *Xcv* Ds1 (EV; compatible) and Ds1 (*avrBsT*; incompatible) strains (Fig. 11). *CaHSP70a* silencing in pepper leaves significantly attenuated induction of the SA-dependent defense genes *CaPR1* and *CaPR10*, the jasmonate-related gene *CaDEF1*, and *CaRBP1* and *CaPIK1* during *Xcv* infection. These results indicate that *CaHSP70a* is involved in defense signaling during compatible and incompatible *Xcv* interactions with pepper.

#### *CaHSP70a* Is Required for Defense Hormone Signaling in Pepper

To determine if *CaHSP70a* silencing affects defense hormone signaling in response to bacterial infection, we quantified the levels of free and total SA and JA in empty-vector control and *CaHSP70a*-silenced pepper leaves during *Xcv* infection (Fig. 12). Accumulation of free and total SA (free SA plus Glc-conjugated SA) significantly declined in *CaHSP70a*-silenced leaves compared with that in empty-vector control leaves at 12 and 24 h after compatible and incompatible *Xcv* infection. JA accumulation was significantly reduced in *CaHSP70a*-silenced leaves compared with that in empty-vector control leaves at 0, 12, and 24 h after compatible and incompatible *Xcv* infection.

#### *CaHSP70a* Overexpression Does Not Enhance Disease Resistance in Arabidopsis

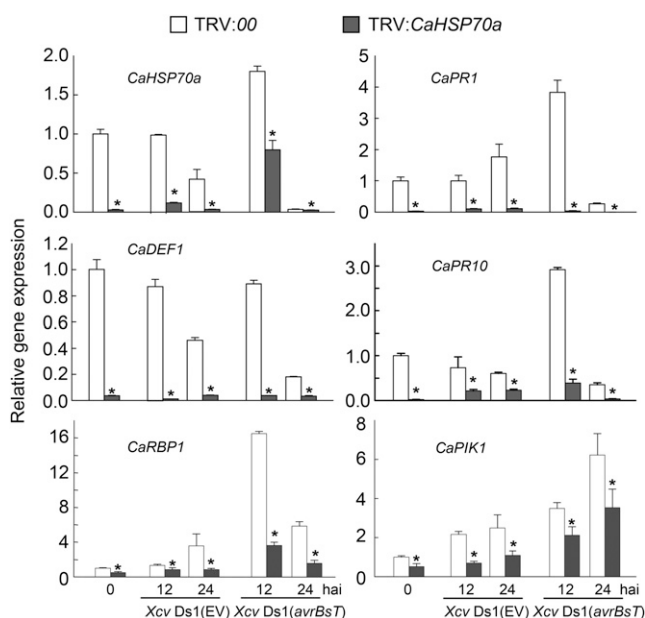
To determine whether ectopic *CaHSP70a* expression induces disease resistance, *CaHSP70a*-overexpressing (OX) transgenic Arabidopsis plants were generated by the floral dip method (Clough and Bent, 1998). Constitutive *CaHSP70a* expression was confirmed in transgenic lines 2, 3, and 4 using RT-PCR (Supplemental Fig. S7A). Four-week-old wild-type and *CaHSP70a*-OX transgenic Arabidopsis plants were infiltrated with *Pseudomonas syringae* pv *tomato* (*Pst*) DC3000 and DC3000 (*avrRpm1*) ( $10^5$  cfu mL<sup>-1</sup>; Supplemental Fig. S7B). There were no significant differences in bacterial titers of *Pst* DC3000 and DC3000 (*avrRpm1*) between wild-type and *CaHSP70a*-OX plants (Supplemental Fig. S7B). Seven-day-old seedlings of wild-type and transgenic Arabidopsis plants were inoculated with *Hyaloperonospora arabidopsidis* Noco2. *CaHSP70a* overexpression did not affect the conidiophore formation of *H. arabidopsidis* (Supplemental Fig. S8).

## DISCUSSION

#### *CaHSP70a* Interacts with AvrBsT in Yeast and in Planta

We showed that the pepper heat shock protein *CaHSP70a* is one of the host targets of AvrBsT, a type III effector protein of pathogenic *Xcv*. AvrBsT contains a putative YopJ-like Ser/Thr acetyltransferase domain





**Figure 11.** Compromised induction of some defense-related genes in *CaHSP70a*-silenced pepper leaves inoculated with *Xcv*. Quantitative real-time PCR was performed for *CaHSP70a*, *CaPR1*, *CaPR10*, and *CaDEF1*. Expression levels of *18S rRNA* were used for the normalization of defense-related gene expression levels. Data represent means  $\pm$  SD of three independent experiments. Asterisks indicate statistically significant differences (Student's *t* test,  $P < 0.05$ ).

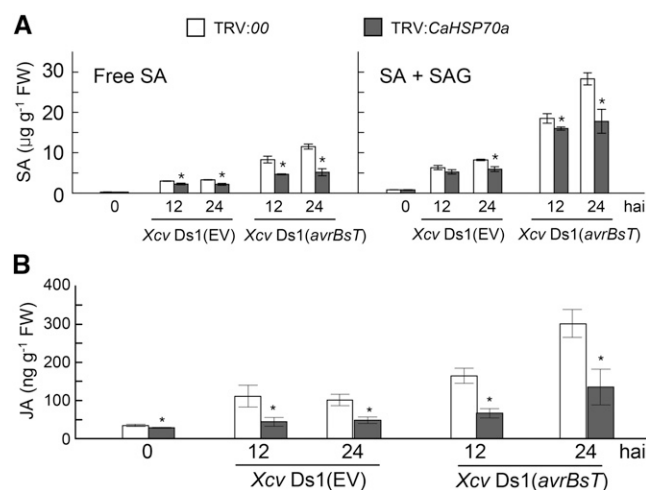
and possesses potent acetyltransferase activity (Mukherjee et al., 2006; Cheong et al., 2014). The YopJ-like AvrBsT activates ETI in Arabidopsis Pitztal 0 plants (Cunnac et al., 2007). The *Xcv* effector protein AvrBsT triggers HR and cell death in pepper and *N. benthamiana* plants (Orth et al., 2000; Escobar et al., 2001; Kim et al., 2010). Pathogen-derived proteins that interact with the host HSP70 have been identified in different plant-pathogen interactions. HSP70 was shown to bind and regulate the coat protein of *Potato virus A*, which promoted viral infection in *N. benthamiana* plants (Hafren et al., 2010). The *P. syringae* effector protein HopI1 directly interacted with the cytoplasmic HSP70 and hijacked it to the chloroplast to promote virulence (Jelenska et al., 2010). Cheong et al. (2014) suggest that AvrBsT-dependent acetylation in planta alters the ACIP1 defense function, which is linked to the activation of ETI. However, the host proteins and molecular mechanisms mediating AvrBsT recognition remain unclear.

Our results from yeast two-hybrid and coimmunoprecipitation analyses provide convincing evidence that *CaHSP70a* interacts with AvrBsT in yeast and in planta. The BiFC images show that *CaHSP70a* 388–648 interacts with AvrBsT in *N. benthamiana* leaves, suggesting that the *CaHSP70a* peptide-BD binds to AvrBsT. The peptide-BD may be essential for *CaHSP70a* interaction with AvrBsT or other cell death-triggering client proteins. The client proteins for the *CaHSP70a*-AvrBsT complex remain to be identified. In recent studies, we identified

the pepper SGT1 as a host target of AvrBsT, which also interacts with pepper PIK1 (Kim et al., 2014). However, BiFC and coimmunoprecipitation analyses of transient *CaHSP70a* coexpression with *CaSGT1* or *CaPIK1* in *N. benthamiana* leaves revealed that *CaHSP70a* does not physically bind to *CaSGT1* or *CaPIK1* in planta (Supplemental Fig. S9). These findings suggest that the *Xanthomonas* spp. effector AvrBsT interacts physically with some host proteins such as *CaHSP70a* and *CaSGT1* to promote cell death and defense responses, although *CaHSP70a* and *CaSGT1* do not interact with each other in plants.

### **CaHSP70a Expression Triggers Pepper Cell Death under Heat Stress Conditions**

*CaHSP70a* expression was strongly induced in pepper leaves by heat stress (37°C) but not at a normal growth temperature (24°C). Transient *CaHSP70a* expression in pepper leaves revealed that *CaHSP70a* positively regulates cell death signaling pathways. Pepper leaves transiently overexpressing *CaHSP70a* were strongly hypersensitive to high temperature (37°C), which induced a severe cell death response. Cytoplasmic localization of *CaHSP70a* induced cell death under heat stress in pepper. These results suggest that transient *CaHSP70a* overexpression requires elevated temperatures to trigger cell death in pepper leaves. The cellular heat stress response enhances the expression of heat stress genes, multigene families that encode molecular chaperones (Bukau et al., 2006; Nakamoto and Vigh, 2007; Richter et al., 2010). Together with other cochaperones, *CaHSP70a* may activate client proteins downstream of cell death signaling, as observed in the complex interplay



**Figure 12.** Reduced SA and JA accumulation in *CaHSP70a*-silenced pepper leaves inoculated with *Xcv*. A, Free SA and total SA (free SA plus Glc-conjugated SA [SAG]) levels in empty-vector control and silenced leaves. B, JA levels in empty-vector control and silenced leaves. Data represent means  $\pm$  SD of three independent experiments. Asterisks indicate statistically significant differences (Student's *t* test,  $P < 0.05$ ). FW, Fresh weight.

between heat stress transcription factor and HSP networks in tomato (Hahn et al., 2011). Downstream defense markers *CaPR1* and *CaDEF1*, or cell death-inducing *CaRBP1* and *CaPIK1* genes, were simultaneously induced by the combination of transient *CaHSP70a* expression and heat stress treatment. Transient *CaHSP70a* overexpression in *N. benthamiana* did not trigger cell death under prolonged heat stress (37°C) conditions. These results suggest that the heat-inducible cell death-triggering HSP70 clients in pepper may be lacking in *N. benthamiana*.

### Cytoplasmic Localization of the CaHSP70a-AvrBsT Complex Promotes *avrBsT*-Triggered Cell Death in *N. benthamiana*

Infiltration of *CaHSP70a* and *avrBsT* using *A. tumefaciens* titers below those that induce HR strongly boosted *avrBsT*-triggered cell death in *N. benthamiana*. These results suggest that *CaHSP70a* expression promotes *avrBsT*-triggered cell death. The AvrBsT recognition factors may be stimulated by *CaHSP70a*, resulting in elevated cell death levels. AvrBsT interacts with *CaHSP70a* in both the nucleus and the cytoplasm. The AvrBsT-*CaHSP70a* complex promoted *avrBsT*-triggered cell death in *N. benthamiana* leaves only when localized to the cytoplasm by attachment of the NES (Slootweg et al., 2010; Choi et al., 2012). These results suggest that cytoplasmic localization of the *CaHSP70a* and AvrBsT complex is required for the positive regulation of cell death in plants. AvrBsT recognition proteins also may be localized to the cytoplasm.

Transient expression of the 35S:*CaHSP70a* 195–648 deletion construct strongly induced a cell death response in pepper leaves at 37°C, similar to that induced by the full-length *CaHSP70a*. These results suggest that the actin-like ATPase- and peptide-BDs are required for *CaHSP70a* promotion of *avrBsT*-triggered cell death. The actin-like ATPase- and peptide-BDs may be involved in protein folding via substrate-binding cycles regulated by its ATPase activity (Hartl and Hayer-Hartl, 2002). However, the exact molecular and functional roles of these domains remain to be elucidated.

### *CaHSP70a* Functions in Defense and HR-Mediated Resistance to Pathogenic Bacteria

HSP70s appear to be involved in the regulation of cell defense responses during microbial pathogenesis in plants. HSP70s were required for tabtoxinine- $\beta$ -lactam-induced cell death (Ito et al., 2014) but suppressed cell death triggered by SA in *N. tabacum* protoplasts (Cronjé et al., 2004). *CaHSP70a* expression was induced in pepper leaves by *Xcv* infection, especially in the incompatible interaction with avirulent *Xcv* (*avrBsT*). More importantly, cytoplasmic *CaHSP70a* proteins significantly accumulated in pepper leaves to induce the HR cell death response by *Xcv* (*avrBsT*) infection. These results suggest that *CaHSP70a* plays a crucial role for disease resistance

signaling. HSP70 is suggested to be essential for mediating the *P. syringae* effector HopI1 virulence effect and to play a role in basal resistance to a nonpathogenic *P. syringae* strain (Jelenska et al., 2010). Cytosolic HSP70 and HSP90 are essential components of the *P. infestans* protein INF1-mediated HR in *N. benthamiana* (Kanzaki et al., 2003).

*CaHSP70a*-silenced pepper plants exhibited enhanced susceptibility to *Xcv* infection. *CaHSP70a* silencing in pepper plants significantly compromised H<sub>2</sub>O<sub>2</sub> accumulation, defense- or cell death-related gene expression, and cell death. *CaHSP70a* loss-of-function analysis suggests that *CaHSP70a* expression contributes to both defense and ETI in pepper. ROS burst closely parallels plant cell death and defense signaling (Torres et al., 2006; Van Breusegem and Dat, 2006). ROS directly inhibit pathogen growth, stimulate cell wall cross-linking, and mediate signaling that triggers defense- and stress-response gene expression (Lamb and Dixon, 1997; Skopelitis et al., 2006). Levels of plant defense hormones such as SA and JA (Tsuda et al., 2009) are significantly reduced in *CaHSP70a*-silenced pepper. These results suggest that SA and JA are required to fine-tune resistance to *Xcv* infection (Clarke et al., 2000). Interestingly, ectopic overexpression of *CaHSP70a* in transgenic Arabidopsis plants did not confer enhanced disease resistance and cell death responses, suggesting that *CaHSP70a* expression alone is not sufficient to trigger defense and cell death signaling.

### Proposed Model for *CaHSP70a* in Plant Cell Death and Defense Signaling

Combining the data presented in this study, we propose a working model for the role of the *CaHSP70a*-AvrBsT complex, together with the CaPIK1-CaSGT1-AvrBsT complex, in mediating cell death and defense signaling in plants (Supplemental Fig. S10). Heat stress or *Xcv* (*avrBsT*) challenge induces defense signaling and rapidly elevates *CaHSP70a* expression levels. *CaHSP70a* binds to AvrBsT or to unknown host cell death-triggering client proteins in the cytoplasm during avirulent Ds1 (*avrBsT*; incompatible) *Xcv* infection. However, we do not know exactly why AvrBsT targets some host proteins such as *CaHSP70a* and CaSGT1. AvrBsT may promote cell death associated with CaPIK1-mediated phosphorylation by interacting with CaSGT1 (Kim et al., 2014). Another possibility is that *CaHSP70a* affects the folding/complex assembly of defense response proteins (i.e. SA- or JA-dependent proteins). AvrBsT may trigger cell death by actively switching *CaHSP70a* to a mode that facilitates the production or assembly of the cell death-promoting complex. Consequently, pepper plants with increased *CaHSP70a* or CaSGT1 levels may suppress *Xcv* growth due to increased folding/assembly of the defense complex. Such cell death signaling events seem likely to induce early defense responses, including SA and JA accumulation, ROS burst, and defense-related gene expression, which ultimately lead to an HR-like cell death and defense response. Taken together, these results

suggest that CaHSP70a is required for signal transduction in cell death development and defense responses in pepper and *N. benthamiana*. Future detailed studies are required to identify the host cell death-triggering client proteins and to investigate the mode of activation of signaling events that lead to cell death and defense responses.

## MATERIALS AND METHODS

### Plant Materials and Growth Conditions

Pepper (*Capsicum annuum* 'Nockwang') and *Nicotiana benthamiana* were planted in plastic pots (8 cm diameter) containing a soil mix (loam soil:perlite:vermiculite, 3:1:1, v/v/v). Plants were grown at 28°C with a long-day cycle (16 h of light and 8 h of darkness) at a light intensity of 100  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ . Arabidopsis (*Arabidopsis thaliana* ecotype Columbia-0) plants were grown in pots containing vermiculite, peat moss, and perlite (1:1:0.5, v/v/v) at 24°C, 60% relative humidity, and 130  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$  for 16 h of light in a growth chamber.

### Pathogen Inoculation

*Xanthomonas campestris* pv *vesicatoria* virulent Ds1 (EV) and avirulent Ds1 (*avrBsT*) strains (Kim et al., 2010) or *Pseudomonas syringae* pv *tomato* DC3000 and DC3000 (*avrRpm1*) strains were grown in YN (5 g of yeast extract and 8 g of nutrient broth per L) or King's B broth (10 g of peptone, 1.5 g of  $\text{K}_2\text{HPO}_4$ , 15 g of glycerol, and 1 mM  $\text{MgSO}_4$  per L), respectively. The cultured bacteria were harvested and resuspended in 10 mM  $\text{MgCl}_2$ . Bacterial growth in leaves was monitored at 0 and 3 d after *Xcv* infiltration with *Xcv* ( $5 \times 10^4$  cfu  $\text{mL}^{-1}$ ) and *Pst* ( $10^5$  cfu  $\text{mL}^{-1}$ ) using a needleless syringe.

*Hyaloperonospora arabidopsidis* Noco2 was grown on Arabidopsis cotyledons at 16°C, 60% relative humidity, and a 14-h photoperiod. *H. arabidopsidis* conidia ( $5 \times 10^4$   $\text{mL}^{-1}$ ) were suspended in distilled tap water containing 0.05% (v/v) Tween 20 and sprayed onto cotyledons of 7-d-old Arabidopsis seedlings.

### Yeast Two-Hybrid Assay

The *avrBsT* open reading frame was cloned into the *Bam*HI/*Hind*III site of the pGBKT7 vector. The yeast prey library was generated from the pepper cDNA library by ligating cDNA inserts with the pGADT7 vector. The constructs were cotransformed into yeast strain AH109 and plated onto synthetic dropout (SD)-His-Leu-Trp medium (Ito et al., 1983). Colonies grown on SD-His-Leu-Trp medium were transferred onto selection medium (SD-adenine-His-Leu-Trp). Plasmids were extracted from surviving yeast colonies and used to transform *Escherichia coli*. Colonies carrying the pGADT7 vector were selected on Luria-Bertani medium containing 100  $\text{mg L}^{-1}$  ampicillin. Isolated plasmids were sequenced, and sequence homology was analyzed using GenBank BLAST tools (<http://blast.ncbi.nlm.nih.gov/>).

### Agrobacterium tumefaciens-Mediated Transient Expression and BiFC Assay

BiFC analyses were conducted as described previously (Waadt et al., 2008). To generate the BiFC constructs, cDNAs encoding *AvrBsT* and CaHSP70a without termination codons were PCR amplified and subcloned into the binary vectors pVYNE(R) (*Xba*I/*Xho*I) and pSCYCE (*Xba*I/*Xho*I) under the control of the cauliflower mosaic virus 35S promoter (Supplemental Table S1). Oligonucleotides containing NLS and NES sequences (Slootweg et al., 2010; Choi and Hwang, 2011) were inserted into the *Xho*I/*Kpn*I site to create the NLS and NES fusion constructs (Supplemental Table S1). *AvrBsT* and CaHSP70a were coexpressed in *N. benthamiana* leaves by infiltrating *A. tumefaciens* strain GV3101 carrying each construct ( $\text{OD}_{600} = 0.5$ ). *AvrBsT* and CaHSP70a interaction was visualized using a confocal laser scanning microscope (LSM 5 Exciter; Carl Zeiss) operated with the LSM Imager at 40 h after transformation.

### Heat Stress Treatment

Pepper plants transiently overexpressing CaHSP70a were treated with 37°C heat stress or 24°C control. *A. tumefaciens* strain GV3101 ( $\text{OD}_{600} = 0.5$ ) carrying

pBIN35S (empty-vector control) or pBIN35S:CaHSP70a (35S:CaHSP70a) was infiltrated into fully expanded pepper leaves. Twenty-four hours after agro-infiltration, plants were placed into the 37°C or 24°C growth chambers for up to 48 h. Visual cell death responses were scored 48 h after alleviation of the heat stress. For ion leakage and histochemical assays, leaf samples were taken at various time points as indicated.

### VIGS

TRV-based VIGS (Liu et al., 2002) was used to investigate CaHSP70a loss of function in pepper plants. The nonconserved 3' untranslated regions of CaHSP70a and CaSGT1 cDNAs were digested with *Eco*RI, and the resulting fragment was inserted into pTRV2. Fully expanded cotyledons of pepper plants were coinfiltrated with the *A. tumefaciens* strain GV3101 ( $\text{OD}_{600} = 0.2$ ) carrying the VIGS vectors pTRV1 and pTRV2, pTRV2:CaHSP70a, or pTRV2:CaSGT1. The efficacy of CaHSP70a and pTRV2:CaSGT1 silencing in pepper plants was examined by RT-PCR after *Xcv* inoculation at the six-leaf stage.

### RNA Gel-Blot and Real-Time RT-PCR Analyses

Total RNA was extracted from pepper plants using Trizol reagent (Invitrogen). For RNA gel blot, RNA was resolved by agarose gel electrophoresis, transferred onto Hybond N+ membranes (GE Healthcare), and hybridized overnight with  $^{32}\text{P}$ -labeled CaHSP70a cDNA. For real-time RT-PCR, 2  $\mu\text{g}$  of RNA was used in an RT reaction with Moloney murine leukemia virus reverse transcriptase (Enzymatics). Real-time RT-PCR was performed using iQ SYBR Green Supermix and iCycler iQ (Bio-Rad). The 18S ribosomal RNA (*rRNA*) transcript level was used to normalize the transcript level of each gene (Supplemental Table S1). Relative expression levels were determined by comparing these values with that of the uninoculated control.

### Ion Leakage Assay

Pepper or *N. benthamiana* leaves were harvested at various time points after infiltration with *Xcv* or *A. tumefaciens*, respectively. Leaf discs (0.5 cm diameter) were removed with a cork borer and washed in 10 mL of sterile double-distilled water for 30 min. Washed leaf discs were transferred to 20 mL of sterile double-distilled water and incubated for 2 h at room temperature. Leaf ion conductivity was measured in the leaf discs using a Sension 7 conductivity meter (HACH).

### Histochemistry

$\text{H}_2\text{O}_2$  accumulation was visualized by placing healthy or inoculated leaves in 1  $\text{mg mL}^{-1}$  DAB (Sigma) solution overnight (Thordal-Christensen et al., 1997). Chlorophyll was cleared from the stained leaves by boiling in 95% (v/v) ethanol. Cell death was monitored by Trypan Blue staining of healthy or inoculated leaves (Koch and Slusarenko, 1990). Leaves were stained with lactophenol-Trypan Blue solution (10 mL of lactic acid, 10 mL of glycerol, 10 g of phenol, and 10 mg of Trypan Blue, dissolved in 10 mL of distilled water) and destained in 2.5  $\text{g mL}^{-1}$  chloral hydrate solution. The samples were photographed using a light microscope mounted with a digital camera (Olympus). Chlorosis, cell death, and phenolic compound accumulation in leaves were visualized using a hand-held UV lamp (UVP).

### $\text{H}_2\text{O}_2$ Measurement

$\text{H}_2\text{O}_2$  accumulation in pepper leaves was quantified using Xylenol Orange (Gay et al., 1999; Choi and Hwang, 2011). Xylenol Orange assay reagent was freshly prepared by adding 500  $\mu\text{L}$  of solution [25 mM  $\text{FeSO}_4$  and 25 mM  $(\text{NH}_4)_2\text{SO}_4$  in 2.5 M  $\text{H}_2\text{SO}_4$ ] to 50 mL of 125  $\mu\text{M}$  Xylenol Orange in 100 mM sorbitol. Eight leaf discs (0.5  $\text{cm}^2$ ) were floated on 1 mL of distilled water in a microtube for 1 h, centrifuged for 1 min at 12,000g, and 100  $\mu\text{L}$  of supernatant was immediately added to 1 mL of Xylenol Orange assay reagent. The mixture was incubated for 30 min at room temperature.  $\text{H}_2\text{O}_2$  was quantified by measuring  $A_{560}$  using a DU 650 spectrophotometer (Beckman) and comparing with a standard curve for  $\text{H}_2\text{O}_2$ , which was generated from absorbance measurements of a 100-nmol to 100- $\mu\text{mol}$  serial dilution of  $\text{H}_2\text{O}_2$ .

### Immunoblot Analysis

Total soluble proteins were extracted from *N. benthamiana* leaves with 1 mL of denaturing buffer (50 mM Tris-HCl [pH 8.8], 4 M urea, 10 mM sodium



phosphate [pH 7.8], 250 mM NaCl, 0.1% [v/v] Nonidet P-40, 1 mM EDTA, and 0.5% [w/v] SDS). Insoluble debris was pelleted by centrifuging leaf extracts at 15,000g for 20 min at 4°C. Proteins were resolved on 8% (w/v) SDS-PAGE gels and transferred to a polyvinylidene difluoride membrane (GE Healthcare Biosciences). Proteins tagged with HA or cMyc epitopes were detected with anti-HA-peroxidase or anti-cMyc-peroxidase antibodies (Sigma), respectively. Anti-CaHSP70a rabbit antibodies were raised against synthetic peptides corresponding to CaHSP70a residues 185 to 202 and 614 to 629 (AbFrontier; <http://www.abfrontier.com>). Immunoblotting of pepper leaf protein extracts was performed using the anti-CaHSP70a antibody.

Nuclear and cytoplasmic fractionation was conducted as described previously (Shen et al., 2007; Choi et al., 2012). Briefly, leaf tissues were homogenized in Honda buffer (2.5% [w/v] Ficoll 400, 5% [w/v] dextran T40, 0.4 M Suc, 25 mM Tris-HCl [pH 7.5], 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 0.5% [v/v] Triton X-100, 1 mM phenylmethanesulfonyl fluoride, and a complete protease inhibitor cocktail). After centrifugation at 1,500g, the harvested supernatant represented the soluble fraction, and the pellet was purified as the nuclear fraction. The total, nuclear, and cytoplasmic fractions were subjected to immunoblot analysis. Anti-H3 (Abcam) and anti-Hsc70 (Abcam) were used as nuclear and cytosolic protein markers, respectively.

## Measurement of SA and JA

SA and SA glycoside were extracted and quantified as described previously (Lee et al., 2011). Leaf tissues (0.5 g) were homogenized and extracted in 90% (v/v) methanol. 3-Hydroxybenzoic acid (Sigma) was used as an internal standard. SA was detected by fluorescence (excitation at 305 nm and emission at 405 nm) using reverse-phase HPLC in a Waters 515 system using a C18 column.

JA was extracted and quantified as described previously (Hwang and Hwang, 2010). Leaf tissues (0.5 g) were homogenized and extracted in 100% methanol and centrifuged at 10,000g for 10 min. The supernatants were transferred, and pellets were reextracted with 100% methanol. Dihydrojasmonic acid was added as an internal standard for JA quantification. The samples were adjusted to 70% (v/v) methanol and pH 3 using sterile water and HCl, respectively. The samples were passed through a prewashed Sep-Pak C18 cartridge (Waters). Filtered samples were partitioned with CHCl<sub>3</sub>, dried over anhydrous MgSO<sub>4</sub>, and methylated with hexane:tert-butyl methyl ether (1:1, v/v). The supernatants were transferred and analyzed for JA. JA was quantified by gas chromatography-mass spectrometry (Agilent 6890) using a DB-5MS column (30 m × 0.25 mm, 0.25 μm; J&W Scientific) with a temperature gradient of 60°C for 1 min, 60°C to 190°C at 15°C min<sup>-1</sup>, 190°C to 220°C at 5°C min<sup>-1</sup>, 220°C to 290°C at 25°C min<sup>-1</sup>, and 290°C for 1 min.

## Arabidopsis Transformation

Transgenic Arabidopsis plants expressing *CaHSP70a* were generated by the floral dip method (Clough and Bent, 1998). The *CaHSP70a* coding region was subcloned into the binary vector pBIN35S (*Xba*I/*Bam*HI) between the cauliflower mosaic virus 35S promoter and the *nopaline synthase* terminator. This construct was introduced into *A. tumefaciens* GV3101 and used to transform Arabidopsis ecotype Columbia-0. Transformed seed stock was selected for kanamycin resistance by planting seeds on Murashige and Skoog (Duchefa) agar plates containing 50 mg L<sup>-1</sup> kanamycin (Duchefa).

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: *CaHSP70a* (KJ619375), *CaPR1* (AF053343), *CaPR10* (AF244121), *CaDEF1* (AF442388), *CaRBP1* (DQ864657), *CaPIK1* (GU295436), and *Ca18S rRNA* (EF564281).

## Supplemental Data

The following supplemental materials are available.

**Supplemental Figure S1.** Nucleotide and deduced amino acid sequences of *CaHSP70a* cDNA.

**Supplemental Figure S2.** Alignment of *CaHSP70a* with other plant HSP70 proteins.

**Supplemental Figure S3.** RNA gel-blot and immunoblot analyses of *CaHSP70a* expression in pepper plants.

**Supplemental Figure S4.** RT-PCR analyses of defense- and cell death-related gene expression in pepper leaves during transient *CaHSP70a* expression.

**Supplemental Figure S5.** Cell death phenotypes and quantification in leaves 2 d after infiltration with *A. tumefaciens* carrying *avrBsT*, *avrBsT:NLS*, and *avrBsT:NES* (A) and *CaHSP70a*, *CaHSP70a:NLS*, and *CaHSP70a:NES* (B).

**Supplemental Figure S6.** *CaHSP70a* and *CaSGT1* expression contributes independently to the resistance to *Xcv* infection.

**Supplemental Figure S7.** *CaHSP70a* overexpression in Arabidopsis plants does not alter susceptibility to *Pst* infection.

**Supplemental Figure S8.** *CaHSP70a* overexpression in Arabidopsis plants does not alter susceptibility to *H. arabidopsidis* infection.

**Supplemental Figure S9.** *CaHSP70a* interacts with AvrBsT but not with *CaSGT1* or *CaPIK1* in planta.

**Supplemental Figure S10.** Proposed model for the *CaHSP70a* and AvrBsT complex, together with the *CaPIK1*-*CaSGT1*-AvrBsT complex, in plant cell death and defense signaling.

**Supplemental Table S1.** Gene-specific primers for plasmid constructs used in this study.

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