The Expression of Genes Encoding Lipodepsipeptide Phytotoxins by *Pseudomonas syringae* pv. *syringae* Is Coordinated in Response to Plant Signal Molecules

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Submitted 16 August 2005. Accepted 25 October 2005.

Specific plant signal molecules are known to induce syringomycin production and expression of *syrB1*, a syringomycin synthetase gene, in *Pseudomonas syringae* pv. *syringae*. This report demonstrates that syringopeptin production likewise is activated by plant signal molecules and that the GacS, SalA, and SyrF regulatory pathway mediates transmission of plant signal molecules to the *syr-syp* biosynthesis apparatus. Syringopeptin production by B132 was increased two-fold by addition of arbutin (100 μ M) and D-fructose (0.1%) to syringomycin minimal medium (SRM). Among 10 plant phenolic compounds tested, only the phenolic glucosides arbutin, salicin, and phenyl- β -D-glucopyranoside induced substantially the β -glucuronidase (GUS) activity of a *sypA::uidA* reporter from 242 U per 10^8 CFU without plant signal molecules up to 419 U per 10^8 CFU with plant signal molecules. Syringopeptin production was found to be controlled by the SalA/SyrF regulon because no toxin was detected from cultures of B301DSL7 (i.e., *salA* mutant) and B301DSL1 (i.e., *syrF* mutant), and the expression of *sypA::uidA* was decreased approximately 99 and 94% in *salA* (B301DSL30) and *syrF* (B301DNW31) mutant backgrounds, respectively. Subgenomic analysis of transcriptional expression with a 70-mer oligonucleotide microarray demonstrated that the *syr-syp* genes are induced 2.5- to 10.5-fold by addition of arbutin and D-fructose to SRM. This study establishes that plant signal molecules are transmitted through the GacS, SalA/SyrF pathway to activate the coordinated transcriptional expression of the *syr-syp* genes.

Additional keywords: GacA, genomic island, Hrp, stimulon.

Bacteria commonly detect specific plant metabolites, which serve as signals for regulating expression of virulence genes that allow adaptation to the plant environment. For example, several virulence (*vir*) genes in *Agrobacterium tumefaciens* are

transcriptionally activated by specific phenolic signal molecules such as acetosyringone (Melchers et al. 1989). The induction is enhanced by pyranose sugars such as D-fructose, D-mannose, and D-glucose at low concentrations. Accordingly, the inducing sugars enhanced the expression of a *virB::lacZ* fusion more than 10-fold at a 10 mM concentration in the presence of 2.5 μ M acetosyringone (Ankenbauer and Nester 1990; Shimoda et al. 1990). Similarly, plant signal molecules were reported to activate the expression of genes involved in toxin synthesis (i.e., syringomycin and coronatine), cell wall degradation (i.e., *pel*), and the Hrp type III secretion system (Beaulieu et al. 1993; Li et al. 1998; Mo and Gross 1991; Quigley and Gross 1994; Xiao et al. 1992). Thus, plant signal molecules appear to play an important role in the interaction between plants and bacteria.

Syringomycin production is modulated by the perception of specific plant signal molecules (Mo et al. 1995; Mo and Gross 1991; Quigley and Gross 1994). Syringomycin is one of the major virulence factors of *Pseudomonas syringae* pv. *syringae*, which incites stem and leaf diseases in numerous monocot and dicot plants throughout the temperate regions of the world (Scholz-Schroeder et al. 2001a). Inoculations of immature cherry fruit demonstrated a rapid and strong expression of *syrB1*, a synthetase gene for syringomycin. Cherry tissues contain plant signal molecules, such as flavonol glycosides (quercetin and kaempferol 3-rutinosyl-4'-glucoside) and a flavanone glucoside (dihydrowogonin 7-glucoside), that are able to transcriptionally activate expression of a *syrB1::lacZ* fusion (Mo et al. 1995). In a survey of 34 phenolic compounds, only specific phenolic glycosides exhibited *syrB1*-inducing activity. The phenolic glycosides that exhibited *syrB1*-inducing activity included arbutin, salicin, and esculin, which are abundant in the tissues of many plant species (Mo and Gross 1991). Arbutin was shown to be an efficient signal molecule that induced the high expression of the *syrB1* synthetase gene (Mo and Gross 1991). The plant signal molecules that activate toxin production by *P. syringae* pv. *syringae* are different from those that induce the *vir* genes of *A. tumefaciens* and the *nod* genes of rhizobia (Melchers et al. 1989; Mercier and Lindow 2000; Peters and Verma 1990). All *syrB1*-inducing phenolic signal molecules characteristically contain an intact glycosidic linkage. Specific sugars that occur in large quantities in leaf tissues markedly enhance phenolic signal activity (Mo et al. 1995; Mo and Gross 1991). D-fructose is the most active sugar, causing a 10-fold stimulation of signal activity in strain B3AR132 when phenolic signals are present at low concentrations (i.e., 1 to 10

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μM). D-fructose and sucrose also exhibit intrinsic low-level *syrB1*-inducing signal activity in the absence of the phenolic inducer. Certain strains of the bacterium, such as B3A, require plant signal molecules for syringomycin production when grown under defined cultural conditions. Nearly all *P. syringae* pv. *syringae* strains produce higher syringomycin levels when the plant signal molecules arbutin and D-fructose are added to the media, but the extent of induction varies for individual strains. Several strains produce more than 10-fold higher toxin levels in the presence of arbutin and D-fructose, whereas other strains, including B301D, show approximately a twofold increase of syringomycin production (Quigley and Gross 1994).

Syringopeptin is another lipodepsipeptide that contributes to virulence of *P. syringae* pv. *syringae* (Scholz-Schroeder et al. 2001a). Syringopeptin and syringomycin have similar structures consisting of cyclic peptide heads attached to 3-hydroxy fatty acid tails. Both syringomycin and syringopeptin are synthesized by a nonribosomal mechanism of peptide biosynthesis (Grgurina et al. 1996; Scholz-Schroeder et al. 2001b). The syringomycin (*syr*) and syringopeptin (*syp*) gene clusters (Fig. 1) are located adjacent to one another on the chromosome and are approximately 42 and 90 kb in size, respectively (Scholz-Schroeder et al. 2003). The *syr* and *syp* gene clusters include biosynthesis, regulatory, and secretion genes for syringomycin and syringopeptin production (Kang and Gross 2005; Lu et al. 2002; Quigley et al. 1993; Scholz-Schroeder et al. 2001b, 2003). Both syringomycin and syringopeptin production are controlled by the *salA* gene. Based on microarray analysis, the *syr-syp* genes belong to the SalA regulon (Lu et al. 2005). Microarray analysis is applicable to studies of the transcriptional regulation of the *syr-syp* genes simultaneously under defined environmental conditions (Lu et al. 2005).

P. syringae utilizes a well-tuned regulatory and signal transduction system to coordinate the expression of virulence factors in order to adapt to the plant environment. The GacS/A two-component signal transduction system is well suited to sense and recognize environmental signals and to transduce these signals to downstream regulators (Heeb and Haas 2001). GacS is a transmembrane protein which functions as a histidine protein kinase that undergoes phosphorylation in response to environmental stimuli (Hrabak and Willis 1993). GacA is a response regulator protein that is phosphorylated by GacS (Heeb and Haas 2001; Rich et al. 1994). The GacS/A system is highly conserved in bacteria (Heeb and Haas 2001) and is dedicated to a

variety of functions, such as pathogenicity (Hrabak and Willis 1993), quorum sensing (Chancey et al. 1999), secondary metabolite production (Corbell and Loper 1995), and biofilm formation (Parkins et al. 2001). It was reported that production of the lipopeptide amphisin by *Pseudomonas* sp. strain DSS73 is regulated by components of sugar beet seed exudates via the GacS/A two-component system (Koch et al. 2002). Mutational inactivation of either GacS or GacA in *P. fluorescens* CHA0 abolishes the response of exoproduct genes such as *hcnA* (for HCN synthetase) to an undefined solvent-extractable signal (Heeb et al. 2002). In *P. syringae*, the GacS/A two-component signal transduction system positively regulates expression of the *salA* gene (Kitten et al. 1998) which, in turn, is required for expression of *syrF* (Lu et al. 2002). The regulatory gene *syrF* positively controls the expression of the syringomycin *syrB1* synthetase gene (Lu et al. 2002). Previous studies showed that *salA* also is required for syringopeptin production, based on toxin bioassays, microarray analysis, and β -glucuronidase (GUS) assays (Lu et al. 2005). Chatterjee and associates (2003) showed that GacA is a master regulator controlling *P. syringae* pv. *tomato* DC3000 *hrpR*, *hrpS*, and *hrpL*, which are required for the activation of Hrp type III secretion and effector genes. GacA apparently is not required for *hrp* expression in *P. syringae* pv. *syringae* B728a (Chatterjee et al. 2003). Based on the high similarity between *P. syringae* pv. *syringae* B728a and B301D, it was predicted that GacS/A is involved in the signal transduction of the B301D *syr-syp* genes, but not the *hrp* genes. Nevertheless, it is unknown whether plant signal molecules activate *hrp* genes in *P. syringae* pv. *syringae*, presumably through a regulatory network other than the GacA/GacS regulon.

Despite progress made toward understanding the regulation of the *syr* and *hrp* genes, little is known about the regulation of syringopeptin or the signal transduction pathway of the *syr-syp* and *hrp* genes in *P. syringae* pv. *syringae* B301D. In this study, it was hypothesized that specific plant signal molecules induce the coordinated expression of the *syr-syp* and *hrp* genes of *P. syringae* pv. *syringae* and that these two categories of genes use different signal transduction pathways. To test this hypothesis, a subgenomic 70-mer oligonucleotide microarray was used to measure the transcriptional profile of B301D grown on syringomycin minimal medium (SRM) agar medium as compared with SRM agar medium supplemented with arbutin and D-fructose (i.e., SRM_{AF}). Evidence is presented that the addition of arbutin and D-fructose resulted in significant in-

Fig. 1. Physical map of a 132-kb genomic DNA region of *Pseudomonas syringae* pv. *syringae* B301D containing both syringomycin (*syr*) and syringopeptin (*syp*) gene clusters. The positions and orientations of the known and potential open reading frames (ORFs) are shown as horizontal arrows. The solid, diagonally striped, and vertically striped arrows represent genes that are predicted to be involved in the synthesis, regulation, and secretion of the phytotoxins, respectively. The gray arrows represent the potential ORFs for which functions remain unknown.

creases in transcriptional levels of the genes contributing to synthesis, secretion, and regulation of both syringomycin and syringopeptin, as well as the *hrp* genes. Mutational analysis of *gacS* showed that the GacS/A two-component system is involved in the signal transduction of the *syr-syp* genomic island but not representative genes associated with the Hrp type III secretion system. These results are discussed in regard to the involvement of the GacS/A two-component system and two key regulators, SalA and SyrF, in mediating plant signal transduction responsible for the transcriptional activity of genes associated with lipodepsipeptide phytotoxin production.

RESULTS

Induction of syringopeptin production by plant signal molecules.

Syringopeptin production was increased substantially for derivatives of strains B301DR and B3AR (i.e., *syrB1* mutants BR132 and B3AR132) in the presence of arbutin (100 μ M) and fructose (0.1%) as plant signal molecules. B3AR132 did not produce a visible zone of syringopeptin inhibitory to *Bacillus megaterium* growth on SRM agar medium, but the radius of the inhibitory zone was approximately 4.7 mm on SRM_{AF} agar medium (Fig. 2). Addition of arbutin and fructose increased the radius of the inhibitory zone produced by BR132 by 4 mm, representing a 66.7% increase in size. Syringomycin was not detected for B3A in the absence of plant signal molecules in a bioassay using *Geotrichum candidum*, but a small zone of inhibition was observed on SRM_{AF} agar medium. The sizes of the zones of syringomycin production by B301D were 55.6%

larger on SRM_{AF} agar medium than on SRM agar medium. Consistent results were obtained with repeats on three independent occasions.

Induction of *sypA*.

The expression of *syrB1::uidA* fusion strain B301DSL8 was increased 1.66-, 1.53-, and 1.55-fold by addition of 100 μ M of the phenolic β -glucosides arbutin, phenyl- β -D-glucopyranoside, and salicin to SRM liquid medium, respectively (Table 1). Similarly, the expression of a *sypA::uidA* fusion strain (B301DSL29) was increased 1.52- to 1.73-fold on SRM with the phenolic glucosides (Table 1). Arbutin was the most active inducer for both genes (Table 1). Other phenolic compounds (hydroquinone, naringenin, phenol, phenyl- β -D-galactopyranoside, quercetin, rutin, and saligenin) tested did not show significant effect on expression of either *syrB1::uidA* or *sypA::uidA* fusions.

A β -glucosidic linkage was shown to be critical to signal activity. This was evaluated by testing the substituted derivatives of arbutin, phenyl- β -D-glucopyranoside and salicin (i.e., hydroquinone, saligenin, and phenol) (Table 1). All three phenolic aglucones were inactive in induction of both *syrB1::uidA* and *sypA::uidA* fusions.

Once it was confirmed that certain phenolic β -glycosides induced the expression of *sypA*, the effects of the sugar D-fructose on arbutin-mediated induction of *sypA* and *syrB1* were determined. Strain B301DSL8, which carries the *syrB1::uidA* fusion, produced high levels of GUS activity, averaging 1,274 units per 10^8 CFU 3 days after incubation at 25°C in SRM_{AF}

Fig. 2. Bioassay for syringopeptin production by two strains of *Pseudomonas syringae* pv. *syringae* grown on syringomycin minimal medium without (SRM) or with 100 μ M arbutin and 0.1% D-fructose (SRM_{AF}) as shown by zones inhibitory to *Bacillus megaterium*. The strains were grown for 4 days on plates at 25°C. Plates were oversprayed with *B. megaterium* and allowed to incubate for 24 h at 25°C. Zones of inhibition indicate production of syringopeptin. A, B3AR132 (*syrB1* mutant) and B, BR132 (*syrB1* mutant).

Fig. 3. Bioassay for syringopeptin production by different strains of *Pseudomonas syringae* pv. *syringae* B301D grown on syringomycin minimal medium without (SRM) or with 100 μ M arbutin and 0.1% D-fructose (SRM_{AF}) as shown by zones inhibitory to *Bacillus megaterium*. The strains were grown on plates at 25°C for 4 days. Plates were oversprayed with *B. megaterium* and allowed to incubate for 24 h at 25°C. Zones of inhibition indicate production of syringopeptin. A, BR132 (*syrB1* mutant); B, B301DSL7 (*salA* mutant); C, B301DSL1 (*syrF* mutant); and D, B301D.

Table 1. Effects of plant phenolic β -glycosides on induction of *sypA::uidA* and *syrB1::uidA* fusions in strains B301DSL29 and B301DSL8, respectively

Phenolics added to SRM ^y	<i>sypA::uidA</i>		<i>syrB1::uidA</i>	
	GUS activity (U/ 10^8 CFU $\pm$ SE) ^z	Fold increase	GUS activity (U/ 10^8 CFU $\pm$ SE) ^z	Fold increase
None	242 $\pm$ 14 a	...	685 $\pm$ 63 a	...
Arbutin	419 $\pm$ 30 b	1.73	1,140 $\pm$ 73 b	1.66
Phenyl- β -D-glucopyranoside	389 $\pm$ 24 b	1.60	1,050 $\pm$ 44 b	1.53
Salicin	369 $\pm$ 18 b	1.52	1,060 $\pm$ 32 b	1.55
Hydroquinone	298 $\pm$ 19 a	1.23	755 $\pm$ 48 a	1.1
Saligenin	279 $\pm$ 27 a	1.15	732 $\pm$ 33 a	1.07
Naringenin	273 $\pm$ 23 a	1.13	710 $\pm$ 36 a	1.04
Phenol	269 $\pm$ 22 a	1.11	698 $\pm$ 51 a	1.02
Quercetin	268 $\pm$ 29 a	1.10	667 $\pm$ 23 a	0.97
Phenyl- β -D-galactopyranoside	252 $\pm$ 33 a	1.04	638 $\pm$ 57 a	0.93
Rutin	241 $\pm$ 22 a	0.99	686 $\pm$ 40 a	1.00

^y All phenolic compounds tested at a final concentration of 100 μ M in syringomycin minimal medium (SRM).

^z GUS = β -glucuronidase. Activities are the mean of six independent assays followed by the standard error (SE) of mean. Different letters indicate statistically significant differences ($\alpha = 0.01$). Differences between treatments were determined by Tukey's analysis of variance.

liquid medium. Without plant signal molecules, GUS activity of B301DSL8 averaged only 685 units per 10^8 CFU. Similarly, the GUS activity of B301DSL29, which contains the *sypA::uidA* reporter, was increased from 242 to 528 units per 10^8 CFU in the presence of arbutin (100 μ M) and fructose (0.1%). Consequently, the expression of *syrB1* and *sypA* were increased 1.9- and 2.1-fold, respectively, by addition of arbutin and fructose to SRM liquid medium.

The *sala* and *syrF* regulatory pathway mediates induction of syringopeptin production and *sypA* expression.

Neither B301DSL7, a *sala* mutant, nor B301DSL1, a *syrF* mutant, produced detectable syringopeptin zones of inhibition to *B. megaterium* Km on SRM or SRM_{AF} agar media (Fig. 3). B301DSL6, a *syrG* mutant, did not produce detectable zones of inhibition on SRM agar medium, but did produce syringopeptin on SRM_{AF} agar medium (not shown). Expression of the *sypA::uidA* reporter was reduced approximately 99% in *sala* strain B301DSL30 (Lu et al. 2005) in both SRM and SRM_{AF} liquid media. Expression of the *sypA::uidA* reporter was decreased by 93.7 and 96.4% in *syrF* strain B301DNW31

in SRM and SRM_{AF} media, respectively. Neither the *sala* mutant B301DSL30 nor the *syrF* mutant B301DNW31 exhibited a significant increase in GUS activity upon the addition of plant signal molecules.

Microarray analysis of the effects of plant signal molecules on expression of virulence-related genes.

Analysis with a 70-mer oligonucleotide subgenomic microarray showed that 26 of 95 genes were induced significantly greater than twofold by addition of arbutin (100 μ M) and D-fructose (0.1%) (Table 2). In this study, no gene or open reading frame (ORF) included was observed to have an expression ratio below 0.5, which indicates that no gene or ORF included in the microarray was negatively regulated by plant signal molecules. Genes dedicated to biosynthesis, secretion, and regulation of syringomycin and syringopeptin were induced by plant signal molecules. The expression of all of the syringopeptin synthetase genes, including *sypA*, *sypB*, and *sypC* (Scholz-Schroeder et al. 2003) (Fig. 1; Table 2), was increased 5.4- to 7.8-fold by addition of arbutin and D-fructose to SRM. The expression of all of the syringomycin biosynthesis genes, includ-

Table 2. Microarray analysis of the stimulon controlled by plant signal molecules

Genes ^y	Ratio (SEM) ^z	Gene product	References
<i>syrD</i>	4.71 ($\pm$ 0.40)	ATP-binding secretion protein	Quigley et al. 1993
<i>syrP</i>	7.76 ($\pm$ 0.61)	Homologue of histidine kinase	Zhang et al. 1997
<i>syrB1</i>	5.23 ($\pm$ 0.40)	Syringomycin biosynthesis enzyme	Zhang et al. 1995
<i>syrB2</i>	5.41 ($\pm$ 0.80)	Syringomycin biosynthesis enzyme	Zhang et al. 1995
<i>syrC</i>	10.51 ($\pm$ 0.66)	Syringomycin biosynthesis enzyme	Zhang et al. 1995
<i>syrE-1</i>	4.40 ($\pm$ 0.55)	Syringomycin synthetase	Guenzi et al. 1998
<i>syrE-2</i>	6.99 ($\pm$ 0.83)	Syringomycin synthetase	Guenzi et al. 1998
<i>syrE-3</i>	8.03 ($\pm$ 0.89)	Syringomycin synthetase	Guenzi et al. 1998
<i>syrF</i>	3.89 ($\pm$ 0.81)	LuxR family bacterial regulator	Lu et al. 2002
<i>ORF20</i>	6.18 ($\pm$ 0.93)	Putative out membrane protein	Lu et al. 2005
<i>ORF21</i>	2.53 ($\pm$ 0.48)	Hypothetical protein	Lu et al. 2005
<i>ORF22</i>	6.45 ($\pm$ 1.29)	Membrane protein	Lu et al. 2005
<i>syrG</i>	3.08 ($\pm$ 0.69)	LuxR family regulatory protein	Lu et al. 2002
<i>sala</i>	2.50 ($\pm$ 0.19)	LuxR family bacterial regulator	Lu et al. 2002
<i>ORF23</i>	0.99 ($\pm$ 0.07)	Hypothetical protein	Lu et al. 2005
<i>ORF24-1</i>	0.85 ($\pm$ 0.13)	Hypothetical protein	Lu et al. 2005
<i>ORF24-2</i>	0.92 ($\pm$ 0.03)	Hypothetical protein	Lu et al. 2005
<i>ORF25</i>	0.94 ($\pm$ 0.20)	Hypothetical protein	Lu et al. 2005
<i>ORF26</i>	1.02 ($\pm$ 0.14)	Unknown (chemotaxis protein)	Lu et al. 2005
<i>sypA</i>	6.16 ($\pm$ 0.64)	Syringopeptin synthetase	Scholz-Schroeder et al. 2003
<i>sypB-1</i>	7.78 ($\pm$ 0.74)	Syringopeptin synthetase	Scholz-Schroeder et al. 2003
<i>sypB-2</i>	5.41 ($\pm$ 0.80)	Syringopeptin synthetase	Scholz-Schroeder et al. 2003
<i>sypC1</i>	5.79 ($\pm$ 1.09)	Syringopeptin synthetase	Scholz-Schroeder et al. 2003
<i>sypC2</i>	5.75 ($\pm$ 1.32)	Syringopeptin synthetase	Scholz-Schroeder et al. 2003
<i>ORF19</i>	3.49 ($\pm$ 0.16)	Putative membrane protein	H. Kang and D. C. Gross, unpublished
<i>sypD</i>	6.23 ($\pm$ 0.59)	Putative ABC transporter	H. Kang and D. C. Gross, unpublished
<i>dat</i>	6.23 ($\pm$ 0.65)	Aminotransferase	H. Kang and D. C. Gross, unpublished
<i>pseA</i>	4.33 ($\pm$ 0.74)	Putative outer membrane protein	Kang and Gross 2005
<i>pseB</i>	1.56 ($\pm$ 0.15)	Efflux membrane fusion protein	Kang and Gross 2005
<i>pseC</i>	1.64 ($\pm$ 0.14)	Efflux membrane fusion protein	Kang and Gross 2005
<i>ORF14</i>	1.03 ($\pm$ 0.07)	Putative chemotaxis protein	N. Wang and D. C. Gross, unpublished
<i>ORF13</i>	0.91 ($\pm$ 0.08)	Unknown	N. Wang and D. C. Gross, unpublished
<i>ORF12</i>	1.02 ($\pm$ 0.09)	Sensor protein	N. Wang and D. C. Gross, unpublished
<i>ORF11</i>	0.79 ($\pm$ 0.09)	Amino acid deaminase	N. Wang and D. C. Gross, unpublished
<i>ORF10</i>	1.30 ($\pm$ 0.16)	Hypothetical protein	N. Wang and D. C. Gross, unpublished
<i>ORF9</i>	1.20 ($\pm$ 0.11)	Hypothetical protein	N. Wang and D. C. Gross, unpublished
<i>ORF8</i>	1.17 ($\pm$ 0.10)	Hypothetical protein	N. Wang and D. C. Gross, unpublished
<i>ORF7</i>	1.06 ($\pm$ 0.14)	Hypothetical protein	N. Wang and D. C. Gross, unpublished
<i>ORF6</i>	0.84 ($\pm$ 0.19)	Hypothetical protein	N. Wang and D. C. Gross, unpublished
<i>ORF5</i>	0.88 ($\pm$ 0.08)	Hypothetical protein	N. Wang and D. C. Gross, unpublished
<i>ORF4</i>	1.25 ($\pm$ 0.45)	Hypothetical protein	N. Wang and D. C. Gross, unpublished
<i>ORF3</i>	0.75 ($\pm$ 0.08)	Hypothetical protein	N. Wang and D. C. Gross, unpublished
<i>ORF2</i>	1.27 ($\pm$ 0.17)	Hypothetical protein	N. Wang and D. C. Gross, unpublished

(continued on next page)

^y The *psyr* numbers stand for open reading frames listed on the Department of Energy's Joint Genome Institute website.

^z The ratio was determined by comparing gene transcription (measured by signal intensity) in *Pseudomonas syringae* pv. *syringae* B301D cultured on syringomycin minimal medium (SRM) supplemented with arbutin and D-fructose (SRM_{AF}) medium to that of B301D cultured on SRM. Data shown in the table are the means of the three independent experiments with the standard errors of means (SEM).

ing *syrB1*, *syrB2*, *syrC*, and *syrE* genes (Guenzi et al. 1998; Lu et al. 2002) (Fig. 1; Table 2), was increased 4.4- to 10.5-fold by addition of plant signal molecules (Table 2). The expression levels observed from three 70-mer oligonucleotide arrays (sequences available from authors upon request) designed from different locations in *syrE* showed greater than fourfold increases with the addition of arbutin and D-fructose (Table 2). Three major regulatory genes located at the right border of the *syr-syp* genomic island, *salA*, *syrF*, and *syrG*, were upregulated by 2.5-, 3.9-, and 3.1-fold, respectively. Expression of most secretion genes in the *syr-syp* genomic island, including *syrD*, *sypD*, two *mtrC* homologues, one *oprM* homologue, and *pseA*, was increased to higher levels of 2.5 to 6.5 times by plant signal molecules. In addition to the *syr-syp* genes, the *hrp* genes *hrpZ*, *hrpR*, *hrpL*, *hrpW*, *hrmA*, and *hrpK* (Alfano et al. 2000; Collmer et al. 2000) included on the microarray were induced more than twofold in the presence of plant signal molecules (Table 2).

The availability of a transcriptional profile for B301D compared with B301DSL7 on potato dextrose agar, as reported by Lu and associates (2005), and that of B301D on SRM agar

medium with or without exogenously added arbutin (100 μ M) and fructose (0.1%), in this study, facilitated cluster analysis of oligonucleotide microarray data with the self-organizing tree algorithm (SOTA) (Herrero et al. 2001). All of the induced genes were grouped into two clusters based on SOTA analysis (Fig. 4), with the *syr-syp* genes clustered together and the *hrp/lavr* genes forming a second cluster.

Housekeeping genes such as *sigX* (Brinkman et al. 1999), *algT* (Keith and Bender 1999), *algD* (Fakhr et al. 1999), *sodB* (Hassett et al. 1993), and *inaK* (Li et al. 2004), located outside of the *syr-syp* genomic island, were expressed at high levels on both SRM and SRM_{AF} media with no significant differences in expression levels between these two media. Genes involved in siderophore production (i.e., *pvdS* [Mossialos et al. 2002], *pvdE* [McMorran et al. 1996], *fsc* [Nelson et al. 2002], *acsD* [Franza et al. 2005], *cbrB* [Mahé et al. 1995], *cbrD* [Mahé et al. 1995], and *fur* [Hassett et al. 1996]), environmental stress (*rulA*) (Zhang and Sundin 2004), quorum sensing (*ahlI*) (Kinscherf and Willis 1999), global regulation (i.e., *gacS*, *gacA*, *rpoN* [Alarcón-Chaidez et al. 2003; Cui et al. 2001], *rpoS* [Hengge-Aronis 1993], and *rpoD* [Tanaka and Takahashi 1991]), phyto-

Table 2. Continued from preceding page

Genes ^y	Ratio (SEM) ^z	Gene product	References
<i>ORF1</i>	1.03 ($\pm$ 0.10)	Hypothetical protein	N. Wang and D. C. Gross, <i>unpublished</i>
<i>hrpL</i>	2.93 ($\pm$ 0.44)	Alternative sigma factor	Alfano et al. 2000
<i>hrpR</i>	2.10 ($\pm$ 0.18)	Regulatory factor	Xiao and Hutcheson 1994
<i>hrpS</i>	1.87 ($\pm$ 0.26)	Positive regulatory factor	Xiao and Hutcheson 1994
<i>hrpK</i>	2.27 ($\pm$ 0.40)	Unknown	Wang and Gross, <i>unpublished</i>
<i>hrpJ</i>	1.01 ($\pm$ 0.16)	Flagella biogenesis	Lidell and Hutcheson 1994
<i>hrpZ</i>	3.95 ($\pm$ 0.28)	Harpin	He et al. 1993
<i>hrpW</i>	2.63 ($\pm$ 0.23)	Type III effector	Charkowski et al. 1998
<i>queA</i>	1.02 ($\pm$ 0.11)	Exchangeable effector locus	Alfano et al. 2000
<i>EEL-chp</i>	2.0 ($\pm$ 0.28)	Type III chaperone protein	N. Wang and D. C. Gross, <i>unpublished</i>
<i>hrmA</i>	2.24 ($\pm$ 0.28)	Avr (effector) proteins	Alfano et al. 2000
<i>gacS</i>	1.56 ($\pm$ 0.26)	Sensor protein	Willis et al. 1990
<i>gacA</i>	1.57 ($\pm$ 0.35)	Regulator protein	Rich et al. 1994
<i>rpoS</i>	1.41 ($\pm$ 0.08)	RNA polymerase sigma factor	Hengge-Aronis 1993
<i>rpoD</i>	1.13 ($\pm$ 0.06)	Principle sigma factor	Tanaka and Takahashi 1991
<i>rpoN</i>	0.82 ($\pm$ 0.06)	Putative sigma-54 protein	Alarcón-Chaidez et al. 2003
<i>sigX</i>	1.37 ($\pm$ 0.23)	Sigma factor	Brinkman et al. 1999
<i>psyr020094</i>	1.28 ($\pm$ 0.22)	Putative NRPS	JGI Integrated Microbial Genomes website
<i>psyr020651</i>	1.86 ($\pm$ 0.14)	Putative NRPS	JGI Integrated Microbial Genomes website
<i>sylD</i>	1.16 ($\pm$ 0.11)	Putative syringolin synthetase	Amrein et al. 2004
<i>algT</i>	1.16 ($\pm$ 0.05)	Alternative sigma factor	Keith and Bender 1999
<i>algD</i>	0.84 ($\pm$ 0.22)	GDP-mannose dehydrogenase	Fakhr et al. 1999
<i>iaaM</i>	1.21 ($\pm$ 0.14)	Tryptophan monooxygenase	Mazzola and White 1994
<i>iaaH</i>	0.84 ($\pm$ 0.07)	Indoleacetamide hydrolase	Mazzola and White 1994
<i>ahlI</i>	1.97 ($\pm$ 0.20)	Acyl homoserine lactone synthetase	Kinscherf and Willis 1999
<i>inaK</i>	0.81 ($\pm$ 0.09)	Ice nucleation protein	Li et al. 2004
<i>pvdS</i>	0.86 ($\pm$ 0.09)	Putative acetylase	Mossialos et al. 2002
<i>pvdE</i>	0.83 ($\pm$ 0.06)	Pyoverdine synthetase	McMorran et al. 1996
<i>fur</i>	1.19 ($\pm$ 0.14)	Ferric uptake regulator	Franza et al. 2005
<i>lccC</i>	2.09 ($\pm$ 0.66)	Levansucrase	Li and Ullrich 2001
<i>cbrB</i>	1.03 ($\pm$ 0.13)	Ferrisiderophore permease	Mahé et al. 1995
<i>cbrD</i>	0.81 ($\pm$ 0.09)	ATP-binding unit in ABC transport	Mahé et al. 1995
<i>acsD</i>	0.77 ($\pm$ 0.04)	Achromobactin biosynthetase	Franza et al. 2005
<i>fsc</i>	0.81 ($\pm$ 0.07)	Ferric siderophore receptor	Nelson et al. 2002
<i>SR-dat</i>	0.80 ($\pm$ 0.05)	Diaminobutyrate transaminase	Ikai and Yamamoto 1997
<i>psyr020143</i>	1.02 ($\pm$ 0.19)	Putative NRPS	Department of Energy's JGI website
<i>tex</i>	0.85 ($\pm$ 0.07)	S1 RNA binding domain protein	Nelson et al. 2002
<i>gshA</i>	1.27 ($\pm$ 0.09)	Glutamate-cysteine ligase	Nelson et al. 2002
<i>argA</i>	1.15 ($\pm$ 0.09)	N-acetylglutamate synthetase	Lu et al. 2003
<i>argE</i>	0.92 ($\pm$ 0.11)	Acetylornithine deacetylase	Lu et al. 2003
<i>SA-ORF6</i>	0.90 ($\pm$ 0.12)	Unknown	Lu et al. 2003
<i>rulA</i>	0.85 ($\pm$ 0.09)	Radiation tolerance	Sundin and Murillo 1999
<i>sodB</i>	1.11 ($\pm$ 0.05)	Iron-superoxide dismutase	Hassett et al. 1993
<i>gyrB</i>	0.79 ($\pm$ 0.04)	DNA gyrase subunit B	Sawada et al. 1999
<i>recA</i>	1.51 ($\pm$ 0.21)	Principal sigma factor	Kokjohn and Miller 1985
<i>luc</i>	0.86 ($\pm$ 0.10)	Luciferase	Coronado et al. 1994
<i>negc10</i>	0.98 ($\pm$ 0.33)	Random primer	Qiagen
16S rDNA	1 ($\pm$ 0.0)	16S ribosomal RNA	Nelson et al. 2002

hormone synthesis (*iaaM* and *iaaH*) (Mazzola and White 1994), and alginate production (*algD*) (Koopmann et al. 2001) were not affected by plant signal molecules.

Independent microarray validation using GUS assays and quantitative real-time transcription polymerase chain reaction.

In addition to the microarray results of plant signal molecules on *syr-syp* genes, 10 genes (9 induced genes [i.e., *syrB1*, *syrC*, *sypA*, *sypB*, *hrpR*, *hrpZ*, *salA*, *syrF*, and *syrG*] and 1 unaffected gene [i.e., *recA*]) that represent a range of changes observed in microarray studies (0.8- to 10.5-fold changes) were validated by GUS assays and quantitative real-time transcription polymerase chain reaction (QRT-PCR) analysis. GUS assays corroborated the direction of regulation observed from microarray analysis for all three regulatory genes, including *salA*, *syrF*, and *syrG*, located at the right border of the *syr* gene cluster. Based on quantitative GUS assays, expression of pSL40F (*salA::uidA*), pSL38F (*syrF::uidA*), and pSL39F (*syrG::uidA*) was significantly increased by addition of arbutin and D-fructose. Strain B301D, carrying pSL40F, pSL38F, or pSL39F, demonstrated GUS activity of 1,059, 1,700, and 535 units per 10^8 CFU, respectively, 72 h after incubation at 25°C in SRM liquid medium. In contrast, their expression levels reached 1,918, 2,711, and 1,220 units per 10^8 CFU, respectively, in SRM_{AF} liquid medium. Therefore, the expression levels of *salA*, *syrF*, and *syrG* were increased 1.81-, 1.59-, and 2.24-fold, respectively, by addition of plant signal molecules.

The regulation patterns defined by QRT-PCR were similar to those determined by the microarray data. Based on QRT-PCR analysis, the fold induction by arbutin and fructose on *syrB1*, *syrC*, *sypA*, *sypB*, *hrpZ*, *hrpR*, and *recA* were 8.4, 16.3, 4.3, 25.7, 13.8, 6.0, and 1.2, respectively (Table 3). In comparison, the induction levels by microarray analysis were increased by 5.2-, 10.5-, 6.2-, 6.6-, 2.1-, 4.0-, and 1.5-fold, respectively (Table 2). The greatest difference in changes was obtained for *sypB*; the change of transcriptional level determined by QRT-PCR analysis was 25.7-fold, compared with a 6.6-fold change in expression by microarray analysis.

Induction of *syr-syp* and type III genes via different regulatory pathways.

QRT-PCR was used to evaluate the effect of a *gacS* disruption on the induction of *syr-syp* and *hrp* genes. The *gacS* mutant (B301DG12) was inoculated on SRM and SRM_{AF} media for 3 days at 25°C as described above. In a *gacS* mutant, the transcriptional expression increase of *sypA*, *sypB*, *syrB1*, and *syrC* caused by plant signal molecules was reduced to 1.0-, 3.4-,

Fig. 4. Unsupervised hierarchical clustering analysis of *syr-syp* and representative genes. Column A represents comparisons between B301D on SRM and SRM_{AF} agar media. Column B represents comparisons of gene expression between B301D and B301DSL7, a *salA* mutant. The scale of gene activities is represented from green (not induced or induced at low level) to red (induced at high level). The cluster analysis of microarray data was performed with the self-organizing tree algorithm. The *syr-syp* genes that clustered together are in red text. The genes associated with the Hrp type III secretion system that are clustered together are in blue text.

Table 3. Quantitative real-time polymerase chain reaction analysis of expression of representative genes of strains B301D and B301DG12 (*gacS* mutant) as influenced by the presence of plant signal molecules

Gene	Change in transcriptional level ($\pm$ SE) on SRM versus SRM _{AF} ^z	
	B301D	B301DG12
<i>syrB1</i>	8.4 $\pm$ 0.7	1.4 $\pm$ 0.2
<i>syrC</i>	16.3 $\pm$ 1.6	1.8 $\pm$ 0.1
<i>sypA</i>	4.3 $\pm$ 0.1	1.0 $\pm$ 0.2
<i>sypB</i>	25.7 $\pm$ 2.0	3.4 $\pm$ 1.0
<i>hrpR</i>	13.7 $\pm$ 2.1	11.5 $\pm$ 2.7
<i>hrpZ</i>	6.0 $\pm$ 0.5	5.7 $\pm$ 0.4
<i>recA</i>	1.2 $\pm$ 0	1.0 $\pm$ 0

^z Values represent the fold change in transcriptional level for strains grown on syringomycin minimal medium (SRM) supplemented with 100 μ M arbutin and 0.1% D-fructose (SRM_{AF}) compared with growth on SRM. Each value represents the average of three independent assays followed by the standard error (SE) of the mean.

1.4-, and 1.8-fold compared with 4.3-, 25.7-, 8.4-, and 16.3-fold increases by B301D, respectively (Table 3). There was no significant difference in the expression of *hrpR* and *hrpZ* between strains B301D and B301DG12 (Table 3). These data suggest that the plant signal transduction pathway responsible for toxin production is independent of that for the Hrp type III secretion system in B301D. In contrast, the expression of the housekeeping gene *recA* was not affected by the *gacS* mutation. The expression of the *recA* gene was comparable to that of B301D grown on SRM or SRM_{AF} media.

DISCUSSION

The induction of toxigenesis in *P. syringae* pv. *syringae* by specific plant signal molecules reflects an ability of the bacterium to adapt to a dynamic plant environment. It was reported that syringomycin production is activated by specific plant signal molecules in diverse strains of *P. syringae* pv. *syringae* (Quigley and Gross 1994). This study established that plant signal molecules likewise control expression of genes involved in syringopeptin production based on the following evidence: i) syringopeptin production by B3AR132 and BR132 was increased more than 1.6-fold by addition of arbutin and D-fructose to SRM agar medium as revealed by bioassays; ii) GUS assays indicated that expression of *sypA::uidA* is increased substantially by addition of specific phenolic glycosides (i.e., arbutin, phenyl- β -D-glucopyranoside, and salicin) to SRM liquid medium; iii) analysis with a subgenomic 70-mer oligonucleotide microarray demonstrated that the transcriptional levels of the *syr-syp* genes are activated 2.5- to 10.5-fold by arbutin and D-fructose; and iv) QRT-PCR analysis showed that the expression of the representative *syr-syp* genes is induced between 4.3- and 25.7-fold by arbutin and D-fructose. This is the first report that syringopeptin production and genes involved in syringopeptin production are activated by specific plant signal molecules.

Plant signal molecules have the same specificity for induction of the *syp* genes as that for the *syr* genes. Of the 10 phenolic compounds tested, only arbutin, salicin, and phenyl- β -D-glucopyranoside showed significant induction of expression of *sypA::uidA* in B301DSL29. As observed for *syrB1* (Mo and Gross 1991), other representative phenolic compounds (hydroquinone, naringenin, phenol, phenyl- β -D-galactopyranoside, quercetin, rutin, and saligenin) did not affect the expression of *sypA::uidA*. In addition, specific sugars, such as D-fructose, enhanced phenolic glycoside-mediated induction of *sypA* at levels similarly observed for *syrB1*. The similar specificity of induction of the *syrB1* and *sypA* synthetase genes by plant signal molecules is consistent with the fact that the SalA regulon (Lu et al. 2005) transduces the plant signals to the *syr-syp* genes. The efficient induction of *syrB1* and *sypA* genes by phenolic compounds and sugars resembles the process of *vir* gene activation in *A. tumefaciens* even though the active phenolic metabolites are different for the two bacteria (Ankenbauer and Nester 1990; Cangelosi et al. 1990; Shimoda et al. 1990). *A. tumefaciens* responds to acetosyringone (Stachel et al. 1985) to induce *vir* gene expression, whereas *P. syringae* pv. *syringae* responds to phenolic β -glycosides such as arbutin and salicin to activate the expression of syringomycin- and syringopeptin-associated genes. The phenolic β -glycosides are found in leaves, bark, and flowers of many plant species that are hosts of *P. syringae* pv. *syringae* (Mo and Gross 1991). Arbutin occurs in one monocot family, Liliaceae, and more than 10 dicot families, including Rosaceae, Leguminosae, and Saxifragaceae, which all are hosts of *P. syringae* pv. *syringae* (Mo and Gross 1991). The phenolic compounds can occur in high concentrations in plant tissues. For example, pear (*Pyrus communis*) leaves contain approximately 150 mM arbutin (Mo et al. 1995). In

addition, fructose and sucrose composed as much as 1 to 3% of the dry weight of the cherry tissues (Keller and Loescher 1989). It appears that plant signal molecules would be present in sufficient quantity to induce syringomycin and syringopeptin production by *Pseudomonas syringae* pv. *syringae* in the invasion of plants as indicated by the substantial induction of *syrB::lacZ* (Mo and Gross 1991) and *sypA::uidA* by arbutin (100 μ M) and D-fructose (0.1%).

Microarray analysis is a powerful tool for monitoring expression of the *syr-syp* genes (Lu et al. 2005; Rhodius and LaRossa 2003). Analysis with 70-mer oligonucleotide subgenomic microarrays indicates that plant signal molecules induce the coordinated expression of the *syr-syp* genes. Once the effects of arbutin and D-fructose on syringomycin and syringopeptin production in B301D were established, microarray analysis was used to examine the signal activity of arbutin and D-fructose on expression of virulence-associated genes. It was shown clearly in the microarray analysis that not only are the toxin biosynthesis genes positively regulated, but regulatory and secretion genes associated with syringomycin and syringopeptin production also are induced by arbutin and D-fructose. The upregulated *syr-syp* genes belong to the SalA regulon (Lu et al. 2005) as indicated by clustering analysis (Fig. 4), which demonstrates that the plant signal transduction of the *syr-syp* genes is mediated by SalA. It appears that cluster analysis is very useful in identifying specific pathways that are co-regulated based on their similar patterns of gene expression (Bolshakova and Azuaje 2003).

In addition to the induction of syringomycin and syringopeptin, most *hrp* and *avr* genes included in this study were induced by the addition of arbutin and D-fructose. The induction of *hrp* and *avr* genes was reported to commonly occur in plant-bacteria interactions (Boch et al. 2002; Rahme et al. 1992). Induction of *hrp* genes was studied mostly in planta, and the specific plant signal molecules involved in induction were not defined (Rahme et al. 1992). The *hrp* genes were shown to be expressed when bacteria were grown in defined minimal medium but not in complex media and could be induced four- to sixfold when transferred from King's B medium to M63M medium (Arlat et al. 1992; Hendrickson et al. 2001; Rahme et al. 1992; Wei et al. 1992; Xiao et al. 1992). The SRM used in this study is different from the defined *hrp*-inducing medium and supports the expression of the *hrp* genes tested. The transcriptional levels of *hrpZ*, *hrpW*, *hrpL*, *hrpR*, and *hrpK* were increased from two- to fourfold in SRM_{AF} liquid medium, which demonstrates a link between plant signal molecules that induce phytotoxin production and genes associated with the Hrp type III secretion system.

Despite the fact that *syr-syp* and *hrp/avr* genes are activated by plant signal molecules, the two systems use different signal transduction pathways to induce virulence-associated genes. The plant signal stimulon might involve the coordinated expression of numerous regulons, a situation similar to that of the heat shock stimulon (Narberhaus 1999; Yura and Nakahigashi 1999). As observed by cluster analysis (Fig. 4), the induced genes were grouped into the *syr-syp* and the *hrp/avr* clusters. The *syr-syp* genes were demonstrated to belong to the SalA regulon (Lu et al. 2005), whereas the *hrp/avr* genes were clustered together and belong to the HrpL regulon (Fouts et al. 2002). In *P. syringae*, three regulatory genes, *hrpL*, *hrpR*, and *hrpS*, are dedicated to *hrp/avr* gene expression (Fig. 5) (Grimm et al. 1995; Hutcheson et al. 2001; Xiao and Hutcheson 1994). Considerable research has been done to verify the relationship between *hrp* and GacS/GacA systems (Chatterjee et al. 2003). RT-PCR transcriptional analysis revealed that a *P. syringae* *gacS* mutation abolished induction of the four syringomycin/syringopeptin synthesis genes *syrB1*, *syrC*, *sypA*, and *sypB* by plant signal molecules

(Table 3). However, the induction of *hrpR* and *hrpZ* by plant signal molecules is not affected by mutation of *gacS*. This is consistent with the discovery that GacA does not control *hrp* genes in *P. syringae* pv. *syringae* B728a (Chatterjee et al. 2003). Thus, the plant signal induction of syringomycin and syringopeptin in B301D is via the GacS/A pathway, whereas the induction of the *hrp* genes uses a different pathway.

Both the *salA* and *syrF* regulatory genes are required for syringopeptin production. It has been demonstrated that the regulation of syringopeptin and syringomycin is controlled by the same regulatory cascade. SalA is the key regulator in the control of syringomycin and syringopeptin production (Lu et al. 2002, 2005). SyrF, which is controlled by SalA, positively regulates syringomycin production (Lu et al. 2002) and this study demonstrated that SyrF positively regulates syringopeptin production (Fig. 3). Standard phytotoxin bioassays showed that disruption of either *salA* or *syrF* resulted in the failure to produce syringopeptin on both SRM and SRM_{AF} agar media 4 days after inoculation (Fig. 3). Furthermore, the expression of *sypA::uidA* in either *salA* or *syrF* mutants was substantially decreased compared with that of B301D grown in SRM and SRM_{AF} liquid media, respectively. Both SalA and SyrF belong to the LuxR regulatory protein family (Kaiser and Losick 1993; Lu et al. 2002). The LuxR protein contains a helix-turn-helix DNA-binding motif at the C terminus and regulates target genes by binding to the *lux* box in the promoter regions (Egland and Greenberg 1999). It appears that SyrF controls the coordinated expression of the *syr-syp* genes by binding to the promoter regions directly (Wang et al. 2006).

Microarray analysis of the plant signal stimulon in *P. syringae* pv. *syringae* provides a valuable foundation for the study of the regulatory mechanism controlling syringomycin and syringopeptin production. Apparently, the bacterium coordinates the expression of the *syr-syp* and *hrp* genes in response to the plant environment (Fig. 5). As proposed in the model shown in Figure 5, *P. syringae* pv. *syringae* senses plant signal molecules via the GacS/A pathway, which then activates the expression of the *syr-syp* genes through SalA and SyrF. It remains to be determined what genes are directly involved in the sensing of plant signal molecules and how SyrF controls syringomycin and syringopeptin production. It is expected that SyrF might control the coordinated expression of the *syr-syp* genes by directly binding to the promoters, as suggested in the model proposed in Figure 5. Currently, the promoter regions of the *syr-syp* genes (Wang et al. 2006) and the functions of SyrF and SalA regulatory proteins are being characterized in an effort to understand the co-regulatory mechanism important for syringomycin and syringopeptin production.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media.

The bacterial strains and plasmids used in this study are listed in Table 4. The *Escherichia coli* DH10B (Sawahel et al. 1993) used for cloning was cultured at 37°C in Terrific Broth (TB) or on Luria-Bertani (LB) agar media. Strains of *P. syringae* pv. *syringae* were cultured routinely at 25°C in nutrient broth-yeast extract (NBY) liquid or agar medium (Vidaver 1967). To evaluate the effect of plant signal molecules on in-

Fig. 5. Diagram of the stimulon induced by plant signal molecules in *Pseudomonas syringae* pv. *syringae* B301D to activate phytotoxin production. Plant signal molecules in the plant apoplast activate the *gacS/gacA*, *salA*, *syrF*, and *hrpL* signal transduction pathways leading to the transcription of the *syr-syp* toxin genes and genes associated with Hrp type III secretion (TTSS). Strain B301D senses plant signals by GacS, a transmembrane sensor kinase, or some unknown acceptor senses the plant signals and transfers them to GacS. GacS then activates *salA* through the response regulator GacA. SalA transcriptionally activates expression of *syrF* and SyrF, in turn, activates the *syr-syp* genes upon binding to the promoter region as a dimer. B301D also senses plant signal molecules through an unknown mechanism (represented as ?) to activate expression of the Hrp type III associated genes through the *hrpR/hrpS/hrpL* regulatory pathway. Expression of syringomycin (*syr*) and syringopeptin (*syp*) genes is induced by plant signal molecules, and the toxins are secreted through a type I secretion system (TISS). The nonribosomal peptide synthetases for *syr* and *syp* are represented as Esyr and Esyp, respectively.

duction of syringopeptin production, syringomycin minimal SRM and SRM_{AF} (SRM containing 100 µM arbutin and 0.1% fructose) media were prepared as described previously (Mo and Gross 1991). For tests of gene induction by plant signal molecules, *P. syringae* pv. *syringae* was cultured in SRM (Mo and Gross 1991) liquid or agar medium with or without plant signal molecules as identified below. Antibiotics (Sigma-Aldrich, St. Louis, U.S.A.) were added to media in the following concentrations: tetracycline, 25 µg/ml; Km, 100 µg/ml; ampicillin, 100 µg/ml; and gentamicin, 5 µg/ml.

Bioassays for syringopeptin production.

The *P. syringae* pv. *syringae* strains BR132 and B3AR132 were evaluated for syringopeptin production on SRM and SRM_{AF} media by using standard bioassays as previously described by Scholz-Schroeder and associates (2001a). Briefly, *P. syringae* pv. *syringae* strains were grown overnight in 2 ml of NBY liquid medium. Bacterial cells were harvested by centrifugation, washed once with sterile distilled water (SDW), and then resuspended in SDW to a concentration of approximately 2×10^8 CFU/ml. Aliquots (5 µl) of bacterial suspension were spotted onto SRM and SRM_{AF} plates. The strains were cultured on plates for 4 days and then sprayed with *B. megaterium* Km and cultured overnight at 25°C. Because syringomycin inhibits the growth of *B. megaterium*, both strains tested for syringopeptin production were *syrB1* mutants (i.e., BR132 and B3AR132) (Scholz-Schroeder et al. 2001a). Duplicate cultures were prepared for all treatments with all assays repeated three times on separate days.

Construction of GUS transcriptional fusions and procedures for GUS assays.

The regulatory effects of *sala* and *syrF* genes on expression of the *sypA::uidA* gene fusion in cis were quantified by fluorometric analysis of GUS activity. Plasmid pSL19, which carries a disrupted *syrF* gene by insertion of the *nptII* cassette, was electroporated into the *sypA::uidA* reporter strain B301DSL29 (Lu et al. 2005) to generate the double mutant B301DNW31. The disrupted *syrF* gene in the double mutant was amplified by PCR. Primers used in the amplification of *syrF* were PsyrFf: GCCATTCCTTGCGCCCATAAA and PsyrFr: CGAG GCAGAATTCCGACACAAG. The resulting PCR product from B301DNW31 was subcloned into the pGEM-T easy vector to generate plasmid pNWE32. The insertion of the *nptII* cassette into *syrF* was confirmed by sequencing using PsyrFf as the primer.

The effects of different plant signal molecules and regulatory genes (i.e., *sala*, *syrF*, and *syrG*) on expression of *syrB1::uidA* and *sypA::uidA* gene fusions were quantified by fluorometric tests of GUS activity. A modified fluorometric protocol was used for analysis of GUS activity (Lu et al. 2002). Bacterial cells were cultured with shaking in 2 ml of NBY broth overnight at 25°C, harvested by centrifugation, washed twice with SDW, and then diluted with 20% glycerol to an optical density of 0.6 at 600 nm. Stock cell suspensions were stored at -80°C. Cell stock suspensions (50 µl) were used to inoculate 4 ml of potato dextrose broth medium or SRM liquid medium with or without plant signal molecules and incubated for 72 h at 25°C without shaking. Cell cultures (50 µl)

Table 4. Strains and plasmids

Strain or plasmid	Relevant characteristics ^a	Source
<i>Escherichia coli</i>		
DH10B	F <i>mcrA</i> Δ lacX74 (ϕ 80dlacZAM15) Δ (<i>mrr-hsdRMS-mcrB</i>) <i>deoR recA1 endA1 araD139</i> Δ (<i>ara, leu</i>)7697 <i>galU galK</i> λ^- <i>rpsL nupG</i>	Grant et al. 1990
<i>Pseudomonas syringae</i> pv. <i>syringae</i>		
B301D	Wild type, from pear	Cody and Gross 1987
B301DG12	<i>gacS::nptII</i> derivative of B301D; Km ^r	Kang and Gross 2005
B301DSL1	<i>syrF::nptII</i> derivative of B301D; Km ^r	Lu et al. 2002
B301DSL7	<i>sala::nptII</i> derivative of B301D; Km ^r	Lu et al. 2002
B301DSL6	<i>syrG::nptII</i> derivative of B301D; Km ^r	Lu et al. 2002
B301DSL8	<i>syrB1::uidA-aaaC1</i> , derivative of B301D; Gm ^r	Lu et al. 2002
B301DSL29	<i>sypA::uidA-aaaC1</i> , derivative of B301D; Gm ^r	Lu et al. 2005
B301DSL30	<i>sala::nptII</i> derivative of B301DSL29; Gm ^r Km ^r	Lu et al. 2002
B301DNW31	<i>syrF::nptII</i> derivative of B301DSL29; Gm ^r Km ^r	This study
BR132	<i>syrB1::Tn3HoHo1</i> derivative of B301D-R; Pip ^r Rif ^r	Mo and Gross 1991
B3A	Wild type	Mo and Gross 1991
B3AR132	<i>syrB1::Tn3HoHo1</i> derivative of B3A-R; Pip ^r Rif ^r	Mo and Gross 1991
Plasmid		
pBluescriptSK(+)	Cloning vector; Ap ^r	Stratagene, La Jolla, CA
pUCP26	Cloning vector; Tc ^r Ap ^r	West et al. 1994
pBR325	Cloning vector; Cm ^r Tc ^r Ap ^r	Prentki et al. 1981
pLAFR3	Cloning vector; Tc ^r	Staskawicz et al. 1987
pNWE32	pGEM-T easy carrying <i>syrF::nptII</i> cloned from B301DNW31	This study
p29	pLAFR3 carrying a 16-kb fragment of B301D genomic DNA; Tc ^r	Lu et al. 2002
pSL2	pBI101 with the 0.85-kb <i>aacC1</i> gene of pUCGM inserted at the <i>EcoRI</i> site downstream of the <i>uidA</i> gene; Km ^r Gm ^r	Lu et al. 2002
pSL19	pBR325 carrying the 5.8-kb <i>BamHI-KpnI</i> fragment of p29 with <i>nptII</i> insertion at <i>BstEII</i> site of <i>syrF</i> ; Ap ^r Tc ^r Km ^r	Lu et al. 2002
pSL9	pBluescriptSK(+) carrying a 2.5-kb <i>HindIII-EcoRV</i> fragment of pSL5 containing <i>syrG</i> ; Ap ^r	Lu et al. 2002
pSL40F	pLAFR3 carrying the 3.0-kb <i>EcoRI</i> fragment of p29 with the 3.2-kb <i>uidA-aacC1</i> fragment from pSL2 inserted at the <i>KpnI</i> site in-frame of <i>sala</i> in forward orientation; Tc ^r Gm ^r	Lu et al. 2002
pSL39F	pLAFR3 carrying the 2.5-kb <i>HindIII-EcoRV</i> fragment of pSL9 with the 3.2-kb <i>uidA-aacC1</i> fragment from pSL2 inserted at the <i>EcoRI</i> site in-frame of <i>syrG</i> in forward orientation; Tc ^r Gm ^r	Lu et al. 2002
pSL38F	pLAFR3 carrying the 5.8-kb <i>BamHI-KpnI</i> fragment of p29 with the 3.2-kb <i>uidA-aacC1</i> fragment from pSL2 inserted at the <i>BstEII</i> site in-frame of <i>syrF</i> in forward orientation; Tc ^r Gm ^r	Lu et al. 2002

^a Km^r, Gm^r, Pip^r, Rif^r, Tc^r, Ap^r, and Cm^r indicate resistance to kanamycin, gentamicin, piperacillin, rifampicin, tetracycline, ampicillin, chloramphenicol, respectively.

Table 5. Primers used for quantitative real-time polymerase chain reaction analysis

Name	Sequence	Resource
sypAF	TGCGGGTCGAGGCGTTTTG	Kang and Gross 2005
sypAR	GTTGCCGCGTCCTTGTCTGA	Kang and Gross 2005
sypBF	TTCGATCAGGGTCACCGCCAACAATG	Lu et al. 2005
sypBR	AGCTGCTCAATGTCGAAAAGGTC	Lu et al. 2005
syrB1F	TTAGCGCCGCGTCAGCCCCTCTCAAG	Kang and Gross 2005
syrB1R	GCTCAACGTCGCGGCTGCATCGCTCA	Kang and Gross 2005
syrCF	ACCTGCAAGCGATGTTCTCTC	Lu et al. 2005
syrCR	TGCCAGCTCGGTCTTGTTC	Lu et al. 2005
hrpRF	TTCGGCGTGGTCAATGGTGCGTTCA	This study
hrpRR	CGTGTCGATTTCTGCCAGATAGAGG	This study
hrpZF	TCCTGAAACCGAGACGACTGG	This study
hrpZR	GACCGTTGCGCATCAGTTCCTC	This study
recAF	CTTCGGTACGCTGGACA	Lu et al. 2005
recAR	AACTCGGCCTGACGGAAC	Lu et al. 2005
16SF	ACACCGCCCGT CACACCA	Lu et al. 2005
16SR	GTTCCCCTACGGCTACCTT	Lu et al. 2005

were collected by centrifugation, resuspended in 100 μ l of GUS extract buffer (Xiao et al. 1992), and lysed by sonication. Duplicate cultures were prepared for GUS assays with all assays repeated three times on separate days.

Phenolic compounds, sugars, and organic acids tested for *sypA*-inducing activity.

Phenolic compounds tested for plant signal activity were arbutin (ICN Biomedicals Inc., Aurora, OH, U.S.A.), hydroquinone, naringenin, phenol, phenyl- β -D-glucopyranoside, phenyl- β -D-galactopyranoside, quercetin, rutin, salicin, and saligenin (Sigma-Aldrich). D-fructose, which is known to enhance the signal activities of phenolic compounds (Mo and Gross 1991), was purchased from Baker Chem. Co. (Phillipsburg, NJ, U.S.A.).

Microarray analysis.

To test the effects of arbutin and D-fructose on the expression of *syr-syp* genes and genes involved in production of factors associated with plant pathogenesis, microarray analysis was performed as described recently (Lu et al. 2005). Strains of *P. syringae* pv. *syringae* were cultured with shaking at 25°C overnight in NBY medium (2 ml). Cells were harvested by centrifugation, washed twice, and then diluted with SDW to a concentration of approximately 2×10^8 CFU/ml. Cell suspensions (50 μ l) were spread on SRM and SRM_{AF} plates. The inoculated plates were incubated at 25°C for 72 h prior to recovery of cells. Total RNA was purified using a RiboPure-Bacteria kit (Ambion, Inc., Austin, TX, U.S.A.). Total RNA (50 μ g) was labeled with either Cy3-dUTP or Cy5-dUTP as described previously (Lu et al. 2005). Glass DNA microarrays containing a set of 70-mer oligonucleotides (Lu et al. 2005), designed and synthesized by Qiagen (now available at Operon Biotechnologies, Inc., Huntsville, AL, U.S.A.), were produced to represent genes contained in the *syr-syp* genomic island and other genes associated with virulence. The microarrays were used to quantify relative mRNA levels by parallel two-color hybridization according to protocols described in detail elsewhere (Lu et al. 2005). Briefly, hybridization was performed at 60°C overnight in a moist chamber. After washing, the slides were dried by centrifugation and scanned immediately using a GenePix 4000b scanner (Axon Instruments Inc., Foster City, CA, U.S.A.) to visualize the hybridization images.

Signal intensity and ratios were generated using GenePix Pro software provided with the scanner. Microarray data with intensities reproducibly higher than that of the background level were selected for analysis. The raw data was normalized using 16S rRNA as a standard. Hybridization experiments were conducted three times and each slide contained duplicate arrays.

Hierarchical clustering.

Unsupervised hierarchical clustering analysis of *syr-syp* and relevant genes was performed with SOTA. Cluster analysis was visualized with Treeview software (Rhodes et al. 2002).

QRT-PCR analysis.

The effect of arbutin and fructose on *syrC*, *sypB*, *hrpR*, *hrpZ*, and *inaK* observed in microarray analysis was evaluated by QRT-PCR using the QuantiTect SYBR Green RT-PCR kit (Qiagen Inc., Valencia, CA, U.S.A.). QRT-PCR also was used to assess the effect of disruption of the *gacS* global regulatory gene on expression of the *syrC*, *sypB*, *hrpR*, and *hrpZ* genes. Primers used for RT-PCR were designed using the Lasergene Expert Sequence Analysis Package (DNASTar, Madison, WI, U.S.A.) and are listed in Table 5. Consistent with microarray analysis, primers specific for the 16S rRNA gene were used for normalization controls. The linearity of detection of each primer pair was confirmed to have a correlation coefficient of at least 0.98 ($r^2 > 0.98$) over the detection area by measuring a fivefold dilution curve with total RNA isolated from bacterial cells. Reverse transcription was conducted at 50°C for 30 min with 100 ng of total RNA as template, then followed by initial activation of HotStarTaq DNA Polymerase (95°C, 15 min). Totally, 30 cycles of RT-PCR reactions (94°C for 15 s, 55°C for 30 s, and 72°C for 30 s) were performed followed by melting curve analysis.

ACKNOWLEDGMENTS

This work was supported by grant 2001-35319-10400 from the National Research Competitive Grants Program of the United States Department of Agriculture, Science, and Education Administration. We thank Angela Records for critical reading of this manuscript.

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