Opposing dual defense roles for HSC70 and BON1

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**Opposing effects on two phases of defense responses from concerted actions of HSC70 and BON1 in Arabidopsis**

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**Summary:** Antagonized functions between HSC70 and BON1 regulate both pre-invasion and post-invasion phases of defense responses.

**Footnotes:**

This work was supported by the National Science Foundation (IOS-0919914 and IOS-1353738 to J.H.) and the China Scholarship Council (to Z.Z. and Q.H).

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**Abstract**

The plant immune system consists of multiple layers of responses targeting various phases of pathogen infection. Here we provide evidence showing that two responses, one controlling stomatal closure and the other mediated by intracellular receptor proteins, can be regulated by the same proteins but in an antagonistic manner. The heat shock protein HSC70, while previously known as a negative regulator of stomatal closure, is a positive regulator of immune responses mediated by the immune receptor protein SNC1 as well as basal defense responses. In contrast to HSC70, a calcium binding protein BON1 promotes abscisic acid and pathogen-triggered stomatal closure in addition to and independent of its previously known negative role in *SNC1* regulation. BON1 likely regulates stomatal closure through activating SGT1b and inhibiting HSC70. New functions of *BON1* and *HSC70* identified in this study thus reveal opposite effects of each of them on immunity. The opposing roles of these regulators at different phases of plant immune responses exemplifies the complexity in immunity regulation and suggests that immune receptors may guard positive regulators functioning at stomatal closure control.

**Introduction**

The plant immune system consists of multiple layers of recognition that target different phases of pathogen infection. The two major pathogen recognition and defense signaling branches are pathogen-associated molecular pattern (PAMP)-triggered-immunity (PTI) and effector-triggered immunity (ETI) (Dangl and Jones, 2001; Chisholm et al., 2006; Jones and Dangl, 2006; Dodds and Rathjen, 2010). PAMPs such as flagellin and elongation factor Tu (EF-Tu) are recognized by pattern recognition receptors (PRRs) such as FLS2 and EFR (Monaghan and Zipfel, 2012) at the plasma membrane to initiate PTI. The short N-terminal peptides of flagellin and EF-Tu, named flg22 and elf18 respectively, are sufficient to trigger PTI (Felix et al., 1999; Kunze et al., 2004). ETI is engaged following the recognition of microbial effectors via plant intracellular immune receptors that are mostly nucleotide binding leucine-rich repeat (NB-LRR) proteins (Chisholm et al., 2006; Jones and Dangl, 2006). Activation of NB-LRR proteins often leads to rapid and effective defense responses including programed cell death to restrict the growth of biotrophic pathogens.

Closure of stomata is one of the responses activated following PAMP recognition to prevent pathogen entry into plant cells (Melotto et al., 2006; Xin and He, 2013). As a gateway for water vapor and CO2 exchange between the mesophyll cells and the atmosphere, stomata pore is finely controlled in its aperture to maximize photosynthesis while preventing water loss. Stress hormone ABA and CO2 can each be perceived by their receptors and activate signaling pathways involving kinases/phosphatases and secondary messengers to modify ion channel activities that change the aperture of the stomatal pores (Kim et al., 2010). Stomata is also the battle ground between plants and pathogens as plants prevent entry of foliar pathogens by closing the gate upon PAMP perception while pathogens use different strategies to open the stomata for their entries (Melotto et al., 2006). While ABA is largely responsible for abiotic stress induced stomatal closure, oxylipin pathway is thought to mediate biotic stress induced closure (Montillet and Hirt, 2013). Downstream signaling events in response to biotic and abiotic signals share common components including reactive oxygen species, calcium, and nitric oxide, (Montillet et al., 2013; Sawinski et al., 2013). Protein kinases including calcium-dependent protein kinases and mitogen activated protein kinases presumably transduce these signals and regulate activities of ion channels and transporters to control stomata opening (Sawinski et al., 2013).

Chaperone or co-chaperone proteins such as SGT1b (SUPPESSOR OF THE G2 ALLELE OF SKP1 VARIANT B), and HSP70 (HEAT SHOCK PROTEIN 70) are important regulators of plant immunity. SGT1b is part of the Skp1/Cullin/F-box (SCF) ubiquitin ligase complex that targets protein for degradation. It has multiple substrates and regulates multiple processes such as development, defense responses, and abiotic stress responses (Austin et al., 2002; Azevedo et al., 2002; Gray et al., 2003; Noel et al., 2007). SGT1b has opposing roles on NB-LRR protein regulation. On the one hand, it is required for multiple NB-LRRs to mediate defense responses, likely by assisting their proper folding and/or positively regulating their protein accumulation (Austin et al., 2002; Peart et al., 2002; Hubert et al., 2003; Leister et al., 2005; Azevedo et al., 2006). On the other hand, the SGT1b-SCF complex is implicated in coupling NB-LRR proteins to cellular degradation machinery and therefore inhibits defense responses (Liu et al., 2002; Holt et al., 2005).

HSP70 proteins are induced by a rapid temperature rise. They are generally involved in protein folding and degradation of unfolded proteins (Hartl, 1996; Park et al., 2007). Arabidopsis has 14 HSP70s, including five cytosolic HSP70s (HSC70s), three ER localized luminal binding proteins (BiPs), two plastid HSP70s, and two mitochondrial HSP70s (Lin et al., 2001; Sung et al., 2001). The cytosolic HSC70 proteins are important for tolerance to abiotic stress including heat (Sung and Guy, 2003; Cazale et al., 2009). They were recently implicated in regulating plant immunity as well but their reported functions are contradictory from different studies. In one study, HSC70s are shown to physically interact with SGT1b, and overexpression of *HSC70.1* confers susceptibility to the virulent oomycete pathogens *Hyaloperonospora arabidopsidis* (*H. arabidopsidis*) as well as virulent and avirulent strains of *Pseudomonas syringae pv. tomato* (*Pst*) DC3000 (Noel et al., 2007). This study indicates that HSC70s are negative regulators of basal resistance as well as NB-LRR-mediated resistance. In another study, HSC70s, especially HSC70.1 and HSC70.3, are shown to be targeted by the *Pseudomonas syringae* effector protein HopI1, and a *hsc70.1* T-DNA insertion mutant exhibits enhanced susceptibility to virulent *Pseudomonas syringae pv. maculicola* (*Psm* or *Pma*) ES4326 and type III secretion-deficient *Pst* DC3000 *hrcC-* (Jelenska et al., 2010). This study indicates that HSC70s have a positive role in plant basal defense responses. The reason for the apparent contradiction regarding the role of HSC70 in plant immunity is not known. Varying effects of HopI1 or different functions of HSC70 under different environments are suspected to contribute to varying outcomes.

The chaperone and co-chaperone proteins were recently shown to be involved in stomatal control (Clement et al., 2011). *HSC70.1* over-expression lines and a *sgt1b* mutant have delayed stomatal closure compared to wild type under several environmental inductions. The clients of HSC70 or SGT1b in stomata regulation are not known, but they likely regulate shared components between biotic and abiotic responses.

BON1 (BONZAI1) is an intriguing regulator of NB-LRRs. It encodes a calcium-dependent phospholipid-binding copine protein that is conserved in protozoa, plants, nematodes, and mammals (Hua et al., 2001). Copine proteins in diverse species studied so far are involved in signaling although no common processes can be readily deduced (Li et al., 2010). In Arabidopsis, the loss-of-function (LOF) mutant *bon1-1* (hereafter, *bon1*) has a dwarf phenotype due to constitutive defense responses triggered by the NB-LRR protein SNC1 (SUPPRESSOR OF NPR1-1, CONSTITUTIVE 1) (Hua et al., 2001; Yang and Hua, 2004). Mutants with constitutive immune responses such as *bon1* are referred to as autoimmune mutants and some of them are due to activation of NB-LRR proteins (Gou and Hua, 2012). SNC1 is highly similar to RPP4 that confers resistance to pathovars of the oomycete pathogen *H. arabidopsidis* (Noel et al., 1999; van der Biezen et al., 2002). The SNC1 protein is under a complex negative regulation (Gou and Hua, 2012). Although the pathogen effector that SNC1 recognizes remains elusive, SNC1 could be a minor resistance protein for the effector AvrRps4 (Kim et al., 2010). A gain-of-function mutant of *SNC1*, *snc1*, has an autoimmune phenotype (Li et al., 2001; Zhang et al., 2003). The *BON1* gene belongs to a three-member gene family in Arabidopsis, and the knockout of all three genes (*BON1*, *BON2*, and *BON3*) results in lethality (Yang et al., 2006). Genetic dissection of a differential phenotype of the *bon1 bon3* double mutants in two accession backgrounds revealed that multiple *NB-LRR* genes are responsible for the lethality phenotype (Li et al., 2009). Therefore, *BON1* is a negative regulator of multiple NB-LRRproteins in addition to the SNC1.

The BON1 protein is localized to the plasma membrane through N-terminal myristoylation; and its calcium binding property, though not responsible for its localization, is essential for its function (Li et al., 2010). How BON1 at the plasma membrane regulates the transcript level of NB-LRR coding gene *SNC1* is not fully understood. Genetic studies suggest that BON1 modulates *SNC1* transcript through chromatin remodeling factors HUB1 (HISTONE MONO-UBIQUITINATION 1) and HUB2 as well as MOS1 (MODIFIER OF SNC1 1) (Li et al., 2010). It is thus likely that signaling regulated by BON1 at the plasma membrane influences expression level of *SNC1*. From yeast two-hybrid screens, two homologous calcium-binding proteins BAP1 (BON1-ASSOCIATED PROTEIN 1) and BAP2 were found to interact with the full length BON1 and specifically the VWA domain of BON1, and these two proteins function similarly to the BON proteins in the regulation of immunity (Hua et al., 2001; Yang et al., 2006; Yang et al., 2007). Another yeast two-hybrid screen identified BIR1 (BAK1-INTERACTING RECEPTOR-LIKE KINASE 1) as additional BON1 interactor, and BIR1 is also a negative regulator of defense responses (Wang et al., 2011).

To further elucidate the role of BON1 in immunity, we immunoprecipitated (IPed) BON1 and identified associated proteins by liquid chromatography mass spectrometry (LC-MS). Using bimolecular fluorescence complementation (BiFC) and yeast two-hybrid assays, we found that HSC70 and SGT1b are potentially BON1-interacting proteins. With the generation of a double mutant with reduced function of *HSC70.1* and *HSC70.3*, we show that BON1, HSC70, and SGT1b have roles in NB-LRR mediated defense responses as well as stomatal closure regulation. The opposing effects on two layers of defense responses suggest complex regulation of plant immunity as well as connection between two branches of immune responses.

**Results**

**BON1 associates with HSC70 and SGT1b proteins**

We searched for BON1-associated proteins by immunoprecipitation (IP) of hemagglutinin (HA) tagged BON1 protein followed by LC-MS analysis of co-IPed proteins. This BON1-HA was expressed under the strong CaMV 35S promoter and it is functional as it rescued the *bon1* defect (Figure S1). A putative BON1-HA protein complex was stabilized by the crosslinker DSP (dithiobis(succinimidyl propionate)) (Figure S2A) and then IPed with anti-HA antibodies. The putative BON1-HA protein complex was visible after being separated on the SDS-PAGE gel after Coomassie blue staining (Figure S2B). LC-MS identified three cytosolic HSC70 proteins in the top ranking BON1-associated proteins (Figure S2C). We designated them as HSC70.1 (AT5G02500), HSC70.2 (AT5G02490), and HSC70.3 (AT3G09440) respectively instead of their previous names HSC70-1, HSC70-2, and HSC70-3 (Lin et al., 2001; Noel et al., 2007) to follow the conventional usage of ‘-’ to indicate mutant alleles.

The association between BON1 and the three HSC70 proteins were subsequently tested using BiFC and co-IP methods (Schutze et al., 2009). For BiFC assays, the BON1 protein was fused to the HA tag as well as the C-terminal half of yellow fluorescence protein (YFPC). For simplicity, HA:YFPC will be referred to as YFPC in the BiFC assay and as HA in the co-IP assay. The three HSC70 proteins were each fused at their C-termini with a Myc tag and the N-terminal half of YFP (YFPN). Again, the Myc:YFPN tag will be referred to as YFPN in the BiFC assay and as Myc in the co-IP assay. When BON1:YFPC and any one of the three HSC70:YFPN were co-expressed in *N. benthamiana*, YFP signals were detected (Figure 1A). No signal was observed when BON1:YFPC or HSC70:YFPN were co-expressed with the YFPN or YFPC controls (Figure 1A). Because BON1 is a plasma membrane localized protein (Hua et al., 2001), proteins that are directly or indirectly attached to plasma membrane could potentially be isolated as false-positive BON1 interacting proteins. We therefore co-expressed the YFPN fusions of the plasma membrane protein COPT1 (COPPER TRANSPORTER 1) with BON1:YFPC in *N. benthamiana* as an additional control. While both BON1 and COPT1 proteins were expressed (Figure1C), no BiFC signal was detected (Figure 1A), indicating that the BiFC signals from BON1 and HSC70 are unlikely due to physical closeness of BON1 with any proteins on the plasma membrane.

In parallel, we IPed BON1:HA using an anti-HA antibody and could detect signals of HSC70.1:Myc from co-infiltrated leaves (Figure 1B). In contrast, no HSC70.1:Myc signal could be detected from the control sample co-expressed with the HA vector (Figure 1B). As a control, the Myc-tagged membrane protein COPT1 could not be co-IPed with BON1:HA, indicating the specificity of BON1:HA in the co-IP assay (Figure1C). Therefore, BON1 specifically associates with HSC70 proteins in plants.

We further determined the interaction between BON1 and HSC70 by co-IP in *BON1-HA*/*bon1* transgenic plants. BON1-HA was IPed by the anti-HA antibody from total protein extract without crosslinker, and associated proteins were separated on SDS-PAGE for Western blot. Positive signals could be detected by anti-HSC70 antibodies on the blot in the BON1-HA co-IP sample but not the control wild-type Col-0 sample (Figure 1D). Furthermore, the ER-localized HSP70 family protein BiP could not be detected by anti-BIP antibody in BON1-HA co-IP sample (Figure 1D). These results indicate that BON1 is associated with cytosolic localized HSC70 proteins but not all HSP70 family proteins.

However, we did not detect a positive interaction between HSC70 and BON1 in a GAL4 based yeast two-hybrid assay. Full-length BON1 or the C-terminal VWA domain of BON1 (BON1A) were fused to the GAL4 transcription activation domain (AD) while HSC70.1 ,HSC70.2, and HSC70.3 were fused with the GAL4 DNA binding domain (BD). Western blot analysis indicated that the all proteins were expressed at comparable levels in yeasts co-transformed with these constructs (Figure S3). No growth of yeast containing BON1 and HSC70 constructs was observed on the same selection media (Figure S3), while yeasts harboring BON1 and BAP1 constructs grew as previously reported (Hua et al., 2001). This data suggests that BON1 may not have a direct physical interaction with HSC70 proteins, although the possibility of direct interaction could not be excluded.

Because HSC70 is known to associate with SGT1b (Noel et al., 2007), we tested if BON1 and SGT1b can interact. Co-expression of BON1:YFPN and SGT1b:YFPC in *N. benthamiana* leaves led to a strong YFC signal while co-expression of BON1:YFPN with YFPC or YFPN with SGT1b:YFPC did not (Figure 2A). In addition, when the SGT1b:HA protein was IPed with anti-HA antibodies, BON1:Myc was detected from co-infiltrated leaves, while no signal could be detected in leaves co-infiltrated with BON1:HA and the Myc vector (Figure 2B). The association between BON1 and SGT1b was further tested by the yeast two-hybrid assay. Yeast co-expressing BON1-BD and SGT1b-AD fusions grew on the selection medium while yeast co-expressing BON1-AD and BD or AD and SGT1b-BD did not (Figure 2C). Growth from co-expression of BON1 and SGT1b was at a similar extent as that of BON1 and BAP1, a previously identified BON1 interactor (Figure 2C). These results suggest that BON1 and SGT1b proteins potentially could have a direct interaction. We subsequently assayed the interaction of BON1 with the TPR domain (amino acids 1-120) and the CS-SGS domain (amino acids 121-358) that is necessary and sufficient for SGT1b to interact with HSC70 (Azevedo et al., 2002; Li et al., 2010). Neither of these two domains exhibited positive interaction with BON1 in the yeast two-hybrid assay (Figure 2D), suggesting that a full-length SGT1b is required for its interaction with BON1.

**The double mutations in *HSC70.1* and *HSC70.3* partially rescued the *bon1* growth defects**

The interaction among BON1, HSC70, and SGT1b prompted us to look at the functional involvement of HSC70 and SGT1b in *bon1*-triggered autoimmune responses. Because HSC70 overexpression was reported to have reduced disease resistance (Noel et al., 2007), we tested whether or not HSC70 overexpression can suppress the autoimmune responses in *bon1*. The *HSC70.1* and *HSC70.3* were each overexpressed by the strong CaMV 35S promoter in *bon1*, and 18 and 16 transgenic lines were obtained respectively. None of the transgenic lines had reduced autoimmune phenotype compared to *bon1* (Figure S4A). When detected with an antibody against cytosolic HSC70 proteins, a slight increase (1.1 to 1.4 fold) of total HSC70 proteins was observed in four *HSC70.1* transgenic lines analyzed (Figure S4B). We were not able to assess expression levels of HSC70.1 due to the lack of specific antibodies, but we reason that there is a moderate increase of HSC70.1 in some transgenic lines, but that increase does not inhibit the *bon1* phenotype.

We subsequently analyzed the effect of loss of the *HSC70* function on the *bon1* phenotypes. T-DNA insertion mutants were isolated from the Salk collection (Alonso et al., 2003) for each of the three *HSC70* genes, namely *hsc70.1-1* (SALK\_135531C, referred to as *hsc70.1*), *hsc70.2-1* (SALK\_085076C, referred to as *hsc70.2*), and *hsc70.3-2* (SALK\_148168, referred to as *hsc70.3*). Both *hsc70.1* and *hsc70.2* mutants were reported to be LOF mutants earlier (Noel et al., 2007). The *hsc70.3* mutant allele was not characterized previously, and it has a T-DNA inserted in the 3’ UTR of the gene (Figure 3A). The transcription levels of *HSC70.1* and *HSC70.3* in the *hsc70.1* and *hsc70.3* mutants were analyzed by quantitative real-time reverse transcription PCR (qRT–PCR). Consistent with previous reports, *HSC70.1* expression was greatly decreased in *hsc70.1* mutant (Noel et al., 2007). *HSC70.3* expression was reduced to about 20% of the wild type in the *hsc70.3* mutant (Figure 3B), indicating that the *hsc70.3* mutant is a knockdown but not a null mutant. It is also noted that *HSC70.3* was significantly upregulated in *hsc70.1* (Figure 3B), suggesting that *HSC70.3* is induced perhaps to compensate for the reduction of *HSC70.1* function.

Genetic redundancy has been reported for the *HSC70* family members (Sung and Guy, 2003; Noel et al., 2007). Single mutants of individual members did not exhibit an obvious mutant phenotype while RNA silencing of the gene family caused embryo lethality. To reduce genetic redundancy and compensation, we generated double mutants between the three *HSC70* genes by crossing among the single mutants. As *HSC70.1* and *HSC70.2* are next to each other on the chromosome, we were not able to obtain the *hsc70.1 hsc70.2* double mutant. A double null mutant of *HSC70.1* and *HSC70.3* genes was reported to be lethal (Noel et al., 2007), but we were able to obtain the *hsc70.1 hsc70.3* double mutant likely because the *hsc70.3* allele used is a reduction of function allele but not a null allele.

The *hsc70* single and double mutants exhibited wild-type growth phenotypes under standard growth conditions (Figure 3C). However, the double mutant is less heat tolerant than the single mutants or the wild type. We initially noticed a drastically reduced heat tolerance in the *hsc70.1 hsc70.3* double mutant compared to the wild type when very young seedlings were exposed to 45°C for 20 minutes (Figure 3D). We subsequently quantified heat tolerance of single and double mutants in 10-day-old seedlings using 20 minutes of 45°C treatment. After recovery at 22°C for 5 days, the *hsc70.1 hsc70.3* mutant plants had slightly more chlorosis than the wild-type plants or single mutants (Figure 3E). Although the chlorosis phenotype was subtle, quantification of fresh weight revealed that the double mutant had a 40% biomass reduction with heat treatment compared to non-treatment while the wild type and the single mutants had no or less than 10% reduction with heat treatment. Therefore, the *hsc70.1 hsc70.3* double mutant we generated is more reduced in *HSC70* function than the single mutants (Fig 3F).

We then investigated the effect of loss of *HSC70* function on the *bon1* phenotype. Double mutants were constructed between *bon1* and the three *hsc70* single mutants. While neither *bon1 hsc70.2* nor *bon1 hsc70.3* had any morphological differences from the *bon1* single mutant, *bon1 hsc70.1* showed a milder growth defect than that of *bon1* (Figure 4A and Figure S5). We further generated two triple mutants *bon1 hsc70.1 hsc70.2* and *bon1 hsc70.1 hsc70.3*. Plants of *bon1 hsc70.1 hsc70.3* but not *bon1 hsc70.2 hsc70.3* exhibited an even milder growth defect than the *bon1 hsc70.1* plant (Figure 4A and Figure S5), which was verified by biomass quantification of seedlings (Figure S6A). Using complementation assay, we confirmed that the inhibition of *bon1* growth defect is due to the *hsc70* mutations. When a wild-type *HSC70.1* genomic fragment was transformed into *bon1 hsc70.1 hsc70.3,* 12 out of the 13 T1 transgenic plants showed a *bon1* like phenotype (Figure 4C). Therefore, the growth defect of the *bon1* mutant is indeed partially rescued by the loss of *HSC70.1* function and further rescued by additional reduction of *HSC70.3* function.

**The loss of *HSC70.1* and *HSC70.3* function compromises immune responses in *bon1***

The suppression of growth defect by the *hsc70* mutations is associated with a reduced disease resistance in *bon1*. In *bon1*, SA-mediated defense responses are constitutively turned on resulting in highly expressed defense response marker gene *PR1* (Yang and Hua, 2004) (Fig 4B). In the *bon1 hsc70.1 hsc70.3* triple mutant, but not the *bon1 hsc70.1* or *bon1 hsc70.3* double mutants,this elevated expression of *PR1* in *bon1* was greatly reduced as detected by qRT-PCR (Figure 4B). Furthermore, enhanced resistance to the virulent bacterial pathogen *Pst* DC3000 in *bon1* was reduced by the *hsc70* mutations: bacteria grew to a greater extent in *bon1* *hsc70.1 hsc70.3* than in *bon1* (Figure 4D).

The reduction of *HSC70.1* and *HSC70.3* function also inhibited the growth and defense defects in the auto-active NB-LRR *SNC1* mutant *snc1* (Li et al., 2001; Zhang et al., 2003). The *snc1* growth defect was significantly reduced by the *hsc70.1 hsc70.3* doublemutation as supported by the biomass quantification (Figure 4E and Figure S6B). The enhanced resistance to *Pst* DC3000 in *snc1* was also reduced by mutations of *HSC70.1* and *HSC70.3* (Figure 4F). Therefore, the inhibition of the *bon1* autoimmune phenotype by the reduction of HSC70 activity could result from the inhibition of SNC1 activity by the loss of HSC70 activity.

Interestingly, the *hsc70.1 hsc70.3* mutant displayed enhanced susceptibility to the virulent pathogen *Pst* DC3000 (Figure 4D). This phenotype was unexpected because overexpression of *HSC70.1* was shown previously to compromise resistance to both virulent and avirulent bacterial pathogens of *Pst* DC3000 (Noel et al., 2007). We analyzed disease resistance phenotypes in the *hsc70.1 hsc70.3* double mutant we generated as it has a stronger reduction of the *HSC70* family than the single mutants. We monitored the growth of additional *Pst* strains on the *hsc70.1 hsc70.3* double mutant, as single *hsc70.1* and *hsc70.3* mutants did not show changes in disease resistance to the virulent bacterial strains *Pst* DC3000 (Noel et al., 2007). At 3 DPI (days post inoculation), the mutantsupported more bacterial growth for the type III secretion-deficient strain *Pst* DC3000 *hrcU-* compared to the wild type (Figure 5A). This indicates that basal defense is compromised in the *hsc70.1 hsc70.3* double mutant plants.

The *hsc70.1 hsc70.3* double mutant is also compromised in resistance conferred by NB-LRR proteins RPS2 and RPS4. When dip-inoculated with the avirulent strains *Pst* DC3000 AvrRpt2 and *Pst* DC3000 AvrRps4, *hsc70.1 hsc70.3* supported more bacterial growth than the wild type Col-0 (Figure 5B and 5C). Therefore, immune responses conferred by NB-LRR proteins are compromised by the reduction of HSC70 function. Considering the dipping infection method used here, it is yet to be determined whether or not the positive role of HSC70s in ETI is solely due to its positive role in PTI.

**Opposite roles of *HSC70* in pre- and post- invasion phases of immune responses**

Compromised basal and NB-LRR mediated resistance was observed in the *hsc70.1* *hsc70.3* double mutant (this study) as well as in the *HSC70.1* overexpression line (Noel et al., 2007). One scenario to explain the apparent discrepancy is that *HSC70* might have opposite roles in two layers of defense: it inhibits immune responses at the stomatal closure phase while it positively regulates immune responses after pathogen invasion. We tested this hypothesis by comparing pathogen growth in the *hsc70.1 hsc70.3* double mutant from two inoculation methods: dipping and vacuum. With the dipping method, plants presumably mount both pre-invasion and post-invasion defense responses, while with the vacuum method, only post-invasion defense mechanism is effective.

We indeed observed differences in resistance in the *hsc70.1 hsc70.3* mutant by the two methods. The virulent pathogen *Pst* DC3000, with vacuum infiltration, had more growth in the double mutant than the wild type by 1.1±0.1, 0.8±0.1, and 0.6±0.1 of log (lg) value of colony forming unit per mg (cfu/mg) of leaf tissue in three independent experiments (Figure 4D, E, and Figure 6A). With dipping inoculation, it had no more growth in the double mutant compared to the wild type: the growth difference between the mutant and the wild type was 0.1±0.1, 0.1±0.1, and 0.3±0.2 lg cfu/mg in three independent experiments (Figure 6B and Figure S7A, B). For the virulent pathogen *Psm* ES4326, with vacuum infiltration, the differential growth in the mutant and the wild type was 2.2±0.1 and 1.3±0.2 lg cfu/mg in two independent experiments (Figure6C and Figure S7C). By dipping inoculation, the differential growth was 0.2±0.1 and 0.5±0.1 lg cfu/mg in two experiments (Figure 6D and Figure S7D). For nonvirulent pathogen *Pst* DC3000 *hrcU*-, the growth increase in the double mutant compared to the wild type was 0.6±0.2 and 0.9±0.1 lg cfu/mg with vacuum (Figure 6E and Figure S7E) and 0.2±0.1and 0.3±0.1 lg cfu/mg with dipping inoculation (Figure 6F and Figure S7F).

The reduced susceptibility to pathogens in the *hsc70.1 hsc70.3* double mutant with dipping inoculation compared to the vacuum inoculation indicates that the double mutant might be more resistant to pathogens at the pre-invasion phase compared to the wild type. Therefore, the *HSC70* genes very likely have opposing functions in two layers of immune responses during PTI and/or ETI: a positive role after the pathogen invades the apoplastic space and a negative role before invasion likely at the level of stomatal closure.

**BON1 positively regulates pre-invasion resistance via modulating stomatal closure**

We investigated whether or not physical interactions of BON1 with HSC70 and SGT1b have a more direct biological relevance in stomata control. Supporting this idea that *BON1* has role in pre-invasion defense, a difference in resistance level in *bon1* compared to the wild type was observed with vacuum and dipping infiltration methods. With vacuum inoculation, the decrease of pathogen growth in *bon1* compared to the wild type was 1.0±0.2 and 1.9±0.1 lg cfu/mg in two independent assays (Figure 6G and Figure S7G). With dipping, the decrease in growth of *Psm* ES4326 in *bon1* compared to the wild-type Col-0 was 0.1±0.2 and 0.3±0.2 lg cfu/mg at 3 days in two assays (Figure 6H and Figure S7H). Therefore, the *bon1* mutant is likely compromised in resistance at pre-invasion phase indicating a positive role of BON1 in pre-invasion defense response. This is in contrast to the previously known role of BON1 in negatively regulating *NB-LRR* genes at the post-invasion phase (Yang and Hua, 2004).

Because BON1 localizes to the plasma membrane where PAMP receptors reside and has a role in pre-invasion immune response, we asked whether or not BON1 might function as a positive regulator of early perception of PAMPs. Responses towards different PAMP treatments were assayed in *bon1* or *bon1 snc1-11* (where effects from activation of *SNC1* are minimized). One response to the PAMP flg22 is root growth inhibition, which is mediated by the PAMP receptor FLS2 (Zipfel et al., 2004). As expected, treatment of flg22 at 100 nM significantly inhibited root growth of Col-0 but not the *fls2* mutant. Roots of *bon1 snc1-11* and *snc1-11* were inhibited by flg22 similarly to the wild type Col-0 (Figure 7A), indicating that flg22 effects are not significantly altered by the loss of *BON1* function. As a more immediate response to 100 nM flg22, the production of reactive oxygen species (ROS), was also comparable in the *bon1 snc1-11*mutant and the wild type. While the *fls2* mutant had much less ROS at 10 min after treatment compared to the wild-type Col-0, *bon1 snc1-11* only showed a slight reduction of ROS compared with Col-0 and *snc1-11* (Figure 7B). Similarly, in response to 100 nM elf18, another PAMP, ROS accumulation in *bon1 snc1-11* and Col-0 or *snc1-11* also did not exhibit significant differences (Figure 7C). These data indicate that BON1 does not play a major role in flg22 and elf18 perception.

Because stomatal closure is an important defense mechanism at the pre-invasion phase, we investigated whether or not stomatal response is altered in the *bon1* mutants. We first analyzed ABA induced closure because ABA is involved in both abiotic and PAMP induced stomatal response (Fan et al., 2004; Melotto et al., 2006). Because activation of *NB-LRR* genes such as *SNC1* has been shown to inhibit ABA induced stomatal closure (Kim et al., 2011), we analyzed the effect of loss of *BON1* function in two pair of genotypes with no functional *SNC1* to eliminate potential secondary effects from its upregulation. The first pair was the wild-type Ws and a LOF *bon1-2* allele in the Ws accession. No functional *SNC1* exists in Ws, and therefore immune responses are not upregulated in *bon1-2* (Yang and Hua, 2004). The second pair was a *SNC1* LOF mutant *snc1-11* and a *bon1 snc1-11* double mutant (Yang and Hua, 2004). Leaf peels made from these plants were treated with 20 μM of ABA. While Ws wild-type and *snc1-11* plants closed their stomata at 1.5 hours after ABA treatment, no obvious stomatal closure was observed in *bon1-2* or *bon1 snc1-11* (Figure 7D, E, Figure S8). To exclude the possibility that this effect results from activation of genes other than *SNC1* in *bon1* (Li et al., 2009), we measured ABA response in a third pair of plants: the *pad4* mutant where TIR-NB-LRR signaling in general is blocked and the *bon1 pad4* double mutant. Compared with *pad4*, *bon1 pad4* did not respond to ABA by closing stomata (Figure 7F). Therefore, *BON1* has a positive role in ABA response in stomatal closure and this function is independent of its negative regulation of *NB-LRR* genes.

We further tested if the loss of BON1 function also compromises pathogen-induced stomatal closure. We used a coronatine deficient (COR-) *Pst* DC3000 strain for such assay to reveal the early defense response (stomatal closure) to pathogen without the counter-acting effect (stomatal opening) by coronatine from pathogens (Melotto et al., 2006). While *snc1-11* and Col-0 wild-type plants closed their stomata at 1.5 hours after pathogen treatment, very little stomatal closure was observed in *bon1* or *bon1 snc1-11* (Figure 7G). Therefore, *BON1* has a positive role in ABA and pathogen-induced stomatal closure, which likely counts towards its positive role in pre-invasion defense.

**The function of *BON1* at stomata is related to *HSC70* and *SGT1b***

Previous studies have demonstrated the involvements of *HSC70* and *SGT1b* in stomatal control (Clement et al., 2011). We determined whether or not the function of *BON1* in stomata control is related with *HSC70* and *SGT1b* functions by analyzing the ABA response in the *bon1 hsc70.1 hsc70.3* triple mutant. At 1.5 hours after ABA treatment, the wild type closed its stomata and the *hsc70.1 hsc70.3* double mutant had a similar response to the wild type. While the *bon1* mutant kept its stomata open, the *bon1 hsc70.1 hsc70.3* closed its stomata similarly to the wild type (Figure 7H). Similarly, overexpression of *SGT1b* largely suppressed the stomatal closure defect of *bon1*. In a representative *SGT1b-OE/bon1* line where *bon1* growth defect was largely suppressed by *SGT1b-OE*, stomata closed in response to ABA in a similar fashion as the wild type(Figure 7H)*.*

We further tested if the *hsc70.1* and *hsc70.3* double mutationscould also rescue *bon1*’s defect in pathogen triggered stomatal closure. At 1 hour after *Pst* DC3000 (COR-) treatment, *hsc70.1 hsc70.3* closed its stomata similarly to the wild type. While the *bon1* mutant kept its stomata open, the *bon1 hsc70.1 hsc70.3* closed its stomata similarly to the wild type (Figure 7I). Therefore, *BON1* positively regulates stomatal closure in response to ABA and pathogen and this function is closely related to the function of *HSC70* and *SGT1b* in stomata regulation.

***STG1b* negatively regulates immune responses in *bon1***

We next assessed the biological relevance of physical interaction between BON1 and SGT1b. We generated a double mutant between *bon1* and the *SGT1b* LOF mutant allele *edm1.1* (Tor et al., 2002) which we refer here as *sgt1b*. While the *bon1 hsc70.1* mutant had a milder growth defect than *bon1*, the *bon1 sgt1b* double mutant had a more severe growth defect than *bon1* (Figure 8A). The visual observation was verified by biomass measurement where fresh weight of *bon1 sgt1b* was significantly lower than that of *bon1* (Figure S6C). Expression of *PR1* is further up-regulated in the *bon1 sgt1b* double mutant compared to the *bon1* single mutant as assayed by qRT-PCR (Figure 8B), indicating that the defense responses in *bon1* are enhanced by the loss of the *SGT1b* function.

We subsequently over-expressed *SGT1b* in *bon1* by the CaMV 35S promoter using the pHPT vector (Tzfira et al., 2005). Among nine such *SGT1b-OE* independent lines, all showed a milder growth defect compared to *bon1* (Figure 8C), which was verified by biomass quantification (Figure S6C). The *SGT1b* transcript level was increased in two selected transgenic lines as assayed by qRT-PCR (Figure S9). In the progenies of the two T1 lines tested, the close to wild-type phenotype co-segregated with the presence of the *SGT1b*-*OE* transgene, further supporting that overexpression of *SGT1b* inhibited the *bon1* growth phenotype. Although *SGT1b-OE* in Col-0 wild type did not appear to affect disease symptom to *Pst* DC3000 (Uppalapati et al., 2011), it compromised the enhanced disease resistance to *Pst* DC3000 in *bon1* (Figure 8D). Therefore, *SGT1b*, opposite to *HSC70*, inhibits autoimmune responses in *bon1*.

We asked if the partial inhibition of the *bon1* phenotype by *SGT1b* overexpression has resulted from blocking signaling leading to *SNC1* transcript upregulation or from disrupting signaling after *SNC1* upregulation. To this end, *SGT1b* was overexpressed in the autoimmune mutant *snc1*. Among 27 *SGT1b-OE* transgenic lines generated in *snc1*, 26 showed inhibition of the growth defect of *snc1* (Fig 8E), which was also verified by biomass quantification (Figure S6C). The *SGT1b* expression was higher in two representative *SGT1b-OE* lines than in the wild type as assayed by qRT-PCR (Figure S9), and, the growth defect suppression was strictly correlated with the *SGT1b-OE* transgene. Therefore, *SGT1b* modulates *SNC1-*mediated defense responses, likely through SNC1 protein degradation, which could account for its modulation of immune responses in *bon1*.

***STG1b* inhibits SNC1 protein accumulation**

A previous study showed a requirement of *SGT1b* in resistance mediated by a number of NB-LRR proteins but not SNC1(Goritschnig et al., 2007). In fact, the SNC1 protein accumulated to a higher level in the *sgt1b* mutant although no increase of disease resistance was observed in the *sgt1b* mutant compared to the wild type (Li et al., 2010). This prompted us to test whether or not the suppression of *snc1* phenotype by SGT1b overexpression is due to the reduction of SNC1 protein accumulation.

To assay both SNC1 activity and SNC1 protein level, we used a previously established expression system where overexpression of a GFP-tagged SNC1 protein induced cell death in *N. benthamiana* (Zhu et al., 2010; Gou et al., 2012; Mang et al 2012). *SGT1b* and a control gene *COPT1* were over-expressed by the CaMV 35S promoter using the vector pGWB402 (Nakagawa et al., 2007) via Agro-infiltration in *Nicotiana tabacum*. At 3 days post infiltration (DPI), extensive cell death was observed in leaf areas co-infiltrated with *SNC1:GFP* and a pGWB402 empty vector, while neither the empty vector nor the pGWB402:SGT1b construct triggered visible cell death (Figure 9A). When *SGT1b* was co-infiltrated with *SNC1:GFP*, cell death was greatly reduced, while co-infiltration of the control gene *COPT1* with *SNC1:GFP* did not affect cell death (Figure 9A). This indicates that the reduction of SNC1 triggered cell death by SGT1b was not due to a non-specific effect from co-infiltration (Figure 7A). This is consistent with the observation in Arabidopsis transgenic plants where overexpression of *SGT1b* suppressed the *snc1* phenotype (Figure 8E). We then analyzed SNC1:GFP accumulation with or without SGT1b co-expression in *N. tabacum* leaves before the onset of cell death by Western blotting. Quantified with the control protein signal stained by Ponceau S, the SNC1:GFP protein level was reduced in tissues co-infiltrated with SGT1b compared to the vector control (Figure 9B). As a control, we also detected the SNC1 level in the Arabidopsis *sgt1b* mutant using an anti-SNC1 antibody previously described (Cheng et al., 2011). Consistent with previous reports (Li et al., 2010), SNC1 accumulates to a higher level in *sgt1b* than in Col-0 wild type (Figure 9C). Together, these data indicate that *SGT1b* inhibits SNC1 protein accumulation, which likely leads to suppression of *SNC1* mediated disease resistance.

***HSC70.1* positively regulates SNC1 activity but not SNC1 protein accumulation**

We used the same expression system to analyze the effect of *HSC70* on *SNC1*-mediated defense responses. At 2 DPI, neither the pMDC32 empty vector (Curtis and Grossniklaus, 2003) nor *HSC70.1* in pMDC32 triggered any cell death in the infiltrated leaf area (Figure 9D). Mild cell death was observed in leaf areas co-infiltrated with *SNC1:GFP* and a pMDC32 empty vector. When *HSC70.1* was co-infiltrated with *SNC1:GFP*, more extensive cell death was induced (Figure 9D), indicating that *HSC70.1* enhances *SNC1* mediated defense responses.

We subsequently analyzed the effect of *HSC70* on the accumulation of SNC1:GFP protein before the onset of cell death. Analyzed by the relative ratio of GFP to the control protein signal, no difference could be detected between the SNC1:GFP signal from tobacco leaves co-infiltrated with the pMDC32 vector or *HSC70.1* in pMDC32 (Figure 9E), suggesting that overexpressing *HSC70*, though enhancing SNC1 activity, does not increase the accumulation of the SNC1 protein. We further compared the endogenous SNC1 protein level in the wild type and the *hsc70.1 hsc70.3* double mutant by western blot using anti-SNC1 antibody. No significant difference in the SNC1 protein level was observed in the *hsc70.1 hsc70.3* double mutant compared to the wild type after the relative ratio of SNC1 to the control signal was calculated (Figure 9F). As a control, an increase of SNC1 level was observed in *snc1* mutant compared with the Arabidopsis wild-type Col-0 as reported earlier (Figure 9F) (Cheng et al., 2011). Therefore, HSC70 may not regulate the SNC1 protein accumulation to affect its activity.

**Discussion**

***HSC70*s have opposite roles in pre- and post-invasion phases of immune responses**

The roles of HSC70 proteins in plant immunity were not clearly defined due to genetic redundancy and functional compensation among *HSC70* genes and lethality of the knockout mutant of the gene family. In this study, we used a reduction of function mutant combination of *HSC70.1* and *HSC70.3* to sufficiently reduce the total HSC70 activity without causing lethality and consequently revealed opposite roles of HSC70 proteins in pre-invasion and post-invasion defenses. This *hsc70.1 hsc70.3* double mutant is more susceptible than the wild type to virulent and type III secretion-deficient bacterial strains(Figure 4, 5), indicating a positive role of *HSC70s* in regulating PTI. The *hsc70.1 hsc70.3* doublemutant is also more susceptible than the wild type to avirulent bacterial pathogens and the double mutation largely suppresses the defense phenotypes conferred by the active form of SNC1 (Figure 4, 5). This may result from the positive role of HSC70s in PTI (as in resistance to virulent and non-virulent pathogen) or it could be from a positive role of HSC70s in ETI as well. Overexpression of HSC70 can enhance SNC1 triggered cell death in *N. tabacum*, suggesting that HSC70 could have a positive role in ETI in addition to PTI.

In addition to revealing a positive role of HSC70s in PTI/ETI, our data support the negative role of HSC70 proteins on stomatal closure in plant immunity. The *hsc70.1 hsc70.3* double mutant is more susceptible when pathogens are dip-inoculated compared with vacuum infiltrated (Figure 6 and Figure S7), suggesting that the double mutant might more effectively restrict pathogen invasion than the wild type. This is consistent with the earlier finding that *HSC70* overexpression or the loss of *SGT1b* function confers insensitivity in stomatal closure to environmental factors (Clement et al., 2011). However, we did not observe an enhanced sensitivity of stomatal response to ABA or non-virulent pathogen in the *hsc70.1 hsc70.3* double mutant (Figure 7), which can be due to the low detection sensitivity of stomatal measurement and/or limited time points we used in this study. In light of this study implicating opposing roles of HSC70s in two phases of immune responses, contradictory roles reported for HSC70s in disease resistance from earlier studies can now be explained by differential contribution of two layers of defenses in a particular plant-pathogen interaction as well as potential differential roles of each of the *HSC70* family members in specific immune responses.

**Interaction of HSC70 and SGT1b in regulating SNC1**

The role of HSC70 in SNC1 mediated defense response is opposite to that of SGT1b which is implicated in the degradation of SNC1. The SNC1 protein accumulated to high levels in the *sgt1b* mutant(Li et al., 2010) and it is targeted by the F-box protein CPR1/CPR30 for degradation (Gou et al., 2009; Cheng et al., 2011; Gou et al., 2012). Here, we found that defects in *bon1* or *snc1* could be enhanced by the *sgt1b* mutation and inhibited by *SGT1b* overexpression (Figure 8). SGT1b is involved in degrading the SNC1 protein, as seen with a reduced level of SNC1 and *SNC1*-triggered cell death in *SGT1b-OE* tobacco leaves (Figure 9). In contrast, HSC70.1, while enhancing SNC1 triggered cell death when overexpressed, does not affect the protein accumulation of SNC1 (Figure 9). As the HSC70s can assist proper folding of client protein (Hartl, 1996), they may enhance SNC1 activity by aiding the correct folding of SNC1. Alternatively, HSC70s might affect components downstream of SNC1 in defense responses and have no direct effect on the SNC1 protein itself. It is yet to be determined whether or not the opposite function of HSC70 and SGT1b results from direct physical interaction of these two proteins or their independent interactions with the SNC1 protein.

There is no evidence so far to support the hypothesis that BON1 regulates SNC1 primarily at the protein level although physical interaction of BON1 and SGT1b is observed here. Elevated defense response in *bon1* results from upregulation of the NB-LRR gene *SNC1* (Yang and Hua, 2004). Because *hsc70.1 hsc70.*3 compromised defense responses in the active *snc1* mutant, and the *hsc70.1 hsc70.3* mutant is more susceptible to *Pst* DC3000 (Figure 4), the reduction of defense responses in *bon1* by the *hsc70.1 hsc70.3* mutation results from an additive effect from *bon1* and *hsc70.1 hsc70.3* (Figure 4). Therefore, BON1 and HSC70 may not function strictly in a linear pathway. The suppression of *bon1* by *hsc70* mutations or *SGT1b* overexpression likely result indirectly from their effects on the SNC1 protein translated from the *SNC1* transcript that is upregulated in *bon1*.

**Function of BON1 in stomatal closure**

We identified a new role of BON1 in plant immunity, that is, the modulation of stomatal closure. Previous screening of BON1 interacting proteins with yeast two-hybrid assays have identified BAP1 and BIR1 proteins which are involved in negative regulation of plant defense that is dependent on *SNC1* (Hua et al., 2001; Yang et al., 2006; Wang et al., 2011). However, the link between the plasma membrane-localized BON1 and the transcriptional regulation of NB-LRR-encoding genes remained elusive. Here we found that BON1 physically interacts with HSC70 and SGT1b, both of which are involved in regulating stomatal closure induced by environmental stimuli (Clement et al., 2011). The *bon1* mutant is compromised in both ABA and pathogen-triggered stomatal closure, and this effect is independent of SNC1 (Figure 7). Furthermore, the defect in ABA response in the *bon1* mutant is suppressed in the *hsc70.1 hsc70.3* mutant and *SGT1b-OE* lines (Figure 7). Thus the BON1 protein might interact with HSC70 and SGT1b to regulate stomatal closure. How BON1 connects with the known signaling components in stomatal closure control is not known. One possibility is that BON1 interacts with receptor like kinases (RLKs) in addition to BIR1, and some RLKs such as GHR1 have been shown to mediate ABA induced stomatal closure (Hua et al., 2012). Further research should identify the regulatory target(s) of BON1, HSC70, or SGT1b in stomata regulation and reveal how BON1 activity might be regulated by biotic and abiotic environmental signals.

The *bon1* mutants do not appear to have major defects in PAMP perception (Figure 7), and therefore BON1 may not directly regulate the activity of PAMP receptors. As BON1 is a calcium binding protein and calcium binding activity is critical essential for its function (Hua et al., 2001; Li et al., 2010), it could be involved in calcium signal perception and in turn modulates signaling pathways or channels/transporters. Similarly, *HSC70* and *SGT1b* were thought to function not in early signal perception but in later signaling and execution events (Clement et al., 2011).

Previous studies revealed a negative regulation of NB-LRR-encoding genes by BON1 and that this regulation likely occurs at the transcriptional level (Yang and Hua, 2004; Li et al., 2009). The positive role of BON1 in plant immunity at the pre-invasion phase suggests that this function of BON1 at the stomata might be ‘guarded’ by SNC1 and the loss of BON1 function might be recognized as a manipulation by pathogen. As there is no evidence for direct interaction of BON1 and SNC1 proteins, the guarding of BON1 by *SNC1* would be indirect, similar to a NB-LRR gene *SUMM2* guarding the MPK4 activity (Zhang et al., 2012). The regulation of *SNC1* by BON1 may mimic the regulation of RPM1 and RPS2 by RIN4, a classical example of ‘guard’ hypothesis (Chisholm et al., 2006; Jones and Dangl, 2006). Coincidently, both RIN4 and BON1 are plasma membrane-localized proteins (Hua et al., 2001; Kim et al., 2005; Takemoto and Jones, 2005; Li et al., 2010), and RIN4 is also involved in stomatal closure (Liu et al., 2009). It remains to be tested if BON1 is targeted by potential effectors for modification or degradation.

In sum, our study reveals that both BON1 and HSC70 have opposing roles in pre- and post-invasion phases of immune responses (Figure 10). In the pre-invasion phase, HSC70 negatively and SGT1b positively regulate stomatal closure while BON1 perhaps works together with HSC70 and SGT1b to positively regulate pathogen- and ABA-induced stomatal closure. In post-invasion phase, BON1 negatively regulates the transcript level of *SNC1* through an as yet undetermined mechanism, while SGT1b and HSC70 have opposing roles in regulating SNC1 protein activity likely by CPR1-coupled degradation, proper protein folding, or downstream signaling. The opposing roles of the same protein on plant immunity at different stages suggest that plant immunity is an evolving system resulting from targeting positive immune regulators by pathogens and guarding the immune regulators with NB-LRR genes by plants.**Materials and methods**

**Plant growth**

Arabidopsis plants were grown in growth chambers either under constant light for growth phenotyping or at 12/12 hour photoperiod for pathogen growth tests, with light intensity at 100 µmol m-2s-1 and relative humidity at 50-70%. Seeds were planted either on 0.5 x MS (Murashige and Skoog, Sigma) medium containing 0.8% agar and 2% sucrose or directly in soil (Metro-Mix 200; SunGro). *Nicotiana benthamiana* or *Nicotiana tobaccum* plants were grown in the greenhouse at 24°C for 4-6 weeks before use for transient expression studies.

**BON1 protein complex purification and LC-MS analysis**

Wild type and *BON1-HA* transgenic Arabidopsis plants were grown to 5 weeks old and half of each were inoculated with *Pst* DC3000. Ten grams of leaf tissues at 2 hours after inoculation were collected and combined with 10 grams of tissue not inoculated with pathogen. Proteins were extracted and the BON1 protein complex was purified following the protocol previously described (Qi et al., 2007). Protein gels for the control and BON1-HA sample were each cut into 4 parts for the in-gel digestion and manual extraction following a previously reported protocol (Zhang et al., 2003). The tryptic digest was subject to nanoLC-ESI-MS/MS analysis using a LTQ-Orbitrap Velos (Thermo-Fisher Scientific, San Jose, CA) mass spectrometer equipped with “Plug and Play” nano ion source device (CorSolutions LLC, Ithaca, NY). Proteins detected in the BON1-HA sample but not in the wild type control in two biological repeats were selected as candidate BON1 interacting proteins.

**Bimolecular fluorescence complementation (BiFC) assay**

The full-length cDNA fragments (without stop codon) of *BON1*, *HSC70.1(2,3)* and *SGT1b(a)* genes were amplified from the wild-type Col-0 cDNA using primers in Table S1, and cloned into the Gateway entry vector pCR®8 TOPO® TA vector (Invitrogen). For BiFC experiments, *BON1* was cloned into pSPYNE-35SGW or pSPYCE-35SGW (Walter et al., 2004; Schutze et al., 2009) using LR clonase (Invitrogen cat#11791) to generate BON1:YFPC or BON1:YFPN constructs, while *HSC70.1(2,3)* and *SGT1b* were cloned similarly to generate HSC70.1(2,3):YFPN and SGT1b:YFPC constructs, respectively. A previously described protocol (Walter et al., 2004, Schütze et al., 2009) was followed to observe BiFC signals with some modification. The constructs were transformed into the *Agrobacterium* strain GV3101. Overnight cell cultures were collected and re-suspended in 1 ml of AS medium (1 ml of 1 M MES-KOH pH 5.6, 333 μl of 3 M MgCl2, 100 μl of 150 mM acetosyringone) to OD600 at 0.7–0.8. The working suspensions were prepared by mixing at 1:1:1 ratio three *Agrobacterium* strains respectively carrying the YFPN fusion, YFPC fusion, and the gene silencing inhibitor pBA-HcPro (Menke et al., 2005) and let them stand for 2–4 hours on bench. The *Agrobacterium* suspensions were then co-infiltrated into the abaxial surface side of 4-6 week-old *N. benthamiana* plant leaves. Fluorescence of the epidermal cell layer of the lower leaf surface was examined at 2-4 DPI. Images were captured by Leica TCS SP2 Confocal Microscope with excitation wavelength at 488 nm and 496 nm, and emission wavelength between 520-535 nm for YFP signals.

**Co-IP and immunoblot analyses using tobacco and Arabidopsis materials**

An HA tag is present in the pSPYCE-35S GW constructs and a Myc tag is present in the pSPYNE-35S GW constructs (Schutze et al., 2009). Tobacco leaves transiently co-expressing each of the BiFC constructs harvested after 2~4 DPI or 3 week-old soil-grown Arabidopsis seedlings were ground with liquid nitrogen. Total proteins were extracted from tobacco leaves with buffer containing 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 150 mM NaCl, 10% glycerol, 5 mM DTT, 0.25% Triton-X 100, and 1x complete protease inhibitor cocktail. After spinning protein extracts twice at 12,000g for 10 min, the supernatant was subject to de-salt by passing through a Sephadex G-25 column (GE healthcare illustra NAP-5). For immune-precipitation, 15 µl of EZviewRed Anti-HA Affinity Gel Beads (E6779, Sigma) was mixed with crude protein extracts and agitated at 4°C for overnight. The affinity beads were then pelleted by centrifugation for 1 min at 1,000 g, and washed three times with 1ml of IP buffer (25 mM Tris-HCl pH7.5, 1 mM EDTA, 150 mM NaCl, 0.15% NP-40, 1x protease inhibitor cocktail). The bound proteins were eluted from the beads by boiling them in 50 µl SDS-PAGE sample buffer for 5 min, and 10 µl IPed samples were separated by SDS-PAGE gel. Immunoblot analyses were performed according to the ECL Western Blotting procedure (GE Healthcare) using commercial anti-HA antibody HAII clone 16B12 (Covance), anti c-Myc antibody (9E10) sc-40 (Santa Cruz), anti-HSP70 antibody ADI-SPA-817 (Enzo Life Sciences), and anti-Bip antibody COP-080017 (Cosmo Bio Co., Ltd).

**Yeast two-hybrid assay**

The yeast two-hybrid constructs were made in the pDEST-GBKT7 and pDEST-GADT7 gateway vectors as previously described (Rossignol et al., 2007). The *BON1* cDNA was cloned from the entry vector into the pDEST-GBKT7 to generate a BD-BON1 construct, while *HSC70s* and *SGT1b* or truncated *SGT1b* were cloned into pDEST-GADT7 to generate AD-HSC70 or AD-SGT1b constructs, respectively. The yeast two-hybrid assay was performed as previously described (Li et al., 2010).

**Characterization of growth phenotypes of *bon1*, *hsc70*, and *sgt1b* related mutants.**

The *HSC70* mutants *hsc70.1* (Salk\_135531C), *hsc70.2* (Salk\_085076C), and *hsc70.3* (Salk\_148168) were ordered from ABRC and genotyped to obtain homozygous seeds using primers listed in Table S1. Double and triple mutants were generated by crossing *hsc70* and *sgt1b* (*edm1.1*) mutants to *bon1* or *snc1*. Unless stated otherwise, at least 10 3-week-old seedlings grown in soil were used for growth phenotyping and biomass quantification.

**Heat-shock tolerance assay of *hsc70* mutants**

Arabidopsis seedlings were grown in ½ MS (1/2 MS salts, MES, pH 5.7, 1% sucrose) plates in growth chamber as described above. Plates containing the 10-day-old seedlings were sealed with plastic tape and submerged in a water bath at 45°C for 30min, and the heat-shocked seedlings were recovered at 22°C for 3-5 days before the photographs were taken and the biomasses were quantified.

**Plant transformation and transgenic plants selection**

The *SGT1b* cDNA from the gateway entry vector was sub-cloned into the destination vector pHPTN1GW which was modified from the binary vector previously described (Tzfira et al., 2005). pHPTN1:SGT1b was transformed into *bon1* and *snc1* mutants, and transgenic plants were obtained using 50 μg/ml hygromycin for selection on MS plates. The *HSC70.1* and *HSC70.3* full length cDNAs (with stop codons) were cloned into pDONR207 entry vector and sub-cloned into pGWB402 destination vector (Nakagawa et al., 2007) to generate the overexpression constructs that were transformed into *bon1.* Transgenic plants were selected with 50 μg/ml Kanamycin on MS plates. The genomic piece of *HSC70.1* was amplified using primers listed in Table S1, cloned into the pDONR222 entry vector and sub-cloned into the PMDC99 gateway binary vector. The *pMDC99:HSC70.1* construct was transformed into *bon1 hsc70.1 hsc70.3* for the complementation test.

**Bacterial growth assay**

Bacteria grown on plates with King’s B medium were washed and collected with 10 ml of 10 mM MgCl2. They were diluted in 10 mM MgCl2 with 0.02% Silwet L-77 to OD600=0.05 for dipping inoculation and OD600=0.002 for vacuum infiltration. Plants were immersed in inoculums for 10 seconds by dipping or 2 minutes by vacuum. They were covered immediately to keep humidity after inoculation and uncovered one hour later. Bacterial growth in plants was assayed at 1 hour (day 0) and 3 days (day 3) after inoculation. Three whole seedlings were collected as one sample, weighed, grounded in 1 ml of 10 mM MgCl2 with 0.02% Silwet L-77, and shaken at room temperature for 1 hour. Serial dilutions of the ground solution were spotted on growth media, and the number of cfu per fresh weight was determined. Three samples were analyzed for each genotype and condition combination.

**qRT-PCR analysis**

Total RNAs were extracted from soil-grown 3-week-old plants using Trizol reagent (Invitrogen) as instructed. SuperScript® II Reverse Transcriptase (Invitrogen) was used to synthesize cDNA from the mRNA. qRT-PCR was performed using primers listed in Table S1. *ACTIN2* and *TUBULIN2* genes were used as internal controls. SsoAdvanced™ Universal SYBR® Green Supermix (Biorad) was used for qRT-PCR.

**Analysis of responses to PAMP signals**

Seedlings were grown in sterile ½ MS medium with flg22 at 10 µM for one to two weeks for root inhibition assay. ROS responses to PAMPs were carried out as previously described (Schwessinger et al., 2011). Eight leaf discs (4 mm diameter) per genotype were collected in 96-well plates and allowed to recover overnight in sterile water. The water was then removed and replaced with an eliciting solution containing 17 mg/mL luminol (Sigma Aldrich), 200 µg/mL horseradish peroxidase (Sigma Aldrich), and an appropriate concentration of the desired PAMP in water. Luminescence was recorded over a 40 – 60 minute time period using a charge-coupled device camera (Photek Ltd., East Sussex UK). Peptide sequences for flg22, elf18 have been previously described (Felix et al., 1999; Kunze et al., 2004) and were synthesized by EZBiolab (Indiana USA).

**Stomatal assay**

Stomatal closure assays were done as previously described (Zeng and He, 2010) with slight modifications. Plants were grown under a 12/12 hours photoperiod at 22°C for ABA-induced stomata closure assay. Leaves were collected at 4 weeks after germination and placed in MES buffer (25 mM MES-KOH, pH 6.15, 10 mM KCl) or MES buffer with 20 μM abscisic acid in closed Petri dishes for 1.5 hrs. For pathogen-induced stomatal closure assay, leaves from 5-week-old plants were incubated in water for 2 hours, before being transferred into water (control) or *Pst* DC3000 COR- (5×107 cfu/ml) bacteria suspended in water for 1 hour in closed Petri dishes. Epidermis were peeled and imaged with Lecia ICC50HD microscope. At least 20 stomatal apertures were measured for each sample using ImageJ software. Each experiment were repeated at least 3 times.

**Transient expression of SNC1 with SGT1b or HSC70.1 in tobacco leaves**

The *pHPTN1:SNC1* construct (Zhu et al., 2010), the *pMDC32:HSC70.1* construct, the *pGWB402:SGT1b* construct, the *pMDC32* vector, and the *pGWB402* vector were each transformed into the *Agrobacterium* strain GV3101. Overnight cell cultures were collected and resuspended in 1 ml of AS medium (1 ml 1 M MES-KOH, pH 5.6, 333 μl 3 M MgCl2 , 100 μl 150 mM acetosyringone) to OD600 at 0.7–0.8. The *Agrobacterium* suspensions containing different constructs were combined at 1:1 ratio and co-infiltrated into the abaxial surface side *N. tabacum* leaves of 4-6 week-old plants. Picture was taken at 2 or 3 DPI for the cell death phenotype. Protein was extracted and Western blot was performed as described (Gou et al., 2012; Mang et al., 2012). The polyclonal GFP antibody A6455 (Invitrogen) was used to detect the SNC1-GFP fusion proteins.

**Arabidopsis total protein extraction and western blot analyses of SNC1**

Arabidopsis seedlings grown on ½ MS plate for 12 days were used for total protein extraction and western blot using anti-SNC1 antibody following a previously described method (Cheng et al., 2011).

**Acknowledgements**

We thank Dr. Sheng Zhang for the help and discussion of the LC-MS analysis of BON1 protein complex, Dr. Xin Li for the kind gift of the anti-SNC1 antibody and advice on Western blot using this antibody, Dr. Tsuyoshi Nakagawa for the PGWB vectors, Dr. Alan Collmer and Dr. Shengyang He for bacterial pathogen strains, Dr. Hailei Wei and Dr. Yuan Wang for the kind help of flg22-triggered ROS assay. We also thank Arabidopsis Biological Resource Center for the Arabidopsis mutants.

**Author contribution:**

MG and JH designed the experiments; MG, ZZ, NZ, QH, JM, HY, and ZY performed experiments; MG, ZZ, NZ, JM, HY, CZ, and JH analyzed data; MG and JH wrote the paper.

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**Figure legends**

**Figure 1. Characterization of BON1 interaction with HSC70 proteins.**

(A) BiFC assay for BON1 and HSC70 interaction. Shown are confocal images (bright field and YFP fluorescence) of *N. benthamiana* leaves co-infiltrated with BON1:YFPC and HSC70.1 (2, 3):YFPN constructs. YFPN, YFPC, or COPT1-YFPN co-infiltrated with BON1 or HSC70 constructs were used as negative controls. Scale bar=50 µm.

(B) Co-IP assay for BON1 and HSC70.1 interaction in *N. benthamiana* leaves. Crude lysates (Input) were IPed with anti-HA antibody and then detected with anti-HA and anti-Myc antibodies for BON1:HA and HSC70.1:Myc, respectively.

(C) Co-IP assay for BON1 and COPT1 in *N. benthamiana* leaves. Blot is displayed as in (B).

(D) Co-IP assay for BON1 and HSC70 using Arabidopsis BON1-HA/*bon1* (indicated by +) transgenic plants with the wild type (indicated by -) as control. Crude lysates (Input) were IPed with anti-HA antibody and then detected with anti-HSC70 and anti-BiP antibodies for cytosolic and ER HSP70 proteins respectively.

**Figure 2. Characterization of BON1 interaction with SGT1b.**

(A) BiFC assay for BON1 and SGT1b interaction. Experiments were performed similarly as in Figure 1A. Scale bar=50 µm.

(B) Co-IP assay for BON1 and SGT1b interaction. Blot is displayed similarly as in Figure 1B.

(C-D) Yeast two-hybrid assay for BON1 and full-length SGT1b (C) and truncated SGT1b proteins (D).

Yeasts were grown on SD plates without leucine or tryptophan (SD-LT) and SD plates without leucine, tryptophan, adenine, or histidine but with 3 mM of 3-Amino-1, 2, 4-triazole (SD-LTAH+3AT). BD-BON1 and AD-BAP1 were co-transformed as a positive control, while BD-BON1 and AD empty vector, or BD empty vector and AD-SGT1b were co-transformed as negative controls. SGT1b1-120 and SGT1b121-358 denote the AD fusions of the SGT1b domains of amino acids 1-120 and amino acids 121-358 respectively. Shown is yeast growth with a serial dilution of 10-1 to 10-5 at 3 days after being spotted onto the SD plates.

**Figure 3. Characterization of the *hsc70* mutants.**

(A) Diagram of the T-DNA insertion mutant of the *HSC70.3* gene. Arrows point to the translation start (ATG) and stop (TAA) codons. Triangle indicates the site of T-DNA insertion in the SALK\_148168 (*hsc70.3-2*) mutant. Dashed lines mark the regions for qRT-PCR analysis of *HSC70.3* expression.

(B) qRT-PCR analysis of *HSC70* transcripts. *HSC70.1* and *HSC70.3* expression levels were assayed in Col-0 wild type, *hsc70.1-1* (referred to as *hsc70.1* afterwards), and *hsc70.3-2* (referred to as *hsc70.3* afterwards) plants at 3 weeks old. Error bars represent standard deviation (Student’s t-test, \*\*\*p<0.001).

(C) Rosette leaf phenotype of *hsc70.1*, *hsc70.3*, and *hsc70.1 hsc70.3* mutants compared to the wild-type Col-0 before bolting*.*

(D) Heat stress sensitivity of the *hsc70* double mutants. Shown are seedlings of the wild-type Col-0 and *hsc70.1 hsc70.3* after a 45°C heat shock for 20 min followed by 5-day recovery at 22°C.

(E) Heat stress sensitivity of the *hsc70* mutants. Shown are seedlings of the wild-type Col-0, *hsc70.1*, *hsc70.3*, and *hsc70.1 hsc70.3* without (left panel) or with (right panel) a 45°C heat shock for 20 min followed by 5-day recovery at 22°C.

(F) Fresh weights of Arabidopsis seedlings in E. The average fresh weight was calculated from 7 seedlings, and error bars represent standard deviation (Student’s t-test, \*\*\*p<0.001).

**Figure 4. Mutations in *HSC70.1* and *HSC70.3* partially suppress the *bon1* and *snc1* phenotypes.**

(A) Partial rescue of the *bon1* growth phenotype by the *hsc70* mutations. Shown are 40-day-old plants of Col-0, *hsc70.1, hsc70.2, hsc70.3, bon1, bon1 hsc70.1, bon1 hsc70.2, bon1 hsc70.3, bon1 hsc70.2 hsc70.3,* and *bon1 hsc70.1 hsc70.3*.

(B) Down-regulation of *PR1* gene expression in *bon1* by *hsc70* mutations. Shown are relative expression levels of *PR1* gene in Col-0, *bon1*, *hsc70.1*, *hsc70.3*, *bon1 hsc70.1*, *bon1 hsc70.3*, and *bon1 hsc70.1 hsc70.3* assayed by qRT-PCR. Error bars represent standard deviation (Student’s t-test, \*\*\*p<0.001).

(C) Complementation test of *bon1 hsc70.1 hsc70.3* mutant. Shown are Col-0, *bon1*, *bon1 hsc70.1 hsc70.3*, and two independent T2 lines (CT-1, CT-2) of *bon1 hsc70.1 hsc70.3* transformed with the genomic fragment of *HSC70.1* in the vector *pMDC99* (*pMDC99:HSC70.1*).

(D) Partial suppression of resistance against the virulent bacterial strain *Pst* DC3000 in *bon1* by the *hsc70* mutations. Shown is the growth of bacteria as log value of colony formation unit (cfu) per mg tissue of in Col-0, *bon1*, *hsc70.1 hsc70.3*, and *bon1 hsc70.1 hsc70.3* via vacuum inoculation at 0 and 3 days post inoculation (DPI). Values represent averages of 3 biological repeats, and error bars represent standard deviations. Letters indicate statistical difference (*p*<0.001, Bonferonni post-test) of different genotypes.

(E) Partial rescue of the *snc1* growth defect by the *hsc70* mutations. Shown are wild-type Col-0, *snc1, snc1 hsc70.1, snc1 hsc70.3,* and *snc1 hsc70.1 hsc70.3* plants before bolting.

(F) Partial suppression of enhanced resistance against virulent bacterial pathogen *Pst* DC3000 in *snc1* by the *hsc70* mutations. Shown are bacterial growths in Col-0, *snc1, hsc70.1 hsc70.3*, and *snc1 hsc70.1 hsc70.3* plants at 0 DPI and 3 DPI. Letters indicate statistical difference (*p*<0.001, Bonferonni post-test) of different genotypes.

**Figure 5. Enhanced susceptibility of the *hsc70.1 hsc70.3* mutant to type III secretion-deficient and avirulent bacterial pathogens*.***

Shown are growth of pathogen strains in wild-type Col-0 plant and the *hsc70.1 hsc70.3* double mutant at 0 DPI and 3 DPI. Strains are (A) type III secretion-deficient strain *Pst* DC3000 (*hrcU-*), (B) avirulent strain *Pst* DC3000 AvrRpt2, and (C) avirulent strain *Pst* DC3000 AvrRps4. Values represent averages of 3 biological repeats, and error bars represent standard deviation. Star indicates a statistical difference from the wild type at 3 DPI (Student’s t-test, \*\*p<0.01, \*\*\*p<0.001).

**Figure 6. Comparing pathogen growth in *hsc70.1 hsc70.3* and *bon1* mutants with different inoculation methods.**

(A-F) Pathogen growth of *Pst* DC3000 (A, B), *Psm* ES4326 (C, D), and *Pst* DC3000 *hrcU-* (E, F) in Col-0 and the *hsc70.1 hsc70.3* mutant by vacuum (A, C, E) or dipping (B, D, E) inoculations at 0 and 3 DPI.

(G, H) Pathogen growth of *Psm* ES4326 in Col-0 and the *bon1-1* mutant by vacuum (G) or dipping (H) inoculations at 0 and 3 DPI.

Values represent averages of 3 biological repeats, and error bars represent standard deviations. Star indicates a statistical difference from the wild type (Student’s t-test, \* p<0.05, \*\*p<0.01, \*\*\*p<0.001).

**Figure 7. BON1 negatively regulates stomatal closure but not PAMP perception.**

(A) Response to flg22 assayed by root growth inhibition. Shown are root lengths of Col-0, *fls2*, *snc1-11*, and *bon1 snc1-11* at 2 weeks after grown on plates with or without 100 nM flg22.

(B) Analysis of oxidative burst triggered by flg22 measured by luminol. Shown are averages of total photo counts of Col-0, *fls2*, *snc1-11*, and *bon1 snc1-11* leaf disks at 10 minutes after treatment with 100 nM of flg22.

(C) Analysis of oxidative burst triggered by elf18 measured by luminol. Shown are averages of total photo count of Col-0, *snc1-11*, and *bon1 snc1-11* leaf discks 60 minutes after treatment with 100 nM of elf18.

(D)-(F) Stomatal aperture after treatment with 20 μM ABA for *snc1-11* and *bon1 snc1-11* (D), wild type Ws and *bon1-2* in Ws(D), and *pad4* and *bon1 pad4* (F) leaves.

(G) Stomatal aperture after treatment with *Pst* DC3000 COR- for Col-0, *bon1*, *snc1-11* and *bon1 snc1-11* leaves.

(H) Stomatal apertures for Col-0 wild type, *bon1*, *hsc70.1 hsc70.3*, *bon1 hsc70.1 hsc70.3*, and *SGT1b-*OE/*bon1* leaves before or after 20 μM ABA treatment for 1.5 hours.

(I) Stomatal apertures for Col-0 wild type, *bon1*, *hsc70.1 hsc70.3*, *bon1 hsc70.1 hsc70.3* leavesafter treatment with *Pst* DC3000 COR- or H2O for 1 hour.

For (D) to (I), values represent average apertures of at least 20 stomata, and error bars represent standard deviations. Stars indicate statistical differences (Student’s t-test, \*\*p<0.01, \*\*\*p<0.001). Experiments were repeated 3 times with similar results.

**Figure 8. *STG1b* negatively regulates immune responses in *bon1.***

(A) Growth defect in *bon1* is enhanced by the *sgt1b* mutation. Shown are plants of wild-type Col-0, *sgt1b*, *bon1*, *bon1 sgt1b* at 5 weeks old.

(B) *PR1* gene expression in *bon1* is enhanced by the *sgt1b* mutation. Shown is the relative expression of *PR1* in Col-0, *bon1*, *sgt1b*, and *bon1 sgt1b* plants assayed by qRT-PCR. Error bars represent standard deviation (Student’s t-test, \*\*\*p<0.001).

(C) Partial rescue of *bon1* growth defect by overexpressing the *SGT1b* gene. Shown are plants of the wild-type Col-0, *bon1*, and two individual T2 lines (Line 1 and 6) of *SGT1b*-OE/*bon1*.

(D) Partial suppression of enhanced resistance against the virulent bacterial strain *Pst* DC3000 in *bon1* by *SGT1b* overexpression. Shown are bacterial growths in Col-0, *bon1, SGT1b-OE/bon1* plants via dipping inoculation at 0 and 3 DPI. Values represent averages of 3 biological repeats, and error bars represent standard deviation. Letters indicate statistical difference (*p*<0.001, Bonferonni post-test) of different genotypes.

(E) Growth phenotype of *snc1* overexpressing the *SGT1b* gene. Shown are wild-type Col-0, *snc1*, and two independent lines (Line 38 and 2) of *SGT1b-OE/snc1*.

**Figure 9. Antagonistic regulation of SNC1 by SGT1b and HSC70.**

(A) Inhibition of *SNC1*-triggerred cell death by transient overexpression of *SGT1b* in *N. tabacum* leaves. For the left leaf, each half was infiltrated with the *pGWB402* vector (CK) or *pGWB402:SGT1b* (SGT1b). For the middle leaf, each half was co-infiltrated with *pHPTN1:SNC1* (SNC1:GFP) and *pGWB402* (CK) or *pGWB402:SGT1b* (SGT1b). For the right leaf, each half was co-infiltrated with *pHPTN1:SNC1* (SNC1:GFP) and *pGWB402* (CK) or *pGWB402:COPT1* (COPT1). Infiltrated areas were marked with black color. Images were taken at 3 DPI, and the experiments were repeated at least 3 times with similar results.

(B) Western blot detection of SNC1:GFP in the co-infiltrated *N. tabacum* leaves.

(C) Western blot detection of the endogenous SNC1 protein in the Arabidopsis *sgt1b* mutant.

(D) Activation of *SNC1*-triggerred cell death by over-expression of *HSC70.1* in *N. tabacum* leaves. For the left leaf, each half was infiltrated with the pMDC32 (CK) or pMDC32:HSC70.1 (HSC70.1). For the right leaf, each half was infiltrated with pHPTN1:SNC1 (SNC1:GFP) together with pMDC32 (CK) or pMDC32:HSC70.1 (HSC70.1). Images were taken at 2 DPI, and a representative image is shown here. This experiment was repeated at least 3 times with similar results.

(E) Western blot detection of SNC1:GFP in the co-infiltrated tobacco leaves

(F) Western blot detection of the endogenous SNC1 protein in Arabidopsis.

For (B) and (E), total proteins were extracted 40 hours after co-infiltration and an anti-GFP antibody was used to detect SNC1:GFP. For (C) and (F), total proteins were extracted from seedlings of 12-day-old plants, and an anti-SNC1 antibody was used for Western blot. Proteins prominently stained by Ponceau S were used as loading controls. GFP/control or SNC1/control are the relative ratios between the GFP or SNC1 signal and the Ponceau S stained control signal quantified by Image J software. The ratio of CK or Col-0 wild type is set as 1.00.

**Figure 10. Working model for the roles of BON1, SGT1b and HSC70 in pre- and post-invasion defenses.**

In pre-invasion phase, HSC70 negatively and SGT1b positively regulate stomatal closure while BON1 works together with HSC70 and SGT1b to positively regulate pathogen and ABA-induced stomatal closure. In post-invasion phase, BON1 negatively regulates the transcript level of NB-LRR gene *SNC1* through a yet to be determined mechanism, while SGT1b and HSC70 have opposing roles in regulating SNC1 protein activity likely by CPR1-coupled degradation, proper protein folding, or downstream signaling.

**Supplemental Data**

Supplemental Figures:

Figure S1. Complementation of the *bon1* defect by overexpression of *BON1-HA*.

Figure S2. BON1 protein complex purification and mass spectrometry detection of BON1 associated proteins.

Figure S3. Yeast two-hybrid assay of interactions between BON1 and HSC70s.

Figure S4. Characterization of *HSC70.1* and *HSC70.3* overexpression lines in *bon1.*

Figure S5. Growth phenotypes of *bon1* and *hsc70* related mutants after bolting.

Figure S6. Quantification of biomass for *bon1*, *hsc70*, and *sgt1b* related plants.

Figure S7. Additional repeats for growth of *Pst* strains in Col-0 and the *hsc70.1 hsc70.3* mutant by dipping and vacuum inoculation shown in Figure 6.

Figure S8. Stomatal closure responses in *bon1* mutants upon ABA treatment.

Figure S9. Relative expression level of *SGT1b* detected by qRT-PCR.

Supplemental Table1. Primers used in this study.