

Interaction between SGT1 and Cytosolic/Nuclear HSC70 Chaperones Regulates *Arabidopsis* Immune Responses ^W

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The conserved eukaryotic protein SGT1 (for Suppressor of G2 allele of *skp1*) has characteristics of an HSP90 (for heat shock protein 90 kD) cochaperone and in plants regulates hormone responses and *Resistance* gene-triggered immunity. We affinity-purified SGT1-interacting proteins from *Arabidopsis thaliana* leaf extracts and identified by mass spectrometry cytosolic heat shock cognate 70 (HSC70) chaperones as the major stable SGT1 interactors. *Arabidopsis* SGT1a and SGT1b proteins associate with HSC70 in vivo and distribute with HSC70 in the cytosol and nucleus. An intact C-terminal SGT1-specific (SGS) domain that is required for all known SGT1b functions in immunity and development is needed for HSC70 interaction and for the nuclear accumulation of SGT1b. Interaction assays of transiently expressed proteins or their domains in *Nicotiana benthamiana* point to a role of SGT1 as a HSC70 cofactor. Expression of two HSC70 isoforms is upregulated by pathogen challenge, and while loss of function of individual cytosolic HSC70 genes has no defense phenotype, HSC70-1 overexpression disables resistance to virulent and avirulent pathogens. Moreover, mutations in SGT1b lead to a similar degree of heat shock tolerance as deregulation of HSC70-1. We conclude that an HSC70-SGT1 chaperone complex is important for multiple plant environmental responses and that the evolutionarily conserved SGS domain of SGT1 is a key determinant of the HSC70-SGT1 association.

INTRODUCTION

Molecular chaperones are essential for cell viability by ensuring the proper folding of nascent polypeptides, protein complex maturation, and the translocation of proteins through membranes. In eukaryotic cells, the conserved chaperones of the Hsp90 (for heat shock protein 90 kD) and DnaK/Hsc70 (for heat shock cognate 70 kD) families control cellular protein homeostasis through ATP-dependent cycles (Young et al., 2003). Hsc70 predominantly binds nonnative polypeptides and folding intermediates that are either newly synthesized or stress-induced (Erbse et al., 2004; Bukau et al., 2006). By contrast, Hsp90 chaperones bind proteins in their near-native state and mediate the maturation and activation of signaling complexes (Young et al., 2003). In yeast and mammalian cells, the activities of Hsp90 and

Hsc70 are modulated by a complex network of cochaperones that define the balance of protein assembly and degradation by the ubiquitin-proteasome machinery (Esser et al., 2004; Bukau et al., 2006).

Sgt1 (for Suppressor of G2 allele of *skp1*) is a conserved, essential protein in eukaryotes that interacts with multiple protein complexes and has features of a cochaperone (Shirasu and Schulze-Lefert, 2003). The Sgt1 protein has three domains: an N-terminal tetratricopeptide repeat (TPR) domain that resembles the folds of Hop/Sti1 (for Hsp70/Hsp90 organizing protein); a central CHORD-Sgt1 (CS) domain that is similar to the Hsp20/ α -crystallin domain of the human p23 cochaperone family; and a C-terminal Sgt1-specific (SGS) domain that is structurally less well defined although highly conserved relative to the other SGT1 domains (Dubacq et al., 2002; Garcia-Ranea et al., 2002; Lee et al., 2004). Yeast Sgt1p associates with Skp1p (for Suppressor of kinetochore protein) and Hsp90p through its TPR and CS domains, respectively, and is needed to assemble an active centromere binding factor3 kinetochore complex in cell cycle progression (Kitagawa et al., 1999; Bansal et al., 2004; Lingelbach and Kaplan, 2004; Catlett and Kaplan, 2006). Yeast Sgt1p is also required for activation of an SCF (for Skp1-Cul1-F box) ubiquitin ligase complex that mediates the ubiquitination of Sic2p, an inhibitor of Cdc28 kinase (Kitagawa et al., 1999). A Skp1p-independent but essential function of Sgt1p was identified in the activation of the yeast adenylyl cyclase (Cyr1p) protein through interaction between the Sgt1p SGS and Cyr1p leucine-rich repeat (LRR) domains (Dubacq et al., 2002). Thus, Sgt1 has a role in diverse signaling processes.

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Plant SGT1 proteins associate with cytosolic HSP90s in vivo, consistent with a conserved cochaperone activity (Hubert et al., 2003; Takahashi et al., 2003; Liu et al., 2004). Also, in tobacco (*Nicotiana benthamiana*) and barley (*Hordeum vulgare*) extracts, SGT1 coimmunoprecipitated with the SCF structural subunits SKP1 and CUL1 and the COP9 signalosome that regulates the SCF ubiquitin-proteasome degradation system (Azevedo et al., 2002; Liu et al., 2002a). A link between SGT1 in plants and yeast (Kitagawa et al., 1999; Lyapina et al., 2001) with components of the ubiquitin-proteasome machinery suggests that SGT1 may assist in the controlled degradation of target proteins. Indeed, the loss of *Arabidopsis thaliana* *SGT1b* (one of two functional *Arabidopsis* *SGT1* genes, *SGT1a* and *SGT1b*) compromised the functions of SCF^{TIR1} and SCF^{COI1} that mediate the ubiquitination-dependent degradation of proteins in response to the phytohormones auxin and jasmonic acid, respectively (Gray et al., 2003).

Plant SGT1 interacts through its CS domain with another cytosolic HSP90 binding protein, RAR1 (for Required for *Mla12* Resistance) (Hubert et al., 2003; Takahashi et al., 2003; Liu et al., 2004), and both *RAR1* and *SGT1* were identified as components of plant resistance mediated by intracellular nucleotide binding-leucine-rich repeat (NB-LRR) immune receptors (Shirasu et al., 1999; Austin et al., 2002; Azevedo et al., 2002; Liu et al., 2002b; Muskett et al., 2002; Tornero et al., 2002). A body of genetic and molecular evidence points to functions of plant SGT1 and RAR1 as cofactors in HSP90-mediated stabilization of preactivated NB-LRR protein complexes (Tornero et al., 2002; Hubert et al., 2003; Lu et al., 2003; Bieri et al., 2004; Liu et al., 2004; Azevedo et al., 2006). These receptors (also known as R proteins) are present in the cell in a constrained conformation and can be specifically activated by the action of pathogen-derived effectors (Shirasu and Schulze-Lefert, 2003). Pathogen recognition potentiates low-level basal defense that limits the growth of virulent pathogens and is often accompanied by localized programmed cell death (Chisholm et al., 2006). SGT1 can interact with the LRR domains of certain NB-LRR proteins and may assist in their proper folding (Bieri et al., 2004; Leister et al., 2005). There is no evidence for a direct association of RAR1 with NB-LRR proteins; therefore, RAR1 may operate at another level of immune receptor assembly or maintenance. While genetically additive contributions of *SGT1b* and *RAR1* were observed in resistance mediated by the NB-LRR genes *Arabidopsis* *RPP5* and barley *MLA6* (Austin et al., 2002; Azevedo et al., 2002), an antagonistic relationship was found between *SGT1b* and the assembly roles of *RAR1* and *HSP90* in certain *Arabidopsis* NB-LRR conditioned responses (Holt et al., 2005). This likely reflects a fine balance between the assembly and degradative activities of the chaperone/cochaperone machineries in maintaining NB-LRR proteins poised for activation. Also, the *Arabidopsis* *SGT1* homolog *SGT1a* may compensate for the loss of *SGT1b* in controlling the steady state levels of certain NB-LRR proteins, since *SGT1a* has intrinsic SGT1 activity but is expressed at a lower level than *SGT1b* (Azevedo et al., 2006). *SGT1a* and *SGT1b* have redundant essential roles in early embryo development, but only mutations in *SGT1b* compromise plant immunity or auxin signaling (Azevedo et al., 2006).

Therefore, SGT1 is necessary for plant development and disease resistance, but it is unclear how it operates molecularly and

whether its activity as a HSP90 cofactor accounts entirely for its diverse cellular functions. We report here that affinity purification-tagged *Arabidopsis* SGT1 protein interacts stably with cytosolic/nuclear HSC70 chaperones in vivo. This interaction occurs with native SGT1 protein and requires an intact SGS domain for which no direct partners were known. Mutations in *SGT1b* and deregulation of HSC70-1, the predominant cytosolic HSC70 isoform in *Arabidopsis*, disable R protein-specified and basal disease resistance and lead to increased heat shock tolerance. We conclude that the SGT1-HSC70 association is important for the regulation of plant responses to biotic and abiotic stresses.

RESULTS

Functional Characterization of SGT1 Proteins Tagged by the StrepII Epitope

In order to search for biochemical interactors of the *Arabidopsis* SGT1 proteins, *SGT1a* and *SGT1b* were fused to a C-terminal StrepII (Strep) affinity purification tag under the control of the constitutive cauliflower mosaic virus 35S promoter or their respective native promoters. *SGT1b* constructs were transformed into the Landsberg *erecta* (*Ler*) *sgt1b-3* null mutant (Austin et al., 2002), and *SGT1a* constructs were transformed into a *Ler*/*Wassileskija* (*Ws-0*) hybrid that was homozygous for *sgt1b-3* (*Ler*) and heterozygous for *sgt1a-1* (*Ws-0* background) (Azevedo et al., 2006). Multiple transgenic lines were selected that expressed the SGT1a-Strep and SGT1b-Strep fusion proteins in the appropriate mutant backgrounds, as shown for representative lines in Figure 1. The functionality of the SGT1b-Strep fusion proteins was tested based on complementation of the known *sgt1b-3* mutant defects. The SCF ubiquitin E3 ligase-dependent functions of *SGT1b* (root growth sensitivity to auxin and jasmonic acid) were fully complemented irrespective of the promoter used (Figure 1; see Supplemental Figure 1 online). *RPP5* resistance to the oomycete pathogen *Hyaloperonospora parasitica* was not restored (Figure 1; see Supplemental Figure 1 online), because transgenic plants exhibited a delayed cell death response. The double homozygote mutant *sgt1a-1 sgt1b-3* is embryo-lethal (Azevedo et al., 2006). Therefore, we crossed SGT1b-Strep transgenic plants into the *Ler*/*Ws-0* hybrid that was homozygous for *sgt1b-3* and heterozygous for *sgt1a-1*. Double homozygote *sgt1a-1 sgt1b-3* mutants expressing SGT1b-Strep could be selected and were fully viable, indicating that SGT1b-Strep complements the lethality of *sgt1b-3 sgt1a-1* (Figure 1). Therefore, SGT1b-Strep complemented three of the four known *sgt1b* mutant phenotypes in *Arabidopsis*. SGT1a-Strep expressed under its own promoter complemented the embryo lethality of *sgt1b-3 sgt1a-1*, which is the only *sgt1a* mutant phenotype known to date.

In Planta Interaction of StrepII-Tagged *Arabidopsis* SGT1 with Cytosolic HSC70 Chaperones

We previously reported the identification of a 70-kD band that specifically copurified with StrepII- and tandem affinity purification-tagged *Arabidopsis* SGT1b expressed under the control of the 35S promoter in healthy plant tissues (Witte et al., 2004). This protein

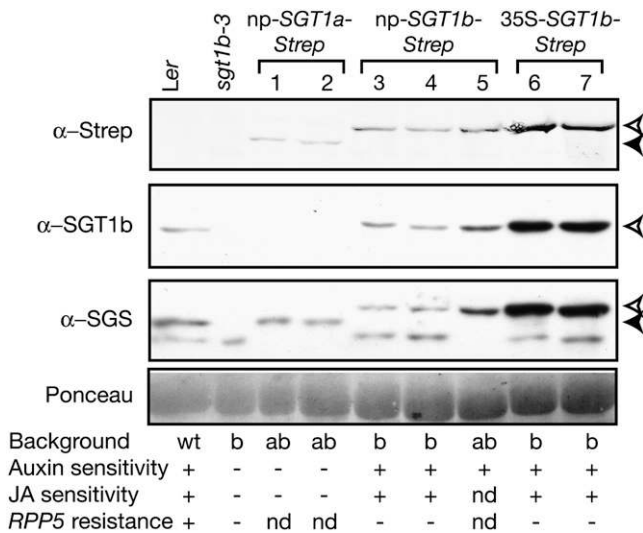


Figure 1. Stability and Functionality of StrepII-Tagged SGT1 Proteins in Transgenic *Arabidopsis*.

Total leaf protein extracts were prepared from 3-week-old plants and analyzed by SDS-PAGE and immunoblotting using anti-SGT1b and anti-SGS antibodies or the Strep-Tactin-AP conjugate, as indicated. The open and closed arrowheads mark SGT1b-Strep and SGT1a-Strep, respectively. 1/2, 3/4, and 6/7 indicate independent transgenic lines. Line 5 is derived from a cross between a Ler/Ws-0 hybrid, *sgt1b-3/sgt1b-3 sgt1a-1/SGT1a*, and plant 4. Equal loading of samples was checked by Ponceau red staining of the blot. Background refers to the *SGT1* alleles: wt, wild-type *SGT1a* and *SGT1b*; a, homozygous *sgt1a-1*; b, homozygous *sgt1b-3*; ab, double *sgt1a-1 sgt1b-3* homozygotes. + indicates plants that have wild-type sensitivity to 0.075 μ M 2,4-D or 10 μ M methyl jasmonate in root growth inhibition assays, and - indicates plants that are insensitive to these hormone concentrations. nd, not determined. *RPP5* gene-mediated resistance assays were performed using *H. parasitica* avirulent isolate Noco2. For *RPP5* resistance, + indicates wild-type hypersensitive response and - indicates trailing necrosis/sporulation. See Supplemental Figure 1 online for detailed phenotypic characterization.

band was processed for mass spectrometric analysis. All 14 tryptic peptides belonged to cytosolic *Arabidopsis* HSC70 (predicted molecular mass of 71 kD; see Supplemental Table 1 online). The *Arabidopsis* genome encodes 14 HSC70 proteins of the DnaK superfamily, five of which (HSC70-1 to -5) are predicted to be cytosolic and/or nuclear due to the presence of predicted nuclear localization signals (Lin et al., 2001). Despite the high sequence similarity within the family (83 to 94% amino acid identity), identification of the HSC70 isoforms in the sample was achieved based on two informative tryptic fragments, C and G (see Supplemental Figure 2 online), whose identities were confirmed by quadrupole time-of-flight tandem mass spectrometry. From this analysis, HSC70-1 (CAB85987) and HSC70-3 (AAF14038) were unambiguously identified and could explain all 14 peptides detected by mass spectrometry. Therefore, HSC70-1 and HSC70-3 are two novel SGT1b-Strep interactors in healthy leaf tissue.

We tested whether the interaction with cytosolic HSC70s is specific to SGT1b compared with SGT1a or is influenced by pathogen challenge by performing a StrepII affinity purification of

SGT1a-Strep and SGT1b-Strep expressed under the control of their respective native promoters (Figure 2). Leaf material was either nontreated or collected at 24 h after inoculation with the avirulent bacterial pathogen *Pseudomonas syringae* pv *tomato* (*Pst*) DC3000 expressing the effector AvrRpm1 (recognized by RPM1) (Figure 2A). As observed by silver staining and on an immunoblot probed with anti-HSC70 antiserum (which does not discriminate between the different isoforms), HSC70s could be purified with SGT1a-Strep or SGT1b-Strep expressed under the control of their native promoters (Figure 2B). These data show that cytosolic HSC70s do not discriminate strongly between SGT1a and SGT1b in their binding and that the observed SGT1-HSC70 interaction is not due to SGT1 overexpression. The amounts of purified HSC70 were globally proportional to the amounts of SGT1a or SGT1b purified. For example, SGT1a-Strep accumulated to higher levels in total extracts after pathogen challenge, and this was reflected in the levels of purified SGT1a and HSC70 proteins (Figure 2B). We reasoned that SGT1 and HSC70 most likely interact directly, since no other protein could be detected by silver staining in several independent purification experiments. This analysis also confirmed indirectly that the tagged SGT1 proteins are soluble and present in the cytosol and/or the nucleus, since they interact with cytosolic/nuclear HSC70s.

We tested whether the spectrum of HSC70 isoforms copurified was affected by pathogen challenge. Unchallenged and pathogen-treated leaf samples were processed as above, and SGT1-Strep protein was collected using the Strep-Tactin-Macroprep resin, which allows higher recovery. Copurified HSC70 protein amounts were too low to perform the analysis with SGT1a-Strep. For SGT1b-Strep, HSC70-1 and HSC70-3 remained the principal interactors of SGT1b expressed under the control of its native promoter in both samples (Figure 2C; see Supplemental Figure 2 online). Weak but reproducible signals were also unambiguously identified as HSC70 isoforms 2 (CAB85986) and 4 (BAB02269) in the pathogen-treated samples only.

Expression of Cytosolic HSC70-2 and HSC70-4 Is Pathogen Inducible

Previous studies showed that *HSC70-1* and *HSC70-3* transcripts are the most abundant of the cytosolic isoforms in young healthy *Arabidopsis* tissue (Lin et al., 2001). In order to establish whether pathogen challenge modulates SGT1 affinity to individual HSC70 isoforms or simply reflects HSC70 abundance in the cell, we measured mRNA accumulation for the different cytosolic HSC70 isoforms by RT-PCR (Figure 3A). Ecotype Columbia (Col-0) plants were infiltrated with MgCl₂ buffer, virulent *Pst* DC3000 containing an empty vector, or avirulent *Pst* DC3000 expressing either AvrRpm1 or AvrRps4 (recognized by RPS4). Expression of *Pathogenesis-Related1* mRNA was also measured to assess the responsiveness of tissues and ensure that unchallenged plants were not stressed prior to infection. Standardization of cDNA samples used for RT-PCR was done by measuring the expression of a constitutive *Tubulin* gene. *HSC70-1* and *HSC70-3* expression did not change significantly after pathogen infection. By contrast, *HSC70-2* and *HSC70-4* expression was barely detectable in untreated samples and was weakly induced in plants

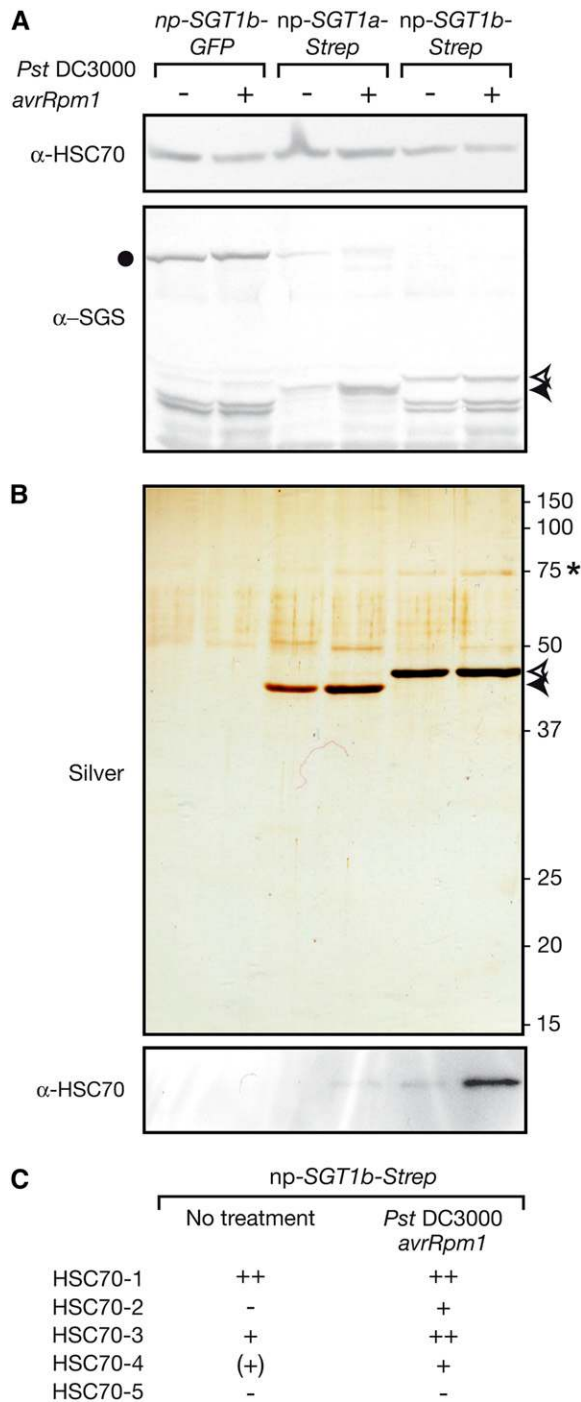


Figure 2. Interactions of StrepII-Tagged SGT1 Proteins with Cytosolic HSC70 Chaperones in *Arabidopsis* Transgenic Lines.

(A) Total leaf protein extracts were prepared from *Ler sgt1b-3* mutants transgenic for SGT1a-Strep or SGT1b-Strep expressed from their native promoters (np). A parallel experiment was performed with leaf tissue sampled at 24 h after infiltration with a bacterial suspension of *Pst* DC3000/*avrRpm1* (10^7 colony-forming units [cfu]/mL). Protein extracts were analyzed by SDS-PAGE and immunoblotting with anti-HSC70 and anti-SGS antibodies. The open and closed arrowheads mark SGT1b-

infiltrated with $MgCl_2$. HSC70-2 and HSC70-4 mRNA levels increased substantially following inoculation with avirulent *Pst* DC3000 strains, the response to AvrRpm1 being earlier than that to AvrRps4. A weaker induction of HSC70-2 mRNA was also observed in samples responding to virulent *Pst* DC3000. HSC70-5 mRNA was barely detectable but was slightly induced at 24 h after inoculation with *Pst* DC3000/*avrRpm1*. SGT1a mRNA accumulated to high levels in avirulent pathogen-treated samples, whereas SGT1b expression was not strongly pathogen-responsive, consistent with earlier analysis of SGT1 promoter: β -glucuronidase (*GUS*) fusions (Azevedo et al., 2006). Samples taken from the same material used for RT-PCR analysis were processed for immunoblot analysis with anti-HSC70 antibody. The results (Figure 3B) reveal that HSC70 levels increased at late time points (6 to 24 h) in the incompatible interactions, consistent with the constitutive expression of the HSC70-1 and HSC70-3 isoforms and the pathogen-inducible expression of HSC70-2 and HSC70-4. Since the HSC70 isoforms interacting with SGT1b broadly mirror the HSC70 expression pattern, we conclude that SGT1b does not discriminate strongly in its interaction with different HSC70 isoforms present in the cytoplasm and/or nucleus.

Arabidopsis SGT1b Interacts with HSC70 in Vivo

We considered that interaction between SGT1-Strep and HSC70s may be due to incorrect folding of SGT1, making it a HSC70 substrate (Erbse et al., 2004). Therefore, we tested whether native SGT1 proteins associate with HSC70 in wild-type tissues. This was examined by coimmunoprecipitation from plant soluble protein extracts using either anti-SGT1b antibodies that bind only SGT1b or anti-SGS antibodies that recognize both SGT1 isoforms (Austin et al., 2002) (Figure 4A). HSC70s could be coimmunoprecipitated in *Ler* and *Col-0* total extracts with anti-SGT1b but not in extracts of the *Ler sgt1b-3* mutant that lacks SGT1b protein (Austin et al., 2002). Anti-SGS antibody coimmunoprecipitated lower amounts of HSC70s from *Ler sgt1b-3* extracts that contain SGT1a. These results indicate that native SGT1a and SGT1b interact with HSC70 in vivo, implying a physiologically relevant association.

In order to characterize the interaction further, soluble protein extracts from healthy *Ler* plants were prepared as for the coimmunoprecipitations and analyzed by size-exclusion chromatography

Strep and SGT1a-Strep, respectively. The closed circle indicates the SGT1b-GFP (for green fluorescent protein) fusion protein used as a negative control in the StrepII purification.

(B) Elution fractions from the purification were analyzed by SDS-PAGE and silver staining or immunoblotting with anti-HSC70 and anti-SGS antibodies. The asterisk marks 70-kD SGT1-interacting proteins. Molecular masses of protein markers are indicated at right in kilodaltons.

(C) SGT1b-Strep expressed from its native promoter was purified using the Strep-Tactin Macroprep resin and boiled off the resin. A parallel experiment was performed with leaf tissue sampled at 24 h after infiltration with *Pst* DC3000/*avrRpm1* (10^7 cfu/mL). Extracts were analyzed by SDS-PAGE and Coomassie blue staining. The 70-kD bands were sampled, digested by trypsin, and analyzed by quadrupole time-of-flight tandem mass spectrometry. The relative abundance of the five cytosolic HSC70 isoforms was determined: -, not detected; (+), weak signal; +, clear signal; ++, strong signal.

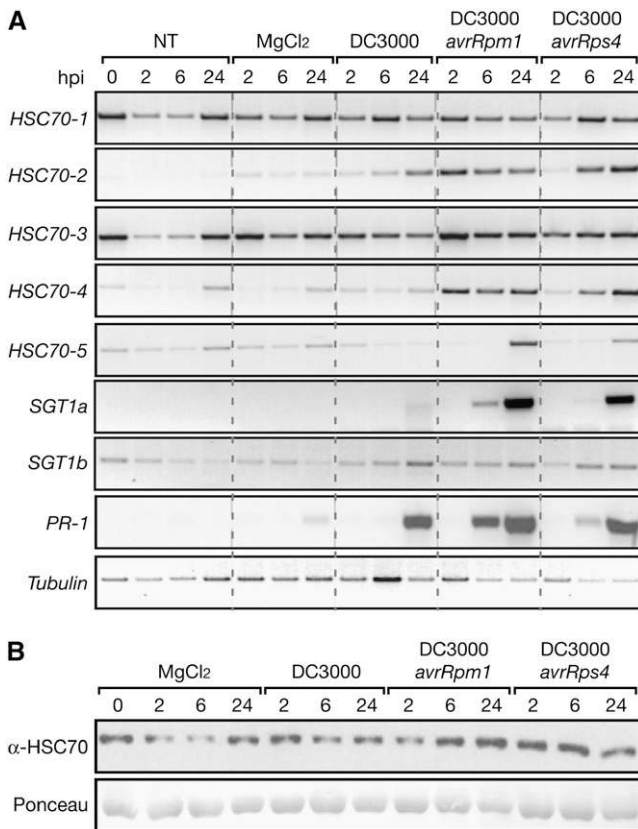


Figure 3. Expression of *HSC70-2* and *HSC70-4* Is Pathogen Inducible.

Four-week-old Col-0 plants were nontreated (NT), hand-inoculated with *Pst* DC3000 strains (10^7 cfu/mL) as indicated, or treated with 10 mM MgCl₂ as a control (MgCl₂). Samples were harvested at 0, 2, 6, and 24 h after inoculation (hpi).

(A) RNAs were isolated from leaves, and RT-PCR products were separated by agarose gel electrophoresis. Ethidium bromide-stained gels are shown.

(B) Total leaf protein extracts were analyzed by SDS-PAGE and immunoblotting with anti-HSC70 antibodies. Equal loading of samples was checked by Ponceau red staining of the blot.

on a Superdex 200 column. Fractions were then collected and analyzed on an immunoblot (Figures 4B and 4C). SGT1a (39 kD) and SGT1b (40 kD) were collected in fractions containing proteins with apparent molecular mass ranges of 41 to 89 kD and 41 to 115 kD, respectively. This observation suggests that SGT1 proteins are either not globular or that their apparent mobility is affected by interaction with other protein partners. Fractions that contained SGT1 proteins (41 to 115 kD) also contained the major HSC70 pool, consistent with a SGT1–HSC70 association and direct interaction in a 1:1 stoichiometric ratio. Under these conditions, we did not detect higher molecular mass complexes containing SGT1.

The SGT1 SGS Domain Is Necessary and Sufficient for Interaction with HSC70

To better define the relationship between SGT1 and HSC70 proteins, we characterized the domains responsible for the SGT1–

HSC70 interaction. First, we tested the effect of the *enhancer of tir1-1 auxin resistance3 (eta3)* mutant allele of *Arabidopsis SGT1b* on HSC70 interaction. The *sgt1b^{eta3}* mutation causes a splicing error that results in a frame shift and a premature stop codon, leading to the synthesis of a truncated SGT1b protein that lacks the last 36 amino acids and therefore has a disrupted 94-amino acid C-terminal SGS domain (Gray et al., 2003). Although the mutant SGT1b^{eta3} protein is more stable than wild-type SGT1b, *sgt1b^{eta3}* phenotypes are indistinguishable from those of the *Ler sgt1b-3* protein null mutant (Gray et al., 2003). Levels of HSC70 recovered in immunoprecipitates of *sgt1b^{eta3}* extracts were reduced compared with those in the wild type in 7 of 11 repetitions, as shown in Figure 4A. This result indicates that an intact SGS domain is needed for efficient HSC70 binding *in vivo*.

The association between SGT1 and HSC70s was confirmed and analyzed further using *Agrobacterium tumefaciens*–mediated transient coexpression of *Arabidopsis* SGT1 (Strep-tagged) and HSC70-1 (hemagglutinin [HA]-tagged) domains in *Nicotiana benthamiana* followed by detection of StrepII copurified protein on immunoblots (Figures 4D and 4E). In this analysis, SGT1b-Strep protein bound significant amounts of HSC70-1, whereas RAR1-Strep or SGT1b^{eta3}-Strep did not (Figure 4D). SGT1a accumulated to similar levels as SGT1b in *N. benthamiana* extracts but bound lower amounts of HSC70-1 protein (Figure 4E). In another *N. benthamiana* interaction assay, we assessed the efficiency with which endogenous HSC70 copurified with approximately equivalent amounts of transiently expressed Strep-tagged SGT1b or the individual CS and SGS domains (Figure 5A). The CS domain from SGT1a (the SGT1b CS domain was not expressed) and a GFP-Strep control protein failed to bind HSC70. By contrast, SGT1b-Strep and SGSb-Strep bound significant amounts of HSC70. These results indicate that the SGS domain is necessary and sufficient for SGT1 association with HSC70. The loss of function of SGT1b^{eta3} is more likely due to the loss of interaction with HSC70-1, since HSP90-1 and RAR1 still interacted with SGT1b^{eta3} tested in a yeast two-hybrid assay (see Supplemental Figure 3 online).

We then tested which of the HSC70-1 domains interacts most efficiently with SGT1b by coexpressing its HA-tagged ATPase domain (N-terminal; 45 kD) or client binding domain (C-terminal; 19 kD) with SGT1b-Strep in *N. benthamiana*. Interaction between SGT1b and HSC70-1 was again strong (Figure 5B). We detected no interaction between SGT1b and the ATPase domain of HSC70-1 and very weak or no interaction with the client binding domain of HSC70-1. These results argue against SGT1b being a substrate of HSC70 and suggest that effective SGT1b binding is probably only achieved with the complete HSC70-1 protein.

Subpools of SGT1b and HSC70 Localize to the Nucleus

We reasoned that colocalization of SGT1 and HSC70 in one or more compartments would be necessary for functional interaction. We first investigated the subcellular localizations of fluorescent protein-tagged HSC70-1 and SGT1b after biolistic transfection of *N. benthamiana* epidermal cells (Figures 6A and 6B). SGT1b fused to Cerulean (a derivative of cyan fluorescent protein) localized to the cytosol but could be seen in nuclei of ~25% of 55 transformed cells examined (Figure 6A), suggesting

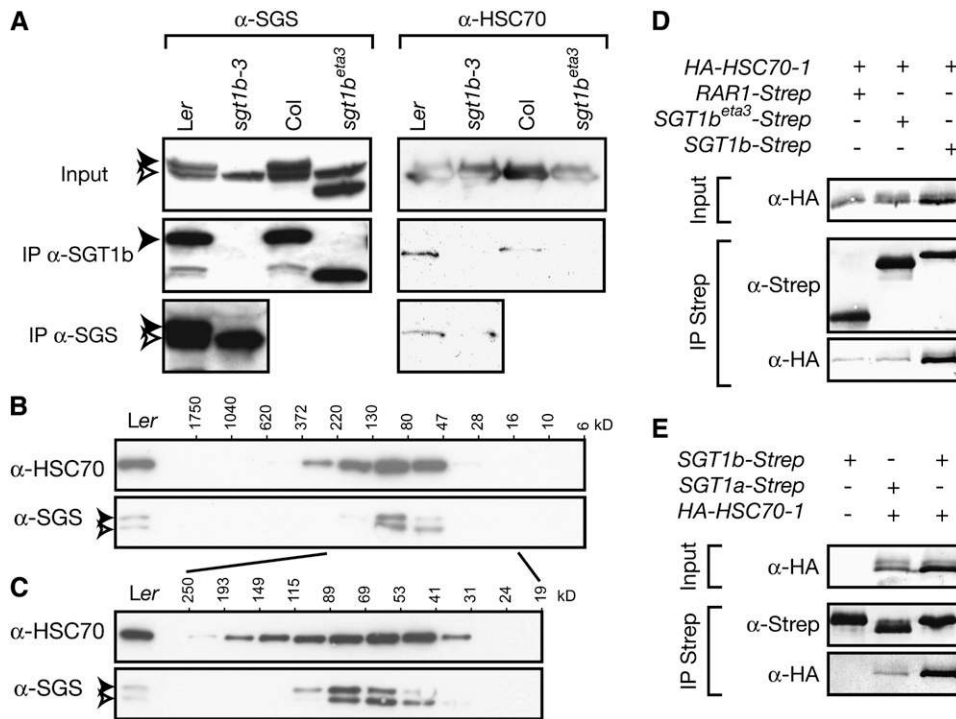


Figure 4. SGT1b Interacts with HSC70s in Vivo.

(A) Total leaf protein extracts of 3-week-old healthy plants were immunoprecipitated with anti-SGT1b or anti-SGS antibodies as indicated. Plant genotypes used were *Ler*, *Ler sgt1b-3*, *Col-0*, and *Col-0 sgt1b^{eta3}*. Total extracts and immunoprecipitates (IP) were analyzed by SDS-PAGE and immunoblotting with anti-HSC70 and anti-SGS antibodies.

(B) and **(C)** Wild-type *Ler* leaf protein extracts were separated by size-exclusion chromatography. Collected fractions were analyzed by SDS-PAGE and immunoblotting with anti-HSC70 and anti-SGS antibodies. Molecular mass ranges of the fractions are indicated in kilodaltons based on the column calibration.

(D) and **(E)** HA-tagged HSC70-1 and Strep-tagged SGT1 were coexpressed using *Agrobacterium*-mediated transient transformation of *N. benthamiana* leaves. Total protein extracts (Input) were subjected to affinity purification of the Strep-tagged protein using Strep-Tactin Sepharose and specifically eluted (IP Strep). Protein extracts were analyzed by SDS-PAGE and immunoblotting with anti-HA antibody or the Strep-Tactin-AP conjugate, as indicated. SGT1b^{eta3} indicates residues 1 to 322.

movement of SGT1b between the cytosol and nucleus. In agreement with a predicted nuclear localization signal in cytosolic HSC70s (Sung and Guy, 2003), yellow fluorescent protein (YFP)-HSC70-1 was detected in the cytosol and nuclei of 100% of cells examined and colocalized with cytosolic and nuclear SGT1b-Cerulean (Figure 6B). We then tested the subcellular distribution of SGT1a and SGT1b and cytosolic HSC70 proteins in *Arabidopsis* by preparing nuclear extracts from leaves of *Col-0*, *eta3*, and the defense signaling mutant *Col eds1-2*. HSC70 and SGT1b signals were detected in both cytosolic and nucleus-enriched fractions of *Col-0* and *eds1-2* (Figure 6C). Notably, SGT1b^{eta3} protein (detected by anti-SGT1b antibody) accumulated only in the nucleus-depleted fraction but stimulated the nuclear accumulation of SGT1a (detected by anti-SGS antibody) compared with wild-type and *eds1-2* tissues (Figure 6C). These data show that subpools of SGT1 and HSC70 protein colocalize in the cytosol and nucleus and that the SGS domain of SGT1b, which is needed for HSC70-1 interaction, is also required for nuclear accumulation. While nuclear import of SGT1b appears to

predominate in wild-type cells, SGT1a has the capacity to enter nuclei in the absence of functional SGT1b.

HSC70-1 Overexpression Disables Plant Immune Responses

To explore whether cytosolic HSC70s are involved in plant processes known to require SGT1b, T-DNA insertion mutants of *HSC70-1* to *HSC70-3* were isolated (see Methods) and characterized for resistance to pathogens. One insertion found in *HSP70-4* did not diminish transcript levels (see Supplemental Figure 4 online) and was not analyzed further. We did not detect alterations in the phenotypes of the *hsc70-1*, *hsc70-2*, and *hsc70-3* mutants after infection with virulent or avirulent strains of *H. parasitica* and *Pst* (see Supplemental Figure 4 online). Gene silencing of the *HSC70* gene family causes embryo lethality (Sung and Guy, 2003) and therefore was not an option to overcome likely functional redundancy within these highly conserved proteins. Also, no specific HSC70 inhibitors have been described

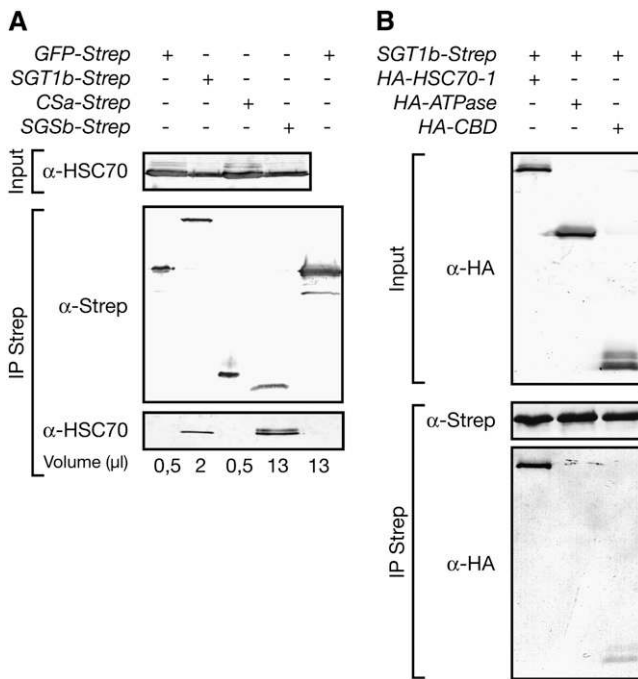


Figure 5. Mapping of HSC70-1- and SGT1b-Interacting Domains in Transient Plant Expression Assays.

Strep-tagged SGT1 domains (**A**) and HA-tagged HSC70-1 domains with Strep-tagged SGT1 (**B**) were expressed using *Agrobacterium*-mediated transient transformation of *N. benthamiana* leaves. Total protein extracts (Input) were subjected to affinity purification of Strep-tagged protein using Strep-Tactin Sepharose and specifically eluted (IP Strep). Protein extracts were analyzed by SDS-PAGE and immunoblotting with anti-HA antibody, the Strep-Tactin-AP conjugate, or the anti-HSC70 antibody to detect endogenous Nb HSC70, as indicated. Different volumes of the elution fraction were analyzed on the gel in order to normalize the amounts of purified Strep-tagged proteins. GFP-Strep was used as a non-HSC70-interacting protein in the purification process. ATPase, ATPase domain of HSC70-1 (residues 1 to 405); CBD; client binding domain of HSC70-1 (residues 374 to 543); CSa, SGT1a CS domain (residues 149 to 260); SGSb, SGT1b SGS domain (residues 268 to 357).

(Brodsky and Chiosis, 2006). In animal and yeast cells, overexpression of HSC70 enhances tolerance to heat stress by increasing protein disaggregation but impairs normal HSC70 cellular function in protein maturation, degradation, and transport (Krobitsch and Lindquist, 2000; Brodsky and Chiosis, 2006). Therefore, we examined the effect of modulating HSC70 function on the plant immune response through overexpression. Several available Col-0 transgenic lines that express *HSC70-1* under the control of the 35S promoter (Sung and Guy, 2003) were characterized, and lines 8-7 (a single T-DNA insertion; sevenfold overexpression) and 8-9 (multiple T-DNA insertions; fourfold overexpression) were selected for further study. These lines exhibited some developmental defects, such as a dwarf stature and a short root system, as described before (Sung and Guy, 2003), but they did not have altered photosynthetic efficiency (Sung and Guy, 2003), root architecture, or responsiveness to auxin (L.D. Noël, unpublished data). The *HSC70-1* overexpression lines displayed a partial break-

down of resistance specified by four different *Arabidopsis* *R* genes tested (Figure 7). *H. parasitica* isolates Cala2 (recognized by *RPP2*) and Emwa1 (recognized by *RPP4*) were able to complete their life cycles in lines 8-7 and 8-9. Pathogen colonization was almost as extensive as that observed in the Col-0 *sgt1b^{eta3}* mutant, measured by the production of conidiospores (Figures 7A and 7B) and trypan blue staining of leaves (Figure 7C). Partial loss of resistance was visualized by the appearance of trailing necrosis around pathogen hyphae, similar to that seen in Col *sgt1b^{eta3}* (Figure 7C). *RPM1*-mediated recognition of *Pst* DC3000/*avrRpm1* and *RPS4* recognition of *Pst* DC3000/*avrRps4* were also compromised (Figures 7D and 7E). Thus, *HSC70-1* overexpression partially disables *R* gene-conditioned resistance to avirulent isolates of *H. parasitica* and *Pst*. Combining the *sgt1b^{eta3}* mutation and *HSC70-1* overexpression caused an additive loss of *RPP4*-mediated resistance (see Supplemental Figure 5 online), in support of a genetic interaction between these two components.

In order to test the effect of *HSC70-1* overexpression on *R* protein accumulation, line 8-7 was crossed with Col-0 plants expressing a functional *RPM1*-myc protein (Boyes et al., 1998), and plants homozygous for the *RPM1*-myc and *HSC70-1* overexpression constructs were selected. As shown in Figure 7F, *HSC70-1* overexpression did not alter *RPM1*-myc steady state levels. It is unlikely, therefore, that perturbation of the assembly or maintenance of preexisting *R* proteins is the cause of the resistance defects arising from *HSC70-1* overexpression.

We considered whether the above phenotypes might reflect a defect in basal resistance that normally restricts the growth of virulent pathogen isolates. Growth of the virulent *H. parasitica* isolate Noco2 (Figure 8A) and *Pst* DC3000 (Figure 8B) was greater in lines 8-7 and 8-9 than in wild-type Col-0 but not as extreme as in the basal defense mutant *eds1-2*. This observation prompted us to investigate the contribution of *SGT1b* to basal defense. Growth of the virulent *H. parasitica* isolate Cala2 was higher in *Ler sgt1b-1* and *sgt1b-3* null mutants compared with the *Ler* wild type (Figure 8C). We conclude that *SGT1b* and *HSC70-1* modulate both basal and *R* protein-specified immune responses.

Mutations in *SGT1b* and Deregulation of *HSC70-1* Lead to Heat Shock Tolerance

We explored whether the interaction between *SGT1* and *HSC70* cytosolic isoforms has broader biological significance by examining the requirement for *SGT1* in the *Arabidopsis* heat shock response, one of several abiotic stresses that recruit *HSC70* chaperone functions (Sung and Guy, 2003). Data from a gene expression microarray experiment that traced the heat shock response of 18-d-old *Arabidopsis* plantlets grown in liquid at 25°C and then incubated at 38°C for 30, 60, or 180 min (L. Nover and P. von Koskull-Doring, http://www.Arabidopsis.org/servlets/TairObject?type=expression_setandid=1007967124) were examined. After 60 and 180 min at 38°C, *SGT1a* mRNA accumulated to 3- and 12-fold higher levels, respectively, than in control samples incubated at 25°C, whereas *SGT1b* and *UBQ10* expression remained unchanged (Figure 9A). *HSP70-1* was moderately and *HSC70-2* was strongly induced by heat shock

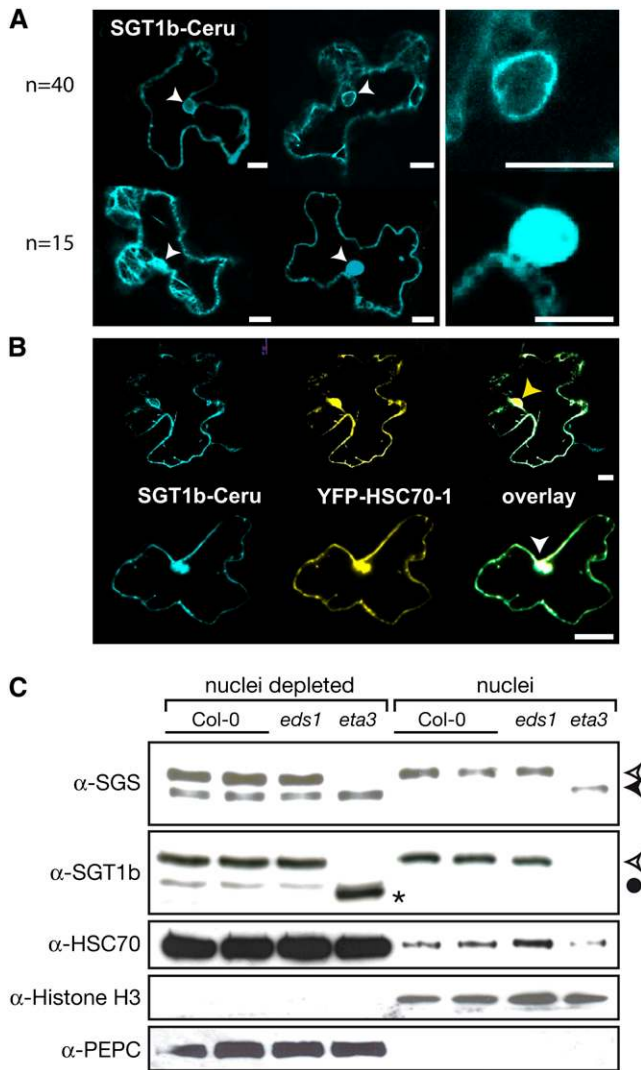


Figure 6. Subcellular Localization of SGT1b and HSC70 Proteins.

(A) and **(B)** Plasmids containing *p35S:SGT1b-Cerulean* **(A)** or *p35S:SGT1b-Cerulean/35S:YFP-HSC70-1* **(B)** were delivered to *N. benthamiana* epidermal cells using a particle gun. Imaging with a confocal laser scanning microscope was done at 18 to 48 h after transformation. Bars = 20 μ m.

(A) SGT1b enters the nucleus of some cells ($n = 15$ of 55 examined). The top panel shows two representative cells with SGT1b excluded from the nucleus, and the bottom panel shows two representative cells with SGT1b inside the nucleus. White arrowheads mark the nuclei. Enlarged views of the right nuclei are also shown.

(B) SGT1b colocalizes with HSC70-1. The top panel depicts the colocalization of both proteins outside the nucleus. The yellow arrowhead indicates the nuclear localization of YFP-HSC70-1 but not SGT1b-Cerulean. The bottom panel shows a representative cell with both proteins colocalized outside and within the nucleus. The white arrowhead indicates the nuclear localization of both YFP-HSC70-1 and SGT1b-Cerulean.

(C) Nucleus-depleted and -enriched protein extracts from Col-0, Col *eds1-2*, and *sgt1b^{eta3}* leaves were analyzed by SDS-PAGE and immunoblotting with anti-SGS, anti-SGT1b, anti-HSC70, anti-histone H3 (nuclear marker), and anti-PEPC (for phosphoenolpyruvate carboxylase; cytosolic marker) antibodies, as indicated. Nuclear samples were 16 times

treatment, as described previously (Lin et al., 2001). There was also a dramatic increase in the activity of the *SGT1a* promoter followed by a GUS reporter gene (Azevedo et al., 2006) after heat shock. In seedlings grown at 25°C, detectable pSGT1a:GUS activity was restricted to two cell lineages of the root pericycle (Figure 9B). After 3 h of incubation at 37°C, GUS activity was detected in cotyledon tissues and was expressed in outer cell layers of the root (Figure 9B). The strong induction of mRNAs for *SGT1a* and the comparative low responsiveness of *SGT1b* after heat shock broadly mirror their modes of expression in response to pathogens (Figures 2 and 3) (Azevedo et al., 2006). We then tested the heat shock tolerance of plants by immersing 4-week-old plants grown at 25°C in a water bath at 41 to 48°C for 10 min, as described (Sung and Guy, 2003). Three days after treatment, extensive tissue collapse was observed in leaves of wild-type Col-0 and in the *sgt1a* mutant after treatment at 42.5°C (Figure 9C). By contrast, *sgt1b^{eta3}* mutant plants displayed reduced tissue damage resembling that of the thermotolerant *HSC70-1*-overexpressing lines 8-7 and 8-9 (Sung and Guy, 2003). The extent of cell collapse in these lines was quantified in five plants by measuring the percentage of total ions that leaked from leaves after heat shock. One hundred percent refers to the total ion content in the plant sample after it had been microwaved to release all ions. *sgt1b^{eta3}* exhibited a similar degree of heat shock tolerance as the *HSC70-1*-overexpressing lines 8-7 and 8-9 (Figure 9D). *Ler sgt1b-3* mutant plants were also more tolerant of heat shock than was the *Ler* wild type (Figure 9E). We conclude that SGT1 and HSC70 proteins have some overlapping functions in modulating responses to biotic and abiotic stresses. A correlation between the loss of HSC70 interaction of the *sgt1b^{eta3}* mutant protein and the increased thermotolerance of *sgt1b^{eta3}* plants further implies that heat shock sensitivity in the wild type involves the interaction of HSC70 with SGT1.

DISCUSSION

We present evidence that two functional isoforms of *Arabidopsis* SGT1, SGT1a and SGT1b, form stable interactions with cytosolic/nuclear HSC70 chaperones in vivo and that these interactions depend on the SGS domain of SGT1. Several observations point to SGT1 behavior as a HSC70 cofactor rather than a substrate. First, SGT1 is not bound strongly by the HSC70 client binding domain. Second, interaction between SGT1b^{eta3} and HSC70 is reduced, arguing against potentially misfolded SGT1 serving as an HSC70 client. Third, the 36 residues that are missing in SGT1b^{eta3} form part of a hydrophilic domain with helices that are not optimally recognized as a substrate by HSC70 chaperones (Rudiger et al., 1997; Lee et al., 2004). Resistance responses in *Arabidopsis* to virulent and avirulent pathogen isolates are compromised both by mutations in *SGT1b* and by deregulation of *HSC70-1* expression. This newly identified connection between SGT1 and HSC70 activities correlates with an enhanced heat

more concentrated than nuclei-depleted preparations. The open and closed arrowheads indicate SGT1b and SGT1a signals, respectively. The asterisk marks the SGT1^{eta3} protein, and the closed circle marks a weak nonspecific signal with anti-SGT1b antibody.

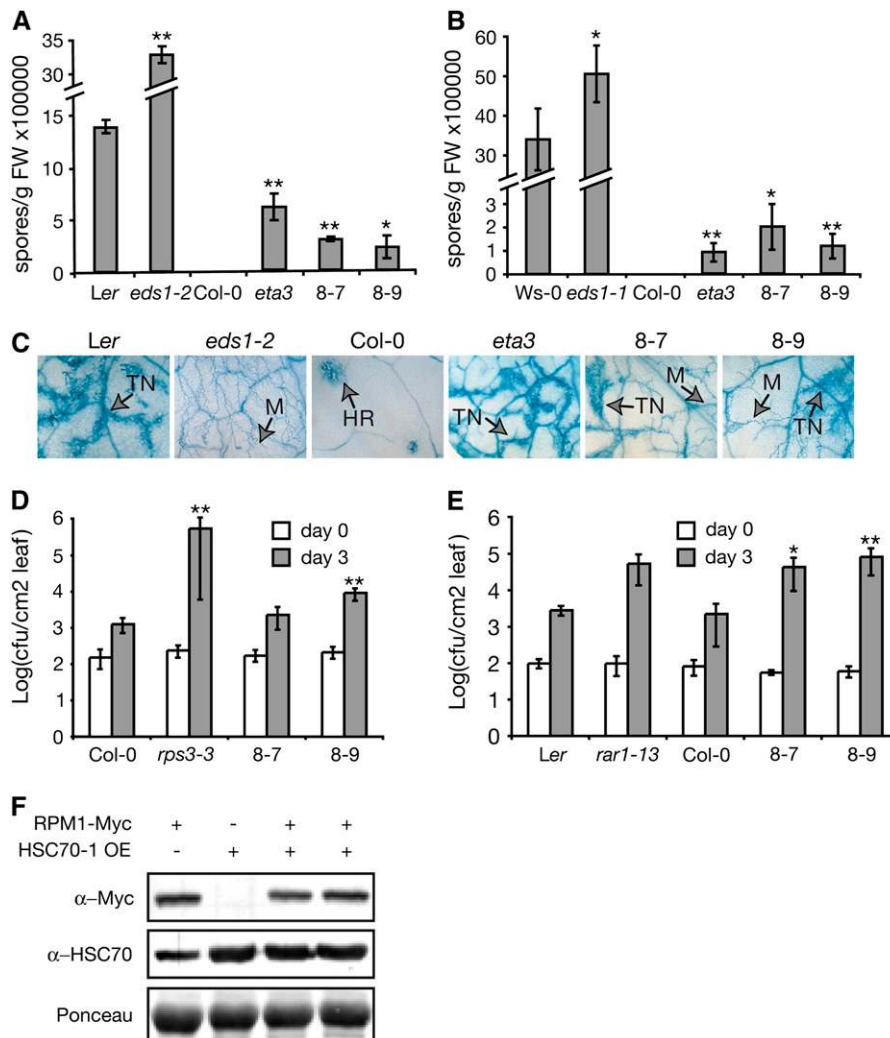


Figure 7. HSC70-1 Overexpression Disables R Gene-Mediated Resistance to Pathogens.

Arabidopsis Col-0 transgenic lines 8-7 and 8-9 overexpressing HSC70-1 were inoculated with avirulent *H. parasitica* (**A**) to **[C]** or strains of *Pst* DC3000 (**D**) and **[E]**). Two-week-old seedlings were inoculated with *H. parasitica* isolates Cala2 (**A**) and **[C]** or Emwa1 (**B**). Spores were counted at 6 d after inoculation (**A**) and **[B]**), and leaves were stained with trypan blue (**C**) to visualize fungal structures and tissue necrosis. *Ler* and *Ws-0* plants are susceptible to Cala2 and Emwa1, respectively. *Ler eds1-2* and *Ws-0 eds1-1* display enhanced susceptibility to Cala2 and Emwa1, respectively. FW, fresh weight; HR, hypersensitive response; M, mycelium; TN, trailing necrosis. Growth of *Pst* DC3000 strains expressing *avrRpm1* (**D**) or *avrRps4* (**E**) was measured at 0 and 3 d after vacuum infiltration of different lines, as indicated. Col-0 *rps3-3* is an *rpm1* mutant that has lost recognition of *Pst* DC3000/*avrRpm1*. The *rar1-13* mutation (*Ler* ecotype) disables *RPS4*. Each data point was analyzed in triplicate, and error bars indicate SE. Three independent experiments gave similar results. Statistically significant differences for values compared with the wild type were determined by Student's *t* test (* *P* < 0.05, ** *P* < 0.005). For (**F**), HSC70-1 overexpression (OE) does not affect RPM1-myc accumulation. Total leaf protein extracts were prepared from 3-week-old plants homozygous for *RPM1-myc* and *HSC70-1* overexpression (line 8-7) transgenes and analyzed by SDS-PAGE and immunoblotting using anti-Myc and anti-HSC70 antibodies. Equal loading was checked by Ponceau red staining of the membrane.

shock tolerance of *sgt1b* mutants, resembling the HSC70-1 overexpression phenotype. Together with the colocalization of SGT1 and HSC70 proteins in the cytosol and nuclei, these data lead us to propose that the interaction between SGT1 and HSC70 chaperones is important for plant defense against pathogens and some other stress responses that are controlled by HSC70 chaperones. Our findings are reinforced by a global study of the yeast Hsp90 interaction network that identified Sgt1 as an Hsc70

(Ssa1, Ssa2) interactor (Zhao et al., 2005). Also, coimmunoprecipitation of human Sgt1 and Hsp70 was recently reported after transfection of cultured cells and in ELISAs of recombinant proteins (Spiechowicz et al., 2007). The analysis by Spiechowicz et al. (2007) and results from our size-exclusion and *N. benthamiana* interaction assays (Figures 4 and 5) support direct chaperone-cochaperone binding. However, we did not observe any specific interaction between recombinant SGT1 and HSC70-1 in vitro or

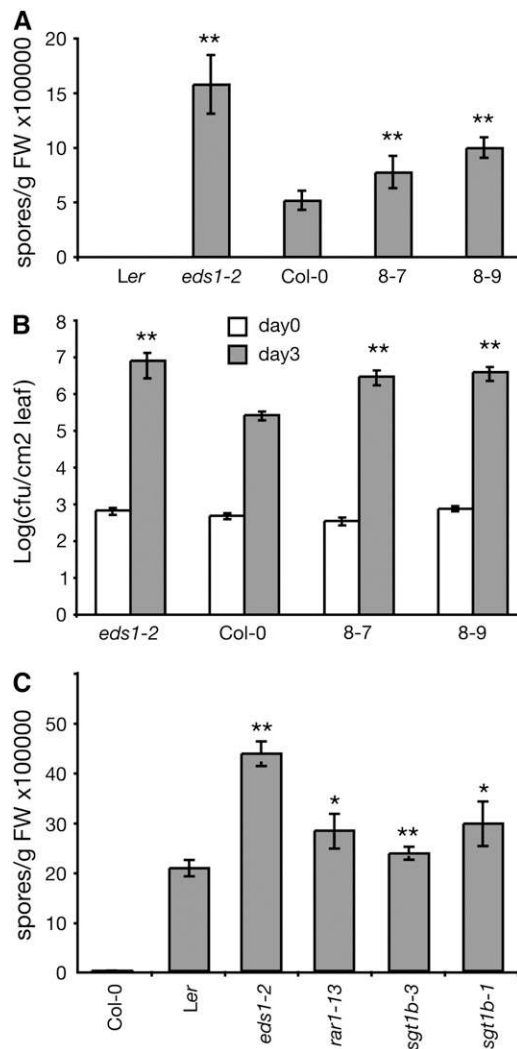


Figure 8. HSC70 and SGT1 Modulate Plant Basal Resistance.

Different *Arabidopsis* lines were inoculated with virulent isolates of *H. parasitica* Noco2 (A) and Cala2 (C) or *Pst* DC3000 (B). Pathogen growth was measured as described for Figure 7. 8-7 and 8-9 are two transgenic lines (Col-0) overexpressing HSC70-1. *Ler eds1-2* and *rar1-13* have compromised basal resistance. *Ler sgt1b-1* and *sgt1b-3* are two independent *sgt1b* null mutants. Statistically significant differences for values compared with the wild type were determined by Student's *t* test (* $P < 0.05$, ** $P < 0.005$). FW, fresh weight.

in yeast two-hybrid assays; therefore, we cannot exclude the possibility that their association is mediated or assisted by another component.

Cytosolic HSC70s and SGT1 Function in Plant Postinvasive Defense

Consistent with a role of cytosolic HSC70s in plant resistance, transcripts of the *HSC70-2* and *HSC70-4* isoforms were responsive to bacterial pathogen infiltration (Figure 3A). Induction of *HSC70-2* and *HSC70-4* mRNAs preceded the appearance of cell death (data not shown) and *SGT1a* mRNA accumulation (Figure

3A) and exhibited slightly faster kinetics than was reported for *Arabidopsis HSP90-1*, which is preferentially recruited in *RPS2* resistance (Takahashi et al., 2003). Therefore, the observed boost in cytosolic HSC70 levels (Figure 3B) is unlikely to be a consequence of plant cell death. There was no impairment of the response of the *Arabidopsis sgt1b^{eta3}* mutant or the *HSC70-1*-overexpressing lines to a host nonadapted fungal pathogen, *Blumeria graminis* f. sp. *hordei* (V. Lipka, personal communication). Resistance to this pathogen in wild-type *Arabidopsis* is expressed prior to the invasion of cells and is not associated with programmed plant cell death (Lipka et al., 2005). Also, virus-induced gene silencing of *SGT1* in *N. benthamiana* showed that it is necessary for multiple cell death-associated disease resistance programs (Peart et al., 2002). Therefore, the activities of both cytosolic/nuclear HSC70s and *SGT1* appear to be most important for plant resistance to pathogens once they have invaded host cells.

We did not observe alterations in resistance phenotypes associated with the depletion of specific cytosolic *HSC70* mRNAs, probably due to high levels of functional redundancy. Consistent with this, combining the *hsc70-1* and *hsc70-3* mutations produced seedling lethality (data not shown). However, overexpression of *Arabidopsis HSC70-1* disabled immune responses (Figure 7) and enhanced tolerance to heat shock (Figure 9). Given that *sgt1b* mutants displayed similar immune response (Figures 7 and 8) and heat stress phenotypes (Figure 9) as *HSC70-1* overexpression and that an *sgt1b* mutation enhanced the disease susceptibility of an *HSC70-1* overexpression line (see Supplemental Figure 5 online), *SGT1* could behave as an important modulator of HSC70, balancing its various activities and interactions within the chaperone-cochaperone network.

Arabidopsis SGT1b did not discriminate in association with particular cytosolic HSC70 isoforms in leaves (Figure 2C), consistent with the presence of stable *SGT1*-HSC70 complexes in healthy and pathogen-treated tissues. However, we observed stronger binding of *SGT1b* than *SGT1a* to HSC70-1 in *N. benthamiana* interaction assays (Figure 4E). *SGT1a* was also reported to bind to HSP90 less strongly than *SGT1b* (Hubert et al., 2003). In healthy *Arabidopsis* leaves, *SGT1b* protein accumulates to approximately fourfold higher amounts than *SGT1a*, although *SGT1a* is strongly pathogen-induced (Figure 3) and can compensate for the loss of *SGT1b* when expressed at high levels (Azevedo et al., 2006). The lower abundance of *SGT1a* coupled with the reduced affinity for HSC70 may account for the preferential genetic recruitment of *SGT1b* in immunity and auxin or jasmonic acid sensing (Austin et al., 2002; Gray et al., 2003). Also, differences in the subcellular distribution of *SGT1a* and *SGT1b* observed here (Figure 6C) may influence their competence as cochaperones. Notably, *SGT1b* import to nuclei predominated over *SGT1a* in wild-type tissues. The finding that *SGT1a* accumulated in nuclear preparations of mutant plants expressing the nonfunctional *SGT1^{eta3}* protein suggests that *SGT1* nuclear accumulation is an important aspect of *SGT1* activity and possibly also HSC70 interaction.

HSC70 and SGT1b Modulate Basal Resistance to Virulent Pathogens

An unexpected finding was the partial disabling of basal resistance to *H. parasitica* isolates in *sgt1b* null mutants and the

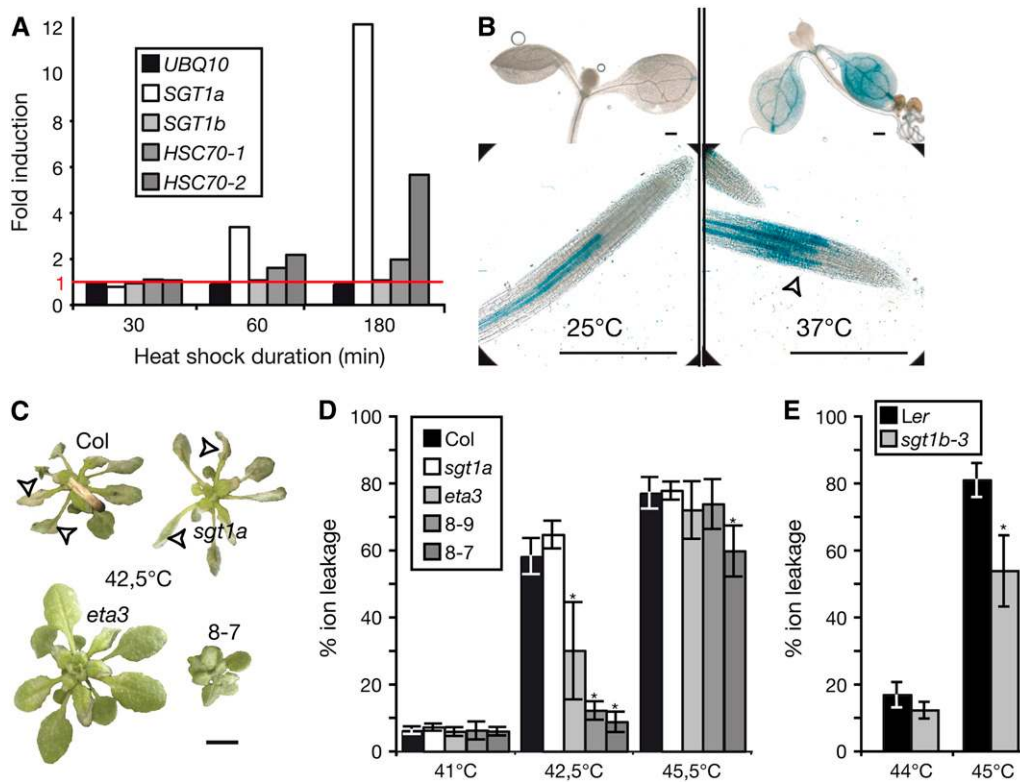


Figure 9. *Arabidopsis sgt1b* Mutants Exhibit Enhanced Tolerance to Heat Shock.

(A) *SGT1a*, *SGT1b*, *HSC70-1*, *HSC70-2*, and *UBQ10* gene expression after heat shock (38°C) relative to control conditions (25°C) was determined by meta-analysis of *Arabidopsis* gene expression microarray hybridization experiments. Samples were harvested after 30, 60, or 180 min. The red line indicates the induction level of genes whose expression is not affected by heat shock at 38°C. UBQ10, ubiquitin constitutive control.

(B) *SGT1a* promoter activity. Transgenic plants expressing the GUS reporter gene under the control of the *SGT1a* promoter were grown for 7 d at 25°C (left panel) and placed at 37°C (right panel) for 3 h before staining for GUS activity (shown in blue). Bar = 0.4 mm. The open arrowhead marks the site of intense GUS activity in the root after heat shock. One representative transgenic line is shown (AB1-1; Azevedo et al., 2006).

(C) Appearance of 5-week-old plants at 3 d after a 10-min incubation at 42.5°C. Open arrowheads indicate tissue collapse. Bar = 1 cm.

(D) and (E) The extent of cell collapse was measured in different lines by ion leakage at 3 d after a 10-min incubation at 41 to 45.5°C (D) or at 44 and 45°C (E). Ion leakage was normalized to the total ion content in the plant sample after it had been microwaved to release all ions. The percentage of total ions leaking from leaf tissue after heat shock reflects the proportion of cells that have lost cell integrity. Values are means from five individual plants. Error bars indicate SD. Statistically significant differences for values compared with the wild type were determined by Student's *t* test (* *P* < 0.005).

HSC70-1 overexpression lines (Figure 8). This form of low-level resistance restricts pathogen colonization in the absence of cell death (Chisholm et al., 2006). Since in yeast and plants, SGT1 affects the functions of several unrelated protein complexes (Muskett and Parker, 2003; Shirasu and Schulze-Lefert, 2003), it is conceivable that SGT1 controls several molecularly independent steps in plant immunity. HSP90 and SGT1 are needed for the steady state accumulation of at least one NB-LRR receptor, Rx (Azevedo et al., 2006), but it is not known whether this is universal for NB-LRR proteins. An absence of obvious effects of *HSC70-1* overexpression on RPM1-myc accumulation (Figure 7F) supports HSC70 action at a site other than NB-LRR protein stabilization. There were no differences in *SGT1a* and *SGT1b* protein accumulation between *HSC70-1* overexpression lines and the wild type (data not shown), arguing against the depletion of SGT1 being an explanation for the loss of resistance. Nevertheless, HSC70 may cooperate with SGT1 (and HSP90) to regulate the levels of numerous low-affinity immune receptors that

together could constitute basal resistance to invasive virulent pathogens (Holt et al., 2005).

A broader biological relevance of an SGT1-HSC70 complex is suggested by the finding that exposure of plants to heat shock elicits strong induction of *SGT1a* expression (Figures 9A and 9B), as found in pathogen responses (Azevedo et al., 2006), and enhanced heat shock tolerance in *sgt1b* leaves, as seen in the HSC70-overexpressing lines (Figures 9C to 9E). We think that these related phenotypes reflect a functional connection between SGT1 and cytosolic/nuclear HSC70 chaperones. However, *HSC70-1* overexpression does not mirror all *sgt1b* loss-of-function phenotypes, since it did not compromise sensitivity to auxin by the root growth inhibition assay (L.D. Noël, unpublished data).

Does SGT1 Bridge HSP90-HSC70 Chaperone Activities?

An association between Sgt1 and Hsp90 in multiple systems and our finding that *Arabidopsis* SGT1 and HSC70 interact in vivo

prompt the question of whether SGT1 serves to coordinate HSP90-HSC70 chaperone functions. Although Hsp90 and Hsc70 proteins have distinct biochemical roles, there is a substantial degree of cooperativity between these two chaperones in regulating protein complex assembly and subcellular trafficking (Pratt et al., 2004). Such coordinated activities are in large part mediated by cochaperones. For example, the TPR protein Hop (known as Sti1 in yeast) binds Hsc70 and Hsp90 and ensures optimal substrate channeling within a single multiple-chaperone complex (Hernandez et al., 2002). Another cochaperone, CHIP (for C terminus of Hsc70-interacting protein) directs Hsp90/Hsc70 chaperone complexes to the proteasome by attaching a ubiquitin signal onto the chaperone-bound client protein (Connell et al., 2001; Esser et al., 2004). Analysis in yeast and human showed that the Hsp90-Sgt1 association is transient (Kitagawa et al., 1999; Lee et al., 2004; Lingelbach and Kaplan, 2004; Catlett and Kaplan, 2006), and this is likely also to be the case in plants, since only a very small proportion of the total SGT1 pool was bound to HSP90 *in vivo* (Hubert et al., 2003). We failed to identify HSP90 in SGT1-Strep affinity purification experiments. We also could not detect RAR1 or ASK1 (the major *Arabidopsis* SKP1 protein in leaves), probably due to the transient and unstable nature of these associations, as reported for interactions between Sgt1p and Skp1p in yeast (Kitagawa et al., 1999; Lyapina et al., 2001). In yeast, human, and plant cells, Sgt1 interaction with the Hsp90 ATPase domain is mediated principally through its central CS (p23-like) domain, although Sgt1 binding does not influence the ATP hydrolysis rate of Hsp90, in contrast with other cochaperones such as Hop/Sti1 and Hip/p60 (Takahashi et al., 2003; Lee et al., 2004; Catlett and Kaplan, 2006). Thus, the Sgt1 CS domain is both necessary and sufficient for Hsp90 binding and should not be affected directly by SGS modifications (Takahashi et al., 2003; Lee et al., 2004). Consistent with this model, the SGT1a CS domain failed to bind HSC70 in *N. benthamiana* interaction assays (Figure 5A) and the SGT1^{eta3} mutant protein retained interaction with RAR1 and HSP90 in a yeast two-hybrid assay (see Supplemental Figure 3 online).

Yeast Sgt1p appears to behave as an adaptor for client proteins and in this capacity is able to link Skp1 to Hsp90 (Catlett and Kaplan, 2006). Hop/Sti1 is one of a number of TPR domain cochaperones that bind HSP90 at its C-terminal EEVD domain and acts as a coupling factor between Hsp70 and Hsp90 to fold proteins such as the glucocorticoid receptor or to assemble protein complexes (Hernandez et al., 2002; Pratt et al., 2004). Hsp70 promotes the initial steps of glucocorticoid receptor folding and transfers its substrate to Hsp90 to complete maturation. Yeast Sgt1p can form ternary complexes with Hsp90 and Hop (Catlett and Kaplan, 2006), and this might influence substrate channeling between the two chaperones as well as their binding of other cofactors. Our finding that the plant SGT1 interaction with cytosolic/nuclear HSC70s requires its SGS domain could allow cooperative binding of HSP90 through the CS domain and an association with SKP1 and the core SCF ubiquitin E3 ligase system through the SGT1 TPR motif. However, the role of the TPR domain in plant SGT1 remains unclear, since it was dispensable for *Arabidopsis* SGT1 activity in R protein-triggered immunity and auxin sensing (Azevedo et al., 2006). If the association between SGT1 and SKP1 is solely through the TPRs (Catlett and Kaplan, 2006), this would argue against the SGT1-SKP1 interaction being im-

portant for SGT1 function in the plant immune and hormone responses. Another possibility is that SGT1 in plants connects with particular SCF E3 ligase complexes through an interaction between its SGS domain and the LRRs of certain F box proteins, such as TIR1 in SCF^{TIR1} and COI1 in SCF^{COI1}. In support of this, yeast two-hybrid interaction analysis by Dubacq et al. (2002) showed that yeast Sgt1p has a propensity to associate with LRRs or WD-40 repeats that are highly represented in F box and other signaling proteins. Also, interactions were observed between SGT1 and the LRR domains of barley MLA1 in yeast two-hybrid assays (Bieri et al., 2004) and pepper (*Capsicum annuum*) BS2 in transient *N. benthamiana* expression assays (Leister et al., 2005). Notably, the SGT1-MLA association was observed with TPR deletants and with a truncated SGT1 protein containing only the SGS domain (Bieri et al., 2004). We propose, therefore, that SGT1 may bridge the HSP90-HSC70 chaperone machinery with a selected number of domains during protein complex maturation and/or activation. The stable interaction of SGT1 with cytosolic HSC70 observed in our study may reflect a default state that is then directed toward assembly or disassembly functions through transient interaction with HSP90 and the activities of other cochaperones.

Significance of the SGT1 SGS Domain

The SGS domain is necessary and sufficient for the binding of SGT1 to HSC70 in *N. benthamiana* transient interaction assays. Several SGS domain mutant alleles (e.g., in *sgt1-5*) were identified in yeast *Sgt1* that block the G1/S transition in cell cycle progression and are affected in the SCF-dependent degradation of Cln1p/Cln2p cyclins and Sic1p Cdk inhibitor (Kitagawa et al., 1999; Bansal et al., 2004). These contrast with mutations in the yeast Sgt1p TPR domain (such as those encoded by *sgt1-3*) that lose interaction with the kinetochore component Skp1p and are blocked in the G2/M transition of the cell cycle. Thus, in yeast, the SGS domain mediates distinct SGT1 functions, consistent with its genetic requirement for adenyl cyclase Cyr1p activation (Dubacq et al., 2002). To our knowledge, HSC70 chaperones are the only direct SGS interactors to date. The SGS domain is the most highly conserved in SGT1 (59% identity between human SGT1 and *Arabidopsis* SGT1b) but is unfolded with a limited degree of helical structure (Lee et al., 2004). Due to the high conservation of the SGS domain in eukaryotes and the ability of an SGT1b TPR domain deletant to complement the *sgt1b-1* mutation (Azevedo et al., 2006), we propose that *sgt1b^{eta3}* may represent a plant *sgt1* G1 allele. This raises the question of whether yeast *sgt1* G1 phenotypes could in part be due to a loss of SGT1-HSP70 interaction. To date, HSP70 has been implicated in cell cycle regulation as a high-copy suppressor of the G2 cell cycle arrest induced by human immunodeficiency virus (Iordanskiy et al., 2004). A role of HSC70 in the cell cycle may be worth exploring.

METHODS

Plant Materials, Growth Conditions, and Pathology Assays

Wild-type *Arabidopsis thaliana* accessions used were Col-0, *Ler*, and *Ws-0*. The *Ler sgt1b-3* and *sgt1b-1* (Austin et al., 2002), *Ler eds1-2* (Falk

et al., 1999), *Ler rar1-13* (Muskett et al., 2002), *Ws-0 eds1-1* (Parker et al., 1996), *Col-0 rps3-3* (Bisgrove et al., 1994), *Col-0 sgt1b^{eta3}* (Gray et al., 2003), *Col-0 pad4-1* (Glazebrook et al., 1997), and *Ws-0 sgt1a-1* (Hubert et al., 2003) mutants have been described. Lines overexpressing SGT1b-Strep (*Ler sgt1b-3* pXCS-SGT1b-Strep) were described previously (Witte et al., 2004). Homozygous *Col-0* plants overexpressing *HSC70-1* (Sung and Guy, 2003) were used: lines 8-7 (single insertion) and 8-9 (multiple insertions). *Col-0* plants expressing functional RPM1-myc protein (Boyes et al., 1998) were crossed with *HSC70-1*-overexpressing line 8-7, and plants homozygous for RPM1-myc and *HSC70-1* transgenes were selected in the progeny. *Col-0* T-DNA insertion mutants in *HSC70-1* (SALK_135531), *HSC70-2* (SALK_085076), *HSC70-3* (GK_758E01), *HSC70-4* (SALK_029571), and *SGT1a* (GK_266H09) were ordered from the SALK and GABI-KAT databanks, and the insertions were verified by PCR and made homozygous. *HSC70* gene expression was measured in these lines by RT-PCR. Two independent transgenic lines expressing the *GUS* gene reporter under the control of the *SGT1a* promoter have been described (Azevedo et al., 2006).

For pathogenicity tests, plants were grown in soil under a 10-h photoperiod at 22°C with light intensity of 180 to 200 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and 65% humidity. *Hyaloperonospora parasitica* and *Pseudomonas syringae* pv *tomato* DC3000 isolates were cultured and prepared for inoculation as described (Aarts et al., 1998; Muskett et al., 2002). Two-week-old seedlings were sprayed with 4×10^4 *H. parasitica* conidiospores/mL in distilled water. Infection phenotypes were scored at 6 to 7 d after infection by lactophenol trypan blue staining and counting of conidiospores as described (Muskett et al., 2002). Bacterial growth tests were performed by vacuum infiltration of a bacterial suspension (1×10^5 cfu/mL) into leaves of 5- to 6-week-old plants. Each data point was analyzed at least in triplicate.

Arabidopsis Hormone and Heat Shock Response Assays

For root growth inhibition assays, seedlings were grown in sterile conditions on vertically oriented Murashige and Skoog (MS/10) medium containing 0.5% sucrose in a white light growth chamber under a 16-h photoperiod at 24/21°C (light/dark). Five-day-old seedlings were transferred to MS/10 medium containing different concentrations of methyl jasmonate (Duchefa) or 2,4-D (Sigma-Aldrich). Root length was measured at 3 d after transfer. To induce a heat shock response, 7-d-old plantlets growing on MS/10 medium at 25°C were incubated at 38°C for 3 h or kept at 25°C (control condition) and stained for GUS activity for 1 to 2 h as described (Jefferson, 1987). Heat shock tolerance was tested as described (Sung and Guy, 2003). Values are means of at least three biological replicates.

Generation of Strep-Tagged SGT1 Variants

In order to express SGT1a-Strep and SGT1b-Strep from their own promoters, SGT1 coding regions with their 1.3-kb upstream promoter sequences (Tor et al., 2002) were amplified from *Col-0* genomic DNA. Primer sequence information is available upon request. Amplicons were cloned into pENTR/D-TOPO (Invitrogen) and transferred into pXCG-Strep by LR recombination (Invitrogen), giving pXCG-SGT1a-Strep and pXCG-SGT1b-Strep, respectively. A pXCSG-Strep derivative, pXCG-Strep (Witte et al., 2004), without the 35S promoter, was obtained by *AscI*/*XhoI* digestion of pXCSG-Strep, fill-in, and religation. To express SGT1 CS and SGS domains and the truncated SGT1b^{eta3} protein with a C-terminal StrepII tag under the control of the 35S promoter, the corresponding SGT1 sequences were PCR-amplified from *Ler* cDNA clones and cloned into pENTR/D-TOPO (Invitrogen). Amplicons were transferred by LR recombination into pXCSG-Strep, giving pXCSG-CSa-Strep, pXCSG-SGSb-Strep, and pXCSG-ETA3-Strep.

Generation of HA-Tagged HSC70-1 Derivatives

Amplicons corresponding to the full-length ATPase domain and the client binding domain (CBD) of HSC70-1 were PCR-amplified from a *Col-0* cDNA library and cloned into pENTR/D-TOPO (Invitrogen). The amplicons were transferred by LR recombination into pJ2B-3HA-GW (N. Medina-Escobar and J.E. Parker, unpublished data), giving pJ2B-3HA-HSC70FL, pJ2B-3HA-HSC70ATPase, and pJ2B-3HA-HSC70CBD.

RT-PCR Analysis

Four-week-old *Col-0* plants were hand-inoculated with *Pst* DC3000 strains (10^7 cfu/mL) in 10 mM MgCl₂ or with 10 mM MgCl₂ alone as a control. RNA was isolated from leaves using Tri reagent (Sigma-Aldrich) for use as RT-PCR template. One microgram of total RNA was subjected to reverse transcription using SuperScript II (Invitrogen). Primer specificity was confirmed by direct sequencing of RT-PCR amplicons. The number of cycles used was determined empirically to be within the linear amplification phase. RT-PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

Plant Transformations

Agrobacterium tumefaciens strain GV3101::pMP90RK was used for transient protein expression in *Nicotiana benthamiana* using pXC/pXN binary derivatives, as described (Witte et al., 2004). Stable transformation of *Arabidopsis* was done by flower dipping as described (Clough and Bent, 1998). Transformants were selected by spraying soil-grown 7-d-old seedlings with 0.1% Basta (Aventis). Resistant T1 transgenic lines expressing detectable amounts of the fusion proteins were self-pollinated to produce T2 seeds. Single insertion lines were selected by segregation analysis of the resistance in T2 populations on MS medium containing 10 $\mu\text{g}/\text{mL}$ phosphinotricin (Duchefa).

StrepII Affinity Purification and Immunoprecipitation

StrepII affinity purifications were performed as described (Witte et al., 2004). For immunoprecipitation experiments, 1 g of leaf tissue was homogenized in 2 mL of W buffer (50 mM Tris, pH 8, 150 mM NaCl, 0.05% Triton X-100, and 2 mM DTT) supplemented with plant protease inhibitor cocktail (Sigma-Aldrich P9599; diluted 1:200). After centrifugation, 500 μL of the cleared protein extract was incubated with 10 μL of antibody with end-over-end rotation at 4°C for 2 h. The following antibodies were used: rabbit anti-SGS (raised against the SGT1a SGS domain; S. Betsuyaku, A. Takahashi, K. Shirasu, and J.E. Parker, unpublished data) and rabbit anti-SGT1b (Austin et al., 2002). Washed Protein A-Sepharose Fast Flow (Amersham Biosciences) was added to samples and incubated for 1 h. Beads were washed three times with 1 mL of W buffer and finally resuspended in 25 μL of Laemmli buffer.

SDS-PAGE and Immunoblotting

Proteins were separated by SDS-PAGE on 10 or 12% gels and transferred onto nitrocellulose or polyvinylidene difluoride membranes. Immunoblots with the Strep-Tactin-AP conjugate (catalog number IBA 2-1503-001) was performed as described (Witte et al., 2004). The following antibodies were used: monoclonal mouse anti-spinach HSP70 (Stressgen SPA-817); rat anti-HA antibody (Roche 1 867 423); mouse anti-c-Myc (clone 9E10; Santa Cruz Biotechnology); anti-SGS antibody (as above); rabbit anti-SGT1b antibody (Austin et al., 2002); rabbit anti-PEPC (Rockland); rabbit anti-histone H3 (Abcam) antibodies; goat anti-rabbit IgG horseradish peroxidase (HRP) conjugate (Santa Cruz Biotechnology sc-2004); goat anti-mouse IgG HRP conjugate (Santa Cruz Biotechnology sc-2005); and goat anti-rat IgG HRP conjugate (Santa Cruz Biotechnology sc-2006). AP and

HRP activities were detected with *p*-nitroblue tetrazolium and enhanced chemiluminescence (SuperSignal West Femto chemiluminescent substrate; Pierce), respectively. Silver staining was performed as described (Shevchenko et al., 1996).

Preparation of *Arabidopsis* Nuclear Extracts

Nuclei-enriched/depleted fractions were prepared from leaves of 4-week-old seedlings as described (Feys et al., 2005).

Yeast Two-Hybrid Analysis

Sequences encoding RAR1, HSP90-1, HSP90^{ATPase}, SGT1b, SGT1b^{eta3}, and HSC70-1 were recombined into Gateway-converted versions of the vectors pGADT7 and pGBKT7 (Clontech) (T. Lahaye, unpublished data). Plasmids were transformed into yeast strains AH109 and Y187 (Clontech). Diploids containing both bait and prey constructs were constructed by mating. Expression of the fusion proteins was checked by protein gel blot analysis using c-Myc and HA antibodies. Dilution series of the obtained diploids were plated on synthetic defined selective medium to screen for interaction according to the manufacturer and grown for 3 d at 28°C.

Construction of Cerulean and YFP Fusions

To generate fluorescent protein destination vectors for Gateway (GW) cloning technology (Invitrogen), PCR-amplified Cerulean (Rizzo et al., 2004) and eYFP (Shah et al., 2002) and a Gateway recombination cassette were inserted into the pXCS-HisHA vector (Witte et al., 2004), resulting in p35S-GW-Cerulean-nos and p35S-eYFP-GW-nos, respectively. The cDNA sequences of SGT1b (Witte et al., 2004) and HSC70-1 cloned into pENTR/D-TOPO (Invitrogen) were recombined into p35S-GW-Cerulean-nos and p35S-eYFP-GW-nos, respectively.

Transient Single-Cell Gene Expression Assays

Detached leaves from 5- to 6-week-old *N. benthamiana* plants cultivated as described (Witte et al., 2004) were used for transient gene expression in epidermal cells mediated by particle bombardment (Bhat et al., 2005). For colocalization, equimolar amounts of plasmids were coated onto the gold particles. After bombardment, leaves were incubated for 18 to 48 h at room temperature prior to microscopic analysis.

Confocal Laser Scanning Fluorescence Microscopy

Transient intracellular fluorescence was observed by confocal laser scanning microscopy using a Leica SP2 AOBS inverted confocal microscope (Leica Microsystems) equipped with argon ion (458-, 476-, 488-, 496-, and 514-nm laser lines) lasers. Additionally, a 405-nm diode laser (BDL 405 SMC; Becker and Hickl) was also installed into the Leica SP2 AOBS system. SGT1b-Cerulean was excited either with the 405-nm diode laser or the 458-nm argon laser line, while eYFP was excited with the 514-nm argon laser line. In cells coexpressing both SGT1b-Cerulean and eYFP-HSC70-1, the imaging was done in sequential mode. Cerulean fluorescence was detected using the Leica AOBS system and a custom 485- to 505-nm band-pass emission filter, while eYFP fluorescence was detected using the Leica AOBS system and a custom 515- to 560-nm band-pass emission filter. A 63× HCX PLAN-APO water-immersible objective lens (numerical aperture = 1.2) was used for imaging.

Mass Spectrometry

Tryptic digestion of proteins separated by SDS-PAGE was performed using a protocol based on that of Shevchenko et al. (2000). Digests were desalted for electrospray mass spectrometry with a C18 reverse-phase

resin (ZipTip; Millipore). A Micromass Q-ToF-2 mass spectrometer operating with nanospray and the Masslynx software (version 3.5) were used for analysis.

Gel-Filtration Analysis

A 25- μ L Ler protein sample prepared as for immunoprecipitation was loaded on a Superdex 200 HR 10/30 column (Amersham Biosciences) at 0.2 mL/min flow with W buffer without Triton X-100. Then, 0.5- and 1-mL fractions were sampled, precipitated using 10% trichloroacetic acid, and analyzed by SDS-PAGE and immunoblotting. Column calibration was performed using the gel filtration low molecular weight and high molecular weight calibration kits (Amersham Biosciences).

Meta-Analysis of *Arabidopsis* Gene Expression Microarray Data

A microarray dataset describing the response of *Arabidopsis* plantlets to heat shock at 38°C was downloaded from The Arabidopsis Information Resource site (for detailed experimental setup and results, see http://www.Arabidopsis.org/servelets/TairObject?type=expression_set&id=1007967124) and processed using Genespring software (Silicon Genetics). Normalization per gene and per chip of the log₂ values was performed, allowing comparison of the two independent replicates available. Normalized gene expression levels of duplicates were averaged and used to determine levels of induction relative to control growth conditions (25°C).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative database under the following accession numbers: At4g16860 (*RPP5*); At3g48090 (*EDS1*); At3g52430 (*PAD4*); At5g51700 (*RAR1*); At4g23570 (*SGT1a*); At4g11260 (*SGT1b*); At5g02500 (*HSC70-1*); At5g02490 (*HSC70-2*); At3g09440 (*HSC70-3*); At3g12580 (*HSC70-4*); At1g16030 (*HSC70-5*); At2g14610 (*PR1*); At5g44340 (*TUB4*); At3g07040 (*RPM1*); At4g05320 (*UBQ10*); At2g39940 (*COI1*); and At5g52640 (*HSP90-1*).

Supplemental Data

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

Supplemental Figure 1. Functionality Tests of the StrepII-Tagged SGT1 Proteins in Transgenic *Arabidopsis* Described in Figure 1.

Supplemental Figure 2. Mass Fingerprinting of Two Peptides Discriminating between Cytosolic HSC70 Isoforms.

Supplemental Figure 3. Interaction Studies between SGT1 and RAR1 or HSP90-1 Using the Clontech Matchmaker GAL4 Yeast Two-Hybrid System.

Supplemental Figure 4. Individual Loss of *Arabidopsis* Isoforms 1, 2, and 3 Does Not Affect Plant Immune Responses.

Supplemental Figure 5. *SGT1b* Loss of Function and *HSC70-1* Overexpression Have Additive Effects on *R* Gene-Mediated Resistance to *H. parasitica*.

Supplemental Table 1. Tryptic Fragments Observed by Mass Spectrometry of 70-kD Proteins Copurified with Overexpressed SGT1b-Strep.

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Interaction between SGT1 and Cytosolic/Nuclear HSC70 Chaperones Regulates Arabidopsis Immune Responses

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