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Cytosolic/nuclear molecular chaperones of the heat shock protein families HSP90 and HSC70 are conserved and essential proteins in eukaryotes. These proteins have essentially been implicated in the innate immunity and abiotic stress tolerance in higher plants. Here, we demonstrate that both chaperones are recruited in Arabidopsis (*Arabidopsis thaliana*) for stomatal closure induced by several environmental signals. Plants overexpressing HSC70-1 or with reduced HSP90.2 activity are compromised in the dark-, CO_2-, flagellin 22 peptide-, and abscisic acid (ABA)-induced stomatal closure. HSC70-1 and HSP90 proteins are needed to establish basal expression levels of several ABA-responsive genes, suggesting that these chaperones might also be involved in ABA signaling events. Plants overexpressing HSC70-1 or with reduced HSP90.2 activity are hypersensitive to ABA in seed germination assays, suggesting that several chaperone complexes with distinct substrates might tune tissue-specific responses to ABA and the other biotic and abiotic stimuli studied. This study demonstrates that the HSC70/HSP90 machinery is important for stomatal closure and serves essential functions in plants to integrate signals from their biotic and abiotic environments.

The control of water vapor and carbon dioxide exchange between the mesophyll and the atmosphere is essential for plant growth and adaptation to varying environmental conditions and is mediated by modulating the aperture of pores at the leaf surface called stomata (Hetherington and Woodward, 2003). Those structures composed of two guard cells respond continuously to environmental signals such as light, CO_2 concentration, and the plant hormone abscisic acid (ABA). While strong light and low CO_2 concentrations favor stomatal aperture and thereby carbon fixation through photosynthesis, it also causes important water losses by transpiration. Thus, water deficit conditions will inhibit stomatal aperture and prevent excessive plant dehydration. ABA plays a central role in physiological processes, including the adaptation of vegetative tissues to water stresses as well as in seed maturation and dormancy. ABA will promote on the one hand a rapid stomatal closure that is mediated by solute efflux in the guard cells and on the other hand specific transcriptional responses for long-term adaptation to drought and dehydration tolerance in vegetative tissues and seeds. In seeds, ABA establishes dormancy and inhibits early seedling development and greening. Forward and reverse genetic analyses have led to the identification of many components that cover the ABA metabolism (Finkelstein et al., 2002; Nambara and Marion-Poll, 2005) and ABA signal transduction (Israelsson et al., 2006; Shinozaki and Yamaguchi-Shinozaki, 2007). Several intracellular ABA receptors such as PYR/PYL/RCAR protein family have recently been identified (Ma et al., 2009; Park et al., 2009;

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Santiago et al., 2009) and allowed the recapitulation of early ABA signaling events within a larger molecular complex composed of PYL/protein phosphatase 2C/SnRK2 (Suc nonfermenting related kinases2) at the atomic resolution (Melcher et al., 2009; Miyazono et al., 2009) as well as in vitro (Fujii et al., 2009). Upon ABA binding to PYL proteins, ABI1/ABI2/HAB1 protein phosphatase 2C activities are inhibited, resulting in a modified phosphorylation of SnRK2.2/3/6 and in the activation of SnRK2.2/3/6 kinase activities. This signaling module is essential for stomatal closure in response to ABA, for transcriptional responses to ABA, for drought tolerance, for seed dormancy, and for ABA-dependent inhibition of seedling development.

Besides controlling gas exchange, stomata also constitute natural entry sites for numerous foliar pathogens (Melotto et al., 2008). The first layer of the plant innate immune system is probably the stomatal closure upon perception of conserved pathogen-associated molecular patterns (PAMPs) since many open stomata mutants show enhanced susceptibility to pathogens (Melotto et al., 2006). For instance, the *Pseudomonas syringae* flagellin peptide flg22 induces rapid stomatal closure in wild-type Arabidopsis (*Arabidopsis thaliana*) but not in the SnRK2.6 mutant *ost1* (Melotto et al., 2006). Thus, PAMP- and ABA-triggered stomatal closure use common signaling components in guard cells. As an attempt to evade this immune response, pathogens have evolved strategies to bypass/corrupt this signaling cascade (Emi et al., 2001; Melotto et al., 2006; Gudesblat et al., 2009).

Molecular chaperones are key components of innate immunity in mammals (Ting et al., 2008) and plants (Shirasu, 2009). These conserved proteins (usually heat shock proteins [HSPs]) are globally essential and define a balance of protein folding, assembly, and degradation in physiological as well as stress conditions (Wegele et al., 2004; Bukau et al., 2006). On the one hand, DnaK/HSP70 (70 kD) chaperones from prokaryotes/eukaryotes mediate ATP-dependent chaperoning of nascent polypeptides, protein addressing, and degradation by somewhat promiscuous interactions to solvent-exposed hydrophobic residues (Erbse et al., 2004; Wegele et al., 2004). On the other hand, HSP90 ATPases are much more selective in their recognition specificity, are essentially involved in protein maturation, and play essential functions in regulating numerous physiological responses (Young et al., 2001; Wegele et al., 2004). In Arabidopsis, there are 14 *HSP70* (also named *HSC70*; heat shock cognate) genes, five of which (*HSC70-1* to *-5*) encode functionally redundant and essential proteins localized in the cytosol and nuclei (Lin et al., 2001; Sung et al., 2001; Noël et al., 2007). There are seven *HSP90* genes in Arabidopsis, four of which encode mostly redundant and essential proteins predicted to be cytosolic/nuclear (Krishna and Gloor, 2001; Hubert et al., 2009). Little is known about HSC70 and HSP90 physiological functions in plants since their essential roles during early embryogenesis have hampered their genetic analyses. The use of *HSC70-1* overexpression (which results in general up-regulation of other *HSC70* gene expression levels) and particular point mutant alleles of *HSP90.2* were of particular interest. These genetic resources were instrumental to perform most of the functional analysis along with two mechanistically related inhibitors of HSP90 ATPase activity, such as geldanamycin and radicicol (Queitsch et al., 2002; Hubert et al., 2003; Sung and Guy, 2003; Takahashi et al., 2003; Noël et al., 2007; Cazalé et al., 2009). Besides the contribution of chaperones to plant innate immunity, HSP90s have been implicated in buffering genetic variation (Queitsch et al., 2002) and drought stress tolerance (Song et al., 2009) while HSC70s are important for meristem function and tolerance to heat shock, heavy metals, γ-rays, and salt (Noël et al., 2007; Cazalé et al., 2009). HSC70 and HSP90 are regulated by a complex network of cochaperones that modulate their enzymatic activities directly or spatially coordinate their functions. For instance, plant SGT1 (suppressor of G2/M transition allele of *skp1*), a conserved eukaryotic protein, presumably acts as a scaffold to bridge HSC70/HSP90 functions (Catlett and Kaplan, 2006) and is important for SCF E3 ubiquitin ligase-dependent signaling (Kitagawa et al., 1999; Gray et al., 2003), plant innate immunity, and heat shock tolerance (Austin et al., 2002; Noël et al., 2007; Uppalapati et al., 2011). In Arabidopsis, *SGT1a* and *SGT1b* encode two SGT1 proteins that are functionally redundant and globally essential (Austin et al., 2002; Takahashi et al., 2003). Because *SGT1a* is much less expressed than *SGT1b* in healthy tissues, the loss of *SGT1a* did not yield any mutant phenotypes but *SGT1a* overexpression complements all known *sgt1b* mutant phenotypes (Austin et al., 2002; Gray et al., 2003; Azevedo et al., 2006; Noël et al., 2007). In contrast to SGT1a and SGT1b that are important for auxin and jasmonic acid phytohormones signaling (Gray et al., 2003), plant HSP90s and HSC70s do not seem to participate in these phytohormone signaling cascades (Cazalé et al., 2009).

In this study, we show that the HSC70/HSP90 machinery is required for stomatal closure and modulates transcriptional and physiological responses to ABA. In addition, our results intimately implicate ABA into plant immunity and the contribution of the SGT1/HSC70/HSP90 proteins to the different layers of plant immunity should be carefully reinvestigated in the light of their newly identified functions in stomata.

RESULTS

Modulation of Whole-Plant Water Losses in Response to Environmental Conditions Is Compromised by HSC70-1 and HSP90.2 Deregulation

We analyzed the kinetic of water loss in darkness for individual mutants affecting the HSC70/SGT1/HSP90 molecular chaperone complex (Fig. 1): *edm1* (*sgt1b* deletion mutant); *eta3* (point mutation in *SGT1b* affecting

HSC70-1 binding); *HSC70*, *SGT1a*, and *HSP90.1* T-DNA insertion mutants, HSC70-1 overexpressing (OE) lines (8.7-, 7-fold OE; 8.9-, 4-fold OE), *hsp90.2-2*, and *hsp90.2-3* (dominant negative mutations in *HSP90.2*). Compared to wild-type Columbia-0 (Col-0) plants and *sgt1*, *hsc70*, and *hsp90.1* mutants, the rate of fresh weight loss for detached rosettes was significantly increased in HSC70-1 OE plants and *hsp90.2* mutants (Fig. 1A). These observations were confirmed using measurements of leaf surface temperatures by infrared imaging

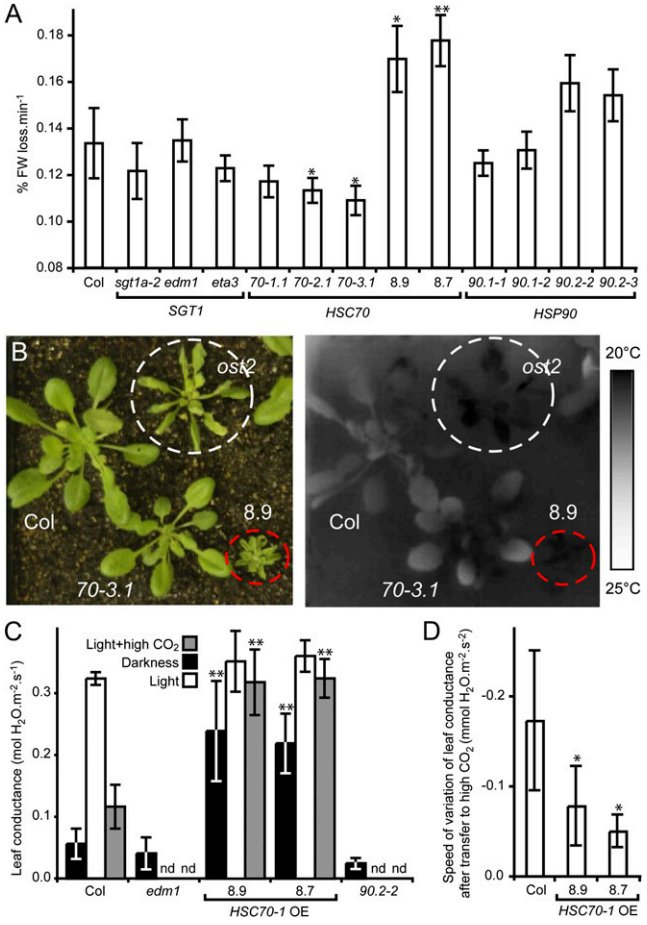

Figure 1. Effect of HSC70 and HSP90 deregulation on plant water losses in response to environmental conditions. A, Rate of fresh weight (FW) loss in darkness of 3- to 4-week-old detached rosettes from different Arabidopsis genotypes was measured after 195 min. Four plants per genotype were used and the experiment performed in triplicate. B, Bright-field (left) and thermal imaging (right) of 5-week-old plants: wild-type (Col), *hsc70-3.1* (*70-3.1*), and *ost2-2D* (*ost2*) mutants and an *HSC70-1* OE line (8.9). C and D, Leaf conductance was measured on attached leaves from 6-week-old plants at 22°C in darkness or light and ambient (400 μL L^{-1}) or high (2,000 μL L^{-1}) CO_2. C, Leaf conductance values were taken at the equilibrium while its speed of variation (D) was measured over a 10-min window following the change of condition. Three independent experiments were performed on at least three different plants. Error bar indicate standard deviations. * and **, Significant differences compared to the wild type (Student's *t* test, $P < 0.05$ and $P < 0.01$, respectively). nd, Not determined.

on intact plants. Leaf surface temperature partially depends on evaporative cooling by transpiration (Merlot et al., 2002). In darkness, the leaves of HSC70-1 OE and *hsp90.2* mutant plants were on average 1°C cooler than the other plant genotypes (Fig. 1B; Supplemental Fig. S1). As a more direct measurement of water loss, continuous recording of the conductance of attached leaves for the two HSC70-1 OE lines and the *edm1* and *hsp90.2-2* mutants was performed (Fig. 1, C and D). Leaf conductance for the HSC70-1 OE lines in darkness was already 4-fold higher than for wild-type or *edm1* and *hsp90.2-2* plants. Responses to dark/light transition and high CO_2 concentrations were strongly hampered both in amplitude (Fig. 1C) and rate of variation (Fig. 1D) as compared to wild-type responses. Thus, dehydration experiments, infrared imaging, and leaf conductance analyses on whole plants showed that a deregulation of *HSC70-1* and *HSP90.2* functions alters physiological responses to darkness and high CO_2 concentrations. Because stomatal densities and morphologies were not significantly different in all the lines studied (Fig. 2A; Supplemental Fig. S2A), our observations suggest that the deregulation of *HSC70-1* and *HSP90.2* causes an aberrant stomatal response to these two stimuli.

Dark-Induced Stomatal Closure Is Compromised by HSC70-1 Overexepression and Requires HSP90 ATPase Activity

Leaf epidermal peels, a classical model to study stomatal opening, were used to measure directly stomatal aperture following a 2-h incubation under light or in darkness. All the different genotypes exhibited similar stomatal apertures under light (Supplemental Fig. S2B). While wild-type plants and *sgt1* and *hsc70* mutants responded to darkness by closing their stomata to 30% of the aperture in light condition, stomata of the HSC70-1 OE and *hsp90* mutants remained opened to 75% and 60%, respectively (Fig. 2B). In a pharmacological approach, radicicol, a specific inhibitor of HSP90 ATPase activity, was used to investigate the role of HSP90s in the stomatal closure: Stomatal apertures on wild-type epidermal peels were measured in response to radicicol in light and darkness conditions (Fig. 2C). Radicicol treatment (100 nM) enhanced stomatal aperture under light by 50%. Darkness-induced stomatal closure was partially and fully suppressed by 1 and 10 nM radicicol, respectively. Altogether these results show that HSC70-1 OE compromises dark-induced stomatal closure and that HSP90 ATPase activity is required for stomatal closure.

Stomata Overexpressing HSC70-1 or with Reduced HSP90 ATPase Activity Are Insensitive to Exogenous ABA

Because ABA triggers stomatal closure, we followed the effect of exogenous ABA application on expression of *SGT1*, *HSC70*, and *HSP90* genes by quantitative reverse transcription (RT)-PCR (Fig. 3). As expected,

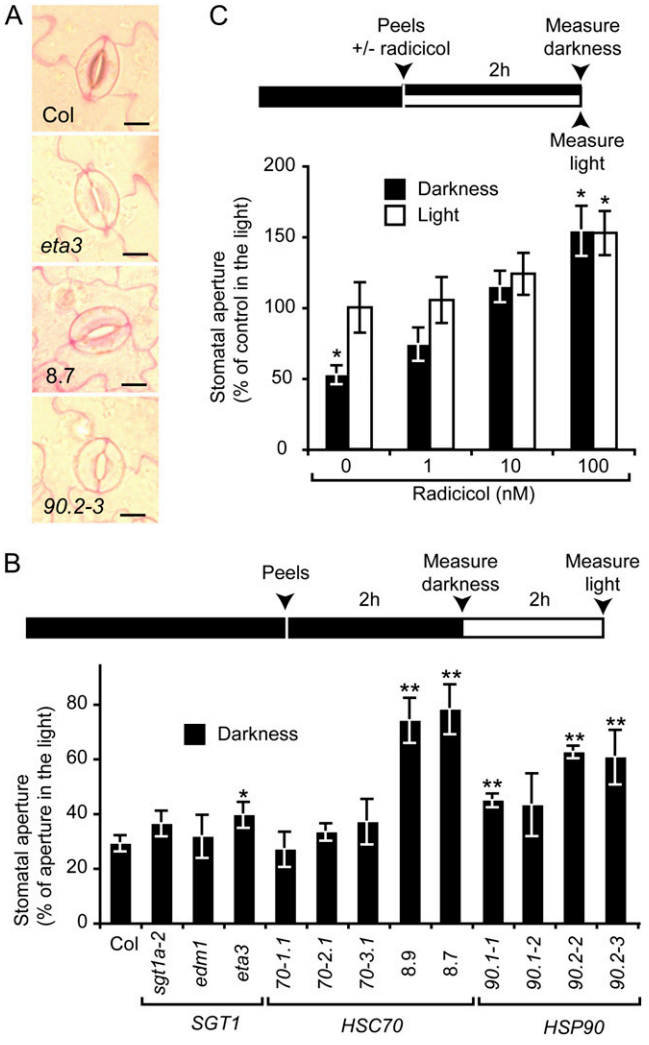

Figure 2. Stomatal closure in response to darkness but not morphology is compromised by HSC70-1 and HSP90 deregulation. A, Representative stomata observed on epidermal peels incubated in darkness and stained with ruthenium red. Bars = 10 μm. B, Stomatal apertures were measured on epidermal peels after 2 h in darkness and subsequently after a 2-h incubation under light conditions. Average stomatal apertures in darkness were expressed as a percentage of aperture compared to light conditions. Average aperture values (μm) under light are available in Supplemental Figure S2B. C, Average stomatal apertures in wild-type Col-0 epidermal peels expressed as percentage of control condition under light was measured and incubated for 2 h in light or darkness with 0 to 100 nM radicicol. Three independent measurements ($n > 50$) were performed per condition on at least three different plants. Experiments were repeated at least twice. Error bar indicate SDS. * and **, Significant differences compared to the wild type (Student's *t* test, $P < 0.05$ and $P < 0.01$, respectively). Chronology of dark (black box)/light (white box) conditions, preparation of peels from plant leaves, treatments with radicicol, and measurements of stomatal apertures are indicated above the corresponding experiments (B and C).

the ABA treatment induced the expression of the ABA-responsive gene *RD29A*. The expression of *HSC70-4* was increased 3-fold by the ABA treatment while no strong effect was observed on other *HSC70* and *HSP90* genes. On the other hand, a 30% reduction in *SGT1a* mRNA accumulation was observed after ABA treatment. These observations suggest that ABA directly or indirectly regulates the expression of specific *HSC70/SGT1* genes.

To test a possible involvement of the corresponding proteins in the ABA signaling and/or ABA-mediated stomatal closure, peels of the different genotypes were first incubated under light for 2 h to preopen stomata and then with 100 μM ABA. After 2h, the ABA-induced stomatal closure was measured (Fig. 4A). ABA promoted stomatal closure for wild-type plants as well as *sgt1*, *hsc70*, and *hsp90.1* mutants. Interestingly, stomata of the HSC70-1 OE lines and *hsp90.2* mutants were still fully opened. Furthermore, inhibition of HSP90 ATPase activity by 100 nM radicicol on wild-type epidermal peels was sufficient to attenuate ABA-dependent stomatal closure (Fig. 4B), indicating that HSP90 ATPase activity is partially epistatic on the ABA signal. The effects of radicicol on stomatal movements in response to environmental conditions are illustrated in Figure 4D. All together our results indicate that HSP90 activity stimulates stomatal closure while HSC70s would inhibit it.

Stomata Overexpressing HSC70-1 or with Reduced HSP90 ATPase Activity Have a Reduced Sensitivity to flg22 Peptide Treatment

HSC70-1 OE has been shown to enhance susceptibility to virulent and avirulent *Pseudomonas* (e.g. Hubert et al., 2003; Noël et al., 2007) and *HSP90* inactivation affects recognition of avirulent *Pseudomonas* (Hubert

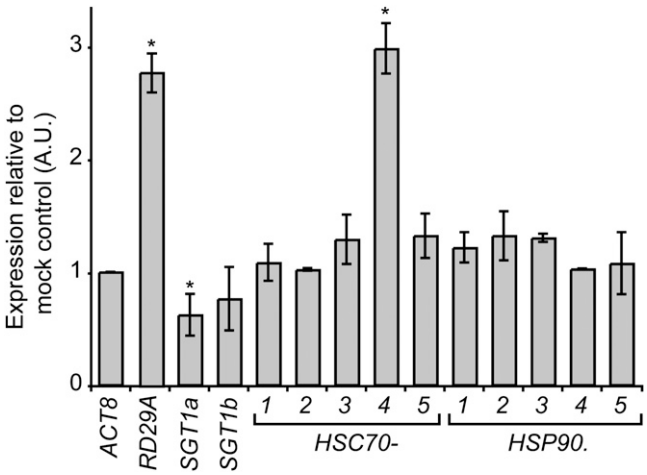

Figure 3. Effect of exogenous ABA treatment relative to mock control on *SGT1*, *HSC70*, and *HSP90* transcripts accumulation. cDNAs were prepared from wild-type 10-d-old Col-0 seedlings 3 h after a 10 μM ABA or mock treatment and transcripts abundance measured by quantitative RT-PCR. The ABA-responsive gene *RD29A* was used as positive control while the *Actin8* (*ACT8*) expression was used to normalize the transcript levels. Three independent RT-PCR experiments were performed on three independent biological samples. The bars represent mean values from three independent experiments and error bars indicate SDS. *, Significant differences compared to the wild type (Student's *t* test, $P < 0.05$).

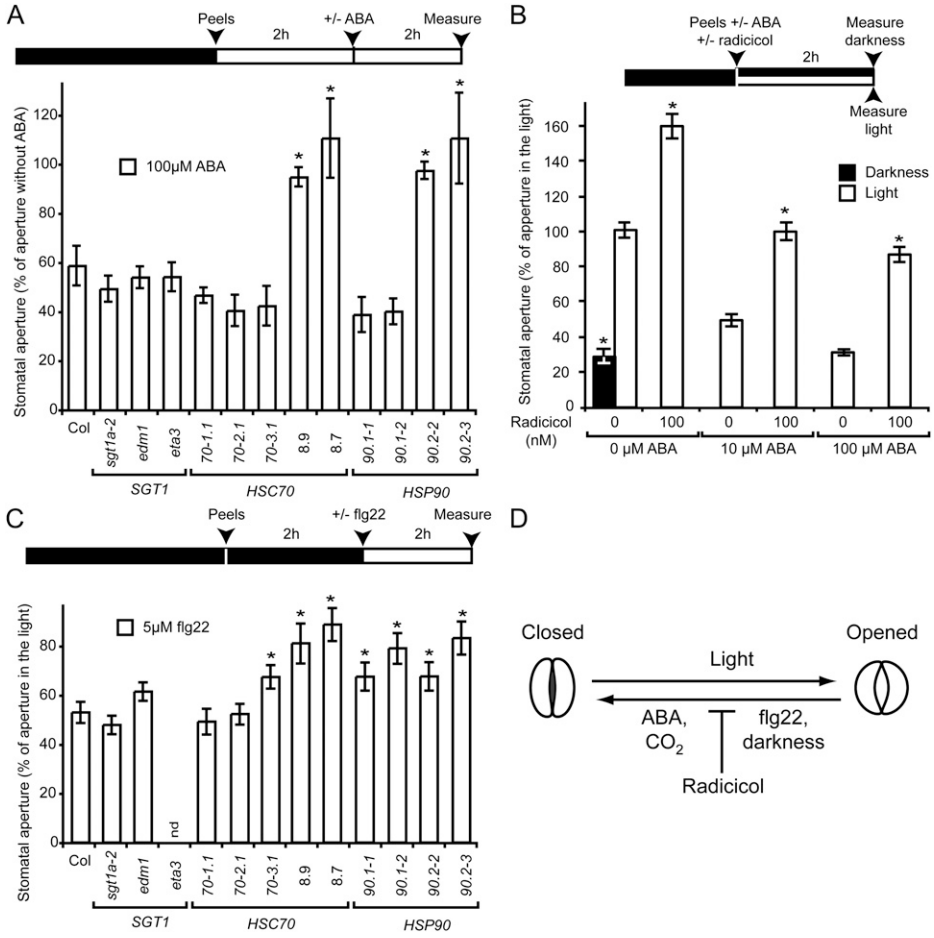

Figure 4. ABA and flg22 treatments cannot block light-induced stomatal opening in plants deregulated for HSC70-1 and HSP90 functions. A, Stomatal apertures were measured on epidermal peels incubated 2 h under light and then 2 additional h with or without 100 μM ABA. Average stomatal apertures in presence of ABA were expressed as a percentage of aperture without ABA. B, Average stomatal apertures expressed as percentage of aperture under light without ABA nor radicicol treatment was measured on Col-0 epidermal peels incubated for 2 h in light or darkness with 0 to 100 nM radicicol and 0 to 100 μM ABA. C, Stomatal apertures were measured on epidermal peels incubated in darkness for 2 h and then transferred under light for 2 h with or without 5 μM flg22. Average stomatal apertures in presence of flg22 were expressed as a percentage of aperture without flg22. Three independent measurements ($n > 50$) were performed per condition on at least three different plants. Experiments were repeated at least twice. Error bar indicate SDS. *, Significant differences compared to the wild type (A and C) or the samples without radicicol treatment for each ABA condition (B; Student's t test, $P < 0.001$). Chronology of dark (black box)/light (white box) conditions, preparation of peels from plant leaves, treatments with ABA/radicicol/flg22, and measurements of stomatal apertures are indicated above the corresponding experiments. D, Schematic representation of the regulation of stomatal opening/closure by biotic and abiotic factors. Radicicol that inhibits HSP90 ATPase activity acts as a general inhibitor of stomatal closure.

et al., 2003; Takahashi et al., 2003). Interestingly, the bacterial PAMP flagellin (or its 22-amino acid peptide flg22) triggers stomatal closure upon infection by a pathway that depends on signaling components shared with the ABA-dependent pathway such as SnRK2.6 (Mustilli et al., 2002; Yoshida et al., 2002; Melotto et al., 2006). To test whether this ancestral immune response might be also affected by HSC70-1 and HSP90 deregulation, we tested the responsiveness of stomata to flg22 treatment. Peels of the different genotypes were first incubated in darkness for 2 h and transferred under light for 2 h with or without 5 μM flg22 (Fig. 4C). flg22 treatment blocked light-induced stomatal opening for wild-type plants as well as *sgt1*, *hsc70*, and *hsp90.1* mutants. Interestingly, stomata of the HSC70-1 OE lines and *hsp90* mutants were significantly more opened under light despite the flg22 treatment. Therefore, HSC70-1 and HSP90s are important components of the flg22-dependent stomatal closure.

HSC70-1 and HSP90 Affect the Expression of Several ABA-Responsive Genes

To study whether HSC70-1 and HSP90 components belong to the ABA/flg22 signaling cascade, different experiments were conducted. We first tested whether

radicicol would inhibit the activation of the SnRK2s, including OST1 (SnRK2.6) that is required for ABA- and flg22-mediated stomatal closure (Mustilli et al., 2002; Yoshida et al., 2002; Melotto et al., 2006). A 2-h pretreatment of a Col-0 cell suspension culture with 10 nM radicicol followed by a 10-min treatment with 30 μM ABA did not affect total SnRK2 in-gel kinase activity, nor SnRK2.6 activity as determined by immunoprecipitation (Fig. 5A). Thus, HSP90 ATPase activity likely acts downstream or independently of ABA-mediated SnRK2 activation.

We then studied the expression of two SnRK2-dependent genes *RD29A* and *COR15A* (Fujii and Zhu, 2009) along with other drought- and ABA-responsive genes (*RD29B*, *RAB18*, *ABI1*, and *ABI2*) by quantitative RT-PCR in the plants deregulated for SGT1, HSC70, and HSP90 functions with or without ABA treatment (Fig. 5, B and C; Supplemental Fig. S3). The level of the ABA-responsive transcripts is reduced after ABA treatment in lines deregulated in *HSC70* and *HSP90* functions (Fig. 5B; Supplemental Fig. S3A). For instance, the expression of *RD29A* and *RAB18* is reduced 3-fold in *hsp90.2* mutants and HSC70-1 OE lines while the *eta3* mutation in *SGT1b* has no impact on expression levels of the studied genes. Interestingly, this effect essentially results from low basal levels of transcription in absence of exogenous ABA treatment in HSC70-1 OE lines and *hsp90.2* mutants as compared to the wild type and the *sgt1b* mutants (Fig. 5C; Supplemental Fig. S3B) and the induction in response to ABA for those genes was similar or greater in the various lines when compared to wild type (Fig. 5D; Supplemental Fig. S3C). These basal expression levels are for example 10 and 100 times lower in *hsp90.2* mutants than those measured in the wild-type plants for *RD29A* and *COR15A*, respectively. Importantly, the basal expression levels of genes that are not ABA regulated (*PCS1*, *FAD8*, *SGT1b*) is not modified in HSC70-1 OE lines and *hsp90.2* mutants relative to wild type (Supplemental Fig. S3D). These results indicate that *HSC70-1* and *HSP90.2* are needed to establish to the basal level of transcription of ABA-responsive genes and suggest a role in the long-term adaptation to drought and dehydration tolerance in vegetative tissues and/or seeds.

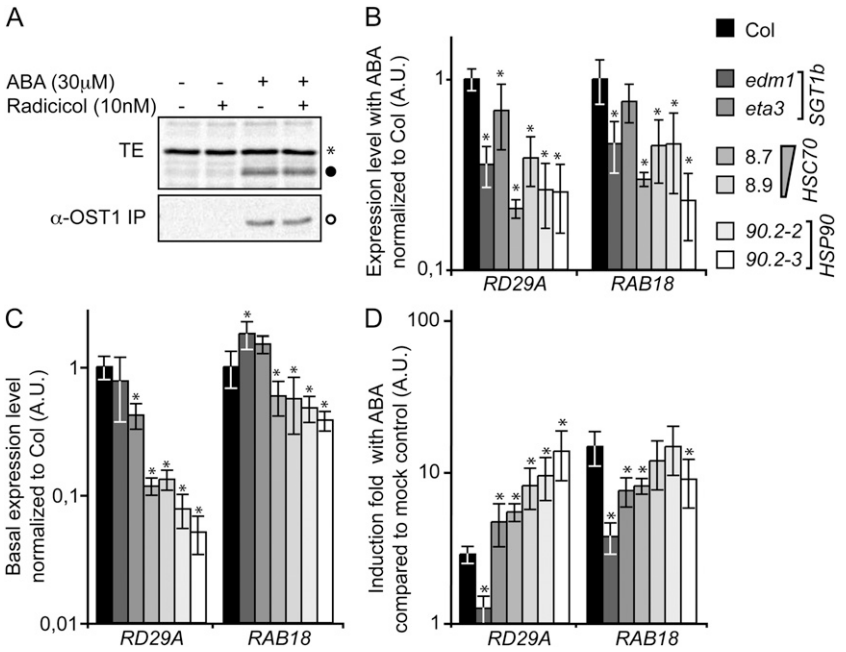

Figure 5. Transcriptional responses to ABA in *sgt1* and *hsp90.2* DN mutants and HSC70-1 OE lines and SnRK activities. A, Autoradiogram of an in-gel kinase assay performed with total extracts (top section) and immunoprecipitated OST1 (bottom section) prepared from Col-0 cell suspension cultures preincubated for 2 h with 0 or 10 nM radicicol and subsequently treated with/without 30 μM ABA for 10 min before harvest. Asterisk, black, and white circle correspond essentially to MPK6, SnRK2.2/3, and SnRK2.6 (OST1) kinase activities. B to D, cDNA was prepared from 10-d-old seedlings 3 h after a 10 μM ABA or mock treatment and its abundance measured by quantitative RT-PCR. Relative cDNA abundance of the ABA-responsive genes *RD29A* and *RAB18* was expressed relative to ABA-treated Col control (B), relative to Col mock control (C), and as induction fold relative to mock control within each genotype (D). *Actin8* (*ACT8*) and *ROC3* expression was used to normalize the transcript levels. Three independent RT-PCR experiments were performed on three independent biological samples. The bars represent mean values from three independent experiments and error bars indicate SDS. *, Significant differences compared to the wild type (Student's *t* test, $P < 0.05$).

SGT1b, HSC70-1, and HSP90 Deregulation Causes Hypersensitivity to ABA in Seed Germination Assays

To test the biological importance of HSC70/SGT1/HSP90 in another ABA-dependent physiological response, we studied the inhibition of seed germination by exogenous ABA treatment. ABA (5 μM) caused approximately 20% inhibition of germination rate for the wild type and the *sgt1a-2*, *hsc70-2.1*, and *hsc70-3.1* mutants relative to the condition without ABA (Fig. 6A). Surprisingly, all the other genotypes showed a stronger inhibition of germination rate, HSC70-1 OE and *hsp90.2* mutations causing the most pronounced hypersensitivity to ABA compared to *hsc70-1.1* and *sgt1b* mutants. Furthermore, while radicicol itself did not affect the germination of wild-type seeds, it strongly enhanced ABA action in inhibiting seed germination (Fig. 6B). These results indicate that HSP90 and SGT1b act as negative regulators in the ABA-mediated inhibition of seed germination and that HSC70 and HSP90 deregulation can affect differentially ABA-dependent responses in seeds compared to guard cells.

DISCUSSION

In this study, we provide genetic and pharmacological evidence for the multiple functions of the HSC70/HSP90 molecular chaperone machinery in the fine-tuned regulation of stomatal aperture in response to various environmental conditions and of physiological responses to the ABA hormone in Arabidopsis.

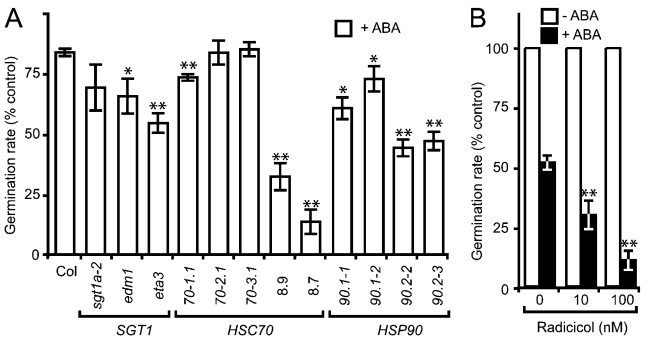

Figure 6. SGT1b, HSC70-1, and HSP90 deregulation causes hypersensitivity to ABA in seed germination assays. A, Fresh seeds harvested simultaneously from different genotypes were sown on Murashige and Skoog/10 in absence or presence of 5 μM ABA. Radicule emergence (germination) was scored after 5 d. The average germination rate is expressed as a percentage of the germination rate in absence of ABA for each genotype tested. * and **, Significant differences compared to the wild type (Student's *t* test, $P < 0.05$ and $P < 0.01$, respectively). B, Fresh wild-type Col-0 seeds were sown on Murashige and Skoog/10 in absence or presence of 3 μM ABA in combination with 0 to 100 nM radicicol and germination scored as in A. The average germination rate is expressed as a percentage of the germination rate in absence of ABA and radicicol. * and **, Significant differences compared to the no radicicol control (Student's *t* test, $P < 0.05$ and $P < 0.01$, respectively). Approximately 50 seeds per condition were scored in triplicate samples. Error bars indicate SDs. Experiments were performed at least twice.

HSC70/HSP90 Are Major Modulators of Stomatal Movement and Responses to ABA in Arabidopsis

The deregulation of cytosolic/nuclear HSC70/HSP90 functions caused very strong insensitivity to darkness, high CO_2, flg22 peptide, and ABA in guard cells that might be explained by a general incapacity in closing stomata. These observations suggest that HSC70/HSP90 could function on one event involved in stomatal closure itself after convergence of those four signaling pathways. In addition to this defect that is not ABA specific, our study and two independent reports also suggest that HSP90s could be implicated in transcriptional responses to ABA for long-term adaptation to drought tolerance: The overexpression of HSP90.2 increased Arabidopsis sensitivity to drought stress (Song et al., 2009) and, transcriptome analysis of *hsp90.2-2/hsp90.2-3* plants and HSP90 RNAi transgenics revealed that the ABA signaling pathway was among the most perturbed (Sangster et al., 2007).

One possible explanation for the late discovery of their involvement in ABA signal transduction could be that both gene families are essential and to some extent functionally redundant thus rendering their functional analysis genetically difficult in Arabidopsis (Sung and Guy, 2003; Noël et al., 2007; Hubert et al., 2009). On the one hand, single loss-of-function mutants in the different *HSC70* and *HSP90* genes did not cause any mutant phenotypes for the different physiological responses tested here and single *hsc70* or *hsp90* loss-of-function mutants displayed no or subtle mutant phenotypes (Hubert et al., 2003; Takahashi et al., 2003; Noël et al., 2007). On the other hand, the mutant screens performed on ABA insensitivity or aberrant guard cell movements did not reach saturation yet so that the identification of HSC70 OE plants or particular *hsp90* alleles was unlikely. Only very saturated screens such as those performed by Hubert et al. (2003, 2009) identified unusual mutant alleles such as *hsp90.2-2* and *hsp90.2-3* that caused very pronounced defects in immunity and physiology when compared to *hsp90.1* or *hsp90.2* loss-of-function mutants. The *hsp90.2-2* and *hsp90.2-3* alleles code for proteins that do not have ATPase activity and are unable to dimerize or interact with SGT1b in vitro but are not null alleles of *hsp90.2* (Hubert et al., 2003, 2009). The use of radicicol that inhibits ATP binding to HSP90s (Roe et al., 1999) confirmed independently that the ATPase activity of HSP90s is essential to mediate ABA signaling for the phenotypes tested here. Single loss-of-function mutants in genes encoding cytosolic/nuclear HSP90s and HSC70s had either no or only mild phenotypes in the different physiological responses investigated here. For instance, the *hsc70-3.1* or *hsp90.1* mutants showed some weakly altered responses to flg22, whole-plant water loss, or dark-induced stomatal closure (Figs. 1A,

2B, and 4C). To overcome genetic redundancy in the *HSC70* gene family and mimic what usually happens during most biotic and abiotic stresses, we globally increased the expression of all cytosolic/nuclear isoforms of the *HSP70* family by OE of the single *HSC70-1* gene (Sung and Guy, 2003). While the molecular consequences of HSC70-1 OE remain elusive and may result in more complex consequences than a single gain of HSP70 activity, this approach has proven to be fruitful to dissect HSP70 functions in vivo in various model organisms including Arabidopsis (Sung and Guy, 2003; Noël et al., 2007; Cazalé et al., 2009; Dokladny et al., 2010). The specificity of such HSP90 and HSC70 deregulation could legitimately be questioned because, DnaK interacts on average every 36 amino acids in the *Escherichia coli* proteome (Rüdiger et al., 1997) and the yeast (*Saccharomyces cerevisiae*) HSP90 interacts physically or genetically with approximately 10% of yeast genes (Zhao et al., 2005). Yet, the *hsp90.2-2* mutants have no reported developmental phenotypes (Hubert et al., 2003) while HSC70 OE lines show only a limited dwarfism (Sung and Guy, 2003). HSC70 OE plants are also fully fertile and most aspects of their physiology (auxin perception, phosphate uptake and signaling, photosynthetic efficiency) and development (flowering time, root architecture) are surprisingly normal considering that cytosolic/nuclear HSC70 are essential proteins (Sung and Guy, 2003; Noël et al., 2007; Cazalé et al., 2009). Furthermore, both chaperones contribute to very specific functions in signal transduction in vivo. For instance, in Arabidopsis, HSP90 compromised only *Resistance* (*R*) gene-specified immunity and HSC70-1 OE was shown to compromise specifically basal and *R*-gene-specified immunity but not nonhost resistance (Hubert et al., 2003; Takahashi et al., 2003; Noël et al., 2007). Similarly, we show that cytosolic/nuclear HSP90/HSC70-1 chaperones are differentially involved in the regulation of ABA-responsive gene expression compared to ABA-mediated inhibition of seed germination. These observations suggest that HSP90/HSC70-1 chaperones modulate specific and distinct signaling events important for the regulation of stomatal closure, seed germination, and transcriptional regulation of ABA-responsive genes in Arabidopsis.

Distinct Chaperone Clients Control Stomatal Closure, ABA Perception/Signaling in Seeds, and mRNA Accumulation of ABA- and Drought-Responsive Genes in Vegetative Tissues

The contrasted phenotypes conferred by the deregulation of cytosolic/nuclear HSC70/HSP90 functions are not incompatible with HSC70/HSP90 controlling the folding of a single target. For instance, the *abo3* mutant is hypersensitive to ABA at the germination and seedling stages while partially insensitive to ABA at the stomatal level (Ren et al., 2010). Since *abo3* partially phenocopies HSC70 OE lines and HSP90DN mutants, ABO3 is a candidate substrate for HSC70/HSP90. Yet, we favor the hypothesis where HSP90/HSC70-1 chaperones act on distinct tissue-specific substrates to mediate ABA-independent stomatal closure, to establish basal levels of ABA- and drought-induced gene transcripts in vegetative tissues, and to modulate ABA-dependent inhibition of seed germination. For instance, the general inability of stomata to close in response to multiple stimuli (high CO_2, darkness, flg22, ABA) suggests that general processes involved in guard cell movements themselves are affected rather than specific signal perception of any of these stimuli. Our results also suggest the existence of a second chaperone substrate in vegetative tissues responsible for the maintenance of basal expression levels of ABA- and drought-responsive genes (Fig. 5). This substrate does not control the ABA responsiveness of those genes but HSC70/HSP90 deregulation would still result in an apparently weakened transcriptional response to ABA and drought conditions since sufficient transcripts levels would not be reached to mount wild-type physiological responses to ABA. In seedlings, HSP90 inhibition did not affect germination rate (Fig. 6B) but strongly sensitized seeds to exogenous ABA treatment. It remains unclear whether HSC70 and HSP90 effect is direct or indirect, but, if in the ABA signaling pathway, this effect would be situated downstream of SnRK2s, since ABA-dependent activation of SnRK2s (including OST1) was not compromised by inhibition of HSP90 activity (Fig. 5A).

The differential requirement of SGT1b for stomatal responses and germination tests also suggests that distinct substrates interacting with distinct chaperone/cochaperone complexes are involved to mediate these responses in the different tissues. Alternatively, the requirements for SGT1b in stomatal responses could be below a threshold where *SGT1a* might be able to compensate for the loss of *SGT1b* as observed for plant immunity (Azevedo et al., 2006) or an SGT1-independent HSC70/HSP90 complex using other cochaperone scaffolds might be involved. We thus tested the involvement of RAR1, an HSP90 cochaperone and SGT1 interactor in ABA-mediated inhibition of seed germination and control of leaf water losses. The *rar1-21* null mutant was not affected in its water loss when analyzed by infrared thermal imaging, rosette dehydration experiments, or stomatal conductance in the dark nor in germination assays in presence of ABA (data not shown). In addition, *HSP90.2-2* retains ability to interact with RAR1 in yeast two hybrid while *HSP90.2-3* loses it. This suggests that RAR1 might not be directly involved in ABA signaling but a yet-to-be-identified cochaperone of HSC70/HSP90.

The HSC70/SGT1/HSP90 Complexes Link Stomatal Closure, ABA Signaling, and Immunity

The identification of ABA mutants by forward and reverse genetic screens with compromised innate immunity have recently highlighted the very contrasted and important effects of ABA on plant immune systems

(de Torres-Zabala et al., 2007; Fan et al., 2009) though the precise molecular mechanisms involved await elucidation (for review, see Asselbergh et al., 2008). This hormone can either act as a positive or negative modulator of plant innate immunity depending on the pathosystem (e.g. Fan et al., 2009). As such, the *sgt1b* and *hsp90.2* mutants that were first isolated in genetic screens for loss of innate immunity are a perfect example of the potential overlap between ABA signaling and immunity. The compromised immunity of those mutants was initially mainly explained in the light of SGT1 and HSP90 functions in the stabilization/activation of several R proteins (Shirasu and Schulze-Lefert, 2003) as observed by specific RPM1 destabilization in *hsp90.2-2* and *hsp90.2-3* mutants (Hubert et al., 2003). While these molecular observations hold true, the careful analysis of these phenotypes in the light of their new stomatal and ABA-dependent functions may help to evidence the precise contribution of these players in the different layers of innate immunity (Lipka et al., 2005). For instance, the stomata are often the first barrier that plant pathogens have to cross to gain access to the leaf intercellular spaces. Thus, several pathogens have evolved important virulence factors to bypass this first layer of innate immunity and prevent stomatal closure upon infection. *Pseudomonas* and *Xanthomonas* both inhibit PAMP-triggered stomatal closure by producing coronatine and the *rpf*/diffusible signal factor, respectively, thus facilitating bacterial penetration inside the leaf tissue (Melotto et al., 2006; Gudesblat et al., 2009). Not so surprisingly, the ABA-insensitive *ost1-2* or PAMP-insensitive plants *eds16-2* and *nahG* that stomata do not close normally showed enhanced susceptibility to a *Pseudomonas* coronatine-deficient mutant after dip inoculation but not infiltration (Melotto et al., 2006). In parallel, *Pseudomonas* virulence factors directly injected inside the plant cells such as AvrRpm1, AvrB, and AvrRpt2 target RIN4, an interactor of the proton pump AHA1 that is directly responsible for stomatal closure (Liu et al., 2009). This further highlights that prevention of stomatal closure upon infection is a key issue for bacterial pathogens (Melotto et al., 2008). Interestingly, *sgt1b* mutants were recently shown to be less sensitive to virulent *Pseudomonas* when spray inoculated and to coronatine that inhibits ABA-dependent stomatal closure (Uppalapati et al., 2011). Still, *sgt1b* mutations did not affect PAMP/flg22-triggered resistance (Zipfel et al., 2004), stomatal responses to ABA, high CO_2, and darkness nor basal immunity when *Pseudomonas* were hand infiltrated into the leaf tissue (Holt et al., 2005). These observations suggest that *SGT1b* is involved in coronatine/jasmonic acid signaling rather than in general pathways leading to stomatal closure. As inferred from their insensitivity to ABA and flg22 treatments and their opened stomata, *HSC70-1* OE lines should also have a compromised basal immunity. Such loss of basal immunity was observed when virulent *Pseudomonas* were syringe infiltrated into leaves thus bypassing the stomatal barrier (Noël et al., 2007). The breakdown of basal resistance against nonpathogenic *Pseudomonas* in the *hsc70-1* mutant is more pronounced when bacteria are spray inoculated than infiltrated (Jelenska et al., 2010). This indicates that HSC70s serve a role in basal immunity before and after the stomatal barrier is crossed. These functions in innate immunity are further confirmed by the identification of the DnaJ domain virulence protein HopI1 used by *Pseudomonas* to modify cytosolic HSC70 ATPase activity and subcellular localization (Jelenska et al., 2007, 2010). For *HSP90s*, no defect in basal immunity was observed in *hsp90.2-2/3* mutants when spray inoculated (Hubert et al., 2003) though on epidermal peels their stomata stay open in all conditions studied here including flg22 treatment. These surprising observations indicate that, besides the regulation of R-protein stabilization/activation in the incompatible interactions (Hubert et al., 2003, 2009), HSP90s might also exert an antagonist effect on another layer of the innate immune system. Interestingly, enhanced susceptibility to *Pseudomonas* is achieved by effector-mediated induction of ABA production and ABA-dependent suppression of SA-dependent defenses in Arabidopsis to which HSP90 could contribute (de Torres-Zabala et al., 2007, 2009).

Yet, the key question remains: What are the HSC70 and HSP90 clients? HSP90 and HSC70-1 deregulation has unraveled the physiological importance of some of their clients for stomatal closure, ABA signaling, and plant innate immunity that identification may have been masked by genetic redundancy in the past. Interestingly, HSP90s and HSP70s were shown to serve as a buffer for genetic variation in *Drosophila* and/or Arabidopsis (Feder et al., 1992; Rutherford and Lindquist, 1998; Queitsch et al., 2002). Thus, exploiting the diversity for the sensitivity to HSP90 inhibitors in stomata and during germination in presence of ABA in the large collection of Arabidopsis ecotypes could serve as a mean to identify genetically the first HSP90 clients in Arabidopsis and novel players in ABA signaling in seeds, stomatal closure, or plant innate immunity.

MATERIALS AND METHODS

Plant Material and Growth Conditions

All Arabidopsis (*Arabidopsis thaliana*) genotypes used in this study are from the Col-0 ecotype but *sgt1b*^{edm1} (Tör et al., 2002) that is in the Col-5 ecotype: *ost2-2D* (Merlot et al., 2007), *sgt1b*^{eta3} (Gray et al., 2003); *sgt1a-2* (Cazalé et al., 2009); *hsc70-1.1*, *hsc70-2.1*, and *hsc70-3.1* (Noël et al., 2007), *rar1-21* (Tornero et al., 2002); *hsp90.1-1* and *hsp90.1-2* (Takahashi et al., 2003), and *hsp90.2-2* and *hsp90.2-3* (Hubert et al., 2003). Two independent homozygous Col-0 plants overexpressing *HSC70-1* (lines 8.7 and 8.9) were studied (Sung and Guy, 2003). Plants were grown in soil in a walk-in chamber under short-day conditions (8 h light/16 h darkness) at 21°C/18°C (light/dark) and a light intensity of 200 μmol photons m^{-2} s^{-1}. Seedlings were also grown in sterile conditions on vertically oriented Murashige and Skoog/10 medium containing 0.5% Suc in a white-light growth chamber under a 16 h photoperiod at 24°C/21°C (light/dark).

Quantitative RT-PCR

Ten-day-old in vitro-grown seedlings were sprayed with 10 μM ABA or water and harvested 3 h later. RNA extraction was performed using RNeasy mini kit according to manufacturer's instructions (Qiagen). The RNA was then

subjected to treatment with TURBO DNase (Ambion, Applied Biosystems) and confirmed by PCR to be free of detectable amounts of DNA. cDNA synthesis was done using the SuperScript VILO cDNA synthesis (Invitrogen). Quantitative RT-PCR was performed in 384-well plates using the Light Cycler 480 SYBR green I master and the LightCycler 480 real-time PCR system (Roche Diagnostics). The specificity of each primer pair was tested on a standard curve based on serial dilutions of the wild-type control cDNA and subsequently by melting curve analysis. The accumulation of each transcript was measured in three independent biological samples with three technical replicates. *Actin8* and *ROC3* expression was used to normalize the transcript levels for each sample. Primer sequences for each real-time reaction are listed in Supplemental Table S1. The bars represent mean values from three independent experiments. Statistically significant differences for values were determined by Student's *t* test analyses.

Kinetics of Water Loss from Excised Rosettes

Hypocotyl of 4-week-old plants was cut and sealed with silicon grease. Water loss was evaluated by weighting rosette each 30 min during the first hour and each hour for the next 4 h. Four plants per lines were used per experiment. Rate of fresh weight loss was calculated over 3 h.

Infrared Thermal Imaging

Thermal imaging was performed using an infrared camera (FLIR, B20HS). The rosettes were imaged at room temperature under low relative humidity on 4- to 5-week-old plants kept in darkness for more than 14 h. The image analysis software provided with the camera (FLIR Researcher) was used to determine the leaf surface temperature from at least 10 positions per rosette for three different plants.

Leaf Conductance Measurement

Stomatal conductance was measured on attached leaves of 6-week-old plants using a LI 6400 portable photosynthesis system with the leaf chamber fluorometer (LI6400-40). The leaf temperature and the relative humidity were 22°C and 70%, respectively. Ambient CO_2 was 400 μmol mol^{-1} unless stated otherwise. Illumination was set to 90% red, 10% blue, and 500 μmol photon m^{-2} s^{-1} irradiance. Three independent experiments were performed on at least three different plants.

Measurements of Stomatal Aperture and Density

Measurements were performed on epidermal peels from mature leaves of 3- to 4-week-old plants essentially as described (Leonhardt et al., 1997). Peels were placed in a solution (30 mM KCl, 10 mM MES, pH 5.6 at 22°C in light/darkness, 0–100 μM ABA, 0–5 μM flg22 peptide, and 0–100 nM radicicol). Stomatal apertures were measured with an optical microscope (Nikon) fitted with a camera lucida and a digitizing table (Houston instrument TG 1017) linked to a personal computer (Bull Micral 30). For each treatment, three peels were analyzed per condition and at least 50 stomatal apertures were measured at a magnification of 1,000. Experiments were performed at least twice. To measure stomatal density, three epidermal peels from three different plants of each genotype were briefly stained in ruthenium red, imaged using a bright-field microscope at a magnification of 400 fitted with a digital camera, and counted manually on the printed image. Experiments were performed at least three times.

Germination Assays

Fresh seeds harvested simultaneously were sown on Murashige and Skoog/10 with 0 to 5 μM ABA and 0 to 100 nM radicicol and stratified 2 d at 4°C. Radicule emergence was observed under the binocular after 5 d. Approximately 50 seeds per conditions were scored in triplicate samples. Experiments were performed at least twice.

Kinase Assays

Col-0 cell suspensions cultured as described (Droillard et al., 2002) were preincubated for 2 h with 0 or 10 nM radicicol and subsequently treated with/without 30 μM ABA for 10 min before harvest. Protein extracts were prepared and used for in-gel kinase assays as described (Boudsocq et al., 2007). Immunoprecipitation of endogenous SnRK2.6 before in-gel kinase assay was performed as described (Vlad et al., 2009).

Supplemental Data

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

Supplemental Figure S1. Impact of SGT1b, HSC70-1, and HSP90 deregulation on average leaf surface temperature measured by thermal imaging.

Supplemental Figure S2. Effect of SGT1, HSC70, and HSP90 on leaf stomatal density and aperture under light.

Supplemental Figure S3. Transcriptional responses to ABA in *sgt1* and *hsp90.2* DN mutants and HSC70-1 OE lines.

Supplemental Table S1. Oligonucleotide primers used for real-time RT-PCR analysis.

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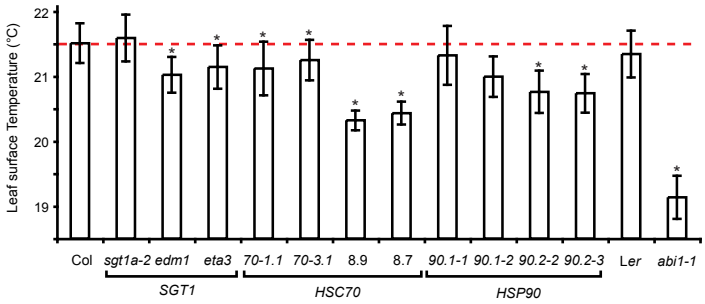
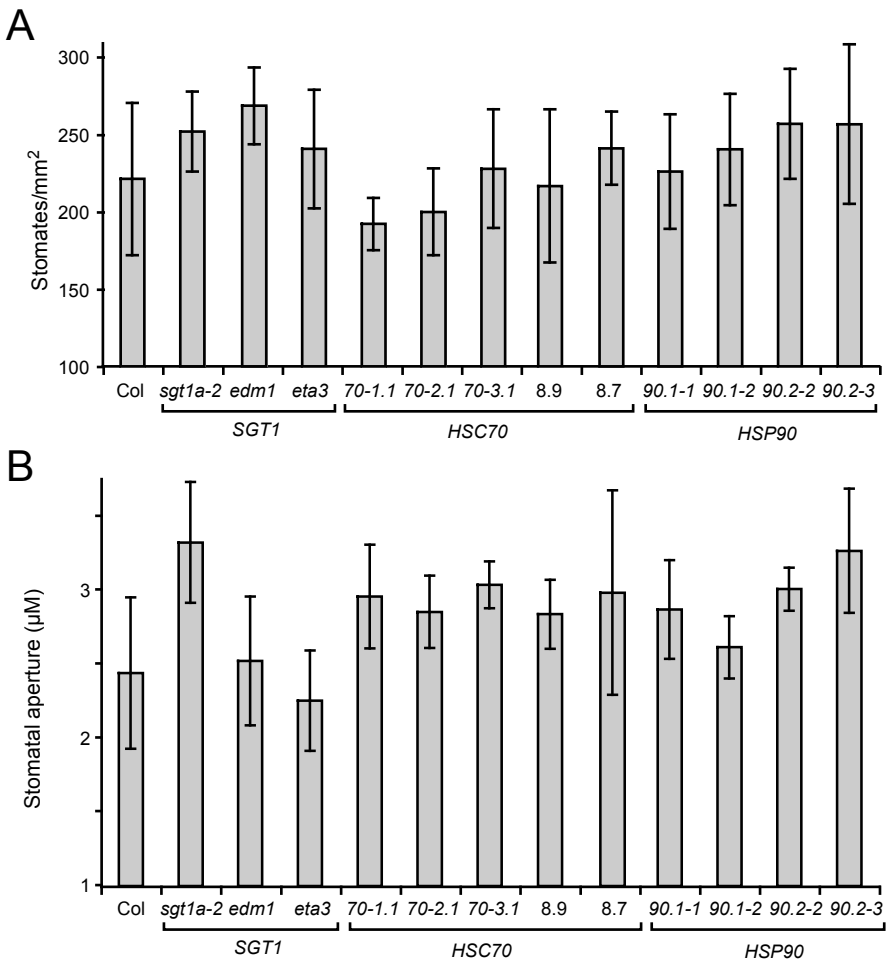
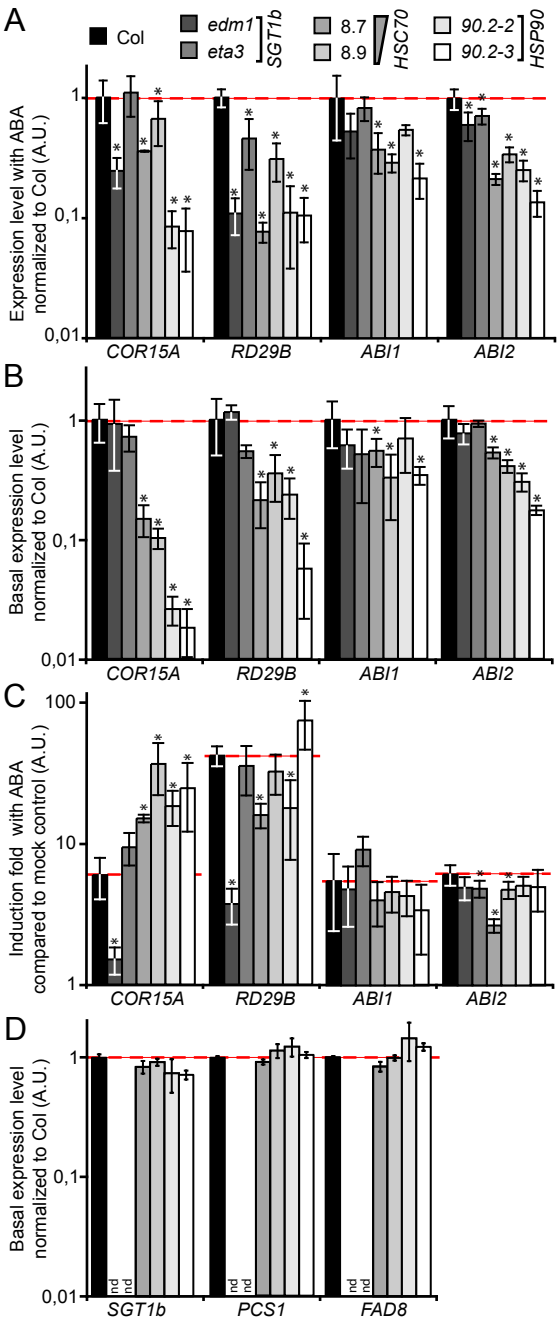


Supplemental Figure S1: Impact of SGT1b, HSC70-1 and HSP90 deregulation on average leaf surface temperature measured by thermal imaging. Values represent the average temperature at 10 locations on different 3 plants. Error bars indicate standard deviations. Asterisks represent values statistically different from wild-type control (p<0,001, student's *t*-test).



Supplemental Figure S2: Effect of SGT1, HSC70 and HSP90 on leaf stomatal density and aperture in the light. The same epidermal peels were used to generate Fig. 2B and Supplemental Fig. S1. (A) Average stomatal density was determined on at least three zones of each epidermal peel used in Fig. 2B and on at least 3 different plants per genotype. (B) Average stomatal aperture (µm) was measured on epidermal peels incubated for 2 h in darkness and 2 h under light conditions. These data used for Fig. 2A permitted to express stomatal aperture as a percentage of stomatal aperture under light. Error bars indicate standard deviations. No statistically significant differences for values compared with wild type could be observed as determined by student's *t*-test ($p<0.05$).



Supplemental Figure S3: Transcriptional responses to ABA in *sgt1* and *hsp90.2* DN mutants and HSC70-1 OE lines. cDNA was prepared from 10-day-old seedlings 3 h after a 10 µM ABA or mock treatment and its abundance measured by quantitative RT-PCR. Relative cDNA abundance of the ABA-responsive genes *COR15A*, *RD29B*, *ABI1* and *ABI2* was expressed relative to ABA-treated Col control (A), relative to Col mock control (B) and as induction fold relative to mock control within each genotype (C). (D) Relative cDNA abundance of *SGT1b, PCS1* and *FAD8* was expressed relative to Col mock control. *Actin8* and *ROC3* expression was used to normalize the transcript levels. Three independent RT-PCR experiments were performed on three independent biological samples. The bars represent mean values from three independent experiments and error bars indicate standard deviations. *, statistically significant differences for values as determined by student's *t*-test ($p<0.05$). nd, not determined.

Table S1: Oligonucleotide primers used for real-time RT-PCR analysis

Gene Name	AGI code	Primer 1	Primer 2
ACT8	At1g49240	5'-CGCTCTCGCTCTTACCTGAG-3'	5'- TCCGAGTTTGAAGAGGCTACA-3'
ROC3	At2g16600	5'-TCGGTGAAAGCTTGATCCTT-3'	5'-ATCGTGATGGAGCTTTACGC-3'
SGT1a	At4g23570	5'-AAGAGTATCGGACTGCTAAA-3'	5'-GCTGTAGGGGTGACAT-3'
SGT1b	At4g11260	5'-GTACCTAAGGAGAACGTAACT-3'	5'-AGGCCCAGGTGATTAT-3'
HSC70-1	At5g02500	5'-TCTATTGAGCAGGCGAT-3'	5'-TCTATTGAGCAGGCGAT-3'
HSC70-2	At5g02490	5'-CACCCGTGCTAGATTCG-3'	5'-GGATCGCAGCCTGAAC-3'
HSC70-3	At3g09440	5'-CGTCAGGTCACTAAGGAT-3'	5'-CTCGGGTTTCCACTAATGT-3'
HSC70-4	At3g12580	5'-TGGGCCTGATATGGGT-3'	5'-AGGTCAGAGCGAGTTT-3'
HSC70-5	At1g16030	5'-TGAGACAGCAGGRGGG-3'	5'-CACCAGCGGTTTTATCC-3'
HSP90.1	At5g52640	5'-CAGCTTTGTTGACGTCTGGA-3'	5'-TCGACTTCCTCCATCTTGCT-3'
HSP90.2	At5g56030	5'-TGATGAGCCAAACACTTTCG-3'	5'-TCGACTTCCTCCATCTTGCT-3'
HSP90.3	At5g56010	5'-GCTGATGCAGACAAGAACGA-3'	5'-CTTCAAGTGGAGGCATGTCA-3'
HSP90.4	At5g56000	5'-CCTTGATGAGCCCAACACTT-3'	5'-TCGACTTCCTCCATCTTGCT-3'
HSP90.5	At2g04030	5'-CGCTGCTTGTAAGAATGCAC-3'	5'-CCTCTGTTTCTCCGCTTTTG-3'
ABI1	At4g26080	5'-GAGGCAGAGAGGGTCCTTTT-3'	5'-AGCCCGGGAAGAAAAATACA-3'
ABI2	At5g57050	5'-AAGATCCTGCAGCAATGTCC-3'	5'-TCCCTTCAAATCAACCACTACC-3'
COR15A	At2g42540	5'-GAGGGTAAAGCAGGAGAGG-3'	5'-ATGAAGAGAGAGGATATGGATCA-3'
RD29A	At5g52310	5'- CTTGGCTCCACTGTTGTTCC -3'	5'-CATCAAAGACGTCAAACAAAACA-3'
RD29B	At5g52300	5'-CACCACCGTTGGGACTATG-3'	5'-GCAAAACCCCAAATCTTCAG-3'
RAB18	At5g66400	5'-GCCGTTAAGCTTCGAACAAT-3'	5'-AAACAACACACATCGCAGGA-3'
FAD8	At5g05580	5'-CTTTGTCATGGGTCCAATCC-3'	5'-TGAGCCCTCCTCTCAGGTAA-3'
PCS1	At5g44070	5′-TCGACGCAAACTGAGTG-3′	5′-AGCAGCGAGATCATCC-3′