Cytosolic HSP90 Regulates the Heat Shock Response That Is Responsible for Heat Acclimation in Arabidopsis thaliana

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Plant survival requires the ability to acclimate to heat. When plants are subjected to heat shock, the expression of various genes is induced, and the plants become tolerant of higher temperatures. We found that transient treatment with geldanamycin and radicicol, two heat shock protein 90 (HSP90) inhibitors, induced heat-inducible genes and heat acclimation in Arabidopsis thaliana seedlings. Heat shock reduced the activity of exogenously expressed glucocorticoid receptor (GR). Since GR activity depends on HSP90, this suggests that heat shock reduces cytosolic HSP90 activity in vivo. Microarray analysis revealed that many of the genes that are up-regulated by both heat shock and HSP90 inhibitors are involved in protein folding and degradation, suggesting that the activation of a protein maintenance system is a crucial part of this response. Most of these genes have heat shock response element-like motifs in their promoters, which suggests that heat shock transcription factor (HSF) is involved in the response to HSP90 inhibition. Several HSF genes are expressed constitutively in A. thaliana, including AtHsfA1d. Recombinant AtHsfA1d protein recognizes the heat shock response element motif and interacts with A. thaliana cytosolic HSP90, HSP90.2. Overexpression of a dominant negative form of HSP90.2 induced the heat-inducible gene. Thus, it appears that in the absence of heat shock, cytosolic HSP90 negatively regulates heat-inducible genes by actively suppressing HSF function. Upon heat shock, cytosolic HSP90 is transiently inactivated, which may lead to HSF activation.

Since plants cannot move or escape to cool places, they have evolved a range of mechanisms that protect them upon exposure to high temperatures. These mechanisms serve to protect the plant from heat-induced tissue damage. One such mechanism involves the accumulation of various types of heat shock proteins (HSPs), including HSP70, HSP90, HSP100/Clp, and small HSP. HSPs play a crucial role in the cell, since they serve as molecular chaperones that not only protect cellular proteins from damage but also refold damaged proteins. It has been shown that prior heat shock elevates the cellular levels of HSPs and that this leads to the acquisition of tolerance to high temperatures that are normally lethal (2–4). This so-called heat acclimation phenomenon enables plants to live without wilting in extremely hot environments. Our understanding of the molecular components responsible for heat acclimation remains limited. A deeper knowledge of this process would be useful, since it could lead to the development of agricultural crop plants that are continuously heat-acclimated and therefore resistant to sudden heat-induced damage.

It has been shown that HSF is responsible for the heat-induced activation of HSP genes in various organisms, including plants (5–8). HSF binds to heat shock response element (HSE), which is a specific DNA sequence (nGAnA) observed in HSP gene promoters. The typical HSF-binding site is composed of three HSE repeats (HSE3) that are oriented head-to-head and then tail-to-tail (5, 6). To bind optimally to HSE3, HSF must form a trimer (9). Although animals and yeast have relatively few HSF genes, plants have many HSF homologues that have been classified into three subfamilies, A, B, and C (7, 8). For example, humans have four homologues, whereas Saccharomyces cerevisiae and Drosophila melanogaster have just one copy each, although Arabidopsis thaliana has 21 homologues. The plant HSF homologues all consist of a highly conserved NH2-terminal DNA-binding domain, a hydrophobic heptad repeat (HR-A/B) region, and a transcriptional activation domain (7). The HR-A/B region is connected to the DNA-binding domain and is responsible for HSF oligomerization, probably due to coiled-coil interactions. The COOH-terminal half of the HSF homologues is variable and includes the activation domain, which recruits transcriptional machineries (7, 8).

Cells constitutively have a pool of HSF proteins, but these proteins do not induce the HSP genes in the absence of heat shock. This indicates that HSF activity is normally negatively regulated. HSF down-regulatory mechanisms have been identified in mammals and yeast, although the mechanisms differ slightly between these two kingdoms. In mammalian cells, HSF, heat shock response element; THX, thioredoxin; GR, glucocorticoid receptor; MES, 4-morpholineethanesulfonic acid; RT, reverse transcription; PBS, phosphate-buffered saline; YFP, yellow fluorescent protein; ER, endoplasmic reticulum.
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HSF1 is maintained in the cytosolic HSP90 complex as a monomer that lacks DNA binding activity (5). Upon heat shock, the complex dissociates and releases the HSF1 monomer, because denatured proteins sequester HSP90. The monomer then forms a trimer capable of DNA binding activity (10). In yeast cells, HSF1 is already in its DNA-binding trimeric form in the absence of heat shock but must become hyperphosphorylated by heat shock before it can activate transcription (11). However, HSP90 also negatively regulates HSF1 activity in yeast (12). With regard to the HSF down-regulatory mechanism in plants, it has been suggested that HSF is regulated by phosphorylation by a cyclin-dependent kinase CDC2 (13) or by binding to HSP70 (14), small HSP (15), or EMP2 (16). However, in vivo evidence for these mechanisms in plants remains limited.

Although HSPs are responsible for refolding denatured proteins and/or for folding newly synthesized proteins, one of these HSPs, HSP90, also plays an additional role in the regulation of various cellular signaling molecules (17, 18). For example, HSP90 regulates glucocorticoid receptor (GR) activity, and inhibition of HSP90 reduces glucocorticoid perception (17–20). The primary structure of HSP90 is well conserved in various species. In A. thaliana, there are seven HSP90 homologues that are localized to the cytosol (HSP90.1 (At5g52640), HSP90.2 (At5g56030), HSP90.3 (At5g56010), and HSP90.4 (At5g56000)), mitochondria (At3g07770), chloroplast (CR88 (At2g04030)), and endoplasmic reticulum (SHD/GRP94 (At4g24190)). Among these, four cytosolic HSP90 proteins are highly homologous to each other (86–99% identity). Cytosolic HSP90 is known to be responsible for disease resistance by interacting with the resistance (R) protein, a pathogen receptor; this interaction is crucial for R protein activity in A. thaliana, Nicotiana tabacum, and Nicotiana benthamiana (21–24). Plant cytosolic HSP90 is also involved in chloroplast protein import, since cytosolic HSP90 delivers precursor proteins to the chloroplast import receptor Toc64 (25). However, it is likely that cytosolic HSP90 also plays other roles in intracellular events in plants, since depletion of HSP90 activity induces a wide variety of morphological changes (21, 26–28). Therefore, we investigated the effect of two HSP90 inhibitors, geldanamycin (GDA) and radicicol (RAD), on A. thaliana to find another function for cytosolic HSP90.

**EXPERIMENTAL PROCEDURES**

**Plant Materials**—We used wild type A. thaliana (Columbia accession) and two transgenic A. thaliana lines, one bearing ProHSP90.1::GUS and the other bearing a dexamethasone-inducible luciferase gene (Pro3SS:GVG and ProGAL4::Luciferase). Both transgenic lines have been described in detail previously (29, 30). We generated a transgenic A. thaliana line harboring the dexamethasone-inducible dominant negative HSP90.2 gene (Pro3SS:GVG and ProGAL4::HSP900.2-D80N). HSP90.2 cDNA was amplified from an expressed sequence tag clone (RZL18h06) obtained from Kazusa DNA Research Institute. The amplified cDNA fragment was introduced into the Gateway entry vector, pDONR221 (Invitrogen), according to the manufacturer’s instructions. Dominant negative mutations were introduced into the HSP90.2 gene by using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and desired primer sets (see supplemental Table S1). We generated a Gateway binary vector, pTAGW72, from pTA7002 (a gift from N. H. Chua) by using the Gateway Conversion System (Invitrogen). pTA7002 was digested with Xhol, blunted with LA Taq (Takara, Shiga, Japan), and Reading Frame Cassette B was inserted. Binary vectors of mutated HSP90.2 genes were introduced into Agrobacterium tumefaciens (EHA105), which was then used to infect A. thaliana (Columbia accession) to make the transgenic line, HSP90.2-D80N.

**Growth Conditions and Heat and Chemical Treatments**—Each well of 96-well microtiter plates received 5–10 surface-sterilized seeds in 150 μl of 0.5× MS liquid medium (0.5× Murashige and Skoog salts, 1.3 mM MES-KOH, pH 5.7). After stratification (4 days at 4 °C), the plates were transferred to an orbital shaker with a 16-h light cycle at 22 °C. After 5 days of growth, the medium was replaced with appropriate treatment media. For heat shock treatment, the seedlings were incubated at 37 °C for 2 h. For HSP90 inhibitor treatment, the seedlings were incubated in medium containing 50 μM GDA (Sigma) or 50 μM RAD (Sigma). For cycloheximide treatment, the seedlings were incubated in medium containing 50 μM cycloheximide (Sigma) for 30 min, followed by chemical or heat treatment under the same conditions. To observe high temperature tolerance, the seedlings were incubated at 22 °C for 2 h after each treatment and then incubated at 45 °C for 1 h (2, 3). For dexamethasone treatment, the seedlings were first subjected to heat shock or HSP90 inhibitors for 30 min before adding 30 μM dexamethasone (Nacalai Tesque, Kyoto, Japan) for 1.5 h. Stock solutions of GDA (1 mg/ml), RAD (1 mg/ml), and dexamethasone (30 μM) were prepared by dissolving the drugs in dimethyl sulfoxide. For β-glucuronidase (GUS) staining, the seedlings were incubated in GUS staining buffer (50 mM potassium phosphate, pH 7.0, 0.5 mM K4Fe(CN)6, 0.5 mM K3Fe(CN)6, 1 mM β-glucuronidase) for 30 min. To stop the reaction and remove chlorophyll, the plants were treated with an ethanol series.

**Reverse Transcription (RT)-Polymerase Chain Reaction**—Total RNA, extracted from 10 seedlings by using Isogen (Nippon Gene, Toyama, Japan), was dissolved in 25 μl of distilled water. cDNA was synthesized from 5 μl of total RNA solution with Ready-to-Go RT-PCR beads (GE Healthcare). Gene fragments were then amplified by PCR using gene-specific primer sets (see supplemental Table S1). For the primers of HSP90 and HSP70, the conserved sequences of all four A. thaliana cytosolic HSP90 or all six HSP70 are used. Quantitative RT-PCR was performed with a SmartCycler (Cepheid, San Diego, CA) and a commercial kit (Ex Taq R-PCR; Takara), as reported previously (31, 32).

**Microarray Analysis**—Microarray analysis was performed as recommended by the manufacturer’s instructions (Agilent Technologies, Palo Alto, CA). Total RNA was used to produce Cy3-labeled cRNA probes. The labeled probes were hybridized to an Agilent Arabidopsis 3 Oligo Microarray (Agilent Technologies). Feature extraction software (GeneSpring; Agilent Technologies) was used to locate and delineate every spot in the array and to integrate the intensity, filtering, and normalization of each spot. Because HSP90 inhibitors were dissolved in di-
methyl sulfoxide, we removed genes whose expression is affected by dimethyl sulfoxide from the data sets. Finally, we obtained the expression features of 12,656 genes.

Expression of Recombinant AtHsfA1d in Bacteria—AtHsfA1d cDNA was amplified by RT-PCR using gene-specific primer sets (see supplemental Table S1) and introduced into the Gateway entry vector pCR8/GW/Topo (Invitrogen). The AtHsfA1d coding region in pCR8/GW/Topo was transferred to pET32a-GW. pET32a-GW was derived from pET32a (Novagen, Madison, WI), which had been modified into a Gateway vector by using the Gateway Vector Conversion System (Invitrogen). The recombinant pET32a-GW vector was then introduced into Escherichia coli (BL21) to produce His-thioredoxin-tagged fusion protein. The recombinant protein (designated as THX-AtHsfA1d) was extracted by 50 mm sodium phosphate, pH 7.2, 0.3 mM NaCl, and 50 mM imidazole and purified with a nickel column. The solvent was replaced with PBS (10 mM sodium phosphate, pH 7.2, and 130 mM NaCl) by dialysis.

Electron Mobility Shift Assay—A mixture of 4.4 μl of THX-AtHsfA1d (10 μg), 1 μl of biotinylated probe (Bio-HSE3; 5 pmol), and 1 μl of poly(dI-dC) (1 pg) (Sigma) was incubated for 30 min at room temperature and subjected to PAGE (5% (w/w) acrylamide) in 1× TBE. For the competition assay, excess amounts of competitor (25 pmol in 1 μl) were added to the mixture. The probe was electrophoretically transferred to a membrane (Hybond-N+; GE Healthcare) and detected by avidin-horseradish peroxidase coupled with the ECL system (GE Healthcare). The oligonucleotide sequences used (see supplemental Table S1) were obtained from a previous paper (33).

Antibody Production—A partial cDNA fragment of HSP90.2, which encodes the amino acid region 471–574, was amplified by RT-PCR using gene-specific primer sets (see supplemental Table S1) and ligated into the pET32a vector (Novagen) to produce His-thioredoxin-tagged fusion protein in E. coli (BL21). The recombinant proteins in inclusion bodies were dissolved in 100 mM sodium phosphate, pH 7.2, and 8 M urea and purified by nickel column chromatography. Purified proteins were injected into rabbits to raise antibodies. Because the anti-HSP90 anti-serum recognized the His-thioredoxin tag, we passed the anti-serum through a His-thioredoxin column several times until the flow-through fraction no longer recognized His-thioredoxin on immunoblots.

Pull-down Assay—Total proteins were extracted from 10 5-day-old A. thaliana seedlings with 20 μl of PBS containing 1 mM phenylmethylsulfonyl fluoride and 2 mM N-ethylmaleimide, followed by removal of cell debris by centrifugation. THX-AtHsfA1d (10 μg, about 0.1 nmol) or THX (2.3 μg, about 0.1 nmol) was then added to the extract and incubated for 1 h at room temperature. After the addition of 15 μl of nickel-nitrilotriacetic acid-agarose beads (Qiagen, Valencia, CA) to the extracts, the beads were recovered by centrifugation and washed several times with PBS. Bound proteins were eluted in 15 μl of PBS containing 500 mM imidazole. Then proteins were separated by SDS-PAGE, transferred to a nylon membrane, and subjected to immunoblot analysis with anti-HSP90 (1:2000 dilution) or stained with Coomassie Brilliant Blue R-250.

Bimolecular Fluorescence Complementation Analysis—cDNAs of A. thaliana HSFs were amplified by RT-PCR using gene-specific primer sets (see supplemental Table S1) and introduced into the Gateway entry vector pCR8/GW/Topo (Invitrogen). These HSF coding regions in pCR/GW/Topo were transferred to the Gateway expression vector, nYFP/pUGW0 (a gift from T. Nakagawa). The construct encodes a fusion protein comprising the amino-terminal half of YFP fused to the amino-terminal side of AtHsfA1d (nYFP-AtHsfA1d). The HSP90.2 coding region in pDONR221 was transferred to the Gateway expression vector, cYFP/pUGW0 (a gift from T. Nakagawa). The construct encodes a fusion protein comprising the carboxyl-terminal half of YFP fused to the amino-terminal side of HSP90.2 (cYFP-HSP90.2). We used the tdTomato gene (a gift from R. Y. Tsien) in the expression vector, ptdGW (a gift from S. Mano) as a marker for positively transformed cells. These plasmid DNAs were bombarded to onion epidermis cells by using the Biolistic Particle Delivery System (Bio-Rad). The onion cells were laid on a 1% (w/v) agar plate containing 1× Murashige and Skoog salts, 1.3 mM MES-KOH, pH 5.7, and incubated at 22 °C in the dark. GDA and RAD were diluted to 50 μM in 0.5× MS liquid medium and applied to the plate. After 1 day, fluorescence was observed under a fluorescent microscope (Axiolmager Z1, Carl Zeiss, Jena, Germany) equipped with a CCD camera (AxioCam HRC; Carl Zeiss).

RESULTS

Geldanamycin and Radicicol Induce Heat-inducible Genes in the Absence of Heat Shock—We used two chemicals, GDA and RAD, to investigate the role of HSP90 in the heat shock response of A. thaliana. Although GDA and RAD are structurally unrelated, both inhibit HSP90 function by binding to multiple residues in its highly unusual, evolutionarily conserved nucleotide-binding pocket (34). To test the effect of GDA and RAD treatments on the expression of known heat-inducible genes, we first employed a transgenic A. thaliana line that bears the GUS gene fused to the HSP90.1 heat response promoter (ProHSP90.1:GUS) (29). When the transgenic seedlings were treated for 6 h with either chemical at germinating temperature (22 °C), both chemicals induced significantly higher GUS staining compared with the untreated plants (Fig. 1, A and B). As expected, heat shock (37 °C for 2 h) also induced higher GUS staining. Thus, GDA and RAD activate the heat-responsive HSP90.1 promoter as well as heat shock. Since the chemicals still activated the HSP90.1 promoter at concentrations as low as 6.25 μM (Fig. 1B), it appears that the promoter is highly sensitive to the chemicals.

To investigate the response of endogenous heat-inducible genes to GDA and RAD treatments, we treated wild-type seedlings with 50 μM of GDA or RAD at 22 °C for 6 h and then subjected their mRNAs to RT-PCR (Fig. 1C). The transcription of cytosolic HSP90, HSP70, and HSP101 was elevated not only by heat shock but also by GDA and RAD treatments, although GDA had a slightly weaker effect than either heat shock or RAD treatment. Lower inhibitor concentrations reduced the accumulation of HSP90 mRNA (Fig. 1D). Thus, GDA and RAD induce the heat shock response in A. thaliana.
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Heat Shock, Geldanamycin, and Radicicol Induce Genes Responsible for Protein Folding, Synthesis, and Degradation—We used microarray analysis to compare the effects of heat shock, GDA, and RAD treatments on the gene expression profile of Arabidopsis seedlings. Of the 12,656 genes that were analyzed, 950, 289, and 2,453 genes were up-regulated more than 2-fold by heat shock, GDA, and RAD treatments, respectively (Fig. 2A). Of these up-regulated genes, 157 were up-regulated by all three conditions (Fig. 2A, and supplemental Table S2). Most of these genes are involved in heat shock protein/protein refolding (45 genes, 28.7%), transcription/translation (16 genes, 10.2%), and protein degradation (17 genes, 10.8%) (Fig. 2B). Only a few (six) of the genes were down-regulated by all three treatments (supplemental Table S3), suggesting that inhibition of HSP90 did not reduce gene expression. Thus, heat shock, GDA, and RAD treatments induce genes involved in the quality control of various proteins.

Plants Treated with Geldanamycin and Radicicol Acquire High Temperature Tolerance in the Absence of Heat Shock—High temperature tolerance results from the induction of various HSPs after heat shock (2, 3). We determined the ability of GDA and RAD to induce this tolerance. As expected, 5-day-old wild-type seedlings died when incubated at 45 °C for 1 h, but they survived these conditions if they had been pretreated with heat shock at 37 °C for 2 h (Fig. 3A). Interestingly, we found that when the seedlings were transiently exposed to GDA and RAD at 22 °C for 6 h, they also developed high temperature tolerance (Fig. 3B). Thus, GDA and RAD treatments are sufficient to induce the acquisition of high temperature tolerance.

Heat Shock Reduces Cytosolic HSP90 Activity in Vivo—The above data suggest that the reduction in HSP90 activity was responsible for the induction of the heat shock response. On the other hand, heat shock induces the accumulation of denatured proteins, which might cause a transient deficiency in the chaperone activity of such proteins as cytosolic HSP90. To investigate this, we performed an experiment using transgenic A. thaliana seedlings that express the GAL4-VP16-GR (GVG) fusion protein by the Pro3SS::GVG gene along with the GAL4 promoter fused to the firefly luciferase (ProGAL4::Luciferase) gene (30). GVG bears a GR domain and a GAL4 DNA-binding domain; therefore, dexamethasone treatment of the transgenic plants activates the GAL4 promoter, which in turn causes the transcription of luciferase (30). Cytosolic HSP90 binds to the GR domain of the GR and keeps it as an active form. As a result, the ability of GR to perceive dexamethasone is completely dependent on cytosolic HSP90 activity (19, 20). Consequently, these transgenic plants can be used to detect defective cytosolic HSP90 activity. The transgenic plants only express luciferase when they had been treated with dexamethasone (Fig. 4). Moreover, as expected, when the plants were treated with GDA or RAD, the dexamethasone-induced expression of luciferase was reduced (Fig. 4). Significantly, heat shock also reduced the dexamethasone-induced expression of luciferase. This suggests that heat shock indeed reduces cytosolic HSP90 activity in vivo.

Most of the Heat Shock-, Geldanamycin-, and Radicicol-induced Genes Bear Heat Shock Response Elements—We next addressed how heat shock, GDA, and RAD induce heat-inducible genes. We searched the promoter regions of the 157 up-regulated genes by microarray analysis for the presence of a conserved motif. The promoters of 148 of the genes were available in AGRIS data base and were queried by using the MEME program, which is frequently used to identify conserved motifs...
The motif identified was HSE3, which is observed in the promoters of many heat-inducible genes. Of the 148 genes examined, most (115 genes, 77.7%) had at least one HSE3-like motif in their promoter region (Fig. 5 and supplemental Table S4). HSE3 is responsible for the expression of heat-inducible genes after heat shock and is bound by HSF in various organisms, including plants (5, 6). These results suggest that the HSE3-like motifs are responsible for the activation of these genes, which are up-regulated not only by heat shock but also by GDA and RAD treatments. We next examined the 33 genes that did not have the HSE3-like motif. We identified the consensus motif (CCACGGCT), which is closely related to the unfolding protein response element in 15 genes (supplemental Table S5). These data suggest that heat shock, GDA, and RAD treatments solicited the unfolding protein response.

Several Heat Shock Transcription Factors Are Constitutively Expressed in A. thaliana—A. thaliana has 21 HSF homologues that have been classified into three subfamilies (36). As shown in Fig. 1C, heat shock, GDA, and RAD treatments up-regulated the mRNA levels of cytosolic HSP90, HSP70, and HSP101, which might be regulated by HSF. Notably, these effects were not down-regulated by co-treatment with cycloheximide, a protein synthesis inhibitor (Fig. 1C). This indicates that HSF is constitutively accumulated as a latent form in the absence of heat shock, GDA, and RAD treatments. We analyzed the expression pattern of six A. thaliana HSF genes in 5-day-old seedlings by RT-PCR (Fig. 6). AtHsfA1d, AtHsfA4c, AtHsfA3, and AtHsfB1 were expressed in the absence of heat shock, GDA, and RAD treatments. Thus, these HSF homologues are candidate transcription factors that may be activated by heat shock, GDA, and RAD treatments.

AtHsfA1d Recognizes Heat Shock Response Element—To investigate the functions of A. thaliana HSF, we expressed AtHsfA1d, AtHsfA4c, AtHsfA7a, and AtHsfB1 in bacteria. Only AtHsfA1d could be expressed as a soluble recombinant protein and purified (Fig. 7A). Fig. 7B shows the ability of recombinant AtHsfA1d to bind to biotinylated HSE3. The ability of recombinant AtHsfA1d to bind to biotinylated HSE3 suggests that AtHsfA1d specifically recognizes HSE3.

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FIGURE 2. Identification of genes that are up-regulated by not only heat shock but also GDA and RAD treatments. The seedlings were subjected to heat shock for 2 h or treated with chemicals for 3 h. Total RNA was then isolated and subjected to microarray analysis. A, a Venn diagram of the genes that were up-regulated more than 2-fold by each treatment. B, a partial list of the genes that are up-regulated by all three treatments. The complete list is presented in supplemental Table S2.
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Heat shock inhibits cytosolic HSP90 activity in vivo. Transgenic Arabidopsis seedlings that harbor Pro35S:GVG and ProGAL4:Luciferase were pretreated with 50 μM GDA or RAD or were heat-shocked at 37 °C for 30 min (HS) and then incubated in 30 μM dexamethasone for 1.5 h. Plants treated with dexamethasone only (DEX) and untreated plants (−) served as controls. RT-PCR was performed with specific primers of luciferase (Luc) and cytosolic HSP70. The numbers on the right indicate the number of PCR cycles used. The βtubulin6 (βTub6) bands serve as loading controls. Quantitative RT-PCR of luciferase was performed. The data were normalized with respect to βtubulin6 (bottom).

A. thaliana HSP90.2 Binds AtHsfA1d—Next, we performed a pull-down assay with recombinant AtHsfA1d in an extract from 5-day-old A. thaliana seedlings to investigate whether cytosolic HSP90 and AtHsfA1d interact in vitro. After adding His-tagged AtHsfA1d to the seedling extract, bound proteins were recovered using Ni²⁺-agarose beads and subjected to immunoblot analysis using anti-HSP90 (Fig. 9A). A Coomassie Blue staining shows that His-tagged AtHsfA1d was recovered with nickel-nitrioltriocetic acid-agarose (Fig. 9A, bottom). Additionally, His-tagged AtHsfA1d efficiently recovered cytosolic HSP90 from the seedling extract (Fig. 9A, top), indicating that AtHsfA1d interacts with cytosolic HSP90. To investigate for a direct interaction between cytosolic HSP90 and AtHsfA1d in vivo, we performed bimolecular fluorescence complementation analysis (37). We transiently expressed AtHsfA1d fused to the amino-terminal half of YFP (nYFP-HsfA1d) and HSP90.2 fused to the carboxy-terminal half of YFP (cYFP-HSP90.2) in onion cells. If nYFP-HsfA1d and cYFP-HSP90.2 physically interact, fluorescence is generated as a result of reconstitution of the YFP molecule. YFP fluorescence was observed in the cytosol and nucleus when nYFP-HsfA1d and cYFP-HSP90.2 were co-expressed in onion cells (Fig. 9B). No fluorescence was observed when cells were treated with GDA or RAD or expressed the dominant negative form of cYFP-HSP90.2 (cYFP-HSP90.2/D80N) (Fig. 9C). These data directly demonstrate that A. thaliana HSP90.2 interacts with AtHsfA1d, and the interaction requires an activity of HSP90.2.

We examined the interaction of HSP90.2 and other A. thaliana HSFs (Fig. 9D). We used three HSFs (AtHsfA4c, AtHsfA7a, and AtHsfB1) instead of AtHsfA1d. Strong YFP fluorescence in nucleus and cytosol was observed when nYFP-HsfA7a was co-expressed with cYFP-HSP90.2. Strong YFP fluorescence in the

FIGURE 3. Pretreatment with heat shock, GDA, and RAD induces high temperature tolerance. A, wild-type seedlings were incubated at 22 °C (−; left) or 45 °C (middle) for 1 h. Alternatively, the seedlings were heat-shocked at 37 °C for 2 h, allowing recovery at 22 °C for 2 h, and then incubated at 45 °C for 1 h (HS/45 °C; right). The panels show the plants 2 days after the treatment. B, wild-type seedlings were treated with 50 μM GDA or RAD for 6 h, and they were washed three times with fresh medium. After plants were allowed to recover at 22 °C for 2 h, they were incubated at 22 °C (−; left) or 45 °C (right) for 1 h. As a negative control, plants were treated with Me₂SO (DMSO), a solvent of inhibitors. The panels show the plants 2 days after the treatment.

FIGURE 4. Heat shock inhibits cytosolic HSP90 activity in vivo. Transgenic Arabidopsis seedlings that harbor Pro35S:GVG and ProGAL4:Luciferase were pretreated with 50 μM GDA or RAD or were heat-shocked at 37 °C for 30 min (HS) and then incubated in 30 μM dexamethasone for 1.5 h. Plants treated with dexamethasone only (DEX) and untreated plants (−) served as controls. RT-PCR was performed with specific primers of luciferase (Luc) and cytosolic HSP70. The numbers on the right indicate the number of PCR cycles used. The βtubulin6 (βTub6) bands serve as loading controls. Quantitative RT-PCR of luciferase was performed. The data were normalized with respect to βtubulin6 (bottom).
nucleus was observed when nYFP-HsfB1 was co-expressed with cYFP-HSP90.2. In contrast, YFP fluorescence was reduced when nYFP-A4c was co-expressed with cYFP-HSP90.2. These data indicate that HSP90.2 interacts with AtHsfA7a and AtHsfB1 but only weakly interacts or does not interact with AtHsfA4c.

Expression of a Dominant Negative Form of HSP90.2 Induces HSP70 Gene—To examine whether the deficiency in cytosolic HSP90 activity induces heat-inducible genes, we generated a transgenic A. thaliana expressing the dominant negative form of HSP90.2 (HSP90.2-D80N) (Fig. 10). We substituted Asp80 of HSP90.2 with an Asn residue, because this mutation of S. cerevisiae HSP90 induces a dominant negative effect when expressed in yeast (38). The A. thaliana transgenic line that expressed HSP90.2-D80N accumulated more of the heat-inducible gene, HSP70, than the control line (Fig. 10). The data indicate that the disturbing HSP90 activity induces the heat-inducible gene. This result also suggests that HSP90.2 is involved in the heat shock response in A. thaliana.

DISCUSSION

Geldanamycin and Radicicol Induce Heat Shock Response and High Temperature Tolerance—Our results show that GDA and RAD activate the pHSP90.1-GUS gene and elevate the endogenous mRNA levels of various heat shock-responsive genes (Figs. 1 and 2). Furthermore, we showed that GDA and RAD induce high temperature tolerance. These observations indicate that these chemicals induce a heat shock response.

Our microarray data indicated that the gene set up-regulated by heat shock included genes that were also up-regulated by GDA and RAD treatments (Fig. 2). This directly demonstrates that these chemicals actually induce heat shock response. The gene sets up-regulated by heat shock, GDA, and RAD treatments did not completely overlap. Indeed, only 157 of the 950 genes (16.5%) that were up-regulated by heat shock were also up-regulated by these chemical treatments. At first glance, this suggests that the genes regulated by heat shock involve the genes that are not regulated by the HSP90 system. However, it should be noted that GDA has a weaker effect than RAD or heat shock (Figs. 1C and 3). Heat shock and RAD treatments both up-regulated 626 genes, which is 65.9% of the 950 genes that are up-regulated by heat shock. Notably, most of these genes (437 of 626 genes) are also up-regulated by GDA but fall below the 2-fold cut-off level. Thus, it appears that over half of all of the genes that are up-regulated by heat shock are up-regulated by these chemicals.

Heat shock helps plants to acclimate to higher temperatures, and this heat acclimation is mediated by the induction of various HSPs (1, 4). For example, it has been shown that HSP101 induction is necessary for the acquisition of high temperature tolerance by A. thaliana (2, 3). To our surprise, we found that transient treatment of GDA or RAD is also sufficient for inducing high temperature tolerance (Fig. 3). GDA and RAD treatments did not significantly induce about one-third (323 genes) of the heat-inducible genes (Fig. 2). However, these chemical treatments were sufficient for the induction of heat acclimation under high temperature conditions (Fig. 3). This implies that the induction of only a subset of heat-inducible genes is required to generate high temperature tolerance. We showed that heat shock, GDA, and RAD treatments induce HSP genes that are responsible for protein synthesis/maintenance. This suggests that plants acquire the heat tolerance by protein synthesis/maintenance, as reported previously (1, 4). Since HSP90 inhibition induces genes involved in protein degradation (Fig. 2B), it appears that these gene products degrade refolding-failed proteins.

GDA and RAD treatments also induced transcription factors, including four HSF genes (Figs. 4B and 6). These HSF genes (AtHsfA3, AtHsfA7a, AtHsfB1, and AtHsfB2a) might be
involved in positive feedback that enhances the heat-responsive gene transcription. Supporting this is the fact that AtHsfA2 has been shown to be induced by heat shock and to amplify the transcription of a subset of genes in the heat shock response (39–41). Heat shock, GDA, and RAD treatments also induce DREB2A (Fig. 2B), which encodes the transcription factor responsible for the dehydration response. Recent research has shown that DREB2A protein is responsible for the heat shock response in A. thaliana (42) and that sunflower DREB (HaDREB2) protein interacts with HaHsfA9 to enhance the induction of heat-inducible genes (43). These observations together strongly suggest that these transcription factors act in the heat shock response as part of a positive feedback system.

The Elevated Transcription Resulting from Heat Shock, Geldanamycin, and Radicicol Treatments Is Mediated by Heat Shock Transcription Factors—A motif search showed that one or more HSE3-like motifs are present in most (115 of 148) of the genes that were up-regulated by heat shock, GDA, and RAD treatments (Fig. 5 and supplemental Table S4). This strongly suggests that the transcription induced by these treatments is mediated by HSF. Trimeric HSF binds HSE in the promoters of heat-inducible genes to activate them (5, 6). HSE3 is composed of three HSEs (nGAAn) that are oriented head-to-head and then tail-to-tail (5, 6) (see Fig. 5). Of the 115 genes with HSE3s, 20 have a complete HSE3 motif (Fig. 5), 55 have one mismatch, 21 have two mismatches, and 19 have three mismatches. It is not known how many motif mismatches are per-
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FIGURE 9. *A. thaliana* HSP90.2 binds AtHsfA1d. *A. thaliana* HSP90.2 binds AtHsfA1d. (A) thioredoxin-His-tagged AtHsfA1d (A1d) or thioredoxin-His (THX) was mixed with (+) or without (−) Arabidopsis extract (ext.), and bound proteins were absorbed to Ni²⁺-nitrilotriacetic acid-agarose beads. Bound proteins were separated by SDS-PAGE and analyzed by immunoblot analysis using anti-HSP90 antibody (top) or stained with Coomassie Blue (bottom). The amount of cytosolic HSP90 in one-tenth of the crude extract used is shown (Input). B, the bimolecular fluorescence complementation analysis of AtHsfA1d and HSP90.2. The expression vectors encoding nYFP-HsfA1d, cYFP-HSP90.2, nYFP, or cYFP are introduced in onion epidermis cells with particle bombardment. nYFP-HsfA1d/cYFP-HSP90.2 (top), nYFP-HsfA1d/cYFP (middle) or nYFP/cYFP-HSP90.2 (bottom) were co-expressed. tdTomato (tdTom) shows the bombarded cells. BF, bright field. C, both nYFP-HsfA1d and cYFP-HSP90.2 were co-expressed in onion cells treated with GDA (top) or RAD (middle). The inactive forms of cYFP-HSP90.2 (cYFP-HSP90.2/D80N) and nYFP-HsfA1d were co-expressed in onion cells (bottom). tdTomato shows the bombarded cells. D, nYFP-HsfA4c (top), nYFP-HsfA7a (middle), or nYFP-HsfB1 (bottom) and cYFP-HSP90.2 were expressed in onion cells. tdTomato shows the bombarded cells. All YFP pictures were taken with same exposure time.

We identified the consensus motif CCACGGCT in 15 of the 33 genes that lack an HSE3-like motif. This motif resembles the unfolding protein response element motif, which has been shown to be responsible for the activation of ER stress-related genes (supplemental Table S5). GDA and RAD inhibit mammalian GRP94, an ER-localized HSP90 homologue, resulting in the induction of ER stress (46, 47). This suggests that GDA and RAD inhibit SHD/GRP94 to induce ER stress, resulting in the activation of these 15 genes in *A. thaliana*. We compared our microarray data with that obtained during ER stress induced by tunicamycin and dithiothreitol treatments (48, 49). We found a large difference in the up-regulated genes between the two analyses; however, seven genes (At1g27350, At1g67360, At2g47180, At3g12050, At4g10040, At5g42020, and At5g47120) were up-regulated in both data sets. This suggests that heat shock and ER stress are closely related and that only a small number of genes are actually up-regulated by ER stress during heat shock, GDA, and RAD treatments.

Heat Shock Reduces Cytosolic HSP90 Activity to Activate Heat Shock Transcription Factors in Plants—We showed that heat shock reduced cytosolic HSP90 activity (Fig. 4) and that overexpression of the dominant negative HSP90 inhibited the HSP70 gene (Fig. 10). This suggests that the reduction of HSP90 activity results in the induction of HSP70 gene during heat shock. We showed that purified AtHsfA1d recognized HSE3 (Fig. 7), and cytosolic HSP90 bound to AtHsfA1d, AtHsfA7a, and AtHsfB1 but not to AtHsfA4c (Fig. 9). AtHsfA1d, AtHsfA4c, and AtHsfB1 genes were expressed in the absence of heat shock (Fig. 6). These results suggest that in the absence of heat shock, AtHsfA1d and AtHsfB1 are inactivated by cytosolic HSP90. AtHsfA4c might be regulated by other mechanisms. Interestingly, HSP90.2 interacts with AtHsfA7a, which is induced by heat shock. This suggests that HSP90.2 down-regulates AtHsfA7a activity when plants are recovered from heat shock. In tomatoes, HsfA1 is responsible for the primary response to heat shock. The expression of tomato HsfA1 is constitutive, and its protein levels are

mitted for the binding of *A. thaliana* HSF. Previous research (33, 44) and our data (Fig. 6C) show that plant HSF homologues specifically recognize HSE3, as do animal and yeast HSF homologues. Moreover, the DNA-binding domains of the 21 *A. thaliana* HSF homologues are very similar to each other and to tomato HsfA1, which recognizes HSE3 but not mutated HSE3 that bears a mutation at three guanine residues (33). This suggests that these HSF homologues have similar DNA binding activities. Although the guanine residue in HSE (nGAAAn) seems to be essential, the two adenine residues are less important and may be substituted, although this does reduce the affinity of the motif for HSF (45). We conclude that not all but many of the HSE3-like motifs we identified probably work as enhancers in the 115 heat-inducible genes during heat shock, GDA, and RAD treatments.

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not changed by heat shock (33). This suggests that, like A. thaliana, tomato HSP90 down-regulates HsfA1 function. It is likely that HSP90 also down-regulates AtHsfA1a and AtHsfA1b, which are constitutively expressed and recognize HSE3 in A. thaliana (44, 50). This negative regulatory system is highly similar to the ones observed in animal and yeast cells. In human cells, Homo sapiens HSP90 negatively regulates H. sapiens HSF1 by directly interacting with it and maintaining it in its monomeric form (10, 12). Upon heat shock, HSP90 dissociates, thereby freeing HSF1 and allowing it to form a trimer (10). In yeast cells, although S. cerevisiae HSF1 exists as a trimer in the absence of heat shock (11), it is also negatively regulated by S. cerevisiae HSP90 due to its direct interaction (12, 51). It is not known whether plant HSF exists as monomers or trimers in the absence of heat shock. Nevertheless, our observations support the notion that in plants, HSP90 directly interacts with HSF in the absence of heat shock, thereby inhibiting its ability to interact with the transcriptional machinery; upon heat shock, denatured proteins deplete HSP90 from the HSP90–HSF complex, which frees the transcriptional activity of HSF. Therefore, the overproduction of HSP90 may cause down-regulation of HSF in A. thaliana, resulting in reduced heat tolerance in yeast cells as well (52). Sangster et al. (28) reported that the A. thaliana mutant lines of HSP90.2 induce the heat-inducible HSP90.1 gene, and McLellan et al. (53) showed that HSP90 inhibitor monoclin I from rhizosphere fungus induces heat acclimation in A. thaliana. Our finding highlights the downstream event after HSP90 inhibition and complements these two recent researches. Interestingly, it has been suggested that in plants, the phosphorylation of HSF by CDC2a or binding of HSF by HSP70 and small HSP may down-regulate their transcriptional activities (13–15). In addition, recent findings indicate that plant HSFs act as coactivator or repressor of the other HSFs. HsfB1 acts as a coactivator of HsfA1 in tomato (54), whereas AtHsfA5 acts as a repressor of AtHsfA4 in A. thaliana (55). HSP90 might regulate HSF activity together with these molecules that fine tune HSF activity in vivo.

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FIGURE 10. Overproduction of dominant negative form of HSP90.2 enhances the expression of HSP70. The mRNA amounts of HSP90 (open bars) or HSP70 (closed bars) in transgenic A. thaliana seedlings were measured by quantitative RT-PCR. The data were normalized to β-tubulin6. HSP90.2/D80N, a transgenic line harboring Pro35S:GVG and the ProGAL4:HSPI.2-D8ON gene. Vector, a transgenic line harboring empty T-DNA. Seedlings were treated with 30 μM dexamethasone (DEX) for 6 h. Error bars, S.E. values of three independent experiments.
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