The Type III Effector HsvG of the Gall-Forming *Pantoea agglomerans* Mediates Expression of the Host Gene *HSVGT*

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The type III effector HsvG of the gall-forming *Pantoea agglomerans* pv. *gypsophila* is a DNA-binding protein that is imported to the host nucleus and involved in host specificity. The DNA-binding region of HsvG was delineated to 266 amino acids located within a secondary structure region near the N-terminus of the protein but did not display any homology to canonical DNA-binding motifs. A binding site selection procedure was used to isolate a target gene of HsvG, named *HSVGT*, in *Gypsophila paniculata*. *HSVGT* is a predicted acidic protein of the DnaJ family with 244 amino acids. It harbors characteristic conserved motifs of a eukaryotic transcription factor, including a bipartite nuclear localization signal, zinc finger, and leucine zipper DNA-binding motifs. Quantitative real-time polymerase chain reaction analysis demonstrated that *HSVGT* transcription is specifically induced in planta within 2 h after inoculation with the wild-type *P. agglomerans* pv. *gypsophila* compared with the *hsvG* mutant. Induction of *HSVGT* reached a peak of sixfold at 4 h after inoculation and progressively declined thereafter. Gel-shift assay demonstrated that HsvG binds to the *HSVGT* promoter, indicating that *HSVGT* is a direct target of HsvG. Our results support the hypothesis that HsvG functions as a transcription factor in gypsophila.

Pantoea agglomerans, a widespread epiphyte and commensal bacterium, has evolved to become a host-specific gall-forming pathogen on various hosts (Barash and Manulis-Sasson 2009; Manulis and Barash 2003). *P. agglomerans* pv. *gypsophila* elicits galls on gypsophila (Cooksey 1986) and hypersensitive response (HR) on beet (Ezra et al. 2000), whereas *P. agglomerans* pv. *betae* causes galls on both beet and gypsophila (Burr et al. 1991). The pathogenicity of *P. agglomerans* pv. *gypsophila* and *P. agglomerans* pv. *betae* is dependent on the presence of indigenous plasmids designated as pPATH_{pag} and pPATH_{pab}, respectively (Manulis et al. 1991). The pPATH_{pag} of strain *P. agglomerans* pv. *gypsophila* 824-1, which has been more extensively investigated, has a size of 135 kb harboring a pathogenicity island of approximately 75 kb that carries a functional *hrp/hrc* gene cluster (Mor et al. 2001; Nizan et al. 1997), six experimentally confirmed type III effectors (re-

ferred to hereafter as effectors) (Barash and Manulis-Sasson 2007), and genes encoding indole-3-acetic acid and cytokinin biosynthetic enzymes (Clark et al. 1993; Lichter et al. 1995). Mutations in any of the *hrp/hrc* genes encoding the type III secretion system (T3SS) or the *hrp* regulatory cascade completely abolished gall initiation (Mor et al. 2001; Nizan-Koren et al. 2003), suggesting that gall formation by *P. agglomerans* pv. *gypsophila* and *P. agglomerans* pv. *betae* is *hrp* dependent.

HsvG and HsvB are two paralogous effectors present in both *P. agglomerans* pv. *gypsophila* and *P. agglomerans* pv. *betae* that are involved in host specificity on gypsophila and beet, respectively (Nissan et al. 2006). Their structure includes a T3SS recognition motif, a region of predicted α helices, two nuclear localization signals (NLS) present at the N- and C-terminal regions of the protein, and a transcription activation domain in yeast (Nissan et al. 2006; Weinthal et al. 2011). The two effectors are mainly distinguished by the presence of two direct repeats (71 and 74 amino acids [aa]) in the transcription activation domain of HsvG as opposed to only one of the repeats in the activation domain of HsvB (Nissan et al. 2006). Exchanging the activation domains between HsvG and HsvB results in a switch in host specificity. A modified yeast two-hybrid assay revealed that either one of the repeats in HsvG or the single repeat of HsvB is sufficient to activate transcription in yeast (Nissan et al. 2006). Transient expression of green fluorescent protein (GFP)-HsvG or GFP-HsvB fusions obtained by particle bombardment of gypsophila, beet, or melon (non-host) showed that these effector proteins are localized to the cell nucleus in host and non-host plants (Nissan et al. 2006). Two NLS present in the N- and C-terminal regions of HsvG and HsvB are responsible for nuclear import of these effectors (Weinthal et al. 2011). By employing random binding-site selection (Okuno et al. 2001) and electrophoretic mobility shift assays, HsvG and HsvB were demonstrated as DNA-binding proteins with a consensus binding site corresponding to ACACC/aAA (Nissan et al. 2006). The characterization of HsvG and HsvB as DNA-binding proteins, which are imported into plant nucleus via the NLS and act as transcription activators in yeast, could suggest that they might also function as transcription factors in their host plants.

Type III effectors of phytopathogenic bacteria that act as specific transcription factors in their hosts are mainly represented by the large AvrBs3/PthA family of *Xanthomonas* spp. (Boch and Bonas 2010). Members of this family have been termed transcription-activator-like (TAL) effectors. They are characterized by an N-terminal T3SS recognition domain, a central domain comprising a variable number of tandemly arranged near-

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*The e-Xtra logo stands for “electronic extra” and indicates that one supplementary figure appears online.

perfect repeats of 33 to 35 aa, C-terminal NLS, and a eukaryotic acidic transcription-activation domain (Bogdanove et al. 2010; Gurlebeck et al. 2006). Specificity of TAL effectors is determined by a novel modular DNA-binding domain (Boch et al. 2009; Moscou and Bogdanove 2009). A few corresponding target genes for TAL effectors have been identified (Boch and Bonas 2010). Noteworthy are the genes upregulated by AvrBs3 (*UPA* genes) in pepper (Marois et al. 2002). A conserved promoter element, designated as the *UPA* box, was found in several induced *UPA* genes which are directly bound and activated by AvrBs3 (Kay et al. 2009; Römer et al. 2009).

The present study was undertaken to test the notion that HsvG functions as a specific transcription factor in gypsophila. To approach this aim, the DNA-binding region of HsvG was delineated and an HsvG target gene that was transcriptionally induced in an effector-specific manner was isolated and characterized.

RESULTS

Delimitation of the DNA-binding region of HsvG.

To locate the DNA-binding domain of HsvG, the *hsvG* open reading frame (ORF) was cloned by polymerase chain reaction

(PCR) into the expression vector pFLAG-ATS and eight deletions were generated (as described below) (Fig. 1A). HsvG deletion mutants were analyzed by gel-shift assay for their ability to bind the DNA consensus sequence ACACAAA, previously determined as the binding site of wild-type HsvG (Nissan et al. 2006) (Fig. 1B). Various deletions between amino acids 107 and 373 abolished binding of HsvG to its target DNA binding site, delineating the maximal site of the DNA-binding motif, whereas deletions of 8 to 107 or 373 to 655 aa did not affect binding, suggesting that neither the repeat domain nor the N- or C-terminal regions of the protein are involved in binding. Consensus secondary structure predictions of HsvG by the bioinformatics servers Porter, Jpred, and PSIPRED (described below) indicated that the DNA-binding domain is located within a critical secondary structure of the protein, which contains 14 α helices and a β strand (Fig. 1A). However, attempts to relate the DNA-binding domain to helix-turn-helix (HTH) or any other canonical DNA-binding structures were negative, suggesting that the DNA-binding domain of HsvG represents a new DNA-binding motif. Although several α helices and β strands were also detected in the N- and C-terminal regions of HsvG outside the DNA binding domain, they did not affect DNA binding (Fig. 1A).

Fig. 1. Delineating the DNA-binding region of HsvG. **A**, Deletion mutants and secondary structures of HsvG. Top panel illustrates the consensus secondary structure prediction of HsvG. Black rectangles indicate α helices whereas vertical open rectangles indicate β strands. The location of the two repeats (RI and RII) is shown by gray rectangles. Middle panel illustrates schematic representation of HsvG deletion mutants ($\Delta 1$ to $\Delta 8$). Positive and negative DNA binding is marked by (+) and (-) signs, respectively. Bottom panel shows results of fold recognition obtained by three different methods showing similarity to RNA polymerase II (amino acids 143 to 553; a, FFASO3). Recombinase (amino acids 50 to 140; b, HHPRED and c, 3D-Jury) and phase integrase (amino acids 52 to 160; c, 3D-Jury). **B**, Gel-shift assay of the corresponding deletion mutants with the ACACAAA consensus binding site. C(+) and C(-) refer to positive (hHsvG with the double-stranded DNA consensus sequence) and negative (DNA without consensus) controls, respectively (Nissan et al. 2006). Arrow points to DNA-bound protein. The DNA-binding region was identified between amino acids 107 and 373 and contained 10 α helices and 5 β strands.

Fold recognition was predicted by three different bioinformatics methods (as described below) and the two top best results of each prediction are shown in Figure 1A (bottom panel). The fold prediction of HsvG by FFAS03 (Fig. 1A-a) was found to match RNA polymerase II between approximately 143 and 553 aa, recombinaise was predicted by HHpred (Fig. 1A-b) and 3D-Jury between approximately 50 and 140 aa, and phase integrase by 3D-Jury (Fig. 1A-c) between 52 and 160 aa. All of the foregoing three proteins are known to interact directly with DNA. The observation that the fold prediction with the matched proteins partially overlapped with the DNA-binding region suggests that it shares similar major secondary structures and topological connections with these proteins.

Identification and characterization of a putative target gene for HsvG in gypsophila.

The identification of an HsvG putative target gene in gypsophila was achieved by employing the binding site-selection (BSS) procedure with hHsvG (HsvG labeled with histidine tag) (as described below) (Fig. 2). Inserts from randomly selected colonies were sequenced and screened for a putative ORF using the software ORF finder (National Center for Biotechnology Information [NCBI]). A 400-bp fragment, which contained the beginning of an ORF and the consensus HsvG binding site (Nissan et al. 2006), was repeatedly isolated. The sequence of 337 bp upstream of the translation start site, predicted to harbor the promoter, is presented in Figure 3A. The full mRNA sequence was obtained by employing the 3' random amplification of cDNA ends (RACE) procedure. A 1.1-kb fragment containing an ORF of 732 bp and a predicted protein

of 244 aa was obtained (Fig. 3B). The isolated target gene of HsvG in gypsophila was designated as *HSVGT* and submitted to the GenBank as accession number GQ923819.

The promoter region of *HSVGT* containing the consensus HsvG-binding site ACACAAA (Nissan et al. 2006) was located 263 bp upstream of the ATG start codon (Fig. 3A). Predictions made by the MEME-ChIP server discovered two motifs which are characteristic of eukaryotic promoters: AAACC[CT] (*E* value $1.1e + 002$), and T[GCT][AC][GT][GC]GTT (*E* value $1.3e + 001$) (Fig. 3A, A and B brackets, respectively). A TATA-box prediction made according to Milanesi and associates (1996) revealed two typical motifs, GTTATAACTC and CCTT TAAATT (Fig. 3A, C and D brackets, respectively).

Sequences homologous to HSVGT protein were found using a CS-Blast search (Biegert and Soding 2009) on NCBI and Uniprot databases. Hypothetical proteins were omitted and the resulting 38 homologs (Supplementary Fig. S1) were aligned using Mafft (Katho et al. 2002). The deduced amino acids of HSVGT showed highest homology to hypothetical DnaJ proteins from various plants but lacked the J-like domain, which is important for the chaperon function (Cheetham and Caplan 1998). Maximal homology was obtained with a DnaJ heat-shock N-terminal domain containing protein from *Helianthus annuus* (GI 211953609) 0.221 (134 aa), heat-shock protein from *Brachypodium distachyon* (GI 193848480) 0.236 (208 aa), DnaJ domain-containing protein from *Oryza sativa* Japonica Group (GI 115488688) 0.208 (125 aa), and DNAJ heat-shock N-terminal domain-containing protein from *Arabidopsis thaliana* (GI 15221189) 0.185 (140 aa). Conservation analysis of the amino acids of HSVGT with proteins of the DnaJ family,

Fig. 2. Schematic presentation of the binding-site selection (BSS) procedure. Genomic DNA of gypsophila was digested with *MseI*, ligated to adaptors, and transferred to a binding mixture containing purified hHsvG. Following incubation, the solution was passed through a nitrocellulose filter, washed three times with the binding buffer, and eluted with an elution buffer. The eluate was phenolized and polymerase chain reaction amplified with primers that complemented the adaptors. This procedure was repeated five times prior to cloning of the recovered DNA and transformation into *Escherichia coli*. Thirty colonies were randomly selected for sequencing. The target gene (*HSVGT*) appeared most frequently (approximately 80%) in two independent experiments.

performed by the Conseq server (Berezin et al. 2004), showed that 45.7% of the amino acids were highly or moderately conserved (Fig. 3B).

A search for domains on the HSVGT protein (Fig. 3B) identified a bipartite NLS predicted by the Predict NLS server, and two DNA-binding domains, namely, a zinc-finger C₂H₂ type (ZnF_C2H2) and a basic leucine zipper (bZIP), were predicted by the DBS-PRED and the DB-Bind servers, respectively. A site for SUMOylation (Fig. 3B) was predicted by the SUMOsp 2.0 server. The HSVGT is an acidic protein with an isoelectric point of 4.97. Stretches of acidic amino acids (Fig. 3B) (namely, glutamate and aspartate, 51 to 62 and 179 to 193 aa, respectively) could serve as potential activation domains (Latchman 2008). All of the above-mentioned domains are characteristic of transcription factors and could support the hypothesis that HSVGT might be involved in transcriptional activation.

HsvG induces HSVGT expression in planta.

The expression of HSVGT in gypsophila cuttings inoculated with the wild-type strain *P. agglomerans* pv. *gypsophila* 824-1 and its *hsvG* mutant (*P. agglomerans* pv. *gypsophila* MxhsvG)

was determined at early stages after inoculation by quantitative reverse-transcription (qRT)-PCR (Fig. 4). Results demonstrate an increase of more than three times in the transcripts level of HSVGT within 2 h postinoculation (hpi), reaching a peak of approximately 6-fold at 4 hpi and a decline to 3.5- and 2-fold after 6 and 8 hpi, respectively. These results suggest that HsvG induces the transcription of its target gene, HSVGT, during a very early stage of disease development.

To investigate whether HsvG might directly activate HSVGT transcription, its binding to the promoter region of HSVGT was examined by a gel-shift assay (Fig. 5). Results obtained illustrate that HsvG binds to double-stranded (ds)DNA generated by annealing the oligonucleotides Bind-Gt-dCo and Bind-Gt-rCo (Table 1), which harbor the promoter region with the consensus sequence ACACAAA (Fig. 5). In contrast, no binding was obtained with dsDNA generated by the oligonucleotides Bind-Gt-d-non and Bind-Gt-r-non that cover the same promoter region but lack the consensus sequence. The findings that HsvG physically binds to the HSVGT promoter, together with the rapid induction in HSVGT transcription, strongly suggest that HsvG acts as a transcription activator in gypsophila.

Fig. 3. Sequence of the HsvG target gene. **A**, Promoter region of HSVGT; 337 bp upstream of the transcription start codon (ATG, marked by clear box) is shown. The promoter contains the consensus HsvG binding site ACACAAA (gray box). Other motif predictions, which are based on bioinformatics tools MEME ChIP motifs, where bracket A = AAACC[CT] (*E* value 1.1e + 002) and bracket B = T[GCT][AC][GT][GC]GTT (*E* value 1.3e + 001), and TATA-box prediction motifs, marked by C and D brackets. **B**, Deduced amino acid sequence of HSVGT. An open reading frame of 244 amino acids (aa) is shown. Conservation score with the DnaJ family was obtained by the neighbor-joining with maximum likelihood method and amino acids are colored by conservation scale (ConSeq) shown below the sequence. Conserved residues are colored magenta and variable residues are cyan. Yellow letters present uncertain conservation prediction. The percentage of amino acids in conservation grade 8/9 is 45.7%. Domain search revealed a bipartite nuclear localization signal (blue line) (6 to 34 aa); zinc-finger C₂H₂ type (ZnF_C2H2) is designated by a green line (65 to 95 aa); and basic leucine zipper (bZIP) (78 to 92 aa) is indicated by a purple line. The highly predicted SUMOylation site is marked by an asterisk. DNA-binding predictions are shown by red (DBS-PRED) and orange (DB-Bind) lines below the sequences. Black lines below the sequence indicate stretches of the negatively charged amino acid residues, glutamic acid (E) (51 to 62 aa) and aspartic acid (D) (179 to 193 aa).

HSVGT is upregulated by HsvG in an effector-specific manner.

The specificity of HsvG as a transcription factor of *HSVGT* in gypsophila was tested by complementation of the *P. agglomerans* pv. *gypsophila* mutant in *hsvG* (*P. agglomerans* pv. *gypsophila* MxhsvG) with *hsvG*, *hsvB*, and *hsvB* converted into *hsvG* (Nissan et al. 2006) (Fig. 6). Results indicate that complementation with *hsvG* could restore transcription of *HSVGT*. In contrast, complementation with *hsvB* (pLHsvB8) could not restore expression of this target gene. However, *hsvB* converted to *hsvG* by exchange of the repeat domain (pLHsvB→G) (Table 2) could fully restore induction of *HSVGT*. Moreover, no significant difference in transcriptional induction could be observed between the *P. agglomerans* pv. *gypsophila*-*hsvG* mutant and mock inoculation (Fig. 4), suggesting that only HsvG and not other effectors that presumably are expressed by the mutant strain can activate *HSVGT*.

DISCUSSION

Deletion analysis of the HsvG protein followed by a binding assay to the DNA consensus sequence delineated the maximal DNA-binding region to 266 aa near the N terminus of the protein. This region is associated with a critical secondary structure, including many α helices and a β strand (Fig. 1). Moreover, fold-recognition analysis predicted similarity to proteins that interact with DNA such as RNA polymerase II, recombinase, and phase integrase, indicating that HsvG might share the same major secondary structures in the same arrangement and with the same topological connections. Previous analysis predicted the presence of the HTH domain in the N-terminus region of HsvG (Nissan et al. 2006). However, an extensive bioinformatics analysis with three different servers could not predict this motif or any other canonical DNA-binding domain within the borders of the identified DNA-binding domain. The precise structure of the HsvG DNA-binding domain should await the solution of the three-dimensional structure of HsvG. It is noteworthy that deletion of the repeat domain, which is

Fig. 4. Transcript levels of *HSVGT* in inoculated gypsophila cuttings with strain *Pantoea agglomerans* pv. *gypsophila* 824-1 and its marker exchange mutant at various time intervals postinoculation. Cuttings were inoculated by dipping in bacterial suspensions (10^8 CFU/ml) or mock inoculated with 10 mM $MgCl_2$ solution. Total RNA was extracted from cutting samples collected at various hours postinoculation and used for determination of *HSVGT* expression by quantitative reverse-transcription polymerase chain reaction analysis. Relative levels of *HSVGT* were normalized with β -actin, glyceraldehyde-3-phosphate dehydrogenase, and ATPase as internal references. Values are the average of three independent experiments with standard deviation. Each experiment included three replicates.

responsible for both host specificity and transcriptional activation of HsvG in yeast (Nissan et al. 2006), did not show any effect on DNA binding to the consensus sequence (Fig. 1). In contrast to HsvG, the DNA-binding domain of the AvrBs3/PthA family of *Xanthomonas* spp. is located in the repeat domain, which was also responsible for host specificity (Boch and Bonas 2010), indicating a fundamental difference between these two classes of effectors.

Although both transcription factors obviously have activation activity in yeast and similar DNA-binding capacities, we are still at a loss to explain the plant specificity of each protein. One possibility could be that the specificity arises not from any specific host factors but simply because the double-repeat protein is unstable in beet and the single-repeat protein is unstable in gypsophila. Thus, the proteins would not be active in the foreign environment. Another possibility would be that additional specific host factors are needed to assure transcriptional activation and these factors would be specific to binding either the single-repeat or double-repeat proteins under in vivo conditions. Proteomic studies of the infected hosts in which HsvG and HsvB can be identified by specific antibodies might be useful in supporting or rejecting this speculation.

The *HSVGT* target gene for HsvG in gypsophila was isolated by a BSS in vitro procedure (Fig. 2) and was shown to be transcriptionally induced promptly following host inoculation in an effector-specific manner (Figs. 4 and 6). Blast analysis indicates that the *HSVGT* protein belongs to the DnaJ family but lacks the J-domain, which is responsible for chaperon activity (Kelly 1998). The DnaJ family proteins are widely distributed in prokaryotes and eukaryotes and act as co-chaperons of the heat-shock protein Hsp70, a major component of the cellular chaperone network and stress response (Hennessy et al. 2005). The J-domain is the major binding site required for the interaction between the DnaJ and Hsp70. It is noteworthy that HopI1, a

Fig. 5. Gel shift assay of hHsvG with the *HSVGT* promoter DNA. Oligonucleotides used to obtain the *HSVGT* promoter are listed in Table 1. Lane 1, oligonucleotides (Bind-Gt-dCo and Bind-Gt-rCo) for generating double-stranded *HSVGT* promoter with the consensus sequence without hHsvG; lane 2, oligonucleotides (Bind-Gt-d-non and Bind-Gt-r-non) for generating double-stranded *HSVGT* promoter without the consensus sequence but with 60 ng of hHsvG; lane 3, as in lane 1 but with 30 ng of hHsvG; lane 4, as in lane 1 but with 60 ng of hHsvG. Arrow points to DNA-bound protein.

virulence effector of *Pseudomonas syringe*, directly binds Hsp70 through its J-domain, leading to Hsp70 ATP hydrolysis, which appears to be essential for virulence (Jelenska et al. 2010). However, the lack of a chaperon activity in HSVGT rules out the possibility that it is involved in activation of Hsp70 and protein folding. The DnaJ family of proteins share the J-domain region but, outside of this region, the family is greatly divergent and may have very different domain structures (Cheetham and Caplan 1998). Many eukaryotic transcription factors are modular proteins composed of distinct functional domains (Latchman 2008). Several structural motifs of the HSVGT protein could suggest that it might act as a transcription factor in plants (Fig. 3B). These include a putative bipartite NLS, suggesting an import to the nucleus; DNA-binding motifs of the C₂H₂ type (or classic) zinc finger and the basic domain leucine zipper (bZIP); as well as the presence of stretches of acidic amino acids (e.g., glutamic and aspartic acids), which are known to be associated with many transcriptional activation domains in eukaryotes (Latchman 2008). C₂H₂ zinc finger is one of the most common DNA-binding motifs in eukaryotic transcription factors. Plant zinc-finger proteins play critical regulatory roles by interacting with *cis*-elements of specific target gene promoters involved in plants stress responses (Ciftci-Yilmaz and Mittler 2008; Tian et al. 2010). bZIP represents a class of transcription factors that regulate expression of genes in a wide variety of developmental and physiological response pathways in plants and animals (Dong 2004; Sibril et al. 2001). In plants, bZIP transcription factors constitute a large family which has been implicated in stress signaling, including UV light and salt or drought signaling (Jakoby et al. 2002). Noteworthy are the TGA/OBF family members that interact with NON-EXPRESSOR OF PR1 (NPR1), a key component in salicylic acid signaling (Zhou et al.

2000). Interestingly, *OsTFX1*, a target gene of the TAL effector *pthXo6* from *Xanthomonas oryzae* pv. *oryzae* PX099^A, was reported to act as a bZIP transcription factor in rice, and is involved in mediating host susceptibility (Sugio et al. 2007). The prediction of sumoylation motif in HSVGT suggests that it might be involved in transcription regulation as well as a variety of other biological processes (Miura et al. 2007).

It is noteworthy that the promoter of *HSVGT* harbors two typical motifs in addition to two TATA boxes (Fig. 3A). Environmentally induced genes almost always contain a TATA box and at least two other *cis*-elements that may be involved in response to activation by external cues (Buchanan et al. 2000). Housekeeping genes, on the other hand, have less diversity in their *cis*-elements and may not even contain a recognizable TATA box. Therefore, the promoter structure of *HSVGT* could reflect characteristics of an environmentally induced gene. Further work will be necessary to find out whether additional target genes for HsvG exist in gypsofila and whether HSVGT does, indeed, act as a transcriptional activator during pathogenesis of *P. agglomerans* pv. *gypsophila*.

The rapid induction of *HSVGT* by HsvG within 2 h (Fig. 4) is in accordance with the anticipated short period required for translocation of effectors into the host cells via the T3SS (Enninga et al. 2005). The observation that HsvG physically binds to the *HSVGT* promoter via the consensus sequence (Fig. 5) suggests that it acts as a direct or primary target gene of HsvG. The demonstration that the consensus of *HSVGT* was responsible for HsvG binding to its promoter raises the hypothesis that it could serve as a conserved promoter element for binding of HsvG to other primary target genes. This could be similar to the *UPA* box, which binds the AvrBs3 of *Xanthomonas campestris* pv. *vesicatoria* to its primary target genes in

Table 1. Sequence of primers used in this study

Primer	Sequence	Used for ^a
1R	5'-AACTCAAACGGCCATGACTCC-3'	...
1L	5'-AAAGACATGGGCGTCGCGCCTC-3'	Creating pFLAG-HsvG _{A115-287}
2R	5'-GCTGTCAGGCTGCTGCACACG-3'	...
2L	5'-CGGGACGGTGACCGGGGTTTC-3'	Creating pFLAG-HsvG _{A530-655}
3R	5'-AGGCGGCGACGCCCATGTCTTTATCG-3'	...
3L	5'-CGGGTTAATGTTTGTTCACATGCTGG-3'	Creating pFLAG-HsvG _{A8-107}
4R	5'-GAACCCGGTACCGTCCC-3'	...
4L	5'-AATGGAGTCATGGCCGTTTGTAG-3'	Creating pFLAG-HsvG _{A373-655}
5R	5'-GGAGAGGACAGCATCACGATACGCG-3'	...
5L	5'-CGGGTTAATGTTTGTTCACATGCTGG-3'	Creating pFLAG-HsvG _{A8-140}
IR	5'-GATACCGCTTACCTGTTCTCGGAGC-3'	...
IL	5'-GTTTTCGTGAAGCCAGGCGCTGAGG-3'	Creating pFLAG-HsvG _{A199-204}
IIR	5'-GGCAGGGTAAACATGATACTGGCCC-3'	...
IIL	5'-CCGGGAGAACGCACCGGCGTC-3'	Creating pFLAG-HsvG _{A224-229}
IIIR	5'-GACTCCATTGACAAAAAGCCTCTCAG-3'	...
IIIL	5'-GGCCTGCCGTGCGAGGTCTTTTCG-3'	Creating pFLAG-HsvG _{A282-293}
Mse-1	5'-GACGATGAGTCCTGAG-3'	...
Mse-2	5'-ATGAGTCCTGAGTA-3'	BSS primers and adaptors
GudR	5'-ACAGTGAGGCTGGTGGTGAT-3'	...
GudL	5'-TCGCGTCCTCCTCCTCAGACC-3'	Cloning of <i>HSVGT</i>
GRt-D	5'-GGGCGTTTCAGGAGCC-3'	...
GRt-R	5'-GCCGCACAATACAGCTCCCTCTTCTCCTC-3'	Real-time PCR of <i>HSVGT</i>
Gg-d	5'-ATGTCTACGATAAGCAAAACAC-3'	...
Gg-r	5'-AAGTTAACACATAGTAGTGTGTC-3'	Glyceraldehydes-3-phosphate dehydrogenase IN
Gc-d	5'-CAGAGAGAAGATGACTCAAATCATG-3'	...
Gc-r	5'-CAGCCTGGATAGCAACATACATAGC-3'	<i>actin</i> IN
Gt-d	5'-ATAAGACGAGAACA-3'	...
Gt-r	5'-GGCAGCAGCATTAATTTG-3'	<i>ATPase</i> IN
5R1-R	5'-TTCCTTATGCTTCTTCGACTG-3'	...
5R2-R	5'-TCGACCTGCTCATGAATCCTCCAC-3'	Primer for 5' RACE
Bind-Gt-dCo	5'-ATGATGGAAATCATGACACAAAAGGTACAGCAAAGGGTGGA-3'	...
Bind-Gt-rCo	3'-TTCCACCCTTTGCTGTACCTTTTGTGTCATGATTCCATCAT-5'	Generating <i>HSVGT</i> promoter region with consensus
Bind-Gt-d-non	5'-GTGATGATGGAAATCATGGTACAGCAAAGGGTGGAAGAA-3'	...
Bind-Gt-r-non	3'-TTCTTTCCACCCTTTGCTGTACCCATGATTTCCATCATCAC-5'	Generating <i>HSVGT</i> promoter region without consensus

^a BSS = binding site selection, PCR = polymerase chain reaction, IN = internal Normalizer, and RACE = random amplification of cDNA ends.

pepper (Kay et al. 2007, 2009). The observation that HsvG but not HsvB could complement the *P. agglomerans* pv. *gypsophila*-*hsvG* mutant for activation of *HSVGT* demonstrates its high specificity to the target gene. Moreover, HsvB converted into HsvG by exchanging their activation domains could fully complement *HSVGT*. Thus, it further support the premise that host specificity of HsvG and HsvB is determined via the activation domain (Barash and Manulis-Sasson 2009; Nissan et al. 2006) rather than by the DNA-binding domain, as in the case of AvrBs3 effectors (Bogdanove et al. 2010).

The induced expression of *HSVGT* measured by qRT-PCR could result from activation of transcriptional initiation as well as post-transcriptional regulation. However, it has been previously shown that HsvG is transported into the nucleus by an NLS-mediated process and it acts as a transcription factor in yeast (Nissan et al. 2006; Weinthal et al. 2011). In the present report, we demonstrate that HsvG physically binds to the promoter of its characterized target gene, *HSVGT*, and induces its expression in a rapid and specific manner. Taken together, these results strongly support the view that HsvG acts as a putative transcription factor in *gypsophila*.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

The bacterial strains, cosmids, and plasmids used in this study are listed in Table 2. Rifampicin-resistant wild-type strain of *P. agglomerans* pv. *gypsophila* 824-1 and its derivatives were used throughout. Bacteria were grown in Luria-Bertani (LB) broth and agar or in a minimal medium (Ausubel et al. 1995) at 28°C with appropriate antibiotics. *Escherichia coli* DH5 α (Invitrogen Co., Carlsbad, CA, U.S.A.) was used as cloning host and was grown at 37°C on LB plates or in LB broth with the appropriate antibiotics. Antibiotics were used in the following concentrations: ampicillin at 150 μ g/ml, kanamycin at 30 μ g/ml, rifampicin at 150 μ g/ml, and tetracycline at 15 μ g/ml.

DNA manipulations.

DNA isolation, agarose gel electrophoresis, electroporation, PCR, and other DNA manipulation methods were performed according to standard procedures (Ausubel et al. 1995) or as recommended by the supplier. DNA fragments were amplified by PCR with *Taq* polymerase (Sigma-Aldrich, St. Louis) or with Phusion hot start DNA Polymerase (Finnzymes, Keilanta, Finland), and synthetic oligonucleotides were synthesized according to specification (Sigma-Aldrich).

PCR-amplified DNA or plasmid DNA was purified by a QIAprep spin miniprep kit (Qiagen, Basel, Switzerland) prior to sequencing. Automated sequencing with *Taq* DNA polymerase was carried out at the Laboratories for Biological Services, Tel-Aviv University using the ABI Prism 3100 Genetic Analyzer. Analysis of sequence data for the DNA and deduced protein sequences was mainly done according to the Program Manual for the Wisconsin Package (version 11; Genetic Computer Group, Madison, WI, U.S.A.).

Deletion mutations in *hsvG* were generated by employing opposite primers (Table 1) that did not include the desired deletion region, as previously described (Nissan et al. 2006). The primers were designed to create in-frame blunt-ended fragments. PCR was performed with Phusion high-fidelity DNA polymerase (Finnzyme, Espoo, Finland) and the amplified fragments were cloned into the expression vector pFLAG-ATS (Sigma-Aldrich) and transformed into *E. coli* DH5 α . The proteins were purified as FLAG tag using ANTI-FLAG resin according to instructions of the manufacturer's protocols (Sigma-Aldrich). The deletion mutants were analyzed by mobility shift assays for DNA binding using the Dig labeling kit

(Roche, Mannheim, Germany) following the manufacturer's protocols and according to Nissan and associates (2005).

Binding-site selection.

HsvG was expressed and purified under native conditions as 6x-his tag HsvG (hHsvG) according to Nissan and associates (2005). *Gypsophila paniculata* var. *perfecta* genomic DNA was extracted, digested with *MseI*, and ligated to *MseI* adaptors according to the online protocol (Carnegie Institution for Science Department of Biology website). BSS was performed with HsvG as a binding protein and primers that complement the adaptors, similar to the random binding-site selection procedure described previously (Nissan et al. 2006, Okuno et al. 2001). The binding mixture contained purified hHsvG (30 ng), *gypsophila* genomic DNA digested with *MseI* and ligated to *MseI* adaptors (1 μ g), bovine serum albumin (5 μ g/ml), EDTA (125 mM), and 3 ml of 1 $\times$ binding buffer before the bound oligonucleotides were eluted with 100 μ l of elution buffer containing 20 mM Tris-HCl (pH 7.5) and 3.5 μ l of 10 $\times$ binding buffer (100 mM Tris-HCl, pH 7.5, 50% glycerol, 10 mM dithiothreitol, and 10 mM EDTA). The mixture was incubated for 30 min at room temperature. Thereafter, this solution was

Fig. 6. Relative expression of *HSVGT* in the *hsvG* marker exchange mutant in *gypsophila* cuttings complemented with *hsvG* or *hsvB* at 4 h postinoculation. Bar a, *Pantoea agglomerans* pv. *gypsophila* MxhsvG (*P. agglomerans* pv. *gypsophila*-*hsvG* mutant); bar b, *P. agglomerans* pv. *gypsophila* MxhsvG/pLHsvG (*P. agglomerans* pv. *gypsophila*-*hsvG* mutant complemented with *hsvG*); bar c, MxhsvG/pLHsvB8 (*P. agglomerans* pv. *gypsophila*-*hsvG* mutant complemented with *hsvB*); and bar d, MxhsvG/pLHsvB $\rightarrow$ G (*P. agglomerans* pv. *gypsophila*-*hsvG* mutant complemented with *hsvB* converted to *hsvG*). Cuttings were inoculated by dipping in bacterial suspensions (10^8 CFU/ml) or mock inoculated with 10 mM MgCl $_2$ solution. Total RNA was extracted from cutting samples collected at various hours postinoculation and used for determination of *HSVGT* expression by quantitative reverse-transcription polymerase chain reaction analysis. Relative levels of *HSVGT* were normalized with β -actin, glyceraldehyde-3-phosphate dehydrogenase, and ATPase as internal references. Results represent the means of three independent experiments with standard deviation. Each experiment included three replicates.

passed slowly through a presoaked BA85, 0.45- μ m, 25-mm nitrocellulose filter (Schleicher & Schuell GmbH, Dassel, Germany). The filter was washed three times with 3 ml of 1 $\times$ binding buffer before the bound oligonucleotides were eluted with 100 μ l of elution buffer containing 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 20 mM NaCl, and 0.1% sodium dodecyl sulfate. The eluate was phenolized and then amplified by PCR using primers Mse-d and Mse-r (Table 1). The amplified product was transferred into the binding reaction mixture and this procedure was repeated. After five rounds of enrichment, the selected oligonucleotides were cloned into pGEM-T Easy (Promega Corp., Madison, WI, U.S.A.), introduced into *E. coli* DH5 α , and sequenced. Thirty DNA fragments were sequenced from randomly selected colonies in each experiment. The sequence related to the target gene (*HSVGT*) was frequently identified (approximately 80%) in two independent experiments. The remaining sequences, which did not show any clear repeated pattern or ORF regions, were abandoned.

RACE cDNA amplification.

Full mRNA sequences were isolated according to the 3' and 5' RACE procedure (Frohman 1993). Total RNA was extracted from gypsophila cuttings using the NucleoSpin RNA Plant kit (Machrey-Nagel GmbH & Co., Duren, Germany) and first-strand synthesis and 3' or 5' RACE PCR were carried out by using the Smart RACE cDNA Amplification kit (Clontech Laboratories Inc., Mountain View, CA, U.S.A.) according to the manufacturer's protocols. PCR was performed using internal primers from the known sequences. The PCR fragment was cloned into the PCR cloning vector pGEM-T Easy (Promega Corp.) and sequenced.

qRT-PCR analysis.

Total RNA was extracted from inoculated cuttings of *G. paniculata* var. *perfecta* as follows. After removal of an ap-

proximately 3-mm section from the bottom of the stem, the cuttings were inoculated by dipping into a bacterial suspension of 10⁸ cells/ml and placed in vermiculite-filled trays in the greenhouse at 26 to 29°C. Total RNA was extracted from 100 mg of tissue per treatment. Gene-specific primers for *HSVGT* (Table 1) were used for PCR amplification. PCR reactions were performed in triplicate and contained template cDNA, 200 nM gene-specific primers, and SYBR Green qPCR master mix (Stratagene, Santa Clara, CA, U.S.A.) in a volume of 15 μ l. Reactions were carried out using an Mx 3000P QPCR System (Stratagene) with the following cycling program: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 54°C, and 30 s at 72°C. Fluorescence was monitored at the end of each cycle. The absence of nonspecific products and primer dimers was confirmed by analysis of melting curves and agarose gel electrophoresis according to Balaji and associates (2008). For data analysis, threshold cycle values of three independent biological replicates were averaged, normalized, and used to calculate relative transcript levels as described by Pfaffl (2001). Data were compared using a Student's *t* test for independent samples (mock versus treated), with the level of significance set at *P* < 0.05.

The gypsophila genes selected as internal standards for normalization were actin, glyceraldehyde-3-phosphate dehydrogenase and ATPase. Because the sequence of the gypsophila genome is not available, specific primers for cloning these conserved genes from gypsophila were taken from tomato (Mayrose et al. 2006). Sequences of the above-mentioned genes from gypsophila were used for planning the primers for the housekeeping genes (Table 1).

Bioinformatics methods.

Secondary structures predictions of the HsvG protein were performed according to Porter (Pollastri and McLysaght 2005), Jpred (Cole et al. 2008), and PSIPRED (Jones 1999). Three

Table 2. Bacterial strains, cosmids, and plasmids used in this study

Strains, plasmids	Relevant characteristics ^a	Reference or source
DH5 α	<i>Escherichia coli</i> , <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>gyrA</i> 96	Invitrogen
<i>Pantoea agglomerans</i> pv. <i>gypsophilae</i> strains		
824-1	Wild-type pathogenic strain with Rif ^r	Manulis et al. 1991
MxhsvG	<i>P. agglomerans</i> pv. <i>gypsophilae</i> 824-1, marker exchange mutant in <i>hsvG</i> (previously <i>P. agglomerans</i> pv. <i>gypsophilae</i> MxB45)	Valinsky et al. 1998
Cosmids		
pLAFR3	Tc ^r , broad-host-range vector <i>IncP-1</i> rixRK2+ <i>lacZa</i> Tra- Mob+ cos	Staskawicz et al. 1987
pLHsvG	Tc ^r , <i>hsvG-Pag</i> under its own promoter cloned into pLAFR3 as an <i>HindIII/BamHI</i> fragment	Valinsky et al. 1998
pLHsvB8	Tc ^r , <i>hsvB-Pag</i> under its own promoter cloned into pLAFR3 as an <i>HindIII/BamHI</i> fragment	Nissan et al. 2006
pLHsvB $\rightarrow$ G	Tc ^r , <i>hsvB-P. agglomerans</i> pv. <i>betae</i> with addition in frame of RI of <i>hsvG</i> cloned into pLAFR3 as an <i>HindIII/BamHI</i> fragment	Nissan et al. 2006
Plasmids		
pQE70	Ap ^r , His-tag expression vector	Qiagen
pHsvG-his	Ap ^r , PCR-amplified <i>hsvG</i> coding sequence cloned into pQE70 as a <i>SphI/BamHI</i> fragment	Nissan et al. 2006
pGEM-T Easy	Ap ^r , PCR cloning vector	Promega
pFLAG-ATS	Ap ^r , Expression and secretion of N-terminal FLAG-fusion proteins under <i>tac</i> promoter	Sigma-Aldrich
pFLAG-HsvG Δ 115-287	Ap ^r , HsvG with a deletion in 115 to 287 amino acids (aa)	This study
pFLAG-HsvG Δ 8-107	Ap ^r , HsvG with a deletion in 8 to 107 aa	This study
pFLAG-HsvG Δ 8-140	Ap ^r , HsvG with a deletion in 8 to 140 aa	This study
pFLAG-HsvG Δ 530-655	Ap ^r , HsvG with a deletion in 530 to 655 aa	This study
pFLAG-HsvG Δ 373-655	Ap ^r , HsvG with a deletion in 373 to 655 aa	This study
pFLAG-HsvG Δ 199-204	Ap ^r , HsvG with a deletion in 199 to 204 aa	This study
pFLAG-HsvG Δ 224-229	Ap ^r , HsvG with a deletion in 224 to 229 aa	This study
pFLAG-HsvG Δ 282-293	Ap ^r , HsvG with a deletion in 282 to 293 aa	This study
pGEM-GP	Ap ^r , cloning of BSS products	This study
pGEM-G3'RACE	Ap ^r , cloning of gypsophila 3' RACE products	This study
pGEM-HSVGT	Ap ^r , PCR-amplified <i>HSVGT</i> coding sequence	This study
pGEM-Gac	Ap ^r , PCR-amplified fragment of Actin gene from the gypsophila	This study
pGEM-GGly	Ap ^r , PCR-amplified fragment of glyceraldehyde-3-phosphate dehydrogenase gene from gypsophila	This study
pGEM-GAT	Ap ^r , PCR-amplified fragment of ATPase gene from gypsophila	This study

^a Rif^r, Tc^r, and Ap^r indicate resistance to rifampicin, tetracycline, and ampicillin, respectively.

different methods were employed to predict the fold recognition of HsvG, namely, 3D-JURY, HHPRED and FFAS03 (Ginalski et al. 2002; Jaroszewski et al. 2005; Söding 2005). Promoter motif prediction for HSVGT was carried out by the MEME-ChIP server according to Baily and Elkan (1994) and Milanese and associates (1996). TATA-box prediction was made by Zeus2 (Milanese et al. 1996).

Sequences homologous to the HSVGT protein were found using a CS-Blast search (Biegert and Soding 2009) on NCBI and Uniprot databases. Hypothetical proteins were omitted and the resulting 38 homologs were aligned using Mafft (Katho et al. 2002). A nuclear localization sequence was predicted by Predict NLS (Nguyen Ba et al. 2009). DNA binding prediction in HSVGT was obtained by the following two servers: DBSPRED predictions (Ahmad et al. 2004) and DB-Bind (Hwang et al. 2007). SUMOylation prediction was made by SUMOsp 2.0 (Ren et al. 2009). Various motifs and domains such as SnF_C2H2 and bZIP were located as presented in the UniProt database. Conservation analysis was performed by the Conseq server (Berezin et al. 2004). Servers used for prediction of helix-turn-helix or helix-loop-helix are listed below.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Carnegie Institution for Science Department of Biology website:
www-ciwdpb.stanford.edu/publications/methods/aflp.html
 Conseq server: conseq.tau.ac.il
 DBS-PRED prediction server:
gibk26.bio.kyutech.ac.jp/jouhou/shandar/netasa/dbs-pred
 EMBOSS Explorer helixturnhelix server: bioinformatica.cecilc.ula.ve/cgi-bin/emboss/helixturnhelix
 GYM 2.0 helix-turn-helix detection program:
<http://users.cis.fiu.edu/~giri/bioinf/GYM2/welcome.html>
 MEME Suite analysis tools:
meme.nbcr.net/meme/intro.html
 Pôle BioInformatique Lyonnais helix-turn-helix motif prediction server:
npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_hth.html
 Prediction of or helix-loop-helix
 SUMOsp server: sumosp.biocuckoo.org/online.php
 UniProt database: www.uniprot.org
 University of Albany DB-Bind server: lcg.rit.albany.edu/dp-bind/
 University of Toronto NLStradamus webpage:
www.moseslab.csb.utoronto.ca/NLStradamus
 Webgene Zeus 2 analysis tool:
zeus2.itb.cnr.it/~webgene/wwwHC_tata.html