Heat Shock Protein 90.1 Plays a Role in Agrobacterium-Mediated Plant Transformation

Dear Editor,

Many bacterial proteins are involved in Agrobacterium-mediated plant transformation. By contrast, relatively little is known about plant proteins that play key roles in transformation. Some of these host proteins interact with Virulence effector proteins, including VirE2, that are transferred from Agrobacterium to plants (Gelvin, 2010; Pitzschke and Hirt, 2010). A recent study indicated that the plant protein SUPPRESSOR OF G2 ALLELE OF SKP1 (SGT1), a co-chaperone of heat shock protein 90 (HSP90), is required for Agrobacterium-mediated transformation (Anand et al., 2012). These studies suggested the involvement of HSP90 in Agrobacterium-mediated transformation.

We investigated whether HSP90.1, a co-chaperone of SGT1, may also be important for Agrobacterium-mediated transformation. We assayed an Arabidopsis hsp90.1 T-DNA insertion mutant and 35S:Myc-HSP90.1-overexpression plants (Supplemental Figure 1A and 1B) for stable root transformation. Compared to controls, the hsp90.1–2 mutant was 1.7-fold less susceptible, whereas 35S:Myc-HSP90.1 plants were twice as susceptible to transformation (Figure 1A). Thus, decreased or increased HSP90.1 expression resulted in altered transformation susceptibility. Genomic DNA blots showed that the amount of uidA DNA, a transgene on the T-DNA, integrated into the hsp90.1–2 mutant genome was four-fold less than that of control plants. However, uidA integration into HSP90.1-overexpression plants was 10.8-fold greater than that of control plants (Supplemental Figure 1C). The greater increase in T-DNA integration than that of stable root transformation may result from silencing of some integrated genes because of epigenetic effects such as DNA methylation (Park et al., manuscript in preparation).

VIP1 (VirE2 interacting protein 1) and VBF (VIP1 F-box binding protein) are host proteins that may be important for T-DNA subcellular trafficking and integration (Tzfira et al., 2001; Djamei et al., 2007; Gelvin, 2010; Zaltsman et al., 2010). We reasoned that VIP1 and/or VBF could interact with HSP90.1. To test this hypothesis, we conducted a Bimolecular Fluorescence Complementation (BiFC) assay in which two A. tumefaciens strains harboring in their T-DNAs genes encoding protein fusions with nVenus or cCFP were co-infiltrated into Nicotiana benthamiana leaves. The results showed an interaction between VIP1 and HSP90.1 (nVenus–VIP1 and cCFP-HSP90.1) as a yellow fluorescence signal in both the cytoplasm and the nucleus (Figure 1B), compared to empty vector combinations (nVenus–VIP1 + cCFP, and nVenus + cCFP-HSP90.1) showing no yellow fluorescence signals (Supplemental Figure 2A). Our BiFC assay did not detect interaction of HSP90.1 with VBF (Supplemental Figure 2A, lower panel), the latter of which recognizes and targets VIP1 and its bound VirE2 for degradation (Zaltsman et al., 2010). These data indicate that HSP90.1 interacts in leaves with VIP1 but not with VBF.

We next conducted co-immunoprecipitation (co-IP) studies. Three Agrobacterium strains individually harboring in their T-DNA regions 35S:Myc-HSP90.1, 35S:YFP–VIP1, or 35S:YFP–SGT1b were co-infiltrated into N. benthamiana leaves. Input proteins were observed using anti-GFP and anti-Myc antibodies, whereas interacting proteins were detected by anti-GFP antibodies (Figure 1C, left panel). The in vivo interaction between Myc-HSP90.1 (83 kDa) and YFP–VIP1 (70 kDa) is strong. YFP–VIP1 alone served as a negative control and did not react with anti-Myc conjugated beads. Our co-IP analysis also detected interaction between VIP1 and SGT1b in N. benthamiana leaves infiltrated with two Agrobacterium strains harboring in their T-DNA regions 35S:Myc–VIP1 or 35S:YFP–SGT1b (Figure 1C, right panel). These results suggest that SGT1b may work in concert with HSP90.1 to protect VIP1. Finally, we conducted yeast two-hybrid (Y2H) analyses to substantiate further the interaction between HSP90.1 and VIP1 (Supplemental Figure 2B). We chose VIP1 as the prey in our assays because VIP1 is a transcription factor and, therefore, VIP1 fused to the gal4 DNA binding domain (BD) was able to auto-activate expression of the reporter genes without interacting with a protein containing an acidic activation domain (AD). Yeast strain AH109 transformed with the BD-HSP90.1 and AD–VIP1 plasmids grew on triple dropout plates (SD/-Leu/-Trp/-His), indicating interaction of the two tested proteins (Supplemental Figure 2B, left four columns). We also detected the expression of the MEL1 reporter gene using an X–α-gal (5-bromo-4-chloro-3-indolyl α-D-galactopyranoside) assay, confirming the interaction between HSP90.1 and VIP1 (Supplemental Figure 2B, last column). Negative control strains containing BD-HSP90.1 and AD-empty vector plasmids, or BD-empty and AD–VIP1

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Figure 1 HSP90.1 Plays a Role in Agrobacterium-Mediated Plant Transformation.

(A) Tumorigenesis assay of Col-0, hsp90.1–2, and 35S:Myc-HSP90.1 root segments inoculated with Agrobacterium tumefaciens A208 at 10^5 cfu ml⁻¹. Lanes 1–3: Col-0, hsp90.1–2, and 35S-Myc-HSP90.1, respectively.

(B) HSP90.1 interacts with VIP1 by Bimolecular Fluorescence Complementation. Bar is 20 μm. mCherry (red) marks both the nucleus and cytoplasm. White arrow indicates the nucleus.
plasmids, did not grow on this medium. Thus, Y2H results further supported the HSP90.1–VIP1 interaction.

The interaction of HSP90.1 with VIP1 prompted us to investigate whether HSP90.1 could prevent VIP1 aggregation in leaves. We compared the pattern of VIP1–YFP fluorescence in infiltrated leaves of wild-type and hsp90.1–2 mutant plants. Large aggregates of YFP–VIP1 appeared in the cytoplasm of hsp90.1–2 mutant leaves, but no such large aggregates were seen in control samples (Figure 1D). Western blots showed similar levels of YFP–VIP1 protein in infiltrated Col-0 and hsp90.1–2 leaves, indicating that the intense YFP fluorescence in hsp90.1–2 leaves did not result from higher amounts of VIP1 protein (Supplemental Figure 3A). A key function of cytosolic HSP90 is to maintain the stability and prevent the aggregation of its client proteins. Geldanamycin (GDA), a specific inhibitor of HSP90, can decrease the stability of HSP90 client proteins, resulting in their aggregation and possible degradation (Theodoraki et al., 2012). Treatment of leaves with GDA resulted in YFP–VIP1 aggregation (Figure 1E, lower panel, and Supplemental Figure 3B). No such aggregates were detected in untreated infiltrated leaves (Figure 1E, top panel). Western blots showed a similar level of YFP–VIP1 in the absence or presence of GDA, suggesting YFP–VIP1 aggregation but not degradation (Supplemental Figure 3C).

VirE2 protein is important for Agrobacterium-mediated transformation (Citovsky et al., 1992). GDA treatment of leaves causes VIP1 aggregation and, because VIP1 interacts with VirE2, we investigated the effect of GDA treatment on VirE2 solubility. Treatment of leaves with GDA caused YFP–VirE2 aggregation in the cytoplasm of infiltrated N. benthamiana leaves (Figure 1F, lower panel; in these cells, the cytoplasm is appressed to the cellular periphery by the large central vacuole), suggesting that HSP90 activity is important for maintaining VirE2 solubility and function, either directly or indirectly by preventing aggregation of the VirE2 interacting protein VIP1.

We have shown that HSP90.1 is important for Agrobacterium-mediated plant transformation. We propose that HSP90.1 functions as a VIP1 molecular chaperone and facilitates transformation through stabilizing VIP1, VirE2, and/or other proteins important for transformation. This new role for a member of the HSP90 family had not previously been described. Gurel et al. (2009) reported that plant transformation susceptibility may be increased by heat treatment. Although heat treatment affects expression of many genes and regulatory pathways, increased expression of HSP90 could thus augment transformation efficiency.

Recently, Shi et al. (2014) indicated that VIP1 does not play an important role in Agrobacterium-mediated transformation. Data presented in that study, along with those presented here, suggest that HSP90.1 plays a role in transformation beyond that of influencing VIP1 function.

SUPPLEMENTARY DATA

Supplementary Data are available at Molecular Plant Online.

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REFERENCES


Title:
Heat shock protein 90.1 plays a role in Agrobacterium-mediated plant transformation

Running Title:
HSP90.1 plays a role in T-DNA transfer

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Supplemental Materials and Methods

Plant materials

Arabidopsis plants were grown at 24°C with a 16-h light/8-h dark photoperiod. HSP90.1 overexpression constructions were introduced into ecotype Col-0 plants by a floral dip transformation protocol (Clough and Bent, 1998). The hsp90.1 mutant hsp90.1-2 (SALK_075596) was obtained from the ABRC (www.arabidopsis.org). Nicotiana benthamiana plants were grown at 24°C with a 16-h light/8-h dark photoperiod.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total mRNA was extracted from Arabidopsis rosette leaves using Trizol Reagent (Invitrogen, La Jolla, CA) following the manufacturer’s instructions. First-strand cDNA was synthesized from total RNA using random primers and reverse transcriptase (Invitrogen). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed using a Bio-Rad CFX96 Real-Time System and 1x of SsoFastEvaGreenSupermix (Bio-Rad). Templates were normalized using EF-1α amplification levels (Remans et al., 2008). Triplicate biological and technical replications were performed. Data were analyzed using BioRad CFX Manager 2.0 Software. The comparative cycle threshold method (ΔΔCt) was used to obtain the relative fold change. Primer information is listed in Supplemental Table 1.

Agrobacterium-mediated stable Arabidopsis root transformation assays

The non-tumorigenic strain A. tumefaciens GV3101 containing pBISN1 (Narasimhulu et al., 1996) was cultured in liquid LB medium plus gentamicin (50 mg l⁻¹), kanamycin (50 mg l⁻¹), and rifampicin (10 mg l⁻¹) until it reached an A₆₀₀ of 0.8. Cut segments from twenty-day old roots of Arabidopsis Col-0, hsp90.1-2 T-DNA insertion mutant, and HSP90.1-overexpression lines were inoculated. For tumorigenesis assays, root segments pooled from five plants were inoculated with the tumorigenic strain A. tumefaciens A208 as described (Nam et al., 1999; Zhu et al., 2003). After 2-days cocultivation, roots were separated on hormone-free MS medium containing Timentin (100 mg l⁻¹). Root segments from a pool of five Arabidopsis plants formed one replicate, and each of three independent replicates was inoculated with bacteria at 10⁵ cfuml⁻¹.
DNA blots
After infection with *A. tumefaciens* GV3101(pBISN1) at 10^8 cfu ml⁻¹, *Arabidopsis* root segments were cultured on CIM containing 100 mg l⁻¹ Timentin without any plant selective agent for four weeks. DNA was extracted from the resulting calli using a CTAB procedure (Weigel et al., 2002). For DNA blots, 10 µg (Col-0 and *hsp90.1-2*) and 5 µg (*35S:Myc-HSP90.1*) of genomic DNA samples were subjected to electrophoresis through a 0.8% agarose gel. To normalize DNA loading, the blots were re-hybridized with a flax rDNA probe. ImageJ was used to calibrate the amount of T-DNA integration following the manual at the NIH ImageJ manual (http://rsbweb.nih.gov/ij/index.html).

Plasmid constructions
Full-length cDNAs of *HSP90.1* (AT5G52640), *VIP1* (AT1G43700), *SGT1b* (AT4G11260) and *VBF* (AT1G56250) were amplified with gene specific primers (Supplemental Table 2), and separately cloned into pCR8/GW/TOPO (Invitrogen). The resulting clones were recombined with pMDC-nVenus, pMDC-cCFP (Bhattacharjee et al., 2011), pEarleyGate104, and pEarleyGate203 (www.arabidopsis.org) using the LR Clonase II enzyme mix (Invitrogen), creating the binary vectors pMDC-nVenus-VIP1, pMDC-cCFP-HSP90.1, pEarley-YFP-VIP1, pEarley-YFP-HSP90.1, pEarley-YFP-SGT1b, pEarley-Myc-HSP90.1 and pEarley-Myc-VIP1. Full-length mCherry from pSAT4A-mCherry-N1 (www.arabidopsis.org) was amplified by specific primers (Supplemental Table 6) and cloned into pEarleyGate100 (www.arabidopsis.org) using the LR Clonase II enzyme mix (Invitrogen). For constructing the YFP-VirE2 binary vector, a full-length *VirE2* gene (accession CP007228) was first amplified from the plasmid pSAT6-N-VirE2-mCherry using primer pairs flanking this gene sequence (Supplemental Table 2). The resulting VirE2 gene was cloned into pEarleyGate104, yielding the T-DNA binary vector construction pEarley-YFP-VirE2. For yeast two-hybrid assays, full-length cDNAs of *HSP90.1* (AT5G52640) and *VIP1* (AT1G43700) were recombined with pDEST-GBKT7 and pDEST-GADT7, respectively, using the Gateway recombination system (Rossignolet al., 2007).

Yeast two-hybrid analysis
pDEST-GBKT7 (DNA binding domain) and pDEST-GADT7 (transcription activation domain) alone (empty vector controls) or containing full-length cDNAs of HSP90.1 and VIP1, respectively, were introduced into the yeast strain AH109 using a small-scale LiAc yeast transformation protocol (Clontech Matchmaker). Yeast transformants were selected on glucose-based synthetic minimal medium (Clontech, #630411) lacking tryptophan and leucine (Clontech, #630417). Selected colonies were incubated on drop-out plates in the absence of tryptophan, leucine, and histidine (Clontech, #630419). All plates and cultures were incubated at 30°C for 72 hours for yeast growth. For β-galactosidase activity, 5-bromo-4-chloro-3-indolyl α-D-galactopyranoside (X-alpha-Gal) was used and activity was detected by an X-Gal overlay assay (Clontech).

Co-immunoprecipitation (Co-IP) and Western blot analysis. For Co-IP, Agrobacterium strains harboring the respective binary vectors were infiltrated into Nicotiana benthamiana leaves. Two days after infiltration, the leaves were collected and homogenized with in vivo binding buffer [50 mMTris-HCl, pH 7.5, 0.1 M NaCl, 0.2% Triton X-100, 1 mM DTT containing Complete protease inhibitor cocktail (Roche)] to extract soluble proteins as described (Park et al., 2007). After centrifugation, one mg of total soluble protein from various leaf samples was incubated with anti-Myc conjugated beads (Thermo scientific, USA) at 4°C for 1 hour to bind Myc-HSP90.1 or Myc-VIP1 protein. Beads were collected and washed at least three times with binding buffer at room temperature, and co-purified proteins were eluted by boiling with Laemmlisample buffer (62.5 mMTris-HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% Bromophenol Blue, and 710 mM β-mercaptoethanol). The eluted proteins were resolved by SDS-PAGE through 12% polyacrylamide gels (Bio-Rad) and transferred to a polyvinylidenedifluoride (PVDF) membrane (Bio-Rad). Proteins were detected by immunoblot analysis using anti-GFP (Invitrogen) or anti-Myc (Abcam) antibodies using the ECL system (Pierce). The membrane was stained with Coomassie Brilliant Blue (CBB).

Agroinfiltration and GDA inhibition
For agroinfiltration, A. tumefaciens GV3101 strains containing T-DNA binary vectors were grown in LB liquid medium with the appropriate antibiotics (gentamycin, 50 mg l⁻¹; kanamycin,
50 mg/l rifampicin, 10 mg/l) overnight at 28°C with shaking (200 rpm) until saturation. After resuspension in infiltration buffer (10 mM MgCl₂, 10 mM MES, pH 5.6, and 150 µM acetosyringone), the bacteria (A₆₀₀=0.6) were infiltrated into intact 30-day old *N. benthamiana* and 20-day old *Arabidopsis* leaves as described (Parket al., 2007). For localization studies, BiFC, and Co-IP assays, agroinfiltrated tissues were analyzed 1-3 days after infiltration.

For agroinfiltration with geldanamycin (GDA) (Sigma-Aldrich, USA), infiltration buffer containing 1 or 5µM of GDA (dissolved in DMSO) or an equivalent amount of DMSO alone was infiltrated into *N. benthamiana* leaves 12-h post-*Agrobacterium* infiltration (Takahashi et al., 2003; Takabatake et al., 2007). After 70 hrs, fluorescence signals were detected using a Zeiss LSM 510 META NLO confocal microscope. *A. tumefaciens* GV3101 containing pEarleyGate100-mCherry was co-transfected as a control. Infiltration experiments were repeated at least three times.

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Supplemental Figure Legends

**Supplemental Figure 1.** *Agrobacterium*-mediated transformation of *Arabidopsis*\(_{\text{HSP90.1}}\) mutant or overexpression lines.\(\text{(A)}\)Schematic representation of the *HSP90.1* gene and the T-DNA insertion mutant analyzed in this study. Gray and white boxes represent exons and introns, respectively. A1 and A2 indicated qPCR primers used to examine *HSP90.1* mRNA abundance. \(\text{(B)}\)Analysis of *HSP90.1* transcripts. Total RNA was isolated from 3-week old plants. *HSP90.1* transcripts were quantified by qRT-PCR using primers A1 and A2. Lanes 1-3: Col-0, *hsp90.1*-2, and 35S-Myc-\(_{\text{HSP90.1}}\), respectively. \(\text{(C)}\)Quantification of integrated transgene DNA using gusA and rDNA probes. The images of the amount of integrated T-DNA were calculated using ImageJ. Lanes 1-3: DNA from Col-0, *hsp90.1*-2, and 35S-Myc-\(_{\text{HSP90.1}}\) roots, respectively. Lane 4, pBISN1 control. Error bars indicate ± SD.

**Supplemental Figure 2.** VIP1 interacts with HSP90.1. \(\text{(A)}\) Negative controls showing interaction results between nVenus-VIP1 and cCFP, nVenus-cCFP and HSP90.1, and nVenus-VBF and cCFP-HSP90.1, respectively, by Bimolecular Fluorescence Complementation. mCherry labels the nucleus and cytoplasm. Bar is 20 µm. White arrow indicates the nucleus. \(\text{(B)}\) VIP1-HSP90.1 interaction in a yeast two-hybrid assay. Dilutions of yeast strains expressing various combinations of VIP1 or HSP90.1 were grown on drop-out plates lacking leucine and tryptophan, or lacking leucine, tryptophan, and histidine, and examined for growth. To test the activation of the *lacZ* reporter gene, yeast cells were spread on medium lacking leucine, tryptophan, and histidine but containing X-gal. Colonies were examined after 3 days.

**Supplemental Figure 3.** VIP1 localization using *Agrobacterium*-mediated leaf infiltration assays. \(\text{(A)}\) Western blot analysis showing similar levels of YFP-VIP1 protein in Col-0 and *hsp90.1*-2. \(\text{(B)}\) Enlargement of large aggregates of YFP-HSP90.1 in the *hsp90.1*-2 mutant. Bar is 20 µm. Arrow indicates nucleus. \(\text{(C)}\) Western blot analysis showing that GDA does not cause YFP or YFP-HSP90.1 degradation in vivo. *N. benthamiana* leaves were infiltrated with *Agrobacterium* strain harboring in the T-DNA cDNAs encoding YFP or YFP-HSP90.1 in the absence or presence of GDA.
Supplemental Table 1. Primers for qRT-PCR

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Supplemental Table 2. Primers for vector constructions

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Supplemental References


Supplemental Figure 1.
Supplemental Figure 2.
Supplemental Figure 3.