Overexpression of the HspL Promotes *Agrobacterium tumefaciens* Virulence in *Arabidopsis* Under Heat Shock Conditions

Hau-Hsuan Hwang, Yin-Tzu Liu, Si-Chi Huang, Chin-Yi Tung, Fan-Chen Huang,
Yun-Long Tsai, Tun-Fang Cheng, and Erh-Min Lai

First author: Department of Life Sciences and Ph.D. Program in Microbial Genomics, National Chung Hsing University, Taichung, 402, Taiwan; second, third, fourth, fifth, and seventh authors: Department of Life Sciences, National Chung Hsing University; fifth author: Ph.D. Program in Microbial Genomics, National Chung Hsing University and Academia Sinica, Taichung, Taiwan, 402; and sixth and eighth authors: Institute of Plant and Microbial Biology, Academia Sinica, Taipei, 115, Taiwan.
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ABSTRACT

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Agrobacterium tumefaciens transfers a specific DNA fragment from the resident tumor-inducing (Ti) plasmid and effector virulence (Vir) proteins to plant cells during infection. *A. tumefaciens* VirB1-11 and VirD4 proteins assemble as the type IV secretion system (T4SS), which mediates transfer of the T-DNA and effector Vir protein into plant cells, thus resulting in crown gall disease in plants. Previous studies revealed that an α -crystallin-type, small heat-shock protein (HspL) is a more effective VirB8 chaperone than three other small heat-shock proteins (HspC,

HspAT1, and HspAT2). Additionally, HspL contributes to efficient T4SS-mediated DNA transfer and tumorigenesis under room-temperature growth. In this study, we aimed to characterize the impact of HspL on *Agrobacterium*-mediated transformation efficiency under heat-shock treatment. During heat shock, transient transformation efficiency and VirB8 protein accumulation were lower in the *hspL* deletion mutant than in the wild type. Overexpression of HspL in *A. tumefaciens* enhanced the transient transformation efficiency in root explants of both susceptible and recalcitrant *Arabidopsis* ecotypes. In addition, the reduced transient transformation efficiency during heat stress was recovered by overexpression of HspL in *A. tumefaciens*. HspL may help maintain VirB8 homeostasis and elevate *Agrobacterium*-mediated transformation efficiency under both heat-shock and nonheat-shock growth.

Infection of susceptible plants by the phytopathogenic bacterium *Agrobacterium tumefaciens* results in crown gall tumors. The disease mechanism involves transfer of a specific DNA molecule (T-DNA) that has been processed from a large bacterial tumor-inducing (Ti) plasmid as well as effector virulence (Vir) proteins to plant cells. *A. tumefaciens* has a VirA/VirG two-component regulatory system that senses important environmental signals, including acidity, monosaccharides, and phenolic compounds, one of the most potent being acetosyringone (AS), to regulate *vir* gene induction (8,17,31,43). The expression of VirD1 and VirD2 proteins leads to the production of single-stranded T-DNA which, when capped at the 5' end with the VirD2 protein, is transported into plant cells. After entering plant cells, proper coordination of several Vir and plant protein functions ensures successful transport, integration, and expression of the T-DNA into the plant genome (17,31,43).

Effector Vir proteins and T-DNA translocation across the bacterial cell envelope is mediated by a type IV secretion system (T4SS) composed of the VirB1-11 proteins, as well as VirD4, a member of a large family of membrane proteins implicated in the coupling of DNA transfer intermediates to the secretion machinery (9,64). The T4SS contains two functional components: a filamentous T-pilus and a membrane-associated transporter complex. Results from genetic, biochemical, and protein-protein

interaction studies have revealed that VirB4 and VirB6 to VirB10 are important for T-pilus and secretion channel assembly and function. The three inner-membrane proteins (VirB3, VirB6, and VirB8), along with the ATPases (VirB4, VirB11, and VirD4), form the cytoplasmic membrane translocon (9,60). VirB2 protein is processed and subsequently cycled to form the T-pili filament (13,32). The minor component of the T-pilus, VirB5, is located at the T-pilus tip and may directly contact host proteins during *A. tumefaciens* infection (2). From results obtained from transfer DNA immunoprecipitation (TrIP) assays, the T-DNA substrate contacts 6 of the 12 subunits of the T4SS machine, including the VirD4 receptor, followed by VirB11 ATPase, VirB6/VirB8, and VirB2 pilin/VirB9 proteins, which further supports the idea that the transfer mechanism has many features in common with conjugational DNA transfer between bacteria (9,10,64).

Early studies revealed that *A. tumefaciens* virulence was strongly reduced at temperatures above 29°C, which agrees well with significant reduction of the assembly and stability of the T-DNA transfer complex composed of 11 VirB and VirD4 proteins at elevated temperatures (6,15,33). The *A. tumefaciens* strain C58 has four α -crystallin-type small heat shock protein (*shsp*) genes: one on the circular chromosome (*hspC*), one on the linear chromosome (*hspL*), and two on the pAtC58 plasmid (*hspAT1* and *hspAT2*) (4). The expression of the linear chromosome- and pAtC58 plasmid-encoded *shsp* genes is induced by heat shock. The heat-induced expression of *hspL* is regulated by RpoH, an alternative σ^{32} -like transcription factor, whereas that of *hspAT1* and *hspAT2* is regulated by the repression of the heat shock gene expression element (ROSE) in their 5' untranslated region (4).

The sHsps are low-molecular-mass chaperones that can stably bind to unfolded proteins and prevent them from heat-induced aggregation (42). The sHsp functions as an ATP-independent mo-

Corresponding author: H.-H. Hwang;
E-mail address: hauhsuan@dragon.nchu.edu.tw

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lecular chaperone and requires the formation of a dynamic quaternary structure by assembling into oligomeric complexes for its activity (7,16). In addition to being induced by heat stress, the expression of *hspL* but not *hspC*, *hspAT1*, and *hspAT2* is induced by the *vir* gene inducer AS at room temperature (59). The HspL protein is mainly localized in the inner membrane and directly interacts with VirB8 in *A. tumefaciens* (57). Thermal aggregation protection assays demonstrated that the VirB8 chaperone activity of HspL is relatively more efficient than that of the other three sHsps (57,58). The absence of HspL affects the tumorigenesis and VirB/D4-mediated conjugal DNA transfer efficiency, which demonstrates the important roles of the sHsp in T4SS stability and *A. tumefaciens* virulence with room-temperature growth (59).

In this study, we further established the important role of HspL in *A. tumefaciens* virulence under heat shock conditions. VirB8 protein accumulation and *Agrobacterium* transformation efficiency were lower under heat stress than at room temperature. Importantly, heat-reduced VirB8 protein level and transformation efficiency was accelerated in the *hspL* mutant. Overexpression of HspL in *A. tumefaciens* enhanced the transient transformation efficiencies of both susceptible and recalcitrant *Arabidopsis* ecotypes. This study further reveals a critical role of HspL in maintaining VirB8 homeostasis and in promoting *A. tumefaciens* transient transformation efficiency during heat stress.

MATERIALS AND METHODS

Plasmid constructions. We first generated the HspL overexpression cassette. *A. tumefaciens* cells grown overnight in 523 medium (24) with appropriate antibiotics were used to isolate genomic DNA with the Qiagen genomic DNA kit (Qiagen, Valencia, CA). The polymerase chain reaction (PCR) product was amplified with use of the Advantage 2 PCR Enzyme System (BD Biosciences Clontech, Palo Alto, CA) with 1 µg of genomic DNA as the template. The PCR amplification cycles were as follows: 95°C for 2 min (1 cycle); 95°C for 1 min, 56°C for 1 min, and 72°C for 1 min/per kb (35 cycles); and 72°C for 10 min (1 cycle). The *hspL* gene was amplified from *A. tumefaciens* genomic DNA with the primer sequences for HspL-rbs-KpnI-F (5'-GTGGT ACCATCGCCAGAATGGGATG-3') and HspL-R-stop (5'-GAA AGCTTACTATTAGTTGACCTGA-3') (with primer sequences of designed restriction enzyme sites shown in bold and comple-

mentary to the specific region in the *A. tumefaciens* genome in italics) and cloned into the pTrc200 vector (48), which resulted in the plasmid pTrc-hspLstop.

The HspL overexpression cassette was digested with *PvuII*/*HindIII* from the plasmid pTrc-hspLstop, and both ends of the DNA fragment were filled with DNA polymerase I (New England Biolabs Inc., Ipswich, MA) to create blunt ends. The plasmid pCambia1305.02 was digested with *SacII* and filled with DNA polymerase I and ligated with the HspL overexpression cassette to create the plasmid pCambia1305.02-HspL. Other plasmids used in this study are in Table 1.

Bacterial strains and culture. *A. tumefaciens* and *Escherichia coli* strains used in this study are in Table 1. *A. tumefaciens* strains were grown in 523 medium or on 523 agar supplemented with appropriate antibiotics (erythromycin at 50 µg ml⁻¹, rifampicin at 50 µg ml⁻¹, gentamycin at 50 µg ml⁻¹, spectinomycin at 100 µg ml⁻¹, and kanamycin at 20 µg ml⁻¹) (MDBio Inc., Taipei, Taiwan) at 28°C. *E. coli* strains were grown at 37°C in 2× YT medium (46) containing appropriate antibiotics (kanamycin at 50 µg ml⁻¹).

Immunoblot analysis. To induce the overexpression of sHsps from pTrc200 (58) in *A. tumefaciens*, bacteria were first grown overnight in 523 medium with appropriate antibiotics and subcultured in acidic AB-MES medium (33) without antibiotics at an absorbance at 600 nm (*A*₆₀₀) = 0.1. After growth at 28°C to mid-log phase for 6 h, bacterial cultures were further cultivated for 24 h at 28°C containing 200 µM AS (Sigma-Aldrich, St. Louis) with or without 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (MDBio Inc.), followed by heat-shock treatments at 37°C for 2 or 6 h or at 42°C for 1 h. To determine the VirB8 protein level in *A. tumefaciens*, bacteria were cultured in acidic AB-MES medium without antibiotics to *A*₆₀₀ = 0.4, cultured for 16 h at 28°C with 200 µM AS, then transferred to 37°C for 15, 30, or 45 min.

Bacterial cultures were collected at different times and protein was isolated as described (22). In brief, bacterial cells were harvested by centrifugation at 10,000 × *g*, 4°C, for 10 min and resuspended in Tris-EDTA buffer (10 mM Tris-HCl and 1 mM EDTA) to *A*₆₀₀ = 10, then boiled for 10 min with 4× sample loading buffer (0.25 M Tris-HCl, 9% sodium dodecyl sulfate [SDS], and 30% glycerol) to disrupt cells. Protein concentration was measured by a BCA protein assay (Pierce Biotechnology, Rockford, IL) with a Power Wave X 340 Microplate Reader (Bio-Tek Instruments, Winooski, VT). In total, 15 µg of crude protein

TABLE 1. Bacterial strains and plasmids

Strains, plasmids	Relevant characteristics ^a	Reference, source
Strains		
<i>Agrobacterium tumefaciens</i>		
NT1RE(pJK270)	Rm ^R Em ^R , Km ^R /Nm ^R , pJK270 is pTiC58Tra ^C with <i>Tn5</i> insertion in T-DNA region without affecting virulence	26
EML1057(Δ <i>hspL</i>)	Rm ^R Em ^R , Km ^R / Nm ^R , markerless <i>hspL</i> deletion mutant in NT1RE(pJK270)	59
EML1280(Δ <i>hspL</i> + <i>hspL</i>)	Rm ^R Em ^R , Km ^R / Nm ^R , Tc ^R , p <i>HspL</i> in Δ <i>hspL</i> for complementation experiment	59
GV3101(pMP90)	Rm ^R Gm ^R , GV3101 harboring pTiC58AT-DNA	29
<i>Escherichia coli</i>		
DH10B	Host for DNA cloning	Invitrogen, Carlsbad, CA
S-17	Host for conjugation	50
Plasmids		
pTrc200	Sp ^R , pVS1 origin <i>lacIq</i> , <i>trc</i> promoter expression vector	48
pTrc-hspLstop	Sp ^R , the <i>HspL</i> gene fragment in pTrc200 vector	This study
pTrcHspL-His	Sp ^R , expression of HspL-His by <i>trc</i> promoter in pTrc200 vector	58
pTrcHspC-His	Sp ^R , expression of HspC-His by <i>trc</i> promoter in pTrc200 vector	58
pTrcHspAT1-His	Sp ^R , expression of HspAT1-His by <i>trc</i> promoter in pTrc200 vector	58
pTrcHspAT2-His	Sp ^R , expression of HspAT2-His by <i>trc</i> promoter in pTrc200 vector	58
pCambia1305.02	Km ^R , a binary vector for plant transformation	18,19
pCambia1305.02-HspL	Km ^R , the HspL overexpression cassette in the binary vector pCambia 1305.02	This study
pCambia 1201-Gm	Cm ^R , Gm ^R , a binary vector for plant transformation	22
pCambia 2201-Gm	Cm ^R , Gm ^R , a binary vector for plant transformation	22
pJZ383	Sp ^R , expression of GFP by <i>tac</i> promoter in pVS vector	56

^a Cm^R, Em^R, Gm^R, Km^R, Nm^R, Rm^R, Sp^R, and Tc^R indicate resistant to chloramphenicol, gentamicin, erythromycin, kanamycin, neomycin, rifampicin, spectinomycin, and tetracycline, respectively.

extract was equally loaded and resolved on 16.5% Tricine SDS-polyacrylamide gels (MDBio Inc.), then transferred to Hybond ECL Nitrocellulose membrane (Amersham Biosciences, Uppsala, Sweden), which was incubated with primary antibodies for HspL (1:20,000) (59), VirB8 (1:10,000) (6), VirB2 (1:1,000) (49), RNA polymerase α subunit (RpoA antibody) (1:7,500) (36,58), or neomycin phosphotransferase II (NptII) (1:50,000) (Sigma-Aldrich), then with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G antibody (1:50,000) (Chemicon International, Inc., Temecula, CA). The membranes were developed by chemiluminescent detection (PerkinElmer Life and Analytical Sciences, Boston) and underwent autoradiography.

Transient transformation assay of *Arabidopsis*. Transient transformation assay of *Arabidopsis* roots was as described (21, 22), with minor modifications. The 523 medium-grown bacterial cells were washed with 0.9% sodium chloride and resuspended in 0.9% sodium chloride at 10^7 or 10^8 colony forming units (CFU) ml⁻¹. Root segments of more than 10 different 3- to 4-week-old *Arabidopsis thaliana* plants were infected with *A. tumefaciens* strains containing the binary vectors pCambia2201-Gm, pCambia1201-Gm (22), pCambia1305.02-HspL, or pCambia1305.02 (18, 19). Prepared roots and bacteria were co-cultivated on Murashige and Skoog (MS) medium at 28°C for 2 days.

For transient transformation assay of *A. tumefaciens* strains containing the pTrc200 vectors, bacterial cells first grown in 523 medium overnight were cultivated for 24 h at 28°C in acidic AB-MES medium with 200 μ M AS and 0.4 mM IPTG to induce *vir* and *sHsps* expression, respectively. After AS and IPTG induction, bacterial cells were washed and resuspended in 0.9% sodium chloride at 10^7 or 10^8 CFU ml⁻¹ for transformation assays. For heat treatments in transient transformation assays, roots and bacteria were first incubated at 37°C for 0.5, 2, or 6 h or at 42°C for 1 h on MS medium, then incubated at 28°C for 2 days.

At least 70 root segments from each plant were examined during each independent transformation assay. At 6 or 7 days after *A. tumefaciens* infection, *Arabidopsis* root segments were stained with 5-bromo-4-chloro-3-indolyl β -D-glucuronic acid staining solution (22) for 1 day at 37°C. Root segments were observed under a stereoscopic microscope to determine transient transformation efficiency. Data represent mean transformation frequency from at least three independent experiments.

***Arabidopsis* root attachment assays.** *Arabidopsis* root attachment assays were as described (56), with minor modification. Roots of 14-day-old *Arabidopsis thaliana* seedlings grown on B5 agar medium were cut into 1-cm segments and transferred to sterile dishes with 1 mM CaCl₂ and 0.4% sucrose solutions. *Agrobacterium tumefaciens* strains were first transformed with a green fluorescent protein (GFP) reporter plasmid, pJZ383 (56), by electroporation. Then, bacteria at 10^7 CFU ml⁻¹ were co-incubated with more than five root segments at 19°C in the dark for 48 h with shaking at very low speed (50 rpm). After co-incubation for 8 h, root segments were recovered, rinsed, resuspended in fresh CaCl₂ and sucrose solutions, placed on slides, and observed under an Olympus IX71 fluorescence microscope (Olympus Optical Co., Tokyo) with the DP Controller program.

RESULTS

The *A. tumefaciens* hspL mutant showed reduced transient transformation efficiency in *Arabidopsis* under heat shock. Previous studies have demonstrated that the HspL protein directly interacts with the VirB8 protein and protects the GST-VirB8 fusion protein against thermal aggregation in vitro (57). The levels of several AS-induced VirB proteins were reduced in the *A. tumefaciens* hspL deletion mutant grown at room temperature (59). To investigate whether the HspL level was associated with VirB8 level in *A. tumefaciens* under heat stress, we determined the VirB8 level in the wild type and hspL deletion mutant during

different times of 37°C heat-shock treatment. The AS-induced VirB8 protein level was decreased in both the wild type and hspL deletion mutant with increasing heat-shock treatment from 0 to 45 min (Fig. 1A). In addition, reduction in AS-induced VirB8 level was greater in the hspL deletion mutant than the wild type (Fig. 1A and 1). After the 15-min heat-shock treatment, the AS-induced VirB8 protein level was reduced to 24% of the original protein level in the hspL mutant but 42% in the wild type (Fig. 1B). After the 45-min heat shock, the AS-induced VirB8 protein level was reduced to 7% in the hspL deletion mutant but 25% in the wild type (Fig. 1B). In contrast, AS-induced VirB2 protein level was not significantly altered by heat shock (Fig. 1A), which agrees with previous studies (6). These data suggest that the HspL protein may be required to maintain VirB8 protein homeostasis in *A. tumefaciens* under heat stress.

Because of the reduced VirB8 protein level in *A. tumefaciens* with heat shock, we next examined the transient transformation efficiency of *A. tumefaciens* in *Arabidopsis* roots with and without a 30-min heat shock. Root segments of *Arabidopsis* plants were infected with the *A. tumefaciens* strains containing the binary vector pCambia1201-Gm on MS medium at 28°C for 2 days. For heat-shock treatments, roots and bacteria were first incubated at 37°C for 30 min on MS medium, then at 28°C for 2 days. The transient transformation efficiency of the hspL deletion mutant was less than 50% of the wild-type efficiency (Fig. 1C), whereas the hspL-complemented strain restored the reduced transient transformation efficiency of the hspL mutant to the wild-type efficiency (Fig. 1C). After heat-shock treatment, the wild type and hspL-complemented strain both showed more than one-third reduction in transient transformation efficiency as compared with no heat-shock treatment (Fig. 1C). In the hspL mutant, transient transformation efficiency was lower, by approximately 45%, with than without heat-shock treatment. In addition, bacterial growth rates did not differ between the hspL deletion mutant, the wild type, and the hspL-complemented strains when grown in acidic minimal medium at 28°C for 24 h with AS induction, followed by 37°C heat-shock treatment for up to 6 h (Supplemental Figure 1). These data suggest that the HspL protein may play a role in enhancing *Agrobacterium* transformation efficiency under heat stress.

Overexpression of HspL but not HspC, HspAT1, or HspAT2 in the wild-type *A. tumefaciens* enhanced transient transformation efficiency in *Arabidopsis*. In *A. tumefaciens*, tumorigenesis and T4SS-mediated RSF1010 transfer efficiency was affected more by the deletion of four *shsp* genes (*HspL*, *HspC*, *HspAT1*, and *HspAT2*) than by single *hsp* deletion (58). All four sHsps equally possess chaperone activities for the model substrate citrate synthase in vitro (58). However, among the four sHsps, only HspL functions as an efficient molecular chaperone in preventing GST-VirB8 proteins from thermal aggregation (58). We determined whether overexpression of each of the four sHsps in wild-type *A. tumefaciens* could enhance the VirB8 protein stability and, therefore, affect *A. tumefaciens* transient transformation efficiency. We successfully induced overexpression of the four His-tagged sHsps from the *trc* promoter in wild-type *A. tumefaciens* by adding IPTG to AB-MES media with AS at 28°C (58) (Fig. 2A). In the wild type, accumulation of His-tagged HspL, HspC, HspAT1, and HspAT2 was greater with than without IPTG (Fig. 2A). The levels of HspL, VirB8, and VirB2 proteins were increased because of the presence of the *vir* gene inducer AS (Fig. 2A). When co-expressed with either one of the four His-tagged sHsps, VirB8 showed a slightly higher level with IPTG than empty-vector (pTrc200) induction. In contrast, VirB2 protein level did not differ with empty-vector or IPTG induction. The level of NptII protein as a loading control was similar in all tested strains with or without IPTG. Therefore, each of four sHsps overexpressed in the wild type may help to stabilize the VirB8 protein, which agrees with previous studies (58).

We determined the transient transformation efficiency of these strains with and without IPTG treatment. Only the wild-type strain with the IPTG-induced His-tagged HspL protein showed significantly higher transient transformation efficiency than all the other four strains in the presence of IPTG (Fig. 2B). In the absence of IPTG, transient transformation efficiency of the wild type with each of the four sHsps did not differ from the wild type with empty vector (Fig. 2B). Overexpression of each of the four sHsps in the wild type may enhance VirB8 accumulation; however, only the wild type with IPTG-induced His-tagged HspL protein could efficiently increase *A. tumefaciens* virulence.

We next determined whether overexpression of HspL protein in the wild type could affect *A. tumefaciens* VirB8 protein level under heat stress. The addition of AS induced HspL, VirB8, and VirB2 protein expression with empty vector or His-tagged HspL grown at 28°C with and without IPTG. With a shift of bacteria to 37°C for 2 h or 42°C for 1 h, HspL protein level was further increased with the empty vector with or without IPTG (Fig. 2C). Although the HspL level was induced by a short period of heat-shock treatment, VirB8 protein stability was still affected by the heat stress. VirB8 level was lower in the wild type with the empty vector grown at 37°C for 2 h or 42°C for 1 h than at 28°C (Fig. 2C). In contrast, both VirB2 and NptII protein levels were not significantly affected by the heat-shock treatments. HspL level

was slightly decreased on transfer of the wild-type bacteria with empty vector from 28 to 37°C for 6 h. VirB8 similarly accumulated at the lowest level in the same strain when grown at 37°C for 6 h (Fig. 2C). Thus, a longer period of heat treatment may affect both HspL and VirB8 protein stability. In the presence of IPTG, the level of His-tagged HspL protein was higher with HspL-His than with empty vector (Fig. 2C). The level of VirB8 protein was also higher in the wild type overexpressing the HspL-His protein than in the wild type containing the empty vector under three kinds of heat-shock treatments. These data further support the idea that overexpression of HspL protein may help maintain VirB8 homeostasis in *A. tumefaciens* during heat stress.

We next examined the effect of heat-shock treatments on transient transformation efficiency during *Agrobacterium* and *Arabidopsis* root co-incubation. Root segments of *Arabidopsis* plants were infected with the *A. tumefaciens* strains containing the pTrc200 vectors and the binary vector pCambia2201-Gm on MS medium at 28°C for 2 days. For heat-shock treatments, roots and bacteria were first incubated at 37°C for 2 or 6 h or at 42°C for 1 h on MS medium, then at 28°C for 2 days. Transient transformation efficiency was reduced nearly one-third in the wild type with an empty vector after heat-shock treatment of 37°C for 2 h or 42°C for 1 h as compared with no-heat-shock treatment (Fig. 2D). Transient transformation efficiency of the wild type

Fig. 1. The *Agrobacterium tumefaciens* *hspL* mutant showed reduced VirB8 protein level and transient transformation efficiency in *Arabidopsis* under heat shock. **A**, Immunoblot analyses of VirB8, HspL, VirB2, and RpoA in the wild type (WT) (NTIRE[pJK270]) and *hspL* deletion ($\Delta hspL$) *A. tumefaciens* strains grown in acidic AB-MES media with 200 μ M acetosyringone (AS) at 28°C for 16 h, then 37°C for 15, 30, or 45 min. RpoA was a loading control. **B**, Quantification of VirB8 protein level over time. The VirB8 protein accumulation of both strains were set to 100% under no-heat-shock treatment. The VirB8 accumulation at different times is relative to that at time 0 for each strain. **C**, Transient transformation efficiency of the WT, *hspL* mutant, and *hspL*-complemented ($\Delta hspL$ + HspL) strains in *Arabidopsis* roots. *Arabidopsis* root segments were infected with bacteria of various *A. tumefaciens* strains at 10^8 CFU/ml on Murashige and Skoog (MS) medium at 28°C for 2 days. For heat-shock treatments, roots and bacteria were first incubated at 37°C for 30 min on MS medium, then at 28°C for 2 days. Root segments were stained with 5-bromo-4-chloro-3-indolyl β -D-glucuronic acid staining solution 7 days after infection. Data are mean $\pm$ standard error from at least three independent experiments. Symbols: * indicates $P < 0.05$ compared with the WT by pairwise Student's *t* test and "a" indicates significant difference ($P < 0.05$) with and without heat treatment within the same strain during infection by pairwise Student's *t* test.

with the empty vector or His-tagged HspL was reduced most with heat-shock treatment of 37°C for 6 h than without heat-shock treatment (Fig. 2D). In the presence of IPTG, transient transformation efficiency was increased more than 1.5-fold with His-tagged HspL overexpression than with the empty vector among the four tested experimental conditions (Fig. 2D). VirB8 level was positively associated with *A. tumefaciens* transient transformation efficiency (Fig. 2C and D). Thus, IPTG-induced His-tagged HspL expression greatly enhanced VirB8 protein stability and transient transformation efficiency of *A. tumefaciens* during heat stress.

Overexpression of HspL in *A. tumefaciens* increased transient transformation efficiency of several recalcitrant *Arabidopsis* ecotypes. We next examined whether overexpression of HspL in the wild type could increase *A. tumefaciens* transformation efficiency in other recalcitrant *Arabidopsis* ecotypes and mutants. We chose four *Arabidopsis* ecotypes (UE-1, Petergof, BL1, and Dijon-G) and two T-DNA insertion mutants, resistant to *Agrobacterium* transformation (*rat3* and *rat4*) (39,41, 65), to determine the transient transformation efficiency of the *hspL* mutant and the HspL overexpression strain at room tem-

perature. The ecotype UE-1 is likely blocked in T-DNA integration, thus resulting in higher transient transformation efficiency than other recalcitrant *Arabidopsis* ecotypes, including Petergof, BL1, and Dijon-G (39,41). The *A. tumefaciens* infection processes of the ecotypes BL1 and Petergof and *rat3* and *rat4* mutants are likely deficient in bacterial attachment, thus causing lower transient transformation efficiency than susceptible *Arabidopsis* ecotypes with *A. tumefaciens* infection (39,41,65). On infection with wild-type *A. tumefaciens*, the transient transformation efficiency of Dijon-G was lowest among the four tested ecotypes and two tested *rat* mutants (Fig. 3A), which agrees with previous studies (39,41). Similar to transient transformation results obtained with the ecotype Ws (Fig. 1C), the *hspL* mutant showed nearly one- to two-third reduction in transient transformation efficiency as compared with the wild type and *hspL*-complemented strains on infection in the four *Arabidopsis* ecotypes and two *rat* mutants (Fig. 3A).

We inserted the *HspL* overexpression cassette into the pCambia1305.02 binary vector and introduced the vector into the disarmed *A. tumefaciens* strain GV3101 for overexpressing

Fig. 2. Overexpression of the His-tagged HspL protein in wild-type (WT) *Agrobacterium tumefaciens* enhanced transient transformation efficiency in *Arabidopsis* under heat-shock treatment. **A**, Immunoblot analysis of the His-tagged sHsps HspL, VirB8, VirB2, and NptII in the WT (NT1RE[pJK270]) with the empty vector (pTrc200) or His-tagged sHsps grown in acidic AB-MES media with 200 μ M acetosyringone (AS) at 28°C for 16 h with and without isopropyl- β -D-thiogalactopyranoside (IPTG). NptII was a loading control. **B**, Transient transformation efficiency of the WT with the empty vector or overexpressing the IPTG-induced His-tagged HspL, HspC, HspAT1, or HspAT2 proteins in *Arabidopsis* roots. *Arabidopsis* root segments were infected with bacteria of various *A. tumefaciens* strains at 10^7 CFU/ml in Murashige and Skoog (MS) medium containing 200 μ M AS with or without 0.4 mM IPTG at 28°C for 2 days. Data are mean $\pm$ standard error from at least three independent experiments. Symbol: * indicates $P < 0.05$ between the WT with empty vector and IPTG-induced His-tagged HspL under the same temperature by pairwise Student's *t* test. **C**, Immunoblot analyses of level of HspL, His-tagged sHsps VirB8, VirB2, and NptII in the WT with the empty vector (pTrc200) or the His-tagged HspL protein grown in acidic AB-MES medium with 200 μ M AS at 28°C for 16 h, then 37°C for 2 or 6 h or 42°C for 1 h with and without IPTG. **D**, Transient transformation efficiency of the WT with the empty vector or overexpressing the IPTG-induced His-tagged HspL protein in *Arabidopsis* roots. *Arabidopsis* root segments were infected with bacteria of two *A. tumefaciens* strains at 10^7 CFU/ml in MS medium containing 200 μ M AS with or without 0.4 mM IPTG. During co-incubation, roots and bacteria were first incubated at 37°C for 2 or 6 h, or at 42°C for 1 h on MS medium, then at 28°C for 2 days. Symbols: * indicates $P < 0.05$ between the WT with empty vector and IPTG-induced His-tagged HspL under the same temperature by pairwise Student's *t* test; "a" or "b" indicate significant difference ($P < 0.05$) with and without heat treatment with the same strain during infection by analysis of variance.

HspL protein without IPTG induction. We detected HspL protein only in the HspL overexpression strain in the absence of AS (at 0 h) (Fig. 3B). The level of HspL was relatively higher in the HspL overexpression strain than in the wild type GV3101 with the empty vector pCambia1305.02 under AS induction. The AS-induced VirB8 protein accumulated at relatively higher levels at an early time of AS induction in the HspL overexpression strain, and its level was also positively associated with HspL level in the same strain. In contrast, the AS-induced VirD2 protein level was similar in both the wild type and the HspL overexpression strain.

We next performed transient transformation assays with these two strains under room-temperature growth on five tested *Arabidopsis* ecotypes (Ws, UE-1, Peterhof, BL1, and Dijon-G) and the two *rat* mutants (*rat3* and *rat4*). Similar to results shown in Figure 2B and D, constitutive overexpression of the HspL protein in the wild type enhanced the transient transformation efficiency 1.25- to 2-fold on infection with four of the *Arabidopsis* ecotypes (Ws, UE-1, Peterhof, and BL1) and *rat3* and *rat4* but did not affect that of Dijon-G (Fig. 3C).

Overexpression of the HspL protein in the wild-type *A. tumefaciens* improved *A. tumefaciens* virulence on the attachment-deficient *Arabidopsis* ecotypes Peterhof and BL1 and attachment-deficient *rat* mutants *rat3* and *rat4*. Therefore, we examined whether overexpression of the HspL protein in *A. tumefaciens* affected bacterial attachment on *Arabidopsis* roots. We used the wild-type and HspL overexpression strains with constitutively expressed GFP to determine bacterial aggregation on roots of the transformation-susceptible *Arabidopsis* ecotype Ws and transformation-recalcitrant *Arabidopsis* ecotype BL1. Previous studies showed that *A. tumefaciens* could not efficiently bind root seg-

ments of the *Arabidopsis* ecotype BL1 (39,41,65). We observed less bacterial aggregation in roots of BL1 than Ws plants (Fig. 3D). However, the bacterial aggregation of both the wild type and the HspL overexpression strains on Ws or BL1 root segments were similar, which suggests that the overexpression of HspL protein in *A. tumefaciens* did not significantly enhance bacterial attachment ability on *Arabidopsis* roots.

DISCUSSION

Temperature is among the major physical factors affecting the growth and development of organisms on earth. Both prokaryotic and eukaryotic cells activate signaling pathways to adjust their metabolism and cell function to reduce heat-associated damage. Many phenotypic effects of heat stress are caused by the unspecific aggregation or incorrect folding of proteins and unbalanced protein homeostasis. In response to heat stress, cells cause the transient expression of a group of heat shock or heat stress proteins (Hsps). The Hsps function as molecular chaperones that bind to partially folded or denatured substrate proteins and prevent the formation of wrong protein aggregates or translocate nonfunctional proteins for degradation in lysosomes or proteasomes (37,42,45). Among different members of Hsp families, the monomers of the sHsps are low, ranging from 15 to 30 kDa, and they have a highly conserved C-terminal α -crystallin domain of 90 amino acids (7,16,54). Depending on the organisms' ambient living environment, environmental temperature variations, and their genome size, the gene numbers encoding sHsps varies (25,38,47,53). Different functions for multiple sHsps in different organelles and cell types under various stresses might have caused

Fig. 3. The *Agrobacterium tumefaciens* HspL overexpression strain enhanced transient transformation efficiency of several *Arabidopsis* ecotypes. **A**, Transient transformation efficiency of the wild-type (WT), *hspL* mutant, and *hspL* complemented ($\Delta hspL + HspL$) strains on roots of different *Arabidopsis* ecotypes. *Arabidopsis* root segments were infected with bacteria of various *A. tumefaciens* strains at 10^8 CFU/ml on Murashige and Skoog (MS) medium at 28°C for 2 days. Data are mean $\pm$ standard error from at least three independent experiments. Symbol: * indicates $P < 0.05$ between WT and *hspL* by pairwise Student's *t* test. **B**, Immunoblot analysis of HspL, VirB8, and VirD2 in the WT (GV3101) strain with the empty vector pCambia1305.02 and the HspL overexpression (HspL O/E) strain with the plasmid pCambia1305.02-HspL grown in acidic AB-MES medium with 200 μ M AS at 28°C for 0, 4, 8, and 12 h. Ponceau S (PS) staining of the membrane was a loading control. **C**, Transient transformation efficiency of the WT (GV3101) and HspL overexpression (HspL O/E) strain on *Arabidopsis* roots. *Arabidopsis* root segments were infected with bacteria of two *A. tumefaciens* strains at 10^7 CFU/ml on MS medium at 28°C for 2 days. Data are mean $\pm$ standard error from at least three independent experiments. Symbol: * indicates $P < 0.05$ between the WT (GV3101) and HspL O/E strain by pairwise Student's *t* test. **D**, Bacterial attachment assays of green fluorescent protein (GFP)-tagged *A. tumefaciens* strains, the WT (GV3101) and HspL O/E strain, on root segments of Ws and BL1 plants. Red arrows indicate bacterial aggregations on *Arabidopsis* root segments. Scale bars, 2 mm.

diversification (34,42,53,55). Four α -crystallin-type small heat shock genes—*hspC*, *hspL*, *hspAT1*, and *hspAT2*—exist in different replicons of *A. tumefaciens* (4). All four sHsps exhibit similar chaperone activity for the model substrate citrate synthase but HspL possesses higher chaperone activity for the VirB8 protein (58). In this study, we successfully established that the absence of HspL protein in *A. tumefaciens* had adverse effects on VirB8 protein homeostasis and bacterial transient transformation efficiency under heat stress. This study corroborated further that the HspL protein functions as the most efficient chaperone for VirB8 and is important in transient transformation efficiency of *A. tumefaciens* under heat stress.

Transient transformation efficiency of the wild type decreased nearly one-third on co-incubation of bacteria and plant roots with a temperature increase from 28 to 37°C for up to 2 h or 42°C for 1 h (Figs. 1C and 2D). By increasing the co-incubation temperature from 28 to 37°C for 6 h, the transient transformation efficiency was reduced nearly half that without heat treatment (Fig. 2D). Previous studies showed that the function and accumulation of several Vir proteins was significantly reduced at elevated temperatures (6,15,23,33). The activation of *vir* genes is regulated by the VirA/VirG two-component regulatory system. With incubation temperature above 32°C, VirA is inactive because of conformational change, which significantly affects downstream *vir* gene expression (23). Furthermore, the levels of several VirB proteins, VirD2, VirE2, as well as the T4SS assembly were substantially decreased with increased temperature (6,15,33). We found that the level of VirB8 but not VirB2 was considerably reduced with increasing heat treatment time (Figs. 1A and 2C). Thus, stability of different VirB proteins in *A. tumefaciens* may differ at temperatures higher than room temperature, which agrees with previous study (6).

We found that, with lack of the VirB8 chaperone protein HspL, VirB8 protein level was significantly affected by heat-shock treatment, and heat-induced VirB8 degradation may furthermore decrease *A. tumefaciens* transient transformation efficiency as compared with the wild type under heat-shock treatment. In addition, IPTG-induced or constitutive overexpression of HspL protein in the wild type enhanced VirB8 protein abundance and *A. tumefaciens* virulence with heat-shock treatment time. Although the IPTG-induced overexpression of the other three sHsps—HspC, HspAT1, and HspAT2—also increased VirB8 protein level in the wild type, only overexpression of HspL could increase *A. tumefaciens* transient transformation efficiency under heat-shock treatment. A previous study (58) also found no significant reduction in tumorigenesis efficiency in the *hspC*, *hspAT1*, or *hspAT2* single-deletion mutants, and HspL protein possessed higher VirB8 chaperone activity than HspAT2. Our data support the idea that HspL protein is the most efficient VirB8 chaperone (58), and it may play a major role in the *A. tumefaciens*-mediated transformation process under heat stress.

Previous studies revealed increased bacterial thermotolerance with overexpression of the recombinant rice OsHsp16.9-CI- β -glucuronidase (GUS) fusion protein or the chestnut Cshsp17.5 protein in *E. coli* (52,62). Heat shock factor (HSF) is essential for the transcription of Hsps. Several gain-of-function studies demonstrated that overexpression of *AtHSF1*-GUS, *AtHSF3*, or *AtHSF3*-GUS in transgenic *Arabidopsis* derepressed the expression of several heat-shock genes at normal temperature and the level of basic thermotolerance was increased (35,44,53). Data from our study and previous studies support the idea that overexpression of sHsps in both prokaryotic and eukaryotic organisms enhances heat stress tolerance.

We used various *Arabidopsis* ecotypes and mutants that are resistant to *A. tumefaciens* transformation to determine whether constitutive overexpression of HspL protein in the wild type could enhance *A. tumefaciens* virulence. The transient transformation efficiency was higher in the HspL overexpression strain than the

wild type on infection of bacteria-attachment-deficient *Arabidopsis* mutants or ecotypes and the T-DNA-integration-deficient *Arabidopsis* ecotype. In addition, the bacterial aggregation of the HspL overexpression strain and the wild type did not differ; therefore, overexpression of the HspL protein may not directly affect bacteria attachment ability. Our data and those from previous studies (57,58) demonstrate that overexpression of HspL in *A. tumefaciens* directly enhances VirB8 protein level. VirB8 protein is one of the core components of the T4SS machinery and may play a key role in maintaining the stability of the T4SS complex (5,11,12,30,61). VirB8 also forms a dimer in *A. tumefaciens*; the dimer interface helps VirB2 protein target the T-pilus assembly sites and is required for proper T4SS functions (9,51, 63). Thus, VirB8 protein may function as a nucleating factor to help the T4SS machinery assembly (1,9). From previous studies of VirB8 protein function, the higher transient transformation efficiency of the HspL overexpression strain may be due to higher VirB8 protein levels and, therefore, may increase T4SS machinery stability and function. Previous studies also demonstrated that sHsps in other organisms may act as membrane-stabilizing factors and may play a role in controlling the integrity of membranes (40). In addition, overexpression of HspL might modify the bacteria membrane property and affect T4SS machinery stability. Hence, overexpression of HspL may increase *A. tumefaciens* virulence at early stages, such as T-DNA and effector Vir protein transfer, during *A. tumefaciens* infection and possibly enhance *A. tumefaciens* virulence when infecting several recalcitrant *Arabidopsis* ecotypes.

A. tumefaciens-mediated plant transformation has been the most commonly used genetic transformation method during the past few decades. Genes within the T-DNA can be replaced by almost any gene of interest; therefore, *A. tumefaciens* is an important and valuable tool for plant biology research and genetic engineering. However, this technique cannot efficiently be used for many economically important plant species or elite varieties of particular crop species. Hence, several strategies have been used to enhance *A. tumefaciens* transformation efficiency, including use of the supervirulent *A. tumefaciens* strain EHA101 that contains the disarmed version of the supervirulent Ti plasmid pTiBo542 or superbinary vectors that carry additional *virB*, *virG*, and *virE* genes of pTiBo542 (3,14,20,27,28). Our study provides another approach to enhance *A. tumefaciens*-mediated plant transformation efficiency by overexpressing the HspL protein in wild-type *A. tumefaciens*. Further investigation of this HspL overexpression strain on different recalcitrant crop species may provide useful information for more efficiently generating transgenic crops.

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