Type III secretion chaperones ShcS1 and ShcO1 from *Pseudomonas syringae* pv. *tomato* DC3000 bind more than one effector

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The hrp-type III secretion (TTS) system is a key pathogenicity factor of the plant pathogen *Pseudomonas syringae* pv. *tomato* DC3000 that translocates effector proteins into the cytosol of the eukaryotic host cell. The translocation of a subset of effectors is dependent on specific chaperones. In this study an operon encoding a TTS chaperone (ShcS1) and the truncated effector HopS1′ was characterized. Yeast two-hybrid analysis and pull-down assays demonstrated that these proteins interact. Using protein fusions to AvrRpt2 it was shown that ShcS1 facilitates the translocation of HopS1′, suggesting that ShcS1 is a TTS chaperone for HopS1′ and that amino acids 1 to 118 of HopS1′ are required for translocation. *P. syringae* pv. *tomato* DC3000 carries two shcS1 homologues, shcO1 and shcS2, which are located in different operons, and both operons include additional putative effector genes. Transcomplementation experiments showed that ShcS1 and ShcO1, but not ShcS2, can facilitate the translocation of HopS1′:AvrRpt2. To characterize the specificities of the putative chaperones, yeast two-hybrid interaction studies were performed between the three chaperones and putative target effectors. These experiments showed that both ShcS1 and ShcO1 bind to two different effectors, HopS1′ and HopO1-1, that share only 16% amino acid sequence identity. Using gel filtration it was shown that ShcS1 forms homodimers, and this was confirmed by yeast two-hybrid experiments. In addition, ShcS1 is also able to form heterodimers with ShcO1. These data demonstrate that ShcS1 and ShcO1 are exceptional class IA TTS chaperones because they can bind more than one target effector.

INTRODUCTION

The bacterial plant pathogen *Pseudomonas syringae* causes disease in a wide variety of plant species. *P. syringae* pv. *tomato* (*Pto*) strain DC3000 has evolved as one of the model strains because it infects the model plant *Arabidopsis thaliana*. The pathogenicity of *P. syringae* is dependent on a type III secretion (TTS) system (Hrp) that spans both bacterial membranes and carries a pilus as an extracellular extension (Galán & Collmer, 1999; Hueck, 1998; Jin et al., 2003). Homologous TTS systems are found in many Gram-negative plant, animal and insect pathogens as well as symbiotic bacteria (Cornelis & Van Gijsegem, 2000; Dale et al., 2001; Galán & Collmer, 1999; Hueck, 1998).

The genes encoding the components of the TTS system are localized in the *hrp* gene cluster. In *P. syringae*, the alternative sigma factor HrpL controls the expression of *hrp* genes and genes encoding substrates for the TTS system via a conserved promoter element (*hrp*-box) (Xiao et al., 1994; Xiao & Hutcheson, 1994).

Two types of protein travel through the TTS system: effector proteins that are translocated directly into the cytoplasm of the plant-host cell and helper proteins that are secreted to the surrounding medium and are presumed to aid in the translocation process (Büttner & Bonas, 2002; Hueck, 1998). Proteins that are secreted by the *P. syringae* TTS system are termed Hop for Hrp-dependent outer proteins. Some effectors are recognized by plants that carry specific resistance proteins and elicit defence reactions including the hypersensitive response (HR), a fast, localized programmed cell death (Keen, 1990; Klement, 1982). These effectors are named avirulence (Avr) proteins (Leach & White, 1996; Staskawicz et al., 1984). Generally, effectors are believed to be virulence determinants, but we have only begun to reveal their molecular functions (Abramovitch & Martin, 2004; Chang et al., 2004; Hotson & Mudgett, 2004).

The secretion signal that directs protein transport through the TTS system is believed to reside in the N-terminus of Hops (Page & Parsot, 2002). Comparison of the amino acid compositions of effector N-termini revealed common...
characteristics, including a high serine content, an isoleucine, leucine or valine at position 3 or 4, and no aspartate or glutamate residues within the first 12 amino acids (Collmer et al., 2002; Greenberg & Vinatzer, 2003). In addition, some proteins are dependent on the function of specific chaperones to be efficiently secreted by the TTS system. These TTS chaperones were identified first in human pathogens (Parsot et al., 2003; Wattiau & Cornelis, 1993) but more recently have also been described in plant pathogens of the genus P. syringae (Badel et al., 2003; Shan et al., 2004; van Dijk et al., 2002; Wehling et al., 2004) and Erwinia amylovora (Gaudriault et al., 2002). TTS chaperones typically exhibit no sequence similarities, but share common features, such as a small size, an acidic pl and an amphipathic C-terminal z-helix (Parsot et al., 2003).

At least nine different TTS chaperones have been postulated or confirmed for Pto DC3000 (Badel et al., 2003; Guttman et al., 2002; Petnicki-Ocwieja et al., 2002; Shan et al., 2004; van Dijk et al., 2002; Wehling et al., 2004), which is far less than the number of effectors (at least 36) that have been identified in this strain so far (Collmer et al., 2002; Greenberg & Vinatzer, 2003). Different functions were assigned to TTS chaperones. They are thought to prevent aggregation and degradation of their substrates within the bacterial cell, to serve as secretion pilots that direct effectors to the TTS system and to impose a hierarchy onto the secretion of effectors by preferentially facilitating the access of their substrates to the TTS system (Feldman & Cornelis, 2003; Parsot et al., 2003). In addition, TTS chaperones were shown to maintain the N-terminal region of effectors in a partially unfolded state that makes them competent for secretion (Birtalan et al., 2002; Cornelis & Van Gijsegem, 2000; Stebbins & Galan, 2001). TTS chaperones have been grouped into several classes (Parsot et al., 2003): class I chaperones associate with one (class IA) or several (class IB) effectors, and class II chaperones bind translocator proteins that aid in the transfer of effectors across the target eukaryotic membrane. Typically, the genes of effector-specific chaperones (class IA) are located in the vicinity of their target effector genes, whereas genes of chaperones that bind more than one effector (class IB) are located within the cluster of the TTS system genes (Parsot et al., 2003).

The putative shcS1/hopS1’ operon from Pto DC3000 was identified in several independent studies (Boch et al., 2002; Guttman et al., 2002; Petnicki-Ocwieja et al., 2002; Zwiesler-Vollick et al., 2002). The genes were shown to be induced upon infection of the plant (Boch et al., 2002), hrpL-dependently regulated (Boch et al., 2002; Zwiesler-Vollick et al., 2002) and preceded by an hrp-box (Boch et al., 2002; Guttman et al., 2002; Petnicki-Ocwieja et al., 2002; Zwiesler-Vollick et al., 2002). It was proposed that ShcS1 (also designated ChpPtoZ, ORF25 and ShcS4) may represent a TTS chaperone and HopS1’ (also designated HplPtoZ, ORF26 and HopPtoS4) a type III effector (Guttman et al., 2002; Petnicki-Ocwieja et al., 2002). Recently, Schechter et al. (2004) used CyaA reporter fusions to confirm that HopS1’ is an effector that is translocated into plant cells. They also reported that HopS1’ is probably truncated due to the insertion of an insertion-sequence (IS) element. Greenberg & Vinatzer (2003) identified two shcS1-homologous chaperone candidate genes, shcS2 and shcO1, and associated putative effector genes in Pto DC3000.

In this study, we functionally characterized the three predicted TTS chaperones from Pto strain DC3000, ShcS1, ShcO1 and ShcS2. We identified their respective target effectors by interaction studies and revealed that although ShcS1 and ShcO1 contain the typical features of specific effector chaperones (class IA), both of them bind two substrates, HopS1’ and HopO1-1, which exhibit very low amino acid sequence similarity. In addition, we showed that ShcS1 forms homodimers and found evidence that ShcS1 and ShcO1 can also form heterodimers. The gene designations throughout this publication have been adapted to the new unified nomenclature of proteins delivered by the Hrp-TTS system (M. Lindeberg, J. Stavriniades, J. H. Chang, J. R. Alfano, A. Collmer, J. L. Dangl, J. T. Greenberg, J. W. Mansfield & D. S. Guttman, unpublished).

**METHODS**

**Bacterial strains, plasmids and growth conditions.** Strains and plasmids are listed in Table 1. Pseudomonas strains were grown in NYG medium (Daniels et al., 1984) at 28°C. Escherichia coli strain DH5α was used as a host in cloning and strain BL21 for overexpression of genes. E. coli was routinely proliferated at 37°C in LB medium (Sambrook et al., 1989). Cultures for protein enrichment were grown in Supermedium (Qiagen). All cloned genes were amplified by Pfu-based PCR from genomic DNA using the primers indicated in Table 2, cloned into the vector pCR-Blunt II-TOPO (Invitrogen) or pBluescript (Stratagene) and verified by sequence analysis prior to cloning into yeast or expression vectors. The following antibiotics were added to the growth medium as appropriate: rifampicin (Rif; 100 μg ml⁻¹), kanamycin (Km; 25 μg ml⁻¹), spectinomycin (Sp; 100 μg ml⁻¹) and ampicillin (Ap; 100 μg ml⁻¹).

**Plants material and inoculation procedures.** Arabidopsis thaliana was grown from seed in growth chambers under an 8 h photoperiod at 21–24°C and 70% relative humidity. To assay for translocation of effector proteins, bacterial strains carrying AvrRpt260–255 fusion constructs were resuspended in 10 mM MgCl₂ at a density of 5 × 10⁷ c.f.u. ml⁻¹ (Pto strains; OD₆₀₀ 0.05; Ultrospec 3000, Pharmacia Biotech) or 2 x 10⁸ c.f.u. ml⁻¹ (Pseudomonas syringae pv. phaseolicola strains; OD₆₀₀ 0.2) and infiltrated into leaves of A. thaliana Col-0 (RPS2) and rps2 mutant plants (Kunkel et al., 1993) using a 1 ml syringe. Leaves were scored for tissue collapse (HR) 24 h after infiltration.

**Construction of AvrRpt2 fusions.** avrRpt260–255 was amplified by PCR from genomic DNA of Pto JL1065 and cloned into pBluescript using EcoRV and HindIII. Sequences from the 5’ end of hopS1’ were amplified by PCR using primers listed in Table 2 and inserted upstream of the avrRpt260–255 gene. The fusions were sequenced to prevent the introduction of PCR mistakes and transferred into the P. syringae-compatible low-copy-number plasmid pVS61 as BamHI/HindIII fragments. The pVS61 derivatives were introduced into P. syringae strains by triparental mating using the helper plasmid pRK2013.
The non-polar deletion of shcS1 was constructed by digesting a pBluescript-derivative carrying shcS1 and hopS1′::avrRpt280–255 with BstXI and PshAI. Subsequent treatment with mung bean nuclease to create blunt ends, and religation. Sequencing of resulting clones revealed two different deletions. Plasmid pBAJ5005.2 carries a deletion that is in-frame with the rest of the shcS1 ORF, and plasmid pBAJ5005.9 carries a deletion of base pairs 60–211 [Δ(shcS1)] that is out-of-frame with the 5′ region of the hopS1 operon and the 5′::Sp′ cassette that is in a frame-shift after amino acid 20 of ShcS1 followed by an early stop codon. The deletions were transferred into pVSP61 as described above yielding plasmids pVJ5005.2 and pVJ5005.9, respectively.

**Construction of the shcS1–hopS1′ deletion strain.** shcS1 and hopS1′ were deleted from the genome of Pto DC3000 by marker replacement. pCJA5 and pCAJ3 are pBluescript subclones (3 kb, SalI and 5.6 kb, HindIII, respectively) from a cosmid carrying the shcS1–hopS1′ operon on a genomic fragment from Pto DC3000 and were chosen for constructing the deletion vector. The coding region of the shcS1–hopS1′ operon and the 5′ region were deleted from pCJA5 by restriction (ClaI, Klenow) and replaced by a spectinomycin-resistance (Ω::Sp′) cassette from pUC4::Ω (SmaI), resulting in plasmid pJ1. The 5′ region of shcS1 was released from pCAJ3 by EcoRI/EcoRV restriction and inserted into pJ1 next to the Ω::Sp′ cassette, resulting in plasmid pJ2. The complete fragment containing the regions upstream and downstream of the shcS1–hopS1′ operon and the Ω::Sp′ cassette was released from pJ2 (EcoRI/KpnI) and inserted into the mobilizable suicide vector pJP5603 (EcoRI/KpnI). The resulting plasmid pJ3 was introduced into Pto DC3000 by triparental mating and single recombinants (Km′, Sp′) were selected. These strains were screened for a second recombination event that eliminated the plasmid and replaced the shcS1 and hopS1′ genes with the Ω::Sp′ cassette resulting in strain J5 (Km′, Sp′). The deletion of shcS1 and hopS1′ in strain J5 was verified by Southern analysis (data not shown).

**Yeast two-hybrid interaction studies.** Protein–protein interactions were analysed using a LexA-based yeast two-hybrid system (Ausubel et al., 1987). Bait constructs were cloned as fusions to the heterologous DNA-binding protein LexA in pMW103 (EcoRI/XhoI), a kanamycin-resistant derivative of pEG202, and transformed in the *Saccharomyces cerevisiae* mating type a strain RFY206 carrying the reporter plasmid pK103. Prey constructs were cloned as fusions to the B42 transcriptional activation domain and a haemagglutinin (HA) epitope tag into pMW103 (EcoRI/XhoI), and transformed in the *S. cerevisiae* mating type a strain RFY231. Interactions between bait and prey constructs were determined by mating of corresponding yeast strains according to Ausubel et al. (1987) and subsequent colorimetric analysis of the activity of the LacZ reporter on selective minimal medium containing X-Gal (20 μg ml⁻¹) as the substrate after 2 days of growth. Expression of the prey construct was induced by the presence of galactose in the growth medium and not induced in the presence of glucose. All constructs were analysed for a possible interaction in liquid culture.

### Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
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<td><strong>Strains</strong></td>
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<td><em>Saccharomyces cerevisiae</em></td>
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<tr>
<td>RFY206</td>
<td>MATa trp1Δ::hisG his3A200 leu-2-3 lys2Δ201 ura3-52 mal⁺, bait strain</td>
<td>Finley &amp; Brent (1994)</td>
</tr>
<tr>
<td>RFY231</td>
<td>MATa his3 trp1Δ::hisG ura3 3LexA1p-Leu2::leu2 MAL⁺, prey strain</td>
<td>Kolonin &amp; Finley (1998)</td>
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<td>DH5x</td>
<td>F⁻ recA ΔlacU169(φ80 lacZAM15) endA hisDR gyrA</td>
<td>Stratagene</td>
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<tr>
<td>BL21</td>
<td>ampT gal [dcm] [lacI] nani hisB (r6K)</td>
<td>Novagen</td>
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<td><strong>Pseudomonas syringae pv. tomato</strong></td>
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<td>DC3000</td>
<td>Wild-type, Rif⁺</td>
<td>Cuppels &amp; Ainsworth (1995)</td>
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<tr>
<td>J5</td>
<td>Derivative of DC3000, ΔshcS1-hopS1′::Ω-Sp′, Rif⁺</td>
<td>This work</td>
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<tr>
<td><strong>Pseudomonas syringae pv. phaseolicola</strong></td>
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<td>RW60</td>
<td>Race 7, derivative of wild-type strain, cured from plasmid pAV511, Rif⁺</td>
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<td><strong>Plasmids</strong></td>
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<td>pGEX-6P-1</td>
<td>Ap¹ expression vector for N-terminal GST-fusions, cleavable by PreScission protease</td>
<td>Amersham Biosciences</td>
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<td>pBluescript II KS+</td>
<td>Ap¹ ColE1 replicon; cloning vector</td>
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<td>Km⁻, pUC ori, cloning vector</td>
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<td>RP4 mob, Km⁺, ori R6K</td>
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<td>pVSP61</td>
<td>Km² pVS1 replicon</td>
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<td>pDSK602 derivative with improved multiple cloning site</td>
<td>Escolar et al. (2001)</td>
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<td>pUC4::Ω</td>
<td>pHG4 derivative carrying Ω-Sp′ cassette</td>
<td>Fellay et al. (1987)</td>
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<td>pRK2013</td>
<td>Km¹ Tra⁺ Mob⁺ ColE1 replicon, helper plasmid</td>
<td>Figurski &amp; Helinski (1979)</td>
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<td>pMW103</td>
<td>ADH-pro::lexA; HIS3 Km⁺; bait vector</td>
<td>Ausubel et al. (1987)</td>
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<td>pG4-5</td>
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<td>Ausubel et al. (1987)</td>
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<td>pRFHM-1</td>
<td>ADH-pro::lexA::bicoid(homoeo domain); HIS3 Ap⁺; negative control for interaction</td>
<td>Ausubel et al. (1987)</td>
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<td>pK103</td>
<td>2 LexA-op::laczZ; URA3 Ap⁺; reporter plasmid</td>
<td>Ausubel et al. (1987)</td>
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<tr>
<td>pV288</td>
<td>pVSP61 derivative carrying avrRpt2</td>
<td>Whalen et al. (1991)</td>
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autoactivation and the expression of the fusions was verified by immunoblot analysis using anti-LexA or anti-HA antibodies.

**Purification of HopS1’ and ShcS1.** HopS1’ and ShcS1 were expressed as glutathione-S-transferase (GST)-fusion proteins and purified by affinity chromatography. Briefly, hopS1’ was released from the pTOPO construct by MfeI/Xhol digestion and inserted into pGEX-6P-1 (Amersham Biosciences) cut with BamHI and XhoI, resulting in construct pGST-HopS1’. This plasmid produces a GST-HopS1’ recombinant protein under lac control. Accordingly, shcS1 was inserted into pGEX-6P-1 using BamHI and XhoI, resulting in construct pGST-ShcS1.

E. coli BL21(pGST-ShcS1) and BL21(pGST-HopS1’) overnight cultures were used to inoculate 5000 ml Supermedium and grown to an OD600 of 0.6–0.8. Expression of the GST-fusion was induced by addition of 1 mM IPTG. The cells were grown for an additional 2 h and harvested by centrifugation at 8000 g for 20 min at 4 °C. The cell pellet was resuspended in 160 ml buffer A, which consisted of 1× PBS (Ausbel et al., 1987), 0.25 M KCl, 10 mM DTT, 1 mM EDTA, and complete protease inhibitor set (Roche). The cells were disrupted with a French pressure cell operating at 130 MPa, and unbroken cells and cellular debris were removed by centrifugation at 21 000 g for 15 min. A 40 ml quantity of this cellular extract was applied to 1 ml column containing Sepharose 4B coupled to glutathione (Amersham Biosciences). The column was washed with 1× PBS and the GST-fusion protein that was retained on the column was cleaved with PreScission Protease (Amersham Biosciences) to release HopS1’ or ShcS1 from the GST portion.

**GST pull-down assays.** E. coli cellular extract containing the GST-HopS1’ fusion protein was expressed as described above and applied to a glutathione-Sepharose column (Amersham Biosciences). The shcS1 gene was cloned into a pBluescript vector that contained a triple cMyc tag (pBcMyc) to produce an ShcS1-cMyc fusion protein under lacPO control (pShcS1-cMyc). Cellular extract of E. coli(pShcS1-cMyc) was prepared as described above and applied to the glutathione-Sepharose column, which was preincubated with GST-HopS1’. After washing the column, the GST-fusion protein and interacting proteins were eluted from the column by the addition of reduced glutathione (10 mM). The presence of GST-HopS1’ and ShcS1-cMyc in the eluted fractions was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Western transfer and immunoblot analysis using anti-GST (Amersham Biosciences) and anti-cMyc (Roche) antibodies. Similar experiments using E. coli expressing GST only from the empty vector pGEX-6P-1 were performed as a negative control.

**Molecular mass analysis.** The molecular mass of ShcS1 was estimated by gel-filtration chromatography on a Superose 12 HR 10/30 column in PreScission protease buffer (50 mM Tris/HCl, pH 7.0, 150 mM NaCl, 1 mM DTT, 1 mM EDTA). Bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), ribonuclease A (13.7 kDa) and cobalamin (1.3 kDa) were used as reference proteins and blue dextran (2000 kDa) was used to determine the V6 of the column.

**Computer analyses.** Secondary structures of proteins were predicted using the PHD (Profile fed neural network systems from Heidelberg) algorithm (Kost, 1996) of the PredictProtein server at the EMBL (www.embl-heidelberg.de/predictprotein/).

### RESULTS

**ShcO1, ShcS1 and ShcS2 show features of TTS chaperones**

The shcS1–hopS1’ operon of *P. syringae* pv. *tomato* (*Pto*) strain DC3000 was identified in an IVET screen (Boch et al.,
Type III chaperones ShcS1 and ShcO1

Fig. 1. Overview of the genes analysed in this study. Genes are drawn to scale as arrows and their designations are given according to the new unified nomenclature. Names and former names of the genes are: shcS1 (shcS4, chpPtoZ); hopS1’ (hopPtoS4’, holPto2); ‘hopS1 (‘hopPtoS4); hopO1-2 (hopPtoO2, hopPtoS3); hopT1-2 (holPtoU2); hopO1-3 (hopPtoO-related); hopT2 (holPtoU3, holPtoU-related); shcS2 (chpPtoO2, orf14); hopS2 (holPtoZ2, orf15); shcO1 (chpPtoO); hopO1-1 (hopPtoS1, hopPtoO1); hopT1-1 (holPtoU). Homologous genes are identically shaded. The localizations and directions of the IVET-fusions ipx69 and ipx70 are indicated by small arrows and hup-boxes are given as black dots. The components of the IS element are: white arrow, transposase gene; small arrowheads, terminal inverted repeats. A premature stop codon within hopO1-3 is indicated by a line. Fragmented genes are symbolized by a zigzag line. The components of the IS element are: white arrow, transposase gene; small arrowheads, terminal inverted repeats. A premature stop codon within hopO1-3 is indicated by a line. Fragmented genes are symbolized by a zigzag line.

ShcS1 is required for translocation of HopS1’ into Arabidopsis cells

The genes of effector-specific chaperones (class IA) are typically located either in the same operon as or adjacent to their cognate effector gene. Because shcS1 and hopS1’ are located next to each other (Fig. 1), we postulated that ShcS1 is the TTS chaperone for the effector HopS1’. To test this we analysed the translocation of HopS1’ into plant cells in the presence or absence of ShcS1. To analyse translocation we used the AvrRpt2-reporter assay which is based on the recognition of the avirulence protein AvrRpt2 by the resistance protein RPS2 in Arabidopsis Col-0 (Guttman & Greenberg, 2001; Mudgett et al., 2000). This assay uses an N-terminal-truncated version of AvrRpt2 (AvrRpt280–255) that can not elicit an HR when expressed by P. syringae because it is devoid of its TTS signals, but still elicits an HR when expressed directly inside the plant cell (Mudgett & Staskawicz, 1999). If the truncated AvrRpt280–255 is fused to the N-terminus of an effector that can facilitate the translocation of the fusion protein into the plant cell, P. syringae expressing the fusion will elicit an HR on wild-type Arabidopsis plants that carry the RPS2 gene (Guttman & Greenberg, 2001; Mudgett et al., 2000).

We cloned the shcS1 and hopS1’ genes including their putative promoter in front of AvrRpt280–255 on a low-copy-number plasmid to generate a HopS1’1–118:AvrRpt280–255 fusion protein. This plasmid (pVAJ5001) was introduced into Pto DC3000 and the resulting strain elicited an HR on A. thaliana RPS2 plants and not on rps2 mutant plants (Fig. 2a, column 4). This indicates that the AvrRpt280–255 moiety of the fusion protein is responsible for eliciting the HR. Truncated AvrRpt280–255 alone did not elicit an HR (Fig. 2, column 1), in contrast to the full-length AvrRpt2, which did (Fig. 2a, column 2). This demonstrated that HopS1’ can be translocated into plant cells as an AvrRpt2-reporter fusion in the presence of shcS1 and confirms experiments which showed that a HopS1’:CyaA reporter fusion was translocated (Schechter et al., 2004). To analyse which part of HopS1’ is necessary to direct translocation, we similarly constructed a plasmid that produces ShcS1 and a fusion of the first 50 amino acids of HopS1’ to AvrRpt280–255 (pVAJ502). Pto DC3000(pVAJ502) elicited only a weak reaction in Arabidopsis (Fig. 2a, column 3), which demonstrates that the first 50 amino acids of HopS1’ can only direct very low translocation in comparison to amino acids 1 to 118 of HopS1’.

2002) by two independent ipx-fusions, ipx69 and ipx70 (Fig. 1). While hopS1’ encodes an effector (Schechter et al., 2004), shcS1 encodes a protein that contains several features reminiscent of TTS chaperones. ShcS1 is a small protein (17.2 kDa) with an acidic pI (5.25) and a predicted amphipathic helix (amino acids 111–138) near the C-terminal end. Homology searches revealed that Pto DC3000 carries two additional shcS1-related genes, shcO1 and shcS2 (Fig. 1; Greenberg & Vinatzer, 2003). The genes shcO1 and shcS2 encode proteins of 15.6 and 17.0 kDa with pI values of 5.45 and 5.91, respectively. The amino acid sequence of ShcS1 displays 79% identity to ShcO1 and 71% identity to ShcS2.

The amino acid sequences of ShcS1, ShcS2 and ShcO1 were used to predict secondary structures. Comparison of these predicted structures with the structural information from other class I TTS chaperones (van Eerde et al., 2004) indicated that α-helices and β-sheets are predicted in ShcS1, ShcS2 and ShcO1 at the positions that would be expected in a TSS chaperone. In addition, the conserved amino acids that have been identified for class IB chaperones were not all present in ShcS1, ShcS2 or ShcO1. Rather, the sequence similarities group them into the class IA group of effector chaperones.
Bacterial strains (a) *P. syringae pv. tomato* (Pto) DC3000, (b) Pto J5 [DC3000 Δ(shcS1–hopS1)], (c, d) *P. syringae pv. phaseolicola* (Pph) RW60 (no shcS1 or hopS1 homologues) carrying various plasmids were infiltrated into *A. thaliana* leaves and plant reactions were documented 24 h post-inoculation. The plasmids were as follows: column 1, pDSK(AvrRpt280–255); column 2, pV288 (AvrRpt21–255); column 3, pVAJ5002 (ShcS1-HopS11–50::AvrRpt280–255); column 4, pVAJ5001 (ShcS1-HopS11–118::AvrRpt280–255); column 5, pVAJ5005.2 [Δ(shcS1::HopS11–118::AvrRpt280–255)]; column 6, pVAJ5005.9 [Δ(shcS12::HopS11–118::AvrRpt280–255)]; column 7, pVAJ5001; column 8, pVAJ5005.2; column 9, pVAJ5005.2 and pDS1 (ShcS1); column 10, pVAJ5005.2 and pDO1 (ShcO1); column 11, pVAJ5005.2 and pDS2 (ShcS2). Leaves displaying a hypersensitive reaction (HR) are marked by white asterisks, weak reactions are indicated in parentheses. Similar results were obtained in at least three independent experiments.

![Image](https://example.com/image.png)

**Fig. 2.** Translocation studies of HopS1 using AvrRpt2 fusions. Bacterial strains (a) *P. syringae pv. tomato* (Pto) DC3000, (b) Pto J5 [DC3000 Δ(shcS1–hopS1)], (c, d) *P. syringae pv. phaseolicola* (Pph) RW60 (no shcS1 or hopS1 homologues) carrying various plasmids were infiltrated into *A. thaliana* leaves and plant reactions were documented 24 h post-inoculation. The plasmids were as follows: column 1, pDSK(AvrRpt280–255); column 2, pV288 (AvrRpt21–255); column 3, pVAJ5002 (ShcS1-HopS11–50::AvrRpt280–255); column 4, pVAJ5001 (ShcS1-HopS11–118::AvrRpt280–255); column 5, pVAJ5005.2 [Δ(shcS1::HopS11–118::AvrRpt280–255)]; column 6, pVAJ5005.9 [Δ(shcS12::HopS11–118::AvrRpt280–255)]; column 7, pVAJ5001; column 8, pVAJ5005.2; column 9, pVAJ5005.2 and pDS1 (ShcS1); column 10, pVAJ5005.2 and pDO1 (ShcO1); column 11, pVAJ5005.2 and pDS2 (ShcS2). Leaves displaying a hypersensitive reaction (HR) are marked by white asterisks, weak reactions are indicated in parentheses. Similar results were obtained in at least three independent experiments.

To test whether ShcS1 is required for translocation of HopS1’, we constructed deletions of shcS1 in the plasmid pVAJ5001 (ShcS1−HopS11−118::AvrRpt280–255). Because it is possible that shcS1 and hopS1’ constitute an operon, we constructed two independent deletions to avoid a polar effect on the expression of hopS1’ (see Methods for details). The corresponding plasmids pVAJ5005.2 and pVAJ5005.9 still conferred to Pto DC3000 the ability to elicit an HR in *Arabidopsis* (Fig. 2a, columns 5 and 6). Pto DC3000 carries a chromosomally encoded copy of shcS1 that might be able to complement the deletion of shcS1 on the plasmid. Therefore, we constructed an shcS1−hopS1’ mutant strain (J5) by replacing the genes in the chromosome of Pto DC3000 with a resistance cassette. We introduced the plasmids expressing the AvrRpt280–255 fusions into Pto J5 and analysed the corresponding strains for elicitation of an HR in *Arabidopsis* (Fig. 2b). Pto J5(pVAJ5001) elicited a strong HR (Fig. 2b, column 4), but Pto J5 carrying the shcS1 deletions (pVAJ5005.2 and pVAJ5005.9) elicited only a weak reaction (Fig. 2b, columns 5 and 6). This indicates that ShcS1 is important for the translocation of HopS1’.

The weak reaction to Pto J5(pVAJ5005.2) and Pto(pVAJ5005.9) of *Arabidopsis* RPS2 plants might indicate that the HopS1’::AvrRpt2 fusion protein is still translocated from this strain but at a very low rate. It is possible that the ShcS1 homologous chaperones, ShcO1 and ShcS2, present in Pto DC3000 and Pto J5 facilitate translocation of HopS1’ to some extent even in the absence of a functional ShcS1. Instead of constructing multiple deletions in Pto DC3000, we chose to analyse this using *Pseudomonas syringae pv. phaseolicola* (Pph) RW60, which does not contain any *shcS1-* or *hopS1’-*homologous genes as indicated by Southern analysis (data not shown). In addition, this strain is non-pathogenic on *Arabidopsis* and therefore allows detection of HR reactions without a potentially interfering disease development.

The native AvrRpt21–255 is translocated from Pph RW60, but the truncated AvrRpt280–255 is not (Fig. 2c, columns 2 and 1, respectively), demonstrating that Pph RW60 is suitable for the translocation assay. Pph RW60(pVAJ5001) elicited an HR on *Arabidopsis* RPS2 plants (Fig. 2c, column 4), indicating that the HopS1’::AvrRpt280–255 fusion is also translocated from Pph RW60 in the presence of ShcS1. In contrast, Pph RW60(pVAJ5005.2) and Pph RW60(pVAJ5005.9), each producing the HopS1’1–118::AvrRpt280–255 fusion without a functional ShcS1, did not elicit an HR (Fig. 2c, columns 5 and 6), clearly demonstrating that ShcS1 is required for translocation of HopS1’.

This set-up also enabled us to test if the three homologous chaperones (ShcS1, ShcS2 and ShcO1) can complement the *shcS1* mutation for translocation of HopS1’1–118::AvrRpt280–255 in *Pph* RW60. We expressed each chaperone from the high-copy-number plasmid pDSK604 (Escolar et al., 2001), in addition to the Δ(shcS11 HopS1’1–118::AvrRpt280–255 reporter construct (pVAJ5005.2), and infiltrated the corresponding strains into *A. thaliana* (Fig. 2d, columns 9–11). Only the strains producing ShcS1 or ShcO1 in addition to the reporter construct elicited an HR in *Arabidopsis* RPS2 (Fig. 2d,
Type III chaperones ShcS1 and ShcO1

columns 9 and 10). This demonstrates that ShcS1 and ShcO1, but not ShcS2, are able to facilitate the translocation of HopS1’ when supplied in trans. Taken together, these data show that ShcS1 and ShcO1 are both able to facilitate the translocation of HopS1’.

**ShcS1 interacts with HopS1’ in yeast and in vitro**

Typically, a chaperone and its cognate effector physically interact. To determine whether ShcS1 interacts with HopS1’, we performed interaction studies using a yeast two-hybrid approach. shcS1 and hopS1’ were each cloned into bait and prey vectors. When transformed into yeast, neither of them alone induced expression of the reporter gene lacZ. However, expression of shcS1 and hopS1’ in the same yeast cell led to a detectable LacZ activity, suggesting an interaction between the two proteins (Fig. 3). LacZ activity was detected irrespective of which protein was used as a bait or prey construct (Fig. 3a, b).

In addition, we used GST pull-down assays to analyse whether ShcS1 and HopS1’ also interact in vitro. HopS1’ was expressed as a GST-fusion in *E. coli*, and the cellular extract was applied to a glutathione-Sepharose affinity column that specifically bound the GST-HopS1’ fusion protein. GST alone was used as negative control. ShcS1 was expressed as a C-terminal cMyc-tagged fusion in *E. coli*, and the cellular extract was applied to the columns containing GST-HopS1’ or GST. Bound proteins were eluted, and the fractions were analysed by SDS-PAGE and immunoblot (Fig. 4). Anti-cMyc antibody detected the ShcS1-cMyc protein only in fractions from the GST-HopS1’ column, but not the GST-column (Fig. 4). This indicates that ShcS1 binds to GST-HopS1’, but not to GST, and that ShcS1 and HopS1’ interact in vitro, supporting the role of ShcS1 as a chaperone for HopS1’.

ShcS1 and ShcO1 both interact with two different effectors, HopS1’ and HopO1-1

TTS chaperones of class IA show a high degree of specificity to their substrates and in general bind only to one effector.

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**Fig. 3.** Interactions between chaperones and effectors in yeast two-hybrid studies. The genes encoding putative chaperones (ShcS1, ShcO1 and ShcS2) and putative effectors (HopS1’, HopS1’ *Pto J1065*, HopO1-1, HopS2 and HopT1-1) were cloned as bait and prey fusions. Diploid yeast strains carrying bait and prey fusions and a lacZ reporter plasmid were incubated on medium containing X-Gal. A blue colour of the colony indicates interaction between bait and prey fusion. (a) Effectors cloned as bait and chaperones cloned as prey fusions. Bicoid (pRFHM-1) was used as a negative control. (b, c) Chaperones cloned as bait and effectors cloned as prey fusions. HopS1’ is truncated by an IS element in *Pto DC3000* and HopS1’ *Pto J1065* is the full-length protein from *Pto J1065*. The empty bait and prey vectors were used as negative controls.

**Fig. 4.** Interaction of the chaperone ShcS1 with its cognate effector HopS1’ in GST-pulldown experiments. GST or the fusion GST-HopS1’ was expressed in *E. coli* (–, no induction; +, induction with 1 mM IPTG) and the cellular extracts were loaded on a glutathione-Sepharose column. Cellular extract from *E. coli* expressing a cMyc-tagged derivative of ShcS1 was applied to both columns. The GST-proteins were eluted by the addition of reduced glutathione and the fractions were analysed for the presence of coeluted chaperone- and GST-proteins by SDS-PAGE and immunoblotting using anti-cMyc and anti-GST antibodies, respectively. Three elution fractions containing proteins are shown.
The similarity of ShcS1, ShcO1 and ShcS2 led us to determine whether the chaperones have common binding specificities. As candidates for target effectors we analysed genes encoded in the same putative operons as shcS1, shcO1 and shcS2, namely hopS1′, hopS2, hopO1-1 and hopT1-1 (Fig. 1). We used the yeast two-hybrid system to analyse interactions between chaperones and effectors, and cloned the genes as bait and prey constructs. The expression of all constructs in yeast was verified by immunoblot analysis to be similar, except for the ShcS2 prey fusion protein, which was present in significantly lower amounts than the other prey constructs. Bait and prey constructs alone did not activate transcription of the lacZ reporter gene. Combinations of chaperones and effectors, and cloned the genes as bait and prey constructs. The expression of all constructs in yeast was verified by immunoblot analysis to be similar, except for the ShcS2 prey fusion protein, which was present in significantly lower amounts than the other prey constructs. Bait and prey constructs alone did not activate transcription of the lacZ reporter gene. Combinations of chaperones and effectors revealed that ShcS1 binds not only to HopS1′ but also to HopO1-1 (Fig. 3) in both bait/prey combinations, demonstrating that ShcS1 has a bivalent effector-binding activity. ShcS1 also showed a weak interaction with the HopS1′ homologue HopS2 but only in one bait/prey combination (Fig. 3a, c).

The hopS1′ allele in Pto DC3000 is truncated due to the insertion of an IS element (Fig. 1; J. Boch, unpublished results; Schechter et al., 2004)). We isolated a non-truncated allele of hopS1 from the Pto strain JL1065 (J. Boch, unpublished results) and compared the binding activity of the long (HopS1p_pro JL1065: 300 amino acids) and short (HopS1′; 118 amino acids) proteins to ShcS1 in yeast two-hybrid studies (Fig. 3b). Both HopS1 proteins interacted with ShcS1 (Fig. 3b).

Surprisingly, ShcS2 did not show an interaction with any of the analysed effectors. The third chaperone, ShcO1, interacted with HopO1-1 and HopS1′ as bait and prey constructs and therefore displayed a similar bivalent binding activity to ShcS1 (Fig. 3). None of the three chaperones showed an interaction with HopT1-1 and it is unclear at this point whether this putative effector relies on a chaperone for translocation. These data demonstrate that ShcS1 and ShcO1 both have a common bivalent binding specificity to the target effectors HopS1′ and HopO1-1.

**ShcS1 and ShcO1 form homo- and heterodimers**

Generally, TTS chaperones function as homodimers (Page & Parsot, 2002). We wanted to determine the molecular mass of ShcS1 under non-denaturing conditions. The ShcS1 protein was affinity purified and subjected to analytical gel filtration. Chromatography of ShcS1 yielded a sharp protein peak in the eluted fractions between the reference proteins chymotrypsinogen A (25 kDa) and ovalbumin (43 kDa; Fig. 5). We conclude from these data that the native ShcS1 is a homodimer with a calculated molecular mass of 34-4 kDa.

ShcS1 and ShcO1 exhibit a high degree of similarity, which prompted us to test whether ShcS1 and ShcO1 can form heterodimers. For this we used the yeast two-hybrid system. shcS1, shcO1 and shcS2 were each cloned as both bait and prey constructs, and all possible combinations were tested in yeast for induction of the lacZ reporter gene (Fig. 6). These experiments revealed that, as expected, ShcS1 and ShcO1 can homodimerize. In addition, ShcS1 and ShcO1 also interact with each other, suggesting that they are able to form heterodimers.

**DISCUSSION**

In this study we describe three novel TTS chaperones from the plant pathogen *Pto* DC3000, ShcS1, ShcS2 and ShcO1, which share significant homologies at the amino acid sequence level. Using AvrRpt2 reporter fusions, we show that ShcS1 and ShcO1, but not ShcS2, facilitate the translocation of the effector HopS1′ into *Arabidopsis* cells. This demonstrates that ShcS1 and ShcO1 act as chaperones in *Pto* DC3000. To identify the target substrates of the three
chaperones, we performed protein-interaction studies. These experiments demonstrate that both ShcS1 and ShcO1 bind to two target effectors, HopS1’ and HopO1-1. The putative chaperone ShcS2 did not interact significantly with any of the tested putative effectors and did not facilitate the translocation of HopS1’. Therefore, it is unclear at this point whether ShcS2 is a functional chaperone.

Typically, TTS chaperones can be grouped into effector-specific chaperones (class IA) and chaperones with a broader effector-binding specificity (class IB). Members of both classes have been crystallized (Page & Parsot, 2002; van Eerde et al., 2004) and although TTS chaperones generally share little amino acid sequence homology to one another, they exhibit similar global structures. Each chaperone monomer is built up of five $\beta$-strands and three to four $\alpha$-helices (Parsot et al., 2003; van Eerde et al., 2004). Analysis of the predicted secondary structures of ShcS1, ShcS2 and ShcO1 suggested that they contain $\alpha$-helices and $\beta$-sheets at similar positions (data not shown). In contrast, several conserved amino acids have been identified for class IB chaperones (van Eerde et al., 2004), but they were not consistently present in ShcS1, ShcS2 or ShcO1. This indicates that these chaperones belong to class IA of TTS chaperones instead. In addition, the shcS1, shcS2, shcO1 genes are located in the vicinity of the genes encoding their respective target effectors (Fig. 1), which is a typical feature of class IA chaperones. However, the bivalent target specificity of ShcS1 and ShcO1 suggests that they are exceptions to the rule that class IA TTS chaperones are specific for only one effector. CesT from E. coli (EPEC) and SycH from Yersinia are two other class IA TTS chaperones that are known to bind more than one target substrate (Creasey et al., 2003; Parsot et al., 2003). However, SycH binds to only one effector and two regulatory proteins, leaving CesT the only other example known to date of a class IA chaperone that binds to more than one effector.

The three chaperone genes shcS1, shcS2 and shcO1 in Pto DC3000 were putatively generated by duplications from an ancient precursor gene (Greenberg & Vinatzer, 2003). This might be the reason for the functional redundancy of ShcS1 and ShcO1 and would explain the unexpected presence of several homologous chaperones in one bacterial strain (Parsot et al., 2003). Subsequent DNA-shuffling events probably created three putative operons that contain one chaperone gene each and a different set of effector genes. The two putative operons shcS1/hopS1’ and shcS2/hopS2 are chromosomally encoded and localized in the same region. In contrast, the putative operon shcO1/hopO1-1/ hopT1-1 is encoded on the large (74 kbp) plasmid of Pto DC3000 (Fig. 1), which additionally contains genes homologous to tra genes (Buell et al., 2003). This opens the possibility that the plasmid-encoded operon could alternatively have been acquired by Pto DC3000 through horizontal gene transfer. Interestingly, genes homologous to the plasmid-encoded effectors hopO1-1 and hopT1-1 (hopO1-2, hopO1-3, hopT1-2, hopT2) are found in the area between shcS1/hopS1’ and shcS2/hopS2 on the chromosome. However, it is unclear whether these genes are expressed because they are separated from the hrp promoter of shcS1/ hopS1’ by an IS element (Fig. 1).

It was surprising to find that two different effectors, HopS1’ and HopO1-1, which share only 16% sequence identity, interact with the same chaperones, and this raises the question of which elements are responsible for chaperone binding. Our functional studies of ShcS1 using the AvrRpt2 assay revealed that the first 50 amino acids of HopS1’ are not sufficient to direct an efficient translocation, but that amino acids 1 to 118 of HopS1’ are necessary. This indicates that the area between amino acids 50 and 118 of the HopS1’ protein is important in addition to the first 50 amino acids for chaperone-mediated translocation and may contain part of the chaperone-binding motif. We compared the amino acid sequences of HopS1’, HopS2 and HopO1-1, but we found that these proteins contain only very few conserved residues. This is in contrast to the class IA chaperone SycH, which interacts with its different substrates YopH, YscM1 (LcrQ) and YscM2 through a stretch of homologous amino acids (Michiels & Cornelis, 1991; Rimpiläinen et al., 1992; Stainier et al., 1997).

Although HopS1’ and HopO1 contain no significant homology at the amino acid sequence level, both proteins are predicted to contain similar secondary structural elements (data not shown). An $\alpha$-helix is strongly predicted in the region that might contain part of the chaperone-binding motif (Fig. 7). A helical-wheel plot revealed that one side of this $\alpha$-helix contains amino acids with strikingly similar characteristics in both proteins (Fig. 7), whereas the other sides of the helices are completely different.

Co-crystallization of the chaperones SicP and SycE (class IA chaperones) with the chaperone-binding domains of their cognate effectors showed that there are two distinct sites on the surface of each chaperone monomer that interact with secondary structural elements of the effector (Birtalan et al., 2002; Stebbins & Galan, 2001). The first interaction site of the chaperones is occupied by an $\alpha$-helix on the effector (Birtalan et al., 2002; Stebbins & Galan, 2001). It is therefore tempting to speculate that the conserved part of the $\alpha$-helix in HopS1’ and HopO1-1 is involved in binding to the TTS chaperones. HopS2 as well contains a predicted $\alpha$-helix between amino acids 84 and 96 displaying most of the conserved residues on one side of the helix (data not shown). It is possible that other parts of HopS2 do not allow an efficient interaction of the protein with any of the tested TTS chaperones. The co-crystallization of SicP/SptP and SycE/YopE (Birtalan et al., 2002; Stebbins & Galan, 2001) revealed that the effector is wrapped around the chaperone dimer. This allows a large area (about 50 amino acids) of the effector to interact with the chaperone surface. It is therefore likely that the chaperone-binding domain of HopS1’ is extended beyond the conserved $\alpha$-helix.
Fig. 7. The effectors HopS1’ and HopO1-1 share similar amino acid sequences on one side of a conserved helix. Part of the amino acid sequence of HopS1’ and HopO1-1 was aligned. Identical amino acids are shaded in black and similar ones in grey. A predicted conserved α-helix is overlined. The helix is drawn as a helical-wheel projection with similar amino acids oriented to the top. The similarities were matched onto the primary sequence with dashed lines using HopS1 as an example. The amino acid characteristics are: hydrophobic (M, I, L, V), aromatic (F, Y, W), hydrophobic and small (G, A, P), acidic (D, E), amide (N, Q), basic (K, H, R), hydroxyl (S, T), thiol (C).

Although our analysis shows that HopS1’ is directly bound by TTS chaperones and efficiently translocated, we realize that this allele is truncated due to the insertion of an IS element in strain DC3000 (Fig. 1; Schechter et al., 2004). We recently isolated a full-length allele of hopS1 from Pto JL1065 (J. Boch, unpublished results). Interestingly, both HopO1-1 and HopS1_pto JL1065 contain common secondary structures and show homology to ADP-ribosyltransferases (J. Boch, unpublished results; Petnicki-Ocwieja et al., 2002). Nevertheless, the amino acid sequence identity between both proteins is still very low (25%), and HopS1 and HopO1 therefore constitute different effector families according to Lindeberg et al. (2004).

We compared the binding of the long and short HopS1 protein to the chaperones in yeast two-hybrid studies. In these studies the longer HopS1 derivative seemed to interact more strongly with ShcS1, which might suggest that additional chaperone-binding regions are present in HopS1_pto JL1065 that are absent in HopS1’. The identification of a longer derivative of HopS1 with putatively functional domains also indicates that hopS1’ and hopS2 are probably pseudogenes and do not encode functional effectors. Nevertheless, HopS1’ still contains elements that facilitate the translocation of this protein. In contrast, HopS2 is not translocated (Schechter et al., 2004), and we additionally showed that it only weakly interacts with one of the three homologous TTS chaperones (ShcS1). HopT1 does not interact with any of the TTS chaperones we analysed. In addition, this protein does not show homology to ADP-ribosyltransferases, and its function remains unknown.

We further analysed ShcS1 to study the properties of the native protein. We purified ShcS1 from E. coli and showed that ShcS1 forms homodimers in vitro. Thus it is similar in its subunit composition to typical TTS chaperones from animal pathogenic bacteria (Page & Parsot, 2002). Our study is one of the first to determine the subunit composition of a TTS chaperone from a plant-pathogenic bacterium. Most TTS chaperones are believed to form homodimers (Page & Parsot, 2002) and this was also recently shown for a TTS chaperone from P. syringae (Singer et al., 2004), but, as an exception, functional SycH from Yersinia enterocolitica forms homotetramers (Neumayer et al., 2004). In addition, we show in yeast two-hybrid analyses that ShcS1 and ShcO1 interact with each other and possibly form heterodimers. From structural studies of other TTS chaperone dimers it became evident that the conserved α-helix 2 of each chaperone monomer lies at the core of the dimerization interface making contact with the other and the adjacent β-sheets (Page & Parsot, 2002; van Eerde et al., 2004). Accordingly, the corresponding predicted α-helix in ShcS1 and ShcO1 (amino acids 68–77) and the downstream β-sheet areas are perfectly conserved between both proteins. Interestingly SycN and YscB, two class IA chaperones from Yersinia, act as a heteromultimer to associate with their substrate effector YopN (Day & Plano, 1998). Future experiments will further characterize the molecular basis for binding of the ShcS1-homologous chaperones to their effector substrates and help us understand the specificity of TTS chaperones.

ACKNOWLEDGEMENTS

We are grateful to B. N. Kunkel for providing the A. thaliana rps2-201 line, J. Mansfield for providing the Pph strain RW60, M.-B. Mudgett for providing plasmid pDSK(AvrRpt2 80–255) and D. Büttner for helpful comments on the manuscript. This work was supported by a grant from the Deutsche Forschungsgemeinschaft awarded to J.B. Sequencing of the P. syringae pv. tomato DC3000 and the P. syringae pv. phaseolicola 1448A genomes by the Institute for Genomic Research (TIGR) was accomplished with support from the National Science Foundation.

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